American Journal of Plant Sciences, 2012, 3, 805-809
http://dx.doi.org/10.4236/ajps.2012.36097 Published Online June 2012 (http://www.SciRP.org/journal/ajps)

Comparative Morphology and Anatomy of Non-Rheophytic and Rheophytic Types of *Adenophora triphylla* var. *japonica* (Campanulaceae)

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Received April 10th, 2012; revised April 30th, 2012; accepted May 18th, 2012

ABSTRACT

The morphology and anatomy of leaves of rheophytic and non-rheophytic types of *Adenophora triphylla* (Thunb.) ADC var. *japonica* (Regel) H. Hara were compared in order to clarify how leaf characteristics differ. Our results revealed that the leaf of the rheophytic type of *A. triphylla* var. *japonica* was narrower than the leaf of the non-rheophytic type because of fewer cells that were also smaller. Moreover, surprisingly, the rheophytic ecotype of *A. triphylla* var. *japonica* was thinner than that of the non-rheophytic type, although the general tendency is that the rheophytic leaf is thicker than the closely related non-rheophytic species, suggesting that the rheophytic type of *A. triphylla* var. *japonica* adapts differently, as compared to other rheophytic plants, to solar radiation and evaporation.

Keywords: Rheophyte; Leaf; Ecotype; *Adenophora triphylla* var. *japonica*

1. Introduction

Morphological diversity is a special feature of angiosperms, and a key challenge in biology is to understand how this extraordinary morphological diversity is generated. Leaves are the main photosynthetic organs of vascular plants, and they show a tremendous degree of natural variation in shape, making them an attractive system for studying the evolution of form. It is important to understand how morphological diversity in leaf forms was generated and whether it arose in response to natural selection. Rheophytic plants, which grow along rivers and streams, are subjected to flash floods after heavy raining, and these act as strong selective pressures. These plants have narrow lanceolate or cuneate leaves as adaptive modifications [1,2]. Moreover, in rheophytes, which grow in sunny riverbeds in forest gaps or margins, the thick cuticles on the leaf surface have been implicated as adaptations to avoiding or resisting thermal damage in open environments [3]. In addition, [4] reported that morphological and anatomical differences in leaf shape between rheophytes and closely related species appear to be correlated with differences in ecological setting. In fact, some studies using rheophytes and closely related species have been conducted as model cases for understanding leaf modification from fern to angiosperm (*Osmunda lancea* Thunb. (Osmundaceae): [5]; *Aster microcephalus* (Miq.) Franch. et Sav. var. *ripensis* Makino (Asteraceae): [6]; *Dendranthema yoshinaganthum* Makino ex Kitam. (Asteraceae): [7]; *Farfugium japonicum* (L.) Kitam. var. *luchuense* (Masam.) Kitam. (Asteraceae): [8]; *Rhododendron indicum* (L.) Sweet f. *otakumi* T. Yamaz. (Ericaceae): [9]).

Adenophora triphylla (Thunb.) ADC var. *japonica* (Regel) H. Hara belongs to Campanulaceae and is distributed in Japan, Korea, and Sahalin [10]. [11] reported that the stenophyllization of *A. triphylla* var. *japonica*, which is a putative rheophytic ecotype, occurs in some areas of Kochi Prefecture in Japan (**Figure 1**). Although comparative morphological and anatomical analyses using the narrow leaves of *A. triphylla* var. *japonica* have not been made so far, the processes involved in the stenophyllization of *A. triphylla* var. *japonica* such as decreases in cell number and/or cell size remain unclear. In general, one of the indicators that is widely used to examine stenophyllization in putative rheophytes is the leaf index, which is the ratio of the length and width of the leaf [12], and some studies indicated that there are significant differences between rheophytic and non-rheophytic taxa in this regard (e.g., [6,7,13]). Therefore, to investigate the morphological and anatomical differentiation of the

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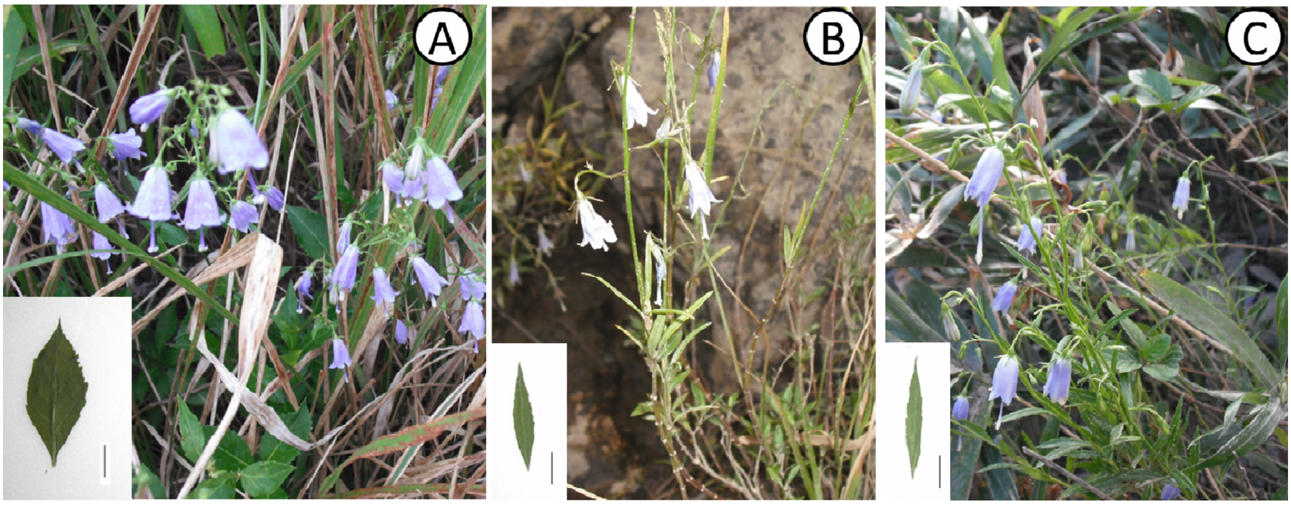

Figure 1. Plants of *Adenophora triphylla* var. *japonica*. (A) Non-rheophytic type in Higashisako; (B) Rheophytic type in Shimanto River; (C) Rheophytic type in Yoshino River. Bar = 1 cm.

rheophytic ecotype of *A. triphylla* var. *japonica*, we conducted comparative morphological and anatomical analyses, using rheophytic and non-rheophytic types.

2. Materials and Methods

All specimens of non-rheophytic and rheophytic types of *Adenophora triphylla* var. *japonica* examined in this study were collected from the fields. A total of 65 individuals of the non-rheophytic type of *A. triphylla* var. *japonica* (Hitsuzan: 35; Higashisako: 30) were analyzed. A total of 61 individuals of the non-rheophytic type of *A. triphylla* var. *japonica*, representing 2 populations of the Shimanto (31 individuals) and the Yoshino rivers (30 individuals), were analyzed. Collecting localities are indicated in **Figure 2** and **Table 1**.

For morphological analysis, individual specimens were measured for the following continuous macromorphological variables of leaves: length and width of the leaf blade and angle of the leaf base. Measurements were made using a pair of digital calipers. Three fully expanded leaves per specimen were measured. We calculated the mean leaf length, width, and thickness of each specimen. For anatomical analysis, the fully expanded leaves from each specimen were collected. The leaves were fixed overnight in a solution of formaldehyde, ethanol, and acetic acid (FAA). To count the number of cells on the blade, the surface of the fixed leaves were peeled off using the Suzuki's Universal Micro-Printing (SUMP) method. The middle part of the blade along the midrib was examined to determine the number and size of the epidermal cells. Replicas of each leaf (1 mm^2) were made to measure the density of the stomata. These copied SUMP images were examined twice for each leaf by using a light microscope.

3. Results

We analyzed the leaf morphology of non-rheophytic and rheophytic types of *Adenophora triphylla* var. *japonica*. **Table 2** presents a summary of these measurements. Leaf lengths of non-rheophytic (2 populations from Higashisako and Hitsuzan) and rheophytic types (from Shimanto and Yoshino Rivers) of *A. triphylla* var. *japonica* were 50.6 ± 9.9, 39.2 ± 13.2, 33.6 ± 10.7, and 25.8 ± 8.2 mm, respectively; their widths were 17.9 ± 2.8, 13.1 ± 5.1, 6.7 ± 2.2, and 6.5 ± 2.6 mm, respectively; leaf thicknesses were 211.0 ± 19.0, 199.6 ± 23.3, 163.0 ± 16.9, and 173.8 ± 12.3 μm, respectively; and the average angles of the leaf base were 46.1 ± 10.6, 46.1 ± 17.5, 23.8 ± 5.7, and 27.0 ± 7.3 degrees, respectively. Each trait differed significantly between the non-rheophytic and rheophytic types, except leaf length. Moreover, we calculated the mean leaf size for the specimens. The leaf sizes of the

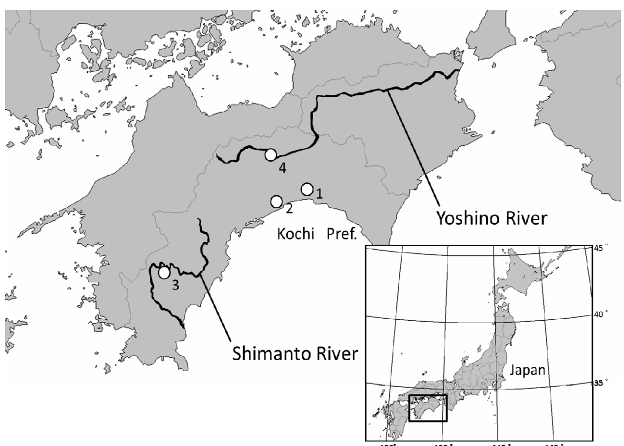

Figure 2. Sampling localities used in this study. For more information, see Table 1.

Table 1. Sampling localities used in this study.

Type	Locality name	Locality no.	Locality	Latitude and longitude
Non-rheophytic	Higashisako	1	Kochi Prefecture, Konan City, Noichi-Cho, Higashisako	33°59'N 133°70'E
	Hitsuzan	2	Kochi Prefecture, Kochi City, Koishigi-Cho, Hitsuzan	33°55'N 133°53'E
Rheophytic	Shimanto River	3	Kochi Prefecture, Takaoka-Gun, Shimanto-Cho, Tohwakawaguchi	33°22'N 132°84'E
	Yoshino River	4	Kochi Prefecture, Nagaoka-Gun, Motoyama-Cho, Motoyama	33°76'N 133°60'E

Locality no. corresponds to that given in **Figure 1**.

Table 2. Morphological and anatomical measurements (average ± standard deviation) of *Adenophora triphylla* var. *japonica*.

Trait	Non-rheophytic				Rheophytic			
	Higashisako		Hitsuzan		Shimanto River		Yoshino River	
Morphological measurements								
Leaf length (mm)	50.6 ± 9.9	a	39.2 ± 13.2	b	33.6 ± 10.7	b	25.8 ± 8.2	c
Leaf width (mm)	17.9 ± 2.8	a	13.1 ± 5.1	b	6.7 ± 2.2	c	6.5 ± 2.6	c
Leaf size (mm^2)	457.4 ± 132.1	a	275.4 ± 176.8	b	119.9 ± 68.6	c	91.3 ± 60.7	c
Leaf index	2.9 ± 0.6	c	3.2 ± 1.1	c	5.2 ± 1.3	a	4.1 ± 1.0	b
Leaf thickness(μm)	211.0 ± 19.0	a	199.6 ± 23.3	a	163.0 ± 16.9	b	173.8 ± 12.3	b
Angle of leaf base (°)	46.1 ± 10.6	a	46.1 ± 17.5	a	23.8 ± 5.7	b	27.0 ± 7.3	b
Anatomical measurements								
Epidermal cell size (μm^2)	2639.7 ± 576.1	a	2912.3 ± 563.3	a	1645.1 ± 502.0	b	1644.5 ± 390.0	b
Epidermal cell number	141121.1 ± 56693.7	a	104530.0 ± 72319.7	b	66501.7 ± 26855.2	c	56000.2 ± 35954.2	c
Stomatal density (N/mm^2)	306.3 ± 74.0	b	256.6 ± 49.7	b	440.4 ± 96.6	a	444.5 ± 70.4	a

Columns marked by different letters differ significantly according to the Tukey's HSD test ($p < 0.05$).

non-rheophytic (2 populations from Higashisako and Hitsuzan) and rheophytic (from Shimanto and Yoshino Rivers) types of *A. triphylla* var. *japonica* were 457.4 ± 132.1, 275.4 ± 176.8, 119.9 ± 68.6, and 91.3 ± 60.7 mm^2, respectively, and the non-rheophytic and rheophytic types differed significantly in this regard.In addition, the leaf index values were calculated as the ratio of leaf length to leaf width similar to the method used by [12]. The non-rheophytic and rheophytic types of *A. triphylla* var. *japonica* also differed significantly in terms of leaf index ($p < 0.05$; non-rheophytic type: 2.9 ± 0.6 (Higashisako), 3.2 ± 1.1 (Hitsuzan) and rheophytic type: 5.2 ± 1.3 (Shimanto River), 4.1 ± 1.0 (Yoshino River)).

We calculated the mean epidermal cell size using the length and width of a cell measured from SUMP samples of non-rheophytic and rheophytic types of *Adenophora triphylla* var. *japonica* from all localities we examined. The cell sizes were 2639.7 ± 576.1 (Higashisako), 2912.3 ± 563.3 (Hitsuzan), 1645.1 ± 502.0 (Shimanto River), and 1644.5 ± 390.0 (Yoshino River) μm^2, and the non-rheophytic and rheophytic types differed significantly in this regard ($p < 0.05$). Based on the leaf size and cell size, we calculated the cell number of a leaf. Total cell numbers of leaves were estimated to be 141,121.1 ± 56,693.7 (Higashisako), 104,530.0 ± 72,319.7 (Hitsuzan), 66,501.7 ± 26,855.2 (Shimanto River), and 56,000.2 ± 35,954.2, (Yoshino River), and the non-rheophytic and rheophytic types differed significantly in this regard ($p < 0.05$). In addition, the stomatal density (440.4 ± 96.6 and 444.5 ± 70.4) of the rheophytic type was significantly higher than that of the non-rheophytic ecotype of *A. triphylla* var. *japonica* (306.7 ± 74.0 and 256.6 ± 49.7).

4. Discussion

[4] reported that rheophytic species are included in more than 60 families, from bryophytes to angiosperms, and some comparative anatomical studies have indicated that stenophyllization occurs in the leaves of these species.

In fern, for example, a comparative anatomical study of a rheophyte (*Osmunda lancea*: Osmundaceae) and its closely related dry land species (*O. japonica* Thunb.) indicated that there is a strong correlation between gross morphology and leaf anatomy [1]. Thus, stenophyllization in *O. lancea* appears to be caused by the decrease in cell size along the leaf-width direction [1]. Regarding angiosperms, [9] reported that the speciation from *Rhododendron indicum* f. *indicum* to rheophytic *R. indicum* f. *otakumi* (Ericaceae) is involved in decreasing the number of leaf cells. A similar process of stenophyllization was indicated in the rheophyte *Farfugium japonicum* (L. fil.) Kitam. var. *luchuense* (Masam.) Kitam. (Asteraceae) [8]. In other rheophytic species of Asteraceae, in contrast, the variation in leaf-width involved both the size and number of leaf cells as in case of the rheophyte *Dendranthema yoshinaganthum* [7] and *Aster microcephalus* var. *ripensis* [6]. Therefore, the developmental processes of narrow leaves in the rheophytes of angiosperms may have evolved from involving only the number of leaf cells into involving both the size and number of leaf cells in Asteraceae. We found that the rheophytic ecotype of *Adenophora triphylla* var. *japonica* has a significantly narrower leaf than the non-rheophytic type, and moreover, our result indicated that the decrease in both the number and size of leaf cells contributed to stenophyllization in a rheophytic ecotype of *A. triphylla* var. *japonica*. According to the overall framework of angiosperm phylogeny [14-18], Campanulaceae species, including *A. triphylla* var. *japonica*, are located at the ancestral position of Asteraceae, suggesting that rheophytic adaptation involves the same process of stenophyllization, and it evolved independently in each lineage of Campanulaceae and Asteraceae.

Comparative analyses of various rheophytic plants of angiosperms can potentially provide a general adaptation scenario for morphological characteristics. Previous studies of rheophytes reported comparative results of various morphological characteristics. In fact, recent studies of rheophytes of angiosperms has revealed some aspects of plant adaptation, with the interaction of environmental factors along streams and rivers being understood best. For example, the stomatal number was higher in the rheophytic *F. japonicum* var. *luchuense* than in inland specimens [19]. Moreover, the angle of the leaf base strongly correlated with both leaf length and width in *As. microcephalus* var. *ripensis*, and the decreasing angle of the leaf base led to the formation of lanceolate leaves [6]. Our results regarding the stomatal number and angle of the leaf base also indicated similar differentiation between rheophytic and non-rheophytic types of *A. triphylla* var. *japonica*, suggesting that with regard to these characteristics, the general tendency in the leaf morphology of rheophytes is to adapt to the environment along streams and rivers. However, surprisingly, the rheophytic ecotype of *A. triphylla* var. *japonica* had thinner leaves than the non-rheophytic ecotype, and it is very interesting the decreased leaf thickness in rheophytic plants. In general, the leaf thickness and stomatal number correlate positively with the mean solar radiation during leaf expansion [20,21], supporting the reports of rheophytic adaptation in some species (e.g., [1,21]). The rheophytic ecotype of *A. triphylla* var. *japonica* grows mainly in riverside habitats; on sunny, moist rocks; and on riverbanks along with other rheophytic plants such as *O. lancea*, *R. ripense,* and *As. microcephalus* var. *ripensis* in the Yoshino and Shimanto rivers. Therefore, although the high irradiance in riverside habitats of the rheophytic ecotype of *A. triphylla* var. *japonica* is the same as that received by other rheophytic plants, increased stomatal number and decreased leaf thickness is observed in this taxa (*A. triphylla* var. *japonica*). This finding suggests that the rheophytic ecotype of *A. triphylla* var. *japonica* shows different adaptation as compared with other rheophytic plants to solar radiation and evaporation. Therefore, further anatomical, ecological, and physiological studies using the rheophytic ecotype of *A. triphylla* var. *japonica* are needed.

5. Acknowledgements

We wish to thank Matsuyama K., Yoshimi Y., Yokoyama N., Isomoto S., Miyata H., Tsuchiya Y., Takei S., Sunami T., Kakimoto N. and Kumekawa Y. for their help. This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and the River Fund in charge of the Foundation of River and Watershed Enviroment Mana- gement (FOREM), Japan (to TF).

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