Plant Physiol. (1986) 81, 390–394
0032-0889/86/81/0390/05/$01.00/0

Respiratory CO_2 as Carbon Source for Nocturnal Acid Synthesis at High Temperatures in Three Species Exhibiting Crassulacean Acid Metabolism[1]

Received for publication September 24, 1985 and in revised form January 16, 1986

KLAUS WINTER*, GABRIELE SCHRÖPPEL-MEIER, AND MARTYN M. CALDWELL[2]
Lehrstuhl für Botanik II, Universität Würzburg, Mittlerer Dallenbergweg 64, 8700 Würzburg, West Germany

ABSTRACT

Temperature effects on nocturnal carbon gain and nocturnal acid accumulation were studied in three species of plants exhibiting Crassulacean acid metabolism: *Mamillaria woodsii*, *Opuntia vulgaris*, and *Kalanchoë daigremontiana*. Under conditions of high soil moisture, nocturnal CO_2 gain and acid accumulation had temperature optima at 15 to 20°C. Between 5 and 15°C, uptake of atmospheric CO_2 largely accounted for acid accumulation. At higher tissue temperatures, acid accumulation exceeded net carbon gain indicating that acid synthesis was partly due to recycling of respiratory CO_2. When plants were kept in CO_2-free air, acid accumulation based on respiratory CO_2 was highest at 25 to 35°C. Net acid synthesis occurred up to 45°C, although the nocturnal carbon balance became largely negative above 25 to 35°C. Under conditions of water stress, net CO_2 exchange and nocturnal acid accumulation were reduced. Acid accumulation was proportionally more decreased at low than at high temperatures. Acid accumulation was either similar over the whole temperature range (5–45°C) or showed an optimum at high temperatures, although net carbon balance became very negative with increasing tissue temperatures. Conservation of carbon by recycling respiratory CO_2 was temperature dependent. At 30°C, about 80% of the dark respiratory CO_2 was conserved by dark CO_2 fixation, in both well irrigated and water stressed plants.

Plants exhibiting CAM are known for their ability to assimilate atmospheric CO_2 at night and to store this carbon in the form of malic acid, usually with 1 mol CO_2 incorporated leading to 1 mol malic acid, *i.e.* to 2 mol H^+ produced (for review, see Ref. 15). In 1973, Szarek *et al.* (10) noted deviations from this stoichiometry for *Opuntia basilaris* during periods of drought: stomatal conductances were very low, reducing nocturnal CO_2 uptake to negligible amounts, although the diurnal fluctuation of malic acid persisted in the chloroplast-containing tissue. To describe these phenomena, a new term was created, 'CAM-idling', which has been defined as follows: stomata remain closed during both the daytime and nighttime, conserving water while restricting the uptake of external CO_2, yet acid metabolism continues by the recycling of respiratory CO_2 (2, 7–9, 12, 13). CAM-idling has been interpreted as a carbon-conserving mechanism allowing succulent plants to survive periods of drought in a moderately active state, which, in turn, enables them to rapidly respond to precipitation and assimilate atmospheric CO_2.

The above conclusions are based largely on intermittent porometer measurements of stomatal conductance and $^{14}CO_2$ uptake during 24-h cycles. This approach only allows one to make qualitative statements about the use of respiratory CO_2 as the substrate for nocturnal acid synthesis and the extent of carbon conservation. In the study presented here, we have attempted a quantitative approach by continuously monitoring nocturnal net CO_2 exchange of plants. Three CAM species were considered which differed in the degree of succulence and in the volume ratio of nongreen water-storage tissue to chloroplast-containing tissue. The role of recycling of respiratory CO_2 in the plants' carbon budgets was assessed for a range of night temperatures, both in well watered and in water stressed plants.

MATERIALS AND METHODS

Plant Material. *Mamillaria*[3] *woodsii* Craig and *Opuntia vulgaris* Mill. were obtained from a local nursery. *Kalanchoë daigremontiana* Hamet et Perr. was grown from bulbils. Plants were kept for at least 2 months in a glasshouse prior to experiments. Daytime temperatures were, on average, 25 to 30°C, nighttime temperatures about 15°C. Photon fluence rates (400–700 nm) were as high as 1.5 mmol m^{-2} s^{-1} on sunny days. Natural daylight was supplemented by 400 W mercury vapor lamps from 7 AM to 7 PM. Plants were watered daily and received nutrient solution containing 24 mol m^{-3} NO_3^- once a week. Plants of *O. vulgaris* consisted of two cladodes, one possessing roots and embedded in the soil, and the second growing above this. The upper cladode, about 15 cm long, 1 cm thick by 2 to 3 cm broad, was used in the experiments. Plants of *K. daigremontiana* were about 3 months old and recently expanded leaves were used. Two groups of experimental plants of *M. woodsii* were available, one with an average initial fresh weight of about 67 g, and a second, older one with an average fresh weight of 113 g.

To induce water stress, roots were removed from *M. woodsii* and plants were kept for up to 1 year on metal grating without substrate in a glasshouse. The terminal cladode of *O. vulgaris* was cut off and did not have access to soil. Cladode orientation was such that both sides received light during the stress treatment. In the case of *K. daigremontiana*, leaves were detached and kept on metal grating. Cut surfaces which were air-dried were small in relation to the total surface area of the plant material.

Experimental Procedures. One week before experiments com-

[1] Supported by the Deutsche Forschungsgemeinschaft.

[2] Permanent address: Department of Range Science, Utah State University, Logan, UT 84322. Supported by A. V. Humboldt-Stiftung.

[3] In the literature, the genus is spelled either *Mamillaria* (orthographically correct) or *Mammillaria* (nomenclatorially correct). The name is based on the Latin diminutive of *mamma* = *mamilla*.

menced, the plant material (either intact plants or, in the case of stress-treatments, detached leaves or cladodes) were transferred to a growth cabinet and exposed to a 12 h light (1.0 mmol m^{-2} s^{-1}, 30°C)/12 h dark cycle (20°C), unless stated otherwise. The dew point of the air in the growth cabinet was 10°C during both the dark and light periods.

Leaves (*K. daigremontiana*), cladodes (*O. vulgaris*), and whole plants (*M. woodsii*) were taken from the growth cabinet at the end of the 12 h light period and kept overnight in a gas exchange chamber (Walz Mess- und Regeltechnik, Effeltrich, F.R.G.). Roots of nonstressed plants of *M. woodsii* were removed prior to enclosure of plants in the chamber. Cut surfaces were sealed with silicone grease. The CO_2 partial pressure of the air entering the chamber was at 350 μbar. Chamber temperature was held between 5 and 45°C using Peltier heat exchangers. The dew point of the air entering the chamber was adjusted by passing water-saturated air through a Peltier-cooled condenser. The tissue-air vapor pressure difference was 2 mbar at 5°C, 3 mbar at 10°C, 7 ± 0.5 mbar between 15 and 35°C, 18 mbar at 40°C, and 36 mbar at 45°C. Dew points of the air entering and leaving the chamber were measured with dew point sensors (Walz Mess- und Regeltechnik, Effeltrich, F.R.G.). Net CO_2 exchange was continuously monitored with an IR gas analyzer BINOS I (Leyboldt-Heraeus, Hanau, F.R.G.) operating in the differential mode. At the end of the 12-h dark period, the plant material was plunged into liquid N_2 and then stored at −20°C.

After thawing, the samples were homogenized with a blender (Ultra-Turrax, IKA-Werk, Staufen, F.R.G.) and boiled in 20% (v/v) methanol for 20 min. Extracts were titrated with 50 mM NaOH to pH 6.5. For calculation of nocturnal increase in tissue acidity (titratable acidity at the end of the dark period minus titratable acidity at the end of the light period), representative samples were harvested at the end of the light period and processed for determination of titratable acidity as described above.

To determine the degree of nocturnal acidification in CO_2-free air, individual detached leaves (*K. daigremontiana*), cladodes (*O. vulgaris*), and whole plants, freed of roots, (*M. woodsii*) were kept in cylindric containers (22 cm long, 7 cm in diameter) overnight which were flushed with CO_2-free air at a flow rate of 1.7 L min^{-1}. The containers were kept in a growth cabinet which allowed for temperature control between 5 and 45°C. CO_2-free air was obtained by passing ambient air through soda lime.

Osmotic potentials were measured using a psychrometric method. Tissue sap was obtained by thawing frozen samples and placed in C-52 Sample Chambers (Wescor, Logan, UT). The psychrometric output was measured with a Wescor HR 33T Microvoltmeter.

For determination of dry weights, tissue samples were kept in an oven at 100°C for 48 h. Chl was measured according to Arnon (1).

RESULTS

Some characteristics of tissue water status of stressed and nonstressed plants are presented in Table I. CO_2 exchange studies on water stressed plants commenced when the original fresh weight of the species was reduced by approximately 50% (Fig. 1) and when the fresh weight/dry weight ratio was 4 to 6. Additional measurements were made on *M. woodsii* after a reduction of the original fresh weight to 30%. Rate of water loss was most pronounced in leaves of *K. daigremontiana* which had the highest surface/volume ratio.

In the absence of water stress, the relationship between nocturnal acidification and net CO_2 assimilation at temperatures from 5 to 45°C showed similar patterns for all three species (Figs. 2A, 3A, and 4A). For example, in *M. woodsii* (Fig. 2A), at low temperatures (5–15°C), uptake of atmospheric CO_2 accounted for essentially all the acid accumulated in the course of the 12 h dark period, *i.e.* the ratio of H^+ produced:CO_2 fixed was about 2:1 (assuming that 1 CO_2 is equivalent to 1 $malate^{2-}$ + 2 H^+). A similar ratio has been reported for other well watered CAM species within this temperature range (3, 4, 6). At higher temperatures, this ratio increased. Above 30°C, *M. woodsii* no longer exhibited net CO_2 dark fixation at any time during the dark period and the net CO_2 balance became increasingly negative with increasing temperature. Yet, net acid synthesis continued to occur up to 45°C. At 30°C and above, the degree of acidification was the same in normal air and in CO_2-free air. It is conceivable that respiratory CO_2 was the exclusive carbon source for dark CO_2 fixation and acid synthesis under these high temperature conditions. Similar results were obtained with well watered plants of *O. vulgaris* (Fig. 3A) and *K. daigremontiana* (Fig. 4A) and are implicit in studies on *Sempervivum montanum* (14). Exposure to CO_2-free air may underestimate the use of respiratory CO_2 for acid synthesis, particularly at low temperatures, due to stomatal opening and consequent loss of CO_2. This, however, was minimized in the experiment shown in Figure 5. Leaves of *K. daigremontiana* were enclosed overnight in airtight 0.16 mm thick plastic wrap to restrict both the availability of external CO_2 and the escape of respiratory CO_2. These exhibited a nocturnal acidification temperature response with an optimum around 30 to 35°C similar to leaves kept in CO_2-free air.

Some physiological differences between species may be related to increased proportions of nongreen water-storage tissue in *O. vulgaris* and *M. woodsii* compared to the leaves of *K. daigremontiana* which have relatively small differences in chloroplast distributions in mesophyll cells. *K. daigremontiana* had a positive CO_2 balance at night up to 35°C. In *M. woodsii* and *O. vulgaris* the CO_2 balance became negative above 28 to 30°C. The temperature at which acidification was twice as high as nocturnal carbon gain, *i.e.* where respiration donates half of the carbon for acid synthesis, was lowest in *M. woodsii* (25°C), intermediate in *O. vulgaris* (27°C), and highest in *K. daigremontiana* (33°C).

Under conditions of severe water stress, dark CO_2 fixation and nocturnal acidification were greatly depressed (Figs. 2C, 3B, and 4B) and temperature compensation points for nocturnal carbon gain decreased. In only one case, *i.e.* in *M. woodsii* kept for 325 d without water was there no longer nocturnal net CO_2 fixation seen at any given temperature (Fig. 2C). Yet, slight nocturnal acidification was still detectable. In all other cases, under severe water stress net CO_2 uptake was still observable at low temperatures, while at high temperatures there was always a negative CO_2 balance. Accumulation of acid at night was observed at all temperatures in all stress treatments. For each species, the degree of acidification remained relatively constant between 15 and 35°C and decreased slightly at the lower and higher temperatures. Obviously, water stress led to a proportionally larger decrease of acid synthesis at low temperatures where acid synthesis relied predominantly on uptake of external CO_2 and, hence, on open stomata. Water stress had proportionally less effect at high temperatures where acid synthesis depends mainly on internal, respiratory CO_2. This is further illustrated in Figures 6 and 7, which show the results from a time-course experiment, in which leaves of *K. daigremontiana* were wilted for up to 7 weeks. The leaf fresh weight decreased to 30% of its initial value and the osmotic potential declined from −2.5 to −8 bar (Fig. 6). Acidification and carbon balance were determined for two night temperatures, 15 and 30°C. At 15°C (Fig. 7A), acid accumulation was mainly a function of external CO_2 uptake and both parameters declined, more or less concomitantly as leaf water content decreased. After 7 weeks of stress, a slightly positive nocturnal carbon balance was detectable accompanied by acid accumulation which was twice as high as carbon gain. At 30°C, a completely different pattern was observed (Fig. 7B). After 3 weeks without irrigation, the nocturnal carbon balance was negative, yet acid accumula-

Table I. *Characteristics of Three CAM Species at Various Stages of a Water Stress Treatment*

Values are for whole plants without roots (*M. woodsii*), cladodes (*O. vulgaris*) and leaves (*K. daigremontiana*). The data are mean values ± SD from three to five samples.[a]

Parameter	Species and Days without Irrigation						
	M. woodsii			*O. vulgaris*		*K. daigremontiana*	
	0	64	325	0	46	0	25
Osmotic potential (bar)	−6.3 ± 0.4	−7.8 ± 0.3	−19.0 ± 1.2	−4.9 ± 0.3	−12.1 ± 0.6	−2.5 ± 0.4	−6.7 ± 0.9
Water content (mg cm^{-2})	ND	ND	ND	602 ± 60	265 ± 11	174 ± 10	125 ± 2
FW DW^{-1}	7.6 ± 2.6	5.0 ± 0.4	3.0 ± 0.3	11.5 ± 1.3	4.0 ± 0.1	14.9 ± 1.4	6.1 ± 0.1
Chl (mg g^{-1} FW)	0.14 ± 0.07	0.21 ± 0.03	0.18 ± 0.04	0.20 ± 0.03	0.37 ± 0.03	0.41 ± 0.08	0.47 ± 0.04

[a] FW and DW, fresh and dry weights, respectively; ND, not determined.

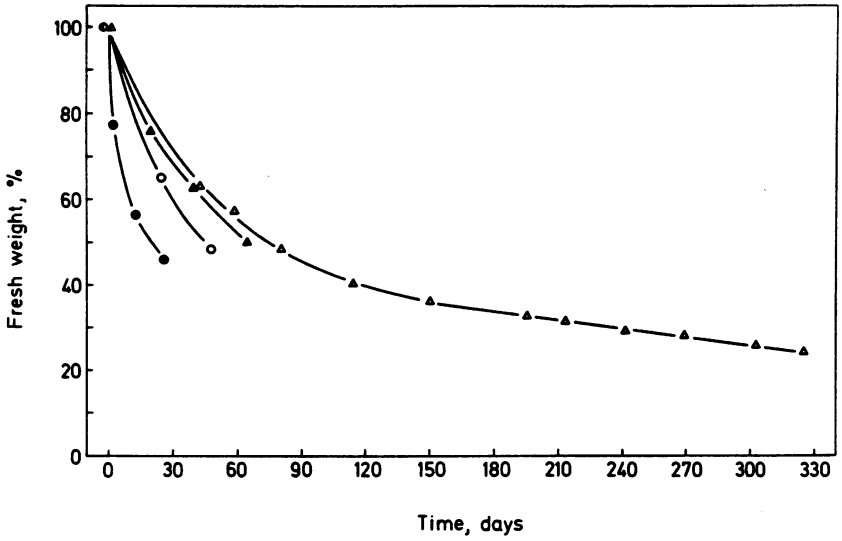

FIG. 1. Relative change in fresh weight as percentage of the original value after withholding water from plants of *M. woodsii* (▲, △), cladodes of *O. vulgaris* (○), and leaves of *K. daigremontiana* (●) for various lengths of time. One hundred percent fresh weight refers to 67 ± 7 g (▲) and 113 ± 10 g (△) for two groups of *M. woodsii* plants, to 15.6 ± 0.4 g for *O. vulgaris*, and to 22.8 ± 0.4 g for *K. daigremontiana*. The first group of *M. woodsii* was used for gas exchange and acidity measurements on d 0 and on d 64, the second group was used after withholding water for 325 d.

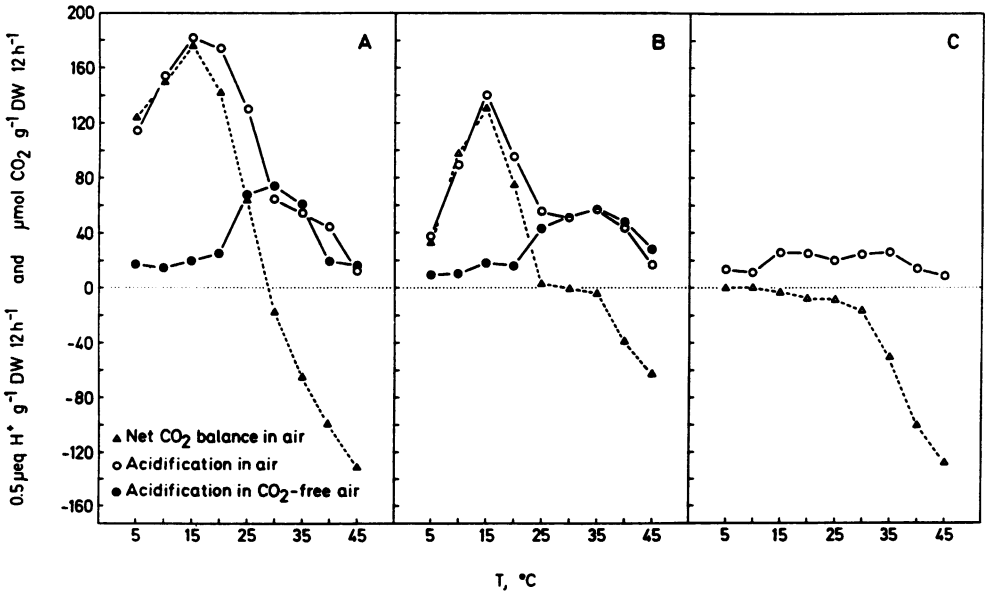

FIG. 2. Effect of nighttime temperature on net CO_2 balance (△) and the concomitant change in titratable acidity (○) (level at the end of the dark period minus level at the beginning of the dark period) in the aboveground tissue of plants of *M. woodsii* during a 12 h dark period in normal air (350 μbar CO_2). The nocturnal change in titratable acidity is also shown for plants kept in CO_2-free air (●). Results are for well-watered plants (A), and for plants after 64 (B) and 325 (C) d without irrigation. Each data point represents the mean of two independent determinations. DW, dry weight.

tion was still relatively high. After 7 weeks of stress, net carbon loss increased but net nocturnal acid synthesis was higher than at 15°C. Thus, due to high rates of respiration, CO_2 supply for acid synthesis was apparently greater at 30°C, than the combined CO_2 supply from atmospheric uptake and respiration at 15°C. At 30°C, higher rates of carboxylation probably also contributed to the higher nocturnal acidification under these water stress conditions (Fig. 7B).

DISCUSSION

High respiration rates at high tissue temperatures resulted in a significant CO_2 recycling via CAM in these plants, irrespective of water status. Under some circumstances this led to the paradoxical result that, under conditions of water stress, nocturnal acid accumulation was more pronounced at high night temperatures when the nocturnal carbon balance was negative than at low temperatures when the carbon balance was positive (Fig. 7).

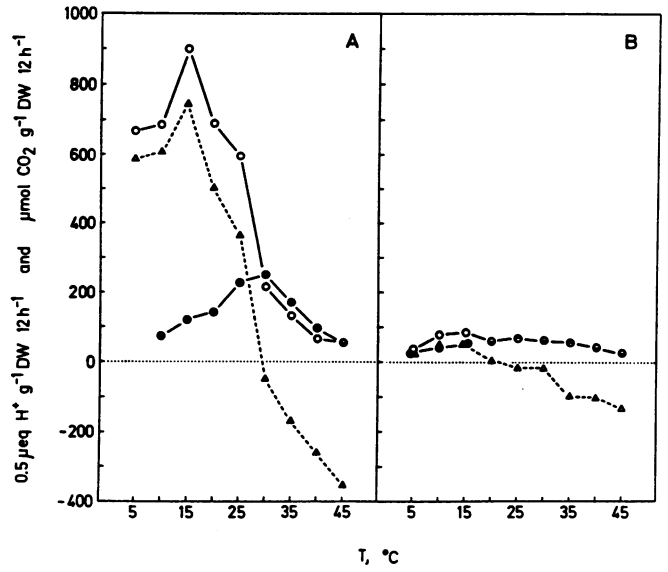

FIG. 3. Nocturnal net CO_2 balance and nocturnal change in titratable acidity in cladodes of *O. vulgaris* before (A) and after 46 d (B) of withholding of water. Each data point represents the mean of two independent determinations. Cladodes of well irrigated plants had a specific weight (g DW m^{-2} (one surface) ± SD, n =3) of 577 ± 100, which was increased to 933 ± 179 after 46 d of water stress. See legend of Figure 2 for further details.

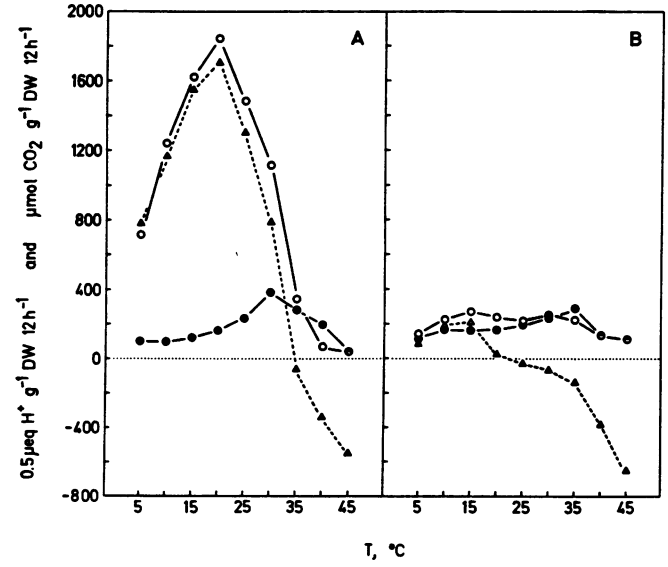

FIG. 4. Nocturnal net CO_2 balance and nocturnal change in titratable acidity in leaves of *K. daigremontiana* before (A) and after 25 d (B) of withholding of water. Each data point represents the mean of two independent determinations. Leaves of well irrigated plants had a specific weight (g DW m^{-2} (one surface) ± SD, n =4) of 126 ± 9, which was increased to 244 ± 3 after 25 d of water stress. See legend of Figure 2 for further details.

A high degree of nocturnal acidification utilizing respiratory CO_2 does not necessarily reflect the efficient conservation of carbon, particularly at temperatures above 35°C, where considerable amounts of carbon may be lost to the atmosphere (Figs. 2–4). In the field, acclimation phenomena probably improve the potential for carbon conservation at high temperatures. Experiments are underway to study the recycling of CO_2 via CAM in intact plants, preconditioned to various temperatures and exposed to drought stress. In *Opuntia basilaris*, the temperature optimum for net dark respiration is 35 to 40°C (11). Biochemical limitations, *e.g.*

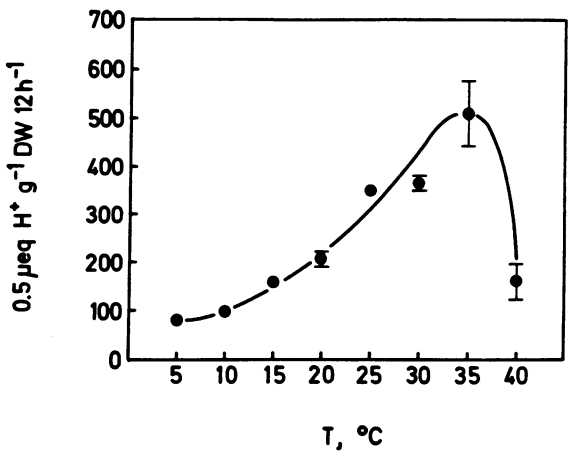

FIG. 5. Effect of nighttime temperature on the nocturnal increase in titratable acidity in leaves of well watered plants of *K. daigremontiana* during a 12 h dark period, during which leaves were enclosed in airtight plastic wrap. Each data point represents the mean of 2 or 5 samples. In the latter case, bars indicate SD. DW, dry weight.

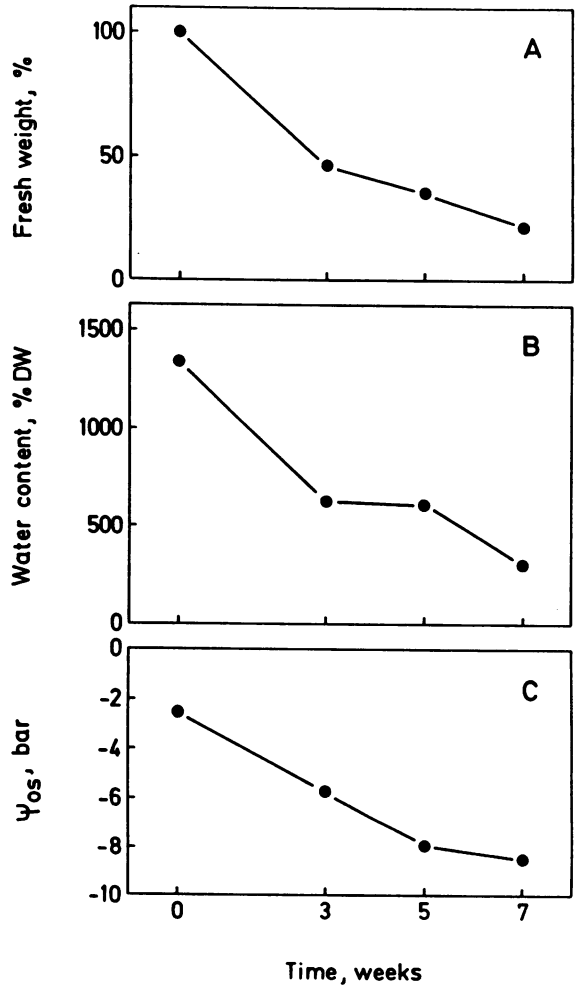

FIG. 6. Relative change in fresh weight as percent of the initial value (A), in the relative water content (B), and in the osmotic potential (C) of leaves of *K. daigremontiana* at various stages of a 7-week water stress treatment (leaves detached). DW, dry weight.

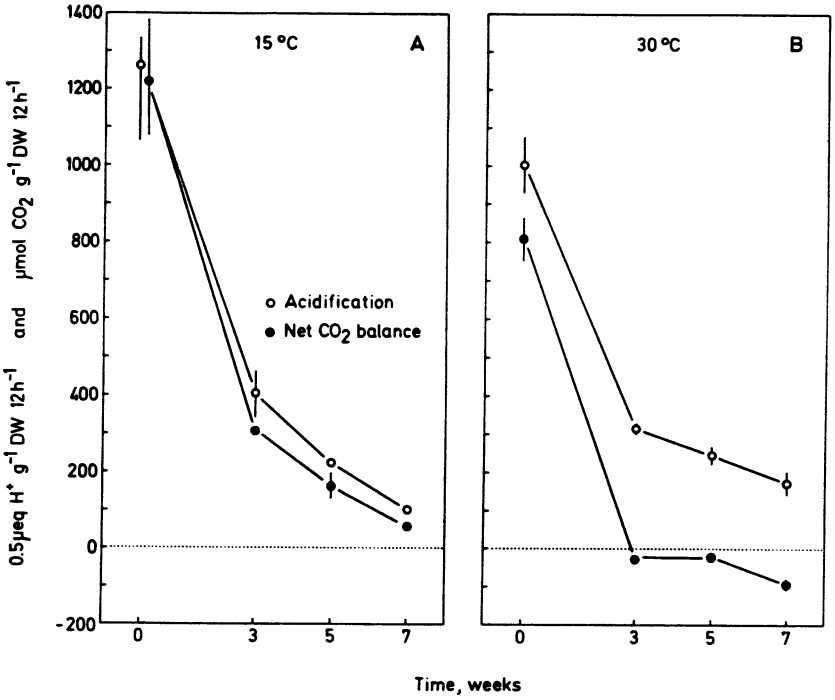

FIG. 7. Effect of water stress (weeks after withholding water) on the net CO_2 balance (●) and the concomitant change in titratable acidity (○) in leaves of *K. daigremontiana* during a 12 h dark period in normal air (350 μbar CO_2) at 15°C (A) and at 30°C (B). During the stress treatment leaves were kept in a growth cabinet under a 12 h light (1.0 mmol m^{-2} s^{-1}, 30°C)/12 h dark (20°C) cycle. Each data point represents the mean of 2 to 4 samples. The bars indicate extremes. When no bars are given, the extremes are smaller than the size of data points. DW, dry weight.

by processes involved in the nocturnal storage of malic acid, may explain why the temperature optimum for acid accumulation, based on respiratory CO_2 alone, may be somewhat lower. At night temperatures around 30°C, approximately 80% of the dark respiratory CO_2 was retained by dark CO_2 fixation, both in well irrigated and in stressed plants of all three species (Figs. 2–4 and 7). High nocturnal temperatures are not unusual in many sites where CAM plants occur, for example in deserts of southwestern North America during summer drought or in epiphytic habitats in the humid tropics (5).

At low nighttime temperatures, recycling of respiratory CO_2 became relatively important only under conditions of water stress as stomatal closure restricted uptake of external CO_2. The magnitude of carbon recycling remained small, however, because of low respiration rates. At these respiration rates, carbon loss from the plant is small in any case.

Cessation of net CO_2 exchange with the atmosphere, a major aspect of CAM-idling as previously defined, was essentially apparent in all three species at the respective temperature compensation points at which the nocturnal CO_2 balance was zero, and this was largely independent of water status. Of course, pronounced net CO_2 uptake for part of the night which is offset by pronounced net CO_2 loss for the other part of the night may principally also result in a net CO_2 balance of zero. The continuous monitoring of net CO_2 exchange throughout the dark periods showed however that, at the temperature compensation points, rates of net CO_2 exchange were around zero for almost the entire dark periods in the stressed plants and for most of the time in the nonstressed plants. With increasing water stress, the temperature compensation points were shifted to lower values. Only when tissue dehydration had reached a state close to severely damaging the plants was there a complete loss in the capacity for net uptake of atmospheric CO_2 at any given night temperature (Table 1 and Fig. 2C). Under these conditions, acid accumulation based on recycling respiratory CO_2 via CAM at both high and low temperatures was only a fraction of that of well watered plants at high tissue temperatures.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxydase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. KLUGE M, IP TING 1978 Crassulacean Acid Metabolism: Analysis of an Ecological Adaptation. Springer-Verlag, Heidelberg
3. MEDINA E 1982 Temperature and humidity effects on dark CO_2 fixation by *Kalanchoë pinnata*. Z Pflanzenphysiol 107: 251–258
4. MEDINA E, CB OSMOND 1981 Temperature dependence of dark CO_2 fixation and acid accumulation in *Kalanchoë daigremontiana*. Aust J Plant Physiol 8: 641–649
5. NEALES TF, CS HEW 1975 Two types of carbon fixation in tropical orchids. Planta 123: 303–306
6. NOBEL PS, TL HARTSOCK 1983 Relationships between photosynthetically active radiation, nocturnal acid accumulation, and CO_2 uptake for a Crassulacean acid metabolism plant, *Opuntia ficus-indica*. Plant Physiol 71: 71–75
7. RAYDER L, IP TING 1983a Shifts in the carbon metabolism of *Xerosicyos danguyi* H. Humb. (Cucurbitaceae) brought about by water stress. I. General characteristics. Plant Physiol 72: 606–610
8. RAYDER L, IP TING 1983b Shifts in the carbon metabolism of *Xerosicyos danguyi* H. Humb. (Cucurbitaceae) brought about by water stress. II. Enzymology. Plant Physiol 72: 611–615
9. SIPES DL, IP TING 1985 Crassulacean acid metabolism and Crassulacean acid metabolism modifications in *Peperomia camptotricha*. Plant Physiol 77: 59–63
10. SZAREK SR, HB JOHNSON, IP TING 1973 Drought adaptation in *Opuntia basilaris*. Significance of recycling carbon through Crassulacean acid metabolism. Plant Physiol 52: 539–541
11. SZAREK SR, IP TING 1974 Respiration and gas exchange in stem tissue of *Opuntia basilaris*. Plant Physiol 54: 829–834
12. TING IP 1985 Crassulacean acid metabolism. Annu Rev Plant Physiol 36: 595–622
13. TING IP, L RAYDER 1982 Regulation of C_3 to CAM shifts. *In* IP Ting, M Gibbs, eds, Crassulacean Acid Metabolism. American Society of Plant Physiologists, Rockville, MD, pp 193–207
14. WAGNER J, W LARCHER 1981 Dependence of CO_2 gas exchange and acid metabolism of the alpine CAM plant *Sempervivum montanum* on temperature and light. Oecologia 50: 88–93
15. WINTER K 1985 Crassulacean acid metabolism. *In* J Barber, NR Baker, eds, Photosynthetic Mechanisms and the Environment. Elsevier Science Publishers, Amsterdam, pp 329–387