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Foliar supplementation of ascorbic acid and glycine boost the growth performance and antioxidant protection in the larvae of tropical tasar silkworm, *Antheraea mylitta*

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Abstract
The present study demonstrates the growth performance and antioxidant defenses of the tasar silkworm, *Antheraea mylitta* (IInd instar larva), in response to feeding on *Terminalia tomentosa* leaves supplemented with different concentrations (0.5%, 1% and 2%) of ascorbic acid or glycine or their combination, for a period of 10 days. Supplementation of 1% ascorbic acid or glycine (individually or combined) was found to be more effective in enhancing the body weight of the larvae compared to control and other supplemented groups. It was also effective in decreasing the H_2O_2 and LPx levels in the larval tissues and in boosting the antioxidant status, as evident from increased activities of SOD and CAT enzymes. The present findings suggest that foliar supplementation of ascorbic acid or glycine enhances the growth rate and antioxidant capacity in the larvae of *Antheraea mylitta* which may improve the quality and quantity of silk production.

Keywords: *Antheraea mylitta*, ascorbic acid, glycine, foliar supplementation, growth, oxidative stress indices, antioxidant defenses

1. Introduction
Silk is well-known for its smooth and gleaming texture. Now a days the importance of silk is not only restricted within textile industry rather it is being used in biomedical applications, biomaterials, tissue engineering, photonics, optics and electronic applications. The tropical tasar silkworm, *Antheraea mylitta* (Lepidoptera: Saturniidae) is an important component of Asian non-mulberry silk industry (Nagaraju and Goldsmith, 2002) [12]. It exhibits a well-defined life cycle consisting of following stages i.e. egg, larva, pupa (inside cocoon) and the moth. The hatchlings from the eggs are normally known as larvae. The developmental process of the larvae usually passes through five instars i.e. Ist, IInd, IIIrd, IVth and Vth instar. The larval stage is the only feeding phase throughout the life cycle. Larvae are generally polyphagous in nature and reared outdoors on different host plants like *Terminalia arjuna*, *Terminalia tomentosa* and *Shorea robusta*. The Vth instar larva after attaining the full growth stops feeding and transforms into pupa inside the cocoon to cope with the extreme environmental conditions. Then the adult moth develops from pupa inside that cocoon. The cocoon is the main source of raw silk and is chiefly consists of two proteins i.e. sericin and fibroin. Silk production primarily depends on the nutritional status of the host plant leaves and the larval health conditions. As silkworms are reared outdoors on host plants, they are more susceptible to plant allelochemicals-mediated oxidative assault, harsh environmental challenges, microbial threat and radiation injury (Shamita and Rao, 2006; Gulevsky *et al.*, 2006; Sahu *et al.*, 2018) [26, 10, 25]. All these factors are likely to cripple the immune effector mechanism and enhance reactive oxygen species (ROS) formation. Superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxides (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) are the major contributors to the reactive oxygen species pool in the cellular environment. Excessive generation of reactive oxygen species leads to oxidative damages to biomolecules and it can alter the tissue redox status of the silkworm, which in turn affect the silkworm health as well as the quality and quantity of silk production. To cope up with the oxidative challenges, antioxidant defense system protects the organisms from oxidative stress-mediated pathogenesis and restores the redox balance. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx) and the nonenzymatic antioxidants such as

vitamin E, C, A and glutathione are responsible for maintaining the redox status of the tissue. A perfect balance between pro- and antioxidants is very important for the proper cellular functions and developmental events. Previously, we have reported that endogenous antioxidant defenses are of critical importance for the developing larvae of *A. mylitta* (Sahoo *et al.*, 2016) [23] and also provide protection to the larvae from the oxidative assaults triggered by host plant-derived allelochemicals (Sahu *et al.*, 2018) [25]. However, scanty reports are available on the role of antioxidant defenses in the improvement of larval health and economic traits of silk fiber obtained from *A. mylitta*. It has been observed that enrichment of host plant leaves by nutrient supplementation is one of the methods by which health management, raw silk and cocoon production of *Bombyx mori* can be improved (Nicodemo *et al.*, 2014; Rao and Devi, 2018; Rahmathulla *et al.*, 2007) [13, 21, 19]. These observations motivated us to take over this study and to bridge the existing research gap for the benefit of non-mulberry silk industry. Ascorbic acid (ASA) is one of the important redox modulators which plays a key role in cellular differentiation, collagen synthesis and development. Similarly, glycine (GLY) is one of the quintessential components of silk protein and the tripeptide, glutathione, the master antioxidant. Therefore, the present study has been designed with dual objectives. The first objective is to evaluate the effect of foliar supplementation of exogenous antioxidants i.e. ASA and GLY (separately or in combination) on the larval growth of *A. mylitta* during the development. The second objective is to trace out the possible involvement of antioxidant defenses in modulating the larval growth in response to foliar supplementation of ascorbic acid and glycine.

2. Materials and Method

2.1 Chemicals

Thiobarbituric acid (TBA), bovine serum albumin (BSA) and sephadex G-25 were purchased from Sigma Chemical Co. USA. Ascorbic acid, glycine, horse radish peroxidase and hydrogen peroxide were obtained from SISCO Research Laboratory, India. All other chemicals used were of analytical grade.

2.2 Insects

Tasar silkworm larvae after hatching were reared on host plant (*Terminalia tomentosa*) of similar age group maintained in the State Government Sericulture field located at Baripada (21^0 56' 6 N, 86^0 43' 17 E), Odisha, India. The IInd instar larvae were collected and brought to the laboratory for conducting experiments as described below.

2.3 Experimental Conditions and Sample Preparation

The IInd instar larvae collected from the rearing field were acclimatized in the laboratory condition for one day. To find out the effect of foliar supplementation of exogenous antioxidants on larval development and their antioxidant defense system, two experiments were conducted with IInd instar larvae of *A. mylitta*.

First, initial body weight of the larvae were taken and divided into different experimental groups. Larvae fed with host plant (*T. tomentosa*) leaves which were supplemented with different concentrations (0.5%, 1% or 2%) of ascorbic acid (ASA), glycine (GLY) or mixture of ascorbic acid and glycine and were served as experimental groups. One group of larvae was supplemented with distilled water sprayed leaves and was kept as control. The supplementation experiment was routinely maintained for 10 days. After the treatment period, the final body weights of the larvae from all groups were recorded to assess the growth performance.

Based on the findings of the above pilot study, the second experiment was carried out to ascertain the possible involvement of antioxidant defense system in modulating the larval growth in response to feeding with *T. tomentosa* leaves supplemented with 1% ascorbic acid or glycine or mixture of both for a period of 10 days. After the experimental period the final body weight of the larvae were recorded and the larvae from control and experimental groups were processed for homogenization for the biochemical analyses. Whole body (WB) of the larva was homogenized (20%) in ice cold phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA and a pinch of phenyl methane sulphonyl fluoride (PMSF) using glass Teflon mechanical homogenizer. The homogenates were centrifuged at 10,000 x g for 20 min at 4 °C and the post-mitochondrial supernatant (PMS) was collected for all biochemical analysis. The endogenous H_2O_2 content and catalase (CAT) activity were immediately measured in the PMS after the centrifugation. The remaining supernatant was kept in -80 °C till further biochemical analysis. All measurements were made in triplicate.

2.4 Oxidative Stress Indices

2.4.1 Measurement of Hydrogen peroxide (H_2O_2) Content

The hydrogen peroxide content in the post-mitochondrial fraction of WB homogenates was determined spectrophotometrically using horse radish peroxidase and H_2O_2 as standard according to the method of Pick and Keisari (1981) [15]. In brief, in each tube 1.7 ml of phosphate buffer (50mM pH 7.4), 0.1 ml of phenol red solution and 50 µl of horse radish peroxidase (50 units) were taken and incubated for 5 min at room temperature. Next 0.1 ml sample was added to it followed by immediate addition of 50 µl of NaOH (1N) to stop the reaction. The absorbance was taken at 610 nm in a UV-Vis spectrophotometer. The H_2O_2 content was expressed as nmoles H_2O_2/mg protein. Pure H_2O_2 was taken as standard and it was linear in the range of 20-80 nmol.

2.4.2 Measurement of Lipid peroxidation (LPx)

Level of LPx in the tissue samples determined by monitoring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa *et al.* (1979) [14]. A 20% (w/v) homogenate of the WB was prepared in 1.15% KCl and centrifuged at 1,000 x g for 10 minutes at 4°C to remove cell debris. Suitably diluted 1,000 x g supernatant of WB homogenates (having around 500 µg protein) were used for the estimation of TBARS. All samples were treated with 0.02% butylated hydroxytoluene to prevent endogenous oxidation. Respective tissue samples were mixed with the TBA mixture and were incubated for 1 hour at 95 °C. Then the samples were centrifuged at 1,000 x g for 10 mins and were carefully decanted into cuvette and absorbance of the reddish-pink chromogen was taken at 532 nm in a UV-Vis spectrophotometer. The amount of TBARS formed was calculated from the extinction coefficient of TBARS i.e. 1.56 x 10^5 M^{-1} cm^{-1} (Wills, 1969) [30] and was expressed as nmol TBARS formed/ mg protein.

2.5 Activities of Antioxidant Enzymes:

2.5.1 Assay of Superoxide dismutase (SOD) activity

For the measurement of total SOD activity, 0.4 ml of post

mitochondrial supernatant containing approximately 10-15 mg of protein was passed through a 2 ml column of sephadex G-25 and the elute was used for the activity assay according to the method of Das *et al.* (2000) [7] as described earlier (Dandapat *et al.*, 2003) [6]. In brief, superoxide radicals generated by photo reduction of riboflavin were allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphanilic acid to produce a diazonium compound, which reacts with naphthylamine to produce a red azo compound having absorption maxima at 543 nm. SOD scavenges superoxide radicals, therefore, nitrite formation in the reaction is inversely proportional to the amount of SOD present in the sample. One unit of enzyme activity is defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. SOD activity was expressed as units/mg protein.

2.5.2 Assay of Catalase (CAT) activity

The CAT activity in the PMS was measured by monitoring the decomposition of H_2O_2 at 240 nm (Cohen *et al*., 1970 [5]; Aebi, 1974 [1]). Prior to assay 5 µl ethanol (1%) was added to 0.5 ml of post-mitochondrial fraction to prevent the formation of the inactive complex of CAT (complex-II) by reacting with H_2O_2. After incubation for 30 mins in ice, 50 µl of triton-x-100 (1%, v/v) was added and again incubated for 15 min to increase the observable CAT activity by releasing it from peroxisomes. Then, this mixture was used immediately for the assay of enzyme activity. 2.9 ml of freshly prepared phosphate buffer (50 mM, pH = 7.0) containing 12 mM H_2O_2 was taken in a cuvette. Then, the above post mitochondrial fraction (0.1 ml) containing around 10-50 µg protein was added to the cuvette and mixed properly. The decrease in absorbance was recorded at 240 nm in every 15 sec intervals for 2 mins in UV-Vis spectrophotometer. Calculation was done by taking the extinction coefficient of H_2O_2 as 43.6 $M^{-1}cm^{-1}$. Activity was expressed as nkat/mg protein. One katal is defined as the amount of enzyme that transforms one mole of substrate per second.

2.6 Protein Estimation

Protein content of the samples for the assay of oxidative stress indices and antioxidant enzymes was estimated according to the method of Bradford (1976) [3] using bovine serum albumin as standard.

2.7 Statistical Analysis

Results of the growth pattern (in terms of body weight gain) of the silkworms are presented as mean ± standard error of mean (SEM) for n=20. Results of the biochemical analysis are presented as mean ± standard error of mean (SEM) for n=10 pooled samples (2 larvae per pool). Difference among the means were analysed by one way analysis of variance (ANOVA), through on line (Vassarstats) followed by Turkey's HSD test. Differences were considered statistically significant at $p < 0.05$.

3. Results

Experiment I

3.1 Changes in the body weight (Fig. 1)

The changes in the body weight of the larvae in response to foliar supplementation are presented in the Fig.1. The body weight of all the larvae fed with host plant leaves (supplemented with different concentrations of ASA or GLY or ASA and GLY) was significantly altered in comparison to control one. The experimental groups those were supplemented with 1% of ASA, GLY or ASA and GLY showed a significant increase in their final body weight in comparison to control one. It is worth mentioning here that the larvae supplemented with 2% of ASA showed a negative impact on the health and weight of *A. mylitta*. Co-supplementation of 1% ASA and GLY (through foliar spray on *T. tomentosa* leaves) showed a significantly incremental effect on both health and weight of *A. mylitta* larvae compared to all supplemented groups.

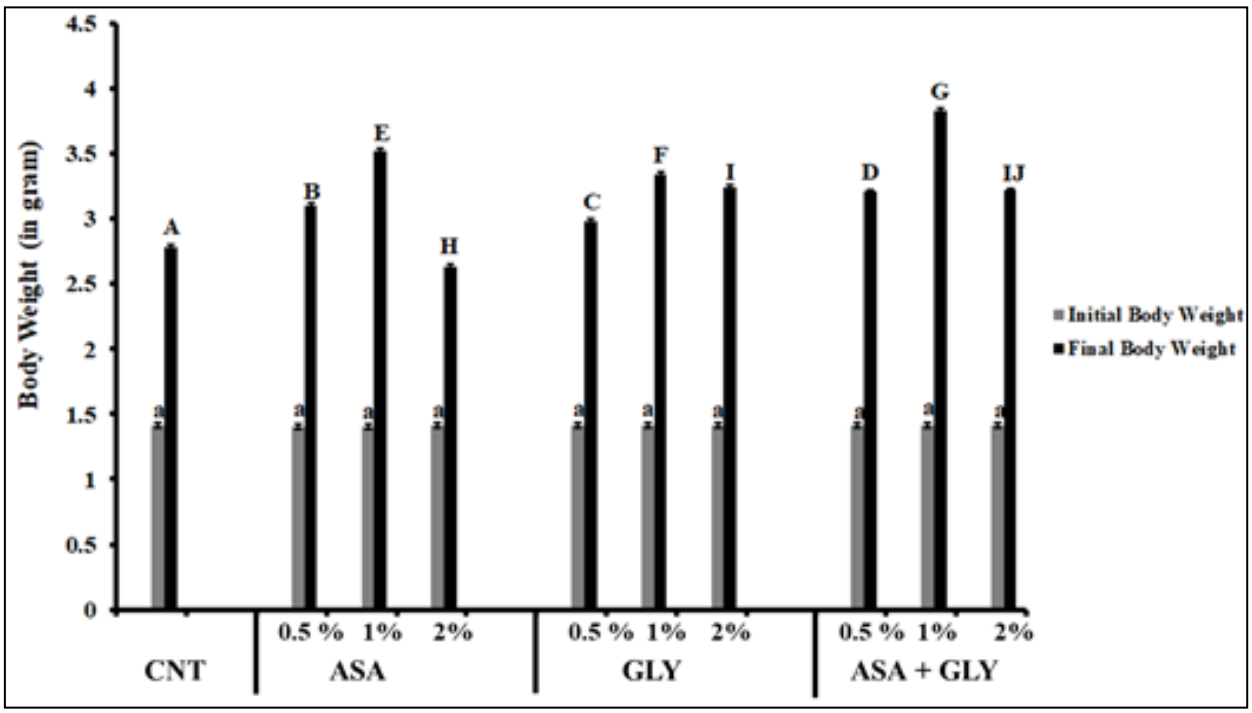

Fig 1: Effect of foliar supplementation of different concentrations of ascorbic acid and glycine (separately and in combination) on the body weight gain of *A. mylitta* larvae. Data are expressed as mean ± SEM (n=20). Bars having superscripts of different letters differ significantly with

respect to control. CNT: Control; ASA: Ascorbic acid; GLY: Glycine; ASA + GLY: mixture of ascorbic acid and glycine.

Experiment II

3.2 Oxidative Stress Indices

3.2.1 H_2O_2 Content (Fig. 2)

The H_2O_2 content was significantly declined in all groups of larvae fed with *T. tomentosa* leaves supplemented with 1% ASA, GLY or mixture of ASA and GLY. Combined supplementation of ASA and GLY showed a better effect on minimizing the H_2O_2 content in the whole body tissue of the larvae.

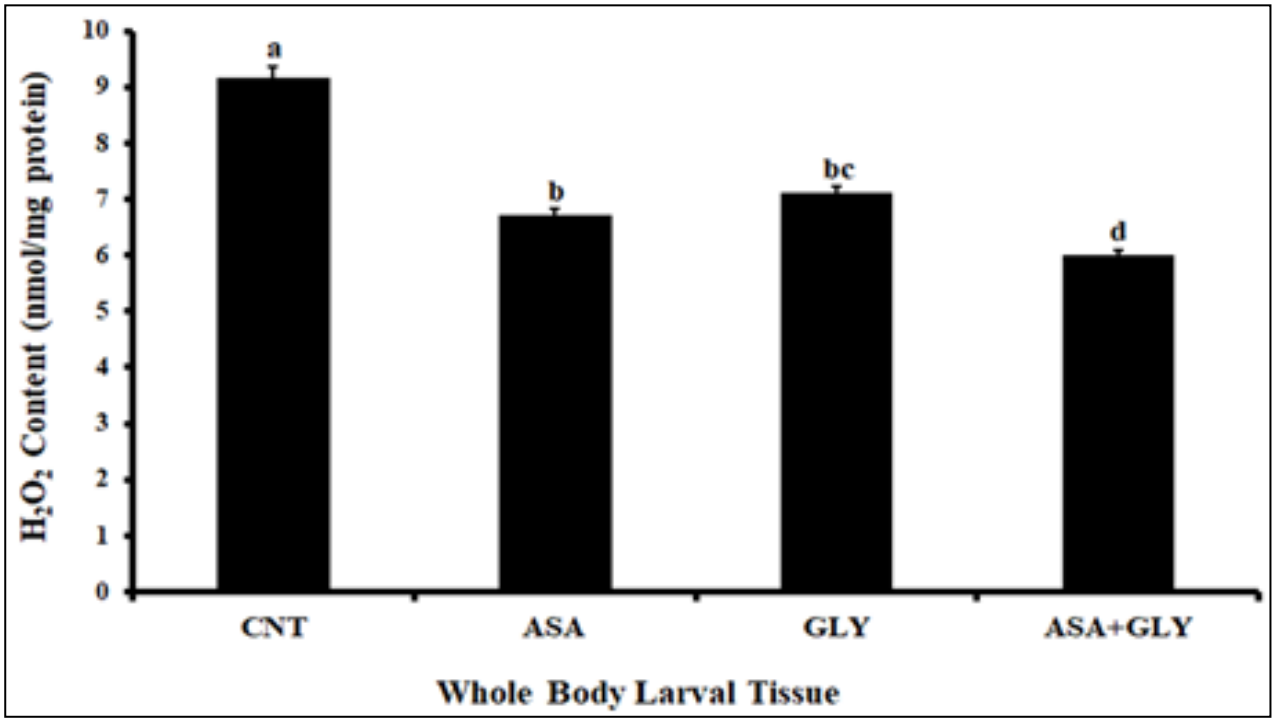

Fig 2: Effect of foliar supplementation of 1% of ascorbic acid or glycine (separately and in combination) on the H_2O_2 content (nmol/mg protein) in the whole body tissue of *A. mylitta* larvae. Data are expressed as mean ± SEM (n=10 pooled samples; 2 larvae per pool). Bars having superscripts of different letters differ significantly with respect to control.

3.2.2 LPx Level (Fig. 3)

Larvae fed with ASA or GLY supplemented leaves exhibited significantly lower level of LPx in their whole body tissue compared to control one. Though the co-supplementation of ASA and GLY was not effective enough in reducing the LPx level further, it indicated a declining trend of LPx compared to other experimental groups.

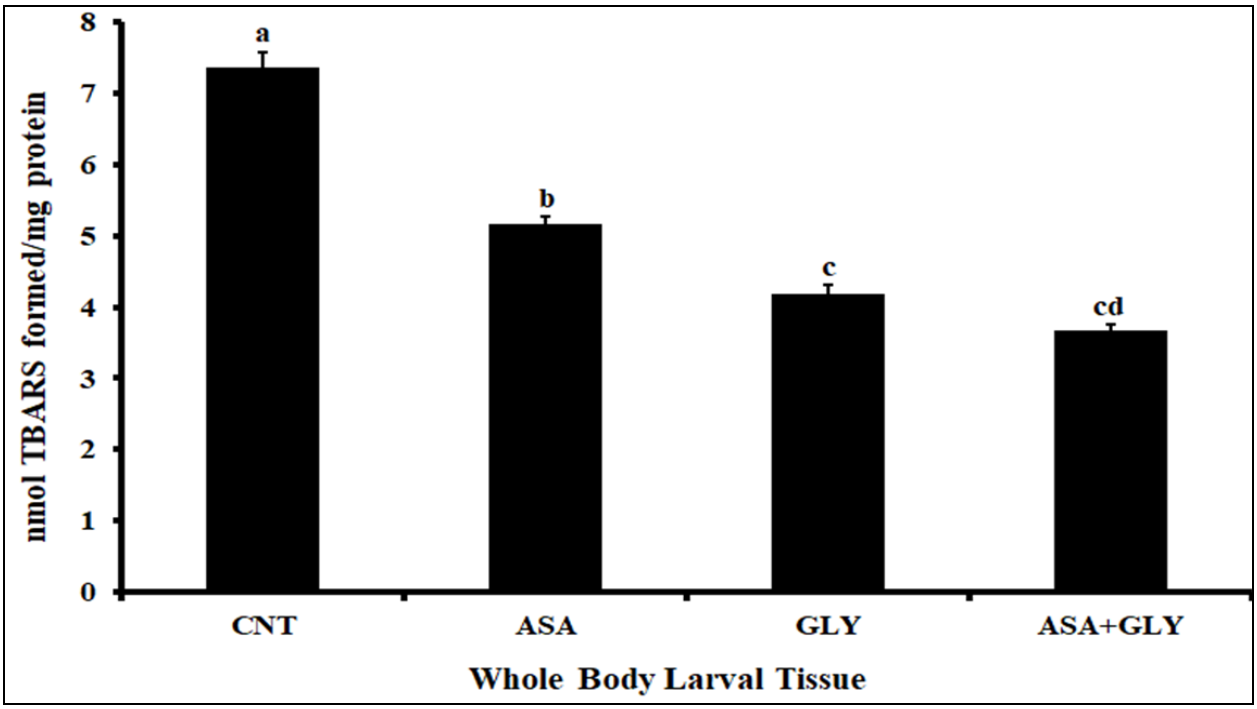

Fig 3: Effect of foliar supplementation of 1% of ascorbic acid or glycine (separately and in combination) on the LPx level (nmol TBARS formed/mg protein) in the whole body tissue of *A. mylitta* larvae. Data are expressed as mean ± SEM (n=10 pooled samples; 2 larvae per pool).

Bars having superscripts of different letters differ significantly with respect to control.

3.3. Changes in the activity of Antioxidant Enzymes (Fig. 4 and 5)

Supplementation of ASA or GLY (separately and in combination) elevated the antioxidant enzyme activities in the whole body tissue of the respective larval groups. SOD and CAT are the major and frontline antioxidant enzymes. SOD activity was significantly increased in ASA and GLY supplemented larval groups (Fig. 4). CAT activity also showed similar trend in all experimental groups of larvae receiving antioxidant supplemented leaves (Fig. 5).

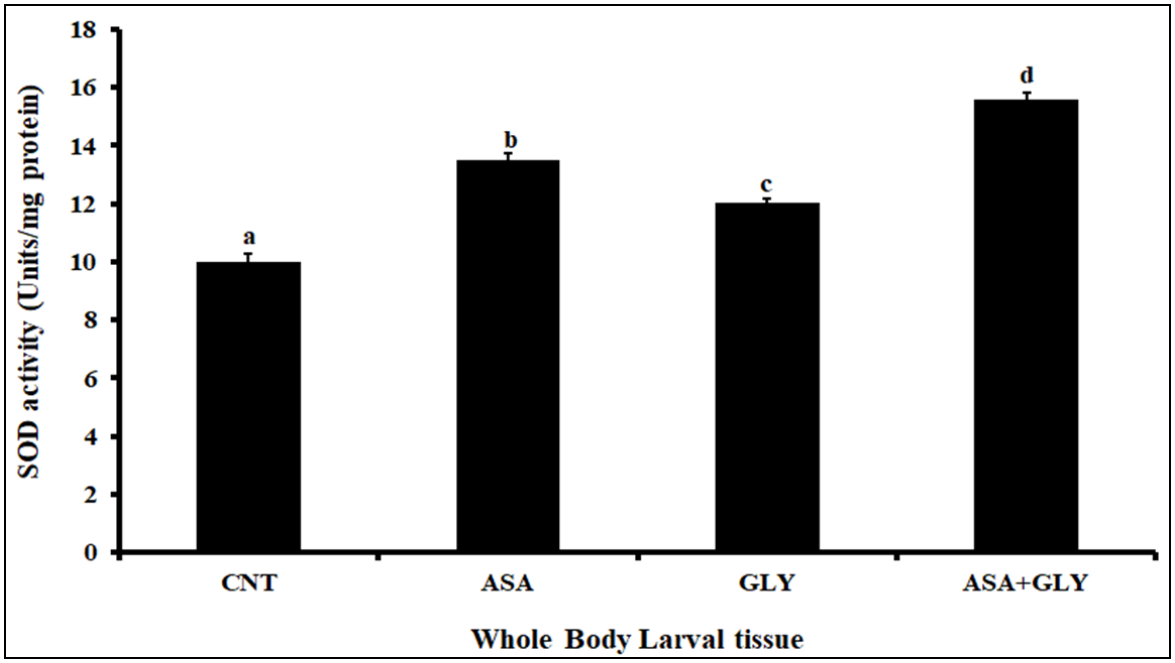

Fig 4: Effect of foliar supplementation of 1% of ascorbic acid or glycine (separately and in combination) on the SOD activity (units/mg protein) in the whole body tissue of *A. mylitta* larvae. Data are expressed as mean ± SEM (n=10 pooled samples; 2 larvae per pool). Bars having superscripts of different letters differ significantly with respect to control.

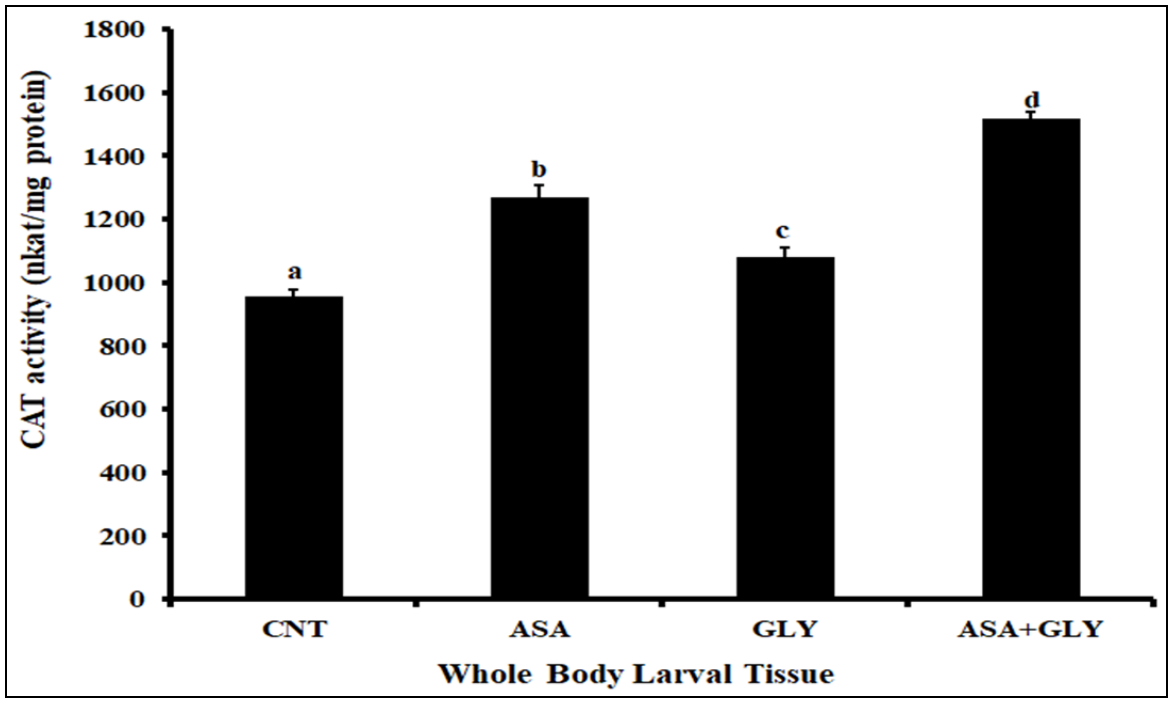

Fig 5: Effect of foliar supplementation of 1% of ascorbic acid or glycine (separately and in combination) on the CAT activity (nkat/mg protein) in the whole body tissue of *A. mylitta* larvae. Data are expressed as mean± SEM (n=10 pooled samples; 2 larvae per pool). Bars having superscripts of different letters differ significantly with respect to control.

4. Discussion

The findings of the first experiment clearly indicated the beneficial role of foliar supplementation of exogenous antioxidants in enhancing the growth of the larvae during development of *A. mylitta*. Earlier findings in this regard also suggested the positive impact of exogenous antioxidant supplementation on the improvement of larval health and silk production (Quraiza *et al*., 2008; Chakrabarty and Kaliwal, 2012; Devi and Yellamma, 2013) [18, 4, 8]. Increase in the growth performance of the silkworm as observed in the

present experiment in response to feeding with *T. tomentosa* leaves supplemented with ASA and GLY is in accordance with the earlier reports. The larval weight of the silkworm has been reported to be increased due to administration of thyroxine (Rajashekargouda *et al*., 1998) [20], folic acid (Rahmathulla *et al.*, 2007) [19] and zinc (Sahu *et al*., 2015) [24]. However, the highest improvement in larval body weight (Fig. 1) of *A. mylitta* was observed in the co-supplemented group of 1% ASA and GLY. In contrast, 2% of ASA or GLY supplemented group of larvae showed a negative impact on the development of larvae (Fig. 1). Specifically, the larvae receiving 2% ASA supplementation showed a negative effect on their health and growth performance (Fig. 1). The high concentration of ASA might be working as a pro-oxidant (Podmore *et al.*, 1998) [16]. Since ascorbic acid increases the iron absorption (Teucher *et al.*, 2004) [28], supplementation of 2% of ascorbic acid might be responsible for the iron accumulation in the tissues and subsequent induction of iron-mediated oxidative stress. It might be one of the reasons for such negative growth rate in 2% ASA supplemented larvae (Fig. 1).

Findings of the second experiment indicated the attenuation of H_2O_2 content (Fig. 2) and LPx level (Fig. 3) in the whole body tissue of the larvae receiving supplementation of 1% ASA or GLY. H_2O_2 is an attractive model oxidant because of its cellular actions and dual role. During development, particularly in insects, the physiological levels of H_2O_2 regulate some of the critical functions like cell differentiation, development, dormancy and lifespan extension (Zhao *et al.*, 1999; Zhao and Shi, 2009; Sahoo *et al.*, 2018) [32, 31, 22]. However, beyond the physiological limits it results in several pathological consequences and developmental abnormalities. In the present study, the H_2O_2 content was decreased in the larval tissues in response to 1% ASA, GLY or co-supplementation of ASA and GLY (Fig. 2). In a recent study, we have observed that the elemental components and allelochemicals of the host plant leaves are the major source for differential rise of H_2O_2 content (Sahu *et al.*, 2018) [25] in the *A. mylitta* larvae. It is clearly observed in the present experiment that the deleterious effect of all such components of host plant leaves might have been minimized by the foliar supplementation of ASA and GLY, therefore a decrease in H_2O_2 is observed in the larvae in response to antioxidant supplementation. Iron is also one of the most critical factors in maintaining the tissue redox balance. It is usually present in host plants of herbivore insects (Welch *et al*., 2002) [29]. Recently we have also observed that iron content is higher in *T. tomentosa* leaves compared to *T. arjuna* leaves (Sahu *et al*., 2018) [25], another host plant of this silk worm. In the presence of iron, hydroxyl radicals ($OH^{\cdot}$) generation from H_2O_2 is normally enhanced and it reduces the nutritive value of food in the gut lumen (Summers and Felton, 1994) [27] and also induces oxidative stress including augmentation of LPx. So, the comparatively higher LPx level in the control larvae might have been associated with the excessive hydroxyl radical formation in the presence of iron and the available H_2O_2 content. As ascorbic acid in optimum level is well-known for its protective role against oxidative stress, the LPx level was significantly decreased in the larvae of ASA and GLY supplemented group (Fig. 3). Therefore, the decrease in H_2O_2 content and consequently the LPx level in the larval tissues of *A. mylitta* in response to antioxidant supplementation is interlinked and clearly suggest the beneficial role of ASA and GLY in an optimum dose (1%), in limiting the oxidative stress level in the larvae.

To minimize the effect of ROS and subsequently the oxidative stress, SOD and CAT are important antioxidant enzymes in insects. We observed that both SOD (Fig. 4) and CAT (Fig. 5) activities were significantly increased in ASA and GLY supplemented larval tissues. These beneficial effects of SOD and CAT in the silkworm larvae have displayed their protective role against the oxidative burden. The protective responses of these antioxidant enzymes have also been well-documented in earlier findings (Aucoin *et al*., 1991; Felton and Duffey, 1992; Lee and Berenbaum, 1990 Pritsos *et al*., 1988) [2, 9, 11, 17]. They have also found that especially SOD and CAT play a defensive role against oxidative stress caused due to plant allelochemicals. The protective role of ASA and GLY supplementation (1%) in attenuating the oxidative stress in the silkworm larvae is well evident in the present experiment and strongly agrees with the previous reports.

Taken together, the present pilot level feeding trial and the subsequent experiment suggest that foliar supplementation of glycine and ascorbic acid in an optimum level is useful in enhancing the growth and antioxidant defences of silkworm than the control one. Our findings further indicate the defensive role of antioxidants through foliar supplementation against the endogenous oxidative stress caused by plant allelochemicals. Further in-depth study on this redox mechanism will enlight the tissue-specific molecular crosstalk in attaining redox homeostasis during larval development of *Antheraea mylitta*, which might be helpful for the improvement of health and physiological fitness of the silkworm. Taking into account the growth rate, decline in oxidative stress and enhancement in antioxidant defences, foliar supplementation of 1% ascorbic acid or glycine (separately or in combination) seems to be promising that could be adopted for large scale rearing of silk worm.

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