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Nitrate Metabolism Affected by Osmotic Stress and Nitrate Supply Level in Relation to Osmoregulation

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Abstract

Eight-week old perennial ryegrass (*Lolium perenne* L. cv. Reveille) plants were exposed to different NO_3^- concentrations or osmotic stress with NaCl. Previously labeled ^{15}N was chased during 14 days of non-labeled $^{14}NO_3$ feeding in order to investigate NO_3^- metabolism in relation to osmoregulation. The short term measurement of osmotic potential showed that the external concentration of NO_3^- had not great effect on the osmotic potential, but that osmotic adjustment was observed in NaCl-treated plants. Total uptake of NO_3^- was largely increased by increasing supply level of NO_3^- while it was depressed by exposing to osmotic stress. Nitrate reduction increased to more than 29% by increasing external NO_3^- concentration from 1 mM to 10 mM. When osmotically stressed with NaCl, nitrate reduction was depressed to about 37% as compared to the control. The decrease in translocation of reduced N into leaves was also observed in NaCl exposed plants. In the medium exposed to 10 mM NO_3^-, osmotic contribution of nitrate to cumulative osmotic potential was decreased, and it was osmotically compensated with soluble carbohydrate. When osmotically stressed with NaCl, the contribution of chloride was much higher than that of nitrate. The present data indicate that NO_3^- in plant tissues, factually affected by the assimilation of this ion, plays an active role in osmotic regulation in correlation with other osmotica such carbohydrate and chloride.

(**Key words** : Nitrate metabolism, Osmotic stress, Nitrate supply level, Osmoregulation)

I. Introduction

The utilization of NO_3^- by higher plants involves the following step : uptake of NO_3^- into the plant, its reduction to NH_4^- and incorporation of NH_4^- to carbon skeletons to produce amino acids. Reduction of NO_3^- to NH_4^+ proceeds by the sequential action of the enzymes nitrate reductase and nitrite reductase (Guerrero et al., 1981). Finally, the ammonium is assimilated by different metabolic pathways.

It is now clear that most of cellular NO_3^- is stored in the vacuolar pool (Martinoia et al., 1981). Only small part of NO_3^- in the cytoplasmic pool and in the xylem flow are readily available for reduction (Shaner and Boyer, 1976). In various plants, the presence of NO_3^- in the medium induces the development of NO_3^- uptake system (Tompkins et al., 1978; Deane-Drummond, 1984; MacKown and McClure, 1988). When the NO_3^- uptake exceeds assimilation as like the condition grown with ample

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supply of NO_3^-, many plants species accumulate nitrate to a high level in the cells, specifically in the vacuoles (Granstedt and Huffaker, 1982). Nitrate may have a reserve function to ensure protein synthesis under unfavorable conditions of nitrogen nutrition (Salsac et al., 1987).

It has been suggested that nitrate has a function unspecifically as an intracellular osmoticum (MacRobbie, 1973). In particular the plants using more carbohydrates and NO_3 ions are used as an osmotic regulators (Blom-Zandstra and Lampe, 1985). Increasing the concentration of polyethylene glycol (PEG) in the medium enhanced NO_3^- uptake by wheat seedling (Talouizte and Champigny, 1988). In contrast, NaCl or PEG added to the medium of Italian ryegrass grown under high light condition, decreased the NO_3^- content of the tissue. In NaCl and PEG treated plants, the osmotic potential was lowed by uptake of chloride and accumulation of soluble carbohydrates (Veen and Kleinendorst, 1985). Under low-light conditions, the decrease in soluble carbohydrate content is osmotically compensated for by an opposite changes in nitrate concentration in ryegrass (Veen and Kleinendorst, 1985), lettuce (Blom-Zandstra and Lampe, 1985) and spinach (Steingrover et al., 1986). Therefore, we could suggest that the content of NO_3^-, which should be regulated by NO_3^- uptake and endogenous NO_3^- reduction, may be closely related intracellular osmoregulation. The availability of external NO_3 has been also been claimed to modify the partitioning of NO_3^- assimilation between root and shoot of many plant species (Andrews, 1986; Kim et al., 1999).

Our interests focussed on the investigation of NO_3^- metabolism as affected by osmotic stress or external NO_3^- concentration in relation to osmotic regulation. The present study was designed to measure the assimilation of endogenous $^{15}NO_3^-$ affected by the external NO_3^- concentration and osmotic stress with NaCl. The osmotic contribution of the relevant biochemical fractions was also estimated.

Ⅱ. Materials and Methods

Plant culture and experimental procedure

Seeds of perennial ryegrass (*Lolium perenne* L., cv. Reveille) were germinated on silicious sand. Two-week old seedlings were transplanted to 3 L pots (5 plants per pot) and grown hydroponically on a nutrient solution containing, in mM, 1.0 NH_4NO_3, 0.4 KH_2PO_4, 0.25 KCl, 0.25 $CaCl_2$, 0.2 $MgSO_4$, 0.15 K_2HPO_4 and micro-nutrient (Fe, B, Mn, Zn, Cu, Mo) with the pH adjusted to 6.4. The nutrient solution was continuously aerated and renewed every 5 days. Photoperiod and temperature were 16 h with metal halide lamp at 24℃ and 8 h in the dark at 18℃.

^{15}N labeling was carried out by replacing NH_4NO_3 with $K^{15}NO_3$ (1.0 mM, 10.43 atom % ^{15}N excess) on the same nutrient solution during 5 days before treatment. Eight-week old plants were sampled as the reference plot (designated t=0). Other 3 plots were differently treated according to NO_3^- supply level and osmotic stress; 1 mM NO_3 non-osmotic stress (control), 10 mM NO_3^- non-osmotic stress (treatment A), 1 mM NO_3^- and NaCl to give osmotic stress to -0.335 MPa (treatment B). The prepared nutrient solutions (non-labeled $^{14}NO_3$) were fed during 14 days. Plants from each treatment were harvest 14 days after treatment (designated t=14) and separated into leaves and roots. One subsample was kept in liquid ntrogen for the measurement of osmotic potential and soluble carbohydrates. Others were dried at 60℃, ground to a fine powder and kept on a vacuum for further analysis.

Chemical fractionation and isotope analysis

About 200 mg of finely ground freeze-dried sample was extracted with 25 mL of 80% ethanol. The ethanol-soluble fraction was filtered, centrifuged, and passed through a H^+ column (Dowex 50W × 8). One-fifth volume of the solution collected from the H^+ column were concentrated to 0.5 mL (nitrate fraction). The remaining four-fifths were passed through a column in the formate form (Dowex 1). The collected solution was concentrated to 2.0 mL (soluble sugars fraction). Soluble reduced N (amino acids + NH_4^+) was eluted with 25 mL of 0.5 N HCl from the Dowex 50W column and concentrated to 1.0 mL. Each fraction was concentrated by drying the collected solutions with rotary vacuum evaporation and the residue was dissolved with distilled water to obtain the final volume of each fraction. The residues of ethanol extraction and filtration were dried for 24 h to obtain dry weight. The resulting dried samples were designated as residue N fraction (protein-N).

The solid samples (total N and residues) were precisely measured into tin capsules. For the solution samples, an appropriate sample volume, usually 0.1 mL, was dropped into tin capsules to give more than minimum sample size for ^{15}N abundance. The tin capsules containing solution were quickly cooled with liquid nitrogen, and then dried in a freeze-dryer. The residues in tin capsules were employed for analysis of N or C content and ^{15}N or ^{13}C abundance. ^{15}N-enriched L-glutamic acid (0.362 ^{15}N atom %) were used as the references for N analysis.

^{15}N amount was calculated by multiplying ^{15}N excess with N content in the sample. The assimilation of $^{15}NO_3^-$ could be estimated comparing with ^{15}N amount recovered in nitrate or reduced N compound. The N uptake was estimated by considering diluted rate of ^{15}N within plant.

Measurement and calculation of osmotic potential

A frozen subsample was thawed in water-bath for 10 min at 50℃ and then centrifuged for 10 min at 40,000 g. The osmotic potential was determined on the supernatant using an osmometer (Roebling, France). The osmotic potential was also calculated on the basis of water, nitrate, sugar, chloride contents, assuming that all these compounds contributed to the osmotic potential. Chloride was extrated from dry power subsample and determined by potentiometric titration using a $AgNO_3$ electrode (Nelson, 1960).

Ⅲ. Results and Discussion

Osmotic Potential

The short term effects of external NO_3^- concentration and NaCl adding on the osmotic potential of plant tissue were investigated. The plants were fed 1 mM NO_3^- and previously placed in darkness for 12 hours in order to decrease the activity of nitrate reduction. Osmotic potentials in plants exposed to different external NO_3^- con-ntration (1 or 10 mM NO_3^-) and osmotic stress (with or without NaCl) were determined in the light during 24 hours. The changes of osmotic potentials in leaves and roots are shown in Fig. 1. No significant changes in osmotic potentials in both leaves and roots occurred in the dark condition. After 24 hours after treatment, the osmotic potentials of leaves decreased to 29, 30 and 52% of initial value (−0.691 MPa) in the mediums exposed to 1 mM (control), 10 mM NO_3^- (treatment A) without NaCl and 1 mM NO_3^- with NaCl (treatment B). However, the osmotic potentials in leaves were not significantly differed by the treatment at each time

course of measurement. In the roots exposed to 1 mM or 10 mM NO_3^- without NaCl, the osmotic potentials remained steady or slightly decreased. When NaCl added to the medium, the osmotic potential decreased from -0.205 MPa (0 h) to -6.610 MPa (24 h). At 24 h after treatment, the difference of -0.379 between control and NaCl-treated plants was found. Therefore we could suggested that, for the plants exposed NaCl, full osmotic adjustment to the external osmotic potential (-0.335 MPa) was achieved in roots. These clearly showed that chloride actively contribute to osmotic adjustment. Veen and Kleinendorst (1985) reported that the osmotic contribution of chloride increased by about 6 times in roots and doubled in leaves when NaCl was added to the medium.

Partitioning of NO_3^- content in plant tissues

NO_3^- uptake was calculated in basis on the diluted rate of ^{15}N by influx of ^{14}N during 14 days after treatment. Total NO_3^- content taken up from the plant supplied with 1 mM NO_3^- without NaCl (control) was 1.71 mgN/plant. By increasing external NO_3^- concentration to 10 mM (treatment A), total NO_3^- uptake increased to 4.77 mg. When added NaCl to medium (treatment B) NO_3^- uptake decreased to 1.57 mg (8 % decrease) compared with control plant.

The changes in ^{15}N amounts in nitrogenous compound during 14 days are given to Table 1. When continuously supplied with same concentration of NO_3^- (1 mM) without osmotic stress (control), ^{15}N amount reduced from nitrate in leaves and roots were respectively 37.7 and 34.2 μg per plant. The decreased ^{15}N in nitrate fraction was recovered in mainly protein and soluble reduced-N fractions in leaves. About 54 μg of ^{15}N in reduced N fraction (soluble reduced N + protein) was translocated from roots to leaves.

When exposed to 10 mM NO_3^- without osmotic stress (treatment A), about 65% of total ^{15}N in nitrate fraction before treatment (t=0) decreased during 14 days. As compared to the control, nitrate reduction was increased to more than 29%. The decrease of ^{15}N amount in nitrate fraction was much higher in leaves than roots. It showed that nitrate reduction accelerated especially in leaves when supplied with high concentration of external NO_3^-. This result confirmed some results, showing that the shoot was the main site of NO_3^- assimilation in herbaceous plants (Beevers and Hageman, 1982; Andrews, 1986). Contrariwise some results for trees reduce showed that NO_3^- almost exclusively in the roots (Scheromn and Plassard, 1988; Gojon et al., 1991). Although data for the site of NO_3^- reduction are still contradictory according to plant species, it could be well accepted that the contribution of the shoots to the whole plant NO_3^- reduction increased with increasing external NO_3^- availability. When supplied with 10 mM NO_3^-, 42.4 μg and 17.6 μg of ^{15}N derived from NO_3^- incorporated into respectively soluble reduced-N and protein fraction in leaves while those in reduced N fraction (soluble reduced N + protein) decreased in roots comparing with control.

When osmotically stressed with NaCl (treatment B), only 21% of total ^{15}N in nitrate fraction before treatment (t=0) decreased during 14 days. It is noteworthy that under osmotic stress ^{15}N amount in nitrate fraction of roots (41.6$\pm$2.2 μg) remained to nearly same level before treatment (36.2$\pm$1.8 μg). Total ^{15}N amount in nitrate fraction 1.6 fold higher than that of control plot. These indicate that nitrate reduction was depressed by osmotic stress especially in roots. The inhibition of nitrate reduction under osmotic stress was also found in the plants treated

Table 1. Changes in ^{15}N amounts (μg plant^{-1}) in nitrogenous compound after two weeks of culture condition exposed to different nitrate concentration or osmotic stress Each value is mean ± S.E for n=3

Treatments	Organs	Nitrogen fraction		
		NO_3^--N	Soluble Reduced-N	Protein-N
t=0				
	Leaves	101.9±3.7	88.9±4.1	30.5±2.6
	Roots	41.6±2.2	79.7±3.3	100.7±4.6
t=14 Control : 1 mM NO_3^-, Non osmotic stress				
	Leaves	64.2±2.5	155.7±5.6	170.2±4.9
	Roots	7.4±0.6	53.4±5.0	72.7±3.4
t=14 Treatment A : 10 mM NO_3^-, Non osmotic stress				
	Leaves	31.3±1.8	198.1±9.2	187.8±9.5
	Roots	19.5±2.0	42.3±1.5	55.9±3.5
t=14 Treatment B : 1 mM NO_3^-, Osmotic stress with NaCl				
	Leaves	77.1±5.4	130.4±7.9	162.7±6.2
	Roots	36.2±1.8	32.3±2.5	92.2±4.8

with polyethylene glycol (Larsson et al., 1989). ^{15}N recovered in the soluble reduced N fraction was significantly decreased in both leaves and roots while that in the protein fraction was much less changed.

The data obtained indicate that the reduced-N resulting from NO_3^- assimilation, principally occurring in the leaves, is mainly incorporated in the leaves of perennial ryegrass. The translocation of reduced-N into leaves was also accelerated with increasing the external NO_3^- concentration while that was significantly depressed as compared to the control. Therefore, it could be suggested that the decrease in translocation in NaCl treated plants was entirely reflected in NO_3^- reduction levels, not in storage. Veen and Kleinendorst (1985) reported that nitrate concentration in plant tissue, factually affected by the assimilation of this ion, was closely linked to osmoregulation.

Osmotic contribution of carbohydrate, nitrate and chloride

The changes in osmotic contribution of nitrate, soluble carbohydrate and chloride as affected by NO_3^- and osmotic stress are shown in Fig. 2. Cumulative osmotic potentials of carbohydrate, nitrate and chloride were -0.3861 and -0.194 MPa in leaves and roots before treatment (t=0). After 14 days of treatment, the cumulative osmotic potentials in both leaves and roots were not significantly differed. In leaves and roots, the contribution of three biochemical compounds to cumulative osmotic potential was responded with a similar pattern.

When supplied with 1 mM NO_3^- without NaCl (control), in both leaves and roots cumulative osmotic potential and the contribution of each compounds were not changed compared with before treatment (t=0). It suggested that, when continuously supplied with same concentration of NO_3^-, no changes in osmotic potential occurred while about 50 % of the previously stored NO_3^- assimilated during 14 days (Table 1).

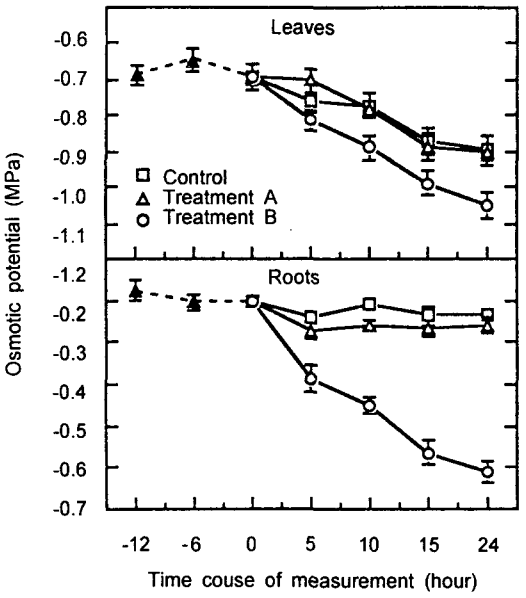

Fig. 1. Changes in osmotic potential in leaves and roots of perennial ryegrass during 12 hours in darkness (dashed line) and 24 hours in light after treatment. Vertical bars give SE for 3 replications. Control, treatment A and treatment B refer to the mediums exposed to 1 mM, 10 mM NO_3^- without NaCl and 1 mM NO_3^- with NaCl.

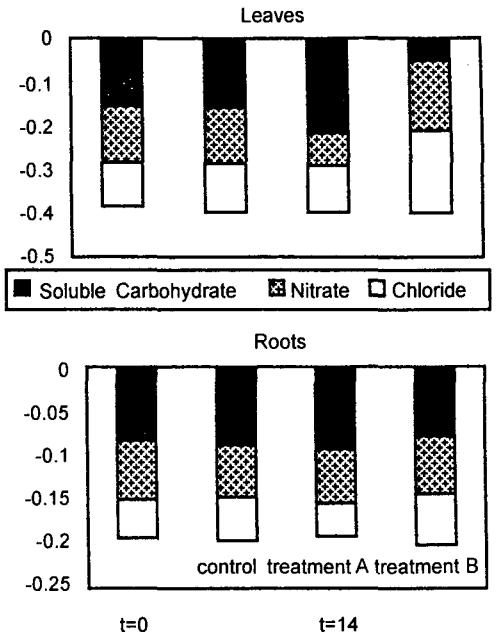

Fig. 2. Cumulative osmotic potential in MPa of soluble carbohydrate, nitrate and chloride in leaves and roots of perennial ryegrass. t=0: before treatment; t=14: 14 days after treatment. Control, treatment A and treatment B refer to respectively the mediums exposed to 1 mM, 10 mM NO_3^- without NaCl and 1 mM NO_3^- with NaCl.

By increasing the concentration of NO_3^- to 10 mM (treatment A), in leaves the percentage of nitrate contribution to cumulative osmotic potential largely decreased from 32.3 % (control) to 18.6 % (treatment A), while that of soluble carbohydrate increased from 40.1 % (control) to 54.8 % (treatment A) although the cumulative osmotic potential was not changed (Fig. 2). Therefore It is suggested that the content of nitrate, which actually affected by uptake, storage and reduction of this ion, inversely related to that of carbohydrate. Claussen (1986) showed that leaves of eggplants, which exported carbohydrate to maturing leaves, had a low nitrate reductase activity and therefore, accumulated nitrate. Similarly, an inverse relationship between carbohydrate and nitrate contents have been observed at different light intensities for lettuce (Blom-Zandstra and Lampe, 1985) and Italian ryegrass (Veen and Kleinendorst, 1985). It could be well accepted that the decrease in osmotic contribution of nitrate, resulting from the increase of whole plant NO_3^- reduction with increasing external NO_3^- availability (Table 1), was osmotically compensated for almost exclusively by the increase of carbohydrate contribution.

When osmotically stressed with NaCl (treatment

B), the percentage of soluble carbohydrate contribution to cumulative osmotic potential largely decreased by 27.2% and 5.9% in leaves and roots as compared to the control, the decreases in soluble carbohydrate were partially compensated for by the increase of nitrate and chloride (Fig. 2). In both leaves and roots, the contribution of chloride to cumulative osmotic potential was much higher than that of nitrate. It clearly showed that chloride plays an active role in osmoregulation in plants exposed to NaCl. Ourry et al. (1989) showed that, in regrowing perennial ryegrass on a nitrate starved medium, the decreases both in soluble carbohydrate and nitrate contents were partially compensated by the increase of chloride. The data obtained indicate that chloride taken up from NaCl treated medium may osmotically contribute by replacing nitrate. In lettuce, an increase in chloride content was observed when nitrate was replaced by chloride in the medium (Blom-Zandstra and Lampe, 1985). Therefore, it could be suggested that both chloride and nitrate might have the osmoregulatory capacity. In conclusion, the function of nitrate as an osmoticum can be related to various factors involving the changes in carbohydrate content and other osmotica under different growth conditions.

IV. References

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