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RESEARCH ARTICLE

Physio-biochemical Evaluation of Mulberry (*Morus* Spp.) Genotypes under High Alkaline Soil for Identification of Stress-Tolerant Genotypes

Thulasy Gayathri*, Subrahmaniam Gandhi Doss, Tanmoy Sarkar, Melur Kodandaram Raghunath and Pankaj Tewary

Abstract

Mulberry (*Morus* spp.) leaf yield and quality are the major factors in sericulture since mulberry foliage is the only source of food for the silkworm (*Bombyx mori* L.). High soil alkalinity will adversely affect the plant's physio-biochemical processes such as nutrient uptake, chlorophyll biosynthesis and photosynthesis, cell membrane integrity, etc. About 21 mulberry genotypes were evaluated under high alkaline soil conditions and estimated chlorophyll, photosynthetic rate and measured activities of antioxidant enzymes (SOD and POX) as well as quantified accumulation of non-enzymatic antioxidants (ascorbic acid, reduced glutathione and phenols) and osmolyte (proline). Based on morphological and physio-biochemical responses of the plants during alkalinity stress for a period of 120 days, four genotypes *viz.,* Sahana (MI 0524), Bheria dangi-1 (MI 0822), T-36 (MI 0226), and Kanthaloor-2 (MI 0449) highly tolerant to soil alkalinity stress (pH> 9.0) were identified in the study along with adaptive responses /traits for future crop improvements in mulberry.

Keywords: Alkalinity, Antioxidants, Adaptive traits, Crop improvement, Mulberry, Osmolyte.

Mulberry Physiology Laboratory, Central Sericultural Research and Training Institute, Manandavadi Road, Srirampura, Mysuru, 570 008, India.

***Author for correspondence:**

gayathrinagasuthan@gmail.com

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Introduction

Growth and development of the silkworm, *Bombyx mori* (L.), solely depend on the mulberry leaf quality. Mulberry leaf yield and quality may largely depend on various environmental factors such as temperature, availability of nutrients, soil moisture content and rainfall and soil pH, relative humidity and other soil parameters. In India, the majority of cultivatable areas are under semi-irrigated or rain-fed conditions and soil alkalinity is a major problem in these areas due to poor rainfall and the presence of soluble salts in the soil. In India, around 7.421 million hectares of land are affected by salinity and alkalinity (Sharma *et al*., 1998). Large areas under semi-irrigated conditions exhibit alkalinity in India due to poor rainfall and poor quality of irrigation water. In India, 3.79 million hectares of area are affected by alkalinity, of which 1.48 lakh hectares are in Karnataka. Salt-affected soils have been categorized into three different types: saline, saline-alkali and alkaline soils, based on total concentration of soluble salts (electrical conductivity), soil pH and exchangeable sodium percentage (ESP). Sodic/alkaline soil is remarkable by high pH, i.e., more than 8.5 or 9.0, exchangeable sodium percentage is greater than 15 and electrical conductivity is less than 4 mmhos/cm. Accumulation of high concentrations of alkaline salts such as $NaHCO_3$ and Na_2CO_3 in the soil results in increased pH and the presence of these salts in excess amounts may cause damage to the plants by ionic toxicity, especially by sodium toxicity. The main problem in salt-affected soils is nutrient deficiency due to the changes in availability, absorption and transport of nutrients

within the plants as a result of salt stress (Munns and Tester, 2008). Availability of nutrients in soil is generally affected by physicochemical properties of the soil such as structure, texture, moisture content, temperature, pH and availability of nutrients (Meena *et al.,* 2017).

High alkaline conditions can adversely affect the plant's physio-biochemical processes such as nutrient uptake, chlorophyll biosynthesis and photosynthesis, cell membrane integrity etc. It will also lead to the production of reactive oxygen species (ROS) and cellular damage. In mulberry, maximum availability of major nutrients is in the range of soil pH 6.5-7.5 and the availability of micronutrients is more in the acidic range than in neutral or alkaline pH (Dandin and Giridhar, 2014). High pH also causes significant damage to the roots, which makes sprouting difficult and if sprouted, then the growth is very much retarded or stunted. Alkalinity causes decreased leaf area and, therefore, during stress, a considerable reduction in the area of energy captured and it decreases the fixing of CO_2/unit area also. Therefore, a low rate of carbon assimilation due to alkalinity and osmotic stress leads to partial stomatal closure, which in turn causes a decrease in stomatal conductance and transpiration. The rate of photosynthesis will also be reduced significantly under alkaline soil (Yang *et al*., 2007).

Plants generally adapt to high pH by accumulating micromolecule metabolites with buffering function, such as organic acid, proline, glycine betaine, etc., for adjusting the internal pH value. Adjusting pH value is a process of consuming energy, which may decrease intracellular pH and inhibit the growth of plants simultaneously. Plants adjust to intracellular pH by maintaining the micro-environment pH around the root to keep a relative balance of metabolism and ions. Plants can adjust pH in the micro-environment outside roots by excreting H^+, organic acid and discharging CO_2 through respiration (Wang *et al.,* 2009). Intracellular pH can be adjusted mainly by accumulating organic acid largely in vacuoles (Shi and Sheng, 2005). The large accumulation of organic acids in the cells of oat seedlings under alkaline stress can compensate for water and adjust intracellular pH. Therefore, it was stated that accumulating organic acids in large amounts was a physiological response mechanism of most plants during alkaline stress.

In the present study, 21 short-listed mulberry genotypes were evaluated under high alkaline soil (pH>9.0), and various physio-biochemical parameters were recorded during the stress period. Chlorophyll content, photosynthetic rate and accumulation of antioxidants were estimated in these genotypes and identified tolerant genotypes and physio-biochemical adaptive traits for alkalinity stress tolerance in mulberry. The information and data generated under this study could be utilized for crop improvement programmes in mulberry. The four alkaline-tolerant genotypes were identified and these genotypes can be utilized as resource materials (parents) for developing stress tolerant varieties through various plant breeding approaches.

Materials and Methods

Saplings of 21 short-listed mulberry genotypes (for low input and alkaline conditions) namely S34, AR12, Sahana, S 1635, RC1, RC2, S13, Mysore Local, K2, RFS 175, V1, Saranath-3 (MI 0764), Bheria dangi-1 (MI 0822), Kanthaloor-2 (MI 0449), T-36 (MI 0226), Pouri-2 (MI 0652), Hosur-C16 (MI 0836), Baragarh -2 (MI 0437), Chirayinkizh (MI 0762), MS-2 (MI 0027) and Madhopur-4 (MI 0670) were raised in nursery beds and after 4 months healthy saplings were transplanted to earthen pots filled with alkaline soil and soil with optimum pH (7.0-7.2, control) and maintained for conducting the experiment. Alkaline soil was collected from an alkaline hot spot area (Kinakanahalli, Karnataka) and filled in earthen pots and used for growing the experimental plants. All saplings (3 saplings per genotype) of short-listed genotypes were simultaneously evaluated under high alkaline soil with pH 9.44 and soil with optimum pH (7.0–7.2). Experimental plants, grown in alkaline soil, were irrigated with alkaline water (initially 15 days with pH 8.5 and from the 16th day onwards with pH 9.0–9.2 water) to maintain the alkaline soil pH. Alkaline water was prepared by adding $NaHCO_3$ and $NaCO_3$ to the irrigation water (10:1 for pH 8.5 and 3:1 for pH 9.2) and used for irrigating the experimental plants.

Control plants were irrigated with normal water (pH 7.5–8.0) and the irrigation frequency was once in three days. Experimental plants were evaluated under alkalinity stress continuously for a period of 120 days. Among the 21 genotypes, 4 susceptible genotypes (Baragarh -2 (MI 0437), Chirayinkizh (MI 0762), MS-2 (MI 0027) and Madhopur-4 (MI 0670) did not survive in high alkaline soil. Fresh leaves were collected from the experimental plants (control and alkaline) for biochemical analysis of antioxidants and osmolytes.

The following physio-biochemical parameters were recorded during the alkalinity stress experiments between the 60th to 75th day of the treatment.

Physiological Parameters

Chlorophyll content

Chlorophyll content was measured using a Soil Plant Analysis Development (SPAD) chlorophyll meter. Chlorophyll content was recorded in the leaves of experimental plants (3 plants per genotype and 3 readings per plant) grown in alkaline soil and the normal soil. Chlorophyll content in leaves was expressed as SPAD value.

Photosynthetic rate

Photosynthetic rate was recorded using a portable photosynthesis system LI-6400 XT (LI-COR Inc., Lincoln, NE, USA) in fully expanded leaves of all experimental plants in pots grown and maintained at alkaline soil and normal soil.

Leaf net photosynthetic rate (P_N) was expressed as µmol $m^{-2}s^{-1}$.

Biochemical Parameters

Antioxidant enzymes

- *Superoxide dismutase (SOD)*

SOD enzyme extraction and activity assay were carried out by the method described by Dhindsa *et al* (1981). SOD was extracted from the leaf tissue using 50mM potassium phosphate buffer (pH 7.8). Enzyme activity was measured by recording the decrease in absorbance of formazan produced by superoxide-nitro blue tetrazolium (NBT) complex. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading of samples by 50% in comparison with tubes lacking enzymes. SOD activity was expressed in Units g^{-1}FW.

Peroxidase (POX)

POX enzyme activity was estimated according to Putter (1974). POX enzyme extracted using 0.1M phosphate buffer (pH 7.0). Peroxidase activity was assayed using guaiacol solution as substrate. Assay mixture consisted of guaiacol solution, crude enzyme extract and H_2O_2 solution. The time required to increase the absorbance from 0.05 to 0.1 at 436 nm was noted and the enzyme activity was calculated using the extinction coefficient of guaiacol dehydrogenation product at 436 nm. Enzyme activity was expressed in Units g^{-1}FW.

Non-enzymatic antioxidants

Reduced glutathione (GSH)

The levels of reduced glutathione (GSH) were estimated by the method of Moron *et al.,* (1979). Plant tissue was homogenized in 5% TCA. The homogenate was immediately acidified by adding 25% TCA to prevent aerial oxidation of glutathione. Precipitate was centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for estimation. Supernatant was diluted with 0.2 M sodium phosphate buffer (pH 8.0), followed by the addition of 2 mL of freshly prepared DTNB (5,5'-dithiobis- (2-nitrobenzoic acid) solution and the intensity of the yellow color was read at 412 nm. A standard curve was plotted using known concentrations of GSH. The quantity of reduced glutathione was expressed in mg g^{-1}FW.

Phenols

Total phenols were estimated according to Malick and Singh (1980). Phenols were extracted with 80% ethanol and quantified by the addition of Folin–Ciocalteu reagent. The absorbance was recorded at 650 nm. Total phenols were calculated using catechol as a standard and the quantity of phenolic compounds in leaves was expressed in mg g^{-1}FW.

Osmolytes

Proline

Proline was extracted with 3% sulfosalicylic acid estimated by the method of Bates *et al*. (1973). Absorbance was recorded at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and the concentration was expressed in µmol g^{-1}FW.

Statistical Analysis

One-way ANOVA (analysis of variance) and Duncan multiple range test were carried out at a significance level of $p < 0.05$ using SPSS (version 16.0; Chicago, IL, USA). Three independent experiments were done in each analysis, and the values were expressed as mean ± standard error.

Results and Discussion

Physiological Parameters

Chlorophyll (SPAD) and photosynthetic rate were recorded in experimental plants under alkalinity stress. All genotypes showed comparatively low chlorophyll (SPAD value) content in alkaline soil. In control (optimum pH), RC1 (40.90), Bheria dangi-1 (37.70), T-36 (36.43), S34 (35.93) and S1635 (34.16) showed high chlorophylls. V1, Saranath-3 and Kanthaloor-2 showed almost similar chlorophyll content. Low chlorophyll was recorded in Sahana (27.93), AR12 (30.86), S13 (31.20) and RC2 (32.16). In alkaline soil, T-36 (31.60) and AR12 (30.30) showed high chlorophyll followed by S 1635 (28.86), Bheria dangi-1 (28.30), Kanthaloor-2 (27.90, V1 (27.33), S34 (26.56), Sahana (25.96) and Saranath-3 (25.71). Low chlorophyll was observed in Pouri-2 (14.22), RC2 (15.23), S13 (17.50) and RC1 (18.16) (Table 1).

Data represented here are the mean value of replicates of three independent analyses and SE within a column, followed by the same letter, are not significantly different ($p < 0.05$) as determined by Duncan Multiple Range Test (DMRT)

The photosynthetic rate was recorded in experimental plants of both the control and the alkaline soil. In control, among the genotypes, maximum photosynthetic rate was recorded in Bheria dangi-1 and Kanthaloor-2 (16.84 µmol $m^{-2}s^{-1}$), Saranath-3 (16.23 µmol $m^{-2}s^{-1}$), Pouri-2 (15.71 µmol $m^{-2}s^{-1}$), T-36 (15.27 µmol $m^{-2}s^{-1}$), S 1635 (13.87 µmol $m^{-2}s^{-1}$) and V1 (13.78 µmol $m^{-2}s^{-1}$). Minimum photosynthetic rate was recorded in RC2, S13 and S34 (5.23-6.90 µmol $m^{-2}s^{-1}$). Under alkaline soil, all genotypes except K2 recorded a reduced photosynthetic rate. In K2, photosynthetic rate was almost same in control (10.57 µmol $m^{-2}s^{-1}$) and treatment (9.75 µmol $m^{-2}s^{-1}$). Photosynthetic rate was high in Kanthaloor-2 (12.14 µmol $m^{-2}s^{-1}$), K2 (9.75 µmol $m^{-2}s^{-1}$), S 1635 (7.74 µmol $m^{-2}s^{-1}$), T-36 (5.65 µmol $m^{-2}s^{-1}$), Bheria dangi-1 (4.35 µmol $m^{-2}s^{-1}$), Sahana (4.23 µmol $m^{-2}s^{-1}$) and AR12 (3.99 µmol $m^{-2}s^{-1}$). Low photosynthetic rate was recorded in Pouri-2 (MI 0652) (0.49 µmol $m^{-2}s^{-1}$),

Table 1: Variation in chlorophyll (SPAD value) content among different mulberry genotypes

Genotype	*SPAD value at Optimum pH*	*SPAD value at Alkaline pH*
S13	31.20 ± 1.30 ^{cd}	17.50 ± 2.72 ^{de}
Mysore Local	37.30 ± 0.40 ^{ab}	22.17 ± 2.79 ^{bcde}
S34	35.93 ± 4.78 ^{abc}	26.56 ± 2.93 ^{ab}
RC1	40.90 ± 1.72 ^{a}	18.16 ± 2.11 ^{cde}
RC2	32.16 ± 0.62 ^{bcd}	15.23 ± 1.50 ^{de}
AR12	30.86 ± 1.04 ^{cd}	30.30 ± 2.54 ^{ab}
V1	33.36 ± 0.75 ^{bcd}	27.33 ± 1.03 ^{ab}
K2	31.26 ± 0.77 ^{cd}	23.03 ± 3.58 ^{abcd}
S1635	34.16 ± 1.04 ^{bc}	28.86 ± 1.14 ^{ab}
Sahana	27.93 ± 0.82 ^{d}	25.96 ± 3.58 ^{abc}
Saranath-3	33.10 ± 0.75 ^{bcd}	25.71 ± 0.81 ^{abc}
Bheria dangi-1	37.70 ± 0.46 ^{ab}	28.30 ± 4.88 ^{ab}
Kanthaloor-2	33.66 ± 2.78 ^{bc}	27.90 ± 0.83 ^{ab}
T-36	36.43 ± 1.28 ^{abc}	31.60 ± 2.52^{a}
Pouri-2	34.10 ± 1.02 ^{bc}	14.22 ± 0.58 ^{e}

Mysore Local, RC2 and S13 (<2 µmol $m^{-2}s^{-1}$) (Table 2). Fully expanded healthy leaves were not present in RFS 175 and Hosur-C16 for recording of photosynthetic rate.

Data represented here are the mean value of replicates of three independent analyses and SE within a column, followed by the same letter, are not significantly different (p <0.05) as determined by Duncan Multiple Range Test (DMRT)

Biochemical Parameters

Antioxidant enzymes (superoxide dismutase and peroxidase) activities were estimated in leaves of experimental plants grown in alkaline (treatment) and normal soil (control). In control plants, maximum SOD was recorded in T-36 (45.79 Units/g), RC1 (32.46 Units/g), Kanthaloor-2 (32.16 Units/g), Hosur-C16 (25.67 Units/g), S13 (22.69 Units/g), Pouri-2 (21.45 Units/g), Bheria dangi-1 (19.88 Units/g), S1635 (19.61 Units/g), Sahana (18.99 Units/g), AR12 (18.55 Units/g) and S34 (18.35 Units/g). Minimum enzyme activity was observed in RC2 (5.73 Units/g) and Mysore Local (8.54 Units/g). SOD enzyme activity was significantly reduced in plants grown under alkaline soil. High activity was recorded in Sahana (28.36 Units/g), T-36 (24.32 Units/g), AR12 (17.71 Units/g), RFS 175 (17.49 Units/g), S 1635 (10.95 Units/g) and Bheria dangi-1 (10.14 Units/g). RC1 (4.31 Units/g), Kanthaloor-2 (5.07 Units/g) and Pouri-2 (5.51 Units/g) showed low SOD activity. In the other seven susceptible genotypes (Mysore Local, S13, S34, RC2, V1, K2 and Hosur-C16), SOD activity is not observed in leaves under alkaline soil conditions (Fig. 1).

Peroxidase enzyme activity was estimated in the leaves of control and alkaline soil-grown experimental plants.

Table 2: Variation in the photosynthetic rate among different mulberry genotypes (P_N-Photosynthetic rate)

Genotype	*P_N at Optimum pH*	*P_N at Alkaline pH*
S13	6.86 ± 1.20 ^{ef}	1.43 ± 0.16 ^{ghi}
Mysore Local	9.47 ± 0.14 ^{def}	0.85 ± 0.41 ^{lj}
S34	6.90 ± 1.82 ^{ef}	3.47 ± 1.45 ^{efgh}
RC1	10.29 ± 1.56 ^{cde}	2.99 ± 0.85 ^{efghi}
RC2	5.23 ± 0.64 ^{f}	1.19 ± 0.40 ^{lj}
AR12	6.63 ± 0.11 ^{ef}	3.99 ± 0.54 ^{defg}
V1	13.78 ± 2.33 ^{abc}	1.43 ± 0.38 ^{hij}
K2	10.57 ± 1.56 ^{cde}	9.75 ± 1.17 ^{b}
S1635	13.87 ± 1.14 ^{abc}	7.74 ± 0.54 ^{c}
Sahana	11.37 ± 0.58 ^{bcd}	4.23 ± 0.64 ^{def}
Saranath-3	16.23 ± 1.47 ^{a}	2.01 ± 0.31 ^{ghij}
Bheria dangi-1	16.84 ± 0.69^{a}	4.35 ± 0.51 ^{de}
Kanthaloor-2	16.84 ± 1.48^{a}	12.14 ± 0.33 ^{a}
T-36	15.27 ± 2.27 ^{ab}	5.65 ± 0.57 ^{d}
Pouri-2	15.71 ± 1.27 ^{ab}	0.49 ± 0.22 ^{j}

Results of the enzyme assay indicated that in control plants. In control plants, high peroxidase activity was recorded in RC1 (51.89 Units/g), followed by V1 (49.56 Units/g), S34 (48.48 Units/g), Sahana (40.59 Units/g), S13 (25.61 Units/g), T-36 (24.25 Units/g), Mysore Local (20.44 Units/g), AR12 (18.12 Units/g), and Bheria dangi-1 (16.38 Units/g). Low enzyme activity as observed in Pouri-2 (3.55 Unit/g), RC2 (5.06 Units/g), S 1635 (6.33 Units/g) and RFS 175 (7.19 Units/g). Under alkaline soil, all genotypes exhibited a low enzyme activity and among the genotypes, higher peroxidase activity was recorded in Bheria dangi-1 (6.65 Units/g), Sahana (5.84 Units/g), AR12 (4.5 Units/g) and K2 (2.3 Units/g). All other genotypes showed a least enzyme activity (<2 Units/g). In RC2 and S 1635, no enzyme activity was detected during the activity assay (Fig. 2).

Non-enzymatic antioxidants, phenols and reduced glutathione were estimated in all experimental plants and results indicated significant variation in the accumulation of these compounds under control and alkalinity stress conditions. In control plants, high phenol content was recorded in T-36 (36.36 mg/g), Sahana (29.76 mg/g), Pouri-2 and RFS 175 (25.50 mg/g), Bheria dangi-1 (24.66 mg/g), Kanthaloor-2 (20.6 mg/g), Hosur-C16 (19.83 mg/g) and AR12 (19.16 mg/g). Low phenols were observed in S13 (13.23 mg/g), RC2 (13.36 mg/g), S34 (14.23 mg/g) and Mysore Local (15.26 mg/g). Under alkaline soil, higher accumulation of phenol was recorded in T-36 (29.73 mg/g), Sahana (28.06 mg/g), Bheria dangi-1 (22.63 mg/g), Pouri-2 (21.16 mg/g), Hosur C16 (18.63 mg/g) and RFS 175 (18.36 mg/g). Mysore Local (11.26 mg/g), S13 (11.33 mg/g), S34 (12.06 mg/g) and RC1 (14.16 mg/g) showed the least phenol content (Fig. 3).

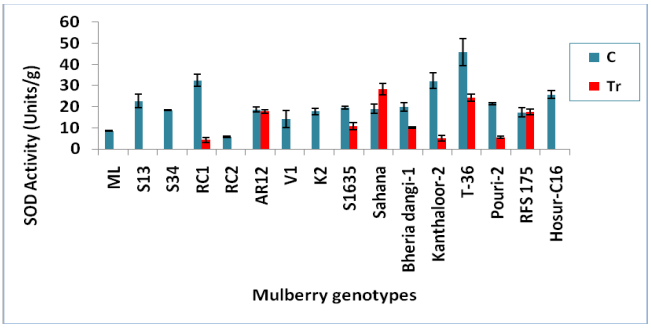

Fig. 1: SOD enzyme activity profile among different mulberry genotypes C- control, T-Treatment (high pH)

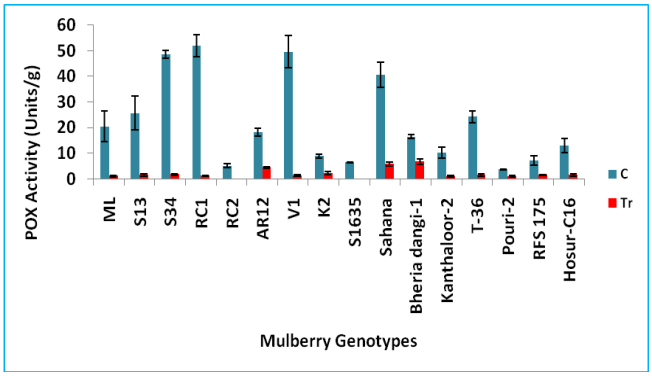

Fig. 2: POX enzyme activity profile among different mulberry genotypes. C- control, T-Treatment (high pH)

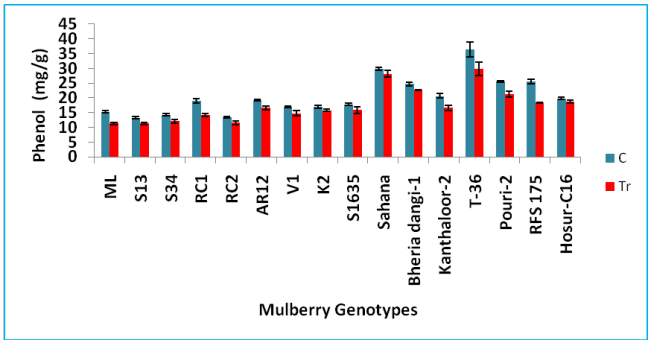

Fig. 3: Variation in quantity of phenols in different mulberry genotypes. C- control, T-Treatment (high pH)

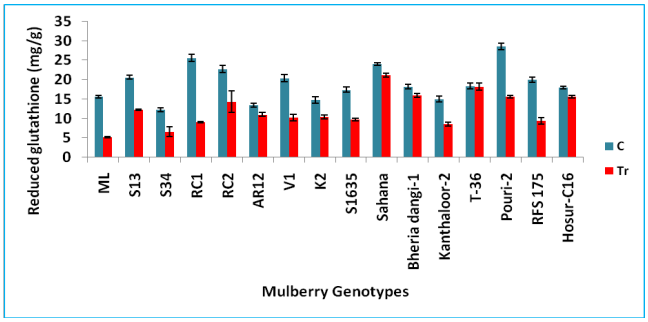

Fig. 4: Variation in quantity of reduced glutathione in different mulberry genotypes. C- control, T-Treatment (high pH).

Reduced glutathione (GSH) was estimated in all genotypes and the results indicated that in control plants, high reduced glutathione content was recorded in Pouri-2 (28.20 mg/g), RC1 (25.6 mg/g), Sahana (24.0 mg/g), RC2 (22.80 mg/g), S13 (20.60 mg/g), V1 (20.40 mg/g) and RFS 175 (20.0 mg/g). Low GSH was observed in S34 (12.20 mg/g), AR12 (13.40 mg/g), K2 (14.80 mg/g), Kanthaloor-2 (15.0 mg/g) and Mysore Local (15.60 mg/g). Under alkaline soil, maximum GSH content was observed in Sahana (21.20 mg/g), T-36 (18.20 mg/g), Pouri-2 and Hosur C16 (15.60 mg/g) and RC2 (14.33 mg/g). Minimum GSH was recorded in Mysore Local (5.14 mg/g), S34 (6.60 mg/g) and Kanthaloor-2 (8.60 mg/g). In other genotypes, it ranged from 9.05 to 12.20 mg/g. In genotypes T-36 (18.40 and 18.20 mg/g), Sahana (24.0 and 21.20 mg/g) and Bheria dangi-1 (18.20 and 16.0 mg/g), the GSH accumulation was almost the same in control and alkalinity stress conditions (Fig. 4).

Accumulation of osmolyte, proline, was estimated in experimental plants grown under optimum pH soil and alkaline soil during the experiment. Under optimal soil pH conditions, high proline accumulation was observed in Bheria dangi-1 (72.58 µmol/g), Sahana (40.45 µmol/g), K2 (39.28 µmol/g), T-36 (35.94 µmol/g), Kanthaloor-2 (34.61 µmol/g) and S1635 (33.97 µmol/g). Low proline was recorded in Mysore Local (9.44 µmol/g) and AR12 (10.82 µmol/g). Under alkaline soil, higher proline accumulation was recorded in Bheria dangi-1 (65.70 µmol/g), Sahana (39.60 µmol/g), Kanthaloor-2 (32.66 µmol/g), RFS 175 (26.48 µmol/g), T-36 (17.49 µmol/g) and V1 (17.06 µmol/g). Low accumulation of proline was observed in Mysore Local (4.88 µmol/g), Hosur C16 (10.82 µmol/g) and S13 (10.13 µmol/g) (Fig. 5).

Physiological parameters such as chlorophyll (SPAD) and photosynthetic rate were recorded in experimental plants grown under optimal soil pH and high alkaline soil. All genotypes showed a reduction in chlorophyll (SPAD value) in alkaline soil. The photosynthetic rate was significantly reduced in plants grown under alkaline soil. However, the tolerant genotypes, T-36 (31.60) and AR12 (30.30), S 1635 (28.86), Bheria dangi-1 (28.30), Kanthaloor-2 (27.90), V1 (27.33), S34 (26.56) and Sahana (25.96) showed high chlorophyll content. In susceptible genotypes such as Pouri-2, RC2, S13 and RC1, chlorophyll content was reduced under alkalinity soil.

Earlier reports confirmed that the photosynthetic rate of a plant decreases with increasing saline stress intensity (Yang *et al*., 2009a,b), and it has been reported that alkaline stress leads to limited photosynthesis in barley (Yang *et al*., 2009a). The present study also reported the low photosynthetic rate in susceptible genotypes and a comparatively higher photosynthetic rate in tolerant genotypes. High rate of photosynthesis was recorded in tolerant genotypes such as Kanthaloor-2, K2, S1635, T-36, AR12 and S34.

In high alkaline soil, deficiency of other nutrients will be observed in the soil due to the high concentration of

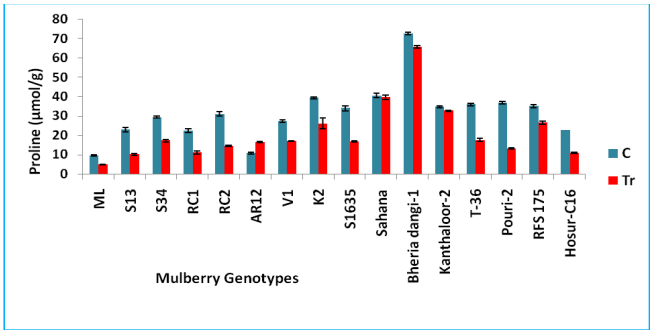

Fig. 5: Variation in accumulation of proline in different mulberry genotypes. C- control, T-Treatment (high pH)

Na^+ and other environmental factors, such as drought, which exacerbate this problem (Silberbush and Ben-Asher, 2001). Besides this, high Na^+ hampers the uptake of other nutrients by: (1) Na^+ interfering with transporters in the root plasma membrane, such as K^+-selective ion channels, and (2) reduction of root growth by high Na^+ concentration (Tester and Davenport, 2003). Thus, the uptake of water and growth-limiting nutrients (such as P, Fe, or Zn) and the growth of soil microorganisms, such as mycorrhizal fungi, can be inhibited and which leads to the stunted growth of the plant as observed in susceptible genotypes.

Normal growth, development, and physiological and biochemical metabolism of plants are severely disrupted during alkalinity stress conditions. The high concentration of sodium ions in the soil under saline-alkali stress can disrupt the dynamic balance of ions in cells, leading to a series of damaging effects on plants, such as destruction of the cell membrane structure, abnormal metabolons in cells and ionic toxicity (Hasegawa, 2013). Plants alleviate the toxicity of sodium ions mainly through excreting sodium ions from cells and sequestering them through ion antiporters such as NHX7 (also named SOS1) within the cell membrane and NHX1 within the vacuolar membrane, both of whose activity is regulated by calcium-dependent SOS2/SOS3 kinase complexes (Bahmani *et al.,* 2015).

Osmotic stress and ionic stress caused by saline-alkali stress lead to the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). These ROS accumulated in the cell during the stress will disrupt the normal physiological functions of cells and which may result in metabolic disorders. Generally, well-developed ROS scavenging systems were identified in tolerant plants, including antioxidant enzymes and antioxidants for reducing the oxidative damage of ROS in plants (Shumei Fang *et al*., 2021). To cope with these adverse stress conditions, plant cells synthesize and accumulate several small-molecule organic compounds, such as proline, soluble proteins, betaine, sugar, polyols and polyamines, to maintain intracellular water potential (Sun *et al*., 2019).

In tolerant plants, a well-organized antioxidant system was developed for mitigating the effects of reactive oxygen species (ROS) generated during the stress conditions. The present study also indicated that the antioxidant enzymes (SOD and POX) showed comparatively higher activities in tolerant genotypes. A recent study in mulberry under optimal growth conditions confirmed that antioxidant enzyme activities were varied among different genotypes and it was higher in stress-tolerant genotypes (Gayathri *et al*., 2022). SOD activity (17.49-28.36 Units/g) was high in genotypes like Sahana, T-36, AR12 and RFS 175. Higher POX activity (4.50- 6.65 Units/g) was recorded in Bheria dangi-1, Sahana and AR-12. SOD is the first line of defense of the antioxidant system in plants and can transform accumulated superoxide molecules into oxygen and H_2O_2, after which catalase, ascorbate peroxidase and peroxidase convert H_2O_2 into water and oxygen. In addition, these enzymes work together to scavenge MDA produced from lipid peroxidation to protect the membrane structure.

Non-enzymatic antioxidants also play an important role in stress tolerance response in higher plants. These antioxidants include mainly glutathione (GSH), ascorbic acid (ASA), mannitol, flavonoids, anthocyanins and vitamin E. These compounds are distributed in different parts of cells to regulate the balance of ROS in cells. Increased levels of these foliar antioxidants were reported during drought stress in mulberry (Guha *et al*., 2011). Antioxidant phenol was estimated in plants grown under alkalinity stress conditions and it was found that high quantity of phenol was recorded in T-36 (29.73 mg), Sahana (28.06 mg) and Bheria dangi-1 (22.63 mg). Low quantity was observed in susceptible genotypes (11.26-11.53 mg) such as Mysore Local, S13 and RC2. Reduced glutathione content was also high in these tolerant genotypes (18.20–21.20 mg) and least in the susceptible one (514–6.60 mg).

Alkalinity stress experiments indicated that in tolerant genotypes, there was a remarkable increase in the foliar antioxidants and compatible solutes (proline), which appear to play a major role in scavenging the reactive oxygen species generated during the stress. Changes in the accumulation of proline and glycine betaine, along with the activities of antioxidant enzymes such as peroxidase, catalase, glutathione reductase, were reported earlier (Ahmed *et al*., 2014) in two mulberry cultivars (S146 and Sujanpur) under sodium carbonate-induced alkalinity stress. This study reported the high activities of antioxidant enzymes in the tolerant cultivar S146. A comparative antioxidant enzyme activity and gene expression analysis were reported in rice (Khare *et al*., 2015) among salt-tolerant (Panvel-3) and salt-sensitive (Sahyadri-3) varieties and confirmed the high level of antioxidant enzymes in the tolerant variety. This report also indicated the additive effect of Na^+ and Cl^- ions under NaCl stress and the tolerant cultivar maintained lower Na^+/K^+ and ROS levels through an effective antioxidant mechanism. A recent study was reported in three halophytic grasses and comparative mineral nutrient analysis was carried out

under saline and alkaline stress conditions (Lata *et al*., 2022). The results of this study indicated that the Na+/K+ ratio is an important indicator of a plant's response to salt stress and low leaf Na+ concentration and low Na+/K+ can be considered as an indicator for stress tolerance.

Proline was an effective osmotic adjusting material of plants for responding to stress situations (Song *et al*., 2006; Yang *et al.,* 2008). A larger accumulation of proline represents a stronger osmotic adjusting ability and resistance to an adverse environment of plants (Yang *et al.,* 2008). Among all compatible solutes, proline and glycine betaine occur widely in higher plants and accumulate in considerable amounts in salt-stressed plants (Ahmad *et al.,* 2006; Koyro *et al.,* 2012; Ahmad *et al.,* 2012). In this study, proline and glycine betaine were increased markedly with an increase in external $NaHCO_3$ level and growth period. Relatively $NaHCO_3$-tolerant cv. S146 was superior to cv. Sujanpuri accumulates both osmoprotectants under alkaline regimes. This pattern of accumulation of the two osmoprotectants clearly shows that they could be used as potential indicators of alkalinity tolerance in mulberry, as was earlier reported in broad bean (Mohamed *et al.,* 2011), mustard (Ahmad *et al*., 2012), and chickpea (Saiema Rasool *et al*., 2012). Stress-tolerant responses in terms of grain yield and associated traits were evaluated in 102 Indian wheat cultivars under terminal heat stress conditions by Singh *et al*., (2020) and identified genotypes with stable yield performance for stress and non-stressed environments. Moisture stress tolerance studies were conducted in Desi, Kabuli and Wild chickpea accessions and confirmed the drought tolerance of the Desi variety due to its high root traits such as root area, root length and root weight. And these root traits were marked as selection indices for drought tolerance with high yield stability (Johal *et al.,* 2021).

The present study also reported the high activity of antioxidant enzymes (SOD and POX), accumulation of antioxidants, as well as proline in tolerant genotypes. Hence, the presence of these antioxidants (SOD, POX, reduced glutathione and phenols) and proline in higher quantities during the alkalinity stress could be utilized as an adaptive trait/response for alkalinity tolerance in mulberry and is useful for selecting tolerant genotypes. Alkalinity-induced symptoms such as necrosis, chlorosis, terminal bud burning, leaf senescence, stunted growth, etc. were observed regularly in the experimental plants grown under high alkaline soil. Based on physio-biochemical responses and appearance of alkalinity-induced symptoms in plant parts, four genotypes (Sahana, Bheria dangi-1, Kanthaloor-2 and T-36) were identified as tolerant to high alkalinity (pH >9).

21 short-listed mulberry genotypes were grown under high alkaline soil and recorded physio-biochemical parameters such as chlorophyll content, photosynthetic rate, activities of antioxidant enzymes and accumulation of antioxidants and osmolytes. Results of the study indicated that tolerant genotypes showed higher photosynthetic rate (>4 µmol $m^{-2}s^{-1}$) even under alkalinity stress. In susceptible genotypes, P_N was significantly reduced during the stress period (0.49 -3.47 µmol $m^{-2}s^{-1}$) and hence, photosynthetic rate can be considered as a physiological adaptive trait for identifying alkalinity stress-tolerant genotypes. Higher activities of superoxide dismutase and peroxidase enzymes were recorded in tolerant genotypes during stress conditions. Non-enzymatic antioxidants (reduced glutathione, ascorbic acids and phenols) and osmolytes (proline and glycine betaine) also exhibited an increased accumulation in tolerant genotypes (Sahana, Bheria dangi-1, Kanthaloor-2 and T-36) under alkalinity stress. Hence, these compounds can be used as biochemical indicators for identifying stress-tolerant genotypes in mulberry. Data on profiling of antioxidant enzymes and non-enzymatic antioxidants as well as the physiological parameters achieved in the study, provide useful insights into the physio-biochemical mechanism of alkalinity stress tolerance in mulberry. The present study also identified four genotypes tolerant to high alkaline soil (pH>9.0) based on physio-biochemical responses and alkalinity-induced symptoms in the leaves and plant parts under alkaline soil conditions.

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