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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.*

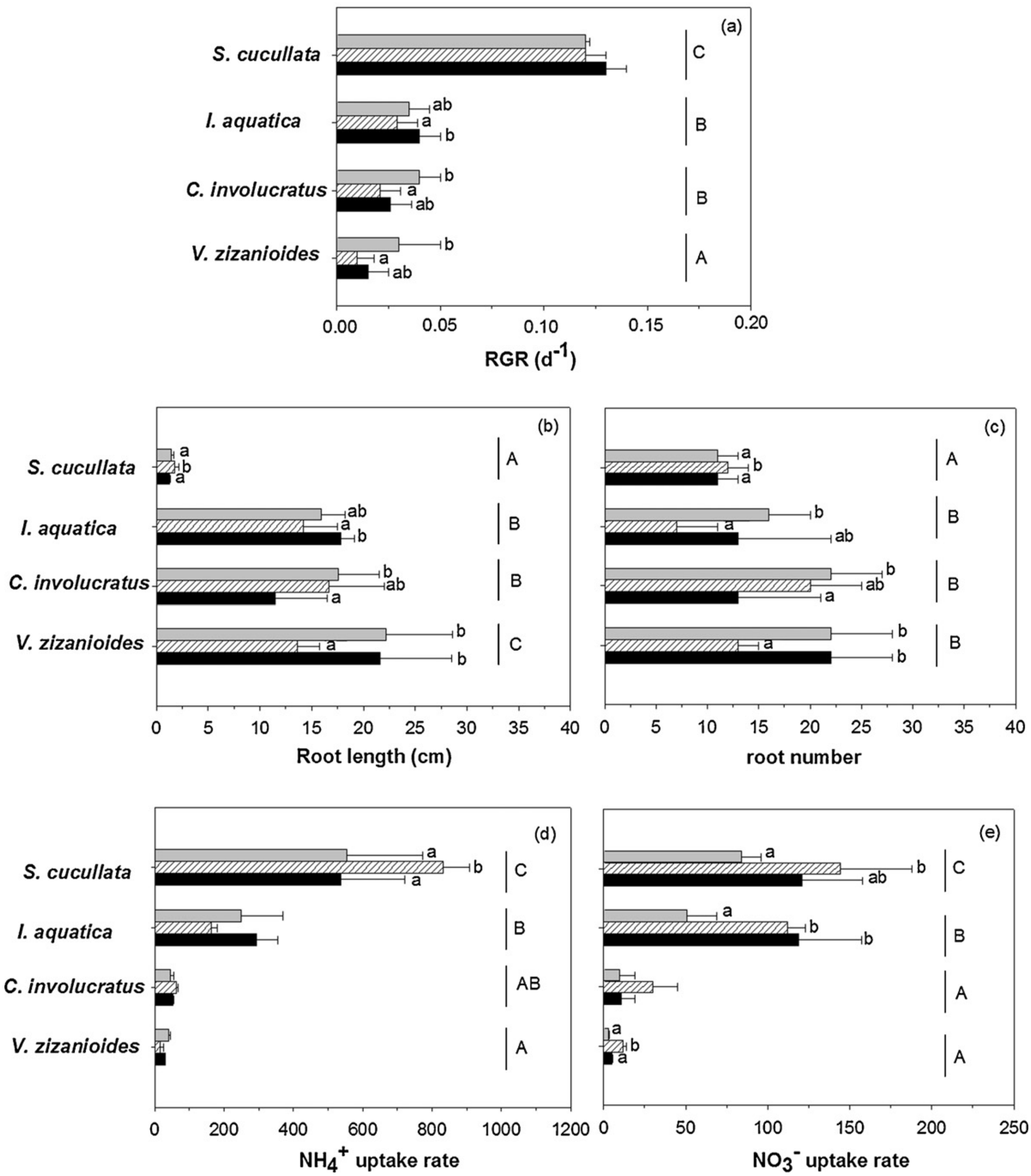

Fig. 1. (a): Relative growth rate, (b): root length, (c): number of roots, (d): NH_4^+ uptake rate (μmol NH_4^+ g^{-1} root DW h^{-1}) and (e): NO_3^- uptake rate (μmol NO_3^- g^{-1} root DW h^{-1}) (mean + SE) of *S. cucullata*, *Ipomoea aquatica*, *Cyperus involucratus* and *Vetiveria zizanioides* supplied with different N sources (▒ NH_4^+, ▨ NO_3^-, ■ NH_4NO_3). Different letters above the columns within each species indicate significant differences between the treatments and different capital letters indicate significant differences between the plant species.

involucratus and *V. zizanioides* when supplied with NH_4^+ (Fig. 1a). There were 43% and 82% increases in the RGRs of NH_4^+ fed plants compared to NO_3^- fed plants in *C. involucratus* and *V. zizanioides*, respectively.

3.2. Morphological characteristics

Both root length and root number was affected by plant species and N source and a significant interaction was observed (Table 1, Fig. 1b and c). The longest roots were found in *V. zizanioides*, followed by *C. involucratus*, *I. aquatica* and *S. cucullata*, (Fig. 1b). New shoot production was different among plant species and was independent of N source. *I. aquatica* and *C. involucratus* produced new shoots faster than *V. zizanioides*.

3.3. Chlorophyll content

Plant species and N source interactively affected Chl *a*, Chl *b* and total Chl *a* + *b* (Table 1). Average chlorophyll content was highest in *I. aquatica*, followed by *S. cucullata*, *C. involucratus* and *V. zizanioides* (Table 2). The chlorophyll contents of NH_4^+ fed plants were consistently higher than that of NO_3^- fed plants, except for *I. aquatica* where the chlorophyll contents were highest in the NH_4NO_3 treatment.

Table 1
Results of a two-way ANOVA (F-ratios) of growth, morphological characteristics, chlorophyll concentrations, N-uptake rate and mineral concentrations of *Salvinia cucullata*, *Ipomoea aquatica*, *Cyperus involucratus* and *Vetiveria zizanioides* grown with different N-sources (500 μM NH_4^+, 500 μM NO_3^- and 250 μM NH_4NO_3).

	Main effect		Interaction
	Plant species	N-source	Plant species × N-source
RGR (d^{-1})	311.8***	0.85	1.42
Root number	14.4***	3.22*	2.42*
Root length (cm)	68.0***	2.5	3.25**
New shoots	80.6***	0.5	1.45
NH_4^+ uptake rate (μmol NH_4^+ g^{-1} root DW h^{-1})	114.0***	2.89*	3.17*
NO_3^- uptake rate (μmol NO_3^- g^{-1} root DW h^{-1})	55.8***	8.92***	2.43*
Chl *a* (mg g^{-1} DW)	27.3***	3.10	5.61*
Chl *b* (mg g^{-1} DW)	47.2***	3.86*	4.04 *
Total Chl *a* + *b* (mg g^{-1} DW)	34.4***	3.43*	5.09***
Chl *a*/Chl *b* ratio	2.53	0.74	5.30***
Total N (mg g^{-1} DW)			
leaves	30.2***	0.51	0.62
roots	49.3***	1.81	0.36
P (mg g^{-1} DW)			
leaves	207.8***	1.05	2.66*
roots	33.8***	1.42	1.61
K (mg g^{-1} DW)			
leaves	22.4***	0.38	0.83
roots	173.8***	6.63**	4.70**
Ca (mg g^{-1} DW)			
leaves	78.4***	1.09	4.05**
roots	38.0***	0.35	1.64
Mg (mg g^{-1} DW)			
leaves	117.8***	2.80*	11.57***
roots	16.1***	1.63	0.49

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

3.4. N-uptake

Nitrogen uptake differed among plant species, but all species had higher uptake rates for NH_4^+ than for NO_3^- (Fig. 1d and e). *S. cucullata* had significantly higher uptake rates for both NH_4^+ and NO_3^- than the other species. NO_3^- uptake was affected by both plant species and N-source and a significant interaction was observed in the ANOVA (Table 1). For all species, NO_3^- uptake rate was high in plants supplied with NO_3^- either alone or in combination with NH_4^+.

3.5. Nutrient and mineral elements

The concentration of most nutrient and mineral elements (N, P, Ca, K, Mg) in both leaves and roots differed among the four species (Fig. 2). The concentration of N and P in leaves and K in roots was significantly affected by plant species and N-source and a significant interaction was observed in the ANOVA (Table 1). The lowest nutrient and mineral contents in leaves and roots were generally found in *V. zizanioides*. The N concentration was consistently higher in leaves than in roots. In *S. cucullata*, the N concentration in leaves was significantly higher in the NH_4^+ and NH_4NO_3 fed plants than in the NO_3^- fed plants. The P concentration in the leaves of *S. cucullata* was significantly lower in the NO_3^- fed plants than in the NH_4^+ fed plants. In the roots of *C. involucratus*, the P concentration was significantly higher in the NO_3^- fed plants than the other treatments. The other mineral cations were only slightly affected by N sources in all four species. In NO_3^- fed *C. involucratus*, Mg concentration in leaves was high, and in leaves of NO_3^- fed *V. zizanioides* Ca concentration was high. NH_4^+ fed plants of *I. aquatica* had significantly higher concentrations of Ca in leaves than plants fed with either NO_3^- or NH_4NO_3.

Table 2
Chlorophyll contents of *Salvinia cucullata*, *Ipomoea aquatica*, *Cyperus involucratus* and *Vetiveria zizanioides* (mean ± SE) grown with different N-sources (500 μM NH_4^+, 500 μM NO_3^- and 250 μM NH_4NO_3). The letter superscripts between columns indicate significant differences between treatments.

	Salvinia cucullata			*Ipomoea aquatica*			*Cyperus involucratus*			*Vetiveria zizanioides*			F-ratio
	NH_4^+	NO_3^-	NH_4NO_3	NH_4^+	NO_3^-	NH_4NO_3	NH_4^+	NO_3^-	NH_4NO_3	NH_4^+	NO_3^-	NH_4NO_3	
1. Chl a (mg g^{-1} DW)	9.0 ± 0.5^{e}	6.4 ± 0.5^{bcde}	6.9 ± 1.9^{cde}	7.1 ± 1.1^{de}	8.1 ± 2.5^{de}	12.7 ± 2.4^{f}	7.3 ± 2.2^{e}	5.5 ± 1.5^{abcd}	4.3 ± 2.1^{abc}	4.1 ± 0.8^{ab}	2.5 ± 1.4^{a}	3.6 ± 1.2^{a}	9.08***
2. Chl b (mg g^{-1} DW)	4.7 ± 5.2^{c}	4.4 ± 0.5^{c}	3.9 ± 1.0^{bc}	4.9 ± 0.6^{c}	4.6 ± 1.3^{c}	6.7 ± 1.0^{d}	3.9 ± 1.1^{bc}	2.6 ± 0.6^{ab}	2.5 ± 0.6^{a}	2.4 ± 0.3^{a}	1.2 ± 0.7^{a}	1.9 ± 0.4^{a}	12.1***
3. Total Chl (mg g^{-1} DW)	14.2 ± 0.6^{c}	10.8 ± 1.0^{bc}	10.8 ± 3.0^{bc}	12.0 ± 1.7^{c}	12.7 ± 3.8^{c}	19.5 ± 3.3^{d}	12.4 ± 2.1^{c}	8.2 ± 2.1^{ab}	6.8 ± 2.7^{a}	6.5 ± 1.1^{a}	4.8 ± 2.0^{a}	5.5 ± 1.6^{a}	10.5***
4. Chl a/b ratio	2.0 ± 0.1^{abc}	1.5 ± 0.1^{a}	1.8 ± 0.2^{ab}	1.5 ± 0.1^{a}	1.7 ± 0.1^{ab}	1.9 ± 0.2^{abc}	2.3 ± 0.7^{c}	2.1 ± 0.1^{bc}	1.6 ± 0.4^{ab}	1.7 ± 0.2^{ab}	1.8 ± 0.7^{abc}	1.8 ± 0.3^{abc}	3.7***

***, $P < 0.001$.

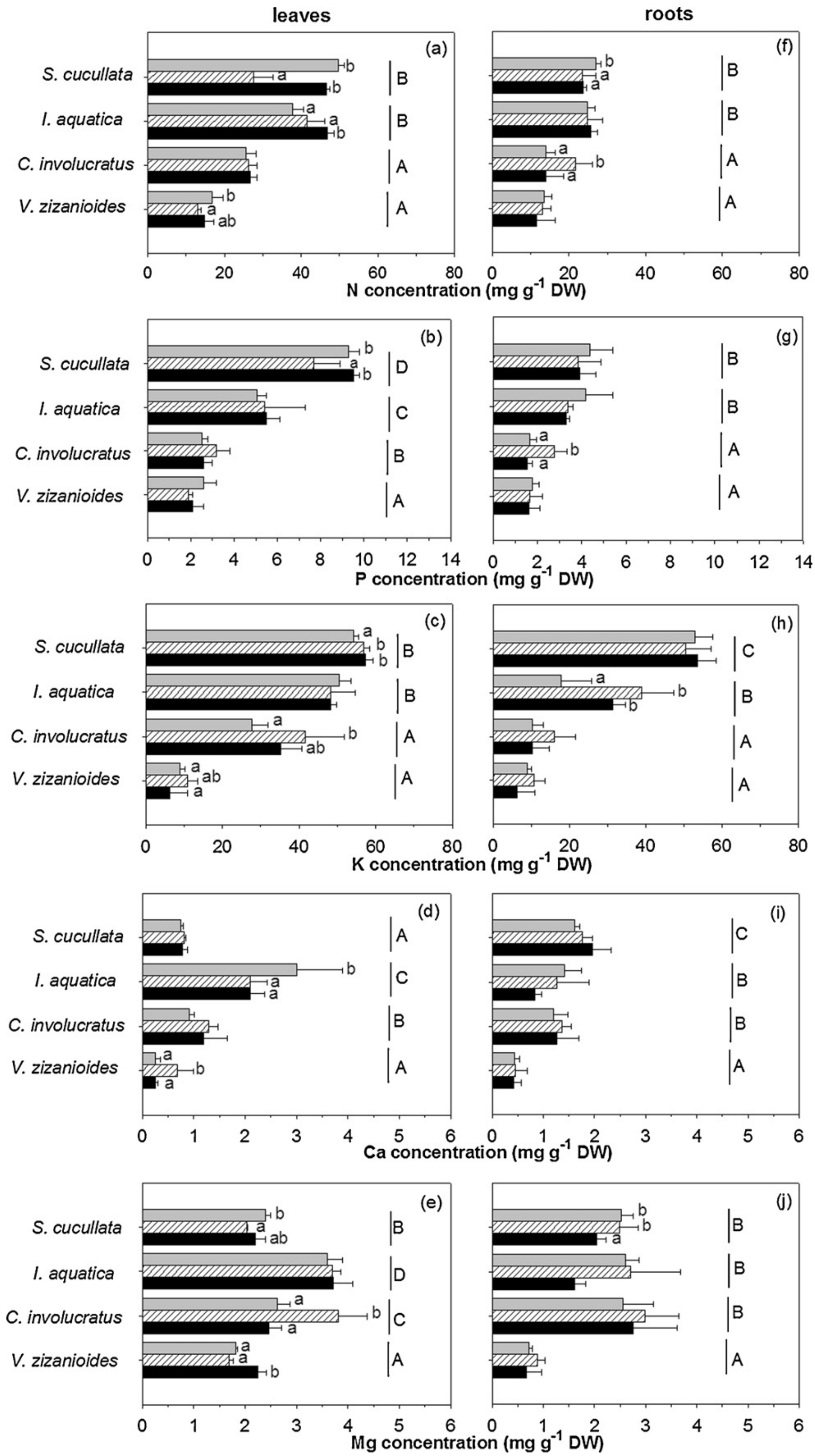

Fig. 2. Concentrations (mean + SE) of total N (a,f), P (b,g), K (c,h), Ca (d,i) and Mg (e,j) in leaves (left panel) and roots (right panel) of *Salvinia cucullata*, *Ipomoea aquatica*, *Cyperus involucratus* and *Vetiveria zizanioides* supplied with different N-sources (□ NH_4^+, ▨ NO_3^-, ■ NH_4NO_3). Different letters above the columns within each species indicate significant differences between the treatments and different capital letters indicate significant differences between the plant species.

4. Discussion

The relative growth rates (RGR) of the free-floating plants were higher than the RGRs of the emergent plants. Free-floating plants obtain nutrients from the water column through root uptake and through the underside of their leaves (Cedergreen and Madsen, 2002; Fang et al., 2007). In the present study most mature leaves of *S. cucullata* were in poor contact with the water as the leaves were raised up above the water surface. Hence, nutrient uptake via leaves was probably insignificant compared to root absorption. Generally, *Salvinia* species grow rapidly and form dense mats with doubling times of two to four days over still waters (McFarland et al., 2004). *S. cucullata* had RGRs ranging from 0.12 to 0.13 d^{-1} in the present study, and the growth rate was unaffected by the N source. This is in contrast to previous studies on *S. natans* and *S. molesta* which had a higher growth rate when grown on NH_4^+ than on NO_3^- (Jampeetong and Brix, 2009a; McFarland et al., 2004). Many species of aquatic macrophytes prefers NH_4^+ over NO_3^- as a N-source, possibly because of the lower energy needed for its uptake and assimilation, and because of the prevalence of NH_4–N in water-saturated anoxic soils (Miller and Cramer, 2005; Fang et al., 2007; Jampeetong and Brix, 2009a; Konnerup and Brix, 2010). The study of Sorrell and Orr (1993) showed that *C. involucratus* had a high net H^+ extrusion and a high N-uptake rate when NH_4^+ was the nitrogen source. Others have reported similar findings for other wetland plants including *Carex rostrata* Stokes, *Typha latifolia* L. and *Phragmites australis* Cav (Trin) ex Steudel (Conlin and Crowder, 1989). In the present study, particularly *C. involucratus* and *V. zizanioides* grew better on NH_4^+ than on NO_3^-, as has also been found for *Glyceria maxima* (Hartm.) Holmb (Tylova-Munzarova et al., 2005). *V. zizanioides* had a deep root system with dense lateral roots, but a low growth rate and low N-uptake rate compared to the other species in this study. Thus, *V. zizanioides* seems to less suitable for removal of N in wastewater treatment systems.

The free-floating plants had higher N-uptake rates than the emergent plants. The N-uptake rate seems to be correlated to the growth rate and hence the N demand. *S. cucullata* had high growth rates and correspondingly high uptake rate for both NH_4^+ and NO_3^-. The average NH_4^+ uptake rate was 640 ± 161 μmol g^{-1} root DW h^{-1} and was similar to published values of 679 ± 97 μmol g^{-1} root DW h^{-1} for *Salvinia natans* (Jampeetong and Brix, 2009a). The N uptake rates of the emergent plants were lower, but the plants had either more roots or greater root length than the free-floating plants. New roots and root laterals are the principal site of nutrient uptake because old roots may develop thick epidermal and hypodermal lignification and suberization that impede nutrient uptake (Sorrell et al., 1993). Hence, a large fraction of the root system of the emergent plants may not have been involved in the N uptake, and uptake rates in the new active roots may have been at a similar level as the uptake by *S. cucullata* roots which are not much lignified (Jampeetong and Brix, 2009c).

As also reported in other studies, the studied species significantly increased NO_3^- uptake rate when external NO_3^- was present (Crawford et al., 1986; Gojon et al., 1994; Cedergreen and Madsen, 2002; Jampeetong and Brix, 2009a; Konnerup and Brix, 2010). Nitrate transporters in the root plasma membranes and nitrate reductase activity (NRA) are generally induced by external NO_3^- availability. Most free floating species have high NRA in roots (Cedergreen and Madsen, 2003), whereas rooted emergent species usually have higher NRA in leaves than in the roots (Munzarova et al., 2006). Nitrate reduction and assimilation in leaves seem to be an advantage as the ATP needed for reduction can be coupled to the photophosphorylation in the chloroplasts. Konnerup and Brix (2010) found that the NO_3^- uptake by *Canna indica* L., which had high NRA in the leaves, was not affected by the type of N-source. This corresponds to the observations in the present study.

Nitrogen nutrition have been reported to affect the concentrations of cations in the plant tissues with lower concentrations in NH_4^+ fed plants than NO_3^- fed plants (Tylova-Munzarova et al., 2005; Jampeetong and Brix, 2009a; Dan and Brix, 2009). In the present study, the N-source only slightly affected mineral concentration in the plant tissues of the studied plant species. Other studies have reported similar findings for *C. indica* and *Sesbania sesban* (L.) Merr. which only responded with small changes in tissue mineral concentration when supplied with different forms of N (Dan and Brix, 2009; Konnerup and Brix, 2010).

We conclude, that the free-floating macophytes *S. cucullata* and *I. aquatica* are good candidate species for use in constructed wetland systems to remove N from domestic or agricultural wastewater because of their high growth rates and high N uptake capacity. Furthermore, their high mineral and protein contents as well as the ease of harvesting makes these species promising for use as feed crops for animals and/or soil fertilizers (Leterme et al., 2009; Somkol, 2009). 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.

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