Environmental factors have a greater influence on photosynthetic capacity in C_4 plants than biochemical subtypes or growth forms

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Summary

- Our understanding of how photosynthetic capacity varies among C_4 species and across growth and measurement conditions remains limited.
- We collated 1696 CO_2 response curves of net CO_2 assimilation rate (A/C_i curves) from C_4 species grown and measured at various environmental conditions and used these data to estimate the apparent maximum carboxylation activity of phosphoenolpyruvate carboxylase (V_{pmaxA}) and CO_2-saturated net photosynthetic rate (A_{max}), two key parameters describing photosynthetic capacity. We examined how V_{pmaxA} and A_{max} vary with species-specific traits, growth and measurement conditions.
- We found little systematic variation of V_{pmaxA} and A_{max} across the classical C_4 biochemical subtypes or growth forms, but showed that growth temperature and measurement conditions are major factors determining C_4 photosynthetic capacity. We found no evidence that common C_4 model species (e.g. maize, sorghum and *Setaria viridis*) differ in photosynthetic capacity from other C_4 species when grown in controlled environments. However, C_4 model

Key words: A/C_i curve, A_{max}, C_4 biochemical subtype, C_4 photosynthesis, environmental response, photosynthesis modelling, V_{pmax}.

species showed up to twice the photosynthetic capacity of other C_4 species when grown in the field.

- Our multivariate model accounts for 47–51% of the variation reported in V_{pmaxA} and A_{max}, and we argue that environmental conditions have a greater influence on C_4 photosynthetic capacity than biochemical subtypes or growth forms.

Introduction

C_4 photosynthesis evolved at least 66 times in the last 35 million years in response to inefficiencies in C_3 photosynthesis under hot, arid and low CO_2 environments that promote photorespiration (Sage, 2016). In C_4 plants, a CO_2-concentrating mechanism increases CO_2 concentrations at the site of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in bundle sheath cells, resulting in an increase in its carboxylase activity, a reduction in photorespiration and an increase in photosynthetic efficiency (Hatch, 1987). Compared with their C_3 counterparts, C_4 plants require one-third of the amount of RuBisCO to achieve the same or higher rates of net CO_2 fixation, leading to greater nitrogen-use efficiency (Ghannoum *et al.*, 2011). C_4 plants also have higher water-use efficiency because they can maintain a lower stomatal conductance relative to the rate of net photosynthetic CO_2 assimilation (A), which supports greater biomass production when water is limited (Evans & von Caemmerer, 1996; Leegood, 2002; Taylor *et al.*, 2010; Ghannoum *et al.*, 2011). Thus, C_4 plants tend to outperform C_3 plants in hot and dry environments, lending them a competitive advantage in subtropical and semiarid ecosystems. As a result, C_4 plants occupy *c.* 20% of the land surface and contribute up to 23% of global gross primary productivity, although they comprise $< 5\%$ of terrestrial plant species (Still *et al.*, 2003; Luo *et al.*, 2024).

Currently, C_4 global primary productivity is typically estimated in Earth System Models using a simplified mechanistic model that uses parameters reflecting photosynthetic capacity (Still *et al.*, 2019; Griffith *et al.*, 2020). The model and its parameter values are largely derived from data collected on a single NADP-dependent malic enzyme (NADP-ME) subtype monocot, *Zea mays* (maize; Collatz *et al.*, 1992). Consequently, the model does not capture substantial variation across C_4 species in the biochemistry underlying CO_2 assimilation (Hatch, 1987), or in their growth forms (Poorter *et al.*, 2009; Liu *et al.*, 2019). The responses of photosynthetic capacity to environmental parameters, such as temperature and irradiance, are also taken from the same study on maize (Collatz *et al.*, 1992). This poses a problem because NADP-ME subtype monocots (e.g. maize) only account for 31% of all C_4 species and are predominantly found in regions with relatively high precipitation, due to their greater drought sensitivity than other C_4 subtypes and growth forms (Ripley *et al.*, 2010; Fan, 2023; Raubenheimer *et al.*, 2023). Thus, it remains unclear whether data from a domesticated C_4 NADP-ME type species can reliably represent the photosynthetic performance of all C_4 plants, including nondomesticated species and those belonging to other C_4 subtypes. Over the last 30 yr, many studies have reported how C_4 photosynthetic parameters vary in response to environmental drivers and described genetic differences within and between species. However, these findings are scattered across publications, except for an analysis by Pignon & Long (2020), and have not been subjected to data synthesis in a manner that could advance the representation of this important plant functional type in Earth System Models.

The most common way to estimate leaf-level photosynthetic capacity is to measure CO_2 response curves of net CO_2 assimilation rate (i.e. A/C_i curves), and assess both the initial slope of the A/C_i curve (a parameter closely related to the apparent maximum activity of phosphoenolpyruvate (PEP) carboxylase, V_{pmaxA}) and the horizontal asymptote of the A/C_i curve (i.e. the rate of CO_2-saturated net photosynthesis, termed A_{max}). The initial slope of a C_4 A/C_i curve is generally insensitive to changes in leaf measurement temperatures (T_{leaf}) for plants grown at moderate temperatures (Long & Woolhouse, 1978; Laisk & Edwards, 1997; Sage, 2002), although it can be reduced in plants grown under chilling conditions, possibly reflecting an increase in the activation energy of PEP carboxylase (PEPc) at low temperatures (Pittermann & Sage, 2001; Kubien & Sage, 2004). By contrast, A_{max} increases with increasing T_{leaf} in C_4 plants grown under moderate temperatures (Sage & Kubien, 2007). As with T_{leaf}, the initial slope of a C_4 A/C_i curve remains largely stable when measured at moderate vs high photosynthetic photon flux density ($PPFD$; e.g. 500 vs 1500 μmol photons $m^{-2}\,s^{-1}$), suggesting that irradiance does not directly change PEPc kinetics, and PEP regeneration may not be the limiting step of photosynthesis at low C_i (Pengelly *et al.*, 2010). However, A_{max} is sensitive to changes in irradiance and increases when measured under high $PPFD$ (Pengelly *et al.*, 2010). This irradiance response of A_{max} may result from the alleviation of chloroplastic electron transport limitation under high $PPFD$ (Ermakova *et al.*, 2019, 2023), thereby enhancing energy availability for PEP or ribulose 1,5-bisphosphate (RuBP) regeneration, leading to higher overall net photosynthesis (von Caemmerer & Furbank, 2016). Taken together, the responses of photosynthetic capacity to measurement conditions are dynamic, with the initial slope of the A/C_i curve tending to be less affected by T_{leaf} and $PPFD$ than A_{max}.

C_4 photosynthetic capacity can also be altered by growth temperatures. There is evidence that when measured under the same conditions, photosynthetic capacity (particularly A_{max}) is reduced in C_4 plants grown at warm to high temperatures (i.e. 30–43°C) compared with control plants. This is resulted from lower RuBisCO content and/or activity, NADP-ME activity, cytochrome *f* content and carbonic anhydrase activity (Pearcy, 1977; Ward, 1987; Dwyer *et al.*, 2007). By contrast, the activity of PEPc (and potentially V_{pmaxA}) may not be affected by high growth temperatures, possibly due to its high thermal stability (Chen *et al.*, 1994; Chinthapalli *et al.*, 2002; Boyd *et al.*, 2015). At low growth temperatures, many chilling-tolerant, cool-adapted C_4 plants show no change in photosynthetic rates or RuBisCO concentrations compared with control plants (Pittermann & Sage, 2001; Cavaco *et al.*, 2003; Naidu *et al.*, 2003; Kubien & Sage, 2004), although the content of other photosynthetic enzymes (e.g. pyruvate, phosphate dikinase (PPDK)) can increase (Wang *et al.*, 2008). These findings highlight that while some C_4 plants can adjust their photosynthetic machinery in response to growth temperature, both high and low temperatures can impose biochemical constraints that limit photosynthetic capacity via acclimation and thermal stress.

Given that C_4 photosynthesis is thought to be an evolutionary response to high photorespiratory loads (Sage, 2004), there has been considerable interest in the response of C_4 photosynthesis to variation in CO_2 concentrations. When C_4 plants are grown in controlled environments, A_{max} can increase under subambient CO_2 conditions, reflecting an up-regulation of RuBisCO capacity to enhance carbon capture (Ripley *et al.*, 2013; Pinto *et al.*, 2014; Cunniff *et al.*, 2017), but remains largely unchanged under the elevated CO_2 levels projected for the coming century (Leakey, 2009; Heckman *et al.*, 2024). By contrast, while the capacity of PEPc in controlled environment-grown plants can also be increased at glacial CO_2 concentrations, it can be reduced at elevated CO_2 concentrations (Wong, 1979; Ghannoum *et al.*, 2000; Pinto *et al.*, 2014). However, when C_4 plants are grown in the field, changes in growth CO_2, concentrations had little effect on photosynthesis or the activity/capacity of key photosynthetic enzymes including PEPc (Leakey *et al.*, 2006; Leakey, 2009; Markelz *et al.*, 2011). Both subambient and elevated CO_2 concentrations influence stomatal conductance, leading to changes in plant water relations and interactions with drought stress that can be complex in nature and create opportunities for crop improvement (Ghannoum *et al.*, 2000; Markelz *et al.*, 2011; Leakey *et al.*, 2019). Although our understanding of how C_4 plants respond to changes in CO_2 concentration and drought, along with their underlying physiological mechanisms, has advanced in recent decades (e.g. Tissue *et al.*, 1995; Wang *et al.*, 2020; Ding *et al.*, 2022), this knowledge has yet to be fully integrated into ecosystem models (Cowling *et al.*, 2007; Still *et al.*, 2019).

There is some evidence suggesting that the response of C_4 photosynthetic capacity to growth environment depends on the biochemistry, life history and plant functional type of the species (e.g. growth form: monocot vs eudicot;Liu *et al.*, 2019). Based on the decarboxylases involved, the C_4 photosynthetic pathway can be categorised into three classical biochemical subtypes: NADP-ME subtype, NAD-dependent malic enzyme (NAD-ME) subtype and PEP-carboxykinase (PCK) subtype (Hatch, 1987; von Caemmerer & Furbank, 2016). Considerable variation in biochemical components (e.g. enzyme abundance) and photosynthetic capacity has been found in C_4 plants of different growth forms within and between C_4 biochemical subtypes. For example, the maximum carboxylation activity of PEPc and RuBisCO, A_{max} and leakiness (i.e. the CO_2 fraction that leaks out from bundle sheath cells) responded to a short-term increase in T_{leaf} differently among the three C_4 biochemical subtypes (Sonawane *et al.*, 2017). In addition, C_4 annual species have been shown to exhibit a greater sensitivity of the chloroplastic electron transport capacity to T_{leaf} than C_4 perennial species (Smith & Dukes, 2017). These findings suggest that differences in C_4 biochemistry and growth form could play a role in determining photosynthetic responses to environmental conditions.

To understand how C_4 photosynthetic capacity responds to environmental cues – and whether these responses differ systematically among biochemical subtypes and growth forms – data are needed from diverse species grown and measured under contrasting environmental conditions. Unfortunately, while numerous studies have evaluated photosynthetic capacity of C_4 plants under various growth and measurement conditions (reference herein), these data are largely siloed in their separate publications, making it challenging to identify broad patterns in photosynthetic traits across C_4 species. In this study, we used published gas-exchange data to explore the drivers of variation in C_4 photosynthetic capacity. Here, A/C_i curves measured from diverse C_4 species across studies were collated to estimate V_{pmaxA} and A_{max} with a widely used C_4 mechanistic model (von Caemmerer & Furbank, 1999; von Caemmerer, 2000, 2021). We assessed how V_{pmaxA} and A_{max} are affected by species-specific traits (biochemical subtype and growth form) and environmental parameters (growth and measurement conditions). For growth conditions, we considered growth temperature and growth CO_2 concentration. We also considered growth location (whether plants were grown in outdoor fields or in indoor controlled environments; see the Materials and Methods section for definitions). Compared with outdoor plants, indoor plants may experience limited growth space and different environments (e.g. fewer pests, relatively steady temperature and light conditions), which can affect overall morphology and physiology (Poorter *et al.*, 2016). For measurement conditions, we were interested in how C_4 photosynthetic capacity varies with measurement T_{leaf} and *PPFD*. An A/C_i curve should be measured under light-saturating conditions, such that *PPFD* is often not considered a key factor in photosynthetic capacity. However, the *PPFD* required for light saturation of net photosynthesis can vary with growth conditions, and A/C_i curves can also be measured under sub-saturating *PPFD* to address specific research questions (e.g. Sonawane *et al.*, 2018).

Using these data, we test the following key hypotheses:

(**1**) V_{pmaxA} and A_{max} are positively correlated, and both vary systematically with C_4 biochemical subtype and growth form.

(**2**) Indoor plants show a higher photosynthetic capacity than their outdoor counterparts due to more optimal growth conditions.

(**3**) Cool-grown plants have a higher photosynthetic capacity relative to their warm-grown counterparts when measured at a common T_{leaf}. While A_{max} increases with increasing T_{leaf}, V_{pmaxA} remains relatively stable.

(**4**) Elevated growth CO_2 concentrations have no effect on photosynthetic capacity in C_4 species.

Lastly, we assess whether the photosynthetic capacity of popular C_4 model species – maize, *Sorghum bicolor* (sorghum) and *Setaria viridis* – the three most abundant species in our dataset, is similar to that of other C_4 species to assess whether data from these three species can be used to represent the broad range of undomesticated C_4 species found in nature. To date, no study has explicitly tested whether model C_4 species differ in photosynthetic capacity from non-model species using a comprehensive dataset.

Materials and Methods

Data acquisition

This work was conceived by members of the C_4 Working Group supported by the US Geological Survey's Powell Center, with data collaboratively sourced within the group and from researchers closely associated with its members. C_4 A/C_i data were compiled from 52 studies published between 2001 and 2024 (Anderson *et al.*, 2001; Pittermann & Sage, 2001; Cousins & Bloom, 2003; Kim *et al.*, 2006; Leakey *et al.*, 2006; Nippert *et al.*, 2007; Ripley *et al.*, 2007, 2008, 2010, 2013; Carmo-Silva *et al.*, 2008; Cunniff *et al.*, 2008; Horst *et al.*, 2008; Mantlana *et al.*, 2008; Osborne *et al.*, 2008; Soares *et al.*, 2008; Kakani *et al.*, 2008a,b; Pengelly *et al.*, 2010; Arena *et al.*, 2011; de Souza, 2011; Pinto *et al.*, 2011, 2014; Soares-Cordeiro *et al.*, 2011; Bloom *et al.*, 2012; Fay *et al.*, 2012; Feng *et al.*, 2012; Sun *et al.*, 2012; Vogan & Sage, 2012; Wang *et al.*, 2012; Chen *et al.*, 2013; Sage *et al.*, 2013; Bissinger *et al.*, 2014; Friesen *et al.*, 2014; Ge *et al.*, 2014; Oakley *et al.*, 2014; Sharwood *et al.*, 2014; Xu *et al.*, 2014; Głowacka *et al.*, 2015; Peixoto & Sage, 2017; Sonawane *et al.*, 2017, 2018; Smith & Dukes, 2018; Li *et al.*, 2019, 2021, 2022; Khoshravesh *et al.*, 2020; Pathare *et al.*, 2020; Danila *et al.*, 2021; Peixoto *et al.*, 2021; Liu *et al.*, 2023; Ermakova *et al.*, 2024; Gan & Sage, 2024) and five unpublished datasets with permission to use. Together, 1696 C_4 A/C_i curves for 74 species of 12 plant families from 57 unique studies were included in the analysis. Raw data files (i.e. direct output files from the measuring instrument) were requested from authors where available. In cases where the raw data files were not available, curves were digitised from published figures as per Pignon & Long (2020). The compiled dataset is provided in Supporting Information Dataset S1.

A/C_i curve analysis

To fit the curves and estimate parameters, the 1696 individual A/C_i curves were consolidated into 543 groups. Replicate measurements of the same species and genotype that were measured within 5 d in a study were considered as one experimental group. This approach allowed us to minimise software crashes due to a lack of model convergence and estimate sampling variance; this grouping approach has been used in other studies (e.g. Wu *et al.*, 2024). The A/C_i data at $C_i < 100\ \mu\text{mol mol}^{-1}$ (i.e. the initial slope of the A/C_i curve) were used to estimate V_{pmaxA}, according to von Caemmerer (2000):

$$A = V_p - R_m = \frac{C_i V_{\text{pmaxA}}}{C_i + K_p} - R_m \qquad \text{Eqn 1}$$

where A is the net CO_2 assimilation rate (μmol CO_2 $m^{-2}\,s^{-1}$), V_p is the rate of PEP carboxylation (μmol CO_2 $m^{-2}\,s^{-1}$), C_i is the CO_2 concentration in the intercellular space (μmol mol^{-1} CO_2), K_p is the Michaelis–Menten constant for CO_2 (μ bar; converted to concentrations using a solubility for CO_2 of 0.0334 mol bar^{-1} and atmospheric pressure at the measurement site; see von Caemmerer *et al.* (1994)), and R_m is daytime mitochondrial respiration in mesophyll cells (μmol CO_2 $m^{-2}\,s^{-1}$). Under low C_i, A is linearly correlated with the maximum PEPc activity in mesophyll cells, given that the leakage of CO_2 from bundle sheath cells is low and is generally ignored (von Caemmerer, 2021). We are aware that leakage of CO_2 from bundle sheath cells could increase when measurements are done at low light (i.e. $PPFD < 100$ μ mol photons $m^{-2}\,s^{-1}$; Yin *et al.*, 2016). Given that our dataset contains no measurements below $PPFD = 100$ μmol photons $m^{-2}\,s^{-1}$, we consider the potential impact of CO_2 leakage on our estimates of V_{pmaxA} to be minimal. We also assumed that PEP substrate concentrations were saturating under the measurement condition, and A was not limited by PEP regeneration, as required by the C_4 model (von Caemmerer, 2000, 2021). K_p was adjusted to account for variation in T_{leaf} according to Boyd *et al.* (2015):

$$K_p = K_{p25} \times e^{E_a(T_{\text{leaf}}-25)/(298.15R(T_{\text{leaf}}+273.15))} \qquad \text{Eqn 2}$$

where K_{p25} is K_p measured at 25°C (assumed to be 80 μ bar; Bauwe, 1986; DiMario & Cousins, 2019), E_a is the activation energy of K_p (36.3 kJ mol^{-1}; Boyd *et al.*, 2015), and R is the molar gas constant (0.008314 kJ K^{-1} mol^{-1}). R_m was taken as half the daytime leaf mitochondrial respiration rate (R_{day}; μmol CO_2 $m^{-2}\,s^{-1}$; von Caemmerer, 2000). Given that there are few R_{day} measurements in C_4 plants, R_{day} was assumed to equal leaf dark respiration such that $R_{\text{day}} = 1.2$ μmol CO_2 $m^{-2}\,s^{-1}$ at 25°C (termed as R_{day25}), according to a data synthesis of dark respiration in 39 C_4 species (Fan *et al.*, 2022). We chose not to scale dark respiration with photosynthetic capacity (i.e. $A_{\max}$) across all C_4 species, as the commonly used scaling relationship was derived from maize (Collatz *et al.*, 1992). Applying this maize-based relationship would undermine our objective of comparing model species (including maize) with non-model species. R_{day} was adjusted for variation in T_{leaf} using a Q_{10} equation (Atkin & Tjoelker, 2003):

$$R_{\text{day}} = R_{\text{day25}} \times Q_{10}^{\frac{T_{\text{leaf}}-25}{10}} \qquad \text{Eqn 3}$$

where Q_{10} is 2 (Huntingford *et al.*, 2013).

Although mechanistic models for enzyme- and light-limited C_4 photosynthesis are available, C_4 photosynthesis could be co-limited by RuBisCO activity, RuBP regeneration and/or PEP regeneration at high C_i, with all three processes requiring energy from light (von Caemmerer & Furbank, 1999). Thus, as no information was provided on the limitation status of photosynthesis at high C_i by individual studies, we estimated $A_{\max}$ using the entire A/C_i curve. The $A_{\max}$ was estimated by the horizontal asymptote of a four-parameter non-rectangular hyperbolic function (Leakey *et al.*, 2006; Li *et al.*, 2022):

$$\theta(A + R_{\mathrm{day}})^2 - (\alpha C_{\mathrm{i}} + A_{\max})(A + R_{\mathrm{day}}) + \alpha C_{\mathrm{i}} A_{\max} = 0 \qquad \text{Eqn 4}$$

where θ is the curvature factor of an A/C_{i} curve (unitless), which was assumed to be 0.7 for C_4 plants (Sonawane *et al*., 2018; von Caemmerer, 2021), and α is the initial slope of an A/C_{i} curve (μmol CO_2 m^{-2} s^{-1}). We did not include mesophyll conductance as a term in Eqn (4) due to a lack of such data in examined studies. However, by assuming $\theta = 0.7$, we considered a potential drop in the C_{i} between the intercellular space and the site of RuBisCO carboxylation (i.e. bundle sheath cells; Evans, 1989). All curve fitting was performed using the non-linear least square (nls) function and sampling variances of every fit were extracted using the vcov function in R (v.4.4.1, R Core Team, 2024). The number of individual curves per group (n_{group}, ranging from 1 to 9) was also reported. Sampling variance of parameters (i.e. squared SE for parameters) was subsequently considered in the statistical models (Nakagawa & Santos, 2012; Noble *et al*., 2017, 2022).

Data classification and summary

Among the 543 groups, eight groups contained fewer than two data points at low C_{i} (i.e. < 100 μmol mol^{-1}), while 131 groups contained fewer than two data points at high C_{i} (i.e. > 500 μmol mol^{-1}). As this lack of data is likely to influence the accuracy of model fitting using these portions of the A/C_{i} curve, the corresponding estimated V_{pmaxA} and $A_{\max}$ values of these groups were discarded. Given that measurements within a single group were made under the same measurement temperature and irradiance, we provided averaged values of T_{leaf} and *PPFD* per group. We further assessed the estimated parameters of each group: if the curve violated the assumption that A is limited by V_{pmaxA} at low C_{i} (as indicated by V_{pmaxA}-limited photosynthesis being higher than $A_{\max}$-limited photosynthesis at low C_{i}), this group of A/C_{i} curves was discarded as per Pignon & Long (2020). This assessment led to 108 additional groups being removed from the final dataset (Dataset S2), many of which included plants subjected to severe abiotic stresses, such as chilling, drought and low nutrients.

The remaining measurements were classified according to their C_4 biochemical subtype (NADP-ME, NAD-ME or PCK subtype), growth form (monocot or eudicot) and growth location (indoor or outdoor). Indoor plants refer to those grown in pots within controlled environment chambers or glasshouses. By contrast, outdoor plants are grown directly in the soil or in common gardens without pot restrictions. Experiments conducted at Free-Air Carbon Dioxide Enrichment (FACE) and Lysimeter CO_2 Gradient (LYCOG) facilities are classified as outdoor-grown, as plants in these settings are planted directly in soil. The growth CO_2 concentration was noted, where the ambient CO_2 level was assumed to be 400 ppm if not given by the study, as 400 ppm represents the average atmospheric CO_2 concentration from 2001 to 2024 (Friedlingstein *et al*., 2025), which covers the period of publications used in this study, although the experiments themselves may have been conducted 1 or 2 yr earlier. For indoor-grown plants, mean maximum ($T_{\max}$) and minimum ($T_{\min}$) growth temperatures were the set daytime and night-time temperatures, respectively. We used the reported measurement T_{leaf} in our analyses, rather than normalising to a rate at a set temperature of 25°C, as we did not want to assume that the V_{pmax} and $A_{\max}$ of all species and growth environments will have the same sensitivity to short-term changes in T_{leaf}. For outdoor-grown plants, mean $T_{\max}$ and $T_{\min}$ were the mean maximum and minimum temperatures observed across the experimental periods at the study sites, respectively. Growth *PPFD* was unavailable in most outdoor studies and was therefore excluded from the analysis. We were unable to consider either fertilisation treatment or water status as potential categories for analysis, as low nutrient treatment and/or water-stressed conditions constituted $< 5\%$ of the data (Fig. 1d,e). A summary of measurements based on plant traits and growth treatments is given in Fig. 1.

Across the dataset, maize, sorghum and *S. viridis* were the three most commonly measured species, accounting for 18, 6 and 4% of the total measurements, respectively. Indeed, maize contributed 43% of the outdoor data and played an important role in the growth location category (Fig. S1). This species skewness is later accounted for in the statistical models (to be described later).

Statistical analysis

To determine how different plant traits and experimental conditions altered V_{pmaxA} and $A_{\max}$, we ran two multivariate linear mixed-effects models with V_{pmaxA} or $A_{\max}$ as the dependent variable in each model. These models accounted for the sampling variance of each parameter, with parameters having greater precision being weighted more heavily to overall means. We included three main fixed effect categories in our models: (1) species-specific traits, including C_4 subtype and growth form; (2) environmental growth conditions, such as growth location, temperature and CO_2 concentration; and (3) measurement conditions, such as T_{leaf} and *PPFD*. Given that mean $T_{\max}$ and $T_{\min}$ were strongly positively correlated with each other (Fig. S2), we used mean $T_{\max}$ in the model. A list of fixed effects is given in Table 1.

In addition, we included six *a priori* interaction terms examining the interactive effects of species-specific traits, temperatures and irradiance: C_4 subtype × mean $T_{\max}$, C_4 subtype × T_{leaf}, C_4 subtype × *PPFD*, growth form × mean $T_{\max}$, growth form × T_{leaf} and growth form × *PPFD*. These terms were designed to test the question of whether the response of C_4 photosynthetic capacity traits to short- and long-term temperatures and measurement irradiance differ among C_4 subtypes or growth forms. We confirmed that our V_{pmaxA} and $A_{\max}$ data per C_4 subtypes and growth form were distributed across the entire range of mean $T_{\max}$, T_{leaf} and *PPFD* (Fig. S3), such that any significant interaction terms would not be biased due to separated data distributions. Data distribution with respect to mean $T_{\max}$ and T_{leaf} is shown in Fig. S4. Random effects included in the models were the different studies (indicated by publications), grouping of the curves (as described in the section A/C_{i} curve analysis) and species. Given that C_4 model species (maize, sorghum and *S. viridis*) made up a significant proportion of the data, we also conducted a

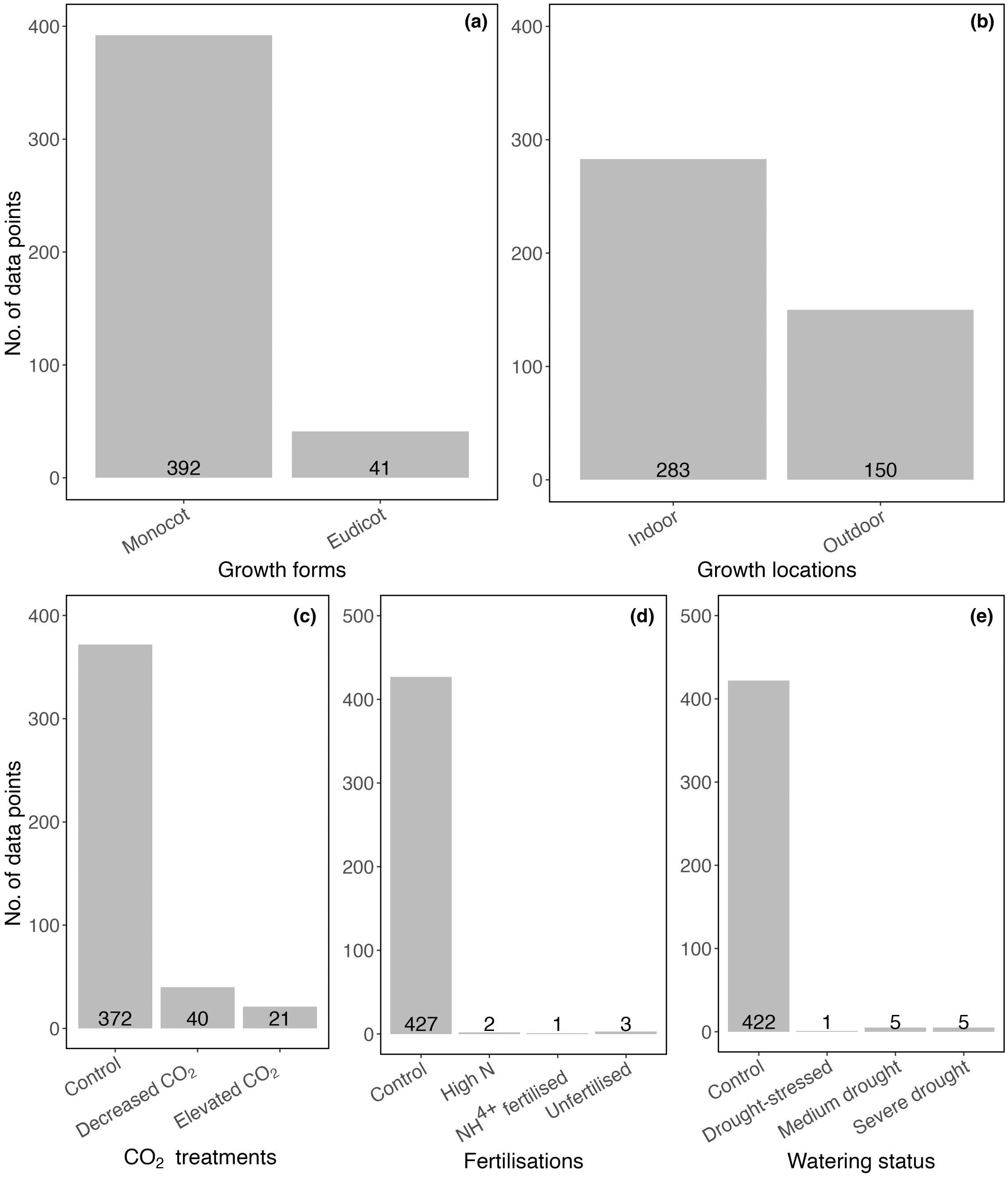

Fig. 1 Summary of data points (by groups) based on (a) growth forms, (b) locations, (c) CO_2 treatments, (d) fertilisation treatments and (e) watering status. Decreased and elevated CO_2 treatments consist of measurements made on plants with a growth CO_2 concentration of < 400 and > 400 ppm, respectively. See the Materials and Methods section for more information.

case study examining to what extent the model results obtained from data of all species match with those of the C_4 model species and visualise our results with consideration of C_4 model species (see the Results section).

The multivariate linear mixed-effects models with restricted maximum likelihood estimation were run using the package METAFOR v.4.6.0 (Viechtbauer, 2010), and plots were made using the package GGPLOT2 v.3.5.1 (Wickham, 2016) and ORCHARD v.2.0 (Nakagawa *et al.*, 2021, 2023) on R program v.4.4.1 (R Core Team, 2024). Estimated effects were considered significant if $P < 0.05$ in the models.

Results

Positive correlations between V_{pmaxA} and $A_{\max}$

In general, V_{pmaxA} and $A_{\max}$ were correlated with each other in a logarithmic manner ($R^2 = 0.81$; Fig. 2a). We found that for

Table 1 Results of multivariate linear mixed-effects models testing the response of V_{pmaxA} and A_{max} against fixed effects of species-specific traits and experimental conditions (see main text for more details).

	V_{pmaxA}					A_{max}				
	Estimate	SE	Lower CI	Higher CI	*P* value	Estimate	SE	Lower CI	Higher CI	*P* value
Fixed effects										
C_4 subtype	−1.299	7.716	−16.470	13.873	0.969	−1.339	3.363	−7.960	5.282	0.727
Growth form	0.270	8.272	−15.996	16.535	0.196	−15.091	11.082	−37.636	7.455	0.295
Growth location	−15.190	5.154	−25.747	−4.634	$\mathbf{0.005}^{**}$	−6.676	2.651	−12.295	−1.057	$\mathbf{0.018}^{*}$
Mean T_{max}	−2.549	1.362	−5.228	0.130	$\mathbf{0.001}^{**}$	0.367	0.754	−1.117	1.851	$\mathbf{0.001}^{**}$
Growth CO_2	−0.018	0.012	−0.042	0.006	0.138	−0.003	0.009	−0.021	0.016	0.761
T_{leaf}	3.580	0.849	1.910	5.250	$< \mathbf{0.0001}^{***}$	2.406	0.476	1.468	3.343	$< \mathbf{0.0001}^{***}$
PPFD	0.014	0.002	0.010	0.018	$< \mathbf{0.0001}^{***}$	0.010	0.001	0.008	0.013	$< \mathbf{0.0001}^{***}$
C_4 subtype × T_{leaf}	0.544	0.519	−0.477	1.565	0.504	0.187	0.261	−0.327	0.700	0.761
C_4 subtype × Mean T_{max}	−0.413	2.268	−4.873	4.047	0.840	−0.943	1.188	−3.283	1.396	0.642
C_4 subtype × *PPFD*	−0.001	0.005	−0.010	0.008	0.674	0.002	0.003	−0.003	0.007	0.750
Growth form × T_{leaf}	−1.412	0.982	−3.342	0.518	0.146	3.590	2.519	−1.368	8.549	0.219
Growth form × Mean T_{max}	3.477	2.132	−0.890	7.845	0.099	0.148	1.207	−2.410	2.707	0.781
Growth form × *PPFD*	0.004	0.008	0.631	−0.012	0.645	−0.023	0.019	−0.066	0.007	0.143
T_{leaf} × Mean T_{max}	0.013	0.036	−0.058	0.083	0.724	−0.043	0.020	−0.082	−0.004	$\mathbf{0.033}^{*}$
Species group	8.209	6.740	−5.385	21.802	0.230	3.467	2.381	−1.376	8.311	0.155
Overall model statistics										
Effect size/sample size	49/401					37/297				
Marginal R^2	0.51					0.47				
Conditional R^2	0.83					0.75				

Effect size denotes the number of studies, while the sample size is the number of V_{pmaxA} or A_{max} data points. Model's marginal R^2 reflects the variance explained by fixed effects only, whereas conditional R^2 considers the variance explained by both fixed and random effects. Bold text indicates statistical significance. Significant codes: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Continuous variables (i.e. mean T_{max}, growth CO_2, T_{leaf} and *PPFD*) were mean-centred before the analysis. Lower and high CI: lower and upper 95% confidence intervals.

leaves measured at a moderate T_{leaf} (i.e. $< 35°C$), changes in V_{pmaxA} were tightly coupled with changes in A_{max} in a nearly linear fashion (Fig. 2a). When leaves were measured at a hotter T_{leaf} (i.e. $> 35°C$), the coupling between V_{pmaxA} and A_{max} broke down, and an increase in V_{pmaxA} was accompanied by a lesser increase in A_{max}. This result was supported by significantly different slopes ($P < 0.001$; standardised major axis analysis) in the linear correlations between V_{pmaxA} and A_{max} for leaves measured at 25–30°C (slope = 0.49; Fig. 2b), 30–35°C (slope = 0.39; Fig. 2c) and 35–40°C (slope = 0.27; Fig. 2d). Together, these findings highlight that at high A_{max} (i.e. high C_i), photosynthesis was less dependent on PEP carboxylation compared with other high-temperature sensitive biochemical processes (e.g. RuBisCO carboxylation), or photosynthesis was constrained by increased limitations in light and bundle sheath (CO_2) at high A_{max} (e.g. affecting RuBP regeneration).

Factors influencing V_{pmaxA} and A_{max} are mostly environmental

Multivariate linear mixed-effects models highlighted several factors that significantly affected both V_{pmaxA} and A_{max} (Table 1). C_4 subtype identity and growth form had little influence on values of either V_{pmaxA} or A_{max} (Fig. 3a–d). Note that the number of studies examining eudicots or PCK-type plants was noticeably lower than studies focusing on monocots or NADP-ME plants, respectively. This highlights an urgent need to further investigate photosynthetic characteristics in eudicots and PCK-type plants. There is no evidence that V_{pmaxA} and A_{max} vary among families or subfamilies ($P = 0.73$ and 0.88 for V_{pmaxA} and A_{max}, respectively) or within each biochemical subtype (Fig. S5).

We found that both V_{pmaxA} and A_{max} were higher when plants were grown indoors than in the field ($P = 0.005$ and 0.018 for V_{pmaxA} and A_{max}, respectively; Fig. 3e,f), where averaged mean T_{max} values were 28.2 and 25.2°C, respectively, for indoor and field plants. Specifically, the models estimated that V_{pmaxA} of indoor plants was 35% higher than their outdoor counterparts (55.3 ± 1.7 vs $40.0 \pm 2.1\ \mu mol\ m^{-2}\ s^{-1}$), while A_{max} of indoor-grown plants was 22% higher than those grown outdoors (33.9 ± 0.9 vs $26.8 \pm 1.1\ \mu mol\ m^{-2}\ s^{-1}$). It is worth noting that C_4 model species (i.e. maize and sorghum) appear to be at the high-end of the V_{pmaxA} and A_{max} spectra, particularly for outdoor-grown plants (Fig. 3e,f). We thus further examined whether there was an interaction between species group and growth location, and found it to be significant ($P < 0.001$). The mean values of V_{pmaxA} and A_{max} were significantly higher in outdoor-grown C_4 model species than in non-model species grown outside ($P < 0.001$; Fig. 4). This result is further supported by comparisons of the photosynthetic capacity of maize and sorghum against other species (Fig. S6). However, no significant differences were observed between model and non-model species grown indoors (Figs 4, S6).

We also found that V_{pmaxA} and A_{max} decreased significantly with increasing mean T_{max} ($P = 0.001$; Fig. 5a,b), with this pattern being stronger for a subset of data measured at a T_{leaf} between 25

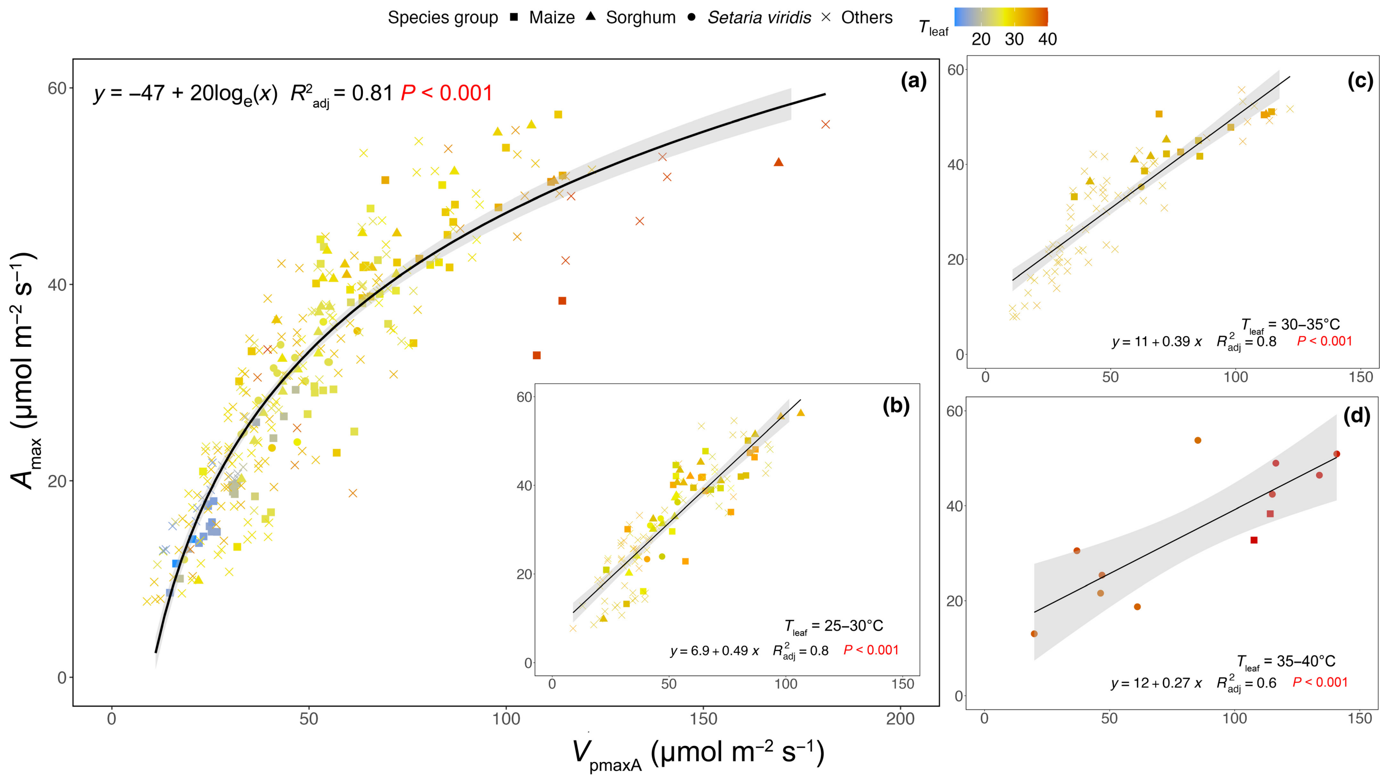

Fig. 2 Correlation between A_{max} and V_{pmaxA} estimated from data collected from a range of species measured at various growth and measuring conditions. Point shapes reflect important species groups, and colours denote measurement temperature (T_{leaf}). (a) All available A_{max}-V_{pmaxA} data; (b–d) data subsets measured at 25–30°C, 30–35°C and 35–40°C, respectively. Equation, adjusted R^2 values and P values of individual fits are shown in each panel. Shaded areas represent 95% confidence intervals around predicted means from the fitted curves.

and 30°C to account for variation in T_{leaf} in the analysis (Fig. S7). No effect of growth CO_2 concentrations was found on either V_{pmaxA} or A_{max} (Fig. 5c,d). In contrast to the negative response to mean T_{max}, V_{pmaxA} and A_{max} increased significantly with increasing T_{leaf} ($P < 0.0001$; Fig. 6a,b) and *PPFD* ($P < 0.0001$; Fig. 6c,d). These results highlight the different responses of V_{pmaxA} and A_{max} to short-term changes in T_{leaf} and long-term acclimation to mean T_{max} (to be described later). To assess whether the differences in V_{pmaxA} and A_{max} between growth locations (Fig. 3e,f) were linked to the distinct growth and measurement conditions experienced by indoor and outdoor plants, we compared the slopes of their correlations with mean T_{max} (Fig. S8), growth CO_2 concentration (Fig. S9), T_{leaf} (Fig. S10) and *PPFD* (Fig. S11) separately for each location (Table S1). We found that, apart from the V_{pmaxA}-T_{leaf} correlation, which differed significantly between growth locations ($P = 0.003$), there was no evidence that growth location explained the effects of mean T_{max} or T_{leaf} on V_{pmaxA} or A_{max} (Table S2). We further examined whether different C_4 subtypes or growth forms varied in their response to mean T_{max}, T_{leaf} and *PPFD* by considering interaction effects. None of the interaction terms was significant (Table 1), suggesting that the responses of V_{pmaxA} and A_{max} to growth and measuring temperatures and irradiance are independent of species-specific traits (such as growth form). Overall, the fixed effects considered in our models accounted for 51 and 47% of the variation in V_{pmaxA} and A_{max}, respectively (Table 1).

Interactive effects of T_{leaf} and mean T_{max} on photosynthetic capacity

The different responses of V_{pmaxA} and A_{max} to changes in long-term mean T_{max} and short-term T_{leaf} pointed towards a possible interaction between these two temperature factors (Figs 5, 6). We thus explored the mean $T_{max} \times T_{leaf}$ interaction and the result showed that the V_{pmaxA} was not affected by the interaction between growth and measurement temperatures ($P = 0.724$; Table 1), although the interaction term was significant for A_{max} ($P = 0.033$). We further illustrated the complex responses of V_{pmaxA} and A_{max} to mean T_{max} and T_{leaf} using contour plots (Fig. 7). The contour plots show that at any given mean T_{max}, the sensitivity of V_{pmaxA} to changes in T_{leaf} was not influenced by acclimation to different mean T_{max} (Fig. 7a). This pattern also means that V_{pmaxA} measured at the predominant leaf temperatures of warm-grown plants is higher than V_{pmaxA} measured at the predominant leaf temperatures of plants grown at lower temperatures. A_{max} also increased with increasing T_{leaf}, but the increase in A_{max} per 1°C increase in T_{leaf} was higher in plants grown in cooler environments (i.e. mean $T_{max} < 25$°C) than in warmer environments (Fig. 7b). This suggests that A_{max} was more sensitive to changes in T_{leaf} in plants acclimated to lower mean T_{max}. This pattern highlights that while A_{max} is lower in plants grown and measured at 20°C, compared with those grown and

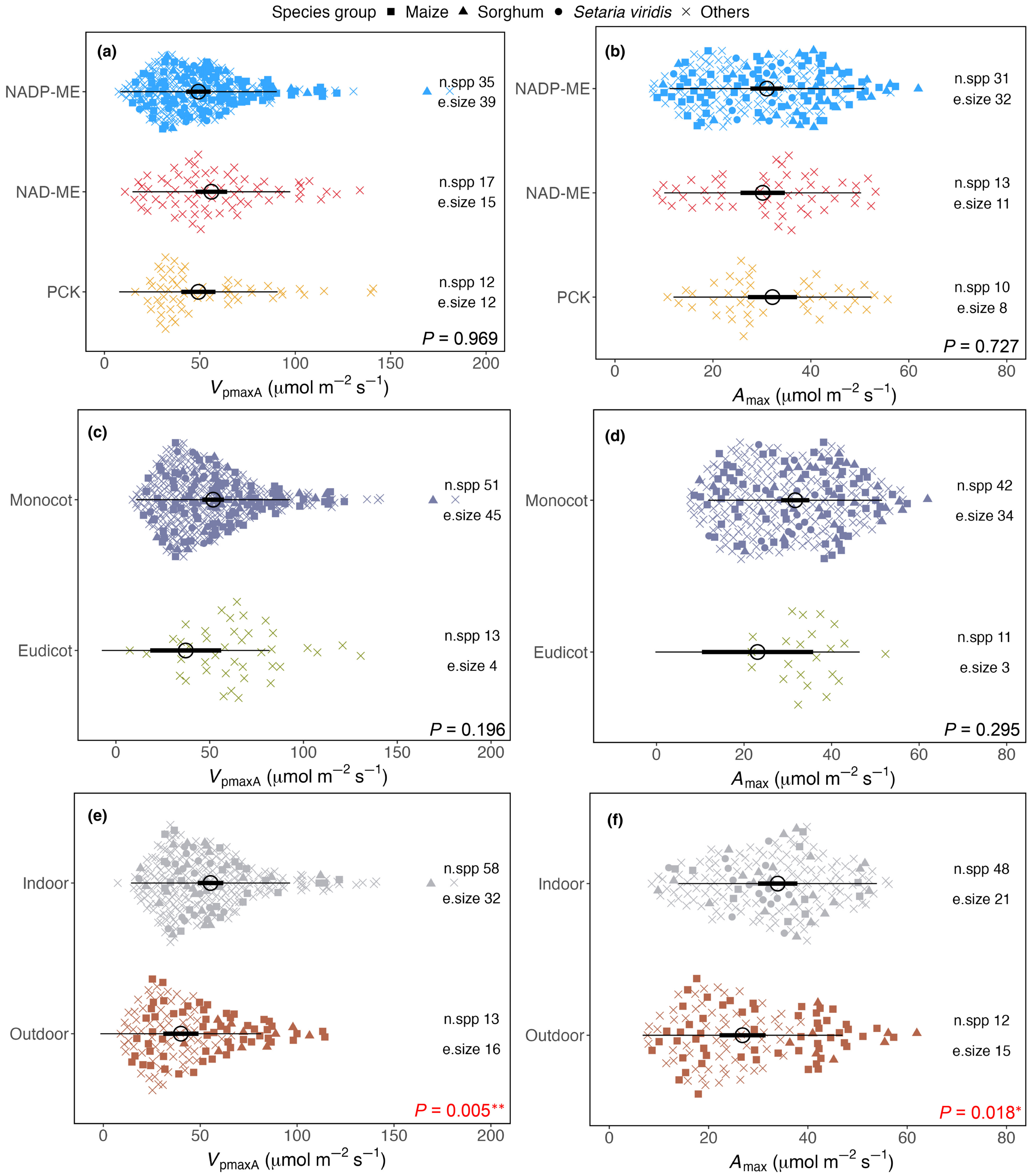

Fig. 3 V_{pmaxA} and A_{max} plotted by C_4 biochemical subtypes (a and b, respectively), growth forms (c and d, respectively) and growth locations (e and f, respectively). Data presented here are a mixture of measurements done at various growth and measuring temperatures and irradiance. Raw data points are plotted as coloured symbols, with different symbol shapes reflecting important species groups. Model-predicted marginal means and intervals are shown as a horizontal line. On this line, black circles indicate model-predicted meta-analytic means of V_{pmaxA} or A_{max}; thick bars are 95% confidence intervals, and thin bars are 95% prediction intervals. On the right-hand side of each panel, the number of unique species per category (n.spp) and the number of individual studies (e.size) are indicated. *P* values of the multivariate linear mixed-effects model are indicated in each panel (see Table 1). Significant codes: *, $P < 0.05$; **, $P < 0.01$.

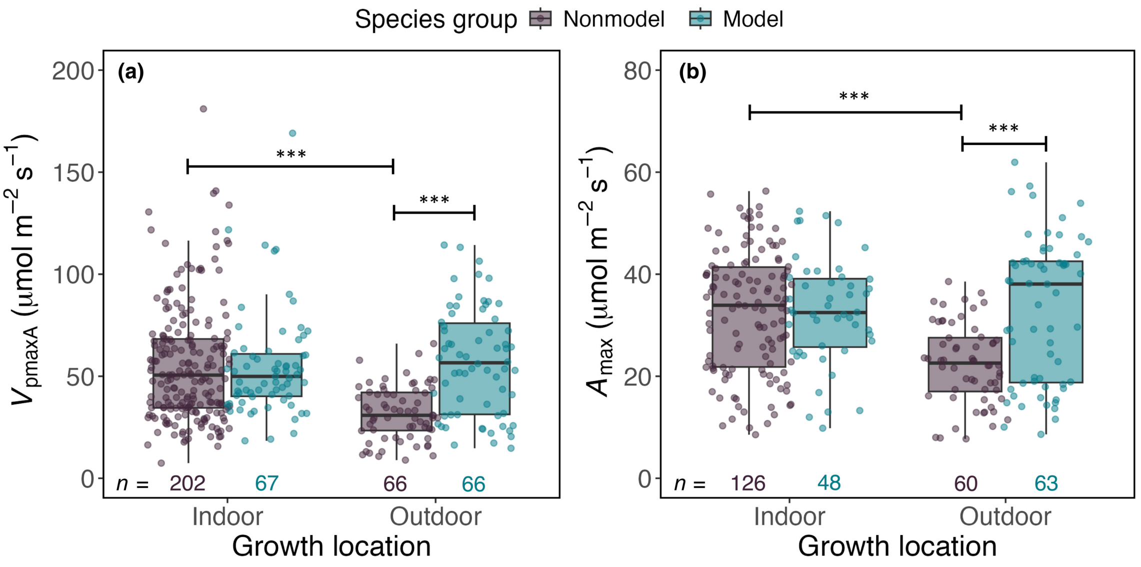

Fig. 4 Comparison between V_{pmaxA} and A_{max} with growth location coloured by species group (C_4 non-model species vs model species). A linear mixed-effect model was conducted to examine V_{pmaxA} and A_{max} between two species groups at a location, or within a species group at both locations. Statistical results of comparisons are denoted with horizontal lines (i.e. the two bars at the beginning and the end of a horizontal line are compared) and asterisks indicate statistical significance (***, $P < 0.001$) from this lnear mixed-effect model. The sample size (*n*) for each group is indicated on plots. Boxplots show the median (horizontal line within each box), interquartile range (IQR; box edges spanning the 25th to 75th percentiles), and whiskers (1.5 × IQR).

measured at 30°C and 38°C, A_{max} in plants grown and measured at 30°C and 38°C is similar (Fig. 7b).

Impact of C_4 model species on the analysis

Maize, sorghum and *S. viridis*, three frequently studied species in C_4 research, dominated our A/C_i dataset (Fig. S1). In fact, data collected from these C_4 model species are widely used to model the response of C_4 non-model species at the ecosystem level (e.g. in the Community Land Model (Lawrence *et al*., 2019)). Consequently, we investigated whether V_{pmaxA} and A_{max} of these C_4 model species are representative of the non-model species. We found no significant difference in V_{pmaxA} and A_{max} between the C_4 model and non-model species ($P = 0.230$ and 0.155 for V_{pmaxA} and A_{max}, respectively; Table 1 – species group). However, it is worth noting that the interaction between species group and growth location was significant due to the lower photosynthetic capacity of non-model species grown outdoors (as described in the previous section and Fig. 4). Therefore, we suggest that while our results show that C_4 model species have similar photosynthetic capacity compared with non-model species, care must be taken when extending this finding to natural outdoor settings, the conditions of most interest to ecologists and Earth System Model modellers. Lastly, our results indicate that our multivariate model was not biased by the weighting of data from C_4 model species.

Discussion

This study aimed to explore general patterns of C_4 photosynthetic capacity, indicated by V_{pmaxA} and A_{max}, among a wide range of species grown and measured at various conditions. We found that V_{pmaxA} and A_{max} were tightly coupled in a way that reflects the limitation states of C_4 photosynthesis at different C_i and T_{leaf} conditions, partially supporting Hypothesis 1 (Fig. 2), although this correlation breaks down at higher temperatures. V_{pmaxA} and A_{max} were similar among the three C_4 subtypes and two growth forms, partially rejecting Hypothesis 1 (Fig. 3a–d), but were influenced by growth and measurement conditions. Indoor plants exhibited higher V_{pmaxA} and A_{max} than their outdoor counterparts, supporting Hypothesis 2 (Fig. 3e,f). Both V_{pmaxA} and A_{max} decreased with increasing mean growth T_{max} (Fig. 5a,b), while they increased with increasing measurement T_{leaf} (Fig. 6a,b), providing partial support for Hypothesis 3. Interestingly, the response of A_{max} to T_{leaf} depended on mean T_{max}, indicating that thermal acclimation alters the thermal sensitivity of photosynthetic capacity in C_4 species (Fig. 7). There was no significant effect of growth CO_2 concentration on photosynthetic capacity, leading us to accept Hypothesis 4, although more data are needed at low and high growth CO_2 concentrations to further confirm this result (Fig. 5c,d). Lastly, we explored whether the more commonly measured C_4 model species (maize, sorghum and *S. viridis*) were good representatives of a wide range of C_4 plants. We found no evidence of generalised superior photosynthetic capacity in C_4 model species when compared to non-model species (e.g. native C_4 grasses and eudicots as a whole; Table 1), although C_4 model species had higher photosynthetic capacity than non-model species when grown outdoors (Fig. 4). Overall, our analysis highlights that C_4 photosynthesis is strongly affected by growth and measurement conditions but is largely similar across C_4 species from different biochemical subtypes and growth forms. We discuss the potential reasons and consequences of our findings below.

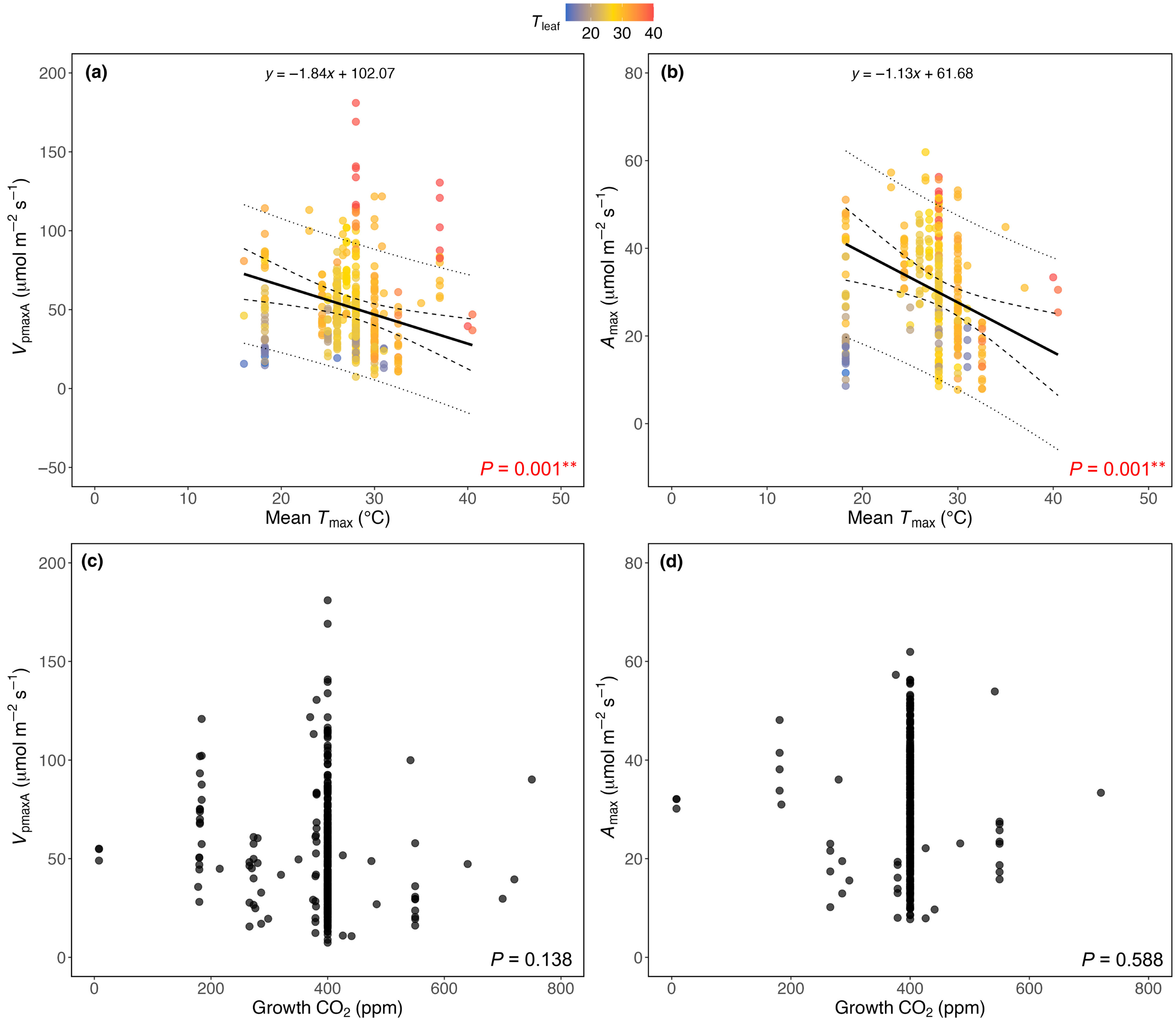

Fig. 5 Relationships between V_{pmaxA} and A_{max} with mean T_{max} (a and b, respectively) and growth CO_2 levels (c and d, respectively). Data points in (a) and (b) are coloured in a gradient by the measurement temperature (T_{leaf}). Solid lines represent model-predicted values of V_{pmaxA} or A_{max} at a given mean T_{max} (equations are shown at the top of each panel), dashed lines indicate 95% confidence intervals, and dotted lines show 95% prediction intervals. *P* values of the multivariate linear mixed-effects models are indicated. Significant codes: **, $P < 0.01$.

Coupling between V_{pmaxA} and A_{max} reflects photosynthetic limitations

We found a strong positive correlation between estimated V_{pmaxA} and A_{max} in the T_{leaf} range of 25–30°C (Fig. 2b). At T_{leaf} above 30°C, the positive correlation between V_{pmaxA} and A_{max} weakened, with A_{max} showing less pronounced change per unit increase in V_{pmaxA} (Fig. 2c,d). The C_4 photosynthetic cycle (reflected as V_{pmaxA}) is generally more efficient than the C_3 cycle (reflected as A_{max}) under high temperatures and light intensities, due to its CO_2-concentrating mechanism that suppresses photorespiration and maintains high RuBisCO efficiency (von Caemmerer & Furbank, 2003). When the efficiency of the C_4 cycle exceeds that of the C_3 cycle (i.e. the rate of CO_2 delivery to bundle sheath cells outpaces the rate of RuBisCO carboxylation), CO_2 may begin to leak back into mesophyll cells, increasing the energetic cost required to refix this CO_2 into C_4 acids in mesophyll cells. This imbalance between C_3 and C_4 cycles, particularly under low light (i.e. $PPFD < 100$ μmol photon m^{-2} s^{-1}; Yin *et al.*, 2016), can reduce overall photosynthetic efficiency (i.e. A_{max}). Given that most of our A/C_i measurements were done at $PPFD > 1500$ μmol photon m^{-2} s^{-1} (Fig. S12), we argue that our observation is more related to different temperature sensitivities of V_{pmaxA} and A_{max} (to be described later).

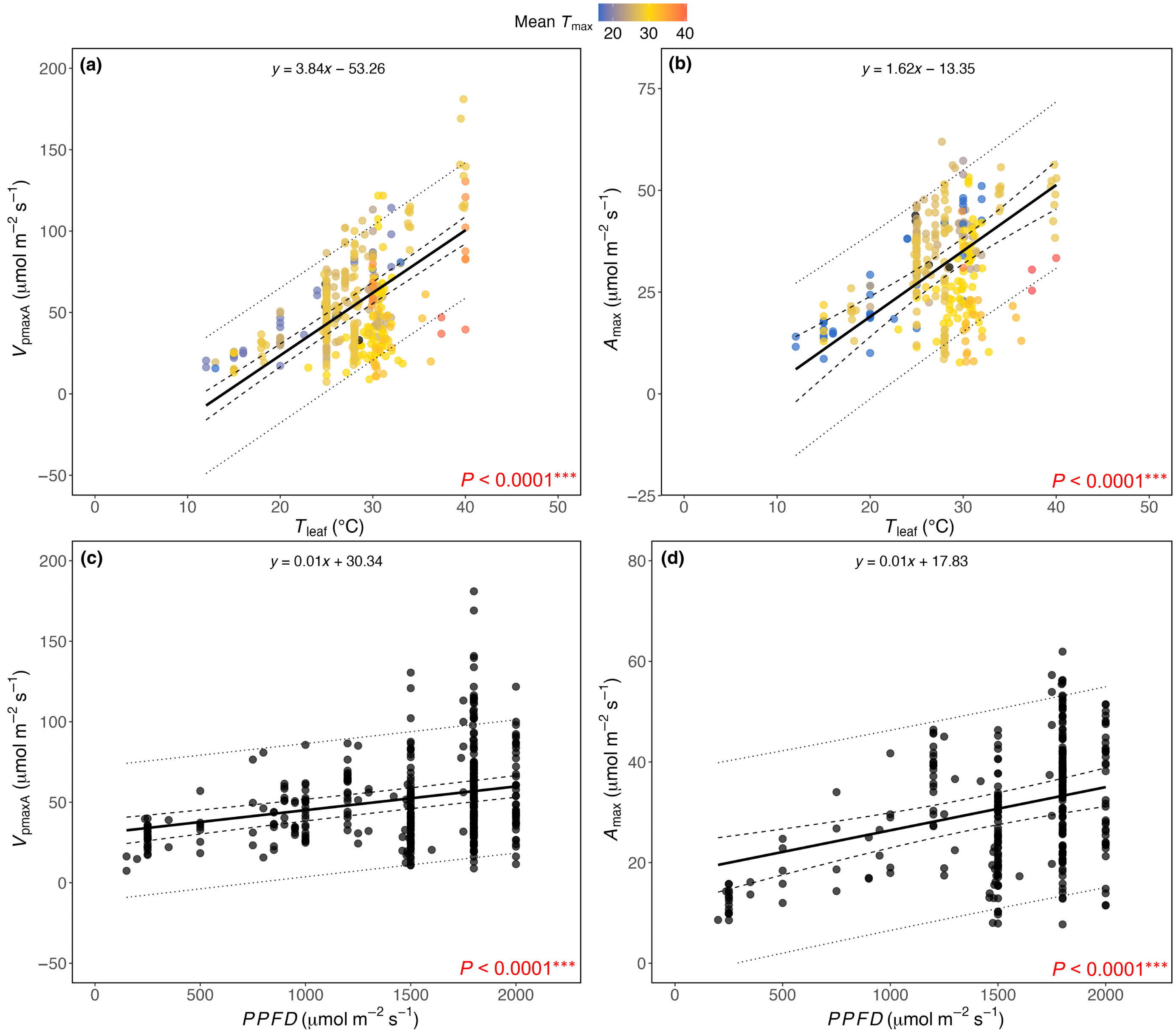

Fig. 6 Relationships between V_{pmaxA} and A_{max} with T_{leaf} (a and b, respectively) and photosynthetic photon flux density (*PPFD*) (c and d, respectively). Data points in (a) and (b) are coloured by the mean maximum growth temperature (mean T_{max}). Solid lines represent model-predicted values of V_{pmaxA} or A_{max} at a given T_{leaf} or *PPFD* (equations are shown at the top of each panel), dashed lines indicate 95% confidence intervals, and dotted lines show 95% prediction intervals. *P* values of the multivariate linear mixed-effects models are indicated. Significant codes: ***, $P < 0.001$.

Our result also agrees with findings derived from flux control analysis (von Caemmerer & Furbank, 2016) and data syntheses (Pignon & Long, 2020), and highlights differences in the temperature-dependent responses of biochemical processes underpinning V_{pmaxA} and A_{max}, such that A_{max} was less temperature sensitive at high T_{leaf} than V_{pmaxA}. The A_{max} in C_4 plants is primarily determined by the maximum carboxylation activity of RuBisCO (V_{cmax}) and the rate of RuBP regeneration at high C_i, with a reduction in either of these leading to a lower A_{max} (Furbank *et al.*, 1996; von Caemmerer *et al.*, 1997; von Caemmerer & Furbank, 1999). At high T_{leaf}, RuBisCO inactivation due to a decrease in RuBisCO activase activity (Hendrickson *et al.*, 2008; Salesse-Smith *et al.*, 2018) reduces V_{cmax} and ultimately A_{max}. The RuBP regeneration rate can be limiting when measurement *PPFD* or the capacity of Calvin cycle enzymes, other than RuBisCO, limit net CO_2 assimilation rate at high C_i, or when the thylakoid membrane is damaged at high temperatures (Peixoto & Sage, 2017). The limitation imposed by RuBP regeneration is expected to be pronounced in C_4 plants, which have lower concentrations of RuBP than C_3 plants (Arrivault *et al.*, 2019). However, this limitation is likely not related to the abundance and activity of sedoheptulose-1,7-bisphosphatase (a rate-limiting enzyme in Calvin cycle; Ermakova *et al.*, 2022). An RuBP limitation could further constrain photosynthesis as C_i increases, as an

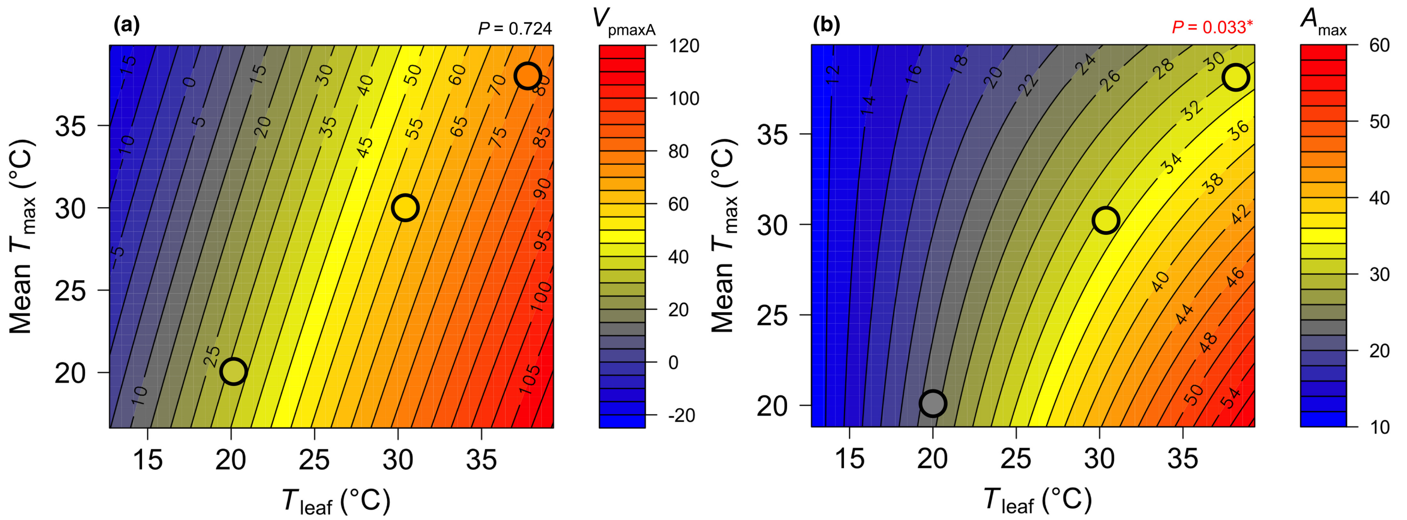

Fig. 7 Contour plots illustrating the model-predicted responses of (a) V_{pmaxA} and (b) A_{max} (μmol m^{-2} s^{-1}) to T_{leaf} and mean T_{max}. P values of linear mixed-effects model testing the interaction between T_{leaf} and mean T_{max} are indicated (see Table 1). Black circles represent comparisons of V_{pmaxA} and A_{max} at the same mean T_{max} and T_{leaf} of 20°C, 30°C and 38°C, with the colour gradient reflecting changes in V_{pmaxA} and A_{max} (see the colour legend on the right-hand side of each panel). Significant codes: *, $P < 0.05$.

increase in C_i promotes RuBisCO carboxylase activity and leads to more RuBP being consumed. In our dataset, the majority of A/C_i measurements were done at $PPFD > 1500$ μmol photon m^{-2} s^{-1}, including those measured at $T_{leaf} > 35$°C (Fig. S12). However, photosynthesis in C_4 plants is usually not light-saturated even at 1500 μmol photons m^{-2} s^{-1} (Ermakova *et al.*, 2019, 2023). There is evidence that due to adaptation to tropical/subtropical climates where high-light environments are common, C_4 plants evolved a series of photosynthetic traits that allow them to grow under high light (Wasilewska-Dębowska *et al.*, 2022). Adaptation to high-light conditions may arguably put C_4 plants at a disadvantage in light conditions that are suboptimal for them but saturating for C_3 plants. These findings highlight the complex interplay between temperature, biochemical limitations and light availability in regulating C photosynthesis, which in part has become a bottleneck for C_4 photosynthesis modelling at the ecosystem level (Knauer *et al.*, 2023).

Photosynthetic capacity is similar across biochemical types and growth forms

Our results show that variation in C_4 biochemical subtypes and growth forms does not lead to systematic variation in photosynthetic capacity (Fig. 3a–d). In general, popular model species (C_4 NADP-ME type grasses: maize, sorghum and *S. viridis*) exhibit similar photosynthetic capacity when compared to non-model C_4 species representing the three C_4 biochemical subtypes (Table 1). However, when growth location is considered, the photosynthetic capacity of C_4 model species is higher than that of non-model species when both are grown outdoors (Fig. 4). These results suggest that photosynthetic capacity data collected from model species could overestimate the productivity of non-model species in outdoor conditions. These findings are particularly important for the modelling community, given that the current C_4 vegetation model at the ecosystem level was developed based on parameters measured in maize grown in controlled environments (Collatz *et al.*, 1992; Lawrence *et al.*, 2019), and likely overestimates the productivity of C_4 native and eudicots grown in the field. The lack of a significant effect of C_4 subtypes on photosynthetic capacity further emphasises the biochemical flexibility in the C_4 pathway. There is growing molecular, biochemical and physiological evidence, suggesting that the three classical subtypes, originally defined based on early ^{14}C-labelling studies (Hatch & Slack, 1970; Hatch, 1971), can be biochemically flexible (Furbank, 2011; Bräutigam *et al.*, 2014; Wang *et al.*, 2014; Sales *et al.*, 2021), which in turn diminishes any potential differences in photosynthetic capacity among the subtypes. This biochemical flexibility may be regulated by developmental and environmental variation, further highlighting the role of environmental effects on C_4 photosynthesis (to be described later).

Growth location affects photosynthetic capacity

Our results showed that growth and measurement conditions are the major factors affecting photosynthetic capacity in C_4 plants. Indoor plants exhibit consistently higher V_{pmaxA} and A_{max} than their outdoor counterparts (Fig. 3e,f), despite indoor plants being grown in warmer environments, which might suppress photosynthetic capacity (Fig. S4a). Plants grown in indoor, controlled environments are usually well-watered and fertilised, and are not challenged with pests and extreme conditions (e.g. heat waves or frost stress; Poorter *et al.*, 2016). The lower photosynthetic capacity in outdoor non-model species is likely due to the more stressful conditions they experience, which are primarily characterised by lower water and nutrient availability than indoor-grown plants. For example, long-term drought (i.e. > 100 d) could cause significant reductions in stomatal conductance, net photosynthetic rate and V_{pmaxA}, with such effects being exacerbated in N-limited C_4 plants (Markelz *et al.*, 2011). Interestingly, the impact of drought on photosynthetic capacity (i.e. A_{max}) was not seen in two well-fertilised C_4 grasses, *Dactyloctenium aegyptium* and *Schoenefeldia gracilis* (Maroco *et al.*, 2000), suggesting changes in photosynthetic capacity may be associated with interactive effects of drought and N limitation on plants. By contrast, C_4 model species, particularly maize and sorghum, have been selectively bred for improved drought tolerance (Lopes *et al.*, 2011) and are typically well-watered and well-fertilised in crop fields due to their high agricultural value. These effects may have contributed to higher photosynthetic capacity in C_4 model species grown outdoors (Fig. 4). Further research is needed to dissect the mechanism underpinning the combined effects of drought and N limitation on photosynthesis and to identify why outdoor growth conditions affect non-model species more than model C_4 species.

Temperature significantly influences photosynthetic capacity

We observed that both V_{pmaxA} and A_{max} decreased with increasing mean T_{max} (Fig. 5a,b). This finding is supported by previous work (Berry & Bjorkman, 1980) and aligns with our knowledge of thermal acclimation (Way & Yamori, 2014). Plants grown in warmer conditions often reduce photosynthetic capacity (i.e. V_{pmaxA} and A_{max}), while maintaining comparable (or even higher) A at their growth temperatures compared with control plants. This decrease in photosynthetic capacity is likely due to reduced photosynthetic enzyme concentrations. Warmer temperatures allow plants to achieve the same rate of photosynthesis with lower enzyme concentrations because higher temperatures enhance enzyme activity (Way & Yamori, 2014; Yamori *et al.*, 2014). Evidence supports this idea in C_4 plants: Dwyer *et al.* (2007) compared C_4 *Panicum coloratum*, *Cenchrus ciliaris* and *Flaveria bidentis* grown at moderate and high temperatures and found that warm-grown plants had reduced photosynthetic capacity (i.e. A_{max}), underpinned by lower concentrations of RuBisCO and chloroplastic electron transport chain proteins. However, the authors did not find a significant effect of growth temperature on the activity or concentration of PEPc (measured at the respective growth temperatures), suggesting that PEPc may have high thermal stability (Chen *et al.*, 1994; Chinthapalli *et al.*, 2002; Boyd *et al.*, 2015). Thus, it is possible that the decrease in V_{pmaxA} with increasing mean T_{max} observed in our study may be reflective of changes in PEP regeneration via PPDK rather than PEPc capacity *per se*. In *Miscanthus* × *giganteus*, the activity and capacity of PPDK per unit leaf area increases in plants grown at chilling temperature, compared with their warm-grown controls (Wang *et al.*, 2008). Furthermore, whether A/C_i measurements are taken at saturated *PPFD* may also affect PEP regeneration, which requires two photosynthetically generated ATP per PEP converted (Hatch, 1987). Although some of the measurements in our dataset were made at $PPFD < 1000\ \mu\mathrm{mol\ m^{-2}\ s^{-1}}$, we found no significant effect of lower measurement *PPFD* on the responses of V_{pmaxA} and A_{max} to intrinsic or extrinsic factors (Tables 1, S3). Further research is needed to explore how PEPc and PPDK capacities are coordinated under thermal acclimation.

Our results highlight that both V_{pmaxA} and A_{max} increase with measurement T_{leaf} (Fig. 6a,b), which is indicative of enhanced enzymatic activities at higher measurement temperatures. According to an *in vitro* study in *S. viridis*, the capacities of major C_4 photosynthetic enzymes generally increase with rising T_{leaf} due to a corresponding rise in enzyme activities (Boyd *et al.*, 2015). The carboxylation activities of RuBisCO and PEPc increase exponentially between 10°C and 40–45°C, before declining (Chen *et al.*, 1994; Chinthapalli *et al.*, 2002; Boyd *et al.*, 2015). However, the extent to which this temperature response of enzymes holds true *in vivo* remains uncertain and needs to be confirmed with high-resolution photosynthesis-temperature response curves across a diverse range of C_4 species.

Finally, our analysis highlights that the response of A_{max} to T_{leaf} is influenced by mean T_{max}, whereas that of V_{pmaxA} is not (Table 1; Fig. 7). We found that A_{max} increases to a greater extent with increasing T_{leaf} in cool-grown plants than the plants grown in warmer conditions, suggesting that A_{max} of cool-grown plants is more sensitive to changes in T_{leaf} (Fig. 7b). Our data suggest that A_{max} of C_4 plants grown under future, hotter climates will be less affected by daily temperature fluctuations compared with plants grown under the current climate (i.e. A_{max} will be more stable; see circles in Fig. 7). This reduced thermal sensitivity of A_{max} at higher temperatures may help buffer photosynthesis in future climates, where heat waves are predicted to occur more frequently and intensely (Brown, 2020). However, this response could also limit the ability of warm-grown C_4 plants to achieve high A_{max} at the high T_{leaf} conditions they will experience (Fig. 7), which may suppress CO_2 uptake in future climate conditions. As we saw no effect of elevated growth CO_2 concentrations on either V_{pmaxA} or A_{max} (Fig. 5c,d), in line with field studies of C_4 species (Leakey *et al.*, 2006; Leakey, 2009; Markelz *et al.*, 2011), these temperature effects are likely to be stronger controls on C_4 photosynthetic capacity in future climates than predicted increases in CO_2 levels. However, further research is needed to fully understand how climate change affects the photosynthetic physiology of C_4 plants.

Future perspectives

Looking forward, our analyses highlight areas where more studies are warranted. For example, the physiology of C_4 eudicots and sedges is underrepresented in our dataset, although eudicots and sedges comprise 38% of C_4 plants (Sage, 2017) and thrive in extreme environments (e.g. *Haloxylon*, a C_4 desert shrub (Feng *et al.*, 2023)). Understanding how C_4 photosynthesis responds to these extreme environments could provide insights into improving how other plants (e.g. C_3 crops) cope with extreme environments. Furthermore, our results show that A_{max} responds differently to growth and measurement temperatures than does V_{pmaxA}. We explored potential biochemical limitations that underpin A_{max} and V_{pmaxA} separately, in addition to the response of these limitations to changes in temperature. However, it is unclear to what extent the individual biochemical limitations may interact and affect overall photosynthetic capacity. If one assumes that PEP regeneration is not limited, V_{pmaxA} is largely determined by the property of PEPc (e.g. activation state and activity of the enzyme) and its temperature sensitivity likely reflects the temperature sensitivity of PEPc. By contrast, A_{max} is determined by a variety of biochemical processes (e.g. RuBisCO carboxylation and rates of electron transport). Therefore, understanding how these processes interact in response to changing temperatures is crucial for fully grasping the overall response of A_{max}.

Conclusion

C_4 plants play a crucial role in carbon exchange and food security on a global scale. A growing number of studies dissect the mechanism of C_4 photosynthesis in specific contexts, yet how C_4 photosynthetic capacity responds to differences in biochemical subtypes, plant functional types, and growth and measurement conditions among a wide range of species remains unclear. Our study uncovers broad patterns of photosynthetic capacity from 74 C_4 species and highlights that environmental conditions play a dominant role in determining C_4 photosynthetic capacity. Importantly, we demonstrate that while the current simplified parameterisation of C_4 NADP-ME-type photosynthesis in leaf and ecosystem-level models likely represents species of all three biochemical types, it overestimates the photosynthetic capacity of C_4 native species in field conditions. Future research should aim to refine current parameter values by incorporating detailed equations that capture the effects of abiotic factors, such as water and nutrient availability, on photosynthetic capacity (Smith *et al.*, 2019), while also examining their link to the leaf economics spectrum (Monson *et al.*, 2025). Additionally, we compile a C_4 A/C_i database for community use, addressing the shortage of accessible raw data for C_4 gas exchange. For example, the TRY leaf-trait database (http://www.try-db.org), one of the most comprehensive databases of plant physiological trait data in the world, contains leaf photosynthetic point measurements for only 591 C_4 species, out of 69,000 terrestrial species measured, and no C_4 photosynthetic response curves (Kattge *et al.*, 2011, 2020). Our database opens new avenues for future studies involving big data analysis for C_4 plants, such as simulation modelling.

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Competing interests

None declared.

Author contributions

DAW conceived the concept for the paper within the discussions provided by the US Geological Survey Powell Center C_4 Photosynthesis Working Group. YF compiled the dataset, coded the scripts and ran the analyses with help from DWAN and BEM. NGS, EAA, FAB, FRD, ME, PF, RTF, SHG, OG, LG, VJ, ADBL, SL, ML, VSP, MMP, BVS, SC, RW and DAW provided the data to this study. YF and DAW wrote the first draft, and received feedback from RKM, RFS, DMG, JK, DLL, KP and CJS. All authors contributed substantially to improving the draft.

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Data availability

The data synthesised in this work are openly available as Supplementary Datasets and can be downloaded from https://github.com/yuzhenfanANU/C4_repository.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 C_4 A/C_i data collated for this study.

Dataset S2 Estimated apparent V_{pmaxA} and A_{max} used in the analysis.

Fig. S1 Species distribution within the categories of growth forms and growth locations.

Fig. S2 Correlation of mean T_{max} and T_{min}.

Fig. S3 Data distribution of V_{pmaxA} and A_{max} in categories of C_4 subtypes and growth forms over T_{leaf}, mean T_{max} and *PPFD*.

Fig. S4 Histograms describing the data distribution across the spectrum of mean T_{max} and T_{leaf} for growth location, growth form and important species group.

Fig. S5 V_{pmaxA} and A_{max} plotted by C_4 biochemical subtypes, with symbols highlighting the effect of phylogeny.

Fig. S6 Comparison between V_{pmaxA} and A_{max} with growth location coloured by species group (maize vs sorghum vs other species).

Fig. S7 Relationships between V_{pmaxA} and A_{max} with mean T_{max} for measurements done at T_{leaf} between 25°C and 30°C.

Fig. S8 Relationships between V_{pmaxA} and A_{max} with mean T_{max} for indoor and outdoor plants.

Fig. S9 Relationships between V_{pmaxA} and A_{max} with growth CO_2 levels for indoor and outdoor plants.

Fig. S10 Relationships between V_{pmaxA} and A_{max} with T_{leaf} for indoor and outdoor plants.

Fig. S11 Relationships between V_{pmaxA} and A_{max} with *PPFD* for indoor and outdoor plants.

Fig. S12 Correlation of mean T_{max} and T_{min} for data with $PPFD > 1500$ µmol photon $m^{-2}\ s^{-1}$.

Table S1 Results of multivariate linear mixed-effects models testing the response of V_{pmaxA} and A_{max} against species-specific traits and experimental conditions for indoor and outdoor plants separately.

Table S2 Correlations between V_{pmaxA} or A_{max} and species traits in indoor and outdoor plants.

Table S3 Results of multivariate linear mixed-effects models for data with $PPFD > 1000$ µmol photon $m^{-2}\ s^{-1}$.