

Analysis of Stomatal and Nonstomatal Components in the Environmental Control of CO₂ Exchange in Leaves of *Welwitschia mirabilis*¹

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ABSTRACT

In well-watered plants of *Welwitschia mirabilis*, grown in the glasshouse under high irradiance conditions, net CO₂ assimilation was almost exclusively observed during the daytime. The plants exhibited a very low potential for Crassulacean acid metabolism, which usually resulted in reduced rates of net CO₂ loss for several hours during the night. In leaves exposed to the diurnal changes in temperature and humidity typical of the natural habitats, CO₂ assimilation rates in the light were markedly depressed under conditions resembling those occurring during midday, when leaf temperatures and the leaf-air vapor pressure differences were high (36°C and 50 millibars bar⁻¹, respectively). Studies on the relationship between CO₂ assimilation rate and intercellular CO₂ partial pressure at various temperatures and humidities showed that this decrease in CO₂ assimilation was largely due to stomatal closure. The increase in the limitation of photosynthesis by CO₂ diffusion, which is associated with the strong decline in stomatal conductance in *Welwitschia* exposed to midday conditions, may significantly contribute to the higher ¹³C content of *Welwitschia* compared to the majority of C₃ species.

Welwitschia mirabilis (class Gymnospermae) is endemic to the Namib desert. The plants are well known for their bizarre appearance as they are characterized by two extremely large leaves only. *Welwitschia* occurs in habitats where annual rainfall is often less than 25 mm and where condensation of coastal fog is equivalent to not more than another 50 mm of precipitation. Morphological and anatomical features which are relevant to the plant's ability to tolerate severe water stress have been studied by Bornman and colleagues (3–6). Physiological work has focused on the photosynthetic pathway by which *Welwitschia* incorporates atmospheric carbon; as yet, the data are ambiguous.

In 1971, Smith and Epstein (25) reported a $\delta^{13}\text{C}$ value of -14.4‰ for *Welwitschia*. Because of the absence of Kranz anatomy, slightly elevated extractable activities of PEP carboxylase and NADP malic enzyme compared to normal C₃ plants, and small nocturnal increases in tissue acidity, it was concluded that *Welwitschia* is a CAM plant (9). The bulk of $\delta^{13}\text{C}$ values published thereafter on samples from the Namib desert were more negative than the first published one. Typically, $\delta^{13}\text{C}$ values around -20‰ were obtained; such values are intermediate between those of regular C₃ plants and those of plants exhibiting pronounced CAM (19, 23, 28, 30). Gas exchange measurements

by Von Willert *et al.* (30) on plants *in situ* did not reveal net CO₂ uptake at any time of the night; instead, net CO₂ assimilation was restricted to daytime. The authors were unable to detect significant diurnal fluctuations in malic acid content of the leaves and concluded that *Welwitschia* is a C₃ plant. Interestingly, the diurnal course of net CO₂ exchange exhibited a two-peak pattern with a maximum CO₂ assimilation rate in the morning, a depression at noon when leaf temperatures were above 30°C and ambient humidity was very low, and a second maximum in the afternoon. Although this daytime gas exchange pattern of *Welwitschia* is similar to that of many CAM plants, midday depressions in CO₂ assimilation have been frequently observed in non-CAM desert shrubs and mediterranean sclerophylls in response to diurnal changes in environmental conditions (15, 26). In a recent laboratory study (27) measurements of ¹⁴CO₂ uptake and of stomatal conductance also suggest that net carbon gain of *Welwitschia* is via C₃ pathway. Small diurnal variations in titratable acidity did occur, however, and were interpreted to indicate that *Welwitschia* recycles respiratory CO₂ via PEP carboxylase into malic acid at night.

In the study presented here, the capacity of *Welwitschia* to perform CAM was examined. In addition, the gas exchange behavior of leaves subjected to the diurnal changes in temperature and humidity as they occur under natural conditions was investigated. The relationship between CO₂ assimilation rate and intercellular CO₂ partial pressure was assessed for a range of conditions to distinguish between environmental effects on stomata and on the photosynthetic apparatus in the mesophyll cells. The data indicate that diffusional limitations of photosynthesis may be a significant factor in contributing to the $\delta^{13}\text{C}$ values of *Welwitschia*, which are less negative than expected for a C₃ plant.

MATERIALS AND METHODS

Growth of Plants. Two plants of *Welwitschia mirabilis* Hook. fil., approximately 5 to 7 years old, were available for the experiments. They had been established from seed (obtained from the Namib desert) at the Würzburg Botanical Garden and were cultivated in cylindrical pots, 80 cm high and 14 cm in diameter, which were filled with a mixture of garden soil and sand (1:1). To allow for continuous water supply to the tap roots, pots were placed in 5 L plastic buckets filled with water. About 6 months prior to onset of experiments, plants received Hewitt's type nutrient solution (34) containing 24 mM NO₃⁻ once per week. Unless stated otherwise, plants were kept in a glasshouse and were exposed to 1500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (400–700 nm), provided by Osram Power Star metal halide lamps (HQI-T 2000 W/D) for 12 h (7 AM–7 PM). Leaf temperatures were between 28 and 32°C during daytime and ranged from 10 to 20°C at night-

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time.

Gas Exchange. A portion of attached leaf, about 20 cm², from the center of leaves was enclosed in a 600 ml leaf chamber made of nickelplated brass. Chamber temperature was controlled by water circulating via a thermostated water bath (Meßgeräte-Werk Lauda, Lauda-Königshofen, F.R.G.) through a water jacket in contact with a heat exchanger inside the cuvette. Laminar air flow within the chamber was provided by a radial fan which forced air through the fins of the heat exchanger and passed it over the leaf. The leaf was illuminated through window glass, using a metal halide lamp (HRI-T 1000 W/D; Radium Elektrizitäts-Ges., Wipperfürth, F.R.G.). Leaf and air temperatures were measured with copper-constantan thermocouples (Omega, Stamford, CT). The CO₂ partial pressure of the air entering the leaf chamber was set by 2 mass flow controllers (Tylan Corporation, Carson, CA) mixing pure CO₂ with CO₂-free air. After humidification, the air was brought to a particular dew point in a constant temperature cold trap. Air flow through the chamber was determined with a mass flow meter (MKS Instruments, Burlington, MA). The dew point of the air entering the chamber was measured with a dew point sensor (Type TMG-1; Walz Meß- and Regeltechnik, Effeltrich, F.R.G.). Changes in water vapor content due to transpiration were recorded with a differential IR gas analyzer Binos II (Leybold-Heraeus, Hanau, F.R.G.). CO₂ concentrations of the air entering and leaving the chamber were measured with an IR gas analyzer Binos II operating in an absolute mode and with a second Binos II CO₂ analyzer operating in the differential mode. The air moisture was reduced to a dew point of 2°C using a Peltier-controlled cold trap before passing through the CO₂ analyzers. Gas pressures in the atmosphere and in various parts of the gas exchange system were monitored with a mercury barometer and a U-tube manometer. Instrumental outputs were recorded with a HP 3421A Data Acquisition Unit (Hewlett-Packard) connected to a HP 41CV calculator for automatic computation of gas exchange parameters. Calculations were made according to Von Caemmerer and Farquhar (29). Data were recorded on a HP 82162A thermal printer. The CO₂ analyzers were calibrated with CO₂ generated from mixing pumps (Wösthoff KG, Bochum, F.R.G.), and the water vapor analyzer calibrated with vapor generated from two condensers kept at different temperatures.

In one preliminary experiment (Fig. 1), a large attached leaf segment (80 cm²) was enclosed into a 5 L leaf cuvette (Walz Meß- and Regeltechnik, Effeltrich, F.R.G.) and gas exchange was measured with a system which was essentially the same as has been described previously (20).

Data shown in Figure 1 refer to one set of experiments. Data shown in Figures 2 to 9 are representative of up to 4 experiments. Nocturnal patterns of net CO₂ exchange were monitored approximately 30 times.

Titrateable Acidity. Leaf material (about 1.5 g fresh weight) was boiled in 20% (v/v) methanol for 20 min. Extracts were titrated with 10 mM NaOH to pH 6.5.

RESULTS

In a series of experiments performed in 1982, clear evidence was obtained that *Welwitschia* has a certain, albeit extremely low, potential to exhibit CAM. In leaves kept at 30°C during the 12 h light period and at 15°C during the 12 h dark period, a minute but definite net uptake of CO₂ was measured for the first 3 h of the dark period (maximal rate 0.1 μmol m⁻² s⁻¹) (Fig. 1). At 20°C in the dark, neither net CO₂ loss nor net CO₂ uptake were observed during the first 5 h. At 25°C, CO₂ was continuously lost to the atmosphere. Yet the nocturnal pattern of net CO₂ exchange at 25°C was similar to that at 20 and 15°C inasmuch as the rate of net CO₂ loss was reduced for the initial part of the night. These data are best explained by a transitory increase in

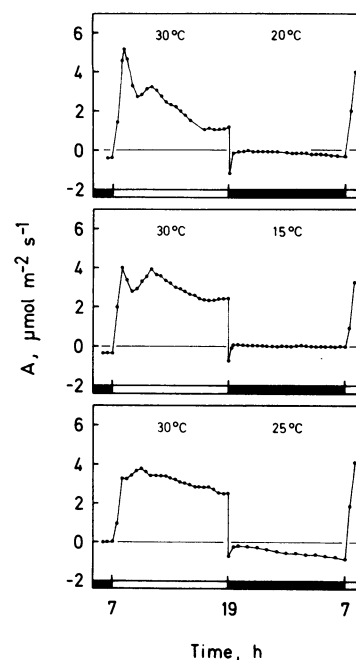


FIG. 1. Net CO₂ exchange, A, of *W. mirabilis* at various temperature regimes during 12-h light/12-h dark cycles. PAR was 1200 μmol quanta m⁻² s⁻¹. The ΔW was 20 mbar bar⁻¹ at 30°C, 10 mbar bar⁻¹ at 25 and 20°C, and 5 mbar bar⁻¹ at 15°C. The p_a was 350 μbar.

the capacity for dark CO₂ fixation, which results in either net carbon gain, zero net gas exchange with the atmosphere, or net carbon loss, depending on the temperature-related intracellular concentration of CO₂ provided by respiration. The low capacity for CAM in *Welwitschia* does not seem insignificant in terms of its impact on total carbon gain. If CAM were completely absent and assuming that the rate of respiratory CO₂ loss measured at the end of the dark period (Fig. 1) had occurred throughout the entire night, then the 24-h carbon gain from 30°C day/20°C night and 30°C day/25°C night regimes would have been reduced by 7.5 and 15.7%, respectively. In leaves kept under a day/night regime of 30/15°C, small nocturnal increases in titrateable acidity were observed; in one experiment acidity increased from 26.1 ± 3.6 to 38.1 ± 2.7 μeq g⁻¹ fresh weight (mean ± SD, n = 3) and in a second from 23.6 ± 4.5 to 36.4 ± 2.1.

In a more detailed investigation performed in 1984, net dark CO₂ fixation was never observed under similar conditions, yet there was always a reduction in the rate of net CO₂ loss, frequently to zero, during the early dark period (Fig. 2). During the early light period, a slight depression in net CO₂ uptake and stomatal conductance was repeatedly monitored (see also Fig. 1). At a p_a² of 350 μbar CO₂, a leaf temperature of 30°C and a moderate ΔW of 21 mbar bar⁻¹, the p_i/p_a ratio remained around 0.65 during the light period which is within the range of values (0.6–0.8) usually observed during photosynthetic CO₂ uptake of C₃ plants (2). At 15°C and a low ΔW in the dark, the initially low stomatal conductance gradually increased in the course of the dark period to reach a value as high as the conductance during the light period (Fig. 2). At 15°C and a higher ΔW, stomatal conductance did not attain such high values at night (data not shown). Although unknown to many, it has long been known that nocturnal opening of stomata is not confined to plants exhibiting pronounced CAM but also occurs in many C₃ plants at night as part of a circadian stomatal rhythm (e.g. 8).

² Abbreviations: p_a, ambient CO₂ partial pressure; A, net CO₂ assimilation; E, transpiration; g, leaf conductance to vapor transfer; p_i, intercellular CO₂ partial pressure; ΔW, leaf-air vapor pressure difference.

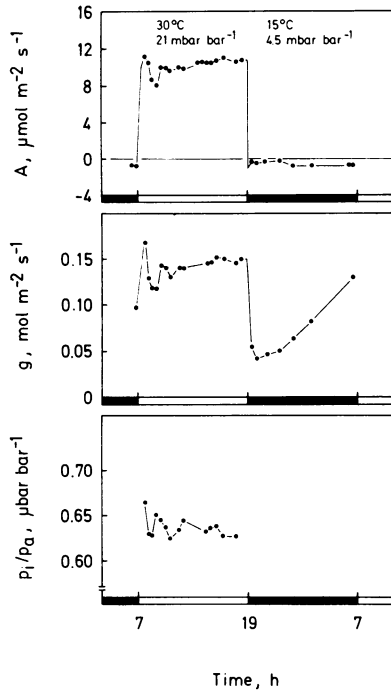


FIG. 2. A , g , and the p_i/p_a of *W. mirabilis* during a 12-h light/12-dark cycle. PAR was $2000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, p_a was $350 \mu\text{bar}$. Leaf temperature and ΔW are given in the uppermost subfigure.

In experiments designed to simulate the diurnal variations in temperature and humidity of the natural habitat in the Namib desert (30), leaf temperature was increased in increments from 20 to 36°C during the first 5 h of the 12 h light period, maintained at 36°C for the following 2 h, and then decreased in increments to 20°C during the last 5 h (Fig. 3). In parallel, ΔW increased from 15 to 50 mbar bar^{-1} . No attempt was made to simulate the natural diurnal change in illumination. The photon fluence density was kept constant at $2000 \mu\text{mol incident quanta m}^{-2} \text{s}^{-1}$. The p_a was $350 \mu\text{bar}$. As leaf temperature and ΔW approached 36°C and 50 mbar bar^{-1} , respectively, CO_2 assimilation rate and transpiration rate dropped, and increased again during the second part of the light period when leaf temperature and ΔW decreased (Fig. 3). The decrease in A was accompanied by a decline in stomatal conductance and by a decline in the p_i/p_a ratio from 0.65 to 0.5.

A similar midday depression in CO_2 assimilation and other gas exchange parameters was observed when the p_a was kept at $1750 \mu\text{bar}$ (Fig. 4). At this p_a , stomatal conductance declined to much lower values and the same applied to p_i/p_a which decreased from 0.75 to 0.35.

Stomata of *Welwitschia* are very responsive to the humidity of the surrounding atmosphere as demonstrated in Figure 5. Leaf temperature was held constant at 30°C and ΔW was changed from 33 to 17 mbar bar^{-1} and vice versa every 2 h. At high ΔW , CO_2 assimilation and transpiration both decreased and at the same time there were decreases in both stomatal conductance and in p_i/p_a .

To determine whether stomatal closure was the main reason for the decline in CO_2 assimilation rate at high leaf temperatures and high ΔW , or whether the photosynthetic capacity of the mesophyll cells also was affected, responses of CO_2 assimilation to intercellular CO_2 partial pressure were measured at various temperatures and humidities. At 20°C and a moderate ΔW of 14 mbar bar^{-1} , CO_2 assimilation was saturated at a p_i of about $1400 \mu\text{bar}$, which corresponds to a p_a of $1750 \mu\text{bar}$ (Fig. 6). At 30°C , saturation of photosynthesis was not achieved at $p_a = 1750 \mu\text{bar}$

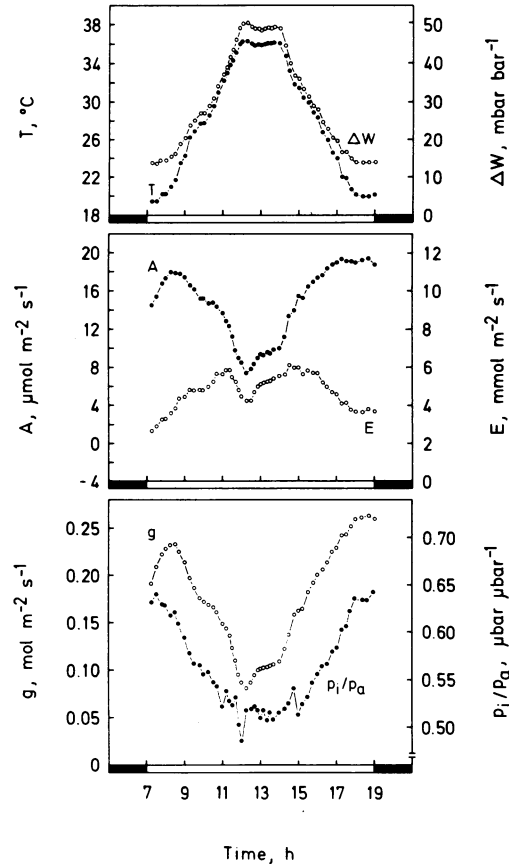


FIG. 3. A , E , g , and the p_i/p_a in *W. mirabilis*, exposed to a diurnal course in leaf temperature, T , and ΔW , characteristic of a natural d in the Namib desert. PAR was $2000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, the p_a was $350 \mu\text{bar}$.

CO_2 (refers to $p_i < 1000 \mu\text{bar CO}_2$) due to an overall reduction in stomatal conductance (Fig. 7). Therefore, the data of Figure 4 (diurnal course in CO_2 assimilation etc. at $p_a = 1750 \mu\text{bar CO}_2$) cannot be interpreted to indicate direct effects of the environmental conditions during midday on the mesophyll photosynthesis reactions. The gas exchange system in its available configuration did not permit photosynthesis measurements to be performed beyond p_a of $1800 \mu\text{bar CO}_2$. CO_2 response curves at 30°C were not different at two values of ΔW , 16 and 33 mbar bar^{-1} (Fig. 7). At the high ΔW , decreased stomatal conductance caused CO_2 assimilation to operate at a lower p_i for a given p_a , with the A - p_i relationship remaining unchanged.

The initial slope of the CO_2 response curves (carboxylation efficiency) was the same when leaf temperature was increased from 20 to 36°C accompanied by an increase in ΔW from 14 to 51 mbar bar^{-1} (Fig. 8). For a p_a of $350 \mu\text{bar CO}_2$, the corresponding p_i decreased from 220 (20°C , 14 mbar bar^{-1}) to $170 \mu\text{bar CO}_2$ (36°C , 51 mbar bar^{-1}) due to reduced stomatal conductance. To assess the relative stomatal limitation of assimilation rate at ambient CO_2 , rates of $p_a = 350 \mu\text{bar CO}_2$ were compared with rates at a higher p_a , at which p_i was $350 \mu\text{bar CO}_2$, i.e. determinations were made of the percentage reduction in photosynthesis below the rate which would occur if stomatal conductance were infinite (11, 14) (Fig. 9). Stomatal limitation of CO_2 assimilation increased from about 30% to more than 50% when leaf temperature and ΔW were increased from 20°C and $14.5 \text{ mbar bar}^{-1}$ to 33°C and 41 mbar bar^{-1} . Similar data can be calculated from the CO_2 response curves shown in Figures 6 and 7. For 20°C and 14 mbar bar^{-1} , stomatal limitation was 29%. At 30°C and a low ΔW of 16 mbar bar^{-1} , stomatal limitation was 34 and 39% in

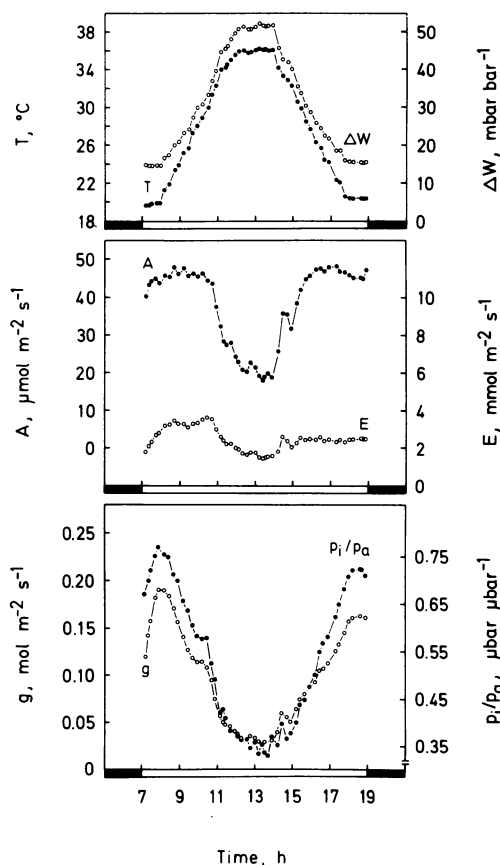


FIG. 4. As in Figure 3, but the p_a was 1750 μbar .

two separate experiments, and increased to 46 and 50% when measurements were conducted at the higher ΔW of 33 mbar bar^{-1} .

DISCUSSION

The reduction in CO_2 assimilation rate in *Welwitschia* exposed to high temperatures and a high leaf-air vapor pressure difference is due primarily to stomatal closure. As a consequence, the limitation of photosynthesis by CO_2 diffusion is increased. This is reflected in a lowered p_i/p_a , which is a measure of an increased gradient in CO_2 partial pressure across the stomata. Both ribulose biphosphate carboxylase and CO_2 diffusion contribute to the fractionation of carbon isotopes during incorporation of atmospheric CO_2 via the C_3 pathway (16). Theoretical considerations indicate that increased diffusional limitation of photosynthesis leads to less discrimination against ^{13}C and, hence, to less negative $\delta^{13}\text{C}$ values of total plant carbon (12, 16). This has been experimentally confirmed in several studies comparing p_i/p_a and $\delta^{13}\text{C}$ value of plants grown for prolonged periods under conditions of water or salt stress (10, 13, 32). Also, growth at low RH has been found to lead to less negative $\delta^{13}\text{C}$ values (33). A mathematical relationship between the average p_i/p_a at which photosynthesis operates in a plant in the long run, and the $\delta^{13}\text{C}$ value has been derived by Farquhar *et al.* (12). According to this relationship, the $\delta^{13}\text{C}$ value of a *Welwitschia* plant, which would operate at an average p_i/p_a of 0.55 under natural conditions (Fig. 3), is less negative by 3.4‰ compared to plants in which photosynthesis is less limited by diffusion and which operate at a p_i/p_a of 0.7, a value most commonly observed in laboratory studies with C_3 plants. The C_3 species found alongside *Welwitschia* in the Namib desert have a mean $\delta^{13}\text{C}$ value of -23.6‰ (23). Thus, a 3.4‰ reduction in $\delta^{13}\text{C}$ value would suffice to explain the

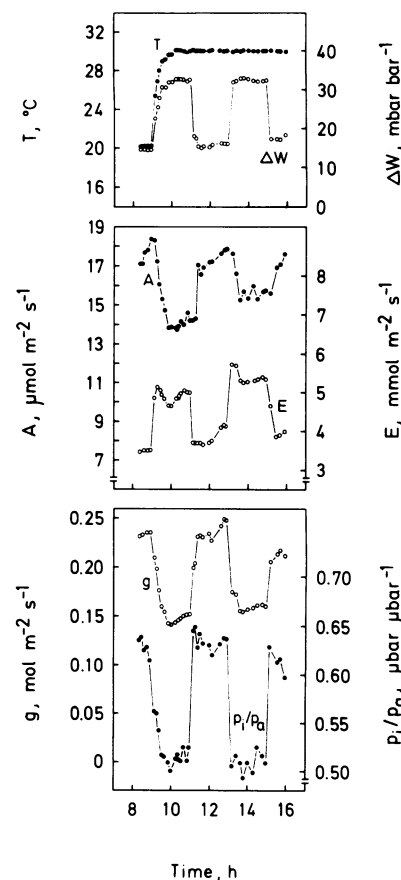


FIG. 5. Effect of short-term changes in ΔW on A, E, g, and p_i/p_a in *W. mirabilis*. Leaf temperature, T, was 30°C as shown, PAR was 2000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and the p_a was 350 μbar .

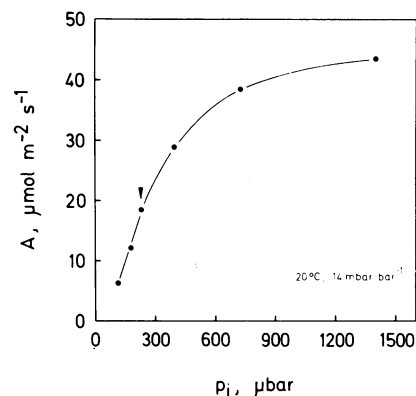


FIG. 6. Response of A to p_i at 20°C leaf temperature and a ΔW of 14 mbar bar^{-1} . PAR was 2000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The arrow indicates measurements made at 350 $\mu\text{bar } p_a$.

majority of carbon isotope ratios observed for *Welwitschia* in the field, although it must be borne in mind that a value of -23.6‰ is already less negative than typically seen in regular C_3 plants from other areas and may itself be indicative of increased diffusional limitation of CO_2 uptake. Further consideration should be given to the fact that these well-watered, high nutrient-status, glasshouse-grown plants exhibit maximum net CO_2 uptake rates higher than those previously reported for plants *in situ* (30). The average p_i/p_a ratios exhibited by both *Welwitschia* and its neighboring C_3 species in the Namib desert have yet to be determined.

A second, ecologically important consequence of a low p_i/p_a

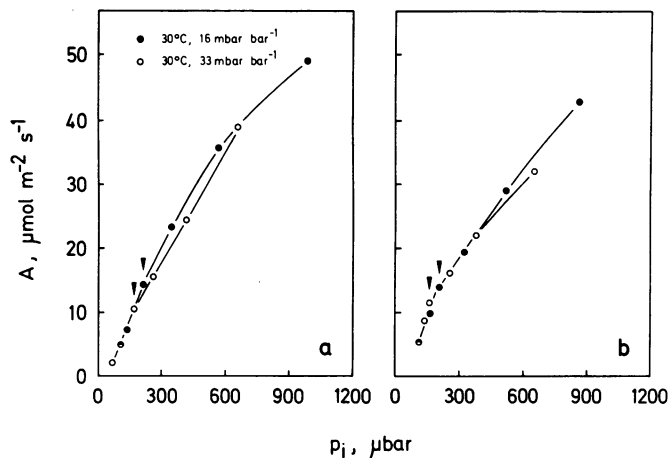


FIG. 7. Response of A to p_i in *W. mirabilis* at 30°C leaf temperature, and a high and low ΔW , respectively. It took 3 to 4 h to obtain a single CO_2 response curve, because gas exchange only slowly reached a new steady state after a change in the p_a . On d 1 (a), the curve at 16 mbar bar^{-1} was established in the morning, followed by the curve at 33 mbar bar^{-1} in the afternoon. To rule out complications due to hysteresis effects and possible diurnal fluctuations in photosynthetic capacity, experiments were repeated in reverse order on d 2 (b). PAR was 2000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Arrows indicate measurements made at 350 $\mu\text{bar } p_a$.

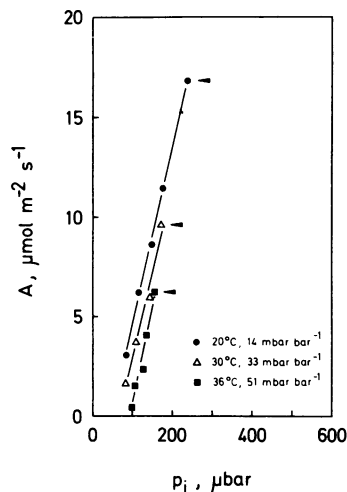


FIG. 8. Relationship between A and p_i in the initial slope region of the CO_2 response curves for *W. mirabilis* exposed to increasing leaf temperature and increasing ΔW . Arrows indicate measurements made at 350 $\mu\text{bar } p_a$. PAR was 2000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$.

is an increased ratio of A/E , i.e. an increased water use efficiency of photosynthesis, provided comparisons are made for a defined set of environmental conditions. Under midday conditions of 36°C and a ΔW of 50 mbar bar^{-1} , a p_i/p_a of 0.5 corresponds to water loss of about 500 mol per mol CO_2 fixed. Water loss would have been 1.4 times higher, had the p_i/p_a remained at 0.65, the ratio observed at the beginning and end of the simulated d (Fig. 3).

In contrast to several other species exhibiting a midday depression in CO_2 assimilation and in stomatal conductance, we did not find evidence that the depression in *Welwitschia* was mediated by direct effects of high temperature and, in particular, high ΔW on the photosynthetic apparatus in the mesophyll cells (e.g. Figs. 7 and 8). High rates of transpiration at high ΔW may cause CO_2 assimilation rate, measured at supra-atmospheric CO_2 pressures, to decrease, possibly because local water deficits develop within leaves (24). In *Welwitschia*, however, transpiration

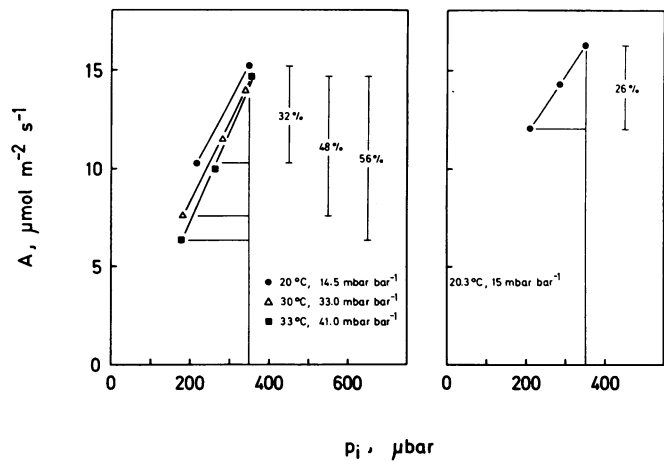


FIG. 9. Percentage stomatal limitation of net CO_2 assimilation at 350 $\mu\text{bar } p_a$ in *W. mirabilis* exposed to increasing leaf temperature and increasing ΔW . For each set of conditions, the response of CO_2 assimilation to intercellular CO_2 partial pressure was established between $p_i = 350 \mu\text{bar } \text{CO}_2$ and $p_i = 350 \mu\text{bar } \text{CO}_2$. Net CO_2 assimilation rate at $p_i = 350 \mu\text{bar } \text{CO}_2$ was set 100%. Experiments were started at 20°C and 14.5 mbar bar^{-1} in the morning. After the increase in temperature and ΔW , the first set of conditions was applied again in the afternoon of the same day (right subfigure). PAR was 2000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$.

decreases at very high ΔW due to the strong decline in stomatal conductance (Fig. 3), which may lead to a more favorable water content of cells rather than to a water deficit (e.g. 21). In those species in which high ΔW has been reported to reduce photosynthetic reactions directly (18, 22, 26), hormonal action might be involved (7, 17). These direct effects on the photosynthetic machinery result in p_i/p_a remaining more or less constant and, therefore, diffusional limitations of photosynthesis do not change.

The least negative $\delta^{13}\text{C}$ values reported for *Welwitschia* thus far (-14.4 and -17.4‰) (23, 25) are probably not due to increased diffusional limitation of photosynthesis alone. If this were so, photosynthetic CO_2 uptake in the light would have to operate at p_i values either below or slightly above the CO_2 compensation point. Data presented here and in two previous communications (9, 27) suggest a very low potential for CAM in *Welwitschia*. Net dark CO_2 fixation was barely observable yet reduced rates of net CO_2 loss for extended periods of the night, obtained under constant temperature conditions, clearly indicate a higher capacity for dark CO_2 fixation in *Welwitschia* than in regular C_3 leaves. In C_3 leaves kept at constant temperature, net CO_2 exchange never changes in such a manner in the course of a night. Rather, net CO_2 loss occurs at a more or less constant rate, or even decreases during prolonged darkness (1). The pattern of nocturnal net CO_2 exchange seen in *Welwitschia* is similar to that of inducible CAM plants like *Mesembryanthemum crystallinum* during early stages of the development of CAM (31). It is conceivable that, under certain conditions, nocturnal CO_2 fixation is much more pronounced than has been hitherto observed, thereby leading to the relatively high ^{13}C content which is occasionally found in this species.

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