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Relationships among rainfall, leaf hydrenchyma, and Crassulacean acid metabolism in *Pyrrosia lanceolata* (L.) Fraw. (Polypodiaceae) in central Taiwan

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ABSTRACT

Leaf succulence is common among drought-adapted plants, including many tropical and subtropical epiphytic species. A prominent anatomical feature of many such succulent leaves is a clear, water-storing tissue often referred to as “hydrenchyma” (water-storage parenchyma). Functionally, hydrenchyma appears to store water for use by the leaf during drought. Although this has been confirmed in several laboratory studies, field studies linking the amount of hydrenchyma in plants with availability of water in their environment are lacking. In this study, the relative amount of leaf hydrenchyma in one of the most widely distributed epiphytes in Taiwan, *Pyrrosia lanceolata*, was measured in plants growing along a gradient of annual mean precipitation from 2048 to 3688 mm. In addition, because *Pyrrosia lanceolata* is a Crassulacean acid metabolism (CAM) plant, the amount of CAM activity was also examined in plants along the gradient. At each of seven sites along the precipitation gradient, leaves were collected, and, using thin mid-leaf slices, the relative areas of the leaf cross-sections occupied by hydrenchyma were determined. CAM, measured as diel changes in leaf acidity, was measured in plants from each site in the field, after 3 days of water-saturation in the greenhouse, and also after 14 days without water in the greenhouse. Regressions of relative hydrenchyma with ten environmental variables in the dry season revealed that the amount of hydrenchyma was significantly and positively correlated with monthly mean number of rainless days, monthly mean number of days with daily mean temperature over 30 °C, and monthly mean temperature. During the wet season, relative hydrenchyma area correlated only with the amount of cloud cover, and the correlation was negative. All plants at all sites exhibited CAM acid fluctuations in the field, under water-saturated conditions, and after desiccation. The largest nocturnal acid accumulations were found when plants were well-hydrated in the field and in the greenhouse, although evidence of drought-induced elevations of CAM was found at the drier sites. The results of this study indicate that the amount of leaf hydrenchyma was greatest in areas with warmer, drier environments. Also, drought-induced elevation of CAM activity occurred in plants from drier sites. This may help to explain the wide range of environments inhabited by this epiphytic fern in Taiwan.

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Introduction

Most epiphytes obtain water primarily from direct interception of rainfall, throughfall, and stem flow, but rarely from soil, and thus experience high variability in moisture availability in

the phorophyte canopy. Some epiphytes possess morphological traits related to drought resistance such as, thickened leaf cuticles, leaf epidermal trichomes, or sunken stomata (e.g., Andrade and Nobel, 1997; Loeschen et al., 1993) or develop specialized organs that store water (e.g., highly succulent stems in cacti or pseudobulbs in orchids). These traits, together with Crassulacean acid metabolism, are found in many epiphytes (Benzing, 1990; Loeschen et al., 1993; Martin et al., 2005; Martin, 1996), and help to maintain positive CO₂ uptake under prolonged periods without water (Herrera et al., 2000; Nowak and Martin, 1997). Many of the traits described above are also found among epiphytes in

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wet tropical or subtropical forests with high amounts of precipitation (e.g., Andrade and Nobel, 1997; Martin, 2010), indicating that water conservation is important for epiphytes even when droughts are infrequent and/or of short duration only (Andrade and Nobel, 1997; Martin, 2010; Stancato et al., 2001).

Within many succulent organs, specialized parenchymatous and chlorophyll-free cells, comprising hydrenchyma tissue, are often found adjacent to the photosynthetic chlorenchyma tissue. Compared to the latter, hydrenchyma has a less negative solute potential (Smith and Lüttge, 1985; Goldstein et al., 1991; Nowak and Martin, 1997; Schmidt and Kaiser, 1987) and/or a lower modulus of elasticity (Goldstein et al., 1991; Nobel, 1988, 2006; Stiles and Martin, 1996), enabling it to absorb water during

wet conditions and then supply water to the chlorenchyma during drought. For example, a progression of desiccation treatments was associated with a continuous decrease in relative size of the hydrenchyma in *Tillandsia ionantha* (Nowak and Martin, 1997), *Hylocereus undatus* (Nobel, 2006), and several species of *Peperomia* (Herrera et al., 2000; Schmidt and Kaiser, 1987), even though the relative size of the adjacent chlorenchyma remained unchanged. Furthermore, although the integrated nocturnal CO₂ uptake of the CAM bromeliad *Tillandsia ionantha* declined slowly, positive CO₂ uptake occurred up to 60 days without water (Nowak and Martin, 1997). Similar results showing the maintenance of physiological function at the expense of a reduced hydrenchyma volume were found for epiphytic species of *Peperomia* (Fondom et al., 2009;

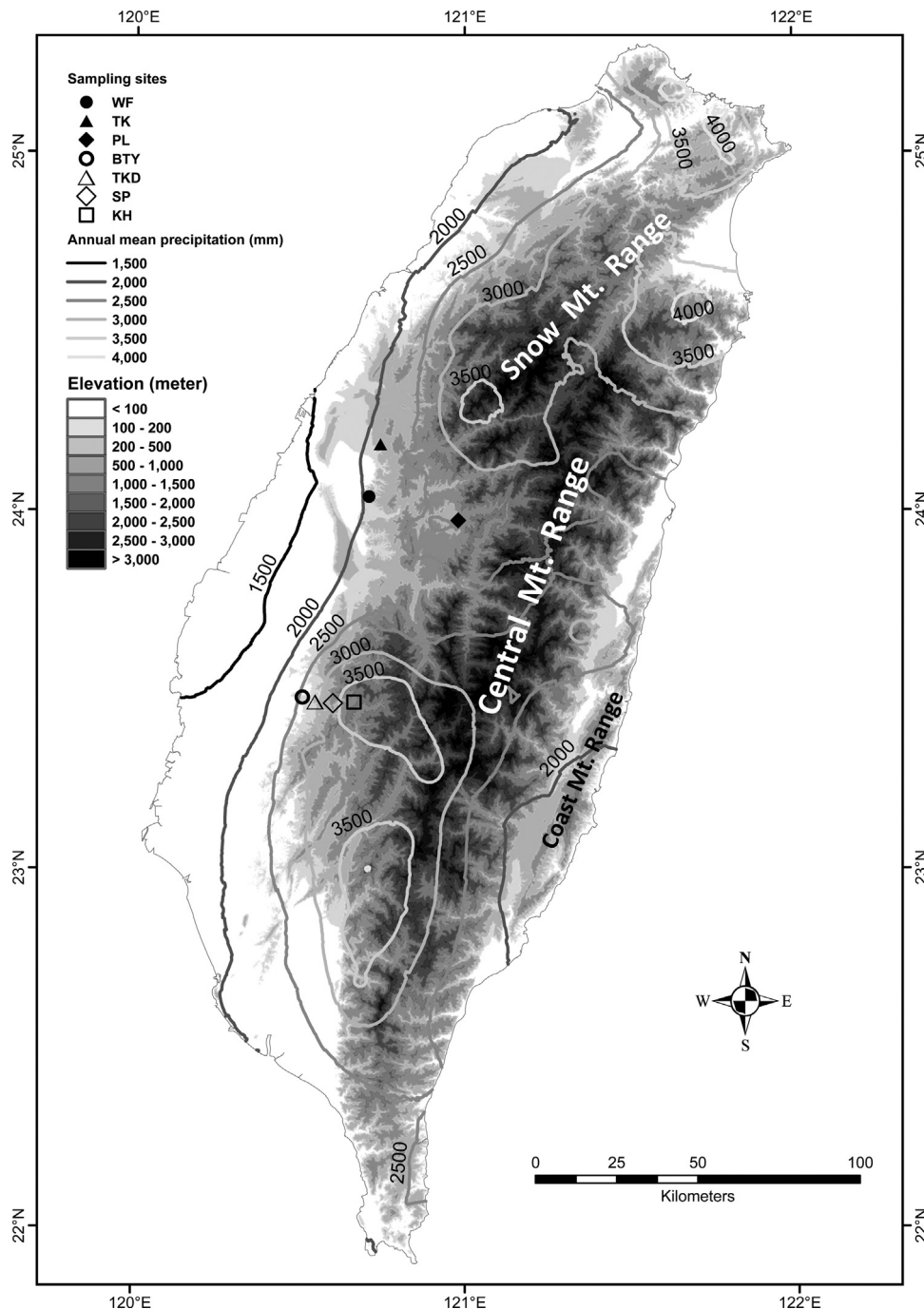


Fig. 1. Sampling locations of *Pyrrhosia lanceolata* in central Taiwan. The annual precipitation of sampling locations ranges from 2048 mm at WF to 3688 mm at KH. Site abbreviations: WF for Wufong, TK for Takeng, PL for Puli, BTY for Bantienyen, TKD for Tsaikongdien, SP for Shiapingtze, and KH for Kunghua.

Herrera et al., 2000; Schmidt and Kaiser, 1987) and cacti (Nobel, 2006).

Although the above studies have examined the function of hydrenchyma in several taxa under controlled conditions, field data in support of this function, to our knowledge, are lacking. Therefore, we investigated the relationships among the amount of hydrenchyma, precipitation, and CAM in a widely distributed epiphytic fern, *Pyrrhosia lanceolata* (L.) Fraw. [Polypodiaceae], in central Taiwan where populations of this species are found along a wide gradient of precipitation (Fig. 1). Furthermore, this species was chosen because the ability of its hydrenchyma to shrink and expand, as is necessary for a water-supply (not just storage) tissue, is supported by the findings of Ong et al. (1992). They reported that the cell walls of leaf mesophyll tissue (hydrenchyma or chlorenchyma not specified) in this epiphytic fern crumpled/folded when plants

were drought-stressed. Such results most likely result from shrinkage of the hydrenchyma of *P. lanceolata* leaves when plants are drought-stressed.

Pyrrhosia lanceolata typically grows on the trunks and major branches of large trees, with its roots and rhizomes lacking soil and being exposed. Its leaves are succulent with a distinct layer of hydrenchyma below the upper epidermis (see Fig. 2 and Warmbrodt, 1984). Like many epiphytes, this fern obtains most of its water from host throughfall and stem flow. In order to provide field data to accompany laboratory studies, the purpose of this study was to determine if the relative size of hydrenchyma in *P. lanceolata* correlates with the degree of drought in its habitat in central Taiwan. If such a correlation exists, this would provide strong evidence from the field that hydrenchyma tissue does indeed serve to store water for use by the leaves during drought. Because this

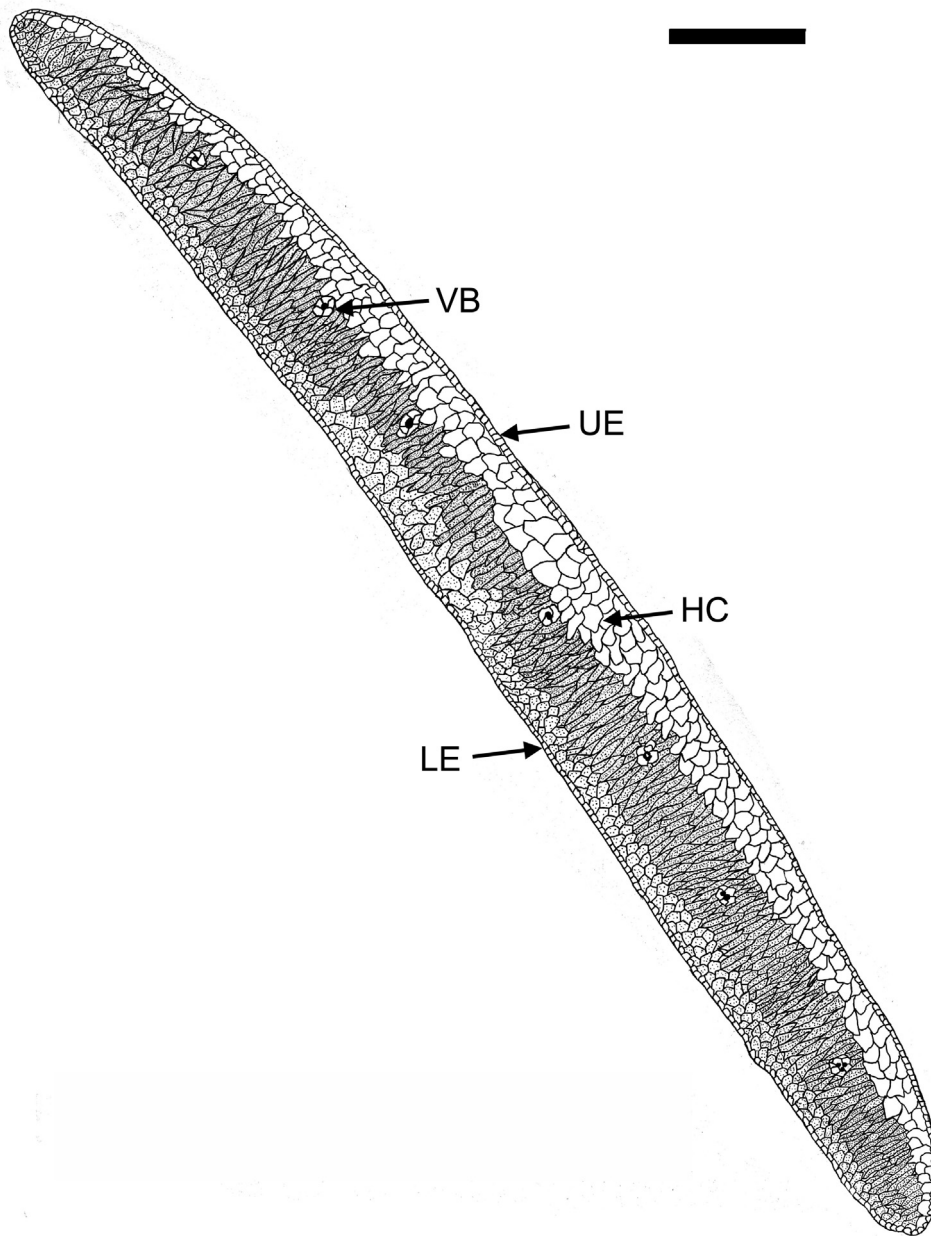


Fig. 2. Drawing of a cross section of a *Pyrrhosia lanceolata* leaf. The cross section in this figure was cut near the distal end of the blade. Dots within cells represent chloroplasts. A clear layer of hydrenchyma, chlorophyll-free parenchymatous cells, below the epidermis of the adaxial surface is found in all leaves. Scale bar 1 mm. HC: hydrenchyma, LE: lower epidermis, UE: upper epidermis, VB: vascular bundle.

epiphytic fern is a CAM plant (Hew, 1984; Holtum and Winter, 1999; Kluge et al., 1989a,b; Ong et al., 1986, 1997; Winter et al., 1983; Wong and Hew, 1976), and CAM clearly constitutes a physiological adaptation to drought (Kluge and Ting, 1978; Lüttge, 1987; Winter, 1985), the amount of CAM activity in populations of *P. lanceolata* along the precipitation gradient in central Taiwan was also investigated.

Materials and methods

Plants and study sites

Individuals of *Pyrrosia lanceolata* were collected from populations growing over a wide range of annual precipitation, from 2048 mm in Wufong (WF) to 3688 mm in Kunghua (KH) – for sampling sites, incl. their abbreviation codes, see Fig. 1. Precipitation, air temperature, number of hours of clear sky, cloud cover (Cloud_{cov}), and relative humidity for each of the 7 sites were interpolated by the universal Kriging method with the spherical model provided by ArcGIS (Chang et al., 2011). The weather data used for Kriging were based on an island-wide climate GIS database (Chang et al., 2011) with meteorological data from 526 stations, covering the period between 2001 and 2009. Cloud cover (Cloud_{cov}) ranged from 0 (cloudless) to 10 (100% overcast).

Determination of relative hydrenchyma area

At each of the seven sites, eight individuals (except at BTY, where only three individuals were found) were collected from the eastern side of the host trunk (each plant was collected from a different tree of several species) to minimize differences in exposure to solar radiation. After soaking in water for three hours in the laboratory, three leaves were excised from each plant, and a thin (<1 mm) cross section was taken near the center of the lamina of each leaf. A digital photograph was taken for each cross section using a dissection microscope. The areas of the entire leaf cross section and the hydrenchyma were traced on the photograph using Photoshop (Adobe Systems Incorporated, San Jose, CA, USA), then measured using Image J (NIH Image, Bethesda, MD, USA; <http://rsbweb.nih.gov/ij/>).

Determination of nocturnal acid accumulation and relative water content

Nocturnal acid accumulation (ΔH^+) and relative water content (RWC) were measured using three different individuals from each of the seven sites *in situ* (d 0), after transplantation in a greenhouse for three days under well-watered conditions (d 3), then throughout two weeks without watering (d 7, d 10, d 13, d 17). Each individual was affixed to an orchid mounting board made of tree fern stems and adventitious roots in the greenhouse. Air temperature and relative humidity inside the greenhouse were modulated by an automatic ventilation system and water walls. The water walls were running continuously throughout the experiment and the ventilation system was automatically turned on when the temperature inside the greenhouse exceeded 27 °C. The daily maximum PPFD inside the greenhouse ranged from 80 to 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, depending on the weather conditions. Nocturnal acid accumulation of plants for each date of measurement was determined by subtracting the titratable acid of leaf tissues obtained at 5 pm from that of the leaves obtained at 7 am. At both collection times, a leaf was excised from a leaf pair previously marked on the same shoot segment, placed in plastic bags in an ice-filled cooler (field samples only), then transported to a freezer in the laboratory and frozen (−10 °C) within 2 h. Upon thawing

Table 1

Pearson's correlation coefficients (*r*) between relative hydrenchyma areas in leaf cross-sections of *Pyrrosia lanceolata* and environmental variables. The dry season lasts from October to April the next year. The wet season lasts from May to September.

Environmental variables ^a	Dry season		Wet season	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Precip.	−0.27	0.059	−0.12	0.399
<i>D</i> _{rainless}	0.38	0.006	0.08	0.564
<i>D</i> _{gt30}	0.30	0.035	0.10	0.491
<i>D</i> _{lt10}	−0.08	0.574	−0.12	0.421
<i>T</i> _{max}	0.15	0.293	0.14	0.344
<i>T</i> _{mean}	0.28	0.050	0.18	0.199
<i>T</i> _{min}	0.09	0.509	0.14	0.333
HR _{sunny}	−0.01	0.932	0.20	0.169
Cloud _{cov}	−0.08	0.596	−0.36	0.009
RH	0.18	0.208	−0.10	0.473

^a Abbreviations: Precip. for mean monthly precipitation in mm; *D*_{rainless} for monthly mean rainless days; *D*_{gt30} and *D*_{lt10} for monthly mean number of days with daily mean temperature > 30 °C and < 10 °C, respectively; *T*_{max}, *T*_{mean}, and *T*_{min} for monthly mean maximum, mean, and minimum daily temperature, respectively. HR_{sunny} for the monthly mean sunny hours; Cloud_{cov} for cloud cover, ranging proportionally from 0 (no cloud) to 10 (fully overcast sky); RH for relative humidity (%).

(within 24 h after leaf collection), a leaf disc of 20.6 mm diameter was removed using a cork borer, weighed, and ground in 50 ml of distilled water using mortar and pestle. The resultant slurry was titrated to pH 7.0 using a pH meter (Microcomputer pH-vision 6071 Jenco Electronics, Ltd, Grand Prairie, TX, USA), then dried at 70 °C until no further weight change occurred to obtain the dry mass.

To determine relative water content, an additional leaf disc of the same size was collected from a detached leaf from each individual at 7 am on each sampling date. After weighing, the leaf discs were dried as above. The relative water content was calculated as:

$$\text{RWC} = \frac{(\text{FM}_a - \text{DM}_a) \times 100}{\text{FM}_i - \text{DM}_i}$$

where FM_a and DM_a are fresh mass and dry mass, respectively, FM_i and DM_i are initial fresh mass and dry mass on d 3 when plants were fully saturated.

Statistical analyses

Differences among means were tested for significance using an analysis of variance, followed by the LSD multiple comparison procedure. Pearson's bivariate correlations were used to determine and test the relationships between the relative hydrenchyma area and site-specific environmental variables. All statistical procedures were performed using R statistical package version 2.12 (R Development Core Team, 2010).

Results

Variation of relative hydrenchyma area along the moisture gradient

Relative hydrenchyma area of the leaf cross sections (Fig. 2) of individuals ranged from 12.2% at the KH site to 29% at the TKD site (Fig. 3). Mean relative hydrenchyma areas of leaves were significantly greater at the BTY and TKD sites than those at the other sites (*p* < 0.001). Among all the environmental variables tested, the amount of leaf relative hydrenchyma area of leaf cross sections significantly correlated with mean monthly number of rainless days (*D*_{rainless}), mean monthly number of days with daily mean temperature greater than 30 °C (*D*_{gt30}), and monthly mean temperature (*T*_{mean}) during the dry season only (Table 1 and Fig. 3). In the

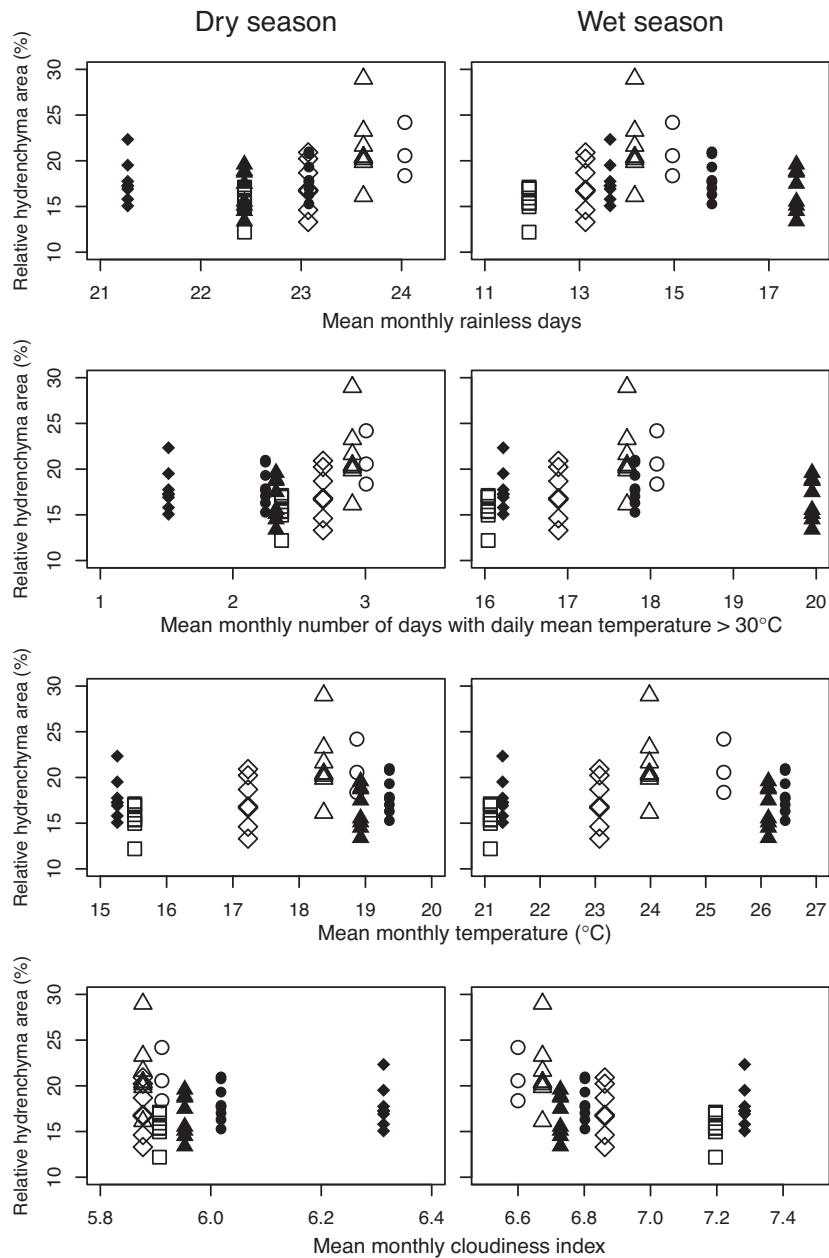


Fig. 3. The relationship between relative hydrenchyma area (%) of *Pyrosia lanceolata* leaves and selected environmental variables (only significant correlations shown, although other environmental variables also tested [see text]). The dry season in this region lasts from October to April the next year. See Fig. 1 for site symbols.

wet season, the relative hydrenchyma area of the leaves correlated only with cloud cover ($Cloud_{cov}$), and this correlation was negative (Table 1 and Fig. 3). The relative hydrenchyma area did not correlate significantly with the amount of precipitation in either dry or wet seasons.

Responses of nocturnal acid accumulation and water status to drought condition

Leaf water content was greatest after 3 days of the saturated water treatment (Fig. 4; $RWC = 100\%$). In comparison, leaf RWC under field conditions ranged from 45% at TK to 85% at KH. At the end of the desiccation treatment (3–17 days), the mean leaf RWC of plants from different populations ranged from 24% (TKD) to 35% (PL).

Regardless of leaf water content, tissue titratable acidity increased overnight in all populations under both field and

greenhouse conditions (Fig. 4). With the exception of the BTY site, ΔH^+ was maximal either in the field (0 day) or under the saturated water condition (3 days). Nocturnal acid accumulation decreased with the progression of the drought treatment (3–17 days) in plants at all sites, although differences were significant only for the KH site.

Discussion

Although the functional significance of leaf hydrenchyma has been elucidated in a number of laboratory studies, few studies have included consideration of these findings for plants in the field. In this study, we used the epiphytic CAM fern *Pyrosia lanceolata* to examine the ecological significance of hydrenchyma along a precipitation gradient in central Taiwan.

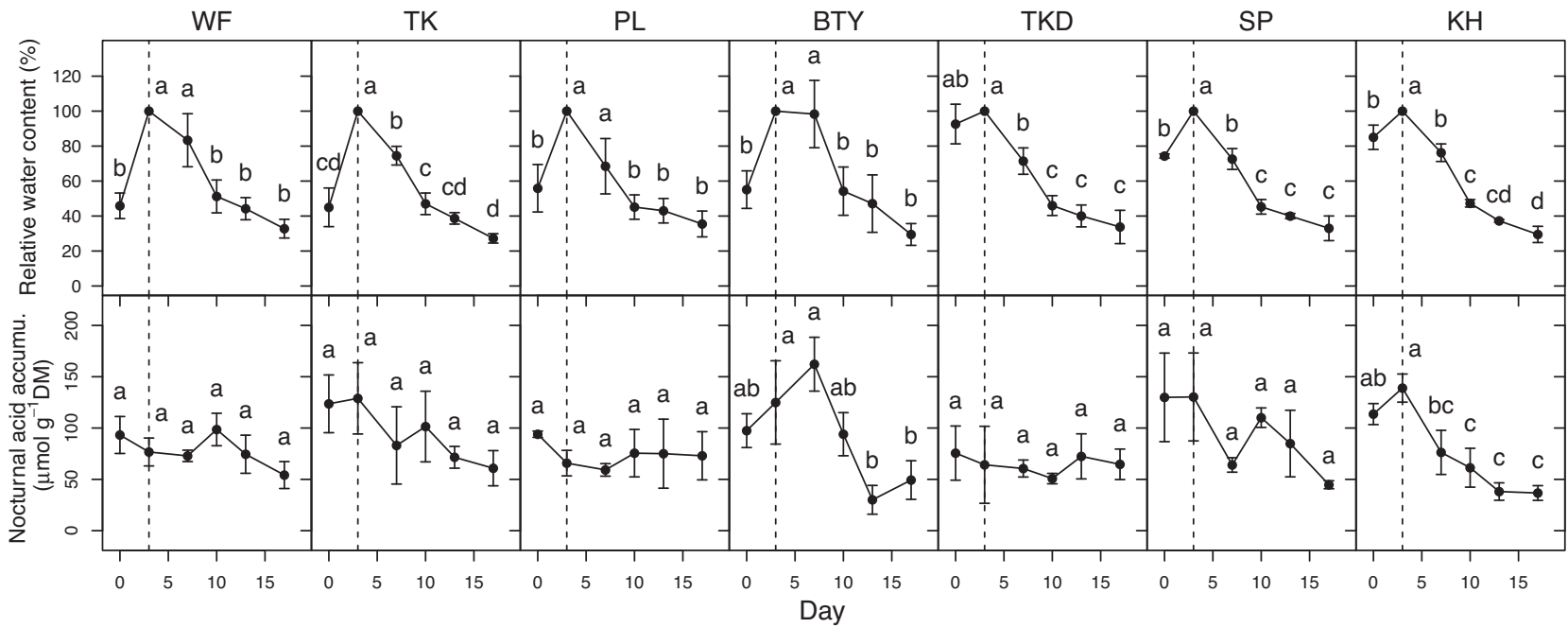


Fig. 4. Nocturnal acid accumulation ($\mu\text{mol g}^{-1}$ DM) and relative water content (%) (\pm standard error) of *Pyrosia lanceolata* in the field (0 day), after three days of saturated water condition (3 days, indicated by vertical dashed lines), and subsequent 14 days of drought (7 days, 10 days, 13 days, 17 days). Different lowercase letters indicate significant differences among dates.

Greater size of hydrenchyma was found at drier and warmer sites

In laboratory studies of several taxa, leaf hydrenchyma has been shown to be a potential adaptation to water scarcity. Thus, the relative area of hydrenchyma in cross sections of *P. lanceolata* leaves was hypothesized to vary across a water supply gradient in subtropical Taiwan, e.g., plants in habitats with less rainfall should have more hydrenchyma than those in wetter habitats. To test this hypothesis, plants were sampled in their natural habitats in central Taiwan along a gradient spanning a nearly 1.8-fold difference in annual and seasonal rainfall. Among the variables that correlated with leaf relative hydrenchyma area, significant relationships were found only with dry season measures of habitat aridity. Greater relative amounts of hydrenchyma should provide the leaf photosynthetic tissue (chlorenchyma) with water during leaf desiccation, presumably allowing prolonged photosynthesis and survival for *P. lanceolata* at sites with more frequent rainless periods (greater D_{rainless}) and warmer temperature (greater $D_{\text{gt}30}$ and T_{mean}) during the dry season. The number of rainless days per month was a better predictor of hydrenchyma amount than was the total amount of precipitation, indicating that the level of drought experienced by these epiphytes was better reflected by the duration of rainless periods than by the quantity of rain.

Levels of CAM in the field and greenhouse

All *P. lanceolata* individuals in this study, regardless of site/elevation of origin, measurement in the field or greenhouse, or leaf water content, exhibited significant nocturnal increases in leaf tissue acidity, supporting past reports of CAM in this epiphytic fern (Ong et al., 1986; Winter et al., 1986; Wong and Hew, 1976), although Sinclair (1984) reported very weak diel changes in tissue acidity in this species, and Holtum and Winter (1999) provided evidence for variable C_3 -CAM intermediacy in the same species. The occurrence and degree of CAM in other species in this genus appears variable or questionable. For example, nighttime stomatal opening in *P. agustata* was accompanied by only very small increases in leaf acidity (Sinclair, 1984).

Maximal levels of CAM in *P. lanceolata* in the current study did not correlate consistently with site location, measurement conditions, or water content. The latter may reflect plant-to-plant variability, population (ecotype?) differences, or environmental differences not measured in this study, e.g., irradiance exposure, atmospheric humidity, etc. Similar to some results of the current study, Sinclair (1984) reported somewhat greater diel changes in leaf titratable acidity in drought-stressed individuals of *P. lanceolata*, relative to well-hydrated plants (note that *P. adnascens* is considered synonymous with *P. lanceolata*; Hovenkamp, 1986). In contrast, although the focus of the work on *P. lanceolata* by Winter et al. (1986) was on sun/shade adaptations, their finding that this epiphytic fern is primarily shade-adapted might be interpreted to predict that plants in exposed conditions, where tissue desiccation is more likely, would exhibit lower levels of CAM (Martin, 1994; Haslam et al., 2003). Whether or not the coarse-scale results of the current study apply at a finer level, i.e., exposed vs. shaded plants, remains a subject for further investigation.

The results of Ong et al. (1986), that CO_2 uptake by *Pyrrosia lanceolata* is particularly sensitive to drought stress, are difficult to compare with those of the current study because the amount of leaf hydrenchyma in the plants used by Ong et al. (1986) was not specified, nor was the exposure level at the collection site. Given the sensitivity to drought stress of the epiphytes reported by Ong et al. (1986), the results of the current study may indicate that these plants were collected from a very moist habitat; thus, had little leaf hydrenchyma. Also, Ong et al. (1986) attributed a quick

drop in CO_2 uptake with drought to high water vapor conductance, although they showed very little change in diel changes in tissue acidity with drought. Similar results were reported by Kluge et al. (1989a,b) for individuals of *Drymoglossum* (= *Pyrrosia*) *piloselloides* in Singapore. Working with the same species, Hew (1984) found greater nocturnal acid accumulations in leaves subjected to small amounts of osmotic stress, but smaller acid accumulations when leaves were further stressed.

Overall, the results of the current study indicate that leaves of plants of the epiphytic CAM fern *Pyrrosia lanceolata* growing in habitats with less available water, have greater amounts of hydrenchyma, which should act to buffer the photosynthetically active portions of the leaves from the effects of whole-leaf water loss during rainless periods. These findings, albeit correlative in nature, provide support, based on plants growing under natural conditions, to past laboratory studies of the importance of leaf hydrenchyma in maintaining leaf physiological activity during periods of drought stress. Furthermore, the CAM mode of photosynthesis, regardless of its variability in response to tissue hydration, should conserve considerable amounts of leaf water, regardless of habitat wetness/aridity, in these plants. Consideration of these findings may help to explain the widespread distribution of *Pyrrosia* in Taiwan and other regions of Southeast Asia.

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