On the nature of facultative and constitutive CAM: environmental and developmental control of CAM expression during early growth of *Clusia*, *Kalanchoë*, and *Opuntia*

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Received 29 January 2008; Revised 21 February 2008; Accepted 26 February 2008

Abstract

The capacity to induce crassulacean acid metabolism developmentally (constitutive CAM) and to up-regulate CAM expression in response to drought stress (facultative CAM) was studied in whole shoots of seven species by measuring net CO2 gas exchange for up to 120 day–night cycles during early growth. In *Clusia rosea*, CAM was largely induced developmentally. Well-watered seedlings began their life cycle as C3 plants and developed net dark CO2 fixation indicative of CAM after the initiation of the fourth leaf pair following the cotyledons. Thereafter, CAM activity increased progressively and drought stress led to only small additional, reversible increases in dark CO2 fixation. In contrast, CAM expression was overwhelmingly under environmental control in seedlings and mature plants of *Clusia pratensis*. C3-type CO2 exchange was maintained under well-watered conditions, but upon drought stress, CO2 exchange shifted, in a fully reversible manner, to a CAM-type pattern. *Clusia minor* showed CO2 exchange responses intermediate to those of *C. rosea* and *C. pratensis*. *Clusia cretosa* operated in the C3 mode at all times. Notably, reversible stress-induced increases of dark CO2 fixation were also observed during the developmental progression to pronounced CAM in young *Kalanchoë daigremontiana* and *Kalanchoë pinnata*, two species considered constitutive CAM species. Drought-induced up-regulation of CAM was even detected in young cladodes of a cactus, *Opuntia ficus-indica*, an archetypal constitutive CAM species. Evidently, the defining characteristics of constitutive and facultative CAM are shared, to variable degrees, by all CAM species.

Key words: Carbon dioxide uptake, *Clusia*, constitutive CAM, crassulacean acid metabolism, development, drought stress, environment, facultative CAM, *Kalanchoë*, *Opuntia*.

Introduction

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation to environmental stress that is exhibited in a large number of vascular plants, from at least 343 genera and 34 families, which typically occupy periodically dry habitats in the tropics and subtropics (Smith and Winter, 1996; Holtum and Winter, 1999; Winter and Holtum, 2002; Crayn et al., 2004; Silvera et al., 2005; Holtum et al., 2007). Compared with C3 and C4 photosynthesis (Cernusak et al., 2007a, b), CAM photosynthesis significantly reduces the water cost of CO2 gain by allowing net CO2 uptake to take place at night when the driving forces for water loss through transpiration are low (Winter et al., 2005). The nocturnally fixed carbon is stored as malic acid, which, during the subsequent day, serves as a CO2 reservoir for C3 photosynthesis in the light (Winter and Smith, 1996; Holtum et al., 2005). The photosynthetic use of CO2 generated within chloroplast-containing tissues is associated with stomatal closure, minimizing water loss during those parts of the day–night cycle when the driving forces for water loss are strongest.

Much of our early understanding of the metabolic control and functional significance of CAM was derived...
from studies with species, many from the Crassulaceae (e.g. Kalanchoe daigremontiana) and Cactaceae (e.g. Opuntia basilaris and Opuntia ficus-indica), in which CAM is considered to be always expressed in mature photosynthetic tissues (synonyms: constitutive or obligate CAM species; Kluge and Ting, 1978; Osmond, 1978, 2007; Nobel, 1988; Winter and Smith, 1996). A second group of species has subsequently provided insight into how the mechanisms responsible for CAM expression have been fine-tuned by ecological selection to respond to seasonal and shorter term environmental changes. In this latter group, CAM is an option rather than a pre-set mode of carbon assimilation. Over the past three decades, research on these photosynthetically flexible C3–CAM species (synonyms: C3/CAM intermediates, inducible CAM species, facultative CAM species) has increased to such an extent that there is now a risk that the ability to choose between the C3 and CAM option of photosynthetic carbon gain is erroneously perceived as the rule and not as the exception among CAM plants (e.g. Schulze et al., 2005).

Well-documented examples of plants in which the expression of CAM is highly flexible include the annual halophytic Mesembryanthemum crystallinum (Winter and von Willert, 1972; Winter and Holtum, 2007) and other species in the Aizoaceae (Winter, 1973; Treichel, 1975), species in the Portulacaceae (e.g. Calandrinia spp., Mooney et al., 1974; Winter et al., 1981; other genera, Martin and Zee, 1983; Herrera et al., 1991; Guralnick et al., 2008) and Crassulaceae (e.g. Sedum spp., Kluge, 1977; Borland, 1996; Smirnoff, 1996), and several long-lived woody species of Clusia (Borland et al., 1992, 1998; Zott and Winter, 1993, 1994a, b; Lüttge, 1999, 2006, 2007, 2008; Gehrig et al., 2003; Holtum et al., 2004).

The common feature of these photosynthetically plastic species is a capacity to induce, or at least to up-regulate, CAM under conditions of stress, particularly drought stress (and/or salinity stress in the case of halophytic species), whereas CO2 fixation proceeds exclusively or predominantly via the C3 pathway in the light when ambient conditions are favourable. In contrast, in so-called constitutive CAM species, the CAM cycle is expressed under essentially all conditions that these plants encounter in nature or in the laboratory, i.e., even when environmental conditions are not particularly stressful.

Constitutive CAM species are recognized by the resilience of the CAM cycle to stress rather than by an up-regulation of CAM. For example, following the imposition of drought stress, dark CO2 fixation in constitutive CAM species usually remains relatively unchanged initially, despite rapid declines of CO2 fixation in the light (Kluge, 1972; Hanscom and Ting, 1978; Kluge and Ting, 1978; Osmond et al., 1979). Although the proportions of light and dark CO2 uptake change in favour of dark CO2 fixation under these circumstances, this does not represent a C3 to CAM shift in the strictest sense, because CAM activity does not increase in absolute terms.

In both constitutive and facultative CAM species, the expression of CAM is most pronounced when photosynthetic tissues are mature. In many constitutive species, a progression from C3 to CAM occurs as leaves mature (Jones, 1975; Gehrig et al., 2005). As a result, young plants, because they have young leaves, tend to function predominantly in the C3 mode, even if older plants exhibit strong CAM (Avadhani et al., 1971). In facultative CAM species, the propensity for the induction, or up-regulation, of CAM also increases as leaves mature, but mature leaves do not necessarily express CAM unless stressed.

The interplay between developmental and environmental factors in controlling the balance between C3 photosynthetic CO2 uptake in the light and CAM-type CO2 uptake in the dark is complex and not well understood. In M. crystallinum, the original report of salinity-induced CAM (Winter and von Willert, 1972) was later interpreted as stress-induced acceleration of a genetically controlled developmental programme (Adams et al., 1998). It was shown recently that unstressed plants are able to complete their life cycle by operating exclusively in the C3 mode (Winter and Holtum, 2005, 2007), thus demonstrating that the shift to CAM in M. crystallinum is a response to environmental triggers, as was suggested originally. Studies of similar detail are not available for other species.

Using CO2 exchange data from a body of >1000 complete day–night measurements, here the contributions of development and environment (the latter exemplified by drought stress) to CAM expression in young plants of the genus Clusia, the second-most studied C3–CAM system after M. crystallinum, are quantified and the observations are compared with species generally accepted to be constitutively CAM. The Clusia species chosen for this study encompass the extraordinary photosynthetic plasticity exhibited by this neotropical group of mostly shrubs and trees. The literature on CAM in Clusia ssp. is substantial, and includes a wealth of information on selected spot measurements of daily CO2 exchange patterns of individual leaves (Lüttge, 2006, 2007). However, in none of these previous studies has the attempt been made to separate carefully, using long-term monitoring of CO2 exchange, the relative roles of ontogeny and environment in the control of CAM expression at the organismal level.

Three of the Clusia species studied here exhibit different degrees of CAM during their early growth when unstressed, and different degrees of up-regulation of CAM when exposed to drought stress. A major and unexpected observation was that drought-induced up-regulation of CAM was also observed during the developmental progression of CAM in species considered to exhibit CAM constitutively, Kalanchoe daigremontiana and Kalanchoe pinnata, and in a stem-succulent cactus, Opuntia ficus-indica.
These observations provide a new perspective on the concept of constitutive and facultative CAM.

Materials and methods

Plant material

Clusia cretosa Hammel ined. [referred to as Clusia sp. A by Gehrig et al. (2003) and Holtum et al. (2004)], Clusia pratensis Seemann, and Clusia rosea Jacq. were grown from seeds collected from plants growing in their natural habitats in Panama, whereas K. daigremontiana Hamet. et Perr. and K. pinnata (Lam.) Pers. were grown from leaf-borne ramets. For O. ficus-indica (L.) Mill., the young cladodes upon which experiments were performed emanated from apical areoles of single cladode mother plants (~5 cm tall, projected areas of 13–16 cm²).

For laboratory-based experiments, the majority of plants were grown in an 80:20 (v/v) mixture of dark loamy potting soil (Novey Tierra, Albion Mall, Panama) and Perlite (Good Earth Horticultural Products, OH, USA) in 2.65 l cylindrical plastic pots. Clusia rosea exposed to stress was grown in Schultz Potting Soil Plus (US Home and Gardens, GA, USA). Opuntia ficus-indica was grown in Cactus, Palm and Citrus Soil (Miracle-Gro Lawn Products, OH, USA) in either a 0.95 l plastic pot or a 1.7 l terracotta pot. All plants were fertilized with 5 g of Osmocote Plus (Scotts-Sierra Horticultural Products, OH, USA).

In field-based experiments, well-irrigated C. pratensis plants in 400 l plastic containers wrapped with reflective insulation were grown outdoors in locally obtained forest top-soil for 2 years at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama (9°07'N, 79°42'W).

Measurements of CO2 exchange in the laboratory

Intact young shoots or cladodes were sealed inside Plexiglass gas-exchange cuvettes (for Clusia and Kalanchoë cuvette dimensions were 30 cm×30 cm×15 cm height or 20 cm×20 cm×15 cm height; for O. ficus-indica dimensions were 11 cm×11 cm×10 cm height). For shoots of Clusia and Kalanchoë, transfer into a cuvette of a young shoot involved removal from the soil and threading the roots and lower portion of the stem through a hole in the bottom of the cuvette. The roots were then replaced in the soil and the stem–cuvette interface was sealed with a non-porous synthetic rubber sealant (Terostat VII, Henkel-Teroson, Heidelberg, Germany). For O. ficus-indica, the cuvette was sealed around an apical cladode such that ~1.5 cm of the 3.0 cm high cladode was exposed inside the cuvette. The cuvettes were located inside controlled-environment chambers (Environmental Growth Chambers, OH, USA) operating either under 12 h light (28 °C)/12 h dark (22 °C) cycles, or, in the case of K. daigremontiana, under 12 h light (23 °C)/12 h dark (17 °C) cycles. Photon flux density (PFD) at the upper outer surface of the cuvettes was 420 μmol m⁻² s⁻¹. When not stressed, plants were irrigated daily.

Drought treatments were imposed by withholding irrigation and, in a severe water stress treatment of O. ficus-indica, removing the plant roots from the soil. The rate at which plants were stressed following the withholding of irrigation was affected by species, plant size, pot size, pot composition, and soil type.

Net CO2 exchange of the shoots or cladodes in the cuvettes was measured using a LI-6252 CO2 analyser (Li-Cor, Lincoln, NE, USA) in a flow-through gas-exchange system consisting mainly of Walz components (Walz GmbH, Effeltrich, Germany) (Holtum and Winter, 2003). Ambient air was supplied to the cuvettes at flow rates of between 2.3 l min⁻¹ and 4.7 l min⁻¹. The dewpoint of air entering the cuvettes was 15, 18, or 20 °C depending on species and plant size.

Measurements of CO2 exchange in the field

For monitoring whole-plant gas exchange, a plant was placed inside an aspirated, naturally illuminated chamber constructed of glass panels and an aluminum framework (internal volume: 8.8 m³). A blower (model 4C054, Grainger Industrial Supply, OH, USA) supplied external air to the chamber at a rate of 10.5 m³ min⁻¹. Within the chamber, air was circulated by four fans, and a split air-conditioning system (model V1124C2H, Innovair, FL, USA) maintained temperatures at close to ambient.

Whole-plant gas exchange was quantified at 30 min intervals from the rate at which the CO2 concentration inside the chamber changed when air flow into the chamber was blocked for 5 min, thereby converting the chamber into a closed system. Changes in the CO2 concentration inside the chamber were measured using a LI-7500 open-path CO2 analyser (LI-COR). Calculations of net CO2 exchange were based upon chamber volume that had been corrected for the volumes of the pot, plant, and other pieces of equipment inside the chamber, and the rate at which the CO2 concentration changed during the period when the chamber was isolated. CO2 measurements were corrected for changes in temperature and humidity.

Results

**Clusia species**

CO2 uptake during the light was a major source of carbon in a young C. rosea shoot monitored for 73 d under well-watered conditions (Fig. 1). Uptake during the light was
the sole contributor to net CO₂ gain until day 37, CO₂ uptake increasing 28-fold between days 1 and 55, and decreasing thereafter by 20%. As the shoot grew, CO₂ uptake in the dark developed. Net CO₂ balance in the dark became positive on day 38, after the initiation of the fourth leaf pair, and progressively increased until day 73.

A second shoot of Clusia rosea exhibited an underlying pattern of CO₂ exchange that was similar to the first, except that the dark CO₂ balance shifted from slightly negative to slightly positive as leaf pair 5 emerged (Fig. 2). Two 13 d drought treatments each resulted in large, reversible decreases of CO₂ uptake in the light. The reductions in CO₂ uptake in the light were accompanied by 1.84-fold and 1.89-fold accelerations, respectively, in the rates of dark CO₂ fixation. Following rewatering after each stress treatment, the daily increment in dark CO₂ uptake returned to the rate observed before the imposition of the stress.

When well watered, net CO₂ uptake by a young shoot of Clusia pratensis was restricted to the light (Fig. 3). Drought treatments of 22 d and 24 d induced pronounced decreases of CO₂ uptake in the light, and shifts from a negative to a positive CO₂ balance at night. The removal of drought stress was rapidly followed by a recovery in CO₂ uptake during the light and the loss of net CO₂ uptake in the dark.

The CO₂ exchange responses to drought of a 2-year-old C. pratensis shrub, measured in a naturally illuminated CO₂ exchange chamber under temperature and light conditions close to those in the field (Figs 4, 5), resembled
those observed for the young shoot of *C. pratensis* (Fig. 3). As in the young shoot, the imposition of water stress in the older plant induced changes in CO2 exchange patterns that were consistent with a C3 to CAM shift. A rhythm of net CO2 uptake in the light and net CO2 loss in the dark was transformed into a rhythm of net CO2 loss for most parts in the light and net CO2 uptake during the dark. As water stress unfolded, net CO2 uptake in the light decreased until it was restricted to a pronounced morning peak and a small afternoon peak between which the CO2 balance was negative. The accompanying development of CO2 uptake in the dark was initially observed as a reduction in CO2 evolution during the night of day 5. Net dark CO2 fixation was first observed during the following night (day 6) and increased gradually until day 12. Following rewatering (day 12), continual CO2 uptake during daylight was restored and the net CO2 balance in the dark became negative again. Because *C. pratensis* produces many stem-borne aerial roots, it was not possible to isolate the pot in order to exclude root and soil respiration from the measurements of net CO2 exchange. Nevertheless, the inclusion of soil and root respiration in the measurements does not affect the principal observation of a reversible switch from a C3- to a CAM-type net CO2 exchange pattern.

Light and dark CO2 exchange patterns exhibited during the early development of *C. minor* resembled those in *C. pratensis*, including the switch from a negative to a positive CO2 balance in the dark following application of drought stress (Fig. 6). However, the CO2 exchange patterns exhibited by *C. minor* differed from those of *C. pratensis* in two significant respects. Unlike *C. pratensis*, *C. minor* exhibited a slightly positive CO2 balance during most dark periods even when well watered and, upon rewatering following stress, the dark CO2 fixation that was induced was not fully reversible. The latter observation is consistent with the onset of developmentally programmed CAM in *C. minor*.

*Clusia cretosa* exhibited CO2 exchange patterns typical of a C3 species (Fig. 7). As in the other *Clusia* spp. studied, drought stress led to a reduction in net CO2 uptake in the light, but, unlike the other *Clusia* spp., stress was not accompanied by the induction of net CO2 uptake in the dark.
When irrigated, a small *K. daigremontiana* shoot exhibited CO\(_2\) fixation in the light and in the dark (Fig. 8), with light CO\(_2\) uptake contributing the majority of the 24 h carbon gain. As the shoot increased in size, the proportion of carbon supplied by CO\(_2\) uptake in the dark increased.

Drought treatments of 34 d and 21 d resulted in substantial reversible decreases of CO\(_2\) uptake in the light. The reductions of CO\(_2\) uptake in the light were accompanied by 1.36-fold and 1.69-fold accelerations, respectively, in the rates of dark CO\(_2\) fixation. Following rewatering after each stress treatment, the daily increment in dark CO\(_2\) uptake returned to the rate observed before the imposition of stress.

Net CO\(_2\) exchange exhibited by a young well-watered developing shoot of *K. pinnata* was outwardly similar to that expressed by *K. daigremontiana* in that both light and dark CO\(_2\) uptake were present, but the rate of CO\(_2\) uptake in the light greatly exceeded that in the dark (Fig. 9). The rate of dark CO\(_2\) fixation increased markedly when water was withheld and decreased transiently following rewatering. Although the timing of the imposition of water stress evidently coincided with the onset of ontogenetically programmed CAM in the experiment shown in Fig. 9, it was possible to calculate that facultative up-regulation

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Opuntia ficus-indica

In a very young cladode of well-watered O. ficus-indica, CO₂ balances in the light and in the dark were positive and similar in magnitude until a drought treatment was initiated (Fig. 10A). Droughting involved 10 d without irrigation followed by 3 d of intensive water stress that was imposed by removing the roots from the soil. During the initial part of the water stress treatment, a slight decline in light CO₂ fixation was accompanied by a continuing strong increase in dark CO₂ fixation. The more severe water stress treatment initiated a precipitous decrease in light CO₂ fixation from 0.235 to 0.063 mmol shoot⁻¹ 12 h⁻¹ and a sharp increase in dark CO₂ uptake. Restoration of the roots to irrigated soil resulted in a substantial increase of CO₂ uptake in the light and a transient 18% reduction in dark CO₂ gain.

In a second experiment, a young cladode of O. ficus-indica was grown in a terracotta pot rather than a plastic pot in order to increase the rate of water loss from the substrate during the droughting treatment (Fig. 10B). During the first 12 d in the cuvette, CO₂ uptake in the light markedly exceeded CO₂ uptake in the dark; in fact, during the initial 3 d, carbon balance was positive only in the light. A drought treatment of 11 d led to a substantial decrease of CO₂ uptake in the light and a 1.92-fold acceleration in the rate of dark CO₂ fixation. Following rewatering, the daily increment in dark CO₂ uptake returned to the rate observed before the imposition of the stress.

Discussion

The assessment of developmental and environmental responses of a species ideally requires observation of that species during its entire life cycle. In a previous study, light and dark CO₂ fixation were continuously tracked throughout the life cycle of the annual C₃–CAM species, M. crystallinum, demonstrating that the induction of CAM is exclusively under environmental control (Winter and Holtum, 2007). Here, long-term gas exchange measurements are reported of seven perennial species, some of which (Clusia spp.) live as trees for decades, rendering life cycle measurements a challenge. Nonetheless, the study of whole shoots during the first few months after germination is meaningful because it provides insights into the relative influences of development and environment on the expression of CAM photosynthesis during a stage critical for establishment and survival.

Reduced water availability was used as a stressor to quantify the degree of environmental control of CAM expression because this is a condition most frequently,
Moreover, the underlying mechanisms for temperature
field by land plants (Zotz and Winter, 1993).
but such conditions are only occasionally experienced in
example, in
Kalanchoe¨
spp. (Kluge
et al.,
1978),
Tillandsia spp. (Kluge et al., 1973), and Clusia spp.
(Haag-Kerwer et al., 1992; de Mattos and Lüttge, 2001),
but such conditions are only occasionally experienced in
field by land plants (Zotz and Winter, 1993).
Moreover, the underlying mechanisms for temperature-
CAM 3 shifts have never been conclusively
identified (Brandon, 1967; Kluge and Schomburg, 1996),
but certainly include direct effects on membrane fluidity
and on catalytic and diffusive processes.

Three major observations derived from this long-term
gas exchange study were that (i) C3 photosynthetic CO2
uptake in the light was initially the dominant pathway of
carbon acquisition in all plants that eventually developed
significant CAM activity; (ii) the expression of CAM in
all of the species was stimulated by water stress to some
degree, including species generally considered to exhibit
CAM constitutively; and (iii) in both seedlings and mature
plants of one species, C. pratensis, the expression of CAM
is essentially exclusively under environmental
control.

C3 photosynthesis dominates CO2 exchange in young plants
In all species, even in those that eventually exhibited
CAM after a certain plant age was reached, CO2 uptake in
the light was the predominant mode of carbon acquisition
in well-watered young shoots. Net CO2 uptake in the dark
was either absent (C. rosea and C. pratensis) or very low
(both Kalanchoe species and C. minor). In O. ficus-indica,
in which the formation of a new cladode originating from
a mother cladode was studied, CO2 uptake in the light was
initially equal to or markedly greater than CO2 uptake in
the dark. In a similar study of O. ficus-indica by Wang
et al. (1998), net CO2 fixation in the light was not
observed during the early development of cladodes.
Rather, development was characterized by a transition
from net CO2 loss in both the light and the dark to net
CO2 uptake in the dark. Mother cladodes in this previous
study were substantially larger, and much of the early
growth in the juvenile cladodes was based on carbon
transported from the parent tissue. Although 1-d-old
cotyledons of cacti can exhibit day–night fluctuations in
acidity typical of CAM (Hernández-González and Briones
Villareal, 2007), the present study with O. ficus-indica
suggests that the contribution of CAM to total carbon gain
is presumably small at this early stage of development.
Consistent with this postulate are observations that in
14 mm seedlings of Agave deserti only 27% of carbon
was fixed during the dark, a value that increased to 81%
in 60 mm seedlings (Nobel, 1988).

A facultative CAM component in ‘constitutive’ CAM species
After an initial C3-type phase, plants induce or up-regulate
CAM in response to internal or external cues. While
internal developmental cues were paramount in the
generation of CAM in C. rosea, both Kalanchoe species,
and O. ficus-indica, an external cue, in this case water
stress, was critical for the induction of CAM in
C. pratensis. Clusia minor exhibited an intermediate
behaviour.

A most significant finding is that a facultative compo-
nent of CAM control was present in all species that were
capable of CAM, even in young shoots of the constitutive
CAM species K. daigremontiana and K. pinnata, and in the
archetypal platyopuntia, O. ficus-indica. The observa-
ition of both developmental and environmental effects on
the expression of CAM appears to relativize the distinc-
tions between constitutive and facultative CAM plants.
Although up-regulation in Kalanchoe and Opuntia was
low in comparison with the background ontogenetically
based CAM, the environmental control was generally
demonstrable as water stress-stimulated dark CO2 uptake
that could be reversed following rewatering. Comparable
responses to drought stress have been seen in fully
expanded leaves of K. daigremontiana, but reversibility
was not documented (Griffiths et al., 2002). In the
experiment with K. pinnata, the stimulation of dark CO2
fixation was difficult to discern from the developmentally
controlled background increase of CAM, but the transient
decrement in nocturnal CO2 uptake upon stress relief is
consistent with an up-regulation of CAM by drought
stress. Stress-related up-regulation of CAM in K. pinnata
was confirmed by CO2 exchange studies with intact
attached leaves during their expansion (data not shown).

It has been reported that A. deserti, a species generally
regarded as a classic constitutive CAM plant, can switch
from a strong CAM-type to a C3-type pattern of CO2 gas
exchange when plants are watered extensively (Hartsock
and Nobel, 1976). Although there has never been a
follow-up study of this intriguing observation, the data
support the notion that strict distinctions between consti-
tutive and facultative CAM are not possible. Increases in
dark CO2 fixation in cacti during the early dry season in
the field do not necessarily reflect drought-induced
up-regulation of CAM because night-time temperatures and daily PFDs became more favourable for dark CO₂ fixation (Pimienta-Barrios et al., 2000).

**C₃ and CAM in Clusia**

The four **Clusia** species studied encompass the wide range of photosynthetic options exhibited by this genus, for which there is a considerable, sometimes bewildering, literature on the interactions between CAM and the environment (Lüttge 1999, 2006, 2007, 2008; Dodd et al., 2002). Early research on *C. rosea* concluded that ‘categorization of this plant as C₃, CAM or an intermediate is impossible’ (Schmitt et al., 1988), but our long-term gas exchange measurements of shoots, together with other studies of leaf gas exchange, titratable acidity, and carbon isotope ratios, show that the expression of CAM in *C. rosea* is predominantly under developmental control (Ting et al., 1985; Ball et al., 1991; Lüttge et al., 1993; Franco et al., 1994; Haag-Kerwer et al., 1996; Borland et al., 1998; Holtum et al., 2004). It is demonstrated for plants with ≥4 leaf pairs (in addition to the cotyledons) that a facultative component is present but small. We do not know whether younger plants which have not yet started to exhibit the developmental component of CAM can exhibit completely reversible CAM induction when droughted. If so, full facultative CAM control would be present at this early stage of development.

**Clusia pratensis** represents the most clear-cut example of facultative CAM reported in the genus Clusia. Both young shoots and mature plants exhibit drought-induced C₃ to CAM shifts that are totally reversible. The latter observation is unique as it has even yet to be reported for *M. crystallinum*, the most intensely studied C₃–CAM system. Measurements of 2-year-old plants of *C. pratensis*, the first that have integrated whole-plant CO₂ exchange for any *Clusia* species under natural tropical conditions, demonstrate that a developmental component of CAM control may be negligible throughout the life cycle of this species.

In *C. minor*, the CO₂ exchange characteristics and drought stress response were between those of *C. rosea* and *C. pratensis*. The experiment shown in Fig. 6 demonstrates drought-induced up-regulation of CAM activity, but the lack of complete reversibility of CAM is consistent with a developmentally controlled CAM component. In contrast to very young *C. rosea*, a low level of net dark CO₂ uptake was measured in young shoots of *C. minor* during several nights prior to the stress treatment (third and fourth leaf pair following the cotyledons). Other experiments (data not shown) demonstrated the capacity for low-level CAM even in plants with only two leaf pairs and the cotyledons. It is possible that the low level of CAM in such very young *C. minor* is the product of transient transpiration-induced water deficits despite the plant being well watered. We noted that the top pair of leaves, which are initially vertical and connected, wilt transiently as they separate and receive higher levels of radiation. Indeed, low rates of dark CO₂ fixation have previously been reported in 60% expanded young leaves of well-irrigated *C. minor* (Borland et al., 1998).

**Clusia minor** has frequently been used as a model C₃–CAM plant because the expression of CAM appears particularly responsive to environmental change (Borland et al., 1994; Lüttge, 2008). In many laboratory experiments, relatively low light intensities, constant day–night temperatures, and high relative humidities have been used to produce an almost exclusively C₃ pattern of day–night net CO₂ exchange (de Mattos and Lüttge, 2001; Grams and Thiel, 2002). Under field conditions, a purely C₃ exchange pattern is less likely and plants would be expected to perform a low background level of CAM under many circumstances, even in the absence of edaphic drought stress. Although field-obtained leaf carbon isotope ratios of between −23‰ and −29‰ are consistent with a predominantly C₃ photosynthetic CO₂ uptake (Ting et al., 1987; Borland et al., 1992; Franco et al., 1994; Holtum et al., 2004), such values do not exclude the possibility that up to 30% of plant carbon may be gained by dark CO₂ fixation (Pierce et al., 2002; Winter and Holtum, 2002) and, indeed, acid accumulation has been reported for *C. minor* in the field during the wet season (Roberts et al., 1998).

**Clusia cretosa** exhibited C₃-type photosynthesis under both well-watered and stressed conditions. Drought stress, although not accompanied by the induction of CAM as observed in the other *Clusia* species studied, led to a decline in CO₂ fixation in the light and a reduction in nocturnal CO₂ loss. For plants of similar size, dark respiration rates were higher in well-watered *C. cretosa* than in the *Clusia* species capable of performing CAM. This pattern may be species specific, but could also indicate refixation of some respiratory CO₂ by the other *Clusia* species, even when they did not exhibit net CO₂ uptake in the dark.

The absence of net dark CO₂ fixation in *C. cretosa*, even when stressed, further strengthens the postulate that species in the ITS and morphology-based *C. multiflora* clade, in which *C. cretosa* and other higher elevation *Clusia* species have been placed, have little or no capacity for net CO₂ uptake in the dark (Hammel, 1986; Grams et al., 1998; Gehrig et al., 2003; Holtum et al., 2004; Gustafsson et al., 2007).

We do not know whether the reversible reduction of nocturnal CO₂ loss in *C. cretosa* which accompanies the strong decrease in diurnal net CO₂ fixation in response to water deficit solely reflects decreased mitochondrial dark respiration of the shoot, or also involves increased nocturnal refixation of respiratory CO₂ via PEP carboxylase. Stress-induced, reversible down-regulation
of mitochondrial respiration, unrelated to the CAM cycle, could contribute to the stress-induced, reversible increase of net dark CO₂ fixation in predominantly constitutive CAM species such as *K. daigremontiana*, *K. pinnata*, and *O. ficus-indica*. Measurements of nocturnal leaf titratable acidity changes, together with expression studies of CAM-associated genes, will thus be crucial to assess the full extent of a facultative CAM component in constitutive CAM species.

**Conclusions**

Distinguishing between facultative and constitutive CAM is a convenient way to categorize the ability of plants to up-regulate environmentally, in a reversible fashion, the contribution of CAM to total carbon gain and growth. However, this ability is not an all-or-nothing phenomenon. The terms constitutive and facultative CAM are not the manifestations of different CAM pathways, rather they represent extremes on a continuum that ranges from the expression of CAM that is fully controlled by ontogeny to the full control of CAM expression by drought stress. All CAM species, irrespective of whether they are designated constitutive or facultative, are genotypically equipped to perform CAM and, as shown in this study, the proportion of carbon gained in the dark depends on both pre-programmed (i.e. developmental) and environmental forces, albeit to varying degrees. As in most biological systems, few CAM species are expected at the extremes of the scale. *Clusia pratensis* appears close to the environmental endpoint because they too exhibit a facultative component.

**Acknowledgements**

We thank Jorge Aranda and Aurelio Virgo for technical assistance. This research was funded by the Smithsonian Tropical Research Institute.

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