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Canopy CO_2 concentrations and Crassulacean acid metabolism in *Hoya carnosa* in a subtropical rain forest in Taiwan: consideration of CO_2 availability and the evolution of CAM in epiphytes

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Abstract

The potential importance of CO_2 derived from host tree respiration at night as a substrate for night time CO_2 uptake during CAM was investigated in the subtropical and tropical epiphytic vine *Hoya carnosa* in a subtropical rainforest in north-eastern Taiwan. Individuals were examined within the canopies of host trees in open, exposed situations, as well as in dense forests. Although night time CO_2 concentrations were higher near the epiphytic vines at night, relative to those measured during the day, presumably the result of CO_2 added to the canopy air by the host tree, no evidence for substantial use of this CO_2 was found. In particular, stable carbon isotope ratios of *H. carnosa* were not substantially lower than those of many other CAM plants, as would be expected if host-respired CO_2 were an important source of CO_2 for these CAM epiphytes. Furthermore, laboratory measurements of diel CO_2 exchange revealed a substantial contribution of daytime CO_2 uptake in these vines, which should also result in lower carbon isotope values than those characteristic of a CAM plant lacking daytime CO_2 uptake. Overall, we found that host-respired CO_2 does not contribute substantially to the carbon budget of this epiphytic CAM plant. This finding does not support the hypothesis that CAM may have evolved in tropical epiphytes in response to diel changes in the CO_2 concentrations within the host tree canopy.

Additional key words: acidity; CO_2 exchange; drought; epiphytic vine; respiration; stable carbon isotopes; tree canopies.

Introduction

Of the three major photosynthetic pathways found among plants, Crassulacean acid metabolism (CAM) is unique in that atmospheric CO_2 is absorbed primarily at night *via* open stomata (Kluge and Ting 1978, Osmond 1978, Winter 1985, Lüttge 1987). As a result of PEP carboxylase activity, the absorbed CO_2 is converted to oxaloacetate, which is then reduced to malate. Throughout the night, the malate is stored in the vacuoles as malic acid. During the day, the malic acid leaves the vacuoles and is de-carboxylated. The resultant accumulation of CO_2 affects daytime stomatal closure while the CO_2 is slowly reduced to saccharides *via* the typical C_3 photosynthetic machinery. By opening their stomata at night and closing them during the hotter and drier day, CAM plants lose considerably less water during photosynthesis, *i.e.* have much higher water-use efficiencies (WUE) relative to C_3 and C_4 plants (Kluge and Ting 1978, Osmond 1978, Winter 1985, Lüttge 1987). This is why many CAM plants grow in arid regions of the world. In addition, even more CAM taxa, primarily in the form of epiphytes, are found in tropical and subtropical areas (Winter 1985, Lüttge 1989, Winter and Smith 1996). This is not

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Abbreviations: CAM – Crassulacean acid metabolism; PDB – Pee Dee belemnite; PPFD – photosynthetic photon flux density; WUE – water-use efficiency.

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surprising, given that drought stress is frequently endured by epiphytic plants between periods of precipitation (Winter 1985, Lüttge 1989, Benzing 1990, Martin 1994).

In the past decade, CAM has also been reported among various taxa of submerged aquatic plants (Keeley 1996). Clearly, minimization of water loss (high WUE) was not a selective force in the evolution of CAM in such plants. Instead, several studies have shown that low daytime CO_2 availability, coupled with high night time availability, constitutes an important benefit of CAM in these aquatic plants (Keeley 1996).

As indicated above, the widespread occurrence of CAM among epiphytes is not surprising as a result of short, but potentially frequent periods of drought stress. On the other hand, reports of CAM in epiphytes that are found in the understory and/or dense canopies of rain forests in tropical and subtropical regions with extremely abundant and frequent rainfall and a short or no dry season are puzzling (*e.g.* Martin *et al.* 1981, 1985, 2005, Winter *et al.* 1983, 1986, Adams 1988, Adams *et al.* 1988, Griffiths 1988, Kluge *et al.* 1989, Carter and Martin 1994, Skillman and Winter 1997). Because atmospheric CO_2 concentrations in the C_3 host canopies are higher at night due to respiration of the canopy leaves, relative to the canopy atmosphere during the day when the host leaves are absorbing CO_2 (see references below), it is tempting to speculate that, as in aquatic CAM plants, CAM might have evolved in such epiphytes in response to CO_2 availability instead of drought stress (Knauft and Arditti 1969, Benzing 1990, Carter and Martin 1994). With this speculation in mind, the goal of the present study was to determine the degree to which an epiphytic CAM plant utilizes CO_2 respired by its host tree at night. Although evidence that extensive usage of such CO_2 might be construed as lending support to the hypothesis that CAM evolved in such plants in response to CO_2 availability, evidence to the contrary would be difficult to reconcile with this hypothesis.

Materials and methods

Study area and plants: The study site was located in a semi-disturbed area (allowing greater accessibility to the plants) in the Fushan Experimental Forest, a subtropical rain forest at 600 m elevation, in north-eastern Taiwan (longitude 121°34’E, latitude 24°26’N). Species of dominant trees at this site were numerous and were primarily in the families Fagaceae and Lauraceae. Climatic conditions at Fushan are subtropical, with monthly average air temperatures of 10–25 °C and monthly rainfall of <10–50 cm, with maxima occurring in the summer months (annual rainfall is 3.56 m). Even in January, the month with the lowest average rainfall, humidities are very high, and rain falls on an average of 20 d of the month; thus, there is no true dry season at Fushan. The average daily humidity throughout the year typically approaches 95 %.

In mid-June 2001, twenty *Hoya carnosa* plants were selected, ten in trees growing in intact, dense stands of forest (referred to here as “closed” canopies) and ten in trees in the open with few neighbors (“open” canopies). The latter trees were often in clearings or near sparsely used roads. Canopy openness and penetration of direct and diffuse radiation into the canopy were measured with a *Nikon 4500* digital camera, *Nikon FC-E8* fisheye lens, and the software program *Delta-T Hemiview* Canopy Analysis Software (Cambridge, UK; Lin *et al.* 2003). Photographs were taken at a height of 2 m in the canopy of each tree by holding the camera adjacent and perpendicular to *H. carnosa* individuals and recording exposures in three directions outward from the tree trunk. These three measurements were then averaged for each tree. In all cases, individuals of *H. carnosa* were epiphytic vines extending vertically along most of the tree trunks.

Atmospheric CO_2 concentrations: Air CO_2 concentrations at mid-day (11:00–13:00 h) and mid-night (00:00–03:00 h) were measured using a *LI-COR* (Lincoln, NE) *LI-6400* Portable Photosynthesis System at a height of 2 m, the same height at which leaves were sampled for acidity and carbon isotope ratios. Air was sampled within 10–20 cm of the *H. carnosa* plants. No rain fell during the night and day of measurements; the day during which air was sampled was partly sunny and warm. The air was calm during both sets of measurements.

Leaf titratable acidity: Shortly before sunset and again shortly after sunrise, a leaf was removed from each of the *H. carnosa* plants and frozen (–10 °C) within 5 min of excision. The days on which the leaves were sampled were partly sunny and warm. After 2 d in the freezer, the leaf was thawed, and a portion was weighed and pulverized in distilled water in a mortar and pestle. The resultant slurry was titrated to pH 7.0 using 0.01 M NaOH. After titrating, the water was evaporated, and the dry mass of the tissue was obtained after a week in an oven at 70 °C.

Leaf carbon isotope ratios: Leaves of the *H. carnosa* individuals and of their host trees were collected and dried for at least a week at 70 °C, then ground into a powder and combusted for determination of the stable isotopic composition of their carbon at the University of Arkansas Stable Isotope Facility using a *Carlo Erba* elemental analyzer (*NA1500 CHN* Combustion Analyzer, *Carlo Erba Strumentazione*, Milan, Italy) coupled to a *Finnigan Delta+* mass spectrometer (*Finnigan MAT*, Bremen, Germany) *via* a *Finnigan Conflo II* interface. The spectrometer had been calibrated using the PDB standard. The instrument error (twice the standard deviation) associated with each measurement was ± 0.1 ‰.

Shoot gas exchange: Plants were collected at the study site, transported to the University of Kansas, and grown in potting soil in a growth chamber under the following conditions: 50–100 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 30/20 °C day/night air temperatures, 2.43/0.52 kPa day/night vapor pressure deficits, and a photo/thermoperiod of 12 h. After three years of growth under these conditions, plants were large, vigorous, and flowering. Shoots with two to four leaves were sealed in gas exchange cuvettes, and net CO_2 exchange was measured continuously for three days under environmental conditions similar to those in the growth chamber. The open gas exchange system comprised: a *LI-COR LI-6262* differential infrared gas analyzer; polycarbonate, water-jacketed gas exchange cuvettes with small fans for air mixing; thermocouple and thermistor temperature sensors and meters for air and leaf temperature measurements; a temperature-controlled humidifier; and a computer for data collection. Further details of this system, as well as methods of data analysis, have been described in Harris and Martin (1991) and Gravatt and Martin (1992).

Statistics: In most cases, pairs of means were compared using the Student's *t*-test or the Mann-Whitney *U*-test whenever the data failed to meet the assumptions of the parametric *t*-test (Sokal and Rohlf 1981). Day and night air CO_2 concentrations measured in the closed and open canopies were compared with a two-way analysis of variance, followed by the Tukey comparison-of-means test (Sokal and Rohlf 1981). In all tests, statistical significance was inferred when $p \leq 0.05$.

Results and discussion

All individuals of *H. carnosa* performed CAM, as evidenced by significant and substantial accumulations of acidity in the leaves at night (Fig. 1; also see gas exchange findings below). Plants in the open canopies exhibited much higher acid accumulations, presumably a result of the increased availability of radiation energy in the more exposed trees (see Fig. 2), although the latter conclusion is in direct contrast to the conclusions of past studies of sun/shade adaptations in three species of *Hoya*, including *H. carnosa* (Winter *et al.* 1983, Adams *et al.* 1987, 1988). On the other hand, some data from these studies support those of the current study; nocturnal acid accumulations for plants growing in the field in Australia were greater in plants growing in full sunlight, relative to values for plants growing in deep shade. Thus, it is clear that more work is required before the sun/shade status of epiphytic species of *Hoya* is fully understood.

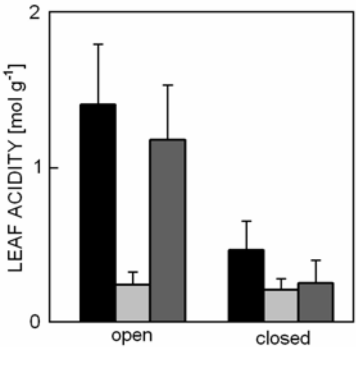

Fig. 1. Mean morning (*black bars*), evening (*light gray bars*), and overnight (*dark gray bars*) acidity of leaves of *Hoya carnosa* in open and closed host tree canopies in a subtropical rain forest in northeastern Taiwan. *The error bars* are standard deviations (*n* = 10). Differences between both sets of morning/evening means and between the mean overnight increases in leaf acidities are highly significant ($p<0.001$).

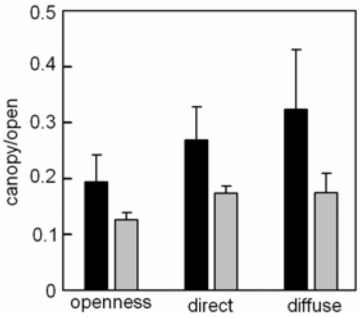

Fig. 2. Mean measures of radiation penetration into the canopy of host trees at a height of 2 m and adjacent to individuals of *Hoya carnosa* in a subtropical rain forest in northeastern Taiwan. *The error bars* are standard deviations (*n* = 10). Canopy openness is the fractional area of all gaps in the canopy; direct irradiance is the proportion of direct PPFD, relative to that in the open; and diffuse irradiance is the proportion of indirect (diffuse) PPFD, relative to that in the open. All values are based on photographic images and are relative to measurements taken in a fully exposed (open) location nearby. In all cases, the open canopies (*black bars*) allowed more irradiance ($p<0.001$) to the *H. carnosa* vines than did the closed canopies (*gray bars*).

The CO_2 concentration of the atmosphere in the host tree canopies in the Fushan rain forest was 40–60 μmol mol^{-1} higher at night than during the day, regardless of the closed or open nature of the forest canopy (Fig. 3). In addition, nocturnal CO_2 concentrations were higher in the closed canopies (trees in dense forest), relative to the open canopies of the more exposed trees. This was not the case during the day (Fig. 3). The day/night changes in canopy CO_2 concentrations at a height of two meters in this Taiwanese subtropical rain forest are not unlike those found at this height in a temperate deciduous forest in Japan (Koike *et al.* 2001). Although the latter study reported somewhat larger day/night differences (around 100 μmol mol^{-1}), CO_2 concentrations were measured at dawn and dusk, as opposed to the mid-day and mid-night measurements in the current study. At another site in Taiwan (approximately 400 km southwest of Fushan), the air CO_2 concentrations at a height of 2 m in the forest ranged from diurnal minima around 345 μmol mol^{-1} to nocturnal maxima of approximately 390 μmol mol^{-1} (Cheng and Kuo 2004), a diel change of 45 μmol mol^{-1}, which is in the range of values reported here for the forest at Fushan.

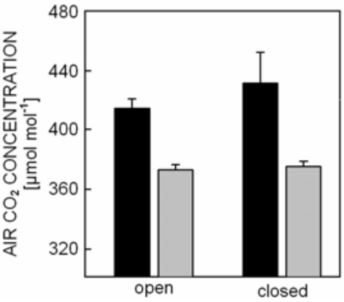

Fig. 3. Mean mid-night (*black bars*) and mid-day (*gray bars*) CO_2 concentrations of the air in the canopies of host trees at a height of 2 m and adjacent to individuals of *Hoya carnosa* in a subtropical rain forest in northeastern Taiwan. *The error bars* are standard deviations (n = 10). The differences in mean CO_2 concentrations between hosts with open and closed canopies were significant ($p<0.05$), as were differences between night and day ($p<0.001$). In addition, the interaction between canopy closure and time of day was significant ($p<0.05$). The data in this figure were obtained in summer 2001. Repeated measurements in summer 2005 revealed nearly identical values.

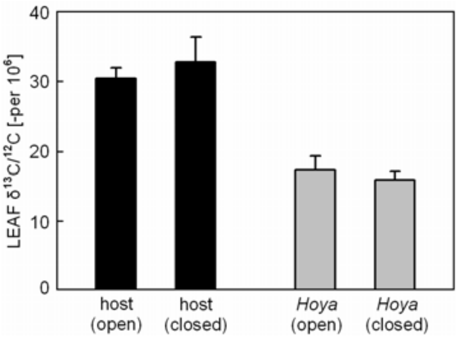

Fig. 4. Mean $\delta^{13}C/^{12}C$ values (–‰) of leaves of *Hoya carnosa* (*gray bars*) and their host trees (*black bars*; open and closed canopies) in a subtropical rain forest in northeastern Taiwan. *The error bars* are standard deviations (n = 10). Differences between open and closed host canopies in both sets of plants were not significantly different ($p>0.05$).

As expected for a CAM plant, the stable carbon isotope ratios of the epiphytes were substantially less negative than those of the host trees (Fig. 4), reflecting, in large part, the discriminatory properties of ribulose-1,5-bisphosphate carboxylase/oxygenase *versus* PEP carboxylase (Kluge and Ting 1978, Holtum *et al*. 1982, Winter 1985, Griffiths 1992, 1993). As was the case with the host trees, the stable carbon isotope ratios of the epiphytes found in the closed canopies were not significantly different from those of the epiphytes in the open canopies (Fig. 4). Values reported in the current study fall between those reported for *H. nicholsoniae* growing in "deep shade" in an Australian rain forest (–14.33 ‰; Winter *et al*. 1986) and those of *H. carnosa* growing at various PPFD levels in a glasshouse also in Australia (–20.1 to –22.2 ‰; Adams *et al*. 1987).

The general goal of this study was to provide evidence that might be used in support of or against the hypothesis that CAM might have evolved in tropical epiphytes in response to increased CO_2 availability at night. Air CO_2 concentrations within the host tree canopies were indeed substantially higher at night than during the day. Because the host trees were all C_3 plants, this respired air would be highly depleted in ^{13}C, and its stable carbon isotope value would be similar to that of the host leaves, *i.e*. around –30 ‰ (Rundel *et al*. 1989, Griffiths 1993). Thus, the air surrounding the epiphytes should have an isotopic composition that represents a mixture of air from above the canopy, presumably with a carbon isotope ratio around –8 ‰ (Rundel *et al*. 1989, Griffiths 1993), with the host-respired air. As a result, although the stable carbon isotope ratio of the canopy air was not measured, its value was presumably more negative, and probably substantially so, than –8 ‰. Indeed, several studies have reported more negative carbon isotope ratios of the air and plant tissues inside tropical forests as a result of respired CO_2 (Medina and Minchin 1980, Schleser and Jayasekera 1985, Medina *et al*. 1986, 1991, Sternberg *et al*. 1989). Therefore, the stable carbon isotope ratios of the *H. carnosa* plants were predicted to be substantially lower than values for most CAM plants, especially those growing in more exposed locations (for a study using a similar rationale and approach, see Treseder *et al*. 1995). Confirmation of this prediction would lend support to the feasibility of the hypothesis about the evolution of CAM in epiphytes stated above.

Despite this expectation, the stable carbon isotope ratios of the *H. carnosa* epiphytes (Fig. 4) were not substantially more negative than values typical of many terrestrial CAM plants that grow in exposed habitats with few neighbors, *e.g*. desert succulents (Troughton *et al*. 1974, Eickmeier and Bender 1976, Sutton *et al*. 1976, Ting 1989). In addition, laboratory measurements of gas exchange using plants of *H. carnosa* collected at the study site revealed substantial amounts of daytime CO_2 uptake (Fig. 5), which would result in carbon isotope ratios more negative than those of CAM plants lacking daytime CO_2 uptake (Winter and Holtum 2002). Furthermore, although nighttime CO_2 concentrations were higher in the closed canopies, the stable carbon isotope ratios of the epiphytes in these canopies were not more negative than the values for the epiphytes in the open canopies. These findings indicate that *H. carnosa* does not utilize CO_2 respired by the host tree canopy, at least to a substantial degree, in spite of the elevated CO_2 concentrations in the canopy at night relative to during the day. Although a different species, the relatively high (less negative) carbon isotope value reported for *H. nicholsoniae* in a dense forest canopy (see above; Winter *et al.* 1986) also indicates a minimal contribution of host-respired CO_2 to the carbon budget of this epiphytic CAM plant. It is surprising that host-respired CO_2 does not contribute more to the carbon composition of these epiphytic CAM plants. Although the weather was calm, and canopy air turbulence was not observed during the measurements made in this study, it is possible that the canopy air is typically less stagnant than this, mixing frequently with the atmosphere above the forest canopy and thereby diluting the contribution of host-respired CO_2 to the canopy air. Canopy air turbulence measurements throughout the year are needed in order to test this explanation.

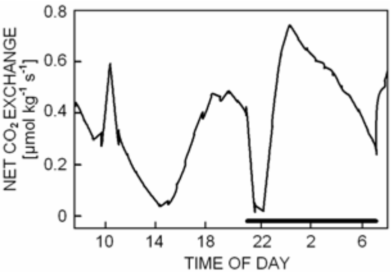

Fig. 5. Net CO_2 exchange for a shoot of an individual of *Hoya carnosa* collected in a subtropical rain forest in northeastern Taiwan, then grown and measured in Kansas. *The thick, horizontal black line* indicates the nighttime. Environmental conditions during measurements are provided in "Materials and methods".

The results of this study do not lend support to the hypothesis that CAM might have evolved in tropical/subtropical epiphytes in response to atmospheric CO_2 availability. Of course, different results might be obtained with other species of epiphytic CAM plants in other forests. The potential for the utilization of substantial amounts of host-respired CO_2 in tree canopies should be investigated in more epiphytic CAM plants before definitive conclusions are drawn. At least in the case of *H. carnosa* in this subtropical rain forest, the likely eco-physiological significance of CAM remains water conservation, which should prove beneficial to such plants despite the infrequent occurrence of drought in this rain forest.

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