Research Article

Crassulacean acid metabolism-cycling in *Euphorbia milii*

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Received: 12 December 2012; Accepted: 14 February 2013; Published: 4 March 2013

Citation: Herrera A. 2013. Crassulacean acid metabolism-cycling in *Euphorbia milii*. AoB PLANTS 5: plt014; doi:10.1093/aobpla/plt014

Abstract. Crassulacean acid metabolism (CAM) occurs in many Euphorbiaceae, particularly *Euphorbia*, a genus with C3 and C4 species as well. With the aim of contributing to our knowledge of the evolution of CAM in this genus, this study examined the possible occurrence of CAM in *Euphorbia milii*, a species with leaf succulence and drought tolerance suggestive of this carbon fixation pathway. Leaf anatomy consisted of a palisade parenchyma, a spongy parenchyma and a bundle sheath with chloroplasts, which indicates the possible functioning of C2 photosynthesis. No evidence of nocturnal CO2 fixation was found in plants of *E. milii* either watered or under drought; watered plants had a low nocturnal respiration rate (R). After 12 days without watering, the photosynthetic rate (PN) decreased 85% and nocturnal R was nearly zero. Nocturnal H+ accumulation (DH+) in watered plants was 18±2 (corresponding to malate) and 18±4 (citrate) μmol H+ (g fresh mass)-1. Respiratory CO2 recycling through acid synthesis contributed to a night-time water saving of 2 and 86% in watered plants and plants under drought, respectively. Carbon isotopic composition (δ13C) was −25.2±0.7‰ in leaves and −24.7±0.1‰ in stems. Evidence was found for the operation of weak CAM in *E. milii*, with statistically significant DH+, no nocturnal CO2 uptake and values of δ13C intermediate between C3 and constitutive CAM plants; ΔH+ was apparently attributable to both malate and citrate. The results suggest that daily malate accumulation results from recycling of part of the nocturnal respiratory CO2, which helps explain the occurrence of an intermediate value of leaf δ13C. *Euphorbia milii* can be considered as a CAM-cycling species. The significance of the operation of CAM-cycling in *E. milii* lies in water conservation, rather than carbon acquisition. The possible occurrence of C2 photosynthesis merits research.

Keywords: CAM-cycling; citrate; transpiration; water saving; water-use efficiency.

Introduction

Crassulacean acid metabolism (CAM) is of frequent occurrence among the Euphorbiaceae and has appeared polyphyletically several times in the family, particularly in the genus *Euphorbia*. In this genus, C4 species seem to be rare, whereas they are abundant in the genus *Chamaesyce* (Webster et al. 1975). In *Euphorbia*, CAM has been reported in 21 species, and values of δ13C suggest its presence in 44 species (Table 1). Several of these species belong to three different clades within the genus (cladograms in Zimmermann et al. 2010).

Twenty-four species can be considered constitutive CAM on the basis of having values of δ13C higher than −17‰, a criterion established by Mooney et al. (1977). In the remaining species, values of δ13C average −24.7‰. A value as low as −28.9‰ found in *E. aphylla* falls into the lower mode of the bimodal frequency distribution of δ13C in CAM plants, designated as low-level (weak) CAM (Winter and Holtum 2002; Silvera et al. 2005).

Since values of δ13C alone are not sufficient to distinguish between C3 species and plants that obtain up to...
Herrera — Weak CAM in Euphorbia milii

Table 1. Carbon isotopic composition of species of Euphorbia with high to intermediate values of $\delta^{13}C$ and CAM mode assigned by authors on the basis of leaf gas exchange, acid accumulation, $\delta^{13}C$ and enzyme activity.

| Species        | $\delta^{13}C$ (%) | Mode          | Reference                     |
|----------------|-------------------|---------------|-------------------------------|
| angusta        | −24.9             | NA            | Webster et al. (1975)          |
| antiquorum     | −14.2             | NA            | Botanouy et al. (1991)         |
| aphylia        |                  | Facultative   | Mies et al. (1996)             |
| avasmontana    | −15.1             | Constitutive  | Mooney et al. (1977)           |
| bcharae        | −14.6             | Constitutive  | Mooney et al. (1977)           |
| bbalina        | −13.2             | NA            | Webster et al. (1975)          |
| burmannii      | −18.3             | NA            | Mooney et al. (1977)           |
| caducifolia    |                  | Constitutive  | Webster et al. (1975), Sayed (2001) |
| caput-medusae  | −13.3             | Constitutive  | Mooney et al. (1977)           |
| cyathophora    | −25.8             | NA            | Mies et al. (1996)             |
| didieroides    | −26.6             | NA            | Webster et al. (1975)          |
| didieroides    | −24.3             | NA            | Winter (1979)                  |
| dregeona       |                  | Constitutive  | Sayed (2001)                   |
| drupifera      | −14.1             | NA            | Webster et al. (1975)          |
| gariepina      | −14.6             | Constitutive  | Mooney et al. (1977)           |
| genoudiana     | −22.7             | NA            | Winter (1979)                  |
| gorgonis       | −12.9             | Constitutive  | Mooney et al. (1977)           |
| gregoraria     | −11.6             | Constitutive  | Mooney et al. (1977)           |
| grandidens     |                  | Constitutive  | Webster et al. (1975), Sayed (2001) |
| inermis        | −13.4             | Constitutive  | Mooney et al. (1977)           |
| ingezalahiana  | −23.6             | NA            | Winter (1979)                  |
| inocua         | −28.1             | NA            | Webster et al. (1975)          |
| leucodendron   | −13.2             | NA            | Winter 1979                    |
| macropodoides  | −28.3             | NA            | Webster et al. (1975)          |
| macropus       | −28.9             | NA            | Webster et al. (1975)          |
| mauritanica    | −16.0             | Constitutive  | Mooney et al. (1977)           |
| milii          | ND                | Non-CAM       | Webster et al. (1975)          |
| milii          | CAM               |               | McWilliams (1970)              |
| nesemnann      | −11.6             | Constitutive  | Mooney et al. (1977)           |
| nivula         | −15.7             | NA            | Webster et al. (1975)          |
| rubica         | −14.5             | NA            | Botanouy et al. (1991)         |
| pentagona      | −14.9             | Constitutive  | Mooney et al. (1977)           |
| peperomoides   | −25.6             | NA            | Webster et al. (1975)          |
| plagiantha     | −13.2             | NA            | Winter (1979)                  |

Table 1. Continued

| Species        | $\delta^{13}C$ (%) | Mode          | Reference                     |
|----------------|-------------------|---------------|-------------------------------|
| polygona       | −10.7             | Constitutive  | Mooney et al. (1977)          |
| pulcherima     | −25.5             | NA            | Mies et al. (1996)            |
| squarrosa      | −12.5             | Constitutive  | Mooney et al. (1977)          |
| stenoclada     | −12.6             | NA            | Winter (1979)                 |
| submamillaris  | ND                | Constitutive  | Webster et al. (1975), Sayed (2001) |
| tetragona      | −14.7             | Constitutive  | Mooney et al. (1977)          |
| thi            | −13.2             | NA            | Batanouy et al. (1991)        |
| tirucallii     | −15.3             | Constitutive  | Mies et al. (1996)            |
| triangularis   | −13.6             | Constitutive  | Mooney et al. (1977)          |
| trigona        | −19.4             | NA            | Webster et al. (1975)         |
| xylophylloides | ND                | Constitutive  | Webster et al. (1975), Sayed (2001) |

NA, no mode assigned; ND, not determined.

one-third of their carbon during the night, which include weak CAM plants (Winter and Holtum 2002), measurements of physiological and biochemical variables are necessary. In order to demonstrate the operation of CAM, routine determinations include, among others, $\Delta H^+$, $\delta^{13}C$ and nocturnal CO2 fixation. Griffiths et al. (2007) devised an ingenious method of ascertaining the occurrence of nocturnal CO2 fixation by examining the response of the night-time CO2 exchange rate to intercellular CO2 concentration (C).

Intermediate values of $\delta^{13}C$ can also suggest the occurrence of C3 metabolism with high water-use efficiency, as the data of Farquhar and Richards (1984) on wheat indicate, or of C2 photosynthesis, as in the case of Euphorbia acuta. In wheat and maize, C2 photosynthesis is responsible for an increase of 8–11% in photosynthetic rate through re-assimilation of photorespired CO2 (Busch et al. 2013).

Plants of Euphorbia milii subgenus Euphorbia, Section Gonioastea, common name crown of thorns, originally from Madagascar, are cultivated worldwide for their ornamental value. Plants are perennial armed shrubs as tall as 1 m, with fleshy stem and branches, and partly succulent leaves. According to observations by Mooney et al. (1977), CAM is present in the weak mode in leafy species of the genus. The medicinal and molluscicidal properties of the latex in E. milii have been extensively investigated (e.g. Mwine and Van Damme 2011); in contrast, literature on the physiology of the species is practically non-existent.
In spite of the succulence of its leaves and the various reports of CAM in the genus, *E. milii* has been reported as non-CAM (Webster et al. 1975). Nevertheless, recalculation of the data of McWilliams (1970) gives a $\Delta H^+$ of 100 $\mu$mol (g fresh mass)$^{-1}$ and a dark CO$_2$ fixation rate of 0.1 $\mu$mol m$^{-2}$ s$^{-1}$, suggesting that CAM in *E. milii* operates in the cycling mode, i.e. nocturnal H$^+$ accumulation and daytime but nearly no night-time CO$_2$ fixation (for the definition of CAM modes, see Cushman 2001).

With the aim of contributing to our knowledge of the evolution of CAM in *Euphorbia*, this study re-examined the possible occurrence of CAM in *E. milii* through daily leaf gas exchange, including $P_N/C_i$ and $R/C_i$ curves (where $P_N$ is the photosynthetic rate and $R$ is the respiration rate), measurements of dawn and dusk H$^+$ content, and determinations of $\delta^{13}$C.

**Methods**

**Plant material and cultivation**

Plants of *E. milii* were propagated from one plant purchased at a nursery by inserting cuttings into the soil of 2-L pots filled with silty clay loam (Viveros Exotica Raphia, S.R.L., Caracas); plants were fertilized monthly with N : P : K 15 : 15 : 15 and grown in the garden for $\approx$ 1 year before the beginning of experiments. Plants, ~50 cm tall, were maintained in the greenhouse under natural light, fully watered every other day and fertilized weekly. Day length was 12 h (06:00–18:00 h), mean maximum daily photosynthetic photon flux density (PPFD) between 09:00 and 14:30 h 507 ± 22 $\mu$mol m$^{-2}$ s$^{-1}$, mean air temperature 32 ± 5/18.4 ± 0.5 °C (day/night) and relative humidity 60 ± 10 %. Water deficit was imposed by withholding watering.

**Anatomy**

Free-hand cross-sections of stems (average thickness 5 mm) and leaves were observed under the microscope at $\times 40$ (stem) and $\times 400$ (leaf). Leaf sections were stained with toluidine blue.

**Succulence**

Leaf and stem water content was determined as the difference between the fresh mass (FM) and the mass after drying for 72 h at 60 °C [dry mass (DM)], divided by the area in the case of leaves (FM/A) and by DM in the case of stems. Leaf thickness was measured with precision calipers. Chlorophyll (Chl) content was determined after Bruinsma (1963) in 80 % cold acetone extracts of leaf or stem sections collected at 18:00 h. The mesophyll succulence index was calculated as $S_m = g$ water (mg Chl)$^{-1}$ after Kluge and Ting (1978).

**Stable carbon isotope composition**

The $\delta^{13}$C was determined with a precision of 0.15 ‰ using a ThermoFinnigan DeltaPlusXL Isotope Ratio Mass Spectrometer (San Jose, CA, USA) and PDB as the standard.

**Nocturnal H$^+$ accumulation**

Whole leaves were weighed fresh and set to boil in 50 mL distilled water for 10 min in a microwave oven at maximum power; samples were sieved through a plastic colander, leaf segments and the colander were rinsed, and the solution was made up to 100 mL. Samples were titrated to pH 7.0 for the estimation of H$^+$ corresponding to malate according to Nobel (1988), and to pH 8.4 for citrate. Since Franco et al. (1990) noted that there was a strong linear relationship between concentrations of malate and citrate determined enzymatically and by titration, in the absence of an enzymatic method for the determination, titration is an adequate alternative. Latex was collected from cut stems and leaves, suspended in distilled water and titrated likewise. The $\Delta H^+$ was calculated as the difference between dawn and dusk H$^+$ contents.

**Leaf gas exchange**

The $P_N$, $R$, stomatal conductance ($g_s$) and transpiration rate ($E$) were measured in the laboratory with a CIRAS 2 IRGA connected to a PLC(B) assimilation chamber (PP Systems, Amesbury, MA, USA) at an incoming CO$_2$ concentration ($C_a$) of 380 $\mu$mol mol$^{-1}$, a chamber temperature tracking ambient (24 ± 1 °C) and an incident PPFD of 200 (the first morning hours) or 1000 $\mu$mol m$^{-2}$ s$^{-1}$ (the rest of the daytime). Records were automatically taken every 30 min. Response curves were done in six different leaves of $P_N$ and $C_i$ between 10:00 and 11:00 h, and of nocturnal CO$_2$ exchange to $C_i$ between 20:00 and 05:00 h.

**Statistics**

Values are mean ± SE ($n = 6$). Statistical significance was assessed where indicated through one- or two-way analysis of variance (ANOVA) ($P < 0.05$) with the Statistica package.

**Results**

Leaf cross-sections showed a dorsiventral anatomy, with a compact palisade parenchyma containing many chloroplasts and a spongy parenchyma with large vacuoles and fewer chloroplasts; the spongy parenchyma constituted 40 % of the whole-leaf thickness (Fig. 1). A bundle sheath with large chloroplasts located centrifugally was observed. Cross-sections of the fleshy stems and leaves, suspended in distilled water and titrated likewise. The $\Delta H^+$ was calculated as the difference between dawn and dusk H$^+$ contents.
have a thick green cortex and colourless pith; the cortex was 54% of the stem thickness on average.

In watered plants, \( S_m \) in both leaves and stem green cortex was 2.7 ± 0.2 g water (mg Chl)\(^{-1} \); \( \delta^{13}C \) was −25.2 ± 0.7‰ in leaves and −24.7 ± 0.1‰ in stems. In the stem green cortex of watered plants, malate- and citrate-H\(^{+} \) content was 48 ± 9 and 29 ± 5 mmol H\(^{+} \) (g FM)\(^{-1} \), respectively, without daily oscillation. Water suspensions of latex showed no acid content.

Leaves had significant amounts of malate- and citrate-H\(^{+} \) at dawn and dusk, contents increasing with time under drought (Fig. 2A and B). A significant accumulation of malate-H\(^{+} \) took place in watered plants, which remained constant up to 16 days of drought (\( P < 0.05 \)). A similar trend in dawn and dusk H\(^{+} \) content and \( \Delta H^+ \) for citrate-H\(^{+} \) was found, except that after 16 days of drought \( \Delta H^+ \) became zero. Mean \( \Delta H^+ \) was 18 ± 2 (malate) and 18 ± 4 (citrate) mmol (g FM)\(^{-1} \). Changes in either morning and evening H\(^{+} \) contents or \( \Delta H^+ \) bore no relationship to changes in FM/A, which remained relatively constant for the duration of the experiment, as did Chl content (Fig. 2). The ratio Chl a/b remained unchanged at 3.3 ± 0.2. Stem water content was 11.2 ± 0.5 g water (g DM)\(^{-1} \), twice as high as in leaves, and did not vary with time under drought (\( P = 0.86 \)).

As shown in Fig. 3A, \( P_N \) of watered plants became saturated at 700 \( \mu \)mol m\(^{-2} \) s\(^{-1} \) PPFD; apparent quantum yield was 0.047 and light-compensation point 48 \( \mu \)mol m\(^{-2} \) s\(^{-1} \). The \( P_N/C_i \) curves (Fig. 3B) show that \( P_N \) did not become saturated by \( C_i \), increasing 47% with an increase in \( C_i \) to 800 \( \mu \)mol mol\(^{-1} \) (\( C_a = 1240 \mu \)mol mol\(^{-1} \)). This lack of saturation could have been due to very low \( g_a \) which remained unchanged by \( C_i \). The CO\(_2\) compensation concentration was 32 \( \mu \)mol mol\(^{-1} \).

Daily courses of leaf gas exchange done in plants progressively under drought showed a decrease in \( P_N \) of 85% with drought; \( R \) became nearly zero after 12 and up to 16 days without watering (Fig. 4). Mean daytime water-use efficiency calculated from these courses of leaf gas exchange was relatively high, decreasing significantly with drought only 18%, from 4.2 ± 0.1 to 3.5 ± 0.1 mmol mol\(^{-1} \) (\( P = 0.00 \)).

Stem cross-sections of 1.5 cm\(^2 \) average area from watered plants introduced in the assimilation chamber showed daytime CO\(_2\) assimilation at rates similar to those determined in leaves on a Chl basis (Table 2). Stem sections, as opposed to leaves, showed dark CO\(_2\) uptake.

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**Figure 1.** Cross-sections of the leaf of *E. milii*. UE, upper epidermis; PP, palisade parenchyma; VB, vascular bundle; BS, bundle sheath; SP, spongy parenchyma; LE, lower epidermis. Arrowheads point at chloroplasts.

**Figure 2.** Time course of changes with drought in leaves of *E. milii* in (A) \( H^+ \) content titrated to pH 7.0 (empty circles, dawn; filled circles, dusk); (B) \( H^+ \) content titrated to pH 8.4 (empty circles, dawn; filled circles, dusk); (C) nocturnal \( H^+ \) accumulation (empty triangles, pH 8.4; filled triangles, pH 7.0), and (D) dawn leaf FM per area (circles) and chlorophyll content (triangles). Values are mean ± SE (\( n = 12 \)). Different letters indicate significant differences at \( P < 0.05 \) after a two-way ANOVA (time under drought × hour of day for each pH in A and B) and a one-way ANOVA (time under drought for each pH in C).
A significant decrease of 65% in $R$ with $C_i$ was observed without significant changes in $g_s$ (Fig. 5).

The regression of $E$ vs. $R$ is shown in Fig. 6. Assuming that the accumulated acids were the products of the recycling of respiratory CO$_2$, the absolute recycling, i.e. the amount of CO$_2$ contained in acids, was calculated. Together with the $E$ vs. $R$ regression, it was found that recycling recovered 10 and 37% of nocturnal CO$_2$ loss in watered plants and after 12 days of drought, respectively, and helped in saving water during the night by 15% in watered plants and 2% in plants under drought. Daytime water saving, calculated as the ratio of the absolute amount of CO$_2$ in acid equivalents to

Table 2. Photosynthetic and respiration rate of stem cross-sections inserted into the IRGA assimilation chamber.

| Organ | PPFD ($\mu$mol m$^{-2}$ s$^{-1}$) | $P_N$ ($\mu$mol m$^{-2}$ s$^{-1}$) | ($\mu$mol (g Chl)$^{-1}$ s$^{-1}$) |
|-------|---------------------------------|---------------------------------|---------------------------------|
| Leaf  | 1500                            | 9.9 $\pm$ 1.2                   | 15.1 $\pm$ 0.3                   |
| Stem  | 1500                            | 10.0 $\pm$ 0.2                  | 9.1 $\pm$ 1.9                   |
| Leaf  | 0                               | $-1.5 \pm 0.2$                 | $-2.3 \pm 0.3$                 |
| Stem  | 0                               | 0.5 $\pm$ 0.2                  | 4.7 $\pm$ 1.3                  |

Values are mean $\pm$ SE ($n = 6$). Incident photosynthetic photon flux density is indicated.

Figure 3. Response curve of the leaf photosynthetic rate to (A) photosynthetic photon flux density and (B) leaf intercellular CO$_2$ concentration in watered plants of $E$. milii. Values are mean $\pm$ SE ($n = 6$). Filled circles, $P_N$; empty symbols, $g_s$. Measurements were made at a CO$_2$ concentration of 380 $\mu$mol mol$^{-1}$ in (A) and a PPFD of 1000 $\mu$mol m$^{-2}$ s$^{-1}$ in (B).

Figure 4. Daily course of the leaf photosynthetic rate, stomatal conductance and transpiration rate in plants of $E$. milii under drought for 0, 7, 12 and 16 days. Measurements were made at a CO$_2$ concentration of 380 $\mu$mol mol$^{-1}$, leaf temperature of 24.0 $\pm$ 1.0°C and PPFD of 200 (06:00–10:00 h) and 1000 $\mu$mol m$^{-2}$ s$^{-1}$ (10:00–18:00 h). The dark bar on the abscissa indicates the length of the dark period.
integrated \( P_N \) (after Fetene and Lützge 1991), was 2 and 86 % in watered plants and plants under drought, respectively.

**Discussion**

Evidence was found for the operation of weak CAM in \( E. \) milii, with statistically significant \( \Delta H^+ \) in watered plants and plants under drought, low \( \delta^{13}C \) and no nocturnal CO\(_2\) uptake; \( \Delta H^+ \) was apparently attributable to both malate and citrate. Results suggest that daily malate and citrate accumulation results from recycling of part (watered plants) or all (plants under drought) of the nocturnal respiratory CO\(_2\). Recycling of CO\(_2\) through malate synthesis, together with the absence of nocturnal CO\(_2\) uptake, helps explain the occurrence of values of leaf \( \delta^{13}C \) intermediate between C\(_3\) and constitutive CAM plants.

Values of \( \Delta H^+ \) determined at pH 7.0 were low compared with CAM species such as Kalanchoe tubiflora and Clusia minor, comparable with the cycling species \( T. \) parviflorum and \( T. \) mengessi and not as low as in \( T. \) teretifolium or \( Z. \) zamifolia (Table 3).

A value of \( S_m \) higher than 1 g water (mg Chl)\(^{-1}\) was also suggestive of CAM, as proposed by Kluge and Ting (1978). The succulent nature of leaves was corroborated by the microscopic observation of cross-sections, in which cells with a large volume and few chloroplasts are present, as in many leaf-succulent CAM plants (Kluge and Ting 1978). In facultative CAM species, values of \( S_m \) are intermediate (Table 4) but, given that

![Figure 5. Response curves to leaf intercellular CO\(_2\) concentration of nocturnal respiration rate and stomatal conductance in watered plants of \( E. \) milii. Filled symbols, \( R \); empty symbols, \( g_s \). Values are mean ± SE (n = 6).](https://www.aobplants.oxfordjournals.org/content/doi/10.1093/aobpla/plt014/160844)

![Figure 6. Change in nocturnal leaf transpiration rate of plants of \( E. \) milii watered and under different degrees of drought as a function of nocturnal respiration rate. Values are data points. The regression line (solid), 95 % confidence intervals (broken lines) and determination coefficient (\( P \), 0.05) are shown.](https://www.aobplants.oxfordjournals.org/content/doi/10.1093/aobpla/plt014/160844)

### Table 3. Values of nocturnal acid accumulation and carbon isotopic composition reported for CAM species.

| CAM mode  | Species                     | \( \Delta H^+ \) (\( \mu \text{mol} \text{ (g FM)}^{-1} \)) | \( \delta^{13}C \) (%) | Reference                  |
|-----------|-----------------------------|--------------------------------------------------------|------------------------|----------------------------|
| Facultative | Clusia minor               | 1400                                                   | -24.6                  | Borland et al. (1992, 1994) |
| Constitutive | Kalanchoe daigremontiana | 152                                                     | -16.7                  | Holtum et al. (1983)       |
| Cycling   | Talinum calycinum           | 39                                                      | -25.2                  | Martin and Zee (1983)      |
| Cycling   | Sedum nutititianum          | 37                                                      | -27.2                  | Gravatt and Martin (1992)  |
| Cycling   | Talinum calcicium           | 29                                                      | -26.0                  | Harris and Martin (1991)   |
| Cycling   | Sedum telephioides          | 23                                                      | -26.2                  | Gravatt and Martin (1992)  |
| **Cycling** | **Euphorbia milii**        | **18**                                                 | **-25.2**              | **This study**              |
| Cycling   | Talinum teretifolium        | 14                                                      | -25.4                  | Harris and Martin (1991)   |
| Cycling   | Talinum parviflorum         | 11                                                      | -25.8                  | Harris and Martin (1991)   |
| Cycling   | Talinum mengessi            | 6                                                       | -24.3                  | Harris and Martin (1991)   |
| Facultative | Zamioculcas zamifolia      | 5                                                       | ND                     | Holtum et al. (2007)       |

Some values were re-calculated from the data in references. ND, not determined.
low as well as high values of $S_m$ can be found in constitutive CAM species (Table 4), it becomes apparent that for a leaf to perform full CAM a large proportion of vacuole volume to chloroplasts is not required. The lack of significant differences in FM/A between five strong CAM and three weak CAM species (Nelson and Sage 2008) lends support to this hypothesis.

The presence in E. milii of bundle sheath cells with chloroplasts is indicative of the possible operation of $C_2$ photosynthesis, as reported in E. acuta. This species shares with E. milii low, $C_3$-like values of $\delta^{13}C$ ($-25.5 \%$ according to Webster et al. 1975, and $-28.5 \%$ according to Sage et al. 2011) and an intermediate value of CO$_2$ compensation concentration (32 mmol mol$^{-1}$; Sage et al. 2011). The occurrence of CAM, Kranz anatomy and $C_4$ photosynthesis in the same leaf has been reported in Portulacaceae (Guralnick and Jackson 2001), but to date no report on CAM together with $C_2$ photosynthesis has been published. Given that the $C_3$ route of carbon fixation has been proposed as an intermediate evolutionary step from $C_3$ to $C_4$ (Lu¨ttge 2007), the role of citrate accumulation in carbon or water balance during CAM remains unclear (Lu¨ttge 2007); citrate does not provide net CO$_2$ fixation. The absence of net dark CO$_2$ fixation was consistent with the proportion of dark CO$_2$ uptake calculated using the regression equations of the proportion of CO$_2$ fixed during the night against $\delta^{13}C$ found by Winter and Holtum (2002) and Pierce et al. (2002). In many facultative CAM species, $\delta^{13}C$ tends towards low values. In a review of 23 facultative CAM species (Herrera 2009), the mean, maximum and minimum $\delta^{13}C$ were $-23.9, -14.0$ and $-30.0 \%$, respectively, indicating that the variability in $\delta^{13}C$ values may lead researchers to classify a species as a $C_3$, facultative or constitutive CAM plant. In Euphorbia aphylla, $\delta^{13}C$ ranged from $-27.1 \%$ for the youngest cladode in the dry season during summer to $-24.5 \%$ for the oldest cladode in the rainy season during winter (Mies et al. 1996), reflecting the effects on $\delta^{13}C$ of day/night temperatures, water availability and developmental stage.

Values of $\delta^{13}C$ higher in stems than in leaves suggest the occurrence of nocturnal CO$_2$ fixation, although this could not be demonstrated in intact plants. The occurrence of an assimilation rate in the dark amounting to a third of PPFD-saturated leaf $P_N$ strongly suggested that stems are capable of nocturnal CO$_2$ fixation. Stem internal CO$_2$ re-fixation in young twigs and branches possessing a green cortex may compensate for 60–90 % of the potential respiratory carbon loss (Pfanz et al. 2002). If stem recycling in E. milii occurred through phosphoenolpyruvate carboxylase (PEPC) activity, that would explain the apparent $^{13}C$ enrichment. There are two alternative explanations for higher $\delta^{13}C$ in the stems of E. milii. One explanation is that this variable was determined in sections comprising all tissues, green as well as non-autotrophic; non-autotrophic organs of $C_3$ plants, such as stems, have been found to be enriched in $\delta^{13}C$ by $\sim1–3 \%$ relative to leaves (Cernusak et al. 2009). Another, simpler, explanation is that barriers to CO$_2$ diffusion into the stem are larger than into leaves, hindering entrance of the heavier $^{13}CO_2$. Actual nocturnal CO$_2$ fixation by stems of E. milii remains to be determined accurately by methods such as carbon labelling, involving all stem tissues.

Table 4. Values of mesophyll succulence index reported for constitutive, facultative- and cycling-CAM species.

| Species               | $S_m$ (mg Chl) | CAM mode | Reference               |
|-----------------------|----------------|----------|-------------------------|
| Kalanchoe daigremontiana | 1.3            | Constitutive | Kluge and Ting (1978)   |
| Euphorbia milii       | 2.7            | Cycling   | This study              |
| Talinum paniculatum   | 3.4            | Facultative | Güere et al. (1996)     |
| Talinum triangulare   | 6.0            | Facultative | Herrera et al. (1991)   |
| Puya flaccosa         | 6.2            | Facultative | Herrera et al. (2010)   |
| Sedum morganianum     | 13.0           | Constitutive | Kluge and Ting (1978)   |
The response of leaf dark respiration to \( C_i \) suggests the operation of a carboxylation system, most likely PEPC, which makes recycling of respiratory \( \text{CO}_2 \) possible. In the constitutive CAM plant \( \text{Kalanchoe daigremontiana} \), a \( P_{\lambda}/C_i \) curve during phase I of the CAM cycle showed a pronounced increase at low \( C_i \) and saturation at a \( C_i \) of \( \sim 250 \mu\text{mol mol}^{-1} \) (Griffiths et al. 2007). Our results show that the response of dark respiration to \( C_i \) was not an artefact caused by changes in \( g_s \), because \( g_s \) remained constant in spite of increasing \( C_i \).

Water saving through respiratory \( \text{CO}_2 \) recycling was significant, as in the case of \( T. \text{paniculatum} \), a facultative CAM species, in which the amount of water saved was 5–12 times that lost by transpiration (Güerere et al. 1996). Similarly, in \( T. \text{calycinum} \), 5–44 % of water was potentially conserved by CAM-cycling (Martin et al. 1988).

Leaf water balance in \( E. \text{milii} \) seems to rest on both recycling of respiratory \( \text{CO}_2 \) and strict stomatal control, rather than on water supply from the succulent stem, as leaf \( F\text{MA} \) remained unchanged after 16 days of drought and stem water content did not vary significantly during this time.

**Conclusions**

In view of the observations presented here, \( E. \text{milii} \) can be considered as a CAM-cycling species that in watered plants shows diurnal, but not nocturnal, \( \text{CO}_2 \) uptake and low \( \Delta H^\ddagger \); plants under drought have very low \( P_{\lambda} \), equally low \( \Delta H^\ddagger \) and no net dark \( \text{CO}_2 \) exchange. The significance of the operation of such a low CAM in \( E. \text{milii} \) resides in water conservation, rather than carbon acquisition. The occurrence of \( \text{C}_2 \) photosynthesis remains to be demonstrated.

**Sources of Funding**

Experiments were done with equipment acquired through grant PG-03.00.6524.2006 and technical assistance provided by grant PG 03.7381.2011-1 (CDCH-UCV).

**Conflicts of Interest Statement**

None declared.

**Acknowledgements**

Thanks are due to the PaleoLab at the College of Marine Science of the University of South Florida and to Dr Enrique Montes for the determinations of \( \delta^{13} \text{C} \). Help with leaf section preparation and the provision of photographs by L. Hermoso, M. Escala and A. Menéndez are gratefully acknowledged.

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