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ORIGINAL ARTICLE

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Expression of crassulacean acid metabolism in *Clusia hilariana* Schlechtendal in different stages of development in the field

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Abstract Expression of crassulacean acid metabolism (CAM) in the obligate CAM-tree *Clusia hilariana* SCHLTDL. was studied in the restinga of Jurubatiba National Park, on the Atlantic coast of Rio de Janeiro state, Brazil, comparing plants at different developmental stages. Between young and mature plants there were trends of differences in six parameters, which are all related to CAM expression. From young to mature plants there were tendencies for a decrease of (1) the degree of succulence, (2) the degree of day/night changes of malic acid levels, (3) titratable acidity with nocturnal acid accumulation, (4) the degree of day/night changes of free hexoses with nocturnal break down, (5) effective quantum use efficiency of photosystem II at high photosynthetic photon flux density, and (6) protection from photoinhibition. These tendencies form a clear pattern which suggests that CAM was somewhat more pronounced in leaves of young plants than in leaves of mature plants. A developmental regulation may be involved. However, the observations are probably best explained by stress, since in the dry soils of the restinga young plants have no access to the ground water table while adult trees develop extensive root systems.

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Introduction

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation for CO_2-acquisition under stress, which is mediated by nocturnal dark-fixation of CO_2 via phosphoenolpyruvate carboxylase (PEPC) (Phase I of CAM sensu Osmond 1978). The malic acid produced via PEPC and stored in the vacuole overnight is remobilized during the subsequent day, decarboxylated and the recovered CO_2 assimilated via ribulose-bis-phosphate carboxylase/oxygenase (RuBISCO) and the Calvin cycle in the light behind closed stomata (Phase III of CAM). In some CAM species, especially in *Clusia*, diurnal oscillations of malate are accompanied by day/night changes of citrate levels (Lüttge 1988).

The expression of CAM is often age dependent. Even in obligate CAM species of the genus *Kalanchoë* CAM increases with leaf age and is only fully expressed in mature leaves (Kluge and Ting 1978). All CAM plants possess flexibility because during transition phases, i.e. Phase II in the morning and Phase IV in the afternoon, direct fixation of atmospheric CO_2 via RuBISCO is also possible, of which CAM plants can make use to smaller or larger extent, depending on environmental conditions. However, there are also many true C_3-photosynthesis/CAM intermediate species. In the annual Aizoaceae *Mesembryanthemum crystallinum* it is a developmental programme which drives a switch from C_3-photosynthesis to CAM as plants age, and this is strongly enhanced by the environmental stress of drought and salinity (Cushman and Bohnert 2002). In the genus *Clusia*, comprising perennial neotropical shrubs and trees, there are also very many C_3/CAM intermediate species. A developmental programme driving a C_3/CAM switch in one direction has not been considered appropriate for the leaves of these plants which are used for several seasons and need to adapt repeatedly to varying environmental conditions.

Indeed, a versatile reversible C_3-CAM-C_3 switch was observed in C_3/CAM-*Clusia*s (Lüttge 1999, 2000; Mattos and Lüttge 2001). Conversely, in the obligate CAM species *C. rosea* Ting et al. (1985) and Sternberg et al. (1987) observed CAM expression especially in juvenile epiphytic plants and less in adult plants. It is not clear if this was due to a developmental programme or to the change of life form from epiphytic to soil-rooted, and hence ecophysiological stress. Moreover, it was not confirmed later, since adult trees of many *Clusia* species including *C. rosea* are excellent CAM-performers (Popp et al. 1987; Lüttge 1999). Wanek et al. (2002) found considerable diurnal acid fluctuations in seedlings of *C. osaensis* and *C. valerii* in a lowland rain forest of Costa Rica that were due to internal CO_2-recycling ("CAM-idling"), and CAM activity increased in both species with age.

To further test the possible operation of a developmental programme in regulating CAM expression in an obligate CAM-*Clusia* under natural environmental conditions in the field we undertook the present study on the CAM-*Clusia C. hilariana* Schlechtendal in the coastal sand dune vegetation of the restinga of Jurubatiba National Park, Rio de Janeiro State, Brazil. A comparative ecophysiological investigation was performed selecting four different developmental stages, namely early growth (seedlings or clonal saplings, which could not be distinguished), young plants, mature plants and senescent plants.

Materials and methods

The study site was the dry restinga of the sand dunes of Jurubatiba National Park at the Atlantic coast of the state of Rio de Janeiro, Brazil (22°00′–22°23′S, 41°15′–41°45′W). The mean annual precipitation is 1,165 mm with a pronounced seasonal distribution showing a minimum in June (40 mm) and a maximum in December (190 mm). The mean annual temperature is 22.6°C with monthly minima and maxima of 20.0°C and 29.7°C. The present study was performed between August and December 2001.

Different developmental stages of the obligate CAM-tree *C. hilariana* were studied, namely early growth, where seedlings and clonal saplings could not be distinguished, young plants, mature plants and senescent plants.

Samples were collected at dawn and dusk. After taking fresh mass (FM), leaf discs were stored on dry ice until further treatment in the laboratory, where the material was killed in a microwave oven for 3–10 s. Samples were then dried at 80°C to constant mass, weighed, ground and extracted with water (10 mg dry mass per ml distilled water) for 1 h at 97°C. Succulence was taken to be the ratio of FM: area. Analyses of malate and citrate were performed enzymatically in aqueous extracts of the microwave-dried leaf samples after

Fig. 1 Developmental stages of *C. hilariana* studied in the restinga of Jurubatiba National Park, Rio de Janeiro state, Brazil. **a** Early growth, **b** young, **c** mature, **d** senescent plant

Table 1 Morphological features of the four different developmental stages of *C. hilariana* studied (mean ± SD)

Developmental stage	Height (m)	Ground cover (m^2)	Circumference (m)	*n*
Early growth	0.68±0.14	0.11±0.09	1.21±0.50	23
Young	1.49±0.20	3.03±1.12	6.13±1.17	5
Mature	3.79±0.80	30.72±12.13	19.40±3.74	5
Senescent	3.79±1.02	47.43±14.19	24.23±3.88	5

Hohorst (1965) and Möllering (1985), respectively. Titratable acidity was obtained in hot water extracts of leaf samples stored at −18°C before use by titration against 0.01*N* NaOH to pH 8.4 (Lüttge 1988). Soluble sugars were analysed enzymatically according to Bergmeyer and Brent (1974). Starch within the pellet was determined as described in Orthen (2001).

Chlorophyll fluorescence was measured using the portable pulse-amplitude modulated fluorometer Mini-PAM of H. Walz, Effeltrich, Germany. The leaf-clip holder coming with the instrument kept the fiber optics at an angle of 60° and a distance of 10 mm from the leaf surface (Bilger et al. 1995). Photosynthetic photon flux density (PPFD) at λ=400–700 nm was measured with a microquantum sensor of the leaf-clip holder calibrated against a Li-COR quantum sensor (LI-COR, Neb., USA). Effective quantum yield of photosystem II (PS II), $\Delta F/F_m'$ was obtained from instant measurements under actual environmental conditions in the field as well as from light curves. The latter were taken from leaves of plants in the field using the light-curve programme of the Mini-PAM, where light intensity was increased in eight steps with 30 s intervals. Potential quantum yield of PS II, F_v/F_m, was measured after darkening the leaves for 10 min at midday to check possible acute photoinhibition indicated by F_v/F_m-values below 0.80 (Björkman and Demmig 1987). The symbols used above refer to ΔF, variable fluorescence of a light-adapted leaf, where $\Delta F = F'_m - F$, F_m' is the maximum and F the minimum or steady state fluorescence of a light-adapted leaf; F_m is the maximum and F_v is the variable fluorescence of a dark-adapted leaf; $F_v = F_m - F_0$, where F_0 is the minimum fluorescence of a dark adapted leaf (Genty et al. 1989; Van Kooten and Snel 1990). F_m and F_m' were determined under saturating light flushes of 600 ms duration.

Results

Morphological features of the four different developmental stages of *C. hilariana* selected for this study are shown in Fig. 1 and Table 1. Early growth refers to seedlings or clonal saplings which could not be distinguished in the field. Naturally, early growth and young plants are much smaller than mature and senescent plants. The latter two are not significantly different in height, ground cover and circumference (Table 1) but are easily distinguished by the much reduced density of the canopy due to drying branches in the senescent trees (compare Fig. 1c,d). Leaf size was not significantly different between young and mature plants but both tended to have a larger leaf area than early growth although not of statistical significance due to the large standard deviation of early growth leaves (Table 2). Succulence (Table 2) tended to be smaller in mature and senescent leaves than in young leaves.

The degree of CAM expression was assessed by analysing night–day changes of both malate and citrate levels and titratable acidity (dawn minus dusk values of determinations). The leaves of plants of the four different developmental stages showed marked dawn/dusk changes in malate and citrate content as well as titratable acidity (Table 3). Although individual comparisons are not statistically significant, there was a trend with the strongest expression of CAM in the early growth and the young plants and a continuous decline towards the mature and senescent plants. With this tendency the expression of CAM was related to the degree of succulence (Table 2). This trend of the degree of CAM expression in young versus old plants may even be underestimated here because (due to logistic problems) samples of the former were taken later in the morning (1100 hours) and earlier in the afternoon (1600 hours) than those of the latter (0900 and 1700 hours).

Carbohydrates are precursors for the glycolytic formation of PEP as a CO_2-acceptor for dark fixation via PEPC. Dawn/dusk changes of the levels of free hexoses and starch (in hexose units) are shown in Table 4. They show trends related to those of the changes of succulence and acidity, dawn/dusk changes of free hexoses are larger in young plants than in early growth, mature and senescent plants.

Data of effective quantum yield of PS II, $\Delta F/F_m'$, of the four developmental stages of *C. hilariana* obtained in measurements during diurnally varying PPFD in the field and from light curves using the light-curve programme of the Mini-PAM are compiled in Fig. 2. The regression lines plotted separately from the data in the bottom panels of Fig. 2, again show a trend that at higher PPFD under natural conditions (left panels) the early growth and the young plants and in the light-curve programme (right panels) the young plants performed better, i.e. had a higher $\Delta F/F_m'$ than the mature and senescent plants.

Acute photoinhibition was assessed by measuring potential quantum yield of PS II, F_v/F_m, at midday (1200–1300 hours) after darkening the leaves for 10 min on 2 consecutive days with very different irradiance due to

Table 2 Leaf sizes and leaf succulence of the four different developmental stages of *C. hilariana* studied (mean ± SD)

Developmental stage	Length (cm)	Width (cm)	Area (cm^2)	Succulence (kg FM m^{-2})	*n*
Early growth	10.8±1.5	5.2±0.8	41.1±11.4	1.48±0.10	23
Young	12.0±1.0	6.3±0.4	54.8±6.2	1.58±0.11	5
Mature	11.8±0.6	6.5±0.2	55.4±3.8	1.39±0.03	5
Senescent	11.4±0.5	6.1±0.6	50.3±6.9	1.39±0.04	5

Table 3 Dawn/dusk changes (Δ) of the levels of malate, citrate and titratable protons of the four different developmental stages of *C. hilariana* studied (mean ± SD)

Developmental stage	Δmalate (mmol m^{-2})	Δcitrate (mmol m^{-2})	$\sum 2\Delta$malate + 3Δcitrate (mmol m^{-2})	ΔH^+ (mmol m^{-2})	*n*
Early growth	182.8±64.5	93.4±16.7	645.7±158.3	655.4±149.3	12
Young	161.4±39.8	120.4±29.0	684.1±149.1	687.5±186.5	5
Mature	134.1±39.5	103.2±12.5	577.9±112.1	492.4±106.0	5
Senescent	100.4±74.5	77.4±39.6	432.9±264.8	380.9±275.2	5

Table 4 Dawn/dusk changes (Δ) of the levels of free hexoses and starch and their relation to dawn/dusk changes in organic acid levels of the four different developmental stages of *C. hilariana* studied (mean ± SD)

Developmental stage	Δhexoses (mmol m^{-2})	Δstarch (mmol m^{-2})	$\sum$ (Δhexoses + Δstarch) (mmol m^{-2})	$\sum$ [(0.5) Δmalate +Δcitrate] (mmol m^{-2})	*n*
Early growth	−154.7±13.6	−23.4±9.7	−178.1	184.8	12
Young	−184.8±28.8	−22.4±4.0	−207.2	201.1	5
Mature	−154.6±19.8	−24.3±6.4	−178.9	170.3	5
Senescent	−94.0±29.8	−21.8±15.2	−115.8	127.6	5

different cloud cover. On the bright day all plants showed clear photoinhibition (F_v/F_m-values below 0.80) which tended to be less pronounced in the young and mature plants (although not statistically significant). On the clouded day photoinhibition was lower (Table 5).

Discussion

It was reported recently that in fast growing tropical trees like *Hyeronima alchorneoides* (Euphorbiaceae), leaf size may change considerably with tree age with young trees having considerably larger leaf area, which may have implications for ecological functioning (Reich et al. 2002). For *C. hilariana*, however, in this study we did not observe such a trend; if anything, early growth leaves were smaller.

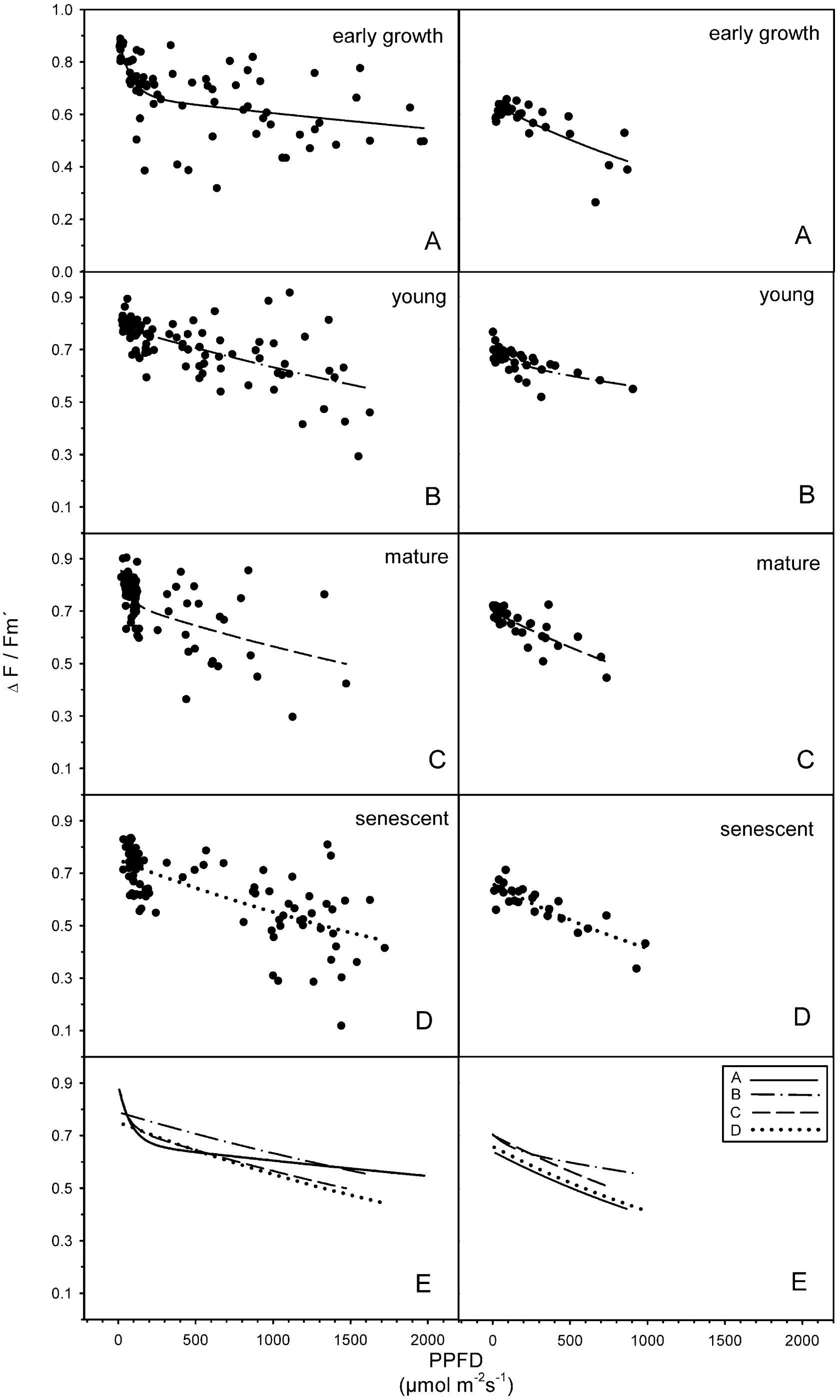
On the other hand, there were trends between developmental groups of *C. hilariana* distinguished here of six physiological parameters all related to the expression of CAM and all pointing in the same direction. Thus, although individual comparisons of averages for the various parameters of the different plants of the age classes compared in this field study were not statistically significant, these trends form a clear pattern. From young to old leaves (1) the degree of succulence, (2) the expression of CAM as given by day/night changes of malic acid levels, (3) titratable acidity, (4) the day night changes of free hexoses possibly serving as precursors for the formation of PEP required in nocturnal fixation of CO_2 in CAM, (5) effective quantum use efficiency at high PPFD, and (6) protection from photoinhibition all show a trend that decreases from young to mature leaves. The methods and approaches required to measure these parameters relied on different techniques. The general patterns obtained agree well with the accepted relations of CAM that (i) a certain degree of succulence is required with the vacuoles serving nocturnal storage of acids, (ii) day-time remobilization of CO_2 from the organic acids leads to high internal CO_2-concentrations in the leaves behind closed stomata (Lüttge 2002) which support high quantum use efficiency of photochemical work especially during hours of high irradiance, and (iii) the high effective quantum use of CO_2-reduction at high internal CO_2-concentrations is contributing to protection from photoinhibition (Herzog et al. 1999; Lüttge 2002).

Stoichiometries of day/night changes of solutes observed agree with CAM performance. Since malate is a dicarboxylate and citrate a tricarboxylate, the night–day changes of titratable acidity should be $\Delta H = 2\Delta\mathrm{malate} + 3\Delta\mathrm{citrate}$. The data match reasonably well with this expectation (Table 3). While the accumulation of citrate in addition to malate has been demonstrated for all CAM-performing *Clusia* species tested so far (e.g. *C. alata*, *C. rosea* Popp et al.1987, *C. minor*, *C. lanceolata* Franco et al. 1992, *C. hilariana* Franco et al. 1996) only three studies addressed the question of the carbon source utilized for night time acid accumulation (*C. alata*, *C. rosea* Popp et al. 1987, *C. rosea* Ball et al. 1991, *C. rosea*, *C.* sp. Franco et al. 1994). From the nocturnal breakdown of one hexose unit stoichometrically two molecules of malate but only one molecule of citrate can be formed, i.e. 1 hexose gives 2 PEP, which plus 2 CO_2 gives 2 malate; and 1 C-6 hexose gives 1 C-6 citrate (Lüttge 1988). Thus, $\sum$ (Δhexoses + Δstarch) should be equal to $\sum$ [(0.5) Δmalate + Δcitrate] when free hexoses and starch are the only precursors for nocturnal acid formation. For *C.*

Table 5 Potential quantum yield, F_v/F_m of PS II (mean ± SD) of the four different developmental stages of *C. hilariana*, at midday (1200–1300 hours) after 10 min darkening on 2 consecutive days with different PPFD [mean ± SD (*n*)]

Developmental stage	PPFD	
	950 µmol m^{-2} s^{-1}	220 µmol m^{-2} s^{-1}
Early growth	0.69±0.05 (19)	0.76±0.02 (22)
Young	0.72±0.04 (20)	0.76±0.02 (22)
Mature	0.73±0.07 (10)	0.77±0.05 (23)
Senescent	0.68±0.06 (20)	0.74±0.04 (21)

Fig. 2 Effective quantum yield, $\Delta F/F_m'$, of PS II of the four developmental stages of *C. hilariana* studied. **A** Early growth, **B** young, **C** mature, **D** senescent plants. **E** Regression lines for **A**–**D**. *Left panels*: measurements during diurnally varying PPFD in the field; *right panels*: measurements of light curves



hilariana we show here that this is well met by the data (Table 4), and hence, dawn/dusk changes of hexoses and starch also match the degree of CAM expression, which is highest in young plants and early growth and declines in mature and senescent plants (Tables 3,4). However, in *C. hilariana* free hexoses play the dominant role. The data demonstrate that in leaves of *C. hilariana* carbon skeleton demand for acid synthesis can be mostly supplied by the breakdown of soluble sugars. The nocturnal breakdown of starch might contribute between 11% (young plants) and 17% (senescent plants) to the demand for acid synthesis.

Zotz and co-workers (Zotz 1997,2000; Schmidt et al. 2001; Zotz et al. 2001) showed that increase in size in epiphytes is generally accompanied by changes in morphological as well as physiological characteristics. *C. hilariana* is also found as a strangler hemiepiphyte in the mata atlântica rain forest that is contiguous to the restinga (Scarano 2002). Additionally to a clear flexibility in life form according to epiphytic or terrestrial habitat type, the trends observed for the expression of CAM in *C. hilariana* in the present study may also indicate a relation to the developmental stage. However, it is equally possible, and perhaps even more likely that it is related to stress. The soil in the restinga can be extremely dry at times. The young plants have no access to the ground water table, while the adult trees develop an extensive far reaching root system.

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