One Year Pilot Study

BPI(PS)010 : Identification of Biochemical markers for thermotolerance in silkworm *Bombyx mori* L.

DURATION

OCTOBER 2016 TO SEPTEMBER 2017

Principle Investigator: Pooja Makwana
Co-Investigator: Dr. Soumen Chattopadhyay

CENTRAL SERICULTURAL RESEARCH AND TRAINING INSTITITE
CENTRAL SILK BOARD
MINISTRY OF TEXTILES (GOVERNMENT OF INDIA)
BERHAMPORE-742101, WEST BENGAL

PART I : GENERAL INFORMATION

1	Name of the Institute / University / Organization submitting the Project proposal	:	Central Sericultural Research & Training Institute, Berhampore, West Bengal
2	Status of the Institute (s)	:	N.A.
3	Name (s) and designation (s) of the Executive Authority of the institute / University forwarding the application	:	Dr. Kanika Trivedy, Director
4	Project Title	:	Identification of biochemical markers for thermotolerance in silkworm *Bombyx mori* L.
5	Category of the Project	:	Applied
6	Specific Area	:	S – Silkworm; I – Improvement; T – Biotechnology
7	Duration	:	1 year (OCT.16 – SEPT.17)
8	Total Cost	:	1.70 Lakh
9	Is the Project single or multi-Institutional	:	Single Institutional
10	If the Project is multi-institutional, please furnish the following :Name, Designation and Address of the Project Coordinator.	:	N.A.

11. (a) Project Summary

Sericulture is practiced in several regions of India. Since India is a tropical country with prevailing hot climatic conditions in most of the regions, sericulture practice becomes difficult in summer seasons. Temperature in most of the traditional sericulture areas in the country goes beyond 35ºC which is far higher than the optimal temperature range 22 to 28ºC to develop silk gland and sustain the process of silk production. In general, silkworm rearing is conducted at 22 to 27ºC with relative humidity (RH) regimen of 65 % to 75% for late age silkworm; whereas young age (chawki) worms are reared at 27 to 28ºC with 80% to 85% of RH. Temperature beyond 30ºC affects survival, fecundity and cocoon traits of mulberry silkworms is reported by many researchers. Efforts have been made to screen the strain(s) of silkworm tolerant to high temperature on the basis of morpho-physiological characters, isoenzymes and/or heat shock proteins (Hsps) expression. Identified strains were used subsequently for hybrid development. Though the outcome of these directions (SK6 x SK7, BCon1 x BCon4, M12(W)x(SK6xSK7), M6DP(C)x (SK6xSK7), APM1xAPS8) are reasonably promising, the long pending demands of high temperature and high humidity tolerant silkworm hybrids are not fulfilled as the yield potential and disease resistance properties of these recently authorized / recommended hybrids are far from being satisfactory.

Thermal stress is related with the production of reactive oxygen species (ROS) which leads to oxidative damage to various cellular components. Hot and highly humid climates during May to September in eastern and north eastern (NE) states of India severely affected the quality / graded silk production. These hot and humid conditions may cause oxidative stress to silkworm and to combat with the stressful condition insects reportedly hasten their antioxidative activities (both enzymatic and non-enzymatic) as protective mechanisms to oxidative damage(s). The objective(s)/aim of the study are to identify oxidative damage and biochemical markers related to antioxidant mechanisms induced against ROSs as defense mechanism in silkworm strains.

(b) Aims and objective:

The study is proposed with the aim to identify stable biochemical markers associated with ROS in response to thermal stress in bivoltine breeds / hybrids. Identified stable biochemical marker(s) will be co-related with yield parameters. Breed/hybrids with such biochemical markers with high antioxidant properties and low oxidative damage will be identified and will be used in future breeding programs to develop temperature tolerant breeds. Also this study will generate essential information about extent of generation of reactive oxygen species in silkworm strains under high temperature stress. This information will be useful in selection of appropriate parents which have better cocoon traits with low oxidative damage due to less generation of ROSs.

PART II : PARTICULARS OF INVESTIGATORS

12	a) Name	**POOJA MAKWANA**
	Date of Birth	16-05-1989
	Sex	F
	Indicate whether Principal Investigator/ Co-investigator	**PI**
	Designation	Scientist – B
	Department	Biotechnology
	Institute/University: Address	CSR&TI, Berhampore, West Bengal
	b) Name	**DR SOUMEN CHATTOPADHYAY**
	Date of Birth	15-05-1961
	Sex	M
	Indicate whether Principal Investigator/ Co-investigator	**CI**
	Designation	Scientist-D
	Department	Biotechnology
	Institute/University: Address	CSR&TI, Berhampore, West Bengal
	d) Name	**DR KANIKA TRIVEDY**
	Date of Birth	
	Sex	F

	Indicate whether Principal Investigator/ Co-investigator	**Executive Authority**
	Designation	Director
	Institute/University: Address	CSR&TI, Berhampore, West Bengal
13	No. of Projects being handled by each investigator at present	
A	Pooja Makwana	PROJECTS: 1 (AS PI)
B	Dr Soumen Chattopadhyay	PROJECTS: 2 (AS PI:1 AND CI:1)

14. Proposed Research Fellows [justification with work sharing is a must]: *NIL*

PART III: TECHNICAL DETAILS OF PROJECT

15. Introduction

The silkworm Bombyx mori L. is an economically important insect that produces silk and contributes to Indian economy. From the total silk production in India mulberry silk contributes 93%. Sericulture is practiced majorly in five states of India- Karnataka, West Bengal, Andhra Pradesh, Tamil Nadu and Jammu & Kashmir. India being the tropical country temperature reaches above 40ºC in summers which adversely affects sericulture in traditional areas.

15.1 Definition of the Problem:

(a) Origin of the project:

India has contributed 18% (approx) to the global silk production which has been achieved mainly with the use of F1 hybrids of multivoltine (female) x bivoltine (male) or multivoltine x multivoltine. These cross breeds produce non-gradable silk despite the fact that they are tolerant to high temperature to some extent. Total quality raw silk production is very limited (~3870kg of bivoltine silk) which is about 18% of the total production of mulberry silk (Anonymous 2015). Scantiness of suitable temperature stress tolerant bivoltine strains is considered as one of the bottlenecks for international grade silk production (Datta and Nanavaty 2005).

In the eastern and north-eastern (NE) states of India, mulberry sericulture is seasonal and practiced mainly in five seasons (February, April, June, August OR September and October-November) under irrigated and three seasons (March–April, June–July and October-November) under rainfed conditions. The optimum conditions of *B. mori* rearing are 22-28^{o}C and 75-85% relative humidity (Krishnaswami 1973).The summer and monsoon rearings are considered unfavorable / adverse due to high temperature (maximum temp. range: 32ºC to 42ºC) and high humidity (maximum RH range: 84% to 91%). Being a poikilothermic insect, survival of the bivoltine and improved cross breeds (Multi x Bi) is badly affected under this hot and humid

climatic conditions. In fact, it is established fact that higher body weight, higher fecundity and longer silk fibre of bivoltine silkworm had negative correlation with survival during hot and humid conditions (Nagraju 2002). So far , efforts made by CSRTI, Pampore and Berhampore to develop silkworm breeds / hybrids suitable to sub-tropical conditions of Northern India resulted in development of new bivoltine breeds namely, YS3 x SF19, SH6 x KA, Pam 101 x SF19, SH6 x NB4D2 during 1980's and CP1B x JP1B, CP1B x J-Plain, CS6 x PAM 101, Dun 6 x Dun21, RSJ3 x RSJ1, RSJ14 x RSJ11 during 1990'sand also, Dun6 x Dun 22 and Dun 16 x Dun 17 in recent times and further, CS6 x PAM 101, Dun 6 x Dun21 and RSJ3 x RSJ1 which were authorized by provincial race authorization committee (Lakshmanan and Suresh 2012). Also several bivoltine hybrids (NB4D2 x NB18; NB18 x P5, P5 x KPGB) are introduced in these zones since 1990s, but all yielded with reasonable success. As a result, rearing of F-1 derived from the crosses of multivoltine x multivoltine and multivoltine x bivoltine is predominant in eastern and some part of NE states India, especially during hot and high humid conditions. These types of hybrids produce low quality of cocoon and silk yarn than bivoltine. But to sustain sericulture over the year and increase the profitability specially in the eastern and parts of NE states, *it is mandatory to introduce suitable bivoltine breeds/ hybrids tolerant to high temperature and high humidity*.

Temperature is an important abiotic parameter which determines the physiologic activity and growth of an organism (Benchamin and Jolly 1986). Variety of physiological pathways may be triggered in insects due to high temperature, which are often associated with enhanced generation of reactive oxygen species (ROS) leading to oxidative damage (Cossins and Bowle, 1987). ROS that are produced naturally during oxidative metabolism include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Livingstone, 2001). During normal metabolic process, a balance exists between the generation of ROS and the antioxidant processes. Physiological use of ROS by cells is now being demonstrated in areas such as intracellular signalling and redox regulation (Imai and Nakagawa 2003).However, during periods of thermal stress (and other abiotic stresses), surplus ROS can trigger severe damage to essential biological molecules like lipids, proteins and nucleic acids (Martindale and Holbrook 2002). ROS induces damage in:

a) Protein, ranges from specific amino acid modifications and peptide breakage to loss of enzyme activity (Stadtman and Levine, 2003).
b) DNA, ranges of mutations, base deletions, degradation, and single-strand scission (Imlay, 2003),
c) Lipid, leads to peroxidation that disrupts cell membrane fluidity and can lead to apoptosis (Green and Reed, 1998).

To prevent thermal stress as well as damage by ROS, insects possess a complex defence system of non-enzymatic scavengers and a range of antioxidant enzymes predominated by the catalase (CAT), different peroxidases (POXs), and superoxide dismutase (SOD). During the enzymatic reactions, SOD removes O_2^-through the process of dismutation to O_2 and H_2O_2 (Fridovich 1995); while CAT and POXs break H_2O_2 into H_2O and O_2.

Concerted efforts have been made during the past two decades to develop high temperature tolerant silkworm strains using conventional breeding strategies. This process has been reasonably successful (cocoon yield range: 42 to 50 kg/100dfls; silk filament length: 700 to 800 meter) with the recent commercialisation of SK6 x SK7 and authorisation of BCon1 x BCon4 in eastern and NE states of India. But most of the other breeds / hybrids performed well only under controlled laboratory conditions with limited success at farmers' fields (Kumar *et al.,* 2011). The major reason for such failure was suggested that the selection of parents was not stable as they solely based on phenotypic characters.

Molecular analysis of thermal stress has been extensively studied in *Drosophila melanogaster* (Sorensen *et al*., 2007), and research has recently included other important insect species (King and MacRae, 2014). However, systemic molecular analysis for stable selection of thermos-tolerant silkworm strains for breeding utilization is very limited (Zhao *et al*., 2010) and attempt to integrate thermal stress tolerance in silkworm with ROS defense is rare.

In this backdrop, we proposed to *analyze the generation of ROS, its key stabilizing enzymes and correlation with the survival of some potential silkworm strains under the hot and humid conditions simulated to the tune of adverse rearing seasons. Subsequently, thermal stress tolerance efficiency of selected strains (those strains shows high correlation of survival with ROS defense) will be assessed by studying the biochemical markers associated with ROS stabilization.*

(b) Expected outcome:

The process would help for identification of stable biochemical markers associated with ROs defense mechanism in response to thermal stress through correlation with yield parameters and survival in bivoltine breeds / hybrids and also future utilization of these biochemical markers for selection of parental strain(s) for breeding programs in hot and highly humid condition.

15.2 Rationale of the project:

Changes in temperature are known to cause a variety of physiological stress responses in insects. The thermal stress depletes antioxidant state of organism and causes oxidative damages (Sahin *et al*., 2001). ROS such as hydroxyl radical ($OH^{\cdot}$), superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), differential lipid radicals, peroxides as well as nitrous oxides are generated endogenously by all living organism during normal oxidative metabolism (Boardman

and Terblanche 2015). Redox homeostasis is also maintained by organisms in normal conditions. Thermal stress responses are usually associated with the increased generation of ROS, resulting in oxidative damage. During thermal stress, when ROSs level rises they tend to react with different molecules in surrounding to stabilize themselves thereby inducing oxidative damage in cells. Insects have developed defense mechanisms comprising antioxidant enzymes and low molecular weight components that plays major role in elimination of ROSs (Kono & Fridovich 1982; Mocket *et al*., 2002). These antioxidant enzymes reported from different insects include- Superoxide dismutases (SOD), catalases (CAT), glutathione peroxidase (GPOX), glutathione reductases (GR), Glutathione S-transferases (GST), Thioredoxin peroxidases and thioredoxin reductases (Blagojevic 2007).In insects including silkworms, genes encoding the antioxidant enzymes are activated under different stress conditions (Krishnan and Kodrik 2006).

Biochemical marker associated with ROS antioxidant enzymes based screening of bivoltine breeds / hybrids under thermal stress is very limited in mulberry silkworm and hitherto effort is lacking to utilize / integrate these differential antioxidative biomarkers with the survival of silkworm strains during thermal stress condition. In this project, an effort would be made to identify stable biomarker among the silkworm strains in association with higher survival, ROS-antioxidant activity and activation of detoxification mechanisms which can be further used for breeding programs.

15.3 Relevance to the Current Issues and Expected outcome:

Indian sericulture is mainly rainfed and rearing is being conducted under hot and highly humid conditions during adverse climatic condition in most of the areas of Eastern and NE areas. Temperature is an important abiotic parameter which determines the physiologic activity and growth of an organism. Changes in environmental factors like temperature and humidity causes changes in silkworm characters which results in poor survival and reduced cocoon weight, shell weight and cocoon shell ratio (Thapa and Ghimire, 2005).

High temperature conditions induce oxidative damage leading to oxidative stress, production of heat shock factors and also affect oxygen delivery in the organism. Though previous some efforts have been done to develop thermos-tolerant breeds / hybrids on the basis of phenotypic characters and expression of heat shock proteins (Howrelia *et al*., 2011). These thermos-tolerant breeds did not perform so well in the field level because of selection of such breeds was done only on basis of phenotypic traits. Since generation of ROS and its stabilization using enzymatic and non-enzymatic pathways are intrinsically associated with thermal and other stress responses of different insects (Ju *et al*., 2013) it seems the process is universal and applicable to mulberry silkworm also. *As scarce information available on biochemical markers*

associated with oxidative damage caused due to ROSs and survival of silkworm breeds of India, the proposal aims to identify silkworm strains with high survival and low oxidative damage ability under hot and humid condition to utilize them in future breeding programs. Successful implementation of the project may generate following outcome:

1. Biocehmical markers associated with higher survival under high temperature conditions will be identified and utilized for screening of germplasm.
2. Silkworm accessions with high survival under high temperature conditions will be identified for future use in breeding programs to synthesize bivoltine silkworms with higher survival rate.

15.4 Objectives:

- ***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.***

16. Review of status of Research and Development on the subject:

Silk is the most preferred fabric over the other fabrics with its traditional importance and properties like water absorbency, heat resistance, dyeing efficiency and luster thereby making sericulture practice important in India. Due to domestication, mulberry silkworm (*Bombyx mori* L.), became very sensitive to environmental fluctuations and unable to survive in extreme fluctuating temperature and humidity conditions. It has been reported that factors like ambient temperature, rearing season, quality of mulberry leaves and genetic constitution of silkworm strains greatly affects the biological as well as cocoon characters. The seasonal differences affect silkworms at genotype level which is observable from phenotypic characters like variation in cocoon weight, shell weight and shell ratio. Multivoltine silkworms being more hardy and resistant, survive extreme climatic factors; while bivoltine silkworms are more susceptible to climatic changes. Besides, poor leaf quality and improper management of silkworm rearing during adverse climatic condition also affect growth, cocoon yield and quality (Suresh *et al.,* 2001).

The young (=chawki) silkworms require relatively high temperature and high humidity for proper growth and survival; whereas late age larvae are susceptible to high temperature and humidity (Pillai and Krishnaswamy 1987). Optimum temperature of 22ºC to 28ºC with 70±5% RH is recommended for normal growth of mulberry silkworm larvae and good quality cocoon yield (Krishnaswami *et al*., 1973). It is reported in several studies that silkworm growth and survival is

affected at higher temperature and humidity conditions mainly in fourth and fifth instars (Pillai and Krishnaswamy 1980).

16.1 National Status:

Effect of temperature on silkworm has been studied in India by many workers for the benefit of farmers. It was reported that under high temperature of 35±1ºC, pupation percentage, cocoon weight and shell weight had decreased in compare to control (Basavaraja *et al.*, 2005). It has also been reported that high humidity affects rearing and increases disease prevalence (Vijaya *et al.,* 2001). Differential levels of thermo-tolerance of multivoltine and bivoltine silkworms have been reported (Joy and Gopinathan 1995; Nagaraju 2002).

Works on Biochemical markers in silkworm under stress

Biochemical parameters like amylase activity, invertase, alkaline phosphatase, trehalose, protease pH 7 and protease pH 10 have been studied and correlated with yield parameters which generated information on importance of digestive amylase activity for survival of silkworm *Bombyx mori* (Chatterjee *et al* 1993). Esterase, G6PD and MDH were used as biochemical marker towards stress tolerance wherein 10 bands were discernible in gut esterase in 24h starved Nistari larvae (Anonymous 1998). In another project nine multivoltine and three bivoltine including one sex-limited syngenic lines were developed from CB5 & M6DPC (multivoltine) and JPN9 & D6 (bivoltine). Esterase was found as marker for shell weight and survival which was utilized as marker to develop congenic line MCon1, MCon4, BCon1 and BCon4 (Anonymous 2004). Esterase isozyme based selection of potential bivoltine parents were crossed with multivoltine along with the back cross breeding and Est-3 marker assisted breeding followed by high temperature treatment to develop temperature tolerant breeds resulted in two promising bivoltine lines SK4C and D6(P)N (Anonymous 2007). Biochemical characterization of silkworm resources for stress tolerance using esterases and heat shock proteins has been done extensively which generated information that Pure Mysore, Nistari, A-25, A4e, MU-11 and LMP can be used as index for thermotolerance (Anonymous 2007).

Works on silkworm thermotolerance and heat shock protein

Thermo-tolerance is the capacity of organism to tolerate high temperature and ability to survive in varied temperature conditions. Reports to integrate heat shock proteins (Hsps), rather than antioxidative enzymes, with thermal stress tolerance of silkworm are more frequent in India. Expression of heat shock proteins (Hsps) of 93kDa, 89kDa and 70kDa have been reported in multivoltine (like *C Nichi*) and bivoltine (like NB_4D_2) silkworm's fat-body, hemolymph and cuticle in time dependent manner with early appearance in multivoltine (Joy and Gopinathan 1995). In

multivoltine silkworm breed Nistari, 70kDa, 64kDa and 39kDa Hsps were reported on exposing to various temperature regimes with maximum tolerance found at 32ºC (Moorthy *et al.*, 2007). It was also reported that in Nistari strain varied high temperature treatment induced production of Hsp72 and Hsp90 as protective mechanism against stress (Sinha and Sanyal 2013). In bivoltine (SK4C and CSR2) and polyvoltine (Nistari) silkworm races gene expression analysis of following heat shock proteins Hsp19.9, Hsp20.1, Hsp20.4, Hsp20.8, Hsp21.4, Hsp23.7, Hsp40, Hsp70, Hsp90 after heat shock treatment resulted Nistari with highest pupation percentage and high expression of HSPs while among bivoltines SK4C had higher Hsps gene expression and pupation percentage in comparison to CSR2 (Chandrakanth *et al.,* 2015).

Limited reports on silkworm thermotolerance and molecular markers

Reports are also available on use of molecular markers for selection of thermo-tolerant breeds and ISSRs and SSRs are most frequently used DNA markers. In an ISSR markers based selection Nistari and CSR2 silkworm strains has been selected as most of thermo-tolerant polyvoltine breeds (Srivastava et al 2007). While in other studies, along with Nistari and CSR2, Cambodge, SK4C and BHR3 were also selected as thermos-tolerance using SSR markers than susceptible breeds (Chandrakanth *et al.,* 2015).

Thermal stress effect on cocoon and silk attributes of mulberry silkworm

Temperature also influences post-cocoon parameters such as cocoon quality, shape and size. Variations in cocoon shape and size affects filament length as well as quality of reeled fibre (Nakada 1993). A study of impact of seasonal variation on cocoon traits carried out in bivoltine breeds reported decreased cocoon traits such as shell weight, shell ratio, filament length, filament size and raw silk percentage in summer season when compared to winter (Kamili and Sharma 2014). Water content of cocoon is recommended to be below 20% for better reelability and this showed that temperature and humidity directly affects the cocoon yield and quality (Akahane and Soubachi 1994).

Importance of ROS stabilizing enzymes during thermal stress of different silkworms

However, inadequate information is available on activation of antioxidants in silkworm in response to temperature stress. A study reported changes in catalase activity in response to high temperature and humidity in fat-body, midgut and hemolymph of CSR4, JROP, NB4D2, KA and CSR2 breeds with maximum catalase acitivity in fat-body over the midgut and hemolymph (Nabizadeh and Jagadeesh 2010). In Tasar silkworm (*Antheraea mylitta*) testes of male pupae antioxidants were studied under thermal stress conditions. Oxygen consumption rate increased with increased levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), ascorbic acid (ASA) and low molecular thiols (L-SH) in testes

under high temperature (40±1°C and above) stress. Also higher levels of TBARs (Thiobarbituric acid reactive substances, an index of lipid peroxidation) were observed indicating oxidative damage in testes of pupae (Jena *et al*., 2013). On above mentioned aspects such extensive investigation has not been done in silkworm. However plentiful literature is available on heat stress induced antioxidant reactions in various insects.

16.2 International status:

Temperature is one of the most important environmental factors that induce physiological changes in insects. All bivoltine silkworms are of temperate origin and introduced in the tropical environment owing to their better silk quality. In contrary, silk quality of multivoltine silkworms is inferior but they are relatively tolerant to thermal stress than bivoltines. Cellular mechanism activated in thermotolerance includes heat shock proteins, some chaperones associated to HSPs and also the formation of free radicals (ROSs) (Bhaumik *et al.,* 1995).

Thermal stress and heat shock proteins

During thermal and other stresses, insects regulate the synthesis of heat shock proteins (Hsps; =stress proteins = molecular chaperones) in the cells. The Hsps are divided into four major families on the basis of their respective molecular masses. These are small heat shock proteins (sHsps), Hsp60, Hsp70, and Hsp90. The sHsp group is considered as first line of cell defence and prevents denaturation of substrate proteins under thermal stress independent of ATP. The remaining three HSPs families interact with proteins and regulate protein synthesis, cell signaling, transcription, and metabolism. Upon exposure of insects to thermal stress, the synthesis of most proteins declines, but HSPs increases (King and MacRae 2014).

When *B.mori* is exposed to high temperature induction of gene expression of heat shock proteins namely BmHsp19.9, BmHsp21.4, BmHsp23.7, BmHsp25.4, BmHsp27.4 and Hsp1 have been studied (Sakano *et al*., 2006; Sheng *et al*., 2010). Similarly a small Hsp BmHsp27.4 has been identified to be expressed in reponse to high temperature conditions in silkworms (Wang *et al*., 2014).There is also evidence that under high temperature stress silk synthesis related proteins expression had been found reduced whereas stress related protein expression increased (Li *et al.,* 2012).

Thermal stress and ROS stabilisation

The thermal stress signal transduction pathways and defence mechanisms are intimately associated with ROS stabilisation (Pnueli *et al.,* 2003).The mitochondria are thought to consume over 90% of the cellular oxygen in unstressed cells and are considered the major sites of aerobic cellular ROS production (Han *et al.,* 2001). ROS produced naturally during oxidative metabolism.

The major ROSs are superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) and others (Livingstone, 2001). In normal situations, a balance exists between the generation of ROS and the antioxidant processes. However, temperature beyond specific tolerance levels may damage cells, increased ROS production and activate antioxidant enzymes (Lopez-Martinez *et al*., 2008).

To maintain homeostasis and prevent oxidative stress as well as damage by ROS, insects possess a complex defence system of non-enzymatic scavengers and a range of antioxidant enzymes. The antioxidant enzymes, including CAT, GSTs, POX and SOD, play an important role in protecting cells and maintaining homeostasis by removing oxidative stress (Neven 2000).

It is known that ROS at low levels are beneficial for cell signaling and activation of defense genes in insects (Wang *et al*., 2001). It has been also reported that ROS generation is also involved along with the production of Hsps in temperature stress (Ando *et al.,* 1994). An antioxidant, thioredoxin peroxidase has been reported to be activated against temperature stimuli and provides protection from oxidative damage in silkworm (Lee *et al.,* 2005).

Thermal stress and DNA markers

SSR markers are tandem repeats of 1-6bp of DNA sequences have also been used to screen thermotolerant breeds in silkworm. One such study in china reports that Dong34 was identified as thermos-tolerant breed and Ou17 as thermo-sensitive breed with the help of SSR markers associated directly with thermotolerance. 5 SSR markers were found to be directly linked with thermotolerance in this study (Zhao *et al*., 2010).

Thermal stress in other insects

It has been reported that in Antarctic midge larvae on rise in temperature SOD and CAT gene expression elevated (Lopez-Martinez *et al.,* 2008). Similarly *Bacterocera dosrsalis* when subjected to thermal stress, CAT, GSTs and SOD levels significantly enhanced while POX and total antioxidant capacity showed non-significant increase. Besides, the rise in lipid peroxidation levels indicated the oxidative damage induced by thermal stress in *Bacterocera dosrsalis* (Jia *et al.,* 2011). There is also evidence high temperature (33-39ºC) induced stress caused significant increase of ROS generation in *Chilo suppressalis* larvae. The antioxidant enzyme activity (CAT and SOD) significantly increased after the exposure to high temperature (Cui *et al.,* 2011). Temperature stress on diapause larvae of *Chilo suppressalis* showed that physiological and biochemical levels like sugar, water, lipids, and antioxidant enzymes like SOD, CAT and POX were affected (Qiang *et al.,* 2012).

In invasive lace bug (*Corythucha ciliata*) production of ROS under oxidative stress in controlled laboratory and field conditions showed same response of antioxidant enzymes activities (Ju *et al.,* 2014). In ladybeetle *Propylaea japonica* cannot survive temperature above 43ºC, and antioxidant enzymes CAT, GST and total antioxidant capacity increased significantly during thermal stress in adults ladybeetle; while the SOD and POD activities decreased at the temperature range of 31-37ºC. However above 37ºC, Malondialdehyde (MDA) concentration (an indicator of oxidative stress), SOD and POX increased significantly. This suggested that there is huge ROS production and activation of antioxidant defense mechanisms in ladybug beetle (Zhang *et al.,* 2015).

The insect cell lines like Sf-9 (*Spodoptera frugiperda*) and Tn-5B1-4 (*Trichoplusia ni*) were reported to have antioxidant enzymes. Sf-9 contained Mn-SOD and Cu-Zn SOD along with APOX for removing superoxide radicles and hydrogen peroxide respectively. Tn-5B1-4 cell line contained catalases for hydrogen peroxide removal. Both cell lines also contained glutathione S-transferase peroxidases activity against hydroperoxides (Wang *et al.,* 2001). Catalases in *Beauveria bassiana* has been studied under thermal tolerance and role of different catalases in oxidative stress. Total catalase activity in *B. bassiana* decreased 89% and 56% in *catB* and *catP* (Wang *et al.,* 2013).

Aforementioned review (national and international) supports that thermal stress increases the production of ROS which leads to oxidative damage if antioxidant enzymes are not activated to eliminate the accumulated ROS. More high temperature might imbalance the ROS levels and can lead to death of an organism.

16.3 **Importance of the proposed project in the context of current status**:

Temperature and humidity are the major limiting abiotic factors which affect silkworm growth and development in non-favorable crop seasons. The proposed study is the first attempt to identify potential silkworm strains on basis of activation of biochemical markers associated with ROS stabilisation following hot and high humidity condition prevails during the adverse rearing seasons of Eastern and NE states of India. The study supplements information to hasten stable selection of strains for development of thermotolerant breeds / hybrids, which is long pending demand of sericulture. The proposal specifically fulfill following concerns of sericulture:

- High temp and high humidity tolerant silkworm breeds / hybrids suitable specially for eastern & NE India.
- The oxidative damage caused in silk gland and other tissues due to temperature and humidity stress will be assessed and the oxidative damage linked induction of

biochemical marker based identification of resources will ensure improved silkworm breeds in this direction.

- Selection will be done based on the correlation of ROS stabilization ability of tested breeds with survival and cocoon yields.

16.4 **Anticipated Products, processes/Technology, Packages/ Information or other outcome from the project and their expected utility**:

Generation of valuable information: Identification of potential silkworm strains on basis of activation of biochemical markers associated with antioxidant defense mechanism against temperature and humidity stress.

Expected outcome from the project and their expected utility: Identified silkworm breed/ hybrids with high antioxidant potential against temperature and humidity stress will be used in future breeding programs for development of tolerant breeds.

16.5 **Expertise available with proposed investigation group/ institution on the subject of the project*:**

Name of the Scientists	Design-ation	Experience
Pooja Makwana	Scientist-B	As a principal Investigator of the proposal, she has 3 years of research experience as research fellow of SBRL, CSB, Bangalore in various aspects of mulberry molecular biology including ---- AOS defense enzymes, gene expression at the level of mRNA and protein. She has prepared her thesis on "Cytotoxicity and detoxification response in the silkworm *Bombyx mori* induced by uzifly *Exorista bombycis*", for submission.
Dr S Chattopadhyay	Scientist-D	As a co-investigator of the proposal, he has 21 years of research experience in various field of mulberry crop improvement. He has expertise (relevant to the proposal) in the areas of- a) ROS and antioxidative enzymes assessment in mulberry and silkworm and published couple of papers in these lines, b) DNA marker analysis of mulberry involving RAPD, ISSR and SSRs , and c) acquainted with basic molecular biological techniques related to molecular marker analysis. He has >45 publications in different International and National level peer review journals. He had concluded three DBT supported projects on: Identification of DNA markers associated with powdery mildew disease, SCAR-markers associated with powdery mildew resistance and development of linkage map of mulberry in collaboration with CCMB since ~10years.

17. Work Plan

17.1 Methodology:

I) *Acitvity 1: Temperature and humidity exposure*

a). Bivoltine strains selected for the study are - SK6, SK7, SK4C, D6(P)N, Gen3, BHR3, BHR2, B.CON1, B.CON4, ATR29, CSR2.

b). **Temperature and humidity stress will be applied to silkworm strains as follows**:

- Silkworm strains (from first activity) will be exposed to high temperature using environmental chamber at 35ºC and 40ºC, 85±5% RH for 6h from 5th instar first day. After 6h exposure larvae will be again reared in optimum temperature and humidity conditions i.e. 25±2ºC and 70±5% RH. Two batches will be maintained for each strain along with one control batch.
- Sample will be collected on 5th day of 5th instar larvae. Hemolymph, midgut, fat-body and silk-gland will be collected and stored separately at -80ºC for protein extraction.

II) Activity 2: Assessment of generation of ROS and oxidative damage caused

Protein will be extracted from tissues (fatbody, silkgland and midgut) and quantified according to protocol of Lowry (1950). ROS generation will be assessed by method of Able *et al* (1998) and Velikova *et al* (2000). Oxidative damage due to ROS generation will be assessed by lipid peroxidation method.

III) Activity 3: Assessment of defense mechanism during the thermal stress induced oxidative damage in silkworm

Antioxidant enzymes like catalases (Sinha 1972), peroxidases (Mathews *et al.,* 1997), Superoxide dismutases (Kono 1978), glutathione-S-transferase (Balmert *et al* 2014) will be assessed in protein extracted from different tissues.

IV) Activity 4: Statistical analysis

All the data will be represented in mean ± SD. Significant difference between variants to be collected at different time points after temperature stress (like catalase activity, SOD, peroxidases, hydrogen peroxidase (H_2O_2 level) between the control and treated sample will be subjected to Analysis of Variance (ANOVA) using Statistica version 9.0 software (Statsoft Inc. Tuscon, USA) .

Biochemical marker levels estimated in selected strains will be correlated with their respective cocoon yields to establish the relationship between the antioxidant potential of strains in response to the oxidative damage caused by ROS and production of silk.

17.2 **Organization of Work Elements**

Name of Scientists	Designation	Time	Organization of work elements
1. Pooja Makwana	PI	80%	• Sample collection and protein extraction. • Biochemical assays in the silkworm protein. • Analysis of data using 'Statistica' & 'SPSS' base 20 softwares and compilation as well as report writing.
2. Dr S	CI	20%	• Analysis of data using 'Statistica' & 'SPSS' base 20 softwares

Chattopadhyay			

17.3 **Proprietary / Patented items, if any, expected to be used for this Project**: Nil

17.4 **Suggested plan of action for utilization of the expected outcome from the project:**

- On basis of correlation of induction of biochemical marker and yield parameters stable biochemical marker associated with ROS stabilisation under thermal stress will be identified.
- Identified potential strains with high antioxidant defense mechanism against temperature stress will be used for developing new breeds through marker- assisted breeding programs using biochemical markers.

17.5 **Time Schedule of activities giving milestones**:

#	Organization of work/ Milestone /Activity	Expected Date of	
		Starting	**Completion**
1.	Screening of silkworm strains on the basis of survival and phenotypic characters after high temperature and humidity stress and sample collection.	Beginning of 1st quarter	End of 1st quarter
2.	Protein extraction from midgut collected from silkworm and quantification of midgut and hemolymph protein	Beginning of 2nd quarter	End of 3rd quarter
3.	Protein extraction from fatbody and silkgland of silkworm and biochemical assays using extracted protein (antioxidant enzyme assays and ROS estimation)	Beginning of 2nd quarter	End of 3rd quarter
4.	Analysis of biochemical assessment result and selection of SW strains for further gene expression studies	Beginning of 3rd quarter	End of 4th quarter

17.6 **Project Implementing Agency /Agencies**:

Name of the agency	Address of the agency	Proposed Research Aspects	Proposed Amount	Cost Sharing %
Central Silk Board	Central Silk Board, Ministry of Textiles, Govt. of India, Bangalore		1.70	100%

PART IV: REFERENCES

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PART-V: BUDGET PARTICULARS

18. BUDGET (in Lakh):

A) Non-Recurring (e.g. equipments, accessories, etc.): Nil

B) Recurring

B.1. Manpower: Nil

B.2. Consumables:

Sl. No.	**Item**	**1st Year**	**Total (in lakh)**
1.	Fine chemicals / chemicals / reagents/ others	0.95	0.95
	Sub Total B2:	**0.95**	**0.95**

Other Items:

Sl. No.	**Item**	**1st Year**	**Total (in lakh)**
B3	Travel	-	-
B4	Contingency	0.75	0.75
	Total	**0.75**	**0.75**
Sub-total (B1+B2+B3+B4 etc.)		1.70	1.70
Grand total (A+ B1+B2+B3+B4)		**1.70**	**1.70**

Justification:

Consumables:

The proposed work is experiment-intensive involving protein extraction and biochemical assays, therefore needs relatively high consumable support.

Contingency

The proposed study requires intensive equipment support. The contingency amount may be utilized for repairing of equipment and other miscellaneous expenditure.

PART-VI: EXISTING FACILITIES

19. Available equipment and accessories to be utilized for the project:

SI. No.	**Name of the Equipment/ Accessory**	**Make**	**Funding Agency**	**Year of Procurement**
1	Palm Cycler	Genetix	CSB	2013
2	NanoDrop	Thermo	CSB	2016
3	Ultra-freezer (-80°C)	Thermo	CSIR	2006
4	Medium speed cold centrifuge	Remi	CSB	2002
5.	Spinwin	Tarson	CSB	2015
5	Quick freezer	Remi	DBT	2011
6	Horizontal gel electrophoresis set with power pack	Atto Corporation	DBT	2007
7	Gel documentation system	Vilber Lourmat(France)	CSB	2012
8	Gel electrophoresis unit	Tarson	DBT	2011
9	Rearing rooms with all rearing facilities and Envinronmental chamber for experiment purpose.			

PART-VI: DECLARATION / CERTIFICATION

It is certified that

a. The research work proposed in the project does not in any way duplicate the work already done or being carried out elsewhere on the subject.

b. The same project has not been submitted to any other agencies for financial support.

c. The emoluments for the manpower proposed are those admissible to persons of corresponding status employed in the institute/ university or as per the Ministry of Science & technology guidelines (Annexure-III).

d. Necessary provision for the project will be made in the Institute in anticipation of the sanction of the scheme.

e. If the project involves the utilization of genetically engineered organism , it is agreed that we will ensure that an application will be submitted through our institutional bio-safety committee and we will declare that while conducting experiments, the bio-safety guidelines of the Department of Biotechnology would be followed in toto.

f. If the project involves field trials / experiments / exchange of specimens etc we will ensure that ethical clearances would be taken from the concerned ethical committees of Biotechnology before implementing the project.

g. It is agreed by us that any research outcome or intellectual property right(s)on the interven (s) arising out of the project shall be taken in accordance with the instructions issued with the approval of the Ministry of Finance . Department of Expenditure as contained in annexure-V

h.. We agree to accept the terms and conditions as enclosed in Annexure-IV. The same is signed and enclosed.

i. The institute agrees that the equipment, the basic facilities and such other administrative facilities as per terms and conditions of the grant will be extended investigators through out the duration of the project .

j. The institute assumes to undertake the financial and other management responsibilities of the project.

Signature of Executive Authority of
Institute with Seal & date

Signature of Project Co-ordinator
[Applicable for inter-institutional
Projects only]
Date:

Signature of Principal Investigator & date	**Signature of Co-Investigator-I & date**	**Signature of Co-Investigator-II & date**