Saline tidal flooding effects on *Spartina densiflora* plants from different positions of the salt marsh. Diversities and similarities on growth, anatomical and physiological responses

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ABSTRACT

Spartina densiflora is a halophytic grass present in many salt marsh ecosystems where it dominates throughout topographical stress-gradients. This work aimed at studying diversities and similarities in ecophysiological responses of *S. densiflora* plants from two contrasting positions in the salt marsh. We simulated a natural tide by exposing plants of *S. densiflora* from upland and lowland sites of a salt marsh to saline intermittent flooding (9 h day^{-1}) during 60 days. Responses in plant growth, biomass allocation, anatomy, ion regulation, and photosynthetic performance were assessed. Saline intermittent flooding caused changes in anatomical and morphological traits of plants from both sites associated with increased root aerenchyma and decreased mass allocation to leaf blades in relation to leaf sheaths, concomitant with reductions of blade size and changes in blade shape. Similar negative effects of saline intermittent flooding were found on physiological traits related to photosynthetic functioning of plants from both sites, like decreases in chlorophyll fluorescence, quantum efficiency and δ^{13}C. However, lowland plants presented unaffected leaf length, better ion regulation (higher Cl$^-$ exclusion, higher K$^+$ concentration, and lower Na$^+$/K$^+$ ratio), as well as later leaf senescence with respect to upland plants, when subjected to saline intermittent flooding. Accordingly, plant biomass production decreased by 15% and 32% for lowland and upland plants, respectively. These results indicate that plants of *S. densiflora* inhabiting in the lowland positions have a better acclimation capacity to the harsh environment imposed by the tide than plants from the upland.

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1. Introduction

Coastal salt marshes are important natural environments that provide relevant ecosystem services like wildlife conservation, coastal protection, erosion control, and carbon sequestration (Barbier et al., 2011). These environments are mainly shaped by two restrictive environmental factors, soil salinity and flooding, whose magnitude is directly associated with the frequency and intensity of tides. Elevation above sea level directly determines a gradient of abiotic stress: daily tides in lowland sites imposes higher salinity and more frequent flooding events than in more elevated uplands, subjected to less frequent tides. *Spartina densiflora* is a perennial tussock grass with C_4 metabolism. In contrast to other species (e.g. *S. anglica*), *S. densiflora* is able to inhabit all along salt marsh spatial gradients, including areas with different tidal regimes (Bortolus, 2006; Maricle et al., 2009). This can be attributed to the existence of different ecotypes, large phenotypic plasticity, or both (Loreti and Oesterheld, 1996; Sultan, 2000). This paper assesses the effect of simulated tides on upland and lowland plants of *S. densiflora*, a halophyte that dominates the entire topology of many temperate salt marshes worldwide, including those on the Atlantic coast of southern South America (Bortolus, 2006; Isacch et al., 2006).

Previous studies have determined ecophysiological plant responses to saline flooding on natural halophytes species (Colmer and Flowers, 2008; see Wetson et al., 2012 for *Suaeda maritima*). These species can deal with flooding because they are able to generate aerenchyma in their roots, which favours internal oxygenation, thereby preventing an energy crisis typical of anoxic tissues. In

this way, these species can maintain their roots well aerated and, therefore maintain an adequate regulation of ions (i.e. Na$^+$, Cl$^-$ and K$^+$) delivered from roots towards shoots (Colmer and Flowers, 2008). However, most studies have focused on relatively tolerant annual crops (as rye, barley or wheat), and bushy/woody perennials species (e.g. *Atriplex amnicola*, *Acacia* ssp., *Eucalyptus* ssp.), reporting that flooding under saline conditions increases Na$^+$ and Cl$^-$ concentration in plant shoots, which can have adverse effects on plant growth and survival (Barrett-Lennard, 2003). In addition to the damage by salt toxicity (ion excess effect), salinity generates dehydration of plant tissues through osmotic or water-deficit effect (Munns and Tester, 2008). Therefore, plants inhabiting saline environments usually have traits and develop responses for minimizing water loss, for example through changes in leaf morphology and anatomy (Maricle et al., 2009). Poorter et al. (2012) stated that flooding generally increases biomass allocation to shoots, whereas salinity minimally affects allocation but, in turn, it negatively affects physiological parameters. Furthermore, salinity negatively affects physiological processes mainly due to the generation of osmotic stress in the roots and ion toxicity (i.e. excessive Na$^+$ or Cl$^-$) at cellular level (Munns and Tester, 2008). Tolerant halophyte species cope with salinity through controlled uptake and compartmentalization of toxic ions, synthesis of organic compatible solutes, and secretion of toxic ions towards the outside of leaves through salt glands (Flowers and Colmer, 2008).

Photoinhibition of photosynthesis can occur in response to several environmental stresses (Takahashi and Murata, 2008), including flooding (Mateos-Naranjo et al., 2007) and salinity (Redondo-Gómez et al., 2007). The damage to the photosynthetic apparatus at PSII level – evaluated through chlorophyll fluorescence (F_v/F_m) and quantum efficiency (θPSII) – leads to a reduction in the leaf photosynthetic capacity (Maxwell and Johnson, 2000) and, therefore such variables can be used as indicatives to infer species tolerance. While highly tolerant to salinity stress, *S. densiflora* is known to show decreases in growth and photosynthesis rates as well as in photochemical efficiency when subjected to high salinity levels (Castillo et al., 2005; but *cf.* Maricle et al., 2007). In C$_4$ plants, increases in ^{13}C discrimination during CO$_2$ assimilation indicate higher CO$_2$ leakiness (the proportion of the CO$_2$ pumped into bundle sheath cells by the photosynthetic carbon reduction cycle that is not fixed by Rubisco and diffuses back to the mesophyll) and/or a higher ratio of intercellular to atmospheric CO$_2$ (for ^{13}C discrimination greater than 4.5‰; Farquhar et al., 1989). Since the latter is an unlikely response to saline intermittent flooding in *Spartina* (Maricle et al., 2007), a higher ^{13}C discrimination would indicate greater CO$_2$ leakiness.

The objective of our study was to determine whether *S. densiflora* plants from upland vs. lowland sites in a salt marsh on the coast of the Rio de la Plata river exhibit significant intraspecific variation according to tidal simulation and whether they differ or not in morphological, anatomical, and physiological traits in response to saline flooding. To achieve this objective, we exposed plants of *S. densiflora* from upland and lowland sites to simulated tidal inundation (saline intermittent flooding treatment) in a greenhouse, and explored diversities and similarities in their response in biomass production and allocation, leaf length, root and leaf anatomy, ion regulation, and photosynthetic performance in relation to control plants. We hypothesized that saline intermittent flooding negatively affects some of the studied parameters in a different extent between plants from both sites, being the lowland plants more tolerant to saline intermittent flooding than the upland ones. These differences would reflect different acclimation capacity of plants from both sites across the topographical gradient of the salt marsh, ecosystems of increasing vulnerability due to sea level rise predictions for the present century.

2. Materials and methods

2.1. Study site and plant material

Individual plants of *S. densiflora* (Brongn.) from two different topographic positions – upland and lowland – were extracted from a salt marsh within the National Park "Campos del Tuyú" (56° 50′ W – 36° 19′ S) on the west margin of the Rio de la Plata estuary (Argentina). This environment is characterized by a surface hydrological system with a subtle topographic gradient that originates a network drainage influenced by tidal fluctuation of the estuary (Carol et al., 2008). Tides are predominantly semidiurnal, with salt water usually ranging less than 2 m into the land, but tidal intensity can vary in relation to seasonality and weather conditions. Tidal water contains NaCl (Carol et al., 2008) with an average electrical conductivity of ca. 26 ± 0.3 dS m^{-1} (this study). Soils belong to the Vertisols order with clay texture, smectite expansible clays, low permeability, and ca. 7% organic matter. According to the information above, flooding and salinity appear as important factors determining a topographic stress gradient, which leads to halophytic plant communities zonation (Cagnoni and Faggi, 1993). In addition, the presence of reduced phytotoxins should not be neglected as they might also affect plant performance in salt marshes (King et al., 1982). However, we focused our attention on the combined effects of flooding and salinity as major stress factors. The upland site is located ca. 20 cm higher than the lowland one, which means that upland positions experience less frequent flooding events (very few times per year). In relation to salinity, upland soils have an electrical conductivity that ranges between 9.1 ± 2.9 and 14.4 ± 2.4 dS m^{-1}, in winter and summer, respectively. Upland site vegetation is dominated by *S. densiflora* in association with *Juncus acutus* and *Cortaderia selloana*, whereas *Apium sellowianum*, *Limonium brasiliense*, *Distichlis spicata* and *Agropyron scabrifolium* are present as subordinate species (Di Bella et al., 2014). Lowland site, where floods occur on a daily basis due to its location beside the tidal channels, is characterized by higher soil salinity, which ranges between 9.3 ± 1.3 and 25.3 ± 2.3 dS m^{-1}, in winter and summer, respectively. Vegetation at this site is almost exclusively dominated by *S. densiflora* and *Sarcocornia perennis*, with ca. 25% bare soil (Di Bella et al., 2014).

In December 2010, we collected 50 plants from both upland and lowland sites. These sites are located at a distance of ca. 85 m apart in the salt marsh under study. To avoid sampling the same individual (because of rhizome growth) collected plants were separated at least 2 m from each other. Plants were transported to an experimental garden at the University of Buenos Aires and they were vegetatively propagated twice (January and August 2011; four–six individuals per original plant) to minimize any influence from the original environment (as in Loreti and Oesterheld, 1996). Afterwards, plants were cultivated in a greenhouse until the beginning of the experiment in October 2011.

2.2. Experimental design and growth conditions

The experiment had two factors: "site" as the classification factor and "flooding" as the treatment factor. Site levels were individuals collected from upland and lowland positions, while flooding treatment levels were control and saline intermittent flooding. Each combination site × flooding had 20 replicates, with 80 experimental units. Plants were grown on washed sand in 2 L plastic pots with drainage holes. The experiment began after 7-days acclimation period, where half the plants were subjected to increasing saline concentration (adding 100 mM NaCl every two days until the final saline concentration was reached, avoiding osmotic shock by sudden imposition of saline conditions). To simulate saline intermittent flooding, we placed pots in plastic containers, simulating

tides with saline solution of $30\,dS\,m^{-1}$ during 9 h per day (from 8 am to 5 pm) and keeping water level 5 cm above the substrate level. Simultaneously, control plants were placed in similar containers without water. The saline solution was obtained adding NaCl (Biopack®, Buenos Aires, Argentina) to tap water to obtain the target saline solution concentration (300 mM NaCl). Saline solution was changed once per week and saline concentration was controlled by using a portable water conductivity meter (HI993310, HANNA®, Rhode Island, USA). Control plants were watered every day using tap water (to field capacity). All plants received 50 ml of quarter strength Hoagland's solution [1.5 mM KNO_3, 1 mM $Ca(NO3)_2$, 0.5 mM $MgSO_4$, 0.25 mM $NH_4H_2PO_4$, 0.025 mM Fe-EDTA, 0.01 mM H_3BO_3, 1.9 μM $MnCl_2$, 0.2 μM $ZnCl_2$, 0.09 μM MoO_3, 0.07 μM $CuCl_2$, 0.04 μM $Co(NO_3)_2$] three times per week to avoid nutrient deficiency. The experiment was performed in a greenhouse with controlled environment conditions with minimum and maximum mean temperatures of $18 \pm 3\,^{\circ}C$ and $32 \pm 4\,^{\circ}C$ respectively, and a minimum and maximum relative humidity of $51 \pm 8\%$ and $95 \pm 5\%$ respectively, during 60 days.

2.3. Biomass and morphological parameters

At the end of the experiment (day 60), adult and young tillers were counted, and the length of the last fully expanded leaf blade and sheath were measured in adult tillers with the same number of developed leaves. Then, all plants were dissected ($n = 20$) into young and old blades, sheaths, rhizomes, roots, young tillers, and senescent material to determine the effect of saline intermittent flooding on biomass production and allocation. Harvested material was weighed after oven drying at $70\,^{\circ}C$ for 72 h. Adult tiller mean weight was calculated as: [(blade biomass + sheath biomass)/number of adult tillers]. Allometric relationships between aboveground and belowground biomass, leaf blade and sheath biomass, rhizome and roots biomass, and leaf blade and sheath length were studied through linear regression using Log_e-transformed data. This way, it was possible to assess unbiased treatment effects, since morphological and biomass ratios could change in conjunction with organ/plant size (Poorter and Nagel, 2000).

2.4. Leaf blade shape and root anatomy

Leaf blade and root samples ($n = 10$) were analyzed applying optical techniques to evaluate changes in leaf and root morphological traits and root anatomy. At the end of the experiment, segments from the middle of the youngest fully expanded leaf blade, and 3–4 cm long segments of roots (from 2.5 cm behind the tip) were carefully washed and preserved in formalin–acetic acid–alcohol (FAA) until needed. The samples were dehydrated in a series of increasing ethanol concentrations and embedded in paraffin wax. Sections 10–12 μm thick were cut, stained with safranin and fast green, and mounted on Canada balsam. Leaf blade morphological traits measured were: width (i.e. abaxial perimeter), maximal and minimal thickness, ridge width, and leaf aperture angle. Maximal thickness was measured as the distance between the top of the five main ridges of each blade and the abaxial side. Minimal thickness was measured as the distance between the bottom of the furrows of the five main ridges of each blade and the abaxial side. Leaf aperture was measured as the angle formed between the central rib and both ends of the blade. Root traits measured were: root diameter, stele diameter, proportion of cortex (%), and proportion of aerenchyma (%). All parameters were determined using an optical microscope connected to a digital camera, while images were processed with ImageJ version 1.47 software (U.S. National Institutes of Health, Bethesda, Maryland, USA).

2.5. Physiological measurements

Leaf greenness ($n = 10$) was measured in young and old, fully-expanded leaves by using a portable chlorophyll meter (SPAD-502, Minolta, Ramsey, NJ, USA), during the experiment. This parameter is useful to examine the effects of saline intermittent flooding on blade yellowing, associated to nitrogen remobilization and senescence in relation to leaf age. For this purpose, we tested the relationship between leaf greenness and nitrogen concentration in blades of *S. densiflora*, obtaining a significant linear regression between both parameters ($y = 18.3 + 20.5x$; $r^2 = 0.36$; $P < 0.001$). Light- and dark-adapted fluorescence parameters were measured in young fully expanded leaves using a portable modulated fluorometer (FMS-2, Hansatech Instruments Ltd., King's Lynn, UK). Maximum quantum efficiency of PSII (F_v/F_m) was measured on dark-adapted leaves for 20 min by using leaf-clips. This parameter is known to correlate with the number of functional PSII reaction centres, so that it can be used to quantify the degree of photoinhibition (Maxwell and Johnson, 2000). Quantum efficiency of PSII (θPSII) was measured on the same leaf section of each plant in light adapted leaves (leaves re-adapted under ambient light conditions for at least 20 min) at midday on clear days (PPFD > 1200 μmol $m^{-2}\,s^{-1}$).

2.6. Chemical and isotopic analyses

Dried material of young and old leaf blades of the final harvest was ground to fine powder. The N concentration and $\delta^{13}C$ isotopic signature [in ‰, with $\delta^{13}C = (^{13}C/^{12}C_{sample}/^{13}C/^{12}C_{V\text{-}PDB\,standard} - 1) \times 1000$] were determined on aliquots of 0.7 mg using an elemental analyzer (NA1110, Carlo Erba Instruments, Milan, Italy) interfaced to a continuous-flow isotope-ratio mass spectrometer (IRMS, Delta Plus, Finnigan MAT, Bremen, Germany). Samples were measured against a working gas standard previously calibrated against a secondary isotope standard (IAEA-CH6, accuracy ± 0.06‰ SD). A laboratory standard (wheat flour) was run after every 10 samples to estimate the precision of the isotope analyses (±0.10‰ SD).

Thereafter, an aliquot of 10 mg was used to estimate the concentration of Na^+ and K^+ by standard flame photometry. For these, ions were extracted in 1 ml of HCl (0.1 N) at $60\,^{\circ}C$ 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 Cl^-, an aliquot of 25 mg of sample was taken using a standard colorimetric method with mercury thiocyanate. The sample was digested with 2 ml of H_2O_2/HNO_3/isoamyl alcohol solution (50/50/1) for 15 min and rinsed to 10 ml with milliQ water. A 320 μl aliquot of supernatant was added to 1 ml colour solution. The solution was made with 15 ml of mercury thiocyanate (4,17 g l^{-1} methanol), 15 ml of $(NO_3)_3$ Fe (202 g l^{-1} water + 21 ml of HNO_3), 50 μl of 4% Brij 35 and milliQ water up to 100 ml. The absorbances of samples were read at 450 nm on a Lambda 25 spectrophotometer (PerkinElmer, Waltham, USA), and quantified by a colorimetric calibration curve using KCl.

2.7. Statistical analyses

Plant biomass, number of tillers, weight per tiller, anatomical variables, ^{13}C signature, and ion concentrations were evaluated by two-way ANOVAs with site and flooding as the main factors. For ion concentrations, different ANOVAs were made for young and old leaf blades. When significant interactions were detected, subsequent LSD Fisher tests were applied to determine the effect of treatments. Normality and homogeneity of variances were previously verified. For allometric analyses, intercept and slope tests were

performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA), to compare the relationships between flooding treatments within sites (Poorter and Nagel, 2000). When intercepts and slopes between regressions did not differ, data were pooled and a single linear equation was presented. Variations in physiological variables along the experiment were evaluated by repeated measures (rmANOVA) within sites, considering flooding as main effect and sampling dates as within-subject effects (Von Ende, 1993). Greenhouse–Geyser adjustment was used when mild violations of the hypothesis of sphericity of the covariance matrices occurred. When interactions between treatment and time were significant, individual contrasts were made on each day by the Bonferroni tests (Von Ende, 1993). Statistical analyses were performed using the package STATISTICA version 6.0 (Stat Soft, Tulsa, OK, USA).

3. Results

3.1. Plant biomass and tillering

Following 60 days of treatment, control plants from upland and lowland sites were indistinguishable in terms of total biomass, number of tillers, and average tiller weight. Saline intermittent flooding decreased final biomass in plants from the upland site more than in those from the lowland site, with a reduction of 32% and 15%, respectively (Fig. 1; two-way ANOVA, site × flooding interaction, $F_{1,76} = 4.65$, $P = 0.034$). There was a decrease in the total number of tillers of ca. 24% in plants from both sites, from 23 ± 0.8 to 17 ± 0.7 tillers per plant (two-way ANOVA, $F_{1,76} = 48.05$, $P < 0.001$), with tillers of upland plants lighter (0.36 ± 0.01 g) than those of lowland plants (0.47 ± 0.02 g) (two-way ANOVA, site × flooding interaction, $F_{1,76} = 4.47$, $P = 0.038$).

3.2. Biomass allocation, leaf blade shape and root anatomy

The relationship between aboveground and belowground biomass (Fig. 2A, B), and between rhizome and root biomass (data not shown), did not change either between flooding treatments or between plants belonging to different sites (intercept and slope tests between sites/flooding: $P > 0.05$). Remarkably, saline intermittent flooding treatment generated a significant reduction in leaf blade biomass for any given leaf sheath biomass in plants from both
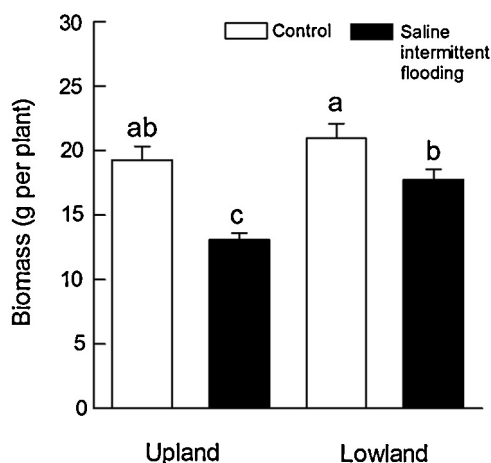

Fig. 1. Biomass of *Spartina densiflora* plants belonging to upland and lowland sites subjected to control and saline intermittent flooding for 60 days. Values are means ± SE ($n = 20$). Different letters indicate significant differences based on LSD Fisher test ($P < 0.05$).

sites (Fig. 2C, D; intercept tests: $P < 0.001$, slope tests: $P > 0.05$). We found a positive relationship between leaf blade length and leaf sheath length that was dependent on the site/flooding combination (Fig. 2G, H): lowland plants did not change this relationship under saline intermittent flooding conditions (intercept and slope tests between flooding treatments: $P > 0.05$), so that they were able to maintain the same blade lengths as controls when subjected to saline intermittent flooding treatment, but in contrast, in upland plants no relationship between blade length and sheath length was found under saline intermittent flooding (linear regression not significant, $P = 0.5$).

Under saline intermittent flooding conditions, abaxial perimeter (i.e. blade width), maximal and minimal thickness, and ridge width were symmetrically diminished in plants belonging to both sites (Fig. 3A, B, E, F; Table 1; two-way ANOVAs, $F_{1,76} = 46.45$, $F_{1,76} = 18.03$, $F_{1,76} = 13.06$, $F_{1,76} = 9.79$ respectively, $P < 0.001$ for all variables). So, narrower and thinner leaves were observed under saline intermittent flooding. Leaf aperture angle was reduced by 53% as a consequence of saline intermittent flooding (compare Fig. 3A, B, E, F; Table 1; two-way ANOVA, $F_{1,76} = 45.19$, $P < 0.001$), in relation to leaf rolling capacity driven by the action of bulliform cells (Fig. 3) under stressful conditions. Thus, saline intermittent flooding influenced leaf morphology quite similarly in plants belonging to both sites, by decreasing blade weight (Fig. 2C, D), and changing leaf blade shape by decreasing their size and aperture angle (Fig. 3A, B, E, F; Table 1). Yet, leaf length was not affected in lowland plants (Fig. 2E, F). Saline intermittent flooding increased root aerenchyma ca. 52% in plants belonging to both sites (two-way ANOVA, $F_{1,36} = 4.56$, $P = 0.04$). This increase was due to the enlargement and/or development of lysigenous aerenchyma lacunae and by an increase in the proportion of cortex from 72% to 81% (Fig. 3C, D, G, H; Table 1; two-way ANOVAs, $F_{1,36} = 72.55$, $P < 0.001$), as a result of a decrease of stele diameter (22%; two-way ANOVAs, $F_{1,36} = 7.82$, $P = 0.008$) and an increase of root diameter (19%; two-way ANOVAs, $F_{1,36} = 9.203$, $P = 0.005$).

3.3. Leaf greenness

Leaf greenness started to be negatively affected by saline intermittent flooding a month after the application of the treatment (Fig. 4). Only old leaves were affected, indicating a saline intermittent flooding-triggered early senescence of basal leaves related to nitrogen mobilization (Fig. 4A, B; rmANOVA, time × flooding, $F_{7,126} = 4.84$, $P < 0.01$ and $F_{7,112} = 2.57$, $P = 0.02$, for plants from the upland and lowland sites, respectively). Notoriously, this occurred even earlier and to a greater extent in plants from the upland site (Bonferroni tests, day 32, $P < 0.05$ and $P > 0.05$ for upland and lowland plants, respectively; compare Fig. 4A and Fig. 4B). Young leaves did not show changes in greenness throughout the experiment (rmANOVA, time and flooding effects: $P > 0.05$).

3.4. Chlorophyll fluorescence

Chlorophyll fluorescence was negatively affected after a month of saline intermittent flooding (Fig. 5). Maximum quantum efficiency of PSII (F_v/F_m) remained constant for approximately 40 days, and then it started to diminish in plants from both sites, which indicates damage by photoinhibition (Fig. 5A, B; rmANOVA, time × flooding, $F_{7,126} = 8.3$, $P < 0.001$ and $F_{7,126} = 15.6$, $P < 0.001$, for the upland and lowland plants, respectively). Such damage was preceded by an earlier decrease in the quantum efficiency of PSII (θPSII) under light environment, which dropped one week before (rmANOVA, time × flooding, $F_{7,126} = 18.52$, $P < 0.001$ and
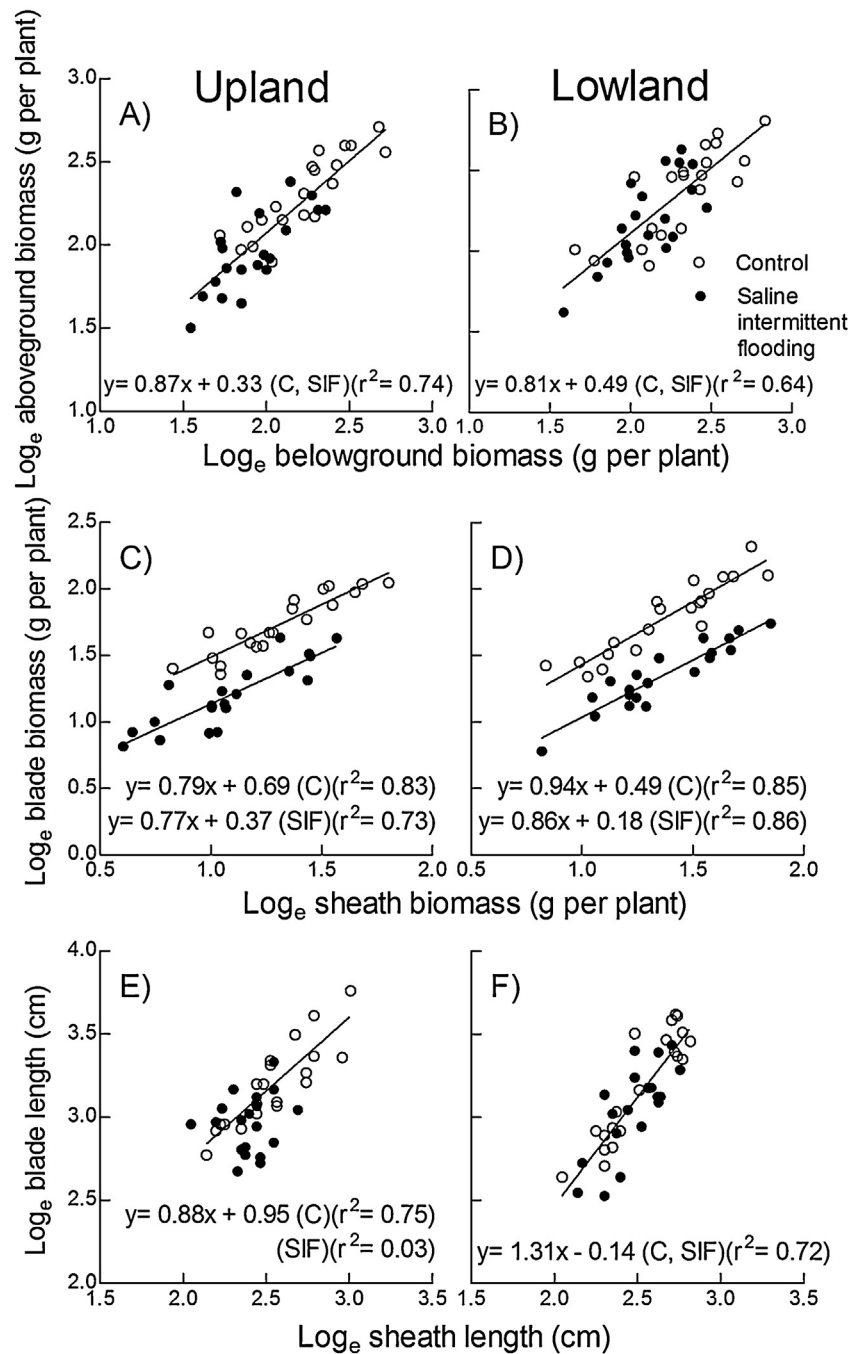
Fig. 2. Allometric relationships between: (i) aboveground vs. belowground biomass (A, B), (ii) leaf blade vs. leaf sheath biomass (C, D), and (iii) leaf blade vs. leaf sheath length (E, F) of *Spartina densiflora* plants belonging to upland (A, C, E) and lowland (B, D, F) sites subjected to control and saline intermittent flooding (SIF) for 60 days ($n = 20$). Note that when parameters between regressions did not differ, data were pooled and a single regression line is presented. All regressions were significant ($P < 0.01$) excepting saline intermittent flooding treatment in (E).

$F_{7,126} = 12.97$, $P < 0.001$, for the upland and lowland plants, respectively).

3.5. ^{13}signature

The $\delta^{13}C$ of young leaves was negatively affected by saline intermittent flooding, decreasing from $-13.5 \pm 0.08‰$ to $-13.9 \pm 0.12‰$ in upland plants, and from $-13.6 \pm 0.08‰$ to $-14.0 \pm 0.09‰$ in lowland plants (two-way ANOVAs, $F_{1,36} = 20.09$, $P < 0.001$). Therefore, saline intermittent flooding decreased $\delta^{13}C$ by 0.4‰ in both upland and lowland plants.

3.6. Ion concentration in leaf tissues

Saline intermittent flooding increased the concentration of Na^+ in both young and old leaves (Table 2; two-way ANOVAs, $F_{1,76} = 1089.2$, $P < 0.001$ for young leaves and $F_{1,76} = 1047.3$, $P < 0.001$ for old leaves). This occurred together with a decrease in K^+ tissue concentrations, which resulted in significant increases in the Na^+/K^+ ratio. Remarkably, plants from the upland site showed greater Na^+/K^+ ratios in leaf tissues than plants from the lowland site (Table 2; site × flooding interaction, $F_{1,76} = 5.5$, $P < 0.05$ for new leaves and site × flooding interaction, $F_{1,76} = 6.5$, $P < 0.05$ for old
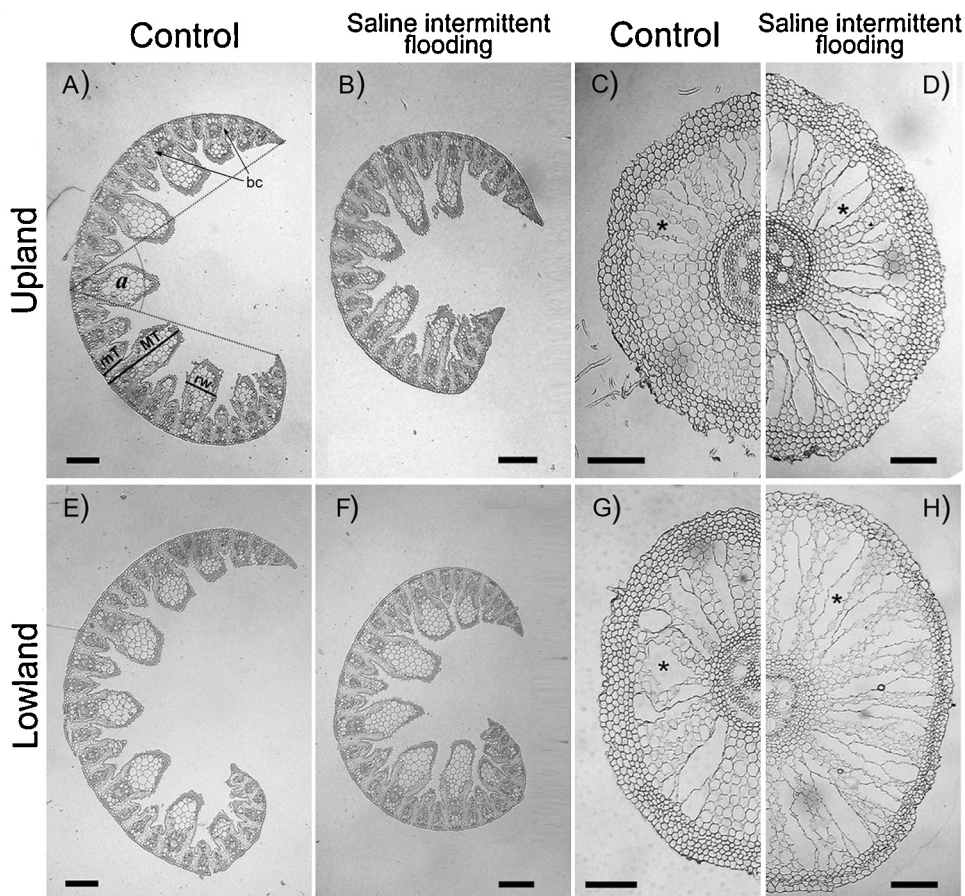
Fig. 3. Leaf blade and root cross sections of *Spartina densiflora* plants belonging to upland (A, B, C, D) and lowland (E, F, G, H) sites subjected to control (A, E, C, G) and saline intermittent flooding (B, F, D, H) for 60 days. Scale bars represent 200 μm for leaves (A, B, E, F) and 100 μm for roots (C, D, G, H). References: a, leaf aperture angle; mT, minimal thickness; MT, maximal thickness; rw, ridge width; bc, bulliform cells. Asterisks indicate aerenchyma lacunae.

leaves). Cl⁻ tissue concentrations only increased in old leaves in plants from both sites (Table 2; $F_{1,76}$ = 20.2, P < 0.001). Importantly, in functional young leaves, plants from the lowland site were more effective in Cl⁻ exclusion and in K⁺ uptake than plants from the upland site (see lower Cl⁻ and higher K⁺ concentrations and 'site effect' significance level in Table 2).

4. Discussion

We found clear differences between plants from upland and lowland sites of the salt marsh. Plants growing in lowlands are better acclimated to saline intermittent flooding than plants from

uplands: saline intermittent flooding reduced biomass production only by 15% in lowland plants, and by 32% in upland plants (Fig. 1). Plants from the lowland site under saline intermittent flooding better regulated Na⁺/K⁺ ratio (Table 2) and were able to keep leaf length unaffected (Fig. 2). Furthermore, associated with these responses, lowland plants triggered the senescence of older leaves under saline intermittent flooding later (Fig. 4). Therefore, we are presenting evidence supporting the idea that the dominance of *S. densiflora* across the whole elevation range in salt marshes – and thus naturally subjected to stress gradient caused by tidal frequency and intensity – could be due to the existence of a different acclimation capacity of upland and lowland plants. Notably, saline

Table 1
Leaf blade shape and root traits of *Spartina densiflora* plants belonging to upland and lowland sites subjected to control and saline intermittent flooding for 60 days.

	Upland		Lowland	
Leaf blade	Control	Saline intermittent flooding	Control	Saline intermittent flooding
Width (mm)	6.0 ± 0.1a	4.9 ± 0.2b	5.5 ± 0.3a	5.1 ± 0.1b
Max. thickness (μm)	547.8 ± 9.4a	516.6 ± 13.1b	567.0 ± 11.5a	498.6 ± 11.8b
Min. thickness (μm)	150.5 ± 2.3a	138.1 ± 3.1b	146.5 ± 2.6a	139.4 ± 2.6b
Ridge width (μm)	237.3 ± 3.4a	227.4 ± 3.6b	247.4 ± 8.7a	217.8 ± 7.3b
Leaf aperture angle (°)	48.1 ± 3.7a	26.3 ± 3.7b	47.9 ± 4.3a	19.3 ± 3.0b
Root				
Root diameter (μm)	947.5 ± 52.6a	1082.4 ± 44.2b	909.6 ± 71.1a	1124.3 ± 55.8b
Stele diameter (μm)	262.7 ± 20.8a	194.6 ± 5.7b	257.7 ± 31.1a	209.9 ± 11.7b
% Cortex	72.2 ± 1.5a	81.7 ± 1.1b	72.1 ± 1.7a	80.8 ± 1.8b
% Aerenchyma	18.1 ± 4.4a	33.0 ± 4.4b	22.9 ± 6.2a	29.3 ± 4.3b

Values are means ± SE (n = 20). Significant differences were detected between flooding treatments for all presented variables based on two-way ANOVAs (P < 0.05).
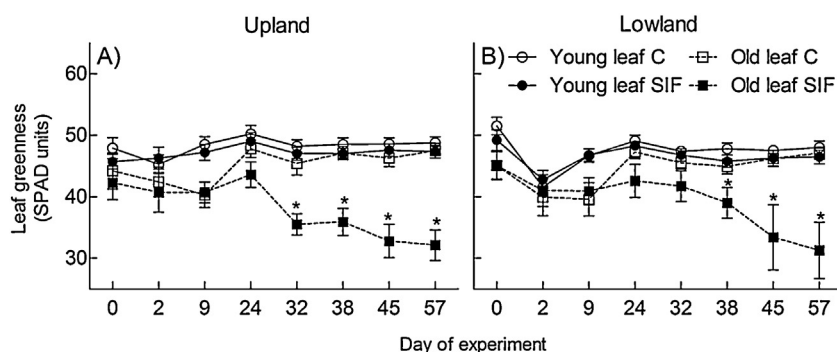
Fig. 4. Leaf greenness (SPAD units) of young and old leaf blades of *Spartina densiflora* plants belonging to upland (A) and lowland (B) sites subjected to control (C) and saline intermittent flooding (SIF) for 60 days. Values are means ± SE ($n = 10$). Asterisks indicate significant differences between treatments in greenness of old leaves for plants from each site based on Bonferroni tests ($P < 0.05$). For young leaves there were no differences between treatments.

intermittent flooding effects on the performance of the photosynthetic apparatus were of equal magnitude in upland and lowland plants (Fig. 5), which suggests that carbon gain is not likely a major determinant of the differential acclimation of these plants to this environment.

4.1. Diversities between plants in leaf growth, senescence and ion regulation

Leaf length (Fig. 2), physiological functioning for ion regulation (Table 2), and timing of senescence of old leaves (Fig. 4) were differentially affected by saline intermittent flooding between plants from both sites. These differences reflect the idea that upland and lowland plants are differentially acclimated to the habitat associated with the regime of saline intermittent flooding of the salt marsh, the plants from lowlands being more tolerant. Naturally, the diversities we found present a narrower range than studies comparing different species from salt marsh ecosystems (Hester et al., 2001), as we screened plants of the same species belonging to two different positions of a gradient and with similar genetic background (see also Nyberg Berglund et al., 2003). We found plastic responses to saline intermittent flooding in plants from both sites. In our study, ion regulation differed between plants from both sites,

with the lowland plants having an innate ability to exclude Cl^- and to sustain higher K^+ concentration in young leaves under saline intermittent flooding treatment than the upland plants. In addition, the Na^+/K^+ ratio was low in young leaves of lowland plants in spite of having a slightly higher Na^+ concentration with respect to upland plants (see Table 2). In this respect, a recent review by Barrett-Lennard and Shabala (2013) highlighted the importance of maintaining low Na^+/K^+ ratios and K^+ homeostasis in functional (young) leaves to define the genotypes most tolerant to saline flooding stress in terms of biomass accumulation (see also Munns and Tester, 2008; Munns, 2002 for salinity alone). In that sense, only plants from the lowland site were able to continue with their leaves elongation without differences when subjected to saline intermittent flooding treatment. By contrast, under saline intermittent flooding conditions, plants from the upland site were unable to sustain their leaves elongation, which denoted that such plants were clearly more stressed (compare Fig. 2E with F). This fact also explained the lighter tiller weight of the upland plants in comparison to those of the lowland plants.

Nitrogen concentration, measured as leaf greenness, was only negatively affected in old leaves subject to saline intermittent flooding (Fig. 4), indicating a presumable remobilization of nitrogen to young leaves and a premature senescence of old leaves
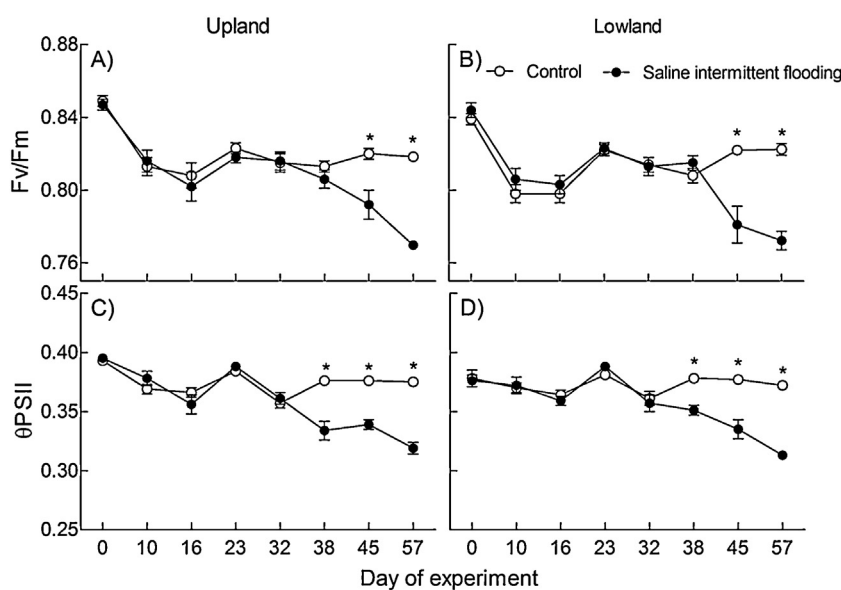

Fig. 5. Chlorophyll fluorescence (F_v/F_m) (A, B) and quantum efficiency (θPSII) (C, D) of *Spartina densiflora* plants belonging to upland (A, C) and lowland (B, D) sites subjected to control and saline intermittent flooding for 60 days. Values are means ± SE ($n = 10$). Asterisks indicate significant differences between treatments for each site based on Bonferroni tests ($P < 0.05$).

Table 2

Ion concentrations and Na⁺/K⁺ ratio in young and old leaf blades of *Spartina densiflora* plants belonging to upland and lowland sites subjected to control and saline intermittent flooding for 60 days.

Young leaves	Upland		Lowland		F-values		
	Control	Saline intermittent flooding	Control	Saline intermittent flooding	Site	Flooding	Interaction
Cl^- (mg g^{-1})	10.29 ± 0.29	11.62 ± 0.65	9.10 ± 0.23	8.90 ± 0.38	14.9**	1.0 ns	1.3 ns
Na^+ (mg g^{-1})	0.46 ± 0.01c	1.29 ± 0.03b	0.44 ± 0.02 c	1.41 ± 0.03a	3.5 ns	1089.2***	6.3*
K^+ (mg g^{-1})	0.85 ± 0.03	0.22 ± 0.01	0.87 ± 0.02	0.29 ± 0.01	5.0*	868.2***	1.2 ns
Na^+/K^+	0.54 ± 0.02c	5.82 ± 0.20a	0.50 ± 0.03c	4.83 ± 0.26b	6.34*	850.9***	5.5*
Old leaves							
Cl^- (mg g^{-1})	14.57 ± 0.61	17.98 ± 0.89	15.50 ± 0.37	18.39 ± 0.75	1.7ns	20.2***	0.2 ns
Na^+ (mg g^{-1})	0.43 ± 0.02	1.47 ± 0.04	0.39 ± 0.03	1.35 ± 0.03	6.6*	1047.3***	1.6 ns
K^+ (mg g^{-1})	0.57 ± 0.03	0.10 ± 0.01	0.53 ± 0.03	0.14 ± 0.01	0.03 ns	403.8***	3.0 ns
Na^+/K^+	0.75 ± 0.06c	14.70 ± 1.60a	0.73 ± 0.09c	9.64 ± 1.04b	6.7*	155.6***	6.5*

Values are means ± SE ($n = 20$). *F*-values of the two-way ANOVAs are presented. Degrees of freedom for each source of variation were: 1 (Site), 1 (Flooding), 1 (P × F) and 76 (error). Significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, $P > 0.05$; based on LSD Fisher tests ($P < 0.05$). Different letters indicate significant differences when site × flooding interaction were detected.

under saline intermittent flooding treatment (Desclos et al., 2009; Hörtensteiner and Feller, 2002). Interestingly, the drop in greenness in old leaves under saline intermittent flooding in the upland plants occurred after 32 days, while in the lowland plants, this was observed after 38 days, indicating a lower tolerance of the former as they entered into early senescence. This response matched with a slightly higher Na^+ concentration, lower K^+ concentration and, thereby a much higher Na^+/K^+ ratio registered in old leaves of the upland plants (Table 2), which could be associated either with an early salt-triggered senescence or with a progressive loss of capacity for ion regulation during senescence (Balazadeh et al., 2010). These responses (leaf length and senescence), taken together, help to explain why biomass accumulation at the whole plant level was lower in plants from the upland site, whose leaves were shorter and entered into senescence earlier, and thereby the total biomass was more reduced than in plants from the lowland site (Fig. 1).

4.2. Similarities in allocation and anatomical changes in response to saline flooding

In a recent meta-analysis Poorter et al. (2012) stated that salinity minimally affects allocation, which in turns negatively affects photosynthesis and transpiration rates. Additionally, they indicated that in tolerant species, flooding usually promotes biomass allocation to aboveground organs, increasing leaf area exposed above water level (Striker et al., 2011). This response allows plants to maintain aerial photosynthesis and facilitates the capture of oxygen by leaves for the oxygenation of submerged tissues through the aerenchyma system transport (Colmer and Voesenek, 2009; Striker et al., 2005). On the one hand, our results agree with Poorter et al. (2012) as we reported saline intermittent flooding effects upon physiological variables; but on the other hand, we found consistent differences in allocation within aboveground organs (Figs. 2–3 and Table 1). Saline intermittent flooding promoted a decreased biomass allocation towards blades in relation to sheaths at the whole plant level in plants from both sites (Fig. 2C, D). Additionally, upland and lowland plants of *S. densiflora* showed considerable (and similar) changes in the blade shape: a decrease of width, thickness, and aperture angle (i.e. leaf rolling) (Table 1). These results suggest that, together with the reduction in the proportion of blades, plants enhanced the ability to tolerate the stress imposed by saline intermittent flooding (particularly the osmotic component due to salinity) by reducing the leaf area exposed to transpiration. This response was driven by the capacity of this species for promoting leaf rolling (see bulliform cells in Fig. 3A), as it was previously reported in a comprehensive anatomical study on *Spartina* species by Maricle et al. (2009), and by the capacity to decrease leaf size. Finally, it is important to note that there was a lack of response in

allocation between aboveground and belowground biomass. This is not an unexpected result as *S. densiflora* is a tall tussock grass. This means it does not need to elongate or increase biomass allocation to aboveground organs to enable the capture of oxygen, as even in the lowland positions of the salt marsh, plants are never completely submerged.

The generation of aerenchyma tissue is another common plant response to flooding, which facilitates the transport of oxygen and other metabolically generated gases between roots and shoots, avoiding adverse effects of soil anaerobiosis (Jackson and Armstrong, 1999; Voesenek et al., 2006). It was stated that species with large volumes of root aerenchyma would be more tolerant to the combination of flooding and salinity than those with less aerenchyma. This is because in the latter, root oxygen deficiency could drastically reduce the energy available for sustaining ion transport across membranes, so that the selective mechanisms favouring the uptake of K^+ and the exclusion of Na^+ and Cl^- would be partly inhibited (Barrett-Lennard and Shabala, 2013; Barrett-Lennard, 2003; Colmer and Flowers, 2008). We agree that the general tolerance of plants of *S. densiflora* from both sites to the combination of flooding and salinity could be related to the high level of constitutive aerenchyma, and the plastic capacity to increase root lysigenous aerenchyma tissue under the imposed stressful conditions (Barrett-Lennard and Shabala, 2013; Justin and Armstrong, 1987; Pezeshki, 2001). These findings concur with other comparative studies where different species showed the plastic ability to increase root porosity due to the generation of aerenchyma tissue in the cortical parenchyma (Barrett-Lennard and Shabala, 2013; Grimoldi et al., 2005; Visser et al., 2000). The results suggested that salinity (as saline intermittent flooding) did not affect oxygen capture (discussed in precedent paragraphs) and transport; thereby the different ion regulation between plants from both sites appears to be innate of each group (discussed above) and unrelated to root aeration potential. In this respect, the lack of differences between plants from upland and lowland sites indicates that anatomical traits related to the generation of root aerenchyma are relatively fixed within the same species, or at least in a relatively subtle range of tidal regime variations.

4.3. Saline flooding effects on photosynthesis functioning

Regarding tolerance of *S. densiflora* to saline intermittent flooding, we found reductions of F_v/F_m and θPSII only after 45 and 38 days of saline intermittent flooding treatment, in the lowland and upland plants respectively (Fig. 5), which denotes *S. densiflora* is highly tolerant to saline intermittent flooding. This ability for maintaining PSII functioning stable for more than one month concurs with reports by Maricle et al. (2007) and Mateos-Naranjo et al. (2007);

in the former study no effects on F_v/F_m were registered in plants flooded with saline water (30‰ salt) for 30 days, while in the latter study, eight weeks of non-saline inundation were needed to reduce F_v/F_m and θPSII. Therefore, taken together, our results strengthen the idea about the high tolerance of *S. densiflora* to hypoxic-saline stress. Regarding the origin of the plants, we did not find consistent differences in the timing and magnitude of the drop in these parameters between them (Fig. 5), which indicates that the functioning of the photosynthetic apparatus, assessed through fluorescence, did not differentiate upland and lowland plants. The reductions in F_v/F_m and θPSII of plants from both sites under saline intermittent flooding should be attributed to the toxic effects provoked by an increased accumulation of Na^+ and Cl^- and a high Na^+/K^+ ratio in young leaves (see Table 2 upper panel) and not to nitrogen-deficiency of such leaves (similar greenness of young leaves; Fig. 4A, B). In this respect, the reduction of K^+ concentration along with Na^+ and Cl^- accumulation under saline stress, could have increased the intracellular level of reactive oxygen species, thus provoking the disruption of PSII functionality (Barrett-Lennard and Shabala, 2013; Munns and Tester, 2008; Takahashi and Murata, 2008).

Saline intermittent flooding decreased $δ^{13}C$ by 0.4‰ in both upland and lowland plants. We do not know reports of saline intermittent flooding effects on ^{13}C discrimination, although shifts of 0.5–1.0‰ have been found in response to salinity in continuously flooded plants of three *Spartina* species (Maricle and Lee, 2006). Flooding *per se* seems to have little effect on ^{13}C discrimination (Waring and Maricle, 2012). Since saline intermittent flooding effects on $δ^{13}C$ were small (O'Leary et al., 1992), and similar in lowland and upland plants, the operation of the C_4 photosynthetic mechanism, while affected by saline intermittent flooding, was not largely altered by it in these *Spartina* plants. This contrasts with far greater effects observed on C_4 species not adapted to salinity (e.g. Barrett-Lennard and Shabala, 2013; Bowman et al., 1989; Meinzer et al., 1994).

4.4. Final comments

Biomass production of grasses depends on three structural characters: blade size, tiller density, and number of green leaves per tiller (Lemaire and Chapman, 1996). Leaf size reduction was observed by the decrease of leaf blade biomass and leaf shape. Tiller density was reduced by saline intermittent flooding. The number of green leaves per tiller was functionally damaged by the premature senescence of old leaves subjected to saline intermittent flooding conditions. Among those characters, leaf length and leaf senescence and – additionally – the higher Na^+/K^+ ratio in young and old leaves were more negatively affected in the upland than in the lowland plants, helping to explain the differences in biomass production between them. Several studies showed a decrease in biomass production of higher (i.e. annual crops and woody perennials) (Barrett-Lennard, 2003) and salt marsh plants (Broome et al., 1995; Cooper, 1982; Wetson et al., 2012) when subjected to salinity and flooding, due to an increase in Na^+ and Cl^- and a decrease in K^+ concentration in the aerial organs (reviewed by Barrett-Lennard and Shabala, 2013). Overall, our results indicate that lowland plants of *S. densiflora* are more tolerant to saline intermittent flooding than plants from the upland, revealing a different acclimation capacity between plants from both sites. This differential acclimation capacity might explain the ability of *S. densiflora* to live along the entire gradient of the salt marsh.

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