Response Of Antioxidant Enzymes in Silkworm, *Bombyx Mori* Supplemented with Ascorbic Acid Under Thermal Stress

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***Abstract*—An imbalance between the formation of free radicals and the antioxidant defense mechanism occurs as a result of oxidative stress. The current research focuses on the role of antioxidant enzymes in silkworms, *Bombyx mori*, under thermal stress and their impact on ascorbic acid supplementation. The fifth instar larvae of silkworms were grown in the lab and exposed to temperature stress (40±2^{0C}) for 1 hour per day. Ascorbic acid significantly boosted antioxidant status (P 0.05). Increased activity of enzymes superoxide dismutase (SOD) and catalase enzymes, as well as lower generation of hydrogen peroxide (H2O2) and lipid peroxide (LPx) in larval tissues, may be seen in the supplemented group (0.2 percent). Ascorbic acid was discovered to have anti-oxidant properties.**

***Index Terms*—Silkworm, Thermal stress; Antioxidant enzymes; Oxidative stress; Ascorbic acid.**

I. INTRODUCTION

Bombyx mori is a lepidopteran insect model and it has become an admirable material for classical genetics because of its abundant germplasm resources. . Temperature is one of the most important environmental elements for larval growth and productivity because it triggers a variety of physiological reactions (Benchamin and Jolly, 1986). Temperature stress can cause oxidative damage by causing an overproduction of reactive oxygen species (ROS). In general, the synthesis of ROS and the antioxidant mechanism are coordinated; but, during times of environmental stress, the balance between these processes may be disrupted, resulting in ROS overproduction (Lopez-Martinez et al., 2008; Lalouette et al.,2011). The accumulation of reactive oxygen species (ROS) during growth predisposes the organism to disease and the deterioration of physiological processes (Sahoo et al., 2016).

Antioxidants are found in all mammals and may catch ROS before they oxidise biomolecules or decrease them instead of being oxidized (Blokhina et al.,2003; Pinnell 2003). Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) are front-line antioxidant enzymes that bind the quantity of reactive oxygen species (ROS) in the cellular environment (Biewenga et al.,1997). Superoxide radicals are split into molecular oxygen and H2O2 by SOD catalase (Dubovskiy et al..2008; Meng et al.,2009). GST is an essential detoxifying enzyme in insects, and it has been related to the neutralisation of a wide range of hydroperoxides, as well as acting as a supplement to the action of selenium (Se) dependent vertebrate GPx (Lukasik 2007).

Invertebrates' immunological response is boosted by ascorbic acid, a cofactor and water-soluble antioxidant vital for animal growth and development (Carr and Frei,1999) (Lee and shiatsu, 2002, 2003). Additionally, by regulating the development of lethal lipid-hydroperoxides, protein carbonyls, and glutathione depletion, ascorbic acid may play a similar protective effect against oxidative damage to insect cellular components (Felton and Summers 1995). The goal of this research is to see how ascorbic acid supplementation affects antioxidant defences in the silkworm *B. mori* when it is exposed to heat. The study's major goals are to learn more about antioxidant enzyme kinetics.

II. MATERIALS AND METHODS

2.1. Rearing

Bivoltine silkworm larvae were received from P2 Basic Seed Farm, National Silkworm Seed Organization, Central Silk Board, Pallatheri, Palakkad, Kerala, and grown in the laboratory at 25±2^0 C temperature and 75± 2% relative humidity according to Krishnaswami's normal rearing conditions (1978). The rearing room, boxes, and instruments were properly cleaned and disinfected using a disinfectant solution (formalin 3 percent) before starting the raising process. Until the fifth instar, the larvae were fed fresh mulberry leaves.

Experimental set up

The healthy and active fifth instar larvae were separated into four groups, each with 25 larvae, using newly moulted 5th instar larvae. The following are the experimental groups: Group I: Control; Group II: Heat shock exposed; Group III: Vitamin C supplementation; Group IV: Heat shock + Vitamin C supplementation. Fresh mulberry leaves were freely distributed to groups I and II. Mulberry leaves were collected and completely immersed in a 0.2 percent ascorbic acid solution for 10 minutes, allowing the ascorbic acid to coat the leaves equally. After that, the leaves were air-dried before being shown to groups III and IV.

During this time, experimental Vth instar (group III and IV) larvae were subjected to heat shocks ranging from (40 degrees C) to (42 degrees C). The larvae were transferred from the growing chamber to the thermostat incubator using appropriate tiny trays. The larvae were returned to their normal rearing conditions after being exposed to heat. For this experiment, a temperature of 250°C was used as the control, and ascorbic acid supplemented larvae (groups I and II). The silk worms were weighed and slaughtered on the fifth day after being exposed for five days in order to collect the haemolymph and other tissues for the evaluation of various biochemical parameters.

Biochemical analysis

The activity of SOD was measured using the Marklund and Marklund technique (1974). The activity of catalase was measured using the method of Luck (1974), as described in Sadasivam and Manikam (1991), which involved monitoring the decrease in the absorbance of H2O2 at 240 nm. The glutathione reductase assay was determined using David and Richard's method (1983). The modified Habig et al. method was used to measure glutathione S-transferase (1974). Rotruck et alapproach.'s was used to determine glutathione peroxidase activity (1973). The levels of lipid peroxidation in the samples were evaluated using Fraga et almethod.'s of detecting the production of thiobarbituric acid (TBARS) (1988). The hydrogen peroxide content in tissue/serum homogenate was determined spectrophotometrically using the method developed by Pick and Keisari, (1981).

Statistical analysis

The data was examined using R programming version 4.0.3, a statistical package. The average standard error (SE) of six replicates was used to calculate the results. One-way analysis of variance (ANOVA) was used to compare the average values of the control and treatment groups, and Duncan's multiple range test was used for *post hoc* analysis (DMRT). P 0.05 was considered significant in all situations.

III. RESULTS

Activity of antioxidant enzymes

3.1. Activity of superoxide dismutase (SOD)

According to the thermal stress and ascorbic acid supplementation employed, the antioxidant enzyme activity in the treated and control groups revealed a varied pattern of response. Haemolymph SOD activity was considerably higher in group III (0.290.003) than in control (0.270.01) and group II (0.230.01). Group IV larvae had the highest SOD activity (0.2570.01) compared to group II larvae. Group II (1.740.02) had the highest SOD activity in the silk gland (Fig. 1)

3.2. Catalase's Activity (CAT)

Catalase activity was significantly reduced in the haemolymph and silk gland of group II (7.930.02 and 27.460.11 respectively) after temperature stress compared to group III (10.510.09 and 33.090.48 respectively). CAT activity was much higher in group IV larvae (10.910.01&40.070.20, respectively) (Fig. 2)

3.3. Glutathione s-transferase activity (GST)

Between group II and group II, GST activity was found to be significant. GST activities of haemolymph and

silkgland were substantially lower in group II (3.170.01&20.480.10 respectively) than group III (4.060.06&24.140.09 respectively) and group IV (4.180.03&22.360.26 respectively) according to statistical analysis. Glutathione S-transferase activity was higher in the haemolymph and silk gland of treated groups (groups III and IV)

3.4. Glutathione reductase activity (GR)

GR activity in the haemolymph and silk gland was significantly higher in group II (4.620.09 and 23.470.10, respectively) than in the control (4.030.01 and 18.190.04). The activity of GR in the haemolymph and silk gland increased significantly in Group III (4.490.01& 23.100.69). Group IV had significantly higher GR activity in the haemolymph and silkgland (4.160.02&21.570.59, respectively) (Fig. 4).

3.5. Glutathione peroxidase activity (GPX)

The temperature-treated and vitamin-c supplemented groups showed substantial differences in Gpx activity. In comparison to the control, group II had significantly lower GPx activity in the haemolymph and silk gland (53.770.21&169.191.48, respectively). The activity of GPx in the haemolymph and silk gland increased significantly in Group III. GPx activity was found to be significantly higher in group IV (Fig 5).

3.6. Changes in oxidative stress indices

Temperature stress resulted in a considerable rise in oxidative stress markers as compared to the control group.

Lipid peroxidation level (LPx)

The haemolymph and silk gland of group 2 larvae had the greatest levels of LPx (MDA content). In comparison to group 1 and group II, group III had much lower levels of LPx in their haemolymph and silk gland. In group IV, there was a considerable drop in MDA content (Fig. 6).

The amount of hydrogen peroxide

Changes in H_2O_2 content in the haemolymph and silk gland of stress-exposed larvae followed a trend similar to that of MDA, with the group II larvae having the highest H_2O_2 concentration. The content of H_2O_2 in the haemolymph and silk gland of groups III and IV was substantially lower (Fig. 7).

IV. DISCUSSION

Insects have evolved various protection mechanisms in response to extreme temperatures, according to many researches on the subject. The role of antioxidant enzymes and stress proteins in this process is crucial. (Yang et al., 2010) (Yang et al., 2010) (Yang et al., An imbalance between the creation of reactive oxygen species (ROS) and the cell's ability to eliminate ROS is referred to as oxidative stress. When fifth instar larvae were exposed to heat stress, they were treated with ascorbic acid, and the physiological oxidative stress responses were investigated. The effect of heat stress on the antioxidant enzymes SOD, CAT, GST, GR, GPX, and LPX, as well as H_2O_2 in B.mori. The larvae were studied.

SOD plays a vital role in antioxidant defence against ROS, reducing levels of superoxide radicle, which is induced by high temperatures (Park M.S *et.al.*, 2009). The haemolymph and silk gland of larvae exposed to high temperatures showed a considerable increase in SOD activity in this investigation. SOD production may have been stimulated in response to temperature variations to protect silkworm larvae from thermal stress. Similar findings have been reported by others (Jia et al., 2011). Excess ROS can be removed directly by SOD and CAT in a coordinated manner. Ascorbic acid supplementation boosts SOD activity in the larvae's haemolymph and silk gland. The findings of this study demonstrated the importance of foliar exogenous ascorbic acid supplementation in promoting larvae health under temperature stress. Exogenous ascorbic acid treatment has been shown to improve larvae development and cocoon generation in previous investigations. (Quraiza and colleagues, 2008; Chakrabarty and Kaliwal, 2012; Devi and Yellamma, 2013).

CAT is the primary H_2O_2 scavenging enzyme, and SOD and CAT collaborate to reduce oxygen in a stepwise manner (Cui, et al., 2011). However, CAT eliminates H_2O_2 only at high concentrations and has minimal effect at low concentrations (Felton, G.W; Duffey, S.S.1992). The activity of CAT in the haemolymph and silk gland was shown to diminish when subjects were exposed to heat stress in this study. Increased free radical generation could lead to CAT

depletion or inactivation. Due to a high level of superoxide radical production under oxidative stress, CAT activity was also found to be reduced. (Dubovskiy *et al*.,2008). Observations similar to these were made by (Arias et.al.,2011). CAT activities were found to be considerably higher in the haemolymph and silk gland of ASA supplemented larvae. These protective actions of SOD and CAT in silkworms demonstrated their protective role against oxidative stress (Dutta et.al. ,2018). In the current experiment, ascorbic acid treatment protected and reduced oxidative stress in the silkworm, which is consistent with previous results.

GST is a family of multifunctional dimer enzymes that may catalyse the release of toxins from a mixture of glutathione and a range of endogenous and heterologous chemicals, as well as isomerization and intercellular transport (Board and Menon, 2013). The activities of GST in the haemolymph were found to be significantly higher in this study compared to the control. However, there was no substantial change in GST activity in the silk gland. Jia et al., (2011), Ali et al., (2016), Yang et al., (2016), and Yang et al., (2016) all accord with our findings (2010). Ascorbic acid supplementation resulted in a considerable increase in GST activity in the haemolymph and silk gland. An increase in GST activity after ascorbic acid treatment is correlated with the findings of Saliu and Bawa-Allah (2012).

When compared to the control group, GR activity increased significantly in the thermal stress group. The GR activity of larvae given an ascorbic acid-fortified diet was considerably higher than that of control and heat-exposed larvae. Increased GR activity may help to protect against oxidative damage. GSH and ascorbic acid both have reducing properties that are somewhat complimentary. GSH deficit should result in ascorbic acid deficiency, which may be linked in our study. According to Tupe et al. (2007), ascorbic acid may play a preventive effect against oxidative stress. By metabolising H2 O2 - H2 O, GPx is another key antioxidant enzyme that decreases the damage caused by free radicals. In this study, the heat-stressed group's GPx activity was considerably lower than the control groups in the haemolymph and silk gland. This is consistent with previous results by Jia et al., (2011), and Ze Qing Miao et al.,2020). In comparison to the control and heat-exposed groups, ascorbic acid supplementation increased GPx activity in the larvae's haemolymph and silk gland. (2020, Moustafa)

MDA is a cellular indicator of oxidative stress. Because MDA is a significant oxidation product of peroxidized polyunsaturated fatty acids, it can be utilised to measure the degree of lipid peroxidation (Rael et al.,2004; Del Rio et al.,2005). When exposed to heat shock, MDA concentrations in the haemolymph and silk gland of larvae increased significantly compared to the control. Thermal stress may increase the rate of lipid peroxidation and associated reactions to oxidative stress in B.mori, according to the findings. Thermal stress has been shown to alter physiological responses in insects (Neven, 2000), potentially increasing the rate of ROS generation and resulting in oxidative stress. The results showed that in the ascorbic acid supplemented group, the degree of lipid peroxidation produced in the haemolymph and silk gland was much lower than in the control group.Because of its cellular activity and dual purpose, H2O2 is regarded as a striking model oxidant.

In insects, H2O2 regulates some of the most important tasks such as cell differentiation, development, dormancy, and lifespan extension (Sahoo et al.,2018). It has numerous clinical and developmental effects when it exceeds physiological limitations. The amount of H 2O2 in the haemolymph and silk gland of larvae exposed to temperature stress was found to be significantly higher in the study. The amount of H2O2 in the haemolymph and silk gland was dramatically reduced when ascorbic acid was supplemented. This is in strong agreement with Dutta *et* alearlier.'s research (2018). Supplementation of ascorbic acid reduced oxidative stress via regulating antioxidant protection under heat stress, according to the findings of the current study.

V. CONCLUSIONS

Thermal stress disrupts the redox balance in *Bombyx mori*, resulting in increased oxidative stress, according to the findings. *B. mor*i is a major potential factor in the generation of oxidative stress products in response to the damages induced by heat stress. Antioxidant enzymes are increased as a defence strategy to reduce potential cellular damage in response to this stress. However, prolonged heat shock resulted in decreased

enzyme activity, as well as reduced antioxidant capacity and elevated levels of oxidative stress. To counteract this stress, the larvae subjected to thermal stress were given an exogenous antioxidant called ascorbic acid. Supplementing with ascorbic acid (0.2 percent) was shown to be more efficient in lowering the levels of LPx and H2O2 in the haemolymph and silk gland of heat-stressed larvae. It also improves antioxidant activity, as seen by enhanced SOD, CAT, GST, GR, and GPx enzyme activity. The current study found that supplementing *B.mori* with ascorbic acid improves growth metrics and antioxidant efficacy, perhaps speeding up the formation of high-quality cocoons.

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FIGURE

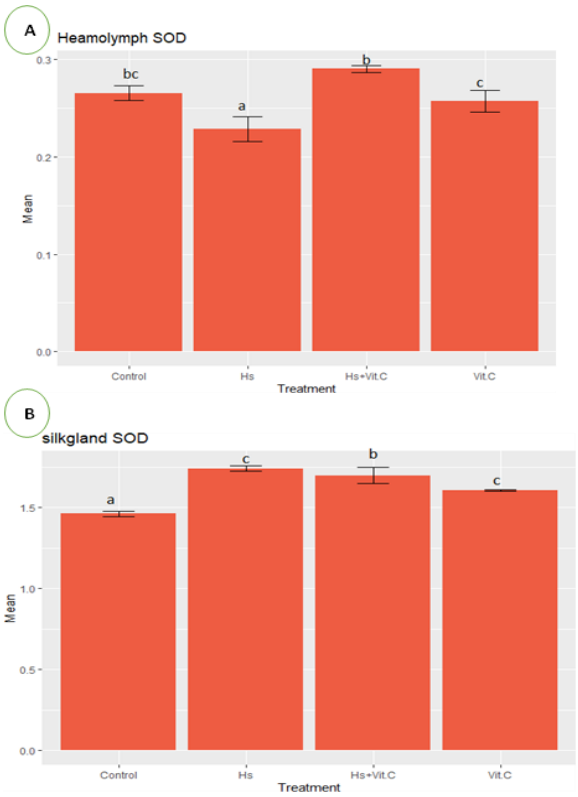


Figure 1: Activity of SOD (units/mg protein) in the (A) haemolymph and (B) silk gland of *B. mori*. Means within a bar followed by the different letters are significantly different in the analysis of variance (ANOVA). Bars having superscripts a,b and c represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM.

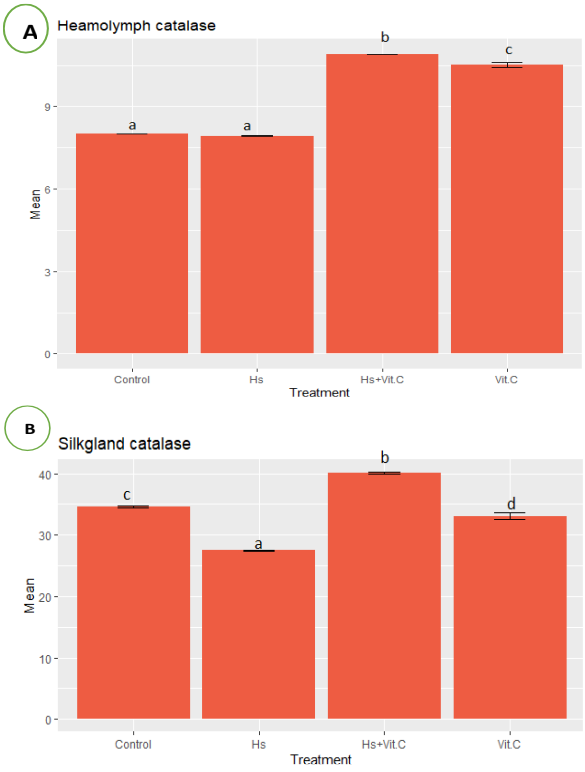
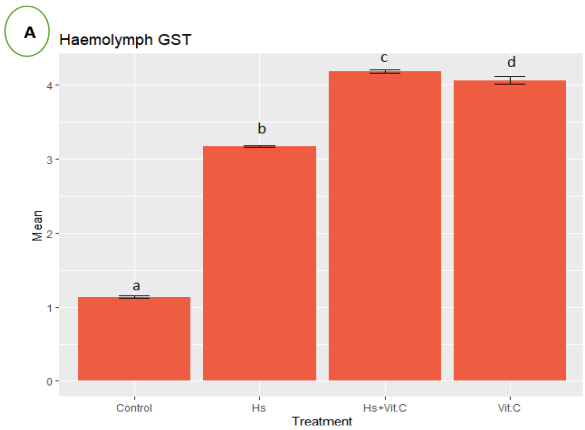


Figure 2: CAT activity (units/mg protein) in the (A) haemolymph and (B) silk gland of *B.mori*. Means within a column followed by the different letters are significantly different in the analysis of variance (ANOVA). Bars having superscripts a,b and c represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM



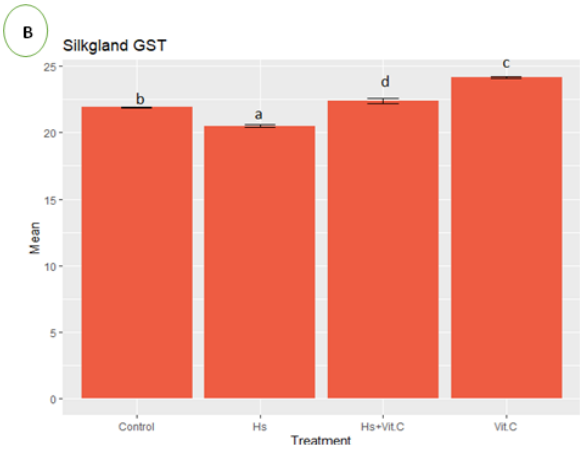


Figure 3. Activity of GST (units/mg protein) in the (A) haemolymph and (B) silk gland of *B.mori*. Means within a column followed by the different letters are significantly different in the analysis of variance (ANOVA). Bars having superscripts a,b and c represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM.

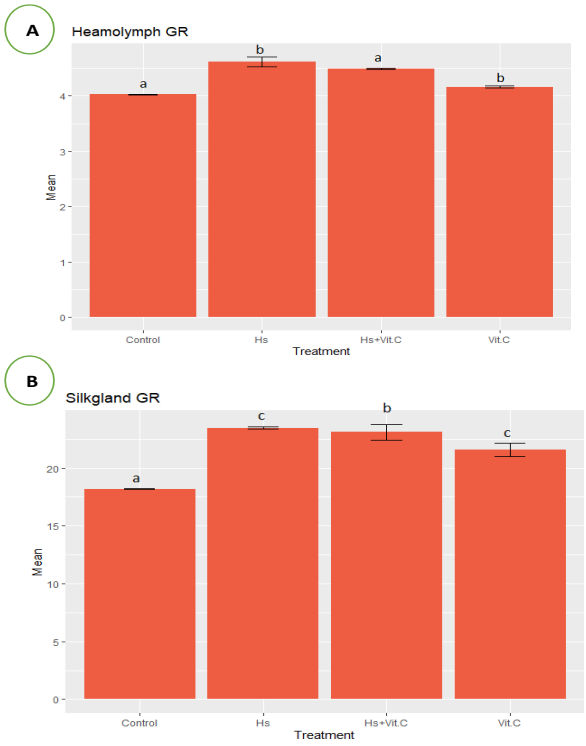
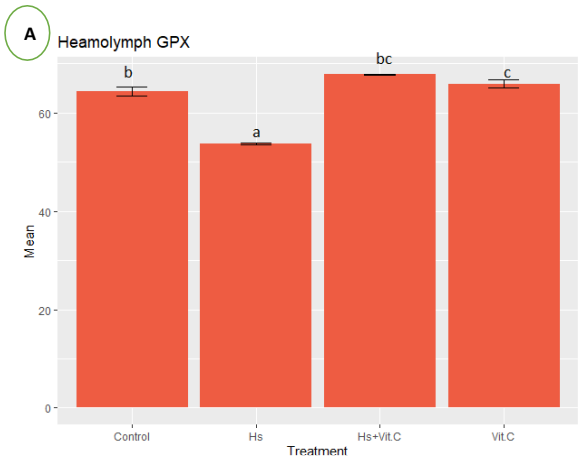


Figure 4. Activity of GR (units/mg protein) in the (A) haemolymph and (B) silk gland of *B.mori*. Means within a column followed by the different letters are significantly different in the analysis of variance (ANOVA). Bars having superscripts a,b and c represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM.



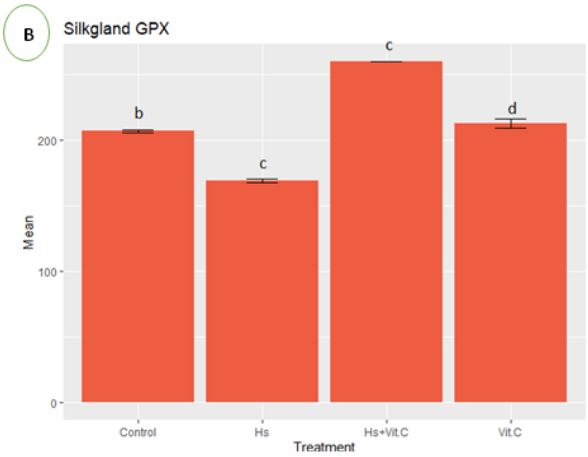
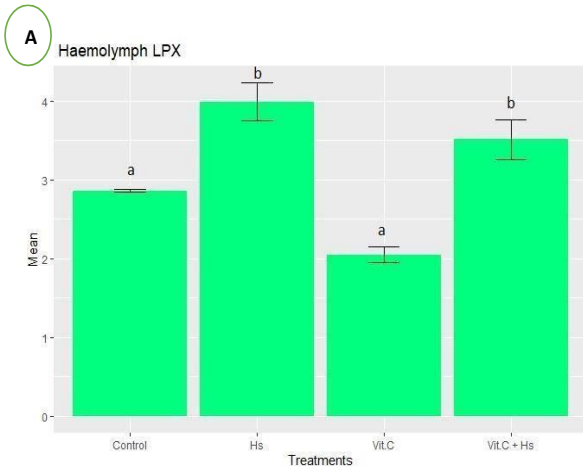


Figure 5.GPX activity (units/mg protein) in the (A) haemolymph and (B) silk gland of *B.mori*. Means within a column followed by the different letters are significantly different in the analysis of variance (ANOVA). Bars having superscripts a,b,cand d represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM.



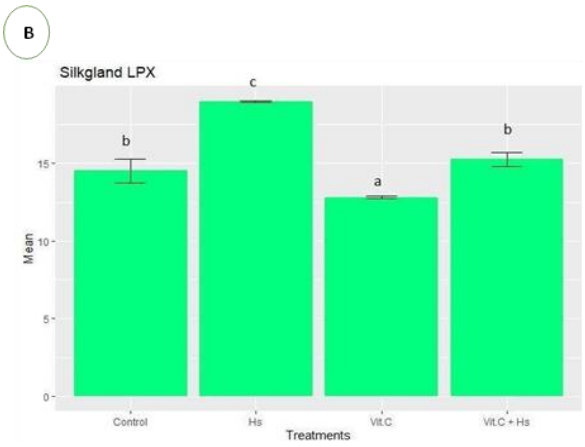


Figure 6. Lipid peroxidation in (A) haemolymph and (B) silk gland of 5th instar larvae *of Bombyx mori.* Bars having superscripts a,b and c represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM.

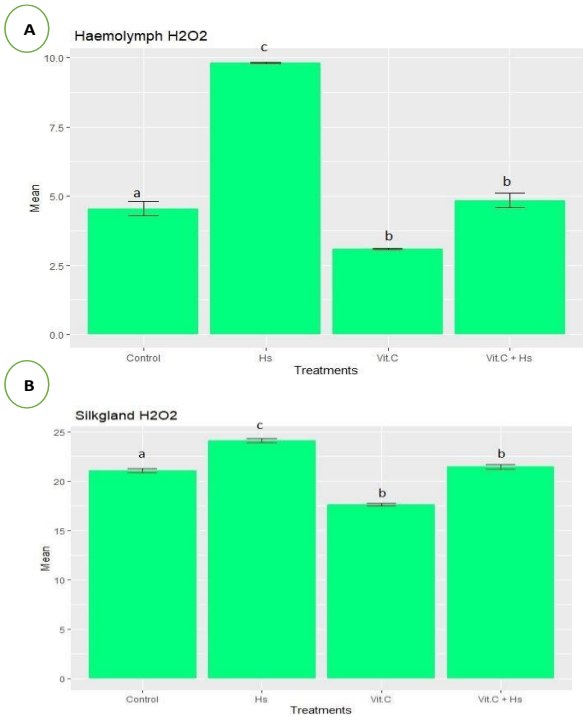


Figure 7. Hydrogen peroxide content in (A) haemolymph and (B) silk gland of 5th instar larvae of *Bombyx mori.* Bars having superscripts a,b and c represents the significant difference ($p<0.05$). Data are expressed as mean ±SEM

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