

Diel leaf growth cycles in *Clusia* spp. are related to changes between C₃ photosynthesis and crassulacean acid metabolism during development and during water stress

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ABSTRACT

This study reports evidence that the timing of leaf growth responds to developmental and environmental constraints in *Clusia* spp. We monitored diel patterns of leaf growth in the facultative C₃-crassulacean acid metabolism (CAM) species *Clusia minor* and in the supposedly obligate CAM species *Clusia alata* using imaging methods and followed diel patterns of CO₂ exchange and acidification. Developing leaves of well-watered *C. minor* showed a C₃-like diel pattern of gas exchange and growth, with maximum relative growth rate (RGR) in the early night period. Growth slowed when water was withheld, accompanied by nocturnal CO₂ exchange and the diel acid change characteristic of CAM. Maximum leaf RGR shifted from early night to early in the day when water was withheld. In well-watered *C. alata*, similar changes in the diel pattern of leaf growth occurred with the development of CAM during leaf ontogeny. We hypothesize that the shift in leaf growth cycle that accompanies the switch from C₃ photosynthesis to CAM is mainly caused by the primary demand of CAM for substrates for nocturnal CO₂ fixation and acid synthesis, thus reducing the availability of carbohydrates for leaf growth at night. Although the shift to leaf growth early in the light is presumably associated with the availability of carbohydrates, source–sink relationships and sustained diurnal acid levels in young leaves of *Clusia* spp. need further evaluation in relation to growth processes.

Key-words: gas exchange; image analysis.

INTRODUCTION

It is now clear that crassulacean acid metabolism (CAM) is a complex ecological, physiological and biochemical process that has been widely selected in diverse plant taxa to conserve water in arid habitats. From the earliest studies, plant physiologists have been preoccupied with the

elucidation of the signature metabolism of CAM, with malic acid synthesis and degradation in mature leaves and stems of these succulent plants in relation to environment and development (Kluge & Ting 1978; Osmond 1978; Black & Osmond 2003; Lüttge 2004; Holtum & Winter 2005). Less attention has been given to the regulation of carbohydrate metabolism and its relation to growth (Sutton 1975a,b; Holtum, Smith & Neuhaus 2005). The fundamental question of how CAM plants preserve carbohydrate reserves in the light for acid synthesis in the dark, while at the same time providing carbon for growth, remains enigmatic (Borland & Dodd 2002). In particular, the evidence for discrete pools of carbohydrates engaged in dark CO₂ fixation and growth (Deléens & Garnier-Dardart 1977; Deléens, Garnier-Dardart & Querioz 1979; Borland *et al.* 1994) challenges all present models of metabolic compartmentation in photosynthetic tissues.

The first detailed diel leaf carbon allocation budgets of a CAM plant (*Sedum telephium* L.) were published by Borland (1996), followed by similar comparisons of several species that switch between C₃ metabolism and CAM (Borland & Dodd 2002). The ‘conflict of interest’ for carbohydrate metabolism in CAM plants (Borland & Dodd 2002) presumably involves three major ‘decision points’ (regulatory compromises): conservation of carbohydrates for CAM; for growth of young leaves; and for export and growth of the plant as a whole. The diel relationships among these processes are likely to be complex and further confounded by shifting patterns during leaf development and plant growth. There have been few comprehensive studies of these relationships. For example, Wang & Nobel (1996) examined the enzymology of CO₂ assimilation and regulated carbohydrate metabolism in *Opuntia ficus-indica* (L.) Mill., and went on to examine the relationship between local CAM and sink activity in growing cladodes and carbon sources in mature cladodes (Wang, Zhang & Nobel 1998). Recently, it has become clear that diel patterns of leaf growth can be examined in detail by imaging methods (Schmundt *et al.* 1998; Walter & Schurr 2005). They can be related to diel patterns in leaf carbohydrate status

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(Walter *et al.* 2005) and to the expression of genes involved in cell division (Matsubara *et al.* 2006). Most annual C₃ plants investigated up to now show the strongest growth activity at night–day transitions or at night when it seems likely that growth is mainly driven by the mobilization of chloroplast starch reserves (Walter & Schurr 2005). In rapidly growing leaves of *Populus deltoides* Bartr. ex Marsh, transient dips in leaf relative growth rate (RGR) were associated with low leaf glucose levels in the afternoon, possibly indicating that demand for sugar export dominated over local requirements for leaf growth (Walter *et al.* 2005).

Clearly, the complex diel patterns of acid metabolism and carbohydrate demand in CAM plants present a challenge to the analysis of diel leaf growth patterns. Initial studies of ‘obligate’ CAM plants, i.e. those that do not show transitions from C₃ to CAM during drought stress or development, showed distinctive diel growth patterns that were dominated by growth during the day in leaves of *Kalanchoe* and cladodes of *Opuntia* spp. (Gouws *et al.* 2005). This was in marked contrast to the nocturnal maximum and diurnal decline in rapidly growing leaves of *Mesembryanthemum crystallinum* L. in C₃ mode, and further studies of the effect of C₃–CAM transitions immediately suggested themselves. However, leaves of *M. crystallinum* become twisted or optically complex (because of epidermal bladders) during the transition to CAM and are not ideal for the imaging analysis of diel patterns of leaf growth.

The CAM literature is now replete with studies of developmentally and environmentally inducible CAM (Dodd *et al.* 2002), and these are nowhere more evident than in the tropical tree genus *Clusia* (Lüttge 2007). For example, Schmitt, Lee & Lüttge (1988) showed that mature leaves of *Clusia minor* (incorrectly identified as *Clusia rosea*; Lüttge 2006) responded rapidly and reversibly to drought stress, converting from a C₃ gas exchange pattern to CAM. Our goal was to relate diel changes in metabolism to diel changes in leaf growth. However, we soon recognized that the bulk of literature on CAM deals with gas exchange and metabolic profiles of fully expanded, mature leaves and their function as source leaves for the growth of the whole plant. Thus, studies of the expression of CAM at different stages of development in the field focused on the youngest fully expanded leaves of *C. minor* L. (Borland *et al.* 1992). Other *Clusia* species, such as *Clusia alata* and *Clusia hilariana* Schltdl. are reputedly ‘obligate’ CAM plants (Lüttge 1999), but again, fully expanded leaves of the same size on individual *C. hilariana* plants of different sizes have been studied (Berg *et al.* 2004). As far as we know, there have been no previous investigations of the patterns of gas exchange or acidification during the expansion growth of young leaves in these C₃–CAM plants.

As *C. minor* and other *Clusia* spp. have planar leaves suitable for growth analysis by imaging methods, we investigated whether the developmental and/or a drought stress-induced transition from C₃ to CAM photosynthesis in these plants was associated with changes in diel cycles of leaf growth.

MATERIALS AND METHODS

Plant material

C. minor L. and *C. alata* Pl. and Tr. plants were raised from seedlings obtained of the *Clusia* collection of the Botanical Garden of the Darmstadt University of Technology, Darmstadt, Germany. *C. alata* is an obligate CAM plant, while *C. minor* is a facultative CAM plant, i.e. it can switch between C₃ and CAM photosynthesis, depending on the external conditions (Haag-Kerwer, Franco & Lüttge 1992; Haag-Kerwer *et al.* 1996; Herzog *et al.* 1999). In the experiments presented here, we controlled the mode of photosynthesis of *C. minor* by withholding soil water.

Plants were grown in the Phytoc greenhouse of the Institute of Phytosphere Research (ICG-3; Research Center Jülich, Jülich, Germany) that was designed to cultivate plants under controlled environmental conditions similar to field conditions. For example, a high transparency for photosynthetically active radiation (PAR) and ultraviolet (UV) radiation (up to 97% in visible light and up to 35% UV-B transmittance) was achieved by installing a specially formulated microstructured glass (Centrosolar Glas, Fürth, Germany), which also led to a homogeneous illumination of the cultivation area. Plants were supplied with slow-release fertilizer and watered automatically with tap water every second day. During growth and during greenhouse experiments, mean midday irradiance (PAR) averaged 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with maximal values of 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$; temperature was set to 21/19 °C day/night but reached 30 °C on sunny days; relative humidity was 60%. When sunlight was lower than 130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ artificial illumination was provided by SON-T Agro 400 W (Philips, Köln, Germany) lamps.

For the analyses of growth and gas exchange in controlled climate conditions, plants were transferred to a growth chamber 2–3 d before the onset of analyses. Chamber relative humidity was kept at 60%, and PAR at the top leaves ranged between 600 and 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by a mixture of SON-T Agro 400 W and HPI-T plus 400 W (Philips) lamps (temperature and day length are specified for each experiment in the Results section). Drought stress was imposed by not watering the plants after transfer to the growth chamber. Leaf growth of these plants stopped completely within 1–2 weeks after the last irrigation, depending on the relation between plant size and soil volume available in the pot.

Gas exchange measurements

Net CO₂ exchange and transpiration was recorded using a CMS 400 minicuvette system of Walz (Effeltrich, Germany). A single leaf of the youngest leaf pair was enclosed in the gas exchange cuvette, when it was about 50% fully expanded (Fig. 1). Gas exchange data were recorded every 5 min and integrated over 60 min to compensate for noisy data. To obtain accurate estimates of gas exchange at low flow rates in the gas exchange cuvette system, it was necessary to keep the cuvette temperature

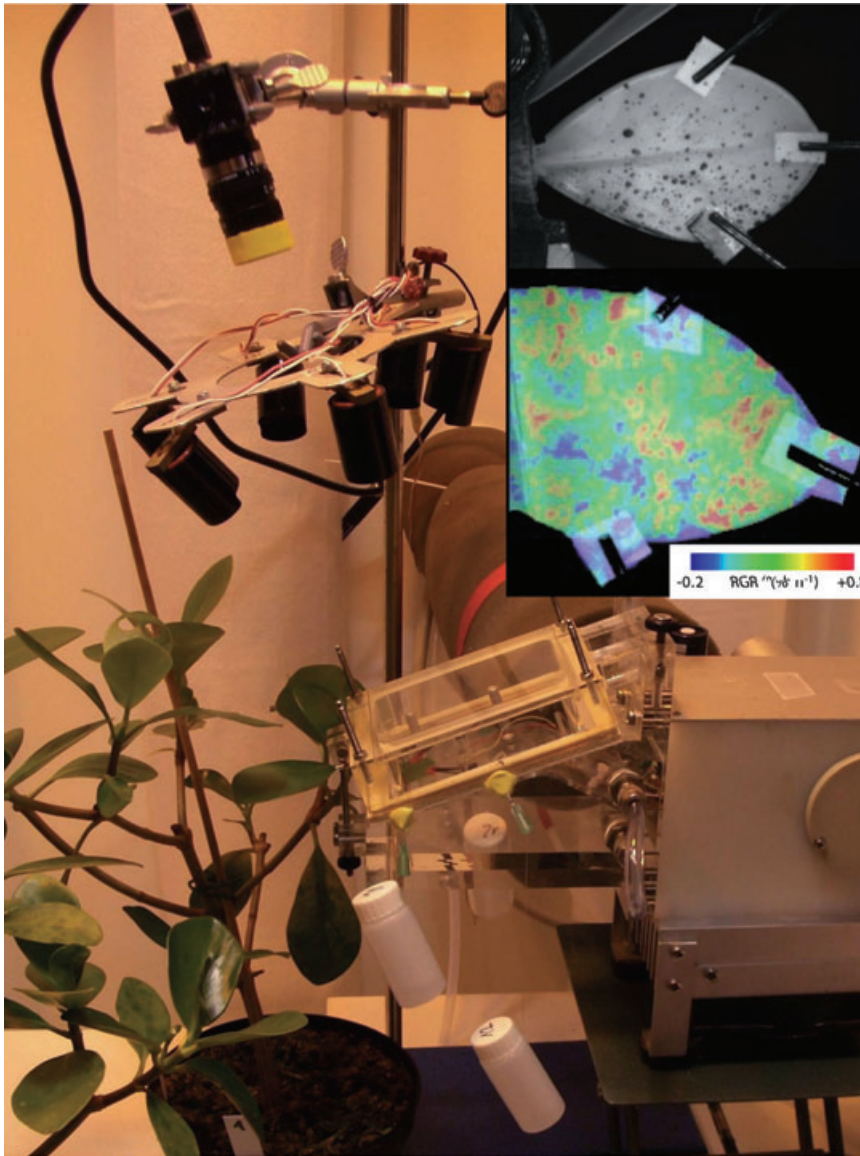


Figure 1. Leaf growth analysis of *Clusia minor*. Set-up for the simultaneous measurement of leaf growth and gas exchange with a CMOS camera (top), near-infrared light-emitting diode NIR-LED illumination assembly (middle) and climate-controlled gas-exchange chamber of the CMS 400 minicuvette containing a leaf of *C. minor* still attached to the plant (bottom). Insets show the original black-and-white image of an ink-sprinkled leaf as produced by the camera (top) and colour-coded spatial growth pattern (24 h average; red: maximal RGR; blue: minimal RGR). RGR, relative growth rate.

constant throughout day and night (25 °C). Dew point temperature was set to 12 °C, leading to a relative humidity of $46 \pm 2\%$. The flow rate was $570 \pm 40 \text{ mL min}^{-1}$. PAR inside the cuvette was $500\text{--}800 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Air pressure data were recorded every 10 min. For gas exchange on growing leaves, the actual leaf area was taken into account by determining the leaf area for every point in time from the optical leaf growth analysis that was performed simultaneously as described further. Gas exchange parameters were calculated according to Farquhar & Sharkey (1982).

Leaf growth measurements

Images were acquired using a CMOS camera (Flea BW, Point Grey, Vancouver, Canada) with constant illumination from near-infrared light-emitting diode (LED) light sources ($\lambda = 880$). The leaf was fixed in the focal plane of the camera

inside the Walz minicuvette system by using nylon fishing lines, which were guided through the cuvette-sealing gasket by injection needles to ensure a dynamic fixation of the expanding leaves in the still air-sealed cuvette system (Fig. 1). The resulting air leakage led to somewhat noisy data, which was smoothed by taking 1-h average values. A set of weights ($3 \times 10 \text{ g}$) was selected to keep the leaf in the focal plane and to prevent movements, still allowing a growth pattern similar to that of a non-fixed leaf (Walter, Feil & Schurr 2002). If leaf growth was not monitored inside the cuvette system, such as for growth analysis in the Phytoc greenhouse, leaves were fixed mechanically in the same way. Grey value images were acquired every 120 or 180 s, and RGR was calculated with algorithms that calculate pixel velocities (Schmundt *et al.* 1998; Scharr 2005; Walter & Schurr 2005; Matsubara *et al.* 2006). Leaves were sprinkled with black ink to improve optical richness (Fig. 1).

Titration of total acidity

Total acidity was estimated in 10-mm-diameter discs punched from growing leaves. Discs were sampled around the time when lights were switched on ('early day') or when lights were switched off ('early night'). Two to four leaf discs from different plants were pooled per replicate. Growing leaves sampled for acidity had the same size and position within the plant as leaves from the plant investigated for growth in the same experiment. The leaf discs were either immediately extracted in boiling 50% ethanol for 5–10 min until all chlorophyll had been converted to pheophytin, or stored at -80°C until extraction. The extract was then cooled, the phenolphthalein indicator was added and the extract titrated with 5 mM NaOH until colour change.

Statistical analysis

Comparisons between RGR at the beginning of the day and beginning of the night and between total acidity at early day and early night were performed using two-tailed Student's *t*-tests (software: Microsoft Excel).

RESULTS

Young expanding leaves on well-watered plants of *C. minor* had a diel leaf growth cycle with maximal activity at the beginning of the night and lower activity in the day, irrespective of whether plants were examined in the Phyttec greenhouse ($21\text{--}30^{\circ}\text{C}$ day/ 19°C night) or in the growth chamber (at 30°C day/night; Fig. 2). Negative RGR (leaf shrinkage) was often observed in the early day following the onset of illumination, as reported previously (Gouws *et al.* 2005).

In well-watered conditions, the amplitude of the diel growth cycle declined with time as the leaf approached full expansion, but maximal growth rates were found early in

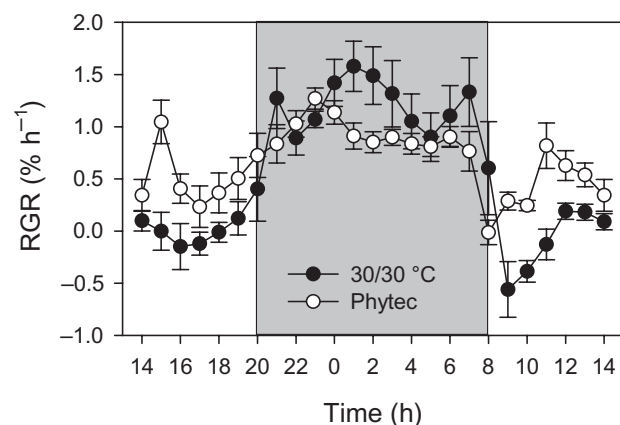


Figure 2. Average diel leaf growth pattern in *Clusia minor*. Diel pattern of relative growth rate (RGR) in the Phyttec greenhouse ($21\text{--}30^{\circ}\text{C}$ day/ 19°C night) versus in the growth chamber (30°C day/night) ($n=6$ for both conditions; mean values and SE). Shaded area indicates night.

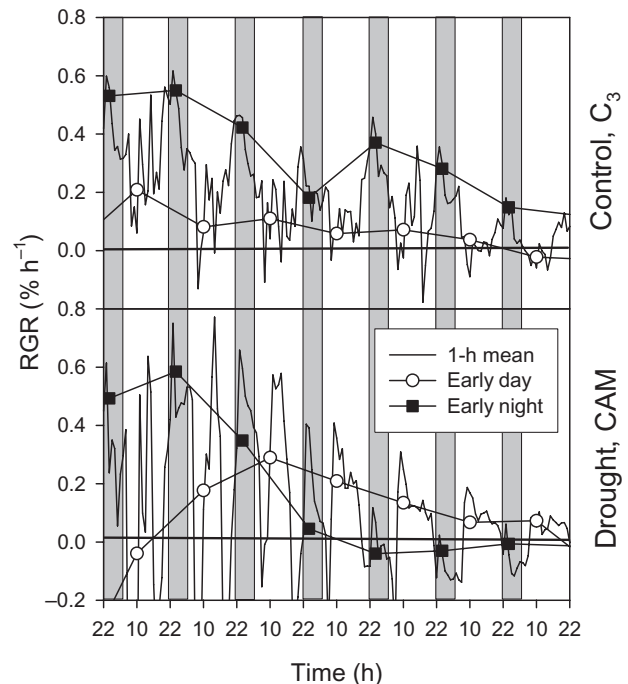


Figure 3. Development of relative growth rate (RGR) in *Clusia minor* throughout 7 d. Hourly means of RGR in expanding leaves of well-watered control (C_3) and drought-stressed (CAM) *C. minor* plants are depicted. Daily average morning (0800–1200 h) and early night values (2300–0100 h) of RGR are shown by open and closed symbols, respectively ($n=4$; mean values).

the night until the end of leaf development (Fig. 3, upper panel). In contrast to this pattern, RGR of growing leaves on drought-stressed *C. minor* decreased rapidly as they approached full expansion and displayed a distinctly different pattern with maximal growth activity during the day (Fig. 3, lower panel). Average morning RGR exceeded that of early in the night, when leaf growth decreased below a value of about $0.3\% \text{ h}^{-1}$. Comparison of diel growth cycles in nearly fully expanded, well-watered and drought-stressed leaves confirms that the differences between control and treatment early in the day and early in the night were highly significant ($P < 0.001$; Fig. 4). Growing leaves of well-watered plants comparable with those studied in Fig. 3 had high early night and early day values of total acidity that were not significantly different (Fig. 5; $P = 0.73$). Growing leaves of drought-stressed plants had a pronounced nocturnal increase and diurnal decrease in total acidity, with early night and early day values that differed significantly ($P = 0.004$). Final leaf size did not differ markedly between control and drought-stressed plants as leaves were affected by the drought stress only late in their development, when they had reached about 80% of their final size. Initiation of the next leaf pair was delayed in drought-stressed plants (data not shown).

The development of CAM and the changes in diel growth cycles in a growing leaf of *C. minor* were examined subsequently by simultaneous CO_2 exchange measurement and

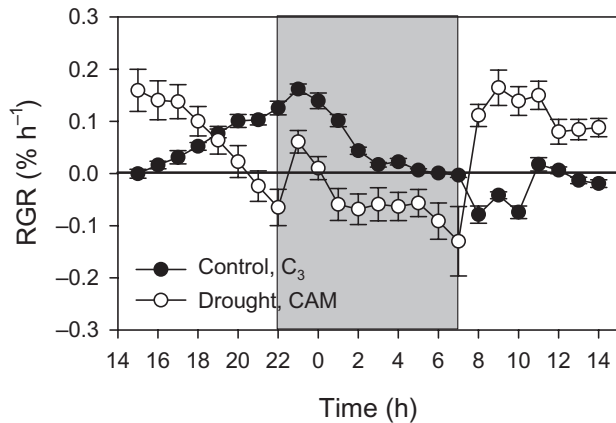


Figure 4. Diel leaf growth patterns in well-watered and drought-stressed *Clusia minor*. Average diel growth cycles are shown for well-watered (control) and drought-stressed leaves of *C. minor* approaching full expansion (maximal RGR $0.3\% \text{ h}^{-1}$; $n = 19$ diel cycles from three control plants; $n = 17$ from four drought-stressed plants; mean values and SE). Shaded area indicates night. CAM, crassulacean acid metabolism; RGR, relative growth rate.

growth imaging analysis for 20 d after cessation of watering (Fig. 6 & Supplementary Fig. S1). At the outset, when the plant was well watered, the stomata were open during the day and CO_2 uptake occurred during the light period, with a slight midday depression. This normal C_3 gas exchange pattern shifted after water was withheld and CO_2 efflux at night and uptake during the day both decreased, consistent with the onset of the CAM gas exchange pattern, which was clearly established by the end of the experiment. Total acidity measured on comparable leaves of an identically treated plant in the growth chamber (but outside the gas exchange system) displayed the characteristic change in early day-early night signature described earlier (Fig. 5), confirming an increase in CAM during the experiment.

Similar experiments were done with *C. alata*, which has been reported to be an obligate CAM plant. Preliminary

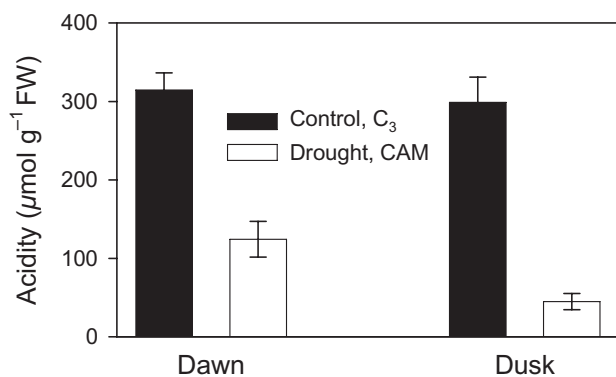


Figure 5. Total acidity in growing *Clusia minor* leaves. Average values at early day and early night for well-watered (control, C_3) and drought-stressed (CAM) leaves ($n = 3 - 6$; mean values and SE). FW, fresh weight; CAM, crassulacean acid metabolism.

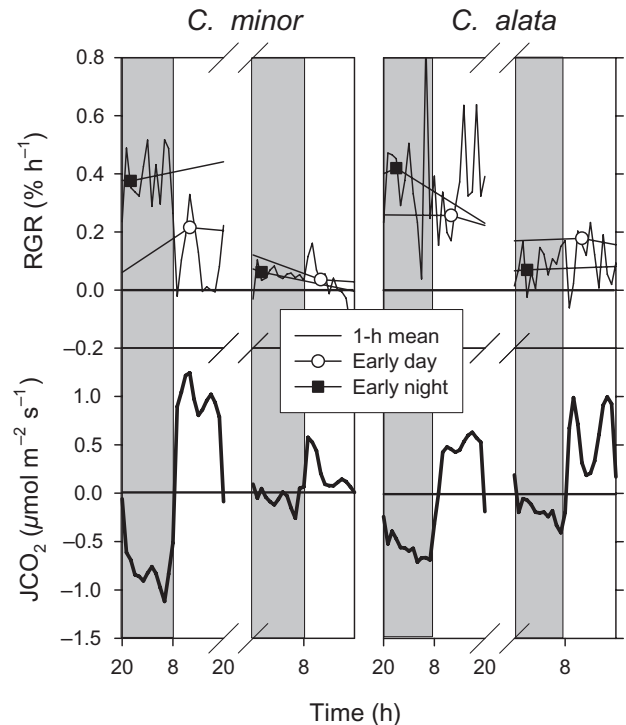


Figure 6. Concurrent measurements of diel leaf growth cycle and gas exchange in *Clusia minor* and *Clusia alata*. Hourly means of leaf relative growth rate (RGR) and net CO_2 exchange (JCO_2) are shown for an initially well-watered plant of *C. minor*, from which water was withheld after the commencement of the experiment (left panels). Open and closed symbols show average morning (0800–1200 h) and early night (2300–0100 h) RGR values, respectively. The same parameters are shown for a developing, well-watered leaf of *C. alata* (right panels). Two exemplary days (day 1 and day 7) are selected to display the typical changes that occurred throughout the experiments. The complete data set including total acidity is shown in Supplementary Figs S1 and S2. Shaded area indicates night.

experiments on a slowly growing, near fully expanded leaf of well-watered *C. alata* in the growth chamber showed maximal RGR during the day and only little growth at night (data not shown). When combined gas exchange and growth analysis were performed on a developing leaf of a well-watered plant of *C. alata*, it was observed that CO_2 was assimilated throughout the day (with a clear midday depression) and was actively respired at night (Fig. 6 & Supplementary Fig. S2). Average growth at the beginning of the night was faster than at the beginning of the day. Comparable leaves on an adjacent plant in the growth chamber contained high levels of acid in the day, and showed little nocturnal increase in acid (Supplementary Fig. S2), all consistent with a C_3 pattern of photosynthesis similar to that in young leaves of *C. minor* (Supplementary Fig. S1). Growth declined throughout the experiment, but from day 4 on, leaf growth was faster in the morning than in the early night period, and the gas exchange pattern also changed. The midday depression of CO_2 uptake increased, and CO_2 release at night was almost zero from day 5 on. By the end

of the experiment (day 10), growth had all but ceased, and the leaf showed all the features of CAM. Net CO₂ release at night was near zero, the midday depression of CO₂ fixation was near complete and a strong diurnal decrease in acid was observed, principally as a result of the decline in acid during the day.

Discussion

Diel variation in leaf growth rate is controlled by a complex network of factors (Walter & Schurr 2005), but the data obtained here show that changed patterns of gas exchange and acid metabolism are associated with changes in the pattern of diel leaf growth, when succulent leaves of the tropical tree genus *Clusia* switch from C₃ to CAM photosynthesis. It is evident that the shift from C₃ to CAM, either because of drought stress (*C. minor*) or development (*C. alata*), is accompanied by a shift from a predominantly nocturnal to a predominantly diurnal pattern of leaf growth (Figs 4 & 6). This is consistent with the results from our earlier study (Gouws *et al.* 2005), in which the RGR of leaves of a shrubby CAM plant (*Kalanchoe beharensis* Drake) and of cladodes of CAM cacti (*Opuntia* spp.) was higher during the day than at night. For mature leaves of CAM plants, it was observed that the nocturnal accumulation of malic acid osmotically drives water influx, and hence can lead to an increase in leaf volume of several percent (Lüttge 1986). Such an increase has not been observed for the young, growing leaves of *C. minor* investigated here. As acidity in growing leaves was low when *C. minor* was engaged in CAM (Fig. 5), an osmotically driven nocturnal increase in leaf volume seems to be negligible for the leaves investigated in this study.

Gouws *et al.* (2005) speculated that the temporal pattern of leaf growth with predominant activity at day might reflect the coincidence in time during the diel cycle of metabolism in CAM plants of three factors propitious for cell expansion growth: (1) the availability of carbohydrates for growth (i.e. over and above those needed to sustain nocturnal CO₂ fixation and CAM) was probably restricted to de-acidification (phase III) in *Kalanchoe* and *Opuntia* spp.; (2) the turgor needed for expansion growth peaked in the early light period (because of high malic acid levels in the vacuole; Lüttge & Ball 1977); and (3) the lower cytoplasmic pH prevailing during de-acidification (Hafke *et al.* 2001) might favour the loosening and deposition of cellulose in leaf cell walls (Cosgrove 1999). However, developing daughter cladodes of the CAM plant *O. ficus-indica* were carbohydrate sinks during the most rapid early growth phase (Wang *et al.* 1998). In 14-day-old cladodes, CO₂ efflux was greatest at night, they contained little starch but were rich in glucose and there was little diel change in the low acid content of the tissues. The daughter cladodes showed no evidence of net nocturnal CO₂ fixation until 18 d after emergence, and the normal pattern of CAM gas exchange (for *Opuntia*) was established only after 28 d. Clearly, source–sink relations complicate the simple interpretation of growth patterns in

terms of the diel carbohydrate metabolism of CAM offered previously.

Leaves of most C₃ plants tend to grow more rapidly at night or during the night–day transition, and it has been suggested that growth is then mainly driven by the mobilization of chloroplast starch and soluble sugar reserves (Walter & Schurr 2005). For example, seasonal changes leading to an afternoon decline in leaf growth of the tree *P. deltooides* at elevated CO₂ were correlated with a pronounced afternoon decline in leaf glucose levels (Walter *et al.* 2005). Moreover, developing leaves of woody plants often remain carbohydrate sinks for many days after emergence, showing high rates of respiration at night and net CO₂ efflux during the day. We can reasonably assume that the young leaves of the woody *Clusia* spp. examined here were engaging in C₃ photosynthesis, with significant net CO₂ fixation in the light. Many previous studies with fully expanded leaves of well-watered *C. minor* showed diurnal C₃ gas exchange patterns that were sensitive to changes in vapour pressure deficit and assumed to involve C₃ photosynthesis (Schmitt *et al.* 1988; Franco, Ball & Lüttge 1992). In the wet season in the field, carbon isotope fractionation during CO₂ fixation in the light by mature leaves of *C. minor* clearly indicated carboxylation by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Borland *et al.* 1993). The young leaves of *C. minor* in our experiments most closely resembled shaded leaves of this species in the field, which contained high, unchanging levels of citric and malic acids in the wet season (Borland *et al.* 1994). The origins of the high levels of acids (presumably a mixture of malic and citric acids) that persisted throughout the 24 h cycle in the young leaves of *C. minor* and *C. alata* are not clear. In spite of the large net efflux of CO₂ in the dark, these leaves may engage in some re-fixation of respiratory CO₂ at night, but further gas exchange studies in combination with isotope labelling are needed to establish this.

Unfortunately, we know little of the carbohydrate status or export capacities of these young *C. minor* leaves. Borland & Dodd (2002) found that mature leaves of this species exported very little carbon when engaging in CAM, but the slightly less negative $\delta^{13}\text{C}$ of young leaves of *C. minor* in the field led Borland, Maxwell & Griffiths (2000) to suggest that carbon fixed by CAM was used for growth of these leaves. Our data suggest it is unlikely that this carbon was derived from endogenous CAM in young leaves, and more likely that it was delivered as sugar or acids from mature leaves engaged in nocturnal CO₂ fixation. A C₃ pattern of gas exchange in young leaves was also found in *C. alata*, a species previously thought to be ‘obligate’ CAM, and in C₃ mode, leaves grew at about the same rate at day and at night. Could it be that in spite of a diurnal C₃ gas exchange pattern, the sustained high levels of acids in young leaves conspire to modify the typical C₃ diel growth pattern in *Clusia* spp. by maintaining high turgor and low cytoplasmic pH propitious for growth throughout the diel cycle?

We suspect that the slowing of leaf growth and the shift in the diel cycle with increasing CAM is primarily driven by the reduced availability of carbohydrates resulting from the

primary demand of CAM for substrates for nocturnal CO₂ fixation and acid synthesis. Although the complexities of carbohydrate–acid relationships in young growing leaves of *C. minor* and *C. alata* cannot be further defined at this stage, we conclude that when well watered and engaging in C₃ photosynthesis, the diel leaf growth cycle is predominantly nocturnal. When drought stress induces CAM in *C. minor*, leaf growth slows and becomes predominantly diurnal. Much the same transition is observed with the slowing of leaf growth and onset of CAM with leaf development in *C. alata*. These plants seem well suited to a further detailed evaluation of the relationships between diel carbon availability, turgor and cytoplasmic pH as factors determining diel patterns of leaf growth in these closely related, but physiologically and biochemically plastic, tropical tree species.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Figure S1. Concurrent measurements of diel leaf growth cycle and gas exchange in *Clusia minor* during the onset of drought. Hourly means of leaf relative growth rate (RGR) and net CO₂ exchange (JCO₂) are shown for an initially well-watered plant of *C. minor*, from which water was withheld after the commencement of the experiment. Open and closed symbols show the average morning (0800–1200 h) and early night (2300–0100 h) RGR values, respectively. Insets show early day and early night total acidity ($\mu\text{mol g}^{-1}$ fresh weight) for leaf disc samples from a growing leaf at the beginning and at the end of the experiment, respectively.

Figure S2. Concurrent measurements of diel leaf growth cycle and gas exchange in a developing, well-watered leaf of *Clusia alata*. Hourly means of leaf relative growth rate (RGR) and net CO₂ exchange (JCO₂) are shown. Open and closed symbols show the average morning (0800–1200 h) and early night (2300–0100 h) RGR values, respectively. Insets show early day and early night total acidity ($\mu\text{mol g}^{-1}$ fresh weight) for leaf disc samples from a growing leaf at the beginning and at the end of the experiment, respectively.

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