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REVIEW

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Unsaturation in the air spaces of leaves and its implications

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Abstract

Modern plant physiological theory stipulates that the resistance to water movement from plants to the atmosphere is overwhelmingly dominated by stomata. This conception necessitates a corollary assumption—that the air spaces in leaves must be nearly saturated with water vapour; that is, with a relative humidity that does not decline materially below unity. As this idea became progressively engrained in scientific discourse and textbooks over the last century, observations inconsistent with this corollary assumption were occasionally reported. Yet, evidence of unsaturation gained little traction, with acceptance of the prevailing framework motivated by three considerations: (1) leaf water potentials measured by either thermocouple psychrometry or the Scholander pressure chamber are largely consistent with the framework; (2) being able to assume near saturation of intercellular air spaces was transformational to leaf gas exchange analysis; and (3) there has been no obvious mechanism to explain a variable, liquid-phase resistance in the leaf mesophyll. Here, we review the evidence that refutes the assumption of universal, near saturation of air spaces in leaves. Refining the prevailing paradigm with respect to this assumption provides opportunities for identifying and developing mechanisms for increased plant productivity in the face of increasing evaporative demand imposed by global climate change.

KEYWORDS

humidity, intercellular air space, leaf mesophyll, photosynthesis, stomatal conductance, transpiration, water potential

1 | INTRODUCTION

Research into the role of stomata in controlling the rate of transpiration from leaves came into focus in the early twentieth century. Initially, there was debate as to whether the extent of the stomatal opening was key in controlling rates of water loss (Darwin, 1898; Darwin & Pertz, 1911; Lloyd, 1908), but careful work established that this was the case (Loftfield, 1921). Alongside this, a theory to explain the liquid-phase ascent of water through plants, including tall trees, was developed (Dixon & Joly, 1895). The concept of water potential was applied in an effort to link liquid and gas-phase transport on a common scale. By applying the analogy of an electric current, water movement through the plant to the atmosphere was conceived as a catenary process (Gradmann, 1928; van den Honert, 1948), with its velocity in the steady-state equal to the water potential difference between any two points of the pathway

divided by the resistance between the same two points. The attraction of this simple formulation was that it could apply to both the liquid water in the capillaries of the xylem and to the diffusion of water vapour in the air spaces of leaves. It was subsequently pointed out that this was not entirely correct because while the liquid flow of water can be linearly related to water potential gradients, gaseous diffusion of water vapour is instead linearly related to gradients of water vapour concentration (Cowan, 1965; Philip, 1966; Rawlins, 1963; Ray, 1960), the latter described by Fick's law. For an isothermal system, water potential and water vapour concentration are logarithmically related. This can be seen in the relationship between water potential and the relative humidity (h) of air:

$$\Psi = \frac{RT}{V_w} \ln h, \tag{1}$$

where Ψ is water potential (Pa), R is the universal gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature (K) and V_w is the partial molar volume of water (18×10^{-6} m^3 mol^{-1}). The equation can be conveniently rescaled with Ψ in MPa. The relationship between Ψ and h at 25°C is shown in Figure 1.

Although the conceptual model of Gradmann (1928) and van den Honert (1948) was not wholly correct, it did provide a useful approximation for exploring where the dominant resistance to water transport through the plant was likely to occur (Philip, 1966). It was argued that this must involve the gas phase, and indeed the stomatal pores and their associated apertures were seen as paramount (van den Honert, 1948). Cowan and Milthorpe (1967) later corrected the analysis for the discontinuity at the liquid–air interface and showed that van den Honert's assertion was still qualitatively correct. Thus, if the resistance to gas-phase diffusion is primarily associated with stomatal pores, then it stands to reason that the largest drop in water potential from plant to atmosphere should take place across these pores and the leaf boundary layer. Due to the relationship between Ψ and h (Figure 1), a corollary to this is that the relative humidity in the intercellular air spaces of the leaf (h_i) should be near saturation and then decline along the pathway through the stomatal pores out into the turbulent atmosphere surrounding the leaf (Rockwell et al., 2022). Capitalising on this argument, Gaastra (1959) suggested that it should be possible, for purposes of analysing leaf gas exchange, to assume that the vapour pressure in the intercellular air spaces of leaves (e_i) can be estimated as the saturation vapour pressure (e_s) at leaf temperature. The e_s for a given leaf temperature (T_l) can be calculated as (Buck, 1981),

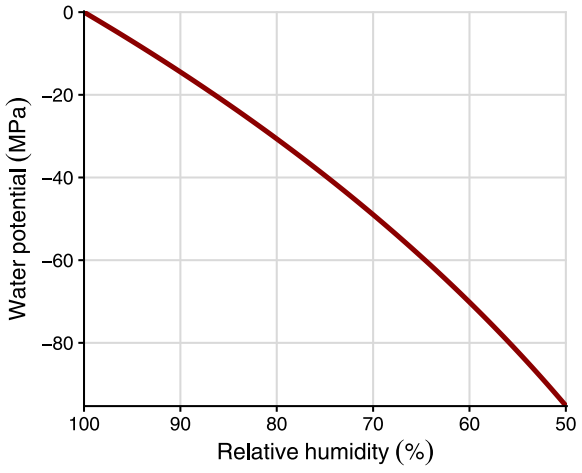

FIGURE 1 Water potential calculated as a function of relative humidity according to Equation (1) of the main text. The relationship shown here is for an air temperature of 25°C, and the relative humidity is shown as a percentage. Corresponding proportional values for relative humidity on the x-axis would range from 1.0 to 0.5. [Color figure can be viewed at wileyonlinelibrary.com]

$$e_s = 0.61121 e^{\left(\frac{17.502 T_l}{240.97 + T_l}\right)}, \tag{2}$$

where T_l is in °C. The h_i is defined as e_i/e_s, where e_i is the actual intercellular vapour pressure and e_s is the saturated value at leaf temperature. Thus, the approach that Gaastra (1959) advocated is to assume that $e_i = e_s$, and therefore that $h_i = e_i/e_s = 1$. With this estimate of e_i in hand, it was then possible to calculate leaf conductance to water vapour (g_t) continuously for a leaf in a gas exchange cuvette (Gaastra, 1959; von Caemmerer & Farquhar, 1981):

$$g_t = \frac{E(P - \bar{e})}{(e_i - e_a)}. \tag{3}$$

In Equation (3), E is the transpiration rate of the leaf measured in the cuvette, P is the atmospheric pressure, e_i and e_a are the vapour pressures in the leaf intercellular air spaces and in the air surrounding the leaf, respectively, and $\dot{e}$ is $(e_i + e_a)/2$. The leaf conductance to water vapour is the inverse of the diffusion resistance of the same path. The stomata and leaf boundary layer occur in series in this path, and their resistances are, therefore, additive (Campbell & Norman, 1998). Thus, expressed as its inverse, the leaf conductance can be decomposed into stomatal (g_s) and boundary layer (g_b) conductances:

$$\frac{1}{g_t} = \frac{1}{g_s} + \frac{1}{g_b}. \tag{4}$$

Equation (4) can be used to calculate g_s from the g_t determined by gas exchange measurements and an estimate of g_b, which can be approximated by measuring the evaporation rate from a saturated filter paper in the shape of a leaf inserted into the same gas exchange apparatus (Gaastra, 1959). It was soon realised that the determination of g_t, when combined with the photosynthetic rate (A), also allowed for the calculation of c_i, the intercellular CO_2 concentration (Moss & Rawlins, 1963; von Caemmerer & Farquhar, 1981):

$$c_i = \frac{c_a - \frac{A}{g_{tc}} - \frac{Ec_a}{2g_{tc}}}{1 + \frac{E}{2g_{tc}}}. \tag{5}$$

For Equation (5), the stomatal conductance to CO_2 (g_{sc}) can be calculated from that determined for water vapour as $g_s/1.6$, and the boundary layer conductance to CO_2 (g_{bc}) can be calculated from that determined for water vapour as $g_b/1.37$ (Cowan, 1972). The leaf conductance to CO_2 (g_{tc}) can then be calculated by analogy to

Equation (4). The c_a in Equation (5) is the CO_2 concentration in air surrounding the leaf. The transpiration rate E appears in Equation (5) due to its role in the ternary effect; that is, the influence of the convective flux of water out of the leaf on the diffusion of CO_2 into the leaf (Jarman, 1974). The equations given here apply to a single leaf surface or an amphistomatous leaf for which any differences between the two sides in fluxes and conductances are negligible (Márquez et al., 2023). They also do not include gas exchange through the cuticle. An updated version that includes cuticular fluxes for water and CO_2 has been recently presented (Márquez et al., 2021).

Calculation of g_s and c_i as described above provided major breakthroughs in leaf gas exchange analysis. These advances eventually contributed to pivotal innovations, such as validation of a biochemical model of photosynthesis (Farquhar et al., 1980; von Caemmerer & Farquhar, 1981). These breakthroughs depended on the assumption that the intercellular air spaces are saturated with water vapour ($h_i = 1$), and this assumption is now employed in all commercial portable photosynthesis systems to calculate g_s and c_i (Cernusak et al., 2018).

In the mid-20th century, there were also developments in the measurement of leaf water potential that supported the idea that h_i was not likely to deviate far from saturation. Thermocouple psychrometers were developed (Spanner, 1951), which enabled the estimation of the water potential of whole leaves or leaf discs. The Scholander pressure chamber was also invented (Scholander et al., 1965, 1964), which has become a standard tool among plant ecophysiologists for quantifying the water potential of whole leaves or shoots. The measurements suggested that mesic plants typically operate with leaf water potentials above about −2 MPa, which would equate to h_i of 0.98 or higher. In extremely arid environments, whole-leaf water potentials as low as −8 MPa can be measured with the Scholander pressure chamber (Scholander et al., 1965). Even these rare values would indicate h_i only as low as about 0.95 if the intercellular air spaces are assumed to be in equilibrium with the whole-leaf water potential (Figure 1). Because the relationship between temperature and saturation vapour pressure is exponential, such typically small deviations from saturation have generally been considered within an acceptable range for gas exchange analysis, given the uncertainty in leaf temperature measurements that would typically lead to variations of a similar magnitude (Mott & Peak, 2011; Rockwell et al., 2022).

Thus, the paradigm of universal, near saturation of leaf intercellular air spaces was reinforced and incorporated into the general corpus of plant physiological theory. In textbooks that describe plant ecophysiology, it is common to encounter a statement such as, "Under most physiological conditions, the air within the leaf is at or near saturation (relative humidity ~99%)"(Smith & Smith, 2015). Our intention here is not to criticise the adoption of this idea; as we have described above, it was based on reasonable and compelling assumptions and observations. Our intention is rather to point out that while the paradigm of $h_i = 1$ was taking hold over the last century, there were also occasional reports of significant unsaturation. When combined with recently presented evidence, these reports collectively suggest that there is indeed reason to doubt the correctness of the century-old paradigm of universal, near saturation of leaf intercellular air spaces.

2 | EVIDENCE OF h_i LESS THAN UNITY

In Table 1, we provide a summary of notable reports of unsaturation of the intercellular air spaces of leaves. The first striking feature in Table 1 is that values as low as 0.80 have been reported for h_i, the intercellular air space relative humidity. This would correspond to a liquid water potential of approximately −30 MPa, more than an order of magnitude more negative than what is commonly observed for whole leaf tissues with a thermocouple psychrometer or a Scholander pressure chamber. The second striking feature is the wide range of experimental approaches that have been brought to bear on the question. The methods for inferring h_i have been diverse and creative in their conceptualisation and implementation.

So far, there has been no method established for directly measuring the vapour pressure of air within intercellular air spaces of leaves, and therefore, all approaches have, by necessity, been indirect to a greater or lesser extent. The first approach taken historically was to assess the vapour pressure of the air surrounding a leaf at which zero transpiration takes place, taking care to ensure that stomata are open and that the intercellular air space is, therefore, connected to the surrounding air via a diffusive pathway through the stomatal pores. In practice, this has involved assessing transpiration rates in air of several vapour pressures and extrapolating the observed relationship to the relative humidity at which transpiration crosses the zero line. This was the approach taken by Thut (1939) and by Whiteman and Koller (1964). In the former case, the openness of stomata during the experiments was confirmed by microscopy, and in the latter case, by observing that the CO_2 assimilation rate was maintained while the vapour pressure around the plant was varied.

An alternative elaboration of this approach was devised by Canny and Huang (2006). They used a cryo-scanning electron microscope to observe the shrinkage of leaf palisade cells of *Eucalyptus pauciflora* in leaf discs equilibrated with air of differing relative humidities. The leaf discs were sectioned perpendicular to the palisade cells, such that the cross-sectional area of palisade cells could be quantified, and they termed this the cell area fraction. A relationship was then developed between cell area fraction and the relative humidity of the air with which the leaf disc had equilibrated. Leaf discs were then collected through a diurnal cycle from plants growing outdoors, immediately frozen in liquid nitrogen, and then assessed for cell area fraction. In this way, the cell area fraction provided a proxy measurement of the relative humidity in the intercellular air spaces at the time of leaf disc collection in the field. Application of these three examples of what could be called an equilibration approach indicated significant unsaturation of intercellular air spaces, with h_i values ranging as low as 0.80 (Table 1).

A second approach has been to quantify g_s by a means which is independent of the measurement of transpiration rate and then to

TABLE 1 Reports of unsaturating relative humidity in the intercellular air spaces of leaves and associated experimental details.

Source	Species investigated	Methodology	Minimum of the observed range of intercellular air space relative humidities
Thut (1939)	*Helianthus annuus* *Lantana camara* *Petunia hybrida* *Phaseolus vulgaris*	Air relative humidity needed to induce zero transpiration was determined for intact leaves—stomatal openness confirmed by microscopy	0.85
Klemm (1956)	*Sedum ellacombianum*	Transpiration was measured gravimetrically for leaves stripped of their epidermis, and transpiration rates compared to those of model leaves containing solutions of varying water potentials	0.85
Shimshi (1963)	*Zea mays*	Transpiration was measured gravimetrically in plants for which stomatal resistance was determined by microscopy—intercellular relative humidity was then calculated	0.93
Whiteman and Koller (1964)	*Reaumuria hirtella*	Gas exchange measurements used to infe rair relative humidity at point of zero transpiration—stomatal openness confirmed by the rate of CO_2 uptake	0.80
Jarvis and Slatyer (1970)	*Gossypium hirsutum*	Two-sided leaf gas exchange measurements were combined with measurements of N_2O diffusion from one leaf side to the other to quantify stomatal resistance	0.70 (with stomatal opening induced by CO_2-free air)
Ward and Bunce (1986)	*Helianthus annuus* *Glycine max*	Two-sided leaf gas exchange; unsaturation inferred from declining transpiration but no change in photosynthesis at the upper surface when the humidity of the lower surface chamber was decreased	0.90
Egorov and Karpushkin (1988)	*Citrus limon* *Cucumis sativus* *Phaseolus vulgaris* *Zea mays*	Leaf gas exchange was measured in normal air and then in air with N_2 replaced by He; CO_2 and water vapour concentrations were adjusted to give the same rates of photosynthesis and transpiration—the two sets of equations then solved for h_i	0.90
Canny and Huang (2006)	*Eucalyptus pauciflora*	Cross-sectional area of palisade cells measured with a cryo-scanning electron microscope for leaf discs in equilibrium with differing relative humidities—the resulting calibration curve was applied to leaves collected in the field throughout the diurnal cycle	0.90
Cernusak et al. (2018)	*Juniperus monosperma* *Pinus edulis*	Gas exchange measured along with the stable isotope composition of CO_2 and water vapour before and after entering the gas exchange cuvette—isotope discrimination provided an additional constraint to solve for vapour pressure in the intercellular air spaces	0.80
Holloway-Phillips et al. (2019)	*Vicia faba*	The method of Cernusak et al. (2018) was improved upon by repeating measurements with CO_2 of two different isotopic compositions—this provided an additional constraint to allow simultaneous estimation of intercellular vapour pressure and mesophyll conductance to CO_2	0.94
Cernusak et al. (2019)	*Populus* × *canescens*	The stable isotopic method of Cernusak et al. (2018) was applied to wild-type plants and plants genetically transformed to be insensitive to abscisic acid (ABA)	0.60 (in a transgenic line with non-closing stomata resulting from ABA insensitivity)
Wong et al. (2022)	*Gossypium hirsutum* *Helianthus annuus* *Eucalyptus pauciflora* *Phaseolus vulgaris* *Xanthium strumarium*	Two-sided leaf gas exchange was employed and the CO_2 concentration at the lower surface was reduced until zero assimilation at that surface, establishing a c_i gradient across the leaf—the gradient provided a constraint for estimating h_i as the vapour pressure deficit (VPD) in both upper- and lower-surface cuvettes was increased	0.80

TABLE 1 (Continued)

Source	Species investigated	Methodology	Minimum of the observed range of intercellular air space relative humidities
Márquez et al. (2023)	*Gossypium hirsutum*	A relationship between assimilation rate and the intercellular CO_2 concentration at the mesophyll cell wall (c_w) was established at low VPD—this was used to estimate c_w as VPD increased, which in turn was used to calculate vapour pressure in the intercellular air spaces	0.92
Márquez et al. (2024)	*Zea mays* *Panicum miliaceum* *Sorghum bicolour*	The technique of Wong et al. (2022), using two-sided leaf gas exchange, was applied to three species that employ the C_4 photosynthetic pathway. All three species are monocots	0.80

use this estimate of g_s along with the measured transpiration rate to solve for e_i. This approach was taken by Shimshi (1963), who used a combination of microscopy and a viscous flow porometer to estimate g_s in maize plants at different soil moistures, and for which g_s had been modified in some plants by application of phenylmercuric acetate, a foliar spray which had been shown to induce stomatal closure (Zelitch & Waggoner, 1962). It was observed that in the plants held at different soil moistures, different relationships between g_s and transpiration rate occurred, with transpiration being lower for a given g_s as soil moisture was decreased. This indicated a lowering of h_i as the plants were exposed to progressively lower soil moisture, with values as low as 0.93 recorded.

An alternative approach to quantifying g_s independently of the Gaastra (1959) approach has been to diffuse a biologically inert gas across a leaf and then to relate the diffusion resistance for the inert gas to that for water vapour. This has been accomplished by having gas exchange chambers independently on the upper and lower surfaces of a leaf, such that the inert gas could be introduced on one side and then detected on the other side. This approach was taken by Jarvis and Slatyer (1970), Farquhar and Raschke (1978), and most recently by Wong et al. (2022). Working with N_2O as the inert gas in CO_2-free air, Jarvis and Slatyer (1970) deduced h_i as low as 0.70 in leaves of cotton. It was assumed that this value was lower than what might be expected in normal air because the introduction of CO_2-free air into the cuvette would promote stomatal opening beyond that which might occur at ambient CO_2 concentrations. A challenge in this type of experiment is that the intercellular air space resistance to diffusion (r_{ias}), that is, the diffusion resistance encountered as the inert gas diffuses across the leaf after it has entered the stomata on one side and before it leaves the stomata on the other side, must be inferred by some means for the system of equations to be solved for e_i.

Approaches have also been developed in which an additional set of measurements is made alongside standard gas exchange measurements, to provide an additional set of constraints through which e_i can be estimated. One example is the experiment of Egorov and Karpushkin (1988). They first measured CO_2 and water vapour fluxes in a gas exchange cuvette in synthetic air comprising 21% O_2 and 79% N_2, in addition to CO_2 and water vapour as trace gases. They then repeated the measurement by replacing N_2 with He. They adjusted the CO_2 and water vapour concentrations in the heliox background to achieve the same CO_2 assimilation and transpiration rates as in the previous step. This meant there were effectively two sets of gas exchange equations for which the CO_2 and water vapour fluxes were the same, and therefore, for which g_s and e_i could also be assumed to be the same. Then, the ratio of diffusivities of the trace gases in $N_2 + O_2$ as compared to $He + O_2$ could be introduced as the additional constraint to allow the equations to be solved for e_i. In so doing, Egorov and Karpushkin (1988) demonstrated significant unsaturation, with h_i as low as 0.90 (Table 1).

In the case of Cernusak et al. (2018), the additional set of measurements was of the stable oxygen isotope compositions of CO_2 and water vapour entering and exiting the gas exchange cuvette. From these measurements, the oxygen isotope composition ($\delta^{18}O$) of the CO_2 in the intercellular air spaces and of the water at the evaporative sites could be inferred. Because the enzyme carbonic anhydrase rapidly catalyses the exchange of oxygen atoms between CO_2 and water, there should be locations within the leaf near the mesophyll cell surfaces where these two isotopic signals converge. By describing this process mathematically, it was again possible to have a system of equations with an additional constraint imposed, in this case, that the CO_2 in mesophyll cells and evaporative site liquid water should be in isotopic equilibrium, which then allowed solving for e_i (Cernusak et al., 2018). Carbonic anhydrase activity is thought to be greatest at the chloroplast surface (Gillon & Yakir, 2000), and therefore the conductance for CO_2 from the intercellular air spaces to the chloroplast surface is an additional parameter that is required in this method. The approach of Cernusak et al. (2018) was to estimate this from measurements at low vapour pressure deficit (VPD), for which $h_i = 1$ was assumed. Holloway-Phillips et al. (2019) improved upon this by making a second set of measurements for each leaf in which the $\delta^{18}O$ of CO_2 entering the gas exchange cuvette was altered from that of the first set. This provided yet another constraint, from which both the mesophyll conductance to CO_2 from the intercellular air space to the site of carbonic anhydrase activity and e_i could be simultaneously solved. Application of these isotopic approaches across multiple studies resulted in estimates of h_i as low as 0.80, and even 0.60 in a transgenic plant with paralysed stomata (Table 1).

Independent measurements of leaf gas exchange on upper and lower leaf surfaces have provided further insights into unsaturation in intercellular air spaces. Ward and Bunce (1986) were able to transiently demonstrate negative transpiration in leaves in which both sides were initially exposed to a low VPD, with the VPD in the upper leaf surface chamber then suddenly increased in one large step. The transpiration rate in the lower leaf chamber then became negative, demonstrating that the vapour pressure in the intercellular air spaces had become lower than that in the lower leaf chamber air and, therefore, appreciably less than the saturated value. They also demonstrated decreasing transpiration rate in the upper leaf surface chamber when VPD was increased in the lower leaf surface chamber, whereas the photosynthetic rate in the upper leaf surface chamber remained unchanged. This indicated that the decreasing transpiration in the upper chamber was the result of h_i declining below one rather than the result of decreasing g_s.

Wong et al. (2022) also made use of independent gas exchange measurements of upper and lower leaf surfaces to present the most comprehensive evidence to date for unsaturation in leaf intercellular air spaces. Results of experiments conducted over about 10 years were presented. The main type of experiment took advantage of two-sided leaf gas exchange measurements. The experiment began at a low VPD (less than 1 kPa), at which stage the CO_2 concentration entering the gas exchange cuvette on the lower leaf surface was reduced until the net CO_2 assimilation rate was zero. The CO_2 concentration entering the upper leaf surface cuvette was adjusted so that the CO_2 concentration within this cuvette was maintained near the ambient concentration of about 400 ppm, such that the leaf continued to photosynthesise, with CO_2 effectively being fed into the leaf through the upper surface only. The intercellular CO_2 concentration, c_i, could then be calculated for each surface according to Equations (3) and (4) above, employing the assumption of $h_i = 1$. Logically, the c_i should be higher for the upper surface than for the lower surface in this scenario, as the CO_2 must diffuse across the leaf to provide the substrate for the lower leaf mesophyll, and this requires a diffusion gradient in that direction, with resistance imposed by the intercellular air space. The VPD was then increased in similar steps in both chambers.

The tell-tale sign of air space unsaturation in this experiment was the apparent inversion of the c_i gradient at higher VPDs, suggesting that the CO_2 concentration in the intercellular air spaces of the lower leaf mesophyll had become higher than that in the upper leaf mesophyll. This apparent inversion of the c_i gradient was an artefact caused by the incorrect assumption of $h_i = 1$. The intercellular air space resistance calculated from the lowest VPD measurements, when h_i was likely near unity, could then be applied to solve for the h_i that would restore the c_i gradient to have the correct sign from upper to lower surface and to have a value proportional to the CO_2 assimilation rate. Values of h_i deduced from these experiments were compared to values deduced by the stable isotope technique of Cernusak et al. (2018), which was conducted concurrently on a subset of the same leaves; estimations of h_i by the two methods were found to compare favourably to each other and to range as low as 0.80 (Wong et al., 2022).

Márquez et al. (2023) further developed the approach of employing two-sided gas exchange by developing a theory for estimating the CO_2 concentration at the mesophyll cell wall surfaces (c_w) from the two-sided measurements. They then measured a CO_2 response curve relating the CO_2 assimilation rate (A) to c_w at low VPD, assuming saturating h_i under the low VPD conditions. Next, for measurements at higher VPDs, h_i was adjusted in the calculations of c_w until the estimate matched that which corresponded to the A-c_w curve. In this way, unsaturating h_i could also be explored under conditions in which the CO_2 concentrations in the chambers of both leaf surfaces were close to the ambient concentration. With this approach, h_i was estimated to decline to 0.92 at a VPD of about 2 kPa in *Gossypium hirsutum* (Table 1).

Returning to Wong et al. (2022), they took the additional step of conducting experiments in which three inert gases He, Ne, and Ar were used in the manner described above, introduced to the air stream entering the cuvette on one side of the leaf, and then detected on the other side. In this way, the sum of the boundary layer, stomatal, and intercellular air space resistances could be estimated independently of the measurements of water vapour and CO_2 fluxes. With these measurements, the authors were able to show that what they termed the unsaturation resistance (r_{unsat}) increased with increasing VPD, but did not appear to exceed the intercellular air space resistance estimated with the noble gases. This indicated that there likely exists a water vapour saturation front within the leaf that is near the substomatal cavities under low VPD conditions, but which then retreats deeper into the leaf as the VPD increases (Figure 2). In such a way, the results of Wong et al. (2022) could also be reconciled with those of Farquhar and Raschke (1978).

Finally, Wong et al. (2022) also measured whole-tissue water potentials by thermocouple psychrometry in leaves in which unsaturation had been established, according to the experimental protocol above. The leaf discs were collected immediately upon opening the gas exchange cuvette after measurements at the highest VPD step. These measurements demonstrated that the bulk leaf water potential for the leaves did not decline below −1.5 MPa, while the water potential of liquid water in equilibrium with h_i, calculated with Equation (1), was as low as −13 MPa. The striking implication, consistent with the other reports reviewed here, was that most of the water within the leaf, largely comprising water inside living cells, remained at a benign water potential, while that in the cell walls lining the intercellular air spaces declined to much lower values—values that would indeed be lethal if experienced within the living cells.

There is one final type of experiment that has been conducted that warrants mentioning. This is an experiment in which the epidermis of a leaf has been stripped away, and the evaporation rate from the leaf with exposed mesophyll is observed (Canny, 2012). The advantage of this approach is that the influence of stomata is completely removed, and any modification of the transpiration rate can be ascribed to processes in the leaf mesophyll. Klemm (1956) took this approach and found that the transpiration rate in a leaf stripped of its epidermis indicated a decline in the conductance of the remaining leaf tissue in response to either a reduction in the leaf

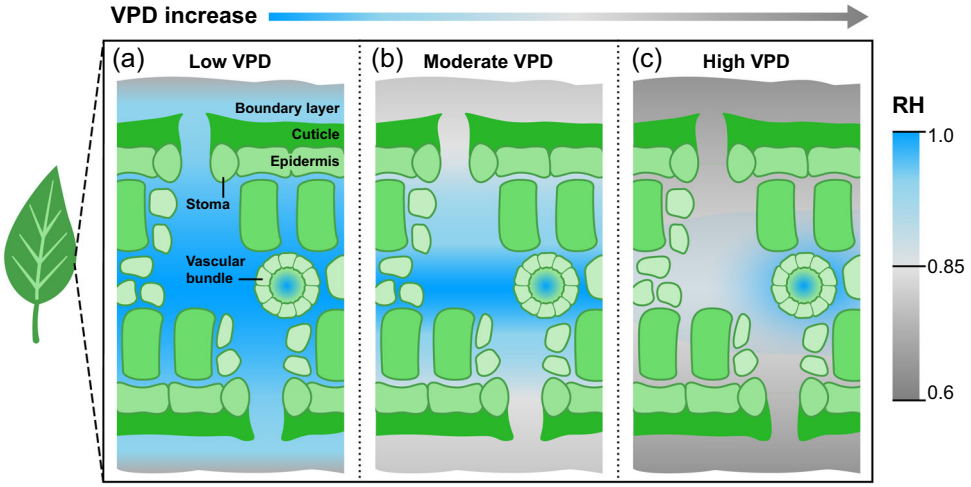

FIGURE 2 A depiction of the retreat of the zone of saturating relative humidity into the interior of the leaf with increasing vapour pressure deficit (VPD) in the air surrounding the leaf, based on data and interpretation in Wong et al. (2022). Relative humidity (RH) is shown here as a proportion, with a value of one representing the saturated value. Figure 2 (a) shows low VPD surrounding the leaf, (b) moderate VPD, and (c) high VPD. [Color figure can be viewed at wileyonlinelibrary.com]

water content or exposure to air of lower relative humidity. This suggested that the mesophyll retained some mechanism of control over the transpiration rate even when the stomata were completely removed from the leaf. Farquhar and Raschke (1978) also confirmed this result, demonstrating that when a leaf was stripped of its epidermis and exposed to dry air, the transpiration rate decreased rapidly, while the whole-leaf water potential remained within a relatively narrow range. One experiment conducted by Klemm (1956) now appears to be prescient: he found that the control of transpiration rate in response to decreasing leaf water content in a leaf stripped of its epidermis was stronger in a living leaf than in one that had been killed chemically, suggesting that some component of mesophyll-based control over transpiration resides in living cells.

3 | A VARIABLE, LIQUID-PHASE RESISTANCE IN THE LEAF MESOPHYLL

van den Honert (1948), in crafting his argument for why stomata should be considered master controllers of transpiration, as conceived by Gradmann (1928), commented on the possibility of unsaturation in intercellular air spaces. He referred to a departure from a state of nearly full saturation as 'incipient drying', using the term as first employed by Livingston and Brown (1912). van den Honert remarked, 'Another deduction refers to the possible significance of incipient drying of the cell walls bordering the intercellular spaces of the leaf. To reduce the transpiration rate materially, the resistance in these single-cell walls, negligible before, should increase to such an extent that it would become greater than the resistance in all the rest of the plant, which is hard to visualise'. This nicely summarises the challenge of understanding how the leaf mesophyll can be an important controller of transpiration, in addition to the control by stomata. It requires two things. First, that there is some mechanism of variable resistance that can respond on physiological time scales rather than simply a fixed resistance. And second, that the magnitude of such resistance is able to increase to such an extent that it becomes several times larger than the resistance associated with the entire transport pathway through the plant up until that point. To appreciate this, consider the example of h_i of about 0.90 in a plant growing in well-watered soil, as shown for cotton (*G. hirsutum*) in Wong et al. (2022). Here, the drop in water potential from the soil to the leaf, as inferred from the bulk leaf water potential, could be no more than about −1.5 MPa. The drop in water potential from the bulk leaf to the intercellular air spaces must then exceed −10 MPa. For steady-state transpiration, this implies that the resistance associated with the movement of water across the cell walls lining the intercellular air spaces should have increased to become more than six times the summed resistance from the root surface to the mesophyll cells. Moreover, the transition of the mesophyll resistance from negligible to very substantial had to have taken place over the time course of hours, as the VPD around the leaf was increased and h_i decreased from near 0.99 to approximately 0.90.

The discovery of aquaporins, nearly half a century after van den Honert (1948) published his paper, now provides an attractive possibility for such a variable, liquid-phase resistor (Scoffoni et al., 2017). Unlike what was imagined by Livingston and Brown (1912), and which van den Honert (1948) found hard to visualise, this resistor would not reside in the cell walls themselves, but rather in the plasma membranes that control the trafficking of water and solutes into, out of, and through the bundle sheath and mesophyll cells. Aquaporins are membrane-integrated proteins that provide channels for the movement of water and other molecules from one side of the membrane to the other. Elucidation of the structure and function of aquaporins began with discoveries in the early 1990s (Chrispeels & Agre, 1994; Maurel et al., 1993; Preston & Agre, 1991; Preston et al., 1992), and they are now recognised to play important

roles in a range of processes central to plant water relations (Byrt et al., 2023; Maurel et al., 2015; Tyerman et al., 1999). Plasma membrane intrinsic proteins (PIPs) make up one group of aquaporins in the larger family of major intrinsic proteins (Park & Saier, 1996; Verdoucq & Maurel, 2018). A feature that makes PIPs attractive as a potential mechanism to explain mesophyll-based control over transpiration is that the water channels that they form can be gated open or closed on time scales that would be relevant to leaf physiological responses to changes in VPD or leaf water content (Ding et al., 2022; Prado et al., 2019). Such posttranslational modification of PIPs can occur through several mechanisms, including phosphorylation (Johansson et al., 1998; Nyblom et al., 2009; Törnroth-Horsefield et al., 2006). Understanding the signalling pathways that lead to the gating of the water channels formed by aquaporins through phosphorylation is an active area of research, with reactive oxygen species appearing to play a pivotal role (Maurel et al., 2021).

The supposition that aquaporins can provide some measure of control over transpiration by the leaf mesophyll requires that at least a fraction of water transport through the leaf occur through the symplast, that is, through the living cells. Water is transported to the leaf mesophyll predominantly by minor veins (Sack & Holbrook, 2006). Once it reaches the mesophyll, it can move to the sites of evaporation either apoplastically along cell walls or symplastically through cells and across plasma membranes (Canny, 1990). Note that symplastic transport through plasmodesmata still requires passage across plasma membranes for water to reach the evaporative sites because plasmodesmata connect neighbouring cells, but do not connect the cell symplast to the intercellular air spaces. Although it has been argued that the majority of water flow through leaves likely occurs through apoplastic pathways (Brodribb et al., 2007; Buckley, 2015), it has also been recognised that at least some of the transpiratory water flux must also be transcellular, thus occurring symplastically (Boyer, 1985; Buckley, 2015; Rockwell & Holbrook, 2017; Rockwell et al., 2014; Scoffoni et al., 2017, 2023). The results that we have catalogued, both recent and historic, indicating significant unsaturation of mesophyll air spaces do not presuppose the extent to which the movement of water through the leaf mesophyll is symplastic, only that some symplastic flux is likely, and that it is likely to be dynamic. A major additional implication, however, is that the permeability of the plasma membrane of a leaf mesophyll cell must be capable at times of declining to such an extent that it can hold a water potential difference between the cytosol and the cell wall in the range of −10 to −25 MPa, as shown conceptually in Figure 3. Reconciling this with reported ranges of membrane permeabilities has presented a challenge to accepting significant unsaturation of leaf air spaces as a true phenomenon (Buckley et al., 2017; Buckley & Sack, 2019; Rockwell et al., 2022).

The idea that the liquid water potential in the cell walls lining the intercellular air spaces could differ dramatically in a transpiring leaf from that of the cytosol of the mesophyll cells bounded by these cells walls is challenging to test directly. The water potential of the whole leaf or of a leaf disc represents a weighted average of the pools of water within the tissue being measured. Because most of the water within a leaf is located within living cells, this likely dominates the determination of whole-tissue water potential (Scoffoni et al., 2023). A typical value for the apoplastic water fraction of a leaf is in the range of 20% (Arndt et al., 2015; Chimenti & Hall, 1994; Wardlaw, 2005). This includes water in xylem conduits, and so mesophyll cell wall water will be an even smaller subset of this. The upshot is that a determination of the bulk water potential of a leaf or leaf disc is probably a reasonable approximation of the cytosolic

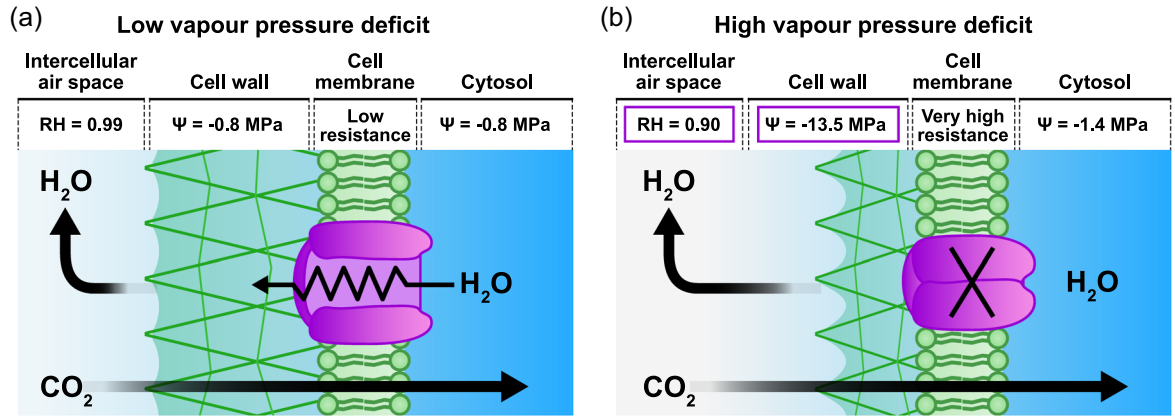

FIGURE 3 Hypothesised mechanism leading to a large difference in water potential (Ψ) between cytosolic water and that in the cell wall lining the intercellular air space at high vapour pressure deficit (VPD). Figure 3 (a) shows that at low VPD, aquaporins integrated into the plasma membrane, shown in purple, are expected to be gated open, facilitating water transport between the two locations. As a result of the high permeability of the plasma membrane, facilitated by aquaporins, the water potentials of the cytosol and cell wall would be in equilibrium. At high VPD, as shown in Figure 3 (b), the plasma membrane-integrated aquaporins would be gated closed, sharply increasing the resistance to water movement across the membrane. As the leaf continues to transpire, this will lead to much lower water potential at the evaporating sites in the cell wall than in the cytosol. The abbreviation RH refers to relative humidity, expressed here as a proportion, equivalent to h_i in the main text. [Color figure can be viewed at wileyonlinelibrary.com]

water potential, but will contain only very little information about the water potential of the cell walls lining the intercellular air spaces. If this localised water potential has departed strongly from that of the symplastic water, as would be indicated by h_i less than one (Figure 3), a determination of the bulk water potential of the leaf would be unlikely to detect it.

Recently, an exciting new technique has been developed, which could shed light on the localised water potential of mesophyll cell walls in actively transpiring leaves (Jain et al., 2021). The approach makes use of hydrogel nanoparticles, termed AquaDust, which are injected through the stomata. Introduced in this way, the particles lodge at the interface between the intercellular air space and the mesophyll cell walls. AquaDust does not enter the symplast or the xylem. The particles emit fluorescence signals that can be calibrated to infer highly localised water potentials (Jain et al., 2021). A lower water potential results in less swelling of the Aquadust, and a smaller distance of separation between dye particles contained within the AquaDust. This altered distance between dye particles affects the Förster resonance energy transfer, which changes the fluorescence wavelength that is emitted. Application of AquaDust in maize (Jain et al., 2024a) and in tomato (Jain et al., 2024b) has shown that the liquid water potential in the mesophyll apoplast can fall to values well below that which would be associated with turgor loss if observed at the whole-tissue level, and that this is associated with a sharp decline in the hydraulic conductance of the leaf mesophyll. Thus, the results so far from research with AquaDust are consistent with the development of localised, liquid-phase water potentials in mesophyll cell walls of transpiring leaves that are significantly more negative than the contemporaneously observed whole-leaf water potentials, which presumably are approximately indicative of the water potential of the mesophyll cell symplast (Scoffoni et al., 2023).

4 | IMPLICATIONS OF h_i LESS THAN UNITY

If h_i at a point in time is less than one, but is incorrectly assumed equal to one, this will lead to bias in estimates of stomatal conductance, g_s, and the intercellular CO_2 concentration, c_i. As noted above, the standard approach for gas exchange calculations since the mid-20th century has been to calculate the intercellular vapour pressure, e_i, as the vapour pressure at saturation, e_s, at the given leaf temperature. In the case of g_s, a faulty assumption that $h_i = e_i/e_s = 1$ will lead to an underestimate of g_s, because what Wong et al. (2022) termed the unsaturation resistance (r_{unsat}) will be incorrectly added to the stomatal resistance. At moderate to high VPD, when h_i departed to its lowest values, g_s was shown to be underestimated by as much as 20%–30% (Cernusak et al., 2018; Holloway-Phillips et al., 2019; Wong et al., 2022). Underestimates of c_i under these conditions were of a similar proportional magnitude or even more marked in species with generally low g_s (Cernusak et al., 2018). Such biases in c_i have important implications, for example, for estimating mesophyll conductance from online measurements of carbon isotope discrimination, typically leading to overestimates of g_m compared to unsaturation-corrected estimates (Holloway-Phillips et al., 2019; Wong et al., 2022). In the data of Cernusak et al. (2018), where underestimation of c_i was particularly pronounced because of unsaturation, the bias resulted in negative estimates of mesophyll conductance from carbon isotope discrimination, a result which cannot physically exist.

Recent studies shown in Table 1 have highlighted the role of VPD in causing the departure of h_i from unity. The measurements typically involved placing a leaf in a gas exchange cuvette and measuring first at a low VPD of less than 1 kPa and then increasing VPD in a stepwise fashion, making measurements after gas exchange rates became steady at each step. So far, only the approach of Holloway-Phillips et al. (2019) provides a means of independently estimating h_i at low VPD, with other recent approaches requiring parameterisation for a given leaf by assuming air space saturation at the lowest VPD at which measurements were made (Cernusak et al., 2018; Wong et al., 2022). Low water potential in the rooting zone, for example, caused by drying soil, also seems a likely driver of leaf air space unsaturation, insofar as it might be expected to trigger an increase in resistance between the mesophyll cell symplast and the adjacent evaporative sites. This was observed to be the case in the study of Shimshi (1963) with maize, and drying soil was also the driver of increasing hydraulic resistance in the mesophyll in the recent studies using AquaDust (Jain et al., 2024a, 2024b). Those results are consistent with a larger body of work demonstrating that declining plant water potential caused by soil drought leads to declining hydraulic conductivity in the outside-xylem zone of the leaf, that is, the hydraulic pathway between the xylem in the minor veins of the leaf and the evaporative sites (Prado & Maurel, 2013; Scoffoni et al., 2023).

The underestimation of stomatal conductance caused by unsaturation as VPD increases has a particular relevance for characterising the phenotypes of transgenic plants with altered stomatal signalling or other disruptions to the functioning of stomata. Such molecular physiological approaches have been key for demonstrating critical features of leaf gas exchange regulation (Assmann et al., 2000; von Caemmerer et al., 1994, 2004). In the case of plant phenotypes that have functionally paralysed stomata, assuming $h_i = 1$ and then assessing stomatal response or nonresponse to humidity by increasing VPD will lead to an incorrect result if unsaturation does indeed occur as VPD increases (Pantin & Blatt, 2018). This was demonstrated in an abscisic acid insensitive line of *Populus* × *canescens* (Cernusak et al., 2019). Assuming saturating h_i, as in the standard approach to calculating g_s, indicated a stomatal closure response to increasing VPD (Figure 4). However, when progressively larger reductions of h_i with increasing VPD were accounted for in the calculations of g_s, the opposite was shown; g_s actually increased in response to increasing VPD. This counter-intuitive result is, in fact, consistent with theory. Epidermal cells have a mechanical advantage over guard cells, such that if water potential decreases evenly across the cell types, the epidermal cells will prise the stomata open as the cells shrink. An active response of guard cell osmotic pressure in angiosperms prevents this from happening and allows for stomatal closure (Buckley, 2005; Buckley et al., 2003;

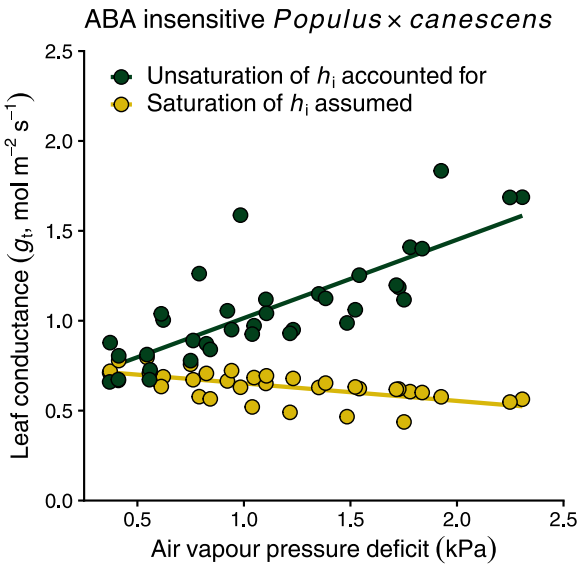

FIGURE 4 Stomatal conductance (g_s) plotted against the air vapour pressure deficit (VPD) in the gas exchange cuvette for a transgenic line of *Populus* × *canescens* (grey poplar) that is insensitive to abscisic acid (ABA). Two sets of calculations for stomatal conductance are shown, one set in which the relative humidity in the intercellular air spaces (h_i) was assumed saturated ($h_i = 1$), and one set in which measurements of the stable oxygen isotope discrimination ($\Delta^{18}O$) by the leaves were used to estimate h_i, which was then allowed to decrease below unity to satisfy the constraints imposed by the $\Delta^{18}O$ measurements. If $h_i = 1$ is assumed in the calculation of g_s, an apparent stomatal closure response to VPD is indicated, even though the stomata are effectively paralysed. However, accounting for unsaturation ($h_i < 1$) shows that this is an artefact caused by the incorrect assumption of full saturation. The counterintuitive stomatal opening response to increasing VPD in these ABA-insensitive plants is consistent with theory regarding the mechanical advantage of epidermal cells over guard cells in an angiosperm plant in which the active stomatal response has been disabled by ABA insensitivity. Data are from Cernusak et al. (2019). [Color figure can be viewed at wileyonlinelibrary.com]

Franks, 2013; Franks & Farquhar, 2007). This active response is mediated by abscisic acid; and it was disrupted in the abscisic acid insensitive *Populus* × *canescens*. Thus, it is critical to take a reduction of h_i into account to correctly characterise the stomatal responses of plants to VPD using gas exchange analysis, particularly in phenotypes likely to show prolonged stomatal opening with increasing VPD for one reason or another.

There is much to be gained by recognising the unsaturation of intercellular air spaces as an important process in the functioning of leaves. One benefit to a plant of using dynamic mesophyll hydraulic conductance to partly control transpiration rate is that the plant can temper its increase in transpiration with increasing VPD while at the same time holding stomata open so that photosynthesis is not diminished by a restricted diffusive supply of CO_2 to the chloroplasts. This can be illustrated by considering two examples, one in which a plant exhibits little to no indication of decreasing h_i as VPD increases, thereby controlling transpiration only by stomatal closure. This behaviour was observed for the wild-type of *Populus* × *canescens* from the same experiment as described above in relation to the abscisic acid insensitive line. In the case of the wild-type, no tendency toward reduction of h_i below its saturating value was detected as VPD increased (Cernusak et al., 2019). Instead, transpiration was controlled within a narrow range as VPD increased in the cuvette, and this appeared to be accomplished solely through progressive stomatal closure. That is, concurrent measurements of the oxygen isotope composition of CO_2 and water vapour, and the application of the method of Cernusak et al. (2018) for calculating h_i, did not indicate that h_i declined substantively below unity as VPD increased. The impact of stomatal closure on the photosynthesis rate was clear; it led to a linear decrease in photosynthesis as VPD increased, such that the photosynthesis rate at a VPD of about 3 kPa was 39% ± 3% (mean ± 1 SE, $n = 6$) lower than that at a VPD of about 1 kPa (Cernusak et al., 2019).

This behaviour can be contrasted with that of a plant, which instead does experience reduction of h_i in response to increasing VPD. For this example, we refer to the data for cotton presented by Wong et al. (2022). Here, the transpiration rate was also held relatively constant as VPD increased from around 1 kPa to near 3 kPa (Figure 5a). However, unlike in the case of the *Populus* × *canescens* wild-type, part of the control over transpiration rate was achieved through a reduction in h_i and part of it through a reduction in stomatal conductance. Because the cotton plants allowed h_i to decline below unity, their stomata could remain more open than they would have been if the control over transpiration were achieved through stomatal closure only. In this way, the resistance to diffusion of CO_2 to the interior of the leaf did not increase to the extent it otherwise would, and the photosynthesis rate was therefore not diminished to the same extent as in the example of the *Populus* × *canescens* wild-type. For cotton, the photosynthesis rate was only reduced by 22% ± 5% (mean ± 1 SE, $n = 5$) at a VPD of about 3 kPa compared to its value at a VPD of about 1 kPa. Thus, tolerance of mesophyll air space unsaturation can allow plants to maintain photosynthetic rates as VPD increases because stomata can remain relatively more open compared to the situation when transpiration is solely controlled by stomatal closure (Márquez et al., 2024). As VPD increases with global warming (Grossiord et al., 2020; Yuan et al., 2019), selecting for or engineering such tolerance could allow plants to maintain photosynthetic rates, thereby boosting food and fibre production in the face of global climate change with accompanying increased transpirational demands on plants.

The ability of the cotton plants in the example above to maintain photosynthetic rates relatively undiminished as h_i decreased implies something intriguing about the behaviour of the mesophyll plasma membranes. They would appear to have the ability to sharply reduce their conductivity to liquid water while at the same time maintaining permeability to CO_2 for transport into the cell to support photosynthesis (Figure 3). This may suggest that the pathways that facilitate the transport of CO_2 across the membrane are different from those that control the water flux; however, a full understanding of such a putative system of differential gating of CO_2 and water transport channels is yet to be realised (Chen et al., 2023; Kromdijk

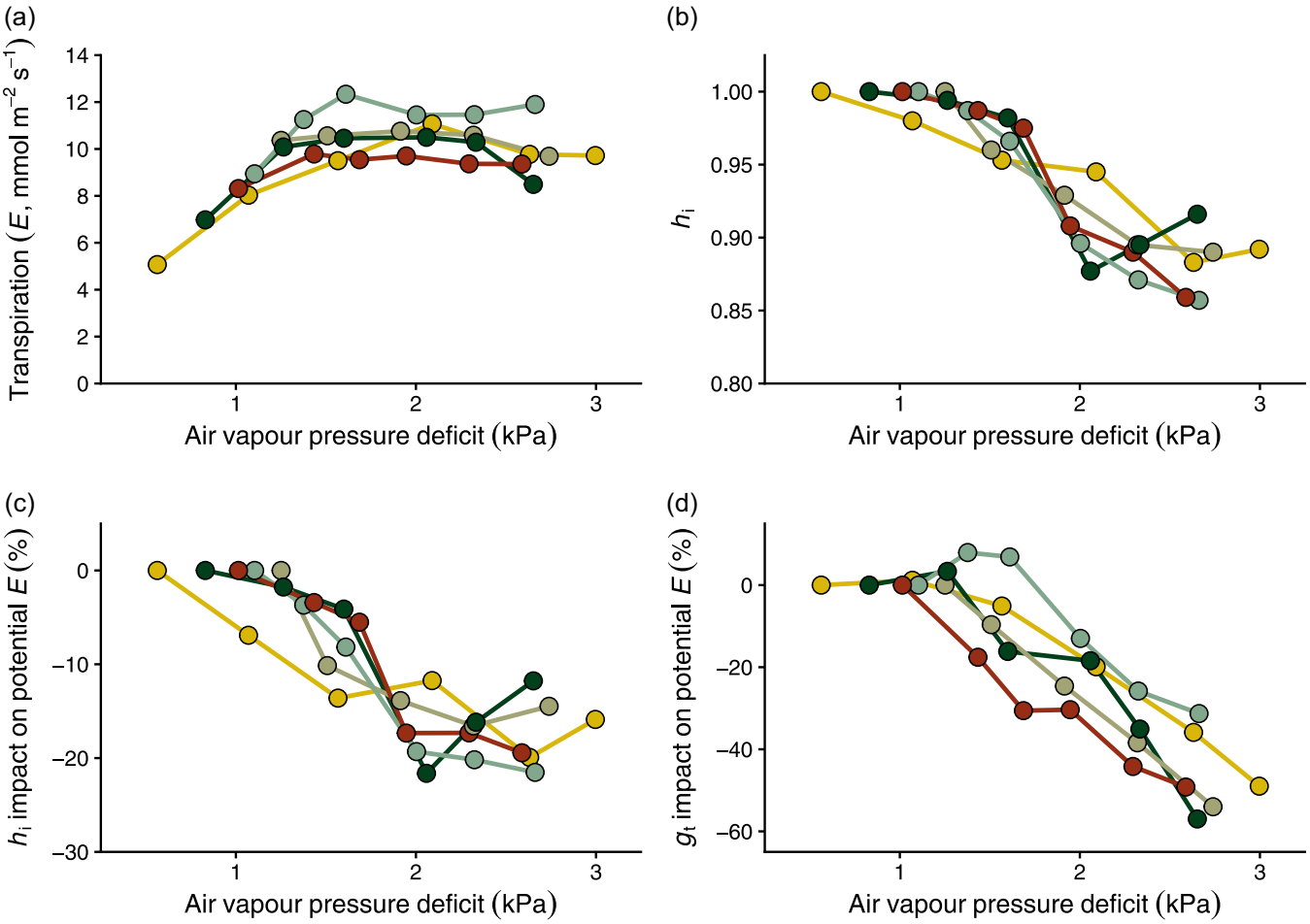

FIGURE 5 (a) Transpiration, E, (b) relative humidity of the intercellular air spaces, h_i, (c) impact of h_i on potential E and (d) impact of leaf conductance, g_t, on potential E plotted against the air vapour pressure deficit during gas exchange measurements. Data are for cotton (*Gossypium hirsutum*) from Wong et al. (2022); each colour represents a different individual plant. [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2020; Zhao et al., 2017). There are few assessments of the VPD response of mesophyll conductance to CO_2 (g_m) that have also accounted for declining h_i, which is necessary to correctly estimate g_m in such a situation. The results of Holloway-Phillips et al. (2019) stand out in this regard. In that experiment, after correcting for declining h_i, calculations of g_m showed that it also tended to decrease with increasing VPD. This could indicate some co-dependence between declining mesophyll conductivity to water and mesophyll conductance to CO_2 because there would seem to be no advantage to the plant in decreasing g_m in this situation if it could be avoided. Further research is clearly needed into the mechanisms of mesophyll cell control over water and CO_2 transport in response to VPD.

Finally, with reliable data about the unsaturation of intercellular air spaces in hand, it is worth examining the question of how much control over transpiration might be exerted by the leaf mesophyll in response to increasing VPD compared to the extent of control exerted by stomata. For this, we again turn to the data for cotton from Wong et al. (2022). Figure 5 shows that h_i declined to a lower range of about 0.85 in response to increasing VPD from about 1 kPa to about 3 kPa. The impact on the transpiration rate of this decline in h_i can be calculated by considering transpiration rate, E, as the product of the total leaf conductance to water vapour, g_t, and the leaf to air vapour pressure difference, where the intercellular vapour pressure is defined as the saturation vapour pressure (e_s) multiplied by h_i:

$$E = g_t\left(\frac{e_s h_i - e_a}{P - \bar{e}}\right), \tag{6}$$

with P being atmospheric pressure, and $\dot{e}$ here being $(e_s h_i + e_a)/2$. As VPD increases, the impact of unsaturation of h_i on E can be quantified as the difference between the E that would occur if $h_i = 1$ and that which does occur when h_i declines below unity. For a leaf in a cuvette in which the boundary layer conductance does not change, the impact of stomatal conductance on E as VPD increases can be calculated as the difference between the E, which would occur if g_t remained at its initial value measured at the lowest VPD and that which does occur as g_t decreases through the sequence of measurements at increasing VPD. Calculations of these proportional reductions in potential E resulting from h_i and g_t are shown in

Figure 5c,d. The impact on potential E resulting from the reduction of h_i ranges to about −20%, while that from declining g_t ranges to about −55%. This shows that while h_i can make an important contribution to the control of transpiration rate in response to increasing VPD, declining stomatal conductance is still a stronger controller. Thus, the argument of van den Honert (1948), that stomata must be the master controllers of transpiration, is still shown to be substantially correct. At the same time, we now consider it indisputable that unsaturation of leaf intercellular air spaces also plays an important, although lesser, role.

5 | CONCLUSIONS

Our review of observations of the unsaturation of the intercellular air spaces of leaves highlights a history of compelling evidence throughout the development of our modern understanding of leaf gas exchange. This evidence was mostly put aside in favour of a seemingly more parsimonious explanation of leaf water relations, that the intercellular air spaces must be saturated with water vapour, or nearly so. The time has come to revise this longstanding assumption. The case for significant unsaturation of intercellular air spaces is substantive and multifaceted. It indicates that localised, liquid-phase water potentials in cell walls lining intercellular air spaces must be considerably more negative than observed for the same leaves by thermocouple psychrometry or the Scholander pressure chamber. Those techniques likely return water potentials close to those of the mesophyll cell symplast, but they will significantly underestimate the tensions in the mesophyll cell walls when unsaturation occurs. Plasma membrane-integrated aquaporins likely provide the variable resistor that holds the water potential inside mesophyll cells within a narrow, benign range, while that in the contiguous cell walls exposed to evaporation reaches values as low as −30 MPa. Incorrectly assuming saturation of the intercellular air spaces results in underestimates of stomatal conductance and intercellular CO_2 concentrations. Unsaturation in intercellular air spaces is advantageous to plants because it enables control of transpiration while at the same time retaining the diffusion pathway for CO_2 to enter the leaf to fuel photosynthesis. Recognising this critical role for unsaturation will create opportunities for increasing plant productivity under the relentless rise in VPD caused by global warming.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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