Recycling of nitrogen from shoots to underground parts in hypernodulation mutant lines of soybean by split-root experiment.

Takuji OHYAMA[1, 2, 3]*, Manabu UENO[2], Yuki ONO[1], Norikuni OHTAKE[1, 2], Kuni SUEYOSHI[1, 2], Takashi SATO[4] and Sayuri TANABATA[5]

(Received June 1, 2017)

Summary

Soybean plants can fix atmospheric dinitrogen (N_2) in root nodules by the association with soil bacteria rhizobia. Soybeans also absorb soil N from the roots, mainly in the form of nitrate in the upper fields. However, it has been known that nitrate strongly inhibits nodulation, nodule growth and nitrogen fixation activity of soybean nodules. Many hypotheses are proposed for the cause of nitrate inhibition of nodulation and nitrogen fixation, i.e., carbohydrate deprivation in nodules, decreased O_2 diffusion into nodules, which restricts the bacteroid respiration, feedback inhibition by a product of nitrate metabolism such as glutamine or asparagine. In this report, split-root system was employed for investing the recycling of ^{15}N in the opposite side of the half-roots supplied with $^{15}NO_3^-$ (10mgN/L) in hypernodulation mutants and the parent Williams for 2 days. The percentage distribution of ^{15}N in nodules were higher in NOD1-3 (0.20%), NOD2-4 (0.47%), NOD3-7 (0.26%) than that in Williams (0.09%) possibly due to large mass of nodules of hypernodulation mutant lines. On the other hand, the percentage distribution of ^{15}N in roots was relatively lower in NOD1-3 (1.36%), NOD2-4 (1.20%), NOD3-7 (1.26%) than that in Williams (1.58%). The sum of the percentage distribution of ^{15}N in the nodules and roots were almost the same between hypernodulation lines and the parent Williams. These results indicate that recycling of N from shoot to underground parts may not be the main cause of nitrate tolerance of hypernodulation mutant lines.

Bull.Facul.Agric.Niigata Univ., 70:1-8, 2018

Key words : soybean, hypernodulation mutant, nitrate inhibition, split-root system, ^{15}N recycling

Introduction

Soybean [*Glycine max* (L.) Merr.] is very important food and feed crop all over the world. Soybean seeds contain a large amount of protein, therefore, they require a large amount of nitrogen (N) relative to cereal grains, such as rice, wheat, barley and corn (Ohyama et al. 2013). Soybean plants can fix atmospheric dinitrogen (N_2) in root nodules by the association with soil bacteria rhizobia. Soybeans also absorb soil N from the roots, mainly in the form of nitrate in the upper fields. However, it has been known that nitrate strongly inhibits nodulation, nodule growth and nitrogen fixation activity of soybean nodules (Ohyama et al. 2011).

The inhibitory effects of nitrate on nodule development were documented about a century ago, however, there is no convincing evidence to explain the mechanism for the effects (Streeter, 1988). Three responses can be separated for the nitrate inhibition, the number of nodules, nodule growth, and nitrogen fixation activity. In addition, there are two effects; "localized" and "systemic" effects on nodulation and nitrogen fixation in legume nodules. The local or direct effects of nitrate inhibition was observed in split-root experiments where strong and rapid nitrate inhibition of the nodules was restricted to the nodules attached to the root portions in direct contact with nitrate, and no or milder inhibition was induced in the other part of the roots absent of nitrate (Hinson 1975, Tanaka et al. 1985). Yashima et al. (2003) employed a vertical sprit root system using two-layered pot system to analyze the effects of the placement, concentration and period of nitrate application (Yashima et al., 2003, 2005), and the systemic inhibition occurred both on N_2 fixation activity and nodule growth but not on the nodule number in the upper part of roots when 5mM nitrate was supplied to the lower roots (Yashima et al., 2003).

Many hypotheses are proposed for the cause of nitrate inhibition of nodulation and nitrogen fixation, i.e., carbohydrate deprivation in nodules (Streeter 1988), decreased O_2 diffusion into nodules which restricts the bacteroid respiration (Schuller et al., 1988), feedback inhibition through phloem by a product of nitrate metabolism in the shoot, such as glutamine (Neo and Layzell 1997) or asparagine (Becanamwo and Harper 1996). Rapid and reversible inhibition on nodule growth and nitrogen fixation activity were observed by direct localized application of nitrate on nodules (Fujikake et al. 2002, 2003,

[1] Graduate School of Science and Technology, Niigata University
[2] Faculty of Agriculture, Niigata University
[3] Faculty of Applied Biosciences, Tokyo University of Agriculture
[4] Faculty of Bioresource Sciences, Akita Prefectural University
[5] College of Agriculture Field Science Center, Ibaraki University
* Corresponding author: ohyama@agr.niigata-u.ac.jp

Saito et al. 2014). The diameter of individual nodules was measured in a culture solution with 0 mM or 5mM nitrate. The increase in nodule diameter was completely stopped after 1 day of 5 mM nitrate supply. However, the nodule growth quickly returned to the normal growth rate following withdrawal of nitrate from the solution (0 mM nitrate). From ^{11}C or ^{14}C labeled carbon dioxide exposure to soybean plants, it was suggested that the decrease in photoassimilate supply to nodules may be involved in the quick and reversible nitrate inhibition of soybean nodule growth and nitrogen fixation activity.

In 1980th several soybean mutant lines were selected after chemical mutagen treatment to overcome the nitrate inhibition of nodulation (Carroll et al. 1985 a, b, Gremaud and Harper 1989, Akao and Kouchi 1992, Francisco and Akao 1993). Gremaud and Harper (1989) selected three independent nodulation mutants, NOD1-3, NOD2-4 and NOD3-7 from Williams using ethyl methanesulfonate or *N*-nitroso-*N*-methylurea as mutagens. All three mutant lines had 2 to 4 times higher number of nodules than the Williams parent, indicating that these mutants were affected autoregulatory control of nodulation. Moreover, these mutants were partially tolerant to nitrate (NO_3^-) in culture solution compared with the parent Williams. Cho and Harper (1991 a,b) found that isoflavonoid concentration in hypernodulation mutant lines are higher than wild type, and this may be related to the lower sensitivity to nitrate inhibition.

In this paper we used split root sytems to evaluate recycling of N absorbed from one side of split-root to another side of roots in hypernodulation mutant lines and wild type Williams.

Materials and Methods

Plant cultivation

Seeds of hypernodulation soybean mutant lines; NOD1-3, NOD2-4, and NOD3-7 isolated from Williams, and the parent Williams were inoculated with a suspension of *B. diazoefficiens* (strain USDA110), and sown in vermiculite on 22th June, 1989. Seedlings were transplanted on 13 days after planting (DAP) to the hydroponic culture in a glasshouse under natural conditions. Each plant was cultivated with 3 L of nutrient solution with K_2HPO_4 95.5, K_2SO_4 28.8, $CaCl_2$•$2H_2O$ 262, $MgSO_4$•$7H_2O$ 245, H_3BO_4 10, $CuSO_4$ $5H_2O$ 0.25, $MnSO_4$ 1.32, $ZnSO_4$ $7H_2O$ 0.25, $(NH_4)_6Mo_7O_{24}$ 0.066, $NiSO_4$ $6H_2O$ 0.066, EDTA 2Na 37.2, $FeSO_4$ $7H_2O$ 27.8, and $NaNO_3$ 60.7 (mg L^{-1}). Culture solution was renewed at 2 or 3 days intervals.

^{15}N treatment with split-root experiment

On 25th July at 33 DAP, soybean roots were separated into two pots. Each pot contains 3 L of culture solution. On 26th July ^{15}N labeled culture solution containing $Na^{15}NO_3$ (10 mgN L^{-1}, 31.2 atom%^{15}N) was replaced for one side of pot (Figure 1). Non-labeled solution containing $NaNO_3$ (10 mgN L^{-1}) was replaced for the other side of pot. At 28th July after 2days treatment, the plant samples are washed and separated into roots and shoots, and frozen with liquid N_2, then the samples were freeze-dried and separated into the roots and nodules of ^{15}N feeding side (^{15}N labeled NO_3^-), the roots and nodules of non-labeled N feeding side (Non-labeled NO_3^-), basal part of roots and nodules from underground part. Leaves and stems are separated from the shoots. The dry weight of each part was measured. Experiment was carried out by 4 replications

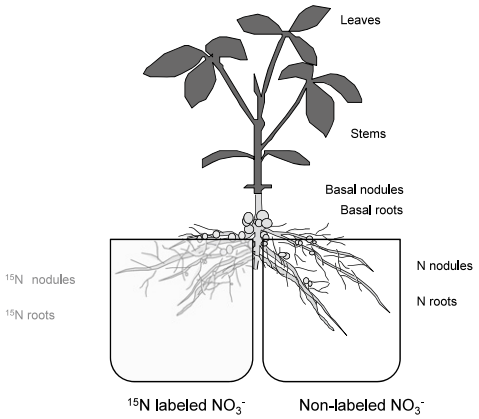

Fig 1. Split-root system in this experiment. A half-root was supplied with ^{15}N labeled culture solution and the opposite side of half-root was supplied with non-labeled nitrate for 2days.

Sample analysis

Each part of samples was ground into a fine powder by a vibration mill. Nitrogen concentration was determined using the modified Kjeldahl digestion method (Ohyama et al. 1991). Aliquot of Keldahl digested solution filled up to 25 mL was put into a Pyrex glass tube and evaporated to dryness in vacuo. The glass tubes are evacuated and sealed with an oxidizer (CuO) and absorber of water and CO_2 (preheated CaO). The ammonium in the sealed glass tubes were converted to gaseous nitrogen (N_2) by heating the tubes at 560 ℃ for 30 minutes. The ^{15}N abundance (atom%excess) of N_2 gas was analyzed by an emission spectrometry with ^{15}N analyzer (N150 JASCO, Ltd. Japan) (Ohyama et al. 2004). Percentage of N from labeled ^{15}N was calculated by the equation:

100 × ^{15}N atom%excess of sample / ^{15}N abundance of labeled NO_3^- where the ^{15}N abundance of labeled NO_3^- is 30.8 atom%excess in this experiment.

Results

Dry weight of each part of plants

Table 1-1 shows the dry weight (DW) of each part of soybean lines. Average total DW was Williams (5.25g), NOD1-3 (6.37g), NOD2-4 (6.50g) and NOD3-7 (5.02g), but these values are not statistically significant. The DW of the basal nodules was higher in NOD1-3 (0.483g), NOD2-4 (0.370g) and NOD3-7 (0.305g) compared with Williams (0.168g).

Table 1-1. Dry weight of each part of soybean plants (gDW/plant)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	2.118 (0.224)	2.743 (0.085)	2.843 (0.115)	2.285 (0.141)
stems	1.228 (0.119)	1.635 (0.079)	1.535 (0.073)	1.263 (0.051)
basal nodules	0.168 (0.023)	0.483 (0.012)**	0.370 (0.040)*	0.305 (0.057)
basal roots	0.655 (0.068)	0.703 (0.038)	0.680 (0.030)	0.473 (0.054)
split-nodules (^{15}N)	0.013 (0.068)	0.033 (0.013)	0.150 (0.028)*	0.063 (0.023)
split-roots (^{15}N)	0.495 (0.074)	0.340 (0.050)	0.400 (0.052)	0.278 (0.044)
split-nodules (N)	0.028 (0.009)	0.050 (0.007)*	0.190 (0.041)*	0.058 (0.010)
split-roots (N)	0.540 (0.083)	0.378 (0.038)	0.340 (0.048)	0.300 (0.054)
Total	5.245 (0.548)	6.365 (0.228)	6.508 (0.271)	5.025 (0.265)

Numbers are average (standard error).
*, ** Significantly different to Williams at >5% and >1% levels by T-test

Table 1-2. Percentage distribution of dry weight of each part of soybean plants (%)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	40.4	43.1	43.7	45.5
stems	23.4	25.7	23.6	25.1
nodules	4.0	8.9	10.9	8.5
roots	32.2	22.3	21.8	20.9

Table 2. Nitrogen concentration of each part of soybean plants (mgN/gDW)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	32.63 (0.80)	38.15 (0.30)**	38.31 (0.63)*	37.24 (1.02)
stems	13.98 (0.36)	14.98 (0.20)	15.29 (0.65)	15.03 (0.21)
basal nodules	43.47 (0.57)	45.29 (0.46)	46.01 (0.57)	48.09 (1.17)*
basal roots	8.36 (0.37)	11.95 (0.49)**	10.87 (0.40)*	11.50 (0.54)**
split-nodules (^{15}N)	43.33 (0.00)	38.63 (0.00)	40.58 (1.54)	38.17 (0.15)**
split-roots (^{15}N)	21.32 (0.25)	25.89 (0.48)**	23.04 (0.61)	24.22 (0.86)
split-nodules (N)	44.6 (0.00)	39.6 (0.00)	39.81 (0.62)**	38.18 (0.33)**
split-roots (N)	21.54 (0.30)	24.94 (0.28)**	23.04 (0.61)*	26.86 (1.02)*

Numbers are average (standard error).
*, ** Significantly different to Williams at >5% and >1% levels by T-test.

Table 1-2 shows the percentage distribution of DW of each part in soybean lines. Distribution of DW in leaves and stems in three hypernodulation mutant lines tended to be slightly higher than those in Willliams. The distribution of DW of nodules are more than twice in NOD1-3 (8.9%), NOD2-4 (10.9%), NOD3-7 (8.5%) compared with Williams (4.0%). The reverse was true for the DW of roots, and distribution of DW of roots were lower in NOD1-3 (22.3%), NOD2-4 (21.8%), NOD3-7 (20.9%) compared with Williams (32.2%).

Nitrogen concentration in each part of plants

Table 2 shows nitrogen concentration (mgN/gDW) of each part of soybean lines. The N concentration in leaves, stems, and basal roots are higher in NOD1-3. NOD2-4, and NOD3-7 than that of Williams. The N concentration of basal nodules are higher in hypernodulation mutant lines compared with Williams. Although the N concentration in split-nodules tended to be higher in Williams than NOD lines, those in split-roots trended to be higher in NOD lines than Williams parent.

Nitrogen content in each part of plants

Table 3-1 shows the N content in each part of soybean lines. Total N in NOD1-3 (179 mgN/plant) and NOD2-4 (188 mgN/plant) were higher than that of Williams (123 mgN/plant) and NOD3-7 (144 mgN/plant). The N contents in leaves and stems in NOD1-3 and NOD2-4 were higher than those in Williams. The total N in basal nodules and basal roots in NOD1-3 and NOD2-4 were also higher than those in Williams.

Table 3-2 shows the percentage distribution of N content in each part of soybean plants. Distribution of N in leaves (56.5-59.3%) and stems (12.4-13.8%) are relatively similar among mutant lines and Williams parent. Distributions of N in nodules are higher in NOD1-3 (13.9%), NOD2-4 (16.3%) and NOD3-7 (13.5%) than that in Williams (7.4%). Reverse was true in distribution of N in roots. The distribution of N in roots of Williams was 22.3% and higher than those in NOD lines about

Table 3-1. N content of each part of soybean plants (mgN/plant)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	69.30 (9.40)	104.59 (3.18)*	108.87 (6.07)	85.25 (5.77)
stems	16.99 (1.34)	24.44 (1.15)*	23.33 (1.12)	19.02 (1.15)
basal nodules	7.33 (1.19)	21.8 (0.48)**	17.13 (2.34)*	14.97 (3.49)
basal roots	5.20 (0.72)*	8.39 (0.81)*	7.37 (0.38)	5.32 (0.44)
split-nodules (^{15}N)	0.58 (0.19)	1.25 (0.55)	6.06 (0.84)**	2.38 (0.97)
split-roots (^{15}N)	10.57 (1.85)	7.30 (1.92)	9.06 (1.20)	6.65 (0.98)
split-nodules (N)	1.155 (0.52)	1.96 (0.32)	7.47 (1.87)*	2.14 (0.46)
split-roots (N)	11.55 (1.87)	9.36 (1.02)	8.54 (1.23)	8.06 (1.76)
Total	122.66 (15.32)	179.08 (4.67)*	187.82 (9.18)*	143.76 (11.58)

Numbers are average (standard error).
*, ** Significantly different to Williams at >5% and >1% levels by T-test.

Table 3-2. Percentage of N content in each part of soybean plants (%)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	56.5	58.1	58.0	59.3
stems	13.8	13.6	12.4	13.2
nodules	7.4	13.9	16.3	13.5
roots	22.3	14.4	13.3	13.9

Table 4. Percentage of ^{15}N (^{15}N%) in each part of soybean plants (%)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	3.87 (1.03)	4.55 (0.58)	4.21 (0.60)	5.07 (0.36)
stems	5.95 (1.13)	7.32 (1.13)	6.04 (0.75)	7.26 (0.71)
basal nodules	0.41 (0.02)	0.65 (0.07)	0.65 (0.07)*	0.70 (0.07)*
basal roots	1.95 (0.55)	3.00 (0.47)	2.83 (0.42)	3.44 (0.47)
split-nodules (^{15}N)	1.23 (0.00)	4.64 (0.00)	1.85 (0.08)	5.86 (1.44)
split-roots (^{15}N)	14.04 (1.74)	21.42 (1.06)	16.36 (1.41)	18.59 (1.53)
split-nodules (N)	0.28 (0.00)	0.92 (0.00)	0.53 (0.07)	0.95 (0.15)
split-roots (N)	0.74 (0.21)	1.35 (0.21)	1.19 (0.21)	1.26 (0.17)

Numbers are average (standard error).
*, ** Significantly different to Williams at >5% and >1% levels by T-test.

14%.

Percentage of N derived from ^{15}N labeled nitrate

Table 4 shows the percentage of N derived from ^{15}N labeled NO_3^- (^{15}N%). The ^{15}N% was the highest (14-21%) in the split-roots in the pot with ^{15}N labeled NO_3^- in all lines. The ^{15}N% in stems (6.0-7.3%) and leaves (3.9-5.1%) were relatively high among organs. The ^{15}N% in split nodules in the pot with ^{15}N labeled NO_3^- was 1.23-5.86%. The ^{15}N% in basal roots (2-3.4%) were higher than basal nodules (0.4-0.7%). Although ^{15}N% was low but it was detected in the split roots (0.7-1.3%) and nodules (0.3-1.0%) with non-labeled NO_3^- in all lines.

^{15}N content in each part of soybean plants

Table 5-1 and Table 5-2 shows the ^{15}N content and percentage distribution of ^{15}N in each part of soybean plants. After 2 days treatment of the ^{15}N labeled NO_3^- to a half roots, about 51-58 % of the absorbed ^{15}N was transported to leaves and 17-20% was transported to the stems.

Percentage of ^{15}N in nodules and roots with non-labeled nitrate

The percentage of ^{15}N in split nodules and roots with non-labeled NO_3^- is shown in Figure 2. The percentage distribution of ^{15}N in nodules were higher in NOD1-3 (0.20%), NOD2-4 (0.47%), NOD3-7 (0.26%) than that in Williams (0.09%). On the other hand, the percentage distribution of ^{15}N in roots are lower in NOD1-3 (1.36%), NOD2-4 (1.20%), NOD3-7 (1.26%) than that in Williams (1.58%). The sum of the percentage distribution of ^{15}N in the nodules and roots were relatively same between hypernodulation lines and the parent Williams.

Discussion

Characteristics of autoregulation control in soybean plants

Kosslak and Bohlool (1984) reported that suppression of nodule development of one side of split-root system of

Table 5-1. ^{15}N content in each part of soybean plants (mgN) (mgN/plant)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	2,990 (1,266)	4,768 (704)	4,620 (821)	4,393 (662)
stems	1,055 (293)	1,793 (313)	1,400 (185)	1,405 (233)
basal nodules	28 (5)	143 (16)**	143 (30)*	103 (26)*
basal roots	118 (48)	248 (44)	213 (45)*	180 (35)
split-nodules (^{15}N)	8 (3)	55 (25)	113 (16)**	145 (93)
split-roots (^{15}N)	1,583 (501)	1,915 (359)	1,528 (302)	1,225 (200)
split-nodules (N)	5 (3)	18 (3)*	38 (14)	20 (4)
split-roots (N)	93 (44)	123 (20)	98 (22)	95 (15)
Total	5,880 (2,143)	9,063 (1,446)	8,153 (1,188)	7,566 (1,116)

Numbers are average (standard error).
*, ** Significantly different to Williams at >5% and >1% levels by T-test.

Table 5-2. Percentage of ^{15}N in each part of soybean plants (%)

	Williams	NOD1-3	NOD2-4	NOD3-7
leaves	50.9	52.6	56.7	58.1
stems	17.9	19.8	17.2	18.6
basal nodules	0.5	1.6	1.8	1.4
basal roots	2.0	2.7	2.6	2.4
split-nodules (^{15}N)	0.1	0.6	1.4	1.9
split-roots (^{15}N)	26.9	21.1	18.7	16.2
split-nodules (N)	0.1	0.2	0.5	0.3
split-roots (N)	1.6	1.4	1.2	1.3

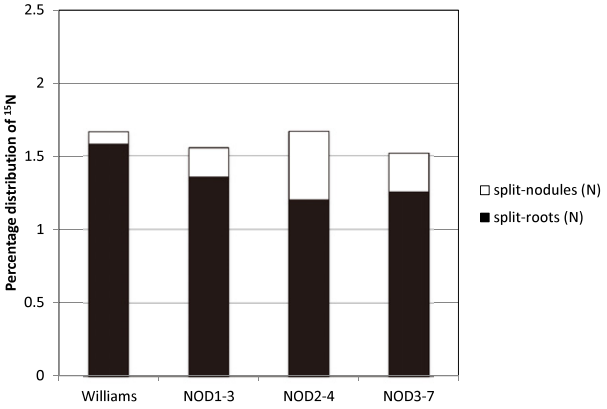

Fig 2. Percentage distribution of ^{15}N underground part of soybean in the non-labeled nitrate.

soybeans caused by prior inoculation of the other side. This indicated that soybean nodulation is controlled by prier inoculation or nodule formation systemically. The hypernodulation mutant lines of soybean were separated, (Carroll et al, 1985a, 1985b, Gremaud and Harper, 1989, Akao and Kouchi, 1992, Francisco and Akao, 1993). All the mutant lines produced profound nodulation and decrease the nitrate inhibition of nodulation. By reciprocal grafting of roots and shoots of hypernodulation mutant lines and wild type, hypernodulation phenotype was controlled by shoot genotype and not root genotype (Delves et al., 1987, Barbera and Harper 1993, Sheng and Harper 1997). The control of nodulation by prior inoculation was named "autoregulation of nodulation", where initial nodule growth induce the synthesis and transport of some signals (infection signal) from roots to shoots, and then the shoots received this signal make some other signal (autoregulation signal) and transport to the roots through phloem and suppress the growth of nodules. Sato et al. (1997) observed that mature leaf blade is the controlling part of shoot by using the rooted single leaf system.

Genes responsible for regulation of nodule number have been identified as *LjHAR1* in model legume *Lotus japonicus* (Nishimura et al. 2002) and *GmNARK* in soybean (Searle et al. 2003). These genes encode leucine-rich repeat receptor kinases similar to *CLAVATA1* in *Arabidopsis thaliana* which controls shoot meristem development. Small peptide named CLE peptides are postulated as a candidate of the infection signal from roots to shoot, based on the similarity of CLAVATA1 and CLE peptide signaling (Okamoto et al. 2009, Reid et al 2011, 2013, Soyano T. et al. 2014). Recently nitrate inducible CLE peptides have been found in addition to rhizobial infection induced CLE peptides (Okamoto et al. 2009, Reid et al 2011). It is postulated that inhibitor is synthesized in the shoot when NARK protein receive CLE peptides. It is transported from shoot to roots, and it prevents differentiation of nodules. However, growth of nodules already differentiated is not prevented by AON. So that mechanism of inhibition of nodule growth by nitrate might be different from that of nodule formation by nitrate and AON.

Although hypernodulation mutant lines have a lot of nodules, plant growth and seed yield tended to be lower than

parent line (Ohyama et al. 1993, Suganuma et al. 2001), although there was no difference in growth of hypernodulation mutant lines and Williams with or without inoculation at initial growth at 8 days after planting (Ito et al. 2006b). The nodule characters such as nodule size, percentage of infected region in nodules, leghemoglogin concentrations are lower than the parent Williams (Nishiwaki et al. 1997, Sato et al. 2001). In addition, hypernodulation mutant lines show some differences such as leaf development (Ito et al. 2008). A microscopic study showed that NOD1-3 and NOD3-7 produced small size leaves due to the smaller number of leaf cells, compared with Williams parent, suggesting that the autoregulation signaling may be related to the leaf cell proliferation.

Nitrate tolerance in hypernodulation mutant lines.

As mentioned before, all the hypernodulation mutant lines show the tolerance of nodulation to the nitrate. Characteristics of the assimilation and distribution of ^{15}N from fixed $^{15}N_2$ and absorbed $^{15}NO_3^-$ were invested in hypernodulation mutant lines, NOD1-3, NOD2-4, NOD 3-7 and parent Williams cultivated with 0 mM nitrate or 5 mM nitrate in culture solution (Ohyama et al. 1993). The Williams plants cultivated with 5 mM nitrate exhibited 95-97% decrease in nodule mass and $^{15}N_2$ fixation, while hypernodulation mutant lines retained about 30 to 40% nodule mass and 17-19% $^{15}N_2$ fixation. Nitrate absorption was less in the hypernodulation mutant lines than Williams parent. However, the distribution of ^{15}N among organs either from $^{15}N_2$ or $^{15}NO_3^-$ were similar between mutant lines and Williams.

In this report, the sum of the percentage distribution of ^{15}N in the nodules and roots were relatively same between hypernodulation lines and the parent Williams. These results indicate that recycling of N transported from the shoot to underground parts may not be the main cause of nitrate tolerance of hypernodulation mutant lines. The precise mechanism of partial tolerance in hypernodulation mutant has not been elucidated.

Neo and Layzell (1997) reported that the application of ammonia to the soybean shoot caused a 2.6-fold increase in total N and 10.5-fold increase in glutamine N, and apparent nitrogenase activity and total nitrogenase activity began to decline within 4 hours and reached about 54% of its initial activity within 6 hours. They suggested that the changes in the N composition of the phloem sap, particularly the glutamine content, may increase nodule resistance to O_2 diffusion, and down-regulate nodule metabolism and nitrogenase activity. Bacanamwo and Harper (1996) reported that by nitrogenase inhibition by nitrate, the C/N ratio in shoots decline but not in nodules.

In this report the recycling of N from shoot to the underground parts in hypernodulation mutant were almost the same as parent Williams, suggesting that this is not the cause of the nitrate tolerance and hypernodulation traits. The distribution of N in nodules are higher in hypernodulation mutant lines may be due to the larger mass of nodules in hypernodulation lines compared with Willliams.

References

Akao, S., and Kouchi, H., 1992. A supernodulation mutant isolated from soybean cultivar enrei., Soil Sci. Plant Nutr., 38, 183-187.

Bacanamwo, M., Harper, J.E., 1996. Regulation of nitrogenase activity in *Bradyrhizobium japonicum*/soybean symbiosis by plant N status as determined by shoot C:N retio. Physiol. Plant., 98, 529-538.

Berbera, A.C., Harper, J.E. 1993. Interaction of shoot and root on nodulation control of grafted soybean and mung bean. Riv. Di Agron., 27, 445-450.

Carroll, B.J., McNeil, D.L., Gresshoff P.M. 1985a. A supernodulation and nitrate-tolerant symbiotic (nts) soybean mutant. Plant Physiol., 78, 34-40.

Carroll, B.J., McNeil, D.L., Gresshoff P.M. 1985b. Isolation and properties of soybean (*Glycine max* L. Merr.) mutants that nodulate in the presence of high nitrate concentrations. Proc. Natl. Acad. Sci. USA., 82, 4162-4166.

Cho, M.-J., Harper, J.E., 1991a. Effect of inoculation and nitrogen on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. Plant Physiol., 95, 435-442.

Cho, M.-J., Harper, J.E., 1991b. Effect of localized nitrate application on isoflavonoid concentration and nodulation in split-root systems of wild-type and nodulation-mutant soybean plants. Plant Physiol., 95, 1106-1112.

Delves A. C., Higgins, A.V., Gresshoff, P.M., 1987. Shoot control of supernodulation in a number of mutant soybeans, *Glycine max* (L.) Merr., Aust. J. Plant. Physiol., 128, 473-478.

Francisco, PB, J.R., Akao, S., 1993. Autoregulation and Nitrate Inhibition of Nodule Formation in Soybean cv. Enrei and its Nodulation Mutants. J. Exp. Bot., 44, 547-553.

Fujikake, H., Yashima, H., Sato, T., Ohtake, N., Sueyoshi, K., Ohyama, T., 2002. Rapid and reversible nitrate inhibition of nodule growth and N_2 fixation activity in soybean (*Glycine max* (L.) Merr.). Soil Sci. Plant Nutr., 48, 211-217.

Fujikake, H., Yamazaki, A., Ohtake, N., Sueyoshi, K., Matsuhashi, S., Ito, T., Mizuniwa, C., Kume, T., Hashimoto, S., Shiketa Ishioka, N., Watanabe, S., Osa A., Sekine, T., Uchida, H., Tsuji, A., Ohyama, T., 2003. Quick and reversible inhibition of soybean root nodule growth by nitrate involves a decrease in sucrose supply to nodules. J. Exp. Bot, 54, 1379-1388.

Gremaud, M.F., Harper J.E., 1989. Selection and initial characterization of partially nitrate tolerant nodulation mutants of soybean. Plant Physiol., 89,169-173.

Harper J.E., Cooper, R.L., 1971. Nodulation response of soybeans [*Glycine max* (L.) Merr.] to application rate and placement of combined nitrogen. Crop Sci. 11, 438-440.

Hinson, K. 1975. Nodulation responses from nitrogen applied to soybean half-root systems. Agron. J., 67, 799-804.

Ito, S., Kato, T., Ohtake, N., Sueyoshi, K., Ohyama, T., 2008. The autoregulation of nodulation mechanism is related to leaf development, Plant Cell. Physiol., 49, 121-125.

Ito, S., Ohtake, N., Sueyoshi, K., Ohyama, T. 2006a. Allocation of photosynthetic products in hypernodulation mutant of

soybean NOD1-3 in the early stages of nodule formation. Bull. Facul. Agric. Niigata Univ., 59, 33-38.

Ito, S., Ohtake, N., Sueyoshi, K., Ohyama, T. 2006b. Comparison of initial growth of hypernodulation soyubean mutants, NOD1-3, NOD2-4, and NOD3-7, and their parent cv. Williams. Bull. Facul. Agric. Niigata Univ., 59, 39-43.

Kosslak, R.M., Bohlool, B.B., 1984. Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side. Plant Physiol. 75, 125-130.

Neo, H.H., Layzell D.B., 1997. Phloem glutamine and the regulation of O_2 diffusion in legume nodules. Plant Physiol., 113, 259-267.

Nishimura, R., Hayashi, M., Wu, G.J., Kouchi, H., Imaizumi-Anraku, H., Murakami, Y., Kawasaki, S., Akao, S., Ohmori, M., Nagasawa, M. Harada, K., Kawaguchi, M., 2002. HAR1 mediates systemic regulation of symbiotic organ development. Nature, 420, 426-429.

Nishiwaki, T., Sato, T., Yashima, H., Ikarashi, T., Ohyama, T., Harper, J.E., Akao, S., Kouchi, H., 1997. Changes in concentration of leghemoglobin components in hypernodulation mutants of soybean. Soil Sci. Plant Nutr., 43, 1091-1096.

Ohyama, T., Ito, M., Kobayashi, K., Araki, S., Yasuyoshi, S., Sasaki, O., Yamazaki, T., Soyama, K., Tanemura, R., Mizuno, Y., Ikarashi, T., 1991. Analytical procedure of N, P, K contents in plants and manure materials using H_2SO_4–H_2O_2 Kjeldahl digestion method. Bull. Fac. Agric., Niigata Univ., 43, 111–120.

Ohyama, T., Nicholas, J.C., Harper, J.E. 1993. Assimilation of $^{15}N_2$ and $^{15}NO_3^-$ by partially nitrate-tolerant nodulation mutants of soybean. J. Exp. Bot., 44,1739-1747.

Ohyama T, Tewari, K., Abdel-Latif, S., Ruamrungsri, S., Komiyama, S., Ito, S., Yamazaki, A., Sueyoshi, K., Ohtake, N., 2004. Direct Analysis of ^{15}N abundance of Kjeldahl digested solution by emission spectrometry. Bull. Fac. Agric., Niigata Univ., 57, 33–144.

Ohyama, T., Fujikake, H., Yashima, H., Tanabata, S., Ishikawa, S., Sato, T., Nishiwaki, T., Ohtake, N., Sueyoshi, K.,Ishii, S., Fujimaki, S. 2011. Effect of Nitrate on Nodulation and Nitrogen Fixation of Soybean, in Soybean Physiology and Biochemistry, Ed. El-Shemy, 333-364.

Ohyama, T., Minagawa, R., Ishikawa, S., Yamamoto, M., Hung, N.V.P., Ohtake, N., Sueyoshi, K., Sato, T., Nagumo, Y., Takahashi, Y., 2013. Soybean seed production and nitrogen nutrition. In A Comprehensive survey of international soybean research-genetics, physiology, agronomy and nitrogen relationships. Ed. By Board, J.E. 115-157.

Okamoto, S., Ohnishi, E., Sato, S., Takahashi, H., Nakazono, M., Tabata, S., Kawaguchi, M., 2009. Nod factor/nitrate-inducible *CLE* genes that drive systemic regulateon of nodulation. Plant Cell Physiol., 50, 67-77.

Reid, D.E., Ferguson, B.J., Gresshoff, P.M., 2011. Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation.Molecular mechanisms controlling legume autoregulation of nodulation. MPMI, 24, 606-618.

Reid, D.E., Li, D., Ferguson, B.J., Gresshoff, P.M., 2013. Structure-function analysis of the *Gm*RIC1 signal peptide and CLE domain required for nodulation control in soybean. J. Exper. Bot., 64, 1575-1585.

Saito, A., Tanabata, S., Tajima, S., Ueno, M., Ishikawa, S., Ohtake, N., Sueyoshi, K., Ohyama, T., 2014. Effect of Nitrate on Nodule and Root Growth of Soybean (*Glycine max* (L.) Merr.). International Journal of Molecular Sciences, 15, 4464-4480.

Sato, T., Yashima, H., Harper, J.E., Akao, S., Ohyama, T., 1997. Nodule formation and N_2 fixation traits of the rooted-single leaf isolated from hypernodulating mutants and the wild type. Jpn. J. Soil Sci. Plant Nutr., 68, 444-447.

Sato, T., Onoma, N., Fujikake, H., Ohtake, N., Sueyoshi, K., Ohyama, T., 2001. Changes in four leghemoglobin components in nodules of hypernodulating soybean (*Glycine max* [L.]Merr.) mutant and its parent in the early nodule developmental stage. Plant and Soil, 237, 129-135.

Schuller, K.A., Minchin, F.R., Gresshoff, P.M., 1988. Nitrogenase activity and oxygen diffusion in nodules of soybean cv. Bragg and a supernodulation mmutant: effects of nitrate. J. Exper. Bot., 39, 865-877

Searle, I.R. Men., A.E., Laniya, T.S. Buzas, D.M., Iturbe-Ormaetxe, I., Carroll, B.J., Gresshoff P.M., 2003. Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinese. Science, 299,109-112.

Sheng, C., Harper, J.E., 1997. Shoot versus root signal involvement in nodulation and vegetative growth in wild-type and hypernodulating soybean genotypes. Plant Physiol., 113,825-831.

Soyano, T., Kawaguchi, M.; Systemic regulation of root nodule formation. In Advances in Biology and Ecology of Nitrogen Fixation. Ed. Ohyama, T., InTech, Rijeka, Croatia, 2014, pp.89-109.

Streeter, J., 1988, Inhibition of legume nodule formation and N_2 fixation by nitrate. CRC Crit. Rev. Plant Sci., 7, 1-23.

Suganuma, T., Fujikake, H., Ohtake, N., Sueyoshi, K., Ohyama, T., 2001. Comparison of the growth and nitrogen fixation activity of the hypernodolation soybean mutant NOD1-3 and its parent cv. Williams in field cultivation. Bull. Fac. Agric., Niigata Univ., 53, 123-132.

Tanaka, A., Fujita, K., Terasawa, H., 1985. Growth and dinitrogen fixation of soybean root system affected by partial exposure to nitrate. Soil Sci. Plant Nutr., 31, 637-645.

Yashima, H., Fujikake, H., Sato, T., Ohtake, N., Sueyoshi, K., Ohyama, T., 2003. Systemic and local effects of long-term application of nitrate on nodule growth and N_2 fixation in soybean (*Glycine max* [L.] Merr.). Soil Sci. Plant Nutr., 49, 825-834.

Yashima, H., Fujikake, H., Yamazaki, A., Ito, S., Sato, T., Tewari, K., Ohtake, N., Sueyoshi, K., Takahashi, Y., Ohyama, T., 2005. Long-term effect of nitrate application from lower part of roots on nodulation and N_2 fixation in upper part of roots of soybean (*Glycine max* [L.] Merr.) in two-layered pot experiment. Soil Sci. Plant Nutr., 51, 981-990.

根分法によるダイズ根粒超着生変異株の茎葉部から地下部への窒素の再循環

大山卓爾[1, 2, 3]*, 上野　学[1], 小野雄基[2], 大竹憲邦[1, 2], 末吉　邦[1, 2], 佐藤　孝[4], 七夕小百合[5]

（平成29年６月１日受付）

要　約

ダイズは、土壌微生物の根粒菌と共生して大気中の窒素を固定利用できる。また、ダイズは、根から畑条件では主に硝酸態として、土壌由来の窒素を吸収できる。しかしながら、硝酸により、ダイズの根粒着生、根粒の生長、窒素固定活性が強く阻害されることが知られている。根粒形成と窒素固定に対する硝酸阻害機構として、根粒中の炭水化物の減少、根粒の酸素拡散の低下によるバクテロイドの呼吸低下、地上部の硝酸代謝産物、例えばグルタミンやアスパラギンによるフィードバック阻害など、多くの仮説が提出されている。本報告では、Williams と根粒長着生変異株について、根分法により、片側の根から与えた$^{15}NO_3^-$の反対側の根への循環を調べた。2日間の処理後、非標識条件の根粒の^{15}N 分配率は、NOD1-3で0.20％、NOD2-4で0.47％、NOD3-7で0.26％と Williams の0.09％より高かった。これは､根粒超着生株の根粒が大きかったことによるのかもしれない。一方、非標識条件の根の^{15}N 分配率は、NOD1-3で1.36％、NOD2-4で1.20％、NOD3-7で1.26％と Williams の1.58％より低かった。根と根粒の分配率の合計は、変異株と親株で同様であった。この結果は、茎葉部から地下部への窒素の再循環の違いが根粒超着生変異株の硝酸耐性の原因ではないことを示唆した。

新大農研報, 70:1-8, 2018

キーワード：ダイズ、根粒超着生変異株、硝酸阻害、根分法、^{15}N 再循環

[1] 新潟大学大学院自然科学研究科

[2] 新潟大学農学部

[3] 東京農業大学応用生物科学部

[4] 秋田県立大学生物資源科学部

[5] 茨城大学農学部附属農業フィールドセンター

* 代表著者 : ohyama@agr.niigata-u.ac.jp