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Phenoloxidase activity in haemolymph of *Spodoptera litura* (Fabricius) mediating immune responses challenge with entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillmin

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ABSTRACT

The present study was carried out to investigate the effect of entomopathogenic fungus, *Beauveria bassiana* on immune response mediated Phenoloxidase enzyme of *Spodoptera litura* larvae. To measure the PO enzyme activity, haemolymph of 3rd, 4th and 5th instar larvae of *S. litura* were treated with different doses of 1.0×10^8, 2.0×10^7, 4.0×10^6 and 8.5×10^5 spores/ml of *B. bassiana*. The results indicated increase in PO activity with increase in *B. bassiana* infection but subsequently decreased in all instars. Significant increase in PO level was observed @ 4.0×10^6- 2.0×10^7 spores/ml after 24 hrs of infection in 3rd and 4th instar larvae. However, no significant effect of *B. bassiana* was observed on 5th instar larvae after 24 hr of treatment but there was a remarkable increase in PO level after 48 hrs. At higher concentration, after 48 hr of infection 3rd and 4th instar larvae showed significant decrease in PO activity. After72 hrs of infection all the treatments showed suppression in PO activity as compared to control. We would like to conclude that PO was an important key enzyme that triggered immune response in *S. litura* larvae against the invasion of *B. bassiana*.

Keywords: *Beauveria bassiana*, *Spodoptera litura*, Phenoloxidase enzyme, Immune response.

1. Introduction

Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae), is a polyphagus sporadic pest[1]. It is commonly known as tobacco caterpillar, a most devastating pest of tobacco, cotton, groundnut, jute, lucerne, maize, rice, soybeans, tea, cauliflower, cabbage and castor [2]. *S. litura* is widely distributed throughout the world and resulted huge crop losses in cruciferous crops [3]. However, insects possess a highly dynamic and efficient immune system that varies with age, food quality and reproductive traits across and within individuals [4].It is known to develop resistance against all major group of insecticides used for its control [5] and demands sustainable alternative strategies.

Unlike vertebrates, they do not rely on true antibodies and lack adaptive immune response [6]. With the result, they depend totally on innate immune system comprises of three types of defence: physical, cellular and humoral [7]. Physical defence mechanism in insects triggered by the presence of cuticle act as a barrier to invading microorganisms. When cuticular defence mechanism fails to overcome the invading microorganisms, initiating cellular defence regulated via interaction of different hemocytes [8].

The hemocyte cells circulate freely in the hemolymph and able to bind pathogens causing phagocytosis and encapsulation of foreign bodies. Haemocytes respond to microbial infection by trapping foreign bodies within aggregates of many haemocytes called nodules [9]. Insects also exhibit extracellular humoral compounds in response to immune challenges. Phenoloxidase (PO) is the one of the key and ubiquitous enzyme activated in the cuticle or the haemolymph of many invertebrates in response to immune challenge or wounding and is activated via prophenoloxidase (PPO) cascade [10]. In defence process, peptidoglycan recognition protein or β-1, 3-glucan recognition protein binds to its respective elicitor and results in production of inactive zymogen prophenoloxidase (proPO) and its conversion into the enzyme PO [11]. PO is involved in the conversion of phenols to quinones and the subsequent production of melanin.

The production of melanin is likely to cut off the pathogen's supply of oxygen and nutrients, eventually killing it [12]. Further, PO also performs various function as it is involved in sclerotization and tanning of damaged cuticular tissue. The second most important component is lysozyme synthesized in the fat body of lepidopterans, which exhibits antibacterial as well as antifungal activities [13].

Biological control seems to be best solution within IPM program to overcome insecticide resistant population of *S. litura*[14]. Previous studies indicate that entomopathogenic fungi (EPF) particularly *Beauveria bassiana*, *Metarhizium anisopliae, Verticillium lecanii,* has been exploited for several agricultural pest controls [15]. *B. bassiana* (Balsamo) (Ascomycota: Hyphocreales) is an insect pathogenic fungi and found to be effective against *S. litura* [16]. It is a ubiquitously distributed and has broad host range towards a diverse number of insect species [17] belonging to Lepidoptera, Coleoptera, Hymenoptera, Diptera and Orthoptera[18]. As a result, *B. bassiana* is used as a promising biocontrol agent for insect control. Hung and Boucias [19] studied PO activity in haemolymph of *S. exigua* after injection of *B. bassiana* blastospores. During the course of infection, they [19] observed a significant change in PO activity in 6th larval instar. The topical application of *B. bassiana* (strain GHA) to adult mormon crickets, *Anabrus simplex* (Haldeman) significantly induced PO activity than uninfected controls [20]. However, a significant decrease in PO activity was reported by Glupov[21] after 24 hrs of treatment of *Galleria mellonella* (Linnaeus) larvae with 2.0×10^6 conidia/ml of *M. anisopliae*. Similarly, Gillespie [22] demonstrated suppression of enzymatic activity in *S. gregaria* when *M. anisopliae var acridium* was topically applied @7.5×10^4 conidia.

The objective of study was to investigate the effect of *B. bassiana* on the enzymatic activity of PO triggered immune responses of *S. litura* larvae and to address question that how different larval stages of *S. litura* have successfully survive the infection from injected conidia of *B. bassiana.*

2. Materials & Methods

2.1 Rearing of insect Culture

The egg masses of *S. litura* were collected from the cauliflower fields at Amritsar district of Punjab, India. The life cycle of *S. litura* was completed approximately in 25 days. After egg hatching, rearing was carried out in battery jars (15cm×10cm) on fresh castor (*Ricinus communis*) leaves were provided for larval feeding till the pupal stage under laboratory conditions (25±2 ºC and 65±70% RH).The jars were covered with muslin cloth fastened with rubber bands and cleaned daily to avoid any type of infection. The pupae were transferred to pupation jars containing 2-3 cm layer of moist sterilized sand covered with filter paper. On emergence, adults were transferred to oviposition jars having 2-3 cm layer of moist sterilized sand covered with filter paper to facilitate egg laying. The adults were fed with 10 % honey solution with multivitamins to increase rate of fecundity. On hatching, the larval rearing was carried out on artificial diet as recommended by Koul [23].The process of culture rearing was repeated and maintained throughout the experimental studies.

2.2 Fungal culture and preparation of spore suspension

The culture of *B. bassiana* (NBAII-Bb-5a) was procured from National Bureau of Agriculturally Important Insects (NBAII), Bangalore and maintained in the laboratory on potato dextrose agar (PDA) medium. Two-three weeks old fungal culture was used for biochemical assay to analyse Phenoloxidase (PO) enzyme activity in haemolymph. Spores were harvested by scrapping the surface of culturein 10 ml of sterilized water containing one drop of 0.01 percent Tween 80, result in homogeneous suspension was obtained. The spore suspension was then filtered through muslin cloth to remove mycelia. From this concentrated spore suspension, serial dilutions were made. Spore count was obtained with the help of haemocytometer. Four concentration i.e, 1.0×10^8, 2.0×10^7, 4.0×10^6 and 8.5×10^5 spores/ml were used for Enzyme assay.

2.3 Biochemical Studies

Biochemical studies were carried out to analyse phenoloxidase (PO) activity in the hemolymph of 3rd, 4th and 5th instar larvae. The larvae were treated with 1.0×10^8, 2.0×10^7, 4.0×10^6 and 8.5×10^5 spores/ml of *B. bassiana*. PO activity in haemolymph of respective challenged larvae was estimated after 24, 48 and 72 hrs of treatments. Three replications were taken for each treatment as well as control and the experiments were repeated twice.

2.3.1 Phenoloxidase (PO) assay

PO assay was estimated according to the method of Cotter and Wilson [24] with certain modifications. The hemolymph was collected from five larvae of each instar (3rd, 4th and 5th) treated with various concentration of *B. bassiana* was pooled into eppendorf tubes by piercing the prothoracic legs with a fine and sterile needle. From pooled hemolymph 8 was added to 400µl of PBS (Phosphate Buffer Saline) with pH=7.4. A 100µl sample of the hemolymph and PBS mixture was added to 100µl of 20mM L-DOPA.PO was monitored spectro-photometrically as the formation of dopachrome. The increase in absorbance was measured on a microplate reader 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 enzyme activity was calculated by using the following formula:

$$b = \frac{\Delta A \times V}{E \times d \times v \times \Delta t} \times d.f$$

Where;

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

2.4 Statistical Analysis

Experimental data for PO activity were analyzed using one way analysis of variance (ANOVA) and significance among the treatments was compared at $P\leq0.05$ using Tukey's test.

3. Results

The application of *B. bassiana* induces differential PO activity in treated larvae as compared to untreated (Table 1). In comparison among the means, different larval instars of *S. litura* treated with *B. bassiana* had greater PO activity than uninfected controls but none of the fungal treatments differed from one another (Fig 1). After 24 hrs of infection in 3rd instar larvae, all the concentrations resulted in significant increase in PO activity as compared to control. With increase in spore concentration the level of PO increased and

ranges from 0.62 to 0.17 U/µl/min with dose of1.0×10^8 to 8.5×10^5 spores/ml. Similarly, after 48 hr of treatment significant differences were observed in enzyme activity of treated and control larvae. The analysis of enzymatic activity after 72 hrs revealed significant decline at higher concentration (1.0×10^8 spores/ml) (Fig 1). However, no particular trend was observed after 72 hrs of infection. The 24 hr post treatment of 4th instar showed highest PO activity at 1.0×10^8 spores/ml which was significantly higher than other concentrations. As compared to control enzymatic activity was increased by 41% at highest concentration (8.5×10^5 spores/ml). After 48 hrs of treatment, the highest PO activity (0.75 U/µl/min) at 4.0×10^6 spores/ml followed by drastic decline was observed at higher concentrations. However, with the increase of infection time, the enzymatic activity decreased. In, 4th instar larvae, after 72 hrs of *B. bassiana* infection all the treatments showed suppression in PO activity as compared to control. Except for lowest concentration the decrease was statistically significant (P=0.34). No significant effect was observed on 5th instar larvae of *S. litura* after 24 hrs of treatment. But with the increase in time interval PO activity increased. The results clearly indicated that a significant difference was recorded between control and various concentrations except for lowest concentration at 48 hrs post treatment. The PO activity was approximately 52.2% higher @1.0×10^8 spores/ml than control. Similarly, the level of PO increased after 72 hr *B. bassiana* infection except for higher concentration. However, significant differences were recorded only at 2×10^7 spore/ml (Table 1).

4. Discussions

The results indicated that *S. litura* responded to *B. bassiana* infection with an increase in PO activity but subsequently decreased in all instars. Significant increase in PO level was observed @ 4.0×10^6- 2.0×10^7 spores/ml after 24 hrs of infection in 3rd and 4th instar larvae. The results showed that PO activity of 3rd instar was lowered than 4th and 5th instar both in fungal treated and control larvae of *S. litura* at different time intervals. At higher concentration, after 48 hrs of infection 3rd and 4th instar larvae showed significant decrease in PO activity. However, no significant effect of *B. bassiana* was observed on 5th instar larvae after 24 hr of treatment but there was a remarkable increase in PO level after 48 hr. surprisingly, with the development of the larvae PO levels in the hemolymph were found to be significantly increased. The defensive responses to fungal infection leads to elevated levels of PO in haemolymph and other enzyme cascade [22]. In addition, PO activity in *A. simplex* infected with *B. bassiana* was higher than uninfected controls as reported by Srygley and Jaronski [20]. *Metarrhizum* infection may results in declining haemolymph protein and phenoloxidase titres over the course of infection until the death of *Schistocerca gregaria* and *Locusta migratoria* [25] whereas *Beauverria* infection increases PO activity in grasshopper, *Melanoplus sanguinipes* and army cutworm, *S. exigua* as demonstrated by Hung and Boucias [19]. However, immune response of insects varies from species to species. Contrary to this, Barabas and Cytrynska [26] revealed that PO activity in *G. mellonella* larvae was much higher when challenged with *Aspergilus oryzae* than control ones. Our results were in conformity with findings, Dorrah [27] who observed an increase in PO activity after 1 hr that gradually reached to a maximum at 24 hr post infection and then decreased again till it reaches the baseline after 72 hrs in fleshfly, *Parasarcophaga hertipes* (Wiedeman) which breeds in carrions that were densely infected with bacteria. Current study showed that PO levels declined with the increase in duration of *B. bassiana* infection. However, Zibaee and Bandani [28] documented decrease in PO activity with the increase in concentration in the adults, *Eurygaster intergriceps* (Puton) injected with 1µl *B. bassiana*. The rate of hemolymph PO activity was significantly diminished in several species of Lepidopteran larvae during the parasitism by ecto and endo-parasitoids [29]. Significant inhibition of PO activity at 48 hr post parasitization with *Campoletis chlorideae* (Uchida) in *H. armigera* was observed in 2nd and 3rd instar larvae [30].

In present studies, death of the larvae started after 3 days of treatment consequently the PO levels in *Spodoptera litura* dramatically declined. This indicated suppression of insect immune response. According to previous findings fungal proteins or toxic metabolites result in suppression of immune system and leading to death of host. The depression of PO activity in hemolymph of larvae by mycosis possibly results from the depletion of requisite enzyme activators or due to increase of protease inhibitors amount that may decrease or fully suppress the activity of serine protease. The proteases work in stepwise process of activation of proPhenoloxidase (proPO) system [31]. Destruxins prevent PO production by locust hemocytes [32] probably by destroying the cells that produce proPO [33]. Shi and Sun [34] revealed that PO activity significantly increased in red turpentine beetle, *Dendroctonus valens* (LeConte) from 2nd instar larvae to final instar and then decreased in pupae as it occurs in the form of proenzyme. PO activity in forager ant *Cataglyphis velox* (Forster) was higher as compared with intra-nidal workers [35]. Schmid [36] also reported an increase in PO activity as adult bees, *Apis mellifera* (Linnaeus) develop from nurses to foragers. On the contrary, PO activity decreased with age in bumblebee, *Bombus terrestris* workers [37].

Table 1: Effect of *B. bassiana* on Phenoloxidase activity of different larval instars of S. litura.

Conc. (spores/ml)	Phenoloxidase activity (U/µl/min) (Mean ± S.E.)								
	3rd Instar			4th Instar			5th Instar		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Control	0.08^{a} ±0.04	0.12^{a} ±0.01	0.44^{a} ±0.03	0.51^{a} ±0.006	0.52^{a} ±0.01	0.56^{a} ±0.01	0.41±0.04	0.44^{a} ±0.03	0.59^{a} ±0.02
8.5×10^5	0.17^{ab} ±0.01	0.29^{b} ±0.03	0.43^{b} ±0.03	0.52^{ab} ±0.01	0.54^{a} ±0.02	0.55^{a} ±0.02	0.40±0.03	0.49^{a} ±0.04	0.61^{ab} ±0.01
4.0×10^6	0.26^{b} ±0.03	0.44^{b} ±0.02	0.41^{ab} ±0.02	0.59^{ab} ±0.02	0.75^{b} ±0.01	0.47^{bc} ±0.02	0.50±0.01	0.59^{b} ±0.02	0.63^{ab} ±0.01
2.0×10^7	0.47^{c} ±0.04	0.40^{b} ±0.02	0.30^{b} ±0.03	0.62^{b} ±0.03	0.69^{c} ±0.01	0.41^{bc} ±0.03	0.54±0.09	0.61^{b} ±0.01	0.66^{b} ±0.01
1.0×10^8	0.62^{d} ±0.05	0.33^{b} ±0.06	0.018^{c} ±0.02	0.72^{c} ±0.01	0.59^{ca} ±0.05	0.34^{c} ±0.04	0.61±0.05	0.67^{c} ±0.007	0.58^{a} ±0.01
F-value	57.76**	15.05**	17.85**	17.09**	10.77**	13.42**	N.S	13.80**	3.36*

**Significant at 1%,* Significant at 5%, N.S (Non-significant)
Values followed by different letters are significantly different at P≤0.05

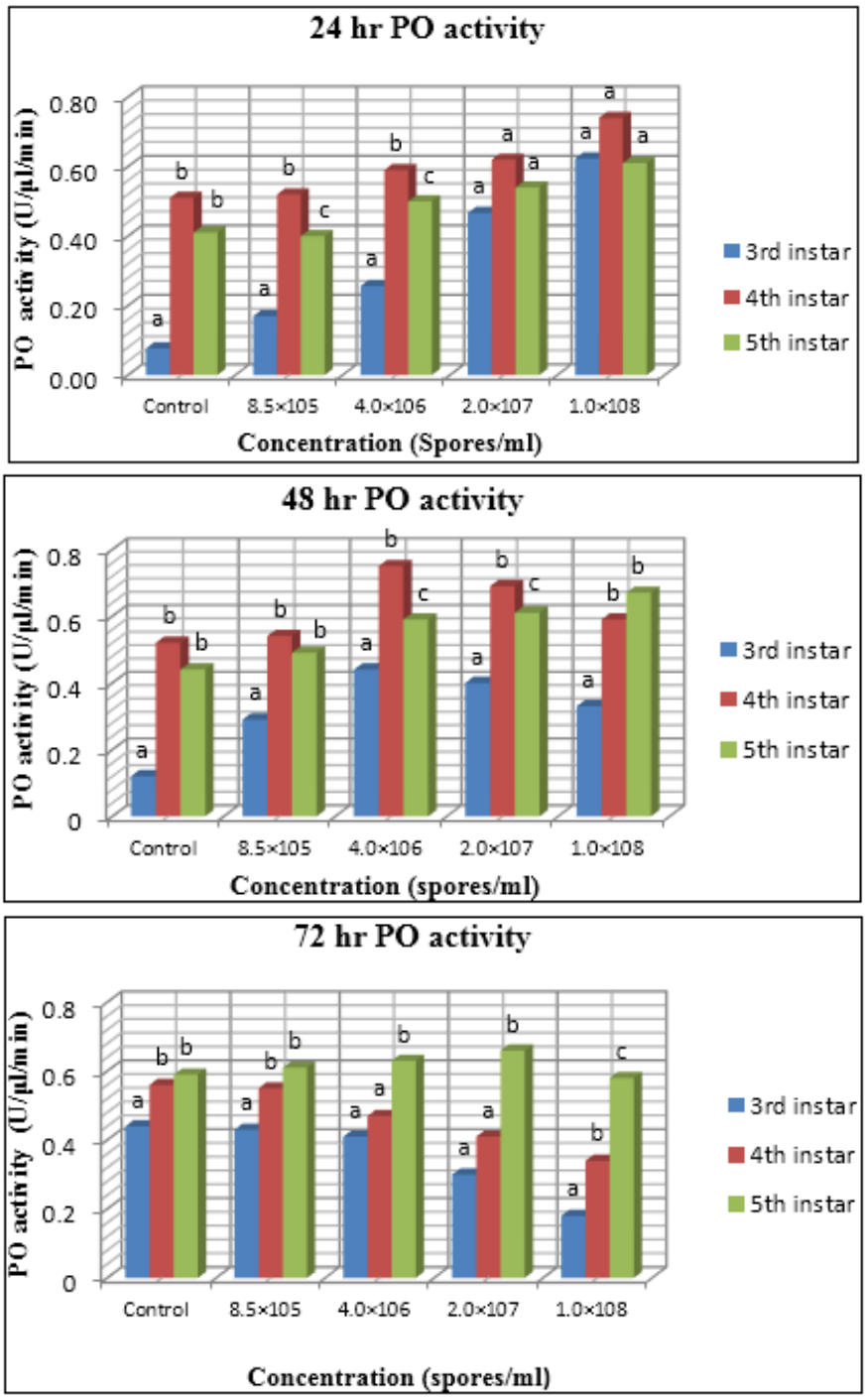

Fig 1: PO activity of different larval instars of Spodoptera litura relative to the dose of B. bassiana applied. Bars with different letters is significantly different from each other taking the level of significance (P≤0.05) as given in Table 1.

5. Conclusions

The present study suggested that insect immune response to fungal infection was not constant or EPF caused significant variation in immune response modulated by PO enzyme in different larval stages of *S. litura*. However, PO levels declined with augment of *B. bassiana* infection. As a result, *B. bassiana* was found to be highly virulent which suppressed the immune response of the host later and resulted in death of the host. Variations in the susceptibility of insect species to fungal invasion may result from several factors, including differences in the structure and composition of exoskeleton, the presence and activity of antifungal protein in hemolymph as well as efficiency of cellular and humoral defense reactions. It is hoped that present study would help to investigate the mechanism underlying in regulation of PO levels triggering immune response and to shed further light on intriguing aspects of insect immunity.

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