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Discrimination Processes and Shifts in Carboxylation during the Phases of Crassulacean Acid Metabolism[1]

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The magnitude and extent of Crassulacean acid metabolism (CAM) activity in two *Clusia* species was manipulated to investigate the regulation of the distinct CAM phases. First, in response to leaf-air vapor pressure deficit at night, changes in leaf conductance altered on-line carbon-isotope discrimination throughout the theoretical range for dark CO_2 uptake during CAM. These ranged from the limit set by phospho*enol*pyruvate carboxylase (PEPc) (−6‰, $\delta^{13}C$ equivalent of −2‰) to that imposed by diffusion limitation (+4‰, $\delta^{13}C$ equivalent of −12‰), but the lowest carbon-isotope discrimination occurred when p_i/p_a was only 0.7. Second, when the availability of external or internal sources of CO_2 was reduced for both field- and greenhouse-grown plants, CO_2 uptake by day via PEPc during phase II largely compensated. Third, by reducing the dark period, plants accumulated low levels of acidity, and CO_2 uptake occurred throughout the subsequent light period. Discrimination switched from being dominated by PEPc (phase II) to ribulose 1,5-bisphosphate carboxylase/oxygenase (phase III), with both enzymes active during phase IV. Under natural conditions, photochemical stability is maintained by extended PEPc activity in phase II, which enhances acid accumulation and delays decarboxylation until temperature and light stress are maximal at midday.

CAM is usually considered to be a property of photosynthetic leaves or stems that engage in CO_2 assimilation predominantly in the dark, although the entire day-night cycle can be succinctly described in terms of four phases (Osmond, 1978). Malic acid is the product of CO_2 fixation by PEPc at night during phase I of CAM. Following storage in the vacuole, malic acid is decarboxylated during the subsequent light period to release CO_2 internally for refixation via Rubisco during phase III of CAM. Day- and nighttime processes are normally tightly regulated to minimize any overlap (Nimmo et al., 1987; Smith and Bryce, 1992; Smith and Winter, 1996). However, two transitional phases occur, with phase II representing the early morning transition from PEPc to Rubisco. Late in the photoperiod, stomata may reopen. Direct C_3 photosynthesis occurs during phase IV of CAM (Osmond, 1978), although C_4 carboxylation can be detected over the latter part of this phase (Griffiths et al., 1990; Borland and Griffiths, 1996).

CAM has been traditionally viewed as an adaptation of succulent plants to arid and semi-arid environments. Recently, there has been a reexamination of these notions because of the unique characteristics of CAM expressed in the genus *Clusia*, consisting predominantly of hemiepiphytic strangling trees in the neotropics. Among the 150 to 200 species of the genus, CAM appears to be widespread (Lüttge, 1996) and is even present in rainforest species in parts of the world where precipitation may be over 4 m per year (Borland et al., 1992).

Certain species of *Clusia* display a dynamic plasticity in the expression of CAM on both a seasonal and daily basis. In response to increased light levels and water deficit as the dry season progressed in Trinidad, *Clusia minor* showed a rapid switch between C_3 photosynthesis and CAM, leading to overnight accumulation of acidity exceeding 1.4 M titratable protons (Borland et al., 1992, 1993). Increases in dark CO_2 uptake can be stimulated in well-watered plants by reducing the daytime CO_2 partial pressure from 35 to 17 $PaCO_2$ (Winter et al., 1992). Thus, carbon gain is maintained and even supplemented in *Clusia* species on a daily basis by increasing the magnitude of CAM when daytime photosynthesis is curtailed. The enhancement of CAM, together with cycling of carbon through citric and malic acid (Popp et al., 1988; Borland et al., 1992; Franco et al., 1992), is also thought to play an important role in preventing photoinhibition at midday (Haag-Kerwer et al., 1992; Borland and Griffiths, 1996).

Recently, the analysis of Δ instantaneously during gas-exchange measurements has been used to identify the contribution that C_3 and C_4 carboxylation makes to the daily carbon balance of CAM plants noninvasively (Griffiths et al., 1990; Griffiths, 1992). Previous studies using on-line discrimination in *Clusia* species (Borland et al., 1993; Roberts et al., 1996) have noted substantial C_4 activity during the early morning phase II and afternoon phase IV. In *Clusia fluminensis*, the PEPc signal dominated CO_2 uptake throughout phases II and IV in the light (Roberts et al., 1996), whereas the activity of PEPc is conventionally thought to be activated by phosphorylation at night and

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Abbreviations: Δ, carbon-isotope discrimination; F_m, maximum yield of fluorescence; F_m', the maximal fluorescence yield reached when a light-adapted leaf is exposed to a pulse of saturating light; g_s, stomatal conductance; PEPc, PEP carboxylase; PFD, photon flux density, 400 to 700 nm; p_i/p_a, the ratio of internal to external partial pressure of CO_2; Φ_{PSII}, photon-use efficiency; VPD, vapor pressure deficit.

down-regulated at dawn by dephosphorylation (Winter, 1982; Nimmo et al., 1984, 1987; Carter et al., 1996; Borland and Griffiths, 1997).

At both biochemical and ecological levels, the genus *Clusia* appears to be unique in regulating photosynthetic mechanisms that compensate carbon gain and maintain photosynthetic integrity when environmental conditions become demanding. We have manipulated the magnitude and duration of nighttime phase I of CAM, and investigated subsequent effects on daytime gas-exchange characteristics in the C_3-CAM intermediate *C. minor* and the CAM species *C. fluminensis*.

Measurements of instantaneous Δ, gas exchange, and fluorescence characteristics were used to assess the interplay between C_3 and C_4 carboxylation and photosynthetic competence. First, we investigated the effect of VPD on g_s and the extent of discrimination by PEPc during phase I. Second, the extent of CO_2 uptake was manipulated at night to determine whether daytime PEPc activity can compensate and maintain daily carbon balance. The experimental approach used a combination of field- and laboratory-based studies to show how these species alter C_3 and C_4 carboxylation processes, depending on the availability of external and internal sources of CO_2.

MATERIALS AND METHODS

Plant Material, Habitat, and Growth Conditions

Seedlings growing from bromeliad tanks and young, freestanding trees of *Clusia fluminensis* Pl. and Tr. were collected from the "restinga" (Brazilian coastal sandy plains) of Barra de Maricá (22° 53'S, 42° 52'W) in Rio de Janeiro, Brazil, in March of 1994. Plants were maintained in free-draining, peat-based compost at Moorbank Botanical Gardens, Newcastle-Upon-Tyne. Plants were grown under tropical greenhouse conditions providing seminatural conditions with respect to gradual diurnal changes in PFD, temperature, and VPD. Work was conducted on young trees of *C. fluminensis* (0.5 m: 4 years old) under greenhouse conditions with supplementary lighting supplied through the day (integrated PFD: 25.6 mol photon m^{-2} d^{-1}). Mean day/night temperatures in the tropical greenhouse were maintained at 30/20°C. Plants remained well-watered throughout the experiment. The C_4 grass *Cynodon dactylon* (L) Pers. was propagated from tillers and grown under a photoperiod of 12 h (integrated PFD: 20 mol photon m^{-2} d^{-1}) for comparative purposes.

Field work on *Clusia minor* L. was conducted on the Island of Trinidad, West Indies. The island is situated 15 km from mainland South America and is characterized by an annual dry season that usually extends from late February to April. Measurements were taken on March 1, 1995, during the dry season on a stand of *C. minor* growing 500 m from the Simla Research station (10° 41'N, 61° 17'W; grid reference PS869 823) in the Arima Valley, where annual rainfall is approximately 2.5 m per year. The population of *C. minor* (previously used by Borland et al., 1992) grew terrestrially on a rocky limestone outcrop and was surrounded by deciduous seasonal forest (for full description, see Broadmeadow et al., 1992). Here plants were growing fully exposed 6 to 8 m high, and access was gained to the most exposed branches overhanging a cliff-face by means of a bamboo platform. Mean day and night temperatures for the week of February 26 to March 3, 1995, were 32 and 23°C, respectively. Integrated PFD was 36.5 mol photon m^{-2} d^{-1} on February 28, and 35.5 mol photon m^{-2} d^{-1} on March 1, 1995. All measurements were conducted on the third fully expanded leaf pair, which showed no signs of senescence. Measurements were carried out at a time when plants were clearly exhibiting all four phases of CAM.

Gas Exchange and Instantaneous Δ

Gas-exchange measurements were obtained from plants of both species using a portable combined IR gas analysis system (CIRAS-1, PP Systems, Hitchin, UK). Air supply to the system was drawn through Teflon tubing from a 25-L mixing volume placed 15 m away from the field site, and air intake was 6 m above the ground and surrounding canopy. For *C. fluminensis*, air supply came from a 100-L mixing volume placed externally to the tropical greenhouse. A leaf was clamped in the leaf chamber, and gas-exchange rates were instantaneously calculated and displayed on an LCD screen by the use of two microprocessors in the gas analysis system. An integrating quantum sensor (Delta T Devices, Ltd., Cambridge, UK) measured the daily integrated PFD; incident PFD and air and leaf temperatures were measured using the PFD and temperature sensors of the leaf chamber during gas exchange measurements.

CO_2 was collected for on-line discrimination during gas-exchange measurements. Samples of CO_2 were collected from the analytical stream (following passage through the leaf chamber) or from the reference stream (air supply) over 15-min intervals during the day and/or night when depletion of CO_2 from the leaf chamber was between 2 and 4 Pa. The CO_2 collection line (as described in Griffiths et al., 1990) was located within the tropical greenhouse or underneath the platform used for access to exposed plants of *C. minor* in the field (for collection methods, see Roberts et al., 1996).

Calculation of Instantaneous Δ

Instantaneous Δ measured during gas exchange was calculated according to the method of Evans et al. (1986):

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \quad (1)$$

where $\xi = p_e/(p_e - p_o)$ and δ_e, p_e and δ_o, p_o are the isotopic composition and CO_2 partial pressures, respectively, entering and leaving the cuvette.

The predicted Δ values for C_3 and C_4 models were calculated using a simple formulation derived in Farquhar et al. (1989):

$$\Delta = a + (b - a)\frac{p_i}{p_a} \quad (2)$$

where a is the isotopic fractionation occurring due to diffusion in air (4.4‰), and b is the net isotopic fractionation caused by carboxylation (respectively, 27‰ as discrimination by Rubisco during C_3 carboxylation or −5.7‰ by PEPc during C_4 carboxylation). The ambient and intercellular partial pressures of CO_2, p_a and p_i, can be measured during gas exchange. It is important to note that changes in p_i/p_a alter the direction of discrimination for PEPc as opposed to Rubisco: during C_3 carboxylation, a lower g_s and reduced p_i/p_a decreases discrimination ($\delta^{13}C$ less negative) as Δ tends toward the lower limit set by diffusion (Δ = 4.4‰, equivalent to $\delta^{13}C$ = −12.4‰). Net discrimination by PEPc is low and in favor of ^{13}C, such that when g_s and p_i/p_a are high, ^{13}C in the C-4 of malate is enriched relative to source CO_2 with a minimum Δ of −6‰, equivalent to a $\delta^{13}C$ of −2‰. In contrast to Rubisco, Δ tends toward the upper limit of 4.4‰ set by diffusion, as p_i/p_a decreases when stomata close.

Leaf Sap Titratable Acidity and Organic Acids

The magnitude of CAM activity was assessed as the dawn-dusk difference of titratable acidity. Five replicate samples from different plants were collected at dawn and dusk, and at regular intervals throughout the experimental period. For *C. fluminensis*, acids were extracted by boiling leaf discs in a known volume of distilled water for 20 min, and extracted acids were then titrated against 2.5 mol m^{-3} NaOH with phenolphthalein as indicator. In the field the extent of CAM activity in *C. minor* was determined from leaf sap prepared from leaves of exposed plants. Extracts of bulk leaf sap were prepared from freeze-thawed tissue using a garlic press. Samples (50 μL) of sap were titrated against 10 mol m^{-3} NaOH, again, with phenolphthalein as indicator.

For analyses of organic acids, aqueous solutions of both species were neutralized with K_2CO_3, and malic and citric acids were determined enzymatically using the methods of Hohorst (1965) and Möllering (1985).

Chlorophyll Fluorescence

Measurements of chlorophyll fluorescence were obtained using a pulse amplitude modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany; Schreiber et al., 1986). The end of the fiber optic cable was fixed at a distance of 1 cm from the upper surface of the leaf at an angle of 60°. Care was taken to ensure that measurements were taken on the same area of leaf each time, and that when positioning the fiber optic cable, the area of leaf where measurements were taken was never shaded.

The F_m induced by a pulse of saturating light in a dark-adapted leaf was ascertained before dawn. Throughout the light period measurements were then taken of F_m'.

The Φ_{PSII} of PSII is obtained by the equation:

$$\Phi_{PSII} = (F_m' - F_s)/F_m' \quad (3)$$

where F_s is the measured light-adapted steady-state fluorescence yield at any given time.

Nonphotochemical fluorescence quenching ($\Delta F_m/F_m'$) is obtained from:

$$q_N = (F_m - F_m')/F_m' = \Delta F_m/F_m' \quad (4)$$

which represents thermal dissipation from the light-harvesting complex of PSII antenna.

Experimental Manipulations

Effect of VPD on Short-Term Changes in Δ during Phase I of CAM

During dark CO_2 uptake, leaves of *C. fluminensis* and *C. minor* were subjected to a range of VPDs to determine the effect of conductance on instantaneous Δ expressed by PEPc. Each leaf was allowed an acclimation period of 1 h at each VPD prior to collection for isotopic analysis. Measurements of instantaneous Δ were also taken for the grass *C. dactylon* to compare carboxylation within a C_4 plant to that of phase I of CAM.

Effect of Varying Nighttime CO_2 Supply

Plants of both *Clusia* species were subjected to the following overnight experimental treatments during phase I: (a) Control treatment (+CO_2): *C. minor* and *C. fluminensis* were allowed normal atmospheric CO_2 supply overnight. (b) CO_2-free treatment (−CO_2): 60 cm of terminal branches of *C. minor* were sealed into polythene bags with a CO_2-free environment maintained by sachets of soda lime (Carbasorb, BDH, Poole, UK). This treatment ensured that only internal CO_2, generated via dark respiration, was used as a substrate for CAM during phase I. (c) O_2- and CO_2-free treatment (−O_2, −CO_2): 60 cm of terminal branches of *C. minor* and *C. fluminensis* were sealed into polythene bags as above, with leaves subjected to an atmosphere of N_2 overnight, thus preventing net CO_2 uptake and respiratory CO_2 release.

Reduction in Phase I Duration

Plants of *C. fluminensis* were illuminated 5 h before normal dawn, and the effects of reduced dark CO_2 uptake on daytime gas exchange and discrimination were investigated.

RESULTS

Ecophysiological Characteristics of CAM and C_3-CAM Intermediacy in the Clusiaceae

Both species displayed all four phases of CAM and showed considerable nighttime CO_2 uptake (phase I), with the resultant accumulation of titratable acidity (Fig. 1). Instantaneous Δ was around 0‰ for *C. fluminensis* during phase I (Fig. 1a), but was more variable in *C. minor*, ranging from −4 to +4‰ (Fig. 1b). During phase II, both species continued to accumulate organic acids until stomatal closure. Such an increase in acidity must have been the result of CO_2 fixation via PEPc, which was supported by low values of Δ, around −5‰ in *C. fluminensis* (Fig. 1a). However, for the C_3-CAM intermediate *C. minor*, the

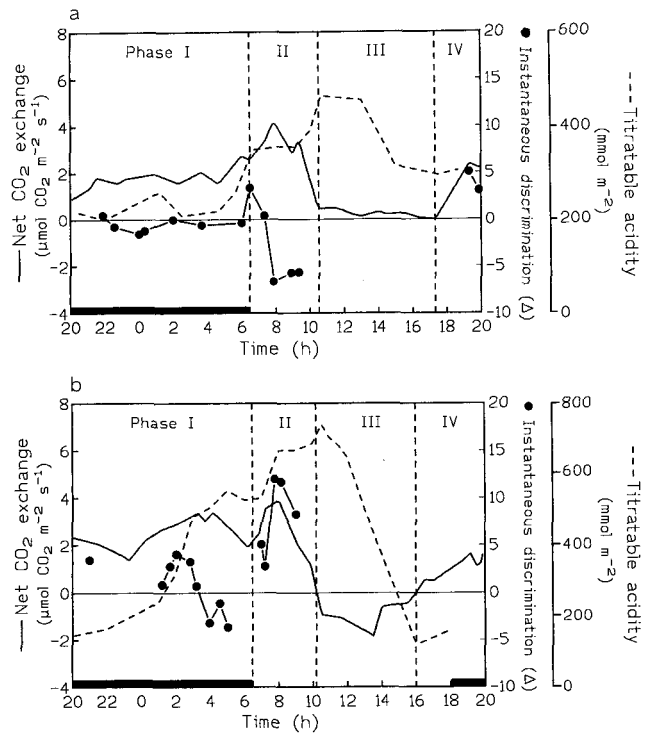

Figure 1. Daily time courses of leaf gas-exchange characteristics for leaves of greenhouse-grown *C. fluminensis* (a) and *C. minor* (b) from Trinidad throughout the four phases of CAM (dashed lines). CO_2 exchange (solid line), leaf sap titratable acidity (dashed line), and instantaneous Δ (●). Solid bars indicate the periods of darkness.

higher values of Δ (2.5–11.6‰; Fig. 1b) indicated that the proportion of CO_2 taken up by Rubisco during phase II was greater than in the constitutive CAM plant *C. fluminensis*.

Extent of Δ Processes Expressed by PEPc during CO_2 Uptake in Phase I

There was a direct correlation between C_4 (PEPc) discrimination processes and environmental conditions, such that Δ was linearly related to VPD (Fig. 2) over a much greater range than seen under natural conditions (Fig. 1). For the two *Clusia* species, the slope of the response was similar, with instantaneous Δ in saturated air close to the theoretical minimum for PEPc at −6‰ (equivalent to a $\delta^{13}C$ of −2‰). There was a linear increase in Δ up to +4‰ (i.e. $\delta^{13}C$ of −12‰) under a VPD of 3 kPa, which is equivalent to a RH of 25% at 30°C (Fig. 2, a and b). Although Δ for the C_4 grass *C. dactylon* also increased under high VPD, the range was much lower, from 0‰ in saturated air to 5‰ under a VPD of 4 kPa (Fig. 2c; compare with Fig. 2, a and b).

It was not always possible to measure g_s at night, when VPD was very low, because of the decreased sensitivity of IR gas analyzers and humidity sensors. The remaining gas-exchange data for both *Clusia* species have been combined for comparison with data for the C_4 grass *C. dactylon* to provide an analysis of Δ, VPD, and p_i/p_a (Fig. 3). There was a much greater range of p_i/p_a for the CAM species (0.7–0.2) across a smaller range of VPD than seen for the C_4 plant (Fig. 3, a and b). As predicted by theory, Δ was linearly related to p_i/p_a (Fig. 3, c and d), but the measured response was steeper than that predicted by theory in the CAM species (Fig. 3c). Thus, as g_s increased and p_i/p_a tended toward unity (Fig. 3c), the minimum Δ associated with PEPc activity (−6‰) occurred at a p_i/p_a of 0.7. In contrast, for the C_4 grass, Δ was tightly coupled to p_i/p_a and showed a consistent offset above the theoretical C_4 plant discrimination signal (Fig. 3d).

Manipulations of Phase I Magnitude

Effect on Subsequent Phases

In both CAM species, depriving leaves of external and/or internal sources of CO_2 at night resulted in enhanced or modified rates of CO_2 uptake during phases II and IV compared with control leaves. Under field conditions, control leaves of *C. minor* attained maximum acidity and CO_2 uptake soon after dawn, whereas these activities

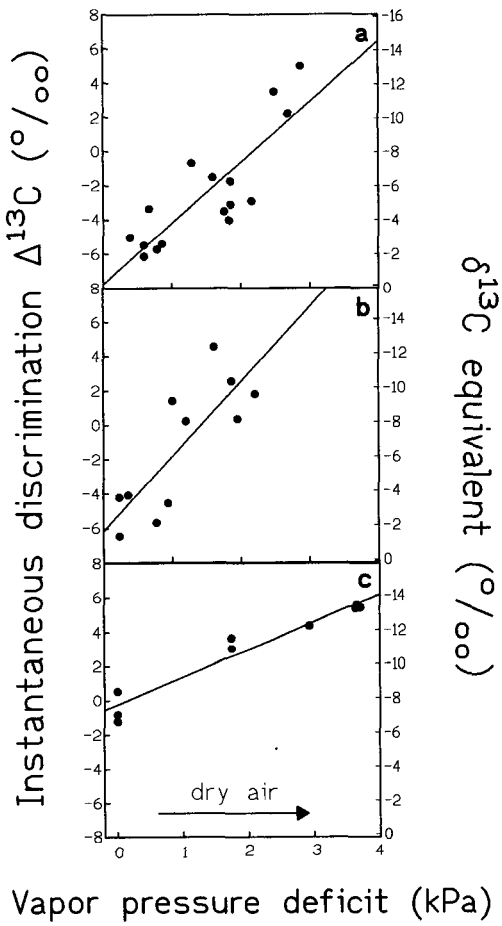

Figure 2. Relationship between instantaneous Δ measured by a change in isotopic composition of CO_2 passing over a leaf, and leaf-air VPD for dark CO_2 uptake in *C. fluminensis* (a) and *C. minor* (b), and for daytime C_4 CO_2 uptake in *C. dactylon* (c). Data points represent simultaneous measurements of the two parameters on individual leaves.

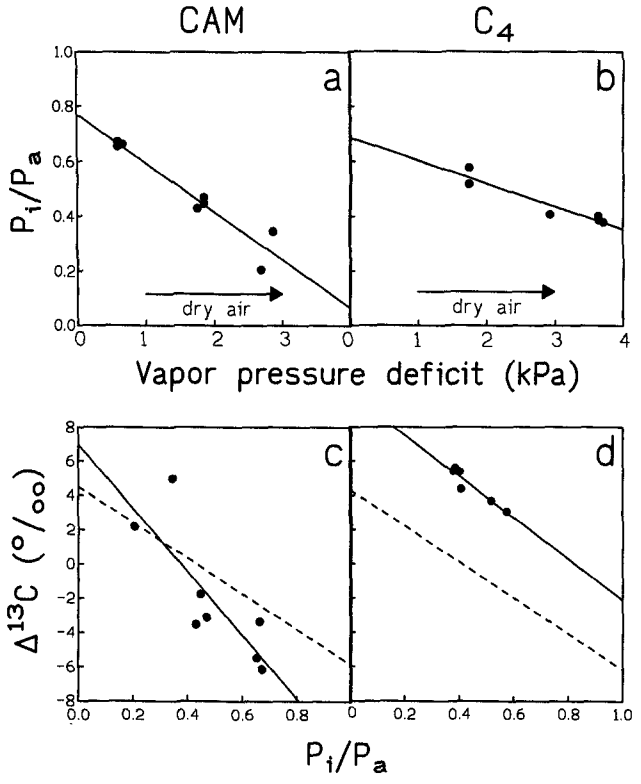

Figure 3. Relationship between p_i/p_a and leaf-air VPD for dark CO_2 uptake during CAM (a) and daytime C_4 CO_2 uptake (b). Relationship between instantaneous Δ measured by a change in isotopic composition of CO_2 passing over a leaf and p_i/p_a for dark CO_2 uptake during CAM (c) and daytime C_4 activity (d), respectively. The dashed line represents the theoretical relationship between Δ and p_i/p_a. Data points represent simultaneous measurements of the two parameters on individual leaves.

were stimulated during phase II in proportion to the degree of limitation suffered overnight (Fig. 4, a and b). When decarboxylation commenced at around 11:00 AM in all leaves, titratable acidity had recovered to some 66% of control leaves in the CO_2-free and N_2 treatments (see Table I). Finally, net CO_2 uptake was also stimulated during phase IV in these treatments (Fig. 4a).

Having observed this phenomenon in the field, the N_2 regime was imposed on *C. fluminensis* under less severe environmental conditions in the greenhouse so that the implications for discrimination could also be explored (Fig. 5). Compared with control leaves, phase II CO_2 uptake was stimulated and extended for 3 h by the N_2 regime (Fig. 5a), accounting for 75% of the daily total uptake (Table II). Titratable acidity continued to accumulate in the N_2-treated leaves, but recovered to a lower proportion than equivalent *C. minor* leaves (Tables I and II). During phase II, the on-line Δ signal shifted from PEPc domination at dawn (−6‰) to a maximum of +7‰ at 12:00 noon (Fig. 5c), showing the onset of Rubisco activity. This was also suggested by the rapid decarboxylation, with only a brief period of stomatal closure representing phase III in the N_2-treated leaves. Phase IV gas exchange then extended throughout the afternoon for 8 h, but, remarkably, the associated isotope signal was dominated by PEPc. Measured Δ values of −2.18 to −3.49‰ matched closely those predicted (−1.82 to −3.77‰) for C_4 activity from p_i/p_a (Eq. 2). However, at this time there was no substantial increase in titratable acidity, which would be associated with PEPc activity. With the higher acidity accumulation overnight, phase III was extensive in control leaves, with relatively high rates of CO_2 uptake in phase IV recommencing after 4:00 PM. It should also be noted that the signal obtained from CO_2 released in control plants at the start of phase III (Fig. 5a) was in the range of −1.6 to −2.4‰, similar to the Δ measured during CO_2 uptake of phase II (see Table II). This would suggest that the CO_2 released from decarboxylation, and subsequently leaking from stomata, has not been subjected to any further fractionation processes (i.e. refixation via Rubisco), suggesting possible inactivation of the enzyme at this time.

Leaf Carbon Balance and Efficiency of Light Utilization

In terms of the daily carbon budget for *C. minor* in Trinidad, net CO_2 uptake in the light was stimulated some 2- to 3-fold by CO_2-free or N_2 treatments, respectively. However, over the 24-h cycle, control leaves took up an equivalent amount of CO_2 (Table I). If it is assumed that one mole of malate is decarboxylated to provide 1 M CO_2, then regard-

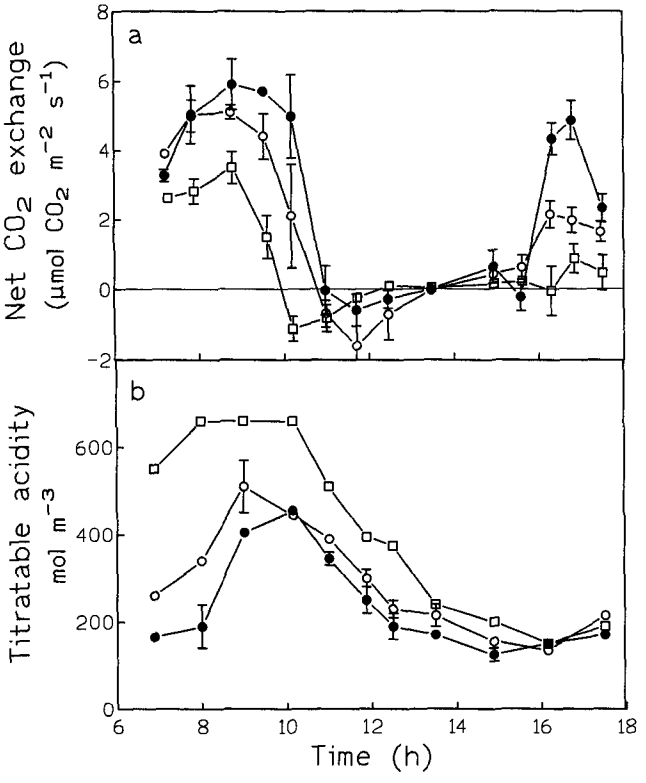

Figure 4. CO_2 exchange (a) and titratable acidity (b) for plants of *C. minor* growing in Trinidad when plants were clearly exhibiting CAM. Symbols represent different nighttime conditions: □, control (ambient air); ○, CO_2-free (soda lime); and ●, CO_2- and O_2-free (N_2) atmosphere.

Table I. *Integrated carbon budgets for leaves previously exposed to different overnight treatments and the dissection of carbon fluxes during the different phases of CAM for C. minor*

The amount of CO_2 taken up over 24 h includes net photosynthesis, together with the amount of CO_2 released internally from the decarboxylation of malate.

Phase of CAM	Overnight Treatment		
	Control (ambient air)	CO_2-free atmosphere	N_2 atmosphere
Phase I		*mmol kg^{-1}*	
Malate accumulation	136.3	17.3	12.3
Citrate accumulation	12.1	0	0
Phase II			
Net CO_2 uptake	53.5	93.2	133.1
Percentage of days net CO_2 uptake	88.1	78	73
Malate accumulation	92	104	49
Citrate accumulation	68	43.5	76
Citrate decarboxylation	24	–	12
Average Δ (‰)	−0.82	–	–
Phase III			
Net CO_2 uptake	3.2	2.6	4.5
Percentage of days net CO_2 uptake	5.2	2.2	2.6
Net CO_2 release	9.3	13.5	5.1
Malate decarboxylation	242.6	143.1	86
Citrate decarboxylation	76	74.5	82
Phase IV			
Net CO_2 uptake	4.1	23.7	42.6
Percentage of days net CO_2 uptake	6.7	19.8	23.7
Malate accumulation	17.6	23.1	66
Citrate accumulation	–	26.9	–
Average Δ (‰)	−2.56	–	–
Total net CO_2 uptake over day	60.8	119.5	180.2
CO_2 uptake over 24 h	303.4	262.6	266.2
Total CO_2 fixed over 24 h including that from citrate breakdown	412.5	345.5	392.1

less of the magnitude of CAM activity, all leaves had similar CO_2 gain over 24 h (Table I). However, for *C. minor* in the field, citric acid breakdown was also observed during phase III (where malate:citrate decarboxylation ratios were 3:1, 2:1, and 1:1 for control, CO_2-free, and N_2 treatments, respectively). The breakdown of citric acid, whether partial or complete (producing 3 or 6 CO_2 per citrate, respectively), would considerably augment the internal supply of CO_2 and may help to alleviate photoinhibition at midday in these plants. A similar situation held for *C. fluminensis*; over a 24-h period, carbon balance was similar in control and N_2-treated leaves (Table II).

Given this compensatory CO_2 uptake in the daytime, curtailing nocturnal CAM activity had little effect on leaf photochemical efficiency in *C. minor* over the following day. There were similar changes in Φ_{PSII}, the intrinsic photochemical efficiency of PSII (F_V/F_M), and nonphotochemical quenching through thermal dissipation (Fig. 6, a–c). In contrast, the changes in chlorophyll fluorescence reflected levels of high light incident on leaves. However, we note that Φ_{PSII} and q_N were restored at dusk (Fig. 6, b and c), indicating that no long-term photoinhibitory damage had occurred under these conditions, and that such recovery re-occurred throughout the dry season (A. Roberts, unpublished observations).

Manipulations of Phase I Duration: Implications for CAM Rhythm and the Activity of Carboxylation Enzymes

Having manipulated the magnitude of phase I, we then investigated the effect of reducing the duration of nighttime CO_2 uptake on subsequent gas-exchange and discrimination patterns in *C. fluminensis*. The plants were illuminated at 2:00 AM, which shifted phase II forward by 5 h (Fig. 7), with the magnitude of CO_2 uptake (maximum 7 μmol m^{-2} s^{-1} at 3:00 AM) and Δ initially similar to the phase II under control conditions (Fig. 1a). Thus, Δ values were C_4-like (0.82‰) and identical to those predicted by the C_4 model (Fig. 7); however, after 4 h stomata did not close, as would have been expected. The instantaneous Δ associated with CO_2 uptake during the extended part of phase II (19.2‰) reflected direct C_3 carboxylation by Rubisco (Fig. 7), indicating that PEPc was then downregulated.

There was continuous CO_2 uptake throughout the light period, with the CO_2 regenerated from organic acids insufficient to cause stomatal closure. Values of Δ through the latter part of phase III and phase IV (9:00 AM to 3:00 PM) were between those predicted for C_3 and C_4 models (10.6 to 6.7‰), suggesting concurrent carboxylation by both PEPc and Rubisco in the light.

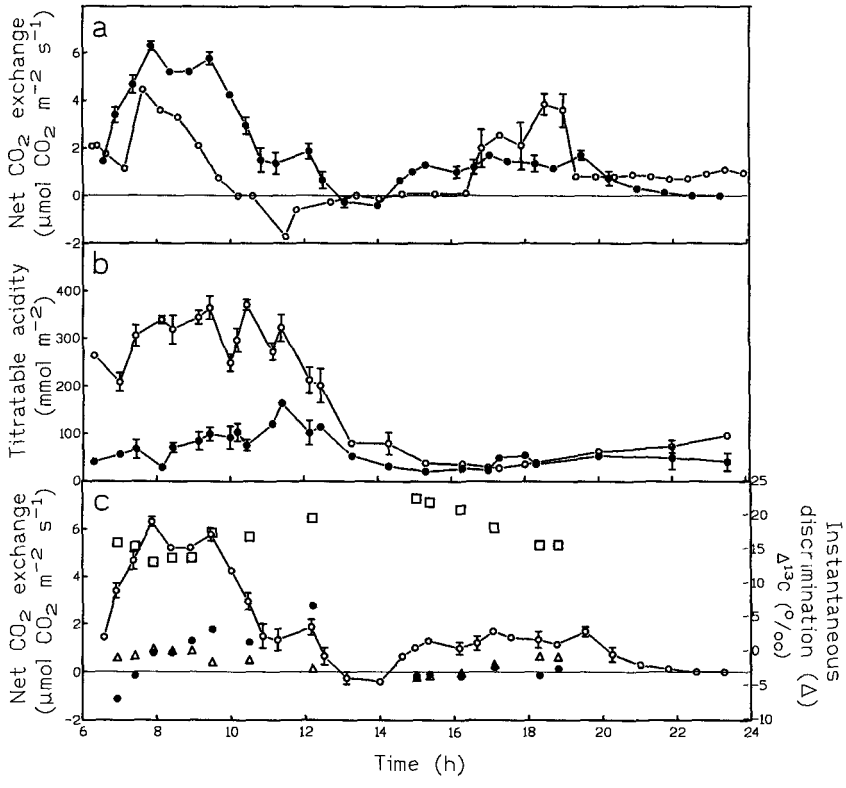

Figure 5. CO_2 exchange (a) and titratable acidity (b) for well-watered plants of *C. fluminensis* under seminatural greenhouse conditions. Symbols represent different nighttime conditions: O, control; ●, CO_2- and O_2-free (N_2). Figure 5c shows gas exchange (O) and instantaneous discrimination (●) for leaves of *C. fluminensis* maintained overnight in N_2. □ and Δ represent predicted instantaneous discrimination for a C_3 and C_4 model, respectively.

DISCUSSION

Phase I and Discrimination by PEPc

Measurements of instantaneous Δ were within the theoretical limits for PEPc-mediated dark CO_2 uptake for the CAM species *C. fluminensis* and the C_3-CAM intermediate *C. minor*. Direct measurements of the isotopic signal associated with the C-4 of malate fell midway within the theoretical range of Δ under normal growth conditions, suggesting co-limitation by carboxylation and diffusion (Holtum et al., 1983). However, most measurements of on-line, instantaneous Δ for constitutive CAM bromeliads have until now shown higher values that more closely reflect the usual range of organic Δ values for CAM plants, i.e. +2 to +10‰, perhaps because of the degree of recycling of carbon inherent in the CAM cycle in these plants (Griffiths et al., 1990).

When leaf conductance was manipulated to provide a broad range of p_i/p_a, the direct relationship between C_4 discrimination processes and environmental conditions was seen across the theoretical range. In the model (Farquhar et al., 1989), the limits of Δ are set first by PEPc activity (−6‰: stomata wide open, p_i/p_a at unity) and second when products tend toward the diffusion-limited isotope composition of CO_2 in the substomatal cavity (+4.4‰). It was interesting that the lowest Δ values were close to the theoretical minimum and were attained at a p_i/p_a of 0.7, indicating the carboxylation strength of PEPc.

In both C_3 and C_4 plants, g_s generally decreases with increasing VPD during daytime CO_2 uptake (Morrison and Gifford, 1983). Measurements on the C_4 grass *C. dactylon* showed a much narrower range of Δ compared with nighttime CAM CO_2 uptake. In addition, the dependence of Δ on p_i/p_a in C_4 plants (typically leading to a narrow range of C_4 isotopic compositions) is also related to the proportion of carbon that leaks from the bundle sheath cells (to be refixed by PEPc), allowing discrimination by Rubisco to be expressed (Farquhar, 1983; Evans et al., 1986; von Caemmerer and Hubick, 1989; Henderson et al., 1995). While a negative correlation between p_i/p_a and Δ (whether on-line or organic) has been shown (von Caemmerer and Hubick, 1989; Madhaven et al., 1991), an increase in bundle-sheath leakiness after stress treatments can also alter Δ independent of p_i/p_a (Bowman et al., 1989). In this study, the constant offset between measured and predicted Δ across the range of imposed conductances suggested a constant rate of leakage.

Phases II and IV

By experimentally manipulating the magnitude of phase I CO_2 uptake during CAM, we have demonstrated a role for phase II, and to a lesser extent phase IV, in maintaining carbon balance directly by enhanced daytime PEPc-mediated CO_2 uptake. The extended C_4 activity during phase II probably reflects a delay in the deactivation of PEPc by dephosphorylation, characteristic of CAM in the genus *Clusia* (Borland and Griffiths, 1997). The extent of Rubisco activation during phase II in these *Clusia* species is less clear. The measured Δ for CO_2 released from *C. fluminensis* at the end of phase II suggests that Rubisco may not be activated until phase III is under way. Experiments to

Table II. *Integrated carbon budgets for leaves previously exposed to different overnight treatments, and the dissection of carbon fluxes during the different phases of CAM for C. fluminensis*

Phase of CAM	Overnight Treatment	
	Control (ambient air)	N_2 atmosphere
Phase I	*mmol kg^{-1}*	
Accumulation of titratable acidity	229.1	5.9
Phase II		
Net CO_2 uptake	45.5	140.0
Percentage of days net CO_2 uptake	28.3	75.1
Accumulation of titratable acidity	107.2	124.0
Average Δ (‰)		
Early phase II	3.7	−2.6
Late phase II	−5.1	3.3
Phase III		
Net CO_2 uptake	11.3	
Percentage of days net CO_2 uptake	7.1	
Net CO_2 release	9.5	1.89
Decarboxylation of titratable acidity	344.0	144.0
Δ of evolved CO_2 (‰)	−2.0	
Phase IV		
Net CO_2 uptake	45.1	34.9
Percentage of days net CO_2 uptake	28.1	18.7
Accumulation of titratable acidity	35	33
Average Δ (‰)	4.5	−3.0
Phase I		
Net CO_2 uptake	58.9	11.6
Percentage of days net CO_2 uptake	36.5	3.2
Accumulation of titratable acidity	156	190
Total net CO_2 uptake over 24 h	160.92	186.9

establish the activation state of Rubisco in these species throughout the day are currently in progress. However, it is also possible that the markedly C_4 discrimination signal measured in *C. fluminensis* during phases II and IV is a result of high PEPc activity, which masks Rubisco activity as in C_4 plants. The fixation of CO_2 by PEPc and Rubisco during phase II would account for CO_2 uptake being greater than malate accumulated in leaves of *C. minor* exposed to N_2 overnight. This could represent futile cycling through malate production and decarboxylation (Borland and Griffiths, 1996), but a recent study on *C. minor* suggests that futile cycling is negligible during phase II in both control and N_2-treated plants, although it may occur during phase IV (Borland and Griffiths, 1997; see also Osmond et al., 1996).

In terms of overall carbon balance, regardless of the magnitude of phase I dark CO_2 uptake imposed by the various treatments, leaves took up comparable amounts of CO_2 over 24 h (see also Winter et al., 1992). Given this "compensatory" CO_2 uptake by day, curtailing nocturnal CAM activity had little effect on leaf photochemical efficiency in *C. minor* over the following day. Therefore, the changing patterns of Φ_{PSII} and nonphotochemical quenching are driven by acclimation to PFD, as was observed previously for sympatric species of *Clusia* with different photosynthetic pathways (Roberts et al., 1996). We conclude that diverse CO_2 uptake mechanisms (via PEPc and/or Rubisco), together with regulated nonphotochemical processes that safely dissipate excess (potentially damaging) radiation, confer protection to the photochemical apparatus of this hemi-epiphytic strangler, even under extreme conditions.

Phase III

During conventional phase III the elevated levels of internal CO_2 cause stomatal closure and discrimination expressed by Rubisco cannot be measured. Reducing the duration of phase I by early illumination effectively reset the phases of CAM, while the low levels of decarboxylation permitted stomata to remain open. Direct determination of discrimination expressed by the eventual onset of Rubisco activity indicated that PEPc had been down-regulated some 4 h after illumination. Moreover, the down-regulation of PEPc in this instance did not seem to be determined solely by the storage capacity of the vacuole, as suggested elsewhere (T.E.E. Grams, personal communication), because the malate content of leaves that received early illumination was significantly lower than that of control leaves at a compara-

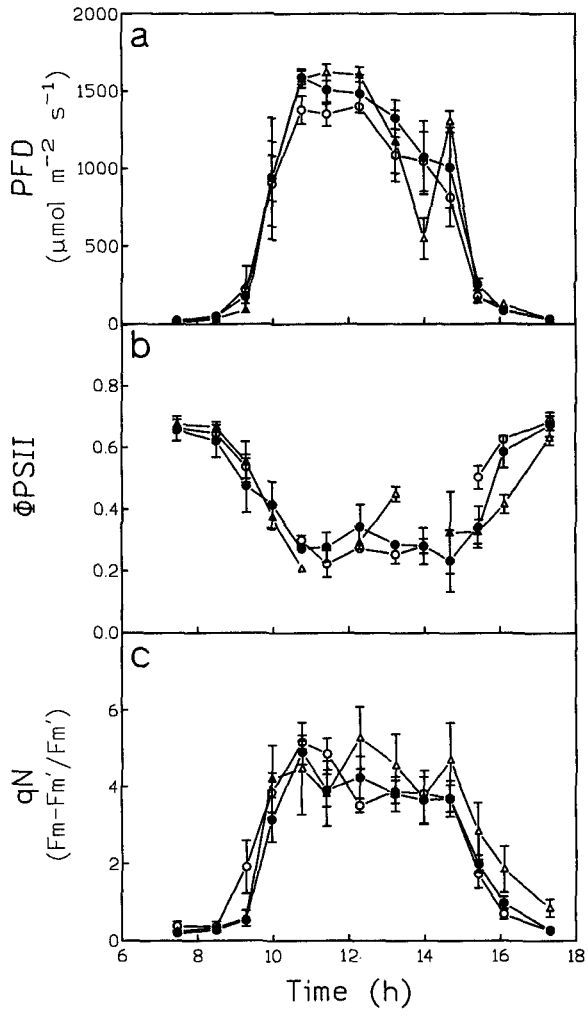

Figure 6. Measurements of incident light (a), Φ_{PSII} (b), and nonphotochemical quenching (qN) (c) in leaves of *C. minor*, where symbols represent different nighttime conditions: ○, control (ambient air); Δ, CO_2-free (soda lime); and ●, CO_2- and O_2-free (N_2).

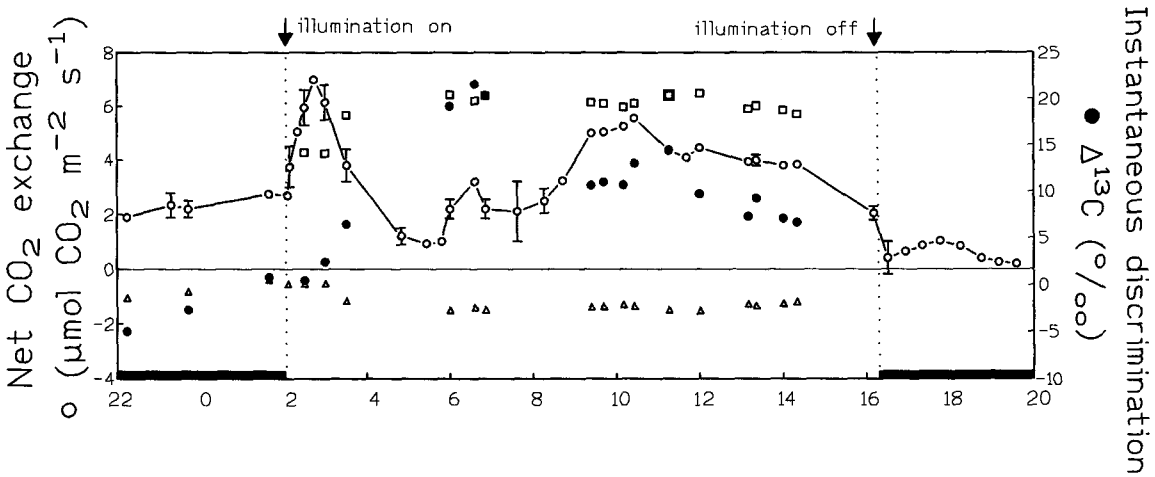

Figure 7. The result of early illumination during the dark period on *C. fluminensis*. Symbols represent net CO_2 exchange (○) and instantaneous Δ (●) measured by a change in isotopic composition of CO_2 passing over a leaf. □ and Δ represent predicted Δ for C_3 and C_4 models, respectively. Note lights on at 2:00 AM. Solid bars indicate the periods of darkness.

ble stage in the cycle (data not shown). It is possible, however, that the early illumination and accompanying increase in temperature may have affected the properties of the vacuole (Kluge and Scomburg, 1996), with malate accumulating in the cytoplasm and inhibiting PEPc.

Uptake of CO_2 continued throughout phase III, reflecting the reduced level of decarboxylation and high carboxylation capacity maintaining drawdown of the internal partial pressure of CO_2 and stomatal opening. Throughout this period, the measured values of Δ suggested co-carboxylation of CO_2 by both PEPc and Rubisco. It is a distinct possibility that futile cycling through malate synthesis/degradation was also occurring at this time. As suggested elsewhere, perhaps futile cycling in CAM plants serves as an additional mechanism for dissipating ATP and reductant derived when excess photons are absorbed (Borland and Griffiths, 1996).

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