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**INTERNATIONAL JOURNAL OF
 ADVANCED RESEARCH (IJAR)**

Article DOI:10.21474/IJAR01/6482
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/6482>



RESEARCH ARTICLE

STUDIES ON BIOCHEMICAL CHANGES DURING TROPICAL TASAR SILKWORM-PEBRINE INTERACTION.

*Madhusudhan. K. N.¹, Manila², Moorthy. S. M.¹, Gupta. V. P.², Sinha. A. K.² and Sivaprasad. V.¹.

1. Central Sericultural Research and Training Institute, Central Silk Board, Srirampura, Mysore-570008, Karnataka, INDIA.
2. Central Tasar Research and Training Institute, Central Silk Board, Piska Nagri, Ranchi-835303, Jharkhand, INDIA.

Manuscript Info

Manuscript History

Received: 09 December 2017
 Final Accepted: 11 January 2018
 Published: February 2018

Keywords:-

Nosema mylitta, *Antheraea mylitta*,
 Hydrogen peroxide, Peroxidase,
 Catalase, Acid phosphatase, Amylase.

Abstract

Tropical Tasar Silkworm, *Antheraea mylitta* D is infected by different pathogens. Among the different pathogens infecting tasar silkworm, pebrine disease caused by protozoa, *Nosema mylitta* is most destructive due to its transovarial nature. In the present study, the variation in the protein, peroxidase, catalase, acid phosphatase, amylase and endogenous hydrogen peroxide concentration during early stages of pebrine infection to tasar silkworm was analyzed. The results revealed the up-regulation in protein concentration, peroxidase, acid phosphatase, amylase enzymes activities and endogenous hydrogen peroxide concentration where as down-regulation in catalase activity. Based on the results of the present study, the changes in the reactive oxygen species and antioxidant enzymes can be used as markers to analyze the health status of the tropical tasar silkworm infected with pebrine disease.

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Introduction:-

Tropical tasar culture is the main livelihood for the poor tribal people in central and north eastern regions of India. Due to outdoor rearing of the tasar silkworm, the insect is accessible to diseases and pests. Among the diseases, the pebrine disease caused by protozoa, *Nosema mylitta* is the most devastating because of the transovarial transmission of the pathogen from one generation to another.

Insects are known to have both cellular and humoral immune system which together forms a potent defence against invading pathogens. Hemocytes are very vital component of the insect immune system and are biochemically very sensitive having multiple functions such as nodule formation, phagocytosis and encapsulation as defence mechanism; synthesis and transport of nutrients and hormones for proper growth and wound healing by way of connective tissue formation (Pandey et al., 2010). Humoral reactions involve slow synthesis of antibacterial and antiviral principles and require several hours for full expression. Cellular responses are direct interactions between circulatory haemocytes and invading non-self materials. The interaction is immediate and includes phagocytosis, nodulation and encapsulation (Singh et al., 2011). In insects, six types of haemocytes are observed in the haemolymph. A variety of functions like mechanization and immobilization of invading organism by encapsulation and/or phagocytosis, wound repair, coagulation have been reported to haemocytes (Pech et al., 1995). The

Corresponding Author:-Madhusudhan,K.N.

Address:-Central Sericultural Research and Training Institute, Central Silk Board, Srirampura, Mysore-570008, Karnataka, INDIA.

susceptibility test for three ecoraces of *Antheraea mylitta* against AmCPV. They also noticed that, ecoraces showing reduced number of haemocytes are tolerant to pathogen. They also opined that total haemocyte counts may indicate the susceptibility status of the insect especially in tasar silkworm. Studies on impact of pebrine on haemocytes are insufficient in tropical tasar silkworm (Singh *et al.*, 2008).

In order to manage the pebrine disease, the host-pathogen interaction between tasar silkworm-pebrine needs to be understood systematically. Hence, the important digestive enzymes such as amylase, regulatory enzymes such as peroxidase and catalase, hydrolytic enzymes such as acid phosphatase along with reactive oxygen species, H₂O₂ were studied and their activity level was recorded in the haemolymph, midgut and fatbody of control and pebrinized larvae.

Materials and Methods:-

Collection of pebrine infected larvae:-

Nosema mylitta infected silkworms (*Antheraea mylitta*) were collected from the rearing plots of Central Tasar Research and Training Institute, Ranchi and they were maintained in the laboratory conditions.

Purification of pebrine spores from pebrinized larvae:-

The infected fifth instar larvae were homogenized and centrifuged. Spores were purified on a discontinuous percoll gradient (25, 50, 75 and 100% v/v) by centrifugation at 10,000 rpm for 10 minutes. The pellets were rinsed and stored at 4°C for later use.

Inoculation of pebrine spores to healthy larvae:-

The healthy Vth instar larvae were fed with pebrine spores (1x10⁸ concentrations) smeared *Terminalia arjuna* leaves under indoor condition. The pebrinized larvae were harvested at different time intervals. The harvested larvae were kept at -20°C for further experimentation.

Preparation of samples for biochemical studies:-

Different tissues viz., midgut, haemolymph and fat bodies of both control and pebrinized were collected separately for the biochemical studies. Haemolymph was collected by cutting the pro-leg of silkworm larvae in a pre-cooled micro-centrifuge tube containing a pinch of phenylthiourea as an anticoagulant and centrifuged at 5,000 rpm for 5 minutes at 4°C. The supernatant collected at -20°C until further use. Dissection was carried out for the tissues like midgut and fatbodies. Homogenization of tissues was done in a homogenizer using phosphate buffer (PB, 0.1M, pH 7.0). The homogenate was transferred to a clean centrifuge tube and centrifuged at 5,000 rpm for 5 minutes at 4°C. The supernatant was collected in a clean centrifugation tube at -20°C until further use.

Estimation of total proteins:-

The larval tissue (0.1g) was extracted in pre-chilled pestle and mortar by using phosphate buffer (PB, 0.1M, pH 7.0). The protein content in the supernatant was estimated by using dye-binding method (Bradford, 1976). The protein concentration was recorded from the standard curve prepared for bovine serum albumin (10-100µg). The protein concentration was expressed in mg/ml of protein.

Time course expression of antioxidant enzymes:-

The different tissues samples collected were used for the analyzing temporal changes in antioxidant enzymes such as peroxidase and catalase activity at different time intervals were analyzed.

Determination of peroxidase activity:-

The tissue sample (0.1 g) was extracted with phosphate buffer (pH 7.0, 0.1M) in a pre-chilled pestle and mortar. The extracted sample was centrifuged at 4°C at 10,000 rpm for 10 minutes. The protein content in the supernatant was estimated by using dye-binding method (Bradford, 1976). The reaction mixture, three ml of phosphate buffer along with Guaiacol, was taken in the spectrophotometer sample cuvette along with 40µl crude extract sample and 40µl of substrate H₂O₂ (10mM) was added. The reaction was measured spectrophotometrically at 470nm (Hammerschmidt *et al.*, 1982). Peroxidase activity was expressed as change in A₄₇₀ mg protein⁻¹ min⁻¹.

Determination of catalase activity:-

By using phosphate buffer (0.1 M, pH 7.0), tissue sample (0.1g) was extracted in pre-chilled pestle and mortar. The extracted sample was centrifuged at 4°C at 10,000 rpm for 10 minutes. The protein content in the supernatant was estimated by using dye-binding method (Bradford, 1976). The reaction mixture containing three ml of Phosphate buffer along with 40µl crude extract was taken in the spectrophotometer sample cuvette and 40µl of H₂O₂ substrate was added. The reaction was measured spectrophotometrically at 240nm. Catalase activity was expressed as change in A₂₄₀ mg protein⁻¹ min⁻¹ (Havir and Mettala, 1987).

Spectrophotometric determination of H₂O₂:-

Endogenous H₂O₂ present in the tissues of healthy and pebrinized tasar silkworm larvae were estimated according to the procedure of Frew *et al.* (1983). The sample was prepared by homogenizing the tissue in 0.1M phosphate buffer pH.6.9. The extracted sample was centrifuged at 4°C at 10,000 rpm for 10 min and the supernatant was taken. The extract (40 µl) is taken for estimating H₂O₂ and 3ml of reagent solution (100ml contains 0.234g of phenol, 0.1g of 4-aminoantipyrine, 1ml of 0.1 M phosphate buffer pH 6.9) was used to estimate the H₂O₂. The quantified amount of H₂O₂ is compared with standard graph. Quantified H₂O₂ is expressed in micromoles/ gram tissue.

Determination of amylase activity:-

Amylase activity was measured with the Dinitrosalicylic acid procedure using soluble starch as substrate (Baker, 1991). Briefly, 20 µl haemolymph was incubated at 1.5ml of 20mM phosphate buffer (pH 6.8) containing 0.2% soluble starch and 1mM CaCl₂ at 37°C for 30 minutes. Similarly, 100mg of midgut tissues was homogenized in 1.5ml of 20mM phosphate buffer (pH 6.8) containing 0.2% soluble starch and 1mM CaCl₂ at 37°C for 30 minutes. Released maltodextrins were estimated by boiling with 2ml Dinitrosalicylic acid solution for 10 minutes and by measuring the absorbance at 540nm. For controls enzyme was added after the solution of Dinitrosalicylic acid. Amylase activity was expressed in unit mole of maltose released/min/mg protein.

Determination of acid phosphatase activity:-

The midgut/fatbody tissues were homogenized in 0.25M sucrose containing 1mM EDTA. Similarly, 0.1ml of haemolymph was used as enzyme source. The homogenate was centrifuged at 4°C at 10,000 rpm for 10 minutes. The obtained pellets were discarded and the supernatant was diluted approximately with 150mM sodium acetate buffer and immediately used for the assay. The tissue sample (0.2ml) was taken and 0.8ml P-nitro-phenyl phosphate pNPP as substrate was added and incubated for 30 minutes at 40°C. After incubation, 4ml of NaOH was added and absorbance was measured at 405nm. Acid phosphatase activity was expressed in micromole/hour/mg protein (Staykova *et al.*, 2010).

Results:-**Protein concentration:-**

The protein concentration in the different tissues samples was analyzed at different time intervals. At the constitutive level, more protein concentration was noticed in haemolymph in comparison with other tissues. After inoculation of pebrine to tasar silkworm, more increase in protein was noticed in the haemolymph in comparison with fatbodies and midgut (Fig.1).

Peroxidase activity:-

The variable level in peroxidase activity was recorded in different time intervals in both control and pebrinized larvae. All the tissues after inoculation with pebrine showed the gradual increase in peroxiase activity in comparison with control (Fig.2).

Catalase activity:-

The gradual decrease in catalase activity was noticed in all tissues viz., midgut, fatbody and haemolymph of pebrine inoculated tasar silkworm larvae in comparison with control. Among the tissues, both haemolymph and fatbodies showed maximum decrease in catalase activity from 4th day post inoculation (Fig.3).

Endogenous Hydrogen Peroxide concentration:-

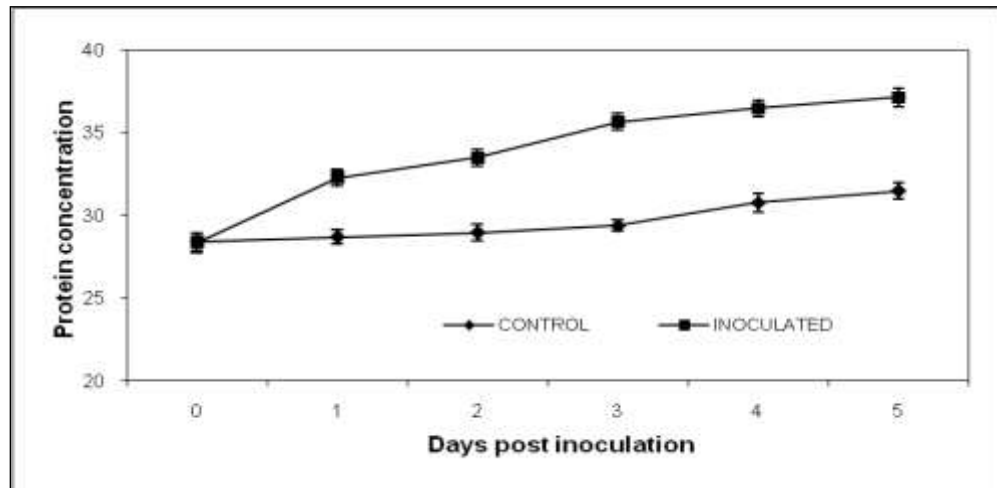
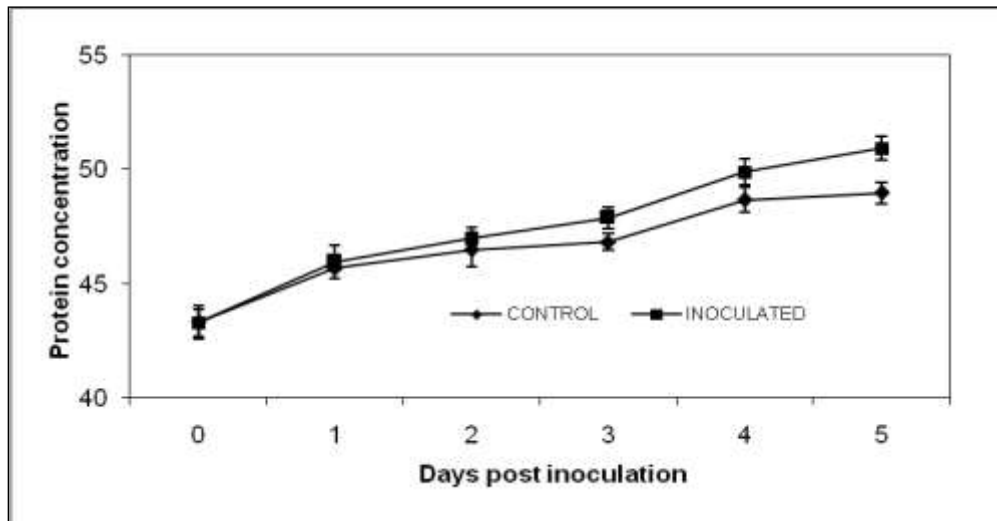
The H₂O₂ level was estimated at different time intervals in pebrinized and control tasar silkworm larvae. A sharp increase in level of H₂O₂ was noticed in haemolymph of pebrinized larvae in comparison with midgut and fat body tissues at 1 day post inoculation (Fig.4).

Acid phosphatase activity:-

The inoculation of pebrine t tasar silkworm showed increased acid phosphatase activity in all the tissues analyzed. Among them, midgut showed increased acid phosphatase activity after 2nd day post inoculation. (Fig.5).

Amylase activity:-

The midgut tissue showed more amylase activity at constitutive level when compared to haemolymph. After inoculation of pebrine, decrease in amylase activity was noticed in both the tissues from 1st day post inoculation. More decrease in amylase activity was noticed after 4th day post inoculation in midgut tissue of pebrinized larvae. (Fig.6).



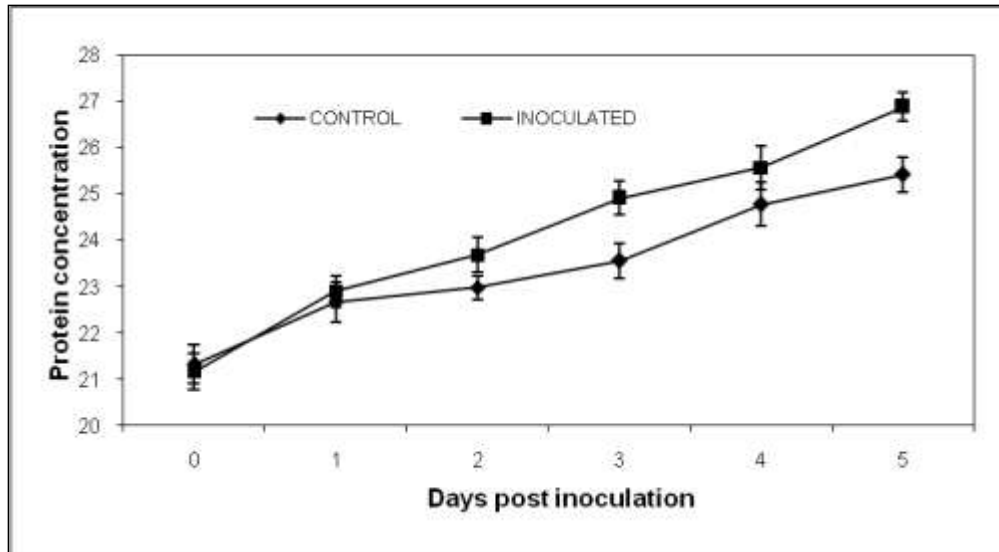
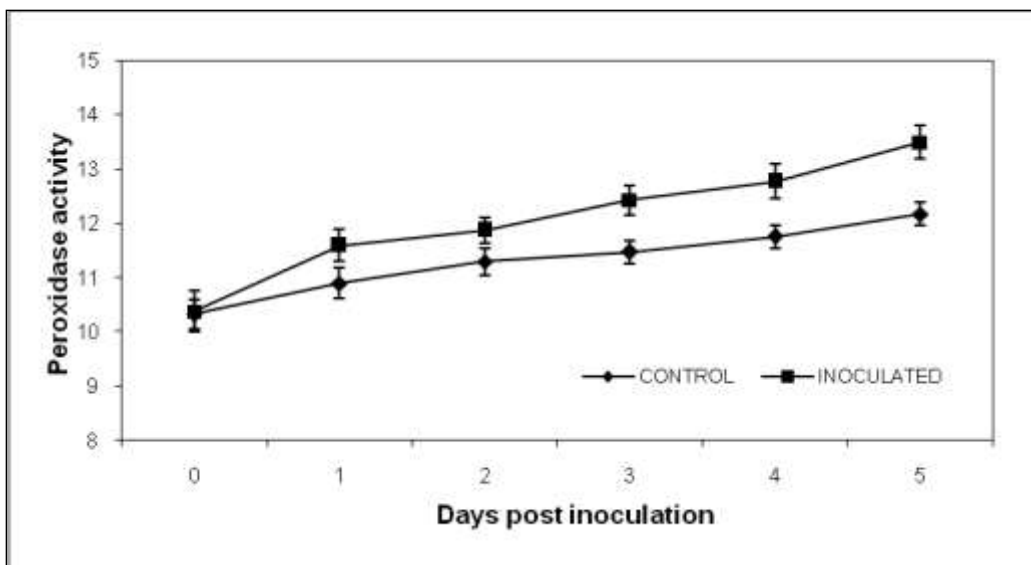


Fig. 1:-Temporal changes in protein concentration in haemolymph, midgut and fatbodies of healthy and pebrinized tasar silkworm larvae.



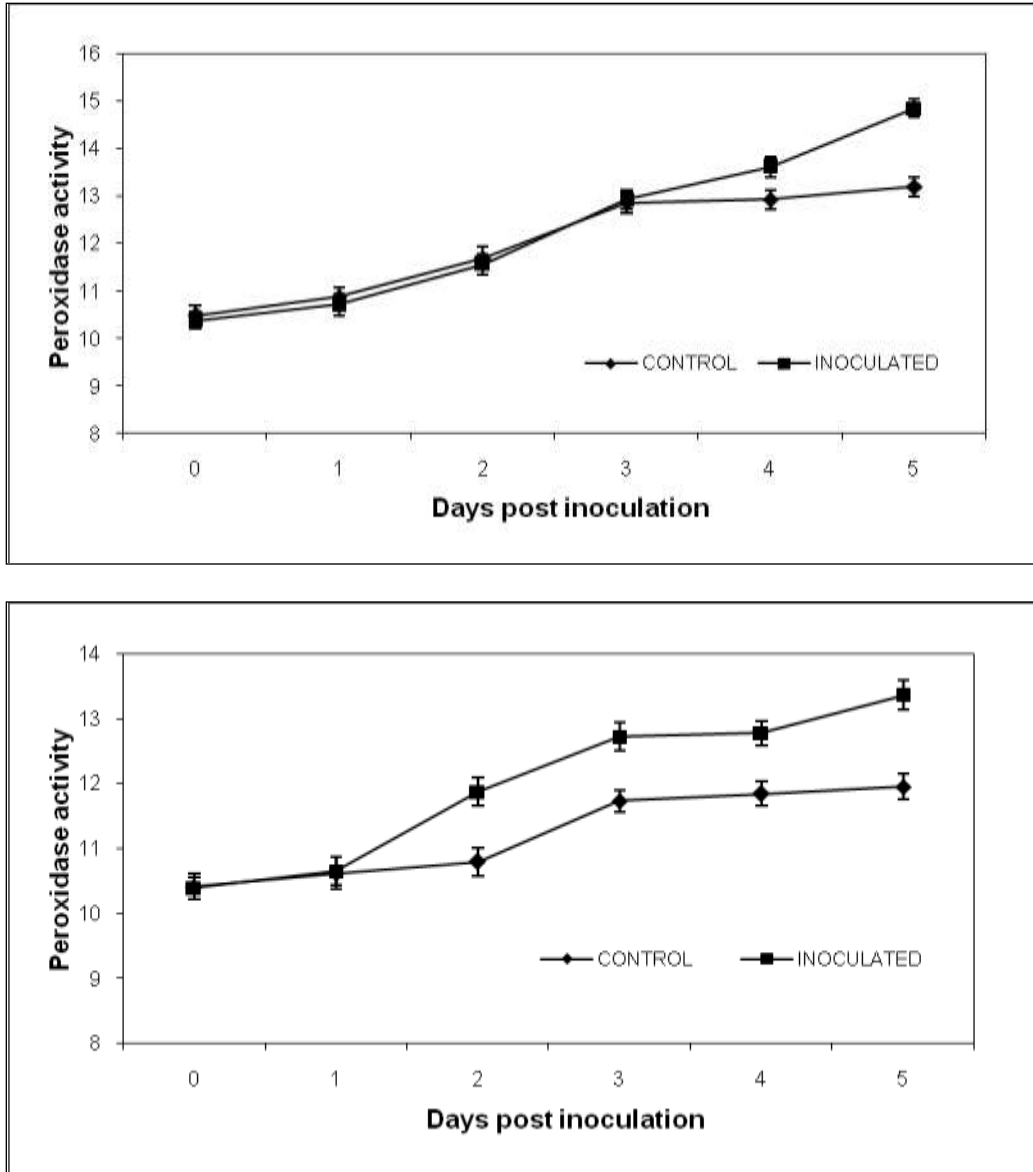
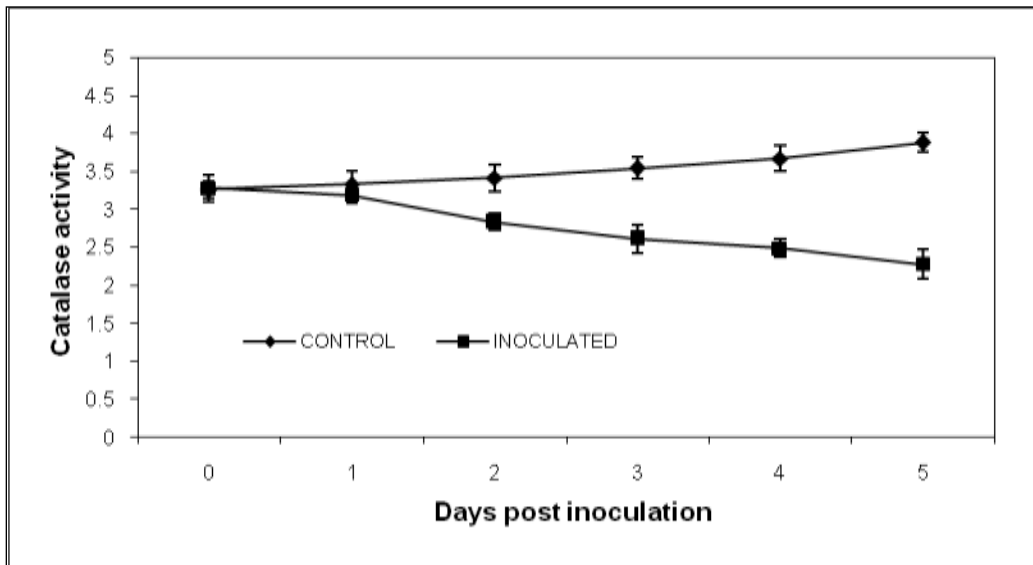
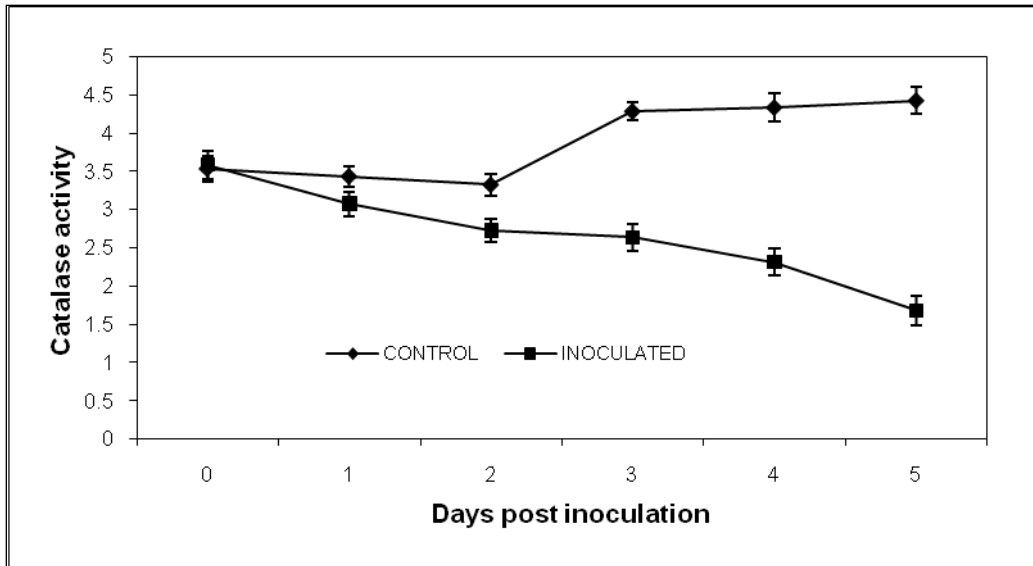


Fig. 2:-Temporal changes in peroxidase activity in haemolymph, midgut and fatbodies of healthy and pebrinized tasar silkworm larvae.



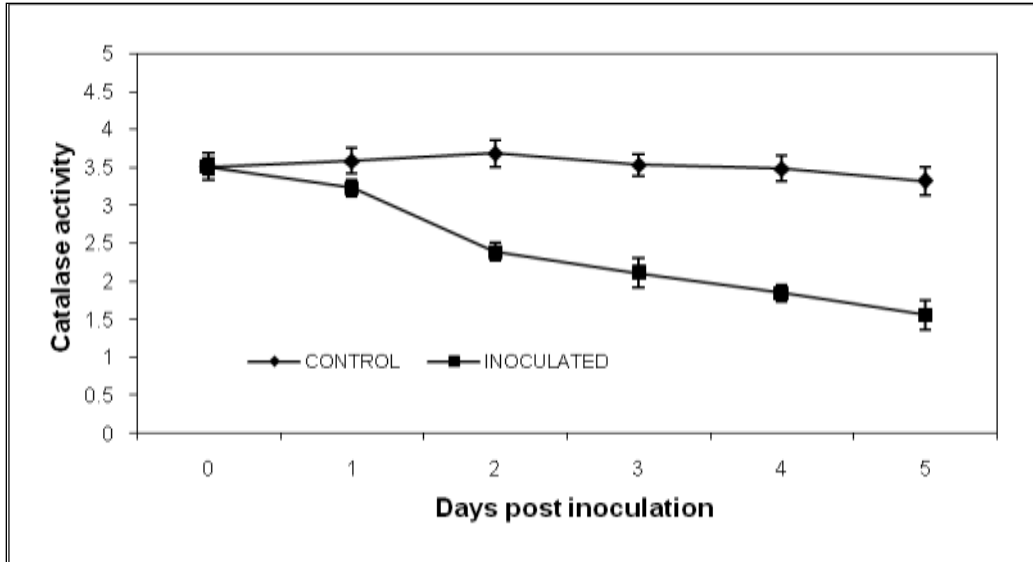
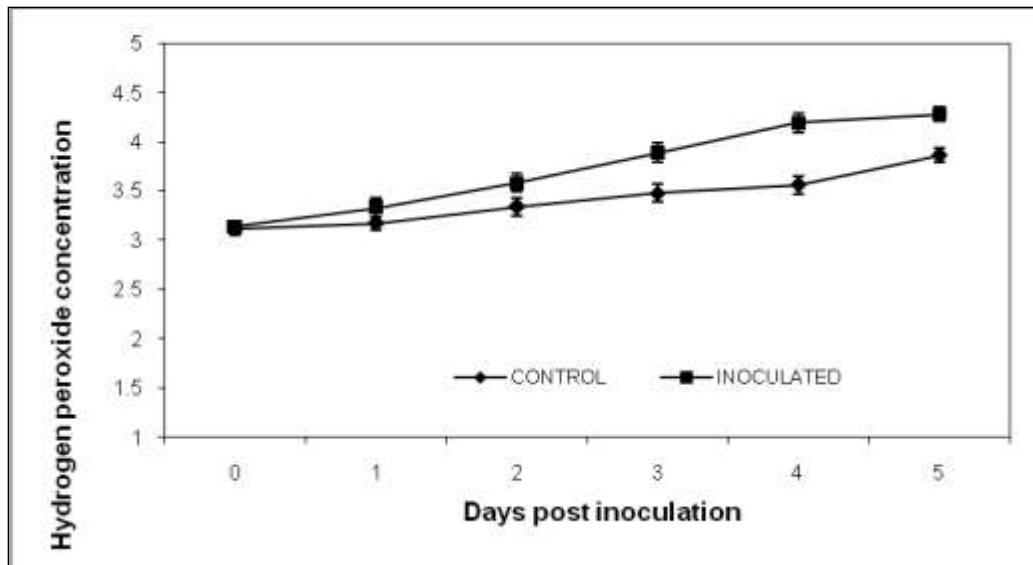


Fig. 3:-Temporal changes in Catalase activity in haemolymph, midgut and fatbodies of healthy and pebrinized tasar silkworm larvae.



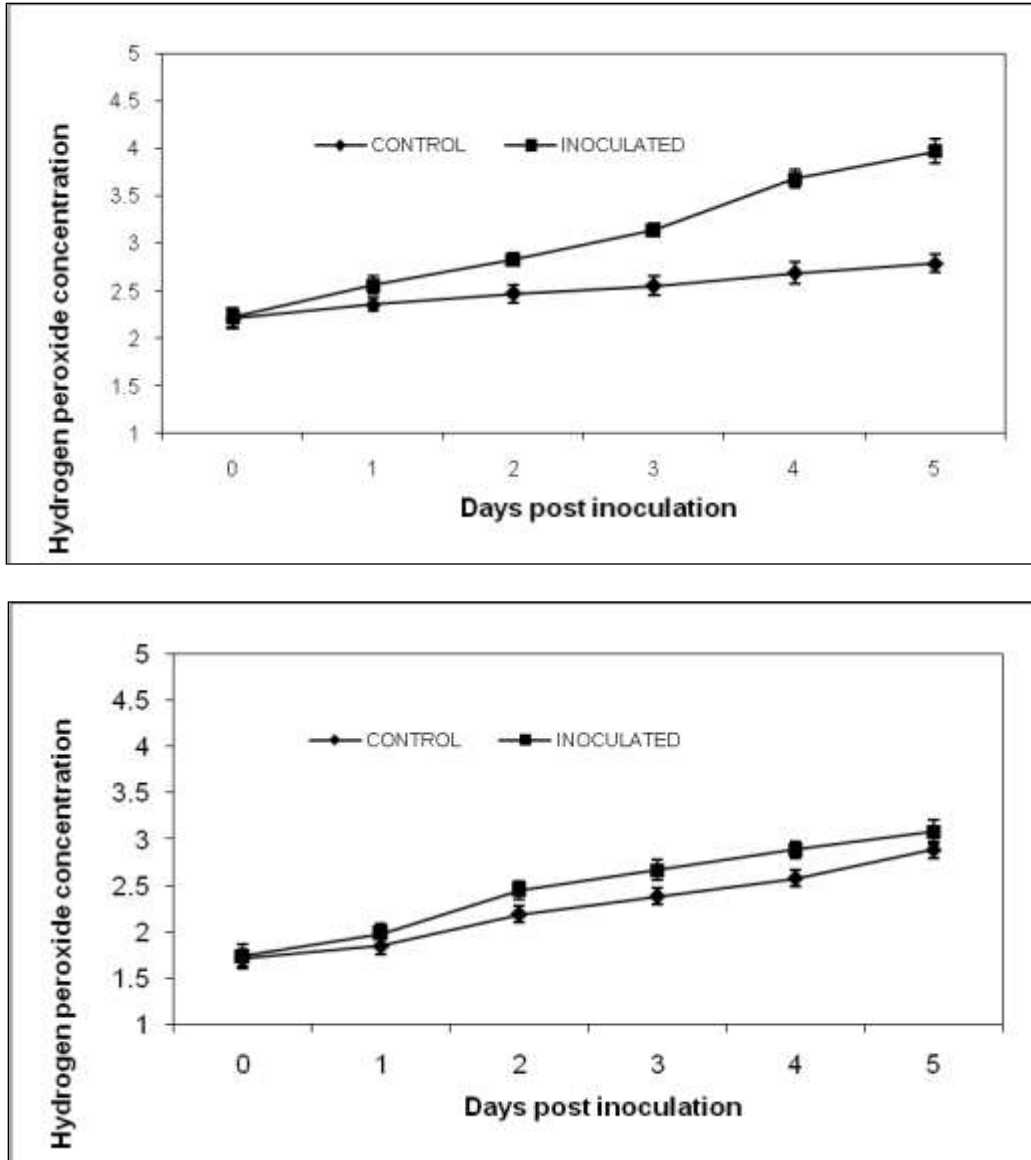
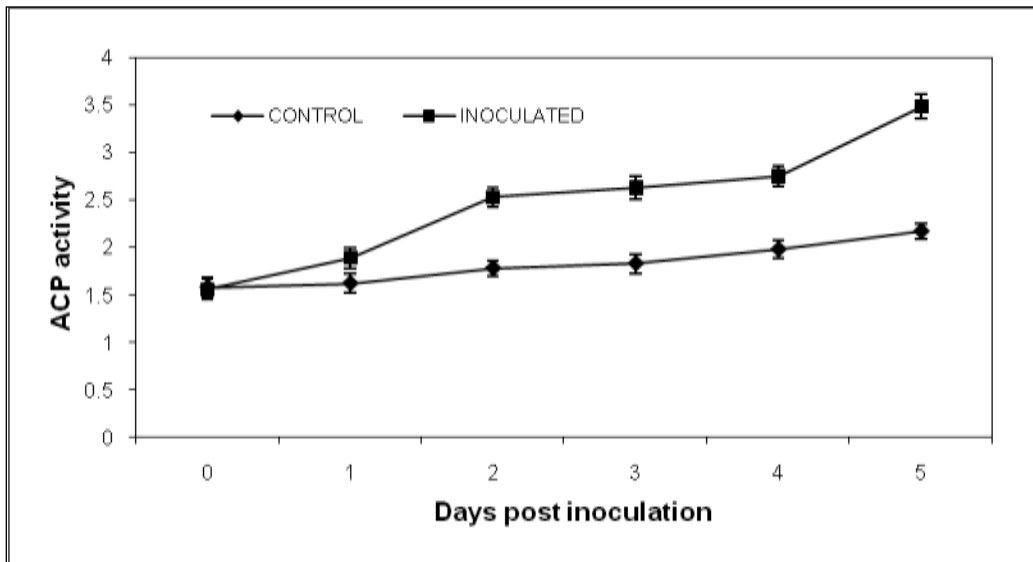
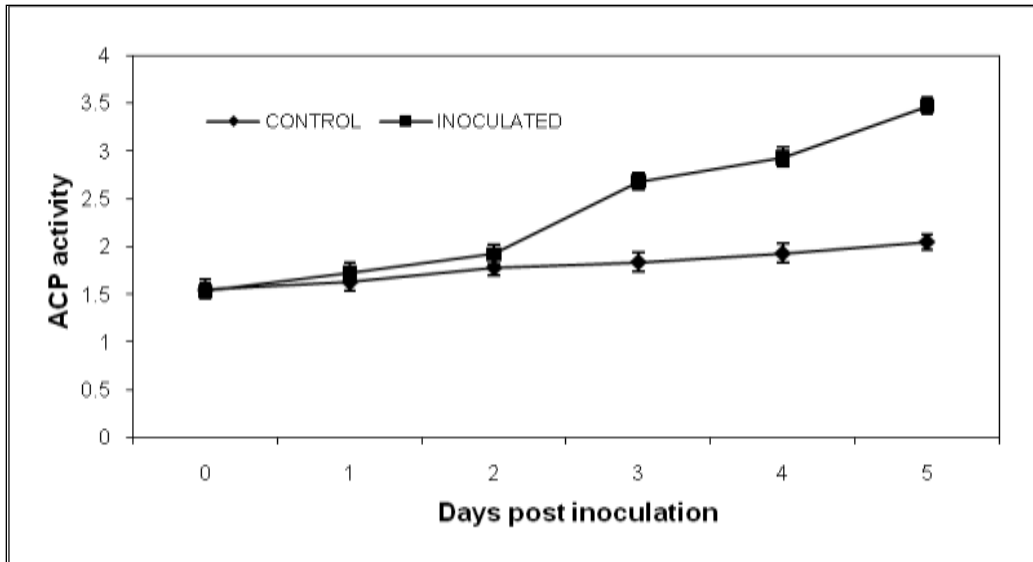


Fig. 4:- Temporal changes in hydrogen peroxide concentration in haemolymph, midgut and fatbodies of healthy and pebrinized tasar silkworm larvae.



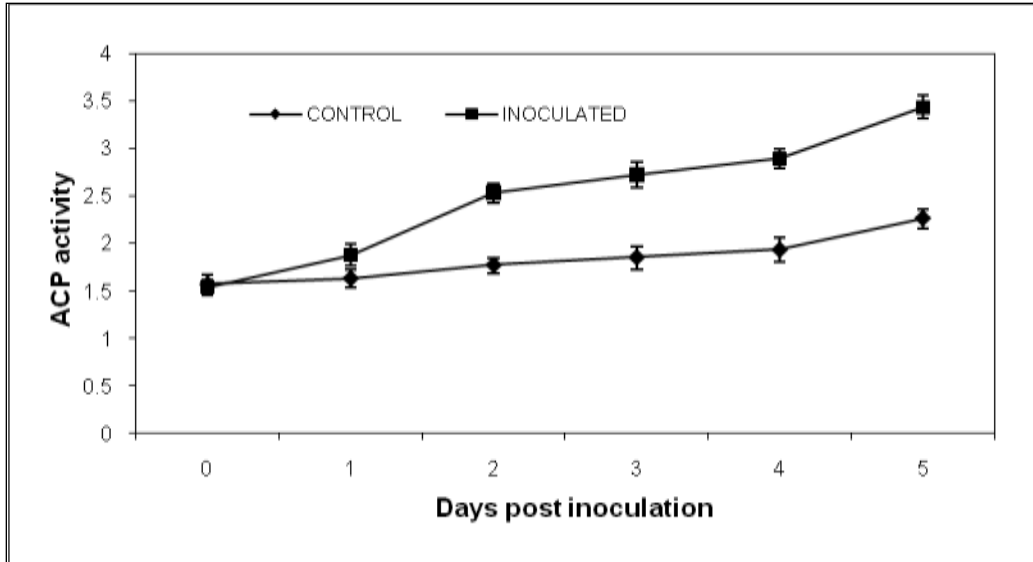
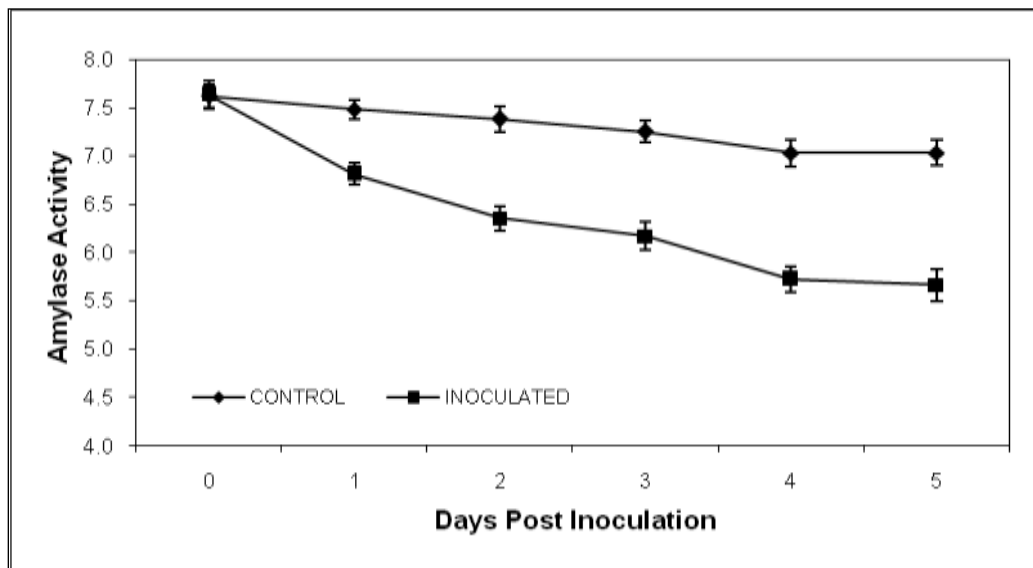


Fig. 5:-Temporal changes in Acid phosphatase activity in haemolymph, midgut and fatbodies of healthy and pebrinized tasar silkworm larvae.



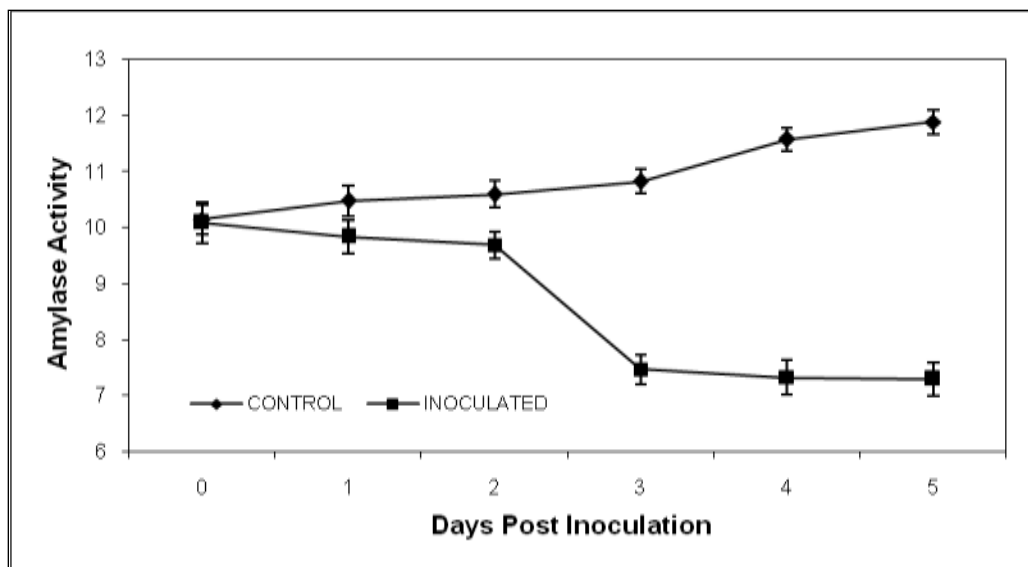


Fig. 6:-Temporal changes in amylase activity in haemolymph and midgut tissues of healthy and pebrinized tasar silkworm larvae.

Discussion:-

Tasar silkworm is infected by different pathogens including pebrine disease. The pathogen is causing considerable yield loss upto 40% in combination with other pathogens (Sahay *et al.*, 2000). Before targeting pathogen to control the disease, there is need to understand the changes in ROS and antioxidant enzymes in pebrinized tasar larvae.

The present study results revealed that, there is a significant increase in total protein concentration in pebrine infected tasar silkworm. Similarly, earlier works carried out on Tropical Tasar Silkworm, *Antheraea mylitta* D during pathogen infected condition showed reduced protein content (Madhusudhan *et al.*, 2011; Ali and Sharma, 2014).

Catalase has an important biological function such as catalyzing the breakdown of hydrogen peroxide, a toxic substance to organisms, to water and molecular oxygen, which are both harmless and useful. The results obtained in our study showed that increase in H_2O_2 level in pebrinized larvae in all the tissues. The recent study on the tasar silkworm revealed differential expression of reactive oxygen species in haemolymph of pebrinized and healthy worms (Jena *et al.*, 2016). In the present study decrease in the catalase activity in the different tissues analyzed at different time intervals in pebrinized tasar silkworm larvae. But, the catalase activities in the *Antheraea* species declines when subjected to stress (Madhusudhan *et al.*, 2012; Pandey *et al.*, 2015). The results of the present study confirm that, *Antheraea* species shows similar trend in catalase expression when exposed to stress condition.

The study revealed that, increase in peroxidase activity in tasar silkworm inoculated with *Nosema mylitta*. Bhavane *et al.* (2013) reported that, treatment of plant extracts to BmNPV inoculated larvae increases the activity of enzymes such as Superoxide Dismutase, Peroxidase, Acid Phosphatase and decrease in Catalase activity. The results of the present study confirms that, increased peroxidase higher than control, implied an important roles of peroxidase in defending against the pathogens.

The results of the present study showed that, acid phosphatase increased in pebrinized larvae. The observation of the present study co-relates that, increase in acid phosphatase noticed in silkworm under stress condition (Pandey *et al.*, 2015).

Amylase is a hydrolytic enzyme found in all organisms including silkworm which are majorly involved in the digestion and carbohydrate metabolism in silkworm including carbohydrates available in the form of starch in mulberry leaves. The reduction in amylase activity in pebrinized larvae in both midgut and haemolymph tissues was noticed. The effect of virus infection resulted in the reduction of activities of amylase, invertase, trehalase and protease were analysed in the infected *Bombyx mori* was studied by Gururaj *et al.* (1999).

Conclusion:-

Pebrine is one of the most common diseases infecting tropical tasar silkworm which contributes majorly for the reduced cocoon production. Tasar silkworm and pebrine interaction studies revealed up-regulation in protein, peroxidase, acid phosphatase and endogenous salicylic acid concentration and down-regulation of catalase activity.

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