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Cryptic crassulacean acid metabolism (CAM) in *Jatropha curcas*

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Abstract. *Jatropha curcas* L. is a drought-tolerant shrub or small tree that is a candidate bioenergy feedstock. It is a member of the family Euphorbiaceae in which both CAM and C_4 photosynthesis have evolved. Here, we report that *J. curcas* exhibits features diagnostic of low-level CAM. Small increases in nocturnal acid content were consistently observed in photosynthetic stems and occasionally in leaves. Acidification was associated with transient contractions in CO_2 loss at night rather than with net CO_2 dark fixation. Although the CAM-type nocturnal CO_2 uptake signal was masked by background respiration, estimates of dark CO_2 fixation based upon the 2 : 1 stoichiometric relationship between H^+ accumulated and CO_2 fixed indicated substantial carbon retention in the stems via the CAM cycle. It is proposed that under conditions of drought, low-level CAM in J. *curcas* stems serves primarily to conserve carbon rather than water.

Additional keywords: biofuel, Euphorbiaceae, photosynthesis, stem respiration.

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Introduction

Jatropha curcas L. (Euphorbiaceae) is a slightly stem-succulent shrub or small tree native to Central America (Maes *et al*. 2009*b*). The plant is extensively cultivated in many tropical and subtropical regions and is viewed by some as a promising bioenergy crop because the seeds are rich in oils that can be processed to produce biofuel (Berchmans and Hirata 2008; van Eijck *et al*. 2014). The high drought tolerance of *J. curcas* (Yin *et al*. 2014) has generated speculation that the water-conserving CAM pathway of photosynthesis might contribute to the plant's ability to withstand severe water limitation (Jongschaap *et al*. 2009; Maes *et al*. 2009*a*) but experimental evidence for CAM is lacking.

CAM is not unusual among plants: approximately 6% of vascular plant species are believed to exhibit at least some degree of CAM, especially those with succulent photosynthetic tissues (Smith and Winter 1996). Overall, CAM has evolved more than 60 times in at least 35 families, including lycophytes, ferns, gymnosperms and angiosperms. The CAM cycle is characterised by nocturnal uptake of CO_2, followed by the storage of CO_2 as malic acid in the vacuoles of chloroplast-containing cells, and the liberation of CO_2 through decarboxylation of malic acid during the following light period. The CO_2 derived from decarboxylation then enters the photosynthetic carbon reduction cycle via Rubisco (Osmond 1978). CO_2 gain over 24 h occurs at low water cost (Neales *et al*. 1968; Winter *et al*. 2005) because at night, when atmospheric CO_2 is acquired and stomata are open, the driving forces for water loss are low. During the day, when the driving forces for water loss are high, the risks of excessive water efflux are averted because photosynthesis is independent of external CO_2 and stomata do not need to open. Depending upon species and the environment, the CAM cycle is either the predominant mode of carbon assimilation as is the case in most cacti and agaves (Nobel 1988), or CAM is only weakly expressed, with C_3 photosynthesis remaining the main provider of carbon, as has been demonstrated for many species of orchids (Silvera *et al*. 2005, 2014). Certain species such as *Mesembryanthemum crystallinum* L. (Aizoaceae), *Calandrinia polyandra* Benth. (Montiaceae) and *Clusia pratensis* Seem. (Clusiaceae) are distinctive in that they are photosynthetically highly plastic and use CAM in an optional, facultative fashion: while employing the C_3 pathway when growing under optimal, non-stressed conditions, they can reversibly switch to, or upregulate, CAM in response to drought stress (Winter and von Willert 1972; Winter and Holtum 2008, 2014).

CAM is well represented in the Euphorbiaceae (Smith and Winter 1996; Horn *et al*. 2014), for example, in the petroleum plant *Euphorbia tirucalli* L. (Winter *et al*. 2005; Hastilestari *et al*. 2013), one of ~850 xerophytic stem-succulent species many of which are known to exhibit CAM. Furthermore, C_4 photosynthesis has evolved once in *Euphorbia* (section Anisophyllum) (Pearcy and Troughton 1975; Yang and Berry 2011; Horn *et al*. 2014). Although the leaves of *J. curcas* are not particularly succulent, the stems have a relatively thick green cortex (Gupta 1985) potentially conducive of CAM. In *Frerea indica* Dalzell, a species in the Apocynaceae, the succulent stems exhibit CAM, while the relatively thin leaves are C_3 (Lange and Zuber 1977).

In the study presented here, CO_2 exchange and titratable acidity measurements were used to explore whether or not

characteristics of CAM were detectable in leaves and stems of *J. curcas*, and whether these were induced or upregulated by drought stress.

Materials and methods

Jatropha curcas L., *Ochroma pyramidale* (Cav. ex Lam.) Urb. (Malvaceae), *Ormosia macrocalyx* Ducke (Fabaceae), and *Kalanchoë pinnata* (Lam.) Pers. (Crassulaceae) were grown in pots 24 cm tall–10 cm wide (*J.c.*, *O.p.*, *K.p.*) and 35 cm tall–11 cm wide (*O.m.*) filled with potting mix (Miracle Gro, Marysville, OH, USA) to which small doses of Osmocote Plus fertiliser (Scotts-Sierra Horticultural Products, Marysville, OH, USA) were added occasionally. Plants of *J. curcas* were grown outdoors either under full solar radiation or beneath a rainshelter at ~60% of sunlight. *O. macrocalyx* and *K. pinnata* were also grown beneath the rainshelter for 1 month before experiments. *O. pyramidale* was grown outdoors under full sunlight. Plant heights were ~22 cm (*J. curcas*), 40 cm (*O. macrocalyx*), 10 cm (*O. pyramidale*) and 18 cm (*K. pinnata*).

Net CO_2 exchange was measured using two open-flow gas-exchange systems assembled with components (CO_2 mixing unit, Peltier-regulated cold traps, mass-flow controlled air pumps, dew-point mirrors) from Walz GmbH (Effeltrich, Germany). CO_2 was monitored using either a LI-6252 or a LI-6262 gas analyser (Li-Cor, Lincoln, NE, USA). Air containing 400 µmol mol^{-1} CO_2 was delivered at flow rates of 1.26 or 4 L min^{-1} to leaf or stem cuvettes depending on surface area studied and magnitude of CO_2 fluxes. Intact attached leaves were either inserted into a Peltier-temperature regulated GWK-3M cuvette (Walz GmbH), or a portion of an intact attached leaf was clamped into the porometer head of a CQP 130 portable photosynthesis system (Walz GmbH). Stem gas-exchange was measured for 12.0–17.5 cm long stem segments enclosed in tubular acrylic-glass cuvettes (Fig. 1). Stems were rooted in all cases. During measurements, the study plants and gas-exchange cuvettes were located in a growth chamber (GC8, EGC, OH, USA) maintained at 28°C during the 12 h light period and 22°C during the 12 h dark period. Illumination was provided by LED lights (SS-GU300-w, Sunshine Systems; GrowPro300 LL4 L-GP300, Greners, Boulder, CO, USA). Leaf CO_2 exchange was measured at a PFD of 1000 µmol m^{-2} s^{-1}, which was also the photosynthetic flux density (PFD) incident on the top of the stem-chamber. Stem surfaces were exposed to diffusive light PFD of 90 µmol m^{-2} s^{-1}.

For measurements of titratable acidity, entire mature leaves or 4–10 cm segments of stems were snap-frozen and stored in liquid nitrogen. After thawing, samples were boiled in 50% ethanol for 15 min, and after addition of water to maintain the initial extraction volume, boiled again for 15 min. After cooling to room temperature, samples were titrated with 5 mM NaOH to pH 6.5.

Results

Figs 2 and 3 depict leaf net CO_2 exchange of two plants of *J. curcas*. Under well watered conditions, maximum CO_2 uptake in the light ranged from ~6.0 to 7.5 µmol m^{-2} s^{-1} and net CO_2 loss in the dark was ~0.4 µmol m^{-2} s^{-1}. Cessation of watering was followed by reduced CO_2 uptake during the light and reduced

Fig. 1. Mature stem of *Jatropha curcas* enclosed by a cylindrical gas-exchange cuvette (17 × 5 cm inner dimensions). The young foliated stem-tip is outside the cuvette. The air inlet is at the bottom, the air outlet at the top.

CO_2 loss in the dark. CO_2 uptake in the light almost completely ceased 4 days after water was withheld. Dark respiration decreased by ~50% during water limitation. Upon rewatering, leaf gas exchange almost completely recovered within 2 days (Fig. 3). During most nights, especially when plants were drought stressed, CO_2 efflux was greater at the beginning and at the end of the night than in the middle of the night, resulting in a concave nocturnal CO_2 exchange pattern. In contrast, nocturnal CO_2 exchange by leaves of seedlings of well watered and drought stressed *Ochroma pyramidale*, a C_3 tree species, was stable throughout the night and did not show the concave nocturnal pattern observed in *J. curcas* (Fig. 4).

Of eight different *J. curcas* plants for which stem gas exchange was examined, we present three examples (Figs 5–7). In contrast to the leaves, the chloroplast-containing stems evolved CO_2 during the light and the dark, although the rates of CO_2 loss were much lower in the light. In almost all cases, rates of net CO_2 loss during the night were not constant in that a concave efflux pattern was evident, with reduced CO_2 loss during the middle of the night. In comparison, chloroplast-containing stems of the C_3 tree species, *Ormosia macrocalyx*, showed a contrastingly convex pattern of CO_2 efflux in the dark (Fig. 8).

In the short- to mid-term, drought did not have a strong effect on the magnitude and pattern of nocturnal stem CO_2 loss from *J. curcas* (e.g. Fig. 6*a*). Following prolonged drought during which leaves abscised, stem gas exchange approached zero, i.e. neither net CO_2 loss nor net CO_2 uptake was detected (Fig. 6*b*). Rewatering resulted in the re-establishment of day and night gas-exchange patterns observed in well watered plants within 2 days.

When gas exchange included the younger parts of the stem in well watered plants, the stem was defoliated immediately beforehand. In these experiments, the rates of nocturnal CO_2 loss were elevated for ~2 days, presumably due to wound respiration (Fig. 7). The concave-shaped pattern of nightly CO_2 flux persisted following defoliation and during the post-defoliation recovery.

In two separate replicated experiments, no significant nocturnal increase in titratable acidity was detected in leaves from well watered *J. curcas* (Table 1). Following drought stress, leaves of plants in one experiment exhibited a small significant nocturnal increase in titratable acidity whereas in a second experiment with longer-lasting drought, titratable acidity did not increase significantly.

Significant nocturnal increases in titratable acidity were detected in young and in mature stems of both well watered and drought stressed plants. Mean acidification values were

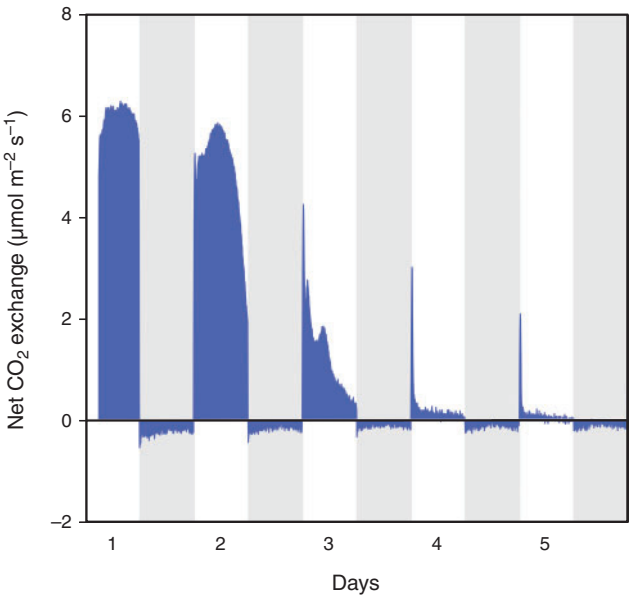

Fig. 2. Effect of drought stress on leaf net CO_2 exchange of *Jatropha curcas* during 12 h light/12 h dark cycles using a clamp-on cuvette. Areas shaded in grey represent the dark periods. The plant was last watered on day 1.

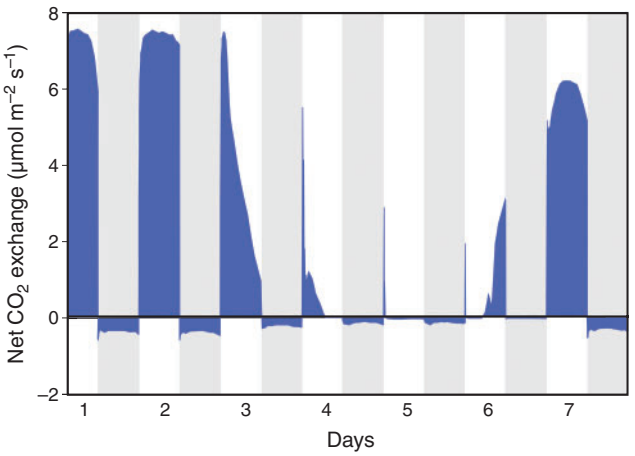

Fig. 3. Effect of drought stress and rewatering on leaf net CO_2 exchange of *Jatropha curcas* during 12 h light/12 h dark cycles using a whole-leaf gas-exchange cuvette. The plant was watered on days 1, 6 and 7.

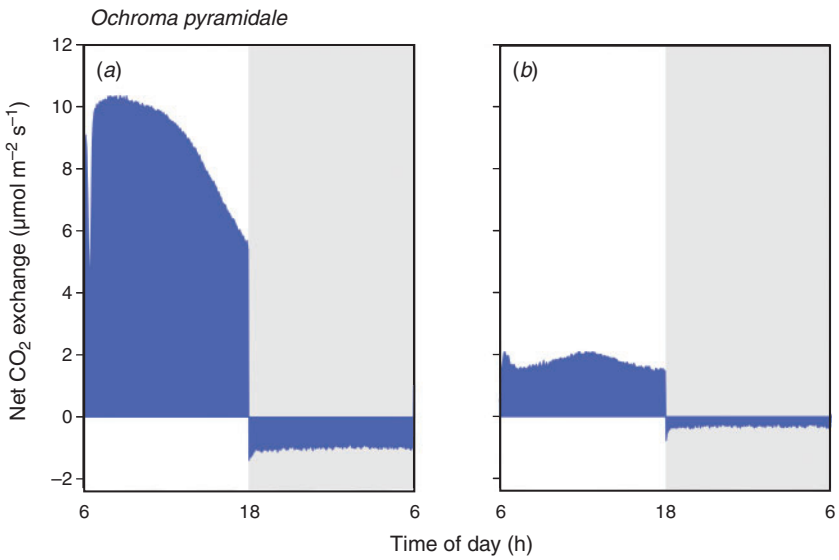

Fig. 4. Leaf net CO_2 exchange during 12 h light/12 h dark cycles of a seedling of the C_3 tree species *Ochroma pyramidale* using a clamp-on cuvette. Data are shown before (*a*) and after (*b*) watering was withheld for 3 days.

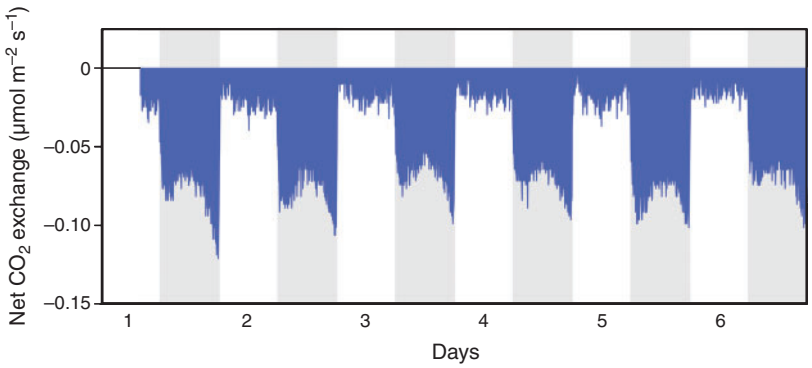

Fig. 5. Stem net CO_2 exchange of the *Jatropha curcas* plant featured in Fig. 1 during 12 h light/12 h dark cycles. Fresh mass and surface area of the stem section measured were 58 g and 114 cm^2 respectively.

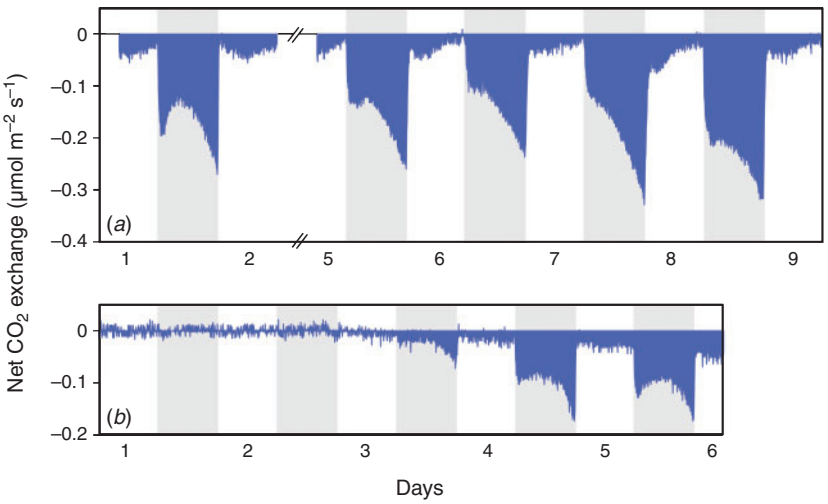

Fig. 6. Effect of drought stress on stem net CO_2 exchange during 12 h light/12 h dark cycles of *Jatropha curcas*. (*a*) Water was withheld for 4 weeks before measurements. The plant was rewatered on day 7. As shown in Fig. 1, leaves were attached and outside the cuvette. Fresh mass and surface area of the stem section measured were 55 g and 111 cm^2 respectively. (*b*) Water was withheld until all leaves had dropped and net CO_2 exchange of the ultimate 16.6 cm of the stem was zero (days 1 and 2). The plant was rewatered on day 3. Fresh mass and surface area of the stem section measured were 46 g and 102 cm^2 respectively.

greater in drought stressed plants. On a fresh mass basis, nocturnal acidification in leaves of the extensively studied CAM species *K. pinnata* was ≥48-fold that in leaves of *J. curcas*, whereas acidification in stems was of a similar magnitude in both species.

Discussion

Our study has shown that C_3 photosynthesis is the principal pathway of carbon fixation in *J. curcas*. However, *J. curcas* also exhibits features diagnostic of CAM that set it apart from a C_3 species as defined in its narrowest sense. These CAM features include small nocturnal increases in acid content consistently observed in stems and occasionally in leaves. Acidification was not accompanied by net CO_2 uptake at night, but rather, was associated with a transient contraction in CO_2 loss that most likely reflects an increase and subsequent decrease in the rate of dark CO_2 uptake superimposed upon a respiratory background. The transient contraction of CO_2 loss at night is reminiscent of CO_2 exchange patterns exhibited by plants with weakly expressed CAM that have been termed 'CAM-cyclers' (Harris and Martin 1991; Herrera 2009), such as *Platycerium veitchii* (Holtum and Winter 1999).

On the basis of acidification values of *J. curcas* that were statistically significant, it can be calculated that CO_2 fixation across the night averaged 0.01 μmol CO_2 m^{-2} s^{-1} for leaves and between 0.07 and 0.36 μmol CO_2 m^{-2} s^{-1} for stems. While dark CO_2 uptake rates of 0.01 μmol CO_2 m^{-2} s^{-1} would be difficult to discern against much larger respiration rates, the rates calculated for stems are well within the range of net CO_2 fluxes shown in

Figs 5–7, suggesting that in stems the contribution of dark CO_2 fixation to reducing nocturnal CO_2 loss is substantial, and far greater than indicated by the transient contractions observed in net CO_2 efflux. CAM may nonetheless be considered 'cryptic' in *J. curcas* because nocturnal acidification is at the limit of detection and what we believe is the CAM CO_2 signal is greatly diluted and masked by respiration.

Our interpretation that the CAM cycle occurs in *J. curcas* is supported indirectly by the presence of CAM in other Euphorbiaceae. First, there is one other report of a CAM feature in the subfamily Crotonoideae (~2000 species; Fig. 9). McWilliams (1970) observed a nocturnal H^+ increase of 10 µmol g^{-1} FM, but no dark CO_2 fixation, in the succulent-stemmed *Jatropha spathulata*, a synonym of *Jatropha dioica* native to Texas and Mexico. The published data did not specify whether leaves or stems were examined. Second, in

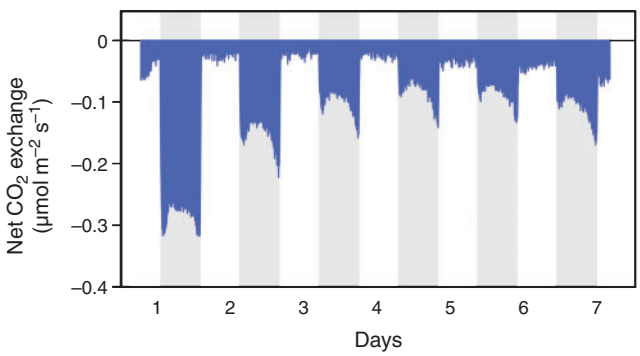

Fig. 7. Net CO_2 exchange during 12 h light/12 h dark cycles by the ultimate 17.5 cm of a stem of *Jatropha curcas* from which all the leaves had been excised on day 1, i.e. immediately before measurements commenced. Fresh mass and surface area of the stem section measured were 46 g and 102 cm^2 respectively. Inner dimensions of the gas-exchange cuvette were 18 × 9 cm.

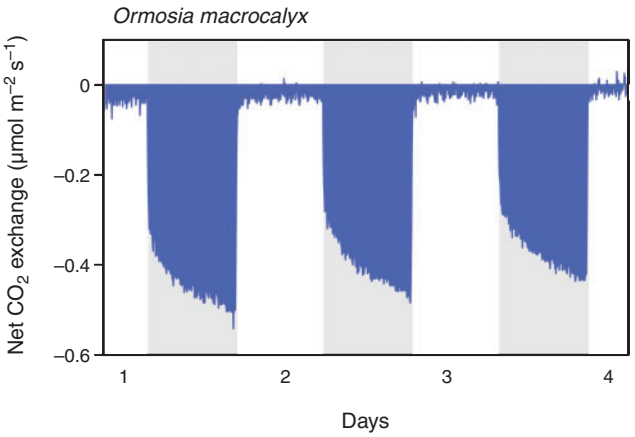

Fig. 8. Stem net CO_2 exchange during 12 h light/12 h dark cycles of a 40 cm tall sapling of the C_3 tree species *Ormosia macrocalyx* with two leaves still attached outside the cuvette featured in Fig. 1. Fresh mass and surface area of the stem section measured were 30 g and 75 cm^2 respectively.

Table 1. Nocturnal acidification in *Jatropha curcas*

Titratable acidities at the end of the day (L) and night (D), and the change during the night (D–L) in leaves and stems of *Jatropha curcas* that had been well watered or drought stressed. Watering was withheld for 13 days (experiment 1) or 11 weeks (experiment 2). For comparison, titratable acidities are presented for stems and leaves of well watered *Kalanchoë pinnata*, a constitutive CAM species. Titratable acidity is expressed on fresh mass and leaf area bases. *P*-value is the probability of acid accumulation during the dark as determined by *t*-tests (non-paired, 1-tailed, equal variance); ± values are s.d. ($n = 3$ in experiment 1, $n = 5$ in experiment 2); ND indicates not determined; n.s. indicates not significant, i.e. $P \geq 0.05$

Species and water status	Titratable acidity							
	µmol H^+ g^{-1} FM				µmol H^+ cm^{-2}			
	L	D	D–L	*P*-value	L	D	D–L	*P*-value
Experiment 1								
Jatropha curcas, well watered								
Leaves	3.84 ± 0.90	5.03 ± 0.75	1.19	n.s.	0.07 ± 0.02	0.09 ± 0.01	0.02	n.s.
Stems, mature	4.94 ± 0.17	6.21 ± 0.70	1.27	<0.05	1.61 ± 0.22	1.70 ± 0.10	0.09	n.s.
Jatropha curcas, drought stressed								
Leaves	3.33 ± 0.37	8.67 ± 2.85	5.34	<0.05	0.06 ± 0.01	0.16 ± 0.06	0.10	<0.05
Stems, mature	4.36 ± 0.72	6.43 ± 0.88	2.07	<0.05	1.63 ± 0.41	2.21 ± 0.20	0.58	<0.05
Experiment 2								
Jatropha curcas, well watered								
Leaves	2.32 ± 0.67	3.65 ± 1.85	1.33	n.s.	0.05 ± 0.01	0.08 ± 0.04	0.03	n.s.
Stems, mature	5.91 ± 0.65	7.72 ± 0.38	1.81	<0.001	2.85 ± 0.28	3.54 ± 0.51	0.69	<0.05
Stems, young	5.72 ± 0.94	11.10 ± 0.76	5.38	<0.0001	2.38 ± 0.25	3.07 ± 0.87	0.69	n.s
Jatropha curcas, drought stressed								
Leaves	5.07 ± 1.31	6.57 ± 1.76	1.50	n.s.	0.10 ± 0.03	0.14 ± 0.04	0.03	n.s.
Stems, mature	5.95 ± 0.77	8.25 ± 0.73	2.30	<0.001	2.97 ± 0.47	4.29 ± 0.55	1.32	<0.05
Stems, young	5.59 ± 0.96	13.12 ± 0.72	7.53	<0.0001	2.09 ± 0.63	5.16 ± 0.40	3.07	<0.0001
Kalanchoë pinnata, well watered								
Leaves	5 ± 2	239 ± 28	234	<0.0001	0.60 ± 0.10	26.90 ± 2.80	26.30	<0.0001
Stems	12 ± 0	19 ± 2	7	<0.0001	ND	ND	ND	

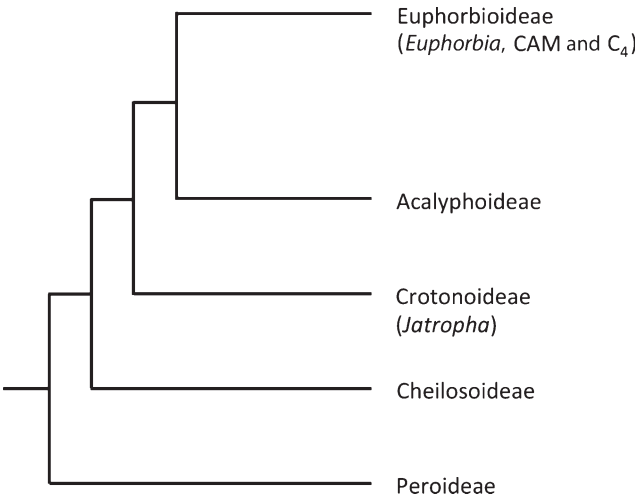

Fig. 9. The sub-family relationships within Euphorbiaceae (modified from Wurdack *et al*. 2005; and Tokuoka 2007), showing the positions of *Jatropha* and the known genus with CAM (*Euphorbia*) or C_4 (*Euphorbia* subgenus *Chamaesyce*). The CAM-containing genera *Monadenium*, *Pedilanthus* and *Synadenium* have recently subsumed within *Euphorbia*.

the subfamily Euphorbioideae, a major CAM lineage which houses all other CAM-exhibiting euphorbs including the iconic candelabra 'trees' (Evans *et al*. 2014), CAM has evolved at least 16 times within the genus *Euphorbia* (~2100 species; The Plant List 2013; Horn *et al*. 2014). In *Euphorbia*, net lineage diversification rates of CAM clades are approximately 3-fold greater than for C_3 clades (Horn *et al*. 2014). Corresponding studies of *Jatropha* species with and without low-level CAM could be informative.

At this point we can only speculate on the extent to which low-level CAM contributes to the well established high degree of drought tolerance in *J. curcas*. At the leaf level, CAM activity seems to be too low to significantly alter the ratio of net CO_2 assimilation to transpirational water loss, that is, water-use efficiency. However, the presence of the CAM cycle in the stems aids carbon retention which may enhance survival under conditions of drought. Therefore CAM in *J. curcas* is most likely a carbon-conserving rather than a water-conserving mechanism.

We cannot provide unequivocal evidence for a facultative component to the CAM signal, although a trend towards higher acidification values was seen in droughted plants. Furthermore, in experiment 1 (Table 1), nocturnal acidification in leaves was only detected in drought stressed plants. Confirmation of a facultative component requires a much larger number of experimental plants than used in the study presented here, and necessitates sampling at multiple intervals during wet–dry–wet cycles.

If the nocturnal fluctuations in acidity and the CO_2 efflux patterns in *J. curcas* indeed reflect low activity of the CAM pathway, then inadvertently *J. curcas* becomes the first species with a CAM cycle for which the entire genome has been sequenced (Sato *et al*. 2011; Yang *et al*. 2015). It is also noteworthy that *Jatropha* is in the order Malphigiales, which contains C_3 trees of the genus *Populus* (Salicaceae) in which efforts are underway to bioengineer the CAM pathway (Borland *et al*. 2014, 2015; DePaoli *et al*. 2014). Genomic and transcriptomic studies of plants with low-level CAM are likely to provide essential information on the minimum requirements for a functional CAM cycle (Winter *et al*. 2015) and thus may be key for our understanding of how plants have transitioned from C_3 to CAM.

Acknowledgements

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