

Adaptation to water level variation: Responses of a floating-leaved macrophyte *Nymphoides peltata* to terrestrial habitats

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Abstract – A straightforward experimental approach was carried out to study the adaptation responses of a typical floating-leaved aquatic plant *Nymphoides peltata* to changes in water availability. *N. peltata* grown in terrestrial habitat was approximately 88.77% lower in total biomass, 62.75% higher in root biomass allocation, 80.9% higher in root-shoot ratio, and 54.5% longer in leaf longevity compared with *N. peltata* grown in aquatic habitats. Anatomical analyses suggest that aquatic-grown *N. peltata* exhibits a well-developed lacunal system in leaf, petiole, and coarse root. Moreover, aquatic-grown *N. peltata* had approximately a higher in lacunal system in leaf, petiole, and coarse root by 28.57%, 56.41% and 82.35%, respectively, than those of terrestrial-grown *N. peltata*. These results indicated that *N. peltata* was well adapted to the terrestrial habitat because of its biomass allocation, morphological, and anatomical strategies that depended on the increase in root biomass allocation and leaf longevity, as well as the decrease in the lacunal system volume in leaf, petiole, and coarse root. This indicates that *N. peltata* can develop multiple morphological and anatomical strategies, an integrated approach to enhance survival in dynamic and unpredictable environments.

Key words: Trade-off / adaptation / resource allocation / floating-leaved aquatic plant

Introduction

In the natural environment, organisms must cope with change or heterogeneity both within and between generations (Cook and Johnson, 1968; Hourdin *et al.*, 2006). Adaptive plasticity to temporal environmental variations is common and may be important for adaptation to varying environments (Schlichting and Pigliucci, 1995; Bazzaz, 1996; Yamamoto and Tsukada, 2010). In many wetland and floodplain ecosystems, periodic flooding and drought occurrences play an important role in the composition, and productivity of species (Li *et al.*, 2004; Leira and Cantonati, 2008). Wetland plants possess various characteristics in order to survive and function in fluctuating and frequently adverse conditions of wetland ecosystems (*e.g.*, Shangguan *et al.*, 2000; Sultan, 2001; Li *et al.*, 2004; Mommer *et al.*, 2005, 2006). In response to changing water levels, many species of higher plants have developed an amphibious existence (Robe and Griffiths,

1998). Previous works on the adaptation of aquatic plants to water level fluctuations focused mainly on how plants adapt to submersed growth, including morphological and anatomical changes in leaf, root, and shoot (Navas and Garnier, 2002; Lynn and Waldren, 2003; Mommer *et al.*, 2005), life history adaptations (Blom *et al.*, 1994), short-term metabolic adaptations (Pedersen and Sand-Jensen, 1997), and hormonal regulation of adaptive responses (Voesenek *et al.*, 1993). However, only few studies have been carried out on adaptations that allow floating-leaved plants to withstand sudden emersion.

Floating-leaved plants show higher plasticity in life form depending on the developmental stages and/or environmental conditions (Tsuchiya, 1988). Some may even develop into small, terrestrial plants on temporarily dried sediments (Tsuchiya, 1991). For floating plant species, the transition from aquatic to terrestrial environments generally involves adjusting to extremely different conditions (Robe and Griffiths, 1998). Floating-leaved plants would more likely develop numerous adaptation strategies in order to cope with the temporal terrestrial habitat.

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Nymphoides peltata, a typical floating-leaved aquatic plant, is native to Eurasia and is widely distributed in temperate regions of the Northern Hemisphere. In China, *N. peltata* grows in small to medium lakes distributed across the Yangtze River Basin, particularly in the transitional areas from the sub-littoral zone to the open waters. Depending on the slope and depth to base flow, *N. peltata* may be exposed to continuous flooding, periodic flooding, and periodic drought. It also can grow in damp marshes. However, in response to temporal terrestrial habitats, *N. peltata* shows longer leaf longevity and smaller specific leaf areas (Tsuchiya, 1988). Aside from the altered leaf longevity and specific leaf area, *N. peltata* might develop numerous adaptation forms and features that interact with each other to help the plant cope with the temporal terrestrial habitats. Hence, a straightforward experimental approach was employed to study the responses of *N. peltata* to temporal terrestrial habitats by comparing morphological and anatomical traits of *N. peltata* under the aquatic and terrestrial growth conditions. The aim of this study is to elucidate whether the species exhibit high flexibility with sets of characters functioning in concert to confer adaptation to particular conditions.

Materials and methods

Culture of plants

The study was conducted at the State Field Station of the Freshwater Ecosystems of Liangzi Lake (30°18'N, 114°26'E). Twelve short shoots (root stocks) of uniform size (6.5–7.7 g) without the leaves but with roots intact were collected from the different sites of Liangzi Lake. The shoots were then transplanted on March 9, 2006 and placed in six 0.5 m³ (1.0 m × 0.5 m × 1.0 m) plastic containers filled with quartz and sandy soil (about 10 cm). Six plants were used in each of the two treatments (*i.e.*, two plants per container), and each treatment was replicated in three blocks. The water depths of the terrestrial and aquatic treatments were 0 cm (soil saturated with water) and 50 cm (above soil level), respectively. The duration of this experiment was 140 d. During the experiment, lake water was added to every plastic container if need to maintain water level and soil saturation.

Harvest and measurement

After plant culture in aquatic and terrestrial habitats for 60 d, leaf life span was determined by marking five newly emerged leaves for each clone with vinyl tape tied loosely around the petiole. The leaves were observed daily until more than half of the lamina area was yellowed or has disappeared (Tsuchiya, 1988). None of the marked leaves was eaten by insects during the investigation.

The final harvest took place on July 29, 2006. During final harvest, the number of ramets and branches were counted for each clone. Then, the plants were transported

to the laboratory and divided into leaves, petioles, stolons, short shoots, fine roots (<2 mm in diameter), coarse roots (>2 mm in diameter), flowers and fruits. All the parts were gently washed, blotted dry, and weighed. Next, fresh weight was recorded. At the same time, the leaf area was measured for presence of fresh leaves using an LI-3100 leaf-area meter (LiCor, Lincoln, NE, USA). All of the leaves for each clone were measured. Likewise, the lengths of the fine and coarse roots were measured to the nearest millimeter. Moreover, three fresh leaves, petioles, and coarse roots of each clone were selected randomly for lacunal volume measurements; there were fixed at 4 °C for 48 h with a solution containing 1.85% (v/v) formaldehyde, 5% (v/v) acetic acid, and 63% (v/v) ethanol. The fixed tissues were sequentially dehydrated with butanol, embedded in paraffin, sliced into 10 µm sections, stretched onto slides coated with Vectabond Reagent, deparaffinized with xylene and ethanol, rinsed, and stained with 0.05% (w/v) Toluidine blue O, as described previously by Hayakawa *et al.* (1994). Thin transverse sections of leaf, petiole, and coarse root materials were viewed and photographed under a Leica[®] DMRB microscope. Lacunal volume, measured as a percentage of leaf, petiole, and root volume, was estimated from the surface areas by stereology (Steer, 1981), with a 5 or 10 mm point/line lattice applied to enlarged photographs of magnified sections (Robe and Griffiths, 1998). In measuring leaf thickness (LT), five mature, healthy, fully exposed leaves were selected randomly from each clone. Each leaf was cut into eight pieces, and these pieces were piled up. Then, LT was obtained by measuring the pile with a vernier caliper. Finally, leaves, petiole, stolons, short shoots, fine roots, coarse roots, flowers and fruits were dried to constant weight at 80 °C for 72 h and reweighed to determine dry weight (d. wt.) using an analytical balance.

Also, five randomly selected, mature, healthy, fully exposed laminas were selected from each clone to determine the specific leaf area (SLA) according to measured leaf dry weight and area. The specific leaf area was expressed in mm² lamina area.mg⁻¹ d. wt. Likewise, the specific root length (SRL) was determined according to measured root dry weight and lengths. The specific root length was expressed in cm root length.mg⁻¹ d. wt.

Data analysis

The mean and standard deviation values were calculated for each treatment. All parameters were analyzed using one-way ANOVA between two treatment groups using the SPSS statistical package (version 17.0, SPSS, Chicago, IL, USA). Independent-sample T-test was applied to estimate the difference between the two treatments.

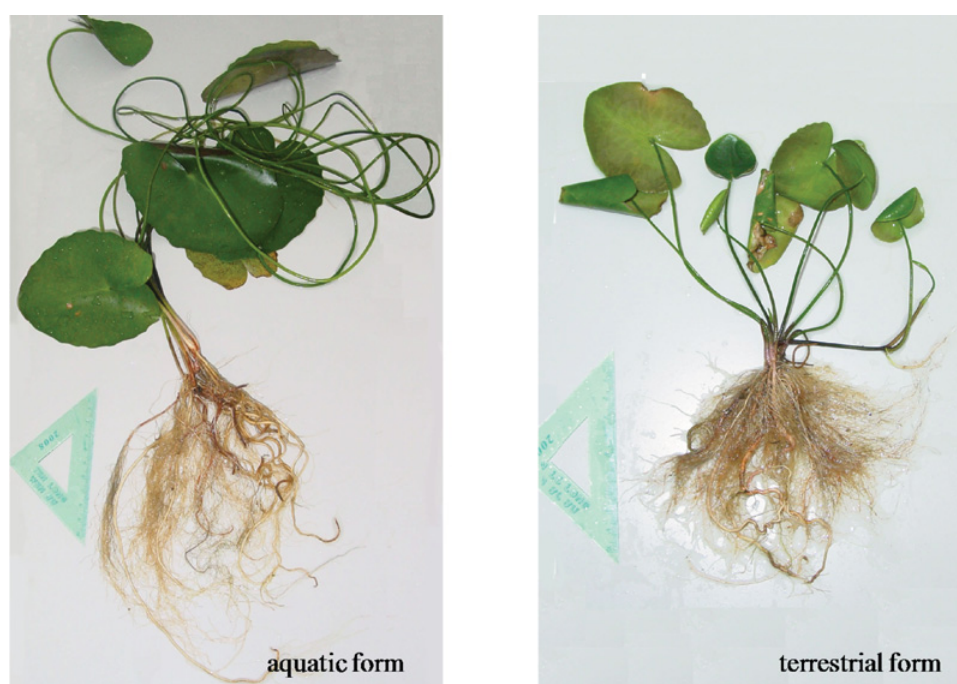
Results

Total biomass, biomass allocation and root-shoot ratio were significantly affected by the experimental treatments (Table 1). The total biomass of aquatically grown

Table 1. Results of independent sample *T* test on total biomass and biomass allocation ratio (dry weight %) of different organs per clone of *N. peltata* grown under aquatic and terrestrial conditions (mean \pm SD, $n = 6$).

	Aquatic environment	Terrestrial environment	<i>P</i>
Total biomass (gDW)	34.20 \pm 5.96	3.84 \pm 1.44	**
Leaf (DW%)	24.35 \pm 3.12	20.69 \pm 1.49	*
Petiole (DW%)	28.92 \pm 2.59	15.84 \pm 0.76	***
Stolon (DW%)	19.54 \pm 1.55	5.12 \pm 1.33	***
Flower and fruits (DW%)	4.95 \pm 0.76	0	***
Short shoot (DW%)	1.92 \pm 0.15	5.03 \pm 1.15	**
Root (DW%)	20.31 \pm 3.67	54.53 \pm 2.52	***
Fine root (DW%)	3.88 \pm 1.44	7.34 \pm 2.03	*
Above-ground biomass (DW%)	77.77 \pm 3.75	40.57 \pm 2.14	***
Below-ground biomass (DW%)	22.23 \pm 3.75	59.43 \pm 2.14	***
Root-shoot ratio (DW%)	0.27 \pm 0.06	1.41 \pm 0.14	***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

**Fig. 1.** *N. peltata* grown under the aquatic and terrestrial conditions.

N. peltata was almost eight times more than that of terrestrially grown *N. peltata*. For biomass allocation, *N. peltata* grown under the terrestrial environment generally allocated more biomass to below-ground parts (59.4% of total biomass) compared with the above-ground. Root biomass accounted for an average of 54.5% of total biomass, while leaf, petiole and stolon biomasses accounted for averages of 20.7%, 15.8% and 5.1%, respectively. In contrast, more biomass was partitioned to above-ground tissue (77.8% of total biomass) in the aquatic habitat, especially for petioles and stolons. Root biomass of *N. peltata* grown under the aquatic condition only accounted for an average of 20.3% of the total biomass, while petiole and stolon biomasses accounted for averages of 28.9% and 19.5%, respectively. Moreover, the fine root allocation ratio under the terrestrial condition was about twice as high as that in

the aquatic habitat (Table 1). The root-shoot ratio of *N. peltata* grown in the terrestrial habitat was five times high as those grown under aquatic conditions (Table 1).

During the experiment, *N. peltata* grew successfully under both aquatic and terrestrial conditions. The appearances of *N. peltata* grown aquatically and terrestrially are shown in Figure 1. Although no significant difference in branch number was observed between the two treatments, they significantly affected plant height and number of ramets ($P < 0.001$) (Table 2). The height and number of ramets in *N. peltata* growing under aquatic conditions were 72% and 71% higher than their counterparts in the terrestrial habitat, respectively (Table 2).

The difference in aquatic or terrestrial conditions greatly influences the morphology of fully expanded leaves and root characteristics. In this study, the leaf area and thickness of floating leaves were considerably higher

Table 2. Results of independent sample *T* test on the morphological characteristics of *N. peltata* grown in aquatic and terrestrial habitats (mean ± SD, *n* = 6).

	Aquatic environment	Terrestrial environment	<i>P</i>
Height (cm)	57 ± 8.5	16 ± 4.3	**
Branch number	1.67 ± 0.50	1.44 ± 1.13	ns
Ramet number of clones	14.57 ± 2.82	4.43 ± 2.37	**
Leaf area (cm ²)	66.48 ± 24.73	18.48 ± 10.64	**
Leaf thickness (mm)	0.47 ± 0.04	0.37 ± 0.05	**
Leaf life span (D)	20.30 ± 2.40	44.55 ± 7.38	**
Specific leaf area (cm ² .g ⁻¹ DW)	277.03 ± 23.42	294.81 ± 64.48	ns
Specific coarse root length (cm.g ⁻¹ DW)	403.67 ± 86.13	191.63 ± 55.45	***
Specific fine root length (cm.g ⁻¹ DW)	22763 ± 6239	14633 ± 5985	*

ns: *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

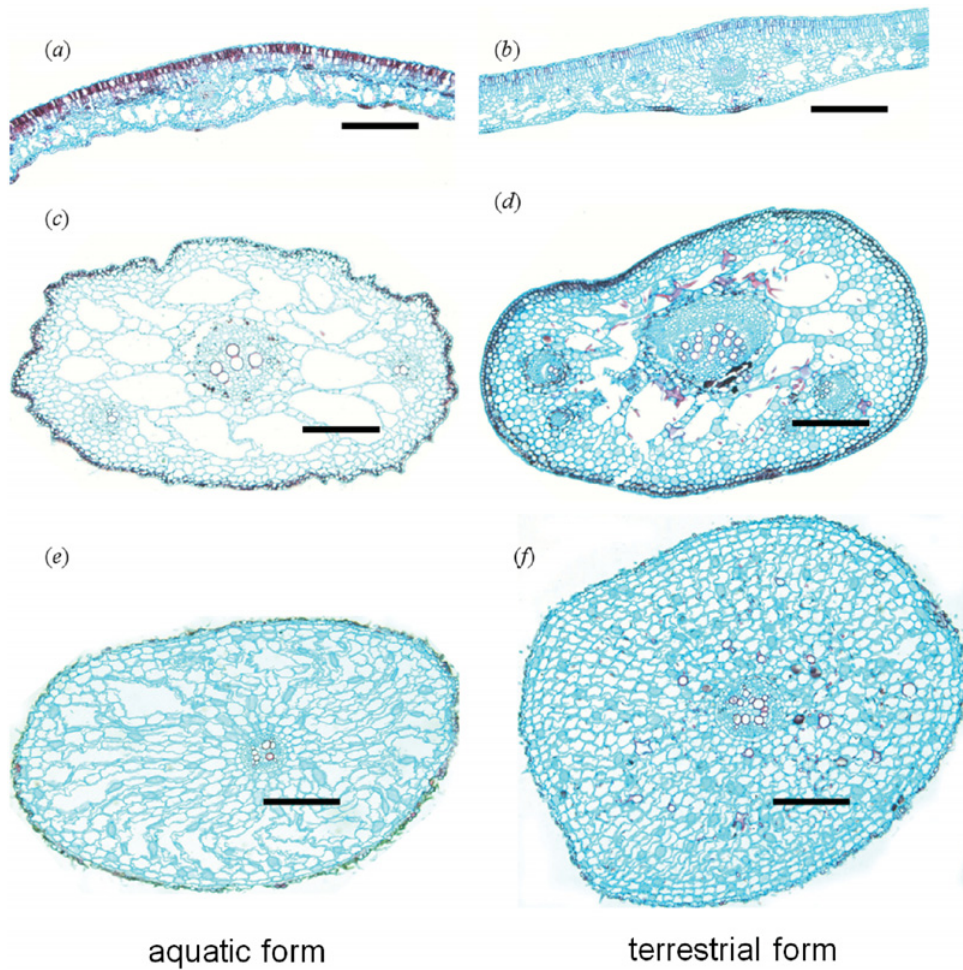


Fig. 2. Light micrographs of the cross-section of lamina (a and b), petiole (c and d), and coarse root (e and f) of *N. peltata* grown under aquatic and terrestrial conditions: a, c and e are grown in aquatic habitat; b, d and f are grown in the terrestrial habitat. Magnification ×50, scale bar 0.05 mm.

than those of emerged leaves (Table 2). And, specific coarse root length and specific fine root length in the terrestrial habitat were significantly lower compared with those in the aquatic habitat (*P* < 0.001 and *P* < 0.1, respectively) (Table 2). Moreover, the life span of the terrestrial leaves was significantly (*P* < 0.001) longer than that of the aquatic leaves. The mean life span and standard deviation was 20.3 ± 2.4 and 44.6 ± 7.4 days for the

aquatic and terrestrial leaves, respectively (Table 2). However, no significant difference in specific leaf area existed between the two treatments (*P* = 0.12, Table 2).

The lacunal volume of leaves, petioles, and coarse roots was significantly affected by the experimental treatments at *P* < 0.01, *P* < 0.001, *P* < 0.001, respectively (Figs. 2 and 3). The transverse sections of leaves, petioles, and coarse roots of aquatic-grown *N. peltata* exhibited well-developed

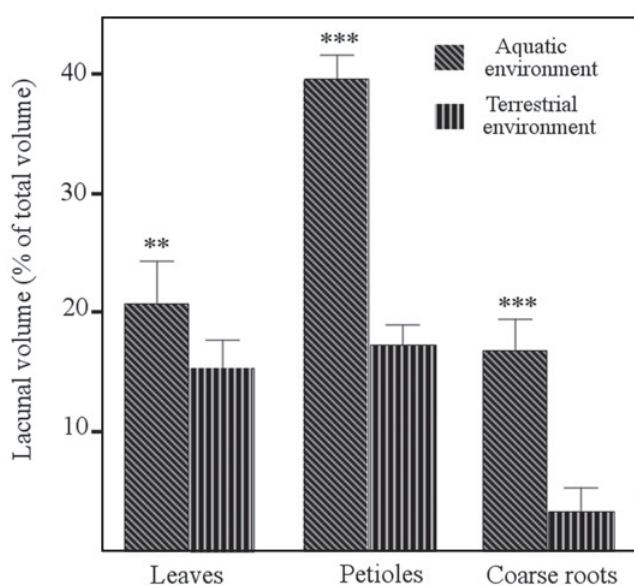


Fig. 3. Results of independent sample *T* test on lacunal volume as a percentage of total volume estimated from transverse section photographs of leaf, petiole, and coarse root using stereology (mean \pm SD, $n = 18$). (** $P < 0.01$, *** $P < 0.001$.)

lacunal system typical of this species, with gas channels occupying about 21%, 39% and 17% of the total leaf, petiole, and coarse root volume, respectively. In contrast, the terrestrial leaf, petiole and coarse root contained a lacunal volume covering only about 15%, 17% and 3% of its total volume, respectively.

Discussion

Biomass allocation is closely linked to the survival strategies of plants (Xie *et al.*, 2005). *N. peltata* species growing in terrestrial habitats allocate more biomass to below-ground parts, possibly because *N. peltata* can adjust its biomass allocation pattern in order to minimize imbalance in water shortage due to terrestrial conditions. Presumably, there may be a trade-off between root biomass allocation to coarse root and fine root. *N. peltata* growing in terrestrial habitats allocates more biomass to fine root parts. Generally, coarse roots are considered the frame of whole root systems while fine roots are recognized as closely related to nutrient and water acquisition (Jackson *et al.*, 1997). Thus, a trade-off between root biomass allocation to coarse root and fine root might be related to the differences in function and biomass cost between coarse and fine roots (He *et al.*, 2004).

The correlation of leaf life span to resource availability have been documented by many studies (*e.g.*, Center and Van 1989; Oikawa *et al.*, 2006). Similar to a previous work (Tsuchiya, 1988), this study has revealed that *N. peltata* grown in a terrestrial environment can significantly prolong its leaf life span and can decrease leaf area to adapt to terrestrial conditions. One explanation is that the

leaf life span is related to the balance between costs and benefits associated with leaf construction and maintenance (Kikuzawa, 1991). Another explanation would be that leaf life span does not present clearly adaptive traits; that is, leaves decay more easily if surrounded by water (*i.e.*, a strong indication that leaves on land is the better approach to maintain life span). Similarly, a smaller leaf area may be an effective adaptive mechanism to the terrestrial habitat, possibly because a smaller leaf area can result in decreasing water loss by transpiration (Oikawa *et al.*, 2006). Therefore, the production of smaller, longer-lived leaves in lower-quality environments may be a more adaptive approach to increase resource use efficiency in response to stress (Chapin *et al.*, 1993; Kikuzawa, 1991; Moriuchi and Winn, 2005). The present study also suggests that terrestrially grown plants have thinner lamina than aquatically grown plants. A possible explanation for this is that the thinner emerged leaves also have shortened diffusion pathways within it and thus result in increased gas exchanges (Mommer *et al.*, 2005). An analogous type of morphogenetic plasticity could occur in root systems. *N. peltata* have different specific root lengths, indicating that they have different strategies for growth and survival in contrasting habitats. In aquatic habitats, high SRL can maximize root surface area and root growth, thus enabling rapid exploitation of resources; this approach is crucial for fast growth and completion of short life cycle (Roumet *et al.*, 2006). In contrast, in terrestrial habitats, the diversion of resources to fine roots and high-density tissues may be more appropriate to enhance survival; therefore, this special modification may be considered as an adaptive against relative water-deficiency.

Another interesting result in the present study is the relatively low lacunal volume of leaves, petioles and roots of *N. peltata* in the terrestrial environment. As a potentially important morphological modification, lacunal volume for gas transport could present functional difference between divergent growth forms of *N. peltata*. Aerenchyma might also be related to mechanical support. Possibly, terrestrial growth forms require relatively more petiole area (*i.e.*, relatively less aerenchyma volume of petiole) for mechanical support due to lack of water buoyancy. Therefore, these special modifications in lacunal system might be considered as another adaptive approach against relative water-deficiency.

The specific differences in these important species in terms of survival and growth under two water level conditions imposed in this experiment suggest that the floating-leaved macrophyte is a species with a wide ecological niche (*i.e.*, fundamental niche). Differences in biomass allocation, as well as morphological and anatomical traits, indicate high potential for species niche differentiation under the two different levels of water supply. This suggests further that *N. peltata* grown in terrestrial habitats can develop many strategies on morphological and anatomical traits in order to adapt to stressful environments. These results also support the hypothesis that *N. peltata* exhibits high phenotypic

flexibility and therefore possess broad tolerance to water (*i.e.*, wide ecological amplitude).

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References

- Bazzaz F.A., 1996. *Plant in Changing Environment: Linking Physiological, Population, and Community Ecology*, Cambridge University Press, Cambridge.
- Blom C.W.P.M., Voesenek L.A.C.J., Banga M., Engelaar W.M.H.G., Rijnders J.H.G.M., van de Steeg H.M. and Visser J.W., 1994. Physiological ecology of river-side species: adaptive responses of plants to submergence. *Aquat. Bot.*, 38, 29–47.
- Center T.D. and Van T.K., 1989. Alternation of water hyacinth (*Eichhorina crassipers* (Mart.) Solms.) leaf dynamics and phytochemistry by insect damage and plant density. *Aquat. Bot.*, 35, 181–195.
- Chapin F.S. III, Autumn K. and Pugnaire F., 1993. Evolution of suites of traits in response to environmental stress. *Amer. Natural.*, 142, S78–S92.
- Cook S.A. and Johnson M.P., 1968. Adaptation to heterogeneous environments. I. Variation in heterophylly in *Ranunculus flammula* L. *Evolut.*, 22, 496–516.
- Hayakawa T., Nakamura T., Hattori F., Mae T., Ojima K. and Yamaya T., 1994. Cellular localization of NADH-dependent glutamate-synthase protein in vascular bundles of unexpanded leaf blades and young grains. *Planta*, 193, 455–460.
- He W.M., Zhang H. and Dong M., 2004. Plasticity in fitness and fitness-related traits at ramet and genet levels in a tillering grass *Panicum miliaceum* under patchy soil nutrients. *Plant Ecol.*, 172, 1–10.
- Hourdin P., Vignoles P., Dreyfuss G. and Rondelaud D., 2006. *Galba truncatula* (Gastropoda, Lymnaeidae): effects of daily waterlevel variations on the ecology and ethology of populations living upstream from a dam. *Ann. Limnol. - Int. J. Lim.*, 42, 173–180.
- Jackson R.B., Mooney H.A. and Schulze E.D., 1997. A global budget for fine root biomass, surface area, and nutrient contents. *PNAS*, 94, 7362–7366.
- Kikuzawa K., 1991. A cost-benefit analysis of leaf habit and leaf longevity of trees and their geographical pattern. *Amer. Natural.*, 138, 1250–1263.
- Leira M. and Cantonati M., 2008. Effects of water-level fluctuations on lakes: an annotated bibliography. *Hydrobiologia*, 613, 171–184.
- Li S.W., Pezeshki S.R. and Goodwin S., 2004. Effects of soil moisture regimes on photosynthesis and growth in cattail (*Typha latifolia*). *Acta Oecologia*, 25, 17–22.
- Lynn D.E. and Waldren S., 2003. Survival of *Ranunculus repens* L. (Creeping Buttercup) in an amphibious habitat. *Ann. Bot.*, 91, 75–84.
- Mommer L., Pons T.L., Wolters-Arts M., Venema J.H. and Visser E.J.W., 2005. Submergence-induced morphological, anatomical, and biochemical responses in a terrestrial species affect gas diffusion resistance and photosynthetic performance. *Plant Physiol.*, 139, 497–508.
- Mommer L., Lenssen J.P.M., Huber H., Visser E.W. and Kroon H.D., 2006. Ecophysiological determinants of plant performance under flooding: a comparative study of seven plant families. *J. Ecol.*, 94, 1117–1129.
- Moriuchi K.S. and Winn A.A., 2005. Relationships among growth, development and plastic response to environment quality in a perennial plant. *New Phytol.*, 166, 149–158.
- Navas M.-L. and Garnier E., 2002. Plasticity of whole plant and leaf traits in *Rubia peregrina* in response to light, nutrient and water availability. *Acta Oecologia*, 23, 375–383.
- Oikawa S., Hikosaka K. and Hirose T., 2006. Leaf lifespan and lifetime carbon balance of individual leaves in a stand of an annual herb, *Xanthium canadense*. *New Phytol.*, 172, 104–116.
- Pedersen O. and Sand-Jensen K., 1997. Transpiration does not control growth and nutrient supply in the amphibious *Mentha aquatica*. *Plant Cell Envir.*, 20, 117–123.
- Robe W.E. and Griffiths H., 1998. Adaptations for an amphibious life: changes in leaf morphology, growth rate, carbon and nitrogen investment, and reproduction during adjustment to emersion by the freshwater macrophyte *Littorella uniflora*. *New Phytol.*, 140, 9–23.
- Roumet C., Urcelay C. and Díaz S., 2006. Suites of root traits differ between annual and perennial species growing in the field. *New Phytol.*, 170, 357–368.
- Schlichting C.D. and Pigliucci M., 1995. Gene-regulation, quantitative genetics and the evolution of reaction norms. *Evolut. Biol.*, 9, 154–168.
- Shangguan Z.P., Shao, M.A. and Dyckmans J., 2000. Nitrogen nutrition and water stress effects on leaf photosynthetic gas exchange and water use efficiency in winter wheat. *Environ. Exp. Bot.*, 44, 141–149.
- Steer M.W., 1981. *Understanding cell structure*, Cambridge University Press, Cambridge.
- Sultan E.S., 2001. Phenotypic plasticity for plant development, function and life history. *Trends Plant Sci.*, 5, 537–542.
- Tsuchiya T., 1988. Comparative studies on the morphology and leaf life span of floating and emerged leaves of *Nymphoides peltata* (GMEL.) O. Kuntze. *Aquat. Bot.*, 29, 381–386.
- Tsuchiya T., 1991. Leaf life span of floating-leaved plants. *Vegetat.*, 9, 149–160.
- Voesenek L.A.C.J., Banga M., Their R.H., Mudde C.M., Harren F.J.M., Barendse G.W.M. and Blom C.W.P.M., 1993. Submergence induced ethylene synthesis, entrapment and growth in two plant species with a contrasting flooding resistance. *Plant Physiol.*, 103, 783–791.
- Xie Y.H., An S.Q. and Wu B.F., 2005. Resource allocation in the submerged plant *Vallisneria spiralis* related to sediment type, rather than water-column nutrients. *Freshwat. Biol.*, 50, 391–402.
- Yamamoto Y. and Tsukada H., 2010. Morphological variation in largemouth bass *Micropterus salmoides* in Lake Biwa, Japan. *Ann. Limnol. - Int. J. Lim.*, 46, 41–45.