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Original Article

FEEDING PREFERENCE OF SILKWORM LARVAE DEPENDING ON BIOCHEMICAL ATTRIBUTES RELATED TO MULBERRY GENOTYPES

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ABSTRACT

Objective: The silkworm rearing was influenced by different mulberry cultivars according to the biochemical properties of leaves. In this present study, a comparative analysis was made to investigate feeding preference of silkworm larvae depending on biochemical attributes of mulberry genotypes.

Methods: For this purpose, seven different mulberry cultivars and one germ plasm namely Dudhiya was selected. F1 hybrid (Nistari × bivoltine) of silkworm larvae was reared under selected cultivars of mulberry leaves at different seasons. Biochemical assessment of all leaves was also done.

Results: Among these, S1, V1 and S1635 mulberry cultivars showed higher amount of total protein, total sugar and chlorophyll, also exhibited better feeding response on economic attributes of silkworm. Maximum accumulation of ascorbic acid and glutathione was recorded during winter in Dudhiya leaves. The accumulation of H_2O_2, superoxide and lipid peroxidation was comparatively higher than other cultivars during stress period in Dudhiya. Statistical analysis revealed that larval growth and economical parameters depend on biochemical properties of leaves and inversely associated with excessive production of Reactive oxygen species (ROS).

Conclusion: The scavenger and ROS ratio was properly maintained in S1, V1 and S1635 leaves which might help leaf metabolic homeostasis. Proper metabolic activities of leaves possibly will produce higher proteins and carbohydrates which were required for larval growth and silk production as established from the PCA plot analysis. Therefore S1, V1 and S1635 might be recommended for silkworm rearing or commercial cultivation purpose throughout all season.

Keywords: Silkworm, Mulberry cultivars, Proline, Reactive Oxygen Species (ROS), MDA, Single cocoon weight.

INTRODUCTION

Silkworm, *Bombyx mori* L. is a domestic lepidopteron insect. Silkworm larval growth depends on the nutritive value of mulberry leaves [1]. The Foliar nutritional value of leaves and biomass production depends on the weather and agricultural practices [2], and it was also different according to cultivars. On the other hand, Susheelamma *et al.* [3] reported that mulberry genotypes produced high biomass and due to more rapid growth rate and higher metabolic activities, mulberry cultivars had a fabulous water demand. Water scarcity can clutch mulberry plant growth and metabolism. Therefore plants experience oxidative stress that reduces plant primary and secondary metabolite production [4, 5] and reduction of plant production directly affects larval development and silk production. Various reports were published on oxidative stress of mulberry plant, and scientists have concentrated on the responses of enzymatic antioxidants [5, 6 and 7]. Kotresha *et al.* [8] investigated some of the most important non-enzymatic antioxidants in different mulberry leaves in response to drought and high-temperature stress. Guha *et al.* [9] analyzed non-enzymatic antioxidative defense under water and drought stress. It was hypothesized that foliar production of mulberry leaves differ under various stress periods. Silkworm larvae may choose superior mulberry cultivars on the basis of their nutritional values. Therefore, in the present work, an attempt was made to find out superior mulberry genotypes on the basis of biochemical leaf quality and feeding response of silkworm from Malda district of West Bengal, India. For the said purpose, seven different cultivars of mulberry leaves were selected namely S1, V1, K2, S1635, Mandalaya, Jaysree and Bombay along with primitive germplasm Dudhiya as a feeding source for 5th instar larvae. Also, we worked on different biochemical attributes of selected leaves which might assist in determining the partial role of antioxidants in leaves related to the larval choice of feeding. Our observation might assist farmers involved in sericulture for selection of mulberry cultivars to rear silkworm larvae at a different season.

MATERIALS AND METHODS

Study location

The study area, Malda district of West Bengal is located at 25.00 ˚N and 88.15 ˚E. The weather is usually extremely humid and tropical. Temperatures can reach as high as 46 ˚C during the day in May and June and fall as low as 4 ˚C overnight in December and January. The winter season arrives in Malda district in the middle of November and continues till the last of February. Winter is succeeded by summer in the months from March to May. After the summer season, the city witnesses a rainy season that begins in the month of June and ends by the middle of September. The rains in this city are the result of the south-west monsoons. Normally, the rainfall in the area is 1453.1 mm. The brief season after rains and before the arrival of winter is the period referred to as the post-monsoon period. This season lasts for about one and a half month and is characterized by cool weather.

Study methods

Feeding experiment

We conducted overall rearing procedure under an optimal temperature (27˚-29 ˚C), humidity (70±5%) and overall sterilized environment in our laboratory. Feeding trial with these eight selected cultivars of mulberry leaves was conducted at three different seasons, spring, summer and autumn. Larvae were fed with young, mature and senescent leaves of all selected cultivars of mulberry. According to Gangwar [10], larval weight, mortality percent, single cocoon weight, single shell weight and other economic parameters were calculated at three different seasons separately.

Study of biochemical attributes

Estimation of free proline

Free proline content in leaf tissue was determined according to Bates *et al.* [11]. Fresh leaf sample (0.5 g) was homogenized in 10 ml of 3% sulfosalicylic acid. The homogenate was centrifuged at 9000 g for 15 min at room temperature. The reaction mixture containing 1 ml leaf extract, 2 ml acid ninhydrin, and 2 ml glacial acetic acid was incubated for 1 h in boiling water bath. After incubation, 4 ml of toluene was added to the reaction mixture and mixed vigorously by vortexing for 15-20 s. The upper reddish pink colored toluene layer was separated, and the absorbance was read at 520 nm in a UV–visible spectrophotometer. Proline content was determined from the standard curve prepared by using authentic proline (Sigma) and was expressed in mg/g Frish weight (FW).

Estimation of chlorophyll content

Chlorophyll was extracted in 80% acetone, and the amount of total chlorophyll were estimated according to Arnon method [12].

Estimation of total carotenoids

For quantification of total carotenoids, fresh leaf sample (0.5 g) was homogenized in 10 ml of 80% (v/v) acetone. The homogenate was centrifuged at 10 000 g for 5 min. The supernatant was collected, and the extraction was repeated twice with 80% acetone. The absorbance of the extract was read at 663.2, 646.8 and 470 nm by using UV–visible spectrophotometer. The total carotenoid content was calculated using the extinction coefficients given by Lichtenthaler [13], and the results were expressed in mg/g FW.

Estimation of total carbohydrate (soluble sugars) contents and reducing sugar content

100 mg of leaves were crushed in 10 ml of 80 % hot ethanol using mortar and pestle and filtered through filter paper. After evaporation of ethanol by heating the sample, the final volume of filtrate was made to 10 ml by adding distilled water.

Total soluble sugars were measured by anthrone method [14]. The mixture of 1 ml extraction and 4 ml anthrone reagent was incubated at 100 ˚C for 10 min. The mixture was cooled to room temperature and absorbance (resultant blue-green colour) was measured at 620 nm. Using a standard curve prepared from sucrose, a total soluble sugar present in the extract was calculated.

Reducing sugars were estimated by DNS method [15]. To 1 ml alcohol-free extract, 1 ml DNS reagent was mixed and boiled in a water bath for 5 min. After the development of the coloured product, 1 ml 40 % Rochelle salt solution was added and mixed well. After cooling the mixture, absorbance was read at 510 nm using reagent blank adjusted to zero absorbance.

Estimation of total protein content

Total protein content in leaves was estimated by Lowry's method [16]. The blue colored complex was formed after well mixing 5 ml alkaline copper solution and Folin-ciocalteu reagent with 1 ml protein sample. The color that is formed in biuret test of alkaline copper reacts with protein and reduction of phosphomolybdic-phosphotungstic compounds occurs in FCR by aromatic amino acid tryptophan and tyrosine present in the protein sample. The intensity of the color is measured at 660 nm.

Estimation of glutathione content

Total glutathione content in mulberry leaves was determined according to Griffith and Meister [17]. Fresh leaf tissue (0.2 g) was homogenized with 0.8 ml of 10% sulphosalicylic acid and centrifuged at 15 000 g for 5 min at 4 ˚C. The supernatant was neutralized by adding 0.6 ml of 10% sodium citrate. 1 ml reaction mixture was prepared by adding 100 µl extracts, 100 µl double distilled water (ddw), 700 µl of 0.3 mM NADPH in potassium phosphate buffer (20 mM, pH 7.5) and 6 mM 5 -dithio-bis(2-nitrobenzoic acid) (DNTB). The reaction mixture was stabilized at 25 ˚C for 3-4 min. Then 10 µl glutathione reductase (GR) was added to the reaction mixture, and the absorbance of the resulting colour was read at 412 nm in a UV–visible spectrophotometer. The results were expressed in µmol/g FW.

Estimation of MDA content

The extent of lipid peroxidation was determined by quantifying malondialdehyde (MDA) formation [18]. Fresh leaf sample (0.5 g) was homogenized in 5 ml of 0.1% (w/v) TCA at 4 ˚C. The homogenate was centrifuged at 5000 g for 10 min at 4 ˚C. The reaction mixture contained 500 µl of the supernatant and 4 ml of 0.5% (w/v) Thiobarbituric Acid (TBA) in 20% (w/v) Trichloroacetic acid (TCA). The reaction mixture was incubated at 95 ˚C in a shaking water bath for 30 min and the reaction was stopped by quickly cooling the tubes in an ice water bath. The samples were centrifuged at 5000 g for 15 min and the absorbance of the supernatant read at 532, 600 and 440 nm. MDA concentration was calculated using an extinction coefficient of 155/mM/cm.

Estimation of H_2O_2 and superoxide anion ($O_2^{\cdot -}$)

H_2O_2 was estimated according to Becana *et al.* [19] with minor modifications. Fresh leaf tissue (0.5 g) was homogenized in liquid nitrogen with 5% (w/v) TCA. The homogenate was centrifuged at 12 000 g for 10 min at 4 ˚C. The supernatant was collected in fresh eppendorf and once again centrifuged at 12 000 g for 2 min and used immediately for assay. H_2O_2 concentration was determined spectrophotometrically at 508 nm in a reaction mixture that contained 50 mM phosphate buffer (pH 8.4), 0.6 mM 4-(-2 pyridylazo) resorcinol and 0.6 mM potassium–titanium oxalate in 1:1 proportion.

Superoxide accumulation was determined according to Doke [20] with minor modifications. Fresh leaf sample (0.5 g) was placed in test tube containing 7 ml of the reaction mixture which contained 50 mM phosphate buffer (pH 7.8), 0.05% nitroblue tetrazolium (NBT) and 10 mM of NaN_3. The test tubes were then incubated in dark for 5 min, and subsequently, 2 ml of the solution was taken from the tube and heated for 10-15 min at 85 ˚C. The sample was cooled on ice for 5 min and the absorbance (A) was measured at 580 nm.

Estimation of ascorbic acid (AA)

Ascorbic acid was determined according to Omaye *et al.* [21] with some modifications. Fresh leaf tissue (0.5 g) was homogenized with 5 ml of 10% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at 10 000 g for 20 min at room temperature. The pellet was re-extracted twice; supernatants were combined and used for the assay. To 0.5 ml of extract, 1 ml of 2% 2, 4-dinitrophenyl hydrazine (DNTPH in 0.5 N H_2SO_4), a drop of 10% thiourea (in 70% ethanol) were added and incubated at 37 ˚C for 3 h. After incubation, 1.75 ml of ice-cold 65% H_2SO_4 was added, allowed to stand at 30 ˚C for 30 min and the absorbance of the resulting colour was detected at 520 nm in UV–visible spectrophotometer. The AA content was determined from the standard curve prepared with authentic L-AA (Sigma) and was expressed in mg/gFW.

Statistical analysis

Differences and interaction between cultivars and seasonal effects were determined by two-way analysis of variance (ANOVA). Separation of Mean was performed by Duncan's multiple range test (DMRT) at $p<0.05$. The correlation between different biochemical attributes of mulberry leaves and economic parameters of the silkworm rearing system was done by using Statistical package for social sciences (SPSS) correlation matrix. Principal component analysis (PCA) of biochemical attributes of different cultivars and economical attributes of the rearing system at different season was analyzed by using XLSTAT 2015 software. Pearson (n) type PCA was used for data analysis.

RESULTS AND DISCUSSION

Biochemical attributes

Silkworm larvae require leaf nutrients in the exact ratio for their growth. Imbalance in nutrients from mulberry leaves also affects larval metabolic activities. Furthermore, the nutritional content of leaves might influence the silk production and silk quality.

Biochemical component of leaves depend on various factors, namely cultivars or genotypes, soil nutrients, water, cultural practices and seasonal variation [22]. In this study, the genotypic selection was made on the basis of leaf nutritional values and feeding preference by larvae associated with seasonal variation.

Plants suffer various stresses at a different season. During November and February (winter season) mulberry plants experience serious osmotic stress due to a significant drop of water potential in soil. Again, due to excess rain, the flood situation and hypoxic stress might be created during mid-July (rainy season). During rainy season soil contains low microelements. Mulberry plants also suffer oxidative stress due to deficiency of microelements like Mn, N, P, and K in soil [23, 24]. Besides these two stressful points, the climatic conditions during other periods are favorable for plant growth.

Oxidative stress parameters of mulberry leaves were evaluated by considering the seasonal fluctuations of free radical accumulation in the plant body. We categorized genotypes into two classes: one acclimated and other non-acclimated. Acclimated genotypes are those who can build up high free radical scavengers and reduce free radicals, which produced in plant body during the stress period. If we consider the genotypic responses in this circumstance, it is clear that S1, V1 and S1635, variety can successfully manage the minimum accumulation of free radicals like peroxide, superoxide and MDA whereas moderate accumulation of the same in Mandalay, Jayasree, Bombay cultivars and Dudhiya accumulate high free radicals. Both the ascorbic acid and glutathione was sufficiently accumulated during winter and rainy season in V1 and S1 varieties, but this accumulation was significantly lesser in Dudhiya genotypes (Tables: 1a, 1b and 2a, 2b). This indicates that the glutathione-ascorbate pool gives sufficient feedback for the regeneration of other antioxidant molecules among stress tolerant cultivars like V1, S1 and S1635 during the crisis. The other ways of defense are an accumulation of compatible osmolytes like proline and protection from photobleaching due to carotenoid pigment, and sufficient accumulation of photoassimilates due to the presence of adequate chlorophyll pigments. Among disease and drought tolerant plant species, proline could form vital amino acid residues which were accumulated in the organism at that time [25]. Maximum proline was obtained from mature leaves of the V1 cultivar than others. Sarkar *et al.* [26] reported that level of proline in mulberry plants increased under water stress. It was stated that in bean plant, proline was high under water stress condition [27]. Similarly, the proline content was found to be high enough during November to February (considered as winter) and July (rainy season). In the case of all three responses, also, these stress-tolerant varieties or acclimated genotype adapts better than non-acclimated Dudhiya genotype.

Table 1a: Pigment member of eight mulberry cultivars at different season

Variety	Pigment member (mg/g FW)	February	April	July	September	November
V1	Carotenoids	0.76±0.008^{a}	0.63±0.013^{c}	0.65±0.007^{c}	0.6±0.01^{d}	0.7±0.016^{b}
	Chlorophyll	15.42±0.41^{d}	20.46±0.53^{a}	19.11±0.32^{b}	21.22±0.47^{a}	18.05±0.55^{c}
S1	Carotenoids	0.71±0.009^{b}	0.65±0.01^{c}	0.75±0.007^{a}	0.61±0.009^{d}	0.72±0.02^{b}
	Chlorophyll	18.28±0.74^{e}	25.11±0.61^{b}	22.82±0.59^{c}	27.23±0.52^{a}	20.11±0.46^{d}
Dudhiya	Carotenoids	0.58±0.004^{a}	0.52±0.006^{c}	0.54±0.004^{b}	0.5±0.009^{d}	0.55±0.007^{b}
	Chlorophyll	11.38±0.81^{d}	15.46±0.69^{bc}	16.54±0.72^{ab}	17.12±0.74^{a}	14.46±0.56^{c}
S1635	Carotenoids	0.65±0.005^{a}	0.58±0.004^{d}	0.61±0.011^{c}	0.55±0.009^{e}	0.63±0.006^{b}
	Chlorophyll	17.05±0.37^{e}	22.84±0.42^{c}	23.68±0.31^{b}	27.25±0.28^{a}	19.09±0.25^{d}
K2	Carotenoids	0.61±0.005^{a}	0.59±0.008^{b}	0.58±0.006^{bc}	0.57±0.006^{c}	0.59±0.004^{bc}
	Chlorophyll	16.22±0.51^{d}	20.59±0.57^{b}	21.12±0.36^{b}	23.67±0.64^{a}	18.42±0.47^{c}
Mandalaya	Carotenoids	0.55±0.007^{ab}	0.53±0.005^{c}	0.56±0.009^{a}	0.5±0.008^{d}	0.54±0.007^{bc}
	Chlorophyll	14.42±0.61^{c}	18.26±0.66^{b}	18.54±0.67^{b}	20.12±0.59^{a}	15.33±0.53^{c}
Jayasree	Carotenoids	0.58±0.006^{a}	0.56±0.009^{b}	0.5±0.012^{c}	0.48±0.005^{d}	0.51±0.006^{c}
	Chlorophyll	12.82±0.44^{c}	16.38±0.37^{b}	15.33±0.39^{b}	18.12±0.61^{a}	13.11±0.53^{c}
Bombay	Carotenoids	0.6±0.008^{a}	0.52±0.007^{c}	0.49±0.004^{d}	0.45±0.009^{e}	0.56±0.008^{b}
	Chlorophyll	13.11±0.34^{d}	21.15±0.39^{b}	20.67±0.28^{b}	24.15±0.37^{a}	18.49±0.41^{c}

Note: Results are represented as mean±SEM, n=3. Values with different letters (a, b, c, d & e) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT)

Protein content

In the case of Lepidopteron larvae, leaf protein plays a vital determinant of leaf nutrient. Larval growth, silk gland development, cocoon production and cocoon quality in silkworm depends on the mulberry leaf protein [28]. Several studies were conducted for finding the varietal difference in leaf protein content [29, 30]. In this study, S1635 contain high protein (table 3). Higher protein content was measured in mature leaves but according to Matei *et al.* [31], protein content in mulberry leaves decreased with the increasing leaf maturity. It was reported that S-41 cultivars of mulberry leaves contain higher protein and low sugar, which gave a better larval duration and decreased molting ratio [32].

Table 1b: Non-enzymatic antioxidant member of eight mulberry cultivars at different season

Variety	Non-enzymatic antioxidant member	February	April	July	September	November
V1	Ascorbic acid (mg/g FW)	2.4±0.15^{a}	1.8±0.22^{bc}	2.1±0.16^{ab}	1.5±0.27^{c}	2.2±0.24^{ab}
	Glutathione (µmol/g FW)	8.1±0.47^{a}	7.2±0.58^{ab}	7.9±0.61^{a}	6.2±0.66^{b}	8±0.49^{a}
S1	Ascorbic acid (mg/g FW)	2.7±0.17^{a}	1.7±0.48^{bc}	2.2±0.36^{ab}	1.4±0.28^{c}	2.3±0.22^{ab}
	Glutathione (µmol/g FW)	8.5±0.23^{a}	7.5±0.37^{b}	8.1±0.34^{ab}	5.9±0.18^{c}	7.8±0.29^{b}
Dudhiya	Ascorbic acid (mg/g FW)	1.5±0.008^{b}	1.2±0.014^{d}	1.6±0.008^{a}	1.1±0.007^{e}	1.4±0.016^{c}
	Glutathione (µmol/g FW)	5.2±0.16^{a}	4.5±0.11^{c}	4.8±0.18b^{c}	4±0.2^{d}	4.9±0.17^{ab}
S1635	Ascorbic acid (mg/g FW)	2.2±0006^{a}	1±0.015^{d}	1.8±0.095^{b}	1.5±0.084^{c}	1.8±0.087^{b}
	Glutathione (µmol/g FW)	7.5±0.23^{a}	6.5±0.27^{b}	6.9±0.21^{ab}	5.5±0.26^{c}	7.1±0.34^{ab}
K2	Ascorbic acid (mg/g FW)	2.3±0.098^{a}	1.3±0.12^{c}	2±0.094^{b}	1.5±0.17^{c}	1.9±0.14^{b}
	Glutathione (µmol/g FW)	8.3±0.17^{a}	7.3±0.19^{c}	7.8±0.11^{b}	6±0.13^{d}	7.6±0.16^{bc}
Mandalaya	Ascorbic acid (mg/g FW)	2.5±0.007^{a}	1.7±0.005^{e}	2.1±0.007^{c}	1.8±0.006^{d}	2.2±0.007^{b}
	Glutathione (µmol/g FW)	7.1±0.13^{a}	6.2±0.17^{c}	6.6±0.16^{b}	5.3±0.12^{d}	6.8±0.19^{ab}
Jayasree	Ascorbic acid (mg/g FW)	1.9±0.016^{a}	1.2±0.013^{d}	1.9±0.02^{a}	1.3±0.019^{c}	1.6±0.015^{b}
	Glutathione (µmol/g FW)	6.8±0.09^{a}	5.3±0.15^{c}	6.2±0.13^{b}	4.5±0.07^{d}	6.5±0.02^{ab}
Bombay	Ascorbic acid (mg/g FW)	2±0.016^{a}	1±0.02^{e}	1.8±0.018^{b}	1.1±0.014^{d}	1.7±0.023^{c}
	Glutathione (µmol/g FW)	6.3±0.007^{a}	4.8±0.008^{d}	5.9±0.007^{b}	4.8±0.059^{d}	5.4±0.076^{c}

Note: Results are represented as mean±SEM, n=3. Values with different letters (a, b, c, d & e) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT)

Chlorophyll content

Chlorophyll content of the leaf is an essential factor for the determination of the photosynthetic efficiency of the plant. Highest chlorophyll content was recorded in S1635 mature leaves followed by young and senescent leaves. Lowest chlorophyll content was observed in young, mature and senescent leaves of Dudhiya. Similarly, Hotta [33] said that chlorophyll content was lesser in top (young) and bottom leaves than middle one (mature). Several works were performed on the chlorophyll content on different mulberry varieties. Santosha Gowda [34] reported that S1635 and S1 had highest chlorophyll content.

Sugar content

Sugars play an important role in silkworm growth, and it acts as one of the essential biting factors of larvae. As a result, sugar is an essential biochemical attribute for mulberry genotype selection for silkworm rearing. Soluble sugar content was higher in Bombay leaf followed by S1635, S1, V1, Jayasree, K2, Mandalaya and Dudhiya. In the present study, S1635 also showed higher carbohydrate content than the remaining seven cultivars of mulberry leaves. Similarly, Purohit and Kumar [35] also obtained highest carbohydrate content (22.83%) in S1635 cultivar. Our experiment revealed that mature leaves had highest soluble sugar in all mulberry cultivars. But Murthy *et al.* [25] reported contradictory results. According to Murthy *et al.* [25] total sugar content was high in the tender or young leaves which reduced gradually with increasing leaf maturity. From this study, one correlation was reflected in between soluble sugar and economic attributes of silkworm rearing (table 4). Murthy *et al.* [25] reported that sugars help to produce main energy for metabolic activity.

Table 2a: ROS (Lipid peroxidation member) accumulation in eight mulberry cultivars at different season

Variety	ROS: lipid peroxidation member	February	April	July	September	November
V1	H_2O_2 (µmol/g FW)	2.8±0.16^{a}	1.3±0.098^{cd}	2.1±0.11^{b}	1.2±0.16^{d}	1.6±0.14^{c}
	MDA (nmole/g FW)	25±0.74^{a}	16±0.85^{c}	20±0.83^{b}	14±0.73^{d}	17±0.91^{c}
	Superoxide Anion (µmol/g FW)	1.01±0.016^{a}	0.45±0.014^{d}	0.7±0.019^{c}	0.21±0.009^{e}	0.88±0.019^{b}
S1	H_2O_2 (µmol/g FW)	3.1±0.24^{a}	1.5±0.16c^{d}	2.4±0.22^{b}	1.3±0.11^{d}	1.9±0.17^{c}
	MDA (nmole/g FW)	23±0.91^{a}	15±0.68^{d}	21±0.82^{b}	12±0.71^{e}	18±0.83^{c}
	Superoxide Anion (µmol/g FW)	1.09±0.16^{a}	0.51±0.083^{c}	0.65±0.072^{bc}	0.25±0.055^{d}	0.76±0.049^{b}
Dudhiya	H_2O_2 (µmol/g FW)	6.4±0.36^{a}	3.2±0.21^{d}	5.3±0.41^{b}	1.9±0.15^{e}	3.9±0.26^{c}
	MDA (nmole/g FW)	48±0.81^{a}	26±0.34^{d}	40±0.94^{b}	19±0.42^{e}	34±0.25^{c}
	Superoxide Anion (µmol/g FW)	1.84±0.11^{a}	0.82±009^{b}	0.4±.09^{c}	0.65±.09b^{c}	1.62±0.16^{a}
S1635	H_2O_2 (µmol/g FW)	4.9±0.27^{a}	2.8±0.12^{c}	4.2±0.31^{b}	1.5±0.11^{d}	3.2±0.18^{c}
	MDA (nmole/g FW)	32±0.84^{a}	17±0.32^{d}	26±0.29^{b}	15±0.17^{e}	22±0.26^{c}
	Superoxide Anion (µmol/g FW)	1.36±0.16^{a}	0.52±0.09^{cd}	0.69±0.09^{c}	0.41±0.06^{d}	1.04±0.11^{b}
K2	H_2O_2 (µmol/g FW)	4.3±0.3^{a}	2.5±0.2^{c}	3.5±0.31^{b}	1.4±0.18^{d}	2.8±0.24^{c}
	MDA (nmole/g FW)	38±0.61^{a}	20±0.37^{d}	33±0.3^{b}	17±0.24^{e}	25±0.29^{c}
	Superoxide Anion (µmol/g FW)	1.22±0.05^{a}	0.44±0.009^{d}	0.63±0.009^{c}	0.38±0.007^{e}	0.83±0.01^{b}
Mandalaya	H_2O_2 (µmol/g FW)	3.6±0.21^{a}	2.2±0.14^{c}	3±0.27^{b}	1.6±0.19^{d}	2.5±0.22^{c}
	MDA (nmole/g FW)	33±0.67^{a}	18±0.14^{d}	25±0.29^{b}	16±0.22^{e}	20±0.24^{c}
	Superoxide Anion (µmol/g FW)	0.94±0.01^{a}	0.39±0.007^{d}	0.58±0.007^{c}	0.28±0.007^{e}	0.75±0.009^{b}
Jayasree	H_2O_2 (µmol/g FW)	5.4±0.43^{a}	2.6±0.28^{c}	3.8±0.36^{b}	1.7±0.19^{d}	2.9±0.22^{c}
	MDA (nmole/g FW)	37±0.24^{a}	19±0.13^{d}	28±0.22^{b}	17±0.13^{e}	23±0.2^{c}
	Superoxide Anion (µmol/g FW)	1.47±0.022^{a}	0.46±0.009^{d}	0.66±0.009^{c}	0.39±0.005^{e}	0.92±0.011^{b}
Bombay	H_2O_2 (µmol/g FW)	5.6±0.34^{a}	2.4±0.27^{c}	4.1±0.38^{b}	1.5±0.25^{d}	2.7±0.22^{c}
	MDA (nmole/g FW)	42±0.29^{a}	21±0.22^{d}	34±0.31^{b}	17±0.13^{e}	28±0.21^{c}
	Superoxide Anion (µmol/g FW)	1.53±0.03^{a}	0.6±0.007^{d}	0.98±0.009^{c}	0.42±0.007^{e}	1.38±0.019^{b}

Note: Results are represented as mean±SEM, n=3. Values with different letters (a, b, c, d & e) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT). Hear, ROS means Reactive oxygen species

Table 2b: Proline accumulation (mg/g FW) in eight mulberry cultivars at different season

Variety	February	April	July	September	November
V1	3.8±0.12^{b}	2.6±0.14^{c}	3.5±0.17^{b}	1.8±0.09^{d}	4.3±0.16^{a}
S1	3.5±0.07^{b}	2.9±0.01^{c}	3.7±0.013^{a}	1.5±0.009^{d}	3.7±0.006^{a}
Dudhiya	1.4±0.01^{b}	1.1±0.009^{d}	1.2±0.009^{c}	0.8±0.005^{e}	1.8±0.01^{a}
S1635	2.5±0.16^{b}	2.4±0.11^{b}	2.6±0.16^{b}	1.5±0.09^{c}	3±0.19^{a}
K2	2.6±0.09^{b}	2.2±0.068^{cd}	2.3±0.073^{c}	2.1±0.066^{d}	2.9±0.08^{a}
Mandalaya	2.2±0.024^{b}	2±0.026^{c}	2±0.024^{c}	1.8±0.027^{d}	2.4±0.019^{a}
Jayasree	1.4±0.016^{a}	1±0.01^{d}	1.1±0.011^{c}	1.1±0.014^{c}	1.2±0.011^{b}
Bombay	1.6±0.014^{a}	0.7±0.009^{d}	0.8±0.01^{c}	0.6±0.012^{e}	0.9±0.011^{b}

Note: Results are represented as mean±SEM, n=3. Values with different letters (a, b, c, d & e) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT)

Table 3: Different biochemical attributes of eight mulberry cultivars

Name of the cultivars	Chlorophyll (mg/g FW)	Protein (mg/g FW)	Soluble sugars (mg/g FW)	Proline (mg/g FW)	Reducing sugar (mg/g FW)
S1	27.23±0.73^{a}	11.23±0.33^{k}	37.34±0.61^{ab}	63±0.55^{d}	2.14±0.06^{ab}
V1	21.22±0.66^{cd}	22.12±0.37^{c}	36.45±0.66^{bc}	81±0.53^{b}	1.78±0.09^{de}
K2	23.67±0.71^{b}	19.45±0.24^{ef}	34.45±0.63^{de}	51±0.59^{gh}	1.67±0.07^{ef}
DUDHIYA	17.12±0.65^{hij}	12.89±0.2^{i}	30.78±0.55^{g}	33±0.61^{k}	1.13±0.04^{hi}
S1635	27.25±0.73^{a}	24.98±0.29^{a}	37.65±0.67^{ab}	53±0.57^{f}	2.24±0.09^{a}
MANDALAYA	20.12±0.64^{de}	22.12±0.26^{c}	34.45±0.59^{de}	43±0.55^{i}	2.01±0.09^{bc}
JAYSREE	18.12±0.61^{gh}	21.89±0.3^{c}	35.56±0.57^{cd}	23±0.53^{m}	1.98±0.08b^{c}
BOMBAY	24.15±0.67^{b}	23.34±0.31^{b}	37.78±0.6^{a}	14±0.64^{o}	2.02±0.07^{bc}

Note: Results are represented as mean±SEM, n=3. Values with different letters (a to k) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT)

Table 4: Correlation between biochemical attributes of mulberry leaves with different economic parameters of silkworm rearing system

Season	Name of Biochemical attributes of leaves	Weight of 10 larvae	Larval mortality	Yield/100 larvae	Survival rate of larvae	Single cocoon weight	Shell weight (100 nos)
Spring	Reducing sugar (mg/g FW)	0.829*	-0.836**	0.662ns	0.803*	0.744*	0.807*
	Chlorophyll content (mg/g FW)	0.623ns	-0.440ns	0.746*	0.643ns	0.480ns	0.657ns
Summer	Soluble sugar content (mg/FW)	0.776*	-0.454ns	0.950**	0.816*	0.792*	0.793*
	Reducing sugar (mg/g FW)	0.624ns	-0.615ns	0.847**	0.782*	0.770*	0.755*
Autumn	Soluble sugar content (mg/FW)	0.847**	-0.763*	0.770*	0.822*	0.825*	0.860**
	Reducing sugar (mg/g FW)	0.803*	-0.821*	0.777*	0.834*	0.773*	0.820*

Note: ns = not significant, * = significant at p<0.05, **=significant at p<0.01

Feeding response

The feeding performance with eight mulberry cultivars at three different seasons was shown in table 5. Larval weight depends on the nutritive values of mulberry leaves which differ according to different cultivars of mulberry [10]. Highest larval weight was recorded in S1 followed by S1635, V1, Jayasree, Bombay, K2, Mandala, and Dudhiya. In our experiment, the observed mortality percent of larvae were greater in summer than two other seasons by all leaf nourishment. It was quite differing from the observations of Gangwar [10], where the mortality rate was high in spring. The weight of single cocoon was enhanced by feeding S1 mulberry leaves followed by V1, S1635, Mandala, Bombay, Jayasree, K2, and Dudhiya at the autumn season and gradually declined during spring and summer. Similarly, Kumar *et al.* [36] obtained highest cocoon weight by feeding S1 leaves. Likewise cocoon weight, higher single shell weight was recorded in autumn. At all three different seasons, shell weight was highest during harvest in cocoons nourished with S1 leaves. Earlier Gangwar [10] found better larval growth and increased the weight of shell influenced by BR_2 mulberry cultivars at Uttar Pradesh, India.

Table 5: Feeding response after nourishment by leaves of eight selected mulberry cultivars

Parameter	Season	S1	V1	Dudhiya	S1635	Mandala	Jayasree	Bombay	K2
Weight of 10 matured larvae (gm)	Spring	48±0.023a	44±0.02c	22±0.024g	45±0.023b	34±0.02f	44±0.021c	43±0.022d	40±0.023e
	Summer	43±0.029a	35±0.03b	21±0.028g	33.13±0.031c	23±0.028f	35±0.031b	32±0.032d	30±0.027e
	Autumn	39±0.24e	38±0.022f	15±0.026h	39.12±0.027d	35±0.021g	42±0.025b	44±0.024a	41±0.026c
Larval Mortality %	Spring	0.8±0.002d	0.58±0.003f	10±0.002a	0.82±0.002c	0.9±0.003b	0.66±0.003e	0.5±0.004g	0.45±0.003h
	Summer	21.5±0.03d	30.5±0.03a	30±0.04b	21.5±0.025d	22.5±0.034c	19.5±0.038f	20±0.035e	18.99±0.036g
	Autumn	2.8±0.021c	3.22±0.026b	8±0.028a	2.58±0.024d	2.2±0.031e	1.89±0.03g	1.67±0.029h	2±0.031f
Survival rate of pupae (%)	Spring	95±0.05b	96±0.055a	50±0.051f	96±0.054a	88±0.052d	90±0.055c	84±0.05e	95±0.053b
	Summer	92±0.048a	90±0.05c	45±0.051h	91±0.047b	75±0.049g	87±0.05e	78±0.053f	89±0.051d
	Autumn	93±0.056b	94±0.061a	34±0.05e	90±0.06d	90±0.057d	90±0.055d	90±0.054d	92±0.06c
Yield/100 larvae-by No	Spring	90±0.045c	95±0.048a	55±0.043e	90±0.039c	70±0.042d	90±0.038c	95±0.04a	91±0.044b
	Summer	65±0.033c	66±0.037b	43±0.038f	75±0.033a	55±0.034e	60±0.031d	66±0.036b	55±0.034e
	Autumn	91.56±0.055b	92±0.052a	54±0.05e	85±0.057d	90±0.06c	90±0.061c	90±0.062c	92±0.06a
Single cocoon weight (gm)	Spring	2.2±0.024a	2.1±0.026b	1.1±0.022g	1.82±0.027e	1.67±0.03f	1.88±0.025d	2±0.024c	1.85±0.026de
	Summer	1.58±0.022de	1.9±0.03a	0.5±0.032g	1.69±0.026b	1.55±0.035ef	1.66±0.029bc	1.5±0.022f	1.62±0.03cd
	Autumn	2.5±0.03a	2.3±0.034b	0.9±0.033d	1.93±0.037c	1.9±0.032c	1.88±0.038c	1.9±0.037c	1.85±0.034c
Cocoon shell weight (gm/ 100 nos)	Spring	37±0.044b	35±0.05c	10±0.046g	37±0.049b	30±0.051f	33±0.049e	34±0.05d	38±0.053a
	Summer	32±0.05b	32±0.044b	11±0.041f	32±0.04b	28±0.048e	29±0.05d	30±0.047c	35±0.053a
	Autumn	38±0.06a	37±0.058b	12±0.062f	36±0.057c	32±0.055e	32±0.055e	34±0.059d	36±0.06c

Note: Results are represented as mean±SEM, n=3. Values with different letters (a, b, c, d, e, f, g & h) are significantly (p<0.05) different from each other by Duncan's Multiple Range Test (DMRT)

Table 6a: Two-way ANOVA analysis (with replication) of different non-enzymatic antioxidant members of eight mulberry leaves with seasonal variation

Source of variation	df	*F*-crit	Ascorbic acid			Glutathione			Chlorophyll			Carotenoids		
			MS	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*
Cultivars	7	2.13	1.05**	130.71	0.000**	16.58**	3709.49	0.000**	122.92**	866.55	0.000**	0.07*	31.66	0.000**
Seasons	4	2.49	3.06**	380.44	0.000**	13.44**	3006.61	0.000**	201.13**	1417.89	0.000**	0.03*	15.13	0.000**
Interaction	28	1.62	0.07**	8.74	0.000**	0.20**	44.29	0.000**	3.38**	23.80	0.000**	0.00	1.09	0.369
Within	80		0.01**			0.00**			0.14*			0.00		

**Significant at p<0.01 and p<0.05 level

Table 6b: Two-way ANOVA analysis (with replication) of different ROS members and compatible osmolyte member of eight mulberry leaves with seasonal variation

Source of Variation	df	*F*-crit	ROS members									Compatible osmolyte member		
			H_2O_2			Superoxide			MDA			Proline		
			MS	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*
Cultivars	7	2.13	8.61**	830.61	0.000**	0.43**	105.37	0.000**	406.27**	622.92	0.000**	11.32**	128.77	0.000**
Seasons	4	2.49	31.96**	3081.98	0.000**	3.50**	857.33	0.000**	1356.19**	2079.38	0.000**	4.77**	54.30	0.000**
Interaction	28	1.62	0.62**	59.40	0.000**	0.08**	20.46	0.000**	22.23*	34.08	0.000**	0.454**	5.17	0.000**
Within	80		0.01**			0.00**			0.6522*			0.088*		

**Significant at p<0.01 and p<0.05 level. ROS = Reactive oxygen species

Dendogram analysis

All eight mulberry cultivars were categorized into four groups or cluster. Dudhiya germplasm was separately placed in Group I (fig. 1). S1 and V1 formed another cluster, Group II. Jayasree and Bombay occupied the third group. Mandalaya, S1635 and K2 were placed in Group IV. Dendrogram cluster analysis was performed on the basis of dissimilarities among eight cultivars. S1 and V1 showed similar a kind of ROS, free radical scavengers (FRS) ratio and others biochemical attributes. On the basis of above characters, S1 and V1 are categorized into the acclimated group. Mandalaya, S1635 and K2 are considered as moderately acclimated groups due to moderate accumulation of ROS and scavenger. Dudhiya was placed under non-acclimated group.

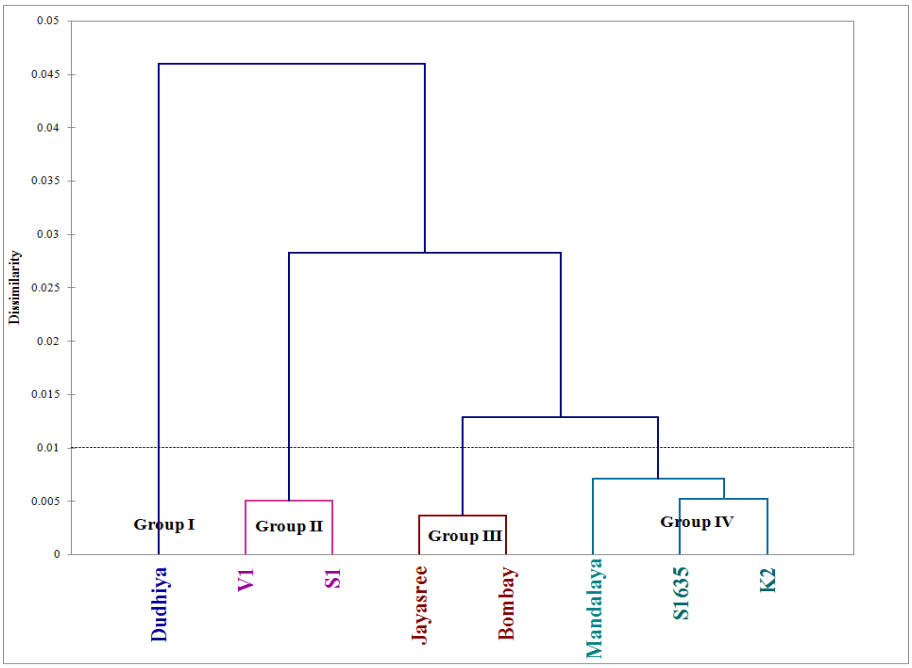

Fig. 1: Dendrogram cluster analysis of eight mulberry cultivars

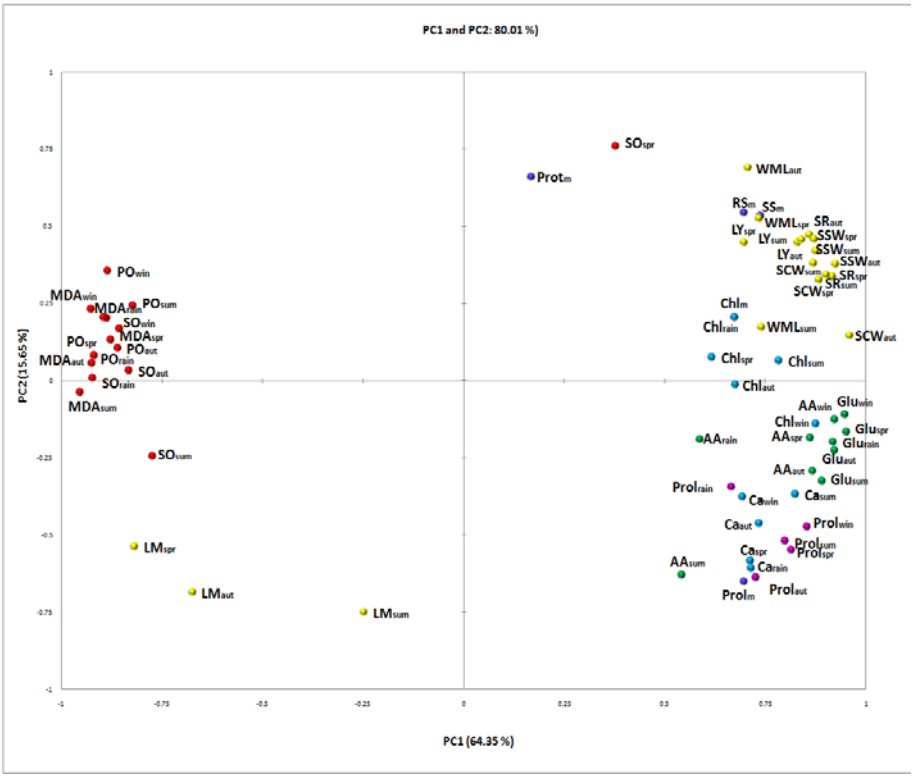

Fig. 2: PCA analysis of free radical scavengers (FRS): Non-antioxidant member (green dot), Pigment member (sky blue dot), and compatible osmolytes member (pink dot), ROS: lipid peroxidation member (red dot), biochemical attributes of mature mulberry leaves (purple dot) and economical attributes of rearing system (yellow dot)

Note: SO = Superoxide, Prot = Protein, RS = Reducing sugar, SS = Soluble sugar (total), Prol = Proline, Chl = Chlorophyll, Ca = Carotenoid, AA = Ascorbic Acid, Glu = Glutathione, MDA = Malondialdehyde, PO = Hydrogen Peroxide, WML = weight of mature larvae, SR = shell ratio, SCW = single cocoon weight, SSW = single shell weight, LY = larval yield. Sum = Summer, Win = Winter, Rain = Rainy Season, Spr = Spring, Aut = Autumn, M = Mature leaves

PCA analysis

Principal components analysis (PCA) is used mainly to condense dimensionally the multiple features to two or three dimensions. PCA helps to summarize the variation of correlated multi-attribute with respect to the uncorrelated components set; each has a meticulous linear combination of the original variables. From this PCA analysis (fig. 2) it was found that all economic attributes of the silkworm rearing system at different season formed a clustering pattern. It was also revealed that commercial attributes were influenced by chlorophyll, protein, reducing sugar and total soluble sugar content of young and mature mulberry leaves. On the other hand, free radical scavengers like ascorbic acid, glutathione (non-enzymatic antioxidant member), carotenoids and chlorophyll (pigment member) formed another cluster. ROS such as lipid peroxidation member (H_2O_2, MDA, superoxide, and proline content) occupied the third cluster on this PCA plot. The lipid peroxidation cluster had a negative impact on economic attributes of the rearing system. This PCA analysis helped to visualize our results.

After compiling all the experimental data, it can be stated that silkworm larvae, particularly rejects the Dudhiya germplasm due to the accumulation of excess ROS and peroxidized product generated from membrane lipid particularly during the stress period. In contrast, nutritional and antioxidant rich genotypes were preferred by larvae. Homeostatic action between ROS production and scavenging activities might facilitate proper ROS signalling, which can directly or indirectly help in maintaining optimum plant protein and carbohydrate production. Serres and Mittler reported that ROS-mediated signalling is controlled by balance between ROS production and scavenging [37]. In plant, ROS function as signalling molecules in different cellular processes are also essential for defense, remodelling of the cell wall and polar cell growth [38].

ANOVA analysis

Here, two-way ANOVA analysis with replication was performed to find out the interaction of this two variable, one is cultivars of mulberry leaves, and another is a seasonal variation of biochemical attributes of mulberry leaves. From ANOVA analysis (Table: 6a and 6b) it can be stated that the main effects of both the variance, i.e. cultivars and seasonal variation, have a significant impact on ascorbic acid, glutathione, chlorophyll, H_2O_2, superoxide, and MDA content. Interactions between them were also significant at $p<0.05$ level.

CONCLUSION

The present study revealed that S1 mulberry cultivar showed comparatively high nutritional superiority in respect to the quantity of leaf protein, total sugars, proline, leaf dry matter and chlorophyll content. V1 and S1635 come next as established from their better quality of foliar nutrition. S1, V1 and S1635 were also more tolerant among selected eight mulberry cultivars. Silkworm larvae choose most nutritionally tolerant cultivars like S1, V1 and S1635 and reject susceptible germplasm like Dudhiya for their feeding. Lastly, S1, V1 and S1635 may be recommended for commercial cultivation for better silkworm rearing by nourishing S1, V1 and S1635 leaves.

Authors' contributions

The authors Suchisree Jha, Dr. Palash Mandal and Dr. Amitava Ghosh designed the research work and performed the laboratory experiments along with data analyses; the manuscript was drafted by Suchisree Jha and Dr. Phalguni Bhattacharyya. All authors read and agreed on the final manuscript.

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ABBREVIATION

ROS: Reactive Oxygen Species, FRS: Free Radical Scavenger, DNTB: 5′-dithio-bis (2-nitrobenzoic acid), NADPH: Nicotinamide adenine dinucleotide phosphate oxidase, DNS: Dinitrosalicylic acid, TCA: Trichloroacetic acid, TBA: Thiobarbituric acid, MDA: Malondialdehyde, H_2O_2:Hydrogen peroxide, AA: Ascorbic acid, DMRT: Duncan's multiple range test, PCA: Principal component analysis, SPSS: Statistical package for social sciences (software)

COMPETING INTERESTS

None of the authors have any competing interests to declare

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