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

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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 311B-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 Graham 1998). 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
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,
Udolic 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 kanamycetus 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, 3Igb-110, 3Igb-76, 3Igb-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 1-110 and 311B-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 antibiotic 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
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 IIb-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 cm³ mason jars, and sealed with a screw capped lid. One hundred cm³ 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 intrinsic 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 3II-B-110 was most inhibited by *S. kanamycetius* and *S. coruleoprinus*, while *A. mediteranei* had no inhibitory effect. Strain 3II-B-76 showed no inhibition by *S. species*. Strain 3II-B-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 \textit{A. mediterranei}, \textit{S. coruleoprunus}, \textit{S. kanamycetius} and \textit{S. species} although not inhibited by \textit{S. rimosus}. Strain 123 was not inhibited by \textit{S. species}, but was significantly inhibited by \textit{S. rimosus}. Strain 127 was inhibited by \textit{S. coruleoprunus} and \textit{S. kanamycetius} and showed no inhibition by either \textit{S. rimosus} or \textit{S. species}. The average percent inhibition of \textit{B. japonicum} by the five actinomycete strains was of the order \textit{S. coruleoprunus} > \textit{S. kanamycetius} > \textit{S. rimosus} > \textit{S. species} > \textit{A. mediterranei}, ranging from 22 to 9.2\% inhibition.

\textit{Testing strains for competency in nodulation and N$_2$ fixation activity}

A total of 81 antibiotic resistant strains of \textit{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 \textit{B. japonicum} for nodule number and occupancy, shoot N content, and nitrogen fixation (acetylene reduction) activity were measured in Trial 1. In Trial 2, strain KT 311B-1 was greater than strains KT311B-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.

\textit{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. mediterranei* 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 1-110 and 311B-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.
japonicum* 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 KN311B-3, KT311B-1 and KT311B-2, and *S.
kanamycetius* plus KN1-110-1, KN311B-2, or KT311B-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, KN311B-2, and KT311B-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 KT311B-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 KN311B-3, KT311B-1, or
KT311B-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.
japonicum* 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.
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Table 1. Selected chemical properties of the soil (Stoy silt loam) used in the soil-pot co-inoculation greenhouse study.¹

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>Soil pH</th>
<th>Bray P&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Extractable P</th>
<th>Exchangeable K</th>
<th>Organic matter content</th>
</tr>
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<tr>
<td>SIUC Agronomy</td>
<td>6.7 ± 0.2</td>
<td>42 ± 1</td>
<td>127 ± 14</td>
<td>2.1 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

¹Values in table are means of five replicates ± the standard deviation (SD).
Table 2. Plate count of seed inocula for the soil-pot co-inoculation greenhouse study.†

<table>
<thead>
<tr>
<th>Inoculum treatment</th>
<th>count (log_{10}/ml)</th>
<th>CV (%)(^t)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. kanamycetius</em></td>
<td>8.36 ± 0.05</td>
<td>0.6%</td>
</tr>
<tr>
<td>KNI-110-1</td>
<td>8.62 ± 0.06</td>
<td>0.7%</td>
</tr>
<tr>
<td>KNI-110-2</td>
<td>8.16 ± 0.13</td>
<td>1.6%</td>
</tr>
<tr>
<td>KNI-110-3</td>
<td>8.73 ± 0.09</td>
<td>1.0%</td>
</tr>
<tr>
<td>KNI-110-5</td>
<td>8.51 ± 0.05</td>
<td>0.5%</td>
</tr>
<tr>
<td>KN3I1B-2</td>
<td>8.63 ± 0.08</td>
<td>0.9%</td>
</tr>
<tr>
<td>KN3I1B-3</td>
<td>8.09 ± 0.15</td>
<td>1.9%</td>
</tr>
<tr>
<td>KT3I1B-1</td>
<td>8.87 ± 0.06</td>
<td>0.7%</td>
</tr>
<tr>
<td>KT3I1B-2</td>
<td>8.87 ± 0.06</td>
<td>0.7%</td>
</tr>
<tr>
<td>KT3I1B-3</td>
<td>8.63 ± 0.04</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

†Values in table are means of three replicates ± the standard deviation (SD)
\(^t\)Percent coefficient of variation.
Table 3. Percent inhibition of *B. japonicum* from soybean nodules.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>ARC$^1$</th>
<th>BRC$^1$</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>83.3a*</td>
<td>96.5a</td>
<td>89.9</td>
</tr>
<tr>
<td>Neomycin</td>
<td>54.1c</td>
<td>76.5c</td>
<td>65.3</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>71.4b</td>
<td>90.1b</td>
<td>80.8</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>75.4b</td>
<td>73.5c</td>
<td>74.0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>69.6b</td>
<td>95.3ab</td>
<td>82.5</td>
</tr>
</tbody>
</table>

$^1$100 μg/ml growth medium  
$^1$Values in table are means of 4,000 nodules  
*Means in the same column followed by the sample letter are not significantly different (P ≤ 0.05) according to the Duncan’s Multiple Range Test.
<table>
<thead>
<tr>
<th>Actinomycete</th>
<th>Test Strain of B. japonicum&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I- 110</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------</td>
</tr>
<tr>
<td>A. mediterranei</td>
<td>3.3c*</td>
</tr>
<tr>
<td>S. coeruleoprunis</td>
<td>23.3b</td>
</tr>
<tr>
<td>S. kanamycetius</td>
<td>46.7a</td>
</tr>
<tr>
<td>S. rimosus</td>
<td>23.3b</td>
</tr>
<tr>
<td>S. species</td>
<td>10.0bc</td>
</tr>
</tbody>
</table>

<sup>1</sup>Three replications per strain.
*Means in the same column followed by the same letter are not significantly different (P≤0.05) according to the Duncan Multiple Range Test.
Table 5. Symbiotic characteristics of selected antibiotic resistant strains of *B. japonicum*: competence study.

<table>
<thead>
<tr>
<th><em>B. japonicum</em></th>
<th>Trial Number</th>
<th>Nodule Number</th>
<th>Nodule Occupancy*</th>
<th>Shoot N Content</th>
<th>Acetylene Reduction Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental Strain (311b-110)</td>
<td>1</td>
<td>44ab*</td>
<td>10b</td>
<td>2.38a†</td>
<td>3ab</td>
</tr>
<tr>
<td>KN311B-2</td>
<td>40.2ab</td>
<td>80a</td>
<td>2.10ab</td>
<td>3ab</td>
<td></td>
</tr>
<tr>
<td>KN311B-3</td>
<td>31.2bcd</td>
<td>95.9a</td>
<td>1.91abc</td>
<td>3ab</td>
<td></td>
</tr>
<tr>
<td>Parental Strain (1-110)</td>
<td>50a</td>
<td>0b</td>
<td>2.43a</td>
<td>1b</td>
<td></td>
</tr>
<tr>
<td>KNI-110-1</td>
<td>31bcd</td>
<td>98.6a</td>
<td>1.38cde</td>
<td>5a</td>
<td></td>
</tr>
<tr>
<td>KNI-110-2</td>
<td>37abcd</td>
<td>97.3a</td>
<td>1.19e</td>
<td>5a</td>
<td></td>
</tr>
<tr>
<td>KNI-110-3</td>
<td>29cd</td>
<td>100a</td>
<td>1.53cde</td>
<td>6a</td>
<td></td>
</tr>
<tr>
<td>KNI-110-5</td>
<td>23d</td>
<td>100a</td>
<td>1.05c</td>
<td>4a</td>
<td></td>
</tr>
<tr>
<td>Parental Strain (311B-110)</td>
<td>2</td>
<td>22</td>
<td>0b</td>
<td>2.04§</td>
<td>59</td>
</tr>
<tr>
<td>KT311B-1</td>
<td>32</td>
<td>100a</td>
<td>2.23</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>KT311B-2</td>
<td>26</td>
<td>19.4b</td>
<td>2.17</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>KT311B-3</td>
<td>25</td>
<td>0b</td>
<td>2.11</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

*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 + tetracycline (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≤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.

<table>
<thead>
<tr>
<th>Inoculum Treatment</th>
<th>Nodule Number</th>
<th>Nodule Occupancy</th>
<th>Shoot N Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KN</td>
<td>KT</td>
</tr>
<tr>
<td>Non-Inoculated Control</td>
<td>28b*</td>
<td>6.1b</td>
<td>0b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.50efg</td>
</tr>
<tr>
<td>N Fertilizer Control</td>
<td>6c</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.97a</td>
</tr>
<tr>
<td><em>S. kanamycetius</em></td>
<td>31b</td>
<td>11.4b</td>
<td>6.3ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.43efg</td>
</tr>
<tr>
<td>KNI-110-1</td>
<td>28b</td>
<td>1.0b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.29g</td>
</tr>
<tr>
<td>KNI-110-2</td>
<td>46a</td>
<td>5.2b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.56efg</td>
</tr>
<tr>
<td>KNI-110-3</td>
<td>31b</td>
<td>3.3b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.21g</td>
</tr>
<tr>
<td>KNI-110-5</td>
<td>29b</td>
<td>6.6b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.41g</td>
</tr>
<tr>
<td>KN311B-2</td>
<td>32b</td>
<td>0b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.40fg</td>
</tr>
<tr>
<td>KN311B-3</td>
<td>28b</td>
<td>19.6b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.99bcd</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KNI-110-1</td>
<td>28b</td>
<td>44.8a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.13bc</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KNI-110-2</td>
<td>32b</td>
<td>14.1b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.81cde</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KNI-110-3</td>
<td>29b</td>
<td>12.0b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.40fg</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KNI-110-5</td>
<td>32b</td>
<td>48.0a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.77cdef</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KN311B-2</td>
<td>26b</td>
<td>55.0a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.05bcd</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KN311B-3</td>
<td>24b</td>
<td>16.4b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.41fg</td>
</tr>
<tr>
<td>KT311B-1</td>
<td>35b</td>
<td></td>
<td>12.2ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.21b</td>
</tr>
<tr>
<td>KT311B-2</td>
<td>35b</td>
<td></td>
<td>18.3a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.07bc</td>
</tr>
<tr>
<td>KT311B-3</td>
<td>32b</td>
<td></td>
<td>10.4ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.30g</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KT311B-1</td>
<td>28b</td>
<td></td>
<td>16.3a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.43fg</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KT311B-2</td>
<td>30b</td>
<td></td>
<td>4.7ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.40fg</td>
</tr>
<tr>
<td><em>S. kanamycetius</em> + KT311B-3</td>
<td>28b</td>
<td></td>
<td>14.4a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.24b</td>
</tr>
</tbody>
</table>

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

1ND = No Data.