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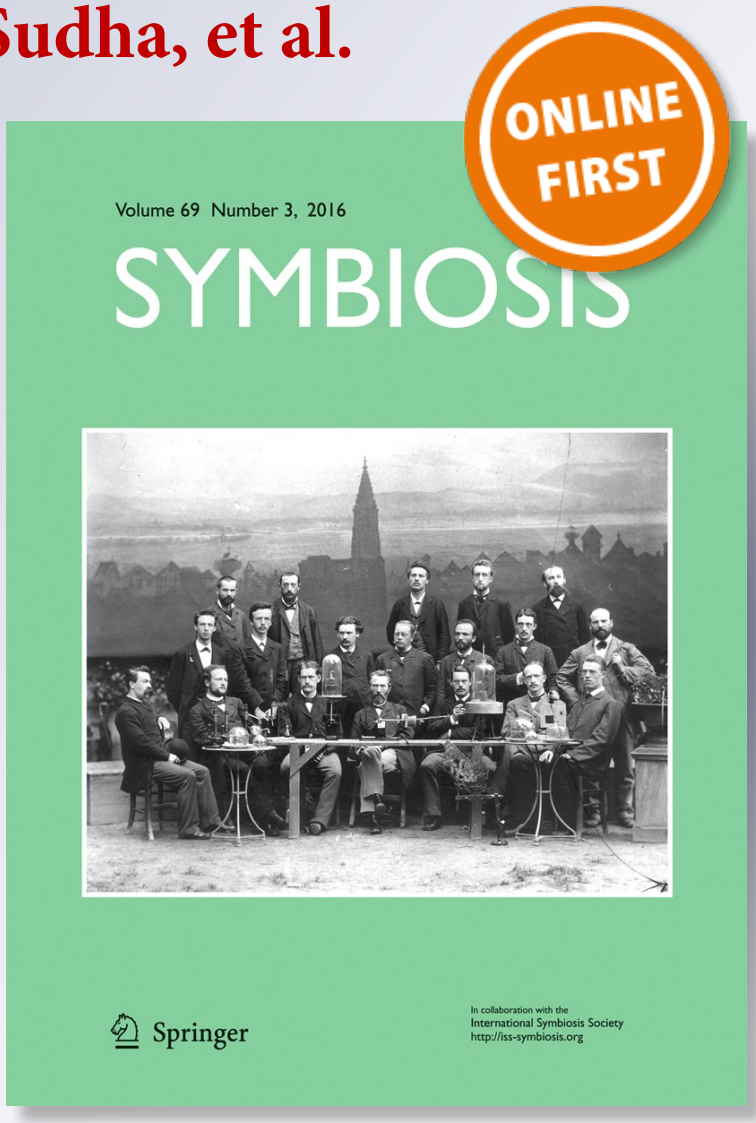

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Intraspecies variation in sodium partitioning, potassium and proline accumulation under salt stress in *Casuarina equisetifolia* Forst

R. K. Selvakesavan[1] · N. N. Dhanya[1,2] · P. Thushara[1] · S. M. Abraham[1] · R. S. C. Jayaraj[1,3] · A. Balasubramanian[1] · B. Deeparaj[1] · S. Sudha[1] · K. S. Sowmiya Rani[1] · V. K. W. Bachpai[1] · D. Ganesh[4] · N. Diagne[1,5,6] · L. Laplaze[6,7] · H. Gherbi[7,8] · S. Svistoonoff[6,7,8] · V. Hocher[7,8] · C. Franche[7] · D. Bogusz[7] · M. Nambiar-Veetil[1]

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Abstract *Casuarina equisetifolia* Forst., a member of the *Casuarinaceae* family, is widely planted in coastal areas due to its ability to function as potential barrier against wind and waves. Significant variation has been reported in the ability of *C. equisetifolia* to grow under salinity stress. In the present study, 82 clones of *C. equisetifolia* were assessed for their response to 50 mM incremental NaCl concentrations ranging from 50 mM to 550 mM in Hoagland's solution and clones with contrasted salt tolerance were identified. Several earlier reports attribute salt sensitivity in *Casuarina* species to the toxic effect of sodium. Intraclonal variation in the levels of sodium accumulation was therefore analysed. However, sodium content in the shoots and roots, showed little correlation (0.351 and −0.171) with salt tolerance in *C. equisetifolia*. Similarly, sodium to potassium ratio in the shoots and roots of NaCl treated and untreated clones also did not show correlation with mortality although certain tolerant clones exhibited selectivity of potassium over sodium under salt stress. Analysis of the shoot to root ratio of sodium however, showed better correlation (0.448) with salt tolerance, suggesting that restricted translocation of sodium to shoots and its relative retention in roots might play a crucial role in the salt tolerant clones of *C. equisetifolia*, and that shoot to root ratio of sodium could be a better parameter for salt tolerance in *C. equisetifolia* clones. The higher salt tolerance observed in certain clones despite higher sodium accumulation or shoot to root ratio of sodium suggests the presence of different multiple adaptive mechanisms that may be operating in different clones to help protect the cells from the toxic effects of sodium. The tolerant clone, TNIPT 4,

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✉ M. Nambiar-Veetil
nvmathish@yahoo.com

[1] Division of Plant Biotechnology, Institute of Forest Genetics and Tree Breeding, Forest Campus, R. S. Puram, Coimbatore 641002, India

[2] Present address: Department of Bioinformatics, Nirmala College for Women, Coimbatore 641018, India

[3] Present address: Rain Forest Research Institute, Jorhat, Assam 785010, India

[4] Department of Plant Biotechnology, School of Biotechnology, Madurai Kamraj University, Madurai 625 021, India

[5] Centre National de Recherches Agronomiques (CNRA/ ISRA), Bambey, Senegal

[6] Laboratoire Commun de Microbiologie, Laboratoire mixte international Adaptation des Plantes et microorganismes associés aux Stress Environnementaux (LAPSE), Centre de Recherche de Bel Air, Dakar, Senegal

[7] Institut de Recherche pour le Développement (IRD), UMR DIADE, 911 Avenue Agropolis, 64501, 34394 Montpellier, Cedex 5, BP, France

[8] Present address: IRD, UMR LSTM (IRD/INRA/CIRAD/Université Montpellier/Supagro), Campus International de Baillarguet, F-34398 Montpellier Cedex 5, France

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which accumulated high concentrations of Na^+, had low shoot to root ratio of Na^+, and also a higher constitutive as well as NaCl induced accumulation of the compatible osmolyte, proline. The study thus emphasizes the need for characterising the genetic components involved in sodium transport, proline metabolism and other mechanisms contributing to salinity tolerance. The identified clones with contrasted stress tolerance mechanisms would thus be a valuable resource for transcriptomic, proteomic and metabolomic exploration in addition to their utility for field evaluation in flooded and coastal saline tracts.

Key words Abiotic stress tolerance · Coastal forestry · Salt tolerant trees · Waste land management · Sodium potassium ratio

1 Introduction

Salinity and sodicity are the major factors limiting crop productivity, and worldwide around 800 Mha are affected by salinity (Witcombe et al. 2008). Twenty percent of the world's irrigated land, corresponding to one-third of the world food production is affected by salinity (Chinnusamy et al. 2005; FAO 2008). Higher temperature and low rainfall regimes are expected to impose high evapotranspiration losses, decreased availability of irrigation water, accelerated use of saline water for irrigation and concomitant secondary salinization (Yeo 1999). Salt stress initially results in an initial osmotic phase wherein there is a rapid reduction in water uptake capacity and thus relative water content and growth rate followed by an ionic phase characterized by accumulation of salt in the leaves, with concomitant reduction of the supply of photosynthates to the plant and senescence of leaves (Munns and Tester 2008; Batista-Santos et al. 2015; Duro et al. 2016). Salt affected soils are characterized by an abundance of toxic sodium ions (Tester and Davenport 2003). To withstand high soil concentrations of sodium, plants have developed diverse mechanisms for transport, exclusion and compartmentalization of sodium ions (Tester and Davenport 2003; Apse and Blumwald 2007; Munns and Tester 2008) in addition to production of osmoprotectants such as betaines and allied compounds, polyols and sugars, and amino acids such as proline (Hong-Bo et al. 2006; Khan et al. 2014). Proline accumulates during various stresses and helps to preserve structural integrity and cellular osmotic potential within different compartments of the cell (Iyer and Caplan 1998; Wang et al. 2011). Once stress is relieved, accumulated proline is degraded to provide energy for growth (Kavi Kishor and Sreenivasulu 2013). Proline accumulation under salinity and drought stress conditions has also been reported in woody species such as *Acacia nilotica* (Nabil and Coudret 1995), *Populus euphratica* (Watanabe et al. 2000) and *C. equisetifolia* (Tani and Sasakawa 2006).

The species of *Casuarinaceae* are able to thrive under salt stressed conditions making them a preferred species for plantation in coastal areas where they function as protective barriers against wind and waves. The symbiotic associations with *Frankia*, and endo- and ectomycorhizal fungi enables them to grow in nutrient deficient soils. Fast growth and their utility as pulp and fuel wood make them a preferred agroforestry and plantation species in southern India. It has been reported that the innate ability of *C. glauca* to tolerate high concentration of NaCl under controlled growth condition is independent of the presence of symbiotic *Frankia* strain although salt tolerant *Frankia* genotypes might improve salt stress tolerance of *C. glauca* (Batista-Santos et al. 2015; Duro et al. 2016). *Casuarina* species show wide variation in their salt stress response. Studies by Clemens et al. (1983) and Aswathappa and Bachelard (1986) revealed that salt tolerant species like *C. equisetifolia* and *C. glauca* exclude both Na^+ and Cl^- from their shoots. The species, that accumulated the highest concentration of Na^+ and Cl^- in the shoot tip, viz., *C. inophloia*, *C. stricta*, *C. instata*, and *C. decaimeana* were those that suffered the greatest reduction in growth, shoot tip chlorosis and/ or death, while the more tolerant species like *C. equisetifolia* and *C. glauca* showed higher accumulation of Na^+ and Cl^- in the roots (Clemens et al. 1983). In the highly tolerant species, Na^+ and Cl^- concentration decreased from lower (older) branchlets to the apical shoot suggesting an accumulation of ions in older branchlets (Aswathappa and Bachelard 1986). Elevated proline levels have been reported in different *Casuarina* species subject to salt stress. It has been shown that the more tolerant species, *C. junghuhniana*, accumulated more proline than *C. cunninghamiana* and *C. equisetifolia* in stressful environment (Reddy 2001). In *C. equisetifolia*, Tani and Sasakawa (2006) showed that salt stress induces proline accumulation but not glycine betaine, other amino acids or total sugars. The capacity of *C. glauca* to tolerate high NaCl has been linked to maintenance of high tissue hydration and photosynthetic adjustments (Batista-Santos et al. 2015).

In India, *C. equisetifolia* is the most widely planted species amongst the *Casuarinaceae*, and shows marked intra species variation in salt stress response (Balasubramanian 2001). Understanding the genetic and physiological bases of these variations would enable development of technologies that enable enhanced salt tolerance breeding programmes. The present study was therefore taken up to screen for highly tolerant and highly susceptible *C. equisetifolia* clones, and analyse the intraclonal variations in the concentrations of Na^+, K^+ and proline and their correlation to salt susceptibility.

2 Materials and methods

2.1 Plant material and salt treatment

Cladode cuttings of 82 *C. equisetifolia* clones (Supplementary Table. 1) from the germplasm collection of the Institute of

Table 1 Effect of salt stress on *Casuarina equisetifolia* clones

S.No	Clone	Survival Percentage (after 500 mM NaCl treatment)	*Net difference*[a] in number of branchlets (%)	Sodium in shoots (mmol/g)(after 550 mM NaCl treatment)	Sodium in Roots (mmol/g)(after 550 mM NaCl treatment)	Shoot to root ratio of sodium
Tolerant Clones						
1	TNIPT 4	95	35.44	0.942 ± 0.01	0.952 ± 0.01	0.99
2	TNKBM 407	90	62.42	0.929 ± 0.27	0.794 ± 0.16	1.17
3	APKKD 10	90	43.02	0.739 ± 0.18	0.596 ± 0.11	1.24
4	APVSP 14	90	48.45	0.309 ± 0.10	0.675 ± 0.35	0.46
5	TNMT 2	90	12.40	0.439 ± 0.05	1.013 ± 0.38	0.43
6	TNKP 2	90	39.19	0.955 ± 0.05	0.649 ± 0.12	1.47
7	TNPP 2	90	30.71	0.736 ± 0.11	0.446 ± 0.19	1.65
8	TNKBM 406	85	39.05	0.774 ± 0.09	0.765 ± 0.02	1.01
9	TNMT 6	85	43.02	0.043 ± 0.00	0.919 ± 0.04	0.05
10	TNIPT 2	85	25.48	1.114 ± 0.07	0.755 ± 0.12	1.48
11	APVJM 39	81.25	51.98	0.659 ± 0.07	0.980 ± 0.03	0.67
12	TNKBM 408	80	55.33	0.022 ± 0.00	0.535 ± 0.09	0.04
13	JKCE 2	75	41.84	0.507 ± 0.04	0.684 ± 0.09	0.74
14	TN 501	75	96.73	0.687 ± 0.13	0.622 ± 0.15	1.10
15	TNRM 8	75	39.67	0.419 ± 0.02	0.374 ± 0.15	1.12
16	JKCE 3	70	65.72	0.204 ± 0.03	0.257 ± 0.01	0.80
17	TNVCR 412	60	86.59	0.338 ± 0.04	0.677 ± 0.07	0.50
	Average for 17 tolerant clones		48 ± 4.9 %	0.577 ± 0.03	0.69 ± 0.05	0.88 ± 0.11
Sensitive clones						
18	TNIPT 3	40	72.51	0.839 ± 0.13	0.699 ± 0.13	1.20
19	TNIPT 1	20	72.26	0.283 ± 0.05	0.319 ± 0.11	0.89
20	TNRM 7	20	31.51	0.426 ± 0.04	0.326 ± 0.04	1.31
21	PY 75	20	77.62	0.241 ± 0.17	0.580 ± 0.05	0.42
22	JKCE 8	20	80.37	1.449 ± 0.31	0.422 ± 0.06	3.44
23	TNPP 4	15	73.43	0.936 ± 0.27	0.546 ± 0.01	1.71
24	TNVM 5	15	89.97	1.030 ± 0.21	1.155 ± 0.14	0.89
25	TNRM 2	15	103.36	0.783 ± 0.22	1.261 ± 0.36	0.62
26	APVJM 33	15	113.05	0.830 ± 0.14	0.438 ± 0.09	1.90
27	TNPV 2	10	73.39	1.365 ± 0.18	0.680 ± 0.02	2.01
28	TNRM 4	10	85.82	0.541 ± 0.04	0.571 ± 0.04	0.95
29	TNVM 3	10	89.36	1.236 ± 0.13	0.786 ± 0.13	1.57
30	PYN	0	101.80	1.359 ± 0.43	0.372 ± 0.18	3.65
	Average for 13 sensitive clones		81.9 ± 5.6 %	0.87 ± 0.04	0.63 ± 0.08	1.58 ± 0.27
	γ		0.731**	0.352	−0.171	0.448**

[a] *Net difference* is the difference in the number of branchlets in the treated plants relative to the control plants when the salt concentration was increased from 200 mM to 500 mM and was calculated by the formula [(B-A)/A x 100]control-[(B- A)/A x 100]treated. For control plants, A and B represent the average branchlet numbers present in the ramets after 4 weeks and 10 weeks respectively; for treated plants, A and B are the average branchlet numbers present in the ramets after 200 mM NaCl and 500 mM NaCl respectively

γ Correlation coefficient observed for the parameters with mortality

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

Forest Genetics and Tree Breeding, Coimbatore, India (Jayaraj and Savio 1998), were rooted in vermiculite using 9.842 mM IBA, in the shade house and after nine months the ramets were transferred to Hoagland solution (Hoagland and Arnon 1950) and allowed to acclimatize for two months under shade nets. Concentration of NaCl was gradually increased at an increment of 50 mM each week from 50 mM to 500 mM. The clones were then left in same concentration for one month after which the salt concentration was again increased to 550 mM. Each *C. equisetifolia* clone was

represented by 28 ramets, of which 20 ramets were subjected to NaCl stress and 8 ramets used as control.

2.2 Impact of salt stress on branchlets

Data on the number of branchlets in each ramet and the number of ramets that survived were recorded following NaCl treatments. The percent difference in the growth was calculated separately for the control and treated ramets using the formula [$(B\text{-}A)/A*100$]. For control plants, A and B represent the average branchlet numbers present in the ramets after 4 weeks and 10 weeks respectively; and for treated plants, A and B are the average branchlet numbers present in the ramets after 200 mM NaCl and 500 mM NaCl treatments, respectively. To study the growth of ramets under salt stress (when the salt concentration was increased from 200 mM at the 4th week to 500 mM at the 10th week) relative to the control ramets, the *net difference* was calculated by using the formula $[(B\text{-}A)/A*100]_{control}$-$[(B\text{-}A)/A*100]_{treated}$. For control plants, A and B represent the average branchlet numbers present in the ramets after 4 weeks and 10 weeks respectively; for treated plants, A and B are the average branchlet numbers present in the ramets after 200 mM NaCl and 500 mM NaCl treatments, respectively.

2.3 Sodium and potassium analysis

Branchlets collected from 3 ramets of each of the 17 tolerant and 13 sensitive clones treated at 200 mM, 400 mM and 550 mM NaCl were used for sodium and potassium determination. Sodium and potassium analyses were also carried out in roots of 3 ramets of these clones treated at 550 mM NaCl. Sodium and potassium contents were analysed using flame photometer (Mediflame 127) by Ind-Ag Inspection and Testing Laboratory, Coimbatore, according to Indian Standards IS: 3025 as described here. To 0.3 g dried sample of roots or branchlets, 5 ml of 3:1 concentrated nitric acid and concentrated perchloric acid were added and digested until a clear solution was obtained. The residue was washed and filtered and the volume was made up to 100 ml with distilled water. This solution was diluted 10 times and used for analysis. The flame photometer reading was set to zero using distilled water and standardized using standard sodium chloride and potassium chloride solutions.

2.4 Proline estimation

In a separate experiment in greenhouse condition, the highly tolerant clone, TNIPT 4, and the highly susceptible clone, PYN, were analysed for proline accumulation. Three months old ramets of these clones maintained in the glass house conditions were subjected to 50 mM incremental NaCl concentrations from 50 mM to 650 mM over a period of 4 months. Proline was estimated in the branchlets collected from twenty ramets of each of the clones just before each NaCl increment by ninhydrin test (Bates et al. 1973).

2.5 Statistical analysis

All the average values are presented as mean value ± standard error. Correlation coefficient was calculated by Pearson's method using the software**,** SPSS 16.0 (SPSS Inc. 2007).

3 Results

3.1 Phenotypic effects of NaCl treatment on *C. equisetifolia*

Eighty-two clones were screened for their salt stress response in hydroponic condition. The survival percentage, the *net difference* in the number of branchlets on increasing the salt concentration from 200 mM to 500 mM in each clone relative to the control plants, and the visual observation on the overall health of the plants were used to identify 13 salt sensitive clones (PYN, TNVM 3, TNRM 4, TNPV 2, APVJM 33, TNRM 2, TNVM 5, TNPP 4, JKCE 8, PY 75, TNRM 7, TNIPT 1, TNIPT 3) and 17 tolerant clones (TNIPT 4, TNKBM 407, APKKD 10, APVSP 14, TNMT 2, TNKP 2, TNPP 2, TNKBM 406, TNMT 6, TNIPT 2, APVJM 39, TNKBM 408, JKCE 2, TN 501, TNRM 8, JKCE 3, TNVCR 412). The sensitive and tolerant clones had a survival percentage ranging from 0 % to 40 % and 60 % to 95 % respectively (Table 1). The tolerant clones generally showed a smaller *net difference* (48 ± 4.9 %) in the number of branchlets after salt stress when compared to the sensitive clones (81.9 ± 5.6 %) (Table 1).

The clones showed visible symptoms of salt stress like drooping and yellowing of the branchlets followed by wilting and falling off of the branchlets from the plants after five weeks of salt treatment. Yellowing was observed in the lower branchlets at 250 mM NaCl in case of the sensitive clone "PYN" and at 300 mM NaCl in case of the tolerant clone, TNIPT 4. The branchlets fell off the plant rapidly at 400 mM and 550 mM for PYN and TNIPT 4 respectively. It was also observed that PYN could not survive beyond 400 mM NaCl while TNIPT 4 was able to survive beyond 550 mM NaCl concentrations.

3.2 Effects of NaCl treatment on Na^+ and K^+ concentration in shoots and roots

The 17 tolerant and 13 sensitive clones identified were analysed for sodium and potassium concentrations in branchlets and roots (Table 1 and Fig. 1). Under salt treatment, the average sodium content in the shoots of 13 sensitive clones (0.87 ± 0.04 mmol/g) were higher than that for the 17 tolerant

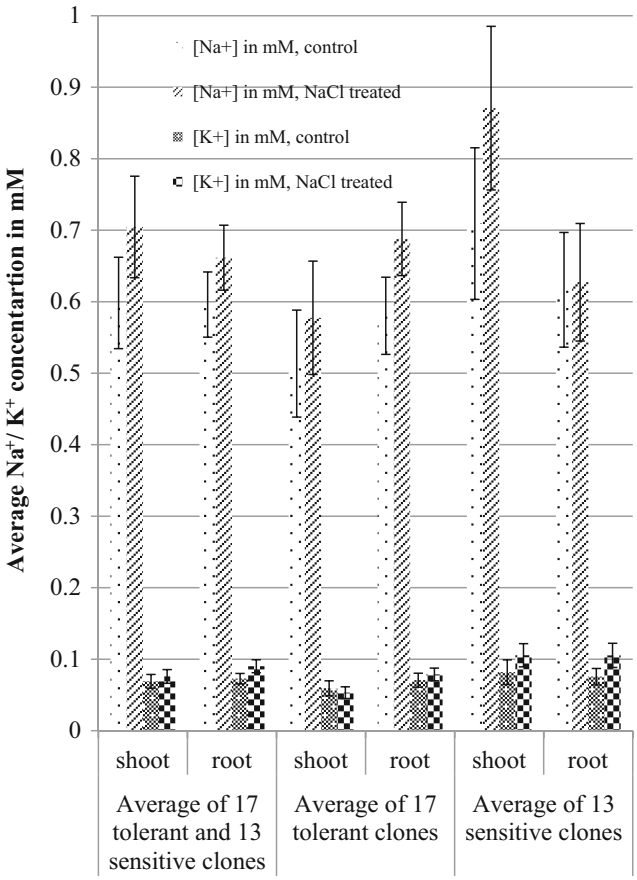

Fig. 1 The average sodium and potassium concentration in the shoots and roots of identified tolerant and sensitive *C. equisetifolia* clones at 550 mM NaCl treatment

clones (0.577 ± 0.03 mmol/g) (Fig. 3). Sodium content in the tolerant clones ranged from 0.021 mmol/g to 1.114 mmol/g and for the 13 sensitive clones it ranged from 0.24 mmol/g to 1.449 mmol/g. Similar to the sensitive clones, shoots of a few tolerant clones (TNIPT 4, TNKBM 407, TNKP 2, TNIPT 2) also showed high sodium accumulation in treated plants (0.942 mmol/g, 0.928 mmol/g, 0.955 mmol/g, 1.114 mmol/g). Shoot sodium content in control and treated plants also showed a non-significant correlation of 0.255 and 0.352 respectively with mortality. Similarly, no correlation was observed for the root sodium content and mortality (Table 2). Na^+ content in shoots or roots thus was not a reliable parameter for screening of salt tolerance in *C. equisetifolia*.

In the 550 mM NaCl treated clones, the shoot to root ratio of sodium ranged from 0.05 to 1.65 at an average of 0.88 ± 0.11 for the 17 tolerant clones and in case of the 13 sensitive clones it ranged from 0.42 to 3.64 at an average of 1.58 ± 0.27 (Fig. 2). For the 5 most tolerant clones selected based on survival data viz., TNIPT 4, TNKBM 407, APKKD 10, APVSP 14 and TNMT 2, the shoot to root ratio of sodium ranged from 0.43 to 1.24 at an average of 0.85 ± 0.17, and in case of the 5 most sensitive clones (PYN, TNVM 3, TNRM 4, TNPV 2, and APVJM 33) the ratio ranged from 0.94 to 3.64 at an average of 2.01 ± 0.4. The shoot to root sodium ratios for the 30 NaCl treated clones showed a highly significant correlation of 0.448 ($p < 0.01$) with mortality (Table 1). Thus the shoot to root Na^+ ratio in NaCl treated plants was found to be a better determinant of salt stress tolerance than Na^+ accumulation in shoots. Similar to the treated clones, we observed that in the untreated controls, tolerant clones showed a lower average shoot to root Na^+ ratio (0.96 ± 0.14) when compared to the susceptible clones (1.76 ± 0.75). To test if shoot to root Na^+ ratio estimated in untreated plants could serve as a diagnostic marker, its correlation with mortality in treated clones was calculated. However, shoot to root Na^+ ratio in untreated controls showed a non-significant correlation of 0.299 with mortality in treated clones.

Potassium content in the tolerant clones ranged from 0.01 mmol/g to 0.14 mmol/g, and for the 13 sensitive clones it ranged from 0.03 mmol/g to 0.21 mmol/g. In order to analyse if the tolerant clones showed any preferential exclusion of sodium in favour of potassium under salt stress, sodium to potassium ratio in the shoots and roots of NaCl treated and untreated clones were analysed. In the shoots of *C. equisetifolia* clones, the tolerant clone, TNIPT 4, showed lower Na^+: K^+ ratio (3.875) compared to sensitive clone, PYN (10.433). Sodium to potassium ratio however did not show significant correlation with mortality. In the shoots of NaCl treated and untreated clones, sodium content showed a significant correlation of 0.684 ($p < 0.01$) and 0.753 ($p < 0.01$) with potassium content. Although in the roots of untreated clones, sodium content showed a significant correlation of 0.464 with the potassium content, under salt stress the root sodium content showed no significant correlation (0.328) with root potassium content (data not shown) suggesting that there could be a preferential retention of Na^+ in the roots during salt treatment.

3.3 Effect of salt treatment on proline accumulation

In a separate experiment under greenhouse conditions, proline accumulation in response to NaCl treatment was estimated in one of the tolerant and sensitive clones, respectively. The highly tolerant TNIPT 4 and the sensitive PYN clones showed marked variation in the levels of proline accumulation with increasing NaCl concentrations (Fig. 4). In untreated plants of the PYN clone, the proline content showed a gradual increase till 9th week from 0.112 ± 0.15 μmoles/g tissue to 8.996 ± 0.040 μmoles/g tissue while it increased till 12th week in the untreated plants of TNIPT 4 (from 2.12 ± 0.04 μmoles/g tissue to 10.06 ± 1.05 μmoles/g tissue). The proline content was thus constitutively higher in the control plants of TNIPT 4 (2.12 ± 0.04 μmoles/g tissue) when compared with those of PYN (0.112 ± 0.15 μmoles/g tissue). In treated plants, proline increased more rapidly in TNIPT 4 and at higher levels when compared to the sensitive clone, PYN. The sensitive clone PYN showed a very gradual increase in proline till 400 mM

Table 2 Correlation analysis of sodium and potassium accumulation with mortality in 30 clones of *C. equisetifolia* contrasting for their survival under salt stress

Parameter	Plant Material	Parameter vs Mortality in treated clones
Sodium content	Treated shoots	0.351
	Control shoots	0.255
	Treated roots	−0.171
	Control roots	−0.056
Na^+/K^+ ratio	Treated shoots	−0.216
	Control shoots	0.205
	Treated roots	−0.219
	Control roots	−0.155
Shoot to root ratio of Na^+	Treated	0.448**
	Control	0.299
Shoot to root ratio of K^+	Treated	0.363*
	Control	0.131

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

NaCl treatment followed by a spurt to 17.646 ± 0.33 μmoles/g tissue at 450 mM NaCl treatment. This was followed by a rapid decrease in the levels of proline and death of plants (Fig. 4). Contrastingly, the tolerant clone, TNIPT 4, had a more rapid increase in proline, with the highest proline concentration of 25.26 ± 0.24 μmoles/g tissue observed at 450 mM salt concentration. This was followed by a more gradual decline in proline levels.

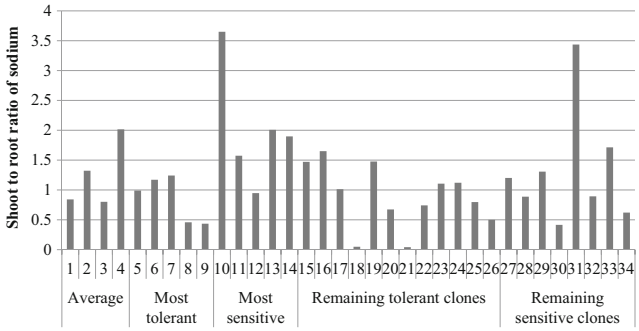

Fig. 2 The shoot to root ratio of sodium content of selected tolerant and sensitive *C. equisetifolia* clones. 1- Average of 17 tolerant clones, 2- Average of 13 sensitive clones, 3- Average of 5 most tolerant clones based on survival data (viz., TNIPT 4, TNKBM 407, APKKD 10, APVSP 14 and TNMT 2), 4- Average of 5 most sensitive clones based on survival data (viz., PYN, TNVM 3, TNRM 4, TNPV 2, APVJM 33), 5–9: Five most tolerant clones (viz., 5- TNIPT 4, 6- TNKBM 407, 7- APKKD 10, 8- APVSP 14 and 9-TNMT 2), 10–14: Five most sensitive clones (viz., 10 - PYN, 11- TNVM 3, 12- TNRM 4, 13- TNPV 2, 14- APVJM 33). 15–26: Remaining tolerant clones (15 - TNKP 2, 16 - TNPP 2, 17 - TNKBM 406, 18 - TNMT 6, 19 - TNIPT 2, 20 - APVJM 39, 21 - TNKBM 408, 22 - JKCE 2, 23 - TN 501, 24 - TNRM 8, 25 - JKCE 3 and 26 - TNVCR 412), 27–34: Remaining sensitive clones (27 - TNIPT 3, 28 - TNIPT 1, 29 - TNRM 7, 30 - PY 75, 31 - JKCE 8, 32 - TNVM 5, 33- TNPP 4, 34 - TNRM 2)

4 Discussion

4.1 Phenotypic effects of NaCl treatment on *C. equisetifolia*

The long term goal of research on understanding salt tolerance mechanisms in plants is to breed for crops with enhanced ability to tolerate moderate salinity. The existence of salt-tolerant plant species, and the intraspecies variation in the form of salt sensitive and tolerant genotypes as in groundnut (Singh et al. 2008), tomato (Rush and Epstein 1976) or wheat (El-Hendawy et al. 2005), indicate that there is a genetic basis for salinity tolerance (Bartels and Dinakar 2013). Halophytes like *Atriplex vesicaria* and *Salicornia bigelovi* can tolerate up to 1 M and 1.2 M NaCl respectively (Glenn et al. 1999; Ayala and O'Leary 1995). In saline soils, studies by Tomar and Gupta (1984–1994) categorized *Casuarina glauca* and *C. equisetifolia* as moderately tolerant (EC 25–35 dS/m). Wide variation in the response of *C. equisetifolia* clones to salt stress has been observed with certain clones being able to survive under high salinity (Balasubramanian 2001). In the present study, 82 *C. equisetifolia* clones were assessed for their salt stress response so that clones with contrasted salt tolerance could be selected and the physiological bases of intraspecies variation in salt tolerance understood. Initial symptoms observed were yellowing in the older branchlets. Swelling of older branchlets was observed in a few of the identified tolerant as well as sensitive clones. However, no red phylloclades were observed. This is in contrast to the studies by Dutt et al. (1991), wherein salinity stress resulted in reddish colouration in the older phylloclades that accumulated higher levels of Na^+, which is likely to be a provenance variation. Seventeen tolerant and 13 sensitive clones were identified based on the percentage of survival, relative growth under salt stress (*net difference* in the number of branchlets after salt stress) and visual observations on the overall health of the plants. The sensitive clones showed greater reduction in branchlets [*net difference* = 81.9 ± 5.6 %] when compared to the tolerant clones [*net difference* = 48 ± 4.9 %]. Although the tolerant clone TN 501 was visually healthy and had a survival of 75 % when the salt concentration was increased from 200 mM to 500 mM, the *net difference* of 96.72 % between the number of branchlets in treated and control plants was similar to those observed for sensitive clones (Table 1). Analysis of the data in the untreated plants revealed that when compared to other clones, TN 501 had a faster rate of generation of branchlets during the period (Fig. 5). During salt stress the number of branchlets remained the same during the treatment period indicating that the rapid rate of generation of new branchlets matched with the rate of shedding of salt accumulating branchlets. The new branchlets that have a lower Na^+ concentration could have helped the ramets to cope with the loss of old branchlets during salt stress. The most sensitive clone PYN wilted at a salt concentration of

400 mM, while the most tolerant clone TNIPT 4 survived for 45 days post 550 mM NaCl stress. Moezel van der et al. (1989) had reported that the highly tolerant *C. glauca* and *C. obesa* could survive up to a concentration of 5600 mS m^{-1} (~ 660 mM NaCl). As the selected tolerant clones of *C. equisetifolia* identified in the study could survive even at 550 mM NaCl in hydroponic condition, these could be good candidate clones for further testing in waterlogged saline and coastal saline areas. The selected contrasting phenotypes (highly salt tolerant: TNIPT 4, TNKBM 407, APKKD 10, APVSP 14 and TNMT 2 and highly sensitive clones: PYN, APVJM 33, TNPV 2, TNRM 4, and TNVM 3) provide a valuable resource for understanding the physiological and genetic basis of variation in salt tolerance in *C. equisetifolia*.

4.2 Variation in Na^+ content in *C. equisetifolia* clones and correlation with mortality

Na^+ and Cl^- taken up in large amounts through the roots, negatively affects plant growth by impairing metabolic processes and decreasing photosynthetic efficiency (Maser et al. 2002; Deinlein et al. 2014). Previous studies analysing interspecies differences in salt stress responses in *Casuarina* reported that tolerant species accumulated lesser sodium in the roots and shoots. El-Lakany and Luard (1984) observed that the shoots and roots of the tolerant species, *C. equisetifolia*, had lower concentrations of Na^+ (127 mmol/L tissue water in shoots and 63 mmol/L tissue water in roots) and Cl^- (158 mmol/L tissue water in shoots and 74 mmol/L tissue water in roots), than the more sensitive species *C. cristata* (220 mmol/L tissue water of Na^+ in shoots, 69 mmol/L tissue water of Na^+ in roots, 280 mmol/L tissue water of Cl^- in shoots and 72 mmol/L tissue water of Cl^- in roots). In the young cladodes of *C. obesa*, a species reported to be more salt tolerant than *C. equisetifolia*, Reddell et al. (1986) reported lower concentrations of Na^+ ranging from 0.052 mmol/g to 0.304 mmol/g. In another study, Moezel van der et al. (1989) reported that the salinity tolerance of *C. obesa*, *C. glauca* and *C. equisetifolia* were associated with exclusion of Na^+ and Cl^-, while relatively sensitive species, *C. cunninghamiana* and *C. cristata*, accumulated salt in the shoots when they were grown under non-saline drained, saline drained, non-saline waterlogged and saline waterlogged conditions in a glasshouse. Tani and Sasakawa (2006) reported that Na^+ concentration in the shoots gradually increased with prolonged treatment, and the fresh weight Na^+ concentration reached a value of 150 mmol/L in *C. equisetifolia* seedlings grown in 500 mmol/L NaCl.

In this study, the dry weight sodium content analysed in the 17 tolerant clones treated at 550 mM NaCl, ranged from 0.021 mmol/g to 1.114 mmol/g and for the 13 sensitive clones it ranged from 0.24 mmol/g to 1.449 mmol/g. Despite the higher accumulation of sodium in salt treated sensitive *Casuarina* clones, no significant correlation was observed between shoot or root Na^+ content and mortality of *C. equisetifolia*. A few tolerant clones like TNIPT 4, TNKBM 407, TNKP 2 and TNIPT 2 had shoot sodium concentrations comparable to that of the sensitive clones suggesting that other mechanisms of salinity tolerance like sequestration of sodium into vacuoles or accumulation of osmoprotectants may be in operation to make these clones tolerant to salinity despite the high sodium content in them.

4.3 Variation in shoot to root ratio of Na^+ in *C. equisetifolia* clones and correlation with mortality

It was observed that the average shoot sodium concentrations in untreated controls (0.77 ± 0.08 mmol/g) were lower in the 17 most tolerant clones when compared to the 13 most sensitive clones (0.89 ± 0.08 mmol/g) suggesting that the tolerant clones had an innate mechanism to constitutively exclude Na^+ from the shoots even during the absence of salt stress (Fig. 3). It was also observed that the average shoot sodium concentrations in the 17 most tolerant clones reduced from 0.77 ± 0.08 mmol/g in the untreated plants to 0.58 ± 0.07 mmol/g at 550 mM NaCl treatments, while the average sodium concentrations in shoots of the 13 most sensitive clones did not change significantly at 0, 200 mM, and 400 mM NaCl treatments (Fig. 3). Thus the tolerant clones were also more efficient in excluding Na^+ from shoots during salt stress, thereby suggesting the recruitment of salt inducible tolerance mechanisms. While the sensitive clones accumulated 0.87 ± 0.11 mmol/g sodium in shoots and 0.62 ± 0.08 mmol/g in the roots, tolerant clones accumulated lesser sodium (0.58 ± 0.07 mmol/g) in shoots and higher sodium (0.68 ± 0.05 mmol/g) in roots (Fig. 1). Thus the sensitive clones had significantly lower concentrations of Na^+ in roots than in shoots. Aswathappa and Bachelard (1986) and Tani and Sasakawa (2006) have reported that Na^+ concentration in roots of salt treated *C. equisetifolia* seedlings were lower than that in shoots. Contrastingly, in the present study the tolerant clones of

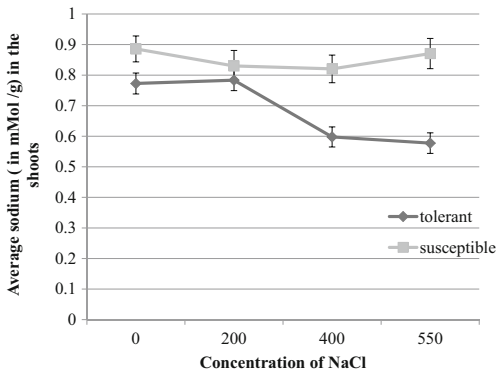

Fig. 3 Average sodium accumulation in the shoots of the 17 tolerant and 13 sensitive clones at different salt concentrations

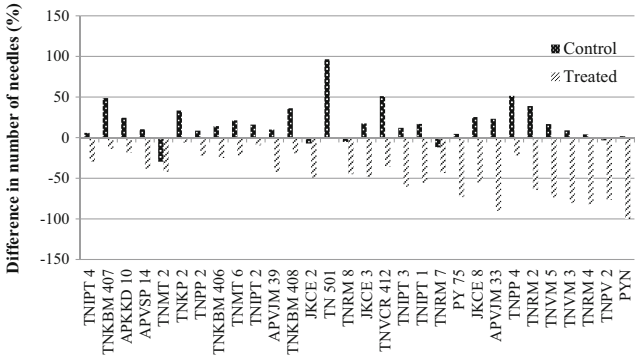

Fig. 5 The *percent* difference in the number of branchlets calculated by the formula [(B-A)/A*100]. For control plants, A and B represent the average branchlet numbers present in the ramets after 4 weeks and 10 weeks respectively; for treated plants, A and B are the average branchlet numbers present in the ramets after 200 mM NaCl and 500 mM NaCl respectively

C. equisetifolia showed a higher accumulation of Na^+ in roots than in shoots. In studies comparing different species of *Casuarina*, tolerant species were reported to have higher root sodium concentrations when compared to susceptible species. El-Lakany and Luard (1982) had reported that the Na^+ and Cl^- concentrations in roots were higher in tolerant species (eg. *C. equisetifolia*) than in sensitive species (eg. *C. inophloia*). Similarly, in studies by Clemens et al. (1983), the roots of tolerant species, *C. equisetifolia*, were shown to have higher concentrations of Na^+ (0.66 % of dry weight) and Cl^- (0.63 % of dry weight) than the susceptible species, *C. inophloia*, *C. cristata* (0.22–0.60 % Na^+ and 0.49–0.59 % Cl^-). The species that accumulated the highest shoot tip concentrations of Na^+ (2.07–3.17 % of dry weight) and Cl^- (2.89–4.90 % of dry weight) viz., *C. cristata*, *C. inophloia*, were those that suffered the greatest reductions in growth, shoot tip chlorosis and/ or death, while the more tolerant species like *C. equisetifolia* showed lower levels of Na^+ (0.62 % of dry weight) and Cl^- (0.52 % of dry weight) in the shoots.

With shoot or root Na^+ content not showing a significant correlation with mortality of the *C. equisetifolia* clones in the present study, and with the observation that roots of tolerant clones had a higher Na^+ content than shoots, another parameter shoot to root ratio of Na^+ was analysed for the individual tolerant and sensitive clones. A higher shoot to root ratio of Na^+ concentration was observed in sensitive clones when compared to tolerant plants. In the NaCl treated plants, the shoot to root ratio of sodium content was 0.88 ± 0.11 in tolerant clones, and 1.58 ± 0.27 in the case of sensitive clones (Fig. 2). A highly significant correlation of 0.448 ($p < 0.05$) between shoot to root sodium ratio and mortality percentage, and 0.130 ($p < 0.05$) between shoot to root sodium ratio and relative growth (net difference in number of branchlets) was observed (Table 1) indicating that shoot to root ratio of sodium could be a better parameter correlating with salt tolerance of *C. equisetifolia* than the sodium content in shoots or roots taken individually. The tolerant clone TNIPT 4 showed highest survival percentage (95 %) and low shoot to root ratio of sodium (0.99) when compared to the sensitive clone PYN, which showed the lowest survival percentage (0 %) and higher shoot to root ratio of sodium (3.65 %). Interestingly, similar higher shoot to root ratio of sodium (1.75 ± 0.75) was also observed in untreated sensitive clones when compared to that of the untreated tolerant clones (0.96 ± 0.14), although no significant statistical correlation with mortality of the treated plants was observed. This implies that shoot to root ratio of Na^+ needs to be estimated under salt stress for being used as a reliable marker for screening for salt tolerance. While previous reports have quantified the sodium concentration in shoots and roots, the values for shoot to root ratio of sodium were not reported in *Casuarina*. Based on the data reported at 150 mM NaCl salt treatment by Clemens et al. (1983), we calculated the shoot to root ratio in *C. equisetifolia* (0.939), *C. cunninghamiana* (1.931), *C. stricta* (3.45), *C. inophloia* (105.66). The susceptible species like *C. stricta* and *C. inophloia* thus showed a high shoot to root ratio of sodium, indicating that this parameter is a differentiating characteristic among species of *Casuarinaceae* for salt tolerance. Low shoot to root ratio of Na^+ concentration reflects the plant's capacity to limit the Na^+ transport to the shoots and compartmentalise them in the roots. It has been shown in potato cultivars that tolerant cultivars distribute Na^+ equally between stems and leaves while sensitive cultivars transport relatively more Na^+ to leaves (Jaarsma et al. 2013). Similarly, salt tolerance in *Casuarina* has been attributed to a more prolonged intake of ions by older branchlets via the transpiration stream and reduced mobility into younger tissues (Greenway 1962). In the highly tolerant species (*C. equisetifolia* and *C. glauca*), the Na^+ and Cl^- concentration decreased from lower (older) branchlets to the apical shoot suggesting an accumulation of ions in the older branchlets (Aswathappa and Bachelard

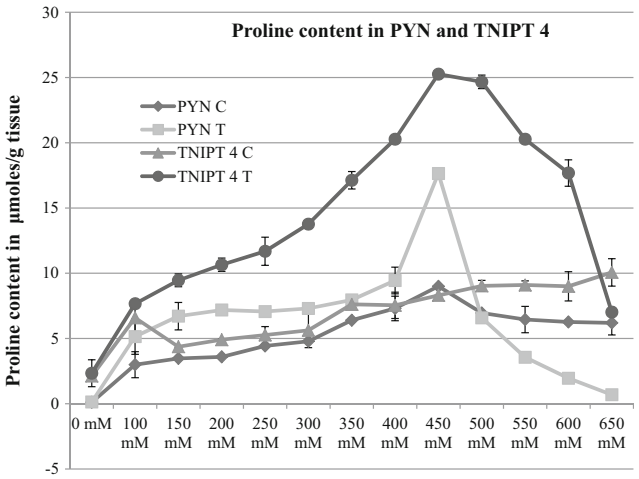

Fig. 4 Proline accumulation in TNIPT 4 and PYN (PYN C - PYN control; PYN T - PYN treated; TNIPT 4 C - TNIPT 4 control; TNIPT 4 T- TNIPT 4 treated)

1986). Analysis of Na^+ and Cl^- concentrations in xylem sap of *C. equisetifolia* and *C. cunninghamiana* showed that lower concentrations of Na^+ and Cl^- in shoots of *C. equisetifolia* are due to restricted translocation of these ions into shoots. In model crops, sodium transporters like *HKT1* have been implicated in restricting translocation of Na^+ into shoots. Members of the *HKT* gene family in rice and Arabidopsis are expressed in xylem parenchyma cells and protect leaves from salinity stress by unloading sodium from the xylem sap (Ren et al. 2005; Sunarpi et al. 2005; Platten et al. 2006). Although, in our study most of the tolerant clones showed lower shoot to root ratio of sodium, few tolerant clones viz., TNKP 2, TNPP 2, and TNIPT 2 showed high shoot to root ratio of sodium at 1.47, 1.65 and 1.48 respectively, similar to the sensitive clones. The high sodium content and high shoot to root ratio of sodium observed in such clones suggests that the tolerance exhibited by these clones may be due to the protection conferred at the cellular level by multiple stress tolerance mechanisms like higher expression of one of the osmoprotectants like proline. Increased soil salt concentrations reduce the ability of a plant to take up water. In tolerant clones, the high sodium concentration observed in shoots and roots may contribute to the osmotic potential (El-Lakany and Luard 1984) thereby facilitating water uptake and associated tolerance. Vacuolar sequestration of sodium is an important strategy that reduces the Na^+ concentration in the cytosol, and overexpression of vacuolar Na^+ / H^+ transporters have been shown to confer salt tolerance in a wide variety of species including *Arabidopsis* (Apse et al. 1999), *Brassica napus* (Zhang et al. 2001) and wheat (Saqib et al. 2005).

4.4 Variation in Na^+: K^+ ratio in *C. equisetifolia* clones and correlation with mortality

Lower Na^+/K^+ ratio has been reported as an indicator for lower Na^+ toxicity and as a determinative trait in salt tolerance in different plants (Tammam et al. 2008; Mahmood 2011; Fakhrfeshani et al. 2015). In *Casuarina*, El-Lakany and Luard (1984) observed that tolerant species had lower Na^+: K^+ ratios (3.58–7.59) than the more sensitive species (15.08). In a study in the tolerant species, *C. obesa*, Na^+: K^+ ratios increased from 0.03 in controls to 0.8 in 400 mM NaCl treatment while K^+ concentration remained stable (Carter et al. 2006). In our study in the shoots of *C. equisetifolia* clones, the tolerant clone, TNIPT 4, showed lower Na^+: K^+ ratio (3.875) compared to sensitive clone, PYN (10.433), indicating selectivity for K^+ over Na^+ in the tolerant clone. The 17 tolerant clones showed a higher average Na^+: K^+ ratio of 7.2 when compared to 5.44 for the sensitive clones. However, no correlation between Na^+: K^+ ratio and mortality was observed after analyses of the treated shoots or roots of these 30 *C. equisetifolia* clones indicating that sodium exclusion may not be the only salt tolerance determining trait in *C. equisetifolia*. Similar results have been observed in *H. vulgare* (Mahmood 2011), in which Na^+/K^+ ratio did not correlate with salt tolerance in all cases.

4.5 Effect of salt treatment on proline accumulation in tolerant and sensitive clones of *C. equisetifolia*

In *C. equisetifolia*, Tani and Sasakawa (2006) had reported that salt stress induces accumulation of proline but not that of glycine betaine, other amino acids or total sugars. In the present study on two *C. equisetifolia* clones with contrasted salt tolerance, a progressive increase in proline content was observed with increasing NaCl concentration up to 450 mM after which there was a decline in both TNIPT 4 and PYN. Proline accumulation showed a 3 fold increase in TNIPT 4 when compared to 1.96 fold increase for the sensitive clone PYN. Proline concentration increased in TNIPT 4 by 1.163, 2.447 and 2.731 folds at 100 mM, 300 mM and 500 mM NaCl concentration respectively. Further increase in the duration of NaCl treatment beyond 2 weeks did not result in an appreciable increase in proline content in the shoots or roots. Similar results were reported in *C. equisetifolia* by Tani and Sasakawa (2006), wherein seedlings after treatment at 100, 300 and 500 mmol L^{-1} NaCl for a period of 2 weeks, showed 1.8, 6.7 and 15-fold increase in proline levels (1.48, 5.35 and 12.05 μmoles/g tissue) respectively when compared to the control (0.80 μmoles/g tissue). The present study also showed that the tolerant clone TNIPT 4 had constitutively higher levels of proline (2.34 ± 1.03 μmoles/g tissue) than the sensitive clone, PYN (0.136 ± 0.34 μmoles/g tissue). Stress tolerance mechanisms are often constitutively different between contrasting varieties as has been observed in a transcriptomic study in grapes (Henderson et al. 2014).

In stressful environment, elevated proline levels have been observed in the more tolerant species, *C. junghuhniana*, than in *C. cunninghamiana* and *C. equisetifolia* (Reddy 2001). However, several studies, as in acacia species (Yokota 2003), reported no relation between proline accumulation and salt tolerance despite the observed positive correlation between salt stress and proline accumulation. In a study (Jayaraj 2014) in 20 clones of *C. equisetifolia* subjected to increasing concentrations of NaCl from 0 to 350 mM, no consistent pattern in proline accumulation, or correlation between proline accumulation and intraclonal variation in salt tolerance were observed. This lack of correlation was attributed to varying presence of adaptive mechanisms other than proline accumulation among the sub-species and provenances.

In the present study, the highly tolerant clone TNIPT 4 had low shoot to root Na^+ ratio (0.99) indicating restricted transport of Na^+ to the shoots as one of the adaptive mechanisms. The high salt tolerance of TNIPT 4, despite the higher than average shoot Na^+ concentration (0.942 ± 0.01 mmol/g),

could be attributed to the accumulated proline providing osmotic balance in the cytoplasm while Na^+ may have been sequestered in the vacuoles. In *C. obesa*, Carter et al. (2006) observed a general relationship whereby samples with high foliar Na^+ and Cl^- concentration also had high foliar proline, consistent with the hypothesis that proline was probably involved in osmotic adjustment of the cytosol as Na^+ and Cl^- were presumably partitioned in the vacuole.

5 Conclusion

Intraclonal variations in salt stress response of 82 different selections of *C. equisetifolia* were studied. The selected contrasting phenotypes provide a valuable resource for understanding the genetic basis of variation in salt tolerance in *C. equisetifolia*, in addition to the selected tolerant clones being used as candidates for further testing in coastal saline areas and in waterlogged saline areas that are also confounded by anaerobic stresses. No significant correlation was observed for Na^+ content and mortality in the thirty clones selected for their contrasting salt stress response, despite the high shoot and root Na^+ content in sensitive clones. Shoot to root ratio of sodium was found to be a better differentiating parameter for the variation observed in salt tolerance in *C. equisetifolia* than shoot or root sodium content or Na^+:K^+ ratio, suggesting that restricted translocation of sodium to shoots and its compartmentalization in roots play a crucial role in the salt tolerant clones like TNIPT 4. The higher salt tolerance despite higher sodium accumulation or shoot to root ratios of sodium observed in certain clones suggest the presence of multiple different tolerance mechanisms operating in these clones. The tolerant clone, TNIPT 4, also showed higher constitutive levels of the osmoprotectant proline as well as a rapid 3-fold increase in proline during salt stress in comparison to the sensitive clone, PYN. In case of TN 501, a faster rate of generation of branchlets was observed to be contributing to the observed tolerance. Molecular characterisation of genes involved in sodium transport and proline biosynthesis, and their regulation could provide greater insights on the salinity tolerance mechanisms in this species that is widely used in coastal afforestation.

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