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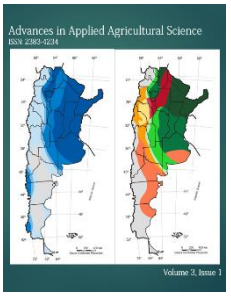

Research Article

Microbiological and soil analysis of the growth-promotion effect of hairy vetch on velvet bean

Shin Okazaki [1*], Kun Yuan [1], Maki Iizuka [1], Michiko Yasuda [1], Yosei Oikawa [1], Sonoko Dorothea Bellingrath-Kimura [1] and Yoshiharu Fujii [1]

[1] *Graduate School of Agriculture, Department of International Environmental and Agricultural Science, Tokyo University of Agriculture and Technology, Japan.*

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[*] Corresponding Author;
E. Mail: sokazaki@cc.tuat.ac.jp
Tel and Fax: +81-42-367-5847

ABSTRACT

Hairy vetch (*Vicia villosa* Roth) is a leguminous plant that is widely used as a green manure and a cover crop. Recent observations have shown that previous hairy vetch cultivation on a plot promotes the subsequent growth of velvet bean (*Mucuna pruriens*). Here we investigated soil nutrients and microorganisms in an attempt to identify the factors responsible for the growth-promotion effect of hairy vetch on velvet bean. Field experiments showed that the dry weight of velvet bean plants was six times heavier in plots that had previously been sown with hairy vetch than in previously unplanted control plots. Soil analysis revealed that the concentrations of nitrate and ammonium nitrogen (N) in hairy vetch plots were up to 20% and 10% higher, respectively, than those in control plots. The root-nodule bacteria (rhizobia) isolated from the hairy vetch nodules were all closely related to *Rhizobium leguminosarum*, whereas those isolated from velvet bean nodules were *Bradyrhizobium* species in both types of plot. There was no significant difference in the infection rate of arbuscular mycorrhizal fungi in velvet bean roots between the two types of plot. These findings suggest that the growth-promoting effect of hairy vetch on velvet bean is not caused by the propagation of symbiotic microorganisms, but rather is due to other factors including an increase in soil nutrients.

Hairy vetch (*Vicia villosa* Roth) is a leguminous plant that is utilized as a green manure and a cover crop around the world. It can grow at temperatures as low as –20 °C, tolerates a wide range of soil pH values, and provides as much as 20–25 kg N per 10 a to the soil (Hoffman *et al.*, 1993; Fujii, 2001). As well as acting as a green manure, hairy vetch efficiently suppresses weed growth when it is used as a cover crop (Teasdale *et al.*, 1991). Recently, cyanamides were identified as the allelochemicals responsible for the weed-suppressing effects (kamo *et al.*, 2003). Cyanamides break down into urea after about 1 week in the soil, providing a N source for plants. These favorable characteristics make hairy vetch a valuable green manure and cover crop.

Hairy vetch has been reported to show growth-promoting effects in combination with several crops including maize, sorghum, and rice. Ebelhar *et al.* (1984) reported that the average yields of corn following hairy vetch cultivation with no N fertilizer were about 2.5 Mg ha^{-1} more than those following cultivation with corn residue or a rye cover crop (Ebelhar *et al.*, 1984). The authors estimated that hairy vetch supplied N equivalent to approximately 90–100 kg ha^{-1} of N fertilizer annually to the corn. These growth-promotion effects are dependent on the nutrient demands of the subsequent crops, as well as the decomposition efficiency of the hairy vetch plant residues in the soil.

Soil microorganisms can affect the growth of various crops. Well-known plant growth-promoting microorganisms include root-nodule bacteria or rhizobia. Leguminous plants such as hairy vetch form root-nodule symbioses with rhizobia in the soil. The symbiotic rhizobia in the root nodules fix atmospheric N_2 into ammonia, which is then exploited by the host plants. Nitrogen is often a limiting factor for plant growth; hence, N_2 fixation by symbiotic rhizobia can greatly affect the growth and yield of leguminous crops. The cultivation of leguminous plants enhances the populations of their symbiotic rhizobia, which could potentially benefit subsequent leguminous crops grown on the same plots. However, so far, little is known about the propagation of symbiotic bacteria via the successive cultivation of leguminous crops including hairy vetch.

Velvet bean (*Mucuna pruriens*) is an annual herbaceous legume that is cultivated widely in Africa and Southeast Asia. It is used in food, livestock feed, and pharmaceutical products, especially in developing countries. It is also employed extensively as a green manure for improving soil fertility and controlling weeds in the savanna of West Africa (Akobundu, 1987; Versteeg and Koudokpon, 1990). Velvet bean contains important raw materials that are used in both Ayurvedic and folk medicine. Among these is L-DOPA, a precursor to the neurotransmitter dopamine, which has been used for the management and treatment of Parkinson's disease (Katzenschlager *et al*., 2004; Tharakan *et al*., 2007). It is therefore a valuable source of income for farmers in South Asia and West Africa, and inexpensive means of cultivation are needed for developing countries.

Plant growth-promoting microbes, such as rhizobia and arbuscular mycorrhizal (AM) fungi, are an important part of cost-effective cropping systems in many countries. Velvet bean has been assumed to acquire N from symbiotic rhizobia via root nodules, and phosphate from AM-fungi associated with roots; however, in practice, it is often not effectively nodulated, and so depends largely on soil available N. Indeed, Sanginga *et al*. (1996) reported that velvet bean did not form nodules in 21% of farmer's fields in Benin and West Africa (Sanginga *et al*., 1996). Inoculation of compatible rhizobia appears to be the most promising approach for enhancing symbiotic N_2 fixation. Some researchers have suggested that velvet bean symbiotic rhizobia are fast-growing, acid-producing strains (Kumar *et al*., 2006; Aparecida de Lima, 2012); however, data on these species remain sparse, and considerable research remains necessary to establish effective inoculation practices for velvet bean.

We recently observed that hairy vetch cultivation promoted the subsequent growth of velvet bean. Two explanations were considered for this growth-promoting effect: first, that decomposition of hairy vetch increased the availability of soil nutrients that were utilized by velvet bean; and second, that hairy vetch cultivation increased the number of symbiotic microorganisms that

also benefitted velvet bean. The symbiotic rhizobia for hairy vetch have been reported as *R. leguminisarum* and *R. etli* (Spaink 1998); however, the corresponding species in velvet bean have not yet been characterized, and it remains unclear whether they include *R. leguminisarum* and *R. etli*.

Here we examined the role of hairy vetch in promoting growth in velvet bean, from the viewpoints of soil nutrition and symbiotic microorganisms. Elucidating the mechanism by which hairy vetch promotes velvet bean growth could be useful for developing simple and inexpensive cropping systems for use in developing countries.

Materials and Methods

Field experiment

Two plots (15 m × 5 m) were prepared in the experimental fields of Tokyo University of Agriculture and Technology (35 °N, 139 °E) in November 2012. There was no known history of cultivation of hairy vetch or velvet bean on these plots. Hairy vetch was sown in one plot (hairy vetch plot), and the other was left untouched (control plot). Velvet bean plants were grown in a nursery for 2 months, and then transplanted to the sites on 17st June 2013. The plants were spaced at 0.8-m intervals in rows that were positioned 1.5-m apart. Velvet bean plants were sampled 60 days after transplantation on 19st August 2013, and the plant dry weight, shoot length, leaf number, nodule number and nodule weights were measured. In total, eight velvet bean plants were sampled from the control plot and seven from the hairy vetch plot. Data were analyzed using the Student's *t*-test.

Soil analysis

Soil samples were taken 10 days after the velvet bean plants had been transplanted, in 28th June 2013. The soil was sampled (at a depth of 10 cm) from five locations in the control plots and the hairy vetch plots using a diagonal line-sampling method. The soil was air dried for 1 week and then filtered using a 5-mm sieve. Total N and carbon were analyzed using an NC analyzer (Sumigraph NC-22F; Sumika Chemical Analysis Service, Tokyo, Japan). To measure the available N, 10 g dried soil was resuspended with 50 ml distilled water, and then filtered (No. 6 filter paper; Advantec MFS, Inc. CA, USA). The nitrate N in the soil suspension was measured by ultraviolet spectroscopy (UVmini-1240; Shimadzu Co., Kyoto, Japan) at an absorbance wavelength of 220 nm. The ammonium N in the soil suspension was measured by the indophenol method. The color was measured at an absorbance wavelength of 635 nm (UVmini-1240). Phosphate and potassium were measured using an ICP spectrometer (iCAP6000; Thermo Fisher Scientific, MA, USA). In total, six measurements were performed, and data was analyzed using the Student's *t*-test at a significance level of 5%.

Soil *microorganisms*

Rhizobia were isolated from the nodules of hairy vetch and velvet bean plants taken from both types of plot. Nodules were surface sterilized with 70% ethanol for 30 s and 1% sodium hypochlorite for 30 s, and then rinsed three times in sterile water. The nodules were crushed in 0.5 ml of 0.9% sodium chloride, and the suspension was streaked on Rhizobium-defined medium (RDM) agar plates (Ronson and Primrose 1979). Colonies that appeared after incubation for 3–7 days at 28 °C were grown in a 2-ml liquid culture, and genomic DNA was extracted using a Wizard Genomic DNA Purification kit

(Promega, WI, USA). The 16S ribosomal RNA (rRNA) genes were amplified by the polymerase chain reaction (PCR), using 10 ng purified DNA, LA taq (Takara, Tokyo, Japan), and primers 16AF (5'-AACTGAAGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') in 25 µl reaction liquid for 30 cycles (94 °C for 1 min, 98 °C for 10 s, and 68 °C for 1.5 min), followed by a final stage at 72 °C for 10 min. The PCR products were purified, and the nucleotide sequence was determined using the 16AF and 1492R primers. The most closely related species was identified by a BLAST search against the National Center for Biotechnology Information (NCBI) database. DNA sequences were deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB931130 to AB931156. A phylogenetic analysis was performed by comparing the 16S rRNA sequences. The sequences were aligned by using the CLUSTAL W program and neighbor-joining tree were constructed by using MEGA version 5.2 software (Tamura *et al.*, 2011). One thousand bootstrap replicates were used to generate a consensus tree.

In order to examine the infection of mycorrhizal fungi, velvet bean roots were cut into 1-cm pieces, immersed in 10% KOH, and heated in boiling water for 15–30 min until they were discolored. The roots were then washed with water and immersed in 5% HCl to neutralize the pH. After washing in distilled water, the roots were immersed in 0.05% tryptan blue lactic acid, and stained by heating in boiling water for 15 min. Stained roots were examined under a light microscope, and the colonization rates of mycorrhizal fungi, vesicles, and arbuscules were counted. In total, 50 roots were observed from five plants at each site (a total of 250 samples).

Results and Discussion

Growth promotion of velvet bean by hairy vetch

Velvet bean plants were grown in both types of plot, and sampled 60 days after cultivation. The plant dry weight, shoot length, and leaf number for the velvet bean plants grown in the hairy vetch plot were significantly higher than those in the control plot (Fig. 1a–c). The plant dry weight was increased by about six fold in the hairy vetch plot, probably due to the drastic increase in leaf number.

Conversely, the nodule numbers and nodule weights of the velvet bean plants were significantly decreased in the hairy vetch plot compared with the control plot (Fig. 1d and e). These results indicated that hairy vetch cultivation did not increase the nodulation of subsequent velvet bean plants. This contradicted a previous observation by Sato *et al.* (2007) that prior hairy vetch cultivation significantly increased the nodule numbers and N_2 fixation activity of subsequent soybean plants (Sato *et al*. 2007). This inconsistency is probably due to the differences in soil type of the fields studied. Sato *et al*. conducted their experiment in a field with heavy-clay soil converted from a paddy field, which had saturated soil with low permeability. When hairy vetch was grown, its roots extended deeply into the clay soil and improved the soil structure and water retention. The hairy vetch also absorbed and transpired excessive moisture in the soil, promoting soil dryness and expanding the oxide layer. These factors led to an increase in the number of root nodules and an improvement in N_2 fixing activity. In the present study, however, the experimental field had an andosol soil with moderate water permeability and water retention, which did not cause moisture damage to the velvet bean plants. The reduced nodule formation in the hairy vetch plot might have been due to the higher

Table 1. *Selected soil characteristics in control and hairy vetch plots.*

Parameters	Control plot	Hairy vetch plot
Total N (g/kg)	5.3 ± 0.1	5.0 ± 0.2
Total C (g/kg)	73.2 ± 1.4	67.3 ± 1.5
C/N ratio	13.7	13.4
Nitrate nitrogen (mg/L)	62.9 ± 3.8	74.6 ± 1.4 *
Ammonium nitrogen (mg/L)	0.70 ± 0.03	0.76 ± 0.02
Available phosphate (mg/kg)	6.6 ± 0.2	6.1 ± 0.2
Potassium (mg/kg)	4.1 ± 0.4	4.6 ± 0.3

*Asterisks denote significant differences between control plot and hairy vetch plot (Student's t-test, $P < 0.05$).

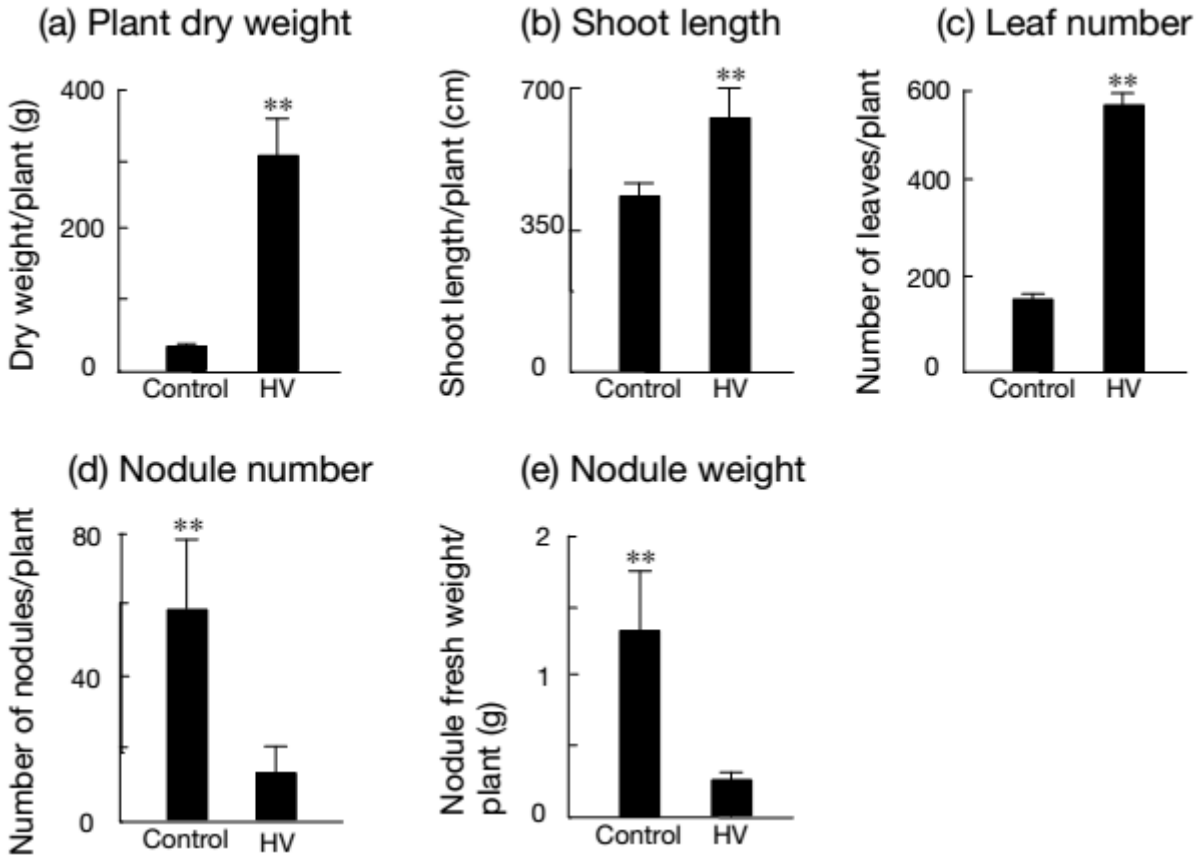

Fig. 1. Effect of hairy vetch cultivation on growth and nodulation of subsequent velvet bean. Velvet bean plants were sampled from the hairy vetch plot (n=7) and an unplanted control plot (n=8) 60 days after cultivation. Data are presented as mean ± standard deviation (s.d.). Statistical analyses (Student's *t*-test) were performed to compare each mutant with the wild-type strain. A single asterisk (*) represents $P < 0.05$ and double asterisks (**) represent $P < 0.01$.

amount of available N (see below) in the soil, as nodule formation by legumes is generally inhibited by the available N (Carrol. *et al*, 1990).

Soil analysis

Soil samples were taken 10 days after the velvet bean plants were transplanted, and the soil properties were compared between control and hairy vetch plots. The total N and total carbon concentrations did not significantly differ between the two types of plot (Table 1). The concentration of nitrate N in the hairy vetch plot was significantly higher than in the control plot, and the hairy vetch plot showed a slightly higher ammonium N level, although the difference was not statistically significant. The soil C/N ratio of the control plot (13.7) was slightly higher than that of the hairy vetch plot (13.4), suggesting that the organic N in hairy vetch was converted to inorganic forms. A low C/N ratio suggests that microorganisms rapidly convert organic N to inorganic compounds, which could explain why the nitrate N and ammonium N levels were higher in the hairy vetch plot. The concentrations of available phosphate and potassium in the soils were not significantly different between the control and hairy vetch plots, suggesting that hairy vetch cultivation had a limited effect on these nutrients under our field conditions.

Symbiotic microorganisms

Rhizobia were isolated from the nodules formed on the roots of hairy vetch and velvet bean plants, respectively. In total, 14 strains were isolated from hairy vetch, and eight and five strains were isolated from velvet beans in the control plot and hairy vetch plot, respectively (Table 2). The 16S rRNA gene sequence analysis revealed that all of the bacteria isolated from the hairy vetch root nodules were closely related to *Rhizobium leguminosarum* (Table 2). The 16S rRNA sequences of each strain exhibited 98% or higher similarity, indicating limited genetic diversity in the field. By contrast, the bacteria isolated from the velvet bean root nodules in both control and hairy vetch plots were identified as *Bradyrhizobium* species (Table 2). Relatively slow growth speed and absence of acid production in RDM medium also indicated the isolates belong to *Bradyrhizobium.* These results suggested that it was unlikely that the hairy vetch increased the numbers of rhizobia that could nodulate and promote the growth of subsequent velvet bean plants.

The 16S rRNA genes of the *Bradyrhizobium* species isolated from velvet bean were relatively divergent. Phylogenetic analysis revealed that they are grouped with *B. cytisi*, *B. elkanii*, and *B. japonicum* (Fig. 2). So far, little is known about velvet bean rhizobia, and no studies have been reported in Japanese soils. Kumar *et al.* (2006) reported that rhizobia isolated from velvet bean nodules in India were identified as *Sinorhizobium* species by physiological tests and amplified 16S rDNA restriction analysis (Kumar *et al.*, 2006). Aparecida de Lima *et al.* reported that isolates from *Mucuna pruriens* in Brazil grew rapidly and acidified the culture medium, suggesting that they were closely related to *Sinorhizobium* or *Rhizobium* species (Aparecida de Lima *et al.*, 2012). By contrast, the present study identified *Bradyrhizobium* species as symbiotic rhizobia for velvet bean. *B. elkanii* and *B. japonicum* are well known soybean symbionts and has not been reported as velvet bean rhizobia. *B. cytisi* was reported as a symbiotic rhizobium of *Cytisus villosus*, which is a perennial shrub of the Fabaceae family that is native to northern Africa and widely distributed in the Mediterranean Basin (Chahboune *et al.*, 2011). Our present study in Japan is

Table 2. *Root nodule isolates of hairy vetch and velvet bean.*

Plant origin	Isolates	Growth speed		Acid productivity	Closest relative based on 16S rRNA gene sequence	DDBJ accession
		2 days	7 days			
Hairy vetch	HV2	+	+	+	*Rhizobium leguminosarum*	AB931130
	HV6	+	+	+	*Rhizobium leguminosarum*	AB931131
	HV7	+	+	+	*Rhizobium leguminosarum*	AB931132
	HV8	+	+	+	*Rhizobium leguminosarum*	AB931133
	HV9	+	+	+	*Rhizobium leguminosarum*	AB931134
	HV10	+	+	+	*Rhizobium leguminosarum*	AB931135
	HV11	+	+	+	*Rhizobium leguminosarum*	AB931136
	HV12	+	+	+	*Rhizobium leguminosarum*	AB931137
	HV13	+	+	+	*Rhizobium leguminosarum*	AB931138
	HV14	+	+	+	*Rhizobium leguminosarum*	AB931139
	HV15	+	+	+	*Rhizobium leguminosarum*	AB931140
	HV16	+	+	+	*Rhizobium leguminosarum*	AB931141
	HV17	+	+	+	*Rhizobium leguminosarum*	AB931142
	HV22	+	+	+	*Rhizobium leguminosarum*	AB931143
Velvet bean (control plot)	MC3	-	+	-	*Bradyrhizobium cytisi*	AB931144
	MC8	-	+	-	*Bradyrhizobium* sp.	AB931145
	MC9	-	+	-	*Bradyrhizobium* sp.	AB931146
	MC10	-	+	-	*Bradyrhizobium elkanii*	AB931147
	MC11	-	+	-	*Bradyrhizobium cytisi*	AB931148
	MC12	-	+	-	*Bradyrhizobium* sp.	AB931149
	MC5	-	+	-	*Bradyrhizobium elkanii*	AB931150
	MC13	-	+	-	*Bradyrhizobium japonicum*	AB931151
Velvet bean (hairy vetch plot)	MH3	-	+	-	*Bradyrhizobium elkanii*	AB931152
	MH4	-	+	-	*Bradyrhizobium cytisi*	AB931153
	MH6	-	+	-	*Bradyrhizobium cytisi*	AB931154
	MH9	-	+	-	*Bradyrhizobium cytisi*	AB931155
	MH10	-	+	-	*Bradyrhizobium japonicum*	AB931156

Table 3. *Colonization rate of mycorrhizal fungi in velvet bean roots grown in control and hairy vetch plots.*

	Control plot (n=5)	Hairy vetch plot (n=5)
Colonization rate (%)	84.8	85.6
Vesicles (%)	62.8	49.6
Arbuscules (%)	65.2	56.8

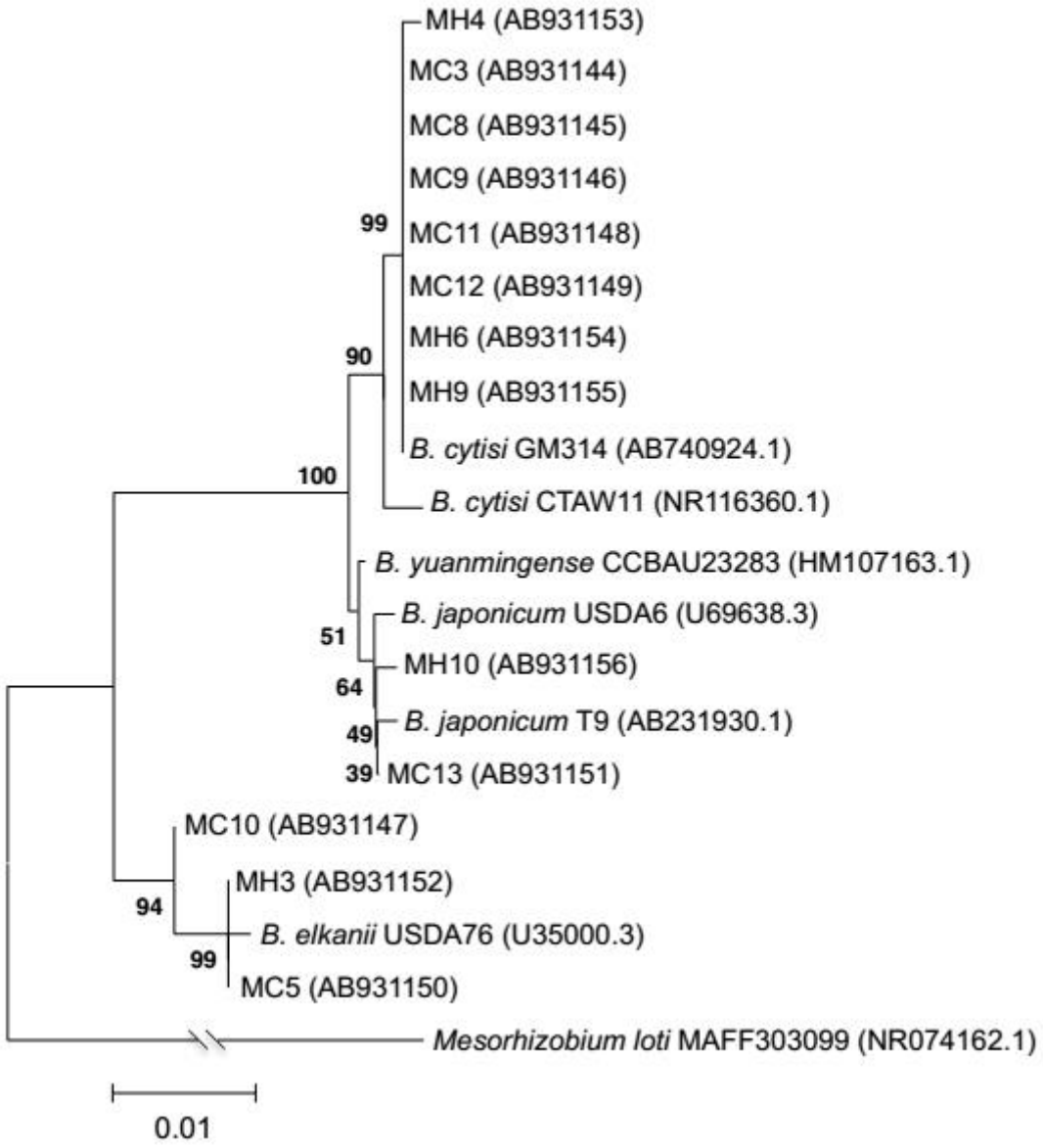

Fig. 2. Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences of strains from nodules of *M. pruriens*. Bootstrap values are indicated as percentages derived from 1000 replications.

the first report that *B. cytisi* was isolated as a velvet bean rhizobium.

We also examined the colonization of AM fungi in velvet bean roots grown in control plots and hairy vetch plots (Table 3). In both sites, the colonization rate of AM fungi in velvet bean roots was approximately 85%, and there was no significant difference between sites, suggesting that this is unlikely to be a factor in the growth-promotion effect. However, differences were observed in the numbers of symbiotic structures, such as vesicles and arbuscules. AM fungi form different numbers of symbiotic structures depending on the species. It is possible that sowing hairy vetch altered the AM fungal flora in the soil, such that different species infected the

velvet bean plants in different types of plot. Further studies will be required to identify the AM fungi species that interact with both hairy vetch and velvet bean.

In conclusion, we revealed that prior planting of hairy vetch strongly promoted growth of velvet bean plants compared with unplanted control plots. We also identified the velvet bean rhizobium in Japan including *B. cytisi* for the first time in the world. Soil available nitrate and ammonium N levels were increased by hairy vetch cultivation, whereas symbiotic microorganisms did not appear to be propagated. These results suggest that the growth promotion effect of velvet bean by hairy vetch was likely associated with the availability of N in the soil. However, the increases of available N levels were limited and did not appear adequate to explain the growth-promotion mechanism. Further studies are needed to explore other related factors including weed-suppression effects and soil-structure improvement, which will provide insights into the growth-promotion effect of hairy vetch, and help to develop sustainable cultivation techniques for velvet bean and other crops.

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