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EFFECT OF *BACILLUS THURINGIENSIS* ON THE SURVIVAL OF *LUCILIA CUPRINA* (WIED) (CALLIPHORIDAE : DIPTERA)

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Abstract: The effect of *Bacillus thuringiensis* on the survival of *Lucilia cuprina* were observed. LD_{50} was found to be $14x10^6$ cells/ml. The treatment resulted in vacuolization of plasma and destruction of haemocytes. Morphological studies of treated flies revealed that bacterial attack caused no apparent decrease in the size of haemocytes as compared to control ones. Differential haemocytes counting (DHC) of the treated flies showed a significant quantitative differences in the haemocyte number as compared to the control flies.

Key words: *Lucilia cuprina, Bacillus thuringiensis*, haemocyte morphology.

INTRODUCTION

Insect pests are major cause of damage as they cause diseases in animals and humans. Current strategies are aimed at reducing crop loses by using chemical pesticides. But these are becoming ineffective against insects, as insects have developed resistance against them because of their extensive use. The death of target as well as non-target species is a major drawback of these chemical insecticides. These are also expensive and cause health hazards (Estruch *et al.*, 1999). Due to above mentioned disadvantages scientists are now moving towards the biological control, which broadly means any method of control that utilizes living organisms or their natural products (Price, 1975).

Over the past few decades many members of different groups have been evaluated as vector control agents. Out of all the bacteria tested for this purpose the only one considered an operational success is the bacterium *Bacillus thuringiensis* (Federici, 1999). *Bacillus thuringiensis* is a rod shaped, gram-positive, catalase positive, spore forming bacterium that is used for special control of some Lepidopteran and Coleopteran insect pests as well as Dipteran vectors of infectious diseases. Its insecticidal activity is connected with parasporal crystalline proteins delta endotoxins produced during sporulation (Knowles, 1994). *Bacillus thuringiensis* insecticidal proteins have been used commercially for over forty years and now represent 98% of all biopesticides. These compounds give fast, drastic but short-lived results. They attack against the target species, and are harmless to mammals. The first known strains of *B. thuringiensis* produced

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proteins toxic to Lepidopteran, but there are now many strains that affect Dipterans, Coleoptrans, Orthopterans and Hymenopterans (Malla, 1997).

Dipteran pests are serious nuisance as well as vectors of many diseases in humans and animals such as malaria, onchocerciasis, equire encephalitis and dog heat worm. Dipteran pests are also a major problem in poultry and cattle industries. They also infest plants, *e.g.*,. Hesian fly, Medfly and Mexfly for which a *B. thuringiensis* product would be valuable. Pests are normally killed by *B. thuringiensis* in larval stages (Beegle, 1978).

The aim of present work was to find the susceptibility of *Lucilia cuprina* to *Bacillus thuringiensis*. *Lucilia cuprina* chosen for the present study belongs to order Diptera and family Calliphoridae. It is also known as sheep blow fly, and is indigenous to Africa but now has spread to other continents like Asia and Australia. Its medical importance was mainly associated with myiasis. It breeds in meat or carrion and may cause facultative myiasis in man by infecting festering sore and wounds (Service, 1980). Now it is also a well known vector of Anthrax affecting sheep, goats, cattle and even humans, causing death if left untreated. Their practice of sitting on dog faces, decaying matter and then on human food means that they can easily transmit undesirable organisms and can readily spread diseases through a community.

Our present work is concerned with the microbial control of *L. cuprina* through a Dipteran specific strain of *B. thuringiensis*.

MATERIALS AND METHODS

Collection and maintenance of insects

The Australian blow flies, *Lucilia cuprina* used for the present work were collected from the meat shops from different localities around Quaid-e-Azam Campus, Punjab University, Lahore. These adult flies were fed on fresh minced beef and pieces of banana peels. The eggs obtained from these flies were kept in sterilized glass jars covered with muslin cloth. The 3rd instar larvae obtained settled down and this marked the onset of the puparial life. The adults emerged nearly five days after the start of puparial life. The larvae were reared on beef throughout their life. The colonies of flies were maintained at 30°C, 12 hours photoperiod and relative humidity ranging from 65% to 75%.

The bacteria used were *Bacillus thuringiensis* Kurstaki (Abbott : Diptera strain). These bacteria were reared on nutrient agar medium at 37°C. Nutrient broth was used as the inoculation medium.

Experimental procedures

Following steps were taken in regard to the entire experimental set-up:

Preparation of bacterial inoculum

The overnight cultures of *B. thuringiensis* were prepared in nutrient broth. These were then used for inoculation studies.

Biotoxicity assays

Milk sugar (1 ml) solution was poured in each sterilized jar having cotton pad and filter paper at the bottom. Then 7 ml of bacterial inoculum was added in each jar. Jars used as control contained 7 ml of autoclaved nutrient broth. Five flies were placed in each jar. Three replicates were set up for the experimental purpose.

Blood film formation and fixation

The flies were given the fumes of glacial acetic acid. A fine sterilized needle was used to puncture the abdomen of the flies, which was gently pressed to squeeze out the blood. The blood thus obtained was evenly spread out into a film by sliding the edge of another glass slide at an angle of 45°C. After air drying, it was fixed in methanol for 5 minutes, and then stained in Giemsa's stain for one minute. After differentiation and dehydration, mounting was carried out in Canada Balsam.

Experimental studies

The sizes of different type of blood cells were measured by using an ocular micrometer. 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 160 cells per experimental stage were counted and classified.

Estimation of LD_{50}

LD_{50} was determined by computerized probit analysis. Three concentrations ($12x10^6$ cells/ml, $14x10^6$ cells/ml and $16x10^6$ cells/ml) were selected for calculating LD_{50}.

RESULTS

The blood or haemolymph of *Lucilia cuprina* is contained in the general body cavity, as in case of all the insects and has two components, the plasma, which is the liquid part and the haemocytes or the blood cells (Fig.1D-F). Three types of blood cells were distinguished on the basis of light microscopy, which were as follows:

1. Prohaemocytes.
2. Plasmatocytes.
3. Granular cells.

Prohaemocytes are small to medium sized cells. These 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 are deeply basophilic but nucleus is eosinopilic. These are germ or stem cells (Ratcliffe and Rowley, 1981) (Fig.1B).

Plasmatocytes are highly polymorphic haemocytes and large in size as compared to prohaemocytes. These cells have basophilic cytoplasm but their nuclei are eosinophilic with granular chromatin. Plasmatocytes have more cytoplasm surrounding their nuclei as compared to prohaemocytes. Very few plasmatocytes were seen dividing (Fig.1A).

Granular cells 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. Their nuclei are eosinophilic and smaller in size as compared to those of plasmatocytes (Fig.1C).

When *L. cuprina* was fed on different concentrations of *B. thuringiensis* LD_{50} was found to be $14x10^6$ cells/ml of *Bt* liquid culture after 24 hours.

The blood films of control as well as treated flies were studied at 8 hours, 16 hours and 24 hours of intervals. After 8 hours of treatment, the direct microscopic observation of the blood film showed that many bacterial cells were scattered in the vicinity of the haemocytes (Fig.2A). After 16 hours of treatment, many bacteria approached the haemocytes and ultimately they were found attached to the cell membranes of the haemocytes (Fig.2B). After 24 hours of treatment, many bacteria were seen to be residing inside the haemocytes (Fig.2C).

Morphological study and DHC

The size of prohaemocytes in control flies ranged from 7.5 to 8.5 μ and the size of their nuclei ranged from 7.0 to 8.0 μ, whereas in *Bt* treated flies blood, the size of prohaemocytes ranged from 5.5 to 6.5 μ and that of their nuclei ranged from 5.0 to 6.0 μ. It showed that there is 2% decrease in the size of prohaemocytes in treated flies as compared to control ones.

The size of plasmatocytes in control flies of blood ranged from 11.0 to 16.0 μ and that of their nuclei ranged from 7.0 to 7.6 μ, whereas in *Bt* treated flies the size of plasmatocytes ranged from 10.3 to 12.6 μ and that of their nuclei ranged from 6.3 to 7.0 μ. It showed that there was 3% decrease in the size of the whole cell and 0.6% decrease in the size of the nuclei of plasmatocytes.

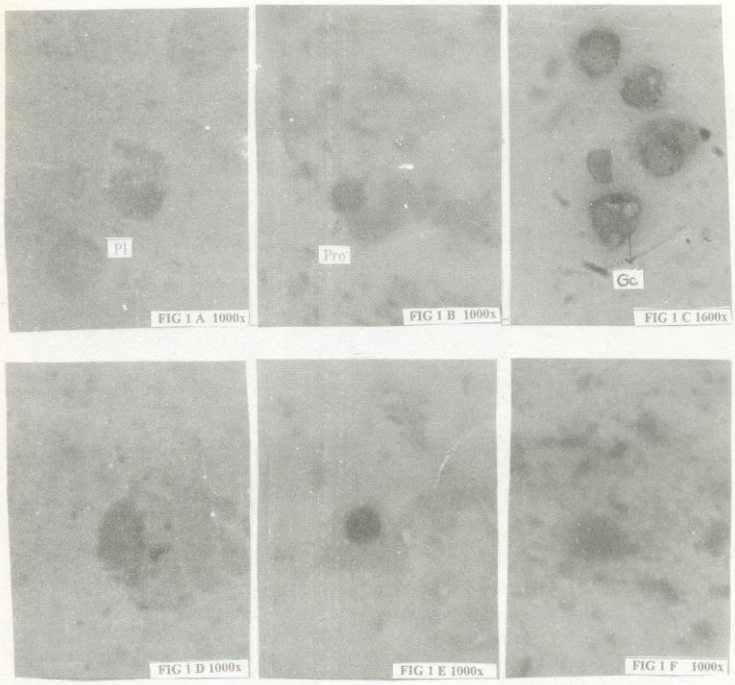

Fig. 1: Control (A-C), A: Pl; Plasmatocytes; B: Pro; Prohaemocytes; C: Gc; Granular cells. General blood picture of control (D-F), D: after 8 hr; E: after 16 hr; F: after 24 hr.

The size of granular cells ranged from 12.6 to 16.0 μ and that of their nuclei ranged from 7.0 to 8.6 μ in control flies, whereas in *Bt* treated flies the size of granular cells ranged from 8.6 to 11.0 μ and that of their nuclei ranged from 5.3 to 6.3 μ. It showed that there was 4% decrease in the size of the whole cell and 2% decrease in the size of the nuclei of granular cells.

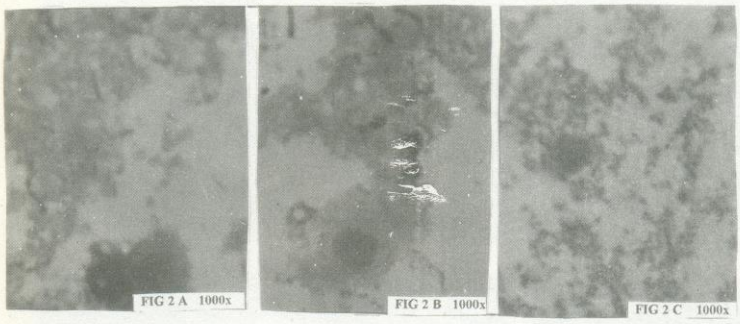

Fig. 2: ***B. thuringiensis*** **(A-C), A: after 8 hr; B: after 16 hr; C: after 24 hr.**

DHC showed that in the control flies prohaemocytes were found to be 15% after 8, 6 and 24 hours, whereas prohaemocytes were found to be 7.5% after 8 hours of treatment, 9% after 16 hours of treatment and 10% after 24 hours of treatment. The statistical analysis showed that there was a significant difference in the prohaemocytes number of control and treated flies blood.

Plasmatocytes were found to be 55% after 8, 16 and 24 hours in control flies blood, whereas in *Bt* treated flies blood, plasmatocytes were found to be 45% after 8 hours, 48% after 16 hours and 52.5% after 24 hours. Statistical analysis showed that there was a significant difference in the plasmatocyte number of control and *Bt* treated flies.

Granular cells were found to be 10% in control flies blood after 8, 16 and 24 hours of treatment, whereas in *Bt* treated flies they were found to be 4% after 8 hours, 6% after 16 hours and 7.5% after 24 hours. Statistical analysis showed that there was a significant difference in granular cells number of control and *Bt* treated flies.

Table I: Showing the effect of *Bt* treatment on the sizes of different types of haemocytes and their nuclei (at 100x).

(30 cells of each type were measured)

Type of cell	Control		*B.t.* Treated	
	Size (μ)	S.D.	Size (μ)	S.D.
Prohaemocytes	7.5-8.5	7.5±0.58	5.5-6.5	5.8±0.46
Nuclei of prohaemocytes	7.0-8.0	7.5±0.44	5.0-6.0	5.5±0.44
Plasmatocytes	11.0-16.0	13.5±1.87	10.3-12.6	11.4±0.92
Nuclei of plasmatocytes	7.0-7.6	7.2±0.25	6.3-7.0	6.6±0.24
Granular cells	12.6-16.0	14.5±1.27	8.6-11.0	9.7±0.97
Nuclei of granular cells	7.0-8.6	7.7±0.70	5.3-6.3	5.8±0.49

Table II: Showing percentages of different types of haemocytes after *Bt* treatment.

Duration of treatment	Cell Types					
	Prohaemocytes		Plasmatocytes		Granular cells	
	Control		Control		Control	
	Total	%	Total	%	Total	%
	30	15	110	55	20	10
	Treated		Treated		Treated	
	Total	%	Total	%	Total	%
8 hours	15	7.5	90	45	8	4
16 hours	18	9	96	48	12	6
24 hours	20	10	105	52.5	15	7.5

Table III: Showing the results of effects of *Bt* treatment on haemocytes size at different durations.

ANALYSIS OF VARIANCE					
Source	DF	SS	MS	F	P
Factor	1	12.32	12.32	1.27	0.293
Error	8	77.84	9.73	-	-
Total	9	90.16	-	-	-

Table value of F = 5.32

There is no significant difference in the size of haemocytes of control and *Bt* treated flies.

Table IV: Showing the results of effects of *Bt* treatment on prohaemocyte number at different durations.

ANALYSIS OF VARIANCE

Source	DF	SS	MS	F	P
Factor	1	228.17	228.17	72.05	0.001
Error	4	12.67	3.17	-	-
Total	5	240.83	-	-	-

Table value of F = 7.71

There is a significant difference in the plasmatocyte number of control and *Bt* treated flies.

Table V: Showing the results of effects of *Bt* treatment on plasmatocyte number at different durations.

ANALYSIS OF VARIANCE

Source	DF	SS	MS	F	P
Factor	1	228.17	228.17	72.05	0.001
Error	4	12.67	3.17	-	-
Total	5	240.83	-	-	-

Table value of F = 7.71

There is a significant difference in the plasmatocyte number of control and *Bt* treated flies.

Table VI: Showing the results of effects of *Bt* treatment on granular cell number at different durations.

ANALYSIS OF VARIANCE

Source	DF	SS	MS	F	P
Factor	1	104.17	104.17	16.89	0.015
Error	4	24.67	6.17	-	-
Total	5	128.83	-	-	-

Table value of F = 7.71

There is a significant difference in the granular cell number of control and *Bt* treated flies.

DISCUSSION

Microbiological control of insects is an important aspect of biological control. Nearly all entomopathogen bacteria are from class "Schizomycetes".

In the present study treatment of *L. cuprina* with *B. thuringiensis* Kurstaki resulted in certain abnormalities *e.g...* disruption of haemocytes and ultimate death of flies after 24 hours of treatment.

The blood film studies revealed that haemolymph was affected both in its plasma and the cellular contents. The plasma became coagulated due to scattering of cytoplasmic contents and the bacteria became entangled in this thickened plasma. This seems to be the first defense against the invasion of these foreign particles. Phagocytosis .is the most important function of haemocytes. The different steps in this process could be seen clearly such as attachment of *B. thuringiensis* to the cell membranes of various haemocytes and their ingestion by these cells. Detoxification of poisons is done by haemocyte phagocytosis. encapsulation of entomopathogenic microorganisms and storing the antibacterial enzyme. Lysozyme (Hoffman and Frodsham. 1993). Most affected cells were plasmatocytes. but granular cells were also affected to a lesser extent. The prolonged treatment resulted in disruption of cells (Salt. 1970).

DHC of the control and the infected flies was done in order to correlate the resultant quantitative changes. During the present study. DHC revealed that nearly 20% of the haemocytes burst and their contents became scattered around them in infected haemolymph. Upto 90% cell lysis has been reported by other workers in some other insects depending upon the pathogenicity 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. The haemocytes decreased in number in response of treatment.

The bacteria belonging to *B. thuringiensis* Kurstaki produce entomocidal crystal proteins (ICP). The protein is proteolytically cleaved in alkaline circumstances of the larval gut juices into smaller fragments. Thus a proteolytically resistant core has toxicity against insect larvae. Characterization of the core is very important in the elucidation of the entomocidal mechanism of crystal proteins (Ogiwara *et al*.. 1971).

Toxic effects of *B. thuringiensis* have been found in insects other than crop pests. *e.g...* in 1997. Akhurst found that the larval of *L. cuprina* were susceptible to some strains of *B. thuringiensis*.

Statistical analysis revealed that there existed a significant difference between the haemocytes number of control and *Bt* treated insects. But a non-significant difference was found in size of haemocytes of the control and *Bt* treated insects.

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