

# Differential growth of *Spartina densiflora* populations under saline flooding is related to adventitious root formation and innate root ion regulation

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**Abstract.** Global change anticipates scenarios of sea level rise that would provoke long lasting floods, especially in lowland areas of salt marshes. Our aim was to evaluate the morpho-physiological adjustment ability to deal with continuous saline flooding of *Spartina densiflora* Brogn. plants from lowlands and uplands along a subtle topographical gradient (0.2 m differential altitude). Plants from both origins were subjected to continuous saline flooding (300 mM NaCl) for 35 days. Responses associated to adventitious rooting, aerenchyma formation, concentration of Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> in roots and shoots tissues, tillering and growth were assessed. Root responses differentiated populations given that lowland plants showed higher ability for adventitious root formation and innate superior root ion regulation than upland plants. High constitutive K<sup>+</sup> concentration plus high Na<sup>+</sup> exclusion in root tissues led to significant low values of Na<sup>+</sup>:K<sup>+</sup> ratios in lowland plants. Better root functioning was, in turn, related with more consistent shoot performance as lowland plants maintained plant tiller number and shoot relative growth rate unaltered while upland plants decreased both parameters by 35 and 18%, respectively, when in saline flooding. The superior performance of lowland plants indicates that locally adapted populations can be promoted in salt marsh habitats with subtle differences at topographic level.

**Additional keywords:** aerenchyma, leaf gas exchange, Na<sup>+</sup>:K<sup>+</sup> ratio, salt marsh, tillering.

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## Introduction

Coastal salt marshes are grassland ecosystems located in intertidal areas that provide valuable ecosystem services such as coastal protection, erosion control, water purification and carbon sequestration (Barbier *et al.* 2011; Brisson *et al.* 2014). As a consequence of their location, salt marshes are influenced by tidal movements (sea water) making of saline flooding a major abiotic stress for plants growing in these environments (Pennings *et al.* 2005). Plant species in salt marshes are often distributed along subtle topographic gradients where, as elevation increases, the frequency and duration of saline flooding decreases (Pennings *et al.* 2005; Di Bella *et al.* 2014a). A future scenario of sea water level rise as a consequence of global warming (Kirwan *et al.*

2010) is expected to provoke long-lasting floods in the low areas of salt marshes. In this paper, we examined the effects of continuous saline flooding, emulating such environmental perspectives and their effect on morpho-physiological responses of *Spartina densiflora* Brogn. plants from lowland and upland areas of a salt marsh.

*Spartina densiflora* is a dominant species along the entire topographical gradient of many salt marshes in South America (where it is native) and also in salt marshes in Southern Europe, North America and North Africa as an invader species (Nieva *et al.* 2003; Bortolus 2006; Mateos-Naranjo *et al.* 2008; Castillo *et al.* 2014). Castillo *et al.* (2014) attributed its invasiveness to a high degree of phenotypic plasticity given that all differences

observed for foliar traits in the field (e.g. leaf size and rolling ability, chlorophyll and nitrogen concentration) among plants collected from California (USA) to British Columbia (Canada) disappeared when cultivated in a common environmental garden. In another study, Álvarez *et al.* (2010) pointed out the existence of different ecotypes for leaf anatomical traits and specific activity of phosphoenolpyruvate carboxylase (PEPC), in plants collected from sites located at lower and higher latitudes – Spain, in the Iberian Peninsula, South Brazil, Centre of Argentine and Patagonia – when grown in a common greenhouse environment. Both possibilities mentioned above are not mutually exclusive, as ecotypes might in turn have different degrees of phenotypic plasticity (Loreti and Oosterheld 1996). Despite the deeper insight contributed by these studies into the knowledge of traits enabling this species to colonise salt marshes, they have focussed their attention on comparing plants collected across ample latitudinal gradients, which widens heterogeneity as for temperature, photoperiod, sediment type and tidal regime. As an alternative, in this paper we present a detailed comparison of physiological responses to saline flooding of 2-year greenhouse-acclimated plants collected from the same salt marsh where the environmental heterogeneity is local, and it is associated with differences in tidal flooding frequency along a subtle gradient of elevation.

Saline flooding imposes severe restrictions for plant growth as the presence of high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  – potentially toxic – is added to the lack of oxygen at root zone (Barrett-Lennard and Shabala 2013). Plant growth depends on the co-ordinated functioning of shoots and roots. Although roots experience stresses directly, shoots are also affected as a result of impairment in root functioning. From the root perspective, when flooding is prolonged (>2 weeks), formation of new adventitious roots of high porosity helps plants to continue with nutrient and water uptake, replacing, to some extent, the functioning of the pre-existing root system (Colmer and Voesenek 2009). So, new adventitious rooting and root aerenchyma (a surrogate for porosity) are important traits to evaluate tissue aeration potential at the plant level. Root aeration in saline environments is also essential because it enables sustained ATP production that is needed for a proper functioning of channels and carriers, thus regulating the entry and compartmentalisation of  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  into the plant (Barrett-Lennard and Shabala 2013; Zeng *et al.* 2014). Therefore, ion concentration in tissues also helps to understand the ion regulation by plants in a stressful environment. A better ion regulation is generally associated to a better plant growth (see Striker *et al.* 2015; for *Melilotus siculus* accessions). For *S. densiflora*, there is no available record of adventitious root formation and ion regulation by roots. From the shoot perspective, Castillo and Figueroa (2009) have shown that *S. densiflora* plants subjected to increasing frequency of tidal flooding (i.e. lower positions on the marsh) decreased their relative growth rate in line with progressive reductions in the number of live shoots per plant. At leaf level, Nieva *et al.* (2003) showed a decrease in net photosynthesis rate when subjecting *S. densiflora* to anoxia (especially in tidal populations) or extreme salinity (during summer in non-tidal populations).

In the salt marsh located in the Samborombón Bay (Argentina), *S. densiflora* is a key dominant species along the

entire topographical gradient from uplands to lowlands (Di Bella *et al.* 2014a). In this paper we examined whether *S. densiflora* plants from the uplands and lowlands on this salt marsh are able to adjust their morphology, physiology, and growth when exposed to a stressful scenario provoked by continuous saline flooding (300 mM NaCl) for 35 days. We hypothesised that plants from uplands and lowlands are locally adapted populations ('biotypes' *sensu* Lowry 2012) with different acclimation capacity to saline flooding. In this respect, we expected that growth of plants from lowlands would be less affected by saline flooding than that of plants from the uplands as a result of a better capacity for root aeration (increased adventitious rooting with high aerenchyma proportion), superior ion regulation in tissues and less decline in leaf physiological activity. The imposition of continuous flooding helped us to simulate the raise in the sea water level expected due to global warming (Kirwan *et al.* 2010), which would provoke long lasting floods in the low areas of the salt marsh. In addition, a better understanding of the morpho-physiological adjustment of locally adapted populations of *S. densiflora* to this extreme event could also help to improve the conservation and restoration efforts in salt marshes.

## Materials and methods

### *Study site, plant material and growth conditions*

Plants of *Spartina densiflora* Brong. from upland and lowland topographic positions were collected from a salt marsh located within the National Park 'Campos del Tuyú' (56°50'W, 36°19'S) on the west margin of the Río de la Plata estuary (Argentina). This salt marsh occupies an extensive area of 96 101 ha, ~40% of the total bay area (Isacch *et al.* 2006), declared as a 'Ramsar site' since 2007 for being a wetland of international importance for conservation purposes (Ramsar Convention Secretariat 2011). This environment is characterised by a surface hydrological system with a subtle topographic gradient, and tidal fluctuations (Carol *et al.* 2008). For this reason, upland positions, located ~20 cm higher than lowland ones, experience sporadic flooding events (only few times per year), whereas lowland positions are flooded on a daily basis. Tidal water contains NaCl (Carol *et al.* 2008) with an average electrical conductivity of  $\sim 26 \pm 0.3 \text{ dS m}^{-1}$  (equivalent to  $\sim 260 \text{ mM NaCl}$ ; Di Bella *et al.* 2014b).

Fifty individual plants were collected from both the upland and lowland sites respectively. The choice of places to collect plants representative of each position was based on our previous characterisation of this marsh ecosystem (see floristic and soil description of uplands and lowlands in Di Bella *et al.* (2014a) and Di Bella *et al.* (2015)). In addition, to avoid sampling the same individual resulting from the rhizomatous growth habit of this species, collected plants were always at least 2 m away from each other. Afterwards, plants were transported to an experimental garden at the University of Buenos Aires (UBA), where they were vegetatively propagated three times during a period of 2 years in order to eliminate any influence from the original environment (as in work by Loreti and Oosterheld 1996). Later, plants were transferred to a greenhouse where they were grown for 2 months in 3 L plastic pots with substrate composed of washed sand and soil (1 : 1 v/v). Greenhouse mean minimum and maximum temperatures were  $17 \pm 2^\circ\text{C}$  and  $30 \pm 5^\circ\text{C}$

(respectively), and RH was  $69 \pm 10\%$ . To avoid nutrient deficiencies in plants, the substrate was fertilised with N, P and K (15% N, 15% P, 15% K + micronutrients – Triple 15, Ciudad Floral, Escobar, Argentina) with a dose of 16 g per pot.

#### *Experimental design and treatment application*

After the plants acclimation period in the greenhouse, 20 homogeneously-sized individuals of each site were selected (out of the 50 individuals collected) to perform a factorial experiment ( $2 \times 2$ ) following a completely randomised design with plants 'origin' as classification factor and 'saline flooding' as treatment factor. Each factor had two levels: 'upland' and 'lowland' (origin), and 'control' and 'saline flooding' (treatment). The combination of origin  $\times$  treatment had 10 true replicates, conforming 40 experimental units. Saline flooding was imposed in two steps to prevent osmotic shock regardless of the fact that *S. densiflora* is a halophytic species. First, all pots with individual plants assigned to this treatment were placed in plastic containers ( $50 \times 80 \times 50$  cm) filled with non-aerated saline solution of 100 mM of NaCl (Biopack, Buenos Aires, Argentina). Two days later, NaCl was progressively added until reaching a final concentration of 300 mM in the saline solution. The water level of the saline solution was maintained 4 cm above the surface during all the experiment. The dissolved oxygen of saline water ranged between 1.0 and  $1.6 \text{ mg O}_2 \text{ L}^{-1}$  (equipment LT Lutron DO-5510, Taipei, Taiwan), indicating the hypoxic environment caused by the treatment. Saline solution was changed twice during the experiment (days 10 and 20) and saline concentration was periodically checked by using a portable water conductivity meter (HI993310, HANNA, Woonsocket, RI, USA). Control plants were placed in similar containers without water and daily watered to field capacity with tap water. Plastic containers were weekly rotated within the greenhouse.

#### *Plant biomass and tillering responses*

Plant harvests were carried out at the beginning of treatments (day 0) in randomly chosen individuals ( $n = 10$ ), and at the end of the experiment (day 35,  $n = 10$ ). Plant biomass was separated into shoots, rhizomes and roots. Shoots of plants (i.e. tillers) were dissected into leaf sheaths and leaf blades. Leaf blades were also classified into young and mature depending on whether they presented unexpanded leaves or fully expanded leaves, respectively. Roots were separated in new adventitious roots (white spongy roots developed from tillers' basis) and pre-existing roots. All harvested material was weighed after oven drying at  $80^\circ\text{C}$  for 72 h.

At the beginning (day 1), middle (day 16) and end (day 35) of the experiment, three easily distinguishable categories of tillers were counted to determine the effect of saline flooding on tillering dynamics over time. Categories of tillers were 'mature tillers' (with at least three fully expanded leaves), 'young tillers' (with one or two fully expanded leaves) and 'daughter tillers' (typically with one or two growing leaves per tiller without any fully expanded leaf).

#### *Aerenchyma measurements*

At the end of the experiment, 4–8 cm long segments of adventitious roots (from 2.5 cm behind the tip) were carefully

washed and preserved in formalin acetic acid alcohol (FAA) until needed ( $n = 10$ ). Segments of roots at 2.5 cm from the tip were dehydrated in a series of increasing ethanol concentrations and embedded in paraffin wax. Sections 10–12  $\mu\text{m}$  thick were cut, stained with safranin and Fast Green and mounted in Canada balsam. Proportion of aerenchyma (%) was determined from digitalised images captured with an optical microscope (Zeiss Axioplan, Zeiss, Oberkochen, Germany) connected to a digital camera (Canon Power Shot G9; Canon, Melville, NY, USA). Images were processed with ImageJ ver. 1.47 free software (USA National Institutes of Health, Bethesda, MA, USA). The cross-section analysis of aerenchyma was used to infer on the potential of root aeration instead of measuring root porosity by pycnometric techniques (see also Striker *et al.* 2014). The large quantity of root tissue required for the latter method prevented the additional measurement of root porosity as we used most of those tissues to quantify tissue ion concentrations (see below). In this respect, Visser and Bögemann (2003) showed that for ranges of porosities between 10 and 30%, values that overlapped with our measurements of aerenchyma, the correlation between these two methods is high.

#### *Physiological measurements*

Stomatal conductance ( $g_s$ ), transpiration rate ( $E$ ) and net photosynthesis rate ( $A$ ) were measured in fully expanded leaf blades using a portable infrared gas analyser system (IRGA) model Li-Cor 6400 (Li-Cor Inc., Lincoln, NE, USA) under saturating light (i.e.  $2000 \mu\text{mol m}^{-2} \text{ s}^{-1}$  PPFD provided by the 6400-40 leaf chamber fluorometer, using a mix of 80% red and 20% blue light). Air flow,  $\text{CO}_2$  concentration in the reference chamber and block temperature were automatically controlled by the equipment at  $300 \mu\text{mol s}^{-1}$ ,  $400 \mu\text{mol mol}^{-1}$  (ppm) and  $24^\circ\text{C}$  respectively. At the same time, air temperature and relative humidity of the greenhouse were monitored (Temperature and RH Probe HUMICAP H, Vaisala, Finland). Both parameters were used to calculate air vapour pressure deficit ( $\text{VPD}_{\text{air}}$ ) as the difference between the saturated vapour pressure (Clausius-Clapeyron equation) and the actual air vapour pressure. The  $\text{VPD}_{\text{air}}$  varied between 1.93 and 3.36 kPa on the measurement days (mean of 2.76 kPa).

#### *Tissue ion concentrations*

Dried material of young and mature leaf blades and pre-existing roots and new adventitious roots of the final harvest were ground to fine powder. An aliquot of 10 mg was used to estimate the concentration of  $\text{Na}^+$  and  $\text{K}^+$  by standard flame photometry. Ions were extracted in 1 mL of HCl (0.1 N) at  $60^\circ\text{C}$  for 1 h. The extract was read in a ZF250 flame photometer (Zeltec, Buenos Aires, Argentina). For quantification, a calibration curve was constructed using a NaCl-KCl solution. To determine the concentration of  $\text{Cl}^-$ , a sample aliquot of 25 mg was taken using a standard colourimetric method with mercury thiocyanate. The sample was digested with 2 mL of  $\text{H}_2\text{O}_2/\text{HNO}_3$ /isoamyl alcohol solution (50 : 50 : 1) at room temperature ( $\sim 25^\circ\text{C}$ ) during 15 min and rinsed to 10 mL with milliQ water. A 320  $\mu\text{L}$  supernatant aliquot was added to 1 mL colour solution. The solution was made of 15 mL of mercury thiocyanate ( $4.17 \text{ g L}^{-1}$  methanol), 15 mL of  $(\text{NO}_3)_3\text{Fe}$  ( $202 \text{ g L}^{-1}$  water + 21 mL of  $\text{HNO}_3$ ), 50  $\mu\text{L}$

of 4% Brij35 and milliQ water up to 100 mL. The absorbance of samples was read at 450 nm on a Lambda 25 spectrophotometer (Perkin Elmer, Waltham, MA, USA), and quantified by a colourimetric calibration curve using KCl.

### Statistical analysis

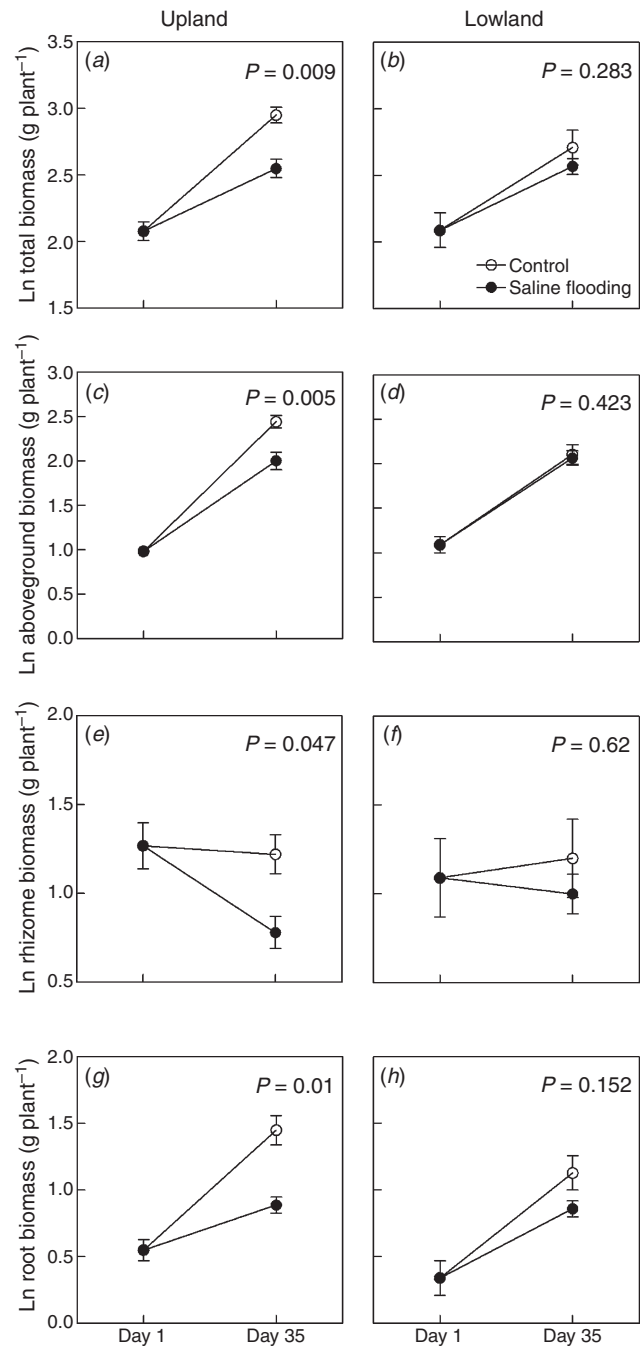
Plant biomass and its compartments (i.e. aboveground, root and rhizome) were ln-transformed to calculate the corresponding relative growth rates (RGR). RGR was calculated following the classical approach by Hunt (1982), which is based on the equation:  $RGR = (\ln(B_f) - \ln(B_i)) / (T_f - T_i)$ , where  $B_f$  and  $B_i$  are the average plant (or compartment) weights of the corresponding treatment at final and initial times (respectively), and  $T_f - T_i$  is the number of days between harvests (i.e. 35 days). Since biomass harvest is a destructive measure, initial biomass values were not paired with final biomass values, then, RGR values were calculated as the average of all possible combinations of values between initial and final harvests among plants of each origin  $\times$  treatment combination. Finally, calculated RGR values were compared between treatments from each origin by means of Student's *t*-test (d.o.f. = 9). Variations in number of tillers and physiological variables along the experiment were evaluated by repeated-measures (rmANOVA), considering 'origin' and 'treatment' as main effect and sampling dates as within-subject effects (Von Ende 1993). When interactions were significant, multiple comparisons among origin  $\times$  treatment combinations were performed using Bonferroni tests (Von Ende 1993). To examine the relationship between net photosynthesis and stomatal conductance, a quadratic equation was adjusted using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). New adventitious root biomass, aerenchyma proportion, and ion concentration of blades and roots were evaluated by two-way ANOVAs with 'origin' and 'treatment' as the main factors. When significant interactions were detected, subsequent l.s.d. Fisher tests were applied to determine the effect of treatments. Normality and homogeneity of variances were previously verified. Statistical analyses were performed using the package Statistica ver. 6.0 (Stat Soft, Tulsa, OK, USA).

## Results

### Plant growth and tillering as affected by saline flooding

RGR under saline flooding was lower than control in plants from the uplands but not in plants from the lowlands, which remained unaltered evidencing their higher tolerance to this stress (Fig. 1*a–h*). The impaired growth of plants from the uplands led to a 13.5% lower final biomass with respect to controls (Fig. 1*a*) as a result of a lower biomass for all compartments: shoots (18%), rhizomes (36%) and roots (38%) (Fig. 1*c, e, g*). Interestingly, these less tolerant plants also showed 38% lower final rhizome biomass than at the beginning of the experiment (compare day 1 and day 35 in Fig. 1*e*).

The dynamics of tiller numbers was consistent with biomass responses. Saline flooding determined a reduction in the number of tillers only in plants from the uplands but not in plants from the lowlands, when compared with controls (Fig. 2). In plants from the uplands this response was observed in mature and young tiller categories and it was marginally significant for daughter tillers (rmANOVA, treatment  $\times$  origin effect, Table 1). At the end of the

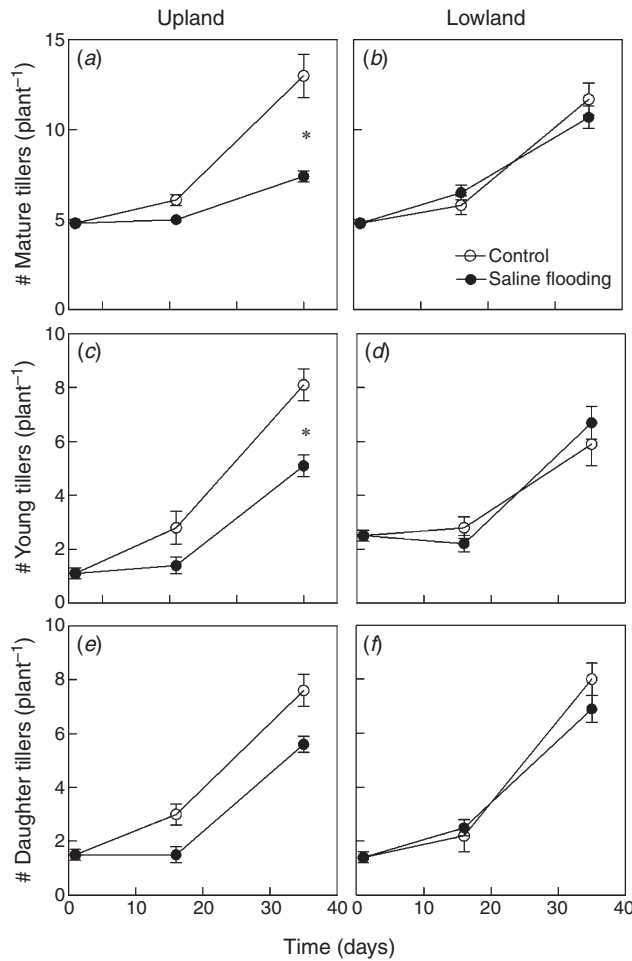


**Fig. 1.** Total (*a, b*), aboveground (*c, d*), rhizome (*e, f*) and root (*g, h*) biomasses of *Spartina densiflora* plants from upland (*a, c, e, g*) and lowland (*b, d, f, h*) sites subjected to control (white circles) and saline flooding (black circles) treatments for 35 days. Values are means  $\pm$  s.e. ( $n = 10$ ). Slopes represent average relative growth rates (RGR) of each plant compartment during the experiment. *P*-values of Student's *t*-test comparisons of RGR between treatments are informed within each figure part.

experiment, upland plants subjected to saline flooding registered 43.1, 37.0 and 26.3% lower number of tillers in the mature, young and daughter categories (respectively) in comparison to control plants (Fig. 2*a, c, e*). In contrast, plants from the lowlands that



were subjected to saline flooding retained the same number of tillers in either category (averaging 11, 6 and 7 for mature, young and daughter tillers) as those that grew under control conditions



**Fig. 2.** Mature (a, b), young (c, d) and daughter (e, f) tillers evolution of *Spartina densiflora* plants from upland (a, c, e) and lowland (b, d, f) sites subjected to control (white circles) and saline flooding (black circles) treatments for 35 days. Values are means  $\pm$  s.e. ( $n = 10$ ). Asterisks indicate significant differences between treatments based on Bonferroni tests ( $P < 0.05$ ) when significant origin  $\times$  treatment interactions were detected.

(Fig. 2b, d, f). Differences along the experiment were observed for all tiller categories (time effect; Table 1).

*Adventitious roots and aerenchyma formation*

Plants from the uplands failed to increase the mass of new adventitious roots as an adaptive response to flood-induced hypoxia, while plants from the lowlands increased the mass of new adventitious roots by  $\sim 64\%$  with respect to their controls (treatment  $\times$  origin interaction,  $P = 0.006$ ; Table 2). Furthermore, in plants from the uplands there was a trend towards a reduction of new adventitious root biomass when flooded in saline water (0.30 g per plant under saline flooding vs 0.36 g per plant under control conditions; Table 2).

High values of constitutive aerenchyma in new adventitious roots were found in both populations (19.9 and 29.2% for lowland and upland, respectively, without significant origin effect; Table 2). Additionally, when subjected to saline flooding, plants from both origins responded similarly by increasing the proportion of root aerenchyma – on average – by 80% with respect to their controls (no significant interaction treatment  $\times$  origin; Table 2, see Fig. S1, available as Supplementary Material to this paper). Thereby, plants from both origins reached absolute final values of root aerenchyma higher than 40%, when flooded in saline water (Table 2), which suggests no limitations in the potential for aeration of individual roots in plants of either of these two origins.

*Plant physiological responses*

Stomatal conductance ( $g_s$ ), transpiration rate ( $E$ ) and net photosynthesis rate ( $A$ ) were similarly reduced by saline flooding in plants from uplands and lowlands (no treatment  $\times$  origin interaction in all parameters, Table 1; Fig. 3a–f). Variations in average values of  $g_s$  and  $E$  during the experiment under control conditions were correlated with  $VPD_{air}$  measured in the greenhouse (Fig. 3g;  $r^2 = 0.88$  for  $g_s$  and  $r^2 = 0.95$  for  $E$ , data not shown). We noted that after an initial shock observed during the first week, all physiological variables were reduced by saline flooding (rmANOVA, treatment effect, Table 1). Plants from both origins were able to similarly maintain their  $g_s$ ,  $E$  and  $A$  in values ranging from 48 to 55% with respect to their controls, without progressive reductions along time, all of which is typical of a saline-hypoxic tolerant species (no significant interaction treatment  $\times$  origin  $\times$  time; Table 1;

**Table 1.** *P*-values and *F*-values for the rmANOVA factors (origin, treatment, O  $\times$  Tr, Time, T  $\times$  O, T  $\times$  Tr and T  $\times$  O  $\times$  Tr) for tillering (mature, young and daughter tiller) and physiological ( $g_s$ ,  $E$  and  $A$ ) variables of *Spartina densiflora* plants belonging to upland and lowland sites subjected to control and saline flooding treatments by 35 days

Main effects and interactions were considered significant as indicated:\*,  $P < 0.05$

Variable	Origin (O)		Treatment (Tr)		O $\times$ Tr		Time (T)		T $\times$ O		T $\times$ Tr		T $\times$ O $\times$ Tr	
	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>
Mature tiller (number plant <sup>-1</sup> )	0.099	2.9	0.001*	13.3	0.002*	11.5	<0.001*	78.8	0.657	0.2	0.008*	8.1	0.219	1.6
Young tiller (number plant <sup>-1</sup> )	0.891	0.02	0.025*	5.5	0.005*	9.3	<0.001*	187.8	0.273	1.2	0.904	0.01	0.010*	7.6
Daughter tiller (number plant <sup>-1</sup> )	0.257	1.3	0.007*	8.4	0.122	2.5	<0.001*	269.1	0.164	2.0	0.164	2.0	0.500	0.5
$g_s$ (mmol m <sup>-2</sup> s <sup>-2</sup> )	0.017	7.1	<0.001*	113.6	0.224	1.6	<0.001*	23.0	0.287	1.3	0.563	0.6	0.962	0.03
$E$ (mmol m <sup>-2</sup> s <sup>-2</sup> )	0.067	3.9	<0.001*	106.7	0.500	0.5	<0.001*	21.0	0.335	1.1	0.648	0.4	0.987	0.01
$A$ (mmol m <sup>-2</sup> s <sup>-2</sup> )	0.016	7.3	<0.001*	150.0	0.378	0.8	<0.001*	54.3	0.227	1.5	0.984	0.02	0.747	0.3

**Table 2.** New adventitious root biomass and proportion of aerenchyma tissue of *Spartina densiflora* plants belonging to upland and lowland sites ('origin') subjected to control and saline flooding treatments by 35 days

Different letters indicate significant differences based on l.s.d. Fisher tests when origin  $\times$  flooding interaction was detected. Values are means  $\pm$  s.e. ( $n = 10$ ).  $F$ -values of the two-way ANOVAs are presented. Degrees of freedom for each source of variation were: 1 (Origin), 1 (Treatment), 1 ( $O \times T$ ) and 36 (error). Significant differences: \*\*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.001$ ; ns,  $P > 0.05$

New adventitious roots	Upland		Lowland		Origin	$F$ -values	
	Control	Saline flooding	Control	Saline flooding		Treatment	Interaction
Biomass per plant (g)	0.36 $\pm$ 0.03ab	0.30 $\pm$ 0.03bc	0.25 $\pm$ 0.03c	0.41 $\pm$ 0.05a	0.00 ns	1.76 ns	8.68 **
Aerenchyma (%)	29.18 $\pm$ 6.22	45.13 $\pm$ 4.36	19.87 $\pm$ 4.02	43.59 $\pm$ 3.44	0.83 ns	17.18 ***	0.43 ns

Fig. 3a–f). In addition, a strong correlation between  $A$  and  $g_s$  ( $r^2 = 0.84$ ,  $P < 0.05$ ) indicated that stomatal resistance provided significant control over gas exchange (Fig. S2).

#### Ion concentrations in roots and leaves

The concentration of  $Na^+$  in pre-existing and new adventitious root tissues when plants grew in saline water was higher in upland than lowland plants. In pre-existing roots,  $Na^+$  concentration increased from  $0.12 \pm 0.01 \text{ mg g}^{-1}$  to  $1.22 \pm 0.06 \text{ mg g}^{-1}$  and from  $0.14 \pm 0.01 \text{ mg g}^{-1}$  to  $1.10 \pm 0.05 \text{ mg g}^{-1}$  in upland and lowland plants respectively (Table 3). Similarly, in new adventitious roots,  $Na^+$  concentration increased from  $0.14 \pm 0.01 \text{ mg g}^{-1}$  to  $1.27 \pm 0.08 \text{ mg g}^{-1}$  and from  $0.18 \pm 0.01 \text{ mg g}^{-1}$  to  $1.16 \pm 0.03 \text{ mg g}^{-1}$  in upland and lowland plants respectively (Table 3). The concentration of  $K^+$  in root tissues was constitutively higher in plants of the lowlands than in those of the uplands (significant 'origin' effect in Table 3). Pre-existing and new adventitious roots of lowland plants innately had 31 and 16% higher  $K^+$  concentration than that of plants from the uplands (Table 3). As a consequence, plants from the lowlands registered 25 and 18% lower  $Na^+ : K^+$  ratio than that of the uplands for pre-existing roots and new adventitious ones (Fig. 4c, d). This provides evidence for their better innate ion regulation ability at root level, also in line with their high capacity for adventitious roots formation already commented (Table 2). The concentration of  $Cl^-$  in root tissues (pre-existing roots and new adventitious roots) presented a 3.9- to 4.6-fold increase with respect to their controls, irrespective of plants origin (Table 3).

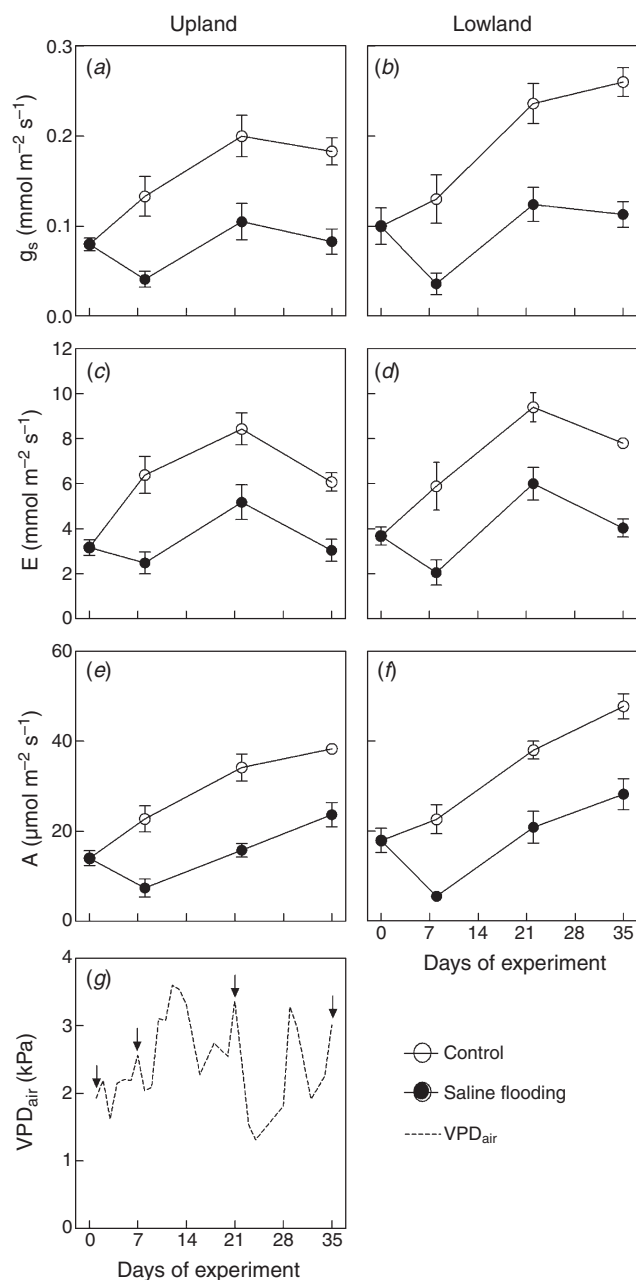
In young and mature leaf blades saline flooding evenly increased  $Cl^-$  and  $Na^+$ , and decreased  $K^+$  in plants from both origins (significant 'treatment' effect but no interaction treatment  $\times$  origin; Table 3). Young leaf blades presented a 1.8-fold and 6-fold increase in  $Cl^-$  and  $Na^+$  concentration, respectively, whereas  $K^+$  concentration decreased by 40%. In mature leaf blades,  $Cl^-$  and  $Na^+$  concentrations were 2.4-fold and 5-fold increased, respectively, and  $K^+$  concentration decreased by 45% (Table 3). These changes in ion concentrations led to higher  $Na^+ : K^+$  ratio, both in young and mature leaves, regardless of plants origin; although, as expected, young leaves had lower values for this ratio than those of mature leaves ( $Na^+ : K^+ = 1.28$  in young leaves vs  $Na^+ : K^+ = 1.66$  in mature leaves; Fig. 4a, b).

#### Discussion

This work is the first to show the presence of populations of *S. densiflora* locally adapted to saline flooding (biotypes *sensu*

Lowry 2012), inhabiting along a subtle topographical gradient within the same salt marsh in South America, where this species is native (Bortolus 2006). Despite the proximity between upland and lowland areas of the salt marsh and the well-known high plasticity of this species (Castillo *et al.* 2014; Di Bella *et al.* 2014b), plants from each origin exhibited clear differences in morphological shoot and physiological root adjustments when growing under continuous saline flooding, an expected stressful scenario given the rise in sea level resulting from the effects of climate change (Kirwan *et al.* 2010). This differential ability between populations to deal with the combined effects of hypoxia and high NaCl concentration was evidenced by the better performance of lowland plants in comparison with upland plants. The lowland population was able to develop more new adventitious roots during saline flooding, exhibit an innate better root ion regulation, and maintain unchanged plant tiller number in comparison to plants from the uplands. Together, these superior morpho-physiological adjustments to saline flooding led plants from the lowlands to sustain the RGR of shoots and roots unaltered when exposed to hypoxic saline stress at the root zone. From an ecological perspective, our identification of two populations of *S. densiflora* with different aptitude to deal with saline flooding in this salt marsh provides further evidence that even subtle differences in stress pressure are able to promote differentiation of biotypes. Similar results were also found in *Paspalum dilatatum*, where two populations inhabiting grassland ecosystems were identified according to the inundation frequency experienced, resulting from its location along a small gradient of elevation (see Mollard *et al.* 2010). In addition, our results show that relatively small differences in traits and/or responses to saline flooding (particularly in roots), appear to be sufficient to trigger this process of differentiation, although genotyping of plants from upland and lowland population is advised as a next step to gain insight in the genetic differences between these two biotypes.

Plants from the lowlands displayed a higher capacity for new adventitious rooting than those from uplands. Individuals from the lowlands increased the biomass of new adventitious roots by 64% when flooded in saline water whereas plants from the uplands showed the opposite trend towards a reduction in this compartment, both compared with their controls (Table 2). The generation of new adventitious roots under prolonged flooding conditions (saline or not) is crucial to continue with water and nutrient uptake (Colmer and Voesenek 2009). In addition, new adventitious roots are located in the upper layer of the soil (near the surface) where gas exchange (i.e. diffusion of oxygen, thereby  $O_2$  availability) is higher than in deeper soil positions



**Fig. 3.** Stomatal conductance ( $g_s$ ; *a, b*), transpiration rate ( $E$ ; *c, d*) and net photosynthesis rate ( $A$ ; *e, f*) of young leaves *Spartina densiflora* plants from upland (*a, c, e*) and lowland (*b, d, f*) sites subjected to control (white circles) and saline flooding (black circles) treatments for 35 days. Treatment effects were detected for all variables ( $P < 0.001$ ) based on Bonferroni tests without significant origin  $\times$  treatment interactions ( $P > 0.05$ ). In (*g*) it is shown the air vapour pressure deficit ( $VPD_{air}$ ) along the experiment where arrows indicate the days on which measurements were performed. Values are means  $\pm$  s.e. ( $n = 5$ ).

(Visser *et al.* 1996; Colmer and Voesenek 2009). So, better aeration can be expected for the root system of plants from the lowlands when compared with that from uplands due to the generation of a more profuse system of new adventitious roots. Importantly, these differential root mass responses between

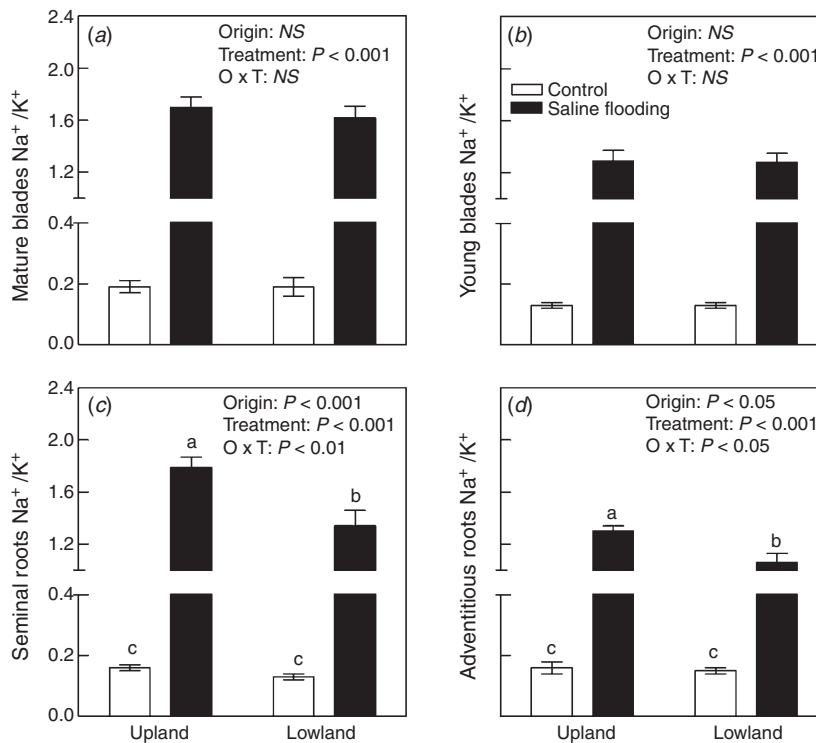
origins of plants cannot be attributed to differences in tissue porosity as root aerenchyma was high and similar (43–45%) between plants from both populations (see Table 2; Fig. S1). Thus, improved root aeration expected at plant level for lowland plants is attributable to the commented high new adventitious root mass, usually linked to high number of new adventitious roots (Visser *et al.* 1996), and not to differences in responsiveness for aerenchyma formation of individual roots. These ideas are concurring with the outstanding performance in root growth of lowland individuals – with unaffected root RGR by saline flooding, also consistent with the relative poor root growth of the upland plants when assessing the whole root system.

Plants from the lowlands also exhibited a better root ion regulation than those of the uplands under the combined effects of salinity and flooding in terms of  $Na^+$  exclusion, innately high  $K^+$  concentration in tissues and thereby, a lower  $Na^+ : K^+$  ratio. This better root ion regulation by plants from the lowlands was true for both pre-existing roots and new adventitious roots types (Table 3). Two non-exclusive potential mechanisms might help to explain a better ion regulation under hypoxic-saline growing conditions. One is to have innately superior ability to regulate ion concentrations in tissues under saline conditions irrespective of the level of root-aeration (see Barrett-Lennard 2003; Striker *et al.* 2015), and the other is possessing physiological adaptations which improve root aeration to sustain energy (ATP) production for ion regulation and growth (i.e. aerenchyma development and/or induction of a barrier to radial oxygen loss; Colmer 2003; Grimoldi *et al.* 2005; Barrett-Lennard and Shabala 2013). Our results from work with *S. densiflora* support the notion of an innate better ability to regulate ions by the lowland population, rather than differences in internal aeration of individual roots between populations, as aerenchyma was similar in roots of uplands and lowlands plants. In both cases final aerenchyma values were sufficiently high (>43%) to assume non-limiting oxygen diffusion along roots through the aerenchymatic pathway (Colmer 2003; Colmer and Voesenek 2009). Similarly, Striker *et al.* (2015) demonstrated that differences in growth under hypoxic-saline conditions in fifteen *Melilotus siculus* accessions (glycophyte legume) were related to ability of accessions to regulate ions (higher  $Na^+$  exclusion and  $K^+$  incorporation, thereby low  $Na^+ : K^+$ ) and not to variations in root porosity, which was high in all accessions (18–25%). It is noticeable that, unlike those registered in leaves, the concentrations of  $K^+$  in new adventitious root tissues slightly decrease by saline flooding, but it was always higher in the more tolerant lowland population ('origin' effect in Table 3). This concurs with data highlighted by Teakle *et al.* (2013), who showed for the first time the importance of  $K^+$  retention in roots to explain the high tolerance to combined anoxia and salinity in the halophyte *Puccinellia ciliata*. Similar results were observed for a closely related species like *Spartina alterniflora*, which displayed a strong ability to keep  $K^+$  concentration in tissues, even up to 550 mM NaCl in hypoxic conditions (see fig. 3 in work by Barrett-Lennard and Shabala 2013). It is possible that, as it occurred in *P. ciliata* and *S. alterniflora*, the innate high  $K^+$  concentration in roots of *S. densiflora* plants from the lowlands provided an advantage over plants from the uplands, helping to maintain more

**Table 3. Ion concentrations ( $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ) in young and mature leaf blades, pre-existing roots and new adventitious roots of *Spartina densiflora* plants belonging to upland and lowland sites ('origin') subjected to control and saline flooding treatments for 35 days**

Different letters indicate significant differences based on l.s.d. Fisher tests when origin  $\times$  flooding interaction was detected. Values are means  $\pm$  s.e. ( $n = 10$ ).  $F$ -values of the two-way ANOVAs are presented. Degrees of freedom for each source of variation were: 1 (Origin), 1 (Treatment), 1 ( $O \times T$ ) and 36 (error). Significant differences: \*,  $P < 0.1$ ; \*\*,  $P < 0.05$ ; \*\*\*,  $P < 0.01$ ; \*\*\*\*,  $P < 0.001$ ; ns,  $P > 0.05$

	Upland		Lowland		Origin	F-values		
	Control	Saline flooding	Control	Saline flooding		Treatment	Interaction	
<i>Young leaves</i>								
$\text{Cl}^-$ ( $\text{mg g}^{-1}$ )	5.80 $\pm$ 0.52	9.89 $\pm$ 0.95	6.04 $\pm$ 0.54	11.91 $\pm$ 1.37	1.5ns	29.7***	0.90ns	
$\text{Na}^+$ ( $\text{mg g}^{-1}$ )	0.19 $\pm$ 0.01	1.07 $\pm$ 0.06	0.18 $\pm$ 0.01	1.04 $\pm$ 0.06	0.3ns	412.6***	0.07ns	
$\text{K}^+$ ( $\text{mg g}^{-1}$ )	1.43 $\pm$ 0.03	0.87 $\pm$ 0.02	1.39 $\pm$ 0.03	0.81 $\pm$ 0.03	3.2*	461.9***	0.04ns	
<i>Mature leaves</i>								
$\text{Cl}^-$ ( $\text{mg g}^{-1}$ )	6.26 $\pm$ 0.35	13.76 $\pm$ 0.95	5.51 $\pm$ 0.59	14.75 $\pm$ 1.14	0.0ns	101.7***	1.10ns	
$\text{Na}^+$ ( $\text{mg g}^{-1}$ )	0.28 $\pm$ 0.02	1.4 $\pm$ 0.07	0.28 $\pm$ 0.03	1.38 $\pm$ 0.06	0.1ns	511.9***	0.09ns	
$\text{K}^+$ ( $\text{mg g}^{-1}$ )	1.51 $\pm$ 0.06	0.81 $\pm$ 0.02	1.51 $\pm$ 0.05	0.84 $\pm$ 0.02	0.1ns	268.4***	0.20ns	
<i>Pre-existing roots</i>								
$\text{Cl}^-$ ( $\text{mg g}^{-1}$ )	3.84 $\pm$ 0.48	17.82 $\pm$ 1.02	4.64 $\pm$ 0.32	18.20 $\pm$ 1.32	0.4ns	247.9***	0.05ns	
$\text{Na}^+$ ( $\text{mg g}^{-1}$ )	0.12 $\pm$ 0.01c	1.22 $\pm$ 0.06a	0.14 $\pm$ 0.01c	1.10 $\pm$ 0.05b	1.5ns	734.5***	3.40*	
$\text{K}^+$ ( $\text{mg g}^{-1}$ )	0.70 $\pm$ 0.07	0.69 $\pm$ 0.03	1.00 $\pm$ 0.07	0.82 $\pm$ 0.07	11.3****	2.4*	1.70ns	
<i>New adventitious roots</i>								
$\text{Cl}^-$ ( $\text{mg g}^{-1}$ )	4.81 $\pm$ 0.41	21.93 $\pm$ 1.54	5.18 $\pm$ 0.43	22.35 $\pm$ 1.01	0.1ns	300.7***	0.00ns	
$\text{Na}^+$ ( $\text{mg g}^{-1}$ )	0.14 $\pm$ 0.01b	1.27 $\pm$ 0.08a	0.18 $\pm$ 0.01b	1.16 $\pm$ 0.03a	0.7ns	597.3***	3.20*	
$\text{K}^+$ ( $\text{mg g}^{-1}$ )	0.93 $\pm$ 0.10	0.94 $\pm$ 0.05	1.16 $\pm$ 0.09	1.01 $\pm$ 0.06	3.7*	0.7ns	0.90ns	



**Fig. 4.**  $\text{Na}^+/\text{K}^+$  ratio in mature (a) and young (b) leaf blades, pre-existing roots (c) and new adventitious (d) roots of *Spartina densiflora* plants from upland and lowland sites subjected to control (white bars) and saline flooding (black bars) treatments for 35 days. Values are means  $\pm$  s.e. ( $n = 10$ ).  $P$ -values of ANOVA analyses are shown in the graphs. Different letters indicate significant differences between treatments when significant origin  $\times$  treatment interactions were detected based on l.s.d. Fisher tests.



favourable (lower)  $\text{Na}^+ : \text{K}^+$  ratios in tissues to sustain growth. In this respect, we hypothesise that roots of plants of both populations might differ in the specificity of  $\text{K}^+$  uptake and transport – high specificity and/or constitutive ability to uptake  $\text{K}^+$  in roots of plants of lowland population – as demonstrated for barley cultivars differing in waterlogging (WL) tolerance (WL intolerant cv. Naso Nijo vs WL tolerant variety TX9425) by Pang *et al.* (2006). Nevertheless, such idea deserves further experimental investigation, likely using microelectrode ion flux measurements (MIFE) and membrane potential measurements on saline-hypoxic roots of our *S. densiflora* populations (see also Zeng *et al.* 2014).

The co-ordination between root and shoot functioning as sources and sinks of water/mineral nutrients and photo-assimilates, respectively, allows an adequate balance of resources to support a continued plant growth (Arru *et al.* 2013). In favour of this coordinated/balanced functioning, we observed that the better root functioning of the plants from the lowlands was also associated with a better shoot functioning in terms of unchanged aboveground biomass accumulation and tiller number per plant when in saline flooding conditions. In relation with rhizomes, which function as storage organs, saline flooding decreased their biomass in upland plants but not in lowland plants (compare Fig. 1e with Fig. 1f). This supports the greater tolerance of lowland population as more sensitive individuals (or biotypes) often decrease rhizome biomass to a greater extent than tolerant individuals (or biotypes), and although the greater the stress, the greater the biomass decrease (see Striker *et al.* 2008, 2011 for flooding; Hellings and Gallagher 1992 for salinity; and Kavanová and Gloser 2005 for defoliation). To illustrate, it was observed in work by Striker *et al.* (2008) that the grass *P. dilatatum* (highly flooding tolerant) did not reduce rhizome biomass under 15 days of flooding whereas the legume *Lotus tenuis* (less tolerant than the grass) reduced its crown biomass (analogous compartment to grass rhizomes) by 30%. Similarly, the relatively poor performance of the root system of plants from the uplands was in line with a clear decline not only of root biomass but also of plant tiller number. In such plants, the most affected tillers were the larger-sized and with high leaf number belonging to the category of mature tillers (as also mentioned by Castillo and Figueroa 2009; and by Striker *et al.* 2008 for *P. dilatatum*; see Fig. 2a) – putatively with the major contribution to carbon fixation at plant level, which also concurs with the lower aerial biomass of plants of the upland population (Fig. 1c). Nevertheless, we note that neither physiological activity at leaf level (Fig. 3) nor ion concentration in leaves (Table 3) were helpful for discriminating differences between populations. In this regard, the lack of differences in physiological behaviour matched with the similar  $\text{Cl}^-$  and  $\text{Na}^+$  concentration in leaves observed between populations after 35 days of saline flooding (Table 3). This might be related with the fact that, being *S. densiflora* an halophyte species with salt glands in leaves (see fig. 4 in work by Maricle *et al.* 2009), the extra  $\text{Cl}^-$  and  $\text{Na}^+$  delivered to the shoots could have been extruded, in contrast to expected for glycophytes (see review by Colmer and Flowers 2008). However, to be conclusive in this aspect, further studies on ion extrusion in these *S. densiflora* populations should be conducted.

## Conclusions

This work incorporates new information to the well-known high phenotypic plasticity of *S. densiflora* demonstrating the existence of locally adapted populations (biotypes *sensu* Lowry 2012) to deal with saline flooding by assessing plants from lowland and upland sites within the same salt marsh. In particular, this study identified crucial traits explaining the outstanding ability of the lowland population to face saline flooding. Root responses clearly differentiated populations as lowland plants exhibited a higher ability for root adventitious formation and a better capacity in the physiological regulation of ions in root tissues than those from uplands. The high innate  $\text{K}^+$  concentration in root tissues along with a slight high capacity for  $\text{Na}^+$  exclusion allowed the lowland plants to sustain significant low values of  $\text{Na}^+ : \text{K}^+$  ratios. In addition, the better root functioning was transmitted to better shoot responses by lowland plants, which were able to maintain the number of tillers per plant and their RGR unaltered.

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