Functional Plant Biology, 2021, **48**, 683–690 https://doi.org/10.1071/FP20151

Low-level CAM photosynthesis in a succulent-leaved member of the Urticaceae, *Pilea peperomioides*

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Abstract. *Pilea peperomioides* Diels (Urticaceae) is a semi-succulent herbaceous species native to south-western China that has become popular in cultivation as an ornamental plant. To investigate whether this species possesses the capacity for CAM photosynthesis, measurements were made of CO₂ gas exchange and titratable acidity in plants under both well-watered and water-deficit conditions. Plants were found to assimilate CO₂ almost exclusively in the light via C₃ photosynthesis. However, distinct transient reductions in the rate of net nocturnal CO₂ release were consistently observed during the course of the dark period, and under water-deficit conditions one plant exhibited a brief period of net nocturnal CO₂ uptake, providing unequivocal evidence of CAM activity. Furthermore, nocturnal increases in titratable acidity in both leaf laminas and petioles were observed in all plants exposed to wet–dry–wet cycles. This is the first report of CAM in the family Urticaceae. The results are discussed in relation to the phylogenetic position of *Pilea* and the partially shaded montane habitats in which this species is typically found. An updated list of all plant families currently known to contain species with CAM is presented.

Keywords: CAM photosynthesis, Chinese money plant, CO₂ fixation, drought stress, nettle, photosynthesis, *Pilea peperomioides*, succulence, tissue acidity, Urticaceae.

Received 2 June 2020, accepted 28 October 2020, published online 8 December 2020

Introduction

Pilea is the largest genus in the nettle family Urticaceae, with 604 accepted species native to tropical and temperate Asia, North, Central, and South America, and Africa. A phylogenetic analysis of *Pilea* was presented by Monro (2006). The majority of species are shade-tolerant annual or perennial herbs, often with fleshy and turgescent stems. Some have horticultural value, such as *Pilea cadierei* Gagnep. & Guillaumin (the aluminium plant), *Pilea pubescens* Liebm. (= *P. involucrata* Urb.; the friendship plant), *Pilea nummulariifolia* (Sw.) Wedd. (creeping Charlie), and *Pilea microphylla* (L.) Liebm. (the artillery plant, so named because of the puffs of pollen ejected by the exploding anthers).

Another increasingly popular ornamental species worldwide is *Pilea peperomioides* Diels, variously known as the Chinese money plant, the missionary plant, and mirror leaf. The plant is an evergreen perennial with distinctive semi-succulent, peltate leaf laminas, shiny and glabrous on the upper surface, borne on long petioles (Fig. 1). The species is native to Yunnan and Sichuan provinces in south-western China, where it grows lithophytically in montane forests on damp rocks such as humus-covered boulders and cliff-ledges. The plant first became known to the western world in the early 20th century through specimens collected in Yunnan province in 1906 and 1910 by the Scottish botanist and explorer George Forrest, which were formally named by Ludwig Diels in 1912 (Diels 1912). Following a more detailed description of the species and the first published image in 1984 (Radcliffe-Smith 1984), interest grew in explaining how this plant came to be so widely cultivated as an ornamental. It appears that living material collected in Kunming, Yunnan Province, was first introduced into Europe by the Norwegian missionary Agnar Espegren in 1946 (the material having survived transportation in a box for over a year), after which the plant was widely propagated and became popular as a house plant (Kers 1985). Despite its widespread cultivation (Dransfield and Dransfield 1984; Lancaster 1985; Walker and Levers 1993; Zou *et al.* 2017), this species remains little investigated.

Because of the succulent foliage of these plants, we decided to investigate whether the species exhibits features of CAM, since the CAM pathway has never previously been demonstrated in the family Urticaceae.

CAM photosynthesis is one of three photosynthetic pathways and is typically found in succulent plants that occupy semiarid terrestrial and epiphytic habitats in the subtropics and tropics. The CAM cycle is defined by nocturnal uptake of atmospheric CO_2 via phospho*enol*pyruvate carboxylase (PEPC) leading to the accumulation of malic acid in the vacuoles of chloroplast-containing cells. During the subsequent light



Fig. 1. Photograph of the shoot of a potted 19-cm tall *Pilea peperomioides*. The width of the complete foliage is 13 cm.

period, malic acid is released from the vacuoles, decarboxylated, and the liberated CO2 re-fixed by Rubisco into the Calvin cycle (Osmond 1978; Winter and Smith 1996a; Holtum et al. 2005). Like C₄ photosynthesis, CAM is a modification of the ancestral C₃ pathway, serving to concentrate CO₂ in the vicinity of Rubisco, thereby enhancing its CO2 fixation capacity. Whereas C4 plants are more water-use efficient than C₃ plants, CAM allows for a further increase in water-use efficiency, because stomata are typically closed for most part of the day, and at night, when stomata open, the driving forces for water loss are strongly reduced owing to a low tissue-air vapour-pressure difference (Neales et al. 1968; Osmond et al. 1979; Winter et al. 2005). Although well-known archetypal CAM plants like cacti and agaves gain carbon mostly at night (Neales 1973a; Hanscom and Ting 1978; Nobel 1988), many CAM-exhibiting species still maintain a strong C₃ component of CO₂ assimilation during the day (Borland et al. 2011; Winter et al. 2015). In fact, in many species with low levels of CAM activity, the contribution of dark CO₂ fixation to total carbon gain may be less than 5%, with CAM merely offsetting the loss of respiratory CO2 and, at the very low end of the CAM spectrum, not necessarily being detectable as net nocturnal CO₂ uptake (Holtum and Winter 1999; Winter et al. 2019a). The ratio of dark to light CO₂ fixation is speciesdependent, and increases as photosynthetic organs mature. Furthermore, this ratio is strongly influenced by environmental conditions. For example, dark CO₂ fixation is less sensitive to water-deficit stress than CO₂ fixation in the light (Kluge and Fischer 1967; Hanscom and Ting 1978; Smith and Lüttge 1985; Winter 2019). Moreover, in plants with facultative CAM, dark CO₂ fixation is induced or enhanced in response to reduced soil water availability or high salinity stress (Winter and Holtum 2011, 2014), with the halophyte Mesembryanthemum crystallinum L. being one of the most studied species (Winter and von Willert 1972; Winter and Lüttge 1976; Winter and Holtum

2007). Hence, unlike a fully developed C_4 pathway, the presence and magnitude of CAM in a species is rarely an allor-nothing phenomenon and the flexibility of many CAMexhibiting species in terms of CO_2 fixation in the dark and light provides some of the most spectacular examples of metabolic plasticity in the plant kingdom (Winter 2019).

In the research presented here, we examined 24-h changes of CO_2 gas-exchange and acid content in *Pilea peperomioides* exposed to wet–dry–wet cycles. We provide evidence for the presence of the CAM cycle in this species, and thus report for the first time the occurrence of CAM in the family Urticaceae.

Materials and methods

Potted plants of Pilea peperomioides Diels were purchased in a local supermarket in Panama City, Republic of Panama. Plastic pots had an upper diameter of 15 cm and a height of 10 cm. Shoots were ~20 cm tall. For measurements of titratable acidity, plants were kept outdoors underneath a transparent polycarbonate-roofed rain shelter for 4 weeks at the Tupper Centre of the Smithsonian Tropical Research Institute (8.963284°, -79.543474°). Daily PFD was ~70% of full natural radiation. Titratable acidity was determined in plants exposed to a wet-dry-wet cycle. Leaf laminas and petioles were excised at dusk and dawn and their fresh mass determined. The area of leaf laminas was measured with a LI-3100 leaf area meter (LI-COR Biosciences). Samples were stored in liquid nitrogen and then freeze-dried for 3 days (FreeZone 4.5, Labconco). After determination of dry mass, samples were boiled in 50 mL of 50% ethanol (v/v) for 10 min. Evaporated solution was replaced with water and samples were boiled again for 10 min. After extracts attained room temperature, their volume was increased with water to 50 mL, and H⁺ content determined by titration with 5 mM NaOH to pH 6.5.

Several attempts were made to measure gas exchange of single attached leaves, either by enclosing leaf laminas into a PMK 10 clamp-on cuvette (Walz GmbH), or by inserting them via their petioles into a 11 cm \times 11 cm \times 10 cm (internal dimensions) Perspex cuvette. In five of six cases, no CO₂ signal was measurable for several days, i.e. net CO₂ exchange was \pm 0. In only one case did a leaf show a photosynthetic-respiratory response. We therefore switched to CO₂ gas-exchange measurements of whole shoots.

Plants for CO₂ gas exchange were established from plantlets that grew out of the base of purchased mother plants. Plants were grown in 1.0 L terracotta pots filled with potting mix (Miracle-Gro Lawn Products). Shoots of entire plants were enclosed inside a Perspex cuvette (internal dimensions 11 cm \times 11 cm \times 15 cm) that rested on the terracotta pot in which the plants grew. The roots and the pot remained outside the cuvette. After initial daily irrigation with water, water deficit treatments were imposed by withholding irrigation until net CO₂ uptake during the light period had decreased markedly, after which plants were rewatered daily. The gas-exchange cuvette, pot and plant were located inside a controlled environment chamber (GC8-T, EGC) operating under 12 h light (25°C):12 h dark (20°C) cycles. Illumination was supplied by an LED grow light (SS-GU300-w, Sunshine Systems). Photon flux density was 200 μ mol m⁻² s⁻¹. The cuvette was supplied with air containing 400 μ L L⁻¹ CO₂ by adding pure CO₂ to CO₂-free air using a GMA-3/10 gas mixing system (Walz). The air flow rate was 1.1 L min⁻¹. Net CO₂ exchange was measured in a flow-through gas-exchange system consisting of Walz components such as mass-flow controlled air pumps (one LD-10R, two LD-1R) and a gas-switch unit (GU-2), and an LI-820 or LI-7000 CO₂ analyser (LI-COR Biosciences) operating in the absolute mode. A CR1000 data-logger (Campbell Scientific) controlled the gas-switch unit and recorded the CO₂ concentration of air entering and leaving the plant cuvette at 2-min intervals.

Results

A total of 125 days of whole-shoot CO_2 exchange were monitored for four separate plants. All experiments provided evidence of low-level CAM. Fig. 2 depicts the data for one of the plants. Net CO_2 uptake was almost exclusively restricted to the light period, but unlike typical

C₃ species, especially when maintained under relatively low light-conditions as employed in the current study, neither the rates of daytime nor night-time CO2 exchange in Pilea peperomioides were constant during their respective parts of the light/dark cycle. This was particularly true for nocturnal CO₂ exchange, which showed a strongly curved pattern throughout the experiment. Following an initial overshoot at the beginning of the night, net CO₂ loss decreased and then increased. While in the first part of the experiment the lowest rates of net CO₂ loss were observed during the very early part of the dark period (days 1-14), this point of minimal CO₂ loss shifted towards the middle of the night and the curvature became more symmetrical as the experiment proceeded (days 15-28). Following cessation of watering on day 4, overall rates of daytime and night-time CO₂ exchange continued to increase as the plant grew further. Daytime CO₂ fixation began to decrease sharply on day 14, indicating the onset of drought stress. Simultaneously, overall rates of net CO₂ loss decreased, and from day 19 onwards the nocturnal CO2 exchange trace shifted transiently to net uptake of CO_2 during the middle of the dark period.





Fig. 2. Twenty-eight days of net CO_2 exchange of a whole shoot of *Pilea* peperomioides maintained under the conditions described in Materials and methods. Watering was withheld on day 4 (red arrow) and recommenced on day 23 (blue arrow). Positive values indicate net CO_2 uptake; negative values indicate net CO_2 loss. Shaded areas represent the 12 h dark periods. At the end of the experiment, total leaf area was 164 cm². Dry mass of leaf laminas, petioles, and stem were 0.86, 0.16, and 0.09 g respectively.

Fig. 3. Twenty-eight days of net CO_2 exchange of a whole shoot of *Pilea* peperomioides maintained under the conditions described in Materials and methods. Watering was withheld on day 4 (red arrow) and recommenced on day 25 (blue arrow). Shaded areas represent the 12 h dark periods. At the end of the experiment, total leaf area was 152 cm². Dry mass of leaf laminas, petioles, and stem were 0.75, 0.12, and 0.19 g respectively.

Upon re-watering on day 23, daytime CO_2 uptake and nighttime CO_2 loss rapidly recovered, while the curvature of nocturnal net CO_2 loss was maintained.

Gas-exchange responses of three other plants exposed to wet–dry–wet cycles were similar to the responses depicted in Fig. 2 in that nocturnal CO_2 loss consistently showed a strongly curved pattern. This is illustrated in Fig. 3 for one of these three plants. However, during severe water deficit stress, rates of net nocturnal CO_2 exchange merely approached zero at the point of minimal CO_2 loss (Fig. 3, days 20–24), and net CO_2 exchange never crossed the compensation line and became positive.

Leaf laminas (Fig. 4) and petioles (Fig. 5) of plants exposed to a wet–dry–wet cycle consistently showed significant nocturnal increases of titratable acidity content, irrespective of treatment or whether acidity was expressed on a fresh mass, dry mass or, in the case of leaf laminas, area basis. Compared with the well-watered initial state, water-deficit treatment led to a significant increase in leaf titratable acidity per unit fresh mass at dawn (P = 0.046), whereas dusk values did not differ



Fig. 4. Titratable acidity at dusk (red columns) and dawn (blue columns) in mature leaf laminas of *Pilea peperomioides*. Well-watered = daily watering. Not watered: 28 days without irrigation. Re-watered: 4 days with irrigation. Acidity values are expressed on a fresh mass (FM) basis (upper panels), dry mass (DM) basis (middle panels), and area basis (lower panels). Values are means \pm s.e. (n = 6; each sample refers to the leaf from a different plant). Numbers are *P*-values (one-tailed *t*-test); all mean values at dawn were significantly greater than those at dusk at $P \le 0.05$.

significantly between these two states (P = 0.287). However, expressed on a dry mass or area basis, neither the dawn nor dusk values of titratable acidity differed significantly between water-deficit and well-watered conditions.

Water-deficit treatments as applied in Figs 2–4 did not lead to visible wilting of leaf laminas. Detached leaf laminas maintained for seven days on a laboratory bench under dim light (PFD of 20 μ mol photons m⁻² s⁻¹ during daytime) at 23°C and 50% RH lost only 22 \pm 3% of their original fresh mass (mean \pm s.d., n = 5), and leaf water content per unit area declined by only 21% from 89 \pm 8 mg H₂O cm⁻² to 70 \pm 6 mg H₂O cm⁻².

Discussion

Although light-driven C₃ photosynthesis is the principal pathway of carbon acquisition in P. peperomioides, the species is not 100% C₃ as both CO₂ gas exchange and titratable acidity measurements indicate the capacity for low-level CAM. The temporal pattern of nocturnal CO₂ exchange shows a strong curvature, with the rate of net CO₂ loss decreasing towards the middle of the dark period followed by a gradual increase thereafter (Fig. 2, predominantly under water-deficit and re-watered conditions; Fig. 3, throughout entire experiment). This pattern would be consistent with a transient increase in dark CO₂ fixation that reduces net CO₂ loss against a more or less constant background of dark respiration. In plants without CAM, under the constant nighttemperature conditions used in this study, rates of respiratory net CO₂ loss would typically remain steady and unchanged throughout the course of the dark period. Moreover, the finding that in one of the plants the nocturnal CO₂ exchange trace transiently crossed the compensation line and shifted to net CO₂ uptake under conditions of severe water-deficit stress (days



Fig. 5. Titratable acidity at dusk (red columns) and dawn (blue columns) in petioles of *Pilea peperomioides*. For further information see legend of Fig. 4.

Table 1. Plant families containing species capable of CAM photosynthesis, a representative CAM genus for each family, and key references

Isoetaceae belongs to lycopods, Polypodiaceae and Pteridaceae are ferns, and Welwitschiaceae and Zamiaceae are gymnosperms; all other families are angiosperms. Families are arranged linearly according to their phylogenetic position. Angiosperm families are listed in the order suggested by APG IV (2016). *Families with aquatic CAM species; in Isoetaceae, Alismataceae, Hydrocharitaceae, and Plantaginaceae, all known CAM-exhibiting species are aquatic, whereas in Crassulaceae, except for aquatic species of *Crassula*, all other CAM-exhibiting species are land plants

Family	Representative genus	References
Isoetaceae*	Isoetes	Keeley (1998)
Polypodiaceae	Pyrrosia	Wong and Hew (1976)
Pteridaceae	Vittaria	Martin <i>et al.</i> (2005)
Zamiaceae	Dioon	Vovides et al. (2002)
Welwitschiaceae	Welwitschia	Smith and Epstein (1971); von Willert et al. (2005)
Piperaceae	Peperomia	Sipes and Ting (1985); Holthe et al. (1992)
Araceae	Zamioculcas	Holtum et al. (2007)
Alismataceae*	Sagittaria	Keeley (1998)
Hydrocharitaceae*	Ottelia	Zhang et al. (2014); Shao et al. (2017); Huang et al. (2020)
Orchidaceae	Dendrobium	Nuernbergk (1961); Winter et al. (1983); Silvera et al. (2005)
Asphodelaceae	Aloe	Nuernbergk (1961); Kluge et al. (1979)
Asparagaceae	Agave	Nobel (1988); Heyduk et al. (2016)
Commelinaceae	Callisia	Martin et al. (1994)
Bromeliaceae	Ananas	Neales (1973b); Medina (1974); Crayn et al. (2015)
Crassulaceae*	Kalanchoë	Nuernbergk (1961); Borland and Griffiths (1997); Keeley (1998)
Vitaceae	Cissus	Virzo de Santo et al. (1980)
Urticaceae	Pilea	This publication
Cucurbitaceae	Xerosicyos	de Luca et al. (1977)
Oxalidaceae	Oxalis	Kluge and Ting (1978)
Clusiaceae	Clusia	Tinoco Ojanguren and Vásquez-Yánes (1983); Lüttge (2006)
Passifloraceae	Adenia	Mooney et al. (1977)
Euphorbiaceae	Euphorbia	Nuernbergk (1961); Horn et al. (2014)
Geraniaceae	Pelargonium	Schütte et al. (1967); Jones et al. (2003)
Aizoaceae	Mesembryanthemum	Winter and von Willert (1972); Winter (2019)
Montiaceae	Calandrinia	Winter and Holtum (2011)
Didiereaceae	Alluaudia	Winter (1979)
Basellaceae	Anredera	Holtum et al. (2018)
Talinaceae	Talinum	Herrera et al. (1991); Brilhaus et al. (2016)
Portulacaceae	Portulaca	Koch and Kennedy (1980); Winter et al. (2019b)
Anacampserotaceae	Anacampseros	Guralnick and Jackson (2001); Winter and Holtum (2017)
Cactaceae	Opuntia	Nobel (1988)
Rubiaceae	Myrmecodia	Winter et al. (1983); Tsen and Holtum (2012)
Apocynaceae	Dischidia	Winter et al. (1983)
Gesneriaceae	Codonanthe	Guralnick et al. (1986)
Plantaginaceae*	Littorella	Keeley (1998)
Lamiaceae	Coleus	Winter <i>et al.</i> (2020 <i>b</i>)
Asteraceae	Kleinia	Schütte et al. (1967); Ruess and Eller (1985)

19–22 in Fig. 2) provides unequivocal evidence of CAM activity, and suggests that low-level CAM in *P. peperomioides* has a facultative component.

Titratable acidity measurements provide further evidence for the presence of CAM, as significant nocturnal increases in H^+ were consistently observed in both leaf laminas (Fig. 4) and petioles (Fig. 5) irrespective of plant water status. The magnitude of acidification in leaf laminas and petioles was similar on a fresh mass and dry mass basis, respectively. Consistent with a facultative CAM component, average ΔH^+ , i.e. the difference between dawn and dusk values, appeared to be slightly enhanced on a fresh mass basis in leaf laminas of water-stressed compared with well-watered and re-watered plants, although this could not be confirmed statistically with the limited sample size available in this investigation.

The observation of CAM in petioles of *P. peperomioides* is in line with the increasing number of recent reports of CAM in small fleshy-stemmed herbs such as *Basella* (K. Winter, unpublished), *Coleus* (Winter *et al.* 2020*b*), *Portulaca* (Winter *et al.* 2019*b*), *Sesuvium* (Winter *et al.* 2019*a*), and *Trianthema* (Winter *et al.* 2020*a*). Although CAM is well known in perennial stem-succulents (Nobel 1988), the relatively short-lived leaf-bearing herbs with CAMexhibiting fleshy stems and petioles may be larger contributors to CAM biodiversity than previously thought.

The adaptive significance of low-level CAM in *P. peperomioides* is presently unclear. Its full evaluation

would require detailed studies of photosynthetic activity of plants and microclimate *in situ*. Although the majority of CAM plants occupy relatively open and periodically dry habitats, partially shaded montane sites where *P. peperomioides* has been collected in China (Radcliffe-Smith 1984) are typically not associated with CAM. However, the energetic cost of the CAM cycle is only moderately higher than that of C_3 photosynthesis and does not represent an extra heavy burden on light-use efficiency (Winter and Smith 1996*b*; Cheung *et al.* 2014; Shameer *et al.* 2018). For example, the bromeliad *Aechmea magdalenae* is a wellknown example of a plant with full CAM that can exist and flourish in the shaded understory of Neotropical forests (Skillman and Winter 1997; Skillman *et al.* 1999).

The Urticaceae belong to the large rosid clade of eudicots and is currently the only family in the order Rosales known to exhibit CAM. Other orders with CAM in the rosids are Vitales, Cucurbitales, Geraniales, Oxalidales and Malpighiales, the last of these containing three families with CAM: Clusiaceae, Euphorbiaceae, and Passifloraceae (Stevens 2001).

Table 1 presents an updated list of the families currently known to contain species with CAM. The list is a revised version of Table 1 in Winter and Smith (1996a) (see also Smith and Winter 1996) incorporating new evidence for presence and absence of CAM in taxa, and taking into account recent changes in plant relationships and taxonomic designations through mainly molecular phylogenetic studies. With addition of the Urticaceae, CAM has now been observed in a total of 37 families. Table 1 is a conservative estimate of the number of CAM families. For the time being, and in contrast to Winter and Smith (1996a), Apiaceae - a family with potentially aquatic CAM species - is no longer listed based on the assessment of Keeley (1998). As in Winter and Smith (1996a) and Smith and Winter (1996), we have not included Celastraceae, Ebenaceae, Flacourtiaceae (now Salicaceae) and Sapindaceae. Rao et al. (1979) reported substantial day-night changes in malate content in thin-leaved species of these families (one species of each). However, these plants all showed C₃-type δ^{13} C values and more work is needed to evaluate whether the CAM cycle genuinely operates in these species. Similarly, more information is required on the possible occurrence of CAM in leaves and stems of two non-succulent xerophytic tree species, Salvadora persica (Salvadoraceae) and Prosopis juliflora (Fabaceae) (Gaur 1968). In the study of Winter et al. (1976), leaves of Salvadora persica showed a C₃-type δ^{13} C value and there was no significant nocturnal accumulation of malate. We believe more work is also needed to confirm recent suggestions of fungal-induced CAM in Camellia oleifera (Theaceae) (Yuan et al. 2012) and of CAM in the coastal C₄ grass Spinifex littoreus (Poaceae) (Ho et al. 2019). In both cases, continuous measurements of day/night CO2 exchange of the type shown in Fig. 2 are warranted to reveal the subtleties of weakly expressed CAM. Furthermore, in Camellia, the reported large difference between nocturnal increase of the malate anion and of H⁺ is stoichiometrically unusual and needs to be re-examined. Another potential 'new' CAM family not considered in Table 1 is the monotypic Halophytaceae, which contains the leaf-succulent Halophytum ameghinoi endemic to Patagonia. Its δ^{13} C value of -18.6 % (Holtum *et al.* 2018; E.

J. Edwards, unpublished) is consistent with CAM, but gasexchange and titratable acidity measurements have not yet been performed on this species. Once CAM is confirmed in these and other species, the total number of CAM families could well exceed 40.

Conflicts of interests

The authors declare no conflicts of interest.

Acknowledgements

This research was supported by the Smithsonian Tropical Research Institute. JACS acknowledges support from the Oxford Martin School.

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Handling Editor: Suleyman Allakhverdiev