

Facultative crassulacean acid metabolism (CAM) in four small C₃ and C₄ leaf-succulents

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Abstract. Measurements of whole-plant gas exchange and titratable acidity demonstrate that the Australian native species *Anacampseros australiana* J.M.Black (Anacampserotaceae), *Crassula sieberiana* (Schult. & Schult.f.) Druce (Crassulaceae) and *Portulaca australis* Endl. (Portulacaceae) and the widespread naturalised tropical exotic, *Portulaca pilosa* L., exhibit facultative crassulacean acid metabolism (CAM). In well-watered plants, net CO₂ uptake was restricted to the daylight hours and occurred via the C₃ pathway (*A. australiana* and *C. sieberiana*) or the C₄ pathway (*P. australis* and *P. pilosa*). Leaves of well-watered plants did not accumulate titratable acidity during the night. Following drought treatment, CO₂ uptake in the light by shoots decreased markedly, nocturnal gas-exchange shifted from net CO₂ loss to a CAM-type pattern that included net CO₂ uptake, and leaves acidified at night. Nocturnal CO₂ uptake by shoots and leaf acidification were most pronounced in *A. australiana* and least so in *C. sieberiana*. The induction of dark CO₂ uptake and tissue acidification was fully reversible in all four species: upon rewatering, nocturnal CO₂ uptake and acidification ceased and the rates of CO₂ incorporation in the light were restored. We suggest that, hitherto considered relatively exceptional globally, facultative CAM may be more common than previously suspected, particularly among the generally small ephemeral leaf-succulents that characterise Australia's succulent flora.

Additional keywords: *Anacampseros*, C₄ photosynthesis, *Crassula*, functional diversity, photosynthetic pathway, *Portulaca*.

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Introduction

Defining characteristics of the crassulacean acid metabolism (CAM) photosynthetic pathway are the incorporation of CO₂ in the dark and the nocturnal sequestration of the carbon as malic acid within vacuoles. CAM expression may be constitutive, in that it is always present in mature tissues of well-watered plants, or it may be facultative (Winter and Holtum 2014). In species with facultative CAM, dark CO₂ fixation is upregulated or induced in response to drought or high soil salinity. Demonstration of the reversibility of the upregulation or induction proves that the response is environmentally triggered rather than associated with stress-accelerated irreversible ontogeny (Winter *et al.* 2008).

Since its discovery in the halophyte *Mesembryanthemum crystallinum* L. (Winter and von Willert 1972; Winter and Holtum 2007) facultative CAM (including reversibility) has been documented in perhaps only 20 species worldwide (Winter and Holtum 2014), creating an impression that it is at best an uncommon trait. All known facultative CAM plants inhabit environments subject to stochastic or a distinct seasonality of water availability. Under such conditions, the employment of C₃ or C₄ photosynthesis when water is available permits

relatively high rates of CO₂ fixation and vegetative growth. The shift to CAM following stress is associated with lower overall rates of CO₂ fixation but net carbon gain is prolonged at low water cost, thereby aiding reproduction (Winter *et al.* 1978, 2015).

The demonstration of facultative CAM in *Calandrinia polyandra* Benth. (Montiaceae) (Winter and Holtum 2011), a succulent-leaved native Australian herb of sandy or stony nutrient-poor soils in winter rainfall habitats, prompted us to explore whether facultative CAM might be more widely expressed by small terrestrial succulents within the Australian flora. Although devoid of large succulents (Ellenberg 1981), Australia supports a significant flora of small succulents that colonise clay-pans, small depressions in sandy or gravelly soils, saline soils, rock seepage lines, skeletal soils on rocky hillsides, and dunes (coastal or inland), all environments where water availability may fluctuate substantially between seasons and over relatively short periods (Holtum *et al.* 2016).

Here we report the presence of facultative CAM *sensu stricto* (i.e. including the demonstration of reversibility) in four small leaf-succulents that inhabit a range of Australian climates and

habitats. The four taxa, from three families (Anacampserotaceae, Crassulaceae and Portulacaceae), include two *Portulaca* species known to express C_4 photosynthesis.

Materials and methods

Species

Anacampseros australiana J.M.Black (Anacampserotaceae *sensu* Nyffeler and Eggli 2010; Hernández-Ledesma *et al.* 2015) is a diminutive tuber-forming leaf-succulent of arid shrubland where it typically grows to 100 mm in skeletal soils on rocky hillsides and plains (Kapitany 2007). It inhabits Mediterranean winter-rainfall climates, tropical summer-rainfall regions, and parts of Central Australia where rainfall is highly unpredictable (Fig. 1).

Portulaca australis Endl. (Portulacaceae *sensu* Nyffeler and Eggli 2010; Hernández-Ledesma *et al.* 2015) is a prostrate tuber-forming annual or short-lived perennial of summer-rainfall areas in tropical and sub-tropical Western Australia, Northern Territory and Queensland (Fig. 1). It is found on granite rock platforms in open forest, skeletal soils or gravels that overlay hard clay-pans, and occasionally on coastal sand flats and sand dunes.

Portulaca pilosa L. (Portulacaceae *sensu* Nyffeler and Eggli 2010; Hernández-Ledesma *et al.* 2015) is a pan-tropical sprawling annual or short-lived perennial that is weedy and most likely not native to Australia (CHAH 2016). With fibrous roots that are rarely tuberous, in Australia it favours recently disturbed sandy or gravelly ground in summer-rainfall regions (Fig. 1).

The mainly perennial form of *Crassula sieberiana* (Schult. & Schult.f.) Druce (Crassulaceae) inhabits moist crevices between rocks of mountains in temperate to subtropical SE mainland Australia and Tasmania (Fig. 1; Toelken 1981, 1983). A principally annual inland form grows sheltered among rocks or in partial shade of trees or shrubs in more inland areas of SE Australia and in SW Western Australia (Toelken 1981, 2002).

Plant material and net CO_2 exchange

Seeds of *A. australiana* (origin: Wilpena Pound, SA), *C. sieberiana* (origin: Mt Cordeaux, Qld) and *P. australis* (origin: Cannonvale, Qld) were obtained from plants grown from cuttings or seeds supplied by A. Kapitany (australiansucculents.com, accessed 27 January 2016). For *P. pilosa*, seeds were obtained from plants grown from seeds collected from Townsville, Qld, Australia. Seeds were germinated in terracotta pots (10 cm upper diameter, 0.4 L volume) containing potting mix (Miracle-Gro Lawn Products, OH, USA). For each experiment, the shoots of a plant were enclosed inside a Perspex cuvette (internal dimensions of 11 × 11 × 10 cm). The attached roots plus pot remained outside the cuvette.

A plant, with the gas-exchange cuvette sealed around the shoots, was located inside a controlled environment chamber (EGC, OH, USA) operating under 12 h light (28°C):12 h dark (22°C) cycles. Photon flux density at the top of the cuvette was 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The flow rate of air through the cuvette was 1.26 L min^{-1} . By adding CO_2 to CO_2 -free air using a GMA-3/10 gas mixing system (Walz, FRG), air containing 400 $\mu\text{mol mol}^{-1}$

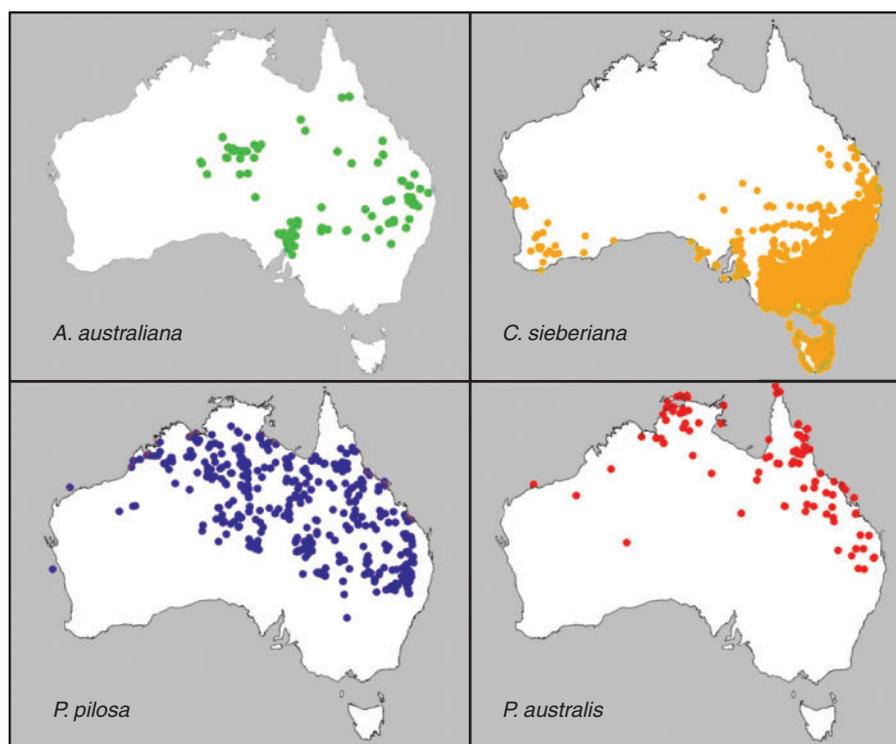


Fig. 1. Collection locations of specimens of *Anacampseros australiana*, *Crassula sieberiana*, *Portulaca australis* and *Portulaca pilosa* lodged in Australian herbaria (ALA 2016).

CO₂ was delivered to the cuvette. Net CO₂ exchange of the plant enclosed in the cuvette was measured continuously for up to 16 day/night cycles with data-points obtained every four minutes. The flow-through gas-exchange system consisted of Walz components and a LI-6252 CO₂ analyser (LI-COR Biosciences, NE, USA) (Holtum and Winter 2003). Drought treatments were imposed by withholding irrigation which was daily in the well-watered treatment. Gas exchange was monitored for two separate individuals subjected to the watering, drought and rewatering treatments. For each species, the responses of the replicates to the treatments were extremely similar. Data are shown for one of the two replicates.

Determination of titratable acidity

Mature leaves were excised at dusk and dawn from 3–6 individuals of well-watered plants, from plants that had been stressed for between 11 and 15 days, and from previously droughted plants that had been re-watered for 4–5 days. The leaves were weighed and stored in liquid nitrogen. Subsequently, the leaves were boiled sequentially in 50% ethanol and in water. Titratable acidity was quantified as the amount of 5 mM KOH required to titrate the extracts to pH 6.5.

Results

In well-watered plants of *A. australiana*, *C. sieberiana*, *P. australis* and *P. pilosa*, net CO₂ uptake by attached shoots was restricted to the light period (Figs 2–5). Daily CO₂ uptake

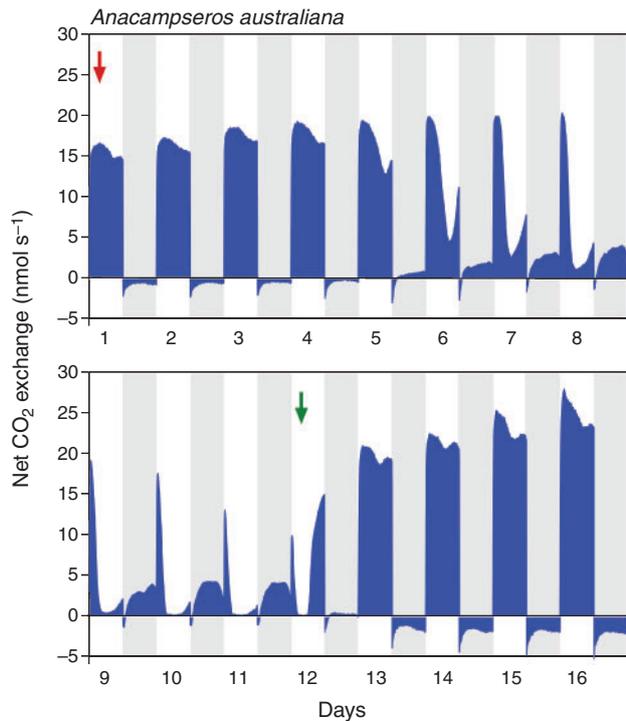


Fig. 2. Sixteen days of net CO₂ exchange by the aboveground shoot of a potted, 3.5-month-old *Anacampseros australiana* under 12 h light/12 h dark cycles. Watering was withheld on day 1 (red arrow) and recommenced on day 12 (green arrow). Shaded areas represent the dark periods.

increased as the shoots grew. After watering ceased, plants continued to grow until the plant-available water in the pot was depleted. In all species, the subsequent onset of drought was associated with a gradual reduction in CO₂ uptake in the light. The decline of CO₂ uptake during the day was accompanied by a progressive decrease in respiratory CO₂ loss at night that eventually culminated in net CO₂ uptake. Net CO₂ assimilation in the dark was most pronounced in *A. australiana* (Fig. 2) and least evident in *C. sieberiana* (Fig. 5). With the exception of *P. australis*, the general decrease in CO₂ fixation during the light was accompanied by a marked mid-day depression of uptake typical of CAM. Following rewatering, all species reattained their original pattern of net CO₂ uptake restricted solely to the light. The rates of CO₂ uptake following rewatering were greater than at the onset of the experiment because the plants continued to grow inside the cuvette throughout the experiment.

Consistent with the drought-induced change in CO₂ exchange patterns, droughted plants of all four species showed significant nocturnal increases in acid content (Table 1). No such increases were observed in well-watered plants or in plants that had been re-watered after drought.

Discussion

The four species studied here exhibit facultative CAM. In plants with C₃ or C₄ photosynthesis, nocturnal CO₂ uptake and acid

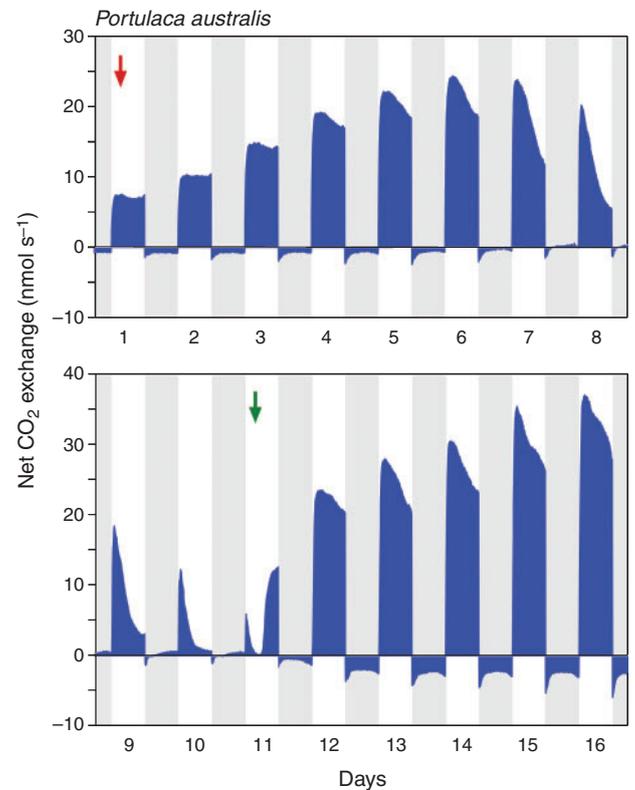


Fig. 3. Sixteen days of net CO₂ exchange by the aboveground shoot of a potted, 5-week-old *Portulaca australis* under 12 h light/12 h dark cycles. Watering was withheld on day 1 (red arrow) and recommenced on day 11 (green arrow). Shaded areas represent the dark periods.

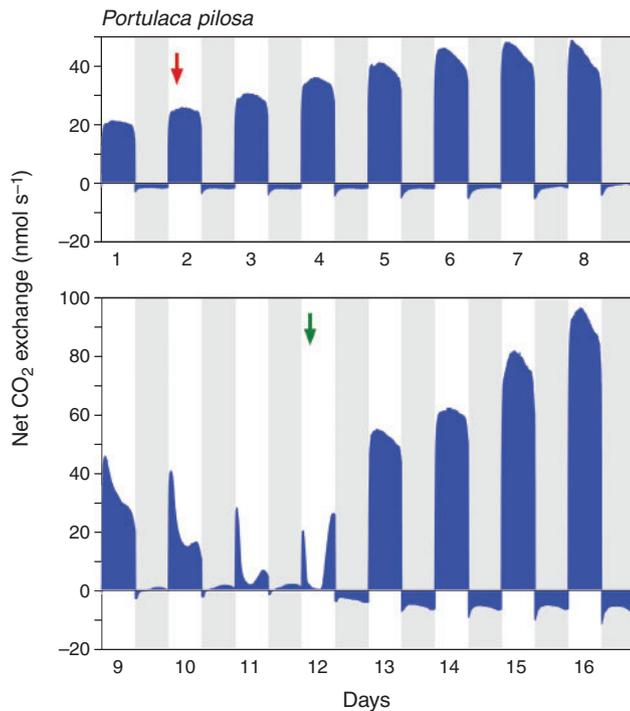


Fig. 4. Sixteen days of net CO₂ exchange by the aboveground shoot of a potted, 3-month-old *Portulaca pilosa* under 12 h light/12 h dark cycles. Watering was withheld on day 2 (red arrow) and recommenced on day 12 (green arrow). Shaded areas represent the dark periods.

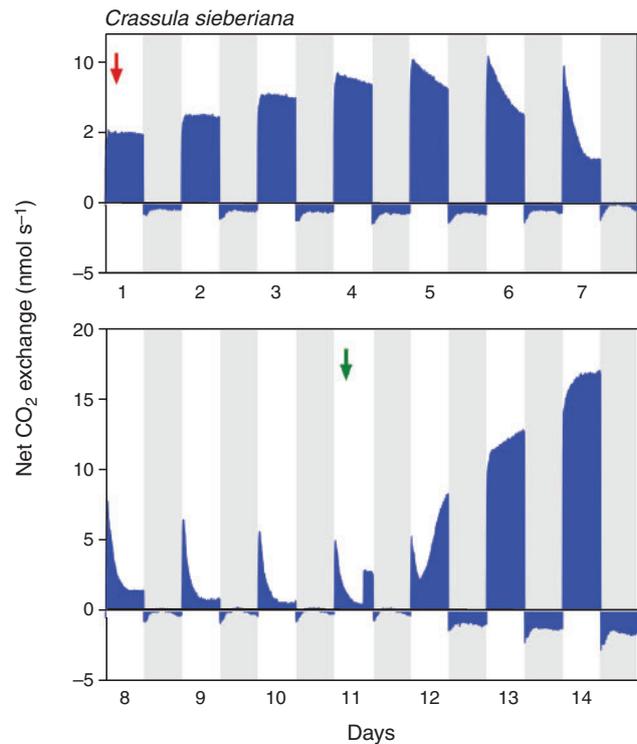


Fig. 5. Fourteen days of net CO₂ exchange by the aboveground shoot of a potted, 2-month-old *Crassula sieberiana* under 12 h light/12 h dark cycles. Watering was withheld on day 1 (red arrow) and recommenced on day 11 (green arrow). Shaded areas represent the dark periods.

accumulation were initiated following the imposition of drought, and were lost after rewatering.

Facultative CAM has not been described previously in the C₄ species *P. australis* and *P. pilosa*, nor in any of the other 14 *Portulaca* restricted to Australia (ALA 2016). However, in *P. mundula*, a taxon from SE and SW USA that is possibly synonymous with *P. pilosa* (Matthews *et al.* 1992), a C₄-type $\delta^{13}\text{C}$ value for cellulose of -11.9‰ was accompanied by a δD value of $+1\text{‰}$. The latter value was intermediate between the mean for 14 C₄ species, -33‰ , and the mean for 10 species with pronounced CAM, $+51\text{‰}$. (Sternberg *et al.* 1984). In *P. mundula* (Kraybill and Martin 1996) and in both *P. mundula* and *P. pilosa* (Guralnick and Jackson 2001) nocturnal acidification and nocturnal CO₂ fixation were reported subsequently in droughted but not well-watered leaves, though reversal of CAM was not demonstrated. CAM is also documented in *P. grandiflora* Hook. (Guralnick *et al.* 2002) and facultative CAM has long been known in *P. oleracea* L. (Koch and Kennedy 1980; Christin *et al.* 2014; D'Andrea *et al.* 2014; Winter and Holtum 2014). In the latter two species CAM is expressed both in leaves, which also perform C₄ photosynthesis, and in stems, which do not.

Facultative CAM is now known in two of the seven *Portulaca* clades described by Ocampo *et al.* (2013): in the 'Oleracea' clade (*P. oleracea* has been introduced to Australia but endemic populations are probably also present (CHAH 2016)) and in the 'Pilosa' clade in which CAM is represented by species with origins as diverse as South America (*P. grandiflora*),

Table 1. Day-night titratable acidities for leaves from well-watered, droughted and re-watered *Anacampseros australis*, *Portulaca australis*, *Portulaca pilosa* and *Crassula sieberiana*

Values are means \pm s.d. ($n = 3$ to 6); significance calculated using an unpaired *t*-test (ns = not significant)

Treatment	Titratable acidity ($\mu\text{mol H}^+ \text{g}^{-1}$ fresh mass)			Significance
	End of day	End of night	Difference	
<i>Anacampseros australis</i>				
Well-watered	204 \pm 10	205 \pm 8	+1	ns
Droughted	57 \pm 6	282 \pm 37	+225	<0.05
Re-watered	154 \pm 14	162 \pm 11	+8	ns
<i>Portulaca australis</i>				
Well-watered	24 \pm 5	20 \pm 4	-4	ns
Droughted	116 \pm 40	188 \pm 53	+72	<0.05
Re-watered	74 \pm 22	70 \pm 22	-4	ns
<i>Portulaca pilosa</i>				
Well-watered	13 \pm 2	9 \pm 1	-4	<0.05
Droughted	13 \pm 3	89 \pm 16	+76	<0.05
Re-watered	14 \pm 2	12 \pm 2	-2	ns
<i>Crassula sieberiana</i>				
Well-watered	11 \pm 2	11 \pm 3	0	ns
Droughted	13 \pm 2	39 \pm 6	+26	<0.05
Re-watered	14 \pm 3	11 \pm 2	-3	ns

Australia (*P. australis*) as well as the pan-tropical *P. pilosa*. CAM has yet to be detected in any members of the 'Australian' clade which includes the Australian natives *P. bicolor*, *P. digyna*,

P. oligosperma and *P. armitii* (recognised as *Sedopsis armitii* in the Australian Plant Census (CHAH 2016)).

A. australiana, the only Australian taxon in an otherwise southern African genus (Nyffeler and Eggli 2010; Hernández-Ledesma *et al.* 2015), exhibits a pronounced facultative CAM gas-exchange signal with the maximum rate of nocturnal CO₂ uptake during drought approaching 20% of the maximum rate of CO₂ uptake in the light before the imposition of drought (Fig. 2). The facultative nature of CAM *sensu stricto* has not been established previously in *A. australiana* although a nocturnal acidification in droughted leaves of 58 µeq g⁻¹ fresh mass indicated CAM is present (Guralnick *et al.* 2008). In general, CAM appears widespread in *Anacampseros* with a survey of 20 of the 36 species revealing a δ¹³C range from a C₃/CAM-type -24‰, for *A. australiana*, to a strong CAM-type of -12.6‰ for *A. arachnoides* (Guralnick and Jackson 2001; Guralnick *et al.* 2008). It remains to be explored whether facultative CAM is present in the African taxa.

C. sieberiana is the only native Australian *Crassula* for which CAM has been shown (Brulfert *et al.* 1991). Induction of CAM following droughting has been demonstrated but the reversibility of the induction was not tested and thus facultative CAM *sensu stricto* was not proven until this report. Although CAM is weakly expressed in droughted *C. sieberiana*, the associated conservation of both carbon and water presumably assists this species to survive water limitation in the shaded habitats it typically frequents. Parallels between habitats occupied by *Crassula* in southern Africa and Australia are indicated by observations that five of the 11 small, mostly ephemeral, taxa indigenous to Australia also occur in southern Africa, and five small southern African *Crassula* are naturalised in Australia (Toelken 1981).

The unequivocal demonstration of facultative CAM in the four species studied here brings the total number of known Australian terrestrial species capable of CAM to 14, of which 10 exhibit facultative CAM (Winter *et al.* 1981; Brulfert *et al.* 1991; Winter and Holtum 2011; Holtum *et al.* 2016). Clearly more Australian terrestrial species need to be surveyed for CAM but it appears that facultative CAM and not constitutive CAM is the predominant form of CAM in the Australian terrestrial flora. An improved understanding of the functional significance of facultative CAM to plants of Australian landscapes in which water supply is ephemeral and seasonally unpredictable awaits life-cycle ecophysiological studies of plants growing *in situ*.

It is becoming evident that facultative CAM is expressed in plants with two patterns of background acidity in their leaves. In one group of plants, exemplified by *C. sieberiana*, *P. australis* and *P. pilosa*, well-watered plants operating in the C₃ mode have low leaf titratable acidities during the day-night cycle. In stressed plants with leaves operating in the CAM mode titratable acidity increases during the course of the night and, during the day, returns to the low level seen in C₃ leaves. Other facultative CAM species in which the low background levels of acidification are observed include *Mesembryanthemum crystallinum* (Winter *et al.* 1978), *Calandrinia polyandra* (Winter and Holtum 2011) and *Talinum fructicosum* (= *T. triangulare*; Herrera 1999).

In a second group, exemplified by *A. australiana*, in well-watered plants the level of titratable acidity is high during the

light and dark, possibly close to the capacities of the vacuoles to accumulate organic acids. When plants are stressed, the background acidity decreases. As a result, in comparison to the leaves in the C₃ state, the CAM tissues have lower acidities in the evening and similar or only slightly greater acidities in the morning. This pattern, originally reported in *Portulacaria afra* (Didiereaceae; Guralnick *et al.* 1984; Guralnick and Ting 1986), appears to be widespread in the Anacampserotaceae (*Anacampseros*, *Grahamia* and *Talinopsis*; Guralnick and Jackson 2001; Guralnick *et al.* 2002) and in *Clusia* (Zotz and Winter 1993; Holtum *et al.* 2004).

In species in which leaves have an elevated background acidity in the well-watered state it would be interesting to undertake a detailed time-course analysis of the quantity and identity of organic acids (e.g. malic acid versus citric acid; Lüttge 1988; Franco *et al.* 1992) during the stress-induced transition from C₃ to CAM, and vice versa. Such a study would add significantly to our understanding of organic acid metabolism in general and would help to explain the physiology behind the switch from a high 24 h acid status in the C₃ mode to the temporarily high acid status typical of CAM.

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