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METABOLIC IMPLICATIONS OF SALT INDUCED OSMOLYTE ACCUMULATION IN *AVICENNIA MARINA*

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Abstract

Salt induced solute accumulation is a well known feature for osmotic adjustment in plants under salt stress. Concomitantly, synthesis of compatible osmolytes requires energy which may compromise plant growth. This study highlights the contribution of different solutes to leaf osmolality in *Avicennia marina* seedlings and saplings and possible metabolic shifts under saline conditions. Salinity was imposed using different seawater strengths (0, 50% & 150% seawater salinity) to evaluate contribution of ions and organic osmolytes and their possible effects on plant growth. Optimum growth was found in 50% seawater salinity (SWS) and decreased under salt stress (150% SWS). Low values for water and osmotic potential could be accounted for an increase in the amount of Na^+ which appeared to contribute the most in leaf osmolality in both seedlings (38%) and saplings (43%) under saline conditions. Among tested organic solutes, the contribution of total soluble sugars (TSS) was higher in non-saline control and moderate salinity, followed by glycinebetaine (GB) while proline (PRO) values were lowest. The amount of GB increased to a 4 fold in seedlings and 2 fold in saplings under salt stress. The increased ratio of GB:TSS in seedlings under salt stress indicates their sensitivity over saplings and a likely metabolic shift at the cost of growth.

Key words: Glycinebetaine, Ions, Mangroves, Sugars, Osmolytes, Stress.

Introduction

Soil salinity not only results in reduced growth of plants due to osmotic or ionic effects (Munns & Tester, 2008) but also bring about several changes in physiological responses (Flowers *et al*., 1977; Glenn *et al*., 1999) and metabolic shift during the course of development. Although in most of the obligate halophytes an optimum growth is observed under moderate salinity (Flowers & Colmer, 2008) there is a general trend of reduced growth rates in plants under highly saline conditions. An excessive build up of salts in hyper saline conditions could have serious implications on growth and plant metabolism by hindering nutrient uptake, enzyme activities and impaired photosynthesis (Greenway & Munns 1980; Demir & Kocakalikan 2002; Munns 2005). Moreover, growth restriction could also be a consequence of energy investment in physiological and biochemical processes to avoid excessive ion accumulation (Munns, 2005). To avert ionic toxicity (particularly Na^+), halophytes effectively manage to use them as potential osmotica by sequestering into the vacuole or apoplast using ion exchangers at plasma membrane or tonoplast (Shabala & Shabala, 2011). Another possibility for defense against osmotic challenge is to accumulate a variety of compatible solutes which are zwitterionic and does not interfere with metabolism (Munns & Tester, 2008). Both ion sequestration and synthesis of osmolytes are energy requiring processes which may utilize large amounts of ATP molecules (Lee *et al*., 2008; Slama *et al*., 2007; Slama *et al*., 2015).

Widespread researches have focused on the role of low-molecular weight carbohydrates and nitrogenous compounds (e.g. glycinebetaine, trigonelline, choline etc.) in osmotic adjustment (Downton, 1982; Clough, 1984; Munns, 1988; Popp & Albert, 1995; Aziz & Khan, 2001a; Yang *et al*., 2007). These osmolytes are accumulated in cytosol as well as cell organelles (Ashraf & Harris, 2004) to achieve an osmotic balance. Recent reports suggest that even their low concentration in leaf tissues may have some role in osmoprotection (Türkan & Demiral, 2009; Moghaieb *et al*., 2004) which may be achieved by thylakoid / plasma membrane integrity and protein stabilization besides ROS scavenging (Parida & Das, 2005). Substantial differences in the capacity of osmotic adjustment (OA) have been reported between species and even cultivars (Morgan, 1984; Rhodes & Samaras 1994). The type of major solutes contributing in osmotic potential could vary both with the duration and quantum of stress (Shangguan *et al*., 1999), age of the plant, type of organ as well as leaf age (Munns & Weir, 1981; Kameli & Lo¨ sel, 1995). This raises the argument whether the osmolytes have individual or combined mode of action against environmental stresses (Yancey, 2005). A number of researchers have published articles on OA in halophytes in general and mangroves in particular (Downton, 1982; Clough, 1984; Munns, 1988; Popp & Polania, 1989; Popp & Albert, 1995; Aziz & Khan, 2001a, b; Yancey *et al*., 2005; Slama *et al*., 2015) yet nature and contribution of solutes under different salinity regimes and plant age have not been discussed in detail.

Avicennia marina, a salt secreting mangrove is considered as an obligate halophyte for its physiological requirement of salt to optimize growth although under higher salinities growth response slows down (Wang *et al*., 2011). This true mangrove is known to accumulate large amount of leaf Na^+ and synthesize glycinebetaine (GB) for its osmotic adjustment under salt stress (Popp, 1984b). Some other workers have reported that sugars and polyols may also have a contribution in osmotic adjustment among mangroves (Rhodes *et al*., 2002; Aziz & Khan, 2014). Contribution and nature of solutes to osmotic adjustment has long been debated among mangroves however, studies on glycinebetaine, proline, carbohydrates and other solutes are limited to field conditions (Popp & Albert, 1995; Gil *et al*., 2013) and highly inconclusive in lab grown plants. Assuming that an increased synthesis of organic solutes occurs at the expense of growth it would be interesting to know the effect of varying degrees of salinity on the type and extent of solutes in *A. marina* at different plant ages. The objectives of the present investigation were (i) To specify the contribution of inorganic and constitutively involved organic solutes for osmotic adjustment in *Avicennia marina* (ii) To evaluate possible metabolic shift for the synthesis of organic solutes under variable salinity regimes (iii) to check whether plant sensitivity to inorganic ions is age related (i.e. seedlings and saplings).

Materials and Methods

Propagules of *Avicennia marina* were grown in plastic pots (containing sand) in a netted green house under ambient environmental conditions. They were sub-irrigated with nutrient solution fortified with nitrogen (Popp and Polania, 1989) till twenty weeks for seedling stage and about 40 weeks for sapling stage. Plants were then treated with three seawater salinities (SWS) using commercial sea salt. Preliminary experiments suggested that mangrove seedlings could survive up to 150% SWS and begins to die with a further increase in salinity (Aziz & Khan, 2014), while optimum growth is observed in 50% SWS (Aziz & Khan, 2001a, b). Based on these findings, 50% SWS (half strength of sea water salinity; EC = 26 dSm^{-1}) was taken as moderate salinity, while 150% SWS (1.5 times of sea water salinity; EC = 85 dS m^{-1}) as stressed condition and 0% SWS (EC = 6 dS m^{-1}) was taken as control. The pots were arranged in a randomized block design and five replicates for each treatment were used. Fresh water was added daily to correct water levels due to evaporation. The solution was renewed every 7 days to avoid built up of salinity in pots. Concentration of saline treatment was gradually increased by incrementing 25% sea salt at 2 d interval to reach the maximum salinity of 150% SWS after 12 days following our preliminary tests. Plants were finally harvested 4 weeks after highest salinity was reached.

Growth: Plants were harvested for height, number of leaves and leaf area immediately while dry weight was taken after drying vegetative parts for 48 h in a forced-draft oven at 60◦C.

Water relations: Leaf water potential (Ψ_w) was evaluated immediately using C-52 sample chamber in a Wescor HR-33 T, dew point microvoltmeter (Wescor International, USA) from each treatment (n = 5). Osmotic potential was determined by the method of Koyro & Huchzermeyer (2004). Leaf samples were frozen with liquid nitrogen and homogenized in a mortar. After thawing, the samples were centrifuged (at 4 °C, for 5 min at 3000 x g). The osmotic potential was determined in the supernatant (leaf sap) by vapor pressure osmometer (VAPRO) and converted to mosmol kg^{-1} solution by using the Van't Hoff equation. Relative water content (RWC) was estimated using the following formula: RWC = (FW-DW)/ (TW-DW) ×100, where FW= weight of freshly collected material, TW= weight after rehydration for 20–24 h at 4°C in the dark and DW= weight after drying at 60°C for 48 h. The relative contribution (RC) of each solute in percent was calculated separately using the values of leaf osmotic potential with the help of the following formula:

RC = OP of solute x 100 /Total leaf OP

Determination of ions: Inorganic solutes i.e. potassium (K^+), sodium (Na^+), calcium (Ca^{++}) and magnesium (Mg^{++}) were determined on hot water extracts using Atomic absorption spectrometry (Perkin Elmer Analyst AA-700).

Determination of osmolytes: 500 mg dried plant material was used to make hot water extracts for osmolyte determination. Total soluble sugars (TSS) were by the method of Yemm & Willis (1954). Free sugars were determined on Shimadzu HPLC system (LC – 20 AT) coupled to RI detector (RID – 10 A), equipped with CLC - NH_2 column (4.6 x 250 mm) and auto-sampler (SIL – 20 A) using the method of Harada *et al.* (2004) modified by Barros *et al.* (2007). The sugar standards used for identification were purchased from Sigma chemical Co.

Glycinebetaine (GB) was determined by the method of Khan *et al.* (1999), using a Jasco HPLC system with diode array detector. Proline (PRO) was determined by spectrophotometric method of Bates *et al*. (1973).

Data were analyzed by using SPSS 11.0 for Windows and means were compared using Bonferroni test at the 5% level of significance.

Results

Growth in *A. marina* plants was promoted in moderate salinity (plant height, leaf area and number) and reduced under stress (150% SWS) (Fig. 1). A two-way ANOVA showed significant individual effects of salinity ($p<0.01$), growth stage ($p<0.05$) and their interactions ($p<0.001$) on height and total fresh weight of plants with higher values in saplings (Table 1). Relative water content (%RWC) remained similar (84 – 86%) in non-saline and moderate salinity and a 6-8% decrease was noted under stress in both seedlings and saplings. Leaf water and osmotic potentials significantly ($p < 0.05$) decreased with the increases in salinity with lowest OP (osmotic potential) values (~ 6 MPa) in hypersaline stress (Fig. 2).

Results of two way ANOVA showed significant individual effect of salinity and interaction of growth stage and salinity ($p<0.01$) on leaf Na^+ but individual effect on growth stage appeared non significant (Table 1). A 2 fold increase of Na^+ in seedlings and ~ 3 fold in saplings of *A. marina* leaves was found in hypersaline stress compared to control (Table 2). Moreover, its contribution to the leaf osmolality was highest (38-43%) among all tested cations, followed by Mg^{++} (ca 3 - 4%), K^+ & Ca^{++} (ca 2-3%) (Fig. 3). Accumulation of all cations showed an increasing trend with the increases in salinity with the exception of Ca^{++} which was slightly increased in saplings (Table 2). Leaf Na/K ratio was also increased in both seedlings and saplings under salt stress (Table 2).

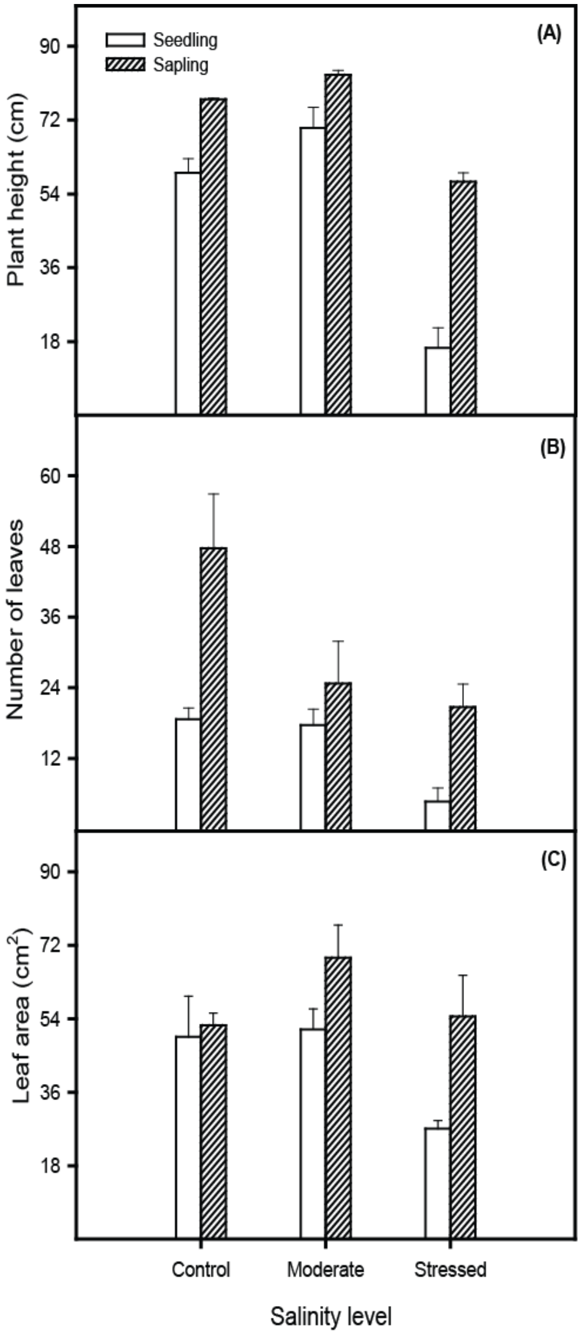

Fig 1. Plant height, Number of leaves and leaf area in seedlings and saplings of *Avicennia marina* under different salinity levels.

Table 1. Results of two way ANOVA for the effect of Salinity (S), Growth stage (G) and their interactions (S x G).

Parameter	S	G	S x G
Plant height	78.34^{**}	31.25^{*}	53.41^{**}
Fresh weight	67.21^{**}	9.25^{*}	21.20^{**}
Sodium	48.10^{**}	$21.20^{n.s.}$	19.62^{**}
Glycinebetaine	101.25^{***}	61.62^{**}	38.41^{**}
Total soluble sugars	39.4^{***}	22.32^{**}	43.61^{***}

Numbers represent F-values. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

A significant increase ($p<0.05$) in leaf GB of *A. marina* was observed with the increases in salinity (Table 3). Results of two way ANOVA revealed a significant individual effect of salinity, growth stage and their interaction ($p<0.001$) on leaf GB (Table 1). Although, trend for GB accumulation was similar in seedlings and saplings it was about 4-fold higher in seedlings and 2 fold in saplings in highest salinity compared to the control (Table 3). In hypersaline conditions the relative contribution of GB increased to ~ 8% in seedlings compared to 3% in control which was highest among tested organic solutes (Fig. 3). On the contrary, its contribution was ~ 6% in saplings compared to those of TSS (~ 9%) in hypersaline stress (Fig. 3). The amount of TSS in *A. marina* was greatly affected by plant age (Table 3). Results of two-way ANOVA indicated significant individual effect of salinity, growth stage and their interaction ($p<0.001$; Table 1). Apparently, both the amount and contribution of TSS was highest among tested organic solutes in all salinity regimes at sapling stage (Table 3; Fig. 3). Among hexose sugars the amount of sucrose was almost twice the amount of glucose and their values were higher in moderate salinity compared to both control and hypersaline stress (Table 3). On the basis of leaf osmolality values, contribution of sucrose was highest (~ 3.5%) in moderate salinity compared to 0.7% in control. Contribution of glucose was 1% in moderate salinity (Fig. 3) compared to the non-saline control (0.3%). Proline (PRO) content increased both in seedlings and saplings under salt stress (Table 3) but its relative contribution to the total osmolality was lowest (< 1%) among all tested organic solutes in all salinity treatments (Fig. 3).

Discussion

Plant growth seems to be highly variable in different species where little amount of salt may result in reduced biomass while tolerant species optimize their growth (Suarez, 2011). While there appears a general trend of growth decline with the increases in salinity (Ahmed & Khan, 2010), some halophytes grow well in low to moderate salinities (Koyro *et al*., 2013; Abideen *et al*., 2014) and mangroves show a similar trend (Aziz & Khan, 2014; Aziz & Khan, 2001b; Khan & Aziz, 2001). Increase in height and plant biomass at moderate salinity corresponds to the previous findings on most of the dicot halophytes (Khan *et al*., 2000; Debez *et al*., 2013). Although not fully understood, this improvement in growth at 50% seawater may be a consequence of an increase in tissue water content (Nerd & Pasternak, 1992) which is revealed through an increase in percent fresh weight. Juvenile plants are considered sensitive to salt stress than adult individuals and this trait is usually reflected in their biomass and energy metabolism (Flowers & Colmer, 2008; Flowers *et al*., 2014). Higher biomass at sapling stage also indicates that mature plants are relatively less sensitive than juvenile ones (Aziz & Khan, 2001a, b; Aziz & Khan, 2014).

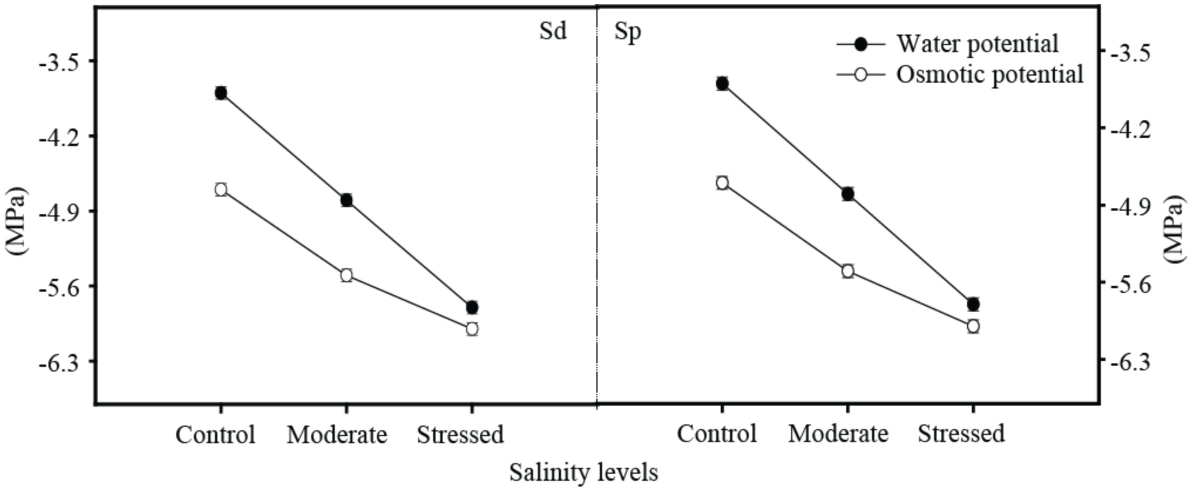

Fig. 2. Leaf water (Ψw) and osmotic potential (Ψs) in seedlings (Sd) and saplings (Sp) of *Avicennia marina* under different salinity levels.

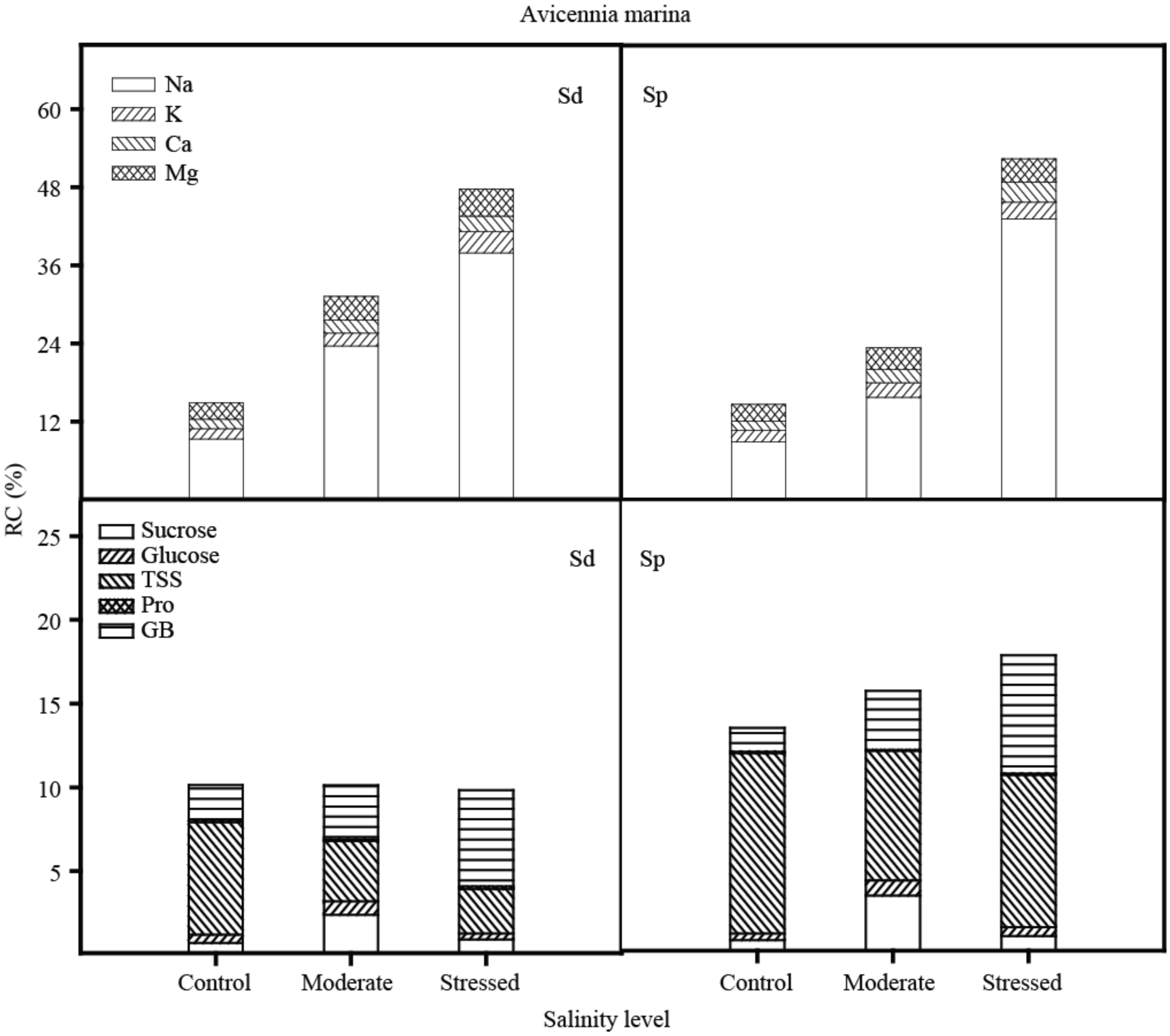

Fig. 3. Percent contribution of inorganic (Na^+ , K^+, $Mg^{++}$and Ca^{++}) and organic (total soluble sugars, sucrose, glucose, Proline and glycine betaine) solutes to measured osmotic potential in *Avicennia marina* leaves at different salinity levels (Sd = seedlings; Sp = saplings).

Table 2. Concentration of inorganic solutes (Na^+, K^+ , Ca^{++} and Mg^{++}) and Na/K ratio in leaves of *Avicennia marina* seedlings and saplings under different salinity levels.

Growth stage	**Solutes (mmol/kg DW)**	**Salinity level**		
		Control	Moderate	Stressed
Seedling	Sodium	969^a ± 59	1648^b ± 10	1919^c ± 3
	Potassium	160^b ± 7	137^a ± 13	167^b ± 3
	Calcium	152^b ± 0.7	137.8^b ± 6	117^a ± 1.1
	Magnesium	265^b ± 5.2	255^b ± 14	211.7^a ± 17.8
	Na/K	6.1^a ± 0.4	12.03^b ± 0.7	11.5^b ± 0.8
Sapling	Sodium	916^a ± 12	1448^b ± 41	2559^c ± 6
	Potassium	182^b ± 3	188^b ± 5	136^a ± 10
	Calcium	145^a ± 1.5	172^b ± 8	167^b ± 3.5
	Magnesium	261^b ± 8	263^b ± 8	187^a ± 2
	Na/K	5.1^a ± 0.3	7.7^b ± 0.4	18.8^c ± 1.4

Means ± standard errors followed by different letters are significantly different at $p<0.05$ (Bonferroni test)

Table 3. Concentration of organic solutes in leaves of *Avicennia marina* seedlings and saplings under different salinity levels.

Growth stage	**Solutes (mmol/kg DW)**	**Salinity level**		
		Control	Moderate	Stressed
Seedling	Sucrose	23.2^a ± 0.3	98.7^c ± 0.4	39.7^b ± 1.2
	Glucose	15.49^a ± 0.3	22.7^c ± 0.7	18.1^b ± 0.8
	Glycine betaine	102^a ± 9.1	279^b ± 2.1	399^c ± 3.7
	Total soluble sugars	560^c ± 9.8	348^b ± 7.6	143^a ± 6.3
	Proline	3^a ± 0.08	3.2^a ± 0.05	6.2^b ± 0.03
Sapling	Sucrose	24^a ± 1.5	118.7^c ± 0.2	41.7^b ± 0.1
	Glucose	14.2^a ± 0.2	34^c ± 0.3	20^b ± 0.2
	Glycine betaine	142^a ± 9.8	244^b ± 9.3	317^c ± 4.2
	Total soluble sugars	779^c ± 15.4	612^b ± 13.9	530^a ± 12.8
	Proline	2.26^a ± 0.05	2.02^a ± 0.06	3.4^b ± 0.02

Means ± standard errors followed by different letters are significantly different at $p<0.05$ (Bonferroni test)

Growth in supra-optimal salinities is usually influenced either due to cellular dehydration or low turgor (Munns & Tester, 2008). A higher variability in leaf RWC (relative water content) to salt exposure hint towards cellular dehydration while little changes in this parameter indicates accumulation of ions for increased water uptake (Suarez, 2011). Minor changes in leaf RWC in this study suggest that ion accumulation provides an osmotic driving force hence, net accumulation of salts appeared to contribute in lowering leaf osmotic potential in salinity treatments than cellular dehydration. Moreover, water potential gradient in plants ensures enhanced water uptake and this is done by solute accumulation (Aziz & Khan, 2001 Mention a/b; Koyro *et al*., 2006). Although, mangroves are usually found growing near the inter-tidal zones and they are not subjected to physical drought they have to cope with salt water which often have lower osmotic potential than seawater (Scholander, 1968). Due to this inherent property they appear to maintain very low OP (osmotic potential) of ≥ 2 MPa even in non-saline conditions. Under extremely saline conditions (higher than seawater) values for water and osmotic potential could be as low as -6 MPa which is essential to maintain turgor under prolonged salt stress (Aziz & Khan, 2001; Khan & Aziz., 2001; Hassine *et al*., 2008). To avoid toxic effect of salts osmotic adjustment in plants is usually achieved either by cellular adaptive mechanisms such as salt compartmentation (Munns, 2002; Munns & Tester, 2008) or by producing organic osmolytes (Volkmar *et al*., 1998; Gagneul *et al*., 2007; Slama *et al*., 2007). Both are energy requiring processes hence, plant growth may be compromised (Khan *et al*., 1998; Rhodes *et al*., 2002; Slama *et al*., 2007).

A substantial increase in leaf Na^+ in both seedlings and saplings indicated by their highest contribution to osmolality is in agreement to the reports on other halophytes which use this ion to develop water potential gradient under saline conditions (Flowers *et al*., 1977;

Shabala *et al*., 2005; Shabala & Mackay, 2011). Decreased amounts of K^+ and increased Na/K ratio in leaves could be linked both to the down-regulation of genes involved in K^+ transport (Maathuis & Amtmann, 1999), or competition between Na^+ and K^+ under saline conditions (Flowers *et al*., 1977). An efficient salt secretion mechanism is, therefore, important in maintaining salt balance in the foliage at physiologically acceptable levels (Flowers *et al*., 2014). Calcium is known to stabilize cell wall by activating various enzymes and regulate ion transport besides maintaining the structural and functional integrity of plant membranes (Epstein, 1998; Zhang *et al*., 2007). It is also considered as an important signaling molecule for activating SOS pathway to enhance ion sequestration (Mahajan *et al*., 2008). Increased amount of Ca^{++} in this study indicates its possible role at the apoplast level for Na^+ exclusion via salt glands, or at tonoplast level using different NHx exchangers (Shabala & Mackay, 2011). However, experimental evidences are required for the extent and comparative expression levels of PM and V-NHx under varying salinities. Higher amount of Mg^{2+} in *A. marina* leaves indicates the inability of plants to remove it from their vegetative parts as observed for various other salt secreting species (Popp, 1984a; Boon & Allaway, 1982; Glenn & Brown, 1998). In the presence of excessive ions, plants usually synthesize and accumulate organic solutes either to achieve an osmotic balance between cytoplasm and vacuole (Flowers *et al*., 1977; Munns, 2002) or for the purpose of osmoprotection to avoid ionic toxicity (Flowers & Colmer, 2008). These organic solutes include quaternary ammonium compounds such as trigonelline, choline, GB, PRO and its derivatives (Munns & Tester, 2008). It is also believed that the contribution of Na^+ to OA is not always direct but it may sometimes trigger GB synthesis under stress (Subbarao *et al*. (2003). GB synthesis is widely reported in chenopods and some of the coastal grasses (Khan *et al*., 1998, Moinuddin *et al*., 2014) as well as mangroves and other salt marsh plants (Popp, 1984b). Recent reports have suggested sub-cellular localization of GB as helpful in protecting PSII functioning under both water and salt stress besides taking part in ROS scavenging (Sakamoto & Murata, 2000; Martinez *et al*., 2004; Yang *et al*., 2007; Chen & Murata, 2008). In the present study, GB accumulation pattern was similar in *A. marina* seedlings and saplings but it was about 4-fold higher in seedlings and 2 fold in saplings in highest salinity compared to the control. These results indicate sensitivity of seedlings to salt exposure for triggering enhanced GB accumulation. Proline (PRO) in *A. marina* increased with the increases in salinity but its relative contribution to the total osmolality was lowest among all organic solutes which is in accordance to the previous findings that lower proline values are not sufficient enough to balance large amount of salts in vacuole (Aziz & Khan, 2001b) and that it has little role in osmotic adjustment (Popp & Polania, 1989). Significantly lower levels of PRO in *A. marina* also supports the view that GB accumulators have low levels of PRO and vice versa (Tipidermaz *et al*., 2006). Although it is yet to be confirmed, lower amount of PRO in mangroves could have a possible role in ROS scavenging. Total soluble sugars (TSS) were substantially decreased in seedlings while GB content was significantly increased indicating a possible survival strategy of plants at the cost of growth (Lee *et al*., 2008; Rhodes *et al*., 2002). On the contrary, the amount of TSS was much higher in saplings with comparatively lesser amount of GB indicating sensitivity of young seedlings than mature saplings. A number of outcomes have been proposed for the extent of carbohydrate accumulation in literature. Increased amount of carbohydrates could be a result of decreased export due to shortage of energy source (Munns & Weir, 1981) or due to the disturbance in metabolism which is regulated by enzymes (Marschner, 1995; Dubey & Singh, 1999). Increased amount of carbohydrates is known to play important role in osmotic adjustment under salt stress (Popp & Albert, 1995; Gagneul *et al*., 2007). Slight changes in TSS and other organic solutes on dry weight basis cannot explain the role of these osmolytes (Gil *et al*., 2013) as they are already stored in cells even without salt treatments and their synthesis may be partly induced under stress (Gil *et al*., 2013). Hence, re-distribution and sub-cellular localization is suggested, the evidences of which are still lacking. Salt stress usually increases the amount of reducing sugars (glucose, fructose), sucrose and fructans in some species (Dubey & Singh 1999). This variation could be due to the increased activity of sucrose phosphate synthase (Huber & Huber, 1996), whereas, starch phosphorylase activity decreases under saline conditions (Dubey & Singh, 1999). Although, both hexose sugars (sucrose & glucose) increased in moderate salinity, sucrose content was 2 fold higher in this study. Apparently, plants showed an optimum growth with the increases in hexose sugars and vice versa under stressed condition. Decreasing amount of hexose sugars in highest salinity could be an indication towards their possible conversion into sugar alcohols for intracellular osmotic adjustment (Parida & Das, 2005; Popp & Albert, 1995).

In conclusion, the major contribution to OP of leaves was accounted by inorganic solutes in all salinity regimes but the role of constitutive osmolytes (sugars and GB) in the intracellular osmotic adjustment cannot be ruled out because most of the organic osmolytes are thought to be restricted to the cytosol which is about 10 – 20% of the total cell volume (Hussine *et al*., 2008). However, decreasing amount of TSS & hexoses in salinity stress hints at the conversion of sugars into polyols. Lowest contribution of PRO among all tested solutes indicates their possible involvement in osmoprotection or ROS scavenging rather than intracellular osmotic adjustment. Higher amount of GB in stressed conditions indicate a possible metabolic shift in *A. marina* plants at the cost of growth. Higher biomass and variation in TSS and hexoses and low values of GB in *A. marina* saplings indicate better growth response while seedlings appear to invest more towards intracellular osmotic adjustment showing their relative sensitivity under salt stress.

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