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Yamakawa, Takeo
Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University

Fukushima, Youko
Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University

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# Low Inoculum Densities of *Bradyrhizobium japonicum* USDA 110 is Effective on Production of Soybean (*Glycine max* L. Merr.) Cultivar Fukuyutaka

**Takeo YAMAKAWA* and Youko FUKUSHIMA[1]**

Laboratory of Plant Nutrition, Division of Molecular Biosciences, Department of Biosciences & Biotechnology, Faculty of Agriculture, Kyushu University, 6–10–1 Hakozaki, Higashi–ku, Fukuoka 812–8581, Japan.
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Root occupation of rhizobia inoculated on seed coat was known to be low because of their low competitiveness against indigenous rhizobia. This study was carried out to clarify the effect of inoculation methods and inoculum density of *Bradyrhizobium japonicum* USDA 110 on the production of soybean. Five experimental plots with three replications corresponded to: no inoculation (NI), seed coating inoculation at $10^5$ cells seed$^{-1}$ (SI5) and $10^7$ cells seed$^{-1}$ (SI7), root zone inoculation at $1.7\times10^3$ cells g$^{-1}$ dry soil (PI7) and $1.7\times10^5$ cells g$^{-1}$ dry soil (PI9). PI plots were plowed after treatment application. Our results indicated a significant higher occupation of serotype USDA 110 in SI5, SI7, and PI9 plots, compared to the other treatments. Their yield (g m$^{-2}$) was significantly increased. There was no yield increase above $10^5$ cells seed$^{-1}$, and this density was considered most effective for seed inoculation. Furthermore, results of PI9 treatment indicated that inoculation of $1.7\times10^5$ cells g$^{-1}$ dry soil using BM2 (neutralized peat moss) was effective to compete with indigenous rhizobia for nodulation.

This study revealed the significant yield increase in SI5 and PI9 treatments with lower inoculation concentrations of $10^5$ cells seed$^{-1}$ and $1.7\times10^5$ cells g$^{-1}$ dry soil, respectively. Increased inoculum density above these levels did not increase seed yield. We concluded that it was possible to increase soybean yield by considering proper inoculum densities of efficient rhizobia and inoculation methods.

**Key words**: Indigenous rhizobia, Inoculation, Soybean production, *Bradyrhizobium japonicum* USDA 110

## INTRODUCTION

Inoculation with efficient rhizobia at the ordinary dose does not increase appreciably the seed yield of soybean because the occupation ratio of the inoculated rhizobial strains in the nodules is very low due to competition with less efficient indigenous rhizobia (Weaver and Frederick, 1974; Kyien *et al*., 1981). In order to increase the seed yield by rhizobial inoculation, the occupation ratio of the inoculated strains must be increased. The increase of the occupation ratio has been examined from various viewpoints such as improvement of inoculation method (Takahashi *et al*., 1996). For the screening of efficient and competitive strains, a large number of useful strains had been isolated from mutagenized and recombinant rhizobia (Maier and Brill, 1978; Williams and Phillips, 1983; Maier and Graham, 1990).

Caldwell and colleagues (Caldwell, 1966; Caldwell *et al*., 1966) discovered that soybean (*Glycine max* L. Merr.) cultivar Hardee was able to nodulate ineffectively with *Bradyrhizobium japonicum* strains 3–24–44 and 122 serogroups. This demonstrated that the ineffective nodulation was controlled by a host dominant gene, $Rj_2$. Furthermore, this cultivar was found to nodulate ineffectively with the *R. japonicum* strain 33 due to the presence of another *Rj*–gene, $Rj_3$ (Vest, 1970). Cvs. Hill and Amsoy 71 harbor a gene ($Rj_4$) that was responsible for the ineffective nodulation. These $Rj_4$–cultivars were nodulated ineffectively with *B. japonicum* strain 61 (Vest and Caldwell, 1972). Soybean plants harboring these *Rj*–genes (*Rj*–cultivars) were considered to restrict effective nodulation with appropriate serogroups of strains and to prefer certain types of rhizobia for nodulation. If this assumption holds true, planting of *Rj*–cultivars could increase the populations of rhizobial strains highly compatible with those cultivars in soils. Therefore, the relationship between the *Rj*–genotypes of soybean and the preference of the *Rj*–cultivars for various types of *Bradyrhizobium* strains was examined (Ishizuka *et al*., 1991a; 1991b; 1993). These *Bradyrhizobium* strains were classified into three nodulation types (A, B, and C), based on the compatibility with *Rj*–cultivars. Nodulation type A strains nodulated with most cultivars except with the $rj_1$–ones (non–nodulating lines, William and Lynch, 1954), and were preferred by non–*Rj*–ones. Type B or type C strains nodulated soybean cultivars except the $Rj_2Rj_3$–ones or $Rj_4$–ones, respectively and were preferred by $Rj_4$–ones or $Rj_2Rj_3$–ones, respectively.

In order to analyze in more detail the relationship between the *Rj*–genotypes of soybean and the preference of *Rj*–cultivars for various types of rhizobia, the isolation of $Rj_2Rj_3Rj_4$–lines from the cross between the $Rj_2Rj_3$–cultivar IAC–2 and the $Rj_4$–cultivar Hill was conducted (Ishizuka *et al*., 1993; Yamakawa *et al*., 1999). The relationship between the *Rj*–genotypes of soybean and their preference for various types of rhizobia for nodulation was investigated (Yamakawa *et al*., 2003). It resulted that in the rhizosphere of the $Rj_2Rj_3Rj_4$–genotypes, the growth of the type A rhizobia was enhanced, while that of types B and C rhizobia was repressed. An experiment was conducted to test the

[1] Division of Bioresource and Bioenvironmental Sciences, Graduate School, Kyushu University, 6–10–1 Hakozaki, Higashi–ku, Fukuoka 812–8581, Japan

* Corresponding author (Email: yamakawa@agr.kyushu–u.ac.jp)

effects of inoculation into a cylindrical soil block from just above the seed using bacterial suspension of *B. japonicum* USDA 110 as useful rhizobia. The results showed a high occupation ratio of USDA 110 in the taproot (70–100%) and the occupancy ratio in the lateral root was 44–77% lower than that of taproot in all soybean genotypes including $Rj_4$–gene. In particular, an extreme high occupancy ratio of USDA 110 was observed in soybean variety Fukuyutaka ($Rj_4$–genotype), which was cultivated as a promoted variety in the warm southwestern region of Kyushu, Japan. These results were thought that Fukuyutaka has a high compatibility with USDA 110.

Sufficient rhizobium population in the rhizosphere of legume seedlings is required for early and enough setting of root nodules. However, there was no positive correlation in the plants inoculated with a high rhizobium density (Iizuka *et al*., 2002). A major limiting factor in commercial inoculant use is competition from indigenous rhizobial populations in the soil (Triplett and Sadowsky, 1992). The timing and intensity of root nodulation are considered to be affected by the rhizobium population in soil (Weaver and Frederick, 1974; Herridge *et al*., 1987).

Application of the rhizobium inoculation technique is essential for soybean cultivation in a field with low rhizobium population, because early setting of sufficient number of nodules on seedling roots is required for soybean production depending on symbiotic nitrogen fixation. Many studies founded the inoculum density–dependent restriction of nodulation in soybean varieties with *Bradyrhizobium japonicum* symbiosis (Lohrke *et al*., 2000; Loh *et al*., 2002; Ferrey *et al*., 1994). In the studies of Loh *et al*. (2002) and Ferrey *et al*. (1994), using of soybean cvs. Essex and Peking, respectively, the density–dependent nodulation suppression is not limited to particular soybean genotypes but is a phenomenon that can be generalized to different soybean cultivars. In soils with a significant population of rhizobia, the exogenously applied inoculant strain ($10^8$–$10^9$ cells $ml^{-1}$) rarely occupies more than a few percent of the nodules formed (Loh *et al*., 2001). Recently, Jitacksorn and Sadowsky (2008) stated that nodulation on two soybean genotypes, cv. Lambert and PI417566, was inhibited when plants received a high–density inoculum ($10^9$ cells $ml^{-1}$) of strain USDA110 grown in complex medium, and more nodules were produced on plants receiving a low–cell–density inoculum ($10^5$ cells $ml^{-1}$).

In 2005, a field experiment was conducted to clarify the effect of the difference between the inoculation of rhizobia on seed surface and to the plow layer for soybean production (Fukushima and Yamakawa, 2006). Non–inoculated plot (NI), seed coat inoculation (SI) plot, and plow layer rhizobial solution inoculation (RI) plot were tested in three replications. Rhizobial concentration for inoculation was $10^7$ cells $seed^{-1}$ in both plots. The results showed a higher number of nodule occupancy rates of serotype USDA 110 in SI plots. However, yield (kg $10a^{-1}$) was higher in RI plots than in the other plots. Because the inoculum density in SI plots was high, many nodules were formed. Hence, a competition for photosynthetic products occurred between the growth of the soybean and the nodulation; suppressing the initial growth in these plots. This explains the lower yield in SI plots in comparison to RI plots. For SI plots, the effect of the inoculation was expected in the case of lower inoculum density as compared to the higher inoculation density of $10^7$ cells $seed^{-1}$ used in this study. From the above–mentioned results, it is necessarily to examine the relationship between inoculum concentration and inoculation method.

Therefore, the purpose of this study was to clarify the effect of the difference of inoculation methods and inoculum densities of *B. japonicum* USDA110 with high compatibility against soybean variety Fukuyutaka on its production.

## MATERIALS AND METHODS

### Site description

The field experiment was conducted in 2006 at Kyushu University Farm in Fukuoka Prefecture, Japan (33°37'N, 130°25'E). Soil samples were collected from 6 locations (15 cm soil depth) of the experimental field for physicochemical analyses. The soil texture was clay loam and its physico–chemical properties are shown in Table 1. Nutrient content of the soil was determined by $H_2SO_4$–salicylic acid–$H_2O_2$ digestion (Ohyama *et al*., 1991) followed by the indophenol method (Cataldo *et al*., 1974) for total N; ascorbic acid method (Murphy and Riley, 1962) for total phosphorus (P); and atomic absorption spectrophotometry (Z–5300, Hitachi, Tokyo, Japan) for total potassium (K). Cation exchangeable capacity (CEC) was determined by the ammonium acetate shaking extraction method (Muramoto *et al*., 1992). Analysis of mineralizable N was performed using the soil incubation method (Inoko, 1986) followed by the indophenol method (Cataldo *et al*., 1974) for $NH_4$–N and by the method of Cataldo *et al*. (1975) for $NO_3$–N. The available P of soil samples was analyzed using Truog's method (Truog, 1930) and Bray Ⅱ method (Bray and Kurtz, 1945) followed by the ascorbic acid method (Murphy and Riley, 1962).

**Table 1.** Phsicochemical characteristics of soil

| Total N | Total $P_2O_5$ | Available nitrogen | Available $P_2O_5$ Bray II | Available $P_2O_5$ Toruog | CEC | Exchangeable cation K | Exchangeable cation Ca | Exchangeable cation Mg |
|---|---|---|---|---|---|---|---|---|
| g $kg^{-1}$ dry soil | | mg $kg^{-1}$ dry soil | | | | $cmol_c$ $kg^{-1}$ dry soil | | |
| 1.17 | 0.77 | 39 | 154 | 90 | 20.20 | 0.94 | 6.31 | 1.69 |

These analyses were performed in 6 replications.

**Plant and rhizobium**

Soybean (*Glycine max* L. Merr.) variety Fukuyutaka and *Bradyrhizobium japonicum* USDA 110 harboring uptake hydrogenase ($Hup^+$) were used in this experiment. *B. japonicum* USDA 110 cultured in HM medium (Kuykendall, 1979) for eight days with a shaking at 30˚C was used for inoculation.

**Plant cultivation and inoculation treatment**

The field used in this study was previously cultivated with barley, and no history of soybean cultivation was reported for the past five years. The amount of lime carbonate for adjusting soil pH to 6.5 was estimated by the buffer curve method (Chiba and Shinke, 1977). After incorporating the lime carbonate to the cultivating layer on July 11, 2006, compound fertilizer (Mame Kasei®) was applied at a rate of 80 kg per 10 a (3.0% ammonia nitrogen, 10.0% acid soluble phosphorus, 10.0% water–soluble potash) to the layer and plowed. Five experimental plots (9.6 $m^2$ $plot^{-1}$: 4.0 m length × 2.4 m width) with three replications each were established. The treatments included a non–inoculation (NI) plot, seed coat inoculation (SI) plots at $10^5$ cells $seed^{-1}$ (SI5) and $10^7$ cells $seed^{-1}$ (SI7), and plow layer rhizobial peat moss inoculation (PI) plots at $10^7$ cells $seed^{-1}$ (PI7) plot and $10^9$ cells $seed^{-1}$ (PI9). Peat moss inoculation was conducted using mixture of BM2 (neutralized peat moss, Group Berger Peat Moss Ltd., Canada) and USDA 110 culture to the plow layer before seed sowing.

The inoculum for SI plots for 100 seeds was made by mixing 1.5 mL deionized water, 10 mL of 12% aqueous solution of gum arabic, 10 g of BM2, and 0.015 mL (SI5) or 1.5 mL (SI7) of USDA 110 culture solution ($1 \times 10^9$ cells $mL^{-1}$). For PI plots inoculum (per $m^2$), 200 g of BM2 was spread on a plastic sheet; tap water (40% of the maximum water holding capacity of BM2) and USDA 110 culture solution ($1 \times 10^9$ cells $mL^{-1}$) with 0.25 mL (PI7) or 25.0 mL (PI9) were added, and the whole was well mixed. After spraying the mixture to the rows, the rows were plowed (approximately 15 cm depth) by a tractor. USDA 110 inoculum for PI7 ($10^7$ cells) or PI9 ($10^7$ cells) in the plowed layer after inoculation occupied a volume of 18,000 $cm^3$ (60 × 20 × 15 cm) of soil in the root zone of one hill (three seeds sown). Consequently, the inoculum density of USDA 110 in the plow layer of these two plots (PI7 and PI9), was estimated to be $1.7 \times 10^3$ and $1.7 \times 10^5$ cells $g^{-1}$ dry soil, respectively. Inoculation of PI plots was done on July 28, 2006, seeds of Fukuyutaka were sown in rows of 60 cm spacing and 20 cm width between hills to a depth of approximately 2–3 cm. The seedlings were thinned to two seedlings at the two–trifoliate stage (V2, growth stage was according to Fehr *et al*. 1971), and at V5 stage, the inter row was cultivated and ridged. Some pesticides were timely sprayed according to the occurrence of disease. At both V6.4 and R5.7 stages, plants of one hill per plot were sampled, and 10 hills were harvested by cutting the stem at the R8 stage (November 8, 2006). Also, in order to see the effects of inoculation on the growth of soybeans, the main stem lengths were measured up to harvest.

**Indigenous rhizobia density**

To estimate the density of indigenous rhizobia, soils were collected from two locations in the experimental field before fertilization and the rhizobia density was measured by the most probable number (MPN) method using soybean cultivars, Orihime (non–*Rj*–genotype) and Fukuyutaka ($Rj_4$–genotype).

**Plant sampling and acetylene reduction activity (ARA) measurement**

Plants sampled with roots at V6.4 and R5.7 stages were divided into shoots and roots. Roots were used for the measurement of ARA and the counting of nodules. ARA was basically assayed according to Haider *et al*. (1991). Plant roots with intact nodules were cut at the cotyledonary nodes and individually placed in 100–mL conical flasks. Flasks were sealed with a serum stopper and 12 mL of the air was replaced with acetylene ($C_2H_2$) gas. The nodulated roots in the flasks were incubated at room temperature and 1.0 mL of subsamples were analyzed for ethylene ($C_2H_4$) production at 5 and 30 min after incubation by using a flame ionization gas chromatograph (GC–14A, Shimadzu, Kyoto, Japan) equipped with a stainless steel column (3 mm diameter, 0.5 m length). The column was filled with Porapak R, 60–80 meshes (Nacalai Tesque, Inc., Kyoto Japan). Column and injector temperatures were 35ºC and 45ºC, respectively. Carrier gas was $N_2$ (flow rate: 45 mL $min^{-1}$).

**Dry matter accumulation, nodulation, and occupation**

After ARA measurement, roots and shoots (leaves and stems + petioles) were air–dried at 80˚C for 24–48 h. The number of nodules was counted in the following five root parts: the upper 3 cm part of taproot (TU), the lower part of taproot (TL), the lateral root part generated from the upper taproot (LU), the lateral root part generated lower taproot (LL) and the superficial root (SR) part. The harvested nodules were freeze–dried. In order to see the effect of the inoculation, the freeze–dried nodules were used for a serological test performed using the USDA 110 antiserum previously produced in the same laboratory according to Yamakawa *et al*. (2003). Significant differences of occupancy of USDA110 serotype were analyzed by the $\chi^2$ test at $P<0.05$ level.

**Yield component and plant analysis**

After examining yield components (effective number of pods per $m^2$, seeds number per pod, ripening seed percentage, one hundred seed dry weight (g), seed dry yield (g $m^{-2}$) as well as measuring the plant dry weight of plants at each period, the dry matter was ground by Cyclotec 1093 sample mill (100–120 mesh, Tecator AB, Hoedanaes, Sweden). Nitrogen content of the ground sample was analyzed according to the indophenol method (Cataldo *et al*., 1974) after a $H_2SO_4$–salicylic acid–$H_2O_2$ digestion (Ohyama *et al*., 1991).

**Statistical analysis**

ANOVA was carried out for the collected parame-

ters. Means of each parameter were compared by Turkey's HSD test at $P<0.10$ level using Excel Statistics 2010 Software (Social Survey Research Information Co., Ltd., Tokyo, Japan).

## RESULTS AND DISCUSSION

### Crop conditions of Fukuyutaka

According to the statistics of Agriculture, Forestry and Fisheries, that was announced by Kyusyu Regional Agricultural Administration Office in February 2007, the yield of soybean Fukuyutaka in Fukuoka Prefecture in 2006 decreased by 39% compared to the previous year due to the impact of unfavorable weather conditions and Typhoon 13.

### Effects on growth parameter

Between the R2 tand R6.5 stages, the length of the main stem of SI7 plots was significantly higher than that of NI and PI7 plots. However, at harvest, the difference on the main stem length was not significantly different among the treatments (Fig. 1). In this experiment, the length of the main stem remained low compared to that of the average year. The little precipitation during the sowing period that created unfavorable hydric conditions was considered the main cause. It resulted in a delay of the early vegetative growth and an inhibition of the subsequent reproductive growth. With regard to dry matter accumulation during the growing season, there was no significant difference among the treatments in the total dry weight up to the V6.4 stage (Fig. 2). In stage R5.7, the total dry weight of SI5 plots was lower than that of the other plots, but the dry weight of seeds and pods was not different among all plots. The distribution of dry matter to pods and seeds during the pod filling stage was higher in SI5 plots than that in the other plots.

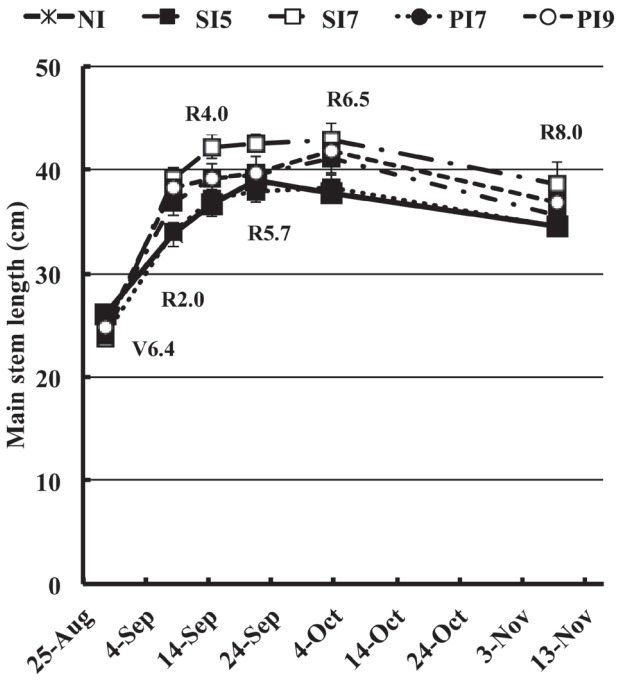


**Fig. 1.** Transition of main stem length of each plot during soybean growth period.
NI, SI5, SI7, PI7 and PI9 indicate experimental plots. V6.4, R2.0, R4.0 R5.7, R6.5 and R8.0 refer to growth stages according to Fehr *et al.* (1971). Error bar of each symbol represents standard error of the mean. This measurement was conducted with 6 plants of one hill per plot and three plots.

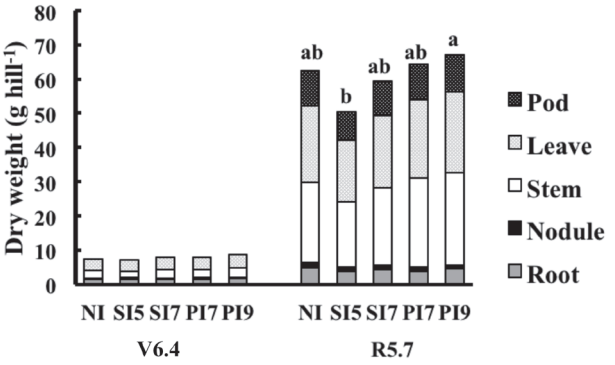


**Fig. 2.** Dry matter accumulations of each plot at each growth stage.
NI, SI5, SI7, PI7 and PI9 indicate experimental plots. V6.4 and R5.7 refer to growth stages according to Fehr *et al.* (1971). No and same letters above the bars within same growth stage are not significantly different at $P<0.10$ (Turkey's test). This measurement was conducted with plants of one hill per plot and three plots.

### Effects on nodulation

There was no significant difference in the total nodule dry weight per hill among the all plots at the V6.4 stage (Fig. 3). At the R5.7 stage, because the upper lateral root nodule dry weight of NI and PI7 plots was higher compared to that of the other plots, total nodule weights of NI and PI7 plots were significantly greater than that of SI5, SI7 and PI9 (Fig. 3A). The total nodule number per hill was not significantly different among all plots (Fig. 3C), but the nodule number on upper lateral roots was highest in PI9 plot at the V6.4 stage. There was no significant difference among the other plots. For PI plots, 45.1 to 52.7% of the total nodule number was observed in the lateral roots, while for NI and SI plots, the lateral roots contained 16.2% to 32.6% of the total nodule number.

### Effects on occupation of serotype USDA 110 and ARA

Table 2 shows the effect of inoculation methods on nodulation. At the V6.4 stage, the nodulation efficiency of PI plots was high in lateral roots, but at the R5.7 stage, no efficiency was observed regardless to the root position and inoculation methods. The occupation ratio of serotype USDA 110 on total root was significantly highest in SI7 and PI9 and lowest in PI7 at the V6.4 stage. At the R5.7 stage, the occupation of ratio *B. japonicum* USDA 110 in superficial and upper lateral roots increased, except in the superficial roots of PI7. Considering the

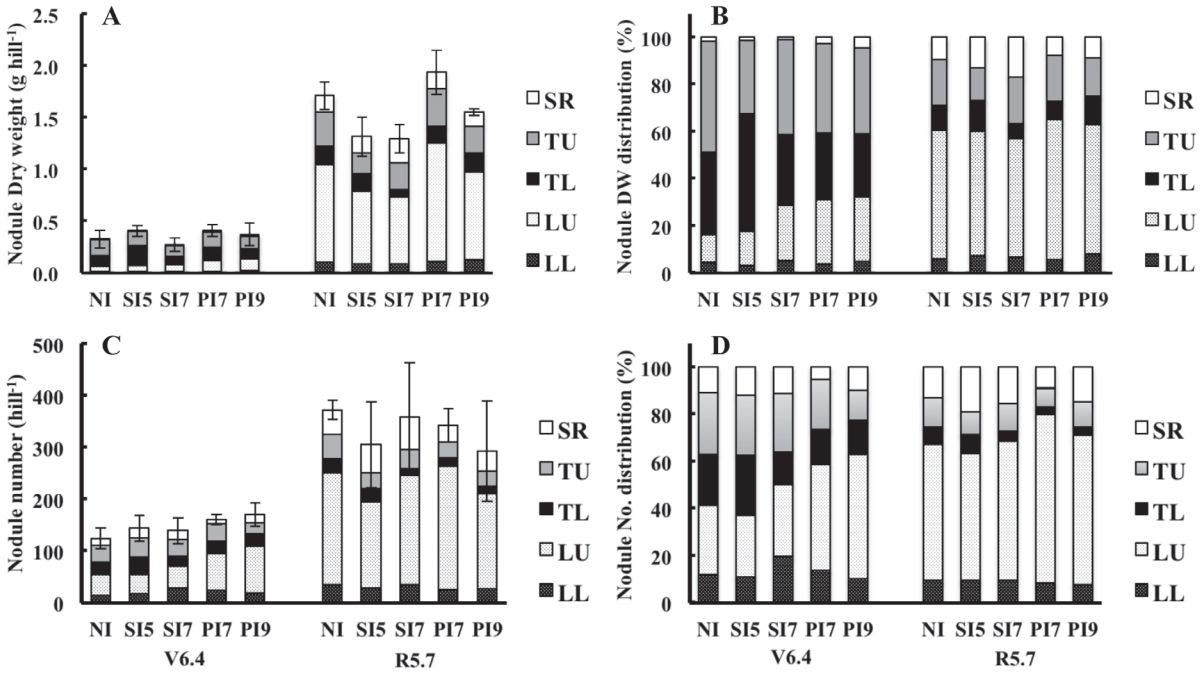


**Fig. 3.** Nodule number and dry weight, and the distribution of each plot at each growth stage.
NI, SI5, SI7, PI7 and PI9 indicate experimental plots. V6.4 and R5.7 refer to growth stages according to Fehr *et al.* (1971). SR, TU, TL, LU and LL indicate superficial root, upper taproot, lower taproot, upper lateral root and lower lateral root, respectively. Error bar of each symbol represents standard error of the mean. This measurement was conducted with plants of one hill per plot and three plots.

**Table 2.** Occupation ratio of serotype USDA 110 in nodules at each growth stage as affected by inoculation methods

| Growth stage | Plot | Superficial root | Tap root | | Lateral root | | Total |
|---|---|---|---|---|---|---|---|
| | | | Upper | Lower | Upper | Lower | |
| V6.4 | NI | 12.5 | 15.0 bc | 25.0 ab | 20.5 bc | 5.6 b | 17.3 b |
| | SI5 | 16.7 | 23.1 bc | 29.7 b | 27.5 b | 12.5 b | 24.0 b |
| | SI7 | 0.0 | 70.3 a | 42.9 a | 34.8 ab | 16.7 b | 27.3 a |
| | PI7 | 0.0 | 6.3 c | 9.1 b | 11.8 c | 5.0 b | 8.7 c |
| | PI9 | 6.7 | 27.8 b | 31.8 ab | 46.3 a | 53.3 b | 38.7 a |
| R5.7 | NI | 10.0 b | 31.6 | 50.0 | 35.7 | 35.7 | 26.0 b |
| | SI5 | 44.8 a | 38.5 | 23.1 | 50.0 | 50.0 | 43.3 a |
| | SI7 | 47.8 a | 58.8 | 28.6 | 28.6 | 28.6 | 50.0 a |
| | PI7 | 27.3 ab | 25.0 | 40.0 | 25.0 | 25.0 | 24.7 b |
| | PI9 | 50.0 a | 53.0 | 33.3 | 54.0 | 54.0 | 53.3 a |

In a column, means followed by the same letter are not significantly different at $P<0.10$ (Chi square test). When letters are absent, the statistics analysis was not significant. The measurement was carried out with plants of one hill per plot for three replicated plots.

total roots, the occupation ratio was significantly increased by inoculation of *B. japonicum* USDA 110, except in PI7 plots. However, it appeared that high–density inoculum tends to decrease ARA (Table 3). The highest seed yield of Fukuyutaka was obtained in SI5 and PI9 plots (Table 5). In SI5, SI7, PI9 plots at the R5.7 stage, the occupation of serotype USDA110 was significantly higher, and it was assumed that the fixed nitrogen was distributed in large amount to pods and seeds. Consequently, seed yield (g $m^{-2}$) was significantly increased in these plots. The density of $10^5$cells $seed^{-1}$ appeared to be most effective for seed inoculation, as no higher effect was observed with the increase of the inoculum density. Furthermore, results of PI9 plots indicated

**Table 3.** Acetylene reduction activity (ARA) at each growth stage

| Plot | Specific ARA | | ARA per hill | |
|---|---|---|---|---|
| | V6.4 | R5.7 | V6.4 | R5.7 |
| | $\mu$mol $C_2H_4$ $g^{-1}$ DW | | $\mu$mol $C_2H_4$ $hill^{-1}$ | |
| NI | 48.0 ab | 9.2 c | 15.7 | 16.8 b |
| SI5 | 50.0 a | 16.3 b | 21.0 | 21.3 ab |
| SI7 | 39.1 ab | 7.9 c | 12.3 | 9.9 b |
| PI7 | 48.0 ab | 28.4 a | 19.9 | 38.4 a |
| PI9 | 33.3 b | 10.1 c | 12.5 | 12.8 b |

In a column, means followed by the same letter are not significantly different at $P<0.10$ (Chi square test). When letters are absent, the statistics analysis was not significant. V6.4 and R5.7 indicate the growth stages. The measurement was carried out with plants of one hill per plot for three replicated plots.

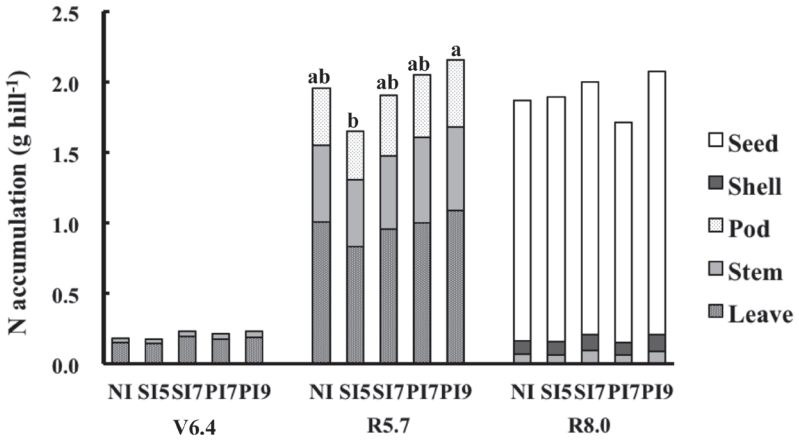


**Fig. 4.** Total nitrogen accumulation on shoot of each plot at each growth stage. NI, SI5, SI7, PI7 and PI9 indicate experimental plots. V6.4 and R5.7 refer to growth stages according to Fehr *et al.* (1971). No and same letters above the bars within same the growth stage are not significantly different at $P<0.10$ (Turkey's test). This measurement was conducted with plants of one hill per plot and three plots at V6.4 and R5.7, and ten hills per plot and three plots at R8.0.

that previous inoculation using BM2 ($1.7 \times 10^5$ cells $seed^{-1}$) was effective in competition with indigenous rhizobia for nodulation.

The highest specific ARA during the period of growth was observed at the V6.4 stage in all plots, followed by a decrease at later stages (Table 3). In comparing the specific ARA within all plots, we noticed that ARA of PI9 plots was significantly lower at the V6.4 stage. At the R5.7 stage, ARA of PI7 and SI5 plots were significantly higher than that of the other plots. ARA per hill increased at the R5.7 stage, due to the increase of nodule mass at the later plant growth stage (Fig. 3A). At the V6.4 stage, ARA per hill within all plots was not significantly different, and at the R5.7 stage, the tendency of ARA per hill was similar to that of specific ARA. Symbiotic nitrogen fixation was especially superior in SI5 and PI7, which corresponded to the treatments with $10^5$ cell $seed^{-1}$ inoculum concentration. However, PI7 showed higher efficiency.

**Nitrogen and dry matter accumulation**

Nitrogen (N) accumulation of soybean shoots in SI plots was greatest at R8 stage. In the other plots, the highest N accumulation was observed at the R5.7 stage, and it decreased thereafter (Fig. 4). Mainly, N was accumulated in leaves from the V6.4 to the R5.7 stage and in pods (seed + shell) from the R5.7 to the R8 stage. The N accumulation of pods in SI5 plots at the R5.7 stage was lower than that of PI9 and PI7 plots. Results in Table 4 of pod N accumulation at the R5.7 and R8.0 stages and the corresponding N fluctuation showed a non–significance of the Turkey's test. However, the pod N accumulation was relatively higher in PI9 plots at the R5.7 stage, where SI5 plots had the lower value. Nitrogen accumulation in shoots at the R5.7 stage was significantly higher in PI9 plots, and significantly lower in SI5 plots.

**Table 4.** N accumulation of pod and shoot at R5.7 and R8.0 stage, and N fluctuation from R5.7 to R8.0 stages

| Plot | Pod (g N hill$^{-1}$) | | | Shoot (g N hill$^{-1}$) | | |
|---|---|---|---|---|---|---|
| | R5.7 | R8.0 | Fluctuation | R5.7 | R8.0 | Fluctuation |
| NI | 0.41 | 1.81 | 1.40 | 1.96 ab | 1.87 | –0.08 ab |
| SI5 | 0.34 | 1.83 | 1.49 | 1.65 b | 1.90 | 0.26 a |
| SI7 | 0.44 | 1.91 | 1.47 | 1.91 ab | 2.00 | 0.09 ab |
| PI7 | 0.45 | 1.65 | 1.21 | 2.05 ab | 1.71 | –0.33 b |
| PI9 | 0.48 | 1.99 | 1.51 | 2.16 a | 2.07 | –0.08 ab |

In fluctuation, R5.7 and R8.0 indicate the growth stages. "+" indicates the increase of N in a plant part from R5.7 to R8.0 stages, and "−" indicates the decrease of N. In a column, means followed by the same letter are not significantly different at $P<0.10$ (Turkey's test). When letters are absent, the statistics analysis was not significant. For R5.7 stage, the measurement was carried out with plants of one hill per plot for three replicated plots, while for R8.0, 10 hills per plot were considered for three replicated plots.

**Table 5.** Seed yield and yield components of each plot affected by inoculation methods

| Plot | Pod number | Seed number Pod$^{-1}$ | Full seed ratio | 100 seed gDW | Yield g DW m$^{-2}$ | Yield index |
|---|---|---|---|---|---|---|
| NI | 477.5 | 1.81 | 0.93 | 21.2 | 165.5 b | 0.57 |
| SI5 | 545.5 | 1.85 | 0.94 | 21.5 | 210.2 a | 0.58 |
| SI7 | 527.5 | 1.80 | 0.92 | 22.7 | 195.9 ab | 0.55 |
| PI7 | 486.7 | 1.84 | 0.94 | 20.6 | 127.7 b | 0.57 |
| PI9 | 577.8 | 1.79 | 0.93 | 21.8 | 208.8 a | 0.56 |

In a column, means followed by the same letter are not significantly different at $P<0.10$ (Chi square test). When letters are absent, the statistics analysis was not significant. V6.4 and R5.7 indicate the growth stages. The measurement was carried out with plants of ten hills per plot for three replicated plots.

At the R8.0 stage, the difference between treatments was no longer observed. Considering the shoot N fluctuation between the two stages, there was a significant increase in SI5 plots. This indicates that nitrogen fixation was active under this period in this treatment. Contrary results were observed on PI7 plots. Thus, the increase in seed yield in the SI5 treatment (Table 5) was presumably related to the maintenance of high nitrogen content in shoots due to a high nitrogen fixation activity under the late ripening period, and a good photosynthetic activity.

The number of effective pods per m$^2$ in SI5, SI7 and PI9 plot was 545.5, 527.5 and 577.8, respectively, and was greater than that in NI plot (477.5) as shown in Table 5. The yield obtained from of SI5, SI7 and PI9 plots were 210.2, 195.9 and 208.8 g dry weight seed m$^{-2}$, respectively. These yields were significantly higher than that of the of NI plots with 165.5 g m$^{-2}$. These results indicated an increase of soybean yield rhizobial inoculation except for the PI7 plots.

From the above results, it is likely that the yield in SI5 plots was due the high occupancy of USDA 110, and good ARA during the reproductive growth that led to a good N distribution to seeds during the pod filling stage. In PI9 plots, despite a large number of nodules infected by the serotype USDA 110 as was observed on upper lateral roots from the early stage of growth, ARA per hill had remained low throughout the growing season. The main reason for the yield increase of this plot is not known so far. Therefore, this N distribution pattern must be clarified in future trials. In PI7 plots, the occupancy of serotype USDA 110 was low, and many nodules infected by indigenous rhizobia other than USDA 110 had been formed. ARA in these plots was higher than in the other plots, but this did not result in yield increase. These results suggest that mostly fixed nitrogen was directed to the nodulation (Fig. 3), and that most of the photosynthetic products were rather consumed for $N_2$ fixation and not directed to the production of seed. In addition, assuming that many of the nodules were infected by rhizobial strains that do not have uptake hydrogenase (Hup–) (Arp, 1992), it is likely that the energy efficiency of $N_2$ fixation was low (Evans *et al*., 1987), and the efficiency of N distribution to seeds got smaller. The reason for the lower occupancy of serotype USDA 110 in PI7 plots compared to that in NI plots must be clarified in future studies.

In this study, the sampling was carried out at V6.4, R5.7, and R8 stages. We think it is necessary that the sampling interval be longer to observe the changes in nodulation and $N_2$ fixation activity. Therefore, in the future it may be necessary to narrow the interval of sampling

and to increase the number of sampling times. In addition, the measurements of ARA carried out in this study, regardless of $Hup^-$ or $Hup^+$, must assess the maximum activity of $N_2$ fixation that does not take into account the release of $H_2$. Furthermore, there is a need to evaluate the efficiency of $N_2$ fixation by simultaneously measuring the release of ARA and $H_2$.

**Suitable inoculation method**

In assessing the presence rate of indigenous rhizobia in soils cultivated with Orihime (non–*Rj*) and Fukuyutaka ($Rj_4$) genotypes, we found they were present at concentrations of $1.4 \times 10^6$ and $1.4 \times 10^5$ cells $g^{-1}$ dry soil, respectively. Based on the difference of the specificity for *Rj*–genotype of host soybean cultivar, *Bradyrhizobium* can be classified into different nodulation types, A, B, and C (Ishizuka *et al*., 1991a; 1991b; 1993). Non–*Rj*–genotype soybean cultivars can be nodulated by any types of bacteria. Fukuyutaka harboring $Rj_4$–gene can be nodulated by type A and type B strains. *B. japonicum* strains of type A can form effective nodules on soybean root of most soybean cultivars. Orihime can be nodulated by all indigenous *Bradyrhizobium* strains. Thus, indigenous rhizobia able to nodulate roots of Fukuyutaka were at least 1/10 of the total indigenous rhizobia. Inoculation of USDA 110 with a lower concentration compared to indigenous type A and type B rhizobial strains resulted to an increased occupancy of serotype USDA 110 and to an enhancement of the competitiveness of *B. japonicum* USDA 110. Ishizuka (1992) described how research to improve BNF was progressing through the breeding of efficient $N_2$–fixing organisms and host plants, selection of the best combinations of host plant and microsymbiont, and by the improvement of inoculation techniques and field management. To increase plant production through enhanced BNF, the constraints in establishing effective $N_2$–fixing systems in the field should be understood and eliminated. Therefore, the inoculation methods using the soybean *Rj*–gene were useful to support soybean production and must be established in the future.

Considering the inoculation methods in this study, with the fact that there was an increase of the occupancy of serotype USDA 110 and yield in PI9 plots, where the inoculum concentration was $1.7 \times 10^5$ cells $g^{-1}$ dry soil, the effectiveness of the inoculation into the plowed layer using the BM2 medium was confirmed. However, the yield and occupancy did not increase in PI7 plots because the inoculum concentration of $1.7 \times 10^3$ cells $g^{-1}$ dry soil was lower than that of the indigenous rhizobia with a concentration of $1.4 \times 10^5$ cells $g^{-1}$ dry soil. It appears that for a good competition, the concentration of inoculated rhizobia should be at least comparable to that of the indigenous rhizobia in the plowed layer. In this study, BM2 made from peat moss was used as the medium for inoculation.

For seed inoculation, it was suggested that to compete for nodulation with indigenous rhizobia, the density of the inoculated rhizobia should be 1000 times higher than that in soil. Higashida (1974) reported that the occupation was increased by the inoculation with corresponding density. However, in Canada, standard inoculum concentration of soybean, kidney bean, and pea was $10^5$ cells $seed^{-1}$ (Smith, 1992). In addition, it was reported that nodulation was suppressed when the inoculation was carried out at a high concentration of *B. japonicum* USDA 110 (Lohrke *et al*., 2000). Many studies noted that soybean plant nodulation was inhibited when plants were inoculated with high–density cell inoculum (Takats, 1986; Smith, 1992; Jitacksorn and Sadowsky, 2008). However, there is little study about the effect of low density inoculum on the yield of soybean. In this study, the increase in yield was observed in SI5 compared to SI7. Thus the increase in inoculum density on seed coat did not increase seed yield. Moreover, the inoculation into the plowed layer by a slightly higher concentration of *Bradyrhizobium* strains than indigenous rhizobia ($1.7 \times 10^5$ cells $g^{-1}$ dry soil) increased the yield of soybean and the occupancy. From the above result, it appears that the seed inoculation at a concentration of $10^5$ cell $seed^{-1}$ and the inoculation into the plow layer by a slightly higher concentration than indigenous rhizobia were able to increase the yield of soybean. In the future, we hope that successful inoculation with some efficient rhizobia to soybean will be able to be carried out.

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