

Temperature Dependence of Dark CO₂ Fixation and Acid Accumulation in *Kalanchoë daigremontiana*

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Abstract

Kalanchoë daigremontiana was grown at 25/15 and 34/24°C and net CO₂ fixation and acid synthesis were measured at 8, 15 and 24°. The ratio of net dark CO₂ fixation to acid accumulation was nearly 1 at all temperatures tested. Although the increased contribution of respiratory CO₂ to total acid synthesis at high temperatures was demonstrated by incubating leaf discs in CO₂-free air, it was not enough to account for the reduction of dark CO₂ fixation. Plants grown at 25/15° temperature regime showed maximal rates of nocturnal acid accumulation at 15°, the rates decreasing markedly at 24°. Plants grown at 24/34° showed similar rates at 15 and 24°, but acid accumulation was significantly lower at 8°. Measurements of dark acid accumulation in CO₂-free air indicated that 34/24° plants have reduced respiration rates in comparison with 25/15° plants. These observations are discussed in relation to hypotheses seeking to account for effects of temperature on crassulacean acid metabolism.

Introduction

In some leaf succulents with crassulacean acid metabolism (CAM), a distinct temperature optimum for dark CO₂ fixation and malic acid synthesis is observed (Wolf 1960; Medina and Delgado 1976) whereas in other stem succulents acidification is relatively independent of temperature (Osmond *et al.* 1979; Gulmon and Bloom 1979). Furthermore, optimum temperature for dark CO₂ fixation may change in relation to temperature during growth. This effect has been shown experimentally in the Crassulaceae (Queiroz 1965) and Cactaceae (Gerwick and Williams 1979; Gulmon and Bloom 1979), and has also been observed to occur under natural conditions (Nisbet and Patten 1974; Medina and Delgado 1976; Medina *et al.* 1977; Gulmon and Bloom 1979).

Reduction in net dark CO₂ fixation above the temperature optimum has been attributed to several causes:

- (1) An interaction between the activity of carboxylating and decarboxylating enzyme systems which differ in temperature optima, so that at high temperatures decarboxylation increases relative to carboxylation (Brandon 1967);
- (2) An increase in respiration with temperature which provides a significant internal source of CO₂ for dark fixation, thereby reducing amounts of CO₂ fixation from surrounding air (Kaplan *et al.* 1976);

- (3) An increase in the permeability of the tonoplast membrane to malate with temperature, so that retention of malate in the vacuole is decreased and the increased malate concentration in the cytoplasm inhibits phosphoenolpyruvate carboxylase (Queiroz 1974; Lüttge *et al.* 1975).
- (4) Reduction in the mobilization of glucan at high temperature and hence reduction in available substrate for acid synthesis (Kluge 1969).

Evaluation of these alternative hypotheses is difficult, yet the stoichiometry between net CO₂ exchange and acid synthesis should provide some insight into alternatives (1) and (2). Given that only one carboxylation step is involved in malic acid synthesis (Sutton and Osmond 1972; Cockburn and MacAuley 1975), a 1:1 molar stoichiometry of acid synthesis and CO₂ fixation is expected. This has been observed in some leaf succulents (Björkman and Osmond 1974; Medina and Delgado 1976; Nobel and Hartsock 1978; O'Leary and Osmond 1980) but, in *Opuntia*, CO₂ fixation accounted for only 40–55% of the acid accumulated (Osmond *et al.* 1979, figure 9). In this paper we report the stoichiometry of net CO₂ fixation and acid synthesis at different temperatures in leaves of *Kalanchoë daigremontiana* grown under different temperature regimes. These experiments, and other observations on enzyme activity in these plants, argue against interpretations (1) and (2) above, but establish that the temperature optimum for acidification in these plants changes (acclimates) in response to temperature during growth.

Materials and Methods

K. daigremontiana plants, 4–5 cm tall and with three or four leaf pairs, were placed in naturally lit growth chambers in the CSIRO phytotron in Canberra. Temperature regimes (day/night) were: 18/8°, 25/15° and 34/24°. Photoperiod was 10 h (0700–1700 hours). Relative humidity during the night was 83–87% in the three cabinets. Leaf temperature at night corresponded within 1°C to air temperature as measured with a ventilated psychrometer and an infrared surface thermometer (Micron). The leaf–air vapour pressure difference during the night therefore increased from 1.8 mbar in the low temperature treatment to 3.9 mbar in the high temperature treatment.

The experiments to measure the amount of acid accumulation at different temperatures were performed by shifting plants from one cabinet to another for one night. Leaf acid content was measured at the beginning and at the end of the dark period (or at shorter intervals). Leaf discs were punched with a cork borer and maintained at –10° until extraction the following day. Sampling of leaf discs was convenient for the calculation of free acid accumulation both on a fresh weight and an area basis. Discs were homogenized in a mortar with boiling distilled water; the homogenate was centrifuged and titrated to pH 6.5 with 0.01 N NaOH. The amount of malic acid in the extracts was measured enzymatically following the Hohorst procedure (1965). The variation in acidity during the night could be attributed entirely to changes in malate concentration, 1 equivalent of malate accumulated during the night corresponding to 1 equivalent acidity titrated in the extract (Δ malate μ equiv./g fresh wt = 2.981 + 0.936 Δ μ equiv. free acid/g fresh wt; $n = 22$, $r = 0.965$, $P < 0.01$). A similar relationship was observed by Osmond *et al.* (1979) and O'Leary and Osmond (1980).

Dark CO₂ fixation was measured in detached mature leaves with an infrared gas analyser in the open system described by Powles and Osmond (1978). At the end of the gas exchange measurement, leaves were extracted to measure the total acid content. Leaves were detached shortly before the beginning of the dark period and several experiments showed that there were no differences between the amount of acid accumulated by attached and detached leaves (from the same leaf pair) under identical conditions. During the experiments, the leaf–air vapour pressure difference was maintained as low as possible to avoid stomatal closure in response to low humidity (Lange and Medina 1979).

Acid accumulation of leaf discs exposed to CO₂-free air at different temperatures was measured. Leaf discs were enclosed in air-tight containers humidified with wet filter paper. The containers were flushed with CO₂-free air for 5 min and then sealed and incubated in the dark at different temperatures.

Results

Dry Matter Production and Leaf Succulence

At the end of the experiments, after 104 days, one average plant (as judged by height and number of leaves) of each cabinet was sampled to measure dry weight, leaf area and degree of succulence of leaves.

Table 1. Dry weight of average plants of *Kalanchoë daigremontiana*
Growing period was July 15–October 27 1979

Temp. (°C)	Stem	Dry weight (g)			Max. area/ leaf (cm ²)	Number of leaf pairs
		Leaves	Roots	Total		
18/8°	1.5	9.5	1.0	12.0	93	6
25/15°	5.2	22.0	2.5	29.7	150	8
34/24°	3.2	19.4	1.4	24.0	127	10

The different temperature treatments markedly affected plant growth. The optimum temperature regime for dry matter production was 25/15°, while the 18/8° regime resulted in the slowest growth. The largest leaves were produced in the treatment 25/15°, but more leaf pairs were produced in the 34/24° treatment (Table 1).

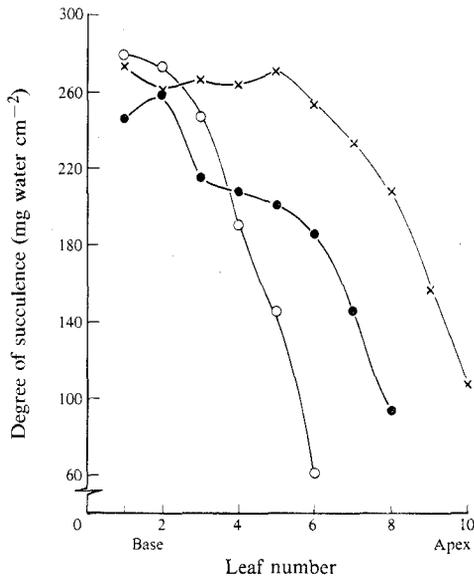


Fig. 1. Variation of the water content per unit leaf area (degree of succulence) with age. Plants were grown for 104 days under temperature regimes as follows:
○ 18/8°C.
● 25/15°C.
× 34/24°C.

The degree of leaf succulence (water content/leaf area) varied with leaf age, and the most succulent leaves were produced in the 34/24° treatment (Fig. 1). The first three leaf pairs produced in the 18/8° treatment also showed a high degree of succulence, but the rest of the leaves were not comparable to the leaf pairs in the same position in the other two treatments. Because leaves were produced at a much slower rate in the 18/8° treatment, most of the experiments were conducted only with plants grown at 25/15° and 34/24°.

Acid Accumulation Rate at Different Temperatures

The pattern of acid accumulation during the night was similar in both 25/15° and 34/24° plants. At high temperature, acid accumulation tended to be slower during the first half of the dark period, and the rate increased during the second half of the dark period. At low temperatures the rate during the first half of the dark period

Table 2. Rate of acid accumulation during the linear phase

Rate of increase in acid content was linear between 2 and 11 h after the beginning of the dark period (Fig. 2). Data are for five data sets, four replications each; the rate is the corresponding regression coefficient for each temperature. Statistical analysis: linear regression and significance of regression coefficients tested with *F* test (Sokal and Rohlf 1966, p. 420). Differences among regression coefficients tested with *F* test (Sokal and Rohlf 1966, p. 450). Homogeneity of variances tested with F_{max} test (Sokal and Rohlf 1966, p. 371). Values with the same letter are not statistically different at $P = 0.01$

Growth temp. (°C)	Accumulation rate ($\mu\text{equiv. cm}^{-2} \text{ h}^{-1}$) for treatment temperature:		
	8°C	15°C	24°C
25/15	2.07 ^{a,c}	2.53 ^a	1.06 ^b
34/24	1.07 ^b	1.47 ^d	1.75 ^{c,d}

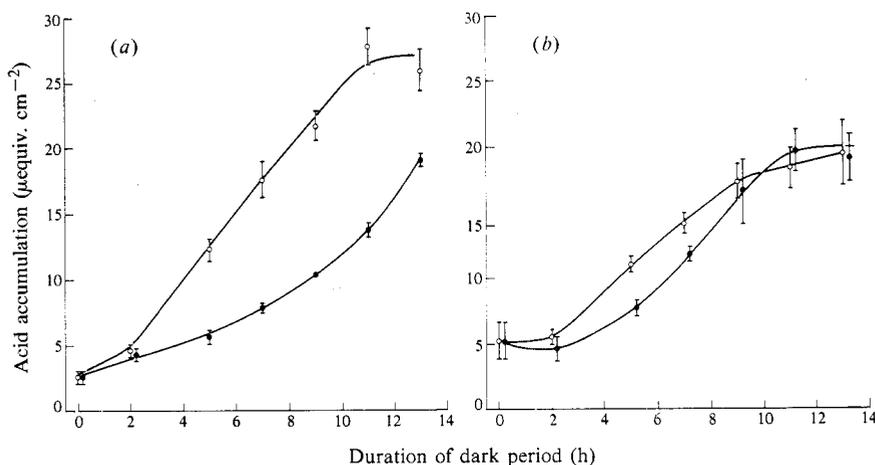


Fig. 2. Time course of acid accumulation in the dark by mature, fully expanded leaves of plants grown at (a) low (25/15°C) and (b) high (34/24°C) temperature regimes. Dark period temperatures were: ○, 15°C; ●, 24°C.

was higher than during the second half. The experiment illustrated in Fig. 2 shows that 25/15° plants displayed significantly reduced dark CO₂ fixation capacity at 24°, while 34/24° plants performed similarly at 15 and 24° (see Table 2). Data points in Fig. 2 indicate that the rate of increase in acid content was linear from 2 to 11 h after the beginning of the dark period. A regression analysis performed with this part of

the curve (Table 2) showed that 25/15° plants did not differ in rate of acid accumulation at 8 and 15°, while there was a significant reduction of the rate at 24°. The 34/24° plants, on the other hand, had similar rates at 15 and 24°, while the rate of acid accumulation at 8° was significantly lower. This pattern reflects the acclimation effect of CAM to low and high temperatures. In this experiment, acid accumulation

Table 3. Net free acid accumulation in attached leaves

Values are means of four replicates ± s.e.

Growth temp. (°C)	Treatment temp. (°C)	Acid accumulation per night ($\mu\text{equiv. cm}^{-2}$)	
		45-day plants ^A	60-day plants ^B
25/15	8	24.1 ± 3.2	23.5 ± 3.2
	15	23.3 ± 4.3	22.0 ± 1.0
	24	16.4 ± 1.8	14.4 ± 2.4
34/24	8	12.7 ± 4.0	15.2 ± 4.0
	15	17.9 ± 7.2	26.0 ± 4.2
	24	15.8 ± 4.9	33.9 ± 5.9

^A3rd and 4th leaf pairs.

^B4th leaf pair.

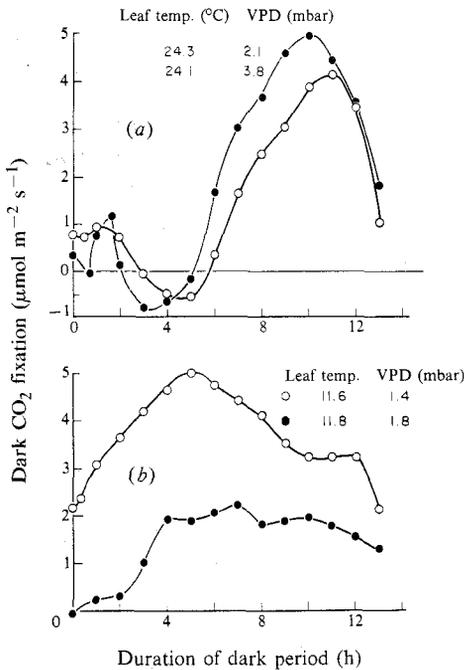


Fig. 3. Net dark CO₂ exchange of mature, detached leaves from plants grown at different temperature regimes. ○ 25/15°C regime. ● 34/24°C regime.

did not increase during the last 2 h of the dark period in the 34/24° plants, but several other experiments performed subsequently showed that 34/24° plants can accumulate high amounts of free acid (Table 3). Data in Table 3 show again that the acclimation pattern is identical to the one in Table 2; that is, 34/24° plants have comparatively lower acid accumulation rates at 8°, and 25/15° plants have reduced acid accumulation rate at 24°C.

Dark CO₂ Fixation and Acid Accumulation

Typical CO₂ exchange curves for high and low temperature plants are shown in Fig. 3. At 24°C there was a delay in the commencement of the increase in rate of CO₂ fixation which was not observed at lower temperatures. Besides, the time to reach maximal rate of dark CO₂ fixation was shorter in the low temperature experiments. When total CO₂ fixation was computed, the gas exchange measurements indicated that 2 equivalents of free acid were produced per mol of CO₂ fixed. This relation remained almost constant at the different temperatures and with plants grown under

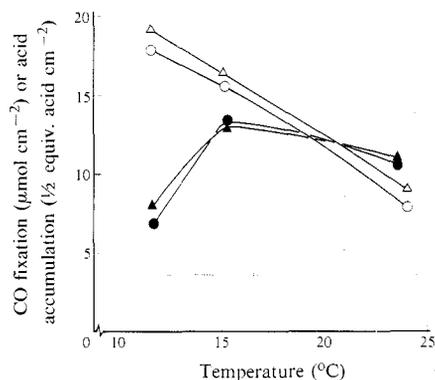


Fig. 4. Net dark CO₂ fixation and net acid accumulation during the night in detached leaves of plants grown at high (34/24°C) and low (25/15°C) temperature regimes.

△,▲ Acid accumulation.
○,● CO₂ fixation.
△,○ 25/15°C.
▲,● 34/24°C.

different temperature regimes (Fig. 4). The regression of free acid accumulation on CO₂ fixed during the dark period was highly significant ($\frac{1}{2}$ μequiv. free acid cm⁻² = 1.08 + 0.97 μmol CO₂ cm⁻²; $r = 0.99$; F ratio = 177.44), and the regression coefficient was not significantly different from 1 (F test, Sokal and Rohlf 1966). On a molar basis, the experimental points of Fig. 4 show that net dark CO₂ fixation was 4.5–14.8% lower than the amount of acid accumulated, the larger differences corresponding to the lower CO₂ fixation values. There is no evidence, however, of an increase of the difference between net CO₂ fixation and amount of free acid accumulated with increasing temperature.

Table 4. Net free acid accumulation by leaf discs incubated in CO₂-free air

Dark period was 13 h. Values are means of three replicates ± s.e.

Treatment temp. (°C)	Accumulation rate (μequiv. cm ⁻² h ⁻¹) for growth temperature:	
	25/15°C	34/24°C
8	17.7 ± 0.0	13.8 ± 3.1
15	54.7 ± 10.0	38.3 ± 8.9
24	86.1 ± 3.4	46.1 ± 5.6

The acid accumulation measured in the detached leaves during the gas exchange experiments was usually similar to the range of values measured with attached leaves in the phytotron. Net acid accumulation by 34/24° leaves at 24° was not as high as in experiments shown in Table 3. The main point, however, is that both CO₂ fixation and acid accumulation changed in parallel in the different experimental conditions.

Accumulation of Organic Acids in CO₂-free Air

Leaf discs exposed to CO₂-free air in air-tight containers showed a significant increase in acidity over the initial predarkness value (Table 4). The amount of acid accumulated during the night in CO₂-free air increased with temperature, as would be expected if respiratory CO₂ is used as a source for acid synthesis. Cutting discs from thick leaves may result in higher respiration rates, but in the experiment of Table 4 this effect should have been similar in both treatments. It appears that 34/24° plants have lower acid accumulation on a fresh weight basis than 25/15° plants when incubated in air-tight containers with CO₂-free air. This result indicates that *K. daigremontiana* plants have lower respiration rates when grown at relative higher temperature, but further studies of temperature regulation of respiration in CAM plants are required.

Discussion

Kaplan *et al.* (1976) argued that decreased net CO₂ fixation in CAM plants at high temperatures is due mainly to an increase in the rate of CO₂ evolution via respiration. They calculated that gross dark CO₂ fixation in *K. daigremontiana* does not respond to temperature. However, if their data are calculated over the whole dark period, rather than to the time taken to reach maximum rate of gross dark CO₂ fixation, it is clear that both net dark CO₂ fixation and gross dark CO₂ fixation are reduced at high temperatures. This reduction is greater than the corresponding increase in respiration. Kaplan *et al.* (1976) did not measure acid synthesis in their experiments, and we have not measured respiration. Even though our data show that at higher temperatures respiratory CO₂ permits more extensive acid synthesis (Table 4), we suspect it makes only a minor contribution to total acid synthesis. If, as Kaplan *et al.* (1976) imply, respiratory CO₂ can provide more than twice as much CO₂ for gross dark CO₂ fixation at 24°, then the molar stoichiometry of net CO₂ fixation/malic acid synthesis should be in the vicinity of 0.3. Fig. 4 shows that it is scarcely different from 1.0, and is not much influenced by temperature. We conclude that, although increased respiration at high temperature can contribute to acid synthesis, this contribution is quite small in leaf succulents such as *K. daigremontiana*, *Echeveria columbiana* (Medina and Delgado 1976) and *Agave desertii* (Nobel and Hartsock 1978). This conclusion needs further testing in experiments which embody a complete balance sheet of net CO₂ uptake, carboxylation products other than malic acid, O₂ uptake, acid synthesis and glucan uptake.

The stoichiometry of near unity implies that the effect of temperature on net CO₂ fixation and acid synthesis must be associated with the biochemistry of these processes alone. Sutton (1975) showed that the relationship between glucan utilization and acid synthesis remained more or less unchanged between 15 and 25°, suggesting that Brandon's hypothesis of more rapid malic acid synthesis and degradation at high temperatures (Brandon 1967) was not tenable in *K. daigremontiana*. We have studied the temperature responses of purified phosphoenolpyruvate carboxylase and NADP malic enzyme from *K. daigremontiana*, which Brandon believed control the balance of carboxylation and decarboxylation, and find them identical over the range 10–30° (Osmond and Nott, unpublished data; see Osmond and Holtum 1981). The observations thus far tend to eliminate hypotheses (1) and (2) set out in the Introduction and encourage us to investigate further the effects of temperature on malic acid

efflux (Lüttge *et al.* 1975), on feedback inhibition of phosphoenolpyruvate carboxylase by malic acid (Kluge and Osmond 1972; Queiroz 1974), and on control of starch utilization (Kluge 1969).

Comparisons of acid accumulation in *K. daigremontiana* grown at different temperatures show clear evidence for temperature acclimation of this complex set of processes which control acid synthesis and CO₂ fixation. Low temperature grown plants (25/15°) show significant high temperature (24°) inhibition of net CO₂ fixation and acid synthesis, as discussed above. High temperature grown plants (34/24°) do not show a high temperature inhibition, but rather a significant inhibition at low temperature (8°) (Table 2; Fig. 4). These results confirm earlier observations on the acclimation of CAM to temperature conditions during growth of leaf succulents (Queiroz 1965; Medina and Delgado 1976). Similar behaviour has been reported in the stem succulent *Opuntia* (Gulmon and Bloom 1979) and evidently does not involve changes in the thermal responses of carboxylation enzymes (Gerwick and Williams 1979). The observation (Table 4) of reduced respiration in high temperature grown plants needs further evaluation in the light of the above discussion. The role of temperature acclimation of dark CO₂ fixation in the ecology of CAM plants remains to be assessed.

Acknowledgments

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Corrigendum

Volume 8, Numbers 4, 5

Involvement of phosphorus in nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.).

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p. 432, caption to Fig. 4. The symbols for phosphorus supply should read '(■, 1.2 g P per pot; ● 0.2 g P per pot)'.