Enhanced attachment of *Bradyrhizobium japonicum* to soybean through reduced root colonization of internally seedborne microorganisms

Nathan W. Oehrle, Dale B. Karr, Robert J. Kremer, and David W. Emerich

Abstract: Internally seedborne microorganisms are those surviving common surface sterilization procedures. Such microbes often colonize the radicle surface of a germinating soybean (*Glycine max*) seed, introducing an undefined parameter into studies on attachment and infection by *Bradyrhizobium japonicum*. Bacterial isolates from surface-sterilized soybean seed, cv. Williams 82 and cv. Maverick, used in our studies, were identified as *Agrobacterium radiobacter*, *Aeromonas* sp., *Bacillus* spp., *Chryseomonas luteola*, *Flavimonas oryzihabitans*, and *Sphingomonas paucimobilis*. Growth of these microbes during seed germination was reduced by treating germinating seeds with 500 μg/mL penicillin G. The effects of this antibiotic on seedling development and on *B. japonicum* 2143 attachment, nodulation, and nitrogen fixation are reported here. Penicillin G treatment of seeds did not reduce seed germination or root tip growth, or affect seedling development. No differences in nodulation kinetics, nitrogen fixation onset or rates were observed. However, the number of *B. japonicum* attached to treated intact seedlings was enhanced 200–325%, demonstrating that other root-colonizing bacteria can interfere with rhizobial attachment. Penicillin G treatment of soybean seedlings can be used to reduce the root colonizing microbes, which introduce an undefined parameter into studies of attachment of *B. japonicum* to the soybean root, without affecting plant development.

Key words: internally seedborne microorganisms, penicillin G, *Bradyrhizobium japonicum*, microbial attachment, soybean (*Glycine max*).

Résumé : Les microorganismes associés et transmis directement par les graines de semence sont ceux qui résistent aux méthodes habituelles de stérilisation de la surface des graines. Ces microbes colonisent souvent la surface des radicules des germes de la fève de soja (*Glycine max*), ce qui introduit un paramètre incontrôlable dans les études sur l'adhérence et l'infection par le *Bradyrhizobium japonicum*. Dans cette étude, nous avons utilisé des bactéries isolées de la surface de graines de fèves de soja stérilisées, cv. Williams 82 et cv. Maverick, et ces bactéries ont été identifées *Agrobacterium radiobacter*, *Aeromonas* sp., *Bacillus* spp., *Chryseomonas luteola*, *Flavimonas oryzihabitans* et *Sphingomonas paucimobilis*. Lors de la germination des graines, le développement des bactéries a pu être réduit en traitant les graines germantes avec 500 μg/mL de pénicilline G. Notre étude rapporte les effets de ce traitement antibiotique sur le développement des jeunes plants et l'adhérence du *B. japonicum*, la formation de nodules et la fixation de l'azote. Le traitement par la pénicilline G n'a pas diminué la germination des graines ou la croissance à la pointe de la racine et n'a pas influencé le développement des jeunes plants. Aucune différence n'a été observée au niveau des cinétiques de formation de nodules, du début et des taux de fixation de l'azote. Par contre, le nombre de cellules de *B. japonicum* fixées aux jeunes plants traités augmentait de 200 à 325 %, ce qui confirme que d'autres bactéries qui colonisent les racines peuvent interférer avec l'adhérence des rhizobiums. Le traitement par la pénicilline G pourrait être appliqué aux jeunes plants de fève soja pour réduire le nombre de microorganismes qui colonisent les racines, ce qui introduit un paramètre non quantifiable lors d'études d'adhérence du *B. japonicum* aux racines de la fève de soja mais qui ne modifie pas le développement des plants.

Mots clés : microorganismes transmis par des graines de semence, pénicilline G, *Bradyrhizobium japonicum*, adhérence bactérienne, fève soja (*Glycine max*).

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Seeds of many different plant species can act as the means of survival for pathogens in successive growing seasons. Seedborne bacteria become associated with the seed via the plant vascular system, reproductive structures, cracks or openings in the seed coat (McGee 1981), and through imbibition or storage practices (Mundt and Hinkle 1976). Their interactions with respective plant surfaces range from beneficial to detrimental (Dowling and Broughton 1986; Kremer 1987; Lynch and Wood 1988; Liu and Sinclair 1993; Zhang et al. 1997) and their presence is determined by the structure, physiology, and variety of the plant (Mundt and Hinkle 1976). These internally seedborne microbes (infecting the seed within the seed coat as opposed to externally seedborne, infesting only the outside of the seed; Sinclair 1993) can survive seed surface sterilization (Mundt and Hinkle 1976; Schiller et al. 1977; Tenne et al. 1977; Grybauskas et al. 1979; Kremer 1987).

Soybean (*Glycine max*) seed is often infested with a multitude of microbes (Sinclair 1993), and few laboratory investigations of early symbiotic events between rhizobia and legumes have accounted for the impact of internally seedborne microbes on the initiation of attachment and infection. Three facts remain however; (*i*) all seedborne microbes can infect seedlings and potentially colonize the root surface (Sinclair 1993); (*ii*) competition between rhizobia and root-colonizing microbes exists (Kosslak et al. 1983; Moawad et al. 1984; Dowling and Broughton 1986; Polonenko et al. 1987; Caetano-Anollés and Bauer 1988); and (*iii*) host rhizosphere populations can greatly outnumber rhizobia inoculants (Moawad et al. 1984; Dowling and Broughton 1986), introducing an important, but difficult-to-define, parameter in rhizobia attachment studies. Here we report on (*i*) the isolated species of internally seedborne microbes infecting two field-grown soybean cultivars, Williams 82 and Maverick; (*ii*) a protocol that reduced their growth on germinating seed and ultimately the seedling radicle; and (*iii*) the effects of this protocol on the symbiosis between *Bradyrhizobium japonicum* 2143 (USDA 3I1b-143 derivative) and soybean.

Detection and identification of internally seedborne microorganisms on our surface-sterilized soybean seeds was assessed by monitoring microbial growth directly from whole seedling radicles and from samples of attachment buffer taken after 5 min incubation with seedlings. Seeds were surface-sterilized with 0.5% sodium hypochlorite (diluted aqueous, Clorox) for 4 min, rinsed extensively with sterile distilled water, soaked in 70% ethanol for 4 min, and finally rinsed extensively with sterile distilled water (Grybauskas et al. 1979; Tenne et al. 1977). Following imbibition, seeds were germinated on sterile distilled water agar (0.6% agar) 48 h prior to microbial growth assessment. The attachment buffer consisted of 0.5 mM MES (2-[N-morpholino]ethanesulfonic acid); 0.5 mM MOPS (2-[N-morpholino]-propanesulfonic acid); 0.2 mM NaCl; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.5 mM $CaCl_2 \cdot 2H_2O$; 0.3 mM $K_2HPO_4 \cdot 3H_2O$; at pH 6.8. This buffer was used consistently during *B. japonicum* attachment to soybean to maintain the rhizosphere at a constant pH. Radicles were embedded in potato dextrose agar (PDA; Grybauskas et al. 1979) pH 5.6; Luria-Bertani (LB; Sambrook et al. 1989) pH 7.0; yeast extract mannitol (YEM; Karr and Emerich 1989) pH 6.8; or Tully's defined media with 25 mM gluconate (TG; Karr and Emerich 1989) pH 7.0; all of which were semi-solid at 0.8% agar. Aliquots of the 5-min seedling incubation in attachment buffer were plated on identical solid media at 1.5% agar. Growth was recorded between 24 and 96 h incubation at 30°C. Isolates were tested for Gram stain, oxidase reaction, motility, morphology, and fluorescence by subculturing on King's B medium (King et al. 1954). Gram-negative isolates were identified using the API 20 NE diagnostic kit (Bio-Mérieux Vitek Inc., Hazelwood, Mo., 63042). Substrates were inoculated with a suspension from each isolate, and after 24–48 h incubation, reactions were scored as positive or negative. Scores, along with the oxidase reaction of the isolate, were used to determine a numerical code matched to profile codes for representative bacterial taxa in the database within the API identification codebook. Classification of both Gram-negative and Gram-positive isolates was verified with gas chromatography – fatty acid methyl ester analysis (GC–FAME; Sasser 1990).

Microbial growth appeared in the surrounding media directly along the radicle surface of surface-sterilized soybean seeds when seedlings were imbedded in PDA, LB, YEM, and TG, and incubated at 30°C. Colonies of seedborne microbes appeared within 24–48 h on solid media spread with aliquots of attachment buffer, sampled after incubation with whole seedling radicles. Those microbes surviving seed surface sterilization were considered to have been internally seedborne. Other sodium hypochlorite concentrations (1.25 and 5.0%) and the inclusion of 0.1% sodium dodecylsulfate, in conjunction with the ethanol treatment, did not eliminate the contamination. Soybean seed, cv. Williams 82 originating from Missouri (MO) and Kansas (KA), and seed cv. Maverick originating in MO, all tested positive for internally seedborne microbes. Typically, 60% of the germinating seeds were infected. Freshly harvested dried seed, and seed stored 6–12 months at 4°C, were infected at similar levels, although the seed germination rate dropped from 80% to 50% over this time, similar to a previous report by Sinclair (1993).

Most isolates were non-pigmented bacteria, and none fluoresced on King's B media. Isolates from cv. Williams 82 MO were identified as *Bacillus pumilus* and *Sphingomonas paucimobilis*. Isolates from cv. Williams 82 KA were identified as *Bacillus megaterium*, *Chryseomonas luteola*, and *Sphingomonas paucimobilis*. Isolates from cv. Maverick MO were classified as *Agrobacterium radiobacter*, *Aeromonas* sp., *Bacillus macerans*, *Flavomonas oryzihabitans*, *Chryseomonas luteola*, and *Sphingomonas paucimobilis*. Most of these species of bacteria have not been previously reported as internally seedborne on soybean. *Aeromonas* sp., *C. luteola*, and *S. paucimobilis* have been isolated from fresh water, streams, and soils, as well as from acclimated sludge along with *F. oryzihabitans* (Holt and Krieg 1984; Holt et al. 1994; Buitron and Gonzales 1996; Collier et al. 1998). *Agrobacterium radiobacter*, a very common non-pathogenic soil microbe, is often isolated internally or from the rhizosphere of legumes (Holt and Krieg 1984; Holt et al. 1994) and *Bacilli* spp. are common to the soybean rhizosphere (Liu and Sinclair 1993) and seed (Mundt and Hinkle 1976; Ellis et al. 1977; Schiller et al. 1977; Grybauskas et al. 1979; Kremer 1987; Sinclair 1993).

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In an attempt to eliminate these internally seedborne microorganisms, surface-sterilized seeds were imbibed for various times in sterile distilled water containing antibiotics, and transferred onto sterile distilled water agar (0.6% agar) to germinate for 48 h. Seedlings were then monitored for microbial growth on the radicle using methods identical to the aforementioned microbial infestation detection. Kanamycin, at the lowest concentration tested (50 μg/mL, 750 U/mg) severely inhibited root tip growth and seedling viability. Neither kanamycin nor streptomycin (30 μg/mL) eliminated the seedborne bacteria. Chloramphenicol (20 μg/mL and 40 μg/mL) eliminated approximately 50% of the contaminating microbes from the seed. Rifampicin (80 μg/mL and 160 μg/mL) treatment was not detrimental to either root growth or seedling development, but did not eliminate seedborne microbial growth unless seeds were soaked for 5 h.

Based on previous publications (Ellis et al. 1977; Hepperly and Sinclair 1977; Schiller et al. 1977; Takats 1984; Kneifel and Leonhardt 1992), concentrations of penicillin G (PEN-NA, benzylpenicillin sodium salt, 1670 U/mg) between 250 μg/mL and 1000 μg/mL, and seed treatment times from 20 min to 5 h were tested. The most effective antibiotic treatment protocol involved soaking seeds for 20 min in penicillin G (PenG) at 500 μg/mL dissolved in sterile distilled water. Seeds were gently shaken for 20 min in the PenG solution, then transferred onto the surface of sterile distilled water agar (0.65% agar) also containing PenG at 500 μg/mL (added after autoclaving), and allowed to germinate 48 h in the dark at 30°C. Seedlings from PenG-treated seed were free of visible microbial growth, on all four media used previously to isolate the seedborne bacteria, for at least 5 days. Microbial growth was visible within 24 to 48 h from radicles not treated with PenG, indicating clearly that the PenG treatment was effective.

The results, demonstrating that PenG could be used to surface sterilize our soybean seeds prior to their use in attachment studies, required *B. japonicum* 2143 to be unsusceptible to any residual PenG (Takats 1984). *Bradyrhizobium japonicum* 2143 (an isolate from USDA strain 3I1b-143 selected for natural resistance to 80 μg/mL rifampicin and 120 μg/mL sodium nalidixate; Karr and Emerich 1989) at 1000 cells/plate was spread onto different concentrations of the antibiotic contained in TG or YEM solid media (1.5% agar). The number of colony forming units (CFU) per plate (determined after 8–10 day incubation at 30°C on TG or YEM containing 500 μg/mL PenG) was the same as that grown on media without the antibiotic. Colonies of *B. japonicum* 2143 appeared within 10 days on either medium. Additionally, a PenG-soaked filter disc was placed onto TG solid media and overlaid with a soft agar (0.8% agar) suspension containing *B. japonicum* 2143 at 1×10^5 cells/mL. No inhibition of growth was observed around the disc. A final procedure tested for any residual effect of PenG on *B. japonicum* 2143 from treated seeds. In this procedure, the zone of inhibition around the radicle of a PenG-treated seed was determined (Hepperly and Sinclair 1977) using whole seedlings (1.5–3.0-cm-long radicle) placed into soft agar overlays on TG semi-solid media (1.0% agar). The overlay contained 1×10^6 cells/mL of *B. japonicum* 2143 and plates were incubated at 30°C. The width of a clear area surrounding the radicle (the zone of inhibition) was measured when bacterial growth in the overlay became opaque (7–8 days). No zone of inhibition or delay in growth was visible, demonstrating again that *B. japonicum* was not susceptible to any residual component of the PenG at the surface of treated roots.

To determine the effects on attachment of *B. japonicum* 2143 to intact soybean seedlings treated with PenG, a method similar to Vesper and Bauer (1985) was used. The seedlings were germinated as described above, but were then placed in an aerated sterile attachment buffer solution with or without 500 μg/mL PenG for an additional 24 h in the dark at 30°C to ensure maximum root hair development. *Bradyrhizobium japonicum* inoculum was grown in YEM liquid medium to mid-log phase (A_{630}, 0.2–0.4), collected by centrifugation at 6500 × *g*, resuspended and diluted to 1×10^7 cells/mL in attachment buffer. After 0, 5, and 10 min separate incubations of inoculum with the radicles of four whole seedlings (i.e., four separate seedling inoculations), loosely adhered bacteria were removed by two vigorous washings of each individual seedling in attachment buffer, and one washing in PBS-Tween 20 (0.02%), pH 6.8. Roots from the 4 seedlings were cut from their cotyledon at each time point, and combined into a 15-mL Corex tube containing 2 mL of PBS-Tween 20, pH 6.8 at 4°C. Those cells firmly attached to the combined root segments were freed by sonication (25 W for three 1-min pulses with 30-s intervals on ice) using a Sonifier cell disruptor with special stepped microtip (model W185, Heat Systems Ultrasonics, Inc., Farmingdale, N.Y.). Aliquots of the diluted 2-mL sonicate (now containing the total number of *B. japonicum* 2143 that attached to four separately inoculated seedlings) were replicate-plated onto 6 YEM plates containing 80 μg/mL rifampicin and 120 μg/mL nalidixic acid. Colony forming units (CFU) were counted after 8 days incubation at 30°C. Two replicate trials showed variations, but only in the total number of *B. japonicum* 2143 bound to treated and non-treated seedlings alike at 5 and 10 min. The reason for this is unknown at this time. The number of CFU recovered from roots of PenG-treated seeds was consistently 200%–325% greater than that from untreated seeds at 5 and 10 min inoculation periods (Table 1) in both experiments (trial 1: $\chi^2 = 2.18 \times 10^5$, $P < 0.001$, $\nu = 1$, $\alpha = 0.05$; trial 2: $\chi^2 = 1.8 \times 10^4$, $P < 0.001$, $\nu = 1$, $\alpha = 0.05$). Identically treated uninoculated controls yielded no CFU, and adequate controls were run to ensure that no disruption of *B. japonicum* cells occurred due to sonication for this length of time at this power level. Plate contamination was assessed by monitoring colony growth rate and morphology.

Although a substantially higher number of *B. japonicum* cells adhered to the radicle (Table 1), nodule emergence in three out of four replicate experiments with PenG-treated and untreated seed showed no statistically significant difference (Trial 1: $0.25 > P > 0.10$; Trial 2: $0.05 > P > 0.025$; Trial 3: $0.50 > P > 0.25$; Trial 4: $0.99 > P > 0.995$; all trials at a significance level α of 0.05) (Fig. 1). Germinated seedlings were inoculated with *B. japonicum* 2143 (1×10^7 cells/mL) in attachment buffer and transferred to autoclaved, clear plastic pouches containing sterilized nitrogen–free nutrient solution and were maintained asceptically (Green and

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Table 1. Attachment of *B. japonicum* 2143 to radicles of penicillin G treated and untreated seedlings.[a]

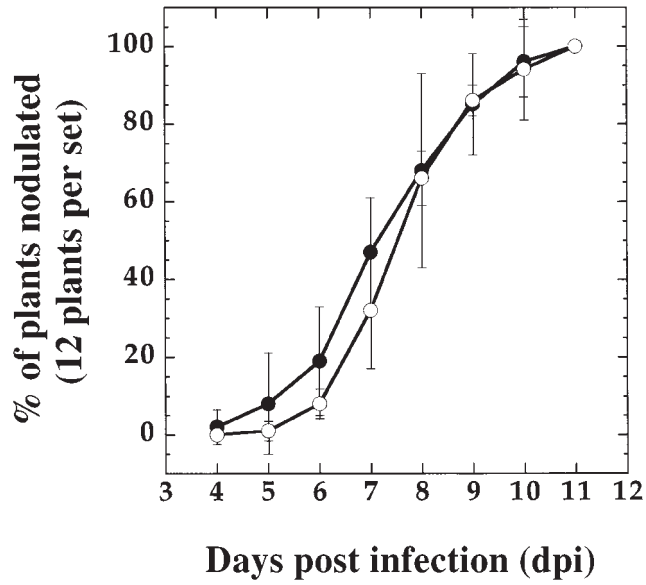
Inoculation time (min)	Untreated	Treated
(Trial 1) 5	26 133 ± 670	66 360 ± 1 040 (250% increase)
(Trial 2) 5	5667 ± 778	11 556 ± 778 (203% increase)
(Trial 1) 10	30 893 ± 426	100 530 ± 3 880 (325% increase)
(Trial 2) 10	8333 ± 611	21 270 ± 1 167 (255% increase)

[a]Colony forming units recovered per radicle from germinated 72 h soybean seeds used in the attachment assay. Seeds were surface sterilized and treated where applicable with 500 µg/mL penicillin G. Results are from two duplicate assays using cv. Maverick MO. Replicate assays gave variations in the actual values of recovered bacteria, but treated seedlings had consistently 2- and 3-fold greater numbers of attached cells. Uninoculated controls had no recovered cells on identical media. The CFU are reported as one experimental mean ± standard deviation (sampling error) from 6 plates representing a combined sample containing 4 seedlings per time point per treatment inoculated separately.

Emerich 1997). Seedlings were grown under a 16 h light, 8 h dark regimen at 26°C. As nodules became visible, the date of their emergence and location were recorded. After 18 days, plants were removed from the pouches and the number of nodulated plants and number of tap root nodules per plant were recorded. Four replicate trials showed that >50% of the total plants in each set (12–24 plants per set) had nodules on the tap root (>1 mm diameter) by 8 days post infection (dpi) (Fig. 1). Nodulation occurred no later than 11 days, with the average number of nodules per plant from treated and untreated seed 9.7 ± 2 (standard deviation; SD) and 9.1 ± 2 (SD), respectively ($0.75 > P(\chi^2 = 1.442) > 0.5$, $\nu = 3$, $\alpha = 0.05$).

Indication of nitrogenase activity was qualitatively determined by measuring acetylene reduction activity on the intact root–nodule system of soybean, cv. Williams 82 MO and cv. Maverick MO, treated and untreated with PenG. Due to the substrate differences between acetylene and dinitrogen, acetylene reduction activity in intact root nodules was not used as an indicator of specific nitrogenase activity levels, but provided a qualitative comparison of nitrogenase activity between treated and untreated seedlings. For these experiments seedlings were surface sterilized as described above, treated with 500 µg/mL PenG where applicable, and inoculated for 10 min in 1×10^7 cells/mL *B. japonicum* 2143 in attachment buffer. Seedlings were grown in sterilized perlite:vermiculite (1:1) nitrogen-free potting medium and nutrient solution, contained in Leonard jars (Karr and Emerich 1989), and grown under a 16 h light, 8 h dark regimen in an environmental growth chamber at 26°C. Eight plants were harvested at each designated day and individually analyzed for acetylene reduction activity (Schwinghammer et al. 1970). Acetylene reduction activity in nodules from PenG-treated and untreated seed, cv. Williams 82 MO ($0.25 > P(\chi^2 = 5.215) > 0.10$; $\nu = 3$, $\alpha = 0.05$) and cv. Maverick MO ($0.50 > P(\chi^2 = 3.122) > 0.25$; $\nu = 3$, $\alpha = 0.05$), showed no significant difference (data not shown), with peak activity occurring at 21 dpi. The average dry weight per nodule from treated and untreated cv. Williams 82 MO seed was

Fig. 1. Nodulation kinetics of penicillin G treated (○) vs. untreated (●) seeds inoculated with *B. japonicum* 2143. Seedlings, cv. Williams 82 MO and cv. Maverick MO, were inoculated with *B. japonicum* 2143 (1×10^8 cells/mL) by dipping an intact root into inoculum for 5 min and then transferring it to the growth pouch. Tap root nodules were counted, collected, and weighed after 18 days. Combined results are from four duplicate experiments (2 of each cultivar) with the % of plants nodulated error calculated as the standard deviation of the mean.



2.54 ± 0.67 mg (SD) and 2.38 ± 0.45 mg (SD) respectively, with the average number of nodules per plant at 24 ± 6 (SD) with treated seed and 22 ± 8 (SD) with the untreated controls. The average dry weight per nodule from treated and untreated cv. Maverick MO was 2.34 ± 0.65 mg (SD) and 2.01 ± 0.48 mg (SD), respectively, with the average number of nodules per plant at 24 ± 9 (SD) with treated seed and 29 ± 6 (SD) with the untreated controls. Hence there was no apparent difference (nodule weight: $0.90 > P(\chi^2 = 0.065) > 0.75$; $\nu = 1$, $\alpha = 0.05$; nodule number: $0.50 > P(\chi^2 = 1.044) > 0.25$; $\nu = 1$, $\alpha = 0.05$) between treated and untreated seed for either cultivar in nodule production or acetylene reduction activity.

Assessment of the effect of PenG on seedling development also required that root and shoot vegetative components be compared between PenG treated and untreated seedlings. Treated and non-treated seedlings, inoculated with *B. japonicum* 2143, were grown in clear plastic, autoclaved pouches and compared to those not inoculated with symbiont. Root tip advancement was recorded daily. Differences in root tip advancement between treated and untreated, inoculated ($0.25 > P(\chi^2 = 8.570) > 0.10$; $\nu = 5$, $\alpha = 0.05$) and uninoculated ($0.50 > P(\chi^2 = 4.846) > 0.25$; $\nu = 5$, $\alpha = 0.05$) seed was not statistically significant and measured root lengths of all four sets were within one standard deviation of the corresponding control and each other (Fig. 2). Root tip advancement of the untreated, inoculated set did however appear to be somewhat delayed. Further vegetative developmental stages were monitored closely and determined by the onset and maturation (i.e., full leaf development) of the first

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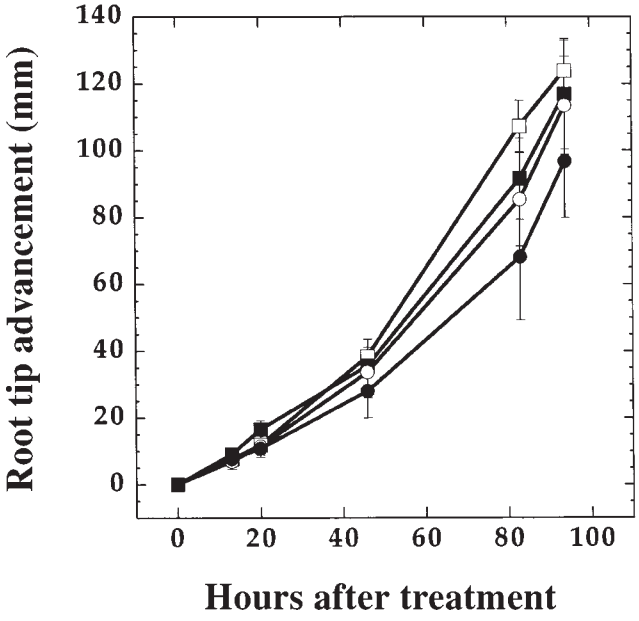
Fig. 2. Root tip advancement of penicillin G treated (○) vs. untreated (●) seed, inoculated with *B. japonicum* 2143, compared to uninoculated treated (□) and uninoculated untreated (■) seed. Surface-sterilized cv. Williams 82 seeds were gently shaken for 20 min in sterile distilled water containing 500 µg/mL penicillin G and germinated in its presence, then transferred to autoclaved, clear plastic pouches containing sterile nitrogen-free nutrients. Untreated controls were treated as above, but without PenG. Seedlings were grown in a 16 h light, 8 h dark regime at 26°C. Results are from one experiment. Replicates were handled identically and yielded very similar results. Root tip advancement error is reported as the standard deviation (sampling error) from 12 roots per time point per treatment.



three stem nodes. Complete unifoliate leafs, second and third trifoliate leaf development were identical between treated and untreated seedlings with unfurling at 7, 11, and 16 days, respectively. Further development of these same seedlings and of those grown in all other PenG treatment experiments showed no visual difference in growth rate. It was also observed that a consistently higher percentage of PenG treated seeds germinated compared to non-treated seeds. Ellis et al. (1977) and Schiller et al. (1977) noted this previously and attributed it to the selective removal of *B. subtilis* from soybean seed.

The results from our studies using PenG to eliminate internally seedborne microbes as an undefined parameter in attachment of *B. japonicum* to soybean, confirm the previous conclusion by Polonenko et al. (1987) that, in general, root-colonizing bacteria on soybean do not interfere with nodulation, but can interfere with the successful establishment of the inoculant on the root surface, the critical step preceding infection and nodulation (Kosslak et al. 1983) and possibly attachment (Mathews et al. 1993). Using our protocol to remove the root-colonizing bacterial strains, we found that these bacteria do interfere with attachment of *B. japonicum* 2143 to soybean. When they are not allowed to colonize the root, 200%–325% more *B. japonicum* 2143 cells attach to the seedling radicle (Table 1). However, we found no difference in nodulation (Fig. 1), nodule mass or nodule number in various experiments. In conjunction with no differences in acetylene reduction activity in mature nodules from PenG-treated or untreated seeds, our results suggest that enhanced attachment of *B. japonicum* to soybean does not result in enhanced nodulation, an idea supported by Moawad et al. (1984).

Other studies have shown that certain culturable and superior root-colonizing bacterial strains used as co-inoculum with *B. japonicum* enhanced nodulation of soybean (Polonenko et al. 1987; Liu and Sinclair 1993; Shabayev et al. 1996; Zhang et al. 1997). As suggested by several of these authors, increased nodule number or mass with the addition of plant growth promoting rhizobacteria may be an indirect effect occurring after attachment, and therefore not related to any beneficial enhancement of attachment or infection for the nitrogen fixing symbiont afforded by strains already colonizing the root surface. Examination of the particular strains of bacteria which were used in those studies, in conjunction with our results with the bacterial strains we found (many not previously known to associate with soybean), demonstrates that a wide range of different root-colonizing strains can affect legume-rhizobium interactions differently and at very different times in the attachment, infection, proliferation process. This concept, introduced by Polonenko et al. (1987), that successful rhizobium establishment is dependent on compatible microbial populations in the soybean rhizosphere, is supported by our results. Single strain co-inoculum studies, it appears, demonstrate only that certain strains may promote nodulation enhancement and that certain strains may be antagonistic to not only colonization by rhizobium, but to infection and (or) nodulation as well. What they do not provide is clues as to what effects multiple species co-inhabiting the rhizosphere can have on the establishment of rhizobium on legumes. Looking at this parameter then becomes the next step in deciphering what effects internally seedborne bacteria can have on rhizosphere establishment by rhizobia.

Interpretation of the results is presently difficult. The possibility exists that the increase in the number of *B. japonicum* attached to penicillin G treated soybean in our study resulted from increased available attachment sites (Vesper and Bauer 1985) for the symbiont after reducing the root-colonizing microbes originating from the seed. This idea is supported by Bowen and Rovira (1961) who found that elimination of rhizosphere microorganisms in clover, and many other plant species, increases root health, length and can greatly increase the population of root hairs. To make an analysis even more difficult is the contrasting results of Mathews et al. (1993), who found a strong relationship between the number of root-colonized *B. japonicum* and the number of nodules on soybean, where our results show none. Hence, our findings further emphasize the complexity of rhizosphere interactions with rhizobium attachment and infection.

In summary, our protocol for soybean seed treatment with penicillin G, and germination in its presence, was effective in eliminating visible growth and root-colonization of internally seedborne microbes for at least 96 h on the field grown soybean cultivars Williams 82 and Maverick used in our lab-

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oratory. In addition, our protocol can be used without any apparent detrimental effects on the seedling itself or *B. japonicum* 2143-soybean symbiotic development, and allows for elimination of an undefined parameter in our attachment studies. The major findings of our work support evidence that soybean internally seedborne microbes can survive surface sterilization and can affect root-colonization by the symbiont *B. japonicum*.

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