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The Effects of Nitrogen as NO_3^- and NH_4^+ on the Growth and Symbiont (*Anabaena azollae*) of *Azolla pinnata* R. Brown

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ABSTRACT

The growth, morphology, and symbiont (Anabaena azollae) of Azolla pinnata R. Brown were investigated under different external N-supply regimes to inform the plant's potential in wastewater treatment. Azolla pinnata plants were supplied with nitrogen as NO_3^- or NH_4^+ at four different concentrations (0, 0.5, 1, and 5 mM) and incubated in a greenhouse for 14 days. The relative growth rates of NO_3^--fed plants were not significantly different between treatments, but decreased significantly at the highest NH_4^+ concentration. Moreover, the NO_3^- concentration did not affect root number. The highest NH_4^+ concentration (5 mM) decreased both the root length and number of symbionts (Anabaena azollae) in the mature leaves of Azolla pinnata. Because Azolla pinnata continued to grow well with supplied NO_3^- and NH_4^+, and retained their ability to absorb nitrogen, they offer potential for treating wastewater, except at the highest NH_4^+ concentration, which led to toxicity.

Keywords: *Azolla pinnata, Anabaena azollae,* Heterocyst, NH_4^+ toxicity, Symbiont

INTRODUCTION

Azolla pinnata R. Brown is a free-floating aquatic fern belonging to the family Azollaceae. It is widely distributed in Asia and along the coast of tropical Africa (Wagner, 1997). The plant consists of alternately arranged leaves on a prostrate, floating rhizome, with one or two roots hanging in the water column. The leaf is bilobed, consisting of a chlorophyllous floating dorsal lobe and a colorless and partially submerged ventral lobe. A cavity in the ventral leaves houses symbiotic cyanobacteria, *Anabaena azollae* (Pabby et al., 2003). This symbiont fixes N_2 from the atmosphere and produces a high N level in the plant tissue of *Azolla pinnata*, making the plant useful as green manure for rice fields, where it has been used for several centuries (Shi and Hall, 1988; Peters and Meeks, 1989; Forni et al., 2001; de Macale and Vlek, 2004).

More recently, *Azolla* spp. have been used in water treatment (Kitoh et al., 1993; Forni et al., 2001; Nahlik and Mitsch, 2006; Costa et al., 2009). Several studies have shown that *Azolla* spp. can both grow in and remove nutrients from wastewater (Reddy and DeBusk, 1985; Kitoh et al., 1993; Vermaat and Hanif, 1998; Costa et al., 2009). In general, two forms of inorganic nitrogen – NH_4^+ and NO_3^- – are commonly found in wastewater at concentrations of 1 to 5 mM (Kadlec and Wallace, 2009). Both the form and concentration of N may affect plant growth, morphology, and the symbiont. A previous study by Ito and Watanabe (1983) showed that NO_3^- and NH_4^+ at concentrations of 1 mM did not inhibit the acetylene reduction activity of the symbiont *Anabaena azollae* in the leaves of *Azolla pinnata*; however, they did not determine its effects on the growth and morphology of the plants.

Several studies have shown that many aquatic or wetland plants, such as *Phragmites australis* (Cav.) Trin. ex Steudel and *Salvinia natans* (L.) All., prefer NH_4^+ over NO_3^-, but that NH_4^+ is toxic at high concentrations (Kitoh et al., 1993; Britto and Kronzucker, 2002; Tylova et al., 2005; Cao et al., 2009; Jampeetong and Brix, 2009a, 2009b; Jampeetong et al., 2012). Because symbionts furnish *Azolla pinnata* with nitrogen, external nitrogen in wastewater may affect the symbiotic relationship and subsequent plant growth. To study this, we examined the growth, morphology, and symbiotic response of *Azolla pinnata* R. Brown to two different forms – NH_4^+ and NO_3^- – and concentrations of inorganic nitrogen; the results can be applied to developing better water treatment systems.

MATERIALS AND METHODS

Plant material and experimental set up

Azolla pinnata was obtained from a natural pond at Chiang Mai University, Chiang Mai, Thailand. The plants were cleaned and grown on a standard N- and P-free growth medium prepared according to Smart and Barko (1985), to which 0.5 mM of NO_3^- or NH_4^+, 100 µM KH_2PO_4, and a commercial plant micronutrient solution (Tropica, Egaa, Denmark) (1 mL: 10 L growth solution) were added. The pH was adjusted to 6.6±0.1.

After the plants had acclimated for 14 days, approximately 2 grams of the ramet from the stock culture was placed in a plastic pot (4 pots per treatment) containing 2 liters of a standard N- and P- free growth medium prepared according to Smart and Barko (1985), to which 100 µM KH_2PO_4, and a commercial plant micronutrient solution (Tropica, Egaa, Denmark) were added. The pH was adjusted to 6.6±0.1 using HCl and NaOH. The treatments consisted of two N forms: NH_4^+ or NO_3^- prepared from $(NH_4)_2SO_4$ or KNO_3, respectively, at different concentrations (0, 0.5, 1, and 5 mM). The plants were incubated in the greenhouse at the Department of Biology, Faculty of Science, Chiang Mai University, Thailand. The growth medium was changed every 3 days and the plants were cleaned gently by hand. At the beginning of the experiment, plant ramets similar to experimental plants (n=10) were selected to estimate the fresh weight and dry weight ratio. The fresh weight of all plants was measured, and then they were dried until they reached a constant weight. The fresh to dry weight ratio was calculated and used for the relative growth rate calculation.

Growth and morphological study

After 14 days, root number and root length of the plants in each treatment were measured. The plants were then harvested and freeze dried. The relative growth rate ($g\ g^{-1}d^{-1}$) was calculated by the formula:

$$\text{Relative growth rate} = \frac{\text{In } W_2 - \text{In} W_1}{(t_2 - t_1)} \qquad g\ g^{-1}d^{-1}$$

where W_1 and W_2 are the initial and final dry weights (g) of plant material from each pot, and t_1 and t_2 are the initial and final time (days).

The shoot area (cm^2) was estimated from digital photos taken of each pot at the same angle and distance. The relative shoot area growth rate (RSGR, cm^2cm^{-2} day^{-1}) was calculated in a similar way to the relative growth rate.

Counting *Anabaena* and heterocysts

Anabaena azollae in both young (1^{st} in position) and mature (6^{th} in position) leaves was determined. The leaves were broken using a needle, and then wet mount slides were made. *Anabaena azollae* filaments in each leaf were counted. The heterocyst frequency was measured by randomly counting 200 cells of *A. azollae* and recording the number of heterocysts found.

Inorganic nitrogen in the whole plant

Five milligrams of freeze-dried plant materials from each replicate were extracted with 15 mL of distilled water at 98°C in a water bath for 20 minutes. Then the NH_4^+ and NO_3^- in the extracts were analysed by a modified salicylate method (Quikchem Method no. 10-107-06-3-B; Lachat Instruments, Milwaukee, WI, USA). The absorbance of the extracts was measured at 690 and 220 nm using a UV-VIS spectrophotometer (Lambda 25 version 2.85.04, USA) to determine NH_4^+ and NO_3^-, respectively.

Statistics

The data were analyzed by one-way and two-way analysis of variance (ANOVA) using Statgraphics Plus ver. 4.1 software (Manugistics, Inc., MD, USA). The normality of the distribution and homogeneity of variance were tested using Cochran's C-test. If necessary, data was log-transformed to ensure homogeneity of variance. Multiple comparisons of means were identified by Scheffe's test ($p<0.05$).

RESULTS

Growth and morphology

Both the form and concentration of N affected relative growth rates (RGRs) of *Azolla pinnata*, with significant interactive effects between these two factors observed (Table 1, Figure 1a). The plants grown on the three lowest concentrations of NH_4^+ had higher relative growth rates than that grown on NO_3^-. However, increasing NO_3^- concentrations did not affect plant growth, whereas the highest NH_4^+ concentration (5 mM) decreased growth significantly. Varying the concentrations of either NH_4^+ or NO_3^- did not significantly affect relative shoot area growth rates (RSGRs) (Fig.1b).

Neither the N form nor concentration significantly affected root number (Figure 2a). However, the higher NH_4^+ concentrations negatively affected root length (Figure 2b), with significant interaction between N form and N concentration (Table 1).

Table 1. Degrees of freedom (d.f.), *F-ratios*, and significance of a two-way ANOVA of relative growth rate (RGR), relative shoot area growth rates (RSGR), root number, root length, number of *Anabaena azollae*, number of heterocyst, and NH_4^+ in the plant tissue of *Azolla pinnata* grown on NO_3^- or NH_4^+ at four different concentrations (0, 0.5, 1, and 5 mM) for 14 days.

		Main effects		Interaction
	d.f.	**N form (A) (NO_3^- / NH_4^+)**	**N concentration (B)**	**A x B**
RGR ($g\ g^{-1} d^{-1}$)	3	1.96	2.34	4.13*
RSGR ($cm^2\ cm^{-2}\ d^{-1}$)	3	8.33	0.05	0.18
Root number	4	3.50	0.37	1.20
Root length (mm)	4	3.16	3.10*	5.17**
Number of *Anabaena azollae* (filament)				
- young leaves	7	2.87	12.38***	5.40**
- mature leaves	7	24.33***	12.4***	5.29**
Number of heterocyst (cell)				
- young leaves	7	6.42*	10.72***	2.07
- mature leaves	7	12.13**	9.16*	4.04*
NH_4^+ in the plant tissue ($mg\ g^{-1}$ dw)	7	1360.58***	15.45***	15.87***

Note: *(in column) indicate significant differences between factors, * ($P<0.05$), ** ($P<0.01$), and *** ($P<0.001$).

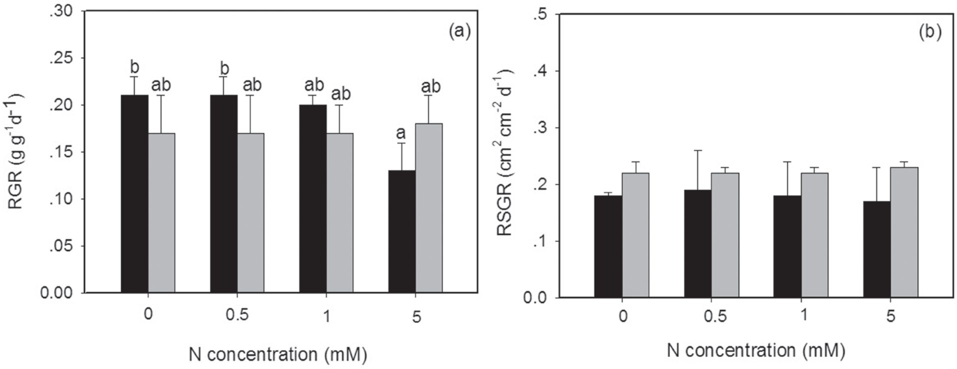

Figure 1. The relative growth rate (RGR) (a) and the relative shoot area growth rate (RSGR) (b) of *Azolla pinnata* (mean±SD) grown with NO_3^- (grey column) or NH_4^+ (dark column) as the nitrogen source at four different concentrations (0, 0.5, 1, 5 mM) for 14 days. Different letters above columns indicate significant differences between treatments.

***Anabaena* and heterocyst**

Both the form and concentration of N affected the amount of *Anabaena azollae*; the interaction between these two factors was observed (Table 1). Fewer *Anabaena azollae* were found in young leaves than mature leaves. In young leaves, the amount of *Anabaena azollae* was mildly affected by the form and concentration of N compared to mature leaves, in which high concentrations of NH_4^+ reduced the number of *Anabaena azollae* (Figure 3a, b). Similarly, heterocyst counts per 200 vegetative cells of *Anabaena* significantly decreased in plants fed with 5 mM NH_4^+; NO_3^- had no affect on either the young or mature leaves (Figure 3b, d). The effects of N concentration depended on the N form, as shown by the significant interaction term in the ANOVA results (Table 1).

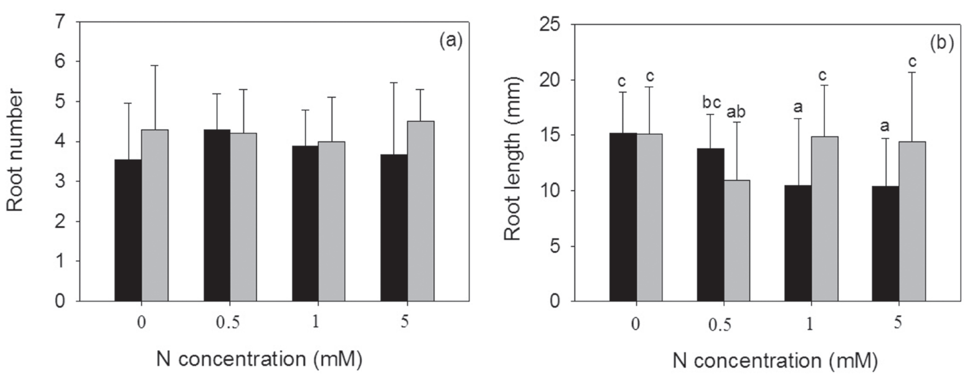

Figure 2. Root number (a) and root length (b) of *Azolla pinnata* (mean±SD) grown with either NO_3^- (grey column) or NH_4^+ (dark column) as the nitrogen source at four different N concentrations (0, 0.5, 1, 5 mM). Different letters above columns indicate significant differences between treatments.

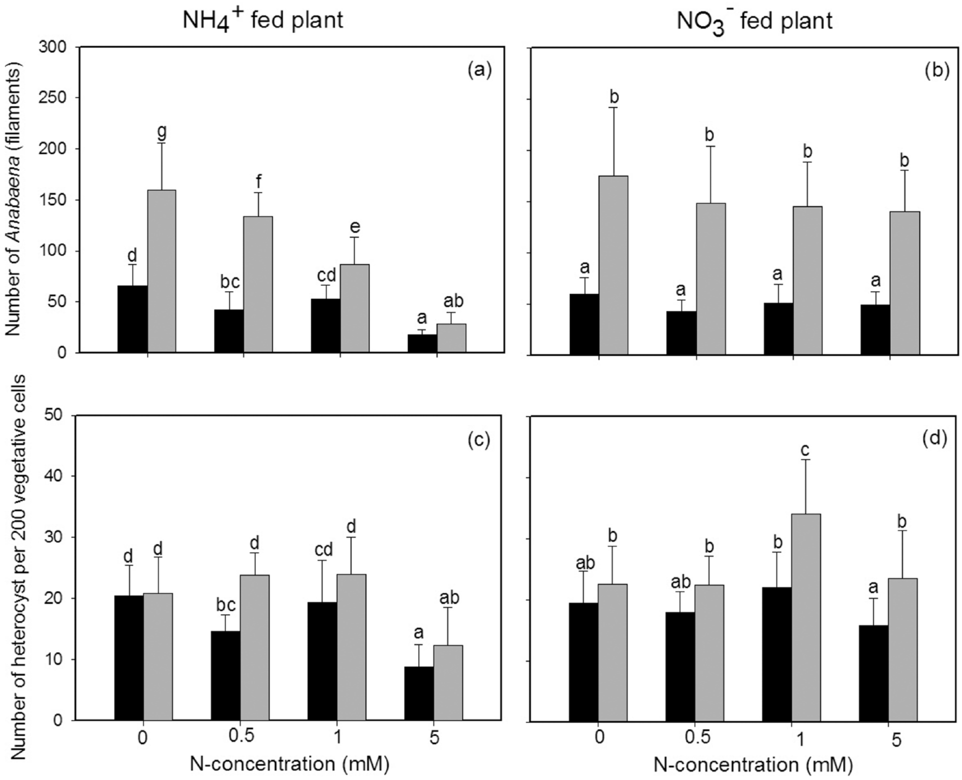

Figure 3. Number of *Anabaena azollae* (a, b) and heterocyst (c, d) in young leaves (dark column) and mature leaves (grey column) of *Azolla pinnata* (mean±SD) grown with either NH_4^+ or NO_3^- as the nitrogen source at four different N concentrations (0, 0.5, 1, 5 mM) for 14 days. Different letters above columns indicate significant differences between treatments.

Inorganic nitrogen in the whole plant

The NH_4^+ in the plant tissue of *Azolla pinnata* treated with NO_3^- at different concentrations did not significantly differ. In contrast, in the NH_4^+-fed plants, the concentration of NH_4^+ in the plant tissue increased when NH_4^+ was supplied at high concentration (Figure 4a). NO_3^- in the plant tissue was unaffected in the NO_3^--fed plants, even as the external concentration of NO_3^- increased. The NO_3^- in the plant tissue of the NH_4^+-fed plants was not determined (Figure 4b).

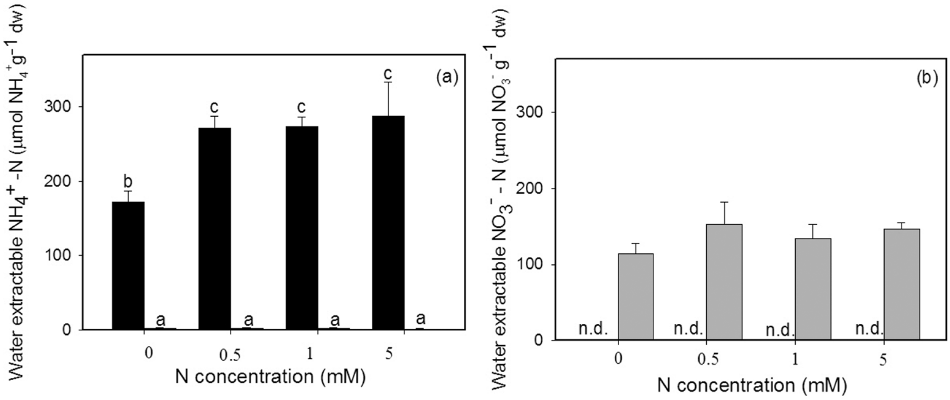

Figure 4. Water extractable NH_4^+ -N (a) and NO_3^- -N (b) in the tissue of *Azolla pinnata* (mean±SD) grown with either NH_4^+ (dark column) or NO_3^- (grey column) as the nitrogen source at four different N concentrations (0, 0.5, 1, 5 mM) for 14 days. Different letters above columns indicate significant differences between treatments. n.d. = not determined.

DISCUSSION

The form (NO_3^- or NH_4^+) and concentration (0, 0.5, 1 and 5 mM) of externally supplied nitrogen affected the growth and morphology of *Azolla pinnata* R. Brown. The effects were lower in the NO_3^--fed plants. Most aquatic plants prefer inorganic nitrogen in the form of ammonium (NH_4^+), because of the lower energy needed for uptake and assimilation (Cedergreen and Madsen, 2002; Tylova-Munzarova et al., 2005; Jampeetong and Brix, 2009a). However, in our study, *Azolla pinnata* showed a negative response – low growth rate, short roots, and leaf chlorosis, especially in mature leaves – to the highest concentration (5 mM) of NH_4^+. Others have found similar effects of high NH_4^+ concentrations in other free-floating plants, such as *Azolla filiculoides* (Kitoh et al., 1993) and *Salvinia natans* (Jampeetong and Brix, 2009b). Ito and Watanabe (1983) reported that biomass decreased after *Azolla pinnata* was exposed to 10 mM NH_4^+ for 4 days, but growth and morphology data were not precisely determined. They also reported that both the form and concentration of nitrogen affected the symbiont *Anabaena azollae* in the leaves of *Azolla pinnata*; a high NH_4^+ concentration (10 mM) inhibited acetylene reduction activity, indicating decreased nitrogenase enzyme activity. However, they did not determine the amounts of *Anabaena* and heterocyst. In our results, we found that *Anabaena azollae* and its heterocyst decreased with increasing external NH_4^+ concentration, particularly in mature leaves. Maejima et al. (2001) found a similar result; both *Anabaena azollae* and heterocyst decreased more than 50% in mature leaves, while young leaves were not affected. In contrast, externally supplied NO_3^- did not affect *Anabaena azollae* and its heterocyst. Therefore, the plants can obtain NO_3^- from both externally supplied NO_3^- and from the atmosphere by fixing N_2. Costa et al. (2009) found similar results with *Azolla filiculoides* grown on combined nitrogen wastewater.

Even though we did not determine the NH_4^+ uptake of *Azolla pinnata*, the NH_4^+ concentration in the plant tissue increased when the plants were supplied with high concentrations of external NH_4^+. This indicated that the ability of the plants to take up external NH_4^+ was not suppressed. Jampeetong et al. (unpublished data) found that *Azolla pinnata* grown on 1 mM NH_4NO_3 showed that the uptake of NH_4^+ was 27 times higher than NO_3^-. Moreover, Cary and Weerts (1992) showed that *Azolla pinnata* and *Azolla filiculoides* can obtain nitrogen from an external supply, even though both species benefit from a symbiotic association. However, in our study, *Azolla pinnata* appeared not to have a mechanism to prevent over-accumulation of NH_4^+ in its cells. This over-accumulation has been shown to be toxic (Britto and Kronzucker, 2002). Similar results were recorded in several NH_4^+ intolerant species, including *Thalassia hemprichii* and *Zostera marina* (van Katwijk et al., 1997; Christianen et al., 2011).

Many aquatic plants have been used for wastewater treatment. Most species had high growth rates and high acquisition of nitrogen (Koerselman and Meuleman, 1996; Abe and Ozaki, 1998). According to our results, *Azolla pinnata* had high growth rates and biomass production. Hence, this species offers potential for treating various types of wastewater in constructed wetland systems.

In conclusion, both the form and concentration of N affected the growth and morphology of *Azolla pinnata*. The plants grown with NH_4^+ up to 1 mM had higher growth rates than NO_3^--fed plants, but the growth rate and root length of the plants decreased at the highest concentration (5 mM). *Anabaena azollae* and its heterocyst also decreased in the mature leaves of the plants fed with a high NH_4^+ concentration, whereas the youngest leaves were not affected. *Azolla pinnata* was able to take up external NO_3^- or NH_4^+, but high NH_4^+ concentrations may cause NH_4^+ toxicity and lead to plant destruction. *Azolla pinnata* offers potential for removing nutrients from wastewater, but exposure to NH_4^+ contamination must be less than 5 mM in order to maintain plant growth and the potential of N uptake from the polluted water.

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