Activity of key enzymes in the late V instar of silkworm (*Bombyx mori* L.); an important ~~assesment~~ assessment~~criteria~~ criterion in silkworm breeding for stress tolerance

ABSTRACT:

Astudyonenzymeactivityinthermotolerantbivoltinesilkwormbreeds under *Beauveria bassiana* disease was conducted at the Department of Sericulture, UAS, GKVK, Bengaluru during 2021-23. Thermotolerant bivoltine breeds *viz.*,B1,B4,andB8, were resistant to muscardine andCSR4, a muscardine susceptible breedswere used in this study. A batch of ~~silkworms of all the breeds~~ all breeds of silkworms ~~were~~ wasinoculatedwith6.86×10^4~~spores/ml~~spores/mlof*B.bassiana*@0.5mlpersilkwormimmediatelyafter4thmoult and another batch ~~were~~wasreared under normal ~~condition~~conditions. The haemolymph was collected fromthese breeds at 48, 96, and 144 hours post inoculation (hpi) with *B.bassiana*sporesinbothinoculatedandcontrolbatches.Biochemical estimationofamylase, protease, and trehalase enzyme activities in the haemolymph. The amylase activityincreasedfrom48hpito144hpiinallthebreeds underbothcontroland muscardine inoculation, but being lesser under inoculation. The protease activity was foundhigherinthe B1breedfrom48hpito144hpiunderbothcontrolandinoculatedconditions. Trehalaseactivitywasenhancedatalltimeintervals under control conditions in B1, B4 and B8 breeds and CSR4 breed ~~shows decreased~~shows decreasedat144hpiunder both control and inoculation conditions.~~Thus~~Thus, increased enzyme activity in B1 and B4 breeds couldbe associated with their better performance for survival and economic parameters underinoculated~~condition~~conditions. The correlational studies revealed that haemolymph amylase, protease, and trehalase were found to be positively ~~corelated~~ correlatedwith cocoon weight (g) and negatively correlated with larval mortality (%). Thus, the study revealed that silkworm breeds like B1, B4, and B8 as productive breeds and hence may be used for future breeding programmes for the evolution of new robust silkworm breeds.

KEYWORDS: Silkworm,Thermotolerant, Amylase, Protease, Trehalase and Muscardine Inoculation

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Introduction :

Diseases in silkworm, *Bombyx mori* L. are fairly common in occurrence and are serious in inflicting losses. Silkworm diseases are grouped under four major categories, namely the microsporidian, viral, bacterial and fungal diseases, which are popularly known as pebrine, grasserie, flacherie and muscardine, respectively. Among these diseases, muscardine is ~~a~~ themost contagious one caused by *Beauveria bassiana*, which accounts for 30-40 per cent of cocoon crop ~~loss (~~loss ((Chandrasekharan and Nataraju, 2008). Haemolymph ~~is plays~~plays on every physiological activity of the insect body ~~that include~~ includingmaintenance of correct moisture ratio, body shape, optimal body temperature, protection against insect, pathogens, etc~~.~~. The organic constituents of haemolymph (proteins, carbohydrates, free amino acids, lipids, enzymes *etc*.) play an important role in biochemical processes underlying the growth and development of insects, thus changes in the composition of ~~haemolymph~~ hemolymphreflect the physiological and biochemical transformations taking place in the insect tissues. Enzymes play a very important role in the growth and development of all organisms as they are involved in various biochemical reactions. The growth of the silkworm during the larval stage is enormous, an increase in growth by size and weight necessitates various enzymes.Amylase is one such key enzyme responsible for disease resistance and is also involved in the digestion and metabolism of carbohydrates present in the mulberry leaves in the form of starch. It is well known that amylase hydrolyses alpha-1, 4-glycosidic bonds of starch to produce maltose units in the silkworm.protease enzymes are integral to the digestive processes, silk production, immune ~~defense~~defence, and growth regulation in Bombyx mori. Their multifaceted roles underscore their importance in the lifecycle and economic significance of silkworms in sericulture.Trehalose, a non-reducing disaccharide, is the major blood sugar in insects playing a crucial role as an instant source of energy and in dealing with abiotic stresses. The hydrolysis of trehalose is under the enzymatic control of trehalase. The enzyme trehalase is gaining interest in insect physiology as it regulates energy metabolism and glucose generation via trehalose catabolism. The two forms of insect trehalase namely, Tre-1 and Tre-2 are important in energy supply, growth, metamorphosis, stress recovery, chitin synthesis and insect flight.In insects, trehalose forms the major hemolymph sugar and is synthesized in the fat body following a pathway that involves two enzymes, namely, trehalose-6-phosphate synthase and trehalose 6-phosphate phosphatase.To understand the response of thermotolerant bivoltine silkworm breeds to biotic stress, activity in four breeds, all subjected to 48, 96 and 144 hpiat different time intervals.

MATERIALANDMETHODS

Breedsusedfortheexperiment

Four silkworm breeds *viz*., B1, B4, B8 and CSR4 were procured fromCentral Sericultural Research and Training Institute, Mysore (Table 1). These breeds were reared by following appropriate rearing practices(Dandin and Giridar, 2014). The fifth

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instar silkworms were topically inoculated with *B.bassiana*spores (LC_{50},6.86×10^4spores/ml at the rate of0.5 ml per worm).

Table 1: Larval and cocoon parameters of the thermotolerant breeds used in theexperiment

Sl. No.	Genotypes	Breed traits	Response to muscardine infection
1	B1	Plain larva spinning ~~oval shaped~~oval-shaped cocoon	Thermotolerant and ~~resistance~~ resistantto muscardine infection
2	B4	Plain larva spinning ~~oval shaped~~oval-shaped cocoon	
3	B8	Marked larva spinning peanut cocoon	
4	CSR_4	Plain larva spinning peanut cocoon	Productive but susceptible to muscardine infection

Source of white muscardine ~~fungi, *B.*~~fungi, *B. bassiana*

The pathogen source was originally collected from Sidlaghatta, Karnataka, India and has been characterized using ITS 1 and ITS 4 markers and named ~~as~~ SHDL isolate (Sahana, 2022). Diseased silkworm cadavers preserved in the Department of Sericulture were used for the present study. The samples were first microscopically examined ~~in order to~~to confirm the presence of conidia and conidiophores of the white muscardine pathogen.

Inoculation of the silkworms

Appropriate dilutions were done ~~so as to~~to make the final concentration to 68,625.00 spores/ml, which was based on earlier work done in the department with same breeds (Keerthana, 2018). The silkworms were inoculated with conidia of the fungus by topical application. The fifth instar silkworms immediately after fourth moult were topically infected with the ~~above mentioned~~above-mentioned concentration at the rate of 0.5 ml per silkworm by spraying uniformly with an atomizer. High relative humidity of 95 ± 5% and a temperature of 25 ± 1ºC were maintained in the rearing room. White muscardine incidence was recorded upto ten days ~~post inoculation~~post-inoculation. Untreated worms of fifth instar were taken as the control.

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Collection and storage of haemolymph

The haemolymph was collected ~~after 48~~after 48h, 96h and 144h post inoculation *i.e.*, on the second, fourth and sixth day of fifth instar in each treatment (Chart.1). The haemolymph was collected from randomly selected fifth instar larvae of each set by cutting the third pair of prolegs. The haemolymph, thus coming out were collected and stored in pre- cooled Eppendorf's tube containing a few crystals of phenylthiourea to prevent oxidation. The samples were labelled and then preserved in deep freezer at -20^0 C until further analysis. The samples were centrifuged at 3000 rpm for 15 minutes to separate out the phenyl thiourea crystals and haemocytes. The supernatant was used for the estimation after proper dilution (Mahesha *et al*., 2000).

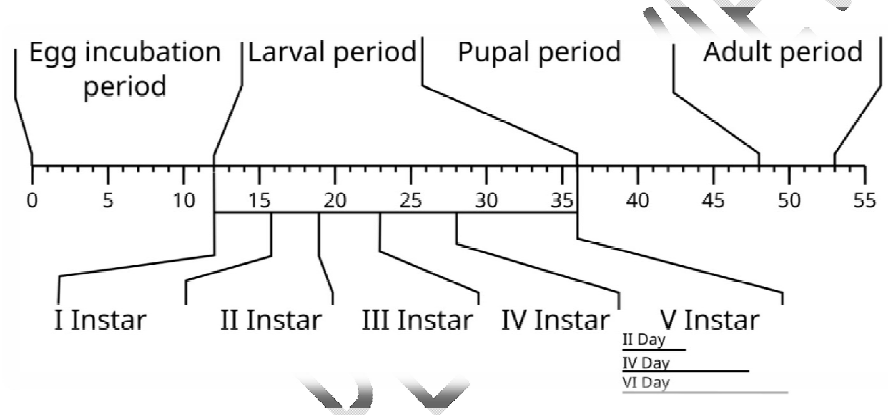

Chart. 1: Sampling scheme of haemolymph on 2nd, 4th and 6thday of V instar larvae

Quantitativeestimationofenzymeactivity inhaemolymph

The amylase and trehalase activity in the haemolymph was determined by measuring the amount of reducing sugar released from the soluble starch substrate by the method reported by Noetling and Bernfeld (1948) using the 3, 5 dinitro salicylic acid (DNS) reagent as modified by Ishaaya and Swirsiki (1976).Also, ~~and t~~the protease activity was measured according to the procedure of Eguchi and Iwamotu (1976) in the haemolymph of both infected and healthy silkworms.The data obtained were analysed using Completely Randomized Design (Sundarraj *et al*.,1972). The mean values of the experiments were compared by using Duncan's MultipleRangeTest(DMRT) (Duncan,1955)and presentedbelow.

Correlation

The nature and magnitude of correlation between cocoon weight, larval mortality, amylase activity, protease activity and trehalase activity were figured out.

Statistical analysis

The statistical analysis of the experimental data wascarriedoutusing computer software OPSTAT.The data obtained from the laboratory experiments wereanalysedanalyzed statistically with Completely Randomized Design (CRD). Different treatmentswere compared using critical difference (CD)~~ value at~~value ~~0.05~~at 0.05 (1%) level of significance.

RESULTSANDDISCUSSION

Amylase enzyme activity in the haemolymph

To understand the response of thermotolerant bivoltine silkworm breeds to biotic stress, amylase activity by quantitative estimation using spectrophotometric analysis in four breeds, all subjected to 48, 96 and 144 hpi at different time intervals.

Forty-eight hpi with *B. bassiana*

Among the four breeds amylase activity under control conditions varied at 48hpi. The enzyme activity was maximum in B1 breed with 2.160 µM/mg protein/min/ml, which was followed by B4 and B8 breeds (2.036 and 1.144µM/mg protein/min/ml), respectively~~)~~. Lower amylase activity was observed in CSR4 (0.718µM/mg protein/min/ml), which were found to be statistically different. Under *B. bassiana* inoculation, the amylase enzyme activity among the breeds was significantly different at 48hpi and significantly maximum enzyme activity was recorded in B1 breed (2.074µM/mg protein/min/ml) followed by B4 breed (1.865µM/mg protein/min/ml). Lower enzyme activity was inCSR4 breed (0.471µM/mg protein/min/ml) followed byB8 breed (0.881µM/mg protein/min/ml) (Fig. 1).

Ninety-six hpi with *B. bassiana*

Among the breeds amylase activity under control conditions varied at 96 hpi. Maximum enzyme activity was seen in B1 breed (2.751µM/mg protein/min/ml) followed by the B4 breed (2.343µM/mg protein/min/ml). Lower enzyme activity was in CSR4 breed (0.867µM/mg protein/min/ml) followed by B8 breed (1.472µM/mg protein/min/ml). Under *B. bassiana* inoculation, the amylase enzyme activity varied among the four breeds with maximµ enzyme activity being in B1 breed (2.569µM/mg protein/min/ml) followed by B4 breed (2.054µM/mg protein/min/ml). Lower enzyme activity was observed inCSR4 breed (0.351µM/mg protein/min/ml) followed by B8 breed (0.895µM /mg protein/min/ml) (Table 1) (Fig. 1).

One hundred and forty-fourhpi with *B. bassiana*

Among the four breeds amylase activity under control conditions varied at 144 hpi. Maximum enzyme activity was seen in B1 breed (3.317µM/mg protein/min/ml) followed by B4 breed (2.595µM/mg protein/min/ml). Lower enzyme activity was in CSR4 (1.215µM/mg

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protein/min/ml) followed by B8 breed (1.638µM/mg protein/min/ml). Under *B. bassiana* inoculation, enzyme activity varied among the four breeds and maximum enzyme activity was in B1 breed (2.729µM/mg protein/min/ml) followed by the B4 breed (1.893µM/mg protein/min/ml). Low enzyme activity was found in CSR4 breed (0.261µM/mg protein/min/ml) followed by B8 breed (0.938 µM/mg protein/min/ml) (Fig. 1).

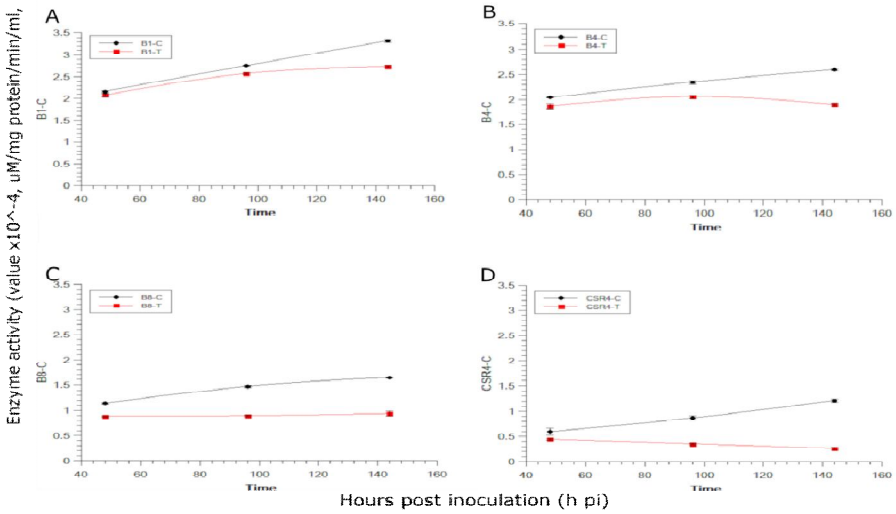

Fig. 1: Amylase activity (µM/mg protein/min/ml) in selected thermotolerant bivoltine silkworm breeds as influenced by *B. bassiana* inoculation at different time intervals

The reports on mulberry leaves shows that the mulberry leaf contains starch, glucose, fructose etc. The monosaccharides glucose and fructose are anticipated to get converted into a readily usable and less reactive ~~(non-reducing)~~ form of carbohydrate called as trehalose. Similarly, the starch fed by silkworms also needs to be converted into trehalose that require the initial breakdown of starch into glucose by amylase enzyme in subsequent conversion to trehalose. Therefore, trehalose is an important low molecular weight stable oligosaccharide in silkwormtowards the rapid and large amount of energy requirement during cocoon formation. Hence, the assessment of amylase activity is an indication of the productivity of silk as well as the silkworm's ability to tolerate/resist stress during its early and late stage of 5th instar.

Among the breeds evaluated for amylase activity ~~as a function of inoculation with *B. bassiana*~~ from the early stage to the later stage of 5th instar showed that the breed B1 and B4 showed better total amylase activity. On the other hand, B8 and CSR4 showed relatively less

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basal total amylase activity. However, when the silkworm matures towards 5th instar a proportional increase in total amylase activity was also found. Upon inoculation with *B. bassiana* the total enzyme activity was found almost similar in all the breeds compared to control. However, as the time pass after inoculation the amylase activity was found gradually declining. The extent of decline was more in B8 and CSR4 compared to B1 and B4. The amylase activity in nutshell, provide knowledge about the preparedness of silkworm breeds for the extent of energy demand during cocooning. Amylase is one of the key enzymes involved in digestion and carbohydrate metabolism in insects.

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The presence of two types of amylase activities in digestive (gut) juice and haemolymph was reported by (Daone *et al*., 1975; Buonocore *et al*.,1976; Horie and Watanabe, 1980). Yokoyama (1959)&~~.~~ Chatterjee *et al*. (1989) reported the presence of two different forms of amylase activity in digestive fluid and haemolymph. Abraham *et al*. (1992) noticed that amylase activity of digestive fluid was ~~40-fold~~40-fold higher than that of haemolymph. The presence of this enzyme is in abundance during larval development in both diapausing and ~~nondiapausing~~non-diapausing strains imply that this enzyme has some important physiological role. The function of haemolymph amylase is not fully understood although Wyatt, (1967) suggested its possible involvement in the degradation of fat body glycogen.

The results showed the decreased amylase activity in the haemolymph of infected silkworm ~~with reference to~~regarding the control. It was directly related to low intake of food as a consequence of fungal infection. Christopher and Mathavan, (1985) suggested that the rational food ~~consuption~~consumption by lepidopteran larvae was correlated directly with the activity of amylase. The larva receiving 100 % food found to have the highest amylase activity, which declined as the percentage of food offered was reduced. In contrast to this, Gururaj *et al*. (1999) found that the activity of amylase increased significantly in the haemolymph from 48 hpi to 144 hpi of infection with *Bm*NPV.

Correlation of haemolymph amylase activity with cocoon weight and larval mortality

During the study, it was found that positive correlations were obtained between haemolymph amylase activity and cocoon weight at 48 hpi (0.563), amylase activity and cocoon weight at 96 hpi (0.626), amylase activity and cocoon weight at 144 hpi (0.726)and negetive correlations were obtained between amylase activity and larval mortality at 48 hpi (-0.367), amylase activity and larval mortality at 96 hpi (-0.472), amylase activity and larval mortality at 144 hpi (-0.589).

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Protease enzyme activity in the haemolymph

Forty-eight hpi with *B. bassiana*

Among the four breeds protease activity under control conditions varied at 48hpi. The enzyme activity was maximum in B1 breed with 0.039µM/mg protein/min/ml, which

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was followed by CSR4 and B4 breeds (0.010and 0.008µM/mg protein/min/ml, respectively). Lower protease activity was observed in B8 (0.005µM/mg protein/min/ml), which were found to be statistically different. Under *B. bassiana* inoculation, the protease enzyme activity among the breeds was significantly different at 48hpi and significantly maximum enzyme activity was recorded in B1 breed (0.030µM/mg protein/min/ml) followed by CSR4 breed (0.008µM/mg protein/min/ml). Lower enzyme activity was in B8 breed (0.005µM/mg protein/min/ml) followed by B4 breed (0.006µM/mg protein/min/ml) (Fig.2).

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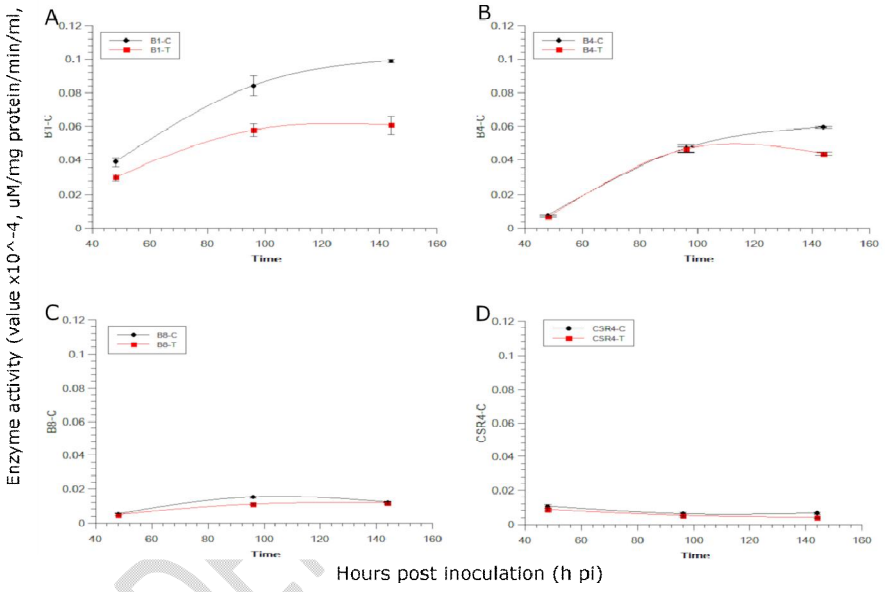

Fig. 2: Protease activity (µM/mg protein/min/ml) in selected thermotolerant bivoltine silkworm breeds as influenced ~~by *B.*~~by *B. bassiana* inoculation at different time intervals

Ninety-six hpi with *B. bassiana*

Among the breeds protease activity under control conditions varied at 96hpi. Maximum enzyme activity was seen in B1 breed (0.084µM/mg protein/min/ml) followed by the B4 breed (0.047µM/mg protein/min/ml). Lower enzyme activity was inCSR4 breed (0.006µM/mg protein/min/ml) followed by B8 breed (0.015µM/mg protein/min/ml). Under

B. bassiana inoculation, the protease enzyme activity varied among the four breeds with maximum enzyme activity being in B1 breed (0.058 µM/mg protein/min/ml) followed by B4 breed (0.046µM/mg protein/min/ml). Lower enzyme activity was observed in CSR4 breed (0.005µM/mgprotein/min/ml), which was followed by B8 breed (0.011µM/mg protein/min/ml) (Fig.2).

One hundred and ~~forty four~~forty-four hpi with *B. bassiana*

Among the four breeds protease activity under control conditions varied at 144 hpi. Maximum enzyme activity was seen in the B1 breed (0.099µM/mg protein/min/ml) followed by B4 and B8 breeds (0.059 and 0.012µM/mg protein/min/ml, respectively). Lower enzyme activity was in CSR4 (0.006 µM/mg protein/min/ml). Under *B. bassiana* inoculation, enzyme activity varied among the breeds and maximum enzyme activity was in the B1 breed (0.061µM/mg protein/min/ml) followed by the B4 breed (0.044 µM/mg protein/min/ml). Low enzyme activity was found in the CSR4 breed (0.004µM/mg protein/min/ml) followed by the B8 breed (0.011µM/mg protein/min/ml) (Fig.2).

According to (Harper *et al*., 1979), the decrease of protease activity in infected breeds ~~compare~~ compared to control batch, a lysosomal enzyme, could be due to the damage caused to lysosomes or due to the destruction of organ systems, there by disturbing the biochemical functions of the cell organelles.

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Srinivas, (1986) described the stimulation of proteolysis in tissues by activating protease enzymes which are responsible for the protein depletion in the tissues of silkworms under phosphomidon toxicity. Protein depletion in tissues may constitute a physiological mechanism and may play a role in compensatory mechanisms under insecticidal stress, to provide intermediates to the Krebs cycle, by retaining free amino acid content in haemolymph, to compensate for osmo-regulatory problems encountered due to the leakage of ions and other essential molecules during the insecticidal stress.

Kobayashi *et al*. (1985) and Nath *et al*. (1997) reported increase in protease activity in various tissues of silkworm. *B. mori* under pathological and induced insecticidal stress conditions. Concurrent to the decrease in total proteins, the significant increases in protease activity in the haemolymph and fat body of both races of silkworm under the influence of fluoride clearly document the domination over protein synthesis.

Karel and Saxena, (1975) reported the increase in proteolysis activity and disturbs the biochemical functioning of cellular activities and impairs protein sysnthetic potentials. Due to the lysosomal instability impaired protein and synthetic potential, cellular distruption might be the reason for the decreased protein levels, as observed in the haemolymph of fifth instar PM and NB4D2 races subjected to the lethal and sublethal doses of fluoride. (Sreedevi *et al*., 1972).

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Rajitha *et al*. 2013 found similar results when interaction between protein content and protease activity was examined in the haemolymph of 5th instar silkworm *Bombyx mori* L. during the progress of fungal pathogen *Beauveria bassiana.* Protein content was elevated significantly in the initial stage of experimental larvae *i.e.* from 1st to 3rdday (168.7 to 200.8 mg/ml), from 4th day to 6th day the biomolecules shown decreased trend (189 to 152.58 mg/ml). Whereas gradual elevation of protease activity was recorded from 1st day of inoculation to 6th day of inoculation (0.037 µg/ml to 0.043 µg/ml).

Correlation of haemolymph protease activity with cocoon weight and larval mortality

During the study, it was found that positive correlations were obtained between haemolymph protease activity and cocoon weight at 48 hpi (0.447), protease activity and cocoon weight at 96 hpi (0.605), protease activity and cocoon weight at 144 hpi (0.664) and negetive correlations were obtained between Amylase activity and larval mortality at 48 hpi (-0.201), protease activity and larval mortality at 96 hpi (-0.332), protease activity and larval mortality at 144 hpi (-0.371).

Trehalase enzyme activity in the haemolymph

Trehalose, a non-reducing disaccharide, is the major blood sugar in insects playing a crucial role as an instant source of energy and in dealing with abiotic stresses. The hydrolysis of trehalose is under the enzymatic control of trehalase. The enzyme trehalase is gaining interest in insect physiology as it regulates energy metabolism and glucose generation via trehalose catabolism. The two forms of insect trehalase namely, Tre-1 and Tre-2 are important in energy supply, growth, metamorphosis, stress recovery, chitin synthesis and insect flight.

In insects, trehalose forms the major hemolymph sugar and is synthesized in the fat body following a pathway that involves two enzymes, namely, trehalose-6-phosphate synthase and trehalose 6-phosphate phosphatase.To understand the response of thermotolerant bivoltine silkworm breeds to biotic stress, trehalase activity in four breeds, all subjected to 48, 94 and 144 hpiat different time intervals.

Forty-eight hpiwith *B.bassiana*

Among the four breeds,trehalase activity varied significantly in the control (without inoculation) at 48hpi. The enzyme activity was found maximum in B1 breed with 1.653 µM/mg protein/min/ml, followed by B4 and B8 breeds (1.345and 1.203µM/mg protein/min/ml, respectively). Lower trehalase activity was observed in CSR4 (0.646µM/mg protein/min/ml), which were found to be significantly different.*B. bassiana* inoculation, the trehalase enzyme activity was measured at 48hpi.Among the breeds, maximum enzyme activity was recorded in B1 (1.565 µM/mg protein/min/ml)followed by B4 (1.199µM/mg protein/min/ml). Lowest enzyme activity was observed in CSR4 breed (0.207µM/mg protein/min/ml) followed by B8 breed (0.962µM/mg protein/min/ml). (Fig.3).

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Ninety-six hpi with *B. bassiana*

Among the breeds trehalase activity under control conditions varied at 96 hpi. Maximum enzyme activity was seen in B1 breed (1.920µM/mg protein/min/ml) followed by the B4 breed (1.711µM/mg protein/min/ml). Lower enzyme activity was inCSR4 breed (0.531 µM/mg protein/min/ml) followed by B8 breed (1.594 µM/mg protein/min/ml). Under *B. bassiana*inoculation, the trehalase enzyme activity varied ~~amongthe~~ among four breeds with the maximum enzyme activity being in B1 breed (1.843µM/mg protein/min/ml) followed by B4 breed (1.722µM/mg protein/min/ml). Lower enzyme activity was observed inCSR4 breed (0.150 µM/mg protein/min/ml), which was followed byB8 breed (0.732µM/mg protein/min/ml) (Table 3) (Fig. 3).

One hundred and ~~forty four~~forty-four hpi with *B. bassiana*

Among the four breeds studied here, the specific activity of trehalase varied significantly from activity under control conditions varied at 144 hpi. Maximum enzyme activity was seen in B4 breed (2.455µM/mg protein/min/ml) followed by B1 breed (2.308µM/mg protein/min/ml). Lower enzyme activity was in B8 breed (1.862µM/mg protein/min/ml) followed by CSR4 (0.296µM/mg protein/min/ml). Under *B. bassiana* inoculation, enzyme activity varied among the breeds and maximum enzyme activity was in B1 breed (1.855µM/mg protein/min/ml) followed by the B4 breed (1.671µM/mg protein/min/ml). Low enzyme activity was found inCSR4 breed (0.107µM/mg protein/min/ml) followed byB8 breed (0.615 µM/mg protein/min/ml) (Fig. 3).

Trehalase plays an important role in energy supply to an insect (Wyatt, 1967) and trehalase serves as an indicator of energy reserves resulting from the availability of carbohydrate nutrients. Trehalase is the one of the most important carbohydrolases in insects occurring in the gut, flight muscles, fat bodies, labial glands, haemolymphand also in the silk glands of silkworm.

It causes the breakdown of trehalose into glucose for internal supply for chitin synthesis, muscular activity during flight, cocoon formation and other metabolic process. The enzyme catalyzes the hydrolysis of trehalose into two glucose molecules.Significant elevation of trehalase activity was observed in the initial stage of infection, then the activity of the enzyme was declined in inoculated larvae compared to healthy ones. It appears that the energy demands are stepped up in the host in initial stage of infection, when the physiology of the host is altered to combat the disease as a natural response. The decrease in the trehalase activity in the *B.bassiana* inoculated larvae could be attributed to decreased metabolic capabilities of infected larvae.

This was also interpreted as due to decreased hydrolysis of trehalose to release glucose molecules under drastic stress conditions and high energy demand (Hawakawa and Chino, 1981)as trehalase activity and trehalose levels are inversely related. In contrast to the present study Sasikala (2007) observed progressively higher trehalase activity in uzi infected 5^{th} instar silkworm larvae. This was attributed to active breakdown of trehalose presumably to meet the energy demands. Higher trehalase activity in the uzi infested tissues over the normal is indicative

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of higher conversion of glucose during energy needs of both the host and parasite. Yaginuma *et al.* (1990) observed trehalase activity tends to increase during the middle stage of CPV infection in infected midgut. Gururaj *et al.* (1999) noticed no significant change in the haemolymphtrehalase activity between *Bm*NPV infected and control larvae till 96 h then enzyme activity was increased in the rest of the instar. He suggested that the increase in the enzyme activity is associated with decreased levels of trehalose.

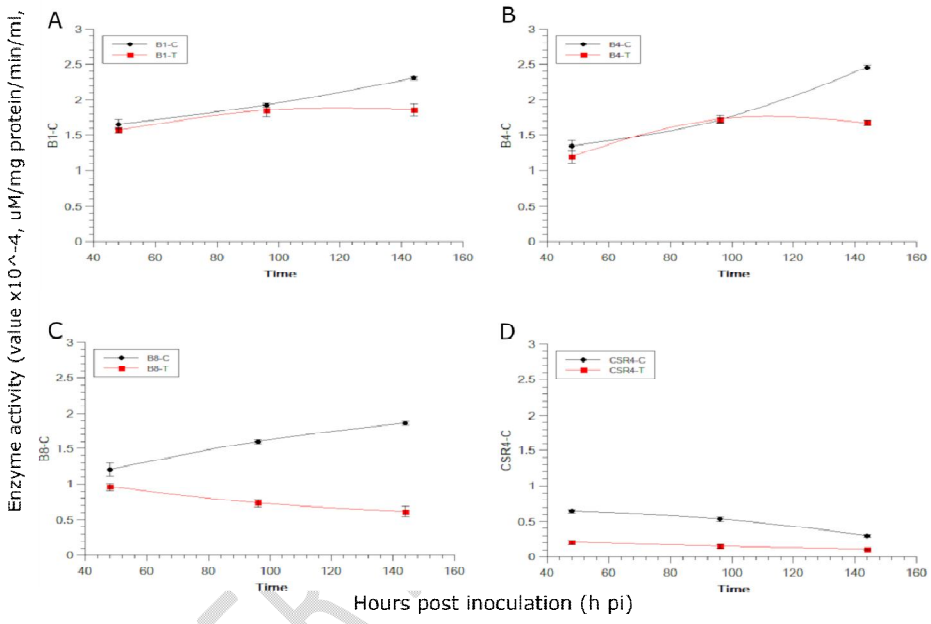

Fig. 3: Trehalase activity (µM/mg protein/min/ml) in selected thermotolerant bivoltine silkworm breeds as influenced ~~by *B.*~~by *B. bassiana* inoculation at different time intervals

Correlation of haemolymph trehalase activity with cocoon weight and larval mortality

During the study, it was found that positive correlations were obtained between haemolymph trehalase activity and cocoon weight at 48 hpi (0.574), trehalase activity and cocoon weight at 96 hpi (0.544), trehalase activity and cocoon weight at 144 hpi (0.682) (Fig.4) and negetive correlations were obtained between Amylase activity and larval mortality at 48 hpi (-0.546), trehalase activity and larval mortality at 96 hpi (-0.520), trehalase activity and larval mortality at 144 hpi (-0.3) (Fig.5) (Table 2).

Conclusion

The study investigated biochemical responses in thermotolerant as well as thermoliable bivoltine silkworm breeds infected with *Beauveria bassiana*. Among the four breeds studied B1, B4 and B8 were found to have ~~a~~ better disease resistance compare to CSR4 in terms of survivability and cocoon characteristics etc.

Biochemical studies ~~shows~~show higher activity in amylase, trehalase and protease in infected B1, B4 and B8 compared to their respective control and susceptible CSR4 showed biochemical evidences for disease resistance. Therefore, ~~these biochemical assessment~~this biochemical assessment can be reliable approach to provide scientific evidence to disease resistance in silkworm infected with pathogen *B. bassiana*. However, as biochemical/enzymatic markers are measurable at a later stage of cellular response, more ~~early stage~~early-stage markers including metabolite markers and transcription markers may be further investigated for rapid assessment as disease resistance screening and parental line selection for breeding purpose.

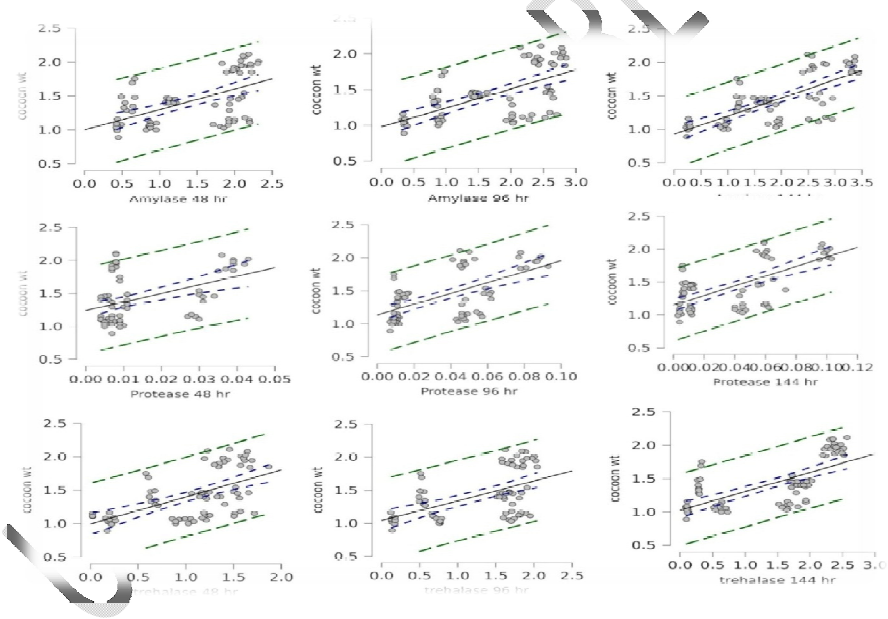

Fig. 4: The correlation analysis between different enzyme activity and cocoon weight

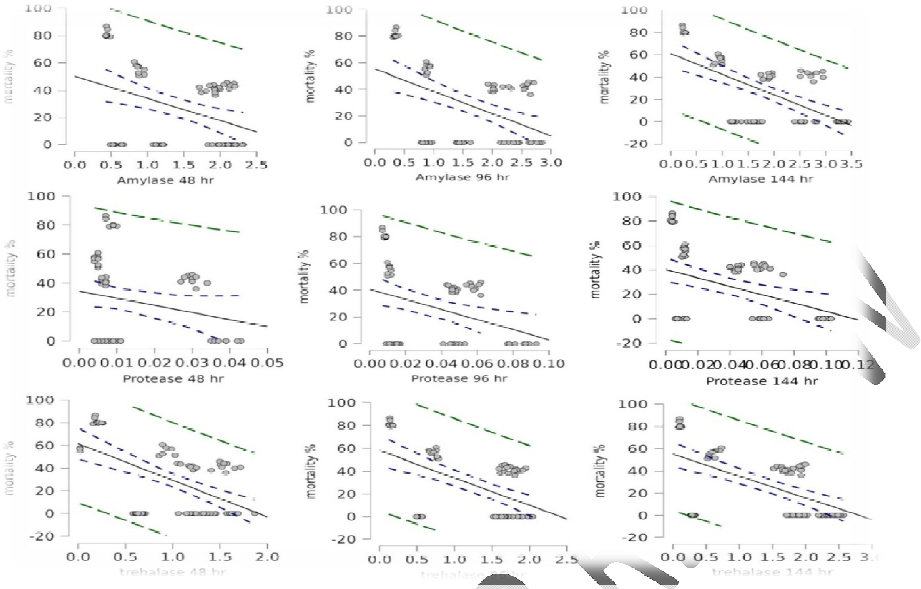

Fig. 5: correlation analysis between different enzyme activity and larval mortality

Table 2: Pearson's correlations between different enzyme activities and silkworm economic characters

Enzyme activity	Cocoon parameters	Pearson's r	p
Amylase 48 h	Cocoon weight	0.563***	< .001
Amylase 96 h	Cocoon weight	0.626***	< .001
Amylase 144 h	Cocoon weight	0.726***	< .001
Protease 48 h	Cocoon weight	0.447***	< .001
Protease 96 h	Cocoon weight	0.605***	< .001
Protease 144 h	Cocoon weight	0.664***	< .001
Trehalase 48 h	Cocoon weight	0.574***	< .001
Trehalase 96 h	Cocoon weight	0.544***	< .001
Trehalase 144 h	Cocoon weight	0.682***	< .001
Amylase 48 h	Mortality (%)	-0.367**	0.002

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Amylase 96 h	Mortality (%)	-0.472***	< .001
Amylase 144 h	Mortality (%)	-0.589***	< .001
Protease 48 h	Mortality (%)	-0.201	0.09
Protease 96 h	Mortality (%)	-0.332**	0.004
Protease 144 h	Mortality (%)	-0.371**	0.001
Trehalase 48 h	Mortality (%)	-0.546***	< .001
Trehalase 96 h	Mortality (%)	-0.52***	< .001
Trehalase 144 h	Mortality (%)	-0.568***	< .001

* p < 0.05, ** p <0.01, *** p <0.001

Comment [SS13]: ??? Are they non-significant

Comment [SS14]: Please check the data of table 5.

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