Chapter – III

Biochemical attributes of mulberry leaves with seasonal variation

3.1 INTRODUCTION

In sericulture, major costs of cocoon production go towards mulberry leaf production alone. Therefore, recently major focus has given on the improvement of mulberry leaves in terms of quality and quantity. Silkworm larval growth depends on the nutritive value of mulberry leaves (Radjabi *et al.,* 2010). The foliar nutritional value of leaves and biomass production depends on the weather and agricultural practices (Ito, 1978), and it was also changing according to cultivars. On the other hand, it was 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 (Susheelamma *et al.,* 1990). Water scarcity can grab mulberry plant growth and metabolism. Hence, plants experience oxidative stress that reduces production of both primary and secondary plant metabolites (Terman and Brunk, 2006; Reddy *et al.,* 2005). Reduction of plant production affects larval growth, development and ultimately silk production.

Various reports were published on oxidative stress of mulberry plant, and scientists have concentrated on the responses of enzymatic antioxidants (Reddy *et al.,* 2005; 2004 and Chaitanya *et al.,* 2003). Several evidences were reported on important non-enzymatic antioxidants present in different mulberry leaves in response to drought and high-temperature stress (Kotresha *et al.,* 2007). Guha *et al.* (2012) analyzed non-enzymatic antioxidative defense under water and drought stress. Foliar production of mulberry leaves might also 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 nutritional values of leaf and feeding response of silkworm larvae. For the said purpose, seven different mulberry cultivars were selected namely S1, V1, K2, S1635, Kosen and Bombay local with primitive germplasm Dudhiya as a feeding source for silkworm larvae. Different biochemical attributes of selected cultivars 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 different season.

3.2 MATERIALS AND METHODS

3.2.1 Study location

Mention in section 2.2.1

3.2.2 Study methods

3.2.2.1 Rearing of silkworm larvae

Mention in section 2.2.2.1. Feeding trial with seven 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.

3.2.2.2 Study of biochemical attributes

3.2.2.2.1 Estimation of free proline

Free proline content in leaf tissue was determined according to Bates *et al.* (1973). 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 15-20s. 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 fresh weight (FW).

3.2.2.2.2 Estimation of chlorophyll content

Chlorophyll was extracted in 80% acetone, and the amount of total chlorophyll was estimated according to Arnon method (Arnon, 1949).

3.2.2.2.3 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 and Wellburnn (1983), and the results were expressed in mg/g FW.

3.2.2.2.4 Estimation of total carbohydrate (soluble sugars) 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 up to 10ml by adding distilled water. Total soluble sugars were measured by anthrone method (Thimmaiah, 2004). 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 Sadasivam and Manickam, 1996). 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.

3.2.2.2.5 Estimation of total protein content

Total protein content in leaves was estimated by Lowry's method (Lowry *et al.,* 1951). The blue coloured complex was formed after well mixing 5ml alkaline copper solution and Folin-ciocalteu reagent (FCR) with 1 ml protein sample. The colour 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 colour is measured at 660 nm.

3.2.2.2.6 Estimation of glutathione accumulation

Total glutathione content in mulberry leaves was determined according to Griffith and Meister (1979). 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.

3.2.2.2.7 Estimation of MDA accumulation

The extent of lipid peroxidation was determined by quantifying malondialdehyde (MDA) formation (Fu and Huang, 2001). 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 5000g 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.

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

H_2O_2 was estimated according to Becana *et al.* (1986) 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 (1983) with minor modifications. Leaf extract was placed in the 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.

3.2.2.2.9 Estimation of ascorbic acid content (AAC)

Ascorbic acid was determined according to Omaye *et al.* (1979) 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/g FW.

3.2.2.3 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) Principal component analysis (PCA) of biochemical attributes of different cultivars and economical attributes of the rearing system at different season and correlation matrix was analyzed by using XLSTAT 2015 software. Pearson (n) type PCA was used for data analysis.

3.3 RESULTS AND DISCUSSION

3.3.1 Biochemical attributes of mulberry leaves

Silkworm larvae require foliar nutrients in the precise ratio for their growth and development. Imbalance in foliar nutrients 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 (Das *et al.,* 2001). 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 different seasons. During winter season (November and February) mulberry plants experience serious osmotic stress due to a significant drop of water potential in soil. Due to excess rain, the flood situation and hypoxic stress again might be created during rainy season (mid-July). During this time soil contains low microelements. Mulberry plants also suffer oxidative stress due to deficiency of microelements like Mn, N, P, and K in soil (Tewari *et al.,* 2007; 2013). Besides these two stressful points, the climatic conditions during remaining periods are favourable for plant growth. Oxidative stress parameters of mulberry leaves were evaluated by considering the seasonal fluctuations of free radical accumulation in the plant body.

The mulberry genotypes were categorized into two classes: acclimated and non-acclimated. Acclimated genotypes are those who can build up high free radical scavengers and reduces much more free radicals, which produced in plant body during the stress periods. If the genotypic responses of mulberry in this situation were considered, it was clearly found that S1, V1 and S1635 variety could successfully manage the minimal accumulation of free radicals like peroxide, superoxide and MDA (Figure 3.1a-h, 3.2a-h and 3.3a-h). Whereas, moderate accumulations of the free radicals were recorded in Bombay local along with Kosen cultivars and Dudhiya cultivar accumulated high free radicals.

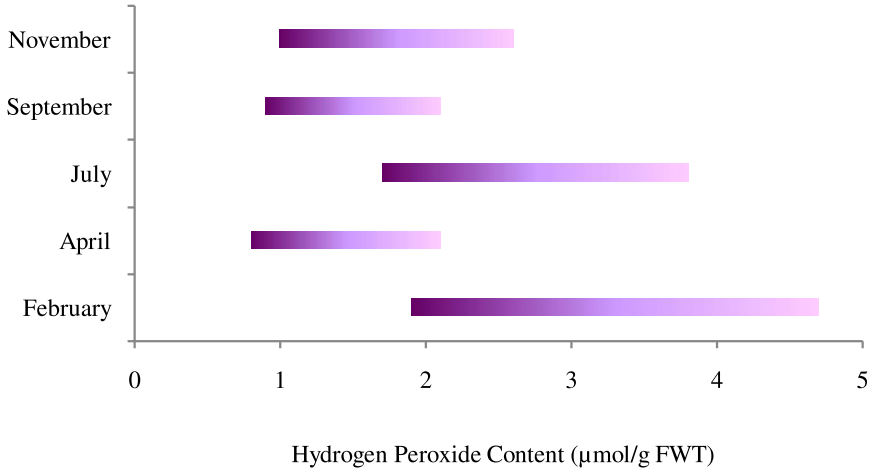


Figure 3.1a: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in V1 mulberry variety

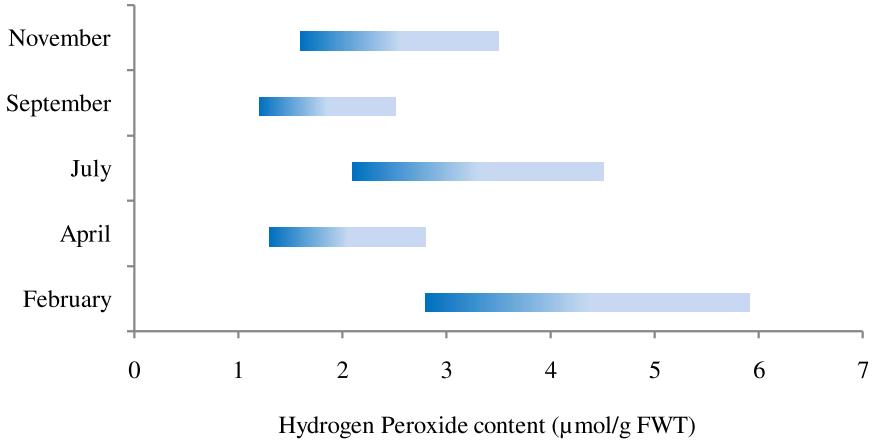


Figure 3.1b: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in S1 mulberry variety

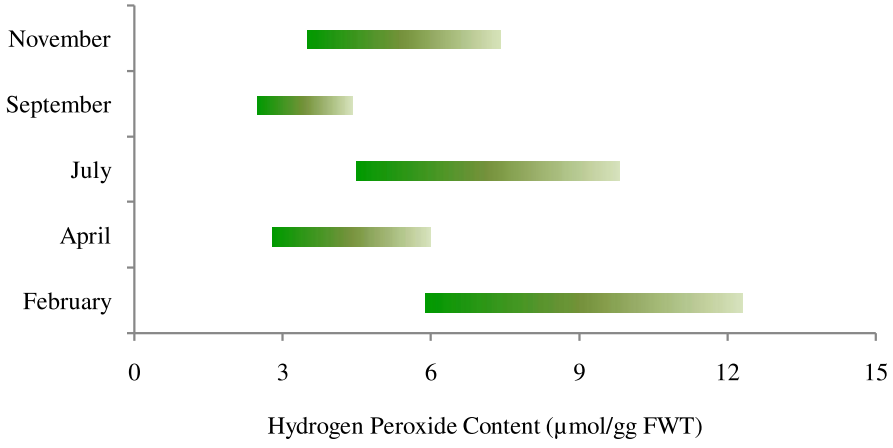


Figure 3.1c: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in Dudhiya mulberry variety

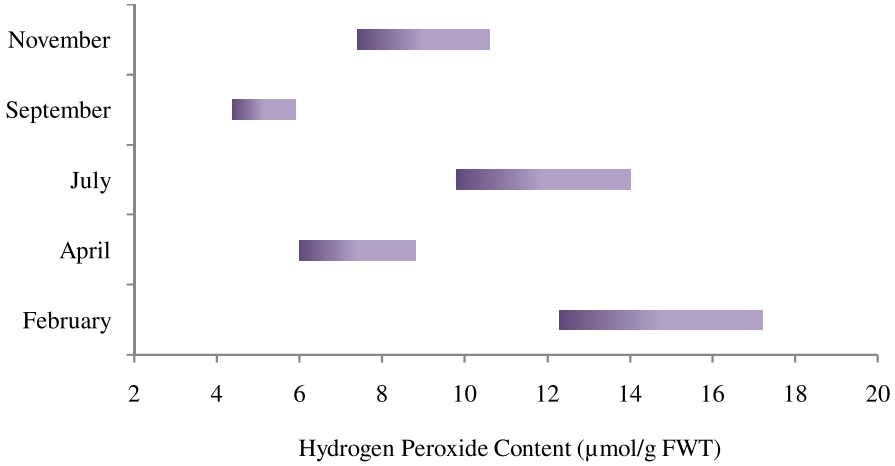


Figure 3.1d: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in S1635 mulberry variety

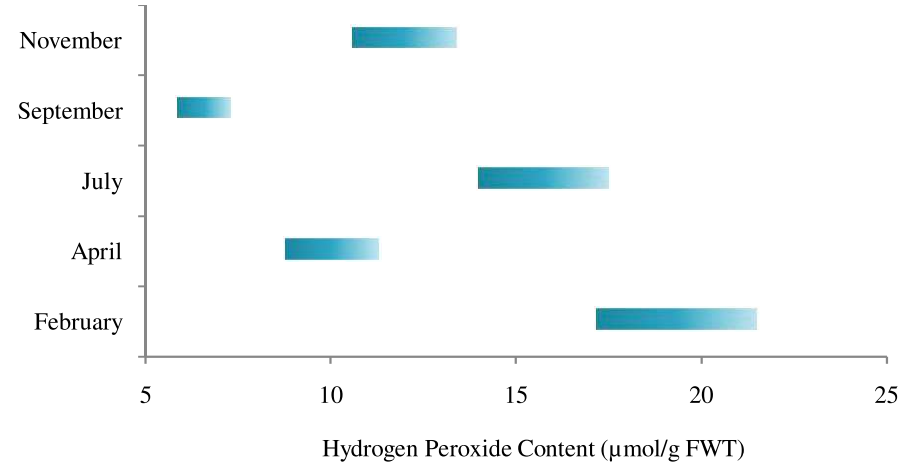


Figure 3.1e: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in K2 mulberry variety

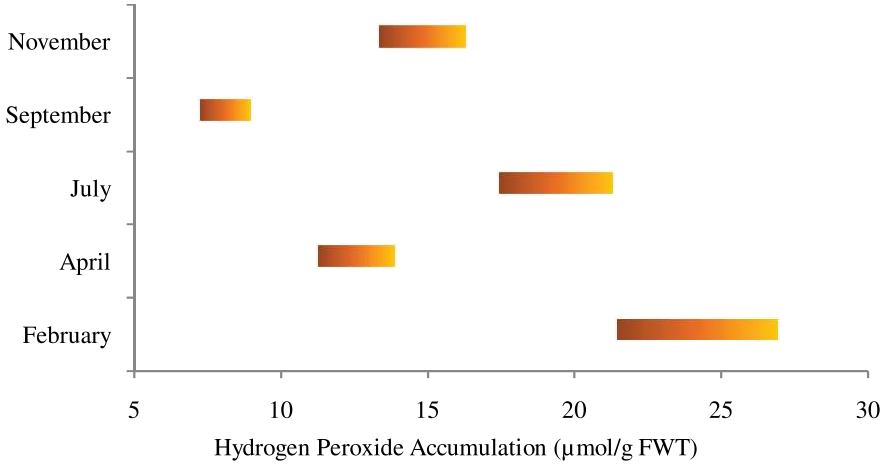


Figure 3.1f: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in Bombay local mulberry cultivar

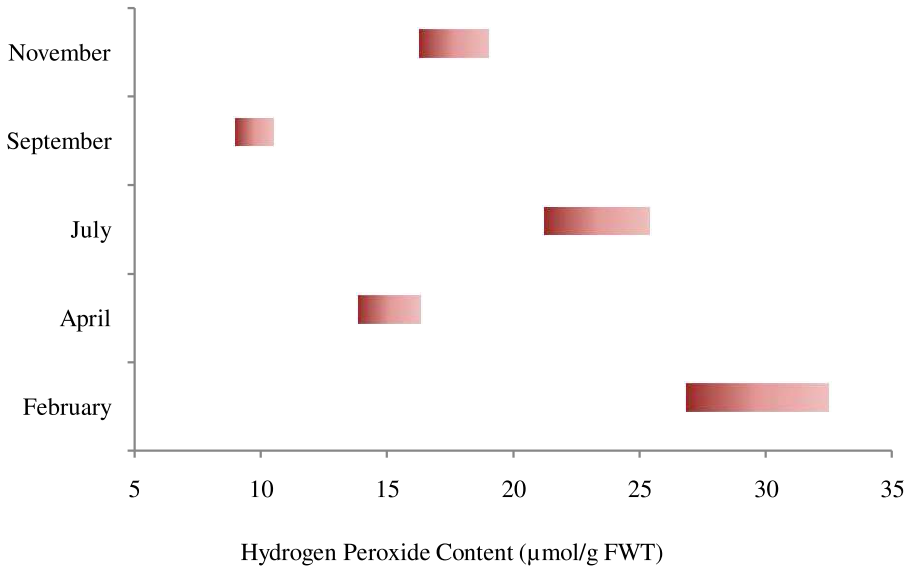


Figure 3.1g: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in Kosen mulberry variety

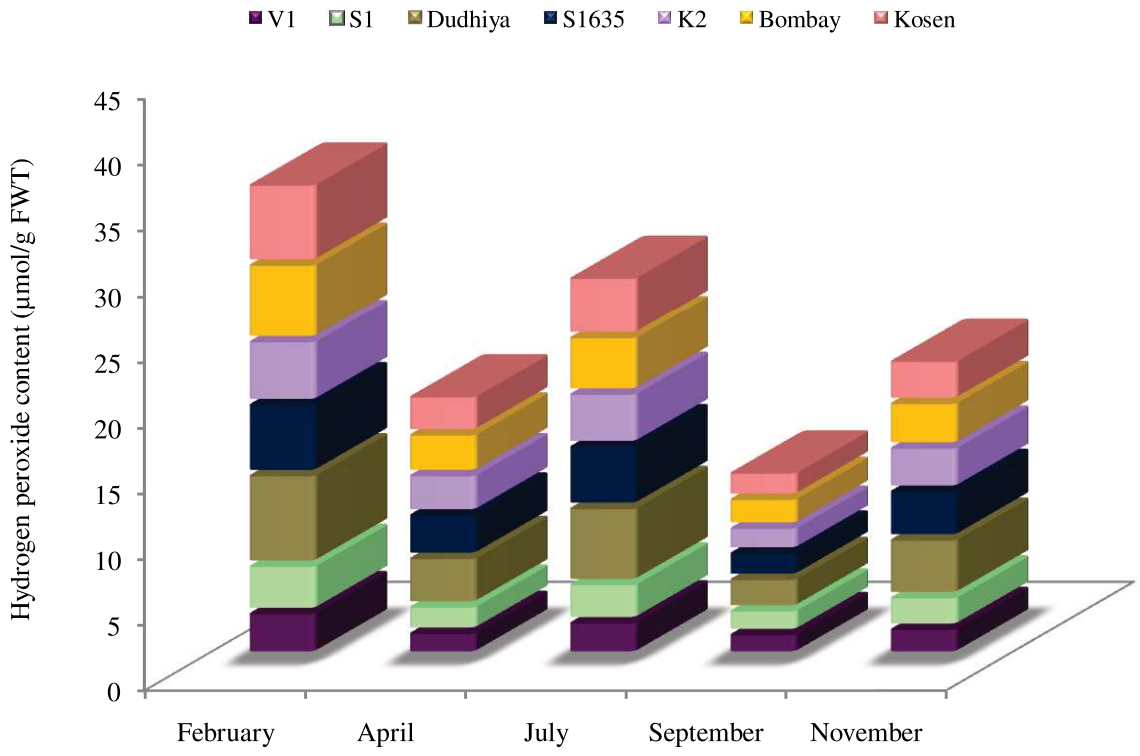


Figure 3.1h: Range of hydrogen peroxide (H_2O_2) accumulation at different seasons in all seven mulberry varieties: a comparative account

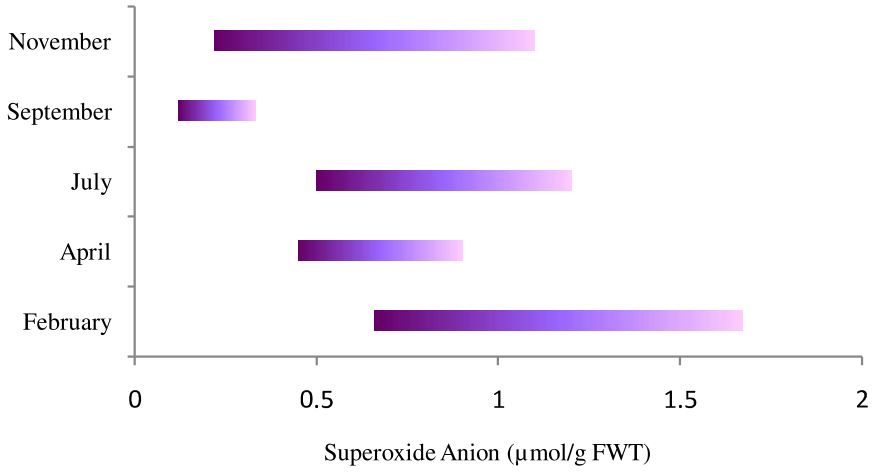


Figure 3.2a: Range of superoxide anion accumulation at different seasons in V1 mulberry variety

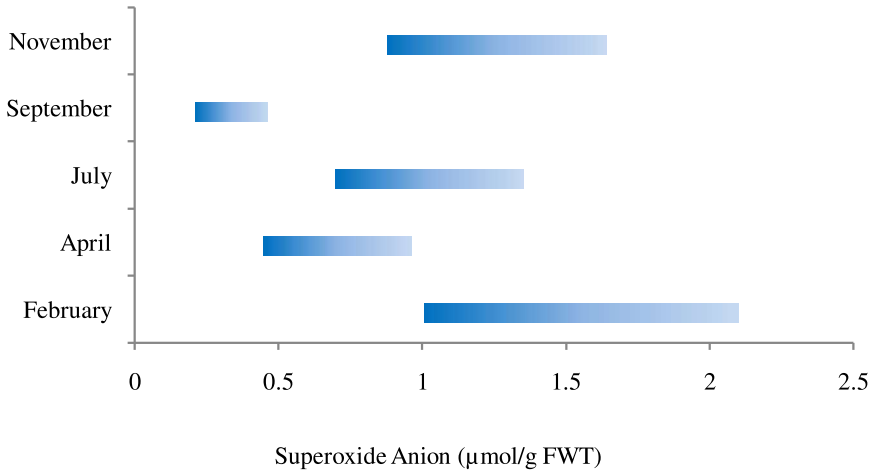


Figure 3.2b: Range of superoxide anion accumulation at different seasons in S1 mulberry variety

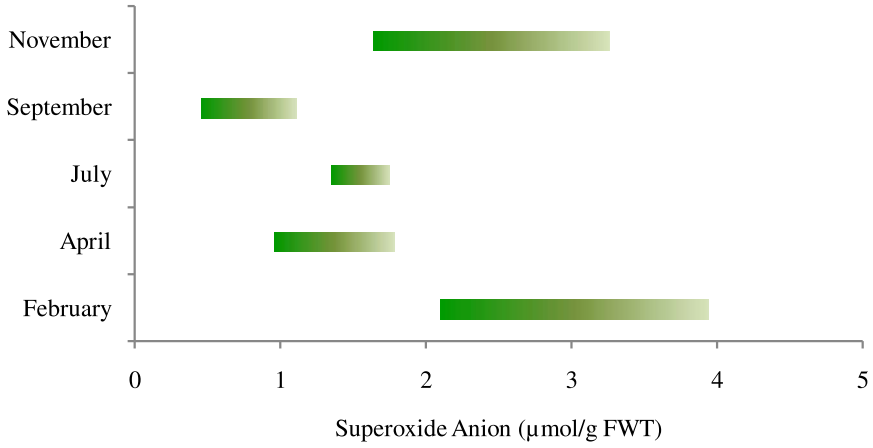


Figure 3.2c: Range of superoxide anion accumulation at different seasons in Dudhiya mulberry variety

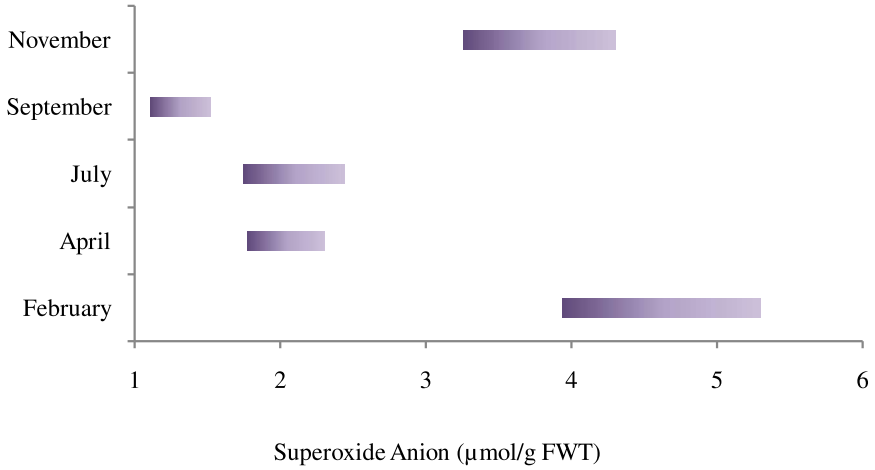


Figure 3.2d: Range of superoxide anion accumulation at different seasons in S1635 mulberry variety

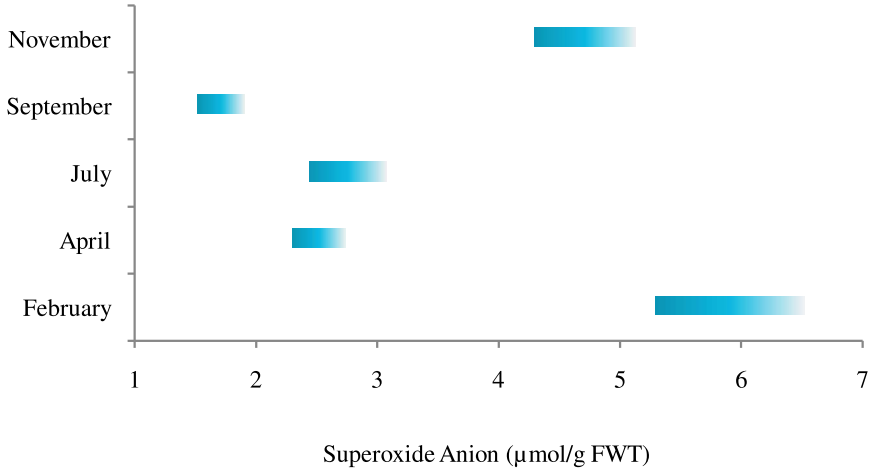


Figure 3.2e: Range of superoxide anion accumulation at different seasons in K2 mulberry variety

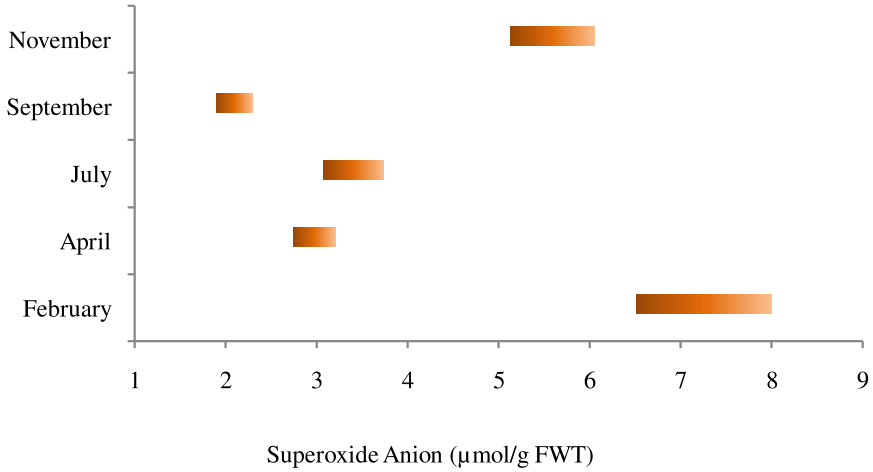


Figure 3.2f: Range of superoxide anion accumulation at different seasons in Bombay local mulberry cultivar

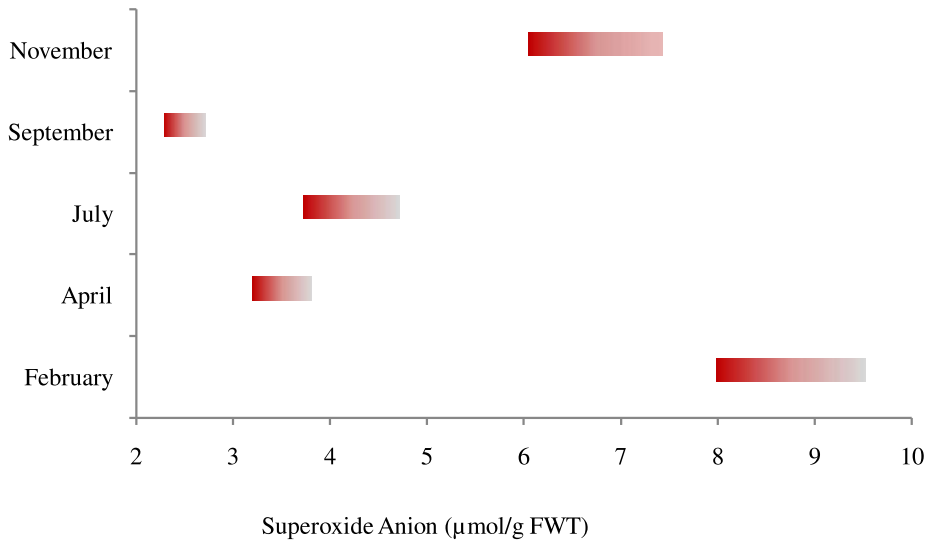


Figure 3.2g: Range of superoxide anion accumulation at different seasons in Kosen mulberry variety

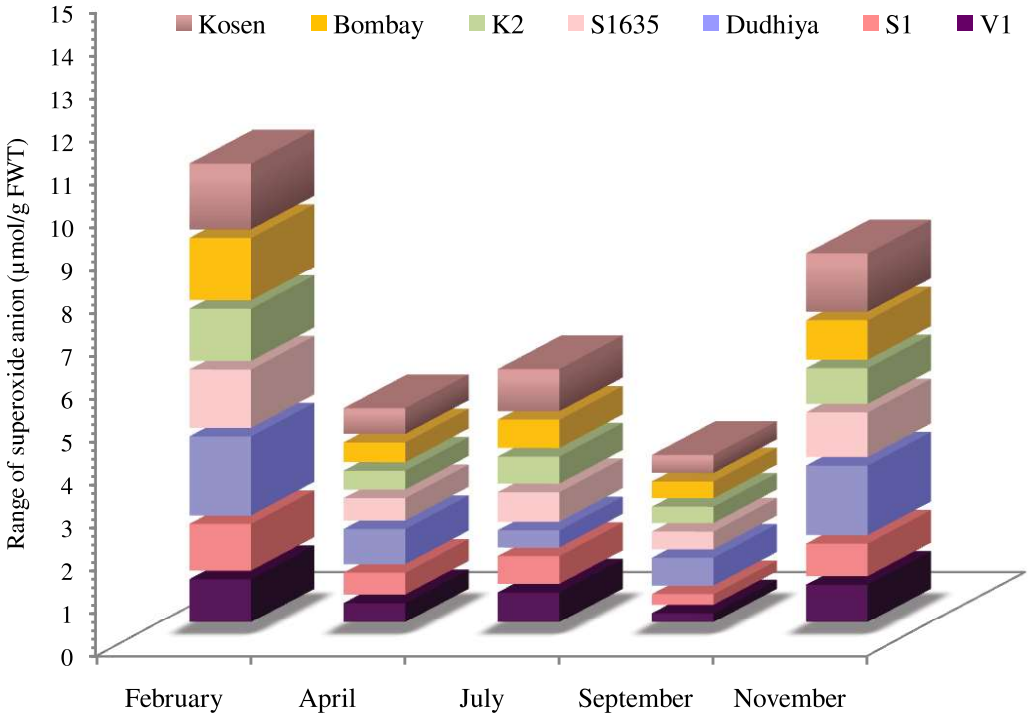


Figure 3.2h: Range of superoxide anion accumulation at different seasons in seven mulberry cultivars: a comparative account

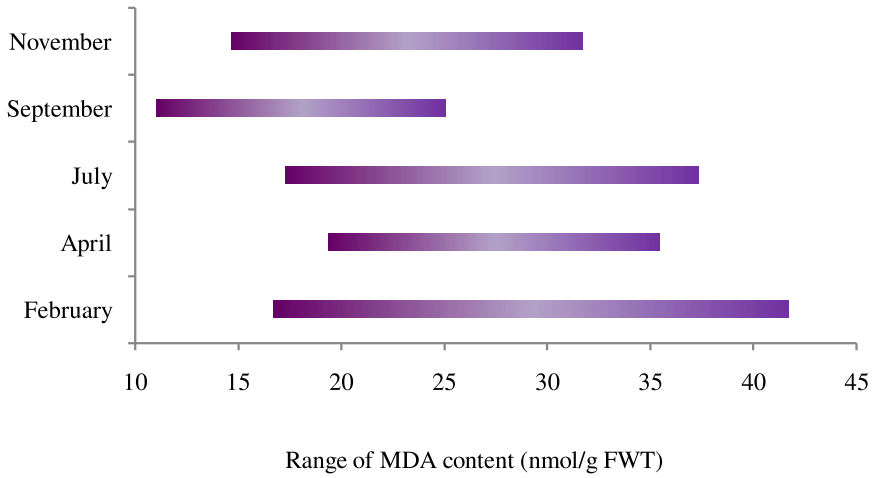


Figure 3.3a: Range of MDA accumulation at different seasons in V1 mulberry variety

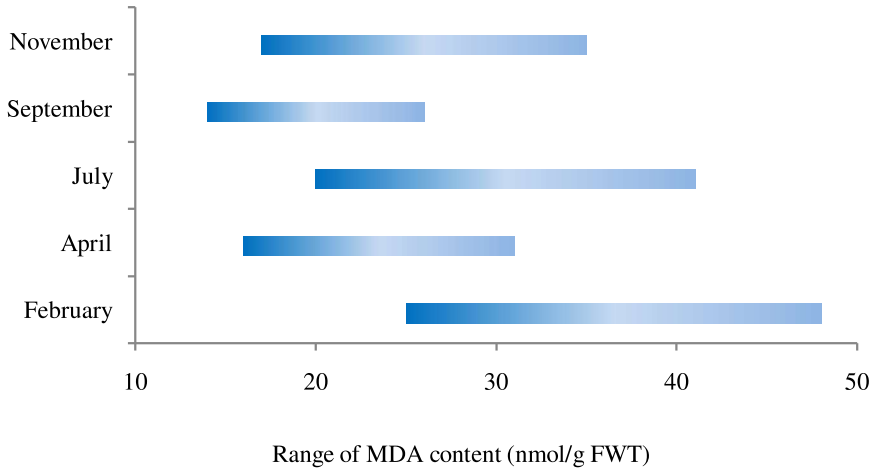


Figure 3.3b: Range of MDA accumulation at different seasons in S1 mulberry variety

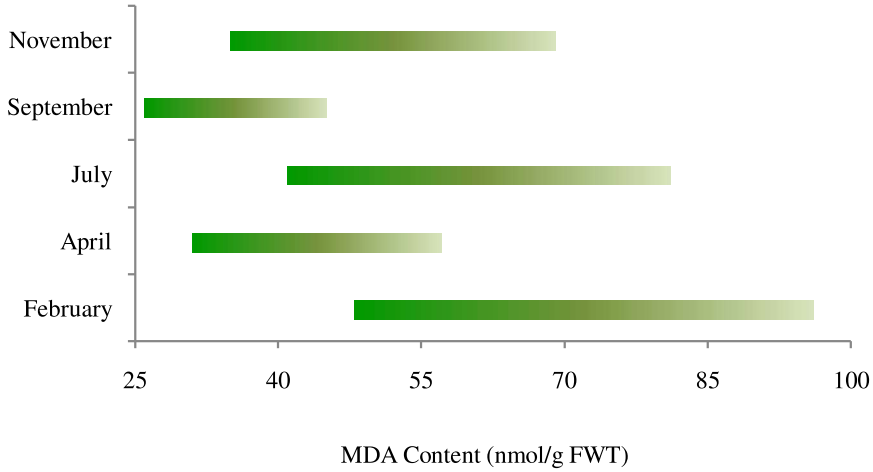


Figure 3.3c: Range of MDA accumulation at different seasons in Dudhiya mulberry variety

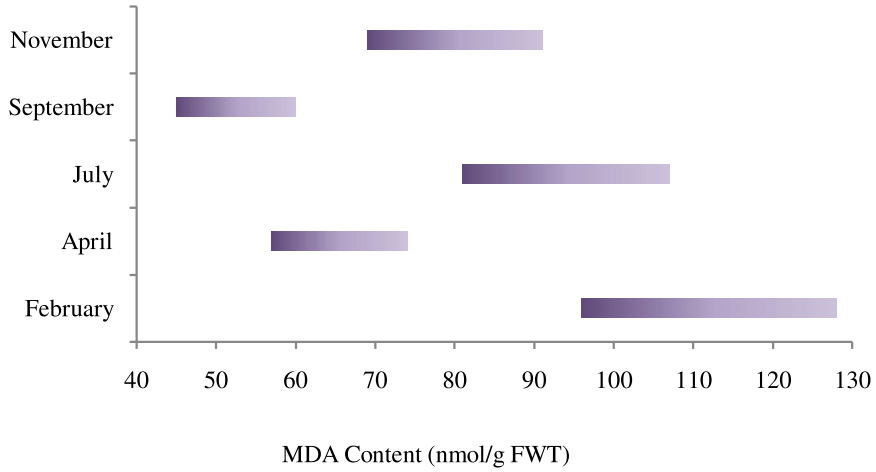


Figure 3.3d: Range of MDA accumulation at different seasons in S1635 mulberry variety

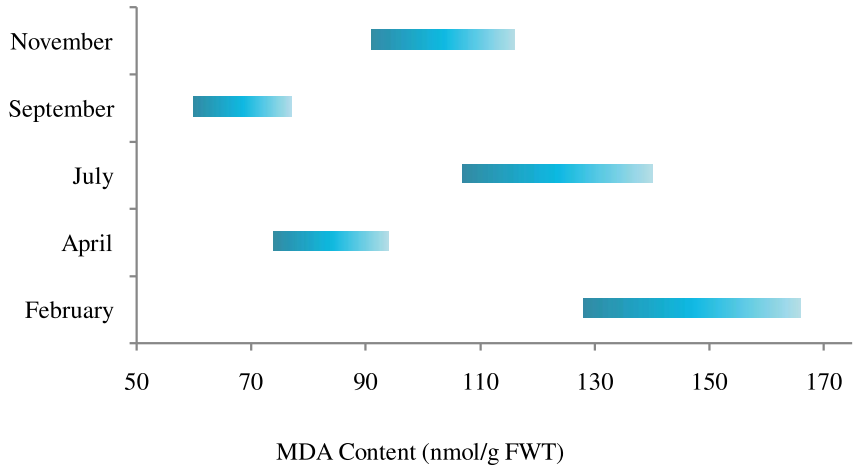


Figure 3.3e: Range of MDA accumulation at different seasons in K2 mulberry variety

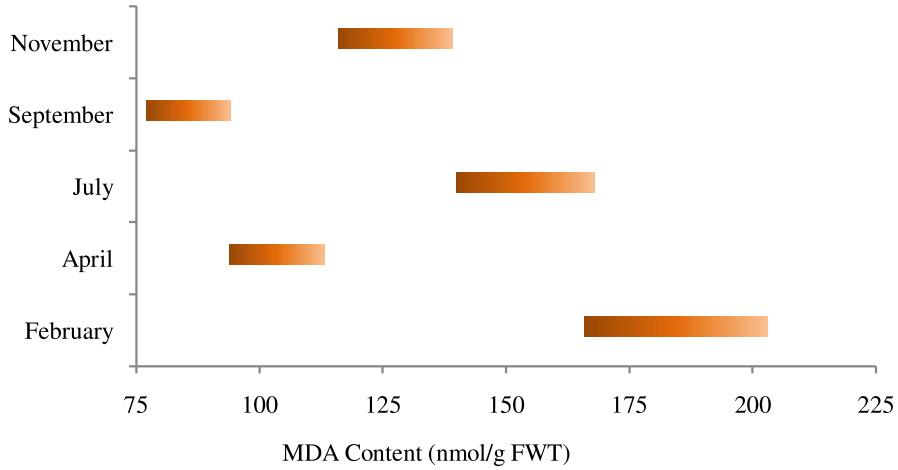


Figure 3.3f: Range of MDA accumulation at different seasons in Bombay local mulberry cultivars

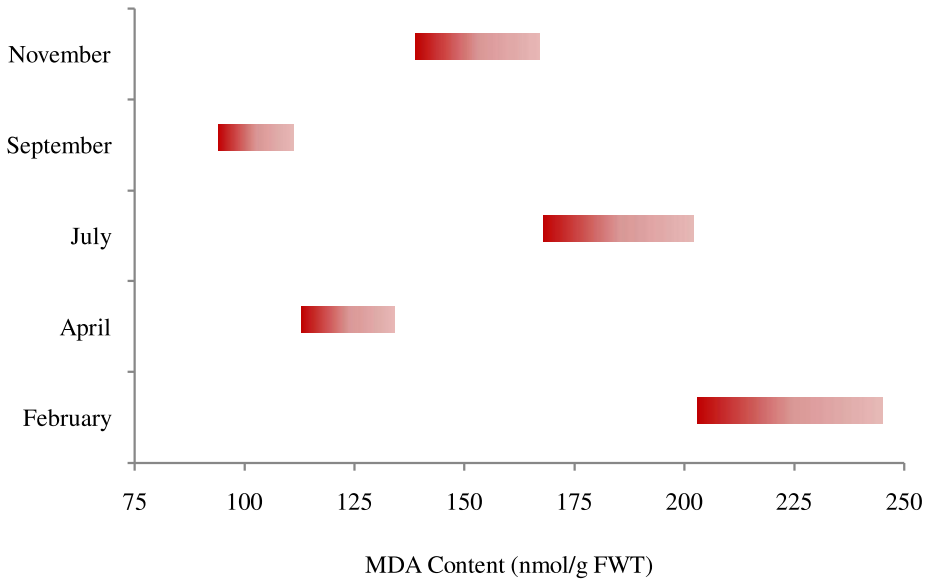


Figure 3.3g: Range of MDA accumulation at different seasons in Kosen mulberry variety

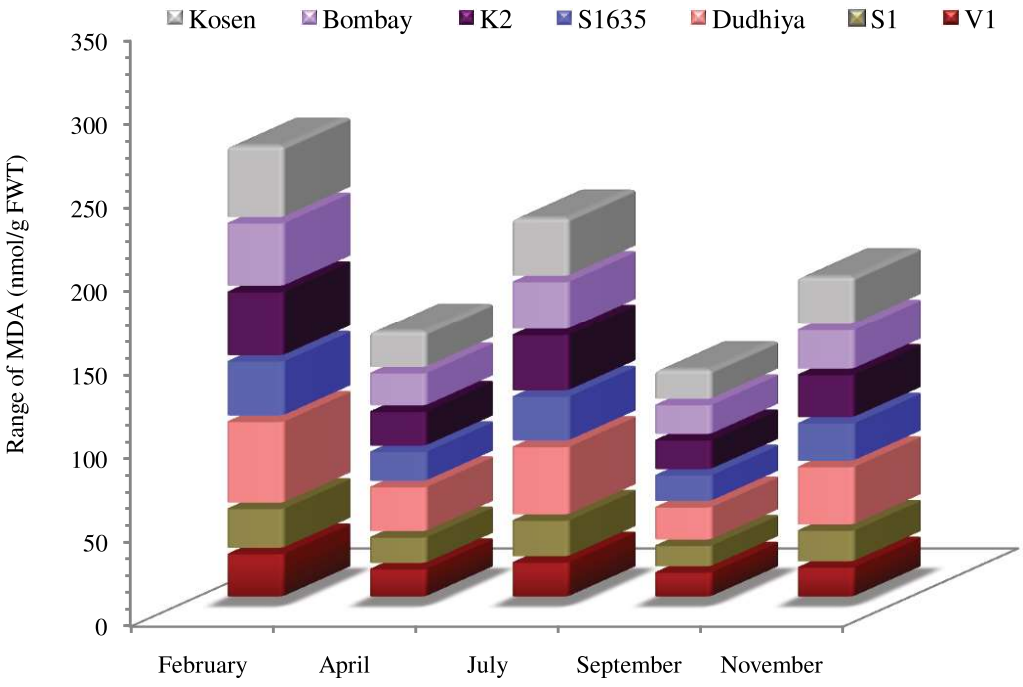
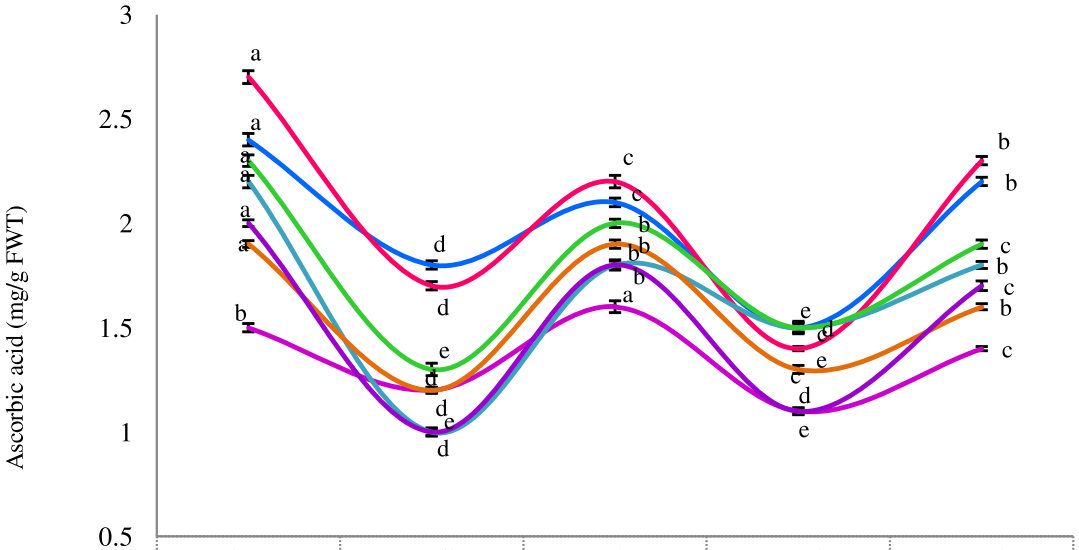
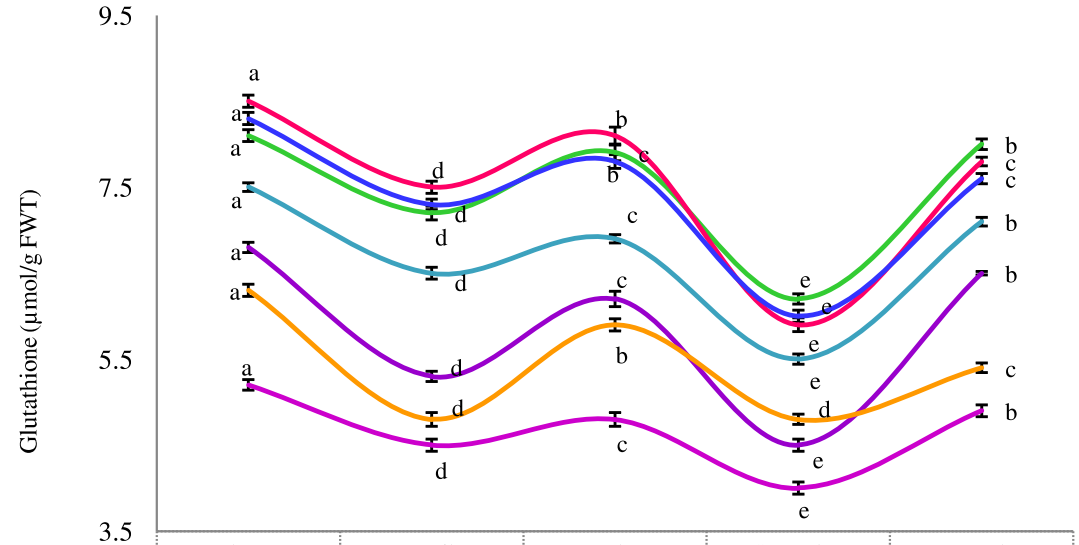


Figure 3.3h: Range of MDA accumulation at different seasons in seven mulberry leaves: A comparative account



	February	April	July	September	November
V1	2.4	1.8	2.1	1.5	2.2
S1	2.7	1.7	2.2	1.4	2.3
Dudhiya	1.5	1.2	1.6	1.1	1.4
S1635	2.2	1	1.8	1.5	1.8
K2	2.3	1.3	2	1.5	1.9
Kosen	1.9	1.2	1.9	1.3	1.6
Bombay	2	1	1.8	1.1	1.7

Figure 3.4: Ascorbic acid accumulation at different seasons in seven mulberry variety



	February	April	July	September	November
V1	8.1	7.2	7.9	6.2	8
S1	8.5	7.5	8.1	5.9	7.8
Dudhiya	5.2	4.5	4.8	4	4.9
S1635	7.5	6.5	6.9	5.5	7.1
K2	8.3	7.3	7.8	6	7.6
Kosen	6.8	5.3	6.2	4.5	6.5
Bombay	6.3	4.8	5.9	4.8	5.4

Figure 3.5: Glutathione accumulation at different seasons in seven mulberry variety

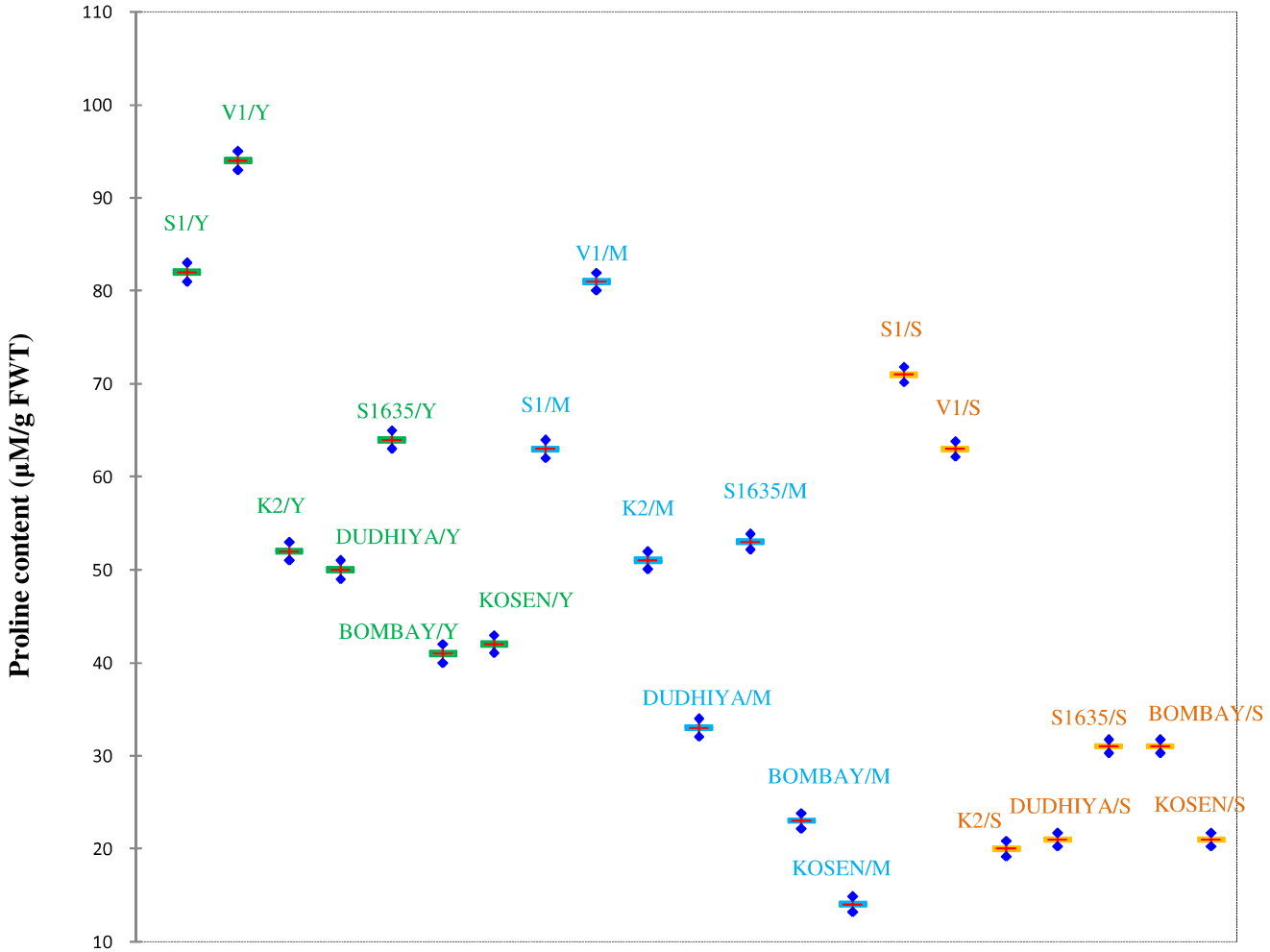


Figure 3.6: Proline content of seven mulberry varieties at different maturity stages

Abb. Used: Y: young; M: mature; S:senescence

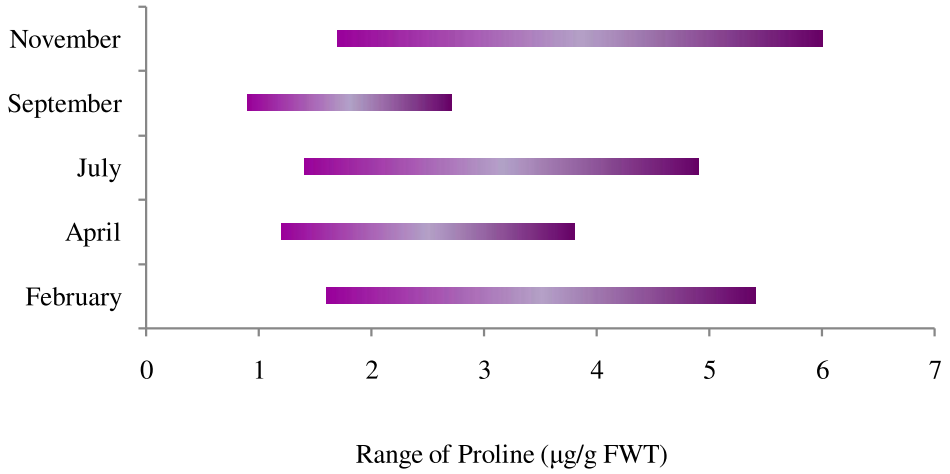


Figure 3.7a: Range of proline accumulation at different seasons in V1 mulberry variety

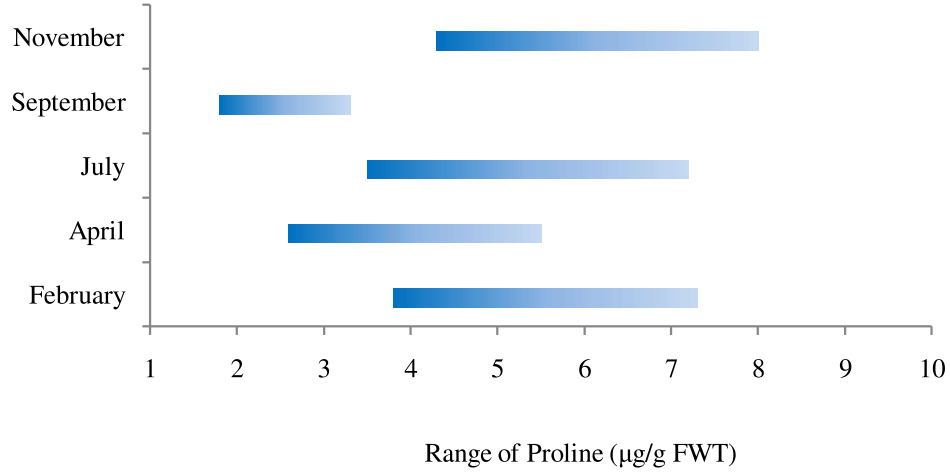


Figure 3.7b: Range of proline accumulation at different seasons in S1 mulberry variety

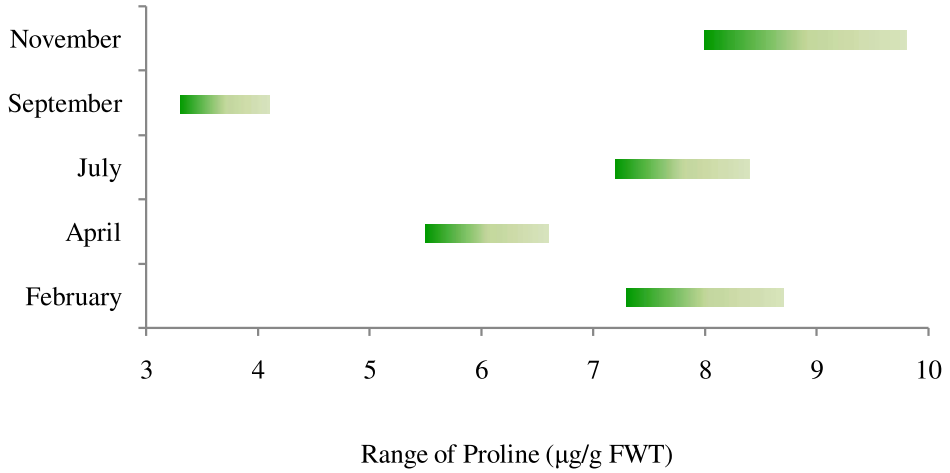


Figure 3.7c: Range of proline accumulation at different seasons in Dudhiya mulberry variety

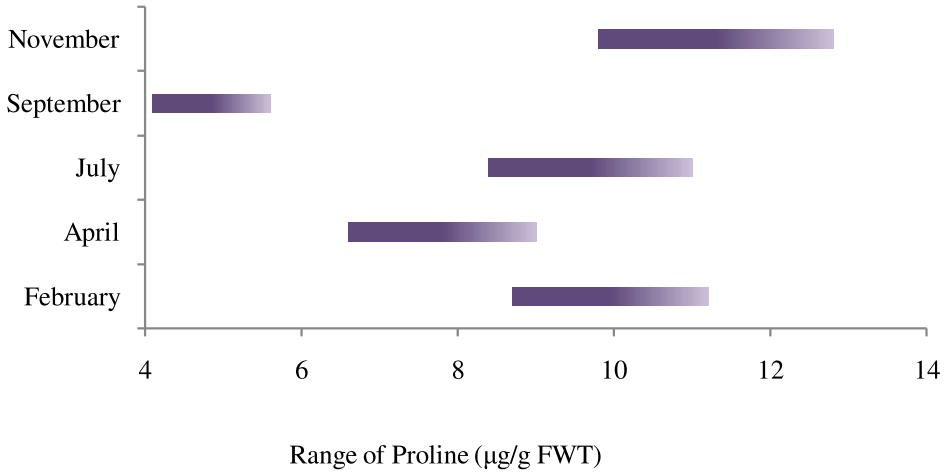


Figure 3.7d: Range of proline accumulation at different seasons in S1635 mulberry variety

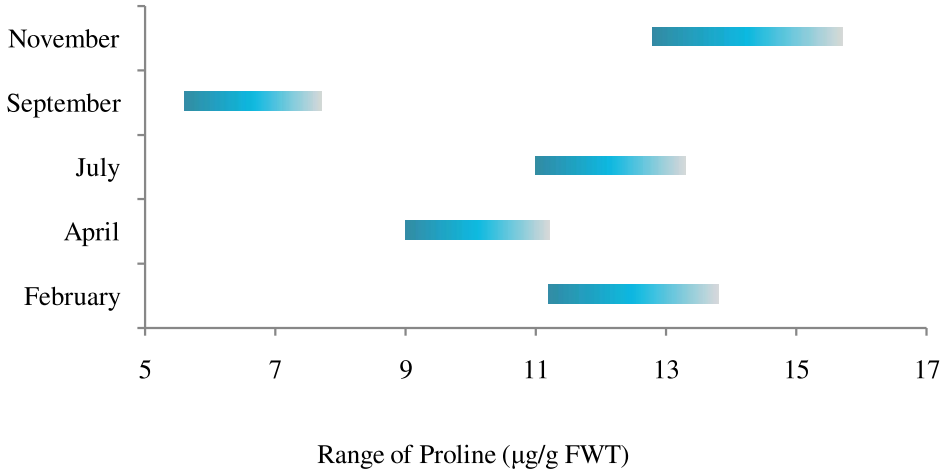


Figure 3.7e: Range of proline accumulation at different seasons in K2 mulberry variety

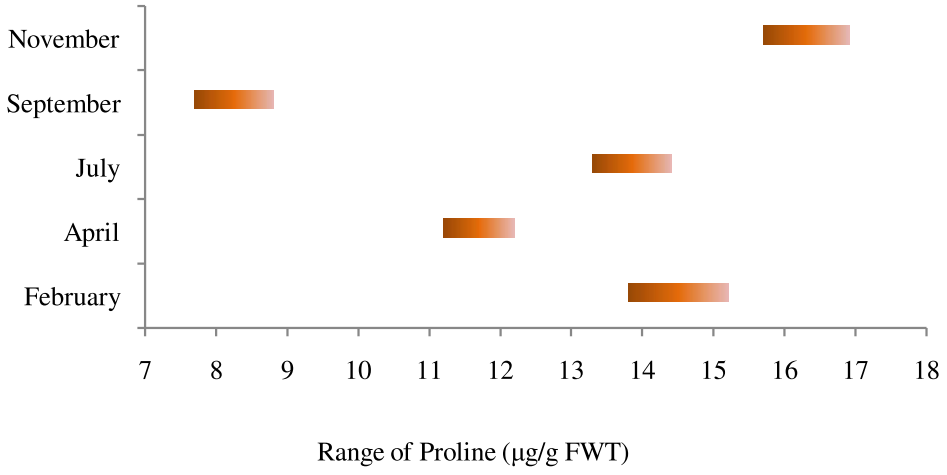


Figure 3.7f: Range of proline accumulation at different seasons in Bombay local mulberry cultivar

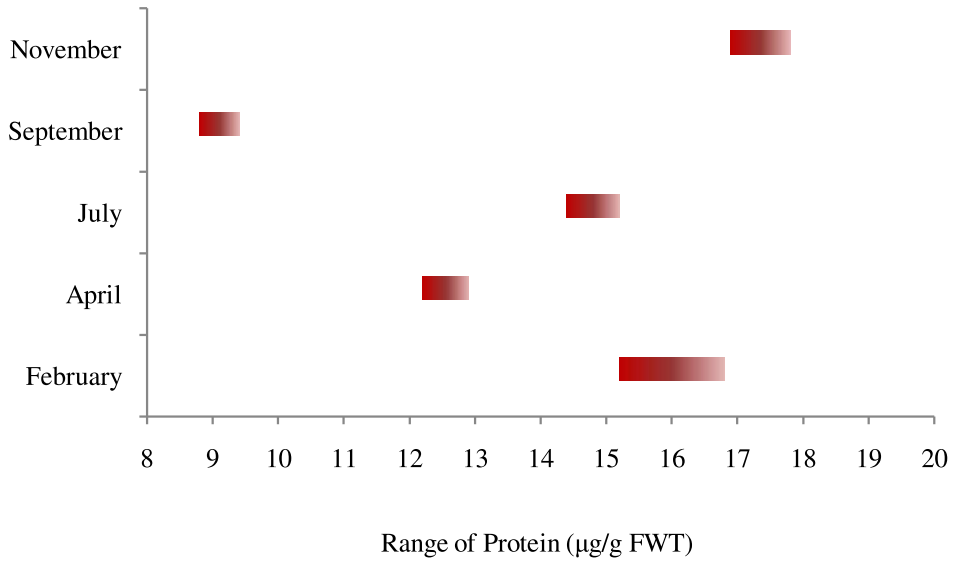


Figure 3.7g: Range of proline accumulation at different seasons in Kosen mulberry variety

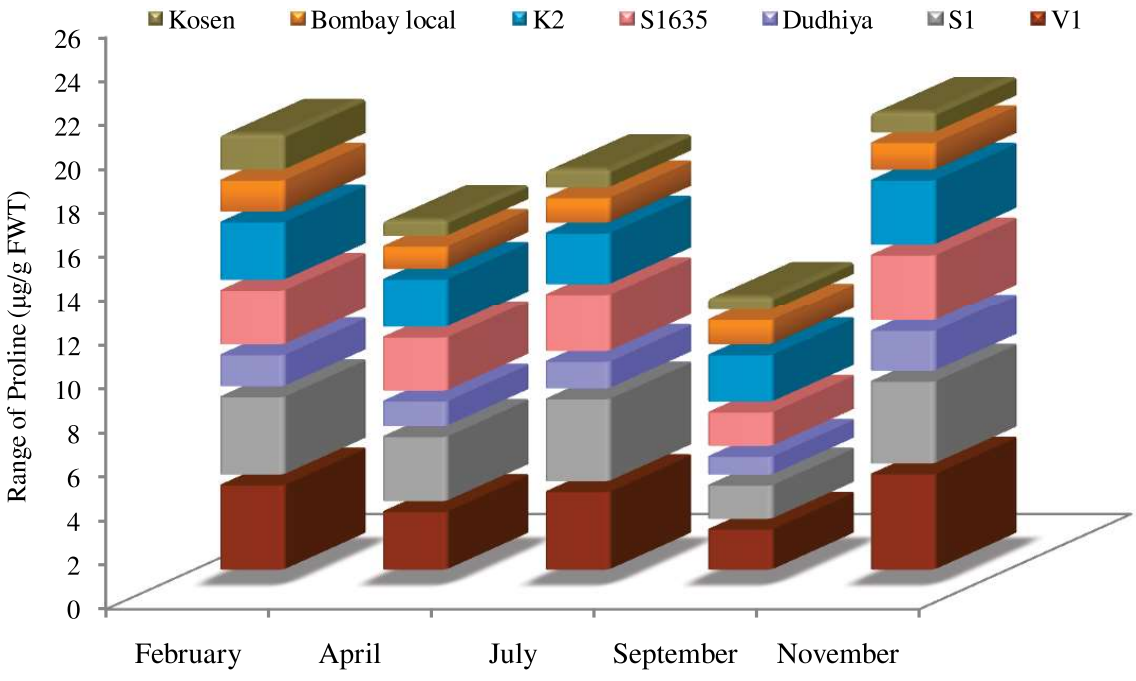
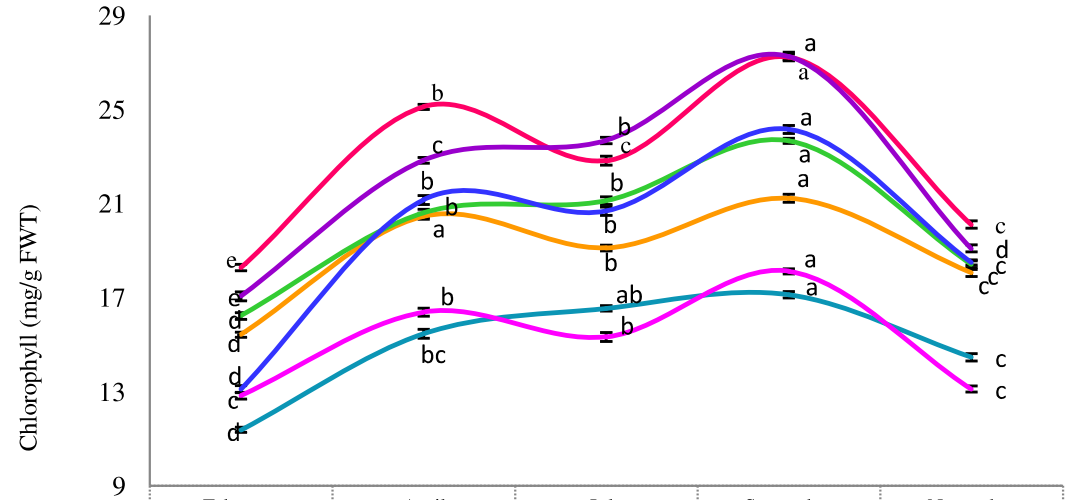
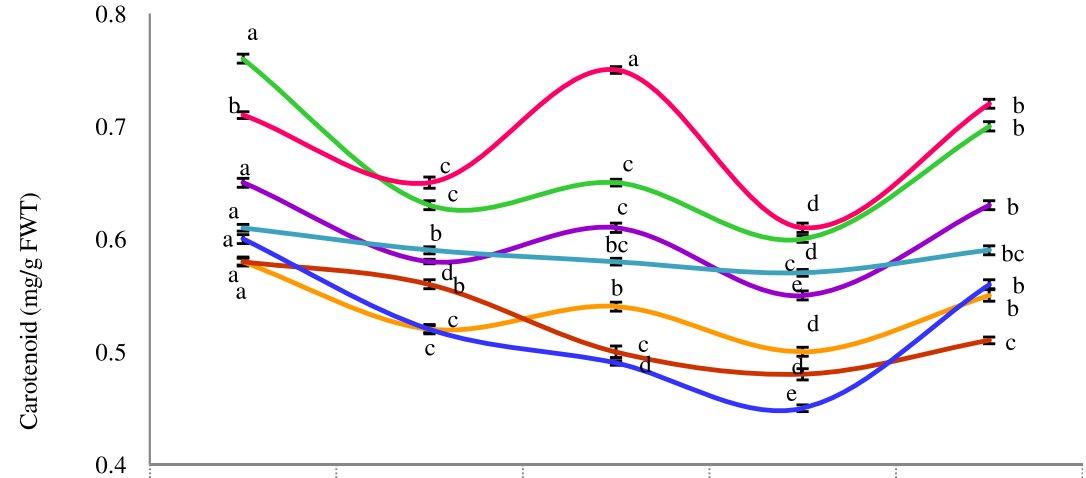


Figure 3.7h: Range of proline accumulation at different seasons in seven mulberry leaves: a comparative account



	February	April	July	September	November
V1	15.42	20.46	19.11	21.22	18.05
S1	18.28	25.11	22.82	27.23	20.11
Dudhiya	11.38	15.46	16.54	17.12	14.46
S1635	17.05	22.84	23.68	27.25	19.09
K2	16.22	20.59	21.12	23.67	18.42
Kosen	12.82	16.38	15.33	18.12	13.11
Bombay	13.11	21.15	20.67	24.15	18.49

Figure 3.8: Chlorophyll (photoassimilates) accumulation at different seasons in seven mulberry variety



	February	April	July	September	November
V1	0.76	0.63	0.65	0.6	0.7
S1	0.71	0.65	0.75	0.61	0.72
Dudhiya	0.58	0.52	0.54	0.5	0.55
S1635	0.65	0.58	0.61	0.55	0.63
K2	0.61	0.59	0.58	0.57	0.59
Kosen	0.58	0.56	0.5	0.48	0.51
Bombay	0.6	0.52	0.49	0.45	0.56

Figure 3.9: Carotenoid (photo-bleaching system) accumulation at different seasons in S1 mulberry variety

The ascorbic acid and glutathione, both were sufficiently accumulated during winter and rainy season in V1 and S1 cultivars, but this accumulation was significantly lesser in Dudhiya genotypes (Figure 3.4 and 3.5). This indicated that the glutathione-ascorbate pool gave sufficient feedback for the regeneration of other antioxidant molecules among stress tolerant cultivars like V1, S1 and S1635 during the crisis phase.

The other ways of defense were an accumulation of compatible osmolytes like proline and protection from photo-bleaching due to carotenoid pigment, and sufficient accumulation of photo-assimilates 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 (Yogananda Murthy *et al.,* 2013). Highest proline was obtained from young leaves of the V1 cultivar than others (Figure 3.6). Maximum proline was accumulated during winter and rainy seasons (Figure 3.7a-h). It was reported that level of proline in mulberry plants increased under water stress (Sarkar *et al.,* 1993). Raggi (1994) observed high proline content in bean plant under water stress condition. Sufficient chlorophyll was accumulated during September and April (Figure 3.8) whereas carotenoid was highly accumulated during winter (February) and rainy (July) season (Figure 3.9). In the case of all three responses also, these stress-tolerant varieties or acclimated genotypes adapted better than non-acclimated Dudhiya genotype.

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 (Bongale, 1995). Several studies were conducted for finding the varietal difference in leaf protein content (Mishra *et al.,* 1996; Ram Rao *et al.,* 2000). In this study, when cultivars were compared, it was observed that S1635 contained higher amount of protein (Figure 3.10). Higher protein content was also measured in mature leaves but according to Matei *et al.* (2006), 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 (Chaluvachari and Bongale, 1996).

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 (Figure 3.11). Lowest chlorophyll content was observed in young, mature and senescent leaves of Dudhiya. Similarly, Hotta (1975) 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 (2002) reported that S1635 and S1 had highest chlorophyll 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 mature leaves of Kosen followed by S1635, S1, V1, Bombay Local, K2, and Dudhiya (Figure 3.12). In the present study, S1635 also showed higher reducing sugar content than the remaining six cultivars of mulberry leaves (Figure 3.13). Similarly, Purohit and Pavankumar (1996) also obtained highest carbohydrate content (22.83%) in S1635 cultivar. Present experiment revealed that mature leaves had highest soluble sugar in all mulberry cultivars. But Yogananda Murthy *et al.* (2013) reported contradictory results. According to Yogananda Murthy *et al.* (2013) 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 3.2). Yogananda Murthy *et al.* (2013) reported that sugars help to produce main energy for metabolic activity.

3.3.2 Feeding response

The feeding performance with seven mulberry cultivars at three different seasons was shown in Table 3.1. Larval weight depends on the nutritive values of mulberry leaves which differ according to different cultivars of mulberry (Gangwar, 2010). Highest larval weight was recorded in S1 followed by S1635, V1, Bombay local, Kosen, K2 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 (2010), where the mortality rate was high in spring. The weight of single cocoon was enhanced by feeding S1 mulberry leaves followed by V1, S1635, Kosen, Bombay local, K2, and Dudhiya at the autumn season and gradually declined during spring and summer. Similarly, Kumar *et al.* (2013) obtained highest cocoon weight by feeding S1 leaves. Likewise cocoon weight and 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 (2010) found better larval growth and increased the weight of shell influenced by BR2 mulberry cultivars at Uttar Pradesh, India.

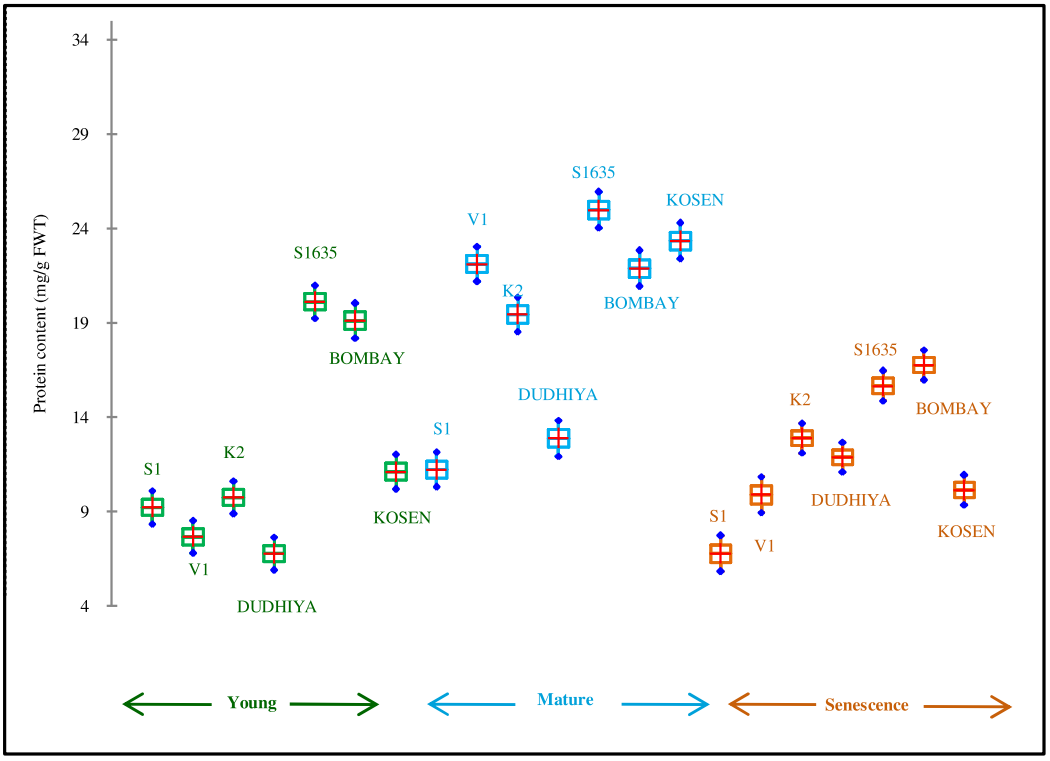


Figure 3.10: Total protein content of seven mulberry leaves at different maturity stages

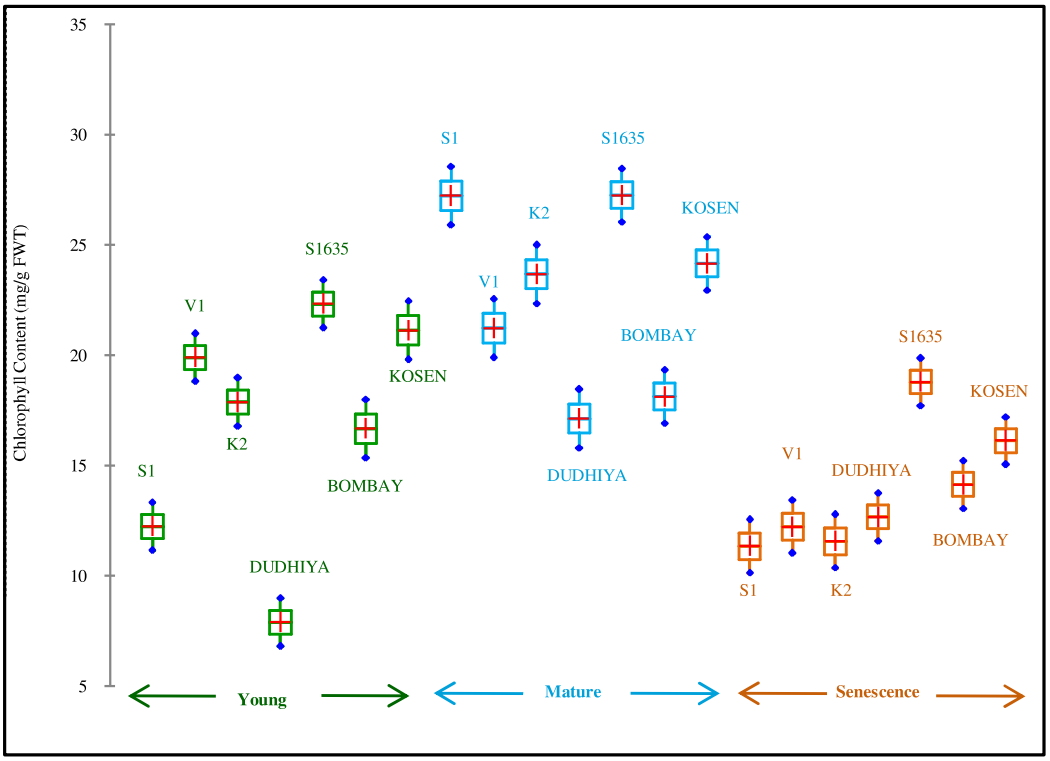


Figure 3.11: Total chlorophyll content of seven mulberry leaves at different maturity stages

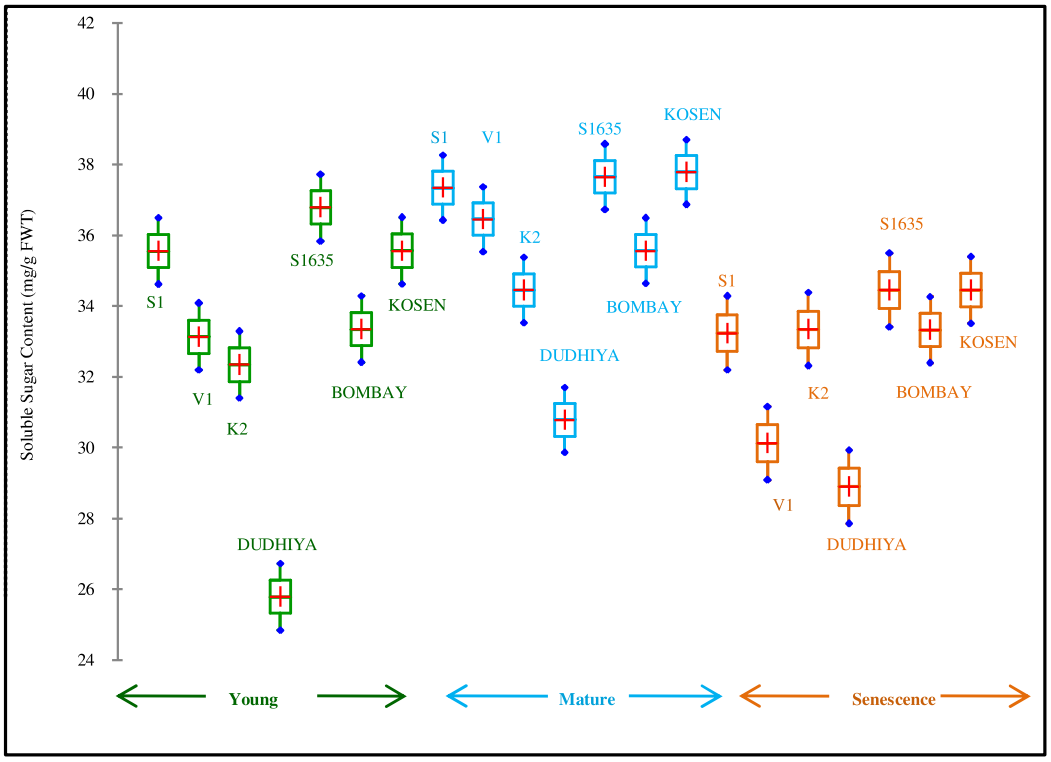


Figure 3.12: Total soluble sugar content of seven mulberry leaves at different maturity stages

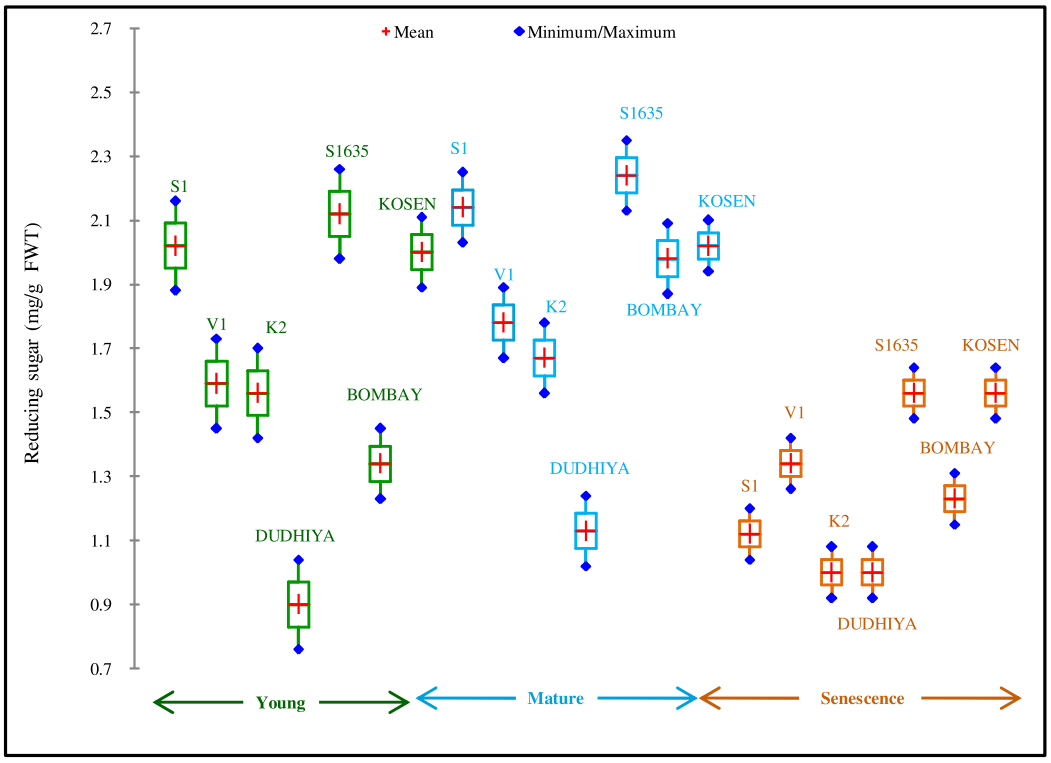


Figure 3.13: Total reducing sugar content of seven mulberry leaves at different maturity stages

Table 3.1: Feeding response under nourishment with seven selected mulberry varieties

Parameter	Season	S1	V1	Dudhiya	S1635	Bombay local	Kosen	K2
Weight of matured larvae (gm/20 nos)	Spring	48±0.53^{a}	44±0.81^{c}	22±0.84^{g}	45±0.93^{b}	44±0.71^{c}	43±0.82^{d}	40±0.83^{e}
	Summer	43±0.89^{a}	35±0.63^{b}	21±0.58^{g}	33.13±0.61^{c}	35±0.51^{b}	32±0.62^{d}	30±0.67^{e}
	Autumn	39±0.74^{e}	38±0.92^{f}	15±0.86^{h}	39.12±0.87^{d}	42±0.75^{b}	44±0.74^{a}	41±0.76^{c}
Survival rate of pupae (%)	Spring	95±0.65^{b}	96±0.55^{a}	50±0.41^{f}	96±0.654^{a}	90±0.65^{c}	84±0.65^{e}	95±0.63^{b}
	Summer	92±0.68^{a}	90±0.65^{c}	45±0.61^{h}	91±0.67^{b}	87±0.55^{e}	78±0.63^{f}	89±0.61^{d}
	Autumn	93±0.56^{b}	94±0.51^{a}	34±0.45^{e}	90±0.56^{d}	90±0.58^{d}	90±0.654^{d}	92±0.56^{c}
ERR%	Spring	90±0.65^{c}	95±0.68^{a}	55±0.43^{e}	90±0.79^{c}	90±0.68^{c}	95±0.74^{a}	91±0.74^{b}
	Summer	65±0.53^{c}	66±0.67^{b}	43±0.58^{f}	75±0.63^{a}	60±0.51^{d}	66±0.56^{b}	55±0.64^{e}
	Autumn	91.56±0.65^{b}	92±0.72^{a}	54±0.55^{e}	85±0.77^{d}	90±0.71^{c}	90±0.82^{c}	92±0.76^{a}
Single cocoon weight (gm)	Spring	2.2±0.24^{a}	2.1±0.26^{b}	1.1±0.22^{g}	1.82±0.27^{e}	1.88±0.25^{d}	2±0.24^{c}	1.85±0.26^{de}
	Summer	1.58±0.22d^{e}	1.9±0.13^{a}	0.9±0.12^{g}	1.69±0.26^{b}	1.66±0.29b^{c}	1.5±0.22^{f}	1.62±0.23^{cd}
	Autumn	2.5±0.13^{a}	2.3±0.24^{b}	0.9±0.13^{d}	1.93±0.27^{c}	1.88±0.28^{c}	1.9±0.27^{c}	1.85±0.24^{c}
Single shell weight (gm)	Spring	0.37±0.004^{b}	0.35±0.005^{c}	0.10±0.002^{g}	0.37±0.004^{b}	0.33±0.004^{e}	0.34±0.003^{d}	0.38±0.003^{a}
	Summer	0.32±0.005^{b}	0.32±0.004^{b}	0.11±0.001^{f}	0.32±0.004^{b}	0.29±0.003^{d}	0.30±0.005^{c}	0.35±0.004^{a}
	Autumn	0.38±0.006^{a}	0.37±0.005^{b}	0.12±0.002^{f}	0.36±0.003^{c}	0.32±0.002^{e}	0.34±0.003^{d}	0.36±0.004^{c}
Shell Ratio (%)	Spring	16.82±0.34^{b}	16.67±0.35^{c}	9.09±0.16^{g}	20.33±0.39^{b}	17.55±0.39^{e}	17±0.32^{d}	20.54±0.33^{a}
	Summer	20.25±0.35^{b}	16.84±0.34^{b}	12.22±0.24^{f}	18.94±0.34^{b}	17.47±0.35^{d}	20±0.34^{c}	21.61±0.33^{a}
	Autumn	15.2±0.36^{a}	16.09±0.38^{b}	13.33±0.12^{f}	18.65±0.37^{c}	17.02±0.35^{e}	17.89±0.34^{d}	19.46±0.32^{c}

Results are represented as mean ± SEM, n=20. 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 3.2: Correlation between biochemical attributes of mulberry leaves and different economical attributes of silkworm rearing system

Seasons	Name of Biochemical attributes of leaves	Weight of larvae	ERR%	Survival rate of larvae	Single cocoon weight	Shell weight (100 nos)
Spring	Soluble sugar content (mg/FW)	0.928**	0.867**	0.791*	0.882**	0.845**
	Reducing sugar (mg/g FW)	0.829*	0.662^{ns}	0.803*	0.744*	0.807*
Summer	Chlorophyll content (mg/g FW)	0.623^{ns}	0.746*	0.643^{ns}	0.480^{ns}	0.657^{ns}
	Soluble sugar content (mg/FW)	0.776*	0.950**	0.816*	0.792*	0.793*
	Reducing sugar (mg/g FW)	0.624^{ns}	0.847**	0.782*	0.770*	0.755*
Autumn	Soluble sugar content (mg/FW)	0.847**	0.770*	0.822*	0.825*	0.860**
	Reducing sugar (mg/g FW)	0.803*	0.777*	0.834*	0.773*	0.820*

ns = not significant, * = significant at $p<0.05$, **=significant at $p<0.01$ and $p<0.05$

Table 3.3: Two-way ANOVA analysis (with replication) of non-enzymatic antioxidant members of seven mulberry leaves with seasonal variation

Source of Variation	df	F crit	Ascorbic acid				Glutathione			
			SS	*MS*	*F*	*P-value*	*SS*	*MS*	*F*	*P-value*
Cultivars	6	2.231	5.744	0.957	118.378	1.2E-34	116.043	19.340	4175.908	2.5E-87
Seasons	4	2.503	11.148	2.787	344.644	3.0E-45	48.128	12.032	2597.897	2.8E-75
Interaction	24	1.674	1.869	0.078	9.631	5.3E-14	5.403	0.225	48.608	1.6E-34
Within	70		0.566	0.008			0.324	0.005		
Total	104		19.327				169.898			

**Significant at $P<0.01$ and $P<0.05$ level

Table 3.4: Two-way ANOVA analysis (with replication) of pigment members of seven mulberry leaves with seasonal variation

Source of Variation	df	F crit	Chlorophyll				Carotenoids			
			SS	*MS*	*F*	*P-value*	*SS*	*MS*	*F*	*P-value*
Cultivars	6	2.231	822.78	137.130	897.919	3.9E-64	0.414	0.069	31.300	5.5E-18
Seasons	4	2.503	743.36	185.841	1216.874	7.2E-64	0.124	0.031	14.078	1.8E-08
Interaction	24	1.674	87.79	3.658	23.951	6.9E-25	0.058	0.002	1.096	0.3714493
Within	70		10.69	0.153			0.154	0.002		
Total	104		1664.62				0.750			

**Significant at $P<0.01$ and $P<0.05$ level

Table 3.5: Two-way ANOVA analysis (with replication) of ROS members of seven mulberry leaves with seasonal variation

Source of Variation	df	F crit	H_2O_2				Superoxide Anion			
			SS	*MS*	*F*	*P-value*	*SS*	*MS*	*F*	*P-value*
Cultivars	6	2.231	58.488	9.748	1004.857	8.1E-66	2.400	0.4001	98.3539	4.17E-32
Seasons	4	2.503	123.280	30.820	3177.001	2.5E-78	13.390	3.3476	822.9856	5.0E-58
Interaction	24	1.674	14.999	0.625	64.424	1.6E-38	2.113	0.0880	21.6458	1.42E-23
Within	70		0.68	0.010			0.285	0.0041		
Total	104		197.45				18.189			

**Significant at $P<0.01$ and $P<0.05$ level

Table 3.6: Two-way ANOVA analysis (with replication) of ROS members and compatible osmolyte of seven mulberry leaves with seasonal variation

Source of Variation	df	F crit	MDA (ROS member)				Proline (compatible osmolyte member)			
			SS	*MS*	*F*	*P-value*	*SS*	*MS*	*F*	*P-value*
Cultivars	6	2.231	2783.425	463.904	740.044	3.1E-61	79.229	13.205	137.185	1.1E-36
Seasons	4	2.503	4893.643	1223.411	1951.649	5.7E-71	19.188	4.797	49.835	8.9E-20
Interaction	24	1.674	591.650	24.652	39.326	1.5E-31	11.930	0.497	5.164	3.8E-08
Within	70		43.880	0.627			6.738	0.096		
Total	104		8312.599				117.085			

**Significant at $P<0.01$ and $P<0.05$ level

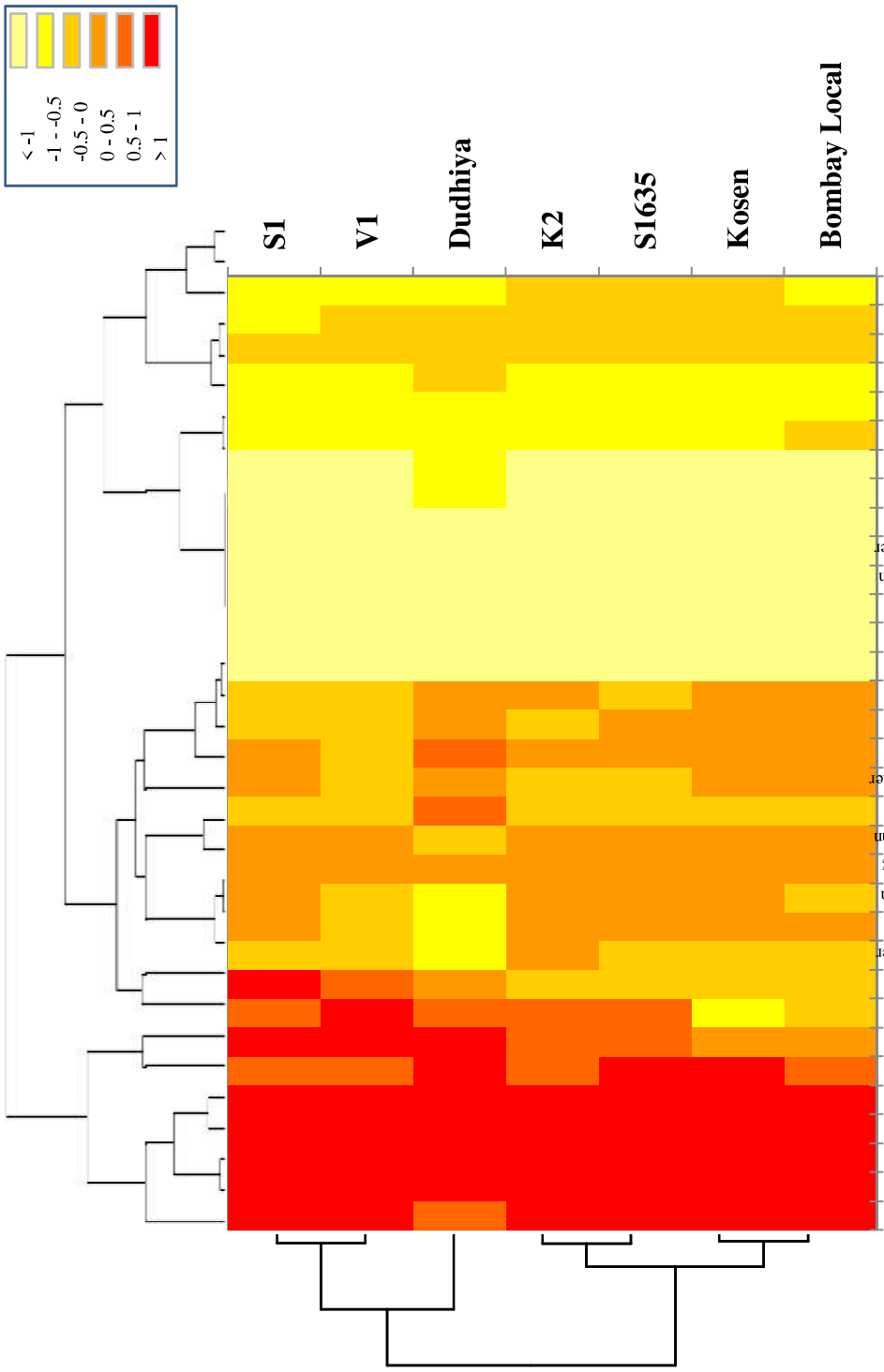


Figure 3.14: Heat map of seven mulberry cultivars on the basis of their biochemical attributes and economical features of silkworm rearing

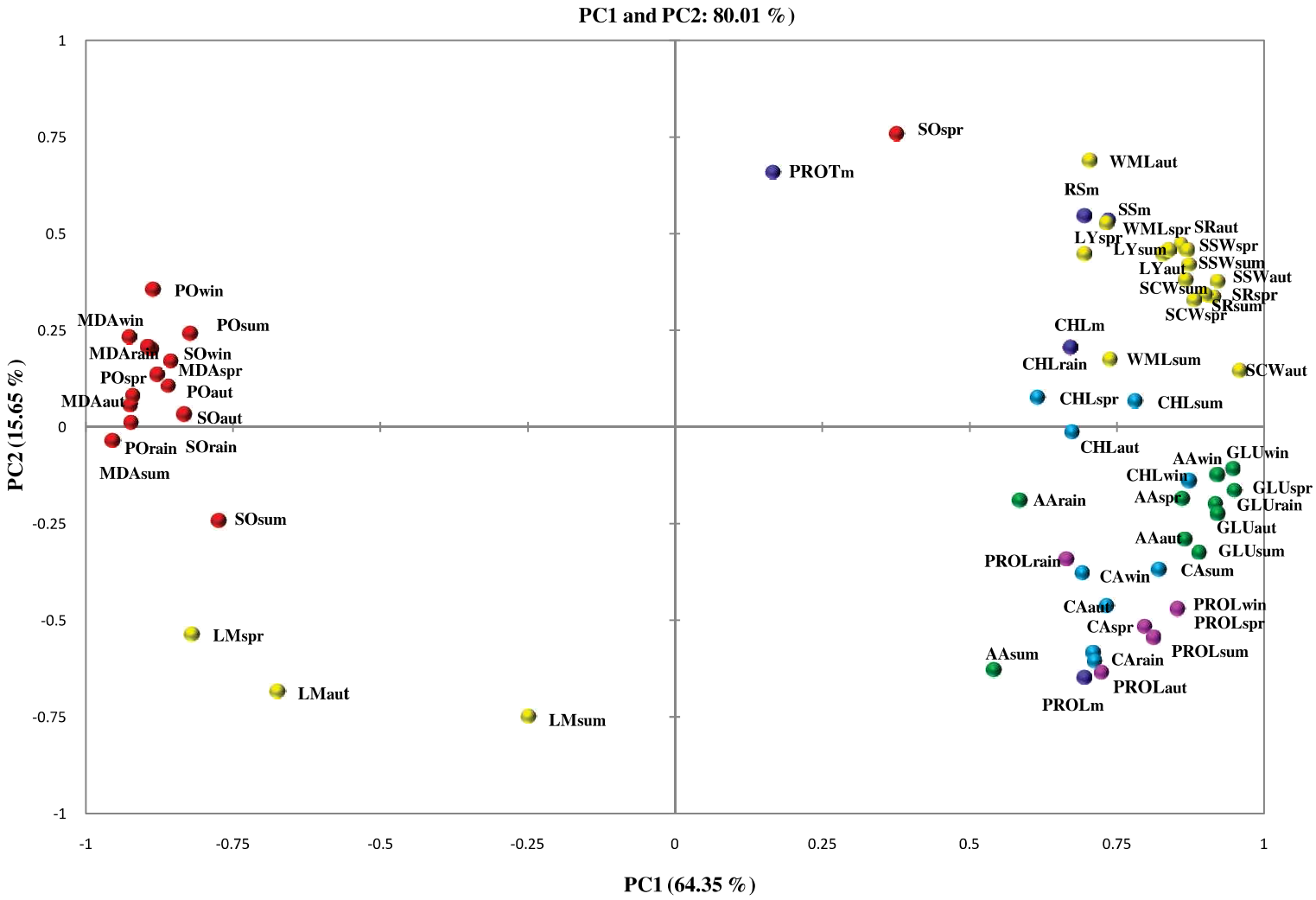


Figure 3.15: PCA analysis of free radical scavengers (FRS): Non-antioxidant member (green dot), Pigment member (sky blue dot), and compatible osmolytes member (pink dot), ROS: lipid peroxidation member (red dot), biochemical attributes of mature mulberry leaves (purple dot) and economical attributes of rearing system (yellow dot)

3.3.3 Dendogram analysis

All seven mulberry cultivars were categorized into four groups or cluster. Dudhiya germplasm was separately placed in Group I (Figure 3.14). S1 and V1 formed another cluster, Group II. Kosen and Bombay local occupied the third group. S1635 and K2 were placed in Group IV. Dendrogram cluster analysis was performed on the basis of dissimilarities among seven 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, V1, S1635 and K2 are categorized into the acclimated group. Kosen and Bombay local are considered as moderately acclimated group due to moderate accumulation of ROS and scavengers. Dudhiya was placed under non-acclimated group.

3.3.4 PCA analysis

Principal components analysis (PCA) was 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 (Figure 3.15) 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 cluster of free radical 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. Bailey-Serres and Mittler (2006) reported that ROS-mediated signalling is controlled by balance between ROS production and

scavenging. 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 (Gapper and Dolan, 2006).

3.3.5 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 3.3-3.6) 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.

3.4. 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 seven mulberry cultivars. Silkworm larvae choose most nutritionally tolerant cultivars viz. S1, V1 and S1635 and rejected 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.