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Atmospheric CO_2 enrichment effect on the Cu-tolerance of the C_4 cordgrass *Spartina densiflora*

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ABSTRACT

A glasshouse experiment was designed to investigate the effect of the co-occurrence of 400 and 700 ppm CO_2 at 0, 15 and 45 mM Cu on the Cu-tolerance of C_4 cordgrass species *Spartina densiflora*, by measuring growth, gas exchange, efficiency of PSII, pigments profiles, antioxidative enzyme activities and nutritional balance. Our results revealed that the rising atmospheric CO_2 mitigated growth reduction imposed by Cu in plants grown at 45 mM Cu, leading to leaf Cu concentration bellow than 270 mg Kg^{-1} Cu, caused by an evident dilution effect. On the other hand, non-CO_2 enrichment plants showed leaf Cu concentration values up to 737.5 mg Kg^{-1} Cu. Furthermore, improved growth was associated with higher net photosynthetic rate (A_N). The beneficial effect of rising CO_2 on photosynthetic apparatus seems to be associated with a reduction of stomatal limitation imposed by Cu excess, which allowed these plants to maintain greater $_i$WUE values. Also, plants grown at 45 mM Cu and 700 ppm CO_2, showed higher ETR values and lower energy dissipation, which could be linked with an induction of Rubisco carboxylation and supported by the recorded amelioration of N imbalance. Furthermore, higher ETR values under CO_2 enrichment could lead to an additional consumption of reducing equivalents. Idea that was reflected in the lower values of ETR_{max}/A_N ratio, malondialdehyde (MDA) and ascorbate peroxidase (APx), guaiacol peroxidase (GPx) and superoxide dismutase (SOD) activities under Cu excess, which could indicate a lower production of ROS species under elevated CO_2 concentration, due to a better use of absorbed energy.

1. Introduction

Climatic change and environmental pollution, due to heavy metal, are two of the major challenges to which humanity will to face for the conservation of ecosystems worldwide (Occhipinti-Ambrogi, 2007). Climate change is likely to alter plants species composition, structure and distribution (Allen et al., 2010). In fact, there is a consensus that atmospheric CO_2 enrichment could stimulate development and growth of hundred plants species (Ghannoum et al., 2000). However, it have been stated that this positive effect is highly dependent on the plant photosynthetic metabolism, being in certain degree less evident in plants species with C_4 photosynthesis metabolic pathway compared with their C_3-conterparts (Ghannoum et al., 2000). Also, this effect it could be influenced by other environmental factors, such as salinity (Lenssen et al., 1993, 1995; Rozema, 1993; Geissler et al., 2009, 2010; Mateos-Naranjo et al., 2010a,b), drought (Calvo et al., 2017), flooding (Setter et al., 1989; Duarte et al., 2014a), etc. Recently there are increasing reports indicating that the co-occurrence of heavy metal contamination in natural soils, due to anthropogenic activity, and rising atmospheric CO_2, due to climate change, may have important consequences for plants development. Thus, few of those studies have indicated that metal stress diminished in non-metal tolerance plants when grown under elevated atmospheric CO_2 concentration (Tian et al., 2014), while other have found that phytoremediation efficiency increased in those which have demonstrated high tolerance and metal uptake capacity (Li et al., 2012). However, despite of these positives evidences, there is not a general consensus, since metal tolerance may vary depending on specific plants species and metals.

Up-to-date, most studies regarding the effect of atmospheric CO_2 fertilization on plant tolerance and biomass production have been

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reported in response to Cd, Cd/Zn and Cd/Pb pollution (Guo et al., 2011, 2015; Jia et al., 2011, 2016; Li et al., 2010, 2012; Song et al., 2013, 2015), being certainly scarce in response to an excess in essential elements for plant, such as Cu. In fact, CO_2 enrichment effect along with Cu pollution has been only reported in some crops species, such as *Brassica juncea*, *Helianthus annus* and *Brassica napus* (Tang et al., 2003; Tian et al., 2014), five forage species (Tian et al., 2014) and few ferns (Zheng et al., 2008). In addition, there is a lack of knowledge about the physiological and biochemical mechanisms involved in these responses, especially in plants with C_4 metabolism photosynthetic pathway and in those which have evolved natural ability to cope with extreme metal polluted environments. Therefore, this experiment was arranged and developed to fill these gaps of knowledge.

The C_4 halophytic cordgrass, *Spartina densiflora* Brongn is a suitable model plant to study in detail the effect of atmospheric CO_2 enrichment on C_4 metal tolerant plant performance in the presence of high Cu amounts in the grown medium, due to its ability to tolerate metal contamination based in several physiological mechanisms which allows it to tolerate wide range of environmental factors (Mateos-Naranjo et al., 2007, 2011), including heavy metal concentrations (Mateos-Naranjo et al., 2008a). Therefore, this study aimed to: (1) assess the growth *S. densiflora* plants in experimental treatments ranging from 0 to 45 Mm Cu at ambient and elevated CO_2 concentrations (400 and 700 ppm CO_2, respectively); (2) determine the extent to which responses could be linked with the photosynthetic apparatus responses, in terms of CO_2 fixation, PSII efficiency, photosynthetic pigments and electron transport energy fluxes; (3) with water balances and nutrient and Cu accumulation patterns; and (4) its relationship with the antioxidant defence abilities consequent of the jointly effect of rising CO_2 and Cu.

2. Material and methods

2.1. Plant material

Seeds of *S. densiflora* were collected during January 2015 at Odiel estuary (37°15′N, 6°58′W; Huelva SW Spain) from 20 different patches randomly chosen, located in a well-drained gently sloping intertidal lagoon (mean sea level +1.70 m relative to SHZ). In the laboratory seeds were dried in a desiccator for 5 days to remove the humidity and stored in the dark for 4 months at 4 °C. In May 2015, seeds placed agar-filled petri dishes and germinated inside a germinator (ASL Aparatos Científicos M-92004, Madrid, Spain), under a photoperiod 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 18 °C, for 15 days. The resulting seedlings were then transferred to individual pots filled with pearlite and placed in a glasshouse (during summer 2015) for plant development and biomass increase (minimum-maximum temperatures of 21–25 °C, relative humidity 40–60% and natural daylight of 250 as minimum and 1000 μmol m^{-2} s^{-1} as maximum light flux). During this period pots were irrigated with 20% Hoagland's solution.

2.2. Experimental design description

In October 2015, after four months of seedlings culture, plants were randomly divided in six block and subjected to three Cu concentrations (0, 15 and 45 mM) in shallow trays and in combination with two atmospheric CO_2 concentration (400 ppm and 700 ppm) in controlled-environment chambers (Aralab/Fitoclima 18.000EH, Lisbon, Portugal) for one month (n = 10 pots per treatment). Chambers were programmed with alternating diurnal regime of 16 h of light (maximum photon flux rate, 300 μmol m^{-2} s^{-1}) at 25 ± 0.5 °C and 8 h of darkness at 18 ± 0.5 °C and relative humidity 50 ± 5%. Atmospheric CO_2 concentrations in chambers were continuously recorded by CO_2 sensors (Aralab, Lisbon, Portugal) and maintained by supplying pure CO_2 from a compressed gas cylinder (Air liquide, B50 35 K).

At the beginning of the experiment, 2 L of each Cu treatments, obtained combining 20% Hoagland's solution with adequate amount of $CuSO_4{\cdot}7H_2O$, were placed in each of the trays down to a depth of 1 cm. During the experiment, the levels were controlled to limit the variation of Cu concentration due to water evaporation. In addition, complete solution (including $CuSO_4{\cdot}7H_2O$) was changed weekly. The control treatment, 0 mM Cu, had exactly 0.0005 mM of Cu, derived from Hoagland's solution composition. These Cu concentrations were the same used in our previous experiments with *S. densiflora* (Mateos-Naranjo et al., 2008a, 2015), being evident differences in Cu uptake capacity and symptoms of stress in *S. densiflora* respect to the control treatments.

2.3. Growth analysis

At the end of the experiment (n = 10) plants were collected and divided in shoots and roots and dried at 60 °C for 48 h until constant weight. Leaf elongation rate (LER) was measured in randomly selected young leaves (n = 10, per treatment) according to Mateos-Naranjo et al. (2008b).

2.4. Gas exchange analysis

Instantaneous gas exchange measurements were performed in randomly selected fully expanded leaves 15 and 30 days after treatment initiation (n = 10) using an open infrared gas analyzer system (LI-6400XT, LI-COR Inc., Neb., USA) equipped with a light leaf chamber (Li-6400-02B, Li-Cor Inc.). Net photosynthetic rate (A_N), stomatal conductance (g_s), intrinsic water use efficiency ($_i$WUE) and intercellular CO_2 concentration (C_i) were determined under the following leaf chamber conditions: light photon flux density of 1500 μmol m^{-2} s^{-1}, leaf temperature of 25 °C, 50 ± 2% relative humidity and CO_2 concentration surrounding leaf (C_a) 400 and 700 μmol mol^{-1} air for plants grown at 400 and 700 ppm CO_2, respectively. Before to record each measurement, gas exchange was allowed to equilibrate (300 s). Photosynthetic area was approximated as the area of a trapezium. Intrinsic water use efficiency ($_i$WUE) was calculated as the ratio between A_N and g_s.

2.5. Stable isotope analysis and C and N concentrations in leaves

At the end of the experiment, carbon isotopic composition of the pulverized dry leaf samples randomly collected (n = 3) was determined according to Duarte et al. (2014b), using A Flash EA 112 Series elemental analyzer couple on line via Finningan conflo III interface to a Thermo delta V S mass spectrometer. The carbon isotope ratio was expressed in delta (δ) notation as the parts per thousand (‰) considering its deviation from a standard material (PDB limestone) through the formula: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 10^3$, where R is $^{13}C/^{12}C$. The analytical precision for the measurement was 0.2‰. Carbon and nitrogen contents (%) were attained during the same analysis (n = 3).

2.6. Gauss peak-spectra pigment analysis

At the end of the experiment period, photosynthetic pigments in leaf samples randomly collected (n = 5), flash-frozen in liquid N_2 and freeze-dried for 48 h in the dark to avoid photodegratation processes (Duarte et al., 2014b). Samples were subsequently grinded in pure acetone and pigments extracted at −20 °C during 24 h in the dark to prevent its degradation, centrifuged at 4000 rpm during 15 min at 4 °C and the resulting supernatant scanned in a dual beam spectrophotometer (Hitachi Ltd., Japan) from 350 to 750 nm at 0.5 nm step. The resulting absorbance spectrum was use to the determination of all the target pigments, after application of the using Gauss-Peak Spectra (GPS) algorithm according to Küpper et al. (2007). For this Sigma Plot