The relationship between CAM and leaf succulence in two epiphytic vines, *Hoya carnosa* and *Dischidia formosana* (Asclepiadaceae), in a subtropical rainforest in northeastern Taiwan[#]

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Abstract

Past reports of correlations between Crassulacean acid metabolism (CAM) and leaf succulence are based on multi-species comparisons. When different individuals of the same species were compared in two epiphytic CAM vines growing in a subtropical rainforest in northeastern Taiwan, the degree of CAM was not correlated with leaf thickness, a measure of succulence. Leaf chlorophyll (Chl) *a* and *b* concentrations and ratios correlated well with leaf succulence, indicating that differences in leaf succulence were likely a result of sun/shade adaptations, not photosynthetic pathway. These findings challenge the assumption that CAM-succulence correlations are causal.

Additional key words: chlorophyll; Crassulacean acid metabolism; leaf thickness; sun/shade adaptation; titratable acidity.

Introduction

Plants with CAM, one of three widely recognized photosynthetic pathways (Black 1973, Salisbury and Ross 1992), are unique in their restriction of stomatal opening to the nighttime. As a consequence, rates of water loss during the cooler and more humid night are highly reduced, relative to rates of water loss during the daytime (Osmond 1978, Kluge and Ting 1978, Winter 1985, Winter and Smith 1986). Not surprisingly, the majority of CAM plants are found in arid environments and microenvironments (*e.g.*, that of tropical epiphytes). Thus, the CAM pathway is considered to be a complex adaptation for the avoidance of drought stress (Kluge and Ting 1978, Osmond 1978, Winter 1985). Often highly correlated with CAM photosynthesis in such plants is succulence of the photosynthetic tissue (Kluge and Ting 1978), *i.e.* morphological features of the photosynthetic organs that allow the retention of large reserves of water.

A precise definition of plant tissue or organ succulence has escaped universal acceptance among plant scientists, and, consequently, measuring the degree of succulence in plant tissues is not a trivial challenge (Kluge and Ting 1978, Gibson 1982, Sundberg and Zahn 1985). Unquestionably, succulent plant organs are thick, and leaf thickness provides a simple, if not simplistic, surrogate for quantifying tissue succulence. This is not the first study to examine the relationship between leaf thickness and CAM, although previous studies included different species having leaves of different thicknesses, while different individuals of the same species were compared in the current study. For example, Teeri *et al.* (1981) found a strong correlation between leaf thickness and level of CAM in different species of *Crassula* in the Crassulaceae, as was also found with species of *Aeonium* (Lösch 1987), also in the Crassulaceae. In addition,

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Abbreviations: CAM – Crassulacean acid metabolism; Chl – chlorophyll; DMF – dimethylformamide.
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Hew (1976), Avadhani *et al*. (1978, 1982), Earnshaw *et al*. (1987), Kluge *et al.* (1995), and Sanders (1979) reported good correlations between leaf thickness and CAM across a wide diversity of orchid taxa, and Winter *et al*. (1983) found that leaf thickness correlated with stable carbon isotope values, an indicator of CAM, in a number of orchid and fern genera and species. Nuernbergk (1960) reported CAM in leaves of a succulent species of *Hoya*, while a non-succulent species did not have CAM leaves. In addition, similar differences were noted in the genus *Kalanchoe* (Crassulaceae; Nuernbergk 1960). The correlation between CAM and leaf succulence has also been reported for different leaves of the same plant, *e.g*., species of *Kalanchoe* and *Mesembryanthemum,* when subjected to different water regimes, stresses, or photoperiods (Kluge and Ting 1978, Osmond 1978, Winter 1985, Lüttge 1987). Contrasting with the results of many of the above studies, correlations between several measures of leaf succulence and CAM were less consistent for a number of species of *Peperomia* (Piperaceae; De Santo *et al*. 1978, 1983) and in species of two genera in the Vitaceae (De Santo *et al*. 1983, 1987). Furthermore, the relationship between tissue succulence and CAM did not always conform with expectations in 18 species in the Cactaceae (Martin and Wallace 2000).

The correlation of CAM and succulence has been considered by some to be a causal relationship (Larcher 2003), given the requirement of CAM for large cells to accommodate large vacuoles in which malic acid, the primary end-product of nocturnal CO_2 fixation, is stored throughout the night (Kluge and Ting 1978, Osmond 1978, Lüttge 1987). Although large cells and vacuoles are certainly necessary for proper CAM functioning, these cellular and organellar features are by no means unique to CAM plants (Salisbury and Ross 1992, Raven *et al.* 1999). Therefore, it is plausible that tissue succulence in CAM plants may simply reflect another adaptation of these plants that minimizes or avoids drought stress in their arid habitats or microhabitats. This view was espoused by De Santo *et al*. (1983). In all previous studies of the CAM-succulence relationship, different species in the same genus or family were compared, while comparisons of CAM and tissue succulence among different individuals of the same species are non-existent or rare. The primary reason for this is undoubtedly the lack of substantial variation in leaf succulence among individuals of the same species. This is unfortunate because inclusion of different species, although related, potentially introduces a host of other phylogenetic, physiological and morphological features that may obscure any causal relationships between CAM and succulence. Examination of the relationship between CAM and tissue succulence among different individuals of the same species should be less prone to co-correlations of other physiological and/or morphological features, and, as a result, should provide greater insight into the causal mechanisms underlying variations in the succulence-CAM correlation. Therefore, it was the purpose of this study to determine the relationship between the degree of CAM and succulence of the photosynthetic tissue among individuals of the same species in two epiphytic CAM species that are abundant in a subtropical rainforest in northeastern Taiwan. In addition, another major determinant of leaf thickness, sun/shade adaptation, was investigated. *Hoya carnosa* and *Dischidia formosana*, epiphytic vines in the Asclepiadaceae (or the Apocynaceae; Wanntorp *et al*. 2006), were selected for this study because both species are obligate CAM plants (Martin, unpublished; Winter *et al*. 1983), and the leaves of both species vary considerably in thickness.

Materials and methods

Plants and study site: Leaf thickness was measured, and leaves were sampled for acidity and Chl analyses for ten individuals each of *H. carnosa* (L. f.) R. Br. and *D. formosana* Maxim. *in situ* at the Fushan Experimental Forest, a comparatively pristine tract of subtropical rain forest (121°34′E, 24°46′N) at an elevation of ~600 m located 40 km southeast of Taipei in northeastern Taiwan. For general climatic conditions at the Fushan site, *see* Martin *et al*. (2004); environmental conditions during the week of measurements (11-15 July 2005) were: 25.1 ºC average daily air temperature (29.8 ºC average daily maximum; 21.3 ºC average daily minimum), 86.8 % average daily air relative humidity, and 20.0 mol m^{-2} d^{-1} average daily photosynthetic photon flux density.

Plants were selected in a partially disturbed section of the forest to allow easy access to the plants. Species of dominant trees at this site were numerous, primarily in the families Fagaceae and Lauraceae; examples include *Litsea acuminata* (Bl.) Kurata (Lauraceae), *Machilus zuihoensis* Hayata (Lauraceae), *Castanopsis cuspidata* (Thunb. *ex* Murray) Schottky var. *carlesii* (Hemsl.) Yamazaki (Fagaceae), and *Pasania hancei* (Benth.) Schottky (Fagaceae).

All plants were large epiphytic vines growing on a variety of host trees, including those listed above. Most plants were flowering at the time of this study. Leaves of both species are arranged opposite each other on the shoots. Leaf pairs are widely separated on shoots of *H. carnosa*, while those of *D. formosana* grow close to each other. Three (*H. carnosa*) or six (*D. formosana*) pairs of leaves per plant were included for analyses. Adjacent leaf pairs on shoots were used to the degree possible to minimize large variations in microenvironmental conditions among the leaf pairs. More than one leaf pair per plant was included to ensure variation in leaf thickness on each plant, and more leaf pairs were

sampled for *D. formosana* as a result of their small size (*see* below). Although plants often grew to much greater heights, all leaves were sampled no higher than three meters from the ground. Only mature, non-senescent leaves lacking substantial insect damage were sampled; very young and very old leaves were avoided to the degree possible. Leaves were selected without regard to host tree species, height from the ground (except as noted), degree of canopy shade (although plants on fully exposed trees were avoided). The thickness of all six (*H. carnosa*) or twelve (*D. formosana*) leaves was measured *in situ* at midday, then one leaf from each pair was removed one hour before sunset for acidity and Chl analyses, and the remaining leaf was removed for acid and pigment analyses one hour after sunrise the following morning. Different portions of the same leaf were used for the two analyses for each sampling time in *H. carnosa*, while two different whole leaves were analyzed separately for the two analyses for each sampling time in *D. formosana*. Weather conditions during the day preceding the evening sampling included periods of full sun, some cloud cover, and an afternoon rain shower, which ended prior to nightfall.

Determination of leaf thickness: In order to ensure inclusion of a wide range of leaf thicknesses for this study, leaf thickness was measured on attached leaves in the field prior to sampling for physiological analyses; however, to ensure the most accurate thickness value for each leaf sampled for physiological measurements, especially with *H. carnosa* (*see* below), leaf thickness was also measured on detached leaves in the laboratory. In both cases, thickness was measured with a caliper (measurement resolution of 0.1 mm) at the center of the leaf, avoiding the midrib (in *H. carnosa*; leaves of *D. formosana* lack a clear midrib). Leaves of *D. formosana* are small and oval-shaped (approximately 0.01 m in diameter) and are thickest at the center, while leaves of *H. carnosa* are large (typically near or exceeding 0.1-0.2 m in length and 0.05-0.1 m in width) and more variable in shape, but fairly constant in thickness across the leaf. Because the leaf margins of *H. carnosa* were seldom flat, leaf thickness was difficult to measure accurately in the field with intact leaves. Thus, the thickness of each leaf sampled for acid titration or Chl concentration was also measured in the laboratory, except leaves of *H. carnosa* were sliced longitudinally alongside the midrib, providing easy access for the caliper; the thickness of one of the leaf halves was then easily measured. Confirming the greater accuracy of leaf thickness measurements in the laboratory *versus* in the field, the mean thickness of the *H. carnosa* leaves measured in the field (0.00148 m) was slightly greater (p=0.012) than the mean of values obtained in the laboratory with the same leaves (0.00128 m). Such field/lab differences in leaf thickness were not observed for *D. formosana* [the mean thickness in the field of leaves used for acidity analysis was 0.00154 m, while the lab mean was 0.0016 m; the mean thickness in the field of leaves used for pigment analysis was 0.00150 m, and the lab mean was 0.0015 m; both sets of means are not significantly different (p=0.64 and p=0.70), respectively].

Determination of titratable acidity: At both collection times, a leaf was excised from a leaf pair on a previously marked shoot, placed in a plastic bag, then frozen at −10° C in the laboratory (within 5 min of leaf detachment) until analysis within the next two days. Upon thawing, the whole leaf (*D. formosana*) or three (0.009 m diameter) or four (0.005 m diameter) leaf discs punched from the leaf blade, avoiding the midrib, of *H. carnosa* were weighed, then ground in a small amount (30-50 cm^3) of deionized water using a mortar and pestle, and the resultant slurry was titrated to pH 7 with a *Metrohm* model *632* pH meter (*Metrohm*, Riverview, FL, USA).

Determination of Chl concentration: For *H. carnosa*, three or four punches were removed (as above) from the same leaf collected for acidity analysis, weighed, then extracted in 10 cm^3 of N,N-dimethylformamide (DMF) at 5 °C overnight for 2-3 d (but *see* below). One intact leaf of *D. formosana* was collected in the evening and another in the morning, weighed, and extracted similarly except in 5 cm^3 of DMF and occasionally for a longer extraction period. After extraction, the majority of leaf tissue samples of both species lacked dark green pigmentation, although some *D. formosana* samples retained green portions, so were extracted an additional 6 d until the tissue was clear). Absorption of the extracts was measured at 603, 647, 664, and 750 nm using a *Hitachi U-2010* spectrophotometer (*Hitachi*, Tokyo, Japan) (zero checked frequently with pure solvent), and Chl concentrations were calculated according to Moran (1982). Both the Chl concentration and the titratable acidity data are expressed on a fresh mass basis. In all cases (both measurements, both species), the fresh mass/dry mass ratio did not change (t-test; p>0.05) between the evening and morning sampling times.

Statistical analyses: Pairs of means were compared with the Student's t-test when the data met the assumptions for using parametric statistics or with the Mann-Whitney U-test when the data failed to meet those assumptions (Sokal and Rohlf 1981). Correlation analysis, including determination of R^2 was performed by the *SigmaPlot* (*SPSS, Inc*., Chicago, IL, USA) graphical software program.

Results and discussion

In the current study, nearly all leaf pairs of all individuals of the two epiphytic vines examined *in situ* performed CAM; however, the degree of CAM, measured as the amount of acid accumulated overnight, correlated poorly with leaf thickness when 15 leaf pairs (from ten individuals) with highly varying leaf thicknesses were compared (Fig. 1*A,B*).

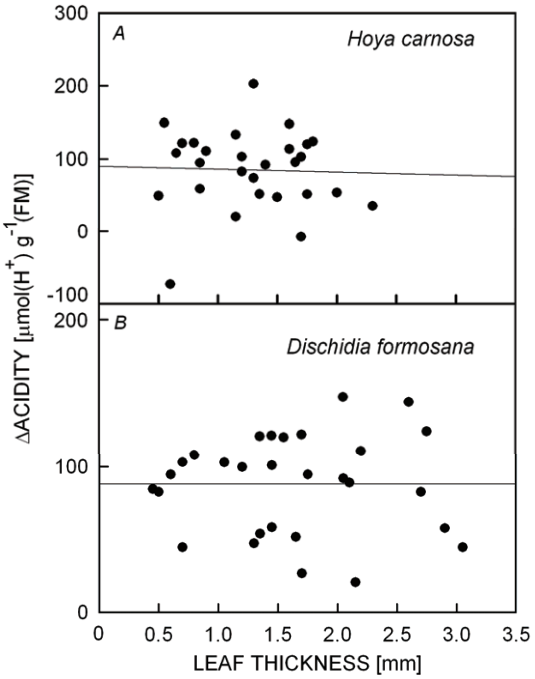

Fig. 1. Correlation (R^2 = 0.001) between overnight increase in leaf tissue titratable acidity; evening and morning means are significantly different (p<0.001) and leaf thickness (average of the two leaves sampled in the evening and the following morning for acidity analysis; mean leaf thicknesses are not significantly different between the two sampling times) for ten individuals (three leaves per plant per time) of *A*: *Hoya carnosa* (p=0.46) or *B*: *Dischidia formosana* (p=0.62), two epiphytic CAM vines (Asclepiadaceae), in a subtropical rainforest in northeastern Taiwan. A leaf lacking CAM would have a Δacidity of zero. FM – fresh mass.

The results of this study contrast with those of most previous studies in which CAM and leaf succulence among different species of a genus or family were compared. Past correlations between CAM and tissue succulence, however quantified, might be subject to complications as a result of other morphological or physiological differences that correlate with either the degree of CAM or tissue succulence among the different species compared, potentially clouding the meaning of a CAM-succulence correlation, or lack thereof. In fact, the leaf pigment data for both species in the current study provide strong support for this statement. Because leaves of both species were collected without regard to incident irradiances, leaf thickness apparently reflected sun/shade differences among the different microhabitats from which the leaves were collected. Thus, in both species, thick leaves had lower Chl concentrations (on a mass basis; Fig. 2*A,B*) and higher Chl *a/b* ratios (Fig. 3*A,B*), relative to pigment data for thinner leaves. These pigment findings comprise typical sun/shade adaptations (Boardman 1977, Björkman 1981). Assuming, therefore, that leaf thickness in these two epiphytic CAM vines reflects sun/shade adaptations, the lack of correlation between CAM and succulence (leaf thickness) may be surprising, as CAM is often associated with high light exposure. On the other hand, epiphytes, including species with CAM, are often shaded by the canopy of their host trees, resulting in morphological and physiological adaptations to shade (Martin *et al*. 1985, 1986, 1989, 1999, Winter *et al.* 1986, Skillman and Winter 1997).

The finding that leaf thickness varies dramatically, over three-fold (Figs. 1-3), and that this variability correlates well with classic sun/shade pigment adaptations

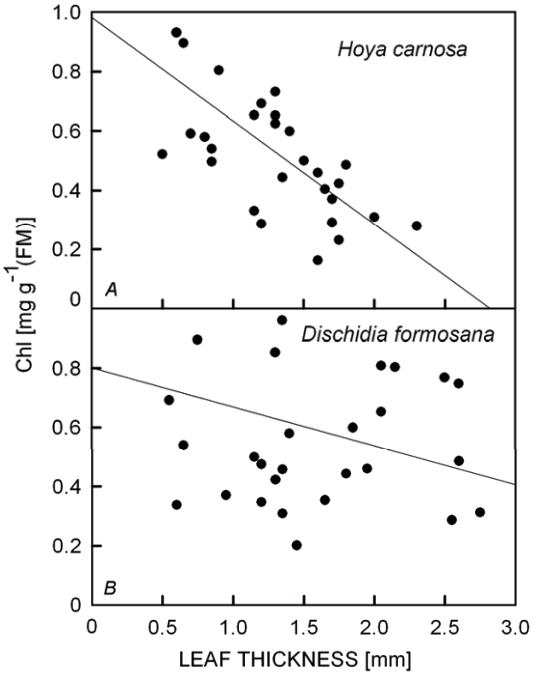

Fig. 2. Correlation between leaf chlorophyll (Chl) concentration and leaf thickness [for both data sets, each value is an average of two leaves sampled in the evening and the following morning along with those sampled for acidity analysis. The evening and morning mean Chl concentrations are not significantly different, nor are the mean leaf thicknesses at these two times (*A*: p = 0.84 and p = 0.46, respectively; R^2 = 0.473, *Hoya carnosa*; *B*: p=0.98 and p=0.62, respectively; R^2 = 0.093; *Dischidia formosana*)] for ten individuals (three leaves per plant per time) of the epiphytic CAM vines (Asclepiadaceae), in a subtropical rainforest in northeastern Taiwan. FM – fresh mass.

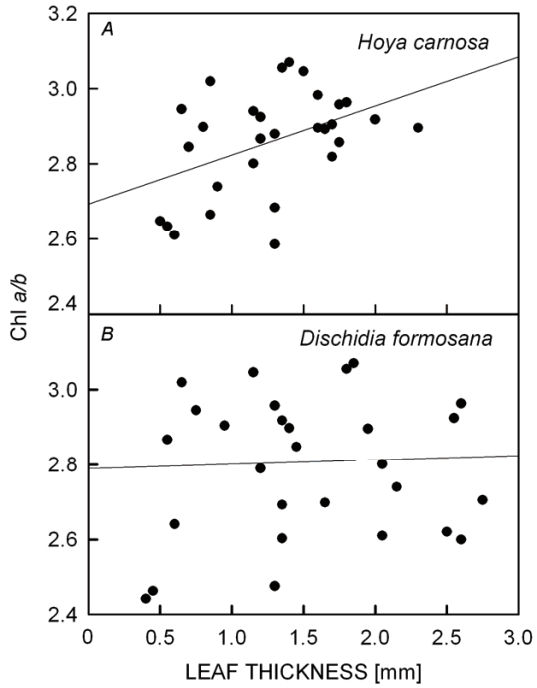

Fig. 3. Correlation between leaf chlorophyll (Chl) *a/b* ratio and leaf thickness [for both data sets, each value is an average of two leaves sampled in the evening and the following morning along with those sampled for acidity analysis. The evening and morning mean Chl *a/b* ratios are not significantly different, nor are the mean leaf thicknesses at these two times (*A*: $p = 0.11$ and $p = 0.46$, respectively; $R^2 = 0.001$, *Hoya carnosa*; *B*: p=0.62 and p=0.37, respectively; $R^2 = 0.001$; *Dischidia formosana*)] for ten individuals (three leaves per plant per time) of two epiphytic CAM vines (Asclepiadaceae), in a subtropical rainforest in northeastern Taiwan.

in the two subtropical vines in this study is not surprising, given the wide range of irradiances occurring in the diverse microhabitats in which leaves are found along their long, crawling shoots that ascend from the base to the canopy of their host trees, typically to heights of dozens of meters. On the other hand, other factors must also account for some of the variation in leaf thickness in these two epiphytic CAM vines, as very thick leaves were occasionally found in the shade and very thin leaves in the sun.

Given the limitation of the CAM-succulence comparison to different individuals of the same species, as well as the inclusion of data reflecting another potential determinant of leaf thickness, *i.e*. adaptation to sun or shade, the results of this study reveal that variations of leaf thickness in these two epiphytic CAM vines apparently reflect sun/shade adaptations, not photosynthetic pathways or relative activity of a pathway, *e.g*., CAM. Extrapolation of the findings here lends little support to the contention that a correlation between CAM and tissue succulence is causal.

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