Photosynthesis Gas Exchange System with Internal CO₂ Directly Measured

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In this study, a leaf gas exchange system was constructed and tested over an extended range of external CO_2 concentrations (c_a) while the concentration inside the leaves (c_i) was directly and continuously determined. For c_a, an infrared analyzer was used to compare c_a with a reference gas at concentrations as high as 50,000 μmol・mol^{-1}. For c_i, a glass cup sealed to the abaxial leaf surface equilibrated with internal CO_2, and the gas was circulated to another infrared analyzer. When stomata were open in the light in a sunflower (*Helanthus annuus* L.) leaf, c_i was about 275 μmol・mol^{-1} in c_a of 400 μmol・mol^{-1}. If stomata closed in the dark, c_i increased until it exceeded c_a but in the light the reverse occurred and c_i decreased to only 60 μmol・mol^{-1}. When c_a increased in light, stomata began to close but c_i could be increased to 50,000 μmol・mol^{-1}, overcoming the closure. But inward CO_2 diffusion was further inhibited by water vapor diffusing outward. The inhibition agreed with theoretical calculations of von Caemmerer and Farquhar (1981). The system expanded the CO_2 concentrations at which CO_2 fixation could be measured while avoiding complications from calculating c_i when stomata close.

Keywords : diffusion interactions, gas diffusion, *Helianthus annuus* L., high CO_2 concentration, leaf CO_2 gradient, stomatal conductance

INTRODUCTION

In leaves, the CO_2 in the intercellular spaces is in close proximity to the mesophyll cells where it dissolves in water and diffuses to the fixation sites for the photosynthetic process. This gas phase or internal CO_2 (c_i, see definitions in Table 1) is at a critical position in the diffusion path between the leaf exterior and fixation sites. Outside, the CO_2 concentration is higher because the CO_2 must diffuse down a gaseous concentration gradient through the leaf boundary layer and stomatal pores to the air spaces inside. From there, the CO_2 must dissolve in the cell water and diffuse down another concentration gradient to the interior of the chloroplasts, often mediated by various forms of carbonic anhydrase and aquaporins (Tanz et al., 2009).

CO_2 concentrations outside of leaves are currently near 400 μmol mol^{-1}, and for technical reasons most methods of measuring CO_2 fixation operate near this level, extending to as high as 2,000 μmol mol^{-1}. The c_i is calculated from the diffusion outward of water vapor in air while CO_2 diffuses inward. Many photosynthetic properties depend on this calculation (Long and Bernacchi,

Table 1 Definition of symbols used in equations.

Symbol	Definition
Fluxes $(mol \cdot m^{-2} \cdot s^{-1}$, based on projected leaf area)	
A_S	Leaf CO_2 flux through stomata
E_S	Leaf transpiration flux through stomata
Concentrations (mole fraction, $mol \cdot mol^{-1}$)	
$\bar{a} = 0.5(a_i + a_a)$	average mole fraction of gases other than water vapor and CO_2
$\bar{c} = 0.5(c_i + c_a)$	average mole fraction of CO_2
$\bar{w} = 0.5(w_i + w_a)$	average mole fraction of water vapor
c_a	CO_2 in bulk air outside of boundary layer around leaf
c_i	CO_2 in intercellular spaces inside the leaf
w_a	water vapor in bulk air outside of boundary layer around leaf
w_i	water vapor in intercellular spaces inside the leaf
Conductances $(mol \cdot m^{-2} \cdot s^{-1}$, based on projected leaf area)	
g_{ca}	diffusive conductance for CO_2 in air
g_{cw}	diffusive conductance for CO_2 in water vapor
g_{wa}	diffusive conductance for water vapor in air

2003), notably the conductance through the stomata (Farquhar and Sharkey, 1982; Parkhurst, 1994; Evans and von Caemmerer, 1996; Jones, 1998; Evans and Loreto, 2000; Massacci and Loreto, 2001), the internal conductance to the fixation sites (Warren, 2006; 2008; Pons et al., 2009; Flexas et al., 2008; Terashima et al., 2011) and the biochemical models of photosynthesis (von Caemmerer, 2000; Sharkey et al., 2007).

In leaves with open stomata, the calculation appears reasonably accurate (Sharkey et al., 1982) but as stomata close, the cuticle increasingly controls diffusion and the calculation becomes less certain (Boyer et al., 1997; Meyer and Genty, 1998). Graan and Boyer (1990) sought to avoid this problem with external CO_2 concentrations as high as $50,000 \, \mu mol \cdot mol^{-1}$ that would overcome stomatal closure and force CO_2 into the leaf. But c_i was not measured and it was unclear how much CO_2 actually entered. Lauer and Boyer (1992) adapted the method of Sharkey et al. (1982) to measure c_i continuously but did not determine CO_2 fixation.

It would be worthwhile to combine these measurements in order to allow photosynthesis to be determined despite diffusion barriers (closed stomata, cuticle, etc). Although such a method might retain the capabilities of prior methods, it does not appear to have been reported. Therefore, the objective of the present work was to construct and test an instrument with this extended capability allowing it to operate at CO_2 concentrations as high as $50,000 \, \mu mol \cdot mol^{-1}$ while c_i was directly measured. The resulting system readily detected c_i and CO_2 fixation while stomata closed. It also measured the inhibition of CO_2 diffusion by water vapor diffusing out of the leaf.

MATERIALS AND METHODS

Plant material

In order to test the system, sunflower plants (*Helianthus annuus* L. hybrid IS897 from Interstate Seed Company, Box 338, Fargo, ND, USA) were grown individually from seed in 1 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 $Ca(NO_3)_2 \cdot 4H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 2 mM KH_2PO_4, 0.5 μM $CuSO_4 \cdot 5H_2O$, 10 μM $MnSO_4 \cdot H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 25 μM H_3BO_3, 0.5 μM H_2MoO_4 and 50 μM Fe-citrate. Whenever the soil surface was dry, this solution was resupplied until drainage occurred. The plants were grown in a Plexiglass enclosure in a controlled environment chamber with day/night temperatures and relative humidities of $25/20 \pm 1°C$ and $60/95 \pm 5\%$, respectively. Fluorescent lamps (cool white) provided a 14 h photoperiod with an irradiance of 850

to 1,000 μmol photons•m^{-2}•s^{-1} of PAR throughout the day at the top of the plants. The plants were used when they were four weeks old.

Gas exchange instrument

The petiole of an attached, recently fully expanded leaf was sealed into the wall of an assimilation chamber with the lamina inside (Fig. 1). Typically, projected leaf areas were 250 to 350 cm², and the air was stirred with a muffin fan (Comair-Rotron Model ST24P3, Saugerties, NY, USA) to give a boundary layer conductance to water vapor of about 650 mmol•m^{-2}•s^{-1}, which was always larger than the leaf conductance. The chamber walls were aluminum with glass plates on the top and bottom. Light was supplied with a quartz iodide bulb (1,000W, 3,200 K filament) filtered with an infrared mirror and 1 cm of water, which removed virtually all infrared radiation between 750 nm and 1,600 nm (Sheoran and Boyer, 1989). All measurements were made at an irradiance of 2,000 μmol photons•m^{-2}•s^{-1} of PAR, air temperature of 25±0.1°C, and air dewpoint
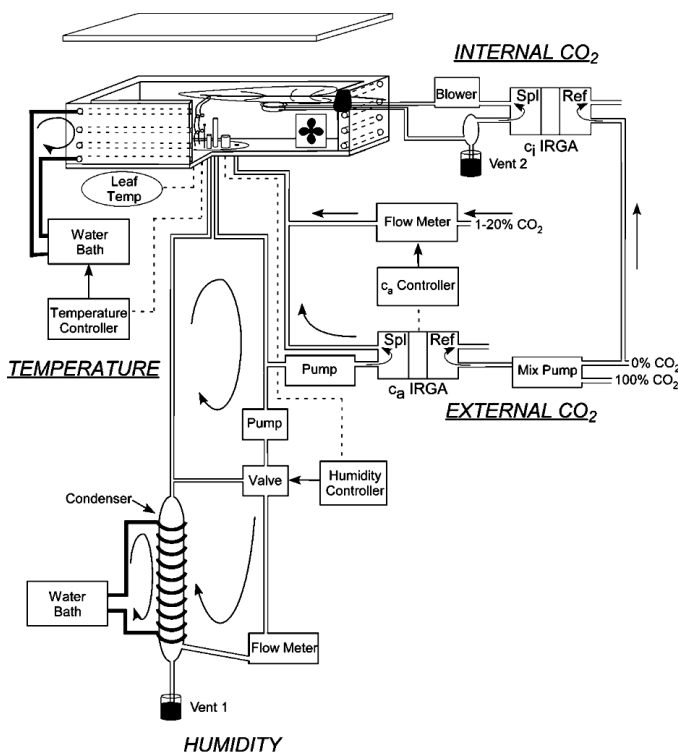

Fig. 1   Gas exchange system with internal CO₂ (c_i) directly measured. Leaf petiole is sealed into an assimilation chamber while the rest of the plant remains outside (plant not shown). Temperature, humidity, and external CO₂ are controlled around the leaf by proportional controllers acting on a water bath, valve, or flow meter, respectively. As a result, these conditions are steady while the rate of CO₂ exchange is determined from the rate of CO₂ injection (1−20% CO₂), and transpiration from the rate of condensation (Condenser). The c_aIRGA is used as a CO₂ comparator and allows c_a control to concentrations as high as 50,000 μmol•mol^{-1}. The c_i is directly measured in a glass cup sealed to one side of the leaf in the assimilation chamber. The air in the cup equilibrates with CO₂ inside the leaf and is circulated by a small blower to a c_iIRGA and back to the cup. The circuit is shown in the absolute mode in which c_i is measured relative to a reference gas containing no CO₂ (c_i−0). The circuit also can be arranged in a differential mode to measure c_i relative to c_a (c_i−c_a) by feeding gas from the sample cell (Spl) in the c_aIRGA to the reference cell (Ref) in the c_iIRGA. Mix pump mixes CO₂-free air with pure CO₂ to provide any concentration of CO₂ continuously to IRGA reference cells as well as to calibrate 1−20% CO₂ mixtures.

of $13.5\pm0.1°C$. Leaf temperature was measured with a fine thermocouple (0.15 mm junction, TFCC-003 & TFCP-003, Omega Engineering, Stamford, CT, USA) clamped to the underside of the leaf (Fig. 1, Leaf Temp). Chamber environmental conditions were held constant with proportional controllers that injected or removed components of the air around the leaf to keep the conditions constant (air temperature$\pm0.1°C$, relative humidity$\pm0.1\%$, CO_2 concentration in air (c_a) $\pm$ $1\,\mu$mol·mol^{-1}). Heat was removed with a water bath (Fig. 1, Temperature), CO_2 consumed by photosynthesis was replaced with air containing concentrated CO_2 (Fig. 1, External CO_2), and water vapor was removed with a condenser (Fig. 1, Humidity).

The system had the following features critical to its operation. The first was the measurement of assimilation by using the c_a control system as a comparator to keep the c_a constant in the chamber. This was achieved by measuring the CO_2 concentration in the assimilation chamber with an infrared gas analyzer (Fig. 1, sample cell Spl in c_aIRGA, LI6251, Li-Cor, Lincoln, NE, USA) signaling a proportioning controller (Fig. 1, c_a Controller, Electromax V, Leeds & Northrup, North Wales, PA, USA) that controlled a mass flow meter (FC202, Tylan Corporation, Torrance, CA, USA) moving CO_2 into the chamber at a high concentration from a cylinder of pre-calibrated compressed gas (1$-$20% CO_2). Any desired c_a to 50,000 μmol·mol^{-1} was obtained by feeding CO_2 and air to a mixing pump (1SA27/3a and 1M3--/a-F, Wösthoff, Bochum, Germany) and then to the reference cell (Fig. 1, Ref in c_aIRGA). The control system acted to bring the concentration in the sample cell to that in the reference cell. The analyzer was unaffected by interference from water vapor but responded to CO_2 absorption band broadening and to dilution by water vapor. With the same or similar concentration in the reference and sample cells, the broadening effects cancelled. By using the IRGA as a comparator with controller, the dilution effect was automatically corrected by the controller.

The assimilation rate was determined from the rate of CO_2 injection multiplied by the concentration difference between the injected CO_2 and the CO_2 exhausting from the assimilation chamber through a bubble seal (Fig. 1, Vent 1). Because this measure of assimilation rate was independent of the c_aIRGA, which acted only to maintain c_a constant, rates could be measured as easily at 50,000 μmol·mol^{-1} CO_2 as at lower CO_2 concentrations. Typically, assimilation rates had a certainty in the third significant place.

The second critical feature was the measurement of transpiration by using the humidity controller to keep the humidity constant in the chamber, thus eliminating interactions with the CO_2 control system. This was achieved by measuring the humidity with a dewpoint sensor (DEW10, General Eastern, Watertown, MA, USA) signaling a proportional controller (Fig. 1, Humidity Controller, Electromax V, Leeds & Northrup, North Wales, PA, USA) operating a valve (252A, MKS Instruments, Burlington, MA, USA) that allowed air to move from the assimilation chamber through a mass flow meter (0259B-50000SV, MKS Instruments, Burlington, MA, USA), then a condenser (Fig. 1, Condenser), and back to the assimilation chamber. The rate of transpiration equaled the rate of condensation determined from the condenser flow and the dewpoint difference between the air entering and departing from the condenser. The condenser drained through a tube whose end was under water, creating a bubble seal (Fig. 1, Vent 1) for the assimilation chamber that maintained the chamber at the surrounding atmospheric pressure while allowing excess gas to escape under the water.

Air temperature was held constant in the assimilation chamber with a platinum resistance thermometer (RTD PR-11-2-100-1/4-9-E, Omega Engineering, Stamford, CT, USA) signaling a proportioning controller (Fig. 1, Temperature Controller, Electromax V, Leeds & Northrup, North Wales, PA, USA) adjusting the heat supplied to a refrigerated water bath (RTE-4, Neslab, Newington, NH, USA) that circulated water through the walls of the chamber. The walls were ribbed to increase the surface area in contact with air in the chamber. The constant air temperature helped to hold the other environmental conditions steady.

Internal CO$_2$

The c_i was measured with a glass cup sealed with lanolin to one surface of the leaf (usually abaxial, as shown in Fig. 2) in the assimilation chamber (Fig. 1). The lanolin provided a seal that was flexible and remained leak-free when the leaf shrank or swelled with changes in water content. The ability to shrink or swell is important because stomatal opening is affected (Tang and Boyer, 2007). In the cup, the CO$_2$ equilibrated with that in the stomatal pores adjacent to the cup (Sharkey et al., 1982; Lauer and Boyer, 1992). The air in the cup was gently circulated by a small blower (SJ-40A12BT, Seiko Electronic Components Ltd., Japan) in a sealed chamber in the circuit that extended through the petiole seal to the sample cell (Fig. 1, Internal CO$_2$, Spl) of the c_iIRGA outside and back to the cup inside. The air moved continuously without pulses. The cup was located at a position where the pressure was atmospheric in the circuit to prevent air from flowing though the stomata of the amphistomatous leaf (Fig. 3). Atmospheric pressure was ensured with a bubble seal at the base of a condenser (Fig. 1, Vent 2). For some experiments, the c_iIRGA (LI6251, Li-Cor, Lincoln, NE, USA) operated as an absolute detector of CO$_2$, calibrated with gas mixtures from the mixing pump, and compared to CO$_2$-free air as shown in Fig. 1. In this configuration, the c_iIRGA indicated ($c_i - 0$). For other experiments, air from the assimilation chamber was circulated from the c_aIRGA to the reference cell in the c_iIRGA, and back to the assimilation chamber. In this configuration, the c_iIRGA indicated the difference ($c_i - c_a$). The c_iIRGA was calibrated to eliminate effects of band broadening, and dilution by water vapor was never more than 1%, which was ignored.

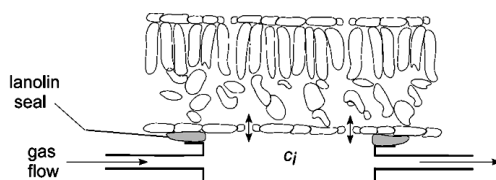In some experiments, the cup sizes were small and covered only a small part of the underside

Fig. 2 Glass cup sealed to abaxial surface of amphistomatous leaf with lanolin.
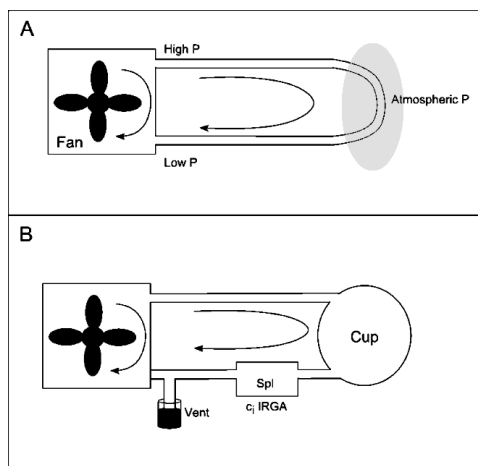

Fig. 3 Cup location in c_i circuit to ensure pressure in cup was identical to that in the assimilation chamber. A) Pressures in circuit. Blower generated pressure differential of about 2 mm water. Shaded part indicates region where pressure was identical to that in the assimilation chamber. B) Cup located in the shaded region. Vent measures pressure in circuit relative to atmosphere.

of the leaf (4 cm², Fig. 2). In other experiments, they were large and covered the entire underside of the leaf. The small cup was glass and round with a flange for the lanolin. The large cup was Plexiglas in the shape of the leaf with a lanolin edge. The c_i were measured continuously with these systems. By keeping the c_iIRGA circuit small, the CO_2 in the small cup equilibrated with that in the intercellular spaces of the leaf within 8 min unless the diffusive conductance of the leaf became less than 25 mmol•m^{-2}•s^{-1}, which lengthened the equilibration time. In the large cup, equilibration times were typically less than 1 min.

Stomatal closure

For some experiments, the stomata were closed with abscisic acid (ABA). The petiole seal was modified to include a 50 ml cup on the outside of the assimilation chamber filled with degassed water through which the petiole extended. The petiole was excised under the water, the remaining plant was removed, and gas exchange was measured in the excised leaf. After 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 ($\pm$)-cis,trans-abscisic acid was purchased from Sigma (St. Louis, MO, USA). The stock solution was made by bringing the pH to 10-11 with KOH to dissolve the ABA, then neutralizing to pH 7 with HCl.

For other experiments, the stomata were closed by darkening the leaf. Upon darkening, the c_a was maintained by absorbing CO_2 from the assimilation chamber. The CO_2 controller was set to an on-off configuration, the air in the chamber was diverted from the CO_2 injection circuit (Fig. 1) to a column of silica gel and a column of Ascarite (not shown) and the CO_2-free air was returned to the chamber. The controller diverted the air from the injection circuit whenever CO_2 in the chamber rose. As the c_a fell in response, the diversion circuit was closed by the controller and the cycle was repeated. This control action caused c_a to rise and fall slightly (about 5 μmol•mol^{-1}). The respiration rate was determined from the rate of rise of c_a in each cycle.

Statistics

The data were scanned every 6 s and stored every 60 s in a computer. All calculations were done with a spreadsheet. Each experiment was repeated at least 3 times and typical individual experiments are shown.

RESULTS

c_i in small and large cups

The c_i measuring system gave continuous results while other features of gas exchange were monitored. The lanolin was an effective seal that caused no visible harm during the measurements. No leaks occurred but were readily detected if they were artificially induced because c_i changed abruptly to approach c_a.

The cup enclosed one leaf surface and allowed gas exchange only through the other surface after equilibrium. The effect of this restriction was determined by first measuring gas exchange in a leaf attached to a small cup, then rapidly transferring the same leaf to a large cup (Fig. 4). With the small cup, most of the gas exchange was through both surfaces of the free leaf because only 1.5% to 2% of the projected surface was covered by the cup. After transfer to the large cup, the entire abaxial surface of the leaf was enclosed, and the leaf exchanged gases only through the adaxial surface. Measuring the gas exchange by the latter surface revealed the effect of cup attachment.

With the small cup, c_i became stable at about 230 μmol•mol^{-1} when c_a was 400 μmol•mol^{-1}, and assimilation (A) was 34 μmol•m^{-2}•s^{-1} (Fig. 4). The large cup was prepared prior to the transfer, and the transfer was accomplished in 3–5 min by opening the assimilation chamber briefly. Transferring the leaf from the small cup to the large one caused A to decrease. However, A gradually resumed and after about 1 h returned close to the original rate. The c_i returned toward its
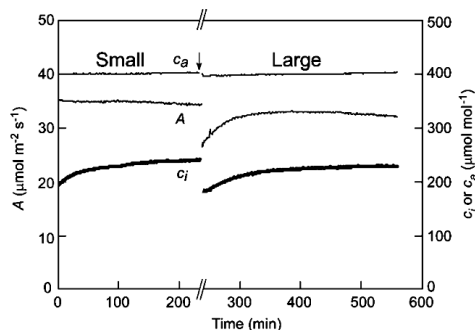
Fig. 4 The assimilation (A), internal CO_2 (c_i) and external CO_2 (c_a) when a small cup covered 1.5–2% of a sunflower leaf surface and the remainder of the leaf surface exchanged gases freely (Small), compared to the same measurements in the same leaf when the entire abaxial surface was covered by the cup (Large). Leaf was moved from small to large cup at the arrow. Representative experiment from four replications.

original concentration ($220\,\mu\text{mol·mol}^{-1}$) at about the same time. Evidently, the leaf above the cup adjusted to cup attachment, and gas exchange gradually returned to that expected for a free leaf (i.e., with the small cup attached and most of the leaf free). Because c_i was essentially the same in the small and large cups, the small cup was used in all the subsequent experiments.

Transpiration also occurred in the cups but slowly because the humidity was saturated. The vapor condensed in the coolest part of the circuit outside of the assimilation chamber where temperature was not controlled. The rate of condensation was about 0.07 ml per 24 h in the small cup and 3 ml per 24 h in the large cup, which was considered negligible and removed once each day.

Stomatal closure in dark

Using the small cup that allowed most of the leaf to exchange gases freely, the c_a was held steady at $400\,\mu\text{mol·mol}^{-1}$ and A became steady at about $23\,\mu\text{mol·m}^{-2}\text{·s}^{-1}$ while c_i gradually increased to $275\,\mu\text{mol·mol}^{-1}$ (Fig. 5A and 5B). The CO_2 conductance for the leaf (g_c) was calculated from these data according to:

$$g_c = \frac{A}{c_a - c_i} \tag{1}$$

and was $125\,\text{mmol·m}^{-2}\text{·s}^{-1}$ soon after the day began, and rose to $190\,\text{mmol·m}^{-2}\text{·s}^{-1}$ after 5 h (Fig. 5C), suggesting the stomata were opening gradually. When the plant was darkened, the g_c rapidly decreased to 1 to $2\,\text{mmol·m}^{-2}\text{·s}^{-1}$, indicating the stomata had closed. A became -1 to $-2\,\mu\text{mol·}$ $\text{m}^{-2}\text{·s}^{-1}$ because CO_2 was released during respiration, and c_i rose above $400\,\mu\text{mol·mol}^{-1}$, gradually increasing to $800\,\mu\text{mol·mol}^{-1}$ by the middle of the night. Upon re-illumination on the second day, each of these properties returned to the levels of the previous day but began to decline as water was depleted in the soil. The experiment was terminated at 25 h and the plant was re-watered.

Stomatal closure in light

By contrast with stomatal closure in darkness, closing stomata in the light caused c_i to decrease (Fig. 6). The leaf had been excised with its petiole in de-gassed water where it displayed g_c of about $120\,\text{mmol·m}^{-2}\text{·s}^{-1}$. When ABA ($50\,\mu\text{M}$) was added to the water around the petiole (Fig. 6, arrow), g_c became only about $15\,\text{mmol·m}^{-2}\text{·s}^{-1}$ and indicated the stomata had closed (Fig. 6C). The A was about $27\,\mu\text{mol·m}^{-2}\text{·s}^{-1}$ before the addition and decreased to $5\,\mu\text{mol·m}^{-2}\text{·s}^{-1}$ afterward (Fig. 6A). The c_i was about $175\,\mu\text{mol·mol}^{-1}$ before the addition but declined to $60\,\mu\text{mol·}$ mol^{-1} afterward as CO_2 was depleted inside the leaf (Fig. 6B). The depletion brought c_i close to the compensation point, which was $41\,\mu\text{mol·mol}^{-1}$ at this temperature and oxygen concentration (shown in Fig. 7A, inset).
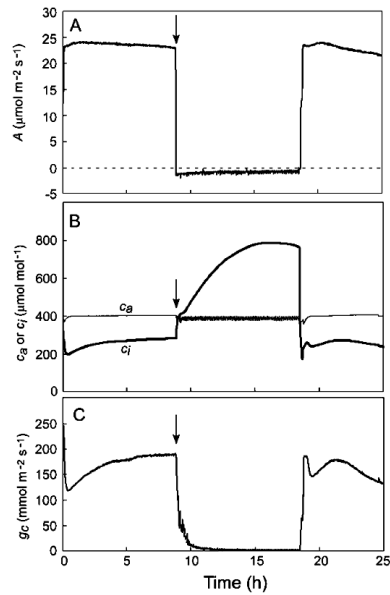
Fig. 5 Diurnal gas exchange of a sunflower leaf. A) assimilation (A), B) internal CO_2 (c_i) and external CO_2 (c_a), and C) leaf conductance to CO_2 (g_c). Leaf was darkened at the arrow and re-illuminated 10 h later. Representative experiment from five replications.
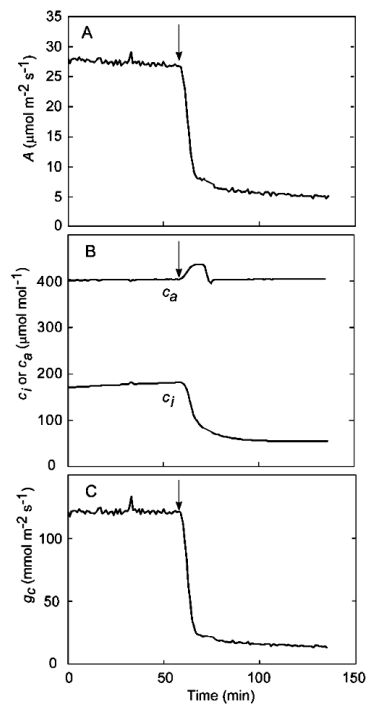

Fig. 6 Gas exchange in excised sunflower leaf fed ABA at the arrow to close stomata in the light. A) assimilation (A), B) internal CO_2 (c_i) and external CO_2 (c_a), and C) leaf conductance to CO_2 (g_c). Representative experiment from nine replications.

Assimilation at various c_i

In an attached leaf with open stomata, an A-c_i response curve revealed that A was saturated at c_i of about 800 μmol•mol^{-1} in sunflower (Fig. 7A). At saturation, A was 37 μmol•m^{-2}•s^{-1} where it remained while c_i rose nearly to 50,000 μmol•mol^{-1}. The transpiration rate (E) and leaf conductance to water vapor g_w decreased as A approached saturation, indicating that stomata closed as CO$_2$ increased (Fig. 7C). However, these properties did not change afterward, between c_i of 1,000 and 50,000 μmol•mol^{-1}, indicating no further stomatal closure. Despite the lack of further closing, the difference between c_i and c_a continued to become larger as c_i rose (Fig. 7B). For example, at c_i of 1,000 μmol•mol^{-1} the difference was 350 μmol•mol^{-1}, but at 50,000 μmol•mol^{-1} the difference had increased to 1,500 μmol•mol^{-1} (Fig. 7B).

Since this effect could not be explained by stomatal closure, it appeared that CO$_2$ diffusion inward was being hindered by water vapor diffusing outward, and the hindrance was greater as c_i increased (Jarman, 1974; von Caemmerer and Farquhar, 1981). If so, the von Caemmerer and Farquhar (1981) equations should predict the hindrance effect. Figure 8 compares the observed c_i with the one predicted by von Caemmerer and Farquhar (1981), shown as Eq. A17 in the Appendix:

$$c_i = c_a - 1.58 \frac{A_s + E_s c_a}{E_s (1 + 0.29 w_i - 1.29 w_a)} (w_i - w_a) \qquad \text{(A17)}$$
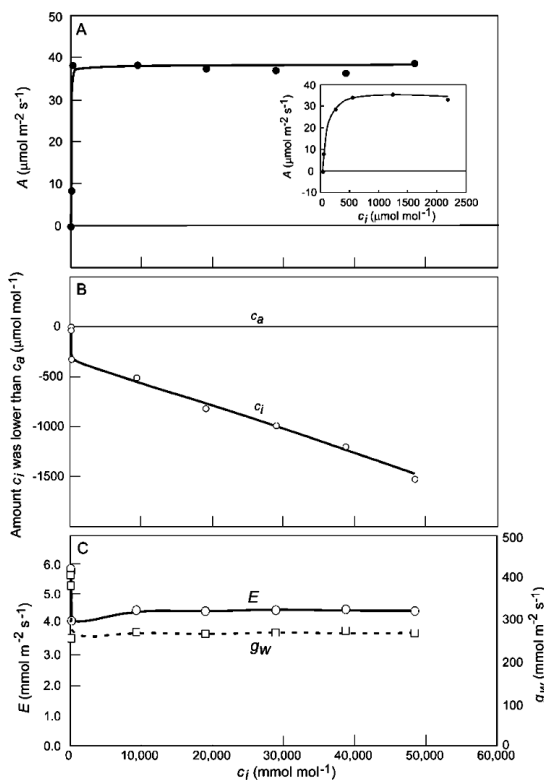

Fig. 7 (A) Assimilation (A) in sunflower leaf over a wide range of internal CO$_2$ concentrations (c_i). Inset in (A) shows details of assimilation at low c_i. (B) amount by which c_i was lower than the external CO$_2$ concentration (c_a) in the experiment of (A). (C) Stomatal behavior for the experiment of (A), indicated as transpiration rate (E) and leaf conductance to water vapor (g_w, calculated according to Appendix Eq. A11). Representative experiment from four replications.
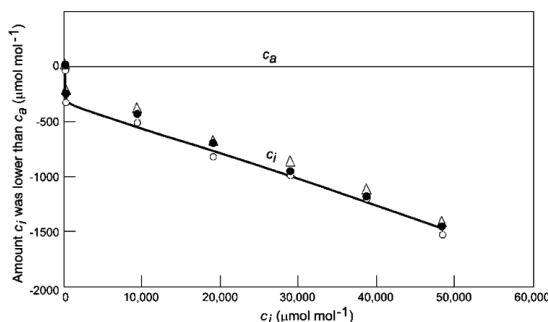
Fig. 8 Comparison of directly measured c_i with calculated c_i for the experiment in Fig. 7. Directly measured c_i are open circles ($\bigcirc$) from Fig. 7B. Calculated c_i are according to von Caemmerer and Farquhar (1981, see Appendix Eq. A17)(closed circles, $\bullet$), or according to the Appendix Eq. A16 (open triangles, $\triangle$). Representative experiment from four replications.

There is a close correspondence between the theoretically calculated c_i and the c_i directly measured. The von Caemmerer and Farquhar (1981) equation was derived only for trace concentrations of CO_2, but as shown in the Appendix Eq. A16 and in Fig. 8, it also applies without significant error at the high CO_2 concentrations used in this work.

<div align="center">DISCUSSION</div>

Most gas exchange systems use infrared gas analyzers to determine photosynthesis rates from the CO_2 difference between incoming and outgoing air flowing across the leaf or the change in CO_2 concentration in a sealed system. As the CO_2 concentration climbs and the sensitivity of the analyzer falls, the signal to noise ratio is degraded until it becomes unusable at concentrations above about 2,000 $\mu mol \cdot mol^{-1}$. In the present work, the analyzer was not used to measure photosynthesis rate. It compared the CO_2 concentration in air around the leaf with that in a reference gas. Relative to other systems, the signal to noise ratio could decline much farther without preventing the analyzer from making its comparison. The rate of photosynthesis was determined from the rate that CO_2 was injected to keep the concentration around the leaf the same as in the reference gas. Consequently, the rate could be measured at much higher CO_2 concentrations than with most other systems.

For c_i measurements at these elevated CO_2 concentrations, the outwardly diffusing water vapor inhibited the inward diffusion of CO_2 and caused the concentration in the cup to be much lower than it otherwise would be (as much as 1,500 $\mu mol \cdot mol^{-1}$ lower). This created a large signal despite the high background concentration and loss of sensitivity inherent in the analyzer.

With the small cup attached to the leaf, the gas exchange system detected c_i whether stomata were open or closed in light or dark over a wide range of CO_2 concentrations. The c_i was below the atmospheric concentration in light but rapidly rose above the atmospheric concentration in dark as expected when stomata close and photosynthesis ceases. When stomata closed in light (ABA), the reverse occurred because c_i was drawn down by the sustained photosynthesis demand for CO_2, requiring a steeper gradient for diffusion through the closing stomata. The instrument also detected stomatal closure caused by increasing c_a, and could measure c_i while the closure occurred.

The extended range of CO_2 concentrations allowed the interaction between CO_2 and water vapor to be plainly seen, as predicted by theory (see Appendix). The outward moving water vapor hindered CO_2 entry much more than the reverse because the diffusive flux of water vapor was much greater than that for CO_2 (see Fig. 7 and Appendix). But the net result was that the instrument could distinguish the hindrance to CO_2 entry caused by stomatal closure on the one hand and water
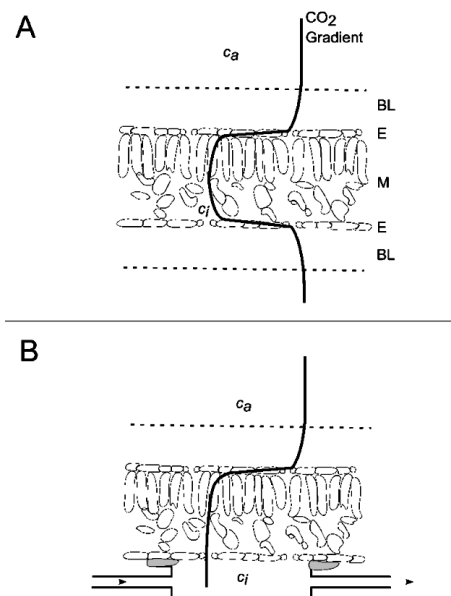
Fig. 9   Likely CO_2 gradients through an amphistomatous leaf in light but without a c_i cup attached (A) or with a c_i cup attached to the abaxial surface (B). Higher concentration is to the right. BL, boundary layer; E, epidermis; M, mesophyll.

vapor on the other.

Characteristics of c_i

The measurement of c_i was tested in a sunflower leaf, which is amphistomatous. At equilibrium, the cup sealed one surface so that gas was exchanged only through the other leaf surface. Inevitably, this altered the CO_2 conditions in the leaf. However, with the small cup, only about 2% of the leaf was enclosed by the attachment, so gas exchange occurred freely for most of the leaf, with gradients proposed in Fig. 9A. In this representation, the epidermis is the main barrier to gas diffusion, and CO_2 moves first through the boundary layers lining both surfaces (BL), then down a steep concentration gradient across the epidermis (E) caused by the impervious cuticle perforated by stomatal pores having limited gaseous cross-section. Once inside, the gradient is not as steep through the mesophyll as through the epidermis, as shown by Mott and O'Leary (1984), Parkhurst et al. (1988) and Parkhurst and Mott (1990). The diffusion paths meet near the middle of the leaf where the gradient ends.

By contrast, the part of the leaf attached to the cup has a diffusion path twice as long as in the free leaf, with a proposed gradient shown in Fig. 9B. Consequently, the longer gradient might cause measured c_i to be lower than that in the free part of the same leaf. If so, 275 μmol•mol^{-1} inside the leaf would be too low in air containing 400 μmol•mol^{-1} (stomata open). Sharkey et al. (1982) and Mott and O'Leary (1984) tested this possibility and concluded that the c_i was similar whether gas exchange was through one or both surfaces, typically within 20 μmol•mol^{-1}. In high c_i, these small differences probably would be undetected. Consequently, the c_i measured with an attached cup may be similar to that in the free leaf as shown by comparing Fig. 9A and 9B.

Within these macroscopic gradients are microscopic ones in the substomatal cavities and between patches of uneven stomatal apertures (Buckley et al., 1997; West et al., 2005). Based on the sizes of the substomatal cavities and the images of uneven stomatal behavior (Buckley et al., 1997; West et al., 2005), the microscopic gradients appear too small to affect the macroscopic ones

determined with the small cup, which probably averages the microscopic variation. This conclusion is supported by similar readings in the small and large cups on the same leaf despite vastly different areas for the measurements.

   Impact of stomatal closure

   A frequent problem with calculations of c_i is the increasing influence of the cuticle as stomata close. The cuticle does not transmit CO_2 and water vapor in the gas phase (Jarman, 1974; Terashima et al., 1988; Mansfield et al., 1990; Terashima, 1992; Boyer et al., 1997; Meyer and Genty, 1998). Instead these molecules diffuse through wax that creates most of the resistance to water loss and forms the outer cuticle layer (Schreiber and Schönherr, 2009). Although pores of small diameter exist in the cutin beneath the waxes, there is little evidence for pores in the wax itself (Schreiber and Schönherr, 2009). Water moves outward through the cuticle while CO_2 moves in the opposite direction and further through the water of the epidermal cells. It appears that the conductance for water vapor, while diminished by the wax, may be higher than for CO_2 (Boyer et al., 1997). If so, relying on water vapor for calculating c_i may over-estimate CO_2 mobility and cause c_i to be too high when stomata close. Direct measurements of c_i are not affected because the measurement does not rely on water vapor, the cup equilibrates regardless of the diffusion path, and the fluxes of water vapor and CO_2 are too low in the cup to influence c_i significantly.

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Appendix

CO₂ diffusion in leaves when water vapor is present

The diffusion of CO₂ into a leaf is hindered by the diffusion of water vapor outward. Therefore, for the same rate of CO₂ diffusion, the concentration difference between inside and outside must be larger than in a dry air system. The interaction between diffusing gas molecules was treated generally by Chapman and Cowling (1970) and elaborated for leaves by Jarman (1974) and von Caemmerer and Farquhar (1981). The theory uses binary gas mixtures to predict the diffusion in ternary mixtures that in the leaf consists of air, water vapor, and CO₂. CO₂ is usually present at trace concentrations in air, but the equipment described in the present work can operate at much higher CO₂ concentrations. In the following, the effect of the higher concentration is explored.

The fundamental equation is B10 of von Caemmerer and Farquhar (1981):

$$c_a - c_i = \left(\frac{\bar{a}}{g_{ca}} + \frac{\bar{w}}{g_{cw}} \right) A_s + \frac{\bar{c}E_s}{g_{cw}} \tag{A1}$$

where the symbols are defined in Table 1, and subscripts ca and cw indicate diffusive conductances of CO_2 in air or water vapor, respectively. The total conductance g (bulk air to leaf interior) is defined as the product of the total gas concentration C and the diffusivity D of each gas in an unstirred binary mixture according to the "effective" pathlength ℓ for diffusion:

$$g = \frac{CD}{\ell} \tag{A2}$$

Jarman (1974) indicates that D for CO_2 in water vapor is the same as for CO_2 in air, and therefore g_{ca} may be considered equal to g_{cw}. When substituted in Eq. A1, this gives:

$$c_a - c_i = \frac{(\bar{a} + \bar{w})A_s + \bar{c}E_s}{g_{ca}} \tag{A3}$$

The sum of the average gas mole fractions in a ternary mix is:

$$\bar{a} + \bar{w} + \bar{c} = 1 \tag{A4}$$

and because the CO_2 concentration cannot be ignored in the present context, $\bar{a} + \bar{w} = 1 - \bar{c}$ can be substituted in Eq. A3 to give:

$$c_a - c_i = \frac{A_s - \bar{c}A_s + \bar{c}E_s}{g_{ca}} = \frac{A_s - \bar{c}(A_s - E_s)}{g_{ca}} \tag{A5}$$

Because E_s is more than a hundred-fold larger than A_s, Eq. A5 can be simplified to:

$$c_a - c_i = \frac{A_s + \bar{c}E_s}{g_{ca}} \tag{A6}$$

and

$$A_s = g_{ca}(c_a - c_i) - \bar{c}E_s \tag{A7}$$

This result is identical to that of von Caemmerer and Farquhar (1981) despite considering CO_2 in the ternary mix $\bar{a} + \bar{w} + \bar{c} = 1$ rather than in trace amounts (i.e., as a binary mix $\bar{a} + \bar{w} = 1$). The interaction between CO_2 and water vapor is $\bar{c}E_s$.

Water vapor diffusion in leaves when CO_2 is present

High CO_2 in a ternary mix also inhibits the diffusion of water vapor. From eq. B9 of von Caemmerer and Farquhar (1981):

$$w_i - w_a = \left(\frac{\bar{a}}{g_{wa}} + \frac{\bar{c}}{g_{cw}} \right) E_s + \frac{\bar{w}A_s}{g_{cw}} \tag{A8}$$

where the subscript wa indicates the diffusive conductance of water vapor in air. In contrast to the situation in Eq. A1, the diffusivities for water vapor and CO_2 are different. Massman (1998) gives D of 2.55×10^{-5} $m^2 \cdot s^{-1}$ for water vapor in air with uncertainty of $\pm 7\%$, and 1.62×10^{-5} $m^2 \cdot s^{-1}$ for CO_2 in air with uncertainty of $\pm 5\%$ at 25°C. The ratio of these diffusion coefficients is 1.58, which gives $g_{wa} = 1.58g_{cw} = 1.58g_{ca}$ assuming the diffusivities are the same through the boundary layer and into the leaf. If the diffusivities differ (a possibility cited by von Caemmerer and Farquhar, 1981), the effect would diminish 1.58 slightly, perhaps affecting calculated c_i by as much as 3% in the present work. But for simplification, this possibility is ignored and $g_{wa}/1.58$ is substituted for g_{cw} in Eq. A8 to give:

$$w_i - w_a = \frac{\bar{a}E_s + 1.58\bar{c}E_s + 1.58\bar{w}A_s}{g_{wa}} \tag{A9}$$

Substituting $\bar{a} = 1 - \bar{w} - \bar{c}$ in Eq. A9 gives:

$$w_i - w_a = \frac{E_s - \bar{w}E_s + 0.58\bar{c}E_s + 1.58\bar{w}A_s}{g_{wa}} \tag{A10}$$

Because E_s is more than a hundred-fold larger than A_s, Eq. A10 can be simplified to:

$$w_i - w_a = \frac{E_s(1 - \bar{w} + 0.58\bar{c})}{g_{wa}}$$

and

$$E_s = g_{wa}\frac{(w_i - w_a)}{1 - \bar{w} + 0.58\bar{c}} \tag{A11}$$

Eq. A11 differs from that of von Caemmerer and Farquhar (1981) by the additional interaction $0.58\bar{c}$ that accounts for the effects of high CO₂.

Using water vapor diffusion to estimate internal CO₂

Moss and Rawlins (1962) were the first to use water vapor diffusion to calculate the CO₂ concentration inside leaves, and von Caemmerer and Farquhar (1981) extended the treatment to include interactions between CO₂ and water vapor when CO₂ was present at trace concentrations. However, the impact of high CO₂ concentrations on the treatment can be explored by first substituting $g_{wa}/1.58$ for g_{ca} in Eq. A7 to give:

$$g_{wa}(c_a - c_i) = 1.58(A_s + \bar{c}E_s) \tag{A12}$$

Placing the identity $\bar{c} = 0.5(c_i + c_a) = 0.5(c_i - c_a) + c_a$ into Eq. A12 and rearranging the left hand term to $(c_i - c_a)$:

$$g_{wa}(c_i - c_a) = -1.58(A_s + 0.5E_s(c_i - c_a) + E_s c_a) \tag{A13}$$

Replacing g_{wa} with the relation in Eq. A11 gives:

$$E_s\frac{(1 - \bar{w} + 0.58\bar{c})}{w_i - w_a}(c_i - c_a) = -1.58(A_s + 0.5E_s(c_i - c_a) + E_s c_a) \tag{A14}$$

At high CO₂ concentrations, the average CO₂ concentration for the inside and outside of the leaf is within 3% of c_a, so $(1 - \bar{w} + 0.58\bar{c})$ can be approximated by $(1 - \bar{w} + 0.58c_a)$. Placing this relation in Eq. A14 gives:

$$\frac{E_s(1 - 0.5w_i - 0.5w_a + 0.58c_a)}{(w_i - w_a)}(c_i - c_a) = -1.58(A_s + 0.5E_s(c_i - c_a) + E_s c_a) \tag{A15}$$

Solving for c_i gives:

$$c_i = c_a - 1.58\frac{A_s + E_s c_a}{E_s(1 + 0.29w_i - 1.29w_a + 0.58c_a)}(w_i - w_a) \tag{A16}$$

The equivalent expression from von Caemmerer and Farquhar (1981) for CO₂ as a trace gas would be:

$$c_i = c_a - 1.58\frac{A_s + E_s c_a}{E_s(1 + 0.29w_i - 1.29w_a)}(w_i - w_a) \tag{A17}$$

which differs from Eq. A16 by the lack of the term $0.58E_s c_a$ to account for high CO₂ interacting with water vapor diffusion. The magnitude of this interaction can be seen by setting realistic conditions for $E_s = 100$ A_s, $w_i = 0.03$, and $w_a = 0.015$. If c_a is 0.05, Eq. A16 gives $c_i = 0.04860$ and Eq. A17 gives $c_i = 0.04856$, a difference small enough to ignore.