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Leaf Characteristics and Photosynthetic Performance of Floating, Emergent and Terrestrial Leaves of *Marsilea quadrifolia*

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ABSTRACT: Individuals of *Marsilea quadrifolia*, an amphibious fern, experiencing extreme variation in environment develop heterophyll. In this study, we compared stomatal and trichome density on upper and lower surfaces, leaf and petiole area mass ratio, spectral properties and photosynthetic performance of floating, emergent and terrestrial leaves of *M. quadrifolia*, to explore the ecological advantages of producing different leaf types. Morphological measurement reveals that these three types of leaf display highly differences in stomatal density on lower epidermis, trichome density on both surfaces and petiole dry mass per length, and reflectance coefficient between 500 and 650 nm. In contrast, no significant difference was found in the PSII electron transport rate of the three types of leaves. The analysis of stable carbon isotope ratio of the three types of leaves indicates that they all use C_3 photosynthetic pathway.

KEY WORDS: amphibious fern, heterophyll, *Marsilea quadrifolia*, PSII electron transport rate, stable carbon isotope ratio, stomatal density, trichome density.

INTRODUCTION

Marsilea is an amphibious fern which has the ability to develop heterophyll. In a completely submerged condition, rhizomes of the plant produce fork-like water leaves with leaflets, 1 to 4, expanding parallel to the long axis of the petiole. When the leaflets were raised above the surface of water, they expand on a plane perpendicular to the petiole, and the leaf is referred to as aerial form. Many studies have focused on the factors governing formation of submerged and aerial leaves in this fern (Allsopp, 1962; Gaudet, 1964; Lin and Yang, 1999; Liu, 1984). Some other studies have examined morphological and anatomical difference between submerged and aerial leaves of *Marsilea* (Allsopp, 1953, 1954, 1955; Gaudet, 1964). In addition to submerged and aerial leaves, it is not rare to find *Marsilea* spp. develop leaves with long petiole and floating on water surface. Growing in dry land, *M. quadrifolia* produces terrestrial leaves. Accordingly, *Marsilea* spp. could potentially develop submerged, floating, and emergent leaves in aquatic habitats while develop terrestrial leaves in terrestrial conditions. In comparison to studies of submerged and emergent leaves, relatively less attention has been made on floating and terrestrial leaves in *Marsilea*. Floating leaves of *Marsilea*, aside from occasional mentioned in some reports (Gaudet, 1964), have often being ignored by researchers. However, floating leaves might represent an intermediate between submerged and emergent leaves, while terrestrial leaves an adaptation to completely terrestrial condition. Hence, these two types of leaf deserve additional investigation.

Differences in leaf morphology, such as differentiation of stomata and trichomes and length of petioles have been found between submerged and land leaves of *M. quadrifolia* (Liu, 1984). Are there also differences in these parameters among floating, emergent and terrestrial leaves? This is the first question we would like to answer in this study.

Optimized carbon gain may represent one of the driving forces for the development of heterophyll in the *Marsilea* spp. (Lin and Yang, 1999). However, compared to morphological and structural studies, little information is available as to the photosynthesis of this genus. Chlorophyll fluorescence techniques provide a rapid and convenient method for estimate photosynthetic performance (Björkman and Demmig-Adams, 1994). In this study, we also used the technique to obtain non-destructive estimates of photosynthetic performance of the study materials.

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Elocharis vivipara, an amphibious plant, has been shown to express C_3 and C_4 modes of photosynthesis in contrasting environment (Ueno et al., 1988). Stable carbon isotopes ratio ($\delta^{13}C$) have been used as indicators of photosynthetic pathways in terrestrial plants (O'Leary, 1988). In this study we also analyzed leaf carbon isotope ratio to investigate the photosynthetic pathway utilized by these three types of leaves.

The objectives of this study are to compare leaf characteristics and photosynthetic performance of floating, emergent and terrestrial leaves of *M. quadrifolia*. Specifically, we asked are there differences in leaf characteristics among floating, emergent and terrestrial leaves? If so, what could be the ecological advantages of the morphological adjustment in these three types of leaves? Do these three types of leaves have different photosynthetic rate and/or utilize different photosynthetic pathways? We test the hypothesis that *M. quadrifolia* adjusts its leaf characteristics to achieve optimal photosynthetic performance in different environment.

MATERIALS AND METHODS

Plant materials

To obtain terrestrial grown *M. quadrifolia*, we transplanted submerged sporophytes, which were originally established from sporocarps and propagated in aseptic cultures (Liu, 1984; Lin and Yang, 1999), into potting soil and grew them in a glasshouse under natural sunlight condition. After producing a few terrestrial leaves, ten rhizomes of terrestrial grown *M. quadrifolia* were transferred to plastic tanks (41 x 36 x 36 cm) filled with potting soil and subjected to different watering regimes. Water was injected into tanks to maintain water level at 5 and 10 cm above the soil, under these conditions, plants developed emergent leaves and floating leaves, respectively. Plants were grown in a glasshouse and fertilized with Hyponex 2 (N:P:K = 20: 20: 20).

Methods

Characteristics of leaves, including stomatal density, trichome density, leaf and petiole area mass ratio and spectral properties, were compared in leaves produced in September 2002 (summer leaves) and those in Jan. 2003 (winter leaves). Fully expanded leaflets (ca. 5 days old) were excised from the plants (n = 10) for the examination of stomatal distribution and trichome density. To measure stomatal density, we used nail polish to make imprints of epidermis. Due to the difficulty in peeling the imprints of both surfaces from the same leaflet, each leaflet was cut lengthwise into two segments, one half for the measurements of stomatal density of upper surface and the other for the lower surface. The imprint of the whole segment was scanned at 600x magnification with a light microscope equipped with a calibrated ocular micrometer, for estimates of stomatal density. The ratio of stomatal density on upper surface to that on lower surface (SU/SL) was then calculated. Trichome density on both surfaces was estimated with a dissecting microscope.

After being excised from shoots, leaflets and petioles were separated, blot dry and then weighted. Leaflet area and the length of petioles were subsequently measured using a leaf area meter (Li-3100, Licor, Nebraska) and a stick meter, respectively, then dried at 60℃ for at least 48 hrs. Dry weight (DW) of leaflets and petioles were then measured again. Leaf mass area (LMA) was calculated as DW/area, and petiole mass per length (PML) as petiole dry weight/length.

Only summer leaves were used for following measurements, including spectral properties, photosynthetic performance and the ratio of stable carbon isotope.

After leaf reflectance and transmittance coefficient between 400 – 700 nm were measured using a spectroradiameter (Li-1800, Licor, Nebraska), the absorption coefficient was calculated as: 1- reflectance coefficient - transmittance coefficient.

To investigate the fluorescence-PPFD (photosynthetic photon flux density) response, potted plants (n = 3, for each type of leaf) were brought into the laboratory and leaflets were held in a leaf-clip holder (2030-B, Walz, Effeltrich, Germany). The PPFD on leaves, provided by a halogen lamp, was adjusted at 20, 40, 90, 130, 220, 330, 500 and 620 $\mu molem^{-2}\ s^{-1}$. After the leaflet was exposed to the desired PPFD for 10 min, the chl *a* fluorescence of PSII was measured using a portable, pulse amplitude modulated fluorometer (PAM 2000, Walz, Germany). The quantum yield of PSII (yield) and the apparent electron transport rate (ETR = yield * PPFD * 0.5 * 0.86) were computed from the Chl. fluorescence measurement.

Leaflets were excised following fluorescence measurement, oven dried and ground to a fine powder with a mortar and pestle. $\delta^{13}C$ values were then determined using continuous flow isotope mass spectrometry (CF-IRMS) consisting a Carlo Erba Elementary Analyzer (NA 1500, Fisons, Italy) connected to an isotopic ratio mass spectrometer (delta S, Finnigan). $\delta^{13}C$ values were calculated in the

Table 1. Leaf mass per area (LMA, mg cm^{-2}), petiole mass per length (PML, mg cm^{-1}), trichome density (no. of trichome mm^{-2}) on upper (TU) and lower surface (TL) and total trichome density (TU + TL), stomatal density (no. of stomata mm^{-2}) on upper (SU) and lower (SL) surface, total stomatal density (SU+SL) and the ratio of stomatal density on lower surface to that on upper surface (SL/SU, %) of floating, emergent and terrestrial leaves of *M. quadrifolia*. Mean ± s. e., n = 6-10. On the same sampling day, values within the same column followed by different superscripts represent significant difference at P = 0.05. ---: not measured.

Sampling date	*Leaf type*	*LMA*	*PML*	*TU*	*TL*	*TU + TL*	*SU*	*SL*	*SU + SL*	*SU/SL*
Sept. 14, 2002	*Floating*	*1.74 ±0.07^a*	*0.26 ±0.01^a*	*1.1 ±0.3^a*	*0.2 ±0.1^a*	*1.3 ±0.3^a*	*387.1 ±26.1^a*	*54.9 ±18.3^a*	*436.5 ±22.4^a*	*15.6 ±6.5^a*
	Emergent	*2.13 ±0.12^b*	*0.75 ±0.03^b*	*8.5 ±1.6^b*	*10.8 ±2.3^b*	*19.3 ±2.2^b*	*266.0 ±23.0^b*	*198.1 ±14.9^b*	*464.2 ±38.4^a*	*75.5 ±3.3^b*
	Terrestrial	*3.08 ±0.08^c*	*1.10 ±0.08^c*	*19.4 ±2.7^c*	*22.7 ±2.6^c*	*42.2 ±4.5^c*	*427.0 ±27.1^a*	*311.3 ±23.8^c*	*738.4 ±52.8^b*	*72.6 ±2.2^b*
Jan. 5th, 2003	*Floating*	*2.51 ±0.09^a*	*0.39 ±0.01^a*	---	---	---	*321.7 ±12.1^a*	*22.0 ±5.5^a*	*343.7 ±11.0^a*	*7.2 ±2.0^a*
	Emergent	*2.82 ±0.09^a*	*0.54 ±0.02^a*	---	---	---	*350.0 ±14.9^a*	*77.7 ±11.8^b*	*427.7 ±19.0^b*	*22.5 ±3.6^b*
	Terrestrial	*2.74 ±0.10^a*	*1.17 ±0.10^b*	---	---	---	*204.7 ±9.5^b*	*103.1 ±6.4^c*	*307.9 ±14.0^a*	*50.7 ±2.7^c*

usual manner as: $\delta^{13}C = [(R_{sample}/R_{standard}) -1] * 1000$. Where R is the corresponding ratio of ^{13}C: ^{12}C. The universally agreed standard for $\delta^{13}C$ is Pee Dee Belmnite.

RESULTS

Though summer leaves (produced in September of 2002) showed significant difference in their LMA, with floating < emergent < terrestrial leaves, however, no difference in this parameter was found among leaf types produced in January of 2003 (Table 1).

Significant difference in petiole mass per length (PML) was also found among leaf types with that measured highest in terrestrial leaves, medium in emergent and lowest in floating leaves (Table 1).

The three types of leaves all have trichomes on both surfaces (Table 1). No significant difference was found in trichome density between upper and lower surface in leaves of each type. In contrast, trichome density, either on upper or on lower surface, was found significantly different among leaf types with the highest in terrestrial leaves, medium in emergent leaves and least in floating leaves. As a result, terrestrial leaves have the highest total trichome density, emergent leaves the medium and floating leaves the least. Trichome density was not measured in leaves produced in January, 2003.

Among the characteristics measured in summer and winter leaves, stomatal density on lower surface (SL) was consistently lower than that on upper surface (SU) for all three types of leaves (Table 1). As a result, the ratio of SL to SU (SL/SU) was less than 1 for the three types of leaves. In both seasons, the SL was found to be highest in terrestrial leaves, medium in emergent and lowest in floating leaves. In September, terrestrial leaves have significantly higher total stomatal density than floating and emergent leaves. For all three types of leaves, SL/SU was higher in summer leaves than in winter leaves.

In general, leaves had the highest reflectance and transmittance coefficient at ca. 550 nm between the measurement wavelengths ranging from 400 to 700 nm (Figs. 1a & b), as a result, the lowest absorptance coefficient was measured at this wavelength (Fig. 1c). Between 500 and 650 nm leaf types had different reflectance coefficient, with terrestrial leaves the highest, emergent the intermediate, and floating leaves the least. Within the same range of spectrum, floating leaves and emergent leaves had similar transmittance coefficient while terrestrial leaves had significantly lower transmittance than the other two. As a result, no significant difference was found in the absorption coefficient among the three leaf types in the measurement wavelengths ranging from 400 to 700 nm. In general, the highest absorptance was recorded in wavelength between 400 to 490 nm and at 650 nm, ca. 0.93. The lowest absorptance coefficient was found at 550 nm, ca. 0.7. The average absorptance coefficient for the range of spectrum from 400 to 700 nm was 0.86.

No significant difference was found in $\delta^{13}C$ values of floating and emergent leaves, -27.4 ± 0.2 and -27.1 ± 0.1 ‰ (mean ± s.e., n = 10), respectively. $\delta^{13}C$ of terrestrial leaves (-28.2 ± 0.1 ‰) was significantly more negative than those of the other two leaf types. Measurements of chlorophyll *a* fluorescence reveal that three types of leaves had similar effective quantum yield of PSII at any given PPFD (Fig. 2a). Consequently, the ETR (estimated as the effective

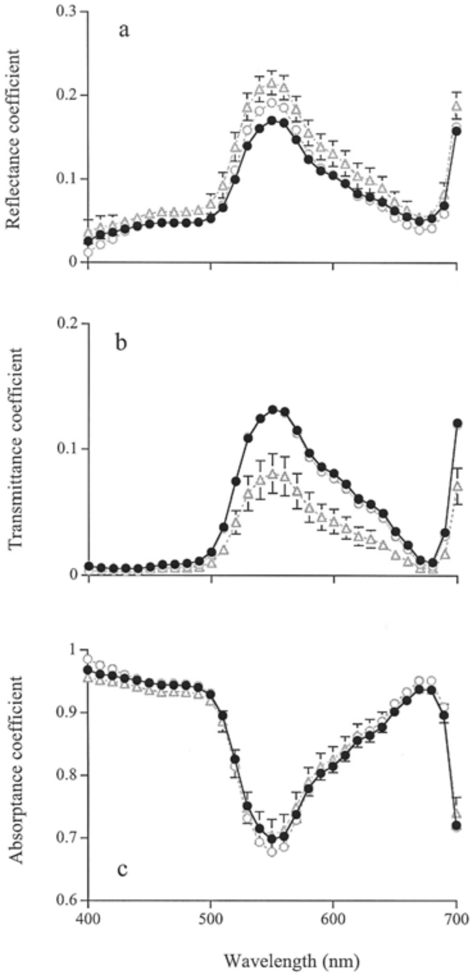

Fig. 1. The reflectance (a), transmittance (b) and absorptance (c) coefficient of floating (●), emergent (○) and terrestrial (△) leaves of *M. quadrifolia* measured in the spectral range of 400 to 700 nm. Each point represents the average of 6 replicates.

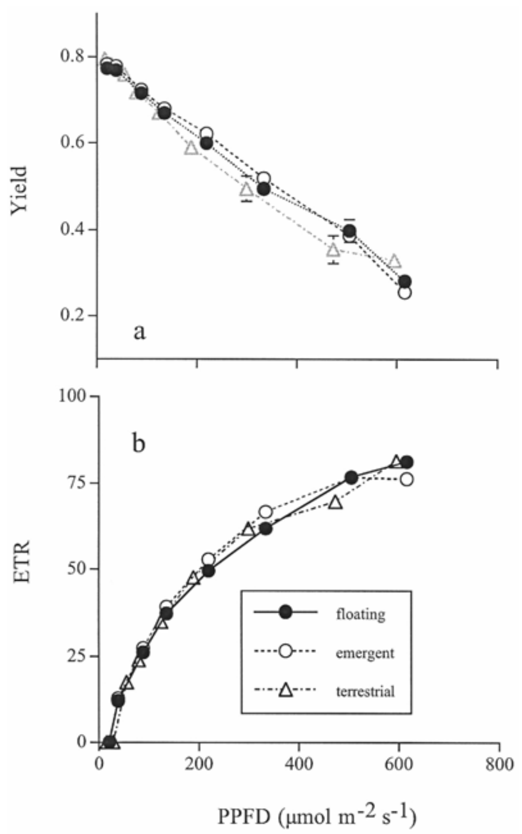

Fig. 2. The response of quantum yield of PSII (a) and relative electron transport rate (b) of floating (●), emergent (○) and terrestrial (△) leaves of *M. quadrifolia* to increase of photosynthetic photon flux density (PPFD). Each point represents the average of 3 replicates.

quantum yield * PPFD *0.5 * 0.86) at any given PPFD did not differ significantly among the leaf types (Fig. 2b).

DISCUSSION

Significant differences in leaf characteristics, i.e. stomatal density on lower surface, trichome density on both surfaces and PML are found among the three types of leaves, which might represent morphological acclimation of *M. quadrifolia* to aquatic and terrestrial environment. Transpirational water loss is an photosynthetic CO_2 uptake through opening stomata. Under water limiting condition, one way to reduce transpiration water loss without hampering CO_2 uptake unavoidable process when terrestrial plants perform is to distribute stomata on lower surface. In addition, by increasing leaf reflectance incident solar radiation on leaf surface can be reduced. Consequently, leaf temperature and transpiration water loss can also be reduced (Ehleringer et al., 1976). Chances of facing a combination of drought and temperature stress would be greater in leaves of terrestrial grown *M. quadrifolia* than in emergent and floating leaves. By adjusting stomatal distribution and producing more trichome (increase leaf reflectance) would help terrestrial leaves to reduce the chance of being damaged by drought and temperature stress. While drought and temperature stress may limit plant performance in terrestrial environment, water current and limitation of CO_2 supply play important roles in affecting plant performance in aquatic habitats. The adjustments of petiole and stomata distribution found in the floating leaves of *M. quadrifolia* would help to mitigate the environmental constrains. For example, by distributing stomata mostly on upper surface would enhance CO_2 uptake while producing elongating and flaccid petiole could reduce disturbance by water current and allow plants to better adjust its leaf position on the water surface.

Since terrestrial leaves have higher trichome density than emergent and floating leaves. It is expected that terrestrial leaves would have higher reflectance than the other two types of leaves. Results reveal that terrestrial leaves do have higher reflectance in the range of 400 to 700 nm (photosynthetic active radiation) than the other two types of leaves. However, due to their difference in transmittance, the three types of leaves have similar absorptance in the range of 400 to 700 nm (Fig. 1). To evaluate the effect of trichome on the absorptance of leaf to solar radiation hence on leaf temperature it is necessary to measure the reflectance of leaves in response to the wavelength ranging from 300 to 4000 nm (corresponding to wavelength of solar radiation incident on leaves). In addition to reflect incident solar radiation, trichomes also increase boundary layer resistance which would further reduce rate of transpirational water loss.

Results of Table 1 suggest that seasonal variation in environmental factors was also involved in affecting the distribution pattern of stomata of *M. quadrifolia*. In general, stomatal density on lower epidermis, total stomatal density and the ratio of stomatal density were higher in summer leaves than those in winter leaves. Variation in light intensity and temperature are the most likely factors contributing to differences in stomatal density and distribution of leaves produced at different seasons. For example, stomatal density was found higher in plants grown in high photon flux density than in plants grown in shade (Miskin and Rasmusson, 1970; Friend and Pomeroy, 1970). Terrestrial leaves of *M. quadrifoia* grown at higher temperature were found to have higher ratio of SU to SL than those grown at lower temperature (Lin and Kao, unpublished). Accordingly, *M. quadrifolia* is not only able to adjust its leaf characteristics in response to interchanges between aquatic and terrestrial environment but also in response to seasonal variation in environmental factors.

In contrast to their differences in growing habitats and in leaf characteristics, the three types of leaves showed similar photosynthetic performance. Accordingly, it is possible that the morphological adjustments in different leaf types enable the plants to achieve optimal photosynthetic performance in various environmental conditions.

$\delta^{13}C$ values of C_3 plants have been reported varying from -23 to -34 ‰, whereas C_4 plants have values of approximately -10 to -15 ‰ (O'Leary, 1988). Though *Elocharis vivipara*, an amphibious plant, growing in contrasting environment has $\delta^{13}C$ values representing C_3 and C_4 modes of photosynthesis (Ueno et al., 1988). The $\delta^{13}C$ values of the three types of leaves of *M. quadrifolia* were within the range of typical C_3 plants indicating that they all use the C_3 photosynthetic pathway.

In conclusion, in response to environmental changes *M. quadrifolia* produces different leaf types with different leaf characteristics. The plastic response may contribute to the ability of *M. quadrifolia* to occupy a continuum of habitats from terrestrial soils to continuously flooded substrates.

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田字草浮水葉、挺水葉和陸生葉葉部特徵和光合作用表現

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摘　　要

田字草是一種水陸兩棲的蕨類，在不同環境下會長出異形葉。本研究比較田字草浮水葉、挺水葉和陸生葉上、下表皮氣孔和表皮毛密度、葉和葉柄單位重量比、葉反射、穿透和吸收光譜特性以及光合作用表現；目的在探討異形葉的形成在生態上的意義。結果發現這三型葉在下表皮氣孔密度、表皮毛密度、葉柄單位重量以及在波長 500 和 650 nm 間之葉反射光譜係數有顯著差異；但在不同光度下的電子傳遞鏈速率則沒有顯著差異；穩定性碳同位素分析顯示三者都使用 C_3 型光合途徑進行固碳作用。

關鍵詞：水陸兩棲蕨類、異形葉、田字草、電子傳遞鏈速率、穩定性碳同位素、氣孔密度、表皮毛密度。

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