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Key Words:	Climate change, Helianthemum, Terfezia, mesophyll conductance, water use efficiency

Elevated atmospheric CO_2 modifies responses to water-stress and flowering of Mediterranean desert truffle mycorrhizal shrubs

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Abstract

Predicted increases in atmospheric concentration of carbon dioxide (CO_2) coupled with increased temperatures and drought are expected to strongly influence the development of most of the plant species in the world, especially in areas with high risk of desertification like the Mediterranean basin. *Helianthemum almeriense* is an ecologically important Mediterranean shrub with an added interest because it serves as the host for *Terfezia claveryi* mycorrhizal fungus, which is a desert truffle with increasingly commercial interest. Although both plant and fungi are known to be well adapted to dry conditions, how the increase in atmospheric CO_2 influences them is still unknown. In this manuscript we have addressed physiological responses of *H. almeriense* x *T. claveryi* mycorrhizal plants to increases in atmospheric CO_2 coupled with drought and high vapor pressure deficit. This work supposes one of the few estimations of mesophyll conductance in a drought deciduous Mediterranean shrub and evaluated its role in photosynthesis limitation. High atmospheric CO_2 concentrations help desert truffle mycorrhizal plants to cope with the adverse effects of progressive drought during Mediterranean springs by improving carbon net assimilation, intrinsic water use efficiency and dispersal of the species through increased flowering events.

Abbreviations: Net carbon assimilation rate (A_N), stomatal conductance (g_s), mesophyll conductance (g_m), intrinsic water use efficiency ($_iWUE$), maximum carboxylation rate of the Rubisco (V_{cmax}), maximum rate of electron transport (J_{max}), vapor pressure deficit (VPD), soil water potential control (Ψ_{soil}), shoot water potential (Ψ_{shoot}), leaf mass per area (LMA), control chamber (CC), high CO_2 concentration chamber (HC), CO_2 concentration surrounding the shoot (C_a), effective photochemical efficiency of photosystem II (ΦPSII), substomatal CO_2 concentration (C_i), chloroplastic CO_2 concentration (C_c), triose phosphate use (TPU).

Keywords: Climate change, *Helianthemum*, *Terfezia*, mesophyll conductance, water use efficiency

Introduction

Atmospheric concentration of carbon dioxide (CO_2) has historically fluctuated on planet Earth between 200 and 280 ppm. However, since the Industrial Revolution, this value has been increasing and has reached values of 400 ppm in the current days and will probably increase up to values between 700 and 800 ppm over the next 50 years, depending on anthropogenic emissions and global policies (IPCC 2018). This increase in atmospheric CO_2 concentration will probably be accompanied by increases in average global temperatures and will cause shorter, less frequent and less widespread precipitation events which will result in expansion of drylands (Schlesinger et al. 1990, Huang et al, 2016).

It is known that all these predicted changes will affect the physiology of plants in different ways. Water-stress negatively affects the gas-exchange parameters of plants, such as the net assimilation rate (A_N), stomatal conductance (g_s) and mesophyll conductance (g_m), although to a different extent, and also depending on plant species (Bartels and Sunkar 2005, Chaves et al. 2009, Flexas et al. 2012). Temperature also affects A_N, since high temperatures decrease total carbon gain, by promoting photorespiration rather than photosynthesis, mainly due to decreases in affinity of Rubisco for CO_2 (Crafts-Brandner and Salvucci 2000). Moreover, supra-optimal temperatures also affect mesophyll conductance (Lambers 2008) and disrupt the proper functioning of the Calvin-cycle (Pastenes and Horton 1996). On the other hand, normally, the increase in the concentration of CO_2 in the atmosphere affects the plants positively, although great differences can be observed depending on the plant species. Some of the general effects of high concentration of CO_2 on the parameters of gas exchange of the plant are decreases in g_s and g_m, increases in A_N and intrinsic water use efficiency ($_i$WUE) and from no effects to decreases in the maximum carboxylation rate of the Rubisco (V_{cmax}) or the maximum rate of electron transport (J_{max}) (Ainsworth and Rogers 2007, Flexas et al. 2014a). The combined effect of CO_2 increases and warmer and/or drought conditions has not been studied in depth, since historically, the effects of these different factors have been analyzed separately. The combination of all these factors must be taken into account, since the increased water use efficiency by plants under a rising CO_2 atmosphere could reduce predictions of future global warming and drought stress due to climatic change (Swann et al. 2016). Studies such as those carried out by Robredo et al. (2011) stating that elevated CO_2 improves nitrogen metabolism by increasing photosynthesis and mitigating water-stress, or by Rodrigues et al. (2016) in coffee crops, which concluded that the elevated atmospheric concentration of CO_2 mitigates the negative impact of supra-optimal temperatures, support this idea. To sum up, all the changes related to climatic change are expected to alter plant physiology, as well as crop yields, and the positive effects caused by the CO_2 enriched atmosphere may be countered by the negative effects caused mainly by high temperatures and drought (DaMatta et al. 2010).

Excess carbon generally results in accumulation of starch and sugar, and it is known that roots are the main organs for starch storage (Loescher et al. 1990, Verdaguer and Ojeda 2002). Taking into account that most of terrestrial plants form intimate interactions with mycorrhizal fungi that are basically based in the delivery of nutrients and water in exchange of photosynthetic carbon (Smith and Read 2008), it is expectable then that the climatic change and the concomitant increase in carbon accumulation will also affect the relationship between mycorrhizal symbionts. Knowledge on this matter was reviewed by Alberton et al. (2005) concluding that, under high CO2 and without any other stressful condition, positive responses could be expected in general terms, both for plants and fungi.

Desert truffles are a group of edible ectendomycorrhizal fungi that grow in arid and semiarid environments (Kovács and Trappe 2014). Because of its great flavor, nutritional and antioxidant properties (Murcia et al. 2002, 2003) and because it is cultivable (Honrubia et al. 2001, Morte et al. 2008), *Terfezia claveryi* Chatin is one of the best known and appreciated desert truffles. From the last 20 years, *T. claveryi* cultivation using *Helianthemum almeriense* Pau, as host plant, has expanded gradually and it is currently considered an alternative crop for arid and semiarid regions (Morte et al. 2017, Andrino et al. 2019). Furthermore, its adaptation to drylands makes it a crop with a great future, as the potential areas for its cultivation are expected to increase due to climatic change (Schlesinger et al. 1990, Lavee et al. 1998, Huang et al. 2016). The host plant itself, *H. almeriense*, is a drought deciduous shrub that appears in open places, in dry, stony, limestone, mica, marl, marl with gypsum or sandy soils, between 0 and 1200 m altitude (López-González 1993). It is well represented in the arid southeast of the Iberian Peninsula forming part of the low and open Mediterranean calcicolous scrub called tomillar, where it plays an important role as a plant cover preventing erosion in these areas (Alados et al. 2006).

According to desert truffle farmers and gatherers, plant phenology plays a crucial role in desert truffle yields, since fungal the starting of fructification season usually coincides with flower appearance and it ends parallel to leaf shedding at the end of spring / start of summer. Since another of the predicted changes related to climatic change is the advancement of the spring (Badeck et al. 2004, Corell et al. 2005, Cleland et al. 2007), it is important to study how the phenology of *H. almeriense*, especially flowering, will be affected by increases in atmospheric CO_2 concentrations. Although several studies have been carried out on desert truffle plants to understand their physiological and molecular responses to water-stress (Morte et al. 2000, 2010, Navarro-Ródenas et al. 2012, 2013, Marqués-Gálvez et al. 2019), the effect of a higher concentration of atmospheric CO_2 together with water-stress has not yet been assayed. In addition, there are also recent studies aiming to improve the yields of desert truffle cultivation (Andrino et al., 2019), but again the forecasted effects of climatic change had not been taken into account. To the best of our knowledge, the only approach to how the climatic change will affect a similar plant species is the experiments carried out by León-Sánchez et al. (2016, 2018), with *Helianthemum squamatum*. In a manipulative field study, the authors concluded that the combination of

warming and reduced irrigation causes decreases in A_N and $_iWUE$, advanced plant phenology and affected mycorrhizal population. Nonetheless, the authors did not consider how the forecasted increase in atmospheric CO_2 concentration will combine with all the predicted changes.

Therefore, the aim of the present work is to study the combined effect of progressive warming and drought, together with the high concentrations of atmospheric CO_2 concentrations ~~in~~ to the cultivation of *H. almeriense* mycorrhizal plants. For this purpose, we have studied those effects in four different scenarios that mimic four environmental stages in the transition from winter to summer. We hypothesize that the negative effects on the physiology of *T. claveryi* x *H. almeriense* plants caused by the increase in drought and VPD will be attenuated due to increased atmospheric CO_2 concentrations. At the same time, we aim to elucidate how these conditions affect *H. almeriense* root starch and sugar content and *T. claveryi* mycorrhizal colonization.

Materials and methods

Biological material and experimental procedure

H. almeriense seeds were collected in Zarzadilla de Totana, Lorca, Murcia and germinated according to Morte et al. (2008). Two months after germination, plants were transferred to bigger 230 cc pots, and inoculated with *T. claveryi* mature spores, extracted from truffles collected at the same place, which were previously mixed with the new substrate (Morte et al. 2008). After 3 months, a total of 72 mycorrhizal plants were transferred to 24 clay-pots of 12,000 cc (3 plants per pot). Pots containing eight-month-old mycorrhizal plants were placed in two different growth chambers (36 plants in each chamber) located at "Servicio de Experimentación Agroforestal" in the University of Murcia (Fig. 1). Temperature (ºC), relative humidity (%), light intensity (W/m^2), photoperiod and CO_2 concentration were controlled in both chambers. Two different treatments were established: "control chamber" (CC) maintained at atmospheric CO_2 concentration (400 ppm) during the whole experimental period and "high CO_2 concentration chamber" (HC) maintained at a CO_2 concentration of 800 ppm. For both treatments, different scenarios with different environmental conditions were assayed. We matched these conditions with the transition from winter to summer by mimicking temperature, relative humidity, vapour pressure deficit (VPD), photoperiod and soil water potential (Ψ_{soil}) from January (winter), March (early spring), May (late spring) and July (summer). Environmental data were retrieved from a meteorological station located in La Alberca (Lorca, Spain IMIDA MU62, http://siam.imida.es) from 1999 to 2017 (Table 1, for detailed environmental design of the chambers see Appendix S1). Environmental parameters were automatically controlled, equally for both chambers and registered using SCADA system. At first, mycorrhizal plants were maintained in winter conditions for two weeks, for acclimation purpose. After that, the assay started by maintaining the conditions showed in Table 1 for three weeks each season. All the measurements were made in the same mycorrhizal plants, on the

morning of the last day of the season and during the same day, except for A/Ci curves that were made the last two days due to time constraints. Ψ_{soil} was measured every day with a portable data logger from Watermark tensiometers (Irrometer; Riverside, CA, USA) and irrigation was applied according to the measurements made in order to maintain the values from Table 1 for each season.

Water potential measurements

Shoot water potential (Ψ_{shoot}) was measured in six plants per season and treatment. To this aim, 5-cm-long plant apices were covered with aluminum foil in dark one hour before the measurement, cut and immediately placed in a pressure chamber (Soil Moisture Equipment Co; Santa Barbara, CA, U.S.A.) according to Scholander et al. (1965).

Plant biomass and morphological parameters

The entire plant was collected and its aerial part of the plant was separated from the root system. The total dry weight (72 h at 60 ºC) of the aerial part from each plant was measured in six plants from each chamber, once every season (winter, early spring, late spring and summer). In addition, flower buds and number of flowers per plant were counted in both chambers during the course of the experiment. The root system was separated into two parts for different measurements: fungal colonization and sugars determination.

For the morphological determinations of the leaves, three leaves were cut per plant from the second and third node from the apex, of six plants per treatment and season and their areas without petiole were measured using image software, ImageJ (https://imagej.net). Then, the leaves were dried at 60 ºC for 72h and their dry weights were measured. Leaf mass per area (LMA) was calculated as the dry weight per area ratio (g m^{-2}). In addition, a non-destructive determination method (Morte and Andrino, 2014) with the help of a SPAD-502 (MINOLTA, Japan) device was used to estimate the total leaf chlorophyll concentration (mg g^{-1}) in three leaves from each plant and six plants per season and treatment.

Sugar content

Free sugars and starch content were determined in roots obtained from six plants per chamber and season, following the protocol of Knudsen (1997). Briefly, roots were pulverized in liquid nitrogen with the help of a pestle and a mortar. 100 mg of pulverized roots were incubated twice in 1 mL 80 % ethanol at 80 ºC for five minutes, then centrifuged and the collected supernatant was subsequently used to determine free sugars. Root pellets were rinsed and incubated in 0.1M pH 5.0 acetate buffer with 100 µL of a thermostable α-amylase (53,7 U mg^{-1} from *Bacillus licheniformis*, Sigma Aldrich, San Luis, MO, USA) at 100ºC for one hour in a thermostatic bath. After that, the thermostatic bath was cooled down to 60 ºC, and 200 µL of amyloglucosidase (3260 U mL^{-1}, from "starch assay reagent", Sigma Aldrich; San Luis, MO, USA) was added. The solution was incubated at 60 ºC for two hours, and then

the enzyme was inactivated by heating at 100 ºC for fifteen minutes. The free sugars of the supernatant and starch were determined as glucose equivalents using the glucose oxidase method (Trinder 1969, Lott and Turner 1975) with the QCA sugar determination kit (Química Clínica Aplicada SA, Spain), following manufacturer's instructions. The reaction product was measured at 505 nm in a Shimadzu UV-1700 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and the resulting data were compared with those of the glucose standard.

Fungal colonization

Total fungal colonization was measured in six plants per chamber and season under an Olympus BH2 microscope, after staining their roots with trypan blue as described in Gutiérrez et al. (2003). To calculate the mycorrhization status, 100 secondary and tertiary root sections per sample were observed under the microscope and classified as "mycorrhizal" or "non-mycorrhizal" depending on the presence/absence of *T. claveryi* mycorrizal structures.

Leaf gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange parameters were estimated simultaneously with chlorophyll fluorescence measurements using a portable photosynthesis system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) equipped with an integrated fluorescence chamber head (Li-6400–40; Li-Cor). Six CO_2 response curves (A_N-C_i curves) of *H. almeriense* leaves, placed in a 2-cm^2-leaf cuvette, were obtained, once for each treatment and in each season. Due to the small foliar area of this species, several fully expanded leaves were carefully placed in the cuvette and after the measurements they were later collected to measure their area using the image analysis software Image J (www.imagej.com). Measurements were taken at an air flow of 200 µmol s^{-1}, in light-adapted mature leaves at a CO_2 concentration surrounding the shoot (C_a) of 400 µmol^{-1} for CC plants and 800 µmol^{-1} for HC plants, and a saturating PPFD (Photosynthetic photon flux density) of 1500 µmol m^{-2} s^{-1}. Once the steady-state gas exchange rate was reached under these conditions, net assimilation rate (A_N), transpiration (E), stomatal conductance (g_s) and the effective quantum yield of photosystem II (PSII) were estimated. Intrinsic water use efficiency (iWUE) was calculated as the ratio between A_N and g_s. Afterwards, C_a was gradually reduced down to 50 µmol mol^{-1}. After completion of measurements at low C_a, it was increased again to 400 or 800 µmol mol^{-1}. Then, C_a was increased stepwise to 1800 µmol mol^{-1}. Leakage of CO_2 in and out of the cuvette was determined for the same range of CO_2 concentrations with a photosynthetically inactive leaf (obtained by heating the leaf until no variable chlorophyll fluorescence was observed) and used to correct measured leaf fluxes (Appendix S2) (Flexas et al. 2007). The effective photochemical efficiency of photosystem II (ΦPSII) was measured simultaneously with A_N and g_s. For ΦPSII, the steady-state fluorescence (F_S) and the maximum fluorescence during a light-saturating pulse of ~8000 µmol m^{-2} s−1 ($F`_M$) were estimated, and ΦPSII was calculated as ($F`_M$ - F_S)/$F`_M$ following the procedures of Genty et al. (1989). The photosynthetic electron transport rate (J_{flu}) was then calculated according to Krall and Edwards (1992),

multiplying ΦPSII by PPFD, by the leaf absorptance (the ratio of the absorbed to the incident radiant power) and by 0.5 (because we assumed an equal partitioning of absorbed quanta between photosystems I and II). The actual leaf absorptance was measured simultaneously with the portable photosynthesis system (Valentini et al. 1995).

Estimation of mesophyll conductance by gas exchange and chlorophyll fluorescence

Mesophyll conductance (g_m) was estimated according to the method of Harley et al. (1992), as follows:

$$gm = \frac{A_N}{(C_i - [\frac{\Gamma^*(J_F + 8(A_N + R_L))}{J_F} - 4(A_N + R_L)]}$$

Equation 1. Calculation of mesophyll conductance (g_m) according to Harley methodology (Harley et al., 1992).

Where A_N and the substomatal CO_2 concentration (C_i) were taken from gas exchange measurements at saturating light, and Γ* (the chloroplastic CO_2 photocompensation point in the absence of mitochondrial respiration) and R_L (the respiration rate in the light) were taken from Bernacchi et al. (2002).

The values of g_m were used to convert A_N-C_i curves into A_N-C_c (chloroplastic CO_2 concentration) using the equation $C_c = C_i - A_N/g_m$. Maximum carboxylation rate (V_{cmax}), maximum electron transport capacity (J_{max}) and triose phosphate use (TPU) were calculated from the A_N-C_c curves, using the Rubisco kinetic constants and their temperature dependencies described by Bernacchi et al. (2002). The Farquhar model was fitted to the data by applying iterative curve fitting (minimum least-square difference) using the Solver tool from Microsoft Excel.

Analysis of partitioning changes in photosynthetic rate

We estimated the different limitations on A_N from stomatal conductance (*ls*), mesophyll conductance (*lm*) and biochemical capacity (*lb*), using the quantitative limitation analysis of Grassi and Magnani (2005) as applied by Tomás et al. (2013). Different fractional limitations, *ls*, *lm* and *lb* (*ls* + *lm* + *lb* = 1), were calculated as:

$$ls = \frac{\frac{g_{tot}}{g_s} \times \partial A_N/\partial C_c}{g_{tot} + \partial A_N/\partial C_c}$$

Equation 2. Calculation of stomatal limitations (*ls*) according to Grassi and Magnani (2005).

$$lm = \frac{\frac{g_{tot}}{g_m} \times \partial A_N/\partial C_c}{g_{tot} + \partial A_N/\partial C_c}$$

Equation 3. Calculation of mesophyll limitations (*lm*) according to Grassi and Magnani (2005).

$$ls = \frac{g_{tot} \times \partial A_N/\partial C_c}{g_{tot} + \partial A_N/\partial C_c}$$

Equation 4. Calculation of biochemical limitations (*lb*) according to Grassi and Magnani (2005).

Where g_s is the stomatal conductance to CO_2, g_m is the mesophyll conductance according to Harley et al. (1992, Equation 1) and g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts (sum of the inverse CO_2 serial conductances gs and gm). $\partial A_N/\partial C_c$ was calculated as the slope of A_N-C_c response curves over a C_c range of 50–100 µmol mol^{-1}. Quantitative limitations of photosynthesis were estimated for at least six different leaves for each species and average estimates of the component photosynthetic limitations were calculated.

Statistical analyses

Statistical analysis (ANOVA, post-hoc Tukey test, Student's t test, normality Saphiro-wilk test and homoscedasticity Levene's test) were performed using R programming language in R studio (Team 2018).

Multivariate analysis methods, such as principal coordinate analysis (PCoA), allow data to be interpreted as a whole, instead of each variable independently, and together with statistical methods such as perMANONVA and post-hocs analysis, allows to infer statistical differences between groups. Therefore, to visualize the dissimilarities between groups of plants with respect to the seasonal conditions and the treatment, a two-dimensional principal coordinate analysis (PCoA) calculated in a Euclidean dissimilarity matrix was performed. To confirm the effect of the two different factors, a permutational multivariate analysis (perMANOVA) was conducted in the same Euclidean matrix, using the *adonis* function of the *vegan* package (Oksanen et al. 2016), and 999 permutations. A post-hoc pairwise analysis with Bonferroni correction was conducted on the perMANOVA results (Martínez-Arbizu 2017).

Results

A two-way perMANOVA analysis was applied to look for significant differences between groups of mycorrhizal plants, taking into account all the plant variables at the same time. The results confirmed that the seasonal conditions ($F = 22.93$, $p < 0.001$) and CO_2 treatment ($F = 8.15$, $p < 0.001$), as well as their interaction ($F = 2.05$, $p = 0.028$), significantly influenced the plant behavior, especially in terms of A_N, C_i, C_c, iWUE and V_{cmax} (Fig. 2A). The subsequent post-hoc pairwise analysis showed that, when comparing both treatments (CC and HC) there were significant differences in plant behavior between almost all the seasons (Fig. 2B), while within each treatment, the number of differences between seasons was higher in the HC treatment (4 statistical differences, Fig. 2C) than in CC treatment (2 statistical differences, Fig. 2D).

Among the different plant parameters measured across the treatments and seasonal conditions, plant biomass was not significantly different among seasonal conditions nor CO_2 treatment, with the exception of summer, when greater shoot dry weight was observed in HC treatment compared to CC treatment (Fig. 3A). Interestingly, neither floral buttons nor flowers were observed for any of the treatments until the end of the experiment, when the number of flower buds and flowers per plant was 6 and 10 times, respectively higher for HC treatment (Fig. 3B). Ψ_{shoot} decreased from winter to summer almost identically in both treatments and this decrease was highly correlated with Ψ_{soil} and VPD from winter to summer for both treatments (Appendix S3). LMA was relatively constant along the seasons (no statistical differences), although slightly increased in HC treatment from winter to summer conditions. No statistical differences were found in leaf chlorophyll content, leaf area nor in leaf weight (Appendix S4).

Gas-exchange parameters were highly affected by seasonal conditions and CO_2 treatment (Fig. 4). A_N, and g_s presented maximum values in winter conditions, and then, decreased until summer conditions, both for CC and HC treatments. In the case of g_s, there were no differences in any condition between CC and HC, while for A_N, and subsequently C_i and C_c, these values were higher in HC than in CC. The progressive decrease in g_s throughout the seasons supposed a significant increase in iWUE only in HC during early spring, late spring and summer, but not in winter. Unlike g_s, g_m reached its maximum in early spring and then decreased until summer in both treatments, although this appreciated decrease was significant only in CC. Positive correlations were found between A_N, g_s and g_m (Figs 5A and 5B), and a good correlation was found at low values of g_s between g_s and g_m, but no correlation was found when taking into account all the data available (including winter) (Fig. 5C). During the course of the seasons, V_{cmax} and TPU remained the same in CC treatment, but increased significantly in HC treatment, while J_{max} was the same in every treatment and condition, although J_{max}/V_{cmax} ratio decreased from winter to summer conditions in both treatments (Appendix S4).

When a two-way ANOVA was performed to the partition analysis on the photosynthetic limitations data, only seasonal influence ($p = 0.00822$), but no influence in the CO_2 treatment or the interaction between both factors were found. For both treatments, stomatal limitations increased and mesophyll limitations decreased as the seasonal conditions advanced from winter to summer (Fig. 6A). Furthermore, when all the data were grouped, gs and the stomatal limitations were linearly correlated: the lower the gs, the greater the stomatal limitations (Fig. 6B).

Mycorrhizal *T. claveryi* colonization increased from winter to summer conditions, but no differences were observed in root starch and free sugar contents. In addition, no effect on the mycorrhizal status nor sugar content was found between any of the CO_2 treatments (CC *vs* HC) at any given moment (Table 2).

Discussion

Atmospheric CO_2 interaction with water-stress influences physiology of *H. almeriense* desert truffle plant

So far, *H. almeriense* physiology has been studied mainly because of its interest as the host species for the cultivation of *T. claveryi*, focusing solely on its adaptation traits to arid environments and its responses to water-stress (Morte et al, 2000, 2010, Navarro-Ródenas et al. 2012, 2013, Marqués-Gálvez et al. 2019), which is considered the primary environmental constraint to the productivity and distribution of Mediterranean vegetation (Gil-Pelegrín et al. 2017). In this study we have evidenced that atmospheric CO_2 in combination with these important seasonal conditions influences the physiology of the mycorrhizal plant as a whole (Fig. 2). As we hypothesized, the increase in atmospheric CO_2 concentrations improves A_N, and, ultimately, the $_i$WUE of *T. claveryi* x *H. almeriense* plants, indicating that this species has an active response to the increase in atmospheric CO_2 concentrations (McCarrol et al. 2009). The increases in plant biomass, A_N and $_i$WUE in HC plants show that the high concentration of atmospheric CO_2 helps plant cope, at least partially, with the negative effects of the water-stress and warming when these stresses are more severe, as in the summer scenario.

H. almeriense mycorrhizal plants find their maximum values of A_N and g_s during the winter scenario (Figs 4A and 4B), similarly to what occurs to *Cistus albidus* (Gulías et al. 2009). The climatic conditions during winter (i.e. the absence of water stress and mild temperatures) of the ecological niches of this species could be the main responsible for this phenomenon. It must be highlighted that g_m has been estimated in *H. almeriense* for the first time in the present study. In contrast to A_N and g_s, the maximum g_m was not found in winter, but in the early spring scenario (Fig. 4C). This differential response can explain that the strong reduction experienced by g_s from winter to early spring (ca. 40% and 63% for CC and HC, respectively) only induced a slight decrease in A_N (ca. 17% and 13% for CC and HC, respectively), as it was partially compensated by the increase in g_m (ca. 68% and 33% for CC

and HC, respectively). Overall, these findings evidenced that – together with g_s – g_m plays an outstanding role in influencing net CO_2 assimilation of this species for both treatments, which resulted in a dependence between A_N and g_s, A_N and g_m (Fig. 5). These dependences are similar to those reported in Mediterranean oaks, whose g_s and g_m are highly correlated to A_N (Peguero-Pina et al. 2017). However, while for Mediterranean oaks, together with other Mediterranean species, g_m is the most correlated factor to A_N and the most limiting factor for CO_2 assimilation (Flexas et al., 2014b; Niinemets & Keenan, 2014; Peguero-Pina et al., 2017), this only occurs in winter conditions for *H. almeriense*. By contrast, stomatal limitations increased at early spring and were more limiting from that point until the end of the vegetative period (Fig. 6). Furthermore, this limitation is correlated with the stomatal conductance, *i.e.* the more closed the stomata, the higher is the stomatal limitation, similarly to what happens in the rockrose *C. albidus* (Galle et al., 2011). Finally, the biochemical limitations were almost negligible for this species, irrespective of the treatment and seasonal condition, which reinforces the predominant role of stomatal and non-stomatal CO_2 diffusion in the photosynthetic activity.

Higher atmospheric CO_2 concentration results in increased *H. almeriense* flowering events

The active plant response to HC treatment resulted in more carbon being assimilated, but the only measured consequence of this was a slight increase in plant biomass in summer and an increase in flowering events at the end of the vegetative period (Fig. 3). Springer and Ward (2007) reviewed papers about this issue and concluded that the general response to the increase in atmospheric CO_2 concentration was earlier flowering. More recently, León-Sánchez et al. (2016) also described an earlier phenology under warmer and more water-stressful conditions for the related *H. squamatum* plants. It should be noted that this manuscript shows one of the few reports of increasing number of flowers as a response to high CO_2 concentrations, similarly to what occurs to *Phlox drummondii*, which showed twice more flowers at 700 ppm than in control conditions (Garbutt and Bazzaz 1984).

The increase in flowers under high CO_2 concentration may have a double importance in the study of desert truffles. On the one hand, the accumulated knowledge of farmers and pickers together with recent phenology studies (Marques-Gálvez et al. 2020) point to a relationship between plant phenology, including flowering, and desert truffle yield. Since we proved that flowering is affected by higher CO_2 concentrations, desert truffle natural and cultivated productions could be also affected under these conditions. On the other hand, *Cistaceae* seeds have in general a series of features (small, hard-coated, short-distance dispersal, long-term persistence in soil seed banks, fire- or heat-induced germination, with an opportunistic strategy of germination, and a slow germination rate) that endow them a considerable ecological advantage in the summer-dry and fire-prone Mediterranean climatic conditions (Thanos et al. 1992). An increased amount of flowers represent an increased amount in seed production, which could lead to an even greater ecological advantage for this species in the much more

drier and fire-prone climatic change conditions in the Mediterranean niches and to improve the restricted dispersal of this vulnerable species, which is common in arid regions (Alados et al. 2009).

Fungal colonization is influenced by water-stress but not directly by atmospheric CO_2

The increased fungal colonization found in control CO_2 treatment from winter to summer, in parallel to the increase in water-stress, has been previously observed ore in this particular mycorrhizal association (Navarro-Ródenas et al. 2013, Marqués-Gálvez et al. 2019). We have demonstrated that this phenomenon is maintained under high CO_2 conditions but no effect of differential CO_2 treatment was found (Table 2). There are several reports indicating that fine root production is increased under high atmospheric CO_2 (Rogers et al. 1999, Norby et al. 2004, Sanchez-de Leon et al. 2018). Since fine roots serve as accommodation sites for *T. claveryi* colonization (Gutiérrez et al. 2003), it may be possible that even without a direct effect, there would be an indirect increase in potential mycorrhizal colonization sites. Because of the pot system used in this particular experiment (3 plants grew in the same pot and, therefore, the roots from different plants were intermingled and part of the root system was retained in the pot after harvest), it was impossible to precisely measure root biomass and fine root production, but this possible indirect effect should be taken into account. Moreover, this experiment was carried out under controlled conditions and only *T. claveryi* colonization was studied. In field conditions, climate change will likely modify mycorrhizal populations inhabiting in the rhizosphere of plant species, as occurs with the close *H. squamatum* species (Leon-Sánchez et al. 2018). How *H. almeriense* mycorrhizal-associated populations will change under high CO_2 and increased water-stress conditions is also of interest for future research on desert truffle distribution, cultivation and eco-physiology.

In conclusion, we proved that high CO_2 atmospheric concentrations affect the overall physiological parameters of *H. almeriense* x *T. claveryi* during spring. We have described for the first time the mesophyll conductance of a mycorrhizal Mediterranean shrub like *H. almeriense* and found that it may play an important role in limiting photosynthesis, especially in absence of water-stress. Our data suggest that the improvement in net assimilation and water use efficiency of *H. almeriense* mycorrhizal plants and the fact that mycorrhizal colonization is not negatively affected will help this plant to cope with water-stress when atmospheric CO_2 concentration increases. Furthermore, we showed an increase in flowering events under high atmospheric CO_2 conditions, probably as a result of the increased influx of photoassimilates, which may influence desert truffle production and ecological distribution and fitness of this species in climate change conditions. Some of the differences observed, such as those in plant biomass or in flower development, which are expected to change slowly over time, may have been underestimated because of the shortened times between seasons. Others, like gas-exchange parameters, could remain the same or even decrease because of adaptation processes when studied under field conditions and/or under longer transition times between seasonal scenarios. To

unravel these questions and more, fFurther field studies using different approaches such as free-air CO_2 enrichment experiments, coupled with metagenomics and population studies will help to understand the fitness of desert truffle natural distribution and cultivation in the future.

Author contributions

AM and ANR conceived, managed and coordinated the project. AM, JEMG and ANR planned and managed the research. JPG and EGP provided advice and knowledge about gas-exchange related measurements. JEMG, ANR, FA and ALG carried out the experiments. JEMG, ANR and JPG carried out analyses and interpretation of the data. JEMG drafted and wrote the manuscript. AM, ANR, JPG and EGP contributed to sections of the manuscript. All the authors read and improved the manuscript.

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Data availability statement

Data sharing is not applicable to this article as all new created data is already contained within this article.

Supporting information

Appendix S1. Environmental design of the chambers for the transition from winter to summer simulation.

Appendix S2. IRGA's leak curves.

Appendix S3. Relationships of shoot water potential (Ψshoot) with soil water potential (Ψshoot) and vapor pressure deficit (VPD).

Appendix S4. Physiological and gas-exchange plant parameters in different conditions and CO2 treatments.

Figure legends

Figure 1. *H. almeriense* mycorrhizal plants during the course of the experiment. (A) Detail of the growth chamber with control *H. almeriense* mycorrhizal plants in clay-pots. **(B)** Gas exchange measurements during summer in high CO_2 plants.

Figure 2. Differences in plant behavior comparing CO_2 treatment and seasonal conditions. (A) Principal coordinate analysis on plant behavior regarding CO2 treatments and seasonal conditions. The arrows represent the greatest eigenvalues from a larger set of plant variables that better explain the differences in plant behavior across the different conditions. Dark blue = control chamber (CC) winter; black = CC early spring; red = CC late spring; green = CC summer; gray = high CO_2 chamber (HC) winter; light blue = HC early spring; pink = HC late spring; yellow = HC summer. **(B)** High CO_2 (HC) *vs* control (CC); **(C)** HC *vs* HC; and **(D)** CC *vs* CC are the graphical representation on post-hoc pairwise analysis of principal component analysis and perMANOVA from Fig. 2A. Cells filled with red color represent significant p values ($p < 0.05$).

Figure 3. Plant biomass and flowering differences between CO_2 treatments. Black bars represent Control Chamber (CC) data, while grey bars represent High CO_2 Chamber (HC) data. The mean of n=6 replicates and the standard error are represented. Asterisks represent statistical differences ($p < 0.05$) between different CO_2 treatments, from the same season, when a t-test was performed. **(A)** Graphical representation of the aerial part dry weight. **(B)** Graphical representation of the number of flower buds and flowers at the end of the experiment for both CO_2 treatments.

Figure 4. Gas exchange parameter variation during the course of the experiment in both CO_2 treatments. Black circles represent Control Chamber (CC) data, while grey circles represent High CO_2 Chamber (HC) data. Each point is the result of the mean of 6 biological replicates and it associated standard error. Different letters represent statistical differences ($p < 0.05$) between seasonal conditions from the same CO_2 treatment when a variance analysis (ANOVA) and a Tukey's post-hoc test were performed. Asterisks represent statistical differences ($p < 0.05$) between different CO_2 treatments, from the same season, when a t-test was performed. **(A)** Net assimilation; **(B)** Stomatal conductance; **(C)** Mesophyll conductance; **(D)** intrinsic water use efficiency (iWUE).

Figure 5. The relationship between A_N, g_s and g_m in *H. almeriense* mycorrhizal plants under different CO_2 treatments and seasonal conditions. Circles represent CC, while triangles represent HC. Data from (**A**) and (**B**) were fitted to a single rectangular hyperbola. Data from (**C**) fitted to a linear regression when only values from summer, late spring and early spring were taken into account.

Figure 6. Partition analysis on the limitations to A_N. (**A**) stomatal (black) and mesophyll (grey) limitations to A_N in every season and CO_2 treatment. Biochemical limitations were not represented as they were less than 0.5 % for each condition and treatment. CC = control chamber; HC = high CO_2 chamber. (**B**) Relationship between stomatal limitations to A_N and g_s.

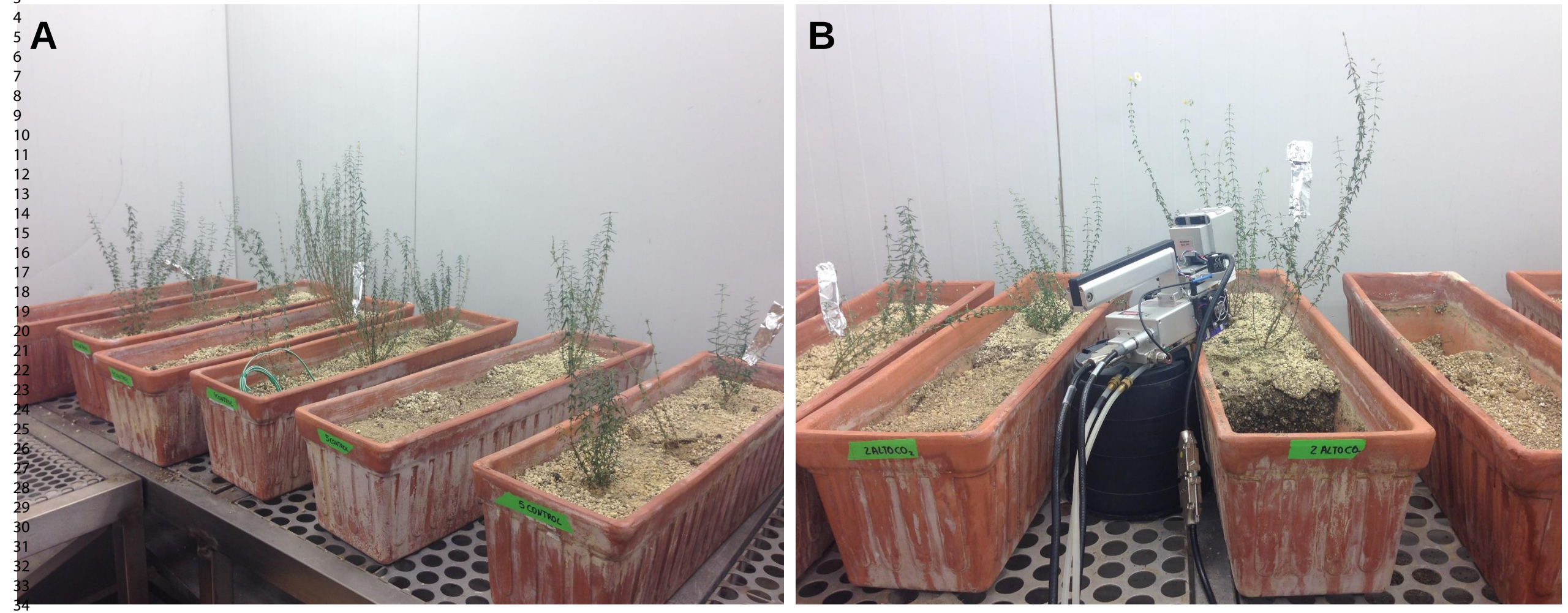
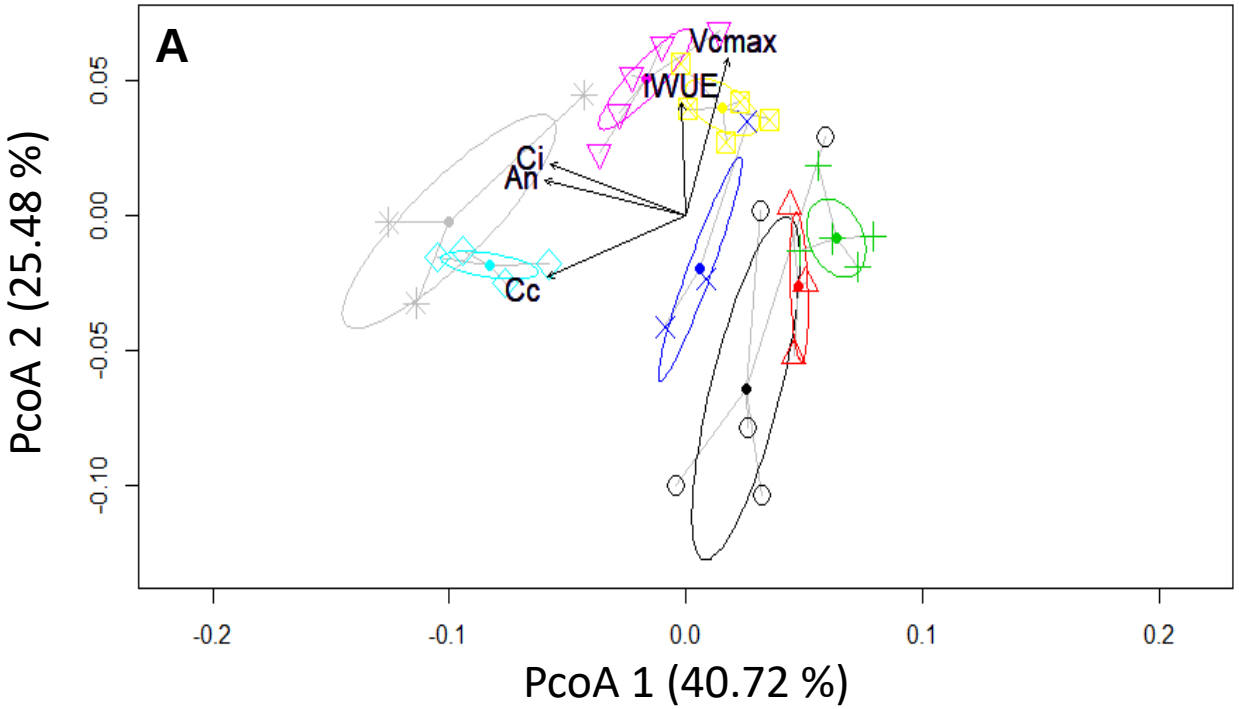
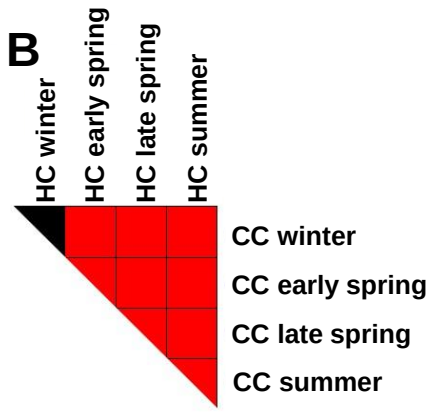
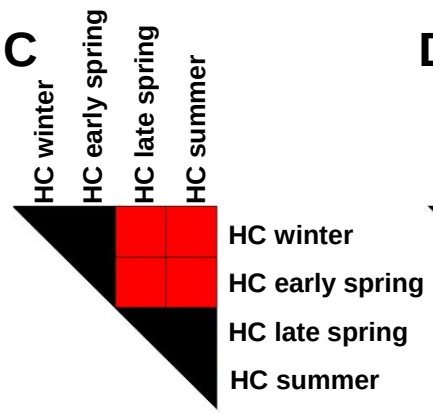

A
5 CONTROL
B
2 ALTO CO2
2 ALTO CO2

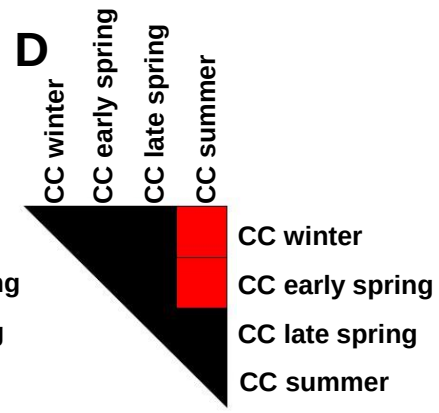
Table 1. Environmental data for each treatment and seasonal condition.

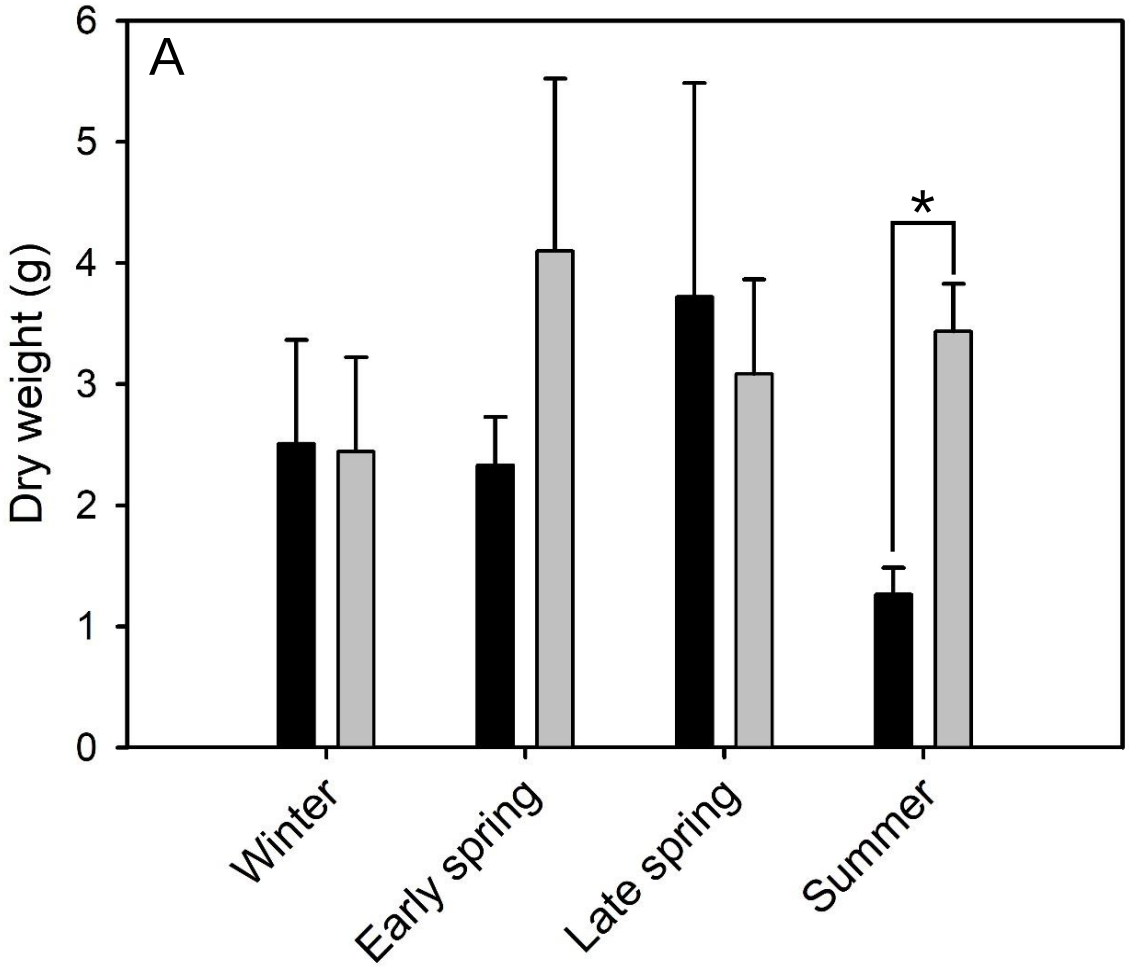
	CONTROL CHAMBER (CC)	HIGH [CO_2] CHAMBER (HC)
SCENARIO 1: WINTER		
Mean temperature (ºC)	12.64	12.64
Relative humidity (%)	60	60
Mean VPD (kPa)	0.61	0.61
Photoperiod (light/darkness)	10/14	10/14
Average Ψ_{soil} (kPa)	-14.33	-19.33
[CO_2] (ppm)	400	800
SCENARIO 2: EARLY SPRING		
Mean temperature (ºC)	14.47	14.47
Relative humidity (%)	60	60
Mean VPD (kPa)	0.80	0.80
Photoperiod (light/darkness)	12/12	12/12
Average Ψ_{soil} (kPa)	-70.44	-53.22
[CO_2] (ppm)	400	800
SCENARIO 3: LATE SPRING		
Mean temperature (ºC)	20.95	20.95
Relative humidity (%)	50	50
Mean VPD (kPa)	1.38	1.38
Photoperiod (light/darkness)	14/10	14/10
Average Ψ_{soil} (kPa)	-103.66	-107.57
[CO_2] (ppm)	400	800
SCENARIO 4: SUMMER		
Mean temperature (ºC)	27.28	27.28
Relative humidity (%)	50	50
Mean VPD (kPa)	2.04	2.04
Photoperiod (light/darkness)	14/10	14/10
Average Ψ_{soil} (kPa)	-160.4	-160.66
[CO_2] (ppm)	400	800

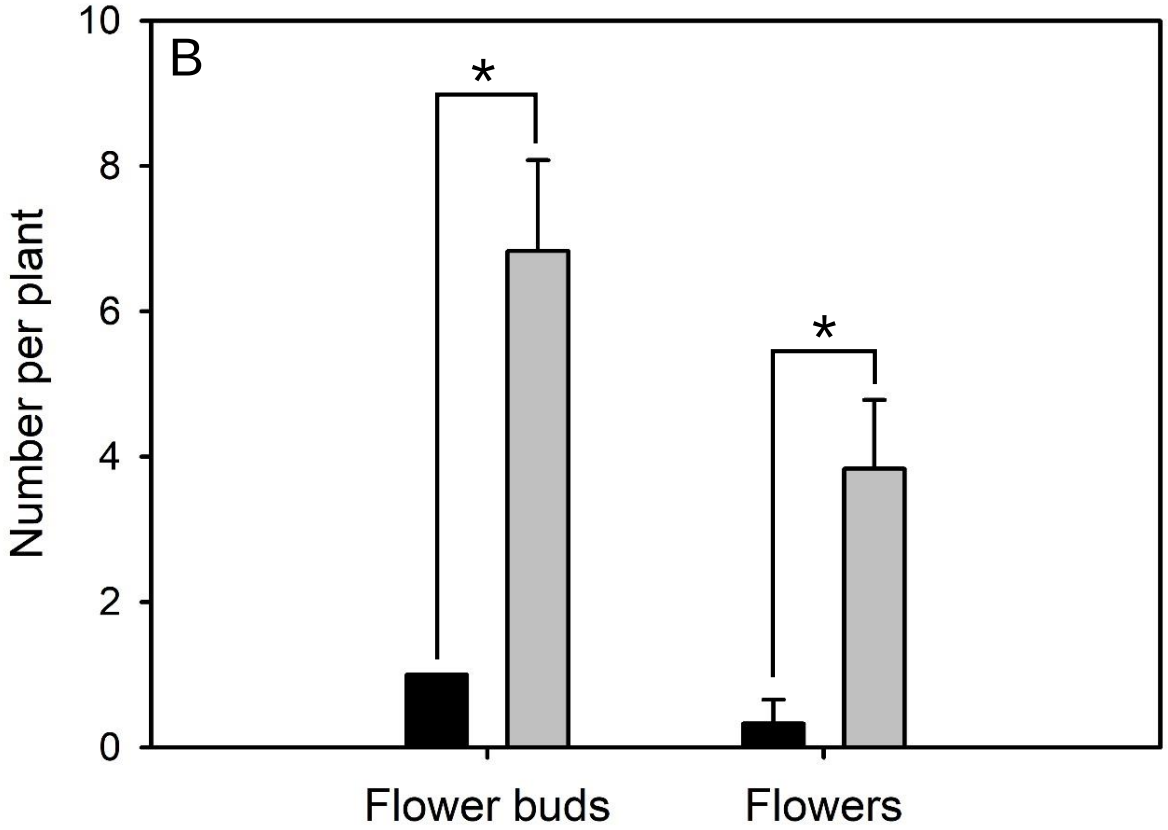

A
Vcmax
iWUE
Ci
An
Cc
PcoA 2 (25.48 %)
PcoA 1 (40.72 %)
0.05
0.00
-0.05
-0.10
-0.2
-0.1
0.0
0.1
0.2

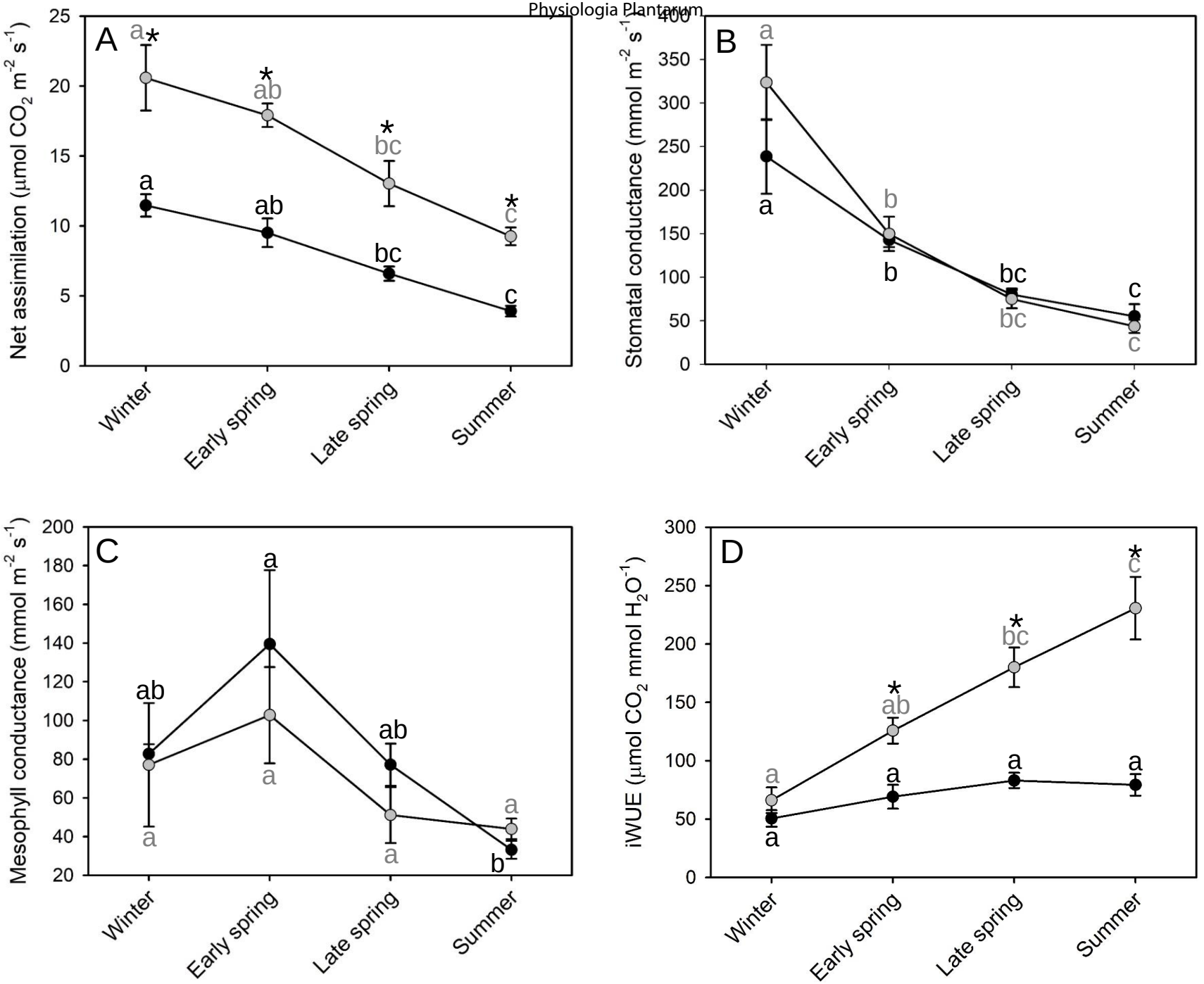

B
HC winter
HC early spring
HC late spring
HC summer
CC winter
CC early spring
CC late spring
CC summer

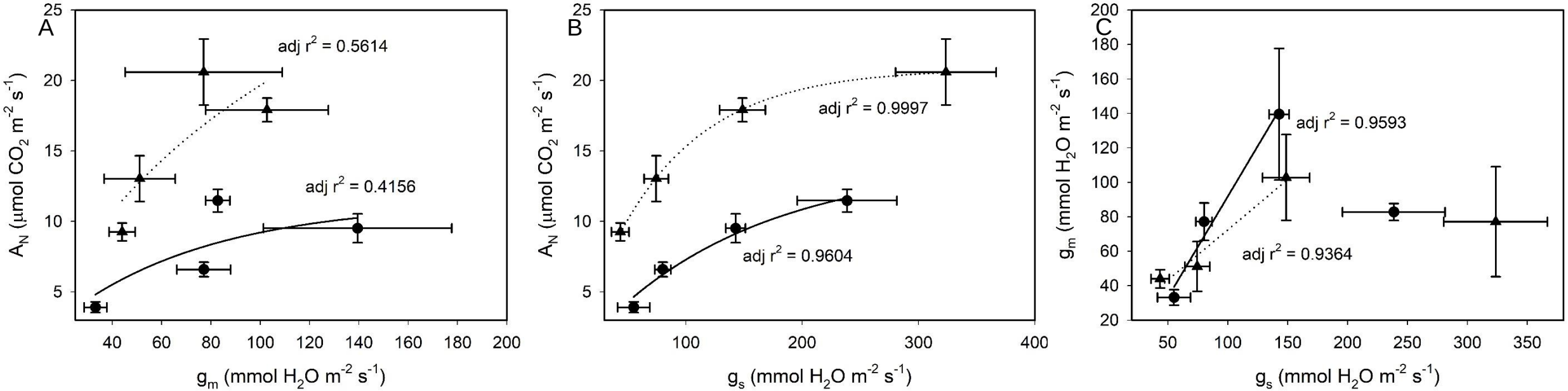

C
HC winter
HC early spring
HC late spring
HC summer
HC winter
HC early spring
HC late spring
HC summer

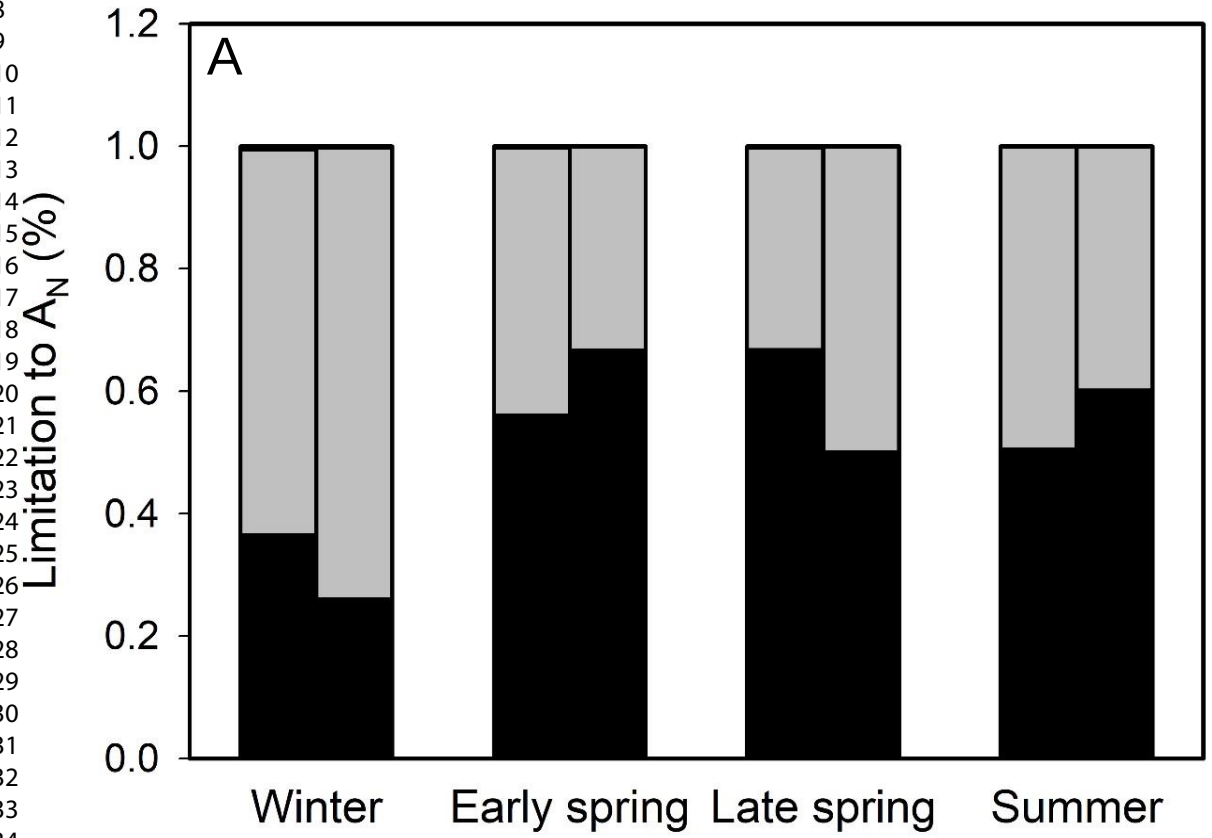

D
CC winter
CC early spring
CC late spring
CC summer
CC winter
CC early spring
CC late spring
CC summer


A
Dry weight (g)
0
1
2
3
4
5
6
*
Winter
Early spring
Late spring
Summer


B
Number per plant
0
2
4
6
8
10
*
*
Flower buds
Flowers


A
Net assimilation (μmol CO2 m-2 s-1)
B
Stomatal conductance (mmol m-2 s-1)
C
Mesophyll conductance (mmol m-2 s-1)
D
iWUE (μmol CO2 mmol H2O-1)
Winter
Early spring
Late spring
Summer


A
adj r2 = 0.5614
adj r2 = 0.4156
AN (μmol CO2 m-2 s-1)
gm (mmol H2O m-2 s-1)
B
adj r2 = 0.9997
adj r2 = 0.9604
AN (μmol CO2 m-2 s-1)
gs (mmol H2O m-2 s-1)
C
adj r2 = 0.9593
adj r2 = 0.9364
gm (mmol H2O m-2 s-1)
gs (mmol H2O m-2 s-1)


A
Limitation to A_N (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Winter
Early spring
Late spring
Summer

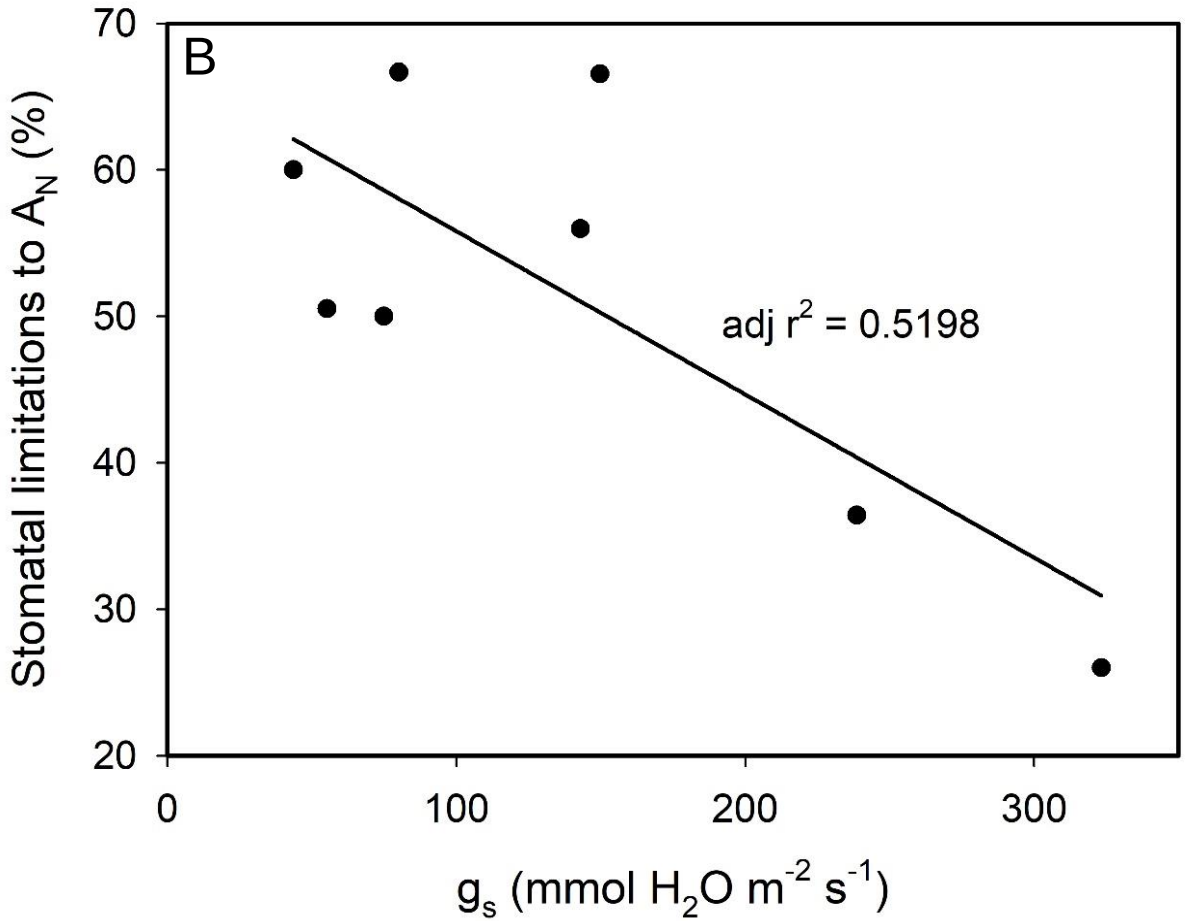

B
Stomatal limitations to A_N (%)
70
60
50
40
30
20
0
100
200
300
adj r^2 = 0.5198
g_s (mmol H_2O m^{-2} s^{-1})

Table 2. Root colonization and sugar content in different seasonal conditions and CO_2 treatments. Mean values ± standard errors are represented. Different letters represent statistical differences ($p < 0.05$) between seasonal conditions from the same CO_2 treatment when a variance analysis (ANOVA) and a Tukey's post-hoc test were performed. Asterisks represent statistical differences ($p < 0.05$) between different CO_2 treatments, from the same season, when a t-test was performed.

	CONTROL				HIGH CO_2			
	Winter	**Early spring**	**Late spring**	**Summer**	**Winter**	**Early spring**	**Late spring**	**Summer**
Mycorrhiza (%)	21.2±3.7a	31.3±3.9a	38.3±7.2ab	46.7±1.8b	24.4±6.7a	26.9±2.7a	44.2±7.0b	39.3±3.6b
Root starch (mM glucose g^{-1})	2.2±0.6a	2.7±0.8a	3.3±0.6a	2.3± 0.1a	3.3±1.1a	2.2± 0.3a	2.5±0.4a	2.2±0.7a
Root free sugars (mM glucose g^{-1})	0.5±0.1a	1.0±0.3a	1.2±0.3a	0.8± 0.1a	0.9±0.2a	1.3± 0.2a	0.83±0.1a	0.8±0.1a