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DIFFERENTIAL RESPONSES TO SALINITY STRESS IN SEEDLINGS OF THREE *EUCALYPTUS* SPECIES

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SUMMARY

Ascorbate and foliar contents of proline, glycine betaine and abscisic acid (ABA) along with lipid peroxidation rates, electrolytic leakage were determined in three different *Eucalyptus* species, viz. *E. camaldulensis* Dehn., *E. citriodora* Hook., *E. tereticornis* Sm., subjected to salinity stress (Mixed salts: NaCl, $MgSO_4$ and $CaCl_2$) of different concentrations (0, 50, 100, 150mM). Sampling was done after 35 days of treatments in leaves. The contents of ascorbate and monodehydroascorbate in the leaf extracts of all *Eucalyptus* species showed variations to salinity stressed conditions. Lower rates of membrane lipid peroxidation and electrolytic leakage were noticed in the leaves of *E. camaldulensis* under salinity stress. Quantitative differences were also noticed in foliar proline, glycine betaine and ABA contents among *Eucalyptus* species in response to salinity. The leaves of *E. camaldulensis* accumulated more proline, glycine betaine and ABA under salinity stress compared to *E. tereticornis* and *E. citriodora*. Our data demonstrate that *E. camaldulensis* have efficient antioxidative characteristics and ABA accumulation which could provide better protection against oxidative stress in leaves under salinity stress conditions.

Key words: **Abscisic acid, antioxidants, ascorbate, compatible solutes, electrolytic leakage, *Eucalyptus*, salinity stress**

INTRODUCTION

Eucalyptus is among the world's important hardwoods and the principal source of timber, fuelwood and pulpwood. It is most popular species in the farm forestry programmes in India. *Eucalyptus* has a wide geographic distribution extending over a range of environmental conditions and it is likely that such species will have levels of genetic diversity. However, as other major crops in India, *Eucalyptus* is also subjected to environmental stresses, particularly salinity stress. Salinity is an environmental stress that limits growth and development in plants (Hasegawa and Bressan 2000, Sairam and Tyagi 2004). The stresses most commonly associated with water deficits are drought, high salinity and low temperature (Bohnert *et al.* 1994). When CO_2 fixation is limited because of stomata closure caused by water deficit, the rate of active oxygen formation increases in chloroplasts because an excess of excitation energy that is not dissipated by the protective mechanisms, is used to form reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) (Asada and Takahashi 1987, Scandalios 1993). Plants possess antioxidant defense mechanisms, which can overcome this oxygen toxicity and delay the deleterious effects of free radicals (Foyer *et al.* 1994). These ROS attack lipids, proteins and nucleic acids, causing lipid

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peroxidation, protein denaturation and DNA mutation (Bowler *et al*. 1992). Plants are endowed with a complex antioxidant system to cope with ROS (Smirnoff 1995, Noctor and Foyer 1998), which includes enzymatic and non-enzymatic, lipid or water soluble antioxidants molecules.

Ascorbate (AsA) occupies a central role in protecting plant cells against the action of the ROS and generally, its content is high in leaf tissue (Heber *et al.* 1996). When ascorbate functions as an antioxidant in cells, it is univalently oxidised to the monodehydroascorbate radical (MDA). In chloroplasts, the thylakoid-bound and stromal AsA peroxidases (Miyake and Asada 1992) generate MDA (Hossain *et al.* 1984) in scavenging the hydrogen peroxide produced via the superoxide dismutase catalyzed disproportionation of the photogenerated superoxide in PSI (Nagano and Asada 1981, Ogawa *et al*. 1995). Accumulation of protective solutes like proline and glycine betaine is an unique plant response to environmental stresses, specifically to salinity stress (Girija *et al.* 2002, Sairam and Tyagi 2004, Claussen 2005). Proline has a prominent role as an osmoticum and because of its zwitterionic and high hydrophilic characters, it acts as a compatible solute also (Hayashi *et al.* 2000). Further, the phytohormone, ABA plays prominent role in various physiological and biochemical processes related to environmental stresses (Gibson *et al*. 1991, Sauter and Hartung 2000).

Eucalyptus now occupies 7.5 lakh hectares of forest and farm lands in India with an average productivity of $12m^3$/ha. *Eucalyptus* subjected to various types of environmental stresses, in particular, salinity stress (Van der Moezel *et al*. 1988). Several farmers favour planting *Eucalyptus* in the coastal areas because of its commercial value. Although, agronomic traits of *Eucalyptus* are well characterized, physiological and biochemical characteristics of *Eucalyptus* under salinity stress conditions are elusive. The hypothesis of this research is that increases in foliar contents of AsA, proline, glycine betaine and accumulation of ABA were part of the reason for higher salinity tolerance of *Eucalyptus* species. The long term goal of our work is to help breed a salt-tolerant *Eucalyptus* to be grown in the salinity areas. The results provide information for selecting the *Eucalyptus* species with the best tolerance to salinity stress which could be used for producing better biomass yields even under adverse environmental conditions.

MATERIALS AND METHODS

The economically valuable *Eucalyptus* species, such as *Eucalyptus camaldulensis* Dehn., *Eucalyptus citriodora* Hook. and *Eucalyptus tereticornis* Sm. were employed in the present study. The *Eucalyptus* seeds were procured from IFGTB (Institute of Forest Genetics and Tree Breeding) Coimbatore, Tamil Nadu, India. Seeds were surface sterilized with 5% sodium hypochlorite, thoroughly washed with distilled water and germinated in trays containing a red coarse sand, watered daily with a half-strength Hoagland nutrient solution as described by Hoagland and Arnon (1950). Separate trays were used for each *Eucalyptus* species. Once a week, trays were rinsed with tap water to avoid salt accumulation. After three months, small seedlings in the trays were transferred to pots. The pot soil had pH of 7.5 and seedlings were allowed to grow in the pots for three months in the open air. Six month old seedlings were used for all the experiments. The average photoperiod during the growth period was approximately 12 hours. The average temperature were 28-32^{0}C during day and 22-25^{0}C during night.

The salts used to obtain the required salinities for the treatments were NaCl, $MgSO_4$ and $CaCl_2$ mixed to give a Na:Mg:Ca ratio of 10:2:1 by weight and applied to the plant with Hoagland nutrient solution as basal dose. The experiment was arranged in a complete randomized design and replicated thrice. The concentrations of salinity (Mixed salts: NaCl, $MgSO_4$ and $CaCl_2$) treatments were chosen in such a way that control, 50mM as low salinity, 100mM as medium salinity and 150mM as high salinity. Each pot was irrigated every day with 750ml of salinity solution for treated plants and control plants were maintained without salinity. Sampling was done in leaves at 35 days after the salinity treatments.

Ascorbate content was determined by following the method of Wise and Naylor (1987). The leaf discs homogenized with 6% perchloric acid and centrifuged at 10000xg for 10 min at 4^0 C. The supernatant was

incubated with equal volume of 10mM HEPES-KOH buffer (pH 7.5) for 10min at 25^{0}C. A 100µl aliquot of the solution was added to 900µl of 200mM succinate buffer (pH 6.0) and the absorbance was measured at 265nm. Monodehydroascorbate content was measured according to Heath and Packer (1968). One gram of the leaf tissue was homogenized in 5ml of 5% trichloroacetic acid (TCA) and centrifuged at 12000xg for 15min. The supernatant was mixed with an equal volume of thiobarbituric acid [0.5% in 20% (w/v) trichloroacetic acid] and the mixture was incubated for 25min in boiling water bath, followed by centrifugation for 5min at 7500xg. The absorbance of the supernatant was measured at 532nm.

Lipid peroxidation rates were determined by measuring the malondialdehyde equivalents according to Hodges *et al*. (1999). The leaf tissue (0.5g) was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3000xg for 10min at 4^{0}C. The pellet was extracted twice with the same solvent. The supernatant were pooled and 1ml of sample was added to a test tube with an equal volume of either the solution comprised of 20% TCA and 0.01% butylated hydroxy toluene (BHT) or solution of 2% TCA, 0.01% BHT and 0.65% TBA. Samples were heated at 25^{0}C for 25min and cooled to room temperature. Absorbances were read at 440, 532 and 600nm. Lipid oxidation rate equivalents (nmol malondialdehyde ml^{-1}) were calculated by using the formulae given by Hodges *et al.* (1999).

The total inorganic ions leaked out in the leaves during salinity stress were measured as described by Sullivan and Ross (1979). Twenty leaf discs were taken in a boiling tube containing 10ml of deionized water and electrical conductivity (EC) was measured (EC_a). The contents were heated at 45 and 55^{0}C for 30 min each in a water bath and EC was measured (EC_b). Later, the contents were boiled at 100^{0}C for 10 min and the EC again recorded (EC_c). The electrolytic leakage was calculated using the formula

$$\text{Electrolytic leakage (\%)} = \frac{EC_b - EC_a}{EC_c} \times 100$$

The content of proline was estimated according to Bates *et al.* (1973). Leaf tissue (5 g) was homogenized in 30% sulfosalysilic acid and filtered. To 2 ml of filtrate, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added and incubated for 1h in a boiling water bath followed by ice bath. To this, 4 ml of toluene was added and mixed vigorously and the chromophore containing toluene was aspirated from aqueous phase and the absorbance was measured at 575nm.

Glycine betaine content in the leaf extracts was determined according to Storey and Wyn Jones (1977). Five grams of leaf tissues was homogenized in 25ml of isopropyl alcohol and centrifuged at 3000xg for 10min. The supernatant was dried in vacuum at 4^{0}C. The residue was washed successively twice with chloroform (20ml) and distilled water (5ml). The two phases of six bulked washings were partitioned by centrifugation and the upper aqueous layer was removed. The remaining lipid layer was washed thrice with 20ml of McOH: H_2O (1:1). The combined aqueous layers were evaporated to dryness in a hot water bath and re-dissolved in distilled water (5ml). Potassium triiodide solution (0.2ml) was added to 1ml of the above extract and the mixture was incubated for 90min on ice bath with intermittent shaking. Two milliliters of ice cold distilled water was added to the mixture followed by 2 ml of 1,2-dichloroethane and the two layers were mixed by constant stream of air bubbles for 5min, while the temperature was maintained at 4^{0}C. The absorbance of the lower organic layer was measured at 365nm.

Extraction and quantification of ABA was done following Daie and Wyse (1982). One gram of the leaf tissue was homogenized with 15ml of extraction medium containing 80% methanol, 100mg/1 butylated hydroxy toluene (BHT) and 0.5g/l citric acid monohydrate (Daie and Wise 1982). The suspension was centrifuged at 1000xg for 20min at 4^{0}C. The supernatant was passed through Sep-Pak-C-18 cartridge and the pooled washings were evaporated under vacuum. The residue was partitioned three times against equal volume of ethyl acetate (pH 3.0). Resulting organic phase was evaporated and the residue was re-dissolved in 2ml of TBS buffer (pH 7.5). The aliquots were conjugated with bovine serum albumin (BSA) and the conjugate was

administered subcutaneously to albino rabbits (1kg body weight). The antibodies were extracted from rabbit's blood and were used for the estimation of ABA.

ELISA was performed on a 96 well micro titration plate following Daie and Wyse (1982). Each well on the plate was coated with 100µl coating buffer (1.5gl^{-1} Na_2CO_3, 2.93gl^{-1} $NaHCO_3$ and 0.02gl^{-1} NaN_3, pH 9.6) containing 0.25µgml^{-1} antigen of the hormone. The coated plates were incubated for overnight at 4^0C and then kept at room temperature for 32-40min. After washing four times with PBS-tween 20 (0.1%, v/v) buffer (pH 7.4), each well was filled with 50µl of 20µgml^{-1} antibody raised against ABA antigens, respectively. The plate was incubated for three hours at 28^0C and then washed as given before. One hundred microliters of 1.25µgml^{-1} IgG horse radish peroxidase substrate was added to each well and incubated for 1h at 30^0C. The plate was rinsed five times with above PBS-tween 20 buffer and 100µl of solution containing 1.5mgml^{-1} O-phenylenediamine and 0.008% (v/v) H_2O_2 was added to each well. The reaction was stopped by adding 50µl of 6N H_2SO_4. The colour development in each well was detected using ELISA reader (Multiscope, Labsystems, Finland) at optical density $A_{490.}$

Statistical analysis was performed using Student's t test. Significant differences from control values were determined at $p<0.05$. All results were represented as means±SE from six independent determinations.

RESULTS AND DISCUSSION

Adaptation to salinity may depend on different mechanisms, including the capacity to maintain high levels of antioxidants and/or through the induction of antioxidants. Fig. 1 and 2 shows non-enzymatic antioxidant levels (AsA, MDA) in leaf extracts of three different *Eucalyptus* seedlings under salinity stressed conditions (50, 100, 150mM). Efficient destruction of O_2^{*-} and H_2O_2 in plant cells requires the concerted action of antioxidants. Among the non-enzymatic antioxidants, AsA is found to be one of the best characterized compounds, required for many key metabolic functions in plant cells (Smirnoff and Wheeler 2000). AsA acts as an antioxidant, protecting cells against oxidative stress. AsA has the capacity to eliminate different ROS

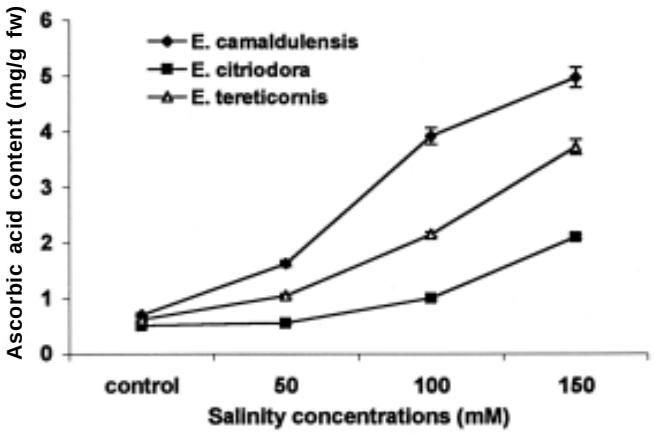

Fig. 1. Effect of salinity stress on ascorbic acid content in the leaves of three different *Eucalyptus* species. Each value is the mean ± SE of six independent determinations (P<0.05)

including singlet oxygen, superoxide and hydroxyl radicals (Foyer 2001). When AsA reduces ROS in non-enzymatic and enzymatic reactions, MDA is formed. The major reaction where MDA is formed in chloroplast is Ascorbate peroxidase reaction. In our present study, the contents of both AsA and MDA increased in three *Eucalyptus* species subjected to salinity stress. However, *E. camaldulensis* showed significantly high amount of AsA and MDA compared to *E. tereticornis* and *E. citriodora*.

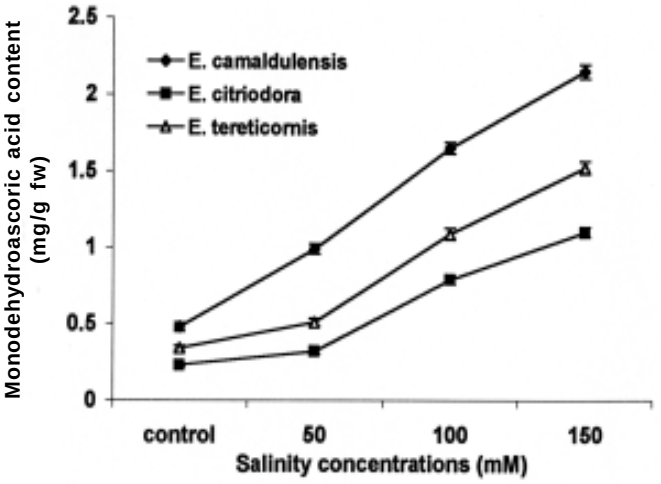

Fig. 2. Salinity stress-induced changes on the content of monodehydroascorbic acid in three different *Eucalyptus* species. Each value is the mean ± SE of six independent determinations (P<0.05)

The electrolytic leakage and lipid peroxidation under salinity stress was more in species *E. citriodora* compared to *E. tereticornis* and *E. camaldulensis* (Fig. 3 and 4). Lipid peroxidation is a destructive chain reaction and it can directly damage the structure of membranes

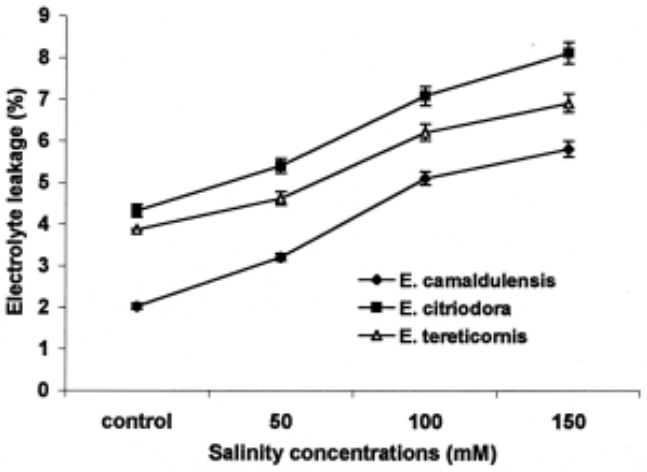

Fig. 3. Influence of salinity stress on electrolytic leakage in the leaves of three different *Eucalyptus* species. Each value is the mean ± SE of six independent determinations (P<0.05)

(Shah *et al.* 2002, Dewir *et al.* 2005). Induction of oxidative stress in salinity stressed plants is known to increase the accumulation of metal ions available for the Haber-Weiss reaction, enhance the oxidative damage of lipids and cell membrane proteins (Mano 2002, Bor *et al.* 2003). Cell membrane are the first target of many plant stresses and maintenance of their integrity and

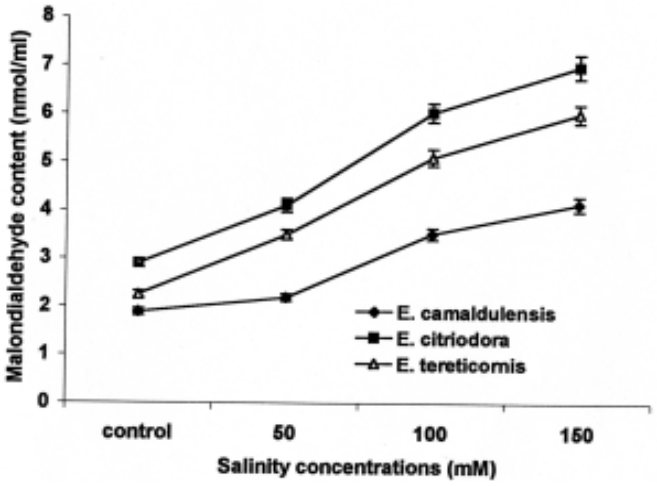

Fig. 4. Salinity stress effects on lipid peroxidation in the leaves of three different *Eucalyptus* species. Each value is the mean ± SE of six independent determinations (P<0.05)

stability under stress conditions (salinity/water) is a major component of tolerance in plants (Bajji *et al.* 2002, Bor *et al.* 2003). Lipid peroxidation of biological membranes might lead to structural alterations in salinity stressed plants. Experimental evidence suggests that lipid peroxidation reactions of cellular membranes may play an important role in radical mediated cell injury (Bhaunik *et al.* 1995). Our results suggest that salinity stress can induce membrane lipid peroxidation resulting membrane fluidity leading to enhanced electrolytic leakage (Fig. 3 and 4). Our data also indicate that the degree of cell membrane injury and levels of membrane lipid were relatively less in *E. camaldulensis* under salinity stress conditions.

Quantitative estimation of proline and glycine betaine among the *Eucalyptus* species were depicted in Table 1 and 2. *E. camaldulensis* species accumulated significantly high amount of proline and glycine betaine compared to *E. tereticornis* and *E. citriodora* grown under salinity stressed regimes. Proline and glycine betaine are known to serve as nitrogen and carbon sources, which can be used during water limited conditions as well as during recovery from the stress. These compatible solutes are also involved in cell osmoregulation and protection of proteins during dehydration (Rantein *et al.* 2002, Claussen 2005).

ABA accumulation was investigated in the leaves of three *Eucalyptus* species under salinity stressed

Table 1. Influence of salinity stress on foliar proline (mg/g fw) content in three different *Eucalyptus* species

Species	Salinity treatments (mM)			
	control	50	100	150
E. camaldulensis	3.45 ±0.16	4.73 ±0.19	7.22 ±0.23	8.84 ±0.28
E. citriodora	2.12 ±0.06	2.98 ±0.11	3.47 ±0.15	4.62 ±0.19
E. tereticornis	2.99 ±0.08	3.83 ±0.16	5.55 ±0.20	7.51 ±0.23

The data are expressed as mean ±S.E. for six independent determinations (P<0.05).

Table 2. Glycine betaine (mg/g fw) content in three different *Eucalyptus* species subjected to salinity stress.

Species	Salinity treatments (mM)			
	control	50	100	150
E. camaldulensis	3.91 ±0.15	4.79 ±0.18	6.75 ±0.20	7.21 ±0.24
E. citriodora	1.99 ±0.06	2.32 ±0.07	2.83 ±0.09	3.62 ±0.14
E. tereticornis	2.55 ±0.08	3.63 ±0.15	4.47 ±0.17	5.74 ±0.20

The data are expressed as mean ±S.E. for six independent determinations (P<0.05).

conditions (Fig. 5). Significant differences in ABA content were found among the species. There was approximately two-three fold increase in ABA content in salinity stressed leaves compared to control plants. However, *E. camaldulensis* possessed nearly two fold higher amount of ABA compared to *E. citriodora* under salinity stressed conditions. Accumulation of ABA in higher plants is well known to limit the transpirational losses particularly under salinity conditions (Kitsaki and Drossopoulos 2005). Our results clearly suggests that accumulation of ABA is a sensitive indicator to change in soil water availability to the *Eucalyptus* species. ABA accumulation in higher plants was reported to be related to oxidative stress tolerance in plants (Bellaire *et al.* 2000). Further, ABA is also known to induce the expression of antioxidant enzyme genes in plant tissue (Guan *et al.* 2000). Our data in this study demonstrate the differential responses to non-enzymatic antioxidants (ascorate, monodehydroascorbate), osmolytes (proline, glycine betaine) and membrane lipid peroxidation along with electrolytic leakage among three different *Eucalyptus* species, in which *E. camaldulensis* was superior with respect to the antioxidative defence system and accumulation of ABA. In conclusion, selection of *Eucalyptus* species with genetic traits like antioxidants and accumulation of ABA might be certainly useful in assessing the adaptive responses of *Eucalyptus* to salinity stress.

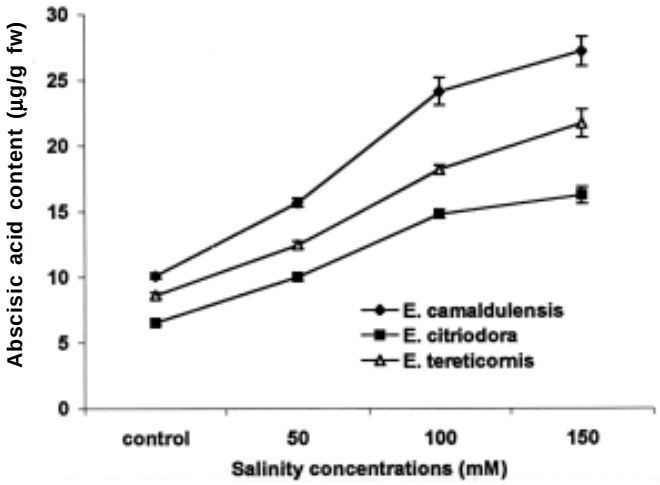

Fig. 5. Effect of salinity stress on abscisic acid content in the leaves of three different *Eucalyptus* species. Each value is the mean ± S.E. of six independent determinations (P<0.05)

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