Mesophyll airspace unsaturation drives C$_4$ plant success under vapor pressure deficit stress

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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 | C4 | 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.

diminishing resistance to CO_2 diffusion between the adaxial and abaxial substomatal cavities (R_{ias}). The apparent decrease in R_{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 C_3 species. Until now, airspace substomatal cavity unsaturation has been demonstrated in the 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 C_3 plants and raises the question of whether such a phenomenon is exclusive to C_3 plants or extends to other photosynthetic pathways, particularly the C_4 strategy.

C_4 plants, with their distinctive carbon fixation mechanism, are traditionally considered more water-use efficient than their 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 C_3 plants (10, 11).
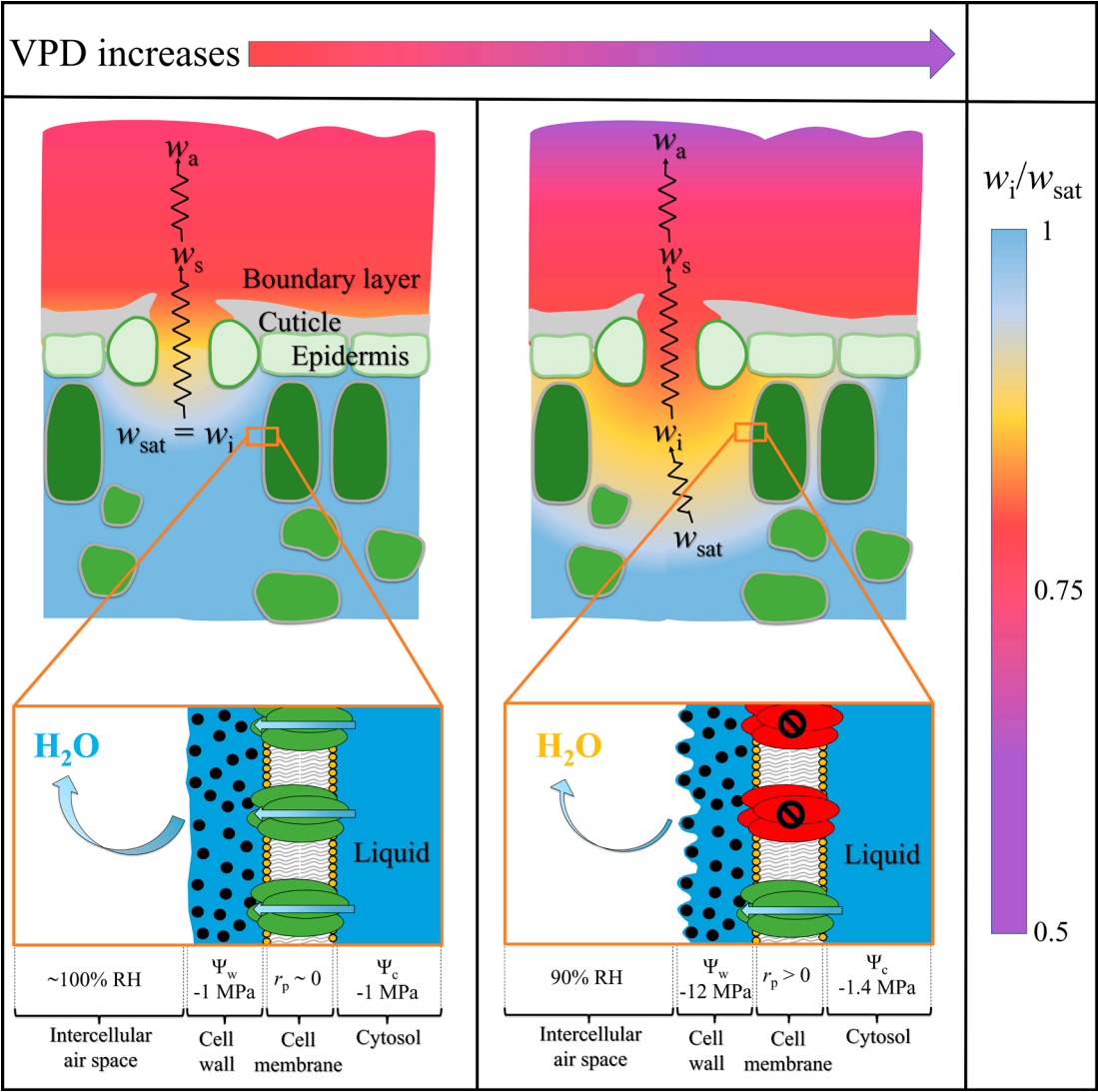

Fig. 1. Diagram of the mechanism of nonstomatal control of transpiration as VPD increases, generating unsaturation in the substomatal cavity. The parameters in the figure are atmospheric water vapor concentration (w_a), water vapor concentration on the leaf surface (w_s), substomatal cavity water vapor concentration (w_i), water vapor concentration at saturation (w_{sat}), resistance to water diffusion through the plasma membrane (r_p), cell wall water potential (Ψ_w), and cytosolic water potential (Ψ_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	$\mu mol\ m^{-2}\ s^{-1}$
A_s	Assimilation rate through stomata	$\mu mol\ m^{-2}\ s^{-1}$
ad and ab subscripts	Adaxial and abaxial	–
c_a	Atmospheric CO_2 concentration	$\mu mol\ mol^{-1}$
CCM	Carbon concentrating mechanism	–
c_s	CO_2 concentration on the leaf surface	$\mu mol\ mol^{-1}$
c_i	Substomatal cavity CO_2 concentration	$\mu mol\ mol^{-1}$
E	Total transpiration rate	$mol\ m^{-2}\ s^{-1}$
E_s	Transpiration rate through stomata	$mol\ m^{-2}\ s^{-1}$
g_{sw}	Stomatal conductance to water vapor	$mol\ m^{-2}\ s^{-1}$
$iWUE$	Intrinsic water use efficiency	$\mu mol\ mol^{-1}$
RH	Relative humidity	–
R_{ias}	Leaf internal airspace resistance to CO_2 diffusion	$m^2\ s\ mol^{-1}$
VPD	Vapor pressure deficit	kPa
w_a	Atmospheric water vapor concentration	$mol\ mol^{-1}$
w_i	Substomatal cavity water vapor concentration	$mol\ mol^{-1}$
w_s	Water vapor concentration on the leaf surface	$mol\ mol^{-1}$
w_{sat}	Water vapor concentration at saturation	$mol\ mol^{-1}$
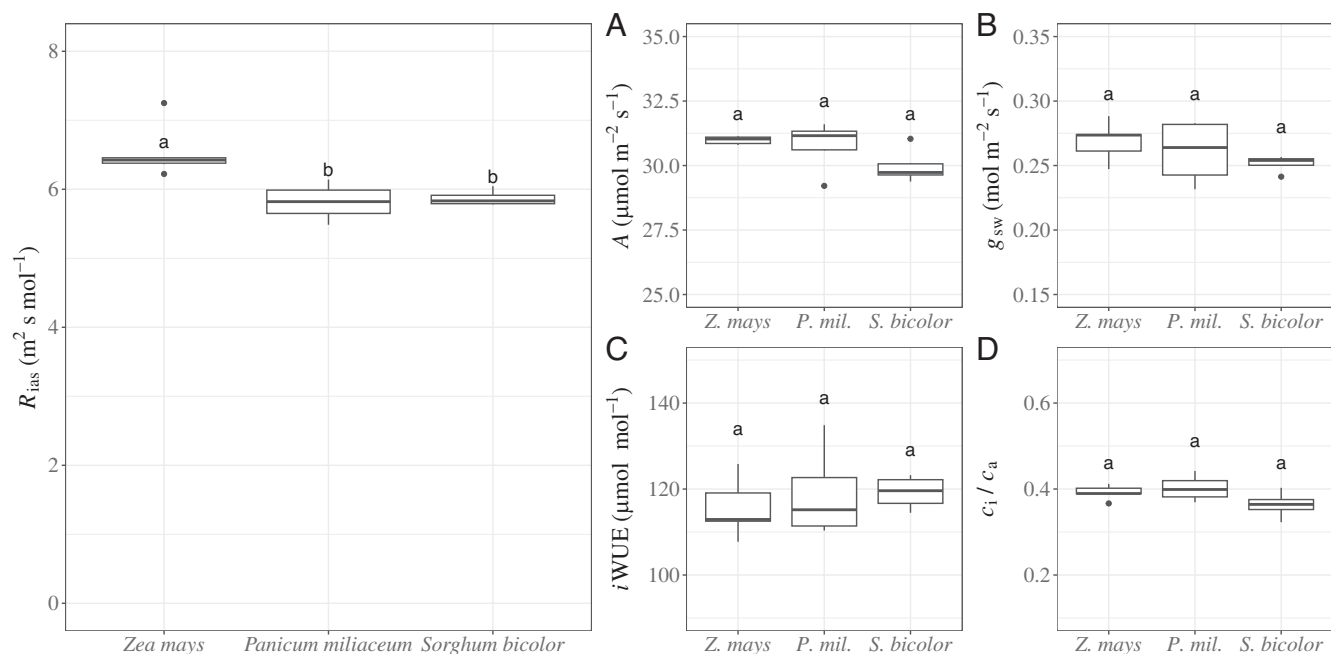
Fig. 2. Average gas exchange parameter values from the plants evaluated. Average internal leaf air space resistance (R_{ias}) found in three C_4 species: *Z. mays, P. miliaceum*, and *Sorghum bicolor*. Panels *A–D* are measurements under the typical setup of a LI-6800 for assimilation rate (*A*), stomatal conductance to water (g_{sw}), *i*WUE, 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. 5*A*. Consistent with the observed decrease in *A*, Fig. 5*B* 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 *i*WUE (*i*WUE = A/g_{sw}) calculated with actual w_i responding less sharply, while the
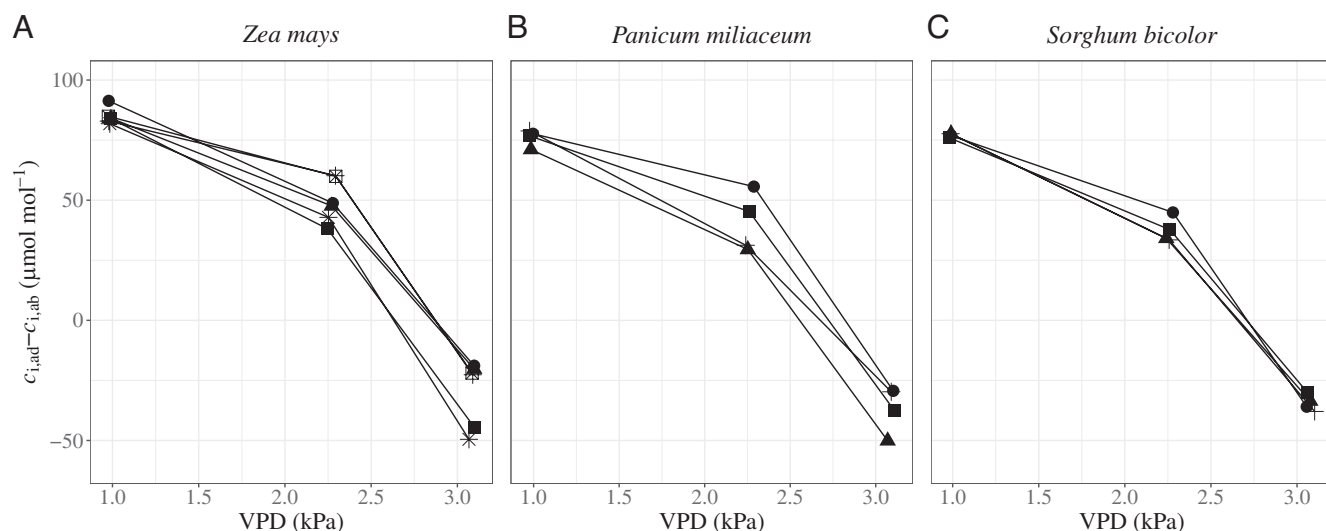

Fig. 3. Difference between adaxial and abaxial substomatal cavity CO_2 concentration ($c_{i,ad} - c_{i,ab}$) of (*A*) *Zea mayz*, (*B*) *Panicum miliaceum* and (*C*) *Sorghum bicolor* leaves. Values presented assume a saturated condition within the substomatal cavity ($w_i = w_{sat}$) in three C_4 species. Each line is a single leaf. Raw data and calculations are provided in *SI Appendix*, Supporting text and Dataset S1.
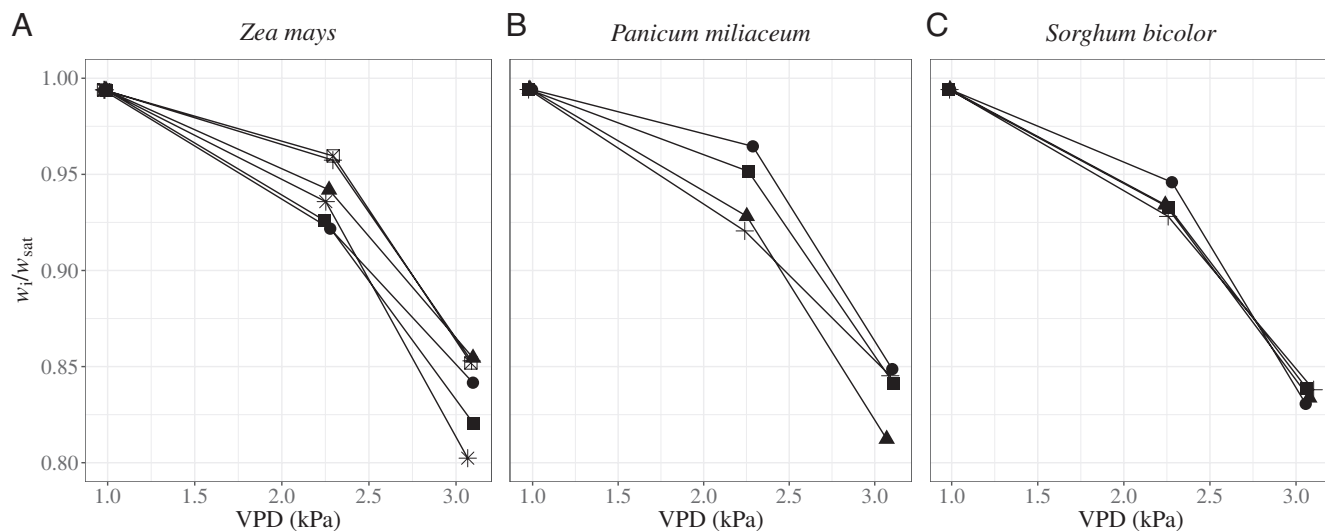
Fig. 4. Unsaturation estimation at different VPDs for (*A*) *Zea mays*, (*B*) *Panicum miliaceum*, and (*C*) *Sorghum bicolor* leaves. Relative humidity in the leaf substomatal cavity (w_i/w_{sat}) was estimated with increasing VPD, tested in three C_4 species. Each line is a single leaf. Raw data and calculations are presented in *SI Appendix*, Supporting text and Dataset S1.

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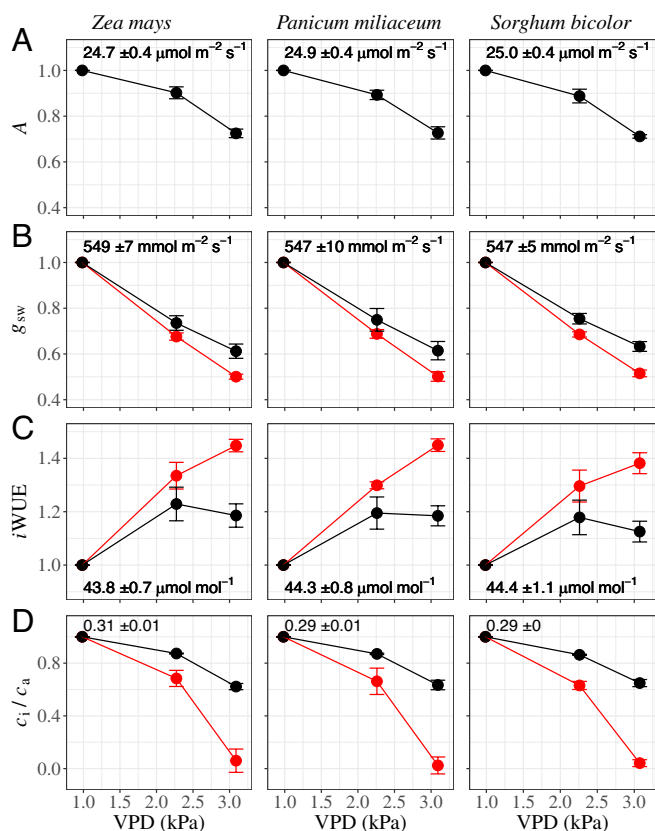

Fig. 5. Comparison of the estimations of gas exchange parameters when accounting for unsaturation (black symbols) versus assuming saturation (red symbols). Assimilation rate (*A*, panel *A*), stomatal conductance (g_{sw}, panel *B*), iWUE (iWUE = A/g_{sw}, panel *C*), and intercellular to atmospheric CO_2 concentration ratio ($c_{i,ad}/c_{a,ad}$, panel *D*) in response to VPD increases. Values are normalized to values at VPD of 1 kPa presented in each graph as the average ± 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 $w_i = w_{sat}$. 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.

complexity to interpreting *i*WUE, as maintaining g_{sw} during VPD increase would not necessarily result in increased water loss.

Importantly, the ratio of internal to atmospheric CO_2 concentration ($c_{i,ad}/c_{a,ad}$) shows a less marked decrease with VPD when actual w_i is considered (Fig. 5*D*), indicating a less severe impact on internal 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_{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 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. 2*D* and the Ac_i curves presented in Fig. 6. The differences between these two approaches under the same conditions are usually small, in the range of 10 µmol 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_{ias} (20).

In line with Márquez, Stuart-Williams, Cernusak, and Farquhar (20), the minimum CO_2 concentration within the leaf ($c_{i,ab}$ in our experiments) showed a small difference with the c_i estimated under standard 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 Ac_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 Ac_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 Ac_i relationship that overestimates the drop in c_i, in our observations by an average of 28% at 2 kPa, estimated as $[1 - c_i(w_{sat})/c_i(w_i)]$, 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 ($c_{i,Wong}$) are expected
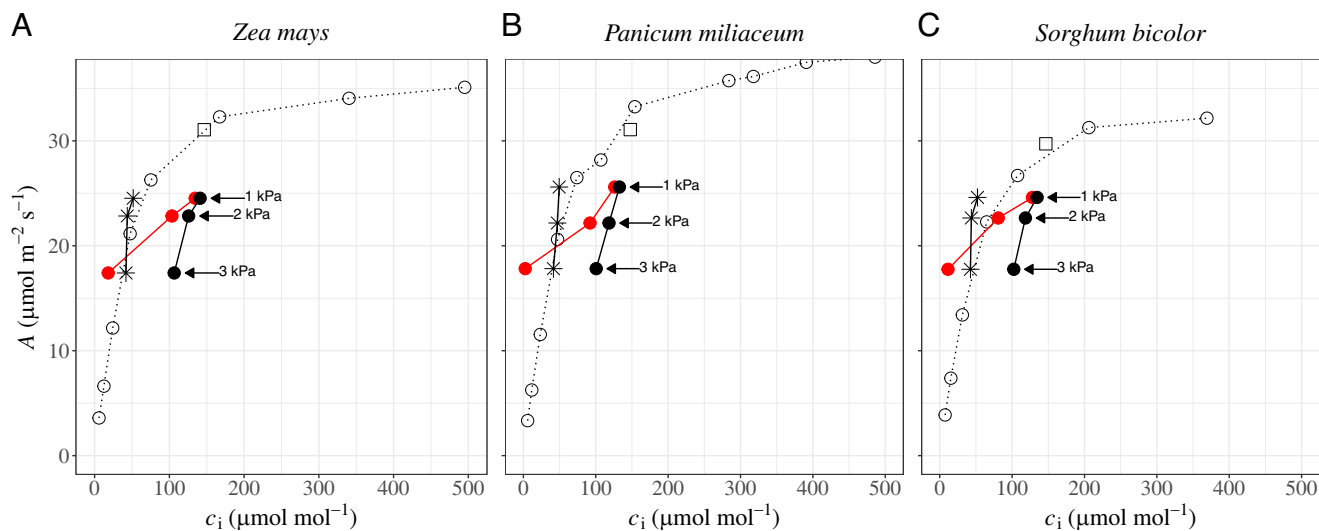
Fig. 6. Comparison of CO_2 response trends of (*A*) *Zea mays*, (*B*) *Panicum miliaceum*, and (*C*) *Sorghum bicolor* leaves. Comparison of a standard CO_2 response curve (open circles) with the relation between assimilation rate and adaxial c_i estimated during VPD increases accounting for unsaturation (black circles) and assuming $w_i = w_{sat}$ (red circles). The asterisks are the minimum CO_2 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_2 response curve ($c_{i,standard}$). This is due to the roughly half extra R_{ias} added to the path length for CO_2 diffusion from the adaxial surface when the CO_2 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_2 sourced exclusively from the adaxial surface, $c_{i,Wong}$ must inherently be higher than $c_{i,standard}$, which benefits from CO_2 sourced from both surfaces. Thus, for a given A, $c_{i,Wong}$ is expected to be roughly $c_{i,Wong} \approx AR_{ias}/2 + c_{i,standard}$, 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 $c_{i,ad}$ 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_2 flux, as the photosynthetic capacity through the adaxial surfaces effectively doubles. For a detailed discussion about this reasoning behind the CO_2 gradient within the mesophyll air space and R_{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_3 species, refuting a longstanding assumption of plant physiology. Here, we showed that substantial unsaturation of the substomatal cavity occurs in C_4 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_4 species evaluated here were from relative humidities of 80% to 85% (w_i/w_{sat}) at 3 kPa VPD, aligning with those found in C_3 species *H. annuus* (4) and *P. edulis* (3) under similar VPDs. However, other C_3 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_4 plants evaluated here exhibit a greater contribution of nonstomatal control of transpiration than most C_3 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_{sw} reduction (38% drop at 3 kPa; Fig. 5) than their C_3 counterparts evaluated in ref. 4 with equivalent unsaturation (55% g_{sw} drop). Considering that C_4 plants have lower g_{sw} and usually operate at a lower c_i than C_3 plants, the observed coordination between nonstomatal control and g_{sw} in C_4 plants points to fine-tuning water loss and CO_2 availability. Notably, our results show significant deviations in the Ac_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_3 plants (4) with similar degrees of unsaturation. These findings are consistent with previous observations that C_4 plants usually suffer a greater reduction in A than C_3 plants when their c_i drops from their normal operational level (17).

Without nonstomatal control of transpiration, C_3 and C_4 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 Ac_i curve can be used to evaluate the expected drop of A proportional to the drop between $c_i(w_i)$ and $c_i(w_{sat})$ 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_3 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_3 Ac_i responses. While C_3 and C_4 plants clearly benefit from having a nonstomatal control of transpiration under VPD stress, our data on three C_4 species suggest that the C_4 strategy achieves a proportionally larger advantage by enhancing CO_2

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 CO_2 for the CCM in the evaluated 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 C_4 species. It follows that not accounting for unsaturation results in overestimating the ability of C_4 plants to sustain high assimilation rates despite a significant apparent reduction in g_{sw} and, consequently, in c_i. This overestimation aligns with the commonly held theory that the CCM alone allows C_4 plants to maintain high A even when g_{sw} is reduced under high VPD or drought conditions. The theory is based on standard measures, i.e., assuming $w_i = w_{sat}$, of A and g_{sw} in C_4 plants at different VPDs, showing that A is not as responsive as g_{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 C_4 species, *Z. mays* and *Paspalum plicatulum*, responding to changes in VPD at atmospheric 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_{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 CO_2 concentration, the assimilation rate was reduced by only 15%, but g_{sw} dropped by about 45%. Ward, Tissue, Thomas, and Strain (23) did not provide c_i values, but this variation of A and g_{sw} would imply a 15% drop in c_i as a rough estimate. Nevertheless, this seems contradictory when contrasted to results comparing 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 CO_2 saturation of 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 Ac_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 Ac_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 C_4 species; and from Ward, Tissue, Thomas, and Strain (23), the g_{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 CO_2 response of C_4 plants. This analysis offers a mechanistic reconciliation between the expected behavior based on 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 C_4 plants under increasing VPD is a function of mesophyll leaf hydraulic properties and the CCM, and not solely the CCM.

Interestingly, if C_3 and 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 C_4 photosynthetic pathway, which has emerged separately many times in the past 35 million years (29). The fact that both C_3 and 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 C_3 and 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 C_4 physiological responses to water stress.

Our research underscores that the 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 CO_2 availability in the mesophyll airspace. Improving 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 C_4 plants evaluated exhibit stronger degrees of unsaturation than many 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 C_3 and C_4 plants.

Conclusion

We have demonstrated that nonstomatal control of transpiration in the 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 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 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 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_{sw} at increased VPD. This insight necessitates a reexamination of the role of the CCM in the ecophysiological strategies of 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. miliaceum*, 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 ± 2 mmol m^{-2} s^{-1} (average $\pm$ SD). To apply the Red-light method, cuticular conductance to 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 CO_2 concentration in the upper and lower cuvettes. Additional experiments were conducted using an LI-6800 with a 6 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 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR), 400 μmol mol^{-1} of 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 constant, with stomatal conductance variations maintained below 5 mmol m^{-2} s^{-1} and assimilation rate fluctuations peak to peak not exceeding 0.5 μmol m^{-2} s^{-1} over 5 min. The stable conditions were logged, and then a standard CO_2 response curve (Ac_i) 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 μmol mol^{-1}, and PAR set at 1,500 μmol m^{-2} 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 ± 0.5 μmol CO_2 m^{-2} s^{-1} (zero).

R_{ias} Estimation. Determining the internal air space resistance to CO_2 diffusion (R_{ias}) involves maintaining a steady CO_2 concentration in the upper cuvette, roughly at atmospheric levels (400 μmol mol^{-1}), while reducing the 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 CO_2 concentration in the substomatal cavity (c_i) of the abaxial surface corresponds to the leaf's minimum CO_2 concentration. Using this premise, we used Eq. **1** derived by Márquez, Stuart-Williams, 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 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 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_a 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 μmol mol^{-1}. Then, the positive root is taken as w_i from Eq. **2**:

$$a w_i^2 + b w_i + c = 0$$

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

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

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

where E_s is the transpiration rate through the stomata (mol m^{-2} s^{-1}), c_s is CO_2 concentration at the leaf surface (μmol mol^{-1}), A_s is the assimilation rate through the stomata (μmol m^{-2} s^{-1}), w_s is the vapor concentration at the leaf surface (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}.

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