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EFFECT OF FLUBENDIAMIDE AND SPIROTETRAMAT ON THE HAEMOCYTES OF AMERICAN BOLLWORM, *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE)

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ABSTRACT

The effect of flubendiamide and spirotetramat (LC_{40}, LC_{50} and LC_{60}) on total and differential haemocyte was evaluated on 3rd and 5th instar larvae of *Helicoverpa armigera*. The total haemocyte count at LC_{50} of flubendiamide increased just after application and became (69300 cells/mm^3), decreased (52300 cells/mm^3) after half an hour, and increased again (68980 cells/mm^3) from normal (68175 cells/mm^3) after an hour. The differential haemocyte count, plasmatocytes decreased from normal 18% to 12%, whereas the percentage of prohaemocytes and, spherulocytes increased from normal (42%, 29% to 51%, 33%). The percentage of cystocytes and granulocytes decreased from normal (2.75% and 8.25% to 0%, 2%, respectively after the application of insecticide in 3rd instar larvae. Similar results were recorded with 5th instar larvae. The total haemocyte count just after the application of spirotetramat in 3rd instar larvae increased (78900 cells/mm^3) from the normal count, while decreased (64700 cells/mm^3) after half an hour, and increased again (80900 cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3). The differential haemocyte count, plasmatocytes increased from normal 18% to 28%. The percentage of prohaemocytes and granulocytes decreased from normal (42% and 8.25% to 34% and 5.5% repectively), whereas percentage of cystocytes increased from normal (2.75% to 4%) after the application of insecticide in 3rd instar larvae. Similar results were recorded in 5th instar larvae of *H. armigera*.

Keywords: Flubendiamide, Haemocytes, *Helicoverpa armigera*, spirotetramat

INTRODUCTION

Insects have cellular and humoral defensive systems within their body which protect them against the infectious organisms. These immune mechanisms eliminate the foreign tissues after identification (Panagiotakos, 2000). The cellular immune system comprises of haemocytes which circulate within the blood or often attach with the tissues of the insect body, while humoral immune response includes soluble molecules production (Strand, 2008). Haemocytes orignates from the embryonic median mesoderm tissues and then through embryogensis are differentiated into different types (Klowden, 2002). The number of circulating cells present in the haemolymph of insect is generally from 25000-100,000 per cubic mm. In small insects this number is much more less than the large insects *e.g.* mosquito female has 10000 haemocytes cells while 9000,000 cells in the adult of *Periplanata spp.* (Champman, 1998).

Immunosurvillence cells (haemocytes) play pivotal role in various physiological processes and insect immunity. These cells, through wound signals contribute in wound healing (Yamashita and Iwabuchi, 2001). These cells aid in the transport of nutrients to different tissues and can also store them. Phagocytosis, encapsulation, nodule formation and coagulation are also important function (Sabri and Tariq, 2004). Phagocytosis is an important function of these cells through which micro-organisms and foreign particals are engulfed. Among various other cell types which are responsible for phagocytosis, thus plasmatocytes play most important role. These cells are also involved in detoxification mechanisms, hormones secretion helping for growth stimulation, protein synthesis, phenol metabolism and

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nutrients storage (Parkash, 2008). Generally haemocytes are damaged by various ways but a number of authors have also observed the defensive mechanism against the toxicants. Most of the authors indicated that in recovery mechanism metabolism of lipids is involved but few claims that haemocytes themselves are involved in detoxication. They play an important role in insecticide detoxification in the transport action of hormones in endocrine system (Patnaik, 2002). These hormones are carried through haemocytes to the target organs and tissues. Haemocytes synthesize and store proteins, glycol-proteins, lipoproteins, amino acids and several other nutrients (Parkash, 2008).

Helicoverpa. armigera is one of the most important and destructive pest of many crops that had a wide geographical distribution (Cunningham *et al*., 1999). It is widely distributed in Asia, Europe, Australia, Canada, Africa, Manitoba, Mexico, USA, Brazil, Peru, Argentina, and America (Gowda, 2005). This polyphagous pest can damage more than 182 plant species including cotton, sunflower, chickpea, sorghum, groundnut, tobacco, alfalfa, clover, flax, maize and various vegetable crops like tomato, pepper, okra, lettuce, cabbage, eggplant, broccoli, beans, pea, fruits i.e. strawberry, watermelon and forest trees. (Gowda, 2005; Annonymous, 2007).

The objective of the present study was to examine the effect of flubendiamide and Spirotetramat, on the total haemocyte count, differential haemocyte count, and to observe the abnormalities caused by these insecticides in the haemocytes of *H. armigera.*

MATERIALS AND METHODS

Collection and rearing *of H. armigera*:

A field population of *H. armigera* (third to fifth instar larvae) was collected from the fields of cotton, tomato in August 2011. This population was reared at 25 ± 2 C, 60 ± 5% RH with 16:8 (light : dark) cycle. The larvae were provided with artificial diet until pupation. The pupae were shifted in a plastic box lined with tissue paper. On adult emergence, individuals were transferred to transparent rearing jars and fed with 10% sugar solution. Nappy liner strips were hanged in the rearing cages for egg laying. Eggs were collected on daily basis. After hatching, neonates were shifted to the artificial diet. 3rd and 5th instar larvae of *H. armigera* were used in experiments.

Insecticides:

Two different insecticides, Belt® 48SC, (Flubendiamide) and Movento® 240SC, (Spirotetramat) were used in the experiment.

Toxicity Bioassays:

Topical bioassays were conducted with 3rd and 5th instar larvae. Five concentrations of each insecticide were prepared in distilled water with Triton X-100 (50 µg / ml) as an additional surfactant. A control containing only water was also used. Each concentration was applied on individual insect with the help of micro-applicator at the rate of 3µl per insect. After the treatment one individual insect were shifted in a Petri dish containing artificial diet. Mortality data was noted after 7 days. Larvae that failed to respond to gentle contact with a fine brush was considered as dead.

3µl of LC_{40}, LC_{50} and LC_{60} concentrations of each insecticide were applied topically to 3rd and 5th instars larvae with the help of a microapplicator for heamocytes studies. Effect of these insecticides was observed immediately after application, after 30 minutes and after 60 minutes in terms of abnormalities in total haemocyte count and differential haemocyte count.

Differential Haemocyte Count:

DHCs were estimated by taking a drop of haemolymph on a clean glass slide and preparing the smear. The air-dried smears were dipped in methyl alcohol for 5-7 minuts. The smears were then air dried. Immerse the dried film in the Wright's stain for 15 minutes. Then Wash the slides with distilled water at pH 6.8. Neutralize the haemocyte contents in freshly prepared buffer solution of pH 6.6 for 15 minutes and air dried the slides. Counting was done with the help telecounter under Phase contrast microscope 10X. A minimum of 200 cells were counted each time and the percentage of various classes were calculated as described by Mahmood and Yousaf (1985).

Total Haemocyte Count:

Neubauer haemocytometer was used for total haemocyte counting. Standard sampling of the haemolymph was carried out with a Thoma white blood cell diluting pipette. Haemolymph from the abdominal leg was collected on a glass slide and then quickly drawn into Thoma white blood cell diluting pipette upto mark 0.5. This was diluted 20 times with Toisson's solution (NaCl = 1.0 gm, Na_2SO_4 = 8.0 gm, neutral glycerine = 30ml, Methyl violet = 0.025 gm and distilled water =160ml) and Thoma white blood cell diluting pipette was filled upto mark II (Mahmood and Yousaf, 1985). This solution was properly stained with staining shaker for 5 minute it prevents the blood to coagulate. Three initial drops of haemolymph mixture were disposed off and one drop of haemolymph mixture was placed near the edge of the coverslip of the Neubauer haemocytometer (Jones, 1962; George and Ambrose, 2004). The counting chamber was filled automatically by capillary action. Haemocytometer was left for 5 minutes so that the blood cells could settle down and then observed the haemocytes under Phase contrast microscope 10X. The four corner squares of both chambers of haemocytometer were counted under low power followed by high power of microscope focusing the counting chamber. Cells of each group of 16 squares, touching bordering the bottom left hand side and the central line were counted. Cells touching the central line in the top and right hand side were not included in the count (Anonymous, 1984). Total haemocyte counting (THC) was done following the formula described by Jones (1962).

Actual number of cell per cubic mm = Average number of cell counted per square millimeter × depth × dilution per square millimeter.

Statical Analysis

Lc_{40}, LC_{50} and LC_{60} values of the insecticide were determined with R version 2.9.0 (R. Development Core Team, 2009).

RESULTS AND DISCUSSION

Toxicity to 3rd and 5th instar larvae of *H.armigera.*

Lc_{40}, LC_{50} and LC_{60} values of flubendiamide against 3rd instar larvae were 0.0002, 0.0003 and 0.0005 respectively ppm respectively (Table 1). Silmilarly, LC_{40}, LC_{50} and LC_{60} values for 5th instar larvae were 0.0008, 0.001 and 0.002 ppm respectively (Table 1). LC_{40}, LC_{50} and LC_{60} values of Spirotetramat against 3rd larval instar were 0.70, 1.44 and 3.00 ppm respectively (Table 1). Similarly LC_{40}, LC_{50} and LC_{60} values for 5th instar larvae were 2.33, 7.92 and 27.11 ppm respectively (Table 1).

Total Haemocyte Count For 3rd and 5th Instar Larvae Of untreated *H. armigera*

On an average there were 68175 blood cells/mm in the haemolymph of 3rd larval instar of *H. armigera* were observed (Table 2). Similarly, in 5th instar larvae on an average 7428.5 blood cells/mm^3 haemocytes were recorded (Table 3).

Differential Haemocyte Count For 3rd and 5th Instar Larvae Of untrated *H. armigera*

Percentages of differential haemocyte counts (DHC) in control larvae of 3rd instar were prohaemocyte (42.00%), spherulocytes (29%), plasmatocytes (18.00%), cystocytes (2.75%) and granulocytes (8.25%). The percentage of prohaemocyte is the highest (41.00%) followed by spherulocytes (25.75%), plasmatocytes (15.00%), oenocytoids (16.00%), cystocytes (3.5%) and granulocytes (2.25%) in 5th instar larvae.

Total and differential haemocyte counts in 3rd and 5th instar larvae of *H.armigera* treated with flubendiamide.

a) Effect at LC_{40}

The total haemocyte count just after the application of flubeniamide in 3rd instar larvae increased (68800 cells/mm^3), while decreased (55400 cells/mm^3) after half an hour, and increased again (67200 cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3) (Table 2). Similar results were reportd in another study in which total haemocytes increased just after the application and then decresed after half an hour and again increased after one hour of application of acetamaprid against *Dysdercus koenigii* adults (Haq *et al.,* 2005) The differential haemocyte count, plasmatocytes decreased from normal 18% to 15%, whereas

Table 1.
Lc_{40}, LC_{50} and LC_{60} Values of flubendiamide and spirotetramat against 3rd and 5th Instar larvae of *H. armigera.*

Insecticides	3rd instar				5th instar			
	LC_{40} ppm (95%CI)	LC_{50} ppm (95% CI)	LC_{60} ppm (95% CI)	Slope±SE	LC_{40} ppm (95%	LC_{50} ppm (95% CI)	LC_{60} ppm (95% CI)	Slope±SE
spirotetramat	0.70 (0.38-1.29)	1.44 (0.71- 2.9)	3.00 (0.98-9.18)	0.55±0.21	2.33 (0.44-12.3)	7.92 (0.42-150)	27.11 (0.26-28)	0.33±0.22
flubendiamide	0.0002 (6.49-0.001)	0.0003 (0.001-0.0002)	0.0005 (.0002-0.0014)	1.07±0.34	0.0008 (0.0005-0.001)	0.001 (0.0008-0.002)	0.002 (0.001-0.003)	1.88±0.37

Table 2
Total number of haemocyte /mm in control and treated (with flubendiamide) 3rd and 5th instars larvae of *H. armigera.*

Time (Minutes)	Control		LC_{40}(ppm)		LC_{50} (ppm)		LC_{60} (ppm)	
	Instars							
	3rd	5th	3rd	5th	3rd	5th	3rd	5th
0	68175	7428.5	68800	8157.5	69300	8605	70000	8975
30	68175	7428.5	55400	5815	52300	5147	50200	4727.5
60	68175	7428.5	67200	10025	68980	11570	72800	13087.5

Table 3
Differential haemocytes in control and treated with flubendiamide 3rd and 5th instar larvae of *H. armigera.*

Differential haemocytes %	Control		LC_{40}(ppm)		LC_{50} (ppm)		LC_{60} (ppm)	
	Instars							
	3rd	5th	3rd	5th	3rd	5th	3rd	5th
PR	42	41	47	48	51	50	53	53
SP	29	25.75	32	31.25	33	33	37	36
PL	18	15	15	13.25	12	10	8	8.5
CO	2.75	3.5	1	0	0	0	0	0
GR	8.25	2.25	3	0	2	0	0	0
OE	0	16	0	7.25	0	6	0	4

the percentage of prohaemocytes, spherulocytes increased (42% and 29% to 47% and 32% repectively) and percentages of cystocytes, and granulocytes decreased from normal 2.75% and 8.25 to 1 and 3 respectively after the application of insecticide in 3rd instar larvae (Table 3). The total haemocyte count just after the application of flubendiamide in 5th instar larvae increased (8157.5 cells/mm^3), while decreased(5815 cells/mm^3) after half an hour, and increased again (10025cells/mm^3) after one hour of application from the normal count (7428.5 cells/mm^3) (Table 2). The results of the present study are in accordance with the results of Abdin *et al*., (2002) who recorded the effect of Nimbokill 60 EC on the last larval instar of *Chrysoperla carnea*. The percentage of plasmatocytes prohaemocytes, spherulocytes, oenocytoids, cystocytes and granulocytes fluctuated from normal 15%, 41%, 25.75%, 16%, 3.5% and 2.25% to 13.25%, 48%, 31.25%, 7.25%, 0% and 0% respectively (Table 3). These results are similar with the findings of Abbas (2008) who noted that percentages of plasmatocytes decreased after the application of Lorsbn 40 EC, Match50 EC and Abamectin 1.8 EC used against *Papilio demoleus* L.

Effect at LC_{50}

The total haemocyte count just after the application of flubeniamide in 3rd instar larvae increased (69300cells/mm^3), while decreased (52300 cells/mm^3) after half an hour, and increased again (68980 cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3) (Table 2). The differential haemocyte count, plasmatocytes decreased from normal 18% to 12%, whereas the percentage of prohaemocytes and, spherulocytes increased from normal (42%, 29% to 51%, 33%). The percentage of cystocytes and granulocytes decreased from normal (2.75% and 8.25% to 0%, 2%, respectively after the application of insecticide in 3rd instar larvae (Table 3). The total haemocyte count just after the application of insecticide in 5th instar larvae increased (7428.5cells/mm^3), while decreased (5147cells/mm^3) after half an hour, and increased again (11570 cells/mm^3) after one hour of application from the normal count (7428.5 cells/mm^3) (Table 2). The percentage of plasmatocytes, prohaemocytes, spherulocytes, oenocytoids , cystocytes, and granulocytes decreased from normal 15%, 41%, 25.75%, 16% ,3.5% and 2.25% to 10%, 50%, 33%, 6%, 0% and 0% respectively after the application of insecticide in 5th instar larvae (Table 3).

b) Effect at LC_{60}

The total haemocyte count just after the application of insecticid in 3rd instar larvae increased (70000cells/mm^3), while decreased (50200cells/mm^3) after half an hour, and increased again (72800cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3) (Table 2). The differential haemocyte count, plasmatocytes decreased from normal 18% to 8%, whereas the percentage of prohaemocytes and, spherulocytes increased from normal (42%, 29% to 53%, 37%). The percentage of cystocytes and granulocytes decreased from normal (2.75% and 8.25% to 0%, 0%) respectively after the application of insecticide in 3rd instar larvae (Table 3). The total haemocyte count just after the application of in 5th instar larvae increased (8975cells/mm^3), while decreased (4727.5 cells/mm^3) after half an hour, and increased again (13087.5 cells/mm^3) after one hour of application from the normal count (7428.5 cells/mm^3) (Table 2). The percentage of plasmatocytes, prohaemocytes, spherulocytes, oenocytoids , cystocytes, and granulocytes flucuated from normal 15%, 41%, 25.75%, 16% ,3.5% and 2.25% to 8.5%, 53%, 36%, 4%, 0% and 0% respectively after the application of insecticide in 5th instar larvae (Table 3).

Total and differential haemocyte counts in 3rd and 5th instar larvae of *H.armigera* treated with spirotetramat.

a) Effect at LC_{40}

The total haemocyte count just after the application of spirotetramat in 3rd instar larvae increased (78900 cells/mm^3) from the normal count, while decreased (64700 cells/mm^3) after half an hour, and increased again (80900 cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3) (Table 4). These results are in accordance with the findings of Sabir (1994) who treated Larvin 80DF on *H.*

armigera and reported that after 0 minutes the THC increased, decreased after half an hour and again increased after the an hour. The differential haemocyte count, plasmatocytes increased from normal 18% to 24.5%, whereas the percentage of prohaemocytes, spherulocytes and granulocytes decreased from normal 42%, 29%, and 8.25% to 36%, 27%, and 7.75% respectively after the application of insecticide in 3rd instar larvae (Table 5). These results are similar to the findings of Bibi (2001) who noted that percentages of plasmatocytes increased, prohaemocytes, spherulocytes and granulocytes decreased after the application of Polo and Endosulfan on adult *Apis mellifera* L.

Similarly, the total haemocyte count just after the application of spirotetramatin 5th instar larvae increased (9187.5 cells/mm^3), while decreased (4825 cells/mm^3) after half an hour, and increased again (11550 cells/mm^3) after one hour of application from the normal count (7428.5 cells/mm^3) (Table 4). This is in partial accordance with those of Sabir (1994) who treated Larvin 80DF and Detaphos-R 350 +10EC against the last instar larvae of *H. armigera*. The differential haemocyte count, the percentage of plasmatocytes increased from normal (15% to 26%). These results are in conformity with the findings of Abbas (2008) who noted that the percentages of plasmatocytes increased after the application of Emamectin 1.9 EC on the last instar larvae of *Papilio demoleus*. The percentage of prohaemocytes, oenocytoids, cystocytes and granulocytes decreased from normal, 41%, 16%, 3.5% and 2.25% to 38.5%, 12%, 3% and 2% respectively (Table 5).

Effect at LC_{50}

The total haemocyte count just after the application of insecticide in 3rd instar larvae increased (81200 cells/mm^3), while decreased (62200 cells/mm^3) after half an hour, and increased again (83200 cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3) (Table 4). The differential haemocyte count, plasmatocytes increased from normal 18% to 28%. The percentage of prohaemocytes and granulocytes decreased from normal (42% and 8.25% to 34% and 5.5% repectively), whereas percentage of cystocytes increased from normal (2.75% to 4%) after the application of insecticide in 3rd instar larvae (Table 5). Similarly, The total haemocyte count just after the application of spirotetramat in 5th instar larvae increased (8200 cells/mm^3), while decreased (5725 cells/mm^3) after half an hour, and increased again (11375 cells/mm^3) after one hour of application from the normal count (7428.5 cells/mm^3) (Table 4). The percentage of plasmatocytes increased from normal, 15% to 30%, whereas the percentage of prohaemocytes, oenocytoids, cystocytes and granulocytes decreased from normal, 41%, 16%, 3.5% and 2.25% to 32%, 13.5%, 2.25% and 1% respectively in 5th instar larvae (Table 5).

b) Effect at LC_{60}

Similarly, the total haemocyte count just after the application of spirotetramat in 3rd instar larvae increased (83400

Table 4
Total number of haemocyte/mm in control and treated with spirotetramat 3rd and 5th instars larvae of *H. armigera.*

Time (Minutes)	Control		LC_{40}(ppm)		LC_{50} (ppm)		LC_{60} (ppm)	
	Instars							
	3rd	5th	3rd	5th	3rd	5th	3rd	5th
0	68175	7428.5	78900	9187.5	81200	8200	83400	7700
30	68175	7428.5	64700	4825	62200	5725	60900	9500
60	68175	7428.5	80900	11550	83200	11375	85800	12370

Table 5
Differential haemocytes in control and treated with spirotetramat 3rd and 5th instar larvae of *H. armigera.*

Differential haemocytes %	Control		LC_{40}(ppm)		LC_{50} (ppm)		LC_{60} (ppm)	
	Instars							
	3rd	5th	3rd	5th	3rd	5th	3rd	5th
PR	42	41	36	38.5	34	32	32	31
SP	29	25.75	27	30.5	29	26	30	20
PL	18	15	24.5	26	28	30	30	34
CO	2.75	3.5	3.75	3	4	2.25	4.5	1
GR	8.25	2.25	7.75	2	5.5	1	4	0.75
OE	0	16	0	12.5	0	13.5	0	18.5

cells/mm^3), while decreased (60900 cells/mm^3) after half an hour, and increased again (85800 cells/mm^3) after one hour of application from the normal count (68175 cells/mm^3) (Table 4). The differential haemocyte ount, plasmatocytes increased from normal 18% to 30%. The percentage of prohaemocytes and granulocytes decreased from normal (42% and 8.25% to 32%, 4% respectively) whereas, the percentage of spherulocytes and cystocytes increased from normal (29% and 2.75% to 30% and 4.5% respectively) after the application of insecticide in 3rd instar larvae (Table 5).

Similarly, the total haemocyte count just after the application, after half an hour and after one hour in 5th instar larvae increased (7700 cells/mm^3, 9500 cells/mm^3 and 12370 cells/mm^3 respectively) from the normal count (7428.5 cells/mm^3) (Table 4). The percentage of plasmatocytes increased from normal, 15% to 34%, whereas the percentage of prohaemocytes, spherulocytes, cystocytes and granulocytes decreased from normal, 41%, 25.75%, 3.5% and 2.25% to 31%, 20%, 1% and 75% respectively in 5th instar larvae (Table 5). The plasmatocyres play more efficient role in phagocytosis. When the foreign particals were injected in the insects, these free blood cells increased in number, phagocytosed the foreign materials and enhanced the defensive system of the insect making it more resistant against insecticides. Belt proved to be more affective against both instars of *H. armigera.* A decrease in the number of plasmatocytes was observed in 3rd and 5th instar larvae of *H. armigera* after the application of Belt. In conclusion both the insecticide effect the haemocytes in the haemolymph of *H.armigera*.

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