Physiological responses to ultra-high CO_2 levels in an evergreen tree species

Ben-El Levy[1], Yedidya Ben-Eliyahu[1], Yaniv-Brian Grunstein[1], Itay Halevy[2], Tamir Klein[1]

[1] Department of Plant and Environmental Sciences, Weizmann Institute of Science, 76100 Rehovot, Israel

5   [2] Department of Earth and Planetary Sciences, Weizmann Institute of Science, 76100 Rehovot, Israel

Corresponding author: Tamir Klein, Department of Plant and Environmental Sciences, Weizmann Institute of Science, 76100 Rehovot, Israel. ORCID: 0000-0002-3882-8845. Tamir.klein@weizmann.ac.il

10   **Abstract**

Although numerous experiments have been dedicated to studying plant response to elevated CO_2, almost none crossed the level of 1000 ppm. Plant responses to high CO_2 levels importantly inform our understanding of plant physiology in ultra-high CO_2 environments, e.g., in Earth history, in the case of unmitigated anthropogenic emissions, and for future colonization of Mars.

15   Here, we challenged two-year old seedlings of fruit trees grown in soil in a mesocosm, with CO_2 levels of 400, 1600 and 6000 ppm, the highest of which is approximately equivalent to that of Mars' atmosphere. Plant growth, and leaf gas exchange (transpiration, stomatal conductance, and CO_2 assimilation) were measured on a weekly basis for 23-25 consecutive days. We hypothesized that elevated CO_2 levels will induce a decrease in transpiration, primarily attributed to reduced stomatal conductance. Indeed, leaf transpiration was decreased at 1600 ppm CO_2 and remained low at 6000 ppm,

20   concurrent with a 50% decrease in stomatal conductance. The CO_2-induced stomatal closure appears to have saturated between 850 and 1600 ppm CO_2. Due to this effect, net assimilation was only mildly changed at 1600 ppm CO_2, but significantly increased at 6000 ppm. As a result, water-use efficiency quadrupled at 6000 ppm CO_2. Stem height increment did not change significantly across the CO_2 treatments.

Taken together, our measurements demonstrated both the potential and limit of CO_2-induced stomatal closure, with positive

25   implications for fruit tree growth in ultra-high CO_2 environments, as on Earth in the case of unmitigated anthropogenic CO_2 emissions and on Mars.

Plain language summary

As atmospheric CO_2 increases globally, plants increase the rate of photosynthesis. Still, leaf gas exchange can be downregulated by the plant. Here we tested the limits of these plant responses in a fruit tree species under very high CO_2

30   levels, relevant to future Earth and to contemporary Mars. Plant water use decreased at 1600 ppm CO_2 and remained low at 6000 ppm. Photosynthesis significantly increased at 6000 ppm. In summary, ultra-high CO_2 may partly compensate for water shortage.

Introduction

Understanding the implications of elevated atmospheric CO_2 levels on plant physiology is paramount, as it has profound
consequences for global ecosystems, water economy, and the very fabric of life (Bazzaz et al. 1990). At the microscopic
level, the sensitivity of plants to altered CO_2 levels directly impacts carbon assimilation through photosynthesis. When the
level of CO_2 within the plant diminishes, a response is triggered, and the stomata open, allowing diffusive uptake of
atmospheric CO_2 (Lawson and Morison 2004). This gas exchange comes at a cost. When the stomata are open, the plant
loses water to the air in transpiration (Brodribb et al. 2009). This loss of water, while essential for photosynthesis and
temperature regulation, can become a significant challenge for the plant in water-limited environments (Wagner et al. 2021,
2022).

The concentration of CO_2 in the leaf and the surrounding atmosphere is a critical factor for photosynthetic carbon fixation.
Changes in CO_2 levels can trigger adjustments in stomatal conductance to optimize photosynthesis and water-use efficiency
(Klein et al. 2013). Yet, stomata are sensitive to many additional factors. Stomata generally open in the presence of light, as
photosynthesis, the process that converts CO_2 and light energy into sugars, is active during daylight hours. Moreover, the
relative humidity inside and outside the leaf affects stomatal conductance. Lower external humidity levels can downregulate
stomatal conductance, leading to a decrease in water loss through transpiration (Wagner et al. 2022). Leaf temperature can
also directly impact stomatal conductance. Warmer conditions often lead to increased transpiration rates, causing stomata to
open wider to cool the leaf (Uni et al. 2023). Adequate nutrient availability, particularly potassium, can influence stomatal
conductance by affecting the turgor pressure of guard cells, which control stomatal opening and closing (Lebaudy et al.
2008). Often, these responses act together in shaping stomatal conductance at a given atmospheric CO_2 concentration
(Bartlett et al. 2016).

On a macroscopic scale, elevated CO_2 exerts significant influence over planetary climate, presenting both challenges and
opportunities for life on Earth and beyond (Forget et al. 2013, Ozak et al. 2016). For example, on Mars, where the partial
pressure of CO_2 is over an order of magnitude higher than that of Earth's atmosphere, understanding the impact of high CO_2
on plant physiology and growth may aid efforts of human colonization, for which suitable conditions are thought to exist
(Slobodian et al. 2015). Mars' atmosphere is about 95% CO_2, and at an average surface pressure of $\approx$6.5 mbar, this is
equivalent to $\approx$6200 ppm CO_2 on Earth (Franz et al. 2017). At the same time, Mars' atmosphere contains only trace amounts
of O_2 (0.16%) and H_2O (0.03% on average, but highly variable). Water is not only rare in the Martian atmosphere, but also
on its surface, present mainly as ice or in hydrated salts, inaccessible to plants (Diez 2018, Nazari-Sharabian 2020). Despite
the growing interest in colonizing Mars, plant growth under such conditions has been explored in only a few studies (e.g.,
Richards et al. 2006, Wamelink et al. 2014).

Ultra-high atmospheric CO_2 levels are also relevant on Earth, past and future. Looking ahead, if industrial development continues as usual (IPCC emission scenario RCP8.5), the CO_2 concentration may rise to 1000 ppm by the end of this century and double that by the end of the year 2200. The effects of these changes on plant development are unknown, and so are the implications for the carbon and water cycles on Earth. This said, such high CO_2 levels existed in Earth's deep past. Recent reconstructions indicate that between ≈65 and ≈40 million years ago atmospheric CO_2 levels were close to ≈1000 ppm, peaking at ≈1600 ppm around 51 million years ago (CenCO2PIP et al. 2023). Over this time interval, all extant plant families already existed (Li et al. 2019). Therefore, we can assume that extant plant species have evolved through large changes in atmospheric CO_2 levels, up to four-fold the current level or higher. At a CO_2 level of 1000 ppm, net assimilation is close to saturation, with values typically at 79-91% of the maximum rate, depending on temperature (Kirschbaum 1994). However, at 400 ppm and 25 ℃, net assimilation is at 64% of the maximum rate.

Interestingly, the high abundance of CO_2 in the Martian atmosphere (comparable to ≈6200 ppm on Earth) may compensate for water shortage on the planet's surface. Plants open their stomata to capture CO_2 from the atmosphere, inevitably losing water vapor (Brodribb et al. 2009). Under rising CO_2 levels, stomatal opening can be minimized, thus saving water for the plant and providing an advantage in dry conditions (Elliott-Kingston et al. 2016, Paudel et al. 2018). Preliminary research hints at the potential outcomes of our exploration: short-term (minutes) responses to an increase in atmospheric CO_2 levels, often involve a decrease in stomatal conductance (Medlyn et al. 2001, Paudel et al. 2018). This initial reduction is a reversible response and is linked to an increase in carbon fixation during photosynthesis. Essentially, when more CO_2 is available, the plant can achieve the same level of carbon uptake at lower stomatal opening, thereby conserving water in the process. In addition, leaf surface pH might decrease due to increased concentrations of dissolved inorganic carbon in leaf surface solutions (Zeebe and Wolf-Gladrow 2001). However, leaves can regulate their leaf surface pH, through mechanisms that are still under study (Gilbert and Renner 2021). On longer timescales, from weeks through the lifetime of an individual plant (Hasanuzzaman et al. 2023) to generations over evolutionary timescales (Steinthorsdottir et al. 2019), plants can undergo morphological and developmental changes in response to altered CO_2 concentrations. Fossilized leaves from times in Earth history when CO_2 levels were higher display a lower areal density of stomata (Beerling et al. 1993, Haworth et al. 2012). As atmospheric CO_2 concentrations decreased over time, many plant species evolved to increase stomatal density. It is believed that this long-term adaptation to slowly evolving CO_2 levels enabled plants to thrive under changing environmental circumstances. Stomatal density has been hence used extensively as a paleo-CO_2 proxy (Steinthorsdottir et al. 2019, Konrad et al. 2021). However, this use has been rarely validated experimentally, but rather calibrated against other paleo-CO_2 proxies (Konrad et al. 2021; but see Brownlee 2001).

Despite the decrease in leaf gas exchange, on a timescale of weeks, carbon assimilation still increases under elevated CO_2, due to passive diffusion, as shown in both fruit trees (Paudel et al. 2018) and forest trees (Dror and Klein 2022) grown under controlled conditions. Among plant species, trees demonstrate the highest CO_2-induced photosynthetic increase, about 45%

100 increase from 370 to 570 ppm CO_2, as shown in a meta-analysis of free-air CO_2 enrichment experiments (Ainsworth and Rogers 2007). The higher assimilation can lead to increased carbon storage (Kinsman et al. 1997, Paudel et al. 2018) and increased growth. For example, elevated CO_2 levels resulted in elongation of branches and stems by 33% in *Garcinia mangostana* (Downton et al. 1990) and by 15% in *Glycine max* (Rogers et al. 1992). However, in contrast to growth in controlled environments, it has been established that under field conditions, where competition over limited resources

105 prevails, tree growth is mostly unaffected by elevated CO_2 (Korner et al. 2005, Klein et al. 2016, Jiang et al. 2020, Norby et al. 2022). Yet, increased tree growth is still observed in some cases (Kim et al. 2020, Norby et al. 2024). Common to all these experiments is the moderate level of elevated CO_2, up to 1000 ppm, motivated by the gradual increase of atmospheric CO_2 concentrations on Earth (Klein and Ramon 2019). An exception, a few studies were conducted at ultra-high CO_2 levels of up to 50,000 ppm. Experimenting with seedlings in small chambers, grown on artificial media, it was shown that the fresh

110 weight of lettuce, mint, and thyme increased up to 10000 ppm CO_2, decreasing somewhat at 30000 ppm, but still five-fold higher than at ambient CO_2 (Tisserat and Silman 2000, Tisserat and Vaughn 2001). A similar trend was observed in young seedlings of loblolly pine grown on artificial media (Tisserat and Vaughn 2003). The effect was smaller in plants grown in soil, with a two-fold increase in fresh weight at 3000 ppm CO_2 (Tisserat and Vaughn 2001). These experiments importantly constrained aspects of plant growth at ultra-high CO_2 levels, but they did not address the matter of the plant's water budget.

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High concentrations of CO_2, which increase CO_2 assimilation and decrease the stomatal conductance, lead to a notable reduction in water loss and an increase in the plant's water-use efficiency (WUE), the ratio of net CO_2 assimilation to transpiration, representing the plant's ability to maximize carbon gain while minimizing water loss (Field et al. 1995, Mathias and Thomas 2021, Dror and Klein 2022). Experiments have demonstrated that tree seedlings exposed to double the

120 ambient CO_2 levels exhibit a decrease in stomatal conductance ranging from 12% to 36% (Klein and Ramon 2019). This reduction in stomatal conductance signifies that at elevated CO_2 levels, fewer signals are sent to keep stomata open, resulting in reduced plant water loss. However, it is crucial to recognize that the response to elevated CO_2 is not uniform across all plant species. Factors such as the duration of exposure to high CO_2, the availability of other essential resources like water and nutrients, and inherent genetic differences between species can modulate the plant's response. Some plant species may

125 even exhibit reduced growth or alterations in their architecture, including shorter stems or smaller leaves, in response to elevated CO_2 levels.

Among tree species, evergreen broadleaf species of tropical biomes have shown higher sensitivity to CO_2 levels in terms of reduced stomatal conductance (Klein and Ramon 2019). Taking advantage of this sensitivity, in this study, an experiment

130 was designed to investigate the response of dwarf guava (*Psidium cattleyanum*), an evergreen broadleaf of the Amazon rainforest, to ultra-high CO_2 levels. This short-stature tree species (growing up to 6 m in height) is also well-adapted to medium light levels. Dwarf guava is an economically important species in some areas where it is cultivated (Patel 2012) and an invasive species in others, like Hawaii (Huenneke et al. 1990). We used an isolated mesocosm chamber that was uniquely

constructed to maintain CO_2 carbon dioxide as the only variable while the other variables (temperature, relative humidity, illumination intensity, nutrient supply) remained constant. In this mesocosm chamber we placed ten two-year-old guava seedlings and increased the CO_2 levels to about four times the ambient atmospheric concentration, 1600 ppm, and to a Mars-like atmospheric concentration, 6000 ppm (although not attempting to simulate Mars' conditions *per se*). We hypothesized that elevated CO_2 concentrations would induce a decrease in leaf transpiration, primarily by a reduction in stomatal conductance. This research may advance our understanding of plant growth and water-use efficiency under conditions expected on Earth by the year ≈2100 if anthropogenic CO_2 emissions are unmitigated, and beyond the terrestrial realm, it serves as a steppingstone towards ecological solutions in planetary environments.

Materials and methods

Experimental Design

Ten two-year-old dwarf guava plants (*Psidium cattleyanum*) of similar size were transferred to 1-liter pots. Plants were placed in a sealed mesocosm chamber ($0.90 \times 0.65 \times 0.60$ m = 0.351 m^3; COY, MI, USA; Rog et al. 2020). This chamber is designed to control humidity and temperature conditions. The humidity was maintained at 70% and the temperature at 24-26 ˚C, producing a vapor pressure deficit of 1.3-1.4 kPa. Tubes from CO_2 and N_2 cylinders were connected to the chamber to regulate gas concentrations, allowing for three experimental treatments with varying CO_2 levels: ambient, high, and ultra-high (400, 1600, and 6000 ppm CO_2, respectively). Experimenting with such high CO_2 levels required special safety precautions, and a CO_2 detector alarming of leaks was installed outside the chamber. This was complemented by periodic CO_2 measurements around the chamber, constantly showing levels of 400-480 ppm, typical of an indoors environment and assuring no leak from the chamber. To eliminate potential effects of differences among plants and chambers, the entire experiment was conducted on the same plants within the same chamber. Therefore, treatment periods were limited to just over three weeks (23-25 days), so that all treatments were applied on plants at the same developmental stage. This period is sufficient to allow for plant acclimation to the CO_2 treatment (Poorter et al. 2022). The experiment was conducted between 4 June and 27 August 2023. Plants were first exposed to 400 ppm CO_2, followed by 1600 ppm, and then 6000 ppm, i.e., from ambient to high and ultra-high CO_2. Prior to each treatment, plants underwent a one-week acclimation period within the mesocosm chamber. Chamber CO_2 was regulated using a SCD30 sensor with measurement accuracy of ±30 ppm at a range of 400-10000 ppm (Sensirion, Chicago, IL, USA) and a ESP32 controller (Espressif Systems, Shanghai, China). Notably, at 400, 1600, and 6000 ppm CO_2, fluctuations of ±30 ppm are equal to ±7.5%, 1.9%, and 0.5%, which are small. For this reason, we consider the CO_2 levels to have been steady. We utilized an HLG-320H-36A solar lamp (Agro Light, Beit Yehoshua, Israel) as the primary light source, positioned on top of the chamber to provide a photosynthetically active radiation (PAR) range of 550-700 µmol m^{-2} s^{-1} at the canopy height. However, most leaves grew below the canopy leaves, under lower PAR of 50-400 µmol m^{-2} s^{-1}, which was still adequate for this tree species (see next paragraph). The day/night cycle was set to 12h/12h. A light distribution map within the mesocosm chamber showed that plants were exposed to a

homogeneous light regime. Individual plant positions were rotated every other day to mitigate any positional effects within the chamber.

Plant response to light intensity and instantaneous changes in CO_2 level

To validate that *Psidium cattleyanum* is suitable for growth in the mesocosm chamber conditions, a photosynthetic light response curve was constructed in a pilot experiment. Six plants were exposed to increasing light levels and their net assimilation rate (μmol CO_2 m^{-2} s^{-1}) was measured. Determination of assimilation rate was achieved with a LI-6800 infrared gas analyzer (IRGA; Li-Cor Biosciences, Lincoln, NE, USA). Temperature, relative humidity, and CO_2 level within the leaf cuvette were adjusted to ambient during the measurement. PAR was adjusted to 1000, 800, 600, 400, 300, 200, 100, 50, and 0 μmol m^{-2} s^{-1}, in this order. The value of assimilation rate increased linearly from -1.89 at darkness (PAR=0) to 5.93 μmol CO_2 m^{-2} s^{-1} at PAR of 200 μmol m^{-2} s^{-1} (Fig. S1). The compensation point was at 45 μmol m^{-2} s^{-1}. At PAR >600 μmol m^{-2} s^{-1}, increases in assimilation rate were minor, and assimilation rate saturated. A second pilot experiment tested the plant response to instantaneous changes in CO_2 level. Here, three plants grown at ambient CO_2 level were exposed to increasing CO_2 levels and their net assimilation rate (μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (mol m^{-2} s^{-1}) were measured with the LI-6800 infrared gas analyzer. Temperature, relative humidity, and PAR level within the leaf cuvette were set to 26 ℃, 75%, and 600 μmol m^{-2} s^{-1}, respectively. CO_2 level was adjusted to 400, 100, 50, 0, 150, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000 and 400 μmol CO_2 m^{-2} s^{-1}, in this order.

Plant growth

Plants were actively growing throughout the experiment. We measured 4 growth parameters on a weekly basis: plant height (cm), leaf surface area (cm^2), branch number and shoot number. Stem diameter growth was too small for a manual measurement, as expected in saplings of this size. The height of each guava plant was measured from the base of the stem to the tip of the plant using a tape measure. The number of new branches was counted manually, with the appearance of clear protrusions consisting of four or more leaves considered as new branches. The number of shoots extending from the ground was recorded separately. The leaf surface area was determined by counting the number of leaves on each plant and multiplying by an average leaf size. The average leaf size was obtained using the Easy Leaf Area application developed by Hsien Ming Easlon (https://github.com/heaslon/Easy-Leaf-Area). In this process, leaf tops were photographed close to a 2×2 cm^2 area painted red over a white background. The leaf area was then derived from the ratio of green to red pixels present in the image.

Leaf gas exchange

Stomatal conductance and transpiration were measured using a LI-600 infrared gas analyzer (Li-Cor Biosciences, Lincoln, NE, USA). The LI-600 was applied to determine the gas exchange during photosynthesis in fresh leaves, with a known leaf surface area of 1 cm^2. The rates of transpiration (E in mmol m^{-2} s^{-1}) and stomatal conductance (gs in mmol m^{-2} s^{-1}) were

measured between 9:00 a.m. and 11:00 a.m. The rate of CO_2 assimilation was measured with a leaf cuvette at 400 ppm µmol CO_2 using the LI-6800 infrared gas analyzer (see under *Plant response to light intensity*) but could not be measured using the leaf cuvette at the higher CO_2 levels, due to the instrument limitations. Instead, assimilation rate was measured across the three CO_2 levels by analyzing the rate of decrease in the CO_2 concentration (ppm) within the controlled mesocosm chamber over a known duration (measured in s), which was then divided by the corresponding leaf surface area of all plants (m^2). The amount of assimilated CO_2 was transformed from the change in ppm CO_2 into µmol CO_2 by multiplying the ppm value by 14.558, which is the number of moles of air in the chamber, according to the ideal gas law, considering atmospheric pressure and mesocosm volume and temperature (above). The SCD30 sensor data, responsible for monitoring the CO_2 levels within the chamber, was collected at regular intervals of 1.5-2.3 minutes. Notably, a decrease in the CO_2 level was considered when the sensor data showed a negative difference persisting for 3 or more consecutive intervals, and the net change in the CO_2 level was computed and subsequently divided by the total duration of the decrease. Data collection for this process was conducted between 8:00 a.m. and 4:00 p.m. exclusively, during daylight hours and on days with no disturbances within the mesocosm chamber. For each CO_2 level, assimilation rate was calculated for 20 periods, from which the mean and standard error were calculated. These measurements facilitated a comprehensive evaluation of the plants' CO_2 assimilation. Water-use efficiency (WUE) was calculated as the ratio of the rate of carbon assimilation to the rate of transpiration (E) in the guava plants on four different days for each CO_2 concentration.

Leaf parameters

Leaf stomatal density was determined on newly matured leaves formed in the new CO_2 environment (3 days old leaves from the top of the canopy, grown at PAR of 550 µmol m^{-2} s^{-1}; *n* = 10 per treatment) by calculating the number of stomata per unit area (mm^2). The lower epidermal layer was carefully affixed to a slide using a transparent contact adhesive. Subsequently, the slide was captured under a light microscope (Leica DM500, Wetzlar, Germany) at a 20× magnification. Utilizing the ImageJ software, images were analyzed, and the number of stomata was divided by the defined area obtained from the slide, with a 0.005 mm line reference. The pH level on leaf surfaces was measured non-invasively using a Hanna device (Hanna Instruments Inc. Woonsocket, USA). The leaves were carefully positioned on a stable cardboard surface, and the device was affixed directly to them.

Statistical analysis

We tested for significant differences among CO_2 treatments for specific parameters. Prior to statistical tests, the distribution of data was presented as a histogram for each parameter. Then, a normal distribution was fit to each histogram, and the goodness of fit was tested by a Shapiro-Wilk test. *P*-values of the Shapiro-Wilk tests were >0.05 (often >0.2), indicating that all parameters were normally distributed. Differences in transpiration and stomatal conductance were analyzed using ANOVA at a significance level α = 0.05. For the analysis, CO_2 treatments were transformed into a nominal parameter (ambient, elevated, and ultra-high). All analyses were performed in JMP software (SAS, Cary, NC, USA). Differences in

stomatal density, A, WUE, height increment, and leaf surface pH were analyzed by t-tests on pairwise comparisons. *P*-values are reported in the figures.

240 **Results**

Examining leaf transpiration (Fig. 1a), a 20% decrease was observed upon the transition from 400 to 1600 ppm CO_2 (from ~4.4 to ~3.5 mmol m^{-2} s^{-1}; F=44.9, *P*<0.0001). The subsequent increase in CO_2 concentration to 6000 ppm resulted in no statistically significant changes in leaf transpiration compared to the 1600 ppm treatment, suggesting a potential threshold effect in the modulation of transpiration rates by elevated CO_2. In terms of stomatal conductance (gs), the transition from 400

245 to 1600 ppm CO_2 resulted in a 50% reduction in gs, which persisted at the 6000 ppm treatment (from ~0.85 to ~0.42 mol m^{-2} s^{-1}; F=22.6, *P*<0.0001; Fig. 1b). Overall, both transpiration and gs fluctuated across measurement days; yet, only under 400 ppm CO_2 was there an increasing trend. Considering that this treatment was applied immediately after plants were brought in from the greenhouse, values during the first 10 days under 400 ppm CO_2 might still reflect acclimation to the chamber conditions. In contrast to the decrease in gs with CO_2 increase, stomatal density increased mildly with CO_2, with a significant

250 20% increase between the 400 and 6000 ppm CO_2 treatments (Fig. 2).

The assimilation rate of CO_2 (A) was calculated for the entire mesocosm and showed a value of 1.5 µmol CO_2 m^{-2} s^{-1} at 400 to 1600 ppm CO_2. While this value is relatively low, it was relatively close to the photosynthetic rate of *Psidium cattleyanum* leaves under PAR of ~100 µmol m^{-2} s^{-1}, which is close to the light intensity experienced by most leaves in the chamber (Fig.

255 S1 and Methods). The assimilation rate exhibited a mild, yet significant decrease over the transition from 400 to 1600 ppm CO_2, demonstrating a major increase only at 6000 ppm (Fig. 3a). Water-use efficiency was unchanged upon the shift from 400 to 1600 ppm CO_2 and displayed a four-fold increase upon the subsequent shift to 6000 ppm (Fig. 3b). Measurements of the weekly growth height did not reveal a significant change (Fig. 4), nor was the weekly increment of leaf surface area (Fig. S2). The height increment was ~1.6 cm week^{-1}, which is equal to 4-5% growth, regardless of CO_2 level. Stalled branch

260 growth or even branch degradation appeared to have occurred upon the change to 6000 ppm CO_2 (data not shown).

Plants that were grown at ambient CO_2 level were also tested for their A and gs responses to instantaneous changes in CO_2 level (Fig. S3). A increased linearly from -2.3±0.6 µmol CO_2 m^{-2} s^{-1} at 0 ppm CO_2 to 12.9±1.0 µmol CO_2 m^{-2} s^{-1} at 800 ppm CO_2 (with a compensation point around 100 ppm CO_2). At higher CO_2 levels, A nearly saturated, yet continued to increase

265 up to 15.7±0.8 µmol CO_2 m^{-2} s^{-1} at 2000 ppm CO_2, the highest level permitted by the IRGA instrument. In parallel, gs decreased in two measured plants from 0.14 to 0.11 mol m^{-2} s^{-1}, and from 0.09 to 0.05 mol m^{-2} s^{-1}.

Discussion

Decades of research on physiological tree responses to elevated CO_2 have shaped our understanding of vegetation feedbacks to the increasing level of atmospheric CO_2 on Earth (Medlyn et al. 2001, Korner et al. 2005, Klein et al. 2016). However, since most experiments were limited to $CO_2 < 1000$ ppm, the limit of stomatal sensitivity to elevated CO_2 has remained unclear, and specifically, the limit of the effect of CO_2-induced decrease in stomatal conductance on tree transpiration is unknown. Previous experiments with ultra-high CO_2 levels indicated consistent benefits in small plants grown in small chambers, mostly on artificial media (Tisserat and Silman 2000, Tisserat and Vaughn 2001, 2003). Yet these former experiments focused mostly on plant growth, leaving the physiological mechanisms and plant water use unresolved. Here, we challenged seedlings of fruit trees grown in a mesocosm, with ultra-high CO_2 levels of 1600 and 6000 ppm. We hypothesized that elevated CO_2 levels will induce a decrease in plant water use, primarily by a reduction in stomatal conductance. Indeed, transpiration was decreased at 1600 ppm CO_2 and remained low at 6000 ppm (Fig. 1a), due to reduced stomatal conductance (Fig. 1b). The effects on assimilation, WUE, and growth were also measured (Figs. 3, 4). Taken together, our measurements demonstrate both the potential and limit of CO_2-induced stomatal closure, with positive implications for fruit tree growth in ultra-high CO_2 environments, as on Earth in the case of unmitigated anthropogenic CO_2 emissions (e.g., IPCC emission scenario RCP8.5) and on Mars (Franz et al. 2017; although not attempting to simulate Mars' conditions *per se*).

Experimenting with lemon tree saplings, we previously showed that CO_2-induced stomatal closure can decrease transpiration, while still increasing assimilation, at CO_2 levels as high as 850 ppm (Paudel et al. 2018). Here we showed that this effect was active upon an increase from 400 to 1600 ppm CO_2 but that no further change occurred upon a further increase to 6000 ppm. On the contrary: stomatal conductance was sometimes higher at 6000 than at 1600 ppm CO_2 (but still lower than at 400 ppm; Fig. 1b). Across the measurements, stomatal conductance at 1600 ppm CO_2 was mostly ~50% its value at 400 ppm. The calculated change in stomatal conductance within this range (400-1600 ppm) was -33 mmol m^{-2} s^{-1} per 100 ppm CO_2 increase. For comparison, this CO_2-induced stomatal closure is higher than calculated for lemon (-19 mmol m^{-2} s^{-1} per 100 ppm CO_2 increase; Paudel et al. 2018), very similar to that measured in mango (-34 mmol m^{-2} s^{-1} per 100 ppm CO_2 increase) and identical with the mean calculated for broadleaf evergreen tree species across 39 different experiments (-33; Klein and Ramon 2019). However, due to the absence of a significant change in stomatal conductance from 1600 to 6000 ppm CO_2, the calculated change in stomatal conductance over the range 400-6000 ppm was only -5 mmol m^{-2} s^{-1} per 100 ppm CO_2 increase. Integrating our current and previous experiments, we can conclude that for an evergreen fruit tree such as guava, CO_2-induced stomatal closure probably saturates between 850 and 1600 ppm CO_2. Do these responses reflect an adaptation to elevated CO_2, or an instantaneous response? Experimenting with trees with no previous exposure to elevated CO_2, we observed a similar trend of stomatal closure (a 20-50% reduction in gs between 400 to 1600 ppm CO_2; Fig. S3) which seemed to level off at higher CO_2 levels. Despite this reduction, net carbon assimilation increased, mostly below 800 ppm CO_2, while measurements were limited to 2000 ppm CO_2, due to the IRGA constraints.

The measured stomatal response to the ultra-high CO_2 levels can explain the observed changes in carbon assimilation. Since assimilation of CO_2 into the leaf occurs by passive diffusion, most tree species increase assimilation despite CO_2-induced stomatal closure (Dror and Klein 2022). However, in our case the strong decrease in stomatal conductance at 1600 ppm was not compensated by the higher CO_2 availability, and the assimilation rate mildly decreased (Fig. 3a). Only at 6000 ppm, the surplus CO_2, which was not accompanied by additional stomatal closure, resulted in a significant increase in assimilation. This indicates that the maximum rate of carboxylation increased under ultra-high CO_2 level, as previously shown in lemon under 650 and 850 ppm CO_2 (Paudel et al. 2018). The CO_2-induced photosynthetic increase observed here agrees with earlier studies at lower levels of elevated CO_2, which showed that photosynthesis is far from saturation at 400 ppm (Kirschbaum 1994), and that trees, more than shrubs, grasses, and crop plants, increase their photosynthesis under elevated CO_2 (Ainsworth and Rogers 2007). In turn, these physiological mechanisms yielded a major increase in WUE (Fig. 3b). Noteworthy, the stable WUE (400-1600 ppm CO_2) was driven by proportional reductions in assimilation and transpiration, whereas the major WUE increase (at the 1600-6000 ppm CO_2 transition) was driven by increased assimilation. The stable WUE (400-1600 ppm CO_2) contrasts an overall increase of WUE in the terrestrial biosphere at this CO_2 range (Walker et al. 2021), however can be expected due to the reduced stomatal conductance in a broadleaf tropical tree species (Paudel et al. 2018), which is not a ubiquitous response across tree species (Klein and Ramon 2019). Finally, seedling growth, measured here as the height increment, was consistent across the large CO_2 range (Fig. 4), meaning that the surplus carbon may have been allocated to sinks other than stem growth, such as storage (Paudel et al. 2018), respiration, or root growth (Dror and Klein 2022). In addition, there was an increase in new shoot production at 1600 ppm (data not shown). Our results are in apparent disagreement with those of the early ultra-high CO_2 experiments, in which the fresh weight of seedlings of several herb species and pine increased at ultra-high CO_2 and saturated only at levels of 10000 ppm (Tisserat and Silman 2000, Tisserat and Vaughn 2001, 2003). Several differences between the early studies and our experiments may account for the different results. Unlike our experiments, in the earlier studies plants were grown on artificial media, sometimes supplemented with sucrose. The different growth conditions and substrate availability are expected to lead to differences in carbon assimilation of the observed sense (i.e., a greater biomass increase under the near-optimal conditions of the past studies). Indeed, the fresh weight increase measured in past experiments was smaller when plants were grown in soil, with only a two-fold increase in fresh weight at 3000 ppm (Tisserat and Vaughn 2001). Lastly, at least for pine, stomata are almost insensitive to increases in CO_2 levels (Klein and Ramon 2019) and especially in loblolly pine (Will and Teskey 1997). Therefore, in pine variants, little or no CO_2-induced stomatal closure is expected and increases in carbon fixations are expected to be larger.

In contrast to the expected decrease in stomatal density in response to higher CO_2 (Beerling et al. 1993, Ainsworth and Rogers 2007), stomatal density increased modestly, but significantly, at the higher CO_2 levels in our experiment (Fig. 2b). For example, stomatal density increased by 15% between 400 and 1600 ppm CO_2, while stomatal size did not change. This was unexpected, since overall, stomatal conductance decreased (e.g., by 50% between 400 and 1600 ppm CO_2). Therefore, it

can be deduced that stomatal aperture was in fact reduced by 65%. Previous studies showed that stomatal conductance of mature leaves has a regulatory effect on the stomatal development of expanding leaves (Brownlee 2001, Miyazawa et al. 2005). We do not fully understand why stomatal density increased, and whether this change represents a long-term response. However, previous research shows that even for plants that were exposed for generations to elevated CO_2, responses can vary by species. Among 17 plant species growing in a naturally enriched CO_2 spring in Italy, stomatal density was higher under the higher CO_2 levels in 7 species, including two tree species (*Buxus sempervirens* and *Ruscus aculeatus*; Bettarini et al. 1998). Similarly, stomatal density increased in 10 out of 27 free-air CO_2 enrichment experiments (Ainsworth and Rogers 2007). More importantly, the assumption that plants decrease stomatal density in response to higher CO_2 has not been rigorously tested in biological experiments. Rather, stomatal density was tested against other paleo-CO_2 proxies such as contemporaneous CO_2 measurements from glacial ice cores, and showed limited utility, especially under warm and moist conditions as in our experiment (Konrad et al. 2021).

Other leaf traits that were examined did not show any sensitivity to CO_2 levels, including leaf surface pH, which was expected to decrease due to a higher dissolved inorganic carbon content in leaf surface solutions (Zeebe and Wolf-Gladrow 2001), but remained at 5.0 (Fig. 4). Variations among plant species in leaf surface pH range between 1.0 and 11.0, yet the pH of 5.0 measured here is lower than the pH values of other Rosids, typically between 6.0 and 10.0 (Gilbert and Renner 2021). In a preliminary experiment we observed black stains on some higher canopy leaves at 6000 ppm CO_2 (not shown), but these were probably related to high light conditions at the top of the mesocosm chamber. Finally, we did not detect any signs related to oxygen deficiency caused by the ultra-high CO_2 levels, as reported in humans at $CO_2 > 1,000$ ppm (Azuma et al. 2018).

Our experiment is not free of limitations. For example, a relatively low number of seedlings ($n = 10$) with natural interplant heterogeneity yielded high variation in some parameters (e.g., net assimilation; Fig. 3a). Our calculation method for net assimilation was also unique, since a cuvette gas analyzer could not be used at the high CO_2 levels studied here. A comparison between the methods, permitted at 400 ppm CO_2, indicated an underestimation by the mesocosm method (1.5 µmol CO_2 m^{-2} s^{-1} compared with 2.4 µmol CO_2 m^{-2} s^{-1} under PAR of µmol CO_2 m^{-2} s^{-1} (Fig. S1). It is possible that, due to self-shading of the leaves, PAR levels were <100 µmol CO_2 m^{-2} s^{-1} for most leaves. Regardless, our calculation method was identical across the three CO_2 levels, and hence the relative changes persist. In addition, our choice of running the experiment over three consecutive periods (one for each of the three CO_2 concentrations), meant that plants were slightly different at each period. Such a difference was minimized by limiting the periods to just slightly over three weeks each, so that all treatments were applied on plants at the same developmental stage. As a result, the study results cannot be generalized to longer timescales of months and years. We reiterate that this choice of continuous experimentation with the same plants eliminated potential effects of differences among plants and chambers, and that prior to each treatment, plants underwent a one-week acclimatization period within the chamber.

Studying the physiological responses to ultra-high CO_2 levels in evergreen tree species can inform our basic understanding of plant physiology as well as the cultivation of fruit trees under high anthropogenic CO_2 emission scenarios and in extreme

375   environments, as on Mars (although not attempting to fully simulate Mars' conditions). Except for a few pioneering studies in small plants grown on artificial media in small chambers, it has been unknown whether plants can thrive or even survive under such high-CO_2 conditions, as has the effect of ultra-high CO_2 levels on plant water use. In this respect, our results bring encouraging prospects of three sorts: (1) *Guava* seedlings were actively growing at 6000 ppm CO_2, at a rate similar to their growth rate under ambient CO_2 levels. We note that other tree species may even show growth benefits, as observed at

380   CO_2 between 400 and 1000 ppm (Paudel et al. 2018, Dror and Klein 2022, Norby et al. 2024). (2) Net assimilation increased significantly at 6000 ppm CO_2. This means that cultivation at elevated CO_2 can serve as a carbon sink, with a possible role in mitigation of anthropogenic CO_2 emissions. (3) Transpiration at 6000 ppm was 20% lower than at 400 ppm CO_2. Together with the increase in assimilation, we measured an almost four-fold increase in WUE across this CO_2 range. Our results are valuable for both future Earth (e.g., emission scenario RCP8.5) and Mars colonization (Richards et al. 2006, Wamelink et al.

385   2014, Slobodian et al. 2015). On Earth, indoor environments are already experiencing excessive CO_2 levels, and plants in green walls help to decrease indoor CO_2 levels (Agra et al. 2021). On Mars, low temperature and low atmospheric O_2 levels mean that plant cultivation will require an engineered environment. In this sense, our mesocosm can be regarded as a simulation of an artificial greenhouse (though we made no attempt to simulate Mars conditions). Considering the scarcity of water on Mars (Diez 2018, Nazari-Sharabian 2020) and in some regions on Earth, especially under climate change scenarios

390   (Gosling et al. 2016, Rosa et al. 2020), the increase in WUE at high CO_2 levels observed in this study is a major advantage stemming from plant physiology. Overall, the high abundance of CO_2 in Earth's future atmosphere and in the present Martian atmosphere might compensate for water shortage.

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Authors' contributions

IH and TK initiated the study. YBG and YBE established the experimental system and the first measurements. BEL

400   performed the experiment and the analyses. BEL, TK, and IH wrote the manuscript.

Competing interests

The authors declare no conflict of interest in preparing this manuscript

405   **Open research**

Data will be available in an open repository upon manuscript acceptance

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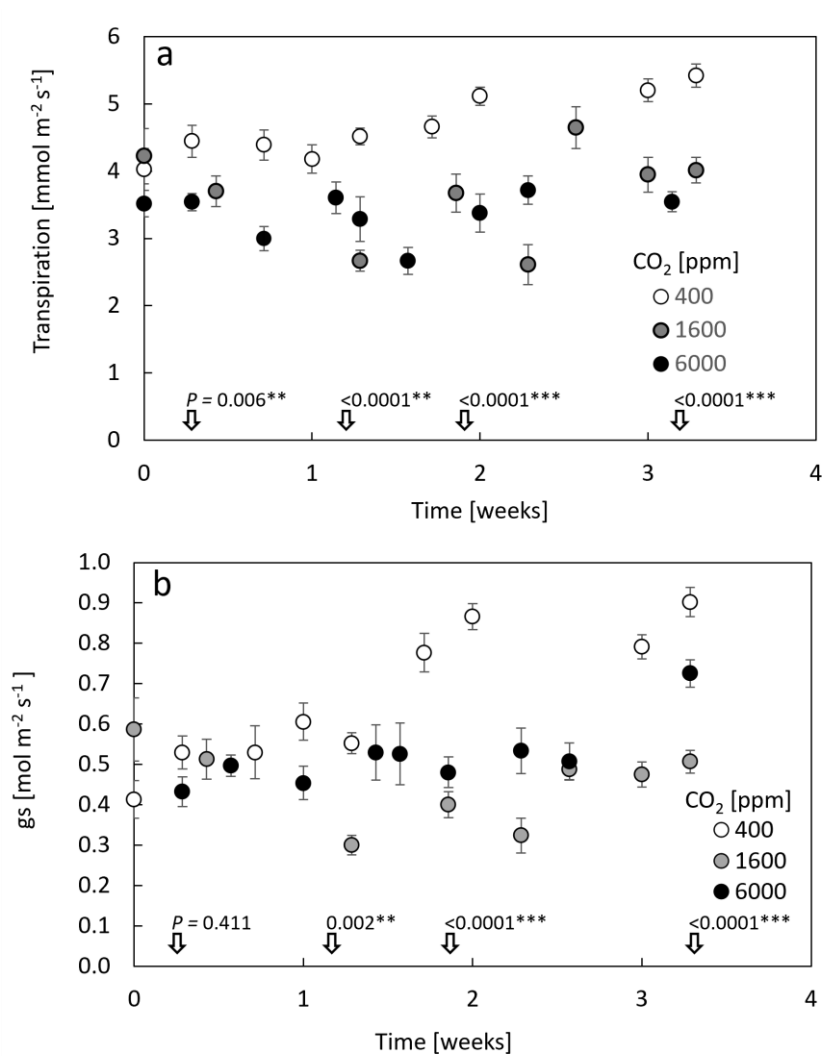
545 **Fig. 1. Leaf transpiration and stomatal conductance are decreased at 1600 ppm CO₂ and remain low at 6000 ppm.** Data points are means of 10 guava saplings subjected to different CO_2 concentrations. Measurements were made with a leaf cuvette. Error bars represent standard errors. *P*-values are from ANOVA on transpiration and stomatal conductance levels at specific dates, and *, **, and *** indicate differences among CO_2 levels at 0.01, 0.001, and 0.0001 significance levels.

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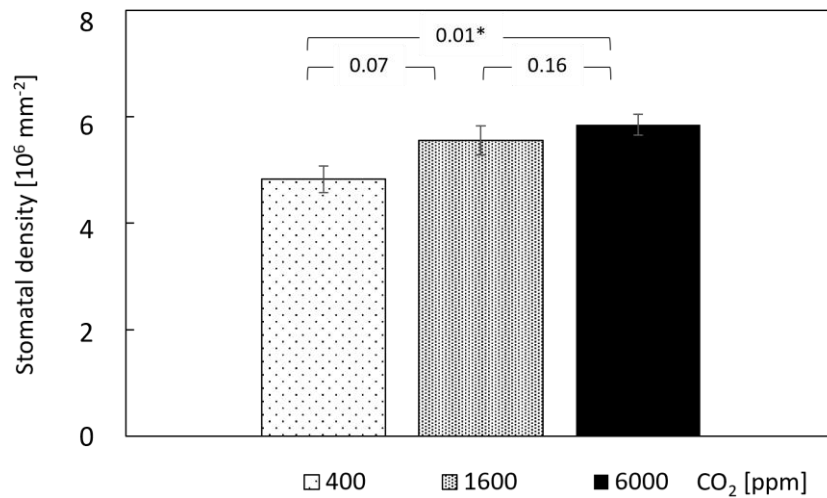
Fig. 2. Stomatal density mildly increased under elevated CO$_2$. Data points and bar heights are means of 10 guava saplings subjected to different CO$_2$ concentrations. Error bars represent standard errors. *P*-values are from paired t-tests and * indicates significant differences among CO$_2$ levels. Stomatal density was measured under a light microscope.

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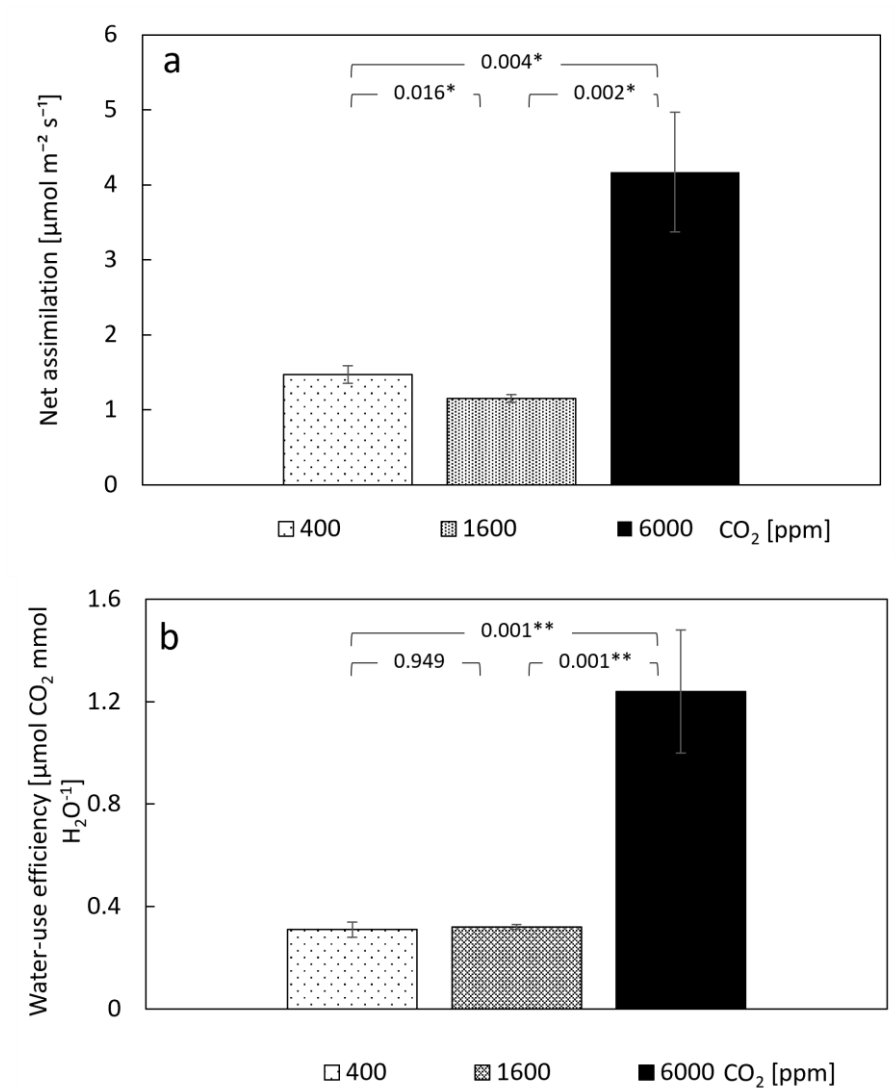
Fig. 3. Net assimilation is mildly changed at 1600 ppm CO₂ and increases at 6000 ppm, driving a major increase in water-use efficiency. Bar heights are means of 10 guava saplings subjected to different CO_2 concentrations. Error bars represent standard errors. *P*-values are from paired t-tests and * and ** indicate differences among CO_2 levels at 0.01 and 0.001 significance levels. Net assimilation (a) was calculated from chamber CO_2 dynamics. Water-use efficiency (b) was calculated as net assimilation divided by transpiration.
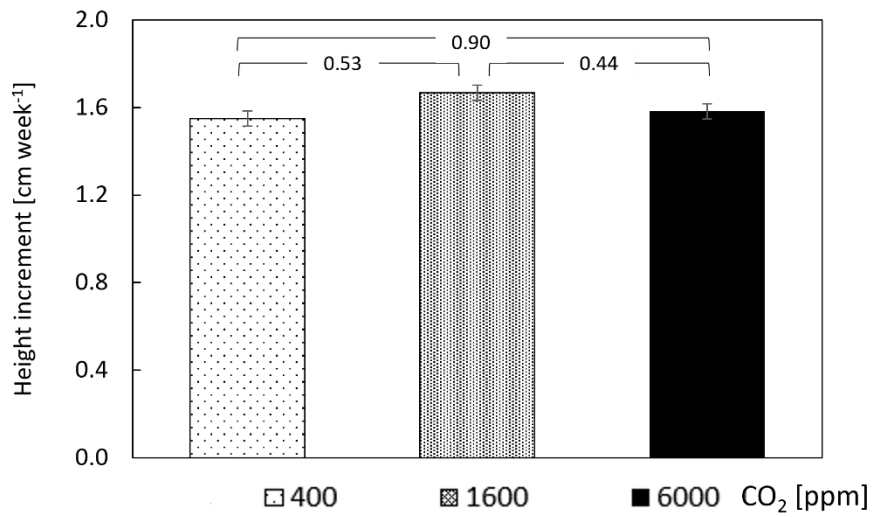
Fig. 4. Plant growth is similar under different CO$_2$ concentrations. Data points are means of 10 guava saplings subjected
to different CO$_2$ concentrations. Error bars represent standard errors. *P*-values are from paired t-tests.