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Symbiotic Potential, Competitiveness, and Serological Properties of *Bradyrhizobium japonicum* Indigenous to Korean Soils†

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The symbiotic potential of *Bradyrhizobium japonicum* isolates indigenous to seven Korean soils was evaluated by inoculating soybeans with 10- and 1,000-fold-diluted soil suspensions (whole-soil inocula). At both levels, significant differences in the symbiotic potential of the indigenous *B. japonicum* isolates were demonstrated. The relationship between rhizobial numbers in the whole-soil inocula (x) and nitrogen fixation parameters (y) was best predicted by a straight line ($y = a + bx$) when the numbers in the inocula were 100 to 10,000 ml^{-1}, while the power curve ($y = ax^b$) predicted the variation when the numbers were 1 to 100 ml^{-1}. Thirty isolates from three soils showed wide differences in effectiveness (measured as milligrams of shoot N per plant), and several were of equal or greater effectiveness than reference strain *B. japonicum* USDA 110 on soybean cultivars Clark and Jangbaekkong. On both of the soybean cultivars grown in a Hawaiian mollisol, the Korean *B. japonicum* isolate YCK 213 and USDA 110 were of equal effectiveness; USDA 110 was the superior strain in colonization (nodule occupancy). Korean isolates YCK 117 and YCK 141 were superior colonizers compared with USDA 110. However, *B. japonicum* USDA 123 was the superior colonizer compared with isolates YCK 213, YCK 141, and YCK 117. In an immunoblot analysis of 97 indigenous Korean isolates of *B. japonicum*, 41% fell into the USDA 110 and USDA 123 serogroups. Serogroups USDA 110 and USDA 123 were represented in six of the seven soils examined. In one Korean soil, 100% of the *B. japonicum* isolates reacted only with antisera of YCK 117, an isolate from the same soil.

The source of rhizobial strains for testing in a strain selection program can range from local isolates to strains already tested in other parts of the region or country to cultures from various overseas collections or to isolates from a "center(s) of diversity" of the intended test legume (33). The center(s) of diversity which is also as a "gene center(s)" harbors valuable genetic material for symbiotic nitrogen fixation both of the host legume and rhizobia (23). The value of gene centers for rhizobial germ plasm is evidenced by the discovery of highly specialized strains of *Rhizobium leguminosarum* bv. viceae which nodulate the pea (*Pisum sativum*) (22) and the fast-growing, soybean-nodulating rhizobia (19) now classified as *Sinorhizobium fredii* (7) from China which is one of the gene centers for the soybean (18, 37).

The domesticated soybean (*Glycine max*) is believed to be a cultigen of the wild soybean (*Glycine usurriensis*) and has a long history of domestication in Korea, China, Japan, the USSR, and Taiwan (15). In Korea, the soybean has been known to be cultivated for hundreds of years and is known locally as the "meat of the upland" because of its historical and present-day significance in the diet of the Korean people. Although nodulation of soybean occurs without inoculation in most Korean soils, the extent of variability in the symbiotic potential of the indigenous *Bradyrhizobium japonicum* populations is not known.

By using the plant infection method (11), Bonish (3) made visual assessments of nitrogen fixation on white clover plants inoculated with increasing dilutions of soil suspensions. This approach, termed the whole-soil inoculation method by Brockwell et al. (4), was recently evaluated as an expeditious assay to determine the symbiotic potential of soil rhizobial populations for subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*).

In this article we report the application of the whole-soil inoculation method to examine the symbiotic potential of the indigenous population of soybean rhizobia from seven Korean soils and the isolation of strains that are highly effective and superior colonizers with distinctive serological properties.

MATERIALS AND METHODS

Collection of soils and MPN measurements. Soils were sampled during winter in different upland regions of two counties in the southeastern part of South Korea. At the time of sampling, the top 2 cm of the soil was icy and was removed before the soil was collected to a depth of 20 cm by use of a sterile hand shovel. Soil was sampled along a 28-m line transect with 1 m between samples. Twenty to 25 samples were made along the transect and pooled in clean plastic bags. Soil samples were later passed through a 5-mm screen and stored at 4°C until used. At each new collection site, the shovel was rinsed clean with water, dried with paper towels, and sprayed with alcohol and flamed to sterilize it prior to soil collection. Soil characteristics and collection sites are shown in Table 1.

To determine the abundance of indigenous *B. japonicum*, a plant infection most-probable-number (MPN) enumeration was done by using plastic pouches (35). Korean soybean (*G. max* cv. Jangbaekkong) seeds were surface sterilized and germinated in moistened sterile vermiculite. Germinated seeds were planted in plastic growth pouches. Soil samples were thoroughly mixed, and 10 g of soil (dry-weight basis, 110°C) was shaken (wrist-action shaker) in 90 ml of sterile water for 15 min. Tenfold serial dilutions were prepared, and

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TABLE 1. Location, characteristics, and size of associated indigenous populations of *B. japonicum* of seven Korean soils

Soil sample no. (location)	Soil type	pH (diluted 1:5 in H_2O)	Soil use	No. of *B. japonicum*[a]
3 (Bubuk, Milyang)	Typic hapludult	4.5	Uncultivated	3.0
4 (Sangnam, Milyang)	Dystric fluventic eutrachrept	5.4	Uncultivated	4.8
6 (Dong, Uichang)	Typic hapludult	4.7	Cultivated	4.8
7 (Chinbuk, Uichang)	Typic dystrochrept	5.1	Cultivated	4.8
8 (Bubuk, Milyang)	Typic hapludult	5.8	Cultivated	4.0
9 (Sangnam, Milyang)	Dystric fluventic eutrachrept	4.7	Cultivated	4.0
10 (Chinbuk, Uichang)	Dystric fluventic eutrachrept	5.8	Cultivated	5.0

[a] Population size ($\log_{10}$ g of soil^{-1}) estimated by the MPN plant infection technique by using soybean cultivar Jangbaekkong as trap host.

1.0-ml aliquots of the appropriate dilutions were inoculated onto the roots of 1-week-old soybean seedlings. Each inoculation treatment was set up in quadruplicate. Plants were grown in a growth room, and MPN estimations based on nodulation were determined 3 weeks after inoculation.

Isolation and authentication of *B. japonicum* strains. Isolates of *B. japonicum* were made from nodules on soybean plants set up for MPN determinations. Nodules were randomly selected from nodulated plants at the lowest and highest dilutions of the MPN series of the seven soils (Table 1). Standard isolation, purification, and authentication procedures were followed (31, 34) for obtaining pure cultures of *B. japonicum* isolates. Pure cultures were maintained on yeast mannitol agar slants (34) at 4°C. All Korean isolates of *B. japonicum* were given a YCK (Yeongnam Crop Experiment Station, South Korea) prefix and a number for identification of isolates.

Assessment of symbiotic potential by using the whole-soil inoculation method. The effectiveness of *B. japonicum* isolates indigenous to each Korean soil was tested on soybean cultivar Jangbaekkong. Modified Leonard bottle-jar assemblies (34), in which horticultural-grade vermiculite was used as a rooting medium, and an N-free nutrient solution (5) for irrigation were used to grow the soybean plants. Suspensions (10-fold dilutions) of the seven soils were used as whole-soil inocula (3, 4). The whole-soil inocula were prepared by suspending 10 g of soil (dry-weight basis, 110°C) in 90 ml of sterile water. Four pregerminated soybean seeds were planted in each jar, and each seed was inoculated with 1 ml of the whole-soil inoculum. Only 10^{-1} and 10^{-3} dilutions of the soil were used as whole-soil inocula. These dilutions were selected for evaluation on the basis of the MPN determinations of the soils done earlier. It was hypothesized that the two dilutions would cause significant changes in the numbers of rhizobia and diversity to demonstrate differences in the symbiotic potential. To compare the levels of effectiveness of the indigenous rhizobia in the whole-soil inocula, soybean seeds were planted and inoculated with peat inoculant water suspensions containing standard *B. japonicum* strains (USDA 110, USDA 138, and CB 1809) of known effectiveness (30). The peat inoculant of the standard strains was prepared as described elsewhere (31) and contained 4.4×10^9 viable cells g^{-1}. Ten- and 1,000-fold dilutions of the peat inoculant were used to inoculate the soybeans. Uninoculated controls were also included. Each treatment was set up in quadruplicate. At day 7, plants were thinned to two per jar. The experiment was terminated at 5 weeks. At harvest, the plants were assayed for nitrogenase activity (acetylene reduction) as described previously (32). Root systems were later washed free of vermiculite, and nodules were picked for counting and dry-weight determination. Plant tops were oven dried (70°C for 48 h), weighed, and later ground for total N determination (24).

Effectiveness of individual isolates of *B. japonicum*. Thirty isolates of *B. japonicum* made from the three soils (soils 4, 6, and 10) were screened for effectiveness on soybean cultivars Clark and Jangbaekkong grown in Leonard jars. *B. japonicum* strains were grown in yeast mannitol broth (YMB) medium (34). Four germinated seeds were planted per jar as described above, and each was inoculated with 1.0 ml of the broth culture of the appropriate strain. Uninoculated controls and standard strain (*B. japonicum* USDA 110) treatments were included. The experiment was a randomized complete block design with four replications per treatment. The experiment was set up in a naturally illuminated greenhouse and was terminated at 35 days. The photosynthetically active radiation in the greenhouse was 1,400 to 1,600 microeinsteins $(m^2)^{-1}$ s^{-1}. The maximum day temperatures in the Leonard jars and potted soil were 28 to 30°C. At harvest, nodule and shoot dry weights and shoot total N were determined as described earlier.

Superiority of Korean *B. japonicum*. Of the 30 *B. japonicum* isolates screened for effectiveness on the two soybean cultivars, YCK 117, YCK 141, and YCK 213 (Fig. 1) were selected for competition against American *B. japonicum* strains USDA 110 and USDA 123 in nodulation. The three Korean isolates were selected on the basis of effectiveness (milligrams of shoot N per plant) and absence of serological cross-reactions by using fluorescent antibodies of USDA 110 and USDA 123. The *B. japonicum* isolates compared were as follows: USDA 110 versus YCK 117; USDA 110 versus YCK 141; USDA 110 versus YCK 213; USDA 123 versus YCK 117; USDA 123 versus YCK 141; and USDA 123 versus YCK 213. Competing rhizobia were cultured individually in YMB medium for 6 days on a rotary shaker. Populations of the fully grown culture were determined by direct counting with a Helber counting chamber (31). Mixtures containing equal numbers of the competing rhizobia (in 25% YMB medium) were prepared by using data from the direct counts. The mixed strain inoculum, containing 3×10^8 cells ml^{-1}, was inoculated at the rate of 1 ml seed^{-1} at planting.

Competition studies were done with inoculated soybean plants grown in a mollisol (Torroxic Haplustoll), Keahua series (pH 6.8, 0.3% total N). Black plastic pots (3 liter) were filled with 2.7 kg (oven-dry basis) of sieved soil. Soil nitrogen was immobilized by incorporation of finely milled sugarcane bagasse (26) at the rate of 10 g kg of soil^{-1}. The procedure of Singleton et al. (29) was followed for adjusting the soil moisture level and for macro- and micronutrient addition. Four seeds of the appropriate soybean cultivar were planted per pot and inoculated immediately. All treatments were set

TABLE 2. Symbiotic potential of native Korean *B. japonicum* populations in a 10-fold-diluted (10^{-1}) whole-soil inoculum inoculated on *G. max* cv. Jangbaekkong

Soil sample	No. of rhizobia (log_{10})[a]	No. of nodules per plant	Nodule dry wt (g) per plant	Shoot dry wt (g) per plant	Shoot total N (mg) per plant	Nitrogenase activity[b]
Soil no.						
3	2.0	12	0.04	0.49	3.9	0.4
4	3.8	34	0.34	1.64	46.0	9.7
6	3.8	40	0.31	1.92	59.6	15.2
7	3.8	35	0.28	1.67	49.0	15.3
8	3.0	33	0.25	1.60	47.7	12.1
9	3.0	21	0.21	1.41	43.3	14.1
10	4.0	40	0.34	2.44	74.3	13.9
Peat culture[c]	7.8	19	0.13	1.51	36.7	6.6
Uninoculated		0	0	0.51	5.3	0
LSD[d] (0.05)		7	0.05	0.47	17.7	4.23

[a] Number of *B. japonicum* ml of diluted whole-soil or peat inoculum^{-1}. Original populations are given in Table 1.
[b] Micromoles of C_2H_4 plant^{-1} h^{-1}.
[c] Peat inoculant contained equal numbers of strains USDA 110, USDA 138, and CB 1809.
[d] LSD, Least significant difference.

up in triplicate. Plants were thinned to two per pot at day 7 after planting. At harvest (day 30), nodule and shoot dry weights (oven dried at 70°C, 48 h) and total shoot N were determined. Nodule occupancy by the various strains was determined by using the oven-dried nodules (previously used in dry-weight determination) and the immunoblot procedure.

Antigen and antisera preparation. Preparation of antigen preparation and production of antisera and fluorescent-antibody were done according to procedures described elsewhere (27, 31). Cells were washed three times in 0.85% saline to remove soluble antigens. Antisera and fluorescent antibody were produced for *B. japonicum* USDA 110 and USDA 123 and Korean isolates YCK 117, YCK 141, YCK 150, and YCK 213.

Strain identification by immunoblot. The oven-dried nodules from the competition experiment were soaked (overnight at 4°C) in 0.2 ml of distilled water in wells of microtiter U-plates (Cooke Laboratory Products, Alexandria, Va.). The bacteroid antigen was released by piercing the nodules with a sterile toothpick and then pressing out the nodule contents. Care was taken to minimize maceration of the nodule. The nodule tissue was removed with the same toothpick. The nodule antigen and cultured cell controls were spotted onto a nitrocellulose membrane (9.2 by 15 cm) (provided in the immunoblot kit from Bio-Rad Laboratories, Inc., Richmond, Calif.) with a 96-pronged multiple inoculator-applicator (West Coast Scientific, Emeryville, Calif.). This procedure was followed by drying the spot blots on the nitrocellulose membrane at room temperature. The identity of the membrane-bound antigens (nodule bacteroids and cultured cell controls) was determined by using the detection procedure described in the Bio-Rad immunoblot kit. Both the primary antiserum and the goat anti-rabbit alkaline phosphate conjugate were diluted 1:4,000 for the detection assay. The fluorescent-antibody technique (27) was used to verify ambiguous immunoblot reactions. A total of 60 nodules (20 nodules × three replications) per treatment were sampled for strain identification.

Antigenic relatedness of indigenous isolates. Ninety-seven isolates of *B. japonicum* from the Korean soils were studied to examine the relatedness of their insoluble somatic antigens by using antisera of USDA 110, USDA 123, YCK 117, YCK 141, and YCK 150. The immunoblot kit procedure used earlier for strain identification was followed. The primary (rabbit) antisera of the six strains were diluted in saline to 1:8,000, and the secondary antiserum (goat anti-rabbit alkaline phosphate) was diluted in saline to 1:4,000. Antigens were diluted to give approximately 10^8 cells ml^{-1}, and 0.2 ml of each antigen was placed in wells of microtiter plates. Homologous antigen controls were included. Cells were transferred to four nitrocellulose membranes by using a multiple inoculator. Of the four, two membranes were not reacted with the primary antisera and served as negative controls.

RESULTS

Two uncultivated and five cultivated Korean soils were sampled for the presence of indigenous *B. japonicum* (Table 1). The numbers in the two uncultivated soils ranged from log_{10} 3.0 to 4.8 rhizobia g of soil^{-1}, while in the five cultivated soils, the populations were in the range of log_{10} 4.0 to 5.0 rhizobia g of soil^{-1}.

The symbiotic potentials of the indigenous *B. japonicum* in 10- and 1,000-fold-diluted soil suspensions (whole-soil inocula) were evaluated by using the Jangbaekkong cultivar. The indigenous *B. japonicum* in the 10-fold-diluted whole-soil inocula of the seven soils showed large differences in their symbiotic potentials. This was evident in all of the nodulation and nitrogen fixation parameters measured (Table 2). The symbiotic potential of *B. japonicum* populations from soil 3 was the lowest. Although 100 rhizobia ml^{-1} was present in the whole-soil inocula of soil 3, the nodules were ineffective and the total shoot N was not significantly different from the uninoculated control. Indigenous *B. japonicum* in soils 6 and 10 showed high symbiotic potential. The total N in shoots of plants inoculated with the whole-soil inoculum of soil 10 was significantly higher (102%) than in plants inoculated with the standard peat inoculant (log_{10} 7.8 rhizobia ml^{-1}) containing a mixture of *B. japonicum* USDA 110, CB 1809, and USDA 138.

After 1,000-fold dilution, the numbers of indigenous *B. japonicum* in the whole-soil inocula ranged from 1 to 100 rhizobia ml of suspension^{-1} (Table 3). Significant differences in most of the whole-soil inocula still persisted, although there was a general trend towards a decrease in the symbiotic potential in all soils after a 1,000-fold dilution. That the dilution contributed towards significant differences in the

TABLE 3. Symbiotic potential of native Korean *B. japonicum* populations in a 1,000-fold-diluted (10^{-3}) whole-soil inoculum inoculated on *G. max* cv. Jangbaekkong

Soil sample	No. of rhizobia ($\log_{10}$)[a]	No. of nodules per plant	Nodule dry wt (g) per plant	Shoot dry wt (g) per plant	Shoot total N (mg) per plant	Nitrogenase activity[b]
Soil no.						
3	0	13	0.03	0.47	5.3	0.4
4	1.8	34	0.27	1.37	38.2	6.9
6	1.8	34	0.22	1.53	44.2	14.9
7	1.8	20	0.17	1.10	23.5	7.4
8	1.0	28	0.24	1.48	40.2	8.9
9	1.0	18	0.13	0.98	24.0	5.7
10	2.0	30	0.26	1.66	57.3	11.1
Peat culture[c]	5.8	20	0.15	1.40	31.1	10.1
Uninoculated		0	0	0.51	5.3	0
LSD[d] (0.05)		6	0.05	0.36	11.42	6.0

[a,b,c,d] See footnotes to Table 2.

symbiotic potential of the whole-soil inocula of the seven soils was confirmed by a paired *t* test done on the data sets in Tables 2 and 3. The *t* test yielded the following results: nodule number, $t = 2.55$, $P < 0.05$; nodule dry weight, $t = 4.35$, $P < 0.01$; shoot dry weight, $t = 3.74$, $P < 0.01$; shoot total N, $t = 3.39$, $P < 0.05$; and total nitrogenase activity, $t = 2.58$, $P < 0.05$.

Since the paired *t* tests indicated significant differences in the symbiotic potential of the indigenous *B. japonicum* in 10-fold-diluted (Table 2) and 1,000-fold-diluted (Table 3) whole-soil inocula, the relationship between the size of the population and indicators of symbiotic potential was analyzed by regression. Results indicated that, when the size of the *B. japonicum* population varied from 100 to 10,000 ml^{-1} (Table 2), the relationship was linear ($y = a + bx$), with the coefficient of determination (R^2) ranging from 0.82 to 0.93 for all the parameters except nitrogenase activity (Table 4).

However, when the population of *B. japonicum* in the whole-soil inocula was 1 to 100 ml^{-1} (Table 3), the relationship between rhizobial numbers and nitrogen fixation could best be described by a power curve ($y = ax^b$); the values of R^2 are shown in Table 4. The corresponding linear R^2 values for the data in Table 3 were lower and were as follows: shoot total N, $R^2 = 0.60$; shoot dry weight, $R^2 = 0.64$; nodule dry weight, $R^2 = 0.67$; nodule number, $R^2 = 0.57$; and nitrogenase activity, $R^2 = 0.60$.

TABLE 4. Fitted models to explain the relationship between nitrogen fixation parameters of soybeans and levels of indigenous *B. japonicum* in whole-soil inocula

Parameter (*y*)	Whole-soil inoculum (*x*)[a]	Fitted model	Coefficient of determination (R^2)
Shoot total N	10^{-1}	$y = -47.67 + 27.74x$	0.83
	10^{-3}	$y = 32.43x^{0.26}$	0.86
Shoot dry wt	10^{-1}	$y = -0.89 + 0.74x$	0.82
	10^{-3}	$y = 1.25x^{0.14}$	0.83
Nodule dry wt	10^{-1}	$y = -0.22 + 0.14x$	0.93
	10^{-3}	$y = 0.19x^{0.23}$	0.88
No. of nodules	10^{-1}	$y = -14.2 + 13.48x$	0.85
	10^{-3}	$y = 25.12x^{0.15}$	0.61
Nitrogenase activity	10^{-1}	$y = -7.98 + 5.81x$	0.62
	10^{-3}	$y = 7.33x^{0.42}$	0.95

[a] Aqueous soil suspension was diluted to 10^{-1} or 10^{-3} and used as inocula to inoculate soybeans grown in Leonard jars. Inocula (10^{-1}) contained 100 to 10,000 cells ml^{-1} (Table 2) and 10^{-3} inocula contained 1 to 100 cells ml^{-1} (Table 3).

The effectiveness (total shoot N) of 30 strains of *B. japonicum* from the nodules of soybean plants inoculated with soils 4, 6, and 10 is shown in Fig. 1. There were several strains that were of equal or better effectiveness than *B. japonicum* USDA 110. Strains in soils 6 and 10 were more variable than those in soil 4.

Inoculation of the Clark and Jangbaekkong cultivars of soybean grown in the mollisol indicated that all inoculation treatments were able to significantly increase the total shoot N in comparison with that of the noninoculated plus bagasse control (Table 5). A two-variable factorial analysis of variance of the shoot N indicated that the effects due to

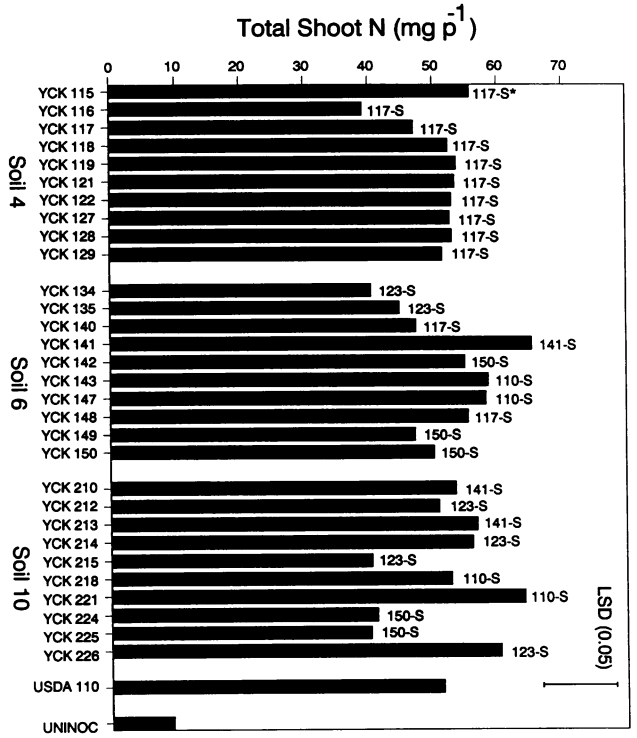

FIG. 1. Effect of Korean isolates of *B. japonicum* on the mean total shoot N of Clark and Jangbaekkong soybean cultivars. Serogroup assignment of isolates is indicated. For example, 117-S designation indicates that isolate YCK 117 reacted positively with antisera YCK 117 in immunoblot reactions.

TABLE 5. Effectiveness of Korean *B. japonicum* as single-strain inoculants and in combination with *B. japonicum* USDA 110 and USDA 123[a]

Inoculation with:	Clark		Jangbaekkong	
	Shoot total N	N fixed	Shoot total N	N fixed[b]
USDA 110	97.9	85.4	102.6	92.6
USDA 123	88.5	76.0	86.7	76.8
YCK 117	72.6	60.1	63.2	53.3
YCK 141	94.2	81.7	75.7	65.8
YCK 213	98.9	86.4	101.6	91.7
USDA 110 + YCK 117	90.3	77.8	64.8	54.9
USDA 110 + YCK 141	89.5	77.0	101.4	91.5
USDA 110 + YCK 213	106.8	94.3	84.1	74.2
USDA 123 + YCK 117	90.1	77.6	83.6	73.7
USDA 123 + YCK 141	83.8	71.3	101.4	91.5
USDA 123 + YCK 213	85.6	73.1	91.9	82.0
Noninoculated + bagasse[c]	12.5	0	9.9	0
Noninoculated[c]	73.3	0	70.3	0
LSD[d] (0.05)	12.1	12.2	11.3	11.3

[a] Experiments were done in *B. japonicum*-free mollisol. Values are in milligrams per plant.
[b] N fixed is the difference between the inoculated and noninoculated plus bagasse.
[c] Not included in statistical analysis.
[d] LSD, Least significant difference.

inoculation were highly significant ($P < 0.001$) and that the soybean cultivars were significantly different but to a lesser degree ($P = 0.05$). A highly significant ($P < 0.001$) host × inoculation interaction indicated that the total shoot N (N assimilation) was affected by specific combination of soybean cultivar and inoculum. *B. japonicum* YCK 213 and USDA 110 were of similar high effectiveness on both soybean cultivars. The mixed inoculum of these two strains was highly effective on the Clark but not on the Jangbaekkong cultivar.

The superiority of the Korean *B. japonicum* isolates in comparison with USDA 110 and USDA 123 is shown in Table 6. Generally, USDA 110 was a poor colonizer in contrast to all of the Korean isolates on both cultivars of soybean. In contrast to USDA 110, USDA 123 was a superior colonizer compared with the three Korean isolates. Korean isolates YCK 117 and YCK 213 formed 23.2 and 18% of the nodules, respectively, in comparison with USDA 123. Mixed (double) infected nodules containing USDA 123 and YCK 117 were almost of equal frequency (13%) when mixed inoculants of these two rhizobia were applied to the two soybean cultivars.

TABLE 7. Serogroup assignment (immunoblotting) of 97 isolates of *B. japonicum* from six Korean soils

Soil sample no. (location)	No. of strains	% Of antisera				
		YCK 117	YCK 141	YCK 150	USDA 110	USDA 123
4 (Sangnam, Milyang)	16	100	0	0	0	0
6 (Dong, Uichang)	20	15	5	35	30	15
7 (Chinbuk, Uichang)	17	0	24	29	6	41
8 (Bubuk, Milyang)	18	28	6	17	44	6
9 (Sangnam, Milyang)	10	40	20	10	20	10
10 (Chinbuk, Uichang)	16	13	13	13	31	31
Mean		31	10	19	23	18

The 30 isolates that were tested for effectiveness (Fig. 1) together with an additional 67 isolates of *B. japonicum* were examined for their distribution in six soils by immunoblotting (Table 7). In soil 4, 100% of the isolates cross-reacted only with the antisera of YCK 117. Isolates cross-reacting with antisera of YCK 141, YCK 150, USDA 110, and USDA 123 were present in variable proportions in all soils except soil 4. It was interesting to note that 44% of the isolates in soil 8 cross-reacted with the antisera of USDA 110, and USDA 123 had a 41% representation in soil 7.

DISCUSSION

The whole-soil inoculation technique was used successfully to demonstrate the symbiotic potential of indigenous *R. leguminosarum* bv. trifolii and *Rhizobium meliloti* (3, 4). We have applied this methodology to provide background in-

TABLE 6. Competition for nodule occupancy between Korean *B. japonicum* isolates and *B. japonicum* strains USDA 110 and USDA 123[a]

Competing strains	Soybean cultivar[b]	% Nodule occupancy by:					Mixed[c]	Unidentified[d]	F ratio[e]
		USDA 110	USDA 123	YCK 117	YCK 141	YCK 213			
USDA 110 + YCK 117	J	0.5		99.5			0	0	***
	C	0		99.0			0.5	0.5	***
USDA 110 + YCK 141	J	23.5			67.0		8.8	0.7	***
	C	13.0			58.5		12.0	16.5	**
USDA 110 + YCK 213	J	2.0				82.5	0	15.5	***
	C	0.3				99.7	0	0	***
USDA 123 + YCK 117	J		78.0	9.0			13.0	0	***
	C		64.0	23.2			12.8	0	*
USDA 123 + YCK 141	J		99.8		0.2			0	***
	C		100.0		0			0	***
USDA 123 + YCK 213	J		92.0			7.0	1.0	0	***
	C		73.0			18.0	9.0	0	***

[a] See Table 5, footnote *a*.
[b] J and C indicate Jangbaekkong and Clark soybean cultivars, respectively.
[c] Proportion of nodules occupied by both *B. japonicum* strains.
[d] Nodules showing negative reactions against antisera of competing pair.
[e] ***, **, and * indicate significant difference at 0.001, 0.01, and 0.05 levels of probability for competing pairs, respectively.

ormation on the variability in the symbiotic potential of indigenous *B. japonicum* in seven Korean soils (Tables 2 and 3).

To obtain a reliable indication of the diversity of symbiotic potential of an indigenous population of rhizobia at a specific site, a systematic but tedious procedure would involve isolating individual strains, testing them for effectiveness on a test legume, and then integrating the various measurements to obtain a single estimate for the whole population (4). The whole-soil inoculation technique avoids this lengthy and laborious approach. However, inherent in this technique is the effect of dilution which does not preserve the original indigenous population. The effect of dilution cannot be prevented since it is necessary in this technique to make a suspension of the soil so that it is practical enough to be manipulated and applied as inocula. However, the original composition of the indigenous soil rhizobia can be conserved and be acceptable if the soil dilution is minimized. Therefore, the lowest level of dilution of the whole-soil inocula that is chosen for inoculation is an important factor that determines the results of the assay. In this study, whole soil diluted 10-fold was more reliable because, at this dilution, the symbiotic potential of the indigenous *B. japonicum* was best expressed. This was supported by the observation that all parameters that were measured were significantly higher in the assay that used 10-fold-diluted whole-soil inocula as analyzed by the paired *t* test.

The 1,000-fold-diluted whole-soil inoculum was helpful in measuring the inoculum potential of smaller numbers of indigenous rhizobia, but this dilution may not represent the full diversity of the indigenous rhizobia originally present in the soil. However, it would allow detection of more effective indigenous rhizobia which may be present in smaller numbers. The diversity of isolates, as indicated by the differences in their effectiveness and serological distinctness (Fig. 1), is supportive of this conclusion. It was shown that rhizobia of different effectiveness were isolated from clover and alfalfa nodules depending on the dilution of the soil applied as inocula (3, 4). Furthermore, it was shown that the frequency (percentage) of isolation of *S. fredii* and *B. japonicum* from the nodules of soybean plants varied with the dilutions of the rhizosphere soil suspension used as inoculum (14).

The effect of rhizobial numbers on nodulation and nitrogen fixation has been reported for large-seeded species such as soybeans (36), peanuts (25), and chickpeas (32). However, these studies analyzed the nitrogen fixation data in relationship to the full range of inoculation rates (rhizobial numbers per seed) instituted. In this investigation, a detailed analysis was made to describe the relationships (Table 4) between nitrogen fixation and large numbers (Table 2) and small numbers (Table 3) of *B. japonicum* in the whole-soil inocula. Our data suggest that, when the numbers in the whole-soil inocula range from 1 to 100 ml^{-1}, the response is curvilinear (Table 4). For example, 86% of the variability in the shoot total N was accounted for by the power curve, while the linear curve accounted for only 60%. Our suggestion that the response curves may be different for small and large numbers of rhizobia in the whole-soil inocula was further supported by the low values for the coefficient of determination for the curves (linear, $R^2 = 0.52$; and power, $R^2 = 0.42$) when the shoot total N data in Tables 2 and 3 were combined for regression analysis. Further research with pure cultures of rhizobia are needed to verify the relationships between nitrogen fixation and inoculation with low numbers of rhizobia, as observed in our study.

The power curve suggests the occurrence of an initial lag phase in the nodulation during which one or a few viable cells present in the inoculum need to multiply to build up sufficient numbers of infective *B. japonicum* to cause root hair infections that develop into nitrogen-fixing nodules. This lag phase in the nodulation was partly responsible for the delayed greening of the plants. The need to multiply a single cell or a few cells resulting from serial dilutions into thousands of cells to greatly enhance the probability of seedling nodulation was demonstrated in the *R. meliloti*-alfalfa symbiosis (2).

At numbers ranging from 100 to 10,000 rhizobia ml^{-1}, a lag phase preventing prompt nodulation and nitrogen fixation may not occur. Hence, the response is best predicted by a linear curve. Our conclusion is in agreement with the earlier report that maximum total N fixed in plant tops of soybeans is achieved at inoculation rates of 2×10^3 and 1×10^5 *B. japonicum* $seed^{-1}$ (36).

The serological (immunoblot) distinctness (Table 7) and the statistically similar effectiveness profiles (Fig. 1) of the *B. japonicum* isolates from soil 4 indicated the presence of a unique indigenous *B. japonicum* population which would facilitate inoculation response and ecological studies with *B. japonicum* USDA 123. The significantly higher nitrogen-fixing ability of USDA 123 compared with that of YCK 117 with both of the soybean cultivars (Table 5) and the complete superiority of USDA 123 as compared with YCK 117 in competition studies (Table 6) support this conclusion. However, these observations were made in inoculation studies with a Hawaiian soil which does not harbor indigenous *B. japonicum*. The potential of USDA 123 as a suitable inoculant strain for soybean inoculation needs to be evaluated further in studies with soil 4 to which the YCK 117 group of isolates are indigenous.

The serological homogeneity of the strains in soil 4 can only be resolved in further studies analogous to that done on *B. japonicum* serogroup 123 where, when detailed studies were carried out by using immunoabsorbed antisera (10, 16, 28), it was shown that this serocluster actually consisted of serotypes 123, 127, 129, and several others.

Reports of a strain of *B. japonicum* of equal effectiveness and superiority in colonization as strain USDA 110 are rare. *B. japonicum* USDA 110 has been a widely recognized reference strain in numerous ecological studies involving soybeans. It produced the greater soybean yield and dominated the serological pattern when applied with strains 38 and 76 in equal numbers to Kent soybeans grown in soil (6). In a recent study, *B. japonicum* USDA 110 maintained its high effectiveness and superiority in colonization in a comparison with strains USDA 138 and CB 1809 under conditions of soil mineral N availability and immobilization (30). In the present study, we report the symbiotic potential of *B. japonicum* YCK 213, which was not only of equal effectiveness as USDA 110 but also completely superior to USDA 110 in colonization (nodule occupancy) (Table 5).

Given the narrow genetic base of the soybeans in the United States (12) and also the narrow genetic base of *B. japonicum* present in the soils of the United States (20), strain YCK 213 presents a new and valuable addition to the rhizobial germ plasm to improve nitrogen fixation in soybeans. However, the poor colonizing ability of YCK 213 in comparison with USDA 123 (Table 6) makes it unattractive for evaluation in most midwestern soils of the United States where members of the *B. japonicum* USDA 123 serogroup are the most successful colonizers (8, 9, 17, 21).

B. japonicum serogroups USDA 110 and USDA 123 are

among the seven most predominant serogroups represented in most soils of the United States (1). Of the 97 indigenous Korean soil isolates tested, 41% belonged to the USDA 110 and 123 serogroups (Table 7). *B. japonicum* strains belonging to the USDA 110 and 122 (USDA 136b) serogroups have also been found in Chinese soils (14). Our study documents for the first time the presence of *B. japonicum* isolates in soils of Korea which fall into the USDA 123 serogroup. The occurrence of *B. japonicum* serogroups common to soils of the United States and Asia (e.g., China and Korea) lends support to the conclusion of Devine and Breithaupt (13) that the soybeans and *B. japonicum* strains evolved in different areas of Asia which is the center of origin of the soybean-rhizobial symbiosis.

In summary, application of the whole-soil inoculation technique permitted the identification and mapping of soils which contained indigenous populations of *B. japonicum* of high symbiotic potential. This approach, in combination with a strain identification technique such as immunoblot, is a significant improvement in rhizobial strain selection and ecology, besides being useful in predicting inoculation responses under field conditions, as demonstrated by Brockwell et al. (4).

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REFERENCES

1. **Bezdicek, D. F.** 1972. Effect of soil factors on the distribution of *Rhizobium japonicum* serogroups. Agron. J. **36**:305–307.
2. **Biederbeck, V. O., and B. D. Walker.** 1981. PEPI: an improved plant infection technique for enumeration of rhizobia, p. 536–561. *In* K. W. Clark and J. H. G. Stephens (ed.), Proceedings of the 8th North American *Rhizobium* Conference. Printing Services, The University of Manitoba, Winnipeg, Canada.
3. **Bonish, P. M.** 1979. Clover rhizobia in soils: assessment of effectiveness using the plant infection count method. N. Z. J. Agric. Res. **22**:89–93.
4. **Brockwell, J., R. A. Holliday, and A. Pilka.** 1988. Evaluation of the symbiotic nitrogen-fixing potential of soils by direct microbial means. Plant Soil **108**:163–170.
5. **Broughton, W. J., and M. J. Dilworth.** 1971. Control of leghaemoglobin synthesis in snake beans. Biochem. J. **125**:1075–1080.
6. **Caldwell, B. E.** 1969. Initial competition of root-nodule bacteria on soybeans in a field environment. Agron. J. **61**:813–815.
7. **Chen, W. X., G. H. Yan, and J. L. Li.** 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. Int. J. Syst. Bacteriol. **38**:392–397.
8. **Cregan, P. B., and H. H. Keyser.** 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123 in soybean. Crop Sci. **26**:911–916.
9. **Damirgi, S. M., L. R. Frederick, and I. C. Anderson.** 1967. Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. Agron. J. **59**:10–12.
10. **Date, R. A., and A. M. Decker.** 1965. Minimal antigenic constitution of 28 strains of *Rhizobium japonicum*. Can. J. Microbiol. **11**:1–8.
11. **Date, R. A., and J. M. Vincent.** 1962. Determining the number of root-nodule bacteria in the absence of other organisms. Aust. J. Exp. Agric. Anim. Husb. **2**:5–7.
12. **Delanney, S., D. M. Rodgers, and R. G. Palmer.** 1983. Relative genetic contributions among ancestral lines to North American soybean cultivars. Crop Sci. **23**:944–949.
13. **Devine, T. E., and B. H. Breithaupt.** 1981. Frequencies of nodulation response alleles Rj2 and Rj4 in soybean plant introduction and breeding lines. USDA Technical Bulletin 1628. U.S. Government Printing Office, Washington, D.C.
14. **Dowdle, S. F., and B. B. Bohlool.** 1985. Predominance of fast-growing *Rhizobium japonicum* in a soybean field in The People's Republic of China. Appl. Environ. Microbiol. **50**:1171–1176.
15. **Duke, J. A.** 1981. Handbook of legumes of world economic importance. Plenum Press, New York.
16. **Gibson, A. H., W. F. Dudman, R. W. Weaver, J. C. Horton, and I. C. Anderson.** 1971. Variation within serogroup 123 of *Rhizobium japonicum*. Plant Soil Special Volume, p. 33–37.
17. **Ham, G. E., V. B. Cardwell, and H. W. Johnson.** 1971. Evaluation of *Rhizobium japonicum* inoculants in soils containing naturalized populations of rhizobia. Agron. J. **63**:301–303.
18. **Hymowitz, T., and C. A. Newell.** 1981. Taxonomy of the genus *Glycine*, domestication, and uses of soybean. Econ. Bot. **35**: 272–288.
19. **Keyser, H. H., B. B. Bohlool, T. S. Hu, and D. F. Weber.** 1982. Fast-growing rhizobia isolated from the root nodules of soybeans. Science **215**:1631–1632.
20. **Keyser, H. H., M. J. Sadowsky, and B. B. Bohlool.** 1985. Fast-growing soybean rhizobia, p. 926–934. *In* R. Shibels (ed.), Proceedings of the World Soybean Research Conference III. Westview Press, Boulder, Colo.
21. **Kvien, C. S., G. E. Ham, and J. W. Lambert.** 1981. Recovery of introduced *Rhizobium japonicum* strains by soybean genotypes. Agron. J. **73**:900–905.
22. **Lie, T. A.** 1981. Gene centres, a source for genetic variants in symbiotic nitrogen fixation: host-induced ineffectivity in *Pisum sativum* ecotype *fulvum*. Plant Soil **61**:125–134.
23. **Lie, T. A., D. Goktan, M. Engin, J. Pijnenborg, and E. Anlarsal.** 1987. Co-evolution of the legume-*Rhizobium* association. Plant Soil **100**:171–181.
24. **Mitchell, H. L.** 1972. Microdetermination of nitrogen in plant tissues. J. Assoc. Off. Anal. Chem. **55**:1–3.
25. **Nambiar, P. T. C., H. N. Ravishankar, and P. J. Dart.** 1983. Effect of *Rhizobium* numbers on nodulation and dinitrogen fixation in groundnut. Exp. Agric. **19**:243–250.
26. **Norris, D. O., E. S. Lopes, and D. F. Weber.** 1970. The use of organic mulch and lime pelleting in field testing of *Rhizobium* strains under tropical conditions. Pesqui. Agropecu. Bras. **5**:129–146.
27. **Schmidt, E. L., R. C. Bankole, and B. B. Bohlool.** 1968. Fluorescent antibody approach to the study of rhizobia in soil. J. Bacteriol. **95**:1987–1992.
28. **Schmidt, E. L., M. J. Zidwick, and H. M. Abebe.** 1986. *Bradyrhizobium japonicum* serocluster 123 and diversity among member isolates. Appl. Environ. Microbiol. **51**:1212–1215.
29. **Singleton, P. W., H. M. Abdel-Magid, and J. W. Tavares.** 1985. The effect of phosphorus on the effectiveness of strains of *Rhizobium japonicum*. Soil Sci. Soc. Am. J. **49**:613–616.
30. **Somasegaran, P., and B. B. Bohlool.** 1990. Single- strain vs. multistrain inoculation: effect of soil mineral N availability on rhizobial strain effectiveness and competition for nodulation on chickpea, soybean and dry bean. Appl. Environ. Microbiol. **56**:3298–3303.
31. **Somasegaran, P., and H. J. Hoben.** 1985. Methods in legume-*Rhizobium* technology. NifTAL Project, University of Hawaii, Paia.
32. **Somasegaran, P., H. J. Hoben, and V. Gurgun.** 1988. Effects of inoculation rate, rhizobial strain competition, and nitrogen fixation in chickpea. Agron. J. **80**:68–73.
33. **Thompson, J. A.** 1988. Selection of *Rhizobium* strains, p. 207–222. *In* D. P. Beck and L. A. Materon (ed.), Nitrogen fixation by legumes in Mediterranean agriculture. Developments in plant and soil sciences, vol. 32. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
34. **Vincent, J. M.** 1970. A manual for the practical study of

root-nodule bacteria. IBP handbook No. 15. Blackwell Scientific Publications Ltd., Oxford.
35. **Weaver, R. W., and L. R. Frederick.** 1972. A new technique for most-probable-number (MPN) counts of rhizobia. Plant Soil **36**:219–222.
36. **Weaver, R. W., and L. R. Frederick.** 1972. Effect of inoculum size on nodulation of *Glycine max* (L.) Merrill, variety Ford. Agron. J. **64**:597–599.
37. **Zohary, D.** 1970. Centers of diversity and centers of origin, p. 33–42. *In* O. H. Frankel and E. Bennett (ed.), Genetic resources in plants—their exploration and conservation. Blackwell Scientific Publications Ltd., Oxford.