MATERIALS AND METHODS
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The present investigation deals with the morphological, physiological and biochemical responses of certain mulberry cultivars. Six varieties of mulberry, namely, S1, S13, S30, Berr 776, K2 and ATP were screened on the basis of morphological parameters. The varieties S1 and ATP were classified as salt-tolerant and salt-sensitive respectively to different regimes of salt stress over a period of 12 days.

A separate set of experiments was carried out to study the morphological parameters, such as root length, leaf area, relative leaf expansion rate and dry mass accumulation. Certain widely cultivated and local varieties of mulberry (*Morus alba* L.) viz., S1, S13, S30, Berr 776, K2, and ATP were selected and procured from the Regional Sericultural Research Station, Central Silk Board, Anantapur, as experimental material. The plant cuttings of the six varieties were prepared from nine-month-old healthy hardwood branches of at least half-inch in diameter. These were prepared for plantation according to Krishnaswamy (1986). The stem cuttings made of approximately five to six inches long with a minimum of 3-4 active buds. While preparing the cuttings, care was taken to see that the ends of the cuttings were clean without splits and bark peeling-off. The cuttings were brought to the laboratory and immediately planted in earthen pots containing 8 kg of air-dried red-loamy soil and farmyard manure in 3:1 proportion. The pots were maintained in the department botanical garden under natural photoperiod (10-12 hrs 28 ± 4°C) by supplying water daily.

Three-month-old plants of all cultivars of approximately equal height and number of leaves were taken for the experimentation. Pots of each cultivar were divided into four sets. For inducing salt stress four levels of NaCl, 0.0 (control), 0.5,
1.0 and 1.5 % were used. Electrical conductivity (EC) of soil saturation extract was 1.7, 2.0, 4.0 and 7.8 mhos/cm² respectively. These pots were arranged in Randomized complete Block experimental Design (RBD) with five replications. Precautions were taken to avoid the drainage of solution during treatment by adding water slightly less than field capacity. The E.C. of the soil saturation extract was checked in alternate days and adjusted, if necessary. The plants were maintained under same conditions as above. The plants were taken for the experimentation.

Morphological parameters

Leaf area and relative leaf expansion rate

The area of the 3rd leaf, from the apex of the plant, was measured on the 0, 4, 8 and 12th day after inducing stress (since the selection of the 3rd leaf in mulberry measurements was used for many eco-physiological studies, Chen, 1992).

The selected leaf was traced on to a graph paper, and area was measured. From the leaf area data, the relative leaf expansion rate (RLER; cm² cm⁻² d⁻¹) was calculated from the formula according to Sobrado (1986).

\[
RLER = \frac{\log_2 A_2 - \log_2 A_1}{(T_2 - T_1)}
\]

Where \(T_1\) and \(T_2\) represents the initial and final day respectively, \(A_1\) and \(A_2\) represents the leaf area of initial and final day respectively. The results were average of five replicates.

Root length

The plants were uprooted carefully on the day-12 after imposing stress. The roots were thoroughly washed with deionised water and roots and leaves were separated. Root length was measured. The results were average of five replicates.
Fresh Weight and Dry weights

The plants were washed with deionized water and blotted dry with filter paper. Root and leaf parts were separated and the material was dried at 80°C in a hot air oven for 48 hours and dry weights were recorded.

Experiments on physiological parameters

The varieties S1 and ATP were selected as salt tolerant and salt sensitive respectively and were subjected to different regimes of salt stress (0.05, 1.0 and 1.5% NaCl). After induction of stress the pots were maintained under similar conditions as mentioned above for 12 days and the experimental data were collected at different time intervals i.e., 4th, 8th and 12th - day. The data were average of five replicates.

Total Chlorophyll Content

The total chlorophyll content was estimated in the leaves of control and stressed varieties of according to the method of Arnon (1949).

Fresh leaves were taken, washed and blotted to dry. The leaf material was homogenized in a prechilled mortar using cold 80% acetone. The homogenate was centrifuged at 3000 rpm for 30 min and the supernatant was collected. The sediment was re-extracted with cold 80% acetone. All the supernatants collected at each time were pooled and made to known volume with 80% cold acetone. The optical density of the acetone extract was measured at 645nm; 663nm using 80% acetone as blank in a spectrophotometer (Baush and Lomb, spectronic 88). Total chlorophyll content was calculated by employing the following formula.

\[ 20.2 \times \text{O.D. (at 645 n.m)} + 8.02 \times \text{O.D (at 663 n.m)} \]

Photosynthesis and related parameters

Rate of photosynthesis, stomatal conductance, inter cellular CO₂ concentration and the rate of transpiration were monitored by using a portable photosynthesis system (LCA-3) equipped with a Parkinson leaf chamber (6.2 cm²), (PLC) (Analytical
Development Co., Hoddesdon, U.K). The measurements were made between 8.00 to 10.00 A.M at photosynthetic photon flux density of approximately 1100 ± 100 μmol m⁻²s⁻¹. The leaf temperature was between 30 ± 2°C. The measurements were done in the third leaf (fully expanded) from the top, since this leaf was found to possess maximum photosynthetic capacity. The selection of third leaf from top in mulberry for ecophysiological studies has also been suggested by Chen (1992).

Water use efficiency (WUE) was calculated as the ratio between net photosynthesis and transpiration are suggested by Blum and Sullivan (1986).

**Carbohydrate Fractions**

200 mg of oven dried plant material was extracted with 80% ethanol according to the method of Highkin and Frankel (1962). The alcoholic extract was evaporated to 10ml. on a water bath and cooled to room temperature. It was centrifuged and the supernatant (alcoholic extract) was used for the estimation of reducing and non-reducing sugars, while the sediment was used for the estimation of starch.

**Estimation of reducing sugars**

The reducing sugars were estimated by Nelson's method (1944) as modified by Somogyi (1952).

One ml of alcoholic extract was taken into a test tube. To this one ml of a freshly prepared mixture of 25 parts of reagent ‘A’ and 1 part of reagent ‘B’ was added and mixed the solutions. The test tubes were incubated in a boiling water bath for 20 min and cooled under a running tap water. One ml of arsenomolybdate reagent was added. The color was developed very rapidly. The mixture was diluted to 10 ml after 15 min and the absorbance was measured in a Baush & Lomb spectronic 88 spectrophotometer at 500 nm. Reagent blanks were used to adjust absorbance to zero. Reducing sugar content was estimated by using a standard curve prepared with dextrose.
Copper Reagent A

25 g of sodium carbonate (anhydrous), 25 g of sodium potassium tartrate and 200 g of sodium sulphate (anhydrous) were dissolved in 800 ml of distilled water and diluted to a litre. The reagent was stored at room temperature.

Copper Reagent B

15g of CuSO$_4$.$7$H$_2$O was dissolved in 100 ml of distilled water and 1 drop of conc. H$_2$SO$_4$ per 100 ml was added.

Arsenomolybdate color reagent

25 g of ammonium molybdate was dissolved in 450 ml of distilled water and 21 ml of conc. H$_2$SO$_4$ was added and mixed. 3g of Na$_2$HAsO$_4$$7$H$_2$O was dissolved in 25 ml of water. Both solutions were mixed and placed in an incubator at 37°C for 48h.

Non-reducing sugars

From the alcoholic extract the non-reducing sugars were estimated by following the method of Scott (1960). 10 ml of original alcoholic extract was hydrolyzed with 10ml of 3N HCl for 30 min at 100°C in a water bath. Later it was cooled and neutralized with 30% NaOH . The total non-reducing sugars in the hydrolyzed extract were estimated with Nelson’s method (1994) as described earlier.

Starch

The sediment left behind after the alcoholic extraction of original material was taken for starch estimation, by following the method of Mc Cready et al., (1960). Starch was solubilised with 52% perchloric acid for 30min, filtered and was made up to known volume. To 1.0 ml of perchloric acid extract, 4.0 ml of freshly prepared anthrone reagent was added in cold. The test tubes were heated for 5 min at 100°C in a water bath. The test tubes were cooled rapidly to room temperature and the color
intensity was measured at 630 nm. Reagent blanks were used to adjust the absorbance to zero.

A standard curve was prepared with known amounts of dextrose and starch content was calculated by multiplying the glucose equivalent present in the sample with 0.9.

**Anthrone reagent**

200 mg of anthrone was dissolved in 100 ml of cold 95% H₂SO₄.

**Anti-oxidative enzymes**

**Superoxide dismutase**

Leaf material was homogenized in 50mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone. The homogenate was filtered and centrifuged in a refrigerated centrifuge at 15000g for 15 min and the supernatant obtained was used as source of enzyme. All steps in the preparation of enzyme extract were carried out at 4°C. An aliquot of 0.1 ml enzyme extract was used for the determination of the protein.

**Assay**

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (Beauchamp and Fridovich, 1971).

The reaction mixture (3 ml) consisting of 50 mM phosphate buffer (pH 7.8), 13mM methionine, 75μM nitro blue tetrazolium, 0.1mM EDTA, 2μM riboflavin and 0.1 ml of enzyme extract. Riboflavin was added lastly and test tubes were shaken and placed 30 cm below a light source (15 W fluorescent lamps). The reaction was started by switching-on the lights. The reaction was allowed for 30 min and then stopped by switching-off the lights. The tubes were covered with black cloth. The reaction mixture which was not exposed to light did not develop color and served as control. The absorbance was measured at 560nm in a spectrophotometer. Log A<sub>560</sub> was plotted.
as function of the volume of enzyme extract used in the reaction mixture. From the resultant graph, the volume of the enzyme extract corresponding to 50% inhibition of the reaction was read and considered as one enzyme unit.

**Extraction for Catalase and Peroxidase**

The plant material was placed in a pre-cooled mortar and ground with cold 0.005M Tris-HCl buffer pH 7.0. The extract was passed through cheese cloth and centrifused at 1000 rpm to remove cellular debris. The supernatant solution was centrifused again at 34,800 g for 20 min. The supernatant was used as crude enzyme extract source for the assay of catalase and peroxidase.

**Catalase**

Catalase activity was assayed and estimated as per the method of Barber (1980). The reaction mixture consisted of enzyme extract 0.005M H$_2$O$_2$ and 0.05M Tris-buffer, pH 7.0. After incubating it for 1 min at 25°C, the reaction was stopped by adding 1.0 ml of 2.5N H$_2$SO$_4$. The residual H$_2$O$_2$ was titrated with 0.01N KMnO$_4$. A blank was maintained with the reaction mixture at zero min. Catalase activity was expressed as mg H$_2$O$_2$ oxidized per gram fresh weight per min.

**Peroxidase**

Peroxidase activity was assayed as per the method of Kar and Mishra (1976). The reaction mixture containing 0.1M Tris-buffer pH 7.0, 0.01M pyrogallol and 0.005M H$_2$O$_2$ and the reaction was started by adding enzyme extract and the mixture was incubated at 25°C for 5 min. The reaction was stopped by adding 1.0 ml 2.5 N H$_2$SO$_4$. The amount of purpyrogallin formed was estimated by measuring the absorbance at 425 nm in a spectrophotometer. The enzyme activity was expressed as change in absorbance units.
Extraction of Glutathione reductase and Glutathione-s-transferase

The plant material was powdered in liquid nitrogen using a pestle and mortar and extracted in 2.0 ml of 100 mM potassium phosphate buffer pH 7.0. The homogenate was centrifuged for 10 min at 11000g in a microcentrifuge. The supernatant was used as enzyme source for the assay of glutathione reductase and glutathione-s-transferase.

Glutathione reductase

Glutathione reductase activity was assayed and estimated as per the method of Foster and Hess (1980).

The reaction mixture containing enzyme extract 100mM potassium phosphate buffer, pH 7.0, 1.0mM EDTA, 150μM NADPH and 500μM oxidised glutathione. The enzyme activity was measured at 340nm. Sample activity was calculated by using the extinction coefficient for NADPH of 6.22 mM⁻¹cm⁻¹. The enzyme activity was expressed as μM NADPH min⁻¹mg⁻¹ protein.

Glutathione-s-transferase

Glutathione-s-transferase activity was determined using the method of Habig and Jacoby (1981). The reaction mixture containing enzyme extract, 100mM potassium phosphate buffer pH 6.5, 1.0mM 1-chloro 2,4 dinitrobenzene (CDNB), 1.0mM reduced glutathione and 1.0mM EDTA. The enzyme activity was measured at 340 nm and activity was calculated using the extinction coefficient of the conjugate 9.6 mM⁻¹cm⁻¹. The enzyme activity was expressed as μ mol GSH min⁻¹ mg⁻¹ protein.