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Reduction in Haemocyte Mediated Immune Response in *Danais chrysippus* Following Treatment with Neem-Based Insecticides

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Abstract: The bioefficacy of some neem-based insecticides (NBIs) (neemazal, multineem and nimbecidine) was evaluated using fifth instar larvae and pupae of *Danais chrysippus*. All the NBIs caused reduction in total haemocyte count and much variation in their normal profile. This caused disintegration of haemocytes leading to reduction in immune response. In addition, the NBIs produced ecdysial stasis thereby resulting in the larval-pupal intermediates, pharate adults and deformed imagoes. A reduction in body weight of treated larvae was recorded too.

Key words: Haemocytes, development, immunity, neem-based insecticides, *Danais chrysippus*

INTRODUCTION

The importance of neem tree (*Azadirachta indica* A. Juss) as antimicrobial and insect repellent is known to Indians since time immemorial. The azadirachtin, an active ingredient of this tree, because of its bioefficacy and biodegradability has now been considered as an alternative to conventional insecticides that are known to cause hazardous effects on human health and environment. Despite having an array of biological effects like antifeedancy, repellency, antifecundity, growth regulatory and prothoracicotropic hormone (PTTH) inhibitory activities as reported by a number of workers (Schmutterer, 1990; Singh, 1996; Cowles, 2004; Medina *et al*., 2004; Pandey *et al*., 2006; Shah *et al*., 2007), the effects of neem-based insecticides (NBIs) on haemocytes, the cells responsible for cellular immunity, have not been much studied (Azambuja *et al*., 1991; Sharma *et al*., 2003; Tiwari *et al*., 2006). The present study was, therefore, undertaken to investigate the effect of some NBIs on haemocyte's population, their morphology as well as on metamorphic development of plain tiger butterfly, *Danais chrysippus*.

MATERIALS AND METHODS

The early larval instars of the plain tiger butterfly, *Danais chrysippus* Linn (Lepidoptera: Nymphalidae) were collected from Ak plant (*Calotropis gigantea*) from the field in the month of February and September. These larvae were reared on fresh leaves of the said plant in a BOD incubator maintained at 27±1°C, 75±5% RH and 16L: 8D photoperiod in laboratory (Pandey and Tiwari, 2005; Pandey *et al*., 2008). Fifth instar larvae (24 h old) and pupae (4-6 h old) from the said lab culture were used. The NBIs used in the present study were neemazal T'S (Azadirachtin 1%, other limonoids 3%, oil fatty acids glycerol esters 46.3%, polyethylene monosorbitol oleate 49.7%-EID Parry India Ltd.),

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multineem (seed extract containing 0.03% Azadirachtin-Multiplex Fertilizers Pvt. Ltd., Bangalore, India) and nimbecidine (0.03% Azadirachtin, neem oil 90.57%, hydroxyl 5%, epichlorohydrate 0.5%, aromax 3%). To examine their effects on haemocytes and metamorphic moulting, various concentrations (2.5, 5, 7, 10, 15, 20, 25 and 30%) of these insecticides were prepared by diluting them with acetone. While all the dilutions of insecticides were applied topically in different doses (vide Tables) with the help of glass micropipette on the dorsal and ventral surfaces at three different body regions (cephalic, thoracic and abdominal) of V instar larvae, prepupae and pupae of experimental group to investigate their effects on metamorphic moulting, only 50 µL of 5% of all the NBIs was used to study their toxic effect on haemocytes. The controls were treated with acetone only. Total haemocyte count (THC) and differential haemocyte count (DHC) were made at 24 hourly intervals in both the groups. The methods of haemolymph collection, smear formation, staining and counting of cells were similar to those applied earlier (Tiwari *et al*., 2006). The mean number of circulating haemocytes per mm^3 was calculated using the formula of Jones (1962). Besides, the effects of NBIs on haemolymph cell's morphology along with survival, moulting, degrees of development and wing shape in test insects were also studied. The body weight of the larvae of both the groups was also recorded at 24 h intervals till pupation to assess the toxic effect of NBIs.

RESULTS

Effect on Haemocyte Count

Application of 50 µL of 5% NBIs prevented the normally occurring increase in the number of haemocytes during fifth instar larval development resulting in over-all reduction in THC compared to control larvae (Table 1). Besides, the DHC revealed a much variation (Table 2). While a reduction

Table 1: Effects of neem based insecticides (5% conc.) on THC in V instar larvae of *D. chrysippus* (Values are mean±SD for 20 larvae)

			THC (No. of cells mm^{-3} of haemolymph)				
			Hours after treatment				
Treatments	Dose (µL)	Before treatment	24	48	72	84	108
Neemazal	50	3240±360	2675±140	4970±245	6785±480	7056±268	9672±510*
Nimbecidine	50	3250±345	2750±105	5495±276	7540±425	8640±520	10360±496*
Multineem	50	3245±320	2856±60	5639±305	7465±396	8325±475	10967±540*
Control (Acetone)	50	3250±340	6515±270	11208±435	13345±575	15300±650*	-

* = Indicates prepupa formation

Table 2: Effects of topical application of some neem based insecticides (5% conc. and 50 µL dose) on DHC in V instar larvae of *D. chrysippus* (Values are mean±SD for 10 larvae)

	Haemocyte percentage					
	After 24 h			After 48 h		
Haemocyte types	Neemazal	Control	Nimbecidine	Neemazal	Control	Nimbecidine
PRs	3.4±0.3 (39%)⁻	4.8±0.7	3.5±0.2 (27%)⁻	2.1±0.2 (53%)⁻	4.5±0.3	3.1±0.3 (31%)⁻
PLs	22.6±1.1 (24%)⁻	29.8±2.6	24.7±1.7 (17%)⁻	20.4±1.3 (34%)⁻	30.7±1.4	22.4±2.2 (27%)⁻
GRs	16.2±1.5 (34%)⁻	24.7±1.9	18.8±1.5 (24%)⁻	16.7±0.9 (28%)⁻	23.2±0.9	18.9±0.7 (19%)⁻
SPs	35.6±2.5 (45%)⁺	24.5±1.2	32.5±3.5 (33%)⁺	28.3±2.6 (41%)⁺	20.1±2.2	28.7±2.3 (43%)⁺
ADs	15.3±0.9 (22%)⁺	12.5±0.8	14.3±1.0 (14%)⁺	18.2±1.5 (38%)⁺	13.2±1.4	16.8±1.5 (27%)⁺
OEs	6.9±0.7 (86%)⁺	3.7±0.4	6.3±0.6 (70%)⁺	14.3±2.0 (72%)⁺	8.3±0.5	10.3±1.3 (24%)⁺

Value in parenthesis showed % decrease (⁻) and % increase (+) in number of different haemocyte types occurred in the counts of prohaemocytes (PRs), plasmatocytes (PLs) and granulocytes (GRs); the spherulocytes (SPs), adipohaemocytes (ADs) and oenocytoids (OEs) showed rise in their counts in treated larvae. Neemazal caused 28% reduction in GRs and 53% in PRs after 48 h of treatment. Further, this NBI caused 72% rise in OEs, 41% in SPs and 38% in ADs respectively.

Effect on Haemocyte Morphology

NBIs caused different degrees of deformity in almost all the cell types in a dose dependent manner. Cellular clumping, vacuolization of all cells, loss of pseudopods in PLs and nuclear fragmentation especially in GRs were seen. Phagocytic capsules were not frequently seen showing apparent reduction in their number. Some GRs and SPs were found with lysed cellular contents coming out of them leaving empty spaces within the cells and many exocytotic vesicles on their periphery. A few GRs were seen only with their outer skeleton but their nuclei and other cytoplasmic contents were not visible perhaps due to degradation.

Effect on Morphogenetic Development

Topical application of NBI on ventral surface of cephalic and thoracic regions of the larvae was more effective than that on dorsal surface of abdominal region. The treatment caused movement of the head on the wall of rearing glass vials, increased defecation and jerking of the body intermittently. The effects of NBIs of different concentrations and doses on insect's survival, moulting, degrees of development and wing shape were observed and the results were presented in Table 3-5.

Table 3: Effects of nimbecidine on morphogenetic development of *D. chrysippus* *

			Effects on		
Insect groups	Concentration (%)	Dose (µL)	Larval-pupal moulting	Pupal-imaginal moulting	Morphogenetic development
V instar larvae	10	100	All died	-	-
	10	125	All died	-	-
	15	50	Occurred	Did not occur	Pharate adults
Prepupae	10	50	Occurred	Did not occur	Anterior half of pupa black and posterior green in colour, died 2nd day
	10	75	Occurred	Pupae died	-
	10	100	Occurred	Pupae died	-
	25	100	Occurred	-	LPI
Pupae	10	50	-	Occurred	Partial eclosion of adults
	10	100	-	Did not occur	Anterior half of pupa black and posterior green, died 2nd day
Control (Acetone)	Undiluted acetone	As per experiment	Occurred	Occurred	Normal butterfly

*: 15 insects were used in each experimental and corresponding control groups separately

Table 4: Effects of multineem on morphogenetic development of *D. chrysippus* *

			Effects on		
Insect groups	Concentration (%)	Dose (µL)	Larval-pupal moulting	Pupal-imaginal moulting	Morphogenetic development
V instar larvae	2.5	25	Occurred	Occurred	Complete eclosion of adults, crumpled wings
	2.5	50	Occurred	Occurred	Complete eclosion of adults, wings more crumpled
	5.0	25	Occurred	Occurred	More eclosion of adults, crumpled wings
	5.0	50	Occurred	Occurred	Partial eclosion of adults
	5.0	100	Occurred	Did not occur	Pharate adult
	10.0	25	Occurred	Occurred	Complete eclosion of adults, crumpled wings
	15.0	100	Occurred	-	LPI

Table 4: Continued

Insect groups	Concentration (%)	Dose (µL)	Effects on Larval-pupal moulting	Pupal-imaginal moulting	Morphogenetic development
	20.0	100	Occurred	-	LPI
	30.0	100	All died	-	-
Pupae	2.5	25	-	Occurred crumpled wings	More eclosion of adults,
	2.5	100	-	Did not occur	Pupae shrank
	10.0	25	-	Did not occur	-
Control	Undiluted acetone	As per experiment	Occurred	Occurred	Normal butterfly

*: 15 insects were used for each experimental and corresponding control groups, separately

Table 5: Effects of neemazal on morphogenetic development of *D. chrysippus**

Insect groups	Concentration (%)	Dose (µL)	Effects on Larval-pupal moulting	Pupal-imaginal moulting	Morphogenetic development
V instar larvae	2.5	150	All died 3rd day	-	-
	5	50	Occurred	Did not occur	Pupa abnormal
	5	100	Occurred	-	LPI
	5	150	All died 3rd day	-	
	7	50	All died same day	-	
Pupae	7	100	-	Did not occur	Pharate adults
	10	25	-	Occurred	Adults enclosed partially
	10	50	-	Did not occur	Pharate adults
	10	100	-	Did not occur	All pupae died after 3 days
Control	Undiluted acetone	As per experiment	Occurred	Occurred	Normal butterfly

*: 15 insects were used for each experimental and corresponding control groups, separately

Table 6: Effects of neem-based insecticides on body weight of *D. chrysippus* larvae (Values are mean±SD for 20 insects)

Treatments	Percent/ dose (µL)	Body weight (mg) of V instar larvae at 24 h intervals 0	24	48	72	96	108	120	144
Multineem	2.5/25	105±5	125±10	225±10	350±8	455±5	550±15	470±5*	340±120**
Nimbecidine	2.5/25	104±5	125±15	230±5	345±15	460±10	560±10	475±10*	350±15**
Neemazal	2.5/25	102±6	120±10	208±10	315±26	435±20	540±25	465±32*	315±20**
Control (Undiluted acetone)	As per experiment	106±6	150±20	280±30	440±25	715±15	665±35*	480±25**	-

*: Prepupa formation, **: Pupa formation

Effect on Body Weight

Besides a delay of about one day in pupation, NBI treated larvae showed less consumption of food in comparison to acetone treated controls. While control larvae fed *ad libitum* attained a body weight of 715±25 mg, neemazal treated larvae could attained only 540±25 mg causing a reduction of 175 mg in their weight. Multineem and nimbecidine treated larvae, on the other hand, were heavier in weight than neemazal treated larvae (Table 6).

DISCUSSION

The response of haemocytes against biological agents as well as toxins has been studied in many insects. The resulting effects thereof are phagocytosis, encapsulation and distortion of cell contour or cellular disintegration (Saxena and Tikku, 1990). The drastic reduction in THC in present insect following NBIs application is similar to the reports of Azambuja *et al.* (1991) in *Rhodnius prolixus*, Sharma *et al.* (2003) in *Spodoptera litura* and Tiwari *et al.* (2006) in *Dysdercus koenigii*. The decrease in THC number may be due to the clumping of haemocytes, the toxic effect of NBIs and/or to their inhibitory effect on endocrine glands. Sharma *et al.* (2003) reported about 50% reduction in THC after 72 h of oral feeding of neem gold in *S. litura* but 61% decline was seen in *D. koenigii* following topical application of neemazal (Tiwari *et al.*, 2006). Present results reveal that neemazal produced 59 and 56% reduction in THC after 24 and 48 h, respectively, which is more effective as compared to two other NBIs (vide Table 1). It could be probably because of more azadirachtin content (1% in the former and 0.03% in the latter two). However, Figueiredo *et al.* (2006) found no significant difference in THC between azadirachtin treated *R. prolixus* nymphs and their controls. The maximum drop of PR percentage (53%) was followed by PLs (34%) and GRs (28%) respectively, 48 h after neemazal treatment (Table 2). This indicates the maximum participation of PRs in over-all decline in cell count more likely by the inhibition of their mitotic divisions (Salehzadeh *et al.*, 2003). The decline in PL- and GR-percentage was reported to be caused by their involvement in phagocytosis and nodule formation (Sharma *et al.*, 2003). But the reduction in number of phagocytotic capsules in NBI treated *D. chrysippus* larvae led to suggest some other reasons for decline in their counts. Similar drastic reduction in encapsulation/ phagocytosis response were also found in *Drosophila* larvae infected by eggs of parasitic wasps (Sorrentino *et al.*, 2002) and azadirachtin treated *R. prolixus* nymphs (Figueiredo *et al.*, 2006). Based on their studies, Figueiredo *et al.* (2006) have suggested for the first time that the phagocytosis is modulated by ecdysone. The reduction in number of these cell types in the present study, therefore, seems to be due to the toxic effect of NBIs rather than their involvement in phagocytic actions.

A large number of abnormalities observed in NBIs treated *Danais* larvae at cellular and developmental levels (see Results) have also been reported by earlier workers (Schmutterer, 1988; Singh, 1996; Sahayaraj and Paulraj, 2001; Sharma *et al.*, 2003; Tiwari *et al.*, 2006). Further, the synthesis and release of PTTH by the brain was also reported to be deficient in azadirachtin treated *R. prolixus* nymphs (Garcia *et al.*, 1990). The factors for these developmental and cellular defects are reported to lie in the endocrine system and the hormones secreted by them (Hoffmann, 1970; Schmutterer, 1990; Koul and Isman, 1991; Koul, 1996; Tiwari *et al.*, 2006). Further, our results reveal the reduction of body weight in NBIs treated larvae. This is suggestive of their antifeedant property as reported for azadirachtin treated cutworm, *Peridroma saucia* (Koul and Isman, 1991) and Aza treated larvae of the lemon-butterfly, *Papilio demoleus* (Pandey *et al.*, 2006).

It is, therefore, suggested that the NBIs might act negatively on the prothoracic glands via brain thereby causing reduction in phagocytic response of haemocytes and reduction in body weight. Further, NBIs might have direct toxic effects on haemocytes leading to necrotic results. The over-all effect of the NBIs could be adverse on the immune activity of the present insect threatening its survival.

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