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Physiological and Biochemical Responses of Eight *Eucalyptus* Species to Salinity Stress

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ABSTRACT: The effect of salt stress on the pysiological and biochemical responses of the seedlings of eight *Eucalyptus* species viz. *E. kingsmillii, E. tetragona, E. salubris*, *E. occidentali*, *E. microtheca*, *E. camaldulensis*, *E. globules* and *E. sargentii* was analyzed. Four month-old seedlings grown in greenhouse were watered by five levels of salt solution (0, 50, 100, 150 and 200 mM of NaCl) in five replications with a factorial experimental design. The results indicated that salinity delayed and inhibited the seedlings' growth after one month, and induced gradual decline in most of the criteria such as leaf area, relative water content and specific leaf area. Moreover, a significant reduction of chlorophyll a, b and total chlorophyll content was observed. Salinity stress raised the content of soluble sugars, proline and glycine betaine. *Eucalyptus sargentii* as the most tolerant species had the optimum growth up to 200 mM NaCl but *E. globulus* presented the most sensitive species to salinity stress. At 200 mM NaCl, proline and glycine beatine raised to 10.57 and 27 µg g^{-1} in the tolerant species (*E. sargentii*), respectively while proline in the sensitive species (*E. globulus*) dropped to 0.003 µg g^{-1}. These results suggest that high tolerance of *E. sargentii* to salinity stress is closely related to lower specific leaf area and enhancement of compatible solutions such as proline, soluble sugar, glycine beatine. This would encourage the possibility of propagating *E. sargentii* in the southern coastal area of Iran. Furthermore, these results provided further biochemical support for the specific abiotic stress tolerance mechanism of *Eucalyptus* species.

Keywords: *Compatible solute, Osmoprotectants, Photosynthetic pigments, Salinity tolerance*

1 INTRODUCTION

Salinity is a major abiotic stress, suppressing crop production worldwide (Verslues *et al*., 2006; Mosaddek *et al*., 2013; Gupta and Huang, 2014). A major emphasis is now being given to growing trees on saline lands to prevent desertification (Singh, 2009). Increased forestation can improve soil health in a number of ways including its impact on soil organic matter, microclimate, reducing evaporation, releasing protons and organic acids in the rhizosphere, decomposition of roots, changing water infiltration, and improving soil aeration and porosity (Nasim *et al*., 2007). Therefore, understanding the mechanisms of plant tolerance to salinity stress is a crucial environmental research topic. Excessive salinity causes hyperosmotic stress and ion

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disequilibrium, leading secondary effects (Gupta and Huang, 2014). It is believed that plant species should possess distinctive indicators of salt tolerance at the whole plant, tissue or cellular level (Lawlor and Cornic, 2002; Akhzari and Ghasemi Aghbash, 2013). There is strong evidence that glycine betaine and proline play an adaptive role in mediating osmotic adjustment, and protecting the sub-cellular structures in plants under stress condition (Ben Ahmed *et al*., 2012 and; Iqbal *et al*., 2014). A positive correlation was recorded between the accumulation of these two osmolytes and stress tolerance (Wani *et al*., 2013). *Eucalyptus* species constitute the dominant canopy in many forest and woodland ecosystems across the Oceania continent. Over 1250 species of *Eucalyptus* are formally recognized, and together occupy a broad range of habitats (Bell *et al*., 1994; Assareh and Sardabi, 2006). Some sections of the genus are renowned for their tolerance to saline conditions and capability to tolerate high salinity (Houle *et al.*, 2001; El-juhany *et al*., 2008; Assareh and Shariat, 2009; Ramírez-valiente *et al*., 2014). *Eucalyptus camaldulensis*, the most widespread Australian eucalypt, has the ability to tolerate both waterlogging and salinity, and expresses a considerable genotypic variation (Farrell *et al*., 1996). *Eucalyptus raveretiana*, *E. spathulata*, *E. sargentii and E. loxophleba* are other species that grow well under moderately saline conditions. These four eucalypt species showed variable osmotic adjustment and accumulated a range of low molecular weight carbohydrates and other potential osmolytes in response to saline conditions (Adams *et al*., 2005). *Eucalyptus* species with the capability to produce aerenchyma in root tissues can be used to rehabilitate the lower regions of catchments affected by increasing periods of soil anoxia. Some eucalypts such as *E. camaldulensis* excluded salt from root zone when salinity levels elevated (Leksungnoen *et al*., 2014). Increased salinity is often associated with reduced plant growth, which is manifested in decreased stem diameter crown volume. Stomatal conductance and photosynthetic rates decrease under saline conditions (Barrett *et al*., 2005; Pita and Pardos, 2001; Lawlor and Cornic, 2002; Ngugi *et al*., 2004; Kawakami *et al*., 2006; Suriyan and Chalermpol, 2009; Noreen and Ashraf, 2009; Mosaddek *et al*., 2013; Akhzari and Ghasemi Aghbash, 2013).

This study aims to investigate distinctive indications of salt tolerance at the whole plant, tissue and cellular level, and also the biochemical mechanisms of *Eucalyptus* tolerance facing salinity stress to provide plant breeders with appropriate indicators. The results strongly support the hypothesis that the biosynthesis of osmoprotectants increases under stress conditions due to the enhancement of salinity stress.

2 MATERIALS AND METHODS

2.1 Plant materials and culture

Seeds of *E. kingsmillii*, *E. tetragona*, *E. salubris*, *E. occidentali*, *E. microtheca*, *E. camaldulens*, *E. globules* and *E. sargentii* were obtained from Kim Seed Co., Wangara, Australia. These species were selected because of their economic importance and faster growth in comparison to other *Eucaluptus* species. The seeds were germinated in pots filled with sterilized marble chips under controlled green house (20°C day/15°C night) in the Biotechnology Research Department of Institute of Forests and Rangelands of Iran. The experimental design was completely randomized with five replications for five treatments. When the seedlings reached at the two-leaf stage, half-strength Hoagland solution was used for irrigation (Rubio *et al*., 2011). Only one good seedling per pot was kept, and the others were eliminated. Four month-old seedlings were watered by five levels of salt solution (0, 50, 100, 150 and 200 mM of NaCl) (EC equal to 0, 3.1, 7.9, 12.3 and 19.4 dS m^{-1}, respectively); electrical conductivities were measured with a Model Mi 180 bench meter; Martini instrument; Romania) used in five replications with a factorial experimental design. To do this, 25 seedlings were assessed for each species. Salt concentrations were gradually increased by 25 mM NaCl increments at 2 d intervals to reach the maximum salinity level of 200 mM NaCl. Samplings were carried out from the stamen leaves of different treatments with one month interval (Adams *et al*., 2005).

2.2 Measurement of physiological and growth parameters

For biomass analysis, the leaves, branches and stems of every harvested seedling were separated and dried at 70 ºC for 48 h before weighing. At each harvest, 10 fully-expanded leaves per plant were collected, and leaf area, specific leaf area (SLA) and weight ratios were calculated (Assareh and Shariat, 2009). The single side area of fresh leaves was measured using a leaf area meter, and then weighed after drying at 70 ºC for 48 h (Shariat and Assareh, 2008). Relative water content (RWC) was measured through incubating 0.5 g leaf samples in 100 ml of distilled water for 6 h, and calculated using Eq. 1 applied by Beadle *et al*. (1993):

$$\mathrm{RWC}=\frac{(\mathrm{FM}-\mathrm{DM})}{(\mathrm{TM}-\mathrm{DM})} * 100 \qquad (1)$$

Where, FM, DM and TM stand for fresh mass, dry mass, and turgid mass, respectively. Chlorophyll a and b levels together with carotenoid content were assessed using the method employed by Jason (1978) in 0.25 g leaf samples homogenized in 4.5 ml of 80% acetone. Light absorbance of the leaves was recorded at 645, 663 and 470 nm using a CECIL Model 3000 spectrophotometer (Cambridge, UK). Glycine betaine was measured applying the method used by Grattan and Grieve (1994). Accordingly, 0.1 g of dried ground material was added to 5 ml of toluene–water mixture (0.5% toluene). All the test tubes were shaken mechanically for 24 h at 25˚C. The extract was filtered and made up to a volume of 100 ml. To 1 ml of filtrate, 1 ml of hydrogen chloride (HCl) solution (2 M) was added. Then an aliquot of 0.5 ml from the earlier extract was taken, and 0.1 ml of potassium triiodide (I_3K) solution was added. It was then shaken in an ice bath for 90 min, and then ice-cooled water (2 ml) was added along with 4 ml of 1,2 dichloroethane ($C_2H_4Cl_2$). By stirring, two layers were formed. The lower colored layer was taken for reading. The optical density was read at 365 nm using a CECIL Model 3000 spectrophotometer (Cambridge, UK). Reference standards of Glycine betaine (50-200 µg ml^{-1}) were prepared in 2 M sulfuric acid. Free proline content was determined using the method of Bates *et al.* (1973). Total soluble sugar was measured by Anthrone method (Irigoyen *et al*. 1992).

2.3 Statistical analysis

Variables were tested for normality using the Shapiro-Wilk test. Homogeneity of the variances was tested using Levene statistic, and transformations were performed when necessary to meet the underlying statistical assumptions of ANOVA using SPSS 17. Least Significant Difference (LSD) test at confidence level of 99% was used to separate means when interaction between the salinity levels and the species was significantly different. Standard error of mean (SE) was employed to indicate the variability of the data. The simple correlation coefficient was calculated to determine the relationships between the studied physiological traits using Pearson's correlation coefficient in the SPSS 17 software.

3 RESULTS

3.1 Proline, soluble sugar and glycine betaine contents

Analysis of variance indicated significant ($P<0.01$) effects of species and salinity on all parameters and significant species and salinity interaction effect for most of the traits (Table 1). LSD test at confidence level of 99% was used to separate the means (Table 2). High proline content was observed in *E. sargentii* grown under sodium chloride (NaCl) treatment; however, it is not clear whether this proline accumulation was indirectly induced due to osmotic stress or the direct effect of NaCl ions. The net increase of proline for *E. sargentii* seedlings peaked 13 fold compared to that of *E. salubris,* and the concentration of proline in *E. globolus* was zero suggesting that *E. globolus* is the most susceptible among the studied species. The soluble sugars' content extracted from the shoots increased progressively by increasing the intensity of salt stress (Table 2 and Figure 1). The accumulation of soluble sugars in the leaves of all species is shown in Figure 1, and the significancy ($P<0.01$) among the treatments analyzed by LSD methods is shown in Table 2. Soluble sugar was comparatively lower for *E. salubris* and *E. globolus* than other species but for *E. sargentii* it was the highest. Accumulation of sugars in *E. salubris* and *E.kingsmilli* increased at 100 mM NaCl; however, it decreased at 150 and 200 mM NaCl. Soluble sugars increased from 624 ± 39.6 to 1729 ± 58.9 in *E. occidentalis*. Glycine betaine concentration of the leaves was also affected by salinity depending on the level of salinity, and increased significantly as salinity increased (Table 2 and Figure 1). Simple correlation coefficient analysis showed the existence of significant positive or negative correlations among the physiological characteristics (Table 3). Osmoprotectants, which are important characters, exhibited positive correlation with each other.

Table 1: ANOVA for NaCl treatments (0, 50, 100, 150 and 200 mM) on the different parameters of eight *Eucalyptus* species

	Proline (µg g^{-1} F.W.)	Soluble sugar (µg g^{-1} D.W.)	Glycine betaine (µgg^{-1} D.W.)	Carotenoid (mg g^{-1} F.W.)	RWC (%)	Biomass (g)	Leaf Area (mm^2)	SLA	Chlorophyll (mgg^{-1} F.W.)		
									Total	a	b
Species	92.32**	1640472**	100603**	69.43**	706.4**	329**	1079411**	3541**	15.5**	5.9**	2.93**
Salinity	10.42**	718811**	45904**	12.00**	2127**	953**	925879**	282**	7.1**	2.2**	1.39**
Species *Salinity	2.07**	35797**	2184**	9.48**	125.8**	32ns	75829 ns	195**	0.37**	0.11**	0.10**
Error	0.21	2640	47	0.02	31.6	33.5	107288	61.4	0.05	0.02	0.01
CV %	4.7	8.4	9.1	8.9	7.8	10.3	9.5	6.8	5.8	4.3	6.1

ns: non-significant difference (P>0.05) and ** significant difference (P<0.01)
RWC: relative water contents, SLA: specific leaf area

Table 2: Effect of NaCl (0, 50, 100, 150 and 200 mM) on the growth and physiological parameters of eight *Eucalyptus* species. Mean±SE, n=5, LSD for all pair comparisons at P=0.01 in each column are shown.

Species	Salinity level	Proline (μgg^{-1}F.W.)	Soluble sugar (μgg^{-1}D.W.)	Glycine betaine (μgg^{-1}D.W.)	Carotenoid (mg g^{-1}F.W.)	RWC (%)	Biomass (g)	Leaf Area (mm^2)	SLA	Chlorophyll (mg g^{-1} F.W.)		
										Total	a	b
E. kingsmillii	0	0.33 ± 0.04	523 ± 4.2	97 ± 3.2	1.3 ± 0.00	97.0 ± 0.0	22 ± 1.6	1,780 ± 137	71 ± 6.4	3.3 ± 0.15	1.8 ± 0.08	1.5 ± 0.09
E. kingsmillii	50	0.33 ± 0.02	979 ± 46.1	144 ± 9.2	3.7 ± 0.01	96 ± 0.4	23 ± 1.9	1,885 ± 433	69 ± 5.3	3.4 ± 0.24	1.9 ± 0.05	0.9 ± 0.02
E. kingsmillii	100	0.55 ± 0.02	1,152 ± 51.0	152 ± 11.2	3.1 ± 0.05	85 ± 0.4	23 ± 2.4	1,620 ± 156	53 ± 4.5	2.9 ± 0.15	0.7 ± 0.01	0.5 ± 0.04
E. kingsmillii	150	0.94 ± 0.02	967 ± 68.2	293 ± 12.4	3.9 ± 0.23	71± 0.7	21 ± 2.0	1,450 ± 212	51 ± 2.3	2.9 ± 0.13	1.3 ± 0.01	1.0 ± 0.04
E. kingsmillii	200	0.83 ± 0.04	1,146 ± 51.8	248 ± 15.2	2.5 ± 0.07	91 ± 0.6	20 ± 5.9	1,370 ± 217	44 ± 6.2	2.7 ± 0.15	1.0 ± 0.37	1.1 ± 0.03
E. tetragona	0	0.56 ± 0.05	1,047 ± 32.8	184 ± 14.2	7.7 ± 0.01	98 ± 0.2	39 ± 5.0	2,210 ± 125	70 ± 3.5	2.4 ± 0.35	2.2 ± 0.04	0.5 ± 0.01
E. tetragona	50	0.61 ± 0.01	1,176 ± 48.8	186 ± 12.3	7.9 ± 0.03	87 ± 0.5	40 ± 1.3	2,146 ± 146	59 ± 1.7	2.4 ± 0.14	0.7 ± 0.05	0.4 ± 0.00
E. tetragona	100	1.77 ± 0.18	1,256 ± 64.3	192 ± 18.9	8.2 ± 0.09	90 ± 0.3	31 ± 2.3	19,027±137	55 ± 1.9	2.4 ± 0.13	2.9 ± 0.09	2.2 ± 0.10
E. tetragona	150	0.59 ± 0.02	1,263 ± 57.2	219 ± 21.3	6.4 ± 0.06	95 ± 0.4	26 ± 1.2	1,850 ± 103	51 ± 4.9	2.2 ± 0.49	1.1 ± 0.03	1.0 ± 0.06
E. tetragona	200	0.64 ± 0.05	1,045 ± 67.2	229 ± 12.6	9.2 ± 0.11	97 ± 0.2	24 ± 4.9	1,730 ± 235	45 ± 4.9	1.7 ± 0.18	0.7 ± 0.06	0.7 ± 0.02
E. salubris	0	0.26 ± 0.01	1,342 ± 27.8	172 ± 12.3	1.7 ± 0.06	77 ± 0.4	30 ± 6.1	1,980 ± 184	60 ± 9.5	2.7 ± 0.03	0.5 ± 0.01	0.4 ± 0.02
E. salubris	50	0.79 ± 0.02	1,358 ± 22.1	176 ± 15.6	2.1 ± 0.06	92 ± 0.7	29 ± 3.8	1,841 ± 177	55 ± 2.2	2.6 ± 0.05	0.9 ± 0.01	0.9 ± 0.05
E. salubris	100	0.82 ± 0.03	1,505 ± 40.7	184 ± 9.6	1.3 ± 0.04	91 ± 0.6	28 ± 5.6	1,620 ± 207	47 ± 1.6	2.6 ± 0.04	1.1 ± 0.06	1.1 ± 0.00
E. salubris	150	1.21 ± 0.05	1,288 ± 20.4	210 ± 11.2	1.9 ± 0.01	92 ± 0.7	26 ± 5.4	1,610 ± 126	39 ± 5.1	2.2 ± 0.06	2.0 ± 0.04	0.7 ± 0.01
E. salubris	200	0.63 ± 0.00	1,263 ± 29.1	215 ± 8.4	1.9 ± 0.07	76 ± 0.4	25 ± 6.0	1,590 ± 93	31 ± 1.6	1.8 ± 0.03	0.6 ± 0.02	0.4 ± 0.01
E. occidentali	0	0.73 ± 0.21	624 ± 39.6	147 ± 11.1	8.5 ± 0.06	90 ± 0.5	25 ± 2.2	1,390 ± 147	61 ± 1.7	4.4 ± 0.15	2.5 ± 0.06	1.9 ± 0.10
E. occidentali	50	0.74 ± 0.04	587 ± 43.2	141 ± 14.2	7.4 ± 0.07	92 ± 0.3	26 ± 2.0	1,246 ± 165	56 ± 3.9	4.6 ± 0.17	2.0 ± 0.07	0.9 ± 0.10
E. occidentali	100	0.83 ± 0.05	791 ± 82.1	186 ± 13.2	6.9 ± 0.03	90 ± 0.8	24 ± 3.5	1,210 ± 135	54 ± 5.5	3.8 ± 0.07	1.5 ± 0.05	0.4 ± 0.03
E. occidentali	150	1.70 ± 0.04	1,148 ± 61.8	180 ± 21.5	7.4 ± 0.03	89 ± 0.8	24 ± 1.7	1,201 ± 69	51 ± 2.3	3.7 ± 0.05	1.4 ± 0.03	0.3 ± 0.02
E. occidentali	200	2.60 ± 0.04	1,729 ± 58.9	243 ± 16.2	7.1 ± 0.11	86 ± 0.9	22 ± 3.4	1,160 ± 49	47 ± 0.5	3.7 ± 0.07	0.5 ± 0.04	0.4 ± 0.03
E. microtheca	0	0.35 ± 0.03	1,267 ± 38.4	198 ± 11.4	8.2 ± 0.11	91 ± 0.7	12 ± 2.4	1,361 ± 355	66 ± 0.8	1.7 ± 0.04	0.5 ± 0.03	0.3 ± 0.02
E. microtheca	50	0.43 ± 0.04	1,637 ± 43.3	209 ± 23.1	1.4 ± 0.05	88 ± 0.9	12 ± 1.2	1,280 ± 506	49 ± 1.5	1.6 ± 0.04	0.3 ± 0.03	0.2 ± 0.03
E. microtheca	100	0.94 ± 0.02	1,410 ± 11.5	227 ± 27.3	1.6 ± 0.06	76 ± 0.8	11 ± 2.1	1,226 ± 231	32 ± 5.8	1.5 ± 0.02	0.5 ± 0.00	0.3 ± 0.02
E. microtheca	150	1.28 ± 0.06	1,415 ± 17.8	239 ± 18.1	1.3 ± 0.04	76 ± 0.5	10 ± 0.4	1,072 ± 151	25 ± 5.4	1.3 ± 0.02	0.4 ± 0.01	0.3 ± 0.01
E. microtheca	200	1.56 ± 0.13	1,449 ± 9.7	245 ± 31.1	1.2 ± 0.03	74 ± 1.1	9 ± 1.5	910 ± 88	25 ± 3.2	1.1 ± 0.01	0.4 ± 0.01	0.2 ± 0.01
E. camaldulens	0	1.02 ± 0.03	1,299 ± 15.1	279 ± 15.2	2.3 ± 0.06	90 ± 1.2	16 ± 2.0	1,242 ± 136	53 ± 3.7	2.3 ± 0.03	0.7 ± 0.01	0.6 ± 0.02
E. camaldulens	50	1.36 ± 0.06	1,320 ± 45.8	350 ± 19.5	1.9 ± 0.06	89 ± 0.9	17 ± 2.8	1,180 ± 93	45 ± 2.8	2.3 ± 0.03	0.6 ± 0.00	0.6 ± 0.03
E. camaldulens	100	1.85 ± 0.07	1,246 ± 24.7	359 ± 27.4	1.6 ± 0.04	83 ± 0.06	14 ± 1.0	1,100 ± 82	44 ± 1.3	2.2 ± 0.02	0.5 ± 0.01	0.4 ± 0.03
E. camaldulens	150	1.80 ± 0.10	1,555 ± 35.0	363 ± 23.5	1.1 ± 0.04	83 ± 1.3	12 ± 0.6	980 ± 290	38 ± 1.9	2.1 ± 0.05	0.8 ± 0.06	0.7 ± 0.03
E. camaldulens	200	1.36 ± 0.15	1,661 ± 26.0	386 ± 36.1	1.1 ± 0.10	73 ± 0.9	10 ± 2.6	920 ± 67	34 ± 1.1	1.9 ± 0.04	0.8 ± 0.02	0.4 ± 0.00
E. globulus	0	0.0 ± 0. 0	1,120 ± 29.7	142 ± 11.2	0.7 ± 0.05	92 ± 0.1	30 ± 1.9	1,750 ± 102	69 ± 4.3	2.0 ± 0.08	0.6 ± 0.04	0.3 ± 0.00
E. globulus	50	0.0 ± 0. 0	1,287 ± 20.3	224 ± 18.2	1.9 ± 0.02	87 ± 2.5	28 ± 4.6	1,690 ± 97	68 ± 3.0	1.5 ± 0.12	1.8 ± 0.04	0.6 ± 0.01
E. globulus	100	0.0 ± 0. 0	1,451 ± 32.8	255 ± 15.7	2.1 ± 0.02	83 ± 1.6	24 ± 4.2	1,682 ± 111	67 ± 4.9	1.2 ± 0.09	1.7 ± 0.03	0.5 ± 0.01
E. globulus	150	0.0 ± 0. 0	1,520 ± 36.6	224 ± 12.4	2.1 ± 0.32	72 ± 1.4	21 ± 2.2	1,650 ± 212	64 ± 1.8	0.9 ± 0.08	1.4 ± 0.08	0.5 ± 0.01
E. globulus	200	0.0 ± 0. 0	1,376 ± 3.1	252 ± 16.1	1.5 ± 0.06	63 ± 0.9	17 ± 2.3	1,600 ± 291	64 ± 8.6	0.8 ± 0.13	0.5 ± 0.02	0.3 ± 0.01
E. sargentii	0	0.01 ± 0.00	1,395 ± 12.6	223 ± 16.3	7.4 ± 0.05	95 ± 1.3	19 ± 1.8	1,257 ± 84	34 ± 2.4	3.7 ± 0.02	0.5 ± 0.02	0.3 ± 0.00
E. sargentii	50	0.00 ± 0.00	1,445 ± 17.6	291 ± 23.4	6.1 ± 0.02	92 ± 2.5	20 ± 0.7	1,260 ± 56	31 ± 1.7	3.8 ± 0.02	0.4 ± 0.03	0.3 ± 0.01
E. sargentii	100	4.30 ± 0.14	1,922 ± 12.7	371 ± 18.1	7.9 ± 0.04	89 ± 2.1	19 ± 4.2	1,233 ± 100	30 ± 0.3	3.2 ± 0.07	2.3 ± 0.03	1.8 ± 0.07
E. sargentii	150	6.20 ± 0.17	1,963 ± 59.2	387 ± 26.3	4.2 ± 0.01	89 ± 1.7	19 ± 2.9	1,117 ± 69	27 ± 0.6	3.2 ± 0.08	1.8 ± 0.05	1.3 ± 0.06
E. sargentii	200	8.20 ± 0.15	2,024 ± 30.7	442 ± 27.1	5.7 ± 0.01	86 ± 1.2	18 ± 2.3	1,103 ± 102	23 ± 2.0	2.9 ± 0.04	1.6 ± 0.06	1.3 ± 0.07
LSD 1%		**0.17**	**64.42**	**8.58**	**0.18**	**7.05**	**7.26**	**410.7**	**9.82**	**0.18**	***0.16**	**0.09**

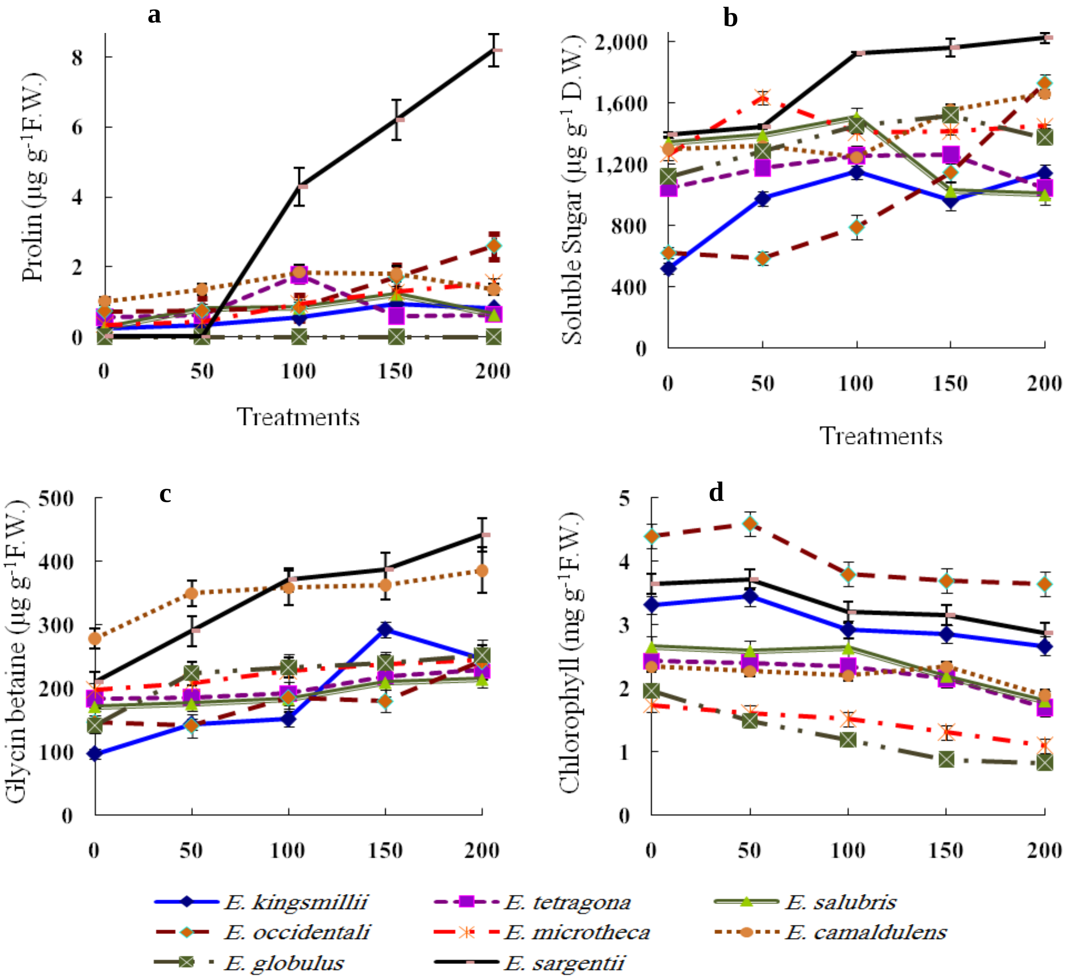

Figure 1: Effect of Nacl level on the accumulation amount (µg g^{-1} fresh weight) of proline (a); soluble sugar (b), glycine betaine (c) and total chlorophyll (d) content (mg g^{-1} fresh weight) in 8 *Eucalyptus* species.

3.2 NaCl treatment and the photosynthetic pigment content of the leaves

There was an inverse relationship between the salinity and total pigments of the leaves (Table 2). The lowest NaCl level (50 mM) favored chlorophyll production in *E. kingsmilii*, *E. sargentii* and *E. occidentalis* but the higher level of salinity was inhibitory. The species expressed a significant variation. Total chlorophyll, as well as chlorophyll a and b concentrations were comparatively higher for *E. occidentalis* than for other species (Table 2), and *E. globolus* and *E. microtheca* had the lowest amounts of pigments. The reduction of total chlorophyll, and chlorophyll a and b in comparison to the control plants was 45%, 44%, and 50%, respectively (Table 2). The differences among *Eucalyptus species* regarding chlorophyll content observed in this study may have been due to the differences of the age of the leaves, despite our efforts to choose leaves with similar ages. Total chlorophyll exhibited significantly positive correlation with soluble sugar, carotenoid, biomass and RWC (Table 3).

Table 3: Pearson correlations for the growth and physiological parameters of eight *Eucalyptus* species

	Proline	Soluble sugar	Glycine betain	Total chloroh	Carotenoid	Biomass	RWC	Leaf area	SLA
Proline	1	0.62**	0.67**	0.60**	0.28**	-0.01^{ns}	0.01^{ns}	-0.14^{ns}	-0.32**
Soluble sugar	0.62**	1	0.57**	0.17^{ns}	-0.09^{ns}	-0.12^{ns}	-0.37**	0.02^{ns}	-0.55**
Glycine betain	0.66**	0.57**	1	0.16^{ns}	0.06^{ns}	0.06^{ns}	-0.19*	-0.13^{ns}	-0.11^{ns}
Total chloroh	0.60**	0.17^{ns}	0.16^{ns}	1	0.34**	0.38**	0.42**	0.05^{ns}	-0.11^{ns}
Carotenoid	0.28**	-0.09^{ns}	0.06^{ns}	0.34**	1	0.08	0.24**	0.23*	0.06^{ns}
Biomass	-0.01^{ns}	-0.12^{ns}	0.06^{ns}	0.38**	0.08^{ns}	1	0.41**	0.23*	0.03^{ns}
RWC	0.01^{ns}	-0.37**	-0.19*	0.42**	0.24**	0.41**	1	0.19*	0.24**
Leaf area	-0.15^{ns}	0.02^{ns}	-0.13^{ns}	0.05^{ns}	0.23*	0.23*	0.19*	1	-0.01^{ns}
SLA	-0.32**	-0.55**	-0.11^{ns}	-0.11^{ns}	0.06^{ns}	0.03^{ns}	0.25**	-0.01^{ns}	1

ns: non-significant difference (P<0.05) and the significance is indicated: * P<0.05, ** P<0.01
RWC: relative water contents, SLA: specific leaf area

3.3 Growth parameters, relative water contents (RWC) and specific leaf area (SLA)

We observed a slight increment in the dry matter and biomass weight of *Eucalyptus* species under all levels of salinity treatment except for 50 mM. Generally, the lower NaCl concentration favored plant growth, and higher salinity concentration was inhibitory. The mean total biomass of the plants increased at 50 mM salt level (Table 2), and decreased at higher salt concentrations in all the eight examined species (Table 3). Comparatively, *E. tetragona* exhibited higher height growth and biomass production in most ranges of the imposed salinity than the other species, and *E. camaldulensis* and *E. microtheca* exhibited the lowest biomass under the mentioned salinity treatments. Simple correlation coefficient analysis showed the existence of significant correlations among the RWC with other characters except soluble sugar (Table 3). Leaf area and SLA were comparatively higher for *E. globolus* in contrary to RWC, which was the lowest. *E. sargentii* exhibited the highest percentage of RWC but the lowest for SLA.

4 DISCUSSION

The tolerance of *E. sargentii* and *E. occidetalis* seedlings to salinity was correlated with changes in osmoprotectants, photosynthetic pigments, RWC, SLA and biomass. Consistently low photosynthetic pigments (chlorophyll a, b and total), as in *E. globolus*, is characteristic of salinity-sensitive species, whereas the maximum chlorophyll content, as in *E. occidentalis*, can occur in salinity-tolerant species. By increasing the salinity, the mean of biomass and leaf area of the treated plants were correspondingly declined (Table 2). Suriyan and Chalermpol (2009) reported that biomass decline can be correlated with the decrease of photosynthetic rate. Furthermore, Mosaddek *et al*. (2013) obtained similar results, suggesting the leaf dry weight is correlated with osmotic potential level. Pita and Pardos (2001) correlated the osmotic potential with SLA. Correlation between photosynthetic rate and osmotic potential was reported by Ngugi *et al.* (2004). The reduction of leaf area could be attributed to the negative effect of stress on the rate of cell elongation, cell volume and cell number (Kawakami *et al*., 2006). At the cellular level, reduced water potential and RWC affect the physiological activity of the cells in several ways, including changes in intercellular organelle positions, transport channels, enzymatic activity, and cell wall shrinkage (Lawlor and Cornic, 2002). This result is in agreement with the findings of Akhzari and Ghasemi Aghbash (2013) who stated salinity had a significant effect on the leaf area and growth of the leaves by reducing the rate of photosynthesis. Variations in salt tolerance have been reported previously in different species and genotypes of woody plants such as eucalypt (Adams *et al*., 2005), almond (Zrig *et al*., 2015) and palm (Yaish and Kumar, 2015). Clonal *Eucalyptus* lines of Australian tree species have been developed for tolerance to saline and or waterlogged conditions. Selected and cloned (*E. camaldulensis*, *E. spathulata* subspecies *spathulata*, *Casuarina obesa* and *C. glauca*) showed higher survival rates, and the surviving plants grew faster than provenance matched seedlings (Bell *et al*., 1994). Moreover, different stages of growth, irrigation and climatic conditions, as well as soil fertility are also known to influence salt tolerance (Assareh and Shariat, 2009). Increasing the salt concentration in the present study decreased the SLA of eight *Eucalyptus* species that concurs with other results (Salter *et al*., 2007). El-juhany *et al*., 2008 found the SLA of *E. camaldulensis*, *E. microtheca* and *E. intertexta* decreased in high salinity treatment. In contrast, Houle *et al.* (2001) reported that salinity treatment had no effect on SLA. In the present study *E. sargentii* and *E. microtheca* had the lowest SLA, that it was related to common mechanism of adjustment to salinity stress, indicating the capacity of *Eucalyptus* to adjust to the environmental conditions morphologically and physiologically. The reduction of SLA could be along with an increase in leaf thickness or tissue density, which was reported by Ramírez-Valiente *et al*. (2014). Nitrogen concentration, light and water availability and salinity stress could affect SLA (Ramírez-valiente *et al*., 2014).

The decrease in chlorophyll content under salinity conditions has been reported by Kusvuran (2010), and Nazarbeygi *et al.* (2011). The negative correlation between leaf chlorophyll concentration and salinity can indirectly occur as a result of stomatal closure (Syvertsen and Garcia-Sanchez, 2014) due to increased activity of the chlorophyll degrading enzyme, chlorophylase (Noreen and Ashraf, 2009). In the salt tolerant species, the chlorophyll content was protected probably because of the high antioxidant enzyme activities that prevented degradation of leaf chlorophyll. Pearson's correlation coefficient analysis showed the existence of significant positive or negative correlations among most of the characteristics. These achievements will help us for future selection program in order to produce seedlings, which are potentially suitable for salinity stress tolerance. Compatible solute accumulation as a response to osmotic stress is a ubiquitous process in organisms. However, the solutes that accumulate vary in the organisms and even in different plant species (Ben Ahmed *et al*., 2012). A major category of organic osmotic solutes consisting of sugars, glycerol, amino acids, sugar alcohols and other low molecular weight metabolites is one of mechanisms evolved by plants to overcome salt stress (Verslues *et al*., 2006; Gupta and Huang, 2014). The role of reducing sugars (glucose and fructose) in the adaptive mechanism is more controversial, and even their accumulation can be detrimental from several points of view (Kerepesi and Galiba, 2000). Moreover, the current results indicate that total soluble sugar content might be a useful trait to select salt tolerant species. The highest accumulation of glycine betaine was observed during the salinity stress in *E. sargentii* and *E. camaldulensis* that coincides with the highest values of RWC. During the salinity stress, averages of glycine betaine and proline content in the leaves of eucalypt treated plants were 50% higher than those grew in normal treatments. These results are in accordance with the idea of Ben Ahmed *et al*. (2012) and Iqbal *et al*. (2014) indicating that proline is known to accumulate in large quantities in higher plants in response to the environmental stresses. Glycine betaine is another extensively studied compatible solute that protects the plant by maintaining the water balance between the plant cell and the environment by stabilizing macromolecules (Wani *et al*., 2013) and preserves thylakoid and plasma membrane integrity after exposure to saline solutions or freezing or high temperatures (Rhodes and Hanson, 1993). Since salt tolerant natural populations meet demands for stress tolerant plants in the modern time's Agro-forestry, this material will prove very useful for revegetation of salt-affected forests, rangelands and prairies by direct growth of such salt tolerant species.

5 CONCLUSION

This research was carried out to estimate the substances produced by most *Eucalyptus* species that behave as anti-stress metabolites pre-accumulated to caution the whole plant against the stresses without interference of soil types and characteristics. The leaves of *E. sargentii* accumulated more proline, soluble sugar and pigments under salinity stress as compared to other species. The results demonstrated that *E. sargentii* has efficient osmoprotectants characteristics' accumulation, which could provide better protection against oxidative and osmotic stress in leaves under salinity stress conditions. Also significant differences in SLA, biomass and leaf area were found in *Eucalyptus* species. The most tolerant species *Eucalyptus sargentii* exhibited the lowest values for SLA. Likewise, reduced SLA had fitness benefits in terms of growth for plants under salinity conditions. This result is important ecologically and economically regarding the advantageous of *Eucalyptus*. Further research is recommended on the salinity tolerance mechanisms in the field with considering natural soil body.

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پاسخ‌های فیزیولوژیکی و زیستی شیمیایی هشت گونه اکالیپتوس به تنش شوری

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چکیده در تحقیق حاضر، اثرات تنش شوری بر برخی صفات فیزیولوژیکی و زیستی شیمیایی نهال‌های هشت گونه اکالیپتوس (*E. kingsmillii, E. tetragona, E. salubris, E. occidentali, E. microtheca, E. camaldulens, E. globules, E. sargentii*) مورد بررسی قرار گرفت. نهال‌های چهار ماهه استقرار یافته در گلخانه با پنج سطح ۰، ۵۰، ۱۰۰، ۱۵۰ و ۲۰۰ میلی مولار نمک طعام به‌مدت یک ماه در قالب آزمایش فاکتوریل بر پایه طرح کاملاً تصادفی در پنج تکرار آبیاری شدند. نتایج بیان‌گر آن بود که شوری منجر به تاخیر و کاهش رشد و نیز کاهش تدریجی اکثر متغیرهایی نظیر سطح برگ، محتوای نسبی آب و سطح ویژه برگ شد. علاوه بر این، مقدار کلروفیل کل، a و b کاهش قابل توجهی یافت. تنش شوری محتوای قندهای محلول، پرولین و گلیسین بتایین را افزایش داد. در میان گونه‌های مورد مطالعه *Eucalyptus sargentii* به‌عنوان مقاوم‌ترین گونه، تا شوری ۲۰۰ میلی مولار دارای رشد مطلوب بود در حالی که *Eucalyptus globulus* بیش‌ترین حساسیت را در برابر تنش شوری نشان داد. در گونه متحمل *E. sargentii* در شوری ۲۰۰ میلی مولار مقدار پرولین و گلیسین بتائین تا ۱۰/۵۷ و ۲۷ میکروگرم بر گرم افزایش یافت در حالی‌که در گونه حساس مقدار پرولین تا ۰/۰۰۳ میکروگرم بر گرم کاهش یافت. این نتایج بیان‌گر تحمل بالای *E. sargentii* به تنش شوری است که با پایین بودن سطح ویژه برگی، افزایش حلال‌های سازگار نظیر پرولین، قندهای محلول و گلیسین بتائین مربوط می‌شود که می‌تواند عامل ترغیب‌کننده‌ای برای تکثیر *E. sargentii* در کناره‌های جنوبی ایران باشد. علاوه بر این، نتایج تحقیق حاضر در مکانیزم‌های خاص تحمل به تنش‌های غیر زنده در گونه‌های اکالیپتوس قابل کاربرد است.

کلمات کلیدی: تحمل شوری، حفاظت کننده‌های اسمزی، رنگیزه‌های فتوسنتزی، محلول‌های سازگار کننده