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The Effects of Anion Replacement on Proteins, Sugars and Nitrate Concentration in Italian Ryegrass (*Lolium multiflorum* L.)

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음이온 대체공급이 이탈리안 라이그라스의 단백질, 당 및 질산염 농도에 미치는 영향

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Abstract

To investigate the effects of anion replacement on NO_3^- concentration in Italian ryegrass. Plants were grown hydroponically to the full vegetative stage. NO_3^- supply(control) was replaced with SO_4^- (T1), Cl^- (T2) and water (T3) to during 14 days. The determination of inorganic nutrient uptake and quantification of principal metabolites (nitrate, protein and sugar) followed. Relatively high uptake of Na^+ and Ca^{++} for control plants, K^+ and PO_4^- for T2 plants, and Cl^- for T1 plants was observed, respectively. Proteins in shoot and stubble were relatively higher in control and T1 plants, which coupled N source. In root proteins largely decreased (especially in T3 plants) during experimental period. Sugars in shoot of all four treatments tended to decrease during the first 7 days and recovered afterward. Sugars in stubble also markedly decreased during the first 7 days, while those in root was much less varied during experimental period. After 14 days of treatment, nitrate concentration in shoot of control plants was 13mg/g FW. Comparing to control, nitrate in shoot reduced by 27%, 46% and 50% in T1, T2 and T3 plants, respectively. Dry weight was slightly increased or not significantly changed in control, T1 and T2 plants, while a significant decrease(31.3% of control) occurred in T3 plants.

(Key words : Italian ryegrass, Anion replacement, Ion uptake, Protein, Sugar, Nitrate)

Ⅰ. INTRODUCTION

Nitrate accumulation in forages can have serious deleterious effects on livestock performance, even leading to death depending on the concentration of nitrate in the forage and the level of consumption

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(Heath et al., 1978). Within the gastrointestinal tract, NO_3^- is reduced to NO_2^-, which is absorbed into the bloodstream where it binds with hemoglobin, oxidizing ferrous iron to ferric iron to form methemoglobin (Wright and Cavison, 1964). This form of the hemoglobin as methemoglovinemia. Additionally, dangers to humans can occur when forages with high NO_3^- levels are ensiled and microbial denitrification results in the release of NO gas which can accumulate to toxic levels in the silo.

It have been well established the cellular NO_3^- is separated with two pools, one metabolizable pool in cytoplasm and the other stored pool in vacuole (Ferrari et al., 1973; Martinoia et al., 1981). Most of cellular NO_3^- in vacuolar pool, while only small part of NO_3^- in the cytoplasmic pool and in the xylem flow are readily available for reduction (Shaner and Boyer, 1976).

Bibliographic analysis indicate that numerous and diverse factors involved in the regulation of activity of nitrate reductase. As endogenous factors, the flow of NO_3^- into shoots (Shaner and Boyer, 1976), the concentration of ammonium and amino acid in plant tissue (Schrader and Thomas, 1981; Campbell, 1990) have been documented. The exogenous factors mediating the activity of nitrate reductase were much more numerous, such as light intensity, wavelength of light, CO_2 concentration, temperature, growth regulators, and osmotic stress.

Different methods were reported to reduce nitrate content with or without yield variation. Some of them used negative correlation between NO_3^- and radiation (Steingrover et al., 1993), or temperature (Santamaria et al., 1996). Other methods are related to nutrient changes, for example NH_4^+/NO_3^- ratio (Boon et al., 1990; Santamaria et al., 1996), and reducing the use of N-NO_3 before harvest (Marlogio, 1995; Carrasco, 1992). Also other authors have introduced the use of intracellular osmoticum (Macrobbie, 1973). The previous study (Kim, 2000) indicated that NO_3^- in plant tissue play an active role in osmotic regulation in correlation with other osmotic such as carbohydrate and chloride. Our interests focused on the investigation of the different behavior for NO_3^- reduction affected by replacement NO_3^- with other anions during full vegetative growth period.

Ⅱ. MATERIALS AND METHODS

Plant culture and experiment procedure

Seeds of Italian ryegrass (*Lolium multiflorum* L.) were germinated on sand. Two-weeks old seedlings were transplanted to 3L 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 7 days. Photoperiod and temperature were 16h at 24℃/ 8h at 18℃ for day/night. Plants were grown with 1.0 mM NH_4NO_3 for 6 months, then increased N supply level to 6.0 mM NO_3^- to induce NO_3^- accumulation in plant tissues. After 2 weeks of growth on complete nutrient solution with NO_3^-, four treatments were applied during 2 week. Control plants were grown without changing the composition of nutrient solution. Three groups were treated by replacing NO_3^- with SO_4^-[$(NH_4)_2SO_4$, T1], with Cl^- [3mM $CaCl_2$, T2] and Water [T3]. Plants were harvested after 7 and 14 days after treatment, respectively. Plants were cut leaving a stubble of 6 cm above root base and separated with 3 organs (root, stubble and shoot). Samples were immediately

frozen in liquid nitrogen. Freeze-Dried samples were finely ground stored under vacuum for further analysis.

Determination of inorganic ion uptake

Inorganic ion uptake was estimated by depletion method, which determined the reduced concentration of specific ion in the nutrient solution. Sampling of nutrient solution was carried out when each treatment applied, and consecutively every 7 days. After 7 and 14 days of treatment, the volume of nutrient solution was corrected to 3L with distilled water and 5mL of well mixed solution was taken for analysis. The cumulative uptake was expressed as milligram per gram of shoot fresh weight. The nutrient solutions were filtered through a 25um syringe(ADVANTEC MFS. Inc.) filter. The concentration anion (NO_3^-, PO_4^-, Cl^- and SO_4^-) and cation (NH_4^+, K^+, Mg^{++}, Ca^{++} and Na^+) in sample solution were determined by ion chromatography (Dionex, DX-120 Summyvale Co. USA).

Chemical analysis

About 25mg of finely ground freeze-dried sample was extracted with 1mL of 100mM $NaPO_4$ buffer (pH 6.8). Tubes were vortexed for 15 sec and placed on ice for 5min, and centrifuged on 14,000 rpm, at 4℃ for 10 min. This procedure repeated four times. Soluble proteins in the supernatant were quantified using dye-binding (Bradford, 1976).

Sugars were extracted with of 92%(v/v) ethanol. Tubes were agitated for 10min, centrifuged on 14,000 rpm, at 4℃ for 10min. The ethanol extraction was repeated three times and the supernatant was removed diluted to a final volume of 10mL with 92% (v/v) ethanol. The sugar concentration in the ethanol extracts was determined with anthrone reagent (Van Hande, 1968) using glucose as a standard.

Nitrate was extracted with 80% (v/v) ethanol. About 200mg of finely ground freeze-dried sample was mixed added with 25mL of 80% ethanol, 1mL of 0.1N H_2SO_4 was added to avoid ammonium volatilization. The combined aqueous samples were placed on the horizon shaker and agitated at 250rpm for 1hr. The extracts were filtered through a whatman NO. 1 filter paper. Nitrate in aqueous extract was determined as described cawse (Cawse, 1967).

Ⅲ. RESULTS

Inorganic ion uptake

The uptake of anion and cation during 14days of treatment is shown in Table 1 and Fig. 1, respectively. Comparing to control plants, PO_4^- uptakes of the plants supplied with $(NH_4)_2SO_4$ (T1) and $CaCl_2$ (T2) were 1.37 and 1.63 fold higher. In the T2 plants, total NH_4^+ uptake during experimental period was 4.25 mg/g FW. K^+ uptake in all treatments largely higher than other cations, range from 2.21mg to 6.99 mg/g FW of shoot. Total K^+ uptake in T1 and T2 plants was 31.6% and 51.5% of control plants. Na^+ uptake in control plant showed a marked increase, while that in T1 and T2 remained at low level (below 0.05 mg/g FW). Ca^{++} uptake in control plants was 0.5mg/g. FW (40.9% of T2 plants supplied with $CaCl_2$), but that in T1 plants was negligible. During the first 7 days of treatment, Mg^{++} uptake in control and T1 plants increased with a similar pattern until about 0.2mg/g FW, but relatively lower in T2 plants. Total uptake

Table 1. Total uptake of anion (NO_3^-, PO_4^-, SO_4^- and Cl^-) and cation (NH_4^+) during 14 days of anion replacement. NO_3^- supply (control) was replaced with SO_4^- (T1), Cl^- (T2) and water(T3) for 14 days

Treatment	Ion uptake (μg/g FW shoot)				
	NH_4^+	NO_3^-	PO_4^-	SO_4^-	Cl^-
Control	301	1,858	135	13.4	16.7
T1	4,247	6.7	185	294	46.4
T2	103	-	221	12.9	1,159
T3	-	-	-	-	-

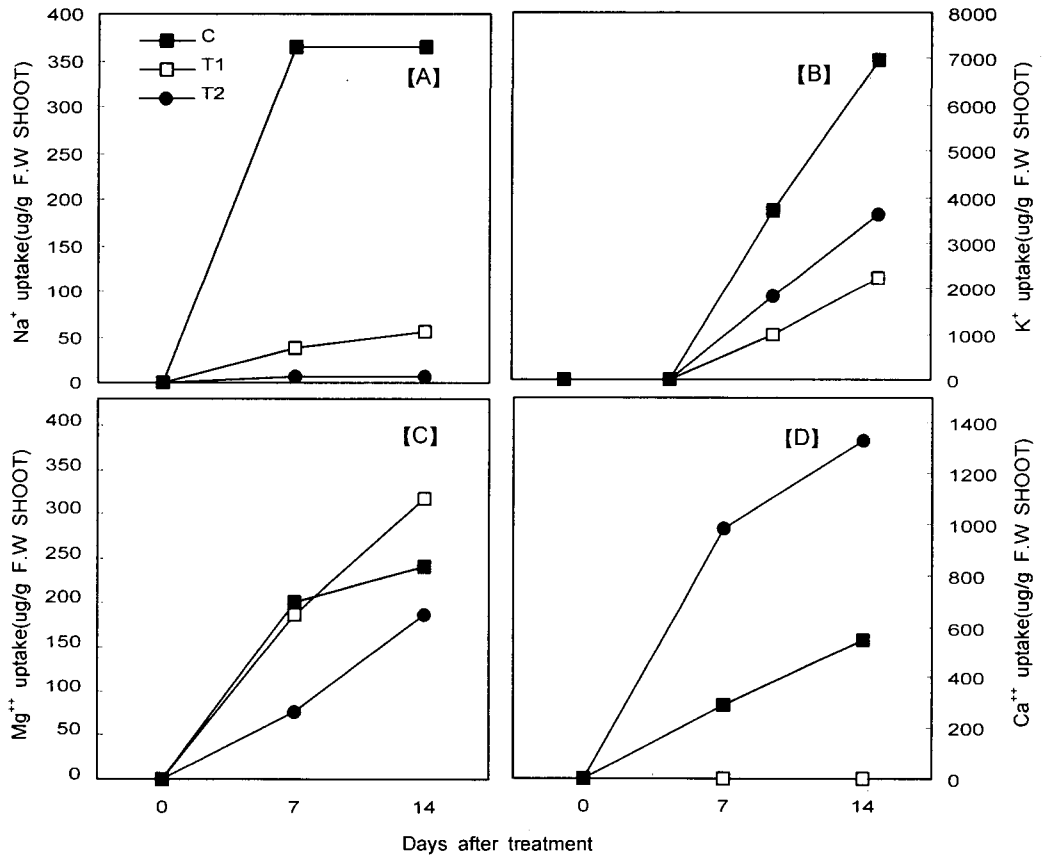

Fig. 1. Changes in cumulative amounts of Na^+[A], K^+[B], Mg^{++}[C] and Ca^{++}[D] uptakeduring 14 days of anion replacement. NO_3^- supply (control) was replaced with SO_4^-(T1), Cl^-(T2) and water(T3) for 14 days.

of Mg^{++} was the highest in T1 plants.

Protein concentration in plant tissue

Changes in protein concentration in shoot, stubble and root during 14 days of treatment are shown in Fig. 2. After 7 day of treatment proteins in shoot in control, T2 and T3 plants significantly decreased by 17.5%, 54.1% and 69.8% of the initial level (D 0), while increased by 16.8% in T1 plant. At day 14 (D 14) protein in shoot of T2 and T3 plants, non-supplied N source, greatly decreased to 12.6mg/g FW (25% of D 0) and 14.9mg/g FW (29.6% of D 0). Proteins in stubble of control and T1 plants, which were received 6 mM N, largely increased during the first 7 days. But in T2 and T3 plants proteins remained at same level or slightly decreased. After 14 days of treatment (D 14) protein concentrations in control and T1 plants was similar at about 18mg, while those in T2 and T3 were significantly lower. Proteins in roots of all treatments continuously decreased during 14 days. The decreasing rate in T3 plants during the first 7 days (D 7) was remarkable (about 89.4% of decrease). The protein concentration in root at day 14 (D 14) showed a similar level four all treatments.

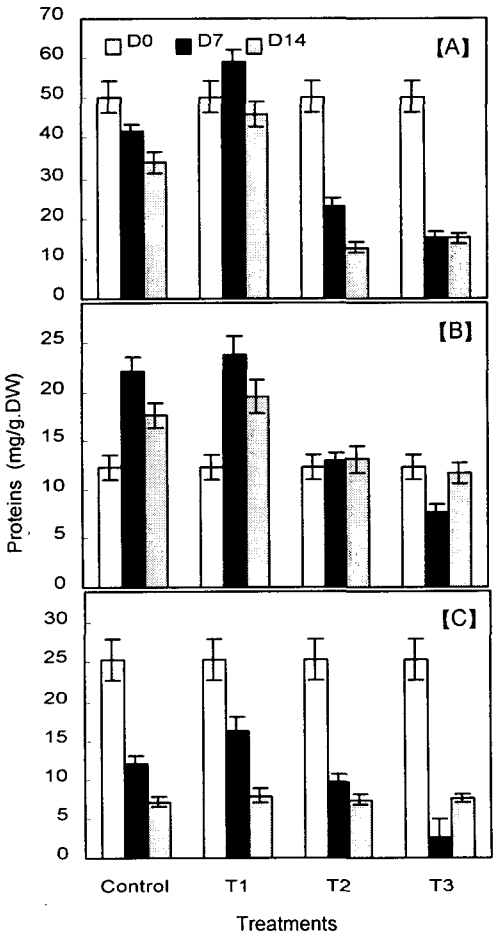

Fig. 2. Changes in protein concentration of shoot[A], stubble[B] and root[C] during 14 days of anion replacement. NO_3^- supply (control) was replaced with SO_4^-(T1), Cl^-(T2) and water(T3) for 14 days. Each value is mean ± s.e. for n=3.

Sugar concentration in plant tissue

The concentrations of sugars in three organs affected by anion replacement during 14 days are summarized in Fig. 3. For the first 7 days of treatment sugars in shoots significantly decreased by 38.1% and 34.9% in control and T1 plants, while not significantly changed level of D 7 in T2 and T3 plants. During the second 7 days, sugars in shoots of all treatments similar without significant difference. At day 14 (D 14) sugars in control, T1, T2 and T3 plants were changes 31.1, 29.0, 41.0 and 42.1 mg/g FW, respectively. In stubble of sugars greatly decreased to about half of the initial (D 0). During the second 7 days the concentration of sugars continued to decrease in T1 plants, while tended to increase in control, T2 and T3 plants.

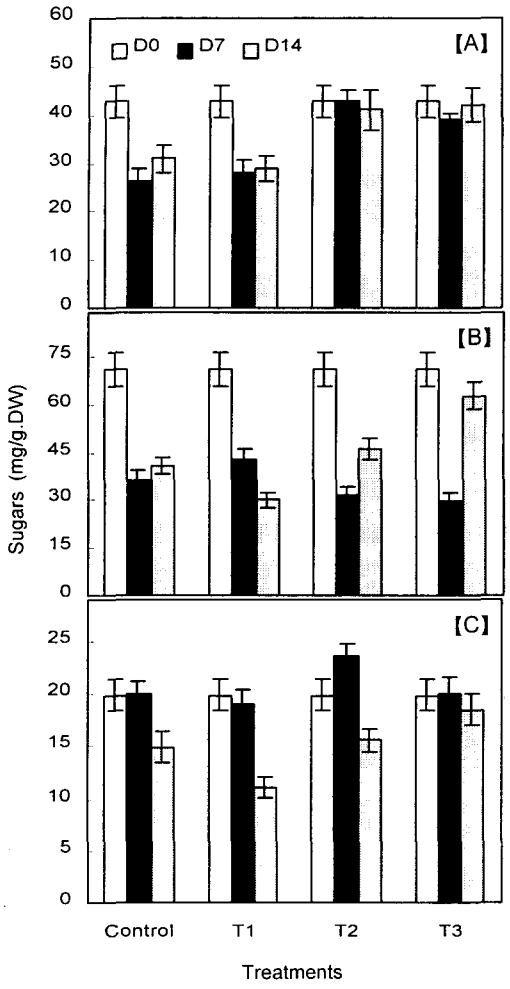

Fig. 3. Changes in sugar concentration of shoot[A], stubble[B] and root[C] during 14 days of anion replacement. NO_3^- supply (control) was replaced with SO_4^-(T1), Cl^-(T2) and water(T3) for 14days.
Each value is mean±s.e. for n=3.

Especially in T3 plants an active recover sugars occurred and arrived at nearly same level of the initial (D 0). The concentration of sugars in roots was much less varied compared to other organs. During the second 7 days sugars in roots significantly decreased at all treatments.

Nitrate concentration and Dry weight in shoot

Changes in nitrate concentration in shoot affected by anion replacement during 14 days are presented in Fig 4A. The initial concentration of nitrate at day 0 was 10.7±0.89 mg/g FW in average of four treatments. After the first 7 days of treatment, NO_3^- concentrations in control plants increased about 30%, while those of T1, T2 and T3 plants decreased by 11.2%, 34.5% and 38.5% respectively. During the second 7 days the concentration of NO_3^- in control and T1 plants were not

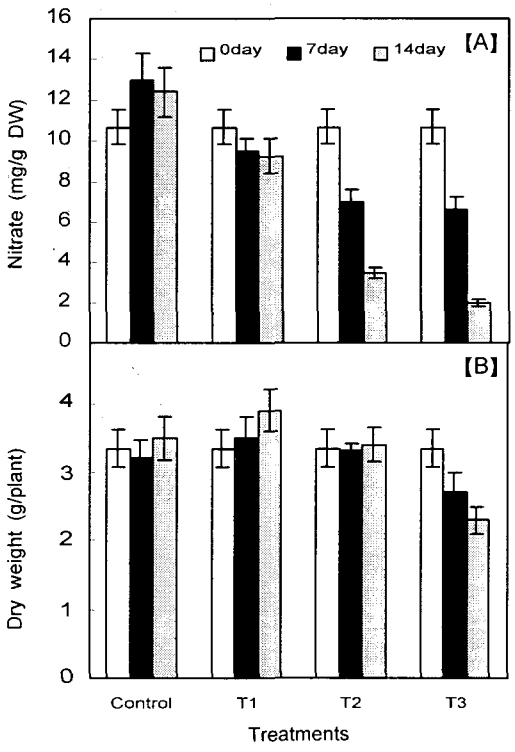

Fig. 4. Changes in nitrate concentration[A] and dry weight[B] of shoot during 14 days of anion replacement. NO_3^- supply (control) was replaced with SO_4^-(T1), Cl^-(T2) and water(T3) for 14 days.
Each value is mean ± s.e. for n=3.

significantly changed, while continuously decreased in T2 and T3 plants. The concentrations of NO_3^- at day 14 (D14) were 12.3, 9.2, 3.5 and 2.0 mg/g FW in control, T1, T2 and T3 plants, respectively. These values corresponded +15.0 %, −13.5 %, −67.4 % and −81.4% of the initial NO_3^- level at day 0 (D 0). When compared to control plants (NO_3^- supply), 27.3%, 71.9% and 83.9% of decrease were estimated in the plants replaced with SO_4^- (T1), Cl^- (T2) and water (T3). Dry matter of shoots during 14 days of treatment is presented in Fig. 4B. The dry weight per plant at day 0 was 3.4±0.2 g/plant in average. After 14 days of treatment, there was not significant change in control and T2 plants. But 16% of increase in T1 and 31.3% of decrease in T3 plants were observed.

IV. DISCUSSION

The concentration of soluble proteins in shoots of control and T2 plants (6 mM N supply) was largely higher than those of T2 and T3 plants (non-external N supply) during 14 days of treatment. Its decreasing rate was predominant in T2 and T3 plants (Fig. 2A). These suggest that protein synthesis and transport of reduced N into growing shoot actively occur when a large enough N was externally supplied as like control and T1 plants. However the pre-accumulated proteins largely hydrolyzed in shoots when non-additional N supplied. These might be associated with the concentration of N uptake (Table 1 and Fig. 1) and the relative low reduction of NO_3^- in shoot of the plants continuously received N as NO_3^- (control) or NH_4^+ (T1), and an active reduction in T2 and T3 plants (non-external N supply, Fig. 4A). These are consistent with some results that shoot contribution to whole plant NO_3^- reduction increases with NO_3^- availability in medium (Andrews, 1986) and uptake rate of this anion (Gojon et al., 1991).

In stubble during the first 7 days, the concentration of proteins increased in control and T1 plants while remained at same level or decreased slightly in T2 and T3 plants. It was observed the accumulation of proteins in the plants continuously received 6 mM N (control and T1) and no more accumulation in T2 and T3 plants. This clearly show that stubble has of a storage organ for soluble proteins in agreement with the results of Ourry et al. (1989) and Prudhomme et al. (1992) who reported that the stubble of ryegrass played a major source organ for soluble protein and fructans. In roots the concentration of proteins in all treatments continuously decreased during experimental period. From these results it could be assumed that roots have low activity of protein synthesis derived from newly absorbed NO_3^-. Much higher decrease of proteins in roots and no more compensatory increase in shoots and stubbles were observed in T2 and T3 plants. However proteins in stubble and shoots were observed in control and T1 plants. Those indicate that the reduced N from external N source in roots readily transported into growing shoots although the absolute quantity of N assimilation and transport could not be estimated from the method of this experiment. Cooper et al. (1989) reported that NO_3^- export to shoots was closely related to the rate of NO_3^- uptake, and it had influence on the competition between reduction of NO_3^- in the root epidermis and cortex and NO_3^- diffusion to the stele. The result obtained clearly indicates that xylem transport of reduced N and NO_3^- into shoots much actively occur rather than protein incorporation in the root although external N is largely available as like control and T1 plants in this experiment. Gojon et al. (1986; 1991) reported that a large

portion of newly reduced N was transported to the shoots regardless of the level of N nutrition

The concentration of sugars in shoots responded much more prominently to treatment (Fig. 3). Sugars in shoots were decreased by about 30% on average in control and T1 plants (6 mM N supply). However in T2 and T3 plants (non-external N supply) the concentration was much less varied during 14 days of treatment (Fig. 3A). The significant and distinct difference between two groups, one supplied with 6 mM N (control and T1) and another without external N supply (T2 and T3), clearly indicates that the external N nutrition has a closer influence on carbohydrate metabolism in shoots rather than anion replacement treatment. Much higher accumulation of sugars in T2 and T3 plants is well agreed with other results (Fishbeck and Phillips, 1981; Ourry et al., 1994) reported that in alfalfa lower availability of combined N increased carbohydrate synthesis. Sugars re-accumulation in T3 plants (water supply treatment) during the second 7 days likely to be related to sugars formation in response to the stress of nutrient deficiency (Volenec et al., 1997). A large decrease of sugars (Fig. 3A) and non-significant change of nitrate (Fig. 4A) in shoots of control and T1 plants were observed. But an inverse trend occurred in T2 and T3 plants (non-significant change in sugars and significant decrease in nitrate). From these data it could be suggested that when the uptake of external N exceeds assimilation as like control and T1 plants accumulate nitrate to a high level in the cells, especially in vacuoles, and that the stored nitrate have a reserve function to ensure protein synthesis under unfavorable conditions of nitrogen nutrition (Salsac et al., 1987). The continuous decrease of nitrate in T2 and T3 plants might be related to this suggestion.

These results clearly showed a negative correlation between the concentration of sugars and nitrate in shoots. In control and T1 plants nitrate accumulation (Fig. 4A) likely to be associated with osmotic regulation to compensate for the lack of sugars (Fig. 3A). The role of nitrate in osmoregulation in perennial ryegrass has been widely reported (Veen and Kleinendorst, 1985; Ourry et al., 1989; Kim, 2000). Kim (2000) reported that at low level of NO_3^- supply osmotic contribution of nitrate to cumulative osmotic potential was decreased, and it was osmotically compensated with soluble carbohydrates. Ourry et al. (1989) found that an over-compensation by nitrate at lower concentration, attributed to a concomitant decrease in other organic solutes such as amino acids and organic acids. Similarly, inverse relationships between carbohydrate and nitrate contents have been observed at different light intensities for ryegrass (Veen and Kleinendorst, 1985) and lettuce (Blom-Zandstra and Lampe, 1985).

Compared to NO_3^- supply (control), the concentration of nitrate was decreased to 27.3% or 71.9% without limiting the production of dry matter by replacing NO_3^- with SO_4^- or Cl^- during 14 days. In the plants supplied with water (T3) nitrate concentration reduced in the largest scale (83.9% of decrease) but the decrease of dry matter (31.3%) was accompanied. Urrestarazu et al. (1998) reported a similar result in lettuce. The decrease of nitrate when replace NO_3^- with Cl^- seems to be associated with an important role in osmotic regulation (Ourry et al., 1989), and Cl^- function for the induction of nitrate release from vacuoles (Santamaria et al., 1996). Overall data obtained indicate that chloride replacement for short time before harvest is a proper strategy to decrease nitrate content without limiting shoot yield.

V. 적 요

음이온의 대체공급이 이탈리안 라이그라스 (*Lolium multiflrum* L.)의 가식부위내 축적된 질산염의 농도에 미치는 영향을 규명하기 위해 NO_3^- 공급구를 대조구로 하고 SO_4^- (T1), Cl^- (T2) 및 water(T3)로 14일 동안 대체 공급한 후 무기영양이온의 흡수, 단백질, 당 및 질산염 농도를 분석하여 비교하였다. 무기영양이온의 흡수는 대조구에서 Na^+와 Ca^{++}, T2 처리구는 K^+와 PO_4^-, T1 처리구는 Cl^- 흡수가 상대적으로 높게 나타났다. 엽신과 그루터기의 단백질 함량은 질소를 공급해 준 대조구와 T1 처리구에서 높았다. 뿌리의 단백질 함량은 모든 처리구에서 감소하는 경향을 보였으며 특히 T3 처리구에서 가장 많이 감소하였다. 모든 처리구에서 엽신의 당 함량은 처리 후 7일 동안 감소하다가 이후 다시 증가하였다. 그루터기의 당 함량은 처리후 7일동안 감소하는 반면 뿌리에서는 변화폭이 낮았다. 처리후 14일차 대조구에서 엽신의 질산염 농도는 13 mg/g FW 이었다. 대조구에 비해 T1 처리구에서 27%, T2 처리구에서 46%, T3 처리구에서 50% 각각 감소하였다. 건물함량은 처리간 유의적인 차이는 없었지만 T3 처리구에서는 대조구에 비해 30% 감소하였다.

(**Key words** : 이탈리안 라이그라스, 음이온 대체공급, 이온 흡수, 단백질, 당, 질산염)

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