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A comparative study on the regulation of C_3 and C_4 carboxylation processes in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* and the C_3-CAM intermediate *Clusia minor*

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Abstract. A comparison of carbon metabolism in the constitutive crassulacean acid metabolism (CAM) plant *Kalanchoë daigremontiana* Hamet et Perr. and the C_3-CAM intermediate *Clusia minor* L. was undertaken under controlled environmental conditions where plants experience gradual changes in light intensity, temperature and humidity at the start and end of the photoperiod. The magnitude of CAM activity was manipulated by maintaining plants in ambient air or by enclosing leaves overnight in an atmosphere of N_2 to suppress C_4 carboxylation. Measurements of diel changes in carbon-isotope discrimination and organic acid content were used to quantify the activities of C_3 and C_4 carboxylases in vivo and to indicate the extent to which the activities of phosphoenolpyruvate carboxylase (PEPCase), ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and decarboxylation processes overlap at the start and end of the photoperiod. These measurements in vivo were compared with measurements in vitro of changes in the diel sensitivity of PEPCase to malate inhibition. The results demonstrate fundamental differences in the down-regulation of PEPCase during the day in the two species. While PEPCase is inactivated within the first 30 min of the photoperiod in *K. daigremontiana*, the enzyme is active for 4 h at the start and 3 h at the end of the photoperiod in *C. minor*. Enclosing leaves in N_2 overnight resulted in a two- to threefold increase in PEPCase-mediated CO_2 uptake during Phase II of CAM in both species. However, futile cycling of CO_2 between malate synthesis and decarboxylation does not occur during Phase II in either species. In terms of overall carbon balance, C_4 carboxylation accounted for ≈ 20% of net daytime assimilation in *both* species under control conditions, increasing to 30–34% after a night in N_2. Although N_2-treated leaves of *K. daigremontiana* took up 25% more CO_2 than control leaves during the day this was insufficient to compensate for the loss of CO_2 taken up by CAM the previous night. In contrast, in N_2-treated leaves of *C. minor*, the twofold increase in daytime PEPCase activity and the increase in net CO_2 uptake by Rubisco during Phase III compensated for the inhibition of C_4 carboxylation at night in terms of diel carbon balance.

Key words: Carbon isotope – Carbon metabolism – *Clusia* (carbon metabolism) – Crassulacean acid metabolism – *Kalanchoë* (carbon metabolism) – Phosphoenolpyruvate carboxylase

Introduction

The biochemical and physiological complexities which comprise the day-night cycle of crassulacean acid metabolism (CAM) were first dissected into four main phases by Osmond in 1978. Since then, invasive and non-invasive measurements have shown that C_4 carboxylation and malate synthesis are generally confined to the dark period (Phase I). Phosphoenolpyruvate carboxylase (PEPCase) is rapidly down-regulated at the start of the light period as a result of feedback inhibition from malate (Phase II; Winter 1982; Nimmo et al. 1984) thereby avoiding futile cycling of CO_2 and refixation during decarboxylation of malic acid. The decarboxylation of organic acids during Phase III releases CO_2 which is refixed via the C_3 carboxylation enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) when stomata are usually closed. With decarboxylation complete, stomata may reopen during Phase IV and atmospheric CO_2 can be fixed directly by Rubisco, although PEPCase may be activated before the end of the light period (Osmond and Allaway 1974; Ritz et al. 1986).

Abbreviations and definitions: CAM = crassulacean acid metabolism; c_i/c_a = internal, external concentration of CO_2; $\delta^{13}C$ = carbon-isotope ratio, ‰ relative to Pee Dee Belemnite (vs. PDB); Δ = discrimination against ^{13}C; PEPCase = phosphoenolpyruvate carboxylase; PFD = photon flux density; Rubisco = ribulose 1,5-bisphosphate carboxylase/oxygenase; VPD = vapour pressure deficit

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It has been a traditional dogma of CAM that Phases II and IV merely represent transitions between carboxylation processes and that PEPCase activity at these times makes a negligible contribution to net carbon gain. However, measurements of short-term changes in carbon-isotope discrimination in hemi-epiphytic stranglers of the genus *Clusia*, which are known to possess facultative CAM, have demonstrated that PEPCase may be active for 4–5 h after dawn under field and greenhouse conditions (Borland et al. 1993; Roberts et al. 1996). Over recent years, the physiological plasticity displayed by various lifeforms of *Clusia* has made this genus the centre of considerable interest (Franco et al. 1990, 1994; Lüttge 1996; Borland et al. 1992, 1994, 1996). In *Clusia*, CAM may be constitutive or, where inducible, appears to serve primarily as a means of maintaining or even supplementing carbon gain at times when photosynthesis is curtailed as a consequence of reduced water availability, higher vapour pressure deficits or lower partial pressures of CO_2 (Borland et al. 1992; Winter et al. 1992). Such observations suggest that the capacity for daytime C_4 carboxylation could provide *Clusia* species with unrivalled flexibility in tailoring CO_2 uptake to match demands imposed by changes in the environment.

By considering the various findings described above, two possible hypotheses are proposed: (i) extended PEPCase activity in the morning in *Clusia* simply reflects gradual increases in photon flux density (PFD) and temperature that occur naturally at dawn in the field/greenhouse or (ii) regulation of carboxylation/decarboxylation processes in *Clusia* are fundamentally different from those in traditionally studied CAM plants, with futile cycling representing a significant proportion of daytime carbon flux. In order to test these hypotheses, a comparative study of carbon fluxes in *C. minor* and the classically studied CAM plant *Kalanchoë daigremontiana* was undertaken under controlled environmental conditions where plants experience gradual changes in PFD, temperature and humidity at the start and end of the photoperiod. In both species the magnitude of CAM activity was manipulated by either maintaining plants in ambient air or by enclosing leaves overnight in an atmosphere of N_2, thus suppressing C_4 carboxylation at night. The photosynthetic characteristics of plants were subsequently assessed by measuring short-term changes in carbon isotope discrimination (Δ) concurrently with leaf gas exchange as a means of illustrating the balance between diffusional and carboxylation limitation to CO_2 uptake over the diel cycle. In addition, measurements of organic acid content and assays in vitro of the regulatory properties of PEPCase were made over the course of the day/night cycle. This combination of invasive and non-invasive measurements provides quantitative evidence for differences in leaf carbon budgets between *K. daigremontiana* and *C. minor*. Firstly, we estimate the contribution of futile cycling during Phases II and IV of CAM; secondly, we compare PEPCase activity measured in vivo with in-vitro measurements of changes in the diel sensitivity of PEPCase to malate inhibition. From this we can infer that there are significant differences in the timing of enzyme activation/inactivation between these two species.

Materials and methods

Plant material and growth conditions. Rooted cuttings of *Clusia minor* L. were potted in commercial compost in 127-mm-diameter pots and grown up in a plant growth chamber (see below) for at least 3 months prior to experimentation. All measurements were conducted on the third leaf pair from the growing tip. Plants of *Kalanchoë daigremontiana* Hamet et Perr., which were approximately one year old and growing in 100-mm-diameter pots, were acclimated in the growth chamber for at least 2–3 months prior to experimentation. All measurements were conducted on the fourth leaf pair from the growing tip. All plants received nutrients on a weekly basis in the form of a modified Hoaglands solution.

The plants were maintained in a Fitotron growth chamber (Sanyo Gallenkamp, Leicester, UK) which was programmed to provide gradual changes in temperature, humidity and PFD at the start and end of the photoperiod in an attempt to mimic conditions in the field. From 08:30 hours until 12:00 hours, PFD increased to a maximum of 530 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at leaf height, temperature increased from 19 °C to 27 °C and relative humidity decreased from 80% to 60% (vapour pressure deficit, VPD, increased from 1.8 to 2.9 kPa). These conditions were maintained until 16:00 hours when PFD decreased gradually until lights-off at 19:30 hours, temperature decreased to 19 °C and relative humidity increased to 80% (VPD = 1.8 kPa). Over the 13-h dark period, temperature (19 °C) and relative humidity (80%) remained constant.

Manipulation of CAM. As a means of inhibiting C_4 carboxylation at night, individual leaves of both species were enclosed in an atmosphere of N_2 overnight thereby preventing access to external, and inhibiting the release of internal (respiratory), sources of CO_2. Control leaves were exposed to the ambient atmosphere in the growth chamber.

Gas exchange and instantaneous discrimination. Gas-exchange measurements were made continuously on the same leaf over 24–36 h with three separate runs made for each treatment. The leaf was enclosed in a porometer head which tracked the environmental conditions in the growth chamber with gas-exchange parameters measured using an open infra-red gas-exchange system (H. Walz, Effeltrich, Germany) with a Binos gas analyser. Reference air (isotopic signature, $\delta^{13}C$ of approx. −9‰) was supplied from a cylinder of compressed air (British Oxygen Company, Guildford, Surrey, UK). Gas-exchange parameters were calculated using DIAGAS software supplied by Walz.

Carbon dioxide was collected on-line for instantaneous discrimination over 15-min intervals using a gas collection line located adjacent to the gas-exchange system (Roberts et al. 1996). Samples were subsequently purified in a vacuum line to remove N_2O and H_2O. The purified CO_2 was then analysed using an isotope-ratio mass spectrometer (Isospec 44-VG602; modified by Provac Services, Crewe, UK). Instantaneous discrimination (Δ) during gas exchange of leaves, was calculated from the difference in the carbon isotope composition of the air leaving the chamber with (δ_o) and without (δ_e) a leaf enclosed (Evans et al. 1986):

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

where $\xi = p_e/(p_e - p_o)$ and p_e and p_o are the CO_2 partial pressures of the air entering and leaving the chamber, respectively, when a leaf is enclosed. Collections of CO_2 were only made when depletion of CO_2 in the leaf cuvette was ≥ 20 $\mu l \cdot l^{-1}$. At lower depletions the accuracy of discrimination measurements is reduced since a shift of only 0.1‰ in $\delta_o - \delta_e$ corresponds to a shift in Δ of 1.5‰. In order to calibrate the reference CO_2 (δ_e), CO_2 was collected from the cylinder of compressed air over 15-min intervals at various times

throughout the diel cycle for the analysis of the carbon-isotope composition.

These direct measurements of discrimination (Δ) were compared with values of Δ predicted for C_3 and C_4 carboxylation using a simple formulation derived in Farquhar et al. (1989) whereby:

$$\Delta = a + (b - a)c_i/c_a$$

where a is the isotopic fractionation occurring due to diffusion in air (4.4‰), b is the net isotopic fractionation resulting from carboxylation (27‰, discrimination by Rubisco or −5.7‰, discrimination by PEPCase) and c_i, c_a are the internal and ambient concentrations of CO_2 measured during gas exchange. It should be noted that the model sets the limits of Δ using diffusion (alone) and carboxylation as the constraints. The theoretical range for PEPCase therefore extends from −6 to +4‰ (equivalent to $\delta^{13}C$ vs. PDB of −2 to −12‰), with the working range for C_3 plants usually extending from 16 to 22‰ (equivalent to $\delta^{13}C$ vs. PDB of −24 to −30‰).

Organic acid determination. Discs were punched from leaves of both species at intervals over the diel cycle and immediately plunged into hot (80 °C) methanol (80‰ v/v). The methanol extracts were heated for 1 h at 70 °C before being evaporated to dryness, taken up in distilled water and neutralised using K_2CO_3. The concentrations of malate and citrate in this extract were determined enzymatically using the methods of Hohorst (1965) and Mollering (1985).

Quantification of C_3 and C_4 carboxylation in vivo. There was close agreement between measured Δ and that predicted for C_4 carboxylation at night and between measured Δ and that predicted for C_3 carboxylation over the middle part of the day (see *Results*). At other times of the day (i.e. Phase II and IV) the relative amounts of CO_2 taken up by PEPCase and Rubisco were estimated by calculating the extent to which measured discrimination deviated from discrimination predicted for C_3 and C_4 carboxylation. Thus:

$$X \text{ (predicted } C_3\ \Delta) + Y \text{ (predicted} C_4\ \Delta) = \text{measured } \Delta$$

where X = % of C_3-mediated CO_2 uptake, Y = % of C_4-mediated CO_2 uptake, and X + Y = 100%. The percentage of C_4 carboxylation was thus obtained for various time points throughout the day and was converted to μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ from the gas-exchange curves to give an estimate of PEPCase activity in vivo during the photoperiod.

The net amount of CO_2 taken up directly via C_3 carboxylation was estimated by converting the percentage of C_3 carboxylation at various time points to μmol $CO_2 \cdot m^{-2} \cdot s^{-1}$ from the gas-exchange curves.

The estimated activity of Rubisco in vivo was obtained by adding the amount of C_3-mediated CO_2 uptake (see above) to the rate at which CO_2 was released from organic acid decarboxylation assuming that 1 mole malate → 1 mole CO_2 and 1 mole citrate → 3 moles CO_2 (Borland et al. 1994).

Sensitivity of PEPCase to malate inhibition in vitro. The extraction and assay of PEPCase was based on the method described by Nimmo et al. 1984. Leaves were homogenised in extraction buffer [200 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1 mM dithiothreitol (DTT); 2% (w/v) polyethylene glycol 20 000] with 60 mg sodium bicarbonate. The homogenate was filtered through three layers of muslin and centrifuged for 2 min at 13 000 · *g*. The extract was then desalted into 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM DTT using columns of Sephadex G25. All steps were carried out at 4 °C and the extraction was completed within 5 min. The activity of PEPCase was assayed and its K_i for malate estimated using different malate concentrations in an assay mix containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM NADH, 10 mM $NaHCO_3$, 2 mM phosphoenolpyruvate. The assay was initiated by the addition of 50 μl of extract.

Results

Photosynthetic characteristics of Clusia minor and Kalanchoë daigremontiana. For the C_3-CAM intermediate *C. minor*, approx. 15% of daily net CO_2 uptake occurred at night (Fig. 1a). Inhibiting CAM at night by enclosing leaves in an atmosphere of N_2 resulted in a substantial increase in rates of CO_2 uptake during Phases II and III but over the subsequent dark period net CO_2 uptake was almost completely abolished.

In the constitutive CAM plant *K. daigremontiana*, approx. 60% of daily net CO_2 uptake occurred at night (Fig. 1b). Inhibition of CAM at night resulted in a substantial stimulation in the magnitude and duration of Phase II. Whilst control leaves of *K. daigremontiana* showed net release of CO_2 for 3 h over the middle part of the photoperiod, in the N_2-treated leaves Phase III was considerably shortened with negligible net release of CO_2. However, in N_2-treated leaves, rates of net CO_2 uptake during Phase IV and the subsequent dark period were lower than those recorded in control leaves.

The contribution from C_4 carboxylation to the net assimilatory capacity of both species was qualitatively assessed by comparing instantaneous measurements of

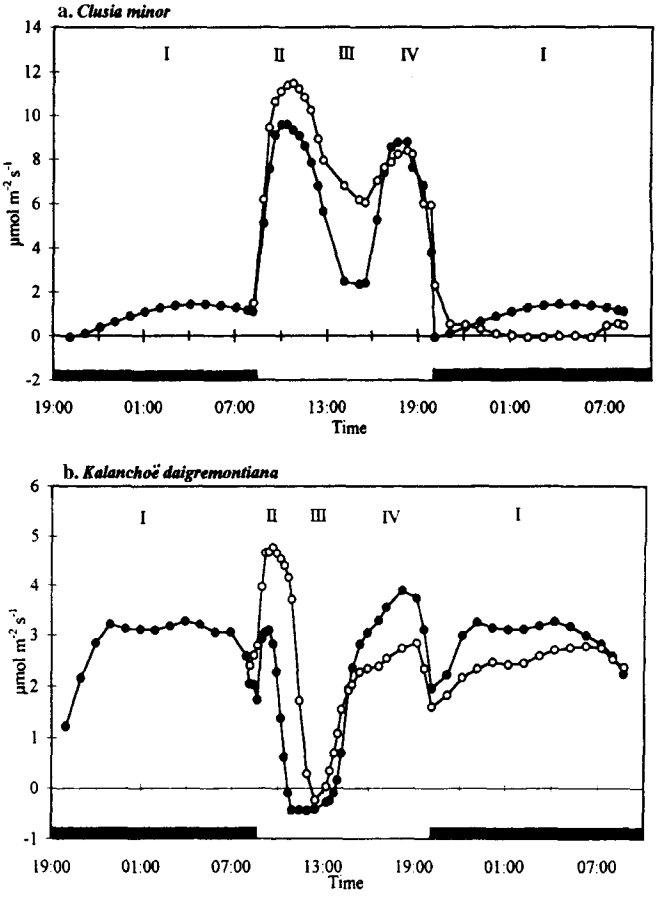

Fig. 1a,b. Rates of net CO_2 assimilation in control (●) or N_2-treated (○) leaves of *C. minor* (**a**) and *K. daigremontiana* (**b**) with the phases of CAM indicated. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM. The *solid bar* on the x-axis represents the period of darkness. Each gas exchange curve is representative of 3 replicate runs with SE < 10% of mean

carbon isotope discrimination (Δ) with Δ predicted from gas-exchange measurements of c_i/c_a using a C_3 or C_4 model (Figs. 2, 3). The substantial deviations between measured and predicted Δ demonstrate changes in carboxylation pathway rather than changes in stomatal or internal conductance (Borland and Griffiths 1996). For control leaves of *C. minor* (Fig. 2a), measured values of Δ at night corresponded closely with the negative values of Δ predicted for C_4-carboxylation (see *Materials and methods*). During the day however, measured Δ showed a substantial divergence from Δ predicted for C_3 carboxylation for 4 h at the start and 3 h at the end of the photoperiod, indicating that PEPCase was active at these times.

For leaves of *C. minor* in which CAM was prevented by enclosing leaves in N_2 overnight (Fig. 2b), the difference between measured and predicted Δ, which reflects the contribution from C_4 carboxylation, was even more marked over the first few hours of the photoperiod compared with control leaves. Over the latter part of the photoperiod, however, the differences between measured and predicted Δ were comparable in both control and N_2-treated leaves. For control leaves of *K. daigremontiana* (Fig. 3a), discrimination measured at night was close to the theoretical values predicted for PEPCase-mediated CO_2 uptake. However, measured Δ increased rapidly to the value predicted for C_3 carboxylation within 30–40 min of the subsequent photoperiod, directly illustrating the down-regulation of PEPCase. Although it was not possible for technical reasons to directly measure Δ over the middle part of the photoperiod when there was no or little net uptake of CO_2, the deviation between measured and predicted Δ over the latter 2–3 h of the photoperiod indicated an increase in C_4 carboxylation (Fig. 3a). In leaves previously exposed to N_2 overnight (Fig. 3b), C_4 carboxylation was in evidence for the first 2–3 h of the photoperiod.

Organic acids. In control leaves of *C. minor*, approx. 70 mol · m^{-2} malate was accumulated over the dark period, with a further 40 mol · m^{-2} accumulated over the first 4 h of the photoperiod (Fig. 4a). In leaves exposed to N_2 overnight there was a marked stimulation in malate accumulation during Phase II with up to 70 mol · m^{-2} accumulated over the first 4 h of the photoperiod. Net breakdown of malate commenced shortly after 12:00 hours in both treatments. In control leaves of *K. daigremontiana*, 100 mol · m^{-2} malate was accumulated over the dark period and, unlike *C. minor*, net breakdown of malate commenced early in the photoperiod (Fig. 4c). In leaves previously exposed to

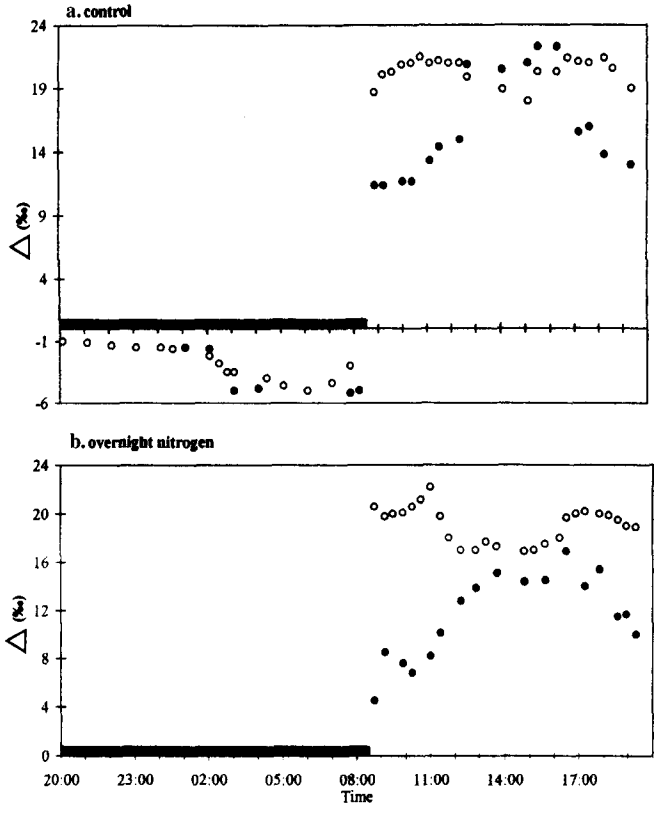

Fig. 2a,b. Comparison of instantaneous discrimination (Δ) measured directly 'on-line' (●) with Δ predicted from gas-exchange measurements of c_i/c_a (○) in control (**a**) or N_2-treated (**b**) leaves of *C. minor* using a C_4 model at night (represented by the *solid bar* on the x-axis) and a C_3 model during the photoperiod. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM. The results presented are representative of 3 replicate runs with SE < 10% mean

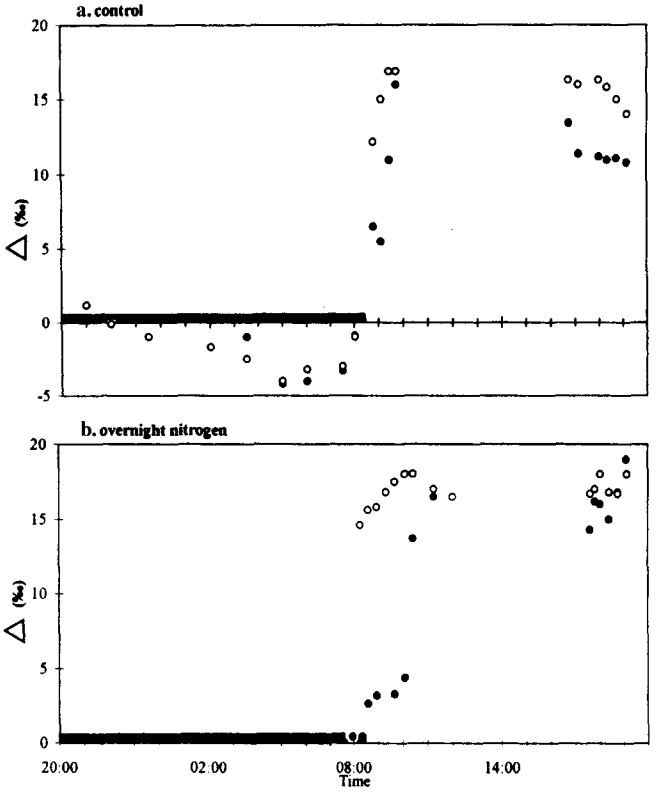

Fig. 3a,b. Comparison of instantaneous discrimination (Δ) measured directly 'on-line' (●) with Δ predicted from gas-exchange measurements of c_i/c_a (○) in control (**a**) or N_2-treated (**b**) leaves of *K. daigremontiana* using a C_4 model at night (represented by the *solid bar* on the x-axis) and a C_3 model during the photoperiod. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM. The results presented are representative of 3 replicate runs with SE < 10% mean

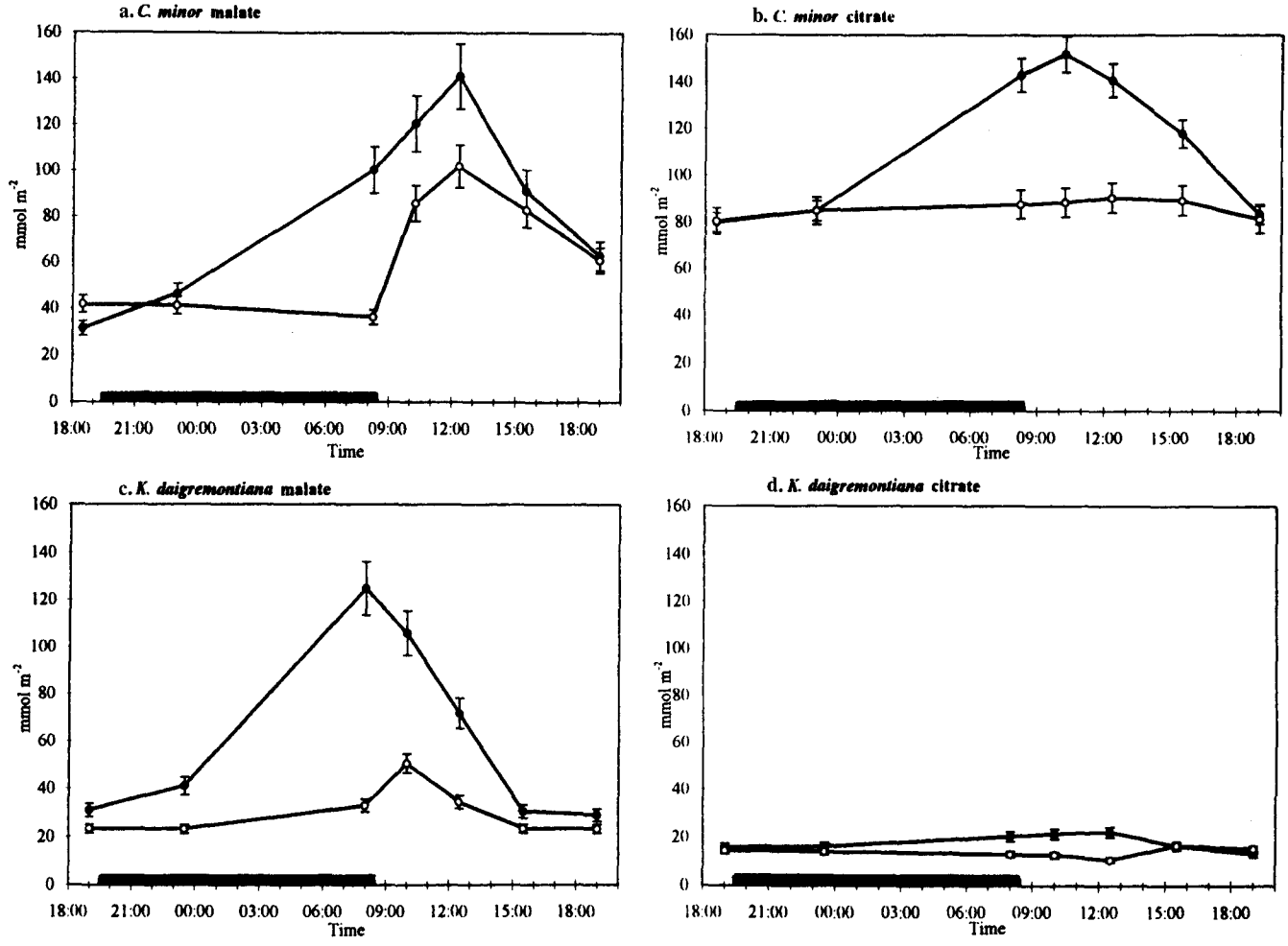

Fig. 4a–d. Changes in malate (**a, c**) and citrate (**b, d**) contents in control (●) and N_2-treated (○) leaves of *C. minor* (**a, b**) and *K. daigremontiana* (**c, d**) over a 24-h dark/light cycle. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM. The *solid bar* on the x-axis represents the period of darkness and each point is the mean of 4 replicates ± SE

N_2 however, there was a net accumulation of approx. 20 mol · m^{-2} malate over the first 2 h of the photoperiod before net breakdown commenced.

Approximately 70 mol · m^{-2} citrate was accumulated over the dark and initial part of the photoperiod in control leaves of *C. minor* with net breakdown commencing shortly after 10:00 hours (Fig. 4b). There was no net accumulation/depletion of citrate in leaves exposed to N_2 overnight. In *K. daigremontiana* there was negligible accumulation/depletion of citrate and background levels were approx. fivefold lower than those in *C. minor* (Fig. 4d).

Quantification of C_3 and C_4 carboxylation in vivo. From the qualitative measurements of instantaneous discrimination (Figs. 2, 3), it is apparent that both C_3 and C_4 carboxylation are operating simultaneously for varying periods, depending on species and treatment, at the start and end of the photoperiod. An attempt was made to quantify how much CO_2 uptake (Fig. 1) can be directly attributable to PEPCase, how much external CO_2 was taken up via C_3 carboxylation and, by including organic acid decarboxylation, what was the estimated activity of Rubisco (see *Materials and methods*). For *C. minor* there was a marked stimulation in the estimated PEPCase activity over the first 2 h of the photoperiod with the maximum rate of PEPCase activity in N_2-treated leaves approx. twofold higher than that in control leaves (Fig. 5a). By 13:00 hours there was negligible C_4 carboxylation in either control or N_2-treated leaves but in N_2-treated leaves PEPCase activity was detected at approx. 15:30 hours and increased steadily until the end of the photoperiod. Resumption of PEPCase activity in control leaves was delayed until 17:00 hours but by the end of the photoperiod estimated PEPCase activity was similar in leaves from both treatments (Fig. 5a). In control leaves of *C. minor*, despite the significant PEPCase activity during Phase II, most CO_2 was taken up directly by Rubisco and over the course of the day C_4 carboxylation constituted only approx. 22% of daytime net CO_2 uptake (Fig. 5a, b). After N_2 treatment, comparable amounts of CO_2 were taken up by C_3 and C_4 carboxylation pathways during Phase II but over the course of the day, C_4 carboxylation accounted for only 33% of daytime net CO_2 uptake. Estimated Rubisco activity was higher in control leaves than in N_2-treated leaves, mainly as a result of the increased availability of CO_2 from organic acid decarboxylation (particularly citrate) in control leaves (Fig. 4a,b).

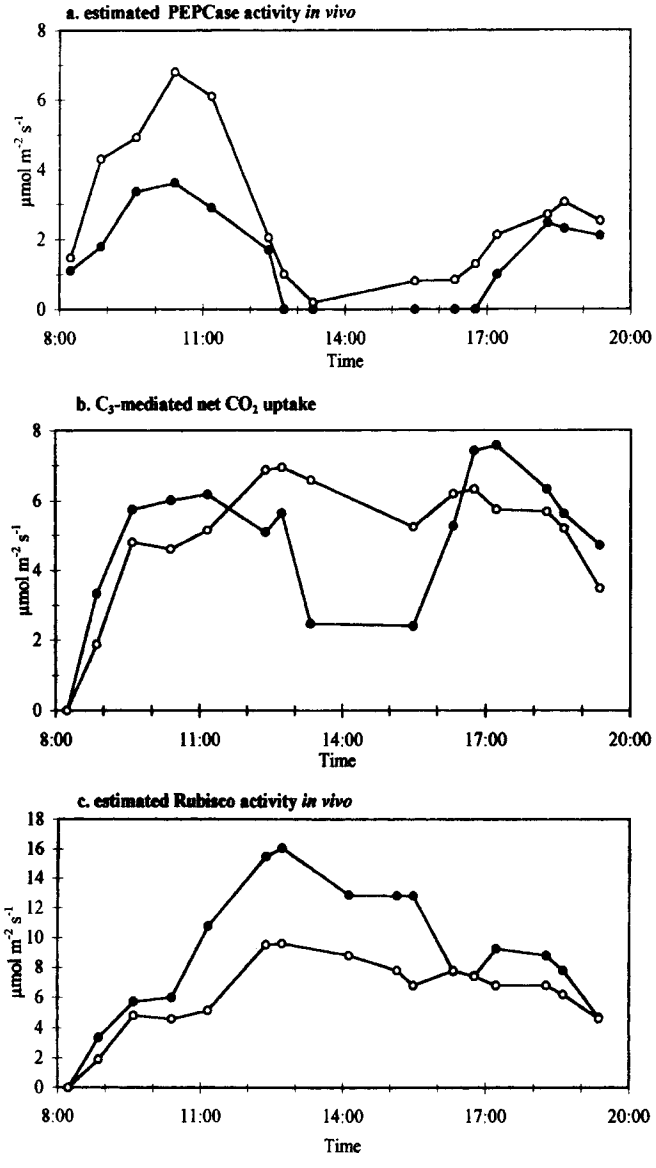

Fig. 5a,b. Estimates of the amounts of external CO_2 taken up by PEPCase (**a**) and C_3 carboxylation (**b**) during the day in control (●) and N_2-treated (○) leaves of *C. minor*. Estimates of Rubisco activity in vivo (**c**) are the sum of C_3-mediated CO_2 uptake (**b**) and the rates of organic acid decarboxylation. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM

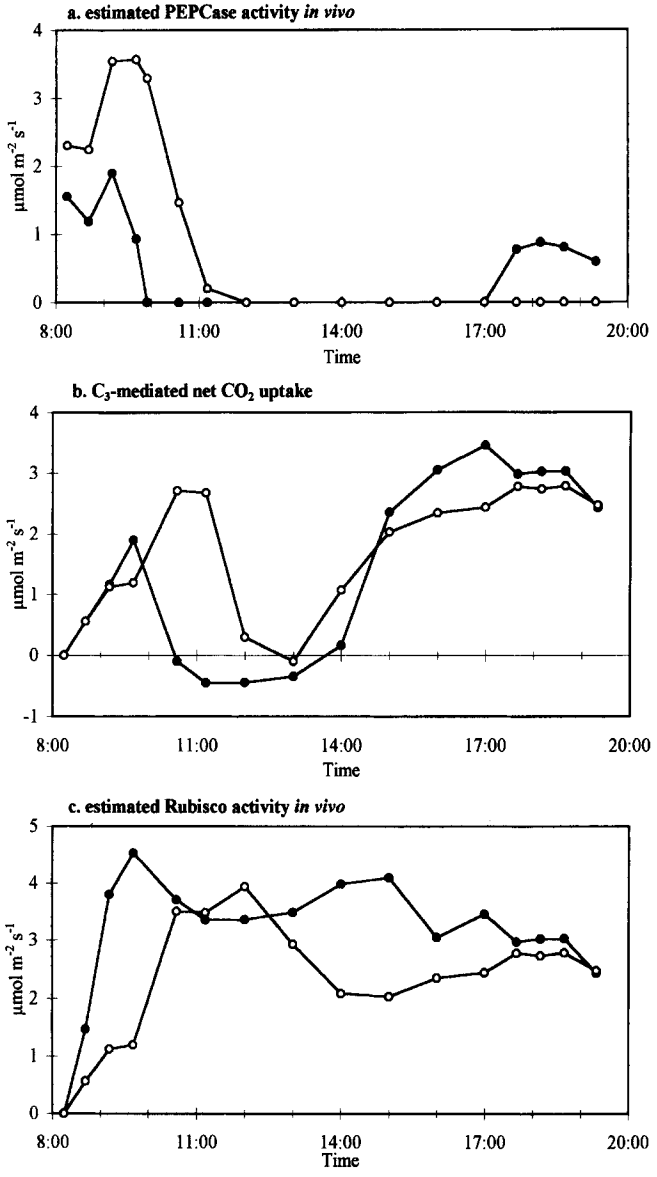

Fig. 6a,b. Estimates of the amounts of external CO_2 taken up by PEPCase (**a**) and C_3 carboxylation (**b**) during the day in control (●) and N_2-treated (○) leaves of *K. daigremontiana*. Estimates of Rubisco activity in vivo (**c**) are the sum of C_3-mediated CO_2 uptake (**b**) and the rates of organic acid decarboxylation. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM

In *K. daigremontiana,* PEPCase activity reached a maximum approx. 30 min into the photoperiod with the maximum rate of activity in N_2-treated leaves some 1.5-fold higher than in controls (Fig. 6a). In control leaves comparable amounts of CO_2 were taken up via C_3 and C_4 carboxylation during Phase II (Fig. 6a, b) but over the course of the day C_4 carboxylation accounted for only 20% of net CO_2 uptake. After a night in N_2, the contribution from daytime C_4 carboxylation had increased to approx. 28% of net daytime CO_2 uptake. The higher Rubisco activity in control leaves over the second half of the photoperiod compared with N_2-treated leaves was again mainly due to increased CO_2 supply from malate decarboxylation.

Activity of PEPCase in vitro. Phosphoenolpyruvate carboxylase was extracted from control and N_2-treated leaves of both species at intervals over the day/night cycle. Diel changes in the regulatory properties of the enzyme were demonstrated via measurements of the K_i malate (i.e. [malate] which inhibits V_{max} by 50%). The activity of PEPCase from *C. minor* remained relatively insensitive to malate inhibition until 12:00 hours when there was a fivefold increase in malate sensitivity (Fig. 7a) coinciding with the commencement of net malate breakdown (Fig. 4a). In contrast, PEPCase from *K. daigremontiana* became approx. tenfold more sensitive to malate inhibition within the first 30 min of the photoperiod. However, after N_2-treatment, during

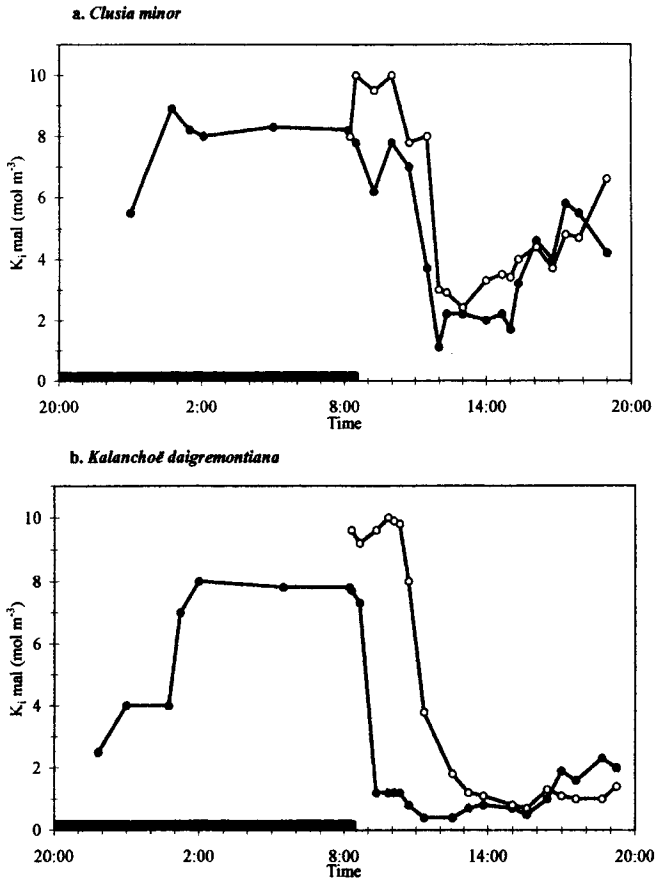

Fig. 7. Diel changes in K_i malate for extracts of PEPCase prepared from control (●) or N_2-treated (○) leaves of *C. minor* (**a**) and *K. daigremontiana* (**b**) where K_i malate is [malate] which inhibits V_{max} by 50%. Leaves of N_2-treated plants were enclosed overnight in an N_2 atmosphere to prevent CAM

Phase II PEPCase was less sensitive to malate inhibition compared with the enzyme extracted from control leaves of both species. Moreover, this malate insensitivity persisted for the first 4 h of the photoperiod in both species. In *C. minor* the sensitivity to malate inhibition persisted for only 3–4 h over the middle part of the photoperiod and, later in the day, PEPCase extracted from control and N_2-treated leaves of this species was less sensitive to malate inhibition than that of *K. daigremontiana*.

Futile cycling and carbon balance. The estimates of PEPCase activity in vivo were integrated to give values for the total amount of CO_2 fixed via C_4 carboxylation during Phases II and IV. This was compared with changes in malate concentration over the same periods (Table 1). Citrate was omitted from these calculations since day/night changes in the concentration of this organic acid are unlikely to result in net CO_2 exchange (Lüttge 1988). During Phase II, the net accumulation of malate by both control and N_2-treated leaves compared very closely with the integrated activity of PEPCase over this period. Thus it would appear that there was negligible futile cycling of malate accumulation/decarboxylation at this time. However, during Phase IV the estimates of net CO_2 uptake via PEPCase were in excess of the net accumulation of malate over this period, indicating that some futile cycling of malate was occurring. In leaves of *K. daigremontiana*, there was an indication of only limited futile cycling during Phases II and IV.

In terms of carbon balance over the entire 24-h diel cycle, during the dark (Phase I) control leaves of *C. minor* accumulated approx. 1.6-fold more malate than could be accounted for by net CO_2 uptake (Table 2). Thus approx. 40% of malate that accumulated overnight was derived from the refixation of respiratory CO_2. This was effectively prevented by enclosing leaves in an atmosphere of N_2 overnight. However, due to the increase in net CO_2 uptake over the subsequent light phases, over the 24-h dark-light cycle, leaves from both treatments took up similar amounts of CO_2. If carbon balance is calculated for the light period and night following the N_2 treatment, comparable amounts of CO_2 were taken up and transpiratory water loss was also very similar in leaves from both treatments.

In control leaves of *K. daigremontiana* the amount of malate which accumulated overnight corresponded closely with the net uptake of CO_2 during Phase I, indicating that respiratory recycling played a negligible role in terms of overall carbon balance in this species. During the subsequent photoperiod, despite a 25% increase in net CO_2 uptake in N_2-treated leaves, over the 24-h dark-light period control leaves of *K. daigremontiana* took up almost twice as much CO_2 as the leaves in which CAM had been prevented overnight (Table 2). However if net CO_2 uptake is calculated over the light period and subsequent dark period, then the

Table 1. The contribution from estimated PEPCase activity in vivo to daytime carbon balance in leaves of *C. minor* and *K. daigremontiana*. The magnitude of futile cycling is calculated as the difference between CO_2 fixed via PEPCase and malate accumulation

	CO_2 fixed or recycled (mmol · m^{-2})					
	Phase II			Phase IV		
	PEPCase	Malate	Futile cycling	PEPCase	Malate	Futile cycling
Clusia minor						
Control	40.2	40.1	0	16.0	0	16.0
+ N_2	74.1	76.0	1.9	26.7	20	6.7
Kalanchoë daigremontiana						
Control	5.6	0	5.6	5.6	0	5.6
+ N_2	22.0	20	2.0	0	0	0

Table 2. Carbon and water balance in leaves of *C. minor* and *K. daigremontiana* ± N_2 overnight. Net uptake of CO_2 and malate accumulation at night (Phase I) are shown alongside net amounts of CO_2 taken up over the subsequent day (Phases II–IV). The implications for diel carbon balance are summarised by (i) calculating total net CO_2 uptake over the night (± N_2) and the subsequent day and (ii) total net CO_2 uptake over the day plus the subsequent night. The amount of H_2O lost over the 24-h light-dark cycle was calculated by integrating transpiration rates (not shown) over this time

	CO_2 fixed or malate accumulated (mmol · m^{-2})					H_2O loss (mol · m^{-2})
	Phase I		Phases II–IV	(i) 24 h	(ii) 24 h	24 h
	Net CO_2	Malate		(dark-light)	(light-dark)	(light-dark)
Clusia minor						
Control	42.1	69.5	258	327.5	328	48.7
+ N_2	0	0	338	338.0	340	49.4
Kalanchoë daigremontiana						
Control	104.4	100.0	80	184.4	209	15.2
+ N_2	0	0	100	100.0	206	18.8

leaves from both treatments took up similar amounts of CO_2 and lost comparable amounts of H_2O (i.e. full recovery and no evidence of N_2-induced narcosis).

Discussion

All chloroplast-containing cells of CAM plants contain high activities of PEPCase, Rubisco and decarboxylase enzymes. Clearly, tight regulation of these activities is essential to achieve the temporal separation of C_3 and C_4 carboxylation processes which distinguishes CAM from C_4 photosynthesis. In the present study, measurements of diel changes in carbon-isotope discrimination and organic acid content were used to quantify the activities of C_3 and C_4 carboxylases in vivo and to indicate the extent to which the activities of PEPCase, Rubisco and decarboxylation processes overlap at the start and end of the photoperiod.

A major finding to emerge from this approach concerns fundamental differences in the diel regulation of flux through PEPCase in the constitutive CAM plant *K. daigremontiana* compared with the C_3-CAM intermediate *C. minor*. Thus, despite the gradual changes in PFD, temperature and humidity at the start and end of the photoperiod in the growth chamber, both in-vivo and in-vitro measurements demonstrated that PEPCase in *K. daigremontiana* showed the conventional rapid down-regulation within the first hour of the photoperiod (Nimmo et al.1984), whilst in *C. minor* PEPCase activity continued for approx. 4 h into the photoperiod. Such observations support previous studies of *C. minor* growing in the field (Borland et al. 1993). The diel changes in the malate sensitivity of PEPCase demonstrated for both species, are achieved via reversible phosphorylation of the enzyme (Nimmo et al. 1984, 1986). It is now widely accepted that a Ca^{2+}-independant protein kinase is activated at night and that this activation is responsible for the post-translational modification of PEPCase (Carter et al. 1991, 1996). The activity of the PEPCase kinase is in turn regulated by a circadian clock at the level of translatable mRNA (Hartwell et al. 1996). At the start of, the photoperiod it is likely that PEPCase kinase is switched off' in *K. daigremontiana* by high concentrations of malate in the cytosol (Carter et al. 1996). The extended C_4 carboxylation during Phase II in *C. minor* might therefore suggest that, unlike in *K. daigremontiana*, the storage capacity of the vacuole has not been exceeded. However, in leaves exposed to N_2 overnight, the subsequent effects on daytime C_4 carboxylation are markedly different in the constitutive CAM plant and C_3-CAM intermediate. Thus, in *K. daigremontiana* whilst the amplitude and duration of PEPCase activity during Phase II was substantially increased, in *C. minor* only the amplitude of PEPCase activity increased (approx. twofold) in leaves treated with N_2. Moreover, the kinase in N_2-treated leaves of *K. daigremontiana* appears to be switched off at a time when the amount of malate accumulated is only 30% of that accumulated under control conditions or in N_2-treated leaves of *C. minor*. Clearly, the partitioning of malate between vacuole and cytosol will be important in regulating flux through PEPCase and it is interesting to note that the down-regulation of PEPCase in N_2-treated leaves of both species occurs at a time when temperature in the growth cabinet reaches a maximum (i.e. 27 °C). Previous studies have demonstrated that high temperature enhances malate efflux from the vacuole (Kluge and Schomburg 1996). Additionally, temperature controls the amount of PEPCase kinase-translatable mRNA and the circadian disappearance of kinase activity can be accelerated by high temperature (Carter et al. 1995; Hartwell et al. 1996). Whilst the factors governing the down-regulation of PEPCase are complex, the two species also show clear differences in the extent to which PEPCase is reactivated during the latter part of the photoperiod. Thus, whilst the reactivation of PEPCase during Phase IV in *K. daigremontiana* was inhibited by previous exposure to N_2, in *C. minor* the reactivation of PEPCase was advanced by 2–3 h compared with controls. The results presented here indicate that *C. minor* and *K. daigremontiana* show clear differences in the timing of PEPCase activation/inactivation under control conditions and after overnight exposure to N_2. Whether this is due to differences in properties of the vacuole (e.g. storage capacity, tonoplast fluidity) and/or differences in the regulation of PEPCase kinase between the species

remains to be determined. However, the inhibition of C_4 carboxylation at night via N_2 provides an excellent experimental system in which the regulation of PEPCase kinase can be investigated, and studies on the molecular and biochemical aspects of this phenomenon are currently in progress.

As discussed, a complex interplay of regulatory processes permits the operation of C_4 carboxylation for substantial periods of time during the day in *C. minor* with up to 4 times more CO_2 taken up by PEPCase during the day in this species than in *K. daigremontiana*. However, net CO_2 uptake during the day was still dominated by C_3 carboxylation in both species and if daytime C_4 carboxylation is expressed as a percentage of net daytime CO_2 uptake then, remarkably, C_4 carboxylation accounted for approx. 20% of net daytime assimilation in *both* species under control conditions, increasing to 30–34% after a night in N_2. Thus, in *C. minor*, the enhanced capacity for C_4 carboxylation over *K. daigremontiana* was also matched by a greater capacity for C_3 carboxylation. In plants like *K. daigremontiana* where the expression of CAM is determined by ontogeny, increases in the amount of PEPCase protein may be accompanied by decreased investment of N in Rubisco protein (Winter et al. 1982). For a C_3-CAM intermediate like *C. minor*, however, it may be beneficial to maintain investment in Rubisco protein in order that C_3 carboxylation can maximise net CO_2 uptake when conditions permit (Borland 1996).

The amount of external CO_2 fixed directly via C_3 carboxylation during the CAM cycle will largely depend on the timing of stomatal opening/closure. Under well-watered conditions, changes in stomatal conductance in CAM plants are generally determined by the generation and consumption of CO_2. In the conventional four-phase model for CAM, the commencement of malate efflux from the vacuole early in Phase II effectively inhibits C_4 carboxylation whilst decarboxylation releases CO_2, thereby increasing the internal concentration of CO_2 (c_i) with resulting stomatal closure (Kluge et al. 1981). In some CAM plants the internal concentration of CO_2 during Phase III may increase to such an extent (up to 4% v/v has been reported; Cockburn et al. 1979) that some CO_2 may leak out of the leaf at this time (Friemert et al. 1986), as seen here for control leaves of *K. daigremontiana*. In N_2-treated leaves of this species, the length of time that stomata remained closed during Phase III was considerably reduced with negligible leakage of CO_2, probably as a result of the marked reduction of internal CO_2 generated from malate decarboxylation. Despite the fact that N_2-treated leaves of *K. daigremontiana* took up 25% more CO_2 during the day than control leaves, this was insufficient to compensate the N_2-treated leaves for the loss of CAM the previous night. However if carbon balance is calculated for the light period and *following* dark period then the increase in daytime PEPCase activity and, to a lesser extent, C_3 carboxylation in N_2-treated leaves meant that 24 h carbon gain and water loss recovered to a remarkably similar level to that noted for control leaves of this constitutive CAM plant.

In *C. minor*, stomata did not close completely during Phase III, suggesting that c_i was maintained at a lower level than that in *K. daigremontiana* despite the potential generation of CO_2 from organic acid decarboxylation being some 3–4 times higher in *C. minor*, largely as a result of citrate breakdown (i.e. assuming 1 citrate $\rightarrow$ 3 CO_2). The resulting estimates of Rubisco activity in vivo, which were up to 4 times higher in *C. minor* than in *K. daigremontiana*, suggest that Rubisco capacity was sufficiently high in the C_3-CAM intermediate to prevent a large increase in c_i during Phase III (Borland and Griffiths 1996; Borland 1996). In N_2-treated leaves of *C. minor*, the reduction in the amount of CO_2 generated from organic acid decarboxylation (particularly citrate) would tend to reduce c_i compared with the control leaves. The resulting increase in net CO_2 uptake during Phase III, together with the twofold increase in daytime PEPCase activity, meant that, unlike *K. daigremontiana*, leaves of *C. minor* exposed to N_2 were able to compensate for the inhibition of C_4 carboxylation at night in terms of diel carbon balance. It has been shown previously that the magnitude of CO_2 uptake at night in *C. uvitana* is inversely related to the magnitude of day-time CO_2 uptake (Winter et al. 1992). Similarly, the increased rates of day-time assimilation in N_2-treated leaves of *C. minor* were accompanied by a significant inhibition of net CO_2 uptake during the subsequent dark period compared with the controls. The signalling processes which modulate these shifts in light:dark CO_2 uptake are still unknown. However, it is possible that increased net CO_2 uptake during the day and the accompanying increase in transpirational water loss in N_2-treated leaves of *C. minor* resulted in a higher water deficit in these leaves at the end of the day compared with controls, with a subsequent reduction in net dark CO_2 uptake.

The timing of malate efflux from the vacuole and its residence time in the cytosol play a pivotal role in determining how shifts in C_3 and C_4 carboxylation contribute to diel carbon balance and to what extent futile cycling of malate synthesis and decarboxylation occurs. Winter and Tenhunen (1982) suggested that the onset of malate efflux from the vacuole and subsequent decarboxylation coincide with the time of maximum CO_2 uptake. However, in the present study, the estimated activity of PEPCase during the 4 h of Phase II compared closely with the amount of malate accumulated over this time in both species under control and N_2 treatments, and it can be concluded that futile cycling is negligible at this time. In *K. daigremontiana*, exposure to N_2 overnight resulted in a 1-h delay in the commencement of decarboxylation compared with control leaves. In *C. minor* it was some 4 h into the photoperiod before decarboxylation commenced in both control and N_2-treated leaves. Thus, in the C_3-CAM intermediate decarboxylation occurs when temperature, light and VPD are at a maximum in the growth chamber. Such a mechanism seems to ensure that generation of internal CO_2 in *C. minor* is maximal during periods when potential photoinhibition and water stress may be maximal (Borland et al. 1993).

Returning to the original hypotheses proposed in the *Introduction*, the results presented here suggest that regulation of carboxylation/decarboxylation processes in *Clusia* is indeed different from that in the model CAM plant *K. daigremontiana*. With a high inherent Rubisco capacity and by increasing daytime PEPCase activity, *C. minor* can compensate for reduced dark CO_2 uptake over the following day. Conversely the constitutive *K. daigremontiana* seems more constrained by the day/night rhythm of CAM and cannot compensate for the loss of night-time C_4 carboxylation over the subsequent day. Mechanisms which coordinate the timing of up/down regulation of C_3/C_4 carboxylation, vacuolar malate flux, decarboxylation and carbohydrate metabolism are now being determined. However, we show here that such processes are tightly regulated within a physiological framework which maximises net CO_2 uptake, conserves water and restricts futile cycling in both species in response to demands imposed by the environment.

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