Research Article

Haemolymph Assay and Morphological Changes of Silkworm, *Bombyx mori* L. (Bombycidae: Lepidoptera) Infected with *Bacillus thuringiensis* var. *sotto*

Muhammad Zeeshan Nazar*, Ayyan Umer, Zahid Mahmood Sarwar, Faiz Ullah Faiz and Sikandar Hussain

Department of Entomology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan.

Abstract | Sericulture is a distinctive field of agriculture in which silkworms, *Bombyx mori* (Bombycidae: Lepidoptera), are reared on wide range for commercial purpose. Silkworm is susceptible to various pathogenic diseases. Among the all bacterial diseases of *B. mori*, flacherie is injurious disease that is initiated by entomopathogenic bacteria, *Bacillus thuringiensis* var. *sotto* (*Bts*). The impact of this infectious disease is weight loss, reduction in cocoon quality as well as quantity and source of infestation for healthy larva. In current study, the 3rd instar larva of *B. mori* were fed on the leaves of *Morus alba* treated with *B. thuringiensis* until the start of 5th instar and the Total Haemocyte Count (THC) of the *Bts* infected silkworm's haemolymph were counted. The findings of current research showed that THC was significantly increased at 1st and 2nd day i.e., 5128/ml and 5704/ml as compared to control 3047/ml, respectively. Afterwards, THC were rapidly decreased and THC were recorded 1928/ml (3rd day) and 344/ml (4th day). The results also exhibited the morphological changes of silkworm i.e., infected larva fully filled with *B. thuringiensis* spores with flaccid body and haemolymph liquid impelled leading to mortality. Provide the required temperature and humidity with appropriate hygienic condition to *B. mori* are advised to produce healthy silk.

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***Correspondence** | Muhammad Zeeshan Nazar, Department of Entomology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan; **Email:** mzeeshan_93@yahoo.com
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Introduction

Many insects are involved in the production of silk but usually silkworm larva are used at large scale. Approximately, 2500 silkworms are required to produce one pound of raw silk. To meet the need of the massive quantity of silk, these worms are reared worldwide. China followed by India are the leading countries for the silk production and considered the pioneer country to initiated sericulture about 5,000 and 3,000 BC. (Gangopadhyay and Singh, 2008). In Changa Manga, Punjab, Pakistan, sericulture industry is established in 2018 by Chinese private companies (Published in Dawn, April 15, 2019*)*.

Several diseases are produced in silkworm which results mortality that are mostly occur at 3rd and 4th instar stage of silkworm which may disappoint the Sericulturists. Consequently, by the effects of diseases, both the quality and quantity of cocoon crops are severely septic and losses of revenue (Aruga, 1994).

In the world, no silkworm biotypes are exist that are resistant to diseases or pests. Among all the pathogenic disorders, bacteria play a fatal role to cause diseases and ruin the sericulture industry. The most important

diseases that infecting the silkworm are, flacherie, grasserie, muscardine and pebrine (Das Gupta, 1950). These diseases cause 70% loss in silk production. The flacherie is the most vulnerable disease, also known as '*Sotto*' which is caused by *B. thuringiensis* that is a spore forming, soil living and gram-positive bacterium. It is also found naturally on leaf surface, aquatic environment and the midgut of many Lepidopterans caterpillars (Aronson et al., 1986).

Mostly *B. thuringiensis* are used as biological insecticides to control insect pests by making crystal protein or Cry protein that are attached to epithelial layer of midgut and rupture it (Mullins, 1985). Several physiological and biochemical functions of the insect are accomplished by the extracellular fluid which act as a reservoir and transportation of minerals, moulting, excretion and metamorphosis, furthermore, performed several vital activities including immune system or cellular defence in insect body through phagocytosis and encapsulation (Gad and Alzahofi, 2010).

This detrimental bacteria is considered to the causative agent of 'Flacherie' disease in *B. mori* that enforce the extreme risk to Sericulture. Due to their distinction in the symptoms, it is often known by many names as shrinking disease, softening disease and faecal disease. Silkworm larvae infected with flacherie stop to feeding by alteration in haemolymph composition along with normal physiological and biochemical transformation in insect tissues also are change (Pawar and Ramakrishnan, 1977; Begum et al., 2004) which lead to convulsions even at very low concentration (0.01%) and it cause 50% mortality at the 4th instar stage of lepidopterans larva (Aruga, 1994). Therefore, flacherie is a challenge for silkworm disease management, because it is awkward contest to eliminate pathogens from the silkworm rearing environment (Glaser and Lacaillade, 1924).

The aim of this study to find out the lethal effects of *Bacillus thuringiensis* var. *sotto* against silkworm larvae to exhibited the haematological changes in the silkworm and exhibited the morphological changes in the body of infected *B. mori* larva.

Materials and Methods

Rearing of B. mori

The disease free eggs (roughly 1000) of *B. mori* (Brazil origin) were obtain in packets from Forest, Wildlife and Fisheries Department Multan, Punjab, Pakistan. These eggs were brushed into small petri dishes and reared in the laboratory under 26±2 °C with R.H. 72±5% and allowed it to hatching. Immediately after hatching, the young ones larva were shifted to trays and fed on chopped *M. alba* leaves until 5th instar larva started as per the recommended rearing practice (Ram, 2000).

Culture division

At the time of inoculation, (3rd instar larva), silkworm larvae were divided into two groups, one group for inoculation and other control group. Each group have five replications and ten larvae in each replication.

Purification of bacteria

The purification of bacteria includes following steps:

Components of nutrient agar: For isolation and purification of bacterial culture, the following material was taken from the Plant Pathology Lab, Bahauddin Zakariya University (BZU) Multan, Punjab, Pakistan, (i) 0.5% Peptone; (ii) 0.3% beef extract/yeast extract; (iii) 1.5% nutrient agar; (iv) 0.5% NaCl; (v) 500 ml distilled water.

Preparation of nutrient agar: In 500 ml of distilled water, 14 g of nutrient agar powder was mixed and allowed it to heating for entirely dissolve all the components at 100 °C for 5 min. The dissolved mixture are autoclaved at 121°C for 15 minutes and allowed it to cool at 4°C for 30 minutes. At the end, transferred the nutrient agar into sterile petri dish until the agar has solidified and stored the dish in the refrigerator for processing the study (Daniel and Rusch, 1961).

Bacterial culture: The *B. thuringiensis* spores were taken from the Plant Pathology Lab, BZU Multan, Punjab, Pakistan. The spores and crystals of *B. thuringiensis* were purified with 1.0M NaCl and 0.01 percent Triton X-100 and washed repeatedly with purified water via centrifugation and then shifted to petri dish containing nutrient agar for multiplication.

Inoculation of bacillus thuringienesis: At the time of inoculation, very low concentation of *thuringiensis* (1×10^{-5} Cells/ml) smeared on fresh mulberry leaves and allowed to fed on second day of 3rd instar larva until the start of 5th instar (Pramanik, 2001).

Preparation of haemolymph sample: After every 24

hours post-inoculation, punctured the caudal horn of silkworm larva and collected haemolymph in 1.8ml PT test vials. The sample were collected from 4 larvae of each replication (2 larvae haemolymph/tube) by insulin syringe (2ml) (Figure 1b) and preserved the sample in refrigerator at 4 °C by adding 3.8% Sodium Citrate as an anti-coagulant to avoid melanisation. A total tube represents one replication collections.

Counting the total haemocyte: Before counting the THC, added 1 ml solution of Phosphate Buffer Saline (anti-coagulant) in it for more clear results and counted the THC of both groups with the help of Neubauer Hemocytometer by taking 10µl sample in micro-pipette. We compared and analysed their differences by counting the cells under Stereo Microscope at 40x magnification.

Statical analysis

Graph Pad Prism 6 software was used for plotting the graph and Latin Square Design on Statistix 8.1 software was applied on data to find mean value, degree of freedom and standard error.

Results and Discussion

Fluctuations of total haemocytes count in infected B. mori

In *B. thuringiensis* infected silkworm, the THC decreased significantly i.e. 5128/ml to 344/ml from day 1st to day 4th post-treatment respectively, as compared to control group of silkworm (Figure 2). The experiment showed that THC decreased 8.86 times in bacterial infected silkworms as compared to healthy worms. In current experiment, it was also find out that initially THC increased 5158/ml to 5704/ml in silkworm (Table 1) due to defensive mechanism and afterwards rapidly decreased (Perveen and Ahmad, 2017).

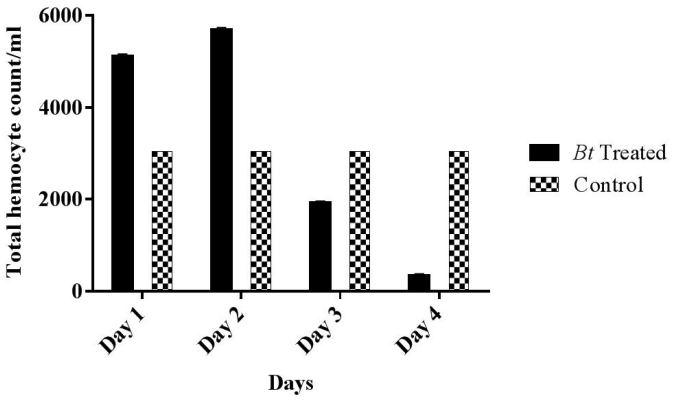

Figure 2: *Concentration of total haemocyte count/ml after infection with B. thuringiensis* var. *sotto.*

Table 1: *Effect of B. thuringiensis on total haemocyte count and mortality data of B. mori larva.*

Days	Bioassay groups	THC/ml ± S.E[a]	df[b]	N[c]	N[d]	Mortality (%)
1st	*Bt* Treated	5128±8.258	4	50	06	12
2nd	*Bt* Treated	5704±8.619	4	50	16	32
3rd	*Bt* Treated	1928±2.408	4	50	37	74
4th	*Bt* Treated	344±2.607	4	50	48	96
	Control group	3047±1.924	4	50	0.00	0.00

[a] Standard error; [b] degree of freedom; [c] Total number of larva exposed to B.t for bioassay; [d] Total number of dead larva.

Flacherie is a bacterial disease that infects the silkworm by rupturing the midgut as the spores of *B. thuringiensis* bind to the epithelial layer of midgut from by producing toxic chemical and proliferate in the haemolymph, disturbing the normal physiological function of the insect. In severe stage, the body is completely filled with spores (Mullins, 1885).

In current examination, it was found that the THC decreased significantly when treated with *B. thuringiensis* that feasts the infection from diseased to healthy silkworm larva and the present results confirm the rapidly propagation of bacteria. Due to defensive mechanism of *B. mori* against *B. thuringiensis,* the Total Haemocytes Count was initially increased in 2nd day after treatment and then sudden decreased of cells on 3rd and 4th days as compared to control group. On 4th day, he THC reduced in disastrous as a result larva died.

Flacherie infected silkworms are weak and died within 4th to 5th days regarding to the level of infestation. Initially the colour of flacherie diseased larva are light to dark brown (Figure 1c) and lastly turn black (Figure 1d) as compared to control (Figure 1a). Various factors that are involved in flacherie disease like inappropriate condition of environment during incubation of eggs, poor leaves quality, improper handling during rearing of *B. mori* and lack of food. Due to physiological weakness of silkworm, larva becomes susceptible to various pathogenic diseases due to loss of immunity (Balavenkatasubbaiah and Sivaprasad, 2014). In the current study, it was also examined that due to infection in digestive system and physiological changed in the body of silkworm; the body became fragile, flaccid and shrink. Afterward, yellowish body fluid discharged from the midgut of silkworm by rupturing the integument (Figure 1e). Our results support the findings of

Ericsson et al. (2009) who studied the response of immune system of the cabbage lopper *Trichoplusia ni* against *B. thuringensis*. He observed that reduction in Total Haemocytes Count after inoculation of *B. thuringensis*.

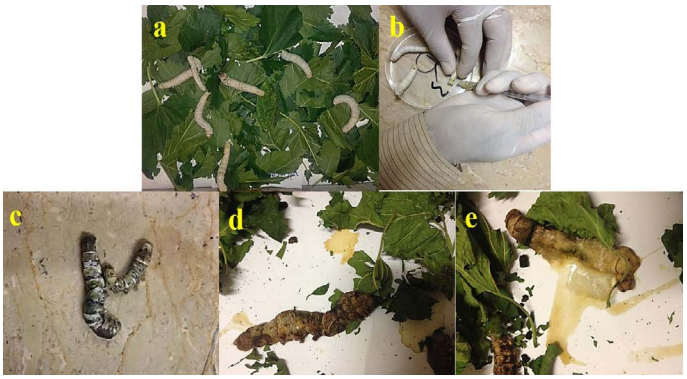

Figure 1: *(a) Healthy and uninfected larva of B. mori. (b) Collecting of Haemolymph from caudal horn of B. mori. (c) Infected larva of 2nd day with light brown colour. (d) Larval color changed to coffee brown on 3rd day. (e) Yellowish body fluid discharged from the midgut of B. mori by rupturing the integument after highly infected with B. thuringiensis.*

As in current study, we examined the morphological changes in infected silkworm that were similar to Poonia (1979) who observed that infected larva with flacherie are entirely lethargic and inactive. The body colour of the larva turned blackish brown, melanisation of the blood and produces bad odour. He also observed that biochemical components are changes in the haemolymph of the silkworm due to flacherie. Lian (1991) also reported that flacherie infected silkworm larva stop to feeding, trembles, paralysis and death. Before death, caterpillar lifts its head. The body colour turned pale and stops the growth and development. At the end, the midgut ruptured and the larval body liquefied. Total haemocytes from *B. thuringiensis* treated larvae of gypsy moth were significantly decreased in number and signs of integument rupture (Broderick et al., 2010).

Anandakumar and Michael (2011) found approximately the same result as described in present study. He reported that when silkworm larvae were fed orally with *Bacillus thuringensis*, the total haemocyte count reduced significantly. Flacherie infected silkworm showed 15.3% decrease in total haemocyte as compared to healthy silkworm larva.

On the basis of above observation, feasibly, the silkworms were not able to feed normally when infected by bacteria and gradually weal down, finally died.

Conclusions and Recommendations

Present experiment indicates that flacherie disease mostly spread at 3rd and 4th instar stage due to contaminated food and suffocation due to over population. Total haemocyte counts increase at initial stage due to defence mechanism in insect then rapidly reduced. Furthermore, change in morphology and feeding behaviour of flacherie-infected silkworm has also been observed. Initially the colour of flacherie diseased larva is dull white, but it gradually changes to coffee brown or light brown and finally dark brown or almost black. Maintain the required temperature and humidity with proper sanitation are suggested to produce healthy silk.

Author's Contribution

MZN is the corresponding author. Conceived the idea, performed research, wrote Introduction, methodology, result and discussion and conclusion. AU helped in Haematological assay and rearing of *B. mori* culture. ZMS wrote Abstract and overall management of the research. FUF helped in statistical analysis and set the References accordingly. SH proof checking of technical Input at every step.

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Conflicts of interest

The authors declare no conflicts of interest.

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