IMPORTANCE OF ANTIBIOTIC PRODUCTION IN ANTAGONISM OF SELECTED *STREPTOMYCES* SPECIES TO TWO SOIL-BORNE PLANT PATHOGENS

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Antagonism of ten *Streptomyces* spp., five of which produce antibiotics, to the plant pathogens *Rhizoctonia solani* and *Phytophthora megasperma* var. *sojae* was studied. Antibiotic activity was detected in culture for the five antibiotic producers. *S. griseus*, *S. hygroscopicus* var. *geldanus*, and *S. noursei* produced wide zones of inhibition to *R. solani* and *P. megasperma* var. *sojae*. Similar activity was found for *S. reticuli* var. *protomycicus* to *P. megasperma* var. *sojae*. *S. cellulosae* reduced Rhizoctonia root rot on pea when sterile soil was infested simultaneously with the antagonist and *R. solani*. *S. hygroscopicus* gave almost complete disease control when the streptomycete was added 7 days prior to infesting the soil with *R. solani*. Several of the *Streptomyces* spp. reduced Phytophthora root rot on soybean when the streptomycetes were added to soil at the same time as *P. megasperma* var. *sojae* or 7 days prior to adding the pathogen. *S. herbaricolor* and *S. coeruleofuscus* gave the most consistent control. No relationship was found between reported antibiotic activity or antagonism on agar media and reduction in disease severity. Only *S. hygroscopicus* var. *geldanus* gave both control of Rhizoctonia root rot and large zones of inhibition on agar media when the streptomycetes were preincubated in soil for 7 days.

The means by which one organism inhibits the growth of another in soil is still a moot point and various mechanisms have been postulated to explain the process. Since some antagonistic microorganisms produce antibiotics *in vitro* which inhibit susceptible organisms, the general tendency is to point to the synthesis of antibiotics as one mechanism involved in antagonism in soil. Whether antibiotics are produced in normal unamended soils is not certain. Usually soil sterilization, or the addition of amendates or both are required for the synthesis of antibiotics in soil[1~7]. Even if antibiotics were produced in soil, they might be inactivated by the physical, chemical, or microbial properties of soil[2,3,8~12]. In this study, the antagonistic ability of ten *Streptomyces* spp. to two soil-borne plant pathogens is looked at in terms of their antibiotic producing ability.

Materials and Methods

Ten species of *Streptomyces* were used in this research. Five had been reported to produce antifungal antibiotics and five species had not been reported to produce antifungal antibiotics. All were obtained from Dr. T. G. PRIDHAM of the Northern Regional Research Center, Peoria, Ill., U.S.A. (Table 1). The root rotting fungi were *Rhizoctonia solani* Kuhn (our own isolate) and *Phytophthora megasperma* Drechs *sojae* Hildeb, race 1, obtained from Dr. L. E. GRAY, U.S.D.A. collaborator at the University of Illinois. Eight streptomycetes were maintained on yeast extract-malt extract agar[13]. *Streptomyces cellulosae* and *Streptomyces herbaricolor* were maintained on glycerol-asparagine agar[13] because of their poor sporulation on yeast extract-malt extract agar. *R. solani* was maintained on glucose-yeast extract agar[14] and *P. megasperma* in either soybean broth or on V-8 agar #248[15]. Soybean broth was made by adding 1 soybean seed for each 10 ml of distilled water.

In Vitro Experiments

Four media were used to measure antagonism. The media compositions are expressed as amount of ingredient per liter. Media were Medium 1) Corn steep-soybean (CSSA); cerelose, 10 g; soybean meal, 15 g; NaCl, 5.0 g; $CaCO_3$, 2.0 g; corn steep, 10 ml; agar 20 g; adjusted to pH 7.0 before autoclaving. Medium 2) EMERSON's Agar; yeast extract, 1.0 g; beef extract, 4.0 g; Bacto-peptone, 4.0 g; NaCl, 2.5 g; cerelose, 10 g; agar 20 g. Medium 3) Potato dextrose agar (PDA) (Difco), adjusted to pH 7.0 before autoclaving. Medium 4) Soil extract agar (SEA)[16].

The ability of a streptomycete to inhibit the pathogen was determined by first streaking each medium with spores of the antagonist in a 1 cm wide band and incubating these cultures at 26°C for 5 days. A plug from the edge of a culture of *P. megasperma* var. *sojae* on V-8 agar or *R. solani* on glucose-yeast extract agar was then placed 1.5 cm or 3.0 cm, respectively, from the streak and these plates incubated at 26°C for different durations depending on the pathogen and the medium.

Table 1. Streptomycete isolates used in the study.

Streptomyces spp.	NRRL number*	Antibiotic
Streptomyces griseus	B-150	Cycloheximide
Streptomyces reticuli var. *protomycicus*	2875	Protomycin
Streptomyces kitazawaensis	B-5488	Antimycin
Streptomyces noursei	B-1714	Nystatin
Streptomyces hygroscopicus var. *geldanus*	3602	Geldanamycin
Streptomyces phaeochromogenes	B-1248	
Streptomyces coeruleofuscus	B-2704	
Streptomyces cellulosae	B-2889	
Streptomyces violaceoruber	B-3319	
Streptomyces herbaricolor	B-3299	

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Antagonism between *Streptomyces* spp. and the fungi was measured by the zone of inhibition, the distance between the edge of the streak and the point the pathogen stopped growing toward the streak. Percent inhibition of linear growth of the pathogen was also measured by comparing growth to non-streaked controls.

Greenhouse Experiments

R. solani inoculum was grown in glucose-asparagine liquid medium[14] at 26°C on a reciprocal shaker for 3 days. *P. megasperma* var. *sojae* inoculum was grown in soybean broth in stationary culture for 14 days at 26°C. The cultures were filtered, mycelium resuspended in sterile distilled water, and homogenized. Inoculum levels used to infest soil were 0.45 g and 0.15 g per pot, equivalent to about 600 g oven dry weight of soil, for *R. solani* and *P. megasperma* var. *sojae* respectively.

Streptomycete inocula were grown in tryptone-yeast extract broth[13] on a reciprocal shaker for 48 hours at 26°C. Inoculum was prepared by centrifugation at 4,500 rpm (GSA rotor) for 10 minutes, decanting and the process repeated using sterile distilled water. Mycelium was suspended in sterile distilled water and homogenized. In all experiments 0.31 g of mycelium (wet weight) was used per pot.

The soil used in all experiments was a sterilized 3: 2 mixture of field soil and sand (v: v). Inoculum was added to enough soil for a treatment, the soil evenly infested, dispensed into 10 cm diameter unglazed clay pots and five seeds planted per pot. Test plants were *Glycine max* L. 'Harasoy' for Phytophthora root rot and *Pisum sativum* L. 'Wando' for Rhizoctonia root rot. Soybean seeds were obtained from Dr. J. D. PAXTON, University of Illinois. Pea seeds were purchased from W. Atlee Burpee Co. Pots were watered to saturation daily or when the soil surface became dry.

For experiments involving the incubation of the streptomycetes in soil prior to planting the seeds, the equivalent of 600 g oven dried soil was dispensed into a 1 liter Erlenmeyer flask and sterilized. Inoculum for each pot was added to the soil in the flask and shaken to infest the soil evenly. Sterile distilled water was added to bring the soil to 60% of the water holding capacity and the flasks incubated at 26°C. All soils for a treatment were then combined and the soil treated as described earlier.

Emergence data were taken at weekly intervals following planting. Twenty-one days after planting, seedlings were removed and rated for disease severity. A 6 point disease severity index (DSI):

0) No visible symptoms
1) Small superficial lesions on epicotyl
2) Large lesions girdling or almost girdling the epicotyl
3) Damping-off of some shoots
4) Postemergence damping-off
5) Preemergence damping-off

was used for rating the severity of Rhizoctonia root rot on peas. The disease severity index for Phytophthora root rot of soybeans was based on fresh root weight per pot, which was calculated as follows: average fresh root weight of the plants in a pot × percent plant stand. These data were transformed to a 0~5 disease severity index consistent with the disease severity index for Rhizoctonia root rot by the following formula:

$$DSI = 5 - \frac{5}{\text{average fresh root weight per pot for uninfested treatment}} \times \text{average fresh root weight per pot}$$

One seedling from each pot still containing living plants at the end of an experiment was selected to reisolate the pathogen and confirm its involvement in the disease.

Completely randomized designs were used in all experiments both in the greenhouse and in the laboratory. The data were statistically analyzed using DUNCAN's multiple range test. For greenhouse experiments, a replicate consisted of one pot containing five seeds. All the experiments were done at least twice and usually more, and those described in this paper are examples of these results. Results were repeatable in many different experiments.

Results

Antagonism on Agar Media

Antagonism between some of the *Streptomyces* spp. and *R. solani* occurred on all the media used (Table 2). However the amount of inhibition varied with the medium. On EMERSON's agar wide zones of inhibition were produced by *S. hygroscopicus* var. *geldanus*, *S. griseus*, and *S. noursei* (Table 2). On PDA the same three species produced zones of inhibition. A small zone of inhibition was produced on PDA by *S. coeruleofuscus*. Least inhibition occurred on SEA with only *S. hygroscopicus* var. *geldanus* producing a large zone of inhibition (Table 2). *S. phaeochromogenes* produced a small zone of inhibition. Those *Streptomyces* spp. producing zones of inhibition also inhibited the growth of *R. solani* to the greatest degree. All *Streptomyces* spp. inhibited the growth of *R. solani* to some degree on EMERSON's agar and PDA.

To determine antagonism to *P. megasperma* var. *sojae* the fungus was placed closer to the streak and incubated longer than *R. solani* because of its slower growth. On CSSA and PDA *S. noursei*, *S. griseus*, and *S. hygroscopicus* var. *geldanus* produced wide zones of inhibition (Table 3). *S. reticuli* var. *protomycicus* also produced a large zone of inhibition on PDA while *S. herbaricolor* produced a small zone (Table 3). No zone of inhibition was recorded for SEA because of the poor growth of the pathogen. Those *Streptomyces* spp. producing zones of inhibition also showed the greatest inhibition of growth. All of the streptomycetes inhibited the growth of *P. megasperma* to some degree on CSSA. On SEA only *S. hygroscopicus* var. *geldanus* and *S. griseus* significantly inhibited growth of the pathogen ($P=0.01$).

The presence and size of the zones of inhibition were used as criteria for the production of antibiotics by the various *Streptomyces* spp. There was a close relationship between the production of antibiotics as reported in the literature and our own data (Tables 1, 2 and 3). Thus the antifungal activities (probably antibiotics) of the *Streptomyces* spp. were confirmed for one or more media against either *R. solani*

Table 2. Antagonism by *Streptomyces* spp. to *Rhizoctonia solani* on agar media.[1]

Streptomyces spp.	Zone of inhibition[2]		
	EMERSON'S agar	Potato dextrose agar	Soil extract agar
S. cellulosae	0	0	0
S. herbaricolor	0	0	0
S. violaceoruber	0	0	0
S. kitazawaensis[3]	0	0	0
S. reticuli var. *protomycicus*[3]	0	0	0
S. phaeochromogenes	0	0	0.10
S. coeruleofuscus	0	0.20	0
S. griseus[3]	0.73	0.27	0
S. noursei[3]	1.47	1.03	0
S. hygroscopicus var. *geldanus*[3]	2.10	2.43	0.50

[1] Each treatment consisted of 3 replicates.
[2] The zone of inhibition was determined by measuring the distance (cm) between the pathogen and streptomycete streak after 5 days incubation of the pathogen.
[3] Reported antibiotic producer.

Table 3. Antagonism by *Streptomyces* spp. to *Phytophthora megasperma* var. *sojae* on agar media.[1]

Streptomyces spp.	Zone of inhibition[2]	
	Corn steep-soybean agar	Potato dextrose agar
S. cellulosae	0	0
S. coeruleofuscus	0	0
S. phaeochromogenes	0	0
S. violaceoruber	0	0
S. kitazawaensis[3]	0	0
S. herbaricolor	0	0.37
S. reticuli var. *protomycicus*[3]	0	0.63
S. hygroscopicus var. *geldanus*[3]	1.00	1.10
S. griseus[3]	1.50	0.70
S. noursei[3]	1.50	1.00

[1] Each treatment consisted of 3 replicates.
[2] The zone of inhibition was determined by measuring the distance (cm) between the pathogen and streptomycete streak after 9 days incubation of the pathogen for corn steep-soybean agar and 14 days for potato dextrose agar. The zone of inhibition on corn steep-soybean agar was calculated from two replicates.
[3] Reported antibiotic producer.

or *P. megasperma* in four of the five reported producers. The fifth reported producer of this group, *S. kitazawaensis* did inhibit the plant pathogen *Colletotrichum dematium* (PERS. ex FR.) Grove var. *truncata* (SCHW.) ARX.

Antagonism in Soil

When *R. solani* was added to soil at the same time as the various *Streptomyces* spp. and peas planted, only *S. cellulosae*, a non-producer gave significant disease control (P=0.05) (Table 4). The DSI was 3.40 for *S. cellulosae* infested soil and 3.98 in the absence of streptomycetes. When the 10 *Streptomyces* spp. were incubated separately in soil 7 days prior to adding the pathogen and planting, only *S. hygroscopicus* var. *geldanus* reduced the disease on peas significantly, (P=0.01) from a DSI 4.10 to 1.55 (Table 4). In the same experiments *S. cellulosae* did not decrease the disease.

The streptomycetes controlling Phytophthora root rot were more variable between experiments as was disease severity. When soil was infested at the same time with both the antagonist and pathogen, *S. herbaricolor, S. coeruleofuscus, S. phaeochromogenes,* and *S. reticuli* significantly reduced disease (P=0.01) (Table 5). *S. herbaricolor* and *S. coeruleofuscus* were the most consistent at controlling disease. More species inhibited Phytophthora root rot than Rhizoctonia root rot. Incubation of the *Streptomyces* for 7 days prior to infesting the soil with Phytophthora resulted in significant disease control by *S. coeruleofuscus, S. cellulosae, S. herbaricolor, S. violaceoruber,* and *S. kitazawaensis* (P=0.01) (Table 5).

Table 4. Effect of infesting soil with *Streptomyces* spp. on Rhizoctonia root rot of pea.[1]

Streptomyces spp.	Time of incubation of *Streptomyces* spp. in soil	
	0 days[2]	7 days[3]
S. cellulosae	3.40 a	4.30 bc
S. hygroscopicus var. *geldanus*[4]	3.55 ab	1.55 a
S. griseus[4]	3.62 ab	4.55 bc
S. violaceoruber	3.70 abc	4.50 bc
S. herbaricolor	3.78 abc	4.68 bc
S. coeruleofuscus	3.80 abc	4.75 c
S. noursei[4]	3.80 abc	4.52 bc
S. kitazawaensis[4]	3.85 abc	4.25 bc
none	3.98 bc	4.10 b
S. phaeochromogenes	4.05 bc	4.30 bc
S. reticuli var. *protomycicus*[4]	4.22 c	4.55 bc

[1] Each treatment consisted of 8 replicates.
[2] Means accompanied by the same letter are not significantly different at P=0.05 according to DUNCAN's multiple range test. For the disease severity index see Methods. Uninfested soil had a DSI of 0.38.
[3] Means accompanied by the same letter are not significantly different at P=0.01 according to Duncan's multiple range test. For the disease severity index see Methods. Uninfested soil had a DSI of 0.70.
[4] Reported antibiotic producer.

Table 5. Effect of infesting soil with *Streptomyces* spp. on Phytophthora root rot of soybean.[1]

Streptomyces spp.	Time of incubation of *Streptomyces* spp. in soil	
	0 days[2]	7 days[2]
S. herbaricolor	1.412 a	1.405 ab
S. coeruleofuscus	1.680 ab	0.881 a
S. phaeochromogenes	1.740 ab	2.373 bc
S. reticuli var. *protomycicus*[3]	1.786 ab	2.254 bc
S. violaceoruber	2.079 abc	1.472 ab
S. hygroscopicus var. *geldanus*[3]	2.122 abc	2.791 c
S. cellulosae	2.416 bcd	1.113 a
none	2.680 cde	2.815 c
S. griseus[3]	2.992 de	2.262 bc
S. kitazawaensis[3]	3.010 de	1.584 ab
S. noursei[3]	3.213 de	2.610 c

[1] Each treatment consisted of 8 replicates.
[2] Means accompanied by the same letter are not significantly different at P=0.01 according to DUNCAN's multiple range test.
[3] Reported antibiotic producer.

Establishment of the *Streptomyces* species in soil was evident by the growth and sporulation of some species on the seed coats of pea and soybean during the experiments. The streptomycetes also grew on the soil during the incubation period. There was no apparent relationship between the degree of growth on the seed coats or on the soil during the period of incubation and disease severity.

No relationship was found for *R. solani* or *P. megasperma* var. *sojae* between reported antifungal antibiotic production and disease severity in soil that had been infested with the *Streptomyces* spp. and the pathogens. A poor correlation between antagonism on agar media and disease severity in soil infested with the respective *Streptomyces* spp. was also found.

Discussion

Although the terms antagonism and antibiosis have been differentiated in their use until now, the data (Tables 2 and 3) indicate that the antagonism of the *Streptomyces* species against the pathogens *in vitro* is due primarily to the diffusion of an antibiotic into the medium. Four of the five species of *Streptomyces* that have been reported to produce an antifungal antibiotic greatly inhibited fungal growth and caused relatively large zones of inhibition to at least one of the pathogens. Though *S. kitazawaensis* had no activity against the two pathogens used, it did inhibit a species of *Colletotrichum*. The inhibition of growth by the remaining species that had not previously been reported to make antifungal compounds could be attributed either to the synthesis of very low concentration of antibiotics or the effect of some other factors limiting the growth of the two fungi.

The data in Tables 2 and 3 also illustrate the importance of nutrients in the synthesis of antibiotics. Soil extract agar supported only moderate growth and caused few zones of inhibition and little inhibition

of growth. This is in sharp contrast to the large amount of inhibition produced on the "rich" media, EMERSON's agar, CSSA, and PDA. This data support the results of our previous studies indicating that in soil *Streptomyces* spp. did not produce antibiotics unless either some plant products were added to soil, the soil sterilized or both.

A few of the *Streptomyces* spp. gave significant reduction of the two diseases (Tables 4 and 5). No obvious relationship was found between the ability of a species to reduce disease and its reported antibiotic activity or antagonism *in vitro*. Proponents of antibiotic production playing a role in antagonism in soil have suggested that antibiotic production may play a significant role in antagonism at the microhabitat level and thus not be present in sufficient levels for detection. Our data suggest that those *Streptomyces* spp. which produce antibiotics *in vitro* show no greater antagonism in soil than those which fail to produce antibiotics suggesting that antibiotics don't play a significant role in antagonism. Situations in which both occur such as when *S. hygroscopicus* var. *geldanus* was incubated in soil prior to adding *R. solani* and the inhibition of the fungus on media are probably fortuitous. The use of previously sterilized soil would have favored antibiotic production in soil and thus the role of antibiotic production in antagonism should have been exhibited if it occurred. The ecological significance of antibiotic producing ability is still uncertain but it appears to play no significant role in antagonism.

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