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# Effect of Systemic Fungicide on Total Hemocyte Count and Hemolymph Biochemical Changes in Silkworm, *Bombyx mori* L., infected with *Beauveria bassiana*

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**Silkworm diseases are major constraint in silk cocoon production. Among silkworm diseases, white muscardine is highly contagious and most common in winter and rainy seasons. It is suggested that hemocytes involve in defense against invasion of *Beauveria bassiana* and systemic fungicide/chemicals prevent the proliferation of fungi in the hemolymph or preventing the growth of the fungi in the body cavity through enhancing the hemocyte mediated defense response. In the present study the influence of systemic fungicide on hematological changes in silkworms infected with *Beauveria bassiana* was reported. It is observed that the total hemocyte counts increased in the hemolymph up to 5 days post inoculation in systemic fungicide treated batches while in the inoculated control the increase was up to 3 days indicating the positive hemocyte mediated response in silkworm treated with systemic fungicide. After 2 days in the inoculated control as the multiplication and growth of mycelia increased, defense capacity of the silkworm was decreased. The biochemical changes were also observed in the hemolymph of silkworm infected with *B. bassiana*. In silkworm infected with *Beauveria bassiana*, the total protein content increased whereas total carbohydrate and total lipids decreased as the infection progresses. In the case of systemic fungicide treated batches the increase in total protein content was comparatively higher and decrease in total carbohydrate and lipids were comparatively lower than the inoculated control.**

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## Introduction

White muscardine in silkworm is caused by the intrusion of the pathogenic fungus *Beauveria bassiana*. The fungal conidia penetrate through the skin and enters into the haemolymph and begin to develop and proliferate. Kawakami (1965) reported that hemocytes of *Bombyx mori* are capable of phagocytizing conidia and hyphal bodies of the muscardine fungi, *Isaria fumororosea* and *Harziella contomophila*, but they do not phogocytise the conidia of *Beauveria bassiana*. Hou and Chang (1985) reported that the hemocytes of *B. mori* are able to phagocytize and encapsulate viable conidia of *B. bassiana* and eventually undergo the nodule formation, but they fail to suppress the spore germination in the nodules. Inset immune responses to bacterial infection are relatively well understood when compared with responses to fungal infection (Kanost *et al.*, 1990). Systemic fungicides has been screened for cure of white muscardine in silkworm, *B. mori* L. (Virendrakumar *et al.*, 1997) and these fungicides are reported to prevent the proliferation of *B. bassiana* in the hemolymph, the main site of multiplication of the fungi.

It is suggested that the systemic fungicide apart from inducing the hemocyte mediated responses, there may be influence on biochemical defense against invading pathogen. However, the information on the influence of systemic fungicide on hematological and biochemical defense in the insect infected with *B. bassiana* is limited. The fungal cell wall components is also reported to activate immune response in silkworm (Bidochika and Hajek, 1998). In the present study, the influence of systemic fun-

gicide (Bayleton) on the hemocyte mediated responses and biochemical defense is reported through study of total hemocyte count and biochemical changes in the hemolymph in the silkworms infection with *B. bassiana*.

## Materials and Methods

Silkworm breed, NB4D2 was received from Germplasm Bank, Central Sericultural Research and Training Institute, Mysore and reared following standard method. Silkworms immediately after four$^{th}$ moult were divided into two sets having 4 batches of larvae each. These larvae were subjected to treatments as per the experimental requirement.

### Prepartion of pathogen inoculum

The pathogen inoculum for experimentation was prepared by suspending the conidia of *Beauveria bassiana* ($4 \times 10^6$ conidia/ml) in sterilized distilled water.

### Preparation of systemic fungicide solution

Bayleton (25% W. P. Triadiamefon, a triazole compound, Rallis, India, Ltd., India) was used as a systemic fungicide in the present study. 0.1% of Bayleton was prepared by dissolving 1 g of Bayleton fungicide in 1,000 ml of sterilized distilled water. The solution was sprayed on known quantity of K2 mulberry leaves at the rate of 200 ml/kg leaf and air dried.

In first set of first batch (T1), larvae were inoculated by topical application with 10 ml *B. bassiana* inoculum ($4 \times 10^6$ conidia/ml) per 100 larvae and fed with normal leaves. After 24 hrs of post inoculation, the larvae were fed five times with systemic fungicide treated leaves and followed it with feeding normal untreated leaves. Again on 4$^{th}$ day, four feedings were given with systemic fungicide treated leaves and it was followed with normal untreated leaves till spinning.

Second batch (T2) larvae were fed five times with systemic fungicide treated leaves for 24 hrs. After 24 hrs, larvae were inoculated with 10 ml of *B. bassiana* inoculum ($4 \times 10^6$ conidia/ml) per 100 larvae and was followed with normal untreated leaves. Again on 4$^{th}$ day four feedings were given with systemic fungicide treated leaves and followed it with normal untreated leaves till spinning. The third batch (T3) larvae were inoculated with 10 ml of *B. bassiana* inoculum ($4 \times 10^6$ conidia/ml) per 100 larvae and fed with normal untreated leaves till spinning. This served as inoculated control. The fourth batch (T4) larvae were served as normal control. The larvae were fed with normal untreated leaves till spinning.

Each treatment had 3 replications of 100 larvae each. Every day from 0 to 6$^{th}$ day, 6 larvae were collected from each replication, the hemolymph from all the 6 larvae was collected into two eppendoff tubes (3 larval hemolymph/tube) on ice and stored at 4°C. A total of 6 tubes representing 3 replications were collected. The following estimations were conducted from the collected hemolymph samples.

### Estimation of total hemocyte counts (THC)

Every day THC was estimated in the hemolymph of all treated and control batches were determined following the method described by Tauber and Yeager (1934, 1935) using hemocytometer. The THC per 1 mm$^3$ of haemolymph was estimated according to the formula suggested by Jones (1962). Second set of larvae were also treated and maintained similar to first set till spinning. Every day from 0 to 6$^{th}$ day, 6 larvae were collected from each replication, the hemolymph from all the 6 larvae was collected into two eppendoff tubes (3 larval hemolymph/tube) on ice and stored at 4°C. A total of 6 tubes represented 3 replication collections. The following estimations were conducted from the collected hemolymph samples.

### Estimation of total proteins

The total protein content in the hemolymph was estimated by the method of Lowry *et al.* (1951). To 100 $\mu$l of hemolymph, 100 $\mu$l of 20% trichloro acetic acid was added and kept for 30 min. The contents were centrifuged at 3,000 rpm for 5 min and the pellet was washed twice with 10% trichloro acetic acid. Finally the pellet was dissolved in 0.1 N sodium hydroxide. To the 10 $\mu$l of pellet sample 5 ml of alkaline copper sulphate reagent (Reagent A: 2% sodium carbonate in 0.1 N sodium hydroxide; Reagent B: copper sulphate in 0.1% potassium sodium tartrate in 1:1 ratio) was added. To make alkaline copper sulphate reagent, reagents A and B were mixed well in the ratio of 50:1. After 10 min, 0.5 ml of folin-phenol reagent (the commercial solution was diluted once with distilled water) was added and shaken well. After 30 min, the colour intensity was read at 660 nm in a Jasco V-530 spectrophotometer. The blank sample contained 10 $\mu$l of distilled water, 5 ml of alkaline copper sulphate reagent and 0.5 ml of folin-phenol reagent. The protein content was recorded from the standard curve prepared for bovine serum albumin (10 - 100 $\mu$g). The protein content in the samples were expressed as mg/ml of hemolymph.

### Estimation of total carbohydrates

The total carbohydrates present in the hemolymph of all treatment samples were estimated by phenol-sulphuric acid method (Dubois *et al.*, 1956). To 10 $\mu$l of hemolymph sample, 0.4 ml of 5% phenol was added and mixed well.

To this 2 ml of concentrated sulphuric acid was added and mixed well. The tubes were cooled at room temperature by keeping in running tap water. The colour intensity was measured at 490 nm in a Jasco V-530 spectrophotometer. The blank sample contained 10 $\mu$l of distilled water, 0.4 ml of 5% phenol and 2 ml of concentrated sulphuric acid. The total carbohydrates present in each sample were recorded from the standard curve prepared by taking 20 - 200 $\mu$g of glucose. The total carbohydratea present in each sample were expressed as mg/ml of hemolymph.

### Estimation of total lipids

The total lipids in the hemolymph were estimated following gravimetry method (Folch *et al.*, 1951). To 0.5 ml of hemolymph, 0.5 ml of chloroform - methanol mixture (2:1; V/V) was added and mixed thoroughly. After 30 min the mixture was centrifuged at 3,000 rpm for 5 min. The chloroform layer containing lipid was collected into a pre-weighed plastic vial. To the upper aqueous layer 0.5 ml of chloroform-methanol mixture was added and lipid extraction was repeated twice. The pooled chloroform-lipid mixture was air dried and the weight of vial was recorded until there is no changes in the weight of plastic vial. The differences between initial and final weight gives the lipid content. The amount of lipid present was expressed in mg/ml of hemolymph.

## Results

The data on the effect of systemic fungicide on *B. bassiana* infection in silkworm are presented in Fig. 1. The mortality due to white muscardine in T1 batch was significantly lower than T3 (inoculated control) batch. The mortality was 38.00% compared to 100% in inoculated control. In the case of T2 the mortality was only 13.33% which was significantly lower than T1 and T3 batches. In T4 (normal control) batch there was no mortality due to white muscardine.

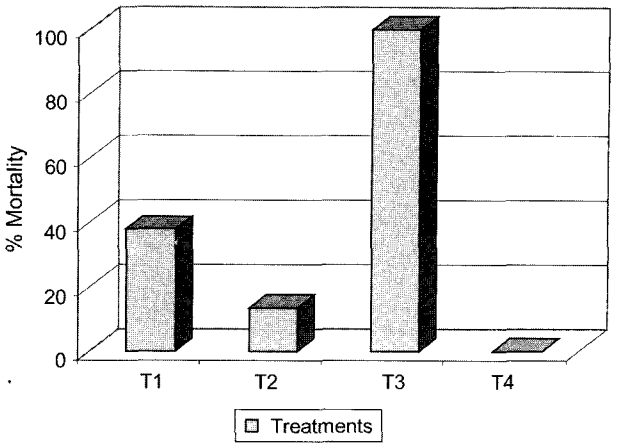


**Fig. 1.** Effect of systemic fungicide on *B. bassiana* infection in silkworm.

The data on the effect of systemic fungicide on total hemocyte counts in *B. bassiana* infected silkworm is presented in Table 1. In treatments (T1, T2 and T4), the total haemocyte counts increased significantly from 1$^{st}$ day to 5$^{th}$ day and decreased later. In T1 batch, the total haemocyte counts were 9,567/mm$^3$ of hemolymph during 1$^{st}$ day and increased to 11,517/ mm$^3$ by 5$^{th}$ day. On 6$^{th}$ day the counts were 10,508/ mm$^3$. The trend was same in the case of T2 and T4 batches. In the case of T3 the total haemocyte counts were increased up to 2$^{nd}$ day and decreased on 3$^{rd}$ day onwards and 100% mortality was recorded on 7$^{th}$ day.

The data on the effect of systemic fungicide on hemolymph total protein content in *B. bassiana* infected silkworm are presented in Fig. 2. The hemolymph proteins in normal control (T4) larvae increased from 25.02 mg/ml on 0 day to 47.10 mg/ml by 6$^{th}$ day. In T1 batch there was an increase in proteins from 28.97 mg/ml (1$^{st}$

**Table 1.** Effect of systemic fungicide on total hemocyte counts in *B. bassiana* infected silkworms

| Sample no. | Treatment | Days | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | T1 | 9,567 | 10,850 | 10,942 | 11,242 | 11,517 | 10,508 |
| 2 | T2 | 9,908 | 11,308 | 11,400 | 11,542 | 11,850 | 10,833 |
| 3 | T3 | 9,558 | 10,417 | 10,358 | 8,092 | 5,767 | 3,067 |
| 4 | T4 | 9,033 | 9,167 | 9,450 | 10,208 | 10,667 | 9,800 |
| **S. E. ±** | | **43.78** | **68.88** | **74.10** | **86.52** | **69.02** | **4.04** |
| **C. D. at 5%** | | **131.94** | **207.58** | **223.31** | **260.75** | **208.01** | **132.69** |

T1 = *B. basssiana* inoculation + After 24 hrs 5 fungicide feeds
T2 = 5 fungicide feeds + After 24 hrs *B. basssiana* inoculation
T3 = Inoculated control
T4 = Normal control

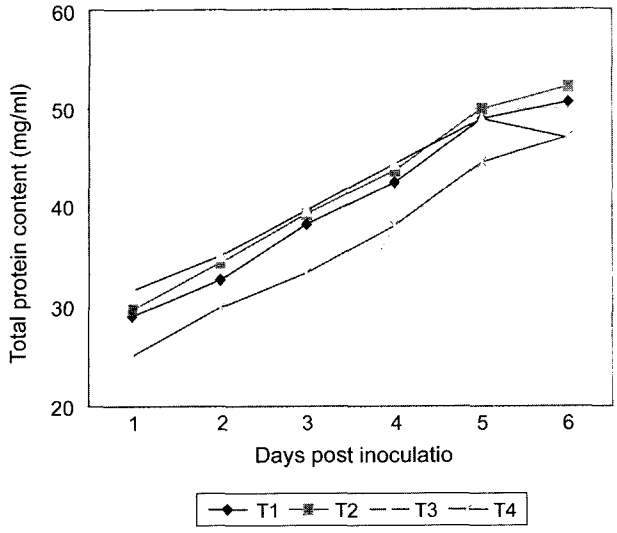


**Fig. 2.** Effect of systemic fungicide on hemolymph total protein content in *B. bassiana* infected silkworm.

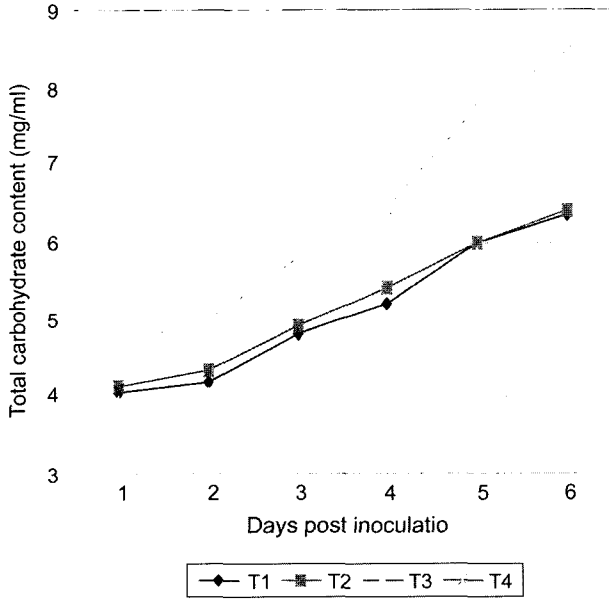


**Fig. 3.** Effect of systemic fungicide on hemolymph total carbohydrate content in *B. bassiana* infected silkworm.

day) to 50.58 mg/ml (6th day). In T2 batch also there was an increase in proteins from 29.70 mg/ml (1st day) to 52.10 mg/ml (6th day). In T3 (inoculated control) batch the protein content increased from 31.73 mg/ml (1st day) to 46.96 mg/ml (6th day). In general, the total protein content increased in T1 and T2 batches compared to normal control. In T3 batch, the total protein content increased up to 5th day and decreased on 6th day.

The data on the effect of systemic fungicide on haemolymph total carbohydrate in *B. bassiana* infected silkworm is presented in Fig. 3. The hemolymph total carbohydrate content of normal control (T4) larvae increased from 4.68 mg/ml on 1st day to 8.45 mg/ml by 6th day. In T1 batch there was an increase in carbohydrate content from 4.06 mg/ml (1st day) to 6.35 mg/ml (6th day). In T2 batch also there was an increase in carbohydrate content from 4.13 mg/ml (1st day) to 6.41 mg/ml (6th day). In T3 (inoculated control) batch the carbohydrate content increased from 3.91 mg/ml (1st day) to 6.10 mg/ml (6th day). In general the carbohydrate content decreased in T1, T2 and T3 batches compared to normal control batch.

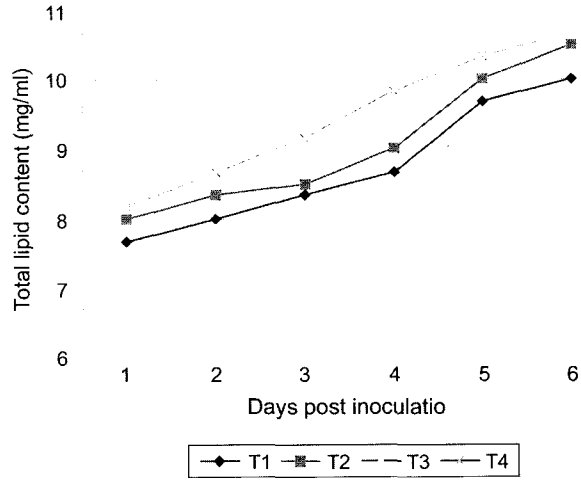


**Fig. 4.** Effect of systemic fungicide on hemolymph total lipid content in *B. bassiana* infected silkworm.

The effect of systemic fungicide on haemolymph total lipid content in *B. bassiana* infected silkworm is presented in Fig. 4. The hemolymph total lipid content was increased from 1st day to 6th day in all the treatments. The hemolymph total lipid content of normal control (T4) larvae increased from 8.17 mg/ml on 1st day to 10.67 mg/ml by 6th day. In T1 batch there was an increase in total lipid content from 7.67 mg/ml (1st day) to 10.00 mg/ml (6th day). In T2 batch also there was an increase in total lipid content from 8.00 mg/ml (1st day) to 10.50 mg/ml (6th day). In T3 (inoculated control) batch the total lipid content increased from 7.50 mg/ml (1st day) to 9.17 mg/ml (6th day). There was a gradual decrease in lipid content in T1, T2 and T3 batches compared to normal control batch. The decrease was more in T3 batch.

## Discussion

It is observed from the results that the systemic fungicide reduced the mortality due to white muscardine by preventing the multiplication of vegetative mycelia in the hemolymph. The multiplication of vegetative mycelia and growth was high in T3 batch and less in T1 and T2

batches. In T3 batch the total hemocyte counts were increased in initial stage of *B. bassiana* infection (up to 2nd day) and decreased up to 6th day. But in the case of T1 and T2 batches the total haemocyte counts were significantly increased up to 5th day and decreased. The increase may represent the defense response of silkworm against the invading pathogen. Once entomogenous fungi have penetrated in the host integument and gained access to the nutrient-rich hemocoel, they are confronted with host humoral and/or cellular defenses (Butt *et al.*, 1988; Butt and Humber, 1989; Vey and Gotz, 1986). As humoral reponse, the phenoloxidase system will be activated to induce the phagocytic processses and melanisation which work as toxin to invading microorganisms (Tanada and Kaya, 1993). The cellular responses to infection have been reported in many insects (Chain and Anderson, 1982; Dunn and Drake, 1983; Horohov and Dunn, 1983). Haemocytes are extremely efficient in removing pathogens by accomplishing a series of reactions designated as phagocytosis, nodule formation or encapsulation (Salt, 1970). It is possible that the systemic fungicide induce the defense response through the proliferation of hemocytes as is indicated by the increase in THC.

The results of the study also indicated that changes occur in the hemolymph protein, during the course of *B. bassiana* infection. The difference in protein concentration between normal (T4) and inoculated control (T3) becomes more pronounced as the disease progresses. This would probably indicate that during infection the synthesis and release of proteins from fat bodies are greatly increased. In several lepidopteran insects infected with *B. bassiana*, the hemolymph proteins increase up to the last stages of infection and then decrease when the insects cease feeding prior to death (Gardner *et al.*, 1979). There are also reports of production of anti-microbial substances such as lectin, defensin and attacin with the entry of foreign bodies (Wago, 1995). These may be induced by the systemic fungicide which may be represented by the increase biochemical protein in the hemolymph. The present results concur with the above findings. Pombo *et al.*, (1998) reported that several viral-induced proteins were also produced during infection of baculovirus and a sharp decrease in over all protein synthesis was observed. Kusunoki and Watanabe (1982) studied the changes in the amino acid composition of the hemolymph form the fifth instar larvae *B. mori* infected with *B. bassiana.* In lepidoptera both carbohydrates and lipids constitute important sources of energy during larval development and during increased physical activities (Chino and Gilbert, 1965). In conformity with the above, in the present study, the total carbohydrate and lipid contents decreased steadily as the disease developed and it is reasonable to assume that the carbohydrates and lipids were used as a source of energy required for the growth and development of fungus.

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