CONCLUDED PILOT STUDY REPORT

i	**Project code & title :**	**BPI (PS) 010: Identification of biochemical markers associated with thermotolerance in silkworm *Bombyx mori* L.**
ii.	**Names of the project investigators (including coordinator in case of collaborative projects):**	**Executive Authority:** Kanika Trivedy **Principal Investigator:** Pooja Makwana **Co-Investigator:** S Chattopadhyay
iii.	**Duration**	October 2016 – September 2017
iv.	**Name(s) of the institute(s) and addresses**	Central Sericultural Research & Training Institute, Central Silk Board, Berhampore 742101, Dist: Murshidabad, West Bengal Phone: 03482-224712 FAX: 03482-224714

v. Objective / goals

- ***To identify biochemical markers associated with ROS defense under thermal stress in bivoltine silkworm***
- ***To establish correlation between ROS defense associated enzymes and yield parameters***

vi. Introduction

The silkworm *Bombyx mori* is an insect of economic importance as it produces silk which is known for its luster, water absorbency and heat resistant properties. The optimum conditions of rearing are 22-28°C and 75% relative humidity (Krishnaswami *et al* 1973). Physiological changes occur in organisms according to the change in environmental factors like temperature, humidity, air and light. Being a poikilothermic insect, *Bombyx mori* is highly sensitive to seasonal fluctuation of temperature and unable to survive under supra-optimal temperature and humidity. Indeed physiology of silkworm depends largely on factors like temperature, humidity, air circulation and light (Hussain *et al.* 2011). Different stage of development and growth are affected by these factors. Literature shows that cocoon production is enhanced and the quality of cocoon is comparatively better when silkworms are reared at temperature range of 22-28°C (Krishnaswami *et al.*1973).

Many studies in silkworm reported that heat shock proteins (HSPs) are activated with the onset of thermal stress. Abiotic stress also depletes antioxidant state of almost all organisms and causes oxidative stress. During respiration process reactive oxygen species (ROS) like hydroxyl radical ($OH^{\cdot}$), superoxide anion radical ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2), differential lipid radicals, peroxides as well as nitrous oxides are generated but redox homeostasis is maintained by organisms in normal conditions. Under various oxidative stresses, redox equilibrium is disturbed leading to oxidative damage. When ROSs level rises these antioxidative constituents of the cell tend to react with different surrounding molecules to stabilize the oxidative damage(s) of cellular components. Due to oxidative stress, lipid peroxides, aldehydic products and protein carbonyls are also produced.

Organisms have developed defense mechanisms comprising of various antioxidant enzymes and low molecular weight components that plays major role in elimination of ROSs. Among the antioxidant enzymes Superoxide dismutases (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductases (GR), Ascorbate peroxidases (APOX) are noteworthy. SOD converts superoxide anion to hydrogen peroxide. Catalase is the main enzyme which catalyses the hydrogen peroxide to water and oxygen. It has been observed that thermal stress can deplete the antioxidant state which can lead to oxidative stress in the organism. The imbalance of reactive oxygen species (ROS) generation and its removal causes oxidative stress which results in the accumulation of hydrogen peroxide, superoxide anion, and singlet oxygen commonly known as reactive oxygen species (ROS).

Though all commercially exploited silkworm hybrids and its progenitor breeds are notoriously vulnerable to thermal stress but association of these breeds' performances with ROS stabilization process with temperature stress are not yet comprehensively studied.

With this background present study was conducted with the objectives to assess the generation of reactive oxygen species (ROSs), its major stabilizing enzyme activities in haemolymph plasma of eleven bivoltine silkworm larvae. Besides, probable correlation of ROS components with the survival and other rearing parameters of these breeds were also examined.

vii. Methodology adopted:

a) Insect materials:

- Ten putative thermotolerant breeds selected for the study were- SK6, SK7, BCon1, BCon4, BHR2, BHR3, D6(P)N, NB18, Gen3, SK4C along with a susceptible breed CSR2 (not available in institute germplasm). All the mentioned breeds were available in institute germplasm.

b) Temperature and humidity stress applied to silkworm strains:

- Silkworm strains (from first activity) were exposed to high temperature using environmental chamber at 35°C and 40°C, 75±5% RH for 4h from 5th instar first day. After 4h exposure larvae were again reared in optimum temperature and humidity conditions i.e. 25±2°C and 70±5% RH. Two batches were maintained for each strain.
- Control larvae were reared in optimum rearing conditions 25±2°C and 70±5% RH. Rearing was conducted in two seasons Falguni and Shravani.
- Haemolymph was collected on 5th day of 5th instar control and temperature stressed larvae. Prolegs of larvae were pricked to collect hemolymph in microfuge tubes containing pinch of phenylthiourea. Hemolymph samples were centrifuged at 3000rpm for 10 minutes to remove hemocytes. Haemolymph plasma was collected in fresh microfuge tubes and stored at -80°C for further experiments.
- Larvae were weighed before and after heat treatment on each day of treatment and also phenotypic characters (like cocoon weight, shell weight, pupation percentage, etc.) were assessed.

c) Assessment of generation of ROS and oxidative damage caused:

- ***Total soluble protein:*** Protein was estimated in hemolymph plasma spectrophotometrically using BSA as standard according to Lowry et al (1950).
- ***ROS estimation and oxidative damage:*** Hydrogen peroxide (H_2O_2) was estimated from thermal stressed and control haemolymph plasma. Generation of H_2O_2 will be measured according to the method of Velikova *et al* (2000). Oxidative damage was assessed by lipid peroxidation assay by measuring the amount of malondialdehyde produced according to the method of Yagi (1998).

d) Assessment of defense mechanism during the thermal stress induced oxidative damage in silkworm:

- ***Catalase (CAT):*** CAT activity was estimated in haemolymph plasma by following the spectrophotometric assessment procedure of Sinha (1972). The reduction of dichromate/acetic acid reagent into the chromic acetate in the presence of hydrogen peroxide (H2O2) was read at 540nm.
- ***Peroxidases (POXs):*** Ascorbate peroxidases was estimated in all four tissues. Ascorbate peroxidase activity was estimated by procedure of Mathews et al., 1997.
- ***Superoxide dismutase (SOD):*** SOD assay was done following the method of Kono (1978) using NBT (nitro blue tetrazolium) as substrate. The reduction of NBT was followed by an absorbance increase at 540nm.

e) Data analysis:

Analysis of variance was performed using OPSTAT (HAU, Hisar). When *F*-values were significant ($P<0.05$), Fisher's least significant differences were calculated. Pearson's correlation coefficients were calculated to compare the association among ROS stabilizing parameters and survival of breeds according to the method of Gomez and Gomez, 1984.

viii. Results:

Evaluation of temperature and humidity stress on bivoltine silkworm

Eleven bivoltine breeds selected for the study based on the previous reports / claims of different workers as putative thermotolerant breeds. All the selected bivoltine breeds were exposed to thermal stress regimes (35°C and 40°C) with 75±5% relative humidity for 4h a day from 2nd day of fifth instar to 5th day. Control batches were maintained at 25°C temperature with 75±5% relative humidity. Larval weight, survival (%), pupation (%), single cocoon weight, single shell weight and shell ratio were recorded for control and temperature stressed larvae.

At 40°C temperature, survival (%) of ten bivoltine breeds ranged 8 to 14.8% (data not shown). All ten breeds treated at 40°C temperature failed to pupate hence no further data was obtained. Notably CSR-2 (susceptible check) was the only breed which could not survive even at 35°C temperature stress. Therefore, further experiments were carried out with ten breeds treated at 35°C (temperature stress) and 25°C (control). Survival (%) of the breeds at 35°C was in the range of 44 to 67.8%; while in control survival (%) ranged from 79 to 89.2%. Larvae exposed to 35°C temperature showed significant ($P \leq 0.01$) reduction in survival (%) from their respective control values. Among the temperature stressed larvae BCon4 showed highest survival of 67.8% followed by BCon1 and BHR3. Similarly, high significant ($P \leq 0.01$) reduction in larval weight was observed in temperature stressed larvae.

Table 1: *Variation in pupation (%) and shell ratio of control and temperature stressed silkworms*

Breeds	Pupation (%)		Shell ratio	
	25°C	35°C	25°C	35°C
SK6	88.50	64.29	17.82	13.90
SK7	90.00	64.70	18.07	15.65
BCon1	88.00	50.00	17.82	16.16
BCon4	91.00	60.16	17.39	14.77
BHR2	87.50	50.00	17.51	16.22
BHR3	89.00	60.00	18.08	15.09
D6PN	87.58	46.16	18.88	15.05
NB18	86.37	54.43	19.32	15.94
Gen3	88.43	55.53	21.24	17.86
SK4C	89.80	58.71	18.28	15.76
	CD(0.05)	SE(m)	CD(0.05)	SE(m)
Temp	0.38	0.14	0.53	0.19
SW	0.86	0.30	1.19	0.42
T X SW	1.21	0.43	*ns*	0.60

Data are mean of two seasonal experiments with 3 observations per season

Pupation (%) is considered as one of the factors for thermotolerance in silkworm. All ten breeds showed high pupation percent (range: 86.3 to 91%) at 25°C; while significantly

(P<0.01 or better) lower pupation percent (range: 46.1 to 64.7%) was observed in 35°C temperature stressed breeds. Single cocoon weight and shell weight were also reduced significantly (P<0.001 or better) in temperature stressed breeds compared to their respective control values. Shell ratio in control breeds ranged from 17.3% to 21.2%; while in temperature stressed breeds significantly (P<0.01 or better) lower shell ratios were (range: 13.9 to 17.8%) observed (Table1).

Assessment of ROS generation and oxidative damage:

Generation of ROS in control and temperature stressed larvae was measured by estimation of hydrogen peroxide (H_2O_2) levels in haemolymph plasma of silkworm breeds (Table-2). Noticeably, H_2O_2 levels were significantly ($P \leq 0.01$ or better) high in all temperature stressed breeds compared to control. At 35°C of temperature treatment, H_2O_2 levels were found maximum in BCon4 (8.39μM) and minimum in SK4C (4.4μM). Besides, significant breed variations of H_2O_2 levels were also observed.

Oxidative damage caused due to ROS in silkworm was also assessed by lipid peroxidation by measuring the malodialdehyde (MDA) levels in haemolymph plasma of control and temperature stressed larvae. Variation of MDA levels were highly significant ($P \leq 0.001$) in hemolymph of temperature stressed larvae compared to their respective control (Table-2). MDA level was 8 folds higher in temperature stressed larvae of BCon1 breed (233.55 nmol mg^{-1} protein) than the control (27.09 nmol mg^{-1} protein) indicating the level of oxidative damage silkworm due to high temperature stress.

Table 2: *Variation in Hydrogen peroxide and MDA levels in hemolymph of control and temperature stressed silkworms.*

Breeds	Hydrogen peroxide (μM)		MDA (nmol mg^{-1} protein)	
	25°C	**35°C**	**25°C**	**35°C**
SK6	6.44	7.99	33.12	91.18
SK7	5.27	6.29	45.59	143.66
BCon1	4.34	8.26	27.09	233.55
BCon4	3.77	8.39	21.07	206.45
BHR2	2.48	7.10	25.37	53.33
BHR3	4.99	7.54	27.53	66.77
D6PN	4.93	5.62	15.48	169.03
NB18	3.06	5.96	11.61	27.09
Gen3	2.73	5.45	15.48	26.24
SK4C	3.72	4.40	13.33	35.69
	CD(0.05)	SE(m)	CD(0.05)	SE(m)
Temp	0.22	0.08	3.61	1.28
SW	0.50	0.18	8.07	2.87
T X SW	0.71	0.25	11.42	4.06

Data are mean of two seasonal experiments with 3 observations per season

Assessment of defense mechanism during the thermal stress induced oxidative damage in silkworm:

In order to stabilize ROS generation in response to the temperature stress induced oxidative damage, antioxidant defense enzymes superoxide dismutases (SOD), catalases (CAT) and ascorbate peroxidases (APOX) are reportedly induced in silkworm. Three antioxidative enzymes were assessed.

SOD activity in control breeds ranged from 2.3 to 5.6 mol mg^{-1} protein; while in temperature stressed condition breed variation of SOD activity ranged 6.3 to 8.1 mol mg^{-1} protein (Fig.1). In temperature stressed larvae BCon4 showed the highest SOD activity followed by BHR3, BHR2 and BCon1. Significantly (P<0.01 or better) higher SOD activity was observed in temperature stressed breeds.

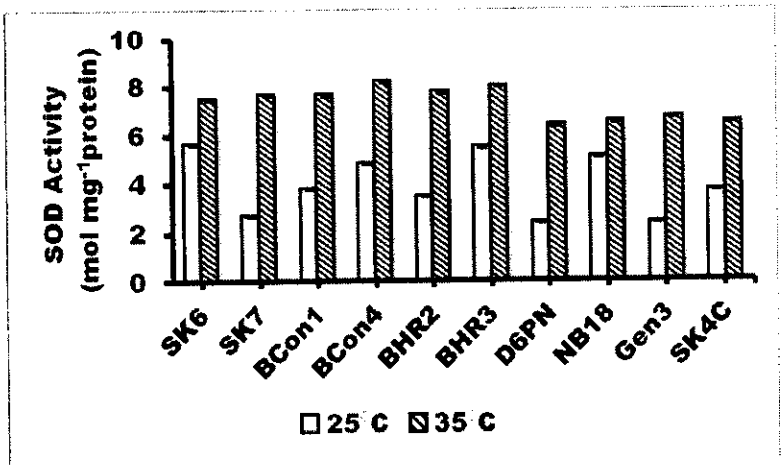

Figure 1: *Variation in SOD activity in hemolymph of control and temperature stressed silkworms (Data are mean of two seasonal experiments with 3 observations per season)*

Similarly, catalase activity and ascorbate peroxidases activity was significantly (P<0.01 or better) higher in hemolymph of temperature stressed larvae compared to control. Catalase activity in control larval hemolymph ranged from 0.09-0.488 μM H_2O_2/min/ml and in temperature stressed larvae 0.233-0.654 μM H_2O_2/min/ml. Ascorbate peroxidases levels were in range 0.073-0.368 mmol UA/mg protein in control and 0.279-1.675 mmol UA/mg protein in temperature stressed breeds.

Pearson correlation matrix was determined to ascertain the relationship among the tested larval / cocoon (survival rate, larval weight, single cocoon weight, single shell weight, shell ratio, pupation rate) and ROS response (H_2O_2 levels, MDA levels, SOD, CAT and APOX) parameters. Significant correlation of survival rate with SOD activity ($p \leq 0.01$; $r = 0.47$*) and H_2O_2 ($p \leq 0.05$; $r = -0.26$*) were observed. This indicated the strong relation of SOD activity and endogenous titer of H_2O_2 with survival of silkworm under temperature stress.

x. Inference / recommendation:

- ✓ None of the 11 tested breeds survived at 40°C temperature indicated the suitable temperature range for better performance of putative thermotolerant breeds ranged between 25°C to 35°C. CSR2, the susceptible check could not survive even at temperature stress of 35°C.

- ✓ Among the tested ROS defense associated biochemical parameters, lipid peroxidation, catalase (CAT) and ascorbate peroxidase (APOX) showed non-significant relation with the survival of the breeds. Therefore these biochemical parameters may not be useful as selective markers for the assessment of temperature stress in mulberry silkworm.

- ✓ However, two other tested biochemical parameters SOD activity and endogenous H_2O_2 titer showed highly significant correlation ($P \leq 0.05$) with survival of the silkworm alongwith pronounced breed variations of obtained values, hence indicated prospective role of these ROS components with the survival of the silkworm under temperature stress alongwith the possibility to use as selective markers.

- ✓ BCon4 followed by BCon1 and BHR3 are found to be the better performing breeds than others in respect of higher survival and ROS defense associated SOD activity. Therefore, these three breeds will be the ideal choice for more focused study to elucidate temperature stress associated ROS stabilization process in silkworm *Bombyx mori*.

Future plan:

These better performing breeds (BCon4, BCon1 and BHR3) need to be studied further for their consistent performance under specific intermittent thermal stress regime beyond 25°C to 35°C and to elucidate the protective role of SOD as an additional marker of thermotolerance in silkworm.

xi. Applications made for patenting / commercialization, if any:

Nil

xii. References:

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xiii. Paper published: NIL

xiv. Summary:

1. Reared ten bivoltine silkworm breeds in two seasons Falguni and Shravani. Temperature stress treatment was given to all ten breeds and effect of temperature stress was studied. No seasonal variations were observed as experiments were conducted in controlled conditions.
2. Assessed ROS and MDA levels in ten bivoltine silkworm breeds to study the oxidative damage which indicated the high level of oxidative damage induced by ROS production in temperature stressed breeds compared to control breeds.
3. Studied antioxidant defense enzymes produced in response to ROS in silkworm breeds under temperature stress. Antioxidant enzyme levels were high in temperature stressed breeds compared to control.
4. Established Pearson's correlation among the tested parameters indicated significant correlation of survival rate with SOD.

xv. Budget utilized:

(Rs.in lakh)

Item	Allocation	Fund utilized	Remarks
1.Non- Recurring i) Equipment	--	--	--
2.Recurring ii) Consumables	0.95	0.95	--
iv) Contingency	0.75	0.36	--
Total	1.70	1.31	

xvi. Certification / Authentication by the Investigators & Head of the Institution

Signature of Principal Investigator (CSR&TI Component):

Pooja Makwana.
Scientist - B, Biotechnology Section,
CSR&TI, Berhampore

Signature of Co-Investigator:

Dr. Soumen Chattopadhyay,
Scientist - D, Biotechnology Section,
CSR&TI, Berhampore

Signature of the Director & Executive authority
CSR&TI, Berhampore

(डॉ. कणिका त्रिवेदी)
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Comments/ Decision of 47th RAC held on 9th January 2018:

#	Comments/ Decision	PIs response
1	How biochemical markers are useful in improvement of breed?	• Biochemical markers are utilized to serve as *rapid and stable additional screening tools* for identification of genotype / breed with better performance in respect of specific character(s). It has been successfully demonstrated in various plant (Nevo 1983) and animal (Ferrell et al.1980) systems including silkworm (Chatterjee et al.,1993). • Indeed, several enzyme protein based markers such as amylase, esterase, alkaline phosphatase, acid phosphatase and heat shock proteins were utilized as reliable tools for screening of silkworm resources for stable selection of better performing breeds (Etebari et al 2005; Ashok et al., 2013).
2	Whether it is appropriate to use the term biochemical markers instead of biochemical parameters which were studied?	• The study was a preliminary search of '*reliable biochemical parameter(s) of silkworm genetic resources*' to '*identify stable biochemical marker*(s)' for rapid screening of thermo-tolerant silkworm breed(s). The study was taken-up based on the guideline CO (CSB-31/2(BER-NP)/2013-14-RCS dt 27-05-2016). The study certainly showed that atleast one tested parameter (SOD) had a potential of biochemical markers to hasten the screening process of thermos-tolerance breeds of mulberry silkworm. However, to ascertain the marker properties of SOD, further in-depth study is essential and that was clearly mentioned the 'conclusion' part of the report.

Comments of the Director:

The project was completed successfully within the stipulated time period of October 2016 to September 2017 and concluded before 47th RAC on 9-1-2018. Significant leads were:

- Three better performing thermo-tolerant breeds (BCon4 > BCon1 > BHR3) were identified at thermal stress regime of 35°C with75-80% RH.
- The susceptible check, CSR2 breed could not survive even at temperature stress of 35°C with75-80% RH.
- The SOD activity showed significant correlation with survival and pupation rate of thermo-tolerant silkworm breed(s).

Utilizing above leads, further study is needed to ascertain the potential of SOD activity as a selective marker to elucidate temperature stress associated feature(s) in silkworm *Bombyx mori*.

6/4/18

Director

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