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EFFECT OF A BIOINSECTICIDE AGAINST *LUCILIA CUPRINA* (WIED) (CALLIPHORIDAE : DIPTERA)

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Abstract: The toxic effect of *Bacillus thuringiensis* on the haemolymph of adult *Lucilia cuprina* was observed. LD_{50} was calculated. The treatment resulted in the destruction and vacuolization of plasma and haemocytes. Differential haemocyte counting of the treated flies showed quantitative changes in the haemocytes as compared to the control ones.

Key words: *B. thuringiensis, Lucilia cuprina*, haemolymph.

INTRODUCTION

The unplanned and indiscriminate use of various insecticides to control them is a serious threat to human health as well as to other biotic factors of the ecosystem and environment. The death of the target as well as the non-target organisms is also a major drawback of these insecticides (Edward *et al.*, 1987; Khillare and Waghs, 1988; Reddy and Bashamohideens, 1989). The other major drawback is the development of resistance which has been reported for a variety of insects *e.g.*, insecticides such as organophosphates, carbamates and pyrethroid have failed to control German cockroach (Schal, 1988; Cochran, 1989; Zhai and Robinson, 1994), Diamond back moth (Tabashnik, 1994), sheep blow fly (Kotze, 1995; Levot, 1995), house fly (Lalah *et al.*, 1995) and mosquito larvae (Mazarri and Georghiou, 1995; Karunaratue *et al.*, 1995) due to the development of resistance.

Keeping in view the hazards of the chemical insecticides, the alternate and relatively safe means *i.e.*, biological pesticides are now kept in focus. One of the strategies involves the use of microorganisms to control those insects whose activities pose serious problems for the mankind (Mittal *et al.*, 1991; Pietrantonio and Gill, 1992; Orduz *et al.*, 1992).

The stress has been imposed on the spore forming bacteria, some of which have proved to be the best pathogens against them. *Bacillus thuringiensis* Kurstaki, HD-1 is one of the widely used bioinsecticides (Teiltelson *et al.*, 1992). The crystal proteins of *B. thuringiensis* have been extensively studied because of their pesticidal properties (Crickmore *et al.*, 1998). It is a rod shaped aerobic, gram-positive spore forming bacterium, which during sporulation produces crystalline structure. These crystals possess insecticidal crystal proteins ICP or endotoxin (Hofte and Whitlley, 1989; Lereclus *et al.*, 1989; Adang, 1991). It is non-pathogenic for mammals including man.

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These proteins can be mixed with food attractants, which increase the normal rate of feeding. Manasherob *et al.* (1994) found the toxicity of *B. thuringiensis* israelensis increased three times when used after encapsulation in *Tetrahymena pyriformis*. This decreased the natural life span and gave efficient control of *Aedes aegypti* larvae.

The object of the present work was to find the susceptibility of *Lucilia cuprina* to *B. thuringiensis* Kurstaki. This blow fly was selected because of its veterinary and medical importance. It is major contributor to fly strike in Australia (Graham, 1979) and is found in more than 70% of all strikes in New Zealand (Heath and Bishop, 1995) and other sheep rearing countries.

MATERIALS AND METHODS

Rearing of the insects

The colonies of flies were maintained at 30°C, 12 hours photoperiod and relative humidity ranging from 65% to 70%. The larvae hatched out from the eggs after about 24 hours. Two more moultings occurred at two days interval. The 3rd instar larvae thus obtained moved away from the food approximately at the end of the second day but remained mobile for 4-6 hours after which they settled down. It indicated the onset of the pupal life. The adults emerged nearly after five days.

Bacterial inoculation

The strain of *Bacillus thuringiensis* Kurstaki, HD-1 was obtained from Centre of Excellence in Molecular Biology (CEMB) (Fig.1). These bacteria were reared on nutrient agar. For the inoculation of bacteria into the insects 10,000 times dilution of the nutrient broth was prepared and number of colonies obtained after 24 hours on the nutrient agar plate were calculated.

One ml of sterilized milk-sugar solution along with 8 ml of bacterial culture was poured in each jar. This was considered to be the treated dose. For the control only 8 ml of nutrient broth was given along with 1 ml of milk sugar solution.

Estimation of LD_{50}

Three concentrations (2 ml, 4 ml, 6 ml) were selected for calculating LD_{50}. Three replicates were set up. Mortality was noted after 48 hours.

Blood film formation

For the blood film formation the insects were exposed to hot glacial acetic acid vapours for 5 to 10 min. Then a drop of blood was obtained on the slide. After spreading it with the help of a coverslip, the film was stained with Giemsa's or Wright's stain. It was then cleared in xylene and mounted in Canada balsam.

Differential haemocyte count

DHC was done by marking a spot in a film randomly. All the cells in the marked spot were counted and categorized. Approximately 200 cells/ experimental stage were

counted and classified. Those cells which appeared to be intermediate between any two types were divided equally between two types following the method adopted by Nappi (1970).

RESULTS

The blood or haemolymph of *Lucilia cuprina* is contained in the general body cavity, as is the case in all the insects and has two components, the plasma, which is the liquid part and the haemocytes or the blood cells.

Eight types of haemocytes were distinguished on the basis of light microscopy, which are, prohaemocytes, plasmatocytes, podocytes, granular cells, cystocytes, oenocytoids, vermiform cells and spherule cells.

Prohaemocytes (Fig.2a)

These are small to medium sized cells varying from 6.0 to 11.0 μm. They are round or ellipsoidal and occasionally fusiform. The nucleus is central and occupies almost all the cell body so that cytoplasm forms only a narrow rim around it. These cells have smooth and regular boundary. The nucleus is usually spherical or ovoidal ranging from 3.5 to 6.5 μm in diameter. These cells are usually deeply basophilic but the nucleus always stains more intensely. In the large cells however, it is slightly eosinophilic and chromatin is then clearly granular and evenly distributed. Many of these cells can be seen undergoing both equal and unequal division. These are germ or stem cells (Rowley and Ratcliffe, 1981).

Plasmatocytes (Fig.2b)

They are highly polymorphic haemocytes and larger in size as compared to prohaemocytes. Although typically they tend to be ovoidal in shape but round, fusiform, spindle-shaped and irregular forms are also common. They have generally centrally located large nucleus which in some cells almost fills the entire cell body. The round plasmatocytes are less than 17 μm in diameter. These cells have moderately basophilic cytoplasm but the nuclei are eosinophilic with granular chromatin. The vacuoles are probably the result of the release of the granules from the cell body. In this preparation many intermediate forms between prohaemocytes and plasmatocytes can be seen. Plasmatocytes have more cytoplasm surrounding their nuclei as compared to that of prohaemocytes, very few haemocytes of this class were seen dividing.

Podocytes (Fig.2h)

These cells have long and tapering cytoplasmic extensions of variable length. The length of these extensions, arms or filopodia varies from 5 to 25 μm beyond the cell body which is in these cases oval. These arms were fixed in position and are not pseudopodial in nature. Some of these cells have fusiform bodies with the two tapering sides extending into long arms. The cytoplasm of these cells is basophilic and finely granular, while nucleus is eosinophilic. Fusiform cells vary from 16x8 μm to 25x10 μm in dimensions.

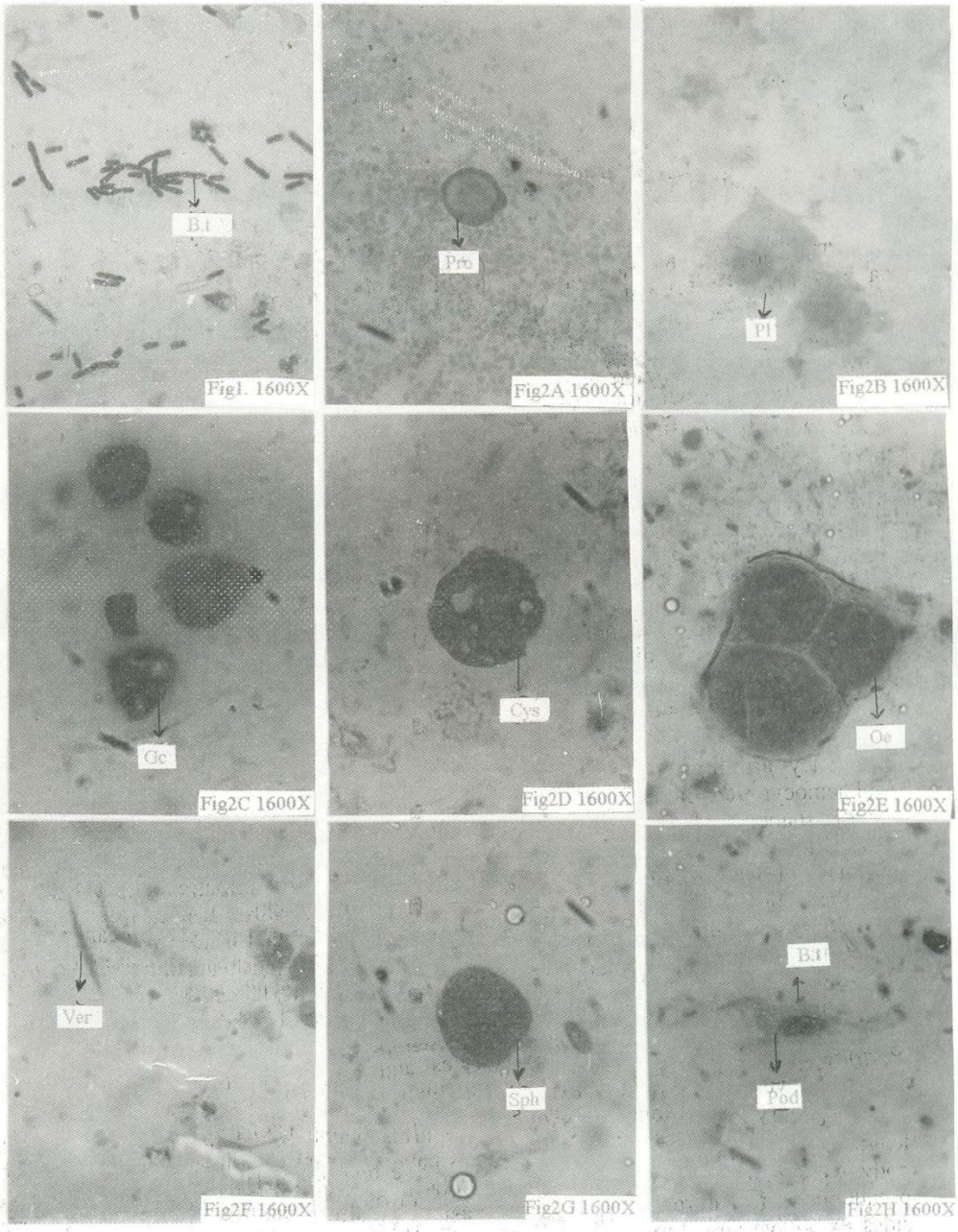

Fig. 1: Clusters of *B. thuringiensis* chains

Fig. 2: Control (a-h) a, Prohaemocyte; b, Plasmatocyte; c, Granulocyte; d, Cystocyte; e, Oenocytoid; f, Vermiform cells; g, Spherule; h, Clumping of cells.

Granular cells (Fig.2c)

These are compact cells of variable size, usually round or disk-shaped, with a relatively small nucleus enveloped in a large volume of cytoplasm which characteristically contains many prominent granules. The size of these granules can vary between 2 and 3 μm in diameter. These cells when round vary from 10 to 25 μm in diameter but when oval they are from 10 to 25 μm wide and 15 to 35 μm long. Their nuclei are eosinophilic and smaller in size as compared to plasmatocytes nuclei and vary from 5 to 10 μm in diameter. Apart from the granules, the cytoplasm also contains vacuoles and vesicles of different sizes.

Many intermediate forms between plasmatocytes and granular cells were observed. Some of the transitional forms were difficult to place as the resemblance was so close to the plasmatocytes.

Cystocytes (Fig.2d)

These cells are round, ellipsoidal or slightly irregular in shape with an occasional broken cell wall. They have a small nucleus as compared to that of the plasmatocytes or granular cells. The cytoplasm is either moderately or slightly basophilic with a round acentric eosinophilic nucleus. These cells vary from 2 to 4 μm in width and 8 to 12 μm in length.

Oenocytoid cells (Fig.2e)

These are usually slightly larger in size than granular cells and are round, ovoidal and sometimes irregular in shape. Their size varies from 12 to 30 μm in width and 15 to 40 μm in length and when round from 12 to 35 μm in diameter. They have a large quantity of homogenous cytoplasm which is almost neutrophilic. The nucleus range from 5 to 8 μm in width and 6 to 10 μm in length.

Vermiform cells (Fig.2f)

These cells are extremely elongated and thin with finely granular basophilic cytoplasm that extends and tapers into long arms. They vary from 16 to 50 μm in length but their width is very small varying from 3 to 6 μm. The comparatively thick central part sometimes also houses an elongated eosinophilic nucleus ranging from 6x3 μm to 9x5 μm in dimensions.

Some of these cells are without any apparent nucleus and have a finely granular cytoplasm in their bodies. Granules are not closely packed. Tuzet and Manier (1959) have called them the "giant fusiform cells".

Spherule cells (Fig.2g)

These cells are very conspicious of their large size and spherular inclusions. They are round, ovoidal and sometimes irregular in shape. They vary from 20 to 100 μm in diameter when round and are from 20 to 80 μm wide and from 25 to 120 μm in diameter. Spherule cells have very little amount of cytoplasm as most of the cell body is filled up with spherules, the large number of which often obscures the nucleus and also

distend the cell periphery. All the cell contents are basophilic. The nucleus is more deeply stained than the cytoplasm.

When *Lucilia cuprina* was fed on different concentrations of *B. thuringiensis*, LD_{50} was noted to be 7 ml of its liquid culture.

Blood smear analysis

The blood of the flies fed with the liquid culture of *B. thuringiensis* was studied at twelve hourly intervals upto 48 hours when death occurred. A large number of abnormalities were noted which have been explained here in some detail.

After the first 12 hours of treatment, the direct microscopic observations of the blood showed many of the bacteria approaching the haemocytes and ultimately attaching themselves to the cell membranes (Fig.3a). After 24 hours, many bacteria were seen entering into the haemocytes themselves (Fig.3b,c). After 36 hours, clumping of

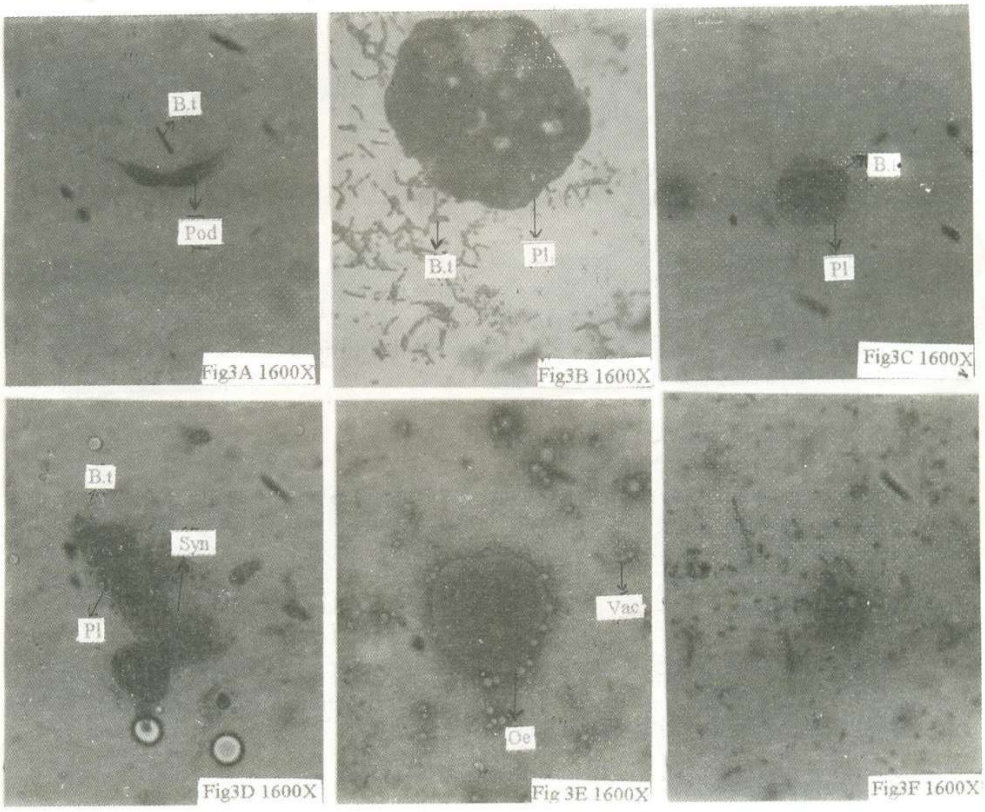

Fig. 3: *B. thuringiensis* treatments (a-f) a, *B.t* in the vicinity of Podocytes after 12 hours; b, Attachment of *B.t* to cell membrane of Plasmatocyte and vacuolization after 24 hours; c, *B.t* ingested by Plasmatocyte after 24 hours; d, Scattered cytoplasmic contents and synctium formation after 36 hours; e, Vacuolization in plasma after 36 hours; f, Destruction of plasmatocyte after 48 hours.

Table 1: **Effect of *B. thuringiensis* treatment on differential haemocytes count (DHC) at 40 x**

	Cell types																
	Pro		PC		GC		OE		POD		Sph		Cys		Verm		No.of cells Counted
	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	Total	%	
Control																	
	20	10	112	56	6	3	8	4	16	8	6	3	8	4	24	12	200
Experimental (B.t. fed)																	
12h	12	6	114	57	10	5	2	1	14	7	10	5	10	5	28	14	200
24h	18	9	124	62	8	4	4	2	16	8	8	4	8	4	14	7	200
36h	20	10	126	63	6	3	-	-	14	7	8	4	4	2	22	11	200
48h	32	16	128	64	4	2	-	-	12	6	4	2	-	-	20	10	200

Pro: Prohaemocytes; GC: Granular cells; Pod: Podocyte; Cys: Cystocyte; PC: Plasmatocytes; OE: Oenocytoids; Sph: Spherule cells; Ver: Vermiform cells.

Table 2: **Results of effects of haemocyte number at different durations (Mean of three replicates was taken).**

Types of haemocytes	Treatments								L.S.D. values of different durations of treatments	L.S.D. values of different durations of strains
	Control				B.t treatment					
	12 hrs	24 hrs	36 hrs	48 hrs	12 hrs	24 hrs	36 hrs	48 hrs		
Prohaemocytes	20	20	20	20	12	18	20	32	13.317	18.833
Plasmatocytes	112	112	112	112	114	124	126*	128*	9.89	13.98
Granulocytes	6	6	6	6	10	8	6	4	4.11	5.80
Oenocytoids	8	8	8	8	2	4	0*	0*	3.046	4.30
Podocytes	16	16	16	16	14	16	14	12*	2.59	3.67
Spherale cells	6	6	6	6	10	8	8	4	6.75	9.55
Cystocytes	8	8	8	8	10	8	4	0	7.05	9.98
Vermiform cells	24	24	24	24	28	14	22	20	9.19	12.98

Note: L.S.D. value at p=0.05

haemocytes took place. The membranes of these cells broke up and synctia were formed (Fig.3d). Plasma became thickened and vacuolization occurred (Fig.3e). After 48 hours, most of the haemocytes were found to be distorted which made it difficult to distinguish the different types of the blood cells (Fig.3f).

The bacteria were found to be mostly entering the plasmatocytes and granular cells, although former were affected more.

Differential haemocytes counting (DHC) (Table 1)

DHC showed that in the control male flies, prohaemocytes were found to be 10% plasmatocytes were 56%, granular cells were 8%, spherule cells were 3%, cystocytes were 4% and vermiform cells were 12%.

Prohaemocytes increased in percentage from 10% to 15% while plasmatocytes also increased from 56% to 64% as compared to the control. Granular cells decreased from 3 to 2%. Oenocytoids also decreased to 2% as compared to the 4% of the control specimen and disappeared altogether after 24 hours of the treatment.

Podocytes also decreased to 6% as compared to its 8% ratio in control flies. Similarly, spherule cells, cystocytes and vermiform cells also decreased to 2%, 2% and 10% respectively as compared to the 3%, 4% and 12% ratio in the corresponding controlled flies.

Statistical analysis

When compared statistically, it was found that there existed significant difference in the number of plasmatocytes, oenocytoids and podocytes in the treated as compared to the controls.

DISCUSSION

Microbiological control of insects is considered as an important aspect of biological control and can be defined as the use of entomopathogenic microorganisms for insect control (Ertola, 1988). One of the important reasons for current interest in the entomopathogens lies in the facts that they are sufficiently specific and do not affect beneficial insects. Nearly all entompathogen bacteria are from class "Schizomycetes".

Bacteria belonging to genus *Bacillus* produce endotoxins which are toxic to insects. The different species of *Bacillus* have already been used as bioinsecticides by various scientists. Chak and Young (1990) found *B. thuringiensis* toxic against *Bombyx mori*, *Aedes aegypti* and *Heliothis* sp.

Bioinsecticidal activity of *B. sphaericus* have also been tested against various insects (Krammer, 1990; Rady *et al.*, 1990). Several strains of *B. thuringiensis* have been found to be toxic to *Aedes aegypti*, *Anopheles gembiae* and *Culex quinquefasiatus* (Federici, 1995; Smith *et al.*, 1996).

The microbial insecticides are also facing resistance problem but rate of the development of resistance has been found to be very slow (Rao *et al.*, 1995). The

diamond back moth *Plutella xylostella* was the first insect to evolve resistance against *B. thuringiensis* in open field population (Tabashnik *et al.*, 1997).

Recently insecticidal toxins from *B. thuringiensis* have also found to be toxic to some non-target species (Tapp and Stotzky, 1997). But on the other hand, this is highly effective against some important crop pests (Broza and Brauch, 1994; Moar *et al.*, 1995; Perez *et al.*, 1995).

The toxic effects of *B. thuringiensis* have been found in insects other than crop pests, *e.g.*, in 1997, Akhurust *et al.* found that larvae of *L. cuprina* were susceptible to some strains of *B. thuringiensis*.

Haemolymph

The blood film studies revealed that the haemolymph was affected both in its cellular contents and the plasma.

Plasma

The plasma became coagulated due to the scattering of the cytoplasmic contents and fragmentation of the cells and the bacteria became entangled in this thickened, coagulated plasma. This seemed to be the first defence against the invasions by these foreign particles.

Haemocytes

Phagocytosis is by far the most spectacular of the haemocyte function. The different steps involved in this process could be seen clearly such as attachment of *B. thuringiensis* to the cell membranes of the various haemocytes and their ingestion by these cells. Insect haemocytes have been found to be implicated in the immune responses against invading microorganisms and the detoxification of poisons by other workers and also by Gupta (1985). This is done by haemocytes phagocytosis and encapsulation of entomopathogenic microorganisms and also storing the antibacterial enzyme, lysozyme (Zachary and Hoffman, 1984).

The most affected cells were found to be the plasmatocytes. These cells have been shown to be involved in phagocytosis also by different scientists for various insects (Salt, 1970; Rowley and Ratcliff, 1981). Granular cells were also involved in this process, but to a lesser extent. The cystocytes also disintegrated. These changes involved the rapid degranulation and the loss of cytoplasmic contents. In most of the insects studied in the past, cystocytes were found to be performing the major role in coagulation (Gregoire, 1974). The oenocytoids did not seen to take part in the phagocytosis in this fly as also found by other workers like Gupta (1979).

Differential haemocyte count (DHC)

DHC of the control and the infected flies was done in order to correlate the resultant qualitative changes. During the present study the DHC revealed that nearly

20% of the haemocytes burst and their contents became scattered around them in the infected haemolymph. Upto 90% cell lysis has been reported by other workers in some other insects depending upon the pathogenecity of the bioagents (Pearson and Ward, 1988).

The control *L. cuprina* blood showed only 1% distorted haemocytes which is a natural phenomenon in all the insects. Plasmatocytes and the prohaemocytes increased in numbers in response to the treatment. Plasmatocytes played a major role against the bacteria. They became disrupted but their constant transformation from the prohaemocytes could easily explain the increase in their number while the prohaemocytes probably increased in number by cell division.

Statistical analysis

Statistical analysis revealed that a significant difference existed between the plasmatocyte oenocytoid and podocyte cell number of the control and *B. thuringiensis* treated flies.

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