



# Mesophyll airspace unsaturation drives $C_4$ plant success under vapor pressure deficit stress

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Affiliations are included on p. 8.

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A fundamental assumption in plant science posits that leaf air spaces remain vapor saturated, leading to the predominant view that stomata alone control leaf water loss. This concept has been pivotal in photosynthesis and water-use efficiency research. However, recent evidence has refuted this longstanding assumption by providing evidence of unsaturation in the leaf air space of  $C_3$  plants under relatively mild vapor pressure deficit (VPD) stress. This phenomenon represents a nonstomatal mechanism restricting water loss from the mesophyll. The potential ubiquity and physiological implications of this phenomenon, its driving mechanisms in different plant species and habitats, and its interaction with other ecological adaptations remain to be fully explored. In this context,  $C_4$  plants spark particular interest for their importance as crops, bundle sheath cells' unique anatomical characteristics and specialized functions, and notably higher water-use efficiency relative to  $C_3$  plants. Here, we confirm reduced relative humidities in the substomatal cavity of the  $C_4$  plants maize, sorghum, and proso millet down to 80% under mild VPD stress. We demonstrate the critical role of nonstomatal control in these plants, indicating that the role of the  $CO_2$  concentration mechanism in  $CO_2$  management at a high VPD may have been overestimated. Our findings offer a mechanistic reconciliation between discrepancies in  $CO_2$  and VPD responses reported in  $C_4$  species. They also reveal that nonstomatal control is integral to maintaining an advantageous microclimate of relatively higher  $CO_2$  concentrations in the mesophyll air space of  $C_4$  plants for carbon fixation, proving vital when these plants face VPD stress.

unsaturation |  $C_4$  | maize | millet | sorghum

Plants have evolved many mechanisms to adapt to diverse environmental challenges, especially those related to water availability and transpiration. One of the basic assumptions in plant sciences has been that the leaf air space remains saturated under most conditions (1). This assumption implies that water evaporates so rapidly from the mesophyll surface that saturation is maintained in the mesophyll air space due to stomatal control, apart from perhaps in the throat of the stoma, where there is a gradient from internal to external humidity. This concept has given rise to the widely accepted view that stomata represent the sole dynamic control of leaf transpiration (2). The assumption of saturation within the leaf has played a pivotal role in plant physiology research, as it has been fundamental for calculating stomatal conductance and the  $CO_2$  concentration inside the leaf from gas exchange measurements. Thus, it has formed a cornerstone for many applications in plant physiological research, especially in relation to photosynthesis and water-use efficiency.

The recent discovery that the leaf air space of  $C_3$  plants can experience unsaturation under slight and severe vapor pressure deficits (VPDs) (3, 4) has brought a paradigm shift in our comprehension of plant–water relations (5, 6). This revelation has significant implications not just for  $C_3$  plants, where this phenomenon has been observed, but potentially for the broader spectrum of plant species (7). Crucially, the occurrence of unsaturation indicates the presence of an internal control of transpiration that we were not aware of before, a nonstomatal control of transpiration.

Based on well-established gas exchange procedures and calculations, the Wong et al. (4) method presents compelling evidence of unsaturation. Wong et al. (4) conducted an experiment which consisted of reducing the  $CO_2$  concentration on the abaxial surface of the leaf until the assimilation rate at that surface was zero, while maintaining atmospheric  $CO_2$  concentration on the adaxial surface. This directly measures the abaxial substomatal cavity  $CO_2$  concentration ( $c_i$ ) while allowing assimilation through the adaxial surface. Then, adaxial  $c_i$  is estimated using conventional calculations for gas exchange. When adaxial and abaxial  $c_i$  were compared, it was observed that as VPD increases, the calculations indicate a decrease in the difference between adaxial and abaxial  $c_i$  if the water vapor concentration in the substomatal cavity ( $w_i$ ) was assumed to be at saturation ( $w_{sat}$ ). This decline would suggest a

## Significance

$C_4$  plants such as maize, millet, and sorghum are crucial for global food security, and our research marks a significant advance in understanding their physiological mechanisms. We demonstrate the existence of nonstomatal control of water loss in  $C_4$  plants and its vital role in maintaining favorable  $CO_2$  conditions to achieve high assimilation rates under increased evaporative demand. Our findings reveal substantial unsaturation within the substomatal cavity even under moderate vapor pressure deficit (VPD), challenging traditional interpretations of  $C_4$  photosynthetic efficiency under VPD stress. This research reframes water use efficiency in  $C_4$  plants, highlighting the crucial role of mesophyll hydraulic properties (i.e., nonstomatal control of water loss) beyond the usual focus on  $CO_2$  concentration mechanisms.

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The authors declare no competing interest.

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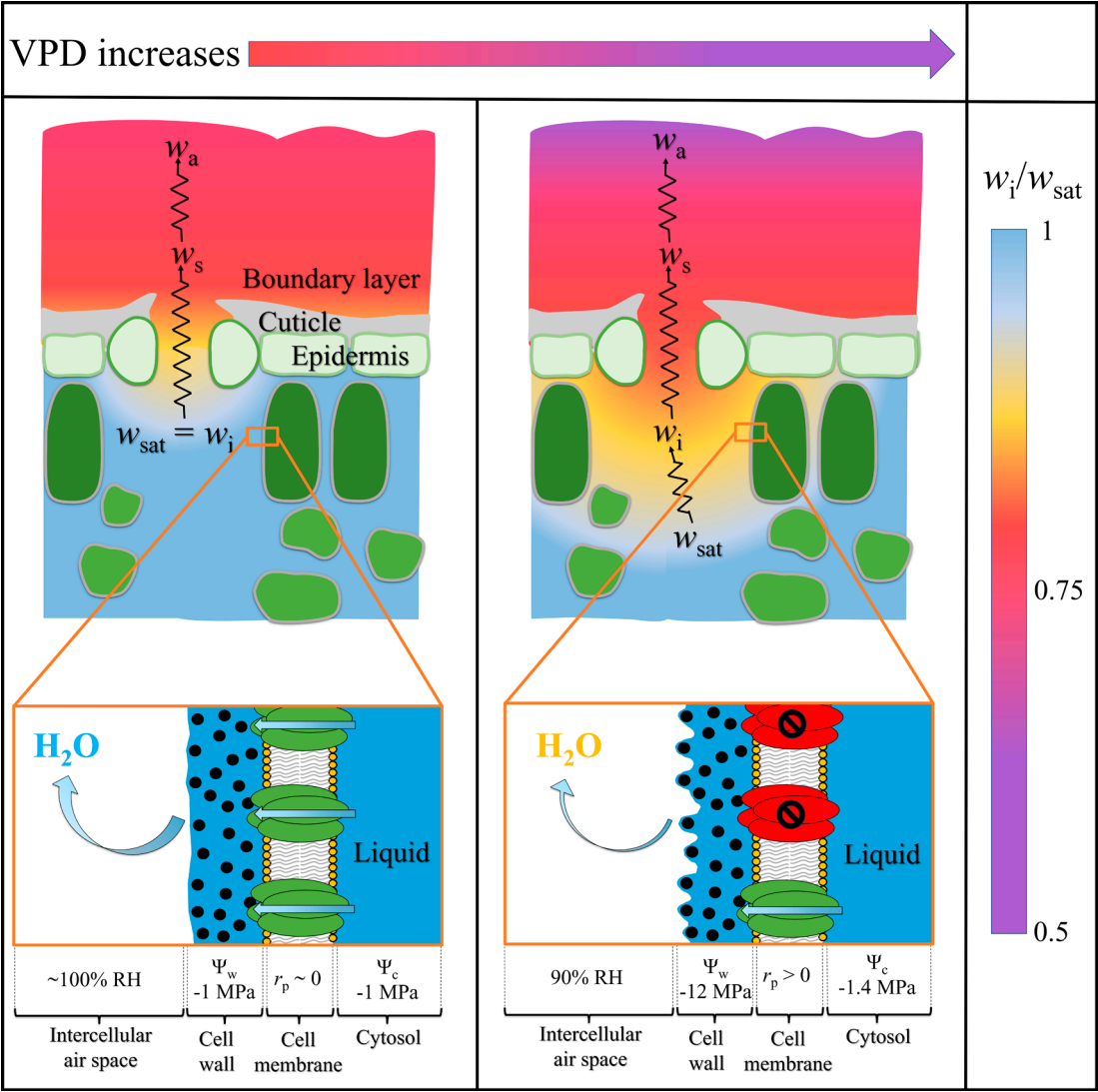
diminishing resistance to  $\text{CO}_2$  diffusion between the adaxial and abaxial substomatal cavities ( $R_{\text{ias}}$ ). The apparent decrease in  $R_{\text{ias}}$  leads to physically unrealistic values, including negative resistance when the adaxial  $c_i$  was computed to be lower than abaxial  $c_i$  at high VPD levels. The reduction in the  $c_i$  difference under increased VPD directs us to the variable  $w_i$  as the modifiable factor. With all other parameters measured and accounted for, the dependency of  $c_i$  on  $w_i$  and unsaturation becomes apparent.

Evidence shows that the nonstomatal control of transpiration has the form of resistance to liquid water movement in the plasma membranes of mesophyll cells ( $r_p$ ), allowing them to maintain a modest water potential in the cytosol while the cell wall dries out (4) (Fig. 1). Wong et al. (4) suggest, based on their observations, that a saturation front moves inward as  $r_p$  develops, creating humidity gradients within the leaf air space. The authors observed an apparent increased resistance to water vapor diffusion in the leaf air space, named unsaturation resistance. They noted that the air space must have a water vapor concentration that matches the relative humidity in equilibrium with the water potential of the nearby cell wall. Thus, the humidity gradient must be due to

the varying water potentials in the cell walls of different mesophyll cells. For this to be possible, a gradual increase in  $r_p$  along the mesophyll cell membranes inward of the leaf must occur as VPD rises. The Wong et al. (4) analysis focused on the  $r_p$  of the cells adjacent to the substomatal cavity, showing how this hydraulic property impacts the leaf air space water vapor concentration.

Research on humidity in mesophyll air space has been centered on  $\text{C}_3$  species. Until now, airspace substomatal cavity unsaturation has been demonstrated in the  $\text{C}_3$  plant species *Gossypium hirsutum*, *Helianthus annuus*, *Phaseolus vulgaris*, *Xanthium strumarium*, *Eucalyptus pauciflora*, *Glycine max* (4), *Juniperus monosperma*, and *Pinus edulis* (3). This suggests a common occurrence in  $\text{C}_3$  plants and raises the question of whether such a phenomenon is exclusive to  $\text{C}_3$  plants or extends to other photosynthetic pathways, particularly the  $\text{C}_4$  strategy.

$\text{C}_4$  plants, with their distinctive carbon fixation mechanism, are traditionally considered more water-use efficient than their  $\text{C}_3$  counterparts (8, 9), reflecting an evolutionary adaptation to hot and arid conditions, where high light intensities and temperatures would promote excessive photorespiration in  $\text{C}_3$  plants (10, 11).



**Fig. 1.** Diagram of the mechanism of nonstomatal control of transpiration as VPD increases, generating unsaturation in the substomatal cavity. The parameters in the figure are atmospheric water vapor concentration ( $w_a$ ), water vapor concentration on the leaf surface ( $w_s$ ), substomatal cavity water vapor concentration ( $w_i$ ), water vapor concentration at saturation ( $w_{\text{sat}}$ ), resistance to water diffusion through the plasma membrane ( $r_p$ ), cell wall water potential ( $\Psi_w$ ), and cytosolic water potential ( $\Psi_c$ ). Black dots represent cell wall fibers. The current theory suggests that aquaporins acting as transmembrane conduits close when VPD rises, increasing the resistance to water movement through the membrane of mesophyll cells ( $r_p$ ) and triggering unsaturation in the substomatal cavity.

The prevailing understanding is to attribute this to the  $C_4$  photosynthetic energy-driven  $CO_2$  concentrating mechanism (CCM), which enhances  $CO_2$  concentration at the sites where it is fixed, increasing the assimilation rate ( $A$ ) and suppressing photorespiration (12).  $C_4$  uses the enzyme phosphoenolpyruvate carboxylase to capture  $CO_2$ , reducing the concentration in the mesophyll air space of the leaf to drive greater fluxes of  $CO_2$  from the atmosphere even when their stomatal conductance ( $g_{sw}$ ) is comparatively lower than in  $C_3$  plants (12, 13).

The accepted hypothesis is that the CCM also allows  $C_4$  plants to maintain a high assimilation rate even when stomatal conductance is reduced under high VPD or drought conditions (14–16). This ability to maintain robust photosynthesis while conserving water underpins the superior water-use efficiency of  $C_4$  plants. Yet, this hypothesis appears to conflict with the  $CO_2$  response observed in  $C_4$  plants, which are known to significantly reduce their assimilation rate at low  $CO_2$  concentrations (e.g., ref. 17). Despite this conflicting evidence, no mechanistic explanation for the observed relatively high assimilation rates at high VPD has been tested or offered. In practice, the presumed efficiency of the CCM at low  $CO_2$  concentrations suggests that  $C_4$  plants require little to no nonstomatal control of transpiration. However, if nonstomatal control of transpiration exists in  $C_4$  plants, it could offer a mechanistic explanation for the observed conflicting evidence between their  $CO_2$  and VPD responses. This would challenge existing paradigms and prompt a reevaluation of how  $C_4$  plants manage water and carbon dioxide under high VPD conditions. The evolutionary advantage of  $C_4$  plants in hot and dry climates makes the potential existence of the nonstomatal control mechanism an exciting avenue of exploration extending beyond scientific interest into important practical applications such as commercial plant breeding.

According to the United Nations Food and Agriculture Organization, the  $C_4$  plant *Zea mays* (maize) and *Saccharum spp* (sugar cane) dominate the world's crop production, with another  $C_4$  species, *Sorghum bicolor*, as the fourth most important cereal in the world (18). *Panicum miliaceum* (proso millet) is also a significant  $C_4$  crop for human consumption and animal feed (18). Given their economic and nutritional significance, understanding the water-use mechanisms of  $C_4$  crops is crucial, and exploring the existence of unsaturation can give us unique insights into their strategies. The species maize, sorghum, and proso millet, highlighted in our study, are not only agriculturally significant but largely used as model plants for  $C_4$  studies. These three species also represent a diverse range of the  $C_4$  plant spectrum: Proso millet belongs to the photosynthetic subtype nicotinamide adenine dinucleotide dependent malic enzyme, sorghum to the nicotinamide adenine dinucleotide phosphate dependent malic enzyme (NADP-ME) subtype, and maize is an NADP-ME subtype with additional provision from the PEP carboxykinase pathway (19).

In light of these considerations, we aimed to explore this uncharted territory, investigating the presence of unsaturation as a mechanism of gas exchange regulation in prominent  $C_4$  species. The findings could have significant implications for understanding plant physiology and applications such as agriculture, where water-use efficiency is paramount.

Results

Our key results reveal 1) a consistent total leaf internal air space resistance to  $CO_2$  diffusion ( $R_{ias}$ ) across the species examined; 2) the three species experienced significant unsaturation of their mesophyll air space at relatively moderate VPD; 3) failure to account for unsaturation significantly impacts the estimation of crucial  $C_4$  physiological trends; and 4) the three  $C_4$  plant species evaluated

present a remarkable interplay between mesophyll  $CO_2$  availability and water loss control mediated by nonstomatal control of transpiration, aiding the maintenance of high assimilation rates under VPD stress. The comprehensive details of these findings are elaborated in the following sections.

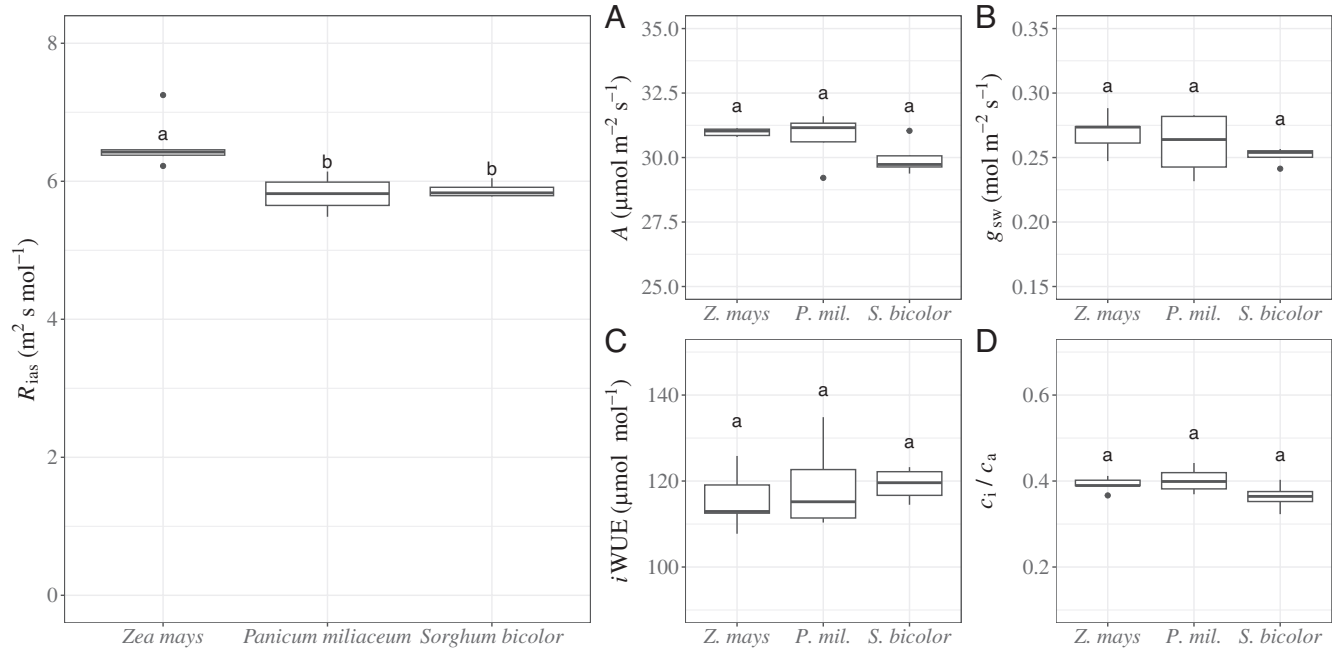
A list of symbols used in the text is provided in Table 1.

**Leaf Air Space Resistance ( $R_{ias}$ ).** The leaf's internal air space resistance ( $R_{ias}$ ) is the resistance to  $CO_2$  diffusion between the adaxial and abaxial substomatal cavities. No difference in the average  $R_{ias}$  was found between *P. miliaceum* and *Sorghum bicolor* (Fig. 2).  $R_{ias}$  values from *Z. mays* leaves were statistically larger than for panicum and sorghum; however, this difference was trivial in practice. In general, it was found that this difference in  $R_{ias}$  was not related to other metrics, such as the assimilation rate ( $A$ ), stomatal conductance to water ( $g_{sw}$ ), intrinsic water use efficiency ( $iWUE = A/g_{sw}$ ), and the ratio of internal to atmospheric  $CO_2$  concentrations ( $c_i/c_a$ ) (Fig. 2 A–D). Throughout our experimental observations,  $R_{ias}$  remained remarkably consistent across species. In line with the findings of Wong, Cowan, and Farquhar (8), the  $c_i/c_a$  ratio was about 0.4 for these  $C_4$  species.

**Observed Unsaturation.** In line with observations of  $C_3$  plants by Wong et al. (4), there was a consistent trend in the variation of the difference between the adaxial and abaxial  $c_i$  ( $c_{i,ad} - c_{i,ab}$ ) with increases in VPD across the three  $C_4$  species studied (Fig. 3). As the VPD increased,  $c_{i,ad} - c_{i,ab}$  diminished, eventually turning negative at a VPD of 3 kPa. Such negative values are physically impossible as the  $CO_2$

Table 1. List of symbols

| Symbol               | Description                                           | Units                                |
|----------------------|-------------------------------------------------------|--------------------------------------|
| $A$                  | Total assimilation rate                               | $\mu\text{mol m}^{-2} \text{s}^{-1}$ |
| $A_s$                | Assimilation rate through stomata                     | $\mu\text{mol m}^{-2} \text{s}^{-1}$ |
| ad and ab subscripts | Adaxial and abaxial                                   | –                                    |
| $c_a$                | Atmospheric $CO_2$ concentration                      | $\mu\text{mol mol}^{-1}$             |
| CCM                  | Carbon concentrating mechanism                        | –                                    |
| $c_s$                | $CO_2$ concentration on the leaf surface              | $\mu\text{mol mol}^{-1}$             |
| $c_i$                | Substomatal cavity $CO_2$ concentration               | $\mu\text{mol mol}^{-1}$             |
| $E$                  | Total transpiration rate                              | $\text{mol m}^{-2} \text{s}^{-1}$    |
| $E_s$                | Transpiration rate through stomata                    | $\text{mol m}^{-2} \text{s}^{-1}$    |
| $g_{sw}$             | Stomatal conductance to water vapor                   | $\text{mol m}^{-2} \text{s}^{-1}$    |
| $iWUE$               | Intrinsic water use efficiency                        | $\mu\text{mol mol}^{-1}$             |
| RH                   | Relative humidity                                     | –                                    |
| $R_{ias}$            | Leaf internal airspace resistance to $CO_2$ diffusion | $\text{m}^2 \text{s mol}^{-1}$       |
| VPD                  | Vapor pressure deficit                                | kPa                                  |
| $w_a$                | Atmospheric water vapor concentration                 | $\text{mol mol}^{-1}$                |
| $w_i$                | Substomatal cavity water vapor concentration          | $\text{mol mol}^{-1}$                |
| $w_s$                | Water vapor concentration on the leaf surface         | $\text{mol mol}^{-1}$                |
| $w_{sat}$            | Water vapor concentration at saturation               | $\text{mol mol}^{-1}$                |



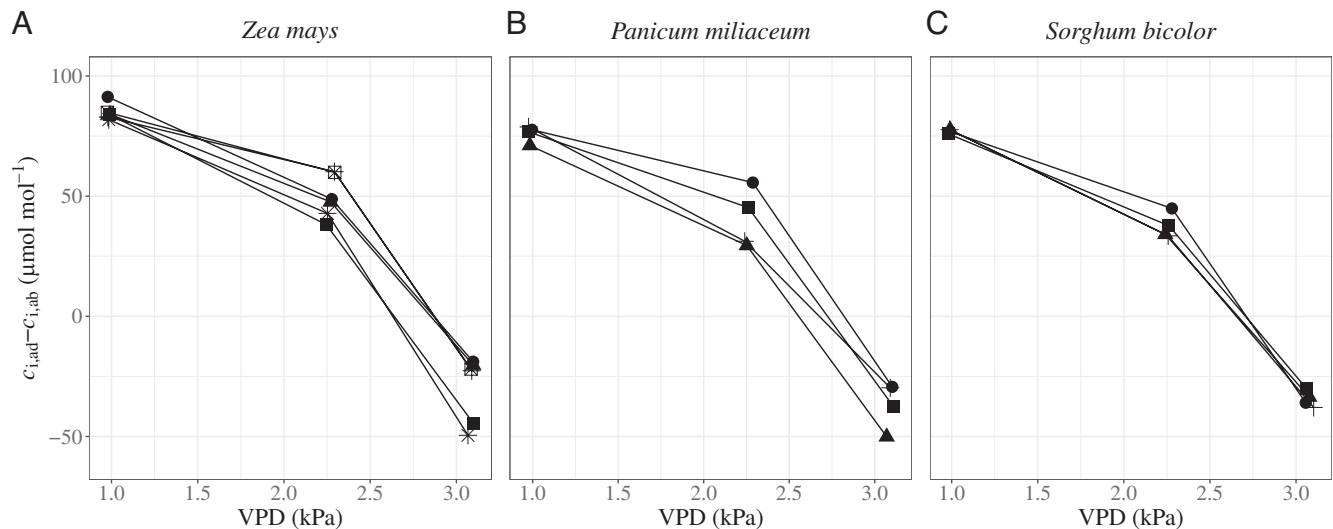
**Fig. 2.** Average gas exchange parameter values from the plants evaluated. Average internal leaf air space resistance ( $R_{ias}$ ) found in three  $C_4$  species: *Z. mays*, *P. miliaceum*, and *Sorghum bicolor*. Panels A–D are measurements under the typical setup of a LI-6800 for assimilation rate ( $A$ ), stomatal conductance to water ( $g_{sw}$ ), iWUE, and  $c_i/c_a$ , respectively. Distinct letters indicate statistical differences with a  $P$ -value  $< 0.05$  using Student's  $t$  test and Tukey's Honest Significant Difference at 95% confidence. Raw data and calculations are provided in [SI Appendix, Supporting text and Dataset S1](#).

is sourced from the adaxial surface (i.e.,  $c_{i,ad} > c_{i,ab}$ ), confirming the error in assuming a saturated condition within the substomatal cavity.

The corrected  $w_i$  values estimated using (Eq. 2) (see derivation in [SI Appendix, Supporting text](#)) showed that the three species experienced unsaturation in their substomatal cavities. As shown in Fig. 4, relative humidity in the substomatal cavity ( $w_i/w_{sat}$ ) fell to between 85% and 80% under the highest VPD evaluated. In more detail, *Z. mays* and *P. miliaceum* leaves experienced internal relative humidity fluctuating between 96% and 92% at a VPD of 2 kPa, and a decline to 85% to 80% at VPD of 3 kPa. Following the same trend, *Sorghum bicolor* experienced slightly lower levels of unsaturation with 95% to 93% relative humidity at 2 kPa and 84% to 83% relative humidity at 3 kPa VPD. Interestingly, all

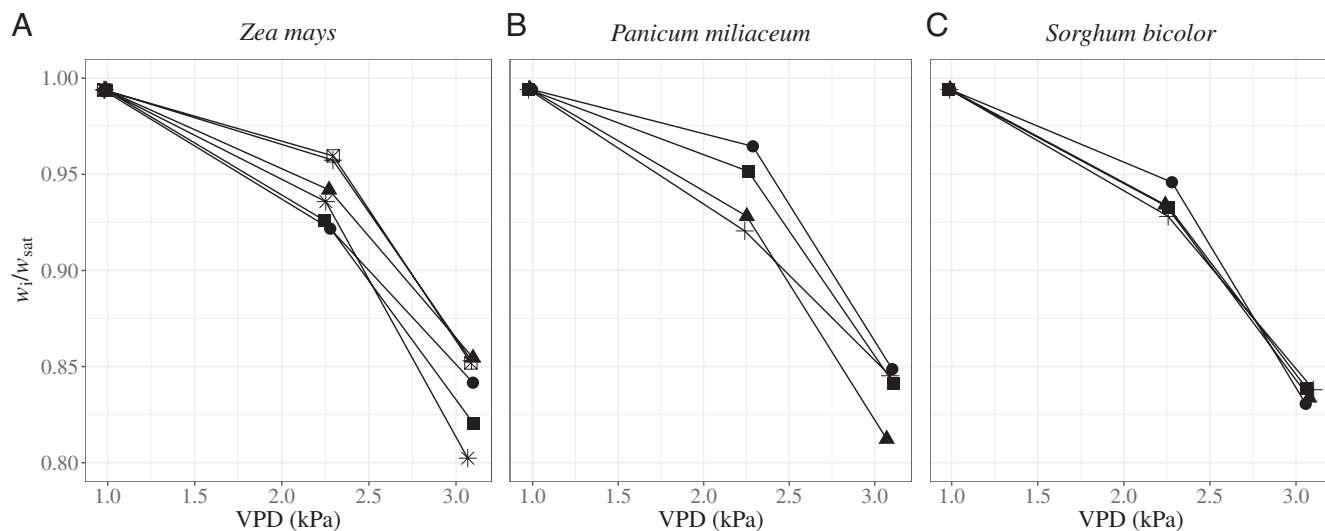
the leaves evaluated achieved a plateau in transpiration rate where a further increase in VPD did not generate a significant increase in transpiration rate ([SI Appendix, Fig. S1](#)). Similar behavior was also observed by Wong et al. (4) in  $C_3$  plants within the same time frame between measurements (30 to 45 min).

**Impact of Unsaturation.** As VPD rose, a consistent decline in  $A$  was observed across all species, as shown in Fig. 5A. Consistent with the observed decrease in  $A$ , Fig. 5B shows that  $g_{sw}$  decreases with increasing VPD; however, the rate of decline is less steep when using actual  $w_i$ , rather than assuming saturation. The difference in the rate of  $g_{sw}$  decline with VPD increase translates to an iWUE ( $iWUE = A/g_{sw}$ ) calculated with actual  $w_i$  responding less sharply, while the



**Fig. 3.** Difference between adaxial and abaxial substomatal cavity  $CO_2$  concentration ( $c_{i,ad} - c_{i,ab}$ ) of (A) *Zea mays*, (B) *Panicum miliaceum* and (C) *Sorghum bicolor* leaves. Values presented assume a saturated condition within the substomatal cavity ( $w_i = w_{sat}$ ) in three  $C_4$  species. Each line is a single leaf. Raw data and calculations are provided in [SI Appendix, Supporting text and Dataset S1](#).





**Fig. 4.** Unsaturation estimation at different VPDs for (A) *Zea mays*, (B) *Panicum miliaceum*, and (C) *Sorghum bicolor* leaves. Relative humidity in the leaf substomatal cavity ( $w_i/w_{\text{sat}}$ ) was estimated with increasing VPD, tested in three  $C_4$  species. Each line is a single leaf. Raw data and calculations are presented in [SI Appendix, Supporting text and Dataset S1](#).

saturation assumption predicts a continuous rise (Fig. 5C). It is worth noting that the unsaturation phenomenon introduced by nonstomatal control of transpiration adds an additional layer of

complexity to interpreting  $iWUE$ , as maintaining  $g_{\text{sw}}$  during VPD increase would not necessarily result in increased water loss.

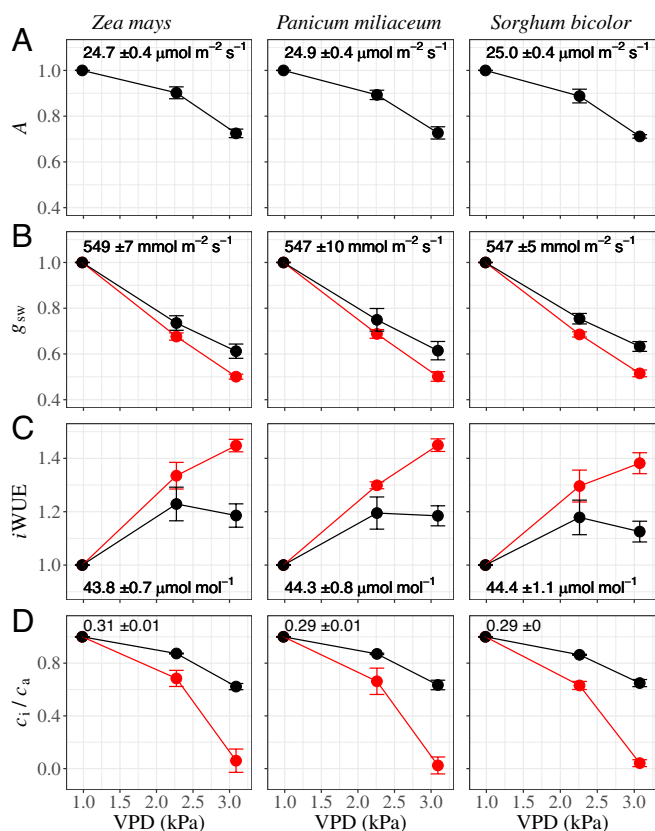
Importantly, the ratio of internal to atmospheric  $\text{CO}_2$  concentration ( $c_{i,\text{ad}}/c_{a,\text{ad}}$ ) shows a less marked decrease with VPD when actual  $w_i$  is considered (Fig. 5D), indicating a less severe impact on internal  $\text{CO}_2$  availability than when assuming saturation. Overall, comparing the calculations using the actual  $w_i$  or assumed saturation highlights the risk of underestimating  $g_{\text{sw}}$  and overestimating the drop in  $c_i/c_a$  when leaf unsaturation is not considered.

**Unsaturation and Photosynthesis.** Note that stomatal conductance used to estimate  $c_i$  accounts for the diffusion path from the substomatal cavity to the external surface of the leaf, so  $c_i$  is the  $\text{CO}_2$  concentration in the substomatal cavity where  $w_i$  is found (Fig. 1). This estimation can be approached in two ways: by calculating  $c_i$  for each leaf surface independently, such as in Fig. 3, or by combining both adaxial and abaxial fluxes. The latter method is the more common approach employed by commercial instruments, examples of which are in Fig. 2D and the  $A/c_i$  curves presented in Fig. 6. The differences between these two approaches under the same conditions are usually small, in the range of  $10 \mu\text{mol mol}^{-1}$ , where the standard approach estimates an average of the adaxial and abaxial  $c_i$  weighted by the assimilation rate of each surface and the leaf  $R_{\text{ias}}$  (20).

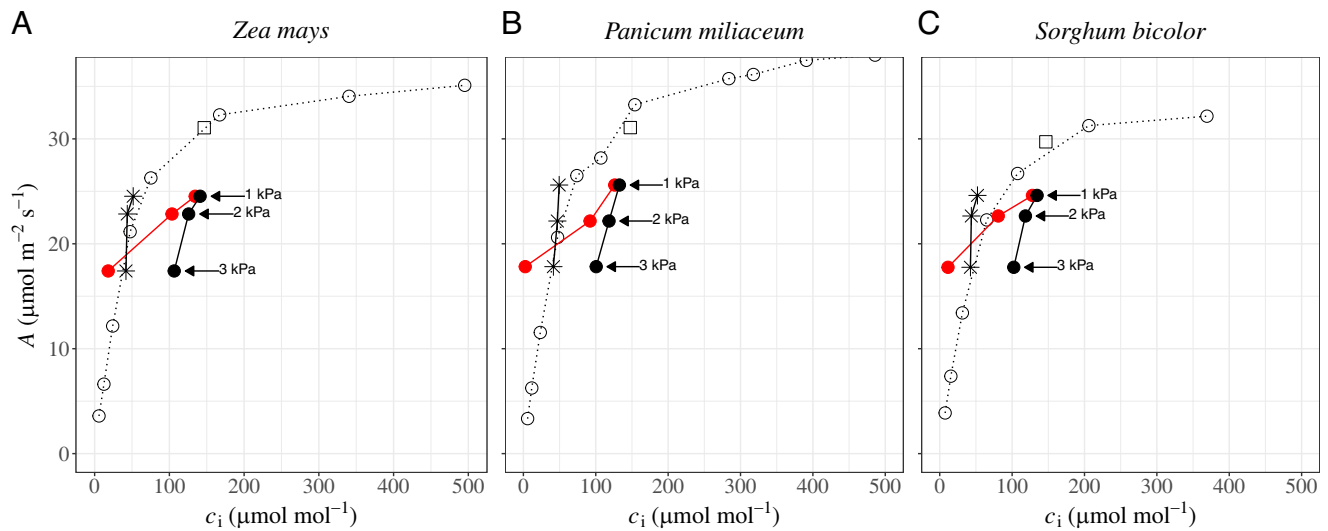
In line with Márquez, Stuart-Williams, Cernusak, and Farquhar (20), the minimum  $\text{CO}_2$  concentration within the leaf ( $c_{i,\text{ab}}$  in our experiments) showed a small difference with the  $c_i$  estimated under standard  $\text{CO}_2$  response experiments, both presenting the same trend (Fig. 6, asterisks and open circles). This observed behavior aligns with expectations, as biochemical processes driving photosynthesis are known not to be influenced by changes in VPD other than those related to the reduction of  $c_i$  due to stomatal closure (21).

On the other hand, notable departures of the adaxial  $c_i$  from the  $A/c_i$  trends (slope) are found when unsaturation is not accounted for, underestimating  $c_i$  levels (Fig. 6, red lines). Estimations of adaxial  $c_i$  track the  $A/c_i$  curve again when corrected for unsaturation (Fig. 6, dotted and black lines). This makes it evident that neglecting unsaturation leads to a markedly altered  $A/c_i$  relationship that overestimates the drop in  $c_i$  in our observations by an average of 28% at 2 kPa, estimated as  $[1 - c_i(w_{\text{sat}})/c_i(w_i)]$ , and 94% at 3 kPa.

The  $c_i$  values from the adaxial surface obtained using the method from Wong et al. (4) to estimate unsaturation ( $c_{i,\text{Wong}}$ ) are expected



**Fig. 5.** Comparison of the estimations of gas exchange parameters when accounting for unsaturation (black symbols) versus assuming saturation (red symbols). Assimilation rate (A, panel A), stomatal conductance ( $g_{\text{sw}}$ , panel B),  $iWUE$  ( $iWUE = A/g_{\text{sw}}$ , panel C), and intercellular to atmospheric  $\text{CO}_2$  concentration ratio ( $c_{i,\text{ad}}/c_{a,\text{ad}}$ , panel D) in response to VPD increases. Values are normalized to values at VPD of 1 kPa presented in each graph as the average  $\pm$  SD of measurements in six *Z. mays*, four *P. miliaceum*, and four *Sorghum bicolor* plants. Black circles and lines represent estimation using  $w_i$  shown in Fig. 4, and red circles and lines represent estimations using  $w_i = w_{\text{sat}}$ . Circles represent the average value, and the error bars represent the SD. Raw data and calculations are presented in [SI Appendix, Supporting text and Dataset S1](#).



**Fig. 6.** Comparison of CO<sub>2</sub> response trends of (A) *Zea mays*, (B) *Panicum miliaceum*, and (C) *Sorghum bicolor* leaves. Comparison of a standard CO<sub>2</sub> response curve (open circles) with the relation between assimilation rate and adaxial  $c_i$  estimated during VPD increases accounting for unsaturation (black circles) and assuming  $w_i = w_{\text{sat}}$  (red circles). The asterisks are the minimum CO<sub>2</sub> concentration within the leaf (abaxial  $c_i$ ). The open square is the steady-state measurement at benign operational conditions presented in Fig. 2. Arrows indicate the VPD on which the measurement was performed. Measurements shown in each panel were conducted on the same leaf. Raw data and calculations are presented in [SI Appendix, Supporting text and Dataset S1](#).

to be higher than those from a standard CO<sub>2</sub> response curve ( $c_{i,\text{standard}}$ ). This is due to the roughly half extra  $R_{\text{ias}}$  added to the path length for CO<sub>2</sub> diffusion from the adaxial surface when the CO<sub>2</sub> concentration from the abaxial surface is dropped until the assimilation rate at that surface is zero. Consequently, to achieve the same assimilation rate with CO<sub>2</sub> sourced exclusively from the adaxial surface,  $c_{i,\text{Wong}}$  must inherently be higher than  $c_{i,\text{standard}}$ , which benefits from CO<sub>2</sub> sourced from both surfaces. Thus, for a given  $A$ ,  $c_{i,\text{Wong}}$  is expected to be roughly  $c_{i,\text{Wong}} \approx AR_{\text{ias}}/2 + c_{i,\text{standard}}$ , which is the offset we observed in all our measurements after accounting for unsaturation, indicating that the correction using  $w_i$  is found in the expected magnitude of  $c_{i,\text{ad}}$  for all our measurements (Fig. 6 and [SI Appendix, Figs. S2–S4](#)). Similarly, the smaller  $c_i/c_a$  ratio observed in Fig. 5 compared to Fig. 2 can be explained by the need for either a higher  $c_a$  or a lower  $c_i$  to sustain the CO<sub>2</sub> flux, as the photosynthetic capacity through the adaxial surfaces effectively doubles. For a detailed discussion about this reasoning behind the CO<sub>2</sub> gradient within the mesophyll air space and  $R_{\text{ias}}$  in the path between the adaxial and abaxial substomatal cavities, see for example refs. 20 and 22.

## Discussion

Wong et al. (4) and Cernusak et al. (3) have provided compelling evidence for the occurrence of unsaturation in the substomatal cavity of turgid leaves of several C<sub>3</sub> species, refuting a longstanding assumption of plant physiology. Here, we showed that substantial unsaturation of the substomatal cavity occurs in C<sub>4</sub> species as well (Fig. 4), presenting a unique insight into the phenomenon and suggesting its extended occurrence among plants regardless of their photosynthetic pathway. The unsaturation levels reported for the three C<sub>4</sub> species evaluated here were from relative humidities of 80% to 85% ( $w_i/w_{\text{sat}}$ ) at 3 kPa VPD, aligning with those found in C<sub>3</sub> species *H. annuus* (4) and *P. edulis* (3) under similar VPDs. However, other C<sub>3</sub> species have been shown to exhibit less unsaturation at similar VPDs, with relative humidities from 95% to 85%. For example, *P. vulgaris* and *X. strumarium*, presented unsaturation with relative humidities of around 95%, and *G. hirsutum*, *E. pauciflora*, *G. max*, and *J. monosperma*, showed unsaturation with levels of relative humidities between 90% and 85%. These variations, possibly influenced by

species characteristics and environmental conditions during growth, underscore the need for further research to clarify the underlying causes of differential unsaturation across species.

The C<sub>4</sub> plants evaluated here exhibit a greater contribution of nonstomatal control of transpiration than most C<sub>3</sub> plants evaluated until now, avoiding a  $c_i$  drop by maintaining their stomata proportionally more open at high VPD. That is, they experienced a lower  $g_{\text{sw}}$  reduction (38% drop at 3 kPa; Fig. 5) than their C<sub>3</sub> counterparts evaluated in ref. 4 with equivalent unsaturation (55%  $g_{\text{sw}}$  drop). Considering that C<sub>4</sub> plants have lower  $g_{\text{sw}}$  and usually operate at a lower  $c_i$  than C<sub>3</sub> plants, the observed coordination between nonstomatal control and  $g_{\text{sw}}$  in C<sub>4</sub> plants points to fine-tuning water loss and CO<sub>2</sub> availability. Notably, our results show significant deviations in the  $A/c_i$  relationships when unsaturation was not considered, compared to when it was (Fig. 6). The differences in  $c_i$  estimations with actual  $w_i$  compared to assuming saturation in the maize, sorghum, and proso millet were consistently three to four times larger than those found in C<sub>3</sub> plants (4) with similar degrees of unsaturation. These findings are consistent with previous observations that C<sub>4</sub> plants usually suffer a greater reduction in  $A$  than C<sub>3</sub> plants when their  $c_i$  drops from their normal operational level (17).

Without nonstomatal control of transpiration, C<sub>3</sub> and C<sub>4</sub> plants would have to close stomata to maintain the observed transpiration, resulting in a decrease of  $A$  or experiencing a higher transpiration rate to sustain the observed  $A$ . To analyze the role of nonstomatal control of transpiration in sustaining  $c_i$ , the standard  $A/c_i$  curve can be used to evaluate the expected drop of  $A$  proportional to the drop between  $c_i(w_i)$  and  $c_i(w_{\text{sat}})$  observed during our experiments. From our measurements at VPD of 3 kPa in maize, sorghum, and proso millet, to maintain the observed transpiration rate, plants would have to close stomata, suffering an 80% to 100% assimilation rate reduction or experience a 24% to 35% higher transpiration rate to sustain the observed assimilation rates ([SI Appendix, Fig. S5](#)). The same analysis for C<sub>3</sub> plants from ref. 4 indicates they would have experienced an approximately 25% lower assimilation rate or a 50% higher transpiration rate, which is within expectations considering the typical shape of C<sub>3</sub>  $A/c_i$  responses. While C<sub>3</sub> and C<sub>4</sub> plants clearly benefit from having a nonstomatal control of transpiration under VPD stress, our data on three C<sub>4</sub> species suggest that the C<sub>4</sub> strategy achieves a proportionally larger advantage by enhancing CO<sub>2</sub>

availability and would suffer a greater  $A$  loss without nonstomatal control. This emphasizes the critical importance of nonstomatal control of transpiration in improving the availability of  $\text{CO}_2$  for the CCM in the evaluated  $\text{C}_4$  plants during VPD stress (Fig. 5) and how plants, in general, further optimize water-use efficiency.

We have provided compelling evidence that the nonstomatal control mechanism is fundamental to maintaining an advantageous microclimate for carbon fixation within leaves of three agronomically important  $\text{C}_4$  species. It follows that not accounting for unsaturation results in overestimating the ability of  $\text{C}_4$  plants to sustain high assimilation rates despite a significant apparent reduction in  $g_{\text{sw}}$  and, consequently, in  $c_i$ . This overestimation aligns with the commonly held theory that the CCM alone allows  $\text{C}_4$  plants to maintain high  $A$  even when  $g_{\text{sw}}$  is reduced under high VPD or drought conditions. The theory is based on standard measures, i.e., assuming  $w_i = w_{\text{sat}}$ , of  $A$  and  $g_{\text{sw}}$  in  $\text{C}_4$  plants at different VPDs, showing that  $A$  is not as responsive as  $g_{\text{sw}}$ , suggesting that the CCM dealing with a reduced  $c_i$  is the leading cause of  $A$  stability (14–16).

As an example, Morison and Gifford (14) presented data from two  $\text{C}_4$  species, *Z. mays* and *Paspalum plicatulum*, responding to changes in VPD at atmospheric  $\text{CO}_2$  concentrations. The results are stunning: From a VPD of 0.9 kPa to 2 kPa, both plants lost about 50% of their  $g_{\text{sw}}$  with practically no effect on their assimilation rate. This translates to a reduction in  $c_i/c_a$  in the range of about 25% to 30%, which was interpreted as an ability of the CCM to sustain photosynthesis. Similar results are presented by Ward, Tissue, Thomas and Strain (23) in *Amaranthus retroflexus* plants after 2 d of drying: At about ambient  $\text{CO}_2$  concentration, the assimilation rate was reduced by only 15%, but  $g_{\text{sw}}$  dropped by about 45%. Ward, Tissue, Thomas, and Strain (23) did not provide  $c_i$  values, but this variation of  $A$  and  $g_{\text{sw}}$  would imply a 15% drop in  $c_i$  as a rough estimate. Nevertheless, this seems contradictory when contrasted to results comparing  $\text{CO}_2$  responses at benign conditions, where a fall from the operational  $c_i$  of that order (15% to 30%) triggers a much larger drop in the assimilation rate [see, for example, von Caemmerer (24), Sage (17), and Ghannoum, Caemmerer, Ziska, and Conroy (25)]. A similar contradiction has been noted during water stress treatments (26), where the assimilation rate was generally not correlated with  $c_i$ , suggesting  $\text{CO}_2$  saturation of  $\text{C}_4$  photosynthesis under most conditions. However, the  $c_i$  levels recorded during these experiments operated below the saturating  $c_i$  as determined by the  $A/c_i$  curves. No mechanistic explanation has been provided for this paradox.

Our data also show such a paradox when unsaturation is not considered, but this gets resolved when nonstomatal control is taken into account (Fig. 5); then the observed reduction in assimilation aligns with the expected response from the standard  $A/c_i$  curve trend (Fig. 6 and *SI Appendix*, Figs. S2–S4). Assuming that unsaturation follows the same patterns as our measurements during the experiments performed by Morison and Gifford (14) and Ward, Tissue, Thomas and Strain (23) we can reanalyze their data and explore alternative outcomes. In the data from Morison and Gifford (14), stomatal conductance would have dropped about 15%, which implies less than a 5% drop in  $c_i/c_a$  for both  $\text{C}_4$  species; and from Ward, Tissue, Thomas, and Strain (23), the  $g_{\text{sw}}$  would have dropped 35% with an approximated fall in  $c_i/c_a$  of only 7%. Values of 5% to 7% drop from their normal operational  $c_i$  without major changes in  $A$  are more aligned with the expected  $\text{CO}_2$  response of  $\text{C}_4$  plants. This analysis offers a mechanistic reconciliation between the expected behavior based on  $\text{CO}_2$  responses and the observed response based on drought and VPD stress.

It is important to highlight that nonstomatal control of transpiration is a mesophyll leaf hydraulic property that involves resistance to liquid water movement through the cell membrane (4). In a broader context, this control mechanism is part of the resistance of

the outside xylem zone (27, 28). This highlights its role, as nonstomatal control must exceed the resistance provided by the stomata to generate unsaturated conditions in the mesophyll air space. Therefore, our analysis of previously published data, coupled with our measurements, leads to the conclusion that the observed stability of  $A$  in the evaluated  $\text{C}_4$  plants under increasing VPD is a function of mesophyll leaf hydraulic properties and the CCM, and not solely the CCM.

Interestingly, if  $\text{C}_3$  and  $\text{C}_4$  plants share the structures that enable nonstomatal control of transpiration, it would suggest that the mechanism might predate the evolutionary divergence of the  $\text{C}_4$  photosynthetic pathway, which has emerged separately many times in the past 35 million years (29). The fact that both  $\text{C}_3$  and  $\text{C}_4$  plants experience unsaturation in their substomatal cavities, allowing them to reduce water loss while preserving their assimilation rate, raises intriguing questions. This potential overlap between the water conservation strategies of  $\text{C}_3$  and  $\text{C}_4$  plants could redefine our understanding of their respective adaptations to environmental conditions. Furthermore, it presents an opportunity to delve deeper into the evolutionary drivers behind these mechanisms and calls for a reassessment of our understanding of  $\text{C}_4$  physiological responses to water stress.

Our research underscores that the  $\text{C}_4$  plant species maize, sorghum, and proso millet significantly benefit from nonstomatal control of transpiration, utilizing this mechanism as a fine-tuning method for water management while enhancing  $\text{CO}_2$  availability in the mesophyll airspace. Improving  $\text{CO}_2$  availability while reducing its water cost creates synergy with the high assimilation rate enabled by the CCM. However, it is still intriguing why all the  $\text{C}_4$  plants evaluated exhibit stronger degrees of unsaturation than many  $\text{C}_3$  plants despite the presence of the CCM. These observations suggest that additional, unexplored physiological nuances might influence these differences. Further research is needed to explore how different species, particularly adapted to arid environments, manage these physiological traits under various environmental conditions. Such studies are essential to fill the gaps in our understanding of how evolutionary pressures and ecological contexts shape nonstomatal control of transpiration in  $\text{C}_3$  and  $\text{C}_4$  plants.

## Conclusion

We have demonstrated that nonstomatal control of transpiration in the  $\text{C}_4$  plant species maize, sorghum, and proso millet is key to maintaining an advantageous microclimate within the leaf for high assimilation while evaporative demand increases, upholding the functional advantage of the CCM. We have confirmed the occurrence of substantial unsaturation in the substomatal cavity of  $\text{C}_4$  plants exposed to relatively mild VPDs comparable to those under which they could be expected to operate in the field. Our findings challenge the prevailing interpretation of  $\text{C}_4$  photosynthetic efficiency under VPD stress, which has traditionally been ascribed to the ability of the CCM to maintain high photosynthetic rates despite diminishing intercellular  $\text{CO}_2$  concentrations ( $c_i$ ). We have shown that the perceived reduction in  $c_i$  does not occur as previously thought; rather, it emerges as a consequence of systematically underestimating  $g_{\text{sw}}$  at increased VPD. This insight necessitates a reexamination of the role of the CCM in the ecophysiological strategies of  $\text{C}_4$  plants, accounting for the contribution of mesophyll hydraulic properties without the assumed reduction in  $c_i$ .

## Materials and Methods

**Plant Material.** For our experiments, we utilized six *Z. mays* B73, four *P. miliaecum*, and four *Sorghum bicolor* TX430 leaves, each from a different plant. These plants were cultivated from seeds in 10 L pots using Martins Potting Mix (Martins



Fertilizers, Yass, NSW, Australia). When sowing, we added 5 g of Osmocote Exact slow-release fertilizer (Scotts Australia, Bella Vista, NSW, Australia). Postsowing, the plants were housed in a glasshouse with natural lighting, maintaining temperatures of 28 °C during daytime and 20 °C at night. They were watered daily. Our analyses targeted fully matured leaves.

The total cuticular conductance to water vapor of the leaves was estimated using the Red-light technique introduced by Márquez, Stuart-Williams, Farquhar and Busch (30) by means of a portable photosynthesis system LI-6800 (LI-COR, Lincoln, NE, USA). Determinations were in the range of  $8 \pm 2 \text{ mmol m}^{-2} \text{ s}^{-1}$  (average  $\pm$  SD). To apply the Red-light method, cuticular conductance to  $\text{CO}_2$  was assumed to be 0.05 of the conductance to water vapor (31).

**Equipment and Calculations.** We conducted the main experiments using a gas exchange analysis system developed by S. C. Wong, as detailed in Wong et al. (4). This system incorporates two LI-6251 gas analyzers (LI-COR, Lincoln, NE, USA) to independently measure the  $\text{CO}_2$  concentration in the upper and lower cuvettes. Additional experiments were conducted using an LI-6800 with a 6  $\text{cm}^2$  chamber (6800-01A). The gas exchange parameters from both instruments were determined based on equations presented by Márquez, Stuart-Williams, and Farquhar (31). For a detailed description of common gas exchange calculations, please refer to supplementary material (*SI Appendix, Supporting text*).

**Measurements.** All the leaves were first measured using a LI-6800 in its typical setup (combined upper and lower cuvettes) under  $1,500 \mu\text{mol m}^{-2} \text{ s}^{-1}$  of photosynthetically active radiation (PAR),  $400 \mu\text{mol mol}^{-1}$  of  $\text{CO}_2$  in the cuvette ( $c_a$ ), 1 kPa of VPD and a 25 °C leaf temperature. Plants were left under those conditions until stable gas exchange parameters were achieved for at least 5 min. Stability is defined as a state where the input concentration into the chamber remains

Cernusak, and Farquhar (20) to estimate  $R_{ias}$ , taking  $m = -0.31$  (rate of change of photosynthetic uptake within the width of the leaf), as suggested by the same authors,

$$R_{ias} = \frac{12}{(6-m)} \frac{(c_{i,ad} - c_{i,ab})}{A_s} \quad [1]$$

**Estimations of  $w_i$  (Unsaturation).** The reasoning presented by Wong et al. (4) to assess the water vapor concentration in the substomatal cavity ( $w_i$ ) is that  $R_{ias}$  can be considered constant within a turgid leaf. Wong et al. (4) used this premise to obtain the expected gradient between  $c_{i,ad}$  and  $c_{i,ab}$  via iteration of  $w_i$ . Here, we incorporated the established relationship between  $R_{ias}$  and  $c_{i,ad}$  and  $c_{i,ab}$  from Eq. 1, and then rearranged our equations to reveal the expected  $\text{CO}_2$  concentration gradient given a specific assimilation rate, allowing us to obtain  $w_i$  according to Eq. 2 (see derivation in *SI Appendix, Supporting text*). By inserting the function  $c_i(w_i)$  into the expression for  $R_{ias}$ , we effectively bind the  $\text{CO}_2$  gradient and  $w_i$  to the measured  $R_{ias}$  at the lowest VPD. This allows us to estimate  $w_i$  more accurately, using gas exchange data and assuming saturated  $w_i$  only to estimate  $R_{ias}$  under benign conditions, bypassing the assumption of saturated  $w_i$  under other conditions.

At 1 kPa of VPD, the substomatal vapor concentration ( $w_i$ ) was assumed to equal the saturated vapor concentration at leaf temperature ( $w_{sat}$ ) to estimate  $R_{ias}$ . VPD increases were performed from 1 kPa to 2 kPa and then from 2 kPa to about 3 kPa. Eq. 2 was used to compute the actual  $w_i$  at 2 and 3 kPa. If needed, either the upper or lower cuvette  $c_s$  was adjusted to maintain the assimilation rate of the lower surface equal to zero after changing VPD (decreasing the lower or increasing the upper), maintaining the lower  $c_a$  at not less than  $40 \mu\text{mol mol}^{-1}$ . Then, the positive root is taken as  $w_i$  from Eq. 2:

$$aw_i^2 + bw_i + c = 0$$

$$a = \left[ E_{s,ad} E_{s,ab} \left( \frac{1.44 R_{ias} A_s (6-m)}{12} + 6.24 (c_{s,ad} - c_{s,ab}) \right) + 7.68 (E_{s,ab} A_{s,ad} - E_{s,ad} A_{s,ab}) \right]$$

$$b = \left[ E_{s,ad} E_{s,ab} \left( \frac{R_{ias} A_s (6-m)}{12} [9.6 - 1.44 (w_{s,ad} + w_{s,ab})] + (c_{s,ad} - c_{s,ab}) [16 - 6.24 (w_{s,ad} + w_{s,ab})] \right) + (A_{s,ab} E_{s,ad} - A_{s,ad} E_{s,ab}) (7.68 (w_{s,ad} + w_{s,ab}) - 25.6) \right]$$

$$c = \left[ E_{s,ad} E_{s,ab} \left( (c_{s,ab} - c_{s,ad}) (16 - 6.24 w_{s,ad} w_{s,ab}) + 20.8 (c_{s,ab} w_{s,ab} - c_{s,ad} w_{s,ad}) + 4.8 (w_{s,ab} c_{s,ad} - w_{s,ad} c_{s,ab}) + \frac{A_s (6-m)}{12} [16 + 1.44 w_{s,ad} w_{s,ab} - 4.8 w_{s,ab} - 4.8 w_{s,ad}] \right) + 25.6 (E_{s,ad} A_{s,ab} w_{s,ab} - E_{s,ab} A_{s,ad} w_{s,ad}) + 7.68 w_{s,ab} w_{s,ad} (E_{s,ab} A_{s,ad} - E_{s,ad} A_{s,ab}) \right] \quad [2]$$

constant, with stomatal conductance variations maintained below  $5 \text{ mmol m}^{-2} \text{ s}^{-1}$  and assimilation rate fluctuations peak to peak not exceeding  $0.5 \mu\text{mol m}^{-2} \text{ s}^{-1}$  over 5 min. The stable conditions were logged, and then a standard  $\text{CO}_2$  response curve ( $A_c$ ) was performed under the same benign conditions. Under benign conditions  $w_i$  was assumed to be  $w_{sat}$ . The same leaf was used later to perform the main experiments using the apparatus built by S.C. Wong.

The gas exchange measurement sequence started at low VPD, around 1 kPa,  $c_a$  for both upper and lower leaf chambers at  $400 \mu\text{mol mol}^{-1}$ , and PAR set at  $1,500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . After the gas exchange rates reached a steady state,  $c_a$  for the lower cuvette was reduced until the assimilation rate of the lower leaf surface was  $0 \pm 0.5 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  (zero).

**$R_{ias}$  Estimation.** Determining the internal air space resistance to  $\text{CO}_2$  diffusion ( $R_{ias}$ ) involves maintaining a steady  $\text{CO}_2$  concentration in the upper cuvette, roughly at atmospheric levels ( $400 \mu\text{mol mol}^{-1}$ ), while reducing the  $\text{CO}_2$  concentration in the lower cuvette until the assimilation rate on the lower surface becomes zero. Under such conditions, there is only assimilation through the stomata ( $A_s$ ) of the adaxial surface and the  $\text{CO}_2$  concentration in the substomatal cavity ( $c_i$ ) of the abaxial surface corresponds to the leaf's minimum  $\text{CO}_2$  concentration. Using this premise, we used Eq. 1 derived by Márquez, Stuart-Williams,

where  $E_s$  is the transpiration rate through the stomata ( $\text{mol m}^{-2} \text{ s}^{-1}$ ),  $c_s$  is  $\text{CO}_2$  concentration at the leaf surface ( $\mu\text{mol mol}^{-1}$ ),  $A_s$  is the assimilation rate through the stomata ( $\mu\text{mol m}^{-2} \text{ s}^{-1}$ ),  $w_s$  is the vapor concentration at the leaf surface ( $\text{mol mol}^{-1}$ ), and *ad* and *ab* subscripts refer to the adaxial and abaxial surfaces, respectively.

Then, relative humidity in the substomatal cavity was estimated as  $w_i/w_{sat}$ .

**Data, Materials, and Software Availability.** All study data are included in the article and/or [supporting information](#).

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