Responses of a Supernodulating Soybean Genotype, Sakukei 4 to Nitrogen Fertilizer

Tomiya Maekawa, Motoki Takahashi* and Makie Kokubun

(Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan; *National Institute of Crop Science, National Agricultural Research Organization, Tsukuba 305-8518, Japan)

Abstract: The supernodulating soybean genotype Sakukei 4 is potentially high-yielding. We characterized its leaf nitrogen (N) content, photosynthesis and growth at different developmental stages and under different dosages and types of N fertilizer, and compared it with its parental cultivar Enrei and the non-nodulating line En1282. At the pod-expansion and seed-filling stages, the N contents per leaf dry weight and per leaf area, and apparent photosynthetic rates (AP) were higher in Sakukei 4 than in the normal and the non-nodulating genotypes. The nodule activity per plant was also higher in Sakukei 4 than in Enrei during the reproductive stage. These traits varied less with the growing condition (field- or pot-grown) and dose or type of N fertilizer applied in Sakukei 4 than in the other genotypes. The superior ability of Sakukei 4 to maintain high leaf N and AP, however, did not enhance its growth performance, which tended to be inferior to that of Enrei. Further studies are needed to define the cultivation conditions optimal for an exploitation of the favorable traits of Sakukei 4.

Key words: Glycine max, Leaf nitrogen, Nitrogen response, Photosynthesis, Soybean, Supernodulation.

Soybean (Glycine max (L.) Merr.) has been grown as a nutritionally valuable crop since ancient times in Asian countries. Recently, there have been a number of reports showing that soybean contains several medicinal substances such as isoflavones and saponins (Fukushima, 2000). Therefore, the future demand for soybean is expected to increase worldwide. Despite these prospects, the production of soybean in major eastern Asian countries has substantially decreased in recent decades, because of the low profitability of soybean farming as compared with other major crops such as rice or vegetables. Thus, an increase in the productivity of soybean is required to render its cultivation more lucrative.

Soybean requires more N than gramineous crops because of its specific physiological characters (Sinclair and de Wit, 1975). Cultivated soybean has three N sources: fertilizer N, soil N and symbiotically fixed N\textsubscript{2}. Since application of fertilizer N hinders symbiotic N\textsubscript{2} fixation, total N absorption is not likely to be enhanced by increasing fertilizer N (Harper, 1987). In addition, overuse of N fertilizer can cause environmental problems. Therefore, soybean productivity should be improved by maximizing the amount of symbiotically fixed N\textsubscript{2}, particularly in soils of low fertility.

The genetic improvement of symbiotic N\textsubscript{2} fixation is an option to enhance the N absorption capability of soybean. Supernodulation would be a feasible way of enhancing N\textsubscript{2} fixation capability, and several supernodulating soybean mutants have been isolated (Carroll et al., 1985a, 1985b; Gremaud and Harper, 1989; Akao and Kouchi, 1992). However, most supernodulating lines bred so far grow slowly, and therefore are agronomically inferior compared to conventional cultivars (Herridge and Rose, 2000; Kokubun, 2001). The poor growth performance appears to be due to excessive consumption of photosynthates to form and maintain a large number of nodules, and to a low capacity to absorb nutrients and water due to smaller root systems (Gremaud and Harper, 1989; Hansen et al., 1989; Ohyama et al., 1993; Takahashi et al., 1995). The supernodulating genotype Sakukei 4 (formerly named En-b0-1-2) used in the present study has a high-yielding potential comparable to its parental cultivar Enrei (Takahashi et al., 2003). Clarification of the physiological characteristics of this genotype should help to utilize it as a high-yielding cultivar, and to further improve it genetically.

The objectives of this study were to characterize and compare the N content in the leaf, photosynthetic rate and growth of the supernodulating genotype Sakukei 4 with those of the two related nodulating and non-nodulating lines under different conditions of fertilization.

Materials and Methods

1. Pot experiment (Exp. 1)

(1) Plant material

The supernodulating genotype Sakukei 4 (formerly En

Received 16 January 2003. Accepted 8 April 2003. Corresponding author: M. Kokubun (kokubun@bios.tohoku.ac.jp, fax +81-22-717-8940).

Abbreviations: AP, apparent photosynthetic rate; ARA, acetylene reduction activity; DAS, days after seeding; NAR, net assimilation rate; PPFD, photosynthetic photon flux density.
plants from three pots were selected and separated into umol m$^{-2}$ S$^{-1}$ PPFD. The temperature of the chamber was kept at 25°C. After the photosynthesis measurement was carried out during 1000 and 1300 hr in the plants at the pod-expansion stage (73-79 DAS), the flow rate of air in the leaf chamber was controlled at 500 μmol s$^{-1}$, and the CO$_2$ concentration was maintained at 350 μmol mol$^{-1}$. The irradiance on the measured leaves (6 cm$^2$) was regulated at 1,500 μmol m$^{-2}$ s$^{-1}$ PPFD. The temperature of the chamber was kept at 25°C. After the photosynthesis measurement, the leaflets were excised, oven dried at 80°C for more than two days and weighed. The dried samples were ground in a mill and the N content was analyzed by the Kjeldahl-Gunning procedure (Kimura, 1995). Differences between values were tested by Fisher’s LSD ($P<0.05$).

### Results

#### 1. Leaf nitrogen content
In pot-grown Enrei and Sakukei 4 plants (Exp. 1), leaf N contents per leaf dry weight at the pod-expansion stage and mature stage (146-165 DAS) were measured at the pod-expansion and the seed-filling stage, respectively, in the same way as described for Exp. 1.
and the seed-filling stage were not changed by the amount of fertilizer applied (Fig. 1, left). By contrast, leaf N content per leaf dry weight at the pod-expansion stage in En1282 increased with increasing amount of N fertilizer although that at the seed-filling stage did not. The N contents per leaf area at the pod-expansion stage tended to increase with increasing fertilization in all genotypes (Fig. 1, right), but the response was only moderate in Sakukei 4. At the seed-filling stage, no such dependence on fertilizer dosage was observed. Leaf N content expressed per dry weight or per leaf area was the highest in Sakukei 4, intermediate in Enrei, and lowest in En1282. Generally, leaf N content was higher at the pod-expansion stage than at the seed-filling stage.

When urea or LP-70 was applied, leaf N content per dry weight at the pod-expansion stage tended to be higher in Sakukei 4 than in Enrei and En1282, but when LP-100 was applied, it was higher in Enrei (Fig. 2, left). Enrei had the highest leaf N content expressed on leaf area basis at the pod-expansion stage irrespective of the type of fertilizer (Fig. 2, right). At the seed-filling stage, leaf N content (both per leaf dry weight and per leaf area) of En1282 was significantly lower than that of the other two genotypes. At this stage, leaf N level seemed to be slightly higher in Sakukei 4 than in Enrei. Comparing the effects of the different N fertilizers, only En1282 showed a general tendency; leaf N contents were the highest when fertilized with urea, followed by LP-70, and LP-100, in this order.

2. Photosynthesis

In pot-grown plants (Exp. 1), the photosynthetic rate responded to the amount of N applied (Fig. 3, left) in a genotype-dependent pattern similarly to that of leaf N content (compare Fig. 1). In field-grown plants (Exp. 2), Sakukei 4 showed the lowest photosynthetic activity among all genotypes at the pod-expansion stage, but the highest activity at the seed-filling stage (Fig. 3, right).
treatments were not statistically significant. The highest fertilizer-dependent dry weight increase was observed also in Enrei and Sakukei 4, but the differences between treatments were not statistically significant. The highest leaf N content and AP were markedly low in the absence of fertilizer and increased dramatically with increasing amounts of N fertilizer. A fertilizer–dependent dry weight increase was observed also in Enrei and Sakukei 4, but the differences between treatments were not statistically significant. The highest leaf N content and AP were smallest in Sakukei 4 and En1282; Enrei was intermediate. The variance of leaf N contents per leaf area of Enrei was larger than that of the other genotypes, we observed a linear relation between the two parameters. Generally, the variances of leaf N contents per leaf area and AP were smallest in Sakukei 4 and greatest in En1282; Enrei was intermediate. The variance of leaf N contents per leaf area of Enrei was larger when grown in the field (Exp. 2) than when grown in pots (Exp. 1). By contrast, the variances of the two parameters were independent of growth conditions in Sakukei 4. In general, leaf N content and AP were higher in field–grown than in pot–grown plants, with the exception of the genotype Sakukei 4 at the pod–expansion stage. The AP of Sakukei 4 tended to exceed that of Enrei and En1282 at a given leaf N content, particularly at the seed–filling stage.

3. Growth

Table 1 summarizes the growth and seed yields in pot–grown plants (Exp. 1). The dry weight of En1282 was markedly low in the absence of fertilizer and increased dramatically with increasing amounts of N fertilizer. A fertilizer–dependent dry weight increase was observed also in Enrei and Sakukei 4, but the differences between treatments were not statistically significant. The highest dry weight was reached in the genotype Enrei fertilized with 0.6 g N per pot. Up to this dose, dry weight was generally highest in Enrei, intermediate in Sakukei 4, and lowest in En1282; treatment with 1.5 g N per pot yielded mixed results. There was no significant difference in seed yields between Enrei and Sakukei 4, and both substantially exceeded the yield of En1282. Seed yield in En1282 showed a strong dependence on fertilizer dosage, but that in the other genotypes did not.

Dry weight did not significantly vary with the fertilizer type in any of the genotypes (Exp. 2, Table 2). Seed yield of En1282, but not that of Enrei and Sakukei 4, depended on the type of fertilizer applied. The yield was highest in urea–treated plants. Reflecting the difference in dry weight, seed yield was significantly higher in Enrei than in Sakukei 4.

4. Root dry weight, nodule dry weight, N fixation activity and leaf area

The root dry weight (Exp. 1) increased with increasing dose of N fertilizer in all genotypes, whereas the nodule dry weight significantly declined at higher dosages of N (Table 3). ARA per plant declined at the highest amount of N applied. Nodule dry weights and ARA per plant tended to be greater in Sakukei 4 than in Enrei. ARA per nodule dry weight did not vary signif-

---

Table 1. Effects of the amount of nitrogen fertilizer applied on total dry weights and seed yields of different soybean genotypes (Enrei, Sakukei 4, En1282) grown in pots, Exp. 1.

| Genotype  | Amount of nitrogen applied (g pot⁻¹) | Total dry weight (including root) at the pod-expansion stage | Seed yield (g pot⁻¹) |
|-----------|-------------------------------------|-------------------------------------------------------------|---------------------|
|           | 0                                   | 0.5                                                        | 0.6                 | 1.5                |
| Enrei     | 63.2**                             | (100)                                                      | (107)               | (121)              | (109)              |
| Sakukei 4 | 51.3*                               | (100)                                                      | (125)               | (129)              | (131)              |
| En1282    | 17.4                                | (100)                                                      | (195)               | (276)              | (405)              |

Total dry weight (stem+pod+seed) at the maturity

| Genotype  | Amount of nitrogen applied (g pot⁻¹) | Total dry weight (stem+pod+seed) at the pod-expansion stage | Seed yield (g pod⁻¹) |
|-----------|-------------------------------------|-------------------------------------------------------------|---------------------|
| Enrei     | 58.4                                 | (100)                                                      | (104)               | (107)              | (89)               |
| Sakukei 4 | 50.7                                 | (100)                                                      | (106)               | (112)              | (103)              |
| En1282    | 5.8                                 | (100)                                                      | (191)               | (430)              | (843)              |

*Values followed by the same letter within one line are not significantly different at the 0.05 probability level. Values given are means of five pots. Figures in parentheses indicate percentages to the value in the plants without application of nitrogen fertilizer.

---

Fig. 4 shows the relationship between leaf N content per leaf area and photosynthetic rate of three soybean genotypes (Enrei, Sakukei 4, En1282) at the pod–expansion stage (left) and at the seed–filling stage (right). Equations are regression functions describing the dependence of photosynthetic rate on leaf nitrogen content, with correlation coefficient (r). * and **: significant at 5% and 1% level, respectively.
Table 2. Effects of different types of nitrogen fertilizer on total dry weights and seed yields of different soybean genotypes (Enrei, Sakukei 4, En1282) grown in field, Exp. 2.

| Type of fertilizer | Genotype | Urea | LP-70 | LP-100 |
|--------------------|----------|------|-------|--------|
|                    |          |      |       |        |
| Total dry weight (including root) at the flowering stage | | | | |
| Enrei | 64.3ns | 63.3ns | 64.1ns |
| Sakukei 4 | 36.5ns | 28.2ns | 33.3ns |
| En1282 | 43.7ns | 43.0ns | 52.9ns |
| Total dry weight (including root) at the pod expansion stage | | | | |
| Enrei | 133.9ns | 122.7ns | 160.3ns |
| Sakukei 4 | 90.0ns | 84.4ns | 83.0ns |
| En1282 | 89.9ns | 85.1ns | 105.6ns |
| Total dry weight (stem+pod+seed) at the maturity | | | | |
| Enrei | 108.4ns | 111.5ns | 122.5ns |
| Sakukei 4 | 81.5ns | 78.6ns | 85.0ns |
| En1282 | 79.7c | 62.6b | 94.8a |
| Seed yield | | | | |
| Enrei | 50.9ns | 51.2ns | 57.9ns |
| Sakukei 4 | 40.4ns | 40.0ns | 42.5ns |
| En1282 | 34.0c | 22.6b | 15.3a |

Values followed by the same letter within one line are not significantly different at the 0.05 probability level. Values given are means of five to ten plants.

Table 3. Effects of the amount of nitrogen fertilizer applied on root and nodule dry weights and on ARA in different soybean genotypes (Enrei, Sakukei 4, En1282) grown in pot at the pod-expansion stage, Exp. 1.

| Amount of nitrogen applied (g pot⁻¹) | Genotype | 0 | 0.2 | 0.6 | 1.5 |
|------------------------------------|----------|---|-----|-----|-----|
|                                    |          |  |     |     |     |
| Root dry weight (g pot⁻¹)          | Enrei    | 8.0a | 7.8a | 10.8b | 10.6b |
|                                    | Sakukei 4 | 6.9a | 8.3b | 8.6b | 10.5c |
|                                    | En1282   | 6.2a | 9.5b | 11.0b | 11.3b |
| Nodule dry weight (g pot⁻¹)        | Enrei    | 2.6b | 2.6b | 2.8b | 1.0a |
|                                    | Sakukei 4 | 6.7c | 7.7c | 5.4b | 1.8a |
| ARA per plant (C₂H₄ μ mol h⁻¹ plant⁻¹) | Enrei    | 34.7ab | 24.8ab | 46.6b | 16.2a |
|                                    | Sakukei 4 | 59.4b | 51.3b | 47.6b | 13.2b |
| ARA per nodule dry weight (C₂H₄ μ mol h⁻¹ g⁻¹) | Enrei    | 25.4ns | 21.2ns | 28.9ns | 30.3ns |
|                                    | Sakukei 4 | 17.4ns | 15.8ns | 17.2ns | 15.6ns |

Values followed by the same letter within one line are not significantly different at the 0.05 probability level. Values given are means of five pots.

cantly with the N dosage, and generally was lower in Sakukei 4 than in Enrei.

Root dry weight (Exp. 2) did not depend on the fertilizer type applied at the flowering stage in any of the genotypes (Table 4). However, root dry weight at the pod-expansion stage in Enrei and En1282 was significantly heavier in the plants fertilized with LP-100. In Sakukei 4, nodule dry weight and ARA at the flowering stage were higher in the plants fertilized with LP-100, but at the pod-expansion stage there was no significant difference. ARA per plant measured at the pod-expansion stage was significantly higher than that at the flowering stage in Enrei and Sakukei 4. At the pod-expansion stage, ARA per plant tended to be higher in Sakukei 4 than in Enrei, but the reverse relationship was observed in ARA per a nodule dry weight.

Leaf area per pot (Exp. 1) was increased by N application with the exception of Enrei treated with 1.5 g N (Table 5). Leaf area of Sakukei 4 was always lower than that of Enrei with the exception of the dose of 1.5 g N. The same tendency was observed in Exp. 2.
It is well-known that gramineous crops are more responsive to N application than legumes. On the contrary, the nodulating genotypes Enrei and, in particular, Sakukei 4 did not show a pronounced N response that is typical of leguminous crops. It is well documented that the application of a large amount of nitrogen fertilizer to soybean inhibits the formation and activity of nodules, resulting in little enhancement of total nitrogen uptake (Harper, 1987).

The genotypic difference in N content became more significant at the seed-filling stage (Fig. 4). Sakukei 4 contained more N per leaf area and showed a higher AP than Enrei and En1282, both in pots and in the field. It is well-known that in normally nodulating soybeans, the nitrogen fixing capability generally declines during seed filling (Sinclair and de Wit, 1975). The N content per leaf area, however, sharply declined in En1282 at the seed-filling stage. These results suggest that Sakukei 4 is capable of maintaining a high N level in leaves by continuous N-fixation until late growth stages. Consequently, Sakukei 4 is less likely to exhibit self-destructive N translocation which is commonly observed in soybean (Sinclair and de Wit, 1975).

There was a positive correlation between leaf N content and photosynthetic rate regardless of the dosage and the type of N fertilizer (Fig. 4). A similar correlation has been observed previously (Lugg and Sinclair, 1981). Leaf N content was also found to correlate with AP in F1 progenies of crosses of various soybean cultivars (Ojima and Kawashima, 1970). Moreover, AP has been reported to correlate positively with soluble protein content of leaves in the studies comparing two (Hesketh et al., 1981) and 29 cultivars (Boon-Long et al., 1983). Sinclair and Horie (1989) quantified the correlation between AP and leaf N content, and found a nearly linear correlation between them in the range between 0.10 and 0.24 mg N cm−2. In our study, it is noteworthy that at a given leaf N content per leaf area, Sakukei 4 had higher photosynthetic rates than the other two genotypes (Fig. 4). The reasons for this phenomenon remain to be elucidated.

Nodule dry weight and ARA decreased with increasing dose of N fertilizer in all genotypes (Table 3). However, nodule dry weights and ARA in most cases were higher in Sakukei 4 than in Enrei, and the difference between the two genotypes was greater at lower N levels. A similar genotypic difference was observed at the flowering stage when plants were fertilized with LP-100 which retains parts of its N load over prolonged periods (Table 4). These results indicate that Sakukei 4 is likely to maintain higher nodule activity at low N availability in the soil. Since nitrogen acquisition in Sakukei 4 seems to rely greatly on nodule activity, growth of Sakukei 4 is expected to depend on soil conditions suitable for nodule activity.

The nodule activity (ARA) per plant was higher in Sakukei 4 than in Enrei in many cases. However, the relationship was reverse in ARA per nodule dry weight (Tables 3, 4). This phenomenon, which also was observed in the parental mutant En6500 (Francisco et al., 1992), is probably caused by insufficient photosynthetic...
supply to the nodules, or by excess nodule formation.

In a previous study with Sakukei 4 and Enrei grown in Ibaraki, Sakukei 4 exhibited a superior yield in some cases, particularly when the overall yield level was low (Takahashi et al., 2003). In the present study, however, the high photosynthetic ability of Sakukei 4 did not result in better growth performance and seed yield. This may be attributed to the cooler growing environment in Sendai. The growth performance of crops can be characterized by two factors: leaf area and net assimilation rate (NAR). The NAR of Sakukei 4 was higher than that of Enrei during the flowering to pod-expansion stage in our study (data not shown), whereas leaf area of Sakukei 4 was always lower (Table 5). Thus, Sakukei 4 appeared to accumulate N in the leaf rather than to utilize it for the expansion of the leaf.

In conclusion, the supernodulating genotype Sakukei 4 is characterized by high AP and high leaf nitrogen contents which are maintained during advanced developmental stages, regardless of the dosage and type of N fertilizer applied. Despite this desirable character, the growth performance was inferior to that of the normal nodulating genotype Enrei. In the practical use of Sakukei 4, increased planting density is probably one of the measures to maximize growth and yield. This genotype also has a potential to grow under conditions of low N availability. Field experiments should be conducted to evaluate these possibilities.

Acknowledgments

We thank Dr. S. Akao, National Institute of Agrobiological Resources, for providing the seeds of En1282 and Mr. Kenji Otomo for technical assistance in the field work.

References

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

Boon-Long, P., Egli, D.B. and Legett, J.E. 1983. Leaf N and photosynthesis during reproductive growth in soybeans. Crop Sci. 23: 617-620.

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

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

Classification Committee of Cultivated Soils. 1996. Classification of cultivated soils in Japan, The 3rd Approximation. National Institute of Agro-Environmental Sciences, Tsukuba, Japan. 50.

Fehr, W.R., Caviness, C.E., Burmood, D.T. and Pennington, J.S. 1971. Stage of development descriptions for soybeans, Glycine max (L.) Merrill. Crop Sci. 11: 929-931.

Francisco, P.B.Jr., Akao, S. and Kokubun, M. 1992. Irradiance and nitrate effects on growth and symbiotic parameters of the supernodulating and nitrate-tolerant soybean mutant En6500 and its parent cultivar Enrei. J. Plant Physiol. 140: 453-459.

Fukushima, D. 2000. Recent progress in research and technology for processing and utilization of soybeans. In the Organizing Committee for ISPUC-III ed., Proc. 3rd Int. Soybean Processing and Utilization Conference (ISPUC-III). Korin Publishing, Tokyo. 11-16.

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

Hansen, A.P., Peoples, M.B., Gresshoff, P.M., Atkins, C.A., Pate, J.S. and Carroll, B.J. 1989. Symbiotic performance of supernodulating soybean (Glycine max (L.) Merrill) mutants during development on different nitrite regimes. J. Exp. Bot. 40: 715-724.

Harper, J.E. 1987. Nitrogen metabolism. In J.R. Wilcox ed., Soybeans: Improvement, Production, and Uses, second edition. Madison, ASA, CSSA, SSSA Inc. 497-533.

Herridge, D. and Rose, I. 2000. Breeding for enhanced nitrogen fixation in crop legumes. Field Crops Res. 65: 229-248.

Hesketh, J.D., Ogren, W.L., Hageman, M.E. and Peters, D.B. 1981. Correlations among leaf CO2-exchange rates, areas and enzyme activities among soybean cultivars. Photosynth. Res. 2: 21-30.

Kimura, K. 1995. Total nitrogen. In K. Hinata and T. Hashiba eds., A Manual of Experiments for Plant Biology. Soft Science Publications, Tokyo. 300-304**.

Kokubun, M. 2001. Physiological approaches for increasing soybean yield potential. Jpn. J. Crop Sci. 70: 341-351**.

Lugg, D.G. and Sinclair, T.R. 1981. Seasonal changes in photosynthesis of field-grown soybean leaflets. 2. Relation to nitrogen content. Photosynthetica 15: 138-144.

Ohyama, T., Nicholas, J.G. and Harper, J.E. 1993. Assimilation of 15N2 and 15NO3 by partially nitrate-tolerant nodulation mutants of soybean. J. Exp. Bot. 44: 1739-1747.

Ojima, M. and Kawashima, R. 1970. Studies on the seed production of soybean. 8. The ability of F2 lines having different photosynthesis in their F2 generations. Proc. Crop Sci. Soc. Jpn. 39: 440-445*.

Sinclair, T. R. and de Wit, C.T. 1975. Photosynthate and nitrogen requirements for seed production by various crops. Science 189: 565-567.

Sinclair, T. R. and Horie, T. 1989. Leaf nitrogen, photosynthesis and crop radiation use efficiency: A review. Crop Sci. 29: 90-98.

Takahashi, M., Kokubun, M. and Akao, S. 1995. Characterization of nitrogen assimilation in a supernodulating soybean mutant En6500. Soil Sci. Plant Nutr. 41: 567-575.

Takahashi, M., Arihara, J., Nakayama, N. and Kokubun, M. 2003. Characteristics of growth and yield formation in the improved genotype of supernodulating soybean (Glycine max L. Merr.). Plant Prod. Sci. 6: 112-118.

*In Japanese with English summary.
**In Japanese.