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

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

2 The utilization of actinomycetes as potential soybean co-inoculants were
3 evaluated in this study. Soil samples from Carbondale and Belleville, Illinois were used
4 to inoculate pre-germinated soybeans plants for the determination of antibiotic
5 sensitivity in the native *Bradyrhizobium japonicum* population. Sensitivity was of the
6 order kanamycin > ~~tetracycline~~ > oxytetracycline > rifampicin > neomycin. Antagonism
7 by five actinomycete cultures toward seven test strains of *B. japonicum* was also
8 assessed. The ranking average inhibition (across all seven *B. japonicum* strains) by
9 these actinomycetes was: *Streptomyces kanamycetius* = *S. coruleoprunis* > *S. rimosus* >
10 *S. species* > *Amycolatopsis mediterranei*. A total of ten antibiotic combinations were
11 used to isolate antibiotic resistant mutants of *B. japonicum* stains I-110 and 3I1B-110
12 via successive cycles of mutation. Eighty-one antibiotic resistant strains were isolated
13 and tested for symbiotic competency, and nine of these were selected for further
14 characterization in a greenhouse pot study. Few differences in nodule number were
15 caused by these treatments. Nodule occupancy varied from 0 to 18.3% when antibiotic
16 resistant strains of *B. japonicum* were used as the sole inoculants. However, when three
17 mutant strains of *B. japonicum* were co-inoculated with *S. kanamycetius* significant
18 increases in nodule occupancy (up to 55%) occurred. Increases in shoot N composition
19 (27.1 to 40.9%) were also caused by co-inoculation with *S. kanamycetius*.

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24 *Key Words:* *Bradyrhizobium japonicum*, *Streptomyces kanamycetius*, indigenous
25 bradyrhizobia, co-inoculation, nodule occupancy
26
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Introduction

1
2 Many nitrogen fixing bacteria convert gaseous nitrogen (N₂) to ammonia
3 contributing to an important source of plant-available soil nitrogen. *Bradyrhizobium*
4 *japonicum* forms a symbiotic relationship with soybean [*Glycine max* (L.)], resulting in
5 biological nitrogen fixation. In Illinois it is estimated that 50 to 60 million dollars of
6 nitrogen fertilizer credit is assigned on a yearly basis to soybean-corn or soybean-wheat
7 rotations. Assuming an average seed nitrogen content of 5.5%, the estimated saving in
8 nitrogen fertilizer costs is an additional 125 million dollars. Increasing seed nitrogen
9 content could provide additional value as a protein supplement in foods and feeds.
10

11 Superior N₂ fixing strains of *Bradyrhizobium* have been identified. However,
12 highly competitive native soil bradyrhizobia limit the ability to control infection of
13 soybean roots by an added inoculum strain. This is referred to as the *Bradyrhizobium*
14 competition problem (Streeter 1994; Sadowsky and Graham 1998). Successful
15 inoculation of soybean is dependent upon overcoming competition of native
16 bradyrhizobia and the establishment of the applied strain in soil.
17

18 *B. japonicum* serocluster 123, including the serogroups USDA 123, 127 and 129
19 (Schmidt et al. 1986), alone inhabits 50 to 90% of soybean nodules in the Midwestern
20 USA (Damirgi et al. 1967; Ellis et al. 1984; Kapusta and Rouwenhorst 1973; Moawad
21 et al. 1984). These serogroups are very competitive (Cregan et al. 1989; Ellis et al.
22 1984; Ham et al. 1971; Klubek et al. 1988) and may be inefficient in N₂ fixation
23 (Caldwell and Vest 1970; Ham 1980).
24

25 Inoculation with *B. japonicum* has been successful in increasing soybean
26 nodulation with increases in plant fresh weight, seed protein and seed yield in soils with
27

1 a low or absent native population (Abel and Erdman 1964; Caldwell and Vest 1970).

2 However, in soils with an established bradyrhizobial population, competition severely
3 limits nodulation by an inoculum and it is not easily enhanced (Ham et al. 1971;
4 Kapusta and Rouwenhorst 1973; Weaver and Frederick 1974; Thies et al. 1992).

5 McLoughlin et al. (1990) used inoculum levels of 10^8 cells per 2.5 cm length of row,
6 and obtained a nodule occupancy of less than 42% with little persistence in the soil.

7
8 Ellis et al. (1984) found that the application of high levels of an applied strain increased
9 the population size of that strain in soil but did not increase nodule occupancy.

10 Additionally, Brockwell et al. (1987) and Roughley et al. (1993) found over 90% of
11 their inoculum died within twenty-four hours of seed application.

12
13 The use of bactericidal agents has been shown to inhibit native bradyrhizobia.

14 Hossain and Alexander (1984) found that the addition of the fungicide benomyl and the
15 antibiotics erythromycin and streptomycin effectively enhanced colonization of
16 soybean, when inoculated with a strain of *Bradyrhizobium* resistant to these
17 antimicrobial compounds. These antimicrobial agents were able to effectively reduce
18 predation by protozoa or competition by native bradyrhizobia and allow the introduced
19 strain to nodulate soybean. Jones and Giddens (1984) found that fungicide-resistant
20 mutants of *B. japonicum* USDA strain 110, when used with the appropriate fungicide,
21 experienced enhanced survival in the soil and the bacterial counts within the nodules
22 were increased. Further studies by Li and Alexander (1986) used streptomycin
23 amendments to the soil to limit the growth of *Sinorhizobium meliloti* allowing the
24 resistant inoculum strain to increase nodule number and occupancy versus the treatment
25 without the antibiotic.
26
27

1 Studies conducted by Dashti et al. (1997) indicate that co-inoculation with plant
2 growth promoting rhizobacteria (PGPR) and *B. japonicum* improved plant development
3 and growth, and the grain and protein yield of soybean crops. Co-inoculation with
4 *Azospirillum* has also been successful in increasing root number and length, root
5 biomass, root hair development, shoot biomass, nodule number, and the fresh weight of
6 soybean (Molla et al. 2001). Recently, Tokala et al. (2002) reported on a novel plant-
7 microbe interaction between *Streptomyces lydicus* strain WYEC 108 and peas [*Pisum*
8 *sativum* (L.)]. *S. lydicus* was shown to increase nodulation frequency, nodule size and
9 mass, nitrogenase activity, and root and shoot weight.
10

11 The objectives of this study were to: (1) determine the antibiotic sensitivity of
12 the native bradyrhizobia in southern Illinois soils; (2) isolate mutants of *B. japonicum*
13 that are efficient in nitrogen fixation and resistant to the antibiotics most inhibiting to
14 the native bradyrhizobia; and (3) assess nodule occupancy and effectivity in nitrogen
15 fixation by the antibiotic resistant strains of *B. japonicum* when co-inoculating soybean
16 with the appropriate antibiotic-producing actinomycete strain.
17

18 **Materials and Methods**

19 *Soil Sampling*

20
21 Twenty-five soil samples per location were randomly collected from five
22 soybean research plots from the Southern Illinois University-Carbondale Agronomy
23 Research Center (ARC) and the Belleville Research Center (BRC). Each research plot
24 was subdivided into five subplots where four to six 2.5 cm dia cores (15 cm deep) were
25 collected along a 6 m transect between the center rows of each subplot, and placed into
26 sterile 355 ml Whirl Pack bags. Each composite soil sample from each subplot was
27

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

13 *Testing for intrinsic resistance*

14 Soybean (LS90-1920, a cultivar developed in the SIUC soybean breeding
15 program, Schmidt et al.1999) were grown in washed vermiculite under a 16 hr photo
16 period with fluorescent lighting at the SIUC Horticulture Research Center greenhouse,
17 Carbondale, Illinois. Seven days after planting, 10 g of each soil sample were mixed
18 with a sterile 90 ml dilution blank, shaken for 1 minute, and used as an inoculum (1 ml
19 per plant). Ten plants (replications) were used for each soil sample. The inoculated
20 soybean plants were grown for an additional 35 days and watered as necessary with a
21 nitrogen free mineral nutrient solution. At harvest the plants were removed from their
22 pots with the plant shoots cut just above the roots. The root system of each plant was
23 then washed free of vermiculite, blotted dry and placed in a sterile Whirl Pak bag and
24 stored in a freezer at -20°C . The soybean nodules from these root samples were used to
25 assess the sensitivity of the native *B. japonicum* population to selected antibiotics.
26
27

1 Following the procedure of Lieberman et al. (1986), 16 nodules of each root
2 were randomly selected, washed, surfaced sterilized with 20% (v/v) bleach and 70%
3 (v/v) ethanol, rinsed three times with distilled water, crushed, and plated on yeast
4 extract mannitol (YEM) agar supplemented with one of the following antibiotics at a
5 concentration of 100 µg/ml: neomycin (N), oxytetracycline (O), rifampicin (R),
6 tetracycline (T), or kanamycin (K). Cycloheximide was also added to all of the growth
7 media at 200 µg/ml to suppress the growth of fungi. A set of YEM plates without
8 antibiotics were used as a positive control. All plates were grown for 7 to 14 days at
9 28°C and scored for growth as compared to the control. The number of developing
10 colonies on the YEM-antibiotic plates divided by the number of developing colonies on
11 the complementary YEM-control plates multiplied by 100 determined the percent
12 intrinsic resistance by the native bradyrhizobia.
13

14 *Testing of antagonistic strains*

15
16 Actinomycete cultures that produce the antibiotics used in nodule typing
17 (described above) were obtained from the American Type Culture Collection (ATCC):
18 *Amycolatopsis mediterranei* ATCC #13685 (rifampicin), *Streptomyces coeruleoprunus*
19 ATCC #43681 (neomycin), *Streptomyces kanamycetius* ATCC #12853 (kanamycin),
20 *Streptomyces rimosus* sub sp. *rimosus* ATCC #33022 (oxytetracycline), and
21 *Streptomyces* sp. ATCC #11652 (tetracycline). The test strains of *B. japonicum* used
22 were USDA strains I-110, 3I1b-110, 3I1b-76, 3I1b-24, 123, and 127, and strain An-5 (a
23 streptomycin resistant strain of serogroup 123 received from Dr. R.M. Zablutowicz, the
24 USDA Weed Science Laboratory, Stoneville, MS). Each test strain was grown to
25 stationary phase (5 days) in 100 ml of YEM broth at room temperature on a tabletop
26
27

1 shaker at 170 rpm. YEM agar plates were spread with 0.1 ml of broth culture and then
2 streaked with an actinomycete culture in the form of a plus sign. A YEM agar plate
3 inoculated with individual *B. japonicum* test strains was used in all cases as a positive
4 control allowing a visual comparison. Following five days of growth at 28°C, inhibition
5 by the actinomycete cultures in the four corners of the '+' pattern was visually rated on a
6 scale of 1 to 10 and statistically analyzed after arcsine transformation (Little and Hills
7 1978).
8

9 *Selection of antibiotic resistant strains*

10 Antibiotic resistant mutants of *B. japonicum* strains I-110 and 3I1B-110 were
11 obtained via successive cycles of mutation (Cooper 1979), on YEM agar plates
12 amended with any combination of two antibiotics: kanamycin + neomycin (KN),
13 kanamycin + tetracycline (KT), kanamycin + rifampicin (KR), kanamycin +
14 oxytetracycline (KO), oxytetracycline + tetracycline (OT), neomycin + tetracycline
15 (NT), rifampicin + tetracycline (RT), neomycin + oxytetracycline (NO), rifampicin +
16 oxytetracycline (RO), and rifampicin + neomycin (RN). The *B. japonicum* strains were
17 grown in 100 ml YEM broth culture at room temperature on a tabletop shaker at 170
18 rpm for 5 days, as previously described. YEM agar plates amended with 12.5 µg/ml of
19 each antibiotic combination listed above were inoculated with 0.1 ml of broth culture.
20
21 The plates were then incubated at 28°C for 7 to 14 days. Individual colonies growing
22 on the plates of each antibiotic combination were transferred to new plates containing
23 25 µg/ml of each antibiotic combination streaked for isolation, and grown as
24 previously described. This step-wise successive transfer of isolates was followed until
25
26 five different isolates were obtained from each antibiotic combination at a final
27

1 concentration of 100 µg/ml.

2 *Testing of antibiotic resistant strains for competency in nodulation and N₂ - fixation*
3 *activity*

4 A 0.9 meter by 3.4 meter greenhouse bench was enclosed in clear plastic canopy
5 to eliminate potential contamination of soybean plants by *B. japonicum* bearing. Lights
6 were installed under the canopy to allow for a 16 hour **photo period**. The soybean
7 plants (LS 90-1920) were grown using washed vermiculite in 12 cm x 8.5 cm diameter
8 plastic cups modified with three drainage holes. A lattice board comprised of 2.5 cm
9 wide wood strips was placed on top of the greenhouse bench supported by the bench's
10 sides. The cups were inserted in the lattice holes (7 cm x 7 cm) suspending them above
11 the bench top. Three surface sterilized seeds (an eight minute exposure to 10% v/v
12 H₂O₂) were planted in each pot and this was reduced to one seedling per pot after
13 approximately 5 days after seeding. The plants were inoculated at one week after
14 planting with 1 ml of a 7 day old broth (YEM) culture of an antibiotic resistant strain of
15 *B. japonicum*. The wild type strains I-110 or 3 I1b-110 were included as controls for
16 each trial evaluating eight to ten resistant strains. Each inoculum treatment was
17 replicated five times and watered as needed with a N free mineral nutrient solution. In
18 total, nine trials were required to assess 81 isolated antibiotic resistant strains.
19
20
21
22 Harvesting occurred at 35 days after inoculation to assure for the development of
23 functional nodules.

24 At harvest, plants were removed from their pots and the plant shoots were cut
25 just above the root as previously described. The roots were shaken free of vermiculite,
26 placed in 946 cm³ mason jars, and sealed with a screw capped lid. One hundred cm³ of
27

1 air was removed and replaced with 100 cm³ acetylene using a 50 cm³ syringe with a 25
2 gauge needle. The jars were incubated for one hour at room temperature in the
3 greenhouse. A 10 cm³ sample was then removed and analyzed for ethylene by gas
4 chromatography (Hardy et al. 1968). Plant shoots were dried at 70°C for 48 hours,
5 ground and analyzed for total N by Brookside Laboratories, New Knoxville, Ohio.

6 Nodules were counted and **nodule occupancy** was determined following the **procedure**
7 of Lieberman et al. (1986). The number of developing colonies on the YEM-antibiotic
8 plates divided by the number of developing colonies on the complementary YEM-
9 control plates multiplied by 100 gave the percent nodule occupancy by the applied
10 strain.
11

12 *Soil pot co-inoculation greenhouse study*

13
14 Approximately 0.06 m³ of soil (0 to 15 cm deep) was obtained from the SIU-C
15 Agronomy Research Center. The soil is classified as a Stoy silt loam, and corn was
16 produced at that site the previous year.

17 The soil was allowed to air dry on a greenhouse bench, ground and analyzed for
18 exchangeable potassium (Knudsen et al., 1982), Bray P1 extractable phosphorus (Olsen
19 and Sommers 1982) and pH (McLean 1982). These results are summarized in Table 1.
20 Two hundred thirty, 12 cm x 8.5 cm cups were modified with three drainage holes and
21 lined with cheese cloth on the bottom of each cup. The cups were then filled with the
22 ground soil. Additionally, for a negative-nodulation control, ten cups were filled with
23 soil amended with 75 mg N (as ammonium nitrate)/kg soil. The soils were moistened to
24 field capacity as described by Sabey et al (2003).
25

26 *S. kanamycetius* and ten antibiotic resistant strains of *B. japonicum* selected for
27

1 the co-inoculation study were grown in YEM broth as previously described. Fifty
2 soybean seeds (LS 90-1920) were treated as follows: (1) 0.4 ml of 10% (w/v) gum
3 acacia (used as a seed adhesive for the applied *S. kanamycetius* and/or *B. japonicum*
4 cells) and 0.5 g activated charcoal (a coating of fine solid particles to avoid seed
5 clumping) (**non-inoculated control**); (2) 0.3 ml of *S. kanamycetius* broth culture
6 followed by the addition of gum acacia and activated charcoal as previously described;
7 (3) 0.3 ml (each) of *B. japonicum* and *S. kanamycetius* followed by gum acacia and
8 activated charcoal. The mean plate count of each inoculum strain is given in Table 2.
9

10 The inoculated seeds were immediately planted into the soil pots (3 seeds per pot
11 for 10 replications per treatment) and randomized in a complete block design. Five days
12 following emergence, each experimental unit (pot) was thinned to one seedling per pot.
13

14 Plants were watered as needed with potable water from the greenhouse, and a 16
15 hour photoperiod was used as previously described. Thirty-five days after planting the
16 plants were removed from the soil with the plant shoots severed just above the root
17 system. The roots were washed free of soil and placed in Whirl Pak bags and stored in a
18 freezer at -20°C until measurements could be made. Nodule number and occupancy
19 was determined as previously described. Nodules were plated on YEM agar plates
20 containing KN or KT (100 µg/ml per antibiotic), and YEM agar alone as a positive
21 control. The plates were incubated 10 to 14 days and scored for growth. Plant shoots
22 were dried at 70°C for 48 hours, ground to pass a 100 mesh sieve, and analyzed for total
23 N by Brookside Laboratories, New Knoxville, Ohio.
24

25 *Statistical analysis*

26 All of the data were analyzed by an Analysis of Variance (ANOVA), and mean
27

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

5 **Results**

6 *Determination of intrinsic antibiotic resistance*

7
8 Neomycin had a lower inhibitory effect on the native bradyrhizobia (54.1%)
9 from the ARC nodule samples than the other four antibiotics (Table 3), while
10 kanamycin had the highest inhibitory effect (83.3%). The efficacy of the test antibiotics
11 was in the order: kanamycin > rifampicin > oxytetracycline > tetracycline > neomycin.
12 The bradyrhizobia from the BRC nodule samples were inhibited the least by neomycin
13 and rifampicin (76.5 and 73.5%, respectively). Tetracycline and kanamycin were most
14 inhibitory (95.3 and 96.5%) to the bradyrhizobia from this site. Inhibition for the BRC
15 strains was in the order: kanamycin > tetracycline > oxytetracycline > neomycin >
16 rifampicin. Overall inhibition across the two sites was of the order kanamycin >
17 tetracycline > oxytetracycline > rifampicin > neomycin.

19 *Testing of antagonist strains*

20
21 The percent inhibition by actinomycete strains when inoculated and grown
22 simultaneously with the *B. japonicum* test strains is given in Table 4. *B. japonicum*
23 strain I-110 was most inhibited by *S. kanamycetius*. Strain 3I1B-110 was most inhibited
24 by *S. kanamycetius* and *S. coruleoprunis*, while *A. mediteranei* had no inhibitory effect.
25 Strain 3I1B-76 showed no inhibition by *S.* species. Strain 3I1B-24 was sensitive to all
26 of the actinomycete strains, while strain An-5 (a member of serogroup 123 resistant to
27

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

9 *Testing strains for competency in nodulation and N₂ fixation activity*

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

26 *Soil-pot greenhouse study*

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

1 were no differences among treatments for nodule number (Table 6). The KNI-110-2
2 treatment had a higher nodule count than all other treatments while the N fertilizer
3 control (as expected) had the lowest nodule count. Nodule occupancy varied from 0 to
4 18.3% when the KN or KT strains were used as sole inoculants. However, an increase
5 in nodule occupancy occurred with co-inoculation by *S. kanamycetius* and strain KNI-
6 110-1, KNI-110-5, or KN3I1B-2, improving from 1.0, 6.6 and 0% to 44.8, 48.0, and
7 55.0%, respectively. The *S. kanamycetius* treatment resulted in a nodule occupancy that
8 was not different from the non-inoculated control. An increase in shoot N content over
9 the non-inoculated control by the co-inoculum treatments of *S. kanamycetius* and strains
10 KNI-110-1, KN3I1B-2, or KT3I1B-3 was determined (3.13, 3.05, and 3.24%,
11 respectively). However, inoculation by strains KN3I1B-3, KT3I1B-1 and KT3I1B-2
12 without *S. kanamycetius* also resulted in a higher shoot N contents (2.99, 3.21, and
13 3.07% respectively) over the non-inoculated control treatment. All other inoculum
14 treatments were equivalent in shoot N content to the non-inoculated control.
15
16

17 Discussion

18 The main objectives of this study were to assess the antibiotic sensitivity of the
19 native bradyrhizobia in southern Illinois soils, and to develop antibiotic resistant strains
20 of *B. japonicum* which may be used to co-inoculate soybean crops with the appropriate
21 antibiotic-producing actinomycete strain.
22

23 At the ARC and BRC research centers, the native bradyrhizobia showed greater
24 sensitivity to kanamycin than neomycin despite their similarities in modes of action.
25 This may be attributed to a plasmid encoded resistance to neomycin, but not kanamycin,
26 at these locations and/or the presence of streptomycetes which synthesize neomycin or
27

1 neomycin-like agents resulting in the selection for neomycin resistance. Tetracycline
2 and oxytetracycline were similar in their degree of inhibition at both the ARC and BRC
3 locations. These results suggest that varying populations of actinomycetes (which
4 produce these antibiotics) exist at the Carbondale and Belleville locations. However,
5 similar results for rifampicin inhibition were obtained for both locations, implying
6 similar populations of *Amycolatopsis mediterranei* may exist at both field sites.
7

8 Collectively, the inhibition of *B. japonicum* from soybean nodules derived from
9 these two locations was not identical, indicating that antibiotic sensitivity varies from
10 location to location. However, on average, kanamycin was most effective in suppressing
11 the growth of the native bradyrhizobia. Marciniak (1984) reported a greater incidence
12 of intrinsic resistance to streptomycin, kanamycin and rifampicin at Brownstown,
13 Illinois but found a greater sensitivity by the native bradyrhizobia to these same
14 antibiotics at Belleville, Flora and Vergennes, Illinois. Mueller et al. (1988) reported
15 that the intrinsic resistance of *B. japonicum* isolated from South Carolina soils to
16 antibiotics was of the order: streptomycin >> streptomycin + neomycin > streptomycin
17 + rifampicin > kanamycin + rifampicin = kanamycin + nalidixic acid. These authors
18 concluded that intrinsic resistance by native bradyrhizobia was the norm for South
19 Carolina soils.
20
21

22 The efficacy of the actinomycete cultures in suppressing the growth of the *B.*
23 *japonicum* test strains was variable. On average, the lower antibradryzobial activity
24 expressed by *S.* species and *A. mediteranei* versus *S. coeruleoprunis*, *S. kanamycetius*,
25 and *S. rimosus* when grown in the presence of the *B. japonicum* test strains (mimicking
26 co-inoculation on the seed coat) suggests that the former strains require the
27

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

15 Only nine isolated strains, resistant to either kanamycin + neomycin or
16 kanamycin + tetracycline, were chosen for additional greenhouse studies. The
17 remaining resistant strains produced few or no nodules indicating that resistance to the
18 other combinations of selected antibiotics interfered with the root-hair infection process.
19 Tetracycline and oxytetracycline are more aggressive protein synthesis inhibitors than
20 neomycin and kanamycin (Stryer 1995.). The strains resistant to oxytetracycline plus
21 neomycin or kanamycin, and neomycin plus tetracycline may have acquired an altered
22 30s subunit of rRNA to survive the mode of action of these antibiotics (Stryer 1995). A
23 mutation of this kind would most likely lead to numerous alterations of proteins
24 including the cell wall structure interfering with host recognition and eventual
25 nodulation. Resistance to an antibiotic may also be due to an alteration of the antibiotic
26
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1 itself, rendering it nonfunctional, pumping the antibiotic out of the cell, or altering the
2 site of action of the antibiotic. The KN and KT strains summarized in Table 5 may have
3 evolved on these latter mechanisms without interfering with the root-hair infection
4 process.

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

16 The addition of nitrogen as a fertilizer control was used to compare the
17 differences in shoot N content between biologically fixed and applied N. Although this
18 fertilizer treatment resulted in a higher N content than all other treatments, the benefits
19 of soybean inoculation by strains KN3I1B-3, KT3I1B-1 and KT3I1B-2, and *S.*
20 *kanamycetius* plus KNI-110-1, KN3I1B-2, or KT3I1B-3 were realized with a 19.6 to
21 29.6% increase in shoot N content over the non-inoculated control. For the latter three
22 co-inoculum treatments, a 27.1 to 40.9% increase in shoot N content was determined
23 over the same *B. japonicum* strains without *S. kanamycetius*. The increase in nodule
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1 occupancy by strains KNI-110-1, KNI-110-5, KN3I1B-2, and KT3I1B-3 when co-
2 inoculated with *S. kanamycetius* further demonstrates the potential synergistic benefits
3 of co-inoculation by any combination of these strains. The 4% increase in nodule
4 occupancy by strain KT3I1B-3 when co-inoculated with *S. kanamycetius* may also have
5 contributed, in part, to the increase in shoot N content. Conversely, the lack of an
6 increase in shoot N content by the co-inoculum treatment KN3I1B-3, KT3I1B-1, or
7 KT3I1B-2 with *S. kanamycetius* suggests that any positive interaction between *S.*
8 *kanamycetius* and a strain of *B. japonicum* may be highly specific. Thus, the most
9 compatible strains of *B. japonicum* in association with *S. kanamycetius* offers the
10 greatest potential for successful soybean inoculation.
11

12 The higher nodule number following inoculation with KNI-110-2 inoculum
13 treatment did not result in an improvement in nodule occupancy by this strain or shoot
14 N content. Although co-inoculation by this strain with *S. kanamycetius* reduced the
15 number of nodules, it did improve nodule occupancy 2.7 fold and shoot N content by
16 9.8%. It may also be that the strain caused an increase in nodule occupancy by a more
17 efficient strain, resulting in a greater shoot N content, and also a reduction in the number
18 of nodules required to fix N. These results show that no relationship exists between
19 nodule number and nodule occupancy or shoot N content, but that co-inoculation with *S.*
20 *kanamycetius* may improve the occupancy of an applied strain.
21

22 The problem of successful soybean inoculation by an applied strain of *B.*
23 *japonicum* still exists. It is clear from this study that the co-inoculation of soybeans
24 with an appropriate actinomycete strain may be a novel approach in overcoming
25 competition by the native bradyrhizobia, and the establishment of an applied strain.
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1 Thus, it may also be important to test the ability of actinomycete strains to colonize
2 soybean roots to overcome the problem of competition for root-hair infection sites in
3 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.[†]

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 inocula for the soil-pot co-inoculation greenhouse study.[†]

Inoculum treatment	count (log ₁₀ /ml)	CV (%) [‡]
<i>S. kanamycetius</i>	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 <i>B. japonicum</i> [†]							Average
	I-110	3I1B-110	3I1B-76	3I1B-24	An-5	123	127	
-----% Inhibition-----								
<i>A. mediterranei</i>	3.3c*	0c	10.0a	43.3bc	6.7ab	10.0ab	3.3b	9.2
<i>S. coeruleoprunis</i>	23.3b	26.7ab	13.3a	76.7a	10.0a	6.7a	10.0a	22.0
<i>S. kanamycetius</i>	46.7a	40.0a	13.3a	36.7c	10.0a	6.7b	13.3a	20.4
<i>S. rimosus</i>	23.3b	13.3b	13.3a	76.7a	0b	20.0a	0b	19.6
<i>S. species</i>	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.

<i>B. japonicum</i>	Trial Number	Nodule Number	Nodule Occupancy [†]	Shoot N Content	Acetylene Reduction Activity
			-----%-----	--%--	-- $\mu\text{mol C}_2\text{H}_4$ 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 $\mu\text{g/ml}$ (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 ($P \leq 0.05$) 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		Shoot N Content
		KN	KT	
Non-Inoculated Control	28b*	6.1b	0b	2.50efg
N Fertilizer Control	6c	ND [†]	ND	3.97a
<i>S. kanamycetius</i>	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
<i>S. kanamycetius</i> + KNI-110-1	28b	44.8a	–	3.13bc
<i>S. kanamycetius</i> + KNI-110-2	32b	14.1b	–	2.81cde
<i>S. kanamycetius</i> + KNI-110-3	29b	12.0b	–	2.40fg
<i>S. kanamycetius</i> + KNI-110-5	32b	48.0a	–	2.77cdef
<i>S. kanamycetius</i> + KN3I1B-2	26b	55.0a	–	3.05bcd
<i>S. kanamycetius</i> + 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
<i>S. kanamycetius</i> + KT3I1B-1	28b	–	16.3a	2.43fg
<i>S. kanamycetius</i> + KT3I1B-2	30b	–	4.7ab	2.40fg
<i>S. kanamycetius</i> + 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.