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Photosynthetic Oxygen Evolution at Low Water Potential in Leaf Discs Lacking an Epidermis

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Land plants encountering low water potentials (low Ψ_w) close their stomata, restricting CO_2 entry and potentially photosynthesis. To determine the impact of stomatal closure, photosynthetic O_2 evolution was investigated in leaf discs from sunflower (*Helianthus annuus* L.) plants after removing the lower epidermis at low Ψ_w. Wounding was minimal as evidenced by O_2 evolution nearly as rapid as that in intact discs. O_2 evolution was maximal in 1 % CO_2 in the peeled discs and was markedly inhibited when Ψ_w was below –1·1 MPa. CO_2 entered readily at all Ψ_w, as demonstrated by varying the CO_2 concentration. Results were the same whether the epidermis was removed before or after low Ψ_w was imposed. Due to the lack of an epidermis and ready movement of CO_2 through the mesophyll, the loss in O_2 evolving activity was attributed entirely to photosynthetic metabolism. Intact leaf discs showed a similar loss in activity when measured at a CO_2 concentration of 5 %, which supported maximum O_2 evolution at low Ψ_w. In 1 % CO_2, however, O_2 evolution at low Ψ_w was below the maximum, presumably because stomatal closure restricted CO_2 uptake. The inhibition was larger than in peeled discs at Ψ_w between –1 and –1·5 MPa but became the same as in peeled discs at lower Ψ_w. Therefore, as photosynthesis began to be inhibited by metabolism at low Ψ_w, stomatal closure added to the inhibition. As Ψ_w became more negative, the inhibition became entirely metabolic. © 2002 Annals of Botany Company

Key words: Photosynthesis, water potential, epidermis, oxygen evolution, stomata, dehydration, carbon dioxide, *Helianthus annuus* L., sunflower.

INTRODUCTION

When plants encounter water deficits their stomata close, thus reducing water loss from the leaves. This limits CO_2 entry and could potentially limit photosynthetic carbon reduction. In some (Robinson *et al*., 1988; Cornic *et al*., 1989; Frederick *et al*., 1990; Quick *et al*., 1992), but not all studies (Ben *et al*., 1987; Graan and Boyer, 1990; Wise *et al*., 1992; Saccardy *et al*., 1996; Kanechi *et al*., 1998; Escalano *et al*., 1999; Tezara *et al*., 1999), raising the CO_2 concentration to overcome this diffusive barrier resulted in a complete recovery of photosynthesis. Even in a single species such as sunflower, some reports suggest a stomatal limitation to photosynthesis (Ben *et al*., 1987; Quick *et al*., 1992), while others do not (Boyer, 1971*a*; Kanechi *et al*., 1998; Tezara *et al*., 1999). There is thus general agreement that an inhibition is present but the reasons for it are controversial.

Studies of desiccated algae show that photosynthesis can be inhibited even though no stomata are present (Davison and Pearson, 1996; Kawamitsu *et al*., 2000). Therefore, direct metabolic limitations clearly occur, and much of the controversy rests on whether stomatal closure in leaves is large enough and early enough to restrict CO_2 uptake, and thus photosynthesis, before metabolism is inevitably inhibited (Boyer, 1976, 1990; Mansfield *et al*., 1990; Chaves, 1991).

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In an effort to resolve this issue, Vassey *et al*. (1991) reasoned that low CO_2 caused by stomatal closure might trigger metabolic inhibition. When CO_2 was kept high during dehydration, they found high activity of the enzyme sucrose phosphate synthase, which was otherwise inhibited in dehydrated *Phaseolus* leaves. Vassey *et al*. (1991) therefore concluded that stomatal closure could initiate metabolic inhibition, and that closure was the primary cause of reduced photosynthesis.

These concepts can be tested directly by removing the epidermis. If stomatal closure results in insufficient CO_2 for photosynthesis, removing the epidermis will restore CO_2 levels and will prevent the inhibition. If CO_2 is low initially and triggers metabolic inhibition later, removing the epidermis beforehand will prevent inhibition. In both cases, however, if photosynthesis is still inhibited, it must be limited by factors other than stomatal closure.

Removal of an epidermis caused increased CO_2 fixation in sweet potato (Kubota *et al*., 1992) and mungbean leaves over a wide range of CO_2 supply and temperature (Kubota *et al*., 1996; Tokuda *et al*., 1999). In the CAM plant *Bryophyllum daigremontianum*, removing the epidermis had no effect on CO_2 fixation when the stomata were open in the dark, but increased fixation when the stomata were tightly closed during the day (Nishida, 1977). The same technique was used to study the effect of abscisic acid (ABA) on photosynthesis of sunflower and broad bean (Terashima *et al*., 1988). High leaf ABA concentrations

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closed the stomata, reducing photosynthesis. When the epidermis was removed, photosynthesis recovered completely indicating that stomatal closure accounted for all the photosynthetic inhibition. However, none of these studies have involved water deficit. The only studies involving water deficit of which we are aware (Dietz and Heber, 1983; Schwab *et al*., 1989) showed that photosynthesis in *Primula palinuri* and the 'resurrection' plant *Ramonda mykoni* recovered only partially when the epidermis was removed from the dehydrated leaves. Here, we applied the same technique to investigate the role of stomata in the photosynthetic activity of dehydrated sunflower plants.

MATERIALS AND METHODS

Plant materials and growth conditions

Sunflower (*Helianthus annuus* L. hybrid IS897 from Interstate Seed Company, Box 338, Fargo, ND, USA) plants were grown singly from seed in 19 cm diameter pots filled with a 2 : 1 soil : peat moss mixture with a pH of 6·5. Nutrient solution was provided twice a week and consisted of 6 mol m^{-3} $Ca(NO_3)_2$, 4 mol m^{-3} KNO_3, 2 mol m^{-3} KH_2PO_4, 2 mol m^{-3} $MgSO_4$, 25 mmol m^{-3} H_3BO_3, 10 mmol m^{-3} $MnSO_4$, 2 mmol m^{-3} $ZnSO_4$, 0·5 mmol m^{-3} $CuSO_4$, 0·5 mmol m^{-3} H_2MoO_4 and 100 mmol m^{-3} FeNa-ethylenediamine-tetraacetic acid. Water was supplied as needed. For plants grown in a glasshouse under natural daylight, average maximal day/night temperatures and relative humidity were 35/15 °C and 30/85 %, respectively. A growth chamber (Environmental Growth Chambers, OH, USA) was also used to grow sunflower at a day/night temperature of 30/20 °C and relative humidity of 60/90 % with a 12 h photoperiod of 700–900 μmol m^{-2} s^{-1} of photosynthetically active radiation. In all but one experiment, dehydration was imposed by withholding nutrient solution from the soil for several days. Fully expanded, mature leaves from 5–6-week-old plants were sampled for measurements of photosynthesis and water status.

Photosynthesis

The rate of photosynthesis was measured in air as O_2 evolution using a gas-phase Walker-type oxygen electrode system equipped with a Björkman lamp as a light source [Delieu and Walker (1981), sold as Model LD-2 from Hansatech Limited, Pentney, UK]. A leaf disc with an area of 4·52 cm^2 was placed in the chamber, and the volume of the chamber was calibrated as described by Delieu and Walker (1981). The volume of the chamber was small and, in order to avoid depleting the CO_2 during the measurement, we sealed the tissue in the chamber for a short time only. The method involved exposing the tissue to light in a water-saturated gas mixture flowing through the open electrode chamber for 5 min, then sealing the chamber for 30–60 s to take a measurement, opening the chamber, flushing and repeating the measurement after 5 min. During each 30–60 s measurement, O_2 evolution was linear with time and the rate was calculated from the slope of the line. The CO_2 partial pressure of the incoming humidified air was controlled by mixing CO_2-free air with air containing either 10 or 60 % CO_2. Because leaf temperature rose upon illumination, the temperature of water circulating around the leaf chamber was maintained at 22·1 °C to keep the leaf temperature at 25 ± 0·2 °C under illumination, measured with a fine thermocouple inserted into the leaf disc.

Leaf water status

Immediately after measuring O_2 evolution, the leaf water potential (Ψ_w) was determined on the same disc by isopiestic thermocouple psychrometry in a vapour chamber coated with melted and re-solidified petroleum jelly (Boyer and Knipling, 1965). The peeled surface of leaf discs was placed face-down in the psychrometer so that the thermocouple measured vapour coming through the intact epidermis. Measurements were corrected for heat of respiration. When experiments were performed to determine the change of Ψ_w during an O_2 electrode measurement, another disc from the same leaf was used to measure the initial Ψ_w.

Leaf peeling

A 9·6 cm^2 disc was removed from the leaf and fine forceps were used to carefully remove the epidermis under a dissecting microscope inside a sealed glove box containing an atmosphere saturated with water vapour. After peeling, a smaller disc (4·52 cm^2) was removed and used to measure O_2 evolution and Ψ_w. In most of the experiments, plants were dehydrated to varying degrees by withholding water from the soil, and afterwards the abaxial epidermis was peeled from the disc used for the measurements. In one experiment, the order was reversed and the disc was peeled before dehydration was imposed.

Microscopy

Leaf tissues of hydrated plants were fixed in CRAF (chromic acid, acetic acid and formalin) solution according to Jensen (1962). The tissues were embedded, sliced and stained with safranin, crystal violet, fast green and gold orange (Triarch Inc., Ripon, WI, USA).

Statistical analysis

All experiments were replicated as indicated. In those experiments where a Ψ_w was established and monitored for various times or at varying CO_2 concentrations, the Ψ_w differed somewhat between replicates, and single representative experiments are reported. In the other experiments involving single measurements of O_2 evolution and Ψ_w (in the same disc), many measurements were made and all of them are reported to show the total range of variation.

RESULTS

Leaf cross-sections showed that peeling completely removed the abaxial epidermis and resulted in the collapse of only a few spongy mesophyll cells (Fig. 1B). Evidence of a collapsed cell could be seen, on average, about every 100 μm

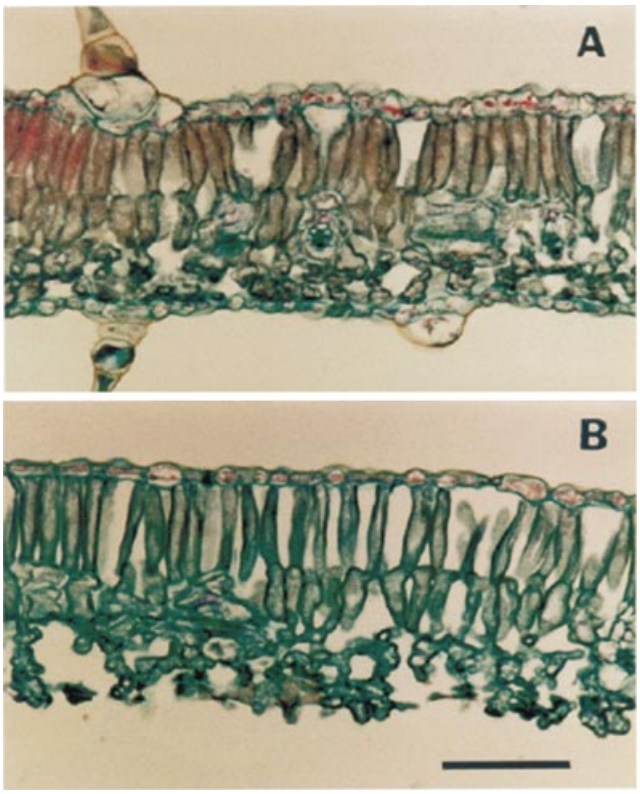

FIG. 1. Cross-section of sunflower leaves: intact leaf (A) and peeled leaf after removal of the abaxial epidermis (B). Bar = 100 µm.

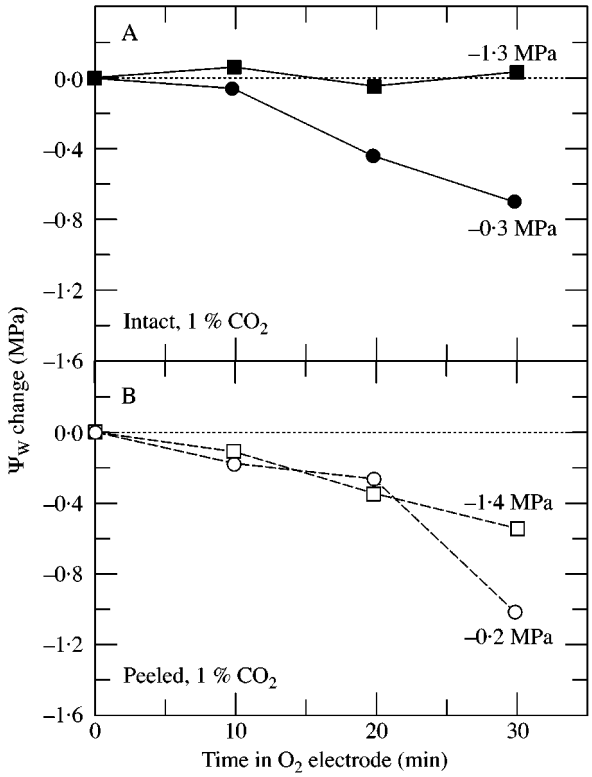

FIG. 2. Change in Ψ_w at various times after leaf discs were placed in the O_2 electrode. A, Intact disc. B, Peeled disc lacking the abaxial epidermis. For each point, two discs were obtained from the same leaf. One was used to measure photosynthetic O_2 evolution followed immediately by Ψ_w. The other disc was used to measure Ψ_w at time zero. The difference between the initial and final Ψ_w is shown on the ordinate. The figure shown alongside each curve is the average of all the values of initial Ψ_w measured for that treatment. This is a single representative experiment from two replicate experiments. O_2 evolution was 47–52 µmol m^{-2} s^{-1} (intact) and 42–49 µmol m^{-2} s^{-1} (peeled) in discs from plants with high Ψ_w, and 9–16 µmol m^{-2} s^{-1} (intact) and 34–43 µmol m^{-2} s^{-1} (peeled) in discs from plants with low Ψ_w. O_2 evolution was measured at an irradiance of 1200 µmol m^{-2} s^{-1} at 25 °C in saturated air containing 21 % O_2 and 1 % CO_2.

on the abaxial surface of the mesophyll. Otherwise, in comparison with the intact disc (Fig. 1A), there was no damage and the remaining parts of the peeled disc were unchanged. Because so few cells were broken, intact and peeled sections had nearly the same thickness.

Effect of peeling on measurements of leaf Ψ_w and O_2 evolution

When the lower epidermis was removed from discs, water was lost more rapidly than from intact discs, and it was necessary to determine the effect this had on the measurements. During peeling, the Ψ_w of the discs decreased by less than 0·2 MPa. When subsequently exposed to the inside of the electrode chamber (high irradiance with water-saturated gas flowing), the decrease in Ψ_w accelerated. For the first 10 min, the acceleration was modest and Ψ_w decreased by 0·2 MPa or less in all discs (Fig. 2). O_2 evolution was stable during this period for all treatments (Fig. 3). In the next 20 min, Ψ_w decreased by 0·6 MPa in the intact hydrated discs (initial Ψ_w = –0·3 MPa; Fig. 2A), or by 0·5–1·0 MPa in the peeled discs (initial Ψ_w –0·2 and –1·4 MPa; Fig. 2B). Only in intact discs with a low initial Ψ_w was dehydration negligible for the entire 30 min, probably because the stomata were somewhat closed (–1·3 MPa; Fig. 2A). O_2 evolution was essentially stable for the first 10 min in the O_2 electrode (Fig. 3A and B) and tended to decrease slightly in the peeled discs during the following 20 min, probably because the discs were dehydrating (Fig. 3B). However, the stability of both Ψ_w and O_2 evolution in the first 10 min in the electrode allowed measurements to be made during that time, and in all remaining experiments O_2 evolution was measured at 10 min, with Ψ_w being measured immediately afterwards in the same discs.

Maximum capacity for O_2 evolution

The maximum capacity for O_2 evolution occurred at 1 % CO_2 when intact discs had high Ψ_w (Fig. 4A). At 5 % CO_2, the rates were slightly lower and at 10 % CO_2 they were distinctly lower, indicating that sufficient CO_2 had entered the discs to satisfy requirements for fixation, and fixation activity was inhibited by excess CO_2. At low Ψ_w, the maximum capacity shifted to 5 % CO_2 probably because the stomata were closed and created more of a barrier to CO_2 entry; 10 % CO_2 was still inhibitory (Fig. 4A). On the other hand, peeled discs with the peeled surface downward and exposed to the gas volume in the electrode system had no

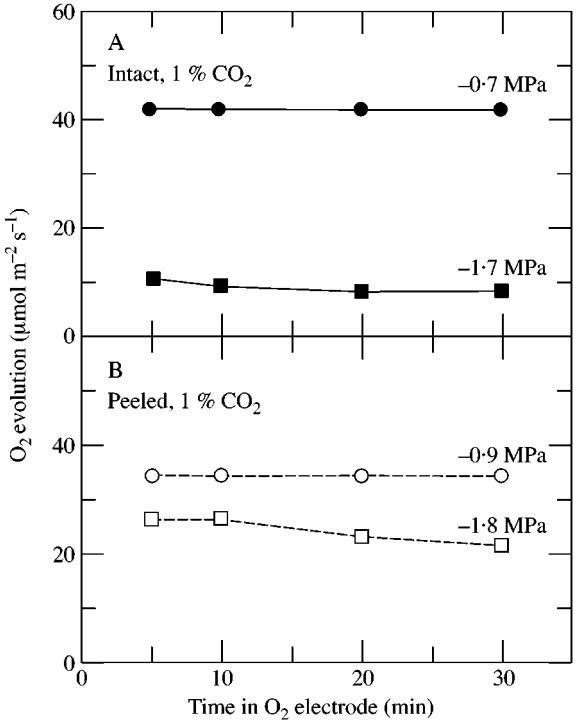

FIG. 3. O_2 evolution at different times in the O_2 electrode in individual leaf discs of sunflower. A, Intact discs. B, Peeled discs lacking the abaxial epidermis. Four discs were sampled from one leaf on plants with either a high or low Ψ_w. Two were used for measuring O_2 evolution or Ψ_w. The other two were used for the same measurements after peeling the epidermis. The initial Ψ_w is shown alongside each curve. This experiment was done twice with six different CO_2 concentrations ranging from 0·4 to 20 % in each experiment. All concentrations gave similar results and a representative experiment is shown only for 1 % CO_2. O_2 evolution was measured at an irradiance of 1200 μmol m^{-2} s^{-1} at 25 °C in saturated air containing 21 % O_2.

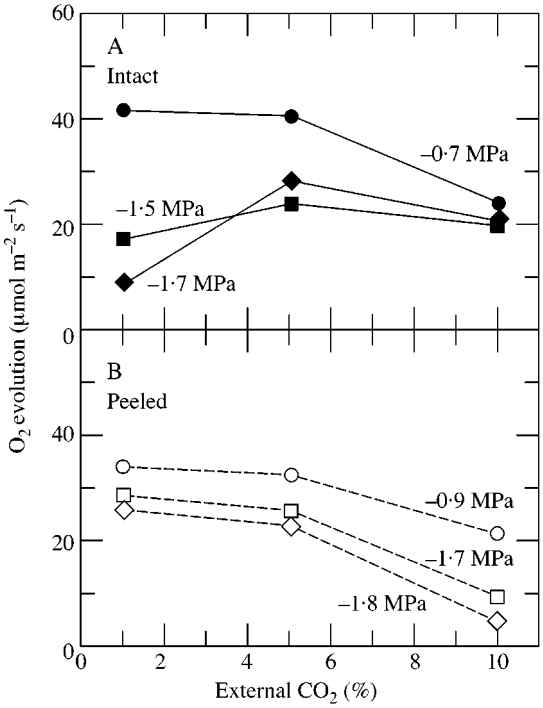

FIG. 4. O_2 evolution at different CO_2 concentrations around sunflower leaf discs in the O_2 electrode. A, Intact disc. B, Peeled disc lacking the abaxial epidermis. For exposure to various CO_2 concentrations, four discs were removed from the same leaf of a plant with a high or low Ψ_w. Three were used for O_2 evolution, one for each CO_2 concentration and one was used to determine Ψ_w at the sampling time, shown alongside each curve. This is a representative experiment from two repetitions with similar results. O_2 evolution was measured after 10 min at an irradiance of 1200 μmol m^{-2} s^{-1} at 25 °C in saturated air containing 21 % O_2.

stomatal barrier to CO_2 entry on the lower surface. The maximum capacity for O_2 evolution occurred at 1 % CO_2 regardless of Ψ_w (Fig. 4B). O_2 evolution was slightly lower at 5 % CO_2 and much lower at 10 % CO_2. These results indicate that the stomatal barrier to CO_2 diffusion can be overcome either by raising the external CO_2 supply to 5 % in intact discs or by removing the lower epidermis from the disc.

Limitations to O_2 evolution at low Ψ_w

When CO_2 concentrations were kept at 1 % in peeled discs and 5 % in intact discs to measure the maximum capacity for O_2 evolution and minimize stomatal effects in both types of disc, the highest rates of O_2 evolution were observed at Ψ_w of –0·9 to –1 MPa and were 45–47 μmol m^{-2} s^{-1} (Fig. 5). The rates were nearly as high in peeled discs as in intact ones, showing that wounding was minimal. As Ψ_w decreased, O_2 evolving activity decreased dramatically at first and more gradually as the plant became drier. The decrease was similar regardless of whether the plants were grown in a glasshouse or in a growth chamber, or whether the discs were intact or peeled. At Ψ_w of –3 MPa, O_2 evolution was approx. 20 % of that at high Ψ_w (Fig. 5).

Does stomatal closure trigger losses in maximum photosynthetic activity?

To evaluate the effect of the stomatal barrier more closely, a similar experiment was conducted on peeled and unpeeled discs at 1 % CO_2. At this concentration, the peeled discs expressed the maximum capacity for O_2 evolution but the intact discs expressed less than the maximum when Ψ_w was low (Fig. 4A), presumably because CO_2 entry was restricted by stomatal closure. As a consequence, the intact discs lost more activity than the peeled discs as Ψ_w became lower (Fig. 6).

In each of these experiments, discs were obtained from dehydrated plants and the epidermis was removed after the stomata had closed. It is possible that stomatal closure diminished internal concentrations of CO_2 and triggered losses in O_2 evolving activity, as demonstrated by Vassey *et al.* (1991) in *Phaseolus*. To test this possibility, the epidermis was removed before discs were subjected to a low Ψ_w by dehydrating them inside the O_2 electrode chamber. Growth chamber conditions were simulated by flowing air containing 0·035 % CO_2 and with a relative humidity of 60 % through the electrode chamber at 25 °C under an irradiance of 800 μmol m^{-2} s^{-1}. At various times, O_2 evolution was measured at 1 % CO_2 and the disc was removed to determine Ψ_w. As dehydration progressed, O_2

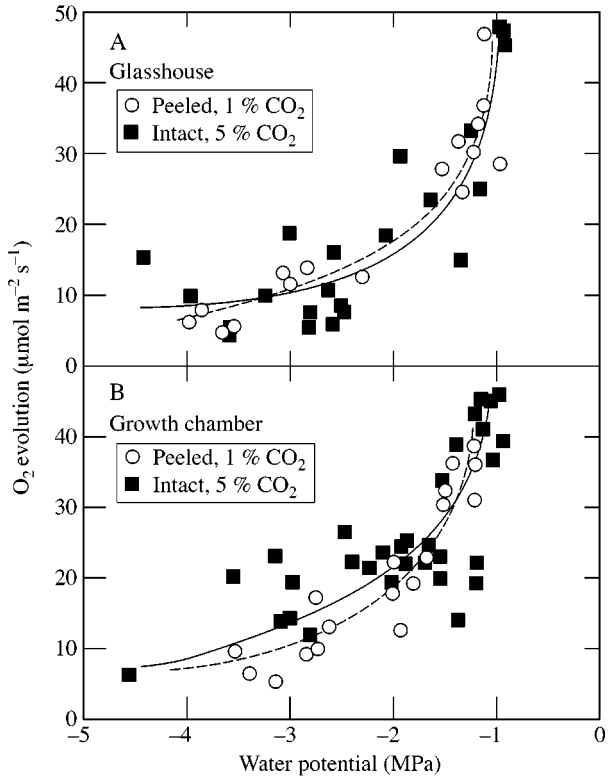

FIG. 5. Maximum O_2 evolution at various Ψ_w in sunflower leaf discs. A, Plants grown in a glasshouse (circles, peeled; squares, intact). B, Plants grown in a growth chamber (circles, peeled; squares, intact). Leaf discs were sampled from the fully expanded leaves after dehydration of the plants by withholding water from the soil for several days. Two discs were taken from the same leaf at the same time, one of which was left intact, the other peeled. O_2 evolution was measured after 10 min with CO_2 supplied at 1 % (peeled) or 5 % (intact) to obtain the maximum rate (see Fig. 4). The same disc was immediately used to determine Ψ_w. The individual measurements were pooled from two (A) or three (B) repeated experiments, each involving a different plant and two adjacent leaves. Other conditions for O_2 evolution measurement were an irradiance of 1200 μmol m^{-2} s^{-1} at 25 °C in saturated air containing 21 % O_2.

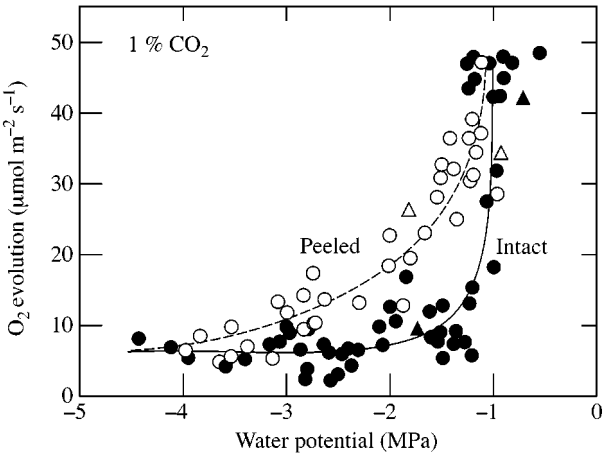

FIG. 6. As for Fig. 5 but O_2 evolution was measured only at 1 % CO_2. Sunflower leaf discs were intact (closed circles) or peeled and lacking the abaxial epidermis (open circles). Also shown are data from Fig. 3 (intact, closed triangles; peeled, open triangles). The individual measurements were pooled from five repeated experiments with plants grown and dehydrated in a glasshouse or growth chamber before the discs were sampled and peeled. Other conditions for O_2 evolution were as described in Fig. 5.

evolution decreased in both peeled and intact discs, but the decrease was much faster in peeled discs (Fig. 7A). At the same Ψ_w, discs peeled before dehydration lost slightly less activity than intact ones (Fig. 7B), a result similar to that of the earlier experiments when discs were peeled after dehydration (Fig. 6). When O_2 evolution was compared in discs peeled before or after dehydration, results were identical (Fig. 8).

DISCUSSION

Maximum capacity for O_2 evolution

There were marked losses in the maximum capacity for photosynthesis when sunflower plants were dehydrating and peeled leaf discs were used for the assay. During the measurements, the discs were pressed against the upper window of the O_2 electrode chamber, causing most of the CO_2 to enter through the lower surface. Because the discs had no lower epidermis, CO_2 entered virtually unhindered by the stomata. Moreover, CO_2 readily reached the cells because the maximum capacity for photosynthesis always occurred at 1 % CO_2 regardless of Ψ_w. This showed that CO_2 entered as readily at low Ψ_w as at high Ψ_w, and the shrinkage of the mesophyll reported by Fellows and Boyer (1978) at low Ψ_w did not hinder CO_2 diffusion significantly. Therefore, when CO_2 supply was adequate, the losses in peeled discs could be attributed only to decreased photosynthetic metabolism.

It is significant that the losses did not depend on whether the epidermis was removed before or after dehydration. Removing the epidermis before dehydration allowed CO_2 to remain at the ambient level as low Ψ_w developed. Removing it after dehydration exposed the mesophyll to possibly depleted CO_2 as the stomata closed. When Vassey *et al.* (1991) supplied 1 % CO_2 during dehydration, reductions in sucrose phosphate synthase activity were prevented. They concluded that low CO_2 triggered metabolic losses because of stomatal closure. In the present experiments, 1 % CO_2 was not supplied during dehydration but removing the epidermis served the same purpose. Without the epidermis, no CO_2 depletion was possible. The results indicate that CO_2 depletion could not account for the losses in photosynthetic metabolism in sunflower, and thus dehydration itself acts directly on photosynthetic metabolism. Perhaps the control of photosynthesis differs in sunflower compared with *P. vulgaris* at low Ψ_w.

The inability of photosynthesis to recover when the epidermis is removed is in contrast to sunflower leaves fed ABA where removing the epidermis completely recovered photosynthesis (Terashima *et al.*, 1988). ABA resulted in stomatal closure, but the complete recovery showed that photosynthetic metabolism was unaffected. In another experiment, Lauer and Boyer (1992) measured the internal CO_2 concentration (C_i) and found that C_i decreased when leaves were fed ABA. The decline in C_i with ABA occurred

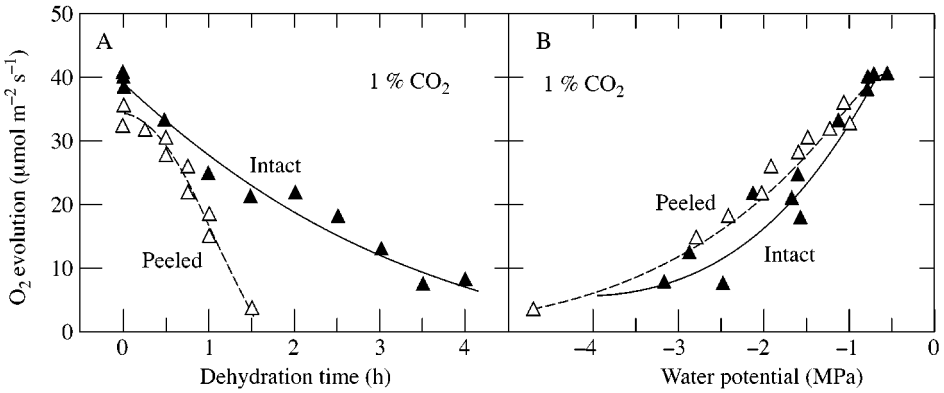

FIG. 7. O_2 evolution in sunflower leaf discs sampled at high Ψ_w and dehydrated afterwards in the O_2 electrode. A, O_2 evolution at various times in 1 % CO_2 (intact, closed triangles; peeled before dehydration, open triangles). B, O_2 evolution at various leaf Ψ_w in 1 % CO_2 (intact, closed triangles; peeled before dehydration, open triangles). Each data point for O_2 evolution is from a separate disc, and Ψ_w was measured in the same disc immediately afterwards. Individual data were pooled from three repeated experiments, and the discs were sampled from two adjacent leaves from each plant. During dehydration in the electrode, irradiance was 800 μmol m^{-2} s^{-1} in air with 0·035 % CO_2 at 25 °C and relative humidity of 60 %. During the O_2 evolution measurements, conditions were as described in Fig. 5.

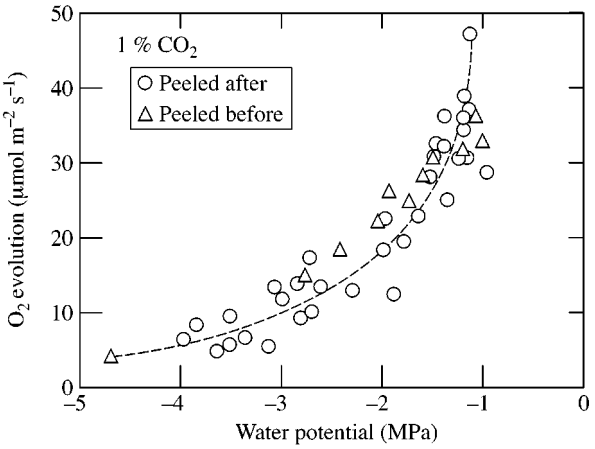

FIG. 8. O_2 evolution at 1 % CO_2 in peeled leaf discs sampled before or after dehydration of the tissue. Circles, Leaf discs obtained and peeled after the plants had developed various Ψ_w. Triangles, leaf discs obtained from plants with a high Ψ_w and peeled before subjecting the discs to various Ψ_w in the O_2 electrode. Ψ_w was measured in the same discs immediately after each O_2 evolution measurement. Individual data were pooled from three repeated experiments, and the discs were sampled from two adjacent leaves from each plant. Conditions during dehydration in the electrode were as described in Fig. 7 and during measurements in the electrode were as described in Fig. 5.

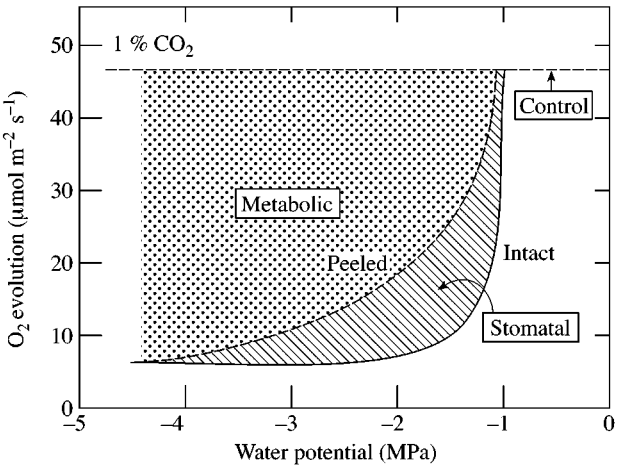

FIG. 9. Metabolic and stomatal components of inhibited O_2 evolution in sunflower leaf discs having low Ψ_w. Dashed line (control) indicates full recovery expected when epidermis is removed if no metabolic inhibition occurs. Dotted area represents the metabolic component and hatched area the stomatal component of inhibition. Adapted from Fig. 6.

because the stomata closed without altering the demand for CO_2 by photosynthesis (Terashima *et al.*, 1988), and CO_2 was depleted inside the leaf. However, at low Ψ_w, Lauer and Boyer (1992) found increasing C_i. This opposite effect could not be attributed simply to stomatal closure. These results indicate that although stomata close at low Ψ_w, large non-stomatal changes also occur and decrease demand for CO_2.

Limitations to O_2 evolution at low Ψ_w

While removing the epidermis allowed direct metabolic influences to be observed, leaving the discs intact gave similar results provided the intact discs were exposed to 5 % CO_2. This concentration saturated photosynthesis at low Ψ_w and should have allowed the maximum activity to be observed in the same fashion as in peeled discs. Because the losses were indistinguishable from those in the peeled discs, they were thus entirely metabolic. Other reports with leaves showed similar inhibition when exposed to high CO_2 at low Ψ_w (Boyer, 1971*a*; Ben *et al.*, 1987; Bunce, 1988; Di Marco *et al.*, 1988; Graan and Boyer, 1990; Wise *et al.*, 1991, 1992; Quick *et al.*, 1992; Kanechi *et al.*, 1998; Escalano *et al.*, 1999; Tezara *et al.*, 1999). Although the degree of metabolic inhibition probably varies with species, water deficit, sampling time and measuring method, our results showed that photosynthetic metabolism was inhibited similarly whether the plants were grown in a growth chamber or glasshouse, or whether plants were dehydrated for several days or discs were dehydrated for a few hours in the laboratory. In contrast, sunflower plants allowed to

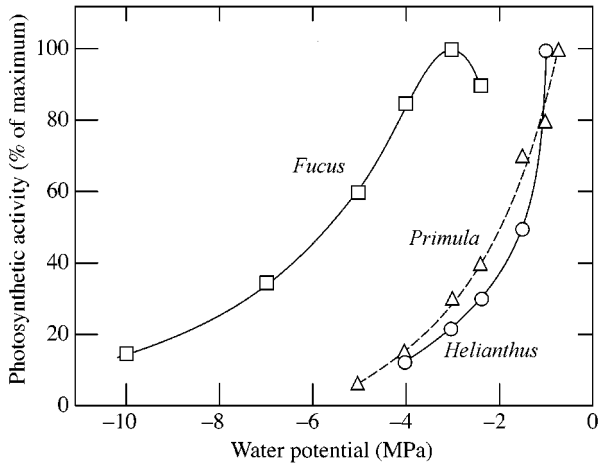

FIG. 10. Comparison of metabolic limitation in three species having low Ψ_w. The limitations are entirely metabolic because in *Fucus vesiculosus* no stomata are present and photosynthesis occurs in the tissue surface (from Kawamitsu *et al*., 2000), while in the leaves of *Primula palinuri* and *Helianthus annuus* the epidermis is peeled away and the stomata have been removed [from Dietz and Heber (1983) and this paper].

acclimate for 2 weeks to moderately low Ψ_w showed less metabolic inhibition than non-acclimated plants, suggesting that conditions in the cytoplasm affect the metabolic response (Matthews and Boyer, 1984).

However, at 1 % CO_2, larger losses in O_2 evolving activity initially occurred in the intact discs than in the peeled ones. Stomata begin to close at Ψ_w around –1 MPa in sunflower (Boyer and Bowen, 1970; Matthews and Boyer, 1984; Sharp and Boyer, 1986). As a consequence, comparing photosynthesis in peeled discs with that in intact discs at 1 % CO_2 allowed the inhibition to be separated into a metabolic component (peeled discs) and an epidermal component that reflected the stomatal contribution (difference between peeled and intact discs). Figure 9 illustrates these components and indicates that the stomatal component was significant at Ψ_w between –1 and –1·5 MPa but the metabolic one dominated below about –1·5 MPa. This finding agrees with other reports of stomatal inhibition during mild dehydration (Kaiser, 1987; Robinson *et al*. 1988; Cornic *et al*., 1989; Schwab *et al*., 1989; Sharkey and Seemann, 1989; Frederick *et al*., 1990; Chaves, 1991; Quick *et al*., 1992; Wise *et al*., 1992).

The response is complicated by the O_2 electrode properties, which are advantageous for rapid measurements but involve large boundary layers because the air is unstirred and the tissue is pressed against the upper window of the chamber. As a result, CO_2 diffusion is restricted, and concentrations often need to be higher than in experiments measuring CO_2 exchange of leaves in stirred air. For example, sunflower leaves at high Ψ_w saturate at CO_2 concentrations around 0·1 % in stirred air (Matthews and Boyer, 1984; Sharp and Boyer, 1986) or about one-tenth of the concentration for discs in the electrode system. On the other hand, when Ψ_w is low, sunflower leaves saturate at about 3 % CO_2 in stirred air (Graan and Boyer, 1990), which is similar to the concentration for discs in the O_2 electrode (5 % at low Ψ_w in the present experiments). Thus, while the maximum capacity is easily measured in the intact discs, the role of the stomata is only approximated in the O_2 electrode system and may be better assessed in intact leaves.

In leaves, tests often rely on the calculated C_i, and some authors have reported decreased C_i (Quick *et al*., 1992; Gunasekera and Berkowitz, 1993), others no change (Di Marco *et al*., 1988; Cornic *et al*., 1989; Wise *et al*., 1991; Gunasekera and Berkowitz, 1993) and still others found increases at low Ψ_w (Frederick *et al*., 1990; Wise *et al*., 1990). Cornic *et al*. (1983) described non-stomatal limitations in *Sinapis* at low Ψ_w using an analysis of calculated C_i. Calculations of C_i are based on transpiration and have been questioned because stomatal closure may not affect transpiration uniformly across the leaf (Terashima *et al*., 1988, but see Wise *et al*., 1991, 1992), and the epidermis may discriminate between water vapour and CO_2 (Boyer *et al*., 1997). When Lauer and Boyer (1992) avoided these problems by directly measuring C_i in several species, they reported a slight decrease in intact sunflower leaves followed by an increase, as Ψ_w became lower. Stomatal closure may have contributed to the slight decrease but could not be responsible for the increase. Clearly, the increase was caused by metabolic inhibition that reduced the demand for CO_2 more than the stomata reduced the diffusion of CO_2. Because the metabolic component became dominant at Ψ_w below –1·5 MPa in the present experiments, the electrode system gave results that were in general agreement with those of Lauer and Boyer (1992) in intact leaves. Thus, it appears that sunflower photosynthesis may have been somewhat influenced by stomata initially but the effect decreased as the metabolic component became dominant at lower Ψ_w.

Causes of metabolic inhibition

The early inhibition of photosynthetic metabolism is curious in view of the comparatively stable photosynthesis at low Ψ_w in species such as *Fucus vesiculosus* that lack stomata (Fig. 10). The early inhibition may be general for land plants because it was also apparent in *Primula palinuri* (Fig. 10). Two factors are likely to contribute to it. First, in sunflower, cells shrink when water is lost, and the shrinkage causes distortions and wrinkling of the cell wall (Fellows and Boyer, 1978). Cytoplasm caught in the folds and distorted by the wall shows breaks in the plasmalemma and/or tonoplast, visible in the electron microscope, and cell lysis follows quickly. At Ψ_w of –1·5 MPa, about 10 % of the cells are lysed and account for about 20 % of the loss in leaf CO_2-fixing activity (Fellows and Boyer, 1978). Upon rewatering, the lysed cells do not contribute to photosynthetic recovery and rates return to less than 100 % in the leaves. With more severe desiccation to the air-dry state (–12 MPa), all cells are lysed and photosynthetic recovery is zero (Fellows and Boyer, 1978).

The second and usually the largest factor is a direct loss of activity in the photosynthesis partial reactions that is reversible upon rehydration. In experiments in which half a sunflower leaf was dehydrated while the other half was supplied with water, isolated chloroplasts were closely

compared and showed decreased electron transport (Boyer and Bowen, 1970; Mohanty and Boyer, 1976; Matthews and Boyer, 1984) and a marked inhibition of cyclic and non-cyclic photophosphorylation (Keck and Boyer, 1974). When measured *in vivo*, there were similar losses in photophosphorylation indicated by membrane energization in sunflower grown in the laboratory (Ortiz-Lopez *et al*., 1987) but not in the field (Ortiz-Lopez *et al*., 1991), probably because of the different growth conditions or illumination in the two environments. Losses in membrane energization were also reported for *Craterostigma plantagineum* and spinach (Schwab *et al*., 1989). In sunflower, Tezara *et al*. (1999) found substantial decreases in ATP synthesis attributable to less ATP synthase (Coupling Factor), and the ability of the chloroplasts to regenerate ribulose-1,5-bisphosphate (RuBP) was lower as a result. Two sunflower hybrids differing in photosynthetic activities at Ψ_w of –1 MPa had differing RuBP contents but not ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content or activity (Giménez *et al*., 1992), and similar observations were reported in tobacco with antisense Rubisco DNA (Gunasekera and Berkowitz, 1993). At low Ψ_w, sunflower leaves also had low quantum yields (Mohanty and Boyer, 1976; Matthews and Boyer, 1984; Sharp and Boyer, 1986) not caused by photoinhibition (Sharp and Boyer, 1986). All of these measurements support the view that losses in the activity of the membrane-associated reactions inhibit CO_2 fixation and account for much of the metabolic inhibition in sunflower. Similar changes are likely to occur in other species but at different Ψ_w, so the closest analysis seems possible within a single species.

Inhibition of photosynthesis was reversed when sunflower leaf tissue was rewatered (except for effects of lysis) (Boyer, 1971*b*; Potter and Boyer, 1973), but not when the chloroplasts were isolated into hydrating media (Potter and Boyer, 1973). In other words, repair or re-configuration of the partial reactions required the intact cell. The loss did not result from low turgor (Boyer and Potter, 1973) and instead was proportional to the increased solute concentration that occurs when water departs and solute is left behind (Potter and Boyer, 1973). As a result, the loss was approximately proportional to the relative water content of the tissue.

Additional insight into the molecular changes resulting from solute were reported in spinach where large losses in photophosphorylation occurred at low Ψ_w (Younis *et al*., 1979), which were also seen *in vivo* (Schwab *et al*., 1989). The conformation of the spinach coupling protein was altered and it was unable to bind substrate ADP (Younis *et al*., 1979) instead of being present in smaller amounts, as in sunflower (Tezara *et al*., 1999). The conformation and activity losses could be reproduced by exposing the protein to Mg^{2+} concentrations at least two-fold higher than normal, or about the concentration expected when 50 % or more of the leaf water is lost (Younis *et al*., 1983). The Mg^{2+} bound to the coupling protein and the low activity and new conformation were not reversed when the protein was returned to media containing normal Mg^{2+} concentrations, i.e. the non-reversibility was similar to that in chloroplasts isolated from leaf tissue at low Ψ_w. To explore whether Mg^{2+} was important in sunflower, Rao *et al*. (1987) grew plants with varying Mg^{2+} concentrations in the leaves and found slightly less photosynthetic inhibition at low Ψ_w when the leaves had low Mg^{2+} than when they had high Mg^{2+} contents, confirming the importance of specific solutes in the metabolic response.

Similar chloroplast effects have been reported for other species (Mayoral *et al*., 1981; Berkowitz and Whalen, 1985; Kaiser *et al*., 1986; Ben *et al*., 1987) and have sometimes been attributed to increased solute concentrations (Berkowitz and Whalen, 1985; Kaiser *et al*., 1986) although dehydration sometimes had to be severe (Sharkey and Seemann, 1989; Scheuermann *et al*., 1991; Martin and Ruiz-Torres, 1992; Gunasekera and Berkowitz, 1993). Thus, if concentrating effects of water loss account for some of the losses in photosynthetic metabolism, the effects should be immediate with no difference between leaf discs peeled before or after imposition of low Ψ_w. In the present experiments, such immediacy was observed, and the time of peeling made no difference.

In other studies, variations on this form of metabolic inhibition have been observed. Sucrose content sometimes increased and was associated with a block in starch synthesis (Vassey and Sharkey, 1989; Chaves, 1991; Quick *et al*., 1992; Kanechi *et al*., 1998). The activity of certain enzymes, such as sucrose phosphate synthase, was reduced (Vassey and Sharkey, 1989; Vassey *et al*., 1991), while that of fructose-1,6-bisphosphatase and Rubisco changed only moderately (Vassey and Sharkey, 1989). Sharkey and Seemann (1989) found that most of the metabolites 3-phosphoglycerate, triose-phosphate, fructose-6-phosphate and RuBP decreased only during severe dehydration.

Cornic and coworkers (Cornic *et al*., 1989; Cornic and Briantais, 1991) measured fluorescence and CO_2 assimilation in *Phaseolus vulgaris* and ascribed losses during water deficits entirely to internal CO_2 depletion by stomatal closure. It is clear that *P. vulgaris* could be more affected by water deficit-induced stomatal closure than sunflower, but the *P. vulgaris* work depends on the stability of the relationship between fluorescence, electron flow and C_i at low Ψ_w. It is well known that considerable chlorophyll fluorescence is absorbed by other chloroplasts, and the signal probably represents mostly surface chlorophyll rather than the entire chloroplast population. Moreover, because electron transport and photophosphorylation can change in chloroplasts from dehydrated plants (described above), it seems risky to assume a stable relationship between fluorescence, electron flow and C_i. In the present work, these assumptions were avoided by removing the epidermis and thus clearly removing stomatal control. Despite the lack of stomata, a marked photosynthetic inhibition remained. A similar experiment in *P. vulgaris* could be illuminating.

CONCLUSIONS

Leaf discs with and without an epidermis provide a straightforward way of identifying the contributions of stomata and metabolism to the rate of photosynthesis. Stomata were closing while metabolism was being inhib-

ited, and the relative severity of each determined which factor was most rate-limiting. Large, direct metabolic losses were clearly seen and stomata contributed only in the early phases of dehydration. However, because discs were used at very high CO_2 concentrations, it was not possible to determine exactly how the limitation would be expressed in intact plants operating in ambient air. If the epidermis could be similarly removed in intact plants, it might be possible to extend this method to a more precise determination of rate-limitation than is currently possible.

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