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Salt secretion, proline accumulation and increased branching confer tolerance to drought and salinity in the endemic halophyte *Limonium linifolium*

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ABSTRACT

Limonium linifolium (L.f.) Kuntze co-occurs with *Bassia diffusa* (Thunb.) Kuntze in the upper intertidal ranges of salt marshes in South Africa. Predictions of climate change indicate that there would be large variations in inundation and salinity in salt marshes in which *L. linifolium* occurs. The aims of this study were to investigate its tolerances to water and salinity variations similar to these predicted conditions. Plants were treated to tidal, drought and submerged conditions each at five salinity levels (0, 8, 18, 35 and 45 psu), for two months. Growth measurements showed that the plant was tolerant to a wide range of salinity under both tidal and drought conditions, but was susceptible to complete submergence, with high membrane damage in tidal-treated plants. Plants died within 2 weeks of complete submergence. Branching increased in drought-treated plants, decreased with increasing salinity and correlated positively with shoot area ($r = 0.927$). Results showed that *L. linifolium* tolerates drought by accumulating large quantities of proline and oxalic acid, and excreting excess salts for water potential balance. Salt secretion is an important adaptation for a plant that thrives in a highly variable saline habitat. Further studies on the reproductive resilience of the species would shed more light on long term resilience of salt marshes in which the species occur, if the climate changes as predicted. This is important for salt marsh species diversity, as this plant is endemic to South Africa.

1. Introduction

Limonium linifolium (L.f.) Kuntze, a tufted upper intertidal-to-supratidal halophyte of the family Plumbaginaceae occurs along the Cape Coast of South Africa from Riversdale to the Transkei (Roux, 2003). It is endemic to South Africa where it occurs in 13 estuaries (Adams et al., 2012) (Fig. 1 shows its distribution in 19 estuaries, following an updated survey by Adams et al. in prep.). It was listed as a species of least concern by Victor and Mucina in 2004 (Raimondo et al., 2009). This means that the species does not fall under the threatened and near threatened categories of the IUCN red list classification, and by implication there is no urgency in its protection/conservation. However, it forms important populations in large estuaries such as the Swartkops and Knysna estuaries in the Eastern Cape Province (Maree, 2000). The plant has been very little studied; it is difficult to grow under glasshouse conditions and its tufted habit renders most classical growth measurements unfeasible. The entire shoot is photosynthetic, with main shoots woody and cylindrical, at the base of which is a basal rosette of lanceolate leaves. This non-succulent tuft co-habits upper intertidal ranges with the succulent *Bassia diffusa* (Thunb.) Kuntze, and mixed stands of the species result in a unique mosaic for those reaches.

Because of low species diversity in salt marshes (Elliott and Whitfield, 2011), this species contributes significantly to salt marsh structure and functioning in estuaries in which it occurs. Species structure in such estuaries is determined by a gradient of physico-chemical drivers (in estuaries with large tidal range) or heterogeneity in these drivers for microtidal estuaries (Bornman et al., 2002, 2004; Taylor et al., 2006; Bornman et al., 2008). Of significant importance are salinity (Pennings and Callaway, 1992; Pennings et al., 2005) and inundation regimes (Riddin and Adams, 2008), and these would vary more under predicted climate change conditions. Such conditions include increased sea levels and submergence due to mouth closure in Temporarily Open/Closed Estuaries (TOCEs), drought in the upper intertidal regions of Permanently Open Estuaries (POE) and in TOCEs when low freshwater inflow is coupled with mouth closure. All these conditions are coupled with salinity variation. Under such conditions, the resulting vegetation structure would depend on the ability of component species to tolerate abiotic changes. Increased submergence in salt marshes with significant populations of submergence-susceptible species would result in bare mud banks or greatly reduced growth of salt marsh species

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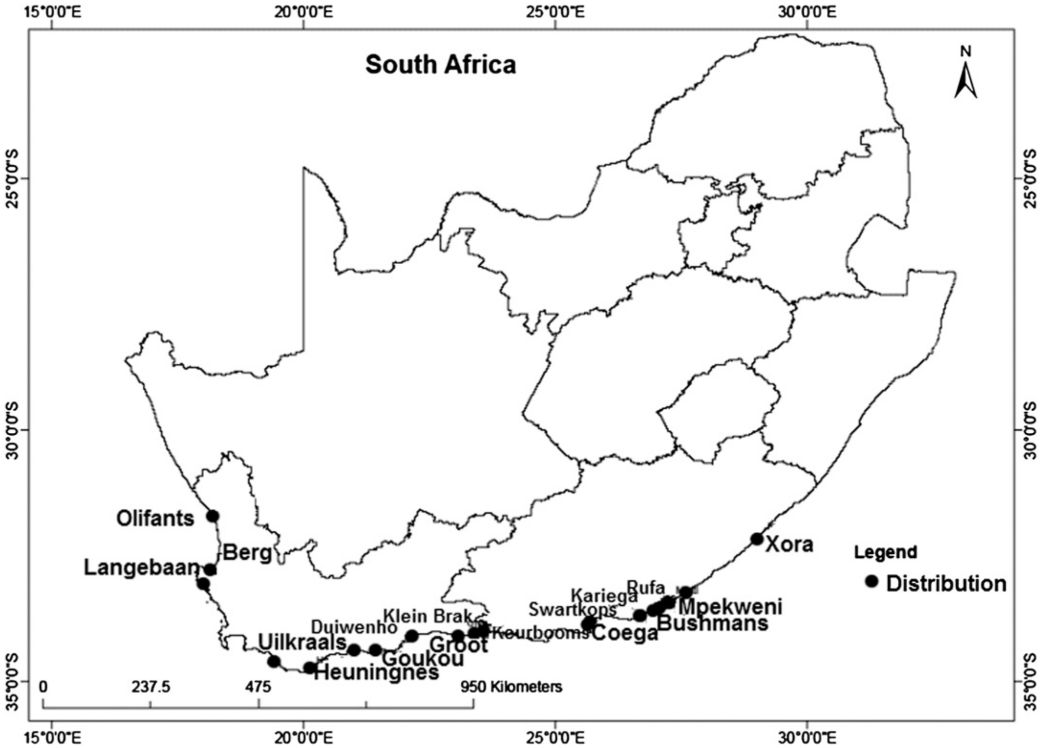


Fig. 1. Distribution of *Limonium linifolium* in South Africa.

(Armstrong et al., 1999; Mauchamp et al., 2001; Colmer et al., 2009), and increase in the cover of submergence-adapted species (Riddin and Adams, 2008). When submergence is coupled with increased or decreased salinity, the pattern of response becomes more complex. Combined with high salinity, increased submergence resulted in reduced salt marsh cover in preference for macroalgae (Riddin and Adams, 2010). On the other hand, drought can occur in middle reaches of Permanently Open Estuaries (POE), River Mouths and TOCEs when prolonged absence of rainfall results in low freshwater inflow. Drought incidents reduce the cover and reproductive ability of species (Chaves et al., 2009). This is further compounded when drought is coupled with increased sediment salinization, as has been shown for *Spartina alterniflora* Loisel. (Brown and Pezeshki, 2007) and *Juncus kraussii* (Naidoo and Kift, 2006). One adaptive morphological strategy for plants growing under such conditions is increased root proliferation to the water table (Bornman et al., 2004, 2008) with trade-offs in reduced above ground growth. To regulate the ionic imbalances that occur under extreme salinity and drought stress, tolerant species have evolved a suite of adaptations including changes in pigment types and/or composition, and the accumulation of compatible solutes in the cytoplasm and organelles. This balances the low osmotic potential in the vacuole and maintains more negative water potential relative to the soil for water and nutrient uptake (Naidoo and Kift, 2006; Ashour and Mekki, 2006; Ashraf and Foolad, 2007; Chen and Jiang, 2010).

Among the most important compatible osmolytes for most halophytes are proline and glycinebetaine. In particular, members of the genus *Limonium* accumulate large quantities of proline and can convert this to more effective osmoprotectants such as prolinebetaine and hydroxyproline betaine depending on the type and/or intensity of stressor (Storey et al., 1977; Hanson et al., 1994). Salt secretion is another adaptive strategy common in most members of the genus on whom research has been done under saline conditions (Salama et al., 1999). Such studies have not been conducted for *L. linifolium* which is endemic to South Africa and the most dominant member of this genus locally; it is especially important to investigate how this species would respond to predicted changes in abiotic stressors as would occur under climate change conditions. This research aimed to answer the following questions:

1. Would *L. linifolium* acclimate to flooding in salt marshes in which increased flooding has been predicted, and if so what would be its tolerance mechanisms?
2. Would the species tolerate increased drought conditions predicted for some salt marshes, and what adaptations does it possess for such tolerance?

The following hypotheses were tested.

H1. Because *L. linifolium* is an upper intertidal species, it survives drought and high salinity for 2 months, and will die if submerged for 2 months.

H2. Proline is a compatible solute in this species, similar to the majority of members of the genus *Limonium*.

The duration of the experiment was set at 2 months because although there are four seasons of three months each in the region, at both extremes of each season there would be transition conditions atypical of the season in question.

2. Materials and methods

2.1. Experimental layout

2.1.1. The tidal system

A tidal tank re-circulating system was set up in the glasshouse of the Botany Department. It consisted of two main tanks, each with five separate chambers, all built using transparent glass. Each chamber measured 52 × 52 × 60 cm. One main tank was designed to be permanently flooded, and the other was designed to simulate tidal cycles with 6 h between high and low tides, as occurs naturally in the region. The functioning of the system was similar to that described in Hoppe-Speer et al. (2011). The only modification was that the salinity was pre-set in all main tanks, and these re-circulated for the duration of the experiment.

There were five tanks set at 0, 8, 18, 35 and 45 psu salinity, and these fed the three inundation states namely tidal, completely submerged and drought. Salinity levels were selected to reflect sediment salinity at the natural range of the species where seedlings were collected (18 psu), salinity conditions under the influence of freshwater inflow (0–8 psu) and hypersaline conditions that would occur under drought and increased sediment salinization (35–45 psu). In the tidal treatment the plants were submerged with a 10 cm depth of water above the sediment, or until the shoots were completely submerged at high tide, but drained at low tide with 6 h between high and low tides. The tides were controlled by a computer programme (Hoppe-Speer et al., 2011). In the complete submergence treatment the plants were submerged with a 10 cm depth of water above the sediment, or until the shoots were completely submerged for the duration of the experiment (see Adams and Bate, 1995; Armstrong et al., 1999; Colmer et al., 2009). Drought conditions were simulated as progressive soil drying; the pots were placed on trays and watered to field capacity with water of the respective salinity and allowed to dry for the duration of the experiment (8 weeks). To simulate natural water uptake from the moisture layer even under drought conditions, partial re-watering was done once from the bottom with 200 ml freshwater.

2.1.2. Seedling establishment and treatment application

Seedlings were collected from the Swartkops Estuary in Port Elizabeth (33°52′S, 25°36′E) in February 2012. They were grown in 2 l pots for a month in estuarine soil collected from the same site. This sediment was at 18 psu salinity, and for seedlings to establish, salinity alleviation was necessary. Hence during establishment the seedlings were watered with freshwater; subsequently they were acclimatised to 18 psu for 1 month, using seawater diluted with distilled water. Prior to treatment application, initial growth measurements were conducted. In April 2012 the treatments were applied to established plants of similar stem length. Seawater, freshwater and Reef Aquarium Sea salts (Reef Aquatics, Cape Town) were used to set up the required salinity in each treatment. Salinity below 35 psu was obtained by diluting seawater with freshwater, and the 45 psu salinity was obtained by the addition of Reef Aquarium Sea salts to seawater. Reef Aquarium salts are formulated for aquaria of marine organisms, and are widely used in research to simulate seawater (see Hoppe-Speer et al., 2011). A hand-held refractometer (Atago, S/Mill-E, Atago Japan) was used in monitoring the salinity during the experiment. The experimental design resolved to a (three inundations + 5 salinity levels) × time experiment. In all there were 15 independent treatments since each water level was coupled with the five different salinity treatments. Each treatment had four replicate pots, and each pot had five plants, three of which were measured every two weeks. The experiment lasted for 8 weeks.

2.2. Plant growth measurements

Stem length was measured prior to the start of the experiment, and every two weeks during the experiment. Stems, branches and branchlets were counted and their lengths and diameters were measured with a digital calliper. The surface area of each individual shoot was calculated arithmetically as follows:

$$A = 2\pi rl + 2\pi r^2$$

where A = surface area, l = stem/branch/branchlet length, r = radius, and π = 3.14.

This calculation was done for the main stem, branches and branchlets and summed per plant. This equation was chosen because of the cylindrical shape of the stems and branches. The method slightly underestimates shoot area because it does not take into consideration the basal rosette of leaves, but these were similar for all treatments and therefore assumed constant. The tufted habit rendered the use of other methods unfeasible. These measurements were repeated at the end of the experiment prior to harvesting. Growth measurements were only completed for the tidal and drought treatments, as the submerged plants at all salinity levels died within the first two weeks.

2.3. Sampling of soil and plant material

At the end of the experiment, soil samples were collected for water potential measurements, and the plants were then harvested. The soil was carefully washed off the roots using a water spray, and the harvested plants were weighed. The roots were then separated from the shoot, which was used for further analyses. Sediments were also collected for determination of effective sediment salinity at the end of the experiment.

2.4. Growth, shoot and leaf mass fraction

The fresh and dry mass of the roots and shoots were measured on a Toledo scale (Mettler Toledo AG 204, Mettler Toledo Products, Switzerland; accuracy ± 0.1 mg). Dry mass was measured after drying the samples at 80 °C for 48 h. Growth was calculated in two ways; as the difference between the initial and final stem lengths, and using the dry masses. The relative growth rate (RGR) was calculated using the equation:

$$\mathrm{RGR} = \frac{(\ln H2 - \ln H1)}{(t2 - t1)}$$

where H2 = final stem length (or dry mass), H1 = initial stem length (or dry mass), t2 = duration of treatments in weeks, and t1 = 0.

It was necessary to use both length-based and biomass-based approaches for these measurements so as to better explain changes in growth.

2.5. Salt excretion in drought-treated plants

During the experiment salt crystals were observed on the stems of the drought-treated plants. After harvest, shoots were immediately rinsed in 25 ml deionised water for 30 min, after which the total dissolved solutes (ppm) were measured using a conductivity/TDS/°C meter (Cyberscan200, Eutech Instruments, Singapore). These readings were then converted to concentrations in mg as follows:

$$\text{Excreted salts (mg)} = \frac{\text{TDS (ppm)} \times 25\ \text{ml}}{1000\ \text{ml}}$$

where 25 ml is the volume of the extract.

Subsequently, the concentration of excreted salts was expressed per unit shoot area and unit shoot mass.

2.6. Relative water content (RWC) and succulence

Shoot segments were weighed fresh, following which they were immersed in excess deionised water for 24 h until turgid, blotted dry and reweighed. They were then oven-dried at 80 °C for 48 h and reweighed. The following formulae were used to calculate shoot relative water content and shoot succulence:

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

$$\text{Succulence} = \frac{(\mathrm{FM} - \mathrm{DM})\ \mathrm{g}}{(\mathrm{DM})\ \mathrm{g}}$$

where FM = fresh mass, DM = dry mass, and TM = turgid mass.

Shoot succulence (dimensionless) is measured as the ratio of the shoot water content to the dry mass.

2.7. Photosynthetic pigment concentration

The method of Lichtenthaler and Buschmann (2001) was used to determine the concentrations of chlorophylls *a*, and *b* and total carotenoid. Shoot segments were weighed, crushed and extracted in 10 ml absolute ethanol (100%). The extract was then filtered through Whatman GFC filters and the absorbance was read from a GBC UV/VIS spectrophotometer (GBC UV/VIS 916, GBC Scientific Equipment Pty Ltd. 1995) at 664.1, 648.6 and 470 nm. All pigment concentrations were subsequently standardised per gramme shoot dry mass, chlorophyll $a + b$ concentration and their ratio was then calculated.

2.8. Electrolyte leakage

Electrolyte leakage was determined by measuring the conductivity, as a percentage of electrolytes leaked from cut cells compared to the total electrolyte pool in the sample (Leopold et al., 1981; Bajji et al., 2001; Pang et al., 2003). Freshly cut shoot segments were washed in double distilled water and rinsed in deionised water; this eliminated any electrolytes on their surface as well as those released during cutting. These samples were then placed in 10 ml deionised water, shaken on a mechanical shaker at 250 rpm for 15 min for exchange of electrolytes (Leopold et al., 1981; Bajji et al., 2001; Pang et al., 2003), and the initial electrical conductivity was read using a conductivity/TDS/°C meter (Cyberscan200, Eutech Instruments, Singapore). Next the beakers containing the leaf samples were placed in a sealed boiling water bath for 15 min, cooled to room temperature, and the total conductivity was read. Electrolyte leakage was calculated as:

$$\mathrm{EL}\ (\%) = \frac{\mathrm{E1}}{\mathrm{Et}} * 100$$

where E1 is the initial conductivity that represents potential ‘natural leakage’ from the cells, and Et is the total conductivity.

2.9. Water potential

Water potential of soil and shoot segments was measured using a Decagon WP4 Dew point Potentiometer (Range −300, resolution −0.1 MPa, Decagon Devices Inc. USA). For soil measurements, the chamber was half-filled with samples while for shoot measurements, an approximately equal quantity of shoot segments was placed in the chamber. The chamber was equilibrated and readings were taken at approximately 15 minute intervals. The final reading for each sample was the mean of three measurements.

2.10. Proline and oxalic acid

These were determined through high performance liquid chromatography (HPLC), using a modification of the method of Colmer et al. (2000). In a previous experiment on *B. diffusa*, oxalic acid was found to have a possible osmoregulatory function; hence it was necessary to assess this possibility in *L. linifolium* as well. Fresh shoot segments were used, but the concentrations were standardised per gramme dry mass using a pre-determined significant ($R^2 > 95\%$) regression equation between dry and fresh mass. The extractant was 3 ml cold 5% (v/v) perchloric acid, the mobile phase was 95:5 (v/v) 0.05 M potassium dihydrogen phosphate in deionised water:methanol adjusted to pH 2.1 with dilute HCl, and readings were made using an Agilent Infinity 1260 HPLC with an autosampler, and a C18 Zorbax column (Agilent Technologies Inc., United States of America). Oxalic acid eluted at a retention time of 1.52 min, and proline eluted at 1.92 min.

2.11. Other observations

Observations were made of chlorotic patterns on the leaves and stems of the species, from 4 weeks to the end of the experiment, and photographs of plants with these lesions were taken.

2.12. Data analyses

Because the salinity treatments were combined with water state, the experiments were resolved into 15 independent (salinity + water state) × time treatments. The Kolmogorov–Smirnov test was used to test all data for normality; Johnson Transformation was used to normalise non-normal data (Chou et al., 1998). When the relevant conditions of normality and homogeneity of variance were met, Two-way General Linear Model ANOVA was used to evaluate treatment effects on the dataset as a whole. To isolate salinity effects for each water state, One Way ANOVA was performed. ANOVAs were conducted in tandem with the Tukey test for mean separation. Two-Factor Analyses of relationships between variables were then conducted, and Pearson correlation was used to confirm the observed relationships. All analyses were conducted at $\alpha = 0.05$ using MINITAB version 16 (Minitab Statistical Software, MINITAB Inc. USA).

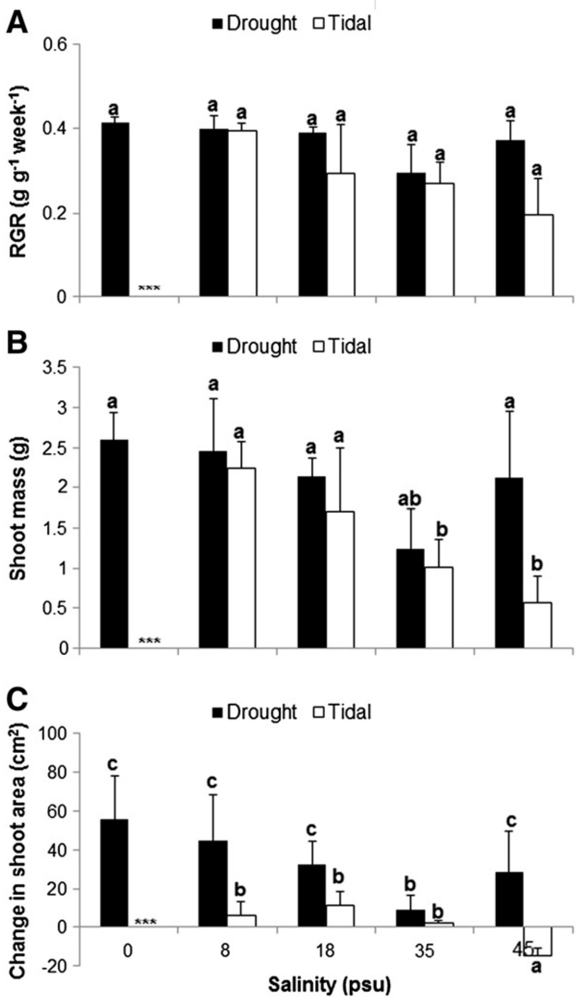


Fig. 2. Growth responses of *Limonium linifolium* to drought and tidal conditions at different salinity concentrations. A: Relative growth rate (RGR); B: Shoot fresh mass (g); C: Change in shoot area (cm^2) over 8 weeks of treatments. Bars represent means ± SE. Means separated through Tukey HSD test at $\alpha = 0.05$; bars with the same letter are not statistically different. ***Plants in the tidal treatment at this salinity were dead by the end of the experiment.

3. Results

3.1. Effect of treatments on growth

Submerged plants died within the first two weeks of submergence. Fig. 2 shows the growth of drought- and tidal-treated plants within 8 weeks of treatment. Two way GLM ANOVA did not show significant difference in relative growth rates between drought treated plants and tidal treated ones; analyses excluded tidal-treated plants at 0 psu which died within 8 weeks. Within the tidal treatments, the best relative growth rate was at 8 psu and growth reduced with salinity increase ($p < 0.05$, DF $= 15$). In the drought-treated plants, relative growth rate did not vary significantly with salinity (Fig. 2A).

Drought-treated plants had a significantly higher increase in shoot area than tidal-treated ones ($p < 0.005$, DF 39). Within the tidal treatments, shoot area increased significantly with salinity between 8 and 35 psu, with negative changes at both extremes (0 and 45 psu) ($p < 0.05$, DF $= 19$). This negative change in shoot area was as a result of the death of some initial shoots; the subsequent tallest shoot of the plant measured would thus provide a lower measurement than the previous one. In the drought-treated plants, the increase in shoot area at different salinity levels was not statistically significant ($p > 0.05$) (Fig. 2C).

Change in the number of branches formed is presented in Fig. 3. The number of new branches formed during the experiment varied significantly with inundation ($p < 0.005$) and salinity ($p < 0.05$). There were significantly more new branches in the drought-treated plants (mean $= 59.3$, N $= 19$) than in the tidal-treated ones (mean $= -6.3$, N $= 16$). Within the drought treatments, the highest number of new branches was in plants at 8 psu (125 ± 44), and branching decreased with increase in salinity. In the tidal treatments, plants at 8 psu also had the highest number of new branches (18 ± 21) which decreased with increase in salinity. Thus there were both drought- and salinity influences on the number of new branches formed (Fig. 3).

The relationship between plant height, branching, absolute growth rate and change in leaf area is presented in Fig. 4. Results show that the change in shoot area was mainly as a result of increased branching and not increase in height. There were strong positive correlations between the final number of branches and number of new branches, with change in shoot area ($r = 0.916$ and 0.927 respectively, $p < 0.001$). The relationship between change in height and change in shoot area was much weaker (Fig. 4D) and no correlations existed between the number of new branches and AGR, and between change in shoot area and AGR (Fig. 4E and F).

3.2. Relative water content and succulence

Relative water content and succulence (data not shown) did not vary significantly with both inundation state and salinity. Within the different inundation states, there were no significant variations in relative water content and succulence with salinity ($p > 0.05$).

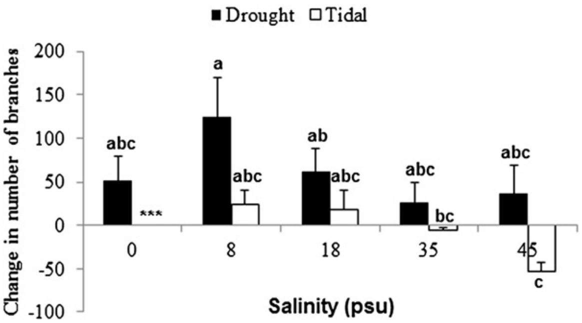


Fig. 3. Effects of salinity on number of branches formed in *Limonium linifolium* under tidal and drought conditions. ***Tidal treated plants at this salinity died during the experiment.

3.3. Response of chlorophyll pigments to the treatments

Chlorophyll concentrations at the end of treatments are presented in Table 1. Across all treatments, chlorophyll concentrations (chlorophylls a, b and $a + b$) did not vary significantly with both water state and salinity ($p > 0.05$), but chlorophyll a/b ratio increased with salinity increase ($p < 0.05$, DF $= 24$). Within the tidal treatments, there were no significant changes in chlorophyll concentration and chlorophyll a/b ratio with salinity increase ($p > 0.05$). In the drought-treated plants, chlorophyll b concentration increased with an increase in salinity to 1.4 ± 0.1 mg l^{-1} at 35 psu. This resulted in a significant decrease in the ratio of chlorophyll a to b as salinity increased ($p < 0.05$, DF $= 14$).

3.4. Electrolyte leakage

Membrane integrity was measured as electrolyte leakage, and results are presented in Fig. 5. Electrolyte leakage significantly increased in the tidal — compared to the drought-treated plants ($p < 0.005$, DF $= 26$). In the tidal-treated plants electrolyte leakage was high and increased with salinity increase (25.3 ± 8.5% at 8 psu to 60.8 ± 3.3% at 45 psu). The range was lower in the plants under drought treatments and without significant variations as salinity increased ($p > 0.05$).

3.5. Oxalic acid and proline

Fig. 6 shows the concentration of oxalic acid (Fig. 6A) and proline (Fig. 6B) in treated plants after 8 weeks. Oxalic acid concentration in plants of the drought treatments was significantly higher than that of the tidal-treated plants ($p < 0.05$, DF $= 27$), but there were no significant interaction or salinity effects. Within the tidal treatments, oxalic acid concentration was highest at 8 and 18 psu, and decreased as salinity increased ($p < 0.05$, DF $= 12$). In the drought treatments, there were high oxalic acid concentrations (62 ± 23.5 to 93 ± 17.1 mM) which did not differ significantly as salinity increased ($p > 0.05$). There were no significant differences in proline concentrations across inundation states and with salinity ($p > 0.05$). Proline concentrations were generally high at both inundation states across plants of all salinity treatments, but with great variability as shown by the large standard errors (256.8–686 mM in the tidal treatments and 507–795 mM in the drought-treated plants).

3.6. Salt secretion from drought-treated plants

Total salts secreted (Fig. 7A) in the drought-treated plants increased as sediment salinity increased, but this increase was not statistically significant when expressed per unit shoot area; however, when expressed per gramme fresh mass of shoot (Fig. 7B), the salt secreted increased as salinity increased ($p < 0.005$, DF $= 13$). There were strong positive correlations between treatment salinity and salt secreted per gramme dry mass ($r = 0.845$, $p < 0.001$) but no correlations were found between treatment salinity and shoot area.

3.7. Relationship between parameters measured

Two Factor Analyses of the relationships between variables for the whole experiment are presented in Fig. 8A. The first two factors accounted for 61% of the observed variations in the data. Sediment salinity was negatively correlated with shoot fresh mass, shoot dry mass, relative growth rates and succulence ($r = -0.579, -0.579, -0.448$ and -0.430 respectively, $p < 0.05$). Growth in height was positively correlated with dry mass ($r = 0.918$, $p < 0.005$). Shoot fresh mass, dry mass and growth rates correlated positively with shoot water potential ($r = 0.788, 0.788$ and 0.871 respectively, $p < 0.005$), while chlorophyll a, b and $a + b$ concentrations correlated negatively with shoot water potential ($r = -0.671, -0.561$ and -0.675 respectively, $p < 0.005$). Electrolyte leakage was negatively correlated with shoot water content

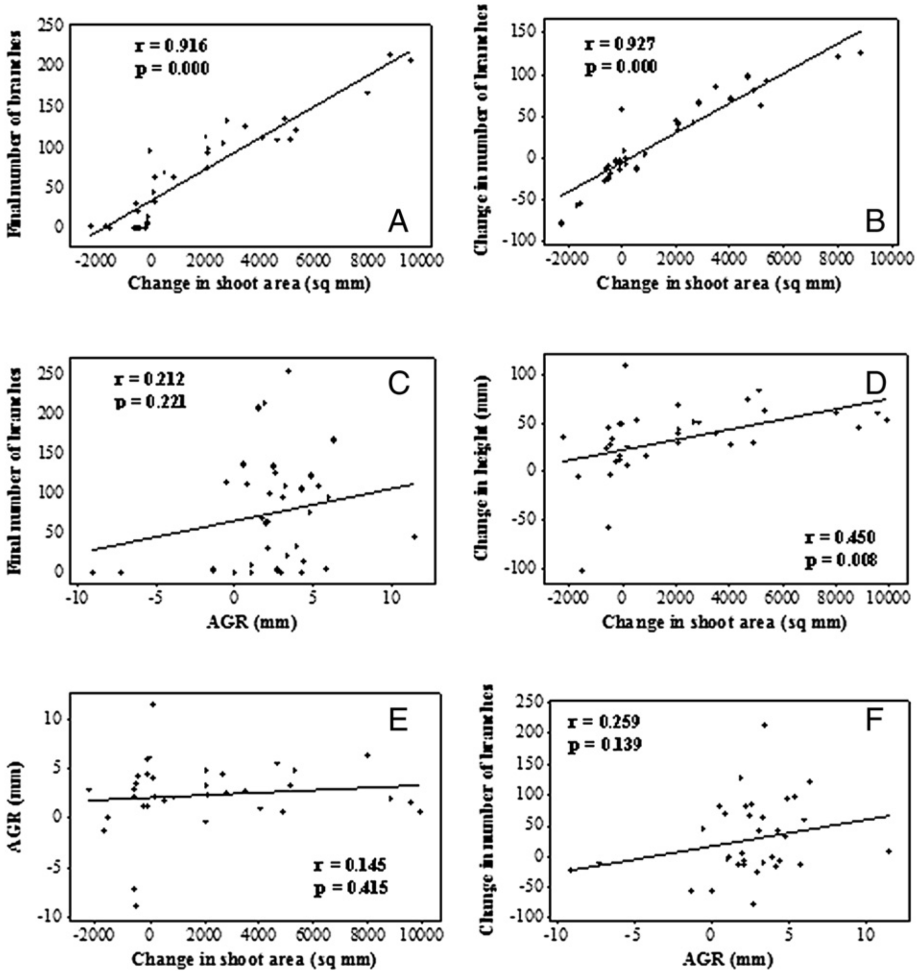


Fig. 4. Relationship between branching, height, RGR and change in shoot area. r = Pearson correlation coefficient, p = probability level.

($r = -0.614$, $p < 0.005$), while more negative shoot water potential correlated positively with higher shoot water content ($r = 0.721$, $p < 0.005$).

Because salt secretion was determined for the drought-treated plants only, it was necessary to analyse these treatments further, without the tidal effects. For these treatments (Fig. 8B), growth was strongly positively correlated with plant water potential ($r = 0.695$, $p < 0.05$), and plants that had higher growth were associated with higher succulence ($r = 0.564$, $p < 0.05$). Increase in sediment salinity negatively correlated with shoot biomass ($r = -0.647$, $p < 0.05$), but significantly positively correlated with the quantity of salt secreted by the species ($r = 0.870$, $p < 0.005$). A more negative plant water potential was associated with less electrolyte leakage ($r = -0.578$, $p < 0.05$).

3.8. Other observations

Fig. 9 shows patterns typical of specific ionic toxicity in drought-treated plants between 8 and 18 psu. After a month of treatment, three chlorotic patterns were observed. Shoots in some plants developed a chlorotic band towards the base, and eventually severed at this point. A second set of plants developed chlorotic lesions on tips of the broad leaves of the basal rosette; while these lesions were random in

Table 1
Effect of drought and tidal conditions at different salinity concentrations on chlorophyll pigments in *Limonium linifolium* after 8 weeks of treatment.

Water state	Salinity (psu)	Chlorophyll concentration (mg l^{-1})			Chlorophyll *a*/*b* ratio
		Chlorophyll *a*	Chlorophyll *b*	Chlorophyll *a* + *b*	
Tidal	0	**	**	**	**
	8	4.2 ± 0.4	1.2 ± 0.2	5.4 ± 0.6	3.7 ± 0.4^{a}
	18	4.0 ± 0.3	1.0 ± 0.03	5.1 ± 0.4	3.9 ± 0.2^{a}
	35	3.6 ± 0.6	1.0 ± 0.4	4.6 ± 1	3.4 ± 0.7^{a}
	45	4.5 ± 0.3	1.7 ± 0.2	6.2 ± 0.5	2.7 ± 0.1^{a}
Drought	0	3.3 ± 0.2	0.8 ± 0.1	4.0 ± 0.3	4.6 ± 0.3^{w}
	8	4.1 ± 0.4	1.1 ± 0.1	5.2 ± 0.5	4.0 ± 0.7^{w}
	18	3.5 ± 0.3	1.2 ± 0.2	4.7 ± 0.4	3.0 ± 0.2^{x}
	35	3.8 ± 0.1	1.4 ± 0.1	5.1 ± 0.2	2.8 ± 0.1^{x}
	45	3.5 ± 0.2	1.2 ± 0.1	4.8 ± 0.3	2.9 ± 0.1^{x}

Values represent means ± SE. Means separated using Tukey post hoc HSD test at $\alpha = 0.05$; means with the same letter for each water state are not significantly different. **There are no values for the tidal plants at 0 psu; these plants died within one month of the treatments.

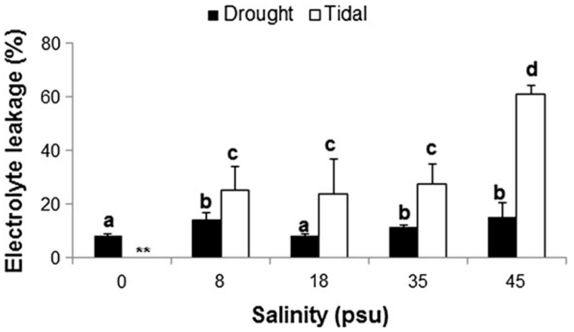


Fig. 5. Effect of various combinations of water and salinity on electrolyte leakage in *Limonium linifolium* after 8 weeks of treatment. Bars represent means ± SE. Means separated using Tukey post hoc HSD test at α = 0.05; bars with the same letter for each water state are not significantly different. **There are no values for the tidal plants at 0 psu; these plants died within one month of the treatments.

some treatments, in other plants these lesions seemed to spread from the bottom- to the top leaves. Plants with leaf lesions survived to the end of the experiment.

4. Discussion

This study showed that *L. linifolium* tolerates wide salinity ranges and grows in both tidal and drought conditions, but with better performance in drained conditions. The species does not grow under tidal freshwater conditions. This explains its occurrence at upper intertidal-to-terrestrial ranges in salt marshes. Analysis of growth using length increase as well as biomass did not yield significant differences under the two inundation states. However, the strong correlations between

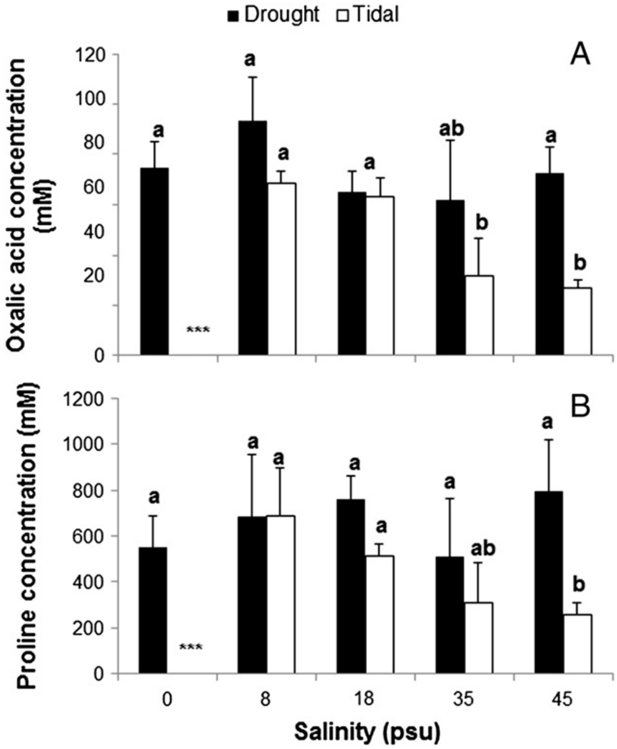


Fig. 6. Effects of various combinations of water and salinity on oxalic acid and proline concentrations in *Limonium linifolium* after 8 weeks of treatments. A: oxalic acid concentration; B: proline concentration. Bars represent means ± SE. Means separated using Tukey post hoc HSD test at α = 0.05; bars with the same letter for each water state are not significantly different. ***There are no values for the tidal plants at 0 psu; these plants died within one month of the treatments. Inclusion of zeros for this treatment would have biassed the analyses.

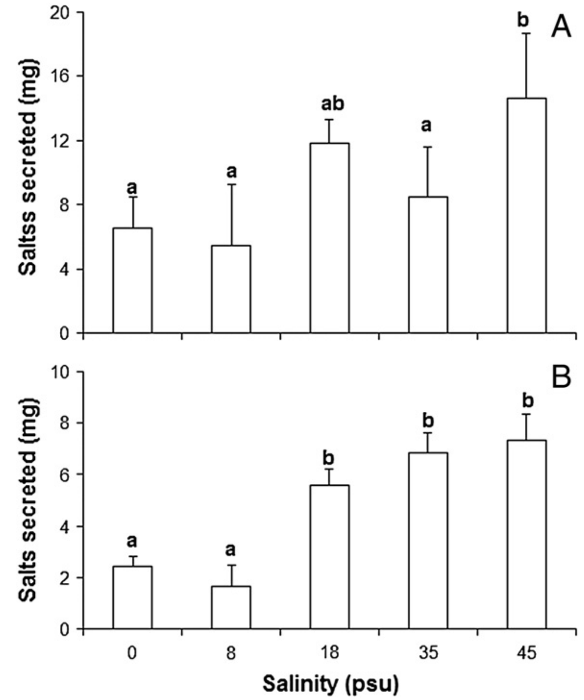


Fig. 7. Effects of drought and salinity on salt secretion in *Limonium linifolium* after 8 weeks of treatments. A: on shoot area basis; B: on fresh mass basis. Bars represent means ± SE. Means separated using Tukey post hoc HSD test at α = 0.05; bars with the same letter are not significantly different. Salt secretion was not done for tidal treatments as these would have been washed by the tidal water.

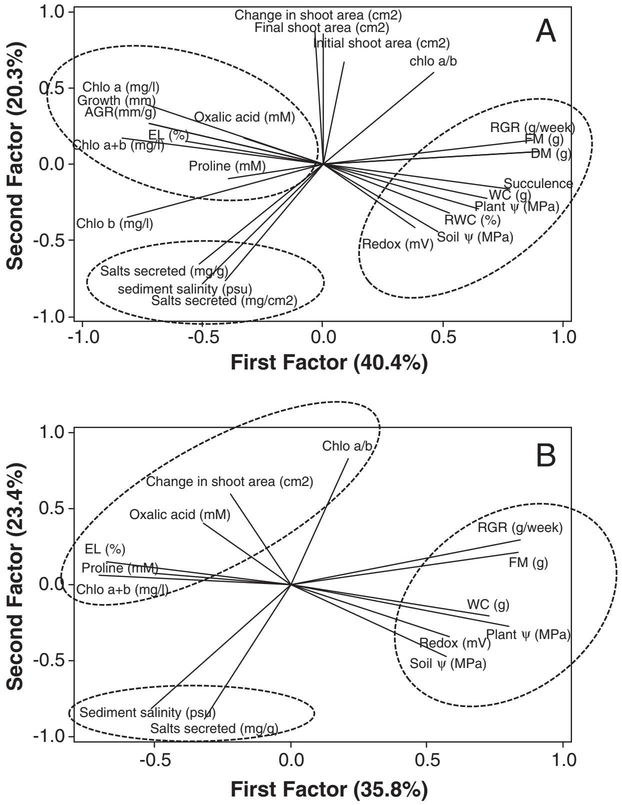
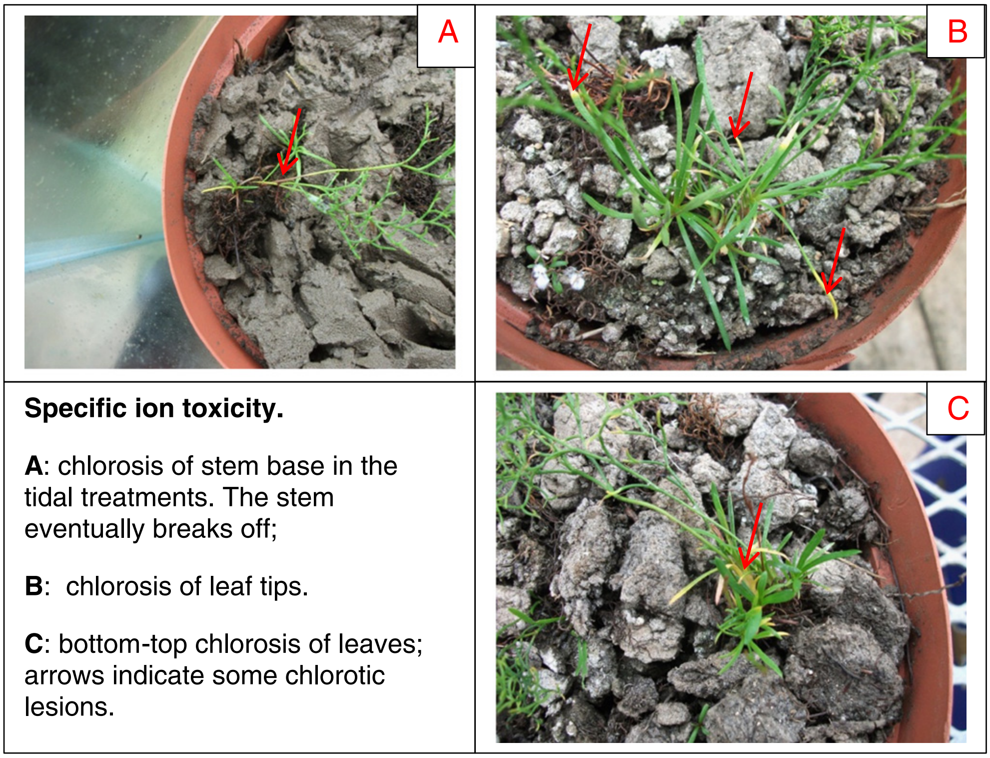


Fig. 8. Principal Components Two Factor Analyses of the relationship between variables, A: for the entire experiment and B: for the drought treatments only. Chlo = chlorophyll, AGR = absolute growth rate, RGR = relative growth rate, FM = fresh mass, DM = Dry mass, WC = shoot water content, RWC = relative water content, ψ = water potential, EL = electrolyte leakage.



Specific ion toxicity.

A: chlorosis of stem base in the tidal treatments. The stem eventually breaks off;

B: chlorosis of leaf tips.

C: bottom-top chlorosis of leaves; arrows indicate some chlorotic lesions.

Fig. 9. Signs of ionic toxicity on *Limonium linifolium* following 8 weeks of drought and tidal conditions under different salinity treatments. Arrows indicate positions of chlorotic lesions.

the number of new branches and change in shoot area, coupled with a weaker correlation between change in shoot area and change in height, and no correlation between change in shoot area and AGR (Fig. 4) suggest that branching is an adaptation for drought hardiness in the species. Few studies exist for comparison of this phenomenon, but our findings on the decrease in new branching with increasing salinity under both tidal and drought conditions are similar to those of Munns and Tester (2008) who showed that under salinity stress “lateral buds develop more slowly or remain quiescent, so fewer branches or lateral shoots form”. However, they do not explain the relative increase in new branching in drought-treated as opposed to tidal-treated plants which we observed in this study. Our results thus show that increased branching under drought stress resulted in the observed change in shoot area; higher number of new branches in drought — compared to tidal-treated plants suggests that this adaptation is more enhanced under drought conditions, and in both inundation states salinity increase negatively influences branching in the species. This phenomenon (increased branching under drought stress) requires further investigation in this and other similar species.

Results further show that tidal saline (18–45 psu) conditions which would occur in its current range under predicted climate change conditions would limit the growth of the species. This is possibly through disruption of water balance and membrane damage. Although shoot relative water content and succulence did not vary significantly between tidal- and drought-treated plants, quantitatively, the higher shoot water content in the drought-treated plants showed that the plant possesses better adaptations for water uptake or water conservation in drained conditions. In both inundation states, shoot water potential was significantly higher than soil water potential. This was necessary for growth as shown by the positive correlations between growth parameters and shoot water potential ($r = 0.788$, 0.788 and 0.871 with fresh mass, dry mass and RGR, respectively, $p < 0.005$). Species tolerant of drought have the capacity to reduce tissue water potential, as well as other physiological and morphological mechanisms (Morgan, 1984 and references therein; Bohnert et al., 1995; Harb et al., 2010).

Physiologically, the ability to take up water under drought and saline conditions also depends in part, on the ability to accumulate compatible solutes. High proline concentrations in the species at both inundation states aid in lowering the shoot water potential and scavenging for reactive oxygen species, consistent with its function as a compatible solute (Ashraf and Foolad, 2007; Lee et al., 2007; Chen and Murata, 2008; Naidoo et al., 2008; Chen and Jiang, 2010). That this accumulation did not vary with salinity increases is consistent with the finding that proline is a natural osmolyte in the genus *Limonium* (Hanson et al., 1994). Oxalic acid also accumulated at significant concentrations in all treatments and it has been shown to function as an osmolyte in other halophytes (Yang et al., 2007; Ma et al., 2011). Beside the osmolytes, salt secretion appears to play a crucial role in tolerance to salinity and drought in the species. The higher salt secreted per gramme shoot mass (and not with increase in shoot area) in plants of the high salinity drought treatments showed that the rate of salt secretion increases as the sediment salinity increases. The strong positive correlations between treatment salinity and salt secreted per gramme shoot mass support the premise that salt secretion is an adaptation to salinity in this species under drought conditions. These findings are consistent with the presence of salt glands in the family Plumbaginaceae, whose members have been shown to possess excretory salt glands which are strikingly similar across genera (Salama et al., 1999). In *Limonium axillare*, *Limonium pruinosum* and *Limoniastrum monopetalum*, several different salt ions were excreted, but with a bias for NaCl (Salama et al., 1999). This may however depend on the effective concentration of different salts in the substrate. Results of the current experiment show that *L. linifolium* tolerates extreme drought by accumulating proline and oxalic acid. This lowers its water potential for uptake of soil water of high salinity and the excess salts are excreted through salt glands. This is an important adaptation for a plant that grows in a highly variable saline habitat.

The lack of significant differences in the concentration of chlorophyll pigments is further indication of the hardiness of the species to drained conditions, and even waterlogged conditions at salinity between 8 and 35 psu. Waterlogged conditions at lower salinity (0 psu) resulted in death of all species; submerged plants all died within two weeks of submergence. The results, through electrolyte leakage of the tidal-treated plants, show that susceptibility to submergence and to waterlogged conditions in general especially at low salinity is in part, through membrane damage. This is consistent with other findings in which electrolyte leakage has been negatively correlated with growth of the species (Agarie et al., 1998; Bajji et al., 2001; Tabot and Adams, 2013).

The results supported both hypotheses, and provide an understanding not only of the plant's responses, but also of how salt marsh habitats could respond to climate change conditions. From Fig. 1 it is evident that the species is principally distributed in a few coastal estuaries of South Africa; these coastal areas are prone to climate change effects. Three predicted conditions could occur in salt marshes in which this species occurs, namely; increased tidal intrusion through sea level rise, increased submergence when high water levels couple with closed mouth conditions in TOCEs, and increased drought in some salt marshes due to low rainfall and low water levels, all with salinity variations. The results show that the salinity effect in this species is secondary to that of the water state, with significant implications. Increased waterlogged conditions (water potential > -0.1 to -2.1 MPa; Redox < -130 to -208 mV) would compromise growth of the species, and prolonged submergence could lead to temporary local extinction in such salt marshes. Through proline and oxalic acid accumulation, salt secretion and increased branching, the species maintains low tissue water potential for water and nutrient uptake, and sufficient photosynthetic area to tolerate extreme drought conditions of up to two months. Where management interventions are possible, for example in TOCEs, complete submergence for more than two weeks should be avoided as a priority. Further studies on the reproductive resilience of the species and its seed bank would shed more light on long term resilience of salt marshes in which the species occur, if the climate changes as predicted.

Submission declaration

The authors declare that the work submitted is original, has not been published in any form, and there are no conflicts of interest. JB designed the experiment, PT conducted the lab work and analyses, and both authors analysed the data, wrote and approved the manuscript.

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