8 Photosynthesis

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8.1 Photosynthetic Physiotypes

One of the great fascinations of the genus *Clusia* is that its single leaf morphotype (Sect. 2.1) expresses different photosynthetic physiotypes. Often different photosynthetic types are even expressed by the same species and even in clones of vegetatively propagated plants or in different leaves of single individual plants depending on environmental conditions. The photosynthetic types observed among *Clusia*s are based on the modes of C_3-photosynthesis and of crassulacean acid metabolism (CAM) and its variants. Figure 8.1 presents a schematic overview of the basic features of the three to four different photosynthetic physiotypes found among *Clusia*s. Figures 8.2 and 8.3 show typical patterns of photosynthetic CO_2-gas exchange for different modes of photosynthesis expressed among four different species under identical conditions in a phytotron (Fig. 8.2) and for different modes of photosynthesis expressed in one species, *Clusia minor* L., under different conditions (Fig. 8.3), respectively.

In C_3-photosynthesis primary fixation of CO_2 by ribulose-bis-phosphate carboxylase/oxygenase (RubisCO) occurs simultaneously with the energy providing light reactions of photosynthesis. Stomata are open during the light period for CO_2-uptake. In the dark period stomatal opening is much reduced or stomata are closed. Some respiratory CO_2 is released. Expression of C_3-type CO_2 exchange is shown by *Clusia venosa* Jacq. in Fig. 8.2A and by well watered *C. minor* under high irradiance (Fig. 8.3A).

In CAM primary fixation of CO_2 occurs in the dark period and is mediated by phospho*enol*pyruvate carboxylase (PEPC). Stomata are open during the dark period for CO_2 uptake. The CO_2 fixed is stored nocturnally in the cell vacuoles in the form of organic acids (Sects. 8.3.2 and 8.3.3). This is called phase I of CAM (Osmond 1978). Organic acids are remobilized from the vacuoles, decarboxylated and the CO_2 regenerated is refixed and assimilated via RubisCO and the Calvin cycle in the light period. This is called phase III of

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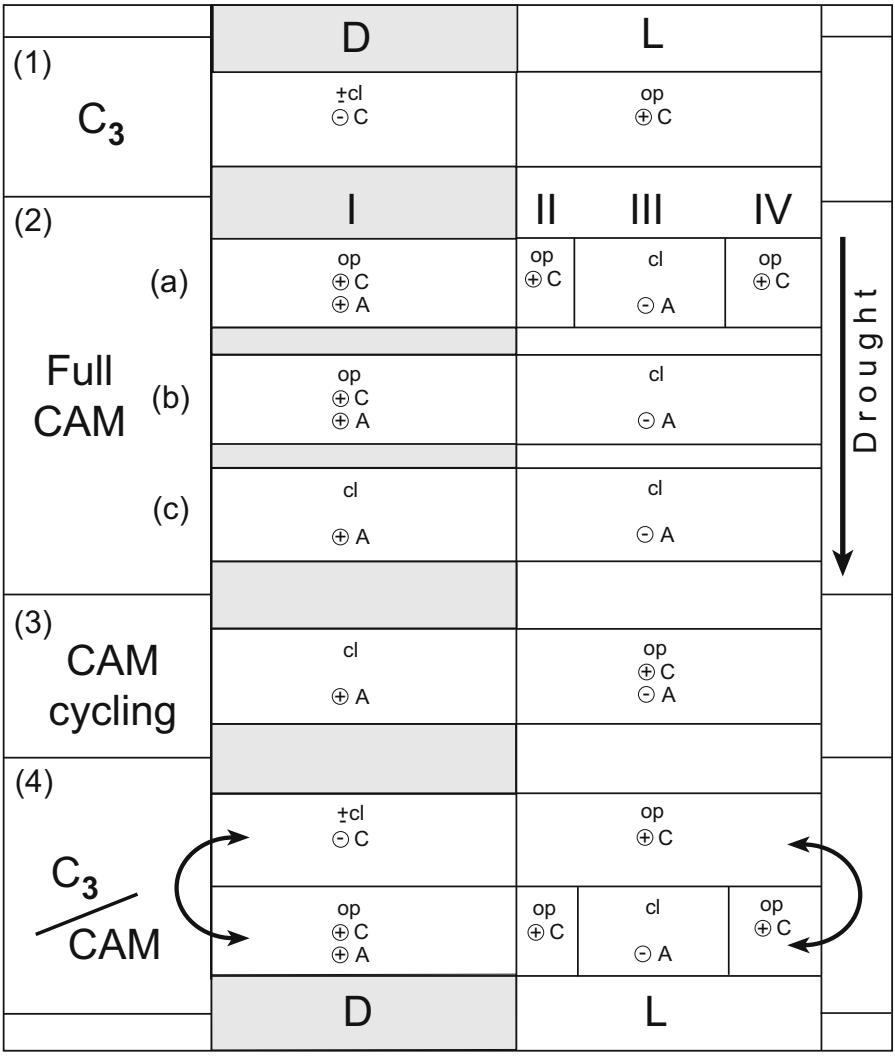

Fig. 8.1. Schematic overview of the photosynthetic physiotypes found among *Clusia* species. **(1)** C_3-photosynthesis; **(2)** full CAM, **(a)** with the four CAM-phases I to IV, **(b)** with phases II and IV suppressed, and **(c)** with CAM-idling as drought increases; **(3)** CAM-cycling; **(4)** C_3/CAM intermediate behaviour. D=dark period, L=light period, cl or op=stomata closed or open, C=net CO_2-exchange (+=uptake, -=release), A net change of organic acid levels (+=accumulation, -=remobilisation), roman numbers=CAM phases

CAM during which stomata remain closed. Since stomata are closed in phase III, its activity cannot be studied by measurements of gas exchange. It can be measured, however, by analyses of declining levels of organic acids, which requires destructive leaf-tissue sampling. In addition phase III activity can be assessed experimentally by non-destructive measurements of chlorophyll flu-

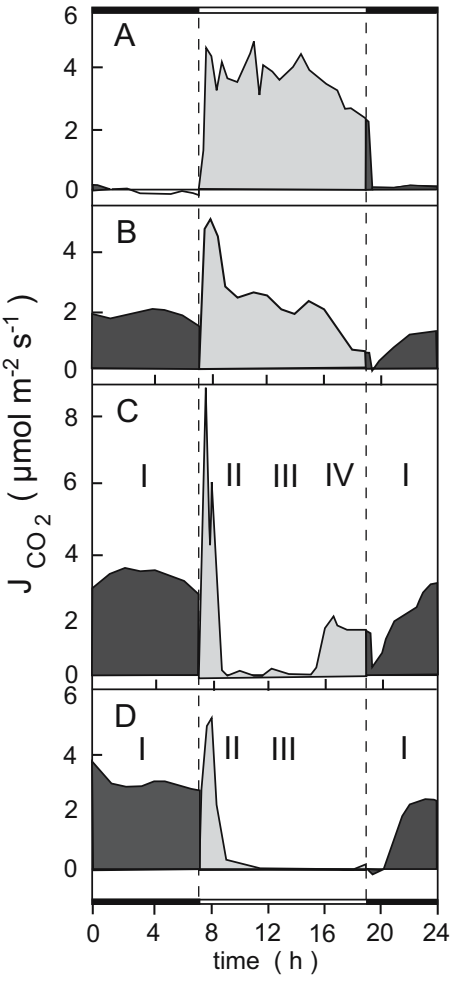

Fig. 8.2A-D. Photosynthetic types expressed by four different species of *Clusia* kept under identical conditions in a phytotron. Net CO_2-exchange, J_{CO2}, of: **A** C_3-photosynthesis: *C. venosa*; **B** CO_2-uptake around the clock: *C. minor*; **C** CAM: *C. major* with phases I to IV; **D** CAM with suppressed phase IV: *C. alata*. *Dark bars* on the abscissa indicate dark periods, *Roman numbers* refer to CAM phases explained in the text (from Lüttge 1991, after data of Franco et al. 1990)

orescence parameters from which apparent photosynthetic electron transport rate, ETR, can be deduced. ETR is determined as

$$ETR = 0.5 \times 0.86 \times PPFD \times (F_m'-F)/F_m' \quad (8.1)$$

(Genty et al. 1990), where F is the ground fluorescence and F_m' the maximum fluorescence of chlorophyll *a* of photosystem II of a light adapted leaf, and

$$\Delta F/F_m' = (F_m'-F)/F_m' \quad (8.2)$$

is effective quantum yield; PPFD is photosynthetic photon flux density; the factor 0.5 accounts for equal distribution of irradiance energy between photosystems II and I, and the factor 0.86 accounts for an estimated average reflec-

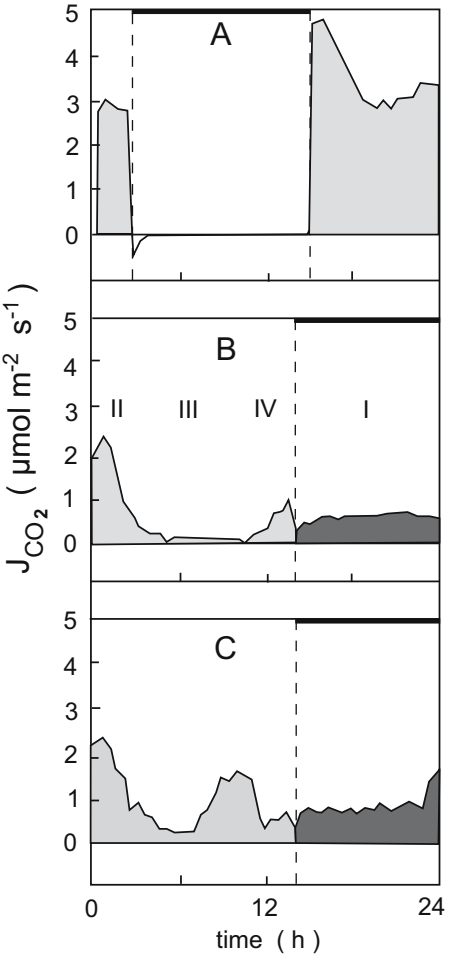

Fig. 8.3A–C. Three different photosynthetic types (net CO_2-exchange, J_{CO2}) expressed by *C. minor* under different conditions in a phytotron: **A** C_3-photosynthesis under well watered conditions with a PPFD of 1700 µmol photons m^{-2} s^{-1} and a leaf/atmosphere VPD of 6.6 mbar bar^{-1}; **B** CAM under drought stress with a PPFD of 400 µmol m^{-2} s^{-1} and a VPD of 13.5 mbar bar^{-1}; **C** CO_2 uptake around the clock under well watered conditions with a PPFD of 400 µmol m^{-2} $s^{-1}$and a VPD of 3.4 mbar bar^{-1}. *Dark bars* on the abscissa indicate dark periods, *Roman numbers* refer to CAM phases. PPFD=photosynthetic photon flux density, VPD=vapour pressure difference. (From Lüttge 1991, after data of Lee et al. 1989, where *C. minor* is wrongly called *C. rosea*)

tion of PPFD of 14 %. In Fig 8.4 the ETR is plotted vs decarboxylation rates calculated from the decline of malate levels at various times during phase III. This example is from *C. minor* growing in the shade of a deciduous dry forest in Venezuela in the dry season and performing weak CAM. The linear correlation between ETR and decarboxylation shows that reduction of CO_2 derived from malate remobilization drives photosynthetic electron transport in phase III.

In a transition phase between phases I and III in the early morning (phase II) stomata are still open and both PEPC and RubisCO may contribute to CO_2 fixation in *Clusia*s in this phase. It appeared particularly in the obligate CAM species *Clusia fluminensis* Planch. et Triana that both carboxylases were active in phase II, while in the C_3/CAM-intermediate species *C. minor* RubisCO made a greater contribution to CO_2-fixation in phase II than PEPC

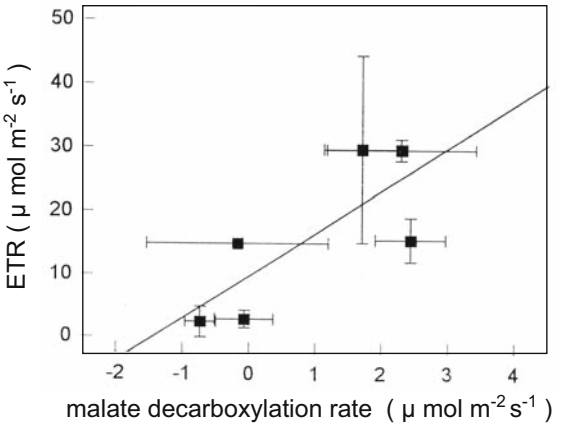

Fig. 8.4. Correlation of apparent electron transport rate of photosystem II, ETR, and internal CO_2 production by decarboxylation of malate in *C. minor* in a semideciduous dry forest in the field in Venezuela in the dry period (Grams et al. 1997)

(Roberts et al. 1997). In most CAM plants phase II is rather short in the early light period and perhaps it is really not much more than a transition phase between the dark period and the light period, where PEPC is gradually down-regulated and RubisCO is activated. However, in some *Clusia* species it has been observed that phase II can be much more extended. In *Clusia rosea* Jacq. in the field on St. John Island, Lesser Antilles, an extension of phase II to almost mid-day was observed (Fig. 8.5). The phenomenon has been studied in *Clusia* in more detail by A. Borland, H. Griffiths and their collaborators. In a comparison with the CAM-plant *Kalanchoë daigremontiana* Hamet et Perrier, in the C_3/CAM-intermediate *C. minor* phase II was more extended and PEPC was much more slowly down-regulated, i.e., it remained active for 4 h after the start of the light period in contrast to *K. daigremontiana* where PEPC was inactivated within the first 30 min (Borland et al. 1993; Borland and Griffiths 1997; Roberts et al. 1998). Roberts et al. (1997) suggest that the extended phase II may have important functions. Extended activation of PEPC in phase II increases overall organic acid accumulation and thus, increases the amount of CO_2 available from organic acid remobilization later in the light period when external conditions such as temperature and irradiance are maximal at midday. Thus, the extended phase II that is typical of several *Clusia* species may have a cardinal role in terms of photosynthetic efficiency.

In the afternoon, when the nocturnally accumulated organic acids are largely consumed depending on conditions, stomata may open and CO_2 is taken up from the atmosphere and fixed directly by RubisCO and assimilated via the Calvin cycle (Phase IV).

Expression of this typical CO_2-exchange pattern of CAM with the four phases is shown by *Clusia major* L. (Fig. 8.2C) and by *C. minor* under drought stress and at medium irradiance (Fig. 8.3B).

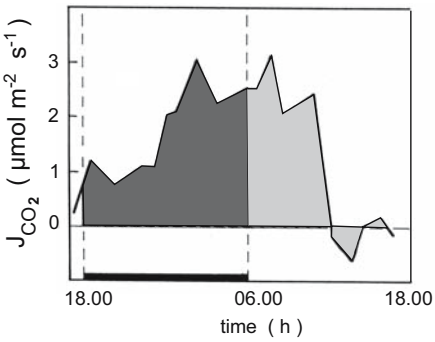

Fig. 8.5. Extended phase II of CAM (net CO_2-exchange, J_{CO2}) observed in *C. rosea* in the field on St. John Island, Lesser Antilles. The *dark bar* on the abscissa indicates dark period (from Lüttge 1991, after data of Ball et al. 1991)

Full CAM with its four phases can be modified particularly in response to drought (Smith and Lüttge 1985). As drought increases, phases II and IV start to be suppressed. A CAM CO_2 exchange pattern without expression of phase IV is shown by *Clusia alata* Planch. et Triana in Fig. 8.2D. With more severe drought, stomatal opening and CO_2 uptake may also be reduced in the dark period (phase I). This may culminate in total stomatal closure night and day. In this condition respiratory CO_2 is recycled via PEPC, organic acids are accumulated in the dark period and are subsequently decarboxylated and the CO_2 is re-assimilated to carbohydrate in the light period, a process that is driven by solar irradiance. Water loss is limited to cuticular transpiration as stomata are closed. Naturally under these conditions there is no carbon gain, but plants can use such respiratory recycling to overcome periods of drought until water is available again. This variation of full CAM is called CAM-idling (Sipes and Ting 1985). It is often observed in succulents of arid habitats during dry periods. *Clusia*s also exploit the photosynthetic flexibility conferred by the plasticity of expressing the different CAM phases to varying degrees (e.g. *C. alata*, Fig. 8.2D). While full CAM idling involves complete closure of stomata in the dark period, partial stomatal closure in phase I will not eliminate but reduce uptake of atmospheric CO_2 and at the same time internal respiratory CO_2 can be re-fixed.

The extent to which this occurs can be measured by comparing CO_2-gas exchange and nocturnal malic acid accumulation. In *Clusia*s in addition to malic acid citric acid may also be accumulated. Citrate does not count in the context of CO_2 recycling, because no net fixation of CO_2 is involved in the formation of citrate, while one CO_2 is fixed per malate accumulated (see Sect. 8.3). If uptake of atmospheric CO_2 accounts for all the malic acid accumulated, recycling of respiratory CO_2 is zero, and conversely, in full CAM-idling recycling is 100 %. Any values in between may be observed. In Table 8.1 examples for a recycling of 28–89 % are given for four species of *Clusia* studied in a phytotron. In *C. minor* 100 % recycling, i.e. full CAM-idling, was observed in plants grown at an irradiance of 260–300 µmol photons m^{-2} s^{-1}

Table 8.1. Recycling of respiratory CO_2 in phase I of CAM in four species of *Clusia* in a phytotron

	CO_2	mal	citr	RCR	RCR %
C. venosa	2.2	19.7	6.9	17.5	89
C. minor	54.1	85.2	37.0	31.1	37
C. major	109.4	151.9	61.0	42.5	28
C. alata	91.2	192.4	103.0	101.2	53

CO_2=uptake of atmospheric CO_2 integrated for the whole dark period
mal=accumulation of malate
citr=accumulation of citrate
RCR=respiratory CO_2 recycling equal to mal minus CO_2
All data are mmol m^{-2} leaf surface, RCR is also given in % of total malate accumulated (Franco et al 1990)

and studied at various temperatures in the day and in the night, i.e. in many cases when day and night temperatures were identical or similar there was nocturnal malate accumulation without a net uptake of CO_2 (Haag- Kerwer et al. 1992). In this study citrate accumulation also occurred in the absence of net CO_2 uptake, i.e. at day/night temperatures of 20/20 and 25/25 °C. Although no net recycling of respiratory CO_2 is involved in citrate accumulation we may call this CAM-idling with a recycling of whole carbon skeletons from hexose to citrate (see also Sect. 8.3). (More detailed results of this elaborate experiment are presented in Fig. 8.29 and Sect. 8.8.3 when the role of temperature as an external control parameter is discussed.)

Another mode of CO_2 assimilation is CAM-cycling (Sipes and Ting 1985). Here stomata are open during the light period and plants perform normal C_3-photosynthesis. Stomata close in the dark period and plants fix respiratory CO_2 via PEPC and store it in the form of organic acids. In the light period CO_2 released from the organic acids supplements CO_2 taken up from the atmosphere for photosynthesis via RubisCO. CAM-cycling has been considered as a kind of an incipient CAM, i.e. in evolution as a first step towards development of full CAM (Lüttge 2004). So far we do not have much information about typical CAM-cycling among *Clusia*s. It appears, however, that *Clusia arrudae* Planch. et Triana under drought conditions in the field is performing gas exchange and organic acid accumulation close to the features of CAM-cycling. We have unpublished data showing that at the end of the dry season *C. arrudae* behaved neither as a typical CAM-cycling plant – since photosynthesis only took place during a couple of hours in the early morning and not throughout the whole day – nor as a typical CAM-idling plant – since there was nocturnal acid accumulation but stomata were not continuously closed.

However, Veste et al. (2001) found a similar photosynthetic pattern in *Monilaria moniliformis* and classified it as a CAM-cycling plant, and Martin (1996) argued that CAM-cycling plants seldom perform CAM sensu stricto, but rather exhibit C_3 gas exchange patterns concomitant with overnight acid accumulation, which also fits the pattern of *C. arrudae*. *C. minor* under certain conditions is able to take up CO_2 from the atmosphere day and night, around the clock (Figs. 8.2B and 8.3C). This is not typical CAM-cycling as this would largely dwell on internal recycling of respiratory CO_2. On the other hand, the combination of night time and day time CO_2 fixation is the characteristic feature of CAM cycling. Thus, we might possibly discuss the CO_2 exchange pattern of *C. minor* shown in Fig. 8.2B in relation to CAM-cycling. Conversely, the pattern of Fig. 8.3C may be closer to a full CAM with strongly expressed phases II and IV. A strong expression of phase II is often observed among *Clusia*s (see above and Fig. 8.5). However, real CAM-cycling was seen in some other experiments with *C. minor*, where the effects of nitrogen nutrition on CAM expression were studied, and which are therefore presented below in Sect. 8.8.4 (Table 8.10; Franco et al. 1991). Plants grown with and without nitrogen at low PPFD and plants grown at higher PPFD without nitrogen showed no CO_2 uptake and a small loss of CO_2 in the dark period. Nevertheless they nocturnally accumulated appreciable amounts of citrate plus a small amount of malate in the case of high PPFD minus nitrogen while daytime CO_2 uptake was substantial (Table 8.10). These are clear indications of CAM-cycling with nocturnal accumulation of mainly citrate and some malate. At the higher PPFD plus nitrogen daytime CO_2 uptake was highly increased and there was only a very low night time CO_2 uptake which, however, was accompanied by quite large accumulations of both malate and citrate, a situation that also comes very close to CAM-cycling, i.e. high C_3-photosynthesis in the light period plus acid accumulation in the dark period with very little CO_2 uptake from the atmosphere.

Finally, there are many species of *Clusia* which are C_3/CAM intermediate. This means that they can readily switch between these two major modes of photosynthesis. We know many C_3/CAM-intermediate species in different phyla of the angiosperms. In well studied cases, such as *Mesembryanthemum crystallinum* L. and *Kalanchoë blossfeldiana* cv. Tom Thumb Poelln., the switch is only in one direction, i.e. from C_3-photosynthesis to CAM, and in addition to environmental factors intrinsic developmental programmes are involved in generation of the switch (see Lüttge 2004). For hemi-epiphytic *Clusia* species it has been assumed initially that CAM would be a particular property of the epiphytic stage and not so much expressed in free standing trees (Ting et al. 1987). However, this has not been supported by much later work. Evidently CAM is expressed in both life forms and there is no developmental programme modulating CAM expression in *Clusia*s (Wanek et al. 2002). Moreover, for a tropical tree like *Clusia* a one-way irreversible switch from C_3 to CAM would confer limited physiological advantages. *Clusia* leaves

are persistent and often used for several vegetation periods. In *C. multiflora* H.B.K. only leaves older than two years in the furthest position relative to the apex are shed (Olivares 1997). If the two modes of photosynthesis, C_3-photosynthesis and CAM, respectively, are different options fit for different environmental conditions only repeated reversible switching between the two modes would suit leaves persistent under varying conditions. Thus, reversible C_3-CAM-C_3 changes are commonly observed among *Clusia*s (Sect. 8.8).

Hence, we can conclude that the *Clusia* morphotype and even individual species, such as *C. minor* in particular (Fig. 8.3) but many others in addition, are very flexible in expressing up to four photosynthetic physiotypes, i.e.

- C_3-photosynthesis
- Full CAM with flexible expression of the different CAM phases including CAM-idling
- C_3/CAM-intermediate behaviour
- CAM-cycling

8.2 Stable Carbon Isotope Signatures

Because of the high flexibility of *Clusia*s with respect to photosynthetic metabolism it is highly desirable to have a tool to determine the extent to which species and individual plants make use of options for C_3-photosynthesis and CAM, respectively, and when performing CAM the relative magnitude of carbon acquisition in phases I and IV. Assessing the contributions made by the different photosynthetic pathways to plant carbon balance may be achieved via stable carbon isotope analysis. The stable carbon isotope ^{13}C in nature has an overall abundance of 1.11 %, while the abundance of ^{12}C is 98.89 %. During carbon acquisition and assimilation from the inorganic CO_2 in the environment there are different mechanisms affecting discrimination against the heavy isotope ^{13}C. Isotope discrimination is influenced by stomatal conductance to CO_2, diffusion of CO_2 through the leaf mesophyll as well as other thermodynamic consequences of discrimination in metabolic reactions. However, all these effects are quantitatively over-ruled by the large differences between the $^{13}CO_2$ discrimination of the two key enzymes of primary CO_2 fixation, namely RubisCO and PEPC (Table 8.2). The discrimination of RubisCO to ^{13}C in CO_2 is +27‰. PEPC has the much lower discrimination of –5‰ to ^{13}C in CO_2, which is determined by the isotope effect of the hydration of CO_2 (–7‰) because the actual substrate of PEPC is not CO_2 but bicarbonate, HCO_3^-. Thus, relative to the actual substrate HCO_3^- PEPC has a ^{13}C discrimination of +2.0‰ (Table 8.2). Stable carbon isotope ratios of plant material are obtained by mass spectrometry of the CO_2 released from combusted samples and expressed as

Table 8.2. ^{13}C-discrimination (‰) in various steps of the CO_2 fixation process (from Ziegler 1994)

Step	^{13}C isotope discrimination
Diffusion of CO_2 in the gas phase	4.4
Dissolution of CO_2 in water	–0.9
Liquid phase diffusion of CO_2 or HCO_3^-	0.0
Hydration of CO_2	–7.0
Carboxylation of phospho*enol*pyruvate	
relative to HCO_3^-	2.0
relative to CO_2	–5.0
Carboxylation of ribulose-bisphosphate	27

$$\delta^{13}C = \left(\frac{^{13}C/^{12}C \text{ of sample}}{^{13}C/^{12}C \text{ of an international standard}} - 1\right) \times 10^3 \text{ (‰)} \qquad (8.3)$$

Thus, the enzyme responsible for the primary fixation of CO_2 grossly determines the carbon isotope signature of the plant material analysed. Therefore, C_3 plants with primary CO_2 fixation via RubisCO have highly negative $\delta^{13}C$ values and CAM plants where PEPC dominates CO_2 fixation have less negative $\delta^{13}C$ values due to the high and low discrimination, respectively, of the two enzymes against $^{13}CO_2$. In this way C_3 and CAM plants can often be readily distinguished.

However, in CAM plants the leaf $\delta^{13}C$ signature is also determined by the extent to which the different phases of CAM contribute to carbon balance. Borland et al. (1993) have measured instantaneous ^{13}C-discrimination concurrently with CO_2 exchange during the four CAM phases in *C. minor*. In their analysis instantaneous discrimination according to Evans et al. (1986) is defined as

$$\Delta^{13}C = \frac{\xi\,(\delta_o - \delta_e)}{\Delta + \delta_o - \xi(\delta_o - \delta_e)} \text{ (‰)} \qquad (8.4)$$

where $\xi = p_e/(p_e - p_o)$ and p_e and p_o are CO_2 partial pressures of the air entering and leaving the gas exchange chamber, respectively, when a leaf is enclosed. δ_o is the carbon isotope ratio (see Eq. 8.3) of the air leaving the gas exchange chamber with a leaf enclosed and δ_e the carbon isotope ratio in control air leaving a gas exchange chamber without a leaf. While for $\delta^{13}C$ negative values

indicate ^{13}C discrimination (Eq. 8.3) in the case of $\Delta^{13}C$ (Eq. 8.4) discrimination is given by positive values. Figure 8.6 shows that discrimination was lowest in phase I of CAM where PEPC is the only CO_2 fixing enzyme, higher in phase II where both PEPC and RubisCO participate in CO_2 fixation, and highest in phase IV where CO_2 fixation is dominated by RubisCO.

Hence, the range of $\delta^{13}C$ values overall obtained from CAM plants is much broader than that of C_3 plants. Moreover, $\delta^{13}C$ signatures also indicate to which extent C_3/CAM-intermediate plants made use of their option for the two different modes of photosynthesis during the life time of the plant material sampled and analysed. In CAM plants, $\delta^{13}C$ values correlate linearly with the proportions of CO_2 taken up during the light and dark (Winter and Holtum 2002). Thus, stable carbon isotope analysis is a very powerful tool for assessing the expression of photosynthetic physiotypes and their variants. The plant material required is easily sampled and transported because only small samples of dried tissue are required and as suggested below the interpretation of $\delta^{13}C$ data can supply very important information. Nonetheless, we must note that $\delta^{13}C$ values per se do not allow to decide conclusively if a plant has an intrinsic potential for CAM or not, because the ability to perform CAM may be hidden by highly negative $\delta^{13}C$ values if the plant actually makes little use of its CAM option and only expresses CAM for limited periods over the life span of the leaf. Additional physiological information is required to assess the inherent capacity for CAM.

$\delta^{13}C$-values for a number of *Clusia* species are shown in Fig. 8.7. The range is continuous from as negative as about –30‰ in *C. parviflora* Saldanha et

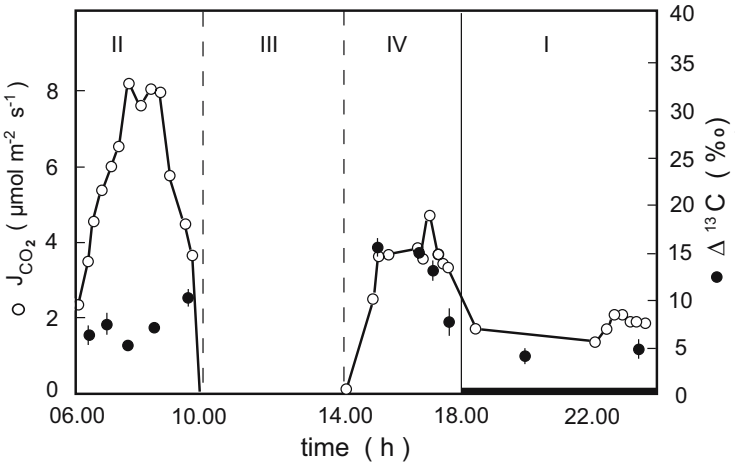

Fig. 8.6. CO_2-exchange (J_{CO2}, *open circles*) and ^{13}C-discrimination ($\Delta^{13}C$ according to Eq. (8.4), *closed circles*) by sun exposed leaves of CAM-performing *C. minor* measured in the dry season in the field in Trinidad. The *solid bar* on the abscissa indicates the dark period, *Roman numbers* give the CAM phases (Fig. 4a in Borland et al. 1993)

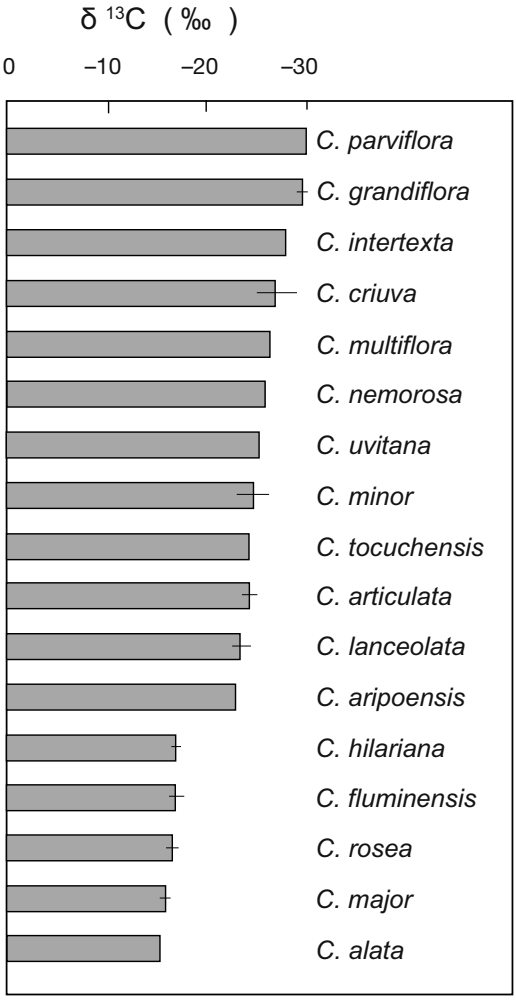

Fig. 8.7. $\delta^{13}C$ values obtained for a range of *Clusia* species (Fig. 1 in Lüttge 1999)

Engl. to the much less negative value of about –15‰ in the obligate CAM species *C. alata*. One can compare these values with results obtained from measurements of gas exchange and night/day changes of organic acid levels. The physiological measurements indicate the capability of the plants to perform C_3-photosynthesis or CAM under the given conditions of the experiments, while the $\delta^{13}C$-values provide information on the actual performance over time. Thus, it is seen in Fig. 8.7 that, for instance, the C_3/CAM-intermediate species *C. minor* under natural conditions makes more use of C_3-photosynthesis than of CAM as its $\delta^{13}C$ values are rather negative.

A more extensive survey was performed on *Clusia* species from Panamá where night/day changes of acidity (ΔH^+) were measured in 25 species and $\delta^{13}C$ values analysed for 38 species (Holtum et al. 2004). The distribution of

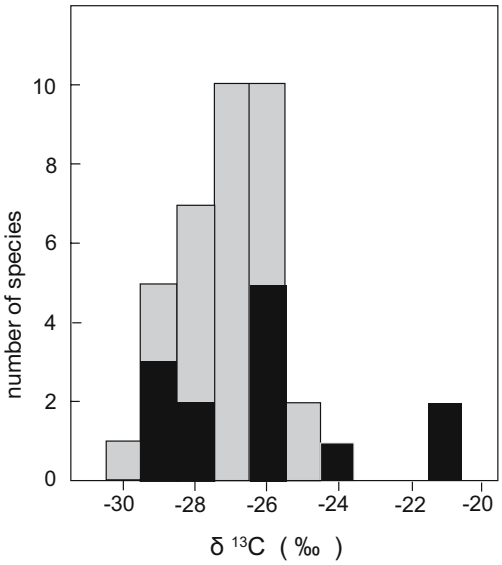

Fig. 8.8. Range of $\delta^{13}C$ values obtained from mature leaves of *Clusia* species growing in their natural environment in Panamá and the presence (*solid bars*) and absence (*shaded bars*) of an intrinsic CAM potential as derived from ΔH^+- measurements (Fig. 3 of Holtum et al. 2004)

values is shown in Fig. 8.8. The values exhibit a predominant C_3-type peak ($\delta^{13}C$ –24 to –30‰), in which the weak CAM performing species are hidden as identified by ΔH^+, and a very small more CAM-like peak at less negative $\delta^{13}C$ values. This suggests that in the field, at least in Panamá, strong CAM is the exception rather than the rule in *Clusia*. Moreover, like *C. minor* mentioned above, many other *Clusia*s which have the intrinsic potential to perform CAM make predominant use of the C_3-option in the field.

8.3 Biochemistry of Crassulacean Acid Metabolism (CAM)

The standard pathway of CAM is that CO_2 is fixed nocturnally via phospho-*enol*pyruvate carboxylase (PEPC), malate (mal) is synthesized by reduction of the oxaloacetate (OAA) formed and stored in the cell vacuoles, from where it is mobilized during the day and decarboxylated, and the CO_2 regenerated is assimilated via ribulose-bis-phosphate carboxylase/oxygenase (RubisCO) in the Calvin cycle (Sect. 8.1). The precursor, phospho*enol*pyruvate (PEP) for nocturnal dark fixation of CO_2 is usually formed from starch stored during the day and broken down to PEP via glycolysis during the night. However, CAM in *Clusia* shows several deviations from this general scheme. Therefore, in this section we need to consider CAM metabolism in more detail. (The reader may note that here just once we have deliberately used the pleonasm, which is so frequently produced in the literature, as a warning: The *M* in CAM, of course, is already standing for *metabolism*.)

8.3.1 Turnover of Carbohydrates

The relevant reactions of intermediary metabolism are summarized schematically in Fig. 8.9, where major intermediates are shown so that turnover of energy equivalents (ATP and reducing potential [2H]) can also be assessed (for details see Holtum et al. 2005). The stoichiometries are based on one C_6-unit (hexose-unit).

In the dark period of the CAM cycle PEP as the acceptor of CO_2 in dark fixation via PEPC is formed glycolytically from carbohydrates. In some CAM species, e.g. pineapple (*Ananas*; Black et al. 1996), in addition to glucans (starch) mobilized from the chloroplasts the precursors for PEP may include free sugars (hexoses or sucrose) mobilized from the vacuoles. An important involvement of free sugars is also very often observed in the CAM of *Clusia* (Fig. 8.10 and Table 8.3). Hexose-phosphate is thought to be mobilized from starch by phosphorolysis. Free hexoses are assumed to be released passively

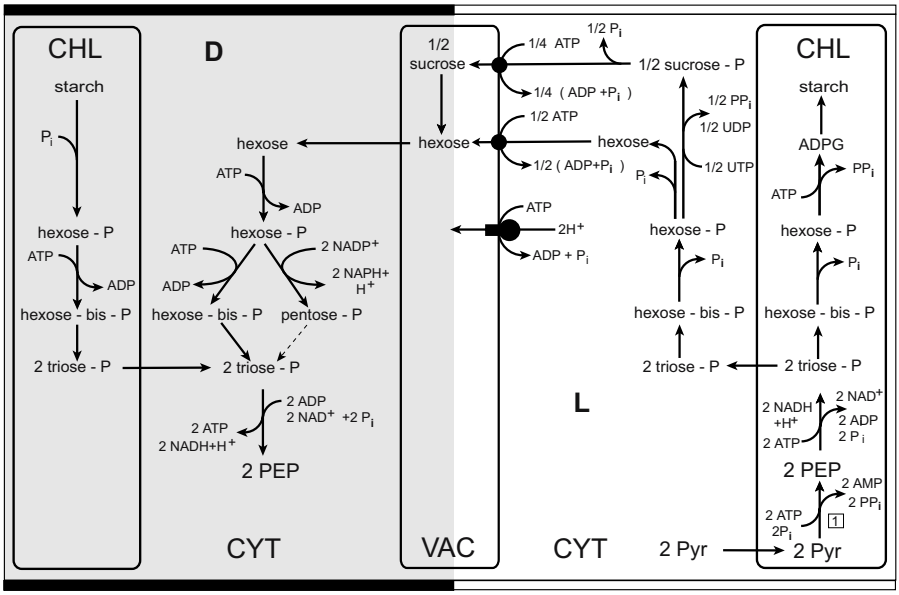

Fig. 8.9. Turnover of carbohydrates in the cycle of CAM, with nocturnal formation of phospho*enol*pyruvate and daytime regeneration of carbohydrate from pyruvate. Nocturnal formation of triose-phosphate is via glycolysis, and an alternative pathway to triose-phosphate is from pentose-phosphate via the oxidative pentose-phosphate cycle. Daytime regeneration of carbohydrates is mediated via gluconeogenesis. Reaction sequences are abbreviated. An important individual key enzyme is (1)=pyruvate-P_i-dikinase. CHL=chloroplast, CYT=cytosol, D=dark period, L=light period, P=phosphate residues, PEP=phospho*enol*pyruvate, pyr=pyruvate, P_i=free inorganic phosphate, VAC=vacuole

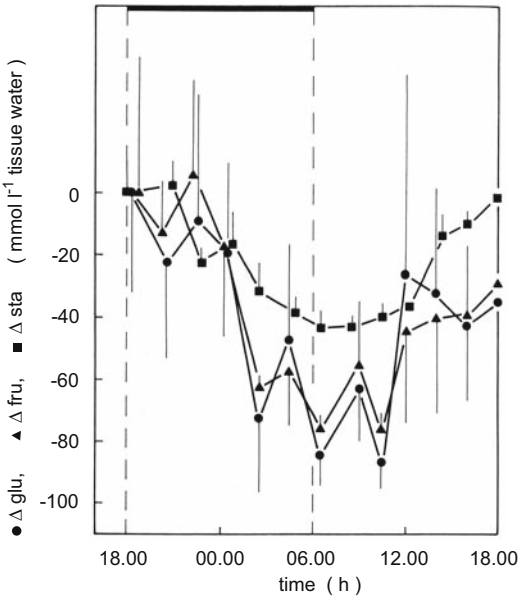

Fig. 8.10. Diurnal changes of the levels of glucose (*circles*), fructose (*triangles*) and starch (*squares*) during the CAM-cycle in *C. rosea* in the field on St. John Island, Lesser Antilles. Starch is given in hexose units. Changes of carbohydrates were related to the levels obtained at 18:30 h at the start of the measurements, namely glucose 98.0 mmol L^{-1} tissue water, fructose 84.7 mmol L^{-1} and starch 50.4 mmol L^{-1}. The *dark bar* on the abscissa indicates dark period (Fig. 4 from Ball et al. 1991)

Table 8.3. Dusk minus dawn values of the levels of free sugars (glucose: glu, fructose: fru, sucrose: su) and of starch (in hexose units) in various species of CAM-performing *Clusia* sampled in the field. Data are in mmol L^{-1} of plant water or fresh weight and were adapted from the references indicated. (*C. hilariana* is not mentioned in the text before, the authority is Schlecht.)

		glu	fru	su	starch	Referencee
C. alata	Dry season	93	94	13	n.d.	Popp et al. 1987
	End of dry season	45	38	–10	22	
C. rosea	Dry season	92	79	5	n.d.	
	End of dry season	174	165	–8	31	
C. rosea		75	85	0	43	Ball et al. 1991
C. hilariana		‖→ 111 ←‖		0	17	Berg et al. 2004
C. minor	Exposed leaves					
	Wet season	‖→	65	←‖	31	Borland et al. 1994
	Exposed leaves					
	Dry season	‖→	81	←‖	39	
	Shaded leaves					
	Dry season	‖→	49	←‖	105	

from the vacuole and sucrose to be split hydrolytically into hexoses by invertase. Thus, there is a net production of one ATP when the precursor is starch but not when it is free hexose or sucrose, and in all three cases 2 [2H] are formed when one hexose unit is broken down to PEP (Table 8.4B).

During the day, the decarboxylation of organic acids formed in the previous night, generates pyruvate (pyr). Therefore, we start the scheme of carbohydrate turnover of CAM in the light period with pyr (Fig. 8.9). PEP is formed from pyr by pyr-Pi-dikinase (PPDK). Reduction equivalents ([2H]) are consumed for reduction of PEP to triose-phosphate. Formation of sucrose and starch is energetically a little more costly than formation of hexose (Table 8.4B, where UTP is taken to be equivalent to ATP). Daytime storage of free sugars, hexoses and sucrose, in the vacuole needs energy for secondary active

Table 8.4. Energy budgets based on one hexose unit of CAM-cycles with different carbohydrates, starch, free hexose or sucrose, and organic acids, malate or citrate, as shown in Figs. 8.9, 8.12 and 8.14. (A) Organic acid turnover, (B) carbohydrate turnover and overall budgets.

(A)	Dark period		Light period	
	ATP	[2H]	ATP	[2H]
2 PEP → 2 vacuolar mal	–2	–2		
2 vacuolar mal → 2 pyr			0	+2
2 PEP → 1 vacuolar citr	–1.5	+1		
1 vacuolar citr → 1 pyr			+1	+4

(B)	**Starch**		**Free hexose**		**Sucrose**	
	ATP	[2H]	ATP	[2H]	ATP	[2H]
Carbohydrates						
Dark period formation of PEP	+1	+2	0	+2	0	+2
Light period recycling of pyr	–5	–2	–4.5	–2	–4.75	–2
Malate and carbohydrates						
Dark period	–1	0	–2	0	–2	0
Light period	–5	0	–4.5	0	–4.75	0
Net	–6	0	–6.5	0	–6.75	0
Citrate and carbohydrates						
Dark period	–0.5	+3	–1.5	+3	–1.5	+3
Light period[a])	–10.5	–3	–10.25	–3	–10.375	–3
Net	–11.0	0	–11.75	0	–11.875	0

[a] Only one pyr is regenerated, 3 CO_2 need to be fixed in the Calvin cycle to regenerate the second C_3 compound.

[2H]=reduction equivalent; citr=citrate, mal=malate, pyr=pyruvate; +=net production, –=net consumption.

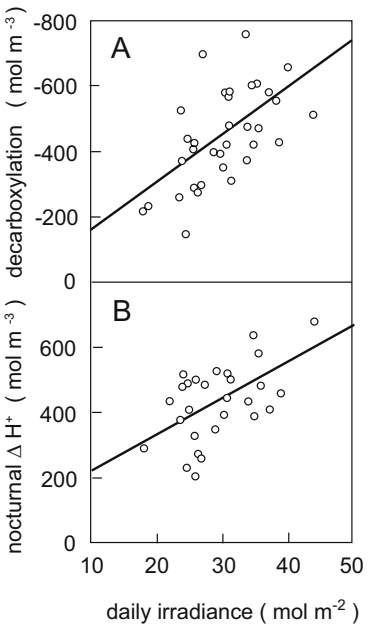

Fig. 8.11A,B. Correlations of: **A** organic acid decarboxylation in phase III of CAM to daily irradiance received; **B** organic acid accumulation in the following dark period (phase I) in *C. minor* (after Fig. 2a,b of Roberts et al. 1998)

transport via sugar translocators at the tonoplast, and it is assumed that free sugars (hexoses, sucrose) are transported into the vacuole by H^+/sugar co-transport driven by the tonoplast H^+-ATPase pumping 2 H^+ into the vacuole per ATP hydrolysed (Holtum et al. 2005).

Energetically the amount of organic acids accumulated during phase I of CAM depends on the irradiance received during the previous light period which determines both the degree of organic acid breakdown in phase III and the accumulation of photosynthetic products, viz. carbohydrates, for the generation of phospho*enol*pyruvate (PEP) as CO_2 acceptor in phase I (Kluge 1968; Nobel 1988). These relations have also been documented in leaves of *C. minor,* where positive linear correlations between decarboxylation and nocturnal acidification, respectively, and light period irradiance have been observed (Fig. 8.11; Roberts et al. 1998). In *C. uvitana* Pittier high acid levels remaining in the leaves at dusk inhibited organic acid accumulation during the following night (Zotz and Winter 1993).

8.3.2 Organic Acid Turnover with Nocturnal Storage of Malic Acid

At night, malate is formed via PEPC and malate dehydrogenase (Fig. 8.12). Nocturnal energy metabolism when starting from PEP is determined by the consumption of [2H] by malate dehydrogenase and of ATP for vacuolar accumulation, where malate is transported into the vacuoles via an inward recti-

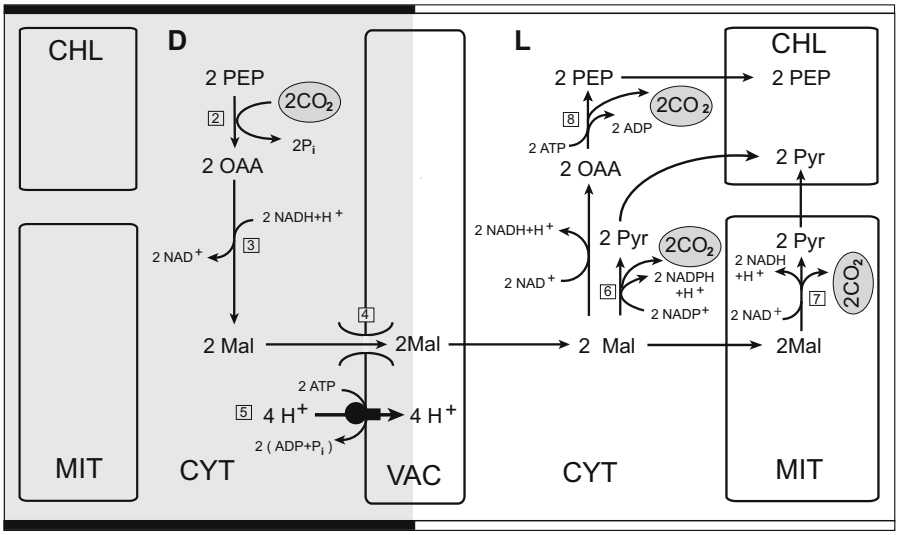

Fig. 8.12. Organic acid turnover in the cycle of CAM with nocturnal storage of malic acid. General explanation as for Fig. 8.9. Abbreviations not yet explained in Fig. 8.9 are as follows: MIT=mitochondrion, Mal=malate, OAA=oxaloacetate. Key enzymes are: (2) PEP-carboxylase (PEPC), (3) malate dehydrogenase, (4) inward rectifier vacuolar malate channel, (5) H^+-transporting ATPase of the tonoplast (V-ATPase), (6) cytosolic NADP-dependent malate decarboxylase or malic enzyme (NADP-ME), (7) mitochondrial NAD-dependent malate decarboxylase (NAD-ME), (8) PEP-carboxykinase (PEPCK)

fier malate channel (Hafke et al. 2003) energetically driven by the H^+-transporting ATPase at the tonoplast (V-ATPase), pumping 2 H^+ per ATP (Table 8.4A; Holtum et al. 2005).

During the light period malate is remobilized passively from the vacuole (Lüttge and Smith 1984). Malate can be decarboxylated to pyr by NADP-dependent malate decarboxylase or "malic enzyme" (NADP-ME) in the cytosol or by NAD-dependent malate decarboxylase (NAD-ME) in the mitochondria. Transport of malate into the mitochondria could occur via energetically neutral exchange systems (Holtum et al. 2005). A third way of malate decarboxylation is via oxidation to OAA and then by PEP-carboxy-kinase (PEPCK) to PEP. This reaction is realized in *Clusia*s (Black et al. 1996; Borland et al. 1998; Holtum et al. 2005). Energetically all three mechanisms are identical. In the scheme of Fig. 8.12 the way via PEPCK appears to be more costly, but it already produces PEP instead of pyr so that the first reaction of light period carbohydrate turnover shown in Fig. 8.9 is saved. Pyruvate is largely transferred to gluconeogenesis and not further broken down to CO_2 (Robinson et al. 1992; Holtum et al. 2005). To avoid futile recycling of CO_2 via PEPC in the light period the activity of PEPC, which has a 60-fold higher affinity for CO_2 than RubisCO, needs to be down-regulated. This is achieved by reversible

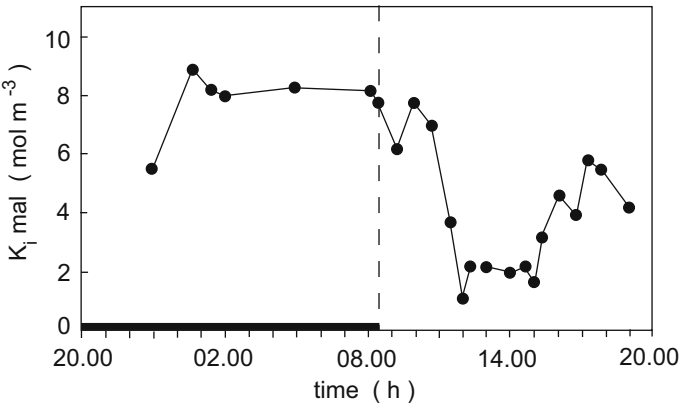

Fig. 8.13. Diurnal changes of $K_{i\text{-}mal}$ for extracts of PEPC prepared from leaves of *C. minor*. (Fig. 7a of Borland and Griffiths 1997)

phosphorylation of the enzyme (Nimmo et al. 1987; Kusumi et al. 1994; Carter et al. 1995a, b, 1996). The night-form of PEPC is phosphorylated and is much more active and less sensitive to lowered pH and to feedback inhibition by its product malate than the de-phosphorylated day form. For *C. minor*, Borland and Griffiths (1997) have demonstrated this phenomenon by analysing the malate inhibitor constant, $K_{i\text{-}mal}$, of PEPC during the diurnal CAM-cycle (Fig. 8.13), showing that inhibition was low (high $K_{i\text{-}mal}$) in the dark period and increased in the light period (lower $K_{i\text{-}mal}$).

Turnover of malate leads to a net gain of carbon. Per malate accumulated 1 CO_2 is fixed (2 CO_2 per hexose unit turned over) in the dark period, which is released in the light period and available for assimilation via RubisCO in the Calvin cycle (Fig. 8.12).

8.3.3 Organic Acid Turnover with Nocturnal Storage of Citric Acid

A number of CAM species are known from the literature, which nocturnally accumulate citric acid in addition to malic acid (Milburn et al. 1968; Lüttge 1988). Generally, citric acid turnover in the CAM cycles is much less important than that of malic acid with day/night changes of citric acid levels of up to 26 mmol kg^{-1} fresh weight in some species of *Kalanchoë*. However, in the CAM cycle of *Clusia*s diurnal changes of citric acid levels may be much higher, i.e. up to 200 mM (Franco et al. 1992), so this is a special trait of CAM in *Clusia*. Citrate metabolism of CAM (Fig. 8.14) is more complex and much less well understood than that of malate.

Starting from two molecules of PEP citrate is formed nocturnally from OAA produced by PEPC in the cytosol and via pyr and generation of acetyl-CoA in the mitochondria. We note that one CO_2 is fixed to one molecule of PEP via PEPC and at the same time one CO_2 is lost by oxidative decarboxylation of pyr obtained from the second molecule of PEP when acetyl-CoA is

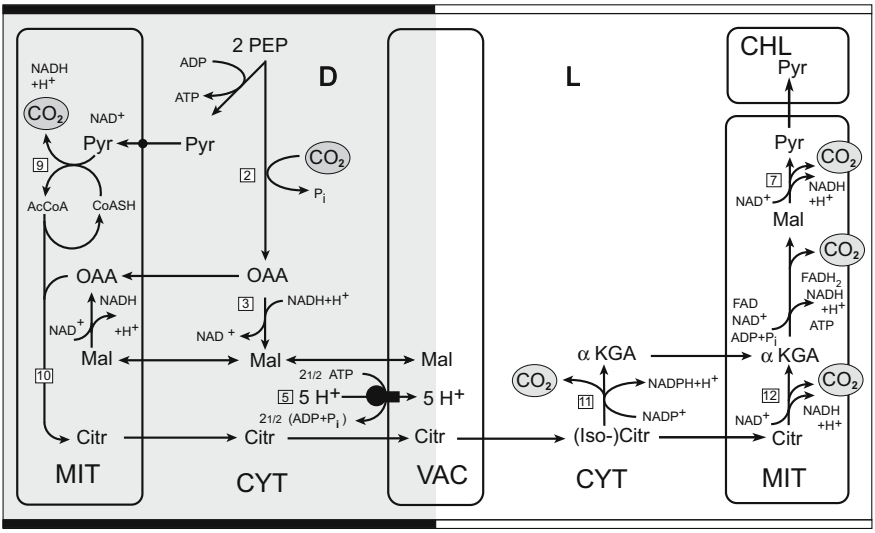

Fig. 8.14. Organic acid turnover in the cycle of CAM with nocturnal storage of citric acid. General explanation as for Fig. 8.9, abbreviations not yet explained in Figs. 8.9 and 8.12 are as follows: AcCoA=acetyl coenzyme A, Citr=citrate, CoASH=coenzyme A, αKGA=α-keto-glutaric acid. Additional key enzymes are: (9) pyruvate decarboxylase, (10) citrate synthetase, (11) cytosolic NADP-dependent iso-citrate dehydrogenase, (12) mitochondrial NAD-dependent iso-citrate dehydrogenase

formed. Hence, in contrast to malic acid, nocturnal accumulation of citric acid is not accompanied by a net gain of carbon. For energy stoichiometry (Table 8.4A) we must note that pyruvate transport into the mitochondria is consuming some of the electrochemical proton gradient at the inner membrane of mitochondria. In the energy budget we take this as being equivalent to one ATP consumed per pyruvate taken up. Moreover, we assume that vacuolar accumulation of citric acid is energized by the V-ATPase in the same way as that of malic acid but using 3 H^+ per citrate transported (see Holtum et al. 2005). Pulse-chase experiments with *C. minor* using radioactively labelled $^{14}CO_2$ have shown that in nocturnal citrate formation malate is formed first and even transported transiently into the vacuoles from where it is re-allocated to the mitochondria to regenerate OAA for citrate synthesis (Fig. 8.15; Olivares et al. 1993; see also Kalt et al. 1990). This peripatetic flow of malate may consume one extra ATP for transport into the vacuoles. The biochemical reactions (OAA to malate in the cytosol and malate to OAA in the mitochondria) together are energetically neutral. Overall, nocturnal citric acid accumulation appears to be energetically somewhat more favourable than malic acid accumulation. ATP consumption is less because one ATP is formed in the reaction from PEP to pyr (pyruvate kinase) and one ATP each is consumed for the peripatetic flow of malate and

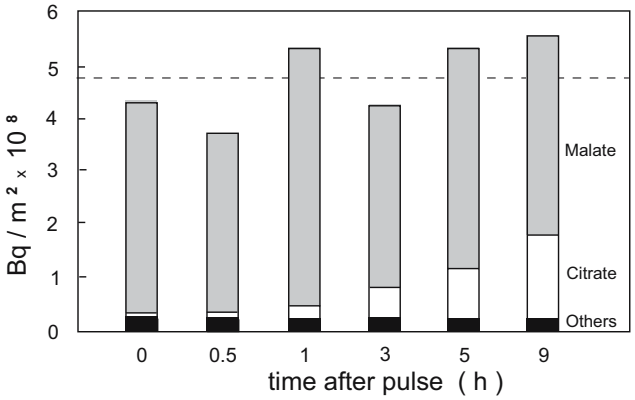

Fig. 8.15. Distribution of ^{14}C label in malate, citrate and other compounds after an exposure of detached leaves of *C. minor* to $^{14}CO_2$ during the last hour of the light period and during a subsequent chase in normal air (0.5 to 9 h). The *horizontal line* gives the average of total radioactivity in the samples taken for the times indicated. It is known that at the end of the light period in the CAM-cycle PEPC already may dominate CO_2 fixation (Kluge et al. 1982). This is corroborated here as almost all of the label of a ^{14}C-pulse at the end of the light period is found in malate. This malate must be sequestered in the vacuole. During the dark period some of the label is gradually transferred to citrate without an overall loss of label from the leaves indicating that some of the malate is transported from the vacuoles into the mitochondria for citrate synthesis and citrate transported back into the vacuoles (From data of Olivares et al. 1993)

citrate during transport into the vacuole. Moreover, starting from PEP in citrate production one [2H] is formed while in malic acid accumulation [2H] is consumed (Table 8.4A). The overall nocturnal budget including carbohydrate breakdown for citrate is a gain of 3 [2H] while for malate it is zero (Table 8.4B). ATP can be formed from the [2H] generated, and overall nocturnal citric acid accumulation remains energetically favourable. It must be noted, however, that in contrast to these theoretical evaluations of paper-biochemistry, measurements of nocturnal citrate accumulation and respiratory O_2 consumption have shown for four species of *Clusia* (*C. venosa, C. minor, C. major* L. and *C. alata*) that O_2-consumption was lower by a factor of 1.3 to 3.1 than theoretically expected from measured citrate accumulation (Franco et al. 1990), and hence, only part of the reducing power generated by the synthesis of citrate enters the respiratory chain.

No investigations are available on metabolic pathways starting with citrate in the light period. This is one of the astonishing gaps in CAM-research. Hence, we are left here with assumptions based on general biochemistry (Fig. 8.14). When released from the vacuole citrate can be decarboxylated via aconitase and iso-citrate by iso-citrate dehydrogenase in the cytosol or in the mitochondrial tricarboxylic acid cycle. Two NADP-dependent iso-citrate dehydrogenases are known in plants, one is located in chloroplasts and only

occurs in ferns and dicotyledons, the other one is cytosolic and appears to be the major form (Chen 1998; Popova and Appenroth 2002; Popova et al. 2002). Another pathway for cytosolic citrate breakdown is via ATP-dependent citrate lyase (Rangasami and Ratledge 2000; Fatland et al. 2002), which generates acetyl-CoA and is involved in fatty acid metabolism (not shown in Fig. 8.14). α-Keto-glutarate generated from citrate can be further processed in the mitochondria to malate and pyr via NAD-ME which then may be used to regenerate carbohydrate (Fig. 8.9).

It has been argued that potentially citrate breakdown in the light period may generate 1, 3 or 6 CO_2. In the first case α-keto-glutarate would accumulate or would have to be used in other reactions. In the case of 6 CO_2 breakdown would have to occur in the tri-carboxylic acid cycle. This would generate a large amount of reduction equivalents, and it would be difficult to envisage how respiratory electron transport might deal with them while photosynthesis is occurring at the same time in the light (Lüttge 1988). One possibility is the cyanide resistant non-phosphorylating alternative pathway of respiration which allows mitochondrial acid breakdown to escape from the control of cellular energy charge (Rustin and Lance 1986). Cyanide-insensitive mitochondrial substrate oxidation was particularly enhanced after CAM induction in the C_3/CAM intermediate species *Kalanchoë blossfeldiana*, and an increase of cyanide resistance of leaf respiration has been observed in the early light period when acid is remobilized in the CAM cycle (Rustin and Queiroz-Claret 1985). In any case, the simplest assumption at this stage appears that 3 CO_2 are generated from each citrate as shown in Fig. 8.14. An indirect way of testing this is via apparent photosynthetic electron transport, ETR, since ETR is correlated to the rate of CO_2-production from acid decarboxylation (Sect. 8.1). In experiments with *Clusia*s in the field, in fact a linear relationship was obtained between ETR and acid decarboxylation in *C. alata* plus an unidentified species when it was assumed that citrate breakdown produces 3 CO_2 per citrate. This corroborates the stoichiometry given in Fig. 8.14. However, it needs to be mentioned that in the case of *C. rosea* a linear relationship was only obtained when assuming the production of only one CO_2 (Fig. 8.16).

With 3 CO_2 produced per citrate broken down only one pyruvate is regenerated in the light period per hexose molecule turned over (Fig. 8.14), which then may be reduced to triose-phosphate in the chloroplasts (Fig. 8.9). This means that to complete the cycle 3 CO_2 need to be fixed via RubisCO and the Calvin cycle with an energy requirement of 3 ATP and 2 [2H] per CO_2 to produce the second triose-phosphate which needs to be considered in the energy budget (Table 8.4B). Overall it is important to stress that in terms of carbon the CAM cycle using citrate is a futile cycle. There is no net gain of carbon. In the dark period the balance of CO_2 liberated and CO_2 fixed is zero. The CO_2 liberated in the light period needs to be refixed and returned into the cycle to maintain the carbon balance. Hence, the expression of a CAM cycle with cit-

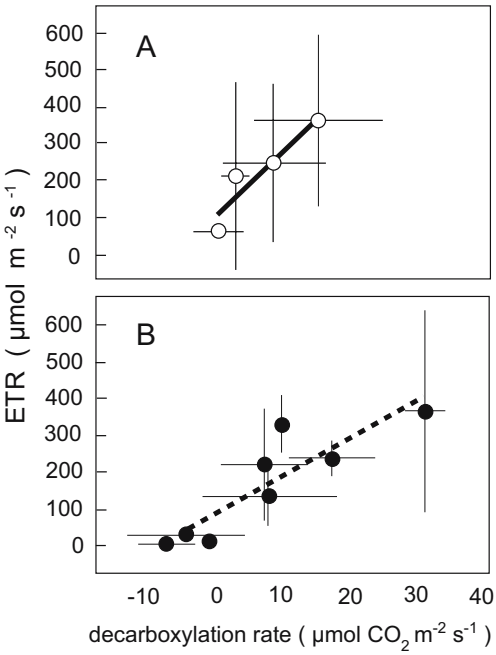

Fig. 8.16A,B. Correlations between rates of CO_2 production from decarboxylation of organic acids and apparent photosynthetic electron transport rates, ETR, (A) for *C. rosea* and (B) for pooled data of *C. alata* plus an unidentified species of *Clusia* measured in the field in Venezuela. To determine decarboxylation rates from measured disappearance of organic acids in the leaf tissue during the light period, it was assumed that one malate releases one CO_2 and that one citrate releases: **A** one CO_2; **B** three CO_2. Correlation coefficients, r, are 0.93 and 0.88 in A and B, respectively (Fig. 6 of Haag-Kerwer et al. 1996)

rate accumulation in *Clusia*s, as large as it is under certain conditions, must have other functions than carbon acquisition, some of which will be discussed below.

8.3.4 Concluding Evaluation

1. The overall energy budgets of the CAM cycle (Table 8.4B) with both malate and citrate are only marginally different for the involvement of starch, free hexose or sucrose as carbohydrates stored during the day for nocturnal PEP production. Hence, the variation of the form of carbohydrate involved can not be well explained by energy metabolism and there may be other reasons.
2. Energetically citrate accumulation is superior to malate in the dark period. However, the recirculation of carbon from citrate to carbohydrate in the light period is much more energy demanding than in the case of malate. It is consistent with this theoretical estimate that observations with *C. minor* in the field have shown a much closer correlation of the citrate breakdown in the light period with irradiance than in the case of malate (Borland et al. 1996). The overall day and night energy budget with citrate is much more costly, i.e. almost twice that with malate (Table 8.4B).
3. Only with malate but not with citrate the CAM cycle leads to a net gain of carbon. Productivity and growth, of course, are only possible if there is car-

bon available for export from photosynthesising source leaves. The two CO_2 produced per hexose unit turned over in phase III of the CAM-cycle with malate may contribute to this. Carbon isotope signatures of exported material have shown, however, that in the C_3/CAM- intermediate species *C. minor* carbon exported from source leaves mainly originates from CO_2-fixation via RubisCO in phase IV of CAM (Borland et al. 1994).

8.4 CO_2 Concentrating Consequences of CAM

The basic function of CAM is that of a CO_2 concentrating mechanism (Lüttge 2002). This is based on the properties of PEPC which has a 60-fold higher affinity for CO_2 than RubisCO. Hence, at prevailing low atmospheric CO_2-concentrations PEPC can support carbon acquisition very effectively. However, this only leads to storage of carbon in the form of the organic acids produced in the dark period phase I of CAM. The actual CO_2-concentrating effect occurs during organic acid remobilization in the light period phase III of CAM when internal CO_2 concentrations ranging from 0.09 % to as much as 2.5 % may build up in the photosynthesising organs of CAM plants, i.e. twice to 60 times atmospheric CO_2-concentration (Lüttge 2002). CO_2 assimilation in the light at high CO_2-concentrations behind closed stomata is also accompanied by a built up of high internal oxygen concentrations of up to over 40 % (Spalding et al. 1979) in various CAM plants. Unfortunately, we have little information in this respect about CAM-performing *Clusia*s. Sternberg et al. (1987) have estimated internal CO_2 concentration in *C. rosea* from gas exchange measurements when stomata were nearly closed at the time of phase III to be about 15 times that of ambient CO_2. Furthermore, we have the very early observation of Alexander von Humboldt in 1800 that *C. rosea* produced internal O_2-concentrations of up to 35 % in its leaves in the light (see Sect. 1.2 and Lüttge 2002), which remains an essential observation for us.

Although the CAM cycle with citrate is futile with respect to carbon gain (Sect. 8.3.3), citrate decarboxylation makes a somewhat larger contribution to the built up of high CO_2-concentrations in the air spaces of leaves than malate since per hexose unit turned over citrate may generate 3 CO_2 and malate only 2 CO_2 in phase III of CAM (Sect. 8.3.3). Whether elevated internal CO_2-concentration from citrate decarboxylation provides any metabolic or physiological advantage or not remains debatable (see Sect. 8.5). High internal CO_2-concentrations in phase III of CAM occurring at day times when solar irradiance is highest have often been considered to protect against high light stress because they support strong photochemical work at substrate saturation of RubisCO (Sect. 8.5). The higher energy demand of organic acid turnover with citrate during the light period as compared to malate (Sects. 8.3.3 and 8.3.4)

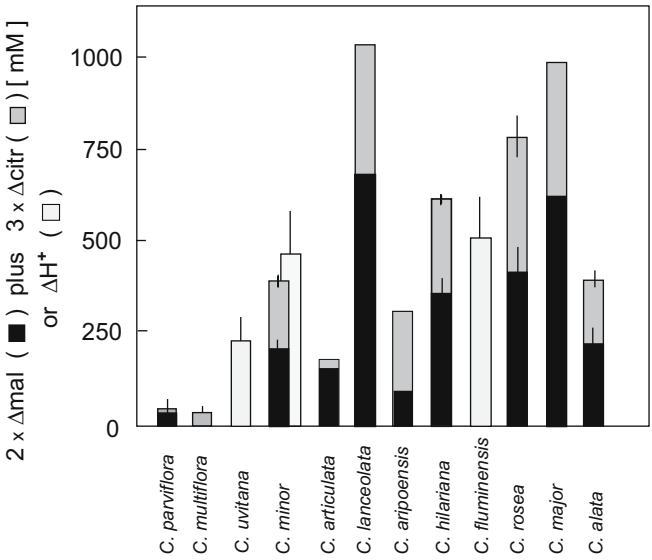

Fig. 8.17. Night/day oscillations (Δ) of organic acid accumulation and remobilisation in various species of *Clusia*. In some cases only night/day changes of titratable protons (ΔH^+) have been analysed. Values for malate (Δmal) and citrate (Δcitr) are given with a factor of 2 and 3 to account for the 2 and 3 carboxyl groups, respectively. The stoichiometry of $2 \times \Delta mal + 3 \times \Delta citr = \Delta H^+$ is realized in many analyses in different species of *Clusia* in the literature (Fig. 1 in Lüttge 1999)

may be an additional advantage for harmless use of energy at excess irradiance (Roberts et al. 1998).

Among the CAM-performing species of *Clusia* we find the strongest nocturnal acid accumulators known. The well studied model plant of CAM research, *Kalanchoë daigremontiana* Hamet et Perrier, may achieve night/day changes of malate (Δmal) of up to about 250 mM amounting to night/day changes of titratable vacuolar protons (ΔH^+) of 500 mM considering the two carboxyl groups of malic acid. With accumulation of malic acid plus citric acid (three carboxyl groups per molecule) in some *Clusia* species nocturnal acidification of a ΔH^+ of more than 1000 mM titratable protons was observed (Fig. 8.17; Franco et al. 1992; see Lüttge 1999, where a large number of analyses presented in the *Clusia* literature are summarized). These plants are also the strongest nocturnal citric acid accumulators known, with Δcitr values of up to 160 mM (Franco et al. 1992). With a nocturnal acid accumulation of ΔH^+=1 410 mM observed in *C. minor* in the field in Trinidad, Borland et al. (1992) certainly make it for the Guinness book of records with the highest nocturnal acidification ever recorded in any CAM plant.

Nocturnal vacuolar accumulation of malic and citric acid is driven by the V-ATPase at the tonoplast (Sects. 8.3.2 and 8.3.3). The capacity of accumula-

tion is limited by the electrochemical proton gradient which is built up at the tonoplast and against which the H^+ transporting V-ATPase must pump (Lüttge and Smith 1984; Franco et al. 1992). The proton gradient is strongly affected by the buffering capacity of the vacuole regulating the concentration of free protons. Citrate is a much stronger buffer compound than malate. Therefore, citrate accumulation itself may facilitate strong accumulation of total acid in the vacuole via a positive feedback effect. Hence, it may be one of the essential functions of citrate accumulation in the *Clusia*s that their very high nocturnal acid accumulation becomes possible and, of course, that such particularly high acid turnover in the CAM cycle of *Clusia*s also supports particularly high CO_2-concentrating in phase III.

8.5 Photorespiration

CAM plants possess the entire biochemical complement for performance of photorespiration. However, it was long assumed that photorespiration would only occur during phase IV of CAM when plants perform C_3-like photosynthesis because the CO_2-concentrating mechanism of phase III would elevate the internal CO_2/O_2 ratio in favour of the carboxylation activity of RubisCO. In view of the concomitant O_2 concentrating effect that occurs behind closed stomata during phase III it is now realized that the situation is more complex. Studies of photorespiration online with photosynthesis are not available for CAM plants in general.

However, data have been obtained for *C. minor* in both the C_3- and the CAM-state using automatic applications of air with only 1 % O_2 for 20 min at intervals during recordings of photosynthetic parameters, such as net CO_2 exchange, J_{CO2}, leaf conductance for water vapour, g_{H2O}, and internal CO_2 partial pressure, p^i_{CO2}, and relative quantum use efficiency of photosystem II, $rel\Phi_{PSII}$ (Fig. 8.18; Duarte 2006, Duarte and Lüttge 2006). Application of 1 % O_2 elicits non-photorespiratory conditions, and therefore, J_{CO2} at 1 % O_2 minus J_{CO2} at 21 % O_2 is a measure of photorespiratory oxygen uptake, J_{O2}. In the C_3-mode a change from 21 % to 1 % O_2 had the expected effects of increased g_{H2O} and CO_2 uptake and decreased p^i_{CO2}. Photorespiration was rather constant over the light period. In the CAM mode photorespiration depended on the CAM phases. In phase II the relative contribution of oxygenase activity to total RubisCO activity was about half that observed in the C_3-mode leaves (32.1–35.7 %) at the beginning of the light period but still 15.6 % of total RubisCO activity, i.e. the onset of the CO_2 concentrating effect reduced photorespiration but did not prevent it. In phase III due to low g_{H2O} there was little effect of 1 % O_2 and the calculated values of p^i_{CO2} are not very reliable and most likely a strong underestimation. At the beginning of phase IV photorespiratory activity was highest in all measurements and even higher than in the

C_3 mode (37.9 %) perhaps due to still high internal O_2 levels. RelΦ_{PSII} is a measure of the photosynthetic irradiance and excitation use. In the C_3-mode it was constant under 21 % O_2 throughout the day but strongly reduced under 1 % O_2 reflecting the particular energy demand of photorespiration (Osmond and Grace 1995; Heber et al. 2001; Heber 2002). Since relΦ_{PSII} was obtained by chlorophyll fluorescence imaging the measure of heterogeneity of relΦ_{PSII} over the leaves could be calculated using an algorithm based on the nearest neighbour matrix concept (Hütt and Neff 2001). In the C_3-mode leaf heterogeneity was generally low and constant under 21 % O_2, but applications of 1 % O_2 dramatically increased heterogeneity. This suggests that photorespiration synchronized the energy demand of different parts of the leaf and had a stabilizing effect on overall energy use of the leaves. In the CAM-mode leaf relΦ_{PSII} increased gradually at the beginning of the light period and decreased again in the late afternoon, in relation to phases II, III and IV (see also Sect. 8.8.1 and Fig. 8.25). As in the C_3-mode reduction of relΦ_{PSII} by 1 % O_2 was also observed in phases II and IV but by contrast to the C_3-adapted plants het-

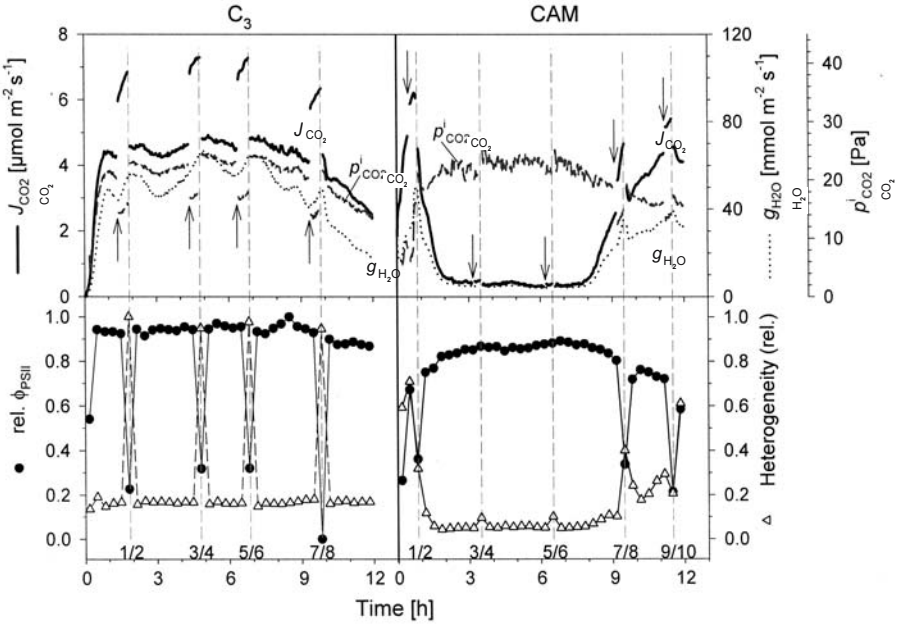

Fig. 8.18. Light period dynamics of photorespiratory activity as revealed by applications of air with only 1 % O_2 (*arrows*) causing non-photorespiratory conditions in plants of *C. minor* adapted to perform C_3-photosynthesis and CAM, respectively. J_{CO2}, net CO_2 exchange, g_{H2O}, leaf conductance for water vapour, p^i_{CO2}, internal partial pressure of CO_2, relΦ_{PSII}, relative quantum use efficiency of photosystem II and its heterogeneity over the leaves. The values obtained during 1 % O_2 (i.e. under non-photorespiratory conditions) minus the values obtained under 21 % O_2 (i.e. under photorespiratory conditions) reflect the effects of photorespiration (from Duarte 2006, Duarte and Lüttge 2006, same plants as in Fig. 8.25)

erogeneity was much more dependent on the CAM phases than on application of 1 % O_2 indicating that under 21 % O_2 heterogeneity is a particular feature of CAM and especially due to desynchronization of leaf parts occurring during CAM phase transitions.

8.6 High Light and Oxidative Stress

We have already mentioned above (Sect. 8.4) that the very high internal CO_2-concentrations prevailing in phase III of CAM at times of the day when solar irradiation tends to be particularly high were thought for a long time to protect from over-energization of the photosynthetic electron transport apparatus. This was even considered to be one of the forces driving the evolution of CAM (Gil 1986). In this hypothesis photosynthesis at CO_2 concentrations saturating RubisCO is assumed to utilize most excitation energy effectively for the photochemical work of CO_2 assimilation. Over-energization is accompanied by oxidative stress where excited electrons cause the formation of reactive oxygen species (ROS) which elicit photochemical damage. Hence, for their protection plants including CAM-species have developed various mechanisms for harmless dissipation of excitation energy not used by the photochemistry of CO_2 reduction as well as antioxidative mechanisms for detoxifying ROS (Lüttge 2000, 2002).

Based on the above hypothesis it was assumed that in CAM plants such mechanisms would be mainly relevant in phases II and IV but not so much in phase III. Indeed, this is suggested in experiments of Winter et al. (1990) on the CAM species *C. rosea*. Also in *C. uvitana* photosynthetic photon use efficiency was highest in phase III (Winter et al. 1992). One of the major mechanisms in plants for harmless dissipation of photosynthetic excitation energy is based on the xanthophyll zeaxanthin (Schindler and Lichtenthaler 1996). Zeaxanthin may divert excitation energy in photosystem II away from the central reaction centers (Horton et al. 1994). Zeaxanthin is also involved in dissipation of excitation energy in the form of heat and is the substrate for the formation of the epoxides antheraxanthin and violaxanthin by protective binding of singlet activated oxygen and consumption of reduction equivalents. Thus, the electrochemical proton gradient at the thylakoid membranes of chloroplasts is used when zeaxanthin is regenerated from the two epoxides in the so-called xanthophyll cycle (Hager 1980; Demmig-Adams 1990; Demmig-Adams and Adams 1992; Pfündel and Bilger 1994). This protective mechanism requires high zeaxanthin levels at times of high light stress. The measurements of Winter et al. (1990) show that in the early light period in phase II of CAM in *C. rosea* the level of zeaxanthin increased substantially over the much lower levels maintained in the dark period, decreased in phase III and subsequently increased again in phase IV. The levels of violaxanthin, the dou-

ble epoxide of zeaxanthin, showed the opposite development during the CAM phases. This supports high energy dissipation as heat in phases II and IV and low dissipation in phase III mediated by the xanthophyll cycle.

This protective thermal energy dissipation is reflected in non-photochemical quenching of chlorophyll fluorescence. The non-photochemical quenching factor, q_N is given as

$$q_N=1-(F_m'-F_o')/(F_m-F_o) \tag{8.5}$$

and non-photochemical quenching (NPQ) is also calculated by the Stern-Volmer equation

$$NPQ=(F_m-F_m')/F_m' \tag{8.6}$$

(Schreiber and Bilger 1993), where F_o and F_o' are the minimal fluorescence of the dark adapted and light adapted sample, respectively, and F_m and F_m' the maximum fluorescence of the dark adapted and light adapted sample, respectively. NPQ values observed in species of *Clusia* can be quite high, e.g. about 3 in *C. minor* and *C. multiflora* at a PPFD of 600–800 μmol m^{-2} s^{-1} (Herzog et al. 1999) or 5 in *C. minor* at 1500 μmol m^{-2} s^{-1} (Roberts et al. 1998). Somewhat lower values were observed for *C. minor* and *C. multiflora* at 1500 μmol m^{-2} s^{-1} by Grams et al. (1997), i.e. 1.5 and 1.8, respectively. The different activities of the xanthophyll cycle in the different CAM phases noted above are also corroborated by the observation of much lower non-photochemical quenching, q_N, of chlorophyll *a* fluorescence of photosynthesis in phase III than in phases II and IV, where non-photochemical energy dissipation is much higher (Fig. 8.19).

However, the relations of high light and oxidative stress in CAM plants generally are not that simple. These stresses always cause photoinhibition, which is reflected in a reduction of the potential quantum yield of photosystem II (PS II), F_v/F_m, where F_v is the variable and F_m the maximum fluorescence of chlorophyll *a* of PS II of a dark adapted leaf (Bilger et al. 1995). The protective mechanism of harmless dissipation of energy in the form of heat described above is a type of acute photoinhibition (Thiele et al. 1998). Chronic photoinhibition is obtained after photochemical damage. Non-photoinhibited leaves show F_v/F_m ratios close to 0.83 because the maximum efficiency of the use of photosynthetically active radiation (PPFD) is generally around 83 % (Björkman and Demmig 1987). Reduced values of F_v/F_m below 0.83 indicate photoinhibition which is chronic when they are not reversible over night and acute if they are not reversible after some time of darkening during the light period but recover over night (Thiele et al. 1998). A survey of *Clusia*s shows that they are often subject to both acute and chronic photoinhibition and that this is particularly prevalent in the CAM species compared to the C_3-species (Fig. 8.20). The C_3/CAM-intermediate *C. minor* allows comparisons for C_3-photosynthesis and

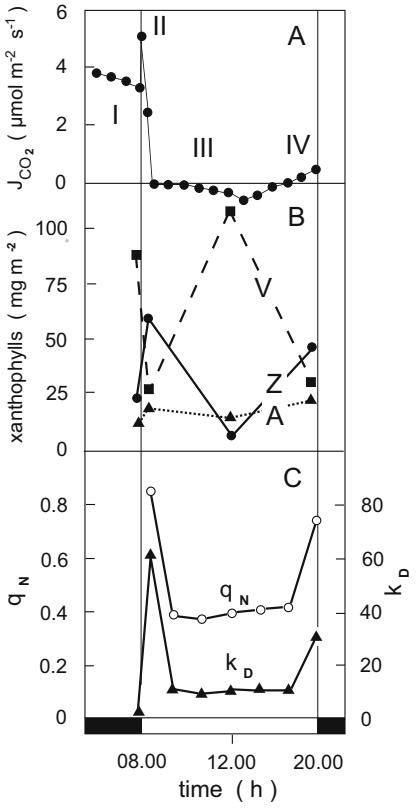

Fig. 8.19A–C. Changes in xanthophyll-cycle compounds in relation to the expression of CAM phases and non-photochemical quenching of chlorophyll *a* fluorescence of photosystem II in *C. rosea*: **A** CO_2-exchange (J_{CO2}), roman numbers refer to CAM phases; **B** xanthophyll-cycle compounds, Z=zeaxanthin, A=antheraxanthin, V=violaxanthin; **C** non-photochemical quenching, q_N, and rate constant of thermal energy dissipation k_D. *Dark bars* on the abscissa indicate dark periods (after data of Winter et al. 1990)

the CAM phases for the same individual plants or plants of clones obtained by vegetative propagation. As expected, acute photoinhibition is highest in phase IV of CAM. However, photoinhibition is not lower in phase III of CAM than in phase II of CAM or in the C_3-state (Fig. 8.21). A very important aspect of phase III that is overlooked by the hypothesis of a protection of leaves from high light and oxidative stress in phase III is the built up of very high internal O_2 concentrations in this phase as noted above.

The combined roles of CO_2-concentrating in phase III of CAM and zeaxanthin in photoprotection are well illustrated by a comparison of the behaviour of the C_3-species *C. multiflora* and the C_3/CAM intermediate species *C. minor* under light stress (Fig. 8.22; Herzog et al. 1999). The two species were grown at low irradiance of a PPFD of 4 mol m^{-2} day^{-1} in a glass house and then transferred to high irradiance of 24.5 or 33.5 mol m^{-2} day^{-1} with and without watering in a phytotron. After five days at the high irradiance of 24.5 mol m^{-2} day^{-1} the C_3-species *C. multiflora* was still capable of using the increased PPFD for increased CO_2 uptake independent of the water supply. However, at the highest irradiance of 33.5 mol m^{-2} day^{-1} it was highly inhibited. When the treatment under 24.5 mol m^{-2} day^{-1} was extended for a longer

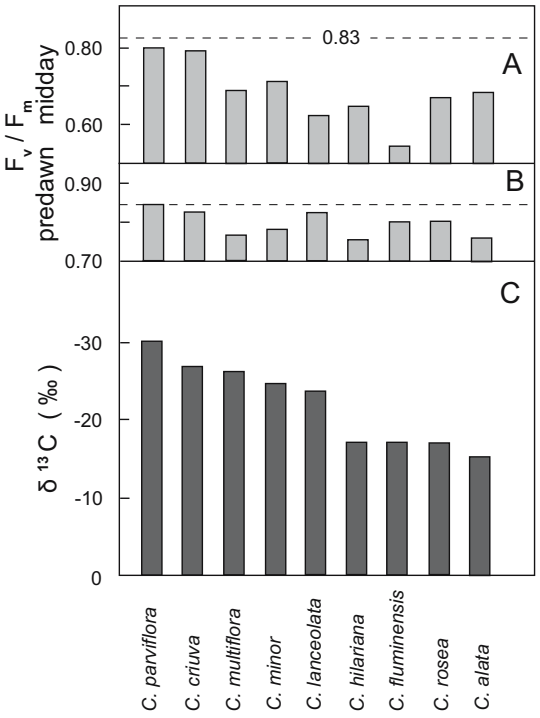

Fig. 8.20A–C. Potential quantum yield of photosystem II, F_v/F_m: **A** at midday after darkening for several tens of minutes; **B** before dawn for various species of *Clusia* with increasingly less negative $\delta^{13}C$-values (**C**) (see Fig. 8.7), i.e. C_3-photosynthesis to CAM performance, *from left to right*, where values below 0.83 indicate acute (**A**) and chronic (**B**) photoinhibition, respectively (Fig. 1B in Lüttge 1999 summarizing a large body of literature; species names not yet mentioned above with authorities are *C. criuva* Camb., *C. lanceolata* Camb.)

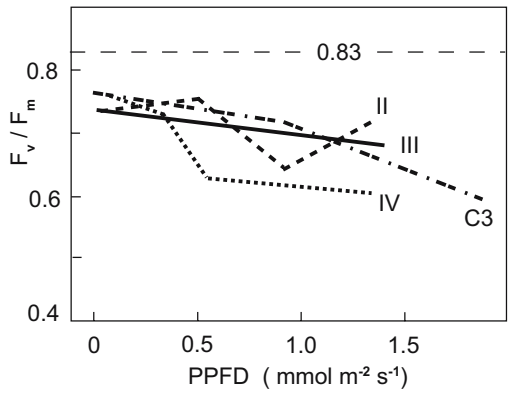

Fig. 8.21. Light response characteristics of acute photoinhibition given by measurements of potential quantum yield of photosystem II, F_v/F_m, in plants of the C_3/CAM-intermediate *C. minor* in the C_3 state (C_3) and in the CAM state during phases II, III and IV of CAM. Plants were kept at the irradiances indicated and darkened for 10 min before F_v/F_m was measured (after Lüttge 2000, with data after Haag-Kerwer 1994)

time leaves became necrotic and died. Conversely, *C. minor* switched to CAM when transferred to the high irradiance and leaves never became necrotic. As shown by F_v/F_m-values both species were not photoinhibited at the irradiance of 4 mol m^{-2} day^{-1}. Chronic photoinhibition at the beginning of the light period and acute photoinhibition building up during the light period at the higher irradiances were similar in both species. However, the responses of thermal energy dissipation via zeaxanthin were different

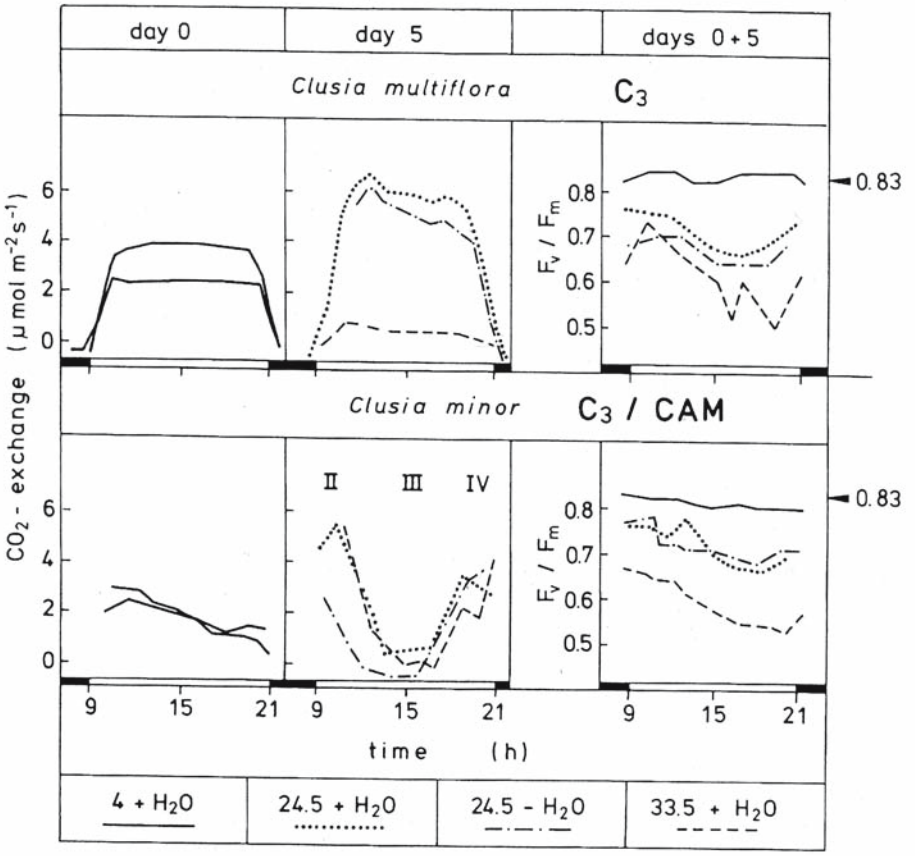

Fig. 8.22. CO_2-exchange and photoinhibition given by measurements of potential quantum yield of photosystem II, F_v/F_m, in the C_3-species *C. multiflora* and the C_3/CAM-intermediate species *C. minor.* Plants were grown at low irradiance and after day 0, the last day at low irradiance, they were transferred to two different high daily doses of irradiance applied in a bell-shaped time course (not shown) with additional stress due to withholding water in one case. The behaviour on day 0 (*solid lines*) and 5 days after the transfer (*other lines*) is shown; numbers at the bottom of the graph explaining the lines are daily doses of photosynthetic photon flux density (mol m^{-2} day^{-1}), roman numbers for gas exchange of *C. minor* on day 5 indicate CAM phases (after Lüttge 2000, with data of Herzog et al. 1999)

(Fig. 8.23). In the phytotron experiments irradiance was increased gradually towards noon to 1260 µmol m^{-2} s^{-1} and then decreased again in a bell shaped fashion over the light period. Accumulation of zeaxanthin and non-photochemical quenching of chlorophyll *a* fluorescence of PS II exactly followed irradiance and this was similar in both species. Short term exposures where different irradiances up to 1200 mol m^{-2} s^{-1} were applied for 20 min or where the irradiance of 1000 mol m^{-2} s^{-1} was applied for various times up to 80 min

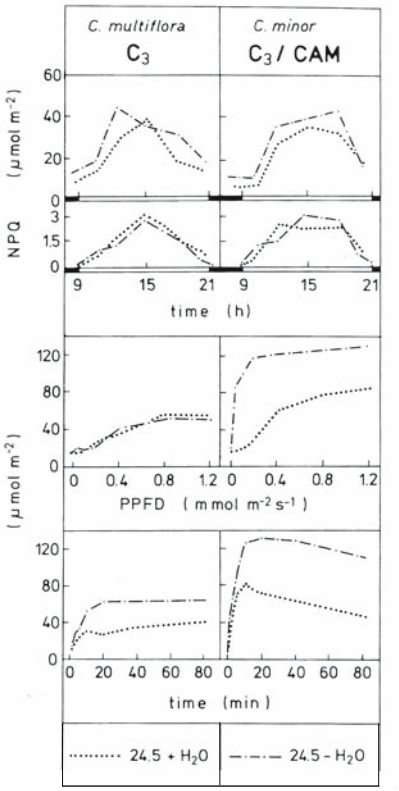

Fig. 8.23. Daily courses of zeaxanthin levels and thermal energy dissipation (non-photochemical fluorescence quenching, NPQ) in the plants of *C. multiflora* and *C. minor* also shown in Fig. 8.22 on day 5 after transfer to high daily doses of irradiance (24.5 mol m^{-2} day^{-1}) with and without drought stress as indicated (*two upper panels*). *Lower panels* show short term experiments with *C. multiflora* and with *C. minor* in the CAM-state, where photosynthetic photon flux density, PPFD, was presented for 20 min as indicated, or a PPFD of 1000 µmol m^{-2} s^{-1} was given for various times up to 80 min, as indicated, before analysis of zeaxanthin levels (after Lüttge 2000, with data of Herzog et al. 1999)

show, however, that the C_3/CAM-intermediate species *C. minor* intrinsically was able to accumulate about three times as much zeaxanthin as the C_3-species *C. multiflora*. However, it did not make use of its full capacity for zeaxanthin accumulation as shown by the day courses where it did not reach higher zeaxanthin levels than the C_3-species. Thus, we may conclude that it was the combination of the induction of CAM and thermal energy dissipation that lead to the overall superior performance of the C_3/CAM-intermediate species under the stress by increased irradiance.

8.7 Osmotic Implications of Night/Day Changes of Organic Acids and Soluble Carbohydrates

Malate/malic acid is an osmotically active solute. In the standard mode of CAM, where starch is broken down to PEP and malate is formed via PEPC and accumulated as malic acid in the vacuole this accumulation generates vacuolar osmotic pressure. Malate acts osmotically, while the accompanying

proton counterions do not count due to the buffering capacity of the vacuolar cell sap. It has been well documented in various CAM plants that nocturnal malic acid accumulation increases cell sap osmolarity, π. Water follows osmotically and this leads to a nocturnal increase in turgor pressure, P. Thus, nocturnal malate accumulation also supports nocturnal water storage (Lüttge 1986; Eller and Ruess 1986; Ruess et al. 1988; Eller et al. 1992; Murphy and Smith 1998).

In *Clusia*s these relationships are more complex due to the larger variety of osmotically active solutes involved. Accumulation of organic acids is only fully effective in increasing π when the precursor is osmotically inert starch, and then malate is twice as effective as citrate because two molecules of malate and only one molecule of citrate are formed per hexose unit of starch. When the precursor is sucrose one osmotically active molecule, i.e. sucrose, is lost while four molecules of malate or two molecules of citrate are produced, and in both cases the process would be increasing π, where again malate would be twice as effective as citrate. When the precursor is free hexose one osmotically active molecule, i.e. hexose, is lost while two molecules of malate or one molecule of citrate are produced, and therefore only in the case of malate the process would be increasing π, while in the case of citrate the bal-

Table 8.5. Diurnal changes of solute levels and calculated osmotic effects during the CAM cycle of various species of *Clusia* in the field. Dawn minus dusk values are presented for malate (Δmal), citrate (Δcitr) and free sugars (Δ free sugars, i.e. hexoses and sucrose) and the calculated osmotic effects (Δπ). Values are in mmol L^{-1} tissue water or kg^{-1} fresh weight

		Δmal	Δcitr	Δ free sugars	Δπ	Reference
C. alata	Dry season	83	77	–200	–40	Popp et al. 1987
	End of dry season	63	35	–73	25[a]	
C. rosea	Dry season	124	90	–176	38	
	End of dry season	240	112	–331	21[b]	
C. hilariana		96	74	–112	58	Berg et al. 2004
C.minor	Exposed leaves Wet season	10	85	–65	30	Borland et al. 1994
	Exposed leaves Dry season	110	61	–81	90	
	Shaded leaves Dry season	105	22	–49	78	
C. rosea		171	106	–160	117	Ball et al. 1991

Measured π was: [a]–46 mosmol L^{-1} and [b]+130 mosmol L^{-1}.

Table 8.6. Water potential, Ψ, osmotic potential, π , and calculated turgor pressure, P, (all in MPa), of *C. uvitana*. Averages of 40 measurements predawn and mid-day, obtained over a whole year with standard errors, SE. (Adopted from data of Zotz and Winter 1994b)

	Ψ	π	P
Predawn	–0.79±0.03	1.38±0.05	0.59
Mid-day	–0.71±0.03	1.26±0.04	0.55

ance is osmotically neutral. Thus, it depends on the balance of malate and citrate produced, which increases π, and free sugars consumed, which decreases π, to which extent the CAM cycle in *Clusia*s would affect osmotic activity of the vacuolar cell sap. A survey of data available in the literature is provided in Table 8.5. In all cases except one the balance is positive, i.e. the turnover of solutes would result in a nocturnal increase of π. However, in contrast to CAM-plants where only malate is accumulated and only starch is serving as precursor, the magnitude of this net osmotic effect in the *Clusia*s in Table 8.5 is much smaller than nocturnal organic acid accumulation, because besides starch free sugars also serve as precursors for acid synthesis. Zotz and Winter (1994a, b) have measured water potential, Ψ, and osmotic potential, π, in *C. uvitana* for over a year at the end of the night period (pre-dawn) and at noon and have not found significant changes of π and turgor pressure , P, calculated from Ψ=P–π (Table 8.6). We have no simultaneous analyses of solutes, i.e. organic acids and free sugars, for this study but we may assume that their balance was neutral in this case and thus there were no diurnal changes in π and P. In two cases of the compilation of data in Table 8.5 π was not only calculated from the theoretical solute balance but actually measured (footnotes in Table 8.5). The differences between calculated and measured π, of course, are explained by transpiration and water equilibration of the cells overriding consequences of changes in solute levels. This shows that when osmotically relevant changes in solute levels do occur they also have direct effects on the water relations of the plants.

8.8 Environmental Factors Regulating Reversible Changes Between the C_3 and CAM-Mode of Photosynthesis and the Degree of CAM Expression

The most important environmental parameters affecting the performance of C_3-photosynthesis and CAM, respectively, in *Clusia*s are water, light, temperature and mineral nutrition, especially nitrogen. After we have now surveyed

basic features of modes of photosynthesis and physiotypes in *Clusia*s (Sects. 8.1–8.6) and before turning to the ecophysiological implications (Chap. 9) we may review the action of these factors on the engagement of CAM. Mostly the environmental parameters do not act singly but interact between each other.

8.8.1 Water

Although it is thought that the environmental pressure originally driving the evolution of CAM was acquisition and concentrating of CO_2 at low ambient CO_2 concentrations (Lüttge 2002, 2004) among extant land plants the balance of water relations is the decisive ecophysiological benefit of CAM. C_3-species may lose considerable amounts of water during the light period and respond to stress by midday closure of stomata and repression of photosynthesis ("midday depression"). CAM plants show much less transpirational loss of water during CO_2-acqusition because evaporative demand is much lower in the dark period when they open stomata for CO_2-uptake. Of course, for daytime CO_2-uptake in phases II and IV of CAM transpirational loss of water will approach that of C_3 plants. C_3/CAM-intermediate plants may switch from C_3-photosynthesis to CAM in response to drought stress.

The relations of CO_2-gain and water loss are expressed as water use efficiency (WUE), i.e. CO_2 taken up and fixed per H_2O transpired. WUE strongly correlates with $\delta^{13}C$ values, which indicate the degree of CAM expression in plants and the nocturnal CO_2 gain (Winter and Holtum 2002; Winter et al. 2005). Table 8.7 gives some examples for WUE in *Clusia*s. The theoretical expectations described above are largely borne out by these experimental observations, i.e. that CAM performance leads to better WUE. However, the results compiled in Table 8.7 also indicate that fine-tuned reactions may be more complex. Night time WUE was always higher than day time WUE in the CAM-performing plants. However, in one experiment *C. minor* in the C_3-state had higher WUE than during the dark period of the CAM-state. When performing CO_2 fixation around the clock (see Fig. 8.2B), in one case *C. minor* as expected showed much higher WUE in the dark period than in the light period, but in another case WUEs were quite similar night and day. This reflects the high flexibility of *C. minor* in multi-factor responses under various experimental and environmental conditions as we shall also see several times again below.

Considering reactions in response to severe drought stress Borland et al. (1998) underlined the importance of genotypic differences in the capacity for CAM which are related to the capacities and amounts of PEPC and PEPCK increasing under stress. The *Clusia* species studied by these authors were categorized as follows: *C. aripoensis* Britt., weak CAM inducible; *C. minor*,

Table 8.7. Water use efficiencies (WUE=mmol CO_2 fixed/mol H_2O transpired[a] or g dry mass produced in growth/kg H_2O transpired[b]) in the light period (L) and the dark period (D) and both periods together for *Clusia* species with different modes of photosynthesis. (Names of species not yet mentioned above with authorities are as follows: *C. cylindrica* Hammel, *C. odorata* Seeman, *C. pratensis* Seeman, *C. valerioi* Standl.)

	L	D	L and D	Reference
C. rosea (CAM)			11.9[b]	Winter et al. 2005
C. uvitana (C3/CAM intermediate)			4.4[b]	
C. cylindrica			3.4[b]	
C. odorata			3.0[b]	
C. pratensis			2.0[b]	
C. valerioi			3.4[b]	
C. venosa (C_3 plus weak nocturnal acid accumulation)	7.1	2.8	6.9[a]	Franco et al. 1990
C. minor (CO_2 uptake around the clock)	6.4	33.8	8.8[a]	
C. major CAM	13.6	24.9	20.2[a]	
C. alata CAM	11.5	29.4	21.6[a]	
C. minor				Lee et al. 1989
C_3	38.8	–	38.8[a]	
CAM	8.0	22.9	23.6[a]	
CO_2-uptake around the clock	9.8	11.1	10.4[a]	

C_3/CAM intermediate; *C. rosea* constitutive CAM (note that these are the same plants also presented in Table 8.9 and discussed below). *C. uvitana* also showed a capacity to increase levels of PEPC under drought stress (Winter et al. 1992). Grams et al. (1998) used drought stress in order to check if even putative obligate C_3 species of *Clusia* may have at least weak capacities for CAM. *C. multiflora* emerged as truly obligate C_3, but the potential for CAM appears to be cryptically wide spread among *Clusia*s.

However, even a given genotype can show different reactions to environmental hydraulic parameters underlining flexibility and plasticity, which probably culminates in *C. minor.* Figure 8.24 shows that even the two opposite leaves at one node of a plant of *C. minor* can simultaneously perform in different ways depending on external conditions (Schmitt et al. 1988, where *C. minor* is wrongly called *C. rosea*). Each of the two leaves was kept in a separate gas exchange chamber. The plant was drought stressed for four days when both leaves showed a CAM pattern of CO_2-exchange. On the fifth day the plant was re-watered while one of the two opposite leaves was kept at a low leaf-to-air water vapour pressure difference (ΔW=6.2 mbar bar^{-1}) and the other leaf at a high ΔW (=13.1 mbar bar^{-1}), i.e. in a drier atmosphere in the gas exchange

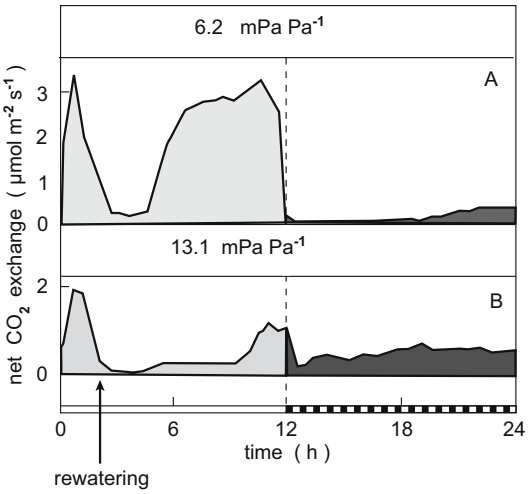

Fig. 8.24A,B. Net CO_2 exchange of two opposite leaves of *C. minor* exposed to two different leaf-to-air water vapour pressure differences, ΔW, of: **A** 6.2 mbar bar^{-1}; **B** 13.1 mbar bar^{-1}. The plant was droughted for several days and re-watered at the time indicated by the *arrow*. The *dark bar* indicates night-time (from Schmitt et al. 1988)

chamber. Only a few hours after re-watering the leaf in the wetter atmosphere changed to a very pronounced C_3-like CO_2 uptake in the light period and drastically reduced nocturnal CO_2 uptake in the following dark period, while the leaf in the drier atmosphere continued to perform full CAM.

Spatiotemporal resolution of the dynamics of photosynthesis using chlorophyll fluorescence imaging suggests that even different parts of one given leaf of *C. minor* may perform C_3-photosynthesis and CAM, respectively Duarte 2006, (Duarte and Lüttge 2006), so that *C. minor* perhaps really is the most flexible and plastic species in which photosynthesis ever has been studied. Figure 8.25 shows experiments, where plants of a clone of *C. minor* were kept at 25 °C night and day and an irradiance of 120 µmol m^{-2} s^{-1} during the day and were adapted to perform C_3-photosynthesis and CAM by watering regularly and by withholding water for three to four days, respectively. Net CO_2-exchange, J_{CO2}, conductance for water vapour, g_{H2O}, and internal partial pressure of CO_2 calculated from these parameters, p^i_{CO2}, integrated over the entire leaves showed the typical C_3- and CAM-characteristics. In the C_3-adapted plants these parameters increased rapidly at the beginning of the light period and were constant over most of the day, starting to decrease slightly towards the end of the light period. In the CAM-adapted plants J_{CO2} and g_{H2O} displayed the daytime phases of CAM with an early morning peak (phase II) followed by stomatal closure and an increase in p^i_{CO2} due to organic acid remobilisation and decarboxylation (phase III) and then again stomatal opening and net CO_2-uptake at the end of the light period (phase IV). Relative quantum yield of photosystem II, $rel\Phi_{PSII}$, as a measure of the use of irradiance and excitation energy could be calculated separately for the interveinal lamina tissue and for the major leaf vein from chlorophyll *a* fluorescence images. In the C_3-mode plants, $rel\Phi_{PSII}$ in the lamina follows J_{CO2}

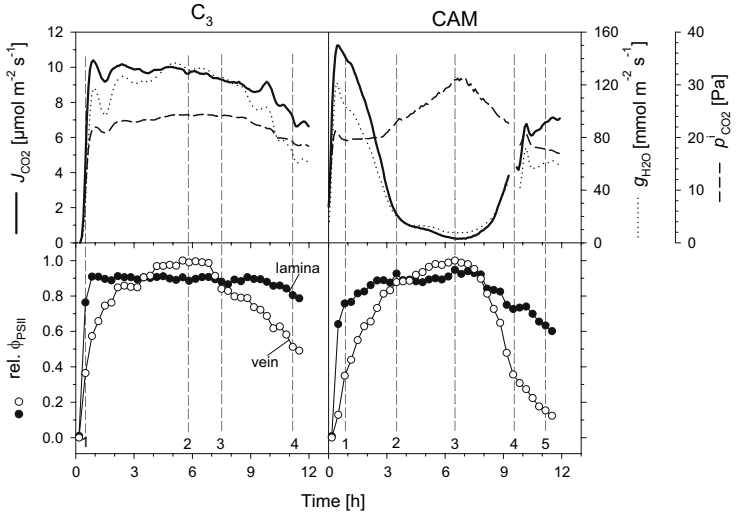

Fig. 8.25. Light period dynamics of photosynthesis parameters in plants of *C. minor* adapted to perform C_3-photosynthesis and CAM, respectively. J_{CO2}, net exchange of CO_2, g_{H2O}, leaf conductance for water vapour, and p^i_{CO2}, internal CO_2 partial pressure, integrated for the entire leaves. RelΦ_{PSII}, relative quantum use efficiency of photosystem II separated for the interveinal lamina tissue (*filled circles*) and the major leaf vein chlorenchyma (*open circles*) (from Duarte 2006, Duarte and Lüttge 2006, i.e. same plants as in Fig. 8.18)

and g_{H2O} with a rapid increase at the start of the light period. Conversely, in the CAM-mode plants relΦ_{PSII} in the lamina increases more slowly and gradually. This can be explained by the fact that in phase II of CAM both carboxylating enzymes PEPC and RubisCO are active in CO_2-fixation (Borland and Griffiths 1996), while only RubisCO carboxylation activity contributes to photosynthetic energy use. The decline of relΦ_{PSII} also is much faster in the afternoon when energy demand decreases with decreasing p^i_{CO2} in phase IV. In the CAM-mode this pattern of relΦ_{PSII} is even more pronounced in the major vein, and even higher values are reached in phase III of CAM than in the lamina tissue. Most interestingly, the vein tissue of the C_3-mode plants in contrast to their lamina tissue shows a very similar pattern to that of the CAM-mode plants. This suggests that in the C_3-mode plants a residual CAM-activity was maintained in the major vein tissue. This is also supported by anatomical observations showing a much more CAM-like anatomy of the vein chlorenchyma and lack of internal air spaces preventing a strong coupling with the lamina tissue by lateral gas diffusion (Sect. 2.2).

As an important response of CAM-species to strong drought stress we have described above internal CO_2 recycling culminating in the expression of full CAM-idling (Sect. 8.1), where CO_2 from respiration is recycled via nocturnal

acid accumulation and daytime decarboxylation behind closed stomata day and night. This now brings us back to considering the role of citrate in the CAM of *Clusia*, which remained enigmatic in the above considerations (Sects. 8.3.3, 8.4 and 8.6). Internal CO_2 recycling and CAM-idling is futile with respect to carbon gain. CO_2 can be recycled via malate (Sect. 8.1, Table 8.1). While citrate accumulation in CAM is always futile with respect to the carbon budget, and there is no carbon gain even if stomata are open and CO_2 exchange with the atmosphere is possible, recycling of carbon via citrate where the entire C_6-carbon skeleton is recycled is more effective than via malate (Sect. 8.3.3, Fig. 8.14). The various potential roles of citrate in CAM in comparison to malate are surveyed in Table 8.8. If recycling in response to drought really were a major advantage of citrate, we would expect the relative importance of citrate turnover to increase in relation to malate turnover, when plants are under drought stress. Table 8.9 summarizes some experimental and analytical results from the literature. Indeed, in many cases the ratios of day/night changes of malate (Δmal) and citrate (Δcitr) decreased in CAM performing species of *Clusia* under drought stress indicating an increasing relative contribution of citrate. However, this pattern was not observed in growth chamber studies of Borland et al. (1998), including *C. minor* which showed a drought elicited increase of Δmal/Δcitr. In the experiments of Borland et al. (1998), however, the Δmal/Δcitr ratios were already rather low without drought stress. Under drought stress absolute Δcitr values increased

Table 8.8. Possible roles of night/day changes in the levels of malate (Δmal) and citrate (Δcitr) during the CAM cycle (see Franco et al. 1992; Lüttge 1996)

	Δmal	Δcitr
CO_2 acquisition	Yes	No
H_2O saving during CO_2 acquisition	Yes	No
Recycling in the night time	CO_2	Carbon skeleton
Recycling in the day time	CO_2	CO_2, more than for malate
Increase in internal CO_2 concentration in the day time	Yes	Yes, more than for malate
Energy budget: dark period	ATP-consumption	ATP-consumption and production of redox power
Energy budget: light period	ATP-consumption	Larger ATP-consumption and consumption of redox power
Osmotic changes	Yes	Yes, less than for malate
Buffer capacity	Yes	Yes, higher than for malate

Table 8.9. Ratios of night/day changes of malate and citrate levels (Δmal/Δcitr) in *Clusia* species in response to drought. (Name of species not yet mentioned above with authority is *C. lanceolata* Camb.)

	Days of drought stress			Reference
	0	10	16	
C. lanceolata	2.8	1.7	1.6	Franco et al. 1992
C. rosea	2.4	0.7		
C. minor	1.8		0.9	
C. minor	5.5		2.1	de Mattos et al. 1999
C. minor				Borland et al. 1998
Young leaves	0.8	1.3		
Mature leaves	0.5	0.5		
C. rosea				
Young leaves	0.2	1.0		
Mature leaves	0.7	0.8		
C. aripoensis				
Young leaves	0.2	0.5		
Mature leaves	0.1	0.5		

slightly in mature leaves of *C. aripoensis* and pronouncedly in mature leaves of *C. minor* and in young leaves of *C. rosea*, but Δmal values also increased and in mature leaves of *C. rosea* Δmal and Δcitr did not differ with and without drought stress. As a result there were no reductions in Δmal/Δcitr-ratios in response to drought and perhaps even some increases. These discrepancies between results in the literature may be explained by the flexible multi-parameter responses of *Clusia*s under various experimental and environmental conditions. Much more strictly comparative work is needed if we really wish to understand the phenomenon of the often quantitatively important role of citrate in night/day oscillations of organic acid levels in the CAM of *Clusia*s. However, currently there does not appear to be much motivation for funding the study of such an exciting problem of biochemical ecology.

8.8.2 Light and Water

High irradiance is often acting in relation to the water factor. One example was described above discussing high light and oxidative stress in the C_3-species *C. multiflora* and the C_3/CAM-intermediate species *C. minor* after transfer from low to high irradiance (Sect. 8.6). In this experiment drought stress was also applied but high light stress alone was the major factor under

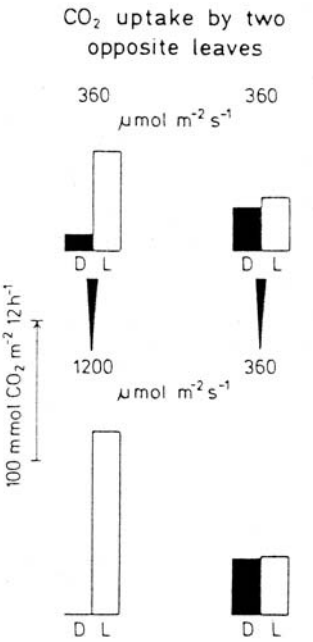

Fig. 8.26. Net CO_2 exchange of two opposite leaves on the same node of a well watered plant of *C. minor* at varied irradiance. One leaf was transferred from a PPFD of 360 µmol m^{-2} s^{-1} during the light period to a PPFD of 1200 µmol m^{-2} s^{-1}, while the other one was kept at 360 µmol m^{-2} s^{-1}. *Columns* give integrated net CO_2 uptake for the light period (L) and the dark period (D), respectively (from Lüttge 1996, after data of Schmitt et al. 1988)

the conditions applied (Figs. 8.22 and 8.23). Another example is given by two opposite leaves of a well watered plant of *C. minor* where one leaf was transferred from an irradiance of 360 µmol m^{-2} s^{-1} to a high irradiance of 1200 µmol m^{-2} s^{-1} while the other one was kept at the lower irradiance (Fig. 8.26). Nocturnal CO_2 uptake was abolished at the higher irradiance but remained at the lower irradiance. This elimination of PEPC mediated CO_2 fixation by a leaf of *Clusia* taking up CO_2 night and day by transfer to high irradiance only occurred in well watered plants. Under limited water availability the opposite effect, i.e. an increase in night time CO_2 uptake would have occurred. Blue light and UV-A radiation are involved in rapid switches from C_3-photosynthesis to CAM in *Clusia* (Grams and Thiel 2002).

8.8.3 Light and Temperature

It is widely assumed that night temperatures lower than day temperatures are favourable for the performance of CAM. This is based on the observation of effects of temperature on the overall performance of the counter acting enzymes of nocturnal carboxylation (PEPC) and daytime decarboxylation of CAM, where lower temperatures favour the former and higher temperatures the latter (Brandon 1967; Buchanan-Bollig and Kluge 1981; Buchanan-Bollig et al. 1984; Carter et al. 1995a). Stimulation of CAM expression by reduced

night temperatures has been confirmed in many experimental observations (Kluge et al. 1973; Neales 1973; Medina et al. 1977; Kluge and Ting 1978; Nobel 1988; Fetene and Lüttge 1991). High night temperatures decrease night-time acid accumulation because of respiratory consumption and loss of accumulated acid (Kaplan et al. 1976) or increased organic acid efflux from the vacuoles (Friemert et al. 1988). On the other hand, in wet tropical forests many CAM species perform well at rather similar night and day temperatures.

In *C. minor* day/night temperature regimes strongly affect C_3-CAM transitions dependent on irradiance during growth. In a very elaborate comparative study Haag-Kerwer (1994, summarized by Haag-Kerwer et al. 1992) measured gas exchange and night/day oscillations of malate and citrate in two sets of regularly well watered plants grown at a low irradiance (PPFD=photosynthetic photon flux density) of 30–50 µmol m^{-2} s^{-1} and a higher irradiance of 260–300 µmol m^{-2} s^{-1}, respectively, for 13 different combinations of day/night temperatures (D/N °C). At similar D/N-temperatures all plants performed C_3-photosynthesis. Plants consistently changed to a CAM pattern in response to

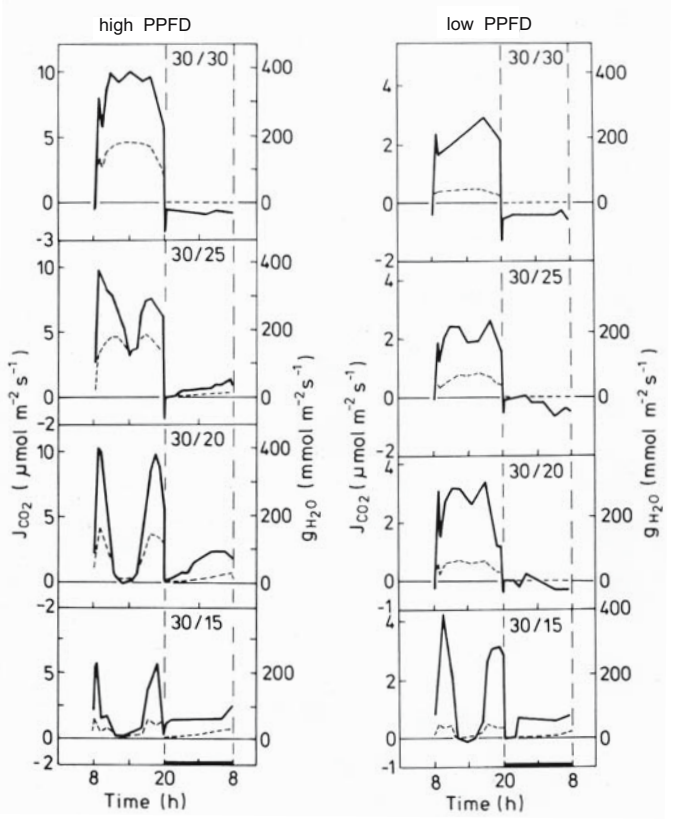

Fig. 8.27. Net CO_2 exchange (J_{CO2}, *solid lines*) and leaf conductance for water vapour (g_{H2O}, *dotted lines*) over a 24-h period for high (260–300 µmol m^{-2} s^{-1}) and low (30–50 µmol m^{-2} s^{-1}) PPFD grown *C. minor* at a day temperature of 30 °C and at various night temperatures. The *dark bars* indicate dark periods (Fig. 1 from Haag-Kerwer et al. 1992)

an increase in the difference between day and night temperatures. With a D/N temperature difference of 10 °C high-PPFD grown plants showed a substantial night-time CO_2 uptake for all temperatures tested, with a marked depression of daytime CO_2 uptake around midday. Low-PPFD grown plants on the other hand needed at least a D/N temperature difference of 15 °C to induce CAM (Fig. 8.27). These changes of the mode of photosynthesis in response to different temperature regimes were induced within a few hours and were fully expressed after three days. They were fully reversible after a return to identical D/N temperatures (Fig. 8.28). Malate and citrate accumulation in the CAM mode responded in different ways to the different temperature regimes. Evidently the temperature optimum for citrate accumulation is different from that of malate accumulation. The summary of the comparison for 13 combinations of D/N temperatures and 4 different parameters (leaf conductance for water vapour indicating stomatal width, CO_2 exchange, Δmal and Δcitr) each for two different growth temperatures of this study presented in Fig. 8.29 is so elaborate that we cannot describe and discuss it here in detail. Thus, we may leave it to the discretion of the respected reader which of the possible comparisons she or he may find particularly interesting. We may note, however, that this in fact provides the recipes for establishing plants of *C. minor* performing CAM with any combinations of nocturnal acids accumulated, e.g. of only malate accumulation (low PPFD, D/N temperatures of 30/15 °C), high malate and low citrate accumulation (high PPFD, D/N temperatures of 25/15 or 30/15 °C), equal malate and citrate accumulation (high PPFD, D/N temper-

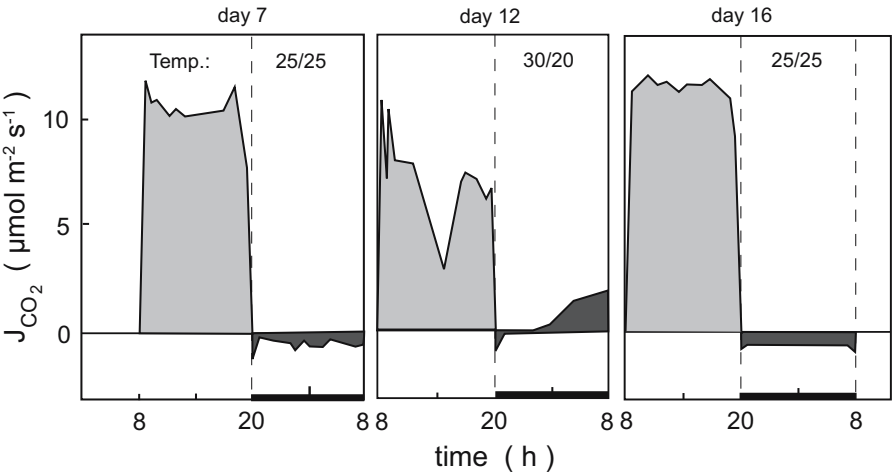

Fig. 8.28. Net CO_2 exchange measured continuously over 17 days for one given leaf of *C. minor* kept for 7 days at D/N temperatures of 25/25 °C, switched to D/N temperatures of 30/20 °C on day 7 and back to D/N temperatures of 25/25 °C on day 13. Recordings of the 7th, the 12th and the 16th day of the experiment are presented. The *dark bars* indicate dark periods (Fig. 3 from Haag-Kerwer et al. 1992)

atures of 25/20 °C), only citrate accumulation (low PPFD, D/N temperatures of 30/30 °C), and high citrate and low malate accumulation (high PPFD, D/N temperatures of 20/20 °C). If and how this may contain an explanation of the citrate enigma discussed above (Sect. 8.8.1) remains a question open to speculation.

Making use of the expression of CAM at D/N temperatures of 30/18 °C and a switch from CAM to C_3-phosotsynthesis under a D/N temperature regime of 30/30 °C, de Mattos and Lüttge (2001) studied the potential ecophysiological

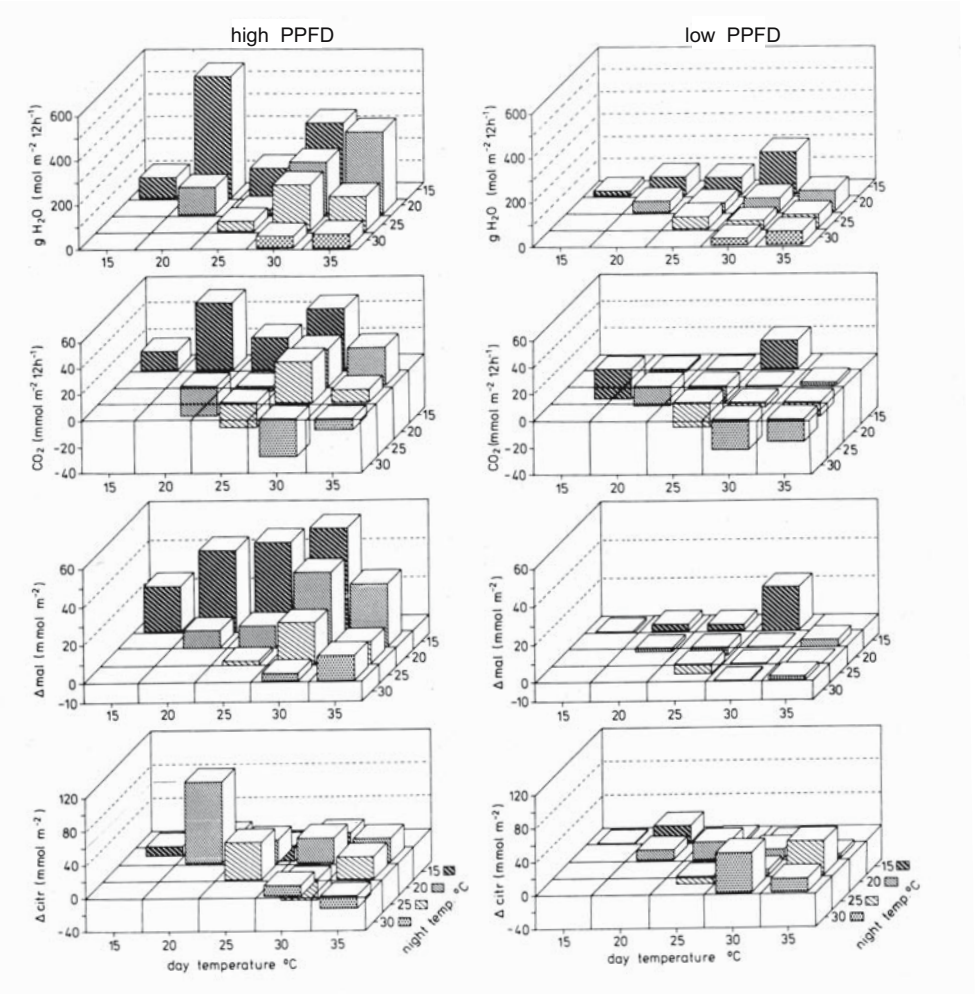

Fig. 8.29. Integrated values of night-time leaf conductance for water vapour (g_{H2O}), integrated net CO_2 exchange for the dark period (CO_2 mmol m^{-2} 12 h^{-1}) and nocturnal malate (Δmal) and citrate (Δcitr) accumulation for 13 different day/night temperature regimes in high PPFD (260–300 µmol m^{-2} s^{-1}) and low PPFD (30–50 µmol m^{-2} s^{-1}) grown plants (Fig. 4 from Haag-Kerwer et al. 1992)

advantage of C_3-photosynthesis over CAM in plants of *C. minor* when they were well watered. During the CAM to C_3 switch followed over seven days nocturnal organic acid accumulation decreased whereas CO_2-uptake during the daytime and integrated over 24 h increased. Water use efficiency was reduced. In contrast to the C_3-like photosynthesis, a pronounced reduction in the effective quantum yield of photosystem II together with a sharp increase in non-photochemical chlorophyll fluorescence quenching were observed during CAM at the beginning and the end of the light period. The results suggested that daily photon utilization increases when there is unrestricted C_3-like CO_2-uptake directly from the atmosphere. Thus, provided water is not limiting, and therefore the plant can afford increased transpiration, *C. minor* performing C_3-photosynthesis may overcome the limitations of the storage capacity of the vacuole for nocturnal organic acid accumulation, improving its daily carbon balance.

8.8.4 Light and Nitrogen

In *C. minor* grown at the lower and the higher PPFD of 60 to 70 and 220 to 320 μmol m^{-2} s^{-1}, respectively, the effect of nitrogen on the expression of the modes of photosynthesis was also investigated (Franco et al. 1991). High and low N-status, respectively, of the plants was maintained by regularly watering

Table 8.10. Effects of nitrogen nutrition (±24 mol m^{-3} NO_3^-) and photosynthetically active radiation (PPFD of 60–70 and 220–320 μmol m^{-2} s^{-1}, respectively) during growth on integrated net CO_2 exchange in the light period and the dark period and nocturnal organic acid accumulation (malate=Δmal, citrate=Δ citr) in *C. minor* (Franco et al. 1991)

	–N		+N	
	Low PPFD	High PPFD	Low PPFD	High PPFD
Integrated net CO_2 exchange (mmol CO_2 m^{-2} leaf area)				
Light period	62.7	59.6	65.8	209.0
Dark period	–4.0	– 0.8	–1.9	12.1
Δorganic acids (mmol m^{-2} leaf area)				
Δmal	–0.4	10.2	–0.2	99.9
Δcitr	24.9	45.9	41.8	32.5
Δorganic acids (mol m^{-3} tissue water)				
Δmal	–0.6	14.4	–0.3	150.7
Δcitr	39.1	64.6	68.9	49.0

with a solution containing 24 mol m^{-3} NO_3^- or no nitrogen. Only a weak nocturnal CO_2-uptake was observed in the high PPFD plants plus nitrogen, and in the other three treatments there was some loss of CO_2 during the night. In all treatments there was considerable nocturnal citrate accumulation which was increased by N-nutrition in the low PPFD plants and decreased in the high PPFD plants. Nocturnal malate accumulation was absent in the low PPFD plants and weak in the high PAR plants without nitrogen but was considerably increased by nitrogen supply in the high PPFD plants (Table 8.10). Here we have yet another example of differential effects of environmental parameters on nocturnal accumulation of malate and citrate (see Sects. 8.8.1 and 8.8.3), but which does not yet allow a clear ecophysiological explanation of the role of citrate.

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