

## The Function of Trichomes of an Amphibious Fern, *Marsilea quadrifolia*

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**ABSTRACT.**—*Marsilea quadrifolia*, an amphibious fern, has the ability to develop heterophyllous, aerial and submerged leaves. In contrast to submerged leaves, aerial leaves have trichomes on both surfaces. To examine if the presence of trichomes can reflect excess light and hence reduce the risk of being damaged by excess light, we compared the optical properties and chlorophyll a fluorescence, in particular the maximum PSII photochemical efficiency ( $F_v/F_m$ ), of *M. quadrifolia* leaves with trichomes (intact) and those having trichomes removed (de-trichomed). Photosynthetic gas exchange measurements were also conducted to quantify transpirational water loss and instantaneous water use efficiency (WUE) of *M. quadrifolia* intact and de-trichomed leaves. The results showed that removal of trichomes neither affected the optical properties in the visible part of the solar spectrum nor the midday depression of  $F_v/F_m$  values of leaflets. In contrast, significantly increased in transpiration rates and decreased rates in WUE were found in de-trichomed leaflets in comparison to intact ones. These results imply that the presence of trichomes is of more importance in reducing water loss than in reflecting light and protecting *M. quadrifolia* against the potentially damaging effect of photoinhibition in aerial environments.

**KEY WORDS.**—amphibious fern, gas exchange, *Marsilea quadrifolia*, optical property, photoinhibition, trichome

How amphibious species cope with contrasting environmental conditions between aquatic and terrestrial habitats is of interests to researchers. *Marsilea*, an amphibious fern genus, has the ability to develop heterophyll. *Marsilea quadrifolia* L. experiencing extreme variation in environment develops submerged, floating, emergent and terrestrial leaves (Liu, 1984; Lin *et al.*, 2007). These different forms of leaves have different morphological and physiological characteristics. For example, in contrast to the glabrous surface of submerged and floating leaves, the adaxial and abaxial surfaces of emergent leaves are covered with dense trichomes. In addition, leaves of terrestrial grown *M. quadrifolia* (terrestrial leaves) have more trichomes than emergent leaves of aquatic grown (Lin *et al.*, 2007). The ecological importance of these trichomes has not been studied.

The two commonly cited functions of trichomes are to increase reflectance and to increase boundary layer resistance (Lambers *et al.*, 1998). Increasing

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reflectance would reduce incident light on leaf surfaces and might reduce the risk of overheating and the potentially harmful effects of excessive light on leaves (Ehleringer, 1984). Increasing boundary layer resistance would reduce transpirational water loss. A combination of drought, temperature and light stress would greatly increase in terrestrial environments in comparison to that in aquatic habitats. Hence, the production of trichomes may represent one of the acclimation responses conferring *M. quadrifolia* the ability to grow in terrestrial conditions (Lin *et al.*, 2007).

The phenomenon of photoinhibition occurs when leaves are exposed to light levels in excess of what can be utilized in photosynthesis (Powles, 1984). Photoinhibition leads to decreases in photon yield and photosystem II is considered the primary site of photoinhibition (Barber and Andersson, 1992). The yield and kinetics of chlorophyll a fluorescence emitted from leaves upon illumination with actinic light have been used as a probe for the primary photochemistry of photosynthesis (Krause and Weis, 1991). In particular, the linear relationship between quantum yield and the ratio of variable fluorescence to maximum fluorescence ( $F_v/F_m$ ) (Kao and Forseth, 1992), suggests that  $F_v/F_m$  can be used as a probe to monitor the activity of photosynthetic carbon assimilation. A decrease in the values indicates reduction in photosynthetic activity.

The objective of this study is to investigate the ecological significance of trichomes in leaves of *M. quadrifolia*. To examine if the presence of trichomes can reflect excess light and hence reduce the risk of being damaged by excess light, we compared the optical properties and chlorophyll a fluorescence of *M. quadrifolia* intact leaves (with trichomes) with leaves having trichomes removed (by de-trichomed treatment). Photosynthetic gas exchange measurements were also conducted to quantify transpirational water loss and instantaneous water use efficiency (WUE) of *M. quadrifolia* intact and de-trichomed leaves. We test the hypothesis that to reduce the risk of drought stress and being damaged by excessive light, *M. quadrifolia* develops trichomes, reducing transpirational water loss and/or reflecting excess light.

#### MATERIAL AND METHODS

Rhizomes of *M. quadrifolia* were planted in 2 L plastic pots filled with a mixture of vermiculite: soil of 1:1 by volume. Plants were grown in a glasshouse receiving natural daylight, watered to soil saturation every other day, and fertilized using inorganic fertilizer (N:P:K of 20:20:20) once every two weeks. The plant produces leaves with four leaflets expanded on a plane perpendicular to the petiole, resembling a four-leaf clover, which is connected to the rhizome. The following measurements were conducted on leaflets.

The morphology of trichomes was observed and the length measured under a scanning electron microscope (TM 1000, Hitachi). The optical properties were measured on the same leaflet before and after partial trichomes being removed. To remove trichomes, we gently brushed both surfaces of leaflets. Trichome density was estimated on both surfaces with a dissecting microscope



before and after the de-trichomed treatment. Leaf optical measurements on adaxial surfaces were made using a custom-built dual integrating sphere system following the method described by Runcie and Durako (2004). Briefly, leaf spectral transmittance ( $T(\lambda)$ ) and reflectance ( $R(\lambda)$ ) were measured from 400 nm to 700 nm at 0.5 nm resolution using a fiber-optic spectrometer (HR2000, Ocean Optics) interfaced with a FOIS-1 (for  $T(\lambda)$  measurement; Ocean Optics) or ISP-REF (for  $R(\lambda)$  measurement; Ocean Optics) integrating spheres. Light source provided by a collimated beam from a tungsten-halogen light (LS-1, Ocean Optics) was directed into the entrance port and to an exit port of the opposite side of the sphere through an optical fiber. For measuring reflectance, measurements were calibrated against a 99% reflectance standard (WS-1-SS, Ocean Optics). After measurements of  $T(\lambda)$  and  $R(\lambda)$ , we then calculated leaf absorptance ( $A(\lambda) = 1 - T(\lambda) - R(\lambda)$ ).

To evaluate if the presence of trichomes can reduce photoinhibition, we measured the characteristics of fluorescence induction on intact and treated leaflets in situ using a portable, pulse amplitude modulated fluorometer (Mini-PAM, Walz, Effeltrich, Germany). Fluorescence was measured on the adaxial side of leaflets with or without trichomes removed ( $n = 6$ ) at 10, 12 and 14 h on a clear day. Leaves were adapted to darkness for 30 minutes before the measurement was taken. Photosynthetic photon flux (PPF) on a horizontal surface at the same height of the leaves and air temperature were also monitored.

Photosaturated photosynthetic rates ( $A_{\max}$ ) and transpiration rates ( $E$ ) were measured with an LI-6400 infrared gas exchange system (LI-Cor, Lincoln, Nebraska, USA) on the most recently expanded, intact and de-trichomed leaflets. The intercellular  $\text{CO}_2$  concentration ( $C_i$ ) was calculated according to Farquhar and Sharkey (1982). Measurement conditions within the cuvette were: photosynthetic photon flux density (PPF) of  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ , cuvette temperature  $30^\circ\text{C}$ , leaf-to-air water vapor pressure difference (VPD) 1.5–1.6 kPa, and ambient  $\text{CO}_2$  concentration  $360 \pm 5 \text{ cm}^3 \text{m}^{-3}$ . The de-trichomed leaflets remained green and looked healthy a few days after the experiment indicating that the brushing treatment did not damage the leaflets. To further make sure that the de-trichomed treatment did not damage the epidermis, we also made paraffin sections of leaflets and found that the epidermis cells remained intact after the detachment of trichomes (data not shown).

All statistical tests were performed with the computer software SYSTAT (Statistical Solution, Cork, Ireland). Significant differences are reported as  $P < 0.05$ .

## RESULTS

*Characteristics of trichomes.*—The morphology of trichomes is shown in the SEM picture (Fig. 1). Grown in terrestrial condition, *M. quadrifolia* produced multicellular, 2–3 cells, trichomes (Fig. 1). Before making the SEM scan, we used liquid nitrogen to fix the samples. Some of trichomes were detached from the surface by the treatment. Hence, we estimated trichome density with a





FIG. 1. Adaxial surface of *M. quadrifolia* leaflet with trichomes.

dissecting microscope instead of calculating the number of trichomes by SEM scanning. The result showed that there was no significant difference in trichome density between the adaxial and abaxial surfaces (Table 1).

*Leaf optical properties.*—In general, intact and de-trichomed leaflets had the highest reflectance and transmittance and the lowest absorptance at ca. 550 nm in the wavelengths ranging from 400 to 700 nm (Fig. 2). In a comparison of intact and de-trichomed leaves, no significant difference was found either in  $T(\lambda)$  or in  $R(\lambda)$ . Consequently, both types of leaves had similar  $A(\lambda)$ . As a result,

TABLE 1. Trichome density ( $\text{cm}^{-2}$ ) on adaxial and abaxial surfaces of intact and de-trichomed leaflets and the optical properties of *M. quadrifolia* (mean  $\pm$  S.E.,  $n = 5$ ). Values within the same row followed by different superscripts represent significant difference at  $P = 0.05$ .

Parameters		Intact leaflets	De-trichomed
Trichome density	Adaxial surface	1433 $\pm$ 69 <sup>a</sup>	149 $\pm$ 47 <sup>b</sup>
	Abaxial surface	1594 $\pm$ 99 <sup>a</sup>	159 $\pm$ 39 <sup>b</sup>
	Total	3027 $\pm$ 164 <sup>a</sup>	308 $\pm$ 77 <sup>b</sup>
Optical properties	Reflectance (%)	5.5 $\pm$ 0.4 <sup>a</sup>	5.3 $\pm$ 1.0 <sup>a</sup>
	Transmittance (%)	6.5 $\pm$ 0.9 <sup>a</sup>	7.8 $\pm$ 1.1 <sup>a</sup>
	Absorptance (%)	88.0 $\pm$ 1.0 <sup>b</sup>	87.0 $\pm$ 1.7 <sup>b</sup>



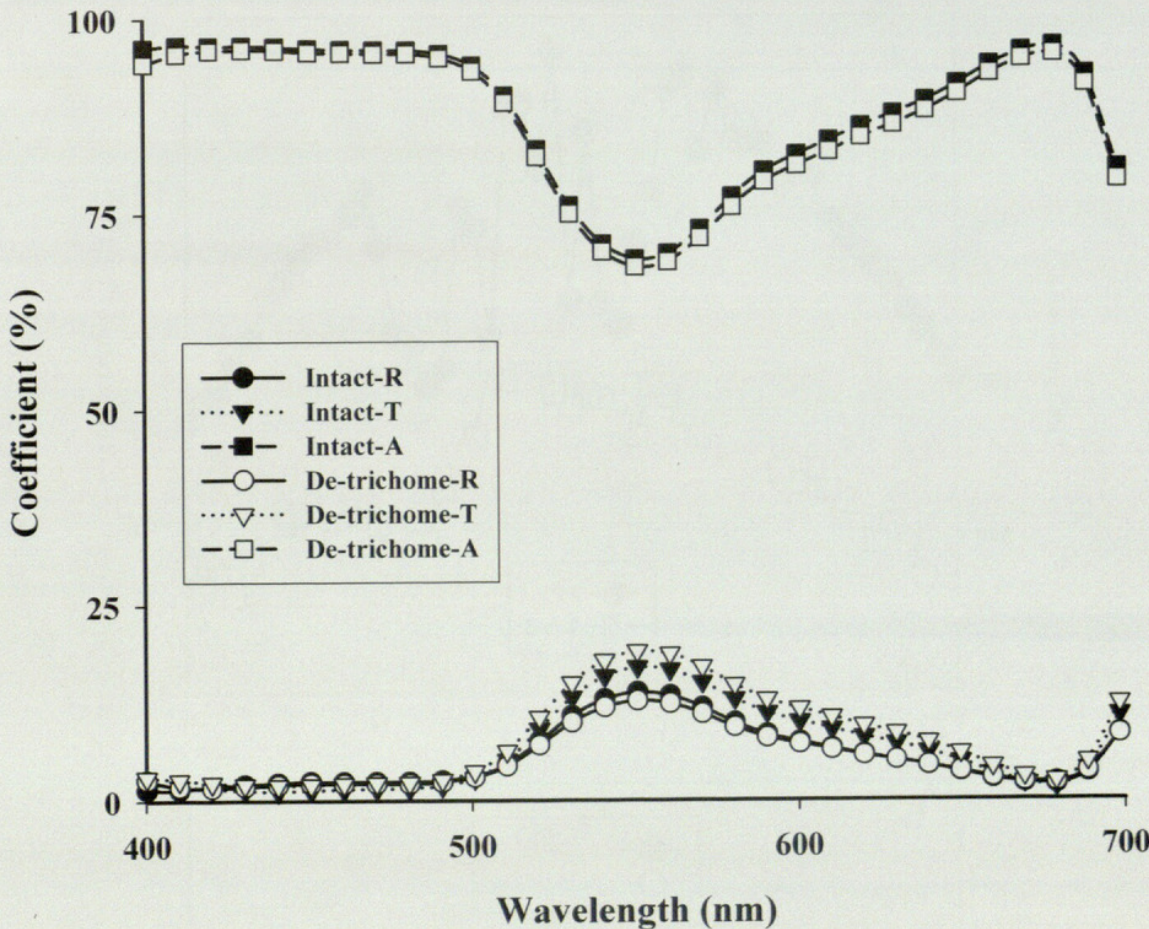


FIG. 2. Average ( $n = 5$ ) spectral reflectance (R), transmittance (T) and absorbance (A) of *M. quadrifolia* leaflets before (Intact) and after the removal of trichomes (De-trichomed).

reducing trichome density did not affect the average reflectance, transmittance and absorbance of visible light in *M. quadrifolia* leaflets (Table 1).

*Chlorophyll fluorescence measurement.*—The diurnal courses of PPF at plant height and air temperature ( $T_a$ ) were recorded on the same day as leaf fluorescence was measured (Fig. 3a). Initial and continuous dark adapted measurements of maximum PSII photochemical efficiency ( $F_v/F_m$ ) indicated that the leaves were healthy and not experiencing stress due to the removal of trichomes (Table 2). Midday depression of  $F_v/F_m$  values was found in the leaflets when exposed to solar irradiation (Fig. 3b). In comparison to continuously dark-adapted leaves, illuminated leaflets showed significant reduction in  $F_v/F_m$  at 1000h, 1200h and 1400h when PPF and air temperature were highest during the day (Fig. 3). No significant difference was found in  $F_v/F_m$  values between intact and de-trichomed leaflets.

*Gas exchange measurement.*—Transpiration rate ( $E$ ) increases with  $A_{max}$  in both intact and detrachomed leaflets; as a result, a significant, positive, linear relationship was found between  $A_{max}$  and  $E$  (Fig. 4a). No significant difference was found between the slopes of these positive relationships for intact and de-



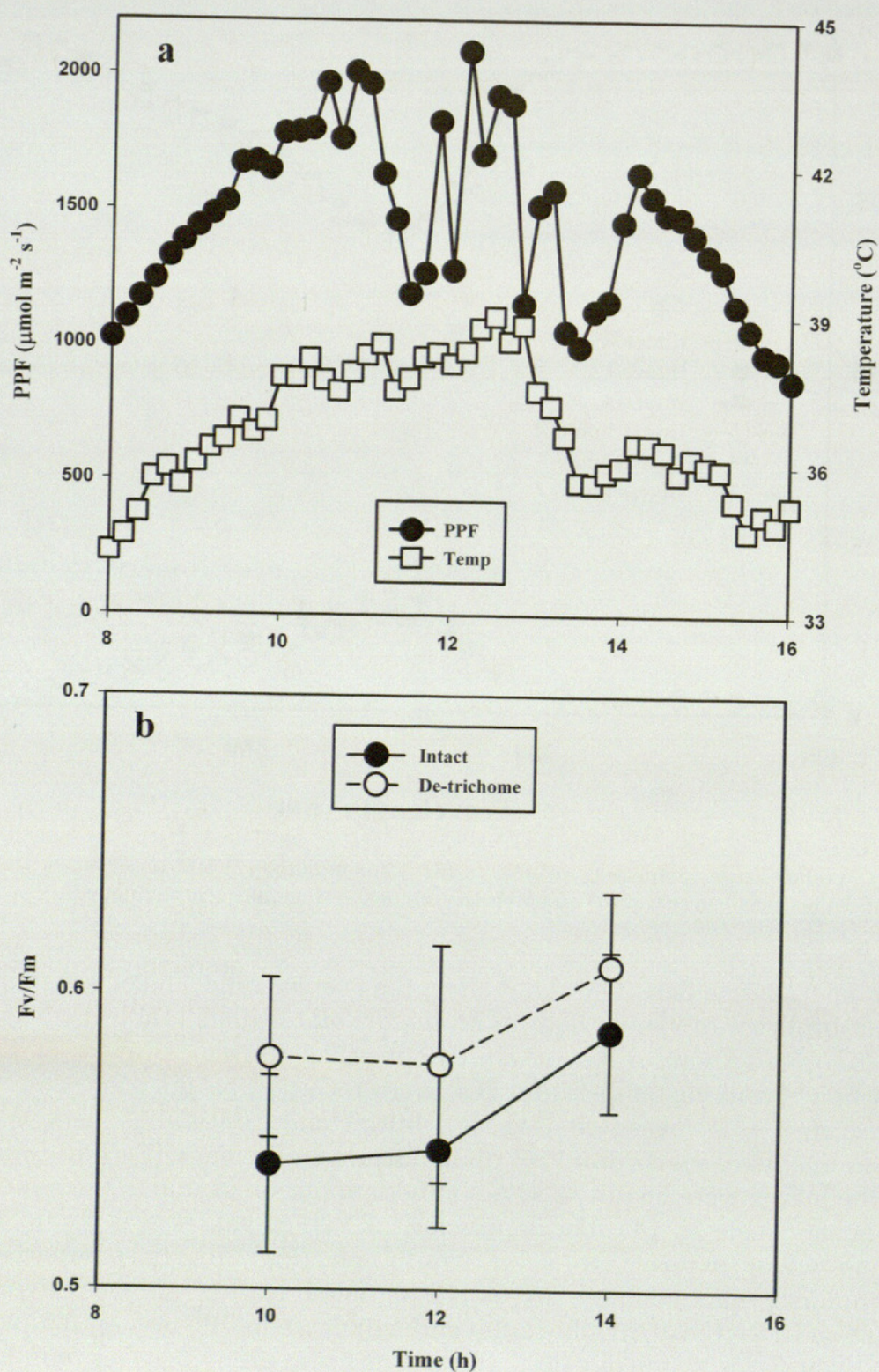


FIG. 3. Diurnal changes in air temperature and PPF incidence on a horizontal surface (a) and the ratio of  $F_v$  to  $F_m$  (mean  $\pm$  s. e.,  $n = 6$ ) measured at 1000, 1200 and 1400 h on naturally orienting leaflets of *M. quadrifolia* with (De-trichomed) and without (Intact) removal of trichomes.



TABLE 2. Maximum PSII photochemical efficiency (Fv/Fm) of initial (before leaflet being exposed to solar irradiation) and continuous dark adapted intact and de-trichomed leaflets of *M. quadrifolia* used in the measurement of chlorophyll fluorescence (mean  $\pm$  s.e., n = 6).

Treatments	Initial	Continuous dark
Intact	0.76 $\pm$ 0.01	0.77 $\pm$ 0.01
De-trichomed	0.77 $\pm$ 0.01	0.77 $\pm$ 0.01

trichomed leaflets. However, de-trichomed leaflets had significantly higher E relative to intact leaflets for a given A<sub>max</sub>.

The calculated Ci values of de-trichomed leaflets were higher than those of intact leaflets (Fig. 4b).

DISCUSSION

Knowledge of how fern species cope with excess light or drought stress in terrestrial environments is of ecological and evolutionary importance. Biochemical mechanisms, such as xanthophyll-mediated energy dissipation, and/or morphological mechanisms, such as leaf curling and laminar scales, have been demonstrated in pteridophytes (Eichmeier *et al.*, 1993; Tausz *et al.*, 2001; Watkins *et al.*, 2006). To our knowledge, the influence of pubescence on the incident radiation and water budget has not been studied in any fern species.

Among other functions (Johnson, 1975; Zvereva *et al.*, 1998), pubescence had been shown to increase reflectance (Ehleringer, 1984; Holmes and Keiller, 2002) and afford protection against excess radiation (Ripley *et al.*, 1999; Morales *et al.*, 2002; Manetas, 2003) in seed plants. Our results, however, showed that *M. quadrifolia* leaflets have a very high absorptance and de-trichomed treatments did not affect the visible part (400–700 nm) of optical properties of the leaflets (Fig. 2). Additionally, the Fv/Fm values of leaflets of *M. quadrifolia* at the midday were not affected by the removal of trichomes (Fig. 3b). It is therefore possible that trichomes on leaflets of *M. quadrifolia* are of less importance in reflecting light and in protecting the plant against the potentially damaging effect of photoinhibition. However, leaf temperatures in de-trichomed leaflets may be reduced due to their increased transpiration rates (Fig. 4a), which may ameliorate the damaging effect of high light and high air temperature on de-trichomed leaflets. For example, the result that the de-trichomed/orienting leaflets had less reduction in Fv/Fm, though not significant, than intact/orienting leaflets (Fig. 3b) might result from the increased transpiration rate in the former. Accordingly, we cannot completely exclude the role of trichomes in providing photoprotection for *M. quadrifolia* leaflets.

Few studies have also shown that the presence of trichomes can reduce leaf transpiration rates (Ripley *et al.*, 1999). The significantly increase in transpiration rates, about 30%, measured in de-trichomed *M. quadrifolia* leaflets compared to intact ones (Fig. 4a) suggests that the presence of



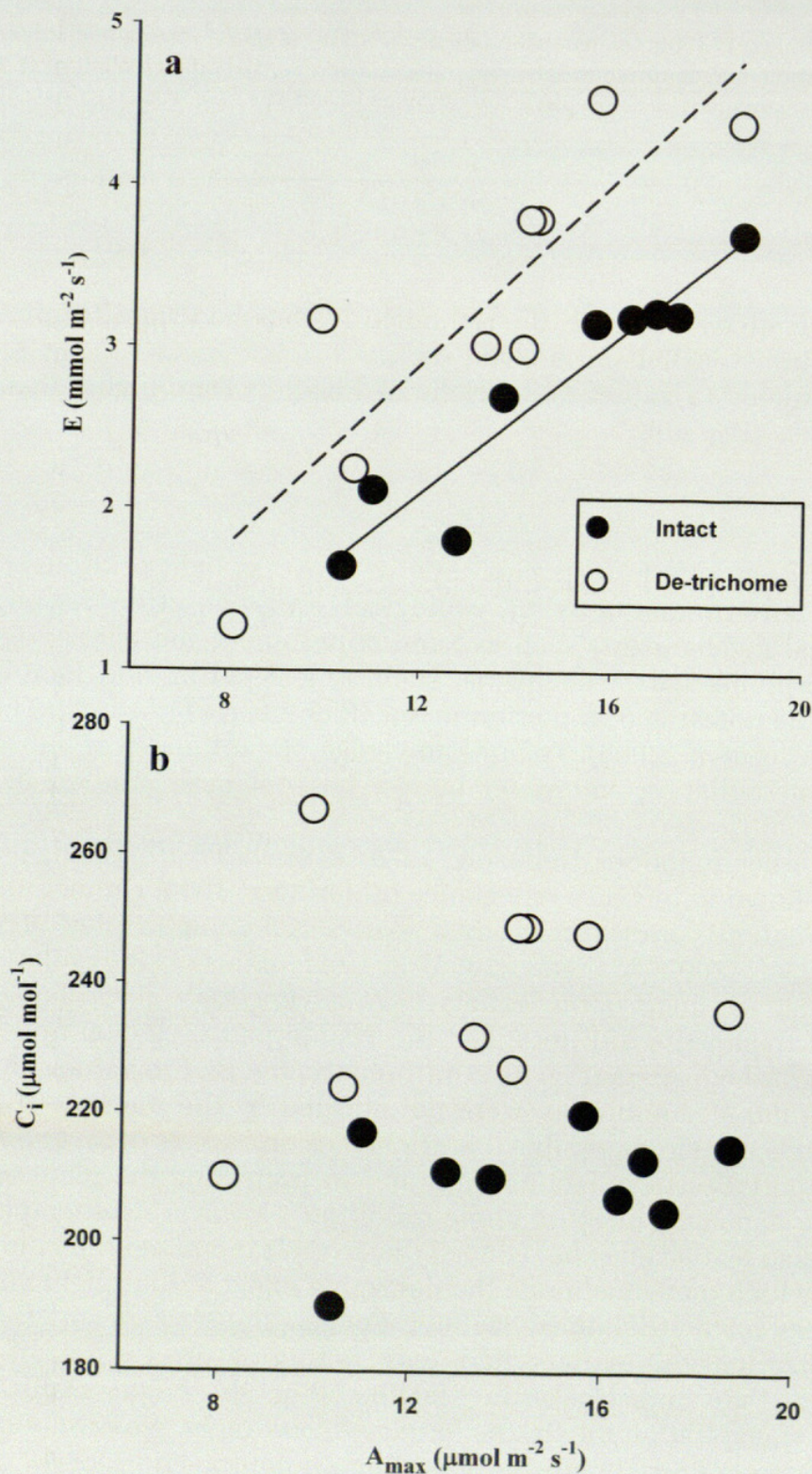


FIG. 4. The relationship between photosaturated photosynthetic rate ( $A_{max}$ ) and transpiration rates ( $E$ ) (a) and intercellular  $CO_2$  concentration ( $C_i$ ) (b) of *M. quadrifolia* leaflets without (Intact) and with removal of trichomes (De-trichomed).



trichomes is of more importance in reducing water loss than in providing photoprotection. At constant leaf-air vapor pressure deficit,  $C_i$  values may be used as a measure of the instantaneous water use efficiency (WUE) of the leaf (Farquhar and Sharkey, 1982), with a lower  $C_i$  indicating a higher instantaneous WUE. De-trichomed treatments resulted in leaflets with higher  $C_i$  (Fig. 4b) implying a lower WUE. These results reveal that the reduction in water loss from *M. quadrifolia* leaflets with trichomes also resulted in increased WUE.

The hair layer on leaves may lead to higher leaf temperatures caused by reducing the transpirational water loss (Ripley *et al.*, 1999). We have observed that leaflets of *M. quadrifolia* have the ability of performing tropic movements (pers. obs.). It is possible that *M. quadrifolia* adjust leaflet angle and azimuth to intercept a smaller quantity of radiant energy, which would allow the plant to moderate leaflet temperature without excessive transpiration. The function of tropic leaf movements in protecting soybean leaves from photoinhibition has been documented (Kao and Forseth, 1992). Thus, the presence of trichomes on both surfaces combined with leaflet movements may provide *M. quadrifolia* mechanisms against drought stress. Accordingly, we hypothesize that the combination of the avoidance mechanisms, leaf movements and the production of trichomes are very important adaptations, conferring the amphibious *M. quadrifolia* ability to grow in terrestrial conditions. The effect of the interaction between water availability and light intensity on trichome density and leaflet movements in *M. quadrifolia* are currently under studied.

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