Elevated atmospheric CO$_2$ modifies responses to water-stress and flowering of Mediterranean desert truffle mycorrhizal shrubs

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Predicted increases in atmospheric concentration of carbon dioxide (CO₂) 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 the *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, it is still uncertain how the increase in atmospheric CO₂ will influences them. In this article we have addressed the physiological responses of *H. almeriense × T. claveryi* mycorrhizal plants to increases in atmospheric CO₂ coupled with drought and high vapor pressure deficit. This work reports one of the few estimations of mesophyll conductance in a drought deciduous Mediterranean shrub and evaluated its role in photosynthesis limitation. High atmospheric CO₂ 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** - Aₙ, Net carbon assimilation rate; Cₛ, CO₂ concentration surrounding the shoot; Cₐ, chloroplastic CO₂ concentration; CC, control chamber; Cₛ, substomatal CO₂ concentration; gₛ, stomatal conductance; gm, mesophyll conductance; HC, high CO₂ concentration chamber; iWUE, intrinsic water use efficiency; Jₘₐₓ, maximum rate of electron transport; LMA, leaf mass per area; TPU, triose phosphate use; Vₖₐₘₓ, maximum carboxylation rate of the Rubisco; VPD, vapor pressure deficit; ΦPSII, effective photochemical efficiency of photosystem II; Ψₛ, shoot water potential; Ψₛₒ, soil water potential control.
Introduction

Atmospheric concentration of carbon dioxide (CO₂) has historically fluctuated on planet Earth between 200 and 280 ppm. However, since the Industrial Revolution, this value has been increasing and has presently reached 400 ppm and will probably increase up to between 700 and 800 ppm over the next 50 years, depending on anthropogenic emissions and global policies (IPCC 2018). This increase in atmospheric CO₂ 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 (Aₙ), stomatal conductance (gₛ) and mesophyll conductance (gₘ), although to different extent, and also depending on plant species (Bartels and Sunkar 2005, Chaves et al. 2009, Flexas et al. 2012). Temperature also affects Aₙ, since high temperatures decrease total carbon gain, by promoting photorespiration rather than photosynthesis, mainly due to decreases in affinity of Rubisco for CO₂ (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 concentration of CO₂ 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 of CO₂ concentration on the parameters of gas exchange of the plant are decreases in gₛ and gₘ, increases in Aₙ and intrinsic water use efficiency (WUE) and from no effects to decreases in the maximum carboxylation rate of the Rubisco (Vₖₑₜₐₓ) or the maximum rate of electron transport (Jₘ₉ₜ₉ₓ) (Ainsworth and Rogers 2007, Flexas et al. 2014a). The combined effect of CO₂ increases and warmer and/or drought conditions has not been studied in depth, 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 CO₂ atmosphere could diminish predictions of the effects of future global warming and drought
stress (Swann et al. 2016). Studies such as those carried out by Robredo et al. (2011) stating that elevated CO₂ 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 CO₂ 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 CO₂ 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 on 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). *Terfezia claveryi* Chatin is one of the best known and appreciated desert truffle, due to 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). 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 CO₂ concentrations. Although
several studies have been carried out on desert truffle plants to understand their physiological and
Marqués-Gálvez et al. 2019), the effect of a higher concentration of atmospheric CO₂ 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 A₅ and ,WUE, advanced
plant phenology and affected mycorrhizal population. Nonetheless, the authors did not consider how
the forecasted increase in atmospheric CO₂ 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 CO₂ concentrations 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 × H. almeriense plants caused by
the increase in drought and VPD will be attenuated due to increased atmospheric CO₂ 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 (37° 52′ 15.5″ N 1° 42′
10.5″ W) and germinated according to Morte et al. (2008). Two months after germination, plants were
transferred to bigger 0.23 l 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 l (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 (°C), relative humidity (%), light intensity (W m⁻²), photoperiod and CO₂ concentration were controlled in both chambers. Two different treatments were established: “control chamber” (CC) maintained at atmospheric CO₂ concentration (400 ppm) during the whole experimental period and “high CO₂ concentration chamber” (HC) maintained at a CO₂ 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 (Ψᵦₒᵢₜ) 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. Ψᵦₒᵢₜ 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 (Ψₑₒᵢₜ) 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, USA) 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°C) 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°C 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°C for 5 min, 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, USA) at 100°C for one hour in a thermostatic bath. After that, the thermostatic bath was cooled down to 60°C, and 200 µl of amyloglucosidase (3260 U ml⁻¹, from “starch assay reagent”, Sigma Aldrich, USA) was added. The solution was incubated at 60°C for 2 h, and then the enzyme was inactivated by heating at 100°C for 15 min. 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 (Quimica Clinica Aplicada SA, Spain), following manufacturer’s instructions. The reaction product was measured at 505 nm in a Shimadzu UV-1700 spectrophotometer (Shimadzu Corporation, 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* mycorrhizal 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., 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 (Photonsynthetic 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$_{lu}$) 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:

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

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 \(\Gamma^*\) (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 \((V_c^{max})\), 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 \((l_s)\), mesophyll conductance \((l_m)\) and biochemical capacity \((l_b)\), using the quantitative limitation analysis of Grassi and Magnani (2005) as applied by Tomás et al. (2013). Different fractional limitations, \(l_s, l_m\) and \(l_b\) \((l_s + l_m + l_b = 1)\), were calculated as:

\[
l_s = \frac{g_t \times \frac{\partial A_N}{\partial C_c}}{g_t \times \frac{\partial A_N}{\partial C_c} + \frac{\partial A_N}{\partial C_c}}
\]

Equation 2. Calculation of stomatal limitations \((l_s)\) according to Grassi and Magnani (2005).

\[
lm = \frac{g_{tot} \times \partial A_N / \partial C_c}{g_{tot} + \partial A_N / \partial C_c}
\]


\[
l_b = \frac{g_{tot} \times \partial A_N / \partial C_c}{g_{tot} + \partial A_N / \partial C_c}
\]

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 \(g_s\) and \(g_m\)). \(\partial A_N / \partial C_c\) 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 perMANOVA 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 \textit{adonis} function of the \textit{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 CO₂ 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 Aₙ, Cᵢ, Cₛ, iWUE and Vₘₐₓ (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 CO₂ 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). Ψᵣᵩₑₑ decreased from winter to summer almost identically in both treatments and this decrease was highly correlated with Ψₛᵋₑ 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 CO₂ treatment (Fig. 4). Aₙ, and gₛ presented maximum values in winter conditions, and then, decreased until summer conditions, both for CC and HC treatments. In the case of gₛ, there were no differences in any condition between CC and HC, while for Aₙ, and subsequently Cᵢ and Cₛ, these values were higher in HC than in CC. The progressive decrease in gₛ throughout the seasons supposed a significant increase in iWUE only in HC during early spring, late spring and summer, but not in winter. Unlike gₛ, gᵣ 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 Aₙ, gₛ and gᵣ (Fig. 5A and B), and a good correlation was found at low values of gₛ between gᵣ and gᵢ, but no correlation was found when taking into account all the data available (including winter) (Fig. 5C).
During the course of the seasons, $V_{\text{cmax}}$ and TPU remained the same in CC treatment, but increased significantly in HC treatment, while $J_{\text{max}}$ was the same in every treatment and condition, although $J_{\text{max}}/V_{\text{cmax}}$ 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 $T. \text{claveryi}$ 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 $H. \text{almeriense}$ desert truffle plant**

So far, $H. \text{almeriense}$ physiology has been studied mainly because of its interest as the host species for the cultivation of $T. \text{claveryi}$, 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 $\text{iWUE}$ of $T. \text{claveryi} \times H. \text{almeriense}$ 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 $\text{iWUE}$ 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 and 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂**

The increased fungal colonization found in control CO₂ 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 CO₂ conditions but no effect of differential CO₂ treatment was found (Table 2). There are several reports indicating that fine root production is increased under high atmospheric CO₂ (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* × *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, further 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**

A.M. and A.N.R. conceived, managed and coordinated the project. A.M., J.E.M.G. and A.N.R. planned and managed the research. J.P.G. and E.G.P. provided advice and knowledge about gas-exchange related measurements. J.E.M.G., A.N.R., F.A. and A.L.G. carried out the experiments. J.E.M.G., A.N.R. and J.P.G. carried out analyses and interpretation of the data. J.E.M.G drafted and wrote the manuscript. A.M., A.N.R., J.P.G. and E.G.P. contributed to sections of the manuscript. All the authors read and improved the manuscript.
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References


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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 CO₂ plants.

**Figure 2. Differences in plant behavior comparing CO₂ 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 CO₂ chamber (HC) winter; light blue = HC early spring; pink = HC late spring; yellow = HC summer. (B) High CO₂ (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 CO₂ treatments.** Black bars represent Control Chamber (CC) data, while grey bars represent High CO₂ Chamber (HC) data. The mean of n=6 replicates and the standard error are represented. Asterisks represent statistical differences (*p* < 0.05) between different CO₂ 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 CO₂ treatments.

**Figure 4. Gas exchange parameter variation during the course of the experiment in both CO₂ treatments.** Black circles represent Control Chamber (CC) data, while grey circles represent High CO₂ 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 CO₂ treatment when a variance analysis (ANOVA) and a Tukey’s post-hoc test were performed. Asterisks represent statistical differences ($p < 0.05$) between different CO₂ 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 CO₂ 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 CO₂ treatment. Biochemical limitations were not represented as they were less than 0.5 % for each condition and treatment. CC = control chamber; HC = high CO₂ chamber. (B) Relationship between stomatal limitations to $A_N$ and $g_s$. This article is protected by copyright. All rights reserved.
Table 1. Environmental data for each treatment and seasonal condition.

<table>
<thead>
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<th>Scenario</th>
<th>Control Chamber (CC)</th>
<th>High [CO₂] Chamber (HC)</th>
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<tbody>
<tr>
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<td>Mean temperature (ºC)</td>
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<td>12.64</td>
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<td>60</td>
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<tr>
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<tr>
<td>Photoperiod (light/darkness)</td>
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<td>10/14</td>
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<tr>
<td>Average Ψₘₕₑᵢₑ (kPa)</td>
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<tr>
<td>[CO₂] (ppm)</td>
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<td>800</td>
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<tr>
<td><strong>Scenario 2: Early Spring</strong></td>
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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.

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<td>Early spring</td>
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<tr>
<td>Mycorrhiza (% )</td>
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<tr>
<td></td>
<td>21.2±3.7a</td>
<td>31.3±3.9a</td>
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<td>Root starch (mM glucose g$^{-1}$)</td>
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<td></td>
<td>2.2±0.6a</td>
<td>2.7±0.8a</td>
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<td>Root free sugars (mM glucose g$^{-1}$)</td>
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<td></td>
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<tr>
<td></td>
<td>0.5±0.1a</td>
<td>1.0±0.3a</td>
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