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Characterization of Vegetative Growth of a Supernodulating Soybean Genotype, Sakukei 4

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Abstract : The supernodulating soybean cultivar Sakukei 4 was previously characterized by its superior ability to maintain a high leaf nitrogen (N) content and high photosynthetic rate. Despite these desirable traits, the growth performance of Sakukei 4 was inferior to that of its normally nodulating parental cultivar, Enrei. The physiological basis for the unique growth characteristics of Sakukei 4 remains unclear. The objective of the present study was to characterize in further detail the vegetative growth of Sakukei 4, particularly during the period before pod expansion. In the first experiment, the growth of Sakukei 4 was compared with that of its parental cultivar Enrei under various rates of N fertilizer. The dry weight of tops, roots and nodules of the plants grown at lower rates of N application was greater in Enrei than in Sakukei 4, but it was vice versa at higher rates of N application. The number and weight of nodules were far greater in Sakukei 4 than in Enrei at any rate of N application. These genotypic differences were significant on 39 days after sowing (DAS) and became greater at the flowering stage. In the second experiment, therefore, more detailed growth analysis was made during an earlier growth stage (DAS 31-46). During this period, relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) were lower in Sakukei 4 than in Enrei and the related non-nodulating line En1282, whereas the leaf photosynthetic rate was higher in Sakukei 4 at all leaf positions. The dry-matter partitioning to each plant part excluding nodules was similar in all three genotypes. The rate of leaf expansion in Sakukei 4 during this period was significantly slower than that in the other genotypes. These results suggest that the inferior growth of Sakukei 4 prior to flowering is probably due to excessive dry-matter partitioning to nodules and depressed capability of leaf expansion and root growth, which might limit dry-matter production of the whole plant during pre-flowering stage.

Key words : Dry matter partitioning, *Glycine max*, Growth rate, Leaf expansion, Nitrogen fixation, Photosynthesis, Soybean, Supernodulation.

Soybean is characterized by requiring a large amount of nitrogen (N) for its growth compared to other major crops (Sinclair and de Wit, 1975). Since soybean seeds accumulate a large quantity of N during the seed filling stage, a large portion of N in the vegetative tissues is translocated to seeds, causing a rapid decline of leaf N content coupled with the decline of photosynthetic capability (Sinclair and de Wit, 1975). This self-destructive N translocation can be one of the factors defining seed yield of soybean. In addition, a lack of N redistributed from vegetative plant parts may limit yield by restricting the duration of seed filling (Sinclair and de Wit, 1975; Boote, 1981; Egli et al., 1984).

Increased application of N fertilizer, either by basal application or top-dressing during growth, is an effective option for enhancing N supply to soybean plants (Yoshida, 1979; Watanabe et al., 1983). However, a high input of N fertilizer can adversely affect the environment. Therefore, soybean productivity should be improved by utilizing biological N_2 fixation. The genetic improvement of symbiotic N_2 fixation is an option for enhancement of the capability of N absorption. In attempts to induce mutations in nodulation capability, several supernodulating soybean lines, capable of forming an extremely large number of nodules compared to normally nodulating cultivars, have been isolated (Caroll et al., 1985a, b; Gremaud and Harper, 1989; Akao and Kouchi, 1992). Most of the supernodulating lines bred so far, however, are inferior in growth and yield performance compared to their normally nodulating parental cultivars, and have not produced practical benefits.

Recently, a supernodulating cultivar, "Sakukei 4", which was derived from a high-yielding cultivar, Enrei, was released (Takahashi et al., 2003a). This genotype's yield and agronomic performance were as good as or better than its parental cultivar under

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Abbreviations : AP, apparent photosynthetic rate; ARA, acetylene reduction activity; DAS, days after sowing; LAR, leaf area ratio; NAR, net assimilation rate; PPFD, photosynthetic photon flux density; RGR, relative growth rate.

certain conditions (Takahashi et al., 2003b). Sakukei 4 is the first soybean cultivar ever bred that has the potential to be grown practically by farmers. In our previous study, Sakukei 4 was characterized by having a superior ability to maintain high leaf N content and high photosynthetic rate, irrespective of the rate and type of N fertilizer applied (Maekawa et al., 2003). Despite these desirable traits, however, the growth performance and yield of Sakukei 4 were inferior to those of the normally nodulating cultivar Enrei. The inferior growth of Sakukei 4 was particularly significant before the seed filling stage. Clarification of the physiological traits involved in the unique characteristics of Sakukei 4 should help improve this genotype further through breeding, as well as develop cultivation methods to maximize yield by exploiting the desirable traits. The objective of the present study was, therefore, to characterize in further detail the vegetative growth of Sakukei 4, and to identify traits responsible for its inferior vegetative growth.

Materials and Methods

1. Experiment 1

(1) Plant materials

The supernodulating genotype Sakukei 4 (formerly En-b0-1-2) and its parent cultivar Enrei were used in this study. This experiment was conducted in 2000 and 2001. We used pot-grown plants because the root and nodules can be collected more precisely in pots than in the field. Pots (16 cm diameter, 19 cm tall) were filled with 4.0 kg of a fine-textured clayey Terrace Yellow soil (Classification Committee of Cultivated Soils, 1996). Prior to sowing, N fertilizer was applied as ammonium sulfate at four rates (0, 0.2, 0.6 and 1.5 g N pot^{-1}). Other fertilizers were applied at fixed rates; 0.6 g P_2O_5, 0.6 g K_2O and 0.5 g of slaked lime per pot. The soil had been used for growing soybeans previously, so inoculation with *Bradyrhizobium japonicum* was unnecessary. Four seeds per pot were sown on 25 May 2000 and 29 May in 2001, and thinned to two plants per pot after emergence. Plants were grown in a greenhouse until V1 (Fehr et al., 1971), then transferred to open air with adequate irrigation. Eight plots (two genotypes fertilized with N at one of the four rates) were arranged randomly, each plot containing 20 pots.

(2) Measurements

The materials grown in 2000 were used for the measurements of dry weights of various plant parts, and the materials grown in 2001 were used for the measurement of acetylene reduction activity (ARA). On 39 (stage V5) and 74 (stage R2 according to Fehr et al., 1971) days after sowing (DAS), five pots (ten plants) of each plot were sampled. The samples were separated into various parts and were dried at 80°C for more than two days in a ventilated oven, then weighed. Nodules were separated from the root, the number was counted, and the dry weight was measured. The leaf color of various leaf positions on the main stem was determined with a SPAD 502 chlorophyll meter (Minolta Co. Ltd., Tokyo, Japan) when the leaves on the main stem had fully expanded.

At flowering stage (73 DAS), three plants showing medium growth were sampled from three pots and separated into shoots and roots. After separation, the root system with nodules was enclosed in 900 mL glass bottles with rubber caps, and incubated with 30 mL acetylene at 25°C. Only one out of two plants in a pot was used in order to ensure adequate volume of air in the bottle. Gas samples (1 mL) were collected after 20 and 30 min of incubation and injected into a HITACHI 163 gas chromatograph (Hitachi Corp., Tokyo, Japan) equipped with a Porapack N glass column with N_2 as carrier. Ethylene produced during a 10 min period between 20 and 30 min of incubation was quantified by comparison with control samples of ethylene gas. After measurement of ARA, nodules were collected from the root system, counted, oven-dried and weighed.

2. Experiment 2

(1) Plant materials

Sakukei 4, Enrei and the related non-nodulating line En1282 were used in this experiment. The same soil as in Exp. 1 was used. Prior to sowing, fertilizer was applied at fixed rates; 0.1 g N, 0.3 g P_2O_5, 0.4 g K_2O and 2 g of slaked lime per pot. We applied 0.1 g N in this experiment because Exp. 1 showed that an application of N at higher rates depressed early growth performance of Enrei. Four seeds per pot were sown on 7 April 2002 and thinned to one plant per pot after emergence. Plants were grown in a greenhouse and temperature in the greenhouse was allowed to vary between 15 and 30°C during growth. Plants were irrigated adequately. The three genotypes, each consisting of 20 pots, were arranged randomly.

(2) Measurements

Six pots (six plants) for each genotype were harvested on 31 (V5 according to Fehr et al., 1971), 35 (V6) and 46 (R1) DAS. The samples were separated into various parts and processed in the same way as described in Exp. 1. The leaf area of a plant of medium size for each genotype was measured with a leaf area meter (automatic area meter model AAM-7, Hayashi Denko Co. Ltd., Tokyo, Japan). After measurement, the leaves were dried and weighed. Leaf area of the other samples was estimated from the leaf dry weight to leaf area ratio. Relative growth rates (RGR), net assimilation rates (NAR) and leaf area ratio (LAR) were calculated on a total dry weight basis.

The apparent photosynthetic rates (AP) of leaves of the just expanded terminal leaflet of six plants from each genotype were measured with an LI 6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA). The flow rate of air in the leaf chamber and the CO_2 concentration were controlled at 500 μmol

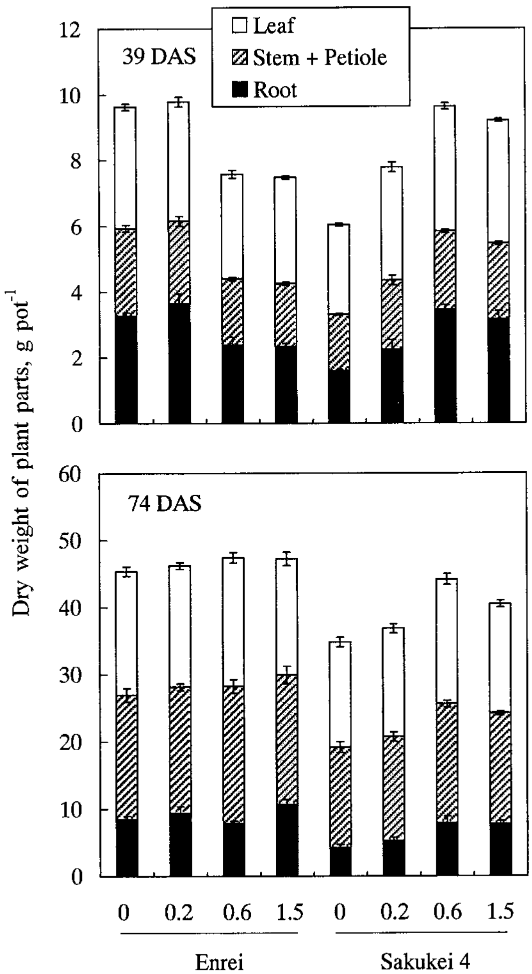

Fig. 1. Effects of the amount of nitrogen applied (0, 0.2, 0.6, 1.5 g pot^{-1}) on the dry weight of plant parts in Enrei and Sakukei 4 on 39 (upper) and 74 (lower) DAS (Exp. 1). Vertical bars indicate SE of five pots.

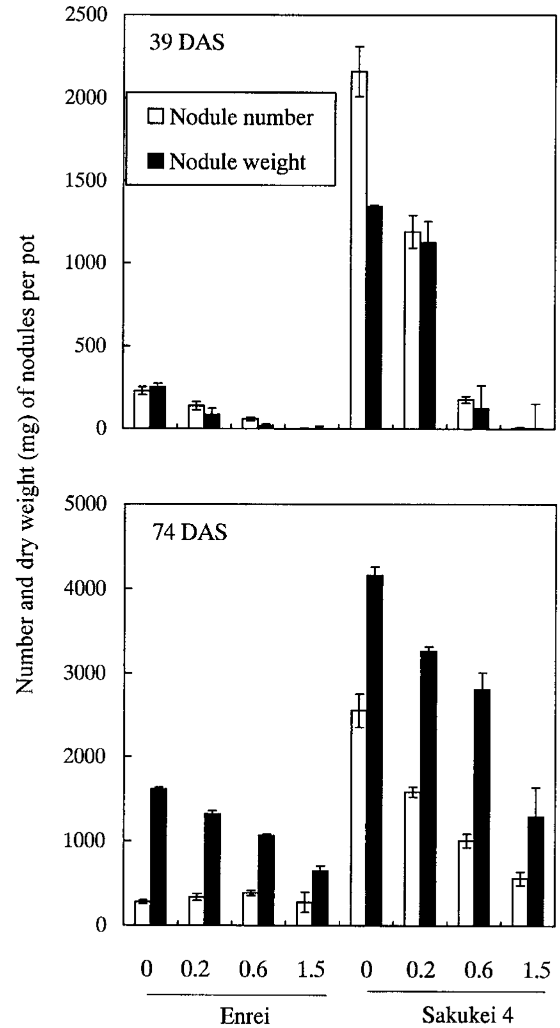

Fig. 2. Effects of the amount of nitrogen applied (0, 0.2, 0.6, 1.5 g pot^{-1}) on the number and dry weight of nodules per plant in Enrei and Sakukei 4 on 39 (upper) and 74 (lower) DAS (Exp. 1). Vertical bars indicate SE of five pots.

s^{-1} and 350 μmol mol^{-1}, respectively. The irradiance on the measured leaf (6 cm^2) was regulated at 1,500 μmol m^{-2} s^{-1} PPFD. The temperature of the chamber was kept at 25°C. The measurements were carried out between 1000 and 1130 hr on 31, 35, 41 and 44 DAS. On 44 DAS, measurements were made on three consecutive leaf positions (the just expanded leaf and two successive lower leaves) on the main stem. The color of the leaves that were used for the measurement of AP was determined with a SPAD 502 chlorophyll meter.

3. Statistical analysis

Each pot was considered as an experimental unit. Data are shown as averages of five (Exp. 1) or six (Exp. 2) pots, except for ARA data in Exp. 1 in which three plants were used. Differences between genotypes were tested using Fisher's LSD ($P < 0.05$).

Results

1. Experiment 1

The two genotypes responded to the amount of N fertilizer differently. On 39 DAS, the total dry weight of Enrei was larger at lower rates of N application (0, 0.2 g N) than at higher rates (0.6, 1.5 g N) (Fig. 1). On 74 DAS, however, the difference among N application rates was negligible in Enrei. In Sakukei 4, the total dry weight tended to increase at higher rates of N application up to 0.6 g N both on 39 and 74 DAS. The root response was most sensitive to the amount of N. The number and dry weight of nodules decreased sharply with increasing amount of N application in both genotypes, but the actual values were far greater in Sakukei 4 (Fig. 2).

Acetylene reduction activity (ARA) per plant on 73 DAS was significantly higher in Sakukei 4 than in Enrei at lower rates of N application (0, 0.2 g N), but the difference was insignificant at higher rates of N application (0.6, 1.5 g N) (Fig. 3).

Generally, SPAD values of the leaves on the main stem were higher in Sakukei 4 than Enrei irrespective of leaf position or N dosage (Fig. 4). This was particularly clear in plots with lower rates of N application (0 and 0.2 g).

2. Experiment 2

On 31 DAS, total dry weight and top dry weight did not vary significantly among the three genotypes, while underground dry weight differed significantly among the genotypes (Table 1). The root dry weight was highest in Enrei, but the nodule dry weight was markedly greater in Sakukei 4 than in Enrei. On 46 DAS, the genotypic difference in the dry weights of each plant part was larger than on 31 DAS; the dry weights of the total plant, top and root were all significantly lower in Sakukei 4 than in Enrei or En1282, but nodule dry weight was higher (Table 1). All the growth parameters (RGR, NAR and LAR), which were calculated based on the values on 31 and 46 DAS, were lower in Sakukei 4 than in the other two genotypes (Table 2). Generally, the ratio of dry-matter partitioning to the aboveground parts was higher in Enrei and En1282 than in Sakukei 4, while the ratio to the root did not vary as much (Fig. 5A). By contrast, the ratio to nodules was markedly higher in Sakukei 4 than in Enrei. Excluding nodules, there was no significant difference in the ratio among the genotypes (Fig. 5B). Leaf area increased linearly during the period from 31 to 46 DAS in all the genotypes, but the rate of increase was smallest in Sakukei 4 (Fig. 6).

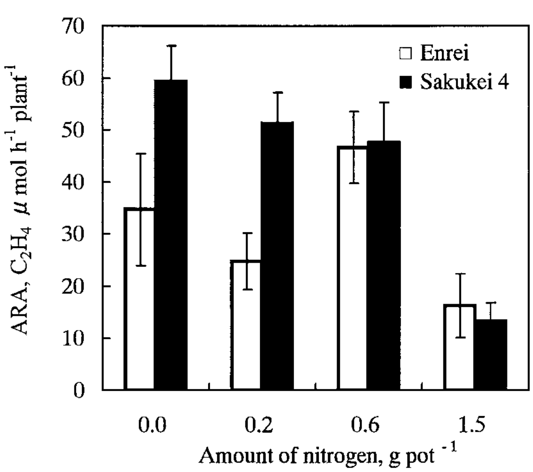

Fig. 3. Effects of the amount of nitrogen applied on acetylene reduction activity (ARA) in Enrei and Sakukei 4 on 73 DAS (Exp. 1). Each point represents mean ± SE of three plants.

Table 1. Dry weights of various plant parts of three soybean genotypes on 31 and 46 DAS (Exp. 2).

DAS	Genotype	Top	Root	Nodule	Total
		g $plant^{-1}$			
31	Enrei	2.85a	0.50a	0.01b	3.36a
	En1282	2.41a	0.36b		2.77a
	Sakukei 4	2.27a	0.41b	0.44a	3.12a
46	Enrei	12.77a	2.01a	0.57b	15.35a
	En1282	11.29a	2.11a		13.40ab
	Sakukei 4	7.31b	1.25b	2.37a	10.92b

Values followed by the same letter within a column of the same sampling date are not significantly different by LSD (P<0.05).

Table 2. Relative growth rate (RGR), net assimilation rate (NAR) and leaf area ratio (LAR) of three soybean genotypes during 31-46 DAS (Exp. 2).

Genotype	RGR	NAR	LAR
	$g\ g^{-1}\ d^{-1}$	$mg\ cm^{-2}\ d^{-1}$	$cm^2\ g^{-1}$
Enrei	0.10	0.80	126.7
En1282	0.11	0.78	134.7
Sakukei 4	0.08	0.70	120.0

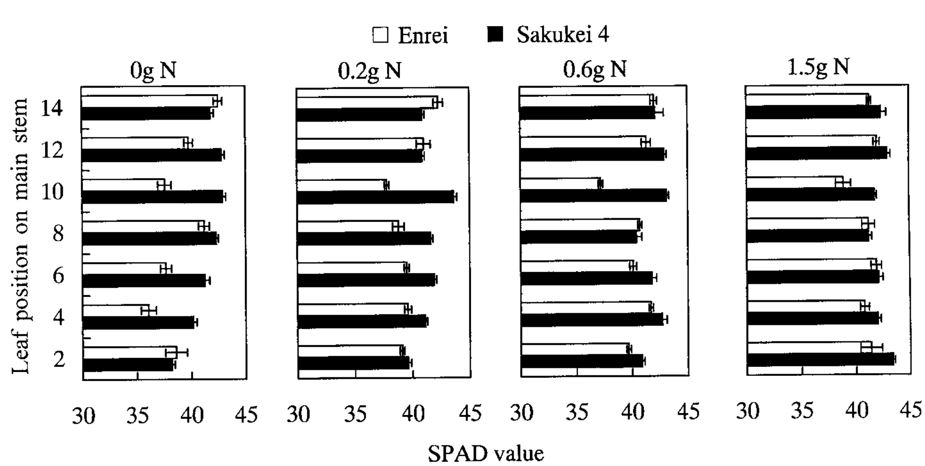

Fig. 4. SPAD values of the leaves at different leaf positions on the main stem in Enrei and Sakukei 4 (Exp. 1). Plants were grown at four rates of nitrogen application (0, 0.2, 0.6, 1.5 g pot^{-1}) and SPAD values were measured on the just expanded leaves. Horizontal bars indicate ± SE of six plants.

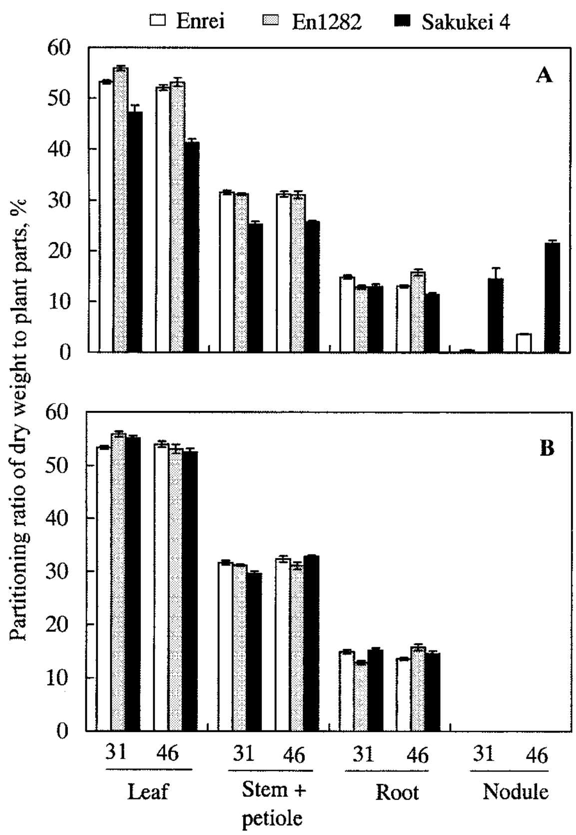

Fig. 5. Partitioning of total dry matter to various plant parts in three genotypes on 31 and 46 DAS (Exp. 2). A : Nodule weight was included in total dry weight. B : Nodule weight was excluded from total dry weight. Each value represents mean ± SE of six plants.

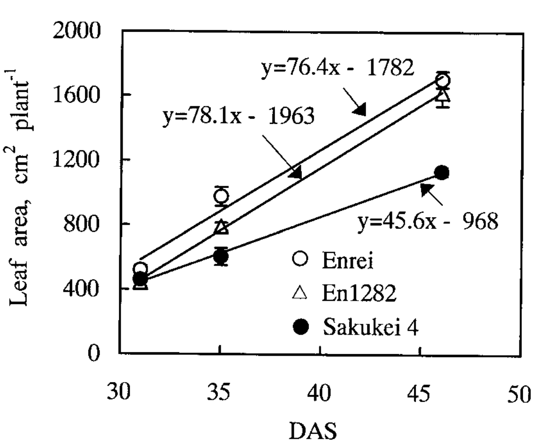

Fig. 6. Change in leaf area per plant of three genotypes during the period from 31 to 46 DAS (Exp. 2). Each point represents mean ± SE of six plants.

The AP of the just expanded leaves was significantly higher in Sakukei 4 than in Enrei or En1282 during the period from 31 to 41 DAS (Fig. 7). This genotypic difference in AP was also observed in the two leaves (L-2, L-3) positioned below the L-1 (Fig. 8, left). The SPAD values of the leaves tended to be higher in Sakukei 4 than in the other two genotypes (Fig. 8, right).

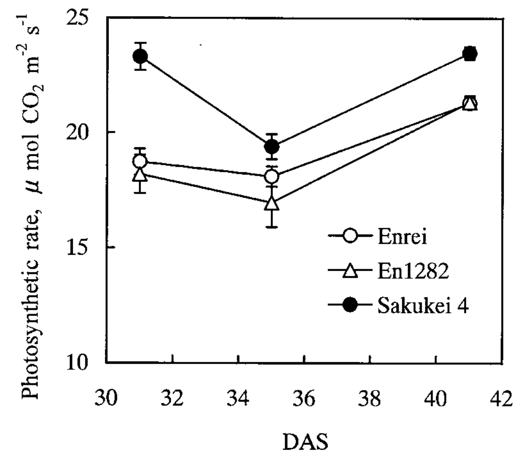

Fig. 7. Change in photosynthetic rate of the just expanded leaf in three genotypes on 31, 35 and 41 DAS (Exp. 2). Each point represents mean ± SE of six plants.

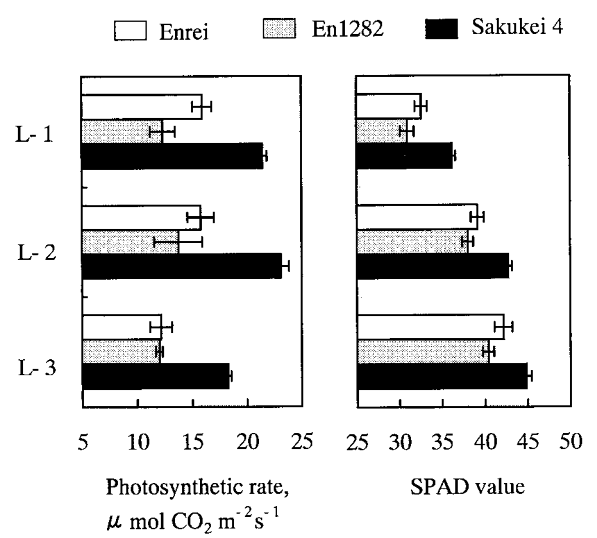

Fig. 8. Photosynthetic rate and SPAD value of leaves at different nodal positions on the main stem of three genotypes on 44 DAS (Exp. 2). Each point represents mean ± SE of six plants. L-1 : just expanded leaf. L-2 : leaf one node below L-1. L-3 : leaf two nodes below L-1.

Discussion

Our previous study indicated that the growth performance of Sakukei 4 was inferior to that of its normally nodulating parental cultivar, Enrei, particularly during the period before pod expansion (Maekawa et al., 2003). In Exp. 1 of the present study, the growth of Sakukei 4 and its parental cultivar Enrei before pod expansion was compared under various rates of N fertilizer. The genotypic difference in growth was significant on 39 DAS and became greater at the flowering stage (74 DAS). In Exp. 2, therefore, growth was analyzed in more detail during an earlier growth stage (31 – 46 DAS). The results suggested that several traits are involved in the inferior vegetative growth of Sakukei 4.

The total dry weight during the early growth period was smaller in Sakukei 4 than in its related normally nodulating or non-nodulating genotypes, and the genotypic difference became significant after 31 DAS (Table 1). The single seed weight was lighter in previously bred supernodulating soybean line En6500 (Takahashi et al., 2003b), and lighter seeds can cause inferior early growth after germination. In the present study, however, there was no significant difference in single seed weight between Enrei and Sakukei 4 (data not shown). In addition, the genotypic difference in growth was negligible before 31 DAS. Therefore, the inferior growth of Sakukei 4 after 31 DAS is not attributable to seed size.

The ratio of dry-matter partitioning to leaf, stem plus petiole, and root was generally lower in Sakukei 4 than in the other two genotypes, while the ratio to nodules was generally higher in Sakukei 4 than Enrei (Fig. 5A). However, dry-matter partitioning to other parts of the plant was very similar in the three genotypes (Fig. 5B). Studies comparing energy costs for NO_3^- assimilation and N_2 fixation showed that the whole-plant energy cost for symbiotic N_2 fixation was greater than that for NO_3^- metabolism (Ryle et al., 1979; Finke et al., 1982). In Sakukei 4, the greater partitioning of photoassimilate to nodules for the maintenance and metabolism of nodules might have lowered NAR and thereby shoot growth despite the higher photosynthetic rate. This excessive partitioning of photoassimilate to nodules appears to be a primary factor responsible for the inferior growth of Sakukei 4 during the pre-flowering stage.

The supernodulating soybean lines NOD1-3, NOD2-4 and NOD3-7 (Ohyama et al., 1993) and En6500 (Takahashi et al., 1995) exhibited restricted root growth and assimilated a smaller amount of nitrate N than their parent cultivars. A similar reduction of growth in supernodulating genotypes compared to parental cultivars has been reported even without inoculation with *Bradyrhizobium japonicum* (Day et al., 1986; Francisco et al., 1992). These results strongly suggest that the lower capacity of N absorption due to a poorly developed root system restricts total N assimilation and thereby overall plant growth in these supernodulating genotypes. As shown in Fig. 1 and Table 1, Sakukei 4 has a smaller root mass than Enrei under a low rate of N application. Therefore, limited root mass might be another critical trait responsible for the inferior early growth of Sakukei 4.

Sakukei 4 had superior photosynthetic ability, which was probably due to a higher concentration of N at all leaf positions (Figs. 4, 7, 8). This desirable trait, however, did not enhance its pre-flowering growth performance. This is consistent with our previous observation that the superior photosynthetic ability of Sakukei 4 does not enhance dry weight at the pod expansion and seed filling stages (Maekawa et al., 2003). The dry-matter production of crops is determined by leaf expansion and leaf photosynthetic rate. An additional reason for the inferior pre-flowering growth of Sakukei 4, therefore, might be its lower capability for leaf expansion; Sakukei 4 accumulates N in the leaves rather than utilizing N for leaf expansion (Figs. 4, 6, 8).

The proportion of N derived from symbiotic fixation is relatively low at an early growth stage (Henson and Heichel, 1984). Therefore, leaf expansion at an early growth stage has to depend largely on N derived from soil and fertilizer. The amount of N from soil and fertilizer may be smaller in Sakukei 4 than in the other genotypes, leading to depressed expansion of leaf, although there is no data available showing the proportion of N derived from soil and fertilizer in supernodulating soybean genotypes. Biochemical characterization of this trait of Sakukei 4 needs to be clarified. For practical use of this cultivar, a high planting density may be one measure to enhance growth and yield. These issues are currently being researched in our laboratory.

Exp. 1 showed that the growth performance of Sakukei 4 was better at higher rates of N application, and the dry weight was close to that of Enrei (Fig. 1). In normally nodulating cultivars, it is common for root development to be depressed with an increasing amount of applied N, as observed for Enrei in this experiment. It is noteworthy that the root weight of Sakukei 4 increased with an increasing amount of N application up to 0.6 g per pot. Since the number and dry weight of nodules, and the nodule activity (ARA), decreased with increasing rate of N (Figs. 2, 3), the N absorption of Sakukei 4 appears to depend largely on nitrogen fertilizer at higher rates of N application. In the previous study conducted in the Kanto region, Sakukei 4 tended to perform better than normally nodulating cultivars, particularly in soils with low N fertility (Takahashi et al., 2003a, b). At low N fertilizer conditions in the present experiment, however, the growth of Sakukei 4 was substantially inferior to that of Enrei, although the formation of nodules and the nodule activity per plant were generally superior in Sakukkei 4. In the experiment using En6500, an ancestral line of Sakukei 4, Takahashi et al. (1995) found that the proportion of fixed N to total N accumulation on 33 (R1) DAS was 37% using plants grown hydroponically at a controlled temperature (28/23°C day/night). The activity of nodules is very sensitive to soil conditions such as temperature, soil moisture and soil N content (Harper, 1987). The diurnal and seasonal variation in nodule activity (ARA) is more closely correlated with soil temperature than with photosynthetically active radiation in field-grown soybeans (Denison and Sinclair, 1985; Sinclair and

Weisz, 1985). The responsiveness of nodule activity to soil temperature might be one of the factors affecting the regional difference in the performance of Sakukei 4.

Significant growth suppression compared to normally nodulating relatives has been commonly observed in all the previously bred supernodulating soybean genotypes (Carroll et al., 1985a, b; Day et al., 1986; Hansen et al., 1989; Gremaud and Harper, 1989; Akao and Kouchi, 1992; Francisco et al., 1992; Ohyama et al., 1993), and this has been the main obstacle to the agricultural use of supernodulating soybeans. Compared to previously bred supernodulating soybean lines, the growth performance and yield have been significantly improved in Sakukei 4, and the yield of this cultivar is superior in certain environments in the Kanto region to that of its parental cultivar Enrei (Takahashi et al., 2003a, b). This capability, however, appears to be difficult to be realized in cooler environments like those in the Tohoku region, probably because lower temperatures limit nodule activity and leaf expansion. Further genetic improvements by breeding, as well as development of appropriate cultural methods, are necessary to exploit the desirable traits of this genotype as a high-yielding cultivar in the Tohoku region.

Acknowledgments

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