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Effects of inorganic nitrogen forms on growth, morphology, nitrogen uptake capacity and nutrient allocation of four tropical aquatic macrophytes (*Salvinia cucullata*, *Ipomoea aquatica*, *Cyperus involucratus* and *Vetiveria zizanioides*)

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ABSTRACT

This study assesses the growth and morphological responses, nitrogen uptake and nutrient allocation in four aquatic macrophytes when supplied with different inorganic nitrogen treatments (1) NH_4^+, (2) NO_3^-, or (3) both NH_4^+ and NO_3^-. Two free-floating species (*Salvinia cucullata* Roxb. ex Bory and *Ipomoea aquatica* Forssk.) and two emergent species (*Cyperus involucratus* Rottb. and *Vetiveria zizanioides* (L.) Nash ex Small) were grown with these N treatments at equimolar concentrations (500 μM). Overall, the plants responded well to NH_4^+. Growth as RGR was highest in *S. cucullata* ($0.12 \pm 0.003\ d^{-1}$) followed by *I. aquatica* ($0.035 \pm 0.002\ d^{-1}$), *C. involucratus* ($0.03 \pm 0.002\ d^{-1}$) and *V. zizanioides* ($0.02 \pm 0.003\ d^{-1}$). The NH_4^+ uptake rate was significantly higher than the NO_3^- uptake rate. The free-floating species had higher nitrogen uptake rates than the emergent species. The N-uptake rate differed between plant species and seemed to be correlated to growth rate. All species had a high NO_3^- uptake rate when supplied with only NO_3^-. It seems that the NO_3^- transporters in the plasma membrane of the root cells and nitrate reductase activity were induced by external NO_3^-. Tissue mineral contents varied with species and tissue, but differences between treatments were generally small. We conclude, that the free-floating *S. cucullata* and *I. aquatica* are good candidate species for use in constructed wetland systems to remove N from polluted water. The rooted emergent plants can be used in subsurface flow constructed wetland systems as they grow well on any form of nitrogen and as they can develop a deep and dense root system.

1. Introduction

The capacity of aquatic macrophytes to remove and assimilate excess nutrients in constructed wetlands (CWs) has resulted in the use of CWs for treating a variety of wastewater types. Ammonium (NH_4^+) and nitrate (NO_3^-) are major forms of inorganic nitrogen found in wastewater runoff from households and farmlands that degrade the water quality. Plants have an important role in CWs for removing nutrients. The ability to take up nitrogen, a vital nutrient for plant growth, is different among plant species. Free-floating macrophytes directly obtain nitrogen and other nutrients from the water column through their roots. Many species of these plants such as *Eichhornia crassipes* (Mart.) Solms, *Pistia stratiotes* L., *Lemna* spp., *Salvinia* spp., etc. have been used in free water surface systems. In contrast, subsurface flow system and vertical flow system are dominated by emergent macrophytes, which obtain nutrients from the substrate via roots (Brix and Schierup, 1989; Ran et al., 2004; Iamchaturapatr et al., 2007; Kantawanichkul et al., 2009).

Plant selection for water treatment is an important component of CW system design, especially selecting suitable plants for different types of wastewater. Various nitrogen forms have different effects on growth and nitrogen uptake of plants. Several studies have shown that aquatic macrophytes grow well when NH_4^+ is the main nitrogen source probably because less energy is needed for NH_4^+ uptake and assimilation compared to NO_3^- nutrition (Room and Thomas, 1986; Petrucio and Esteves, 2000; Fang et al., 2007; Jampeetong and Brix, 2009a; Konnerup and Brix, 2010). Despite the fact that many tropical plants have been used in water treatment systems (Boonsong and Chansiri, 2008; Kantawanichkul et al., 2009; Konnerup et al., 2009), their nitrogen nutrition is not well understood.

Salvinia cucullata Roxb. ex Bory and *Ipomoea aquatica* Forssk are tropical free-floating macrophytes. Many species of *Salvinia* have a high growth rate and high dispersal rate and have consequently been spreading around the world. A study by Jampeetong and Brix (2009a) showed that *S. natans* has a higher growth rate

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when supplied with NH_4^+ than when supplied with NO_3^-, and the species tolerates NH_4^+ concentration up to at least 5 mM, which is approximately the level commonly found in domestic wastewater (Jampeetong and Brix, 2009b). Similarly, *I. aquatica* grows in water bodies with high N and P concentrations, and can remove nutrients and some heavy metals from polluted waters (Göthberg et al., 2002; Lin et al., 2002; Li et al., 2007; Weerasinghe et al., 2008). Moreover, this species is an edible plant with a high protein content that can be used as animal fodder (Somkol, 2009). However, the responses of *S. cucullata* and *I. aquatica* to various forms of N are unknown. Emergent macrophytes like *Cyperus involucratus* Rottb. and *Vetiveria zizanioides* (L.) Nash ex Small have been used in tropical CWs (Kantawanichkul et al., 2009; Xiao et al., 2009). *C. involucratus* is a common plant in eutrophic tropical wetlands. *V. zizanioides* is widely used to reduce soil erosion and in water treatment systems in Southeast Asia, because of its high tolerance to diverse growth conditions and the fact that it can be harvested and used for many proposes. However, information concerning nitrogen uptake and mineral allocation of these species is limited.

Here, we assess the growth and morphological responses of four tropical aquatic macrophytes to different forms of inorganic nitrogen. We also evaluate nitrogen uptake and nutrient allocation in the plant tissue when the plants are supplied with NH_4^+, NO_3^-, and combined NH_4NO_3 treatment.

2. Materials and methods

2.1. Plant materials and growth conditions

Plants with similar weights and/or height (depending on the species), *S. cucullata* Roxb. ex Bory, *I. aquatica* Forssk., *C. involucratus* Rottb. and *V. zizanioides* (L.) Nash ex Small, were selected and cultivated in hydroponic culture in the greenhouse at the Department of Biology, Faculty of Science, Chiang Mai University, Thailand at a temperature range of 32–35 °C during the day and 22–25 °C at night. The light regime was approximately 80% of full sun and the light:dark cycle was 14:10 h. The growth medium was a half-strength standard nitrogen-free nutrient solution (Smart and Barko, 1985) to which a commercial micronutrient solution for aquarium plants (Tropica Master Grow, Tropica Aquacare, Aarhus, Denmark) was added. The composition of the solution was (μM): K^+ 21, Mg^{2+} 16.7, S^{2-} 32.8, B^{3+} 0.4, Cu^{2+} 0.1, Fe^{2+} 1.3, Mn^{2+} 0.8, Mo 0.02 and Zn^{2+} 0.03. Phosphorus was added as KH_2PO_4 (100 μM). The pH of the growth medium was adjusted to 7.0.

Nitrogen was supplied as NH_4^+ and/or NO_3^- in equimolar concentrations (500 μM) to create the following three treatments: (i) 500 μM NH_4^+ (ii) 500 μM NO_3^-, and (iii) 250 μM NH_4NO_3. Ten replicates of *I. aquatica*, *C. involucratus* and *V. zizanioides* and five replicates of *S. cucullata* were used for each treatment. The initial fresh weights (FW) of all experimental plants were measured and the fresh and dry weights (DW) of ten uniform sized plants were measured to estimate DW to FW ratio. Clonal fragments from *S. cucullata* stock cultures (approximately 2 g FW) and *I. aquatica* (approximately 10 g FW and 30 cm tall) were placed in 5 L containers. *C. involucratus* (approximately 7 g FW and 15 cm tall) and *V. zizanioides* (approximately 6 g FW and 20 cm tall) were placed in 1 L containers. All treatments were arranged in a randomized complete block design in the greenhouse. The growth medium was replaced every two days to avoid depletion of nutrients.

2.2. Growth study

All plants were harvested, cleaned, and their morphological characteristics recorded after four weeks for *I. aquatica* and *S. cucullata* and six weeks for *C. involucratus* and *V. zizanioides* when plants were fully acclimated to the growth conditions. The individual plants were separated into shoots and roots, freeze dried and weighed. The relative growth rate (d^{-1}) for each treatment was calculated using the formula: $RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$, where W_1 and W_2 are the initial and final DW, and t_1 and t_2 are initial and final time (days).

2.3. N uptake rate

After the growth experiment, the NO_3^- and NH_4^+ uptake rates of the acclimated plants were determined. Plants of uniform size ($n = 4$) from each treatment were pre-incubated for 18 h in a container with a N-free growth medium under the same conditions as the growth study. After pre-incubation, *I. aquatica*, *C. involucratus*, and *V. zizanioides* were placed in 240 mL vessels with 500 μM NO_3^- or NH_4^+, *S. cucullata* was placed in a 300 mL beakers. NO_3^- and NH_4^+ uptake was estimated based on N depletion (Konnerup and Brix, 2010). The NH_4^+ concentration in all samples was analysed using a modified salicylate method (Quikchem Method no. 10-107-06-3-B; Lachat Instruments, Milwaukee, WI, USA). The NO_3^- concentration was analysed from the absorbance at 202 nm and 250 nm (Cedergreen and Madsen, 2003). After the uptake experiment (6 h), all plants were separated into shoots and roots, freeze dried and weighed. The N uptake rate was calculated from the depletion curves with linear regression analyses and related to root DW.

2.4. Chlorophyll content

The contents of Chl *a*, Chl *b* and total Chl *a* + *b* in the leaves of each plants were analysed according to Lichtenthaler (1987). Freeze dried leaves from each plant were cut into small pieces and weighed into 5–10 mg samples. Pigments were extracted with 8 mL of 96% ethanol in the dark at room temperature. After 24 h, the absorbance of the extracts was measured at 648.6 nm and 664.2 nm using a UV–vis spectrophotometer (Lambda 25 version 2.85.04, USA).

2.5. Mineral elements

The concentration of total N, phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) in the plant tissue was analysed in subsamples (150–180 mg) of finely ground freeze dried plant material. The samples were digested by mixing with 7 mL of an acid solution (concentrate H_2SO_4 1 L, K_2SO_4 100 g and selenium 1 g) at a temperature range of 100–330 °C. Total N was analysed by the Kjeldahl method (Hanlon et al., 1994) and the concentrations of P, K, Ca and Mg were analysed according to Chapman and Pratt (1978).

2.6. Statistics

All statistics were carried out using Statgraphics Plus ver. 4.1 (Manugistics, Inc., MD, USA). Data were tested for normal distribution and homogeneity of variance using Cochran's C-test. If necessary, data were log-transformed to ensure homogeneity of variance. The data was tested by both one-way and two-way analysis of variance (ANOVA). Differences between treatments were identified by the Tukey HSD's test at a 5% significance level.

3. Results

3.1. Growth study

The relative growth rate (RGR) of plants varied between species and was affected by N-source (Fig. 1a, Table 1). *S. cucullata* had a relative growth rate ($0.12 \pm 0.003\ d^{-1}$) that was substantially higher than the three other species. Increase in RGR was found in both *C.*