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Nitrogen Assimilation and Carbohydrate Concentration as Affected by the N Supply Form and Their Level in Shoot of Perennial ryegrass (*Lolium perenne* L.)

Bok-Rye Lee, Woo-Jin Jung, Dae-Hyun Kim, Kil-Yong Kim* and Tae-Hwan Kim†

페레니얼 라이그라스에서 질소공급형태 및 수준에 따른 질소동화와 탄수화물 대사산물의 변화

이복례 · 정우진 · 김대현 · 김길용* · 김태환†

ABSTRACT

To investigate the short-term effects of N-supply form (NO_3^- or NH_4^+) and their level (0.2, 1.0 and 6.0 mM) on N assimilation and C metabolism were examined in perennial ryegrass (*Lolium perenne* L.). The increase in shoot fresh for NH_4^+-fed plants much less than NO_3^--fed ones. Nitrate concentration in NO_3^--fed plants tended to increase with increasing the supply level, while that of NH_4^+-fed plants was nearly stable. Nitrate reductase activity (NRA) responded much quickly, showing a proportional increase within 24 h of feeding. NRA in NO_3^--fed plants at 72 h increased by 13.7, 40.3 and 84.0% in 0.2, 1.0 and 6.0 mM NO_3^-, but it was not changed in NH_4^+-fed plants regardless of the supply level. After 72 h of treatment, the sugar accumulation in the plants supplied with 0.2 and 1.0 mM NH_4^+ was remarked. After 72 h of feeding, fructan hydrolysis was observed in all levels for NH_4^+-fed plants, but only in 6.0 mM for NO_3^--fed plants.

(**Key words** : Nitrate, Nitrate reductase activity, Sugar, Fructan, Perennial ryegrass)

I. INTRODUCTION

Nitrogen is a major limiting nutrient for plants in most ecosystems. It is taken up from soils mainly as nitrate (NO_3^-) and/or ammonium (NH_4^+) by the roots of higher plants(Marschner, 1995). The use of nitrate or ammonium as an N-source may have fundamental consequences for the growth, development and metabolism of the plants (Pearson and stewart, 1993; Marschner, 1995). Although N assimilation is associated with reduction of NO_3^- to NH_4^+, many plants

전남대학교 농업생명과학대학 동물자원학부(Department of Animal Science & Institute of Agricultural Science and Technology, College of Agriculture and Life Science, Chonnam National University, Gwanju 500-757, Korea)
* 전남대학교 농업생명과학대학 응용생물공학부(Department of Biological & Environmental Chemistry, College of Agriculture and Life Science, Chonnam National University, Gwangju 500-757, Korea)
† Corresponding Author: Prof. Tae-Hwan Kim, phone; +82-62-530-2126, fax; +82-62-530-2129, email: grassl@chonnam.ac.kr

show growth inhibition when NH_4^+ is supplied as the exclusive N source (Gerendas et al., 1997; Raab and Terry, 1994). Also ammonium as the sole nitrogen source leads to physiological disorders when compared with nitrate or mixed nitrogen nutrition (Goyal and Huffaker, 1984). Growth inhibition has been attributed to various factors, such as NH_4^+-induced disorders in pH regulation and toxic effects of free ammonia (Claussen and Leaz, 1995).

The interaction between the two organic nitrogen from NH_4^+ and NO_3^- on plant growth was related to photo-assimilate carbon. Nitrogen assimilation is an energy-intensive process, requiring the transfer of two electrons per NO_3^- converted to NO_2^-, six electrons per NO_2^- converted to NH_4^+ (Bloom et al., 1988). Ammonium taken up was usually assimilated by *glutamine synthase* (EC 1.4.1.14) and the resulting glutamate can then be transformed by transaminase to other amino acids (Beevers and Hageman, 1983). Also a continual supply of reducing *mine synthetase* (EC 6.3.1.2) and *glutamate* equivalents, ATP and carbon skeletons is required for sustained enzymatic assimilation of N into protein and nucleic acids (Beevers and Hageman, 1980). Nitrogen assimilation is tightly linked to energy/C-metabolism as energy and C-skeletons are needed to convert inorganic nitrogen to organic compounds. Nitrogen assimilation is a high demand for carbon regardless of in root or shoot, independent of the N-source.

With these backgrounds, in the present work, short-term effects of N-supply form and their level on nitrogen assimilation and carbohydrate metabolism related to shoot growth in perennial ryegrass have been investigated.

Ⅱ. MATERIALS AND METHODS

Plant culture and experiment procedure

Sterilized seeds of perennial ryegrass (*Lolium perenn* L.) were germinated on wet filter paper. Germinated seedlings were transplanted to 3 L pot (5 plants per pot). Plants were grown with a complete nutrient solution containing 0.2 mM NH_4NO_3. After 60 days of vegetative growth (0 h), plants were exposed to two different N-forms [KNO_3^- or $(NH_4^+)_2SO_4^{-2}$] with three different N levels (0.2, 1.0 or 6.0 mM). Plants were harvested at 0, 24 or 72 h after treatment. Harvested plants were immediately frozen in liquid nitrogen. The analysis in the present study were carried out for only shoot tissues. Freeze-ried samples were finely ground and stored under vacuum for further analysis.

Chemical analysis

1. Nitrate

Nitrate was extracted with 80% (v/v) ethanol. About 200 mg of finely ground freeze-dried sample was homogenized with 25 mL of 80% ethanol, and added with 1 mL of 0.1 N H_2SO_4 to avoid ammonium volatilization. The combined aqueous samples were placed on the horizon shaker and agitated at 500 rpm for 1 h. The extracts were filtered through a whatman No. 2 filter paper. Nitrate in aqueous extract was determined as described by Cataldo (1975).

2. Nitrate Reductase Activity

For the determination of *Nitrate reductase* activity (NRA), about 200 mg of fresh tissues

were placed in 5 mL of the incubation medium containing 0.1 M of photassium phosphate buffer (pH 7.2), 0.1 M of KNO_3 and 3% n-propanol. The tubes were vacuum infiltrated three times for 1 min, and incubated under dark condition at 30℃ for 45 h. Aliquots of 2 mL were removed from the incubating medium and the released NO_2^- was determined with a mono modification of the method of Cawse (1967). The activity of *Nitrate reductase* was expressed as μmol NO_2^- released for 1 h from 1 g of fresh tissue.

3. Carbohydrate analysis

About 25 mg of finely ground sample was extracted with 1 mL of 92% (v/v) ethanol. Tubes were shaken for 10 min at room temperature, and centrifuged at 10,000 g at 4℃ for 10 min. The ethanol extraction was repeated three times. The combined supernatant was diluted to a final volume of 10 mL with 92% (v/v) ethanol.

The sugar concentration in the supernatant was determined with anthrone [9 (10H)-anthracenone] (Van Hande, 1968) with glucose as a standard.

Starch in the ethanol extracted residue was dried at 80℃ to evaporate ethanol, and the tubes were sealed and heated in a boiling water bath for 10 min to gelatinize the starch. The pH was adjusted to 5.1 by adding 0.2 N Na-acetate buffer. Starch was hydrolyzed by adding 0.2 U of amyloglucosidase (Sigma product A3514) and 40 U of α-amylase (Sigma product A0273) in the acetate buffer to each sample. The tubes were incubated at 50℃ for 24 h with occasional shaking. Tubes were centrifuged as previously described, and glucose in the supernatant was determined using glucose oxidase (Glucose Trinder, Sigma product 315-100). Starch concentrations were estimated as 0.9 X glucose concentration.

Fructan existing in the starch extracts was hydrolyzed with 0.1 N H_2SO_4, and the fructose released was quantified using resorcinol (Davis and Gander, 1967). Glucose liberated from the fructan was determined as described above. Fructan concentration was calculated by multiplying the sum of fructan-glucose and fructose with 0.9.

III. RESULTS

1. Plant growth

For the early 24 h, shoot growth in NO_3^--fed plants slightly increased, but that of NH_4^+-fed plants was not significantly changed in all three levels (Fig. 1). By a comparison with the initial fresh weight (0 h), shoot fresh weight of plants grown with NO_3^- was significantly increased to 23.7, 24.7 and 29.6% by 72 h feeding with 0.2, 1.0 and 6.0 mM. NH_4^+-fed plants slightly increased plant growth during 24 h, and then kept on same level.

2. Nitrate concentration

Nitrate concentration in 6.0 mM NO_3^--fed plants increased by 41% within 24 h compared with the initial level, while the concentration in 0.2 and 1.0 mM was not significantly changed (Fig. 2). No significant difference was observed in 0.2 mM NO_3^--fed plants. Comparing the nitrate concentration at 72 h of treatment, nitrate concentration in 1.0 and 6.0 mM NO_3^- 23% and 62% higher than 0.2 mM NO_3^--fed plants.

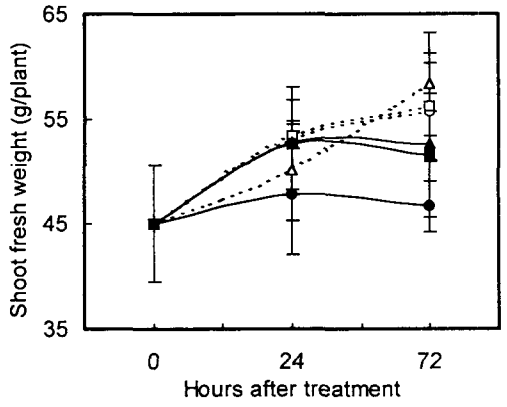

Fig. 1. Short term effects of different N-supply form and their level on shoot growth of perennial ryegrass. Dashed and continued line refers to NO_3^- and NH_4^+-supply, respectively, with 0.2 mM (○, ●), 1.0 mM (□, ■) and 6.0 mM (△, ▲). Each value is the mean ± S.E. for n=3.

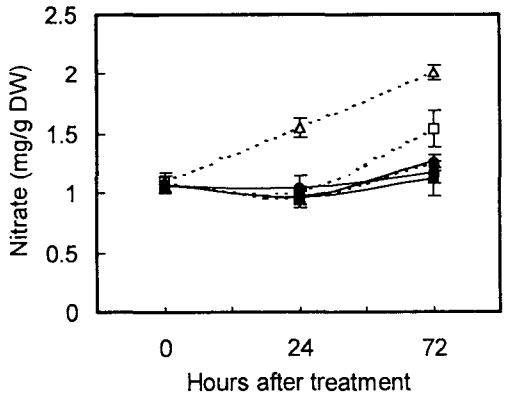

Fig. 2. Changes in nitrate concentration as affected by the nitrogen supply in the form of NO_3^- (dashed line) or NH_4^+ (continuous line) with 0.2 mM (○, ●), 1.0 mM (□, ■) and 6.0 mM (△, ▲). Each value is the mean ± S.E. for n=3.

Nitrate concentration in NH_4^+-fed plants was not significantly changed during 72 h regardless of its supply level.

3. NRA

NRA was rapidly responded to NO_3^- application, showing a significant increase with 0.7, 1.5 or 4.8-fold, respectively, within 24 h in 0.2, 1.0 and 6.0 mM (Fig. 3). NRA at 72 h in 0.2 mM NO_3^--fed plants was 151.5 μmol NO_2^-/g F.W/h. The activity at 72 h in 1.0 and 6.0 mM NO_3^- was 3.1 and 5.6-fold higher than that of 0.2 mM NO_3^--fed plants. NRA in NH_4^+-fed plants much less varied in all three levels.

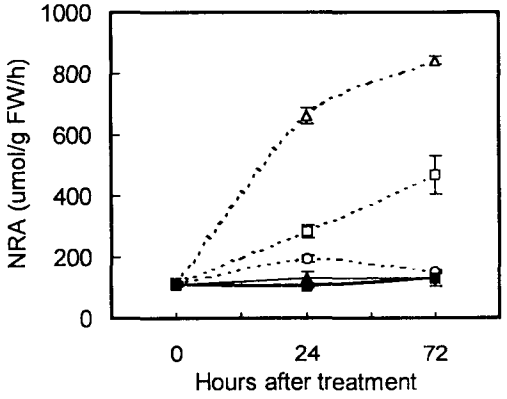

Fig. 3. Changes in NRA as affected by the nitrogen supply in the form of NO_3^- (dashed line) or NH_4^+ (continuous line) with 0.2 mM (○, ●), 1.0 mM (□, ■) and 6.0 mM (△, ▲). Each value is the mean ± S.E. for n=3.

4. Sugar concentration

Sugar concentration in both N supply forms tended to decrease after 24 h of each treatment (Fig. 4). Sugar concentration in NO_3^--fed plants at 72 h was found to recover the initial level or to be slightly lower. Sugar concentration in 0.2 and 1.0 mM NH_4^+ largely increased by 69.2% or 26.6% compared to the initial level, but maintained at the same concentration in 6.0 mM.

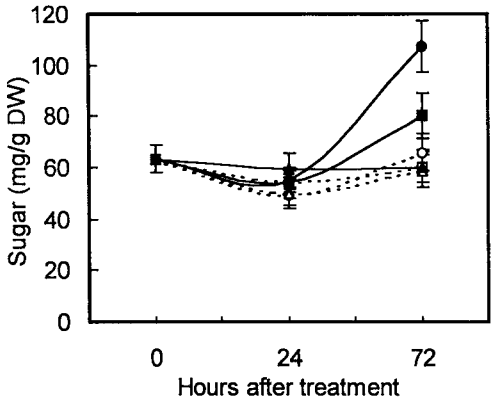

Fig. 4. Changes in sugar concentration as affected by the nitrogen supply in the form of NO_3^- (dashed line) or NH_4^+ (continuous line) with 0.2 mM (○, ●), 1.0 mM (□, ■) and 6.0 mM (△, ▲). Each value is the mean ± S.E. for n=3.

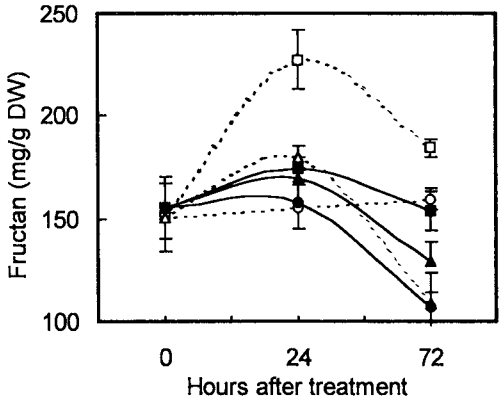

Fig. 5. Changes in fructan concentration as affected by the nitrogen supply in the form of NO_3^- (dashed line) or NH_4^+ (continuous line) with 0.2 mM (○, ●), 1.0 mM (□, ■) and 6.0 mM (△, ▲). Each value is the mean ± S.E. for n=3.

5. Fructan concentration

Fructan concentration in 0.2 mM NO_3^--fed plants was not significantly changed throughout experimental period (Fig. 5). However, the frcutan concentration in 1.0 and 6.0 mM NO_3^- significantly increased within 24 h, and then decreased. The absolute accumulation and decrease in furctan was largely higher in 1.0 mM than other N-supply levels. In the plants grown with NH_4^+, little change in fructan concentration occurred within 24 h, and then decreased significantly in all three NH_4^+-levels. By overall observation, the decrease in fructan concentration between 24 and 72 h was remarkably higher in NO_3^--fed with 1.0 and 6.0 mM than NH_4^+-fed plants.

Ⅳ. DISCUSSION

Shoot growth in NH_4^+-fed plants was significantly lower than that of NO_3^--fed ones (Fig. 1). These results are consistent with various reports postulating that growth reduction of NH_4^+-fed plants might be caused by a lack of NO_3^- as an important osmotic anion for leaf cell expansion (Raab and Terry, 1994; Salsac et al., 1987). Some other reports suggested that the growth reduction in NH_4^+-fed plants may be due to reduced cell numbers (Macadam et al., 1989) or smaller cell size (Snir and Neuman, 1997).

It has been demonstrated that ammonium toxicity or growth reduction caused by ammonium can be alleviated by nitrate (Deignan and Lewis, 1988). This effect has approved in our experiment, since nitrate was detected in the plant tissue even when ammonium was applied as a sole nitrogen source (Fig. 2). Shoot nitrate concentration increased proportionally with increasing the NO_3^- supply level, showing 13.7, 40.3 and 84.0 % in 0.2, 1.0 and 6.0 mM NO_3^- during 72 h. Nitrate reductase activity (NRA) in NO_3^--fed plants sharply increased within 24 h, and continued to increase, especially in 1.0 and

6.0 mM (Fig. 3). The increasing rate in NRA was higher as the NO_3^- supply level was increased. However, NH_4^+-supply level had not a significant influence on NRA. This data indicate that NRA was closely related to NO_3^- concentration in plant tissue in agreement with the results of Gojon et al. (1991) who showed a shift of relative nitrate reduction in the shoots with increasing nitrate supply.

Sugar accumulation was remarkably higher in NH_4^+-fed plants than NO_3^--fed ones (Fig. 4). Similar results have been reported recently by Kndlbinder et al. (1997) and Walch-Liu et al. (2000). Sugar concentration increased proportionally with decreasing the NH_4^+-supply level. These results suggest that the carbon cost for NH_4^+ assimilation is much less than that for NO_3^-, and that the excessive ammonium in higher NH_4^+ level may be preferentially at the expense of root growth. Therefore, it is likely that short-term response of sugar concentration in NH_4^+-fed plants might be distinct in lower NH_4^+-supply levels. In the long-term case, photosynthesis might be decreased by a feedback repression in response to increased sugar accumulation, which has been similarly reported for N deficiency (Paul and Dricoll, 1997). After 72 h, Fructan, polysaccharide, in NH_4^+-fed plants significantly decreased in all three level, while its concentration in NO_3^--fed plants slightly increased or maintained at the initial level (Fig. 5). Fructan reduction in NH_4^+-fed plants may be associated with a net carbon fluxes from the shoot to the root (Schortemeyer et al., 1997). It has been frequently stated that root growth in NH_4^+-fed plants is restricted by low availability of carbohydrates due to excessive consumption of soluble sugars for NH_4^+ assimilation (detoxification) in the root tissue (Cramer and Lewis, 1993; Kafkafi, 1990). Because the assimilation of ammonium into amides and amino acids requires carbon skeletons from the tricarboxylic acid cycle (Oaks, 1992), roots of ammonium-fed plants may form a stronger sink for carbohydrate than the roots of nitrate-fed plants, if nitrate is assimilated in the shoot (Barta, 1976).

In conclusion, The requires for the assimilation of N supplied are different between NH_4^+- and NO_3^--fed plants according to their levels. It may be a rapid flow of carbon from shoot to root for detoxifying ammonia in root for NH_4^+-fed plants.

V. 요 약

페레니얼 라이그라스에서 질소의 공급형태 (NO_3^- or NH_4^+) 및 수준 (0.2, 1.0 and 6.0mM)에 따른 질소동화와 탄수화물 대사산물에 미치는 영향을 알아보기 위해 nitrate, nitrate reductase, sugar 농도와 Fructan 농도를 조사하였다. NH_4^+ 공급구에서 잎의 생체량은 약간 증가하다가 같은 수준으로 유지되는 반면 NO_3^- 공급구에서는 농도가 증가함에 따라 처음수준에 비해 약 25%에서 30% 증가하였다. NO_3^- 공급구에서 Nitrate 농도는 Nitrate 농도가 더 높을 때 현저히 증가하는 반면에 NH_4^+ 공급구에서는 유의적인 변화가 없었다. Nitrate reductase activity(NRA)는 초기수준에 비해 0.2, 1.0 and 6.0mM NO_3^- 공급구에서 13.7, 40.3 and 84.0% 각각 증가하였다. NH_4^+ 공급구는 실험기간 동안 유의적인 차이가 없었다. Sugar의 축적은 NH_4^+ 공급구에서 뚜렷히 나타났으며 특히 공급수준이 가장 낮은 0.2mM에서 107.2 mgg^{-1} DW로 다른 공급수준에 비해 가장 높게 나타났다. NO_3^- 공급구에서 sugar의 농도는 초기수준에 비해 같은 수준으로 유지되거나 약간 감소하는 경향을 보였다. 처리 후 72시간에서 fructan 농도를 비교할 때 NH_4^+ 공급구의 경우

3 공급수준에서 공히 유의적으로 감소하였으나, NO_3^- 공급구에서는 약간 증가하거나 초기 수준과 비슷하게 유지되었다.

Key words : Nitrate, Nitrate reductase activity, Sugar, Fructan, Perennial ryegrass.

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