Introduction, Development, and Characterization of Supernodulating Soybean Mutant
−Nitrate Inhibition of Nodulation and Nitrogen Fixation in Supernodulating Soybean Mutant−

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ABSTRACT

Inhibition of nodule formation and nitrogen fixation by soil nitrogen, primarily nitrate, is well known in legume plants. The present study was undertaken to evaluate the effect of NO_3^- on the nodulation, nitrogenase activity, and growth of supernodulating soybean mutant and its wild type. A greenhouse study was conducted to compare two of supernodulating mutants, 'SS2-2' and 'nts 382', with the normal nodulating cultivar 'Sinpaldalkong 2' when grown in a 1-ℓ styroform cup filled with sand, and fertilized with five levels of NO_3^- (0, 2, 4, 8, and 12 mM). During the growth period, each plant was supplied two or three times a week with 50 mL of nutrient solution. Supernodulating soybean mutants, SS2-2 and nts 382, showed more nodules and nodule mass, and greater C_2H_2 activity than the wild type, Sinpaldalkong 2, regardless of the level of exogeneous nitrogen supply. On the other hand, total dry weight of SS2-2 mutant, which was smaller than Sinpaldalkong 2, did not respond to the various NO_3^--N levels. This suggested that supernodulating SS2-2 mutant could maintain fairly high total dry weight at the low NO_3^--N level, even in the absence of exogeneous NO_3^--N in the nutrient solution. From the reduced top growth and high nitrogen fixing ability of supernodulating mutants, it was surmised that supernodulating mutant could potentially protect agricultural environments from pollution through the reduction in nitrogen fertilization as well as maintain fairly high yield with increasing planting density.

Key words : supernodulating soybean, mutant, nitrate inhibition, nitrogen fixation.

Of the two main sources of nitrogen for legume plant growth which include combined nitrogen such as nitrate or ammonia nitrogen and the fixed nitrogen from the atmosphere, recent research have focused on the utilization of nitrogen derived from biological nitrogen fixation in the root nodule. Nodulation and nitrogen fixation in legume plants may be limited by many external factors such as soil moisture, temperature, light, soil oxygen, soil acidity, mineral elements, and fertilization level with combined nitrogen. Of many environmental factors, high level of combined nitrogen in the soil is a potentially important limiting factor (Davis, 1980).

There is enough evidence for the preference of combined nitrogen rather than nitrogen fixed biologically (Streeter, 1988). This concept is in good agreement with the general inhibitory effects of soil nitrogen, primarily soil nitrate, on nodulation and nitrogen fixation (Gibson and Harper, 1985; Malik et al., 1987; Stephens and Neyra, 1983). Generally, soil nitrate is known to inhibit the infection process of nitrogen-fixing bacteria such as root hair curling for trapping bacteria, binding of bacteria to root hairs, and formation of infection threads. Thus, high soil nitrate resulted in reduced nodule growth and nitrogenase activity.

Recently supernodulating soybean mutants even in the presence of high soil nitrate were isolated by several independent research groups. Supernodulating soybean mutants were developed from cultivar 'Bragg' with EMS mutagensis by Carroll et al. (1985a ; 1985b), and from cultivar 'Enrei' by Akao & Kouchi (1992). Hypernodulating soybean mutants, which exhibited nodulation greater than wild type and smaller than supernodulating mutant, were also developed independently by Carroll et al. (1985a; 1985b) and Gremaud & Harper (1989). These super- or hyper-nodulating mutants could be unique biological material to reduce the nitrogen fertilizer requirements for crop production as well as to protect agricultural environments (Lee et al., 1997).

More recently, Lee et al.(1997) isolated a supernodulating mutant, SS2-2, from M_2 families of Sinpaldalkong 2 mutagenized with 30 mM EMS. In the presence of high exogeneous nitrate supply, greater nodulation was observed in SS2-2 like other supernodulating soybean mutants isolated previously such as nts 246, nts 382, and En6500. However, comparison of nodulation between SS2-2 and nts mutants revealed that the mutant, SS2-2, showed faster nodulation than the nts mutants (Lee et al., 1997).

In this study, nodulation, plant growth, and yield in response to exogeneous nitrate supply was compared among three soybean genotypes, consisting of two supernodulating mutants (SS2-2 and nts 382) and a normal nodulating wild-type Sinpaldalkong 2. Two types of experiments, sand culture with supply of nutrient solution and pot experiment with urea fertilization, were performed independently.

MATERIALS AND METHODS

Two supernodulating soybean mutants, SS2-2 and nts 382, and the wild type of SS2-2, Sinpaldalkong 2, were

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used in this study. SS2-2 mutant was previously isolated by Lee et al. (1997), and seeds of nts 382, isolated by Carroll et al. (1985a;1985b), were supplied by Prof. P.M. Gresshoff, Institute of Agriculture and Center for Legume Research in the Univ. of Tennessee, USA. Four seeds, which were surface-sterilized with 50 ml/ℓ NaOCl for 10 min, were planted in each 1-ℓ styroform cup filled with river sand and grown in well-controlled glasshouse. One week later, plants were thinned to grow two plants per pot.

Right after thinning, plants were inoculated with commercial *Bradyrhizobium japonicum* (NITRAGIN, Lipha-Tech Inc., Milwaukee, Wisconsin, USA). Two weeks after thinning, each pot was fertilized two or three times a week with 50 mL of modified Hoagland's solution, in which nitrate concentrations were adjusted to be 0, 2, 4, 8, and 12 mM NO_3^- with KNO_3 and $Ca(NO_3)_2$ during the growth period (Table 1). Soybean plants, treated with each level of nitrate concentration, were placed in a 3×5 factorial experiment with three replications in a completely randomized design.

Eight weeks after planting, the dry weight of each plant part (stem, leaf, root, and nodule) and acetylene reduction activity were measured. Acetylene reduction activity was measured on removed root from the whole plant which was placed in a 1-ℓ glass jar, and sealed with a lid containing a serological stopper. Using a 50-cc syringe, a 50-cc aliquot of air was removed from the jar, and the same amount of C_2H_2 was then injected into the sample jar. The root system was allowed to remain in the jar with C_2H_2 for 30 min, after which a 10-cc aliquot was withdrawn from the jar, and the aliquot was injected into a 10-cc vacutainer tube. From this 10-cc tube, 0.5-cc aliquot was later drawn for gas chromatograph analysis of

Table 1. Composition of modified Hoagland's solution for adjusting NO_3^- concentration.

Stock	Nitrogen concentration (mM)				
	0	2	4	8	12
	…… ml in a liter nutrient solution ……				
1M K_2SO_4	2.5	–	–	–	–
1M $MgSO_4$	2	2	2	2	2
1M $Ca(H_2PO_4)_2$	0.5	–	–	–	–
1M $CaSO_4$	2	2	–	–	–
1M KH_2PO_4	–	1	1	1	1
1M KNO_3	–	1	2	4	6
1M $Ca(NO_3)_2$	–	–	1	2	3
Micronutrients†	1	1	1	1	1
Iron chelate†	1	1	1	1	1

† Stock solutions for micronutrients and iron chelate were made according to Hoagland's solution preparation.

Table 2. Nodulation and acetylene reduction activity of Sinpaldalkong 2 and two supernodulating mutants as affected by nitrogen concentration.

Genotype	Nitrogen concentration (mM)					Mean
	0	2	4	8	12	
	…… Nodule number per plant ……					
Sinpaldalkong 2	63	62	63	62	53	61^{b}†
SS2-2	382	241	230	239	253	269^{a}
nts 382	306	210	340	321	245	284^{a}
Mean	250^{a}†	171^{b}	211^{ab}	207^{b}	184^{b}	
	…… Nodule dry weight (mg/plant) ……					
Sinpaldalkong 2	311	223	203	194	118	210^{c}
SS2-2	444	353	347	364	304	362^{b}
nts 382	554	373	635	580	527	534^{a}
Mean	437^{a}	316^{c}	395^{ab}	379^{b}	316^{c}	
	…… C_2H_2 reduction sctivity ……					
Sinpaldalkong 2	4.5	5.6	2.9	3.5	2.3	3.8^{c}
SS2-2	5.4	6.8	3.7	4.0	4.4	4.9^{b}
nts 382	8.7	6.6	5.4	5.0	3.6	5.9^{a}
Mean	6.2^{a}	6.3^{a}	4.0^{b}	4.2^{b}	4.5^{b}	
	…… Specific C_2H_2 reduction activity ……					
Sinpaldalkong 2	14.6	25.3	14.6	18.3	22.2	19.0^{c}
SS2-2	12.1	19.3	10.7	11.3	14.2	13.5^{b}
nts382	15.6	17.6	8.5	8.7	6.8	11.5^{a}
Mean	14.1^{b}	20.7^{a}	11.3^{b}	12.7^{b}	14.4^{b}	

† Within traits, means (column or row) not followed by the same letter are significantly different at $P \leq 0.05$ based on LSD.

C_2H_4.

A pot experiment was also designed to compare the plant yield and yield components between SS2-2 and its wild type Sinpaldalkong 2 in response to the level of nitrogen fertilization. One plant was grown in each 4-ℓ pot. Plants were fertilized at four levels of nitrogen (0, 2, 4, and 6 g/pot) in the form of urea. At harvest, pod number, seed number, 100 seed weight, and seed yield were measured for each plant.

RESULTS AND DISCUSSION

Supernodulating soybean mutants, SS2-2 and nts 382, produced more nodules and nodule mass than the wild type, Sinpaldalkong 2, regardless of the level of exogeneous nitrogen supply (Table 2). Averaged over five levels of nitrogen concentration, supernodulating mutants showed 4 to 5 times greater in nodule number, and 1.5 to 2.5 times greater in nodule dry weight per plant. There was a significant effect of nitrogen level on the nodule number. However, it was unusual that high nitrate concentration gave little effect on decreasing the nodule number of Sinpaldalkong 2, but did effect the nodule dry weight. This may be due to the fact that nodule number was determined earlier than nodule dry weight in the process of nodulation. In this study, nodulation sites were probably pre-determined prior to the nitrogen application, because nitrogen was supplied two weeks after inoculation of *Bradyrhizobium japonicum*. Significant decrease in nodule dry weight in response to high nitrogen level was observed in Sinpaldalkong 2, whereas slight decreases were found in SS2-2 and nts 382. This was consistent with earlier studies (Carroll et al. 1985a; Eskew et al., 1989; Day et al. 1989) that nitrate inhibition of nodulation was not great but detectable in supernodulating soybean mutants, though supernodulating mutants were highly nitrate-tolerant in nodulation.

Averages of C_2H_2 reduction activity for supernodulating mutants over five levels of nitrogen concentration were 1.3 to 1.5 times higher than that of the wild type, Sinpaldalkong 2 (Table 2). Significant effect of nitrogen concentration was observed in C_2H_2 reduction activity. Regardless of genotypes, nitrate inhibition of C_2H_2 reduction activity was shown above 4mM nitrogen concentration. Based on the nodule dry weight, specific C_2H_2 reduction activity was obtained from the C_2H_2 reduction activity (Table 2). As compared to supernodulating mutants, SS2-2 and nts 382, higher specific C_2H_2 reduction activity was observed in the wild type, Sinpaldalkong 2. As was reported by Day et al. (1989), lower specific nitrogenase activity of supernodulating mutants was mainly due to the reduced amount of symbiotic tissue which was characterized by smaller nodule cells, fewer bacteroids per peribacteroid membrane vesicle, and lowered haem content per nodule.

There was significant effect of soybean genotype on the dry weight of each plant part measured eight weeks after planting, and significant effect of exogeneous nitrogen concentration on the all dry weights of each plant part except pod dry weight (Table 3). Also, all the dry weight of each plant part had significant interaction effects between soybean genotype and nitrogen concentration level, indicating that soybean genotypes showed a different manner in the partitioning of dry matter into each plant part in response to the nitrogen concentration.

Genotypic difference in total dry weight including root, nodule, stem, leaf, and pod dry weight was observed in response to the NO_3^--N level. As the NO_3^--N level was higher, the accumulation of total dry weight for supernodulating mutant, SS2-2, was clearly different from its wild type, Sinpaldalkong 2 (Fig. 1). Total dry weight of Sinpaldalkong 2 increased sharply with increasing NO_3^--N level. On the other hand, total dry weight of SS2-2 mutant did not respond to the NO_3^--N level. This suggested that supernodulating SS2-2 mutant could maintain a fairly high total dry weight at low NO_3^--N level, even in the absence of exogeneous NO_3^--N in the nutri-

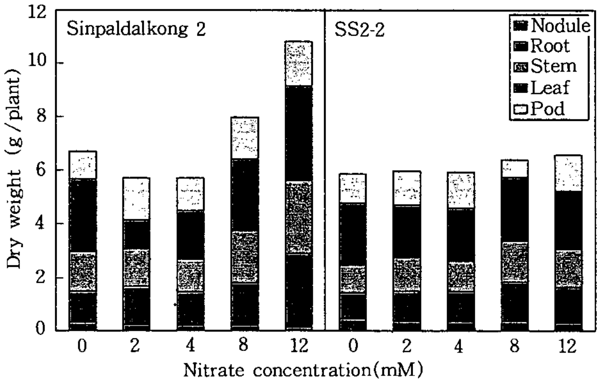

Fig. 1. Genotypic difference in total dry weight as affected by exogeneous nitrogen supply.

Table 3. Analysis of variance for dry weight of each plant part as affected by nitrate level and soybean genotypes.

Source of variation	df	Mean squares				
		Nodule dry wt.	Root dry wt.	Stem dry wt.	Leaf dry wt.	Pod dry wt.
Genotype (G)	2	263**	500**	2144**	614**	5569**
Nitrogen level (N)	2	16**	695**	944**	1000**	95
G×N	4	9**	266**	284**	360**	111*
Error	18	1	34	48	87	39

*, **, *** significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 4. Yield and yield components of Sinpaldalkong 2 and SS2-2 as affected by the level of nitrogen fertilization in the pot experiment.

Genotype	Nitrogen application (kg/ha)				Mean
	0	20	40	60	
	Pod number/plant				
Sinpaldalkong 2	36.4	38.5	47.3	49.8	$42.8^{a\dagger}$
SS2-2	15.8	13.3	12.5	24.3	16.8^{b}
Mean	$26.1^{a\dagger}$	25.9^{a}	29.9^{a}	37.1^{a}	
	Seed number/plant				
Sinpaldalkong 2	45.4	50.5	77.7	57.8	55.9^{a}
SS2-2	21.3	25.3	25.5	26.3	24.3^{b}
Mean	33.4^{b}	37.9^{b}	51.6^{a}	42.1^{ab}	
	100 seed weight (g)				
Sinpaldalkong 2	15.7	16.5	17.0	16.6	16.7^{a}
SS2-2	16.8	16.1	17.6	16.7	16.4^{a}
Mean	16.3^{a}	16.3^{a}	17.3^{a}	16.7^{a}	
	Seed dry weight (g/plamt)				
Sinpaldalkong 2	7.2	8.3	13.2	9.5	9.2^{a}
SS2-2	3.6	4.1	4.5	4.4	4.1^{b}
Mean	5.4^{b}	6.2^{b}	8.9^{a}	7.0^{ab}	

† Within column or row, means not followed by the same letter are significantly different at $P \leq 0.05$ based on LSD.

ent solution. From this result, it was surmised that a supernodulating mutant can maintain its growth by using nitrogen fixed biologically in the root nodule even in the absence of soil nitrogen.

In the pot experiment, increase in nitrogen fertilization enhanced the plant yield, especially of Sinpaldalkong 2, mainly due to the greater seed number per plant. In Sinpaldalkong 2, higher plant yield was observed when more than 40kgN/ha was applied. On the other hand, increased fertilization of nitrogen in the pot experiment did not increase the yield of supernodulating mutant SS2-2, indicating that a significant response of plant yield to nitrogen fertilization was not present in SS2-2. Generally, the plant yield of SS2-2 was lower than that of Sinpaldalkong 2 because of reduced pod and seed number per plant.

Supernodulating mutant, SS2-2, was characterized by greater nodulation and nitrogen fixation as well as fairly equal plant dry weight and yield in the absence of nitrogen fertilization, when compared to the high nitrogen fertilization level. Therefore, supernodulating mutants could be good breeding material to protect agricultural environments from pollution through the reduction in nitrogen fertilization. Also, fairly high yields can be achieved in supernodulating soybean mutant SS2-2 by increasing planting density. Higher population density of SS2-2 may be possible because of its reduced top growth when compared to Sinpaldalkong 2.

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REFERENCES

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

Carroll, B. J., D. L. McNeil, and P.M. Gresshoff. 1985a. 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.

________, ________, and ________. 1985b. A supernodulation and nitrate-tolerant symbiotic(nts) soybean mutant. Plant Physiol. 78: 34-40.

Davis, L. C. 1980. Limiting factors in nitrogen fixation. What's New in Plant Physiol. 11(11): 41-44.

Day, D. A., B. J. Carroll, A. C. Delves, and P.M. Gresshoff. 1989. Relationship between autoregulation and nitrate inhibition of nodulation in soybeans. Physiol. Planta. 75: 37-42.

Eskew, D. L., J. Kapuya, and S. K. A. Danso. 1989. Nitrate inhibition of nodulation nodulation and nitrogen fixation by supernodulating nitrate-tolerant symbiosis mutants of soybean. Crop Sci. 29: 1491-1496.

Gibson, A. H. and J. E. Harper. 1985. Nitrate effect on nodulation of soybean by *Bradyrhizobium japonicum*. Crop Sci. 25: 497-501.

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

Lee, H. S., Y. A. Chae, E. H. Park, Y. W. Kim, K. I. Yun, and S. H. Lee. 1997. Introduction, development, and characterization of supernodulating soybean mutant. I. Mutagenesis of soybean and selection of supernodulating soybean mutant. Korean J. Crop Sci. 42(2): 247-253.

Malik, N. S. A., H. E. Calvert, and W. D. Bauer. 1987. Nitrate induced regulation of nodule formation in soybean. Plant Physiol. 84:266-271.

Stephens, B. D. and C. A. Neyra. 1983. Nitrate and nitrite reduction in relation to nitrogenase activity in soybean nodules and *Rhizobium japonicum* bacteriods. Plant Physiol. 71: 731-735.

Streeter, J. 1988. Inhibition of legume nodule formation and nitrogen fixation by nitrate. CRC Critical Rev. in Plant Sci. 7: 1-23.