Characterization of *Streptomyces* species causing common scab disease in Newfoundland

Agriculture Research Initiative Project #ARI-1314-005

FINAL REPORT

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Executive Summary

Potato common scab is an important disease in Newfoundland and Labrador and is characterized by the presence of unsightly lesions on the potato tuber surface. Such lesions reduce the quality and market value of both fresh market and seed potatoes and lead to significant economic losses to potato growers. Currently, there are no control strategies available to farmers that can consistently and effectively manage scab disease.

Common scab is caused by different *Streptomyces* bacteria that are naturally present in the soil. Most of these organisms are known to produce a plant toxin called thaxtomin A, which contributes to disease development. Among the new scab control strategies that are currently being proposed are those aimed at reducing or eliminating the production of thaxtomin A by these bacteria in soils. However, such strategies require a thorough knowledge of the types of pathogenic *Streptomyces* bacteria that are prevalent in the soil and whether such pathogens have the ability to produce this toxic metabolite. Currently, no such information exists for the scab-causing pathogens that are present in the soils of Newfoundland.

This project entitled "**Characterization of *Streptomyces* species causing common scab disease in Newfoundland"** is the first study that provides information on the types of pathogenic *Streptomyces* species that are present in the province and the virulence factors that are used by these microbes to induce the scab disease symptoms. Pathogenic bacterial strains were isolated from scab lesions on potatoes grown in the province and were subjected to genetic analysis in order to identify each strain and to determine the presence of known virulence genes. Furthermore, the production of the thaxtomin A phytotoxin as well as other known *Streptomyces* phytotoxins was assessed using analytical chemical methods. The results of this work show that a variety of different pathogenic strains exist within the soils of Newfoundland and that while some of these pathogens utilize thaxtomin A as a virulence factor, others appear to produce novel virulence factors that contribute to plant pathogenicity.

The primary team members for this project were Dr. Dawn Bignell (Assistant Professor, Department of Biology, Memorial University) and Dr. Joanna Fyans (Postdoctoral fellow, Department of Biology, Memorial University). In addition, Ruth-Anne Blanchard (Pest Management Development Officer, NL Department of Natural Resources) served as a key collaborator on the project by providing scabby potatoes for pathogen isolation.

The funding request was **$55,000** for the 2013-2014 fiscal year. Of this, $40,000 was used to support the salary and benefits for Dr. Fyans; $9,500 was used to cover the cost of laboratory supplies needed to conduct the proposed research; and $5,500 was used to cover the travel expenses and conference fees to allow Dr. Fyans to travel to national and international scientific meetings for dissemination of her results.

All of the described research was conducted in the laboratory of Dr. Dawn Bignell at Memorial University. The project start date was April 1, 2013, and the end date was February 28, 2014.

The results of this study are expected to benefit the Newfoundland and Labrador economy by providing important information on the scab-causing pathogens that threaten the potato industry in the province. Such information can, in turn, assist in the development of new control strategies that reduce the economic impact of scab disease on potato growers in the province.

Background and Rationale for Investigation

Root and tuber crops such as potato, beet, radish, carrot, turnip and parsnip have long been staple dietary components of the people of Newfoundland and Labrador. As such, diseases that affect these crops have a direct impact on both the local growers and the local consumers. An important disease of root and tuber crops in Newfoundland is common scab (CS), which is characterized by the formation of unsightly lesions on the underground structures of the plant. Such lesions are often variable in appearance and can range from shallow (superficial) brown lesions to raised (erumpent), wart-like lesions to deep, pitted lesions (Fig. 1). Furthermore, the lesions can cover only a small area of the root/tuber surface, or they can cover the entire surface. The main impact of CS disease is on the potato industry where the resulting lesions reduce the tuber quality and therefore the market value of the crop, leading to significant economic losses to growers. Processing, seed and table stock potatoes are all affected by this disease, and although good records on the economic impact of CS in Newfoundland are not available, a published report in 2005 estimated the total economic loss in Canada due to CS in 2002 to be between $15-17 million dollars nationwide (Hill and Lazarovits 2005). Furthermore, the study revealed that the eastern and Atlantic provinces are most affected by this disease compared with other parts of the country.

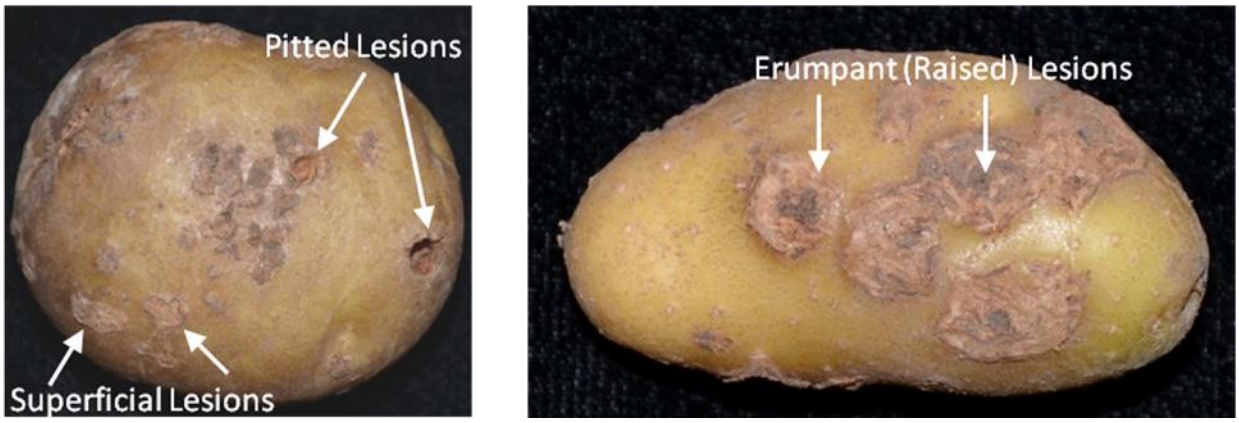

Figure 1: Typical scab lesions on potato tubers harvested in Newfoundland. The different types of lesions that can occur [superficial, erumpent (raised), and pitted] are indicated.

CS disease is a very difficult disease to control as nearly all of the methods that are currently used exhibit inconsistent and/or inadequate results (Dees and Wanner 2012). Although there are some potato varieties that display moderate resistance to the disease, many of the varieties that are preferred by the consumer are highly susceptible. Moreover, no potato variety is completely resistant to CS disease as any variety can become infected given the right environmental conditions and the presence of highly virulent CS - causing pathogens in the soil (Wanner 2009 and references therein).

CS disease is caused by *Streptomyces* bacteria that are naturally found in the soil. The most ancient and best-characterized scab-causing streptomycete is *Streptomyces scabies* (syn. *S. scabiei*), which has a world-wide distribution (Loria et al. 2006). A similar disease called "acid scab" is caused by *S. acidiscabies,* which first emerged in the northeast US in the 1940s (Loria et al. 2006). Acid scab is identical to CS except that it occurs in acidic soils where CS is normally suppressed. In addition, there are at least six other newly emergent plant pathogenic *Streptomyces* species that are now known to cause scab-like symptoms on root and tuber crops (Loria et al. 2006). All scab-causing *Streptomyces* species are neither tissue nor host specific, and in addition to inducing scab lesions on various root and tuber crops, they can infect the fibrous roots of various higher plants resulting in root stunting and browning, and seedling death (Loria et al. 2008). A key virulence factor produced by most scab-causing species is a phytotoxic secondary metabolite called thaxtomin A (Fig. 2; Bignell et al. 2014). This metabolite is essential for disease development as mutant strains of *Streptomyces* that cannot produce the metabolite are avirulent on plants (Healy et al. 2000; Joshi et al. 2007b). Furthermore, pure thaxtomin A has the ability to induce scab-like symptoms on immature potato tubers (King et al. 1989), suggesting that this metabolite is the primary virulence factor contributing to disease symptom development. The importance of thaxtomin A as a *Streptomyces* virulence factor has led some to propose new control strategies focused on controlling the production of this molecule in the field (Legault et al. 2011), or on the use of thaxtomin A in potato breeding programs to select for plants that display enhanced scab resistance (Hiltunen et al. 2011). However, a recently isolated scab-causing *Streptomyces* strain from Iran was found to be unable to produce thaxtomin A; instead, it produces a different metabolite called borrelidin, which also exhibits toxicity towards plants (Cao et al. 2012). This discovery highlights the need to better understand the microbial flora responsible for CS disease in agricultural soils.

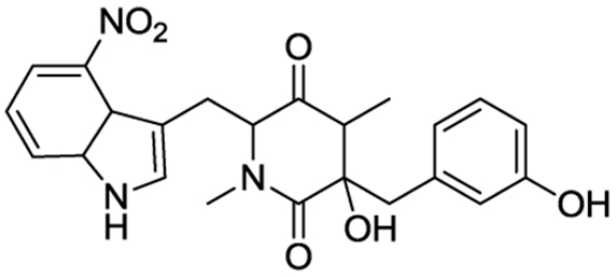

Figure 2: Structure of the phytotoxic secondary metabolite thaxtomin A, which is produced by scab-causing *Streptomyces* species.

Despite the occurrence of CS disease in Newfoundland, there have been no previous studies aimed at characterizing the bacterial strains that cause this disease in the province. It is not known, for example, whether *S. scabies* is the prominent pathogen in Newfoundland soils or whether other pathogenic species are more prevalent. Furthermore, the importance of thaxtomin A in scab disease development in the province is unknown. Such information is not only useful for better understanding the ecology and microbiology of CS disease, but it is also critical for the development of new control strategies that would target the most prevalent pathogens in Newfoundland in order to reduce the economic impact of the disease on local growers.

The goal of this project was to identify and characterize different isolates of *Streptomyces* bacteria that cause CS disease in Newfoundland. This was achieved through the following specific objectives:

(i) To isolate pure cultures of *Streptomyces* bacteria from potato scab lesions harvested in Newfoundland
(ii) To test the pure cultures in a radish seedling bioassay in order to identify those that are pathogenic to plants
(iii) To examine the ability of the pathogenic isolates to produce the known virulence factor thaxtomin A
(iv) To examine the ability of non-thaxtomin producing pathogenic isolates to produce other known *Streptomyces* phytotoxins
(v) To use polymerase chain reaction (PCR) and DNA sequence analysis to identify the closest relatives of the plant pathogenic isolates and to detect the presence of known virulence genes in each isolate

Funding and Partnerships

The primary research team consisted of Dr. Dawn Bignell, who was the principle investigator for the project, and Dr. Joanna Fyans, who conducted all of the described research. Furthermore, Ms. Ruth-Anne Blanchard (Pest Management Development Officer, NL Department of Natural Resources) served as a key collaborator on the project by providing the research team with NL-grown potatoes exhibiting typical scab lesions.

The funding requested was **$55,000** for the **2013-2014 fiscal year**. Of this, $40,000 was used to support the salary and benefits for Dr. Fyans; $9,500 was allocated to cover the cost of laboratory supplies needed to conduct the proposed research; and $5,500 was allocated to cover the travel expenses and conference fees to allow Dr. Fyans to travel to two scientific meetings (one national, one international) for dissemination of her results.

Methods and Implementation

Potatoes exhibiting typical scab lesions were collected from different locations in Newfoundland (Table 1). This was conducted by Ruth-Anne Blanchard after the 2013 summer growing season as well as by Dr. Bignell after the 2011 and 2012 growing seasons. At the start of the project, the Bignell laboratory had already obtained stocks of 18 *Streptomyces* isolates recovered from lesions on potatoes harvested in 2011. These isolates were included in the study as part of Objectives (ii), (iii), (iv) and (v).

Table 1: Sources of scabby potatoes used for bacterial isolations in this study

Growing Season	Locations Where Potatoes Were Harvested
2011	Portugal Cove
2012	Conception Bay South
	Portugal Cove
	Brookfield Road, St. John's
2013	Brookfield Road, St. John's
	Portugal Cove
	Cormack

The following describes the experimental procedures that were conducted for each of the specific objectives.

(i) Isolation of Streptomyces bacteria from potato scab lesions. Bacteria were isolated from potato scab lesions using a protocol that was developed in the Bignell laboratory and which is based on the protocol described by Wanner (2004). Briefly, a small piece of tuber containing a scab lesion was excised and surface-sterilized using 1.5% Chlorox bleach for 2 minutes, after which the tissue was rinsed 10 times with sterile water. Then, the tissue was ground up in 1 ml of sterile water. This homogenate was incubated at 55°C for 30 minutes to eliminate any competing rhizobacteria, after which the homogenate was diluted 100 fold and was plated onto agar (1.5%) water plates containing nalidixic acid (to inhibit the growth of other bacteria) and nystatin (to inhibit the growth of fungi). The plates were incubated at 28°C for up to 1 month, after which the *Streptomyces* – like colonies were picked and cultured several times on ISP-4 agar medium containing nalidixic acid and nystatin in order to obtain pure, isolated colonies. Stocks of each *Streptomyces* –like isolate were then prepared from a single, well-separated colony and were stored at -80°C.

(ii) Radish seedling bioassay for identification of plant pathogenic Streptomyces isolates. The radish seedling bioassay was performed essentially as described previously (Bignell et al. 2010). Briefly, radish seeds were treated with 70% ethanol for 5 minutes followed by 15% Chlorox bleach for 10 minutes in order to surface sterilize them. The seeds were rinsed several times with sterile water and were then germinated by incubating at room temperature (~22-24°C) for 24 hours in the dark in a Petri dish containing moistened filter paper. Germinated seeds were

placed into wells (13 mm in diameter) in 1.5% agar - water plates and were inoculated with *Streptomyces* mycelial suspensions that were prepared from liquid cultures. A total of 18 plants were inoculated with each strain, and control plants were inoculated with known pathogenic *Streptomyces* species (e.g. *S. scabies* 87-22, *S. acidiscabies* 84-104, and/or *S. turgidiscabies* Car8) or were treated with water (uninoculated control). An additional control that was included was the inoculation of seedlings with the *S. scabies* Δ*txtA*/Δ*cfa6* strain, which is significantly reduced in virulence due to the inability to produce thaxtomin A as well as another phytotoxic secondary metabolite (Bignell et al. 2010). The plates were wrapped with parafilm and incubated at 22 ± 2°C under a 16-h photoperiod for 7 days. The total plant size (root + shoot) was measured and averaged for each treatment, and the Student's *t*-test was used to identify statistically significant differences among the treatments.

(iii) Analysis of thaxtomin A production by plant pathogenic Streptomyces isolates. The production of thaxtomin A was assessed by growing each pathogenic strain in oat bran broth (OBB) liquid culture medium, which is known to support the production of this metabolite (Johnson et al. 2007). The strains were cultured at 25°C for 7 days, after which the culture supernatants (ie the liquid portion of the cultures) were harvested. An initial test for bioactivity in the supernatants was performed by filter-sterilizing and then using the supernatants in a radish seedling bioassay as described above. The supernatants were then extracted with 0.5 volumes of ethyl acetate, and the resulting extracts were dried down and redissolved in 100% methanol. To test for bioactivity in the culture extracts, a potato tuber disk assay was performed as described previously (Bignell et al. 2010). The presence of thaxtomin A in the extracts was determined using reverse phase high performance liquid chromatography (HPLC) as described before (Johnson et al. 2007) except that the instrument used was an Agilent 1260 Infinity Quaternary LC system with a Poroshell 120 EC-C18 column (4.6 × 50 mm, 2.7 μm particle size; Agilent Technologies Inc.). Pure, authentic thaxtomin A (Santa Cruz) was used as a standard for the analysis, and quantification of thaxtomin production was assessed by comparing the thaxtomin peak area in the isolate extracts to that in the *S. scabies* 87-22 extract.

(iv) Assessing the ability of non-thaxtomin A-producing pathogenic isolates to produce other known Streptomyces phytotoxins. Pathogenic isolates that did not produce thaxtomin A were analyzed for their ability to produce other phytotoxic secondary metabolites such as concanamycin A and borrelidin. Culture extracts were prepared as described above and were analyzed by HPLC using established protocols for detecting concanamycin A and borrelidin (Cao et al. 2012; Natsume et al. 1998). Pure, authentic standards of concanamycin A (Sigma Aldrich) and borrelidin (kindly provided by Kenji Arakawa, Hiroshima University) were used for identification of the phytotoxins in the culture extracts.

(v) PCR and sequencing analysis of Streptomyces isolates. The identification of pathogenic isolates was performed by extracting genomic DNA from each isolate using the One-Tube Bacteria DNA Isolation Kit (BioBasics) according to the manufacturer's instructions. Then, PCR was conducted to amplify the 16S ribosomal (r) RNA gene sequence and the *rpoB* gene

sequence from each isolate using primers that have been described before (St-Onge et al. 2008). The resulting PCR products were gel-purified and then sent for sequencing to the Centre for Applied Genomics in Toronto. The 16S rRNA and *rpoB* gene sequences obtained were compared to known sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) in order to identify the closest relatives for each isolate. The *rpoB* sequences from the isolates and their closest relatives were also used to generate a phylogenetic tree using the MEGA 5.2 software (Tamura et al. 2011) with the neighbour-joining method.

PCR was also performed using the genomic DNA from each bacterial isolate in order to detect the presence of known *Streptomyces* virulence genes. PCR primers were designed to amplify *txtA* or *txtD,* which are involved in thaxtomin A biosynthesis (Bignell et al. 2014), *nec1,* which is important (but not essential) for the plant pathogenic phenotype of some *Streptomyces* species (Joshi et al. 2007a) and *tomA,* which is a predicted virulence gene (Kers et al. 2005; Seipke and Loria 2008). The primer sequences were based on those previously reported in the literature (St-Onge et al. 2008) or were designed to anneal to highly conserved regions within the genes.

Proposed Timeline

The anticipated timeline and major indictors of success for each of the specific objectives is shown in Table 2.

Results and Discussion

(1) Isolation of *Streptomyces* bacteria from potato scab lesions and assessment of the plant pathogenic phenotype of each (Objectives i and ii). Using the procedures outlined in the Methods section, we were able to successfully isolate a large number of *Streptomyces*-like bacterial strains from the potatoes harvested in 2011, 2012 and 2013 (Table 3). The 2011 and 2012 bacterial isolates were tested in the radish seedling bioassay, and 19 out of 52 (or 36.5%) were determined to be plant pathogenic (Table 3 and Figures 3, 4, 5, 6 and 7). Note that an isolate was considered plant pathogenic if it caused significant stunting of the radish seedlings as compared to the mock control (water treated seedlings). The degree of pathogenicity among the different isolates was found to be quite variable as indicated by the degree of seedling stunting observed in the bioassays (Figures 3, 4, 5, 6 and 7). In the case of the 2013 isolates, these were not tested in the bioassay as we are still in the process of stocking all of these isolates.

Table 2: Timeline and major milestones of success for the proposed research

Project Specific Objective	Start Date[1]	Anticipated Completion Date[2]	Major Milestone(s)/Indicators of Success
(i) Isolate pure cultures of *Streptomyces* bacteria from potato scab lesions	April 1, 2013	November 30, 2013	Freezer stocks of *Streptomyces* isolates obtained
(ii) Test each isolate in the radish seedling bioassay in order to identify those that are pathogenic	June 1, 2013	January 31, 2014	Bioassay results for all *Streptomyces* isolates obtained
(iii) Examine the ability of each pathogenic isolate to produce the known virulence factor thaxtomin A	August 1, 2013	February 28, 2014	HPLC analysis of culture extracts completed for all pathogenic isolates
(iv) Examine the ability of non-thaxtomin producing pathogenic isolate to produce other known *Streptomyces* phytotoxins	August 1, 2013	February 28, 2014	HPLC analysis of culture extracts completed for all non-thaxtomin producing pathogenic isolates
(v) Use PCR and DNA sequence analysis to identify the closest relatives of the plant pathogenic isolates and to detect the presence of known virulence genes in each isolate	August 1, 2013	February 28, 2014	DNA sequence results for the 16S rRNA and *rpoB* genes obtained for all pathogenic isolates; PCR results for the *txtA, txtD, nec1* and *tomA* genes obtained for all pathogenic isolates

[1] The start dates indicated were the anticipated start dates for the work involving the potatoes harvested during the 2011 and 2012 growing season. Work involving the 2013 potatoes was to commence once the potatoes were obtained.

[2] These were the anticipated completion dates for all work that was to be conducted for each specific objective.

Table 3: Summary of the Results Obtained for Objectives i and ii

Growing Season	Total Number of *Streptomyces* –like Bacterial Isolates Obtained from Scab Lesions (Objective i)	Total Number of Isolates Examined Using the Radish Seedling Bioassay (Objective ii)	Total Number of Pathogenic Isolates Detected Using the Radish Seedling Bioassay (Objective ii)
2011	18	16†	5
2012	39	39	14
2013	47	---*	---*
TOTAL	**104**	**52**	**19**

† Only 16 of the 18 isolates from 2011 were tested in the bioassay as the remaining two isolates could not be sufficiently cultured in liquid medium to be assessed.

* The assessment of the 2013 isolates using the radish seedling bioassay still needs to be completed.

(2) Assessing the ability of the plant pathogenic isolates to produce the known phytotoxic secondary metabolite thaxtomin A (Objective iii). The ability of *S. scabies* and other characterized plant pathogenic *Streptomyces* species to cause scab disease is primarily due to the ability to produce the phytotoxic secondary metabolite thaxtomin A (Fig. 2). Given that several of the pathogenic isolates recovered in 2011 and 2012 displayed a similar phenotype as *S. scabies* 87-22 in the radish seedling bioassay, we thought that it was likely that these pathogenic isolates also produce thaxtomin A. To investigate this further, we performed an initial test with the 2011 pathogenic isolates where we first cultured the isolates in a liquid growth medium (OBB) that supports the production of thaxtomin A, and after 7 days incubation we harvested the culture supernatants and filter-sterilized them. We then used the sterile culture supernatants in the radish seedling bioassay to determine whether the supernatants contained any phytotoxic compounds that could cause stunting of the radish seedlings. As shown in Figure 8, the culture supernatants for Isolates 1-2, 1-7 and 2-4 caused significant stunting of the seedlings as compared to the mock (uninoculated control) treatment. Interestingly, the supernatant for Isolate 1-2 was found to exhibit even greater bioactivity than that for the *S. scabies* 87-22, which suggested that if Isolate 1-2 produces thaxtomin A, it might make higher levels of this metabolite than *S. scabies* 87-22.

We next tested whether the bioactivity observed in the culture supernatants from the 2011 isolates could be extracted using an organic solvent. We chose to extract the supernatants using ethyl acetate as it is a very good solvent for many secondary metabolites, and it has previously been used for the extraction of thaxtomin A (Beauséjour et al. 1999). The resulting extracts were dried down and resuspended in 100% methanol, and the bioactivity was tested using a potato tuber disk assay. We chose this particular bioassay over the radish seeding bioassay because the methanol used for redissolving the organic extracts causes adverse effects on radish seedlings whereas it has very little effect on potato tuber tissue. As shown in Figure 9, the extract from the Isolate 1-2 caused significant pitting and necrosis of the tuber tissue in a

similar manner as the *S. scabies* 87-22 culture extract. The extract from Isolate 2-4 also caused necrosis of the potato tuber tissue, while the extracts from Isolates 1-7, 1-10 and 2-1 did not have any effect.

To determine whether any of the isolates produce thaxtomin A, the organic culture extracts were subjected to reverse phase HPLC analysis using a published protocol for detecting this phytotoxin. As shown in Figure 10, thaxtomin A was readily detected in the culture extracts of *S. scabies* 87-22, whereas no thaxtomin A could be detected in the culture extracts from any of the 2011 potato isolates.

Taken together, these results indicate that all of the 2011 pathogenic isolates produce one or more virulence factors that are distinct from the thaxtomin A phytotoxin. Furthermore, the fact that the ethyl acetate extracts from Isolates 1-2 and 2-4 were bioactive suggests that the primary virulence factor(s) produced by these isolates is a secreted secondary metabolite rather than a secreted protein (which would not be soluble in ethyl acetate).

The ability to produce thaxtomin A was also assessed in some of the 2012 pathogenic isolates. As shown in Figure 11, isolates 12-4-1, 12-11-1 and 12-11-3 were all found to produce thaxtomin A whereas other pathogenic isolates (12-11-5, 12-15-1) did not produce this metabolite. Interestingly, isolates 12-11-1 and 12-11-3 were shown to produce much higher levels of thaxtomin A than *S. scabies* 87-22 (Fig. 12). Given that thaxtomin A production levels have been positively correlated with the severity of scab disease exhibited by some pathogenic *Streptomyces* strains (Goyer et al. 1998; Loria et al. 1995), this result suggests that isolates 12-11-1 and 12-11-3 may cause more severe disease symptoms than *S. scabies* 87-22.

(3) Assessing the ability of non-thaxtomin producing pathogenic isolates to produce other known *Streptomyces* phytotoxins (Objective iv). The inability of some of the pathogenic isolates to produce thaxtomin A was a surprise, though it was not entirely unexpected given recent findings that have been published by other labs. In particular, it has been reported that some *Streptomyces* strains have the ability to produce other phytotoxic secondary metabolites such as concanamycin A and borrelidin (Bignell et al. 2014). To determine whether any of our pathogenic isolates could also produce these phytotoxins, we subjected the culture extracts from the 2011 pathogenic isolates to HPLC analysis using published protocols for detecting concanamycin A and borrelidin. To ensure that the detection protocols used were working properly, we included pure, authentic standards of concanamycin A and borrelidin in our analyses. We also included the culture extract from the *S. scabies* 87-22 and Δ*txtA*/Δ*cfa6* strains, which produce low levels of concanamycin A but not borrelidin. As shown in Figures 13 and 14, none of the 2011 pathogenic isolates produced any detectable levels of concanamycin A or borrelidin. In the case of Isolate 1-2, this strain did produce a compound that had a very similar retention time as borrelidin (Fig. 14); however, an analysis of the absorbance spectrum of this compound revealed that it is distinct from borrelidin (Fig. 15). Our results therefore indicate that none of the 2011 pathogenic isolates produce any of the known *Streptomyces* phytotoxins.

(4) Detection of known virulence genes in the pathogenic isolates using PCR (Objective v). To further characterize the pathogenic isolates, PCR was performed on a subset of the isolates in order to determine whether any of them carry one or more of the virulence genes that have been described in other pathogenic *Streptomyces* species. The genes that were chosen for the analysis included *txtA* and *txtD,* which encode thaxtomin A biosynthetic enzymes (Bignell et al. 2014); *nec1,* which encodes a secreted protein that contributes to disease symptom development (Joshi et al. 2007a; Bukhalid and Loria 1997); and *tomA,* which encodes a saponinase that may allow pathogenic streptomycetes to overcome plant defense responses (Seipke and Loria 2008). As shown in Figure 16, none of the 2011 pathogenic isolates appear to harbour the *txtA* gene, a result that is consistent with our inability to detect thaxtomin A production by these isolates (Fig. 10). Amplification of the *nec1* gene was detected in the Isolate 1-10, and this might explain part or all of the pathogenic phenotype of this isolate. Furthermore, Isolates 1-2 and 1-7 both appear to carry the *tomA* gene. In the case of the 2012 pathogenic isolates, strains 12-4-1, 12-11-1 and 12-11-3 were all found to carry the *txtA* and *txtD* genes (Fig. 17), which is consistent with the fact that we detected thaxtomin A production by these isolates (Fig. 11). These isolates were also shown to harbour the *nec1* virulence gene, and Isolates 12-11-1 and 12-11-3 were additionally shown to carry *tomA* (Fig. 17).

A summary of the results obtained for the phytotoxin production analyses and virulence gene detection analyses is given in Table 4. Overall, our results show that the Newfoundland isolates vary in the types of virulence factors that they can produce.

Table 4: Summary of the Results Obtained for Objectives iii, iv and v

Bacterial Isolate	Phytotoxin Production[1] (Objective iii and iv)			Virulence Genes[2] (Objective v)			
	Thaxtomin A	Concanamycin A	Borrelidin	*txtA*	*txtD*	*nec1*	*tomA*
2011 Isolates							
1-2	-	-	-	-	n.d.	-	+
1-7	-	-	-	-	n.d.	-	+
1-10	-	-	-	-	n.d.	+	-
2-1	-	-	-	-	n.d.	-	-
2-4	-	-	-	-	n.d.	-	-
2012 Isolates							
12-4-1	+	n.d.	n.d.	+	+	+	-
12-11-1	+	n.d.	n.d.	+	+	+	+
12-11-3	+	n.d.	n.d.	+	+	+	+
12-11-5	-	n.d.	n.d.	-	-	-	-
12-15-1	-	n.d.	n.d.	-	-	-	-

[1] Presence (+) or absence (-) of phytotoxin production as determined by HPLC

[2] Presence (+) or absence (-) of virulence genes as determined by PCR

n.d., not determined

(5) Preliminary identification of the pathogenic bacterial isolates (Objective v). Although *S. scabies* is the predominant pathogenic streptomycete responsible for CS disease in North America, other *Streptomyces* species are also known to be able to cause this disease (Loria et al. 2006; Wanner 2009). To determine the closest relatives of the 2011 pathogenic isolates obtained in this study, PCR and DNA sequencing of the 16S ribosomal (r) RNA and *rpoB* genes was conducted for each isolate, and the resulting sequences were compared to those present within the NCBI database. The 16S rRNA and *rpoB* genes were chosen for this work because they are highly conserved and are routinely used for phylogenetic analysis of *Streptomyces* species (St-Onge et al. 2008). The results of the 16S rRNA sequence analysis indicated that as expected, all of the 2011 pathogenic isolates belong to the genus *Streptomyces.* Sequencing of the *rpoB* gene provided additional information regarding the most closely related species for each isolate since this gene is not as highly conserved as the 16S rRNA gene. The results of the *rpoB* sequence analysis were used to construct a phylogenetic tree (Fig. 18), which showed that all of the isolates represent distinct species rather than representing different clones of the same species. Furthermore, the results showed that the isolates are most closely related to species that have not been previously described as being pathogenic (Fig. 18). This suggests that the 2011 pathogenic isolates may represent novel pathogenic species.

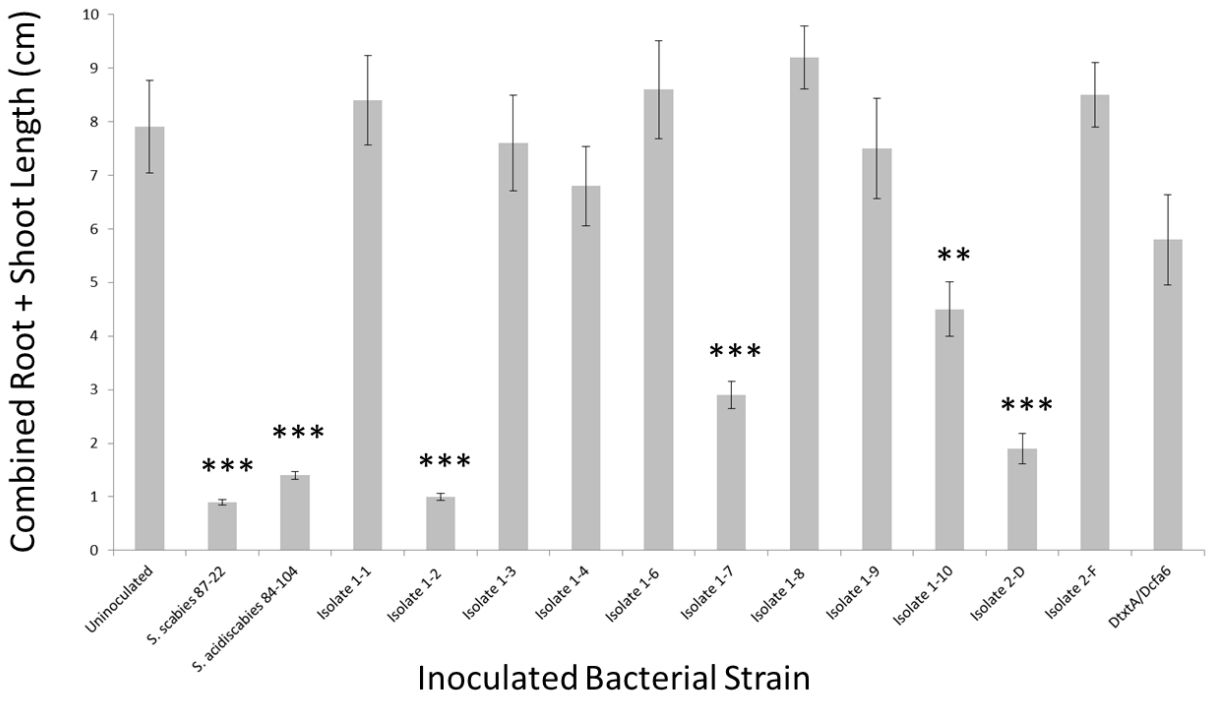

Figure 3: Radish seedling bioassay using Newfoundland bacterial isolates from 2011. Negative control seedlings were treated with sterile water (uninoculated) or with an avirulent *S. scabies* strain (Δ*txtA*/Δ*cfa6*), while positive control seedlings were treated with known scab pathogens (*S. scabies* 87-22, *S. acidiscabies* 84-104). The average measurement per treatment is indicated, with error bars representing the standard error of the mean. The Student's *t*-test was used for statistical analysis of the data, and treatments that produced statistically significant results compared to the uninoculated control are indicated by *** ($p < 0.001$) and ** ($p < 0.01$).

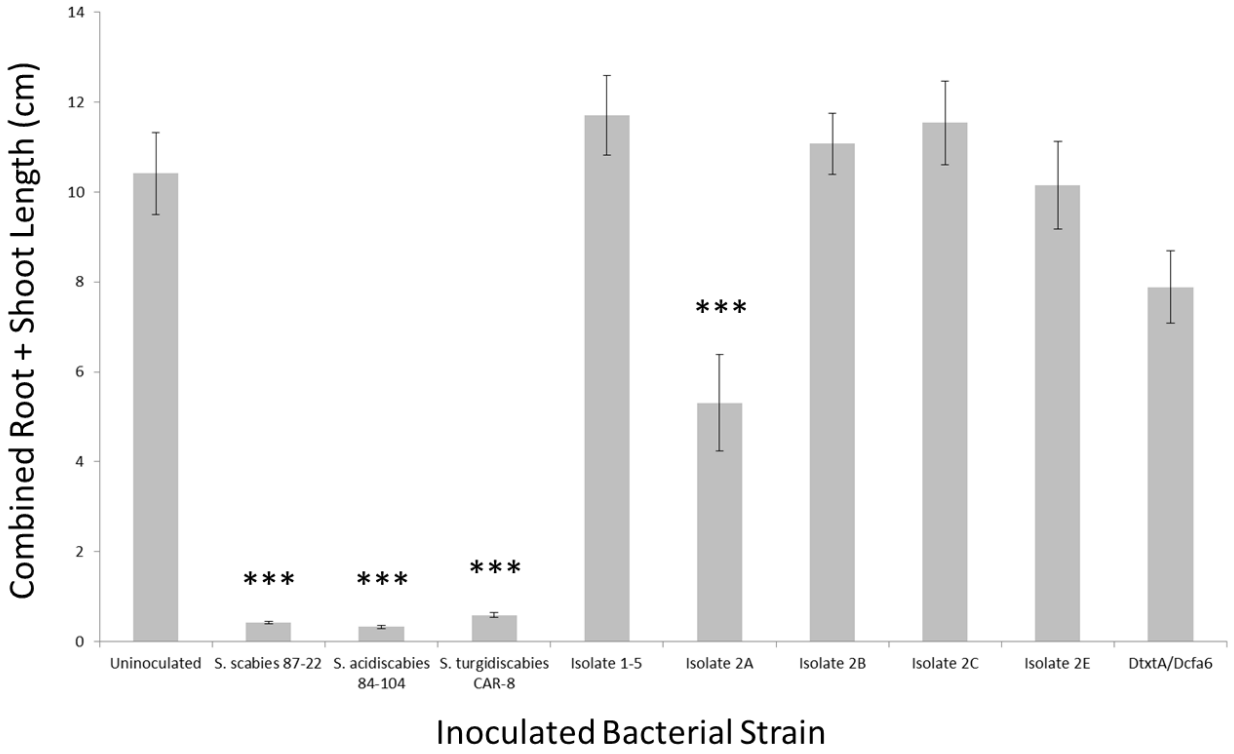

Figure 4: Radish seedling bioassay using Newfoundland bacterial isolates from 2011. Negative control seedlings were treated with sterile water (uninoculated) or with an avirulent *S. scabies* strain (Δ*txtA*/Δ*cfa6*), while positive control seedlings were treated with known scab pathogens (*S. scabies* 87-22, *S. acidiscabies* 84-104, *S. turgidiscabies* Car8). The average measurement per treatment is indicated, with error bars representing the standard error of the mean. The Student's *t*-test was used for statistical analysis of the data, and treatments that produced statistically significant results compared to the uninoculated control are indicated by *** ($p < 0.001$).

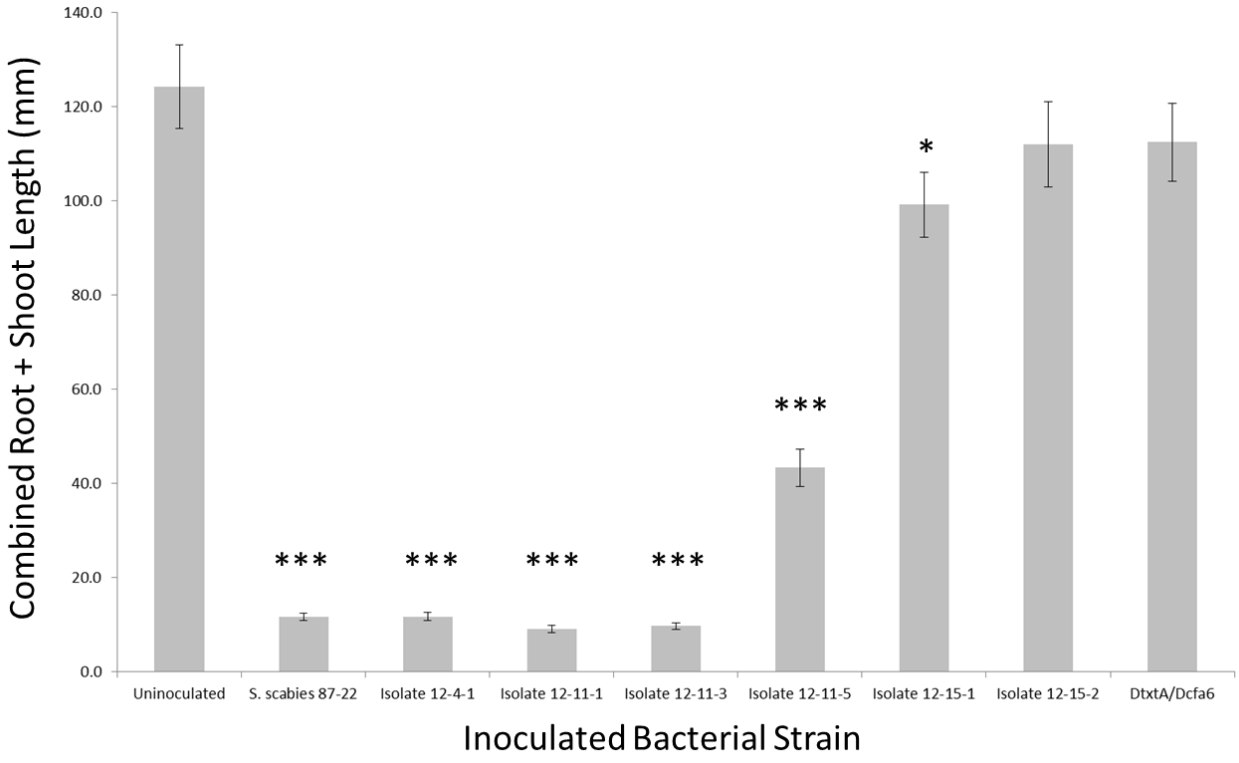

Figure 5: Radish seedling bioassay using Newfoundland bacterial isolates from 2012. Negative control seedlings were treated with sterile water (uninoculated) or with an avirulent *S. scabies* strain (Δ*txtA*/Δ*cfa6*), while positive control seedlings were treated with the known scab pathogen *S. scabies* 87-22. The average measurement per treatment is indicated, with error bars representing the standard error of the mean. The Student's *t*-test was used for statistical analysis of the data, and treatments that produced statistically significant results compared to the uninoculated control are indicated by *** ($p < 0.001$) and * ($p < 0.05$).

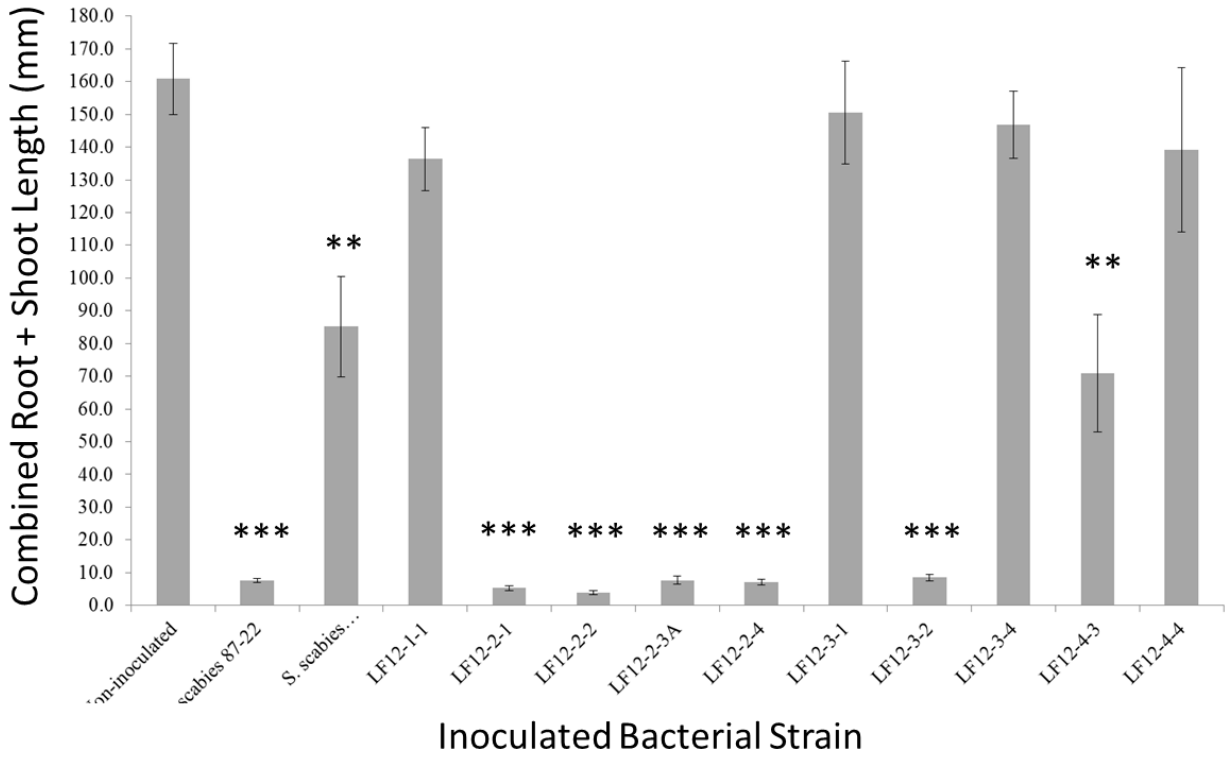

Figure 6: Radish seedling bioassay using Newfoundland bacterial isolates from 2012. Negative control seedlings were treated with sterile water (uninoculated) or with an avirulent *S. scabies* strain (Δ*txtA*/Δ*cfa6*), while positive control seedlings were treated with the known scab pathogen *S. scabies* 87-22. The average measurement per treatment is indicated, with error bars representing the standard error of the mean. The Student's *t*-test was used for statistical analysis of the data, and treatments that produced statistically significant results compared to the uninoculated control are indicated by *** ($p < 0.001$) and ** ($p < 0.01$).

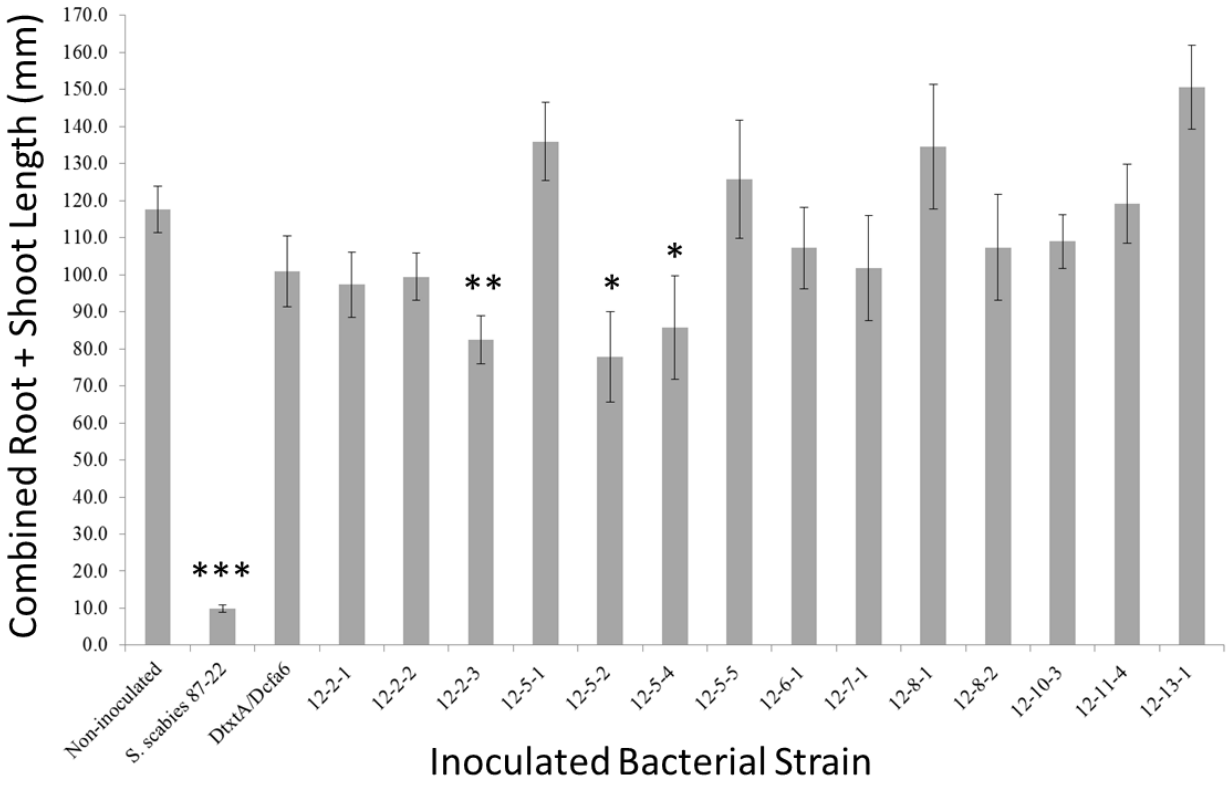

Figure 7: Radish seedling bioassay using Newfoundland bacterial isolates from 2012. Negative control seedlings were treated with sterile water (uninoculated) or with an avirulent *S. scabies* strain (Δ*txtA*/Δ*cfa6*), while positive control seedlings were treated with the known scab pathogen *S. scabies* 87-22. The average measurement per treatment is indicated, with error bars representing the standard error of the mean. The Student's *t*-test was used for statistical analysis of the data, and treatments that produced statistically significant results compared to the uninoculated control are indicated by *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

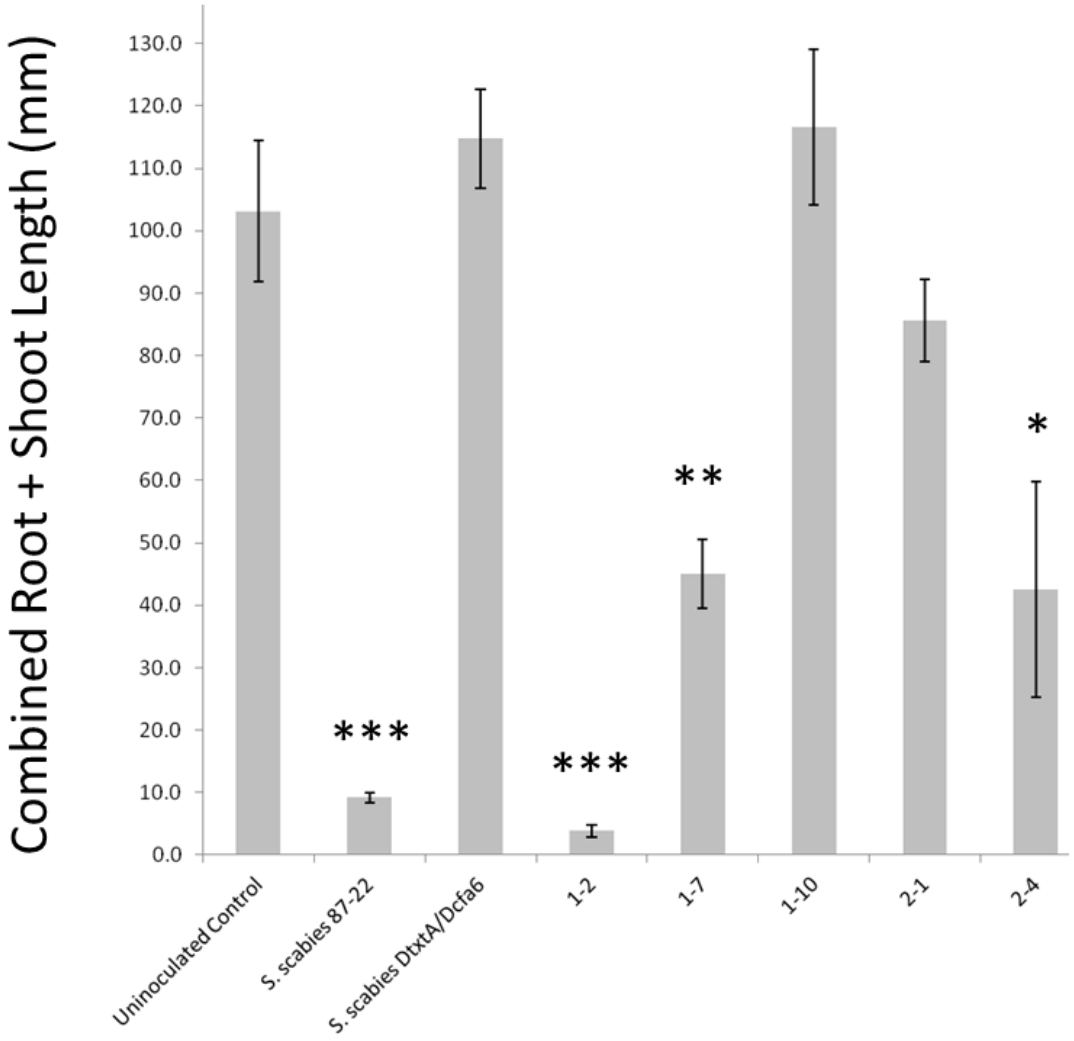

Figure 8: Radish seedling bioassay for detecting bioactivity in the culture supernatants of the 2011 pathogenic Newfoundland bacterial isolates. Bacterial strains were cultured in OBB growth medium for 7 days at 25°C, after which the culture supernatants were harvested, filter sterilized and used to treat radish seedlings. Negative control seedlings were treated with sterile uninoculated OBB medium or with supernatant from the avirulent *S. scabies* strain Δ*txtA*/Δ*cfa6*, while positive control seedlings were treated with culture supernatant from known scab pathogen *S. scabies* 87-22. The average measurement per treatment is indicated, with error bars representing the standard error of the mean. The Student's *t*-test was used for statistical analysis of the data, and treatments that produced statistically significant results compared to the uninoculated control are indicated by *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

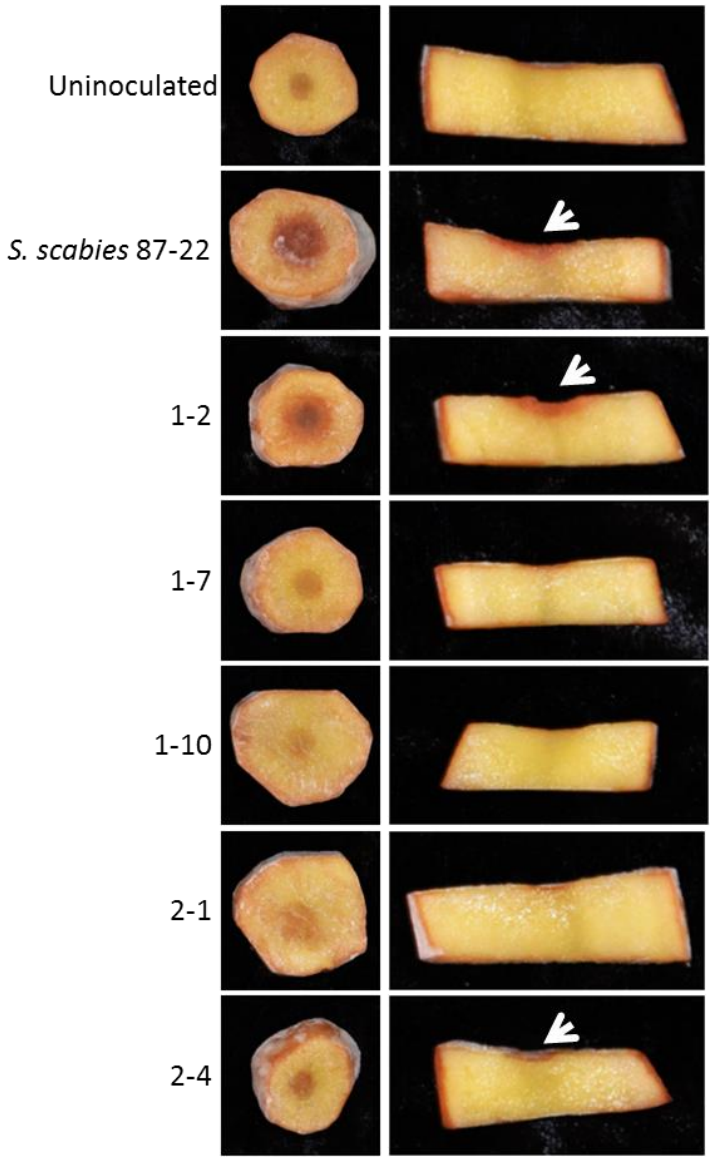

Figure 9: Potato tuber disk assay for detecting bioactivity in culture extracts from the 2011 pathogenic Newfoundland bacterial isolates. Bacterial strains were cultured in OBB growth medium for 7 days at 25°C, after which the culture supernatants were extracted with ethyl acetate. The resulting extracts were then dried down, resuspended in 100% methanol, and used to treat potato tuber disks. Negative control disks were treated with extracted from an uninoculated OBB culture while positive control disks were treated with culture extract from the known thaxtomin-producing scab pathogen *S. scabies* 87-22. The white arrows indicate areas of pitting and/or necrosis of the potato tuber tissue in response to the extracts.

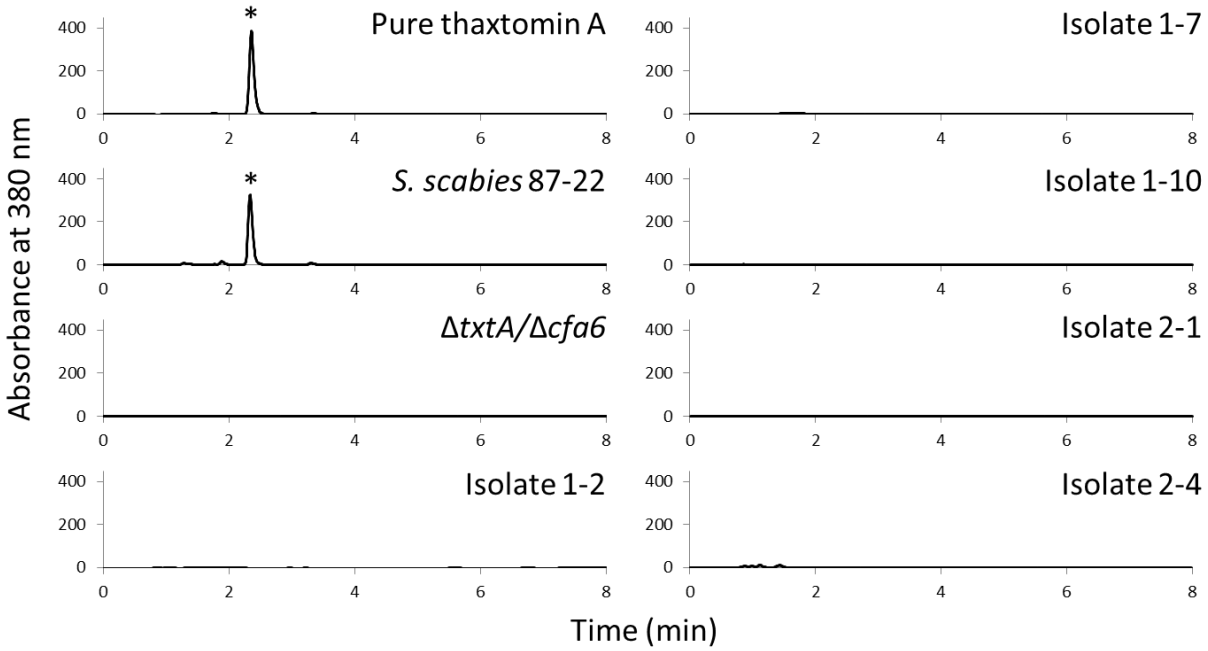

Figure 10: Detection of thaxtomin A production by the 2011 pathogenic Newfoundland bacterial isolates. Bacterial strains were cultured in OBB growth medium for 7 days at 25°C, after which the culture supernatants were extracted with ethyl acetate. The resulting extracts were then dried down, resuspended in 100% methanol, and analyzed by reverse phase HPLC. Extract from the known thaxtomin A producing strain *S. scabies* 87-22 served as a positive control while extract form the thaxtomin A non-producing strain Δ*txtA*/Δ*cfa6* served as a negative control. A pure thaxtomin A standard was also included in the analysis to allow for identification of the thaxtomin A peak in the chromatograms. The peak representing thaxtomin A is indicated with *.

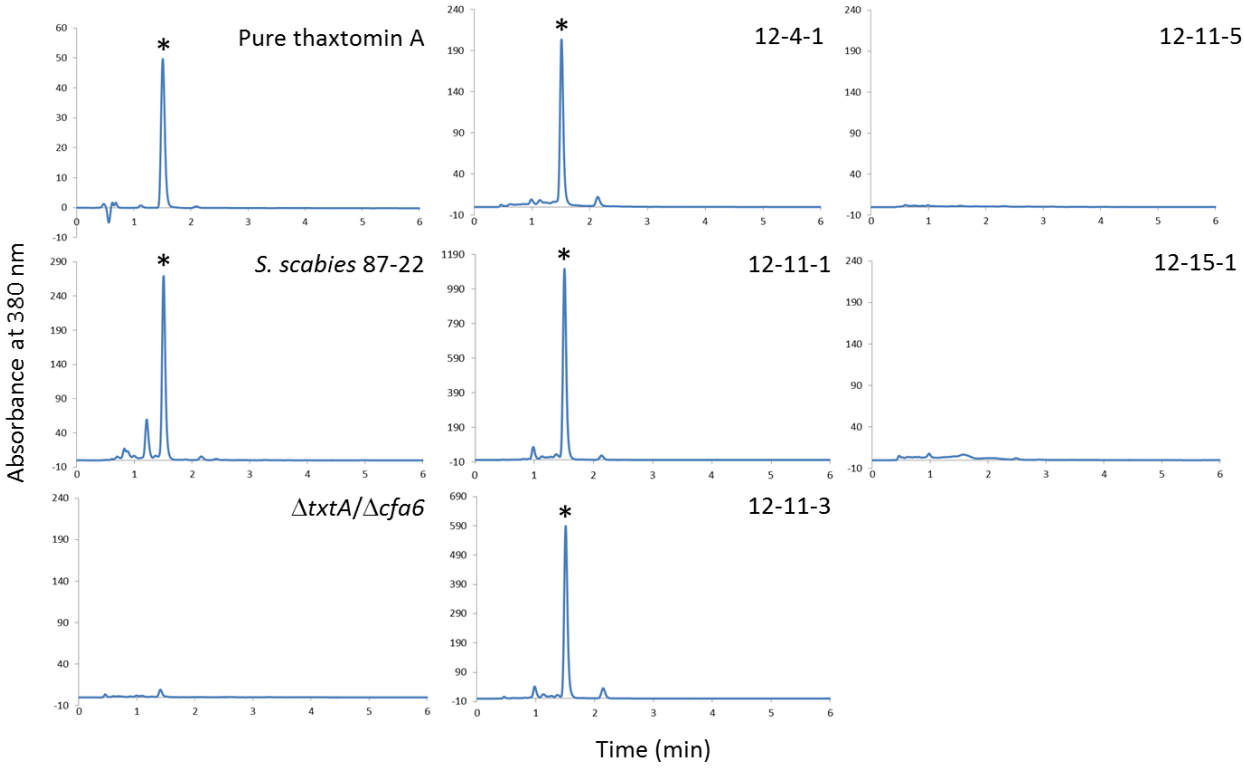

Figure 11: Detection of thaxtomin A production by the 2012 pathogenic Newfoundland bacterial isolates. Bacterial strains were cultured in OBB growth medium for 7 days at 25°C, after which the culture supernatants were extracted with ethyl acetate. The resulting extracts were then dried down, resuspended in 100% methanol, and analyzed by reverse phase HPLC. Extract from the known thaxtomin A producing strain *S. scabies* 87-22 served as a positive control while extract form the thaxtomin A non-producing strain Δ*txtA*/Δ*cfa6* served as a negative control. A pure thaxtomin A standard was also included in the analysis. The peak representing thaxtomin A is indicated with *.

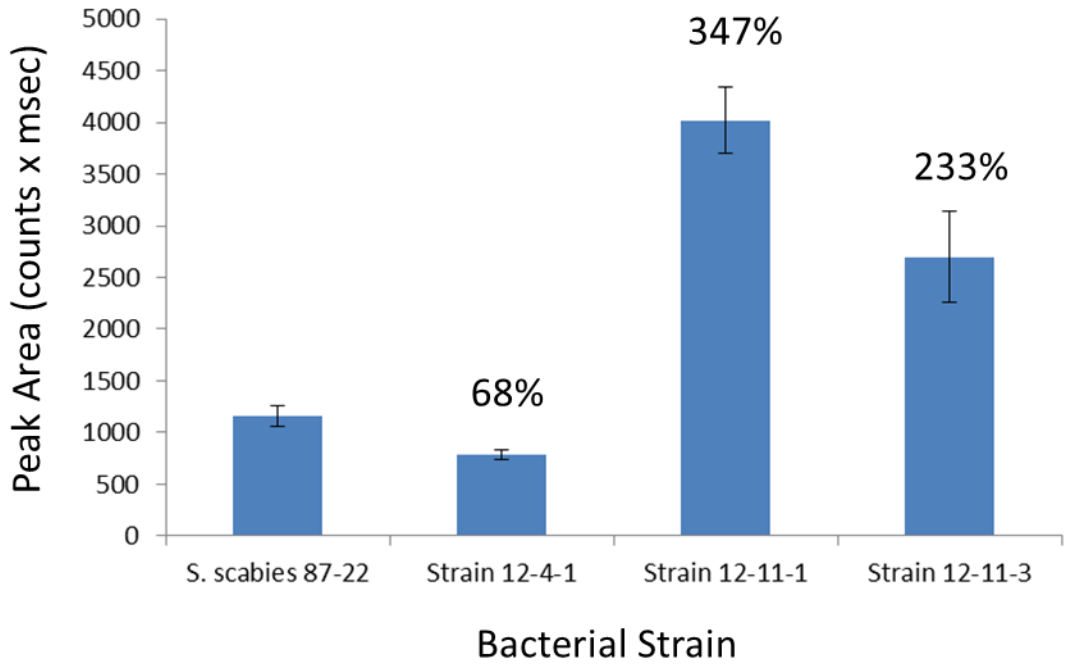

Figure 12: Bar graph showing the relative productions levels of thaxtomin A by *S. scabies* 87-22 and by the 2012 pathogenic isolates. Each bar represents the mean thaxtomin A peak area from triplicate cultures for each strain, and the error bars represent the standard deviation from the mean. The % production in each isolate as compared to *S. scabies* 87-22 is also shown.

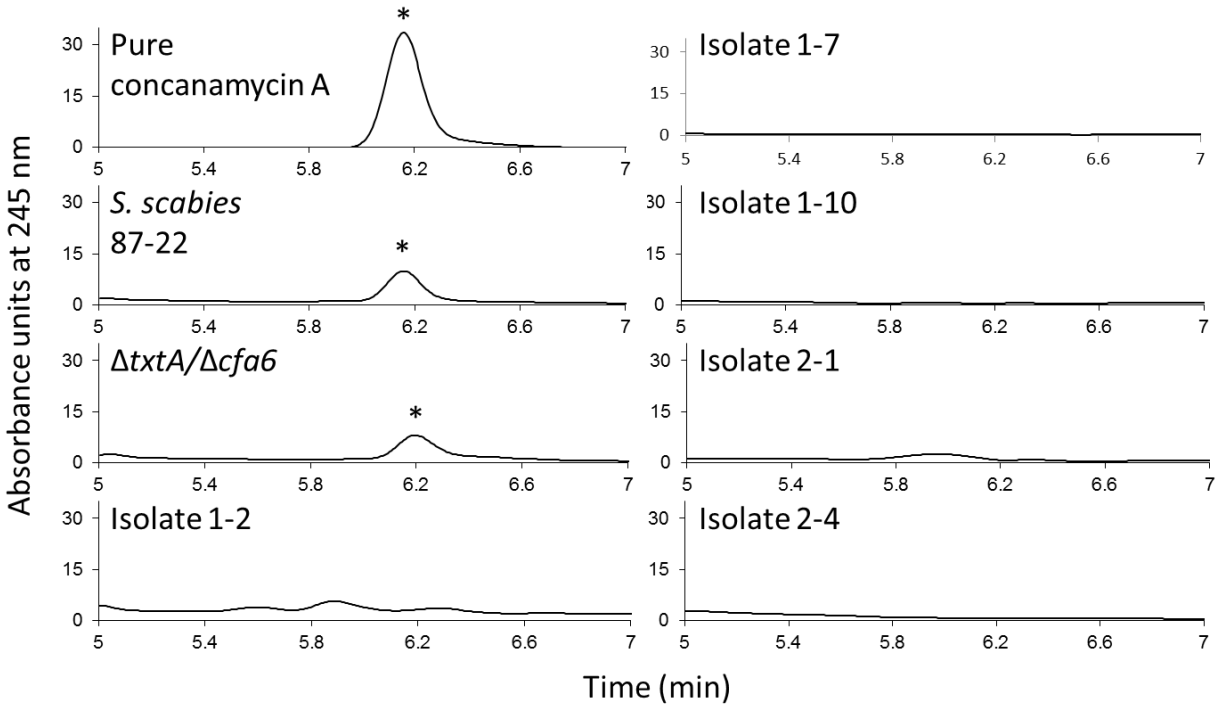

Figure 13: Detection of concanamycin A production by the 2011 pathogenic Newfoundland bacterial isolates. Bacterial strains were cultured in OBB growth medium for 7 days at 25°C, after which the culture supernatants were extracted with ethyl acetate. The resulting extracts were then dried down, resuspended in 100% methanol, and analyzed by reverse phase HPLC. Extract from the known concanamycin A producing strains *S. scabies* 87-22 and Δ*txtA*/Δ*cfa6* served as positive controls. A pure concanamycin A standard was also included in the analysis. The peak representing concanamycin A is indicated with *.

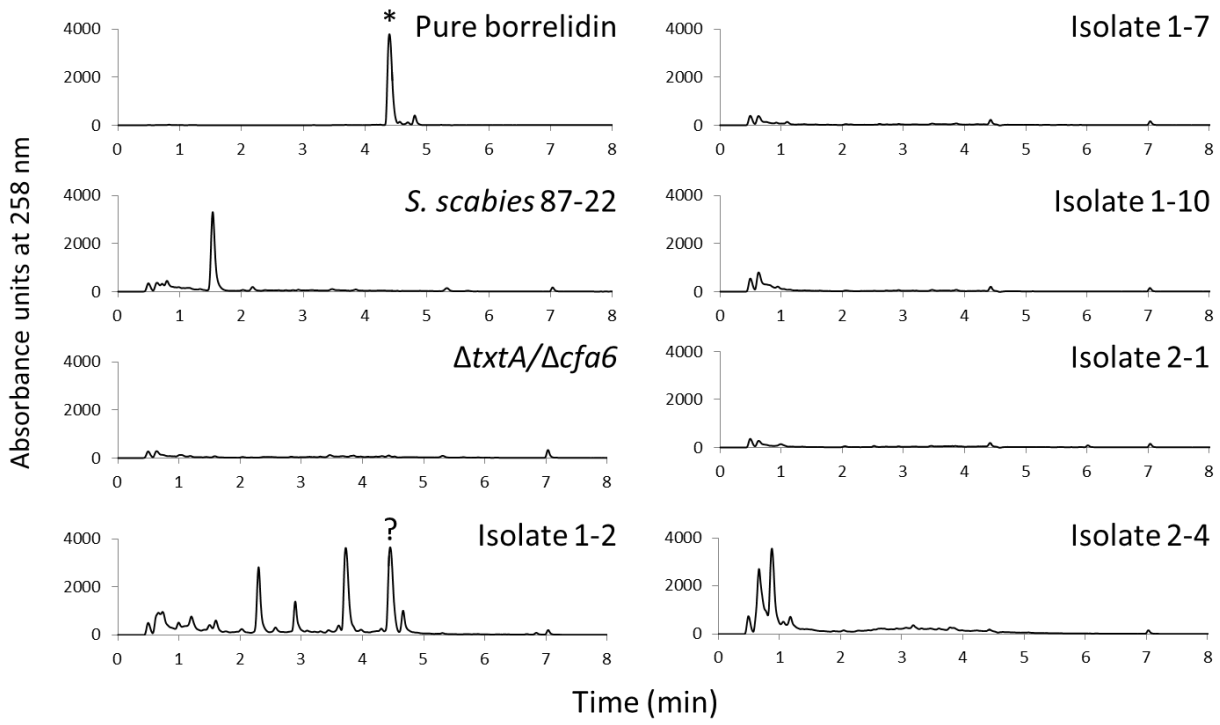

Figure 14: Detection of borrelidin production by the 2011 pathogenic Newfoundland bacterial isolates. Bacterial strains were cultured in OBB growth medium for 7 days at 28°C, after which the culture supernatants were extracted with ethyl acetate. The resulting extracts were then dried down, resuspended in 100% methanol, and analyzed by reverse phase HPLC. A pure borrelidin standard was also included in the analysis, and the peak representing borrelidin is indicated with *. A peak with the same retention time as the borrelidin peak was detected in the culture extract from Isolate 1-2, and this peak is indicated with "?".

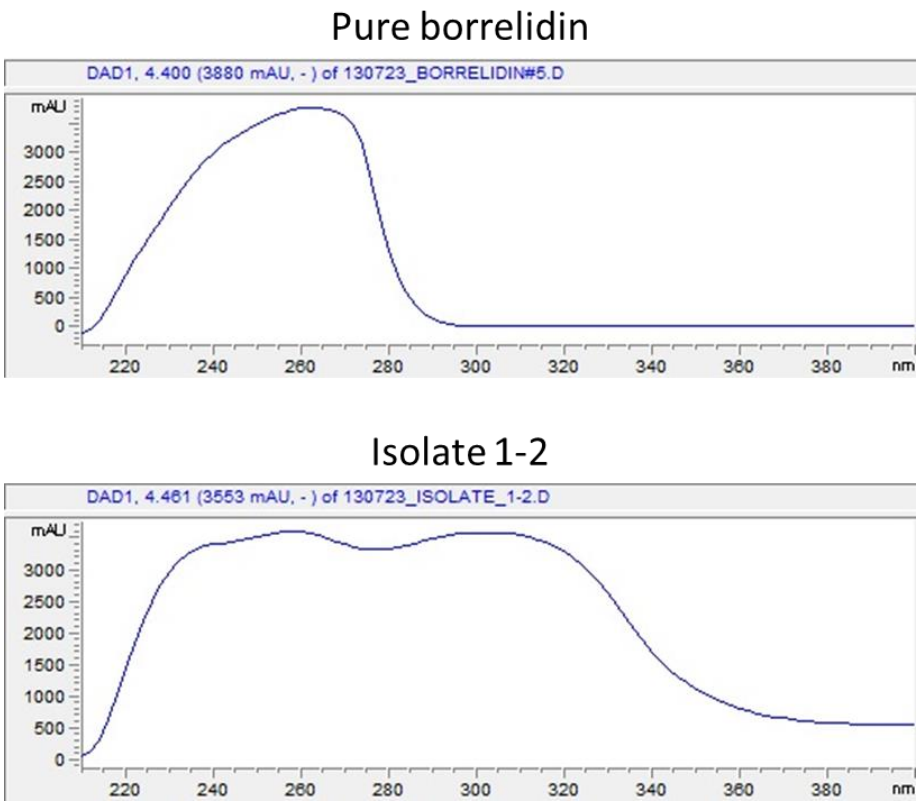

Figure 15: Absorbance spectrum (210 to 400 nm) of the putative borrelidin peak observed in the Isolate 1-2 culture extract. The absorbance spectrum of pure borrelidin is included for comparison. The fact that the two spectra do not match indicates that the compound produced by Isolate 1-2 is not borrelidin.

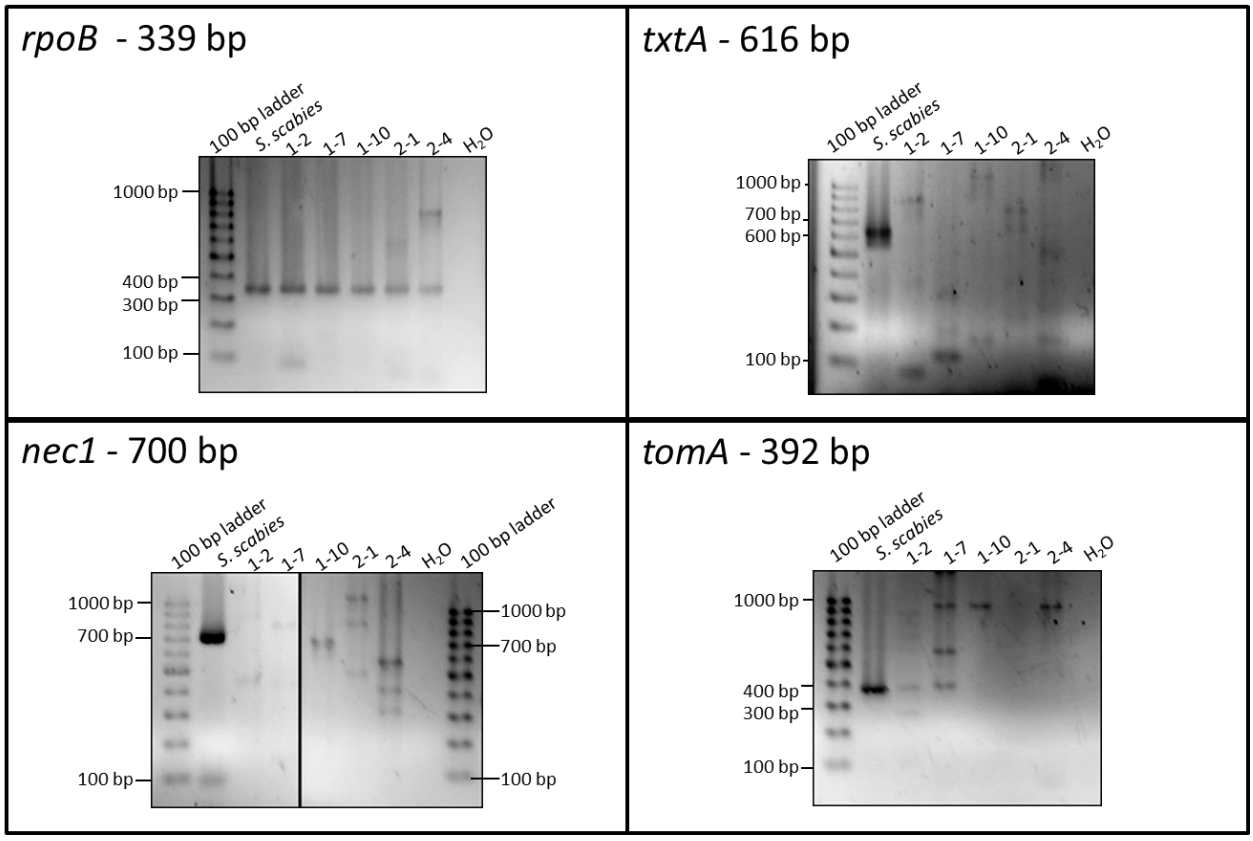

Figure 16: Detection of the known or putative virulence genes *txtA, nec1* and *tomA* in the 2011 pathogenic Newfoundland bacterial isolates. Genomic DNA from each of the isolates was extracted and then used in PCR reactions along with primers specific for each gene. DNA from the known pathogen *S. scabies* 87-22 served as a positive control, while reactions containing water in place of DNA served as a negative control. The expected size of the PCR product for each gene is indicated, and the *rpoB* gene was used as an amplification control.

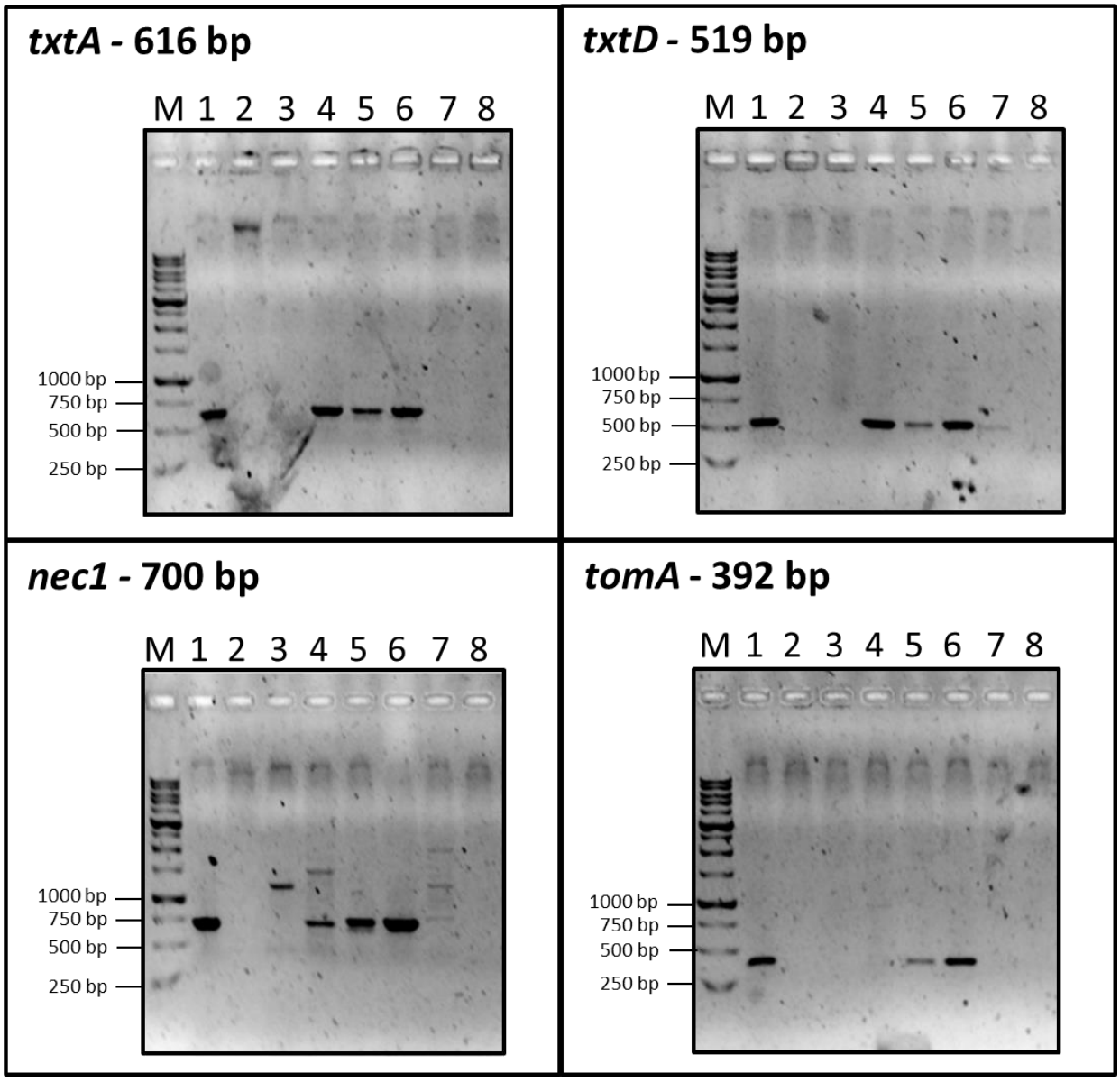

Figure 17: Detection of the known or putative virulence genes *txtA, txtD, nec1* and *tomA* in the 2012 pathogenic Newfoundland bacterial isolates. Genomic DNA from each of the isolates was extracted and then used in PCR reactions along with primers specific for each gene. 1, *S. scabies* 87-22 (positive control); 2, water (negative control); 3, Isolate 2-1 (2011 isolate used as a negative control); 4, Isolate 12-4-1; 5, Isolate 12-11-1; 6, Isolate 12-11-3; 7, Isolate 12-11-5; 8, Isolate 12-5-1. The expected size of each PCR product is indicated. M, 1 kb molecular weight DNA ladder used for product size estimation.

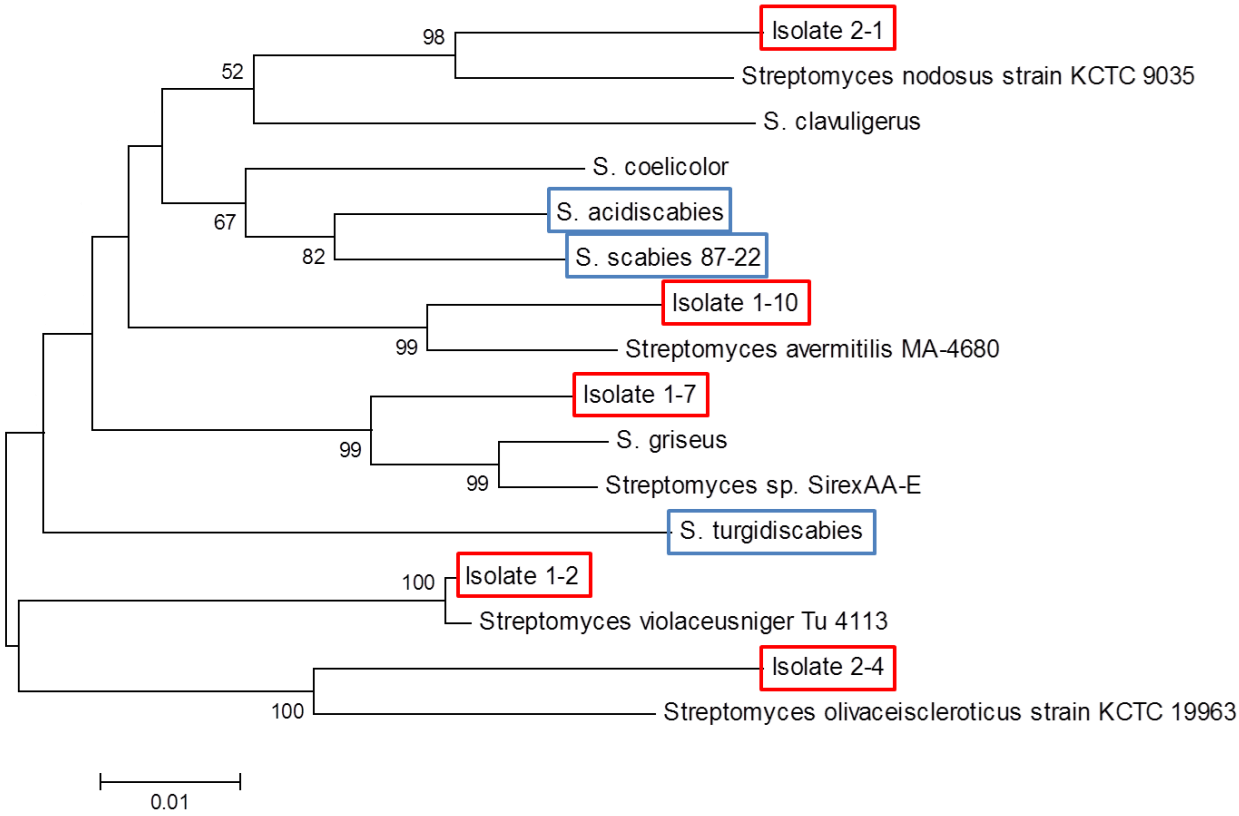

Figure 18: Phylogenetic tree showing the relationships of the 2011 pathogenic bacterial isolates to other *Streptomyces* species. The tree was derived from the variable regions of the *rpoB* gene sequence from each organism and was generated using the MEGA 5.2 software (Tamura et al. 2011) with the neighbour-joining method. Bootstrap values ≥ 50% for 1000 repetitions are indicated. The scale bar indicates the number of nucleotide substitutions per site. The 2011 pathogenic isolates are indicated by the red boxes while known pathogenic *Streptomyces* species are indicated by the blue boxes.

Conclusions and Future Recommendations

The main results obtained from this project can be summarized as follows:

(1) Plant pathogenic *Streptomyces* strains were readily isolated from scab lesions on potatoes harvested in 2011, 2012 and 2013 from different locations in Newfoundland. A total of 19 pathogenic isolates were obtained out of 52 that were tested, and a large variation in the degree of pathogenicity exhibited by these isolates was observed.
(2) Three of the pathogenic isolates from 2012 were shown to produce the main scab-associated virulence factor thaxtomin A, and they were shown to harbour thaxtomin biosynthetic genes as well as the *nec1* virulence gene. Two of the isolates were also found to carry the putative virulence gene *tomA.*
(3) The five pathogenic isolates from 2011 were shown not to produce thaxtomin A, and they did not produce other known *Streptomyces* phytotoxins. Two of the isolates (1-2 and 2-4), were shown to secrete one or more bioactive molecules that are extractable with ethyl acetate and may represent novel phytotoxins.
(4) Only one of the five 2011 pathogenic isolates was found to harbour the *nec1* virulence gene, while two of the isolates were shown to carry the *tomA* gene.
(5) Based on analysis of the nucleotide sequences of the 16S rRNA and *rpoB* genes, it was determined that the five pathogenic isolates from 2011 represent distinct and potentially novel pathogenic *Streptomyces* species.

Overall, the results show that there is a variety of different bacterial strains that are likely contributing to CS disease in Newfoundland, and these strains appear to utilize different virulence mechanisms for causing plant disease. This has implications for the development of new control strategies for CS as such strategies will need to be effective against all of the different CS-causing pathogens that exist in the soils of Newfoundland. In recent years, new scab control strategies have been proposed that are aimed at reducing or eliminating the production of thaxtomin A by pathogenic *Streptomyces* species in soils. However, based on our findings, it is likely that such strategies would have little effect in controlling CS disease in Newfoundland given that there are pathogenic strains here that do not utilize thaxtomin A as a virulence factor.

Although this project can be considered a success in terms of achieving all of the milestones outlined in Table 2 for the 2011 isolates, there is still a significant amount of work that needs to be completed in order to get a complete picture of the microbiology of CS disease in Newfoundland. For example, we still need to finish the characterization of thaxtomin A production and the identification of the other virulence genes in several of the 2012 pathogenic isolates. We also need to complete the identification of the 2012 pathogenic isolates in order to determine whether any are relatives of *S. scabies* or other known pathogenic *Streptomyces* species. Furthermore, all of the 2013 isolates need to be screened for pathogenicity followed by further characterization of those pathogenic isolates that are identified. We also feel that further characterization of the 2011 pathogenic isolates is warranted as these appear to be novel pathogens that may not have been seen elsewhere. Confirmation of their identity, which

will involve morphological and physiological characterization, will be necessary as well as the purification and identification of the phytotoxin(s) produced by the 1-2 and 2-4 strains. Finally, in the age of genomics where it is relatively easy and inexpensive to obtain the complete nucleotide sequence of a bacterial chromosome, we feel that some of the pathogenic isolates obtained in this study will serve as excellent candidates for future genome sequencing so that we can better understand the genetic features that allow these organisms to function as pathogens.

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Communication and Outreach

(i) Peer-Reviewed Publications

Bignell, D.R.D., Fyans, J.K. and Cheng, Z. (2014) Phytotoxins produced by plant pathogenic *Streptomyces* species. Journal of Applied Microbiology 116: 223-235.

- This review article included some of the results obtained for the 2011 pathogenic isolates. A copy of the published article is attached.

(ii) Non-Peer Reviewed Publications

Dixon, B. (2013) Actinobacterial activities. Microbe 8 (9): 342-343.

- This is a scientific commentary that highlighted some of the research that was presented at the 2013 Society for Applied Microbiology meeting in Cardiff, Wales. Among the highlights was the research presented by Dr. Dawn Bignell on the 2011 pathogenic isolates from Newfoundland. A copy of the published article is attached.

(iii) Publications in Preparation

Fyans, J.K. and Bignell, D.R.D. (2014) Characterization of bacterial isolates causing common scab disease in Newfoundland, Canada. PLOS One.

- This publication will include all of the results obtained for the 2011 and 2012 pathogenic isolates. It is anticipated that the completed manuscript will be submitted for per review this coming summer.

(iv) Conference Presentations

(a) Oral Presentations

Bignell, D. Biology of Plant Pathogenic Streptomycetes. Society for Applied Microbiology Summer Meeting. Cardiff, Wales. July 1 – 4, 2013. (182 attendees)

- This was an invited presentation given by Dr. Dawn Bignell on July 4, 2013 at the Society for Applied Microbiology Summer Meeting. The presentation highlighted some of the work that had been completed on the 2011 pathogenic isolates.

Fyans, J.F. and Bignell, D.R. Characterization of *Streptomyces* species causing common scab disease in Newfoundland. Canadian Phytopathological Society Annual Meeting. Edmonton, AB. June 17 – 19, 2013.

- This presentation given by Dr. Joanna Fyans highlighted her work on the 2011 pathogenic isolates.

(b) Poster Presentations

Fyans, J.F. and Bignell, D.R.D. Know your enemy: Insights from plant pathogenic *Streptomyces* species isolated in Newfoundland, Canada. Presidential Meeting for the British Society of Plant Pathology. Birmingham, UK. December 17 – 18, 2013.

- This presentation was given by Dr. Joanna Fyans, and it highlighted her work on the 2011 pathogenic isolates. A copy of the poster presentation is attached.

(v) Other Invited Presentations

Fyans, J.F. Characterization of *Streptomyces* species causing common scab disease in Newfoundland. Department of Biology, Memorial University, St. John's, NL, October 18, 2013.

- This oral presentation was given by Dr. Joanna Fyans as part of the weekly seminar series in the Department of Biology at Memorial University.

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REVIEW ARTICLE

Phytotoxins produced by plant pathogenic *Streptomyces* species

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Keywords
agriculture, pathogenesis, plant diseases, streptomycetes, toxins.

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Summary

Streptomyces is a large genus consisting of soil-dwelling, filamentous bacteria that are best known for their capability of producing a vast array of medically and agriculturally useful secondary metabolites. In addition, a small number of *Streptomyces* spp. are capable of colonizing and infecting the underground portions of living plants and causing economically important crop diseases such as potato common scab (CS). Research into the mechanisms of *Streptomyces* plant pathogenicity has led to the identification and characterization of several phytotoxic secondary metabolites that are known or suspected of contributing to diseases in various plants. The best characterized are the thaxtomin phytotoxins, which play a critical role in the development of CS, acid scab and soil rot of sweet potato. In addition, the best-characterized CS-causing pathogen, *Streptomyces scabies*, produces a molecule that is predicted to resemble the *Pseudomonas syringae* coronatine phytotoxin and which contributes to seedling disease symptom development. Other *Streptomyces* phytotoxic secondary metabolites that have been identified include concanamycins, FD-891 and borrelidin. Furthermore, there is evidence that additional, unknown metabolites may participate in *Streptomyces* plant pathogenicity. Such revelations have implications for the rational development of better management procedures for controlling CS and other *Streptomyces* plant diseases.

Introduction

Organisms belonging to the genus *Streptomyces* are well known for their filamentous morphology, their large genomes and their complex developmental life cycle that involves the production of desiccation-resistant spores. The vast majority of *Streptomyces* spp. are soil-dwelling saprophytes that degrade recalcitrant biological polymers and contribute to the recycling of nutrients in the environment. Furthermore, these organisms are renowned for their ability to synthesize a wide array of medically and agriculturally useful secondary metabolites such as antibiotics, immunosuppressants, anti-tumour agents, insecticides and pesticides (Berdy 2005). Such compounds may provide a selective advantage to the producing organism by allowing it to compete with other micro-organisms for limited nutrients in the soil environment, and/or they may serve as facilitators of inter- and intra-generic communication (O'Brien and Wright 2011). In addition, some secondary metabolites are thought to promote symbiotic relationships between *Streptomyces* spp. and eukaryotic organisms (Seipke *et al.* 2012). An example of this is the involvement of secondary metabolites in parasitic relationships between plant pathogenic *Streptomyces* spp. and various plant hosts, a subject that is the focus of this review.

The ability to colonize living plant tissues and to cause plant diseases is a rare trait among the streptomycetes. Species that have this ability infect the underground portions of a wide variety of economically important crops, while above-ground parts of the plant will generally remain healthy unless nutrient and water transport between the roots and the shoots is hindered by the infection (Dees and Wanner 2012). The most important

host that is affected by plant pathogenic streptomycetes is potato (*Solanum tuberosum*), and as such most of the research to date has focused on the diseases affecting this crop. However, those species causing scab disease of potato are neither tissue—nor host—specific and can infect potato as well as tap root crops such as carrot, beet, radish and parsnip under field conditions (Dees and Wanner 2012). Furthermore, such species can infect the seedlings of a variety of monocot and dicot plants under controlled conditions, leading to root and shoot stunting, cell hypertrophy and tissue necrosis (Leiner *et al.* 1996).

Potato common scab (CS) is considered the most important disease caused by *Streptomyces* spp. and is characterized by the formation of superficial, raised or pitted lesions on the surface of potato tubers (Loria *et al.* 1997). Such lesions reduce the market value of the potato crop and result in significant economic losses to growers. Several *Streptomyces* spp. are responsible for the disease (Table 1), of which *S. scabies* (syn. *S. scabiei*) was the first to be described and is the best-characterized and most widely distributed species. In addition to CS, *S. scabies* is responsible for pod wart of peanut, which is characterized by raised necrotic lesions on the peanut pericarp (Loria *et al.* 1997). Another disease, called acid scab (AS), is caused by *Streptomyces acidiscabies* and results in the same symptoms as CS except that the disease occurs in acid soils where CS is normally suppressed (Loria *et al.* 2006). Netted scab (NS) is a potato disease that has been reported mainly in Europe and is characterized by the formation of brown, superficial lesions with a netted appearance on the tuber surface. Unlike CS and AS, NS also causes severe necrosis of the fibrous roots of the potato plant and results in significant yield losses (Loria *et al.* 1997). Russet scab (RS) is similar to NS in that the lesions on the potato are superficial and are limited to the tuber periderm. However, the lesions do not have the netted pattern that is characteristic of NS, and root necrosis and yield losses have not been reported with this disease (Loria *et al.* 1997). Soil rot of sweet potato is caused by *Streptomyces ipomoeae,* which infects the fibrous roots of sweet potato (*Ipomoea batatas* (L.) Lam.), leading to tissue necrosis and death, and subsequent yield losses. Furthermore, the pathogen induces necrotic lesions on the fleshy storage roots, resulting in reduced marketability (Loria *et al.* 1997).

This review focuses on the recent progress of research into the phytotoxic secondary metabolites that contribute to *Streptomyces*—plant interactions and to the development of plant diseases. Much of the discussion will focus on the thaxtomin phytotoxins, which play a critical role in the development of CS, AS and soil rot of sweet potato; however, recent research has suggested that additional phytotoxic secondary metabolites may also contribute to the development of these and other plant diseases in natural settings, and therefore such phytotoxins will also be addressed here.

Table 1 Pathogenic *Streptomyces* spp. and associated plant disease(s) and phytotoxin(s) produced

Species	Disease(s) caused*	Phytotoxin(s) produced	Reference(s)
S. scabies (*S. scabiei*)	CS, Pod wart of peanut	Thaxtomins, concanamycin A and B, COR-like metabolite(s)	King *et al.* (1989, 1992); Natsume *et al.* (1996, 1998, 2001); Bignell *et al.* (2010b)
Streptomyces turgidiscabies	CS	Thaxtomins	Bukhalid *et al.* (1998)
Streptomyces acidiscabies	AS	Thaxtomins	Bukhalid *et al.* (1998)
Streptomyces europaeiscabiei	CS, NS	Thaxtomins	Loria *et al.* (2006)
Streptomyces reticuliscabiei	NS	Unknown	Bouchek-Mechiche *et al.* (2000)
Streptomyces stelliscabiei	CS	Thaxtomins	Loria *et al.* (2006)
Streptomyces luridiscabiei	CS	Unknown	Park *et al.* (2003)
Streptomyces niveiscabiei	CS	Unknown	Park *et al.* (2003)
Streptomyces puniciscabiei	CS	Unknown	Park *et al.* (2003)
Streptomyces spp. IdahoX	CS	Thaxtomins	Wanner (2007)
Streptomyces spp. DS3024	CS	Thaxtomins	Hao *et al.* (2009)
Streptomyces spp. GK18	CS	Borrelidin	Cao *et al.* (2012)
Streptomyces cheloniumii	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225003	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225004	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225005	RS	FD-891	Natsume *et al.* (2005)
Streptomyces spp. MAFF225006	RS	FD-891	Natsume *et al.* (2005)
Streptomyces ipomoeae	Soil rot of sweet potato	Thaxtomins	King *et al.* (1994); Guan *et al.* (2012)

*CS, Common scab; AS, acid scab; NS, netted scab; RS, russet scab.

Thaxtomins

The first phytotoxic secondary metabolites associated with *Streptomyces* plant pathogenicity were reported in 1989 by King and colleagues (King *et al.* 1989), who described the isolation of two members of the thaxtomin family of phytotoxins associated with CS disease. Thaxtomins are cyclic dipeptides (2,5-diketopiperazines) derived from the condensation of L-phenylalanine and L-4-nitrotryptophan moieties (reviewed in King and Calhoun 2009). Eleven members of the thaxtomin family have been identified and characterized, with each member differing only in the presence or absence of hydroxyl and *N*-methyl groups at specific sites (King and Calhoun 2009). The 4-nitro moiety, together with the L,L configuration of the tryptophan and phenylalanine groups, have been shown to be essential for the phytotoxic activity of these compounds (King *et al.* 1989, 1992). Thaxtomin A (Fig. 1a) is the primary family member produced by *S. scabies, S. acidiscabies* and *Streptomyces turgidiscabies,* although other family members have been shown to be produced in minor amounts (King and Calhoun 2009). Thaxtomin C (Fig. 1a), which is a less modified, nonhydroxylated family member, is the major product synthesized by *S. ipomoeae* (King *et al.* 1994; Guan *et al.* 2012).

Biological activity of thaxtomins

Thaxtomins have the ability to cause necrosis on excised potato tuber tissue (Loria *et al.* 2006), and they can induce scab-like lesions on aseptically cultured minitubers (Lawrence *et al.* 1990). In addition, nanomolar concentrations of thaxtomin A cause root and shoot stunting and radial swelling of monocot and dicot seedlings, effects that mimic the seedling disease symptoms caused by *S. scabies* and *S. acidiscabies* (Leiner *et al.* 1996; Loria *et al.* 1997). A positive correlation has been observed between the ability to produce thaxtomin A and the pathogenicity of scab-causing *Streptomyces* spp. (King *et al.* 1991; Loria *et al.* 1995; Goyer *et al.* 1998; Kinkel *et al.* 1998), and a constructed thaxtomin mutant of *S. acidiscabies* could not induce typical scab lesions on potato minitubers (Healy *et al.* 2000). Recently, it was shown that *S. ipomoeae* thaxtomin C mutants are unable to penetrate the intact adventitious roots of sweet potato plants (Guan *et al.* 2012). Thus, the thaxtomin phytotoxins are an essential

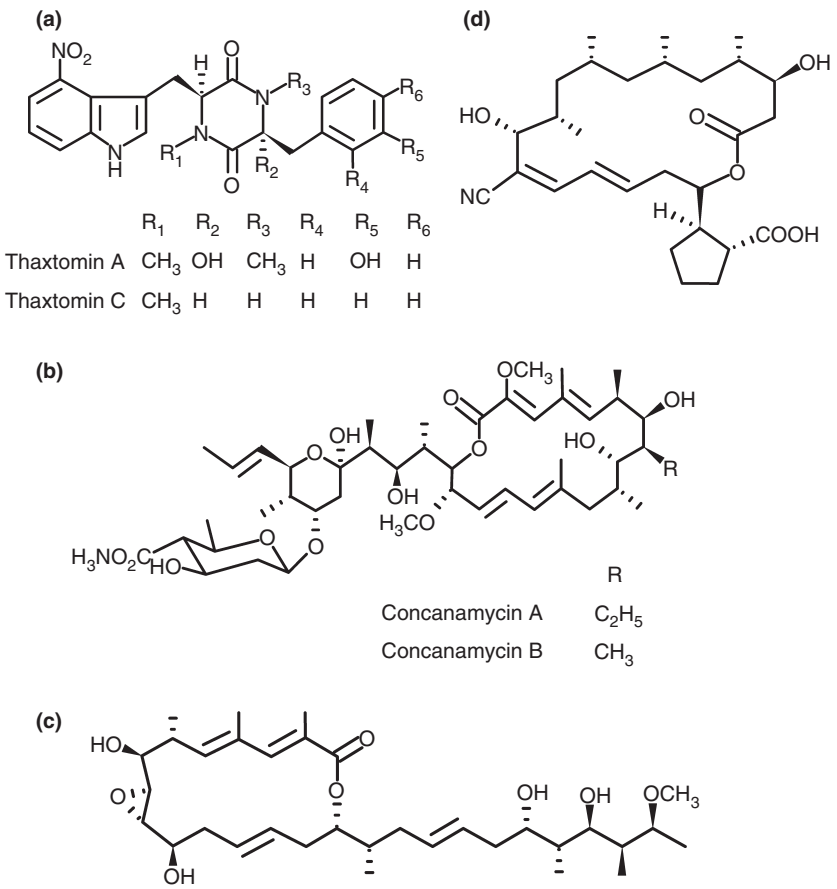

Figure 1 Molecular structure of the thaxtomin A and C (a), concanamycin A and B (b), FD-891 (c) and borrelidin (d) phytotoxins that are produced by plant pathogenic *Streptomyces* spp.

virulence factor in several plant pathogenic *Streptomyces* spp.

A number of physiological effects in plants have been reported to occur in response to thaxtomins, including alterations in plant Ca^{2+} and H^+ ion influx, induction of programmed cell death, and production of the antimicrobial plant phytoalexin scopoletin (Duval *et al.* 2005; Tegg *et al.* 2005; Errakhi *et al.* 2008; Lerat *et al.* 2009). Fry and Loria noted that nanomolar concentrations of thaxtomin A cause plant cell hypertrophy in onion seedling hypocotyls, radish seedling hypocotyls and tobacco suspension cultures (Fry and Loria 2002). It also interferes with cytokinesis in onion root tip cells, and it inhibits normal cell elongation of tobacco protoplasts (Fry and Loria 2002). This, in turn, led the authors to propose that thaxtomin A targets the plant cell wall. Further evidence for a cell wall target was provided by Scheible *et al.*, who demonstrated that thaxtomin A inhibits the incorporation of ^{14}C-glucose into the cellulosic fraction of the cell wall in *Arabidopsis thaliana* (Scheible *et al.* 2003). More recently, Bischoff *et al.* showed that thaxtomin A reduces the crystalline cellulose content of *A. thaliana* plant cell walls, and it affects the expression of cell wall synthesis genes in a similar manner as the known cellulose synthesis inhibitor isoxaben. Furthermore, spinning disc confocal microscopy revealed that thaxtomin A depletes cellulose synthase complexes from *A. thaliana* plasma membranes (Bischoff *et al.* 2009). Duval and Beaudoin used whole genome microarrays to show that thaxtomin A and isoxaben elicit a similar gene expression profile in *A. thaliana* cell suspensions (Duval and Beaudoin 2009). Taken together, the results suggest that the primary mode of action of thaxtomin A is the inhibition of cellulose biosynthesis.

Biosynthesis of the thaxtomin phytotoxins

As with other *Streptomyces* secondary metabolites, the biosynthetic genes for the thaxtomin phytotoxins (*txt*) are clustered together on the chromosome of *S. scabies, S. turgidiscabies, S. acidiscabies* and *S. ipomoeae* (Loria *et al.* 2008; Guan *et al.* 2012). The genes are arranged in at least two operons, with the first likely consisting of *txtA, txtB, txtH* and possibly *txtC,* and the second consisting of *nos*/*txtD* and *txtE,* the co-transcription of which has been confirmed (Barry *et al.* 2012). Analysis of the encoded protein products indicates a high degree of conservation among the four distantly related species, although it is apparent that the conservation is considerably higher among the scab-causing pathogens (Table 2). This, together with the localization of the *txt* gene cluster on a mobile pathogenicity island in *S. turgidiscabies* (Kers *et al.* 2005; Huguet-Tapia *et al.* 2011), suggests that horizontal gene transfer likely played a role in the acquisition of the *txt* gene cluster by *Streptomyces* spp.

The biosynthesis of the thaxtomin phytotoxins begins with the production of nitric oxide (NO) from arginine, a reaction that is catalysed by the TxtD nitric oxide synthase (Kers *et al.* 2004). NO is then used for the site-specific nitration of L-tryptophan by TxtE, which is a novel cytochrome P450 (Barry *et al.* 2012). Deletion analysis of the *txtE* gene in *S. turgidiscabies* confirmed that it is essential for thaxtomin A biosynthesis, while addition of L-4-nitrotryptophan to cultures of the Δ*txtE* strain restored thaxtomin A production (Barry *et al.* 2012). This, together with the fact that L-4-nitrotryptophan accumulates in cultures of the *S. scabies txtA* and *txtB* mutants (Johnson *et al.* 2009), indicates that the production of L-4-nitrotryptophan is the first committed step in the thaxtomin biosynthetic pathway. L-4-nitrotryptophan then serves as a substrate for the TxtB nonribosomal peptide synthetase (NRPS), while L-phenylalanine is the substrate for the TxtA NRPS (Johnson *et al.* 2009). In the case of thaxtomin A biosynthesis, the resulting cyclo-(L-4-nitrotryptophyl-L-phenylalanyl) intermediate (called thaxtomin D) is *N*-methylated on both the nitrotryptophyl and phenylalanyl moieties (Healy *et al.* 2002), and it is presumed that the methylation is

Table 2 Proteins encoded by the *S. scabies* thaxtomin biosynthetic gene cluster and their % identity/similarity to homologues in other plant pathogenic *Streptomyces* spp.

Txt Proteins from *Streptomyces scabies* 87-22	Function	% Identity/similarity to *Streptomyces turgidiscabies* Car8 Txt homologues	% Identity/similarity to *Streptomyces acidiscabies* 84.104 Txt homologues	% Identity/similarity to *Streptomyces ipomoeae* 91-03 Txt homologues
TxtA	Synthesis of thaxtomin backbone	90/93	100/100	50/60
TxtB	Synthesis of thaxtomin backbone	90/93	99/99	60/70
TxtC	Hydroxylation of thaxtomin backbone	90/93	100/100	Absent
TxtD	Nitration of L-tryptophan precursor	91/93	100/100	75/83
TxtE	Nitration of L-tryptophan precursor	88/94	100/100	85/91
TxtH	Unknown	81/85	100/100	63/69
TxtR	Regulation of thaxtomin biosynthesis	78/83	100/100	42/58

catalysed by the *S*-adenosylmethionine-dependent *N*-methyltransferase domain found in both TxtA and TxtB. It has previously been reported that *N*-methyl-L-4-nitrotryptophan can accumulate in the culture supernatants of wild-type *S. scabies* (King and Lawrence 1995) and of a *S. scabies* Δ*txtA* mutant (Johnson *et al.* 2009), which suggests that the *N*-methylation occurs prior to cyclic dipeptide formation. Interestingly, the *S. ipomoeae* TxtAB homologues are also predicted to each contain an *N*-methyltransferase domain, and yet thaxtomin C is only *N*-methylated on the nitrotryptophanyl moiety (Fig. 1a). The final step in thaxtomin A biosynthesis is the addition of hydroxyl groups to the phenylalanyl moiety of thaxtomin D by the TxtC P450 monooxygenase. Deletion analysis of *txtC* in *S. acidiscabies* led to the accumulation of thaxtomin D in the culture supernatant, confirming the role of TxtC in postcyclization hydroxylation (Healy *et al.* 2002). Notably, *txtC* is absent from the *S. ipomoeae txt* gene cluster, and no homologue appears to exist anywhere else in the *S. ipomoeae* genome (Bignell *et al.* 2010a; Guan *et al.* 2012), an observation that is consistent with the fact that this organism does not produce thaxtomin A (King *et al.* 1994).

An additional gene (*txtH*) that was recently identified in the thaxtomin biosynthetic gene cluster of *S. scabies* (Bignell *et al.* 2010a) is predicted to encode a member of the MbtH-like protein superfamily. MbtH-like proteins are small proteins (normally 62–80 amino acids) that are often associated with NRPS gene clusters (reviewed in Baltz 2011). Deletion studies have shown that some MbtH-like proteins are necessary for production of the corresponding metabolite, whereas in other instances, deletion of the MbtH-like protein-encoding gene does not have any effect. The latter is often due to the presence of other MbtH-like protein-encoding genes elsewhere in the genome that can cross complement the deleted gene with varying efficiencies. Recent biochemical studies have shown that some MbtH-like proteins can be co-purified with their cognate NRPS and that they function to facilitate the adenylation reaction catalysed by the NRPS adenylation domain (Baltz 2011 and references therein). The *S. scabies txtH* gene is conserved in the *txt* gene clusters of *S. turgidiscabies, S. acidiscabies* and *S. ipomoeae,* suggesting that it may be important for the biosynthesis of thaxtomins (Table 2). However, it is noteworthy that the genome sequences for all four pathogens contain multiple predicted MbtH-like protein-encoding genes, and therefore the possibility exists for cross-complementation to occur in each organism.

Regulation of thaxtomin biosynthesis

The production of thaxtomin A by scab-causing streptomycetes is affected by several physiological and environmental signals. For example, production does not take place in common microbiological growth media such as LB and tryptic soy broth (Loria *et al.* 1995), whereas it readily occurs in living host tissue or in plant-based media such as potato broth, oatmeal broth or oat bran broth (Babcock *et al.* 1993; Loria *et al.* 1995; King and Lawrence 1996; Goyer *et al.* 1998). Glucose appears to repress the biosynthesis of thaxtomin in liquid growth media (Babcock *et al.* 1993; Loria *et al.* 1995), a phenomenon that has been reported for other *Streptomyces* secondary metabolites (Ruiz *et al.* 2010). Aromatic amino acids such as tryptophan, tyrosine and phenylalanine have also been shown to inhibit phytotoxin biosynthesis, whereas aliphatic amino acids have no effect (Babcock *et al.* 1993; Lauzier *et al.* 2002).

Recent work has identified specific plant-based compounds that are capable of stimulating thaxtomin A biosynthesis. Wach *et al.* (2007) demonstrated that the addition of xylans, glucans and cellobiose to oat bran broth medium stimulates higher levels of thaxtomin A production in *S. acidiscabies* compared to the unamended control, while Johnson *et al.* (2007) showed that the cellobiose and cellotriose could stimulate *txt* gene expression and phytotoxin production in a defined minimal medium. Moreover, suberin, which is a complex plant polymer found on the surface of potato tubers, has been shown to stimulate phytotoxin production in a minimal medium (Beausejour *et al.* 1999), and more recently it was demonstrated that the addition of both suberin and cellobiose to a minimal medium stimulates much higher *txt* gene expression and thaxtomin A production then when cellobiose or suberin are added separately (Lerat *et al.* 2010).

Embedded within the *txt* gene clusters of *S. scabies, S. turgidiscabies* and *S. acidiscabies* is a gene (*txtR*) that encodes an AraC-family transcriptional regulator (Table 2; Joshi *et al.* 2007). Given that regulatory genes are often associated with secondary metabolite biosynthetic gene clusters and that they function to control the production of the corresponding metabolite (van Wezel and McDowall 2011), it was hypothesized that TxtR likely serves as a regulator of thaxtomin biosynthesis in these organisms. This was confirmed by constructing a *S. scabies* Δ*txtR* mutant and showing that it produced only trace levels of thaxtomin A, was reduced in expression of the thaxtomin biosynthetic genes, and was avirulent on tobacco and radish seedlings (Joshi *et al.* 2007; Loria *et al.* 2008). Interestingly, the expression of the *txtR* gene in *S. scabies* and *S. turgidiscabies* was shown to be dependent on cellobiose (Johnson *et al.* 2007; Joshi *et al.* 2007), and cellobiose was demonstrated to serve as a ligand for the *S. scabies* TxtR protein in a pull-down assay (Joshi *et al.* 2007). Given that thaxtomin A targets

cellulose biosynthesis and that cellobiose is the smallest subunit of cellulose, it has been proposed that cellobiose and possibly other cello-oligosaccharides may serve as a signal for the presence of active plant cell growth and tissue expansion where cellulose synthesis takes place, and that stimulation of thaxtomin A production by cellobiose may allow penetration of the expanding tissue by the pathogen (Loria *et al.* 2008). Whether suberin or breakdown products of suberin also serve as signals that are sensed by TxtR remains to be determined; however, as it was recently shown that suberin induces the onset of morphological differentiation and secondary metabolism in both pathogenic and nonpathogenic streptomycetes (Lerat *et al.* 2012), it is likely that the effect of suberin is not specific to the thaxtomin phytotoxins.

Recently, a *txtR* homologue was reported in the *txt* gene cluster of *S. ipomoeae* (Guan *et al.* 2012). The resulting protein product shows only weak similarity to the TxtR protein from *S. scabies* (Table 2), which might reflect differences in the regulation of thaxtomin production in the scab-causing pathogens and in *S. ipomoeae*. Specifically, thaxtomin C in *S. ipomoeae* is not produced in the same plant-based media that induce thaxtomin A production (King *et al.* 1994; Guan *et al.* 2012), which suggests that cello-oligosaccharides do not function as inducers of thaxtomin C production. The exact ligand(s) that interacts with the *S. ipomoeae* TxtR remains to be determined, but it is intriguing to speculate that the ligand(s) is a plant-derived molecule that is specific to the *Convolvulaceae* family, and that this might account for the observed narrow host range of *S. ipomoeae* as compared to the scab-causing pathogens (Guan *et al.* 2012).

Concanamycins

In addition to thaxtomins, *S. scabies* has been reported to produce two members of the concanamycin family of secondary metabolites (Table 1). Concanamycins are polyketide macrolides that were first isolated from the culture medium of *S. diastatochromogenes* (Kinashi *et al.* 1984). They are characterized by an 18-membered tetraenic macrolide ring with a methyl enol ether and a β-hydroxyhemiacetyl side chain (Fig. 1b), and they function as vacuolar-type ATPase inhibitors and exhibit antifungal and anti-neoplastic activity but not antibacterial activity (Kinashi *et al.* 1984; Seki-Asano *et al.* 1994). Natsume and colleagues were the first to report the isolation of *S. scabies* strains from Japan that produced concanamycin A and B, and rice seedling bioassays demonstrated that the pure compounds exhibit root growth inhibitory activity (Natsume *et al.* 1996, 1998). The genome sequence of *S. scabies* 87–22 contains a biosynthetic gene cluster that is highly similar to the concanamycin biosynthetic gene cluster from *Streptomyces neyagawaensis* (Haydock *et al.* 2005), suggesting that this strain of *S. scabies* also produces concanamycins. The contribution of concanamycins to CS disease needs further clarification given that other characterized CS pathogens do not appear to produce these compounds (Natsume *et al.* 1998, 2001, 2005).

COR-like metabolites

Genome sequencing of *S. scabies* strain 87–22 revealed the presence of a biosynthetic gene cluster that is highly similar to the coronafacic acid (CFA) biosynthetic gene cluster from the Gram-negative plant pathogens *Pseudomonas syringae* and *Pectobacterium atrosepticum* (Bignell *et al.* 2010b). CFA (Fig. 2a) is the polyketide component of coronatine (COR) (Fig. 2b), which is a nonhost specific phytotoxin produced by different pathovars of *Ps. syringae* (Gross and Loper 2009). The COR molecule consists of CFA linked via an amide bond to an

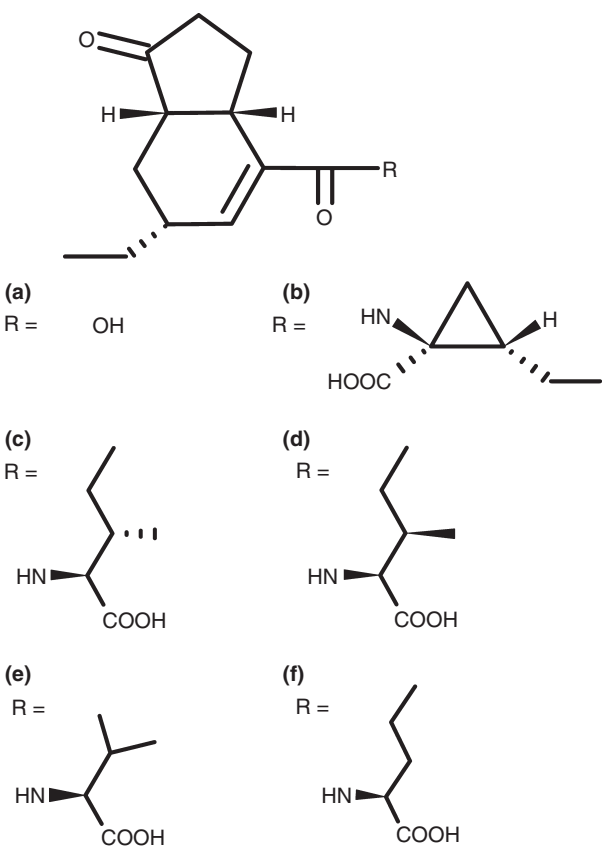

Figure 2 Molecular structure of coronafacic acid (CFA) (a), coronatine (COR) (b), CFA-isoleucine (c), CFA-*allo*-isoleucine (d), CFA-valine (e) and CFA-norvaline (f) produced by *Pseudomonas syringae*.

ethylcyclopropyl amino acid called coronamic acid (CMA), which is derived from L-*allo*-isoleucine (Gross and Loper 2009). Although COR is the primary metabolite produced by *Ps. syringae* and is the most toxic, other coronafacoyl compounds in which CFA is linked to various amino acids have been reported, including CFA-isoleucine, CFA-*allo*-isoleucine, CFA-valine and CFA-norvaline (Fig. 2c–f; Bender *et al.* 1999).

The CFA-like biosynthetic gene cluster identified in *S. scabies* 87–22 consists of at least 15 genes, nine of which are homologous to genes from the CFA biosynthetic gene clusters of *Ps. syringae* pv. *tomato* and *P. atrosepticum* (Bignell *et al.* 2010b). These include the *cfa1-5* genes that encode enzymes believed to synthesize the 2-carboxy-2-cyclopentenone intermediate (CPC), as well as the *cfa6* and *cfa7* genes, which encode the large multidomain polyketide synthases (PKSs) that generate the CFA backbone from CPC (Rangaswamy *et al.* 1998). In addition, the *cfl* gene, which in *Ps. syringae* encodes an enzyme that is believed to catalyse the adenylation of CFA and the ligation of the CFA adenylate to CMA (Bender *et al.* 1999), is also conserved in *S. scabies.* Although *S. scabies* is unable to produce COR due to the absence of the CMA biosynthetic genes in the genome (Bignell *et al.* 2010b), it is likely that this organism produces one or more COR-like metabolites that are similar to the minor coronafacoyl compounds that are generated by *Ps. syringae* (Fig. 2c–f).

It is interesting to note that there are six genes within the *S. scabies* CFA-like biosynthetic gene cluster that are absent from the *Ps. syringae* and *P. atrosepticum* CFA biosynthetic gene clusters, and at least three of these genes are predicted to encode enzymes that could potentially modify the CFA polyketide backbone (Bignell *et al.* 2010b). Furthermore, the *S. scabies* Cfa7 enzyme contains an enoyl reductase domain that is absent from the Cfa7 homologues in *Ps. syringae* and *P. atrosepticum* (Bignell *et al.* 2010b), and if active, this domain would presumably reduce the carbon double bond that is present in CFA (Fig. 2b). Purification and structural analysis of the COR-like metabolite is currently ongoing within our laboratory, and this will provide insight into whether the molecule is novel in structure as compared to COR and the COR analogues produced by *Ps. syringae.*

Bioactivity of the *S. scabies* COR-like metabolite

Gene deletion studies in *S. scabies* have demonstrated that the COR-like metabolite contributes to the development of root disease symptoms in tobacco seedlings (Bignell *et al.* 2010a,b), and this correlates with the observed role of COR as an important contributor of disease symptom development during *Ps. syringae* infections (Xin and He 2013). Whether the COR-like metabolite also influences the severity of CS disease symptoms has not been determined, but is something that does warrant further investigation. However, it is likely that the metabolite is not required for CS disease development as other CS pathogens do not appear to produce it (Bignell *et al.* 2010b). It is noteworthy that the metabolite can cause hypertrophy of potato tuber tissue in a similar manner as COR (Fig. 3), suggesting that it may share the same target(s) in the plant host. It has been determined that COR functions as a molecular mimic of jasmonoyl—isoleucine (JA-Ile), which is the active form of the jasmonic acid (JA) plant hormone (Katsir *et al.* 2008a,b). JA-Ile controls the expression of genes involved in plant growth, development and defence against herbivores and necrotrophic pathogens (Browse and Howe 2008). When JA-responsive genes are activated, this leads to suppression of salicylic acid (SA)—mediated defence pathways, which are important for defence against biotrophic pathogens such as *Ps. syringae* (Koornneef and Pieterse 2008). Thus by functioning as a molecular mimic of JA-Ile, COR suppresses the plant defence response that is most important for combating infection by *Ps. syringae.* It is possible that the *S. scabies* COR-like metabolite also functions in a similar manner to allow the pathogen to overcome the host immune response, an idea that is currently under investigation in our laboratory.

Regulation of COR-like metabolite production in *S. scabies*

Embedded within the CFA-like biosynthetic gene cluster in *S. scabies* is a gene (*scab79591;* referred to herein as *cfaR*) that was previously shown to modulate the expression of the biosynthetic genes within the cluster (Bignell *et al.* 2010b). The encoded protein belongs to a novel family of transcriptional regulators that are only found in actinobacteria and are characterized by a C-terminal LuxR- family DNA-binding domain and an N-terminal

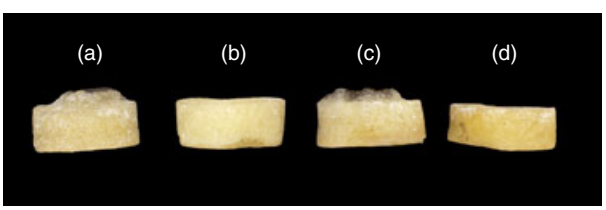

Figure 3 Induction of potato tissue hypertrophy by the *S. scabies* coronatine (COR)-like metabolite. Potato tuber disks were treated with COR (250 ng) (a) or with culture extract from a *S. scabies* COR-like metabolite-producing strain (c). Control treatments included 100% methanol (b) and culture extract from a *S. scabies* COR-like metabolite nonproducing strain (d).

PAS fold domain. The best-characterized member of this family is PimM, which controls the production of the polyene macrolide pimaricin in *Streptomyces natalensis.* PimM is required for expression of the pimaricin biosynthetic genes and for pimaricin production (Anton *et al.* 2007), and it has been shown to directly bind eight promoter regions within the biosynthetic gene cluster (Santos-Aberturas *et al.* 2011b). In addition, a Δ*pimM* mutant can be complemented by other closely related members of the PAS-LuxR family such as *amphRIV, nysRIV* and *pteF,* which are associated with the amphotericin, nystatin and filipin polyene macrolide biosynthetic clusters, respectively, and heterologous expression of *pimM* can enhance the biosynthesis of amphotericin and filipin in the respective producing organisms (Santos-Aberturas *et al.* 2011a). Together, this suggests that there is functional conservation among these members of the PAS-LuxR protein family. Genetic studies have shown that CfaR functions as a positive activator of gene expression in the CFA-like gene cluster (Bignell *et al.* 2010b), and electrophoretic mobility shift assays have confirmed that the protein directly binds to DNA within the cluster (Z. Cheng, unpublished data). It is currently not clear how the DNA binding activity of CfaR is regulated, although this is presumed to somehow involve the associated PAS domain. Interestingly, phylogenetic analysis suggests that CfaR may represent a novel member of the PAS-LuxR family as the protein does not appear to cluster with other family members in the database (Fig. 4).

FD-891

Streptomyces cheloniumii (Table 1) is a new species of *Streptomyces* that was isolated in Japan and causes RS but not CS on potato tubers (Oniki *et al.* 1986). In 2005, Natsume and colleagues reported the isolation of a new phytotoxin produced by *S. cheloniumii* and four other *Streptomyces* strains isolated from Japan (Natsume *et al.* 2005). Bioassays indicated that the phytotoxic compound induces necrosis of potato tuber tissue and causes stunting of rice and alfalfa seedlings, indicating that like thaxtomin A, it is a nonspecific phytotoxin. Purification and structural analysis of the phytotoxic compound identified it as the 16-membered macrolide FD-891 (Fig. 1c; Seki-Asano *et al.* 1994; Eguchi *et al.* 2004). FD-891 was previously reported to have cytocidal activity against animal cells (Seki-Asano *et al.* 1994), and the report by Natsume *et al.* is the first to describe its phytotoxicity (Natsume *et al.* 2005). Although FD-891 has a similar structure to the concanamycins (Fig. 1), the mode of action of the two types of metabolites appears to be different (Kataoka *et al.* 2000). It is currently not clear whether other RS-causing pathogens from other parts of the world also produce FD-891, and the contribution of FD-891 to RS disease symptom development also remains to be determined.

Borrelidin

Recently, a new pathogenic strain of *Streptomyces* was isolated from a scab lesion on a potato grown in Iran (Cao *et al.* 2012). The strain (GK18) was shown to induce deep pitted lesions on potato tubers rather than the raised lesions that are typically caused by *S. scabies* and other thaxtomin-producing species, and it also caused severe stunting of potato plants grown in pots. Interestingly, the authors could not detect thaxtomin A production by this strain, nor could they detect the *txtA* gene using Southern analysis. Instead, the strain was shown to produce the 18-membered polyketide macrolide borrelidin (Fig. 1d), which was first identified as an antibacterial antibiotic produced by *Streptomyces rochei* (Berger *et al.* 1949). Southern analysis confirmed that strain GK18 contains genes involved in the biosynthesis of borrelidin, and bioassays using potato tuber slices and radish seedlings demonstrated that the borrelidin purified from GK18 culture extracts exhibits phytotoxic activity. Interestingly, borrelidin was reported to cause deep, black holes on the potato tuber slices, an effect that is reminiscent of the disease symptoms caused by *Streptomyces* spp. GK18 on mini tubers. Thaxtomin A, on the other hand, produced more shallow, brown lesions on the potato tuber slices. Thus, it appears as though different *Streptomyces* phytotoxins can contribute to the production of distinct types of scab symptoms on potato tubers, and that production of different phytotoxins by different pathogenic streptomycetes might explain in some instances why there are several types of disease symptoms associated with CS disease in natural settings.

Borrelidin has been shown to exhibit anti-bacterial, antiviral, anti-malarial and anti-angiogenic activity (Dickinson *et al.* 1965; Wakabayashi *et al.* 1997; Otoguro *et al.* 2003); however, the report by Cao and colleagues is the first to demonstrate that this metabolite also exhibits phytotoxic activity (Cao *et al.* 2012). Furthermore, the report supports previous findings (Park *et al.* 2003; Wanner 2004) that some CS-causing streptomycetes do not produce thaxtomin A. It is noteworthy that Cao and colleagues were able to isolate 17 additional CS-causing streptomycetes, none of which produced thaxtomin A or borrelidin (Cao *et al.* 2012). Furthermore, research in our own laboratory has led to the isolation of two *Streptomyces* strains from Newfoundland, Canada that are pathogenic on radish seedlings (Fig. 5) and on potato tuber disks (data not shown), and yet they do not appear to produce thaxtomins, borrelidin or concanamycins (J. Fyans, unpublished). It therefore appears as though additional, unknown phytotoxic

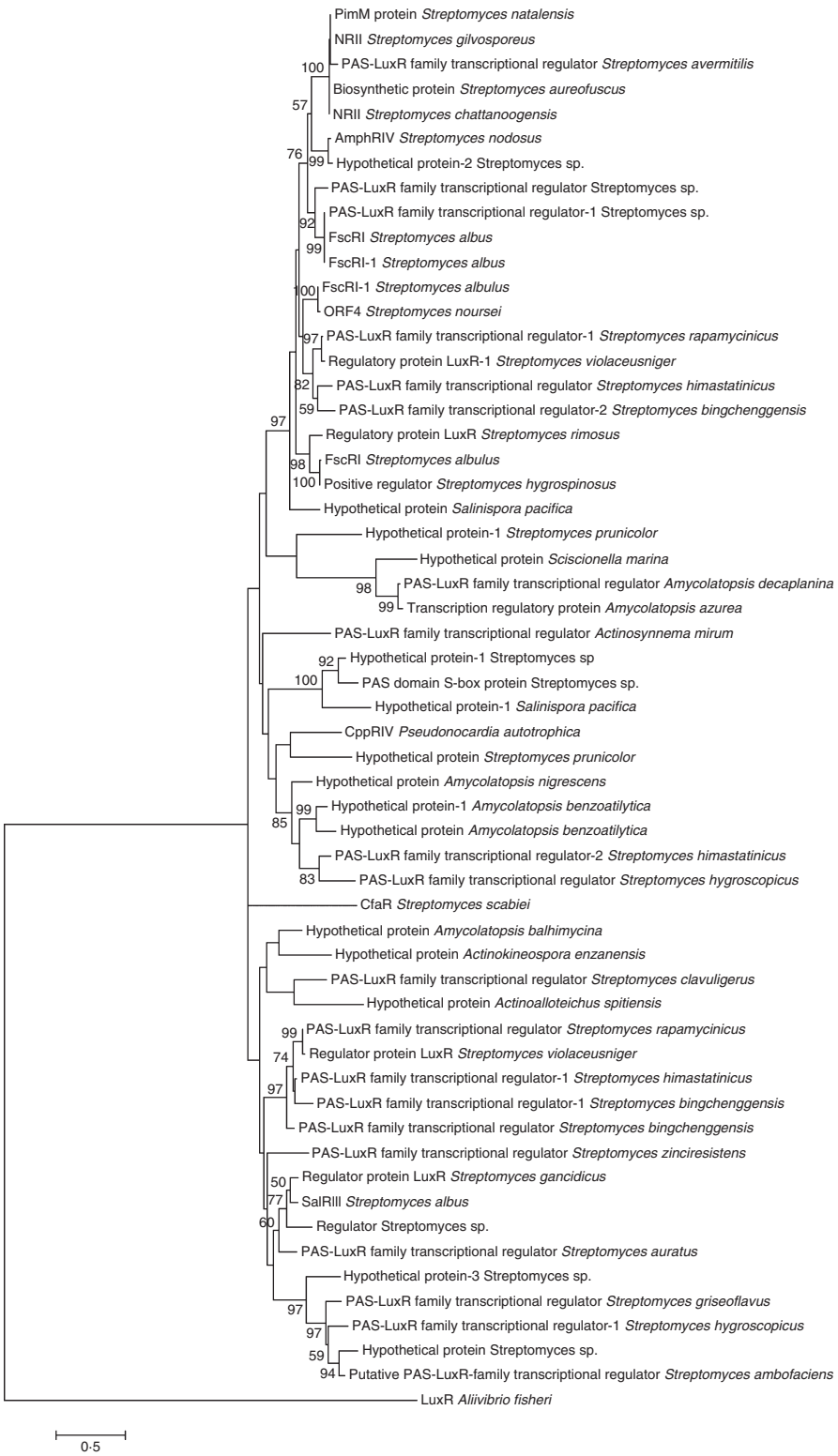

Figure 4 Phylogenetic analysis of PAS-LuxR family proteins from *Streptomyces* and other actinomycetes. The tree was constructed using the MEGA 5.2 software (Tamura *et al.* 2011) with the maximum likelihood algorithm. Bootstrap values ≥50% for 1000 repetitions are indicated. The scale bar indicates the number of amino acid substitutions per site. Accession numbers for the protein sequences used in this analysis are listed in Table S1. The *Aliivibrio fischeri* LuxR protein was included as an outgroup.

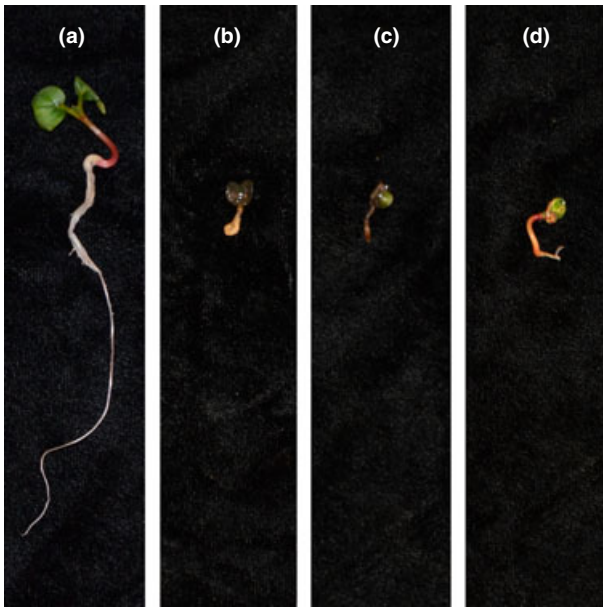

Figure 5 Virulence phenotype of nonthaxtomin-producing plant pathogenic *Streptomyces* strains isolated from Newfoundland, Canada. Radish seedlings were inoculated with *Streptomyces* spp. 11-1-2 (c) and 11-2-4 (d), whereas control seedlings were treated with water (a) or with *S. scabies* 87-22 (b).

secondary metabolites are possibly contributing to *Streptomyces* plant pathogenicity in the environment, and the identification and characterization of such metabolites will undoubtedly contribute to a better understanding of the mechanisms of disease development by these organisms.

Concluding remarks

Research over the last several years has provided important insights into plant pathogenic *Streptomyces* spp. and the phytotoxins that they produce to colonize and infect living plant tissues. Such information has assisted in the development of better procedures for detecting the pathogens in agricultural settings, and has provided new ideas for developing better control methods for reducing the economic impact of CS and other diseases. For example, the thaxtomin phytotoxins are now known to function as key virulence factors that are produced by several different CS and AS pathogens, and recent work using thaxtomin A as a selective agent has provided promising results for the development of potato lines that display elevated resistance to CS (Wilson *et al.* 2010; Hiltunen *et al.* 2011). This is significant given that CS is ubiquitous and notoriously difficult to effectively manage, and there are currently no potato cultivars that are completely resistant to the responsible pathogens (Dees and Wanner 2012). But, as discussed in this review, it is now apparent that multiple phytotoxic secondary metabolites are likely playing a role in the pathogenic phenotype of *Streptomyces* spp. in the environment, and thaxtomins are not always involved in the development of CS disease. This has important implications for control strategies that specifically target thaxtomin as such strategies will likely not be universally effective against all CS pathogens. Therefore, it is vital that we continue to decipher the role of secondary metabolism in the development of economically important crop diseases by *Streptomyces* spp. as this information is critical for the rational development of control strategies that will be effective in the long term. In addition, the functional analysis of *Streptomyces* secondary metabolites will help to further elucidate the complex mechanisms involved in host–pathogen interactions, which are ever evolving and dynamic processes.

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Conflict of interest

No conflict of interest is declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Accession numbers of the PAS-LuxR protein sequences used for construction of the phylogenetic tree.

Actinobacterial Activities

A recent meeting of the Society for Applied Microbiology highlighted organisms involved in potato scabs, nitrogen fixation and desert-based alchemy

Bernard Dixon

Had a local journalist, seeking material for a sensational article, dropped into the summer meeting of the Society for Applied Microbiology (SfAM), held this year in Cardiff, Wales, he or she might have spotted one particularly promising source. It was a poster entitled "Potentially zoonotic viruses circulating in wildlife in China," written by Guangjian Zhu of East China Normal University in Shanghai with other colleagues in China and in the United States and United Kingdom.

Even without journalistic embellishment, the report was of considerable interest. It summarized the results of tests on swabs and blood samples from over 2,000 mammalian species, including bamboo rats, masked palm civets, and Malayan porcupines, caught in the wild or acquired from markets. The main findings, confirmed by sequencing, were an approximately 10% prevalence of astroviruses, and a high incidence of SARS-like coronaviruses in Chinese horseshoe rats. Most of the coronavirus positives showed 90–98% homology with various bat coronaviruses.

If the same journalist had needed enticement to listen to a scientific lecture (a rare scenario these days), he or she might have attended one of four remarkably cogent yet entertaining talks by students at the SfAM meeting. Given by Suzy Moody of Swansea University, Wales, it highlighted what she called "a model organism, and a bit of a show-off." Accompanied by conversational asides ("and that set me thinking . . . I struck lucky . . . something was definitely going on . . . ") which in no way detracted from the scientific importance of her talk, Moody described her efforts to identify the in vivo role of albaflavenone, a sesquiterpene antibiotic synthesized by *Streptomyces coelicolor*.

"We found that a disruption mutant incapable of producing albaflavenone had a specific phenotype when grown under osmotic stress, being unable to generate the pigmented antibiotics for which *S. coelicolor* is renowned," Moody said. "Our aim was to find out how albaflavenone mediated this alteration in phenotype." She and her coworkers used antibiotic assays to quantify the change in pigmented antibiotic production, and qRT-PCR to determine the specific regulators affected by albaflavenone. Bioinformatic analysis and modelling identified a possible araC family transcriptional regulator (AFTR) to which the albaflavenone is a ligand.

Moody and her collaborators next constructed a disruption mutant of the AFTR. Further antibiotic assays indicated that the AFTR-albaflavenone partnership is an important regulatory system for the formation of pigmented antibiotics. "Our work provides evidence that albaflavenone is a novel bacterial hormone," Moody concluded. "The phenotype, assays and qRT-PCR data all point to a new signalling role for this antibiotic."

Actinobacteria were one of the principal themes of the Cardiff meeting, not least for the potential of those found in extreme environments as sources of new drugs. While some would question the assertion by Michael Goodfellow of Newcastle University that "it is rarely acknowledged that bacteria are the dominant forms of life on Earth," most would support his enthusiasm for studying and indeed harnessing actinobacteria living in places such as the Atacama Desert in northern Chile, where he has worked in recent years,

"Actinobacteria have an unrivalled capacity to synthesize a wide spectrum of bioactive compounds, and it is now becoming apparent that taxonomically novel isolates need to be screened in drug discovery programs if we are to avoid the costly rediscovery of known chemical entities," Mike said. "Research on isolates from the Atacama Desert, the oldest and driest desert in the

world, has led to the recognition of innumerable novel actinobacterial species, including some belonging to so-called rare genera. This work not only emphasizes the importance of establishing cultural actinobacterial diversity in extreme habitats. It also paves the way for the selection of candidate strains for genome mining and systems/synthetic biology."

One of Goodfellow's collaborators, Gilles van Wezel of Leiden University in the Netherlands, described a new way of classifying actinomycetes—one that addresses a limitation in current approaches. With large whole-genome bacterial data sets being generated apace these days, rapid and accurate molecular taxonomy is increasingly important. The existing method, based on the sequence divergence of 16S ribosomal RNA, reveals differences that are too small to allow accurate discrimination between strains. The new technique developed by van Wezel is based on what he called the "extraordinary" conservation of SsgA and SsgB proteins.

"SsgA-like proteins are developmental regulators, which streptomycetes require for septum-specific cell division during sporulation-specific cell division," van Wezel explained. "The almost complete conservation of the SsgB amino acid sequence between members of the same genus, and its high divergence even between related genera, provide excellent data for the classification of morphologically complex actinomycetes."

The data obtained in Leiden clearly validate *Kitasatospora* as a sister genus to *Streptomyces* in the family *Streptomycetaceae* and indicate that *Micromonospora, Salinospora*, and *Verrucosispara* represent different clades of the same genus. The amino acid sequence of SsgA is an accurate determinant of the ability of streptomyces to make submerged spores.

Introducing a subsequent speaker, Martha Trujillo of the Universidad de Salamanca, Spain, Goodfellow commented that even five years ago we would not have believed anyone who said that *Micromonospora* was involved in nitrogen fixation. Yet here we were, hearing that species of this actinobacterium were normal inhabitants of the root nodules formed in legumes and actinorhizal plants as a result of symbioses established with rhizobia and *Frankia* respectively. Moreover, most of these actinobacterial populations represent newly recognized species.

"Recent studies suggest that *Micromonospora* is a growth-promoting bacterium, interacting with rhizobia or *Frankia* in a tripartite process." Trujillo said. "Other work indicates that *Micromonospora,* co-inoculated with rhizobia or *Frankia*, can promote both nodulation and plant growth. Reinfection experiments not only show that *Micromonospora* induces nodules but also suggest that a rhizobial organism is necessary for it to penetrate the tissues. It is still too early, however, to explain the exact role which *Micromonospora* plays inside root nodules or how it penetrates."

Trujillo and colleagues have very recently determined the genome sequence of *Micromonospora lupini* strain Lupac 08 from lupine nodules. Their genomic information points to a large number of putative genes coding for hydrolytic enzymes, which may be involved in the process of infection—as suggested for other microorganisms living symbiotically with plants.

This was by no means the only actinobacterial advance reported at the SfAM conference, which in turn raises new questions about their activities. Dawn Bignell of Memorial University, St John's, Newfoundland, Canada, discussed her work on *Streptomyces* species that cause scab disease on economically important root and tuber crops such as potatoes, carrots, radishes and beet. The main known virulence determinant made by *S. scabies* and its relatives is thaxtomin A, which targets cellulose biosynthesis. Although this has prompted suggestions that its suppression might be harnessed to prevent scabs, Bignell and others have found that some of the pathogens work in other ways.

The St John's group recovered strains of *Streptomyces* from scab lesions on infected potatoes, and used plant bioassays to determine the virulence phenotype of each isolate. Morphological characterization plus 16S rDNA sequencing then identified pathogenic isolates, while a combination of genetic and chemical approaches showed the capacity of each strain to produce thaxtomin A. The results clearly demonstrated that some pathogenic streptomycetes do not generate this secondary metabolite but use other virulence factors when infecting hosts and causing disease.

One aspect of the otherwise excellent SfAM conference was less appealing—mobile phones not only ringing during presentations but being answered, leaving people seated nearby to hear one half of a heated conversation. Is such behavior becoming socially and professionally acceptable?