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Understanding the impact of a complex environmental matrix associated with climate change on the European marshes engineer species Spartina maritima

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Abstract:	A challenge exists in the need to understand plant responses in complex environmental matrixes, such as those predicted by climate change models, being this information essential for species that support important ecosystem functions. A factorial climatic chamber experiment was designed to evaluate the impact of atmospheric CO 2 concentration (400 ppm and 700 ppm CO 2) in combination with two maximum and minimum temperature ranges (28/13ºC and 32/17ºC) and salinity concentrations (0 and 171 mM NaCl) on the growth and photosynthetic responses of the ecosystem engineer species Spartina maritima . Plants grown at 32/17ºC showed a reduction ~39% on relative growth rate (RGR) and this was more drastic (i.e. 64%) in those exposed to 700 ppm CO 2 , which also showed an increment in the percentage of dead tillers regardless of salinity. These reductions were explained by the negative impact on net photosynthetic rate (A N), which decreased with temperature increment, being this reduction more acute at 700 ppm CO 2 . This response was associated with an augmentation in CO 2 diffusion limitations, as indicated the lower stomatal conductance (g s), together with a down-regulation photochemical apparatus efficiency, as indicated the lower electron transport rate (ETR) and energy fluxes derived from Kautsky curves. In addition, the greatest g s drop at 700 ppm CO 2 , would limit plant ability to cope with temperature excess through evapotranspiration, a fact that could have boosted temperature-triggered damage and, consequently, leaf senescence. Therefore, we can conclude that temperature and atmospheric CO 2 increments would compromise the development of S. maritima and consequently the maintaining of its ecosystem functions.
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Highlights

- Impact of a complex environmental matrix on *S. maritima* performance was evaluated.
- Temperature and atmospheric CO_2 increments had synergic injury effects.
- These conditions drastically reduced plant growth and increased its senescence.
- Carbon assimilation, light-harvesting and photoprotective impacts were found.
- Atmospheric CO_2 enrichment would limit stomata ability to cope with heat excess.

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Understanding the impact of a complex environmental matrix associated with climate change on the European marshes engineer species *Spartina maritima*

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ABSTRACT

A challenge exists in the need to understand plant responses in complex environmental matrixes, such as those predicted by climate change models, being this information essential for species that support important ecosystem functions. A factorial climatic chamber experiment was designed to evaluate the impact of atmospheric CO_2 concentration (400 ppm and 700 ppm CO_2) in combination with two maximum and minimum temperature ranges (28/13 ºC and 32/17 ºC) and salinity concentrations (0 and 171 mM NaCl) on the growth and photosynthetic responses of the ecosystem engineer species *Spartina maritima*. Plants grown at 32/17 ºC showed a reduction ~39% on relative growth rate (RGR) and this was more drastic (i.e. 64%) in those exposed to 700 ppm CO_2, which also showed an increment in the percentage of dead tillers regardless of salinity. These reductions were explained by the negative impact on net photosynthetic rate (A_N), which decreased with temperature increment, being this reduction more acute at 700 ppm CO_2. This response was associated with an augmentation in CO_2 diffusion limitations, as indicated the lower stomatal conductance (g_s), together with a down-regulation photochemical apparatus efficiency, as indicated the lower electron transport rate (ETR) and energy fluxes derived from Kautsky curves. In addition, the greatest g_s drop at 700 ppm CO_2, would limit plant ability to cope with temperature excess through evapotranspiration, a fact that could have boosted temperature-triggered damage and, consequently, leaf senescence. Therefore, we can conclude that temperature and atmospheric CO_2 increments would compromise the development of *S. maritima* and consequently the maintaining of its ecosystem functions.

Keywords: CO_2 enrichment; Climate change; Chlorophyll fluorescence; Gas exchange; Halophyte; Temperature stress.

1. Introduction

Climate change arises as one of the greatest challenges for worldwide ecosystems conservation. Thus, along with recognized atmospheric CO_2 enrichment to values ~700 ppm by the end of the century (IPCC, 2014), climate models predict a parallel increase in daily temperature range (between 1.8 - 6.0 ºC, depending on the region) and rainfalls pattern alteration which would lead to a decrease in water availability and soil salinization (IPCC, 2014). An important part of the effect of climate change on ecosystems will be related to those variations triggered by environmental conditions in the structure and composition of plant species (Allen and Ort, 2001; Hooper et al., 2005; Short et al., 2016; Zhao et al., 2018), being this impact greater in those ecosystems dominated by few species, which also sustain key functions (such as the control of the fluxes of energy and matter) and contribute to functional diversity and species assemblages (Hooper et al., 2005).

The genus *Spartina* is integrated by grass species widely distributed on every continent except for Antarctica, being many of them frequently found in coastal salt marshes (Bortolus et al., 2019). It has been highlighted the importance of these species in salt marshes ecosystem functions, with a relevant role on coastal accretion and marsh creation, as well as the maintenance of ecosystem primary and secondary production (Bortolus et al., 2019). Among *Spartina* genus species, the European *Spartina maritima* (Curtis) Fernald is an important pioneer and ecosystem engineer in European salt marshes (Castellanos et al., 1994), playing also an important role for the maintenance and conservation of marsh ecosystem biodiversity (Curado et al., 2018). This species develops its populations mainly in the lowest parts of the marshes, acting as a primary colonist contributing to facilitate succession in marsh ecosystems (Castellano et al.,

1994, 1998; Castillo et al., 2008) and playing a key role in shoreline stabilization (Duarte et al. 2014). In addition, this species has demonstrated a high biotechnological potential as a tool for monitoring and phytoremediating metal polluted areas (Padinha et al., 2000; Mesa et al., 2015). Furthermore, many studies have been developed to understand the impact of environmental factors associated with future global change scenarios on the development of this important salt marsh species (Mateos-Naranjo et al., 2010a; Couto et al., 2014; Duarte et al., 2014). Thus, a certain effort has been made to assess the effect of atmospheric CO_2 enrichment on *S. maritima* development, having observed an improvement on plant growth and physiological performance under optimal and suboptimal salinity conditions, despite being a C_4 species (Mateos-Naranjo et al., 2010a). Besides, *S. maritima* has demonstrated the ability to maintain its photosynthetic activity even during prolonged submersion periods associated with the sea level rise due to global warming (Duarte et al., 2014). In addition, a rising temperature model has demonstrated an enhancement in this salt marsh species aboveground biomass (Couto et al., 2014), but it has been identified that an increase in the frequency and duration of high temperature events will lead to a decrease of its photo-biological fitness (Duarte et al., 2016). Despite these efforts, there is great uncertainty about the real effects of climate change on the conservation of this species, since most of those studies only have evaluated plant responses to one or two environmental stressors in combination. Therefore, a challenge exists in determining plant responses in a complex environmental matrix, such as the one predicted by climate models. Consequently, this study was designed and carried out to understand the influence of a complex environmental matrix, characterized by atmospheric CO_2 concentration, air temperature and medium saline concentration variations on *S. maritima* growth and development as well as key photosynthetic parameters. We

hypothesized that, although a direct positive physiological impact of increased atmospheric CO_2 concentration on *S. maritima* has been previously identified (Mateos-Naranjo et al., 2010a), the co-occurrence of other stress factors such as temperature pattern variation and medium salinization could trigger metabolic responses that could jeopardize the development of this important salt marsh species and, consequently, the maintenance of the ecosystem functions in which this plant is involved.

2. Material and Methods

2.1. Plant material

Clumps of *S. maritima* of 20 cm diameter with between 25-30 mature tillers were collected in June of 2019 from a well-established population in a low-marsh site located in the Odiel salt marshes (37º15'N, 6º58'O; SW Spain). Clumps were planted in individual plastic pots (15 cm high × 18 cm diameter) using its own soil as a potting substrate, and placed in a greenhouse under the follow controlled conditions: maximum temperature between 21/25 ºC, minimum temperature between 13/11 ºC, 40-60% relative humidity and natural daylight of 200 μmol m^{-2} s^{-1} as minimum and 1000 μmol m^{-2} s^{-1} as maximum light flux). Pots were irrigated with tap water, and plants were kept for a stabilization period of 7 days under the previously described conditions before the experiment's onset.

2.2. Experimental design

At the beginning of the experiment, the number of tillers in each pot was homogenized to 20 tillers completely developed and healthy in order to standardize our

samples before the experiment onset and to avoid any effect of tiller health and age in our results. Then, pots were randomly assigned to eight different experimental blocks with ten plants in each one, as follows: two concentrations of atmospheric CO_2 (400 ppm and 700 ppm CO_2) in combination with two ranges of ambient maximum and minimum temperature (28/13 ºC and 32/17 ºC) and irrigation with two salinity concentrations (0 and 171 mM NaCl) for 40 days. For the atmospheric CO_2 concentration and temperature range treatments, pots were placed in controlled-environment chambers (Aralab/Fitoclima 18.000EH, Lisbon, Portugal), which were programmed with alternating diurnal regime of 14 h of light and 10 h of darkness with the specific maximum and minimum temperature range, light intensity of 300 μmol m^{-2} s^{-1}, 40–60% relative humidity and the specific atmospheric CO_2 concentration. Atmospheric CO_2 concentrations in chambers were continuously monitored by CO_2 sensors and maintained by supplying pure CO_2 from a compressed gas cylinder (Air liquide, B50 35K). Finally, NaCl concentrations were established by combining tap water with appropriate amounts of NaCl. At the beginning of the experiment, the pots were placed in plastic trays containing the appropriate NaCl solutions for each specific salinity concentration treatment to a depth of 1 cm. In order to avoid changes of the NaCl concentration caused by water evaporation, levels in the trays were monitored continuously throughout the experimental period.

2.3. Plant growth analysis

At the beginning and at the end of the experiment, three and seven plants from each specific treatment were harvested and divided into roots and tillers. Then, these biomass fractions were oven dried at 60 ºC for 48 h and weighed to obtain both initial and final

dry biomass estimates. In addition, the number of dead tillers was recorded at the end of the experiment.

The relative growth rate (RGR) of whole plants was calculated using the formula:

$$RGR = (\ln B_f - \ln B_i) \cdot D^{-1} \ (g\ g^{-1}\ day^{-1})$$

where B_f = final dry mass (the mean of the seven plants from each treatment at the end of the experiment), B_i = initial dry mass (the mean of the three plants from each treatment at the beginning of the experiment) and D = duration of experiment (days).

2.4. Leaf gas exchange analysis

At the end of the experiment, instantaneous leaf gas exchange measurements were taken on fully developed expanded leaves (n = 10) using an infrared gas analyzer in an open system (LI-6400-XT, Li-COR Inc., NE., USA) equipped with a light leaf chamber (Li-6400-02B, Li-Cor Inc.). Net photosynthesis rate (A_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were recorded under the following leaf chamber settings: a photosynthetic photon flux density (PPFD) of 1000 µmol photons $m^{-2}\ s^{-1}$ (with 15% blue light to maximize stomatal aperture), vapour pressure deficit of 2.0–3.0 kPa, 50 ± 5% relative humidity, CO_2 concentration surrounding the leaf (C_a) of 400 or 700 µmol CO_2 mol^{-1} air depending on the atmospheric CO_2 concentration treatment and air temperature of 28 °C or 32 ºC for plants grown at low and high temperature range treatments, respectively. All measurements were made between 10:00 and 13:00 h inserting the order between the different treatments to standardize samples, and before to record each measurement, gas exchange was allowed to equilibrate for 120 s. Finally,

intrinsic water use efficiency ($_i$WUE) was calculated as the ratio between A_N and g_s [mmol (CO_2 assimilated) mol^{-1} (H_2O transpired)].

2.5. Chlorophyll fluorescence analysis

Two different fluorescence protocols were developed at the end of the experiment in the same leaves of gas exchange analysis in order to test how the different combinations of atmospheric CO_2 concentration, air temperature and medium salinity concentration used in this study affect photosystem II (PSII) energy use efficiency. Thus, the saturation pulse method was used to determine the maximum quantum efficiency of PSII photochemistry (F_v/F_m) and quantum efficiency of PSII (Φ_{PSII}; Genty et al., 1989). As described by Schreiber et al. (1986), a 0.8 s saturating actinic light pulse of 10000 µmol m^{-2} s^{-1} was applied at midday (1400 µmol photons m^{-2} s^{-1}) in previously dark- and light-adapted leaves (n = 10) for 30 min using a modulated fluorimeter (FMS-2; Hansatech Instruments Ltd., UK). Using this information, the electron transport rate (ETR) was calculated according to Krall and Edwards (1992).

On the other hand, the chlorophyll *a* fast kinetics, by the OJIP-test (or Kautsky curves), which depicts the rate of reduction kinetics of various components of PSII, was also measured in 30 min dark-adapted leaves (n = 7), using the pre-programmed OJIP protocols of the FluorPen FP100 (Photo System Instruments, Czech Republic). Moreover, absorbed (ABS/RC), trapped (TR/RC), electron transport (ET/RC) and dissipated (DI/RC) energy fluxes per reaction center derived from OJIP were calculated according to Strasser et al. (2004).

2.6. Statistical analysis

R software ver. 4.0.0 (R Core Team, 2020) was used to perform the statistical analyses. Firstly, a multivariate statistical approach using a principal component analysis (PCA) was performed to get an overview of the plant growth and physiological performance in response to the different experimental treatments. Missing values in the PCA dataset appeared as a result of the different number of measurements in each variable. They were handled using the expectation-maximization (EM) algorithm in the 'missMDA' R package (Josse and Husson, 2016). Secondly, a downscaling assessment was carried out through generalized linear models (GLMs) to analyze the main and/or interaction effects of atmospheric CO_2 concentration, maximum and minimum temperature range and low and high NaCl concentration (as categorical factors) on the growth and physiological parameters (as dependent variables) of *S. maritima* plants. In case of significant results, multiple comparisons were analyzed by *post hoc* LSD test (i.e. Fisher's Least Significant Difference). Before statistical analysis, Kolmogorov-Smirnov and Levene tests were used to verify the assumptions of normality and homogeneity of variances, respectively.

3. Results

3.1. Multivariate approach: global overview of S. maritima growth and physiological status

Growth and physiological performance variations of *S. maritima* during the experimental setup were mainly represented in the first two PCA axes. They explained, respectively, 36.0% and 18.4% of the total variation in the recorded data, being both temperature range and atmospheric CO_2 enrichment the experimental factors which led the main groupings (Fig. 1A, B; Table 1). Hence, the PC1 axis reflected a clear divergence of plants grown at 32/17 ºC of maximum and minimum temperature range,

which were located in the left part of the plot (especially those grown at 700 ppm CO_2). This separation was mainly explained by the lower RGR, A_N, F_v/F_m, Φ_{PSII} and ETR values, together with an increment in the percentage of dead tillers and DI/RC in those plants compared to the rest of the treatments (Fig. 1B; Table 1). In addition, the PCA revealed a certain divergence between both atmospheric CO_2 concentration treatments along the PC2 axis, with most of non-CO_2 enriched plants located in the upper part, being this response pattern mainly linked with higher g_s and lower C_i values of plants grown at 400 ppm CO_2 (Fig. 1B; Table 1).

3.2. Effects of atmospheric CO_2 enrichment, temperature augmentation and NaCl concentration on S. maritima growth

A summary of generalized linear model (GLM) results is made available as supplementary material, including fitting tests and confidence intervals (Table A.1). There was a significant effect of temperature range treatment on the RGR of *S. maritima* after 40 days of treatment (GLM: Temp., $p < 0.01$; Table A.1). Additionally, this effect was, to some extent, modulated by atmospheric CO_2 concentration treatment (GLM: [CO_2] x Temp., $p < 0.05$; Table A.1). Thus, RGR decreased considerably in plants grown under the highest maximum and minimum temperature range treatment (i.e. 32/17 ºC), and this effect was more acute in those exposed to 700 ppm CO_2 regardless of saline irrigation treatment (Fig. 2A).

Similarly, the percentage of dead tillers augmented with temperature and atmospheric CO_2 enrichment regardless of saline irrigation treatment (GLM: Temp., $p < 0.01$; [CO_2], $p < 0.05$; Table A.1), being this increase more pronounced in plants grown

at 700 ppm CO_2 and 32/17 ºC compared to the rest of treatments, although no significant differences were found (Fig. 2B).

3.3. Effects of atmospheric CO_2 enrichment, temperature augmentation and NaCl concentration on photosynthetic apparatus performance

There was a significant effect of the environmental factors tested on leaf gas exchange characteristics and PSII photochemical effiency of *S. maritima* after 40 days of exposure (GLM, $p < 0.05$). Thus, A_N values decreased in plants grown at 32/17 ºC (GLM: Temp., $p < 0.01$; Fig. 3A; Table A.1), and these values were overall lower at 700 ppm CO_2 although without significant differences. A very similar trend was recorded for g_s in relation to temperature increment (GLM: Temp., $p < 0.01$) but, in addition, g_s values were significantly lower in plants grown at 700 ppm CO_2 (GLM: [CO_2], $p < 0.01$; Fig. 3B; Table A.1). Contrarily, C_i and $_i$WUE values were significantly higher in plants grown at 700 ppm CO_2 compared to their non-CO_2 enriched counterparts, regardless of temperature and saline irrigation treatments (GLM: [CO_2], $p < 0.01$; Fig. 3C, D; Table A.1).

Regarding photochemical parameters, F_v/F_m values were lower in plants subjected to the highest minimum and maximum temperature range treatment at both salinity irrigation conditions (GLM: Temp., $p < 0.01$), being this reduction also more pronounced in plants grown at 700 ppm CO_2 (GLM: [CO_2] x Temp., $p < 0.05$; Table A.1), and especially in those plants irrigated with 171 mM NaCl (Fig. 4A). Likewise, minimum and maximum temperature augmentation led to a marked reduction in *S. maritima* Φ_{PSII} values, although this overall effect was much more marked in those plants exposed to high atmospheric CO_2 concentration and saline irrigation (GLM: [CO_2] x Temp., $p < 0.05$; Fig. 4B; Table A.1). A very similar response was detected for

ETR, with the lowest values recorded in plants subjected to 700 ppm CO_2, 32/17 ºC and irrigation with 171 mM NaCl (Fig. 4C).

Focusing on the chlorophyll *a* fast kinetics results, there were remarkable differences in the shape of Kautsky curves between plants grown under the different enviromental factors after 40 days of treatments. Thus, overall fluorescence kinetics values were lower in plants subjected to 700 ppm CO_2 and 32/17 ºC, being this trend also more pronounced in plants irrigated with 171 mM NaCl (Fig. 5). These Kautsky curve shape divergence was also accompanied by marked differences in energy fluxes through photosystems. Thus, ABS/RC was lower in plants grown at 700 ppm CO_2 and 28/13 ºC, compared to those exposed to 400 ppm CO_2 (GLM: [CO_2] x Temp., $p < 0.01$; Table A.1). Moreover, ABS/RC showed an increase in plants subjected to 700 ppm, 32/17 ºC and irrigation with 171 mM NaCl compared to the rest of treatments (GLM: [CO_2] x Temp. x [NaCl], $p < 0.05$; Fig. 6A; Table A.1). A very similar trend was recorded for DI/RC (Fig. 6D). Oppositely, both ET/CS and TR/CS tended to decrease in plants exposed to 700 ppm CO_2 compared with their non-CO_2 enriched counterparts (GLM: [CO_2] , $p < 0.05$; Fig. 6B, C; Table A.1). However, this effect was more pronounced for TR/CS in plants grown at 28/13 ºC compared to those grown in a higher temperature range (GLM: [CO_2] x Temp., $p < 0.01$; Fig. 6C; Table A.1).

4. Discussion

Gaining a mechanistic understanding of the effect of the main coexisting factors linked with climate change, such as atmospheric CO_2 enrichment, temperature pattern variations and medium salinization, on the growth and physiological performance of key plant species in ecosystem functionality is crucial to obtain a more realistic view on

how this global phenomenon influences ecosystems conservation and the development of these plant communities (Bernacchi et al., 2013).

Our results demonstrated that atmospheric CO_2 enrichment, as a consequence of climatic change, would not cause a substantial improvement on growth and development of the important pioneer and salt marsh ecosystem engineer species *S. maritima*. However, an increase up to 4 ºC in the maximum and minimum daily temperature range, as expected by the end of the century (IPCC, 2007), would entail a negative effect on the development of this species, being this impact even more acute in a CO_2 enriched atmosphere. Thus, our results revealed that *S. maritima* plants grown at 400 ppm atmospheric CO_2 concentration and 32/17 ºC of maximum and minimum daily temperature range showed a drastic growth reduction, measured as RGR, of ~39% compared to their counterparts exposed to the colder temperature range. This reduction was up to 64% in those plants also exposed to 700 ppm CO_2, regardless of the saline irrigation treatment. The lack of salinity effects on plant growth, as well as for most of the studied parameters, was somewhat expected since *S. maritima* is a halophyte species that has demonstrated a high tolerance to a wide range of salinities (Adams and Bate, 1995; Castillo et al., 2008; Mateos-Naranjo et al., 2010a). However, it should be highlighted that the absence of beneficial effects of atmospheric CO_2 enrichment on plant development contrasts with the previous results obtained by Mateos-Naranjo et al. (2010a) for this plant species, who observed that an increment of atmospheric CO_2 concentration to 700 ppm stimulated plant RGR ~40% through a salinity range between 0 and 510 mM NaCl. Similarly, our results also varied with respect to those shown by other *Spartina* species, such as *S. densiflora*, which experienced an increase in biomass production of 35% and 20%, respectively at 0 and 171 mM NaCl, compared to plants grown at 400 ppm CO_2 (Mateos-Naranjo et al., 2010b). These differences could be

related to the variation in the experimental conditions of each specific study. In this study, plants were subjected to different environmental synergies growing in natural soil whilst, in the aforementioned experiments, plants were grown in pearlite. The use of this substrate would allow the previous studies to assess the direct effect of atmospheric CO_2 enrichment on plant performance avoiding the influence of other factors associated to soil physicochemical properties, which could alter plant direct responses to atmospheric CO_2 enrichment. However, we believe that the use of a natural soil-plant complex, as we have done in this study, would allow a more realistic view of the real impact of climate change factors on plant development. In fact, according to our results, Curtis et al. (1989), in an experiment developed under field conditions using OTC chambers, were unable to detect a response to atmospheric CO_2 enrichment in *Spartina patens*. Furthermore, Rozema et al. (1991) obtained different results regarding the effect of atmospheric CO_2 enrichment on *S. patens* performance, depending on growth medium aeration level in plants grown in hydroponic conditions. To some degree, this lack of response to atmospheric CO_2 enrichment is consistent with multitude of CO_2 enrichment experiments, which indicated that plants with C_4 photosynthetic metabolism, as *S. maritima*, would not be as benefited by the air CO_2 fertilization effect (Ghannoum et al., 2000). However, this overall response could be modified by interactions with other co-occurring stress factors related to climate change, such as temperature pattern variation or medium salinization (IPPC, 2014). Therefore, it has been described that the effect of atmospheric CO_2 enrichment on plant development could be neutral, positive through the improvement of plant tolerance against environmental stress or negative by enhancing environmental stress deleterious effects, being also these responses species-specific (Balfagón et al., 2019). In this respect, as we previously indicated, the increment in temperature together with an atmospheric CO_2

concentration enrichment would substantially reduce *S. maritima* growth. Besides, it is important to emphasize that this lower plant development was accompanied by an increase in senescence level, as reflected by the higher percentage of dead stems at the end of the experiment.

The deleterious effect of increased temperatures and, to a greater extent, CO_2-enriched atmosphere conditions on *S. maritima* development was partly explained by the negative impact of these factors on some essential steps of plant photosynthetic apparatus performance. Thus, our results showed a substantial reduction in plant carbon fixation capacity, measured as A_N, at elevated temperature range treatment being this reduction in certain degree more pronounced at elevated atmospheric CO_2 concentration. This effect was associated with an augmentation in CO_2 diffusion limitations through stoma in plants grown at 32/17 ºC, as indicated their lower g_s values. Temperature is one of the most variable environmental factors and it can affect many plant physiological processes, including plant gas exchange characteristics, but little is known about its effect on g_s, especially at high temperatures (Teskey et al., 2015). In fact, several studies have recorded a wide range of plant responses to temperature increment, including stomal opening (Lu et al., 2000; Mott and Peak, 2010) and closure (Weston and Bauerle, 2007; Lahr et al., 2015) but also non-significant responses (Cerasoli et al., 2014; von Caemmerer and Evans, 2015). Moreover, stomatal behaviour to temperature could be modified by interactions with other environmental factors (Flexas et al., 2012). In this sense, there is a need to highlight that g_s reduction with temperature increment was even more acute in those plants exposed to 700 ppm CO_2. In addition, this reduction was greater than the one reported in A_N, contributing to overall higher $_i$WUE values under rising atmospheric CO_2 conditions. Thus, this decoupling between g_s and A_N in plants grown at 700 ppm CO_2 and 32/17 ºC would reflect the

higher stomatal sensitivity to CO_2 variations. In fact, different studies have shown that one of the most consistent responses of C_4 plant species to atmospheric CO_2 concentration enrichment is a decrease in g_s (Ainsworth and Rogers, 2007). Robredo et al. (2007) explained that C_i increase caused by the high CO_2 concentration could promote partial closure of the stoma, as it was recorded in this study. However, the higher stomatal sensitivity to elevated CO_2 regardless of temperature, could have had also important consequences for plant tolerance to heat stress. Thus, although stomatal behaviour is a key factor to preserve the trade-off between CO_2 acquisition for photosynthetic processes and water losses, it is necessary to emphasize its role for plant adaptation to thermal stress through the regulation of heat excess by increasing leaf evapotranspiration (Feller, 2006). Therefore, the importance of stoma in leaf cooling has been identified, and while the stomata remains open, the evaporative cooling can mitigate the negative effect of temperature excess, positively affecting plant photosynthesis, yield and survival (Lu et al., 1994; Ameye et al., 2012). Therefore, the higher g_s drop in *S. maritima* plants under elevated atmospheric CO_2 concentration could have been limiting their ability to modulate leaf temperature compared to plants grown at 400 ppm, which could also contribute to explain the greater injury effect of temperature increment at these experimental conditions.

On the other hand, the impact of temperature increase, alone or in combination with atmospheric CO_2 enrichment, had a great impact on *S. maritima* photochemical apparatus functionality. According to our results, one of the best recognized effects of high temperature on plant photosynthetic apparatus is the destruction of PSII components (Flexas et al., 2012; Pérez-Romero et al., 2019a; López-Jurado et al., 2020). Likewise, in concordance with this study, Mateos-Naranjo et al. (2010b) and Duarte et al. (2014) also found that CO_2 enrichment affected PSII photochemistry in the

halophytes *S. densiflora* and *S. maritima*, respectively. However, this is the first study that corroborates that this effect would be exacerbated with an increase up to 4 ºC in the maximum and minimum daily temperature range and, to some extent, with the augmentation in medium salinity concentration. Thus, our results showed that F_v/F_m and Φ_{PSII} values were affected by the elevated temperature range and this effect was more acute in plants grown at 700 ppm CO_2 and irrigated with 171 mM NaCl, suggesting that the combination of these environmental conditions would increase photoinhibition (Werner et al., 2002). Likewise, we attributed the negative impact of these environmental factors on *S. maritima* photochemical apparatus to the down-regulation of its electron transport chain functionality, as indicated by the lower ETR values recorded in plants grown at 700 ppm CO_2, 32/17 ºC and irrigated with 171 mM NaCl. This fact indicated that these plants would have had more difficulties to transform the captured energy in their photosystems, suggesting the synergistic impact of elevated temperature and atmospheric CO_2 enrichment on the functionality of reaction centers. Thus, this idea was also supported by the assessment of energy fluxes per reaction center derived from Kautsky curves, which showed that, although plants were exposed to elevated atmospheric CO_2 and air temperature range, they increased their ABS/RC values compared to the rest of treatments. This would mean a greater number of active reaction centers functioning as a heat radiator, hence protecting plant photosystems against high temperature and light intensities (Strasser et al., 2004; Pérez-Romero et al., 2018, 2019a,b). This elevated absorption rate would excess the capacity of photosystems to metabolize energy, as was corroborated by the decoupling with TR/RC and ET/RC values. Moreover, the high ABS/RC would unbalance the trade-off between the energy absorbed and metabolized, being necessary the activation of some defense mechanisms to dissipate the energy excess as heat, as indicated also higher DI/RC

values recorded. As a consequence, much of the absorbed energy would not take the photochemical pathway (Flexas et al., 2012), a circumstance that would affect photosynthetic productivity and, consequently, plant growth, a fact which would contribute to explain the observed growth pattern in response to tested environmental factors. Therefore, our results would suggest that temperature and atmospheric CO_2 increments related to climate change would jeopardize the development and maintenance of natural populations of *S. maritima*. This circumstance would cause significant structural and functional changes in the drainage system of the marshes systems dominated by this grass species, due to its importance in shoreline stabilization and salt marsh accretion during the first phases of ecological succession, with the consequent alteration of sediment, mineral elements and nutrient cycles, as well as the fluxes of energy necessary for other levels of the trophic chain in the ecosystem.

5. Conclusion

This study reveals the high vulnerability of the salt marsh key species *S. maritima* to increases up to 4 ºC in the maximum and minimum daily temperature, as predicted for future scenarios by global change models, being this susceptibility even higher under CO_2 enriched atmosphere conditions. This idea would be motivated by drastic plant growth decrease and leaf senescence increment recorded at 700 ppm CO_2 and 32/17 ºC of maximum and minimum daily temperature range. This response was associated with the negative effect of temperature and atmospheric CO_2 concentration on plant carbon fixation machinery through increased stomatal limitations to CO_2

diffusion and the unbalance between the energy absorbed and metabolized in *S. maritima* photosystems, linked with a down-regulation of the electron transport chain functionality. The higher g_s drop at 700 ppm CO_2 would also limit plant ability to regulate heat excess through leaf evapotranspiration, a fact that could have boosted temperature-triggered damage and, consequently, leaf senescence compared to plants grown at 400 ppm. Finally, from a conservational perspective, these results suggest that temperature increment and atmospheric CO_2 enrichment associated with future climate change scenarios would jeopardize the development of *S. maritima* populations and, consequently, the maintenance of ecosystem functions derived from the presence of this important pioneer and ecosystem engineer salt marshes plant species.

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CRediT authorship contribution statement

Enrique Mateos-Naranjo: Conceptualization, Methodology, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. **Javier López Jurado:** Methodology, Formal analysis, Writing - review & editing. **Jennifer Mesa Marín:** Methodology, Writing - review & editing. **Carlos Javier Luque:** Conceptualization, Methodology, Writing - review & editing. **Eloy Manuel Castellanos:** Conceptualization, Methodology, Writing - review & editing. **Jesús Alberto Pérez-Romero:** Methodology. **Susana Redondo-Gómez:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Fig. 1. Representation of the principal component analysis (PCA) biplot obtained for the growth and physiological data of *Spartina maritima* plants in the experimental set-up. Panel A represents the distribution of the selected variables loading on these axes and the percentage of explained variance for these axes (see Table 1 for a description of these variables and their contribution to the two main axes of PCA). Panel B shows the ellipses (95% confidence level) which encompass occurrence points in the two main axes for the environmental factors tested.

Fig. 2. Relative growth rate, RGR (A) and percentage of dead tillers (B) in *Spartina maritima* plants in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 7. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 3. Net photosynthetic rate, A_N (A), stomatal conductance, g_s (B), intercellular CO_2 concentration, C_i (C) and intrinsic water use efficiency ($_i$WUE) in randomly selected fully developed expanded leaves of *Spartina maritima* in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 10. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 4. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A), quantum efficiency of PSII, Φ_{PSII} (B) and electron transport rate, ETR (C), at midday in randomly

selected fully developed expanded leaves of *Spartina maritima* in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 10. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 5. Transient fluorescence (Kautsky curves) of dark-adapted leaves of *Spartina maritima* plants grown at 400 ppm (A) and 700 ppm (B) atmospheric CO_2 in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean of seven measurements per treatment combination.

Fig. 6. Absorbed energy flux, ABS/RC (A), electron transport energy flux, ET/RC (B), trapped energy flux, TR/RC (C) and dissipated energy flux, DI/RC (D), per reaction centre in randomly selected fully developed expanded dark-adapted leaves of *Spartina maritima* in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 7. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

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Understanding the impact of a complex environmental matrix associated with climate change on the European marshes engineer species *Spartina maritima*

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ABSTRACT

A challenge exists in the need to understand plant responses in complex environmental matrixes, such as those predicted by climate change models, being this information essential for species that support important ecosystem functions. A factorial climatic chamber experiment was designed to evaluate the impact of atmospheric CO_2 concentration (400 ppm and 700 ppm CO_2) in combination with two maximum and minimum temperature ranges (28/13 ºC and 32/17 ºC) and salinity concentrations (0 and 171 mM NaCl) on the growth and photosynthetic responses of the ecosystem engineer species *Spartina maritima*. Plants grown at 32/17 ºC showed a reduction ~39% on relative growth rate (RGR) and this was more drastic (i.e. 64%) in those exposed to 700 ppm CO_2, which also showed an increment in the percentage of dead tillers regardless of salinity. These reductions were explained by the negative impact on net photosynthetic rate (A_N), which decreased with temperature increment, being this reduction more acute at 700 ppm CO_2. This response was associated with an augmentation in CO_2 diffusion limitations, as indicated the lower stomatal conductance (g_s), together with a down-regulation photochemical apparatus efficiency, as indicated the lower electron transport rate (ETR) and energy fluxes derived from Kautsky curves. In addition, the greatest g_s drop at 700 ppm CO_2, would limit plant ability to cope with temperature excess through evapotranspiration, a fact that could have boosted temperature-triggered damage and, consequently, leaf senescence. Therefore, we can conclude that temperature and atmospheric CO_2 increments would compromise the development of *S. maritima* and consequently the maintaining of its ecosystem functions.

Keywords: CO_2 enrichment; Climate change; Chlorophyll fluorescence; Gas exchange; Halophyte; Temperature stress.

1. Introduction

Climate change arises as one of the greatest challenges for worldwide ecosystems conservation. Thus, along with recognized atmospheric CO_2 enrichment to values ~700 ppm by the end of the century (IPCC, 2014), climate models predict a parallel increase in daily temperature range (between 1.8 - 6.0 ºC, depending on the region) and rainfalls pattern alteration which would lead to a decrease in water availability and soil salinization (IPCC, 2014). An important part of the effect of climate change on ecosystems will be related to those variations triggered by environmental conditions in the structure and composition of plant species (Allen and Ort, 2001; Hooper et al., 2005; Short et al., 2016; Zhao et al., 2018), being this impact greater in those ecosystems dominated by few species, which also sustain key functions (such as the control of the fluxes of energy and matter) and contribute to functional diversity and species assemblages (Hooper et al., 2005).

The genus *Spartina* is integrated by grass species widely distributed on every continent except for Antarctica, being many of them frequently found in coastal salt marshes (Bortolus et al., 2019). It has been highlighted the importance of these species in salt marshes ecosystem functions, with a relevant role on coastal accretion and marsh creation, as well as the maintenance of ecosystem primary and secondary production (Bortolus et al., 2019). Among *Spartina* genus species, the European *Spartina maritima* (Curtis) Fernald is an important pioneer and ecosystem engineer in European salt marshes (Castellanos et al., 1994), playing also an important role for the maintenance and conservation of marsh ecosystem biodiversity (Curado et al., 2018). This species develops its populations mainly in the lowest parts of the marshes, acting as a primary colonist contributing to facilitate succession in marsh ecosystems (Castellano et al.,

1994, 1998; Castillo et al., 2008) and playing a key role in shoreline stabilization (Duarte et al. 2014). In addition, this species has demonstrated a high biotechnological potential as a tool for monitoring and phytoremediating metal polluted areas (Padinha et al., 2000; Mesa et al., 2015). Furthermore, many studies have been developed to understand the impact of environmental factors associated with future global change scenarios on the development of this important salt marsh species (Mateos-Naranjo et al., 2010a; Couto et al., 2014; Duarte et al., 2014). Thus, a certain effort has been made to assess the effect of atmospheric CO_2 enrichment on *S. maritima* development, having observed an improvement on plant growth and physiological performance under optimal and suboptimal salinity conditions, despite being a C_4 species (Mateos-Naranjo et al., 2010a). Besides, *S. maritima* has demonstrated the ability to maintain its photosynthetic activity even during prolonged submersion periods associated with the sea level rise due to global warming (Duarte et al., 2014). In addition, a rising temperature model has demonstrated an enhancement in this salt marsh species aboveground biomass (Couto et al., 2014), but it has been identified that an increase in the frequency and duration of high temperature events will lead to a decrease of its photo-biological fitness (Duarte et al., 2016). Despite these efforts, there is great uncertainty about the real effects of climate change on the conservation of this species, since most of those studies only have evaluated plant responses to one or two environmental stressors in combination. Therefore, a challenge exists in determining plant responses in a complex environmental matrix, such as the one predicted by climate models. Consequently, this study was designed and carried out to understand the influence of a complex environmental matrix, characterized by atmospheric CO_2 concentration, air temperature and medium saline concentration variations on *S. maritima* growth and development as well as key photosynthetic parameters. We

hypothesized that, although a direct positive physiological impact of increased atmospheric CO_2 concentration on *S. maritima* has been previously identified (Mateos-Naranjo et al., 2010a), the co-occurrence of other stress factors such as temperature pattern variation and medium salinization could trigger metabolic responses that could jeopardize the development of this important salt marsh species and, consequently, the maintenance of the ecosystem functions in which this plant is involved.

2. Material and Methods

2.1. Plant material

Clumps of *S. maritima* of 20 cm diameter with between 25-30 mature tillers were collected in June of 2019 from a well-established population in a low-marsh site located in the Odiel salt marshes (37º15'N, 6º58'O; SW Spain). Clumps were planted in individual plastic pots (15 cm high × 18 cm diameter) using its own soil as a potting substrate, and placed in a greenhouse under the follow controlled conditions: maximum temperature between 21/25 ºC, minimum temperature between 13/11 ºC, 40-60% relative humidity and natural daylight of 200 μmol m^{-2} s^{-1} as minimum and 1000 μmol m^{-2} s^{-1} as maximum light flux). Pots were irrigated with tap water, and plants were kept for a stabilization period of 7 days under the previously described conditions before the experiment's onset.

2.2. Experimental design

At the beginning of the experiment, the number of tillers in each pot was homogenized to 20 tillers completely developed and healthy in order to standardize our

samples before the experiment onset and to avoid any effect of tiller health and age in our results. Then, pots were randomly assigned to eight different experimental blocks with ten plants in each one, as follows: two concentrations of atmospheric CO_2 (400 ppm and 700 ppm CO_2) in combination with two ranges of ambient maximum and minimum temperature (28/13 ºC and 32/17 ºC) and irrigation with two salinity concentrations (0 and 171 mM NaCl) for 40 days. For the atmospheric CO_2 concentration and temperature range treatments, pots were placed in controlled-environment chambers (Aralab/Fitoclima 18.000EH, Lisbon, Portugal), which were programmed with alternating diurnal regime of 14 h of light and 10 h of darkness with the specific maximum and minimum temperature range, light intensity of 300 μmol m^{-2} s^{-1}, 40–60% relative humidity and the specific atmospheric CO_2 concentration. Atmospheric CO_2 concentrations in chambers were continuously monitored by CO_2 sensors and maintained by supplying pure CO_2 from a compressed gas cylinder (Air liquide, B50 35K). Finally, NaCl concentrations were established by combining tap water with appropriate amounts of NaCl. At the beginning of the experiment, the pots were placed in plastic trays containing the appropriate NaCl solutions for each specific salinity concentration treatment to a depth of 1 cm. In order to avoid changes of the NaCl concentration caused by water evaporation, levels in the trays were monitored continuously throughout the experimental period.

2.3. Plant growth analysis

At the beginning and at the end of the experiment, three and seven plants from each specific treatment were harvested and divided into roots and tillers. Then, these biomass fractions were oven dried at 60 ºC for 48 h and weighed to obtain both initial and final

dry biomass estimates. In addition, the number of dead tillers was recorded at the end of the experiment.

The relative growth rate (RGR) of whole plants was calculated using the formula:

$$RGR = (\ln B_f - \ln B_i) \cdot D^{-1} \ (g\ g^{-1}\ day^{-1})$$

where B_f = final dry mass (the mean of the seven plants from each treatment at the end of the experiment), B_i = initial dry mass (the mean of the three plants from each treatment at the beginning of the experiment) and D = duration of experiment (days).

2.4. Leaf gas exchange analysis

At the end of the experiment, instantaneous leaf gas exchange measurements were taken on fully developed expanded leaves (n = 10~~, one in each plant per treatment and three extra randomly selected for each treatment~~) using an infrared gas analyzer in an open system (LI-6400-XT, Li-COR Inc., NE., USA) equipped with a light leaf chamber (Li-6400-02B, Li-Cor Inc.). Net photosynthesis rate (A_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were recorded under the following leaf chamber settings: a photosynthetic photon flux density (PPFD) of 1000 µmol photons $m^{-2}\ s^{-1}$ (with 15% blue light to maximize stomatal aperture), vapour pressure deficit of 2.0–3.0 kPa, 50 ± 5% relative humidity, CO_2 concentration surrounding the leaf (C_a) of 400 or 700 µmol CO_2 mol^{-1} air depending on the atmospheric CO_2 concentration treatment and air temperature of 28 °C or 32 ºC for plants grown at low and high temperature range treatments, respectively. All measurements were made between 10:00 and 13:00 h inserting the order between the different treatments to standardize samples, and before to record each measurement, gas exchange was allowed to equilibrate for 120 s. Finally,

intrinsic water use efficiency ($_i$WUE) was calculated as the ratio between A_N and g_s [mmol (CO_2 assimilated) mol^{-1} (H_2O transpired)].

2.5. Chlorophyll fluorescence analysis

Two different fluorescence protocols were developed at the end of the experiment in the same leaves of gas exchange analysis in order to test how the different combinations of atmospheric CO_2 concentration, air temperature and medium salinity concentration used in this study affect photosystem II (PSII) energy use efficiency. Thus, the saturation pulse method was used to determine the maximum quantum efficiency of PSII photochemistry (F_v/F_m) and quantum efficiency of PSII (Φ_{PSII}; Genty et al., 1989). As described by Schreiber et al. (1986), a 0.8 s saturating actinic light pulse of 10000 µmol m^{-2} s^{-1} was applied at midday (1400 µmol photons m^{-2} s^{-1}) in previously dark- and light-adapted leaves (n = 10~~, one in each plant per treatment and three extra randomly selected for each treatment~~) for 30 min using a modulated fluorimeter (FMS-2; Hansatech Instruments Ltd., UK). Using this information, the electron transport rate (ETR) was calculated according to Krall and Edwards (1992).

On the other hand, the chlorophyll *a* fast kinetics, by the OJIP-test (or Kautsky curves), which depicts the rate of reduction kinetics of various components of PSII, was also measured in 30 min dark-adapted leaves (n = 7, one in each plant per treatment), using the pre-programmed OJIP protocols of the FluorPen FP100 (Photo System Instruments, Czech Republic). Moreover, absorbed (ABS/RC), trapped (TR/RC), electron transport (ET/RC) and dissipated (DI/RC) energy fluxes per reaction center derived from OJIP were calculated according to Strasser et al. (2004).

2.6. Statistical analysis

R software ver. 4.0.0 (R Core Team, 2020) was used to perform the statistical analyses. Firstly, a multivariate statistical approach using a principal component analysis (PCA) was performed to get an overview of the plant growth and physiological performance in response to the different experimental treatments. Missing values in the PCA dataset appeared as a result of the different number of measurements in each variable. They were handled using the expectation-maximization (EM) algorithm in the 'missMDA' R package (Josse and Husson, 2016). Secondly, a downscaling assessment was carried out through generalized linear models (GLMs) to analyze the main and/or ~~interactive~~ interaction effects of atmospheric CO_2 concentration, maximum and minimum temperature range and low and high NaCl concentration (as categorical factors) on the growth and physiological parameters (as dependent variables) of *S. maritima* plants. In case of significant results, multiple comparisons were analyzed by *post hoc* LSD test (i.e. Fisher's Least Significant Difference). Before statistical analysis, Kolmogorov-Smirnov and Levene tests were used to verify the assumptions of normality and homogeneity of variances, respectively. ~~A summary of generalized linear model (GLM) results is made available as supplementary material, including fitting tests and confidence intervals (Table A.1).~~

3. Results

3.1. Multivariate approach: global overview of S. maritima growth and physiological status

Growth and physiological performance variations of *S. maritima* during the experimental setup were mainly represented in the first two PCA axes. They explained, respectively, 36.0% and 18.4% of the total variation in the recorded data, being both temperature range and atmospheric CO_2 enrichment the experimental factors which led

the main groupings (Fig. 1A, B; Table 1). Hence, the PC1 axis reflected a clear divergence of plants grown at 32/17 ºC of maximum and minimum temperature range, which were located in the left part of the plot (especially those grown at 700 ppm CO_2). This separation was mainly explained by the lower RGR, A_N, F_v/F_m, Φ_{PSII} and ETR values, together with an increment in the percentage of dead tillers and DI/RC in those plants compared to the rest of the treatments (Fig. 1B; Table 1). In addition, the PCA revealed a certain divergence between both atmospheric CO_2 concentration treatments along the PC2 axis, with most of non-CO_2 enriched plants located in the upper part, being this response pattern mainly linked with higher g_s and lower C_i values of plants grown at 400 ppm CO_2 (Fig. 1B; Table 1).

3.2. Effects of atmospheric CO_2 enrichment, temperature augmentation and NaCl concentration on S. maritima growth

A summary of generalized linear model (GLM) results is made available as supplementary material, including fitting tests and confidence intervals (Table A.1). There was a significant effect of temperature range treatment on the RGR of *S. maritima* after 40 days of treatment (GLM: Temp., $p < 0.01$; Table A.1). Additionally, this effect was, to some extent, modulated by atmospheric CO_2 concentration treatment (GLM: [CO_2] x Temp., $p < 0.05$; Table A.1). Thus, RGR decreased considerably in plants grown under the highest maximum and minimum temperature range treatment (i.e. 32/17 ºC), and this effect was more acute in those exposed to 700 ppm CO_2 regardless of saline irrigation treatment (Fig. 2A).

Similarly, the percentage of dead tillers augmented with temperature and atmospheric CO_2 enrichment regardless of saline irrigation treatment (GLM: Temp., $p <$

0.01; [CO_2], $p < 0.05$; Table A.1), being this increase more pronounced in plants grown at 700 ppm CO_2 and 32/17 ºC compared to the rest of treatments, although no significant differences were found (Fig. 2B).

3.3. Effects of atmospheric CO_2 enrichment, temperature augmentation and NaCl concentration on photosynthetic apparatus performance

There was a significant effect of the environmental factors tested on leaf gas exchange characteristics and PSII photochemical effiency of *S. maritima* after 40 days of exposure (GLM, $p < 0.05$). Thus, A_N values decreased in plants grown at 32/17 ºC (GLM: Temp., $p < 0.01$; Fig. 3A; Table A.1), and these values were overall lower at 700 ppm CO_2 although without significant differences. A very similar trend was recorded for g_s in relation to temperature increment (GLM: Temp., $p < 0.01$) but, in addition, g_s values were significantly lower in plants grown at 700 ppm CO_2 (GLM: [CO_2], $p < 0.01$; Fig. 3B; Table A.1). Contrarily, C_i and $_i$WUE values were significantly higher in plants grown at 700 ppm CO_2 compared to their non-CO_2 enriched counterparts, regardless of temperature and saline irrigation treatments (GLM: [CO_2], $p < 0.01$; Fig. 3C, D; Table A.1).

Regarding photochemical parameters, F_v/F_m values were lower in plants subjected to the highest minimum and maximum temperature range treatment at both salinity irrigation conditions (GLM: Temp., $p < 0.01$), being this reduction also more pronounced in plants grown at 700 ppm CO_2 (GLM: [CO_2] x Temp., $p < 0.05$; Table A.1), and especially in those plants irrigated with 171 mM NaCl (Fig. 4A). Likewise, minimum and maximum temperature augmentation led to a marked reduction in *S. maritima* Φ_{PSII} values, although this overall effect was much more marked in those plants exposed to high atmospheric CO_2 concentration and saline irrigation (GLM:

[CO_2] x Temp., $p < 0.05$; Fig. 4B; Table A.1). A very similar response was detected for ETR, with the lowest values recorded in plants subjected to 700 ppm CO_2, 32/17 ºC and irrigation with 171 mM NaCl (Fig. 4C).

Focusing on the chlorophyll *a* fast kinetics results, there were remarkable differences in the shape of Kautsky curves between plants grown under the different enviromental factors after 40 days of treatments. Thus, overall fluorescence kinetics values were lower in plants subjected to 700 ppm CO_2 and 32/17 ºC, being this trend also more pronounced in plants irrigated with 171 mM NaCl (Fig. 5). These Kautsky curve shape divergence was also accompanied by marked differences in energy fluxes through photosystems. Thus, ABS/RC was lower in plants grown at 700 ppm CO_2 and 28/13 ºC, compared to those exposed to 400 ppm CO_2 (GLM: [CO_2] x Temp., $p < 0.01$; Table A.1). Moreover, ABS/RC showed an increase in plants subjected to 700 ppm, 32/17 ºC and irrigation with 171 mM NaCl compared to the rest of treatments (GLM: [CO_2] x Temp. x [NaCl], $p < 0.05$; Fig. 6A; Table A.1). A very similar trend was recorded for DI/RC (Fig. 6D). Oppositely, both ET/CS and TR/CS tended to decrease in plants exposed to 700 ppm CO_2 compared with their non-CO_2 enriched counterparts (GLM: [CO_2] , $p < 0.05$; Fig. 6B, C; Table A.1). However, this effect was more pronounced for TR/CS in plants grown at 28/13 ºC compared to those grown in a higher temperature range (GLM: [CO_2] x Temp., $p < 0.01$; Fig. 6C; Table A.1).

4. Discussion

Gaining a mechanistic understanding of the effect of the main coexisting factors linked with climate change, such as atmospheric CO_2 enrichment, temperature pattern variations and medium salinization, on the growth and physiological performance of key plant species in ecosystem functionality is crucial to obtain a more realistic view on

how this global phenomenon influences ecosystems conservation and the development of these plant communities (Bernacchi et al., 2013).

Our results demonstrated that atmospheric CO_2 enrichment, as a consequence of climatic change, would not cause a substantial improvement on growth and development of the important pioneer and salt marsh ecosystem engineer species *S. maritima*. However, an increase up to 4 ºC in the maximum and minimum daily temperature range, as expected by the end of the century (IPCC, 2007), would entail a negative effect on the development of this species, being this impact even more acute in a CO_2 enriched atmosphere. Thus, our results revealed that *S. maritima* plants grown at 400 ppm atmospheric CO_2 concentration and 32/17 ºC of maximum and minimum daily temperature range showed a drastic growth reduction, measured as RGR, of ~39% compared to their counterparts exposed to the colder temperature range. This reduction was up to 64% in those plants also exposed to 700 ppm CO_2, regardless of the saline irrigation treatment. The lack of salinity effects on plant growth, as well as for most of the studied parameters, was somewhat expected since *S. maritima* is a halophyte species that has demonstrated a high tolerance to a wide range of salinities (Adams and Bate, 1995; Castillo et al., 2008; Mateos-Naranjo et al., 2010a). However, it should be highlighted that the absence of beneficial effects of atmospheric CO_2 enrichment on plant development contrasts with the previous results obtained by Mateos-Naranjo et al. (2010a) for this plant species, who observed that an increment of atmospheric CO_2 concentration to 700 ppm stimulated plant RGR ~40% through a salinity range between 0 and 510 mM NaCl. Similarly, our results also varied with respect to those shown by other *Spartina* species, such as *S. densiflora*, which experienced an increase in biomass production of 35% and 20%, respectively at 0 and 171 mM NaCl, compared to plants grown at 400 ppm CO_2 (Mateos-Naranjo et al., 2010b). These differences could be

related to the variation in the experimental conditions of each specific study. In this study, plants were subjected to different environmental synergies growing in natural soil whilst, in the aforementioned experiments, plants were grown in pearlite. The use of this substrate would allow the previous studies to assess the direct effect of atmospheric CO_2 enrichment on plant performance avoiding the influence of other factors associated to soil physicochemical properties, which could alter plant direct responses to atmospheric CO_2 enrichment. However, we believe that the use of a natural soil-plant complex, as we have done in this study, would allow a more realistic view of the real impact of climate change factors on plant development. In fact, according to our results, Curtis et al. (1989), in an experiment developed under field conditions using OTC chambers, were unable to detect a response to atmospheric CO_2 enrichment in *Spartina patens*. Furthermore, Rozema et al. (1991) obtained different results regarding the effect of atmospheric CO_2 enrichment on *S. patens* performance, depending on growth medium aeration level in plants grown in hydroponic conditions. To some degree, this lack of response to atmospheric CO_2 enrichment is consistent with multitude of CO_2 enrichment experiments, which indicated that plants with C_4 photosynthetic metabolism, as *S. maritima*, would not be as benefited by the air CO_2 fertilization effect (Ghannoum et al., 2000). However, this overall response could be modified by interactions with other co-occurring stress factors related to climate change, such as temperature pattern variation or medium salinization (IPPC, 2014). Therefore, it has been described that the effect of atmospheric CO_2 enrichment on plant development could be neutral, positive through the improvement of plant tolerance against environmental stress or negative by enhancing environmental stress deleterious effects, being also these responses species-specific (Balfagón et al., 2019). In this respect, as we previously indicated, the increment in temperature together with an atmospheric CO_2

concentration enrichment would substantially reduce *S. maritima* growth. Besides, it is important to emphasize that this lower plant development was accompanied by an increase in senescence level, as reflected by the higher percentage of dead stems at the end of the experiment.

The deleterious effect of increased temperatures and, to a greater extent, CO_2-enriched atmosphere conditions on *S. maritima* development was partly explained by the negative impact of these factors on some essential steps of plant photosynthetic apparatus performance. Thus, our results showed a substantial reduction in plant carbon fixation capacity, measured as A_N, at elevated temperature range treatment being this reduction in certain degree more pronounced at elevated atmospheric CO_2 concentration. This effect was associated with an augmentation in CO_2 diffusion limitations through stoma in plants grown at 32/17 ºC, as indicated their lower g_s values. Temperature is one of the most variable environmental factors and it can affect many plant physiological processes, including plant gas exchange characteristics, but little is known about its effect on g_s, especially at high temperatures (Teskey et al., 2015). In fact, several studies have recorded a wide range of plant responses to temperature increment, including stomal opening (Lu et al., 2000; Mott and Peak, 2010) and closure (Weston and Bauerle, 2007; Lahr et al., 2015) but also non-significant responses (Cerasoli et al., 2014; von Caemmerer and Evans, 2015). Moreover, stomatal behaviour to temperature could be modified by interactions with other environmental factors (Flexas et al., 2012). In this sense, there is a need to highlight that g_s reduction with temperature increment was even more acute in those plants exposed to 700 ppm CO_2. In addition, this reduction was greater than the one reported in A_N, contributing to overall higher $_i$WUE values under rising atmospheric CO_2 conditions. Thus, this decoupling between g_s and A_N in plants grown at 700 ppm CO_2 and 32/17 ºC would reflect the

higher stomatal sensitivity to CO_2 variations. In fact, different studies have shown that one of the most consistent responses of C_4 plant species to atmospheric CO_2 concentration enrichment is a decrease in g_s (Ainsworth and Rogers, 2007). Robredo et al. (2007) explained that C_i increase caused by the high CO_2 concentration could promote partial closure of the stoma, as it was recorded in this study. However, the higher stomatal sensitivity to elevated CO_2 regardless of temperature, could have had also important consequences for plant tolerance to heat stress. Thus, although stomatal behaviour is a key factor to preserve the trade-off between CO_2 acquisition for photosynthetic processes and water losses, it is necessary to emphasize its role for plant adaptation to thermal stress through the regulation of heat excess by increasing leaf evapotranspiration (Feller, 2006). Therefore, the importance of stoma in leaf cooling has been identified, and while the stomata remains open, the evaporative cooling can mitigate the negative effect of temperature excess, positively affecting plant photosynthesis, yield and survival (Lu et al., 1994; Ameye et al., 2012). Therefore, the higher g_s drop in *S. maritima* plants under elevated atmospheric CO_2 concentration could have been limiting their ability to modulate leaf temperature compared to plants grown at 400 ppm, which could also contribute to explain the greater injury effect of temperature increment at these experimental conditions.

On the other hand, the impact of temperature increase, alone or in combination with atmospheric CO_2 enrichment, had a great impact on *S. maritima* photochemical apparatus functionality. According to our results, one of the best recognized effects of high temperature on plant photosynthetic apparatus is the destruction of PSII components (Flexas et al., 2012; Pérez-Romero et al., 2019a; López-Jurado et al., 2020). Likewise, in concordance with this study, Mateos-Naranjo et al. (2010b) and Duarte et al. (2014) also found that CO_2 enrichment affected PSII photochemistry in the

halophytes *S. densiflora* and *S. maritima*, respectively. However, this is the first study that corroborates that this effect would be exacerbated with an increase up to 4 ºC in the maximum and minimum daily temperature range and, to some extent, with the augmentation in medium salinity concentration. Thus, our results showed that F_v/F_m and Φ_{PSII} values were affected by the elevated temperature range and this effect was more acute in plants grown at 700 ppm CO_2 and irrigated with 171 mM NaCl, suggesting that the combination of these environmental conditions would increase photoinhibition (Werner et al., 2002). Likewise, we attributed the negative impact of these environmental factors on *S. maritima* photochemical apparatus to the down-regulation of its electron transport chain functionality, as indicated by the lower ETR values recorded in plants grown at 700 ppm CO_2, 32/17 ºC and irrigated with 171 mM NaCl. This fact indicated that these plants would have had more difficulties to transform the captured energy in their photosystems, suggesting the synergistic impact of elevated temperature and atmospheric CO_2 enrichment on the functionality of reaction centers. Thus, this idea was also supported by the assessment of energy fluxes per reaction center derived from Kautsky curves, which showed that, although plants were exposed to elevated atmospheric CO_2 and air temperature range, they increased their ABS/RC values compared to the rest of treatments. This would mean a greater number of active reaction centers functioning as a heat radiator, hence protecting plant photosystems against high temperature and light intensities (Strasser et al., 2004; Pérez-Romero et al., 2018, 2019a,b). This elevated absorption rate would excess the capacity of photosystems to metabolize energy, as was corroborated by the decoupling with TR/RC and ET/RC values. Moreover, the high ABS/RC would unbalance the trade-off between the energy absorbed and metabolized, being necessary the activation of some defense mechanisms to dissipate the energy excess as heat, as indicated also higher DI/RC

values recorded. As a consequence, much of the absorbed energy would not take the photochemical pathway (Flexas et al., 2012), a circumstance that would affect photosynthetic productivity and, consequently, plant growth, a fact which would contribute to explain the observed growth pattern in response to tested environmental factors. Therefore, our results would suggest that temperature and atmospheric CO_2 increments related to climate change would jeopardize the development and maintenance of natural populations of *S. maritima*. This circumstance would cause significant structural and functional changes in the drainage system of the marshes systems dominated by this grass species, due to its importance in shoreline stabilization and salt marsh accretion during the first phases of ecological succession, with the consequent alteration of sediment, mineral elements and nutrient cycles, as well as the fluxes of energy necessary for other levels of the trophic chain in the ecosystem.

5. Conclusion

This study reveals the high vulnerability of the salt marsh key species *S. maritima* to increases up to 4 ºC in the maximum and minimum daily temperature, as predicted for future scenarios by global change models, being this susceptibility even higher under CO_2 enriched atmosphere conditions. This idea would be motivated by drastic plant growth decrease and leaf senescence increment recorded at 700 ppm CO_2 and 32/17 ºC of maximum and minimum daily temperature range. This response was associated with the negative effect of temperature and atmospheric CO_2 concentration on plant carbon fixation machinery through increased stomatal limitations to CO_2

diffusion and the unbalance between the energy absorbed and metabolized in *S. maritima* photosystems, linked with a down-regulation of the electron transport chain functionality. The higher g_s drop at 700 ppm CO_2 would also limit plant ability to regulate heat excess through leaf evapotranspiration, a fact that could have boosted temperature-triggered damage and, consequently, leaf senescence compared to plants grown at 400 ppm. Finally, from a conservational perspective, these results suggest that temperature increment and atmospheric CO_2 enrichment associated with future climate change scenarios would jeopardize the development of *S. maritima* populations and, consequently, the maintenance of ecosystem functions derived from the presence of this important pioneer and ecosystem engineer salt marshes plant species.

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CRediT authorship contribution statement

Enrique Mateos-Naranjo: Conceptualization, Methodology, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. **Javier López Jurado:** Methodology, Formal analysis, Writing - review & editing. **Jennifer Mesa Marín:** Methodology, Writing - review & editing. **Carlos Javier Luque:** Conceptualization, Methodology, Writing - review & editing. **Eloy Manuel Castellanos:** Conceptualization, Methodology, Writing - review & editing. **Jesús Alberto Pérez-Romero:** Methodology. **Susana Redondo-Gómez:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Fig. 1. Representation of the principal component analysis (PCA) biplot obtained for the growth and physiological data of *Spartina maritima* plants in the experimental set-up. Panel A represents the distribution of the selected variables loading on these axes and the percentage of explained variance for these axes (see Table 1 for a description of these variables and their contribution to the two main axes of PCA). Panel B shows the ellipses (95% confidence level) which encompass occurrence points in the two main axes for the environmental factors tested.

Fig. 2. Relative growth rate, RGR (A) and percentage of dead tillers (B) in *Spartina maritima* plants in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 7. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 3. Net photosynthetic rate, A_N (A), stomatal conductance, g_s (B), intercellular CO_2 concentration, C_i (C) and intrinsic water use efficiency ($_i$WUE) in randomly selected fully developed expanded leaves of *Spartina maritima* in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 10. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 4. Maximum quantum efficiency of PSII photochemistry, F_v/F_m (A), quantum efficiency of PSII, Φ_{PSII} (B) and electron transport rate, ETR (C), at midday in randomly

selected fully developed expanded leaves of *Spartina maritima* in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 10. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 5. Transient fluorescence (Kautsky curves) of dark-adapted leaves of *Spartina maritima* plants grown at 400 ppm (A) and 700 ppm (B) atmospheric CO_2 in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean of seven measurements per treatment combination.

Fig. 6. Absorbed energy flux, ABS/RC (A), electron transport energy flux, ET/RC (B), trapped energy flux, TR/RC (C) and dissipated energy flux, DI/RC (D), per reaction centre in randomly selected fully developed expanded dark-adapted leaves of *Spartina maritima* in response to treatment with two atmospheric CO_2 concentrations (400 ppm and 700 ppm) in combination with two temperature ranges (28/13 ºC and 32/17 ºC) and irrigation with 0 mM NaCl (-) or 171 mM NaCl (+) for 40 days. Values represent mean ± SE, n = 7. [CO_2], Temp., NaCl or their interactions in the corner of the panels indicate main or interaction significant effects (GLM test, $^{*}P < 0.05$, $^{**}P < 0.01$).

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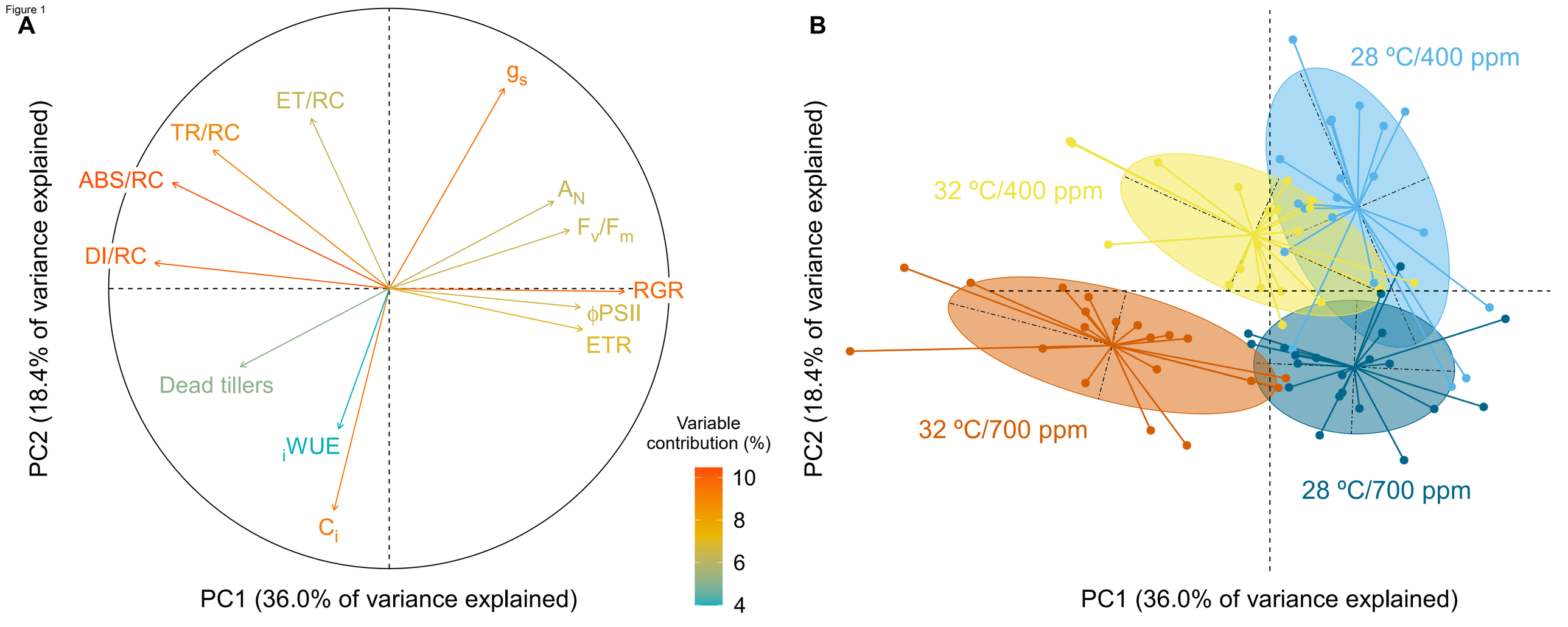
Figure 1


A
B
PC2 (18.4% of variance explained)
PC1 (36.0% of variance explained)
g_s
ET/RC
TR/RC
ABS/RC
DI/RC
A_N
F_v/F_m
RGR
ϕPSII
ETR
Dead tillers
$_i$WUE
C_i
Variable contribution (%)
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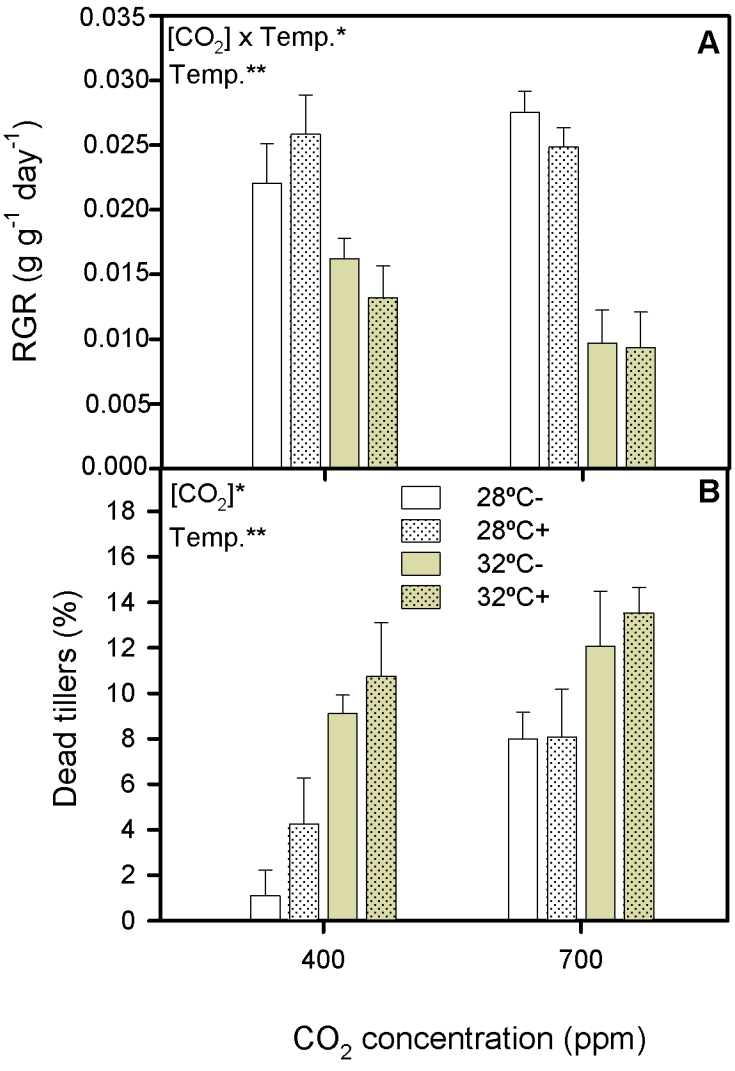
8
6
4
28 ºC/400 ppm
32 ºC/400 ppm
32 ºC/700 ppm
28 ºC/700 ppm

Fig. 2


[CO2] x Temp.*
Temp.**
A
RGR (g g-1 day-1)
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
[CO2]*
Temp.**
B
28ºC-
28ºC+
32ºC-
32ºC+
Dead tillers (%)
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16
14
12
10
8
6
4
2
0
400
700
CO2 concentration (ppm)

Figure 3

Fig. 3

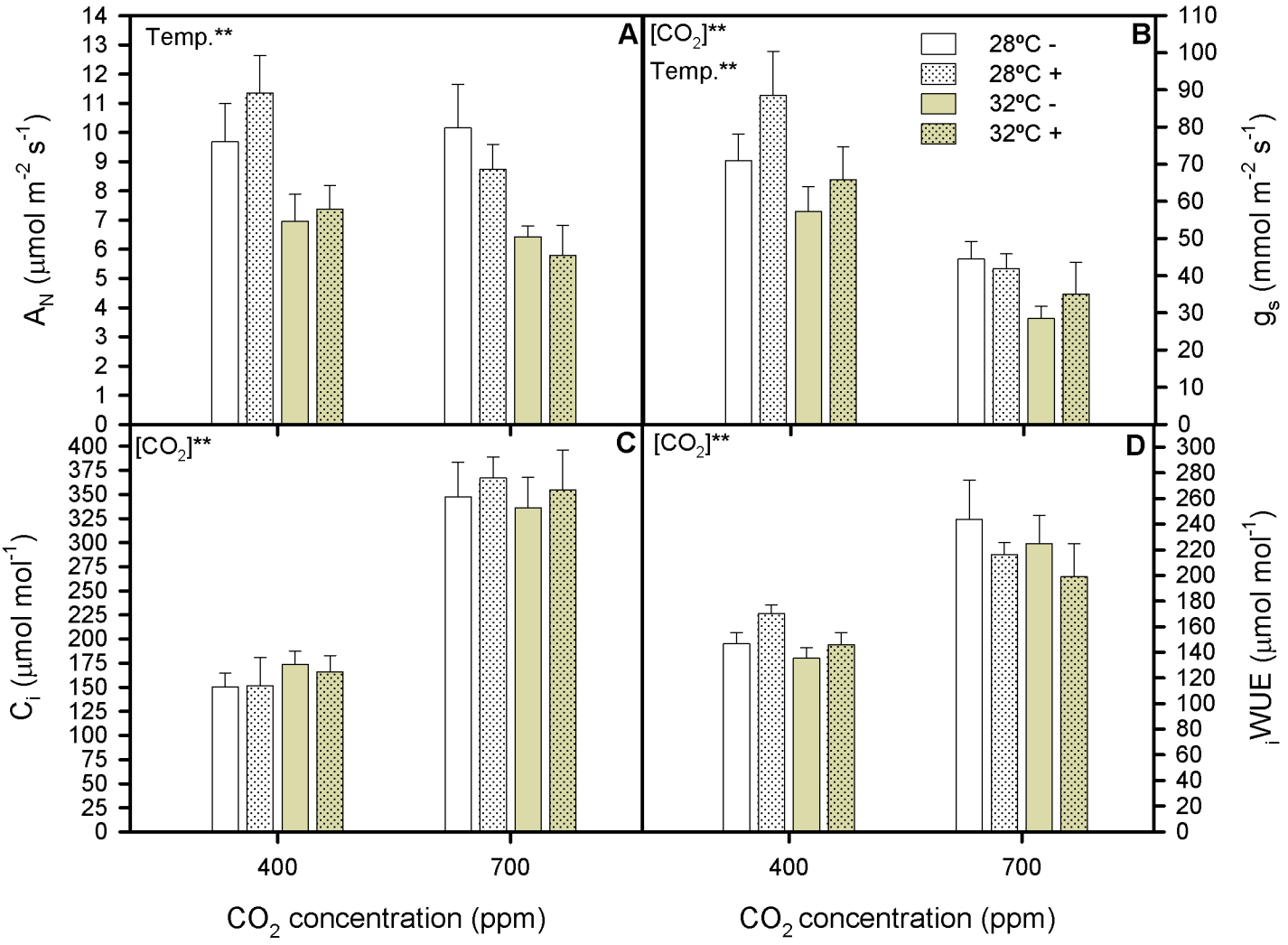

Figure 4

Fig. 4

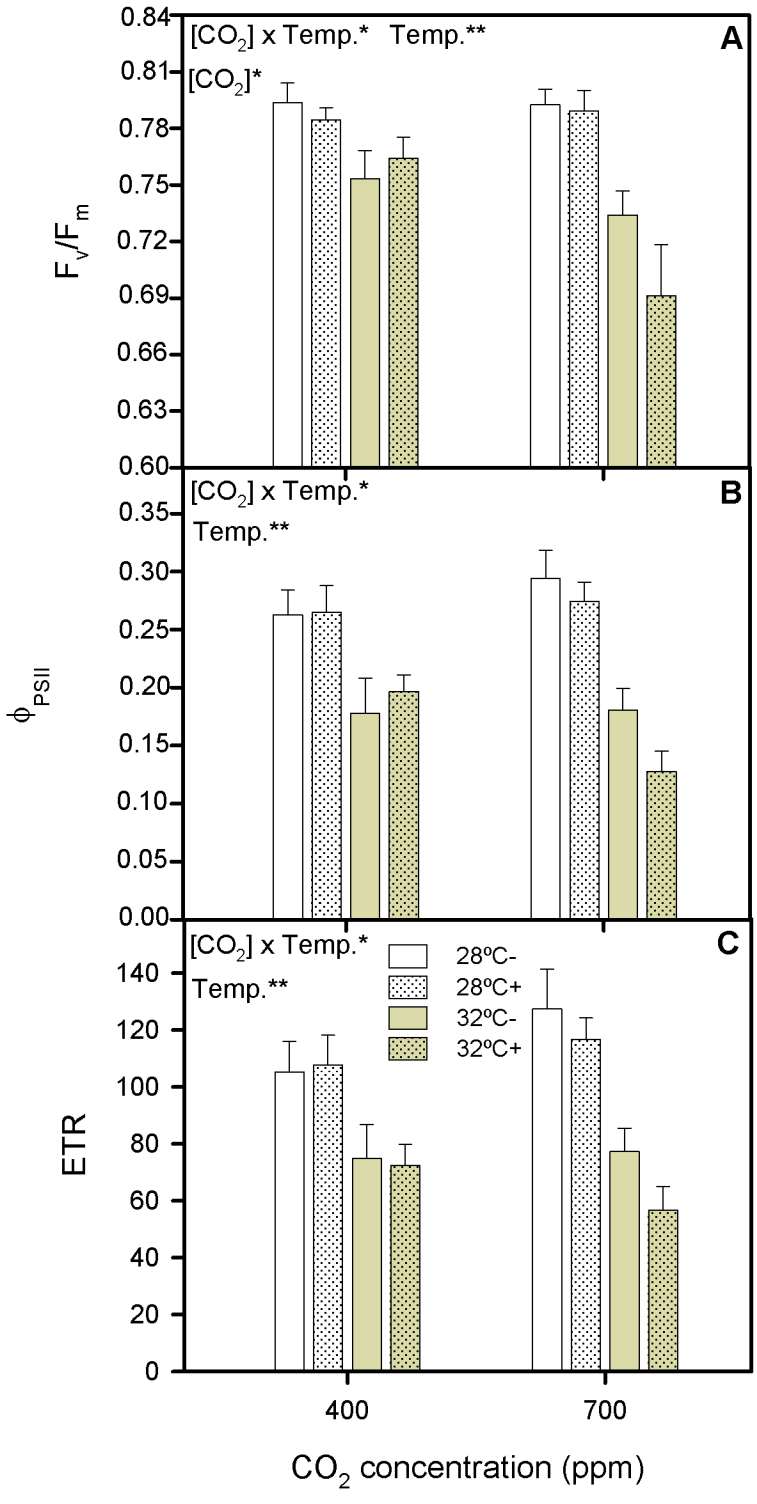

Figure 5

Fig. 5

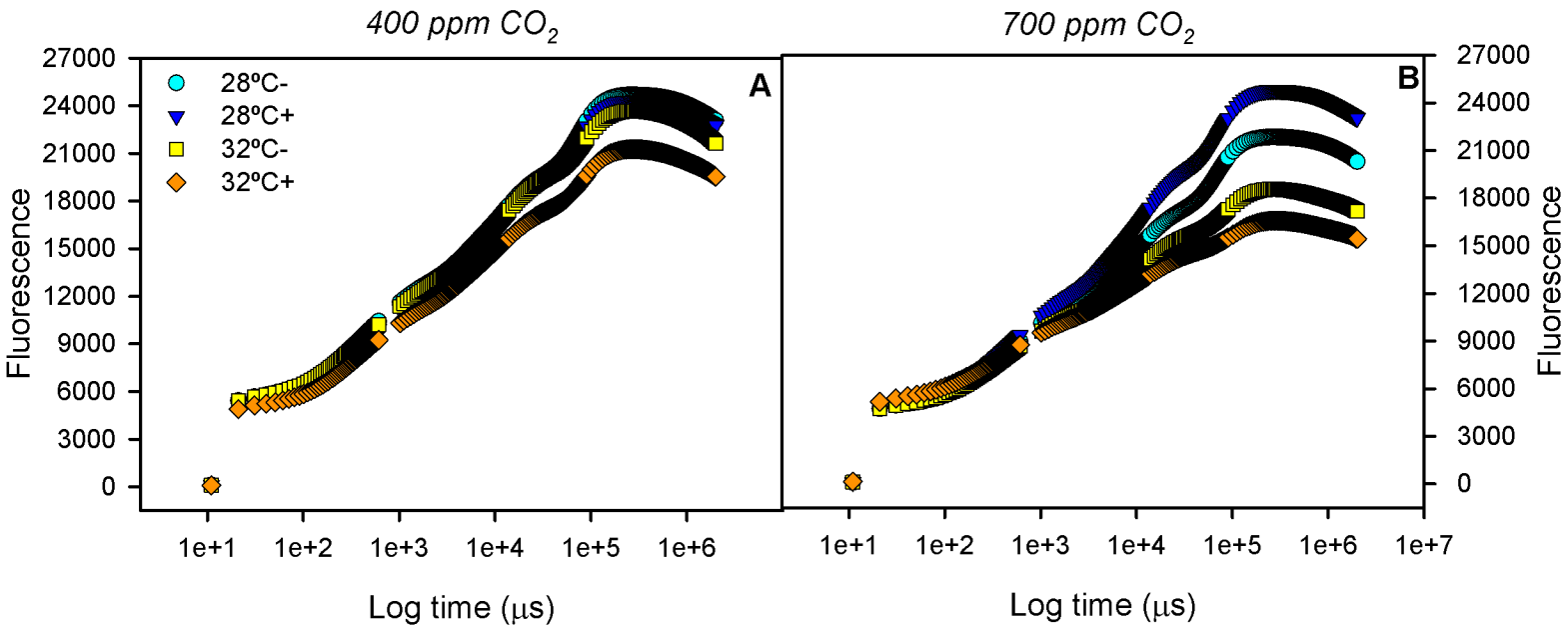

Figure 6

Fig. 6

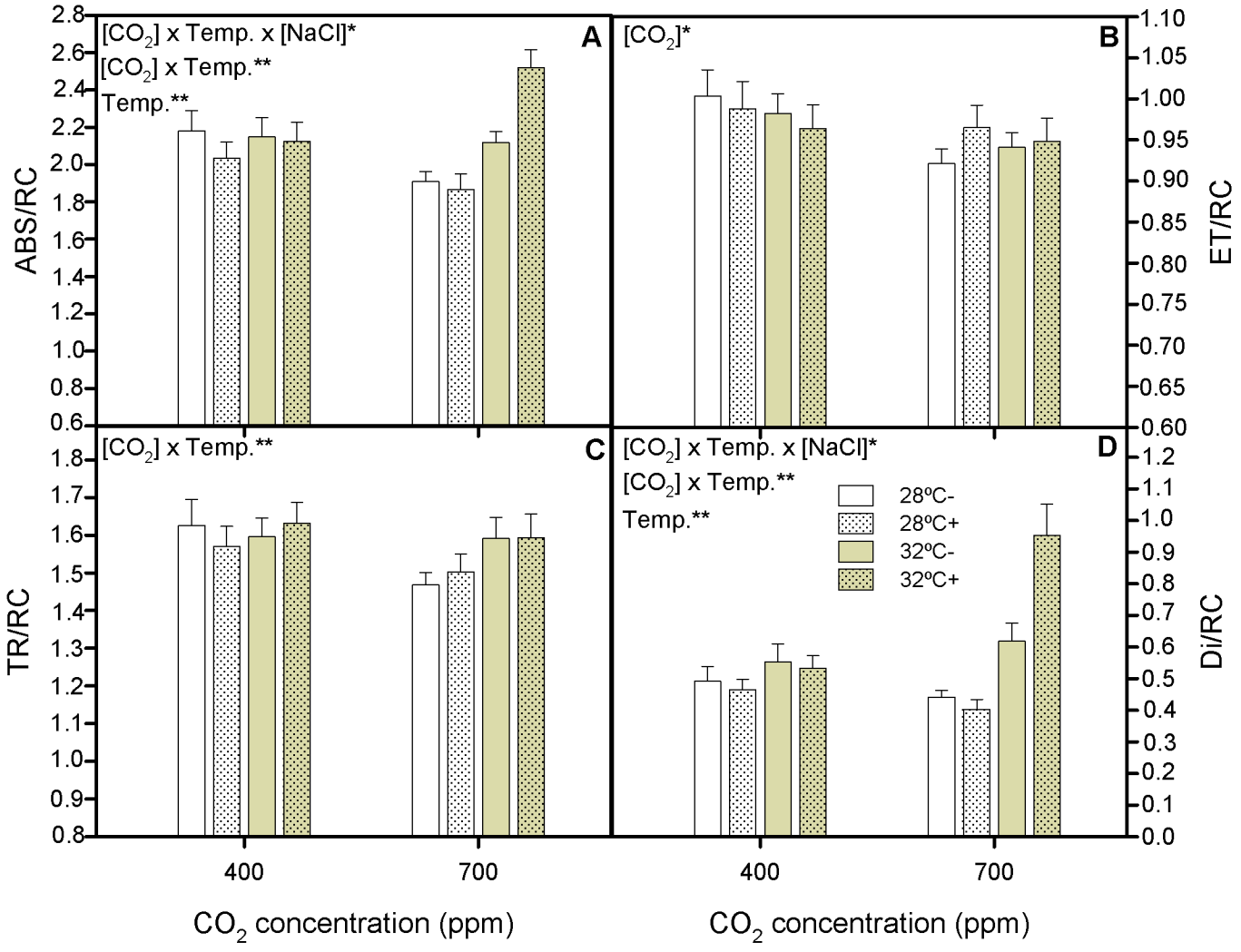

Table 1

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Table

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Table A.1

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Enrique Mateos-Naranjo: Conceptualization, Methodology, Formal analysis, Funding acquisition, Writing - original draft, Writing - review & editing. **Javier López Jurado:** Methodology, Formal analysis, Writing - review & editing. **Jennifer Mesa Marín:** Methodology, Writing - review & editing. **Carlos Javier Luque:** Conceptualization, Methodology, Writing - review & editing. **Eloy Manuel Castellanos:** Conceptualization, Methodology, Writing - review & editing. Jesús Alberto Pérez-Romero: Methodology. **Susana Redondo-Gómez:** Conceptualization, Methodology, Funding acquisition, Writing - review & editing.