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Effects of antibiotic-producing *Streptomyces* on nodulation and leaf spot in alfalfa

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Abstract

The ability of antibiotic-producing streptomycetes to colonize alfalfa (*Medicago sativa* L.) plants and influence the activities of a fungal plant pathogen (*Phoma medicaginis* var. *medicaginis*) and a mutualistic symbiont (*Sinorhizobium meliloti*) was investigated. *Streptomyces* strains were introduced around seeds at the time of planting. Hyphal filaments and spore chains were observed by scanning electron microscopy on roots of alfalfa seedlings receiving the streptomycete amendment. *Streptomyces* strain densities on leaves decreased 10–100-fold over an 8-week period, while densities on roots remained constant over time. The *Streptomyces* strains also colonized alfalfa root nodules. We then tested the ability of 15 antibiotic-producing strains of *Streptomyces* to inhibit in vitro growth of *Phoma medicaginis* var. *medicaginis* Malbr. & Roum., the causal agent of spring blackstem and leaf spot of alfalfa. The majority of the *Streptomyces* strains inhibited growth of three diverse strains of *P. medicaginis*. In a detached leaf assay, one *Streptomyces* strain decreased leaf spot symptoms caused by *P. medicaginis* when inoculated onto leaves 24 h before the pathogen. Two *Streptomyces* strains decreased defoliation caused by *P. medicaginis* when the streptomycetes were introduced around seeds at the time of planting. We also examined inhibitory activity of *Streptomyces* strains against 11 strains of *S. meliloti*. Eight of the 15 *Streptomyces* strains inhibited in vitro growth of five or more of the *S. meliloti* strains, while four *Streptomyces* strains had no effect on growth of any test strains. In a growth chamber assay, two of six *Streptomyces* strains, when inoculated into the planting mix, significantly reduced plant dry weight compared to the treatment with *S. meliloti* alone, but did not significantly reduce the number of nodules. These results suggest that careful selection of *Streptomyces* isolates for use in biological control of plant diseases will limit the potential negative impacts on rhizobia.
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1. Introduction

Alfalfa (*Medicago sativa* L.) is the primary cultivated forage crop in the United States. Growth of alfalfa increases soil fertility, improves soil structure, and reduces erosion, making alfalfa an important component in crop rotations and in sustainable agricultural systems. However, a number of serious diseases affect persistence and yield of alfalfa. In particular, root rot and crown rot diseases, caused by a complex of fungi, as well as several foliar pathogens, can cause significant yield reductions. *Phoma medicaginis* var.

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medicaginis Malbr. & Roum., the causal agent of spring blackstem and leaf spot of alfalfa, infects leaves and stems during cool wet weather (Leath et al., 1988). The disease causes the most severe yield reduction in the first spring harvest, which typically has the highest dry matter production and forage quality. Infection of crowns and roots can cause stand thinning (Leath et al., 1988; Rodriguez et al., 1990), decreasing the productive life of an alfalfa field. Little resistance to *P. medicaginis* has been found in alfalfa germplasm and no highly resistant varieties are currently available.

We have investigated the use of *Streptomyces* spp. as broad-spectrum biological control agents for multiple pathogens in diverse cropping systems including potato, alfalfa, soybean, and corn. Streptomycetes are common filamentous bacteria that are effective, persistent soil saprophytes and often are associated with plant roots. They are well-known producers of antibiotics and extracellular hydrolytic enzymes. These characteristics, as well as their ability to withstand desiccation and high temperatures as spores, make them attractive as biological control agents. Several strains are available as commercial products (Nemec et al., 1996).

A naturally-occurring potato scab suppressive soil was found to have high population densities of antibiotic-producing, non-pathogenic streptomycetes (Lorang et al., 1995; Liu et al., 1996). These antibiotic-producing strains have strong in vitro inhibitory activity against a wide variety of plant-pathogenic bacteria and fungi (Liu, 1992; Jones and Samac, 1996; Xiao et al., 2002). Inoculation of soil with individual strains controls potato scab in greenhouse and field experiments (Liu et al., 1995). Individual strains control root rot caused by *Aphanomyces euteiches* Dresh. and *Phytophthora medicaginis* Hansen et Maxwell on alfalfa in growth chamber and greenhouse studies (Jones and Samac, 1996; Xiao et al., 2002) and reduce population densities of the root-lesion nematode (*Pratylenchus penetrans* (Cobb) Filipjev & Schuurmans Stekhoven) on alfalfa roots (Samac and Kinkel, 2001). Field experiments have shown control of Septoria leaf spot of hybrid poplar by foliar applications of single *Streptomyces* strains originating from the suppressive soil (Gyenis, 2000; Shimizu, 1994).

Streptomyces spp. have the potential to contribute to management of soilborne and foliar plant pathogens on diverse plant hosts. However, their value in a multiple crop, integrated disease management system depends not only on their abilities to control plant disease, but also on their ability to colonize plant host surfaces and their potential inhibitory effects on beneficial microbes. In particular, because of their production of broad-spectrum antimicrobial compounds, *Streptomyces* spp. may have negative impacts on the growth and performance of nitrogen-fixing mutualistic bacteria.

The objectives of this study were to evaluate the potential for *Streptomyces* spp. to contribute to the management of foliar and soilborne plant pathogens of alfalfa. Specifically, we investigated the ability of *Streptomyces* strains to colonize alfalfa leaves and roots following inoculation of the soil at planting. We also examined the ability of *Streptomyces* strains to inhibit *Phoma medicaginis* growth in vitro and quantified the effects of *Streptomyces* strains on leaf spot symptoms caused by *P. medicaginis*. Finally, we evaluated the effects of antibiotic-producing *Streptomyces* strains on in vitro growth of *Sinorhizobium meliloti* and nodulation of alfalfa plants.

2. Materials and methods

2.1. Bacterial and fungal strains

Streptomyces strains were isolated from a potato scab-suppressive soil in Grand Rapids, MN as described previously (Liu et al., 1996). Fifteen strains were selected for study here based on their ability to produce large zones (>10 mm diameter) of growth inhibition in vitro of *Streptomyces scabies*, the causal agent of potato scab (Liu et al., 1996). A subset of 11 strains from the USDA *S. meliloti* strain collection was obtained from M. Sadowsky, University of Minnesota. *Streptomyces* and *Sinorhizobium* strains were stored until use in 20% glycerol at −80 °C. *Phoma medicaginis* strain 866 was obtained from K. Leath, (USDA-ARS, University Park, PA), strain T430 from N. O'Neill (USDA-ARS, Beltsville, MD), and strain NY001 from G. Bergstrom (Cornell University, Ithaca, NY). Fungi were stored on silica gel at 4 °C.

Fresh spore suspensions from *Streptomyces* strains were collected in sterile distilled water with 0.01% Tween 20 from cultures grown for 7–10 days at 28 °C on oatmeal (OM) agar plates (Liu et al., 1995).

Inoculum concentrations were determined by comparing the optical density of the spore suspension at 600 nm with colony forming units (CFU) previously determined by dilution plating of the spore suspension on OM agar plates. *P. medicaginis* cultures were grown on potato dextrose agar (PDA) at 25 °C for 14–21 days for production of conidia. Fresh spore suspensions were collected in sterile distilled water with 0.01% Tween 20 and spore concentrations were determined using a hemacytometer. *S. meliloti* strains were cultured on YEM agar plates (Vincent, 1970) at 28 °C for 2 days and cell suspensions were made by vortexing colonies removed from plates in phosphate buffered saline. Inoculum concentrations were determined by comparing the optical density at 600 nm with CFU previously determined by dilution plating on YEM agar plates.

2.2. Alfalfa colonization

Seeds of the alfalfa variety Agate were surface sterilized by immersion in 70% ethanol for 1 min, followed by continuous agitation in a 10% bleach solution (0.525% sodium hypochlorite) for 10 min, and three rinses with sterile distilled water. Seeds were allowed to imbibe water overnight at room temperature. Plants were grown in 3.8 cm × 19 cm containers (Stuewe & Sons Inc., Corvallis, OR, USA) in a sterilized sand:vermiculite mixture (1:1 (v/v)). A fresh suspension of *Streptomyces* spores (strains 93, GS6-17, and GS43-11) in OM broth with 0.01% Tween-20 was added to the planting mixture immediately before planting so that each *Streptomyces*-amended container received approximately 1×10^6 CFU/cm^3 planting mix. Control treatments received an equal volume of OM broth. Seeds were placed on the surface of the planting mix approximately 2 cm from the top of the container and covered with a 0.5 cm layer of the sterilized sand:vermiculite mixture. Each plant was inoculated with approximately 1×10^4 CFU of *S. meliloti* strain USDA 105F21 in phosphate buffered saline 7 days after planting by pipetting the inoculum onto the soil mix at the base of the plant.

Plants were maintained in a growth chamber with a 16 h day length at 21 °C, approximately 600 μmol m^2/s irradiance, and night temperature of 19 °C. Plants were fertilized weekly with a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994) and watered as needed. Alfalfa tissue samples were harvested for assessment of *Streptomyces* population densities at 3, 6, and 8 weeks after planting. The oldest trifoliolate leaf was removed and roots were then removed from the planting mixture, gently rinsed with distilled water to remove soil particles, and blotted dry. Roots were divided into the upper 5 cm root segment and the remaining lower root system. Nodules were removed from roots at 6 and 8 weeks after planting. Shoot, root, and nodule fresh weights were recorded. The trifoliolate leaf and root sections were sonicated separately for 15 min in 9 ml sterile distilled water with 0.01% Triton X-100 using a Bransonic 8200 water bath sonicator (Branson Ultrasonics Corp., Danbury, CT, USA). Nodules were homogenized in 9 ml sterile distilled water with 0.01% Triton X-100 using a Tissue Tearor (Research Products International Corp., Mount Prospect, IL, USA). Serial dilutions of wash suspensions and homogenates were plated on OM agar containing antibiotics (Loria and Devis, 1988). Streptomycete colony counts were recorded after incubation for 7 days at 28 °C. Five plants per treatment were analyzed, at each time point, and the experiment was repeated twice.

2.3. Scanning electron microscopy

Alfalfa roots from 14-day-old plants grown as described above and inoculated with *Streptomyces* strain 93 at planting were cut into 1 cm sections and fixed overnight in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7. Roots were rinsed three times with 0.1 M phosphate buffer, pH 7.0 and stained for 30 min in 2% osmium tetroxide. After rinsing three times with distilled water, roots were dehydrated in a graded acetone series, then critical-point dried. Samples were mounted on aluminum pin stubs and coated with Au/Pd in a vacuum evaporator. Samples were visualized using a Philips SEM 500.

2.4. In vitro antibiosis assays

A double layer agar method (Vidaver et al., 1972) was used to determine in vitro inhibition of 3 strains of *P. medicaginis* and 11 strains of *S. meliloti* by each of the 15 *Streptomyces* strains. In order to determine the potential of *Streptomyces* strains to influence

the production of antimicrobial compounds by other *Streptomyces* strains in culture, antibiosis assays were carried out initially with one *Streptomyces* strain per agar plate and results were compared with assays in which four strains were cultured on each agar plate. For assays with one isolate per plate, 10 μl of a fresh spore suspension of each *Streptomyces* strain at 10^7–10^8 CFU/ml in sterile water with 0.01% Tween-20 was dotted onto the surface of 10 ml R2YE agar (Hopwood et al., 1985) in a 15 mm × 60 mm Petri-plate. Plates were incubated at 28 °C for 3 days. The *Streptomyces* cultures were killed by inverting cultures over watch glasses containing chloroform in a fume hood for 1 h. Traces of the solvent were removed by placing plates in a laminar flow hood for 30 min. Plates were then overlaid with 3 ml of molten 1% water agar at approximately 55 °C containing either *P. medicaginis* (approximately 10^6 spores/ml) or *S. meliloti* (approximately 10^7 CFU/ml). Each *Streptomyces–Phoma* or *Streptomyces–Sinorhizobium* combination was tested on three individual plates. Clear growth inhibition zones were measured after incubation at 28 °C for 3 days (*S. meliloti*) or 7 days (*P. medicaginis*). The same procedure was used in assays examining four strains on each agar plate, using 35 ml of R2YE medium in 100 mm × 15 mm Petri-plates. The plates were overlaid with 10 ml of molten water agar inoculated with *P. medicaginis* or *S. meliloti* as above. Each *Streptomyces–Phoma* or *Streptomyces–Sinorhizobium* combination was tested on three individual plates with variable combinations of *Streptomyces* strains.

2.5. Disease assays

A single plant from the alfalfa variety Regen-SY (Bingham, 1991) was vegetatively propagated and plants were grown in a growth chamber under the same conditions as described above. Leaves were removed from the oldest three nodes of several clones and placed abaxial side down on sterile moistened filter paper in covered plastic 100 mm × 15 mm Petri-plates, four leaves per plate. Leaves in each plate were sprayed with approximately 1 ml of a 1×10^8 CFU/ml suspension of *Streptomyces* spores in OM broth. Plates were incubated at room temperature for 24 h during which time the free moisture in the *Streptomyces* inoculum dissipated. Leaves in each moist chamber were then sprayed with approximately 1 ml of *P. medicaginis* strain 866 spores at 1×10^6 spores/ml. Control treatments of oatmeal broth followed by *P. medicaginis* inoculation and oatmeal broth followed by spraying with water were included in each experiment. Leaves were incubated at room temperature for 5–6 days; water was added periodically to each chamber to keep filter papers moist. Leaves were scored for leaf spot symptoms, where 0: no symptoms; 0.5:1–10 "tar spots" on each leaflet, no yellowing; 1: 10–15 spots on each leaflet, approximately 25% of leaf yellow, 2: more than 15 spots on each leaflet, approximately 50% of leaf yellow; 3: 75% of leaf yellow, 4: 100% of leaf yellow. For each *Streptomyces* strain, four plates were assayed with leaves within plates treated as subsamples. The experiment was carried out twice.

Eight-week-old plants inoculated with *Streptomyces* strains 93, GS6-17 or GS43-11 and *S. meliloti* USDA105F21 as in Section 2.2 (40 plants per treatment), were sprayed until run-off with a fresh spore suspension of *P. medicaginis* strain 866 at 1×10^6 spores/ml in sterile distilled water with 0.01% Tween-20. Plants were maintained in 100% relative humidity in darkness for 48 h then returned to the growth chamber. Defoliation of the primary stem of each plant was evaluated 7 days after inoculation.

2.6. Nodulation assay

Seeds of the alfalfa variety Agate were surface sterilized and imbibed on sterile filter paper moistened with sterile distilled water at room temperature overnight. Growth containers were assembled from two nested 110 mm Magenta boxes (Magenta Corporation, Chicago, IL, USA). The top Magenta box was modified with a 0.5 cm diameter hole in the base fitted with a rope wick into the bottom box. Approximately 340 cm^3 of a sand:vermiculite (1:1 (v/v)) mixture was placed into the top Magenta box, moistened with 200 ml of a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994), and the containers autoclaved for 30 min on 2 consecutive days. A fresh *Streptomyces* spore suspension (approximately 6.8×10^7 CFU/ml) was added to the planting mixture immediately before seeding to give approximately 1×10^6 CFU/cm^3 planting mix. In the first experiment, the spores were suspended in phosphate buffered saline with 0.01% Tween-20, and in

the second experiment, the spores were suspended in OM broth with 0.01% Tween-20. Five imbibed seeds were planted in each container, approximately 0.5 cm deep, the planting mixture was covered with a plastic lid to maintain humidity, and containers were placed in a growth chamber with a 16 h light cycle at 21 °C. After 5 days, the lids were removed and each plant was inoculated with approximately 1×10^4 CFU *S. meliloti* strain USDA 105F21 in phosphate buffered saline. Additional nutrient solution was added to the bottom reservoirs as needed. Plants were removed 4 weeks after planting. Fresh weight of each plant was measured and the number of nodules on each plant counted. For each *Streptomyces–Sinorhizobium* combination, five containers were evaluated and each plant was analyzed as a subsample. Control treatments of each *Streptomyces* strain alone or *S. meliloti* alone were included in each experiment.

2.7. Statistical analyses

The Student's *t*-test was used to test for differences in plant biomass, nodulation, and disease severity between inoculated and non-inoculated treatments. Percent defoliation of each plant was converted to the arcsin value before analysis. To determine the influence of different strains on in vitro antibiosis, analysis of variance was performed using PROC GLM of SAS (SAS Institute, 1988). Fisher's unprotected least significant difference (LSD) test was used for mean comparisons. The Pearson correlation coefficient was used to evaluate the strength of the relationships between antibiosis against *P. medicaginis* and *S. meliloti* by the *Streptomyces* strains.

3. Results

3.1. Colonization of alfalfa seedlings by Streptomyces strains

Three *Streptomyces* strains, 93, GS6-17 and GS43-11, colonized roots, leaves, and nodules of alfalfa plants in the growth chamber assay (Fig. 1). The results from the two experiments were similar; data from one experiment are shown in Fig. 1. Streptomycete colonies recovered from inoculated plants had the morphology of the strain used for inoculation. Strain 93

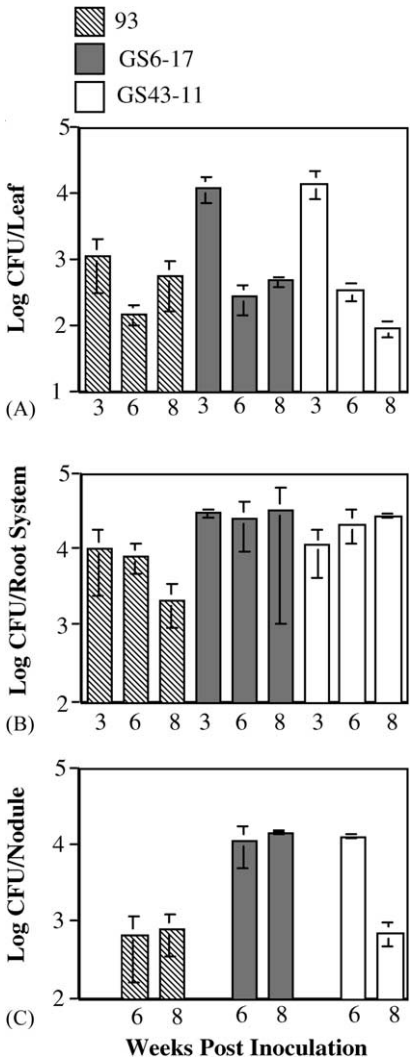

Fig. 1. Colonization of alfalfa plants by three *Streptomyces* strains: (A) mean log CFU per leaf; (B) mean log CFU per root; (C) mean log CFU per nodule. Each bar is the mean of five plants sampled at each time point with the standard deviation indicated.

had lower initial colonization densities than strains GS6-17 and GS43-11 on all organs. For all strains, colonization of the oldest trifoliolate leaf decreased significantly between 3 and 6 weeks after inoculation and colonization density remained low at 8 weeks after inoculation (Fig. 1A). The mean CFU recovered from individual root systems decreased over time on plants inoculated with strain 93 but remained constant on root systems inoculated with strains GS6-17

and GS42-11 over the 8-week period (Fig. 1B). Colonization of the upper 5 cm of each root system was approximately 10-fold greater (CFU/g root) than the remaining root system at all time points for all strains (data not shown).

The three *Streptomyces* strains were effective colonizers of alfalfa nodules. At 6 and 8 weeks after inoculation the mean CFU per nodule recovered from plants inoculated with strains 93 and GS6-17 were similar (Fig. 1C). From plants inoculated with strain GS43-11, the CFU per nodule decreased significantly from a mean of 1300 CFU per nodule at 6 weeks to 710 CFU per nodule at 8 weeks. There were no significant differences in nodule number or total nodule weight between 6 and 8 weeks for plants inoculated with strain GS43-11 (data not shown).

In one experiment, *Streptomyces* colonies with the morphology of the test strains were recovered from control plants indicating aerial movement of strains. At 6 weeks after inoculation, 20 and 38 CFU were recovered from the trifoliolate of two control plants, respectively. At 8 weeks after inoculation, *Streptomyces* colonies were recovered from the trifoliolate (8, 10, and 20 CFU per trifoliolate, respectively) and upper root system ($1–3 \times 10^3$ CFU per root system) of three control plants. No significant differences in shoot, root or nodule weight were observed between control and *Streptomyces*-inoculated plants.

Scanning electron microscopy confirmed the colonization of alfalfa roots by *Streptomyces*. In contrast to non-inoculated plants (Fig. 2A), a web of streptomycete filaments was observed along the entire root system of *Streptomyces*-inoculated plants (Fig. 2B) and many filaments appeared to be forming spore chains (Fig. 2C).

3.2. In vitro antibiosis

Initial double agar layer antibiosis assays with *P. medicaginis* 866 and *S. meliloti* 105F21 were conducted to test whether culture of multiple *Streptomyces* strains on an agar plate would influence the zone of growth inhibition by individual *Streptomyces* strains. Diameters of the zones of growth inhibition were similar for each specific *Streptomyces-P. medicaginis* or *Streptomyces–S. meliloti* combination, regardless of whether the *Streptomyces* strain was cultured alone or with four strains per plate (data not shown). Therefore,

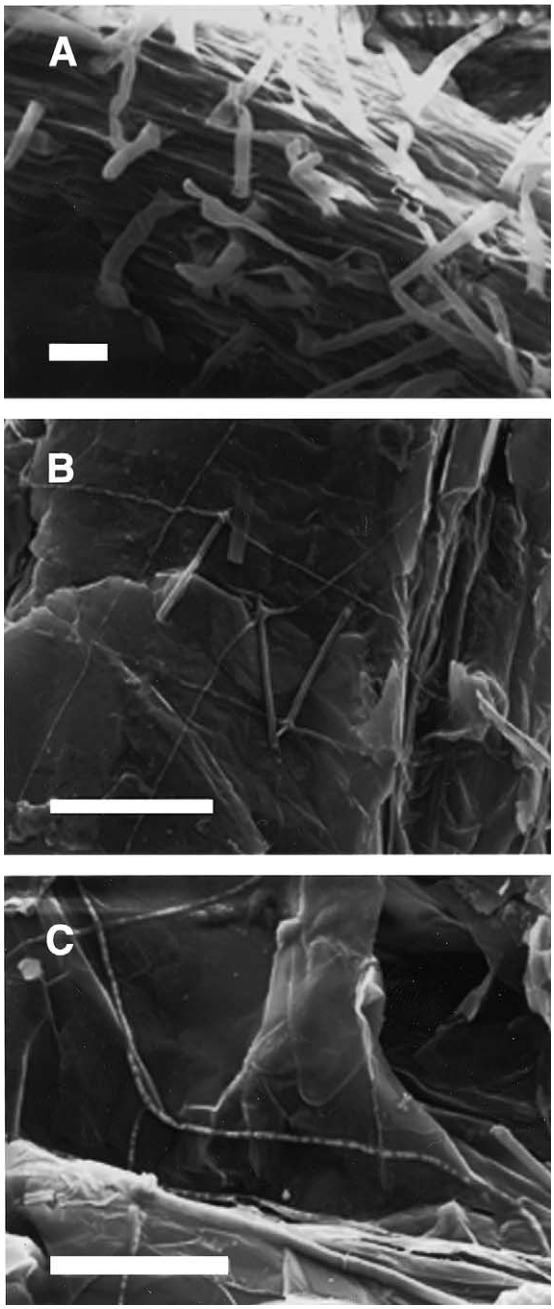

Fig. 2. Scanning electron micrographs of alfalfa roots inoculated with *Streptomyces* strain 93 at planting: (A) non-inoculated root. Bar = 50 μm; (B) *Streptomyces* filaments on inoculated root. Bar = 20 μm; (C) *Streptomyces* spore chains on inoculated root. Bar = 20 μm.

Table 1
Diameters (cm) of zones of in vitro growth inhibition of three strains of *Phoma medicaginis* by *Streptomyces* strains[a]

Streptomyces strain	*Phoma medicaginis*		
	866	T430	NY001
GS2-14	1.59 a	1.54 a	1.83 a
GS2-21	1.59 a	1.38 abc	1.63 abcd
GS2-17	1.59 a	1.35 abc	1.84 a
93	1.55 ab	1.19 bcd	1.39 cd
GS2-11	1.54 ab	1.51 a	1.73 ab
GS4-21	1.52 ab	1.43 ab	1.62 abcd
GS10-16	1.49 ab	1.01 d	0.68 e
GS6-17	1.45 ab	1.36 abc	1.59 abcd
GS43-5	1.35 ab	1.47 ab	1.7 abc
GS8-16	1.33 ab	1.28 abcd	1.43 bcd
GS8-22	1.26 ab	1.11 cd	1.38 d
GS8-1	1.20 b	1.15 cd	1.32 d
GS43-11	0 c	0 e	0 f
GS43-12	0 c	0 e	0 f

[a] Values followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).

antibiosis assays testing sensitivity of 3 strains of *P. medicaginis* and 11 strains of *S. meliloti* to the *Streptomyces* strains were carried out using 4 *Streptomyces* strains per plate.

Growth of each *P. medicaginis* strain was inhibited by 12 of the 14 *Streptomyces* strains tested. There were no significant differences (Pearson's $P < 0.0001$) among the three *P. medicaginis* strains in their response to *Streptomyces*, although diameters of zones of inhibition varied among *P. medicaginis* strains (Table 1). Among the *Streptomyces* strains inhibiting growth, the strains with the greatest inhibitory activity towards *P. medicaginis*, based on the combined average diameters of the zones of inhibition, were GS2-11, GS2-14 and GS2-17. The strains with the least inhibitory activity were GS8-1 and GS8-22. Strain GS43-11 and GS43-12 had no inhibitory activity.

The zones of growth inhibition for *S. meliloti* were much smaller than those for *P. medicaginis* and fewer *Streptomyces* strains had inhibitory activity against *S. meliloti* strains (Table 2). Seven *Streptomyces* strains had no inhibitory activity against any *S. meliloti* strain or had only weak inhibitory activity against *S. meliloti* USDA1005. Two strains caused small growth inhibition zones on several *S. meliloti* strains while six strains inhibited growth of all or most *S. meliloti* strains. Based on combined sizes of zones of inhibition for all *S. meliloti* strains, *Streptomyces* strains GS2-11 and GS2-17 had the greatest inhibitory activity towards *S. meliloti*. The *S. meliloti* strains varied substantially in sensitivity to antibiotic inhibition; the most sensitive strains, based on number of inhibitory interactions, were USDA1005, USDA105F21, and USDA1093 and the most resistant strain was USDA1179.

A comparison of in vitro growth inhibition of *P. medicaginis* and *S. meliloti* by the *Streptomyces* strains tested shows that growth of each *P. medicaginis* strain and *S. meliloti* strain was inhibited by at least one *Streptomyces* strain (Tables 1 and 2). However, two *Streptomyces* strains (GS43-11 and GS43-12) were unable to inhibit the in vitro growth of any strain of either *P. medicaginis* or *S. meliloti*. Finally, there was no significant correlation among *Streptomyces* strains in growth inhibition of *P. medicaginis* and *S. meliloti* (Pearson's R value $= 0.458$, $P = 0.1$). Although some *Streptomyces* strains inhibited growth of both *P. medicaginis* and *S. meliloti* (GS2-11, GS2-17), other strains inhibited the pathogen, but had little to no effect on growth of the mutualistic symbiont (93, GS8-16). Thus, inhibition of the pathogen by a *Streptomyces* strain was not predictive of inhibition of the mutualistic symbiont.

3.3. Effect of Streptomyces strains on leaf spot symptoms

Six *Streptomyces* strains that had similar inhibitory activity against *P. medicaginis* 866 were tested for their effect on leaf spot symptoms in a detached leaf assay. After 5–6 days of incubation in a moist chamber, control leaves treated with only OM broth had a nominal amount of leaf yellowing and no tar spots (Table 3). Leaves treated with OM broth followed by *P. medicaginis* showed a high density of leaf spots and approximately 50% of leaf area was yellowed. Treatment of leaves with *Streptomyces* strain GS6-17 prior to pathogen inoculation caused a significant reduction in disease scores in both experiments compared with the treatment with *P. medicaginis* alone although disease symptoms were still evident. The other strains (GS8-16, GS2-11, 93, GS43-5, and GS2-17), although as inhibitory as GS6-17 against *P. medicaginis* strain 866 in antibiosis assays, did not significantly reduce disease symptoms.

Table 2
Diameter (cm) of zones of in vitro growth inhibition of *Sinorhizobium meliloti* by *Streptomyces* strains

S. meliloti	Streptomyces strains														
	GS2-11[a]	GS2-17	GS43-6	GS2-21	GS6-17	GS2-14	GS4-21	GS43-5	93	GS8-1	GS8-22	GS8-16	GS10-16	GS43-11	GS43-12
105F21	1.27 bc	1.23 abc	0.7 cd	0.78 bcd	0.74 bc	0.15 de	0.33 ab	0.44 ab	0	0	0	0	0	0	0
1005	1.45 b	1.43 a	1.28 ab	0.9 abc	1.18 ab	0.52 c	0.57 a	0.57 ab	0.17 a	0.07 a	0.03 a	0	0	0	0
1021a	1.48 b	1.35 ab	1.1 abc	0.97 ab	1.07 ab	0.15 de	0.23 bc	0 c	0	0	0	0	0	0	0
1031	2.03 a	1.32 ab	1.42 a	1.37 a	1.42 a	0 d	0 c	0.67 a	0	0	0	0	0	0	0
1035	1.45 b	0.97 bc	0.8 bcd	0.92 abc	0.83 bc	0 d	0 c	0 c	0	0	0	0	0	0	0
1045	1.47 b	1.05 abc	1.08 abc	0 e	0.73 bc	0 d	0 c	0.37 ab	0	0	0	0	0	0	0
1093	0.95 cd	0.9 bcd	0.7 cd	0.32 de	0.46 cde	0.27 cde	0.1 bc	0.23 bc	0	0	0	0	0	0	0
1098	0.69 d	0.45 d	0.39 d	0.08 e	0.24 de	0 d	0 c	0 c	0	0	0	0	0	0	0
1171	0.8 d	0.86 cd	0.61 cd	0.54 bcde	0.57 cd	0.29 cd	0.06 c	0 c	0	0	0	0	0	0	0
1179	1.08 cd	1.17 abc	0.72 cd	0.75 bcd	0 e	0 c	0 c	0 c	0	0	0	0	0	0	0
1180	0.95 cd	0.82 cd	0.68 cd	0.48 cde	0.23 de	0.28 d	0 c	0 c	0	0	0	0	0	0	0

[a] Values within a column followed by the same letter are not significantly different (LSD, $P < 0.05$).

Table 3
Effect of *Streptomyces* strains on leaf spot symptoms caused by *Phoma medicaginis*[a]

Inoculum	Mean disease score[b]	
	Experiment 1	Experiment 2
GS8-16	2.75 a	2.03 abc
P. medicaginis control	2.02 b	2.55 ab
GS2-11	1.91 b	2.67 a
93	1.84 b	1.69 bc
GS43-5	1.44 bc	2.19 abc
GS2-17	1.31 bc	1.84 abc
GS6-17	0.91 cd	1.53 c
Control[c]	0.44 d	0.38 d

[a] Numbers followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).

[b] Values are the mean of the disease score of 12 leaves. 0 = no symptoms; 0.5 = 1–10 "tar spots" on each leaflet, no yellowing; 1 = 10–15 spots on each leaflet, approximately 25% of leaf yellow, 2 = more than 15 spots on each leaflet, approximately 50% of leaf yellow; 3=75% of leaf yellow, 4 = 100% of leaf yellow.

[c] Leaves were treated only with oatmeal broth.

Three *Streptomyces* strains were tested for their effect on defoliation caused by *P. medicaginis* using 8-week-old plants. The percent defoliation was greatest in both experiments in the non-inoculated control treatment (Table 4). In the first experiment, there was no significant effect of *Streptomyces* inoculation on defoliation. However, inoculation with strains GS43-11 and 93 at the time of planting significantly reduced defoliation in the second experiment.

Table 4
Effect of *Streptomyces* inoculation on defoliation by *Phoma medicaginis*[a]

Inoculum	Percent defoliation	
	Experiment 1	Experiment 2
Control	57 ab	59 a
93	61 a	41 b
GS43-11	56 ab	41 b
GS6-17	51 b	52 a

[a] Numbers followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).

3.4. Effect of Streptomyces strains on nodulation

Six *Streptomyces* strains with a range of in vitro antibiotic activity against *S. meliloti* USDA105F21 were tested to determine their effect on nodulation and plant weight. In the first experiment, the planting mixture was inoculated with spores of the *Streptomyces* strains suspended in phosphate buffered saline. Neither the number of nodules per plant nor plant dry weight were significantly influenced by *Streptomyces* treatments compared to the treatment with *S. meliloti* alone (Table 5). In the second experiment, spores were suspended in OM broth. In this experiment, the number of nodules per plant varied significantly among *Streptomyces* treatments, although no treatment resulted in a significant difference in nodule number from the *S. meliloti* control. Nonetheless, plant weights were reduced significantly when plants were inoculated with GS2-11 and GS2-17 compared to inoculation with

Table 5
Effect of *Streptomyces* strains on nodulation and plant dry weight[a]

Inoculum	Nodule number per plant		Dry weight per plant (g)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
GS43-6	18.6 a	24.3 a	0.42 a	0.53 b
GS6-17	17.3 ab	15.3 bc	0.39 a	0.52 b
GS8-16	16.6 ab	21.9 ab	0.38 a	0.50 b
S. meliloti control	16.3 ab	20.8 abc	0.43 a	0.62 ab
93	15.5 ab	23.6 a	0.44 a	0.76 a
GS2-17	15.0 ab	14.7 bc	0.41 a	0.26 c
GS2-11	14.5 b	16.4 ac	0.42 a	0.24 c
Control[b]	0 c	0 d	0.10 b	0.10 d

[a] Numbers followed by the same letter within a column are not significantly different (LSD, $P < 0.05$).

[b] Planting mixture treated only with phosphate buffered saline (Experiment 1) or oatmeal broth (Experiment 2).

S. meliloti alone (Table 5). These two strains showed the greatest in vitro growth inhibition against *S. meliloti*. However, other strains with strong in vitro antibiosis activity, GS43-6 and GS6-17, did not affect plant weights significantly.

4. Discussion

Streptomycetes are typically found in soils, but they have shown some potential for biological control of foliar pathogens (Gyenis, 2000). We found that alfalfa leaves, roots, and nodules were colonized by *Streptomyces* following inoculation of the planting mixture. Filaments as well as putative spore chains were observed along the entire root system of inoculated plants indicating active growth rather than passive movement of inoculum with plant growth. Relatively high population densities of *Streptomyces* occurred on alfalfa leaves up to 8 weeks after planting, when leaves had begun to senesce. Older alfalfa leaves are more susceptible to spring blackstem and leaf spot than young leaves, therefore maintaining effective population densities of *Streptomyces* as leaves age would be important for controlling this disease. Specific strains of *Streptomyces* have been found to effectively control foliar pathogens of *Poa pratensis* (Hodges et al., 1993) and hybrid poplar (Gyenis, 2000). Control of *P. medicaginis* with *Streptomyces* is attractive because the fungus attacks foliage, crowns, and roots of alfalfa plants (Leath et al., 1988) and inoculation of soil may be adequate to provide protection both above and below ground. Further experiments are needed to establish the longevity of introduced *Streptomyces* strains on alfalfa leaves and in the rhizosphere under field conditions. All *Streptomyces* strains tested attained relatively high population densities on alfalfa nodules, including those strains with antibiosis activity in vitro. Apparently alfalfa plants, or the rhizosphere soil of the plants, provide sufficient nutrients to support development of relatively high population densities of streptomycetes without affecting plant biomass accumulation. Nonetheless, streptomycete population densities on plant surfaces were affected by inoculum concentration. Preliminary experiments showed that a lower inoculum concentration (5×10^4 CFU/cm^3 of planting mix) resulted in lower population densities on alfalfa leaves and roots than a higher (10^6 CFU/cm^3) inoculum concentration. Thus, while the SEM study showed streptomycete growth on alfalfa root surfaces, some of the CFUs recovered from plants growing in ammended soil may arise from the original inoculum.

Diffusible chemicals from *Streptomyces* cultures have been shown to trigger antibiotic production by pathogen-suppressive *Streptomyces diastatochromogenes* PonSSII (Becker et al., 1997). Such interactions among streptomycetes may influence the antibiotic inhibition of pathogen strains in vitro. We tested whether culturing multiple *Streptomyces* strains in double agar layer antibiosis assays would affect the size of zones of growth inhibition against *P. medicaginis* or *S. meliloti*. Because the sizes of zones of inhibition were similar with single or multiple strains per plate, diffusible or volatile chemicals did not appear to impact in vitro antibiosis assays when test cultures were grown more than 20 mm apart. However, growth of *Streptomyces* strains in a closed atmosphere or at a closer distance to each other than that used here may influence the results.

The majority of the *Streptomyces* strains tested produced compounds highly inhibitory against growth of all three strains of *P. medicaginis* in vitro. The three strains, representing a range in geographic and genetic diversity within the species, responded differently to the panel of *Streptomyces* strains tested. This suggests that individual *Streptomyces* strains are producing distinct antifungal compounds, or different amounts of inhibitory compounds, and that pathogens vary in their sensitivity to these compounds. However, growth inhibition in culture did not correlate directly with control of disease symptoms. In the detached leaf assay, strain GS6-17 significantly decreased leaf spot symptoms caused by *P. medicaginis* on alfalfa leaves compared to the treatment with *P. medicaginis* alone (Table 3). However, in a defoliation assay, strain GS6-17 did not significantly decrease disease symptoms (Table 4). Inoculation of plants with strain GS43-11, which had no antibiotic activity against *P. medicaginis*, significantly decreased defoliation in one experiment. It is possible that antifungal compounds produced by the *Streptomyces* strains may not be produced on leaves or may not be effective once the fungus has penetrated the leaf surface. Furthermore, higher streptomycete population densities may be needed to have a significant effect on

disease control. Additional experiments are required to determine the optimal population density for effective control, to determine the competitive abilities of streptomycete strains against fungal pathogens and timing of antifungal compound production on the leaf surface. These experiments, and others using the same *Streptomyces* strains (Schottel et al., 2001; Xiao et al., 2002), indicate that antibiotic assays alone have a limited capacity to predict whether specific strains will be effective biological control agents for alfalfa diseases.

Overall, the *Streptomyces* strains had modest inhibitory effects on in vitro growth of the collection of *S. meliloti*. However, several strains affected growth of all *S. meliloti* strains to some degree. To determine if *Streptomyces* strains would affect nodulation, strains with different in vitro activities were allowed to colonize alfalfa seedlings for 5 days before inoculating with *S. meliloti*. Strains GS2-11 and GS2-17 showed marked in vitro activity against *S. meliloti*, however neither strain reduced nodule number significantly in either experiment compared to the treatment with *S. meliloti* alone. Interestingly, both strains significantly reduced plant dry weight in the second experiment, suggesting that the number of *S. meliloti* per nodule or their metabolic activity may have been reduced, leading to diminished nitrogen fixation. It is also possible that the *Streptomyces* strains produced plant growth-inhibiting compounds. Similar experiments were carried out with biological control strains of *Pseudomonas fluorescens* CHA0 that inhibit in vitro growth of *S. meliloti* due to production of pyoluteorin (Niemann et al., 1997). Over-production of pyoluteorin did not affect nodulation, however, lack of plant growth promotion by the over-producing strain was observed. In field soil, because of the presence of numerous different strains of *S. meliloti* and the variation in inhibition among *S. meliloti* strains, it is unlikely that inoculation with *Streptomyces* will significantly reduce either nodulation or plant dry matter accumulation.

These results lend further support to the concept that streptomycetes have the potential to contribute significantly to an integrated disease management system that includes alfalfa and other crops such as potato, corn, and soybeans due to their ability to colonize plants and decrease damage from a broad range of pathogens.

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