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Research paper

Drought stress obliterates the preference for ammonium as an N source in the C_4 plant *Spartina alterniflora*

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ABSTRACT

The C_4 grass *Spartina alterniflora* is known for its unique salt tolerance and strong preference for ammonium (NH_4^+) as a nitrogen (N) source. We here examined whether *Spartina*'s unique preference for NH_4^+ results in improved performance under drought stress. Manipulative greenhouse experiments were carried out to measure the effects of variable water availability and inorganic N sources on plant performance (growth, photosynthesis, antioxidant, and N metabolism). Drought strongly reduced leaf number and area, plant fresh and dry weight, and photosynthetic activity on all N sources, but the reduction was most pronounced on NH_4^+. Indeed, the growth advantage seen on NH_4^+ in the absence of drought, producing nearly double the biomass compared to growth on NO_3^-, was entirely obliterated under both intermediate and severe drought conditions (50 and 25% field capacity, respectively). Both fresh and dry weight became indistinguishable among N sources under drought. Major markers of the antioxidant capacity of the plant, the activities of the enzymes superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, showed higher constitutive levels on NH_4^+. Catalase and glutathione reductase were specifically upregulated in NH_4^+-fed plants with increasing drought stress. This upregulation, however, failed to protect the plants from drought stress. Nitrogen metabolism was characterized by lower constitutive levels of glutamine synthetase in NH_4^+-fed plants, and a rise in glutamate dehydrogenase (GDH) activity under drought, accompanied by elevated proline levels in leaves. Our results support postulates on the important role of GDH induction, and its involvement in the synthesis of compatible solutes, under abiotic stress. We show that, despite this metabolic shift, *S. alterniflora*'s sensitivity to drought does not benefit from growth on NH_4^+ and that the imposition of drought stress equalizes all N-source-related growth differences observed under non-drought conditions.

1. Introduction

Under natural conditions of growth and development, plants are inevitably exposed to multiple stresses, such as drought, salinity, flooding, mineral deficiencies, and toxicity (Ben Hamed et al., 2013). Of these, drought is considered one of the most formidable challenges to agricultural productivity (Mahajan and Tuteja, 2005; Hessini et al., 2008, 2009b), and the greatest losses in productivity occur in arid and semiarid regions, where, in addition to scarcity, the quality of irrigation water is often low (Fernández-Cirelli et al., 2009).

Drought inhibits plant growth by disturbing the uptake of ions and water, impeding N-metabolism, and causing oxidative stress (Gonzalez et al., 1998; Bhargava and Sawant, 2013). The extent of damage depends on plant genotype, the severity of the stress, and the type, quantity, and regime of fertilization (Hessini et al., 2009a; Waraich et al., 2011, 2012). The use of fertilizer to enhance crop productivity has increased five-fold since the 1960s, and about 65% of it is used on cereals. However, inadequate or inefficient fertilization perturbs plant growth and contributes to soil degradation

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; EL, electrolyte leakage; FC, field capacity; GR, glutathione reductase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GPX, guaiacol peroxidase; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase.

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(Humbert et al., 2013), and it is predicted that access to fertilization will be one of the main challenges for crop production in drought-prone areas (Shiferaw et al., 2014).

Nitrogen (N), the most important macronutrient obtained by plant roots, is deficient in most soils, and in particular those in arid and semiarid regions (Hernández et al., 1997). It is critical to all principal metabolic processes, including those related to osmotic adjustment, and constitutes almost 80% of the total nutrients absorbed by plant roots (Marschner, 1995). Plants take up N mainly in two forms: nitrate (NO_3^-) and ammonium (NH_4^+), or as mixtures of the two. Great differences exist between species in their preference for the sources of N, with most species growing best on either NO_3^- or a mixed N source (Kronzucker et al., 1999), while only few perform best on NH_4^+ (Kronzucker et al., 1997; Britto and Kronzucker, 2002; Britto and Kronzucker, 2013).

Plant response to N fertilization under drought conditions varies with plant species, climate, N source, and fertilization regime (Waraich et al., 2011). Nitrate may not always be beneficial under drought, as it can accumulate in plant leaves without contributing to biomass or to increasing yield (Martinoia et al., 1981; Bernguer et al., 2009). However, the NO_3^- ion can also serve as an electron sink and potentially alleviate photosystem stress under water limitation conditions (Yi et al., 2014). Plants with high tissue NO_3^- levels also lose nutritional value because, when consumed in excess, NO_3^- can be harmful for human and livestock health (Britto and Kronzucker, 2002; Hessini et al., 2009b). Thus, there is interest in identification and development of drought-tolerant plant genotypes able to utilize NH_4^+ as their principal N source. Indeed, the addition of NH_4^+ to the nutrient solution has been reported to mitigate the adverse effects of drought on growth and development of rice (Gao et al., 2010). NH_4^+ has also been reported to mitigate the effects of salt stress on *Hordeum vulgare*, *Citrange carrizo*, and *Spartina alterniflora* (Kant et al., 2007; Fernández-Crespo et al., 2012; Hessini et al., 2013), although others have observed the opposite effect, as, for instance, in pea (Speer et al., 1994; Speer and Kaiser, 1994).

Although the mechanism by which NH_4^+ may enhance plant tolerance to osmotic stress is not clear, several authors consider it a result of: (i) NH_4^+ assimilation carrying a lower energy cost than that of NO_3^- (Kant et al., 2007); (ii) increased plant water absorption (Gao et al., 2010); (iii) the activation of antioxidant enzymes responsible for some mechanisms of early acclimation to stress (Misra and Gupta, 2006; Fernández-Crespo et al., 2012).

Spartina alterniflora is an interesting test species due to its C_4 photosynthetic habit and high tolerance for environmental stresses. Due to these characteristics, *Spartina* is sometimes an invasive species that can disturb natural ecosystems. In this study, we explore the mechanism for drought tolerance in this species and report the effects of ammonium nutrition on the species in the light of the responsiveness of its antioxidant systems and shifts in N metabolism that may be required for acquisition of drought tolerance.

2. Material and methods

2.1. Plant material and propagation

The plants used in this experiment were obtained from 25-cm-high cuttings transplanted into 4-L blow-moulded pots (one cutting per pot) filled with sandy soil and irrigated with Hewitt (1966) nutrient solution for one month under well-watered conditions, in a greenhouse with an average air temperature of 25/18 °C day/-night, an air relative humidity ranging between 65 and 90%, an average irradiance at mid-day of ~900/1800 μmol m^{-2} s^{-1} PAR, and a natural photoperiod of 12–15 h. In order to prevent nitrification, 4 μL L^{-1} Nitrapyrin (Nserve; Dow Chemical Co., Kings Lynn, England) and 7.5 μL L^{-1} dicyandiamide (DCD; Sigma Chemicals, St. Louis, MO) were added to the nutrient solution. Nitrogen was added as either calcium nitrate [$Ca(NO_3)_2$] or ammonium sulphate [$(NH_4)_2SO_4$] or a mixture of NH_4^+ and NO_3^- in a ratio of 1:1 [NH4NO3] (N concentration was 7.5 mM in total), with three superimposed drought regimes (100%, 50%, and 25% field capacity). The field capacity (FC) of soil was estimated according to the technique of Bouyoucos (1983). To maintain 100% field capacity, plants were rewatered to the corresponding weight every day with the above-mentioned nutrient solution. Soil water contents (SWC, %) determined in the treatments of 100, 50, and 25% FC were 11.5, 5.75, and 2.88%, respectively. Evaporation from the soil surface was prevented by enclosing all pots in plastic bags sealed at the base of each seedling. In addition, ten pots without plants were used to monitor evaporative water loss from the soil surface. The medium containing NH_4^+ as the only N source was buffered with 0.33 g $CaCO_3$ per kg soil DW (Cantera et al., 1999). Ten replicate pots were used and the treatments were arranged in a completely randomized design. Soils of control plants were maintained at 100%, while those of drought-exposed plants were kept at 50% (mild stress) and 25% (severe stress) of FC. Sixty days after the onset of the drought treatments, plants were harvested (between 10:00 a.m. and noon) and separated into leaves and roots. The fresh weights (FW) of leaves and roots of each plant were determined immediately after plant collection, as were the number of leaves and leaf surface area (LI-3000A, Li-Cor Nebraska, USA). Sub-samples of fresh shoots and roots were weighed and frozen in liquid N and stored at −80 °C for later metabolite analysis and enzymatic assays. Root and shoot dry weights (DW) per plant were determined after oven-drying samples to constant weight at 60 °C. The water content (WC) was calculated as follows:

$$WC(\%) = [(FW - DW)/FW] \times 100$$

Measurements were carried out on ten plants per treatment.

2.2. Gas exchange measurements

Gas exchange parameters (net CO_2 assimilation rate – A; transpiration rate – E; stomatal conductance – gs; instantaneous water-use efficiency – WUEi, calculated as the ratio A/E) and leaf surface temperature were determined one day before final harvest using a portable gas exchange system (Li-Cor 6200, Li-Cor Nebraska, USA). Measurements were taken from the first fully expanded leaves after they had acclimated to the leaf chamber conditions for 10 min; all the measurements were performed between 10:00 a.m. and 2:00 p.m. (10 replicates per treatment).

2.3. Free amino acids, proline, and total soluble sugars determination

Free amino acid and proline contents were determined by the ninhydrin method as described by Zivcovic et al. (2005). These compounds were extracted from leaf material (0.25 mg) with 85% ethanol. An aliquot (0.2 mL) from the extract was mixed with 1 mL 0.2 M citrate buffer (pH 5.0) containing 0.4 N NaOH, 0.72 mm $SnCl_2$, and 2% ninhydrin in ethylene glycol. The mixture was incubated in boiling water for 20 min. Samples were cooled and diluted in water:propanol (1:1 v/v). The optical density was read with a spectrophotometer (Spectro UVS-2700, Labomed) at 570 and 440 nm to determine free amino acid and proline content, respectively. Leucine and proline were used for calibration curves. Total soluble sugars (TSS) were extracted in 80% ethanol from 1 g fresh leaf and quantified according to Staub (1963) using a spectrophotometer (Sherwood Scientific Ltd., model 259, Cambridge, UK).

2.4. Electrolyte leakage

Electrolyte leakage (EL) was measured as described by Dionisio-Sese and Tobita (1998). Shortly after harvest, fresh samples from the uppermost fully expanded leaf blades of five plants (200 mg) were incubated in test tubes with distilled water at 25 °C for 2 h, and then initial conductivity (EC_1) of the bathing medium was measured. The tubes containing the leaf material were then boiled for 30 min to release all the electrolytes, cooled to 25 °C, and the conductivity (EC_2) was measured again. The electrolyte leakage was calculated as follows:

$$EL = (EC_1/EC_2) \times 100$$

2.5. Hydrogen peroxide

Tissue H_2O_2 was determined as described by Frew et al. (1983). After harvest, fresh samples from the uppermost fully expanded leaf blades of five plants (0.5 g per plant) were quickly frozen in liquid nitrogen, then ground to powder with mortar and pestle, together with 5 mL of 5% (w/v) TCA and 0.15 g activated charcoal. The mixture was centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was adjusted to pH 8.4 with a 17 M ammonia solution, then filtered. H_2O_2 in the extract was allowed to react with 4-aminoantipyrine and phenol, in the presence of a peroxidase. The product, a quinoneimine dye, with a maximum absorption at 505 nm, was photospectrometrically detected at 505 nm. The H_2O_2 concentration was inferred using a calibration curve.

2.6. Determination of antioxidant enzyme activities

For each enzymatic assay, three samples from the first pairs of fully expanded leaves were weighed and finely ground in a mortar with liquid nitrogen, then homogenized with the respective extraction buffer. Homogenates were filtered through two layers of Miracloth (Calbiochem) before being centrifuged for 20 min at 20,000*g* at 4 °C. Aliquots of the extracts were used for enzyme assays.

2.7. CAT and GPX activities

Frozen leaves (0.2 g) were homogenized in a mortar with 2 mL of extraction medium containing 66 mM potassium phosphate buffer (pH 7.0) and 0.1 mM EDTA. CAT activity was determined by following the decrease in absorbance at 240 nm according to Beers and Sizer (1952). The reaction medium contained 60 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2, and leaf extract. GPX was determined according to Castillo et al. (1984) by following the increase in absorbance at 470 nm as a result of guaiacol oxidation. The reaction medium contained 43 mM potassium phosphate buffer (pH 6.1), 8 mM guaiacol, 2 mM H_2O_2, and diluted extracts. The final volume was 1 mL for all reactions.

2.8. APX activity

Samples of leaves (0.2 g) were homogenized with 0.8 mL of extraction medium containing 50 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, 0.2% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM ascorbate as described by Hossain and Asada (1984). APX activity was followed by the decrease in absorbance at 290 nm of a reaction medium containing 50 mM HEPES-NaOH (pH 7.6), 0.25 mM ascorbate, 0.1 mM H_2O_2, and 10–40 μL extract (leaf). Controls with p-chloromercurybenzoate, an APX inhibitor, were included to test for non-specific peroxidases and spontaneous reactions (Ranieri et al., 1996).

2.9. GR and SOD activities

Samples of plant tissues (0.2 g) were homogenized with 0.8 mL of extraction medium containing 5 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.2% (v/v) Triton X-100, 1 mM PMSF, 2 mM DTT, and 0.1% (w/w) polyvinylpolypyrrolidone (PVPP). The supernatant was desalted using a Bio Gel P6-DG (Bio-Rad laboratories), then used to assay the activities of GR and SOD. GR was assayed by the decrease in absorbance at 340 nm (NADPH oxidation). The reaction mixture contained 0.1 M HEPES (pH 7.8), 3 mM $MgCl_2$, 2.5 mM GSSG, 0.2 mM NADPH, and 200 μL sample extract, as described by Edwards et al. (1990). The oxidation rate was corrected for non-enzymatic oxidation of NADPH by GSSG. SOD activity was measured according to McCord and Fridovich (1969). The superoxide radical was generated during oxidation of xanthine in a reaction catalyzed by xanthine oxidase. The reduction of cytochrome c by the superoxide radical is inhibited by SOD. One unit of SOD activity was required to inhibit the reduction rate, followed at 550 nm, by 50%.

2.10. Determination of GS and GDH activities

Enzymatic assays were carried out on mixtures of the third and fourth pairs of fully expanded leaves. GS (EC 6.3.1.2) was extracted using the same proportion of plant material to volume of extractant (1/1; w/v). The extraction medium contained: 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol, 5 mM dithiothreitol, 10 mM $MgSO_4$, 1 mM cystein, and 0.6% polyvinylpyrrolidone. Extracts were filtered and centrifuged at 35,000*g* for 15 min, and then the supernatants were assayed. GS activity was assayed as the ligase reaction at 30 °C in a medium containing 60 μmol imidazole pH 7.0, 1 μmol $MnCl_2$, 2.6 μmol Na_2HAsO_4, 1.5 μmol ADP, 65 μmol glutamine, 50 μmol NH_2OH, pH 7.0, and enzyme in a total volume of 0.6 mL. The reaction was started by adding NH_2OH to the medium and stopped by adding 0.5 mL of 0.6 M $FeCl_3$ in 2.5 M HCl. Activity was assessed from absorbance at 546 nm.

To analyse GDH, shoots and roots were ground in a mortar with liquid nitrogen, and then the powder (5 g material to 5 mL medium) was extracted with buffer containing 100 mM maleic acid-KOH (pH 6.8), 100 mM sucrose, 2% 2-mercaptoethanol, and 15% ethylene glycol. Extracts were ground in a mortar with liquid nitrogen and then filtered and centrifuged at 35,000*g* and 4 °C for 10 min. The supernatant was used to determine the enzyme in the aminating direction by following the absorption change at 340 nm induced by NADH oxidation (Groat and Vance, 1981).

2.11. Statistical analysis

Data were analysed using an analysis of variance (ANOVA) at a significance level of $p < 0.05$. The model is defined on the basis of fixed effects and hierarchical classification criteria. The main effects were taken to be drought and nitrogen form, as well as their interactions. A similar two-way analysis of variance (ANOVA) was performed for data obtained with three levels of soil drought and form of nitrogen as the main factors. When the ANOVA was significant at $p < 0.05$, Duncan's Multiple Range Test was used to compare means. All statistical analyses were carried out using SAS software.

3. Results

3.1. Growth

Under well-watered conditions (100% FC), plant biomass accrual responded strongly to the N source present in the root medium. The biomass of NO_3^--fed plants was only half that of those fed with NH_4^+, while NH_4NO_3-fed plants had intermediate biomass

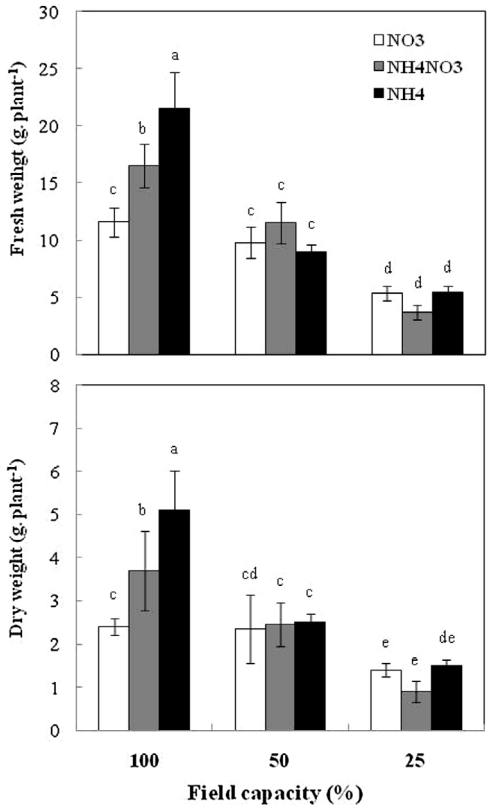

Fig. 1. Effect of drought and nitrogen form on fresh weight (FW, g plant^{-1}) and dry weight (DW, g plant^{-1}) in *S. alterniflora*. Each data point is the mean ± SD of ten replicates per treatment. Values with different letters are significantly different at p = 0.05 level (Duncan's multiple-range test).

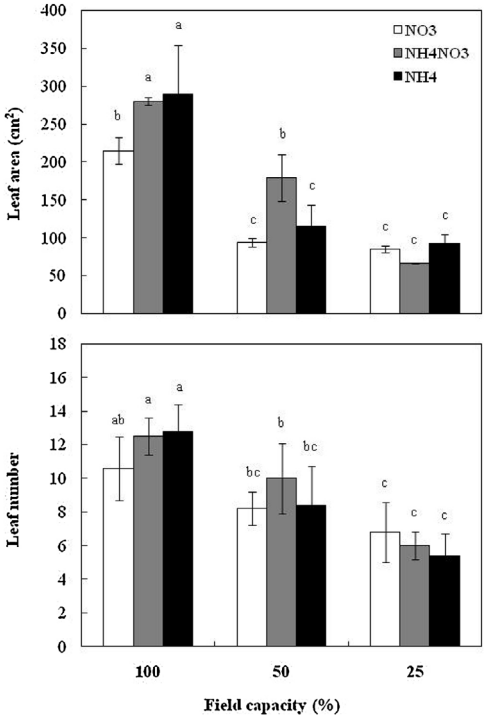

Fig. 2. Effect of drought and nitrogen form on leaf area (cm^2 plant^{-1}) and leaf number, in *S. alterniflora*. Each data point is the mean ± SD of ten replicates per treatment. Values with different letters are significantly different at p = 0.05 level (Duncan's multiple-range test).

(Fig. 1). Under drought conditions (soil moisture 50 or 25% FC), plant biomass accumulation became similar on all three N-source treatments, and at 50% FC no significant differences from control, NO_3^--fed, plants were observed.

The effect of drought on plant growth was dependent on the degree of stress applied (Table 1, $P \leq 0.05$). Based on plant fresh and dry weights, plant leaf area, and numbers of leaves per plant, and with the exception of NO_3^--fed plants grown in soils at 50% FC, the plant response to drought was dependent on the level of stress imposed (Figs. 1 and 2; $P \leq 0.05$). At the end of the experiment (after 60 days of drought), the total fresh and dry plant weights were strongly reduced (Fig. 1, $P \leq 0.05$) by drought, being more pronounced in the severe drought treatment. In accordance with this result, leaf area, number of leaves, and leaf water content were also strongly affected by drought (Figs. 2 and 3; $P \leq 0.05$). Under control conditions, plant growth increased with increasing NH_4^+ in the nutrient solution.

3.2. Photosynthesis, transpiration, stomatal conductance, leaf temperature, and water use efficiency

The effects of N source and drought on photosynthetic capacity, transpiration rate, and leaf temperature of *S. alterniflora* were assessed at the end of the experimental period (Table 2). Irrespective of the N form, net CO_2 assimilation (A), transpiration rate (E), and stomatal conductance (gs) were strongly reduced under drought conditions (Table 2, $P \leq 0.05$). Instantaneous water-use efficiency (WUEi) and the difference between ambient temperature and leaf temperature increased more in plants subjected to severe than to mild drought stress (Table 2, $P \leq 0.05$). This increase

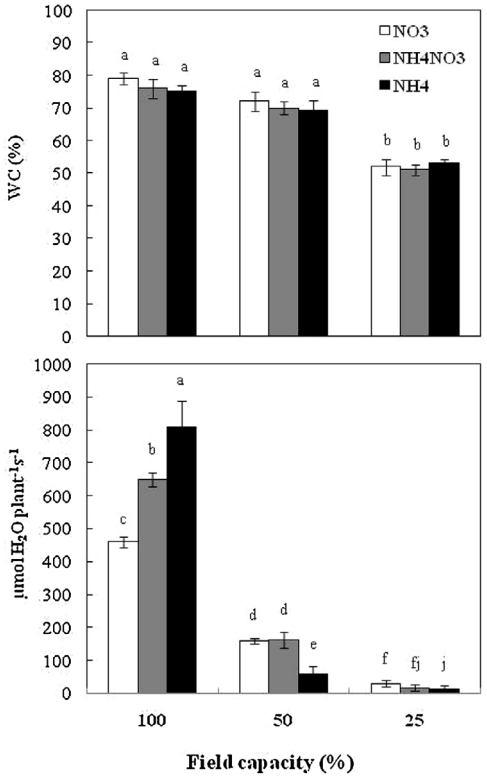

Fig. 3. Effect of drought and nitrogen form on leaf water content (WC, %) and water loss (mmol H_2O plant^{-1} s^{-1}) in *S. alterniflora*. Each data point is the mean ± SD of ten replicates per treatment. Values with different letters are significantly different at p = 0.05 level (Duncan's multiple-range test).

Table 1
Two-way ANOVA analysis of N form, drought, and their interactions for fresh and dry weight, leaf number and area, TAA (total amino acids) TSS (total soluble sugars), proline, ions, organic N, photosynthetic parameters, mean leaf temperature −air temperature (Tc-Ta), H_2O_2, EL, antioxidant and N metabolic enzyme activities.

	Source of variation		
	N form	Drought	Drought × N form
Fresh weight	9.85^{*}	18.42^{***}	2.76^{*}
Dry weight	5.73^{ns}	8.49^{**}	2.04^{ns}
Leaf area	3.32^{*}	30.15^{***}	3.38^{*}
Leaf number	1.96^{ns}	14.86^{***}	0.48^{ns}
A	1.35^{ns}	163.59^{***}	3.64^{*}
E	19.16^{***}	22.65^{***}	2.88^{*}
gs	10.21^{**}	28.17^{***}	0.04^{ns}
WUEi	20.42^{***}	1.49^{ns}	3.12^{*}
Tc-Ta	12.47^{**}	7.23^{**}	0.38^{ns}
H_2O_2	3.77^{*}	190.10^{***}	2.47^{ns}
EL	7.35^{**}	23.25^{***}	0.33^{ns}
TAA	2.34^{ns}	29.03^{***}	11.59^{***}
Proline	8.12^{**}	49.18^{***}	4.11^{*}
TSS	24.84^{***}	48.46^{***}	3.75^{*}
SOD	15.65^{***}	1.41^{ns}	0.08^{ns}
CAT	75.65^{***}	8.32^{**}	2.59^{ns}
APX	21.62^{***}	2.68^{ns}	0.59^{ns}
GR	6.49^{*}	0.16^{ns}	0.52^{ns}
GPX	2.16^{ns}	2.12^{ns}	1.40^{ns}
GPX	11.47^{***}	92.17^{***}	3.68^{*}
GDH	0.06^{ns}	20.73^{***}	1.26^{ns}

ns-non significant, **- significant at $P<0.01$, ***- significant at $P<0.001$.

was more pronounced in NH_4^+- than in NO_3- or NH_4NO_3-fed plants (Table 2, $P \leq 0.05$).

3.3. Organic solute contents

Table 3 shows the effect of nitrogen form and drought on content of organic solutes (total amino acids, proline, and total soluble sugars) in *Spartina alterniflora* leaves. Irrespective of the N form, drought significantly increased the amount of proline (and total amino acids in general). However, it did not influence the content of soluble sugars (Table 3, $P \leq 0.05$).

Under drought, the presence of ammonium enhanced the leaf accumulation of proline and total amino acids (Table 3, $P \leq 0.05$)

3.4. Evaluation of oxidative stress

Regardless of the N form, drought induced a significant increase in the H_2O_2 content of *S. alterniflora* leaves. This effect was more pronounced at severe than at mild drought stress (Fig. 4A, $P \leq 0.05$). Membrane damage also increased, as assessed by solute leakage as drought level increased. The damage to drought-stressed plants

Table 3
Effect of nitrogen form and drought on total amino acids (TAA, μmol g^{-1} FW), proline (μmol g^{-1} FW), and total soluble sugars (TSS, μmol g^{-1} FW) in leaves of *Spartina alterniflora*.

Treatments	N-form	TAA	Proline	TSS
100% FC				
	NO_3^-	2.5 ± 0.1de	0.1 ± 0d	0.1 ± 0
	NH_4NO_3	2.4 ± 0.4de	0.2 ± 0.03d	0.2 ± 0
	NH_4^+	2.3 ± 0.3e	0.3 ± 0.2c	0.2 ± 0
50% FC				
	NO_3^-	2.5 ± 0.5de	0.3 ± 0.1c	0.2 ± 0
	NH_4NO_3	2.9 ± 0.2de	0.6 ± 0.2b	0.2 ± 0
	NH_4^+	5.7 ± 1b	0.6 ± 0.1b	0.3 ± 0
25% FC				
	NO_3^-	4.5 ± 0.5c	0.6 ± 0.1b	0.3 ± 0
	NH_4NO_3	3.1 ± 0.1d	0.8 ± 0.1a	0.3 ± 0
	NH_4^+	6.6 ± 0.3a	0.9 ± 0.2a	0.3 ± 0

Values represent the mean ± SE of ten replicates per treatment. Different letters within the same column indicate significant differences between treatments at $P \leq 0.05$.

was higher with ammonium than with nitrate nutrition (Fig. 4B, $P \leq 0.05$).

3.5. Activities of antioxidant enzymes

Regardless of the N regime, SOD activity did not change significantly in response to drought stress. However, total SOD activity was always higher (±3%) in leaves of NH_4^+- than of NO_3- or NH_4NO_3-fed plants (Fig. 5A). CAT followed the same pattern of activity (Fig. 5C). The activities of APX and GR in NO_3- and NH_4NO_3-fed plants did not change in response to drought. However, ammonium-fed control plants tended to have higher APX and GR activities, which increased under drought conditions (Fig. 5B and D, $P \leq 0.05$). Finally, GPX activity was not observed to respond to N source or drought (Fig. 5E, $P \leq 0.05$).

3.6. Nitrogen metabolism

GS activity in leaves of NO_3-fed plants was higher under mild drought stress than in those not exposed to drought and those under severe drought stress. However, in both mixed-fed and ammonium-fed plants, this activity increased under mild drought stress, but was strongly reduced under severe drought stress. In general, the supply of NH_4^+ to the nutrient solution partially inhibited the activity of GS (Fig. 6A, $P \leq 0.05$).

Regardless of the form of N applied, drought stress induced an increase in GDH activity in leaves of *S. alterniflora*. Under drought, the total activity of GDH was higher in ammonium-fed than in

Table 2
Effect of nitrogen form and drought on photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (gs), instantaneous water use efficiency (WUEi, mol CO_2 m^{-2} s^{-1}/(mmol H_2O m^{-2} s^{-1})) and mean leaf temperature −air temperature (Tc-Ta) in *Spartina alterniflora* plants.

Water treatments	N-form	A	E	gs	WUEi (A/E)	Tc-Ta (°C)
100% FC						
	NO_3^-	12.5 ± 0.7a	2.2 ± 0.2b	31.5 ± 5.4a	5.7 ± 0.6	4.2 ± 0.5e
	NH_4NO_3	13.4 ± 1.2a	2.3 ± 0.1b	32.5 ± 2.1a	5.8 ± 0.4	5.5 ± 0.2 cd
	NH_4^+	13.6 ± 0.9a	2.9 ± 0.4a	35.6 ± 8a	4.7 ± 1.4	4.3 ± 0.6e
50% FC						
	NO_3^-	7.4 ± 1.1b	1.7 ± 0.2c	23.0 ± 2.7b	4.4 ± 0.4	5.0 ± 0.3d
	NH_4NO_3	6.9 ± 0.7b	0.9± 0.1d	22.8 ± 1.3b	7.6 ± 0.5	5.5 ± 0.1 cd
	NH_4^+	4.5 ± 0.3c	0.5 ± 0.1e	28.0 ± 2.8b	9.0 ± 1.0	6.2 ± 0.8bc
25% FC						
	NO_3^-	2.4 ± 0.4d	0.4 ± 0.1e	7.3 ± 1.9c	6.0 ± 0.4	6.5 ± 0.3b
	NH_4NO_3	3.6 ± 0.5 cd	0.3 ± 0.1e	7.3 ± 1.3c	12.0 ± 2.3	6 ± 0.7bc
	NH_4^+	3.3 ± 0.5 cd	0.2 ± 0.1e	10 ± 3.9c	16.5 ± 1.5	7.2 ± 0.1a

Values represent the mean ± SE of ten replicates per treatment. Different letters within the same column indicate significant differences between treatments at $P \leq 0.05$.

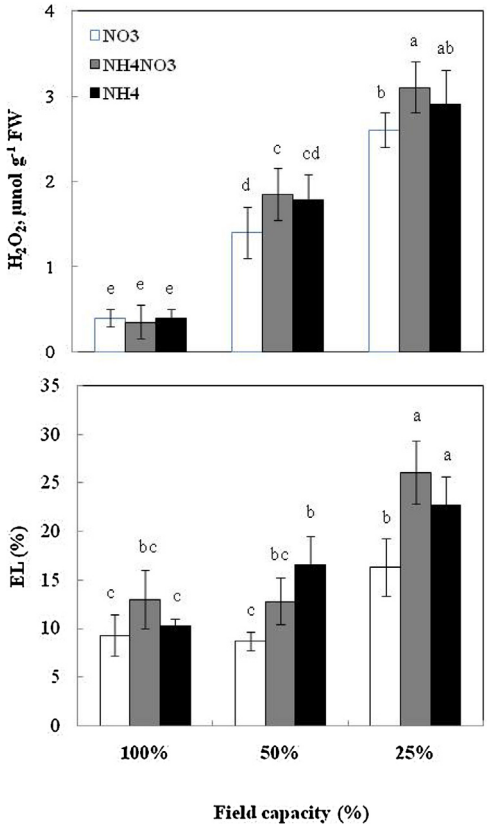

Fig. 4. Effect of drought and nitrogen form on hydrogen peroxide (H_2O_2) concentrations (µmol [g FW]$^{-1}$) and electrolyte leakage (EL) (%) in leaves of *S. alterniflora* plants. Each data point is the mean ± SD of three replicates per plants. Values with different letters are significantly different at the p = 0.05 level (Duncan's multiple-range test).

nitrate-fed plants, being more pronounced under severe drought stress (Fig. 6B, $P \leq 0.05$).

4. Discussion

Drought is a major stress factor for most plants, even for C_4 species, which have a water-use efficiency significantly superior to that of C_3 plants under arid conditions, being able to maintain stomatal closure when water availability is low (Sage, 2004; Osborne and Beerling, 2006). *S. alterniflora*, a C_4 grass, is able to thrive on, acclimate to, and tolerate salinity stress well (Hessini et al., 2013). It is also unique in that it grows best on NH_4^+ as an N source, rather than nitrate or mixed N sources, which are preferred by most species (Kronzucker et al., 1997, 1999, 2000; Britto and Kronzucker, 2002, 2013; Hessini et al., 2013). It is well known that growth on NH_4^+ leads to fundamental shifts in C/N balance, ion homeostasis, hormone balance, and overall performance under stress (Britto and Kronzucker, 2002; Guo et al., 2005; Ariz et al., 2013; Li et al., 2014; Esteban et al., 2016). Ammonium nutrition is associated with higher levels of oxidative stress, and consequent induction of components of the plant antioxidant apparatus, in plants susceptible to NH_4^+ toxicity (Zhu et al., 2000; Bendixen et al., 2001; Guo et al., 2007; Nimptsch and Pflugmacher, 2007; Wang et al., 2010), but it is not known how ammonium-tolerant species respond to this particularly when challenged by drought. Our previous studies demonstrated that, under salt stress, ammonium nutrition results in higher antioxidant defence than nitrate nutrition and thus enhances the tolerance of S. *alterniflora* to salinity (Hessini et al., 2013). In this work we explored the involvement of the antioxidant metabolism of *S. alterniflora* in response to drought stress, and the response of its nitrogen metabolism when *Spartina* is presented with its preferred nitrogen source, NH_4^+, as opposed to NO_3^-, or mixed NH_4NO_3 nutrition. Prior to the imposition of drought stress, NH_4^+ was favourable for the growth of *S. alterniflora* over nitrate-or mixed-fed plants. A similar positive effect of NH_4^+ has been observed in some wetland and marine species (Thursby and Harlin, 1984; Munzarova et al., 2006), as well as in plants in terrestrial habitats where NH_4^+ prevails as N form, such as late-successional temperate and Northern forest soils and flooded rice fields (Kronzucker et al., 1997; Garnett et al., 2001; Britto and Kronzucker, 2002; Munzarova et al., 2006). It must be kept in mind, however, that N-source preference is a complex biological trait without a precise definition (Britto and Kronzucker, 2013), and comparisons of preference are typically conducted under very limited sets of conditions. Strikingly, in our study, despite a clear preference for NH_4^+ by *S. alterniflora*, which produced over twice the biomass of plants grown on NO_3^-, this preference was completely lost when drought stress was superimposed. Indeed, at both mildly and severely lowered water availability, no differences in dry matter accumulation between plants fed with differing N sources could be observed. This result contradicts some previous reports showing that feeding rice with ammonium can significantly increase its drought tolerance (Yang et al., 2012), but is in agreement with many studies that describe the additional stress NH_4^+ can impose when other significant stresses, such as salinity stress, co-occur (Speer et al., 1994; Speer and Kaiser, 1994). Thus, in terms of drought tolerance, *Spartina*'s preference for NH_4^+ fails to translate into improved performance under salinity challenge, as observed in this study. Indeed, the biomass decline under drought was especially pronounced when combined with NH_4^+ nutrition. The reductions in plant biomass were accompanied by changes in plant leaf area (number of leaves and leaf size; Fig. 2), which may have had two consequences: 1) a reduction of carbon fixation due to a decrease in the photosynthetic rate per plant, which may reduce plant tolerance to drought; and 2) a reduction of water loss, which may help the plant cope with drought stress. We observed water loss by *S. alterniflora* plants to be significantly reduced by drought. Ammonium-fed plants were by far the most water-requiring under control (non-drought) conditions, with water loss nearly twice that of nitrate-fed plants, and, notably, the adjustments between morphology (leaf number and leaf area per plant) and physiology (transpiration rate and conductance) brought about by even mild drought stress transformed these into the least water-requiring of all the plants under the various N-source-drought treatments applied (Figs. 2 and 3, Table 2), thus partly explaining the resistance of this species to drought stress.

The decline in plant growth resulting from drought is in part related to changes in whole-plant carbon status (Lopes et al., 2011). The CO_2-concentrating mechanisms of C_4 plants usually allow their photosynthesis to be competitive under conditions that promote carbon loss, by virtue of a lack of photorespiration and superior capacity for stomatal closure. Such conditions, which cause excessive light reactions and limited carbon flux (i.e. "excitation pressure" or "acceptor limitation" in the photosynthetic apparatus), include extreme temperatures, high irradiance, and water deficit (Edwards et al., 2004). We observed that drought reduced the rate of photosynthesis to a greater extent than stomatal conductance, the reduction being more pronounced when ammonium was the sole N source. This decline in photosynthetic activity may therefore be due to not only decreased stomatal conductance, but also non-stomatal factors, including the reduced activity of photosynthetic enzymes, inhibition of chloroplast activity, chlorophyll breakdown, inhibition of nitrate assimilation, induction of early senescence, and changes to leaf anatomy and ultrastructure (Ghannoum, 2009; Ashraf and Harris, 2013). Such behaviour was not observed in salt-treated *S. alterniflora* grown with NH_4^+, even with a 50% drop in

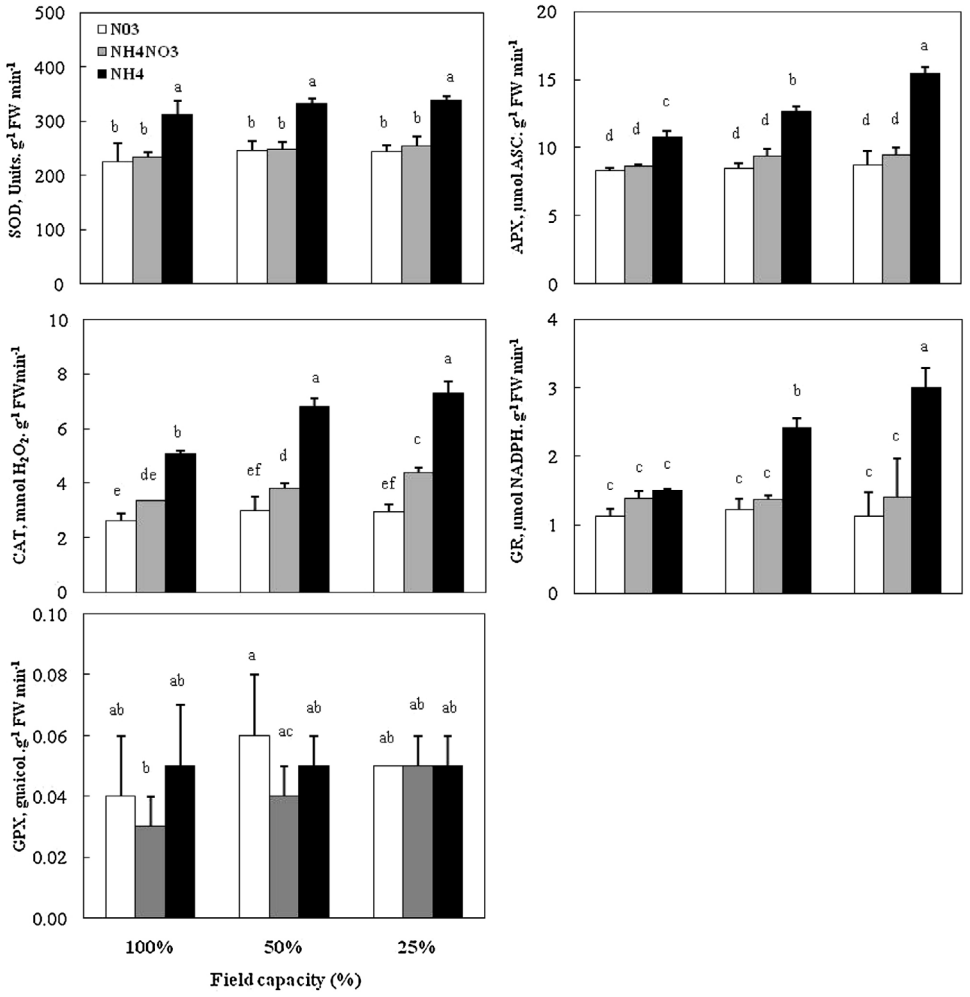

Fig. 5. Effect of drought and nitrogen form on activities of superoxide dismutase (SOD), ascorbateperoxidase (APX), catalase (CAT), glutathione reductase (GR), and andguaiacol peroxidase (GPX) (E) in leaves of *S. alterniflora*. Each data point is the mean ± SD of three replicates per plants. Values with different letters are significantly different at the p = 0.05 level (Duncan's multiple-range test).

stomatal conductance (Hessini et al., 2013). Thus, the highly efficient stomatal control of transpiration by this C_4 species observed under salt stress did not occur under drought stress. The sensitivity of C_4 plants to drought stress has been well reviewed by Ghannoum (2009), who argued that, although the CO_2-concentrating mechanism offers C_4 photosynthesis a greater buffering capacity against CO_2 shortages caused by partial stomatal closure under drought, the biochemistry of C_4 photosynthesis is at least as sensitive as that of C_3 photosynthesis. The reasons for this are not clear. Importantly, in plants of any photosynthetic type that grow on NH_4^+, there is a higher carbon (C) flow requirement, as incoming NH_4^+ must be metabolized immediately to a much greater extent than in the case of NO_3^-, which can be sequestered in vacuoles of both root and shoot cells (Martinoia et al., 1981; Kronzucker et al., 1995). Free NH_4^+concentrations typically remain low in tissue, except under toxicity conditions (Barth et al., 2010; Li et al., 2014). This necessitates the upregulation of anapleurotic pathways (Roosta and Schjoerring 2008a; Ariz et al., 2013) to supply sufficient C, in particular when CO_2 entry through stomata is limited on account of the need to reduce water loss under drought stress. This also explains why higher light intensities can counteract NH_4^+ toxicity as long as stomata can remain open to supply C-skeletons via photosynthesis, but this is frequently nullified by lower leaf numbers and areas in plants grown on NH_4^+ (Gerendas et al., 1997; Guo et al., 2007). However, per-leaf-area CO_2 assimilation rate can be enhanced in NH_4^+-grown plants (Guo et al., 2007), and the CO_2 compensation point can be lower under NH_4^+ than NO_3^- nutrition (Guo et al., 2005). The larger biomass, and leaf number and area, in NH_4^+-grown *S. alterniflora* under control (non-drought) conditions is not maintained under drought conditions; thus, NH_4^+-grown *S. alterniflora* lack the ability to prevent C limitation in the leaf apparatus; indeed, under mild drought, plants grown on a mixed N diet had the largest leaf area, with no N-source-dependent differences at the highest level of drought, and per-leaf-area assimilation was not sufficiently enhanced in NH_4^+-grown plants under drought to compensate for leaf-area decline (Table 2).

In many plants, drought is perceived as an oxidative stress (Helena and Cruz, 2008), and several studies show that growth on NH_4^+ can produce a higher oxidant stress load (Misra and Gupta, 2006; Skopelitis et al., 2006; Wang et al., 2010). However, how plants grown on ammonium protect themselves from oxidative stress remains largely unknown (Bendixen et al., 2001). Domínguez-Valdivia et al. (2008) suggest that, independent of the ammonium tolerance of the plants studied, the stress originating from applying ammonium as the only N source is not an oxidative stress. In contrast, Misra and Gupta (2006) found that NH_4^+-fed plants generate more oxygen radicals than those fed with nitrate. Recently, Podgórska et al. (2013) showed that ammonium nutrition in *Arabidopsis thaliana* increases the NAD(P)H/NAD(P)$^{(+)}$ ratio in leaves, ROS content, and the accumulation of biomolecules oxidized

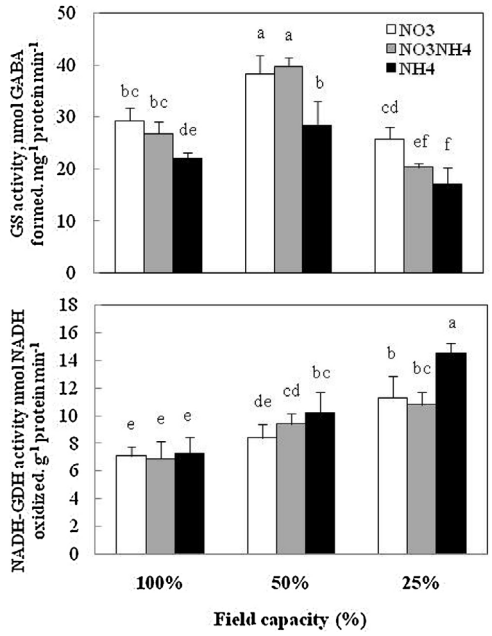

Fig. 6. Effect of drought and nitrogen form on activities of glutamine synthetase (GS, nmol γ-amino butyric acid. mg^{-1} protein min^{-1}) and glutamate dehydrogenase (GDH, nmol NADH oxidized g^{-1} protein min^{-1}) in leaves of individual *S. alterniflora* plants. Each data point is the mean ± SD of three replicates per treatment. Values with different letters are significantly different at p = 0.05 level (Duncan's multiple-range test).

by free radicals. Using H_2O_2 levels in leaves as a proxy for oxidative stress, we observed no clear difference between N sources (Fig. 4a), suggesting that, in terms of oxidative stress, ammonium nutrition is not more stressful for *S. alterniflora* than the other N sources. In all N conditions, a net increase in H_2O_2 and membrane damage was observed when drought stress was imposed, with no significant differences between nitrate- and ammonium-fed plants. Thus, gauged by H_2O_2 production, NH_4^+ appeared to neither aggravate nor improve oxidative stress in *S. alterniflora*.

To counter stress effects, plants have developed oxidation-protection systems such as antioxidant enzymes (SOD, CAT, APX, GPX, and GR). Surprisingly, our study did not observe effects of drought on the activities of these major antioxidant enzymes in *S. alterniflora*, other than when fed with NH_4^+. However, when NH_4^+ and drought were combined, significantly increased activities of principal antioxidant enzymes (SOD, CAT, APX, and GR) were observed. The mechanisms by which ammonium induces an increase in antioxidant activity in NH_4^+-fed plants is unknown, but has been observed in other systems (Wang et al., 2010). Nevertheless, it is documented that the NH_4^+ ion may be a stress signal triggering the activation of enzymes responsible for some early mechanisms of acclimation to stress (Rios-Gonzalez et al., 2002; Misra and Gupta, 2006; Wang et al., 2010). Several studies have indicated that ROS production on NH_4^+ media is higher than under other N regimes (Nimptsch and Pflugmacher, 2007; Wang et al., 2010), while NO_3^-, as an electron sink under photosynthetic C-acceptor limitation, can partially alleviate photostress and subsequent ROS production (Yi et al., 2014). This also leads to the frequently observed higher stress load when other stresses, such as aluminium or salt stress, are combined with growth on NH_4^+ (Tan et al., 1992; Speer et al., 1994; Speer and Kaiser, 1994; *cf.* Hessini et al., 2013). From our study, two points are worth emphasizing: (i) the activities of all major antioxidant enzymes were low under all N and water conditions examined; and (ii) except in NH_4^+-fed plants, these were not upregulated, even under severe drought stress. The non-activation of the SOD, APX, GPX, and GR protective mechanism does not exclude the synthesis of the critical antioxidant metabolites, such as tocopherols, ascorbic acid, and glutathione, and/or the activation of alternative oxidases (Mittler 2002), but it is nevertheless surprising. It should also be emphasized that the synthesis of antioxidants, such as ascorbic acid and glutathione, is carbon-intensive (Ivanov and Edwards, 1997; Wang et al., 2010) and can compete directly with the C-demand for N assimilation; this may, in part, explain the higher antioxidant stress state in NH_4^+-grown plants, as gauged by the antioxidant enzyme response, albeit not by H_2O_2, which appears to serve as a poor proxy for the oxidative stress load. It is also interesting to note that the level of H_2O_2 detected in *S. alterniflora* exposed to drought is similar to that measured when it was exposed to salinity stress, despite very different antioxidant activity states of the enzymes (Hessini et al., 2013). Clearly, despite the preference for the NH_4^+ source of N under non-drought conditions, *S. alterniflora* suffers from elevated oxidative stress on NH_4^+ when carbon flow is impaired by drought, as occurs in non-NH_4^+ specialists.

Drought inhibits plant growth by disrupting several metabolic processes, including N metabolism. However, mild drought can also stimulate the activity of several enzymes involved in N metabolism, such as glutamine synthetase (GS) (Fiasconaro et al., 2012). The observed increase in GS activity in leaves of *S. alterniflora* under mild drought conditions indicates an enhanced capacity to assimilate NH_4^+. Similar findings had already been reported for barley in response to salt stress (Kant et al., 2007). However, during severe or prolonged drought periods, decreased water availability may disturb N metabolism, including GS activity (Foyer et al., 1998). Acclimation to water deficits requires responses that allow primary metabolism to continue. In our study, plants grown on NH_4^+ consistently showed lower GS activities than those grown on NO_3^- or NH_4NO_3; this likely reflects the ability of nitrate to act as a positive signal for GS activity (Foyer et al., 2003; Serapiglia et al., 2008), inducing, in addition to cytosolically-located GS, GS located in plastids (including proplastids in roots), opening up an assimilatory pathway not available on NH_4^+ alone (Redinbaugh and Campbell, 1993; Kronzucker et al., 1999; *cf.* Roosta and Schjoerring, 2008b). However, some flexibilities in the GS machinery, under NH_4^+ nutrition, drawing on two cytosolic forms of the enzyme, at least in the C_4 genus *Sorghum*, have also been reported (El Omari et al., 2010), providing a partial explanation of that genus' superior tolerance for NH_4^+, similar to that of *S. alterniflora,* under non-drought conditions.

There is evidence that NADH-GDH can contribute greatly to the assimilation of NH_4^+ when plants are subjected to harsh conditions (Terce-Laforgue et al., 2004; Kant et al., 2007; Zhonghua et al., 2011). Accordingly, increased GDH activity may be a good indicator of stress. The increased GDH activity observed in ammonium-fed and drought-stressed plants in this study may be due to the ability of the NH_4^+ ion to induce the expression of the gene encoding the a-subunit of GDH and to consequently increase the levels of GDH protein (Turano et al., 1997) and activity (Terce-Laforgue et al., 2004). Furthermore, it has been shown that GDH expression can be induced by elevated ROS levels under abiotic stress (Skopelitis et al., 2006). Our results support this hypothesis since an increase in proline and total amino acids was observed in ammonium-fed and drought-stressed plants. Such shifts toward enhanced GDH activity have been shown to improve performance under salinity in wheat (Wang et al., 2007) and rice (Zhou et al., 2015). In our study, the increase in GDH (Fig. 6) was accompanied by an increase in the stress indicators H_2O_2 and EL (Fig. 4), and in leaf temperature (Table 2), indicating that, under severe water deficit (25% % FC), ammonium-fed *S. alterniflora* was more stressed than when fed with nitrate. For example, the difference between leaf temperature and ambient air temperature increased with the degree of drought stress, particularly in ammonium-fed plants (Table 2),

due to a decrease in the instantaneous transpiration rate and a decrease in plant leaf area (Fig. 2), leading to a drastic decrease in water loss (Fig. 3). The difference between leaf temperature and ambient temperature is a non-destructive, fast and reliable indicator of plant stress (O'Neill et al., 2006). By a variety of measures, therefore, *Spartina* loses the growth and performance advantages normally associated with NH_4^+ as an N source, and, in fact, unlike under salinity stress, the grass performs less well than on other N sources when drought stress is imposed.

5. Conclusion

Although *Spartina alterniflora* is able to thrive on, acclimate to, and tolerate both ammonium and salinity stresses, it is unable to tolerate drought, even when provided with its preferred N form, NH_4^+. Indeed, in contrast to performance under salt stress, drought imposition completely nullifies any of the growth advantages observed in this C_4 grass under normal conditions when provided with NH_4^+ as an N source. The sensitivity of the species to drought is in part due to low constitutive levels of activities of antioxidant enzymes able to combat the oxidative damage caused by osmotic stress. A shift in N metabolism from glutamine synthetase toward higher engagement of glutamate dehydrogenase when fed with NH_4^+, while leading to elevated levels of compatible solutes in our test species, and while beneficial under water-deficit conditions in some species, ultimately proves insufficient as a tolerance mechanism under drought in the C_4 grass.

Author contributions

HK and CC carried out experimental and analysis work.
HK, CC and AC: design and interpretation of all experiments.
HK and KHJ wrote the manuscript.

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References

Ariz, I., Asensio, A.C., Zamarreno, A.M., Garcia-Mina, J.M., Aparicio-Tejo, P., Moran, J.F., 2013. Changes in the C/N balance caused by increasing external ammonium concentrations are driven by carbon and energy availabilities during ammonium nutrition in pea plants: the key roles of asparagine synthetase and anaplerotic enzymes. Physiol. Plant 148, 522–537.
Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: an overview. Photosynthetica 51, 163–190.
Barth, C., Gouzd, Z.A., Steele, H.P., Imperio, R.M., 2010. A mutation in GDP-mannose pyrophosphorylase causes conditional hypersensitivity to ammonium, resulting in Arabidopsis root growth inhibition, altered ammonium metabolism, and hormone homeostasis. J. Exp. Bot. 61, 379–394.
Beers, R.F., Sizer, I.A., 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–134.
Ben Hamed, K., Ellouzi, H., Talbi, O.Z., Hessini, K., Slama, I., Ghnaya, T., Bosch, S.M., Savouré, A., Abdelly, C., 2013. Physiological response of halophytes to multiple stresses. Funct. Plant Biol. 40, 883–896.
Bendixen, R., Gerendas, J., Schinner, K., Sattelmacher, B., Hansen, U.P., 2001. Difference in zeaxanthin formation in nitrate- and ammonium-grown *Phaseolus vulgaris*. Physiol. Plant 111, 255–261.
Bernguer, P., Santiveri, F., Boixadera, J., Lloveras, J., 2009. Nitrogen fertilization of irrigated maize under Mediterranean conditions. Eur. J. Agron. 30, 163–171.
Bhargava, S., Sawant, K., 2013. Drought stress adaptation: metabolic adjustment and regulation of gene expression. Plant Breeding 132, 21–32.
Bouyoucos, 1983. Les propriétés physiques du sol dépendent de sa texture et de sa structure. In: Les Bases De La Production végétale. Tome 1. Collection Sciences et Technique agricoles, pp, pp. 67–87.
Britto, D.T., Kronzucker, H., 2002. NH_4^+ toxicity in higher plants: a critical review. J. Plant Physiol. 159, 567–584.
Britto, D.T., Kronzucker, H.J., 2013. Ecological significance and complexity of N-source preference in plants. Ann. Bot. 6, 957–963.
Cantera, R., Urdaniz, A., Lerga, J., Garcia-Mina, J.M., 1999. Acidifying product as a source of stabilized ammonia nitrogen under greenhouse conditions. In: Bech, J. (Ed.), Soils with Mediterranean Type of Climate. UB Publicaciones, Barcelona, pp. 233–235.
Castillo, F.J., Penel, C., Greppin, H., 1984. Peroxidase release induced by ozone in sedum album leaves. Plant Physiol. 74, 846–851.
Dionisio-Sese, M.L., Tobita, S., 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci. 135, 1–9.
Domínguez-Valdivia, M.D., Aparicio-Tejo, P., Lamsfus, C., Cruz, C., Martins-Loução, M.A., Moran, J.F., 2008. Nitrogen nutrition and antioxidant metabolism in ammonium-tolerant and −sensitive plants. Plant Physiol. 132, 359–369.
Edwards, E.A., Rawsthorne, S., Mullineaux, P.M., 1990. Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum* L.). Planta 180, 278–284.
Edwards, G.E., Franceschi, V.R., Voznesenkaya, E.V., 2004. Single cell C4 photosynthesis versus the dual-cell (Kranz) paradigm. Ann. Rev. Plant Biol. 55, 173–196.
El Omari, R., Rueda-Lopez, M., Avila, C., Crespillo, R., Nhiri, M., Canovas, F.M., 2010. Ammonium tolerance and the regulation of two cytosolic glutamine synthetases in the roots of sorghum. Funct. Plant Biol. 37, 55–63.
Esteban, R., Ariz, I., Cruz, C., Moran, J.F., 2016. Mechanisms of ammonium toxicity and the quest for tolerance. Plant Sci. 248, 92–101.
Fernández-Cirelli, A., Arumí, J.L., Rivera, D., Boochs, P.W., 2009. Environmental effects of irrigation in arid and semi-arid regions. Chil. J. Agric. Res. 69, 27–40.
Fernández-Crespo, E., Camañes, G., García-Agustín, P., 2012. Ammonium enhances resistance to salinity stress in citrus plants. J. Plant Physiol. 169, 1183–1191.
Fiasconaro, M.L., Gogorcena, Y., Muñoz, F., Andueza, D., Sánchez-Díaz, M., Antolín, M.C., 2012. Effects of nitrogen source and water availability on stem carbohydrates and cellulosic bioethanol traits of alfalfa plants. Plant Sci. 191–192, 16–23.
Foyer, C.H., Valadier, M.-H., Migge, A., Becker, T.W., 1998. Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. Plant Physiol. 117, 283–292.
Foyer, C.H., Parry, M., Noctor, G., 2003. Markers and signals associated with nitrogen assimilation in higher plants. J. Exp. Bot. 54 (382), 585–593.
Frew, J., Jones, P., Scholes, G., 1983. Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solution. Anal. Chim. Acta 155, 130–150.
Gao, Y., Li, Y., Yang, X., Li, H., Shen, Q., Guo, S., 2010. Ammonium nutrition increases water absorption in rice. Plant Soil 331, 193–201.
Garnett, T.P., Shabala, S.N., Smethurst, P.J., Newman, I., 2001. Simultaneous measurement of ammonium, nitrate and proton fluxes along the length of eucalypt roots. Plant Soil 236, 55–62.
Gerendas, J., Zhu, Z.J., Bendixen, R., Ratcliffe, R.G., Sattelmacher, B., 1997. Physiological and biochemical processes related to ammonium toxicity in higher plants. Z Pflanzenernähr Bodenkd 160, 239–251.
Ghannoum, O., 2009. C4 photosynthesis and water stress. Ann. Bot. 103, 635–644.
Gonzalez, E.M., Aparicio-Tejo, P.M., Gordon, A.J., Minchin, F.R., Royuela, M., Arrese-Igor, C., 1998. Water-deficit effects on carbon and nitrogen metabolism of pea nodules. J. Exp. Bot. 49 (327), 1705–1714.
Groat, R.G., Vance, C.P., 1981. Root nodules enzymes of ammonium assimilation in alfalfa (*Medicago sativa* L.). Plant Physiol. 67, 1198–1203.
Guo, S., Schinner, K., Sattelmacher, B., Hansen, U.P., 2005. Different apparent CO_2 compensation points in nitrate- and ammonium-grown Phaseolus vulgaris and the relationship to non-photorespiratory CO_2 evolution. Physiol. Plant 123, 288–301.
Guo, S., Zhou, Y., Shen, Q., Zhang, F., 2007. Effect of ammonium and nitrate nutrition on some physiological processes in higher plants – growth, photosynthesis, photorespiration, and water relations. Plant Biol. 9, 21–29.
Helena, M., Cruz, C., 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signal. Behav. 3 (3), 156–165.
Hernández, I., Peralta, G., Pérez-Lloréns, J.L., Vergara, J.J., Niell, F.X., 1997. Biomass and dynamics of growth of Ulva species in Palmones River Estuary. J. Phycol. 33, 764–772.
Hessini, K., Ghandour, M., Albouchi, A., Soltani, A., Koyro, H.-W., Abdelly, C., 2008. Biomass production, photosynthesis, and leaf water relations of *Spartina alterniflora* under moderate water stress. J. Plant Res. 121, 311–318.
Hessini, K., Martínez, J.P., Gandour, M., Albouchi, A., Soltani, A., Abdelly, C., 2009a. Effect of water stress on growth, osmotic adjustment, cell wall elasticity and water use efficiency in *Spartina alterniflora*. Environ. Exp. Bot. 67, 312–319.
Hessini, K., Lachaâl, M., Cruz, C., Soltani, A., 2009b. Role of ammonium to limit nitrate accumulation and to increase water economy in wild swiss chard. J. Plant Nutr. 32, 821–836.
Hessini, K., Ben Hamed, K., Gandour, M., Mejri, M., Abdelly, C., Cruz, C., 2013. Ammonium nutrition in the halophyte *Spartina alterniflora* under salt stress: evidence for a priming effect of ammonium? Plant Soil 370, 163–173.
Hewitt, E.J., 1966. Sand and water culture methods used in the study of plant nutrition. Commonw. Bur. Hort. Tech. Commun. 22, 431–446.
Hossain, M.A., Asada, K., 1984. Inactivation of ascorbate peroxidase in spinach chloroplast on dark addition of hydrogen peroxide: its protection by ascorbate. Plant Cell Physiol. 25, 1285–1295.
Humbert, S., Subedi, S., Cohn, J., Zeng, B., Bi, Y.-M., Chen, X., Zhu, T., McNicholas, P.D., Rothstein, S.J., 2013. Genome-wide expression profiling of maize in response to individual and combined water and nitrogen stresses. BMC Genomics 14 (3), 1–13.

Ivanov, B., Edwards, G.E., 1997. Electron flow accompanying the ascorbate peroxidase cycle in maize mesophyll chloroplasts and its cooperation with linear electron flow to NADP(+) and cyclic electron flow in thylakoid membrane energization. Photosynth. Res. 52, 187–198.
Kant, S., Kant, P., Lips, H., Barak, S., 2007. Partial substitution of NO_3^- by NH_4^+ fertilization increases ammonium assimilating enzyme activities and reduces the deleterious effects of salinity on the growth of barely. J. Plant Physiol. 164, 303–311.
Kronzucker, H.J., Glass, A.D.M., Siddiqi, M.Y., 1995. Nitrate induction in spruce: an approach using compartmental analysis. Planta 196, 683–690.
Kronzucker, H.J., Siddiqi, M.Y., Glass, A.D.M., 1997. Conifer root discrimination against soil nitrate and the ecology of forest succession. Nature 385, 59–61.
Kronzucker, H.J., Glass, A.D.M., Siddiqi, M.Y., 1999. Inhibition of nitrate uptake by ammonium in barley: analysis of component fluxes. Plant Physiol. 120, 283–292.
Kronzucker, H.J., Siddiqi, M.Y., Glass, A.D.M., Kirk, G.J.D., 2000. Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: implications for rice cultivation and yield potential. New Phytologist 145, 471–476.
Li, B.H., Li, G.J., Kronzucker, H.J., Baluška, F., Shi, W.M., 2014. Ammonium stress in Arabidopsis: signaling, genetic loci, and physiological targets. Trends Plant Sci. 19, 107–114.
Lopes, M.S., Araus, J.L., Van Heerden, P.D.R., Foyer, C.H., 2011. Enhancing drought tolerance in C_4 crops. J. Exp. Bot. 62 (9), 3135–3153.
Mahajan, S., Tuteja, N., 2005. Cold, salinity and drought stresses: an overview. Arch. Biochem. Biophys. 444, 139–158.
Marschner, H., 1995. Mineral Nutrition of Higher Plants, 2nd. edition. Academic Press, San Diego, London.
Martinoia, E., Heck, U., Wiemken, A., 1981. Vacuoles as storage compartments for nitrate in barley leaves. Nature 289, 292–294.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzyme functions for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6050.
Misra, N., Gupta, A.K., 2006. Effect of salinity and different nitrogen sources on the activity of antioxidant enzymes and indole alkaloid content in *Catharanthus roseus* seedlings. J. Plant Physiol. 163, 11–18.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7 (9), 405–410.
Munzarova, M., Lorenzen, B., Brix, H., Vojtiskova, L., Votrubova, O., 2006. Effect of NO_3^-/NH_4^+ availability on nitrate reductase activity and nitrogen accumulation in wetland helophytes *Phragmites australis* and *Glyceria maxima*. Environ. Exp. Bot. 55, 49–60.
Nimptsch, J., Pflugmacher, S., 2007. Ammonia triggers the promotion of oxidative stresss in the aquatic macrophyte *Myriophyllum mattogrossense*. Chemosphere 66, 708–714.
O'Neill, P.M., Shanahan, J.F., Schepers, J.S., 2006. Use of chlorophyll fluorescence assessments to differentiate corn hybrid response to variable water conditions. Crop Sci. 46, 681–687.
Osborne, C.P., Beerling, D.J., 2006. Nature's green revolution: the remarkable evolutionary rise of C4 plants. Philos. Trans. R. Soc. B: Biol. Sci. 361, 173–194.
Podgórska 1, A., Gieczewska, K., Łukawska-Kuźma, K., Rasmusson, A.G., Gardeström, P., Szal, B., 2013. Long-term ammonium nutrition of Arabidopsis increases the extrachloroplastic NAD(P)H/NAD(P)(+) ratio and mitochondrial reactive oxygen species level in leaves but does not impair photosynthetic capacity. Plant Cell Environ. 36 (11), 2034–2045.
Ranieri, A., D'Úrso, G., Nali, C., Lorencini, G., Soldatini, G.F., 1996. Ozone stimulates apoplastic antioxidant systems in pumpkin leaves. Physiol. Plant. 97, 381–387.
Redinbaugh, M.G., Campbell, W.H., 1993. Glutamine synthetase and ferredoxin-Dependent glutamate synthase expression in the maize (Zea mays) root primary response to nitrate (Evidence for an organ-Specific response). Plant Physiol. 101, 1249–1255.
Rios-Gonzalez, K., Erdei, L., Lips, S.H., 2002. The activity of antioxidant enzymes in maize and sunflower seedlings as affected by salinity and different nitrogen sources. Plant Sci. 162, 923–930.
Roosta, H.R., Schjoerring, J.K., 2008a. Root carbon enrichment alleviates ammonium toxicity in cucumber plants. J. Plant Nutr. 31, 941–958.
Roosta, H.R., Schjoerring, J.K., 2008b. Effects of nitrate and potassium on ammonium toxicity in cucumber plants. J. Plant Nutr. 31, 1270–1283.
Sage, R.F., 2004. The evolution of C-4 photosynthesis. New Phytol. 161, 341–370.
Serapiglia, M.J., Minocha, R., Minocha, S.C., 2008. Changes in polyamines, inorganic ions and glutamine synthetase activity in response to nitrogen availability and form in red spruce (*Picea rubens*). Tree Physiol. 28, 1793–1803.
Shiferaw, B., Tesfaye, K., Kassie, M., Abate, T., Prasanna, B.M., Menkir, A., 2014. Managing vulnerability to drought and enhancing livelihood resilience in sub-Saharan Africa: technological, institutional and policy options. Weather Clim. Extr. 3, 67–79.
Skopelitis, D.S., Paranychianakis, N.V., Paschalidis, K.A., Pliakonis, E.D., Delis, I.D., Yakoumakis, D.I., Kouvarakis, A., Papadakis, A.K., Stephanou, E.G., Roubelakis-Angelakis, K.A., 2006. Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell 18, 2767–2781.
Speer, M., Kaiser, W.M., 1994. Replacement of nitrate by ammonium as the nitrogen source increases the salt sensitivity of pea plants. 2. Intercellular and intracellular solute compartmentation in leaflets. Plant Cell Environ. 17, 1223–1231.
Speer, M., Brune, A., Kaiser, W.M., 1994. Remplacement of nitrate by ammonium as the nitrogen source increases the salt sensitivity of pea plant. I. Ion concentrations in roots and leaves. Plant Cell Environ. 17, 1215–1221.
Staub, A.M., 1963. Extraction, identification et dosages des glucides dans les extraits d'organes et les corps bacteriens. In: Masson et Compagnie (Ed.), Techniques De Laboratoire. Tome 1 et 2, Paris, pp. 1307–1366.
Tan, K.Z., Keltjens, W.G., Findenegg, G.R., 1992. Effect of nitrogen form on aluminum toxicity in sorghum genotypes. J. Plant Nutr. 15, 1383–1394.
Terce-Laforgue, T., Dubois, F., Ferrario-Mery, S., de Crecenzo, M.A.P., Sangwan, R., Hirel, B., 2004. Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion cells when ammonia is provided either externally or released during photorespiration. Plant Physiol. 136, 4308–4317.
Thursby, G.B., Harlin, M.M., 1984. Interaction of leaves and roots of *Ruppia maritima* in the uptake of phosphate, ammonia and nitrate. Marine Biol. 83, 61–67.
Turano, F.J., Thakkar, S.S., Fang, T., Weisemann, J.M., 1997. Characterization and expression of NAD(H)-dependent glutamate dehydrogenase genes in Arabidopsis. Plant Physiol. 113, 1329–1341.
Wang, Z.Q., Yuan, Y.Z., Ou, J.Q., Lin, Q.H., Zhang, C.F., 2007. Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. J. Plant Physiol. 164, 695–701.
Wang, C., Zhang, S.H., Wang, P.F., Li, W., Lu, J., 2010. Effects of ammonium on the antioxidative response in *Hydrilla verticillata* (L.f.) Royle plants. Ecotox. Environ. Safe 73, 189–195.
Waraich, E.A., Ahmad, R., Ashraf, M.Y., Ahmad, S.M., 2011. Improving agricultural water use efficiency by nutrient management in crop plants. Acta Agriculturae Scandinavica Sect. B Plant Soil Sci. 61 (4), 291–304.
Waraich, E.A., Ahmad, R., Halim, A., Aziz, T., 2012. Alleviation of temperature stress by nutrient management in crop plants: a review. J. Soil Sci. Plant Nutr. 12, 221–244.
Yang, X., Li, Y., Ren, B., Ding, L., Gao, C., Shen, Q., Guo, S., 2012. Drought-induced root aerenchyma formation restricts water uptake in rice seedlings supplied with nitrate. Plant Cell Physiol. 53 (3), 495–504.
Yi, X.P., Zhang, Y.L., Yao, H.S., Zhang, X.J., Luo, H.H., Gou, L., Zhang, W.F., 2014. Alternative electron sinks are crucial for conferring photoprotection in field-grown cotton under water deficit during flowering and boll setting stages. Funct. Plant Biol. 41, 737–747.
Zhonghua, T., Yanju, L., Xiaorui, G., Yuangang, Z., 2011. The combined effects of salinity and nitrogen forms on *Catharanthus roseus*: the role of internal ammonium and free amino acids during salt stress. J. Plant Nutr. Soil Sci. 174, 135–144.
Zhou, Y.B., Zhang, C.S., Lin, J.Z., Yang, Y.Z., Peng, Y.C., Tang, D.Y., Zhao, X.Y., Zhu, Y.H., Liu, X.M., 2015. Over-expression of a glutamate dehydrogenase gene, MgGDH, from *Magnaporthe grisea* confers tolerance to dehydration stress in transgenic rice. Planta 241, 727–740.
Zhu, Z., Gerendas, J., Bendixen, R., Schinner, K., Tabrizi, H., Sattelmacher, B., Hansen, U.P., 2000. Different tolerance to light stress in NO_3^-- and NH_4^+-grown *Phaseolus vulgaris* L. Plant Biol. 2, 558–570.
Zivcovic, T., Quartacci, M.F., Marinine, B.S.F., Navari-Izzo, F., 2005. Low-molecular eight substances in the poikilohydric plant *Ramonda serbica* during dehydration and rehydration. Plant Sci. 168, 105–111.