Effects of Symbiosis with *Rhizobium fredii* on Transport of Fixed Nitrogen in the Xylem of Soybean Plants

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An experiment was conducted to identify the main nitrogenous compound transported in the xylem sap of soybean plants nodulated with *Rhizobium fredii*. Soybean (*Glycine max* L. Merr.) cultivars, wild type Bragg (nod⁺, fix⁺) and its nitrate tolerant, hypernodulating mutant nts1116 (nod⁻⁻, fix⁺) were used for this experiment. These soybean plants were inoculated with a slow-growing rhizobium, *Bradyrhizobium japonicum* USDA110 or fast-growing rhizobia consisting of a mixture of *R. fredii* USDA191, USDA193, and USDA194 and grown in a phytotron under natural light and controlled temperature conditions. Xylem sap was collected from Bragg and nts1116 plants at the flowering and pod elongation stages. Acetylene reduction activity per plant or per nodule weight was not different between soybean lines and inoculums. The composition of the nitrogenous compounds in the xylem sap was compared between the symbionts, with *B. japonicum* and *R. fredii*. At the flowering stage, ureide-N and amide-N accounted for 53 to 70% and 20 to 27% respectively of the total N in the sap collected from the plants inoculated either with *B. japonicum* or *R. fredii*. At the pod elongation stage, ureide-N and amide-N accounted for 74 to 85%, and 7 to 19% of total sap N. With the growth of the soybean plants, the ratio of ureide-N in the xylem sap increased. These results suggest that in the case of wild soybean and the hypernodulating mutant line nodulated by *R. fredii*, ureide is transported as the main nitrogenous compound of fixed nitrogen in the xylem sap in the same way as in plants nodulated with *B. japonicum*.

**Key Words:** N₂ fixation, *Rhizobium fredii*, soybean, ureides, xylem transport.

Symbiotic nitrogen fixation of leguminous plants is performed under conditions of symbiosis with specific rhizobia. Soybean (*Glycine max* L. Merr.) plants nodulate with two genera of rhizobia. One genus of rhizobia (*Bradyrhizobium japonicum*), with a slow growth rate produces an alkaline reaction in mineral salts medium containing mannitol (Jordan 1984). Another genus of rhizobia, *Rhizobium fredii*, is a new group of fast-growing soybean rhizobia was identified by Keyser et al. (1982) from nodules of soybeans domesticated in China and the wild progenitor species of soybean (*Glycine soja* Sieb. and Zucc.). The rhizobia grow fast and produce an acid reaction in mineral salts medium containing mannitol. This fast-growing *R. fredii* (Jordan 1984) induces effective nitrogen-fixing symbio-

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sis in the cultivar Peking and does not nodulate common commercial cultivars of soybean (Keyser et al. 1982).

Nitrogen-fixing legumes can be classified into broad classes based on the nitrogen transport products (Schubert 1986); legumes that transport amides such as alfalfa, lupine, and pea, and legumes that transport ureides such as soybean, cowpea, and kidney bean. An amide-transporting legume forms generally an effective nitrogen-fixing symbiosis with fast-growing rhizobia. A ureide-transporting legume forms generally an effective nitrogen-fixing symbiosis with slow-growing rhizobia. However, the main nitrogenous compound transported in the xylem of soybean that can form effective symbiosis with the fast-growing *R. fredii* has not been identified yet.

This study aims to determine what are the main nitrogen transport products in N₂ fixation by the symbiosis between soybean and *R. fredii*.

However as indicated above, it has not been determined whether common commercial cultivars of soybean form effective nodules with *R. fredii*. Thus, in this study, Bragg and its hypernodulating mutant nts1116 (Carroll et al. 1985) belonging to non-Rj cultivars were used as host plants. These non-Rj cultivars do not harbor the nodulation-conditioning gene (Rj-gene) and do not restrict the nodulation with *Bradyrhizobium* strains (Ishizuka et al. 1991). The nodulation of nts1116 was more active than that of the wild variety Bragg (Gresshoff and Delves 1986; Haider et al. 1991). In addition, the nodule formation by nts1116 may not be suppressed by the mechanism of autoregulation (Delves et al. 1986). Therefore, it was expected that symbionts with a higher nitrogen fixation capacity could be obtained by using nts1116 compared with the wild variety Bragg. As fast-growing rhizobia, mixed inoculums from *R. fredii* USDA191, USDA193, and USDA194 were used because the compatibility between each strain of *R. fredii* and these host plants had not been confirmed. As slow-growing rhizobia, *B. japonicum* USDA110 which is compatible with these host plants was used.

Xylem sap was collected from nodulated soybean plants supplied with N-free nutrient solution from the vegetative stage to the pod-filling stage in a greenhouse. The xylem sap contained seasonal averages of 78 and 20% of total N as ureide-N and amino acid-N, respectively (McClure and Israel 1979). Streeter (1979) analyzed samples of stem exudate collected from field-grown soybean plants for ureides, amino acids, and nitrate throughout the reproductive development. He reported that ureide-N, allantoin, and allantoic acid, were the principal forms of nitrogen transported from nodulated roots to shoots of soybean plants. Moreover, it was shown that the ureide concentration in the exudate from the nodules of greenhouse-grown soybean plants was the highest at the early stages of reproductive development. Therefore, in this experiment conducted in a greenhouse, xylem sap was collected at the early reproductive stages, namely flowering and pod elongation stages. It was considered that the composition of the nitrogenous compounds in the xylem sap reflected the transport forms of fixed nitrogen.

**MATERIALS AND METHODS**

**Plants and rhizobia.** The soybean (*Glycine max* L. Merr.) wild type cultivar Bragg (nod⁺, fix⁺) and its nitrate-tolerant, hypernodulating mutant nts1116 (nod⁺⁺, fix⁺⁺) were used. Seeds of these varieties were harvested at Kyushu University Farm in 1991. *Bradyrhizobium japonicum* strain USDA110 and *Rhizobium fredii* strains USDA191, USDA193, and USDA194 were supplied by Harold Keyser, United States Department of Agriculture,
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Beltsville, Md. These strains were stored at 4°C on yeast extract mannitol agar. USDA110 was cultured in yeast extract mannitol broth (Vincent 1970) on a rotary shaker (100 rpm) at 30°C for 7 d and *R. fredii* strains for 3 d. The culture of USDA110 and the culture of *R. fredii* strains were diluted to one hundredth with one-fourth strength of modified Hoagland nutrient (MHN) solution (Nakano et al. 1997). These cell suspensions (about 10⁷ cells mL⁻¹ per each strain) were used as inoculums as described below.

**Plant growth.** Seeds were sterilized by immersion in a sodium hypochlorite solution (50 g kg⁻¹ as active chlorine) for 5 min, washed five times with ethanol, and rinsed five times in sterile deionized water. Six seeds were sown in a sterilized porcelainous pot containing 2.8 L of vermiculite and 1.4 L of MHN solution. The solution only contained 0.5 mM NH₄NO₃ as the nitrogen source. The pH was adjusted to 6.0. The pot was inoculated with 5 mL of cell suspension per seed and covered with aluminum foil to maintain sterile conditions on July 14, 1992. At 3 d after sowing (DAS), the aluminum foil covering the pots was removed. At 7 DAS, the seedlings were thinned to three plants. Plants were grown in a phytotron under a 12 h-25°C regime in the daytime and 12 h-20°C in the night, and 70% relative humidity under natural light. Sterilized deionized water was supplied according to the demand every 3 to 4 d from 7 to 20 DAS. Sterilized MHN solution and sterilized N-free MHN solution were supplied from 24 to 41 DAS and from 44 to 72 DAS (final sampling day), respectively.

**Xylem sap collection.** The soybean plants at the flowering (55 DAS) and pod elongation (72 DAS) stages were used for xylem sap collection and the measurement of the acetylene reduction activity (ARA). Two out of three plants per pot and three pots were used for xylem sap collection in each treatment and at each stage. The remaining plant per pot was used for ARA measurement after xylem sap collection. For the xylem sap collection, the soybean plants were decapitated in situ midway between the cotyledonary and primary leaf nod with pruning shears. The shoots were stored in an icebox until separation into plant parts. After 30 s, the cut end was wiped with a tissue paper and inserted in a silicon tube. After 2.5 h, the bleeding sap in the tube for each pot was collected with a 1-mL syringe into a 20-mL glass vial, and the volume was recorded. Collection of the bleeding sap was started around 10:30 and was completed at 14:00. The bleeding sap was stored on ice before transportation to the laboratory and diluted with sterilized water to obtain 10 mL. The diluted sap samples were stored in a freezer (−20°C) until analysis.

**ARA measurement.** The plant roots with vermiculite were pulled out from the porcelainous pot. Almost all of the vermiculite around the roots was removed gently to avoid the release of nodules. The roots were washed with tap water to clean up the surface of the roots and nodules. The soybean roots with intact nodules, which were not used for xylem sap collection, were severed at the cotyledonary node and individually placed in 200-mL conical flasks. The shoots were stored in an icebox until separation into plant parts. The other operating conditions were the same as those described by Haider et al. (1991) except that the incubation time with acetylene gas was 5 and 35 min.

After the xylem sap collection and ARA measurement, the plants were separated into leaf, petiole, stem, root, nodule, and flower or pod and lyophilized. The dry weight was measured and the nodule number was counted.

**N analysis.** The nitrogen concentration in the diluted sap samples was determined by the following methods.

Total-N: A volume of 2 mL of diluted sap sample was dried up at 110°C and digested according to the H₂SO₄-H₂O₂ Kjeldahl digestion method modified by Ohyama et al. (1991).
The digest was analyzed colorimetrically for ammonia according to the method of Cataldo et al. (1974).

Ammonia-N: Ammonium was determined colorimetrically by using the reagents of Cataldo et al. (1974). That is, 1 mL of diluted sap sample was placed in a test tube (150 × 15 mm diameter). Then, 2.5 mL of solutions C and D according to the method of Cataldo et al. described above were added and the tube contents mixed. After 1 h of reaction at room temperature, the absorbance was measured at 625 nm.

Amino-N: A volume of 0.5 mL of diluted sap sample was assayed colorimetrically by using the ninhydrin-hydridantin reagent according to the method of Moore and Stein (1954) except that the reagent solution was newly prepared without storage under a stream of nitrogen gas.

Amide-N: Amide-N in the diluted sap sample was determined based on the increase of the ammonia concentration by acid hydrolysis with H₂SO₄. For this analysis, 0.2 mL of diluted sap sample was used.

Ureide-N: Ureide-N was measured in relation to allantoin and allantoic acid by the slightly modified method of Ishizuka (1972). A volume of 0.5 mL of diluted sap sample was used for this analysis. All the reagents for this procedure were used as half volumes of the standard method.

Test of significance. Significant differences in the data between each symbiont were estimated by Duncan’s multiple-range test.

RESULTS AND DISCUSSION

Dry matter accumulation and nodulation

Dry matter accumulation in both soybean lines Bragg and nts1116 by the symbiosis with R. fredii was significantly lower than that by the symbiosis with B. japonicum (p = 0.05). At the flowering stage, Bragg and nts1116 inoculated with R. fredii accumulated 80 and 70% of dry matter compared with the plants inoculated with B. japonicum. At the pod elongation stage, the values were 75 and 66%, respectively (Fig. 1). Dry matter accumulation in both Bragg and nts1116 nodulated with R. fredii was significantly lower than that of the plants inoculated with B. japonicum. These results indicate that the nitrogen fixation activity in the symbiosis with fast-growing rhizobia was low compared with that in the symbiosis with slow-growing rhizobia.

Nodule dry weight per plant was not significantly different between the soybean plants inoculated with B. japonicum and R. fredii (Fig. 2A). Nodule number per plant inoculated with B. japonicum was not significantly different between both soybean cultivars and did

Fig. 1. Effect of inoculation of rhizobia on dry matter accumulation. Bj and Rf on the abscissa in this figure as well as in other figures are abbreviations for Bradyrhizobium japonicum and Rhizobium fredii, respectively. Flowering and pod elongation refer to the growth stages of soybean plants. Columns and bars on top indicate the mean and standard deviation of nine plants, respectively.
not change during the growth of the soybean plants (Fig. 2B). Nodule number per plant inoculated with *R. fredii* was significantly higher at the flowering stage than at the pod elongation stage (*p* = 0.05). That is, the nodule number induced by *R. fredii* decreased with the growth of the soybean plants, presumably due to the fact that soybean plants inoculated with rhizobium with a low compatibility formed a large number of ineffective nodules (Nakano et al. 1997). These results indicate that *R. fredii* displayed a lower compatibility with soybean cultivars in this study than USDA110. Dry weight per nodule of nts1116 inoculated with *R. fredii* at the pod elongation stage was significantly larger compared with all the other symbionts (*p* = 0.01) (Fig. 2C). The induction of large nodules is frequently observed when a small number of nodules are formed. In the symbiosis with *B. japonicum*, nts1116 formed 2 to 4 times more root nodules compared with Bragg (Gresshoff and Delves 1986; Haider et al. 1991). However, nodule formation in nts1116 and Bragg was not different in this experiment. Therefore, in long-term cultivation using a pot without a drain, the accumulation of either the nutrients which did not absorb by the soybean roots or the materials which were secreted from the soybean roots might have suppressed the formation of root nodule.

Determinate nodules were formed on the roots of soybean plants by *B. japonicum*, a slow-growing rhizobium and *R. fredii*, a fast-growing rhizobium. It remained to be determined which strain(s) of *R. fredii* could induce the nodulation of Bragg and nts1116.

**Acetylene reduction activity (ARA)**

ARA per plant and per nodule dry weight was not significantly different between both symbionts inoculated with *B. japonicum* and *R. fredii* in Bragg and nts1116 (Fig. 3).

Thus, ARA per plant or per nodule weight was not different between both soybean lines inoculated with either fast-growing or slow-growing rhizobia.

![Fig. 2. Effect of inoculation of rhizobia on nodule formation. Columns and bars on top indicate the mean and standard deviation of nine plants, respectively. A: Nodule dry weight (g) per plant. B: Nodule number per plant. C: Dry weight (mg) per nodule.](image-url)
Nitrogenous compounds in the xylem sap

The average flow rate of the xylem sap at the flowering stage was 327 and 460 μL plant⁻¹ h⁻¹ in Bragg and nts1116 inoculated with *B. japonicum*, while 46 and 140 μL plant⁻¹ h⁻¹ in the plants inoculated with *R. fredii*, respectively. The flow rate at the pod elongation stage was 287 and 307 μL plant⁻¹ h⁻¹ in the plants inoculated with *B. japonicum*, while 147 and 367 μL plant⁻¹ h⁻¹ in the plants inoculated with *R. fredii*, respectively. The flow rate of nts1116 was higher than that of Bragg. The flow rate of the plants inoculated with *B. japonicum* was higher than that of the plants inoculated with *R. fredii*, except for the symbiosis between nts1116 and *R. fredii* at the pod elongation stage.

At the flowering stage, the transport rate of nitrogenous compounds in the xylem sap was significantly higher in the soybean plants inoculated with *B. japonicum* than in those inoculated with *R. fredii* (p=0.05) (Fig. 4A). At the pod elongation stage, the transport rate in Bragg nodulated with *R. fredii* was significantly lower than that of the other symbionts (p=0.05). However, the main form of transported nitrogenous compound was obviously ureide in the xylem sap of both symbionts with fast-growing and slow-growing rhizobia.

The distribution of each nitrogenous compound in the xylem sap is shown in Fig. 4B. At the flowering stage, ureide-N accounted for 70 and 53% and subsequently amide-N accounted for 22 and 25% of the total N in the xylem sap from Bragg nodulated with *B. japonicum* and *R. fredii*, respectively. In the case of nts1116, ureide-N accounted for 66 and 66% and subsequently amide-N accounted for 27 and 20% of the total N in both symbionts with *B. japonicum* and *R. fredii*, respectively. Especially, the xylem sap of Bragg nodulated with fast-growing rhizobia contained a low level of ureide-N. This finding suggests that the N₂ fixation activity of the symbiont between Bragg and fast-growing rhizobia might be low during the early growth stages.

At the pod elongation stage, ureide-N accounted for 74 to 85% and subsequently amide-N accounted for 7 to 19% of the total N in the xylem sap from both symbionts with *B. japonicum* and *R. fredii*, respectively. The content of ureide-N plus amide-N was constant. The ratio of ureide-N in the xylem sap of nts1116 tended to be higher than that of Bragg. A similar trend was observed in the experiment on the comparison of the nodulation between Bragg and nts1116 by *B. japonicum* (Haider et al. 1991) and between the hyper-nodulation soybean mutant lines (NOD1-3, NOD2-4, NOD3-7) and their parent Williams (Sato et al. 1998) for unknown reasons. In both soybean lines, symbionts nodulated by *R. fredii* accounted for a high percentage of ureide-N. Especially, the ratio of ureide-N to total

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Fig. 3. Effect of inoculation of rhizobia on acetylene reduction activity. Columns and bars on top indicate the mean and standard deviation of three replicates, respectively.
N in the xylem sap of nts1116 nodulated by *R. fredii* was the highest of all the symbionts, presumably due to the nodule size (Fig. 2C). The ratio of ureide-N in the xylem sap increased with the growth of soybean plants.

In the symbiosis of rhizobium and legumes, N$_2$ is reduced to NH$_4^+$ in the bacteroid, and NH$_4^+$ is assimilated in the infection cells immediately. The fixed nitrogen is converted to ureides in the non-infected cells, and transported from root to shoot through the xylem. Purine for the synthesis of ureide is supplied by de novo synthesis (Ohyama and Kumazawa 1978) or by turnover of RNA and DNA (Fujihara and Yamaguchi 1978). Large application of nitrogen fertilizer to the nodulated soybean plants accelerated the synthesis of ureides (Hoshi et al. 1978). In the non-nodulating soybean isolate grown in the nutrient solution containing nitrate, the proportion of ureide-N in total N was low, amounting to 10% or less. Therefore, the main form of transported nitrogenous compound in the xylem sap, which was not associated with nitrogen fixation, was not ureide. In soybean plants, nitrogen fixation started at about 20 d after the infection by rhizobium, and the senescence of the nodules started at about 20 d after the onset of nitrogen fixation (Mellor 1988). That is, the senescence of the nodules may have already begun at about 40 DAS at the vegetative stage. However, in this experiment, nodule decay could not be recognized. Therefore, transport of nitrogen in the xylem sap of the soybean cultivars used in this experiment did not occur as a result of the decomposition of proteins and other components in the nodules, and fixed nitrogen was considered to consist of nitrogenous solutes in the xylem sap.

The results obtained in this experiment suggest that the main form of transported nitrogenous compound of fixed nitrogen in the symbiont between soybean plant and *R. fredii* consists of ureides.
REFERENCES


