Effect of Plant Based Essential Oil on Immune Response of Silkworm, *Antheraea assama* Westwood (Lepidoptera: Saturniidae)

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Botanicals have been regarded as sustainable solution of pest control because of their nominal effect on non-target pest, biodegradability and other environment friendly properties. However, study on impact of botanicals against beneficial insects is less. In this investigation effort has been made to study the effect of essential oils of *Ocimum sanctum*, *Ocimum gratissimum* and *Ageratum conyzoides* against non-mulberry silkworm *Antheraea assama* in terms of percent mortality, total haemocyte count (THC) and phenol oxidase (PO) enzyme activity. After application of essential oil of the plants by contact residual film technique, age dependent decrease of percent mortality was recorded. Topical treatment of oil at higher dosages ($1\ \mu\text{g}^{-1}$ and $0.5\ \mu\text{g}^{-1}$) caused 100% mortality of late instar larvae at 24 hour but the lowest dose ($0.25\ \mu\text{g}^{-1}$) did not cause any mortality till 48 hour of treatment. Initial increase of total haemocyte count (THC) was recorded at early hours of treatment (0.25 hour) at all dosages ($1, 0.5, 0.25\ \mu\text{g}^{-1}$) after topical treatment. A dose dependent increase was observed in phenol oxidase enzyme activity and this might indicate ability of the essential oils to cause induction of immune response in *Antheraea assama* larvae.

Key words: *Antheraea assama*, Phenoloxidase, Haemocyte, *Ocimum sanctum*, *Ocimum gratissimum*, *Ageratum conyzoides*

Introduction

Although botanicals can be used in environment friendly way of pest management, effort has not been made yet to use botanicals in host plant garden of *Antheraea assama* Westwood, producer of the golden yellow muga silk. Use of chemicals in host plant garden of muga silk worm is rare as the larvae are reared in outdoor condition. Moreover, *A.assama* larvae are very much susceptible to synthetic chemical pesticides even at very low dosages. Therefore botanicals are the possible alternatives to be used for the management of pests and parasitoids of silkworm. *Ageratum* and *Ocimum* spp. possess long history of traditional use as medicinal and insecticidal plants. Farmers of Assam also use *Ocimum* leaf water as a holy substance on egg bunch (Kharika) of *A.assama* without any report of adverse effect on larvae. The plants *Ageratum conyzoides*, *Ocimum sanctum* and *Ocimum gratissimum* have been found to be effective against *Exorista sorbitalans*, a parasitoid of the silk worm (Bora *et al.*, 2010; Bora and Khanikor, 2011) which causes 20-80% loss in silk cocoon production round the year. Before exploring the possibility of using them as botanical pesticide in sericulture garden, it is essential to understand how does the silkworm respond to the effect of those botanicals. It has been well documented that insect haemocytes mediate immune response in invertebrates on presence of foreign material, toxin and microorganisms (Gupta, 1991; Pathak, 1993) and the response is exhibited in terms of phagocytosis in case of small sized particle and encapsulation and nodulation in case of large sized material and pathogens (Gillespie and Kanost, 1997). In addition defensin and phenoloxidase are two important protein molecules involved in insect immunity (Pathak, 1993; Hillyer and Christensen, 2005; Liu *et al.*, 2009). Phenoloxidase located in cuticle, haemolymph and oenocyte is involved in sequential conversion of dopa into melanin and thus helps in the fight against nonself. If phenoloxidase plays

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important role in development of resistance against specific insecticides (Liu *et al.*, 2009) or can act as indicator for immunocompetence in host-parasite model system (Pauwels *et al.*, 2011), phenoloxidase activity may be used as a marker for evaluating toxic action as well. Therefore the possible effect of the plants in question were attempted to be evaluated through studies on effect of the plant's essential oil on percent mortality, total haemocyte count and phenoloxidase enzyme activity.

Materials and Methods

Experimental animal

DFL (Disease Free Layings) of *Antheraea assama* Westwood (Lepidoptera: Saturniidae) were brought from Govt. Sericulture Farm of Assam and kept in formalin washed (2%) plastic tray. Newly hatched larvae were maintained by providing fresh tender leaves of host plant Sualo (*Litchea polyantha*). Moisture was provided by covering the tray with transparent polythene paper. From third instar onwards larvae were maintained in host plants grown in the garden of Department of Life Sciences, Dibrugarh University, Assam.

Extraction of oil

Fresh leaves were collected and washed in tap water to remove dust particles or other debris. The leaves were then chopped into fine pieces and hydrodistilled by using Clevenger apparatus (Denny, 1989). After 4 hours of distillation the essential oil was separated from the surface of water. The oil was dried over anhydrous sodium sulphate. The sample was sealed and kept in dark glass vial in refrigerator for treatment.

Effect on percent survival of *A. assama* larvae

Bioassay of crude oil on early (E) and late (L) instar larvae of *A. assama* was carried out by contact residual film technique by using 0.01 to 0.5% concentration of oil extracted from the plants. The solutions were prepared in acetone. 1.5 ml of oil solution was applied per replication to Whatman filter paper in the petriplate and allowed to dry the solvent for five minutes. *A. assama* larvae were then released in the treated plate in room temperature (19-32°C, RH 56-82%). Only acetone was applied for the control against each test concentration. Second day old larvae were used in each case. Observation was made at 1 h, 3 h, 6 h, 24 h, 30 h, 48 h of treatment.

Topical bioassay of essential oil of selected plants were carried out by applying 1, 0.5 and 0.25 µg⁻¹ of oil on late instar larvae and mortality as well as survival was recorded at 1 h, 3 h, 6 h, 24 h, 30 h, 48 h of treatment.

Abbott's correction factor (Abbott, 1925) was considered before computing percent survival of larvae.

Effect on total haemocyte count

Total haemocyte count of late larval instar was done after treatment with oil extracted from *A. conyzoides*, *O. sanctum* and *O. gratissimum* at the dose of 0.25, 0.5 and 1 µl/g body weight. Haemocyte count was done by the method modified after Gupta (1991).

Live insect was dipped into water maintained at 60°C for 5 min. Then the larva was taken out of water and proleg was cut to draw the haemolymph. The first few drops of haemolymph was collected in a cavity block and quickly drawn into Thoma WBC pipette upto 0.5 mark. The physiological saline was sucked upto the 11th mark and was vigorously shaken for 3 minutes. The mixture was then carefully, poured on Neubauer haemocytometer. Observation of cell was done under Olympus light microscope at 40X. Manduca's physiological saline (Dean *et al.*, 2004) was used for total haemocyte count. The composition of the saline was 4 mM NaCl; 40 mM KCl; 18 mM MgCl₂ · 6H₂O; 1.5 mM Na₂HPO₄; 1.5 mM NaH₂PO₄; 192.8 mM Sucrose; 0.05 g Gentian violet. Cells present in the four large squares in the four corners and in the central large square were counted. Each large square has a surface area of one square mm. So total cells present in one square mm (THC/mm³) was calculated by using the following formula

$$\text{THC/mm}^3 = \frac{X \times 10 \times Y}{5}$$

X = Total cells present in 5 large squares.

Y = Dilution = 20 times.

10 = Depth of chamber.

For each treatment, three replication was maintained and there was control to have the normal haemocyte count.

Effect on phenoloxidase

Late instar larvae of *Antheraea assama* were topically applied with three dosages of essential oils of the three plants (1.0, 0.5 and 0.25 µg⁻¹ body weight of insects) to study their effect on phenoloxidase activity by using the method of Mucklow *et al.* (2004). L-Dopa (Merck-Germany) was used as substrate. PO activity was equivalent to dopachrome production which was the product of the oxidation of L-Dopa by the enzyme PO. Absorbance of Dopachrome was observed at 475 nm using UV-Visible spectrophotometer. 1 µl of haemolymph was drawn in to micropipette by cutting the forelegs of the treated larvae and poured into the cuvette containing 150 µl of phosphate-buffered saline (PBS). The composition of PBS was

Fig. 1. Effect of essential oil of *O. sanctum* on percent survival (Mean) of early (E) & late (L) instar larvae of *A. assama* (CRF).

0.15 M of NaCl, 10 mM of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ at pH 7.4 with o-phosphoric acid. Then 450 μl of 20 Mm Dopa was added in the cuvette and absorbance was measured in the spectrophotometer. Absorbance was recorded immediately after adding the L-Dopa and every 15 minutes upto 1h using the same cuvette. Against each treatment, in non-haemolymph controls containing only L-Dopa and PBS, the activity was measured and this activity of the non-haemolymph control was subtracted from the activity of PO obtained in treated haemolymph. Change of PO activity of haemolymph without treatment was also measured. PO activity estimates were then calculated from the change in absorbance over the 1h time period (absorbance after 1h – absorbance at time 0). Enzyme activity was studied at 15min, 1h, 3h and 6h interval after treatment.

Statistical analysis

Percent mortality was expressed in mean $\pm$ standard error (SE). Significance was evaluated using Paired t-test in THC and phenoloxidase enzyme activity experiments ($P < 0.05$ was considered significant).

Results

Application of *O. sanctum* essential oil to early instar larvae did not cause any mortality at lower concentrations. At higher concentrations (0.50 and 0.25%) onset time of mortality was 30 h. 0.50% concentration caused 10% and 20% mortality at 30h and 48 h respectively while 0.25% concentration caused 6.67% and 13.34% mortality at 30 h and 48 h respectively (Fig. 1). For essential oil of *O. grat-*

Fig. 2. Effect of essential oil of *O. gratissimum* on percent survival (Mean) of early (E) & late (L) instar larvae of *A. assama* (CRF).

Fig. 3. Effect of essential oil of *A. conyzoides* on percent survival (Mean) of early (E) and late (L) instar larvae of *A. assama* (CRF).

issimum, onset time of mortality was 24 h at 0.06, 0.125, 0.25, 0.5% concentrations. Percent mortality was higher at 0.5% (maximum 35% at 48 h) and 0.25% (maximum 20% at 48 h) in comparison to those of *O. sanctum* (Fig. 1 and 2). For essential oil of *A. conyzoides*, onset time of mortality was 48 h at the lowest concentration (0.06%) and 24 h for the rest of the concentrations. 0.50% and 0.25% concentration caused 100% mortality at 24 h and 48 h respectively (Fig. 3). LC₅₀ of *A. conyzoides* oil was 0.19% (Fig. 4) ($Y = 7.11 + 2.92X$; $R^2 = 0.989$; $\chi^2 = 172.24$; Fiducial limit = 2.394-2.837) while the same could not be com-

Fig. 4. Regression plot of probit mortality of *A. assama* early instar larvae against log concentration of essential oil of *A. conyzoides*.

Fig. 5. Effect of essential oil of selected plants on percent mortality of late instar larvae of *A. assama* (Topical application).

puted for *Ocimum* spp. for their low toxicity against the silkworm larvae. The results reflected higher toxicity of *A. conyzoides* essential oil against early instar larvae and the order of toxicity of essential oil of these three plants can be shown as *O. sanctum* < *O. gratisimum* < *A. conyzoides*. However 100% survival of late instar larvae was recorded till 48 h at all the concentrations. After topical application of essential oil of the plants, the higher dosages were found toxic to the late instar silk worm larvae (Fig. 5). Application of the oil at the dosages of 1.0 and 0.5 μg^{-1} resulted in 100% mortality of larvae at 24 h of treatment while larvae survived till 48h at the lowest dose (0.25 μg^{-1}). These three toxic dosages were considered for study in order to assess response of immune system of *A. assama* in terms of total haemocyte count and phenoloxidase

Fig. 6. Variation of THC in *A. assama* larvae after treatment of essential oil of *O. sanctum*.

Fig. 7. Variation of THC in *A. assama* larvae after treatment of essential oil of *O. gratissimum*.

enzyme activity.

On application of essential oil of *O. sanctum* at a dose of 1 μg^{-1} on *A. assama* larvae, THC significantly increased at 0.25h (5320 $\pm$ 180*) and 1h (3924 $\pm$ 71*) which then came down to the level of control (2782 $\pm$ 213) at 3 h and 6 h. On application of 0.5 μg^{-1} oil, THC increased significantly from 0.25 h onwards. *O. sanctum* oil at a dose of 0.25 μg^{-1} enhanced THC significantly till 48 h (Fig. 6). Application of 1 μg^{-1} of essential oil of *O. gratissimum* on *A. assama* larvae initially increased THC level at 0.25h (4472 $\pm$ 4*) and then significantly decreased at 1h (1828 $\pm$ 14*). THC then became at par with control at 3h and 6h. At the dose of 0.5 μg^{-1} , initial rise of THC occurring at 0.25 h was followed by a significant decrease at 1h of treatment. THC again significantly rose at 6 h. Application of 0.25 μg^{-1} showed significant rise of THC from 1 h till

Fig. 8. Variation of THC in *A. assama* larvae after treatment of essential oil of *A. conyzoides*.

Fig. 9. Variation of log phenol oxidase enzyme activity in *A. assama* larvae after treatment of essential oil of *O. sanctum*.

Fig. 10. Variation of log phenol oxidase enzyme activity in *A. assama* larvae after treatment of essential oil of *O. gratissimum*.

Fig. 11. Variation of log phenol oxidase enzyme activity in *A. assama* larvae after treatment of essential oil of *A. conyzoides*.

24 h while at 48 h of treatment THC decreased significantly (Fig. 7). Application of essential oil of *A. conyzoides* at a dose of 1 μlg^{-1} and 0.5 μlg^{-1} caused significant rise of THC from 0.25 h to 6h of treatment (Fig. 8). Application at the dose of 0.25 μlg^{-1} enhanced THC till 48 h.

Results presented in the Fig (9-11) showed that there was dose dependent increase in enzyme activity. After application of 1 μlg^{-1} and 0.5 μlg^{-1} dose, the essential oil of *O. sanctum*, *O. gratissimum* and *A. conyzoides* enhanced PO activity in treated larvae in comparison to control larvae. But after application of the lowest dose (0.25 μlg^{-1}) of the three oil types variation of phenoloxidase enzyme activity was found to be not significantly differed from that of control.

Discussion

The results showed that out of the three plants, the essential oil of *A. conyzoides* was the most toxic. The study further revealed that while early instar larvae were susceptible to the action of essential oils, late instar larvae were resistant and could survive after exposure to oil treatments. Such resistance of late larval instars of *A. assama* to the essential oil under consideration may be attributed to age dependent changes in bioconstituents. Insecticides upon entering insect body may be acted upon by detoxifying enzyme system for detoxification before they reach the site of action. Age dependent changes in enzyme activity and their subsequent correlation with insecticide toxicity have been reported for several insects with respect to cyto-

chrome P450, glutathione S-transferase, malathion carboxylesterase and microsomal oxidase enzyme (Yu 1983; Gui *et al.*, 2009; Lee *et al.*, 1996; Rajatileka *et al.*, 2011).

THC is correlated with the rate of phenomena occurring during insect immune response such as phagocytosis, nodule formation, encapsulation, recognition of foreign bodies and wound healing and hence the total number of haemocytes reflect the involvement of immune system to deal with pathogens or chemical molecules (Kraaijeveld *et al.*, 2001). In the present investigation, increase of THC at early hours of treatment at all dosages and at all hours at the lowest dose on treatment with essential oil of *O. sanctum*, *O. gratissimum* and *A. conyzoides* might reflect activation of immune response. Rapid haemocyte division to encounter with the nonself oil compounds may enhance THC. Starvation resulting in low haemolymph volume was considered a reason of increasing THC in *Prodenia eridenia* larva (Rosenberger and Jones, 1960; Sharma *et al.* 2008). The ups and subsequent down regulation of THC level occurring on application of essential oil might suggest an active involvement of the defense system to overcome the toxic action in which the haemocytes in the circulation have been used up for the purpose of defense. At sub lethal dose ($0.25 \mu\text{g l}^{-1}$), although at 24 h post treatment THC was high in cases of all the oil, at 48 h in response to action of *O. gratissimum* THC decreased. Greater stress of *O. gratissimum* was also evident at the time of topical application when it caused immediate shrinkage of integument and darkening of the area of application and vigorous jerking of anterior body part which were not observed on application of the other oils. In case of treatment with both *O. gratissimum* and *A. conyzoides*, greater amount of regurgitants were observed which is considered as symptom of toxicity (Matsumura, 1980). Initial increase followed by decrease in THC, 12-72 h post treatment of insecticides were reported by several workers (Sharma *et al.* 2008; Qadri and Narsaiah, 1978; Ayyangar and Rao, 1990; El-Aziz and Awad, 2010; Prasada Rao *et al.*, 1984). THC is also dependent on ecdyson titre Prasada Rao *et al.*, 1984; Ayyangar and Rao, 1990). Insecticide like abamectin induced release of antidiuretic hormone from neurosecretory cells of the thoraco-abdominal ganglionic mass that slowed down the rate of excretion resulting in increase of blood volume and thereby decrease of total haemocyte count (Suhail *et al.*, 2007). Reduction of haemocytes may also be due to disturbance caused in haemopoetic organs which are responsible for production of haemocytes (Tiwari *et al.*, 2002). Decrease in haemocyte on application of insecticidal chemical after 12 h and 24 h of treatment was reported in other insects also (Qadri and Narsaiah, 1978; Ayyangar and Rao, 1990; El-Aziz and Awad, 2010).

Phenoloxidase enzyme plays an important role in non-self recognition and thereby confers immunity against exogenous chemicals, parasites, parasitoids and pathogens in various arthropods (Ratcliffe *et al.*, 1984; Marmaras *et al.*, 1996; Ashida and Brey, 1997; Shelby, 2000; Shiao *et al.*, 2001; Mucklow and Ebert, 2003; Yu *et al.*, 2003; Ling *et al.*, 2005; Zhu *et al.*, 2009). The lowest dose of the essential oil of the selected plants could not induce phenoloxidase activity in *Antheraea assama* larvae while the higher dosages significantly enhanced the enzyme activity than that of control larvae. This is the first report of activation of phenol oxidase enzyme activity in *Antheraea assama* after oil treatment. Phenoloxidase occurs as an inactive proenzyme i.e. prophenoloxidase in the haemolymph of insects including silkworm *Bombyx mori* and *Antheraea pernyi* (Nappi and Sugumaran, 1993). Phenoloxidase enzyme converts tyrosine into Dopa which is used in synthesis of melanin for subsequent use in wound healing and immune response (Huang *et al.*, 2002). Increase in phenoloxidase activity has long been correlated with increased resistance to pathogen (Brookman *et al.*, 1989; Soderhall and Cerenius, 1998; Sugumaran, 2002; Shelby and Popham, 2006) and decrease of phenoloxidase activity is attributed to weakening of immune system (Hiromori and Nishigaki, 2001). In addition to pathogens, the system is also activated in response to presence of other nonself materials like chemical insecticide, glass beads etc (Pathak, 1993; Liu *et al.*, 2009). Activation of PO activity confers resistance against invading nonself material, but it may not always adequately reflect overall resistance against all insults (Schwarzenbach and Ward, 2007). Although consistent measurement of phenoloxidase activity and haemocyte count have been successfully used in immunity studies in insects (Schwarzenbach and Ward, 2007; Lourenco *et al.*, 2005; Schmid *et al.*, 2008), study of immune related gene expression in haemolymph and fat bodies responsible for conferring immunity are required to understand the complex mechanism of immune response occurring in response to oil treatment (Fang *et al.*, 2010).

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