Biological Control of Fungi Causing Alfalfa Seedling Damping-Off with a Disease-Suppressive Strain of *Streptomyces*

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Biological control of alfalfa seedling diseases by a disease-suppressive strain of *Streptomyces* was evaluated *in vitro,* in controlled environments, and in the field. *Streptomyces* strain 93 inhibited the growth of soil-borne pathogens causing seed rot and seedling damping-off *in vitro* but did not affect the growth of the symbiont *Rhizobium meliloti.* Treating seeds of a susceptible alfalfa variety with *Streptomyces* spores inhibited the development of *Pythium* damping-off in a rolled paper towel assay. Control of Phytophthora root rot was achieved by infesting soil with *Streptomyces* at the time of planting under greenhouse conditions. The frequency of healthy plants increased significantly for the susceptible variety and the average disease severity index decreased significantly for both the resistant and susceptible varieties tested. Field experiments were performed in 1993 and 1994 to evaluate the effect of *Streptomyces* alone and in combination with the fungicide metalaxyl on disease control and plant performance. In 1993, neither treatment alone improved seedling establishment or disease control over controls. However, the combination of fungicide and *Streptomyces* resulted in the highest seedling survival, dry matter production, and frequency of healthy plants with no or slight symptoms of Phytophthora root rot. In the 1994 field experiment, plots receiving the combination of *Streptomyces* and fungicide were not significantly different from the untreated control in seedling survival or in incidence or severity of root rot. These studies indicate that a potential exists for utilizing *Streptomyces* to control alfalfa seedling diseases.

KEY WORDS: alfalfa; *Medicago sativa;* damping-off; *Pythium ultimum; Phytophthora medicaginis;* Phytophthora root rot; biocontrol; disease-suppressive *Streptomyces.*

INTRODUCTION

A number of fungi are associated with seedling diseases of alfalfa (*Medicago sativa* L.). Depending on location and environmental conditions, *Pythium, Aphanomyces, Phytophthora, Rhizoctonia,* and *Fusarium* spp. cause seed rot, preemergence, and postemergence damping-off (Altier and Thies, 1995; Handelsman *et al.,* 1990; Holub and Grau, 1990a; Schmitthenner, 1964) and can lead to significant stand losses during plant establishment. Multiple control measures are required to obtain optimum stand density in the presence of this pathogen complex. Presently, disease-resistant varieties, seed dressing with fungicide, and cultural control are used to minimize damage from seedling diseases. However, these measures do not always provide satisfactory control. Disease-resistant varieties have been the main strategy for minimizing disease losses in alfalfa (Leath *et al.,* 1988). Seedling resistance to *Phytophthora medicaginis* Hansen et Maxwell (=*Phytophthora megasperma* Drechs. f. sp. *medicaginis* T. Kuan & D. C. Erwin) (Irwin *et al.,* 1979), *Aphanomyces euteiches* Drechs. (Holub and Grau, 1990b), and some strains of *Pythium ultimum* Trow and *P. paroecandrum* Drechs. (Altier and Thies, 1995) have been identified. Selecting for resistance to alfalfa seedling diseases involving a single pathogen is straightforward. However, developing a variety with resistance to a shifting disease complex is not practical. Moreover, optimum expression of resistance is dependent on environmental conditions and age of the seedling (Altier and Thies, 1995; Holub and Grau, 1990a; Irwin *et al.,* 1979). Seed dressings with fungicide can augment genetic resistance but do not provide complete control. The fungicide metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methyoxyacetyl)-DL- alanine methyl ester] is effective against *Pythium* spp. and *P. medicaginis* but does not control other fungi in the disease complex. Development of a broad-spectrum biological control agent for alfalfa seedling diseases could enhance the benefits derived from

genetic resistance, chemical treatments, and cultural control methods.

Only a few microbial agents are currently being used for biocontrol of plant diseases. However, the feasibility of using root-associated microorganisms to control soil-borne pathogens in alfalfa and other legume crops has been studied previously (Kraft and Papavizas, 1983; Parke *et al.,* 1991; Handelsman *et al.,* 1990). In the case of alfalfa seedling diseases, antagonists need only protect seeds and seedlings for a relatively limited time, during germination and emergence, to be effective.

In Grand Rapids, Minnesota, disease-suppressive *Streptomyces* strains were isolated from fields that had shown a decline in potato scab (*Streptomyces scabies* (Thax.) Lambert and Loria) after being continuously cropped to potato for many years (Lorang *et al.,* 1995; Menzies, 1959). The suppressive strains protect potato tubers against pathogenic *S. scabies* in field and greenhouse trials (Liu, 1992; Liu *et al.,* 1995, 1996). Laboratory studies indicate that many of these strains produce antibiotics which inhibit *in vitro* growth of pathogenic isolates of *S. scabies.* Some suppressive strains also inhibit the growth of various soil-borne pathogens, including some that are pathogens of alfalfa (Liu, 1992).

The objective of this research was to determine the potential of using a strain of *Streptomyces* for biologically controlling soil-borne pathogens of alfalfa causing seedling damping-off. The effect of the suppressive strain on the growth of a number of pathogens was determined by an *in vitro* plate assay. Protection from damping-off caused by *P. ultimum* and *P. medicaginis* was tested under controlled environmental and field conditions.

MATERIALS AND METHODS

In Vitro Inhibition Assay

Streptomyces strain 93, provided by Dr. Neil Anderson, Department of Plant Pathology, University of Minnesota, was tested *in vitro* for its ability to inhibit the growth of microorganisms pathogenic to alfalfa using a double-layer agar method (Vidaver *et al.,* 1972). Spores of strain 93 were obtained from 10-day-old oatmeal agar cultures, spot-plated onto R2YE regeneration medium (Hopwood *et al.,* 1985) at 1×10^4 to 1×10^5 colony-forming units (CFU), and incubated at 28°C for 3 days. The bacteria were killed by inverting the plates over 3 ml of chloroform for 1 h. After drying for 30 min, the plates were overlaid with 15 ml molten 1% water agar containing a suspension of each pathogen tested. Following 3 to 5 days of incubation at 24°C, the plates were evaluated for growth inhibition of the pathogens, observed as clear zones around the spot of *Streptomyces,* by recording the diameter of the inhibition zone.

Streptomyces strain 93 was tested against the following alfalfa pathogens: *A. euteiches* (2 strains), *Clavibacter michiganensis* subsp. *insidiosus* (McCull.) Davis *et al.* (2 strains), *P. medicaginis* (2 strains), *Pythium paroecandrum* (1 strain), *P. sylvaticum*-like (1 strain), *P. ultimum* (2 strains), *Rhizoctonia solani* Kühn (1 strain), and the bacterial symbiont *Rhizobium meliloti* Dang. (1 strain). The *A. euteiches* and *P. medicaginis* strains were obtained from C. R. Grau, University of Wisconsin-Madison, *R. meliloti* from C. P. Vance, USDA-ARS (St. Paul, MN), and the remaining cultures from D. A. Samac, USDA-ARS (St. Paul, MN). Each pathogen was tested on four plates, and the experiment was repeated three times.

Bioassay for Pythium Damping-Off

A modification of the rolled paper towel assay described by Mitchell *et al.* (1969) was used to evaluate the efficacy of coating alfalfa seeds with spores of *Streptomyces* strain 93 to control infection of alfalfa seedlings by *P. ultimum* strain W3. *Streptomyces* spore suspensions were obtained by flooding 10-day-old oatmeal agar plate cultures with sterile distilled water. Suspensions were diluted with sterile distilled water to obtain a range of spore concentrations for coating seeds. Surface-sterilized seeds of the *Pythium*-susceptible variety Saranac were soaked for 30 min in *Streptomyces* spore suspensions and air dried in a laminar flow hood. To determine the number of spores present on the seed coat after inoculation, a sample of seeds was agitated in sterile distilled water and an aliquot of the resulting suspension was plated onto oatmeal agar. The number of *Streptomyces* colonies was determined after 10 days of incubation at 28°C. Three levels of *Streptomyces* were used; the average numbers of CFU per treatment were: 0, 58, and 7378 CFU per seed. The *P. ultimum* inoculum was produced by infesting 1 kg of sterilized soil amended with 5 g/kg ground dried alfalfa leaves with one finely chopped 3-day-old cornmeal agar culture of the fungus. Infested soil was incubated at room temperature for 10 days and then refrigerated at 5°C until used (Martin, 1992). Inoculum density of *P. ultimum* in this soil was 3–6 CFU/g soil. Seeds were spread on a moistened sterile 26 × 30-cm paper towel, 20 seeds per towel, and covered with 10 g of the *P. ultimum*-infested soil. The towels were rolled, placed in plastic bags, and incubated for 5 days at room temperature. Four towels were used per treatment, and the experiment was carried out three times. After incubation, disease severity was rated using a 5-class scale (1 = healthy seedling; 2 = primary root tip necrotic but firm; 3 = primary root tip soft and rotted; 4 = dead seedling, germinated seed with rotted radicle; 5 = dead seed, ungerminated rotted seed) and expressed as the average disease severity index (ASI) described by Altier and Thies (1995). Data were subjected to analysis of

variance and treatment means were separated with Fisher's protected least square difference (LSD) using the Statistical Analytical System (SAS) (SAS Institute, 1988).

Greenhouse Assay for Biocontrol of P. medicaginis

Greenhouse studies were performed to test the ability of the suppressive *Streptomyces* strain 93 to control the incidence and severity of seedling damping-off and root rot caused by *P. medicaginis.* Seeds of two alfalfa varieties, WAPH-1 and Saranac, resistant and susceptible to Phytophthora root rot, respectively, were planted in ProMix BX (Premier Co., Winnipeg, Canada) in 6-cell plastic packs (cell size $4 \times 6 \times 6$ cm deep), 10 seeds/cell. Granular inoculum of *Streptomyces* strain 93 in a peat base at 1×10^7 CFU/g (The Urbana Laboratories, St. Joseph, MO) was incorporated into the mix at planting at approximately 0, 3×10^5 to 6×10^5 or 1×10^6 CFU/seed by adding 0, 0.5, or 1.0 g inoculum/cell, respectively. As is common with peat-based preparations, the inoculum contained *Streptomyces* and a large number of other microbes. The plants were grown in the greenhouse under a 16-h photoperiod and 22–24°C average temperature.

The fungal inoculum was prepared by homogenizing 9-day-old *P. medicaginis* V-8 agar cultures (strain M2019) in a blender with sterile distilled water, 30 ml/plate, for 30 s. The mycelial suspension was added to the soil at the base of seedlings, 1 ml per seedling, when the first trifoliolate leaf was expanded in the majority of the seedlings (10 to 12 days old). Flooded conditions were maintained for 2 days after inoculation to stimulate infection. Plants were watered daily thereafter. Fifteen days after inoculation, the plants were rated for Phytophthora root rot infection using a 5-class scale (1 = healthy seedling, no necrosis of roots or hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons and moderate stunting of stems; 4 = extensive necrosis of roots, hypocotyls and cotyledons, severe stunting of stem; 5 = dead seedling) (Holub and Grau, 1990b). The experimental design was a factorial with two pathogen levels, two alfalfa varieties, and two *Streptomyces* levels. An average of 120 seedlings per treatment were evaluated in each experiment, and the experiment was carried out three times. Statistical analyses were performed using the general linear model procedure of SAS and treatment means were separated using Fisher's protected LSD.

1993 Field Experiments

Field trials were conducted to assess the ability of the suppressive strain to control *P. medicaginis* under irrigation and to compare the effects of fungicide application and *Streptomyces* treatment on alfalfa establishment, severity of Phytophthora root rot, and yield of resistant and susceptible alfalfa varieties. In 1993, field plots were planted June 1 at the University of Minnesota St. Paul Experiment Station (St. Paul, MN). The site had a sandy loam soil (Waukegan) that for 8 years had been used as an alfalfa disease nursery for evaluating resistance to Phytophthora root rot. The field was left fallow for 2 years previous to the establishment of this experiment. Four alfalfa varieties representing a range in resistance to Phytophthora root rot were planted with a belt seeder at a seeding rate of 150 viable seeds per 3-m row. The varieties were Saranac (susceptible), Alfagraze (low resistance), Agate (resistant), and an experimental variety MWNC-4 (resistant) obtained from D. K. Barnes, USDA-ARS (St. Paul, MN). The fungicide metalaxyl (Ciba-Geigy Corp., Greensboro, NC) was incorporated into soil before seeding at a rate of 2.0 kg active ingredient/ha.

To prepare the bacterial inoculum, spore suspensions of *Streptomyces* were obtained from 10-day-old oatmeal agar cultures and inoculated onto sterile vermiculite (Strong-lite Products Corp., Serena, IL) amended with oatmeal broth (3:1 v/v) (Liu *et al.,* 1996). At the time of planting, after 4 weeks of incubation at 24°C, the inoculum contained 1.9×10^7 CFU/cm³. Inoculum was broadcast in the plots at 97 cm³/m and incorporated by raking the top 10 cm of soil. The target inoculum density was estimated based on the results of Liu (1992) and aimed for 2% (v/v) of the biocontrol agent's inoculum per volume of soil in the alfalfa rhizosphere.

The experimental design was a randomized complete block replicated four times with treatments in a split-split-plot arrangement. The plot size was 0.66×3.0 m with two rows per plot. Whole plots measured the fungicide effect, subplot treatment was *Streptomyces* inoculum, and sub-subplots were alfalfa varieties.

Alfalfa seedling populations were measured 4 weeks after seeding by counting a central 0.305-m² area within each sub-subplot. Daily overhead irrigation for 25 min/day was used for 6 weeks starting 4 weeks after planting to increase the occurrence of Phytophthora root rot. Plots were mowed 9 weeks after planting without taking dry matter yields. The plots were harvested for yield 16 weeks after planting by cutting two 1-m-long rows from each treatment 5 cm above the plant crowns. Fresh weights were recorded in the field, and the whole sample was dried at 140°C for dry matter determination. To estimate disease severity, plants from a 50-cm row were dug and the roots washed and rated for Phytophthora root rot symptoms following the 6-class rating scale used to evaluate Phytophthora root-rot resistance in disease nurseries (1 = no lesions; 2 = small root lesions; 3 = large nongirdling lesion and/or branch root tips rotted off; 4 = extensive lesions

with tap root and branch root ends rotted off; 5 = tap and lateral roots almost destroyed, plant alive; 6 = plant dead, calculated from loss in stand) described previously (Frosheiser and Barnes, 1973). The frequency of healthy plants was expressed as the percentage of plants with no or slight symptoms of infection (classes 1 + 2). Plots were mowed after harvest and new stand counts were recorded in a 0.305-m² area within each sub-subplot. The same areas were counted again during the spring of the second year and foliage was cut as before to determine second-year yield. Data were subjected to analysis of variance and treatment means were separated using Fisher's protected LSD using SAS.

1994 Field Experiments

Two experiments were established during 1994 (May 11 and May 25, 1994) to determine the effect of *Streptomyces* on plant establishment and Phytophthora root-rot symptoms in two alfalfa genotypes. Application of the biocontrol agent and fungicide was carried out in a manner applicable to production field situations. The plot, in a sandy loam, had been used to evaluate Phytophthora root-rot resistance for 26 years and was located on the University of Minnesota St. Paul Experiment Station (St. Paul, MN).

Two alfalfa varieties, Vernal (susceptible to Phytophthora root rot) and Legend (resistant), were planted at a seeding rate of 150 viable seeds per 3-m row. The seeds were either nontreated or coated with metalaxyl (Apron) by Seedbiotics (Bakersfield, CA). A granular inoculum of *Streptomyces* strain 93 in a peat base at 1×10^7 CFU/g (The Urbana Laboratories) was incorporated in the furrow with the seed at planting (0.9 g per 1.5-m row) using a belt planter. The two experiments had completely randomized block designs with four replicates. The plot size was 1×1.5 m containing three rows with a distance of 0.33 m between rows. Irrigation was used to increase the occurrence of *P. medicaginis* infection. Irrigation started June 7, when the plants of the first and second plantings were 23 and 8 days old, respectively. The plots were watered by overhead irrigation for 25 min/day during the following 6-week growing period.

Plant stands were measured 2 weeks after planting for each planting date. Phytophthora root-rot symptoms were assessed as before using plants from the middle row of each plot when plants of the first planting date were 10 weeks old and 8 weeks after the second planting. Due to the short growing period of these tests, the forage yield was not measured. No differences between the two experiments were observed; therefore, the results were analyzed over experiments using Fisher's protected LSD.

RESULTS AND DISCUSSION

In Vitro Inhibition Assay

Streptomyces strain 93 inhibited the growth of all alfalfa pathogens tested in the *in vitro* assays but did not affect the growth of *R. meliloti* (Table 1). There were no significant differences ($P = 0.05$) in the average diameters of the inhibition zones between pathogens or between strains. Similar results were obtained using two other potato scab-suppressive *Streptomyces* strains isolated from potatoes with the exception that one strain inhibited the growth of *R. meliloti* (data not shown). Liu (1992) reported that scab-suppressive *Streptomyces* strains inhibit the growth of a wide range of plant pathogens *in vitro* by production of antibiotics. We found that *Streptomyces* strain 93 inhibited the growth of pathogens causing alfalfa seedling diseases including: *A. euteiches, C. michiganensis* subsp. *insidiosus, P. medicaginis, Pythium* spp., and *R. solani.* These results suggest that *Streptomyces* could be used for effective control of the seedling damping-off disease complex.

The ability to inhibit the growth of a wide range of organisms is not common among biocontrol agents. Antagonists often have a high degree of specificity for a particular pathogen or strains of a pathogen (Kommedahl and Windels, 1980). However, *Streptomyces* isolates have been reported to control plant diseases caused by nematodes (Dicklow *et al.,* 1993), fungi (Crawford *et al.,* 1993; Hodges *et al.,* 1993), and bacteria (El-Abyad *et al.,* 1993; Tanii *et al.,* 1990), evidence that these bacteria have a broad spectrum of activity against other microorganisms. Production of antibiotics has been suggested as the principal mechanism of

TABLE 1

In Vitro Growth Inhibition of Alfalfa Pathogens by *Streptomyces* Strain 93[a]

Microorganism screened	Strain	Inhibition zone (mm)[b]
Aphanomyces euteiches	NC-1	24.8 ± 3.5
A. euteiches	Mf-1	24.2 ± 1.3
Clavibacter michiganensis subsp. *insidiosus*	R1	18.7 ± 1.5
C. michiganensis subsp. *insidiosus*	R9	25.0 ± 6.0
Phytophthora medicaginis	FD1206	22.8 ± 2.9
P. medicaginis	M2019	24.3 ± 1.3
Pythium paroecandrum	L3	15.0 ± 1.7
P. sylvaticum-like	L4	15.7 ± 2.6
P. ultimum	GR1	18.0 ± 3.6
P. ultimum	W3	12.3 ± 1.5
Rhizobium meliloti	102F51	0
Rhizoctonia solani	140	13.2 ± 2.9

[a] *Streptomyces* was grown on R2YE agar plates and killed with chloroform before being overlaid with the microorganisms tested.

[b] Mean diameter of inhibition zones with standard error measured after 3–5 days incubation at 24°C.

action of many microorganisms under study for use as biological control agents (Handelsman *et al.,* 1990; Liu *et al.,* 1995; Parke *et al.,* 1991; Silo-Suh *et al.,* 1994). Research on the use of *Streptomyces* strains for control of potato scab, including strain 93 used in these experiments, indicates that a combination of antibiosis and resource competition with the pathogenic strains may be responsible for the reduction of disease (Eckwall *et al.,* 1994; Liu *et al.,* 1995; Ryan and Kinkel, unpublished). However, the ability of an organism to produce an antibiotic *in vitro* does not necessarily ensure its effectiveness as an antagonist in the field (Kommedahl and Windels, 1980), because *in vitro* antibiosis is not always correlated with antibiosis in the rhizosphere. In addition, competition for resources and induction of host defense responses leading to enhanced systemic resistance may also play a role in successful biocontrol (Deacon, 1988; Mandeel and Baker, 1991).

Biological Control of P. ultimum and P. medicaginis under Controlled Environmental Conditions

Damping-off caused by *P. ultimum* was significantly reduced when seeds were coated with *Streptomyces* spores. In the combined results from the three experiments (Table 2), there was a significant reduction ($P = 0.05$) in the ASI, from 4.5 to 4.0, and a significant increase in the frequency of healthy plants, from 34 to 56%, when seeds were treated with *Streptomyces* compared to the untreated control. Increasing the *Streptomyces* inoculum level, from an average of 58 to 7378 CFU per seed, did not significantly increase the efficacy of biocontrol. This may indicate that only a limited number of spores were able to colonize the treated seed and the surrounding soil. Various inoculation methods should therefore be tested to optimize seed and seedling protection. In the absence of the pathogen, *Strepto-*

TABLE 2

Effect of Seed Coating with *Streptomyces* on the ASI of *Pythium* Damping-Off and Frequency of Healthy Plants in a Rolled Towel Assay

Average number of *Streptomyces* (CFU/seed) [a]	ASI [b]	Frequency of healthy plants (%) [c]
0	4.5	34
58	4.1	54
7,379	4.0	56
LSD (0.05)	0.2	10

[a] Surface-sterilized seeds were coated with a water suspension of *Streptomyces.*

[b] Disease severity was rated using a 5-class scale (1 = healthy seedling, 5 = dead seed, nongerminated rotted seed) and expressed as the average disease severity index.

[c] Percentage of plants with no symptoms or slight symptoms (classes 1 and 2).

myces had no effect on ASI or the frequency of healthy plants (data not shown), indicating that the biocontrol agent had no negative effect on seed germination. These experiments suggest that seed treatment with the suppressive strain is a promising method for controlling seedling damping-off caused by *P. ultimum* and possibly other organisms causing seed rot and damping-off.

The potential of *Streptomyces* strain 93 to control damping-off and root rot caused by *P. medicaginis* was tested in a greenhouse assay. There was a significant decrease ($P = 0.05$) in the ASI for plants from both the susceptible (Saranac) and resistant (WAPH-1) varieties receiving the *Streptomyces* treatment compared to the untreated controls (Fig. 1A). The *Streptomyces* treatment significantly increased the frequency of Saranac plants with no symptoms or a resistant type of reaction (classes 1 and 2), but did not have a significant effect on the percentage of healthy WAPH-1 plants (Fig. 1B). It is possible that a beneficial effect from the biocontrol agent would be observed for the resistant variety if it was exposed to higher pathogen pressure. Due to the genetic heterogeneity in alfalfa varieties, a variety with high resistance contains approximately 50% resistant plants. In highly disease conducive conditions, the expected frequency of plants with a resistant reaction is 35–60% of the population for WAPH-1 and 0–10% for Saranac. The average frequency of resistant plants in the untreated controls in these experiments was 84% for WAPH-1 and 54% for Saranac, indicating low disease pressure. With the low disease pressure observed, there was no significant effect due to concentration of *Streptomyces* on either genotype for ASI or percentage of resistant plants ($P = 0.05$). However, the data indicate a trend of increased disease control with higher levels of *Streptomyces* which may be magnified with higher disease pressure. In the absence of the pathogen, *Streptomyces* treatment had no influence on ASI or percentage of healthy plants (data not shown).

Field Experiments

Trails were conducted in 1993 and 1994 to evaluate the efficacy of *Streptomyces* protection alone and in combination with the fungicide metalaxyl in a field situation against seedling damping-off and root rot caused by *P. medicaginis.* In the 1993 field trial, neither *Streptomyces* nor fungicide treatments alone increased plant populations at establishment (4 weeks), at harvest (16 weeks), or in the second year over the untreated controls (Table 3). However, there was a significant interaction ($P = 0.05$) with the combined *Streptomyces* and fungicide application that affected plant establishment, plant populations in the second year, dry matter yield at 16 weeks, and disease (Table 3). When plant counts were averaged across all alfalfa varieties, plots that received both treatments had the
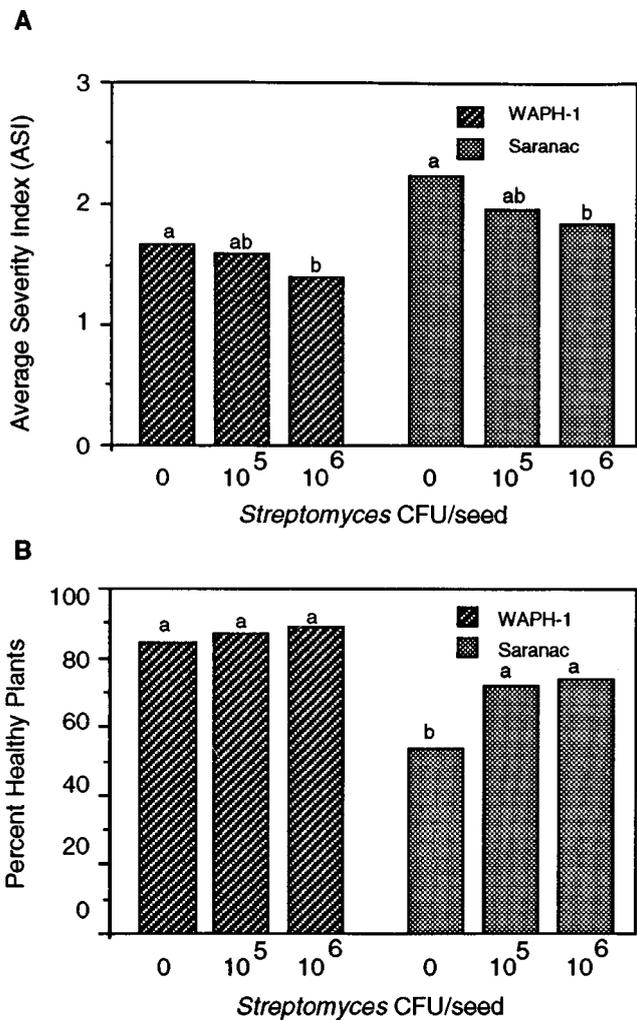
A

Average Severity Index (ASI)

WAPH-1
Saranac

a
ab
b
a
ab
b

Streptomyces CFU/seed

0   10^5 10^6 0 10^5 10^6

B

Percent Healthy Plants

WAPH-1
Saranac

a   a   a
b
a   a

Streptomyces CFU/seed

0   10^5 10^6 0 10^5 10^6

FIG. 1. Effect of *Streptomyces* treatment on Phytophthora root-rot symptoms in plants from resistant (WAPH-1) and susceptible (Saranac) varieties. *Streptomyces* was added to soil at the time of planting. Statistical analyses were performed within alfalfa genotype. Bars with the same letter are not different at $P = 0.05$. (A) Average severity index (ASI) of plants rated on a 0 to 5 scale (1 = no symptoms; 5 = dead plant). (B) Percentage of plants with no or slight symptoms of infection (classes 1 and 2).

highest plant stands at both 4 and 16 weeks after planting, and significant differences in plant numbers were still evident in the spring of the second year. As a result of higher plant stands, dry matter production in the seeding year was significantly higher for the combined treatment over the control and fungicide-treated plots. *Streptomyces* treatment alone tended to decrease dry matter production, although the difference was not significant ($P = 0.05$) from the untreated and fungicide-treated plots. By the spring of the second growing season, the surviving plants from all treatments had compensated for disease losses and dry matter production was not significantly different for any treatment. The combined treatment provided protection from seed-

ling damping-off, as indicated by the significantly higher plant stands, under field conditions. Seedling damping-off is caused by *P. megasperma* as well as a number of other fungi including *A. eutiches, Fusarium* spp., *Pythium* spp., and *R. solani,* depending on location and environmental conditions. Alfalfa plants are susceptible to Phytophthora root rot at the seedling stage and as adult plants. The combined *Streptomyces* and fungicide treatment dramatically increased the frequency of healthy plants with no symptoms or slight symptoms of Phytophthora root rot over untreated plants or plants receiving either treatment alone. Because the fungicide has relatively short-term effects, the suppressive strain may be present on roots and suppressing disease throughout the season or *Streptomyces* may have a long-lasting detrimental impact on the pathogen population. The interaction between *Streptomyces* and fungicide treatments suggests that fungicide treatment favors the establishment of the biological control agent or that each treatment is affecting a different set of soil microorganisms.

The reactions of four alfalfa varieties with different levels of resistance to Phytophthora root rot were examined in the field for interactions with the biocontrol agent, fungicide treatment, or the combined *Streptomyces* and fungicide treatment. No significant variety by treatment interactions were observed. Data were combined across treatments (Table 4) since the ranking of the four varieties was the same in all treatments for plant populations, dry matter yield, and disease resistance. Across all treatments, the resistant varieties, Agate and MWNC-4, had significantly higher plant populations in 1994, higher dry matter yield in 1993, and a higher frequency of healthy plants than the susceptible variety Saranac. The lack of an interaction in this experiment between *Streptomyces* and the alfalfa varieties tested does not rule out the possibility that other varieties, or individual plants within varieties, have characteristics that enhance their interactions with biocontrol agents. *Streptomyces* colonies with the pink–gray coloration of strain 93 were isolated from alfalfa root surfaces 16 weeks after planting from plots receiving the *Streptomyces* treatments (data not shown). Although a systematic sampling was not performed, it appeared that alfalfa genotype affected the recovery of *Streptomyces.* It should therefore be possible to select alfalfa plants that support enhanced disease control and/or colonization by the biocontrol agent.

In the 1994 field trials *Streptomyces* was applied as a granular inoculum in furrow and metalaxyl was applied as a seed coating to approximate on-farm production practices. Experiments were started at two times to evaluate the effect of planting date on disease control. The results from the two experiments were not statistically different and values reported are averages

TABLE 3

Effect of *Streptomyces* Treatment and Metalaxyl Application on Plant Population, Dry Matter Production, and Frequency of Plants with a Resistant Reaction to Phytophthora Root Rot

Treatment	Plant population (number/m²)[a]			Dry matter (g/m²)		Frequency of healthy plants (%)[b]	Disease severity (ASI)
	4-week-old	16-week-old	1994	1993	1994		
Untreated	95[c]	37	27	83.6	367.5	43.3	3.5
Streptomyces	108	38	26	67.8	344.2	43.0	3.4
Metalaxyl	112	40	31	94.9	359.0	59.0	3.3
Streptomyces + metalaxyl	125	58	39	144.1	353.6	88.2	2.1
LSD (0.05)	21	10	5	22.3	NS[d]	17.3	NS

[a] Plant populations were measured at establishment (4 weeks after planting), at harvest (16 weeks after planting), and in the spring of 1994.
[b] Percentage of plants with no or slight symptoms of Phytophthora root rot (classes 1 and 2) at 16 weeks after planting.
[c] Values are averages across alfalfa varieties.
[d] NS, not significant ($P = 0.05$).

across the two planting dates. Results of the 1994 field trials were markedly different from those of the 1993 trial. The highest plant populations were obtained with the combination of fungicide seed coating and *Streptomyces* application (Table 5). However, unlike the results from the previous year, the differences in populations were not significant ($P = 0.05$) between the untreated control and the combined treatment. For both Vernal, the Phytophthora root-rot-susceptible variety, and Legend, the resistant variety, there were not significant differences in initial plant populations from the control in plots with fungicide seed coating or with the combined treatment. The biocontrol treatment alone significantly decreased initial plant populations for Vernal and decreased both initial and final plant populations for Legend, compared to the control and other treatments. For both varieties, none of the treatments significantly increased the frequency of healthy plants or decreased the disease severity compared to the control. The *Streptomyces* treatment alone significantly decreased the frequency of healthy plants from the

variety Legend. Counter to the 1993 field trial, the combination of fungicide and *Streptomyces* did not have a significant effect on disease control compared to the untreated plots and *Streptomyces* treatment alone had a detrimental effect on plant establishment and disease resistance. It is possible that the inoculum, a pure vermiculite-based culture in 1993 and a peat-based culture in 1994 that contained a wide variety of microorganisms, contained pathogenic organisms or influenced the rhizosphere microbial populations and consequently the efficacy of biocontrol observed. Further research into the effects of different methods of *Streptomyces* delivery on microbial populations and efficacy of biological control are clearly warranted. Interestingly, fungicide treatments in 1993, in which metalaxyl was incorporated into the soil at planting, and in 1994, when used as a seed dressing, did not significantly improve plant populations, the frequency of healthy plants, or significantly decrease disease severity. Failure of metalaxyl to improve alfalfa establishment has been observed (Vincelli *et al.,* 1994) and populations of

TABLE 4

Effect of Variety on Plant Population, Dry Matter Production, and Frequency of Plants with a Resistant Reaction to Phytophthora Root Rot

Variety	Plant population (number/m²)[a]			Dry matter (g/m²)		Frequency of healthy plants (%)[b]	Disease severity (ASI)[c]
	4-week-old	16-week-old	1994	1993	1994		
Saranac	117[d]	40	25	78.6	328.5	53.4	3.3
Alfagraze	126	44	33	86.0	350.6	49.5	3.4
Agate	89	45	32	106.7	380.6	61.2	2.8
MWNC-4	108	43	34	121.5	366.7	70.4	2.8
LSD (0.05)	21	NS[e]	5	22.3	48.5	17.5	NS

[a] Plant populations were measured at establishment (4 weeks after planting), at harvest (16 weeks after planting), and in the spring of 1994.
[b] Percentage of plants with no or slight symptoms of Phytophthora root rot (classes 1 and 2) at 16 weeks after planting.
[c] Average disease severity index based on a 6-class scoring system of roots: 1 = healthy root, 6 = roots rotted, dead plant.
[d] Values are averages across treatments.
[e] NS, not significant ($P = 0.05$).

TABLE 5

Effect of *Streptomyces* Treatment and Metalaxyl Seed Coating on Plant Population, Frequency of Resistant Plants, and Phytophthora Root-Rot Severity for Two Alfalfa Varieties

Treatment[a]	Vernal[a]				Legend			
	Plant population (number/m²)[c]		Frequency of healthy plants (%)[d]	Disease severity (ASI)[e]	Plant population (number/m²)		Frequency of healthy plants (%)	Disease severity (ASI)
	Initial	Final			Initial	Final		
Untreated	113[f]	59	14	5.2	127	104	43	3.5
Streptomyces	105	62	18	4.8	119	97	37	3.4
Metalaxyl	113	73	25	4.6	134	119	47	3.1
Streptomyces + metalaxyl	118	73	23	4.8	138	119	49	3.2
LSD (0.05)	10	NS[g]	NS	NS	11	16	10	NS

[a] Vernal, Phytophthora root rot susceptible variety; Legend, resistant variety.

[b] *Streptomyces* was applied as a granular inoculum in the furrow; metalaxyl as a seed coating.

[c] Plant population was determined 2 weeks after planting and at harvest. Experiments were planted May 11 and May 25. Harvest was done 8 and 10 weeks after planting, respectively.

[d] Percentage of plants with no or slight symptoms of Phytophthora root rot (classes 1 and 2) at harvest.

[e] Average disease severity index based on a 6-class scoring system of roots: 1 = healthy root, 6 = roots rotted, dead plant.

[f] Values are averaged across the two planting dates.

[g] NS, not significant ($P = 0.05$).

metalaxyl-resistant *P. medicaginis* have been found (Hunger *et al.,* 1982), underscoring the need for alternative tools for managing alfalfa seedling diseases.

In contrast with the results from the greenhouse assay, *Streptomyces* treatment alone did not efficiently control Phytophthora root-rot symptoms in the field nor was seedling damping-off controlled by the biocontrol treatment alone. Biological control of Phytophthora root rot in alfalfa under field conditions was tested previously with *Bacillus cereus* Frankland & Frankland UW 85 and was also found to be less successful than under controlled conditions (Handelsman *et al.,* 1990). However, *B. cereus* UW 85 demonstrates effective field control of root rot in soybean (*Glycine max* [L.] Merr.) caused by *Phytophthora sojae* M. J. Kaufmann & J. W. Gerdemann (Gilbert *et al.,* 1993). This suggests that at least some of the difficulties found in the effectiveness of biocontrol agents for disease control under field conditions may be associated with the genetic heterogeneity within alfalfa varieties. It is likely that support and action of biocontrol agents is determined to some extent by the genotype of the plant. In that case, selection for strains that interact with all alfalfa genotypes equally well or selection of genotypes that support the action of a broad spectrum of biocontrol microorganisms would provide more effective biological control.

Environmental conditions in the field most likely also influenced the effect of the biocontrol agent through direct effects on colonization by *Streptomyces* or by affecting interactions between the biocontrol agent and the pathogen. Wet environmental conditions are condu-cive to the development of *P. medicaginis* and other fungi in the Class Oomycetes. Growth of plant pathogenic *Streptomyces* strains closely related to the pathogen-suppressive strains is inhibited by excessive soil water potential (Lapwood and Adams, 1975) and this growth characteristic may limit the effectiveness of the biological control agent for managing Phytophthora root rot. Because disease control was observed after saturated soil conditions in the greenhouse assay and in the 1993 field trial, it is possible that the suppressive strains are less affected by soil moisture than pathogenic strains. Alternatively, the presence of the suppressive strains in the rhizosphere of alfalfa may alter the microbial environment, making it less conducive to disease-causing organisms, or the biocontrol agent may induce resistance mechanisms in the alfalfa plants. Understanding the mechanisms of interaction and environmental conditions influencing biocontrol will be critical to developing strategies for enhancing disease control. Research leading to optimization of delivery systems and determinations of appropriate concentrations of the antagonist to be used in each soil and pathogen situation is clearly indicated. In addition, selection of strains active over the wide range of environmental conditions encountered at planting or use of mixtures of strains with different conditions of growth could also improve *Streptomyces* performance.

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