

# Constitutive and facultative crassulacean acid metabolism (CAM) in Cuban oregano, *Coleus amboinicus* (Lamiaceae)

**Klaus Winter<sup>A,C</sup>, Aurelio Virgo<sup>A</sup>, Milton Garcia<sup>A</sup>, Jorge Aranda<sup>A</sup> and Joseph A. M. Holtum<sup>A,B</sup>**

<sup>A</sup> Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Republic of Panama.

<sup>B</sup> College of Science and Engineering, James Cook University, Townsville, Qld 4811, Australia.

<sup>C</sup> Corresponding author. Email: [winterk@si.edu](mailto:winterk@si.edu)

## Running title CAM in *Coleus*

**Abstract.** Plants exhibiting the water-conserving crassulacean acid metabolism (CAM) photosynthetic pathway provide some of the most intriguing examples of photosynthetic diversity and plasticity. Here, a largely unnoticed facet of CAM-plant photosynthesis is highlighted: the co-occurrence of ontogenetically controlled constitutive and environmentally controlled facultative CAM in a species. Both forms of CAM are displayed in leaves of *Coleus amboinicus* Lour. (Lamiaceae), a semi-succulent perennial plant with oregano-like flavour that is native to southern and eastern Africa and naturalised elsewhere in the tropics. Under well-watered conditions, leaves assimilate CO<sub>2</sub> predominantly by the C<sub>3</sub> pathway. They also display low levels of CO<sub>2</sub> uptake at night accompanied by small nocturnal increases in leaf tissue acidity. This indicates the presence of weakly expressed constitutive CAM. CAM expression is strongly enhanced in response to drought stress. The drought-enhanced component of CAM is reversible upon rewatering and thus considered to be facultative. In contrast to *C. amboinicus*, the thin-leaved closely related *Coleus scutellarioides* (L.) Benth. exhibits net CO<sub>2</sub> fixation solely in the light via the C<sub>3</sub> pathway, both under well-watered and drought conditions. However, low levels of nocturnal acidification detected in leaves and stems indicate that the CAM cycle is present. The highly speciose mint family, which contains few known CAM-exhibiting species and is composed predominantly of C<sub>3</sub> species, appears to be an excellent group of plants for studying the evolutionary origins of CAM and for determining the position of facultative CAM along the C<sub>3</sub>–full CAM trajectory.

**Additional keywords:** CO<sub>2</sub> fixation, CAM evolution, drought stress, leaf acidity, photosynthesis.

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## Introduction

*Coleus amboinicus* Lour. (Lamiaceae) (previously *Plectranthus amboinicus* (Lour.) Spreng.; Paton *et al.* 2019) is one of the most widely recognized members of the genus *Coleus*. It is a semi-succulent perennial herb native to southern and eastern Africa that is widely cultivated and naturalized elsewhere in the tropics and sub-tropics. Its leaves are highly aromatic, have many traditional medical uses (Lukhoba *et al.* 2006; Arumugam *et al.* 2016), and serve as a substitute for oregano (*Origanum vulgare* L.) to flavour food. Common names of *C. amboinicus* include Mexican mint and Cuban oregano. *Plectranthinae* is the only subtribus in the mint family in which species with the water-conserving CAM photosynthetic pathway of photosynthesis have been

identified (Kluge and Ting 1978; Smith and Winter 1996). Conclusive photosynthetic pathway information for *C. amboinicus* is not available.

CAM photosynthesis is believed to occur in well over 5 percent of angiosperm species, and is also found in ferns, fern allies, and gymnosperms (Smith and Winter 1996). In most CAM-exhibiting species, the expression of CAM is constitutive (or obligate), i.e. the CAM pathway is always present in mature photosynthetic tissues (Winter *et al.* 2015; Winter 2019). The degree to which plants engage in constitutive CAM relative to C<sub>3</sub> photosynthesis is species-specific and modulated by environment. There is a small, yet steadily increasing, number of species in which CAM can be shown to be facultative: CAM is elicited in response to environmental stress, typically drought, and is downregulated once the stress ceases (Winter and Holtum 2007, 2014). Under well-watered conditions, facultative CAM plants acquire carbon mostly via C<sub>3</sub> photosynthesis or, in some cases, C<sub>4</sub> photosynthesis (Holtum *et al.* 2017; Winter and Holtum 2017).

Building on observations by Kluge and Ting (1978), who reported nocturnal acidification in *Plectranthus prostratus* Gürke (now *Coleus prostratus* (Gürke) A.J.Paton; Paton *et al.* 2019), Herppich and collaborators studied the occurrence of CAM in several species of what was then considered *Plectranthus* native to Yemen and southern Africa (Herppich and Herppich 1996, 1997; Herppich 1997; Herppich *et al.* 1998). Features of CAM were noted in four of seven species. CAM expression ranged from very low levels in a species with predominantly C<sub>3</sub> photosynthetic CO<sub>2</sub> fixation in the light, to relatively high levels in *P. marrubioides* (now *Coleus cylindraceus* (Hochst. ex Benth.) A.J.Paton; Paton *et al.* 2019) where nocturnal CO<sub>2</sub> fixation contributed more than 50 percent to total daily carbon gain in well-watered plants.

More recently, Ramana and Chaitanya (2015) reported CAM-type nocturnal increases in tissue acidity in several species of *Coleus* including *C. aromaticus*, a synonym of *C. amboinicus*, and *C. blumei*, a synonym of *C. scutellarioides* (Paton *et al.* 2019). Well-watered plants of these species showed significant overnight accumulation of total organic acids, a result that would be consistent with obligate CAM. However, nocturnal increases in H<sup>+</sup> were considerably larger than nocturnal increases in malic acid and citric acid combined, raising questions about the nature of the organic acid anion involved in CAM in these plants. Net CO<sub>2</sub> exchange was not determined by these authors, nor did they study the effects of drought stress on photosynthetic pathway physiology.

Facultative CAM *sensu stricto*, i.e., the reversible induction or up-regulation of CAM in response to soil water-deficit stress, has never been explicitly demonstrated in any member of the Lamiaceae, although a close inspection of published data for *P. marrubioides*, classified as an obligate CAM plant by Herppich *et al.* (1998), does reveal evidence of drought-enhanced nocturnal CO<sub>2</sub> fixation. However, drought-enhanced nocturnal malate accumulation was not observed in *P. marrubioides* (figs. 4 A, B in Herppich *et al.* [1998]).

In the study presented here, the possible presence of CAM, in particular facultative CAM, was explored in leaves of *C. amboinicus*. Measurements of day-night CO<sub>2</sub> exchange and of nocturnal changes in tissue acidity demonstrated that this species has the ability to display both strong facultative CAM and weakly expressed constitutive CAM. CO<sub>2</sub> exchange responses of *C. amboinicus* to drought stress were compared with those of *C. scutellarioides*, a thin-leaved species that exhibits net CO<sub>2</sub> fixation solely in the light. Our original hypothesis that *C. scutellarioides* is exclusively C<sub>3</sub> was however proven incorrect during the course of the study.

## Materials and methods

### *Growth of plants*

*Coleus amboinicus* Lour. was grown from 5 cm stem cuttings in 1.6 L, 23 cm high TP49 tree pots (Stuewe & Sons) filled with Miracle-Gro potting mix (Miracle-Gro Lawn Products). Cuttings were obtained from plants purchased at Riba-Smith supermarket in Panama City, Republic of Panama. Plants were kept underneath a rain shelter and received ~ 30% of daily full solar radiation. They were 20– 30 cm tall when used for gas-exchange and nocturnal acidification measurements.

*Coleus scutellarioides* (L.) Benth. was cultivated from seeds of plants collected in a local garden in Panama City. Pot volume was 1.0 L. Soil and growing conditions were as for *C. amboinicus*.

*C. amboinicus* was also grown under natural sunlight and rainfall conditions in a raised garden box at the Smithsonian Tropical Research Institute's Santa Cruz Experimental Research Facility in Gamboa, Republic of Panama, from October 2016 to April 2017 (Fig. 1). The dimensions of the raised garden box were 1.5 x 1.5 x 0.3 m. Its frame was made of 3-cm thick wood panels. Approximately 50 small plants that had been established from cuttings were inserted in forest top soil at the end of September 2016.

#### *Titrateable acidity*

Leaf discs were excised with a cork borer at dusk and dawn (four 1.5 cm diameter disks per sample) and, after fresh mass (FM) determination, were frozen in liquid nitrogen. In one experiment with *C. scutellarioides*, stem samples (1.5– 3 g FM) were also taken. Samples were freeze-dried (Freezone 4.5, Labconco) for dry mass determination, and boiled in 60 mL of 50% (v/v) ethanol for 10 min. The volume was brought back to 60 mL with water, and samples were boiled again for 10 min. After cooling to room temperature, samples were titrated with 5 mM KOH to pH 6.5.

#### *Net CO<sub>2</sub> exchange*

In the experiments depicted in the 'Results' section, the major portion of mature, attached leaves was enclosed into a clamp-on PMK 10 gas-exchange cuvette (Walz GmbH) connected to a through-flow (open) gas-exchange system consisting of Walz components and a LI-6262 CO<sub>2</sub> analyzer (LI-COR Biosciences). Air containing 400  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub> was delivered to the leaf cuvette at a flow rate of 1.26 L min<sup>-1</sup>. Illumination was by a SS-GU300-w LED light (Sunshine Systems). Leaf cuvette and plant were placed inside a temperature-regulated controlled-environment chamber (GC8-T, EGC).

Gas exchange was also measured on entire, attached leaves using a GWK 3M chamber (Walz) (data not shown). In total, leaves of five different plants of *C. amboinicus* and leaves of two plants of *C. scutellarioides* were studied. Results from replicate experiments were consistent. Representative gas-exchange experiments are depicted in the 'Results'.

## **Results**

In well-watered plants of *P. amboinicus*, light-driven C<sub>3</sub> photosynthesis was the principal carbon acquisition pathway (Fig. 2.), but the diel pattern of CO<sub>2</sub> exchange deviated significantly from what one might expect for a typical C<sub>3</sub> species. Net CO<sub>2</sub> uptake exhibited a temporary dip during the light period. Nocturnal respiratory CO<sub>2</sub> loss, rather than staying constant, gradually decreased over the course of the dark period, with CO<sub>2</sub> exchange transitioning to very small rates of net CO<sub>2</sub> uptake towards the end of the dark period. In response to drought stress, net CO<sub>2</sub> fixation in the light dropped to essentially zero on Days 6 and 7 (except for a short peak immediately after the onset of the light period), whereas CO<sub>2</sub> uptake in the dark was rapidly and markedly upregulated. After only 3 days of withholding irrigation, net CO<sub>2</sub> uptake was observed throughout almost the entire dark period. Upon rewatering, CO<sub>2</sub> fixation in the light swiftly recovered and nocturnal CO<sub>2</sub> fixation gradually declined. On Day 11, 5 days after rewatering, the nocturnal CO<sub>2</sub> balance was similar to that observed for the well-watered plant on Day 1. CO<sub>2</sub> exchange was negative during the first two thirds of the night but was positive at the end of the night.

Consistent with leaf gas-exchange, well-watered plants of *C. amboinicus* showed small but significant nocturnal increases in leaf-tissue acidity (Fig. 3, treatment A). Nocturnal H<sup>+</sup> increase ( $\Delta\text{H}^+$ ) rose 5- to 6-fold in response to drought stress (Fig. 3, treatments B and D). Upon rewatering, the component of  $\Delta\text{H}^+$  elevated in response to drought stress reversed fully (treatment C) or almost fully (treatment E).

In well-watered plants, the basal, permanently expressed  $\Delta\text{H}^+$  increased with leaf age, from 4  $\mu\text{mol g}^{-1}$  FM in the youngest leaf (leaf 1) to 17  $\mu\text{mol g}^{-1}$  FM in the oldest leaf (leaf 6) (Fig. 4). The drought stress-triggered increase of  $\Delta\text{H}^+$  also responded to leaf age in that the  $\Delta\text{H}^+$  levels

gradually increased from leaves 1 to 3 and was greatest (up to 115  $\mu\text{mol g}^{-1}$  FM) in fully expanded mature leaves (leaves 4 to 6).

The responses of plants grown outdoors in a raised garden box from November 2016 to May 2017 throughout a wet and a dry season corroborated those of potted plants (Fig. 5). Nocturnal acidification was present but low at the end of the wet season (November and December 2016). The  $\Delta\text{H}^+$  levels, when expressed on the bases of both leaf fresh mass and area, increased markedly with the onset of the dry season in January and February, reaching peak values during March and April, before tailing off in April as the new wet season began.

In contrast to *C. amboinicus*, the diel net  $\text{CO}_2$  exchange pattern of leaves of well-watered *C. scutellarioides* was outwardly consistent with a classic  $\text{C}_3$  photosynthetic phenotype. During the entire wet-dry-wet cycle shown in Fig. 6, net  $\text{CO}_2$  uptake was restricted to the 12-h light periods. In the initial well-watered state, rates of  $\text{CO}_2$  uptake were relatively constant at around 11  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Net  $\text{CO}_2$  loss during the dark period was also relatively constant although the rate decreased during the middle of the night, giving the overall nocturnal trace a bowed appearance. Exposure to drought stress led to a decline of  $\text{CO}_2$  gain during the light and a reduction of  $\text{CO}_2$  loss during the dark. The curved nature of the nocturnal trace of  $\text{CO}_2$  exchange became more pronounced. Upon rewatering, the diel pattern of  $\text{CO}_2$  exchange reverted to the pattern observed for the well-watered plant on Day 1.

The subtle difference in the curved shape of the nocturnal gas-exchange observed for *C. scutellarioides* from a conventional level  $\text{C}_3$ -type trace can be an indicator of the presence of low-level CAM. This possibility was confirmed. Leaves of *C. scutellarioides* exhibited small nocturnal increases in titratable acidity in water-stressed plants but not in well-watered plants. The differences were discernible irrespective of whether acidification was expressed on a fresh mass, dry mass or leaf area basis (Fig. 7). Stem tissues of *C. scutellarioides* also acidified at night. The pattern of acidification in stems differed from leaves in that acidification was present in the stems of well-watered, drought-stressed and rewatered plants (Fig. 8). The level of stem acidification increased when plants were drought-stressed and decreased to levels present in well-watered plants when droughted plants were rewatered.

## Discussion

Although  $\text{C}_3$  photosynthesis is the principal carbon acquisition pathway in *C. amboinicus*, CAM photosynthesis, with both constitutive and facultative features, is clearly present in this species. Whereas leaves always show a background level of weakly expressed constitutive CAM, they also have the ability to strongly upregulate CAM in a facultative manner in response to drought stress. Compared to well-watered plants, nocturnal accumulation of  $\text{H}^+$  was 5- to 10-fold greater in mature leaves of drought-stressed plants. In the fully CAM-induced state, nocturnal  $\text{CO}_2$  assimilation reached 1.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , which is a relatively high rate in comparison to many other facultative CAM species, equivalent to approximately 15% of rates of  $\text{C}_3$ -photosynthetic  $\text{CO}_2$  assimilation of unstressed *C. amboinicus* in the light. Thus, *C. amboinicus* joins the subset of CAM plants that have relatively high facultative CAM-expression, such as *Clusia pratensis* and *Mesembryanthemum crystallinum* (Winter 1973; Winter and Holtum 2014). In many other facultative CAM plants, nocturnal carbon gain in the fully-induced CAM state is typically less than 5 percent of the diurnal  $\text{C}_3$  (or  $\text{C}_4$ ) photosynthetic carbon gain (Winter 2019); species in the Basellaceae, Portulacaceae and Talinaceae belong to this category (Winter and Holtum 2014, 2017; Holtum *et al.* 2018).

It is well established that in species with constitutive CAM, CAM activity increases as photosynthetic tissues mature (Jones, 1975; Winter, 2019). Consistent with these previous observations, background levels of weakly expressed CAM in *C. amboinicus* increased as leaves aged. In the same way, the magnitude of the facultative induction of CAM-type nocturnal acidification increased with leaf age, indicating that facultative CAM, although by definition a strictly environmentally-triggered phenomenon, is still subject to similar ontogenetic constraints as constitutive CAM (Winter 1973; Borland *et al.* 1998).

As more species with facultative CAM are discovered, it is possible that, like *C. amboinicus*, many of these will display the trait in the form of drought-enhanced CAM upregulation on top of pre-existing weakly-expressed constitutive CAM, rather than starting with a relatively clean non-CAM phenotype, as demonstrated for *M. crystallinum*, some *Calandrinia* spp., and *Portulaca* spp. (Winter and Holtum 2007, 2011, 2014). In fact, the extensively studied facultative CAM species *Clusia minor* has long been known to exhibit low-level CAM even when well watered (Borland *et al.* 1998; Lüttge 2006). Future species comparisons will show whether there is correlation between the degree of CAM expression in the fully drought-induced state and the absence or presence of weakly expressed CAM in the well-watered state.

The adaptive significance of CAM in *C. amboinicus* in its natural habitats is currently unclear. To learn more about the ecology of CAM in *C. amboinicus*, *in situ* monitoring of seasonal changes of  $C_3$  and CAM activities, growth, and reproduction is required. In Africa, these plants are found at low altitudes in woodland or coastal bush, on rocky slopes, and loamy or sandy flats (Datiles and Acevedo-Rodríguez 2014). In Panama and elsewhere, plants are typically cultivated under semi-shaded conditions. In the present study, drought-stressed plants maintained at 30 percent of natural sunlight accumulated similar amounts of  $H^+$  overnight as drought-stressed plants outdoors under full sunlight conditions (Figs 3, 4, 5), consistent with a preference of *C. amboinicus* for partial shade.

Facultative CAM species operating in either the  $C_3$  ( $C_4$  in the case of *Portulaca*) or CAM mode are excellent study systems for identifying the molecular and biochemical underpinnings of the CAM pathway (Winter and Holtum 2014; Brilhaus *et al.* 2016; Ferrari *et al.* 2020). Facultative CAM species can also significantly contribute to our understanding of CAM evolution, as facultative CAM may be either an evolutionary 'end point' or an intermediate state along the evolutionary trajectory from the ancestral  $C_3$  pathway to full, strongly expressed CAM. Furthermore, species like *C. amboinicus* raise the interesting question of whether facultative CAM is an addition to weakly expressed permanent CAM or vice versa. Either way, for elucidating how species transitioned from  $C_3$  to CAM, obtaining a clean  $C_3$  control species within a particular lineage for comparative purposes can be a major obstacle, especially when the lineage under investigation is rich in CAM species (e.g., Heyduck *et al.* 2018, 2019). Agavoideae, Cactaceae, or Orchidaceae are examples of such lineages. In the Lamiaceae, the sixth largest angiosperm family with over 7,000 species, most species are  $C_3$  plants and thus far CAM is only known in the genus *Coleus* as all species with CAM previously described as *Plectranthus* can now be considered *Coleus*. The preponderance of the  $C_3$  pathway should greatly facilitate the identification of a suitable, closely related  $C_3$  control species in the Lamiaceae, making it an attractive family for future CAM evolution studies, especially since it is well described taxonomically and phylogenetically (Paton *et al.* 2004, 2018, 2019; Suddee *et al.* 2004; Li *et al.* 2016).

Over the last 30 years, *Coleus* was merged into *Plectranthus* by most authors, but a recent phylogenetic study of the monophyletic subtribe Plectranthinae (Paton *et al.* 2018, 2019; building upon Paton *et al.* 2004) recommends generic recognition of *Coleus* and distinguishes two sister groups: (i) the *Plectranthus* clade that contains *Plectranthus sensu stricto* and the genera *Alvesia*, *Aeollanthus*, *Capitanopsis*, *Equilabium*, *Tetradenia* and *Thorncroftia*, and (ii) the *Coleus* clade that consists of two clades, one of which diversified from ~14.4 million years ago and contains *C. amboinicus* and *C. scutellarioides*, two species in which the magnitude of CAM-expression greatly differs (Fig. 9).

Studies of two closely related *Erycina* species (Orchidaceae), one exhibiting CAM and one ostensibly exhibiting  $C_3$ , indicated similar expression patterns of key CAM pathway genes suggesting that early steps of the transitioning to CAM occurred in the ancestor of both species (Heyduk *et al.*, 2019). In fact, during the early phase of this study we considered using *C. scutellarioides* as a  $C_3$  control species until we discovered that the 24 h gas exchange deviated slightly from a strict  $C_3$ -type pattern and that a small degree of nocturnal acidification was measurable, especially in stems. This experience highlights that informed choices of species for studies of CAM evolution require not just robust phylogenies but also detailed physiological measurements on CAM presence and absence.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

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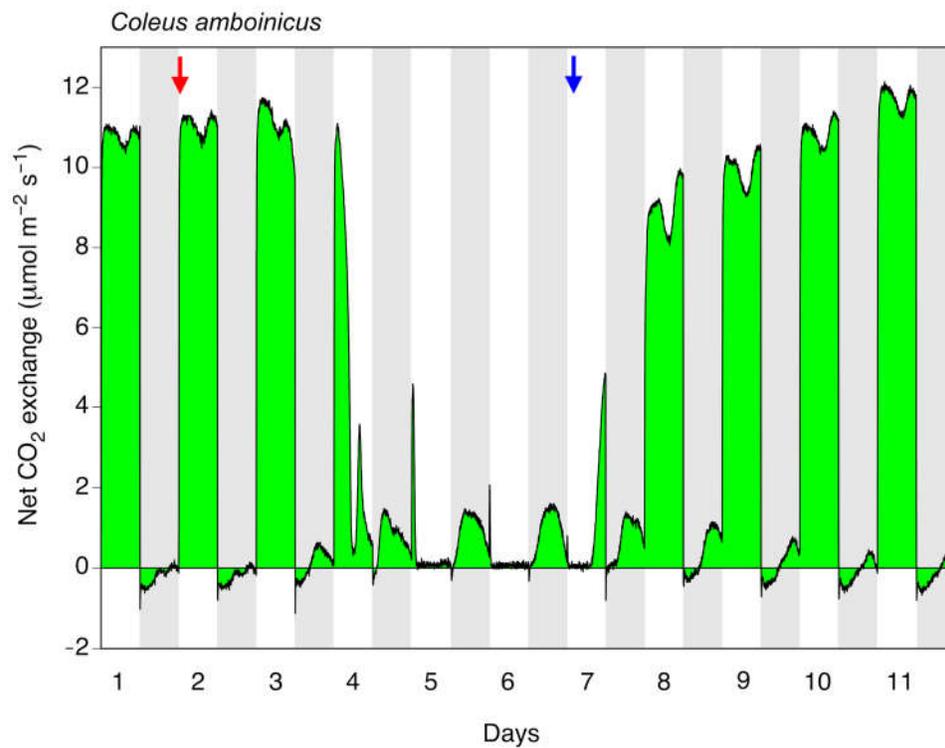
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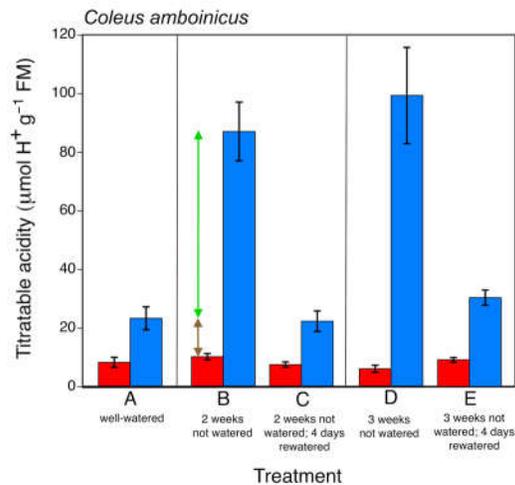
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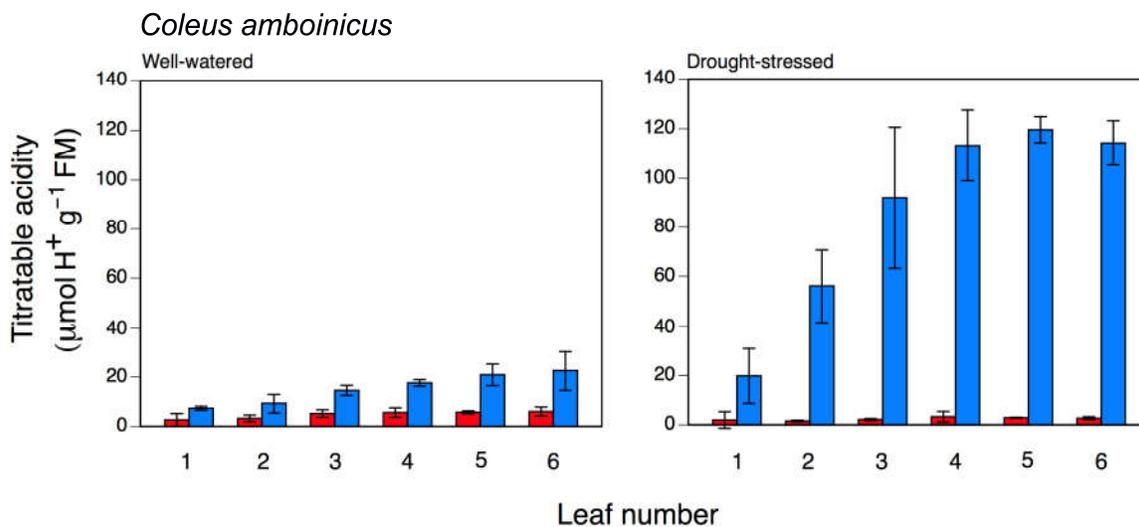
**Fig. 1.** *Coleus amboinicus* growing outside in a 1.5 x 1.5 x 0.3 m raised garden box at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama. Photo from 22 December 2016.



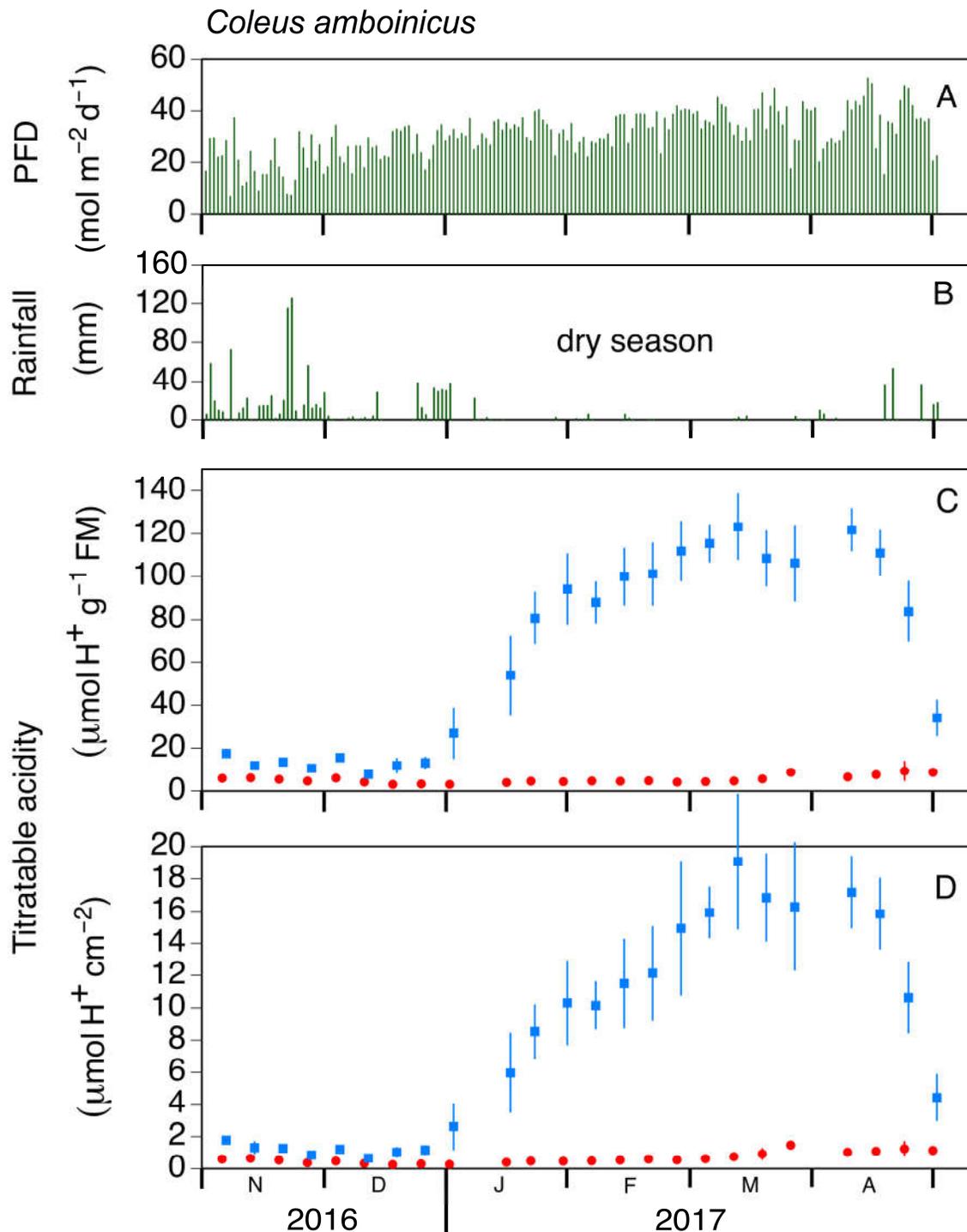
**Fig. 2.** Eleven days of net CO<sub>2</sub> exchange of a fully expanded leaf of *Coleus amboinicus* growing in a 1.6-L pot. Watering was withheld on Day 2 (red arrow) and recommenced on Day 7 (blue arrow). Shaded areas represent 12 h dark periods. Photon flux density was  $640 \mu\text{mol m}^{-2} \text{s}^{-1}$ .



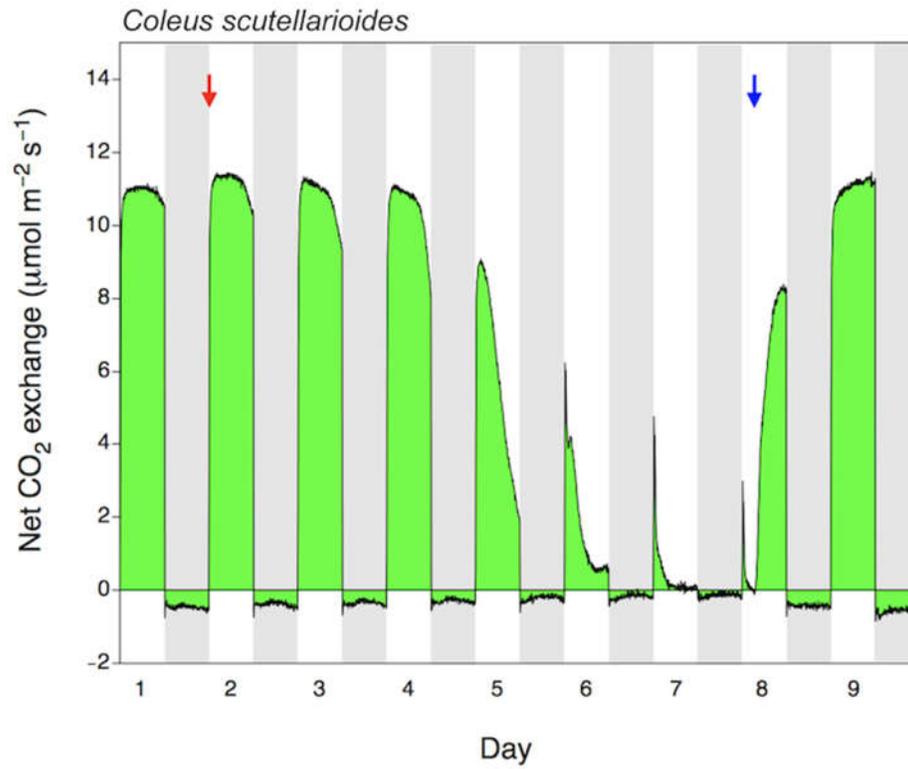
**Fig. 3.** Effect of drought stress on titratable acidity content in mature leaves of *Coleus amboinicus*. Red columns, dusk; blue columns, dawn. Data are means of five independent samples from different plants  $\pm$  s.d. In order to highlight the contributions of constitutive and facultative CAM, for treatment B, the nocturnal increase in  $H^+$  is divided into its constitutive (brown) and facultative (green) component.



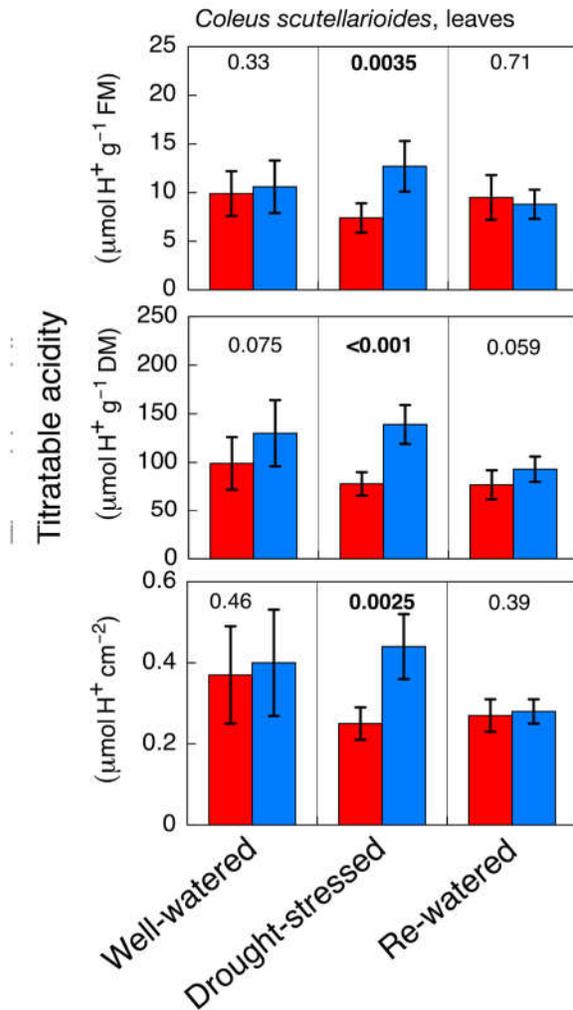
**Fig. 4.** Effect of leaf age on titratable acidity content in well-watered and drought-stressed plants of *Coleus amboinicus*. Leaf 1 is the youngest leaf. Drought stress was imposed by withholding water for 10 days. Red columns: dusk; blue columns: dawn. Data are means of five independent samples from different plants  $\pm$  s.d



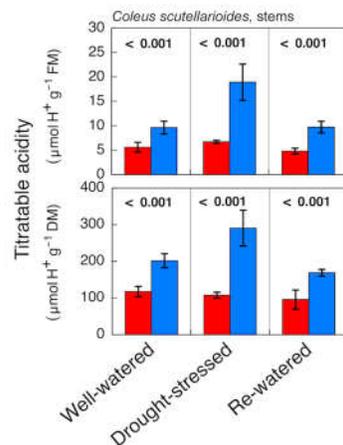
**Fig. 5.** Seasonal changes in photon flux density (A), rainfall (B), and variation of titratable acidity at dusk (red) and dawn (blue) (C, D) in leaves of *Coleus amboinicus* grown in a raised garden box (see Fig. 1). Plants were grown during the latter half of the 2016 wet season and throughout the 2017 dry season. Acidity values are means  $\pm$  s.d. ( $n=5$ ; each sample comprised a leaf from a different plant).



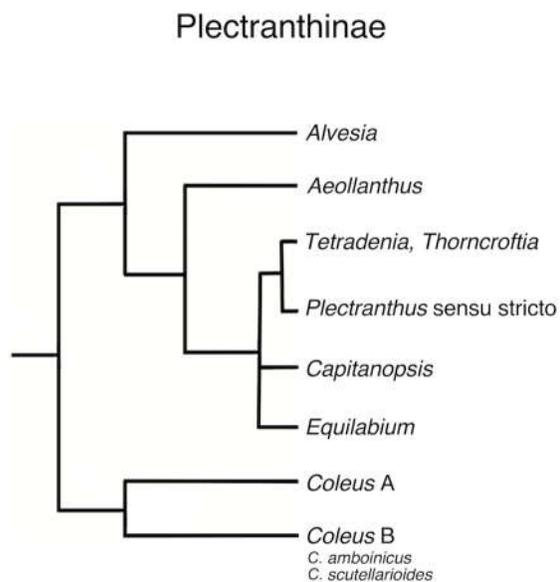
**Fig. 6.** Nine days of net CO<sub>2</sub> exchange of a fully expanded leaf of *Coleus scutellarioides* growing in a 1.0-L pot. Watering was withheld on Day 2 (red arrow) and recommenced on Day 8 (blue arrow). Shaded areas represent 12 h dark periods. Photon flux density was  $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ .



**Fig.7.** Titratable acidity at dusk (red column) and dawn (blue column) in mature leaves of *Coleus scutellarioides*. Well-watered = daily watering. Drought-stressed, 6 days without irrigation; re-watered, 6 days with irrigation. Acidity values are expressed on a fresh mass basis (upper panels), dry mass basis (middle panels), and leaf area basis (lower panels). Bars are standard deviations ( $n = 5$ ; each sample refers to the leaf from a different plant). Numbers are  $P$ -values (one-tailed  $t$ -test, bold letters indicate that the values at dawn were significantly greater than those at dusk at  $P < 0.05$ )



**Fig. 8.** Titratable acidity at dusk (red column) and dawn (blue column) in stems of *Coleus scutellarioides*. Well-watered = daily watering. Drought-stressed, 6 days without irrigation; re-watered, 3 days with irrigation. Acidity values are expressed on a fresh mass basis (upper panels) and dry mass basis (lower panels). Bars are standard deviations ( $n = 5$ ; each sample refers to stem tissue from a different plant). Numbers are  $P$ -values (one-tailed t-test, bold letters indicate that the values at dawn were significantly greater than those at dusk at  $P < 0.05$ ).



**Fig. 9.** Diagram showing the genera in Plectranthinae and the positions of *C. amboinicus* and *C. scutellarioides* after Paton *et al.* (2018). Two other CAM-exhibiting species, *C. hadiensis* and *C. cylindraceus* also belong to clade B. The CAM-exhibiting *C. prostratus* has not yet been sequenced but morphologically is most likely to fall within clade B too (A. Paton, pers. comm.).