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Short communication

Ammonium uptake and excretion in *Azolla-Anabaena* symbiosis

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Summary. The processes of NH_4^+ uptake and induced NH_4^+ excretion in the *Azolla-Anabaena* symbiosis were studied. Uptake rates in accessions of *Azolla microphylla* and *A. mexicana* were similar. No pH optimum for NH_4^+ uptake was observed. Rates of N excretion induced by methionine sulfoximine were also similar. When *A. caroliniana* was subjected to the herbicide Ignite (Hoechst-Roussel), more NH_4^+ was initially released than with methionine sulfoximine treatment. Glutamine synthetase was not completely suppressed.

Key words: *Azolla* – *Anabaena-azollae* – Ammonium uptake – Ammonium excretion – Glutamine synthetase

Azolla Lam.-*Anabaena azollae* Strasb. is the only non-leguminous symbiosis known to have valid potential as an N biofertilizer in flooded rice soils. This fern-cyanobacterial association has been used for centuries to increase yields in Vietnam and China (Liu 1979; Tuan and Thuyet 1979).

The N metabolism of this N_2-fixing symbiosis is scientifically interesting, particularly NH_4^+-N metabolism, because this is the key factor in host-symbiont interactions. N assimilation in the *Azolla-Anabaena* symbiosis has been studied in detail (Ray et al. 1978; Newton and Selke 1981; Meeks et al. 1987).

In the present study we examined rates of NH_4^+ uptake and the potential for induced NH_4^+ excretion in intact *Azolla* associations. Accessions of three New World *Azolla* spp. with particular agronomic value were evaluated.

Materials and methods

Five *Azolla* accessions were selected for this project, *A. microphylla* (MI 4074) from Paraguay, *A. mexicana* from Colombia (ME 2015) and California, USA (ME-UCD 62), and *A. caroliniana* from Brazil (CA 3015 and 3017). These accessions were cultivated in the laboratory and the greenhouse. Artificial growth and experimental conditions included a photon flux density of 150 μmol m^{-2} s^{-1} from cool-white and gro-lux lamps, and a temperature of 25 °C. An N-free growth medium was used (Watanabe et al. 1977).

Two grams fresh weight of *Azolla* were used in most instances, covering a surface area of 50 cm^2. Fifty milliliters of N-free medium buffered with 20 m*M* sodium phosphate or carbonate were used in the NH_4^+ uptake trials. Experiments involving the effects of pH were repeated using distilled water adjusted with HCl or NaOH. Control samples (pH 9.0) without *Azolla* were monitored for NH_3 volatilization. NH_4Cl was added to the medium at 100 or 200 μ*M* before the onset of 5-h uptake trials. The NH_3 content in the medium or water was measured by the phenol hypochlorite method of Solorzano (1969).

NH_4^+ excretion was induced by inhibiting the glutamine synthetase of the *Azolla-Anabaena* symbiosis. The growth medium was amended with 1 m*M* L-methionine-D,L-sulfoximine or fronds were pre-incubated with a 1% spray of the herbicide, monoammonium 2-amino-4-(hydroxymethylphosphinyl) butanoate (trade names: Ignite, Basta; Hoechst-Roussel Agri-Vet Co., Somerville, New Jersey, USA).

The inhibition of glutamine synthetase was assayed in the *Azolla* accessions ME UCD-62 and CA 3015 after exposure to methionine sulfoximine or the herbicide Ignite. The *Azolla* biomass was ground in an extraction buffer containing 50 m*M* TRIS-HCL, 0.1% (v : v) 2-mercaptoethanol, and 1% (w : v) polyvinylpyrrolidone (average molecular weight of 40000 daltons). Plant debris and intact endosymbionts were removed by centrifugation. Enzyme activity was assayed as transferase and biosynthetase (Shapiro and Stadtman 1970).

Results and discussion

NH_4^+ uptake

Studies on NH_4^+ uptake in *Azolla* spp. have mainly centered on growth response, N content, or effects on N_2 fixation (Ito and Watanabe 1983; Okoronkwo and Van Hove 1987; Okoronkwo et al. 1989). In one study, small amounts of NH_4^+ were released by certain *Azolla* strains during active growth, when temperatures were elevated (Watanabe and Berja 1983). There has also been one report citing a significant inhibition of glutamine synthetase (up to 25%) in *Azolla* with a 200 ppm application of the rice herbicide Butachlor (Venkataramanan and Kannaiyan 1984).

The three accessions tested in the present study for NH_4^+ uptake removed NH_4^+ from the growth medium at similar rates. The accessions MI 4074, ME 2015, and ME-UCD 62 had uptake velocities of 17.0±2.2, 18.3±2.6, and 18.4±4.3 μmol NH_4^+ g^{-1} (dry weight) h^{-1}, respectively,

with 100 μ*M* initial NH_4Cl. The velocities increased proportionately to 22.3±3.9, 28.4±2.1, and 27.1±3.3 μmol NH_4^+ g^{-1} (dry weight) h^{-1} with 200 μ*M* initial NH_4Cl (average of four experiments, ±SD).

No pH optimum was defined for NH_4^+ uptake in the intact *Azolla* symbiosis. For example, the *Azolla* accession ME 2016 removed NH_4^+ from the growth medium at rates of 13.1±2.6 (pH 4.0), 13.7±4.9 (pH 5.0), 16.1±8.2 (pH 6.0), 20.3±7.8 (pH 7.0), 28.3±11.7 (pH 8.0), and 31.8±6.1 (pH 9.0) μmol g^{-1} h^{-1} (average of seven experiments). No NH_3 volatilization was detected in the controls (pH 9.0). These results agree with findings that there was no pH optimum for NH_4^+ uptake by a cyanobiont cultured from *Azolla* (Zimmerman and Boussiba 1987).

NH_4^+ excretion

Both *Azolla* spp. and *Anabaena azollae* assimilate NH_4^+ almost exclusively through the glutamine synthetase-glutamate synthase pathway (Meeks et al. 1987; Lee et al. 1988). Approximately 40% of the N_2 fixed by the cyanobiont, chiefly NH_3, is released for consumption by the host (Meeks et al. 1985, 1987). Chemical mutagenesis or repression of glutamine synthetase with methionine sulfoximine can also create "leaky" cyanobacteria isolated from *Azolla* (Zimmerman and Boussiba 1987; Zimmerman 1987, unpublished).

The NH_4^+ excretion rates induced by methionine sulfoximine were linear and similar for MI 4074, ME 2015, and ME UCD-62 over a 24-h period (Table 1). The rate for CA 3015 was somewhat less; unlike the others, this accession was cultivated in a greenhouse, and its mature fronds were less effectively permeated by methionine sulfoximine in the medium. The herbicide spray led to a greater initial release of NH_4^+ than did methionine sulfoximine. The *Lemna* sp., a non-diazotrophic aquatic angiosperm, excreted NH_4^+ more slowly than CA 3015 when treated with the herbicide, and appeared to re-assimilate part of the released N before the end of the 24 h. However, autolysis of all plants, whether *Azolla* or *Lemna* spp., was inevitable.

A linear excretion rate was measured over two successive days in the *Azolla* accession CA 3017 ($r = 0.999$ for day 1 and 0.963 for day 2). The rate slowed in the 2nd 24 h, with <400 μ*M* NH_4^+ accumulating in the medium compared to >500 μ*M* for the 1st day ($n = 8$). The excretion was accompanied by some general biomass degradation and fragility.

Glutamine synthetase activity

The greatest decrease in enzyme activity after exposure of the plants to methionine sulfoximine was observed during 0–2 h for ME-UCD 62 and 0–3 h for CA 3015, or roughly the time estimated for NH_4^+ excretion to begin for each accession. There was no evidence of active N excretion in separate control experiments without exposure to methionine sulfoximine.

After 7 h of exposure to methionine sulfoximine, glutamine synthetase activity had decreased from 100% (no inhibition) to 71.0% for transferase and 71.1% for biosynthetase with the *Azolla* accession CA 3015 ($n = 2$). Rates for ME-UCD 62 dropped to 66.2% and 64.4% after 9.75 h for transferase and biosynthetase, respectively. In CA 3015, glutamine synthetase was repressed to a similar extent after treatment with either methionine sulfoximine or the herbicide. Glutamine synthetase transferase was not totally inhibited even after 22 h of treatment. The initial rates for *Azolla* ranged from 0.83 to 4 μmol γ-glutamylhydroxamate formed g^{-1} (fresh weight) h^{-1}.

These results are in agreement with earlier work with *Azolla* spp., showing that methionine sulfoximine inhibited 70% of the synthesis of labelled products of N_2 fixation in 90 min, but the maximum of 97% inhibition required 7 h of incubation (Meeks et al. 1987).

Conclusions

Despite its rapid assimilation, NH_4^+ cannot maintain normal growth of *Azolla* spp. (Newton and Selke 1981). Further, the N released may not be newly fixed N_2 but decomposition products from the host. An *Azolla* sp. mutant that leaks NH_3 is an interesting laboratory speci-

Table 1. NH_4^+ excretion rates (μmol NH_4^+ g^{-1} dry weight) over time in four isolates of *Azolla* and one of *Lemna*

Time (h)	1 m*M* Methionine sulfoximine				1% (v:v) Ignite	
	MI 4074 (Paraguay)	ME 2015 (Colombia)	ME-UCD 62 (USA-CA)	CA 3015[a] (Brazil)	CA 3015[a] (Brazil)	*Lemna* (USA)
0	0	0	0	0	0	0
2	12.3	13.4	2.7	1.1	34.1	19.7
4	47.4	46.0	36.0	4.3	72.2	41.4
6	114.7	99.9	84.2	13.6	118.4	70.5
8	203.2	173.9	153.9	53.7	143.8	80.6
10	212.6	–	189.2	–	–	108.9
24	984.1	983.7	709.3	484.0	422.9	78.3
n	8	5	7	6	5	6
r	0.978	0.984	0.986	0.969	0.999	0.994[b]

[a] Greenhouse-cultivated accession (others were grown under artificial lighting)
[b] Excluding the data from the 24-h time point

men but is of no use in the field. This type of mutant lacks viability, fitness, and the ability to grow well in the presence of the excreted NH_3. The effects of constant high rates of NH_3 excretion by *Azolla* spp. on heterotrophic microbial populations, or the possibility of obtaining chemically unfavorable field conditions, are also unknown.

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