Taiwania, **53**(3): 237-241, 2008

Can Ethylene Induce Heterophyll in *Marsilea quadrifolia*?

Chia-Hong Lin[(1)], Bai-Ling Lin[(2)] and Wen-Yuan Kao[(1,3,4)]

(Manuscript received 18 March 2008; accepted 12 May 2008)

ABSTRACT: Individuals of *Marsilea quadrifolia*, an amphibious fern, experiencing extreme variations in environment develop heterophyll with different morphological characteristics. The objective of this study is to investigate if ethylene can induce floating type of leaves in this fern. To achieve this goal, ratio of stomatal density on abaxial and adaxial leaf surfaces (stomatal ratio) and the mass per unit length of petiole (PML), on leaves of terrestrial shoots sprayed with an ethylene gas releaser, Ethephon, were compared with those of leaves produced by submergence of terrestrial shoots. Leaves with different stomatal ratio and PML, corresponding to that of terrestrial type and floating type of leaves, were produced when terrestrial shoots of *M. quadrifolia* were submerged. The result reveals that the plasticity of leaves to respond to submergence depends on leaf's age. Application of Ethephon significantly altered the stomatal ratio of young leaves on terrestrial shoot but not their PML. Leaves response to Ethephon treatment was also age dependent. These results indicate that ethylene might be involved in the formation of floating leaves in *M. quadrifolia*.

KEY WORDS: amphibious fern, ethylene, floating leaves, heterophyll, *M. quadrifolia*, stomatal ratio.

INTRODUCTION

Marsilea is a genus of amphibious fern which has the ability to develop heterophyll. Researchers have studied the formation of leaflets produced under water surface (submerged leaves) and those raised above the surface of water (usually referred to land leaves) of this genus (Allosopp, 1953; Bristow and Looi, 1968; Gaudet, 1963; Liu, 1984; Lin and Yang, 1999). It has been shown that submerged shoots can be induced to produce land leaves (Liu, 1984) and vice versa (Allsopp, 1962). In addition to produce submerged and land leaves, *Marsilea* has been observed to produce floating leaves in field (Lin et al., 2007). In contrast to submerged and land leaves, floating leaves receive much less attention. In a previous study, we found that floating leaves have characteristics significantly different from land and submerged leaves (Lin et al., 2007).

Factors and mechanisms related to the switch of submerged and land leaves in *M. quadrifolia* have been studied (Liu, 1984; Lin and Yang, 1999; Hsu et al., 2001; Lin et al., 2005). Studies have found that phytohormones play important roles in mediating the development of heterophyll (Wallenstein and Albert, 1963; Liu, 1984; Young et al., 1987; Goliber and Feldman, 1989). For example, treatment with ABA induced formation of land leaves on submerged shoots of *M. quadrifolia* (Liu, 1984; Lin and Yang, 1999). In contrast, exogenous application of GA elicited submerged-types leaves on shoots of *M. drummondii* grown aerially (Allsopp, 1962). Mechanisms related to the formation of floating leaves have not been studied. Many semi-aquatic plants possess the capacity to elongate upon submergence (Ridge, 1987), and in many cases, this response is mediated by ethylene. *M. quadrifolia* has been shown to be capable of producing ethylene under submergence (Chernys and Kende, 1996). However, the effect of ethylene on formation of heterophyll in *Marsilea* has not been studied. Is it possible that ethylene also participates in inducing heterophyll of *Marsilea*? To answer this question, we compared leaf characteristics of terrestrial shoots sprayed with Ethephon, an ethylene gas releaser, with those of leaves produced by submergence of terrestrial shoots.

1. Department of Life Science, National Taiwan University, 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan.
2. Genomics Research Center, Academia Sinica, 128, Sec. 2, Academia Rd., Taipei 115, Taiwan.
3. Institute of Ecology and Evolutionary Biology, National Taiwan University, 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan.
4. Corresponding author. Tel: 886-2-33662511; Fax: 886-2-2367-3374; Email: wykao@ntu.edu.tw

MATERIALS AND METHODS

Materials

Aseptic cultures of *M. quadrifolia* L. were established from sporocarps and propagated by subculturing according to the method of Liu (1984).

The aseptic cultures of *M. quadrifolia* were transplanted into 10 cm pot filled with potting soil and well watered. Plants were grown in the greenhouse and fertilized with Hyponex 2 (N: P: K = 20: 20: 20) once a week. The transplants started producing, ca.10 days after being transferred into potting soil, and continuously produced land leaves in the terrestrial condition.

Methods

Effect of submergence on terrestrial grown shoot bearing land leaves

Clones of terrestrial-grown rhizomes were transferred into six plastic trays (41 cm * 36 cm * 36 cm) each filled with 2 cm depth of soil and kept in a greenhouse for one month. Before submergence treatment, rhizomes with 6 leaves, age recorded from 0 (the youngest primodium) to 11 days old were marked. Water was then injected into the plastic trays and maintained at a depth of 15 cm above soil surface. The whole plants were immersed into the water. The treatment continued for 12 days until the newly produced leaves were fully developed. The new leaves that developed after the treatment were designated as 0, -1 and -2 days old. The old and the new, fully developed leaves and their petiole were excised for further measurements as followings.

Measurements of leaf characteristics

In a previous study, we found that mass per unit length of petiole (PML) and the ratio of stomatal density of abaxial to adaxial leaf surface (stomatal ratio) are good morphological characteristics to distinguish leaf types of *M. quadrifolia* (Lin et al., 2007). Hence, in this study we also used these two parameters to identify leaf types. The dry weight (DW) and the length of petiole (PL) of fully developed leaves (older than 12 days) were measured then PML calculated as the ratio of DW to PL. A thin layer of nail polish was applied to the upper and lower surfaces of one of the four leaflets from each plant (n = 6). After drying, the nail polish was peeled and scan at 100 x magnification with a light microscope equipped with a calibrated ocular micrometer for estimates of stomatal density. The stomatal ratio was calculated.

Effect of Ethephon on terrestrial grown shoot bearing land leaves

Clones of terrestrial-grown plants were transferred into three plastic trays (41 cm * 36 cm * 36 cm) each filled with 4 cm depth of soil and kept in a greenhouse for one month. Before treatment, rhizomes with 11 leaves, age recorded from 0 (the youngest primodium) to 11 days old, were marked. Plants in the four plastic trays were sprayed with 45 ml of de-ioned water containing 0, 0.01, and 0.05 % of Ethephon, which releases ethylene at neutral pH, in two days. The new leaves developed after 2 days of treatment and were designated as -2 days old. The old and the new, fully developed (about 10 days old) leaves with petiole were excised. Leaf and petiole characteristics were then measured.

Statistical test

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

RESULTS

The effect of submergence

Based on the stomatal ratio, leaves on terrestrial grown shoot after being submerged can be easily classified into two types. One type of leaves, age older than 2 days when the treatment started, had the stomatal ratio higher than 0.6, while the other, age younger than 3 days when the treatment started, the ratio lower than 0.1 (Fig. 1A).

Leaves also had significant difference in their PML (Fig. 1B), however, the pattern was not as distinct as that of stomatal ratio. Among leaves of the treatment shoot, those age of 11 days old had the highest PML, while leaves already one day old and those newly produced when the treatment applied had similar lowest PML. Leaves ages ranging from 3 to 9 days when submerged had the intermediate values of PML and their PML increased with leaf age.

The effect of application of Ethephon

Total density of stomata was not affected by Ethephon application (data not presented). The stomatal ratio of leaves younger than 4 days was significantly reduced by treatment of Ethephon. Though the distribution of stomata on leaves age younger than 4 days responded to Ethephon application, while that on leaves older than 4 days did not (Fig. 2A).

In contrast to the distinct response of stomatal distribution to ethephon application, PML of most of the treatment leaves showed no significant response, while only that of leaves of 3 days old was significant reduced by the application of Ethephon (Fig. 2B).

DISCUSSION

It has been reported that submerged leaves can be distinguished from land leaves by stomatal

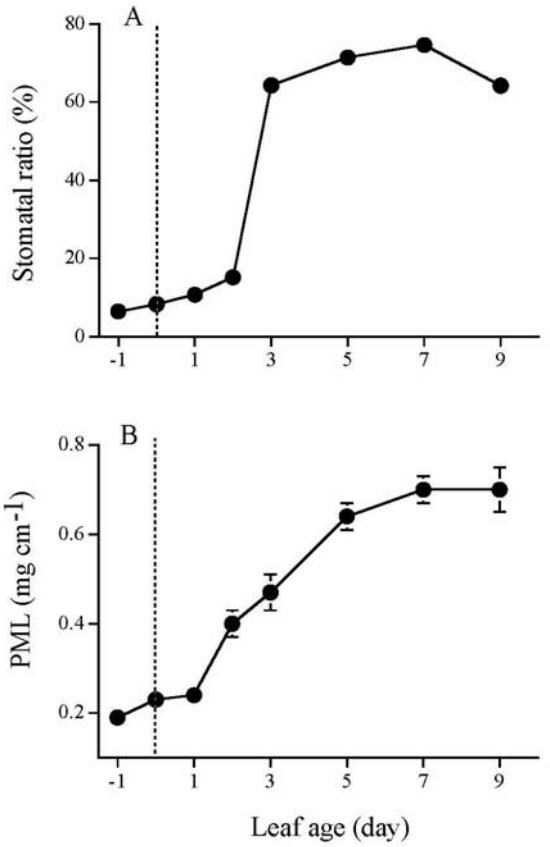

Fig. 1. Effects of submergence on the ratio of stomatal density of abaxial to adaxial surface (stomatal ratio) (A) and petiole mass per unit length (PML) (B) of terrestrial grown *M. quadrifolia* leaves with different age. Dash line indicates the beginning of the treatment. Data presented are mean ± S.E.M.

distribution on leaf surface, the former have few stomata mainly distributed on the upper surface (Liu, 1984). In comparison to land leaves, floating leaves have significantly lower stomatal ratio and less PML (Lin et al., 2007). In this study, when terrestrial shoots of *M. quadrifolia* were submerged, two types of leaves with different stomatal ratio were formed (Fig. 1A). In reference to previous studies (Liu, 1984; Lin et al., 2007), stomatal ratio of these two types of leaf corresponds to that of terrestrial and floating leaves. Hence, terrestrial grown *M. quadrifolia* could be induced to produce floating type of leaves characterized by significantly lower stomatal ratio (Fig. 1A) and lower PML than land leaves (Fig. 1B). However, the plasticity of changing leaf types is age dependent. Leaves older than 4 days did not respond to the changes in water level. The formation of floating leaves can also be triggered by an increase in water level in terrestrial plants such as *Ranunculus sceleratus* and *Sparganium eurycarpum* (Kaul, 1976; Maberly and Spence, 1989).

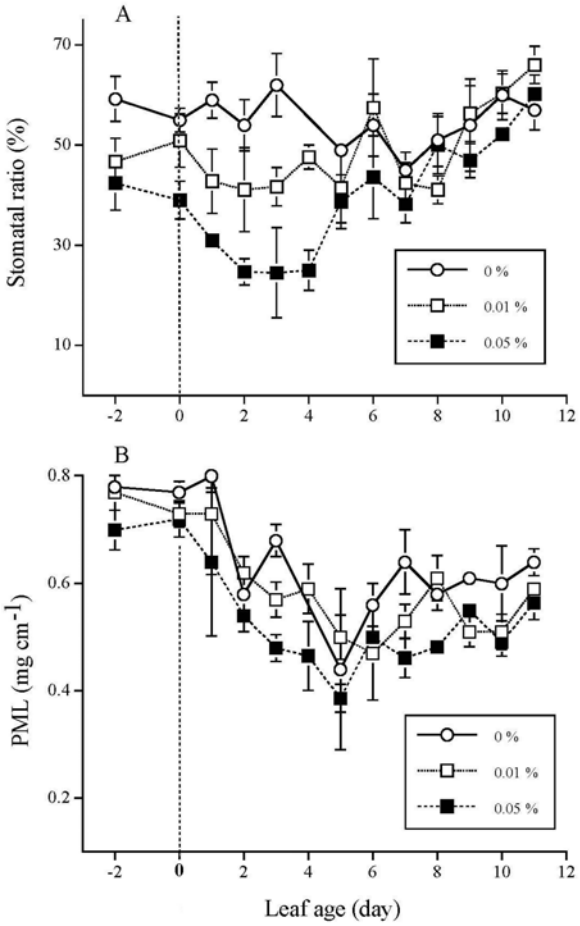

Fig. 2. Effects of different concentration of Ethephon, 0, 0.01 and 0.05 % on the ratio of stomatal density of abaxial to adaxial surface (stomatal ratio) (A) and petiole mass per unit length (PML) (B) of terrestrial grown *M. quadrifolia* leaves with different ages. Dash line indicates the beginning of the treatment. Data presented are mean ± S.E.M.

Treatment with Ethephon significantly reduced the stomatal ratio of some leaves of terrestrial shoots (Fig. 2B). The stomatal ratio of these leaves is comparable to that of floating leaves reported by Lin et al. (2007). The plasticity of leaves to respond to Ethephon treatment also depends on the leaf's age, leaves younger than 4 days but not those older than 4 days showed the response. This result is consistent with results from submergence treatment that only leaves younger than 4 days are responsive to submergence (Fig. 1A). It indicates that ethylene might be one of the endogenous factors responsible for the changes in leaf type when terrestrial grown *M. quadrfolia* were submerged.

The other parameter that can be used to distinguish floating vs land leaves is PML, the former have significantly lower PML than the later (Lin et

al., 2007). Submergence also induced leaves with different PML, although the pattern was not as distinct as that of the stomatal distribution. In contrast to the effect of submergence on PML (Fig. 1B), application of Ethephon did not affect PML (Fig. 2B). Since we have applied Ethephon only twice in two days, the concentration of Ethephon or the duration of the treatment might not be sufficient to induce changes in PML. Alternatively, other factors or other phytohormones in addition to ethylene might be also involved in mediating the changes in leaf types. For example, it has been shown that in addition to ethylene buoyancy also controls rachis elongation in the semi-aquatic fern *Regnillidium diphyllum* (Musgrave and Walters, 1974). In *Callitriche platyarpa* and *Ranunculus sceleratus* the petiole response to ethylene was GA-dependent (Musgrave et al., 1972; Musgrave and Walters, 1973).

It has been reported that applied ethylene gas on terrestrial shoots of *Ludwigia arcuata* resulted in the formation of submerged-type leaves (Kuwabara et al., 2001; Kuwabara et al., 2003). Instead of inducing submerged leaves, ethylene gas induces floating type of leaves on terrestrial grown *M. quadrifoloia*. In comparison to leaves completely submerged, floating leaves should have more access to light, CO_2 and O_2 (Maberly and Spence, 1989), and may represent an intermediate form between submerged and land leaves. The ability to produce a third type of leaves in addition to two may represent adaptive strategy allowing the plant to explore another ecological niche, in this case, the interface between underwater and air. The distinct diversity in the genus of *Marsilea* may originate through an adaptive radiation of sympatric taxa into various ecological niches (Kornas, 1988). Accordingly, a complete study of different types of leaves will not only allow us to understand how *Marsilea* adjust to accommodate the fluctuating of water levels, but also will improve our understanding of the evolutionary process driving the diversification of this genus.

LITERATURE CITED

Allsopp, A. 1953. Experimental and analytical studies of pteridophytes. XX. Investigations on *Marsilea*. 3. The effect of various sugars on development and morphology. Annals of Botany **17**: 447-463.

Allsopp, A. 1954. Experimental and analytical studies of pteridophytes. XXIV. Investigations on *Marsilea*. 4. Anatomical effects of changes in sugar concentration. Annals of Botany **18**: 449-461.

Allsopp, A. 1955. Experimental and analytical studies of pteridophytes. XXVII. Investigations on *Marsilea*. 5. Culture conditions and morphogenesis with special reference to the origin of land and water forms. Annals of Botany **19**: 247-264.

Allsopp, A. 1962. The effects of gibberellic acid on morphogenesis in *Marsilea drummondii*. A. Br. Phytomorphology **12**: 1-10.

Bristow, J. M. and A. H. Looi. 1968. Effects of carbon dioxide on the growth and morphogenesis of *Marsilea*. American Journal of Botany **55**: 884-889.

Chernys, J. and H. Kende. 1996. Ethylene biosynthesis in *Regnellidium diphyllum* and *Marsilea quadrifolia*. Planta **200**: 113-118.

Gaudet, J. J. 1963. *Marsilea vestita*: conversion of the water form to the land form by darkness and by far-red light. Science **140**: 975-976.

Gaudet, J. 1964. Morphology of *Marsilea vestita.* II. Morphology of the adult land and submerged leaves. American Journal of Botany **51**: 591-597.

Goliber, T. E. and L. J. Feldman.1989. Osmotic stress, endogenous abscisic acid and the control of leaf morphology in *Hippuris vulgaris* L. Plant, Cell and Environment **12**: 163-171.

Kaul, R. B. 1976. Anatomical observations on floating leaves. Aquatic Botany **2**: 215-234.

Kornas, J. 1988. Adaptive strategies of *Marsilea* (Marsileaceae: pteridophyta) in the Lake Chad basin of N. E. Nigeria. Fern Gaz. **13**: 231-243.

Kuwabara, A., H. Tsukaya and T. Nagata. 2001. Identification of factors that cause heterophylly in *Ludwigia arcuata* Walt. (Onagraceae). Plant Biology **3**: 98-105.

Kuwabara, A., K. Ikegami, T. Koshiba and T. Nagata. 2003. Effects of ethylene and abscisic acid upon heterophylly in *Ludwigia arcuata* (Onagraceae). Planta **217**: 880-887.

Laetsch, W. M. 1967. Ferns. In: Wilt, F. H. and N. K. Wessells (eds.), Methods in developmental biology, Crowell, New York, USA. pp. 319-328.

Lin, B.-L. 1984. Abscisic acid induces land form characteristics in *Marsilea quadrifolia* L. American Journal of Botany **71**: 638-644.

Lin, B.-L. and W.-J. Yang. 1999. Blue light and Abscisic acid independently induce heterophyllous switch in *Marsilea quadrifolia*. Plant Physiology **119**: 429-434.

Lin, C.-H., B.-L. Lin and W.-Y. Kao. 2007. Leaf characteristics and photosynthetic performance of floating, emergent and terrestrial leaves of *Marsilea quadrifolia*. Taiwania **52**: 195-200.

Maberly, S. C. and D. H. N. Spence. 1989. Photosynthesis and photorespiration in freshwater organisms: amphibious. Aquatic Botany **34**: 267-286.
Minorsky, P. V. 2003. Heterophyll in aquatic plants. Plant Physiol. **133**: 1671-1672.
Musgrave, A., M. B. Jackson and E. Ling. 1972. *Callitriche* stem elongation is controlled by ethylene and gibberellin. Nature **238**: 93-96.
Musgrave, A. and J. Walters. 1973. Ethylene-stimulate growth and auxin transport in *Ranunculus sceleratus* petioles. New Phytologist **72**: 783-789.
Musgrave, A. and J. Walters. 1974. Ethylene and buoyancy control rachis elongation of the semi-aquatic fern *Regnillidium diphyllum*. Planta **121**: 51-56.
Ridge, I. 1987. Ethylene and growth control in amphibious plants. In: Crawford, R. (ed.), Plant life in aquatic and amphibious habitats, Blackwell Scientific Publications, Oxford, UK. pp. 53-79.
Wallenstein, A. and L. S. Albert. 1963. Plant morphology: its control in *Proserpinaca* by photoperiod, temperature, and Gibberellic acid. Science **140**: 998-1000.
Wells, C. L. and M. Pigliucci. 2000. Adaptive phenotypic plasticity: the case of heterophylly in aquatic plants. Perspectives in Plant Ecology, Evolution and Systematics **3**: 1-18.
Winn, A. A. 1999. The functional significance and fitness consequences of heterophylly. International Journal of Plant Science **160**: 113-121.
Young, J., N. G. Dengler and R. F. Horton. 1987. Heterophylly in *Ranunculus flabellaris*: the effect of Abscisic Acid on leaf anatomy. Annals of Botany **60**: 117-125.

乙烯是否可以誘導田字草異形葉的形成

林家弘[(1)]、林白翎[(2)]、高文媛[(1,3,4)]

(收稿日期：2008 年 3 月 18 日；接受日期：2008 年 5 月 12 日)

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，這些異形葉具不同的形態特徵。本研究目的在探討乙烯氣體是否可以誘導田字草浮水葉的形成。我們比較陸生植株經淹水後、以及噴灑 Ethephon（會釋出乙烯氣體）後，不同葉齡的葉片其上、下表皮氣孔密度比和葉柄單位長度重量比。結果發現陸生環境下長出的葉子經淹水後能否轉變成浮水葉特徵和其葉齡有關，較老的葉子維持陸生葉特徵(較高的下/上表皮氣孔比和較重的葉柄單位長度重量比)，幼葉則可以轉而形成浮水葉特徵(較低的下/上表皮氣孔比和較輕的葉柄單位長度重量比)，而淹水後才新長出的葉片具浮水葉特徵。陸生植株噴灑 Ethephon 後有些原已形成的葉子其氣孔比是否會轉變成傾向浮水葉特徵也和其葉齡有關；葉柄單位長度重量比則不受 Ethephon 噴灑的影響，仍維持陸生葉特徵。結果顯示乙烯氣體可以誘導田字草改變葉子的部份特徵，此可能和浮水葉的形成有關。

關鍵詞：水陸兩棲蕨類、乙烯、浮水葉、異形葉、田字草、氣孔比。

1. 國立臺灣大學生命科學系，106 台北市羅斯福路 4 段 1 號，臺灣。
2. 中央研究院基因體研究中心，115 台北市研究院路 2 段 128 號，臺灣。
3. 國立臺灣大學生態學與演化生物學研究所，106 台北市羅斯福路 4 段 1 號，臺灣。
4. 通信作者。Tel: 886-2-33662511; Fax: 886-2-2367-3374; Email: wykao@ntu.edu.tw