LEAF ANATOMY AND CO$_2$ RECYCLING DURING CRASSULACEAN ACID METABOLISM IN TWELVE EPIPHYTIC SPECIES OF TILLANDSIA (BROMELIACEAE)

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The relationship between leaf anatomy, specifically the percent of leaf volume occupied by water-storage parenchyma (hydrenchyma), and the contribution of respiratory CO$_2$ during Crassulacean acid metabolism (CAM) was investigated in 12 epiphytic species of *Tillandsia*. It has been postulated that the hydrenchyma, which contributes to CO$_2$ exchange through respiration only, may be causally related to the recently observed phenomenon of CO$_2$ recycling during CAM. Among the 12 species of *Tillandsia*, leaves of *T. usneoides* and *T. bergeri* exhibited 0% hydrenchyma, while the hydrenchyma in the other species ranged from 2.9% to 53% of leaf cross-sectional area. Diurnal malate fluctuation and nighttime atmospheric CO$_2$ uptake were measured in at least four individuals of each species. A significant excess of diurnal malate fluctuation as compared with atmospheric CO$_2$ absorbed overnight was observed only in *T. schiedeana*. This species had an intermediate proportion (30%) of hydrenchyma in its leaves. Results of this study do not support the hypothesis that CO$_2$ recycling during CAM may reflect respiratory contributions of CO$_2$ from the tissue hydrenchyma.

Introduction

Crassulacean acid metabolism (CAM) constitutes a complex metabolic adaptation which reduces the possibility of drought stress in plants living in arid environments such as deserts, as well as in the potentially stressful microenvironment of tropical epiphytes (Kluge and Ting 1978; Osmond 1978; Winter 1985). Crassulacean acid metabolism plants reduce water loss by closing their stomata during the day when the temperature and vapor pressure deficit are high, and opening their stomata during the night when the evaporative demand of the atmosphere is lower. At night, atmospheric CO$_2$ is absorbed, resulting in the formation of malic acid, which is stored overnight in large vacuoles. During the day, malate is released from the vacuoles and decarboxylated; this CO$_2$ enters the photosynthetic carbon reduction cycle (Kluge and Ting 1978; Osmond 1978; Winter 1985).

In CAM, the stoichiometric ratio between moles of malate formed and moles of CO$_2$ absorbed is one (Kluge and Ting 1978). Therefore, integration of CO$_2$ uptake rates throughout the night should match the total amount of malate accumulated. In general, under nonstressful conditions, this expected relationship has been observed in many CAM species (Medina and Delgado 1976; Nobel and Hartsock 1978, 1983; Eickmeier 1979; Nobel et al. 1984; Winter et al. 1986; Virzo De Santo et al. 1987). Several variations have been observed, however, resulting in divergence from the expected 1:1 stoichiometry.

In terrestrial CAM plants under stress, stomata remain closed day and night while acid fluctuations continue through fixation of internally released, respired CO$_2$ (Szarek et al. 1973; Ting 1985; Winter et al. 1986; Lee et al. 1989). This process, termed "CAM-idling" (Ting 1985), may benefit a plant by preventing photoinhibition (Osmond et al. 1980) or by maintaining metabolic readiness during stress, enabling rapid recovery once the stress is removed (Szarek et al. 1973).

In other species, uptake of atmospheric CO$_2$ occurs throughout the day as in C$_3$ plants, while respiratory CO$_2$ is captured throughout the night and, as in CAM plants, stored in the form of malate. Thus, malate accumulates at night while stomata are closed, and, hence, no atmospheric CO$_2$ is absorbed. The ecophysiological significance of this form of intermediate metabolism, termed "CAM-cycling" (Ting and Rayder 1982; Ting 1985), is poorly understood, although recent work by Martin and co-workers suggests that this process may conserve water by reducing daytime transpiration (Martin et al. 1988; Harris and Martin 1991).

Most recently, another deviation from the 1:1 relationship between nocturnal malate formation and CO$_2$ uptake has been described for several species of epiphytic CAM plants (Griffiths et al. 1986; Smith et al. 1986; Griffiths 1988*a*) and a few terrestrial species (Sale and Neales 1980; Griffiths et al. 1986; Borland and Griffiths 1990). For example, in the genus *Tillandsia*, 50%–90% of the nocturnally accumulated malate could not be accounted for by the amount of atmospheric CO$_2$ absorbed (Griffiths et al. 1986). These plants assimilate atmospheric CO$_2$ at night, while simultaneously fixing respiratory CO$_2$. In the current study, this phenomenon is referred to as "CO$_2$ recycling during CAM." It is actually surprising that all CAM plants do not exhibit CO$_2$ recycling during CAM; apparently, rates of dark respiration

are too slow to contribute measurable quantities of CO_2 to the buildup of malic acid in most CAM plants studied. There are at least two hypotheses why some CAM plants refix such high levels of respired CO_2 (Benzing 1990). Nighttime respiration rates may be higher in these species because of the warm tropical environment characteristic of epiphytic CAM plants as compared with terrestrial CAM plants in arid, temperate regions (Winter et al. 1986; Lüttge and Ball 1987; Benzing 1990; Fetene and Lüttge 1991). Many terrestrial CAM plants, however, also grow in tropical regions and do not exhibit CO_2 recycling during CAM (Medina and Osmond 1981; Medina 1982). Conversely, some CAM plants that reportedly recycle CO_2 through CAM grow in temperate regions (Borland and Griffiths 1990).

Griffiths et al. (1986) have suggested that variations in leaf anatomy may explain this CO_2 recycling phenomenon. Leaves of many of the epiphytic CAM bromeliads that exhibit CO_2 recycling during CAM have two distinct tissue types, water-storage parenchyma ("hydrenchyma") and chlorenchyma. The leaf volumetric ratio of hydrenchyma : chlorenchyma varies widely among epiphytic bromeliads, particularly among species of *Tillandsia* (Tomlinson 1969; Benzing and Renfrow 1971). Hydrenchyma, lacking chlorophyll, should contribute CO_2 through respiration and might, in species with high hydrenchyma : chlorenchyma ratios, constitute a substantial source of CO_2 for fixation by the adjacent chlorenchyma tissue. Thus, an increase in the 1:1 malate : CO_2 stoichiometry would be expected.

The latter possibility has been investigated in two studies. Lüttge and Ball (1987) reported relatively minor contributions of CO_2 from the tissue hydrenchyma to the total leaf dark respiration in four terrestrial CAM plants. Griffiths (1988a), however, found greater amounts of CO_2 recycled during CAM in one epiphytic species of *Aechmea* that had a large amount of hydrenchyma tissue, relative to another species in this genus with less hydrenchyma in its leaves. The possible effects of anatomy on CO_2 recycling during CAM in other epiphytes remains unaddressed. We hypothesize that species with more hydrenchyma, relative to chlorenchyma, exhibit high levels of CO_2 recycling during CAM.

Material and methods

STUDY SPECIES AND GROWTH CONDITIONS

Individuals of *Tillandsia schiedeana* Steudel and *T. ionantha* Planchon were collected from *Taxodium distichum* (L.) Rich. at El Salto Falls, San Luis Potosí, Mexico; *T. usneoides* (L.) L., *T. recurvata* (L.) L., *T. balbisiana* Schultes, *T. paucifolia* Baker (= *T. circinnata* Schlechtendal), *T. fasciculata* Swartz var. *densispica* Mez, *T. val-*enzuelana A. Richard, *T. utriculata* L., and *T. setacea* Swartz were collected in the Everglades region near the Big Cypress National Preserve east of Naples, Collier County, Florida. Most of these species were also collected from *Taxodium distichum,* except *T. usneoides* and *T. recurvata,* which were collected from *Quercus virginiana* Mill. and *Quercus geminata* Small, respectively. *Tillandsia paleacea* Presl and *T. bergeri* Mez were obtained from the collection at the Marie Selby Botanical Gardens in Sarasota, Florida. Most plants were collected in July and November 1988, although some were collected earlier. Nomenclature and authorities of the epiphytes are according to Smith and Downs (1977).

Plants were maintained in a greenhouse at the University of Kansas under natural photoperiods with photosynthetic photon flux density (PPFD) between 500 and 1,000 μmol m^{-2} s^{-1}, depending on time of day, location in greenhouse, and seasonal variability; day/night average air temperatures of ca. 27/20 C (rarely maximum and minimum temperatures of 38 and 10 C were recorded); and day/night relative humidities of ca. 50%/80%. Plants were watered three to five times per week, and a dilute solution of nutrients (18% of each of total N, P_2O_5, K_2O; including trace elements) was applied once per week. All plants used in measurements appeared to be healthy, growing, and some species occasionally flowered.

LEAF TISSUE ANATOMY

Nine samples of midleaf sections of each species were fixed in formalin–acetic acid–50% ethanol (5:5:90, v/v/v), hand-sectioned, stained with toluidine blue and mounted in water, and their images were projected with a Ken-A-Vision (Tech A II) microprojector onto white construction paper using a 10 mm NA.25 lens. Leaf images were traced and cut out, and the tissue ratios calculated by weighing the paper cut-outs of chlorenchyma and hydrenchyma.

GAS EXCHANGE

Gas exchange of all species, with simultaneous sampling for malate, was measured from December 1987 to December 1988 and additionally in November 1989 and December 1990 for *T. paleacea* (Harris and Martin 1991). Nonliving material was removed; plants were wetted, allowed to surface-dry (ca. 1 h), and weighed (species-average FW ranged from 2.5 to 14 g). Individuals were sealed into temperature-controlled polycarbonate chambers and allowed to acclimatize 1 d at 30/20 C day/night, 1,000–1,500 μmol m^{-2} s^{-1} PPFD inside the chambers (12-h photoperiod), and constant dew point of 15.5 C. Net CO_2 exchange was recorded throughout the second day and night. At lights-on of the third day, several leaves or shoots were removed from the cham-

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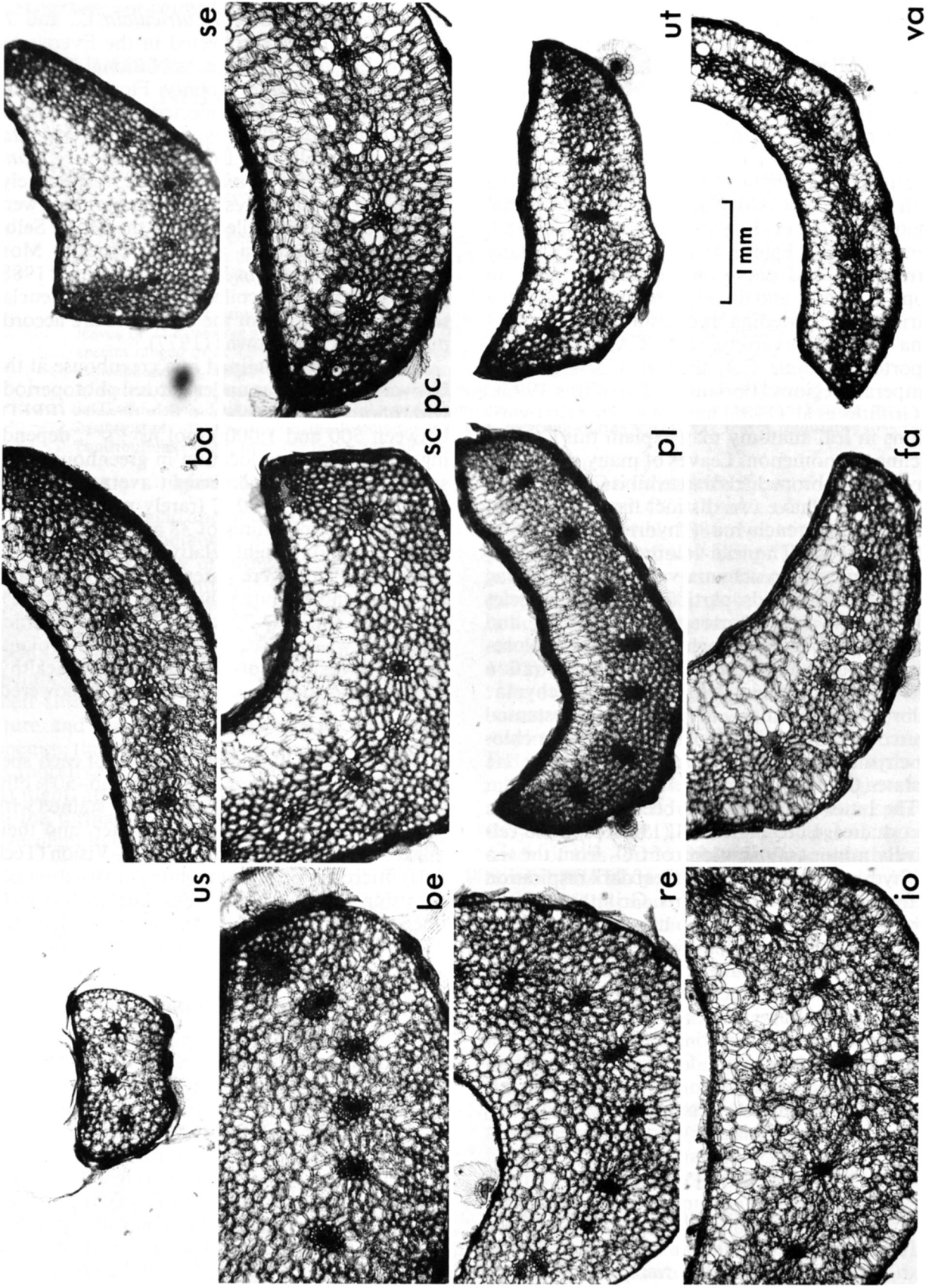
bers and stored at −65 C until malate analysis. At lights-out of the same day, the remaining plant tissue was frozen for malate analysis.

From the CO$_2$ exchange curves, integrated nighttime CO$_2$ uptake was calculated by determining the area of paper cutouts of the nighttime CO$_2$ uptake with a LI-COR (Lincoln, Nebr.) LI-3000 leaf area meter. Occasionally, data from the first night were used because of computer failure on the second night. Comparison of the data from both nights indicated that this substitution resulted in no substantial difference in total nighttime CO$_2$ uptake. Such substitutions were made for two plants in each of the following species: *T. usneoides, T. bergeri, T. recurvata, T. balbisiana, T. paucifolia,* and *T. valenzuelana.*

MALATE ANALYSIS

Sap was expressed from the thawed tissue by centrifugation according to Smith and Lüttge (1985). The tissue was weighed before and after centrifugation and after 3 d at 65 C. Mean (± standard deviation) fraction of tissue liquid extracted ranged from a minimum of 0.03 (± 0.01) for *T. recurvata* to a maximum of 0.23 (± 0.05) for *T. ionantha,* with a grand mean for all species of 0.13 (± 0.08). Malate concentration of the extracted liquid was determined according to Gutmann and Wahlefeld (1974), using standard curves of known malate concentrations.

STATISTICS

For the anatomical data, the hydrenchyma: chlorenchyma ratios were arcsine-transformed and analyzed by single-classification ANOVA and Tukey's HSD (Sokal and Rohlf 1981). Because sample sizes were small for the physiological data, the differences between each datum and its species-average were tested for normality, together with difference data for all means to be compared, using the D$_{max}$ statistic from the BASTAT software program (Rohlf 1985). These differences were normally distributed. If the variances of the means were equal according to an *F*-test, the data were tested further with the parametric Student's *t*-test; otherwise, they were tested with the nonparametric Mann-Whitney *U*-test (*T. paleacea* only). Significant differences between means were ascribed only when *P* < .05. All statistical procedures were according to Sokal and Rohlf (1981).

Results and discussion

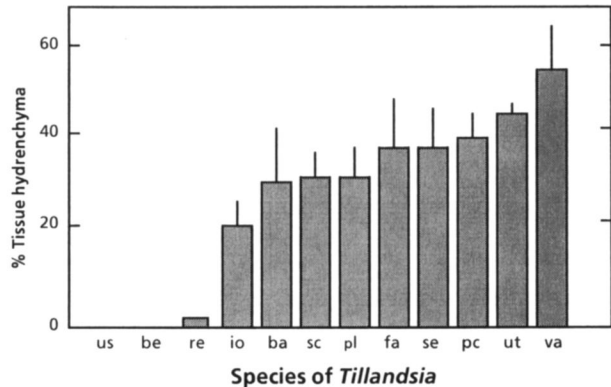The percentage of the leaf tissue attributable to hydrenchyma, estimated from midleaf cross-

Fig. 2 Percentage of the area of midleaf cross sections occupied by hydrenchyma (remainder as chlorenchyma) in 12 species of *Tillandsia.* Data are means of nine individuals; linear extensions represent 1 SD. Species abbreviations as in fig. 1.

sectional areas, varied widely among the 12 species of *Tillandsia* investigated (figs. 1, 2). Both *T. usneoides* and *T. bergeri* exhibited no distinguishable hydrenchyma tissue. The remaining species exhibited as little as 2.9% tissue hydrenchyma in *T. recurvata* to as much as 53% tissue hydrenchyma in *T. valenzuelana* (fig. 2). Tomlinson (1969) reported proportions of tissue hydrenchyma similar to those reported here for *T. usneoides, T. setacea, T. paucifolia,* and *T. fasciculata,* but a higher percentage (approximately 30%) for *T. recurvata.* This high variability between species in the amount of leaf volume occupied by hydrenchyma provided an excellent opportunity to test the hypothesis that respiratory CO$_2$ from the hydrenchyma significantly contributes to CAM in the chlorenchyma and alters the 1:1 stoichiometry expected for these parameters.

All individuals of all species exhibited Crassulacean acid metabolism, with CO$_2$ uptake primarily occurring during the night (fig. 3). Amounts of daytime CO$_2$ uptake (early morning and late afternoon [Osmond 1978]) were variable between individuals within species as well as between species. Reasons for this variability are unclear.

Contrary to expectations based on the above hypothetical relationship, only *T. schiedeana* exhibited a significant difference between the amount of malate formed at night and integrated nocturnal CO$_2$ uptake (fig. 4). Nearly twice as much malate was formed as could be accounted for by atmospheric CO$_2$ uptake. All other species exhibited the 1:1 relationship expected in CAM and previously found in many terrestrial species.

For each species, the ratio of nighttime malate

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Fig. 1 Representative midleaf cross sections of 12 species of *Tillandsia.* × 40. Abbreviations represent the following species: *us = usneoides, be = bergeri, re = recurvata, io = ionantha, ba = balbisiana, sc = schiedeana, pl = paleacea, fa = fasciculata, se = setacea, pc = paucifolia, ut = utriculata, va = valenzuelana.*
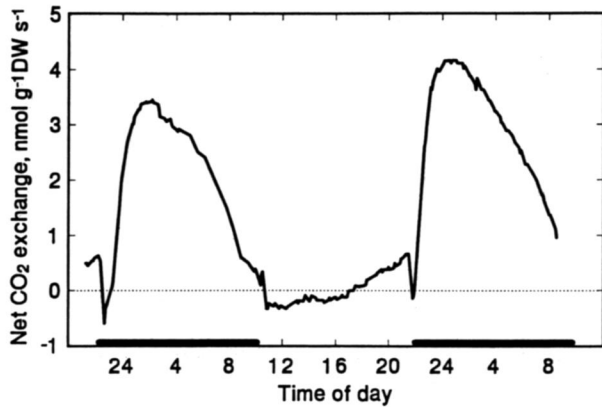
Fig. 3 Net CO_2 exchange of an individual of *Tillandsia fasciculata*. Dark bars indicate nighttime. The area under the curve during the second night was used to calculate integrated CO_2 uptake.
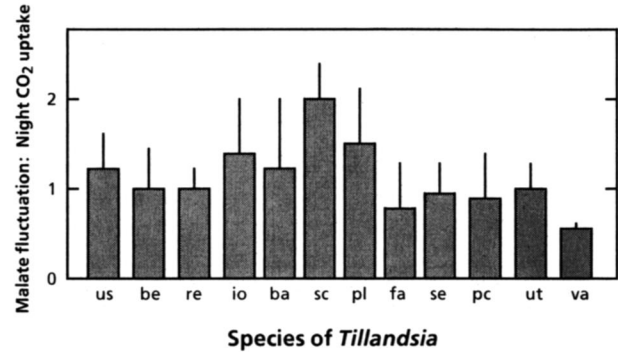

Fig. 5 Mean ratios of diurnal malate fluctuations : integrated night CO_2 uptake for 12 species of *Tillandsia* arranged in order of increasing percentage hydrenchyma in the leaves (from left to right; see figs. 1, 2). Data are means of four individuals (except *Tillandsia ionantha* [8], *Tillandsia paleacea* [7], *Tillandsia utriculata* [6]); linear extensions represent 1 SD. Species abbreviations as in fig. 1.

fluctuation to integrated CO_2 uptake (fig. 5) was calculated for each individual in order to minimize the variability between individuals in absolute nighttime activity. As expected from the above findings, *T. schiedeana* exhibited the highest ratio of 2.0 (fig. 5). Four other species, *T. usneoides, T. ionantha, T. paleacea,* and *T. balbisiana* also exhibited mean ratios higher than 1.0, although, as indicated by the large standard deviations, the variability between plants was

high. These results indicate that some individuals exhibited substantial levels of CO_2 recycling while others maintained the 1:1 stoichiometry expected for CAM. *Tillandsia valenzuelana* consistently exhibited malate : CO_2 ratios less than 1.0, assimilating atmospheric CO_2 that did not contribute to malate accumulation. There was, however, no significant difference between mean malate fluctuation and mean CO_2 uptake for this species as
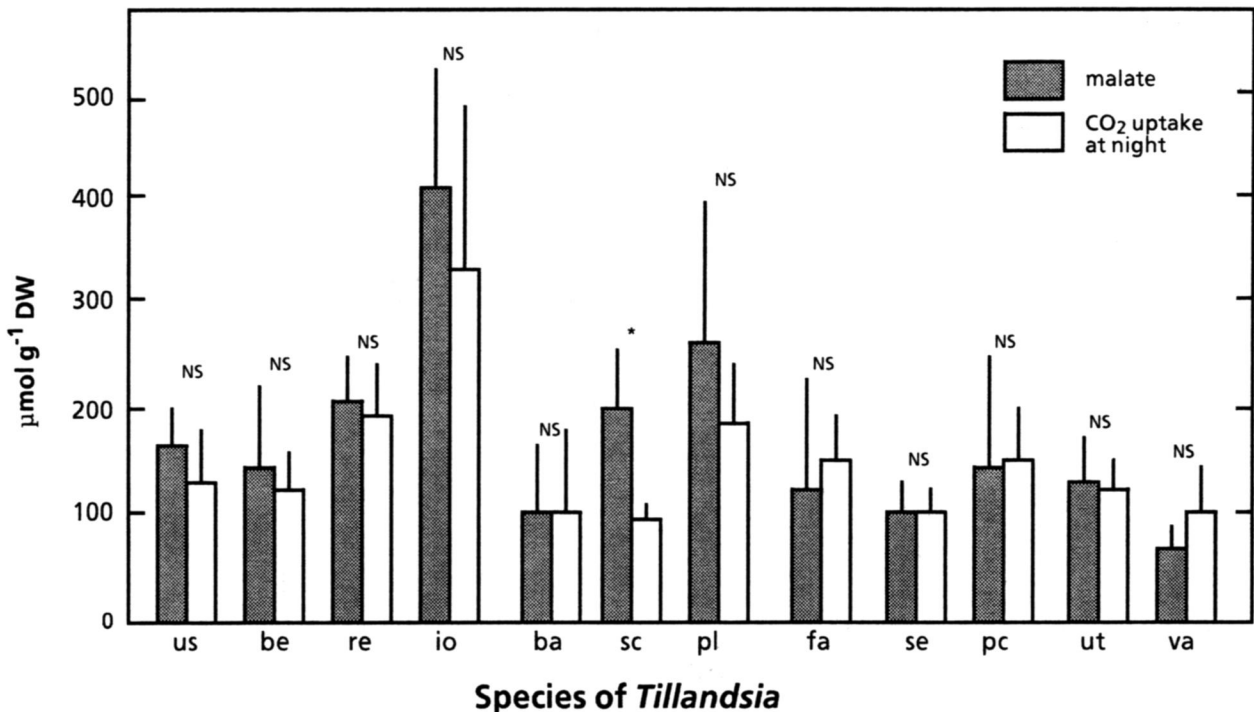

Fig. 4 Comparison of diurnal malate fluctuations (stippled bar) and integrated night CO_2 uptake (open bar) for 12 species of *Tillandsia* arranged in order of increasing percentage hydrenchyma in the leaves (from left to right; see figs. 1, 2). Malate fluctuations were obtained from the same individuals used in gas exchange measurements. Data are means of four individuals (except *Tillandsia ionantha* [8], *Tillandsia paleacea* [7], *Tillandsia utriculata* [6]); linear extensions represent 1 SD. Data for all species except *T. paleacea* were tested with Student's *t*-test. Mann-Whitney *U*-test was applied to *T. paleacea* data. NS = not significant; * = $P < .05$. Species abbreviations as in fig. 1.

a result of high variability between individuals in these photosynthetic parameters (fig. 4). The significance of these results when considered on a per plant basis is unclear. Perhaps CO_2 contributed to the accumulation of another acid such as citrate, although previous theoretical considerations indicate that this is unlikely (Lüttge 1988). *Tillandsia schiedeana,* with intermediate amounts of tissue hydrenchyma, was the only species to exhibit significant CO_2 recycling during CAM.

These findings are similar to those reported by Martin and Adams (1987). Species with the highest amounts of tissue hydrenchyma showed no indications of CO_2 recycling. Thus, the results of this study indicate that respiratory contributions of CO_2 from hydrenchyma tissue to CAM acid formation in the photosynthetic tissue is an unlikely mechanism to explain the general phenomenon of CO_2 recycling during CAM in epiphytes. The findings of this study may indicate that CO_2 recycling during CAM is not as widespread as currently thought (Griffiths 1989).

Past estimations of CO_2 recycling are difficult to interpret because they are often calculated using measurements of photosynthetic parameters from different plants or from field studies of plants with unknown stress levels (Griffiths 1989). The singular nature of consistent CO_2 recycling in *T. schiedeana* among the 12 species of *Tillandsia* studied here may suggest an unusually high level of respiration for this species alone or the presence of nonoptimal environmental conditions resulting in a response similar to CAM-idling (Griffiths 1988a, 1988b, 1989; Borland and Griffiths 1989; Lüttge 1990). *Tillandsia schiedeana* might, therefore, be expected to exhibit a malate : CO_2 uptake stoichiometry of 1:1 under different, i.e., optimal, environmental conditions. This assumes that nonoptimal conditions were used in this and the previous (Martin and Adams 1987) studies, in spite of attempts to ensure otherwise. Carbon dioxide recycling during CAM would thus represent a gradient between CAM and CAM-idling (Griffiths 1988a, 1988b, 1989; Fetene and Lüttge 1991). Further studies are necessary to conclude if either explanation accounts for the observation of CO_2 recycling during CAM in *T. schiedeana,* as well as in other species.

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