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ORIGINAL PAPER

Morphological, physiological, and biochemical responses of *Pistacia atlantica* seedlings to elevated CO_2 concentration and drought stress

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Abstract

Elevated atmospheric CO_2 concentration and changes in precipitation patterns affect plant physiological processes and alter ecosystem functions. In combination, the interactions between these factors result in complex responses that challenge our current understanding. We aimed to investigate the effects of elevated CO_2 and drought stress on the growth and physiology traits of One-year-old *Pistacia atlantica* seedlings. Seedlings of *P. atlantica* were grown at two different CO_2 concentrations (ambient 380 ppm and elevated 700 ppm) and the two irrigation regimes (100% and 50% of field capacity) for one growing season. Seedlings collar diameter, height, leaf area, biomass accumulation, root length and volume, photosynthetic parameters, pigment content, and relative water content increased at elevated CO_2. At the same time, the amounts of proline, electrolyte leakage, malondialdehyde, and antioxidant enzymes decreased at elevated CO_2. Drought stress had negative effects on the measured growth parameters. These, however, ameliorate in the presence of elevated CO_2 through enhanced photosynthesis performance and maintaining better water status, and possibly also by a reduction of oxidative stress. Increased CO_2, as expected in a future climate, might thus mitigate the negative effects of drought in *P. atlantica* trees under natural conditions.

Keywords Elevated CO_2 · Drought stress · Growth · Photosynthesis · Oxidative stress · Antioxidants

Introduction

Atmospheric CO_2 concentration has increased since the onset of the industrial revolution, and a future increase in CO_2 concentration is expected (IPCC 2018). Increasing CO_2, along with other greenhouse gases, is supposed to trigger global warming, changes in precipitation patterns (IPCC 2013), and more frequent, intense, and erratic drought (Sippel et al. 2018; Jiang et al. 2021). Global warming impacts eco-physiological processes in terrestrial plants and ecosystems (Jentsch and Beierkuhnlein 2008; Albert et al. 2011a). The simultaneous drought and warming occurrence emphasize the need to investigate their impact on plants and ecosystems (Adams et al. 2009; Allen et al. 2010). Considering co-occurrences of climate features, studying each factor alone and in combination with others' effects on environmental changes is necessary, especially in interaction investigations.

Available water is a major limiting factor for plant growth because the water restriction induces changes in various physiological and biochemical processes (Farooq et al. 2009; Sippel et al. 2018). Stomata close progressively with increased drought stress, followed by reduced net photosynthetic rates (Reddy et al. 2004). Drought stress also reduces the contents and activities of photosynthetic carbon reduction cycle enzymes, including the critical enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Reddy et al. 2004). In addition, drought stress-induced generation of reactive oxygen species (ROS) is well recognized at the cellular level and is tightly controlled at both the production and consumption levels in vivo through increased antioxidative systems (Reddy et al. 2004). Antioxidant enzyme activity is an adaptive mechanism in plants to reduce ROS damage. In other words, the activity of the antioxidant enzyme scavenges the accumulated Hydrogen peroxide

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(H_2O_2) and reduces it to non-toxic levels, and in this way, alleviates oxidative stress (Gill and Tuteja 2010; Lotfi et al. 2019). Long-term exposure of plants to elevated CO_2 leads to several physiological effects, many of which are interpreted in the context of ameliorating the negative impacts of drought stress (Wullschleger et al. 2002). The direct effects of elevated CO_2 are typically increased photosynthesis and water use efficiency in vegetation (Ainsworth et al. 2020). Acclimation of photosynthesis during long-term exposure to elevated CO_2 reduces critical enzymes of the photosynthetic carbon reduction cycle, increasing nutrient use efficiency (Drake et al. 1997).

More recently, observations of widespread drought-induced tree mortality (Allen et al. 2015) have sharpened the focus on CO_2-induced changes in plant water use as a mechanism to maintain vegetation function during drought (De Kauwe et al. 2021). Numerous studies have reported the mitigation effects of elevated CO_2 on drought stress in different plant species (Drake et al. 1997; Wullschleger et al. 2002). Most elevated CO_2 field studies have addressed the potential for system-wide water savings under mild or moderate drought conditions (De Kauwe et al. 2021). On the contrary, elevated CO_2 may enhance plant performance during drought while water storage is depleted similarly to plants in control environments. For example, Jiang et al. (2021) found that elevated CO_2-grown eucalypts exhibited less drought stress during short-term drought, with less negative leaf water potentials despite having larger biomass and no change in soil moisture content. The positive and extended photosynthetic response to elevated CO_2 during drought stress may provide plants with additional nonstructural carbohydrates (NSC) to maintain lower osmotic potential and sustain metabolic activity (Jiang et al. 2021). Other studies, show a shift of biomass allocation into more roots in coffee plants exposed to elevated CO_2 during moderate drought (e.g. Avila et al. 2020). They suggested that this was associated with a higher transcript abundance of aquaporin genes (Avila et al. 2020). However, studies show different effects of elevated CO_2 on plant water relationships during drought, possibly influenced by experimental treatment (e.g. duration of CO_2 exposure, nutrient availability, and drought severity) as well as species-specific morphological, physiological, and biomass adjustments to the growth conditions (Zhou et al. 2013; Becklin et al. 2017). For example, it was shown that the elevated CO_2 benefits on plant growth, photosynthesis and nonstructural carbohydrates diminished with increasing aridity (Albert et al. 2011b; Duan et al. 2013). In addition, spring and early season leaf responses are most susceptible to elevated CO_2 and are followed by a down-regulation towards the onset of autumn. At the whole-tree level, CO_2 fertilization only causes consistent biomass increments in young seedlings, whereas mature trees show a variable response (Lauriks et al. 2021). Overall, it is necessary to consider various influencing factors to reconcile the disparate experimental evidence on the possible ameliorating role of elevated CO_2 during drought stress.

Atlas mastic tree, Beneh in Iran (*Pistacia atlantica* Desf.) is one of the most important native species distributed extensively in Zagros forests located in western Iran. These forests, characterized by a semi-Mediterranean climate, are one of Iran's most important and sensitive ecosystems (Ahmadi et al. 2014). Many studies have shown the ecological flexibility and tolerance of *P. atlantica* to challenging environmental conditions in Iran. Therefore, natural forests of this species are found throughout Iran but are particularly common in the western and southern parts of the country (Heydari et al. 2016). Although it tolerates and adapts to diverse ecological conditions, the natural regeneration and reforestation of *P. atlantica* have become difficult (Mirzaei and Karamshahi 2015; Sadeghzadeh Hallaj et al. 2022). The harsh climate of Zagros prohibited the natural regeneration of *P. atlantica* (Mirzaei and Karamshahi 2015). In recent years, the mortality of *P. atlantica* has been increasing rapidly and has become a public concern (Attarod et al. 2016; Hosseini et al. 2017). These extreme events are attributed to regional consequences of global climate change and are projected to further increase in intensity and frequency. In order to better adapt forest management, we aim to better understand the functional traits and physiological responses of this species to the expected combined changes in climate and CO_2 concentration.

This study aims to elucidate whether elevated CO_2 concentration mitigates or exacerbates the negative effects of drought stress in *P. atlantica* seedlings. We are particularly interested in responses that increase or decrease the vulnerability of *P. atlantica* in the Zagros region. It is hypothesized that elevated CO_2 concentration leads to increased assimilation and improved water use efficiency due to a higher photosynthetic pigment concentration, resulting in increased diameter and height growth, as well as a relative increase of the root system. These effects will be more expressed in drought stressed plants. In addition, increased CO_2 concentration mitigates the drought stress-induced proline concentration, electrolyte leakage (EL), malondialdehyde, and antioxidant enzyme activity.

Materials and methods

Plant material

The seeds of *P. atlantica* were grown for one year in 5-L pots containing a mixture of natural soil, sand, and manure (1:3:1, v/v). The pot's soil properties are shown in Table 1. The seedlings were watered twice every week at field capacity in the nursery. One-year-aged seedlings in the nursery

Table 1 General physicochemical properties of the pot's soil

Characteristics	Texture	pH	Sand (%)	Clay (%)	Silt (%)	Porosity (%)	EC (µS cm^{-1})	N (%)	P (µg g^{-1})	K (µg g^{-1})	CaO (%)	OC (%)	OM (%)	Density (g cm^{-3})
Pot's soil	Loamy sand	6.31	73	12.75	14.25	60	944	0.37	81.6	159.8	3.5	3.12	5.37	1.6

EC: Electrical conductivity

were transferred to the two growth chambers (3. 3. 2 m, L, W, H) with the two different CO_2 concentrations (the ambient CO_2, 380 ppm, and the elevated CO_2, 700 ppm) for one growing season from April 15 to December 10, 2019. We considered the other climatic factors fixed in the two chambers (170 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 60 ± 5% humidity, 27/16 °C day/night air temperature, and 14/10, 15/9, and 10/14 h day/night photoperiod in spring, summer, and autumn, respectively). *Pistacia atlantica's* natural regeneration commonly occurs under the canopy shade of nurse trees (Sadeghzadeh Hallaj et al. 2022; Jahanpour et al. 2010; Negahdarsaber and Abbasi 2010). Reforestation attempts by *Pistacia atlantica* also could be successful if the seedlings were treated with the shade of nurse trees (Hamzepour et al. 2006). A study on the growth and development of *Pistacia atlantica* seedlings also revealed that full sunlight inhibits growth even for well-watered seedlings (Sadeghzadeh Hallaj et al. 2022). Field observations in the Zagros forest confirm that the early development of *Pistacia atlantica* seedlings depends on the low light intensity. This species grows solely under the shade of light-tolerant trees. It is unclear whether improved performance under shade is due to protection against severe sunlight, moderation of drought stress or both. According to the literature and experiences, we thus applied a very low light intensity (170 µmol m^{-2} s^{-1}) for the experiment. The seedlings were rotated within the growth chambers every week to avoid micro-environmental effects.

Experimental design and treatments

The experiment was conducted in the two growth chambers located at the Faculty of Agriculture and Natural Resources, Lorestan University (33° 26′ 14.4″ N, 48° 15′ 38.7″ E). The experiment was done as a factorial based on a completely randomized design. The experimental treatments consisted of the control or ambient conditions (C), drought stress (D), elevated CO_2 concentration (CO_2), and the two factorial combinations ($CO_2 \times D$) with four replicates. We considered ten seedlings in each replicate, which resulted in 40 seedlings for each treatment. At each CO_2 concentration, seedlings were divided into two groups. One group was subjected to well-watered treatment (100% of field capacity), and the other was subjected to drought stress (50% of field capacity).

Growth measurements

Each seedling's stem diameter and height were measured using a digital calliper and ruler at the end of the growing season. Then, the seedlings were carefully dug out of the pots. The roots were hand-washed to remove all soil particles. Roots' lengths were carefully measured using a ruler. Also, root volume was estimated directly through the transposition of water in the graded container cylinders (Norouzi Haroni et al. 2019). Scanned leaf images were used to determine the leaf area for each seedling using the software Image Tool 3.0 (Wilcox et al. 2002). The seedlings shoot and root were dried at 80 °C for 48 h, and then biomass was measured.

Measurements of gas exchange parameters

Leaf gas exchange parameters were measured using an LC4 portable gas exchange system (ADC Bioscientific, Ltd., Hoddesdon, UK). Net CO_2 assimilation (A_{net}, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), and transpiration (E, mmol H_2O m^{-2} s^{-1}) were simultaneously measured of the first fully expanded leaves. Leaf temperature was held at 27 °C at a relative humidity of 60%. Measurements were made under a PAR of 170 µmol m^{-2} s^{-1}. The CO_2 concentration in the leaf chamber was the same as in the growth conditions. The A_{net}/g_s ratio was used as intrinsic water use efficiency (WUE_i, µmol mol^{-1}), according to Farquhar et al. (1989).

Measurements of photosynthetic pigments

At first, frozen leaves (0.1 g) were extracted at – 80 °C with 0.1 g calcium carbonate and 4 ml 80% acetone in the dark. Since chlorophyll is light-sensitive, the extraction took place in a dark room (Bergstrasser et al. 2015). The resulting extract was centrifuged at 4000 rpm for 10 min at 4.0 °C. The light absorption was measured at 470, 662, and 645 nm wavelengths using a spectrophotometer (Mapada UV-1800, Shanghai, People’s Republic of China). The contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were calculated according to Lichtenthaler (1987).

Relative water content measurement

First, the fresh weights (FW) of the sample leaves were recorded, and the leaves were floated in distilled water in Petri dishes. After 24 h, the leaves were removed, the leaf surface was gently wiped, and the turgid weight (TW) was measured. The samples were dried in an oven at 80 °C for 48 h to measure dry weight (DW). Relative water content (RWC) of leaf tissue was determined using the equation $RWC = 100[(FW - DW)/(TW - DW)]$ (Ritchie et al. 1990).

Proline, electrolyte leakage, and malondialdehyde determination

Free proline content in leaves was quantified following Bates et al. (1973). 0.5 g of fresh leaf tissue was removed and mixed with sulfosalicylic acid and acetic acid. After adding the ninhydrin solution, samples were placed in hot water. Light absorbance was read at 520 nm. 100 mg fresh leaf samples were cut into 5 mm lengths and placed in test tubes containing 10 mL distilled deionized water, then EL was determined. The tubes were placed in a water bath maintained at a constant 32 °C. After two hours, the initial electrical conductivity of the medium (EC_1) was measured using an electrical conductivity meter. Then, the samples were put in an oven at 120 °C for 120 min. Then, samples were cooled to 25 °C, and the final electrical conductivity (EC_2) was measured. We used the equation suggested by Nayyar (2003) to calculate El.

$$EL = 100\left[EC1/EC2\right].$$

Malondialdehyde (MDA) as a measure for oxidative destruction of lipids was determined by forming a pink dye when reacting with thiobarbituric acid (TBA). First, 0.5 g of the fresh leaf was mixed with 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 25 min, and the reaction was stopped by quickly placing it in an ice bath. The absorbance of the supernatant was read by spectrophotometer at 532 nm (Valentovic et al. 2006).

Activities of antioxidant enzymes in leaf extracts

Catalase (CAT) activity was determined in leaf extracts according to Chance and Maehly (1955). Leaf tissue (0.3 g FW) was ground in liquid N_2, homogenized with 1.5 mL of K phosphate buffer (containing 1 mM EDTA and 2% PVPP), and centrifuged at 14,000 rpm for 20 min at 4 °C. The CAT activity in the supernatant was calculated from the decrease in A_{240} and expressed as µmol H_2O_2 reduced min^{-1} g FW^{-1}. Peroxidase (POD) activity was assayed in leaf extracts as in MacAdam et al. (1992). Leaf tissue (0.3 g FW) was ground in liquid N_2, homogenized with 1.5 mL K phosphate buffer (pH 7.0), and centrifuged at 14,000 rpm and 4 °C for 20 min. The POD activity in the supernatant was calculated from the decline in A_{475} and expressed as µmol H_2O_2 reduced min^{-1} g FW^{-1}. Ascorbate peroxidase (APX) activity was assayed in leaf extracts as in Nakano and Asada (1981). Leaf tissue (0.3 g FW) was homogenized with 3 mL 0.05 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% PVPP and centrifuged at 14,000 rpm for 20 min. The APX activity in the supernatant was calculated from the decline in A_{290} and expressed as µmol H_2O_2 reduced min^{-1} g FW^{-1}.

Statistical analysis

We checked the data’s normality with the Kolmogorov–Smirnov test. Results indicated that the normality assumption was met for all variables, and no transformation was necessary. Two-way analysis of variance (ANOVA) was used to test the effects of CO_2 concentrations, drought stress, and their interaction on all dependent variables. The Duncan test at $P \leq 0.05$ was used to compare means. All statistical analyses were conducted using the SPSS software version 21.0.

Results

Growth parameters

Elevated CO_2 concentration and drought stress significantly affected the diameter growth of *P. atlantica* seedlings, while $CO_2 \times D$ had no effect (Table 2). Elevated CO_2 concentration enhanced the mean diameter growth by 19% compared to the control seedlings, whereas the diameter of drought stressed seedlings only reached 57% (Fig. 1a). Height growth and leaf area also increased under elevated CO_2 concentration (Table 2). The height and leaf area increments were 38% and 27% greater under elevated CO_2 than the control seedlings (Fig. 1b, c). Under $CO_2 \times D$, height growth and leaf area decreased by 6% and 30%, respectively, compared to the control (Table 2, Fig. 1b, c). The positive effect of CO_2 could be better revealed when we compare $CO_2 \times D$ with drought stress alone. Under $CO_2 \times D$, height growth and leaf area increased by 24% and 17%, respectively, compared to drought stress alone (Table 2, Fig. 1b, c).

Root length and volume tended to increase relative to the control when *P. atlantica* seedlings were exposed to elevated CO_2 (by 30% and 43%, respectively), while drought stress reduced the root length and volume by 12% and 69%, respectively (Table 2, Fig. 2a, b). Root length and volume were considerably higher under $CO_2 \times D$ than under drought stress alone (13% and 23%, respectively) (Table 2, Fig. 2a, b).

Elevated CO_2 concentration and drought stress also affected shoot, root, and total biomass (Table 2). As expected, elevated CO_2 concentration increased shoot biomass by 73%, root biomass by 47%, and total biomass by 58% in comparison to the control, while drought stress reduced the shoot, root, and total biomass by 76%, 84%, and 80%, respectively (Fig. 3a–c). Under $CO_2 \times D$, shoot and total biomass increased by 50% and 56%, respectively, compared to drought stress alone (Table 2, Fig. 3a, c).

Table 2 F-values obtained from the two-way variance analysis for the elevated CO_2 concentration (CO_2) and drought stress (D) treatments, and their interactive effects on the growth and physiological traits of 1-year-old *P. atlantica* seedlings

Trait	Factor		
	CO_2	D	$CO_2 \times D$
Diameter (mm)	21.0**↑	146.0***↓	1.6
Height (cm)	51.0***↑	75.5***↓	6.2*↓
Leaf area (cm^2)	24.7***↑	165.9***↓	5.1*↓
Root length (cm)	23.1***↑	21.3**↓	4.7*↑
Root volume (cm^2)	29.7***↑	114.3***↓	8.0*↓
Shoot biomass (DW g per plant)	89.2***↑	148.2***↓	17.3**↓
Root biomass (DW g per plant)	17.4**↑	29.5***↓	0.4
Total biomass (DW g per plant)	52.8***↑	88.6***↓	4.8*↓
Net photosynthesis (μmol CO_2 m^{-2} s^{-1})	142.8***↑	138.7***↓	10.7**↑
Stomatal conductance (mol H_2O m^{-2} s^{-1})	47.4***↑	147.2***↓	12.2**↓
Transpiration (mmol H_2O m^{-2} s^{-1})	49.1***↑	65.4***↓	4.9*↓
WUE_i (μmol mol^{-1})	15.6**↑	0.3	0.9
Chlorophyll *a* (mg g^{-1} FW)	208.4***↑	89.6***↓	13.9**↑
Chlorophyll *b* (mg g^{-1} FW)	88.3***↑	40.1***↓	4.8*↑
Chlorophyll $a+b$ (mg g^{-1} FW)	227.3***↑	99.5***↓	14.2**↑
Carotenoids (mg g^{-1} FW)	100.4***↑	24.4***↓	5.1*↑
Relative water content (%)	192.2***↑	146.7***↓	5.5*↑
Proline (mg g^{-1} FW)	84.8***↓	210.1***↑	48.5***↑
Electrolyte leakage (%)	29.4***↓	62.8***↑	4.9*↑
Malondialdehyde (μmol g^{-1} FW)	41.4***↓	97.7***↑	11.6**↑
Catalase (Unit g^{-1} FW)	7.5*↓	21.6**↑	5.7*↑
Peroxidase (Unit g^{-1} FW)	128.5***↓	100.6***↑	0.0
Ascorbate peroxidase (Unit g^{-1} FW)	33.6***↓	52.7***↑	14.2**↑

Asterisks (*) denote the level of significance (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Arrows indicate the increase or decrease of the parameters

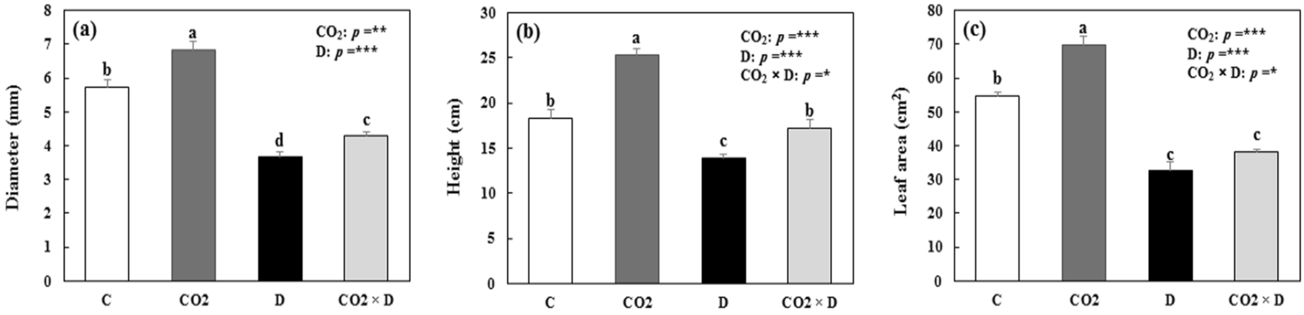

Fig. 1 Mean (±SE) diameter (**a**), height (**b**), and leaf area (**c**) of *P. atlantica* seedlings after 8 months of growth under control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n = 4$). Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

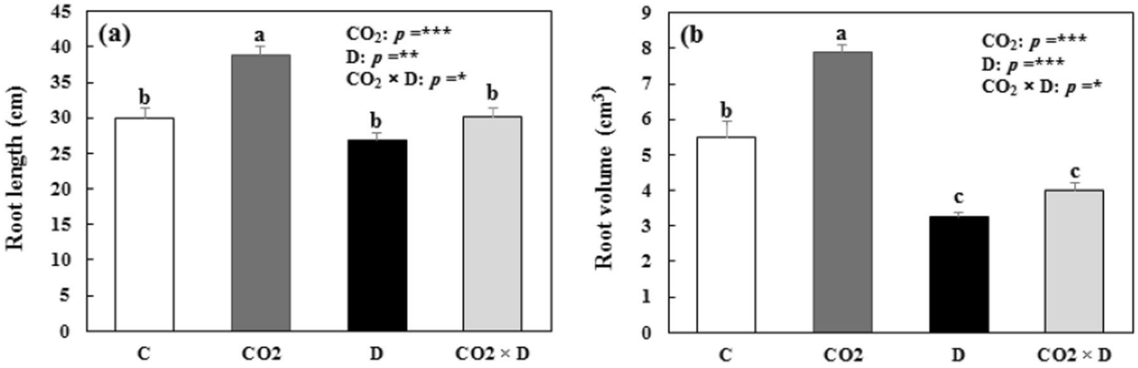

Fig. 2 Mean (±SE) root length (**a**) and volume (**b**) of *P. atlantica* seedlings after 8 months of growth in control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n = 4$). Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

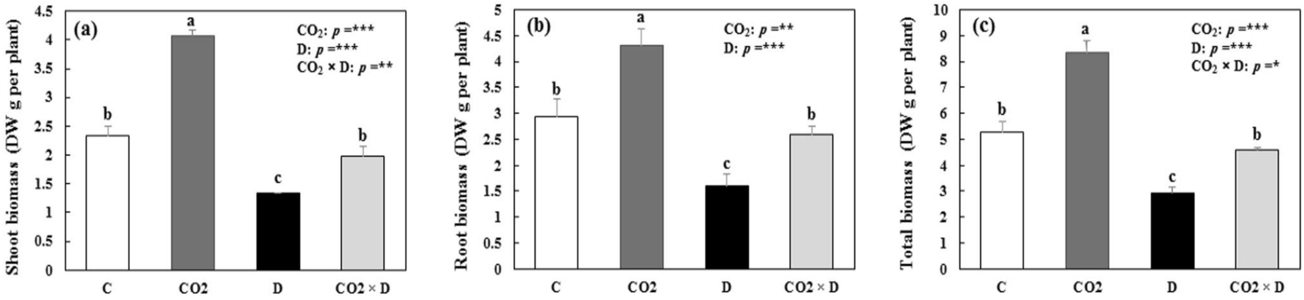

Fig. 3 Mean (±SE) shoot (**a**), root (**b**), and total (**c**) biomass of *P. atlantica* seedlings after 8 months of growth under control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n = 4$). Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Gas exchange

The net photosynthesis (A_{net}), stomatal conductance (g_s), and transpiration (E) rate of *P. atlantica* seedlings were influenced by elevated CO_2, drought stress, and $CO_2 \times D$ treatments (Table 2). The A_{net} (by 81%), g_s (by 43%), and E (by 61%) increased under elevated CO_2 concentration, while their rates decreased under drought stress by 82%, 56%, and 62%, respectively, in comparison with the control treatment (Fig. 4a–c). In the combined treatment, elevated CO_2 concentration alleviated the effect of drought stress so that the reductions in A_{net}, g_s, and E were improved by 84%, 22%, and 51%, respectively (Table 2, Fig. 4a–c). In addition, elevated CO_2 concentration also enhanced intrinsic water use efficiency (WUEi) by 27% compared to the control seedlings, while its rate did not differ between the

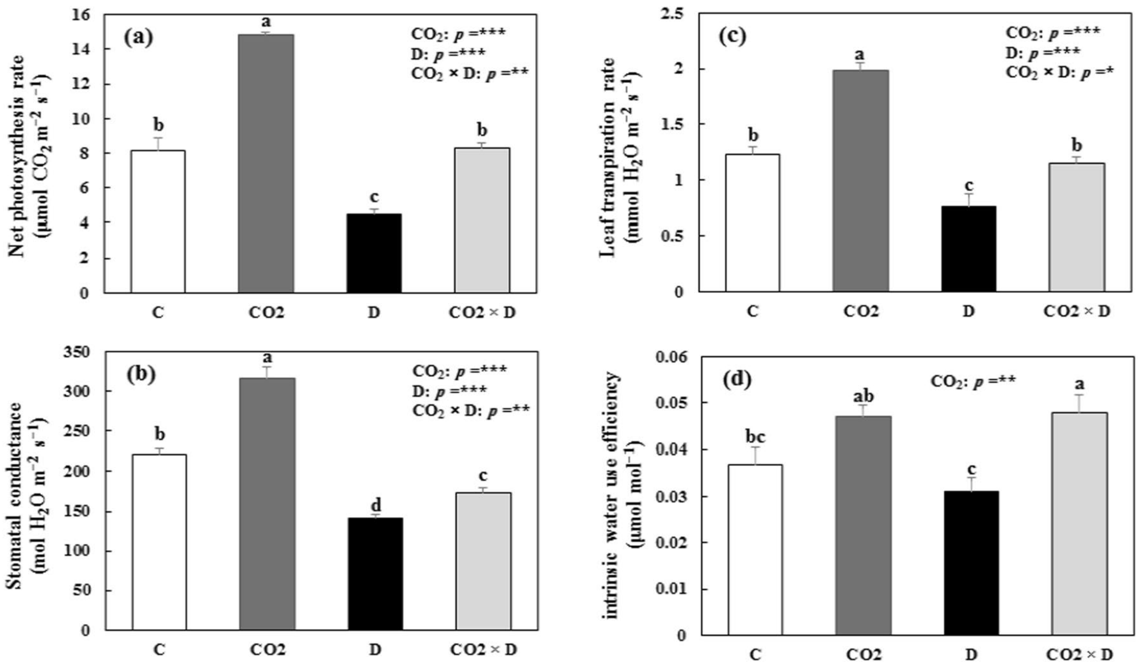

Fig. 4 Mean (±SE) net photosynthesis (A_{net}) (**a**), stomatal conductance (g_s) (**b**), transpiration (E) (**c**), and intrinsic water use efficiency (WUE_i) (**d**) rates of *P. atlantica* seedlings after 8 months of growth under control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n = 4$). Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

two water regimes (Table 2, Fig. 4d). In the $CO_2 \times D$ treatment, although the WUEi was increased in comparison with drought stress alone (by 55%), it was not significant (Table 2, Fig. 4d).

Pigment content

Exposure to elevated CO_2 concentration significantly increased chlorophyll *a* by twofold, chlorophyll *b* by 86%, chlorophyll $a + b$ by twofold, and carotenoids by 87% compared to the control seedlings. In contrast, their concentrations were reduced with increasing drought stress by 46%, 44%, 45%, and 24%, respectively (Table 2, Fig. 5a–d). When elevated CO_2 concentration was combined with drought stress, the chlorophyll *a*, chlorophyll *b*, chlorophyll $a + b$, and carotenoids increased 86%, 77%, 83%, and 68%, greater than drought stress alone treatments, respectively (Table 2, Fig. 5a–d).

Water status, proline, electrolyte leakage, and malondialdehyde

RWC increased by 13% under elevated CO_2 concentration compared to the control treatment, while its level decreased under drought stress by 20% (Table 2, Fig. 6a). Increasing CO_2 levels generally reduced or reversed the impact of drought stress (by 22%; Table 2, Fig. 6a).

In leaves, drought stress increased the mean proline, EL, and malondialdehyde (MDA) concentrations by 85%, 33%, and 84% compared to the control seedlings. However, their contents decreased by 10%, 12%, and 23% with increasing CO_2 concentration, respectively (Table 2, Fig. 6b–d). Elevated CO_2 significantly alleviated the effect of drought stress so that proline, EL, and MDA contents were considerably lower under $CO_2 \times D$ than under drought stress alone treatment (by 53%, 23%, and 51%, respectively; Table 2, Fig. 6b–d).

Enzyme activities

Drought stress also promoted an increase in CAT (twofold), POD (27%), and APX (66%) activities in comparison to control seedlings (Table 2, Fig. 7a–c). In contrast, the CAT, POD, and APX activities, measured at the CO_2 growth concentration, were lower in elevated CO_2 seedlings than in ambient CO_2 seedlings (by 7%, 42%, and 13%, respectively) (Table 2, Fig. 7a–c). Compared to the activity of the antioxidant enzymes of leaves between drought stress treatments, CAT and APX activities were significantly reduced under elevated CO_2 (by 67% and 55%, respectively). However, POD activity remained unaffected (Table 2, Fig. 7a–c).

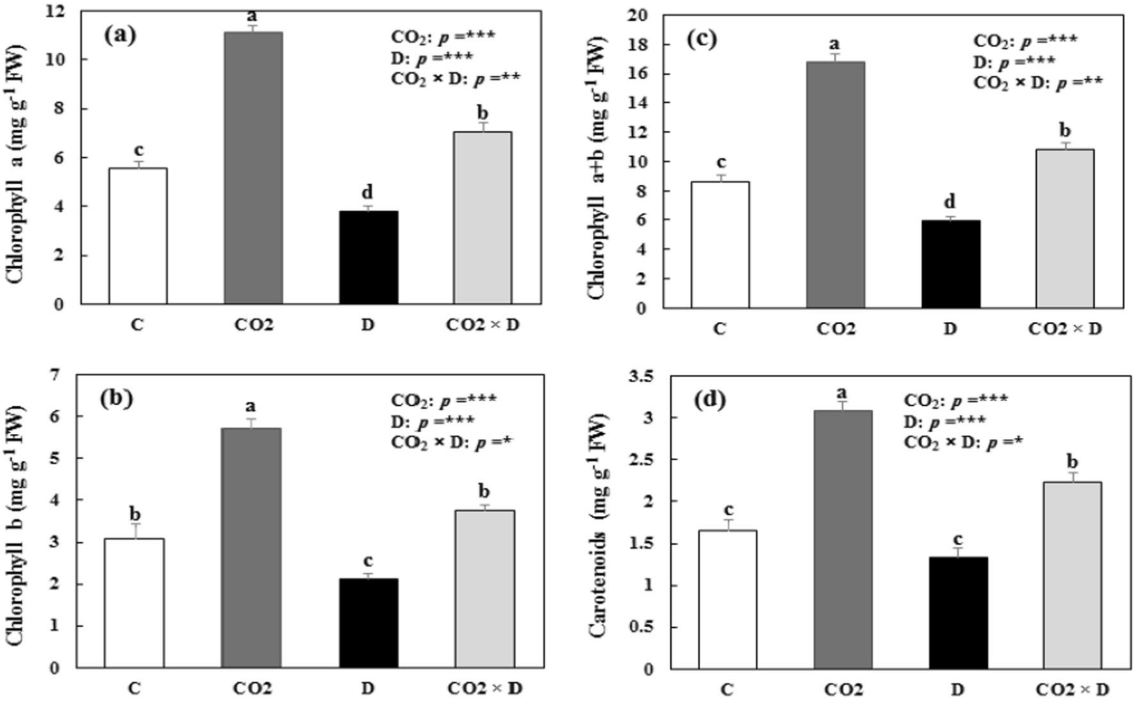

Fig. 5 Mean (±SE) concentrations of chlorophyll *a* (**a**), chlorophyll *b* (**b**), chlorophyll *a*+*b* (**c**), and carotenoids (**d**) in leaves of *P. atlantica* seedlings after 8 months of growth under control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n=4$). Asterisks (*) denote the level of significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$)

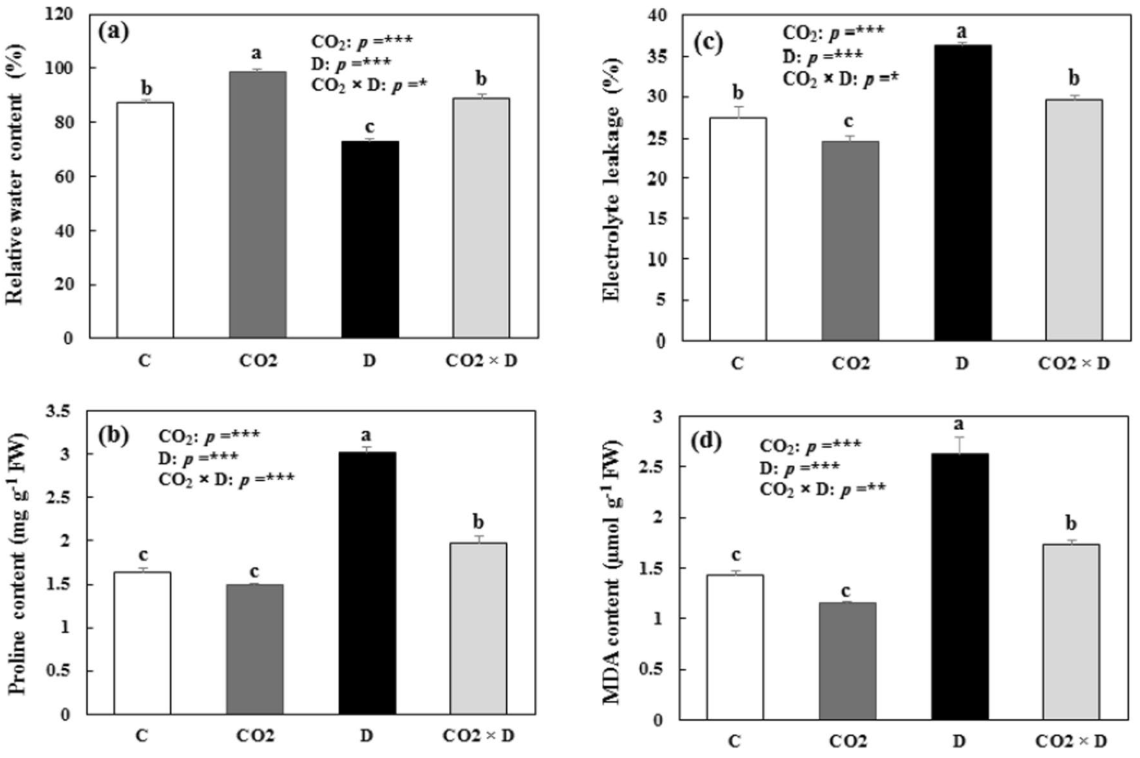

Fig. 6 Mean (±SE) relative water content (RWC) (**a**), Proline (**b**), electrolyte leakage (EL) (**c**), and malondialdehyde (MDA) (**d**) contents of *P. atlantica* seedlings after 8 months of growth in control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n=4$). Asterisks (*) denote the level of significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$)

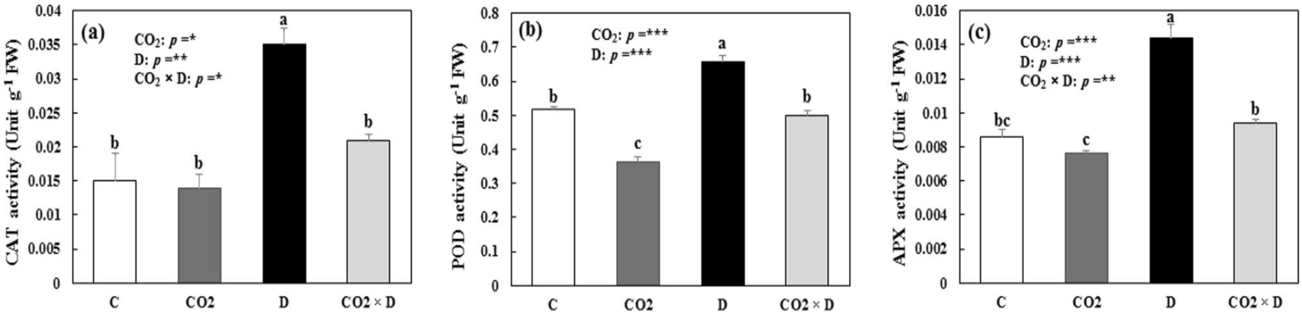

Fig. 7 Mean (± SE) catalase (CAT) (**a**), peroxidase (POD) (**b**), and ascorbate peroxidase (APX) (**c**) activities of *P. atlantica* seedlings after 8 months of growth under control (C), elevated CO_2 concentration (CO_2), drought stress (D), and $CO_2 \times D$ conditions (Duncan test; $P \leq 0.05$; $n = 4$). Asterisks (*) denote the level of significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

Discussion

Results show that increasing CO_2 up to 700 ppm increased growth, including diameter, height, leaf area, root length, root volume, shoot-, root-, and total biomass. Similar results also were observed in the previous studies with other plant species (Vaz et al. 2012; Arab et al. 2018; Song et al. 2020; Lauriks et al. 2021). Increased drought or CO_2 also affects root allocation in many cases. Effects on root allocation are often observed regarding drought (e.g. Santos et al. 2021; Jeong et al. 2021), and sometimes also regarding CO_2 (e.g. Norby et al. 2004). However, the latter response depends on other soil and weather conditions (e.g. Handa et al. 2008) and plant strategies, which also might favour exudation instead of root biomass (Fahey et al. 2005). Many studies have reported that elevated CO_2 stimulates root growth (Crookshanks et al. 1998; Norby et al. 2004; De Graaff et al. 2006). In a meta-analysis, root biomass exhibited more significant increases than shoot biomass with elevated CO_2; therefore, increasing root biomass with elevated CO_2 may enhance the potential to store C (De Graaff et al. 2006; Luo et al. 2006; Nie et al. 2013). In addition, these effects are expected to cause increased amounts of C input into the soil (De Graaff et al. 2006). The observed plant growth increment in elevated CO_2 in our experiment was consistent with the increase in photosynthetic rate during the same period.

We found that drought stress reduced all the measured growth parameters, similar to the findings of Guo et al. (2010), Deligoz and Gur (2015), and Jafarnia et al. (2017). The reductions in the aboveground growth of seedlings under increasing water deficit are well-known adaptations. Optimal partitioning theory suggests that "plants preferentially allocate biomass to acquire the resource that most limits growth" (Kobe et al. 2010). Therefore, under drought stress, plants tend to invest in root growth at the expense of diameter and height growth resulting in aboveground biomass reductions (see, for example, Schall et al. 2012; Jeong et al. 2021; Santos et al. 2021). In our study, elevated CO_2 concentration alleviated the negative effects of drought and promoted plant growth under stress. Owensby et al. (1997) have successfully used a combination of approaches (measurements of leaf water potential to whole-ecosystem gas exchange) to show that reduced water use in a C_4 tallgrass prairie exposed to elevated CO_2 was sufficient to increase above- and below-ground biomass production in years when drought stress was frequent.

Elevated CO_2 is often reported to increase photosynthesis (A_{net}) and intrinsic water use efficiency (WUE_i) in C_3 plants. In this study, A_{net} and WUE_i also increased with increasing CO_2. The same results are observed for *Vitis vinifera* (Moutinho-Pereira et al. 2009), *Kalopanax septemlobus* (Watanabe et al. 2010), *Quercus mongolica* (Yan et al. 2010), *Deschampsia flexuosa* (Albert et al. 2011c), *Quercus suber* (Vaz et al. 2012), and *Phragmites australis* (Mozdzer and Caplan 2018).

Despite many studies that have shown a reduction in stomatal conductance (g_s) and transpiration (E) rates under elevated CO_2 (Wullschleger et al. 2002; Ainsworth and Rogers 2007; Mozdzer and Caplan 2018), the results of this study showed that the rate of g_s and E of *P. atlantica* seedlings increased under elevated CO_2, which is in accordance to (Albert et al. 2011c; Zinta et al. 2014; Sreeharsha et al. 2015; Monda et al. 2016). Thus, the decrease in g_s due to findings in some other species elevated CO_2 is not a universal response but may be due to a species-specific strategy. The different response of g_s due to elevated CO_2 is particularly found in specific species or ecotypes, plant functional types (PFTs), and development stages in cotrast to others (Xu et al. 2016). In addition, Medlyn et al. (2001) reported that g_s response to elevated CO_2 was significantly stronger in young trees than in old trees, deciduous compared to coniferous trees, and drought-stressed to nutrient-stressed trees.

According to the current findings, drought stress significantly decreased A_{net}, g_s, and E rates in *P. atlantica*. This indicates a stomatal closure in response to a reduction in relative water content (Reddy et al. 2004). Stomatal closure decreases the foliar photosynthetic rate and internal CO_2 concentration. In the current study, increasing CO_2 levels generally alleviated the negative effects of drought stress on gas exchange. Previous studies also observed this finding (Zeppel et al. 2012; Bauweraerts et al. 2013; AbdElgawad et al. 2015; Miranda-Apodaca et al. 2015; Jiang et al. 2021).

Drought stress significantly decreased the concentrations of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, chlorophyll $a+b$, and carotenoids (Dutta et al. 2015; Zhang et al. 2018; and Mahmoudian et al. 2021). The chloroplast and thylakoid structures are usually injured under increased oxidative stress due to drought, leading to decreases in chlorophyll and carotenoid content (Asrar and Elhindi 2011). Our findings also support the role of elevated CO_2 in the photosynthetic pigments concentration enhancement of seedlings under drought stress. It may be due to less oxidative stress and less damage to photosynthetic pigments, which is consistent with the findings of AbdElgawad et al. (2015).

RWC decreased under drought stress. Similar results were obtained by Wang (2014) and Cui et al. (2019). The foliar photosynthetic rate of higher plants is known to decrease as the relative water content and leaf water potential decrease (Cornic 2000). In our study, drought stress led to stomata closure, but in the presence of elevated CO_2, while the stomata remained open, the RWC reduction was mitigated. Similar results were shown by Atwell et al. (2007) and Cui et al. (2019). It may be due to better water supply through more root biomass. With increasing drought intensity and decreasing relative water content, plants try to absorb maximum moisture from the soil through osmotic adjustment mechanisms and reduced stem water potential (Sanchez-Blanco et al. 2004). Some authors have affirmed that elevated CO_2 would permit the plant to increase the fine roots and, in general, the root biomass, raising the root-to-shoot ratio and boosting drought tolerance (Xu et al. 2013; Miranda-Apodaca et al. 2018).

In our study, the accumulation of proline, malondialdehyde and EL concentrations increased with drought stress (Wang 2014; Jafarnia et al. 2017; Chiappero et al. 2019; Zhang et al. 2019) and a significant increase was seen in antioxidant enzyme activities such as CAT, POD, and APX (Xu et al. 2008; Patel and Hemantaranjan 2012; Wang 2014). While the seedlings grown under drought stress conditions and simultaneously exposed to elevated CO_2 showed reduced proline, MDA, and EL contents, similar results were obtained by Xu et al. (2014) and AbdElgawad et al. (2015). A significant alleviation was seen in the negative effects of drought stress on CAT and APX activities in the pistacia seedlings exposed to elevated CO_2 (see also Schwanz et al. 1996). In fact, under conditions of oxidative stress, the peroxidation of unsaturated fatty acids increases and various aldehydes, including MDA, are produced by the attack of free radicals on lipids (Gharibi et al. 2016). EL is also an indicator of cell integrity and cellular membrane stability, reflecting the degree of damage to the plant by stress factors (Kocheva et al. 2004). A reduction in oxidative stress effects under elevated CO_2 may originate from reduced ROS generation, with a concomitant reduction of stress impact (relaxation) and/or at the level of increased ROS scavenging (antioxidant) capacity (AbdElgawad et al. 2015). A reduction of ROS levels by elevated CO_2 is biochemically explained by increased rubisco carboxylation capacity, reducing photorespiratory H_2O_2 production. As a consequence of the reduced ROS generation, also antioxidant levels may remain low under stress conditions in elevated CO_2 (AbdElgawad et al. 2015).

Conclusions

The obtained results in this study largely supported our hypotheses. We found that elevated CO_2 positively provokes marked changes in the physio-morphological traits of *P. atlantica* seedlings. On the other hand, drought stress negatively affected the studied traits. We also observed that elevated CO_2 could potentially mitigate the negative effects of drought stress by improving photosynthesis and mitigating drought stress. In summary, plants exposed to drought stress may benefit from future elevated CO_2 conditions.

Author contribution PY: Running the experiment, laboratory analysis, measuring the data, statistical analysis, writing the draft of the manuscript. BP: Supervisor, Conceptualization, Methodology, Monitoring data analysis, and Original manuscript editing. AHN: Advisor, Tips for drought stress. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest No conflict of interest declared.

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