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A Study on Nitrogen Metabolism of Lemnaceae: Assimilation of Nitrate and Ammonia in *Spirodela polyrhiza* and *Lemna aequinoctialis*

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개구리밥科 植物의 窒素代謝에 관한 硏究: 개구리밥(*Spirodela polyrhiza*)과 좀개구리밥(*Lemna aequinoctialis*)의 NO_3^-와 NH_4^+의 同化作用

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ABSTRACT

Spirodela polyrhiza and *Lemna aequinoctialis* often occurred at the sites of high ammonium concentration and at the sites of high nitrate concentration, respectively. We investigated the different distribution between two species in relation to the types of nitrogen sources and their concentrations. Our experiments showed that *L. aequinoctialis* grew faster than *S. polyrhiza* in nitrate media with lower than 15 mM concentration. The nitrate uptake was also faster in *L. aequinoctialis* than in *S. polyrhiza*. However, neither differences in growth nor in uptake patterns between these two species were observed in ammonium media. Glutamine synthetase (GS), glutamate dehydrogenase (GDH) and glutamate synthetase (GOGAT) activities were higher in *L. aequinoctialis*. In particular, nitrate reductase activity (NRA) in *L. aequinoctialis* was 12.1 times as high as that in *S. polyrhiza*. These results showed that the two species responded varyingly to the types of nitrogen sources and their concentrations. Therefore, the difference in geographic distribution between the two species appeared to reflect the interspecific differences in enzyme activities and, subsequently, nitrogen absorption abilities.

INTRODUCTION

The family Lemnaceae is a morphologically and ecologically distinct group from other flowering plants. For example, many species of the Lemnaceae are extremely reduced in the size, belonging to the tiniest flowering plants. They float at or just below the surface of water. It comprises 35 species of the 4 genera, being distributed world-widely (Hillman, 1976). Among the species of the duckweed group, two species, *Spirodela polyrhiza* and *Lemna aequinoctialis*, are reported in Korea (Chung, 1965).

It has been reported that some species of the family Lemnaceae occur only in quite unique habitats. For example, species of the genus *Lemna* live in the water in which NO_3^- is abundant (Landolt and Wildi, 1977; Chang *et al.*, 1990). Certain species of the genus *Spirodela* are present in the water where the organic matters were not fully decomposed. These studies suggest that duck-

weeks may take up certain types of nitrogen sources from the water depending upon the species. Although the two Korean species often do not occur at the same habitats (Chang *et al.*, 1990), we do not know about the dynamics of nitrogen uptake in these two Korean species.

Duckweeds are an ideal material for physiological research. This is because their predominant vegetative reproduction results in genetically uniform clones which can be easily grown in aseptic cultures and be subject to experiments. Duckweeds also possess a potential for waste water treatment, since they can convert the degradable pollutants directly into useful materials (Oron *et al.*, 1986). In this experiment, we investigate the relationship between both the types of nitrogen sources (NO_3^- and NH_4^+) and their concentration and the metabolic responses of *S. polyrhiza* and *L. aequinoctialis*.

MATERIALS AND METHODS

Plant materials and growth conditions. *Spirodela polyrhiza* and *Lemna aequinoctialis* were collected from paddy water in Seongnam city near Seoul. After the plants were carried to the laboratory, they were treated with 0.5% sodium hypochloride for 30 sec (Hillman, 1961). The plants were cultivated in 100 m*l* of 1/2 strength Hutner's medium adjusted to the pH 6.3 (Hutner, 1953) under continuous illumination of white fluorescent tube and light bulbs at 26℃. The light intensity was 3,000 lux. The plants were transferred to the new sterilized medium every three days. The healthy fronds were selected for experiments.

The growth of *S. polyrhiza* and *L. aequinoctialis* at varying concentrations of NO_3^- and NH_4^+. Four fronds of *S. polyrhiza* and 8 fronds of *L. aequinoctialis* were innoculated respectively into the flasks containing 50 m*l* of modified Hunter's media with 5 different concentrations of NO_3^- and NH_4^+. NO_3^- gradient media were prepared as follows: N sources in Hutner's media were excluded, and Ca^{++} and K^+ lost were supplemented as $CaCl_2$ and KCl. Appropriate amount of NO_3^- was then supplied as KNO_3. NH_4^+ gradient media were prepared like the NO_3^- media, but NH_4^+ was supplied as $(NH_4)_2SO_4$. The concentrations of nitrogen sources were adjusted to 0, 2, 4, 8 and 16 mM. On the 13th day from innoculation, the number, total chlorophyll and fresh weight of the fronds were measured.

Measurement of uptake rate. For the measurement of nitrate uptake rate by *S. polyrhiza* and *L. aequinoctialis*, about 0.3 g-0.4 g of each species were transferred to N-free medium. They were innoculated in various nitrate concentrations after 24 h. When the plants were incubated in a growth chamber for 7 h, the loss of nitrate in the media was quantified by a rapid UV method (APHA-AWWA-WPCF, 1981). For the measurement of ammonium uptake rate by *S. polyrhiza* and *L. aequinoctialis*, the same procedure as for the nitrate uptake was conducted for the plants. However, ammonium in the media was determined by phenyl hypochlorite method of Solorzano (1969) after incubation for 4 h, not for 7 h.

Determination of protein. The protein content of crude extract was determined by the method of Lowry *et al.* (1951).

Cell homogenate-preparation of crude extracts. About 0.3-0.4 g of fresh weights (gfw) of *S. polyrhiza* and *L. aequinoctialis* was harvested on the 13th day of innoculation. Fronds were collected by cheese cloths, washed twice with the distilled water, and blotted dry with filter paper. They were weighed and homogenized with the chilled homogenizer using 2-3 m*l* of extraction buffer solution. The extracts were centrifugated at 13,000 g for 15 minutes. The enzyme activity of supernatant fraction was then tested.

Enzyme assays. Nitrate reductase (NR) : Nitrate reductase activity was determined by measuriing the rate of nitrite formation according to the method of Pistorius *et al.* (1976). The enzyme activity was expressed in μmol NO_2^-/gfw/hr. To investigate the protease activity, NR was assayed in the presence of 10 μM of leupeptin, protease inhibitor, and compared with that in the absence of leupeptin.

Nitrite reductase (NiR) : Nitrite reductase activitiy was determined by the modified method of Aslam *et al.* (1985). The plants were homogenized at 0℃ with 0.1 M potassium phosphate buffer containing 0.5 mM EDTA adjusted to the final pH 8.0. The reaction mixture contained in a final volume of 2 m*l*, 10 μM potassium phosphate buffer, 2 mM $NaNO_2$, 0.4 m*l* dithionite [$Na_2S_2O_4$ (1.3058 mg) soluted in 0.29 M $NaHCO_3$], 0.75 mM methylviologen and the 0.2 m*l* of enzyme extract. Reaction was started by addition of dithionite. The enzyme activity was defined by the amount of nitrite loss per hour. Boiled enzyme extract was treated as control.

Glutamine synthetase (GS) : Glutamine synthetase was assayed by the ATP-dependent formation of the γ-glutamyl-hydroxamate from L-glutamate and hydroxylamine (Ahmad and Hellebust, 1984). The enzyme activitiy was expressed in μmol γ-glutamyl-hydroxamate/gfw/min.

NAD (P)H-dependent glutamate dephydrogenase (GDH) :

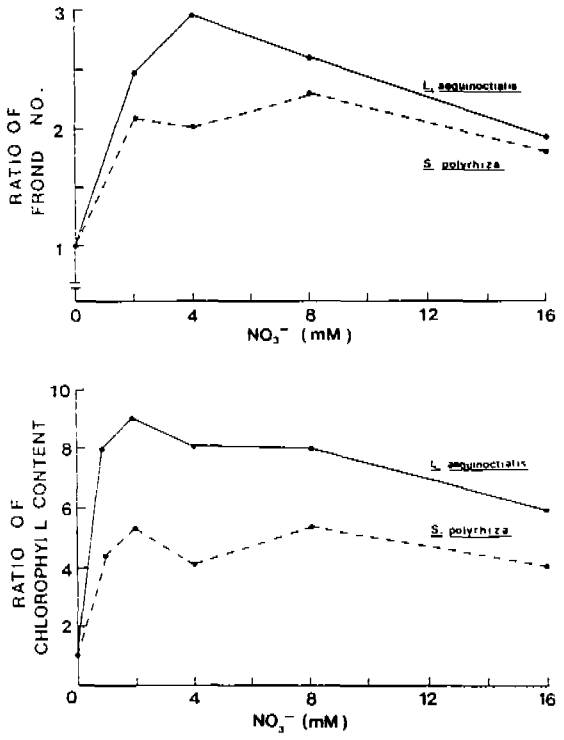

Fig. 1. The effect of concentration of NO_3^- on the growth of *S. polyrhiza* and *L. aequinoctials*. Frond number and chlorophyll content (mg) at each concentration are expressed by the ratio to those at 0 concentration.

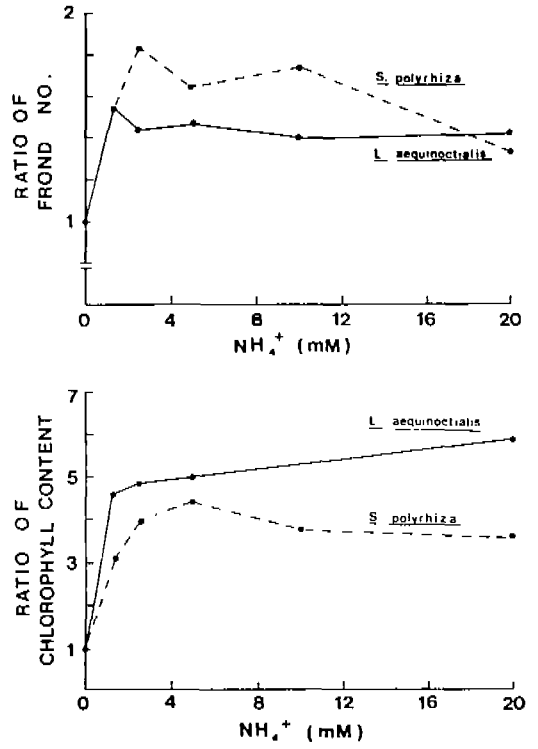

Fig. 2. The effect of NH_4^+ concentration on the growth of *S. polyrhiza* and *L. aequinoctialis*. Frond number and chlorophyll content (mg) at each concentration are expressed by the ratio to those at 0 concentration.

NAD (P)H/GDH activities were determined by Schmidt and Schmidt (1983) with modification. The assay mixture was 0.3 M ammonium sulfate, 6 mM α-ketoglutarate, 1.3 mM EDTA, 0.15 mM NAD(P)H and the enzyme extract. Reaction was started by addition of NAD(P)H. One unit of enzyme activity is defined as the amount of enzyme catalyzing 1 μmol of NAD(P)H/min at 30℃. The oxidation of NAD(P)H was measured at 340 nm using spectrophotometry.

Glutamate synthase (GOGAT) : NADH-dependent Glutamate Synthetase was measured by the absorbance decrease at 340 nm at 30℃. The reaction mixture contained 0.1 M tris buffer (pH 7.7), 10 mM L-glutamine, 10 mM α-ketoglutarate, 0.15 mM NADH and the enzyme extract.

RESULTS

The growth of *S. polyrhiza* and *L. aequinoctialis* in NO_3^- and NH_4^+ media. *L. aequinoctialis* showed a growth rate higher than *S. polyrhiza* in NO_3^- media (Figs. 1 and 2). The initial optimal concentration for growth of both species was 2-4 mM NO_3 . In NH_4^+ media, the growth rates of two species did not show a consistant tendency.

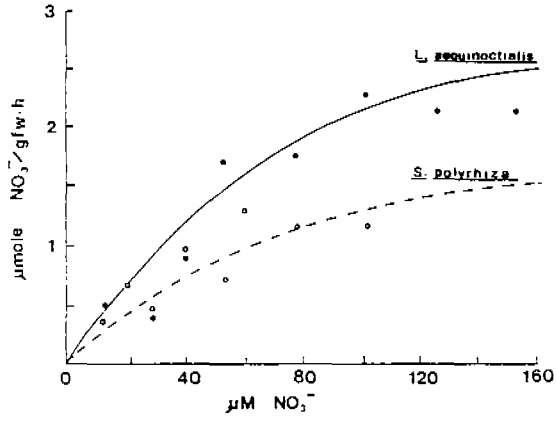

Fig. 3. Nitrate (NO_3^-) uptake in *S. polyrhiza* and *L. aequinocytialis*.

Uptake of nitrate and ammonium. *L. aequinoctialis*

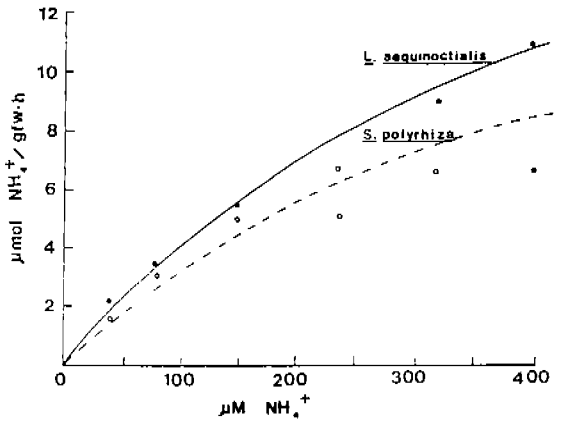

Fig. 4. Ammonium (NH_4^+) uptake in *S. polyrhiza* and *L. aequinoctialis*.

Table 1. K and V_{max} values for the uptake of NO_3^- and NH_4^+ in *S. polyrhiza* and *L. aequinoctialis*

	NO_3^-		NH_4^+	
	K (μmol)	V_{max} (μmol/gfw/h)	K (μmol)	V_{max} (μmol/gfw/h)
S. polyrhiza	31.33	1.508	208.57	10.764
L. aequinoctialis	37.32	2.688	207.71	13.624

Table 2. The activities of NR, NiR, GS, GOD/NAD(P)H and GOGAT in the extracts of *S. polyrhiza* and *L. aequinoctialis*

	In vitro enzyme activity	
	S. polyrhiza	*L. aequinoctialis*
	μmol/gfw/hour	
NR	0.166	2.005
NiR	9.000	18.000
	μmol/gfw/min	
GS	2.033	2.850
GDH/NADH	0.192	0.421
GDH/NADPG	0.070	0.088
GOGAT	0.052	0.105

Samples were harvested at 10 a.m. each day.

took up nitrate faster than *S. polyrhiza*. *S. polyrhiza* showed a maximum uptake rate at 90 μM, but *L. aequinoctialis* showed the maximum uptake rate at 160 μM (Fig. 3). In the experiment of ammonium uptake, the uptake rates of both species were quite similar (Fig. 4). The 1/[S] values of NO_3^- and NH_4^+ uptake were close in both species in any media (Table 1). These results indicate that the affinity for the two substrates was similar in these species. However, the V_{max} of NO_3^- uptake was higher in *L. aequinoctialis* than in *S. polyrhiza*.

Table 3. The NR activities of *S. polyrhiza* and *L. aequinoctialis* in NO_3^- and NH_4^+ supplied media of two different concentrations

N-sources supplied		*S. polyrhiza*	*L. aequinoctialis*
		μmol/gfw/hour	
NO_3^-	2 μM	0.514	1.662
	5 μM	0.964	2.525
NH_4^+	2 μM	0.051	0.121
	5 μM	0.053	0.092

Table 4. The relationship between NR activities extracted and assayed in the absence and presense of leupeptin

	Nitrate reductase activity (μmol/gfw/hour)	
	S. polyrhiza	*L. aequinoctialis*
−Leupeptin	0.116	2.005
+Leupeptin	0.333	6.122
−Leupeptin/ +Leupeptin	0.498	0.328

Table 5. The NIR activities of *S. polyrhiza* and *L. aequinoctialis* on the various bases

Bases	*S. polyrhiza*	*L. aequinoctialis*	L/S
Fresh weight (μmol/gfw/hour)	0.166	2.005	12.1
Protein (μmol/g protein/hour)	3.864	62.637	16.2
Chlorophyll (μmol/mg chl/hour)	0.190	1.604	8.4
Frond number (μmol/frond no.)	0.970	2.851	2.9
Frond area (μmol/cm^2)	4.084	39.309	4.8

Activities of NR, NiR, GOH/NADH and GOGAT. When the enzymes involved in nitrogen metabolism were assayed, the NR activity in *L. aequinoctialis* was about 12 times as high as in *S. polyrhiza* (Table 2). The NR activities of two species were higher in the media with NO_3^- than those with NH_4^+ and increased at higher NO_3^- concentration (Table 3), suggesting substrate- and concentration-sensitive NR activities. On the other hand, NR activity in leupeptin treated media was twice and about three times as high as that in untreated media for *S. polyrhiza* and *L. aequinoctialis*, respectively (Table 4). Al-

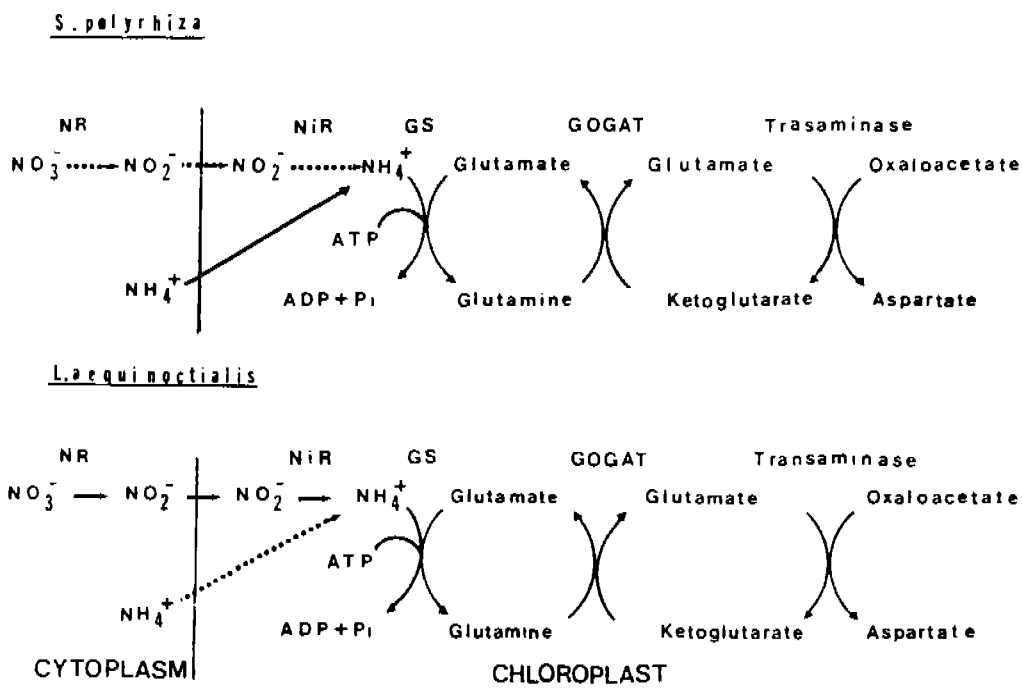

Fig. 5. The proposed scheme of nitrate and ammonia assimilation in *S. polyrhiza* and *L. aequinoctialis*.

though leupeptin inhibits the protease activity, NR activity of *L. aequinoctialis* was higher than that of *S. polyrhiza*. NR activities were calculated on the various other bases (Table 5). In all of the bases, NR activities of *L. aequinoctialis* were higher than those of *S. polyrhiza*.

NiR activities of the two species were higher in an order of magnitude than NR activities (Table 2). NiR activities of *L. aequinoctialis* were twice as high as those of *S. polyrhiza*. NiR activities of *S. polyrhiza* were similar in magnitude to those of barley seedlings (Aslam *et al.*, 1985). GS activities of both species were relatively high compared with NR activities. GDH/NADH activities of the two species were higher than GDH/NADPH. GS activities and GDH/NAD(P)H activities of *L. aequinoctialis* were a bit higher than those of *S. polyriza*, while GOGATG activities of the former species were about twice as high as those of the latter. Based on the data on enzyme activities, a diagram on the distinctive pathways for inorganic nitrogen assimilation between *S. polyrhiza* and *L. aequinoctialis* was proposed (Fig. 5).

DISCUSSION

It has been reported that under certain circumstances, *Spirodela* had a higher growth rate than *Lemna*. However, *Lemna* generally took over and would replace *Spirodela* in pond under equal conditions (Oron *et al.*, 1986). Chang *et al.* (1990) showed that *Spirodela* and *Lemna* were present in habitats with NH_4^+ and NO_3^- as major nitrogen sources, respectively. In our experiment. *L. aequinoctialis* showed a higher growth rate than *S. polyrhiza* in NO_3^- media. This is concordant with previous reports about the distribution of duckweed species. However, *L. aequinoctialis* and *S. polyrrhiza* showed no significant differences in the growth rate for NH_4^+. This may indicate that the growth rate was controlled by the rate of nitrogen supply, not by the nitrogen concentration, under the suboptimal nitrogen nutrition (e.g., Ericsson *et al.*, 1982). On the other hand, the type of nitrogen sources affected the length of the duckweed root (Ericsson *et al.*, 1982). For example, duckweeds cultivated in the NO_3^- and NH_4^+ supplied media had long and short roots, respectively. Biological significance of their results is still doubtful. This is because in aquatic Lemnaceous plants the importance of the root as an absorptive and translocating organ is not clear (Hillman, 1976).

The time taken for introgen uptake varied between the types of nitrogen sources. Our experiment on the uptake of NO_3^- was completed 7 hours after innoculation, but that of NH_4^+ being completed 4 hours after innocula-

tion. Furthermore, it appears that the ability of utilizing NH_4^+ as a sole nitrogen source is similar in these two species. Several authors showed that Lemnaceous species prefer NH_4^+ to NO_3^-, and that the plants take up NO_3^- only in case of low concentration of NH_4^+ (Porath and Pollck, 1982; Ingemarsson, 1987).

In this study, it was also clear that when NO_3^- was given as a sole nitrogen source, *L. aequinoctialis* showed a higher uptake rate of NO_3^- and higher growth rate than *S. polyrhiza*. These data suggest that *L. aequinoctialis* may have a different metabolic capacity for using NO_3^- from *S. polyrhiza*. However, not only was there no great difference in the 1/[S] values of NO_3^- uptake between *S. polyrhiza* and *L. aequinoctialis*, but were the values relatively high compared with other NO_3^- uptake system. Therefore, the duckweeds may have a relatively low affinity to the external NO_3^-. The data are quite interesting because *Lemna* is generally found in the natural habitats with high nitrate concentrations (Ullrich *et al.*, 1981).

Therefore, it is possible that differences in the V_{max} may result from more rapid disappearance of NO_3^- in *L. aequinoctialis* than in *S. polyrhiza*, not from a more powerful uptake system in the former species. This expectation is particularly likely to be true upon considering a high NR activity in *L. aequinoctialis*. The higher NO_3^- uptake rate of *L. aequinoctialis* may be due to a higher reduction rate of NO_3^- inside of the plant cell. In other words, the relatively high NR activity might result in the rapid NO_3^- reduction, subsequently lowering NO_3^- concentration inside the cell. If so. the uptake rate of NO_3^- might be continuously high. Ullrich *et al.* (1981) stated that NO_3 accumulation in the vacuoles and the transport to other cells and tissues are the processes limiting nitrate uptake in more or less starved *Lemna* plants. In barley seedlings, *in vivo* reduction of nitrate was correlated with *in vitro* activity of NR (Chantarotwong *et al.*, 1976), and the net nitrate uptake and its removal from the root to the transpiration stream seemed to be critical to the rate of nitrate reduction in the root (Mattsson *et al.*, 1988). Similary, in the N-limited *Lemna*, the short-term changes in the nitrate assimilation appear to be related to the flux of nitrate to the reducing site (Intemarsson, 1987). In this study, NR activity were induced not by NH_4^+ but by NO_3^-. In addition, the induction of NR by NO_3^- in *L. aequinoctialis* was greater than that in *S. polyrhiza*. Although NR has long been known as a substrate inducible enzyme (Hewitt *et al.*, 1976), NO_2^- which is the end product of NO_3 reduction, also induced NRA in several plant tissues such as tabacco cells, barley leaves and bean seed cotyledens (Aslam *et al.*, 1987). However, the activity of nitrate reductase was rather stabilized by the presence of ammonium in Lemnaceous plants (Ingemarsson et al., 1987).

The difference of the NR activities in the two species seemed to result from the characteristic NR for each species. The NR activity of *Lemna* was appreciably enhanced and stabilized by all of the antiproteolytic compounds tested, with casein and leupeptin being the most effective (Ingemarsson, 1987). Between the two species used in this study, the protease inhibitor activity appeared to affect the NR of *L. aequinoctialis* more than that of *S. polyrhiza*.

NiR activity or nitrite reduction of *Lemna* species was scarcely studied. NiR activities of *S. polyrhiza* and *L. aequinoctialis* were higher than NR activity of both species, indicating that the nitrite produced by NR was almost reduced by NiR. Higher NiR activities than NR activities were also observed in *Kalanchoe fedtschenkoi* (Chang *et al.*, 1981) and *Chenopodium rubrum* (Renner and Beck, 1988). On the other hand, GS activities were not greatly different in the two species. They were relatively high compared with those in other species (Chang and Ha, 1986; Fisher and Klein, 1988) and other enzyme activity such as NR and GDH/NADH. These data reflect the facts that the uptake rate of NH_4^+, the direct substrate of GS, was relatively high compared with other species and that the former was similar in both species (Ingemarsson *et al.*, 1984).

In various photosynthetic tissues, the ammonia assimilation proceeds via activities of GS and GOGAT. Recently, the evidence has been accumulated, indicating an involvement of GDH in nitrogen assimilation during plant growth with ample nitrate supply or in the media containing abundant ammonia. Under the simulated *in vivo* conditions favoring activity of GDH, the glutamate is formed from the ammonia derived from glycine or applied exogenously in the isolated mitochondria (Bergyer *et al.*, 1986). Bergyer *et al.* (1986) suggested that GDH might be operative at the elevated intracellular ammonium levels or during the energy limitations opposed to GS activity.

The response of duckweeds to NO_3^- and NH_4^+ concentration may be strongly affected by the assimilation process of inorganic nitrogen. Overall, the results of our study tend to support the view that there are two forms of enzyme activity in the frond tissues of duckweeds. The differences in the ecophysiological distribution between *L. aequinoctialis* and *S. polyrhiza* may directly ref-

lect the metabolic characteristics of *L. aequinoctialis*, i.e., higher usage of NO_3^- than *S. polyrhiza*.

적 요

암모니아의 농도가 높은 지역에서는 개구리밥(*Spirodela polyrhiza*)이, 질산염의 농도가 높은 지역에서는 좀개구리밥(*Lemna aequinoctialis*)이 주로 분포한다. 이 원인을 규명하기 위하여 질소원의 종류(질산염과 암모늄염)와 농도에 따른 생장을 조사하였다. 질산염의 농도가 15 mM 이하일 때 좀개구리밥의 성장이 더 우세하였으며 질산염의 흡수도 좀개구리밥이 개구리밥에 비하여 2배 정도 빨랐다. 그러나 암모늄염에 있어서는 성장에 있어서나 흡수 정도에 있어서 두 종에서 차이가 없었다. Nitrate reductase의 활성은 좀개구리밥이 2.005 μM/gfw·h, 개구리밥은 0.166 μM/gfw·h로서 좀개구리밥이 12.1배나 높았다. GS(Glutamine synthetase)와 GDH(Glutamate dehydrogenase)의 활성도도 좀개구리밥에서 높았다. 위의 결과는 두 종의 질소원의 종류와 농도에 대한 생리적 차이를 보여준다. 따라서 두 종의 지역적 분포의 차이는 종간에 존재하는 질소대사에 관여하는 효소 활성의 차이와 이에 따른 특정 질소원에 대한 흡수의 차이에 기인하는 것으로 사료된다.

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