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# A Study on Assessments of the Variants of Mulberry Silkworm *(Bombyx Mori L.)* Breeds 'Nistari' using Biochemical & Molecular Markers

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## Abstract

Nistari, is an indigenous poly voltine breed of mulberry silkworm (*B.mori L.*). Though Nistari is not a highly productive, but gene(s) imparting to thermo-tolerance and resistant many pathogens are conserved in this poly voltine silkworm breed. Among poly voltine silkworm breeds, Nistari is the oldest breed that is being reared in India. Sericulture in eastern India is highly dependent on 'Nistari' as the breed can survive extreme climatic variations and disease-resistant properties. In fact, in the high temperature and humidity conditions of West Bengal, the Nistari breed is used as one of the components of hybrids (Multi x Multi and Multi x Bi) commercially reared during five seasons February, April, June, September, and November. On the basis of acclimatization in different regions of eastern India, different variants (=strains) of Nistari are available in West Bengal. Depending upon the locality/ area of availability, such variants are known as Nistari-Batepur, Nistari-Chalsa, Nistari-Shivnivas, and Nistari-Debra. Moreover, some other strains like Nistari-Plain, Nistari-Marked, Nistari-White and Nistari-Sex-limited variants are quite frequent in different areas of West Bengal. These variant names are on the basis of larval and cocoon characteristics. All these mentioned Nistari strains exhibit phenotypic variations like cocoon weight, shell weight and markings on larval surface. With this background following study is proposed to study variation among strains of Nistari at biochemical (using enzymatic and non-enzymatic cellular components, molecular level (using microsatellite markers)) and repairing of DNA damage ability and along with the differences in yield traits of Nistari variants available in Institute's repository.

**Keywords:** Sericulture; Nistari; DNA; Molecular markers; Coccon

**Abbreviations:** ROS: Reactive Oxygen Species; CAT: Catalase Activity; SOD: Superoxide Dismutase Specific Activity; TSS: Total Soluble Sugar Content; LPO: Lipid Peroxidation as a Function of Malonaldehyde Oxidation; APOX: Ascorbate Peroxidase Activity; $H_2O_2$: Hydrogen Peroxide Titre

## Introduction

Sericulture of eastern region is highly dependent on Nistari as it is the hardy polyvoltine breed which can survive extreme climatic conditions of this region. Nistari is used for producing cocoon on commercial scale in West Bengal and other states of eastern India. On the basis of acclimatization in different region of eastern India it has been called by area names such as Nistari-Batepur, Nistari-Chalsa, Nistari-Shivnivas and Nistari-Debra. Moreover there are other strains too named as Nistari-Plain, Nistari-Marked, Nistari-White and Nistari-Sex-limited. 1. All strains of Nistari reportedly conserved genes imparting thermo tolerance and disease resistant though the breed is not productive 2. Some strains of Nistari show better fecundity, larval and cocoon characters 3.Sericulture in Eastern India demands region and season specific breeds due to its fluctuating climatic conditions 4. Nistari plain reportedly having better survival and cocoon yield; while Nistari marked has high shell weight and shell ratio.

Chalsa and Debra have better performance over plain and marked strains of Nistari. Nistari Debra+P which is derived from the crossing of Nistari Plain and marked, has enhanced fecundity and yield parameters 3. Studies suggest that phenotypic variability among Nistari strains is due to environmental variability rather than genetic variability. But inadequate information is available on the reason of variation among Nistari strains found in Eastern India. To study the genetic variability among various available

strains of Nistari molecular markers can be used. Reactive oxygen species (ROS) are produced by cellular metabolism and by exogenous agents in the cells. ROS include a number of chemical reactive molecules derived from oxygen, such as super-oxide anions, singlet oxygen, hydrogen-peroxide and hydroxyl radicals. Over production of ROS can cause oxidative damage of various bio-molecules, especially lipids and proteins. The lipid per oxidation induced cellular leakage due to free radicals is one of the major reasons of cell death .The DNA is also the major target of direct and/or indirect action of generated ROSs. The harmful effect of the free radicals can however, be blocked by antioxidant substances. Like many insects, during biotic and a biotic challenges imbalance between oxidant generation and antioxidant defese occurs in silkworm. Reactive oxygen species (ROS) and associated anti-oxidative enzyme system plays an important role in biotic / abiotic stress resistance in various insects. Various stress inducted ROS usually reacts with proteins, lipids and DNA, causing oxidative damage and impairing the normal functions of cells 4.Many researchers correlated some of the reactive oxygen species (ROS) associated enzymes like superoxide dismutase (ROS), ascorbate per oxidase (APOX), catalyse (CAT) and non-enzymatic component like $H_2O_2$ generations and lipid per oxidation. Besides, stress induced DNA damage is also considered as a major event during a biotic stresses. Though some strains of Nistari showed varying degree of biotic and stress tolerances, but information is lacking on the comparative analysis of these major anti oxidative groups among the available strains of Nistari. The relationship with radical scavenging potentials and oxidative stress induced DNA damage repair abilities of the available Nistari resources are also not known.

## Objectives of Study

a) Assessment of variability of available 'Nistari' variants using ROS stabilizing enzymatic and non-enzymatic components of mulberry silkworm

b) Evaluation of stress induced DNA damage repairing ability of 'Nistari' variants

c) Assessment of genetic variability of 'Nistari' variants using Microsatellite markers.

## Materials and Methods

Six strains (=variants) of poly voltine silkworm *(Bombyxmori L.)* breed Nistari were selected for the study. These six [Nistari strains- plain, marked, chalsa, Debra, white and Sex-limited Y (YSL)] strains were reared under recommended temperature and humidity. While, for different biochemical assessments, haemolymph of fifth instar ($4^{th}$ day) larvae was collected by pricking the $1^{st}$ abdominal proleg of each larva using 1.5mL of sterile microfuge tube contacting 5-10mg of phenylthiourea. Subsequently, haemocytes were separated from haemolymph by centrifugation at 5000rpm for 10min at 4 °C and haemolymph plasma was stored at -35 °C for further use.

## Results and Discussion

### Assessment of Variability of Available 'Nistari' Variants Using ROS Associated Non-Enzymatic and Enzymatic and Components

Six strains of Nistari namely Plain, Marked, Chalsa, Debra+P, White and Y-Sex-limited are maintained in institute's repository which were reared twice during January-February 2018 and April-May 2018 following standardized protocol. Haemolymph was collected from $5^{th}$ instar larvae and store at -35 °C in 0.5mL aliquots for estimation / assessment of different parameters.

Nistari variants (=strain) originated from *B.mori L*. varied significantly for the haemolymph contents of $H_2O_2$, lipid peroxidation (LPO) and total soluble protein (TSS) as well as the activities of ascorbate peroxidase (APOX), catalase (CAT) and superoxide dismutase (SOD; Table 1). Variations of antioxidative enzyme activities in the tested strains of Nistari were generally much higher than the anti-oxidative non-enzymatic components and TSS. Among the enzymes, variation of APOX activity was maximum (5.4 fold), followed by CAT (3.5 fold); while SOD was the least variable (1.6 fold).Among the tested non-enzymatic components, variation of LPO was maximum (3.3fold).

**Table 1:** Range, standard error of mean (SEm), least significant difference (LSD), coefficient of variation (cv) and variation of reactive oxygen species (ROS) associated enzymatic and non-enzymatic components in six strains of polyvoltine silkworm breed Nistari.

| Parameter | Unit | Range | SE(m) | $LSD_{(0.05)}$ | cv% | Variation (fold) |
|---|---|---|---|---|---|---|
| $H_2O_2$ | µmole $ml^{-1}$ | 0.101 – 0.149 | 0.002 | 0.008 | 3.8 | 1.5 |
| APOX | unit $ml^{-1}$ | 0.020 – 0.107 | 0.015 | 0.005 | 12.9 | 5.4 |
| CAT | unit $ml^{-1}$ | 0.016 – 0.056 | 0.002 | 0.006 | 3.5 | 3.5 |
| SOD | Unit$mg^{-1}$ protein | 1.501 – 2.333 | 1.896 | 6.052 | 3.4 | 1.6 |
| TSS | mg $ml^{-1}$ | 19.28 – 21.07 | 0.622 | 3.42 | 7.5 | 1.1 |
| LPO | nmol $ml^{-1}$ | 6.77 – 22.44 | 0.128 | 0.408 | 2.2 | 3.3 |

Data are mean of two seasonal observations with three each of replications (n= 6)

$H_2O_2$ = hydrogen peroxide titre, APOX = ascorbate peroxidase activity; CAT = catalase activity; SOD = superoxide dismutase specific activity; TSS = total soluble sugar content and LPO = lipid peroxidation as a function of malonaldehyde oxidation.

Unit = amount of enzyme that oxidized 1µmol of substrate/min.

### ROS Associated Non-Enzymatic Components

Free radical-induced lipid per oxidation is considered to be an important mechanism of membrane deterioration during ageing of animal tissues. Concentrations of MDA, a product of lipid per oxidation, measured in the haemolymph of all six strains of Nistari breed (Figure 1). The strain variation of MDA was significant (3.3 fold). Lipid per oxidation level was significantly higher in marked Nistari strain followed by Nistari plain and Chalsa. Increased lipid per oxidation, generally considered as an indicator of increased oxidative damage [2].

$H_2O_2$, one of the major ROSs has been postulated to play a central role in insect resistance to oxidative stress. Among the effects, involvement in the induction of cell death, membrane deterioration and impairment of protein cross linking are well documented [3]. But information is limited about the endogenous levels of $H_2O_2$ in different strains of a biotic stress tolerant silkworm breed Nistari. Therefore, we measured endogenous level of $H_2O_2$ from the haemolymph of six strains of Nistari (Figure 2). Variation of $H_2O_2$ titters were highly significant $H_2O_2$ among

the tested strains. Like, lipid per oxidation ability, Nistari marked showed maximum level of haemolymph $H_2O_2$ content followed by 'Chalsa' and 'Debra'; while Nistari strain 'white' exhibited minimum of $H_2O_2$ content. On the other hand endogenous level of the total soluble haemolymph protein was maximum in 'White' and 'Chalsa' strains of Nistari breed (Figure 3). While, ROS generation capacity wise superior strain 'Marked' 'Plain' and 'Debra' exhibited low levels of TSS [4].

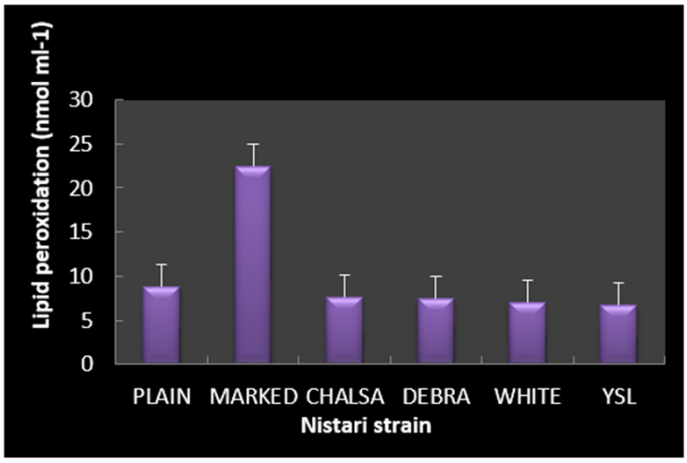



**Figure 1:** Comparative lipid peroxidation abilities of six strains of polyvoltine silkworm breed Nistari.

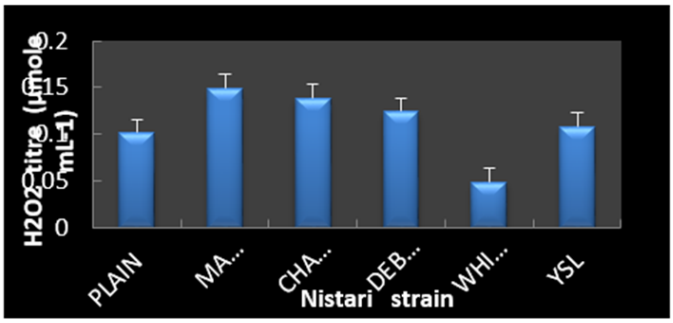



**Figure 2:** Comparative hydrogen peroxide contents of six strains of polyvoltine silkworm breed Nistari.

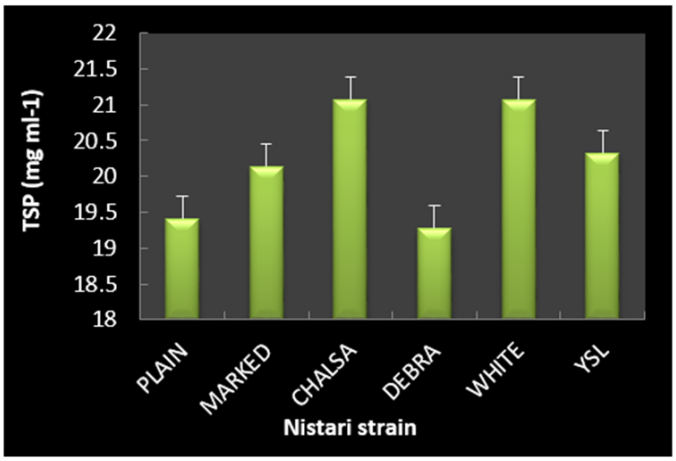



**Figure 3:** Comparative total soluble protein contents of six strains of polyvoltine silkworm breed Nistari.

## ROS Associated Enzymatic Components

The intracellular concentration of ROS depends on the production and/or removal by the antioxidant system. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, and to regulate redox sensitive signaling pathways. Three of the primary antioxidant enzymes contained in mammalian cells that are believed to be necessary for life in all oxygen metabolizing cells are superoxide dismutase (SOD), catalase and a substrate specific per oxidase [5]. Changes in activities of anti-oxidative enzymes have been found in many species when plants or animals are subjected to abiotic stresses [6]. Most studies show that higher activities of the enzymes were positively correlated with the resistance to biotic or abiotic stress. The biotic stress induced changes are also depends on animal genotypes. Catalases and ascorbate per oxidases help in removal of hydrogen peroxide in non-specific and specific ways in insect systems, respective and thereby stabilize the internal balance of toxic free radicals [6].

Corollary to the endogenous higher titters of ROS like $H_2O_2$ and efficiency of lipid per oxidation, lowest level of CAT (Figure 4) and APOX (Figure 5) activities were observed in the Nistari strain 'Marked'. CAT and AOX activities were 5.4 fold and 3.5fold higher than the maximum performing strain 'Chalsa' and 'Plain', respectively. However, APOX and CAT activity levels were significantly different in strain 'Plain' [7,8].

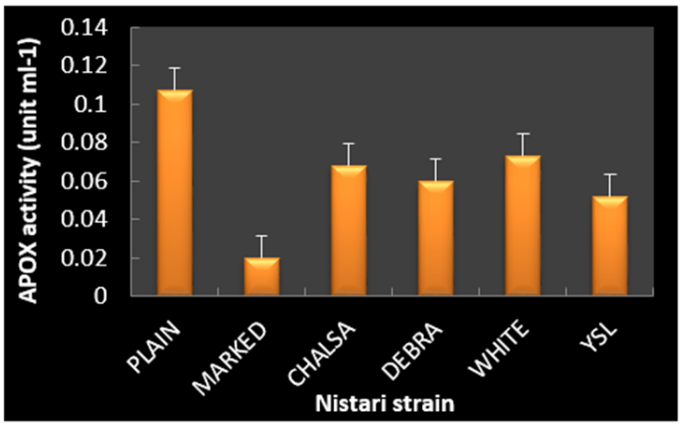



**Figure 4:** Comparative ascorbate per oxidase(APOX) activities of six strains of poly voltine silkworm breed Nistari.

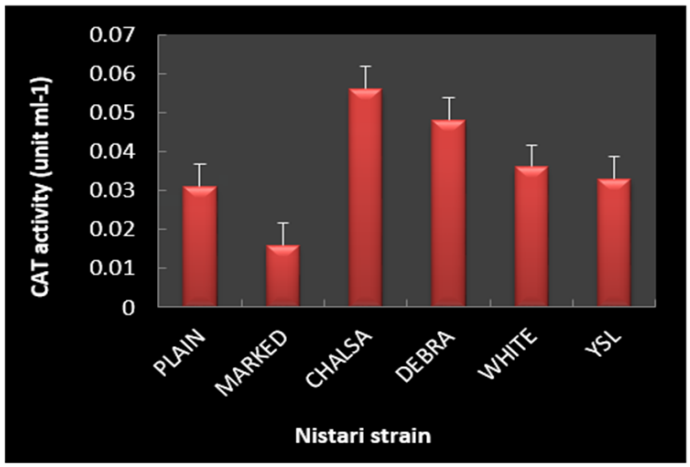



**Figure 5:** Comparative catalase (CAT) activities of six strains of polyvoltine silkworm breed Nistari.

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals by adding or removing an electron thereby converting superoxide radical to less damaging species i.e., molecular oxygen or hydrogen peroxide [9]. Though significant species / genotypic variation of SOD activities has been reported in different animal and plant systems [10]. But among the six Nistari variants / strains, variation of SOD was only 1.6fold (Figure 6), least among other two enzymes tested. The SOD showed maximum specific activity in Marked' followed by very low differences with other strains.

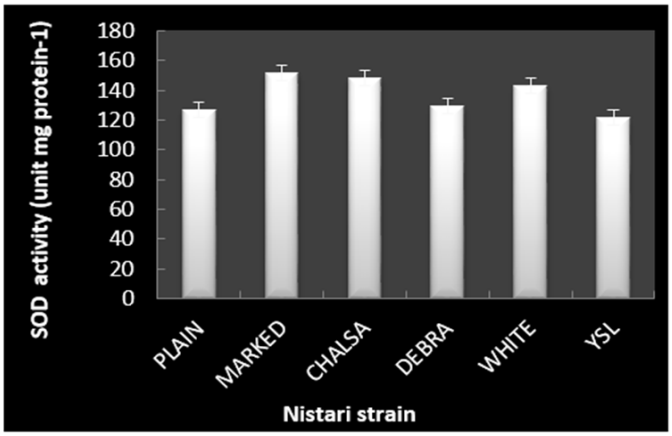



**Figure 6:** Comparative superoxide dismutase (SOD) activities of six strains of polyvoltine silkworm breed Nistari.

## II. Evaluation of stress induced DNA damage repairing ability of 'Nistari' variants

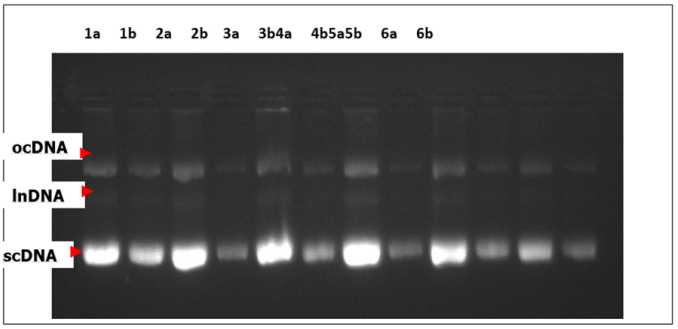
### DNA isolation and quality assessment:

High molecular weight DNA was obtained (Table 2) along with an average of $A_{260}/A_{280}$ ratio of about 1.80 (range: 1.61 to 1.82) with the DNA yield range of indicating purity of the isolated DNA (Table 2). DNA yield was ranged from 0.17to 0.28 µg $mL^{-1}$ haemolymph.

**Table 2:** DNA yield of six variants of poly voltine silkworm breed Nistari.

| Variants | $A_{260}/A_{280}$ | DNA yield (µg $mL^{-1}$haemolymph) |
|---|---|---|
| Plane | 1.72 | 0.23 |
| Marked | 1.97 | 0.28 |
| Chalsa | 1.82 | 0.25 |
| Debra | 1.61 | 0.19 |
| White | 1.66 | 0.22 |
| YSL | 1.74 | 0.17 |

The poly voltine silkworm breed Nistari is considered as most tolerant to different biotic and abiotic stress [11]. Different degree of tolerance of various variants of Nistari to temperature and other stresses are reported by various researchers [12]. It is now recognized that the extremely reactive .OH radical derived from reactive oxygen and $H_2O_2$ is a cause of DNA strand scission in cellular damage [11]. The protective effect of freshly collected haemolymph from different strains of poly voltine silkworm breed Nistari against stress induced DNA damage under simulated oxidative stress condition ( induced by using $H_2O_2$ + UV-treatment) was studied on pBR322 plasmid DNA [13].




[Lane1= Plain, 2= marked, 3 = Chalsa, 4 = Debra, 5 = white, 6 = YSL; a = uv- treated and b = uv-un-treated]

**Figure 7:** Effect of haemolymph extracts of six strains of polyvoltine silkworm breed Nistari on the protection of supercoiled plasmid DNA (pBR322) against free radical generated by UV-treatment and photolysis of hydrogen peroxide.

Figure 7 shows the electro phoretic pattern of DNA after UV-photolysis of $H_2O_2$ in the absence or presence of haemolymph extracts of six Nistari strains.DNA derived from the plasmid showed two bands on agarose gel electrophoresis (lane 1), the faster moving prominent band corresponded to the native super coiled circular DNA (sc DNA) and slower moving faint band was the open circular form (ocDNA). The UV-irradiation in the presence of $H_2O_2$ was responsible for the cleavage of scDNAto give prominent ocDNA and a faint linear DNA (lnDNA) indicating that OH-generated from UV-photolysis of $H_2O_2$ produced DNA strand nick. This damage was considerably suppressed the formation of lnDNA in various intensities by the haemolymph of different Nistari strains (lane1a to 6b).The band intensities of all uv-untreated scDNA (1b to 6b) was more than the uv-treated variants (1a to 6a). The uv-irradiation of DNA in the presence of $H_2O_2$ (lane 1 to 6a) caused the cleavage of scDNA to give ocDNA and the linear form (linDNA), indicating that .OH radical generated by UV-photolysis of $H_2O_2$ produced DNA strand scission. The haemolymph of Plane, Marked, Chalsa and Debra (1 to 4) suppressed the formation of linDNA in compare to their respective uv-untreated control.

## Findings and Future Scope

The present findings indicated that:

- Significant variability of non-enzymatic and enzymatic antioxidative components was present among the six strains/variants of polyvoltine silkworm breed Nistrai.
- Variability of hemolymph ascorbate peroxidase activity was maximum (5.4fold), followed by catalase activity (3.5fold) and lipid peroxidation ability (3.3fold) among the studied strains of Nistari.
- While the variations of superoxide dismutase (SOD) activity, $H_2O_2$ and total soluble protein contents were less than 1.7 fold among the tested strains.
- Relatively higher levels of $H_2O_2$ content were negatively correlated with APOX and CAT activities in the 'Plain' and 'Marked' strains of the polyvoltine silkworm breed Nistari.
- However, the direct relationship between SOD activity and ROS components like $H_2O_2$ titers was not apparent.
- Oxidative stress-induced DNA damage repairing ability of four out of six strains/variants of Nistari was observed at least under simulated oxidative stress conditions.

- Though the good quality of DNA was isolated from all six strains of Nistari, due to paucity of time and / pre scheduling of dissertation report submission, SSR profiling of the variants was incomplete.

Future scope of Research

✓ Assessment of antioxidant potential of the isolated haemolymph of six Nistari variants are essential to establish relationship with ROS stabilization process.

✓ A comparative study of SSR or other codominant maker based profiling of all six strains of Nistari under oxidative stress and non-stressed condition is essential to ascertain thermotolerance ability the breed.

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