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RESEARCH ARTICLE

EVALUATION OF THE EFFECT ON *PRO*PHENOLOXIDASE SYSTEM IN *SPODOPTERA LITURA* (FABRICIUS) EXPOSED TO *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE*

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ABSTRACT

Phenoloxidase activity was detected in the serum of *Spodoptera litura*, which is present as an inactive proenzyme, *pro*Phenoloxidase. Substrate screening was done using different phenolic substrates, in which the PO was high in L-DOPA at 458nm. *S. litura* was treated with fungal pathogens *Beauveria bassiana* and *Metarhizium anisopliae*, Compared to untreated *S. litura*, the *pro*Phenoloxidase activity was high in fungal infected *S. litura*. The fungal pathogen also induced pupal and adult deformities. In both untreated and treated *S. litura*, on addition of activators effectively triggers the *pro*PO activation. The addition of protease inhibitor, chelators and eicosonaids constantly inhibited the activation of *pro*PO, in which PTU, DTT and DEX shows effective inhibition of *pro*PO to PO, EDTA shows little effect even in higher concentration. Malformed pupae and emergence of adults with crippled wings strongly suggest the efficacy of the selected fungi as biocontrol agent.

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INTRODUCTION

Invertebrates are distributed almost in all kinds of the habitat and their dispersal/survival depends on their successful immune responses primarily via innate immune mechanisms, against different microorganisms and parasites. These possess open circulating system, hence holds strong recognition molecule which includes agglutinin, antimicrobial peptides, β-1, 3-glucan binding proteins and Phenoloxidase (PO) activating system. *ProPhenoloxidase* (*pro*PO) activating system is induced on encountering non-selfmolecules, which in turn activates other defence processes (Söderhäll & Cerenius, 1998) such as phagocytosis, melanisation (i.e. synthesis and deposition of melanin around pathogen), synthesis of extracellular matrix, reactive intermediates of oxygen and nitrogen, pro-apoptotic molecules, pro-inflammatory cytokines and encapsulation (González-Santoyo & Lórdobe- Aguilar, 2011). Phenoloxidase is a multifunctional enzyme, distributed in microbes, plants and animals (Ratcliffe *et al.*, 1984), otherwise referred as tyrosinase, catalyzes the hydroxylation of monophenols and oxidizes the resulting *o*-diphenols to quinone intermediates (Sugumaran, 1990). In insects, PO is present in the haemolymph as an inactive proenzyme called *pro*Phenoloxidase (*pro*PO), which could be activated by exogenous proteases and non-self molecules (Ratcliffe *et al.*, 1984; Leonard *et al.*, 1985 a, b; Fisher & Brady, 1983; Dularay & Lackie, 1985; Iwama & Ashida, 1986; Brookman *et al.*, 1989 a, b; Rowley & Rahmet-Alla, 1990). However the enzyme is detected in the serum, plasma as well as haemocytes of some insects (Ashida, 1981; Sugumaran *et al.*, 1985; Saul *et al.*, 1987; Saul & Sugumaran, 1988; Brehélin *et al.*, 1989). The PO activity is assessed in many of the arthropod species using different phenolic substrates in order to understand the physiological and immunological response to design the effective biocontrol agents. *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) is one of the most destructive pest of cauliflower, groundnut, cotton, tomato, cabbage and other cruciferous crops (Anand *et. al.*, 2009). It is commonly referred as tropical armyworm, cluster caterpillar, rice cutworm, and cotton leaf worm or tobacco cutworm. CAB International 2002, referred these pest as polyphytophagous crop pest distributed throughout the south and eastern world. The larvae defoliates many important crops which leads to economic loss as well as difficulty in attaining food sufficiency. Hence, if not controlled timely, it may result in huge crop loss ranging from 25-100% in the various parts of India (Ahmad *et. al.*, 2005). Chemical pesticides are widely used as they react rapidly and easy to use. Besides these pesticide mismanagement like broad spectrum application of the insecticides at high dosage or repeated usage of the same

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active ingredients can lead to the development of resistance in many insects including *S. litura* and also contaminate the environment and the agricultural products (Ramakrishnan *et al*., 1983; Wu *et al.,* 1995; Kranthi *et al.,* 2002; Ahmad *et al.,* 2005). An alternative to the chemicals is the bio insecticides. Biological control of the insect pests using microorganisms is highly specific, relatively lower cost and easily degradable hence leaves no residues in the ecosystem (Castillo *et al.,* 2000). Recent studies has suggested that among microorganisms, fungi play a significant role in controlling various crop pests, mainly Lepidopteran pest. They are also tested against Orthopteran (Anggraeni *et al.,* 2011), Coleopteran (Gindin *et al.,* 2006) and Acaricidans (Ren *et al.,* 2012). Few of the commonly known entamopathogens are *Beauveria bassiana, Metarhizium anisopliae, Verticillum lecanii,* etc.

The study is designed to characterize PO in the serum of circulating hemolymph with the activators and inhibitors of the healthy and fungal pathogen exposed larvae of *S. litura*. Further the development, oviposition and hatachability are determined under laboratory conditions.

MATERIALS AND METHODS

Animal maintenance

Larval instars of the tropical armyworm, *S. litura* were collected from the agricultural fields in and around Gobichettipalayam, Tamil Nadu, India. Larvae were transferred to the laboratory in the ventilated plastic boxes (15 × 10 cm). Different larval instars were maintained in the separate plastic boxes at room temperature (25 ± 2º C) with photoperiod of 16:8 (Light: Dark) hours. The larvae were fed *ab libitum* with fresh leaves of the *Ricinus communis* and maintained under the sterile laboratory condition. II instar were infested with the fungal pathogens whilst larvae without exposure to fungi served as control.

Fungal culture and preparation of the spore suspension

Fungal culture *Beauveria bassiana* and *Metarhizium anisopliae* were obtained from Tamil Nadu Agricultural University, Coimbatore and were cultured in the potato dextrose broth and 10 days old culture was used for collecting the spores. The spores were counted using the haemocytometer and a concentration of 2×10^8 spores/mL were used for experimental analyses. The fungal spore suspension were applied separately on the *R. communis* leaves (1 mL/leaf) using a sterile paint brush. The leaves were held in the sterile conditions till the full dryness. The second instar of *S. litura* were fed with the fungal spores applied leaves for two consecutive days following which they were fed with untreated leaves. Both the fungal treated and untreated larvae were maintained separately and the larvae were observed each day for behavior and morphological changes. The V instar were used for the evaluation of the phenoloxidase activity.

Collection of serum

V instar control larvae (untreated conditions as well as) experimental (fungal treated) were immobilized on the ice and surface sterilized with 70% ethanol. Using a sterile scissors, the pro-leg was cut and the oozing hemolymph was collected in a pre-chilled sterile polypropylene eppendorf tube held on ice. Hemolymph was immediately centrifuged (2000 rpm, 10 min, 4˚C) and the resulting supernatant served as the serum.

Assay of phenolic substrates and wavelength determination

The suitable phenolic substrates were selected by enzyme-substrate reaction and the wavelength was determined by UV spectrum scanning. It was done by incubating 50 µl of serum from untreated V instar with 2 ml of phenolic substrate solutions (5mM *L*–DOPA, 1mM *L*–tyrosine, 1mM protocatechuic acid and 1mM pyrogallol) separately for 10 minutes at RT. Each reaction mixture was scanned using a spectrophotometer (Shimadzu (UV 2450), from 200 to 800 nm to determine the specific wavelength which read the chroma of enzyme-substrate reaction.

Assay of PO activity

PO activity in the serum of *S. litura* V instar was determined spectrophotometrically by recording the formation of dopachrome from 5mM *L*–DOPA at 458 nm. Unless specified, phenoloxidase activity was expressed in units.min^{-1}.mg protein^{-1} following Söderhäll and Smith (1983). One unit of enzyme activity is defined as the amount of enzyme that in 10 min gives an increase of 0.001 in absorbancy at 458 nm.

Effect of the activators on PO activity

Activation of *pro*PO into active PO was achieved by incubating samples from the untreated and fungal treated V instar with different activators such as exogenous proteases (trypsin and α-chymotrypsin), detergents (Triton X 100) and laminarin (a polymer of β -1, 3 glucan). For this, 50 µl of serum was separately pre-incubated with 50 µl of separate activators (1mg/1ml concentration). Controls consisted of substitution of activators with 50 µl of Tris-HCl buffer (250 mM, pH 7.2) incubated with 50 µl of serum. All these reaction mixtures were incubated at RT for 10 min. After incubation, 1 ml of 5mM L-DOPA was added to all the tubes, mixed well and incubated for 10 min. All the samples were read at 458 nm against a suitable reagent blank.

Effect of inhibitors on PO activity

The effect of the inhibitors on the serum sample was performed by incubating samples from the untreated and fungal treated V instar with the different inhibitors namely, phenylthiourea (PTU), dithiothreitol (DTT), dexamethasone (DEX) (1mg/1ml concentration) and ethylene-diamine-tetra-acetic acid (EDTA) (7mg/1ml concentration). For this, 50 µl of serum was separately pre-incubated with 50 µl of the above separate inhibitors. Controls consisted substitution of inhibitors with 50 µl of Tris-HCl buffer (250 mM, pH 7.2) incubated with 50 µl of serum. All these reaction mixtures were incubated at RT for 10 min. After incubation, 1 ml of 5mM L-DOPA was added to all the tubes, mixed well and incubated for 10 min. All the samples were read at 458 nm against a suitable reagent blank.

Protein estimation

Total protein concentration in the serum of the untreated and fungal treated V instar was determined by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

In this study, based on spectrum scan, irrespective of the phenolic substrates tested, 458 nm was the best to detect the PO activity in the *Spodoptera litura.* Results induced the maximum oxidation of the *L*-DOPA by the serum when compared to other phenolic substrates (Table 1). Based on these observations, further spectrophotometric analysis of the oxidation of the *L*-DOPA by serum of *S. litura* was performed.

Table 1. Absorption maxima of enzyme-substrate reaction at 458 nm

S.No	Phenolic Substrates	Optical Density
1.	L – DOPA	0.339
2.	L – Tyrosine	0.273
4.	Protocatechuic acid	0.187
5.	Pyrogallol	ND

ND: Not detectable

Serum showed detectable level of the PO activity using the *L*-DOPA as substrate. Upon challenging *S. litura* II instar with selected fungal pathogens (*Beauveria bassiana* and *Metarhizium anisopliae),* there was an increase in the level of PO in the serum when compared to the unchallenged larvae (Figure 1). The result suggest that the innate immune system of the larvae continuously enhanced to combat fungal infection. The effect of the *B. bassiana* isolates on the lepidopteran pests had shown varied percent mortality rate with varied isolates (Wraight *et al.,* 2010). The PO activity was found to be significantly enhanced in the serum of the larvae exposed to *B. bassiana and M. anisopliae* (Figure 1), suggesting that the PO present in the inactive state was activated on exposure to pathogens. Figure 2 indicates the effect of incubation of serum of unexposed larvae with serine proteases such as trypsin and α–chymotrypsin, wherein enhancement of PO activity was significantly ($p < 0.001$) higher compared to buffer control.

There was no significant enhancement with the Triton X 100, a non-ionic detergent. On the other hand, *pro*PO in the serum was induced by the laminarin. Enhancement of PO activity on the exposure to activators clearly demonstrated that PO exists as a proenzyme i.e. *pro*Phenoloxidase (*pro*PO) which can be activated by the activators. On incubation of the serum of fungal pathogens exposed larvae to various activators showed a significant elevation of *pro*PO levels with α–chymotrypsin (Figure 2). Irrespective of the serum tested and the activators used against *B. bassiana* infected larvae indicated only α–chymotrypsin as found to been efficient activators. This activation of *pro*PO in fungal pathogen exposed serum indicated that the *pro*PO is not completely induced by pathogens and it might require some specific active site for the recognition of the non-self. Preincubation of protease inhibitor or chelators, PTU, DTT and EDTA with serum of control larvae, showed a significant inhibition of PO (Figure 3). Among these inhibitors the PTU, a known inhibitor of PO, significantly ($p < 0.001$) reduced the oxidation of L – DOPA by PO presented in the serum. This significant inhibition of oxidation of L-DOPA by PTU, an activator of tyrosinase and melanin synthesis (Ratcliffe *et al*., 1984), strongly suggests that the observed activity was not due to the peroxidase (Chen *et. al*., 1995). On the other hand EDTA, a known chelator of cations did not inhibit the PO activity in *S. litura* similar to *S. exigua* indicating that this system did not need the Ca^{2+} ions for its activity (Hung & Bousias., 1996). EDTA is known to be a potent inhibitor in other insect systems and are considered to prevent Ca^{2+} mediated serine proteases activation responsible for the *pro*PO to PO cascade (Ashida and Söderhäll, 1984; Leonard *et al*., 1985 a, b; Dunphy, 1991). The effect of inhibitors and chelator on PO activity in serum of Vinstar of different ffungal treated *S. litura* highlights the maximum inhibitory potential of the PTU in serum (Figure 3).

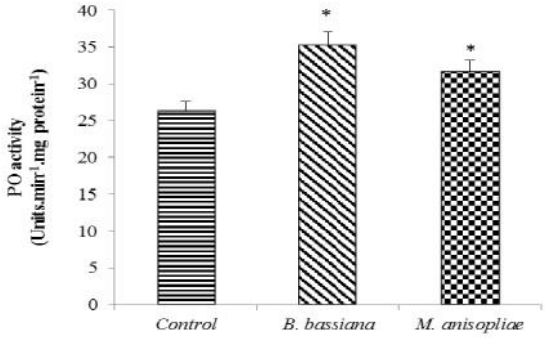

Figure 1. Activation of *pro*PO in the serum of control and experimental V instar *Spodoptera litura*. Values are based on mean±SD of six determinations using the samples from different preparations (*$p < 0.05$)

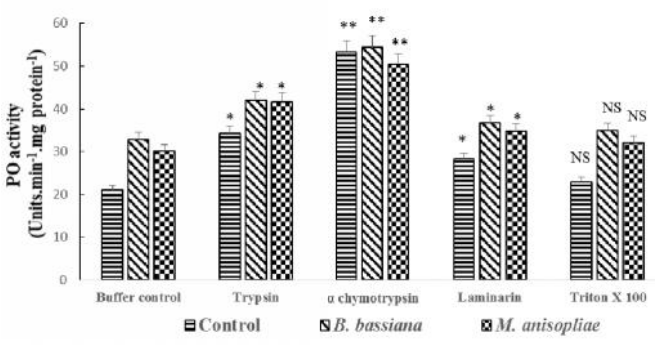

Figure 2. Effect of exogenous proteases, detergents and non-self molecule on PO activity in serum of control and experimental V instar *Spodoptera litura*. Values are based on mean±SD of six determinations using the samples from different preparations ($p<0.001$; *$p<0.05$; NS : not significant)**

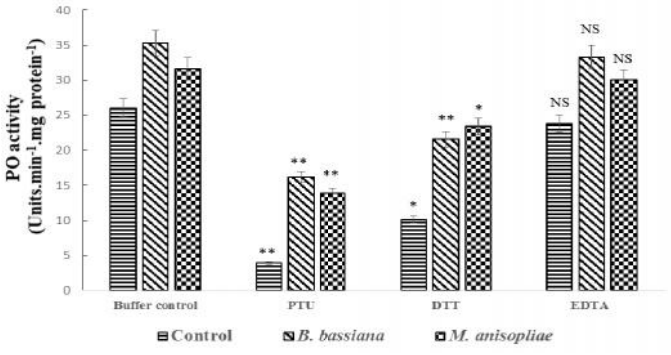

Figure 3. Effect of protease inhibitor and chelator on phenoloxidase activity in serum of control and experimental V instar *Spodoptera litura*. Values are based on mean ± SD of six determinations using th samples from different preparations ($p<0.001$; *$p<0.05$; NS : not significant)**

The effect of dexamethasone, a specific phospholipase A_2 (PLA_2) inhibitor on *pro*PO level of the serum of untreated V instar, showed the varied inhibition levels of PO (Table 2).

Table 2. Levels of the *pro*PO against Dexamethasone in the serum of V instar of untreated and fungal treated *Spodoptera litura*

Inhibitors	Control	*B. bassiana*	*M. anisopliae*
Buffer control	26.0 ± 0.4	35.3 ± 0.2	31.6 ± 0
DMSO control	14.5 ± 0.4	32.9 ± 0.2	24.6 ± 0.2
Dexamethasone	11.0 ± 0.3	24.8 ± 0.2	12.8 ± 0.2

(a) Normal pupa (b) Normal adult

(c) *B. bassiana* infected (d) *M. anisopliae* infected

Figure 4. Pupal and adult deformities in *Spodoptera litura* on treatment with *Beauveria bassiana* and *Metarhizium anisopliae*

(a) (b) (c)

Figure 5. (a) Control (b) Culture of *Beauveria bassiana* treated larval hemolymph (c) Culture of *Metarhizium anisopliae* treated larval hemolymph

Similarly, the effect of dexamethasone on *pro*PO level in serum of V instar of fungal treated *S. litura* was also in accordance with the untreated larvae and it highlights the maximum inhibitory in a (24.8 ± 0.2 in *B. bassiana* 12.8 ± 0.2 in *M. anisopliae*) compared to the DMSO control (32.9 ± 0.2 in *B. bassiana*>24.6 ± 0.2 in *M. anisopliae*; Table 2). These observations suggests that PO along with PLA_2 may involve in clearance of non-self.

Morphological changes

The development of larvae which was continuously monitored for any alterations in the external morphology. It was observed that the pupae were malformed and those which emerged as adults had crippled wings in both *B. bassiana* and *M. anisopliae* treated larvae, rarely those transformed to adults had a contradiction in malformed wings (Figure 4). These observations of malformed developmental stages were also observed with the *B. bassiana* treated *S. litura*, suggested the potency of fugal pathogens. Thus, most of the pupae were not emerged into adult. On culturing the hemolymph of the fungus exposed V instar on PDA plate, correspondingly pure culture of fungal growth was observed in the all the plates (Figure 5) thus it confirms the possible organism for deformity of the larvae.

Conclusion

The current study hypothesized to control the larval stages of *S. litura* using fungal pathogens and further analyses of modulation levels in regard to phenoloxidase (PO) / *pro*Phenoloxidase (*pro*PO) in order to understand the role of humoral immune molecules in polyphagous insects. PO activity is detected in the serum of hemolymph of *Spodoptera litura*, which exists as a proenzyme (*pro*PO) in the serum, where it can be activated by the exogenous proteases,

α-chymotrypsin and laminarin (a polymer of β- 1, 3 glucan) in its active form, and suggesting that this enzyme is involved in the defensive function of the larvae and it plays an effective role for its survival. The oxidation of PO by L-DOPA, in the serum as an evidenced in the findings and suggests that the hemocytes are the repository for PO. The study also confirms the role of innate immune system as an evidenced through increased PO activity after fungal infection which can be due to the activation of recognition sites when compared to the healthy larvae. Further, incubation of the serum with the activators such as trypsin, α- chymotrypsin, Triton X 100 and laminarin proves the existence of PO as a proenzyme, whereas the serum of fungal treated larvae reveales that the induction of *pro*PO is not completely by the pathogens rather it requires some specific active sites for these activators. On the other hand, pre-incubation with the protease inhibitors such as PTU, DTT and EDTA with the serum, PTU is found as the potent inhibitor suggesting the absence of peroxidase mediated activity. EDTA non-inhibition suggest that the system does not solely rely on Ca^{2+} ions for *pro*PO to PO cascade establishment. The evaluation of the PO activity with the dexamethasome (PLA_2 inhibtor) showed a significant inhibitory potency in the serum of both the treated and untreated larvae, thus the suggesting PO along with PLA_2 can be involved in the clearance of non–self. Malformed pupae and emergence of the adults with the crippled wings strongly suggest the efficacy of the selected fungi as a biocontrol agent. These findings strongly recommends the use of selected fungi for *S. litura* control as a part of Integrated Pest Management (IPM) as attributed to its non-toxicity in both the humans and environment after confirmation with the down-regulation of PO genes to enhance formulation of potent fungal toxic molecule as a bio-control agent.

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