

## EFFECT OF VARIOUS TREATMENTS OF AZADIRACHTIN, SPINOSAD AND ABAMECTIN ON THE HAEMOGRAM OF *COCCINELLA SEPTEMPUNCTATA* L. (COLEOPTERA: COCCINELLIDAE)

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### ABSTRACT

Larvae of the seven spotted ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) were treated with 0.3, 0.0096 and 0.0036% azadirachtin (Nimbokill<sup>®</sup> 60 EC), spinosad (Tracer<sup>®</sup> 480 SC) and abamectin (Sure<sup>®</sup> 1.8 EC) respectively and their impact on haemogram was investigated for 1, 30 and 60 minutes after treatment. The haemogram of *C. septempunctata* comprised prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs) and oenocytoids (OEs) with the entire cell size of  $6.15 \pm 0.275$ ,  $18.65 \pm 1.21$ ,  $13.36 \pm 0.339$ ,  $12.95 \pm 0.399$  and  $14.89 \pm 0.479$  micron respectively. Total haemocyte count (THC) increased one minute after treatment with azadirachtin and spinosad, whereas decreased after application of abamectin. Azadirachtin and spinosad resulted in an increased overall THC, whereas spinosad application resulted in a decreased THC 30 minutes after treatment. After 60 minutes, larvae treated with azadirachtin and spinosad had 1.2 and 1.4-fold more THC respectively whereas abamectin-treated larvae had 1.8-fold fewer THC than untreated controls. The differential haemocyte count indicated that azadirachtin application resulted in increased percentage of PLs, GRs and SPs, whereas a decrease percentage of PRs and OEs. Spinosad resulted in an increased percentage of PLs, OEs and SPs, and a decreased percentage of PRs and GRs. Abamectin treatments resulted in an increased percentage of GRs and SPs, and a decreased percentage of PRs and PLs. Overall, azadirachtin was relatively safe for *C. septempunctata* larvae compared with spinosad and abamectin.

**KEYWORDS:** green chemistries, insecticides, ladybird beetle, predator, haemocytes

### INTRODUCTION

Integrated pest management (IPM) includes a combination of chemical, biological and cultural control strategies (Sarfraz *et al.* 2005) although insecticides will continue to comprise important components of such programs. Insecticides applied in an agroecosystem not only control the target insect pests but may adversely affect the non-target organisms including biocontrol agents. New chemical insecticides are introduced rapidly but very little is known about their effects on predators and parasitoids of herbivorous pests. Insecticides should not only suppress the insect pest population but also be safe to their natural enemies. It is, therefore, imperative to screen the insecticides for their selectivity for important biocontrol agents before they could be incorporated into IPM programs (Sarfraz & Keddie, 2005). Successful screening of insecticides largely depends on a better understanding of their toxicological effects on target pests as well as on their natural enemies.

Haematological studies are of crucial importance for insect toxicology. Haemocytes are involved in detoxification (e.g. phagocytosis and encapsulation) of metabolites and biologically active material in addition to transport of nutrients and hormones (Patton, 1983) suggesting that haemogram is a useful tool for investigation of toxic effects of insecticides on biocontrol agents (Rosenberger & Jones, 1960). The number of haemocytes ranges from 100 to 167,000 per unit volume of haemolymph (Romosen & Stofolano, 1998), but has been found to be quite variable depending upon insect species, developmental stage, physiological state, and the protocols followed. On the basis of morphology, functions and histochemical reactions, haemocytes are widely classified as prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SPs), adipohaemocytes (ADs), oenocytoids (OEs) and coagulocytes (COs) (Chapman, 1998); PLs and GRs are involved in phagocytosis and encapsulation (Steinhaus, 1949; Chapman, 1998).

Suitable integration of safe and effective chemicals with other tactics, including biocontrol agents can prove effective strategies for IPM (Gogi *et al.* 2006). Seven spotted ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) is an important generalist biological control agent. It preys on different insect pests namely aphid species (Srivastava *et al.*, 1987; Viola *et al.*, 1988), sugar cane whitefly, *Aleurolobus barodensis* (Maskell) (Kapur, 1940), coffee mealybug, *Planococcus lilacinus* (Cockerell) (Szent-Ivany, 1963), rice brown plant hopper, *Nilaparvata lugens* (Stål) (Samal & Misra, 1982), tobacco caterpillar, *Spodoptera littoralis* (Boisd.) (Noctuidae: Lepidoptera), coccids (El-Ghareeb, 1989), and various jassid species (Singh *et al.*, 1991) in a variety of cropping system. Ladybird beetles have high reproductive potential, and are active predators both in larval and adult stage (Karpacheva, 1991). They have been found tolerant to several insecticides under field conditions (Saharia, 1982) but mechanisms of resistance are unknown.

Azadirachtin-based products are derived from leaves and kernels of neem plant, *Azadirachta indica* A. Jussieu and have been found to be effective against several insect pests, namely whiteflies, aphids, thrips, fungus gnats, caterpillars, beetles, mushroom flies, mealybugs, leafminers and gypsy moths (Thomson, 1992; Gahukar, 2000). Azadirachtin acts both as systemic and contact insecticide and possesses antifeedant, repellent, sterilant and insect growth inhibitor properties (Schmutterer, 1990; Gahukar, 2000). Its exact site of action is unknown, but generally it is believed to disrupt the endocrine system of the treated insects (Schmutterer, 1990). Neem products are considered relatively environmentally-friendly, compatible with modern IPM strategies and have the potential to be adopted on a large scale (Gahukar, 2000). In different studies, azadirachtin-based products were relatively safe to the predatory mite, *Amblyseius fallacies* (Garman) (Phytoseiidae: Acarina) (Kain & Agnello, 2002) and lacewings, *Chrysoperla carnea* (Stephans) (Chrysopidae: Neuroptera) (Medina *et al.* 2003).

Spinosad is a member of the Naturalyte class of pesticides that has been classified as bioinsecticide (Copping & Menn, 2000). It comprises primarily two macrocyclic lactones, spinosyn A and D (Sparks *et al.* 1995; Sparks, 2004; Sarfraz *et al.* 2005) which

are secondary metabolites produced by the soil bacterium, *Saccharopolyspora spinosa* Mertz & Yao (Actinomycetales: Pseudonocardiales), under natural aerobic fermentation conditions (Mertz & Yao, 1990). Spinosad acts as both stomach and contact insecticide, primarily targeting a nicotinic acetylcholine receptor as well as  $\gamma$ -aminobutyric acid (GABA) gated chloride channels and causes a general paralysis of the insect (Salgado *et al.* 1997; Watson, 2001; Sparks, 2004; Sarfraz *et al.* 2005). Spinosad effectively controls pest species of the orders Lepidoptera, Diptera, and Thysanoptera and also shows toxicity to certain species of Coleoptera, Orthoptera, Hymenoptera, Isoptera, Siphonaptera, Dermaptera and Psocoptera (Thompson *et al.* 1995, 2000; Cisneros *et al.* 2001; Thompson & Sparks, 2002; Blanc *et al.* 2004; Sarfraz *et al.* 2005). Spinosad is known to have exceptional safety to non-target organisms compared to synthetic insecticides (Sarfraz *et al.* 2005). Spinosad-treated aphids fed to coccinellid and chrysopid larvae caused no mortality of these predators (Schoonover & Larson, 1995). Additional studies determined that spinosad was practically non-toxic to insect natural enemies such as lady beetle, minute pirate bug, lacewing, and predatory mites (Bret *et al.*, 1997; Jones *et al.*, 2005). Spinosad applied to bell peppers effectively controlled the pepper maggot, *Zonosemata electa* (Say) (Tephritidae: Diptera), but it did not reduce populations of beneficial arthropods including species of Chrysopidae, Coccinellidae, Cecidomyiidae, Syrphidae, Nabidae and hymenopteran-parasitized Aphididae (Boucher, 1999). Spinosad applications were less toxic than lambda cyhalothrin to the majority of predators including chrysopids, coccinellids, syrphids and geochorids in sweet corn (Musser & Shelton, 2003).

Ivermectin (e.g. abamectin) is a group of macrocyclic lactones produced by *Streptomyces avermitilis* sp.nov. (Ex Burg) (Burg, *et al.* 1979; Miller *et al.* 1979; Chabala *et al.* 1980), being largely used as anti-parasitic in the domestic animals (Aziz *et al.* 1982; Campbell *et al.* 1983). Abamectin is a mixture of avermectins containing > 80% avermectin B1a and < 20% avermectin B1b (Meister, 1992); both of these components have similar biological and toxicological properties (Lankas & Gordon, 1989). Abamectin targets GABA receptor (Tanaka, 1987; Sun & Peng, 2000) and is registered for its foliar applications on a

variety of agronomic and horticultural crops (Lankas & Gordon, 1989). Abamectin controls chewing (e.g. leaf miners, diamondback moth, tomato pinworm, Colorado potato beetle, etc.) and sucking insect pests (e.g. psylla and thrips) (Sun & Peng, 2000). It exhibited low toxicity to non-target beneficial arthropods which has accelerated its acceptance for IPM programs (Lasota & Dybas, 1990). Avid<sup>TM</sup> (abamectin), used against mites and leafminers, was found to spare some of the major parasites of leafminers (Parella, 1987) and some predacious mites (Hoy & Conley, 1987). Abamectin did not have any adverse effect on the egg viability and larval development of *Chrysoperla externa* (Hugen) (Chrysopidae: Neuroptera) (Bueno & Freitas, 2004). In potato fields in Java (Indonesia), applications of abamectin led to a reduction in leafminer infestations but did not have any adverse effects on predators and parasitoids (Hidayani, 2005).

Present laboratory study was designed to determine the relative safety of three commercially available bioinsecticides to the larvae of *C. septempunctata*. The effects of treatments of azadirachtin (Nimbokill<sup>®</sup> 60 EC), spinosad (Tracer<sup>®</sup> 480 SC) and abamectin (Sure<sup>®</sup> 1.8 EC) in concentrations of 0.3 %, 0.0036 % and 0.0096 %, respectively were investigated on the total haemocyte count (THC) and differential haemocyte count (DHC).

## MATERIAL AND METHODS

### Insects

Present research was carried out in the Environmental Entomology Laboratory, Department of Agricultural Entomology, University of Agriculture, Faisalabad, Pakistan. The final instar larvae of *C. septempunctata* were collected from the commercial field of wheat crop in Faisalabad, Pakistan (31.30° N, 73.10° E, altitude 213 m). The larvae were brought to the laboratory and kept in plastic containers (2 liter) at 30±1°C, relative humidity ranging from 75 ±5% and photoperiod from 11 to 13 h (George & Ambrose, 2004). These larvae were maintained on mustard aphids, *Lipaphis erysimi* Kalt (Aphididae: Homoptera) which were collected from *Brassica juncea* (L.) Czern. plants grown in the greenhouse.

### Insecticide Treatment

Three insecticides, viz. azadirachtin (Nimbokill<sup>®</sup> 60 EC, STEDEC Technology Commercialization

Corporation of Pakistan, Ministry of Science and Technology, Islamabad), spinosad (Tracer<sup>®</sup> 480 SC, DowAgro Sciences (Ltd.), Sahiwal, Pakistan) and abamectin (Sure<sup>®</sup> 1.8 EC, Food Machinery Corporation (FMC), Multan, Pakistan) were tested in this study. In preliminary tests, the LC<sub>50</sub> values for the last larval instar of *C. septempunctata* for 48 h duration were found to be 0.3, 0.0096 and 0.0036 % for azadirachtin, spinosad and abamectin respectively. Twenty microlitres of 1/10 of the 48 h LC<sub>50</sub> of each insecticide was topically applied to the thoracic region of the final instar larvae of *C. septempunctata* using a micropipette and treated larvae were kept in individual Petri plates; control larvae were treated with distilled water. A total of 120 Petri plates were used in this experiment; 30 plates were used for each tested insecticide and ten served as controls. Each treatment group was further divided into three sub-groups of plates; each sub-group with ten treated larvae. Larvae of first sub-group were subjected to haemogram study immediately (one minute) after treatment, while second and third sub-group were subjected to haemogram analysis 30 and 60 minutes post-treatment (George & Ambrose, 2004).

### Total Haemocyte Count

The haemolymph was collected on a clean glass slide by pricking the needle into the abdominal leg and quickly drawn into Thoma white blood cell diluting pipette up to 0.5 mark and then diluted 20 times with Toisson's solution (NaCl =1.0 g, Na<sub>2</sub>SO<sub>4</sub>= 8.0 g, neutral glycerin =30ml, Methyl violet =0.025g and distilled water = 160 ml) up to mark 11 (Mahmood & Yousaf, 1985). The haemolymph filled pipette was shaken for 10 minutes to prevent coagulation and for uniform staining. The first few drops of haemolymph present below the bulb of the pipette were discarded (Jones, 1962; George & Ambrose, 2004), a drop of haemolymph was placed near the edge of the cover slip of the haemocytometer and the chamber was automatically filled by capillary action and to let the haemocytes settle down, the haemocytometer was left for 5 minutes (Tonapi, 1994) and haemocytes were counted in the four corners ruled squares in each of the two chambers.

### Differential Haemocyte Count

The haemolymph was fixed by glacial acetic acid vapors for 5 to 10 minutes in a small desiccator at

40°C. Abdominal leg was pierced by needle and the haemolymph was drawn into Thoma white blood cell diluting pipette. The haemolymph filled pipette was shaken for 10 minutes to prevent coagulation and for uniform staining. The first few drops of haemolymph present below the bulb of the pipette were discarded; a droplet of blood was placed on the clean white grease free microscope slide and smear was made in the manner by drawing second slide across the first one at 45° angle. The smear was air dried and stained by Wrights stain for four minutes. A freshly prepared buffer solution ( $\text{Na}_2\text{HPO}_4=3.8\text{g}$ ,  $\text{K}_2\text{HPO}_4=5.47\text{g}$  and distilled water =1L) of pH 6.6 was applied for 15 minutes to neutralize the haemocyte contents for differential staining. Differential counting of haemocytes was done under oil immersion phase microscope (10X x 100X). Each time 100 cells were counted and the percentage of various classes was computed (Mahmood & Yousuf, 1985). The preparations were observed under a microscope. Categorization of haemocytes was done according to the identification key of Gupta (1986).

### Statistical Analysis

Means of THC of different insecticide-treated insects were subjected to regression analysis. The DHC were first analyzed by one-way analysis of variance to determine whether differences existed among treatment means. When significant differences among treatment means were found, differences between individual treatment means were tested by Tukey's studentized range test at  $P = 0.05$  (SAS, 1999).

## RESULTS

### Haemocyte Types and Sizes

Five types of haemocytes viz. PRs, PLs, GRs, SPs and OEs were distinguished in the fixed haemolymph preparations of untreated larvae of *C. septempunctata*. The PRs were comparatively small and round cells ( $6.15 \pm 0.275 \mu$ ) with relatively larger nuclei but with lesser perinuclear space ( $4.24 \pm 0.11 \mu$ ). The PLs were larger than PRs and highly polymorphic (rounded, ovoid and spindle-shaped). On an average they were largest of all the haemocytes ( $18.65 \pm 1.21 \mu$ ) with round or elongate centrally located nucleus having perinuclear space ( $4.25 \pm 0.212 \mu$ ). Granulocytes ( $13.36 \pm 0.339 \mu$ ) contained rich quantity of granules in their cytoplasm. They were round, oval or fusiform and contained round or elongate and centrally positioned

nucleus with a perinuclear space of  $6.05 \pm 0.245 \mu$ . Spherulocytes were oval haemocytes ( $12.95 \pm 0.399 \mu$ ) with large spherical granules which obscured the cytoplasm and nucleus; the perinuclear space was  $6.05 \pm 0.245 \mu$ . Oenocytoids were generally large oval or elongate cells ( $14.89 \pm 0.479 \mu$ ) with eccentrically positioned nucleus ( $6.95 \pm 0.401 \mu$ ). The percentage of PRs was the highest (17.77 %) in untreated larvae of *C. septempunctata* followed by PLs (6.55 %), GRs (4.66 %), SPs (2.33 %) and OEs (2.00 %).

### Effect of Insecticide Treatments on the Haemogram of *Coccinella septempunctata*

All the three tested insecticides significantly affected the THC ( $F = 148.62$ ;  $P < 0.0001$ ). Insecticides had significant effects on DHC of PRs ( $F = 21.33$ ;  $P < 0.0001$ ), PLs ( $F = 35.65$ ;  $P < 0.0001$ ), GRs ( $F = 98.63$ ;  $P < 0.0001$ ), OEs ( $F = 3.61$ ;  $P = 0.02$ ) and SPs ( $F = 17.77$ ;  $P < 0.0001$ ).

### Effect of Azadirachtin

Azadirachtin-treated larvae had 1.4, 1.6 and 1.2-fold more THC than those of untreated controls at 1, 30 and 60 minutes after treatment, respectively (Table 1). Azadirachtin at 1 minute post-treatment resulted in an increased DHC of PLs, GRs and SPs (9.27, 6.61 and 3.11 % respectively) than in untreated control larvae (6.55, 4.66 and 2.33 % respectively). However, azadirachtin resulted in a decreased DHC of PRs and OEs 1 minute after treatment (13.05 and 1.27% respectively) than in untreated controls (17.77 and 2.00 % respectively) (Fig. 1)

### Effect of Spinosad

Spinosad-treated larvae had 1.2, 0.6 and 1.4-fold more THC than those of untreated controls at 1, 30 and 60 minutes after treatment, respectively (Table 1). Spinosad at 1 minute post-treatment resulted in an increased DHC of PLs, OEs and SPs (9.44, 2.44 and 6.16 % respectively) than in untreated control larvae (6.55, 2.00 and 2.33 % respectively). However, spinosad applications resulted in a decreased DHC of PRs and GRs after 1 minute (13.94 and 1.33 % respectively) than in untreated controls (17.77, and 4.66 % respectively) (Fig. 1)

### Effect of Abamectin

Abamectin-treated larvae had 1.4- fold more THC than those of untreated controls at 30 minutes after treatment, but 1.9 and 1.8-fold less THC than those of untreated controls at 1 and 60 minutes after

treatment, respectively (Table 1). Abamectin at 1 minute post-treatment resulted in an increased DHC of GRs and SPs (5.38 and 4.94% respectively) than in untreated control larvae (4.66 and 2.33 % respectively). However, treatment with abamectin resulted in a decreased DHC of PRs and PLs (16.44 and 4.72%, respectively) than in untreated controls (17.77 and 6.55 % respectively); OEs in abamectin-treated larvae were similar (2 %) to their untreated counterparts (Fig. 1)

## DISCUSSION

This is the first study of its kind to examine major types of haemocytes and demonstrate the effects of azadirachtin, spinosad and abamectin on the haemogram of the final-instar larvae of *C. septicapunctata*. Five types of haemocytes were identified in the present study viz. PRs, PLs, GRs, SPs and OEs. The THC recorded in the final instar larvae of *C. septicapunctata* were 7612 cells per mm<sup>3</sup> whereas Sanjayan *et al.* (1996), Pelc (1986), Sabir (1994), Rauf (1995), Ayub (1996) and Alhariri (2001) recorded 3263, 15000-22000, 6875, 31150, 18750 and 8690-10330 cells per mm<sup>3</sup> in fifth-instar larvae of *Spilostethus hospes* (Fab.) (Heteroptera: Lygaeidae), larvae and pupae of *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), *Euproctis fraterna* (Moore) (Lepidoptera: Lymantriidae), adults and nymphs of *Drosicha stebbingi* Green (Margarodidae: Homoptera) and nymphs of *Schistocerca gregaria* (Forskål) (Acrididae: Orthoptera) respectively. Prohaemocytes were predominant in *C. septicapunctata* followed by PLs and GRs; SPs and OEs were least in number in *Tryporyza* sp. (Hassan, 1985), *Choristoneura fumiferana* (Clemens) (Tortricidae: Lepidoptera) (Dunphy & Nolan, 1980), *Earias* spp. (Ahmad, 1991), *H. armigera* (Sabir, 1994), *E. fraterna* (Rauf, 1995) and *Rhynocoris kumarii* Ambrose & Livingstone (Hemiptera: Reduviidae) (George & Ambrose, 2004). Silva *et al.* (2002) also reported PRs, PLs and GRs as the most numerous cells in the haemolymph of *Anastrepha obliqua* (Macquart) (Tephritidae: Diptera). The highest percentage of PRs could be attributed to the fact that they are believed to be the basic stem-type cells that divide frequently and give rise to other types of haemocytes (Saxena & Srivastava, 2001). Higher number of PLs could be due to the fact that these cells are highly

polymorphic and might be converted into other types of haemocytes, particularly into GRs (Gupta & Sutherland, 1966); PLs and GRs are involved in phagocytosis and encapsulation (Steinhaus, 1949).

The THC increased after the treatments of azadirachtin and spinosad. The increase in THC by azadirachtin could be attributed to the fact that the main constituent of azadirachtin, tetranortriterpenoid which is a strong antifeedant and also acts as a potent growth inhibitor at microgram levels (Schmutterer, 1990) induces starvation effect in treated insects; the starvation effect has been reported to increase the THC in *Prodenia eridania* (Cramer) (Noctuidae: Lepidoptera) (Rosenberger & Jones, 1960) probably owing to decreased volume of haemolymph (Sharma *et al.* 2003). The increase in THC by azadirachtin and spinosad at different time of post-treatment might also be correlated to the degree of the defensive action of haemocytes involved in detoxification (George, 1996; George & Ambrose, 2004) and also due to decreased blood volume (Feir, 1979) at various time intervals of post-treatment. Sanjayan *et al.* (1996) and Feir & O'Connor (1969) also reported that the haemolymph volume was an important factor influencing total count of circulating haemocytes. Pathak (1991) reported that blood volume increased due to the release of antidiuretic hormone from neurosecretory cells of the thoraco-abdominal ganglionic mass. This hormone slowed down the rate of excretion and this eventually increased the volume of haemolymph. We did not measure the volume of haemolymph in the present study, but we suspect that azadirachtin and spinosad might have inhibited the release of antidiuretic hormone from neurosecretory cells of the thoraco-abdominal ganglionic mass consequently accelerating the excretory system and eventual decrease in blood volume. Khan (1994), Awan (1995) and Tariq (2001) reported that THC increased just after application of insecticides in *Leucinodes orbonalis* Guenee (Pyralidae: Lepidoptera), *Pieris brassicae* L. (Pieridae: Lepidoptera) and *Earias* spp. respectively. As the circulating haemocytes are involved in defensive reactions in insects, so increase in their number after treatment may indicate whether the host defensive system has activated or deactivated (Silva *et al.*, 2002). The increase in THC also shows the activation of the mechanisms responsible for the production of new haemocytes, involved in encapsulation and phagocytosis of foreign particles,

so time dependent increase in THC by azadirachtin and spinosad in the haemolymph indicates that they neither effected the mechanisms, responsible for production of defense cells nor resulted in the severe mortality of the haemocytes responsible for phagocytosis or encapsulation. This increase in THC could be correlated to the enhanced encapsulation of foreign/toxic molecules through process of melanization; melanin deposition during encapsulation is commonly initiated by haemocytes and/or phenoloxidase enzyme circulation in the plasma (Nappi & Christensen, 2005 and Rolff & Siva-Jothy, 2002). According to Lavine & Strand (2002), THC is also positively correlated with the rate of phagocytosis, nodule formation, encapsulation, recognition of foreign bodies and wound healing. As total number of haemocytes in haemolymph is likely to reflect the capability of immune system to deal with pathogens or chemical molecules (Kraaijeveld *et al.* 2001) both in terms of encapsulation and/or other immunological processes (Rolff & Siva-Jothy, 2002) the increase in THC as a result of azadirachtin and spinosad treatments suggests that these two insecticides did not adversely affect the larvae of *C. septempunctata*. It can, therefore, be concluded that azadirachtin and spinosad are comparatively safer to be used in the IPM system where *C. septempunctata* is to use as biocontrol agent. Azadirachtin was safest of all the tested insecticides in this study against *C. septempunctata* as it resulted in elevated THC compared with spinosad and abamectin. The laboratory studies indicated that applications of neem oil did not affect egg viability and larval survival and development of *Cycloneda sanguinea* (L.) (Coleoptera: Coccinellidae) (Silva & Martinez, 2004). When the larvae were sprayed with neem oil, significant mortality was observed only at the higher concentrations, but larval development and predatory capacity were not affected. Also, adults developed from treated larvae exhibited no abnormalities in sex ratios, fecundity, fertility and longevity. Similarly, at concentration of 5 ml/l, the neem oil caused no mortality to the adults, since all of them, sprayed or kept on sprayed twigs, presented a longevity curve similar to their untreated counterparts (Silva & Martinez, 2004). Second-instar larvae of *C. septempunctata* were far less susceptible when feeding 48 h on neem-sprayed aphids than first instars however the time of their development was prolonged, and aphid consumption

reduced. Larvae of *C. carnea* proved to be less susceptible, when feeding on neem-sprayed aphids, than *Episyrphus balteatus* DeGeer (Syrphidae: Diptera) and *C. septempunctata*; however, significant effects were also observed in prey consumption, time of development, mortality, longevity, and rate of deformity (Ahmad *et al.*, 2003). Foliar sprays of neem kernel water extract had less severe effects on the *Diaeretiella rapae* (McIntosh) (Braconidae: Hymenoptera:) a parasitoid of aphid (Ahmad *et al.*, 2003). Three azadirachtin-based insecticides, Agroneem® (4.8 mg a.i. liter<sup>-1</sup>), Neemix® (20 mg a.i. liter<sup>-1</sup>) and Ecozin® (20 mg a.i. liter<sup>-1</sup>) caused 11.1, 16.7 and 5.6% adult mortality in *Cotesia plutellae* Kurdjumov (Braconidae: Hymenoptera), endoparasitoid of *Plutella xylostella* (L.) (Plutellidae: Lepidoptera), respectively (Haseeb *et al.* 2004). This toxicity of azadirachtin to endoparasitoid may be due to the fact that parasites are more susceptible to insecticides than predators. Toxicity trials of pyriproxyfen, spinosad and tebufenozide on survival and reproduction of *C. carnea* adults indicated that spinosad along with pyriproxyfen and tebufenozide were harmless to adult survival and did not exert any negative effects on the fecundity and fertility irrespective of the insecticide or time of application. However spinosad reduced the number of adults by 39.8% and 87.2% in topical and ingestion treatment at the maximum concentration (800 mg a.i. litre<sup>-1</sup>) which was much higher than the recommended rates against different soft bodied insect pests (Medina *et al.* 2003). Methoxifenozone and spinosad had no acute toxicity to adults and nymphs of *Deraeocoris brevis* (Uhler) (Miridae: Hemiptera:), an important generalist predator in pome fruits in the western United States as well as no effect on egg hatch and nymph survival just after hatch (Dong-Soon, 2006). Spinosad appears to be relatively safe to beneficial arthropods, although some effects might be noted on tachinid flies, ladybird beetles and particularly hymenoptera species; however occurrence of refugia may help in limiting the effects of spinosad on these species (Anonymous, 1998). Under laboratory conditions, spinosad (Tracer®), tebufenozide (Mimic®) and azadirachtin (Align®) were found nontoxic to eggs and pupae of *C. carnea*, as fecundity and hatching percentages were similar in treated and controls; larvae developed normally into pupae and pupae developed into normal adults. However, spinosad, at the highest concentrations tested, caused a slight

reduction in the adult life span and fecundity and azadirachtin caused a slight reduction in the number of pupae and adults; however, fecundity and fertility of surviving adults was normal (Medina *et al.* 2001). Spinosad was harmless to *Poecilus cupreus* L. (Carabidae: Coleoptera) (ground dwelling predator) and had limited adverse effects on *E. balteatus* and *C. septempunctata* (foliage dwelling predators (Thompson *et al.* 1995)). Against another foliage dwelling predator, *C. carnea*, spinosad was harmless at 36 g a.i. /h L in an extended laboratory study involving realistic application methods (Thompson *et al.* 1995).

The THC decreased after the treatment of abamectin and could be associated with the fact that some haemocytes fulfill the function of food storage since they contain inclusion of glycogen and fat and decrease on starvation of the insects (Yeager & Munson, 1941; Munson & Yeager, 1944). The decrease in THC due to abamectin treatment could also be due to increase in haemolymph volume caused by abamectin. Because abamectin, possibly induced the release of antidiuretic hormone from neurosecretory cells of the thoracico-abdominal ganglionic mass which slowed down the rate of excretion and this ultimately increased the blood volume. In spite of all these, reduction in haemocyte count may be due to the ablation of haemopoietic organs which is responsible for the production of some haemocytes (PLs, PRs and OEs) as reported by Tiwari *et al.* (2002) in the fifth-instar larvae of *Papilio demoleus* L. (Papilionidae: Lepidoptera). Bhargana & Pillai, (1976) also recorded a drastic reduction in both sexes of *Dysdercus koenigii* (F.) (Pyrrhocoridae: Hemiptera) 48 hours post-treatment of apholate (chemosterilant). Ahmad (1991) and Ayub (1996) reported that THC declined after treatments of different insecticides in *Earias* spp. and *H. armigera*. The decrease in THC treated specimens indicates a severe and drastic decrease in the proportion of metabolically active cells which suggests a reduce ability of insect cellular defense system to phagocytose or encapsulate the foreign particles because effective physiological mechanisms of phagocytosis, encapsulation and other related defense mechanisms primarily depend upon the availability of circulatory immune cells particularly plasmatocytes and granulocytes which the insects need to utilize to feed and survive in the habitat (Sanjayan *et al.*, 1996). A drastic decrease in

THC due to abamectin toxicity also indicates the susceptibility of tested insect as Kraaijeveld *et al.* (2001) recorded two-fold reduction in the density of haemocytes in the haemolymph of susceptible insects' strains. It leads to the conclusion that abamectin is toxic to *C. septempunctata* and should be avoided in the IPM systems where this predator is to be introduced against soft-bodied insect pests. Similar results were recorded in numerous toxicity trials conducted in various parts of the world on certain predators and parasitoids. For instance, toxicity trials conducted on *D. brevis* in pome fruits in the western United States at 10 % and full field rate showed that abamectin at full field rate did not affect egg hatch, but its residues had moderate to high toxicity to hatched nymphs as against methoxifenozone and spinosad (Dong-Soon, 2006). The laboratory studies in Florida showed that abamectin + petroleum oil caused 100 % mortality of *C. sanguinea* and *Harmonia axyridis* Pallas (Coccinellidae: Coleoptera) (Michaud, 2002). Abamectin resulted in 15.55 % mortality in *C. septempunctata* as compared to tebufenozide (19.64 %), cartap (16.66 %) and lambda-cyhalothrin (41.79 %) (Lui & Sengonca, 2002).

The percentage of PLs increased after application of azadirachtin and spinosad as recorded by George & Ambrose (2004) in *R. kumarii* against endosulfan. This increase might be the result of normal mitosis (Mall & Gupta, 1982) and less transformation of these cells into other haemocytes as these cells are involved in phagocytosis directly (Chapman, 1998). This increase could be due to the activation and proper functioning of haemopoietic organs even after the toxification of azadirachtin and spinosad as these organs are responsible for the production of PLs as reported by Tiwari *et al.* (2002) in the fifth-instar larvae of *P. demoleus*. The increase in PLs owing to azadirachtin and spinosad suggests that these two bio-insecticides do not adversely affect the cellular defensive system and can be considered as the safest bio-insecticides to *C. septempunctata*. But Ambrose (2004) recorded reduction percentage in PLs of adult *Dysdercus koenigii* due to azadirachtin which may be due to difference in insect species tested or protocols followed for THC or DHC. However the decrease in PLs in abamectin-treated categories could be due to ablation of haemopoietic organs, responsible for the production of PLs as reported by Tiwari *et al.* (2002) in the fifth-instar

larvae of *P. demoleus* or their transformation into other haemocytes, particularly into GRs as reported by Beaulaton & Monpeysson (1976) and George (1996). Whatever the reason is, this reduction in PLs reveals that abamectin is toxic and lethal to the cellular defensive system of final instar larvae of *C. septempunctata*. Tikku *et al.* (1992) and George & Ambrose (2004) also recorded reduction percentage in PLs of adult *R. kumarii* due to tested organophosphate insecticides that were found to be highly toxic to the treated organism.

In the present study, the percentage of PRs decreased after the application of all tested bio-insecticides as investigated by Tikku *et al.* (1992) and George & Ambrose (2004) in the adult of *Dysdercus koenigii* F. (Pyrrhocoridae: Hemiptera) against azadirachtin and *R. kumarii* against malathion respectively. The PRs serve as stem cells in the haemolymph (Silva *et al.* 2002) and reduction in PRs could be correlated to the greater transformation of PRs into other type of cells which play their role in phagocytosis (Bhatti, 2002; Saxena & Srivastava, 2001) or ablation of haemopoietic organs, responsible for the production of PRs (Tiwari *et al.* 2002).

Granular haemocytes are characterized by the possession of acidophilic granules which are membrane bounded. These are involved in the detoxification of chemicals and killing of microorganism through encapsulation and phagocytosis (Saxena & Srivastava, 2001; Chapman, 1998; Steinhaus, 1949). The increase observed in GRs after exposure to azadirachtin and abamectin might be attributed to the greater transformation of PLs and PRs into GRs during detoxification (George & Ambrose, 2004) and correlated with greater role played by GRs in detoxification through phagocytosis (Jose & Martin, 1989; Kurihara *et al.* 1992). George & Ambrose (2004) and Sharma *et al.* (2003) also recorded the greatest increase in GRs in methyl parathion-exposed individuals of *R. kumarii* and in azadirachtin-exposed sixth-instar larvae of *Spodoptera litura* F. (Noctuidae: Lepidoptera). However decrease in GRs due to spinosad treatments as recorded by George & Ambrose (2004) in endosulfan-exposed individuals of *R. kumarii*, might be due to the disintegration of GRs, initiated by vacuolization and loss of compactness of organelles leading to degranulation and degenerative transformation (Sharma *et al.*

2003). This reduction might be the result of GRs destruction due to the extensive blabbing of their cellular membrane as result of insecticide toxicity as recorded by Sewify & Hashem (2001) in the larvae of *Galleria mellonella* L. (Pyrilidae: Lepidoptera) infected with *Metarhizium anisopliae* (Metsch.).

Spherulocytes and OEs were observed the least affected haemocytes by three bio-insecticides tested in the present investigations; probably these cells are not involved in the detoxification and phagocytosis (Crossley, 1964).

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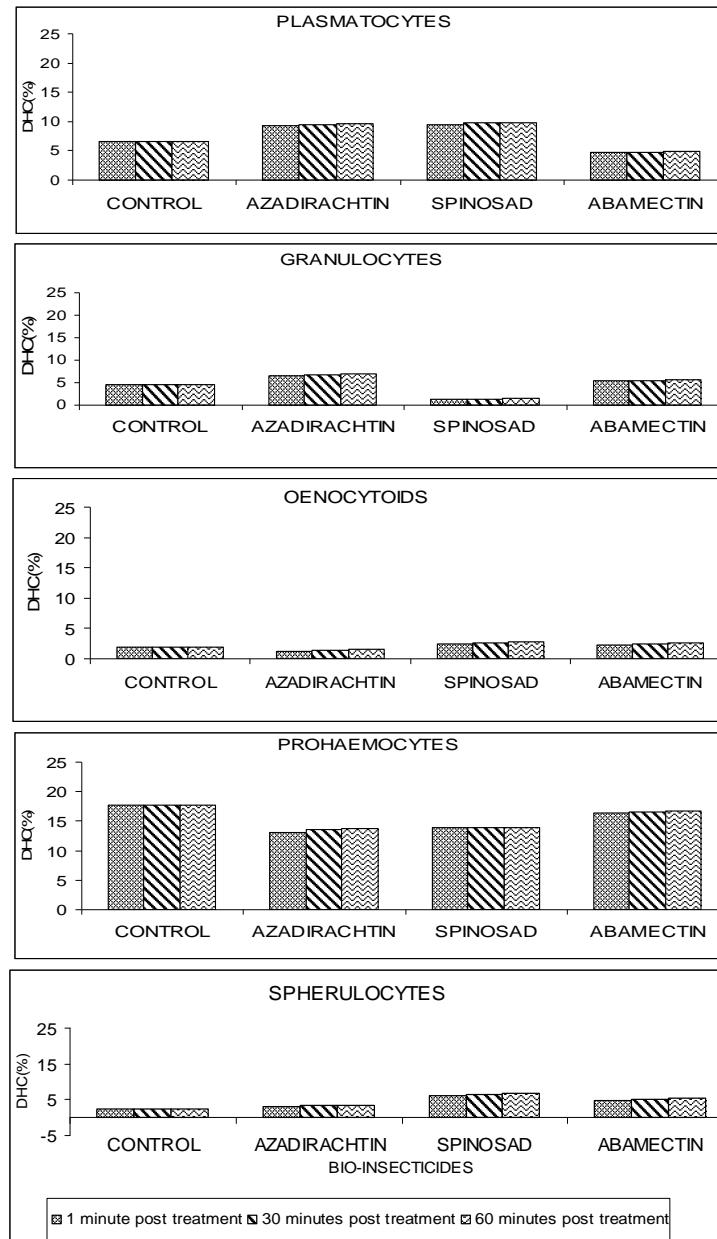
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**Table 1. Effect of various treatments of azadirachtin, spinosad and abamectin on the total haemocyte count per mm<sup>3</sup> of the last instar larvae of *Coccinella septempunctata***

Post-treatment time	Control	Azadirachtin	Spinosad	Abamectin
One minute	7612.17±38.6 <sup>NS</sup>	10811.33±198.1*	8998.17±47.9*	4056.83±224.3*
30 minutes	7612.17±38.7 <sup>NS</sup>	12389.67±513.5*	4859.67±76.5*	10420.83±425.2*
60 minutes	7612.17±38.8 <sup>NS</sup>	9278.17±59.2*	10804.33±552.8*	4327.67±173.6*

<sup>NS</sup> non-significant at P = 0.05; \*significant at P < 0.001



**Fig. 1. Effect of sublethal concentrations of azadirachtin, spinosad and abamectin on the differential haemocyte count in the last instar larvae of *Coccinella septempunctata*.**

