8-2003

Identification and Use of Actinomycetes for Enhanced Nodulation of Soybean Co-Inoculated with Bradyrhizobium japonicum

A. K. Gregor

Brian Klubek
Southern Illinois University Carbondale

E. C. Versa

Preprint version.
Published in *Canadian Journal of Microbiology*, vol.49, no.8, pp.483-491,
at doi:10.1139/w03-061.

Identification and Use of Actinomycetes for Enhanced Nodulation of Soybean Co-Inoculated with *Bradyrhizobium japonicum*

A. K. Gregor, B. Klubek,[1] and E.C. Varsa

Department of Plant, Soil and General Agriculture

Southern Illinois University

Carbondale, IL 62901-4415 U.S.A.

[1]Author to whom correspondence should be addressed. Department of Plant, Soil and General Agriculture, Southern Illinois University-Carbondale, Carbondale, Illinois 62901-4415 USA. Telephone: 618-453-2496; Fax: 618-453-7457; email: bklubek@siu.edu

Abstract

The utilization of actinomycetes as potential soybean co-inoculants were evaluated in this study. Soil samples from Carbondale and Belleville, Illinois were used to inoculate pre-germinated soybeans plants for the determination of antibiotic sensitivity in the native *Bradyrhizobium japonicum* population. Sensitivity was of the order kanamycin > tetracycline > oxytetracycline > rifampicin > neomycin. Antagonism by five actinomycete cultures toward seven test strains of *B. japonicum* was also assessed. The ranking average inhibition (across all seven *B. japonicum* strains) by these actinomycetes was: *Streptomyces kanamycetius* = *S. coruleoprunis* > *S. rimosus* > *S.* species > *Amycolatopsis mediterranei*. A total of ten antibiotic combinations were used to isolate antibiotic resistant mutants of *B. japonicum* stains I-110 and 3I1B-110 via successive cycles of mutation. Eighty-one antibiotic resistant strains were isolated and tested for symbiotic competency, and nine of these were selected for further characterization in a greenhouse pot study. Few differences in nodule number were caused by these treatments. Nodule occupancy varied from 0 to 18.3% when antibiotic resistant strains of *B. japonicum* were used as the sole inoculants. However, when three mutant strains of *B. japonicum* were co-inoculated with *S. kanamycetius* significant increases in nodule occupancy (up to 55%) occurred. Increases in shoot N composition (27.1 to 40.9%) were also caused by co-inoculation with *S. kanamycetius*.

Key Words: *Bradyrhizobium japonicum*, *Streptomyces kanamycetius*, indigenous bradyrhizobia, co-inoculation, nodule occupancy

Introduction

Many nitrogen fixing bacteria convert gaseous nitrogen (N_2) to ammonia contributing to an important source of plant-available soil nitrogen. *Bradyrhizobium japonicum* forms a symbiotic relationship with soybean [*Glycine max* (L.)], resulting in biological nitrogen fixation. In Illinois it is estimated that 50 to 60 million dollars of nitrogen fertilizer credit is assigned on a yearly basis to soybean-corn or soybean-wheat rotations. Assuming an average seed nitrogen content of 5.5%, the estimated saving in nitrogen fertilizer costs is an additional 125 million dollars. Increasing seed nitrogen content could provide additional value as a protein supplement in foods and feeds.

Superior N_2 fixing strains of *Bradyrhizobium* have been identified. However, highly competitive native soil bradyrhizobia limit the ability to control infection of soybean roots by an added inoculum strain. This is referred to as the *Bradyrhizobium* competition problem (Streeter 1994; Sadowsky and Graham1998). Successful inoculation of soybean is dependent upon overcoming competition of native bradyrhizobia and the establishment of the applied strain in soil.

B. japonicum serocluster 123, including the serogroups USDA 123, 127 and 129 (Schmidt et al. 1986), alone inhabits 50 to 90% of soybean nodules in the Midwestern USA (Damirgi et al. 1967; Ellis et al. 1984; Kapusta and Rouwenhorst 1973; Moawad et al. 1984). These serogroups are very competitive (Cregan et al. 1989; Ellis et al. 1984; Ham et al. 1971; Klubek et al. 1988) and may be inefficient in N_2 fixation (Caldwell and Vest 1970; Ham 1980).

Inoculation with *B. japonicum* has been successful in increasing soybean nodulation with increases in plant fresh weight, seed protein and seed yield in soils with

a low or absent native population (Abel and Erdman 1964; Caldwell and Vest 1970). However, in soils with an established bradyrhizobial population, competition severely limits nodulation by an inoculum and it is not easily enhanced (Ham et al. 1971; Kapusta and Rouwenhorst 1973; Weaver and Frederick 1974; Thies et al. 1992). McLoughlin et al. (1990) used inoculum levels of 10^8 cells per 2.5 cm length of row, and obtained a nodule occupancy of less than 42% with little persistence in the soil. Ellis et al. (1984) found that the application of high levels of an applied strain increased the population size of that strain in soil but did not increase nodule occupancy. Additionally, Brockwell et al. (1987) and Roughley et al. (1993) found over 90% of their inoculum died within twenty-four hours of seed application.

The use of bactericidal agents has been shown to inhibit native bradyrhizobia. Hossain and Alexander (1984) found that the addition of the fungicide benomyl and the antibiotics erythromycin and streptomycin effectively enhanced colonization of soybean, when inoculated with a strain of *Bradyrhizobium* resistant to these antimicrobial compounds. These antimicrobial agents were able to effectively reduce predation by protozoa or competition by native bradyrhizobia and allow the introduced strain to nodulate soybean. Jones and Giddens (1984) found that fungicide-resistant mutants of *B. japonicum* USDA strain 110, when used with the appropriate fungicide, experienced enhanced survival in the soil and the bacterial counts within the nodules were increased. Further studies by Li and Alexander (1986) used streptomycin amendments to the soil to limit the growth of *Sinorhizobium meliloti* allowing the resistant inoculum strain to increase nodule number and occupancy versus the treatment without the antibiotic.

Studies conducted by Dashti et al. (1997) indicate that co-inoculation with plant growth promoting rhizobacteria (PGPR) and *B. japonicum* improved plant development and growth, and the grain and protein yield of soybean crops. Co-inoculation with *Azospirillum* has also been successful in increasing root number and length, root biomass, root hair development, shoot biomass, nodule number, and the fresh weight of soybean (Molla et al. 2001). Recently, Tokala et al. (2002) reported on a novel plant-microbe interaction between *Streptomyces lydicus* strain WYEC 108 and peas [*Pisum sativum* (L.)]. *S. lydicus* was shown to increase nodulation frequency, nodule size and mass, nitrogenase activity, and root and shoot weight.

The objectives of this study were to: (1) determine the antibiotic sensitivity of the native bradyrhizobia in southern Illinois soils; (2) isolate mutants of *B. japonicum* that are efficient in nitrogen fixation and resistant to the antibiotics most inhibiting to the native bradyrhizobia; and (3) assess nodule occupancy and effectivity in nitrogen fixation by the antibiotic resistant strains of *B. japonicum* when co-inoculating soybean with the appropriate antibiotic-producing actinomycete strain.

Materials and Methods

Soil Sampling

Twenty-five soil samples per location were randomly collected from five soybean research plots from the Southern Illinois University-Carbondale Agronomy Research Center (ARC) and the Belleville Research Center (BRC). Each research plot was subdivided into five subplots where four to six 2.5 cm dia cores (15 cm deep) were collected along a 6 m transect between the center rows of each subplot, and placed into sterile 355 ml Whirl Pack bags. Each composite soil sample from each subplot was

placed in a portable cooler layered with polyfoam refrigerant packs until returned to the laboratory where they were stored at 5° C until used. The soils at the ARC are classified as a Stoy silt loam (fine-silty, mixed, mesic, Aquic Hapludalf), and a Weir silt loam (fine, smectitic, mesic, Typic Epiaqualf). The soils at the BRC are classified as a Cowden silt loam (fine, smectitic, mesic, Vertic Albaqualf), a Rushville silt loam (fine, smectitic, mesic, Typic Albaqualf), and a Clarksdale silt loam (fine, smectitic, mesic, Udollic Endoaqualf). Previous crop histories of the ARC sampled plots were either a corn [*Zea mays* (L.)]-soybean [*Glycine max* (L.)] or a corn-soybean-sorghum [*Sorghum bicolor* (L.)] rotation. The BRC plots had been in either: (1) a soybean-wheat [*Triticum aestivum (L.)*] or a corn-soybean-wheat rotation.

Testing for intrinsic resistance

Soybean (LS90-1920, a cultivar developed in the SIUC soybean breeding program, Schmidt et al.1999) were grown in washed vermiculite under a 16 hr photo period with fluorescent lighting at the SIUC Horticulture Research Center greenhouse, Carbondale, Illinois. Seven days after planting, 10 g of each soil sample were mixed with a sterile 90 ml dilution blank, shaken for 1 minute, and used as an inoculum (1 ml per plant). Ten plants (replications) were used for each soil sample. The inoculated soybean plants were grown for an additional 35 days and watered as necessary with a nitrogen free mineral nutrient solution. At harvest the plants were removed from their pots with the plant shoots cut just above the roots. The root system of each plant was then washed free of vermiculite, blotted dry and placed in a sterile Whirl Pak bag and stored in a freezer at -20°C . The soybean nodules from these root samples were used to assess the sensitivity of the native *B. japonicum* population to selected antibiotics.

Following the procedure of Lieberman et al. (1986), 16 nodules of each root were randomly selected, washed, surfaced sterilized with 20% (v/v) bleach and 70% (v/v) ethanol, rinsed three times with distilled water, crushed, and plated on yeast extract mannitol (YEM) agar supplemented with one of the following antibiotics at a concentration of 100 µg/ml: neomycin (N), oxytetracycline (O), rifampicin (R), tetracycline (T), or kanamycin (K). Cycloheximide was also added to all of the growth media at 200 µg/ml to suppress the growth of fungi. A set of YEM plates without antibiotics were used as a positive control. All plates were grown for 7 to 14 days at 28°C and scored for growth as compared to the control. The number of developing colonies on the YEM-antibiotic plates divided by the number of developing colonies on the complementary YEM-control plates multiplied by 100 determined the percent intrinsic resistance by the native bradyrhizobia.

Testing of antagonistic strains

Actinomycete cultures that produce the antibiotics used in nodule typing (described above) were obtained from the American Type Culture Collection (ATCC): *Amycolatopsis mediterranei* ATCC #13685 (rifampicin), *Streptomyces coeruleoprunus* ATCC #43681 (neomycin), *Streptomyces kanamycetius* ATCC #12853 (kanamycin), *Streptomyces rimosus* sub sp. *rimosus* ATCC #33022 (oxytetracycline), and *Streptomyces* sp. ATCC #11652 (tetracycline). The test strains of *B. japonicum* used were USDA strains I-110, 3I1b-110, 3IIb-76, 3IIb-24, 123, and 127, and strain An-5 (a streptomycin resistant strain of serogroup 123 received from Dr. R.M. Zablotowicz, the USDA Weed Science Laboratory, Stoneville, MS). Each test strain was grown to stationary phase (5 days) in 100 ml of YEM broth at room temperature on a tabletop

shaker at 170 rpm. YEM agar plates were spread with 0.1 ml of broth culture and then streaked with an actinomycete culture in the form of a plus sign. A YEM agar plate inoculated with individual *B. japonicum* test strains was used in all cases as a positive control allowing a visual comparison. Following five days of growth at 28°C, inhibition by the actinomycete cultures in the four corners of the '+' pattern was visually rated on a scale of 1 to 10 and statistically analyzed after arcsine transformation (Little and Hills 1978).

Selection of antibiotic resistant strains

Antibiotic resistant mutants of *B. japonicum* strains I-110 and 3I1B-110 were obtained via successive cycles of mutation (Cooper 1979), on YEM agar plates amended with any combination of two antibiotics: kanamycin + neomycin (KN), kanamycin + tetracycline (KT), kanamycin + rifampicin (KR), kanamycin + oxytetracycline (KO), oxytetracycline + tetracycline (OT), neomycin + tetracycline (NT), rifampicin + tetracycline (RT), neomycin + oxytetracycline (NO), rifampicin + oxytetracycline (RO), and rifampicin + neomycin (RN). The *B. japonicum* strains were grown in 100 ml YEM broth culture at room temperature on a tabletop shaker at 170 rpm for 5 days, as previously described. YEM agar plates amended with 12.5 µg/ml of each antibiotic combination listed above were inoculated with 0.1 ml of broth culture. The plates were then incubated at 28°C for 7 to 14 days. Individual colonies growing on the plates of each antibiotic combination were transferred to new plates containing 25 µg/ml of each antiobiotic combination streaked for isolation, and grown as previously described. This step-wise successive transfer of isolates was followed until five different isolates were obtained from each antibiotic combination at a final

concentration of 100 µg/ml.

Testing of antibiotic resistant strains for competency in nodulation and N_2 - fixation activity

A 0.9 meter by 3.4 meter greenhouse bench was enclosed in clear plastic canopy to eliminate potential contamination of soybean plants by *B. japonicum* bearing. Lights were installed under the canopy to allow for a 16 hour photo period. The soybean plants (LS 90-1920) were grown using washed vermiculite in 12 cm x 8.5 cm diameter plastic cups modified with three drainage holes. A lattice board comprised of 2.5 cm wide wood strips was placed on top of the greenhouse bench supported by the bench's sides. The cups were inserted in the lattice holes (7 cm x 7 cm) suspending them above the bench top. Three surface sterilized seeds (an eight minute exposure to 10% v/v H_2O_2) were planted in each pot and this was reduced to one seedling per pot after approximately 5 days after seeding. The plants were inoculated at one week after planting with 1 ml of a 7 day old broth (YEM) culture of an antibiotic resistant strain of *B. japonicum*. The wild type strains I-110 or 3 I1b-110 were included as controls for each trial evaluating eight to ten resistant strains. Each inoculum treatment was replicated five times and watered as needed with a N free mineral nutrient solution. In total, nine trials were required to assess 81 isolated antibiotic resistant strains. Harvesting occurred at 35 days after inoculation to assure for the development of functional nodules.

At harvest, plants were removed from their pots and the plant shoots were cut just above the root as previously described. The roots were shaken free of vermiculite, placed in 946 cm^3 mason jars, and sealed with a screw capped lid. One hundred cm^3 of

air was removed and replaced with 100 cm^3 acetylene using a 50 cm^3 syringe with a 25 gauge needle. The jars were incubated for one hour at room temperature in the greenhouse. A 10 cm^3 sample was then removed and analyzed for ethylene by gas chromatography (Hardy et al. 1968). Plant shoots were dried at 70°C for 48 hours, ground and analyzed for total N by Brookside Laboratories, New Knoxville, Ohio. Nodules were counted and nodule occupancy was determined following the procedure of Lieberman et al. (1986). The number of developing colonies on the YEM-antibiotic plates divided by the number of developing colonies on the complementary YEM-control plates multiplied by 100 gave the percent nodule occupancy by the applied strain.

Soil pot co-inoculation greenhouse study

Approximately 0.06 m^3 of soil (0 to 15 cm deep) was obtained from the SIU-C Agronomy Research Center. The soil is classified as a Stoy silt loam, and corn was produced at that site the previous year.

The soil was allowed to air dry on a greenhouse bench, ground and analyzed for exchangeable potassium (Knudsen et al., 1982), Bray P1 extractable phosphorus (Olsen and Sommers 1982) and pH (McLean 1982). These results are summarized in Table 1. Two hundred thirty, 12 cm x 8.5 cm cups were modified with three drainage holes and lined with cheese cloth on the bottom of each cup. The cups were then filled with the ground soil. Additionally, for a negative-nodulation control, ten cups were filled with soil amended with 75 mg N (as ammonium nitrate)/kg soil. The soils were moistened to field capacity as described by Sabey et al (2003).

S. kanamycetius and ten antibiotic resistant strains of *B. japonicum* selected for

the co-inoculation study were grown in YEM broth as previously described. Fifty soybean seeds (LS 90-1920) were treated as follows: (1) 0.4 ml of 10% (w/v) gum acacia (used as a seed adhesive for the applied *S. kanamycetius* and/or *B. japonicum* cells) and 0.5 g activated charcoal (a coating of fine solid particles to avoid seed clumping) (non-inoculated control); (2) 0.3 ml of *S. kanamycetius* broth culture followed by the addition of gum acacia and activated charcoal as previously described; (3) 0.3 ml (each) of *B. japonicum* and *S. kanamycetius* followed by gum acacia and activated charcoal. The mean plate count of each inoculum strain is given in Table 2.

The inoculated seeds were immediately planted into the soil pots (3 seeds per pot for 10 replications per treatment) and randomized in a complete block design. Five days following emergence, each experimental unit (pot) was thinned to one seedling per pot.

Plants were watered as needed with potable water from the greenhouse, and a 16 hour photoperiod was used as previously described. Thirty-five days after planting the plants were removed from the soil with the plant shoots severed just above the root system. The roots were washed free of soil and placed in Whirl Pak bags and stored in a freezer at -20°C until measurements could be made. Nodule number and occupancy was determined as previously described. Nodules were plated on YEM agar plates containing KN or KT (100 µg/ml per antibiotic), and YEM agar alone as a positive control. The plates were incubated 10 to 14 days and scored for growth. Plant shoots were dried at 70°C for 48 hours, ground to pass a 100 mesh sieve, and analyzed for total N by Brookside Laboratories, New Knoxville, Ohio.

Statistical analysis

All of the data were analyzed by an Analysis of Variance (ANOVA), and mean

separation ($P < 0.05$) was achieved by the Duncan Multiple Range Test (Helwig and Council 1979). All numeric differences in the data are considered significantly different at this level of probability.

Results

Determination of intrinisic antibiotic resistance

Neomycin had a lower inhibitory effect on the native bradyrhizobia (54.1%) from the ARC nodule samples than the other four antibiotics (Table 3), while kanamycin had the highest inhibitory effect (83.3%). The efficacy of the test antibiotics was in the order: kanamycin > rifampicin > oxytetracycline > tetracycline > neomycin. The bradyrhizobia from the BRC nodule samples were inhibited the least by neomycin and rifampicin (76.5 and 73.5%, respectively). Tetracycline and kanamycin were most inhibitory (95.3 and 96.5%) to the bradyrhizobia from this site. Inhibition for the BRC strains was in the order: kanamycin > tetracycline > oxytetracycline > neomycin > rifampicin. Overall inhibition across the two sites was of the order kanamycin > tetracycline > oxytetracycline > rifampicin > neomycin.

Testing of antagonist strains

The percent inhibition by actinomycete strains when inoculated and grown simultaneously with the *B. japonicum* test strains is given in Table 4. *B. japonicum* strain I-110 was most inhibited by *S. kanamycetius*. Strain 3I1B-110 was most inhibited by *S. kanamycetius* and *S. coruleoprunis*, while *A. mediteranei* had no inhibitory effect. Strain 3I1B-76 showed no inhibition by *S.* species. Strain 3I1B-24 was sensitive to all of the actinomycete strains, while strain An-5 (a member of serogroup 123 resistant to

streptomycin) was equally inhibited by *A. mediteranei, S. coruleoprumus, S. kanamycetius* and *S.* species although not inhibited by *S. rimosus*. Strain 123 was not inhibited by *S.* species, but was significantly inhibited by *S. rimosus*. Strain 127 was inhibited by *S. coruleoprunus* and *S. kanamycetius* and showed no inhibition by either *S. rimosus* or *S.* species. The average percent inhibition of *B. japonicum* by the five actinomycete strains was of the order *S. coruleoprunus* > *S. kanamycetius* > *S. rimosus* > *S.* species > *A. mediteranei*, ranging from 22 to 9.2% inhibition.

Testing strains for competency in nodulation and N_2 fixation activity

A total of 81 antibiotic resistant strains of *B. japonicum* were isolated and evaluated in nine greenhouse trials of symbiotic competency. Table 5 only summarizes the results of two trials from which 9 strains were selected for the soil-pot greenhouse study. In all of the other trials the antibiotic resistant strains had reduced symbiotic competency and these were discarded. Differences between the KN antibiotic resistant strains and the parental wild type strains of *B. j*aponicum for nodule number and occupancy, shoot N content, and nitrogen fixation (acetylene reduction) activity were measured in Trial 1. In Trial 2, strain KT 3I1B-1 was greater than strains KT3I1B-2 and-3 in nodule occupancy but not in the other characteristics of symbiotic competency. Most of the soybean plants inoculated by the antibiotic resistant strains in Trials 1 and 2 had a nodule number count, shoot N content and nitrogen fixation (acetylene reduction) activity that were equal to or greater than the soybeans inoculated with the parental wild type strains.

Soil-pot greenhouse study

With the exception of treatment KNI-110-2 and the N fertilizer control, there

were no differences among treatments for nodule number (Table 6). The KNI-110-2 treatment had a higher nodule count than all other treatments while the N fertilizer control (as expected) had the lowest nodule count. Nodule occupancy varied from 0 to 18.3% when the KN or KT strains were used as sole inoculants. However, an increase in nodule occupancy occurred with co-inoculation by *S. kanamycetius* and strain KNI-110-1, KNI-110-5, or KN3I1B-2, improving from 1.0, 6.6 and 0% to 44.8, 48.0, and 55.0%, respectively. The *S. kanamycetius* treatment resulted in a nodule occupancy that was not different from the non-inoculated control. An increase in shoot N content over the non-inoculated control by the co-inoculum treatments of *S. kanamycetius* and strains KNI-110-1, KN3I1B-2, or KT3I1B-3 was determined (3.13, 3.05, and 3.24%, respectively). However, inoculation by strains KN3I1B-3, KT3I1B-1 and KT3I1B-2 without *S. kanamycetius* also resulted in a higher shoot N contents (2.99, 3.21, and 3.07% respectively) over the non-inoculated control treatment. All other inoculum treatments were equivalent in shoot N content to the non-inoculated control.

Discussion

The main objectives of this study were to assess the antibiotic sensitivity of the native bradyrhizobia in southern Illinois soils, and to develop antibiotic resistant strains of *B. japonicum* which may be used to co-inoculate soybean crops with the appropriate antibiotic-producing actinomycete strain.

At the ARC and BRC research centers, the native bradyrhizobia showed greater sensitivity to kanamycin than neomycin despite their similarities in modes of action. This may be attributed to a plasmid encoded resistance to neomycin, but not kanamycin, at these locations and/or the presence of streptomycetes which synthesize neomycin or

neomycin-like agents resulting in the selection for neomycin resistance. Tetracycline and oxytetracycline were similar in their degree of inhibition at both the ARC and BRC locations. These results suggest that varying populations of actinomycetes (which produce these antibiotics) exist at the Carbondale and Belleville locations. However, similar results for rifampicin inhibition were obtained for both locations, implying similar populations of *Amycolatopsis mediterranei* may exist at both field sites.

Collectively, the inhibition of *B. japonicum* from soybean nodules derived from these two locations was not identical, indicating that antibiotic sensitivity varies from location to location. However, on average, kanamycin was most effective in suppressing the growth of the native bradyrhizobia. Marciniak (1984) reported a greater incidence of intrinsic resistance to streptomycin, kanamycin and rifampicin at Brownstown, Illinois but found a greater sensitivity by the native bradyrhizobia to these same antibiotics at Belleville, Flora and Vergennes, Illinois. Mueller et al. (1988) reported that the intrinsic resistance of *B. japonicum* isolated from South Carolina soils to antibiotics was of the order: streptomycin >> streptomycin + neomycin > streptomycin + rifampicin > kanamycin + rifampicin = kanamycin + nalidixic acid. These authors concluded that intrinsic resistance by native bradyrhizobia was the norm for South Carolina soils.

The efficacy of the actinomycete cultures in suppressing the growth of the *B. japonicum* test strains was variable. On average, the lower antibradyrizobial activity expressed by *S.* species and *A. mediteranei* versus *S. coeruleoprunis*, *S. kanamycetius*, and *S. rimosus* when grown in the presence of the *B. japonicum* test strains (mimicking co-inoculation on the seed coat) suggests that the former strains require the

accumulation of tetracycline/rifampicin for the suppression of *B. japonicum* growth. The efficacy of *B. japonicum* antagonism expressed by *S. coreuleoprunis, S. kanamycetius,* or *S. rimosus* apparently does not require the accumulation of neomycin, kanamycin, or oxytetracycline, but rather may be associated with the direct suppression of growth. It has been reported that different strains may show different intrinsic resistance patterns to the antibiotic employed (Cole and Elkan 1979; Davis 1962; Graham 1963). Consequently, a variety of intrinsic resistance patterns may result from different locations, strains, and experimental conditions. The use of a good antagonist strain as the co-inoculum was important for this study. Due to the variety of intrinsic resistance patterns that were observed, all combinations of kanamycin, neomycin, oxytetracycline, rifampicin, and tetracycline were used to isolate resistant mutants of *B. japonicum* strain I-110 and 3I1B-110.

Only nine isolated strains, resistant to either kanamycin + neomycin or kanamycin + tetracycline, were chosen for additional greenhouse studies. The remaining resistant strains produced few or no nodules indicating that resistance to the other combinations of selected antibiotics interfered with the root-hair infection process. Tetracycline and oxytetracycline are more aggressive protein synthesis inhibitors than neomycin and kanamycin (Stryer 1995.). The strains resistant to oxytetracycline plus neomycin or kanamycin, and neomycin plus tetracycline may have acquired an altered 30s subunit of rRNA to survive the mode of action of these antibiotics (Stryer 1995). A mutation of this kind would most likely lead to numerous alterations of proteins including the cell wall structure interfering with host recognition and eventual nodulation. Resistance to an antibiotic may also be due to an alteration of the antibiotic

itself, rendering it nonfunctional, pumping the antibiotic out of the cell, or altering the site of action of the antibiotic. The KN and KT strains summarized in Table 5 may have evolved on these latter mechanisms without interfering with the root-hair infection process.

The variation in nodule occupancy, shoot N content, and nitrogen fixation (acetylene reduction) activity may also be attributed to the acquisition of antibiotic resistance. Cole and Elkan (1979) have suggested that a potential for the loss of resistance characters exists for *B. japonicum*. Additionally, the failure to detect an applied inoculum labeled with multiple markers may also be due to slow growth and an inappropriate incubation period. Levin and Montgomery (1974) reported that *B. japoncium* is, in general, not susceptible to the loss of effectiveness in nodulation or efficiency in nitrogen fixation due to antibiotic resistance. The observed differences in shoot N content and nitrogen fixation (acetylene reduction) activity in this study may be related to the acquired resistance to kanamycin and/or neomycin which interferes with nutrient transport (Stryer, 1995).

The addition of nitrogen as a fertilizer control was used to compare the differences in shoot N content between biologically fixed and applied N. Although this fertilizer treatment resulted in a higher N content than all other treatments, the benefits of soybean inoculation by strains KN3I1B-3, KT3I1B-1 and KT3I1B-2, and *S. kanamycetius* plus KNI-110-1, KN3I1B-2, or KT3I1B-3 were realized with a 19.6 to 29.6% increase in shoot N content over the non-inoculated control. For the latter three co-inoculum treatments, a 27.1 to 40.9% increase in shoot N content was determined over the same *B. japonicum* strains without *S. kanamycetius*. The increase in nodule

occupancy by strains KNI-110-1, KNI-110-5, KN3I1B-2, and KT3I1B-3 when co-inoculated with *S. kanamycetius* further demonstrates the potential synergistic benefits of co-inoculation by any combination of these strains. The 4% increase in nodule occupancy by strain KT3I1B-3 when co-inoculated with *S. kanamycetius* may also have contributed, in part, to the increase in shoot N content. Conversely, the lack of an increase in shoot N content by the co-inoculum treatment KN3I1B-3, KT3I1B-1, or KT3I1B-2 with *S. kanamycetius* suggests that any positive interaction between *S. kanamycetius* and a strain of *B. japonicum* may be highly specific. Thus, the most compatible strains of *B. japonicum* in association with *S. kanamycetius* offers the greatest potential for successful soybean inoculation.

The higher nodule number following inoculation with KNI-110-2 inoculum treatment did not result in an improvement in nodule occupancy by this strain or shoot N content. Although co-inoculation by this strain with *S. kanamycetius* reduced the number of nodules, it did improve nodule occupancy 2.7 fold and shoot N content by 9.8%. It may also be that the strain caused an increase in nodule occupancy by a more efficient strain, resulting in a greater shoot N content, and also a reduction in the number of nodules required to fix N. These results show that no relationship exits between nodule number and nodule occupancy or shoot N content, but that co-inoculation with *S. kanamycetius* may improve the occupancy of an applied strain.

The problem of successful soybean inoculation by an applied strain of *B. japoncium* still exists. It is clear from this study that the co-inoculation of soybeans with an appropriate actinomycete strain may be a novel approach in overcoming competition by the native bradyrhizobia, and the establishment of an applied strain.

Thus, it may also be important to test the ability of actinomycete strains to colonize soybean roots to overcome the problem of competition for root-hair infection sites in soil.

Acknowledgments

The authors thank K. Shimizu, E. White, and L. Lorton for their technical assistance, T. Wyciskalla for help with statistical analysis, and L. Hartline for help in the preparation of this manuscript. This research was supported by a grant from the Illinois Council on Food and Agricultural Research (C-FAR).

Abel, G. H., and Erdman, L.W. 1964. Response of Lee soybeans to different strains of *Rhizobium japonicum*. Agron. J. **56**:423-424.

Brockwell, J., Roughley, R.J., Herridge, D.F. 1987. Population dynamics of *Rhizobium japonicum* used to inoculate three successive crops of soybean. Aust. J. Agric. Res. **38**:61-74.

Caldwell, B.E., and Vest, G. 1970. Effect of *Rhizobium japonicum* strains on soybean yields. Crop Sci. **10**:19-21.

Cole, M.A., and Elkan, G.H. 1979. Multiple antibiotic resistance in *Rhizobium japonicum*. App. Environ. Microbiol. **37**:867-870.

Cooper, J.E. 1979. Rapid method for counting antibiotic-resistant rhizobia in soils. Soil Biol. Biochem. **11**:433-435.

Cregan, P.B., Keyser, H.H., and Sadowsky, M.J. 1989. Host plant effects on nodulation and competitiveness of the *Bradyrhizobium japonicum* serotype strains constituting serocluster 123. Appl. Environ. Microbiol. **55**:2532-2536.

Damirgi, S.M., Frederick, L.R., and Anderson, J.C. 1967. Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. Agron. J. **59**:10-12.

Dashti, N. Zhang, F., Hynes, R., and Smith, D.L. 1997. Application of plant-growth promoting rhizobacteria to soybean [*Glycine max* (L) Merr] increases protein and dry matter under short-season conditions. Plant and Soil **188**:33-41.

Davis, R.J. 1962. Resistance of rhizobia to antimicrobial agents. J. Bacteriol. **84**:187-188.

Ellis, W.R., Ham, G.E., and Schmidt, E.L. 1984. Persistence and recovery of *Rhizobium japonicum* inoculum in a field soil. Agron. J. **76**:573-577.

Graham, P.H. 1963. Antigenic affinities of the root-nodule. J. Bacteriol. **84**:187-188.

Ham, G.E. 1980. Interactions of Glycine max and *Rhizobium japonicum*. *In* Advances in Legume Science. *Edited by* R.J. Summerfield and A.H. Bunting. Royal Botanical Gardens, Kew, United Kingdom. pp. 289-296.

Ham, G.E., Cardwell, V.B., and Johnson, H.W. 1971. Evaluation of *Rhizobium japonicum* inoculants in soils containing naturalized populations of rhizobia. Agron J. **63**:301-303.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., and Burns, R.C. 1968. The acetylene-ethylene assay of N_2-fixation: laboratory and field evaluation. Plant Physiol. **43**:1187-1207.

Helwig, J.R., and Council, K.A. 1979. The SAS User's Guide. SAS Institute, Inc., Raleigh, North Carolina. 474 p.

Hossain, A.K.M., and Alexander, M. 1984. Enhancing soybean rhizosphere colonization by *Rhizobium japonicum*. Appl. Environ. Microbiol. **48**:468-472.

Jones, R., and Giddens, J. 1984. Introduction of effective N_2-fixing rhizobial strains into the soybean plant by use of fungicide resistance. Agron. J. **76**:599-602.

Kapusta, G., and Rouwenhorst, D.L. 1973. Influence of inoculum size on *Rhizobium japonicum* serogroup distribution frequency in soybean nodules. Agron. J. **65**:916-919.

Klubek, B.P., Hendrickson, L.L., Zablotowicz, R.M., Skwara, J.E., Varsa, E.C., Smith, S., Islieb, T.G., Maya, J., Valdes, M., Dazzo, F.B., Todd, R.L., and Walgenback, D.D. 1988. Competitiveness of selected *Bradyrhizobium japonicum* strains in the midwestern USA Soils. Soil Sci. Soc. Am. J. **52**:662-666.

Knudsen, D., Peters, G.A., and Pratt, P.F. 1982. Lithium, sodium, and potassium. *In* Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. *Edited by* A.L. Page, R.H. Miller, and D.R. Keeney. American Society of Agronomy, Madison, Wisconsin. Agronomy **9**:225-246.

Levin, R.A., and Montgomery, M.P. 1974. Symbiotic effectiveness of antibiotic-resistant mutants of *Rhizobium japonicum*. Plant and Soil **41**:669-676.

Li, D.-M., and Alexander, M. 1986. Bacterial growth rates and competition affect nodulation and root colonization by *Rhizobium meliloti*. Appl. Environ. Microbiol. **52**:807-811.

Liberman, M.T., Zablotowicz, R.M., Davis-Omholt, N.P. 1986. Improved method of typing *Bradyrizobium japonicum* in soybean nodules. Appl. Environ. Microbiol. **51**:715-719.

Little, T.M., and Hills, F.J. 1978. Agricultural Experimentation. Design and Analysis. John Wiley and Sons, New York, NY. 300p.

Marciniak, W.H. 1984. Isolation, characterization, and recovery of antibiotic mutants of *Rhizobium japonicum* (Kirchner) strain 110. M.S. thesis, Southern Illinois University-Carbondale, Carbondale, Illinois.

McLean, E.O. 1982. Soil pH. *In* Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. *Edited by* A.L. Page, R.H. Miller, and D.R. Keeney. American Society of Agronomy, Madison, Wisconsin. Agronomy **9**:199-224.

McLoughlin, T.J., Alt, S.G., and Merlo, P.A. 1990. Persistence of introduced *Bradyrhizobium japonicum* strains in forming nodules in subsequent years after inoculation in Wisconsin soils. Can. J. Microbiol. **36**:794-800.

Moawad, H.A., Ellis, W.R., and Schmidt, E.L. 1984. Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. Appl. Environ. Microbiol. **47**:607-612.

Molla, A.H., Shamsuddin, Z.H., Halimi, M.S., Morziak, M. and Putek, A.B. 2001. Potential for enhancement of root growth and nodulation of soybean co-inoculated with *Azospirillum* and *Bradyrhizobium* in laboratory systems. Soil Biol. Biochem. **33**:457-463.

Muller, J.G., Skipper, H.D., Shipe, E.R., Grimes, L.W., and Wagner, S.C. 1988. Intrinsic antibiotic resistance in *Bradyrhizobium japonicum*. Soil Biol. Biochem. **20**:879-882.

Olsen, S.R., and Sommers, L.E. 1982. Phosphorus. *In* Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. *Edited by* A.L. Page, R.H. Miller, and D.R. Keeney. American Society of Agronomy, Madison, Wisconsin. Agronomy **9**:403-430.

Roughley, R.J., Gemell, L.G., Thompson, J.A., and Brockwell, J. 1993. The number of *Bradyrhizobium* sp. (*Lupinus*) applied to seed and its effect on rhizosphere colonization, nodulation and yield of lupin. Soil Biol. Biochem. **25**:1453-1458.

Sabey, B.R., Klubek, B.P., Chong, S-K., and Varsa, E.C. 2003. Introductory experimental soil science. Second edition. Stipes Publishing Company, Champaign, Illinois.

Sadowsky, J.J., and Graham, P.H. 1998. Soil biology of the Rhizobiaceae. *In* The Rhizobiaceae. Molecular Biology of Model Plant-Associated Bacteria. *Edited by* H.P. Spaink, A. Kondorosi, and P.J.J. Hooykaas. Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 155-172.

Schmidt, E.L., Zidwick, M.J., Abebe, H.M. 1986. *Bradyrhizobium japonicum* serocluster 123 and diversity among member ioslates. Appl. Environ. Microbiol. **51**:1212-1215.

Schmidt, M.E., Klein, J., Suttner, R.S., and Myers, Jr., O. 1999. Registration of 'LS90-1920' soybean. Crop Sci. **39**:295.

Streeter, J.G. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. Can. J. Microbiol. **40**:513-522.

Stryer, L. 1995. Biochemistry. Fourth edition. W.H. Freeman and Company, New York.

Thies, J E., Bohlool, B.B. and Singleton, P.W. 1992. Environmental effects on competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. Can. J. Microbiol. **38**:493-500.

Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Salove, M.H,, Deobald, L.A., Bailey, J.F., and Morra, M.J. 2002. Novel plant-microbe rhizosphere interaction involving *Sreptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Appl. Environ. Microbiol. **68**:2161-2171.

Weaver, R.W., and Frederick, L.R., 1974. Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. II. Field studies. Agron. J. **66**:233-236.

Table 1. Selected chemical properties of the soil (Stoy silt loam) used in the soil-pot co-inoculation greenhouse study.[†]

Sampling location	Soil pH	Bray P_1 Extractable P	Exchangeable K	Organic matter content
		----------mg/kg----------		-----%-----
SIUC Agronomy	6.7 ± 0.2	42 ± 1	127 ± 14	2.1 ± 0.1

[†]Values in table are means of five replicates ± the standard deviation (SD).

Table 2. Plate count of seed incoula for the soil-pot co-inoculation greenhouse study.[†]

Inoculum treatment	count (log_{10}/ml)	CV (%)[‡]
S. kanamycetius	8.36 ± 0.05	0.6%
KNI-110-1	8.62 ± 0.06	0.7%
KNI-110-2	8.16 ± 0.13	1.6%
KNI-110-3	8.73 ± 0.09	1.0%
KNI-110-5	8.51 ± 0.05	0.5%
KN3I1B-2	8.63 ± 0.08	0.9%
KN3I1B-3	8.09 ± 0.15	1.9%
KT3I1B-1	8.87 ± 0.06	0.7%
KT3I1B-2	8.87 ± 0.06	0.7%
KT3I1B-3	8.63 ± 0.04	0.5%

[†]Values in table are means of three replicates ± the standard deviation (SD)
[‡]Percent coefficient of variation.

Table 3. Percent inhibition of *B. japonicum* from soybean nodules.

Antibiotic[†]	ARC[‡]	BRC[‡]	Average
Kanamycin	83.3a*	96.5a	89.9
Neomycin	54.1c	76.5c	65.3
Oxytetracycline	71.4b	90.1b	80.8
Rifampicin	75.4b	73.5c	74.0
Tetracycline	69.6b	95.3ab	82.5

[†]100 µg/ml growth medium

[‡]Values in table are means of 4,000 nodules

*Means in the same column followed by the sample letter are not significantly different ($P \leq 0.05$) according to the Duncan's Multiple Range Test

Table 4. Percent inhibition of *B. japonicum* growth by antagonistic strains of actinomycetes.

Actinomycete	Test Strain of *B. japonicum*[†] I-110	3I1B-110	3I1B-76	3I1B-24	An 5	123	127	Average
	% Inhibition							
A. mediterranei	3.3c*	0c	10.0a	43.3bc	6.7ab	10.0ab	3.3b	9.2
S. coeruleoprunis	23.3b	26.7ab	13.3a	76.7a	10.0a	6.7a	10.0a	22.0
S. kanamycetius	46.7a	40.0a	13.3a	36.7c	10.0a	6.7b	13.3a	20.4
S. rimosus	23.3b	13.3b	13.3a	76.7a	0b	20.0a	0b	19.6
S. species	10.0bc	13.3b	0b	60.0ab	6.7ab	0c	0b	10.4

[†]Three replications per strain.

*Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test.

Table 5. Symbiotic characteristics of selected antibiotic resistant strains of *B. japonicum*: competence study.

B. japonicum	Trial Number	Nodule Number	Nodule Occupancy[†]	Shoot N Content	Acetylene Reduction Activity
			-----%-----	--%--	--µmol C_2H_4 plant/hr--
Parental Strain (3I1b-110)	1	44ab*	10b	2.38a[‡]	3ab
KN3I1B-2		40.2ab	80a	2.10ab	3ab
KN3I1B-3		31.2bcd	95.9a	1.91abc	3ab
Parental Strain (I-110)		50a	0b	2.43a	1b
KNI-110-1		31bcd	98.6a	1.38cde	5a
KNI-110-2		37abcd	97.3a	1.19e	5a
KNI-110-3		29cd	100a	1.53cde	6a
KNI-110-5		23d	100a	1.05e	4a
Parental Strain (3I1B-110)	2	22	0b	2.04[§]	59
KT3I1B-1		32	100a	2.23	72
KT3I1B-2		26	19.4b	2.17	88
KT3I1B-3		25	0b	2.11	94
		NS[¶]		NS	NS

[†]Number of plated nodules (out of 16) that were positive for growth on YEM agar supplemented with 100 µg/ml (each of kanamycin + neomycin (KN) or kanamycin + tetrcycline (KT).
[‡]Data (Trial 1) normalized for biological fixed N by subtracting the shoot N content of the non-inoculated control, (0.89%), from the inoculated treatment.
[§]Data (Trial 2) normalized for biological fixed N by subtracting the shoot N content of the non-inoculated control, (1.01%), from the inoculated treatment.
*Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test.
[¶]NS = Non-significant

Table 6. Nodule number, nodule occupancy, and shoot N content of selected antibiotic resistant strains of *B. japonicum*: soil-pot co-inoculation greenhouse study.

Inoculum Treatment	Nodule Number	Nodule Occupancy KN	Nodule Occupancy KT	Shoot N Content
		--------%----------		-----%----
Non-Inoculated Control	28b*	6.1b	0b	2.50efg
N Fertilizer Control	6c	ND†	ND	3.97a
S. kanamycetius	31b	11.4b	6.3ab	2.43efg
KNI-110-1	28b	1.0b	–	2.29g
KNI-110-2	46a	5.2b	–	2.56efg
KNI-110-3	31b	3.3b	–	2.21g
KNI-110-5	29b	6.6b	–	2.41fg
KN3I1B-2	32b	0b	–	2.40fg
KN3I1B-3	28b	19.6b	–	2.99bcd
S. kanamycetius + KNI-110-1	28b	44.8a	–	3.13bc
S. kanamycetius + KNI-110-2	32b	14.1b	–	2.81cde
S. kanamycetius + KNI-110-3	29b	12.0b	–	2.40fg
S. kanamycetius + KNI-110-5	32b	48.0a	–	2.77cdef
S. kanamycetius + KN3I1B-2	26b	55.0a	–	3.05bcd
S. *kanamycetius* + KN3I1B-3	24b	16.4b	–	2.41fg
KT3I1B-1	35b	–	12.2ab	3.21b
KT3I1B-2	35b	–	18.3a	3.07bc
KT3I1B-3	32b	–	10.4ab	2.30g
S. kanamycetius + KT3I1B-1	28b	–	16.3a	2.43fg
S. kanamycetius + KT3I1B-2	30b	–	4.7ab	2.40fg
S. kanamycetius + KT3I1B-3	28b	–	14.4a	3.24b

*Means in the same column followed by the same letter are not significantly different ($P \leq 0.05$) according to the Duncan Multiple Range Test.

†ND = No Data.