

Nitrogen Utilization in the Supernodulating Soybean Variety “Sakukei 4” and Its Parental Varieties, “Enrei” and “Tamahomare”

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Abstract: The supernodulating variety “Sakukei 4”, which has improved growth and yield, was recently developed. To evaluate its physiological traits related to the high productivity, we compared dry matter production, nitrogen (N) accumulation and N utilization in Sakukei 4 with those in the parental normally nodulating varieties, Enrei and Tamahomare, and a nonnodulating line, En1282, in pot experiments. The seed yield of Sakukei 4 was similar to that of Enrei and Tamahomare. Leaf area and relative ureide abundance in xylem sap were maintained for longer growth period in Sakukei 4 and Tamahomare than in Enrei. The total amount of nodule N₂ fixation at maturity was also larger in Sakukei 4 and Tamahomare than in Enrei and En1282. In En1282 and Enrei, a larger part of N accumulated in leaves and stems before seed maturation was translocated to seeds during seed filling. However, Sakukei 4 and Tamahomare, more N tended to remain in leaves and stems at maturity. These observations suggested that the physiological traits of dry-matter accumulation and N utilization in Sakukei 4 were quite similar to those in Tamahomare, despite the difference in nodulation. N use efficiency for dry matter production (NUED) was lower in Sakukei 4 than in Enrei and Tamahomare, probably due to the energy cost for sustaining a greater number and mass of nodules. This observation indicated that the low NUED of Sakukei 4 impairs its productivity and seed yield, and therefore Sakukei 4 could not exceed Enrei or Tamahomare in seed productivity.

Key words: Growth, Nitrogen fixation, Root nodulation, Soybean (*Glycine max* (L.) Merr.), Supernodulation.

Soybean requires a large amount of nitrogen (N) for growth compared with other major crops because of its high seed N content (Sinclair and Wit, 1975). N sources for soybean are soil N, including fertilizer N, and symbiotically fixed N₂ (Harper, 1987). However, a large amount of N fertilizer application cannot increase the yield of soybean, since symbiotic N₂ fixation is suppressed by application of N fertilizer (McClure and Israel, 1979; Vessey et al., 1988). In addition, heavy N application would pose environmental problems. Thus, soybean productivity should be improved by enhancing symbiotic N₂ fixation.

The genetic improvement of symbiotic N₂ fixation is an option for enhancement of N absorption. Supernodulation is one way of enhancing N₂ fixation capability, and several supernodulating soybean lines have been isolated (Carroll et al., 1985a, b; Gremaud and Harper, 1989; Akao and

Kouchi, 1992). However, most supernodulating lines are inferior to their normally nodulating parental varieties in growth and yield, and have no agronomical benefit (Song et al., 1988; Wu and Harper, 1991; Ohyama et al., 1993; Pracht et al., 1994; Song et al., 1995).

Recently, a supernodulating variety, “Sakukei 4”, which was produced by chemical mutation and backcrossing of the normally nodulating variety “Enrei”, was released and its growth and yield were superior to Enrei (Takahashi et al., 2003a, b). Sakukei 4 had naturally crossed with Tamahomare, a high-yielding variety, during a period of selection, so that Tamahomare is the true pollen parent of Sakukei 4 (Yamamoto et al., 2004). Reports on the comparison of the growth and yield between Sakukei 4 and its parental varieties have been inconsistent (Maekawa et al., 2003; Takahashi et al., 2005a; Shimamura et al.,

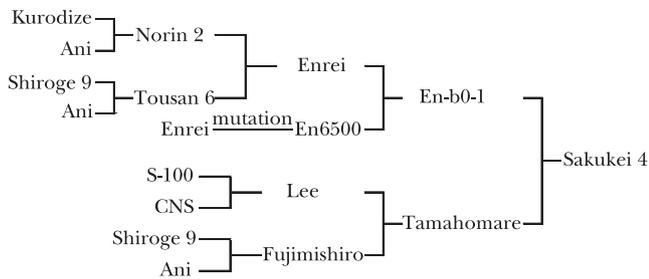


Fig. 1. Pedigree of supernodulating soybean Sakukei 4; figure modified from Yamamoto et al. (2004).

2007). Moreover, whether the improved productivity originates from supernodulation of Enrei or from the productivity of Tamahomare is unclear. This study was conducted to characterize dry matter production, N accumulation and N utilization in the supernodulating variety Sakukei 4, compared with those in the normally nodulating parental varieties, and a nonnodulating mutant line En1282, isolated from Enrei.

Materials and Methods

1. Plant materials and culture methods

Four contrasting varieties were used in this study: the supernodulating variety Sakukei 4 (presently "Kanto 100"), normally nodulating parental varieties, Enrei and Tamahomare, and a nonnodulating line, En1282. Sakukei 4 was originally selected from progenies of an Enrei/En6500/Tamahomare cross (Fig. 1) (Takahashi et al., 2003a, b; Yamamoto et al., 2004). En1282, a nonnodulating line, is an artificially induced mutant of Enrei (Akao and Kouchi, 1992; Francisco and Akao, 1993).

Experiments were conducted with four replicates in a greenhouse at the National Institute of Crop Science (NICS) in Tsukuba, Japan. The minimum air temperature in the greenhouse was 22°C and the maximum was 30°C. 1/5000a Wagner pots were filled with 3 kg of air-dried soil (low-humic andosols) collected from the field at NICS. The soil (pH (H₂O)=5.7) contained 64.2 mg kg⁻¹ inorganic N and 3.78 g kg⁻¹ total N in the dry soil base. Nitrogen (0.6 g N pot⁻¹ as (NH₄)₂SO₄), phosphorus (2 g P₂O₅ pot⁻¹ as Na₂HPO₄), and potassium (2 g K₂O pot⁻¹ as K₂SO₄) were applied to the soil. Nitrogen was added as ¹⁵N-labeled (NH₄)₂SO₄ (3.13 atom %) at 0.6 g N pot⁻¹. Six seeds were sown on 7 June 2004, in each pot. Seedlings emerged on 11 June 2004 and were thinned to one per pot when the first trifoliolate leaf had fully expanded. Pots were irrigated with water to maintain 60% water holding capacity.

2. Samplings

Plants were sampled at five stages, start of blooming (R1), start of pod formation (R3), start of seed production (R5), start of maturity (R7) and full maturity (R8) (Fehr et

Table 1. Sampling day of each soybean variety.

Varieties	Growth stage*				
	R1	R3	R5	R7	R8
Sakukei 4	37	47	54	87	124
Enrei	32	43	52	73	99
Tamahomare	37	53	57	94	124
En1282	37	45	54	73	99

Values are expressed as days after emergence. * Description of growth stages (Fehr et al., 1971).

al., 1971) (Table 1) and separated into leaves, stems (including petioles), pods, roots and nodules (≥ 0.5 mm diameter). Additionally, pods were divided into pod shells and seeds at harvest. The dry weight of each plant part was determined after oven drying at 80°C for 48 hr. After measuring the dry weight, samples were ground and a portion was used for chemical analysis.

3. Chemical analysis

The nitrogen (N) content in the sample was determined with an N/C analyzer (Sumigraph NC-22F, Sumika Chemical Analysis Service Co. Ltd., Japan) and ¹⁵N atom % of the sample, which was weighed into thin capsules containing 50 to 200 μ g N per capsule, was measured by a Finnigan DELTA plusXP mass spectrometer (Thermo Electron Co., USA). The amount of ¹⁵N in each part of the plant at R5 and R8 stages was calculated as follows:

The amount of N derived from ¹⁵N chemicals is $TN \times (^{15}N\text{-BCG}) / (FC\text{-BCG})$,

where TN is the total amount of N in the sample, ¹⁵N is the ¹⁵N atom % in the sample, BCG is the background (¹⁵N atom %) in the blank sample, and FC is the ¹⁵N atom % of the fed ¹⁵N chemical.

The total nitrogen in the plant is the sum of that from the fertilizer, soil, and N₂ fixation; total nitrogen was measured with an N/C analyzer, fertilizer nitrogen was determined by ¹⁵N analysis, and N from the soil was inferred from the amount of N accumulated in En1282 after correcting for that derived from the fertilizer, and thus the amount of N derived from N₂ fixation was determined from the other values (Takahashi et al., 1991).

4. Xylem sap sampling and relative ureide assay

The method of Takahashi et al. (1992) was used to collect the bleeding sap and for relative ureide assays. The bleeding sap was collected by cutting the main stem between 0830 and 1030 at R1, R3, R5, R7 and R8 stages. The concentrations of ureide-N, NO₃-N, and amino-N in the sap were determined. The amount of NO₃-N was measured by colorimetric methods using a TRRACS-2000 autoanalyzer (BLAN+RUBE, Co. Ltd., Germany). The amounts of amino-N and ureide-N were respectively determined by the ninhydrin method (Herridge, 1984)

and Young-Conway’s method (Young and Conway, 1942). Relative ureide abundance was calculated as follow: Relative ureide abundance = ureide-N/(ureide-N+NO₃-N+amino-N) × 100.

Results

Sakukei 4, Tamahomare, and Enrei had similar seed yield (Fig. 2), while En1282 had a lower yield. The whole plant dry weight of Tamahomare increased during the ripening period (after R7 stage) until maturity (Fig. 3),

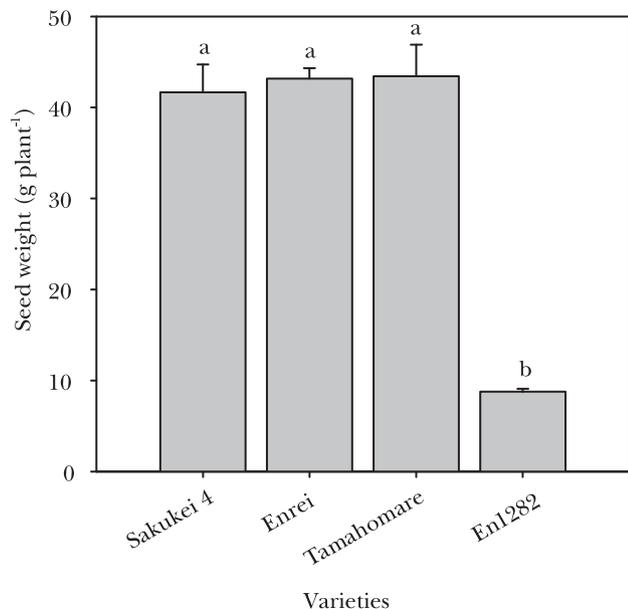


Fig. 2. The seed yield of soybean varieties Sakukei 4, Enrei, Tamahomare, and En1282. Bars with the same letter indicate no significance by Tukey’s HSD test (5% level). Vertical bars represent standard errors of 4 replicates.

whereas that of Sakukei 4 and Enrei increased slowly and that of En1282 decreased during this period. The dry weight of all organs except for pods decreased in Enrei and En1282, but it was maintained during the seed filling period in Sakukei 4 and Tamahomare. The amount of N in the whole plant increased in all varieties except En1282 during the seed filling period (Fig. 4). The amount of N in all organs except for pods in Enrei and En1282 was remarkably lower compared with Sakukei 4 and Tamahomare at maturity. The pattern of N accumulation within Sakukei 4 plants appeared similar to that in Tamahomare.

The nodule dry weight was heaviest in Sakukei 4 and it increased strikingly after flowering (R1 stage) (Fig. 5). The nodule dry weight of Tamahomare increased after R5 stage and was heavier than that of Enrei. The nodule number of Sakukei 4 increased after flowering and followed the order Sakukei 4 >> Tamahomare > Enrei. Relative ureide abundance in xylem sap tended to increase until R3 stage in Enrei and until R5 stage in Sakukei 4 and Tamahomare. After attaining a maximum value it decreased rapidly. The relative ureide abundance decreased after 50 d after emergence more steeply in Enrei than in Sakukei 4 and Tamahomare, since the seed filling period (R5 to R8) was longer in Sakukei 4 and Tamahomare than in Enrei.

The N concentration in the whole plant during the growing period was the highest for Sakukei 4 (Fig. 6). The concentration increased over the growing period in all varieties except En1282, in which it decreased. Figure 7 shows the relationship between whole plant N accumulation and whole dry mass during growing stages in Sakukei 4, Enrei and Tamahomare. The N accumulation in the whole plant was positively correlated with the whole

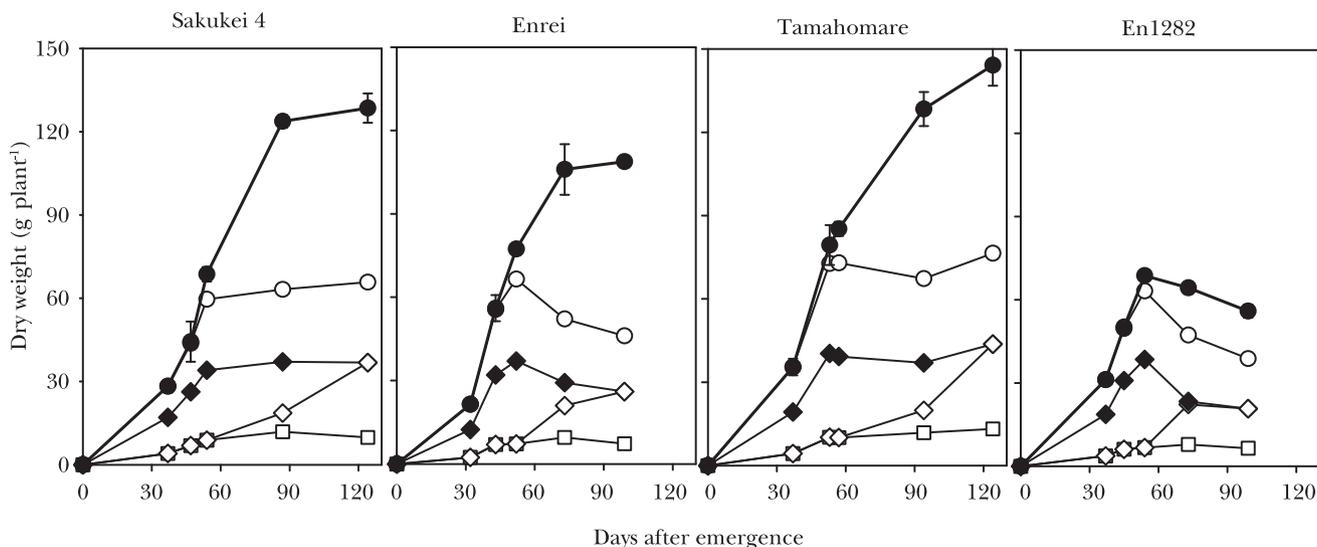


Fig. 3. Temporal changes in dry weight of plant organs of soybean varieties. Vertical bars represent standard errors of 4 replicates. Soybean plants were sampled at R1, R3, R5, R7 and R8 stages. ●—, whole plant; ○—, stems, leaves, dead leaves, roots and nodules; ◆—, leaves, dead leaves, roots and nodules; ◇—, dead leaves, roots and nodules; □—, roots and nodules.

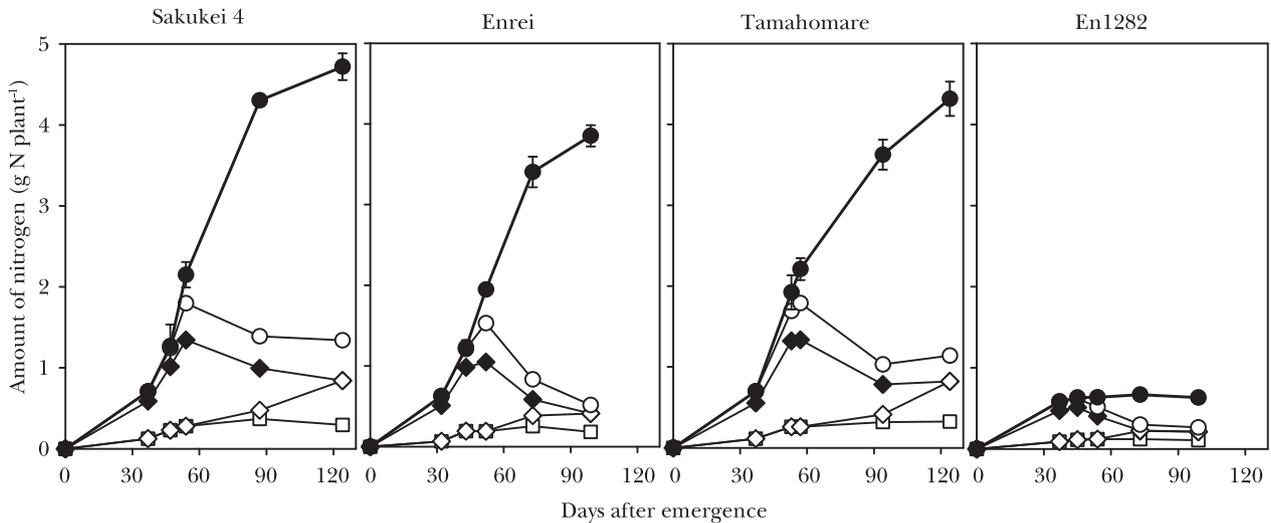


Fig. 4. Temporal changes in amount of nitrogen accumulated in plant organs of soybean varieties. Vertical bars represent standard errors of 4 replicates. Sampling dates were same as in Figure 3. —●—, whole plant; —○—, stems, leaves, dead leaves, roots and nodules; —◆—, leaves, dead leaves, roots and nodules; —◇—, dead leaves, roots and nodules; —□—, roots and nodules.

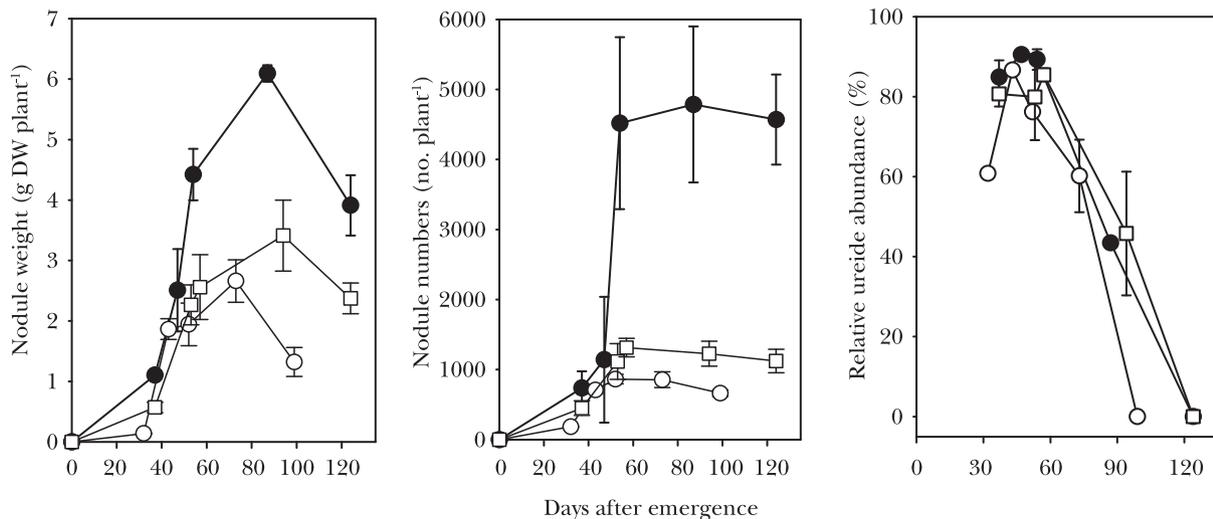


Fig. 5. Temporal changes in nodule weight, nodule number and relative ureide abundance of soybean varieties. Vertical bars represent standard errors of 4 replicates. Sampling dates were same as in Figure 3. —●—, Sakukei 4; —○—, Enrei; —□—, Tamahomare.

dry mass in all soybean varieties and this relationship in Enrei and Tamahomare were expressed by the a same linear regression line with a significant level of 5% by the analysis of covariance (Fig. 7). Therefore, the slope of the regression line would indicate N use efficiency for dry matter production ([NUED], dry mass per unit amount of N in whole plant), and the slope was steeper in Enrei and Tamahomare than in Sakukei 4 in all growing stages.

Crop growth rate (CGR) can be expressed as the product of the net assimilation rate (NAR) and the leaf area (LA). CGR in Enrei was highest at R3, however it declined thereafter more rapidly than in Sakukei 4 and Tamahomare (Fig. 8), CGR of En1282 decreased from R1 stage. All varieties except for En1282 showed a similar NAR

at all stages. LA for Sakukei 4 and Tamahomare maintained a large LA during the seed filling period. The gentle decline of CGR during the seed filling period in Sakukei 4 and Tamahomare could be attributed to their larger leaf area.

Table 2 shows the calculated N redistribution ratio (NRR, the ratio of the N amount in non-reproductive organs decreased during the seed filling period to the amount of N in non-reproductive organs at R5 stage) and the seed N contribution ratio (SNCR, the ratio of the N amount in non-reproductive organs decreased during the seed filling period to the that in seeds at harvest). NRR was lower in Sakukei 4 and Tamahomare than in Enrei and En1282. SNCR was highest in the order of En1282 > Enrei

≧Tamahomare ≧ Sakukei 4.

The amount of N derived from fertilizer and soil was nearly the same at R5 and R8 stages in all varieties (Fig. 9). Thus, most of the N that accumulated in the plant during the seed filling period was from nodule-fixed N. The ¹⁵N distribution ratio at the R5 stage was higher in leaves than in other organs in all varieties (Fig. 10). The ¹⁵N distribution ratio to leaves at the R5 stage was lower in

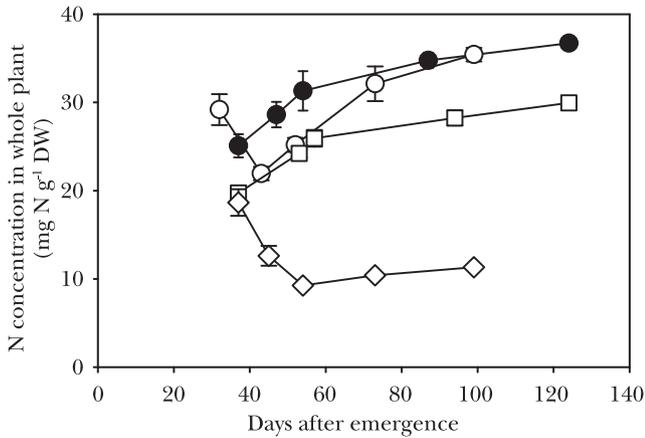


Fig. 6. Temporal changes in N concentration of the whole plant in soybean varieties. Vertical bars represent standard errors of 4 replicates. Sampling dates were same as in Figure 3. ●, Sakukei 4; ○, Enrei; □, Tamahomare; ◇, En1282.

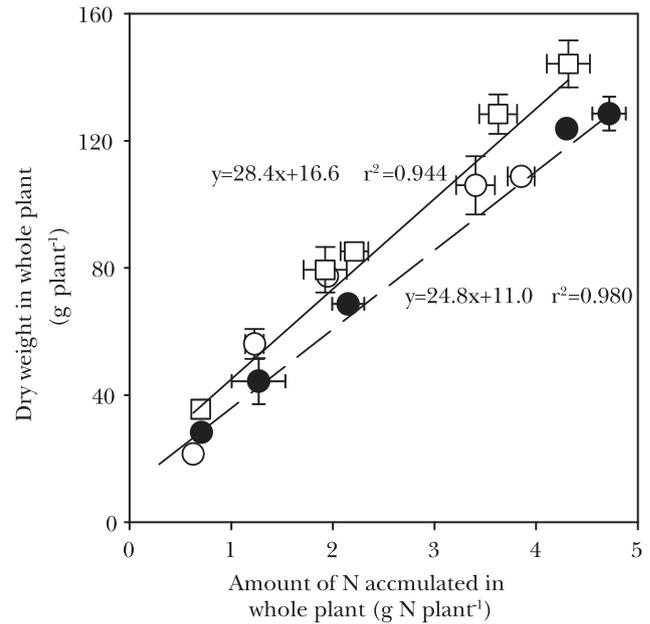


Fig. 7. Relationship between the amount of N accumulated in the whole plant and dry weight of whole plant in soybean varieties during the growth. The regression lines are represented by $y=28.4x+16.6$ ($r^2=0.994$, $p<0.01$) in Enrei and Tamahomare (solid line) and $y=24.8x+11.0$ ($r^2=0.980$, $p<0.01$) in Sakukei 4 (dashed line). Error bars indicate standard errors of 4 replicates. ●, Sakukei 4; ○, Enrei; □, Tamahomare.

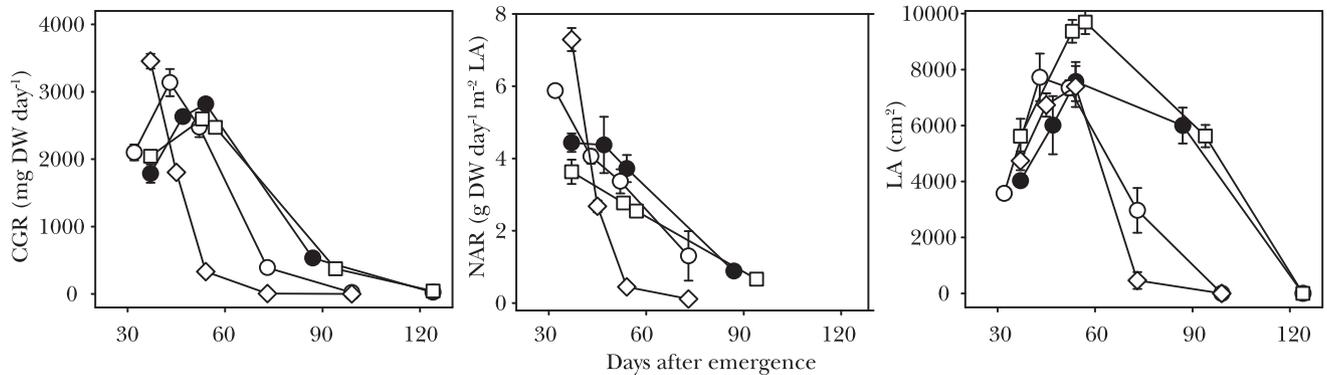


Fig. 8. Temporal changes in crop growth rate (CGR), net assimilation rate (NAR), and leaf area (LA) of soybean varieties. Vertical bars represent standard errors of 4 replicates. Sampling dates were same as in Figure 3. ●, Sakukei 4; ○, Enrei; □, Tamahomare; ◇, En1282.

Table 2. N redistribution ratio (NRR) and N-contribution ratio (SNCR) in non-reproductive organs (all organs except for seeds) during maturation in soybean varieties.

	Varieties			
	Sakukei 4	Enrei	Tamahomare	En1282
NRR	9.3 ± 18.4	59.0 ± 0.5	26.4 ± 8.0	40.9 ± 2.5
SNCR	3.2 ± 8.9	20.2 ± 1.7	15.8 ± 3.8	64.1 ± 2.3

Data are means “±standard error”. $N_{transfer}$, NRR and SNCR can be calculated based on the amount of N in non-reproductive organs at R5 stage (N_{R5}), the amount of N in nonreproductive organs at harvest (N_H), and the amount of N in the seeds at harvest (N_{seed}) as follows: $N_{transfer} = N_{R5} - N_H$, $NRR = N_{transfer} / N_{R5} \times 100$ and $SNCR = N_{transfer} / N_{seed} \times 100$.

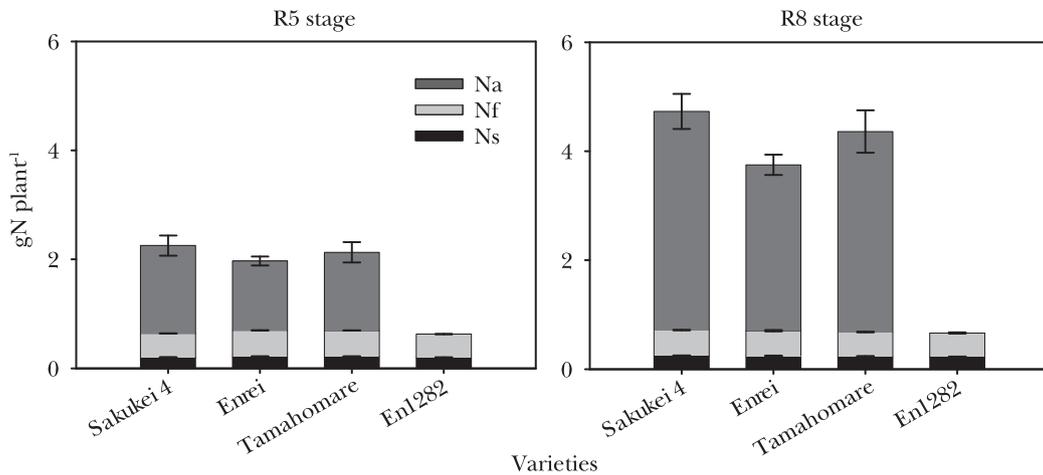


Fig. 9. Amount of N accumulated in the whole plant originating from soil (Ns), fertilizer (Nf), and symbiotic N₂ fixation (Na) at R5 and R8 stages in soybean varieties. Vertical bars represent standard errors of 4 replicates.

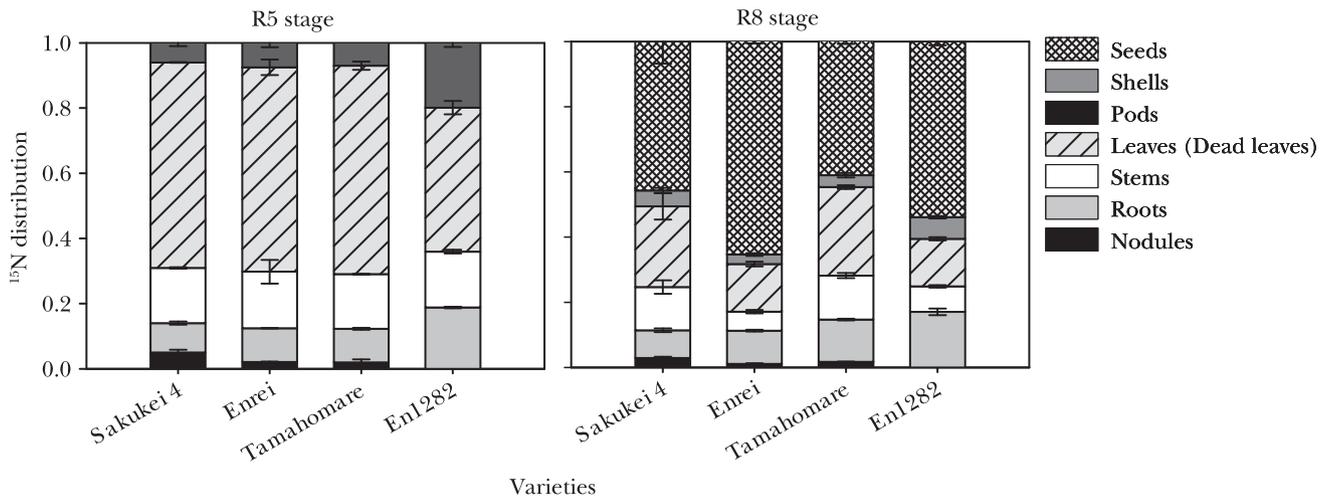


Fig. 10. ¹⁵N distribution ratio in each organ at R5 and R8 stages in soybean varieties. Vertical bars represent standard errors of 4 replicates.

En1282 than in the others. At the R8 stage, it was also higher in Sakukei 4 and Tamahomare than in Enrei and En1282 (Fig. 10).

Discussion

Most of the supernodulating lines bred so far are inferior to their normally nodulating parental varieties in growth and yield. Because Sakukei 4 which is derived from Enrei and Tamahomare is exceptionally high yielding, we determined whether its high productivity is due to characteristics of Enrei or Tamahomare.

In our experiment, seed yield did not differ among varieties, except for the non-nodulating En1282 (Fig. 2). Although Takahashi et al. (2005a) reported that Sakukei 4 had higher productivity than Enrei and Tamahomare, this was not the case in our study. In the preliminary experiment, the yield of Sakukei 4 was the same as that of

Enrei in a green house (data not shown), and in our previous field experiment (Shimamura et al., 2007), it was also almost the same as that of Enrei but was lower than that of Tamahomare. Additionally, although the growth and yield of supernodulating soybeans are generally inferior to the normally nodulating ones (Song et al., 1988; Wu and Harper, 1991; Ohyama et al., 1993; Pracht et al., 1994; Song et al., 1995), the yield of Sakukei 4 was the same as that of the normally nodulating varieties, Enrei and Tamahomare.

Sakukei 4 has been reported to show a higher leaf area index than supernodulated soybeans (En-b0-1, En6500) and Enrei which are predecessors of Sakukei 4 (see Fig. 1) in field conditions (Takahashi et al., 2003a). The larger LA observed during the seed filling period (R5 to R8) may be one of the reasons for the gentle decline of CGR during the seed filling period of Sakukei 4 and Tamahomare

could be smaller (Fig. 8). These growth characteristics of Sakukei 4 seem to have been mainly introduced from Tamahomare. All four varieties appeared to finish absorbing N from both soil and fertilizer by the R5 stage (Fig. 9). The relative ureide abundance positive is related to N_2 fixation activity in the nodule (Herridge and Peoples, 1990). The relative ureide abundance during the seed filling period (R5 to R8) also decreased more slowly in Sakukei 4 and Tamahomare in which the seed filling period was longer (Fig. 5). This implies that N_2 fixation was maintained at a higher level as well, and thus the total N accumulation was larger in these varieties (Figs. 4, 9).

The higher yield potential of Sakukei 4 has been ascribed to a higher nitrogen content and photosynthetic rate in leaves during seed filling (Takahashi et al., 2005a). Thus, N dynamics in the plant during seed filling is needed to evaluate the yield potential of Sakukei 4. Any N shortage during the growth of soybean seeds must be compensated for by the translocation of N from leaves and stems to the seed, which reduces photosynthesis (Sinclair and Wit, 1975). This is a process of self-destruction, because the N demand of soybeans during the seed filling stage is the highest of all crops. The total N accumulated in seeds corresponds to N_{transfer} , the N accumulated in non-reproductive organs (all organs except for seeds) until the start of seed maturation (R5 stage) and then translocated to the seeds, plus the N absorbed from soil and fixed by nodule from the air during the seed filling and maturation (Osaki et al., 1991; Shinano et al., 1991). The N-redistribution ratio (NRR), which is the ratio of N_{transfer} to N_{R5} (N that had accumulated in non-reproductive organs at the start of seed maturation, R5 stage), was lower in Sakukei 4 and Tamahomare, and N-contribution ratio (SNCR), the ratio of N_{transfer} to N_{seed} (N of the seeds at harvest), was highest in En1282 and lowest in Sakukei 4 (Table 2). N from fertilizer and soil was mostly absorbed by the R5 stage in all varieties because the amount of N originating from soil and fertilizer did not increase from the R5 to R8 stage (Fig. 9). Furthermore, the ratio of fertilizer N absorbed before the R5 stage in leaves and stems and then translocated to seeds was higher in Enrei and En1282 than in Sakukei 4 and Tamahomare (Fig. 10). These results suggested that, in En1282 and Enrei, N accumulated in non-reproductive organs, especially in leaves and stems at a higher ratio than in Sakukei 4 and Tamahomare, was translocated to seeds during seed filling and therefore the leaves and stems of the latter would not work as a chief source of N to the seeds. In other words, the degree of self-destruction (Sinclair and Wit, 1975) in Sakukei 4 was lower than that in Enrei and En1282, and was similar to that in Tamahomare. Further, the higher nodulation of Sakukei 4 was expected to cause a larger contribution of fixed N to seed filling, but the relative ureide abundance of Sakukei 4 was not statistically different from that of Tamahomare

(Figs. 5, 9). Thus, the N-fixing ability would not differ from that of Sakukei 4 and Tamahomare. The lower seed SNCR of Sakukei 4 and Tamahomare implies that most of the N in seeds at maturity was derived from nodule N_2 fixation during the seed filling period (Table 2). Thus, the physiological traits of dry matter and N accumulation and utilization of Sakukei 4 were very similar to those of Tamahomare except for number and mass of nodules. Takahashi et al. (2005a) reported that Sakukei 4 tended to show less self-destruction than the normally nodulating varieties (Sinclair and Wit, 1975) and their results differed from our observations. However, their study was done in the early spring, under conditions different from those in the present experiments, especially in day length. In their experiment (Takahashi et al., 2005a), the disturbance of translocation might have occurred because all the varieties examined showed delayed stem maturation at seeds maturity.

There was not a large difference in relative ureide abundance between Sakukei 4 and Tamahomare, and the amount of N originated from N_2 fixation in Sakukei 4 was slightly larger than that in Tamahomare, although the weight and the number of nodules were quite the largest in Sakukei 4 (Figs. 5, 9). This would suggest that the N-fixing activity per unit nodule weight and that per nodule number in Sakukei 4 were lower than in the other two varieties (Takahashi et al., 2005b). The acetylene reduction activity per plant and the respiratory rate in the nodules of Sakukei 4 were reportedly higher than those in Enrei, while the energy efficiency of N_2 fixation was not significantly different (Takahashi et al., 2005b), suggesting that the amount of fixed N_2 was larger and nodule respiratory rate was higher in Sakukei 4 than in Enrei because of its heavier nodule weight and larger number of nodules, although its net N_2 fixation ability was lower.

In our experiment, the N supplied from soil and fertilizer was almost exhausted by R5 (Fig. 9). Thus, the N concentration in the whole plant of the nonnodulating soybean En1282 decreased quickly until R5 stage, and was kept constant thereafter (Fig. 6), whereas the N concentration in Sakukei 4 increased rapidly until R5 stage, and then slowly, reaching a plateau. The pattern of the change in the N concentration in the whole plant of Tamahomare was similar to that of Sakukei 4 except at a lower N concentration. The reduction pattern of relative ureide abundance in Tamahomare was similar to that in Sakukei 4 after R5 stage (Fig. 5). Neo et al. (1996, 1997) reported that a change in N conditions controls the activity of symbiotic N_2 fixation; the N content of phloem sap especially plays a role in feedback regulation of nitrogenase activity. These observations suggest that, following the reduction in supply of N from fertilizer and soil near the R5 stage, the symbiotic N_2 fixation activity of Tamahomare increased to a level comparable to that of Sakukei 4. In the

normally nodulating variety Tamahomare, the reduction in the supply of N from soil and fertilizer would trigger N₂ fixation to the same level as Sakukei 4 and thus secure the amount of N required by the plant. In other words, supernodulation vs. normal-nodulation would not affect N acquisition but only the priority of the N sources.

Sakukei 4 had the highest N concentration among all varieties, and its NUED was lowest. The whole-plant energy cost for symbiotic N₂ fixation is greater than that for NO₃⁻ assimilation (Yamaguchi, 1978; Rlye et al., 1979; Finke et al., 1982). Although Sakukei 4 had low N₂ fixation activity per nodule weight and number, the respiratory loss of photosynthate should be greater because of the extremely large mass and number of nodules, and this greater loss presumably caused the lower NUED in Sakukei 4. In Fig. 7, the amount of N accumulated in plant was linearly related with dry matter accumulation. The slope of this relationship would almost synonymous with NUED. The slope of Sakukei 4 would show that the amount of dry mass per the amount of N in Sakukei 4 was smaller than that in Tamahomare and Enrei. This lower NUED appears to be one of the factors that make the productivity of Sakukei 4 similar to that of Enrei or Tamahomare.

We concluded that the high productivity of Sakukei 4 may be supported by its relatively larger leaf area and high photosynthetic rate during the seed filling period due to low N retranslocation from the leaves to the seeds and high N₂ fixation, but its productivity will not exceed that of the parental varieties because of the need to offset loss of photosynthate to maintain the supernodulating trait. Its favorable physiological traits were probably transmitted from Tamahomare but do not result from its supernodulating characteristics. We therefore conclude that the supernodulating character of Sakukei 4 may not be beneficial for improving soybean productivity.

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