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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).