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Effects of NO_3^- Gradients on Nitrogen Fixation, Nitrate Reduction and Ureide Content of Soybean

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大豆의 窒素固定, 窒酸還元 및 Ureide含量에 미치는 NO_3^-의 영향

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ABSTRACT

Soybean, inoculated with effective *Rhizobium japonicum* 110, were grown by sand culture with nutrient solution containing either of 0,1,3,10 or 30 mM NO_3^-/l, and analyzed growth characteristics, NR activity, N_2-fixation activity, and changes of ureide contents during the growing period.

The amount of nodule formation decreased abruptly by nitrate treatment, the maximum nodule dry weight was 1.59, 1.05, 0.78, 0.09 and 0.008 g plant^{-1}, respectively for each treatment on the 98th day. Specfic activity of N_2-fixation showed the maximum rates of 140, 101, 37, 5 and 2.2 *n*M dw · mg^{-1} · hr^{-1}, respectively for each treatment in the earlier growth period. The maximum acetylene reduction activity on the 98th day after sowing was 81.5, 35.3, 14.3, 0.1 and 0.0045 μM C_2H_4 plant^{-1} · hr^{-1}, respectively for 0,1,3,10 and 30 *n*M of NO_3^- gradients. Nitrate reduction activity increased along with nitrate gradients, and decreased abruptly with age. Relative abundance of ureides in plant organs was high in reproductive growth, and showed the maximum value in fully symbiotic dependent plant. Relative abundance of ureides in stem is a useful indication for the evaluation of nitrogen fixation in nodules of symbiotic plant.

INTRODUCTION

Recently many investigations have been accumulated on the regulation of nodulation, symbiotic nitrogen fixation and assimilation of nitrogenous compounds of leguminous plants inoculated with *Rhizobium* sp.. It is a well known fact that NH_4^+ and NO_3^- of soluble inorganic compounds inhibited nodule formation and N_2-fixation activity(Evans *et al.* 1973). The number and weight of root nodules of soybean decreased abruptly with the increase of N-fertilizer application(Weber, 1966). Herridge(1982 a,b) reported that acetylene reduction activity of nodule was correlated inversely with the level of nitrate.

While, other researchers demonstrated from the analysis of nitrogen economy and detailed tracer experiments using ^{14}C and ^{15}N in legumes that the ureides, allantoin and allantoic acid, were major products of N_2-fixation, responsible for transporting approximately 80% of fixed

N from the root nodules of fully symbiotic plants (Atkins *et al.*, 1982; Fujihara *et al.*, 1977; Herridge *et al.*, 1978; Matsumoto *et al.*, 1977 a,b; Pate *et al.*, 1980; Rainbird *et al.*, 1984).

Ureide contents reflect a symbiotic dependence of plants, provide the basis for a quantitative movement of N_2-fixation, and are used more efficiently in seed protein than N in the form of amino acids, amides and nitrate(Day *et al.*, 1978; McClure *et al.*, 1979. 1980; McNeil *et al.*, 1984; Patterson *et al.*, 1983; Streeter, 1979. 1985).

On the other hand, the uptake of nitrate, reduction of nitrate and assimilation of subsequent amino acid are necessary primary processes of environmental nitrogen utilization in plant development, and provide regulatory mechanisms for nitrogenase and nitrate reductase activity (Elirich *et al.*, 1973; Harper *et al.*, 1972). The relationship between nitrate application and nitrate reductase activity was characterized by a saturation curve in leaves and a sigmoidal curve in roots(Robin *et al.*, 1979). However, further discussions are expected to clarify the regulation mechanism and quantitative relationship between enzymic activities of nitrogen metabolism and nitrogen fertilizer application.

The present study reports the effects of various nitrate gradients on nodulation, activities of nitrogenase and nitrate reductase, and changes of relative abundance of ureides during the growth of soybean plant.

MATERIALS AND METHODS

Soybean(*Glycine max* L. Merr. cv. Kwanggyo) inoculated at sowing with effective *Rhizobium japonicum* 110, were grown in sand culture in plastic pot(20 cm of diameter). Ten seeds of mean size were sown per pot. Treatments of nitrate gradients were made up of 0,1,3, 10 and 30 *m*M NO_3-N with N-free Boysen-Jensen medium. Each pot received 200 ml of culture medium of respective treatment daily.

Triplicate measurements were combined in the sampling design relating plant growth and nitrogenous contents of plant parts. Every fortnight sampling was made for the whole plant and determined the growth characters. Plant parts separated into each organ were oven dried at 80℃ for 72 hrs and used for the analysis of the contents of nitrogenous compounds. Fresh root nodules had been collected and placed into 10ml vials with rubber stopper, added 0.1 atm. of C_2H_2 (1 ml) and incubated at 30℃ to determine N_2-fixation activity.

Gas samples of 0.5 ml were removed with 1ml syringe from incubated vials at intervals of an hour, and analyzed the peak of ethylene production by PYE 304 Unicam gas chromatography equipped with a H_2-frame ionization detector and a column of porapak R(182 cm × 0.32 cm).

N_2-fixation activity was calculated with conversion ration of 1.5: 1(acetylene reduced: N_2-fixed) (Evans *et al.*, 1973). Nitrate reductase activity of leaves was determined by the method of Scott & Neyra(1979). Uppermost trifoliate leaves were punched into discs of 12 mm diameter, and tissue discs were placed into a vial containing reaction solution mixed with 2.5 ml of phosphate buffer (pH 7.5), 0.05 ml of isopropanol, 0.5 ml of 0.5 M KNO_3 and 2 ml of H_2O.

Vials were rubber stoppered and evacuated the air phase and replaced by argon gas to make anaerobic condition. After incubation in dark at 30 for 30 minutes and 1 hour, nitrite formed was determined by adding 0.1% of Gries Romijn reagent and reading absorbance at 540 nm. Total N contents of each plant part were analyzed by micro-kjeldahl method(Song & Monsi, 1974). For the determination of nitrate and ureides, dry samples(0.2 g) of leaves, stems, nodules and roots were extracted with 5 ml boiling water for 2 minutes. The extracts were filtered, made up to 10 ml of volume, and analyzed for nitrate by ultraviolet spectrophotometric method(Rand *et al.*, 1976). Ureides, allantoin and allantoic acid, were determined by Young & Conway method(1942) which degrades these two compounds to urea and glyoxylic acid.

The relative abundance of ureides of plant tissues was calculated as the percentage ratio of ureide-N to the sum of ureide-N plus nitrate-N. The symbiotic dependence was determined with the percentage ratio of N_2-fixation per total N contents of plant. Steps of vegetative and reproductive developments of soybean plant were noted according to the scheme of Fehr *et al.* (1971).

RESULTS AND DISCUSSION

Changes of the height growth of soybean plants with nitrate gradients showed sigmoid curves with little differences among treatments(Fig. 1). The maximum heights attained 73, 77,84,80 and 74 cm, respectively for 0,1,3,10 and 30 mM of NO_3-N treatments on the 98th day after sowing. Statistical analysis of all data showed no significant differences among treatments.

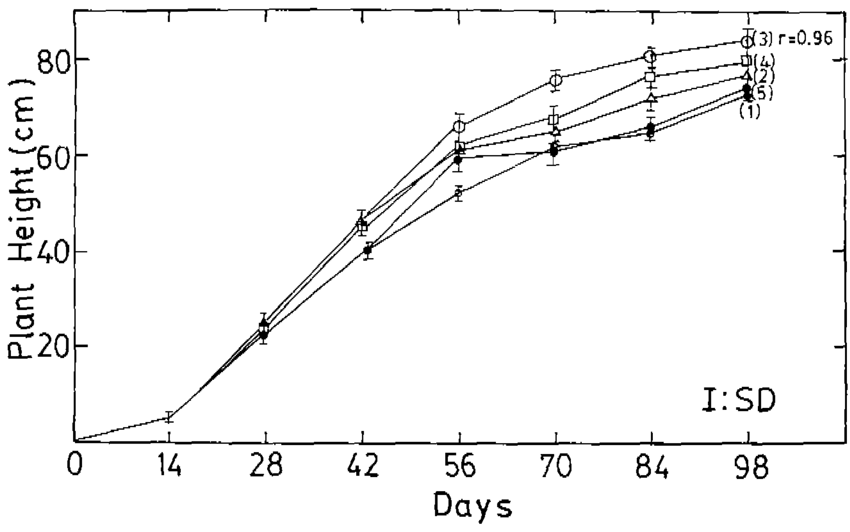

Fig. 1. Effects of NO_3^- gradients on the height growth of soybean: (1) control; (2) 1 mM; (3) 3 mM; (4) 10 mM and (5) 30 mM KNO_3, SD:standard deviation.

Table 1. Steps of vegetative(V)* and reproductive(R)** developments of soybean treated with NO_3^- gradients

KNO₃ Treatment	Days after sowing					
	28	42	56	70	84	98
Control	V4	V5	V8	V11, R2	V12, R3	V14, R5
1 mM	V4	V6	V9	V10, R3	V11, R3	V13, R5
3 mM	V4	V6	V9	V11	V15, R2	V16, R4
10 mM	V4	V5	V8	V11	V12, R2	V14, R3
30 mM	V4	V5	V8	V 9	V11, R1	V13, R3

* Vegetative stage numbers are determined by counting the number of nodes on the main stem, beginning with the unifoliolate nodes. **Reproductive stages are based on flowering, node development, seed development, and plant maturation(Fehr *et al.*, 1971).

Developmental steps of vegetative and reproductive growth changed by nitrate gradients are shown in Table 1. The reproductive growth initiated from 8 weeks after sowing for the plants of control and 1mM nitrate treatments, but delayed a few weeks for the plants of higher nitrate gradients. Nodulation and N_2-fixation activity appeared characteristic change patterns showing remarkable differences among nitrate gradients(Fig. 2). Plants exposed to the highest level of nitrate(30 mM) resulted in the lowest formation of root nodule tissues and the lowest rates of

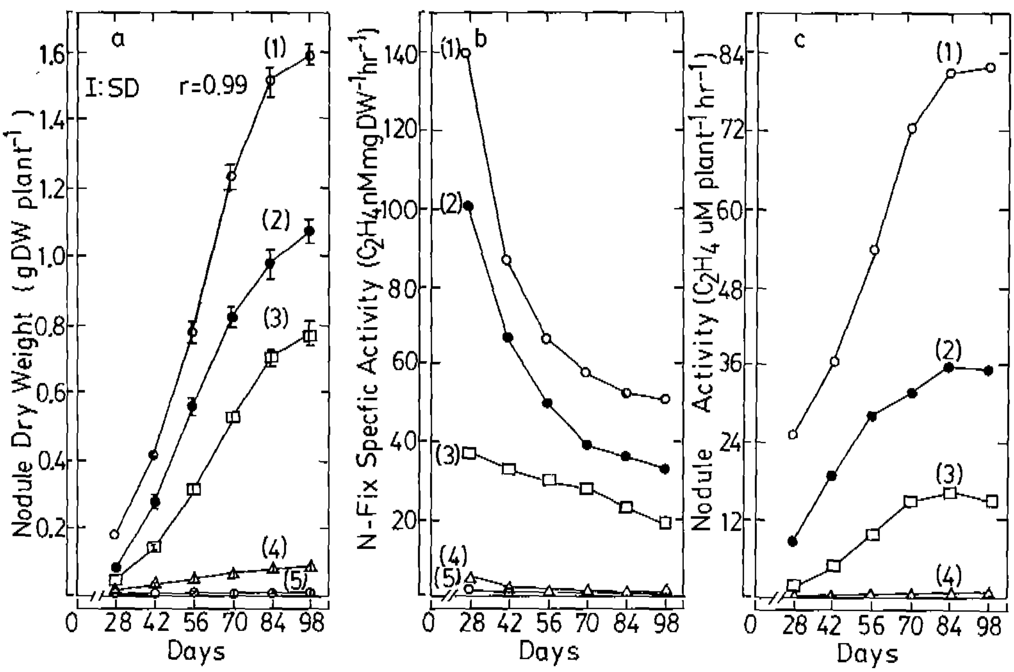

Fig. 2. Effects of nitrate gradients on a) nodule dry weitht increase, b) specific activity(C_2H_4 nM dw · mg^{-1} · hr^{-1}) and c) total acetylene reduction activity(μM plant^{-1}. hr^{-1}); (1) control; (2) 1mM; (3) 3mM; (4) 10mM and (5) 30mM KNO_3, SD:standard deviation.

N_2-fixation activity. The completely symbiotic plant grown without N-sources produced the most abundant nodule tissues and showed the highest acetylene-reduction activity. Nodules began to appear from 18th day after sowing and increased exponentially during the active growing period of plant biomass attaining the maxium amounts after 98th day from sowing. The maximum dry weight of root nodules were 1.59, 1.05, 0.78, 0.09 and 0.008 g per plant, respectively for 0, 1, 3, 10 and 30 mM of nitrate treatments(Fig. 2a). The formation of root nodules was inhibited by 28.2-50.0, 50.9-68.9, 88.9-94.6 and 95.3-99.5%, respectively for 1, 3, 10 and 30 mM of nitrate treatments when it is compared with that of the fully symbiotic plants during the growth period. The specific activity of N_2-fixation of soybean nodules showed apparent decrease by the increase of nitrate gradients and tissue ages, and attained the maximum rates of 140, 101, 37, 5 and 2 *m*M C_2H_4 dw mg^{-1} $hour^{-1}$, respectively for 0, 1, 3, 10 and 30 mM of NO_3-N treatments(Fig. 2b). Therefore, seasonal profiles of acetylene reduction activity of soybean showed remarkable increase between 42th and 70th day after sowing, and characterized with gradual inhibition by the increase of nitrate gradients. The maximum rate of total acetylene reduction per plant on the 98th day after sowing was 81.5, 35.3, 14.3, 0.1 and 0.0045 μM C_2H_4 per hour, respectively for 0, 1, 3, 10 and 30 mM of nitrate treatments(Fig. 2c). In the early growing stage the relationship between root nodule weight(g) and leaf(cm^2) showed a good linear correlation among nitrate gradients.

Effects of nitrate gradients on the percentage allotments of N_2-fixation for total N-contents of soybean plant inoculated with *Rhizobium japonicum* 110 are shown in Table 2. Ratio of

Table 2. Effects of NO_3^- gradients on the percentage allotments of N_2-fixation for total N-contents of soybean

KNO_3	Days after sowing					
Treatments	28*	42	56	70	84	98
Control	100.00	100.00	100.00	100.00	100.00	100.00
1 mM	52.80	66.15	66.90	59.72	58.90	59.00
3 mM	22.14	30.00	35.22	37.08	36.61	34.90
10 mM	5.05	4.12	3.87	2.74	2.54	2.67
30 mM	0.72	0.73	0.77	0.66	0.49	0.54

N_2-fixation to total-N input for the plant growth was decreased abruptly with increasing levels of nitrate gradients due to the inhibition of N_2-fixation activity and the increased amount of absorption from environmental nitrogen source. The more the nitrate gradients decreased, the more the dependence to N_2-fixation increased with the plant development. Nitrate contents and nitrate reductase activity of leaves increased with nitrate gradients as shown in Fig. 3. The maximum nitrate contents and nitrate reductase activity appeared in the early growing stage showing gradual decrease with age. It seems that nitrate contents of plant tissue decreased due to the dilution effect by the quick accumulation of plant biomass and the assimilation of organic nitrogen on account of leaf growth.

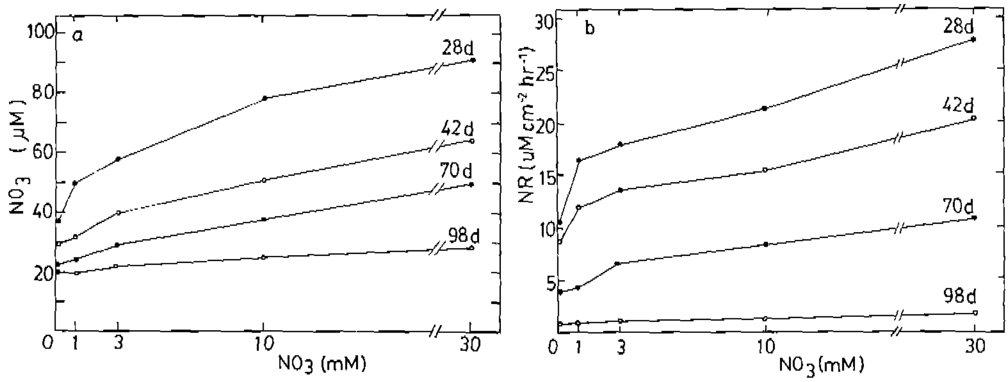

Fig. 3. Effects of nitrate gradients on nitrate concentration(1) and nitrate reduction activity(b) in leaves of soybean.

Changes of relative abundance of ureides in each plant organ are illustrated in Fig. 4. The highest contents of ureides were 46, 68, 55 and 48%, respectively for leaves, stems, roots and nodules in the completely symbiotic plant, and relative abundance of ureides increased abruptly during reproductive growth. The relative abundance of ureides decreased with increasing levels of nitrate gradients.

The ureide contents as major nitrogenous products of N_2-fixation from nodulated soybean

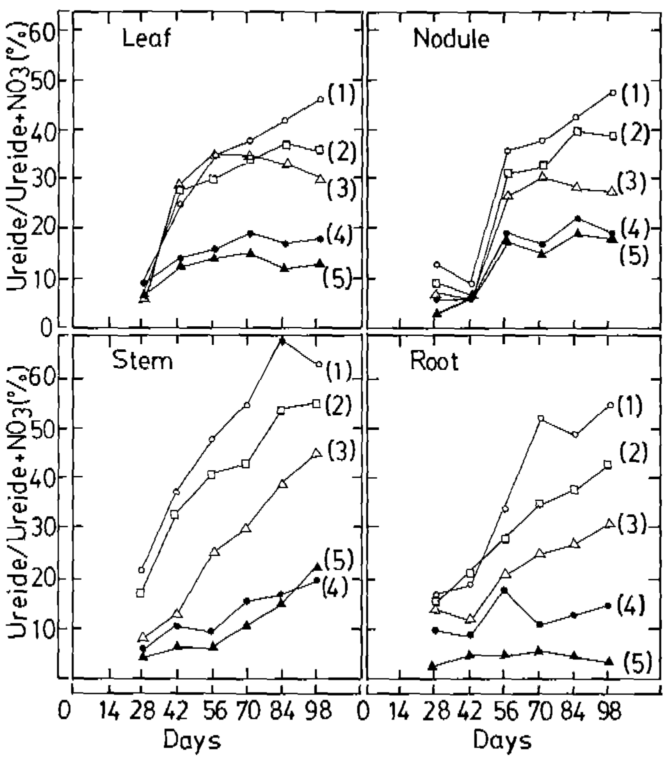

Fig. 4. Effects of nitrate gradients on the relative abundance of ureides(expressed as the percentage ratio ureide-N to the sum of ureide-N plus nitrate-N) of soybean: (1) control; (2) 1mM; (3) 3 mM; (4) 10 mM; (5) 30 mM KNO_3.

increased by the high rate of N_2-fixation activity of the plant. It suggested that ureides are major transport form of nitrogen. Therefore, N_2-fixation activity can be evaluated by relative abundance of ureides of stem and root.

Fig. 5. illustrates the relationship between the relative abundance of ureide content of each organ and the symbiotic dependence of the plant. Actually, relative abundance of ureide increased with the increase of symbiotic dependence and was remarkably high during the

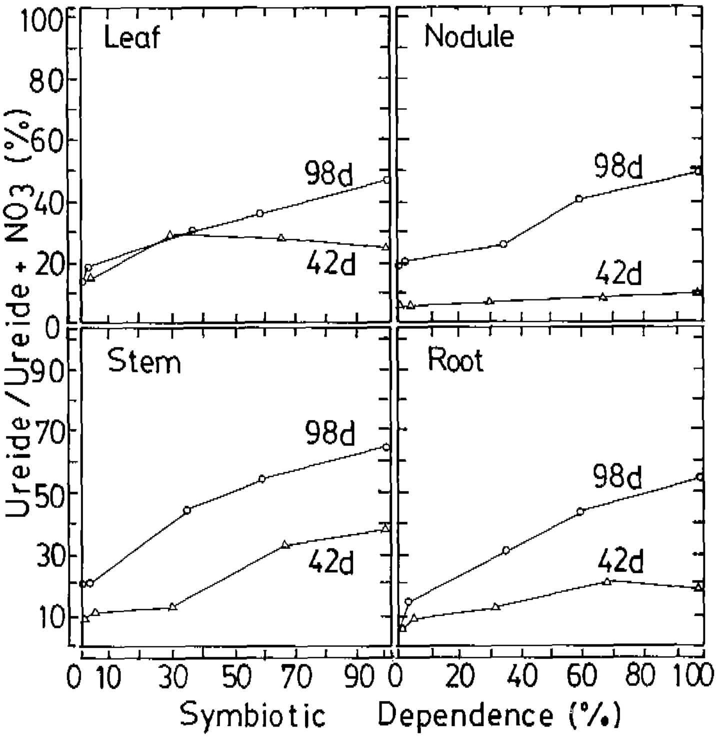

Fig. 5. Relationships between the relative abundance of ureides in plant organs and the symbiotic dependence of plant during the soybean vegetative and the reproductive growth.

reproductive growth(98th day) than the vegetative growth(42th day) for stems, roots and nodules. However, the correlation between relative abundance of ureides and symbiotic dependence in leaf was low accounting for further synthesis of various nitrogenous compounds as the sink, and further work is needed to demonstrate the mechanism of inhibitory and regulatory functions for the nitrogenase system of root nodules by nitrate gradients.

摘 要

대두(*Glycine max* L. Merr. cv. Kwanggyo)를 NO_3^-구배의 배양액으로 사경재배하여 생장과정 중의 생육특성, 질소고정활성, 질산환원활성 및 Ureide 함량변화를 분석하여 얻은 결과는 다음과 같다.

NO_3^- 구배처리에 따라 대두의 근류형성량은 급격히 감소하였으나, 생장과 더불어 증가하여 최대치는 98일째에 0, 1, 3, 10 및 30 *m*M KNO_3구에서 각각 1.59, 1.05, 0.78, 0.09 그리고 0.008 g dw · $plant^{-1}$

였다. 질소고정활성은 생장초기에 각각 140, 101, 37, 5 및 2.2 nM dw · mg^{-1} · hr^{-1}로 최대치를 나타낸 후 급격히 감소하였다. 최대 질소고정량은 98일째에 각각 81.5, 35.3, 14.9, 0.1 및 0.0045 μM $plant^{-1}$.hr^{-1}의 순으로 질소구배에 따라 감소하였다. 질산환원활성은 NO_3^- 처리농도가 클수록 생장 초기에 높았고, 생장과 더불어 급격히 감소하였다. 각 기관별로 ureide 상대함량은 생장단계에 따라 증가하였고, 무질소구에서 최대치를 나타내었으며, 질소농도 구배에 따라 급격히 감소하였다. 특히 뿌리와 줄기에서는 공생의존도가 클수록 ureide 상대함량의 증가가 현저하였고, 영양생장보다 생식생장에서 높은 값을 보여 질소고정활성의 지표로서 이용될 수 있었다.

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