MATERIALS AND METHODS

The study presents certain morphological, physiological and biochemical responses of six mulberry genotypes namely ANANTHA, RFS-175, S-34, RFS-135, S-30 and M-5 to different regimes of water stress over a period of 15 days.

The major objective of the study was to screen and identify drought resistance/incidence sources of mulberry to include in field trails or in breeding programme for evolving drought resistant mulberry varieties for rainfed situations. Many mulberry genotypes have been released as high yielders but majority are well suited for irrigated conditions and relatively few can adapt well under rainfed situations. Understanding the difference in growth rates of varieties differing in their performance under rainfed and irrigated conditions helps in identifying characters suitable for higher productivity in these conditions. Several experiments were conducted to study the varietal differences in extent of rooting and sprouting and the growth under water stress condition. The cuttings of mulberry were procured from Regional Sericulture Research Station (RSRS), Anantapur, Andhra Pradesh; REC, Madivala (Kolar), Karnataka. A separate set of experiments was carried out to study the propagation parameters such as sprouting and rooting. The six elite cultivars (Anantha, RFS-175, S-34, RFS-135, S-30 and M-5) were selected for evaluating their sprouting and rooting

performance under stress conditions. The experimental data on these parameters were collected as suggested by Jolly and Dandin (1986).

The cuttings of six mulberry varieties were prepared eight-month-old healthy hard wood branches of at least half-inch in diameter. The cuttings made were of 5-6 inches long with a minimum of three active buds. While preparing cuttings care was taken to see that the ends of the cuttings were clean, without splits of bark peeling off. The cuttings were brought to the laboratory and immediately planted (three cuttings in each pot) in earthern pots (50 x 50 cm) containing red loamy soil and farm yard manure in 3:1 proportions. After establishment, plantlets were maintained for another 70 days. The pots were irrigated with water once a day. The pots were kept under natural photoperiod of about 12-13 h with a temperature of 28 ± 4° C in the departmental garden. After 70 days, plants (six varieties) with approximately equal height and number of leaves were selected and divided and pots were arranged in Randomized complete Experimental Block Design (REBD). Water stress was induced by adding required volume of water daily in the morning to give 100, 75, 50 and 25% of field capacity and stress level was characterised as control, mild, moderate and severe stresses respectively. After induction of stress the pots were maintained for another 15 days and the experimental data were collected at different time intervals i.e., on the 5th, 10th and 15th day. The data were the average of five replicates.

Soil moisture was measured by gravimetric dry weight basis. Plants were uprooted and washed thoroughly with deionised water, blotted to dry and the lengths of shoot and root were measured separately.

Root length and Shoot length

The length of the root and shoot measured and recorded on day-5, 10 and 15 after inducing water stress. The results were average of five replicates. The length of the root and shoot was expressed in cm plant^{-1}.

Fresh weight (FW) and Dry weight (DW)

The plants were washed with deionized water and blotted with filter paper. Leaves were separated from shoot. The leaf and root fresh weights were recorded separately. The material was dried at 80°C in hot air-oven for 48 hours and dry weights were recorded. The results were the average of five replicates.

Leaf area

The leaf area of the expanding leaf (third leaf from the apex) was measured with leaf area meter (Laser Leaf Area Metr, Model: CI-203) and data was recorded.

Moisture %

The freshly harvested leaves from both control and water stressed plants were washed with deionized water and blotted to dry and then fresh weight of the leaves was recorded. Then the leaves were dried at 80° C in a hot air oven for 48 h and dry weights were recorded. The moisture percentage was calculated from the following formula.

$$\text{Mositure \%} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100$$

Moisture retention capacity (Moisture content after 6 h of harvesting)

The freshly harvested leaves were stored in air-conditioned room (Temp. 24 ± 2° C; RH 80%) for 6 h and then the fresh weight was recorded. Then the leaves were dried at 80° C in a hot air oven for 48 h and dry weights were recorded. Moisture content was determined as explained above.

Leaf water potential

Leaf water potential was measured using a portable PR-55 Psychrometer Microvoltmeter with C-52 sample chamber (Wescor, Logan Utah, USA). The reading were measured between 8.00 AM to 10.00 AM discs of 0.5 cm diameter were punched from the middle portion of the uppermost fully expanded third leaf using a metal punch and were immediately put into leaf chambers. Leaf water potential readings were taken by the Psychrometric method after an equilibration period of 30 min. The measurements were the average of twenty discs to obtain a mean water potential for the leaf.

Relative water content

The leaves were washed in deionized water and blotted dry and fresh weights (FW) were taken. Then the leaves were immersed in distilled water and after 4 hrs, they blotted dry and the turgid weight (TW) was taken. The leaves were kept at 80°C in hot air oven for 48 h and dry weights (DW) were recorded.

The relative water content (RWC) was calculated from the formula, according to Turner (1981)

$$RWC = \frac{(FW - DW)}{(TW - DW)} \times 100$$

Estimation of proteins

The protein content was estimated by the Folin-phenol method according to Lowry *et al.*, (1951).

Extraction of total proteins

500 mg of fresh plant tissue was extracted into 80% hot ethanol by macerating in a mortar with pestle. The homogenate was transferred into centrifuge tubes and centifuged at 2000 rpm for 20 min and the supernatant was discarded. The pellet was suspended into a suitable volume of 10% trichloroacetic acid. Again it was centrifuged and the supernatant was discarded. This process was repeated twice. The pellet was then re-extracted with absolute ethanol and with hot ethanol-ether mixture (3:1). Every time the supernatant was discarded after centrifugation. Total protein content was estimated from the pellet.

Estimation of proteins

The pellet was suspended in 2 ml of 0.1 N NaOH solution and 1.0 ml of this sample solution was taken into a test tube. To this 5.0 ml of alkaline solution

was added, mixed thoroughly and allowed to stand at room temperature for 10 min. Then 0.5 ml of diluted (1:1) Folin-Ciocalteu's reagent was added rapidly with immediate mixing and allowed for incubation for 30 min at room temperature. The colour developed was read at 750 nm against reagent blank in UV-Vis spectrophotometer (Shimadze, 1601). Protein content was calculated from a standard curve prepared from bovine serum albumin.

10% Trichloroacetic acid

10 g trichloroacetic acid was dissolved in 100 ml of distilled water.

Ethanol-ether mixture

75 ml of ethanol and 25 ml of ether were mixed.

0.1 N NaOH

400 mg of NaOH dissolved in 100 ml of distilled water.

Alkaline sodium carbonate solution (Solution A)

2 g of sodium carbonate was dissolved in 100 ml of NaOH.

Copper sulphate - Sodium potassion tartrate solution (Solution B)

50 mg of $CuSO_4.2H_2O$ and 100 mg of sodium potassium tartrate were dissolved in 10 ml of distilled water.

Alkaline solution

This was prepared by mixing 50 ml of solution A with the 1.0 ml of solution B at the time of use.

Folin-Ciocalteu's reagent

The commercial reagent was diluted with the equal volumes of distilled water on the day of use.

Proline

The extraction and estimation of proline was done according to Bates *et al.*, (1973).

Extraction

500 mg of plant material was homogenised in a mortar with pestle using 10.0 ml of 3% aqueous sulfosalicyclic acid. The homogenate was filtered through four layered muslin cloth and the filtrate was collected. The extraction was repeated twice and all the filtrates were pooled and made up to known volume.

Estimation

2.0 ml of the filtrate was taken into a test tube and 2.0 ml of acid ninhydrin and 2.0 ml of glacial acetic acid were added. The tubes were incubated at 100°C for 1 h in a boiling water bath. The tubes, after incubation, were transferred to an ice bath to terminate the reaction. 4.0 ml of toluene was added to the contents of the tubes and mixed thoroughly using a test tube stirrer for 15 sec. Chromophore containing toluene was aspirated from the aqueous phase. Then the absorbance of the solution was measured at 520 nm in a UV-Vis spectrophotometer (Shimadzu, 1601) against reagent blank. Proline was measured from the standard curve prepared with authentic proline and its amount was calculated on fresh weight basis.

Acid ninhydrin

1.25 g of ninhydrin was dissolved in a warm mixture of 30.0 ml of glacial acetic acid and 20.0 ml of 6 M phosphoric acid with agitation. Reagent was stable for 24 h if stored at 4°C.

Extraction for Peroxidase and Catalase

The leaf material was placed in a pre-cooled mortar and ground with cold 0.05 M Tris-HCl buffer pH 7.0 The extract was passed through cheese cloth and centrifuged at 1000 rpm to remove cellular debris. The supernatant solution was centrifuged again at 34,800 rpm for 20 min. The supernatant was used as crude enzyme source for the assay of catalase and peroxidase. All steps were carried out in a cold room

Peroxidase

Peroxidase activity was assayed as per the method of Kar and Mishra (1976). The reaction mixture containing 0.1 M Tris-buffer (pH 7.0), 0.01 M pyrogallol and 0.005 M H_2O_2. The reaction was started by adding enzyme solution and the mixture was incubated at 25°C for 5 min. The reaction was stopped by adding 1.0 ml 2.5 N H_2SO_4. The amount of pyrogallin formed was estimated by measuring the absorbance at 425 nm in a spectrophotometer (Shimadzu 1601). The enzyme activity was expressed as change in absorbance units.

Catalase

Catalase activity was assayed and estimated as per the method of Barber (1980). The reaction mixture consisted of enzyme extract, 0.005 M H_2O_2 and 0.05 M Tris-buffer (pH 7.0). After incubating it for 1 min at 25°C, the reaction was stopped by adding 1.0 ml of 2.5 N H_2SO_4. The residual H_2O_2 was titrated with 0.01 N $KMnO_4$. A blank was maintained with the reaction mixture at zero time. Catalase activity was expressed as mg H_2O_2 oxidized per gram fresh weight per min.

Malondialdehyde (MDA)

The levels of malondialdehyde content was determined by the thiobarbituric acid (TBA) reaction as described by Peever and Higgins (1989). One gram of tissue (FW) was homogenised in 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 10000 g for 5 min and 4 ml of 20% TCA containing 0.5% (w/v) TBA was added to 1 ml of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10000 g for 15 min and the absorbance was measured at 532 nm and 600 nm in Shimadzu 1601 spectrophotometer was read after subtracting the non-specific absorbance at 600 nm. The concentration of MDA was determined by its extinction coefficient of 155 mM^{-1} cm^{-1}. MDA content expressed as μ mol g FW^{-1}.

Statitical Analysis of Data

The data were subjected to Analysis of Variance (ANOVA) and the mean values were compared by Duncan's Multiple Range (DMR) test at 0.05% level (Duncan, 1955).