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Methane Emissions from Seedlings of the Cyprus Variety of Faba Bean (*Vicia faba* L.) Under Drought and Heat Stress Factors at Elevated Carbon Dioxide

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Abstract

Global warming is caused by a group of greenhouse gaseous pollutants emitted by human activities into the atmosphere, mainly carbon dioxide (CO_2) and Methane (CH_4). However, CH_4 is the most potent contributor to the warming of Earth and a changing climate, despite its lower concentration than CO_2. There are many natural sources of methane emission in nature including plants, which have been shown to produce more CH_4 under stressful environmental conditions, particularly heat and drought. However, the combined effects of these two factors on methane emissions from plants have little been investigated. This study was designed to examine the combined effects of heat and drought on CH_4 emissions from Cyprus faba bean (*Vicia faba* L.) seedlings at elevated CO_2. Plants were grown in controlled-glass cabinets under two temperature regimes (20°/17°C day/night for control and 30°/26°C day/night for heat-stressed plants), two CO_2 concentrations of 400 ppm (ambient) and 800 ppm (elevated), and two watering regimes (well-watered and drought-stressed) for 14 days following cultivation of plants for 12 days. The effects of temperature, CO_2 concentration and watering regime on CH_4 emissions, growth, physiological, and biochemical parameters of fava bean seedlings were examined. Our results showed that heat and drought-stressed seedlings emitted significantly more CH_4 under single stress factor or their combination compared to control seedlings. Moreover, stress factors inhibited growth and development of faba bean seedlings. Elevated CO_2, in turn, decreased CH_4 emissions from stressed plants and enhanced their growth under control and stress conditions. Moreover, our results showed that elevated CO_2 enhanced the antioxidant capacity of all seedlings by increasing their flavonoids, chlorophyll and moisture contents. In conclusion, findings of this study corroborated the hypothesis that atmospheric CO_2 enrichment decreased plants' abilities to emit CH_4 in stressful environments and accordingly improve their growth under the predicted trends of climate change.

Keywords: Abiotic stress, Climate change, Faba bean (*Vicia faba* L.), Drought, Elevated CO_2, Heat, Methane emissions, Plant growth.

1. Introduction

Climate change and global warming are major issues of international concerns. Increasing emissions of gaseous wastes due to human actions, particularly CO_2 and CH_4, continuously add to those naturally occurring in the atmosphere, leading to an altered climate and a steady increase in temperature of the globe (Lomborg, 2020). An increase of 0.65-1.06°C in the Earth's temperature was recorded from 1880-2012 with additional increases over the next years (IPCC, 2013). Much of the warming and elevated global temperature has occurred in more recent years, and a temperature rise reaching as high as 4.1°C is highly expected by the end of the century (Lomborg, 2020). The consequences of climate change and global warming are likely catastrophic to the natural environments and human lives if human-induced emissions of gaseous waste products are not diminished. Global warming is linked with severe shifts in other climatic factors including wind and precipitation patterns, enhanced drought, flooding, heat waves, and air pollution (AghaKouchak *et al.,* 2020). These drastic climatic changes have thus become issues of major concern to scientists and international communities around the world. Of all greenhouse gases, CO_2 is the greatest contributor to increasing Earth's temperature; however, CH_4 is more potent, although it is emitted in less quantities and has a shorter atmospheric lifespan than CO_2. It has been reported that CH_4 contributes to about 25% of the change in climate over the last quarter-millennia, with a heat trapping capacity that is up to 23 times greater than that of CO_2 (Chai *et al.,* 2020). Although the largest proportion of CH_4 emissions come from human activities, other organisms (methanogenic archaea, cyanobacteria, fungi, algae, and plants) contribute to CH_4 release as well (Ernst *et al.,* 2022). As major producers of the biosphere and the most diverse, plants have gained special attention in studying their contribution to the atmospheric budget of CH_4, under the environmental challenges imposed by climate change and global warming (Turner *et al.,* 2020).

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A series of studies over the past decade have made it clear that plants produce CH_4 aerobically, and these emissions are influenced by a variety of abiotic stress factors, particularly heat and drought (two key ecological components of climate change) (Wang *et al.,* 2021; Ernst *et al.,* 2022; Gautam *et al.,* 2024). It's well established that abiotic stresses exert deleterious effects on plants and induce CH_4 emissions (Wang *et al.,* 2021; Yao *et al.,* 2023). However, it is evident that environmental factors, temperature and drought stresses in particular, increase aerobic CH_4 emissions, but the interactive effects of stress factors on CH_4 emissions remain poorly understood, though evidence suggests that tailored responses occur in response in plants to the combination of environmental stresses. Moreover, these responses occurring only from the combination of stress factors, cannot be inferred from results of individual stress factor experiments, and therefore multiple-factor studies are essential (Zandalinas *et al.,* 2021). Elevated CO_2 (the third key component of climate change) has experimentally been shown to compensate for stressful conditions by alleviating the negative consequences of abiotic environmental stresses on plants (Abo Gamar *et al.,* 2023; Rakhmankulova *et al.,* 2024; Vijayalakshmi *et al.,* 2024).

It has been reported that the main components of climate change (heat, elevated CO_2 and drought) cause plants to experience changes in their physiology, anatomy, morphology and biochemistry in their natural habitats (Abo Gamar *et al.,* 2023).

Faba bean belongs to the family Fabaceae or Leguminosae, and it is among the oldest crops grown worldwide (Hobdari *et al.,* 2023). Faba bean is also known as broad bean, horse bean and field bean, and it is the fourth most main crop in the world (Davies *et al.,* 2022). The crop has been consumed in different ways, such as dry seeds, green vegetable, or as processed food. Its products, especially seeds, are very rich with high-quality protein for human diet. Also, its dry seeds, green haulm and dry straw have been used as resources for animal feeds (Davies *et al.,* 2022). The seeds of faba bean contain high protein content of 24-33% (Warsame *et al.,* 2022). In Jordan, the crop is cultivated either under irrigation or rainfed for the purposes of fresh pod utilization and dry seed production (Thalji, 2015). Four most common faba bean cultivars have been grown by Jordanian farmers. These include Cyprus (large seed), Equina-Syria-1 (medium seed), Balady and MinorBeck- Giza (small seed) (Thalji, 2015).

The potential of plants to release CH_4 into the atmosphere under normal or stress conditions were investigated in this study using Cyprus faba bean (*Vicia faba* L.), which is commonly grown in Jordan. We hypothesize that heat and drought stresses would reduce growth of Cyprus faba bean seedlings and induce them to emit CH_4, and that elevated CO_2 could enhance their growth and decrease CH_4 emissions under our experimental stress treatments. The specific objectives of this study are to: (1) investigate the individual and combined effects of temperature, CO_2, and watering regimes on CH_4 emission from Cyprus faba bean seedlings; (2) investigate if elevated CO_2 alleviated the negative impacts of heat and drought stresses on Cyprus faba bean seedlings, and thus, CH_4 emissions; and (3) address the possible mechanism (s) by which elevated CO_2 alleviate the negative impacts of heat and drought stresses on Cyprus faba bean seedlings. To the best of our knowledge, this study is the first to be conducted related to CH_4 emission on crops grown in Jordan.

2. Materials and methods

2.1. Plant materials and growth conditions

The experiment was conducted inside the greenhouse of Yarmouk University from March to June 2021 on Cyprus faba bean seedlings to investigate the effects of temperature, CO_2, and watering regimes and their interactions on the growth and CH_4 emission from Cyprus faba bean seedlings. In this study, faba bean seeds (obtained from the National Agricultural Research Center [NARC] in Baqaa, Jordan) were sown in pots containing a mixture of soil, perlite and peat moss (1:1:1, by volume). In each pot, a seed of fava bean was sown to start germination under greenhouse conditions; 20°C day/17°C night, 14 hr light/10 hr dark, photosynthetic photon flux density (PPFD) of 530 photons m^{-2} s^{-1} and relative humidity at 55 % ± 2 %. The germination process, in total, took about 7 days and pots were watered with tap water to help seeds to germinate. Following germination, seedlings were kept in these conditions for a period of 5 days until all had produced at least four true leaves. NPK fertiliser was added (18-18-18, Navarsol IV, Timac Agro Italia). Afterward, seedlings were transferred to four controlled-glass cabinets (90 cm depth × 95 cm width × 70 cm height each) maintained in a naturally-illuminated greenhouse. The temperature, humidity and light were monitored by a cabinet control and logging computer to establish experimental conditions. The first two cabinets represent the non-heat stress treatment (normal temperature treatment; 20°C day/17°C night), and the other two represent the heat stress treatment (30°C day/26°C night). Temperatures were selected to match the current average temperatures in Jordan from March to June. For the two cabinets, which were under the non-heat stress treatment, the first one was provided with the atmospheric CO_2 concentration, which was around 400 ppm (ambient CO_2) and the second one with 800 ppm CO_2 concentration (elevated CO_2). Moreover, half of the seedlings were supplied with water to the field capacity (well-watered) and the other half were drought-stressed. Same treatments were applied for the two cabinets, which were under the heat stress condition. A split-split-plot design with three factors (temperature, CO_2, and watering regime) was used, and in total, eight different experimental treatments were conducted as follows: (1) normal temperature, ambient CO_2 and well-watered (control); (2) normal temperature, ambient CO_2 and drought-stressed; (3) normal temperature, elevated CO_2 and well-watered; (4) normal temperature, elevated CO_2 and drought-stressed; (5) heat stress, ambient CO_2 and well-watered; (6) heat stress, ambient CO_2 and drought-stressed; (7) heat stress, elevated CO_2 and well-watered; and (8) heat stress, elevated CO_2 and drought-stressed. CO_2 circulation in each cabinet was kept constant by using a small electrical fan and the relative humidity was also maintained at 55 % ± 2 %. Flow of gas from the CO_2 cylinder to the cabinets was regulated by a pSense portable CO_2 meter (K30 CO_2 Sensor). Seedlings were grown under their respective treatment for 14 days in cabinets.

2.2. Measurement of methane emissions

After 14 days of growth under experimental conditions and from each condition, three samples of fresh leaves were detached and incubated inside 4ml plastic syringes for 3 hr under the temperature of 20°C and PPFD of 530 photons m^{-2} s^{-1}. Two ml of gas was pulled from each syringe, and was injected manually into Hewlett-Packard 5880 Series II gas chromatograph equipped with a flame-ionization detector (Palo Alto, CA) operated at 200°C, a 2-m stainless steel column packed with 13 XMS (60/60 mesh). The injector temperature was 56°C, and the carrier gas was N_2 flowing at 31 ml min^{-1}. The retention time (~2.9 mins) of external standard was used to identify CH_4, and the standard curve of certified CH_4 gas was used to quantify its emissions rate on basis of dry biomass by drying leaves at 60°C for 48 hr.

2.3. Determination of growth parameters

After 14 days of growth, stem length was measured from the soil surface to apical meristem of each surviving plant using a ruler. Stem diameter was also measured on each plant using a Digimatic caliper (Digimatic CD15DCX; Mitutoyo) placed at the midway point between soil and the apical meristem. Three plants from each trial were harvested and dried at 60°C for 72 hr in a forced air oven in order to determine leaf mass, stem mass, root mass, and leaf area. The leaves, stems, and roots of each plant were weighed separately on analytical balance (Mettler Toledo, Columbus, XP205) in order to determine dry and fresh masses accumulation of each plant. An average-sized leaf from each plant was removed and weighed on an analytical balance (Mettler Toledo, Columbus, XP205) before and after drying in order to determine leaf moisture content. Leaf area of each plant was determined using an image J software (http://rsb.info.nih.gov/ij/). Growth indices were measured using the dry matter data; measurements included leaf area ratio (LAR) (cm^2 g^{-1}), leaf mass area (LMA) (g m^{-2}), leaf mass ratio (LMR), and shoot:root dry mass ratio.

2.4. Stress indicators

2.4.1. Measurement of proline

Proline content was estimated according to Bates et al. (1973). Three fresh leaf samples (60 mg) from three separate plants were collected from each treatment and quickly homogenized in 5 ml of 3% aqueous sulfosalicylic acid by using a mortar and pestle. After centrifuging the homogenate for 10 mins at 4000g, 2 ml of the filtrate were combined with 2 ml glacial acetic acid and 2 ml acid-ninhydrin. The mixture was boiled at 100°C for 30 mins., then cooled in the ice bath, and extracted with 5 ml of toluene. A UV/visible spectrophotometer (UV-550; Jasco, Japan) was used to measure the absorbance of the aqueous upper layer at 520 nm, and toluene was used as a blank. A standard curve was used to determine the proline content on a fresh mass basis (μmol g^{-1} FM).

2.4.2. Measurement of lipid peroxidation

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) using 2-thiobarbituric acid assay procedure according to Abo Gamar et al. (2023) with some modifications. Three fresh leaf samples (50 mg) from three separate plants were collected from each treatment and fast frozen in liquid nitrogen and homogenized, by using a mortar and a pestle in a solution composed of 1.5 ml 0.5% 2-thiobarbituric acid and 1.5 ml 0.1% trichloroacetic acid. After centrifuging the homogenate at 4000g for 15 mins at 4°C, the supernatant was boiled for 10 mins and cooled on ice. A UV/visible spectrophotometer (UV-550; Jasco, Japan) was used to measure the absorbance at 532 nm and 600 nm using 1 ml of the supernatant. The 0.5% 2-thiobarbituric acid and 0.1% trichloroacetic acid were used as a blank. MDA content (nmol g^{-1} FM) was calculated by using the following formula: $[((A532 - A600) \times v) \times 1000]/(\varepsilon \times M)$. In the formula, 'ε' stands for specific extinction coefficient (= 155 mM^{-1} cm^{-1}), 'v' for the volume of extracting medium, 'M' for the leaf fresh mass, and 'A600' and 'A532' for absorbance at 600 and 532 nm wavelengths, respectively.

2.4.3. Measurement of membrane permeability

Membrane permeability was estimated by measuring the electrical conductivity using the method of Abo Gamar et al. (2023). Three leaf samples (100 mg) from three different plants were obtained from each treatment and washed with distilled water before being put in test tubes containing 15 ml of double distilled water and incubated at room temperature for 24 hr. The initial conductivity (C1) of the fresh tissue was measured with a Mettler Toledo MPC 227 equipped with Mettler Toledo Inlab 730 conductivity electrode. Samples then were boiled for 1 hr at 100°C before being allowed to cool to room temperature. The dead tissue's maximal conductivity (C2) was measured, and electrolyte leakage was computed as the percentage ratio of C1–C2.

2.4.4. Measurement of nitrogen balanced index (NBI), flavonoids and anthocyanins by dualex machine

Nitrogen balance index (NBI), chlorophyll, flavonoids, and anthocyanins measurements were taken from at least three leaves from separate plants in each treatment. Each measurement of optical absorbance was recorded for the values of the four components, which were taken using the Dualex Scientific® machine (Dualex Scientific, Force-A, Orsay Cedex, France).

2.5. Analysis of photosynthetic pigments

Chlorophyll (Chl) *a*, Chl *b*, carotenoids, total Chl and Chl *a:b* ratio were measured according to Hiscox and Israelstam (1979). From each treatment, three leaf samples (~ 50 mg) were harvested from three different plants and were incubated at room temperature in 5 ml of dimethylsulfoxide for 24 hr in the dark until the pigments were completely bleached. After that 1 ml of each solution was placed into a cuvette and measured for absorbance at 664 nm, 648 nm, 470 nm using a UV-visible spectrophotometer (UV-550; Jasco, Japan). Concentrations of Chl *a*, Chl *b*, carotenoids, total Chl and Chl *a:b* were calculated from the absorbencies measured (Chappelle *et al.,* 1992).

2.6. Data analysis

The effects of temperature, CO_2, watering regime, and their interactions on the CH_4 emission and growth from Cyprus faba bean plants were determined using a general linear model procedure. A three-way analysis of variance from this output was used to determine the differences between normal (20/17°C) or heat stress (30/26°C) at a well-watered (watered to field capacity) or drought-stressed (watered at leaf wilting point) state without supplying CO_2 or with supplying CO_2 (SAS Institute, 2011). A one-way ANOVA procedure was used to establish significant differences among each of the eight experimental treatments as well as among the three factors being manipulated, using Scheffé's multiple-comparison procedure at the 5% confidence level. For most of the parameters, three trials were used.

3. **Results**

3.1. Methane emissions

Methane emissions were greater in heat-stressed seedlings of faba bean than seedlings grown under normal temperatures (Table 1; Figure 1). However, less CH_4 emissions were detected when seedlings were grown under elevated CO_2 compared to those grown under ambient CO_2 (Table 1; Figure 1). Drought-stressed seedlings emitted more CH_4 than well-watered seedlings (Table 1; Figure 1). Temperature, CO_2, watering regime, the two–way interaction between temperature (T) × CO_2, CO_2 × watering regime (W), and the three–way interaction among T × CO_2 × W significantly affected CH_4 emissions (Table 2). The T × CO_2 interaction revealed that CH_4 emissions were significantly highest for seedlings grown under heat stress at ambient CO_2, but significantly lowest for seedlings grown under normal temperatures at elevated CO_2 (Figure 1). The CO_2 × W interaction showed that CH_4 emissions were significantly highest for drought-stressed seedlings at ambient CO_2, but significantly lowest for well-watered seedlings at elevated CO_2 (Figure 1). Based on the T × CO_2 × W interaction, CH_4 emissions were significantly highest for seedlings grown under heat, ambient CO_2 and drought-stressed, but significantly lowest for seedlings grown under normal temperatures, elevated CO_2 and well–watered (Figure 1).

Table 1. Individual effects of temperature, carbon dioxide and watering regime on methane emissions, growth, stress indicators, biochemical parameters and photosynthetic pigments of Cyprus faba beans (*Vicia faba* L.) seedlings.

	Temperature		Carbon dioxide		Watering regime	
Parameter	Normal	Heat	Ambient	Elevated	Well-watered	Drought-stressed
Methane (ng g^{-1} DM h^{-1})	33.06 ± 2.85B	48.34 ± 5.27A	48.59 ± 5.29A	32.81 ± 2.68B	31.46 ± 2.38B	49.93 ± 5.03A
Stem length (cm)	37.93 ± 3.11A	25.09 ± 1.53B	28.32 ± 2.93B	34.7 ± 3.02A	37.96 ± 3.03A	25.06 ± 1.67B
Stem diameter (mm)	0.47 ± 0.06A	0.30 ± 0.03B	0.33 ± 0.04B	0.44 ± 0.06A	0.49 ± 0.06A	0.28 ± 0.02B
Leaf area (cm^2 $plant^{-1}$)	159.6 ± 15.83A	122.01 ±15.1B	130.04 ± 16.1B	151.57 ± 6.21A	189.71 ± 7.56A	91.89 ± 7.12B
Leaf number ($plant^{-1}$)	10.7 ± 0.79A	7.54 ± 0.57B	8.36 ± 0.68B	9.89 ± 0.9A	10.87 ± 0.79A	7.38 ± 0.46B
Leaf moisture (%)	87.86 ± 2.14A	81.09 ± 2.93B	80.68 ± 2.57B	88.09 ± 2.47A	90.86 ± 1.56A	77.9 ± 2.26B
Root length (cm)	30 ± 3.64A	24.2 ± 1.59B	23.69 ± 2.83B	30.52 ± 2.69A	32.27 ± 2.96A	21.94 ± 1.93B
Stem fresh mass (g)	4.4 ± 0.52A	2.73 ± 0.3B	3.03 ± 0.47B	4.09 ± 0.47A	4.7 ± 0.46A	2.42 ± 0.22B
Leaf fresh mass (g)	3.54 ± 0.36A	2.36 ± 0.22B	2.67 ± 0.27B	3.23 ± 0.39A	3.77 ± 0.31A	2.13 ± 0.14B
Root fresh mass (g)	3.91 ± 0.43A	3.42 ± 0.42A	3.19 ± 0.44B	4.08 ± 0.38A	4.75 ± 0.29A	2.65 ± 0.21B
Total fresh biomass (g)	12.05 ± 1.28A	8.97 ± 0.74B	9.46 ± 1.05B	11.57 ± 1.14A	13.35 ± 1.0A	7.68 ± 0.39B
Stem dry mass (g)	3.6 ± 0.42A	2.15 ± 0.13B	2.56 ± 0.37B	3.19 ± 0.37A	3.59 ± 0.42A	2.17 ± 0.16B
Leaf dry mass (g)	3.02 ± 0.26A	1.73 ± 0.18B	2.16 ± 0.27A	2.59 ± 0.3A	2.86 ± 0.3A	1.88 ± 0.2B
Root dry mass (g)	3.34 ± 0.35A	3.39 ± 0.14A	3.17 ± 0.27A	4.08 ± 0.38A	3.92 ± 0.2A	2.8 ± 0.21B
Total dry biomass (g)	9.77 ± 0.97A	7.26 ± 0.36B	7.89 ± 0.82B	9.14 ± 0.78A	10.37 ± 0.79A	6.66 ± 0.32B
Leaf area ratio (LAR) (cm^2 g^{-1})	16.45 ± 0.62A	16.31 ± 1.21A	16.4 ± 0.52A	16.33 ±1.24A	18.19 ± 0.79A	14.52 ± 1.0B
Leaf mass area (LMA) (g m^{-2})	0.081 ± 0.008A	0.016 ±0.001B	0.018 ± 0.0017A	0.016 ± .0012A	0.014 ±0.0013A	0.019 ± 0.0015A
Leaf mass ratio (LMR)	0.29 ± 0.011A	0.23 ± 0.014B	0.27 ± 0.016A	0.26 ± 0.015A	0.27 ± 0.015A	0.25 ± 0.017A
Shoot:root dry wt. ratio	1.97 ± 0.1A	1.15 ± 0.08B	1.53 ± 0.16A	1.59 ± 0.15A	1.64 ± 0.15A	1.48 ± 0.15A
Proline (µmole g^{-1} FM)	35.2 ± 3.75B	72.38 ±5.52A	59.53 ± 8.99A	48.05 ± 4.53B	44.59 ± 5.97B	62.99 ± 7.61A
MDA (µmole g^{-1} FM)	0.31 ± 0.07A	0.18 ± 0.02A	0.29 ± 0.07A	0.2 ± 0.04A	0.16 ± 0.01B	0.33 ± 0.07A
Electrical conductivity (%)	16.9 ± 1.4B	31.4 ± 1.8A	27.5 ± 2.6A	20.9 ± 2.4B	20.8 ± 2.2B	27.7 ± 2.8A
Nitrogen balance index	89.92 ± 5.98A	80.67 ± 4.09B	86.73 ± 3.89A	83.22 ± 6.33A	98 ± 4.1A	71.95 ± 2.82B
Chlorophyll (µg cm^{-2} FM)	27.83 ± 1.37B	32.65±1.2A	28.37 ± 0.65B	32.11 ± 1.82A	27.73 ± 1.28A	23.75 ± 1.26B
Flavonoids (µg cm^{-2} FM)	0.36 ± 0.04B	0.44 ± 0.4A	0.34 ± 0.02B	0.46 ± 0.05A	0.3 ± 0.02B	0.5 ± 0.04A
Chl *a* (µg mg^{-1} FM)	2.1 ± 0.29A	2.45 ± 0.38A	2.04 ± 0.39A	2.51 ± 0.27A	2.83 ± 0.23A	1.72 ± 0.35B
Chl *b* (µg mg^{-1})	1.58 ± 0.22B	3.37 ± 0.26A	2.6 ± 0.38A	2.35 ± 0.34A	2.58 ± 0.34A	2.37 ± 0.38A
Carotenoids (µg mg^{-1} FM)	0.54 ± 0.08A	0.49 ± 0.05A	0.47 ± 0.06A	0.57 ± 0.07A	0.57 ± 0.07A	0.56 ± 0.08A
Total Chl (µg mg^{-1} FM)	3.68 ± 0.5B	5.82 ± 0.31A	4.46 ± 0.46A	4.86 ± 0.58A	4.86 ± 0.58A	4.09 ± 0.43B
Chl *a/b*	1.37 ± 0.09A	0.89 ± 0.16B	1.06 ± 0.18A	1.19 ± 0.1A	1.19 ± 0.1A	0.98 ± 0.17B

Cyprus faba bean seedlings were grown under normal temperatures (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions. Data are means ± SE of at least 9 samples from three different experiments. Means ± SE followed by different upper-case letters within rows and factors are significantly different ($P < 0.05$) according to Scheffé's test.

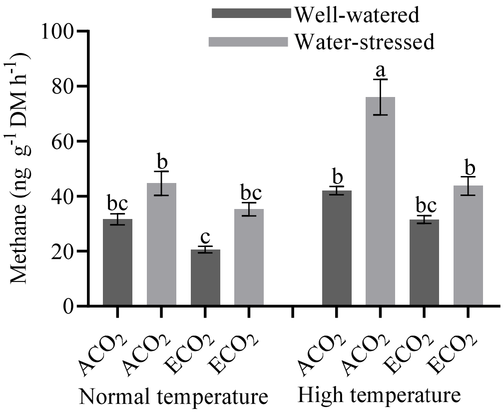

Figure 1. Methane emissions for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. *ACO_2* ambient CO_2, *ECO_2* elevated CO_2

Table 2. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on methane emissions from Cyprus faba bean (*Vicia faba* L.) seedlings.

		Methane (ng g^{-1} DM h^{-1})
Source	df	
Temperature (T)	1	48.99*
Main plot error	2	-
Carbon dioxide (CO_2)	1	61.49**
T x CO_2	1	7.73*
Subplot error	4	-
Watering regime (W)	1	71.91****
T x W	1	4.5
CO_2 x W	1	5.34*
T x CO_2 x W	1	7.15*
Split-subplot error	8	-

Note: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Cyprus faba bean seedlings were grown under normal temperatures (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions.

3.2. Plant growth

Elevated CO_2 increased stem length and diameter, leaf area and number, leaf moisture content and root length of seedlings, while heat and drought stresses decreased them in seedlings (Table 1; Figure 2A-F). Temperature, watering regime and the two–way interactions between T × W significantly affected stem height (Table 3). The T × W interaction revealed that seedlings grown under normal temperatures and well–watered were significantly the tallest, while those grown under heat and drought–stressed were significantly the shortest (Figure 2A). Temperature and watering regimes significantly affected stem diameter of the test plants (Table 3). Temperature, CO_2 and watering regimes significantly affected leaf area and number (Table 3). Moreover, leaf moisture content and root length were significantly affected by CO_2 and watering regimes (Table 3). Furthermore, root length was significantly affected by the two–way interaction between T × W (Table 3). The T × W interaction showed that roots of seedlings grown under normal temperatures and well–watered were significantly the tallest, while roots of seedlings grown under normal temperatures and drought–stressed were significantly the shortest (Figure 2F).

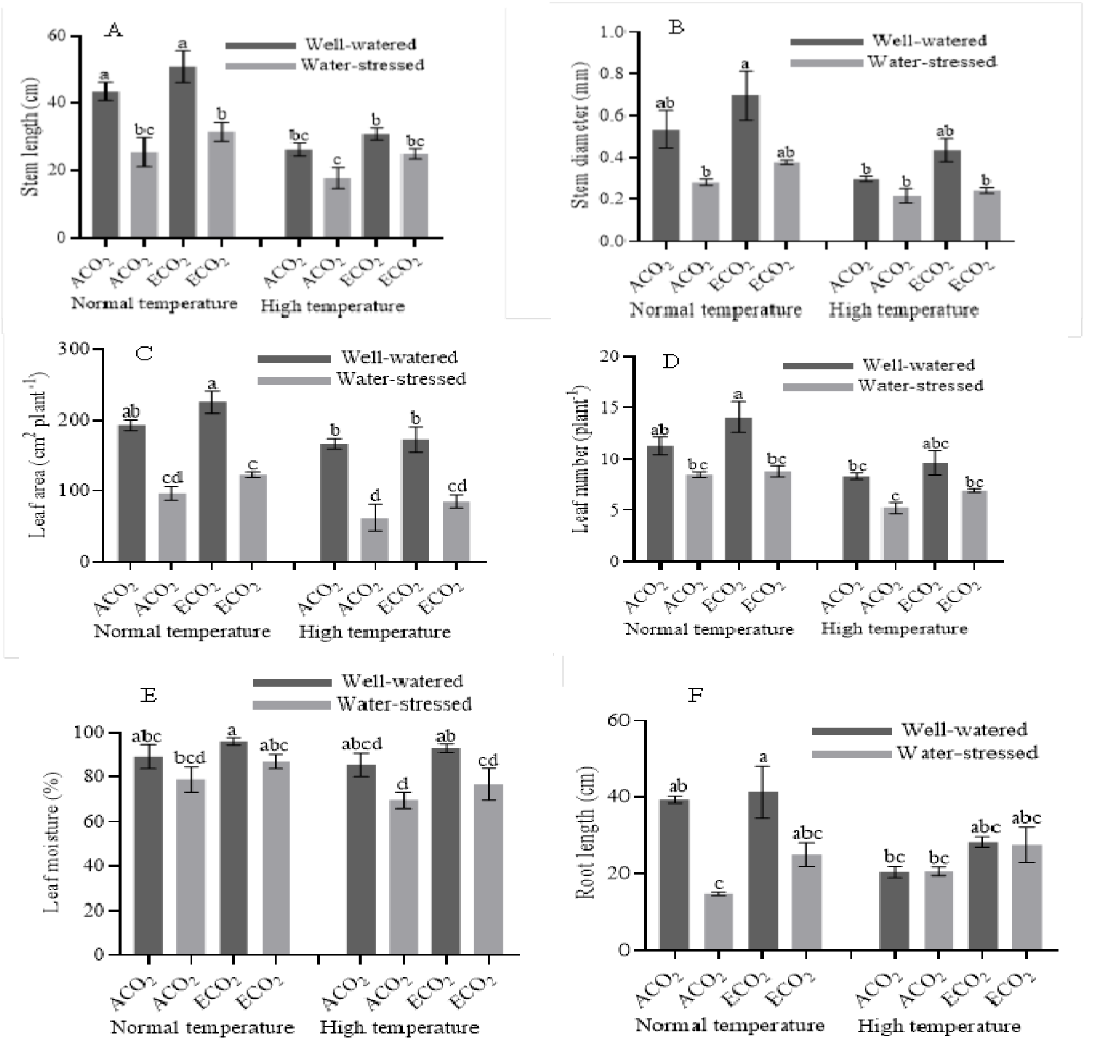

Figure 2. Stem length (A), stem diameter (B), leaf area (C), leaf number (D), leaf moisture content (E) and root length (F) for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. *ACO_2* ambient CO_2, *ECO_2* elevated CO_2

Table 3. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on stem length, stem diameter, leaf area, leaf number, moisture content and root length of Cyprus faba bean (*Vicia faba* L.) seedlings.

		Stem height (cm)	Stem diameter (mm)	Leaf area (cm^2 $plant^{-1}$)	Leaf number ($plant^{-1}$)	Moisture content (%)	Root length (cm)
Source	df						
Temperature (T)	1	1480.4***	57.92*	97.34*	25.07*	5.10	4.27
Main plot error	2	-	-	-	-	-	-
Carbon dioxide (CO_2)	1	7.31	6.30	53.75**	11.47*	15.44*	12.24*
T x CO_2	1	0.03	0.31	6.53	0.00	0.00	0.10
Subplot error	4	-	-	-	-	-	-
Watering regime (W)	1	388.21****	23.87**	234.66****	62.27****	51.29****	16.46**
T x W	1	73.85****	2.89	0.08	1.71	3.14	15.68**
CO_2 x W	1	0.22	1.08	0.19	1.46	0.01	0.52
T x CO_2 x W	1	2.23	0.07	0.67	2.48	0.06	0.81
Split-subplot error	8	-	-	-	-	-	-

Note: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Cyprus faba bean seedlings were grown under normal temperatures (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions.

3.3. Biomass accumulation

3.3.1. Fresh mass accumulation

Heat and drought stresses reduced stem (Table 1; Figure 3A), leaf (Table 1; Figure 3B), root (Table 1; Figure 3C) and total fresh biomass (Table 1; Figure 3D), but elevated CO_2 increased them (Table 1; Figure 3A-D). Stem, leaf, and total fresh biomasses were significantly affected by temperature, carbon dioxide, watering regime and by the two–way interaction between T × W (Table 4). Root fresh mass was only significantly affected by watering regime (Table 4). For the T × W interaction, well-watered seedlings grown under normal temperatures had significantly the highest stem, leaf and total fresh biomass, whereas drought-stressed seedlings grown under heat stress had significantly the lowest stem, leaf and total fresh biomass (Figure 3A,B,D).

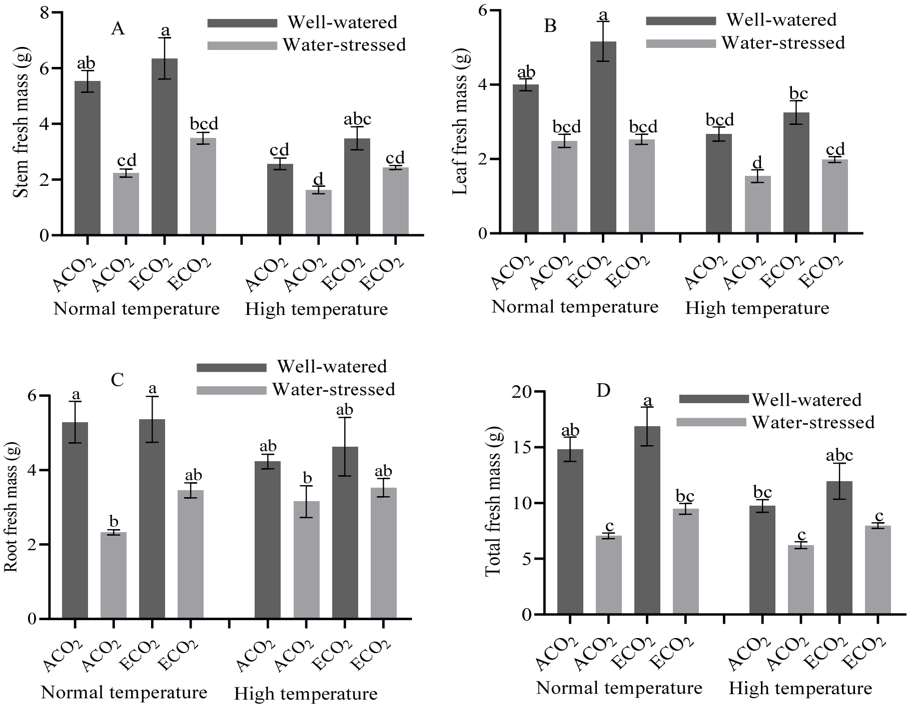

Figure 3. Stem fresh mass (A), leaf fresh mass (B), root fresh mass (C) and total fresh mass (D) for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. *ACO_2* ambient CO_2, *ECO_2* elevated CO_2.

Table 4. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on stem fresh mass, leaf fresh mass, root fresh mass and total fresh biomass of Cyprus faba bean (*Vicia faba* L.) seedlings.

		Stem fresh mass (g)	Leaf fresh mass (g)	Root fresh mass (g)	Total fresh biomass (g)
Source	df				
Temperature (T)	1	71.77*	66.63*	6.29	107.65**
Main plot error	2	-	-	-	-
Carbon dioxide (CO_2)	1	25.13**	14.94*	7.11	26.36**
T x CO_2	1	0.02	0.09	0.12	0.1
Subplot error	4	-	-	-	-
Watering regime (W)	1	91.29****	114.72****	39.21***	93.43****
T x W	1	11.54**	8.12*	1.03	10.67*
CO_2 x W	1	0.02	4.2	0.00	0.00
T x CO_2 x W	1	0.6	2.64	0.00	0.12
Split-subplot error	8	-	-	-	-

Note: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Cyprus faba bean seedlings were grown under normal temperatures (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 and growth period of 5 days under greenhouse conditions.

3.3.2. Dry mass accumulation

Seedlings grown under heat stress had lower stem, leaf and total dry biomass than seedlings grown under normal temperatures (Table 1; Figure 4A,B,D). Seedlings grown under elevated CO_2 had higher stem and total dry biomass than those grown under ambient CO_2 (Table 1; Figure 4A,D).

Drought-stressed seedlings had lower stem, leaf, root and total dry biomass than well-watered seedlings (Table 1; Figure 4A,B,C,D). Stem dry mass was significantly affected by temperature, carbon dioxide, watering regime and the two–way interactions between T × W (Table 5). Leaf dry mass was significantly affected by temperature and watering regime (Table 5). Root dry mass was significantly affected by watering regime and the two–way interactions between T × W (Table 5). Total dry biomass was significantly affected by temperature, carbon dioxide, watering regime and the two–way interaction between T × W (Table 5). The T × W interaction revealed that seedlings grown under normal temperatures and well–watered had significantly the highest stem and total dry biomass, whereas seedlings grown under heat and drought–stressed had significantly the lowest stem and total dry biomass (Figure 4A,D). The T × W interaction showed that seedlings grown under normal temperatures and well–watered had significantly the highest root dry mass, while those grown under normal temperatures and drought–stressed had significantly the lowest root dry mass (Figure 4C).

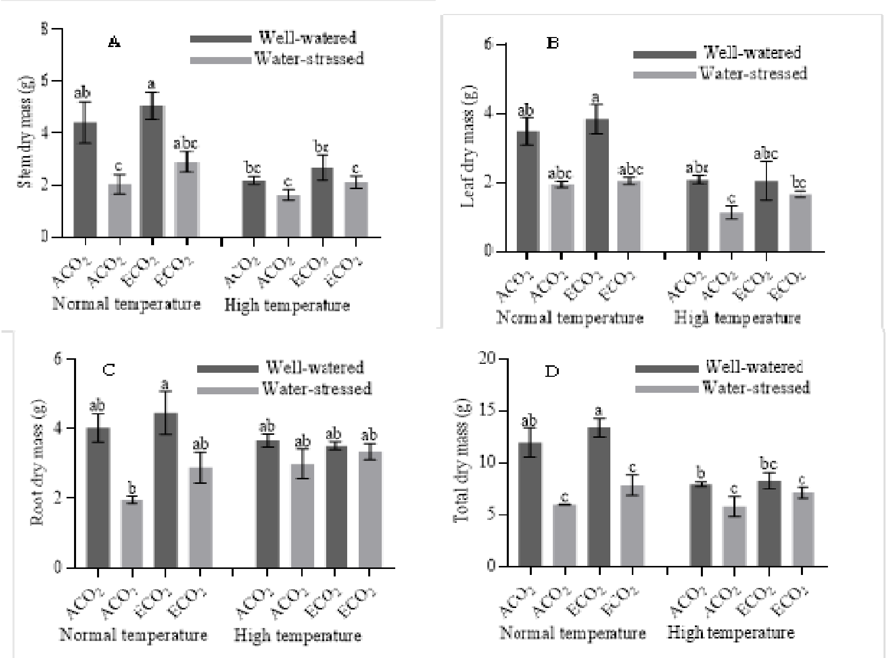

Figure 4. Stem dry mass (A), leaf dry mass (B), root dry mass (C) and total dry mass (D) for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. ACO_2 ambient CO_2, ECO_2 elevated CO_2.

Table 5. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on stem dry mass, leaf dry mass, root dry mass and total dry biomass of Cyprus faba bean (*Vicia faba* L.) seedlings.

		Stem dry mass (g)	Leaf dry mass (g)	Root dry mass(g)	Total dry biomass (g)
Source	df				
Temperature (T)	1	19.07*	18.79*	0.03	27.23*
Main plot error	2	-	-	-	-
Carbon dioxide (CO_2)	1	28.36**	2.28	3.30	11.09*
T x CO_2	1	1.25	0.42	1.82	1.2
Subplot error	4	-	-	-	-
Watering regime (W)	1	37.07***	22.39**	21.12**	72.5****
T x W	1	13.54**	2.21	8.2*	22.27**
CO_2 x W	1	0.05	1.69	1.63	0.74
T x CO_2 x W	1	0.06	0.00	0.00	0.12
Split-subplot error	8	-	-	-	-

Note: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Cyprus faba bean seedlings were grown under normal (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions.

3.4. Growth indices

Seedlings grown under heat stress had lower leaf mass area (LMA), leaf mass ratio (LMR) and shoot:root dry wt. ratio than seedlings grown under normal temperatures (Table 1; Figure 5B,C,D). Drought-stressed seedlings had lower leaf area ratio (LAR) than well-watered seedlings (Table 1; Figure 5A). LAR was only significantly affected by watering regime (Table 6). Shoot:root dry wt. ratio was only significantly affected by temperature (Table 6). None of the interactions among the three main factors significantly affected any of the measured growth indices (Table 6).

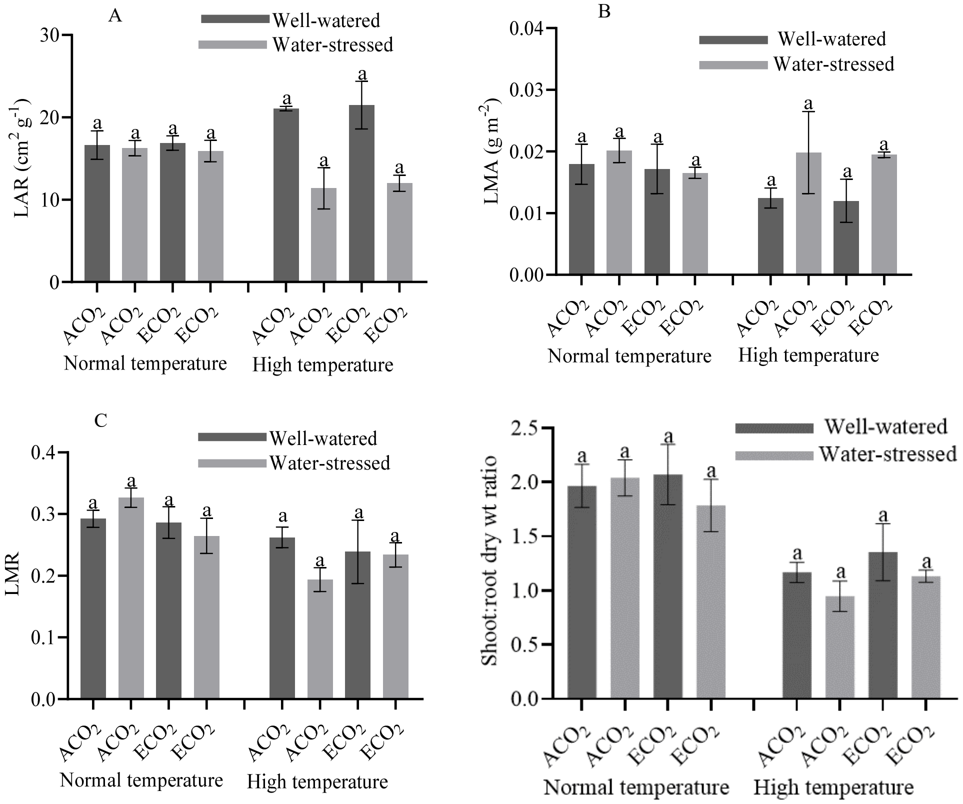

Figure 5. Growth indices for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. LAR (A), LMA (B), LMR (C) and shoot:root dry wt ratio (D). *LAR* leaf area ratio, *LMA* leaf mass area, *LMR* leaf mass ratio, *ACO_2* ambient CO_2, *ECO_2* elevated CO_2.

Table 6. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on leaf area ratio, leaf mass area, leaf mass ratio and shoot:root dry wt. ratio of Cyprus faba bean (*Vicia faba* L.) seedlings.

		Leaf area ratio($cm^2\ g^{-1}$)	Leaf mass area ($g\ m^{-2}$)	Leaf mass ratio	Shoot:root dry wt. ratio
Source	df				
Temperature (T)	1	0.00	0.61	7.92	202.78**
Main plot error	2	-	-	-	-
Carbon dioxide (CO_2)	1	0.08	0.76	0.98	0.15
T x C	1	0.11	0.35	2.66	0.84
Subplot error	4	-	-	-	-
Watering regime (W)	1	15.6**	3.24	0.58	1.38
T x W	1	11.81	2.11	1.15	0.17
C x W	1	0.01	0.09	0.01	0.42
T x C x W	1	0.02	0.11	2.2	0.41
Split-subplot error	8	-	-	-	-

Note: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Cyprus faba bean seedlings were grown under normal (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions.

3.5. Proline, lipid peroxidation and electrical conductivity

Proline content was increased by heat and drought stresses, but decreased by elevated CO_2 in faba bean seedlings (Table 1; Figure 6A). Significant effects of temperature, carbon dioxide, watering regime and the two–way interactions between T × CO_2 and CO_2 × W were observed on proline content (Table 4). For the T × CO_2 interaction, seedlings grown under heat stress and ambient

CO_2 had significantly the highest proline content, while those grown under normal temperatures and ambient CO_2 had significantly the lowest proline content (Figure 6A). The CO_2 × W interaction showed that seedlings grown under ambient CO_2 and drought-stressed had significantly the highest proline content, whereas seedlings grown under ambient CO_2 and well-watered had significantly the lowest proline content (Figure 6A).

Malondialdehyde (MDA) generation increased under drought-stressed condition in faba bean seedlings (Table 1; Figure 6B). MDA generation was significantly affected by carbon dioxide and watering regime (Table 7). None of the interactions among the three main factors had significant effect on the MDA content (Table 7).

Electrical conductivity was increased by heat and drought stresses, but decreased by elevated CO_2 in faba bean seedlings (Table 1; Figure 6C). Effects of temperature, carbon dioxide, watering regime, the two–way interactions between T × CO_2, T × W and the three–way interaction among T × CO_2 × W were significant on the electrical conductivity (Table 7). The T × CO_2 interaction revealed that seedlings grown under heat stress at ambient CO_2 resulted in significantly the highest electrical conductivity, but normal temperatures at ambient CO_2 resulted in significantly the least electrical conductivity in seedlings (Figure 6C). The T × W interaction showed that heat and drought–stressed seedlings had significantly the highest electrical conductivity, whereas those grown under normal temperatures and well-watered conditions had significantly the lowest electrical conductivity (Figure 6C). On the basis of the T × CO_2 × W interactions, seedlings grown under heat stress, ambient CO_2, and drought–stressed conditions had significantly the highest electrical conductivity, whereas those grown under normal temperatures, ambient CO_2 and well-watered conditions had significantly the lowest electrical conductivity (Figure 6C).

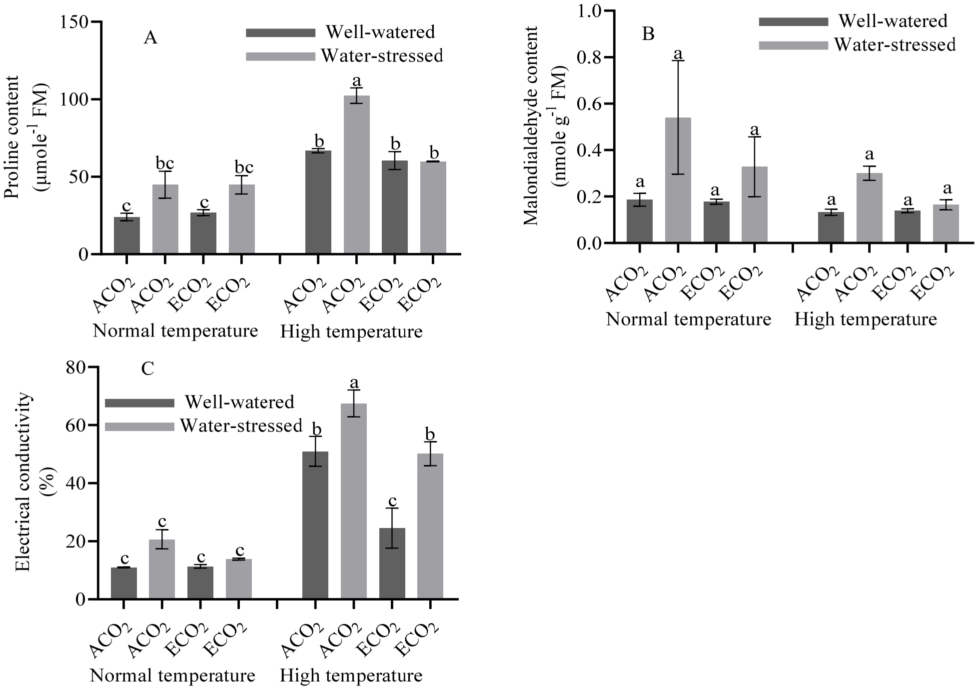

Figure 6. Proline content (A), Malondialdehyde content (B) and electrical conductivity (C) for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. *ACO_2* ambient CO_2, *ECO_2* elevated CO_2.

3.6. Nitrogen balance index (NBI), chlorophyll and flavonoids

NBI decreased under heat and drought stress conditions in faba bean seedlings (Table 1; Figure 7A). NBI was significantly affected by temperature and watering regime, but not significantly affected by the interactions among the three main factors (Table 7).

Chlorophyll content was significantly increased by heat stress and elevated CO_2, but drought stress increased it (Table 1; Figure 7B). Chlorophyll content was significantly affected by all the main factors and the two–way interactions between T × CO_2, T × W and CO_2 × W (Table 7). The T × CO_2 interaction showed that elevated CO_2 caused highest chlorophyll content for seedlings grown under heat stress, but elevated CO_2 caused lowest chlorophyll content for seedlings grown under normal temperatures (Figure 7B). The T × W interaction revealed that seedlings grown under heat stress and drought-stressed had the highest chlorophyll content, while those grown under normal temperatures and well-watered had the lowest chlorophyll content (Figure 7B). The CO_2 × W interaction showed that drought-stressed seedlings grown under elevated CO_2 had the highest chlorophyll content, whereas well-watered seedlings grown under elevated CO_2 had the lowest chlorophyll content (Figure 7B).

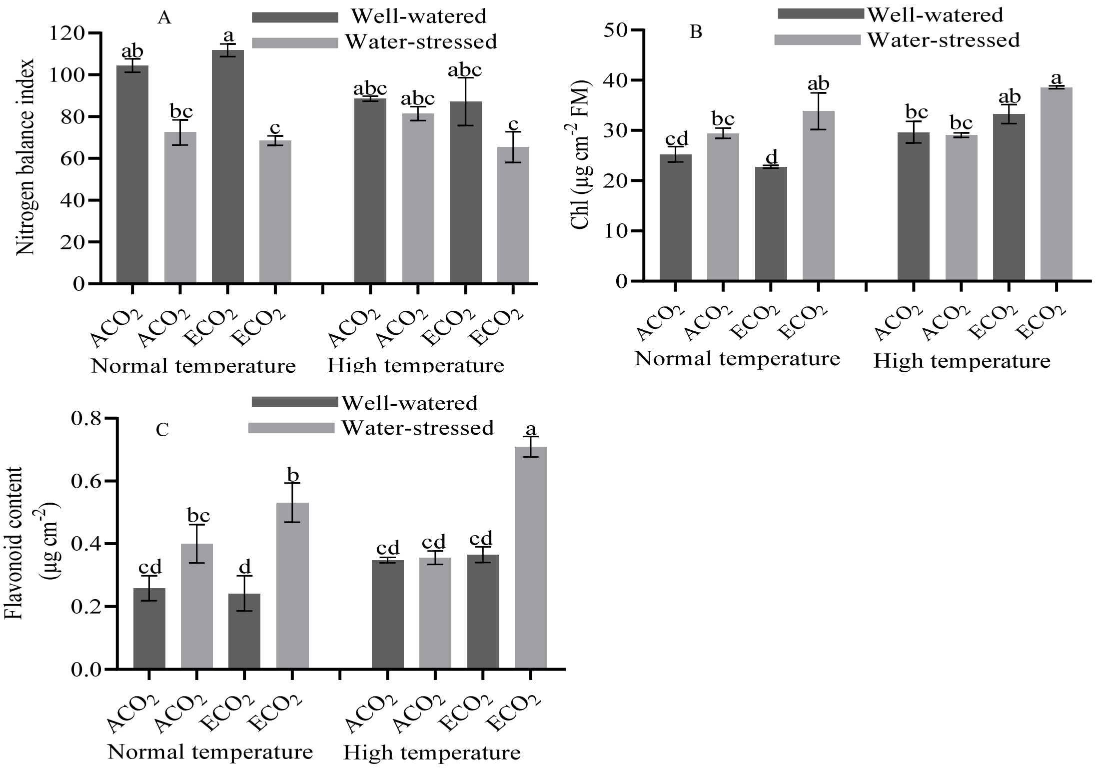

Figure 7. NBI (A), chlorophyll content (B) and flavonoid content (C) measured by Dualex machine for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. *ACO_2* ambient CO_2, *ECO_2* elevated CO_2.

Table 7. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on proline content, MDA content, electrical conductivity, NBI, chlorophyll and flavonoids of Cyprus faba bean (*Vicia faba* L.) seedlings.

		Proline content (µmole g $^{-1}$ FM)	MDA content (µmole g $^{-1}$ FM)	Electrical conductivity (%)	NBI	Chlorophyll (µg cm^{-2} FM)	Flavonoids (µg cm^{-2} FM)
Source	df						
Temperature (T)	1	192.82**	1.91	272.05**	35.84*	21.84*	18.23
Main plot error	2	-	-	-	-	-	-
Carbon dioxide (CO_2)	1	14.59*	10.70*	62.01**	1.52	55.55**	79.15***
T x CO_2	1	18.25*	0.73	34.18**	3.37	31.2**	22.07**
Subplot error	4	-	-	-	-	-	-
Watering regime (W)	1	24.33**	5.41*	75.92****	25.82**	45.26***	101.69****
T x W	1	0.07	1.06	23.13**	5.11	12.33**	1.05
CO_2 x W	1	6.77*	1.33	0.09	1.59	18.41**	39.08***
T x CO_2 x W	1	5.01	0.04	6.91*	0.03	0.11	5.92*
Split-subplot error	8	-	-	-	-	-	-

Note: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Cyprus faba bean seedlings were grown under normal (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions. NBI, chlorophyll content and flavonoid content were measured by Dualex machine.

3.7 Photosynthetic pigments

Overall, heat stress increased Chl *b* and total Chl, but decreased Chl *a:b* ratio (Table 1; Figure 8). Drought stress decreased Chl *a*, total Chl and Chl *a:b* ratio (Table 2; Figure 8).

Chl *a* content was significantly affected by carbon dioxide, watering regime, the two–way interactions of T × CO_2, T × W, CO_2 × W and the three–way interaction of T × CO_2 × W (Table 8). For the T × CO_2 interaction, seedlings grown under heat stress and ambient CO_2 had significantly the highest and lowest Chl *a* content (Figure 8A). The T × W interaction revealed that seedlings grown under heat stress and well–watered had significantly the highest Chl *a* content, while those grown under heat stress and drought-stressed had significantly the lowest Chl *a* content (Figure 8A). The CO_2 × W interaction showed that seedlings grown under ambient CO_2 and well-watered had significantly the highest Chl *a* content, whereas seedlings grown under ambient CO_2 and drought-stressed had significantly the lowest Chl *a* content (Figure 8A). Based on the T × CO_2 × W interaction, seedlings grown under heat stress, ambient CO_2 and well-watered conditions had significantly the highest Chl *a* content, whereas seedlings grown under heat stress, ambient CO_2 and drought-stressed conditions had significantly the lowest Chl *a* content (Figure 8A).

Chl *b* content was significantly affected by the two–way interaction of CO_2 × W and the three–way interaction of T × CO_2 × W (Table 8). For the CO_2 × W interaction, seedlings grown under ambient CO_2 and drought-stressed had significantly the highest Chl *b* content, whereas seedlings grown under elevated CO_2 and drought-stressed had significantly the lowest Chl *b* content (Figure 8B). On the basis of the T × CO_2 × W interaction, seedlings grown under heat stress, ambient CO_2 and drought-stressed conditions had significantly the highest Chl *b* content, whereas seedlings grown under normal temperatures, elevated CO_2 and drought-stressed conditions had significantly the lowest Chl *b* content (Figure 8B).

Carotenoid content was significantly affected by the carbon dioxide and the two–way interaction of CO_2 × W (Table 8). The CO_2 × W interaction showed that seedlings grown under elevated CO_2 and drought-stressed had significantly the highest carotenoid content, while seedlings grown under ambient CO_2 and drought-stressed had significantly the lowest carotenoid content (Figure 8C).

Total Chl was significantly affected by watering regime, but not significantly affected by the interactions among the three main factors (Table 8).

Chl *a*:*b* ratio was significantly affected by the three main factors, the two–way interactions of T × CO_2, CO_2 × W and the three–way interaction of T × CO_2 × W (Table 8). The T × CO_2 interaction indicated that seedlings grown under normal temperatures and elevated CO_2 had significantly the highest Chl *a*:*b* ratio, while those grown under heat stress and ambient CO_2 had significantly the lowest Chl *a*:*b* ratio (Figure 8E). The CO_2 × W interaction revealed that seedlings grown under elevated CO_2 and well-watered had significantly the highest Chl *a*:*b* ratio, while those grown ambient CO_2 and drought-stressed had significantly the lowest Chl *a*:*b* ratio (Figure 8E). Based on the interactions among T × CO_2 × W, seedlings grown under normal temperatures, elevated CO_2 and well-watered conditions had significantly the highest Chl *a*:*b* ratio, whereas seedlings grown under heat stress, ambient CO_2 and drought-stressed conditions had significantly the lowest Chl *a*:*b* ratio (Figure 8E).

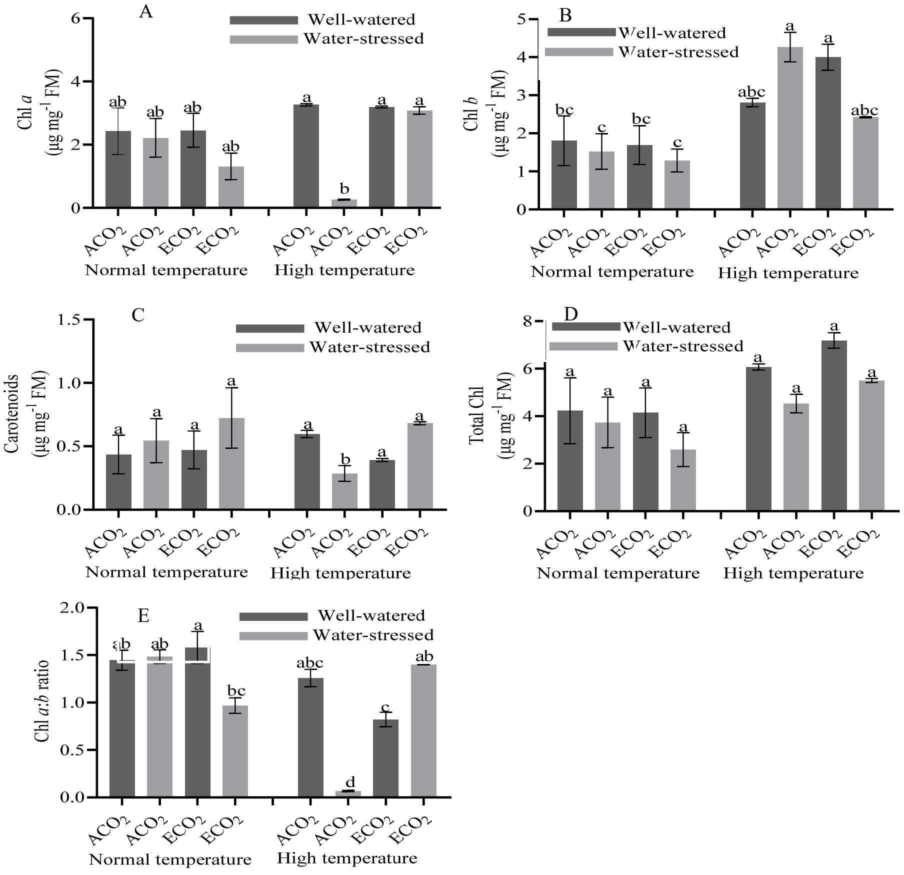

Figure 8. Chl *a* (A), Chl *b* (B), carotenoids (C), total Chl (D) and Chl *a:b* ratio (E) for 26-day-old Cyprus faba bean (*Vicia faba* L.) seedlings grown in controlled-environment glass cabinets for 14 days under eight experimental conditions including two temperature regimes, two CO_2 concentrations and two watering regimes after 12 days of initial germination and growth under greenhouse conditions. *ACO_2* ambient CO_2, *ECO_2* elevated CO_2.

Table 8. Analysis of variance for the individual effects of temperature, carbon dioxide and watering regime and their interactions on Chl *a*, Chl *b*, carotenoids, total Chl and Chl *a/b* ratio of Cyprus faba bean (*Vicia faba* L.) seedlings.

Source	df	Chl *a* (μg mg^{-1} FM)	Chl *b* (μg mg^{-1} FM)	Carotenoids (μg mg^{-1} FM)	Total Chl (μg mg^{-1} FM)	Chl *a:b* ratio
Temperature (T)	1	0.36	14.92	0.10	4.25	29.18*
Main plot error	2	-	-	-	-	-
Carbon dioxide (CO_2)	1	18.55*	2.25	8.34*	0.98	45.14**
T x CO_2	1	69.61**	0.20	0.03	14.45	277.79****
Subplot error	4	-	-	-	-	-
Watering regime (W)	1	444.23****	1.14	2.69	32.89***	17.64**
T x W	1	69.43****	0.56	3.39	1.71	0.02
CO_2 x W	1	85.03****	17.38**	12.91**	1.70	15.62**
T x CO_2 x W	1	325.7****	14.72**	4.87	1.00	73.01****
Split-subplot error	8	-	-	-	-	-

Note: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Cyprus faba bean seedlings were grown under normal (20/17ºC) or heat stress (30/26ºC), ambient CO_2 (400 ppm) or elevated CO_2 (800 ppm) and well-watered or drought-stressed conditions. Plants were grown under experimental conditions in controlled-environment glass cabinets for 14 days, following an initial germination period of 7 days and growth period of 5 days under greenhouse conditions.

4. Discussion

Results from this study show that heat stress significantly increased CH_4 emissions, proline content, electrolyte leakage, Chl content measured by Dualex Scientific, flavonoids, Chl *b* and total Chl, but decreased plant growth parameters, leaf moisture content, fresh and dry mass accumulation except for root fresh and dry mass, LMA, LMR, shoot:root dry wt ratio, NBI and Chl *a:b* ratio in seedlings (Table 1).

In line with our results, heat stress enhanced CH_4 emissions (Table 1) from faba bean seedlings (Gautam *et al.,* 2024). The increase of proline content in seedlings grown under heat stress (Table 1) is inconsistent with previous study on Giza faba bean (Abo Gamar *et al.,* 2023) and other plant species (Jiang *et al.,* 2023). The increase in the electrolyte leakage under heat stress indicates the inability of plants to prevent oxidative damage of their cell membranes (Alamri *et al.,* 2014). Chlorophyll measured by Dualex Scientific, Chl *b*, and total Chl was significantly increased under heat stress (Table 1), which was contrary to expectations. However, Chl *a* has been suggested as precursor of CH4 in phytoplanktons (Ordóñez *et al.,* 2023). Since heat stress increases the level of reactive oxygen species (ROS) within the leaves, increased flavonoids (Table 1) is likely both a method of protection against oxidative damage as well as a mechanism to induce abiotic stress tolerance in faba bean seedlings (Ernst *et al.,* 2022). The reduction in growth parameters and leaf moisture content (Table 1) because of heat stress led to biomass decrease of different plant parts and is consistent with earlier studies on cucumber (*Cucumis sativus* L.) (Hongal *et al.,* 2023). Furthermore, heat stress might have also decreased growth in faba bean seedlings through reducing photosynthesis via the inactivation of the Rubisco (ribulose-1,5-bisphosphate carboxylase/ oxygenase) enzyme (Way and Oren, 2010). Reduced LMA and LMR caused by heat stress (Table 1) could result from the production of smaller and thicker leaves, which might be an adaptation to decrease loss of water from leaves of stressed seedlings. Moreover, reduction in shoot:root dry wt ratio (Table 1) could be another mechanism of adaptation to help stressed seedlings to manage their water balance and survive under heat stress condition. NBI is a patented calculation that estimates the levels of nitrogen nutrition by establishing a ratio of chlorophyll to flavonoids. A lower NBI under heat stress (Table 1) indicates alterations to the allocation of carbon/nitrogen in leaves to produce more flavonoids, which is consistent with the increase in flavonoid content in our plants. Heat stress was shown to increase the chlorophyll a/b contents (Zahra *et al.,* 2023), which is in agreement with our results.

Elevated CO_2 significantly increased plant growth parameters and leaf moisture content, fresh and dry mass accumulation, chlorophyll measured by Dualex Scientific, flavonoids, but decreased CH_4 emissions, proline content and electrolyte leakage in seedlings (Table 1). It has been well acknowledged that elevated CO_2 enhances growth and biomass of plants by increasing the photosynthesis rate of leaves and water use efficiency, and decreasing the transpiration rate (Wei *et al.,* 2022). Increase of chlorophyll content (Table 1) in seedlings grown under elevated CO_2 is inconsistent with previous studies on basil (*Ocimum basilicum* L.) (Barickman *et al.,* 2022). The increase in flavonoid content (Table1) under elevated CO_2 indicated the increase of seedling abilities to reallocate the extra nitrogen, carbon and other crucial materials away from the photosynthetic machinery to synthesize antioxidant-defense materials, such as flavonoids. Moreover, it has been shown that elevated CO_2 concentration mitigates stress-induced emissions of CH_4 from plants by preventing photorespiration (Mattoo and B M, 2023), which has been suggested as one of the main causes for CH_4 emissions from plants under stress conditions. In our study, elevated CO_2 reduced proline content (Table 1), which is in agreement with previous studies on faba bean (*Vicia faba* L.) (Abo Gamar *et al.,* 2023) and maize (*Zea mays* L.) (Vanaja *et al.,* 2024). Seedlings grown under elevated CO_2 had lower electrolyte leakage (Table 1), which indicates that CO_2 protect cell membranes by alleviating oxidative stress enhanced by stress factors (Shabbaj *et al.,* 2021).

Drought stress increased CH_4 emissions, proline, MDA content, electrolyte leakage, and flavonoids, but decreased plant growth parameters and leaf moisture content, fresh and dry mass accumulation, LAR, NBI, chlorophyll measured by Dualex Scientific, Chl *a*, total Chl and Chl *a/b* ratio in seedlings (Table 1). Our results showed that drought stress enhanced plant CH_4 emissions (Table 1), which supports previous results that drought-stressed plants emit more CH_4 than well-watered plants (Zhou et al., 2021). CH_4 has been shown to reduce osmotic stress induced on mung bean (*Vigna radiata* L.) plants by polyethyleneglycol (PEG), through regulating the status of reactive oxygen species (ROS), which enhance homeostasis of sugar, ascorbic acid, and glutathione (Zhang *et al.,* 2018). Drought stress enhanced proline content in faba bean seedlings (Table 1). Proline has been found to have a role in protecting and stabilizing different antioxidant enzymes, such as superoxide dismutase (Khatun *et al.,* 2020). Reactive oxygen species has been found to enhance the MDA content in drought-stressed plants, which might be led to membrane injury (Hassan *et al.,* 2020) and increasing electrical conductivity in tested plants. An increase of flavonoids in drought-stressed seedlings (Table 1) shows an increase in antioxidant contents. Because drought stress increases the level of ROS within the leaves, increased flavonoids is likely both a method of protection against oxidative damage as well as a mechanism to induce abiotic stress tolerance by reducing photorespiration (Bita and Gerats, 2013) and, in turn, reducing CH_4 emissions. Reduction in the growth and biomass of drought-stressed seedlings (Table 1) is expected and in agreement with findings on faba bean (*Vicia faba* L.) (Kibbou *et al.,* 2022; Abo Gamar *et al.,* 2023). Decreased LAR in drought-stressed seedlings (Table 1) may have contributed to the reduced biomass. A significant reduction in nitrogen balance index (NBI) supports the significant changes in flavonoids, chlorophyll measured by Dualex Scientific, Chl *a* and Total Chl (Table 1), as a decreased NBI suggests that nitrogen fertility is low, and the plant is allocating resources to regenerating flavonoids as opposed to making chlorophyll (Tremblay *et al.,* 2012). This could contribute to the decrease in net photosynthesis of drought-stressed faba bean seedlings and, in turn, reduction in their growth and biomass. The reduction in the Chl *a/b* ratio (Table 1) in drought-stressed seedlings could be explained by the decrease of Chl *a* and the increase of Chl *b*.

Climate change is inevitable within our lifetime, even if the production of greenhouse gases is stopped today (IPCC 2013). In this study, from 33 cases of interactions, 26 of two-way (see Tables 2, 3, 4, 5, 7, and 8) and 7 of three-way interactions (see Tables 2, 7, and 8) were significant. Plant parameters were significantly affected by T × CO_2 in 7 cases (see Tables 2, 7 and 8), by T × W in 11 cases (see Tables 3, 4, 5, 7, and 8), by CO_2 × W in 8 cases (see Tables 2, 7 and 8), and by T × CO_2 × W in 7 cases (see Tables 2, 7 and 8).

In agricultural systems, heat and drought stresses, which result in more methane emissions and smaller plants, can have large impacts on crop performance and yield. Elevated CO_2 mitigated many of the negative effects of increased temperature and drought stress on plants. Thus, it is important to study the individual effects of the main climate change factors (temperature, CO_2 and watering regime) along with their combined effects on methane emissions and growth of plants.

The interaction of all three factors (T × CO_2 × W) mainly had significant effects on CH_4 emissions (Table 2), electrolyte leakage (Table 7), flavonoids (Table 7), Chl *a*, Chl *b* and Chl *a/b* ratio (Table8.). CH_4 emissions were significantly highest for seedlings grown under heat stress, ambient CO_2 and drought-stressed, but significantly lowest for seedlings grown under normal temperatures, elevated CO_2 and well–watered (Table 2; Figure 1). It has been suggested that CH_4 could enhance the resistance of plants to different types of abiotic stress by supporting their antioxidant defense system (Gautam *et al.,* 2024). Moreover, it was shown that CH_4 emissions from plants have a major role in protecting them from ROS, functioning as an important signaling molecule, and controlling multiple stress associated genes in plants that influence their growth and development (Gautam *et al.,* 2024).

Seedlings grown under heat stress, ambient CO_2, and water–stressed conditions had significantly the highest electrical conductivity, whereas those grown under normal temperatures, ambient CO_2 and well-watered conditions had significantly the lowest electrical conductivity (Table 7; Figure 6C), which agrees with earlier study on faba bean (*Vicia faba* L.) (Abo Gamar *et al.,* 2023). Flavonoid content was significantly highest for seedlings grown under heat stress, elevated CO_2 and drought-stressed, but significantly lowest for seedlings grown under normal temperature, elevated CO_2 and well–watered (Table 7; Figure 7C). Total antioxidant capacity was shown to significantly enhanced by the combination of heat and drought stresses, and to a higher extent at elevated CO_2 (Zinta *et al.,* 2018). More flavonoid content in seedlings grown under heat and drought stresses, individually and together, indicated increased antioxidant capacity. Because the combination of heat and drought stresses increases the concentration of ROS in plant leaves, increased flavonoids may be protecting plants against being damaged by oxidation (Tourky *et al.,* 2023). Our study revealed that the combination between heat and drought stresses decreased Chl *a* and Chl *a:b* ratio, but increased Chl *b* (Table 8; Figure 8). On the other hand, elevated CO_2 didn't affect Chl *a* under the combination of stress factors and negatively affected Chl *b* under drought stress (Table 8; Figure 8). It is clear, from these results, that the combination of heat and drought stresses had antagonistic effects with elevated CO_2 on photosynthetic pigments, and that elevated CO_2 had negative effects on photosynthetic pigments. It is possible that the variations in photosynthetic pigments, particularly in Chl *a*, could have affected gas exchange and, consecutively, affected faba bean seedlings growth and biomass.

5. Conclusions

Studies focusing on addressing the effects of an individual environmental factor on methane emissions from plants and their growth and development may not give a correct or truthful scenario of the response of plants to future climates. It is important to study the combination of as many environmental components as possible, with superior focus being employed on those environmental factors (heat, elevated CO_2 and drought stress) that have the potential to change under the future climate change scenario. Our results showed that these three factors may modify CH_4 emissions from plant bodies and imposed some consequences for future climate. Heat and drought stresses caused seedlings to emit more CH_4 at present atmospheric CO_2. Elevated CO_2 decreased aerobic CH_4 emissions, but cannot completely inverse the enhancement effects of heat and drought stresses on CH_4 emissions from plants. Moreover, elevated CO_2 antagonize stress effects of heat and drought stresses on seedlings mainly by enhancing their antioxidant activities through increasing flavonoid and improving plant chlorophyll and water contents.

Regardless of the small input of CH_4 emissions to the global CH_4 budget from plants, environmental stressors will proliferate in the upcoming days, resulting in an increased influence of this source of CH_4 to the worldwide budget.

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Conflicts of Interest

Authors declare no conflict of interest.

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