A Quantitative Method for Determining Soil Populations of *Streptomyces* and Differentiating Potential Potato Scab-Inducing Strains

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ABSTRACT

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A procedure is described for estimating *Streptomyces* populations in soil. Soils are air-dried, 10-g quantities are shaken in plastic bags containing 0.1% water agar and homogenized with a Stomacher homogenizer, serial dilutions are plated on a semi-selective culture (STR) medium and incubated for 2 weeks at 22°C, and the *Streptomyces* colonies are enumerated. Use of STR medium reduced the bacterial and fungal colonies recovered from soil to levels below that of the *Streptomyces* spp. while not affecting the number of *Streptomyces* colonies compared with those enumerated on yeast malt extract medium. A procedure for screening large numbers of *Streptomyces* strains for thaxtomin production, a phytotoxin recognized as a virulence marker in *S. scabiei*, is also described. Strains are grown on oatmeal medium, and the thaxtomin is extracted from the medium by facilitated diffusion and detected by miniature thin layer chromatography. *S. scabiei* and *S. acidiscabiei* strains (approximately 130 from Ontario and 70 from other locations in North America) that produced thaxtomin did not form aerial mycelia or sporulate on STR medium within 2 weeks at 22°C. Ontario *S. scabiei* strains that produced thaxtomin A also produced melanin on STR medium. All *S. scabiei* strains from scab lesions that produced thaxtomin A had this colony morphology, whereas only 4 to 9% of strains from soil with this colony morphology produced thaxtomin A. Using these procedures, we determined that the population of thaxtomin-producing *S. scabiei* in soil from a potato field in Ontario with a history of potato scab was about 20,000 CFU/g soil.

Additional keywords: common scab, deep pitted scab, soilborne pathogen

Potato scab is an economically important disease in North America and Europe (20,26). This disease is caused by bacteria belonging to several *Streptomyces* spp. (4,6,7,8,9,26), but *S. scabiei* is the predominant causal agent (21). *S. acidiscabiei* is the predominant causal agent in soils below pH 5 (26). Depending on the *Streptomyces* strain and the environment, shallow, raised, or deep-pitted lesions develop (9,11,26). Pathogenic *Streptomyces* strains produce phytotoxins named thaxtomins, which are thought to be involved in pathogenicity (3,17,22,23,25). Thaxtomin A is the most abundant phytotoxin produced by *S. scabiei* and *S. acidiscabiei*, along with about nine minor thaxtomins (15). Thaxtomin C is the most abundant phytotoxin

produced by *S. ipomoeae*, the cause of soil rot of sweet potato (16).

In Ontario, the incidence of scab is erratic and uneven, even within a field. Tubers from infested sites are often unmarketable, particularly if deep-pitted lesions occur. For some growers, this disease has become a limiting factor in production. Pathogenic *Streptomyces* spp. pose a long-term threat to potato production because they can survive in soil or on plant residues for over a decade (18). Control of this pathogen has not been seriously attempted in Ontario. Development of disease control methods using biological and chemical materials or soil amendments is constrained by the lack of a rapid and quantitative technique for measuring populations of pathogenic *Streptomyces* spp. in soil. Most media for selective isolation of *Streptomyces* spp. from soil or scab lesions use antibiotics such as cycloheximide, nystatin, polymyxin B, and penicillin G to reduce the growth of unwanted bacteria and fungi (7,8,13,14,27). The selection of these antibiotics and the concentrations used was derived from studies where actinomycetes were isolated from a pine forest soil (2,31). These researchers stressed that some actinomycetes

were inhibited by these antibiotics and the importance of testing the effect of antibiotics against target organisms before any new ecological investigation began. However, such tests have never been carried out for isolation of *Streptomyces* bacteria from soil from a potato field. When we tested media containing these antibiotics for isolation of *S. scabiei* from soil from potato fields that had been amended with organic amendments, we encountered two problems. One was that these antibiotics did not provide sufficient selection for *Streptomyces* spp., particularly from soils where organic amendments had dramatically increased bacterial numbers. High populations of nonfilamentous bacteria obscured *S. scabiei* colonies on dilution plates. In addition, *S. scabiei* strains that we isolated from scab lesions did not grow on media containing these antibiotics. Thus, we developed a medium that is selective for *S. scabiei* strains found in soils from Ontario potato fields by modifying the type and quantities of antibiotics used. We also tested the effect of various concentrations of NaCl on selectivity of the medium.

Enumeration of *S. scabiei* in infested soil does not provide an estimation of scab potential, however, since pathogenic *S. scabiei* strains comprise only a small proportion of the total population. Keinath and Loria (13) isolated *Streptomyces* spp. from soil and identified *S. scabiei* based on colony and spore morphology. When these strains were infested into soil in which potato plants were grown for 2 months in the greenhouse, only 3.3% of the *S. scabiei* strains were pathogenic. This method is too time-consuming and labor intensive to be useful for evaluating populations of *S. scabiei* from potato soils. In addition, the characteristic of pathogenicity among these strains can be inaccurately assessed using greenhouse assays. Production of melanin has been used as a diagnostic characteristic of *S. scabiei* (14), but it does not differentiate between pathogenic and nonpathogenic strains. Attempts have been made to find genetic markers that can be used to identify pathogenic *S. scabiei* strains. Analyses of 16S rRNA sequences (29), fatty acid methyl ester profiles (28), restriction fragment length polymorphisms (4), and DNA-DNA hybridization (10,30) have been carried out. The general conclusion from these studies is that pathogenic

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S. scabies strains comprise a genetically diverse population. If this conclusion is correct, it would exclude the use of these techniques for quantifying pathogenic *S. scabies* in soil. We decided to focus on thaxtomin production as a marker for pathogenicity since in North America this appears to be an excellent indicator of pathogenicity of *S. scabies* on potatoes (17,22,23,25). To help screen *S. scabies* and *S. acidiscabies* strains for thaxtomin production, we took advantage of the fact that they can be induced to produce thaxtomins in oatmeal medium (1,25).

The objectives of this research were to (i) develop a semi-selective medium to isolate *Streptomyces* spp. from potato field soil, (ii) develop a rapid procedure for screening strains for thaxtomin production, and (iii) compare colony morphology on our semi-selective medium, thaxtomin production, and pathogenicity of *S. scabies* and *S. acidiscabies* strains from various locations in North America.

MATERIALS AND METHODS

Streptomyces strains. We obtained approximately 70 *S. scabies* and three *S. acidiscabies* strains from various locations in North America. Some of these strains

are listed in Table 1 along with their sources. These strains were stored as spore suspensions in 15% glycerol at -20°C .

Media. All reagents and antibiotics used were supplied by Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. Pseudomonas agar F (PAF) medium was prepared as described by the supplier. Yeast malt extract (YME) medium and peptone-yeast extract iron (PYI) medium were prepared as described by Loria and Davis (27). To prepare oatmeal (OM) broth, rolled oats (40 g/600 ml) in distilled H_2O were brought to a boil in a microwave oven and strained through cheesecloth, yielding 400 ml of filtrate. One ml of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ stock solution (1 g/100 ml) was added, the volume was brought up to 1 liter, and the pH was adjusted to 7.2 before autoclaving. OM medium (1% agar) was also prepared.

Development of Streptomyces selective medium. Our starting point was a *Streptomyces* selective (STR) medium developed by G. Kritzman (Department of Plant Pathology, ARO, The Volcani Center, Bet Dagan, Israel) that he subsequently modified for isolation of *Streptomyces* spp. from peanuts with pod wart in Israel (19). Soil from a potato field near Alliston, Ontario (site M) was used to test STR medium for selective isolation of *Streptomyces* spp. from soil. Soil dilutions were plated on STR medium with the following range of NaCl and antibiotic concentrations: NaCl (0.5 to 3.5%), cycloheximide (dissolved in 70% EtOH; 55 to 1,300 ppm), nystatin (4,420 units/mg; dissolved in 95% EtOH; 2.5 to 20 ppm), rifampicin (dissolved in MeOH; 0.5 to 33 ppm), nalidixic acid (free acid; dissolved in 0.1 N NaOH; 5.6 to 44 ppm), streptomycin sulfate (dissolved in H_2O ; 4 to 16 ppm), polymyxin B sulfate (dissolved in H_2O ; 0.7 to 11 ppm), and penicillin G (sodium salt; dissolved in 70% EtOH; 0.1 to 2.2 ppm). Numbers of *Streptomyces*, nonfilamentous bacteria, and fungi were counted after incubation for 2 weeks at 22°C and compared with the numbers obtained from parallel platings on PAF and YME media. Reference cultures of pathogenic *S. scabies* strains were streaked on the media to determine their growth potential. After incubation for 2 weeks at 22°C , the extent of growth was evaluated based on the relative sizes of isolated colonies. This experiment was conducted three times. The final composition of STR medium used is (per liter of distilled H_2O): soluble potato starch, 5.0 g; yeast extract (Difco, Detroit, MI), 4.0 g; bacto peptone (Difco), 0.6 g; protease peptone no. 3 (Difco), 0.6 g; NaCl, 10 g; K_2HPO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; agar (E. Merck, Darmstadt, Germany), 17 g; and 1 ml of a stock solution of micro-elements comprised of FeSO_4 , 10 mg; ZnSO_4 , 1 mg; and MnCl_2 , 1 mg dissolved in distilled H_2O . The pH of this medium is 7.2. Cycloheximide (200 ppm), nystatin (5.6 ppm),

rifampicin (0.6 ppm), and nalidixic acid (15 ppm) are added after sterilization. STR-M medium has the same composition as STR medium except STR-M medium contains NaCl (2%), cycloheximide (222 ppm), nystatin (10 ppm), rifampicin (1 ppm), and nalidixic acid (22 ppm). Seven thaxtomin-producing *S. scabies* strains from site M were used to determine the highest concentration of NaCl and antibiotics that could be used to select *S. scabies* strains from soil at site M.

Isolation of Streptomyces spp. from soil. Soil samples were collected from two potato fields near Alliston, Ontario (sites M and B). Cores to a depth of 15 cm were taken from 25 locations in each field and pooled. Subsamples were air-dried for 1 week, and any stones present were removed by hand. Samples (10 g) were weighed into plastic pouches (17 × 30 cm; Cinran Plastics Inc., London, ON), and 90 ml of sterile water agar (0.1% wt/vol) was added. Pouches were heat sealed and shaken on an orbital shaker (200 rpm) for 2 h. Soil mixtures were then homogenized in the pouches using a Stomacher blender (Stomacher 400, Seward Medical, UK) set on normal speed for 30 s. Serial 10-fold dilutions were prepared in saline solution (0.85% NaCl). The first dilution was made in 9 ml and the others in 900 μl . Aliquots (100 μl) from each dilution were plated onto various media. Microbial populations in air-dried soil were compared with those in nondried soil. Isolations were made from three soil samples for each experiment, and each experiment was conducted at least three times.

Isolation of S. scabies from scab lesions. Scab infected tubers were collected from three potato fields near Alliston, Ontario (sites M, B, and K), a garden near Paris, Ontario (site P), and a potato field near Simcoe, Ontario (site S). Potatoes were cv. Snowden for all sites except site P, which was cv. Golden Yukon. Tubers were washed to remove soil from the lesions. Pieces of tissue from beneath lesions were excised and macerated in saline solution, and serial dilutions were plated on STR medium. Plates were incubated for 2 weeks at 22°C . Isolations were made from two or three lesions on two or three tubers from each site.

Comparison of Streptomyces spp. on STR medium. Colony characteristics of *Streptomyces* spp. isolated from scab lesions and soil from Ontario potato fields were compared on STR medium. *S. scabies* and *S. acidiscabies* strains from scab lesions from various locations in North America were also compared on STR medium. Rate of sporulation, color, surface features, and melanin production were noted for individual colonies of each strain. Melanin production on STR medium was compared with that on PYI medium. Colonies were classified as nonsporulating if individual colonies did not form aerial

Table 1. *Streptomyces* strains used in this study

Strain ^a	Source
MSS 94-006	MI, USA ^b
MSS 94-035	MI, USA ^b
MSS 95-104	MI, USA ^b
MSS 95-142	MI, USA ^b
DP	MI, USA ^b
Fi II	MI, USA ^b
RL 84-01-232	USA ^c
RL 84-01-110	USA ^c
RL 84-01-95	USA ^c
NRRL B-2796	MT, USA ^d
NRRL B-2801	ME, USA ^d
PonP	MN, USA ^e
Roy	MN, USA ^e
LM5MT-2	NB, CAN ^f
L89-19	NB, CAN ^f
LPit-2	NS, CAN ^f
LQH-4	PQ, CAN ^f
LR90-3	ON, CAN ^f
LWis-76-17-2	AB, CAN ^f
Warba-6	AB, CAN ^f
SP	ON, CAN ^g
PP	ON, CAN ^g

^a *Streptomyces scabies* and *S. acidiscabies* (RL 84-01-110) strains from scab lesions.

^b Obtained from the culture collection of F. Spooner (Michigan State University).

^c Obtained from the culture collection of R. Loria (Cornell University) via F. Spooner.

^d Obtained from the culture collection of D. Lalseda (Microbial Properties Research, USDA, Peoria) via F. Spooner.

^e Obtained from the culture collection of N. Anderson (University of Minnesota) via F. Spooner.

^f Obtained from the culture collection of C. H. Lawrence and R. R. King (AAFC, Fredericton, NB, Canada).

^g Isolated by the authors from site B (strain SP) and site P (strain PP).

mycelia and sporulate on STR medium within 2 weeks at 22°C. When colony numbers were dense, forming a lawn, production of aerial mycelia and sporulation sometimes occurred; therefore, only isolated colonies were rated. Colonies were classified as smooth if they did not form aerial mycelia, sporulate, or have erupted surfaces.

Thaxtomin detection. *Streptomyces* strains were grown on potato tuber slices or OM medium for 7 to 10 days at 27°C. A 2-cm² area of the tuber tissue or OM medium showing dense bacterial growth was cut out, placed in a 2-ml Eppendorf tube, and 1 ml of ethyl acetate added. A toothpick was used to keep the tuber or agar pieces submerged. Thaxtomin was extracted by facilitated diffusion (12), in which Eppendorf tubes containing the tuber or medium pieces were placed in a vacuum chamber and the ethyl acetate was brought to a boil four times by repeatedly evacuating the chamber and breaking the vacuum. Pieces of tuber or medium were removed, and the volume of ethyl acetate was made up to 1.5 ml with fresh ethyl acetate. The ethyl acetate was dried with anhydrous Na₂SO₄ (0.3 g), which was then pelleted by centrifugation for 10 s in a microcentrifuge (13,000 rpm). The ethyl acetate was transferred with a Pasteur pipette to a clean tube and evaporated to dryness overnight in an air stream provided

by a fume hood. The residue was dissolved in 10 µl of ethyl acetate, which was collected by centrifugation for 10 s in a microcentrifuge. The entire extract was loaded onto E. Merck silica gel 60F thin layer chromatography (TLC) plastic plates (EM Separations Technology, Gibbstown, NJ) cut to a dimension of 5.5 × 7 cm. Two outer lanes on each plate were spotted with a mixture of thaxtomin A and B standards supplied by R. R. King (AAFC, Fredericton, NB, Canada). Plates were developed in CHCl₃:MeOH (9:1) in small glass tanks. Thaxtomin A and B appear as bright yellow spots and absorb UV₃₆₆ light. Occasionally, *Streptomyces* strains were grown in OM broth (100 ml/250-ml flask). These cultures were shaken on an orbital shaker (200 rpm) for 7 to 10 days at 27°C, filtered through glass wool, and the supernatant extracted with 2 volumes of ethyl acetate. The ethyl acetate was dried with anhydrous Na₂SO₄ and concentrated on a rotary evaporator. Presence of thaxtomins was determined by TLC.

Pathogenicity assays: Tuber slice. The procedure used was adapted from Loria et al. (25). Potato tubers, cv. Kennebec or Snowden, were peeled, sterilized in 0.05% calcium hypochlorite and 0.1% CaCO₃ for 3 min with stirring, and rinsed twice in sterile H₂O. Tubers were sliced into large disks with a sterile knife, and pieces (2 cm² × 0.5 cm thick) from the middle of the

slices were placed in sterile petri plates. Three tuber slices were used for each strain tested. A piece of medium from a zone of dense growth was cut out from *Streptomyces* strains grown on OM medium for 7 to 10 days at 27°C. The piece of medium was then placed upside down on a tuber slice. Pieces of noninoculated OM medium were used as a control in all tests. *S. scabiei* strains known to cause shallow necrotic lesions and deep-pitted lesions were included as positive controls. Tuber slices were placed in an incubator at 22°C for 3 to 6 days. Pure thaxtomin and thaxtomin extracts were applied to the surface of tuber slices as positive controls. The thaxtomin samples were first applied to sterile paper disks, the ethyl acetate was allowed to evaporate, and the disks were placed on tuber slices with a drop of sterile H₂O. Paper disks that had received only ethyl acetate were used as a control.

Growth of potato plants in *Streptomyces*-infested sand. Spores of *Streptomyces* strains were scraped off YME medium into YME broth (200 ml), which was then added to 300-ml sterile perlite in a flask. Cultures were incubated at 27°C for 6 weeks, mixed with sterile sand at a ratio of 10% vol/vol, and placed in 16-cm pots. Three pots of sand were infested for each strain tested and placed in a greenhouse in a completely randomized design. Sand containing noninoculated perlite was used

Table 2. Effect of NaCl and antibiotics on the growth of some pathogenic *Streptomyces scabiei* strains^a

Media ^b	Relative growth of strains ^c							
	SP	LM5MT-2	L89-19	LPit-2	LQH-4	LR 90-3	LWis-76-17-2	Warba-6
PAF	+++++	+++++	+++++	+++++	++++	+++++	+++++	++++
YME	+++++	+++++	+++++	+++++	++++	+++++	+++++	++++
STR +								
NaCl (0.5%)	++++	+++	+++	+++	++	+++	+++	++
NaCl (1%)	++++	++	++	+++	++	++	++	+
NaCl (1.5%)	++++	+	+	++	+	+	+	-
NaCl (2%)	++++	-	-	+	-	+	+	-
STR + NaCl (1%) +								
Ny (5 ppm)	++++	++	++	+++	+	++	++	+
Ny (20 ppm)	++++	++	++	+++	+	++	++	+
Cy (110 ppm)	++++	++	++	+++	+	++	++	+
Cy (1,300 ppm)	++++	++	++	+++	+	++	++	+
Ny (5.6 ppm) + Cy (200 ppm)	++++	++	++	+++	+	++	++	+
STR + NaCl (1%) +								
Ny (5.6 ppm) + Cy (200 ppm) +								
Nal (11 ppm)	++++	++	++	+++	+	++	++	+
Nal (44 ppm)	++++	++	++	+++	+	++	++	+
Rif (0.5 ppm)	++++	++	++	+++	+	++	++	+
Rif (1 ppm)	+++	+	++	++	-	++	++	-
Pol (0.7 ppm)	++++	++	++	+++	-	++	++	-
Stp (4 ppm)	++	-	-	-	-	-	-	-
Pen (0.26 ppm)	++++	++	++	+++	-	++	++	+
STR + NaCl (1%) + Ny (5.6 ppm) + Cy								
(200 ppm) + Nal (15 ppm) + Rif (0.6 ppm) ^d	++++	++	++	+++	+	++	++	+

^a See Table 1 for sources of *S. scabiei* strains.

^b Pseudomonas agar F (PAF), yeast malt extract (YME), and *Streptomyces* selective (STR) media. Antibiotics tested were nystatin (4,420 units/mg) (Ny), cycloheximide (Cy), nalidixic acid (free acid) (Nal), rifampicin (Rif), polymyxin B sulfate (Pol), streptomycin sulfate (Stp), and penicillin G (sodium salt) (Pen). The concentrations of NaCl and antibiotics shown in this table are only a partial list of concentrations tested.

^c Pure cultures of the *S. scabiei* strains were streaked for isolated colonies on the media. The sizes of isolated colonies were compared after incubation for 2 weeks at 22°C. A dash indicates no isolated colonies grew. Data shown are from one representative experiment. Each NaCl and antibiotic concentration was tested at least three times.

^d This was the final combination of NaCl and antibiotics chosen for STR medium. Reference to STR medium in this paper indicates this medium.

as a control. A known pathogenic *S. scabiei* strain was included as a positive control. Sterile 6-week-old plantlets (cv. Kennebec) grown from nodal explants in tissue culture were planted in the pots. After 2 to 3 months, tubers were evaluated for scab symptoms. *Streptomyces* strains were reisolated from some of the lesions and characterized.

RESULTS

Optimizing NaCl and antibiotic concentrations for STR medium. There was negligible or no growth by most *S. scabiei* reference strains at 1.5% NaCl (Table 2). Strain SP was more resistant, being able to grow at NaCl concentrations up to 3%. Increasing the concentration of NaCl from 1.0 to 1.5% reduced *Streptomyces* numbers from soil at site M, whereas there was no effect on the number of culturable nonfilamentous bacteria or fungi detected (Table 3). However, NaCl did slow the growth rate of many nonfilamentous bacterial and fungal isolates so the 1% NaCl level was kept as a component of the medium.

S. scabiei reference strains grew equally well at all concentrations of nystatin and cycloheximide tested (Table 2). The higher concentrations of these antibiotics slightly reduced the number of *Streptomyces* spp.

detected in soil (Table 3). When only these antibiotics were included in STR medium, fungal colonies could not be enumerated because they were obscured by bacterial colonies. Once the optimal combination of antibiotics was identified for reducing bacterial numbers, various combinations of nystatin and cycloheximide were tested to find the optimal concentrations for reducing fungal numbers while maintaining *Streptomyces* numbers constant. Nystatin at 5.6 ppm and cycloheximide at 200 ppm were selected for STR medium, as they reduced the number of culturable fungi detected from a potential of 300,000 CFU/g of soil (as found on YME medium) to 3,700 CFU/g of soil (on STR medium) while the number of *Streptomyces* spp. was the same on both media (Table 3).

Polymyxin B, streptomycin sulfate, and penicillin G were not useful for our selective medium, as at the concentrations that many *S. scabiei* reference strains exhibited reduced growth (Table 2), no significant decrease in nonfilamentous bacterial numbers was found and/or the number of *Streptomyces* spp. was reduced (Table 3). Strain SP was more resistant to these antibiotics, showing negligible growth at 3 and 2 ppm polymyxin B and penicillin G, respectively. All *S. scabiei* reference strains grew at 0.5 ppm rifampicin (Table 2). This

concentration of rifampicin reduced the number of nonfilamentous bacteria detected from a potential of 21 million CFU/g of soil (as found on PAF medium) to 700,000 CFU/g of soil while having no effect on the number of *Streptomyces* spp. detected (Table 3). Rifampicin thus provided a significant selection for *Streptomyces* spp. All *S. scabiei* reference strains grew equally well at 11 and 44 ppm nalidixic acid (Table 2). These concentrations of nalidixic acid reduced the soil nonfilamentous bacterial numbers detected while not reducing the *Streptomyces* numbers (Table 3). Nalidixic acid was considered a valuable component of STR medium, as it also reduced the colony size of many bacteria. A nalidixic acid concentration of 15 ppm was chosen for STR medium because the 44-ppm concentration did not reduce the nonfilamentous bacterial numbers any further (Table 3).

All *S. scabiei* reference strains grew at the concentrations of NaCl and the mixture of antibiotics selected for STR medium (Table 2). STR medium will refer to this combination of NaCl and antibiotics. The number of *Streptomyces* detected on the final STR medium was the same as found on PAF and YME media tested (Table 3). The number of nonfilamentous bacteria, however, was reduced from a potential of 21 million CFU/g of soil (as detected on PAF medium) to 160,000 CFU/g of soil on STR medium (Table 3). Thus, for this particular soil sample, the numbers of *Streptomyces* and nonfilamentous bacteria detected on PAF medium at the 10^{-5} dilution were 10 and 210, respectively. Below this dilution, it was practically impossible to find any *Streptomyces* spp., as they were obscured by nonfilamentous bacteria. In contrast, on STR medium at the 10^{-4} dilution, the numbers of *Streptomyces* and nonfilamentous bacteria detected were 100 and 16, respectively. This allowed for detection of *Streptomyces* spp. at the 10^{-4} and 10^{-3} dilutions (Fig. 1). On STR medium, the number of fungi detected was 3,700 CFU/g of soil compared with 300,000 CFU/g of soil on YME medium (Table 3), which virtually prohibited any fungal contaminants from interfering with enumeration of *Streptomyces* spp. Air-drying of the soil before plating also helped reduce the number of nonfilamentous bacteria detected while having no effect on the number of *Streptomyces* spp.

Thaxtomin detection. Small TLC plates (5.5×7 cm cut from 20×20 cm TLC sheets) were used to detect thaxtomin, which allowed 90 samples to be run on a 20×20 cm TLC sheet. These small TLC plates required only 6 min to develop in very small volumes of solvent and allowed for the detection of smaller amounts of toxin than on larger TLC plates. Thaxtomin A and B were easily recognized on plates based on their R_f values compared with standards, their bright yellow color,

Table 3. Effect of NaCl and antibiotics on the number of *Streptomyces*, nonfilamentous bacteria, and fungi isolated from a potato field soil near Alliston, Ontario (site M)

Media ^a	CFU/g soil $\times 1,000^b$		
	<i>Streptomyces</i> bacteria	Nonfilamentous bacteria	Fungi
PAF	1,000 $\pm$ 33	21,000 $\pm$ 4,000	— ^c
YME	1,000 $\pm$ 200	9,000 $\pm$ 2,500	300 $\pm$ 0.0
STR +			
NaCl (0.5%)	1,600 $\pm$ 720	5,000 $\pm$ 2,500	150 $\pm$ 50
NaCl (1%)	1,100 $\pm$ 470	8,700 $\pm$ 1,800	67 $\pm$ 20
NaCl (1.5%)	470 $\pm$ 67	8,700 $\pm$ 2,200	130 $\pm$ 33
NaCl (2%)	330 $\pm$ 120	5,900 $\pm$ 2,100	120 $\pm$ 39
STR + NaCl (1%) +			
Ny (5 ppm)	900 $\pm$ 120	8,000 $\pm$ 1,200	—
Ny (20 ppm)	630 $\pm$ 180	12,000 $\pm$ 1,900	—
Cy (110 ppm)	1,700 $\pm$ 330	14,000 $\pm$ 4,400	—
Cy (1,300 ppm)	1,000 $\pm$ 380	7,000 $\pm$ 1,500	—
Ny (5.6 ppm) + Cy (200 ppm)	830 $\pm$ 200	8,300 $\pm$ 1,900	—
STR + NaCl (1%) +			
Ny (5.6 ppm) + Cy (200 ppm) +			
Nal (11 ppm)	1,500 $\pm$ 58	4,800 $\pm$ 420	—
Nal (44 ppm)	1,300 $\pm$ 220	4,500 $\pm$ 490	—
Rif (0.5 ppm)	1,000 $\pm$ 180	700 $\pm$ 200	—
Rif (1 ppm)	700 $\pm$ 250	500 $\pm$ 0.0	—
Pol (0.7 ppm)	1,300 $\pm$ 200	14,000 $\pm$ 6,800	—
Stp (4 ppm)	400 $\pm$ 150	4,300 $\pm$ 1,400	—
Pen (0.26 ppm)	900 $\pm$ 150	4,400 $\pm$ 260	—
STR + NaCl (1%) + Ny (5.6 ppm) +			
Cy (200 ppm) + Nal (15 ppm) +			
Rif (0.6 ppm) ^d	1,100 $\pm$ 120	160 $\pm$ 65	3.7 $\pm$ 2.2

^a Pseudomonas agar F (PAF), yeast malt extract (YME), and *Streptomyces* selective (STR) media. Antibiotics tested were nystatin (4,420 units/mg) (Ny), cycloheximide (Cy), nalidixic acid (free acid) (Nal), rifampicin (Rif), polymyxin B sulfate (Pol), streptomycin sulfate (Stp), and penicillin G (sodium salt) (Pen). The concentrations of NaCl and antibiotics shown in this table are only a partial list of concentrations tested.

^b Means ($\pm$ standard error of the mean) were determined from three soil samples.

^c Number of fungi could not be determined because fungal colonies were obscured by bacterial colonies.

^d This was the final combination of NaCl and antibiotics chosen for STR medium. Reference to STR medium in this paper indicates this medium.

which remained stable for months, and their absorbance of UV₃₆₆ light. Other yellow pigments encountered from various strains did not have all three of these properties. Thaxtomin was detected by this procedure from *Streptomyces*-inoculated OM medium as well as from inoculated tuber slices.

Extracts containing yellow components corresponding to thaxtomin A or B were tested for phytotoxicity using the tuber slice assay. Such extracts produced symptoms identical to pure thaxtomin A or B, confirming the accurate identification of thaxtomin by TLC. Extracts from non-thaxtomin-producing strains caused no symptoms on tuber slices. To determine if this extraction and detection procedure missed any strains producing low amounts of thaxtomin, a number of strains identified as thaxtomin deficient when grown on OM medium (Tables 4 and 5) were tested for thaxtomin production in OM broth (100 ml). All strains that did not produce thaxtomin on OM medium did not produce it in OM broth either. These strains were also tested on potato tuber slices to determine if they produced thaxtomin on tuber slices but not on OM medium. All strains that did not produce thaxtomin on OM medium did not produce it on tuber slices either. Inclu-

sion of an *S. scabies* or *S. acidiscabies* strain known to produce thaxtomin, as a positive control, is recommended and was routinely done in our screening. With this procedure, one person can easily screen 50 strains over the 2-day extraction and detection process.

Characteristics of *Streptomyces* spp. on STR medium. *Streptomyces* spp. grown on STR medium could be readily identified by a distinct circular depression in the medium at the edges of the colonies. This depression was most apparent when plates were examined at a slight angle. *Streptomyces* spp. formed colonies that were hard and adhered strongly to the medium. Most colonies formed aerial mycelia and sporulated on STR medium. However, all pathogenic *S. scabies* reference strains (Table 2) either did not form aerial mycelia and sporulate on STR medium when incubated at 22°C or were slow to do so. If STR medium was inoculated with very large numbers of bacteria, the lawn that arose sometimes showed sporulation, but individual colonies never formed aerial mycelia and sporulated within 2 weeks at 22°C. Most of these strains sporulated on STR medium if incubated at higher temperatures. All thaxtomin-producing strains isolated from scab lesions or soil from five

locations in Ontario had the nonsporulating (NS) colony phenotype on STR medium (Table 4). In addition, all these colonies were smooth, brown, and produced melanin (Mel⁺) (Table 4; Fig. 1). Many of the strains were grown on PYI medium to test for melanin production. Only those that produced melanin on STR medium produced it on PYI medium. These NS colonies could be detected on STR medium after 3 to 4 days along with the presence of melanin. All strains with this colony morphology from scab lesions produced thaxtomin A (Table 4). Thaxtomin-producing strains from soil were also of the same phenotype, but not all strains with this colony morphology produced thaxtomin (Table 4). Only 7 and 9% of strains with this colony morphology isolated from soil at sites M and B, respectively, produced thaxtomin A (Table 4).

Comparison of some North American *S. scabies* and *S. acidiscabies* strains. Comparison of colony morphology on STR medium, thaxtomin production, and pathogenicity of some North American *S. scabies* and *S. acidiscabies* strains is shown in Table 5. All strains grew on STR medium. All strains that produced thaxtomin had smooth NS colonies on STR medium (Table 5). Most of the *S. scabies* strains

Fig. 1. Isolation of *Streptomyces* spp. on a semi-selective culture (STR) and STR-M media from a potato field soil near Alliston, Ontario (site M). STR-M medium had higher concentrations of NaCl and antibiotics than STR medium and was specifically designed for isolation of *S. scabies* from soil from site M. Note that the majority of colonies on these media are *Streptomyces* bacteria. *S. scabies* colonies that produce thaxtomin are characterized by the presence of melanin and lack of aerial mycelia and sporulation (arrows). Photographs were taken after plates had been incubated for 2 weeks at 22°C.

produced melanin, with the exception of some from Michigan (Table 5). Tests of selected strains for pathogenicity using the tuber slice assay revealed a positive correlation between thaxtomin production and necrotic symptoms on tuber slices (Table 5). Two types of symptoms were observed: one was necrosis of cells around the inoculation site identical to that induced by thaxtomin, and the other was a deep-pitted lesion in the tuber slice. There were a few *Streptomyces* strains from soil that did not produce thaxtomin but caused deep-pitted lesions on tuber slices. These strains failed to cause any lesions on tubers when tested in the greenhouse assay. Only thaxtomin-producing strains caused scab symptoms on potato tubers grown under greenhouse conditions (Table 5). Symptoms varied from shallow to deep-pitted lesions. Some thaxtomin-producing strains did not cause scab symptoms in the greenhouse assay (Table 5).

Quantifying thaxtomin-producing *S. scabies* in soil. Soil from a potato field near Alliston, Ontario (site M) was plated on STR and STR-M media, and the smooth, brown, Mel⁺, NS colonies were transferred onto OM medium. These strains were then tested for thaxtomin production. The number of *Streptomyces* detected on STR medium was 1.4 million CFU/g of soil (Table 6). Of these, the *Streptomyces* spp. with the NS colony phe-

notype represented just under half the population, i.e., 570,000 CFU/g of soil (Table 6). Four percent (3/75) of this group produced thaxtomin, which translated to 23,000 CFU/g of soil. On STR-M medium, the number of *Streptomyces* detected was reduced to 350,000 CFU/g of soil, and of these, 130,000 CFU/g of soil had the NS colony phenotype (Table 6; Fig. 1). Fifteen percent (8/54) of colonies with the NS phenotype produced thaxtomin. Thus, the number of thaxtomin-producing *Streptomyces* spp. was 19,000 CFU/g of soil, which was not significantly different from that calculated on STR medium (Table 6).

DISCUSSION

We developed a culture medium that provides selective isolation of *Streptomyces* spp. from soils where potatoes are grown. STR medium reduces the number of unwanted bacteria and fungi on soil dilution plates to levels below that of *Streptomyces* spp., while not affecting the number of *Streptomyces* detected compared with that on PAF or YME media. In addition, the medium allows colonies to express a phenotype (smooth NS) that helps identify potential pathogenic *S. scabies* strains. All pathogenic *S. scabies* and *S. acidiscabies* reference strains we screened had this colony phenotype on STR medium. All pathogenic *S. scabies* strains we isolated from scab lesions or soils from

Ontario produced melanin, which narrowed the phenotype even further. Since melanin production can be detected on STR medium, this eliminates the need to screen strains on PYI medium. Production of melanin has been used as a diagnostic characteristic of *S. scabies* (14). However, some other *Streptomyces* spp. also produce melanin (Table 4), and not all pathogenic *S. scabies* strains in North America produce melanin (Table 5). Thus, melanin production by itself cannot be used as a diagnostic characteristic of pathogenicity of *S. scabies*.

After we were able to isolate *Streptomyces* spp. from soil, we needed a simple procedure to screen hundreds of strains for thaxtomin production. We developed a procedure that allows for rapid extraction of thaxtomin from small amounts of inoculated OM medium and uses minimum solvent volumes and low-cost Eppendorf tubes. Advantages of using inoculated OM medium over tuber slices for detection of thaxtomin are that the medium is easier to prepare and the extracts produce cleaner chromatograms. To confirm thaxtomin production by *S. ipomoeae*, however, tuber slices would be required, since *S. ipomoeae* does not produce thaxtomin on OM medium (16). We did not test any strains of this species. Using this procedure, we found that all *S. scabies* strains with the smooth NS colony phenotype that we isolated from potato scab lesions produced thaxtomin A. However, only 4 to 9% of *S. scabies* strains from soil with this colony morphology produced thaxtomin A. This is a similar result to that found by Keinath and Loria (13) in which only 3.3% of *S. scabies* strains isolated from a soil were found to be pathogenic. The positive correlation between thaxtomin production and smooth NS colonies on STR medium means that only these colonies need to be tested for thaxtomin production and for further characterization. We have not determined yet if all *Streptomyces* colonies with the smooth NS phenotype isolated from these soils are *S. scabies* or a mixture of *Streptomyces* spp. However, all *S. scabies* reference strains producing thaxtomin that we tested had this colony phenotype.

All thaxtomin-producing *S. scabies* and *S. acidiscabies* strains that we tested caused symptoms on tuber slices. There were a few strains from soil that did not produce thaxtomin but caused deep-pitted lesions on tuber slices. *S. scabies* strains that produce thaxtomin and cause deep-pitted lesions must be producing various enzymes to create such lesions. A positive correlation between production of hydrolytic enzymes and the ability to cause deep-pitted lesions has been demonstrated (6,9). It is not surprising that some strains that do not produce thaxtomins, but may produce hydrolytic enzymes, can also cause lesions on tuber slices. However, only thaxtomin-producing *Streptomyces*

Table 4. Colony characteristics on *Streptomyces* selective (STR) medium and thaxtomin production by *Streptomyces* spp. isolated from scab lesions and soil in Ontario

Source of isolates ^a	Sporulation of colonies ^b	Colony color	Melanin production ^c	Colony surface ^d	Thaxtomin A production/total ^e
Site M					
Scab lesions	NS	Brown	+	Smooth	9/9
Soil	NS	Brown	+	Smooth	5/72
	NS	Yellow	–	Smooth	0/18
	Sporulated	Various	+ & –	Powdery	0/119
Site B					
Scab lesions	NS	Brown	+	Smooth	6/6
Soil	NS	Brown	+	Smooth	4/44
	NS	Yellow	–	Smooth	0/33
	NS	Red	+	Smooth	0/2
	NS	Yellow	–	Erupted	0/76
	Sporulated	Various	+ & –	Powdery	0/60
Site P					
Scab lesions	NS	Brown	+	Smooth	48/48
Site S					
Scab lesions	NS	Brown	+	Smooth	44/44
Site K					
Scab lesions	NS	Brown	+	Smooth	12/12

^a Isolated on STR medium from five locations in Ontario.

^b Colonies were classified as nonsporulating (NS) if individual colonies did not form aerial mycelia and sporulate on STR medium within 2 weeks at 22°C. If colony numbers were dense, forming a lawn, production of aerial mycelia and sporulation sometimes occurred, so only isolated colonies were rated.

^c Melanin production on STR medium. These results were the same as those on peptone-yeast extract iron medium.

^d Colonies were classified as smooth if they did not form aerial mycelia, sporulate, or have erupted surfaces.

^e Total *Streptomyces* isolates from scab lesions consisted of isolates from several lesions at each site. All *Streptomyces* colonies from the 10⁻⁴ to 10⁻⁶ dilution plates from soil were tested. Thaxtomin A production was determined by growing the isolates on oatmeal medium, extraction of thaxtomin, and detection by thin layer chromatography. None of the isolates listed produced detectable amounts of thaxtomin B.

strains caused scab symptoms on potato tubers grown under greenhouse conditions. Some thaxtomin-producing *S. scabies* strains did not cause symptoms on tubers grown in the greenhouse. We do not know whether this was due to loss of pathogenicity, poor survival, or inappropriate environmental conditions. The most serious problem with the greenhouse assay is maintaining optimal soil moisture levels for infection by *S. scabies*. Reports from the literature suggest that dry soil conditions provide optimal infection conditions for *S. scabies* (11,20). Plants in the greenhouse were watered every 2 to 3 days, so the soil was wet more than it was dry. The fact that strain SP caused deep-pitted lesions every time we tested it suggests that environmental conditions are not critical to all strains. These experiments reveal, however, that pathogenicity assays utilizing tuber slices or growing plants in the greenhouse are not totally reliable for distinguishing pathogenic from nonpathogenic strains. This was one of the reasons we chose to use thaxtomin production as a pathogenicity marker in our screening of *Streptomyces* strains.

Using STR and STR-M media along with our procedure for detection of thaxtomin, we determined that the number of thaxtomin-producing *S. scabies* in soil from a potato field in Ontario with a his-

tory of potato scab was about 20,000 CFU/g of soil. Use of STR-M medium reduced the number of *Streptomyces* colonies with the smooth NS phenotype that needed to be screened for thaxtomin production. This demonstrates that STR medium can be modified to increase the relative proportion of pathogenic *Streptomyces* strains from specific soils. Use of higher levels of NaCl could certainly be exploited for isolating *S. scabies* from certain soils, because some strains have higher tolerances to salt than the ones we isolated. For example, 40% of *S. scabies* strains from

Quebec grew at 4% NaCl (5), 83% of strains from a variety of locations grew at 5% NaCl (21), and 100% of strains from a Minnesota location grew at 6% NaCl (24). Modification of STR medium will not necessarily provide better isolation of *S. scabies* from every soil, however. We tested STR-M medium for quantifying *S. scabies* from soil from site B. Pathogenic *S. scabies* reference strains from this site grew on STR-M medium, and the number of colonies from soil with the smooth NS phenotype was decreased. However, this soil contained a *Streptomyces* sp. that was

Table 6. Quantifying *Streptomyces* bacteria from a potato field soil near Alliston, Ontario (site M) and determination of the number producing thaxtomin

Media ^a	<i>Streptomyces</i> (CFU/g soil × 1,000) ^b		
	Total	Mel ⁺ , NS ^c	Thaxtomin producing ^d
STR	1,400 ± 370	570 ± 220	23 ± 8.6
STR-M	350 ± 18	130 ± 3.3	19 ± 0.3

^a STR = *Streptomyces* selective medium. STR-M medium had higher concentrations of NaCl and antibiotics than STR medium and was specifically designed for isolation of *S. scabies* from soil from site M.

^b Means (± standard error of the mean) were determined from three soil samples.

^c This group of *Streptomyces* bacteria contains *S. scabies* strains. These bacterial colonies produced melanin (Mel⁺) and did not form aerial mycelia or sporulate (NS) on STR or STR-M medium when incubated at 22°C.

^d The number of *S. scabies* strains that produced thaxtomin was determined by growing Mel⁺, NS colonies on oatmeal medium, extraction of thaxtomin, and detection by thin layer chromatography. The number of *Streptomyces* with Mel⁺, NS colonies was then multiplied by the percentage of thaxtomin producers to get the number of thaxtomin-producing *S. scabies*/g of soil.

Table 5. Colony characteristics on *Streptomyces* selective (STR) medium, thaxtomin production, and pathogenicity of some North American *Streptomyces scabies* and *S. acidiscabies* strains

Strain ^a	Sporulation of colonies ^b	Colony color	Melanin production ^c	Colony surface ^d	Thaxtomin production ^e	Pathogenicity ^f	
						Tuber slice	Greenhouse tubers
MSS 94-006	NS	Brown	+	Smooth	A	+	+
MSS 94-035	Sporulated	Yellow	–	Powdery	–	–	–
MSS 95-104	NS	Brown	+	Smooth	A	+	NT
MSS 95-142	NS	Yellow	–	Smooth	A	+	NT
DP	NS	Yellow	–	Smooth	A	+	+
Fi II	NS	Brown	+	Smooth	–	–	–
RL 84-01-232	NS	Brown	+	Smooth	A	+	–
RL 84-01-110	NS	Yellow	–	Smooth	A,B	+	+
RL 84-01-95	Sporulated	Yellow	+	Powdery	–	–	–
NRRL B-2796	NS	Brown	+	Smooth	A	+	+
NRRL B-2801	Sporulated	Yellow	+	Powdery	–	–	–
PonP	NS	Brown	+	Smooth	A	+	+
Roy	NS	Brown	+	Smooth	A	+	–
LM5MT-2	NS	Yellow	+	Smooth	A	+	NT
L89-19	NS	Yellow	+	Smooth	A	+	–
LPit-2	NS	Yellow	+	Smooth	A	+	NT
LQH-4	NS	Yellow	+	Smooth	A	+	+
LR90-3	NS	Yellow	+	Smooth	A	+	+
LWis-76-17-2	NS	Yellow	+	Smooth	A	+	NT
Warba-6	NS	Yellow	–	Smooth	A	+	NT
SP	NS	Brown	+	Smooth	A	+	+
PP	NS	Brown	+	Smooth	A	+	+

^a *S. scabies* and *S. acidiscabies* (RL 84-01-110) strains from scab lesions. See Table 1 for sources of strains.

^b Colonies were classified as nonsporulating (NS) if individual colonies did not form aerial mycelium and sporulate on STR medium within 2 weeks at 22°C. If colony numbers were dense, forming a lawn, production of aerial mycelium and sporulation sometimes occurred so only isolated colonies were rated.

^c Melanin production on STR medium. These results were the same as those on peptone-yeast extract iron medium.

^d Colonies were classified as smooth if they did not form aerial mycelia, sporulate, or have erupted surfaces.

^e Production of thaxtomins A and B determined by growing isolates on oatmeal medium, extraction of thaxtomin, and detection by thin layer chromatography.

^f + = shallow or deep-pitted lesions. – = no lesions. NT = not tested.

present in high numbers and was not inhibited by this medium. Thus, the colonies with the smooth NS phenotype were obscured by colonies of this *Streptomyces* sp., which prevented their enumeration. We believe, however, that STR medium, or modifications, and the thaxtomin detection procedure described will be useful for investigations into population dynamics of pathogenic *Streptomyces* in soil.

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