

## Measuring the spermosphere colonizing capacity (spermosphere competence) of bacterial inoculants

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Spermosphere establishment by bacteria which were coated onto seeds was studied using soybean seeds treated with four bacterial strains at levels of  $\log_{10}$  1 to 4 colony-forming units (cfu) per seed planted in a field soil mix, and incubated 48 h. Each strain at every inoculum level developed spermosphere population densities of  $\log_{10}$  4 to 8 cfu/seed, demonstrating an average multiplication of  $\log_{10}$  3 cfu/seed. An alternative method was developed to differentially rank bacteria for spermosphere colonizing capacity, based upon incorporation of bacteria into a soil and monitoring the resulting spermosphere population densities around noninoculated seeds after 4 days at 14°C. Fifty-seven bacterial strains which were isolated from soybean roots or from water samples, including *Pseudomonas putida*, *P. putida* biovar B, *P. fluorescens*, *Serratia liquefaciens*, *Enterobacter aerogenes*, and *Bacillus* spp. were tested in the spermosphere colonization assay. Average spermosphere population densities for the 57 strains ranged from 0 to  $\log_{10}$  7.0 cfu/seed. Strains of a given taxon demonstrated marked diversity with ranges from 0 to  $\log_{10}$  6.0 cfu/seed for *Bacillus* spp. and from  $\log_{10}$  1.4 to 7.0 cfu/seed for *Pseudomonas putida*. The relative ranking of representative strains was consistent in repeating experiments. The potential usefulness of the assay for efforts to develop competitive bacterial inoculants for crop seeds is discussed.

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L'établissement d'une spermosphère par des bactéries utilisées lors de l'enrobage de graines a été étudié chez des graines de soya traitées avec quatre souches bactériennes à des niveaux de  $\log_{10}$  1 à 4 unités formant (ufc) des colonies par graine semée dans un mélange de sol de champ et incubées durant 48 h. Pour chaque niveau d'inoculum, chacune des souches a développé une spermosphère et les densités de populations ont varié de  $\log_{10}$  4 à 8 ufc/graine, ce qui indique une multiplication moyenne de  $\log_{10}$  3 ufc/graine. Une autre méthode a été développée pour classifier les bactéries de façon différentielle quant à leur capacité de colonisation des spermosphères. Cette méthode a consisté à incorporer des bactéries dans un soi et de suivre le développement des densités de populations spermosphériques sur des graines non inoculées après 4 jours à 14°C. Cinquante-sept souches de bactéries isolées de racines de soya ou d'échantillons d'eau de délavage ont été testées pour leur aptitude à la colonisation des spermosphères; parmi celles-ci, citons: *Pseudomonas putida*, *P. putida* biovar B, *P. fluorescens*, *Serratia liquefaciens*, *Enterobacter aerogenes* et *Bacillus* spp. Les densités moyennes de populations des spermosphères pour les 57 souches ont varié de 0 à  $\log_{10}$  7,0 ufc/graine. Les souches de taxons particuliers ont présenté une diversité marquée, savoir de 0 à  $\log_{10}$  6,0 ufc/graine pour *Bacillus* spp et de  $\log_{10}$  1,4 à 7,0 pour *Pseudomonas putida*. La classification relative des souches représentatives fut en accord avec les résultats d'expériences successives. L'utilité potentielle de cet essai face aux efforts de développement d'inoculants bactériens compétitifs pour les graines de semence est discutée.

[Traduit par le journal]

Bacterial inoculation of legume seeds with *Rhizobium* spp. is routinely practised in commercial agriculture. A distinct group of nonrhizobial bacteria, termed plant growth-promoting rhizobacteria (PGPR) (Kloepper, Schroth, and Miller 1980), has recently induced increases in plant growth and yield for several crops (Howie and Echandi 1983; Kloepper, Leong, et al. 1980; J. W. Kloepper and M. N. Schroth. 1978. Proceedings of the 4th International Conference of Plant Pathology and Bacteriology, Angers. 1978; Suslow and Schroth 1982) and reductions in pathogen population densities in the rhizosphere (Kloepper 1983) or reductions in plant disease (Scher and Baker 1980). PGPR will, therefore, soon constitute a second major group of commercially available bacterial inoculants. The successful use of either rhizobial or PGPR inoculants in agriculture depends upon the delivery of viable bacteria to the root zone, which is most frequently accomplished by inoculating seeds with a preparation of dormant bacterial cells, by means of coated seeds or bulk inoculants. Such bacteria must be activated during seed germination to establish high population densities in the rhizosphere.

The seed-germination process releases an abundance of carbohydrates and amino acids, in the form of seed exudates (Schlub and Shmittenner 1978; Lynch 1978; Subrahmanyam et al. 1983) to which some bacteria are chemotactically attracted (Scher et al. 1985). A zone of increased microbial

growth, termed the spermosphere (Slykhuis 1947; Lynch 1978; Verona 1963), results from seed exudation. The bean spermosphere extends 7 to 10 mm from the seed (Short and Lacy 1974; Stanghellini and Hancock 1971) and may result in differential stimulation in growth of native soil microorganisms (Keeling 1974).

To efficiently colonize roots, the introduced bacteria must be competitive with native microorganisms. Stimulation of inoculant bacterial population densities in the spermosphere leads to greater competitive ability. *Rhizobium* strains which are highly competitive as a result of motility (Ames and Bergman 1981) or chemotaxis (Hunter and Fehring 1980) form more root nodules than less competitive strains.

In a previous report (Scher et al. 1984) we found that 30 of 54 rhizosphere and soil bacterial strains (all nonrhizobia), inoculated onto maize seed, did not colonize roots within 14 days from planting, suggesting that these strains failed to establish in the spermosphere or failed to transfer from the spermosphere to the developing roots. Therefore, a method to identify strains which are superior spermosphere colonizers and which are transferable to roots would improve selection of root-colonizing strains. We report here the development of an assay for determining the spermosphere colonizing capacity (spermosphere competence) of strains to be used as bacterial inoculants of crop seeds.

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TABLE 1. Identification and isolation source of bacterial strains used in the study

Strain identification	Isolation source*	Strain No.
<i>Pseudomonas putida</i>	Roots	SC 3, 36, 52, 55, 56, 57
	Water	SC 16, 17, 18, 19, 22, 31, 33
<i>Pseudomonas putida</i> biovar B	Roots	SC 42, 43
	<i>Pseudomonas fluorescens</i>	Roots
<i>Serratia liquefaciens</i>	Water	SC 8, 15
	Roots	SC 1, 2, 4, 5, 6, 35, 37, 38
	Water	SC 10, 12, 13, 14, 20, 23, 25, 26, 27, 28, 29, 30, 32, 40, 44, 45, 46, 47, 48, 62
<i>Enterobacter aerogenes</i>	Roots	SC 49
<i>Bacillus</i> spp.	Water	SC 58, 59, 60, 61, 63, 64

\*Isolations were performed as described in the text. Roots were from soybean planted in various soil samples

## Methods

### Bacterial strains

Bacterial strains used in this study were isolated either from water samples (SC7-32, SC58-61, SC63, SC64) or from soybean roots growing in various soil samples (SC1-6, SC33-57, SC62) by dilution plating and purification on tryptic soy agar (Difco Laboratories, Detroit, MI, U.S.A. 48232) with incubation at 30°C. Sixty-four strains were selected from the developing colonies on the basis of maximizing diversity of colony morphologies. Spontaneous, stable mutants resistant to 100 µg/mL rifampicin were selected for each strain.

All 64 strains (rif-mutants) were tested for Gram-stain reaction. Gram-negative bacteria were identified by test reaction profiles on API 20E test strips (Analytab Products, Ayerst Laboratories, Inc., Plainview, NY, U.S.A.). Complementary tests for Gram-negative bacteria included growth on MacConkey medium, the type of metabolism in OF glucose medium, production of DNase, fluorescent pigment production, gelatin hydrolysis, nitrate reduction, starch hydrolysis, oxidase reaction, and lipase production (Tween 80 hydrolysis). Gram-positive bacteria were identified by the following tests: growth on MacConkey medium, the type of metabolism in OF glucose medium, catalase test, gelatin hydrolysis, Voges-Proskauer reaction, indole production, citrate utilization, motility, urease production, endospore formation, and acid production from glucose, saccharose and (or) inositol. The methods for all of the biochemical tests were those approved by the American Society for Microbiology. The identifications of the bacterial strains, on the basis of these tests, are listed in Table 1.

### Bacterial colonization of preinoculated seeds

A series of experiments was conducted to determine the effect of varying the level and bacterial seed inoculation on spermosphere populations of these bacteria in field soil. Bacterial strains SC 1-4 were grown for 48 h at 29°C on Pseudomonas agar F (PAF) (Difco Labs, Detroit, MI, U.S.A. 48232). The resulting lawns were scraped into 10 mL of 0.1 M MgSO<sub>4</sub>; one threefold dilution was prepared followed by two 10-fold dilutions. Three millilitres of each dilution was mixed with 3 mL of sterile 1% methylcellulose (4000 centipoises, Sigma Chemical Co., St. Louis, MO, U.S.A. 63178). The mixtures were placed in plastic bags and rolled for 3 min with 70 soybean seeds (cv. Maple Arrow) each, followed by gentle mixing with 15 g talc. Coated seeds were transferred to open envelopes at 20°C for 24 h, then to

TABLE 2. Spermosphere establishment of bacteria which were preinoculated onto soybean

Bacterial strain	Inoculant level (cfu/seed)	Spermosphere population density* (cfu/seed)
SC-1	1.6 × 10 <sup>4</sup>	3.9 × 10 <sup>7</sup>
	5.3 × 10 <sup>3</sup>	8.6 × 10 <sup>6</sup>
	5.1 × 10 <sup>2</sup>	6.3 × 10 <sup>6</sup>
SC-2	5.2 × 10 <sup>3</sup>	>1 × 10 <sup>5</sup>
	2.1 × 10 <sup>4</sup>	5.7 × 10 <sup>7</sup>
	6.9 × 10 <sup>3</sup>	5.0 × 10 <sup>6</sup>
SC-3	7.1 × 10 <sup>2</sup>	1.0 × 10 <sup>6</sup>
	7.0 × 10 <sup>1</sup>	>1 × 10 <sup>5</sup>
	1.8 × 10 <sup>4</sup>	1.1 × 10 <sup>8</sup>
SC-4	6.0 × 10 <sup>3</sup>	5.7 × 10 <sup>6</sup>
	5.9 × 10 <sup>2</sup>	2.3 × 10 <sup>6</sup>
	6.0 × 10 <sup>1</sup>	>1 × 10 <sup>5</sup>
	1.2 × 10 <sup>4</sup>	5.2 × 10 <sup>7</sup>
	3.9 × 10 <sup>3</sup>	3.9 × 10 <sup>6</sup>
	4.0 × 10 <sup>2</sup>	3.4 × 10 <sup>6</sup>
	3.9 × 10 <sup>1</sup>	>1 × 10 <sup>5</sup>

\*Population density on seed, radicle, and tightly adhering soil particles after 48 h (see text for experimental procedure).

sealed envelopes at 4°C. Bacterial populations were determined for each strain × inoculum level by replicate plating (eight replications per treatment) onto PAF plates containing 100 µg/mL rifampicin (PAF-R) using a spiral plater (Spiral Systems Inc., Bethesda, MD, U.S.A. 20814). After 24- to 48-h incubation at 29°C, colonies were counted with a laser counter (model 500A, Spiral Systems), and bacterial population densities per seed were calculated with a CASBA data processor (model 800, Spiral Systems).

A clay loam soybean field soil was sieved (4.75 mm), adjusted to 15% moisture (w/v), resieved, and 75 g was placed into each of eight replicate 25 × 100 mm petri dishes. Field soil was stored at 10°C for a maximum of 2 months after collection, and the original moisture level was 7 to 10%. One coated soybean seed was placed 1.5 cm deep in the centre of each petri dish, and dishes were incubated for 48 h in the dark at 20°C. Seeds were removed from the petri dishes; excess soil was shaken off; seeds were agitated in 10 mL blanks of 0.1 M MgSO<sub>4</sub>; and blanks were spiral plated onto PAF-R. Colonies were counted after 24- to 48-h incubation at 29°C. Experiments were repeated once or twice with similar results.

### Development of a spermosphere colonization assay

Assay development centred on optimizing conditions for placing a test bacterial strain into soil and monitoring its subsequent establishment in the spermosphere. Accordingly, experiments were designed to determine the effects of bacterial concentrations in soil and of incubation time on the final bacterial population densities in the spermosphere.

The experiments testing the effect of initial soil populations of bacteria on the final spermosphere population densities used strain SC-2. A 48-h PAF slant culture was used to inoculate 40 mL of tryptic soy broth (TSB) (Difco Labs, Detroit, MI, U.S.A. 48232) which was incubated for 24 h inside a 250-mL screw-top Erlenmeyer flask at 30°C and 150 rpm. Cells were aseptically harvested by centrifugation at 5000 × g for 10 min followed by three washings with 10<sup>-2</sup> M phosphate buffer (PB), pH 7.0. The bacterial concentration was aseptically adjusted to an optical density (OD) of 0.6 at 780 nm with a Bausch & Lomb Spectronic 20 spectrophotometer (this OD was determined by plate counts to equal log<sub>10</sub> 9 colony-forming units (cfu/mL). Four serial 10-fold dilutions were prepared.

Five millilitres of each dilution was mixed with 100 g conditioned soil to yield a dilution series of log<sub>10</sub> 7, 6, 5, 4, and 3 cfu/g. Conditioned soil was prepared by mixing 4 parts Pro-mix "C" (Plant

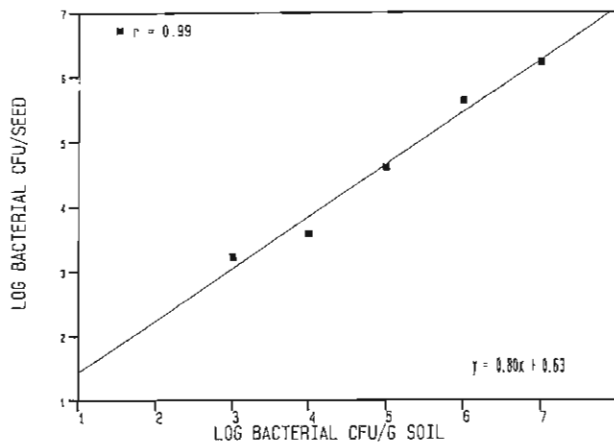


FIG. 1. Correlation between *Serratia liquefaciens* (strain SC-2) density in soil and the subsequent density on untreated soybean seeds. The log bacterial colony-forming units per seed values are the means of eight replicates after incubation for 48 h at 14°C.

Products Co. Ltd., Bramalca, Ont.) with 1 part of a clay loam soybean field soil, using this mix to grow soybeans to the second true leaf stage, discarding soybean plants, and remixing the soil. The resulting soil blend contained field soil microorganisms and had an acceptable texture for seed germination in petri dishes. Each of eight 25 × 100 mm petri dishes were filled with 130 g conditioned soil for each inoculum level. Controls consisted of 5 mL PB/100 g soil. One soybean seed (cv. Maple Arrow) was placed in the centre of each dish, and dishes were incubated 96 h at 14°C (which corresponds to soil temperature at planting time in Southern Ontario). Spermospere population densities were determined by spiral plating on PAF-R as previously described. The experiment was repeated with similar results.

Studies on changes in spermospere population densities with time were conducted with four strains (SC1, 2, 4, 6) at inoculum levels of  $\log_{10}$  3 cfu/g soil; control was PB mixed with conditioned soil. The experimental methods and design were as described above; however, 32 petri dishes were included for each bacterial treatment and control. Eight replications of each treatment were sampled on days 1, 2, 3, and 4 after planting.

#### Application of the spermospere colonization procedure

Fifty-seven individual strains (SC7-64 from Table 1) were selected for testing. Assays were set up as previously described with an initial soil population density of  $\log_{10}$  3 cfu/g. Numbers of bacteria present in the spermospere were determined after 4 days at 14°C by spiral plating onto PAF-R plates. One experiment, consisting of 8 to 10 bacterial treatments and one control, each replicated eight times, was conducted weekly. Twelve representative strains of the 57 strains (4 strains with high, 4 with medium, and 4 with low seed colonization levels) were retested to determine the experimental variation in the final mean of seed population densities. The treatment means were analysed for statistically significant differences using analysis of variance.

## Results

### Bacterial colonization of preinoculated seeds

Each of the four bacterial strains coated onto soybean seed established spermospere population densities of  $\log_{10}$  7 to 8 cfu/seed after 48 h (Table 2) as a result of inoculating seeds with  $10^4$  cfu/seed. Seed coating with low inoculum levels of 39 to 70 cfu/seed also resulted in spermospere population densities of  $>\log_{10}$  5 cfu/seed.

### Development of a spermospere colonization assay

Spermospere population densities of bacteria on untreated

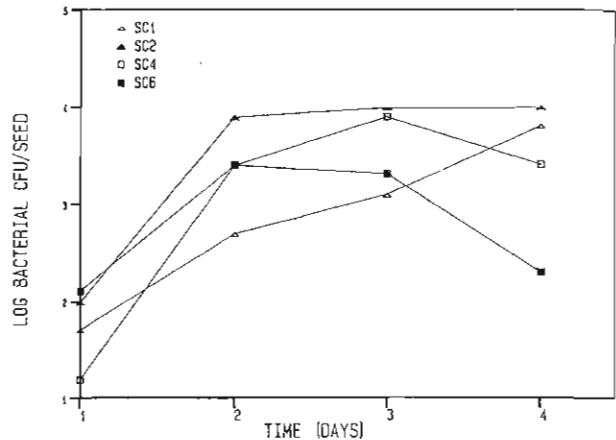


FIG. 2. Spermospere population density over time. The four indicated strains were mixed into soil at  $10^3$  cfu/g, and untreated soybean seeds were planted and incubated at 14°C. The log bacterial colony-forming units per seed values shown are means of eight replications.

TABLE 3. Spermospere establishment of bacteria which were preinoculated into soil at a density of  $10^3$  cfu/g soil\*

Strain	Application of the seed colonization assay		Log cfu/seed
	Log cfu/seed†	Strain	
SC-54	7.0 A	SC-31, 43	4.6 KL
SC-50	6.8 AB	SC-30, 47	4.5 KL
SC-55, 12	6.7 BC	SC-25, 26, 33	4.4 L
SC-10	6.6 BC	SC-23, 35	4.3 LM
SC-52	6.5 CD	SC-37, 49	4.1 LM
SC-45, 46	6.2 DE	SC-20	4.0 M
SC-58	6.0 E	SC-14, 41	3.8 MN
SC-19, 57	5.8 EF	SC-24, 39, 42	3.6 N
SC-11, 51, 56	5.6 FG	SC-9, 13	3.1 O
SC-15	5.5 GH	SC-17	2.5 P
SC-7, 40, 62	5.4 GH	SC-16, 18, 64	2.2 Q
SC-48	5.3 H	SC-36	1.7 R
SC-38	5.0 I	SC-63	1.6 RS
SC-21	4.9 IJ	SC-22	1.4 S
SC-27	4.8 IJ	SC-60	1.0 T
SC-28, 29, 32, 34, 44	4.7 JK	SC-8, 59, 61	0 U

\*The table is a summary of six separate experiments, each with one control and 8 to 10 bacterial treatments.

†Populations shown represent the bacteria, incorporated into soil at  $10^3$  cfu/g, which colonized seeds in 4 days at 14°C; see text for experimental details. Values are means of eight replications. Means with the same letter are not significantly different from each other ( $P = 0.05$ , LSD = 0.3 (coefficient of variation = 6.1).

seeds was directly proportional to the bacterial populations in the soil (Fig. 1). Strain SC-2 developed a spermospere population of  $\log_{10}$  3.2 cfu/seed at the lowest inoculum level ( $\log_{10}$  3 cfu/g soil) in conditioned soil. Therefore,  $\log_{10}$  3 cfu/g soil was chosen as the standard inoculum level for further studies to allow the maximum degree of differentiation of spermospere competence between different bacterial strains.

Spermospere population densities of bacteria mixed into conditioned soil varied among the four tested strains over the 4 day sample period (Fig. 2). The maximum differentiation between strains occurred on the 4th day when 1.9 log units separated strains SC-2 and SC-6. Consequently, we chose to conduct our routine spermospere colonization assays using the methods described previously with final population

determinations being made 4 days after planting at 14°C.

#### Application of the spermosphere colonization procedure

A large diversity in spermosphere colonization (Table 3) occurred with the 57 soil bacteria. Twenty strains colonized seeds at  $\log_{10}$  5 cfu/seed or greater while 11 strains colonized at levels below  $\log_{10}$  3 cfu/seed. Mean spermosphere colonization levels for 12 strains which were retested varied 0.5 log units or less from the originally calculated mean, and the relative ranking of the strains as high-, medium- or low-level seed colonizers remained the same.

#### Discussion

An assay has been developed for determining the spermosphere competence of a bacterial strain by measuring the spermosphere colonizing capacity of the strain in soil which contains a native field soil microflora. The first procedure described herein, where bacteria were coated onto seeds demonstrated that seed-inoculated bacteria have a selective advantage for spermosphere colonization (Table 2) even when the initial inoculant level is less than 100 cfu/seed. While these results are encouraging for efforts designed to deliver viable bacteria into the spermosphere—rhizosphere by means of the use of inoculated seeds, they indicate that the procedure is not useful for ranking bacterial strains for competitiveness in the spermosphere. This procedure does not differentiate between strains' capacities to successfully transfer from the spermosphere to the developing root system. Furthermore, such a procedure does not correspond with a field delivery system of bulk inoculants which are either mixed in the seed hopper box or drilled into furrows without the use of adhesive agents.

These limitations have been partially overcome in the second developed assay described in the methods section. In this assay, a successful spermosphere-colonizing strain must, after incorporation into soil at a relatively low population ( $\log_{10}$  3 cfu/g) compete with the indigenous soil- and seed-coat bacteria for utilization of seed exudates and for colonization sites and must begin transfer from the seed to the radicle which is 1 to 4 cm long at the 4 day sample time. The assay proved useful for clearly identifying bacteria (of those randomly isolated from water or roots, Table 1) which were superior seed colonizers (Table 3).

It is interesting to note that a given taxon such as *Bacillus* contained strains with spermosphere colonization levels ranging from 0 (SC 59 and 61 in Table 3) to  $\log_{10}$  6.0 cfu/seed (SC-58). This variation in spermosphere colonization was also observed with the root isolates of fluorescent pseudomonads which ranged from  $\log_{10}$  1.7 (SC-36) to 7.0 (SC-54) cfu/seed. Hence, spermosphere competence appears to be strain specific and the assay should have broad applicability to studies designed to select superior spermosphere-colonizing bacteria in field soils.

It was beyond the scope of this report to determine the precise relationship between spermosphere colonization by bacterial inoculants and subsequent root colonization. However, using this assay, one can detect strains which do not transfer from the spermosphere to roots as seen in Fig. 2 with strain SC-6 which declined from day 2 to day 4, which is when the radicle has emerged. It is also reasonable to expect that strains with a high spermosphere competence, as measured

with the spermosphere colonization assay, will have a selective advantage for root colonization compared with strains that have a low to moderate spermosphere competence. Hence, this assay should also aid efforts to select bacteria which will be efficient root colonizers in situations where microbial competition impacts upon colonization.

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