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Elevated growth [CO₂] enhances heat stress resistance of photosynthesis in young leaves of avocado (*Persea americana*)

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Climate change is associated with higher atmospheric [CO₂] and more frequent temperature extremes, with the strongest impact expected in the tropics where plants often operate close to their heat stress limit. How the resistance of foliage photosynthetic traits to heat stress varies with [CO₂] elevation remains largely unknown, particularly in tropical species with continuously expanding canopies, where the heat resistance of leaves can vary with age. We studied the impact of heat shock stress resembling heatflecks due to fluctuating light (48 °C for 10 min) on foliage physiological traits and chemical contents in young-mature and old-mature foliage of the tropical species *Persea americana* Mill. plants grown under ambient (400 μmol mol⁻¹) and elevated (800 μmol mol⁻¹) [CO₂]. Leaf characteristics were studied through a 48 h recovery period. Light-saturated net assimilation rate (A) decreased with leaf age in both ambient and elevated [CO₂]. In young-mature leaves, A in plants grown under elevated [CO₂] was greater than A in plants grown under ambient [CO₂]. In old-mature leaves, A was similar under both [CO₂] and this was associated with increased nutrient limitation under elevated [CO₂]. Upon heat stress application, A decreased in all cases due to both reduction in stomatal conductance and inhibition of biochemical photosynthetic capacity (maximum Rubisco carboxylase activity). During recovery, A increased to pre-stress level in all but in young-mature plants grown under ambient [CO₂] where A remained much lower (78% reduction) than in control plants. As young leaves have a longer remaining lifespan and higher future potential contribution to plant carbon gain, preservation of photosynthetic capacity in young leaves under elevated [CO₂] suggests that elevated [CO₂] can enhance long-term photosynthetic production in *P. americana* exposed to heat episodes.

KEYWORDS

carbon dioxide enrichment, leaf developmental stage, nitrogen content, phosphorus content, gas exchange, Rubisco carboxylase activity, tropical fruit trees, thermal tolerance

1 Introduction

Since the industrial revolution, the global atmospheric CO₂ concentration has risen from around 280 mol mol⁻¹ to over 420 mol mol⁻¹. Contingent on socio-economic trajectory taken, [CO₂] is expected to reach 730–1000 μmol mol⁻¹ by the end of this century (Strandsbjerg Tristan Pedersen et al., 2021). Whole plant responses to elevated [CO₂] depend on modifications in plant photosynthesis and respiration rates (carbon balance), carbon allocation and nutrient availability, whereas the importance of individual factors depends on plant species and functional type (Drake et al., 1997; Ainsworth and Long, 2005; Dusenage et al., 2019). Alterations in leaf photosynthesis rates, in turn, are intimately linked to changes in leaf structure and chemical composition (Niinemets et al., 1999; Onoda et al., 2017). As the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is not [CO₂]-saturated at current atmospheric [CO₂], leaf photosynthesis is typically immediately enhanced upon the increase of [CO₂] (Singh and Reddy, 2016; Cummins, 2021). Stimulated photosynthesis rate further leads to improved plant growth rate in short to medium term (Drake et al., 1997; Ainsworth and Long, 2005; Singh and Reddy, 2016; Reich et al., 2018). However, the stimulation of biomass accumulation under longer-term [CO₂] increase may not be sustainable, and plants often reduce photosynthetic capacity through an acclimation process commonly referred to as ‘down-regulation’ due to factors such as limited nutrient and water availability and other sub- or supraoptimal environmental conditions (Sanz-Sáez et al., 2013; Atkin et al., 2015; Terrer et al., 2018; Zheng et al., 2019; Jiang et al., 2020). In addition, the impact of the increase in [CO₂] on whole plant performance can be strongly affected by how [CO₂] affects the rate of new leaf production and leaf senescence and nutrient resorption (Li et al., 2019; Abiola et al., 2025a), and thus, the share of young mature and old mature leaves. The impact of leaf age can be particularly important in species continuously forming new leaves such as common in many tropical long-living species, but there is little information of how elevated [CO₂]-driven photosynthetic downregulation depends on leaf age.

The increase in [CO₂], combined with rising levels of other greenhouse gases, has resulted in a 0.8 °C increase in mean annual global temperature (Hansen et al., 2010) and it is expected to rise further by 2–5 °C before the end of this century (Tian et al., 2020). Overall higher temperatures are associated with increased frequency of heatwaves (Breshears et al., 2021; Ahrens et al., 2021), and consequently, with more severe heat stress impacts on plants, curbing plant growth and survival in future climates. Both stomatal and biochemical factors can inhibit photosynthesis at

temperatures slightly above the thermal optimum, and the contribution of these factors varies among species (Kask et al., 2016; Slot and Winter, 2017; Kumarathunge et al., 2018; Turan et al., 2019; Sulaiman et al., 2023). The thermal optimum itself, and accordingly the severity of stress at the given temperature is also species-dependent and might vary with the plant developmental stage (Ruelland and Zachowski, 2010; Okereke et al., 2021). In this study, the term “heatwave” denotes extreme temperatures that significantly exceed the optimal photosynthetic range of tropical species, typically 30–35 °C (Slot and Winter, 2017). Photosynthesis is highly susceptible to heat stress, especially photosystem II (PSII) and oxygen-evolving complex are the first components to sustain thermal damage (Falcioni et al., 2024; Paethaisong et al., 2025). Temperatures above 45 °C for just a few minutes can cause losses in membrane integrity, and lead to photoinhibition and reactive oxygen species (ROS) generation; ROS propagation and reduction of photosynthetic activity can continue even after returning to lower temperatures (Hüve et al., 2011; Bernacchi et al., 2025). Acclimatory responses to heat stress involve changes in the activities of photosynthetic enzymes, adjustments of stomatal openness for more effective transpiratory cooling, and increases in photoprotective energy dissipation, while redox signals from the photosynthetic electron transport activate heat shock proteins and antioxidant defenses which enhance both thermal protection and recovery capacity (Tarvainen et al., 2022; Diao et al., 2024).

The impact of heat stress varies among leaves of differential developmental stage with mature leaves generally exhibit higher heat tolerance compared to young leaves (Wahid et al., 2007; Marias et al., 2017). However, why heat responses of leaves of different developmental stages vary and how the contribution of stomatal and biochemical modifications to overall heat stress response varies among leaves of different ages are poorly understood (Gu et al., 2012; Zhu et al., 2021; Sun et al., 2024b). Higher heat resistance of older leaves might be attributed to their more developed structural characteristics such as greater leaf thickness, more robust cell walls, and higher mass per area, well-established antioxidant systems and rapid capacity for formation of heat shock proteins as well as non-specific protective compounds such as sugars (Hüve et al., 2011; Wahid et al., 2007; Marias et al., 2017; Yurina, 2023; Watson-Lazowski et al., 2024). Meanwhile, fully-developed photosynthesis apparatus is also essential in regulating plant heat responses by balancing carbon assimilation with protective mechanisms in mature leaves (Zahra et al., 2023; Bernacchi et al., 2025).

Apart from the direct effect of [CO₂] on photosynthesis rate, higher [CO₂] can potentially ameliorate the impact of heat stress (Rodrigues et al., 2016; Abo Gamar et al., 2019; Diao et al., 2024). Improvement of stress resistance by elevated [CO₂] in stressed plants is evident in a reduced cellular oxidative damage (such as lipid peroxidation and protein oxidation), in a lower content of stress-generated reactive oxygen species (ROS) and in a lower degree of reduction in photosynthesis rate (Geissler et al., 2010; Sun et al., 2013a; Zinta et al., 2014; Abdelgawad et al., 2015; Ulfat et al., 2021). A greater stress resistance has been associated with reduced photorespiration and as a consequence, with lower production of active oxygen species and H₂O₂ (Foyer and Noctor,

Abbreviations: A, light-saturated net assimilation rate; C_i, intercellular [CO₂] concentrations; C_M, leaf carbon content per unit dry mass; g_s, stomatal conductance; M_A, leaf dry mass per unit area; N_M, leaf nitrogen content per unit dry mass; P_M, leaf phosphorus content per unit dry mass; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; V_{max}, maximum carboxylase activity of Rubisco.

2009; Munné-Bosch et al., 2013; Zinta et al., 2014), enhanced contents of lipophilic antioxidants and membrane-protecting enzymes through upregulation of antioxidant defense metabolism (Xu et al., 2014; Naudts et al., 2014) and higher content of sugars that stabilize membranes (Sun et al., 2013a; Ulfat et al., 2021). However, how elevated $[\text{CO}_2]$ -dependent improvement of heat stress resistance varies among leaves of different developmental stage has not been studied. Given that elevated $[\text{CO}_2]$ might alter the share of young and old leaves in the canopy, this is a relevant omission.

To the best of our knowledge, interactive effects of heat stress and elevated $[\text{CO}_2]$ on primary metabolism in leaves of different developmental stage have not been studied, especially in tropical plants with continuous leaf formation. We grew the saplings of tropical evergreen fruit tree avocado (*Persea americana* Mill., Lauraceae) under ambient $[\text{CO}_2]$ of $400 \mu\text{mol mol}^{-1}$ and elevated $[\text{CO}_2]$ of $800 \mu\text{mol mol}^{-1}$ and studied the interaction between photosynthetic acclimation to $[\text{CO}_2]$ and heat stress response in leaves of different age. We hypothesized that: (i) elevated $[\text{CO}_2]$ enhances photosynthesis rate, but leads to a down-regulation of biochemical photosynthetic capacity; (ii) the degree of photosynthetic downregulation is greater in older leaves; (iii) elevated $[\text{CO}_2]$ increases heat resistance of photosynthesis; (iv) older foliage sustains less physiological damage and exhibits faster recovery than young-mature foliage, and this difference is more pronounced under elevated $[\text{CO}_2]$. *Persea americana* is an economically important crop species and there is an unprecedented increase in the production and consumption of its products worldwide (Schaffer et al., 2013). Thus, we expect that the results of this study contribute to predicting the avocado performance in future climates. Furthermore, we consider avocado as a representative tropical species with continuous leaf formation, and thus, we argue that this study provides general insight into how a large part of vegetation in humid tropics responds to global change.

2 Materials and methods

2.1 Plant material and growing conditions

We used seeds of *Persea americana* Mill. cv. Hass, a cultivar bred in California from Mexican-Guatemalan ancestry and currently cultivated worldwide. The fruits (origin Aconcagua basin, Chile) were bought from a local fruit shop. The seeds were planted in 2 L clay pots and grown at the plant growth room of the Estonian University of Life Sciences under the following conditions: light intensity of $500\text{--}700 \mu\text{mol m}^{-2} \text{s}^{-1}$ with day/night temperatures of $28/24^\circ\text{C}$ for a 12 h light period and 60% relative humidity. The growth substrate was a commercial organic garden soil and a mixture of peat and sand (0–2 mm, Bauhof, Tartu, Estonia). The plants were watered every other day to soil field capacity and fertilized with 10 g slow-release fertilizer with microelements (N:P:K = 14:11:25, AS Baltic Agro, Tartu, Estonia) once per month. The seedlings were grown under these conditions until they were 50–70 cm tall and had utilized all seed reserves. Before the start of

CO_2 treatments, the plants were transplanted into 5 L pots and the foliated parts of the plants were removed leaving ca. 5 cm long stems. Subsequently, the plants were transferred to the plant growth chamber (FITOCLIMA S600PLLH, Aralab, Lisbon, Portugal) to either ambient ($400 \mu\text{mol mol}^{-1}$) or elevated $[\text{CO}_2]$ ($800 \mu\text{mol mol}^{-1}$). Chamber conditions were consistent with those in the plant growth room, except that light intensity was maintained at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The plants were watered every other day to the soil field capacity and fertilized in the beginning and in the middle of the growth period (see Abiola et al., 2025a for details of the growth conditions). At the time of the experiment, the plants had approximately 30–40 leaves and were 80–100 cm tall, after a growth period of four months. In this experiment, new leaves were formed continuously, and the plastochron (interval between the formations of successive leaves) was 11–12 days. Fully expanded young-mature leaves were ca. 60–70 days old, and old-mature non-senescent leaves were ca. 110–120 days old. We estimated that the available C and N stored in the root system could have been responsible for less than 10% of new growth, and thus, most of the above-ground biomass developed after removal of above-ground plant parts and transfer of plants to the new growth environment resulted from *de novo* carbon fixation and nutrient uptake from soil.

2.2 Gas exchange measurements

A custom-made open gas-exchange system was used to measure the foliage gas-exchange rate (Copolovici and Niinemets, 2010 for the full description of the system). The measurement system was specifically designed for simultaneous measurement of photosynthesis, transpiration, and volatile organic compound (VOC) emission rates, with all components constructed from glass, stainless steel, and Teflon®. The 1.2 L measurement chamber was made with double glass walls. Water with preset temperature circulated between the chamber walls to regulate the chamber temperature (Copolovici and Niinemets, 2010). The CO_2 and H_2O vapor concentrations at the chamber inlets and outlets were measured with an infra-red dual-channel gas analyzer operated in differential mode (CIRAS III, PP-Systems, Amesbury, MA, USA).

After the measurement leaf was enclosed in the chamber, following standard conditions were established: light intensity at the leaf surface of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$, chamber temperature of 24°C , CO_2 concentration of $390\text{--}410 \mu\text{mol mol}^{-1}$ (ambient $[\text{CO}_2]$ measurements), relative air humidity 60%, and leaf-to-air vapor pressure deficit of 1.7 kPa. After the steady-state gas exchange rates were achieved, typically between 25–30 minutes after leaf enclosure, differences in CO_2 and water vapor concentrations were recorded. Net assimilation rate (A), transpiration rate (E), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were calculated according to von Caemmerer and Farquhar (1981). The apparent maximum Rubisco carboxylase activity (V_{cmax}) was computed according to Niinemets et al., 1999 as explained in detail in De Kauwe et al. (2016). In these calculations, average

Rubisco kinetic characteristics for “warm” C_3 species from Galmés et al. (2016) were used. As C_i was used as the substitute for chloroplastic CO_2 concentration, the estimates of V_{cmax} provide an estimate of foliage photosynthetic activity without stomatal effects, but they do not consider possible variation in mesophyll conductance.

2.3 Heat stress treatments

Before the heat treatment, steady-state values of A and g_s were recorded under standard conditions (24 °C). Heat stress was applied by immersing the sample leaves in distilled water at 48 °C for 10 min according to the procedure detailed in previous studies (Copolovici et al., 2012; Kask et al., 2016; Okereke et al., 2022). During immersion, the leaves were enclosed in a chemically inert polyester bag to avoid direct contact with water. The control treatment consisted of leaf immersion in distilled water at 25°C for 10 min. A controlled temperature water bath (VWR International, West Chester, Pennsylvania, USA) was used to maintain a highly stable temperature of the immersion medium. We choose this procedure as it does not depend on stomatal effects that can alter leaf temperature at given air temperature due to transpiratory cooling. We have demonstrated that the heat stress applied this way is highly repeatable and the severity of heat stress scales quantitatively with photosynthetic reduction, volatile emission and ion leakage (e.g., Okereke et al., 2022; Sulaiman et al., 2023).

In these experiments, four replicates of young-mature and old-mature leaves from different plants were used. Foliage gas-exchange measurements were conducted at 0.25, 1, 3, 24, and 48 h after the treatment under the standard conditions (chamber temperature of 24 °C).

2.4 Estimation of leaf dry mass per area, and carbon, nitrogen, and phosphorous contents per dry mass

Data of nitrogen (N_M), carbon (C_M), and phosphorus (P_M) content per dry mass used in this study are those from (Abiola et al., 2025a). Fresh leaves were scanned at 300 dpi and leaf area was measured using ImageJ 1.8.0 (NIH, Bethesda, Maryland, USA). Leaf dry mass was estimated after oven-drying at 70°C for 48 h, and leaf dry mass per unit area (M_A) was calculated.

2.5 Data analyses

The measurements of control and heat-treated plants were conducted in four replicate plants ($n = 4$), with one young-mature and one old-mature leaf measured per plant, using different plants grown under ambient and elevated $[CO_2]$. The data were presented as averages $\pm$ SE. The degree of heat stress

recovery of gas exchange characteristics for each plant age groups was calculated as:

$$R_{C,i} = (x_{i,after\ stress} - x_{i,before\ stress}) / x_{i,before\ stress} \times 100\% \quad (1)$$

where $R_{C,i}$ is the relative change of trait i and $x_{i,before\ stress}$ and $x_{i,after\ stress}$ the trait values assessed before and after stress application (Liu et al., 2022). Linear mixed models (LMM) with heat stress and $[CO_2]$ levels as fixed effects and time as a random effect were used to test the effects of individual and interactive effects of treatments and $[CO_2]$ on gas exchange characteristics through the stress recovery time. Tukey's honestly significant difference (HSD) test following one-way ANOVA was used to compare averages at different heat stress recovery time points. Two-way analyses of variance with leaf age and $[CO_2]$ as main effects and leaf age $\times$ $[CO_2]$ interaction were used to estimate the global effects of growth $[CO_2]$, leaf age and their interaction. All statistical analyses were conducted with R statistical software ver. 4.2.0 (2021) and visualized with OriginPro 2018 (OriginLab Corporation, Northampton, USA). The data used for ANOVA and LMM were tested for normality of distribution (Kolmogorov-Smirnov test), and when necessary, the data were log-transformed to improve the normality of data. All statistical effects were considered significant at $P < 0.05$. All datasets analyzed during this study are available in the EMU DSpace repository (Abiola et al. 2025b).

3 Results

3.1 Effects of growth $[CO_2]$ and leaf age on photosynthetic characteristics

In *Persea americana*, the net assimilation rates ranged from 6.42 to 8.61 $\mu mol\ m^{-2}\ s^{-1}$, with the lowest value observed in mature leaves under ambient $[CO_2]$ and the highest in young leaves under elevated $[CO_2]$ (Figure 1). The net assimilation rate (A) in unstressed young-mature leaves was 14% greater in elevated $[CO_2]$ -grown plants compared to ambient $[CO_2]$ -grown plants, indicated by a significant $[CO_2]$ effect on ($P < 0.05$), whereas A was similar between old-mature leaves, regardless of growth $[CO_2]$ (Table 1). A similar A measured suggest photosynthetic downregulation in high- $[CO_2]$ -grown plants, and this was supported by lower leaf nitrogen content per dry mass (N_M) and leaf phosphorus content per dry mass (P_M) under elevated $[CO_2]$ (Abiola et al., 2025a) for the effect of growth $[CO_2]$ on elemental contents). The apparent (C_i -based) maximum carboxylase activity of Rubisco (V_{cmax}) was higher in young-mature leaves under elevated $[CO_2]$ (mean $\pm$ SE = 51.95 $\pm$ 2.19) compared to young leaves under ambient $[CO_2]$ (42.38 $\pm$ 1.8, $P < 0.001$, Table 1). However, V_{cmax} was similar in old-mature leaves under both $[CO_2]$ conditions ($P > 0.05$, Table 1) despite reduced nutrient content (Abiola et al., 2025a) for the effect of growth $[CO_2]$ on elemental contents). For intercellular CO_2 concentration (C_i), young-mature leaves under elevated CO_2 exhibited significant main effects of growth $[CO_2]$ (mean $\pm$ SE = 175.02 $\pm$ 9.55) compared to ambient young-mature (205.15 $\pm$ 9.84, $P < 0.001$, Table 1). Again, old-

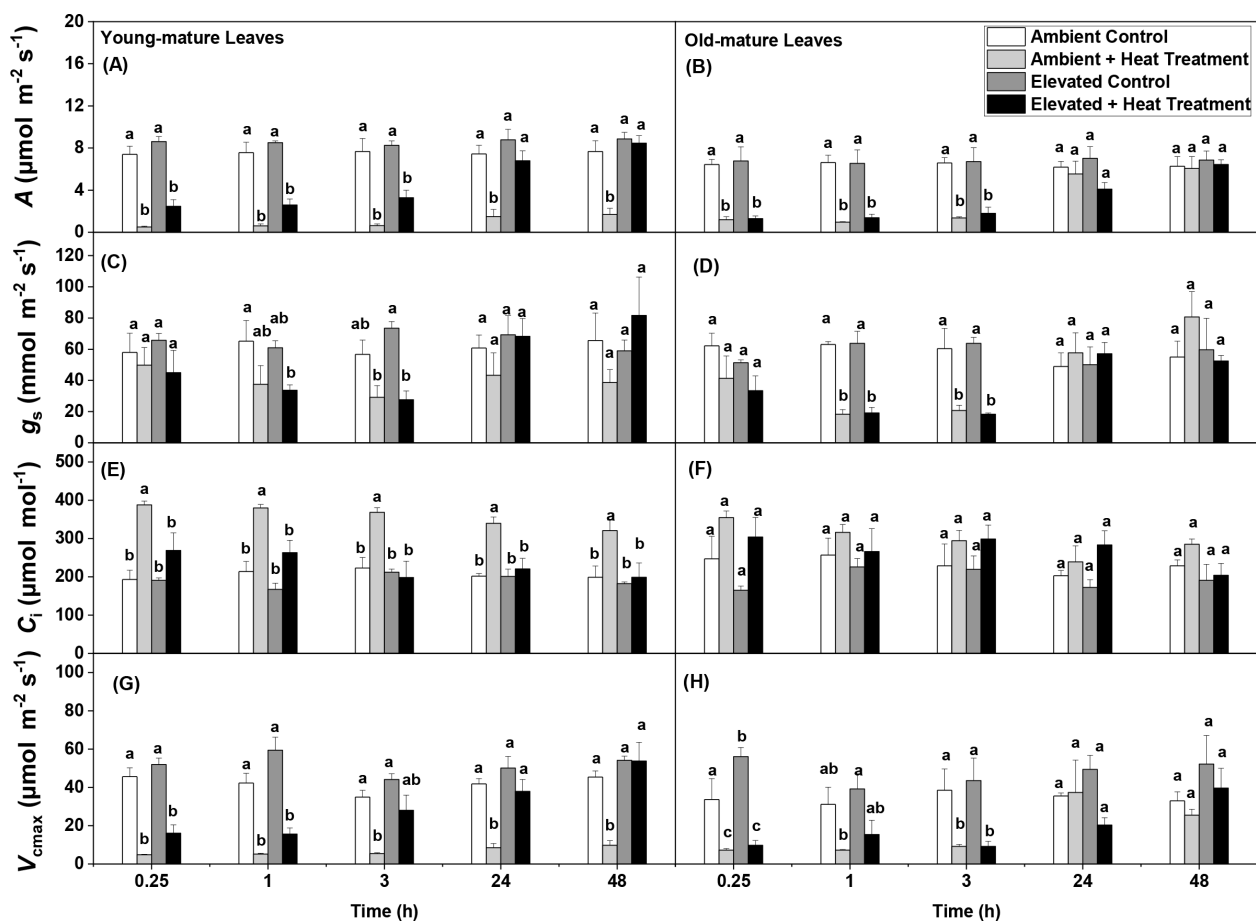


FIGURE 1

Light-saturated net assimilation rate (A) (A, B), stomatal conductance to water vapor (g_s) (C, D), intercellular CO_2 concentration (C_i) (E, F), and maximum carboxylase activity of Rubisco (V_{cmax}) (G, H) in control (25°C) and heat-treated (48°C) leaves of *Persea americana* measured at recovery time points of 0.25, 1, 3, 24 and 48 h. The heat treatment was applied by submerging the leaves in distilled water at 48°C for 10 min. For the control treatment, the water temperature was 25°C . All photosynthetic measurements were conducted at 24°C , at $700 \text{ mmol m}^{-2} \text{ s}^{-1}$ light, and at CO_2 concentration of $390\text{--}410 \text{ mmol mol}^{-1}$. Each bar represents the treatment average $\pm$ SE measured at different recovery times. Averages at each recovery time were compared by one-way ANOVA followed by a Tukey *post-hoc* test. Different lowercase letters denote significant differences ($P < 0.05$) among the treatment groups. $n = 4$.

mature leaves exhibited no C_i difference between $[\text{CO}_2]$ treatments ($P > 0.05$; Table 1).

Meanwhile, in both growth $[\text{CO}_2]$, A decreased with leaf age ($P < 0.05$ in ambient $[\text{CO}_2]$ and $P = 0.001$ for elevated $[\text{CO}_2]$), whereas the interactive effects of leaf age $\times$ $[\text{CO}_2]$ were not significant ($P > 0.05$; Table 2). There was no significant difference in V_{cmax} and stomatal conductance (g_s) between leaf ages in both growth $[\text{CO}_2]$. However, leaf dry mass per unit area (M_A) increased with leaf age, and the interaction between leaf age $\times$ $[\text{CO}_2]$ was significant ($P < 0.05$, Table 2).

3.2 Impacts of heat stress on foliage photosynthetic characteristics as affected by growth $[\text{CO}_2]$ and leaf age

Heat stress (48°C) led to immediate decreases in foliage photosynthetic characteristics in all cases with full or partial recovery depending on growth $[\text{CO}_2]$ and leaf age. The degree of

recovery varied between young- and old-mature leaves and dependence on the growth $[\text{CO}_2]$ as well (Figures 1, 2; Table 1). Heat shock resulted in decreases in light saturated photosynthesis (A) in both young and old-mature leaves grown under different $[\text{CO}_2]$ (Figures 1A, B, 2A, B, Table 1). The degree of reductions in A in all the heat-stressed plants was proportional to reductions in V_{cmax} (Figures 1A, B, G, H, 2A, B, E, F, Supplementary Figure S1, Table 1). The application of heat stress uncoupled the relationship between A and g_s (Figures 1C, D, 2C, D, Table 1), and thus, the heat stress-dependent changes in net assimilation rate through recovery were primarily driven by changes in V_{cmax} (biochemical limitation) for different combinations of leaf age and growth $[\text{CO}_2]$ (Supplementary Figure S1).

Reductions of A due to heat stress were the greatest in young-mature leaves of plants grown under ambient $[\text{CO}_2]$ (Figures 1A, 2A, Table 1). At the end of the 48 h heat stress recovery period, A , g_s and V_{cmax} in all the stressed plants recovered fully, except for the young-mature leaves grown under ambient $[\text{CO}_2]$ (Figures 1A–D, G, H, 2A–F). In heat-stressed young-mature leaves grown under

TABLE 1 Output of linear mixed model for individual and interactive effects of growth [CO₂], ambient (400 $\mu\text{mol mol}^{-1}$) vs. elevated (800 $\mu\text{mol mol}^{-1}$) and treatment, control vs. heat stress, and recovery time on leaf gas exchange characteristics (light-saturated net assimilation rate, *A*; stomatal conductance to water vapor, *g_s*; intercellular CO₂ concentration (*C_i*) and maximum carboxylase activity of Rubisco; *V_{cmax}*) within the same leaf age groups (young- or old-mature leaves grown under ambient and elevated [CO₂]) in *Persea americana*.

Traits	Effects	χ^2	df	P value
<i>A</i> ($\mu\text{mol m}^{-2} \text{s}^{-1}$)				
Young-mature leaves	[CO ₂]	58.9	1	0.000***
	Time	20.78	1	0.000***
	Treatment	260.09	1	0.000***
	Time x Treatment	21.57	1	0.000***
	[CO ₂] x Time	15.00	1	0.000***
	[CO ₂] x Treatment	22.07	1	0.000***
Old-mature leaves	[CO ₂]	0.88	1	0.35 ^{ns}
	Time	33.85	1	0.000***
	Treatment	107.27	1	0.000***
	Time x Treatment	45	1	0.000***
	[CO ₂] x Time	0.26	1	0.61 ^{ns}
	[CO ₂] x Treatment	2.34	1	0.13 ^{ns}
<i>g_s</i> ($\text{mmol m}^{-2} \text{s}^{-1}$)				
Young-mature leaves	[CO ₂]	0.65	1	0.42 ^{ns}
	Time	0.20	1	0.66 ^{ns}
	Treatment	20.38	1	0.000***
	Time x Treatment	3.35	1	0.07 ^{ns}
	[CO ₂] x Time	1.23	1	0.27 ^{ns}
	[CO ₂] x Treatment	0.51	1	0.48 ^{ns}
Old-mature leaves	[CO ₂]	0.32	1	0.57 ^{ns}
	Time	13.72	1	0.000***
	Treatment	8.35	1	0.004**
	Time x Treatment	15.79	1	0.000***
	[CO ₂] x Time	0.64	1	0.42 ^{ns}
	[CO ₂] x Treatment	0.97	1	0.33 ^{ns}
<i>C_i</i> ($\mu\text{mol mol}^{-1}$)				
Young-mature leaves	[CO ₂]	51.91	1	0.000***
	Time	9.53	1	0.002**
	Treatment	54.35	1	0.000***
	Time x Treatment	3.65	1	0.06 ^{ns}
	[CO ₂] x Time	0.08	1	0.77
	[CO ₂] x Treatment	29.10	1	0.000***
Old-mature leaves	[CO ₂]	2.90	1	0.09 ^{ns}
	Time	4.02	1	0.04*
	Treatment	20.30	1	0.000***
	Time x Treatment	1.65	1	0.20 ^{ns}
	[CO ₂] x Time	0.20	1	0.65 ^{ns}
	[CO ₂] x Treatment	0.00	1	0.99 ^{ns}
<i>V_{cmax}</i> ($\mu\text{mol m}^{-2} \text{s}^{-1}$)				
Young-mature leaves	[CO ₂]	68.85	1	0.000***
	Time	25.22	1	0.000***
	Treatment	162.28	1	0.000***
	Time x Treatment	14.04	1	0.000***
	[CO ₂] x Time	15.93	1	0.002***
	[CO ₂] x Treatment	20.63	1	0.000***
Old-mature leaves	[CO ₂]	2.95	1	0.09 ^{ns}
	Time	9.43	1	0.002**
	Treatment	37.71	1	0.000***
	Time x Treatment	6.48	1	0.01*
	[CO ₂] x Time	0.62	1	0.43 ^{ns}
	[CO ₂] x Treatment	1.85	1	0.17 ^{ns}

Experimental treatments as in Figure 1. All photosynthetic measurements were conducted at 24 °C, at 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light and at CO₂ concentration of 390–410 $\mu\text{mol mol}^{-1}$. After application of the heat stress (48 °C for 10 min) or control treatment (25 °C for 10 min) the leaf physiological characteristics were measured at 0.25, 1, 3, 24 and 48 h (Time effect). *n* = 4 for each leaf age/[CO₂] treatment combination. The models were fitted by maximum likelihood method. Data from Abiola et al. (2025b), available at EMU DSpace: <https://doi.org/10.15159/EDS.DT.25.02>.

TABLE 2 Output of two-way ANOVA for individual and interactive effects of leaf age, young-mature vs. old-mature, and growth [CO₂], ambient (400 μmol mol⁻¹) vs. elevated (800 μmol mol⁻¹), on light-saturated net assimilation rate (*A*, μmol m⁻² s⁻¹), stomatal conductance to water vapor (*g_s*, mmol m⁻² s⁻¹), intercellular CO₂ concentration (*C_i*, μmol mol⁻¹), maximum carboxylase activity of Rubisco (*V_{cmax}*, μmol m⁻² s⁻¹), and leaf dry mass per unit area (*M_A*) in *P. americana*.

Species	<i>A</i>	<i>g_s</i>	<i>C_i</i>	<i>V_{cmax}</i>	<i>M_A</i>
F-values for two-way ANOVA					
<i>P. americana</i>					
[CO ₂]	3.97*	0.31ns	4.56*	14.46***	98.64***
Leaf age	17.19***	1.85ns	1.64ns	3.99ns	551.78***
[CO ₂] x Leaf age	0.96ns	0.14ns	0.88ns	0.42ns	9.51*

The young-mature leaves were 60–70 days old, and the old-mature leaves were 110–120 days old. All photosynthetic measurements were conducted at 24 °C, at 700 μmol m⁻² s⁻¹ light and at CO₂ concentration of 390–410 μmol mol⁻¹. The global effects of [CO₂] and leaf age as analyzed by two-way ANOVA are reported in the main text. Statistical significance is shown as: * - *P* < 0.05, ** - *P* < 0.01, and *** - *P* < 0.001. *n* = 3–4. Data from Abiola et al. (2025b), available at EMU DSpace: <https://doi.org/10.15159/EDS.DT.25.02>.

elevated [CO₂], full recovery of *A* (*P* > 0.6 between control and at 48 h after heat shock) was accompanied by a greater recovery of *g_s* and *V_{cmax}* and a decrease in intercellular CO₂ concentration (*C_i*) than in young-mature leaves grown under ambient [CO₂] (Figures 1A–F, 2A–F).

4 Discussion

We grew the saplings of tropical evergreen fruit tree avocado (*Persea americana*) under ambient (400 μmol mol⁻¹) and elevated (800 μmol mol⁻¹) growth [CO₂] and determined the leaf photosynthetic responses to an heat shock treatment (10 min. exposure to 48 °C) in young-mature and old-mature leaves. The heat stress applied is close to the heat stress limit of vascular plants (Kask et al., 2016; Turan et al., 2019; Okereke et al., 2022) and allows gaining an insight into variation in heat stress resistance and recovery in dependence on leaf age and [CO₂] treatment. In nature, it resembles the heat stress the plants might be exposed on hot days upon sunflecks when the leaves can rapidly heat up 5–10 °C above the ambient temperature (Leakey et al., 2005; Hüve et al., 2019).

So far, the effects of heat stress on primary and secondary metabolism have been investigated in numerous studies (Jardine et al., 2015; Teskey et al., 2015; Chatterjee et al., 2020; Okereke et al., 2021; 2022; Sulaiman et al., 2023), however, much less is known about the interaction between elevated [CO₂] and heat stress, and no study has looked at heat stress and growth [CO₂] x leaf age interaction. This is a significant omission as both aging and elevated [CO₂] alter leaf physiological activity, structure and content of protective chemicals such as antioxidants and sugars that collectively can also affect heat resistance.

4.1 Leaf-age and growth [CO₂]-dependent changes in foliage photosynthetic activity as related to leaf elemental contents and structure

Changes in foliage net assimilation rate (*A*) can occur due to alterations in stomatal conductance (*g_s*), mesophyll conductance

(*g_m*) and maximum carboxylase activity of Rubisco (*V_{cmax}*) (Ethier et al., 2006; Niinemets, 2018). In our study we looked at changes in *g_s* and apparent *V_{cmax}* that does not consider possible differences in *g_m*. Generally, *A* decreases in non-senescent leaves with increasing leaf age, but the underlying physiological mechanisms can be species-specific (Ethier et al., 2006; Kositsup et al., 2010; Abiola et al., 2025a). In the current study, *A* was lower in old-mature leaves in comparison to young-mature leaves. Given the similar nutrient contents in the leaves with varying age in *P. americana* (Abiola et al., 2025a), the age-related reduction in photosynthetic capacity was not associated with limiting nutrient contents, but might indicate a decrease in mesophyll conductance due to thickening of cell walls (Niinemets et al., 2005, 2006; Onoda et al., 2017). This is plausible given the increase of leaf dry mass per unit area (*M_A*) with increasing leaf age (Table 2).

Elevated [CO₂] is expected to increase *A* as the result of enhanced CO₂ availability for photosynthesis (Reich et al., 2018; Zheng et al., 2019; Jiang et al., 2020). However, previous studies have shown that *A* in plants under elevated [CO₂] can increase, reduce or even remain unaffected (Urban et al., 2012; Abiola et al., 2025a). Unaffected or reduced *A* is indicative of photosynthetic downregulation (see Introduction). Typically, plants grown under elevated [CO₂] have lower nutrient contents and higher *M_A* (Sun et al., 2013a; Fleischer and Terrer, 2022; Abiola et al., 2025a). In our study, leaf nitrogen content per dry mass (*N_M*) and leaf phosphorus content per dry mass (*P_M*) were higher in both young-mature and old-mature leaves grown under ambient [CO₂] compared to elevated [CO₂]-grown plants (Abiola et al., 2025a). Previous studies have shown that decreases in foliage nutrient contents such as N and P contribute to decreases in photosynthesis under elevated [CO₂]-grown plants (Jifon and Wolfe, 2002; Sanz-Sáez et al., 2013; Reich and Hobbie, 2013; Arrizabalaga-Arriazu et al., 2020). Lower *N_M* is typically associated with lower photosynthetic capacity per dry mass (*A_{mass}*), while the rate of photosynthesis per area (*A_{area}*) also depends on *M_A*, i.e., *A_{area}* = *M_A* x *A_{mass}* (Onoda et al., 2017; Onoda and Wright, 2018). Thus, the enhancement of *M_A* under elevated [CO₂] might compensate for reductions in *A_{mass}*. However, as discussed above, a higher *M_A* can be associated with greater investment in cell walls, which reduces photosynthetic efficiency by lowering N allocation to photosynthetic proteins and decreasing CO₂ diffusion

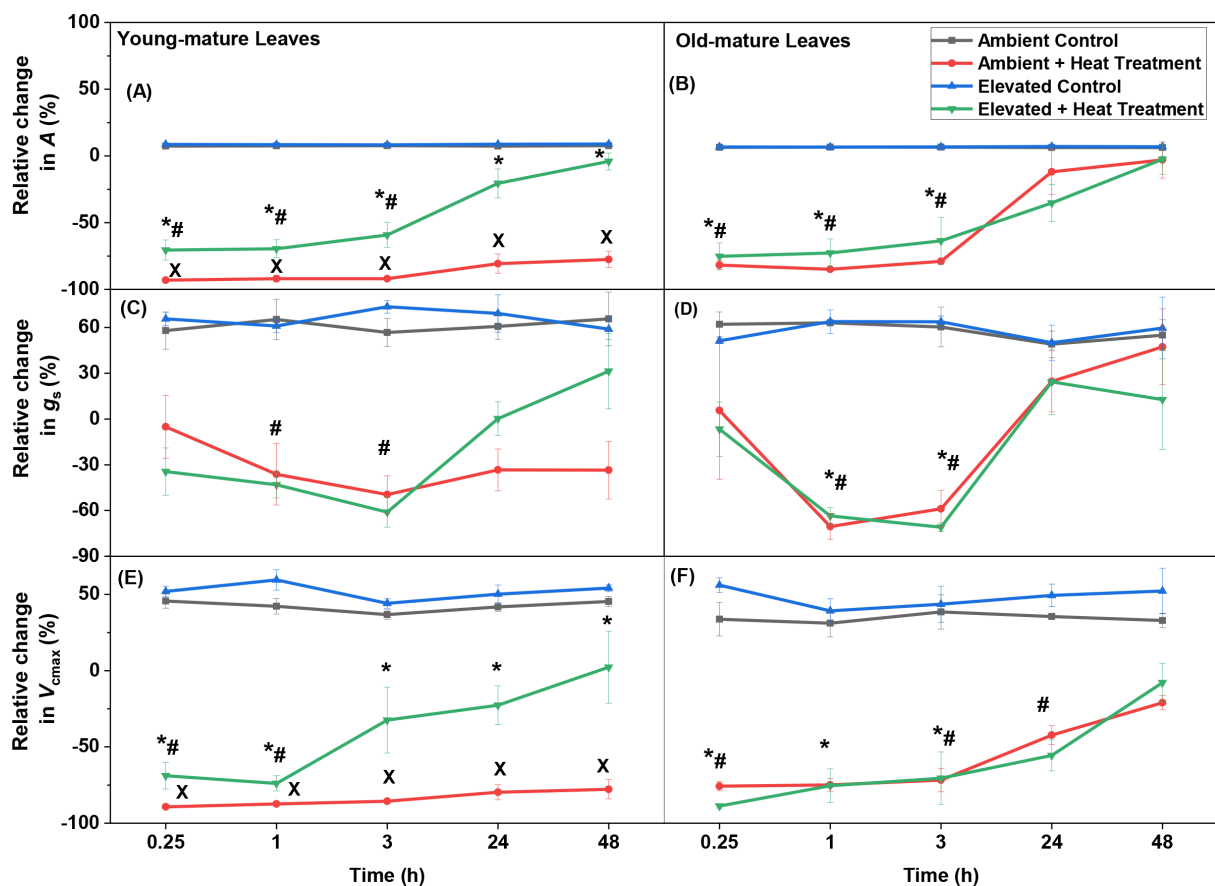


FIGURE 2

The relative change (Equation 1; degree of recovery) of net assimilation rate (A) (A, B), stomatal conductance to water vapor (g_s) (C, D), and maximum carboxylase activity of Rubisco (V_{cmax}) (E, F) after heat stress application ($48^\circ C$ applied for 10 min) in leaves of *Persea americana* with recovery time points of 0.25, 1, 3, 24 and 48 h (average $\pm$ SE). (A–F) show the degree of recovery of leaf age groups from plants grown at ambient ($400 \text{ mmol mol}^{-1}$) and elevated ($800 \text{ mmol mol}^{-1}$) $[CO_2]$, relative to each treatment/leaf age combination prior to heat stress application. The photosynthetic measurements were conducted at $24^\circ C$, at $700 \text{ mmol m}^{-2} \text{ s}^{-1}$ light and at CO_2 concentration of $390\text{--}410 \text{ mmol mol}^{-1}$. Statistically significant differences ($P < 0.05$) of heat-treated leaf trait values relative to control treatment are shown by * for ambient $[CO_2]$ -grown plants and by # for elevated $[CO_2]$ -grown plants. Significant differences among heat-treated ambient and elevated- $[CO_2]$ -grown plants are shown by x (one-way ANOVA, $P < 0.05$; $n = 4$).

due to low mesophyll conductance associated with thick cell walls (Tosens et al., 2016; Osnas et al., 2018). We argue that further studies should look at elevated $[CO_2]$ effects on mesophyll conductance in different aged leaves.

4.2 Leaf maturation and elevated $[CO_2]$ enhanced heat stress tolerance of photosynthesis in *P. americana*

We demonstrated that reductions of A in all heat-stressed *P. americana* were primarily due to non-stomatal factors, specifically, reductions in ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity (Figures 1, 2, Supplementary Figure S1). Rapid reductions in Rubisco maximum activity upon heat stress might be attributed to thermal inhibition of Rubisco enzymatic activity (Hüve et al., 2011; Kask et al., 2016; Djanaguiraman et al., 2018). However, photosynthesis recovered in all the heat shock-treated *P. americana* leaves except in young-

mature leaves of *P. americana* grown under ambient $[CO_2]$ (Figures 1A, E), likely indicating direct thermal damage of photosynthetic components in these leaves (Hüve et al., 2019; Sulaiman et al., 2023).

Previous studies have associated severe heat stress ($>47^\circ C$) with rapid reductions in A in several tropical species (Okereke et al., 2021; 2022). The current study showed that severe heat resulted in an immediate decrease in A by ca. 82% in old-mature leaves of *P. americana* (Figures 1B, 2B; Table 1), similar to the reductions observed previously in foliage of old-mature tropical herbaceous species including *Amaranthus cruentus*, *A. hybridus*, *Solanum aethiopicum*, *Telfairia occidentalis* and *Vigna unguiculata* (Okereke et al., 2021), but less than the reductions observed in the foliage of the tree species *Carica papaya* (Okereke et al., 2022). Also, unlike in *C. papaya* (Okereke et al., 2022), A in old-mature *P. americana* recovered completely after severe heat stress application (Figures 1B, 2B), suggesting greater heat stress tolerance of old-mature *P. americana* leaves. The recovery of old-mature leaves under ambient $[CO_2]$ indicates enhancement of photosynthetic apparatus tolerance of

heat-stressed plants. As suggested in the Introduction, such an enhancement in heat resistance with increasing leaf age might have multiple causes, including greater antioxidative capacity, enhanced repair capacity and enhanced formation of stress-protective compounds, partly as the result of greater physiological activity (Albert et al., 2018; Krause et al., 2014; Drake et al., 2018; Tarvainen et al., 2022). However, in young-mature leaves of *P. americana* grown under ambient [CO₂] (Figures 1A, 2A), photosynthesis did not recover (<78%) after severe heat stress application, suggesting the photosynthetic sensitivity of the young-mature leaves.

Both young and old-mature leaves grown under elevated [CO₂] exhibit greater heat resistance than those grown under ambient [CO₂], as evidenced by their enhanced recovery to control conditions following heat shock (Figures 1A, B, 2A, B). This demonstrates that increased leaf age and elevated [CO₂] independently and synergistically improved heat shock tolerance and recovery of photosynthetic activities in *P. americana*. Typically, heat protection of photosynthesis by elevated [CO₂] is associated with higher concentration of sugars in leaves under elevated [CO₂] (Taub et al., 2000; Zhang et al., 2019). This is because of greater photosynthesis rate under elevated [CO₂], and nutrient limitation of growth (lower sink activity) (Leakey et al., 2009; Habermann et al., 2019).

In conclusion, our study demonstrates that *P. americana* is a relatively heat tolerant species. Nevertheless, under transient heatwaves at current ambient [CO₂], photosynthesis of young leaves is expected to be strongly reduced. Increases in ambient [CO₂] are expected to improve the heat resistance of young leaves and thus, elevation in [CO₂] might improve the whole canopy photosynthesis in heat-exposed avocado. Future research should examine effects of heat stress on a wider range of tropical woody species, including impacts on canopy-level carbon gain, reproductive development, and yield under future climate scenarios.

5 Conclusions

Our study suggests that the interactive effect of elevated [CO₂] and heat stress might in nature contribute to improved heat resistance in plants with actively growing canopy. We demonstrated that leaf developmental stage and the growth [CO₂] play a major role in how plant photosynthetic characteristics respond to heat stress conditions through a 48 h recovery period. The results showed a certain downregulation of photosynthesis in old-mature plants grown under elevated [CO₂]. Upon heat stress application, photosynthetic reductions were mainly associated with Rubisco limitation with a minimal contribution of stomatal conductance. During 48 h recovery under ambient temperature, plants from all leaf age/treatment combinations recovered to the control condition at the end of the experiment, except young-mature leaves under ambient [CO₂]. Thus, elevated [CO₂] is expected to enhance the heat resistance of younger leaves. Given that these leaves have more time left to photosynthesize than older mature leaves, losing these leaves due to a heat stress episode would be highly devastating for the whole plant. Thus, the overall impact of elevated [CO₂] in *Persea americana* would be the increased heat

resistance of the long-term whole canopy photosynthetic production. Although elevated [CO₂] improved the heat resistance of *P. americana*, heat waves during leaf development are expected to have a major impact on foliage photosynthetic activity under the current and future [CO₂]. Future studies should examine the biochemical and physiological mechanisms responsible for greater heat resistance of young leaves in elevated [CO₂]-grown plants and look at the generality of this finding across tropical species with continuously expanding canopies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: EMU DSpace: <https://dspace.emu.ee/bitstreams/3bc232b7-eddc-42fd-ad01-6adbfcd86248/download>.

Author contributions

YA: Visualization, Methodology, Conceptualization, Writing – original draft, Writing – review & editing, Investigation, Formal analysis. HS: Writing – review & editing, Formal analysis. EK: Investigation, Conceptualization, Writing – review & editing, Methodology. ÜN: Funding acquisition, Conceptualization, Supervision, Methodology, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2025.1638556/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Correlations between the apparent (C_i-based) maximum carboxylation rate of Rubisco (V_{cmax}) and net CO₂ assimilation rate (A) after heat treatment (48 °C applied for 10 min) in *P. americana* leaves grown at ambient (400 μmol mol⁻¹ and elevated (800 μmol mol⁻¹) [CO₂]. Each data point represents an average of four independent replicates measured at 0.25, 1, 3, 24, and 48 h after heat stress treatments. Panel A shows young-mature leaves from ambient and elevated growth [CO₂], while panel B shows old-mature leaves from ambient and elevated growth [CO₂].

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