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KINETICS OF PHENOLOXIDASE ACTIVITY IN SILKWORM *BOMBYX MORI* L. INFECTED WITH *BEAUVERIA BASSIANA* (BALS) VUILL.

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ABSTRACT

Highly significant elevation of phenoloxidase enzyme activity was recorded in the integument and reduction of phenoloxidase activity was observed in the midgut and silkgland of Beauveria bassiana inoculated silkworms. The elevation of the enzyme activity in the integument of inoculated silkworm could be attributed to initiation of melanization as a defense response during the course of invasion of fungal pathogen. Decreased phenoloxidase enzyme activity in the midgut and silkgland of the experimental animal might be due to immune-suppressive effect of fungal proteins or toxic metabolites and it may also be due to starvation induced by the fungal pathogen.

Keywords: Bombyx mori, Beauveria bassiana, Phenoloxidase, Integument, Midgut, Silkgland

I. INTRODUCTION

Silkworm *Bombyx mori* is susceptible to diseases owing to complete domestication and consequent loss of resistance. Among the many constraints that influence the success of cocoon production, the menace of disease is the prime one. The major diseases affecting mulberry silkworm *Bombyx mori* can be grouped under four major categories, namely the microsporidian disease, viral diseases, bacterial diseases and fungal diseases. Among the diseases of silkworm, white muscardine caused by *Beauveria bassiana* inflicts heavy economic loss to the sericulturists in India. Muscardine is one of the contagious diseases, which is causing loss to 5-50 percent in total loss due to diseases. Silkworm *Bombyx mori* is very much susceptible to diseases due to centuries of domestication. The remarkable evolutionary success of insects is partly due to their ability to build up a sophisticated, effective, and highly adaptable defense system against numerous microorganisms, including pathogenic fungi. Upon the invader's breaching of the physical barriers, the immediate onset of enzymatic cascades leads to localized blood clotting and melanization, involving the production of cytotoxic molecules. In this response, a pivotal role is played by the prophenoloxidase (proPO)- activating system. Phenoloxidase (PO) is a vital enzyme involved in a number of crucial processes, such as defense, wound healing, sclerotization, and pigmentation. Since active PO generates deleterious quinonoid compounds, most insects preserve this enzyme in the inactive form and activate it upon necessity. The innate immune system in insects is composed of a large variety of specific and non-specific responses i.e., phagocytosis, melanization (i.e., synthesis and deposition of melanin around the pathogen), synthesis of extracellular matrix, adhesion cells, recognition molecules, reactive intermediates of oxygen and nitrogen, proapoptoti molecules, pro-inflammatory cytokines, and antimicrobial peptides (Nappi and Vass (2001), Tunaz *et al* (2003), Bulet *et al* (2004), Nappi and Christensen 2005). These immune reactions are activated in response to the presence of foreign agents. One important element in such responses is the enzyme phenoloxidase (PO). Within this broad range of immune responses, an important immune component used by arthropods is melanogenesis (Asada *et al* (1999), Eleftherianos and Revenis (2011), Amparyup *et al* (2009). Enzymatic and non-enzymatic reactions play an important role in melanogenesis. Phenoloxidase produces indole groups, which are subsequently polymerized to melanin. Melanin is a brown–black pigment that inhibits entomopathogenic

bacterial and fungal enzymatic activity by encapsulation has been observed in Lepidoptera (Jiang *et al* 1998). Melanogenesis is responsible for encapsulating multicellular pathogens, repairing tissues, and defending against other pathogens such as bacteria, fungi, and even viral agents (Boman (1986), Ashida and Brey (1997), Nappi and Christensen (2005). Melanization is an easily observed defense reaction in invertebrates that is initiated by a proteolytic cascade that terminates with cleavage of prophenoloxidase (proPO) to phenoloxidase (PO). PO is a vital enzyme involved in a number of crucial processes, such as defense, wound healing, sclerotization and pigmentation.

II. MATERIALS AND METHODS

PM × CSR2 silkworm strain was selected for the investigation. Silkworms are reared in the laboratory under optimum conditions as suggested by Dandin *et al* (2003). Immediately after fourth moult the heatlhy larvae were selected from the rearing stock and grouped into two sets. Each group consists of 4 replications with 100 larvae for each group. One set of larvae was treated with fungal spore suspension with sub lethal concentration (3.25 x 10^6 spores/ ml @ 50 ml/100 worms) and another set of larvae were treated with double distilled water and used as control. Every day, silkworms from both the sets were randomly selected from 1st day to 7th day of 5th instar silkworms and dissected in physiological saline solution and collected the three tissues viz., integument, midgut and silkgland for phenoloxidase activity. Phenoloxidase activity was determined by the method of Fric and Fuchs (1970).

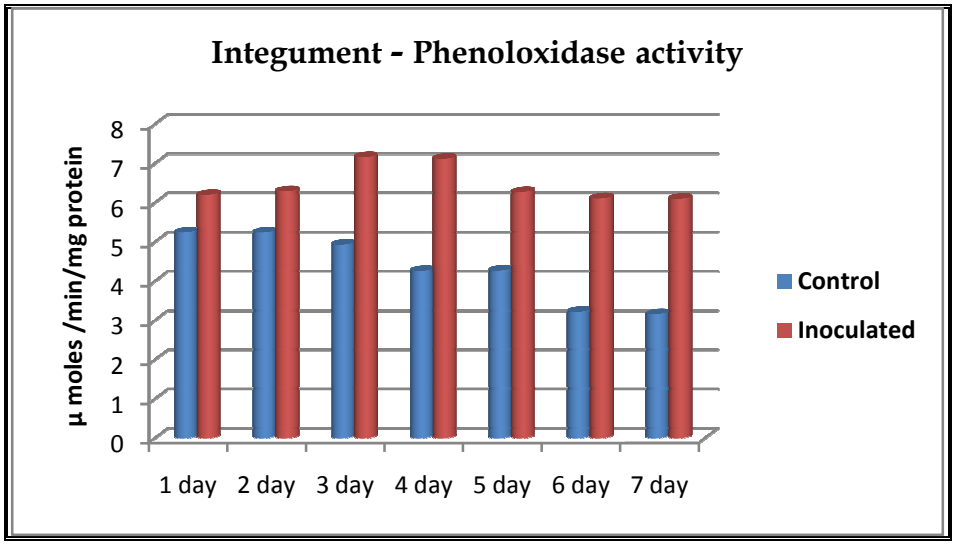
III. RESULTS AND DISCUSSION

The results of phenoloxidase (PO) enzyme activity in the integument of 5th instar silkworm inoculated with *Beauveria bassiana* are presented in Table -1 and Graph -1. Highly significant elevation of phenoloxidase enzyme activity was recorded in *Beauveria bassiana* inoculated silkworms (6.20 to 6.10 µ moles/mg protein/min) than the control (5.24 to 3.16 µ moles /mg protein/min) from the day of inoculation to the end of the instar. Table-2 and Graph-2 show the results of the phenoloxidase activity in the midgut of 5th instar silkworm *Bombyx mori* inoculated with fungal pathogen *Beauveria bassiana.* The decreased trend of phenoloxidase activity was observed in the midgut of both inoculated (5.29 to 1.88 µ moles/mg protein/min) and control (5.92 to 3.24 µ moles /mg protein/min) samples from the 1st to 7th day of 5th instar silkworm. No significant variation was noticed on the day of inoculation compared healthy silkworms then significant reduction of phenoloxidase activity was observed from 2nd day of the inoculation to end of the instar (4.90 to 1.88 µmoles /mg protein/min) with reference to control (5.15 to 3.24 µmoles /mg protein/min). Table -3 and Graph -3 show the results of phenoloxidase activity in the silkgland of *Beauveria bassiana* infected 5th instar silkworm compared to control. Decreased trend of phenoloxidase activity was recorded in silkgland of both inoculated (6.30 to 2.01 µ moles/mg protein/min) and control (6.29 to 3. 24 µ moles/mg protein/min) from the 1st to 7rd day of the instar. No significant variation was noticed in the phenoloxidase activity on the day of inoculation with fungal pathogen *Beauveria bassiana* with reference to control, then significant reduction of phenoloxidase activity was observed in treated silkworms from the 2nd day to till the end of the instar (5.95 to 2.01 µ moles/mg protein/min) with reference to control (5.76 to 3.24 µ moles/mg protein/min).

Table – 1 Day to day variations in phenoloxidase activity (µ moles /min/mg protein) in the integument of 5th instar silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* compared to control

Treatments	Days of 5th instar						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Control	5.24 ±0.02	5.24 ±0.04	4.93 ±0.02	4.26 ±0.05	4.27 ±0.01	3.22 ±0.04	3.16 ±0.05
Inoculated	6.20 ±0.04 ****	6.29 ±0.01 ****	7.16 ±0.02 ****	7.12 ±0.01 ****	6.27 ±0.06 ****	6.11 ±0.01 ****	6.10 ±0.01 ****

Mean±Standard Deviation; NS = Not Significant; *P<=0.05, **P<=0.02, ***P<=0.01, ****P<=0.001

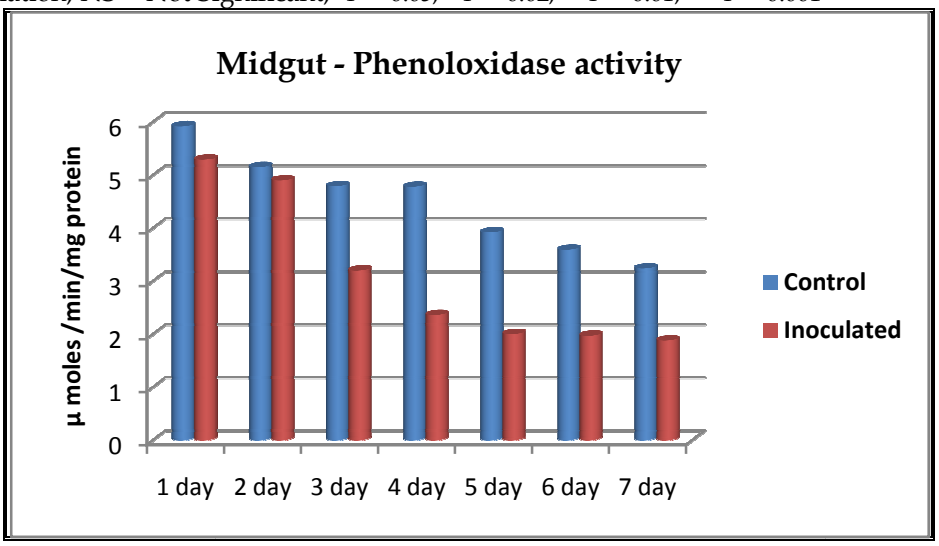


Graph – 1 Histogram showing the day to day variations in phenoloxidase activity (µ moles /min/mg protein) in the integument of 5th instar silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* compared to control

Table – 2 Day to day variations in phenoloxidase activity (µ moles /min/mg protein) in the midgut of 5th instar silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* compared to control

Treatments	Days of 5th instar						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Control	5.92 ±0.01	5.15 ±0.01	4.79 ±0.01	4.78 ±0.03	3.92 ±0.02	3.59 ±0.02	3.24 ±0.04
Inoculated	5.29 ±0.02 NS	4.90 ±0.01 ***	3.20 ±0.03 ****	2.36 ±0.23 ****	2.00 ±0.01 ****	1.97 ±0.03 ****	1.88 ±0.02 ****

Mean±Standard Deviation; NS = Not Significant; *P<=0.05, **P<=0.02, ***P<=0.01, ****P<=0.001

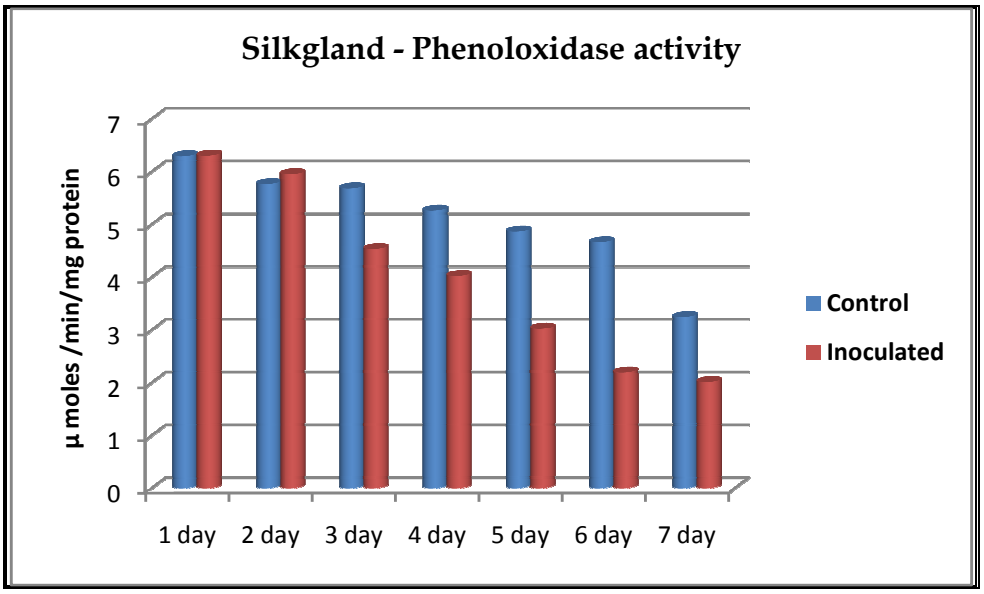


Graph – 2 Histogram showing the day to day variations in phenoloxidase activity (µ moles /min/mg protein) in the midgut of 5th instar silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* compared to control

Table – 3 Day to day variations in phenoloxidase activity (µ moles /min/mg protein) in the silkgland of 5th instar silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* compared to control

Treatments	Days of 5th instar						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Control	6.29 ±0.05	5.76 ±0.01	5.68 ±0.02	5.26 ±0.06	4.86 ±0.05	4.66 ±0.05	3.24 ±0.03
Inoculated	6.30 ±0.03 NS	5.95 ±0.04 ***	4.53 ±0.04 ****	4.02 ±0.03 ****	3.02 ±0.03 ****	2.19 ±0.03 ****	2.01 ±0.01 ****

Mean±Standard Deviation; NS = Not Significant; *P<=0.05, **P<=0.02, ***P<=0.01, ****P<=0.001



Graph – 3 Histogram showing the day to day variations in phenoloxidase activity (µ moles /min/mg protein) in the silkgland of 5th instar silkworm *Bombyx mori* during the progress of fungal pathogen *Beauveria bassiana* compared to control

Significant elevation of phenoloxidase activity was observed in the integument of experimental silkworms compared to control. The elevation could be attributed to initiation of melanization as a defense response in the process of invasion of fungal pathogen. Similar observations were made by several workers. Gillespie *et al* (2000) observed enhanced levels of phenoloxidase enzyme activity in response to fungal infection. Brey *et al* (1993) and Lee *et al* (1994) reported that integument can actively respond to minor injury in the presence of microbial cell wall components (lipopolysaccharide and peptidoglycan) by de novo synthesis of cecropin antibacterial peptides and relaying them to the zone of cuticular aggression. In insects common defense response is cuticular melanization. This response can be elicited by a mechanical scratch or by microbial invasion. This defense response is known to play a role in the sequestration of fungal pathogens as they attempt to penetrate the cuticle (Golkar *et al* 1993). Lai-Fook (1966) and Barrett (1984) suggested that cuticular melanization resulted from injury by the activation of a pro-phenoloxidase by a proteinaceous activator. It is well known that melanin is synthesized from phenolic substances by the action of phenoloxidase (Mason *et al* 1955).

Decreased phenoloxidase enzyme activity was recorded in the midgut and silkgland of the experimental animal this might be due to immune-suppressive effect of fungal proteins or toxic

metabolites and it may also be due to starvation induced by the fungal pathogen. As the cost of production and maintenance of the PO system, including the activation of proPO is likely to be high for two reasons. First, the main compound of the proPO-activating system – tyrosine – is obtained from phenylalanine, which can only be obtained from ingested food (Chapman 1998). Secondly, melanin a final product of proPO - activating system, is a nitrogen-rich compound, which may require substantial nitrogen or protein investment for its synthesis (Blois 1978 and Lee *et al* 2008). Thus, production and maintenance of the proPO-activating system is dietary-dependent.

Rajitha and Savithri (2013) observed significant enhancement of phenoloxidase (PO) enzyme activity in the haemolymph of 5th instar silkworm up to 4th day of the *Beauveria bassiana* inoculated worms then the enzyme activity was declined significantly in the rest of the 5th instar compared to control. Initial enhancement of enzyme activity may be due to the consequence of the invasion of fungal pathogen into the host haemolymph. In lepidopterans, proPO mostly appears in haemolymph and in other groups such as *Locusts* and cockroaches, proPO stored in haemocytes until a pathogen induces its release (Brehe´lin *et al,* 1989 and Durrant *et al* 1993). PO-generated quinones may serve as toxic metabolites that might be harmful to the intruders (Ashida and Yamazaki 1990, Kopacek and Sugumaran 1998). Decreased enzyme activity in the later stage of infection might be due to suppression of host enzyme activity by releasing inhibitor factors by the invading fungal pathogen. Parasitoids are able to suppress the phenoloxidase system through the inhibition of protease activity, blocking of pattern-recognizing proteins and destruction of immunocompetent cells (Vinson 1990, Brehelin 1990 and Shelby *et al* 2000).

PO cascade is a key component of insect immunity and is undoubtedly a major player in the fight, against a wide range of parasites (Christensen *et al* 2005). Agnieszka and cytrynska (2010) suggested that along with synthesis and release of inducible antimicrobial peptides/proteins, immunization of *Galleria mellonella* larvae with filamentous fungus *Aspergillus oryzae* or bacteria *Escherichia coli* induced synthesis and release of inhibitors of proPO system and/or activity. Such inhibitors could control proPO system activation and allow avoiding toxic effects of quinines and other melanization intermediates on the host cells. Prophenoloxidase system prevalent in Crustacea makes them capable of resisting every possible foreign particle entering their body by promoting cell-to-cell communication and subsequently eliminating them. Phenoloxidase (PO) is the terminal enzyme in the proPO system and acts as both recognition and effector component of the arthropod defense system (Ratclilfe *et al* 1985, Vargas Albores *et al* 1993, Ashida and Yamazaki 1990).

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