Original Research Article

Activity of peroxidase and catalase enzymes activity in thermotolerant silkworms *Bombyx mori*. L. in response to *Beauveria bassiana* (Bals. - Criv.) Vuill. infection

ABSTRACT

With an intention to understand the mechanism of dual stress tolerance, a study on antioxidant enzymes activity in thermotolerant bivoltine silkworm breeds and hybrids under *Beauveria bassiana* infection was conducted at the Department of Sericulture, UAS, GKVK, Bengaluru during 2021-22. Thermotolerant bivoltine breeds *viz*., B1, B4 and B8 and their hybrids with CSR4 along with Pure Mysore as a muscardine resistant and CSR4 as a muscardine susceptible breeds were used in this study. All the breeds and hybrids were raised following CRD experimental design and were inoculated with *B. bassiana* immediately after 4th moult and another group was reared under normal condition. The haemolymph was collected from these breeds and hybrids at 24, 48, 72, 96 and 120 hours post inoculation (hpi) from both the batches and utilized for the estimation of total protein content and peroxidase and catalase enzyme activities in the haemolymph. The total protein content increased from 24 h to 120 h in all the breeds and hybrids under both control and muscardine inoculation, but being lesser under inoculation. The peroxidase activity was found higher in B1 breed from 48 hpi to 120 hpi under both control and inoculated conditions, but decreased at 24 hpi under inoculation. Catalase activity was enhanced at all time intervals under inoculated conditions in Pure Mysore, B1, B4 and B8 breeds and B4×CSR4 hybrid compared to control condition. Thus increased catalase activity in B1 and B4 breeds and decreased peroxidase activity in B1 breed could be associated with their better performance for survival and economic parameters under inoculated condition.

Keywords: Silkworm, thermotolerant, antioxidant, muscardine and inoculation

1. INDRODUCTION

An economically significant poikilothermic insect that produces silk is the mulberry silkworm, *Bombyx mori* L. The natural growth and development of silkworms are greatly influenced by environmental conditions, particularly temperature, which can be raised most effectively between 25 and 28 °C. Any difference from the ambient temperature required for proper physiological and metabolic functions is frequently regarded as thermal stress, which is linked to a variety of physiological stress enhances reactive oxygen species (ROS) production, which in turn causes oxidative damage. Silkworms, like all insects, have enzymatic and non-enzymatic antioxidative defence systems to prevent oxidative damage. Silkworm has developed efficient innate immune system to fight against microbial pathogens. The innate immune system plays a crucial biological role in the limiting the microbial infections by using different immune strategies such as production of antimicrobial peptides (AMPs), generation of reactive oxygen species and formation of melanin Abbas *et al*. [1]. Production of

reactive oxygen species under stress and their quenching is a response to get rid of the stress faced by the insect. Superoxide dismutases (SOD), catalases (CAT), peroxidases (POX), and glutathione S-transferases (GST) are the most important antioxidant enzymes secreted by silkworms Pooja *et al*. [2].

The superoxide dismutase (SOD) upon infection with bacteria after an initial burst (10 min), sharply declined at 30 min and then significantly enhanced in larval haemolymph, implying the modulation of superoxide anion generation is an early response as part of the defensive process against pathogen invasion Krishnan *et al*. [3]. Similarly, Catalase activity in the fat body revealed a positive correlation between the control (28±1^0 C) and high temperature (40±1^0 C) Nabizadeh and Jagadeesh Kumar, [4]. The lipid peroxidation (LPO) levels increased significantly in a time-dependent manner under thermal stress in oriental fruit fly, *Bactrocera dorsalis* (Hendel) Jia *et al*., [5]. Increase in hydrogen peroxide generation with concomitant increase in LPO and protein carbonyl levels in midgut and hemocytes was observed in *B. mori* exposed to low temperature, hypoxia and NPV infection indicting a possible transitory defense mechanism to avoid oxidative stress induced cell damage. Silkworms are known to respond to fungal infection through production of serinprotease inhibitors Li *et al*. [6] and prophenoloxidase – phenoloxidase cascade Zhang *et al*. [7].

The studies on stress induced ROS production in silkworm under *B. bassiana* infection and it's controlling mechanism is scanty, though it is known that catalase activity is affected under *B. bassiana* infection Rajitha and Savithri, [8].

Earlier studies revealed that thermotolerant bivoltine breeds to *B. bassiana* infection have reaveled that B1, B2, B4 and B8 to be relatively resistant to fungal infection Keerthana, [9]. To identify productive thermotolerant breeds resistant to *B. bassiana* infection in order to identify dual stress tolerant breeds, the study was intended to further understand the mechanism of defending ROS accumulating in thermotolerant bivoltine silkworm under *B. bassiana* infection.

2. MATERIAL AND METHODS

2.1 Silkworm breeds used

Five silkworm breeds *viz*., B1, B4, B8, CSR4 and Pure Mysore were procured from Central Sericultural Research and Training Institute, Mysore. Three hybrids B1×CSR4, B4×CSR4 and B8×CSR4 were developed by crossing at Sericulture department, GKVK, Bangalore. These breeds and hybrids were raised by following recommend rearing practices Dandin and Giridar, [10]. The fifth instar silkworms were topically inoculated with *B. bassiana* spores (LC_{50}, 9.04×10^4 spores/ml @ 0.5 ml per worm).

2.2 Collection and storage of haemolymph

The haemolymph was collected after 24h, 48h, 72h, 96h and 120h post inoculation *i.e*., on the first, second, third, fourth and fifth day of fifth instar in each treatment. 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 phenylthiourea crystals and haemocytes. The supernatant was used for the estimation after proper dilution Mahesha *et al*. [11].

2.3 Quantitative estimation of protein and enzyme activity in haemolymph

The total proteins in the haemolymph was estimated by following Lowry's method (Lowry *et al*. [12] using crystalline Bovine Serum Albumin (BSA) as standard. Catalase enzyme activity was determined by the method of Abei [13] and Peroxidase enzyme activity was determined according to Sadashivam and Manickam, [14].

The data were analysed using Completely Randomized Design Sundarraj *et al*. [15]. The mean values of the experiments were compared by using Duncan's Multiple Range Test (DMRT) Duncan, [16] and presented below.

3. RESULTS AND DISCUSSION

3.1 Total Protein Content (mg/ml)

In the present study the total protien content was estimated at 24h, 48h, 72h, 96h and 120h time intervals after post inoculation. Total protein content in haemolymph was more under control compared to infected and it was minimum at 24 hpi and maximum at 120 hpi in all breeds and hybrids. It was observed that protein content to be more in hybrids, followed by thermotolerant bivoltine breeds and it was found to be least in Pure Mysore breed. B8 breed among the breeds recorded maximum total protein content of 243.47 mg/ml at 24 hpi under control and 209.61 mg/ml under inoculated conditions. At 48 hpi B4 breed (280.25 mg/ml) recorded maximum protein content among the breeds under control whereas, under inoculated conditions B8 breed (246.98 mg/ml) recorded maximum protein. From 72 hpi to 120 hpi B4 breed (327.24, 357.60 and 435.85 mg/ml under control, 292.22, 340.09 and 356.14 mg/ml under inoculated condition at 72, 96 and 120 hpi, respectively) recorded maximum total protein under both control and inoculated conditions. Among the hybrids B4×CSR4 hybrid (351.47, 417.15, 455.67, 492.16 and 527.48 mg/ml; 310.61, 368.11, 402.55, 467.93 and 490.41 mg/ml under control and infected conditions, respectively) (Table 1) recorded maximum total protein content at all time intervals from 24 hpi to 120 hpi under both control and inoculated conditions.

Ramesh Babu *et al*. [17] suggested that the survival ability of an animal to stress majorly depends on its protein synthetic potential, any stress on an animal invokes compensatory metabolic adjustments in its tissues through modifications of proteins. Malik and Malik [18] reported that in the normal larva of *B. mori*, haemolymph protein content showed relatively constant increase from first to fourth stage. In the fifth instar larvae, it increased nearly two-fold on the day four and reached a maximum on day nine.

Among ten thermotolerant studied under muscardine infection, the total protein content in haemolymph was found to decrease from 24 hpi to 72 hpi and again decreased 120 hpi in control, whereas in *B. bassiana* inoculation, it decreased from 24 hpi to 72 hpi and then increased at 120 hpi. The protein content under the stress conditions were much lesser than that of corresponding control Sreejith, [19]. In the present study the same trend was seen among all the breeds and hybrids where their was an increase in total protein content from 24 hpi to 120 hpi. The decrease in total protein content due to the infected could be that the pathogen may have caused the reduction in metabolic activity in the silkworm thus reduced protein synthesis. This would affect the cocoon production of silk content in the infected silkworm batches. These results are in agreement to the findings of Rajitha and Savithri, [20] in cross breed silkworms.

3.2 Peroxidase enzyme activity (µmole/l)

In all the breeds and the hybrids, the peroxidase enzyme activity was found higher in control compared to that of infected ones. Maximum peroxidase activity was observed at 24 hpi in all breeds and hybrids. Minimum enzyme activity was seen at 120 hpi. Among breeds, B1 breed 152.95, 150.23, 144.78, 72.47 and 59.73 µmole/l, 78.73, 75.99, 67.70, 61.02 and 59.28 µmole/l (Fig 1) has shown highest enzyme activity from 24 hpi to 120 hpi under both control and infected conditions, respectively. Among the hybrids, B1×CSR4 and B8×CSR4 hybrids have shown higher enzyme activity at all time intervals from 24 hpi to 120 hpi.

Reddy and Venkatappa [21] studied the effect of *Staphylococcus aureus* infection in fifth instar silkworm and found gradual decrease in glutathione peroxidase (GPx) activity among infected group, compared to control. The lowest activity was recorded for GPx (0.121 nmol NADPH/min/mg protein) at 24 hours post infection.

3.3 Catalase enzyme activity (µmoles/min/ml)

In Pure Mysore breed initially catalase enzyme activity was higher under inoculated condition at 24 hpi and 48 hpi, then it decreased at 72 hpi and increased at 96 hpi up to 120 hpi compared to control. In CSR4 breed the enzyme activity was low at 24 hpi and also at 48 hpi, then it has showed higher activity at 72 hpi, 96 hpi and 120 hpi compared to that of control.

In B1 breed the enzyme activity was higher compared to control conditions throughout the larval period from 24 hpi to 120 hpi compared to control. In B4 breed initially at 24 hpi and 48 hpi enzyme activity was higher under inoculated conditions then it decreased at 72 hpi and again it increased at 96 hpi and 120 hpi compared to control. In B8 breed at 24 hpi enzyme activity was same under both control and inoculated conditions, then it decreased under inoculated conditions at 48 hpi, increased at 72 hpi, decreased at 96 hpi and again increased at 120 hpi compared to control. In B1×CSR4 hybrid initially enzyme activity was same at 24 hpi under both control and inoculated conditions. Then from 48 hpi to 120 hpi enzyme activity was lower under inoculated condition compared to control. In B4×CSR4 hybrid the catalase enzyme activity was lower under inoculated conditions up to 48 hpi compared to that of control. Then from 72 hpi to 120 hpi enzyme activity was higher under inoculated conditions than that of controlled ones. B8×CSR4 hybrid showed lower enzyme activity lower at 24 hpi under inoculated conditions compared to control, then at 48 hpi enzyme activity increased under inoculated conditions and again from 72 hpi to 120 hpi it decreased under infection compare to control condition (Fig 2).

Catalase enzyme activity was found to be enhanced under inoculated conditions in the breeds Pure Mysore, B1, B4, B8 and hybrid B4×CSR4 compared to control conditions which may be associated with their resistance to the disease. But in CSR4 breed and B1×CSR4 and B8×CSR4 hybrids enzyme activity was maximum under control compared to inoculated conditions. At different time intervals the breeds and hybrids exhibited more fluctuations in catalase enzyme activity, except in B1 breed and B1XCSR4 hybrid which showed higher catalase activity under inoculated and normal conditions, respectively.

According to Praveena and Savithri [22] catalase activity during fifth instar under *Beauveria bassiana* infection in three breeds revealed that the activity gradually decreased in all breeds under infected condition compared to control. Elevated antioxidant enzyme catalase activity in bivoltine double hybrid suggests their higher level of immunity compared to single hybrids. Such enhanced antioxidant enzyme activity may provide defense to the host organism.

Table 1. Total protein content (mg/mL) in haemolymph of selected thermotolerant bivoltine silkworm breeds and hybrids as influenced by *B. bassiana* infection

Breeds **and** hybrids		24 hpi		48 hpi		72 hpi		96 hpi		120 hpi	
		Control	Muscardine inoculation	Control	Muscardine inoculation	Control	Muscardine inoculation	Control	Muscardine inoculation	Control	Muscardine inoculation
Breeds	Pure Mysore	179.26^{g}	160.58^{de}	195.91^{ef}	171.09^{f}	210.20^{f}	172.82^{f}	228.00^{f}	212.24^{e}	266.82^{f}	260.70^{f}
	CSR4	160.58^{h}	150.95^{e}	207.28^{e}	180.72^{f}	237.64^{e}	187.14^{ef}	251.65^{e}	201.73^{e}	329.87^{d}	258.94^{f}
	B1	191.52^{f}	160.29^{de}	192.69^{f}	170.21^{f}	287.55^{d}	214.87^{d}	317.03^{d}	297.18^{d}	311.19^{e}	298.06^{e}
	B4	225.38^{e}	171.96^{d}	280.25^{d}	211.36^{e}	327.24^{c}	292.22^{c}	357.60^{c}	340.09^{c}	435.85^{c}	356.14^{d}
	B8	243.47^{d}	209.61^{c}	268.56^{d}	246.98^{d}	283.46^{d}	196.77^{e}	322.87^{d}	206.99^{e}	318.49^{de}	214.29^{g}
Hybrids	B1×CSR4	263.03^{c}	253.40^{b}	313.82^{c}	271.78^{c}	354.39^{b}	305.35^{c}	430.86^{b}	336.00^{c}	505.00^{b}	434.95^{b}
	B4×CSR4	351.47^{a}	310.61^{a}	417.15^{a}	368.11^{a}	455.67^{a}	402.55^{a}	492.16^{a}	467.93^{a}	527.48^{a}	490.41^{a}
	B8×CSR4	338.63^{b}	243.77^{b}	348.55^{b}	315.57^{b}	345.92^{b}	329.87^{b}	440.79^{b}	358.77^{b}	450.13^{c}	385.91^{c}
F test		*	*	*	*	*	*	*	*	*	*
S.Em ±		3.733	4.126	4.115	4.026	4.035	4.869	4.774	5.773	5.065	4.676
CD @5%		11.287	12.475	12.442	12.174	12.2	14.724	14.435	17.455	15.314	14.14
CV (%)		2.648	3.441	2.563	2.882	2.234	3.21	2.328	3.304	2.231	2.4

*Figures with same superscript are statistically on par; *-Significant @5%*

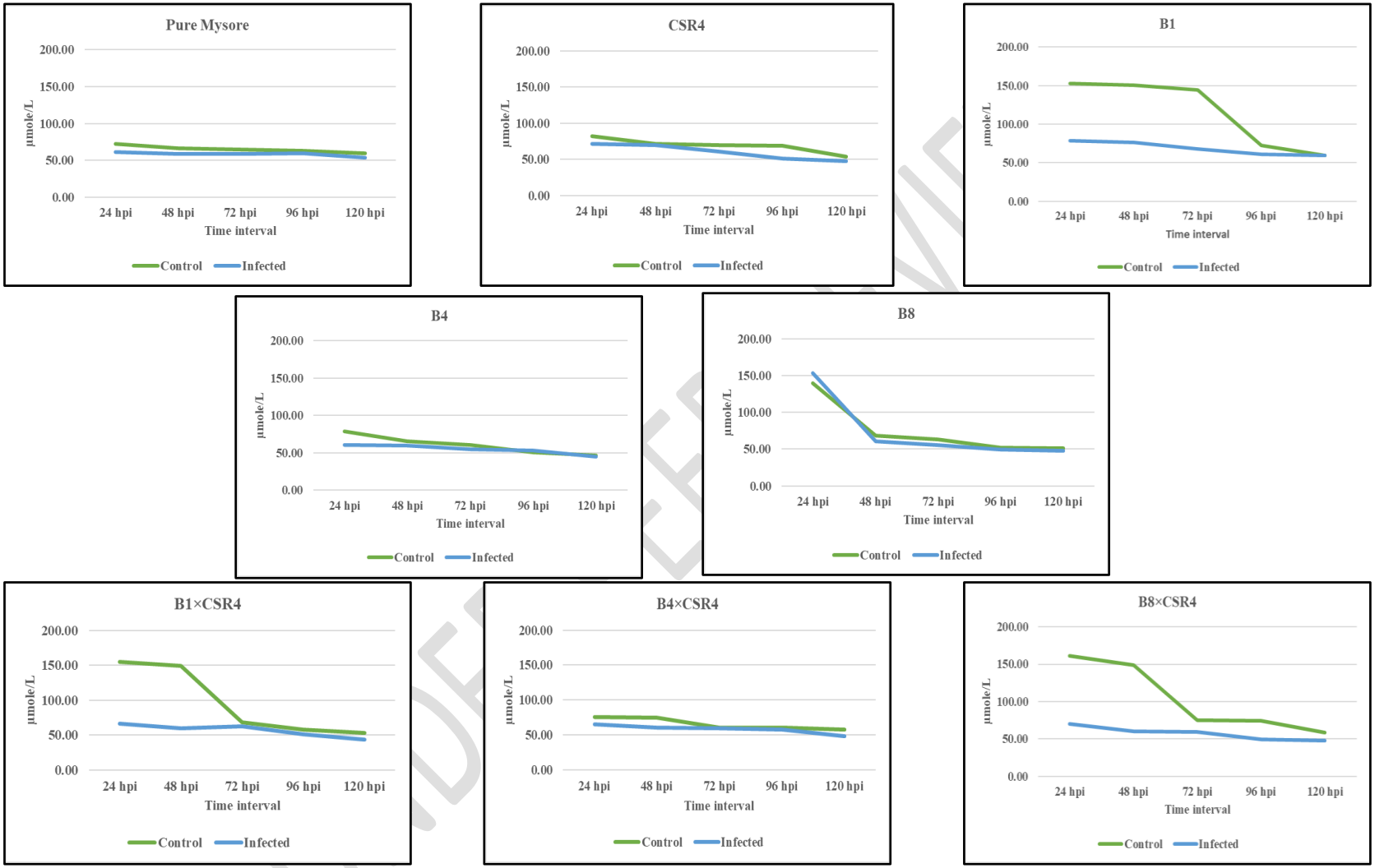


Fig 1. Peroxidase enzyme activity in thermotolerant bivoltine silkworm breeds and hybrids under control and muscardine inoculation at different time intervals.

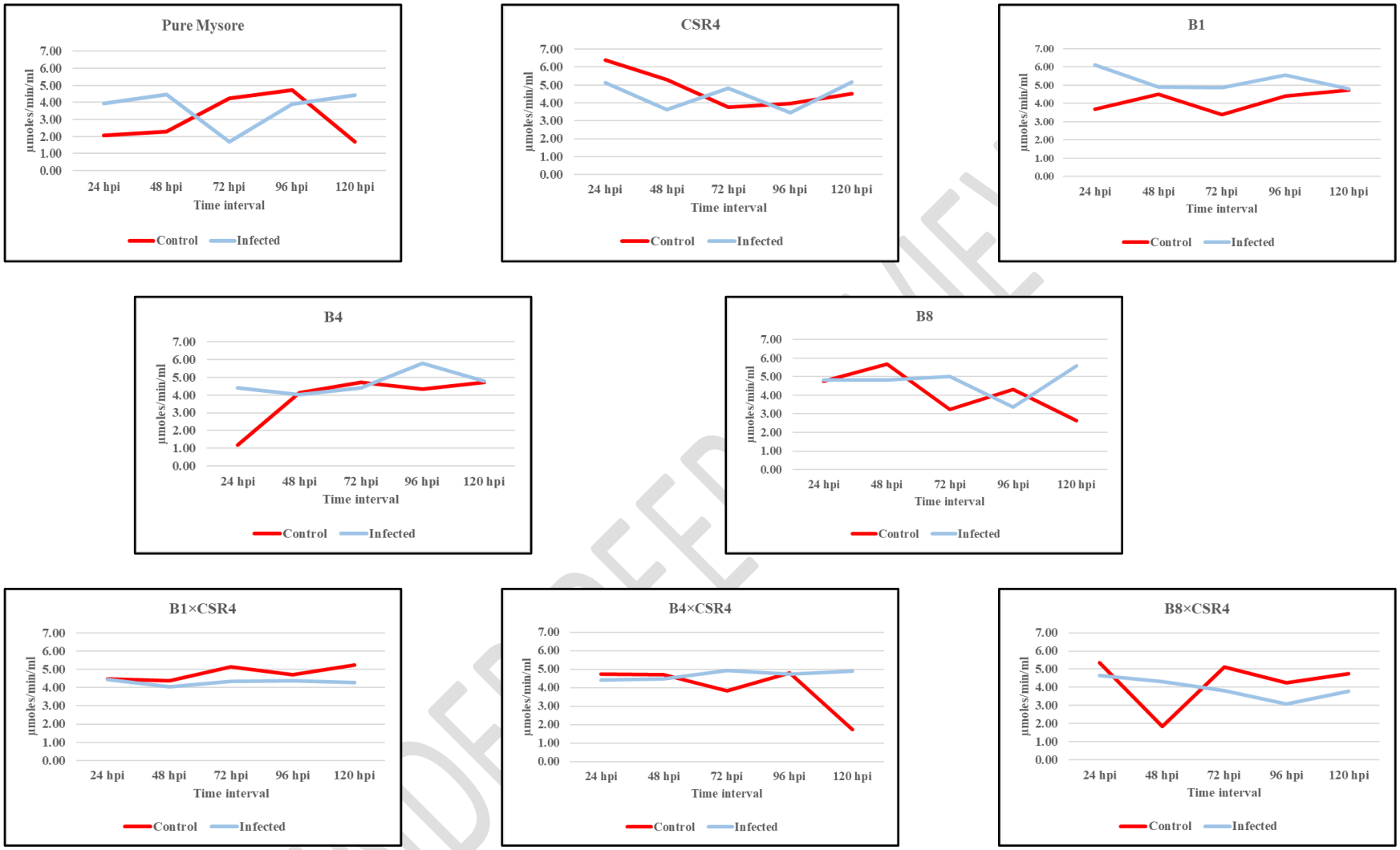
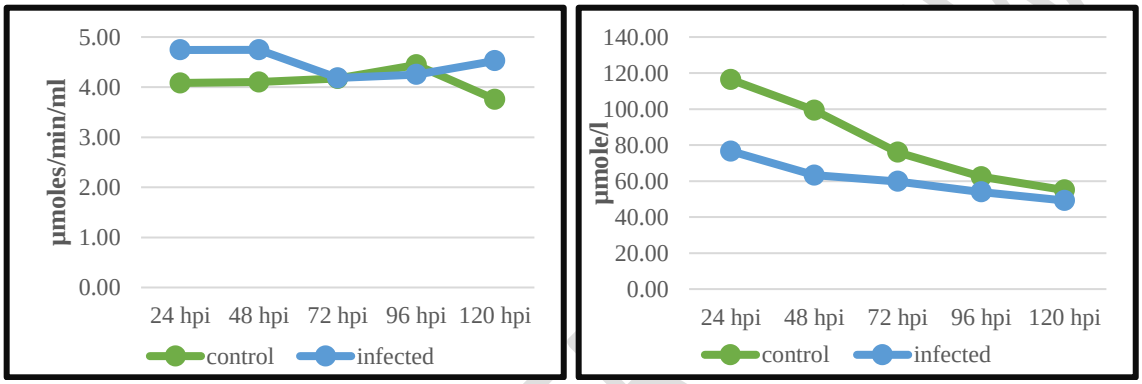


Fig 2. Catalase enzyme activity in thermotolerant bivoltine silkworm breeds and hybrids under control and muscardine inoculation at different time interv

The average catalase and peroxidase enzyme activity over the breeds at different hpi is represented in the Fig 3a and 3b, respectively. These figures indicate that catalase enzyme activity among the breeds under fungal infection was higher compared to that of control condition at 24, 48, 72 and 120 hpi except at 96 hpi. While, the peroxidase enzyme activity was higher in healthy silkworms than under *B. bassiana* infected condition. Hence, it can be presumed that the breeds tried to respond to infection by increasing catalase activity rather the enhancing peroxidase activity.

Catalase activity increased in the larvae of *Spodoptera litura* as a response to *Aspergillus flavus* infection in accordance with increase in load of the fungus Karthi *et al*. [23]. Similar phenomenon is observed in the present study where catalase activity increased over a period of 5 days among thermotolerant bivoltine silkworm breeds in response to *B. bassiana* infection. In the fruit fly, *Bactrocera dorsalis*, the peroxidase activity decreased in response to temperature stress, either lower or higher temperature than 27 °C, regardless of duration of exposure to thermal stress Jia *et al*. [24].



(a) **(b)**

Fig 3. Enzyme activity of the breeds over different time intervals (a) catalase and (b) peroxidase. Each value is derived from 8 breeds/hybrids at 24, 48, 72, 96 & 120 hpi with *B. bassiana* @ 9.04 × 10^4 spores / ml on the first day of 5^{th} instar.

4. CONCLUSION

This study investigated the response of antioxidant enzyme activity in thermotolerant bivoltine silkworm breeds and hybrids under *Beauveria bassiana* infection. Key findings revealed significant variations in total protein content, peroxidase and catalase enzyme activities among different breeds and hybrids under both control and infected conditions. The study suggests that the observed increase in catalase activity, particularly in B1 and B4 breeds, along with the decrease in peroxidase activity in B1, could serve as biochemical markers for better performance and survival under fungal infection. These findings provide valuable insights into the mechanisms of dual stress tolerance in silkworms and could aid in the selection and development of more resilient silkworm breeds for sericulture.

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