RESEARCH PAPER

Turgor and the transport of CO$_2$ and water across the cuticle (epidermis) of leaves

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Received 18 December 2014; Revised 18 December 2014; Accepted 23 January 2015

Abstract

Leaf photosynthesis relies on CO$_2$ diffusing in while water vapour diffuses out. When stomata close, cuticle waxes on the epidermal tissues increasingly affect this diffusion. Also, changes in turgor can shrink or swell a leaf, varying the cuticle size. In this study, the properties of the cuticle were investigated while turgor varied in intact leaves of hypo-stomatous grape (Vitis vinifera L.) or amphistomatous sunflower (Helianthus annuus L.). For grape, stomata on the abaxial surface were sealed and high CO$_2$ concentrations outside the leaf were used to maximize diffusion through the adaxial, stoma-free cuticle. For sunflower, stomata were closed in the dark or with abscisic acid to maximize the cuticle contribution to the path. In both species, the internal CO$_2$ concentration was measured directly and continuously while other variables were determined to establish the cuticle properties. The results indicated that stomatal closure diminished the diffusion of both gases in both species, but for CO$_2$ more than for water vapour. Decreasing the turgor diminished the movement of both gases through the cuticle of both species. Because this turgor effect was observed in the adaxial surface of grape, which had no stomata, it could only be attributed to cuticle tightening. Comparing calculated and measured concentrations of CO$_2$ in leaves revealed differences that became large as stomata began to close. These differences in transport, together with turgor effects, suggest calculations of the CO$_2$ concentration inside leaves need to be viewed with caution when stomata begin to close.

Key words: Cuticle, epidermis, Helianthus annuus, turgor, water potential, Vitis vinifera.

Introduction

In leaves of terrestrial plants, most gas exchange occurs through stomata. As stomata close, the exchange becomes slower and the cuticle of the epidermal tissue begins to control the rate. The physics of gas movement differs for the two paths because, by contrast with movement through the gas phase in the stomatal pores, the cuticle does not transmit CO$_2$ and water vapour through pores. Instead, the molecules diffuse through solid wax forming the outer cuticle layer (Kerstiens, 1996; Schreiber and Schönher, 2009). Pores of small diameter exist in the cutin beneath the waxes but there is little evidence for pores in the wax itself (Kerstiens, 1996; Burghardt and Riederer, 2006; Schreiber and Schönher, 2009).

As a first approximation, Moss and Rawlins (1963) suggested that water vapour diffusing through stomata could be used to calculate the internal CO$_2$ concentration in leaves ($c_i$), and this has become the norm (von Caemmerer and Farquhar, 1981; Boyer and Kawamitsu, 2011). However, the calculations assume an identical gas phase path for CO$_2$ and water vapour that does not hold as stomata close and the cuticle and epidermis become the dominant path. Frequently, it is desirable to determine photosynthetic behaviour despite
stomatal closure by determining $c_i$ (e.g., plotting $A$ versus $c_i$ to eliminate the effects of stomatal closure) (Farquhar and Sharkey, 1982). The accuracy of calculated $c_i$ then becomes critical.

Cuticles have been isolated from leaves for studies of their water permeability. Although many conditions and species have been explored (Kerstiens, 1996; Riederer and Müller, 2006; Schreiber and Schönerr, 2009), much less attention has been directed to CO$_2$ despite its substrate role in photosynthesis. Kirschbaum and Pearcy (1988), Lendzian and Kerstiens (1991), and Meyer and Genty (1998) consider the implications of the cuticle conductance for CO$_2$, and Boyer et al. (1997) reported a lower conductance for CO$_2$ than for water vapour in cuticles of intact grape leaves. A particular problem is that turgor pressures in the epidermis stretch cuticles and are absent after the cuticle is isolated. In leaves such as sunflower, dehydration can shrink the leaf by 50% or more (Tang and Boyer, 2007). Boyer et al. (1997) and Burghardt and Riederer (2003) observed that cuticle gas exchange was affected when leaf water potentials decreased, suggesting that cuticle stretching could be important but turgor pressures were not measured.

The work of Boyer et al. (1997) depended on an indirect method to determine the $c_i$ of grape leaves. The $c_i$ was assumed to remain constant for the experiments. Sharkey et al. (1982) developed a way to measure $c_i$ directly and Boyer and Kawamitsu (2011) incorporated it into a gas exchange system. With this system, the $c_i$ could be continuously monitored while other parameters for gas exchange were determined. Because the leaves remained intact during the measurements, the effect of turgor could be tested. The following study used this instrument to determine the gas exchange properties of the cuticle and epidermis and whether turgor affects them.

**Materials and methods**

**Plant material**

Plants of grape (Vitis vinifera L. cv. Thompson Seedless from Professor MA Matthews, Department of Viticulture and Enology, University of California-Davis, CA, USA) were grown individually from bare root stock and served as a source of hypostomatous leaves. Plants of sunflower (Helianthus annuus L. hybrid IS897 from the Interstate Seed Company, Box 338, Fargo, ND, USA) were also grown separately but individually from seed and were a source of amphistomatous leaves.

Both species grew in 1.0 l pots containing peat, Perlite, and soil in volumes of 1:1:1. Dolomitic limestone was added to adjust the pH to 6.9. At planting, the soil mix was saturated with a nutrient solution (Hoagland and Arnon, 1950) composed of 4 mM KNO$_3$, 6 mM Ca(NO$_3$)$_2$, 0.4 mM MgSO$_4$, 2 mM KH$_2$PO$_4$, 0.5 μM CuSO$_4$, 5H$_2$O, 10 μM MnSO$_4$.H$_2$O, 2 μM ZnSO$_4$.7H$_2$O, 25 μM H$_2$BO$_3$, 0.5 μM H$_4$MoO$_4$.9H$_2$O, and 50 μM Fe-citrate. Whenever the soil surface was dry, this solution was resupplied until drainage occurred. The plants were grown in a controlled environment with day/night temperatures and relative humidities of 25/20 ± 1 °C and 60/95 ± 5%, respectively. Fluorescent lamps (cool white) provided a 14 h photoperiod with an irradiance of 850–1000 μmol photons m$^{-2}$·s$^{-1}$ of PAR throughout the day at the top of the plants. The grape plants were used after growing for 2–3 months, and sunflower after growing for 4–5 weeks. All measurements were made at a leaf temperature of 25 ± 0.1 °C.

**Gas exchange instrument**

The gas exchange system for these experiments was described in detail elsewhere (Boyer and Kawamitsu, 2011). The petiole of an attached, recently fully expanded leaf was sealed into the wall of an assimilation chamber with the lamina inside and the remainder of the plant outside (Fig. 1A). Typically, projected leaf areas were 250 – 350 cm$^2$ for both species, and the air was stirred with a muffin fan (Comair-Rotron Model ST24P3, Saugerties, NY, USA) to give a boundary layer conductance to water vapour of about 1100 mmol·m$^{-2}$·s$^{-1}$, which was substantially larger than the maximum leaf conductance. The chamber environment was kept constant (air temperature ± 0.1 °C, relative humidity ± 0.1%, CO$_2$ concentration in air ($c_0$)

![Fig. 1. Experimental design. (A) Assimilation chamber showing the position of a small cup on a grape or sunflower leaf. (B) Details of the small cup on the grape leaf show double sealing of the entire lower surface except where the cup is attached. Gas exchange was through the upper stoma-free cuticle. (C) Similar details of small cup on a sunflower leaf. Gas exchange was through both surfaces but placing the plant in the dark caused stomata to close and restricted gas exchange to the cuticle primarily.](image-url)
±1 μmol mol⁻¹) by injecting or removing atmospheric constituents with proportional controllers.

This system was not an open one to which standard calculations for open systems would be applied. Unique to it was a CO₂ gas analyser (cₖIRGA, LI6251, Li-Cor, Lincoln, NE, USA) that compared CO₂ in the assimilation chamber with that in a reference gas. The CO₂ controller brought the concentration in the assimilation chamber to that in the reference gas and held it there. Regardless of whether the air was diluted by water vapour or other gases in the assimilation chamber, the CO₂ controller maintained the concentration at the reference concentration automatically. This simplified the calculation of photosynthesis rate because dilution did not need to be considered. In order to determine the rate of photosynthesis, it was necessary only to know the rate of CO₂ injection into the assimilation chamber from the CO₂ control system and multiply that by the concentration difference between the injected CO₂ and the automatically-maintained CO₂ concentration exhausting from the assimilation chamber. Because this measure was independent of the cₖIRGA, which acted only to keep cₛ constant at the reference gas level, rates could be measured as easily at 50 000 μmol mol⁻¹ CO₂ as at lower CO₂ concentrations.

For respiration in the dark, the CO₂ was allowed to rise by about 5 μmol mol⁻¹ around the CO₂ set point. The respiration rate was taken from the rate of rise. The air in the assimilation chamber was then diverted automatically to a column of silica gel and a column of Ascarite in series, and this CO₂-free air brought the external CO₂ concentration (cₑ) back down to the set point. Typically, photosynthesis and respiration rates had a certainty in the third significant place.

A second critical feature of the gas exchange system was the measurement of transpiration by using the proportioning humidity controller to condense water from the air and keep the humidity constant in the chamber, thus eliminating interactions with the CO₂ control system. A similar control system kept air temperature constant by extracting heat in order to minimize interference with the control system. A similar control system kept air temperature constant by extracting heat in order to minimize interference with the control system.

Internal CO₂

In this gas exchange system, the cₛ was directly measured with a glass cup sealed with lanolin to the abaxial surface of the leaf, as shown in Fig. 1A (Boyer and Kawamitsu, 2011). In the cup, the CO₂ equilibrated with that in the stomatal pores enclosed by the cup and was a measure of cₛ. A second gas analyser operated as an absolute detector of this CO₂ (cₖIRGA, LI6251, Li-Cor, Lincoln, NE, USA). The air in the cup was gently circulated with a small fan to the analyser and back to the cup inside the assimilation chamber.

In some experiments, the cup sizes were small and covered only a small part of the underside of the leaf (4 cm², or about 2% of the projected leaf area, as in Fig. 1A). Consequently, most of the leaf exchanged gases freely. By keeping the volume of the cₖIRGA circuit small, the CO₂ in the small cup circuit equilibrated with that in the intercellular spaces of the leaf within 5 min unless the diffusive conductance of the leaf became less than 25 mmol·m⁻²·s⁻¹, which lengthened the equilibration time. In other experiments, cups were large and covered the entire underside of the leaf (100% of the projected area). In this situation, equilibration occurred in less than 1 min.

Stomatal closure

For grape leaves, stomata were closed on the abaxial surface by coating with lanolin and a polyethylene sheet before inserting the lamina into the assimilation chamber. This double seal minimized net gas exchange from that surface (Fig. 1B) so that essentially all gas exchange occurred through the cuticle of the adaxial surface above the seal. The double seal covered the entire abaxial surface except where the cup was sealed to the leaf. Because there was no net CO₂ exchange with the equilibrated cup, the leaf above the cup was in the same condition as the rest of the leaf.

For sunflower leaves, the stomata were on both surfaces and instead of sealing with lanolin/polyethylene, the stomata were closed in the dark. CO₂ then moved out of the leaf mostly through the cuticle because respiratory activity increased cₛ above that in the atmosphere. However, as with grape, the cₛ was monitored continuously using the cup sealed to the abaxial surface of the leaf (Fig. 1C).

For other sunflower experiments, the stomata were closed in the light with abscisic acid (ABA). The petiole was cut in a 50 ml cup on the outside of the assimilation chamber filled with degassed water. After the petiole was excised under the water, the remaining plant was removed and gas exchange was monitored in the excised leaf. When the measurements became steady, the stomata were closed by mixing 0.1 mM ABA with the water in the cup to give a final concentration of 50 μM. The (±)-cis,trans-abscisic acid (from Sigma, St Louis, MO, USA) was dissolved in a stock solution by bringing the pH to 10–11 with KOH, then neutralizing to pH 7 with HCl.

Turgor measurement

Plants were hydrated by supplying nutrient solution until drainage occurred from the soil. Leaf turgor (Ψ_p) was determined in discs excised from the leaves used for gas exchange (disc area 3 cm²). The discs were immediately sealed into an isopiestic thermocouple psychrometer. Isopiestic potentials were measured in the fresh tissue (water potential Ψ_w) and repeated after freezing and thawing the same tissues (osmotic potential Ψ_o) according to Boyer (1995). Turgor was calculated from:

\[ \Psi_p = \Psi_w - \Psi_o \] (1)

Calculations and statistics

Water vapour conductance g_w was calculated according to Boyer and Kawamitsu (2011) who extended the relation to high CO₂:

\[ g_w = \frac{E(1 - \bar{w} + 0.587)}{(w_i - w_a)} \] (2)

where E is the transpiration flux (mol m⁻² s⁻¹), w_i and w_a are the respective water vapour concentrations of the intercellular spaces inside the leaf and the bulk air outside of the leaf boundary layer, respectively, and \( \bar{w} \) and \( \bar{z} \) are the average water vapour and CO₂ concentration in these two locations (mol mol⁻¹). The \( w_a \) were assumed to be saturating at leaf temperature.

The \( \bar{w} \) and \( \bar{z} \) account for interactions between water vapour diffusing out and CO₂ diffusing into the leaf (von Caemmerer and Farquhar, 1981; Boyer and Kawamitsu, 2011). The interactions accounted for less than 1% of \( g_w \) in these experiments.

Similarly, the CO₂ conductance \( g_c \) was calculated from the relation in Boyer and Kawamitsu (2011):

\[ g_c = \frac{A}{c_z - c_i} \] (3)

where \( A \) is the CO₂ flux (mol m⁻² s⁻¹) and \( c_z \) and \( c_i \) are CO₂ concentrations outside the boundary layer and inside the intercellular spaces, respectively (mol mol⁻¹). No term for interactions appears in this equation because \( c_i \) was directly measured and already reflected the interactions (see Discussion).

All experiments were repeated at least three times and the results are given as means with standard errors or standard deviations.

Results

Grape leaves

In grape leaves exchanging gases freely without a seal on the abaxial surface, photosynthesis was rapid under the...
conditions expected to be used when measuring cuticle properties. At \( c_a \) of 10 000 \( \mu \text{mol mol}^{-1} \), \( A \) was about 10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and the conductances of the leaves for water vapour and \( \text{CO}_2 \) were about 40 and 25 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), respectively. When the abaxial surface was sealed, the conductance of the cuticle/epidermis was detected and was low, so steps were taken to maximize the signal. First, the grape leaves were exposed to \( c_a \) of 10 000 \( \mu \text{mol mol}^{-1} \). Inside the leaf, \( c_i \) was only 46 \( \mu \text{mol mol}^{-1} \) and steady (\( \text{CO}_2 \) compensation point was 44 \( \mu \text{mol mol}^{-1} \) for these leaves). The large concentration difference across the cuticle enhanced \( \text{CO}_2 \) uptake. Second, the controllers for the gas exchange system were turned off and sealed during the measurements so that gases were exchanged only in the assimilation chamber.

These enhancements allowed small changes in \( \text{CO}_2 \) and humidity to be detected, as shown in Fig. 2A and B. The slope of the change indicated the rate of \( \text{CO}_2 \) uptake or water vapour release through the stoma-free cuticle on the adaxial side. The walls of the assimilation chamber were quite inert during the measurements, and there was no detectable sorption of water vapour and only small amounts of \( \text{CO}_2 \) (typically 5\% except for one replication at 15\% of the signal). All assimilation rates were corrected for sorption by removing the leaf and repeating the measurement immediately afterward in an empty chamber. As is apparent in Fig. 2A and B, the rate of water loss through the cuticle was always greater than \( \text{CO}_2 \) uptake, causing the cuticular conductance for water vapour to be about 40\times larger than for \( \text{CO}_2 \) (Fig. 2C).

**Sunflower leaves**

By contrast with grape, gas exchange was more rapid in sunflower and the controllers for the gas exchange system could be used continuously. In order to determine whether the gas exchange properties were altered by cup attachment for \( c_i \) measurements, determinations with the small cup were followed by determinations with the entire abaxial surface enclosed in a cup. In the latter situation, gas exchange was measured when only the adaxial surface was operating (similar to the situation in the grape experiments). Figure 3A indicates that sealing the cup to the abaxial surface caused only a slight change in \( c_i \) while \( c_i \) and \( A \) decreased to half or less of what they had been with the small cup. However, \( c_i \) and \( A \) began to rise and after about 1.5h returned to the earlier rate (when most of the leaf was free and attached only to the small cup). Similar results were obtained with \( w_a \), \( w_i \), and \( E \) measured simultaneously in the same leaf (Fig. 3B). Thus it appears that, when a cup was attached, stomata above the cup gradually opened more widely than in the freely exchanging leaf. The wider opening returned gas exchange to the rates in the free leaf.

When the small cup was attached and most of the leaf was free to exchange gases normally, daytime \( c_i \) was less than \( c_a \) (Fig. 4A). \( \text{CO}_2 \) diffused in as \( A \) consumed the internal \( \text{CO}_2 \) (Fig. 4A). By contrast, night-time \( c_i \) was above \( c_a \) because of respiration, and \( \text{CO}_2 \) diffused out. Transpiration also occurred, rapidly during the day but slowly at night because the stomata closed (Fig. 4B). The transpiration became slower as the night progressed, probably because the stomata gradually closed more tightly. Water vapour concentrations were essentially constant day and night (Fig. 4B). The data in Fig. 4A and B allowed the conductances to be calculated as shown in Fig. 4C and indicated that conductance to water vapour was always high during the day with open stomata and low at night when stomata were closing. With most water lost through the cuticle at night, the average cuticular conductance to water vapour was 38 ± 6.9 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at the end of the night (three replications). For \( \text{CO}_2 \) it was 2 ± 0.40 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Therefore, like grape, the conductance for water vapour was larger than for \( \text{CO}_2 \) (about 20\times).

It was also possible to close stomata during the day by allowing ABA to be absorbed through the petiole of excised sunflower leaves. Figure 5 shows that photosynthesis and
transpiration were diminished when ABA entered the leaf. The loss in transpiration indicated that stomata had closed at least partially (Fig. 5B), and $c_i$ decreased to $60 \mu\text{mol mol}^{-1}$ because $\text{CO}_2$ was consumed inside the leaf (Fig. 5A). The $\text{CO}_2$ compensation point was $42 \mu\text{mol mol}^{-1}$ in these leaves, which indicated that the stomata had not closed as tightly as in the dark (i.e. as in Fig. 4). Nevertheless, the ABA-fed leaves displayed a larger cuticular conductance for water vapour than for $\text{CO}_2$, by about $5.5 \times$ (Fig. 5C).

**Cuticle response to turgor**

The right-hand side of Fig. 6A shows a grape leaf having a conductance to water vapour of $4.8 \text{mmol m}^{-2} \text{s}^{-1}$ and $\text{CO}_2$ of $0.13 \text{mmol m}^{-2} \text{s}^{-1}$ while attached to the plant. The water potential was about $-0.9 \text{MPa}$ and turgor was $1.1 \text{MPa}$ (Fig. 6B, right side). After the leaf was detached from the plant, water was lost slowly through the adaxial surface for 3–4 d and the water potential declined to $-3.3 \text{MPa}$. During this time, the leaf turgor declined and the cuticle conductance for water vapour decreased in the same leaf (Fig. 6A, B). The decline also occurred for $\text{CO}_2$ by the same relative amount (Fig. 6A). By the end of 3–4 d, conductances for the gases had diminished to about $0.9 \text{mmol m}^{-2} \text{s}^{-1}$ for water vapour and $0.02 \text{mmol m}^{-2} \text{s}^{-1}$ for $\text{CO}_2$, while turgor had dropped to 0.24 MPa. The leaves appeared normal in every respect and showed no sign of wilt (i.e. a turgor of zero). It should be noted that these measurements were for cuticle/epidermis without stomata.

In sunflower, conductances were higher than in grape when water was available (Fig. 7). But, like grape, a decrease in turgor (Fig. 7B) was associated with a decrease in conductance for water vapour and $\text{CO}_2$ (Fig. 7A). There was more variability...
in conductances in sunflower than in grape, perhaps because the stomata closed to varying degrees in sunflower while there were no stomata on the adaxial surface of grape. Turgor decreased more in sunflower than in grape as water potentials declined. Turgor in sunflower reached zero at a water potential of −0.9 to −1.1 MPa and the leaves appeared wilted. At zero turgor and a water potential of −1 MPa, the leaves had about 80% of their original area. No further declines in conductance were detected after zero turgor despite further decreases in water potential and further shrinkage of the leaves.

Discussion

Gas exchange

The gas exchange system used here was specially designed for experiments like these (Boyer and Kawamitsu, 2011). The operating ranges were 0–4% for water vapour and 0–5% for CO₂, which were used to advantage to increase the rate of diffusion across cuticle/epidermal barriers in grape while the leaves remained intact. These specifications are not currently available in most open gas exchange systems (where air moving across the leaf exits to the open air). As concentrations rise in these systems, CO₂ concentrations must be accurately measured against an increasing background. By contrast, in the system used here, the CO₂ analyser for the assimilation chamber did not participate directly in the measurement and was only a comparator for CO₂ control. This minimized the problem of declining resolution at high CO₂ concentrations and allowed rates to be measured at much higher CO₂ concentrations than usual. In open systems, the gases dilute each other which must be taken into account. In the present work, the comparator automatically corrected for dilution of CO₂ by water vapour, avoiding the need for further correction and simplifying the analysis.

The new system also incorporated a method to measure cᵢ directly, avoiding the uncertainties of calculating cᵢ (Boyer and Kawamitsu, 2011). Consequently, concentrations at both ends of the diffusion paths for CO₂ and water vapour were determined simultaneously while rates of diffusion were measured for both gases. The cᵢ in these experiments were measured by sealing a cup to one surface of the leaf and allowing it to equilibrate with CO₂ in the leaf at that surface. Because volumes were small, equilibration was rapid.
Cuticle conductance and turgor for sunflower leaves having various water potentials. (A) Conductances to water vapour ($g_w$) and CO$_2$ ($g_c$). Note that the y-axis for $g_w$ is 10x that for $g_c$ to facilitate comparison. (B) Turgors in the same leaves became zero at water potentials of −0.9 to −1.1 MPa with detail shown from Boyer and Potter (1973). Conductance data are from six different plants (six replications) in the dark. Turgor data are from five different plants (additional six plants in Boyer and Potter, 1973). Irradiance was 800 µmol m$^{-2}$ s$^{-1}$ PAR.

With this system, it was possible to test whether the $c_i$ measurements automatically reacted to any interactions with water vapour (assumed in Eq. 3), or required interactions between CO$_2$ and water vapour to be included according to Eq. 4 (von Caemmerer and Farquhar, 1981; Boyer and Kawamitsu, 2011):

$$g_c = \frac{A + \bar{c}E}{c_a - c_i}$$  (4)

where $\bar{c}$ is the average CO$_2$ concentration inside and outside of the leaf. Note that transpiration or high $\bar{c}$ cause $g_c$ to be higher than if no transpiration is occurring or $\bar{c}$ is negligible (other terms being unchanged). At $\bar{c}$ approximating that for water vapour in the new system, interactions between the two gases would be apparent.

This test was conducted in leaves with open stomata, illustrated in Fig. 7 of Boyer and Kawamitsu (2011) and reproduced here in Fig. 8. As CO$_2$ concentrations increased outside the leaf, i.e. when the degree of interaction between water vapour and CO$_2$ increased (Eq. 4), leaves with open stomata had progressively lower $c_i$. Moreover, the directly measured $c_i$ without accounting for interactions (Eq. 3) followed the calculated $c_i$ that included the interactions (Eq. 4). As long as stomata were open, this result was obtained whether the calculations were based on trace concentrations of CO$_2$ or high concentrations of CO$_2$ (see Appendix in Boyer and Kawamitsu, 2011). The adjustment for interactions apparent in Fig. 8 for an attached cup indicated that Eq. 3 was correct for $c_i$ measured with an attached cup. In other words, CO$_2$ in the cup automatically responded to any interaction with water vapour departing the leaf.

**Cuticle effects on gas exchange**

For the test in Fig. 8, the leaves had open stomata. Most of the gas exchange occurred along a gaseous pathway. When stomata began to close in the present work, however, rates of gas exchange diminished and the external cuticle became more important. The low conductances for water vapour indicated that stomata were closing in both species and the cuticle and epidermis dominated gas exchange.

Whether stomata occurred on one surface or both surfaces, the cuticle effect was stronger for CO$_2$ than water vapour, perhaps because the CO$_2$ moved through cuticle wax plus the water in the epidermal cells while water moved only through the waxes. This result agrees with the conclusions of Boyer et al. (1997) who found a lower cuticle conductance for CO$_2$ than for water vapour. The cuticle conductance for CO$_2$ was so small (1/20th to 1/40th that of water vapour) that CO$_2$ could be considered essentially to move only through the stomata while water vapour moved through stomata plus the cuticle.

With no stomata on the upper surface in grape, sealing the lower surface was a way to force transport nearly all through the cuticle on the upper surface, and gas movement was unambiguously dominated by the cuticle and epidermis. Sealing a cup to the lower surface acted the same as a direct seal, and the cup indicated photosynthesis had depleted CO$_2$ inside the leaf despite 10 000 µmol mol$^{-1}$ outside. The cuticle and epidermis clearly acted as major barriers to CO$_2$. 

Fig. 8. Comparison of directly measured $c_i$ and calculated $c_i$ in a sunflower leaf with open stomata. Directly measured $c_i$ are open circles (○) that automatically include interactions with water vapour. Calculated $c_i$ account for the interactions in the calculation and are according to von Caemmerer and Farquhar (1981) for trace concentrations of CO$_2$ (closed circles, ●) or Boyer and Kawamitsu (2011) for large concentrations of CO$_2$ (open triangles, △). Details are in Boyer and Kawamitsu, 2011. Photosynthesis as stomata open, resulted in depletion of CO$_2$ from the leaf with open stomata. Directly measured $c_i$ and calculated $c_i$ for leaves with open stomata. Directly measured $c_i$ are open circles (○) that automatically include interactions with water vapour. Calculated $c_i$ account for the interactions in the calculation and are according to von Caemmerer and Farquhar (1981) for trace concentrations of CO$_2$ (closed circles, ●) or Boyer and Kawamitsu (2011) for large concentrations of CO$_2$ (open triangles, △). Details are in Boyer and Kawamitsu, 2011. Photosynthesis gas exchange system with internal CO$_2$ directly measured. Environmental Control in Biology 49, 193–207.
By contrast, this method could not be used for sunflower with stomata on both surfaces. Methods were restricted to those that began to close the stomata and allowed cuticular conductances to start to dominate the gas exchange. Leakage through imperfectly closed stomata could have affected the results, and conductances with closed stomata were greater in sunflower than in grape. In fact, ABA probably did not close stomata completely in sunflower because the directly measured $c_i$ decreased to only about 60 $\mu$mol mol$^{-1}$ while the CO$_2$ compensation point was 42 $\mu$mol mol$^{-1}$. But even with leakage, the discrimination against CO$_2$ was evident with darkness and ABA in agreement with the unambiguous results in grape. ABA was used to close stomata of sunflower because the petioles of the leaf were excised under water in order to feed the ABA. With the petioles in water, turgor was high in the leaf before the feeding, and cuticle properties should have been minimally affected by increases in turgor caused by stomatal closure. This is in contrast to darkening the intact plant where the simple diurnal changes in leaf water potential could have caused similar diurnal changes in turgor as darkness occurred.

Turgor effects

In both species, a change in turgor caused a change in gas exchange of the cuticle. The cuticle appeared to become tighter when it was less stretched by turgor. The effect was largest in sunflower where turgor loss was complete. Sunflower leaves shrink substantially as turgor is lost and can have 50% or less of their original area after turgor falls to zero (Tang and Boyer, 2007). In addition to the obvious effect of shrinkage on cuticle waxes, the shrinkage is also important for stomatal closure because returning leaf area to its original size causes the stomata to open somewhat (Tang and Boyer, 2007).

Grape leaves also showed the same tendency for conductances to decline as turgor diminished, but not as much as in sunflower, perhaps because turgor was not lost as much. Slight turgor remained even at a water potential of $-3.5$ MPa.

In this work, turgor and cuticle gas exchange were measured in the same leaves by repeatedly sampling for turgor while the gas exchange measurements were in progress. The turgor samples were in the dark (enclosed in a psychrometer chamber), causing stomata to close in the samples. Under these conditions, water vapour moved mostly through cuticle. However, by making the measurements isopiestically, the effect of the cuticle was avoided because the detector (a solution on a thermocouple) was in thermal and vapour pressure equilibrium with the unknown (leaf sample), and no net transfer occurred. Whenever the thermocouple is out of equilibrium with the sample, transfer occurs between the sample and detector and thus across the cuticle. Transfer is at a rate determined by cuticle properties and will vary with the turgor in the sample. Since many thermocouple psychrometers are not based on thermodynamic equilibrium, they experience this variability. The isopiestic approach avoided turgor-based variability and was therefore crucial for this study. The turgor pressure measured isopiestically has proved to be quite accurate (Boyer, 1995).

In isolated cuticle, of course, turgor is absent which suggests that conductances will be less than when measured on an intact leaf. In effect, isolated cuticles would be tighter than when they are on the leaf. In support of this conclusion, Kerstiens (1996) surveyed the water conductivity reported for the cuticles of 200 plant species and found those on intact leaves to be about $5 \times$ to $10 \times$ those that had been isolated (from his Fig. 1 and Table 1 recalculated only for isolated cuticles from 36 reports). The author attributes the effect to tightening during storage but turgor effects were unknown when this valuable survey was done.

It seems likely that stretching by turgor alters the molecular orientation in the waxes. Kerstiens (1996) points out that, although cracking might be suggested, there is no evidence for such permanent pore structures. Instead, the evidence implicates a motion of 0.5–1 nm between the wax molecules that allows dynamic diffusion across the wax layer. Stretching would affect this motion and so intact cuticles would tend to be more conductive when turgor is high (e.g. at night) than when turgor is low (e.g. during the day). In fact, anything that alters stomatal opening and, in turn, leaf turgor would change cuticle stretching and thus affect molecular motion and gas conductivity.

Importantly, cuticle tightening at low turgor inhibited gas conductances for both water vapour and CO$_2$, suggesting that the stretching mechanism is indeed physical and would alter the diffusion of other gases as well. The effect was especially apparent in grape where transport was dominated by the wax barrier and not stomata.

Implications for calculated $c_i$

These findings have large implications for leaf gas exchange because the calculations of $c_i$ assume a gaseous diffusion path. The general equation is (Boyer and Kawamitsu, 2011):

$$c_i = c_a - 1.58 \frac{A_s + E_{cs}}{E_s \left(1 + 0.29w_i - 1.29w_a + 0.58c_a \right)} \left(w_i - w_a\right) \quad (5)$$

where the subscript ‘s’ emphasizes the gas path through the stomata, and $E_{cs}, 0.29E_{wi}, 1.29E_{wa},$ and $0.58E_{cs}$ are interactions between CO$_2$ and water vapour. Boyer and Kawamitsu (2011) pointed out that this equation reduces to that of von Caemmerer and Farquhar (1981) but is expressed in terms of primary measurements rather than conductances. All of the primary measurements are included in the data shown in Figs 2–5. Because these data indicate that the cuticle contributes more to $E_s$ in the denominator than to $A_s + E_{cs}$ in the numerator, Eq. 5 tends to overestimate $c_i$. In other words, the cuticle contribution to transpiration, but scarcely to assimilation, causes the term to the right of the negative sign to be too small. The effects are especially apparent when stomata begin to close and gas exchange is increasingly dominated by the cuticle.

This is illustrated in Fig. 9 which compares calculated $c_i$ from Eq. 5 with directly measured $c_i$ as stomata close with ABA. Before ABA is fed, stomata are open and calculated $c_i$ are slightly higher than the measured ones. This was also reported by Sharkey et al. (1982) and can be seen by close
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inspection of Fig. 8 for sunflower. When ABA started to close the stomata in Fig. 9, however, calculated \( c_i \) increased while measured \( c_i \) decreased. The effect of the cuticle on calculated \( c_i \) thus became extreme as stomata began to close.

Although, in principle, it is possible to correct for the cuticle by subtracting the cuticle component from the total transpiration of the leaf, the differences between CO\(_2\) and water transport, as well as the turgor effect on cuticle conductance, prevents this from being easily done. Until these effects can be easily corrected, directly measured \( c_i \) appear to be more accurate than calculated \( c_i \).

Acknowledgements

I thank Dr An-ching Tang for technical assistance and Dr Shinji Shimada for some preliminary experiments.

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