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# Nocturnal sap flow in the C$_3$-CAM species, *Clusia minor*

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**Abstract** *Clusia minor* L. is a C$_3$-CAM species in which Crassulacean acid Metabolism (CAM) is induced, among other factors, by water deficit. We propose that CAM induction by natural drought in *C. minor* shifts the sap flow pattern from daytime to a night-time one, and that the decreased osmotic potential due to increased malate content in droughted plants aids in the increase in nocturnal sap flow. In order to test these hypotheses, we followed for 2 years the seasonal changes in parameters of water relationships and sap flow velocity in one single, freestanding tree growing in Caracas. Leaf water and osmotic potential were measured psychrometrically, nocturnal proton accumulation by titration of aqueous leaf extracts and sap flow density with thermal dissipation probes. Leaf water, osmotic and turgor potential remained relatively high throughout the seasons. Nocturnal proton accumulation was nil under extreme drought or after frequent and heavy rains, and high after moderate rainfall. Estimated malate and citrate concentrations contributed up to 80 and 60%, respectively, of the value of osmotic potential. The shape of the daily courses of sap flow velocity varied seasonally, from mostly diurnal during the dry season to mostly nocturnal after a short dry spell during the rainy season, when nocturnal acid accumulation attained high values. There was a strong positive relationship between the proportion of the integrated sap flow courses corresponding to the night and dawn [H$^+$] ($r^2 = 0.88$). Increased nocturnal sap flow in the CAM stage of the tree of *C. minor* may be explained by a lower osmotic potential due to an increased acid concentration, together with increased stomatal aperture, as suggested by increased nocturnal acid accumulation probably due to nocturnal CO$_2$ fixation.

## Introduction

The occurrence of Crassulacean acid Metabolism (CAM) in the genus *Clusia* (Clusiaceae) is well documented; *Clusia* is the only dicotyledonous tree genus known to possess CAM and C$_3$-CAM species (Lüttge 2006).

Plants of *C. minor* are evergreen small freestanding trees, hemi-epiphytes or strangler holo-epiphytes; they live in a broad range of environments, from forests to savannas, and from the forest floor to tree branches (Lüttge 2006). In *C. minor* CO$_2$ fixation is best characterised as C$_3$-CAM; watered plants of *C. minor* show nil nocturnal acid accumulation ($\Delta$H$^+$), while a short period of drought is capable of inducing the appearance of nocturnal CO$_2$ fixation with concomitantly high values of $\Delta$H$^+$ (Schmitt et al. 1988). CAM is extremely plastic in this species; in one single branch, leaves subjected to drier air performed CAM, whereas the opposite leaves, under humid air, did not (Schmitt et al. 1988). The shift between C$_3$ photosynthesis and CAM is rapid and plastic under both field and controlled conditions (Borland et al. 1996; Haag-Kerwer et al. 1996; Schmitt et al. 1988; Mattos et al. 1999; Mattos and Lüttge 2001).

In plants of the C$_3$-CAM hemi-epiphyte *C. uvitana* growing on a *Ceiba* tree, the seasonal variation in leaf

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water potential ($\psi$), leaf osmotic potential ($\psi_s$) and turgor potential ($\psi_T$) was small and little influenced by water availability or evaporative demand; $\Delta H^+$ was high under mild drought and nil after prolonged drought or rains (Zotz and Winter 1994).

It is currently accepted that CAM is an adaptation to dry environments that helps the plant attain higher water use efficiency than $C_3$ or $C_4$ plants (Winter 1985). CAM may not only serve as a water-saving adaptation but also as a water-collecting mechanism. In droughted plants of the CAM species *Senecio medley-woodii*, water uptake was correlated with an increase in morning osmotic pressure and malate content (Ruess and Eller 1985; Ruess et al. 1988); also, the accumulation of malate in the vacuole of *Kalanchoe daigremontiana* caused a reduction in $\psi_s$, which may favour water absorption (Smith and Lüttge 1985).

Not only malate but also large amounts of citrate accumulate in *C. minor* and citrate accumulation has been suggested to act as a protection mechanism against light or water stress (Franco et al. 1992).

Daily courses of gas exchange in *C. minor* under moderate irradiance and low leaf-air water vapour gradient showed the four phases of CAM defined by Osmond (1978), with 24-h $CO_2$ uptake and open stomata (Lee et al. 1989; Franco et al. 1990; Lüttge 2006). Changes in magnitude and daily pattern of sap flow velocity ($V$) reflect changes in transpiration, provided sap flow is driven by transpiration rather than replenishment of reservoirs. In trees of tropical $C_3$ woody species, sap flow continued through the night and was accompanied by substantial stomatal conductance (Bucci et al. 2005). Therefore, we can expect to evidence the four phases of CAM in daily courses of $V$.

Since in plants of *C. minor* with CAM-induced stomatal aperture occurs mostly during all phases but III (Franco et al. 1990, 1991, 1992; Borland et al. 1996; Zotz et al. 1997; Mattos et al. 1999), sap flow must be faster during the night and the first morning hours than during the day. Nevertheless, up to 4% of daily carbon balance has been found in the field to occur during phase IV (Ball et al. 1991). In addition, acids accumulated during the night would cause a decrease in $\psi_s$; therefore, the magnitude of the soil–plant–atmosphere water potential gradient in plants with CAM induced would favour water absorption during the night and on phase II. The $CO_2$ uptake and acid content in *C. minor* has been shown to be high on phase II for as late into the daytime as 12 h.

We propose that CAM induction by natural drought in *C. minor* shifts the sap flow pattern from daytime to a nighttime one, and that the decreased $\psi_s$ due to increased organic acid content in droughted plants aids in the increase in nocturnal sap flow. In order to test these hypotheses, we followed for 2 years the seasonal changes in water relations and $\Delta H^+$ and for 1 year the changes in $V$ in one single tree growing in the garden in Caracas.

## Materials and methods

### Study site and plant material

One freestanding individual of *C. minor* L., approximately 3-m-high with a 13-cm diameter at breast height, growing in the gardens of the Instituto de Biología Experimental (IBE) in Caracas (10°30′N–66°55′E) at an altitude of 1,000 m, was sampled from March 2003 until August 2004, covering two dry and two rainy seasons. Daily values of rainfall in Caracas were obtained from the Ciudad Universitaria weather station (Universidad Central de Venezuela, Caracas), at approximately 4 km from the IBE. The tree of *C. minor* was not watered throughout the period of study. Air RH, air temperature and total radiation (TR) were collected by a weather station mod. Vantage Pro (Davies Instruments, Hayward, CA, USA) at IBE. The crown of the tree of *C. minor* received an average of 20% of TR incident on the taller trees of *Ficus obtusifolia* and *Hura crepitans* that shaded the tree of *C. minor*; leaves of *C. minor* in the upper canopy received direct sunshine for a small portion of the day.

### Water relations

Morning $\psi$ was measured at 06.30 h placing one leaf disk ($n = 6$, different leaves every time) in each of six C52 chambers previously calibrated with NaCl solutions of known osmolality and connected to an HR33T psychrometer (Wescor Inc., Lincoln, UT, USA); measurements were made at $25 \pm 1°C$ after a 30-min equilibration time. The $\psi_s$ was determined likewise in the same disks frozen in liquid nitrogen. Leaf water content (LWC) was determined in leaf disks ($n = 6$) collected between 06.30 and 07.00 h and between 17.00 and 18.00 h, weighed fresh and dried at 60°C for 72 h.

### Nocturnal proton accumulation

Leaf disks to a total area of 2 cm$^2$ ($n = 6$, different leaves) were collected at dawn (06.00 h) and dusk (18.30 h), weighed and placed in 10-ml plastic syringes maintained in liquid nitrogen until measurements were made. Syringes were placed in 50-ml plastic tubes and centrifuged for 5 min at 8,000 rpm in a centrifuge mod. Multex (MSE, Crawley, UK). The residue in the syringe was rinsed into the tube with distilled water to a final volume of

approximately 20 ml; protons were titrated with 10 mM KOH to pH 7.0. The $\Delta H^+$ was calculated as the difference between dawn and dusk values.

## Sap flow velocity

Sap flow velocity was measured during 2004 with Granier-type thermal dissipation probes (Granier, 1987) mod. TDP-30 (30-mm long) connected to a DL2e data logger (Dynamax Inc., Houston, TX, USA). Two holes 5-cm vertically apart were drilled using a portable drill into the trunk after removing the cortex. Once the probes were inserted in the holes a small piece of art eraser (Faber-Castell, Buenos Aires, Argentina) was used to ensure an adequate seal; probes were secured to the trunk with polystyrene foam half-spheres and duct tape (3M, Buenos Aires, Argentina) and covered with bubble plastic sheets and windshield aluminium foil protectors. On each date, five TDPs were inserted in the trunk at a height of 1.5 m above ground; records were taken for a minimum of 1 week. The output in mV from the probes was converted to °C (dT) by dividing the voltage by 0.04. From the empirical equation of Granier (1987) sap flow velocity was calculated as $V = 10^4 \times 0.0119 \times K^{1.231}$, where $K = (dT_m - dT)/dT$, with dT the temperature difference between the downstream and the upstream thermocouples in the probe and $dT_m$ the maximum obtained, when sap flow is assumed to be nil. Voltage was adjusted to give an average $dT_m$ of approximately 8°C. One representative record per season is presented.

## Transpiration rate

Transpiration rate ($E$) during 2004 was calculated as the difference in LWC (morning-afternoon) divided by 12 h.

## Statistics

Values are mean ± one SE. Regressions were done using Sigmaplot 7.0 at $P < 0.05$.

## Results

The seasonal changes during 2003–2004 in physiological parameters of the tree of *C. minor* are shown and compared to daily rainfall in Fig. 1. The $\psi$ and $\psi_s$ remained relatively high throughout the seasons, maximum decreases amounting to 0.64 and 0.66 MPa, respectively (Fig. 1a, b). Turgor potential varied between 0.10 and 0.42 MPa and

increased almost immediately with rainfall on certain dates, as in mid-April 2003, mid-May 2003 and mid-March 2004; LWC varied no more than 15% throughout the season (Fig. 1c). Dawn as well as dusk $H^+$ content changed seasonally, dusk values responding rapidly to rainfall (Fig. 1d). The $\Delta H^+$ increased with moderate rainfall in April and June 2003, and March and July 2004, and decreased with either drought or heavy and/or frequent rains, as in August 2003 and the end of May to mid-June 2004 (Fig. 1e).

Noon atmospheric water-vapour saturation deficit ($\Delta w$) was twice as high during the dry season and the beginning of rains as during the rainy season (Fig. 2).

Nocturnal acid accumulation decreased with dusk $H^+$ content ($r^2 = 0.32$). Low correlations were found between the cumulative rainfall for the previous seven or 15 days and $\Delta H^+$, $\psi$, $\psi_s$ or $\psi_T$ ($r^2 < 0.2$ in all cases, $P < 0.05$). Similarly, coefficients for the correlations between $\psi$, $\psi_s$, $\psi_T$ and dawn $H^+$ content, dusk $H^+$ content or $\Delta H^+$ were all $\leq 0.12$ ($P < 0.05$).

Malate and citrate concentrations were estimated from the dawn $H^+$ content and the corresponding LWC, using the proportion of 1.3 malate:one citrate reported by Franco et al. (1990) for *C. minor*. The contribution to $\psi_s$ of malic and citric acid increased linearly with dawn $H^+$ content ($r^2 = 0.70$) and explained up to 32 and 24%, respectively, of the variation in $\psi_s$ (Fig. 3).

In Fig. 4 are shown daily courses of $V$ representative of all those recorded. Maximum $V$ was lowest in May, followed by February and highest in July and March. The shape of the daily course varied seasonally, from mostly diurnal during the dry season to mostly nocturnal after a short dry spell during the rainy season, when nocturnal acid accumulation attained high values.

The relationship between the integrated nocturnal sap flow and $\psi$ was poor ($r^2 = 0.19$), whereas a strong positive correlation was found between the proportion of total sap flow corresponding to the night and $[H^+]$ (Fig. 5).

Transpiration rate was solely diurnal until the beginning of the rainy season and solely nocturnal during the rainy season (Table 1).

## Discussion

We have measured for the first time in *C. minor* the seasonal changes due to water availability in parameters of water relations together with xylem sap flow. Changes in $\Delta H^+$ were apparently governed by rainfall, although in an indirect manner. The shape of the daily course of sap flow changed with rainfall and obeyed changes in dawn $H^+$ content.
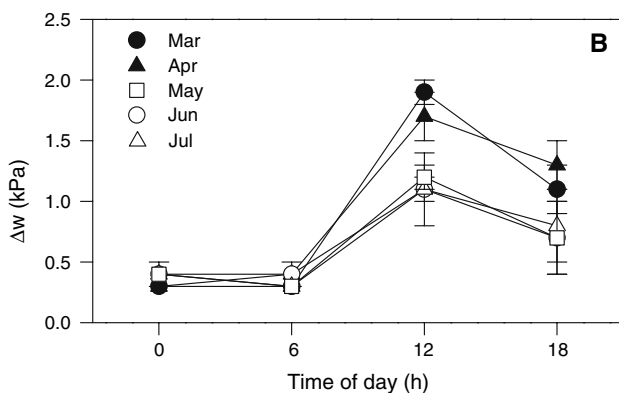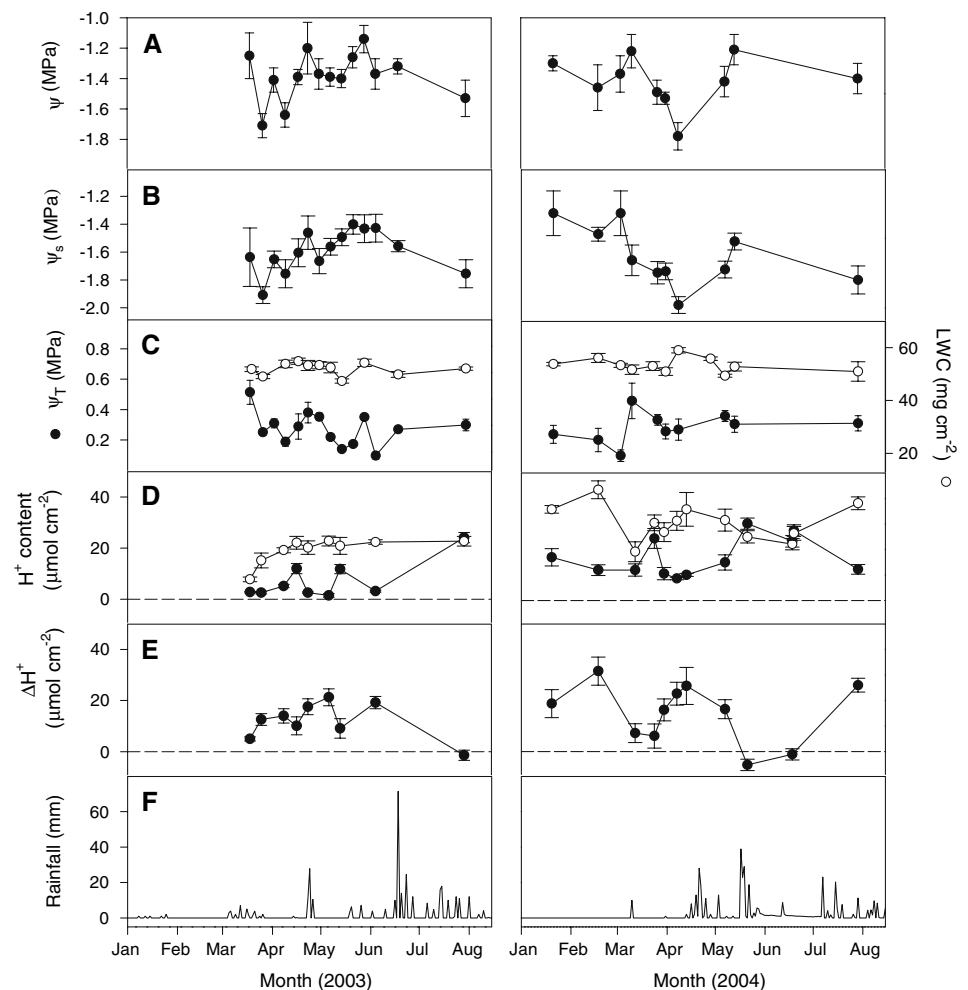
**Fig. 2** Time-course of daily changes in atmospheric water-vapour saturation deficit through the seasons (indicated in the legend). Values are mean ± one SE

Changes in $\psi$, $\psi_s$ and $\psi_T$ with changes in water supply were small, as previously shown in trees of *C. uvitana* growing in Barro Colorado Island, Panama throughout the dry and the rainy seasons, and between a drier and a wetter environment (Zotz and Winter 1993, 1994). Similarly, in

the present study $\psi_T$ reached values as low as 0.1 MPa only after prolonged dry periods. Such maintenance of leaf water status may owe, among other factors, to the succulent nature of leaves of CAM *Clusia* species. In *C. minor*, LWC was similar to values reported by Zotz et al. (1997) and varied less than 15% throughout the seasons; the leaf of *C. minor* has a hypodermis (Borland et al. 1996) that may contribute to the maintenance of leaf water and hence, of $\psi_T$.

During the dry season/beginning of rains, $\psi_T$ increased rapidly in response to showers fallen the previous days, suggesting that water absorption was very efficient. Runoff from the crown and water absorption by the adventitious roots, which are abundant in this tree, may have rapidly improved leaf water status. This assumption is supported by the observation that hydraulic conductivity in *C. uvitana* was found to be higher in aerial roots than in stems (Zotz et al. 1994).
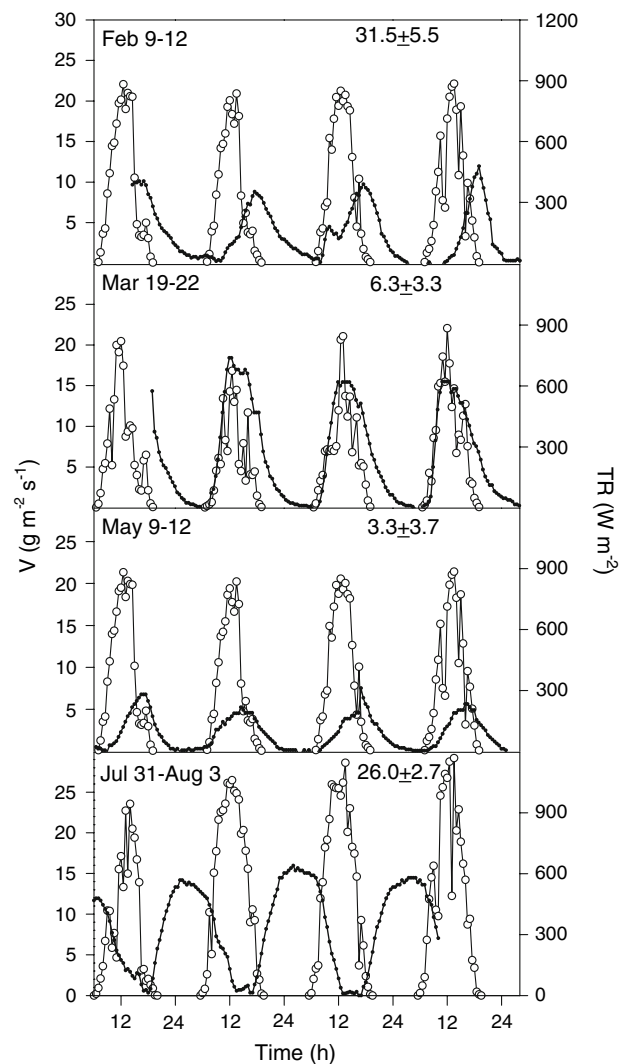
Values of $\Delta H^+$ were similar to those previously reported for *C. minor* at medium PPFD (Franco et al. 1992). The $\Delta H^+$ changed rapidly with rainfall, varying from high
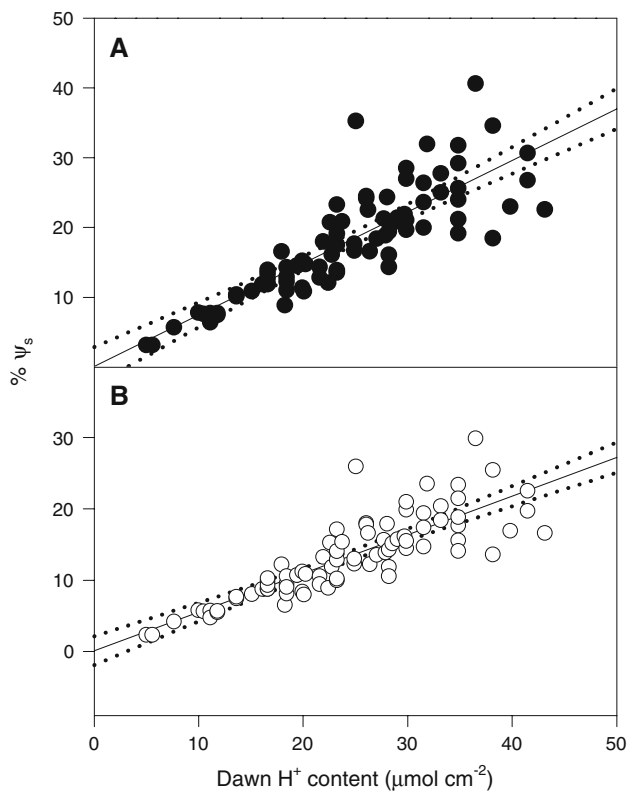
**Fig. 3** Correlation between dawn H$^+$ content in leaves of *Clusia minor* and the proportion of leaf osmotic potential contributed by **a** malate and **b** citrate. Acid concentration was calculated using the proportion 1.3 malate: one citrate reported by Franco et al. (1990). Values are individual data points measured in 2003–2004. The determination coefficient is $r^2 = 0.48$. *Dotted lines*, confidence intervals ($P < 0.05$)



**Fig. 4** Changes during 2004 in the daily courses of sap flow velocity (*dots*) in a tree of *Clusia minor* growing in the garden and total radiation (*open circles*) on the indicated dates. Values are individual data points recorded at half-hourly intervals. The numbers inserted in each panel are values of nocturnal H$^+$ accumulation ($\mu$mol cm$^{-2}$, mean $\pm$ one SE, $6 \leq n \leq 12$)

values during the dry season to a decrease at the end of the dry season (March-April), an increase with rains at the beginning of the rainy season, and a decrease after heavy rainfall during the rainy season. A similar trend of $\Delta$H$^+$ with rainfall was observed in *C. uvitana*, with large values occurring after periods of moderate rains and small values after either prolonged periods of drought or several months of rain (Zotz and Winter 1994).

In this study, when $\Delta$H$^+$ was nil under severe drought, both dawn and dusk [H$^+$] were small but when it was nil after heavy rains, high values of both dawn and dusk [H$^+$] content were measured, in concordance with reports in *C. uvitana* (Zotz and Winter 1993, 1994). The $\Delta$H$^+$ decreased with dusk [H$^+$], as reported by Zotz and Winter (1993), but not in such good correlation as theirs, suggesting that decarboxylation was not always completed. The occurrence of high [H$^+$] well into the morning and as late as 14.00 h during phase III due to delayed decarboxylation reported by Roberts et al. (1997, 1998), but not determined in the present work, may also help water absorption during this part of the photoperiod.

Assuming that part of the [H$^+$] measured in the present study corresponds to citrate, high dawn and dusk [H$^+$], together with low $\Delta$H$^+$ values may have resulted from a large citrate accumulation with little daily fluctuation, as suggested by Franco et al. (1992).

Maximum *V*, whether diurnal or nocturnal, was a fourth of the value in the range reported for 31 tree species, mostly tropical and all C$_3$, with stem diameters of 15 cm (Meinzer 2003); such difference between *C. minor* and these tropical species is to be expected, given the low values of *E* measured and calculated by us and of stomatal conductance reported for *C. minor* (Franco et al. 1990, 1991, 1992; Mattos et al. 1999, Mattos and Lüttge 2001).
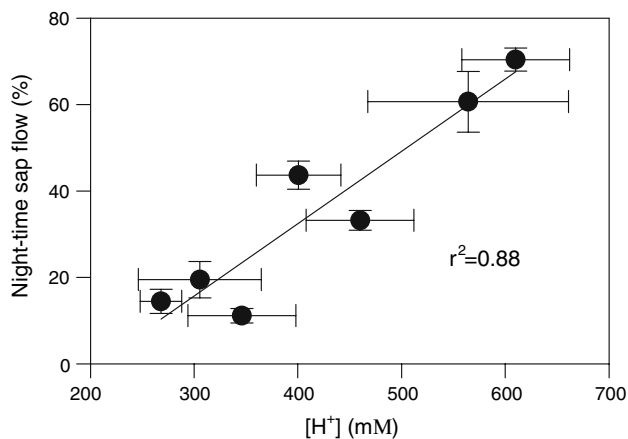
**Fig. 5** Changes in leaves of a tree of *Clusia minor* during 2004 in the proportion of total sap flow corresponding to the night with dawn [H$^+$]. Values are mean ± one SE ($n = 6$ for [H$^+$] and $n = 3$ for daily courses of *V*). The determination coefficient ($P < 0.05$) is indicated

**Table 1** Seasonal changes during 2004 in transpiration rate of leaves of a tree of *Clusia minor*

| Month | $E$ (mmol m$^{-2}$ s$^{-1}$) | |
|---|---|---|
| | Daytime | Night-time |
| January | 0.14 ± 0.01 | 0 |
| February | 0.09 ± 0.00 | 0 |
| March | 0.05 ± 0.00 | 0 |
| April | 0.01 ± 0.00 | 0 |
| May | 0 | 0.03 ± 0.00 |
| June | 0 | 0.06 ± 0.00 |
| July | 0 | 0.25 ± 0.06 |

Values are mean ± SE

The shape of the daily courses of *V* in *C. minor* differed from those for gas exchange previously reported (Roberts et al. 1997, 1998) in that an increase in *V* during phase II was found only in May at the end of the dry season. Nevertheless, *V* was always high in the part of the light period corresponding to phase IV, in agreement with high photosynthetic rates during this phase (Roberts et al. 1997, 1998). During phases II to III a significant proportion of sap flow may have been due to refilling of reservoirs, rather than transpiration, whereas sap flow during phase IV was most probably due to stomatal opening. Such lag between sap flow and transpiration was found in hemi-epiphytic trees of *C. minor* and *C. uvitana* (Zotz et al. 1997). The observation that in July, with high ΔH$^+$ values and presumably high rate of CO$_2$ fixation on phase II, nocturnal sap flow peaked at around midnight to decrease towards dawn supports the hypothesis that a large proportion of this flow was due to transpiration.

Daily patterns of *V* changed seasonally, in concordance with *E*. In savanna trees, nighttime transpiration amounting

up to 28% of daytime values has been reported (Bucci et al. 2005). Daily courses of *E* could aid in clarifying whether changes in *V* obeyed transpiration, rather than refilling, if it were not for the observation that in the same seedling of *C. minor* under controlled conditions one leaf may be performing CAM while the opposite may be performing C$_3$ CO$_2$ fixation (Franco et al. 1992). This situation would be exaggerated in the tree crown, where measurements of leaf *E* in individual leaves may not reflect whole crown transpiration. Stomatal conductance may be strongly decoupled from canopy transpiration due to a large boundary layer, making comparisons between leaf transpiration and whole-tree transpiration incorrect (Wullschleger et al. 1998).

The daily pattern of *V* changed in a consistent way with ΔH$^+$, shifting from a mainly diurnal pattern during phase III of CAM with low ΔH$^+$ to a mainly nocturnal one during phase I with high ΔH$^+$. Similarly, in the hemi-epiphyte *C. uvitana*, 36% of daily sap flow, measured by heat dissipation probes in aerial roots, occurred during the night (Zotz et al. 1997) and, as drought progressed, CO$_2$ fixation shifted from mostly diurnal to mostly nocturnal (Winter et al. 1992).

Maximum *V* was relatively independent of TR, except for the dry season in February, when *V* was half the maximum recorded, and the beginning of rains in May, when it was less than a third. In the first case, the tree had received no water during the previous 2 months and it may have been under severe stress aided by a high Δw, thus decreasing its leaf conductance and sap flow. An increase in Δw has been shown to reduce leaf gas exchange in leaves of *C. minor* (Schmitt et al. 1988). In the second case, the appearance in May of new leaves, with probably lower gas-exchange rate, as shown in young leaves of the same species (Borland et al. 1996), may have reduced whole crown transpiration despite a lower Δw.

The relative contribution of nocturnal sap flow to total daily sap flow was linearly related to [H$^+$], in a manner consistent with results in *S. medley-woodii*, in which nocturnal water uptake was related to malate accumulation (Ruess et al. 1988). A nocturnal decrease of up to 2.2 MPa in leaf sap $\psi_s$ calculated from data of ΔH$^+$ and free sugar contents in *C. minor* (Lüttge 2007) suggests that water absorption is favoured by CAM during phase II. Daily courses of $\psi_s$, acid concentration and *V* should help elucidate the role of acids on osmotic adjustment and sap flow through the daily cycle.

The relationship between nocturnal sap flow and [H$^+$] suggests nocturnal CO$_2$ fixation and stomatal opening. This, together with changes in ΔH$^+$, allows us to conclude that carbon fixation mode in leaves of *C. minor* shifted from CAM idling with very low ΔH$^+$ after a prolonged drought to CAM with high values of ΔH$^+$ under moderate rainfall, to C$_3$ fixation after heavy and/or frequent rains

with low or nil values of $\Delta H^+$, as found previously under field as well as greenhouse conditions (Lüttge 2006). The operation of CAM apparently favoured water absorption through nocturnal stomatal opening, lowering of $\psi_s$ and increase in the soil–plant–atmosphere $\psi$ gradient.

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