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Does the C_4 plant *Trianthema portulacastrum* (Aizoaceae) exhibit weakly expressed crassulacean acid metabolism (CAM)?

Klaus Winter [A,C], *Milton Garcia* [A], *Aurelio Virgo* [A], *Jorge Ceballos* [A] and *Joseph A. M. Holtum* [A,B]

[A]Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Republic of Panama.
[B]College of Science and Engineering, James Cook University, Townsville, Qld 4811, Australia.
[C]Corresponding author. Email: winterk@si.edu

Abstract. We examined whether crassulacean acid metabolism (CAM) is present in *Trianthema portulacastrum* L. (Aizoaceae), a pantropical, salt-tolerant C_4 annual herb with atriplicoid-type Kranz anatomy in leaves but not in stems. The leaves of *T. portulacastrum* are slightly succulent and the stems are fleshy, similar to some species of *Portulaca*, the only genus known in which C_4 and CAM co-occur. Low- level nocturnal acidification typical of weakly expressed, predominantly constitutive CAM was measured in plants grown for their entire life-cycle in an outdoor raised garden box. Acidification was greater in stems than in leaves. Plants showed net CO_2 uptake only during the light irrespective of soil water availability. However, nocturnal traces of CO_2 exchange exhibited curved kinetics of reduced CO_2 loss during the middle of the night consistent with low-level CAM. *Trianthema* becomes the second genus of vascular land plants in which C_4 and features of CAM have been demonstrated to co-occur in the same plant and the first C_4 plant with CAM-type acidification described for the Aizoaceae. Traditionally the stems of herbs are not sampled in screening studies. Small herbs with mildly succulent leaves and fleshy stems might be a numerically significant component of CAM biodiversity.

Keywords: CAM evolution, CO_2 assimilation, C_4 photosynthesis, facultative CAM, Kranz anatomy, Sesuvioideae, stem photosynthesis, *Trianthema portulacastrum*.

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Introduction

C_4 photosynthesis and crassulacean acid metabolism (CAM) are modifications of the ancestral C_3 photosynthetic pathway that have evolved independently in a wide range of families and genera (Smith and Winter 1996; Sage 2016). There are approximately 8100 known C_4 species, which are dispersed across 418 genera in 19 angiosperm families (Sage and Sultmanis 2016), whereas the roughly 16 000 CAM taxa are in 400+ genera from 37 families of angiosperms, gymnosperms, ferns and lycopsids (Smith and Winter 1996; Yang *et al*. 2015; Winter *et al*. 2020*a*).

In both C_4 and CAM plants, the structure and function of photosynthetic organs has been modified from the C_3 template creating CO_2-concentrating mechanisms that suppress photorespiration. In C_4 plants which, like C_3 plants, exhibit net CO_2 uptake during the light only, suppression of photorespiration has led to enhanced photosynthetic CO_2 uptake in warm climates at reduced water cost. Water-use efficiency is further enhanced in CAM plants as water loss by transpiration is reduced when CO_2 is taken up during the cool of the night and stomata close during most of the day (Winter *et al*. 2005). While the CAM cycle is associated with nocturnal increases and diurnal decreases in the organic acid content of chloroplast-containing tissues, 24-h fluctuations in H^+ are typically absent from C_3 and C_4 tissues (Winter 2019). Despite the relatively frequent appearance of C_4 and CAM across the phylogeny of plants, only four families of vascular terrestrial plants contain C_4 species as well as CAM species: Aizoaceae, Asteraceae, Euphorbiaceae, and Portulacaceae (Smith and Winter 1996; Edwards and Ogburn 2012; Sage 2016).

Portulaca L. (Portulacaceae, Caryophyllales) is the only genus of land plants in which C_4 and CAM are known to co-occur in the same plant and, indeed, in the same leaf (Koch and Kennedy 1980). The genus is currently considered to contain 150 species (POWO 2020), overwhelmingly annual succulent-leaved herbs, allocated across six clades (Ocampo *et al*. 2013). Five clades contain C_4 taxa and one contains species with C_3-C_4 intermediate characteristics. CAM has been reported in nine species from five of the six clades (Koch and Kennedy 1980; Kraybill and Martin 1996; Guralnick and Jackson 2001; Guralnick *et al*. 2002; Holtum

et al. 2017*a*; Winter and Holtum 2017; Winter 2019) including the clade with C_3-C_4 intermediates (Winter *et al*. 2019*b*). In all cases reported to-date, the expression of CAM in leaves of *Portulaca* with C_4 photosynthesis has been facultative; CAM is expressed in leaves that have been water-stressed but is absent from leaves of well-watered plants. In the case of *P. oleracea*, in which CAM expression in leaves is facultative but its expression in stems is constitutive with a facultative component, the transcript abundance of genes putatively associated with C_4 and CAM has been studied in well-watered and in drought-stressed plants (Ferrari *et al*. 2020).

Within the Aizoaceae, a family within the order Caryophyllales of close to 1800 species and 119 genera (Hernández-Ledesma *et al*. 2015; Klak *et al*. 2017; POWO 2020), C_3 and CAM photosynthesis are present in three subfamilies, Mesembryanthemoideae, Ruschioideae and Sesuvioideae (Ting 1989; Smith and Winter 1996; Winter *et al*. 2019*a*). C_4 photosynthesis is known in 31 species of the sub-family Sesuvioideae (Bohley *et al*. 2017) where it occurs in the genera *Trianthema* (21 of ~30 species), *Sesuvium* (5 of 13 species) and *Zaleya* (all species). Within the Sesuvioideae, CAM has been reported in two species, the pantropical, predominately C_3-exhibiting *Sesuvium portulacastrum* (Ting 1989; Winter *et al*. 2019*a*) and in *S. maritimum* (Martin *et al*. 1982).

Here we report the presence of low-level CAM-type nocturnal acidification in a C_4 member of the Sesuvioideae, *Trianthema portulacastrum*. Curved patterns of nocturnal net CO_2 loss indicated temporarily enhanced refixation of CO_2 at night, consistent with low-level CAM in this species.

Methods

Plant material

A salt-tolerant annual that has Kranz anatomy in leaves but not in stems, *Trianthema portulacastrum* L. is a fast-growing, succulent-leaved herb that germinates during the wet season in the seasonally dry tropics of Panama, forming prostrate mats or clumps up to 50+ cm in diameter, before it senesces and dies during the dry season. Of uncertain geographical origin, it is widespread in the tropics and subtropics of western and south-east Asia, Africa, America and Australia (ALA 2020) where it inhabits beach dunes, moist or seasonally-dry open wetlands including alkaline flats, clay pans and playa lakes. It is often a weed of disturbed areas including gardens, irrigated soils and road-sides.

Seedlings of *T. portulacastrum* were obtained from the Sarigua National Park, Azuero Peninsula, Republic of Panama (8.013348°N, –80.485658°W), where the species coexists with *Sesuvium portulacastrum* (L.) L. (Winter *et al*. 2019*a*).

Outdoor experiment

In October 2018, the second half of the rainy season, 10 seedlings (diameter of ~5 cm each) were planted in a mixture of 50% (v/v) forest soil: 50% (v/v) sea-sand (Novey, Panama) in a 1.2 × 1.2 × 0.38 m raised garden box (Hummert International) at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama (9.120027°N, –79.702017°W) (Fig. 1). Plants were exposed to full natural solar radiation and received natural rainfall. From mid-December 2018 onwards, samples of leaves and stems were taken at dusk (17:30 – 18:00 hours) and dawn (07:00 hours) for fresh and dry mass determinations and for acid analysis at weekly intervals until the plants senesced and died during the dry season at the end of February 2019. Self-sown seeds germinated following the beginning of the wet-season rains in 2019. Starting at the end of May 2019, once again leaves and stems were sampled at dusk and dawn for fresh mass, dry mass and acid analysis at weekly to biweekly intervals. Samples were weighed for fresh mass determination and stored in liquid nitrogen to be processed for determination of dry mass and measurements of titratable acidity as described below.

Pot experiments

Throughout 2018 and 2019, experiments were conducted with potted plants to study titratable acidity changes and gas-exchange responses during wet–dry-wet cycles. Plants were grown in terracotta pots (0.5 or 3.0 L) containing 2/3 (v/v)

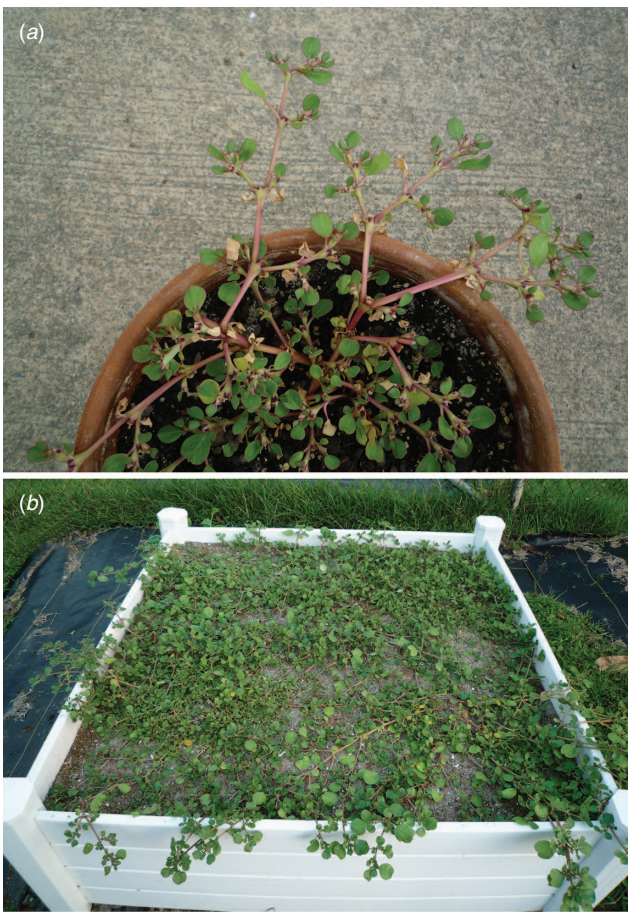

Fig. 1. (*a*) A potted *Trianthema portulacastrum*. Note the prostrate habit, slightly fleshy leaves and succulent stems. The outer diameter of the pot is 20 cm. (*b*) *Trianthema portulacastrum* growing outside in a 1.2 × 1.2 × 0.38 m raised garden box at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama. Photo taken 15 December 2018.

potting mix (Miracle-Gro Lawn Products) and 1/3 quartz sand (Novey). Plants were maintained at the Tupper Centre of the Smithsonian Tropical Research Institute, Panama City (8.962938°N, –79.702017°W) under either full solar radiation or underneath rain-shelters at 70% natural light. Growth conditions are specified in the corresponding figure legends. As in the outdoor experiments, for acidity measurements mature leaves and stems excised from plants at dusk and dawn were weighed for fresh mass determination and frozen in liquid nitrogen. Prior to freezing, leaf area was determined using a LI-3100 leaf area meter (LI-COR Biosciences).

Titratable acidity and dry mass

Samples were transferred from liquid nitrogen to a freeze-drier (Freezone 4.5, Labconco). After 72 h samples were reweighed for dry mass determination. Freeze-dried tissue was extracted sequentially in boiling 50% ethanol and in water (Winter and Holtum 2017). Extracts were titrated with 5 mM KOH to pH 6.5

Net CO_2 exchange

Twenty-four hour CO_2 exchange patterns by intact shoots of whole plants or intact attached branches were studied during wet–dry-wet cycles. In two of the three experiments shown in the results section, the shoot of a small seedling was enclosed inside a Perspex cuvette (internal dimensions 11 × 11 × 16 cm) that rested on the 0.5 L terracotta pot in which the plant grew. The roots and the pot remained outside the cuvette. After initial daily irrigation with water, 9–11 day drought treatments were imposed by withholding irrigation until net CO_2 uptake during the light period had decreased markedly, after which plants were rewatered daily. In another experiment, an intact attached branch of a plant was enclosed into a Walz GWK-3M gas exchange cuvette (Walz GmbH).

The gas-exchange cuvettes, pots and plants were located inside a controlled environment chamber (GC15, EGC) operating under 12 h light (28°C)/12 h dark (22°C) cycles. Illumination was supplied by a LED grow light (SS-GU300-w, Sunshine Systems). Photon flux density is specified in the corresponding figure legends. The cuvette was supplied with ambient air sourced 16 m above ground level and passed through a 2 m^3 buffer at a flow rate of 2.3 L min^{-1}. Net CO_2 exchange was measured in a flow-through gas-exchange system consisting of Walz components and a LI-6252 CO_2 analyser (LI-COR Biosciences) (Holtum and Winter 2003).

Anatomy

Cross-sections of fully expanded healthy leaves (middle between base and tip, and between edge and midrib) and mature stems were prepared either by hand using double edge breakable razor blades or, in some cases, tissue was fixed in 4% (v/v) glutaraldehyde for 48 h at 7°C before sectioning in a cryostat microtome (CM1860, Leica). Chlorophyll auto-fluorescence (682 nm) was examined using a confocal microscope (FV3000, Olympus) with an integrated camera system.

Results

Titratable acidity

Variation in dusk/dawn titratable acidity levels was determined in outdoor-grown plants over ~15 months. The study period included one full wet season and two dry seasons (Fig. 2*a–c*). Consistent with the annual life-form of *T. portulacastrum*, plants died during the latter part of each dry season. Reduced cloud cover during the dry season led to higher daily photon flux densities (Fig. 2*a*). As is typical for tropical rainforest climates, seasonal variations in temperature at the study site were small (Fig. 2*b*).

Throughout the course of the experiment, and irrespective of season, leaf H^+ values, when expressed on a fresh mass basis, were significantly greater at dawn than at dusk for 15 of 30 measurements (Fig. 2*d*). The frequency of significantly greater dawn levels increased to 25 when tissue acidity was expressed on a dry mass basis (Fig. 2*f*), thereby correcting for diurnal decreases in leaf water content and thus reduced fresh mass: dry mass ratios (Fig. 2*h*). Titratable acidity of stems was consistently greater at dawn than at dusk on 27 of 29 days when acidity was expressed per unit of fresh mass, and on all days when expressed per unit of dry mass (Fig. 2*e*, *g*). On the days with significant overnight acidification, the fold-increase (dawn: dusk ratio of H^+ per unit of dry mass) was greater in stems (2.03 ± 0.48, mean ± s.d., n = 29) than in leaves (1.55 ± 0.19, n = 25) (P = <0.001). There was no obvious seasonal change in the degree of nocturnal acidification of both leaves and stems (Fig. 2*f*, *g*). The H^+ values were similar during the wet and dry seasons.

Linear regressions showed that nocturnal acidification of leaves and stems correlated positively with integrated PFD of the previous day (Fig. 3). The relationship was significant for leaves (P = <0.01) and close to significant for stems (P = <0.06).

In a pot experiment under full solar radiation at STRI's Tupper Centre during the 2018 dry season, plants were exposed to a wet–dry-wet cycle. Small but significant nocturnal acidification per unit dry mass was detected in leaves of drought-stressed plants, whereas nocturnal acidification was consistently observed in stems of both watered and drought-stressed plants irrespective of whether the data was expressed on a dry mass or fresh mass basis (data not shown).

CO_2 gas-exchange

Sixty-eight days of CO_2 gas-exchange were monitored for three separate plants. Figures 4 and 5 depict the results for two plants, the shoots of which developed from seedlings inside the gas-exchange cuvette. One plant was exposed to a single watering-drought-rewatering cycle and a second to two watering-drought-rewatering cycles.

Under both well-watered and drought conditions, net CO_2 uptake was restricted to the light during all treatments. In the experiment depicted in Figure 4, CO_2 efflux was initially relatively constant throughout the night following an initial slight overshoot and period of equilibration to night temperature (22°C) which was 6°C below day temperature (28°C). Following cessation of watering on day 3, a small curvature in the nocturnal CO_2 release pattern was detectable

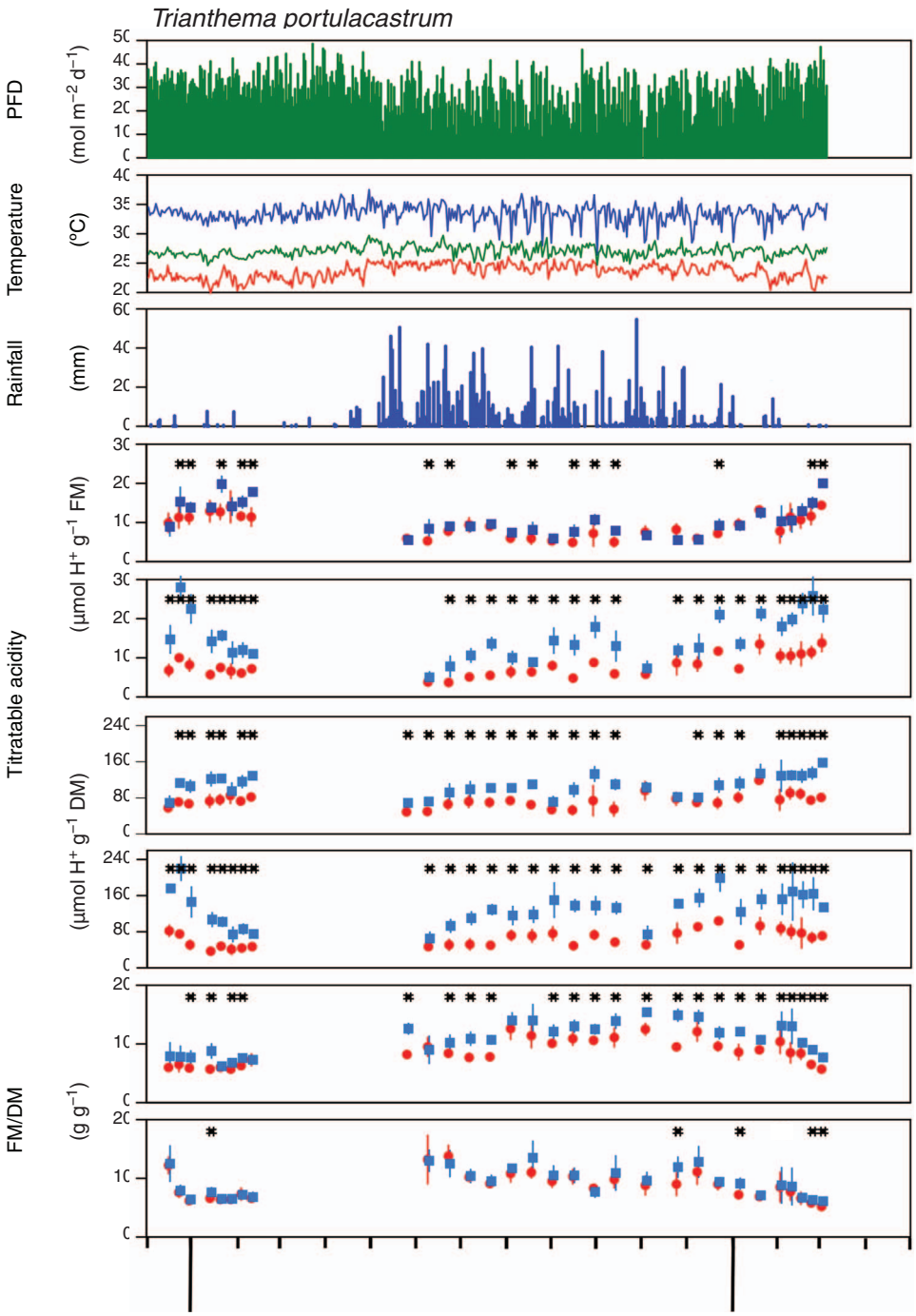

Fig. 2. Seasonal changes in photon flux density (*a*), maximum (upper blue line), minimum (lower red line) and mean (middle green line) temperature (*b*), rainfall (*c*), variation of titratable acidity in leaves and stems at dusk (red dots) and dawn (blue squares) (*d–g*), and variation in fresh mass: dry mass ratio at dusk (red dots) and dawn (blue squares) in leaves (*h*) and stems (*i*) of *Trianthema portulacastrum*. Plants were grown in a raised garden box in a 50% soil : 50% sea-sand mixture. Measurements were taken between December 2018 and March 2020. Plants died during the 2019 dry season (February) and re-grew from previously produced seeds 2 months later at the onset of the rainy season. Acidity levels are expressed per unit fresh mass (leaves, *d*; stems, *e*) and dry mass (leaves, *f*; stems, *g*). Acidity and FM/DM values are means ± s.d. (n = 5; for leaves, each sample comprised 6–9 leaves). Samples were derived from different plants. Error bars are absent if they are smaller than symbols. In (*d–i*) * indicate significant increases of titratable acidity and FM/DM between dusk and dawn values (one-tailed *t*-test, $P \leq 0.05$).

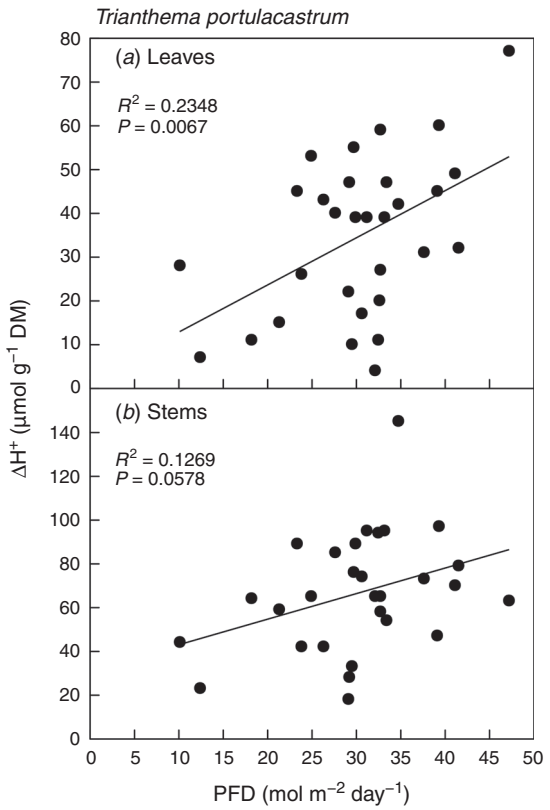

Fig. 3. Relationship between nocturnal increase in titratable acidity and integrated photon flux density of the previous day in leaves (*a*) and stems (*b*) of *Trianthema portulacastrum*. ΔH^+ values were calculated from the data in Fig. 2*f, g* and refer to days with significantly greater acidity at dawn than at dusk. (*a*) $y = 1.077x + 2.1188$; (*b*) $y = 1.1681x + 31.378$.

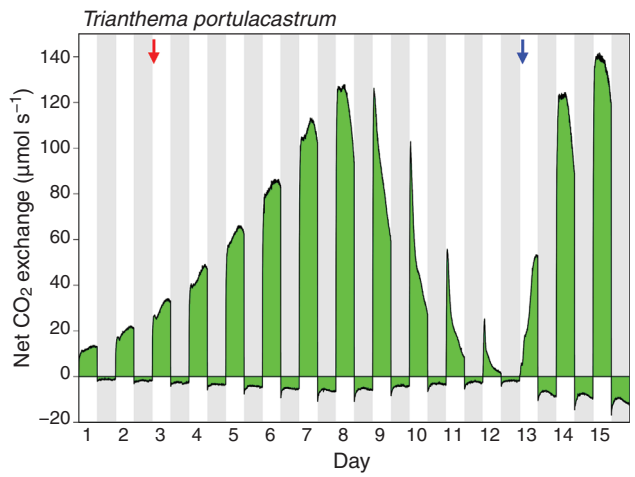

Fig. 4. Fifteen days of net CO_2 exchange by the shoot of a potted *Trianthema portulacastrum*. Watering was withheld on day 3 (red arrow) and recommenced on day 13 (blue arrow). Shaded areas represent the 12 h dark periods. Photon flux density was 700 μmol m^{-2} s^{-1} at the level of the shoot. On the last day of the experiment, total leaf area was 68 cm^2 and leaf and stem dry masses were 0.41 and 0.22 g respectively.

with lower rates in the middle than at the beginning and end of the dark period. CO_2 uptake during the day and CO_2 loss at night continued to increase as the shoot continued to grow utilising water in the pot. From day 9 onwards water-deficit stress developed as indicated by the progressive daily reduction in both CO_2 uptake during the day and CO_2 loss at night. Daytime CO_2 exchange developed a prominent morning peak of uptake. At all times, nocturnal CO_2 exchange remained below the CO_2 compensation point. Following rewatering on day 13, a recovery of CO_2 uptake in the light to pre-drought rates was observed within 3 days. Concomitant with the recovery in rates of daytime CO_2 uptake, nocturnal respiratory CO_2 loss increased to rates greater than observed before drought. The respiratory trace also developed a pronounced curvature that persisted.

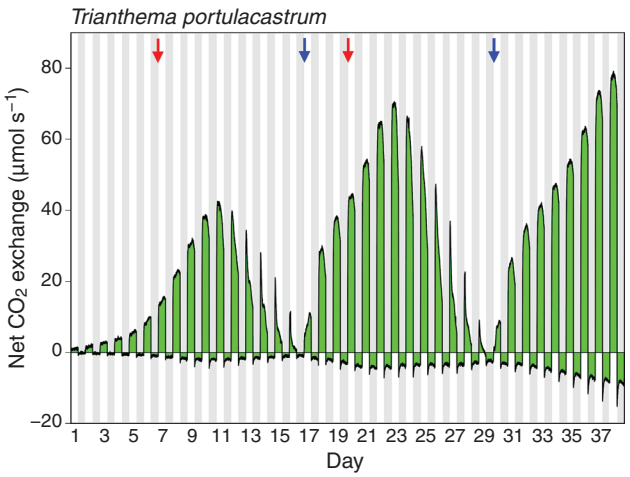

Fig. 5. Thirty-eight days of net CO_2 exchange by the shoot of a potted *Trianthema portulacastrum*. During this period, the plant was subjected to two drying/re-watering cycles. Watering was withheld on days 7 and 20 (red arrows) and recommenced on days 17 and 30 (blue arrows). Shaded areas represent the 12 h dark periods. Photon flux density was 700 μmol m^{-2} s^{-1}. On the last day of the experiment, total leaf area was 21.6 cm^2.

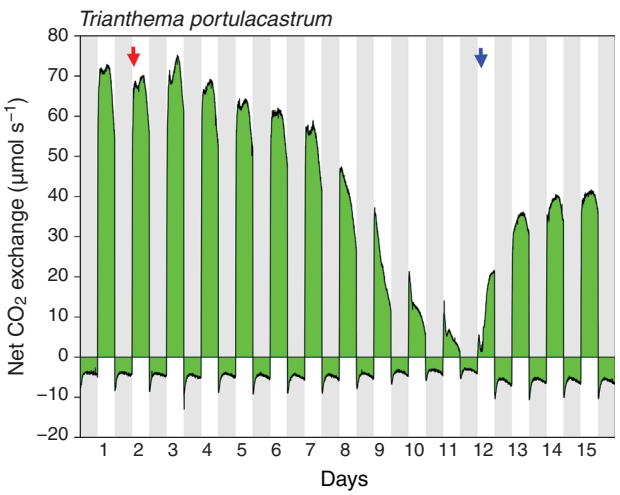

Fig. 6. Fifteen days of net CO_2 exchange by a branch of a potted *Trianthema portulacastrum*. Watering was withheld on day 2 (red arrow) and recommenced on day 12 (blue arrow). Shaded areas represent the 12 h dark periods. Photon flux density was 700 μmol m^{-2} s^{-1}.

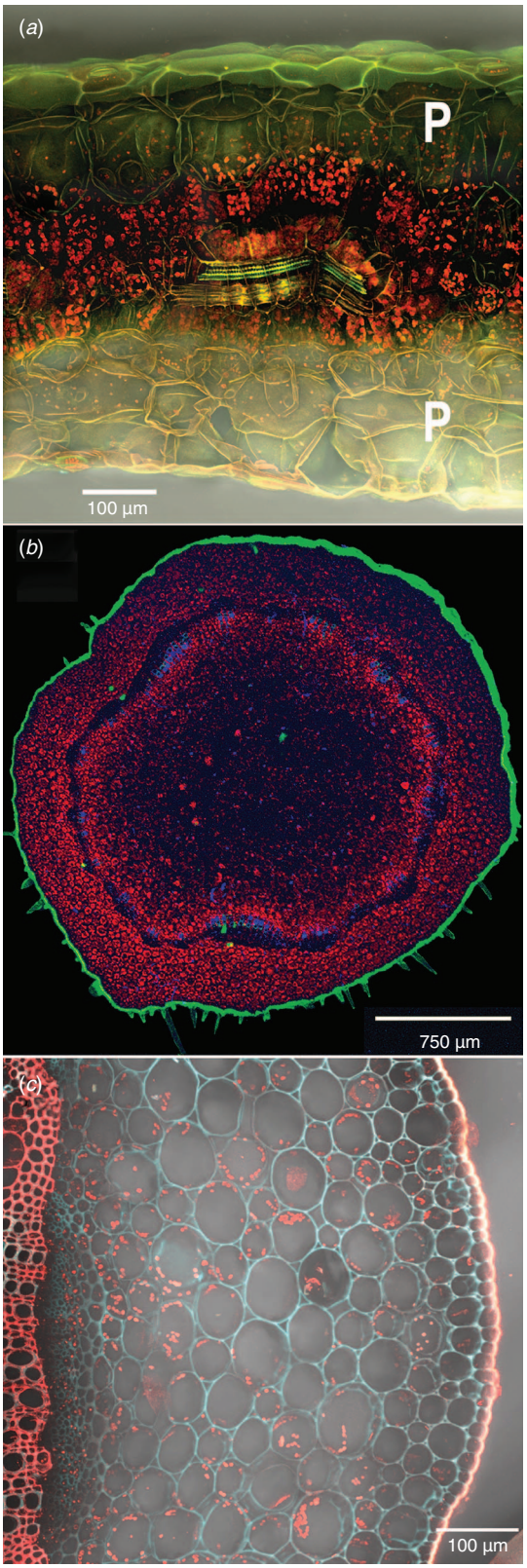

Fig. 7. Hand cross-sections of a leaf (*a*) and a stem (*b*), and a microtome cross-section through the stem cortex (*c*) of *Trianthema portulacastrum*. In (*a*) note in the centre of the leaf the atriplicoid Kranz anatomy with abundant red-fluorescing chloroplasts. In the outer abaxial and adaxial layers of large parenchyma cells (P), red-fluorescing chloroplasts are present but sparse. In (*b*) note the red-fluorescing chloroplasts in the outer pith. In (*c*) note the even distribution of red-fluorescing chloroplasts across the stem cortex.

The gas-exchange trace for the plant that was exposed to two watering-drought-rewatering cycles (Fig. 5) was similar to that for the plant exposed to a single cycle (Fig. 4) except that the absolute rates of CO_2 uptake were lower as it was a smaller plant. In Figure 5, the curvature in the nocturnal respiratory trace that was noted in particular following rewatering of the plant monitored in Figure 4, was again observed throughout most of the 38 day experiment, especially during the entire second watering-drought-rewatering cycle.

The observations for a branch of a third plant subjected to a single watering-drought-rewatering cycle are provided in Figure 6. Net CO_2 uptake in the light started to decline almost immediately when watering was stopped. Again, nocturnal net CO_2 exchange never crossed the compensation line to become positive, but during all 15 dark periods of the experiment a distinct temporal reduction of nocturnal net CO_2 loss occurred.

Leaf and stem anatomy

The leaves contain a central Kranz-type core of chlorophyll-rich bundle-sheath (BS) and mesophyll cells that encircle the leaf vascular bundles (atriplicoid pattern) (Fig. 7*a*; Muhaidat *et al.* 2007). In the BS cells the chloroplasts are arranged centripetally. The Kranz cells are surrounded by chloroplast-sparse parenchyma cells one cell deep on the upper (adaxial) surface and two cells deep on the lower (abaxial) surface. The vascular bundles, chloroplast-dense Kranz cells and associated air spaces together constitute ~38% of the leaf volume, the chloroplast-sparse outer parenchyma cells and associated air spaces together constitute 30% of the leaf volume, and the epidermis/cuticle constitutes the remainder.

In contrast to the leaves, Kranz anatomy is absent from the stems of *T. portulacastrum*. The stems contain a band of vascular tissue encircling a central parenchymatous pith (Fig. 7*b*). The pith cells near the vascular band contain chloroplasts. The vascular tissue is surrounded on the outside by a cortex, ~9–11 cells deep, of parenchyma cells that contain chloroplasts that are relatively evenly distributed across the tissue (Fig. 7*c*).

Stomatal densities were 98 ± 17 mm^{-2} and 89 ± 4 mm^{-2} (mean ± s.d., n = 5) for the upper and lower leaf surfaces respectively. Stem stomatal densities were 18 ± 4 mm^{-2} for the sun exposed upper surfaces and 16 ± 7 mm^{-2} for the lower soil facing surfaces.

Discussion

Nocturnal H^+ increase and CO_2 exchange

The C_4 pathway is the principal mode of carbon acquisition in *T. portulacastrum*. However, *T. portulacastrum* is not 100% C_4. Measurements of titratable acidity and CO_2 gas exchange strongly suggest that this species also exhibits low-level CAM photosynthesis. Increases in H^+ content from dusk to dawn, a defining feature of CAM photosynthesis, were consistently

Table 1. Nocturnal acidification in *Trianthema portulacastrum* as compared to species with weak and strong CAM
Values for *Trianthema portulacastrum* are the means from all days with significant nocturnal acidification in Fig. 2. For *Sesuvium portulacastrum*, corresponding means were calculated from data in figs 2 and 3 of Winter *et al*. (2019*a*). Among the species with weakly expressed acidification, *Bulbophyllum putidum* had a CAM-type $\delta^{13}C$ value of –15.2‰, and *Zamioculcas zamiifolia* and *Oncidium panamense* showed net CO_2 dark fixation. Data for *Zamioculcas zamiifolia* and *Coleus scutellarioides* were obtained from plants exposed to water deficit stress, a treatment that resulted in enhanced CAM expression. ND, not determined

Species, Family, Plant organ	Nocturnal acidification (μmol H^+ g^{-1} fresh mass)	(μmol H^+ g^{-1} dry mass)	Reference
Trianthema portulacastrum, Aizoaceae, leaves	3	40	This study
Trianthema portulacastrum, Aizoaceae, stems	8	67	This study
	Weak CAM		
Sesuvium portulacastrum, Aizoaceae, leaves	3	49	Winter *et al*. (2019*a*)
Sesuvium portulacastrum, Aizoaceae, stems	9	54	Winter *et al*. (2019*a*)
Zamioculcas zamiifolia, Araceae, leaves	5	36	Holtum *et al*. (2007)
Coleus scutellarioides, Lamiaceae, leaves	5	62	Winter *et al*. (2020*b*)
Coleus scutellarioides, Lamiaceae, stems	12	182	Winter *et al*. (2020*b*)
Platycerium veitchii, Polypodiaceae, fronds	8	ND	Holtum and Winter (1999)
Bulbophyllum putidum, Orchidaceae, leaves	14	158	Silvera *et al*. (2005)
Oncidium panamense, Orchidaceae, leaves	22	134	Silvera *et al*. (2005, 2014)
	Strong CAM		
Opuntia cochenillifera, Cactaceae, cladodes	102	1216	K. Winter, unpubl. data
Epidendrum radicans, Orchidaceae, leaves	227	2197	Silvera *et al*. (2005)
Kalanchoë pinnata, Crassulaceae, leaves	234	ND	Winter and Holtum (2015)
Kalanchoë pinnata, Crassulaceae, stems	7	ND	Winter and Holtum (2015)
Agave americana, Asparagaceae, leaves	309	1988	K. Winter unpubl. data
Clusia rosea, Clusiaceae, leaves	327	1136	K. Winter unpubl. data

observed in stems, and very frequently in leaves. Nocturnal acidification was detected in stems of field-grown plants on a regular basis, irrespective of whether acidity was expressed on a fresh mass or dry mass basis, whereas in leaves the lower degree of acid accumulation was most obvious when H^+ was expressed on a dry mass basis because the day-night differences in leaf water content result in underestimates of nocturnal acidification when expressed on a fresh mass basis. Overall, nocturnal increases in H^+ were much smaller than those of archetypal CAM species (e.g. many cacti and agaves) but in the range of other species that operate at the low end of the C_3 – full CAM or C_4 – full CAM spectrum (Table 1). Titratable acidity measurements in leaf and stem extracts are highly sensitive, can resolve differences of as low as 1 μmol H^+ g^{-1} fresh mass between dusk and dawn samples, and are well suited to reliably identify small nocturnal H^+ increases such as those in *T. portulacastrum*. Nocturnal increases of H^+ are most likely the result of CAM-type CO_2 dark fixation, where the uptake of 1 CO_2 typically leads to the accumulation of 1 malate anion + 2 H^+. We are not aware of studies that have demonstrated significant nocturnal H^+ accumulation in photosynthetic tissues that engage 100% in the C_3 or C_4 pathway.

Nocturnal net CO_2 exchange of CAM-exhibiting species reflects the balance between uptake of atmospheric CO_2 via PEP carboxylase and production of CO_2 by mitochondrial respiration. In archetypal CAM species, dark CO_2 fixation greatly outweighs respiration, leading to a highly positive carbon balance at night. Another feature of CAM-gas exchange is that rates of dark CO_2 fixation rarely reach a steady-state: they typically increase in the first half of the dark period and decline in the second half (Osmond 1978). In species with weakly expressed CAM, dark CO_2 fixation is enhanced but often does not exceed rates of CO_2 loss. Hence the nocturnal carbon balance may remain negative. Nonetheless, in analogy to the nocturnal increases and decreases of rates of net CO_2 dark fixation observed in species with strong CAM, enhanced dark CO_2 fixation in species with very weakly expressed CAM is still reflected by a characteristic temporary decrease of net CO_2 loss in the course of the night.

Here we present the results of three independent CO_2 exchange experiments with three different plants that spanned a total of 68 day-night cycles and generated 23380 measuring points at 4 min intervals. We tested whether *T. portulacastrum* has the capacity for net dark CO_2 fixation in the well-watered state, a result which would have been unequivocal evidence of constitutive CAM. We also examined whether *T. portulacastrum* has the capacity to reversibly induce or upregulate net dark CO_2 dark fixation in response to water-deficit stress, a result which would have been unequivocal evidence of facultative CAM. In both well-watered and drought-stressed plants, at no time did shoot or branch CO_2 exchange at night exceed the compensation point and become positive. However, the patterns of nocturnal CO_2 exchange were clearly different from plants that engage 100% in C_3 or C_4 (where the nocturnal CO_2 exchange trace is essentially a straight negative line under constant night-time temperature conditions as employed in the current study of

gas-exchange). In *T. portulacastrum* curved patterns of net CO_2 loss were consistently observed, indicating a temporal reduction of net CO_2 loss likely related to enhanced CO_2 uptake. This curved shape of nocturnal CO_2 exchange has been observed for other species with low levels of CAM expression (e.g. *Platycerium veitchii*, Holtum and Winter 1999; *Portulaca digyna*, Holtum *et al*. 2017*a*; *Sesuvium portulacastrum*, Winter *et al*. 2019*a*). Taken together, the two observations, small nocturnal increases in titratable acidity and the small temporal reductions of nocturnal net CO_2 loss strongly suggest that a weakly expressed CAM cycle is present in *T. portulacastrum*. Utilising $\delta^{13}C$ of plant carbon as an indicator of CAM use in *T. portulacastrum* is not possible because C_4 and CAM photosynthesis lead to similar carbon isotopic signatures.

Overall, considering the large dataset of seasonal changes in H^+, nocturnal acidification in leaves and stems of the annual *T. portulacastrum* appears predominately constitutive with no or little evidence of stress-associated upregulation. The constitutive nature of low-level CAM in *T. portulacastrum* is supported by the positive relationship between overnight H^+ increase and PFD during the previous day (significant in leaves and almost significant in stems), a feature well documented for cacti (Nobel 1982). The nature of CAM in the annual *T. portulacastrum* therefore not only differs in pattern and extent from known annual C_4 *Portulaca* species but also differs from other well-studied CAM annuals such as *Mesembryanthemum crystallinum* (Aizoaceae; Winter and von Willert 1972) and *Calandrinia polyandra* (Montiaceae; Winter and Holtum 2011). In *M. crystallinum* and *C. polyandra* the environmentally-triggered switch from C_3 to CAM involves net nocturnal CO_2 uptake during the CAM phase, assisting the extension of life-cycle into the dry season and enabling succulent leaves and branches to persist, reproductive material to develop and seeds to fill after the soil dries out (Winter *et al*. 1978; Winter and Ziegler 1992). In contrast, nocturnal acidification in *T. portulacastrum* is not restricted to the dry season. Furthermore, nocturnal CO_2 uptake in *T. portulacastrum* merely suffices to reduce CO_2 loss but does not lead to net dark CO_2 fixation.

Anatomical considerations – a comparison with Portulaca

Trianthema portulacastrum is only the tenth vascular C_4 or C_3-C_4 plant species recorded to also exhibit CAM cycle activity. The other C_4-with-CAM species are all *Portulaca*: *P. australis* (Winter and Holtum 2017), *P. cryptopetala* (Winter *et al*. 2019*b*), *P. cyclophylla* (Holtum *et al*. 2017*a*), *P. digyna* (Holtum *et al*. 2017*b*), *P. grandiflora* (Ku *et al*. 1981; Guralnick and Jackson 2001), *P. molokiniensis* (Winter *et al*. 2019*b*), *P. oleracea* (Koch and Kennedy 1980), *P. pilosa* (Winter and Holtum 2017) and *P. umbraticola* (Winter 2019). In an attempt to explain why CAM and C_4 photosynthesis were not known in a given species (with the exception of *Portulaca* spp.) despite sharing many similar metabolic steps and a tendency to cluster in similar regions of the angiosperm evolutionary tree (Edwards and Ogburn 2012), Sage (2002) suggested that each pathway requires a distinct leaf anatomy to function efficiently; C_4 evolution requires enhanced BS size in C_3 ancestors whereas CAM evolution requires succulence. Suggesting that the starting anatomies were mutually exclusive, Sage (2002) further postulated that the outcome of any evolutionary selection pressure may be predetermined, resulting in an incompatibility between the two types of photosynthesis that does not permit co-existence. Almost two decades later, the Sage-hypothesis holds for the most part. There are still no species known that exhibit C_4 in conjunction with the high rates of nocturnal CO_2 uptake typical of plants with pronounced CAM such as an *Agave* or *Opuntia*. In those species where CAM photosynthesis is known to co-exist with C_4, the rates of nocturnal CO_2 uptake are typically low and the two photosynthetic pathways appear to occur in anatomically distinct regions of the leaf, rather than in the same cells. The only evidence for both pathways functioning in the same cell is a report, based on an inability to immunochemically detect Rubisco in the large putatively CAM parenchyma cells, that malate is transported in the light from the CAM cells to Kranz cells in leaves of stressed *P. oleracea* (Lara *et al*. 2004). The postulated metabolic scheme did not consider the more parsimonious exchange of CO_2, rather than malate, between cell types, and it was not clear how PEP would be made available for CO_2 dark fixation in the parenchyma cells. Among C_4 and CAM researchers there is currently considerable interest in understanding traits that might favour the evolution of one photosynthetic pathway over the other (Edwards and Ogburn 2012). There is also much to be learned from localising the components that might enable the co-existence of the two photosynthetic pathways in leaves of species such as *Trianthema* and *Portulaca* (Ferrari *et al*. 2020).

If low-level CAM in the leaves of *T. portulacastrum* occurs in the layers of large parenchyma cells that lie above and below the central C_4 cell layers, one would expect to observe chloroplasts in the cells, but perhaps at low density as the degree of CAM is low. A low density of chloroplasts detected in parenchyma cells of leaves from plants used in this study supports this hypothesis (Fig. 7*a*). However, in several studies, leaf parenchyma cells of *T. portulacastrum* have been reported to lack chloroplasts: Kienholz (1926) reported that the parenchyma tissues he defined as water-storage cells were achlorophyllous; Muhaidat and McKown (2013) stated that the parenchyma is non-chlorenchymatous but showed a photograph in which the abaxial parenchyma cells clearly contained chloroplasts; and Muhaidat *et al*. (2007) also considered the parenchyma cells non-chlorenchymatous and showed a photograph of a leaf cross-section with very poorly developed parenchyma layer. In contrast, similar to our observations, Bohley *et al*. (2015) reported that the spongy parenchyma tissue contains a few chloroplasts. In all likelihood, as with the development of CAM, the development of the water-tissue-type large parenchyma cells and their chloroplast complement may depend upon growth conditions, specifically irradiance. In contrast to the observational variation reported for chloroplasts in leaf parenchyma, chloroplasts are abundant in the cortex and pith cells of stems (Fig. 7*b*, *c*).

Phylogenetic implications

CAM appears unevenly distributed among the Aizoaceae. It is widespread in the sub-families Mesembryanthemoideae and Ruschioideae (Smith and Winter 1996; Winter 2019) yet there is no clear evidence of CAM in the sub-families Acrosanthoideae and Azooideae. Only two species with CAM-type nocturnal acidification have been described among the ~50 species in the sub-family Sesuvioideae, in the predominately C_3 *S. portulacastrum* (Winter *et al*. 2019*a*) and now in the predominately C_4 *T. portulacastrum*. There are no reports of CAM in the C_4 members of *Sesuvium*, or in the only C_3 *Trianthema*, *T. ceratosepala* (Bohley *et al*. 2015). The other genera in the subfamily Sesuvioideae, *Anisostigma* and *Tribulocarpus*, exhibit C_3-type carbon isotope signatures. In both *T. portulacastrum* and *S. portulacastrum*, the expression of CAM features is at the very low end of the CAM spectrum in which, depending upon species and environmental conditions, the contribution of CAM to total carbon gain can vary from values of <1% to close to 100% (Winter 2019), with archetypal CAM species such as cacti gaining close to 100% of their carbon via CAM (Nobel 1988). Despite differences in their principal photosynthetic pathways, *T. portulacastrum* and *S. portulacastrum* exhibit similarities in morphology and habit. Both are prostrate annuals of the seasonally dry tropics with succulent or semi-succulent leaves that are attached to conspicuous green fleshy stems. The more salt-tolerant C_3 *S. portulacastrum* is a plant of coastal habitats whereas the C_4 *T. portulacastrum* is more an opportunistic weed of drier and disturbed landscapes. Although both are successful species in that they have pantropical distributions, it would be inappropriate to ascribe their wide distributions to ecological advantages endowed by the ability to express dual pathways of photosynthesis as both are salt-tolerant, produce prolific quantities of small seed, and in each the contribution of CAM to carbon gain is small.

The relationships between the origins of C_4 and CAM in the Sesuvioideae are uncertain. More than one interpretation is possible for the origin of C_4 (Bohley *et al*. 2015) and information on CAM presence or absence is as yet insufficient for hypothesis testing. A maximum likelihood optimisation of a phylogeny based upon molecular and trait characters favoured the hypothesis of a single early gain of C_4 photosynthesis in a C_3 ancestor of the *Trianthema*/*Sesuvium*/*Zaleya* lineage and two subsequent $C_4 \rightarrow C_3$ reversions, one in *Sesuvium* and one in *Trianthema*. An alternative scenario of C_4 evolution that excluded the possibility of $C_4 \rightarrow C_3$ reversions requires C_4 to have evolved from C_3 ancestors multiple times (at least twice in *Sesuvium* and at least three times in *Trianthema*). A situation similar to the latter alternative appears to have occurred in *Portulaca*, except that CAM is ancestral to C_4 and CAM continued to be expressed after C_4 had evolved (Christin *et al*. 2014).

How widespread is CAM in C_4 species?

Since fewer than 5% of the close to 180 species of *Trianthema* and *Portulaca* have been surveyed for CAM, and several unscreened species exhibit succulent leaves and/or fleshy stems that are similar to *T. portulacastrum* and *P. oleracea* (Hartmann *et al*. 2011; Bohley *et al*. 2015), it would be reasonable to expect that there are more species with features of CAM to be discovered within both genera. There also seems to be a strong possibility of CAM occurrence in C_4 species of the genus *Sesuvium*. We note that it is generally assumed, because of the absence of Kranz anatomy, that the dominant photosynthetic pathway in the stems of *Portulaca* and *Trianthema* with C_4 (or C_3-C_4 intermediate) leaf anatomy is C_3. However, clearly in some species these stems are not 100% C_3 and evidently have evolved some degree of CAM. To our knowledge, the relative contributions of C_3 and CAM to daytime and night-time carbon gain in water-stressed and non-water-stressed stem tissues has yet to be demonstrated experimentally. The presence of stomata, although at low density, suggests some CO_2 exchange with the atmosphere. Separate measurements of diel leaf- and stem-CO_2 exchange and studies of stem stomatal functioning are clearly warranted.

The role of stems

A spate of recent reports of CAM-type acidification in small fleshy-stemmed herbs with mildly succulent leaves such as *Calandrinia* (Montiaceae; Winter and Holtum 2011; Hancock *et al*. 2019), *Coleus* (Lamiaceae; Winter *et al*. 2020*a*), *Portulaca* (Portulacaceae; Holtum *et al*. 2017*a*; Winter 2019; Winter *et al*. 2019*b*), *Sesuvium* (Aizoaceae; Winter *et al*. 2019*a*) and now *Trianthema*, have not only increased our knowledge of CAM diversity in a numeric sense but highlight a hitherto unrecognised aspect of CAM diversity. Although CAM is well known to be associated with small and massive perennial stem-succulents (Nobel 1988; Smith and Winter 1996) it appears that annual herbs with fleshy stems may be larger contributors to CAM biodiversity than previously envisaged. Generally, when screening these plants for CAM by means of acid titrations or gas exchange, only leaves are sampled, but not stems and petioles. It is probable that more intense screening of the angiosperm phylogeny for CAM in stems and petioles, particularly in annual and bi-annual taxa, will uncover more species with CAM. Moreover, in herbs in which the expression of CAM in leaves may be facultative, i.e. absent in leaves of a well-watered plant, stem- or petiole-screening may detect a permanent CAM signal. We do not know the principal function of low-level CAM in stems and petioles. Reducing water-loss in *T. portulacastrum* is likely to be of minor importance as the stems have few stomata. It would be logical if CAM acted principally to reduce carbon loss by recycling respiratory CO_2.

Adaptive significance

The possible contribution of low level CAM to the overall carbon budget of the C_4 plant *T. portulacastrum* can be assessed by estimating nocturnal carbon loss in the absence of the temporary reduction in net CO_2 efflux, i.e. by assuming that after the initial overshoot of net CO_2 loss at the very beginning of the dark period, the rate of net CO_2 loss remained at the same level as was observed at the end of the dark period. For the branch experiment of Fig. 6, where the temporal reductions in nocturnal net CO_2 release were particularly

pronounced, low level CAM contributed only up to ~2% to 24 h carbon gain (= carbon gain in the light minus carbon loss at night) in the well-watered and re-watered states. But under severe water-deficit stress (Fig. 6, day 11), when CO_2 uptake in the light was substantially reduced, the temporal reduction of nocturnal net CO_2 loss by 20% almost doubled 24-h-CO_2 gain, potentially aiding survival.

Conflicts of interest

The authors declare no conflicts of interest.

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