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Effect of Nitrate on Nodulation and Nitrogen Fixation of Soybean

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1. Introduction

1.1 Biological nitrogen fixation and nitrogen nutrition in soybean plants

Biological nitrogen fixation is one of the most important processes for ecosystem to access available N for all living organisms. Although N₂ consists 78% of atmosphere, but the triple bond between two N atoms is very stable, and only a few group of prokaryotes can fix N₂ to ammonia by the enzyme nitrogenase. Annual rate of natural nitrogen fixation is estimated about 232 × 10⁶ t, and the 97% depends on biological nitrogen fixation (Bloom, 2011). This exceeds the rate of chemical nitrogen fertilizer uses about 100 × 10⁶ t in 2009. Soybean can use N₂, though symbiosis with nitrogen fixing soil bacteria, rhizobia, to make root nodules for harboring them.

Soybean (*Glycine max* [L.] Merr.) is a major grain legume crop for feeding humans and livestock. It serves as an important oil and protein source for large population residing in Asia and the American continents. The current global soybean production was 231 × 10⁶ t in 2008 (FAOSTAT). It is a crop predominantly cultivated in U.S.A., Brazil, Argentina and China, which together contribute nearly 87 percent of the total world produce in 2008. Soybean has become the raw materials for diversity of agricultural and industrial uses.

Soybean seeds contain a high proportion of protein, about 40% based on dry weight, therefore, they require a large amount of nitrogen to get a high yield. About 8 kg N is required for 100 kg of soybean seed production. Soybean can use atmospheric dinitrogen (N₂) by nitrogen fixation of root nodules associated with soil bacteria, rhizobia. Soybean plants can absorb combined nitrogen such as nitrate for their nutrition either from soil mineralized N or fertilizer N.

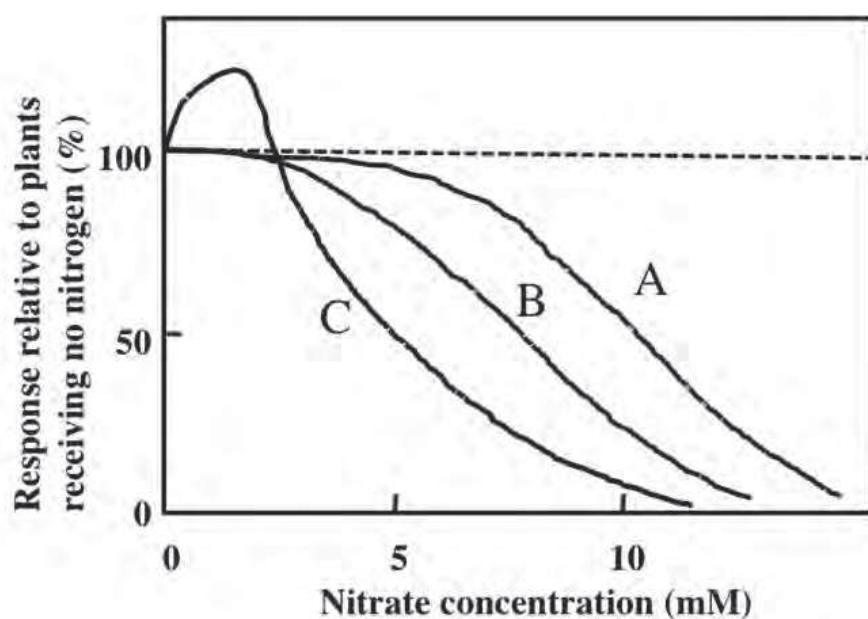
It is well known that heavy supply of nitrogen fertilizer often causes the inhibition of nodulation and nitrogen fixation. Therefore, only a little or no nitrogen fertilizer is

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practically applied for soybean production. However, soybean plants only depend on the nitrogen fixation shows poor growth and low seed yield, because of the early decline in photosynthesis by decreased supply of nitrogen during the pod filling stage. Harper (1974) reported that both soil N and symbiotic N are required for the optimum soybean production.



A: nodule number per a plant, B: Nitrogen fixation activity per g dry weight of nodules, C: Nodule mass per a plant.

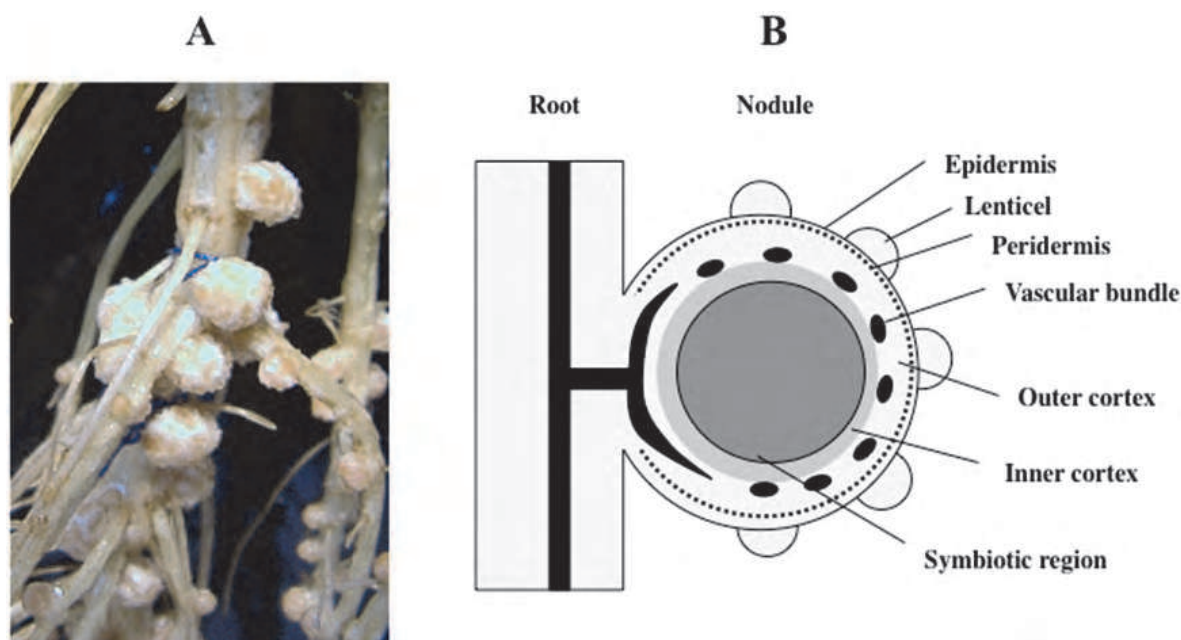
Fig. 1. Response of legume nodules to nitrate proposed by Streeter.

The inhibitory effect of nitrate on nodulation was early reported by Fred and Graul (1916) as cited in Streeter (1988), however, the precise mechanism for the inhibition of nodulation and nitrogen fixation has not been fully understood. In the review article for inhibition of legume nodule formation and N_2 fixation by nitrate written by Streeter (1988), he proposed the responses to nitrate illustrated in Fig. 1. Curve A represents nodule number per a plant, which appears a relatively high nitrate concentration. Curve B is on nitrogen fixation activity per unit mass (g dry weight) of nodules. Curve C shows the growth response (nodule mass per a plant), this response is most sensitive to nitrate concentration, although a low concentration of nitrate as low as about 1-2 mM nitrate promotes nodule growth.

1.2 Nodule structure and function of soybean

Soybean nodule appears about 10 days after sowing when inoculated with compatible strain of rhizobia, and it grows about 3mm until about 20 days after planting (Fig. 2. A.) . The nodules start to fix nitrogen (Sato et al., 2001, Ito et al., 2006). The maximum size of nodule reaches maximum about 6-7 mm diameter, and then they eventually senesce and degrade. Soybean nodule is classified to a determinate type nodule, which has a spherical form, and nodule growth is mainly due to cell expansion after initial cell proliferation and development.

Fig. 2.B shows the structural model of a soybean nodule attached to the root. The soybean nodule has the symbiotic region (or infected region in synonym) in the center, which



A: Photograph of soybean nodules formed in the roots of a plant cultivated by hydroponics. B: Structural model of soybean nodule.

Fig. 2. Soybean root nodules.

consists the mosaic of large infected cells and small uninfected cells. The infected cells are filled with bacteroids (the symbiotic forms of rhizobia) and they are easily recognized by the red color with nodule specific protein, leghemoglobin (Lb). The nitrogenase, an enzyme to fix N_2 in bacteroid, is very susceptible to free O_2 and irreversibly destroyed by O_2 , therefore, free O_2 concentration should be kept very low in symbiotic region of nodules. There are four major components of Lb, Lba, Lbc1, Lbc2, and Lbc3 (Sato et al., 1998, 1999a). The Lb in legume nodules solves the dilemma to keep free O_2 concentration low and sufficient supply of O_2 for bacteroid respiration to support nitrogen fixation and the assimilation. Lb is a most abundant protein in nodules (about 20% of total protein) and it can bind with O_2 to form LbO_2 to decrease free O_2 concentration in the infected cells. On the other hand, nitrogen fixation and assimilation processes require a large amount of energy and reductant produced by O_2 respiration, therefore, nodule respiration is about four times higher than that of roots based on dry weight. To support active respiration, abundant supply of O_2 is essential.

Symbiotic region is surrounded by nodule cortex where the network of vascular bundles surrounding the symbiotic region to supply photoassimilate to bacteroids and to receive N_2 fixation products from them. Nodule cortex consists of inner cortex with small cells and outer cortex with large loosely packed cells. The sclerenchyma cells, which have thick cell wall were located in the outer cortex. O_2 concentration decreases sharply through the inner cortex, and the O_2 permeability is flexibly controlled by this layer (Witty and Minchin 1990, Hunt and Layzell 1993). It is hypothesized that a reversible exchange of intercellular water by the inner cortical cells plays a role in the regulation of nodule conductance to O_2 diffusion (Serraj et al., 1995, 1998, Fleurat-Lessard et al., 2005). There are lenticels outside of nodules and one layer of epidermis. Under the epidermis, there is a peridermis, a tightly packed one layer of cells, which may restrict free diffusion of solutes between inside the nodule and medium solution.

The group of positron-emitting tracer imaging system (PETIS) for plant analysis in Quantum Beam Science Directorate, Japan Atomic Energy Agency, developed a novel method of non-invasive observation and quantification of nitrogen fixation in intact soybean plants (cv. Williams) with nodules using ^{13}N -labeled nitrogen gas ($[^{13}\text{N}]\text{N}_2$) tracer and a PETIS (Ishii et al., 2009, Fujimaki et al., 2010). CO_2 gas was irradiated with a proton beam delivered from a cyclotron (Takasaki Advanced Radiation Research Institute, Japan Atomic Energy Agency) to produce ^{13}N nuclei by the $^{16}\text{O}(\text{p}, \alpha)^{13}\text{N}$ nuclear reaction. $[^{13}\text{N}]\text{N}_2$ was isolated from the resulting gas using gas chromatography and then mixed with appropriate composition of oxygen and (non-radioactive) nitrogen gases for the following feeding experiment. The total time required for the purification procedures was approximately 15 min, which is about 1.5 times the half-life of ^{13}N (only 9.97 min) and short enough to yield sufficient radioactivity of the tracer.

PETIS is one of the most advanced imaging methods today, which provides serial images of movement of positron-emitting radiotracers inside living plant bodies, like a video camera. The root of an intact test plant with nodules was immersed in a hydroponic culture solution in an acrylic box sealed with plastic clay to prevent leakage of the fed gas. This set-up was placed at the midpoint between the opposing detector heads of the PETIS apparatus so that the underground part in the acrylic box was in the field of view (Fig. 3.). The tracer gas was introduced into the box and the solution level was lowered simultaneously, then this was kept for 10 min for exposure of the nodules to the tracer gas. Finally, the tracer gas was flushed out by flowing the ambient air into the box. The two-dimensional distribution of ^{13}N in the field of view was continuously monitored by PETIS for 1 h.

As a result, obvious signals of ^{13}N were observed at the positions of the nodules (Fig. 4.). Moreover, the rates of nitrogen fixation in the whole nodules were quantitatively estimated from the PETIS data. The nitrogen fixation rate of the whole nodules was estimated at $7 \mu\text{g N}_2 \text{ h}^{-1}$ in this case. The largest advantage of this method is that it is non-invasive. The instant response of fixation activities to nitrate application will be examined in a future study.

Soybean nodule is highly organized complex organ as shown by the distribution of minerals examined by EPMA (Electron Probe X-ray Microanalysis) (Mizukoshi et al., 1995). Fig. 5. shows the distribution of minerals in nodulated roots. The concentrations of N and P were higher but those of K and Cl were lower in the symbiotic region compared with nodule cortex. Ca was locally distributed in the surface layer, sclerenchyma cells and inner cortex, but the content was low in the central symbiotic region. Mg specifically accumulated in the inner and outer cortex inside sclerenchyma cells but not outside them (Mizukoshi et al., 1995).

Fig. 6. shows the outline of N metabolism in soybean nodule (Ohyama et al., 2009). Ammonia is known to be the initial product of nitrogenase. After discovering a new enzyme glutamate synthase (GOGAT) in *Aerobacter aerogenes*, it is confirmed that ammonia can be assimilated via alternative of GDH, via glutamine synthetase (GS) and GOGAT pathway (Ohyama et al. 2009). From the result obtained by the $^{15}\text{N}_2$ pulse chase experiment, the ammonia fixed by nitrogenase in bacteroids is rapidly incorporated into of amido-N of glutamine, followed by glutamate, and amino-N of glutamine in this sequence was in accordance with the initial assimilatory pathway be GS/GOGAT pathway rather than GDH. This was supported by the evidence that the rapid decline of ^{15}N in glutamine but not glutamate immediately after changing to the chase period. A major part of fixed N was used for purine synthesis in infected cells then uric acid is

transported to the uninfected cells, then degraded into allantoin and allantoate. All the species in Phaseoleae (soybean, common bean, cowpea etc.) and some species in Robinieae, Indigofoeae and Desmodieae transport ureides (Atkins, 1991). Reviews on ureide biosynthesis in legume nodules were published (Schubert, 1986, Tajima et al., 2004). We compared the labeling patterns of ureides and amino acids from $^{15}\text{N}_2$ and $^{15}\text{NO}_3^-$ (Ohyama & Kumazawa, 1979), and the labeling pattern indicated that most of ureides derived from fixed N rather than absorbed N.

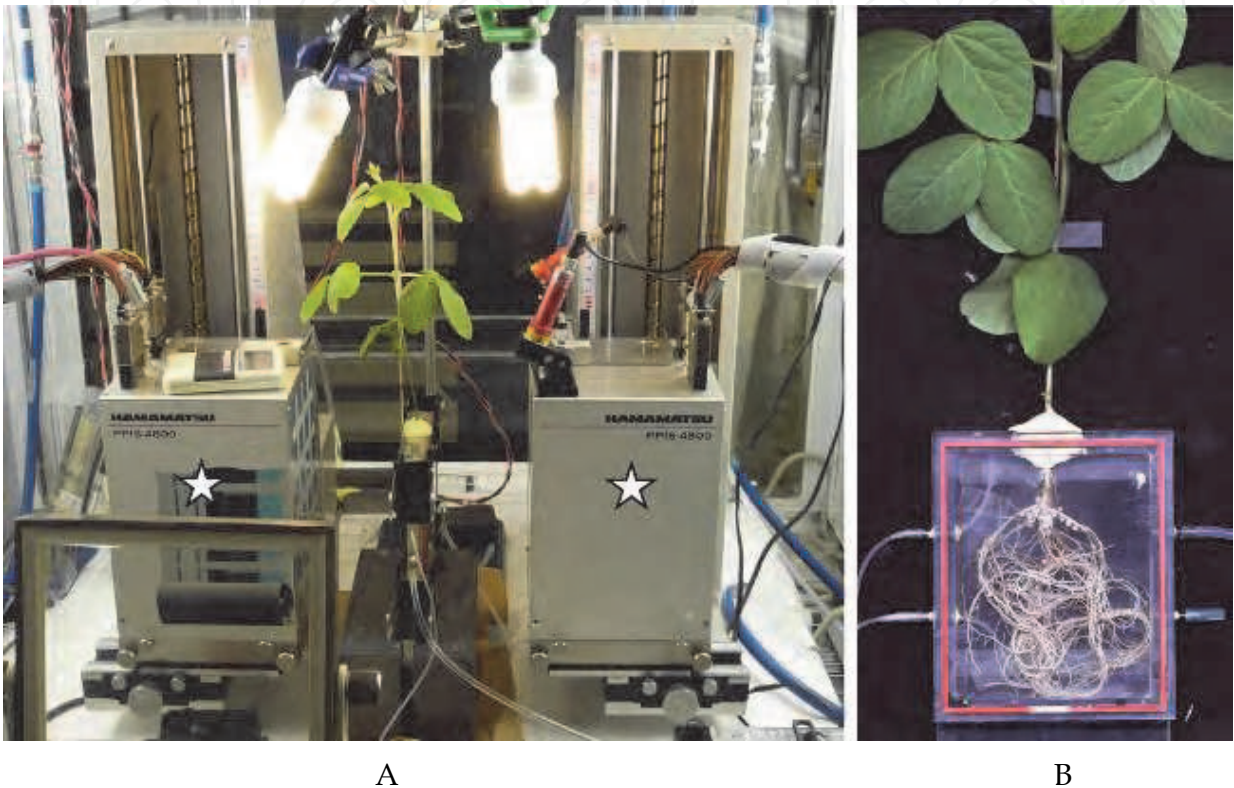
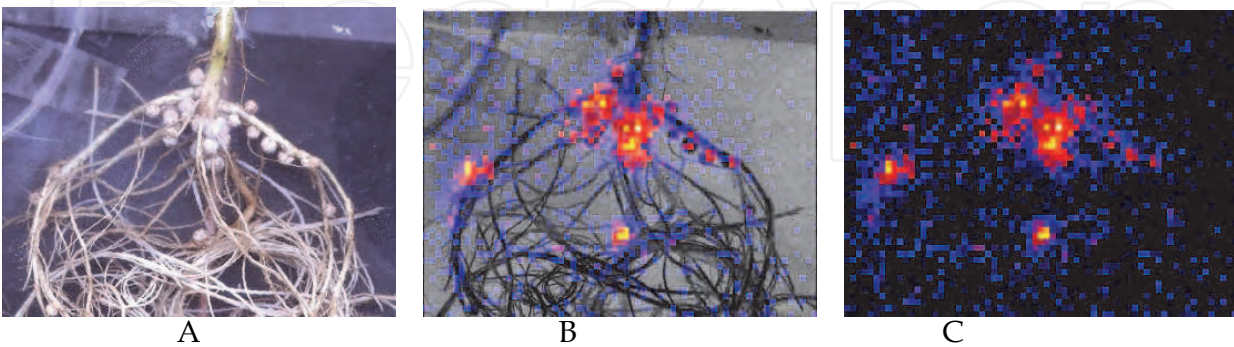


Fig. 3. Set-up for the PETIS experiment (A) and a test plant (B). Star signs indicate the opposing detector heads of the PETIS apparatus.



A: Nodulated roots of a test plant.
B: The merged image of nodulated root and radioactivity in the same view.
C: PETIS image of radioactivity.

Fig. 4. Image of radioactivity after exposure the nodulated soybean roots to $^{13}\text{N}_2$.

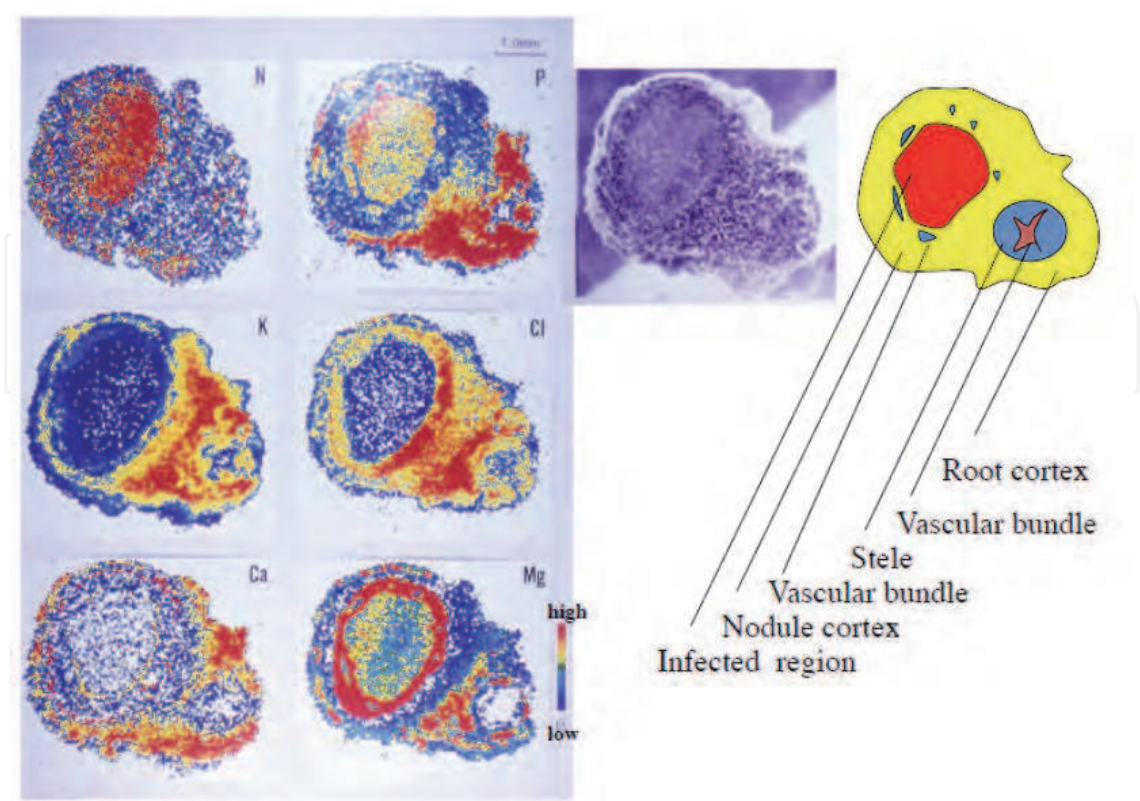
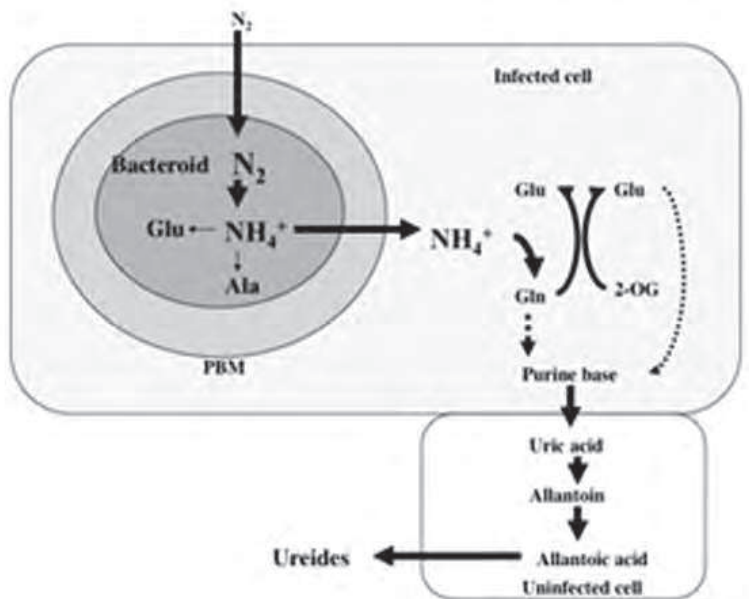


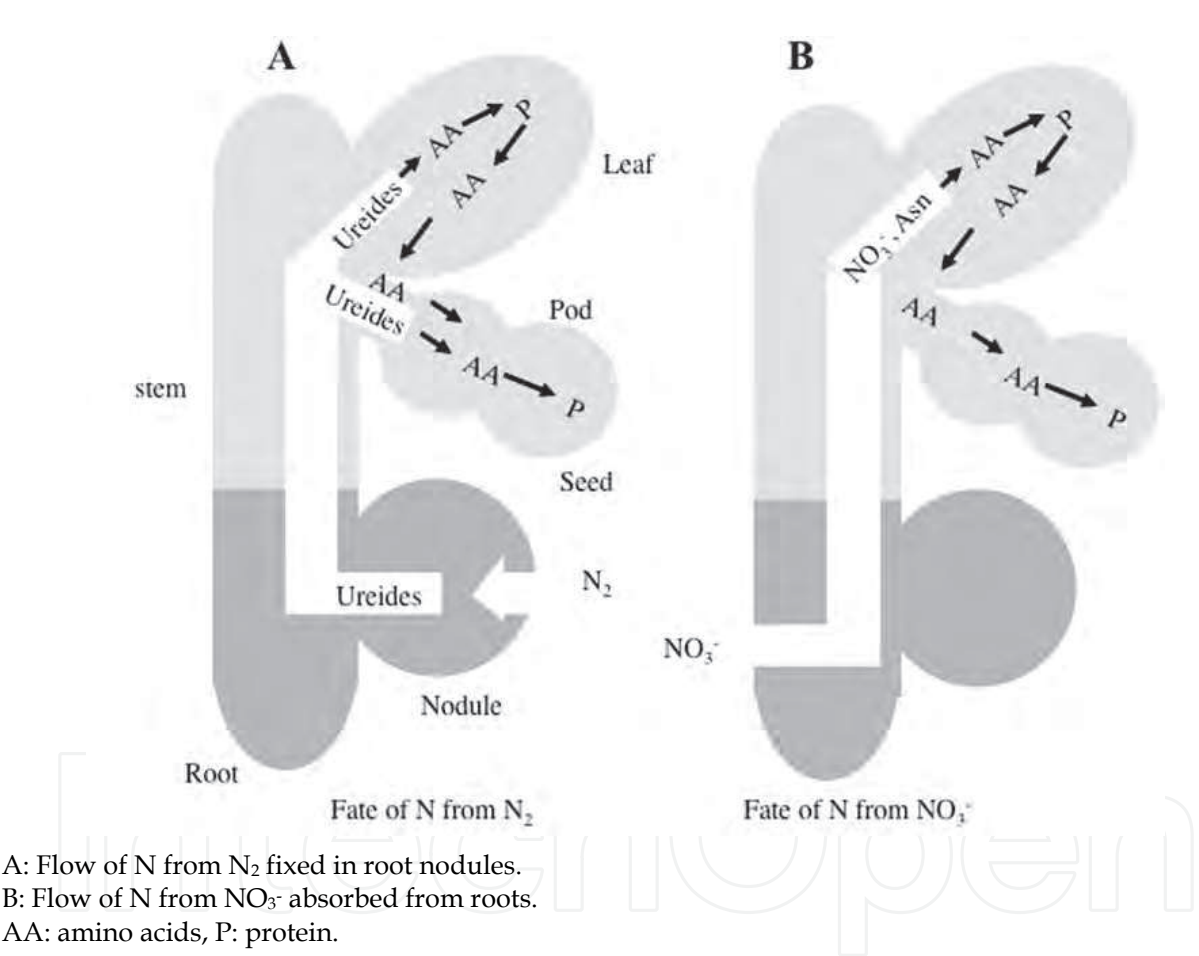
Fig. 5. Distribution of N, P, K, Cl, Ca and Mg in a nodule and root. The concentration is higher in red, orange, yellow, green, blue and white in this sequence.



Gln: glutamine, Glu: glutamate, 2-OG: 2-oxoglutarate, PBM: peribacteroid membrane.

Fig. 6. A model of the N flow of fixed N_2 in soybean nodules.

Fig. 7. shows the model of nitrogen assimilation and transport of N derived from N_2 fixation and NO_3^- absorption in soybean plants (Ohyama & Kumazawa, 1978, 1979, 1980abc, 1981ab, 1983, 1984, Ohyama et al., 2009) . The N fixed in nodule is exported to the host plant as in the form of allantoin and allantamate about 80-90% of total N. On the other hand, some part of the NO_3^- absorbed in the roots are reduced in the roots to NO_2^- by nitrate reductase, then the NO_2^- is further reduced to NH_4^+ by plastidic nitrite reductase, then the NH_4^+ is assimilated by GS/GOGAT pathway in the roots, and mainly metabolized to asparagine then transported to shoot via xylem. Some part of NO_3^- is directly transported through xylem to the shoots and reduced in leaves. Ohtake et al. (1995) reported the seasonal changes in amino acid composition in xylem sap of soybean and they confirmed that asparagine was the principal amino acids in xylem sap collected from basal cut end of the stem at any stages.



A: Flow of N from N_2 fixed in root nodules.
B: Flow of N from NO_3^- absorbed from roots.
AA: amino acids, P: protein.

Fig. 7. A model of N flow in soybean plant.

1.3 Nitrate inhibition of nodule growth and nitrogen fixation

The inhibitory effects of externally supplied N especially NO_3^- have been reviewed (Streeter, 1988, Harper, 1987). The nitrate inhibition is complex and it cannot be explained by a single mechanism. It has been suggested that there are multiple effects of nitrate inhibition, such as the decrease in nodule number, nodule mass, and N_2 fixation activity, as well as the acceleration of nodule senescence or disintegration (Streeter, 1988, Harter, 1987). In addition, nitrate inhibition of nodules is complex, because the effects of nitrate

on nodule formation and growth are influenced by nitrate concentration, placement and treatment period as well as legume species (Harper & Gibson, 1984, Gibson & Harper, 1985, Davidson & Robson, 1986).

Nitrate inhibition is primarily host plant dependent and it is independent of nitrate metabolism of rhizobia (Gibson & Harper, 1984, Carrol & Mathews, 1990). Many hypothesis are proposed for the cause of nitrate inhibition of nodulation and N_2 fixation, i.e. carbohydrate deprivation in nodules (Streeter, 1988, Vessy & Waterer, 1992), feedback inhibition by a product of nitrate metabolism such as glutamine (Neo & Layzell, 1997), asparagine (Bacanambo & Harper, 1996, 1997), and decreased O_2 diffusion into nodules which restricts the respiration of bacteroids (Schuller et al., 1988, Vessey et al., 1988, Gordon et al., 2002). Kanayama and Yamamoto proposed that NO formed from NO_3^- binds to Lb to make nitrosylleghemoglobin and defect the O_2 binding activity (Kanayama & Yamamoto, 1990). On the other hand, Giannakis et al. (1988) suggested that nitrate metabolism does not occur in symbiotic region of soybean nodule, even when a dissimilatory NR is expressed, because of restricted access of nitrate.

Leghemoglobin (Lb) plays a crucial role in N_2 fixation of leguminous nodules by facilitating O_2 supply to the bacteroids. There are four major components of Lb in soybean nodules, Lba, Lbc1, Lbc2, and Lbc3, and different roles are suggested among components (Fuchsman et al., 1976), because Lba has higher affinity for O_2 than has Lbc. The concentrations of Lba and Lbc were separated by Native PAGE (Nishiwaki and Ohyama, 1995). All the four components Lba, Lbc1, Lbc2, and Lbc3 were separately determined by capillary electrophoresis (Sato et al., 1997). The concentration and component ratios in the hypernodulation mutant NOD1-3, NOD2-4, and NOD3-7 from Williams parent, and in En6500 from Enrei parent were compared in relation to their nodulation characteristics. Three mutants (NOD1-3, NOD3-7 and En6500) were controlled by a single recessive allele *rjz*, but NOD2-4 was non-allelic mutant to them (Vuong et al. 1996). Plants were hydroponically cultivated in N free solution, and the nodules were separated by size. Concentration and composition of Lb components in the same size nodules were analyzed by gel-electrophoresis and capillary electrophoresis. In all NOD mutants Lb concentration was about 70% of that in the parent Williams, irrespective of nodule size and growth stages. In the hypernodulation mutant En6500, the total Lb concentration was only 25% of that in the parent Enrei, irrespective of nodule size. In Enrei, relative compositions of Lba, Lbc1, Lbc2 and Lbc3 were 36, 26, 18 and 17%, respectively, and very stable irrespective of nodule size. En6500 had relatively equal amounts of each component in which the relative compositions of Lba, Lbc1, Lbc2 and Lbc3 were 30, 22, 22 and 26%. The concentration of Lbc forms in nodules was decreased by addition of nitrate to Enrei plants, but not to En6500. When the nodule morphology was compared among hypernodulation mutant lines and parent lines, we noticed that mutant line had thick cortical regions relative to the comparable parent nodules. The relative volume of symbiotic regions was about 50-60% of total nodule volume in Williams, but it accounted for only 40-50% in NOD mutants.

Sato et al. (2001) investigated the changes in four leghemoglobin components in nodules of NOD1-3 and its parent in the early nodule developmental stage. The hydroponically grown NOD1-3 and Williams were periodically sampled. All the visible nodules were collected from the roots and then the four Lb components in the largest nodules were analyzed by capillary electrophoresis. In NOD1-3 nodule development was faster than those of Williams. Acetylene reduction activity was detected at 19 days after planting in NOD1-3 and at 22 days after planting in Williams. In addition the Lbs were initially detected at 19 days after

planting in NOD1-3, a few days earlier than in Williams at 22 days after planting. The Lbcs (Lbc1, Lbc2 and Lbc3) were the main components at the earliest nodule growth stage, and the relative proportion of Lba increased with nodule growth in both NOD 1-3 and Williams. The hypernodulation soybean mutant lines (NOD1-3, NOD2-4, NOD3-7) and the parent Williams and mutant line En6500 and the parent Enrei were cultivated in a sandy dune field in Niigata, and the nodules and root bleeding xylem sap were analyzed at 50, 70, 90 and 120 days after planting (Sato et al., 1998). The number of nodules of the hypernodulation mutant lines was about two to three times higher than that of the parent lines irrespective of sampling date. The concentration of Lb components was measured by capillary electrophoresis. The concentration of Lb components in the hypernodulation mutant lines tended to be lower than in the parents, but the component ratios were not different between mutants and the parents.

It is well recognized that plant growth is affected by various environmental factors, such as temperature, moisture, photoperiod, light intensity and quality, as well as physical, chemical, and biological properties of soil. The degree of nitrate inhibition was affected by soil medium composition with vermiculite and perlite, where the proportion of solid, liquid and gas space was changed (Nishiwaki et al., 1995).

It has been reported in alfalfa that the inhibition of nodulation by nitrate was reduced by medication of ethylene production inhibitor aminoethoxyvinylglycine (Ligero et al., 1991). While the exogenous ethylene inhibited nodulation on the primary and lateral roots of pea (Lee & LaRue, 1992ab). Ethylene is one of the important phytohormone regulating plant growth. Ethylene is produced through oxidative decomposition of 1-aminocyclopropane-1-carboxylic acid (ACC), and silver thiosulfate (STS) is a potent inhibitor of ethylene action in plants (Veen, 1983). Sato et al. (1999c) investigated the effect of ethylene action on soybean nodulation using ACC and STS in relation to the inhibitory mechanism of nitrate using hypernodulation mutant NOD1-3 and the parent Williams. The hypernodulation mutant of soybean NOD1-3 and its parent Williams were cultivated in culture solution with or without NO_3^- , and ACC or STS were added in the solution. The nodule dry weight was decreased by both ACC and STS treatments, however, the ratio in nodule dry weight in total plant dry weight were not significantly influenced by these treatments with or without NO_3^- . Therefore, it was concluded that the decrease in nodule dry weight by ACC or STS was caused by inferior growth. In soybean the depression of nodulation and N_2 fixation by nitrate is not mediated through ethylene action. Schmidt et al. (1999) also reported the independence of ethylene signaling on the regulation of soybean nodulation. Moreover, the nodulation of hypernodulation mutant was not specifically influenced by ACC treatments. This suggests that autoregulation of nodulation may not be involved in ethylene action or transduction pathways in soybean plants.

Recently, defective long-distance auxin transport regulation was reported in the *Medicago truncatula* super numeric nodules mutant (Van Noorden et al., 2006). However, similar trend is not observed in hypernodulation mutants of soybean. Terakado et al (2005) reported that systemic effect of brassinosteroid on nodule formation in soybean after the foliar application of brassinolide and brassinazole, the inhibitor of brassinosteroid formation. In addition, they reported that shoot applied polyamines suppressed nodule formation in soybean (Terakado et al., 2006). Suzuki et al. reported that nodule number is controlled by the abscisic acid in *Trifolium repense* (white clover) and *Lotus japonicus* (Suzuki et al., 2004).

2. Local effect of nitrate on nodule growth and nitrogen fixation

2.1 Rapid and reversible inhibition of nodule growth and nitrogen fixation by nitrate

Short-term local effect of nitrate supply on nodule formation and nitrogen fixation was evaluated using hydroponically grown soybean plants (cultivar Williams), which were inoculated with *Bradyrhizobium japonicum*, (strain USDA110) (Fujikake et al. 2002, 2003). In the first experiment (Fujikake et al. 2002), the diameter of nodules on the upper part of nodulated soybean roots in a glass bottle was measured with a slide caliper. Nodulated soybean (cv. Williams) plants were hydroponically cultured, and various combinations of one-week culture solution with 5 mM or 0 mM nitrate were applied using 13 days old soybean seedlings during three successive weeks. The treatments were designated as 0-0-0, 5-5-5, 5-5-0, 5-0-0, 5-0-5, 0-5-5 and 0-0-5, where the three sequential numbers denote the nitrate concentration (mM) applied in the first-second-third weeks. The size of the marked individual nodules was measured periodically using a slide caliper. All the plants were harvested after measurement of the acetylene reduction activity (ARA) at the end of the treatments. In the 0-0-0 treatment, the nodules grew continuously during the treatment period. As shown in Fig. 8., individual nodule growth was immediately suppressed after 5 mM nitrate supply. However, the nodule growth rapidly recovered by changing the 5 mM nitrate solution to a 0 mM nitrate solution in the 5-0-0 and 5-5-0 treatments. In the 5-0-5 treatment, nodule growth was completely inhibited in the first and the third weeks with 5mM nitrate, but the nodule growth was enhanced in the second week with 0 mM nitrate. The nodule growth response to 5 mM nitrate was similar between small and large size nodules.

In this experiment nodule numbers are not significantly affected by nitrate treatments (Fig. 9. A), although the nodule weight was significantly affected by the period of nitrate supply (Fig. 9. B), where 5-5-5 and 0-5-5 treatments depressed nodule dry weight about 1/3 of 0-0-0 plants. After the 5-5-5, 5-0-5, 0-0-5 and 0-5-5 treatments, where the plants were cultured with 5 mM nitrate in the last third week, the acetylene reduction activity (ARA) per a plant and ARA per g nodule dry weight (DW) were significantly lower compared with the 0-0-0 treatment (Fig. 10. A,B). On the other hand, the ARA after the 5-0-0 and 5-5-0 treatments was relatively higher than that after the 0-0-0 treatment, possibly due to the higher photosynthate supply associated with the vigorous vegetative growth of the plants supplemented with nitrate nitrogen. It is concluded that both soybean nodule growth and N_2 fixation activity sensitively responded to the external nitrate level, and that these parameters were reversibly regulated by the current status of nitrate in the culture solution, possibly through sensing of the concentration of nitrate or its assimilates in roots and/or nodules.

The nitrate concentration was analyzed in each organ of soybean harvested at the end of the treatment on 34 days after planting. In the plants supplied with 5 mM nitrate during the last week in both the first and second series of treatments (5-5-5, 5-0-5, 0-5-5 and 0-0-5 treatments), the nitrate concentration was significantly high in each organ. Especially the roots and stems accumulated about 9-14 gN kg⁻¹DW and about 5-9 gN kg⁻¹DW nitrate, respectively. On the other hand, the nitrate concentration in roots (0.19 gN kg⁻¹DW), stems (0.03 gN kg⁻¹DW) and nodules (0.11 gN kg⁻¹DW) was fairly low in the 5-5-0 treatment where nitrate was not supplied during the last third week. All the accumulated nitrate during the first and second weeks was reduced and assimilated during the third week of the 0 mM nitrate treatment under the experimental conditions. The nitrate concentration in the nodules was relatively lower than that in the roots and stems, but in the 5-5-5, 0-5-5, 0-0-5 treatments, the nodules accumulated more than 1 gN kg⁻¹DW nitrate.

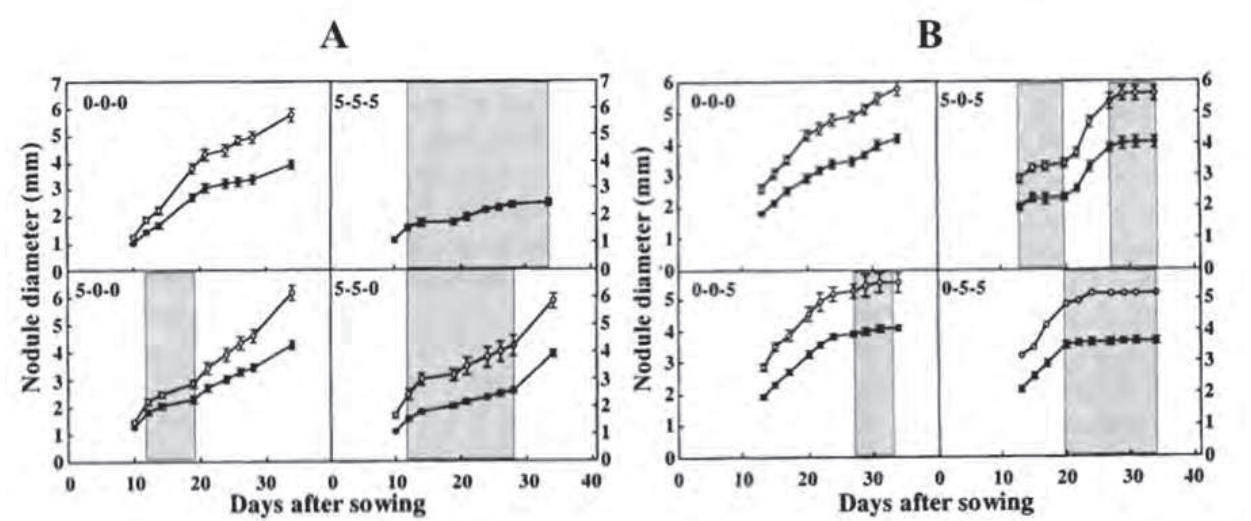


Fig. 8. Changes in nodule diameter of soybean plants with various nitrate treatments. Gray background shows the duration of 5 mM nitrate treatment, and white background shows the 0 mM nitrate. Open circle: large nodules, Closed circle: small nodules.

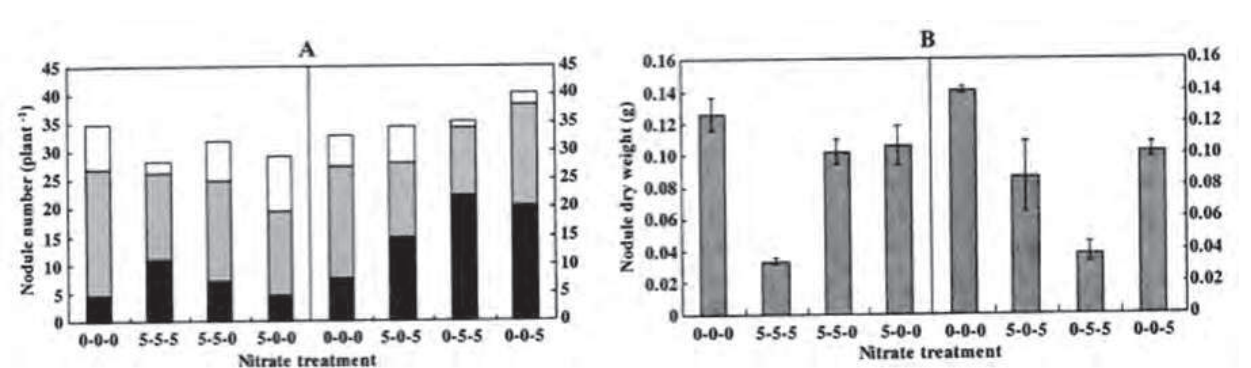


Fig. 9. Number (A) and dry weight (B) of nodules at the end on 34 days after planting. (A) nodule size were indicated by black column (3mm<), gray column (3-5 mm) or white column (>5mm).

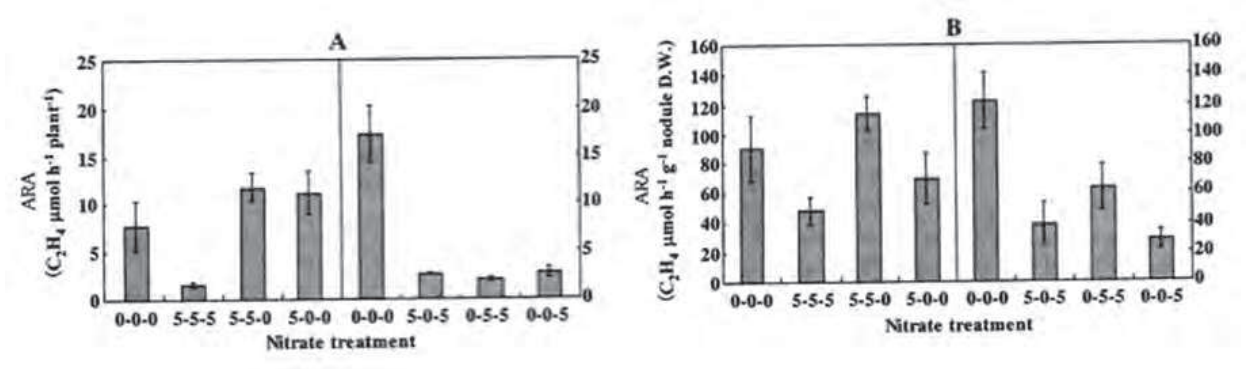


Fig. 10. Acetylene reduction activity per a plant (A) and per nodule dry weight (B) of nitrate treatments on 34 days after planting.

In the second experiment (Fujikake et al., 2003) and the diameter of individual nodules was measured from 10-24 days after planting using a computer microscope under controlled environmental conditions (Fig. 11.). Photos (Fig. 11A) and the diameter changes (Fig. 11B) of nodules were shown. A, nodule growth was rapid only under 0 mM nitrate conditions. The diameter of a nodule attached to the primary roots increased from 1 mm to 6 mm for 2 weeks under N free conditions (Fig. 11. Aa, Ba). The increase in nodule diameter was almost completely stopped after 1 d of supplying 5 mM NO_3^- (Fig. 11. Ab, Bb). However, nodule growth quickly returned to the normal growth rate following withdrawal of NO_3^- from the solution (Fig. 11. Ac, Bc).

The morphology of typical nodule slices of soybean observed by an optical microscope is shown in Fig. 12. (A) and the average size of infected cells, uninfected cells and inner cortex cells were measured (Fig. 12. B). It is conspicuous that the infected cells of nodules became larger under 0 mM nitrate condition (a) from 10 to 18 days after planting. On the other hand, the size of infected and uninfected cells and inner cortex cells remained small with 5 mM nitrate solution. Cell growth recovered rapidly after 2 days of 0 mM nitrate (18 days after planting) after 2 days of 5 mM nitrate (16 days after planting) (c). This result indicates that nodule growth at this stage depends on the cell expansion, rather than cell proliferation. The rapid and reversible nodule growth inhibition is caused by nodule cell growth.

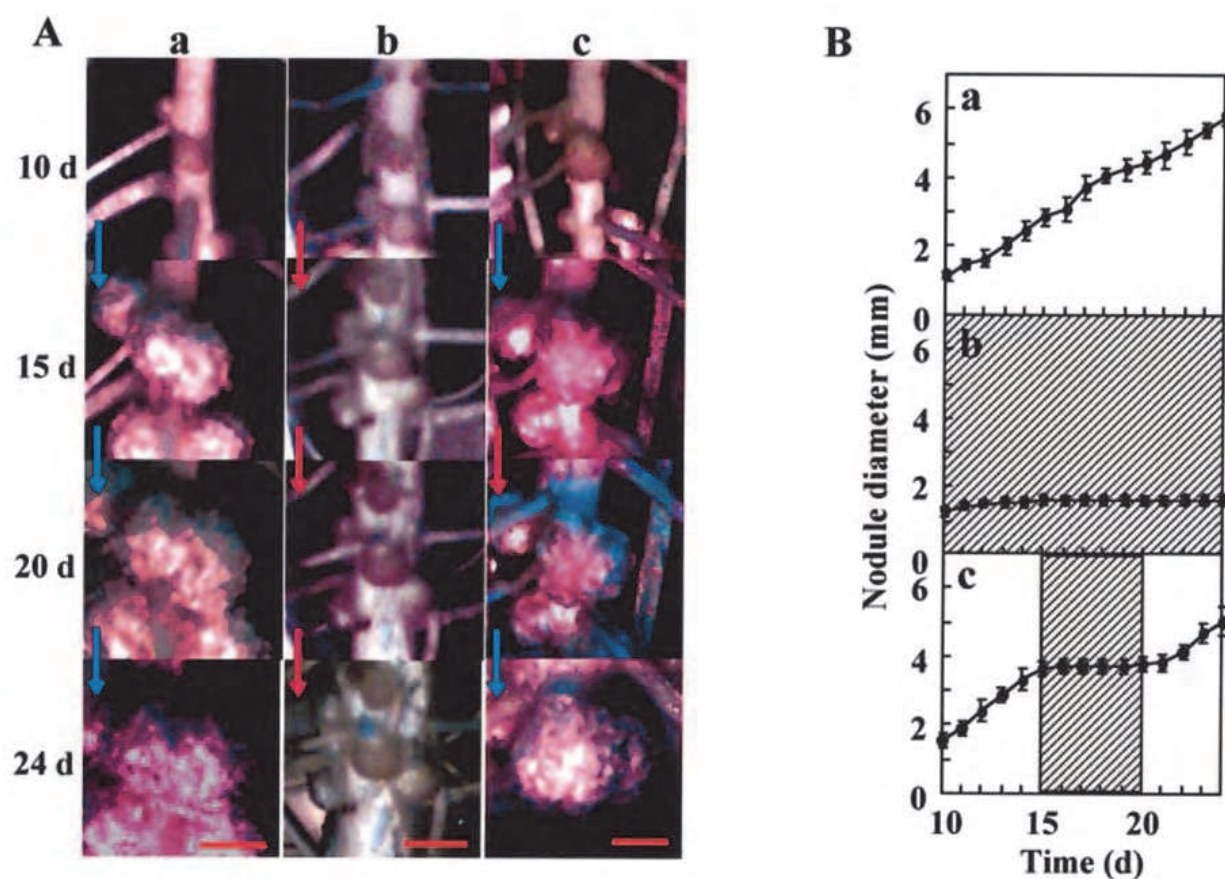


Fig. 11. (A) Growth response of soybean nodules to 0 mM nitrate (blue arrows) or 5 mM nitrate (red arrows) application in the culture solution. (B) Changes in nodule diameter with 0 mM (white background) or 5 mM (hatched background).

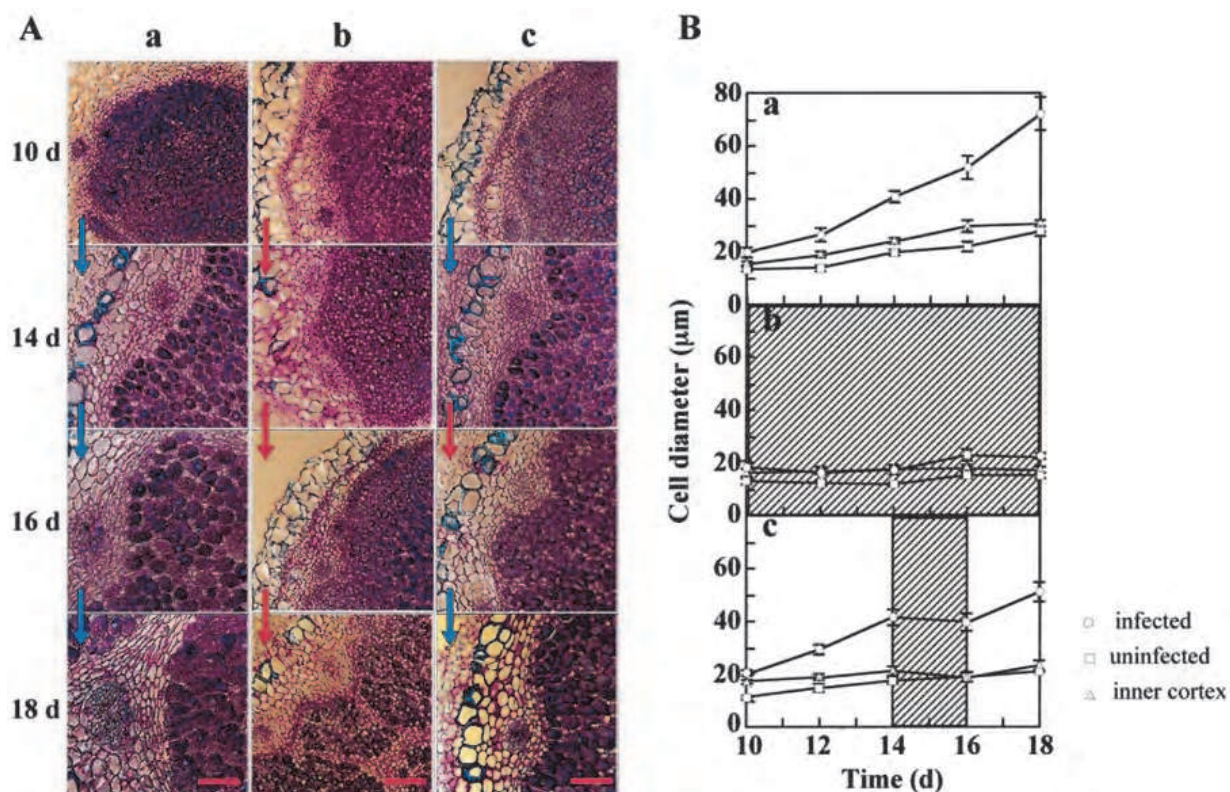


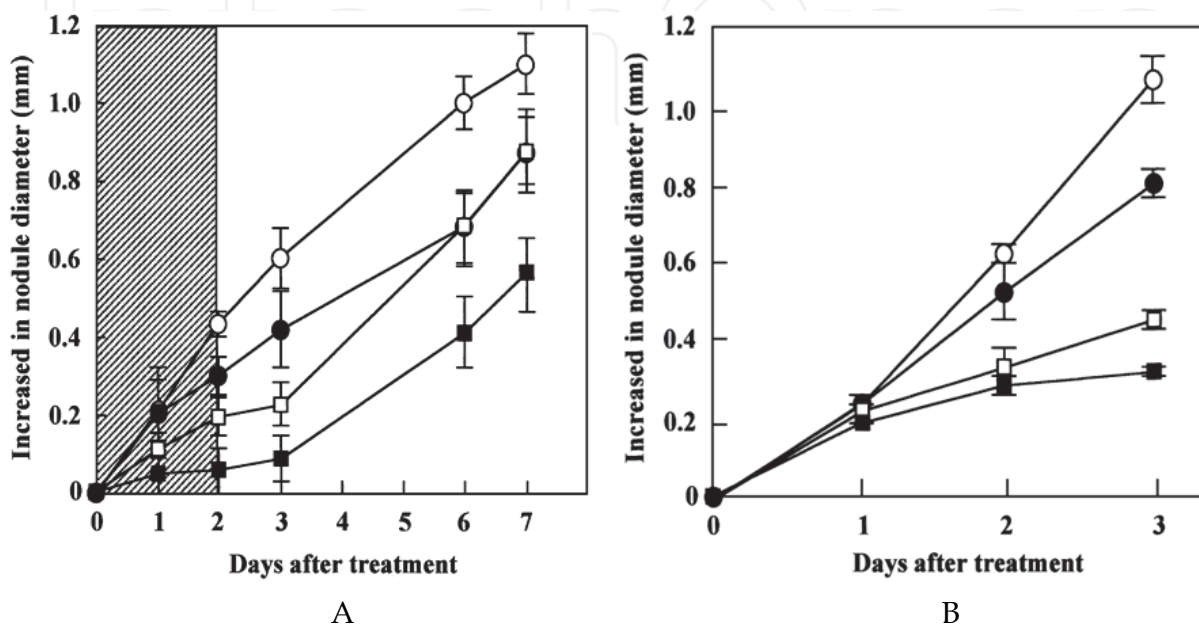
Fig. 12. Effect of 0 mM and 5 mM nitrate treatment on the size of nodule cells. (a) soybean plants were cultured in 0 mM nitrate for 10-18 days. (b) soybean plants were cultured with 5 mM nitrate for 10-18 days. (c) soybean plants were treated with 0 mM from 10-14 days and 5 mM nitrate from 14-16 days and 0 mM nitrate from 16 to 18 days after planting. The size of infected cells, uninfected cells and inner cortex cells were measured in B.

The effect of dark treatment on nodule growth was examined in combination with nitrate treatments for two days followed by normal light/dark conditions (Fig. 13.A.). Under continuous dark conditions, nodule growth maintained on the first day, but depressed on the second day. The reversible depression of nodule growth by NO_3^- was similar to the restriction of the photoassimilate supply under continuous dark conditions for 2 days. The nodule growth with 5 mM nitrate under continuous dark conditions depressed most severely among treatments. When plants were returned to the normal conditions (14 h light and 10h dark) with 0 mM nitrate, all the nodules recovered the growth rate.

The inhibitory effect of 5 mM nitrate was partially alleviated by the addition of 3% sucrose to the culture solution (Fig. 13.B.), suggesting that soybean root nodules are under carbon deficiency.

The positron emitting radioisotope $^{11}\text{CO}_2$ was supplied to the first trifoliolate leaves of 29 days after planting for 10 min, then the movement of ^{11}C was monitored by positron-emitting tracer imaging system (PETIS) (Fujikake et al., 2003). Split-root system was made by cutting the primary root of soybean seedling at 24 days after planting. Each split roots was supplied with solution containing with 0 mM or 5 mM NO_3^- for 3 days from 27-29 days after planting. Both sides of split roots were supplied with 0 mM, 0 mM (a), 5mM, 5 mM (b) or 0 mM, 5 mM (c). In the plants with 0 mM, 0 mM or 5 mM 5mM, the ^{11}C assimilated in the first trifoliolate was translocated both upward to the young developing apical leaf bud and downward to the whole root system (Fig. 14. A). Very little ^{11}C was transported to the fully

developed trifoliate and primary leaves. This means that fully developed leaves are not sink of photoassimilate from other leaves. Compared with the split root system supplied with 0 mM NO_3^- on one side and 5 mM NO_3^- on the other side, the transport rate of ^{14}C was faster in the split-roots supplied with 5 mM NO_3^- than those in 0 mM NO_3^- (Fig. 14. B, C). This result indicates that when NO_3^- is supplied to a part of the roots, photoassimilate flow become faster in this part.



A: Open circle: 0 mM nitrate, light 14h/dark 10h. filled circle: 0 mM nitrate, continuous dark for 2 days. Open square: 5 mM nitrate, light 14h/dark 10h. filled square: 5 mM nitrate, continuous dark for 2 days. B: Effect of 3% sucrose on nodule growth (B). Open Circle: 3% sucrose + 0 mM nitrate. Filled circle: 0% sucrose + 0 mM nitrate. Open square: 3% sucrose + 5 mM nitrate. filled square: 0% sucrose + 5 mM nitrate.

Fig. 13. Nodule growth of soybean plants grown with 0 mM or 5 mM nitrate under light/dark conditions

Quantitative evaluation was conducted using ^{14}C as a tracer for the plants supplied either with 0 mM or 5 mM NO_3^- for one day before supplying $^{14}\text{CO}_2$. Whole shoot of a plant at 22 days after planting was exposed to the $^{14}\text{CO}_2$ for 120 min using circulation system. After $^{14}\text{CO}_2$ feeding, plant samples were immediately dried and the radioactivity in leaves, stems, nodules and roots were determined using Liquid Scintillation Counter. By supplying 5 mM NO_3^- , the partitioning to the underground part were almost the same in 0 and 5 mM nitrate treatments, but the ^{14}C partitioning to nodule decreased from 9.1 % to 4.3%, while that to the roots increased from 5.2 % to 9.1% (Fig. 15).

These results indicate that the decrease in photoassimilate supply to nodules may be involved in the quick and reversible nitrate inhibition of soybean nodule growth and N_2 fixation activity (Fig. 16.). The decrease in starch concentration in nodules (Vessey et al., 1988, Gordon et al., 2002) and the down-regulation of sucrose synthase transcript within 1 day of nitrate treatment (Gordon et al., 2002) may imply that NO_3^- reduces photosynthesis flow into nodules and sucrose utilization in nodules.

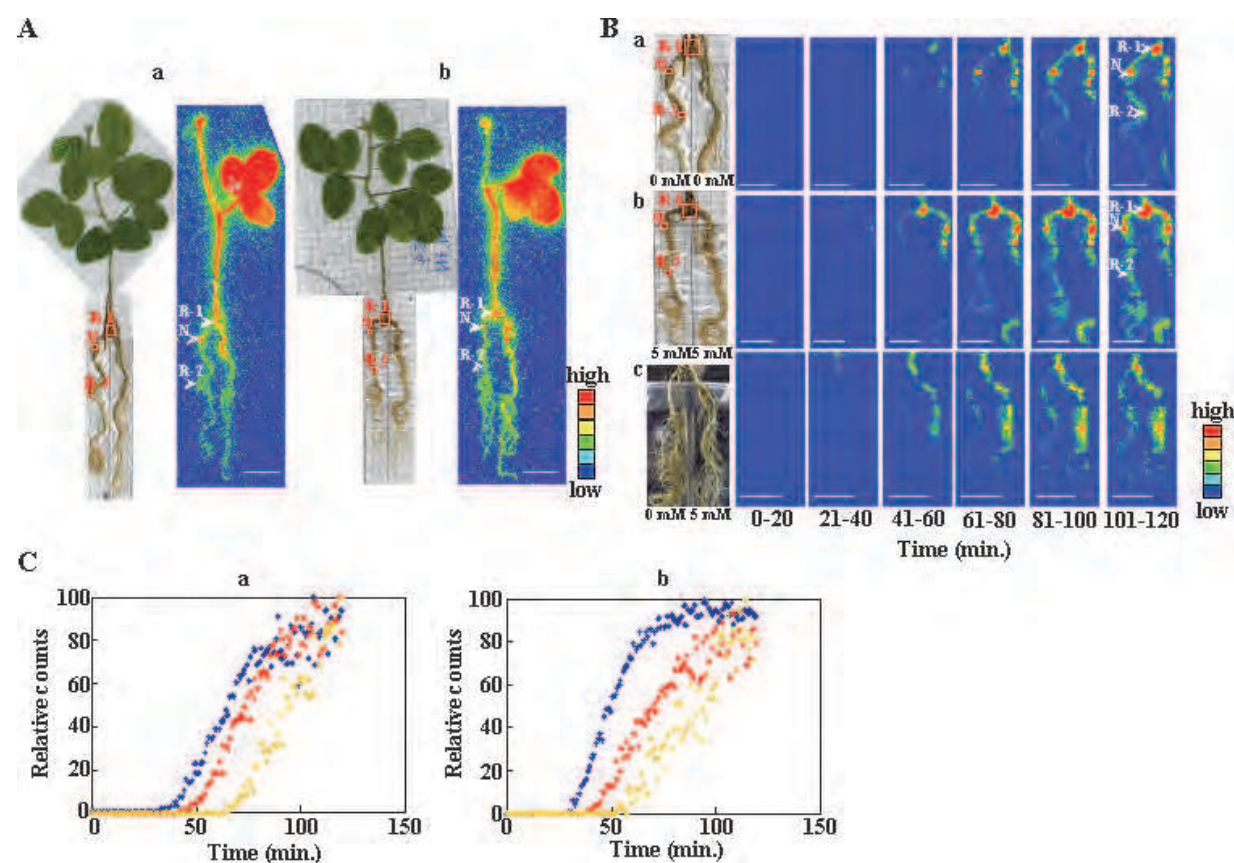


Fig. 14. ¹¹C translocation to the split root systems from first trifoliolate leaves of 29 days old plants. (A) Images of the distribution of ¹¹C in soybean by Bioimaging analyzer. (B) The time course for the accumulation of radioactivity as shown by PETIS. (C) The accumulation of radioactivity for the point of R-1 (blue), N (red) and R-2 (yellow).

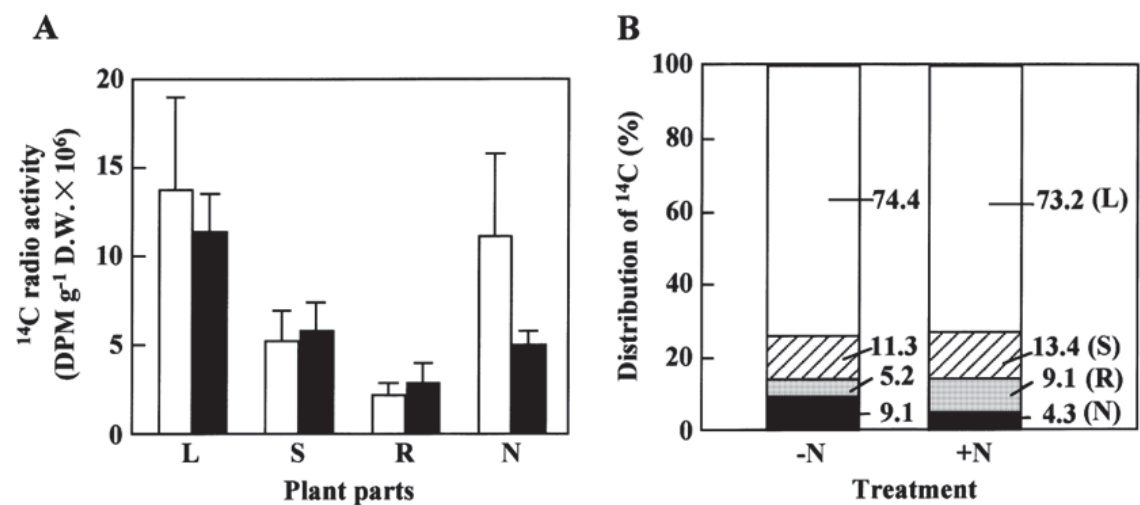


Fig. 15. Partitioning of ¹⁴C labeled photoassimilate in soybean plants. (A) Radioactivity per g dry weight of each part after one day of 2 h ¹⁴CO₂ feedings to a whole shoot, with 0 mM nitrate (white column) and 5 mM nitrate (black column). (B) Distribution of ¹⁴C among organs with 0 mM (-N) or 5 mM (+N) treatment for one day. (L): leaves, (S): stems, (R): roots, (N): nodules.

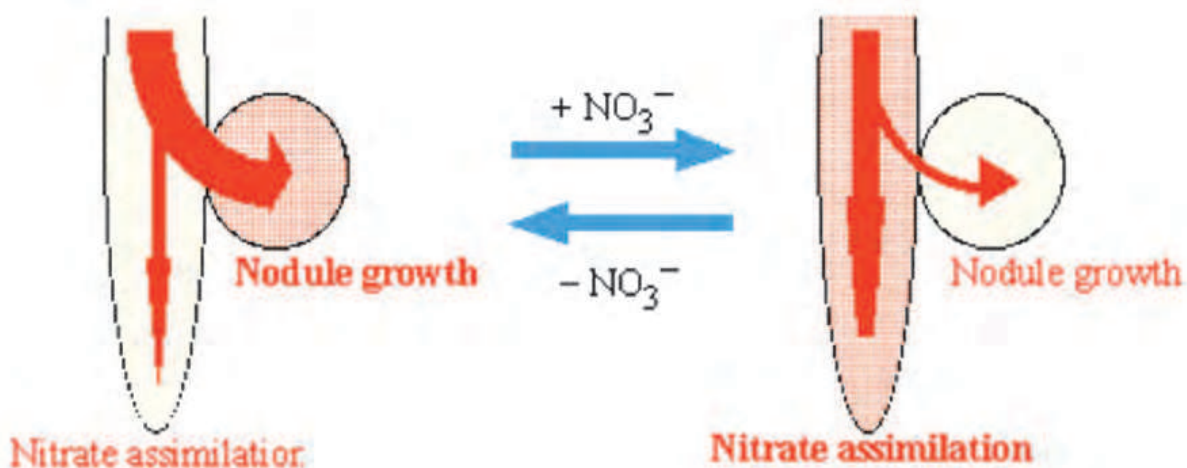


Fig. 16. A model of photoassimilate transport to nodule and roots under no nitrate or presence of nitrate.

2.2 The routes of nitrate entry into nodules

It is well known that nitrate inhibit nodule growth and nitrogen fixation activity, but the routes of nitrate entry into nodule has not fully been understood. It is postulated that there are several routes in nodules. First, NO_3^- is absorbed from the roots and transported to nodules through the xylem. However, xylem transport of NO_3^- to nodules is negligible, because nitrate accumulation is very low in separated nodules in the upper part of roots, when NO_3^- was supplied in the lower roots. In addition the role of xylem in nodule is the export of assimilated nitrogen from nodules to shoots, rather than the import of water and minerals from roots. Most of water and minerals are supplied from shoots to nodules through phloem. Second route is NO_3^- supplied via phloem. However, usually NO_3^- concentration in phloem is very low due to nitrate reduction in leaves. Third is NO_3^- is taken up from the subtending roots and transported from root cortex to the nodule cortex via symplastic pathway (Streeter 1993). Forth, NO_3^- is absorbed from nodule surface.

The nitrate transport pathway into soybean nodules were investigated using tungstate (WO_4^{2-}) and $^{15}\text{NO}_3^-$ as a tracer (Mizukoshi et al. 1995). Tungstate was used as an anion tracer as an analogue of nitrate (NO_3^-). The distribution of tungsten (W) was observed by an Electron Probe X-ray Microanalysis (EPMA). At 3 days after 1 mM tungstate treatment, a large amount of W accumulated in the root cortex, and the import of W into stele was restricted (Fig. 17A, C). It is well known that there is a barrier in endodermis between cortex and stele, where water movement is not allowed. Therefore, solutes should pass through endodermis through inside the cells via symplastic pathway. In addition, the movement of W inside the nodule was negligible (Fig. 17 C). This result suggests that the external anions cannot be readily enter into the cortex of nodules through appoplastic pathway by diffusion. In contrast, nodulated roots were treated with 1.7 mM $^{15}\text{NO}_3^-$ for one day, a relatively large amount of nitrate was accumulated in nodule cortex, although NO_3^- and ^{15}N were negligible in the symbiotic region. This result indicates that nitrate can be absorbed from the nodule surface into cytoplasm of nodule epidermis, and it is transported by symplastic pathway through plasmodesmata, and accumulated in the nodule cortex cells.

As shown in Fig. 19., the accumulation of NO_3^- in nodule cortex may be involved in the restriction of O_2 permeability into symbiotic resion, then it decreases the nitrogen fixation

and nodule growth. Witty and Minchin (1990) reported that the NO_3^- treatment decreased the air space within a oxygen barrier in inner cortex of nodules.

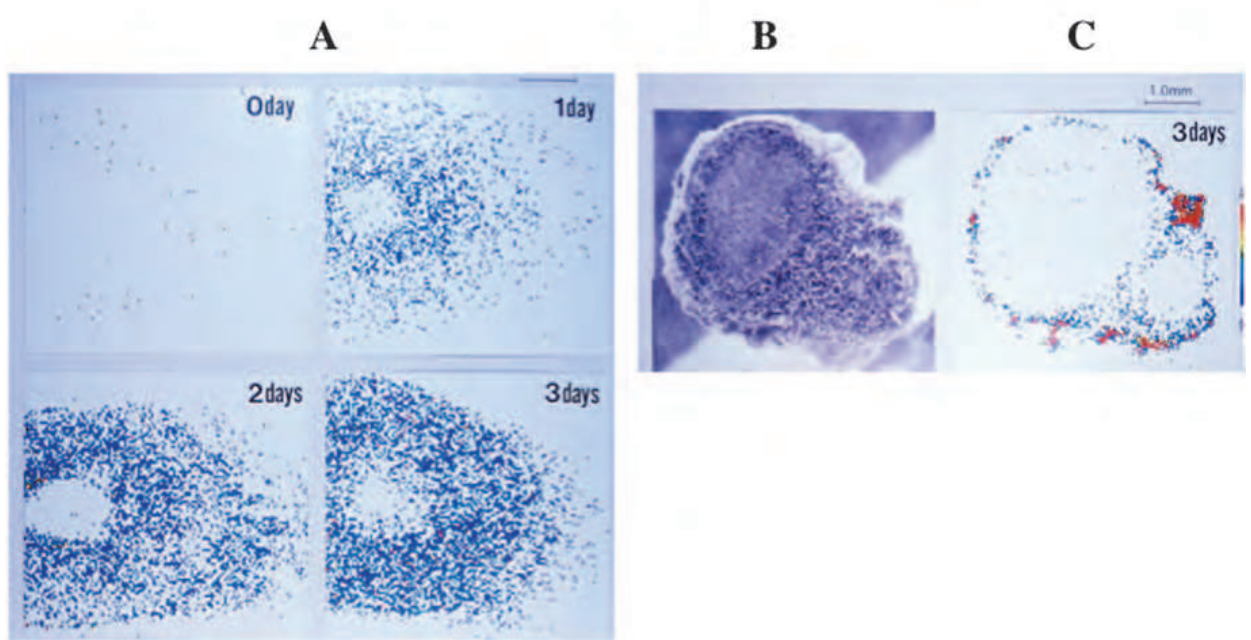


Fig. 17. Distribution of tungsten (W) in soybean root at 0, 1, 2, 3 days after 1 mM tungstate treatment (A). Distribution of tungsten (W) in soybean nodule with root at 3 days after 1 mM tungstate treatment (A). The W concentration is higher indicated by red, yellow , green, blue, and white, in this sequence.

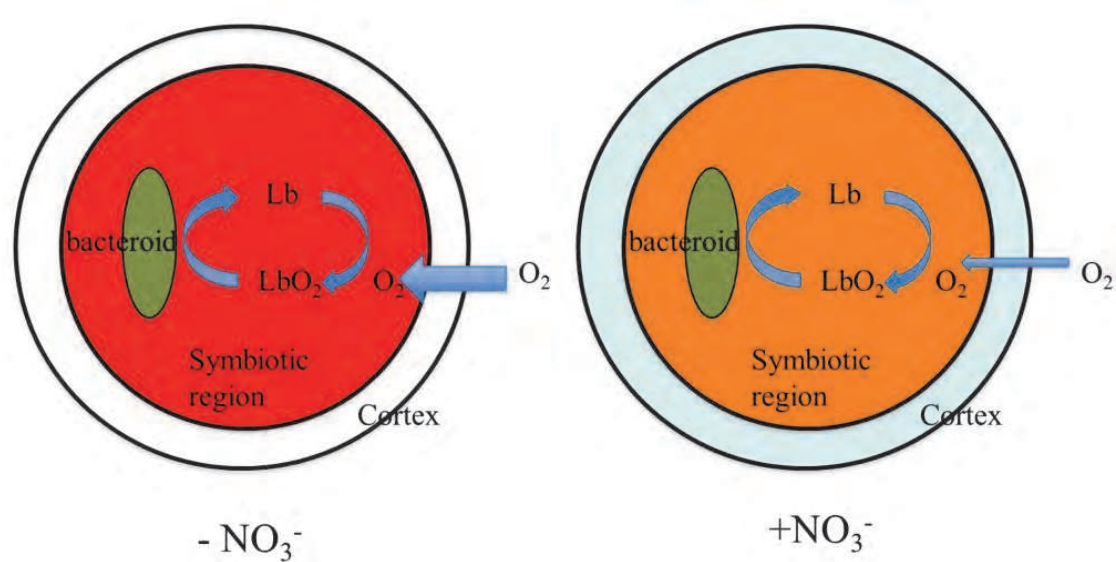
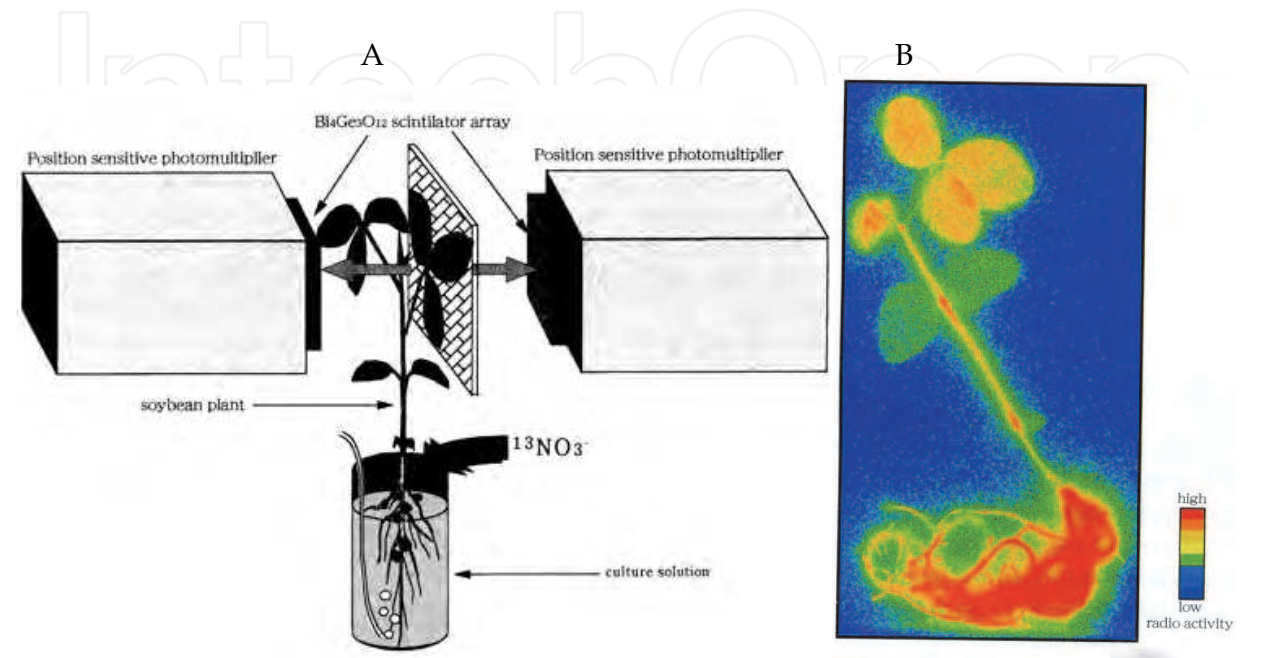


Fig. 18. Effect of nitrate accumulation in cortex of soybean nodule on oxygen permeability and nitrogen fixation activity.

Sato et al. (1999) analyzed nitrogen absorption and translocation in non-nodulated and nodulated soybean plants cultivated with 0 mM or 1 mM nitrate (Fig. 19.). The radioactivity was measured in a first trifoliolate leaf after addition of $^{13}\text{NO}_3^-$ supply to the root solution. The

relative radioactivity from 0 to 40 min after $^{13}\text{NO}_3^-$ supply were similar between non-nodulated and nodulated soybean with 0 or 1 mM nitrate (Fig. 20.). This suggests that nodulation does not change the absorption and transport pattern of nitrate absorbed in the roots. However, quantitative measurement using stable isotope $^{15}\text{NO}_3^-$, total amount of ^{15}N was higher in non-nodulated soybean than nodulated soybean, especially with 1 mM $^{15}\text{NO}_3^-$, this is due to the increase in the root mass in these plants.



A: Positron Emitting Tracer Imaging System. B: Image of ^{13}N radioisotope in non-nodulated soybean plant (T202).

Fig. 19. Real time observation of radioactivity in the first trifoliolate of soybean plant after $^{13}\text{NO}_3^-$ was supplied to the root solution.

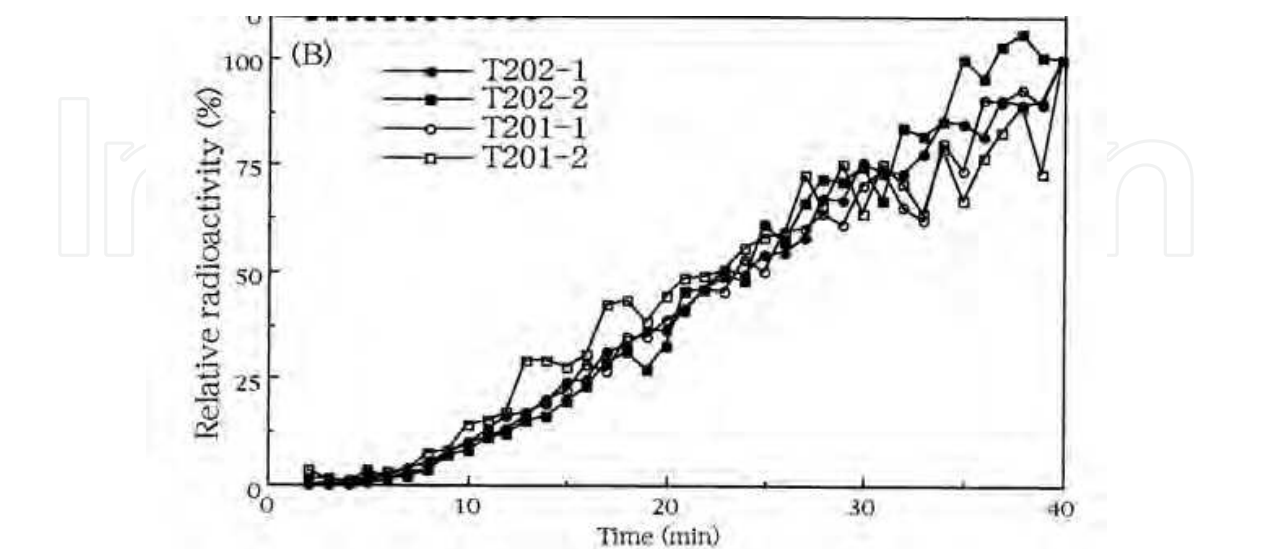


Fig. 20. Changes in relative radio activity in leaf of nodulated (T-202) and non-nodulated (T-201) isolines of soybean after $^{13}\text{NO}_3^-$ was supplied to the root solution. The radioactivity at 40 min is normalized as 100%.

3. Systemic and long-term effect of nitrate on nodule growth and nitrogen fixation

3.1 The effect of 0mM or 5 mM nitrate application in upper and lower part of roots

Local and systemic effects by nitrate on nodulation have been reported in leguminous plants. The local effect of nitrate inhibition was shown in split-root experiments where root systems had been separated into two equivalent parts. The strong and rapid nitrate inhibition of nodule growth and N_2 fixation activity is restricted in the nodules attached to the root portions that are in direct contact with nitrate; and no or milder inhibition is induced in the other part of the root system receiving no nitrate (Tanaka et al., 1985). However, some systemic inhibition of nitrate on nodulation and nitrogen fixation has also been observed with a high concentration of nitrate in clover (Silsbury et al., 1986).

We investigated the local and systemic effects of continuous supply of NO_3^- by using horizontal split-root system in two-layered pot system, where the lower part of roots were supplied with culture solution containing 1mM NO_3^- in the lower pot, and the upper roots were in the vermiculite medium with N-free culture solution in the upper pot (Ohyama et al., 1993). The soybean plants (cv. Williams and Norin No.2) were cultivated with 0 mM or 1 mM NO_3^- solution in the lower pot, and harvested at maturing (R7) stage (Fher et al., 1971). In this stage, there are no nodules remained in the lower part of roots. The dry weight of shoot and upper part of roots were almost the same between 0 mM and 1 mM NO_3^- supply, but nitrate treatment decreased the dry weight of nodules attached in the upper part of roots in both varieties. This result indicates that continuous long-term supply of NO_3^- may impose systemic inhibition of nodulation in soybean plants.

Systemic and local effects of long-term application of nitrate on nodule growth and N_2 fixation in soybean plants were more precisely investigated using two layered pot system. Four treatments were imposed i.e., 0/0, 0/5, 5/0 and 5/5, with the 0 mM or 5 mM NO_3^- treatment in upper pot/ lower pot, respectively. The plants were harvested at the initial flowering (R1) stage and pod setting (R4) stage, and the effect of nitrate placement on nodule number, nodule growth, and N_2 fixation activity in the upper and lower pots were elucidated (Yashima et al., 2003).

The development of the root system in the lower pots was quite different between 0 and 5 mM NO_3^- in the lower pot (Fig. 21.). The root length was longer in 0 mM treatment in lower pot (0/0, 5/0), but a bunch of short lateral roots was formed in the solution with 5 mM NO_3^- in lower pot (0/5, 5/5). In the lower pot where the nodules were in direct contact with 5 mM NO_3^- , the inhibition on the nodule number, nodule size and N_2 fixation was conspicuous. Systemic and local effect on nodule number per a plant did not occur in the upper nodules in vermiculite. On the other hand, systemic inhibition on the nodule dry weight and N_2 fixation activity in the upper pot was apparent. The 5/5 treatment depressed the nodule growth and nitrogen fixation activity in the upper nodules. Nitrate accumulation was observed only in the part of roots and nodules in direct contact with 5 mM NO_3^- either in the upper or lower pot. The concentration of total amino acids was higher in the lower roots in 0/5 treatment than those in 0/0 treatment, however, that was almost the same level in the roots and nodules of the upper pot both at R1 and R4 stage. The soluble sugar concentration in the lower roots in 0/5 treatment was lower than that in the 0/0 treatment. The similar trend was observed in the upper roots of 0/5 treatment, suggesting that the absorption of NO_3^- from the lower roots decrease sugar

concentration in both lower roots in direct contact with nitrate, and the upper roots not contact with NO_3^- .



Fig. 21. Root system in the lower pot treated with 0/0 (A), 0/5 (B), 5/5 (C) and 5/0 (D) treatments (upper pot mM nitrate / lower pot mM nitrate).

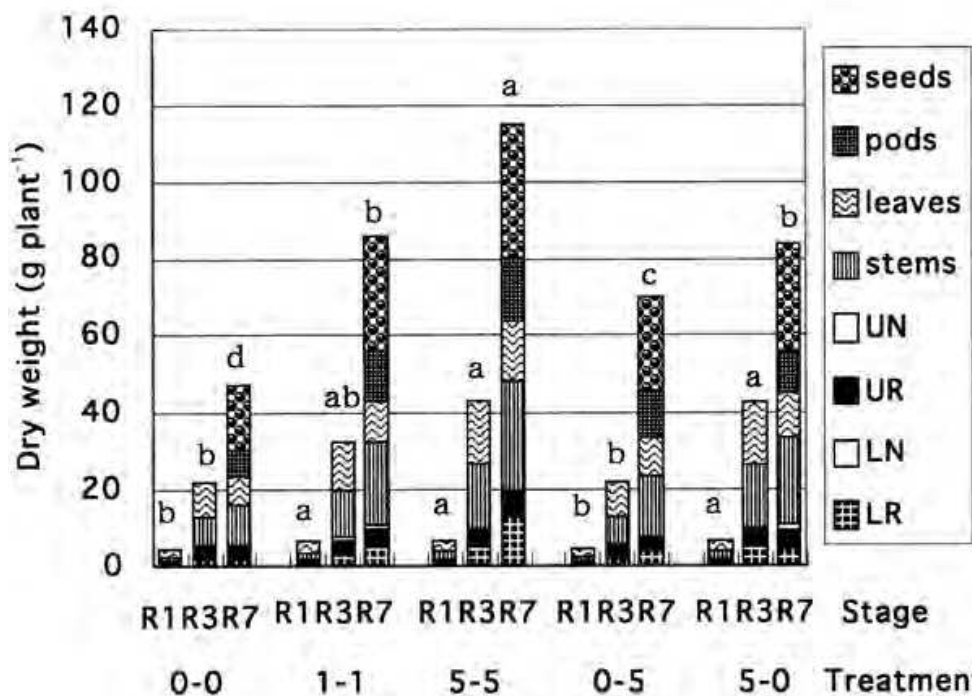
3.2 Long-term effects of 0 mM, 1 mM, 5 mM nitrate application in lower part of roots

Long-term effect of NO_3^- application from the lower part of roots on the nodulation of the upper part of roots was further investigated in relation to concentration and treatment period (Yashima et al. 2005). The solution with 0 mM, 1mM or 5 mM NO_3^- was supplied from transplanting to two-layered pot system at 14 days after planting to R7 stage. Five treatments were imposed that 0-0 treatment (continuous 0 mM NO_3^-), 1-1 treatment (continuous 1 mM NO_3^-), 5-5 treatment (continuous 5 mM NO_3^-), 0-5 treatment (0 mM until R3 then 5 mM NO_3^-), and 5-0 treatment (5 mM until R3 then 0 mM NO_3^-).

Total plant dry weight and seed dry weight at R7 stage was the highest in 5-5 treatment, intermediate in the 1-1, 5-0, 0-5 treatments, and lowest in the 0-0 treatment (Fig. 22.).

Fig. 23. shows the nodule number per a plant classified with nodule diameter. Nodule number in the upper pot was higher in 5-5 and 1-1 treatments than 0-0 treatment, although proportion of the small nodules under 4 mm diameter was higher in 5-5 treatment. The nitrate supply in the lower pot increased the total nodule number in the upper roots, although decreased the number of nodules in the lower roots.

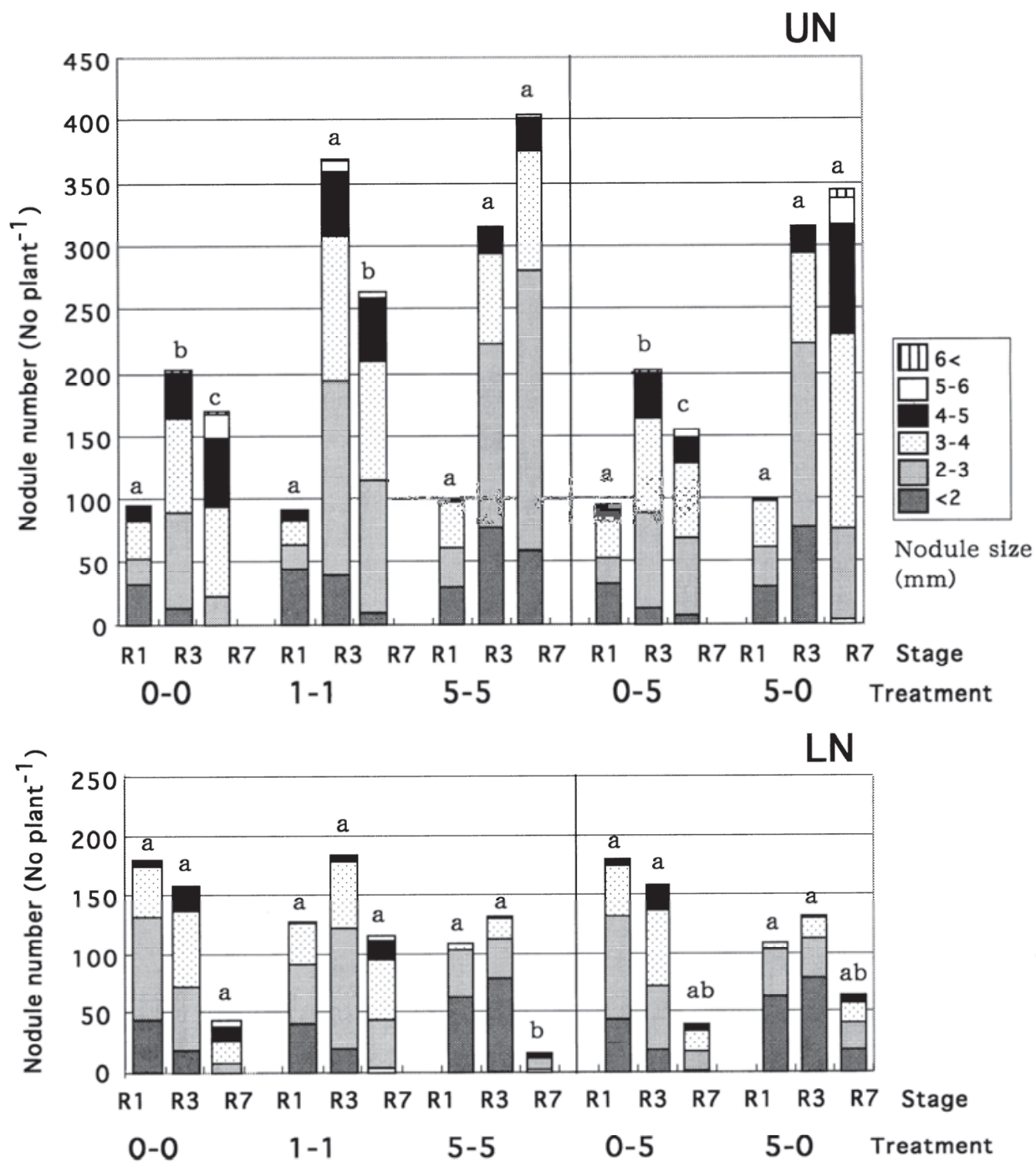
The value of the nodule dry weight per a plant (Fig.24.) and N₂ fixation activity (acetylene reduction activity) per a plant (Fig. 25) and that per nodule dry weight (Fig. 26.) were lowest in the 5-5 treatment. Interestingly, the nodule dry weight in the upper roots was highest in the plants with 1-1 treatment, which exceeded the 0-0 treatment (Fig. 24.). The acetylene reduction activity per a plant of the upper nodules at R3 stage was also the highest in the 1-1 treatment (Fig. 25.). This was due to the nodule dry weight and not ARA per dry weight of nodules. These results indicated that continuous supply of low concentration of NO₃⁻ from the lower roots does not inhibit the nodule growth and N₂ fixation activity, but it can promote nodulation and N₂ fixation. Fig. 27. shows an example of soybean root systems cultivated with continuous supply of 1 mM nitrate at R3 stage. Nodulation was enhanced in the upper roots by supplying 1mM NO₃⁻ from the lower roots (Fig. 27. Ba), where the nodulation was severely depressed in the lower pot (Fig. 27. Bb).



Different alphabet on the column means the statistical difference ($P<0.05$) between treatments.

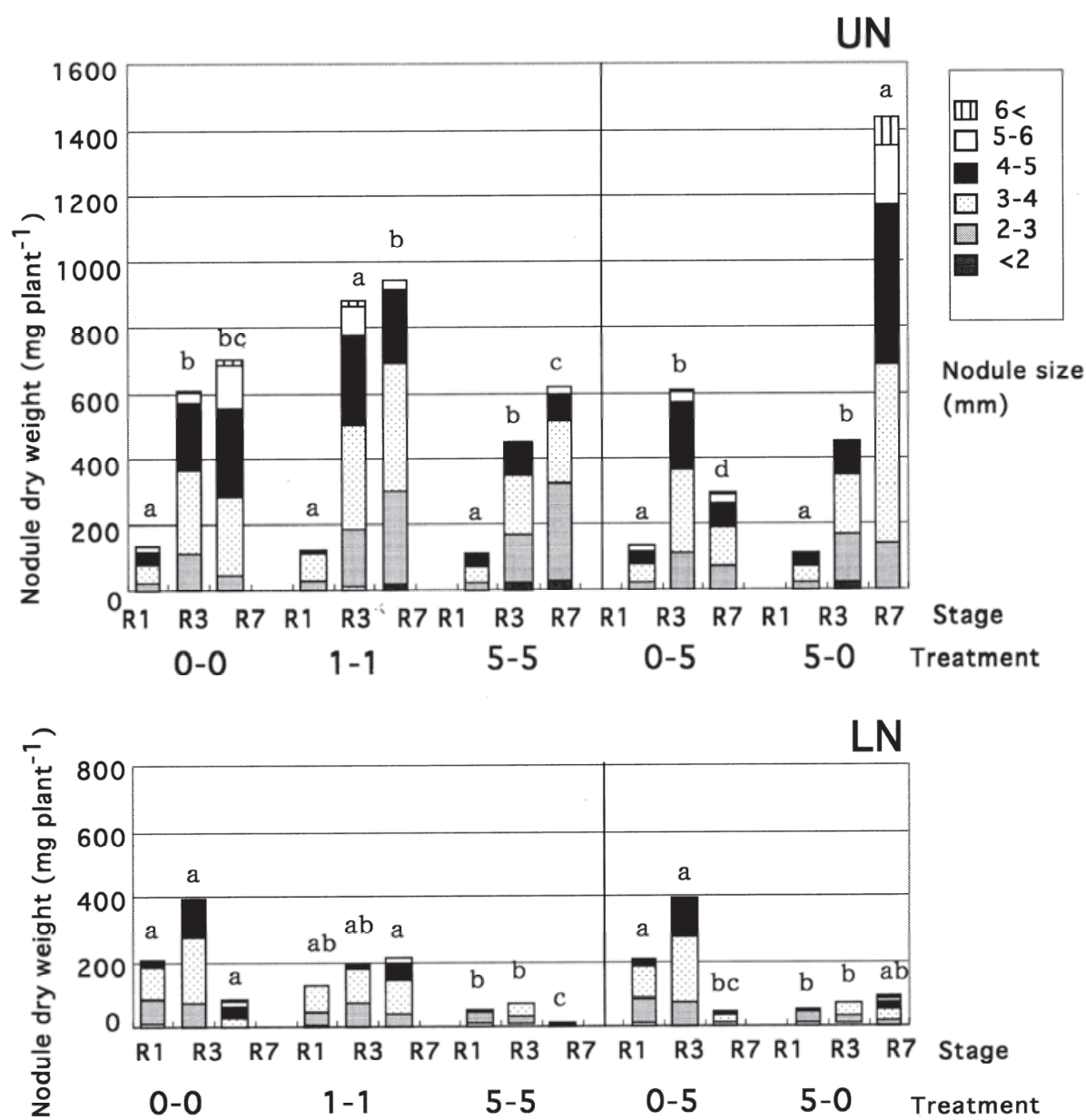
Fig. 22. Changes in dry weight of each part of soybean plants at R1 (initial flowering stage; 40 days after planting), R3 (initial pod setting stage, 60 days after planting), R7 (maturing stage; 100 days after planting) with various nitrate treatments. 0-0, 1-1, 5-5, 0-5, 5-0 indicates mM nitrate concentration from transplanting-R3 stage, and R3-R7 stage respectively.

Withdrawal of 5 mM NO₃⁻ after R3 stage in 5-0 treatment markedly enhanced nodule growth and acetylene reduction activity at R7 stage when the values of both parameters decreased in the other treatments. The nitrate concentration of the nodules attached to the upper roots was very low, including continuous supply of high concentration of NO₃⁻ in 5-5 treatment. This result indicates that the inhibitory effect of 5 mM NO₃⁻, or promotive effect of 1 mM NO₃⁻ was not directly controlled by nitrate itself, but was mediated through some systemic regulation.



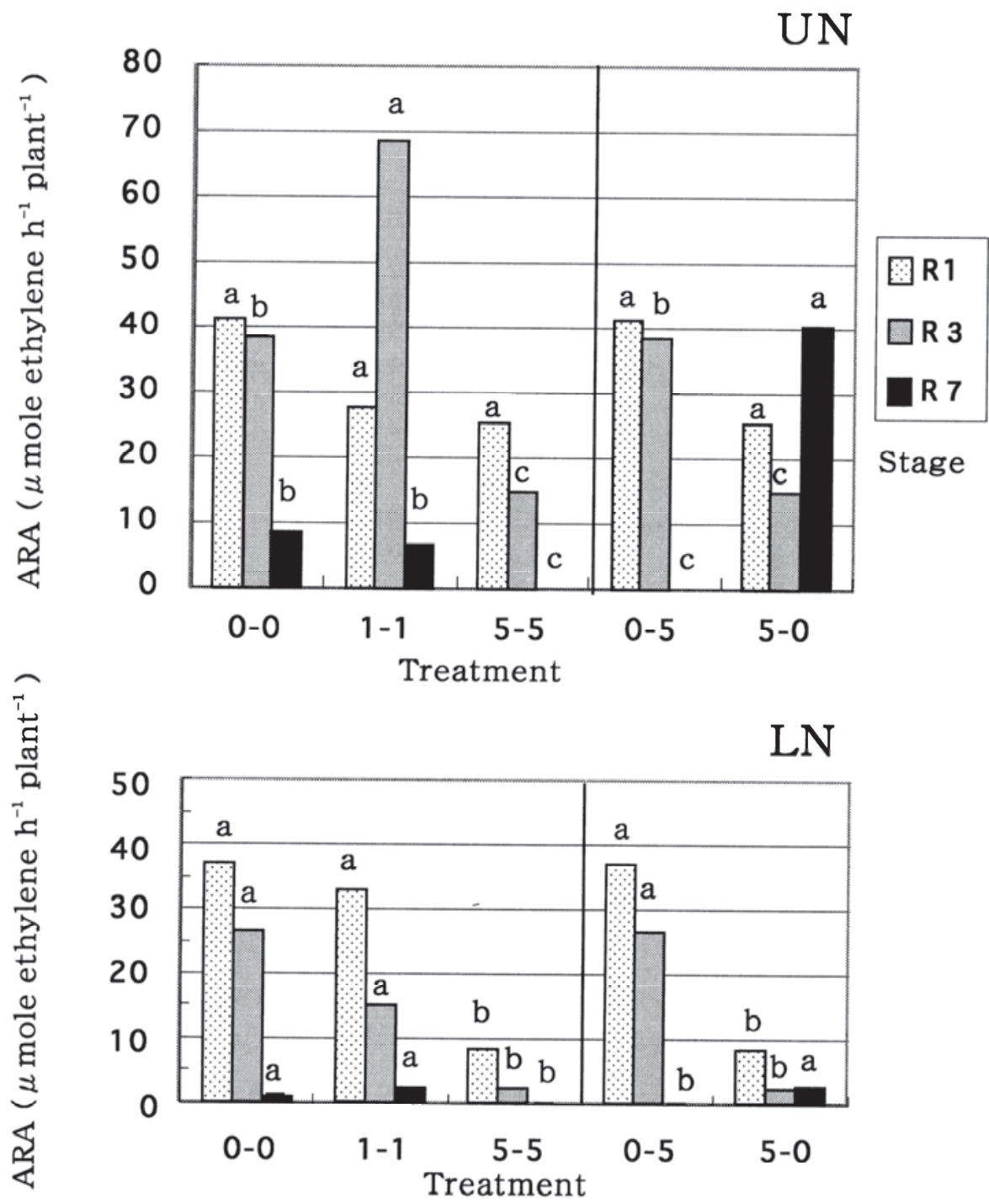
UN: upper nodules, LN: lower nodules.
Different alphabet on the column means the statistical difference ($P<0.05$) between treatments.

Fig. 23. Nodule number per a plant with various nitrate treatment in the lower pot. Size distribution is shown.



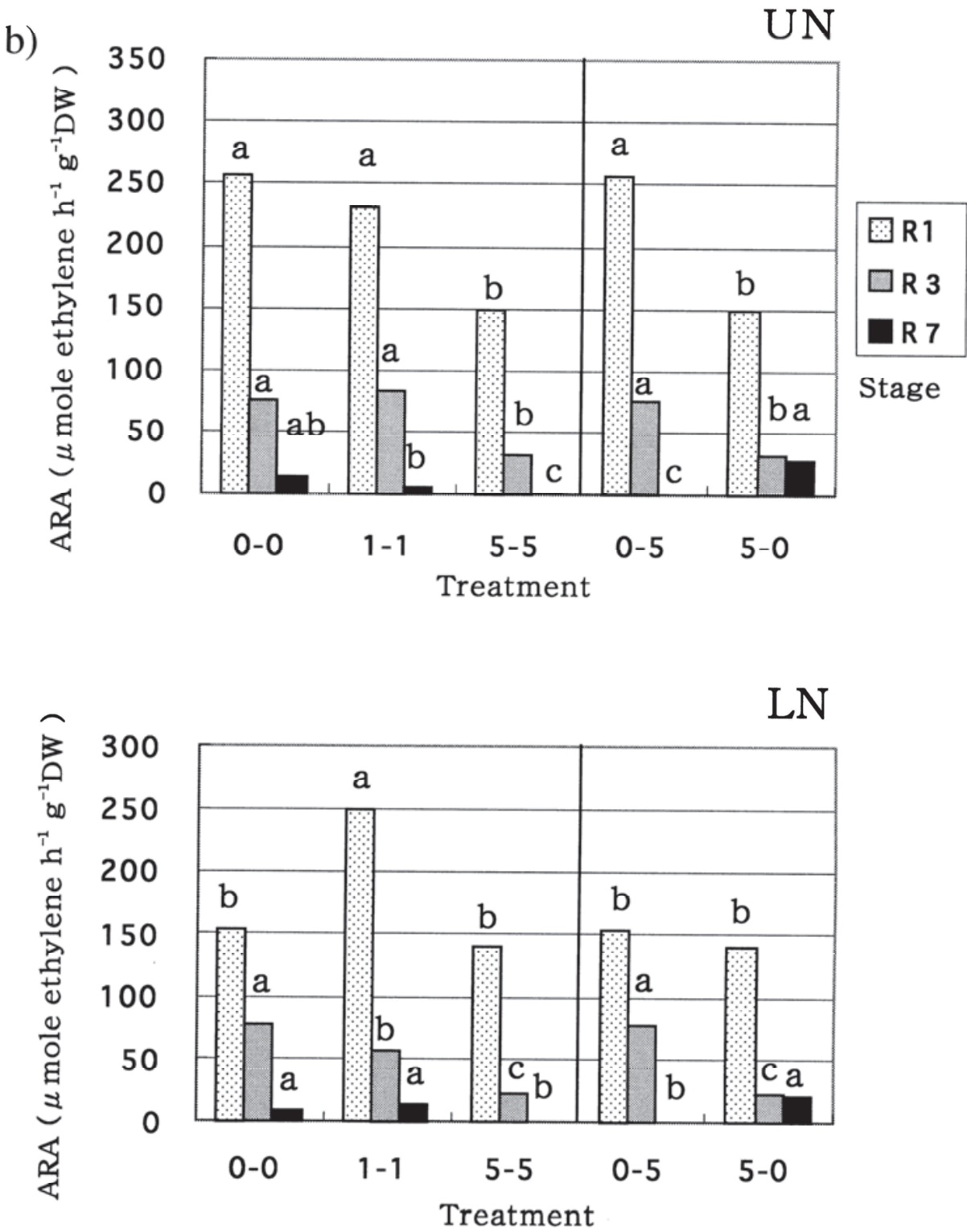
UN: upper nodules, LN: lower nodules.
Different alphabet on the column means the statistical difference ($P<0.05$) between treatments.

Fig. 24. Nodule dry weight per a plant with various treatment of nitrate in a lower pot.



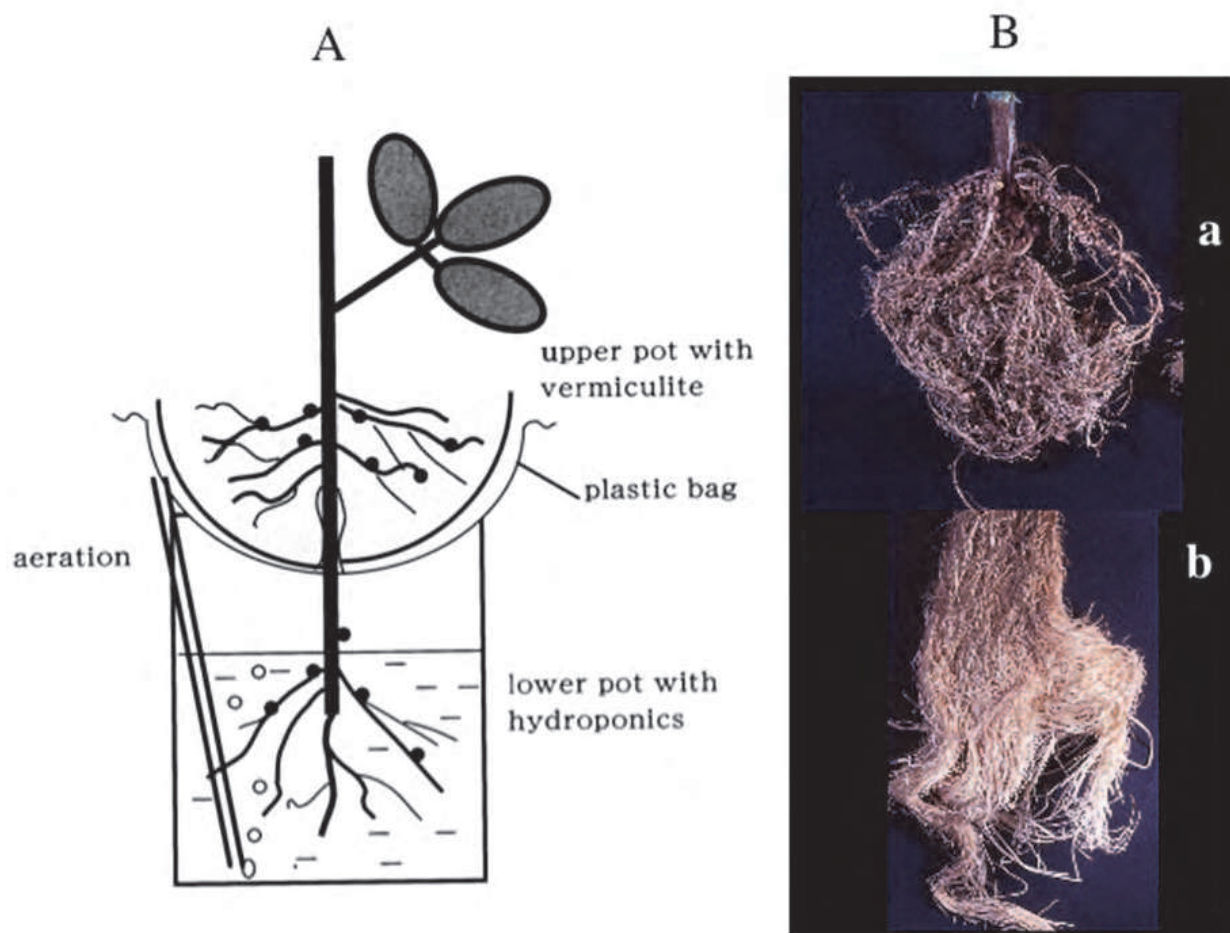
UN: upper nodules, LN: lower nodules.
Different alphabet on the column means the statistical difference ($P<0.05$) between treatments.

Fig. 25. Nitrogen fixation activity per a plant in soybean plants treated with various nitrate supply from lower pot.



Different alphabet on the column means the statistical difference ($P<0.05$) between treatments.

Fig. 26. Nitrogen fixation activity per gram nodule dry weight in soybean plants treated with various nitrate supply from lower pot. UN: upper nodules, LN: lower nodules.



A: Apparatus for two-layered pot.

B: photograph of root system in the upper roots with 0 mM nitrate (a) and the lower roots with 1 mM nitrate(b).

Fig. 27. A vertical split root system used in long-term effect of nitrate from lower pot.

4. Conclusion

In this chapter, we focus on the effects of nitrate supply on nodule growth and nitrogen fixation in soybean plants. First, we found the rapid and reversible inhibition of 5mM nitrate on nodule growth and nitrogen fixation activity in soybean plants. When young soybean plant grown in hydroponic culture was supplied with 5 mM nitrate solution, the nodule growth was completely stopped after one day of application. The culture solution was changed back to nitrogen free solution the nodule growth and nitrogen fixation activity were recovered at one day after changing. The inhibitory effect by nitrate may be due to the changes in photoassimilate supply from nodule to the roots by the experiments exposing the shoot to positron emitting radioisotope ^{11}C or radioisotope ^{14}C labeled CO_2 .

Second, the effect of long-term application of nitrate was evaluated by the vertical split root experiments using two-layered pot, which separates the upper and lower parts of the root system. Both direct and systemic inhibitions were observed for nodule dry weight and nitrogen fixation activity with long-term supply of 5 mM nitrate. Severe inhibition in the root part in direct contact with 5mM nitrate solution was observed, although milder

depression was in the separate parts not in direct contact with nitrate. Nitrate accumulation in nodule was observed only in the root part in direct contact with 5 mM nitrate. When the 1mM concentration of nitrate was continuously supplied from the lower roots, the nodule growth and nitrogen fixation activity in the upper roots were promoted compared with control plants supplied with nitrogen free solution. When 5 mM nitrate supply has stopped from pod setting stage, nodule weight and nitrogen fixation activity were recovered and exceeded over control plant at maturing stage.

The routes of nitrate entry into nodules was investigated. It was suggested that most of nitrate is absorbed through the surface of the nodule, but little is transported from the lower part of roots or shoots, either via xylem or phloem. The accumulation of nitrate in the cortex of nodule may inhibit respiration by decreasing O₂ permeability or suppress the photoassimilate import to the central symbiotic region of nodules, where bacteroid reside and fix nitrogen.

From the characteristics of nitrate effects on soybean nodulation and nitrogen fixation, we have developed a new fertilizer method “deep placement of slow release nitrogen fertilizer” for promoting soybean seed yield without inhibiting nodulation and nitrogen fixation (Takahashi et al., 1991, 1992, 1999, Tewari et al., 2010).

5. References

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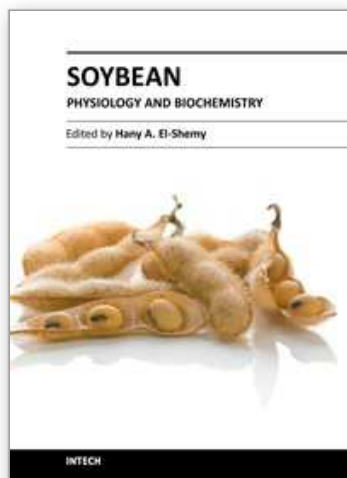
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