Synergic effect of salinity and CO2 enrichment on growth and photosynthetic responses of the invasive cordgrass *Spartina densiflora*

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Received 22 October 2009; Revised 25 January 2010; Accepted 27 January 2010

Abstract

*Spartina densiflora* is a C4 halophytic species that has proved to have a high invasive potential which derives from its clonal growth and its physiological plasticity to environmental factors, such as salinity. A greenhouse experiment was designed to investigate the synergic effect of 380 and 700 ppm CO2 at 0, 171, and 510 mM NaCl on the growth and the photosynthetic apparatus of *S. densiflora* by measuring chlorophyll fluorescence parameters, gas exchange and photosynthetic pigment concentrations. PEPC activity and total ash, sodium, potassium, calcium, magnesium, and zinc concentrations were determined, as well as the C/N ratio. Elevated CO2 stimulated growth of *S. densiflora* at 0 and 171 mM NaCl external salinity after 90 d of treatment. This growth enhancement was associated with a greater leaf area and improved leaf water relations rather than with variations in net photosynthetic rate (*A*). Despite the fact that stomatal conductance decreased in response to 700 ppm CO2 after 30 d of treatment, *A* was not affected. This response of *A* to elevated CO2 concentration might be explained by an enhanced PEPC carboxylation capacity. On the whole, plant nutrient concentrations declined under elevated CO2, which can be ascribed to the dilution effect caused by an increase in biomass and the higher water content found at 700 ppm CO2. Finally, CO2 and salinity had a marked overall effect on the photochemical (PSII) apparatus and the synthesis of photosynthetic pigments.

Key words: Chlorophyll fluorescence, CO2 enrichment, cordgrass, gas exchange, growth rate, PEPC activity, photosynthetic pigments, salinity.

Introduction

Global environmental changes such as climatic change and biological invasions are common conservation problems affecting ecosystems worldwide (Occhipinti-Ambrogi, 2007). Climate change is likely to alter patterns of alien plant invasions through its effect on three general aspects: the invisibility of the ecosystem, climate impacts on indigenious species, and the invasive potential of the alien species (Dukes and Mooney, 1999).

*Spartina densiflora* is a C4 halophytic species with a South American origin that is invading salt marshes as far apart as southern Europe (Tutin, 1980; Mateos-Naranjo *et al.*, 2007), North Africa (Fennane and Mathez, 1988) and North America (Kittelson and Boyd, 1997). In Spain, *S. densiflora* has proved to have a high invasive potential which derives from its prolific seed production and from its clonal growth (Figueroa and Castellanos, 1988; Nieva *et al.*, 2001). In addition, its physiological and morphological versatility apparently allows *S. densiflora* to tolerate a wide range of salinity, tidal submergence, and drainage (Castillo *et al.*, 2005; Mateos-Naranjo *et al.*, 2007). This is consistent with *S. densiflora* having colonized high, middle, and low marshes, with their different characteristic assemblages of
native species (Nieva et al., 2001). Many ecological and physiological aspects of S. densiflora have hitherto been analysed (Castillo et al., 2005; Mateos-Naranjo et al., 2007, 2008a, b). Nevertheless, so far no studies have assessed the influence of climatic change and of rising atmospheric CO₂ concentrations on the invasive potential of S. densiflora.

Predictions regarding climate change indicate that the CO₂ concentration in the atmosphere is expected to have undergone a 2-fold increase by the end of the century with a value of c. 760 ppm by 2100 (IPCC, 2001). There is a general consensus on the direct physiological impact of increasing CO₂ concentration on plant photosynthesis and metabolism, stimulating growth and development in hundreds of plant species (Ghannoum et al., 2000). However, recent evidence from free-air CO₂ enrichment experiments suggested that elevated CO₂ concentration did not directly stimulate C₄ photosynthesis. Nonetheless, drought stress can be ameliorated at elevated CO₂ concentration as a result of lower stomatal conductance and greater intercellular CO₂ concentration (Leakey, 2009). Furthermore, the effects of CO₂ enrichment on plants can be modified by other environmental factors, such as salinity (Lenssen et al., 1993, 1995; Rozema, 1993) and temperature.

The aims of this study were to investigate (i) whether CO₂ enrichment stimulates the growth of the invasive species S. densiflora, and whether this stimulation is mediated by an improvement of photosynthetic activity, and/or (ii) whether salinity stress can be ameliorated at high CO₂ concentration. The specific objectives were to: (i) analyse the growth of plants in experimental salinity concentrations from 0 to 510 mM NaCl at ambient and elevated CO₂ concentrations (380 and 700 ppm, respectively); (ii) determine the extent of the effects on the photosynthetic apparatus (PSII chemistry), gas exchange characteristics, phosphoenolpyruvate carboxylase activity (PEPC), and photosynthetic pigments; and (iii) examine the possible role of concentrations of mineral matter (ash), calcium, potassium, sodium, and zinc accumulated and C/N ratio in response to increasing external salinity at both CO₂ levels.

Materials and methods

Plant material
Seeds of S. densiflora were collected in December 2006 from Odiel Marshes (37°15’ N, 6°58’ W; SW Spain), and subsequently stored at 4 °C (in darkness) for three months. After the storage period, seeds were placed in a germinator (ASL Aparatos Científicos M-92004, Madrid, Spain), and subjected to an alternating diurnal regime of 16 h of light (photon flux rate, 400–700 nm, 35 μmol m⁻² s⁻¹) at 25 °C and 8 h of darkness at 12 °C, for a month. Seedlings were planted in individual plastic pots (9 cm and 11 cm of height and diameter, respectively) filled with perlite and placed in a greenhouse (during spring 2007) with pots (9 cm and 11 cm of height and diameter, respectively) filled with perlite and placed in a greenhouse (during spring 2007) with 12°C, for a month. Seedlings were planted in individual plastic pots (9 cm and 11 cm of height and diameter, respectively) filled with perlite and placed in a greenhouse (during spring 2007) with pots (9 cm and 11 cm of height and diameter, respectively) filled with perlite and placed in a greenhouse (during spring 2007) with

Growth conditions
In April 2007, after a month of seedling cultures, the pots were allocated to three NaCl treatments in shallow trays: 0, 171, and 510 mM in Hoagland’s solution. Afterwards, they were exposed to ambient (380 ppm) or elevated CO₂ concentration (700 ppm), in a controlled-environment chamber, in the same greenhouse and supplied with Hoagland’s solution with or without NaCl (ten pots per tray, with one tray per NaCl and CO₂ treatments) for a further three months. The CO₂ concentration was within 10% of the target concentration for 85% of the time, on the basis of 1 min averages. NaCl concentrations were chosen to cover variations recorded by Mateos-Naranjo et al. (2008a) in the salt marshes of the Odiel River where S. densiflora occurs.

The CO₂ concentration in the greenhouse with ambient CO₂ was not controlled, but it was measured with a CO₂ analyser (Testo 535, Germany). The controlled-environment chamber consists of transparent chamber tops, 3.3 m × 1.4 m × 1.1 m (length × width × height) made with 0.005 thick acrylic glass, and aluminium angular frame elements. The CO₂ level in the enriched chamber was maintained by supplying pure CO₂ from a compressed gas cylinder (Air liquide, B50 35K) into the chamber. The CO₂ concentration in the chamber was continuously recorded by a CO₂ sensor (Vaisala CARBOCAP GMT220, Finland), the signal being received by a computer (ASCON M3, Italy) that activated, if necessary, CO₂ injection into the enriched chamber so as to reach the desired 700 ppm.

At the beginning of the experiment, 3.0 l of the appropriate solution were placed in each of the trays down to a depth of 1 cm. During the experiment, the levels in the trays were monitored and they were topped up to the marked level with 20% Hoagland’s solution as a way to limit the change of NaCl concentration due to water evaporation of the nutritive solution.

Growth analysis
At the beginning and at the end of the experiment, three and seven entire plants (roots and leaves) from each treatment, respectively, were dried at 80 °C for 48 h and then weighed. Dried, ground samples were ignited in lidded, ceramic crucibles and ash weights were recorded; the furnace temperature was raised slowly over 6 h to 550 °C and this temperature was maintained for a further 8 h. Also, the number of tillers was measured.

A classical growth analysis (Evans, 1972) was carried out with ash-free dry mass. The relative growth rate in whole plant dry mass (RGR) was calculated and partitioned into its three components, unit leaf rate (ULR), specific leaf area (SLA), and leaf mass fraction (LMF), using the software tool of Hunt et al. (2002):

\[
(1/W)(dW/dt)=(1/L_A)(dW/dt)=L_A/L_W=W/RGR \quad \text{ULR} \quad \text{SLA} \quad \text{LMF}
\]

where t is time, W is total dry mass per plant, L_A is total leaf area per plant, and L_W is total leaf dry mass per plant. Leaf area was calculated by superimposing the surface of each leaf over a mm-square paper.

Leaf elongation rate (LER) was measured in random leaves (n=14, per treatment; two measurements per plant) at 90 d of treatment by placing a marker of inert sealant at the base of the youngest accessible leaf. The distance between the marker and the leaf base was measured after 24 h (Mateos-Naranjo et al., 2008b).

Gas exchange
Gas exchange measurements were taken on random, fully expanded penultimate leaves (Fig. 1; n=10, one measurement per plant and three extra taken randomly) using an infrared gas analyser in an open system (LI-6400, Li-Cor Inc., Nebraska, USA) after 7, 30, and 90 d of treatment. Net photosynthetic rate (A), intercellular CO₂ concentration (C_i), and stomatal conductance to
CO₂ (Gₐ) were determined at ambient CO₂ concentration of 380 and 700 ppm CO₂, temperature of 20 °C, 50±5% relative humidity, and a photon flux density of 1000 μmol m⁻² s⁻¹. A, Gₐ, and G were calculated using standard formulae of Von Caemmerer and Farquhar (1981). Photosynthetic area was approximated as the area of a trapezoid. The water use efficiency (WUE) was calculated as the ratio between A and transpiration rate (mmol CO₂ assimilated mol⁻¹ H₂O transpired).

**Leaf water content**
Leaf water content (WC) was calculated after 90 d of treatment as:

\[ WC = \frac{FW - DW}{FW} \times 100 \]

where FW is the fresh mass of the leaves, and DW is the dry mass after oven-drying at 80 °C for 48 h.

**Chlorophyll fluorescence**
Chlorophyll fluorescence was measured in random, fully developed penultimate leaves (n=10, one measurement per plant and three taken randomly) using a portable modulated fluorimeter (FMS-2, Hansatech Instrument Ltd., England) after 7, 30, and 90 d of treatment. Light- and dark-adapted fluorescence parameters were measured at dawn (stable, 50 % humidity, and a photon flux density of 1000 μmol m⁻² s⁻¹) and at midday (1600 μmol m⁻² s⁻¹) to investigate whether NaCl and CO₂ concentration affected the sensitivity of plants to photoinhibition.

Plants were dark-adapted for 30 min, using leaf-clips exclusively designed for this purpose. The minimal fluorescence level in the dark-adapted state (F₀) was measured using a modulated pulse (<0.05 μmol m⁻² s⁻¹ for 1.8 μs) which was too small to induce significant physiological changes in the plant. The data stored were an average taken over a 1.6 s period. Maximal fluorescence in this state (Fₘ) was measured after applying a saturating actinic light pulse of 15 000 μmol m⁻² s⁻¹ for 0.7 s. The value of Fₘ was recorded as the highest average of two consecutive points. Values of the variable fluorescence (Fᵥ=Fₘ−F₀) and maximum quantum efficiency of PSII photochemistry (Fₘ/Fₘ) were calculated from F₀ and Fₘ. This ratio of variable to maximal fluorescence correlates with the number of functional PSII reaction centres, and dark-adapted values of Fᵥ/Fₘ can be used to quantify photoinhibition (Krivosheeva et al., 1996).

The same leaf section of each plant was used to measure light-adapted parameters. Steady-state fluorescence yield (Fₛ) was recorded after adapting plants to ambient light conditions for 30 min. A saturating actinic light pulse of 15 000 μmol m⁻² s⁻¹ for 0.7 s was then used to produce the maximum fluorescence yield (Fₘ) by temporarily inhibiting PSII photochemistry.

Using fluorescence parameters determined in both light- and dark-adapted states, the following were calculated: quantum efficiency of PSII (Φₛₚₛₛ=Fₛ/Fₘ) and non-photochemical quenching (NPO=(Fₛ−F₀)/Fₛ, Redondo-Gómez et al., 2006).

**Photosynthetic pigments**
At the end of the experiment period, photosynthetic pigments in fully expanded penultimate leaves (n=5) were extracted using 0.05 g of fresh material in 10 ml of 80% aqueous acetone. After filtering, 1 ml of the suspension was diluted with a further 2 ml of acetone and chlorophyll a (Chl a), chlorophyll b (Chl b) and carotenoid (Cₐ+c) contents were determined with a spectrophotometer (Hitachi U-2001, Hitachi Ltd, Japan), using three wavelengths (663.2, 646.8, and 470.0 nm). Concentrations of pigments (µg g⁻¹ FW) were obtained by calculation, using the method of Lichtenthaler (1987).

**Determination of sodium, potassium, calcium, magnesium, zinc, and nitrogen**
In accordance with protocols of Redondo-Gómez et al. (2007), at the end of the experiment leaf and root samples were dried at 80 °C for 48 h and ground. Leaves and roots were carefully washed with distilled water before any further analysis. Then 0.5 g samples, taken from a mixture of the leaves or the roots belonging to the seven plants used for each treatment, were tripicately digested with 6 ml HNO₃, 0.5 ml HF, and 1 ml H₂O₂. Ca²⁺, K⁺, Mg²⁺, Na⁺, P, and Zn were measured by inductively coupled plasma (ICP) spectroscopy (ARL-Fison 3410, USA). Total N and C concentrations were determined for undigested dry samples with an elemental analyser (Leco CHNS-932, Spain).

**Preparation of desalted protein extracts**
Plants were exposed to 2 h of direct sunlight at midday. To extract PEPC (n=5), 0.2 g of leaf tissue were taken and immediately ground in a chilled mortar with 1 ml of extraction buffer A containing 100 mM TRIS-HCl pH 7.5, 20% (v/v) glycerol, 1 mM EDTA, 10 mM MgCl₂, 14 mM β-mercaptoethanol, 1 mM phenylmethysulphonylfluoride (PMSF), 10 μg ml⁻¹ chymostatin, 10 μg ml⁻¹ leupeptin, and 10 mM potassium fluoride. The homogenate was centrifuged at 15 000 g for 2 min and the supernatant was filtered through Sephadex G-25 equilibrated with buffer A without β-mercaptoethanol. The desalted extract was used rapidly to determine the activity and sensitivity of PEPC to l-malate, as described below.

**Assay of PEPC activity and its inhibition by l-malate**
PEPC activity was measured spectrophotometrically at the optimal and suboptimal pH values of 8 and 7.3, respectively, using the NAD-malate dehydrogenase-coupled assay at 2.5 mM phosphoenolpyruvate (PEP) described by Echevarria et al. (1994). Assays were initiated by the addition of an aliquot of crude extract (n=5).

An enzyme unit is defined as the amount of PEPC that catalyses the carboxylation of 1 μmol of phosphoenolpyruvate min⁻¹ at pH 8 and 30 °C. Malate sensitivity was determined at suboptimal pH 7.3 in the presence or absence of various concentrations of l-malate (IC₅₀, 50% inhibition of initial PEPC activity by l-malate; Echevarria et al., 1994). A high IC₅₀ is related to a high degree of PEPC phosphorylation.
Protein quantification
Protein amounts were determined by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Statistical analysis
Statistical analysis was carried out using Statistica v. 6.0 (Statsoft Inc.). Pearson coefficients were calculated to assess correlation between different variables. Data were analysed using one-, two-, and three-way analysis of variance (F-test). Data were first tested for normality with the Kolmogorov–Smirnov test and for homogeneity of variance with the Brown–Forsythe test. Significant test results were followed by Tukey tests for identification of important contrasts. The comparison between measurements of fluorescence at dawn and midday and between the means of ambient and elevated CO₂ concentration treatments in all parameters were made by using the Student test (t-test).

Results
Growth analysis
Total dry mass showed a broad optimum at 171 mM NaCl external salinity for both 380 and 700 ppm CO₂ (Fig. 2A); dry mass was substantially reduced at the highest salinity (510 mM NaCl) for both CO₂ concentrations. On the other
hand, total dry mass was higher at 700 ppm CO2 and 0 and 171 mM NaCl than at 380 ppm CO2 for the same salinity treatments (two-way ANOVA: CO2×salinity, F2,32=4.1, P <0.05; Fig. 2A). The same trends were evident in mean relative growth rate (RGR; Fig. 2B), and the effects of salinity and CO2 concentration on RGR were highly significant after 90 d of treatment (two-way ANOVA: CO2×salinity, F2,32=4.1, P <0.05). The peak at 171 mM NaCl was associated with higher values of total leaf area (Fig. 2C) and specific leaf area (Fig. 2D), whereas the lower values of RGR at 510 mM were linked to lower values of unit leaf rate (Fig. 2E). Leaf mass fraction did not show any relationship either with salinity or CO2 treatments, showing values c. 0.7 g g−1 in all cases (data not presented).

Finally, RGR was directly correlated with leaf elongation rate (LER; r=0.84, P <0.0001) and number of tillers (r=−0.81, P <0.0001) at 700 ppm CO2. There were not significant correlations between these parameters at 380 ppm CO2, although they showed similar trends (Fig. 2F, G).

Gas exchange

Overall net photosynthetic rate (A) data were similar for all salinity and CO2 treatments at each of the three measurement times; except at the 30 d treatment, where plants grown at 700 ppm CO2 with 0 and 171 mM NaCl showed higher A values than those at ambient CO2 (t test, P <0.01; Fig. 3A–C). Stomatal conductance (Gs) showed a trend that was extremely similar to that of A (Fig. 3D–F). Nonetheless, Gs values were lower at 700 ppm CO2 after 30 d and 90 d of treatment (t test, P <0.05). Intercellular CO2 concentration (Ci) at 700 ppm CO2 responded differently to salinity at the earlier stages of the experiment than at the later stage: salinity had no effect on Ci after 7 d and 30 d but Ci reached a peak at 171 mM NaCl after 90 d (Fig. 3G–I). Ci values were higher at 700 ppm CO2 at each of the three measurement times for all salinity treatments (t test, P <0.05).

Plants grown at 700 ppm CO2 showed consistently higher water use efficiency (WUE), although significant differences were only recorded at 0 mM NaCl (t test, P >0.05). Leaf water content (WC) was higher at 700 ppm CO2 for all salinity treatments after 90 d of treatment (t test, P >0.0001; Fig. 4). Furthermore, WC was higher at 171 mM NaCl at 700 ppm CO2 concentration (one-way ANOVA: F2,17=23.4, P <0.0001).

Chlorophyll fluorescence

Values of Fv/Fm and quantum efficiency of PSII (ΦPSII) at dawn were high at both 380 ppm and 700 ppm CO2 at all external NaCl concentrations after 7, 30, and 90 d of treatment, varying between 0.81 and 0.84 for Fv/Fm, and between 0.80 and 0.83 for ΦPSII. Fv/Fm and ΦPSII, respectively, were always lower at midday and the reductions resulted mainly from lower values of Fm and qP (data not presented), respectively, at midday than at dawn (t test, P <0.05). On the other hand, the midday Fv/Fm values at both CO2 concentrations decreased during the course of the

Fig. 3. Net photosynthetic rate, A (A–C), stomatal conductance, Gs (D–F), and intercellular CO2 concentration, Ci (G–I) in randomly selected, fully expanded penultimate leaves of Spartina densiflora in response to treatment with a range of NaCl concentrations at ambient and elevated CO2 concentration after 7 d (A, D, G), 30 d (B, E, H), and 90 d (C, F, I). Values represent mean ±SE, n=10.
experiment, especially in plants grown with 700 ppm CO₂; again as a consequence of lower values of \( F _ { m } \) (Fig. 5A–C).

The effects of salinity and of CO₂ concentration on \( F _ { / } F _ { m } \) at midday were highly significant after 90 d of treatment (two-way ANOVA: \( \text{CO}_2 \times \text{salinity}, F_{2,41}=5.0, P<0.05; \) Fig. 5C). Moreover, \( \Phi _{PSII} \) values at midday increased with external salinity in plants grown at 380 ppm CO₂ after 90 d of treatment (one-way ANOVA; \( F_{2,46}=4.0, P<0.05; \) Fig. 5F); and \( \Phi _{PSII} \) values were lower at 700 ppm CO₂ in the presence of NaCl than at 380 ppm CO₂ (\( t \)-test, \( P<0.05 \)).

Finally, plants treated with both CO₂ concentrations maintained nearly constant \( NPQ \) at each of the three measurement times, irrespective of the salinity treatment (c. 1; \( P>0.05 \)).

Photosynthetic pigments

The effects of salinity and CO₂ concentration on pigment concentrations (Chl \( a \), Chl \( b \), and Cx+c, all in \( \mu g \ g^{-1} \ FW; \) Fig. 6A–C) were highly significant after 90 d of treatment (two-way ANOVA, \( \text{CO}_2 \times \text{salinity}; \) Chl \( a, F_{2,23}=17.7, P<0.0001; \) Chl \( b, F_{2,22}=4.6, P<0.05; \) Cx+c, \( F_{2,23}=5.0, P<0.05 \)). Pigment concentrations increased with increasing salinity treatment at both CO₂ concentrations (one-way ANOVA, \( P<0.05 \)). Contrary to that, leaf nitrogen content diminished with increasing salinity (Fig. 6D). Furthermore, plants treated with NaCl showed higher Chl \( a \) and \( b \) concentrations at 380 ppm CO₂ than under 700 ppm CO₂ concentration (\( t \)-test, \( P<0.0001 \)). In the case of carotenoids, there were not any differences between CO₂ treatments (\( P>0.05 \)).

Determination of sodium, potassium, calcium, magnesium, zinc, and nitrogen

Overall, the mineral (ash) contents of both leaves and roots were higher at 380 ppm CO₂, and increased with increasing external NaCl concentration. Likewise, ash content was greater in roots than in leaves (three-way ANOVA, \( \text{CO}_2 \times \text{salinity} \times \text{tissue} ; F_{2,23}=3.7, P<0.05 ; \) Table 1).

By the end of the experiment, tissue Na concentrations were greater in leaves than in roots, and increased markedly.
with external NaCl concentration (t test, $P < 0.001$). By contrast, leaf K, Ca, and Mg concentrations decreased with increasing salinity at both CO2 treatments. In addition, leaf and root K, Ca, and Mg concentrations were higher at 380 ppm CO2 (t test, $P < 0.05$).

On the other hand, tissue zinc concentration was greater at 380 ppm CO2 (t test, $P < 0.0001$) and Zn content was higher in the roots than in the leaves at ambient CO2 concentration (Table 1).

Finally, C/N ratio was considerably higher in leaves than in roots for all salinity and CO2 treatments (three-way ANOVA, $P < 0.0001$). Tissue C/N ratio increased with external NaCl concentration at 380 ppm CO2. Furthermore, C/N ratio was greater at 380 ppm CO2 for leaves and at 700 ppm CO2 for roots.

**PEPC activity**

The effects of salinity and of CO2 concentration on PEPC activity were significant after 90 d of treatment (two-way ANOVA: CO2 $\times$ salinity, $F_{2,18}=3.8$, $P < 0.05$). The lowest value of PEPC activity was recorded at 380 ppm CO2 and 171 mM NaCl, and the highest value at the same salinity and elevated CO2 concentration (Fig. 7A). Contrary to that, IC$_{50}$ for malate was higher at 380 ppm CO2 and 171 mM NaCl, and lower at 700 ppm CO2 and the same salinity (Fig. 7B).

**Discussion**

Significant long-term (i.e. months) effects of CO2 concentration on the growth of the C4 Spartina densiflora were observed, with plants grown at elevated CO2 concentration producing 35% and 20% more biomass, at 0 and 171 mM NaCl, respectively, than their ambient CO2-grown counterparts; although this effect was counterbalanced by high salinity (510 mM NaCl), recording similar total dry mass and RGR at ambient and at elevated CO2 concentrations. This response was apparent as the RGR of ash-free dry mass, total leaf area, leaf elongation rate and, by inference, the number of tillers produced. Castillo et al. (2005) found the highest rate of leaf elongation of S. densiflora in distilled water. Nevertheless, in our experiment, the growth at 171 mM NaCl was slightly higher than in the absence of salt under both CO2 concentrations. In this and other studies, the absence of salt has been proved to affect neither the photosynthetic function of S. densiflora nor its growth (Mateos-Naranjo et al., 2008b).

Enhanced growth at elevated CO2 concentration disagrees with results reported by Lenssen et al. (1993), who found that elevated CO2 concentration reduced plant weight of Spartina anglica by 20%, this reduction being associated with decreased SLA. In the current study, the stimulation of growth in S. densiflora at elevated CO2 concentration and 171 mM NaCl was linked to higher SLA, while leaf mass fraction was not affected by either salinity or CO2 concentration. The increase of SLA with CO2 concentration and salinity could be mediated by an improvement in water relations and/or an induction of cell expansion. Rozema et al. (1991) found that a higher turgor pressure might stimulate leaf expansion. In this way, higher leaf water content was observed in S. densiflora at elevated CO2 concentration in the presence of salt. On the other hand, De Souza et al. (2008) found that elevated CO2 concentration in sugarcane induced cell expansion through the action of XTH (xyloglucan endotransglycosylase/hydrolase) on leaf cell walls. A similar effect was observed by Ferris et al. (2001) for Populus species.
Enhanced growth of *S. densiflora* was higher than that reported by Rogers et al. (2008) for C4 invasive plants of *Cyperus rotundus* and *C. esculentus* (10–15%), even higher than the growth stimulation described in the literature for other C4 plants in response to a doubling of the current ambient CO2 concentration under non-saline conditions (22–33%; Ghannoum et al., 2000). On the other hand, Rozema et al. (1991) noted that *Spartina patens* showed higher RGR values at elevated CO2 concentration when plants were treated with low salinity (10 mM NaCl), while RGR of those treated with 250 mM NaCl decreased at 580 ppm CO2 (–48.3%, under aerated conditions in the culture solution). In the case of *S. densiflora*, the decrease of RGR at an elevated CO2 concentration was recorded in plants treated with 510 mM NaCl. Reduced RGR at high salinity can be attributed to lower unit leaf rate. This component of RGR was the most sensitive to salinity and CO2 concentration, underlining the primary importance of the rate of assimilation per unit leaf area.

Little effect of salinity and of CO2 concentration was detected on the net photosynthetic rate of *S. densiflora*. In this regard, starch accumulation has been described as a possible cause of the reduction of a CO2 stimulation of photosynthesis during long-term elevated CO2 concentration levels (DeLucia et al., 1985). However, our results showed lower values of foliar C/N ratio at 700 ppm CO2. Yelle et al. (1989) found in *Lycopersicon esculentum* that

![Fig. 7. PEPC activity (A) and IC50 values for l-malate (B) in crude extracts of illuminated leaves of *Spartina densiflora* in response to treatment with a range of salinity concentrations at ambient and elevated CO2 concentration over 90 d. Values represent mean ± SE, n=5. Different letters indicate means that are significantly different from each other (two-way ANOVA, CO2×salinity; Tukey test, P <0.05).](https://academic.oup.com/jxb/article-abstract/61/6/1643/457966)
acclimation to elevated CO\textsubscript{2} concentration was not a result of starch accumulation but was instead related to decreased activity of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco).

Contrastingly, De Souza et al. (2008) found that \textit{A} of sugarcane decreased because of root growth limitation after 22 weeks at 720 ppm CO\textsubscript{2}, since it is known that root growth limitation can result in lower photosynthetic rates (Arp, 1991). Thus, roots of \textit{S. densiflora} occupied 5% less of the volume of the pot for all treatments after three months, so pot size did not limit root growth.

On the other hand, different works have shown that one of the most consistent responses of C\textsubscript{4} plant species to elevated atmospheric CO\textsubscript{2} concentration is a decrease in \textit{G}_{s} (Ainsworth and Rogers, 2007). In the case of \textit{S. densiflora} this decrease was only recorded after 30 d and 90 d of treatment. Robredo et al. (2007) explained that increased \textit{C}_{i} originated by the elevated CO\textsubscript{2} concentration could promote partial stomatal closure, although the mechanism whereby stomata respond to the CO\textsubscript{2} signal remains unknown. Nevertheless, higher \textit{C}_{i} values of \textit{S. densiflora} at elevated CO\textsubscript{2} were also recorded after 7 d of treatment without there being a lower \textit{G}_{s}, which could be explained by the lower \textit{A} values.

By contrast, the levels of PEPC protein of \textit{S. densiflora} leaves were high at elevated CO\textsubscript{2} concentration. Intermediate salinity and CO\textsubscript{2} concentration enhanced PEPC activity, which could also contribute to the acclimation of \textit{A} to CO\textsubscript{2} enrichment. On the other hand, the lower PEPC activity recorded at 171 mM NaCl and ambient CO\textsubscript{2} concentration was counterbalanced by a higher activation of PEPC (see \textit{IC}_{30} for malate). This response has previously been reported by Echevarria et al. (2001) and García-Mauriño et al. (2003), who found that PEPC-kinase activity of \textit{Sorghum} increased in salt-treated plants. Contrary to this, Sankhla and Huber (1974) found that high concentrations of Na within the plants might limit the activity of PEPC. Nevertheless, PEPC activity of \textit{S. densiflora} was not limited by the progressive accumulation of Na\textsuperscript{+} in root and shoots, with increasing external salinity, at either CO\textsubscript{2} concentration treatment.

Accumulation of Na\textsuperscript{+} with increasing external salinity concentration was accompanied by the decrease of leaf K, Ca, and Mg levels. Similar results have been recorded for other halophytes (Khan et al., 2000; Redondo-Gómez et al., 2007). The higher mineral (ash) contents reported at ambient CO\textsubscript{2} concentration could be accounted for by the higher leaf and root K, Ca, Mg, and Zn concentrations. Rogers et al. (1999) reported that plant nutrient concentrations often decline under elevated CO\textsubscript{2} concentration, and this decline can be ascribed to the dilution effect caused by increases in biomass. In the case of \textit{S. densiflora}, this decline could be also ascribed, to a great extent, to the dilution effect caused by the higher water content found at elevated CO\textsubscript{2}. Ainsworth and Rogers (2007) explained this decline by a reduction of leaf transpiration rate under elevated CO\textsubscript{2} concentration, which may cause a lower flux of nutrient through the soil to the root surface, thereby reducing nutrient uptake. All these possibilities could explain the lower nutrient content recorded in \textit{S. densiflora} under elevated CO\textsubscript{2} concentration.

On the other hand, Randall and Bouma (1973) found that carbonic anhydrase (involved in photosynthesis, facilitating the diffusion of CO\textsubscript{2} through the liquid phase of the cell to the chloroplast) demonstrated a lower efficiency at concentrations approaching CO\textsubscript{2} saturation with Zn deficiency, suggesting that zinc deficiency impairs the biochemical capacity of the plant to fix CO\textsubscript{2}. Nonetheless, net photosynthesis of \textit{S. densiflora} was not affected, instead a lower leaf Zn concentration was recorded at 700 ppm CO\textsubscript{2}.

There was evidence that elevated salinity and CO\textsubscript{2} concentration affected the integrity or function of the photochemical apparatus in the long term, and there was an impact on chlorophyll concentrations in the leaves. Chlorophyll content was enhanced by salinity; although at an elevated CO\textsubscript{2} concentration this enhancement was only recorded at 510 mM NaCl. According to Geissler et al. (2009), plants seemed to use the additional energy supply under an elevated CO\textsubscript{2} concentration for increasing the investment in salinity tolerance mechanisms; for example, for reducing oxidative stress and water loss. The NaCl-induced increase in the chlorophyll level has been previously reported in \textit{S. densiflora} (Castillo et al., 2005). Furthermore, as in our experiment, Chen et al. (1999) found that the impairment of the photosynthetic pigments was greater in crop plants at an elevated CO\textsubscript{2} concentration. These findings seem to point to CO\textsubscript{2} enrichment as an accelerator of pigment degradation and of leaf senescence (Munns, 1993). However, leaf N concentration was not lower at elevated CO\textsubscript{2}, so there was no link between leaf nitrogen and pigment concentration, as would be expected due to the fact that N is mostly contained in chlorophyll molecules (Torres Netto et al., 2005). This may be explained by the previously referred dilution effect, caused by the higher water content found at elevated CO\textsubscript{2}. In fact, the response of leaf N concentration and of water content to salinity and CO\textsubscript{2} concentration were quite similar.

On the other hand, the response of \textit{A} to salinity and to CO\textsubscript{2} concentration did not track that of \textit{Φ}_{\text{PSII}}, since the quantum efficiency of PSII data suggest a long-term negative effect of high CO\textsubscript{2} in the presence of salt. This disparity could have been caused by changes in the relative rates of CO\textsubscript{2} fixation, photorespiration, nitrogen metabolism, and electron donation to oxygen (the Mehler reaction; Fryer et al., 1998). Redondo-Gómez et al. (2010) suggested that photorespiration and cyclic electron transport could be mechanisms to protect \textit{Arthrocnemum macrostachyum} against an excess of radiation under high salinities. Both pathways can lead to the additional consumption of reducing equivalents and can thus function as sinks for excessive excitation energy (Asada, 1996). These two physiological processes could be relevant mechanisms to protect \textit{S. densiflora} against an excess of radiation under high salinities, since, as in the case of \textit{A. macrostachyum}, the relatively stable \textit{NPQ} across the salinity range could
indicate that salt does not cause an increase in thermal dissipation in the PSII antennae. By contrast, Chen et al. (1999) reported enhanced \( \Phi_{\text{PSII}} \) yield at 700 ppm \( \text{CO}_2 \) without additional \( \text{NaCl} \) in the nutrient solution.

The maximum quantum efficiency of PSII photochemistry \( (F_v/F_m) \) did show a significant reduction at midday compared to dawn values. This midday depression of \( F_v/F_m \) is greater in the long term. It was also dependent on salinity treatment, in the same way as \( \Phi_{\text{PSII}} \). The decreased \( F_v/F_m \) values at midday with the duration of treatment and the \( \text{NaCl} \) and \( \text{CO}_2 \) concentration could have been caused by a lower proportion of open reaction centres (lower values of \( F_m \)), which could be attributed to a decrease in chlorophyll \( a \) content. The fact that photoinhibition was not more severe in salt-adapted plants, even when exposed to high light, suggests that they have mechanisms by which excess energy is dissipated safely (Qiu et al., 2003).

In summary, the comparison of growth and photosynthetic responses of \( S. \ densiflora \) has provided a new insight into the response to climatic change and rising atmospheric \( \text{CO}_2 \) concentration in a competitive coastal invader, which experiences salinity levels as high as those present in seawater. Differences in growth rate over this range of salinity can be accounted for by the ability to develop and maintain an assimilatory surface area combined with an improvement of water relations at elevated \( \text{CO}_2 \) concentration, rather than by variations in net photosynthetic rate. Salinity and \( \text{CO}_2 \) concentration have a marked effect on the photochemical (PSII) apparatus in the long term, but not on photosynthesis. However, the absence of a \( \text{CO}_2 \)-enrichment effect on net photosynthesis might be explained by enhanced PEPC carboxylation capacity. By contrast, photosynthetic pigments seem to be adversely affected by elevated \( \text{CO}_2 \) concentration in the presence of NaCl. Finally, our results suggest that the productivity of this invader might increase in a scenario of future increase in atmospheric \( \text{CO}_2 \) concentration for plants growing at salinities ranging between 0 mM and 171 mM NaCl. Nevertheless, the salt stress that may be experienced by plants growing at 510 mM NaCl would offset this fertilizer effect.

**Acknowledgements**

We are grateful to Mr F Fernández-Muñoz for technical assistance. We also thank the Spanish Science and Technology and Environmental Ministries for their support (projects PCI2006-A7-0641 and 042/2007 Organismo Autónomo Parques Nacionales, respectively), and Seville University Greenhouse General Service for collaboration.

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