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Haemolymph Phenoloxidase Activity of Larval *Plodia Interpunctella* and *Galleria Mellonella* in Response to *Beauveria Bassiana* and *Pseudomonas Fluorescens*

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Abstract **- Phenoloxidase (PO) is an important component of immune system in invertebrates. In this study, PO activity was investigated in two pyralid species, *Plodia interpunctella* and *Galleria mellonella* treated with *Beauveria bassiana* and *Pseudomonas fluorescens*. PO activity was enhanced in response to two entomopathogens in both insects, but it was greater in *P. interpunctella* by almost two folds compared to *G. mellonella*. Furthermore, *B. bassiana* managed to induce the immune system two times faster than the *Pseudomonas* in *Plodia*. In contrast, *Galleria* PO activity response time was the same for both pathogens (12 h). Accordingly, it may be postulated that the induction of immune system- or its index genes, *i.e.*, prophenoloxidase, are different in *Galleria* and *Plodia* caterpillars. In *G. mellonella*, the response was quite the same to both pathogens suggesting that the insects take similar routes to withstand the attack, while *P. interpunctella* immune system activation seems to respond by different pathways.**

Keywords **– Phenoloxidase Activity, *Plodia Interpunctella, Galleria Mellonella, Beauveria Bassiana*, *Pseudomonas Fluorescens.***

I. INTRODUCTION

The innate immune system (IIS) of insects is comprised of cellular and humoral reactions (Lehane *et al.,* 2004; Iwanaga and Lee, 2005; Cheng *et al*., 2006; Hancock *et al*., 2006; Mak *et al.,* 2010; Mowlds *et al*., 2010). Analysis of the prophenoloxidases activation can be used as a measure to evaluate the launch of the humoral system and other related immune processes following recognition of invader pathogens by host receptors (Lehane *et al*., 2004; Feldhaar and Gross, 2008; Mak *et al*., 2010; Gonzalez-Santoyo and Cordoba-Aguilar, 2011; Ezzati-Tabrizi *et al*., 2013). Pattern-recognition receptors in insect haemolymph are activated by recognition of microbial cell wall components called pathogen-associated molecular patterns (or PAMPs). Lipopolysaccharides in Gram-negative bacteria, peptidoglycans and lipoteichoic acids in Gram-positive bacteria, and β-1,3-glucans in fungi are the main inducers of the insect's immune system (Soderhall and Cerenius, 1998; Cerenius and Soderhall, 2004). The binding of these elicitors (or PAMPs) to host pattern-recognition receptors in haemolymph generally leads to the activation of a serine protease family of enzymes. Serine proteases, as regulators of some invertebrate defense responses, play a significant role in the activation of prophenoloxidases (Hung and Boucias, 1996; Cerenius and Soderhall, 2004; Kanost *et al*., 2004; Feng *et al*., 2008), signal transduction pathways (Wang *et al*., 2011), and antimicrobial peptide production (Freitak *et al*., 2007; Wang *et al*., 2011). Throughout this process, inactive prophenoloxidase converts into its active form, phenoloxidase (Ashida and Brey, 1998; Cerenius and Soderhall, 2004; Liu *et al*., 2006; Feng *et al*., 2008). The phenoloxidase is involved in the oxidation of monophenols (e.g., tyrosine) to *O*-diphenols (e.g., dopa) and oxidation of *O*-diphenols to *O*-quinone (Rajagopal *et al*., 2005; Ericsson *et al*., 2009). Quinone changes to melanin, which is involved in microbial encapsulation (Cerenius and Soderhall, 2004; Kanost *et al*., 2004; Ling and Yu, 2005). Despite the molecular and physiological analysis of prophenoloxidase in many insects (Hall *et al*., 1995; Chase *et al*., 2000; Rajagopal *et al*., 2005; Feng *et al*., 2008; Wang *et al*., 2011), the full comprehension of its activation cascade has remained elusive (Hall *et al*., 1995; Jiang *et al*., 2003; Feng *et al*., 2008). In the present study, phenoloxidase activity has been compared in larvae of two pyralid species, the Indian meal moth, *Plodia interpunctella* and the greater wax moth, *Galleria mellonella* as target hosts in response to two microorganisms; *Beauveria bassiana* (as a crucial representative of entomopathogenic fungi) and *Pseudomonas fluorescens* (as a secondary entomopathogen).

II. MATERIALS AND METHODS

A. Chemicals

All chemicals were purchased from Merck (Germany), with the exception of L-DOPA obtained from S.D. Fine-Che Limited (India).

B. Insects

P. interpunctella and *G. mellonella* larvae were reared on artificial diets with detailed modifications containing wheat bran, Brewer's yeast, honey and glycerol (Vilcinskas and Wedde, 1997; Hartzer *et al*., 2005). Bees wax was included in *Galleria's* diet and were maintained at 27 ± 1 °C, 50 ± 10 % relative humidity and 12:12 h photoperiod for *Plodia* and lastly absolute darkness for *Galleria*. The last instar larvae of both species were used in the following subsequent experiments.

C. Microorganisms

Beauveria bassiana EUT105 was isolated by the *Galleria*-bait method and *Pseudomonas fluorescens* UTPF68 was isolated from soil. The fungus was cultured on SDAY (Sabouraud's dextrose agar with 1% yeast extract) at 25 °C and the bacterium was grown in YP (yeast peptone) at 30 °C for 16 hours.

D. Bioassay

For immune challenge test, 2 and 10 µl from each pathogen inoculums (10^6 agent ml-1), were injected separately into *Plodia* and *Galleria* larval haemocoel using a Hamilton syringe through left last proleg. Conidia/cells of micro organisms were killed before injection (30 minutes for bacterial cells at 98 °C and 20 minutes for fungal conidia at 121°C). For the control group, the same volume of sterile distilled water was used.

Insect haemolymph was collected at different intervals (6, 12, 24, 48 and 72 hours after injection). Larvae were anesthetized on ice and the surface sterilized with ethanol (70 °C). The haemolymph was collected by cutting the last proleg with a sterile needle and drawing haemolymph into a gel-loading micropipet tip containing anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA, 0.041 M citric acid, pH 4.5; Anggraeni and Ratcliffe, 1991). According to Hung and Boucias (1996), 20 µl of collected haemolymph was mixed with the same volume of phosphate buffer (pH 7) at 4°C. The samples were centrifuged (12,000 ×g) at 4 °C for 5 minutes. The supernatant (plasma) was collected and used immediately for PO activity. This assay was carried out with three replications for each treatment and the whole assay was repeated twice.

E. Phenoloxicase (PO) activiy assay

The phenoloxidase assays were carried out in 96-well plates. Each well contained 20 µl enzyme, 20 µl substrate (20 mM L-DOPA) and 80 µl phosphate buffer, pH 7.0. The absorbance was recorded by a microplate reader (BioTek, USA) over 45 minutes with a one minute interval at 490 nm. Pooled data of two-time repeats of the whole assay for PO activity were analyzed to determine possible significant differences between the control group and treatments via T-test ($P < 0.005$). The protein content of the samples was determined according to the method of Lowry et al. (1951) using bovine serum albumin (Bio-Rad) as the standard.

III. RESULTS AND DISCUSSION

The levels of prophenoloxidase activity in haemolymph of *P. interpunctella* and *G. mellonella* larvae challenged with cells of *P. fluorescens* and conidia of *B. bassiana* were shown in Tables 1 and 2, respectively. There is a difference in the increase rate of PO activity in each insect. In *Plodia* the enzyme activity is two-fold greater than *Galleria*. The T-test results for PO activities between microbial treatments and control treatments in both insects showed significant differences at each time point ($P<0.05$) (Tables 1 and 2). Higher PO activity in microbial treated haemolymph in contrast to the control treatment may be due to the interaction of microbial cell wall components, i.e., β-1, 3-glucan of *B. bassiana* conidia and lipopolysaccharid of *P. fluorescens* cells, with the immune system receptors (Hung and Boucias, 1996). Maximum enhancement of the enzyme activity level was recorded at 12 h post injection in *Plodia* (*Beauveria*-treated) and *Galleria* (*Pseudomonas*- and *Beauveria*-treated) larvae and 24 h for *Plodia* larvae treated with *Pseudomonas*. Comparisons between PO activities in *Plodia* (Table 1) and *Galleria* (Table 2) at each time-point showed a significant difference between bacterium-treated larvae only at 24 h. In contrast to fungus-treated larvae, significant differences were noted at 6, 24, 48 and 72 h. The results indicated that both insects have no considerable difference in immune response to bacterium at 6, 12, 48 and 72 h after injection, but in fungus-treated larvae, notable differences were observed at several time-points. In other words, the immune system of both insects similarly inhibited bacterium development yet in response to fungus acted differently. Since killed microorganisms were used in this experiment, the impact of metabolite or toxic compounds produced by fungal conidia (Cerenius *et al.,* 1990; Hung and Boucias, 1992; Dowd, 1999), and secreted antibiotics by the bacterium (Eleftherianos *et al.,* 2007) on phenoloxidase are most likely out of the picture. The reduction in enzyme activity after initial increase in the case of both pathogens in *Galleria* and bacterium in *Plodia* may be due to the inability of host receptors in recognition of killed pathogens in comparison with live ones. According to Rigby *et al.* (2002), pathogen resistance is dependent on both immune reactions and the ability of the immune system to recognize microbial invaders. The possible existence of protease inhibitors in insect haemolymph may be the reason for enzyme activity reduction or its disturbance (Gonzalez-Santoyo and Cordoba-Aguilar, 2011). Moreover, the degradation of microbial cell wall by various enzymes in host larvae may also be associated with the gradual decrease of Po activity.

In conclusion, a comparative evaluation of PO activities between *Plodia* and *Galleria* larvae revealed that fungal cell wall components in *Plodia* induced insect immune response (expression of prophenoloxidase genes) faster than the bacterium, however in *Galleria* both of the pathogens stimulated larval immune system at the same rate. Although it is fair to say that this study may indicate changes of enzyme activity between two different pathogens in two pyralid caterpillars, broader investigation is clearly needed to understand the specific molecular mechanisms correlated with increased enzyme level in these two organisms.

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Table 1: Phenoloxidase activity (Mean±SE) of haemolymph of *Plodia* larvae treated by *Pseudomonas fluorescens* and *Beauveria bassiana* at different point times

Time	Mean±SE^{a} (*Pseudomonas* -treated)	Mean±SE (Control)	T value^{b}	*P* value	Mean±SE (*Beauveria* -treated)	Mean±SE (Control)	T value ^{c}	*P* value
6h	12.85±1.9	1.66±0.1305	5.85	0.004	15.03±1.28	2.4667±0.1764	9.66	0.000
12h	13.72±2.25	4.243±0.997	4.68	0.009	21.7±3.5	5.6333±0.3756	4.56	0.011
24h	17.78±2.3	2.65±0.41	6.46	0.002	15.23±1.71	4.9667±0.1856	5.95	0.004
48h	13.02±1.09	5.33±0.292	6.79	0.002	17±2.02	4.4333±0.6692	5.90	0.004
72h	18.6±3	7.643±1.643	3.20	0.033	13.9±1.01	4.3667±0.1202	9.37	0.000

[a] Expressed basis on the specific activity (U mg^{-1})
[b] *T*-test results of enzyme activity between bacterium and control
[c] *T*-test results of enzyme activity between fungus and control

Table 2: Phenoloxidase activity (Mean±SE) of haemolymph of *Galleria* larvae treated by *Pseudomonas fluorescens* and *Beauveria bassiana* at different point times

Time	Mean±SE^{a} (*Pseudomonas* - treated)	Mean±SE (*Beauveria* - treated)	Mean±SE (Control)	T value^{b} (B and C)	*P* value (B and C)	T value ^{c} (F and C)	*P* value (F and C)
6h	9.4300±0.7679	8.2833±0.3680	2.2767±0.0698	9.27	0.000	16.03	0.000
12h	10.0000±0.2802	10.2267±0.4402	3.9367±0.2368	16.52	0.000	12.58	0.000
24h	8.8933±0.3595	7.1800±0.6061	4.3367±0.4373	8.04	0.001	3.80	0.019
48h	8.3900±0.4800	7.1967±0.3595	2.1833±0.0869	12.72	0.000	13.55	0.000
72h	5.2400±0.0854	4.8533±0.5613	2.8367±0.3633	6.43	0.002	3.01	0.039

[a] Expressed basis on the specific activity (U mg^{-1})
[b] *T*-test results of enzyme activity between bacterium and control
[c] *T*-test results of enzyme activity between fungus and control