ECOLOGY AND CHARACTERIZATION OF *STREPTOMYCES* SPECIES ASSOCIATED WITH COMMON SCAB DISEASE CONDUCIVE AND BIOFUMIGATED SOILS IN SOUTH AFRICA

By

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OPSOMMING

Bruinskurf is ‘n ernstige kosmetiese siekte op aartappels in Suid Afrika sowel as internasionaal. Die siekte affekteer die voorkoms en kwaliteit van aartappels en lei dikwels tot aansienlike jaarlikse verliese. Aartappel produsente in Suid Afrika, insluitend kommersiële boere, die opkomende landbou sektor en die verwerkings bedryf, vind dit moeilik om bruinskurf voorkoms te bestuur, veral die grondgedraagde inokulum. Bestaande produkte en bestuursprogramme teen bruinskurf is nie voldoende nie. Die twee hoofdoelwitte van hierdie studie was om i) die patogeniese *Streptomyces* spp. in aartappel produksie streke te karakteriseer en ii) die meganismes waardeur die inkorporering van *Brassica* reste in die grond bruinskurf voorkoms kan verminder, te bestudeer en wyses te vind waarop dit ingesluit kan word in ‘n onderhoubare bestuursprogram.

Streptomyces scabiei word steeds beskou as die hoofveroorsakende agent vir bruinskurf in Suid Afrika. Wêreldwyd word die siekte egter veroorsaak deur ‘n kompleks van *Streptomyces* spesies met die dominante spesie wat verskil in elke produksie area. In die lig hiervan is a totaal van 132 *Streptomyces* isolate vanaf ses produksie areas in Suid Afrika versamel en gekarakteriseer. Aartappel potproewe het getoon dat 53 % van hierdie isolate patogenies was. Deur gebruik te maak van spesie spesifieke “primers” en filogenetiese analise (16S rRNA filogenie en multilokus filogenie) is getoon dat *S. scabiei* die mees prominente spesie in Suid Afrika is met 51.4 % van die patogeniese isolate wat positief getoets het vir hierdie spesie, gevolg deur *S. europaeiscabiei* (30 %), *S. caviscabies* (5.7 %), and *S. stelliscabies* (1.45 %). Die oorblywende 11.45 % van die patogeniese isolate bestaan uit drie taksa wat verwant is en inpas binne filogenetiese “clades” wat nie bruinskurf isolate van enige land behalwe Suid Afrika bevat nie. Die taksa word hier genoem *Streptomyces* taxa RSA1 (5.7 %), RSA2 (4.3 %) en RSA3 (1.45 %). *Streptomyces* taxon RSA1, wat voorgekom het in twee produksie areas, is van spesifieke belang omdat hierdie isolate spleetskurf simptome produseer wat lei tot aansienlike kosmetiese knolskade. Spleetskurf is nog nie in enige ander produksie streek in die wêreld gerapporteer nie en is veral van groot belang in Suid Afrika omdat dit op die bruinskurf tolerante kultivar, Mondial, voorkom. PKR analises wat die drie merker patogenisiteit eiland (PAE) gene (*txtAB, nec1, tomA*) teiken, het getoon dat *nec1* 89 %, *tomA*, 81 % en *txtAB* 89 % voorgekom het in die patogeniese isolate. Die

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isolate (11 %) wat nie die *txtAB* gene bevat het nie en ook nie thaxtomin A produseer het nie, het behoort aan *S. caviscabies* en *Streptomyces* taxa RSA2 en RSA3 groepe.

Die inkorporering van *Brassica* reste in die grond het onlangs potentiaal getoon om bruinskurf siekte voorkoms te verminder. *Brassica* reste bevat glukosinolate (GLN) wat tydens selvernietiging gehidroliseer word deur die ensiem mirosinase, om 'n diverse groep biologiese aktiewe hidrolise produkte te lewer wat toksies is vir grond mikrobe. Hierdie meganisme van beheer staan bekend as bioberoking. Die huidige studie het getoon dat bruinskurf voorkoms betekenisvol verminder is deur die inkorporering van vars en lugdroog *Brassica oleracea* var. *capitata* (kopkool) reste onder veld toestande in twee opeenvolgende aartappel aanplantings. Die effek van vlugtige verbindings vanaf verskeie *Brassica* spesies op *Streptomyces* is geevalueer deur middel van twee *in-vitro* bio-analise tegnieke. 'n *In-vitro* agarplaat bio-analise het getoon dat vlugtige verbindings van water-geaktiveerde vriesdroog reste van 'n *B. juncea / S. alba* mengsel en *B. napus* oor die algemeen meer effektief was om *Streptomyces* groei en sporulasie te onderdruk as *B. oleracea* var *italica* en *B. oleracea* var. *capitata* reste. In 'n gaskamer bio-analise waar daar gebruik gemaak is van varsgemaalde *Brassica* reste het *B. oleracea* var. *capitata* en 'n *B. juncea / S. alba* mengsel die sporulasie van *Streptomyces* onderdruk maar nie die hifegroei nie. Die gaskamer bio-analise het getoon dat die bioberoking effek bakteriostaties is omdat die isolaat groei herstel het na blootstelling aan die vlugtige verbindings. Beide bio-analises het getoon dat betekenisvolle komponente van beide die patogeniese (50 %) en nie-patogeniese (20 %) *Streptomyces* populasies wat ondersoek is glad nie deur die *Brassica*-reste geinduseerde vlugtige verbindings geaffekteer is nie.

Meganismes van siekte vemindering deur *Brassica* inkorporering is nie beperk tot bioberoking nie, maar veranderings in die struktuur van mikrobiese gemeenskappe betrokke in ge-induseerde sistemiese weerstand (GSW) en/of algemene onderdrukking kan ook bydra tot siekte onderdrukking. In die hierdie studie het 'n aartappel wortel-split eksperiment waar die nageslagknolle en wortels ruimtelik geskei is in subeenhede wat gevul was met *B. juncea / S. alba* (mosterd mengsel) of *B. oleracea* var *oleracea* (kopkool) behandelde of nie-behandelde grond, getoon dat sistemiese onderdrukking betrokke is in bruinskurf siekte voorkoms. Die rol van toksiese GLN hidrolise produkte is uitgeskakel in hierdie siekte

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onderdrukking omdat die vlugtige verbindings voor grond inkorporering vrygestel is uit die *Brassica* weefsel. Verhoogde mikrobiese aktiwiteit in die *Brassica* behandelde subeenhede was bevestig deur betekenisvolle verhogings in β-glukosidase en urease aktiwiteit. "Principle component" analise het sekere tendense uitgelig in die algehele grond, knol en wortel-geassosieerde mikrobiese genera (*Trichoderma, Pseudomonas, Streptomyces*, totale bakterieë en *Fusarium*) in die *Brassica* behandelde en onbehandelde subeenhede. Die mosterd behandelde, en tot 'n mindere mate, die kool behandelde eenhede, het getoon dat daar 'n verhoging in grond *Fusarium* en *Trichoderma* en wortel *Trichoderma* populasies en 'n afname in totale bakterieë en *Streptomyces* populasies in grond en knolle en *Streptomyces* populasies in wortels was.

Hierdie studie het bygedra tot ons kennis oor *Streptomyces* spesies wat bruinskurf op aartappels veroorsaak in Suid Afrika en meganismes waardeur inkorporering van *Brassica* materiaal in die grond bruinskurf kan verminder. Verskeie *Streptomyces* spesies, insluitend moontlike nuwe patogeniese spesies, is betrokke by bruinskurf voorkoms en hul onderskeie virulensies en reaksie op die inkorporering van *Brassica* materiaal vereis die implementering van 'n geïntegreerde bestuursprogram. Die plant van kopkool as 'n kontant gewas met gevolglike inkorporering van koolreste hou belofte in as 'n bestuurs strategie vir die opkomende landbou sektor. Die meganismes betrokke in bruinskurf onderdrukking deur *Brassica* toevoeging in grond het getoon dat plant geinduseerde sistemiese weerstand sowel as algemene onderdrukking 'n rol speel. Altesaam kan die kennis wat deur hierdie studie ingewin is gebruik word om i) volhoubare aartappel produksie stelsels te optimiseer, ii) meganismes betrokke by siekte onderdrukking verder te verken en iii) molekulêre tegnieke soos "real-time" PKR te ontwikkel vir spoedige identifikasie en kwantifisering van bruinskurf veroorsakende spesies in Suid Afrika.

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SUMMARY

Common scab of potato is a serious cosmetic disease in South Africa as well as internationally. The disease affects the appearance and quality of potatoes resulting in major annual losses. Potato producers in South Africa, in the commercial, emerging and processing potato industries, struggle to manage the incidence of common scab, especially soilborne inoculum. Existing products and management programs against common scab are often insufficient. The two main aims of the study were to i) characterize and determine the pathogenic *Streptomyces* spp. occurring in potato production regions in South Africa and ii) investigate the mechanisms through which *Brassica* soil amendments can reduce common scab and ways in which it can be included in a sustainable management program.

In South Africa, *Streptomyces scabiei* is still regarded as the main causal agent of common scab. However, world-wide, the disease is caused by a complex of *Streptomyces* species, with the dominant species varying in different regions. Therefore, a total of 132 *Streptomyces* isolates collected from six South African potato production regions were characterized. Potato pot trials showed that 53 % of the isolates were pathogenic. Analyses using species specific primers and phylogenetic analyses (16S rRNA phylogeny and multilocus phylogeny) showed that *S. scabiei* was the most prominent species in South Africa comprising 51.4 % of the pathogenic isolates, followed by *S. europascabiei* (30 %), *S. cavisabies* (5.7 %), and *S. stelliscabies* (1.45 %). The remaining 11.45 % of the pathogenic isolates comprised three taxa, which are related and fit within phylogenetic clades that do not contain common scab isolates from any country other than South Africa. The taxa are named here *Streptomyces* strains RSA1 (5.7 %), RSA2 (4.3 %) and RSA3 (1.45 %). *Streptomyces* strain RSA1, which occurred in two production regions, is of special concern since these isolates produce fissure scab symptoms that result in severe cosmetic tuber damage. Fissure scab has not been reported from any other region of the world and is of concern in South Africa since it occurs on the cultivar Mondial that is tolerant to typical common scab. PCR analyses targeting three marker pathogenicity island (PAI) genes (*txtAB, nec1, tomA*) showed that among the pathogenic isolates *nec1* occurred in 89 % of the isolates, *tomA* in 81 % and *txtAB* in 89 % of the isolates. The isolates (11 %) that did not contain the *txtAB* gene and also did not produce thaxtomin, belonged to *S. caviscabies* and *Streptomyces* strains RSA2 and RSA3.

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The incorporation of *Brassica* tissue into soil has recently shown some potential for reducing common scab disease incidence. *Brassica* crop residues contain glucosinolates (GLN) that upon cell disruption are hydrolysed by the enzyme myrosinase to yield a diversity of biologically-active hydrolysis products that are toxic to soil microbes. This control mechanism is known as biofumigation. The current study showed that common scab was significantly reduced under field conditions through incorporation of fresh or air-dried residues of *Brassica oleracea* var. *capitata* (cabbage) in two consecutive potato plantings. The *in-vitro* effect of volatile emissions from various *Brassica* species towards *Streptomyces* was evaluated using two bioassay methods. An *in-vitro* agar plate bioassay showed that, in general, volatile emissions from water activated freeze-dried tissue of a *B. juncea/S. alba* mix and *B. napus* were superior to those from *B. oleracea* var *italica* and *B. oleracea* var *capitata* for suppression of growth and sporulation of *Streptomyces*. In a gas chamber bioassay that used freshly macerated *Brassica* tissue, *B. oleracea* var *capitata* and a *B. juncea/S. alba* mix suppressed sporulation but not hyphal growth of *Streptomyces*. The gas chamber bioassay showed that the biofumigation effect was bacteriostatic, i.e. isolates recovered after volatile exposure. Both bioassays showed that significant components of both the pathogenic (50 %) and non-pathogenic (20 %) *Streptomyces* population examined were unaffected by the *Brassica* tissue derived volatiles.

Mechanisms of disease reduction through *Brassica* amendments are not limited to biofumigation, but changes in the structure of microbial communities involved in systemic induced resistance and/or general microbial suppression may also contribute to disease suppression. In the current study a potato split-root experiment that spatially separated the progeny tubers and roots of *Brassica juncea/ Sinapus alba* (mustard mix) and *Brassica oleracea* var *oleracea* (cabbage) amended soil sub-units from non-amended soil sub-units, showed that induced resistance induced in plants was involved in common scab suppression. The role of toxic GLN hydrolysis products was ruled out in the induced resistance mediated disease suppression, since volatiles were released from *Brassica* amended soil prior to initiating the experiment. Increased microbial activity in the *Brassica* amended units was evidenced by significant increases in ß-glucosidase and urease activities. Principal component analyses revealed some trends in the overall soil, tuber and root associated microbial genera (*Trichoderma, Pseudomonas, Streptomyces,* total bacteria and *Fusarium*) in the *Brassica* amended and non-amended units. The mustard amended treatment, and to a lesser extend the cabbage amended units, showed trends towards increases in soil *Fusarium*

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and *Trichoderma* and root *Trichoderma* populations, and decreases in total bacterial and *Streptomyces* populations in soil and tubers, and *Streptomyces* in roots.

This study has contributed towards our knowledge of the *Streptomyces* species causing potato common scab in South Africa, and mechanisms through which *Brassica* soil amendments can reduce common scab. Several *Streptomyces* species, including novel pathogenic taxa, are involved in causing common scab and their differential virulence, and responses to being suppressed by *Brassica* amendments will require the implementation of an integrated management program. The planting of cabbage as a cash crop, with the subsequent incorporation of residues into soil shows promise as a management strategy for subsistence farmers. The mechanisms involved in common scab suppression through *Brassica* amendments were shown to involve systemic induced resistance in plants and general microbial suppression. Altogether, knowledge obtained in this study can be used to i) optimize management strategies for sustainable potato production, ii) further elucidate the mechanisms involved in disease suppression and iii) develop molecular techniques, such as quantitative real-time PCR for rapid identification and quantification of common scab-causing species in South Africa.

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1. THE GENUS *STREPTOMYCES*: TAXONOMICAL ASPECTS, IDENTIFICATION AND THEIR ROLE IN COMMON SCAB ON POTATO

INTRODUCTION

Potato is classified as a horticultural crop and plays an important role in the South African agricultural marketplace. South Africa is currently rated as the 31st largest potato- (*Solanum tuberosum*) producing country in the world, contributing 0.5 % of the world's total potato production. The total potato production in Africa is estimated at 11.5 million tonnes annually and is cultivated on approximately 1 million hectares of land. In Africa, South African potato growers only plant 5 % of the total hectares, but produce 14 % of the total African crop. The gross value of the potato harvest in South Africa is approximately 43 % of all major vegetables, 13.5 % of horticultural products and 3.5 % of the total agricultural production (Theron, 2003). Potato cultivation takes place throughout the year, since the 16 potato production areas (Fig. 1) have a wide variety of climatic conditions that allow potatoes to be planted at different times in different areas. The utilisation of the South African potato crop can be divided into three categories; 1) table / ware potatoes, 2) seed potatoes, and 3) potatoes for processing. Of these three categories, table / ware potatoes comprise the largest sector (63.5 %). Most of the table potato crop is sold on the fresh produce markets, but a growing portion is distributed to informal markets.

All potato markets are negatively affected by the incidence of common scab (Theron, 2003). Common scab reduces the cosmetic value of ware and seed potatoes, since it causes circular, raised, tan to brown, corky lesions on the surface of tubers. The disease has resulted in an increased trend towards downgrading of consignments on the market because of the growing consumer and grower demand for blemish free produce and planting material, respectively. In 2006/07, 32 % of table potatoes produced were discarded as a result of the consignment downgrading due to common scab symptoms. When taking into consideration that average annual production in South Africa is 180 million 10 kg bags, then 57.6 million 10 kg bags were downgraded and rejected as a result of this disease. This amounts to a total loss of R1.7 billion per year for the potato industry in South Africa.

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Common scab is caused by several *Streptomyces* species, with most isolates producing a phytotoxin, known as thaxtomin. Thaxtomin is a pathogenicity determinant involved in symptom development in common scab pathogens (King *et al.,* 1989; Bukhalid and Loria, 1997). *Streptomyces scabiei* is the most common species and has been reported worldwide (Faucher *et al*., 1992; Loria *et al*., 1997; Miyajima *et al*., 1998; Gouws, 2006). Other major pathogenic species include *S. acidiscabies* (Eastern North America; Japan, Korea) *S. turgidiscabies* (Japan, Korea, Scandinavia), *S. aureofaciens* (Finland) and *S. botropensis* (originally described in Egypt but also reported in North America) (Lambert & Loria, 1989a; Faucher *et al*., 1992; Miyajima *et al*., 1998; Wanner, 2006). There have also been reports from Europe of new pathogenic *Streptomyces* spp. that include *S. reticuliscabiei* (European netted scab), *S. europaeiscabiei* and *S. stelliscabiei* (Bouchek-Mechiche *et al*., 2000), which were subsequently reported in North America (Wanner, 2006). Three novel pathogenic *Streptomyces* spp. were also reported in Korea; *S. luridiscabiei, S. puniscabiei* and *S. niveiscabiei* (Park *et al*., 2003). The most recent report is from the study conducted by Wanner (2007), where surveys in several locations in the United States identified a new *Streptomyces* strain that was pathogenic on potato and radish and was able to infect underground stems and stolons. The morphological and physiological properties of this species are distinct from those of previously described *Streptomyces* spp.

The first reference to the common scab pathogen and its association with potatoes in South Africa was documented by Pole Evans in 1905. He described the causal agent as *Oöspora scabies* and referred to it as a fungus that flourished in sandy soil and was widely distributed in the potato-growing regions of the country. Dippenaar (1933) later studied the epidemiology of the pathogen as well as the agrochemical control measures available at that time. More than 50 years later, a study by Slabbert *et al.* (1994) focused on the role of toxins in the etiology of common scab incidence. They were able to show a positive correlation between the pathogenicity of *S. scabiei* isolates from common scab lesions on field grown potatoes and their ability to produce thaxtomin A. In a more recent MSc study conducted by Gouws (2006), an overview of the disease incidence was established, and the etiology and alternative control measures were investigated. This study also confirmed the findings of Slabbert *et al.* (1994).

Effective management strategies for common scab are limited, and most potato cultivars planted in South Africa are susceptible to the disease. Since chemicals are not always effective, an integrated management strategy has to be implemented. Management strategies that can be considered include irrigation scheduling, cultivar tolerance, agro-chemical applications, crop rotation, green manuring, *Brassica* crop amendments and other organic amendments (Larkin, 2008). Among these strategies, *Brassica* crop amendment is an important consideration, since it is an environmentally friendly approach that can enhance soil health, and it can also be cost effective. The incorporation of *Brassica* crop residues into soil to suppress soilborne pathogens is known as biofumigation that refers to the release of toxic volatiles from *Brassica* tissue when the tissue is macerated. Researchers at the ARC-Roodeplaat VOPI have shown (Gouws, 2006; Gouws & Wehner, 2004) that *Brassica* residues reduced common scab incidence on potatoes when the soil was amended with *Brassica* tissue in greenhouse, tunnel (*B. oleracea* var *oleracea*; *B. oleracea* var *italica*; *B. oleracea* var *botrytis*; *B. oleracea* var *gemmifera*) and field trials (*B. oleracea* var *oleracea*). Therefore, biofumigation seems to be a feasible option for the management of common scab, since it not only reduces disease, but also results in an increase in carbon and thus improved soil health within agricultural systems.

HISTORY OF THE NOMENCLATURE OF *STREPTOMYCES* SPP. CAUSING COMMON SCAB

The genus *Streptomyces* consists of a large number of spp. that are filamentous prokaryotes distinguished by the production of non-fragmenting substrate mycelium that colonise and penetrate organic matter in soil (Loria *et al.* (1997). Most Streptomycetes are soil dwelling saprophytes that produce a range of antibiotics, and extracellular hydrolytic enzymes that allow access to nutrients from organic compounds that are difficult to degrade in soil. Streptomycetes are immobile and produce spores for dispersal purposes through the fragmentation of aerial hyphae that form on substrate mycelium (Fig. 2). Since the 1970s, more than 3000 *Streptomyces* spp. have been described in literature, including mostly non-pathogens and only a few pathogens. This, however, was an overestimation of the number of species due to the poor species definitions that were available, resulting in taxonomic chaos (Guo *et al*., 2008). Subsequently, a revision of the species was conducted, and in 2010 there were 576 validly published species names, which are increasing every year (Labeda, 2011).

For many decades among all these species, only four species were well-recognized as plant pathogens including *S. scabiei, S. acidiscabies, S. turgidiscabies* (common scab on potato) and *S. ipomoeae* (sweet potato rot) (Loria *et al*., 1997; Miyajima *et al*., 1998). More recently a few other species have also been described as plant pathogens, although the validity of some has been questioned.

The causal agent of common scab was first described by Thaxter (1891) as *Oöspora scabies,* a melanin-producing actinomycete bearing grey spores in spiral spore chains. The name was later changed to *Actinomyces scabies* by Güssow (1914), followed by another name change in 1948 (Waksman & Henrici) to *Streptomyces scabies.* In an effort to standardise the terminology for *S. scabies* as the predominant species causing common scab of potato, Lambert & Loria (1989a, b) published two papers in the International Journal of Systematic bacteriology that formally described the type species of *S. scabies* (ATCC 49173) and *Streptomyces acidiscabies* (ATCC 49003), causal agent for acid scab. They also demonstrated that the majority of pathogenic streptomycetes isolated from potatoes form a distinct species, *S. scabies*, consistent with the original description. Phenotypic criteria were provided that differentiated this species from other species. The latest change in nomenclature was brought about by Trüper & De Clari (1997) who changed the epithet from the substantive noun (*scabies*) to the genitive form (*scabiei*) thus renaming the organism to *Streptomyces scabiei*.

The name changes and subsequent uncertainty in species identity stemmed from inadequate descriptions of *Streptomyces* species based on the use of a small number of characteristics in the early studies. To address this problem the International *Streptomyces* Project committee (ISP) was established in 1963. It was a joint international effort to assemble and re-describe authentic type species of the named species in the genera *Streptomyces* and *Streptoverticillium* (Kurylowics *et al*., 1976). The main outputs of the ISP committee were: (i) the establishment of a set of standardised tests and procedures for the identification of *Streptomyces* spp. and (ii) a large number of detailed species descriptions for the classification of *Streptomyces* spp. (Gyllenburg, 1976). The descriptions from the ISP classification system were based on phenotypic characteristics and even though it proved to be very useful it has since been enhanced by more accurate cellular fatty acid analysis and

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DNA-based techniques e.g. DNA-DNA hybridisation, 16S rRNA sequence analysis and whole genome sequencing (Bowers, *et al.*, 1996; Ndwora, *et al.*, 1996; Takeuchi, *et al.*, 1996; Song *et al.*, 2004; Bignell *et al.*, 2010).

IDENTIFICATION OF COMMON SCAB-CAUSING *STREPTOMYCES* SPP.

Since the early 1950s, common scab disease surveys have been conducted in various potato production regions world-wide, focusing on detection and identification of the causative agent. The initial studies were directed at obtaining information on the extent of the disease and its regional distribution (Large & Honey, 1953). Unfortunately, identifying the causal agent(s) was often problematic since detection was hampered by the lack of rapid, reliable identification methods. In an effort to simplify identification and detection, Douglas & Garrard (1954) investigated the use of serological methods. They made use of rabbit antisera and a simple flocculation test, and were able to show a striking similarity in serological behaviour between pathogenic *Streptomyces* species. However, they could not make an absolute separation between pathogenic and non-pathogenic types on the basis of these tests. Since then, several techniques have been used to identify *Streptomyces* spp. associated with common scab. These studies have shown diversity among isolates identified morphologically and molecularly as *S. scabiei,* and in other phytopathogenic streptomycetes. This confirms that common scab on potato can be caused by a complex of *Streptomyces* spp., with the dominant species varying in different potato production regions (Bramwell, *et al.*, 1998; Bouchek-Mechiche, *et al.*, 2000; Doumbou, *et al.*, 2001; Bencheikh & Setti, 2007).

Designation of the neotype strain for comparative characterization studies. In most of the early characterization studies, the *Streptomyces* spp. associated with common scab lesions were isolated from infected tubers and morphologically compared to *Species 17* of Millard & Burr (1926). However, when Waksman (1961) re-described the species he designated a different isolate as neotype, IMRU 3018 = ISP 5078. Unfortunately the selection of this isolate was only based on pathogenicity and therefore Elseway & Szabo (1979) suggested a new neotype culture, ATCC 33282, in accordance with the original description of Thaxter. Since this neotype species was not included in the Approved List of Bacterial Names, Lambert & Loria (1989a) re-described the common scab pathogen as *Streptomyces*

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scabies and assigned neotype strain ATCC 49173 as the type species. Subsequently, this species has been used as the standard for the characterization of common scab-causing pathogens internationally.

Phenotypic characterization. The characterization of pathogenic *Streptomyces* species are based on phenotypic and biochemical evaluations as described by the ISP committee. The attributes that are evaluated include spore chain morphology, colony colour, reverse side colony colour, melanoid pigment formation and carbon utilization (L-Arabinose, D-Fructose, D-Glucose, D-Mannitol, Raffinose, Rhamnose, Sucrose, D-Xylose, *meso*-Inositol) (Shirling & Gottlieb, 1966). Furthermore, these assessments often included evaluation of the effective pH range, sensitivity to a range of antibiotics (penicillin, oleandomycin, streptomycin), pathogenicity and thaxtomin production (Lambert & Loria, 1989b).

Several studies using the ISP identification system were successful in identifying all of the isolates under consideration to the species level. Heinnames & Sepannen (1971) isolated forty-four actinomycetes from scab lesions on field grown potato tubers. Ten isolates were selected based on their origin and melanin pigment reaction and were all identified as *S. scabiei*. Loria *et al*. (1997) conducted surveys in the North Eastern US as well as parts of Canada, and found that the majority of *Streptomyces* spp. isolated from common scab tubers displayed spiral spore chains, produced melanoid pigments, had primarily grey aerial mycelium and were able to utilise the discriminatory carbon sources tested, typically depicting *S. scabiei*.

Although the ISP identification system proved to be very useful for standardizing the identification of *Streptomyces* spp. associated with common scab on potato, several studies found that the method was insufficient for describing the variety of causal agent(s) isolated from symptomatic tubers. Faucher *et al.* (1992) isolated several actinomycetes from common scab lesions on potato from production regions in Quebec, Canada. The isolates they obtained were grouped into six classes according to the ISP method and among these included the pathogenic isolates *S. scabiei*, *S. acidiscabies* and an unidentified streptomycete. The unidentified streptomycete was associated with deep-pitted scab lesions, concurring with

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earlier findings from Archuleta & Easton (1981) and later described as *S. caviscabies* by Goyer *et al*. (1996). Bouchek-Mechiche *et al*. (1998) found that *S. scabiei* was the single causal agent of common scab in France. However, the *S. scabiei* species from their survey were phenotypically heterogeneous, since three subphenons were delineated. The phenon differentiation was based on the utilisation of 1-o-methyl-α-galactopyranoside, trans-aconinate, 5-keto-D-gluconate, betain, D(+)trehalose and gentisate. Phenon 1 contained species of *S. scabiei* from different geographical areas, including five *S. scabiei* isolates from South Africa. In South Africa, Gouws (2006) identified in addition to *S. scabiei,* which composed the largest group (82 % of the pathogenic *Streptomyces* spp.), two other phenotypically distinct groups.

Fatty acid analysis. Fatty acid methyl ester profiles have been widely used to characterize and identify bacteria (Busse *et al*., 1997), but only a few studies have applied this technique to common scab streptomycetes. In a characterisation study conducted by Paradis *et al*. (1994), fatty acid composition was assessed for pathogenic and non-pathogenic *Streptomyces* isolates phenotypically related to *S. scabiei*. Although DNA-DNA hybridisation values suggested that two genetically diverse groups were included in *S. scabiei*, no correlation could be established between fatty acid profile and genetic clusters. Bowers *et al.* (1995) also evaluated the utility of fatty acid analysis to characterise scab-inducing species of *Streptomyces* collected on a broad geographic scale. The analyses indicated that a wide diversity in fatty acid composition existed among pathogenic isolates of *Streptomyces* and that it is a useful tool to cluster closely-related isolates such as the pathogenic *S. scabiei*. Ndwora *et al.* (1995) used fatty acid analysis to identify and differentiate disease suppressive, pathogenic and nonpathogenic species of *Streptomyces* spp. With the exception of *S. acidiscabies*, they were able to distinguish pathogenic *Streptomyces* species from disease suppressive species. Cellular fatty acid analysis also suggested that *S. scabiei* species could be divided into two subgroups although they appeared closely related (Loria *et al*., 1997).

DNA-based characterization. The first DNA studies of common scab associated streptomycetes were conducted in the 1960s. Lawrence & Clark (1966) conducted the first study on the DNA from pathogenic and nonpathogenic species of *S. scabiei.* The two pathogenic and nonpathogenic species could not be distinguished based on their G+C content, purine/pyrimidine and AT/GC compositions. The study could taxonomically,

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however, place the *Streptomyces* group more in line with bacteria instead of an intermediate position between fungi and bacteria.

DNA-DNA hybridisation (DDH) analysis, is an direct measurement of relatedness of bacterial isolates and allows the distinction between species based on the "operational species concept" (i.e. two bacteria belong to different species if their measured DDH value is <70 %, <5 % Δ Tm, and biochemical or other phenotype-based tests can distinguish between them) (Almeida *et al*., 2010). Healy & Lambert (1991) made use of DDH to determine the genomic relationship of streptomycetes to the Diastatochromogenes group and to interpret the significance of common pathogenicity mechanisms among the potato scab pathogens. They found that the genetic diversity of isolates referred to as *S. scabiei* exceeded the genetic diversity found at the species level, and that some of the isolates appeared to be related to phenotypically similar non-pathogens.

DDH has also been applied to describe several new pathogenic *Streptomyces* species. Miyajima *et al*. (1998) described *S. turgidiscabies* and reported that the levels of DNA relatedness of this organism with other *Streptomyces* species that cause common scab, were low. In a similar study of *Streptomyces* species pathogenic to potato in France, Bouchek-Mechiche *et al*. (2000) reported three new genomospecies; *S. europaeiscabiei* and *S. stelliscabiei* associated with common scab, and *S. reticuliscabiei* associated with netted scab. Park *et al*. (2003a) also described three scab-causing *Streptomyces* species, *S. luridiscabiei; S. puniscabiei* and *S. niveiscabiei,* that were associated with common scab symptoms. DDH data supports the suggestion that plant pathogenic *Streptomyces* spp. are not closely related. It also indicates that some isolates of *S. scabiei* have much lower DNA relatedness than expected (Loria *et al*., 1997).

Although DDH is a very reliable technique for delineating species, most streptomycete studies have used sequence data of the 16S ribosomal (r)RNA gene, also used in most bacterial taxonomic studies, for identifying species (Takeuchi *et al*., 1996; Bramwell *et al*., 1998; Kreuze *et al*., 1999; Doumbou *et al*., 2001; Park *et al*., 2003; Song *et al*., 2004; Wanner, 2006; Flores-Gonzales *et al*., 2008; St-Onge *et al*., 2008; Wanner, 2009; Almeida *et al*., 2010; Zhao *et al*., 2010; Huguet-Tapia & Loria, 2012). This is due to the labour-intensive

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nature and expertise required for DDH analyses, when compared to gene sequencing. It is important that in identifying a group of isolates as a new species, not only 16S rRNA data should be used, but a consistency in morphological and physiological characteristics should be shown, and comparison must be made to type cultures that have been used in other studies (Loria *et al.*, 1997).

Takeuchi *et al*. (1996) conducted one of the first phylogenetic studies on 12 *Streptomyces* species, including potato scab pathogens, using complete 16S rRNA sequence data. The phylogeny showed that *Streptomyces* spp. that cause potato scab are distributed on unique branches. The study confirmed the lack of close relationships among *Streptomyces* spp. that cause potato scab, and further suggested that potato scab is caused by phylogenetically diverse *Streptomyces* spp. in which pathogenicity has developed independently. In the myriad of 16S rRNA studies that followed that of Takeuchi *et al*. (1996), teams from all over the globe contributed to the growing database of information on the immense variation among *Streptomyces* spp. that are responsible for inducing a range of scab symptoms on potatoes worldwide. Kreuze *et al*. (1999) could successfully describe, *S. scabiei* (common scab lesions), *S. turgidiscabies* (pitted scab lesions) and *S. aureofaciens* (nette scab lesions) from the survey they performed in Finland. In the same way Park *et al*. (2003) described the presence of *S. scabiei* and *S. turgidiscabies* in their collection from Korea, but added three novel descriptions (*S. luridiscabiei, S. puniciscabiei* and *S. niveiscabiei*) to the list of pathogens.

In addition to 16S rRNA phylogenetic studies, other DNA regions that have been used in single gene phylogenetic studies for taxonomic evaluation of phytopathogenic *Streptomyces* include the *rpoB* (RNA polymerase, beta subunit) genes and the 16S-23 rDNA internally transcribed spacer (ITS) region. Song *et al.* (2004) investigated the ITS region from several phytopathogenic *Streptomyces* species and concluded that the ITS regions are not useful for phylogenetic studies in *Streptomyces.* It was, however, shown that the ITS regions were useful for clear differentiation of *S. scabiei* and *S. europaeiscabiei* (Song *et al.*, 2004). Mun *et al.* (2007) and St-Onge *et al.* (2008) found that the *rpoB* gene provides better discrimination of isolates than the 16S rRNA gene.

The use of multilocus sequence typing (MLST) or multilocus sequence analysis (MLSA) is increasingly being used for bacterial typing, and large internet databases are avialable for comparative studies for several genera such as *Pseudomonas* and *Xanthomonas* (Almeida *et al*., 2010). Only a few MLSA studies investigated the genus *Streptomyces*, mostly focusing on non-pathogenic spp. that are well known producers of antibiotics and many industrially and agronomically important secondary metabolites (Rong & Huang, 2012). The first *Streptomyces* MLSA study was conducted by Guo *et al.* (2008) on the *S. griseus* 16S rRNA gene clade, which is taxonomically one of the most complex groups and only includes two common scab-causing spp., *S. caviscabies* and *S. luridiscabiei* (Rong & Huang, 2010). The latter spp. were, however, shown by Rong & Huang (2010) to be later heterotypic synonyms of *S. fimicarius* and *S. microflavus* respectively.

Guo *et al.* (2008) constructed phylogenetic trees based on six genes [ATP synthase F1, beta sub unit (*atpD*), DNA gyrase B subunit (*gyrB*), recombinase A (*recA*), *rpoB,* tryptophan synthase, beta sububit (*trpB*) and 16S rRNA], which compared 53 reference strains that represent 45 valid species and subspecies. The proportion of variable sites in the alleles of the genes varied, with the highest being in the *gyrB* gene (48 %) and the lowest in the 16SrRNA gene (20 %). The 16S rRNA gene tree was found to be unreliable due to low bootstrap support and small conflicting topologies when compared to the other gene trees. Furthermore, none of the single genes contained enough phylogenetic information to reliably discriminate all species. Therefore, concatenation of multigene sequences were used in the final analyses, since trees from the different single-gene trees were congruent. The multi-six-gene tree was able to show clear differentiation of all strains at the species level (Guo *et al.*, 2008).

Based on this work, an internet database (http://pubmlst.org/streptomyces) was also established (Jolley *et al.* 2004) to assist future MLSA studies (Guo *et al.*, 2008). A second study on the *S. griseus* clade (Rong & Huang, 2010) included 18 additional *S. griseus* clade spp. The study revealed that MSLA of the five housekeeping genes (*atpD*, *gyrB, recA, rpoB* and *trpB*) was better than the previous six-gene scheme of Guo *et al.* (2008) since it provided equally good resolution and stability and is more cost-effective. The multi-gene-trees were suitable for discriminating strains that show >99 % 16S rRNA gene sequence similarity.

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MLSA of three to four of the genes also showed good resolution for differentiating most of the strains and can be of value for everyday use (Rong & Huang, 2010). Rong *et al.* (2009) conducted a MLSA study on the *S. albidoflavus* clade that included 10 species and subspecies, which have identical 16S rRNA sequences, using the same five house-keeping genes used by Guo *et al.* (2008). It was proposed that the 10 species and subspecies should be combined into one genomic species, *S. albidoflavus* (Rong *et al.*, 2009). Rong & Huang (2012) conducted MLSA on the *S. hygroscopicus* clade and a few related species, which did not include any common scab spp., and confirmed that the five-gene tree is a valuable alternative for *Streptomyces* spp. assignment since it correlated with DDH data (Guo *et al.*, 2008; Rong & Huang, 2012). A broader investigation on the three genera of the family *Streptomycetaceae* using the small and large subunit ribosomal RNA genes and the *atpD*, *gyrB, recA, rpoB* and *trpB* genes included some novel findings and supported the findings of all the previous studies; (i) protein genes can give higher resolution than rRNA genes, (ii) combined gene sequences give better phylogenetic resolution with higher stability than any single genes, (iii) rRNA gene-based phylogenies can be misleading, (iv) blind inclusion of more genes for phylogenetic analysis is not the best option since higher levels of bootstrap can be obtained with three or four genes than with five genes, (v) protein coding gene phylogenies have a high correlation with the genome relatedness of spp. and (iv) the *gyrB* gene provides good phylogenetic resolution, since this gene tree and the combined tree were supported by higher bootstrap values than any of the other trees (Han *et al.*¸ 2012). The study of Han *et al.* (2012) included only *S. scabiei* as a phytopathogenic spp.

Only two studies have used MLSA for investigating *Streptomyces* that included 10 or more phytopathogenic species. Weon *et al.* (2011) used a combined RNase P RNA (*rnpB*) and 16S RNA gene tree to investigate relationships among 41 scab-causing *Streptomyces* strains. The combined gene tree had a similar topology to the 16S rRNA tree, but showed more divergent phylogenetic clades. For example *S. scabiei* was diverged from *S. europaeiscabiei.* This is due to the fact that the *rnpB* gene only showed 90.7 % similarity for these two species (Weon *et al.*, 2011). Labeda (2011) conducted MLSA analyses (*atpD, recA, rpoB* ad *trpB*) on 62 *Streptomyces* species, which included the type strains of 10 known phytopathogenic species and six uncharacterized phytopathogenic isolates. The study did not include the *gyrB* gene since the authors observed that the *S. scabiei* RL87.22 genome sequence contained two copies of the *gyrB* operon (one degenerate). Furthermore, Labeda

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(2011) observed that the *gyrB* alignments of previous studies (Guo *et al.*, 2008; Rong *et al.,* 2009; Rong & Huang, 2010) contained some evidence that more than one locus was amplified and sequenced in *Streptomyces*. Labeda (2011) studied not only the type strains of 10 known phytopathogenic species (*S. scabiei, S. acidiscabies, S. europaeiscabiei, S. luridiscabiei, S. niveiscabiei, S. puniciscabiei, S. reticuliscabiei, S. stelliscabiei, S. turgidiscabies* and *S. ipomoeae*), but also 52 other species, including 17 additional type strains that were phylogenetically closely related to the phytopathogenic species, based on 16S rRNA gene sequence analysis. The concatenated four-gene tree showed that the phytopathogenic species are taxonomically distinct from each other despite high 16S rRNA gene sequence similarities. The four-gene tree provided higher bootstrap support for clades than the 16SrRNA tree and also clearly separated *S. scabiei* from *S. europaeiscabiei.* The study that is in agreement with previous 16S rRNA studies, showed that most scab-causing spp. grouped within the so-called *S. diastatochromogenes* 16S rRNA clade. The remaining species, *S. acidiscabiei, S. niveiscabiei* and *S. puniciscabiei* were phylogenetically distant from the *S. diastatochromogenes* cluster. The *Streptomyces* spp. that was most distantly grouped from the *S. diastatochromogenes* 16S rRNA clade, was *S. luridiscabiei* (synonym of *S. microflavus*) that fitted into the *S. griseus* 16S rRNA clade (Labeda, 2010).

HOST RANGE

Although potato is the best known host of common scab-causing *Streptomyces* spp., these pathogens can also affect other crops. The fleshy roots of turnip (*Brassica rapa* L.), radish (*Raphanus sativus* L.), beet (*Beta vulgaris* L.), carrot (*Daucus carota* L.) (Janse, 1988), parsnip (*Pastinaca sativa* L.) (Jones, 1953), mangel (Beta macrorrhiza), salsify (*Tragopogon porrifolius* L.) (www.plantclinic.cornell.edu) and rutabaga (*Brassica napus* L. var. *napobrassica* (L.) (Koronowski & Massfeller, 1972; Hooker, 1981) can be affected by common scab pathogens.

A few studies have specifically investigated the pathogenicity of *Streptomyces* isolates towards seedlings of various crops. Hooker (1949) found a reduction in fresh mass of roots of soybean (*Glycine max* L.), pea (*Pisum sativum* L.), wheat (*Triticum sativum* L.), radish and beet seedlings inoculated with *S. scabiei*. Subsequently, it was reported that *S.*

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scabiei (Leiner *et al*., 1996) and *S. acidiscabies* (Loria *et al*., 1996) can cause disease on seedlings of monocot (wheat) and dicot (crucifer and legumes) plants under laboratory conditions. The symptoms included reduction of shoot and root length, radial swelling and tissue necrosis and chlorosis (Leiner *et al*., 1996). In all these studies a positive correlation was demonstrated between pathogenicity of *Streptomyces* species on the respective seedlings and on potato tubers, suggesting a common mechanism of pathogenicity, later elucidated as thaxtomin production (Loria *et al*., 1997).

The crops that are affected by *S. scabiei* in South Africa include potato, beet (Doidge *et al*., 1953) and groundnut (*Arachis hypogaea* L.) (De Klerk *et al*., 1997). The disease is not a major concern on groundnut, but can be of economic significance where potatoes and groundnuts are grown in rotation in the same field.

SCAB SYMPTOMS ON POTATO AND THE SPECIES AND ENVIRONMENTAL CONDITIONS INVOLVED

Symptoms caused by *Streptomyces* spp. on potato are restricted to tubers and only occasionally occur on roots. There have been no reports of systemic infections, although aerial plant parts can show symptoms of stunting and wilting if plant roots are severly affected (Loria *et al*., 1997). Various scab types have been described e.g. common, netted, russet, deep-pitted and acid scab, causing a variety of deep or shallow-pitted lesions on the host plants. Common scab is regarded as the most prevalent type with netted (Labruyère, 1971; Scholte & Labruyère, 1985; Bouchek-Mechiche *et al*., 2000), russet (Harrison, 1962; Bång, 1979; Oniki *et al*., 1986; Faucher *et al*., 1992), deep-pitted (Archuleta & Easton, 1981; Goyer *et al*., 1996) and acid scab (Manzer *et al*., 1977) occurring to a lesser extent in defined areas. The specific species causing different lesion types can vary, and have not been well-studied except for common scab, deep pitted scab and acid scab.

Common scab tuber symptoms can vary in colour from brown to black. The tuber lesion morphology can range from small, raised or superficial, cork-like tissue to large deep

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sunken pits of up to 7 mm in depth only seen on potato tubers (Archuleta & Easton, 1981; Hooker, 1986; Babcock *et al*., 1993).

Netted scab is displayed as superficial lesions on potato tubers with a typical netted pattern, and is also associated with severe potato root necrosis with a resulting yield loss. Cultivar resistance for netted scab seems to be unique and is restricted to a few cultivars (Scholte & Labruyère, 1985; Loria *et al*., 1997). Netted scab has been reported in various parts of Europe e.g. Netherlands, Sweden, Denmark, Norway and Switerzerland (Bång, 1979; Scholte & Labruyère, 1985), under conditions of cool temperatures and high moisture. This is in contrast with common scab that is favoured by conditions of high soil temperatures and low moisture.

Russet scab can easily be confused with netted scab and is also associated with superficial lesions on the tuber surface. However, lesions do not appear to have a distinct pattern and the disease does not affect potato roots (Harrison, 1962). The symptom has been reported in North America (Harrison, 1962), Northern Sweden (Bång, 1979), Japan (Oniki *et al*., 1986) and Canada (Faucher *et al*., 1993). The disease is favoured by high soil moisture and high temperatures, unlike netted scab that prefers lower temperatures. The *Streptomyces* isolates that cause russet scab are associated with lower soil pH levels (pH 5), are stimulated by nitrate ions and not ammonium ions as compared to common scab isolates. The symptom seem to be restricted to specific potato cultivars (Bång, 1979).

Deep-pitted scab is characterized by deep corky pits on the surface of the potato tubers. Archuleta & Easton (1981) managed to isolate six known (*S. atroolivaceous; S. cinerochromogenes; S. corchorusii; S. diastatochromogenes, S. lydicus; S. malachiticus*) and three unknown *Streptomyces* spp. from deep-pitted scab lesions. They concluded that a number of *Streptomyces* spp. probably including *S. scabiei* may cause deep and shallow scab and that environmental conditions played a role in the expression of the symptoms. In a subsequent study Goyer *et al.* (1996) phenotypically compared deep-pitted-scab-inducing Streptomycetes with representative species of the principal plant pathogenic *Streptomyces* spp. The deep-pitted *Streptomyces* species could be distinguished on the basis of their morphological and physiological properties and was thus classified as a new bacterial species

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named *S. caviscabies* (ATCC 51928). The epidemiology of this pathogen has not been studied extensively, but it seems to be similar to that of *S. scabiei* except for its growth at lower pH levels (4.5).

Acid scab was identified as a novel scab problem in Maine (1953), and was characterized by the ability to develop in an acid soil environment (pH below 5.2) (Bonde & McIntyre, 1968; Manzer *et al.*, 1977). Bonde & McIntyre (1968) reported that the unknown streptomycete that they isolated was not *S. scabiei,* and suggested that further tests be conducted to determine its exact position in the genus. They confirmed the ability of this unknown streptomycete to cause scab at low pH levels. In a follow-up survey conducted on the causal agents of potato scab in the Northeast USA, Loria *et al*. (1986) also found evidence of a pathogenic *Streptomyces* isolate that could grow at pH values as low as 4.0. Lambert & Loria (1989b) later described the species as *S. acidiscabies*.

Very little published literature is available on the lesion types caused by *Streptomyces* in South Africa. Gouws (2006) reported the presence of raised (Fig. 3a), superficial (Fig. 3b, deep-pitted (Fig. 3c) and netted/russet lesions (Fig. 3d).

EPIDEMIOLOGY OF COMMON SCAB ON POTATO

Soil inoculum, survival and spread. The progress and severity of common scab epidemics are greatly influenced by the amount and source of inoculum, and its subsequent spread. The level of inoculum present in the soil determines the severity of common scab (Booth, 1970). Initial inoculum density may thus play an important role in development of the disease (Keinath & Loria, 1991). Virgin soils as an inoculum source of common scab have been well-documented (Millard, 1923; Dippenaar, 1933; Lapwood *et al.*, 1971). Some of the first reports on disease incidence on virgin soil were published by Jones & Edson (1901). Lutman (1914) later elucidated the phenomenon by indicating that the normal microflora of practically all soils includes scab-producing *Streptomyces* species. Another source of common scab inoculum can be infected tubers (Booth, 1970; Rowe, 1993). The pathogen is spread by means of infested soil that is transferred to adjacent fields by wind, rain

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and farm implements. It has also been reported that the pathogen can survive passage through the digestive tract of animals and can be disseminated by manure (Rowe, 1993).

Common scab pathogens can survive as spores or mycelium in crop debris and can remain viable in soil from a decade (Kritzman *et al*., 1996) to up to 20 years or more without any potato cultivation (Dippenaar, 1933). Phytopathogenic *Streptomyces* species only produce spores, and not any specialized survival structures. Vegetative mycelium can, however, survive in soil for long periods on decomposing plant material, the roots of living plants and manure (Pemberton, 1994). Even though *Streptomyces* spores can survive in dry soil for long periods, the vegetative hyphae are intolerant of high moisture tensions (Mayfield *et al.,* 1972). The spores differ from hyphae in having an outer sheath, a thicker wall, greater resistance to heat, and resistance to drought. *Streptomyces* spores are not evenly distributed in the soil and occur in small, localised clusters (Mayfield *et al.,* 1972). These clusters are usually associated with debris derived from previous or existing crops. Germination of spores is also enhanced by the close proximity of organic particles. Hyphae produced by the spores develop radially and it is thought that spread is facilitated in this way (Goodfellow & Williams, 1983).

Environmental conditions favouring disease development. Soil pH is the most reliable parameter for predicting common scab. The first investigations in this regard were conducted by Gillespie & Hurst (1918). In accordance with the *in-vitro* growth response of *S. scabiei* to pH, disease development increases with soil pH from 5.0-8.0 (Goto, 1985). Maintaining the pH of soil at 5.0-5.2 can therefore significantly reduce common scab (Rich, 1983), but would obviously be ineffective against acid scab.

Lowering of soil pH has practical limitations as a stragety for management of this disease. The lower pH could aggravate diseases caused by fungal pathogens (Alexander, 1961), and suppress beneficial bacteria (Williams *et al*., 1971). It could furthermore result in reduced availability of nitrogen, calcium, magnesium, phosphorus, potassium, boron and perhaps sulphur, but increased availability of iron, manganese, zinc, aluminium, copper and cobalt (Brady, 1974).

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Soil moisture is also an important factor that influences common scab development. Lapwood & Lewis (1967) observed a close association between the incidence of common scab and low soil moisture during the early stages of tuber formation. This knowledge was used succesfully to control common scab by appropriate irrigation. Therefore, in the United Kingdom and Europe, common scab is controlled largely by specified irrigation schedules (Lapwood, 1966; Lapwood *et al.*, 1970, 1971, 1973; Wellings & Lapwood 1971; Davis *et al.*, 1976; Adams *et al.*, 1987).

Host infection. The common scab pathogen can infect all underground parts of the potato plant (Labruyére, 1971). Only actively growing potato tubers at the tuber initiation phase are infected through young lenticels, wounds and stomata (Labruyére, 1971; De Klerk, 1989). Initial pathogen growth is intercellular after which the actively growing cells are penetrated and destroyed. Symptoms start off as small (5-8 mm), reddish-brown, water-soaked lesions surrounding the infection site. Infection then spreads laterally and colonization of surrounding tissue ensues with the characteristic corky tissue development in the tuber periderm. The irregular corky areas on the tuber surface may coalesce to form irregularly shaped patches that are usually tan to brown in colour and rough in texture (Rich, 1983). As disease progresses, the corky patches can extend into the tuber tissue taking on a star-like appearance. Pathogen progression can extend even further into the tuber resulting in shallow or deep-pitted scab (Archuleta & Easton, 1981; Ndowara *et al.*, 1996). When the lesions are excised the underlying flesh appears straw-coloured and somewhat translucent (Rich, 1983).

PATHOGENICITY DETERMINANTS IN *STREPTOMYCES*

Thaxtomin production. Although the involvement of a phytotoxin as a pathogenicity determinant in common scab pathogens was suspected, it was not until 1989 that phytotoxic compounds involved in symptom development could be isolated and identified. King *et al.* (1989) and later Lawrence *et al.* (1990) managed to isolate and fractionate two active compounds, designated thaxtomin A and B. The compounds were characterised as unique 4-nitroindol-3-yl containing 2,5-dioxopiperazines, that could be consistently separated from the diseased plant and then used to reproduce the disease in healthy plants thereby satisfying all of the basic criteria of vivotoxins. Thaxtomin A is the predominant phytotoxin produced

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by both *S. scabiei* and *S. acidiscabies* in potato tissue, however minor amounts of other related compounds have been isolated and characterised (Healy & Lambert, 1991; King, *et al*., 1991; King & Lawrence, 1996).

Subsequent to the purification and identification of thaxtomins, investigations continued that found more support for the importance of thaxtomin as a pathogenicity factor. Several studies showed that the production of thaxtomins is only associated with *Streptomycetes* that are pathogenic towards potatoes, thus supporting the importance of thaxtomin production in pathogenicity (Healy & Lambert, 1991; King *et al.,*1991;). Based on this evidence and the genetic diversity in *Streptomyces* species causing scab, it was suggested that thaxtomin biosynthetic genes may have been transferred horizontally among soil inhabiting *Streptomyces* spp. living in close association with potato tubers, and that plant pathogenicity arose more than once within the genus *Streptomyces* (Loria *et al.,* 1995). Further evidence in support of the importance of thaxtomin as a pathogenicity determinant were the findings that (i) the quantity of thaxtomin is associated with the virulence of isolates (Loria *et al.,* 1995) and (ii) mutagenic studies showing that although a few mutated isolates were still pathogenic, they were less virulent than the original isolates, whereas one of the mutants was non-pathogenic (Goyer *et al*., 1998).

Thaxtomin production in streptomycetes can be studied relatively easily using *in-vitro* culture conditions. Pathogenic *S. scabiei* produce phytotoxins, with potato scab inducing activity, when grown on oatmeal agar medium or in oatmeal broth medium (Babcock, *et al*., 1993). The thaxtomin is secreted when cells reach the late exponential to early stationary phase of culture growth (Babcock *et al*., 1993). The presence of thaxtomin can be investigated using standard chromatographic analyses of culture filtrates. Goyer *et al*. (1998) showed that a simplified technique can be used that consists of growing isolates on oat bran agar, with thaxtomin production being indicated by the presence of a yellowish halo surrounding the *Streptomyces* colonies. However, Goyer *et al*., (1998), reported that *S. caviscabies* isolates do not produce thaxtomin on oat bran medium, but will do so on potato slices/peels.

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The biosynthesis of thaxtomin involves several genes. Conserved non-ribosomal peptidase synthetases (NRPS), encoded by the *txtA* and *txtB* genes, are responsible for the production of a N-methylated cyclic dipeptide, the backbone of the toxin. A P450 mono-oxygenase, encoded by *txtC,* is required for the post-cyclization hydroxylation steps (Healy *et al*., 2002). Nitric oxide synthases (NOSs), which are located on the same genome region as the *txtA* and *txtB* genes, are important for the nitration of thaxtomin. These genes have high sequence similarity to the oxygenase domain of mammalian genes. NOSs may have a second function, which is the modulation of host responses (Kers *et al*., 2004; Loria *et al*., 2008). Johnson *et al*. (2007) was the first to suggest this function when they showed that plant pathogenic *Streptomyces* spp. also produce NOS-derived NO at the host-pathogen interface. Since NO is an important signalling molecule in plants (Wilson *et al*., 2007), it has been suggested that NO produced by pathogenic streptomycetes, modulates signalling pathways in the host (Loria *et al*., 2008). The regulator of thaxtomin production is the *txtR* gene that is imbedded in the thaxtomin biosynthetic pathway, and it is thus also located on the PAI. TxtR is regulated by cellobiose and belongs to the AraC/XylS family of proteins (Joshi *et al*., 2007).

Several compounds are important in the induction and repression of thaxtomin production. Babcock *et al*. (1993) showed that glucose, tryptophan and tyrosine repress thaxtomin production. This finding is consistent with the repression of secondary metabolites by other species of *Streptomyces,* and phytotoxins by other bacteria. The repression of thaxtomin production by glucose could explain why *S. scabiei* infection only occurs early in tuber development, since the glucose level in the potato peel during tuber development is less than 0.1 %. This level of glucose does not suppress thaxtomin production, and would thus allow phytotoxin production. Wach *et al*. (2007) first hypothesized that complex carbohydrates are important in the induction of thaxtomin biosynthesis, and that these compounds may act as environmental signals to plant pathogenic *Streptomyces* that enable host colonization by the pathogen. This was confirmed by Johnson *et al*. (2008) who showed that cellotriose was a more effective inducer of thaxtomin than cellobiose. This suggests that the release of this simple sugar trimer is an important plant produced signal molecule that is involved in regulating pathogenicity in *S. scabiei* and other pathogenic *Streptomyces* spp.

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The discovery of a pathogenicity island (PAI). The importance of thaxtomin as a pathogenicity determinant is well recognized, but it was suspected that more genes are involved in pathogenicity. The first evidence for this came from the study of Bukhalid & Loria (1997) that showed that the insertion of a 9.4-kb DNA fragment, of which 1.6-kb was essential, from a pathogenic *S. scabiei* isolate into the non-pathogen *S. lividans,* resulted in *S. lividans* being able to necrotize and colonise potato tuber slices and produce scab like symptoms on potato mini tubers. The symptoms were, however, less severe than those produced by *S. scabiei.* The 1.6-kb region contained three open reading frames (ORF), i.e. ORF*tnp* with high sequence identity to the putative transposases of the *IS1164* element, an ORF designated as *nec1* that has necrogenic activity and ORF2 that was an incomplete ORF. The G+C content of *nec1*, which differs from the overall G+C content of *S. scabiei,* supported the hypothesis that *nec1* might have been mobilised into *S. scabiei* through a transposition event mediated by ORF*tnp* (Bukhalid & Loria, 1997). In a follow-up study, Bukhalid *et al*. (1998) found that both *nec1* and *ORFtnp* occurred in all thaxtomin-producing *Streptomyces* isolates and that the nucleotide sequences of the homologues of *nec1* and ORF*tnp* from *S. scabiei, S. acidiscabies* and *S. turgidiscabies* were identical. It was therefore proposed that *nec1* and ORF*tnp* were horizontally mobilized from *S. scabies* to *S. acidiscabies* and *S. turgidiscabies.* Investigations into the genetic organization of regions adjacent to the 3' end of *nec1* in *S. scabiei,* revealed the presence of a new insertion sequence (IS) element, IS1629. IS1629 was present in multiple copies in *S. scabiei, S. acidiscabies* and *S. turgidiscabies*. This finding also supported *S. scabiei* as the donor species and a unidirectional transfer model of the ORF*tnp*-*nec1*-IS1629 locus from IS1629-containing *S. scabies* to *S acidiscabies* and *S. turgidiscabies* (Healy *et al*., 1999).

Further evidence supporting the presence of a PAI in phytopathogenic *Streptomyces* isolates was attained when larger DNA fragments were examined. Bukhalid *et al*. (2002) found that a 26-kb DNA fragment, including and flanking the virulence gene *nec1*, was conserved among *S. scabiei* and genetically distinct *Streptomyces* species in the Diastatochromogenes cluster, providing further evidence for the horizontal transfer of a PAI. This also indicated that PAI transfer occurred frequently within and among species closely related to *S. scabiei*. The full description of the PAI (325-660 kb) was published by Kers *et al*. (2005), and was the first PAI described in a Gram-positive plant pathogenic bacterium. Mobilization of the PAI (660-kb) through mating from *S. turgidiscabies* to the non-pathogens

S. coelicolor and *S. diastatochromagenes* resulted in a pathogenic phenotype in *S. diastatochromogenes* but not *S. coelicolor* (Kers *et al.*, 2005).

Partial sequencing of the PAI revealed the presence of the thaxtomin biosynthetic pathway, *nec1*, a putative tomatinase (*tomA*) gene and many mobile genetic elements (Kers *et al.,* 2005). The presence of the tomatinase gene is interesting, since it is a well-characterized enzyme from plant pathogenic fungi that detoxifies the anti-microbial saponin, α-Tomatin (Kers *et al.,* 2005), and has been shown to be functional in *S. scabiei* (Seipke & Loria, 2008). α-tomatin assist plant pathogenic fungi such as the tomato pathogens *Septoria lycopersici* and *Fusarium oxysporum* f. sp. *lycopersici* (Seipke & Loria, 2008) by suppressing plant defence responses (Bouarab *et al*., 2002). A homologue of tomatinase has also been identified in *Clavibacter michiganensis* subsp. *michiganensis* (Kaup *et al*., 2005). The importance of the *tomA* in common scab pathogenicity has not been established, but according to Seipke & Loria (2008), the conservation of the gene on a pathogenicity island in *S. acidiscabies* and *S. turgidiscabies* suggests a role in the plant-microbe interaction.

Presence of known pathogenicity determinants in *Streptomyces* species and their use for pathogen detection. Several studies have investigated the presence of the *nec1, tomA* and *txtAB* genes in scab-causing *Streptomyces* species. The presence of these genes may not only hint at their role in pathogenicity, but the universal occurrence could also help to identify and quantify these pathogens from environmental samples.

Investigations into the *nec1, tomA* and *txtAB* genes have shown that the association of these genes with pathogenicity of *Streptomyces* isolates was highest for *txtAB*, followed by *nec1* and then *tomA*. Most studies have found that *txtAB* is present in *S. scabiei, S. acidiscabies* and *S. turgidiscabies* isolates (King *et al*., 1991; Bukhalid *et al*., 1998; Healy *et al*., 2000; Loria *et al*., 2003; Loria *et al*., 2006; Wanner, 2006; Loria *et al*., 2008; St-Onge *et al*., 2008; Qu *et al*. 2008, Wanner, 2009). However several studies have reported that some pathogenic *Streptomyces* isolates, including isolates beloning to *S. luridiscabiei, S. puniciscabiei* and *S. reticuliscabiei,* do not produce thaxtomin (Tashiro *et al*., 1990; Natsume *et al*., 1998; El-Sayed, 2000; Park *et al*., 2003, Wanner 2004; Bouchek-Mechiche *et al*., 2006; Flores-Gonzalez *et al*., 2008). The absence of thaxtomin production in pathogenic

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Streptomyces species could be due to the prolonged preservation and/or repeated subculture of pathogenic isolates, leading to loss of thaxtomin production (El-Sayed, 2000). Alternatively, this may suggest that additional pathogenicity factors or determinants are involved in the disease etiology (Kinkel *et al*., 1998; Leiner *et al*., 1996). The *nec1* gene has been reported in several studies as being absent from pathogenic *Streptomyces* spp. suggesting that it is not essential for pathogenesis (Bukhalid *et al*., 1998; Kreuze *et al*., 1999; Park *et al*., 2003; Wanner, 2004; Wanner, 2007). Although Cullen & Lees (2007) stated that there was a clear correlation between pathogenicity and the presence of the *nec1* gene in the 32 isolates that they investigated, this deviates from the finding of most other publications. This incorrect statement is also reflected in their own finding that a pathogenic *S. albidoflavus* and an uncharacterized pathogenic strain did lack the *nec1* gene.

The *nec1* and *txtAB* genes have been evaluated for detection and quantification of scab-causing *Streptomyces* species from environmental samples. Cullen & Lees (2007) evaluated *nec1* as a marker for the detection of pathogenic *Streptomyces* on potato tuber and soil samples by means of conventional and real-time PCR. They concluded that this method provided a reliable and quantitative technique for detecting pathogenic *Streptomyces* species (Cullen & Lees, 2007), even though several other studies have reported a lack of association of *nec1* with pathogenicity (Bukhalid *et al*., 1998; Kreuze *et al*., 1999; Park *et al*., 2003; Wanner, 2004; Wanner, 2007). Therefore, it is unlikely that the *nec1* gene can be used for detection and quantification of scab-causing isolates in environmental samples. Qu and colleagues (2008) developed a real-time PCR assay using primers that targeted the *txtAB* operon in plant pathogenic *Streptomyces* species. The assay was successful in accurately and reliably detecting and quantifying pathogenic *Streptomyces* species in potato tubers and soil.

Complete genome sequences. The genomes and draft genomes of several *Streptomyces* species are now available, and will allow further investigations among pathogens, and between pathogens and non-pathogens. This will make it possible to garner an understanding of the mechanisms and evolution of plant pathogenicity in the *Streptomyces* genus. The first complete *Streptomyces* genome that was published was that of a non-pathogenic *Streptomyces* species (*S. coelicolor*) (Bentley *et al*., 2002). The *S. coelicolor* chromosome is 8,667,507 bp long, has a linear structure and a G+C content of 72.1 %. Subsequently, the whole genome of *S. avermitilis,* an important industrial species, was

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published by Ikeda *et al*. (2003). Recently the *S. scabiei* (strain 87.22) genome was sequenced and was shown to consist of 10,148,695 bp with a G+C content of 71.45 % (Bignell *et al*., 2010). The draft genome of *S. turgidiscabies* (species Car8) is also now available and was used by Huguet-Tapia *et al*. (2010) to expand on investigations of the PAI in this species. The draft genome sequence confirmed the findings of Kers *et al.* (2005), who first characterized the *S. turgidiscabies* PAI. The *S. turgidiscabies* PAI consists of two non-overlapping modules of ~105 kb and ~568 kb, of which the ~105 kb module is identical to the genomic island in *S. scabiei* 87.22, but the ~588kb module only has a short region of synteny with *S. scabiei* (Fig. 4; Huguet-Tapia *et al*., 2010).

INDUCED RESISTANCE

Induced resistance in plants is a "physiological state of enhanced defensive capacity" (Choudhary *et al*., 2007). Initially the phenomenon was referred to as acquired physiological immunity and the serological basis of the resistance was debated until Ross (1961) could prove the concepts of localized acquired resistance and systemic acquired resistance. Induced resistance in plants can be elicited by specific environmental stimuli and it readies the plant for potential biotic and/or abiotic challenges. Biotic factors that trigger systemic acquired resistance include a range of pathogens (fungi, bacteria, viruses), nematodes, parasitic plants and insect herbivores (Hammerschmidt & Kuc, 1995; Sticher *et al*., 1997; van Loon *et al*., 1998; Walling, 2000; Heil & Bostock, 2002).

There are two clearly defined forms of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Loon *et al*., 1998). The two resistance mechanisms can be distinguished from each other by the nature of the elicitor and the regulatory pathways involved. SAR can be triggered when plants are exposed to virulent, avirulent or non-pathogenic microbes. Establishment of the SAR takes place over a specific time period wherein pathogenesis-related proteins (chitinase and glucanase) as well as salicylic acid are accumulated. ISR, on the other hand, is potentiated by plant growth promoting rhizobacteria (PGPR) and does not involve the accumulation of chitinase, glucanase and salicylic acid (Pieterse *et al*., 2000). Rhizobacteria-mediated ISR is regulated by jasmonic acid and ethylene signalling in the plant. When the jasmonic acid pathway is

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triggered, a cascade of pathogenesis-related proteins is produced e.g. oxidative enzymes. However, with our increased knowledge concerning the regulation of induced resistance, it has become clear that the delineation of ISR and SAR based on the nature of the elicitor and the regulatory pathways involved is not clear cut. For example, ISR induced by some *Bacillus* strains requires salicylic acid and not jasmonic acid (Ryu *et al.*, 2003; Bostock, 2005). The following discussions will only focus on ISR.

Induced systemic resistance (ISR). Several antagonistic PGPR, very often isolated from suppressive soils, elicit ISR in host plants (Harman *et al*., 2004). The process of ISR in plants has three phases; (i) induction, (ii) signalling and (iii) expression. In the induction phase, several bacterial determinants are involved in triggering ISR. Cell surface components that can trigger ISR include lipopolysaccharides and flagella (Erbs & Newman, 2003). Other determinants that have been reported include 2,3-butanediol, pyochelin, pyocyanin, 2,4 DAPG, siderophores and Fe-regulated compounds (Audenaert *et al*., 2002; Ramos-Solano *et al*., 2010). It is important to note that rhizobacterial strains from the same species can differ considerably in their ability to induce resistance (Bostock, 2005). Following the induction phase of ISR, the signalling phase is a very complex process and multiple pathways are involved. Studies using *Arabidopsis* signalling mutants have shown that the ISR and SAR pathways converge at the point of the transcriptional regulator NPR1 (Bostock, 2005; Choudhary *et al*., 2007). The expression phase of ISR is usually depicted by reduced disease severity and a decrease in the number of diseased plants (Choudhary *et al*., 2007). The reduction in disease severity is usually associated with a decrease in pathogen growth as well as a reduction in colonization of induced plant tissues, indicating the ability of the plant to resist the pathogen (Choudhary *et al*., 2007).

Induced resistance triggered by *Streptomyces*. *Streptomyces* species have only recently been investigated for their role in induced resistance and ISR. Galal (2006) screened nine foliage-applied *Streptomyces* species for their antiphytoviral activity against cucumber mosaic virus (CMV). *Streptomyces* culture filtrate applications resulted in a 50-85 % reduction in CMV incidence when applied before viral inoculation. *Streptomyces* have also been shown to suppress bacterial pathogens through induced resistance. *Streptomyces* strain RS70 that was syringe infiltrated on the top leaves of tomato plants, 7 days prior to inoculation of the pathogen *Ralstonia solanacearum* onto the roots of the tomato plants,

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significantly reduced bacterial wilt disease incidence. Northern blot analysis showed that PR-1 gene expression was induced in non-inoculated leaves in plants that were treated with *Streptomyces* RS70 (Teng *et al*., 2006). Shimizu *et al*. (2000) showed that treatment of tissue-cultured rhododendron seedlings with non-antagonistic endophytic *Streptomyces* spp. induced disease resistance against *Pestalotiopsis sydowiana* (causal agent of pestalotia disease). They demonstrated that the seedlings accumulated anthocyanin(s), which suggested that the resistance induction depended on activated defence responses associated with the phenylpropanoid pathway rather than antibiosis. Lehr *et al*. (2007) co-inoculated *Streptomyces* sp. GB 4-2 and *Heterobasidion abietinum* [root and butt rot of Norway spruce (*Picea abies*)] on spruce seedlings, which resulted in a marked reduction of disease incidence. They discovered complex interactions between the *Streptomyces* strain and the pathogen, which indicated that *Streptomyces* GB 4-2 induced both local and systemic defence responses in Norway spruce.

ISR in potato. Rhizobacteria-mediated induced systemic resistance has been studied extensively in various plant systems, however, only a few studies have been conducted in potato plant systems. In one of the first studies on induced systemic resistance on potatoes, live and heat-killed *Rhizobium etli* G12 was shown to induce systemic resistance in potato against the nematode, *Globodera pallida* (Hoffman-Hergarten *et al*. 1997). Hasky-Günther *et al*., (1998) went on to conduct a similar split-root trial to study the ability of rhizobacteria (*Bacillus sphaericus* strain B43 and *Agrobacterium radiobacter* strain G12) to stimulate induced systemic resistance against *G. pallida*. They were able to demonstrate that live and heat-killed bacterial cells of both strains were able to induce systemic resistance and result in a significant reduction of root penetration by *G. pallida* juveniles. It was later confirmed that the lipopolysaccharides of *R. etli* G12 act as the inducing agent of systemic resistance in potato roots (Reitz *et al*., 2000). There has only been one study conducted on induced systemic resistance of common scab on potato (Singhai *et al*., 2011). In this study four Pseudomonad strains were used with or without vermicompost amendments to detect their efficacy in reducing common scab. The results revealed that *Pseudomonas mosselii* strain R1 was able to induce antimicrobial systems and significantly reduce disease incidence when applied to the soil with vermicompost.

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MANAGEMENT STRATEGIES TO REDUCE THE INCIDENCE OF COMMON SCAB

Since effective management strategies for common scab are limited, an integrated disease management (IDM) strategy has to be implemented. Such a strategy is likely to include options such as; (i) irrigation scheduling, (ii) cultivar tolerance, (iii) agro-chemical applications, (iv) crop rotation, (v) green manuring, (vi) *Brassica* amendments (green manure and seed meals) and (vii) amendments other than green manures. A single measure management approach or practice will not be effective for obtaining economically acceptable disease suppression. For example, combining crop rotation (barley and rye) with biological amendments (aerobic compost tea) can result in a significant reduction in common scab incidence, in comparison to the individual application of these strategies (Larkin, 2008)

Irrigation scheduling. Managing common scab disease incidence by means of irrigation scheduling during tuber initiation has been widely applied as an effective control measure for common scab under greenhouse and field conditions (Adams *et al*., 1987). This is due to the fact that (i) pathogenic *Streptomyces* spp. only infect tubers during the tuber initiation phase when lenticels and small growth cracks are present that serve as entry and infection points for the pathogen and (ii) infection during tuber initiation is highly influenced by soil moisture.

As early as the 1970's, observations were made that common scab incidence was high when tuber initiation was marked by dry soil conditions (Lapwood & Hering, 1968; Lapwood & Hering, 1970; Adams & Lapwood, 1978). A series of studies were conducted to investigate the association between soil moisture levels during the tuber initiation phase of potato (*Solanum tuberosum* L.) and the incidence and/or severity of common scab (Lapwood, 1966; Lapwood & Lewis, 1967; Lapwood & Hering, 1970; Lapwood *et al*., 1971; Lapwood & Adams, 1975; Adams *et al*., 1987). It was confirmed that high soil moisture levels during the tuber initiation phase reduced the incidence of common scab, whereas low soil moisture levels during the same period seemed to increase the incidence and severity of the disease. Thus, common scab incidence was inversely related to soil moisture content (Lapwood & Hering, 1968; Singh & Singh, 1981). Several theories were put forward to describe the

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reduction in disease incidence at high soil moisture levels including, the effect of rapid lenticel proliferation, subsequent lowering of soil temperature and reduced oxygen availability to the pathogen, changes in antagonistic microbial activity in soil as affected by the increase in soil moisture and the decrease of calcium levels in the tuber tissue (Labruyère, 1971; Adams, 1975; Davis *et al*., 1976).

Cultivar tolerance. Although common scab resistant/tolerant cultivars have formed an important part of various strategies in managing this disease in commercial and emerging potato production systems, they have some limitations. A lot is known about the variation in response of cultivars to common scab infection, which has allowed for the selection of tolerant cultivars (Goth *et al*., 1993). Even though tolerant cultivars remain an essential factor in managing common scab, only a few tolerant cultivars are commercially viable and the tolerance of these cultivars is not always durable (Haynes *et al*., 2010; Gouws & Mcleod, 2012). For example, in South Africa, Mondial is the potato cultivar most tolerant to common scab and therefore also the cultivar most planted. However, Mondial has shown an increase in common scab disease incidence over the past 10 years of cultivation which is causing serious concerns in the potato production industry in South Africa (unpublished data). Several common scab screening programs are currently employed by potato breeders and have resulted in the identification of new tolerant cultivars.

Various strategies have been employed to evaluate common scab resistance of potato cultivars. Schaal *et al.* (1953) made use of the ferric chloride test based on the measurement of chlorogenic acid present in cells adjacent to the periderm of resistant cultivars and absent in susceptible cultivars. In other programs, promising cultivars are pre-screened in a greenhouse or tunnel. In South Africa the double pot technique, developed by Marais & Vorster (1988) is followed for pre-screening of promising lines and cultivars. Successful lines are usually evaluated further in scab infested fields (Goth *et al*., 1993; Mishra & Srivastava, 2001; Lambert *et al*., 2006).

Agro-chemical application. Several agro-chemical formulations have been evaluated for controlling common scab. The formulations can be applied as foliar sprays (McIntosh, 1979; McIntosh & Burrel, 1980; McIntosh *et al*., 1981; 1982; 1988), tuber treatments (Singh & Soni, 1987; Wilson *et al*., 1999; Pung & Cross, 2000) and soil treatments (Vashisth *et al*.,

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1990; De Klerk & Engelbrecht, 1996), depending on the source of inoculum and level of infestation in the field. Most formulations are applied as soil or seed treatments before planting to reduce the risk of infection, and to limit disease spread since pathogenic *Streptomyces* spp. are usually introduced into clean soils by means of infected seed (Dippenaar, 1933). The most widely used soil treatment is quintozene (PCNB) (Vashisth *et al*., 1990; Wilson *et al*., 1999). The only chemical that is presently registered for the control of soilborne inoculum of common scab in South Africa is quintozene, whereas flusulfamide and mancozeb are registered as tuber treatments (Nel *et al*., 2003). These chemicals are not always effective and are often very costly (unpublished data).

Crop rotation. Rotation cropping is aimed at reducing disease incidence through a reduction in inoculum, since monoculture cropping is usually associated with an increase in pathogen inoculum. In common scab control, this becomes a controversial issue since although short monoculture cycles do lead to an increase in scab incidence (Werner *et al*., 1944), long monoculture cycles can have the opposite effect. Prolonged monoculture of potatoes in a scab conducive soil can lead to a suppressive soil condition (Lorang *et al*., 1989). Short rotations with non-host crops between potato plantings seemed to result in severe scab incidence (Goss & Afanasiev, 1938). Although longer rotation cycles of 4 to 6 years can have a reducing effect on disease incidence (Goss & Afanasiev, 1938), the longer rotation cycles tend to be economically non-viable and very often impractical.

The choice of rotation crop is often challenging since various contradictory findings have been published. This led to the hypothesis that the duration of the rotation cycle was key to successful common scab reduction, rather than the specific crop used (Heeg & Richardson, 1958). Several crops have, however, been identified that can lead to an increase in scab incidence. Wheeler (1964) found that rotation with alfalfa had no influence on scab incidence but that a 2-year sweet clover rotation increased scab incidence. In a similar study Weinholdt *et al*. (1964) reported that there was no difference between alternate year rotations of barley, cotton or sugar beet, but that a 3-year alternate rotation of potatoes, sugar beet and cotton resulted in a rapid increase in scab disease incidence that progressed more rapidly than a rotation with monoculture potato plantings.

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Green manuring. In contrast with crop rotation, green manuring involves the incorporation of cover crops into diseased soils. This practice has the potential to reduce soilborne disease incidence (Mishra & Srivastava, 2004; MacGuidwin *et al*., 2012). It is important to note, however, that pathogen inoculum does not always decrease, even though yield is increased and that the crop utilized as green manure plays an important role (Larkin, 2008). Lazzeri *et al*. (2000) described the reduction of *Pythium* spp. by means of *Cleome hassleriana* green manure in infested soil, but noted that treatment with common green manure crops (non-*Brassica*) increased *Pythium* propagules four-fold.

Several green manure crops have been investigated for their efficacy in suppressing common scab of potato. In one the earliest studies, Millard (1923) demonstrated that the addition of various green manure crops could significantly reduce the incidence of common scab on potato tubers. He conducted several experiments, including amendments with mustard, rye and ryegrass, which showed that incorporation of all of these amendments, resulted in reduced scab incidence. Subsequently, a variety of plants have been studied as possible green manure crops, with contradictory results on the ability of crops (mainly rye, barley and soybean) to reduce disease incidence. Although White (1928) found that incorporation of rye can reduce scab, other studies showed that it had no effect on scab incidence (Sanford, 1926). Another grass species, barley, was even found to increase scab (Weinhold *et al*., 1964; Oswald & Lorenz, 1956). A soybean green manure has mostly been found to reduce scab incidence (Oswald & Lorenz, 1956; Weinhold *et al*., (1964), although Rouatt & Atkinson (1950) found that it only helped in preventing inoculum build-up, but not in reducing scab incidence. Mishra & Srivastava (2004) conducted a comprehensive study on the incorporation of several crops (soybean, sanayee (*Crotolaria juncea*)) and dhaicha (*Sesbania rostrata*)) to reduce scab incidence. They found that soybean green manure was most effective in reducing scab incidence (73 %), followed by sanayee (66 %) and dhaicha (58 %).

Several theories have been proposed for the mechanisms involved in green manure suppression of common scab. Millard (1923) discussed two possible theories, i.e the "Soil reaction theory" (as hypothesized by Gillespie & Hurst (1918)) and the "Preferential food theory" (as proposed by Millard, 1922). The soil reaction theory was based on the hydrogen-

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ion exponent of the various soils. The rationale was that if the soil had a hydrogen-ion exponent of 5.2 or lower, it rarely produced scab, whereas soils with higher hydrogen-ion exponents generally were conducive to scab development. Millard (1923) rejected the theory and stated that some neutral and alkaline soils could produce both clean and scabbed potatoes and therefore soil reaction could not be the determining factor for common scab disease incidence. The Preferential food theory, also known as the "Decoy" theory, was proposed by Millard (1922). It states that when a sufficient quantity of green matter is introduced into a common scab infested soil and incorporated thoroughly, disease incidence would be reduced. The assumption was made that causal agent(s) are primarily saprophytic micro-organisms that feed on decomposing tissue and thus prefer the incorporated green manure crop over the actively growing potato host. Millard (1923) conducted various experiments to substantiate the theory and concluded that this was the most plausible and supported theory.

Another explanation for the reduction of common scab by means of green manure can be the concept of increased antagonism of non-pathogenic soil microbes, including non-pathogenic *Streptomyces*. It has been shown that a green manure crop of canola, sudan grass or buckwheat can cause an increase in the antagonistic *Streptomyces* populations in soil and a reduction in common scab incidence (Wiggins & Kinkel, 2005a, b). When dried grass meal was added to scab infested soils as green manure a significant reduction in disease incidence was reported (Rogers, 1969). This finding could not be ascribed to an increase in soluble manganese, but a marked increase in soil microbial populations was observed as a result of the grass amendments. A significant correlation was established between soybean green manure incorporation and a selective increase in bacterial soil populations with a subsequent reduction in scab incidence. A significant increase in the actinomycete population was also observed (Mishra & Srivastava, 2004).

In an attempt to elucidate the changes in common scab disease incidence due to amendments with alfalfa, rye and wheat straw, Adams *et al.* (1970) conducted a study on the relationship of soil ammonia, nitrates, soil pH, soil temperature and soil moisture with scab incidence after green manuring. They found changes in soil properties in response to amendment incorporation, with the most consistent correlation being that between nitrate levels and the incidence of russet scab. With higher levels of nitrate, lower levels of russet

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scab incidence were recorded. No significant changes in soil pH, soil temperature or soil moisture was observed (Adams *et al.*, 1970).

***Brassica* amendments (green manures and seed meals)**. *Brassica* amendments have been studied since the 1960s for their positive effect on the suppression of plant diseases. Papavizas (1966) already showed suppression of soilborne diseases by incorporating *Brassica* tissue into infested soil in the 1960s (reference cited by Lewis & Papavizas, 1970). In the early 1970's, Lewis & Papavizas (1970) investigated the emission evolution of volatile sulphur compounds from the decomposition of crucifers in soil. Subsequently, rotation with *Brassicaceae* crops and incorporation of *Brassica* residues into soil (biofumigation) (Kirkegaard *et al.*, 1993; Angus *et al.*, 1994) has been reported to suppress a variety of pests and pathogens, including fungi, bacteria, nematodes, insects and weeds.

Biofumigation has been successfully applied in potato production systems for the control of wireworms (Toba, 1984), root-knot nematodes (Motjahedi *et al.*, 1993), weeds (Boydston & Hang, 1995) and wilt caused by *Verticillium dahliae* Kleb. (Davis *et al.*, 1996). Several other potato pathogens have also been suppressed by biofumigation, e.g. *Fusarium sambucinum* (Mayton *et al.*, 1996), *Sclerotium rolfsii* (Stapleton & Duncan, 1998), *Rhizoctonia solani* AG-3 and AG-8 (Lewis & Papavizas, 1974; Sarwar *et al.*, 1998; Harding & Wicks, 2000), *Colletotrichum coccodes*, *Phytophthora erythroseptica*, *Phytophthora cryptogea* (Harding & Wicks, 2000) and *Ralstonia solanacearum* (Akiew *et al.*, 1996). There have also been a few reports on common scab reduction on potatoes (Larkin & Griffen, 2007; Larkin, 2008). In South Africa, greenhouse and tunnel trials showed that *Brassicaceae* residue amendments (fresh and dry) can cause a reduction in common scab incidence on potatoes (Gouws & Wehner, 2004).

Disease suppression by means of biofumigation is based on the presence of ß-D-thioglucosidic compounds known as glucosinolates (GSLs) in the *Brassicaceae* that include broccoli (*Brassica oleracea* var *italica*), cauliflower (*Brassica oleracea*), mustard (*Brassica juncea*)], rapeseed/canola (*Brassica napus*), and horseradish (*Armoracia rusticana*) and other families of the order Capparales (Brown & Morra, 1997; Kirkegaard & Sarwar, 1998). Glucosinolates, which contain sulphur, nitrogen and an R group derived from glucose, also

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give members of the family *Brassicaceae* their characteristic pungent odour (Brown & Morra, 1997). Non-toxic GSLs are hydrolysed in the presence of water and myrosinase (thioglucoside glucohydrolase) to produce biologically active compounds such as organic cyanides, ionic cyanate, oxazolidinethiones and isothiocyanates (ITCs). Myrosinase is an enzyme, which occurs endogenously in *Brassica* tissues (Brown & Morra, 1997). The types of compounds produced, and their spectrum of biological activity, are specific to the respective glucosinolates present in the tissue and conditions under which hydrolysis occurs (Gil & Macleod, 1980; Macleod & Rossiter, 1986). The myrosinase enzyme and GSLs are separated in intact plant tissue and only come into contact as a result of cell disruption in response to stress (biotic or abiotic) or injury. Under these conditions the enzymatic hydrolysis of GSLs results in the production of degradation products of which ITCs are considered the most toxic. ITCs are classified as general biocides that can interact non-specifically and irreversibly with proteins and amino acids (Fenwick *et al*., 1983; Kawakishi & Kaneko, 1987).

Various factors can influence the efficacy of biofumigation. The concentration profile and distribution of GSLs and type of hydrolysis products vary between the *Brassica* species, tissue type and physiological plant age, and these variables can thus all influence the efficacy of biofumigation (Mithen, 1992). GSL extracts from the same *Brassica* species may thus inhibit or stimulate the target organism, depending on the plant part, growth stage, or various other conditions (Jiménez-Orsonia & Gleissman, 1987). The efficacy of biofumigation can also be affected by temperature (Borek *et al*., 1995), soil type (Mason-Sedun *et al*., 1986; Matthiessen *et al*., 1996), moisture (Borek *et al*., 1995) and organic matter content (Smelt & Leistra, 1974; Borek *et al*., 1995). Optimization of pathogen control would thus include the evaluation of different *Brassica* species, type and age of the tissue, mode of application, environmental and cultural parameters, and moisture content of tissue.

Although the mode of action for suppression of plant diseases by biofumigation is in general attributed to the breakdown of GSLs, some studies show clear evidence for the importance of *Brassicaceae* crops causing a shift in certain microbial populations that are responsible for suppressing pathogens. *Brassica* seed meal can stimulate soil microbial biomass, activity, and diversity, and may enhance populations of microorganisms that are antagonistic to pathogens, or alter microbial communities in other beneficial ways (Cohen *et*

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al., 2005). Davis *et al*. (1996) reported that effective reductions of *Verticillium* wilt of potato using *Brassica* green manures were related to increased microbial biomass, activity, and antagonism towards the pathogen. In comparing different potato cropping systems, Larkin (2003) has documented distinct changes in soil microbial communities related to *Brassica* rotation crops, and that these community characteristics are to some degree associated with disease levels. Smolinska (2000) also noted the role of microbial communities in the suppression of disease following *Brassica* amendments. In the suppression of apple replant disease, Mazzola *et al*., (2001) observed that disease suppression is associated with an increase in saprophytic *Streptomyces* populations.

Although most studies use freshly incorporated *Brassica* tissue, a few studies have used dried *Brassica* material, which were also effective in disease suppression. Lewis & Papavizas (1970) made use of air dried leaf and stem tissue, that were ground and amended to loamy soils at a rate of 5 % mass:volume. The *Brassica* species in this study included low glucosinolate containing types e.g. brussel sprouts [(*Brassica oleraceae* var *gemmifera* DC), cabbage (*Brassica oleraceae* var *capitata* L.), kale (*Brassica oleraceae* var *viridis* L.), turnip (*Brassica rapa* L.)] and high glucosinolate containing *Brassicas* e.g. mustard (*Brassica nigra* L.). They found that the decomposition of dried cabbage leaf and stem tissues resulted in the formation of volatile sulphur-containing compounds, methanethiol (CH_3SH), dimethyl sulphide ($(CH_3)_2S$) and dimethyl disulphide ($(CH_3)_2S_2$), which may be responsible for the reduction or suppression of soil borne diseases. The other *Brassica* species decomposed to yield similar products Lewis & Papavizas (1971) conducted a follow-up study to determine the effect of these sulphur containing volatile compounds and vapours from cabbage decomposition on *Aphanomyces euteiches* (root rot of pea). Volatile isothiocyanates (ITCs) were also measured in this study and it was shown that it reduced oomycete growth to a greater extent than the volatile sulphides and disulphides arising from the microbial decomposition of the material. Volatiles from decomposing cabbage completely prevented growth of the pathogen. The effect was evident from as early as 2 days after amendment and lasted up to 26 days. They were also able to demonstrate that the degree of disease suppression increased long after the volatile ITC was emitted from the system, suggesting that an alternative mechanism likely contributed to the overall reduction in root rot. They went on to speculate that the mechanism of disease reduction might have been entirely or

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partly due to increased antagonistic microbial populations, production of antibiotic materials or increased levels of fungistasis (Lewis & Papavizas, 1971).

Brassicaceae plants have potential to be used in crop rotations as a natural pesticide source, thereby decreasing our dependence on synthetic chemicals. Although being a natural pesticide, glucosinolates are well known for their toxic effects (mainly as goitrogens) in both man and animals at high dosages. At high concentrations, ITCs are also general biocides that behave much like commercial pesticides. In fact, several commercial soil fumigants including Dazomet, Vapam, and Vorlex possess ITC-generating chemistries as the mode of action for pest control. At low concentrations ITCs are considered beneficial to human health and are under investigation for the prevention of cancer (with sulforaphane in broccoli being the best known). Additional benefits from the incorporation of *Brassica* residues are that it can have long- and short-term consequences on soil and plant health by influencing complex biological and chemical interactions (Snapp & Borden, 2005; Snapp *et al*., 2007).

Amendments other than green manures. Various types of amendments have been evaluated for their efficacy in reducing common scab incidence on potatoes. The amendments can be grouped into the following categories: (i) high nitrogen amendments, (ii) animal manures, (iii) compost and compost teas, (iv) biological inoculants and (v) natural polymers.

i. High nitrogen amendments. High nitrogen amendments include bloodmeal, fishmeal (Wilhelm, 1951), soymeal, poultry manure (Conn & Lazarovits, 1999), meat and bone meal (Lazarovits *et al*., 1999). A significant reduction in common scab disease incidence was achieved by amending soil with meat and bone meal, soymeal and poultry manure at rates of 37 ton/ha, incorporated to a depth of 15 cm (Lazarovits *et al*., 1999). It was hypothesized that the mechanism of control was in part reliant on the ability of the soil to accumulate ammonia and that the ammonia had a direct toxic effect on the pathogen population. However, it is important to note that the efficacy of these materials for disease control was dependent on soil pH since there was an elevation of soil pH from 6.0-8.5 and a corresponding increase in ammonia levels 2 weeks after incorporation (Lazarovits *et al*., 1999).

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ii. Animal manures. Even though animal manures have in general been associated with an increase in common scab incidence (Loria *et al*, 1997), Conn & Lazarovits (1999) and Mishra & Srivastava (2004) were able to reduce scab disease incidence with manures. Conn & Lazarovits (1999) found that liquid swine manure amendments could reduce common scab, *Verticillium* wilt and nematodes in potato field trials. However, the common scab reduction efficacy was minimized when soil moisture levels increased due to a dilution effect on the active components. Another interesting observation was the increase in *Trichoderma* populations in soils treated with liquid swine manure, which indicates a possible mechanism for the reduction in pathogenic *Streptomyces* (Conn & Lazarovits (1999). Mishra & Srivastava (2004) evaluated the incorporation of cow manure, goat manure and swine manure into a common scab soil, and found that swine manure reduced common scab incidence to a greater extent than goat and cow manure.

iii. Compost and compost teas. Amendment with certain composts has led to disease suppressive soil conditions (Hoitink & Fahy, 1986). Composts and compost teas have also been utilized as biological control agents (Keener *et al*., 2000). The implied mechanisms for disease reduction are based on an increase in soil microbial activity (Lumsden *et al*., 1986) and induced systemic resistance (Kyung & Deok, 2011). However, very little has been published on the efficacy of compost tea as a means to control soil-borne diseases. Cummings *et al*., (2009) evaluated the efficacy of a compost tea drench in combination with a biological seed treatment (*Bacillus pumulis*) on soilborne damping-off pathogens in an organic greenhouse study on spinach. They found that post emergence wilt, caused by *Fusarium oxysporum* f. sp. *spinaciae* was suppressed. In a study conducted by Larkin (2008), he evaluated various biological amendments, including aerobic compost tea (ACT) for i) their efficacy in introducing beneficial microorganisms, ii) affecting soil microbial communities and iii) reducing soilborne diseases of potato in greenhouse and field trials. The soil-applied ACT and the ACT in combination with a mixture of beneficial microorganisms reduced stem canker and black scurf (*Rhizoctonia solani*) as well as common scab (*Streptomyces* spp.) on potato tubers by 18-33 %. Neither of the amendments was effective in the continuous potato cropping system, indicating that the amendments may have different effects on soil microbial communities and disease development depending on the crops and their rotations (Larkin, 2008).

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It is very difficult to compare results between various compost amendment studies, since the quality and composition of the various composts differ considerably. Only one other study has reported on the use of compost for suppressing common scab. Singhai *et al*. (2011) found that vermicompost was effective in suppressing scab. The suppressive effect of vermicompost was hypothesized to be caused by elevated antimicrobial activity.

iv. Biological inoculants. Various biotic and abiotic factors affect the suppressiveness of soil environments and the impact on soilborne disease incidence. Several micro-organisms have been implicated in the suppression of soilborne pathogens. These microbes make use of a variety of mechanisms including, antibiosis, competition for resources, and induced host resistance to suppress disease (Mazzola, 2002).

The focus on biological control of common scab and the involvement of a biological factor that pre-empts the suppressive condition was first hypothesized by Millard & Taylor (1926) and investigated by Menzies (1959). A large component of the biological control organisms evaluated against common scab has been non-pathogenic *Streptomyces* spp. (Liu *et al*., 1995; Ryan & Kinkel, 1997). The efficacy of *S. melanosporofaciens* strain EF-76 in reducing common scab incidence was evaluated in several studies (Beausejour *et al*., 2003; Prevost *et al*., 2006). *S. melanosporofaciens* EF-76 was effective in reducing common scab incidence in greenhouse and field evaluations.

Larkin (2008) assessed a variety of biological agents (*Bacillus* spp., *Trichoderma* spp.) for their capacity to become established in soil and have an effect on soil microbial communities and common scab disease incidence. He also evaluated the effect of the biological inoculants on other potato diseases and potato yield. The combination of aerated compost and a mixture of beneficial microorganisms (*Bacillus* spp., *Streptomyces griseoviridis*, *Trichoderma harzianum*) reduced common scab incidence, but not significantly. However, soilborne fungal disease incidence was reduced and disease control was associated with changes in microbial community composition. Tagawa *et al*. (2010) recently reported on 15 fungal species that were isolated from soil and evaluated for their efficacy against common scab. They found that the phylogenetically diverse group of fungi showed antagonistic activity against the main common scab pathogens. In a field study by

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Al-Mughrabi (2010) it was shown that seed treatment with *Enterobacter cloacae* and *Pseudomonas fluorescens*, individually as well as in combination with a mustard meal amendment significantly reduced the severity of common scab when compared to the non-treated control. Singhai *et al*. (2011) also investigated a *Pseudomonas* spp. for the suppression of common scab. They showed that *Pseudomonas mosselii* species R1 promoted overall plant growth and induced systemic antimicrobial mechanisms in the potato host which reduced common scab incidence and severity.

v. Natural polymers. Chitosan (poly-ß(1-4)-2-amino-2-deoxy-ß-D-glucan) is a natural chitin polymer amendment that elicits plant defence mechanisms (Benhamou *et al*., 1994; Beausejour *et al*., 2003). The polymer has been applied as an organic amendment to reduce disease incidence and symptom severity, and it is often used in combination with biological control agents, where the chitosan is utilized as a carrier for introducing the microorganism to the soil (Jobin *et al.,* 2005). Most such studies have evaluated the efficacy of chitosan for reducing fungal disease incidence (Benhamou & Theriault, 1992; Sathiyabama & Balasubramanian, 1998), with only two studies investigating control of common scab.

Beausejour *et al*. (2003) evaluated the effect of chitosan and *S. melanosporofaciens* strain EF-76 on common scab incidence, individually and in combination. They found that the individual treatments as well as the combination thereof, reduced the incidence and severity of common scab. In a similar study by Prevost *et al*. (2006), the combination of *S. melanosporofaciens* EF-76 and chitosan also effectively controlled common scab. Disease suppression was in part ascribed to the promotion of antagonistic actinomycetes in treated soils.

DISEASE SUPPRESSIVE SOILS

A soil is considered suppressive when, even though conditions are favourable for disease to develop, the pathogen either (i) cannot become established, (ii) does establish but further disease development does not occur, or (iii) the pathogen establishes, disease develops, but declines within a relatively short time period (Weller *et al*., 2002). Soil

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suppressiveness can be classified into two types: (1) general suppression and (2) specific suppression (Weller *et al.,* 2002).

General suppression has a limited ability to suppress the growth or activity of soilborne pathogens and has also been termed non-specific antagonism (Hornby, 1998) or biological buffering (Huber & Watson, 1970). The suppression is thus due to total microbial biomass, and it is not transferable between soils. In this type of suppression the mechanisms by which disease causing organisms are suppressed, include direct parasitism, nutrient competition, and direct inhibition through antibiotics secreted by beneficial organisms (Weller *et al*., 2002). To enhance general suppression, actions can be taken to build up the total microbial population in soil by organic amendments, building up of soil fertility and cultural practices e.g. no-till. The active response of plants to soil microbes also contributes to suppressiveness through induced systemic resistance (Weller *et al*., 2002).

The mechanism of specific suppression is based on the activity of a specific micro-organism or a specialized group of micro-organisms, on a specific pathogen or pathogenic infection cycle (Weller *et al*., 2002). The key to this type of suppression is the transferability of activity between soils, and it is thus also known as transferable suppression (Cook & Rovira, 1976; Stutz *et al*., 1986; Westphal & Becker, 2000). Research on specific suppression has played an important role in our understanding of biological control mechanisms, although we still know very little. However, with the ever increasing speed at which molecular technology develops, we are bound to increase our knowledge on specific suppressive soils more rapidly in future. For example, Mendes *et al.* (2011) used a high-density 16S ribosomal DNA oligonucleotide micro-array and a metagenomic approach coupled with culture-dependent functional analyses to make novel findings on the bacteria and mechanisms involved in *Rhizoctonia solani* suppressive soils. They identified a vast number bacterial and archaeal species, more than 33 000, in the rhizosphere of beet seedlings grown in suppressive soil. Proteobacteria, Firmicutes and Actinobacteria were consistently associated with disease suppression, with the disease-suppressive activity of Proteobacteria being governed by nonribosomal peptide synthases (Mendes *et al*., 2011).

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Suppressive soils can also be classified further according to the longevity of their suppressive activity e.g. long-standing suppression and induced suppression (Hornby, 1983). Long-standing suppression refers to a biological state in which soils have a natural balance and appears to stay active in the absence of plants. Whereas induced suppression is initiated and sustained by means of crop monoculture or introduction of microbial inoculants to target a specific pathogen (Weller *et al*., 2002).

Naturally occurring disease suppressive soils have been reported for various host pathogen systems of which *Fusarium* wilt (Scher & Baker, 1980; Abadie *et al*., 1998; Alabouvette, 1999), take-all decline (Cook & Rovira, 1976; Simon & Sivasithamparam, 1989; Andrade *et al*., 1994), apple replant disease (Mazzola, 1998; Mazzola, 1999; Mazzola & Gu, 2000) and potato scab decline (Menzies, 1959; Lorang *et al*., 1989; Liu *et al*., 1995; Lorang *et al*., 1995; Liu *et al*., 1996; Ryan & Kinkel, 1997; Schottel *et al*., 2001) are the most studied systems. The micro-organisms responsible for disease suppression in most of these host pathogen systems include fluorescent *Pseudomonas* spp., *Fusarium* spp., *Trichoderma* spp. and actinomycetes.

Fluorescent *Pseudomonas* spp. have been investigated as a major component of the biological factors involved in suppressing soilborne diseases (Weller, 1988; Mazzola, 1999; Weller *et al*., 2002; Haas & Defago, 2005; Chang, 2009; Sanguin *et al*., 2009). Several studies have shown that fluorescent *Pseudomonas* spp. play a critical role in the suppression of *Fusarium* wilt (Scher & Baker, 1980; Elad & Baker; 1985; Mazzola, 2002), *Rhizoctonia* root rot of apple (Mazzola, 1999) and take-all decline of wheat (Chang, 2009; Sanguin *et al*., 2009). In root rot suppression, *Pseudomonas putida* was the species that was found to be highly antagonistic to the pathogenic *Rhizoctonia* spp., whereas in the take-all decline suppression model, several antibiotic (2,4-diacetylphloroglucinol) producing fluorescent *Pseudomonas* spp. were implied as the suppressive agents (Mazzola, 2002; Weller *et al*., 2002). Other postulated mechanisms by which *Pseudomonas* suppress soil borne fungal pathogens include the production of siderophores, eliciting induced systemic resistance in the host plant or interfering specifically with fungal pathogenicity (Weller, 1988; Haas & Defago, 2005).

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In *Fusarium* wilt diseases, disease suppression through non-pathogenic *Fusarium* spp. seems to be limited to vascular *Fusarium* wilt, since no effect is evident on non-vascular *Fusarium* plant pathogens e.g. *F. roseum* and *F. solani* (Alabouvette, 1986). Most of the cases have demonstrated long standing-suppression by non-pathogenic *Fusarium* spp. (Mazzola, 2002), but in the case of *F. oxysporum* f. sp. *melonis* on melon (Sneh *et al*., 1987), and *F. oxysporum* f. sp. *niveum* on watermelon (Hopkins *et al*., 1987; Larkin *et al*., 1993) induced suppression were also described.

Trichoderma spp. are often important in disease suppressive soils, with *T. harzianum* being the species that has been described and studied most frequently in biocontrol experiments (Liu & Baker, 1980). On apple, *T. harzianum* plays an important role in the suppression of *Rhizoctonia* in suppressive root rot soils (Mazzola, 1999; Mazzola, 2002) and soils affected by take-all of wheat (Cook & Rovira, 1976). Recently, Weerakoon *et al*., (2012) were also able to demonstrate long-term suppressiveness towards *Pythium* spp. in response to *B. juncea* seed meal amendments, associated with increases in various *Trichoderma* spp.

Trichoderma lignorum has also been recorded as an antagonist of *R. solani* that causes damping-off in citrus seedlings (Shalini *et al*., 2006). A possible mechanism of pathogen suppression by *T. harzianum* was elucidated by Elad *et al*. (1982), where it was shown that the antagonism towards soil-borne pathogens was due to the release of lytic enzymes such as glucanase and chitinase. Other important mechanisms include the production of antibiotics and the induction of induced systemic resistance in plants (Vinale *et al*., 2008). As with many organisms, abiotic environmental factors also play an important role in biocontrol efficacy of *T. harzianum.* For example *T. harzianum* growth and sporulation is stimulated at low soil pH resulting in more effective *Rhizoctonia* suppression (Chet & Baker, 1980).

Various actinomycetes, including *Streptomyces,* have been implied as antagonists in plant disease systems, most likely due to the ability of this genus to produce an array of antibiotics (Liu *et al*., 1996). Actinomycetes have been shown to inhibit fungal root pathogens (Broadbent *et al*. 1971), and are also involved in natural suppression of take-all decline of wheat (Chang, 2009). Non-pathogenic *Streptomyces* spp. have also been shown to play an

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important role in disease suppressive soil systems (Mazzola, 1999; Wiggins & Kinkel 2005b). *Streptomyces* spp. that were isolated from the rhizosphere of *Brassica napus* seed meal amended soil were capable of suppressing *R. solani* and Cohen & Mazzola (2006) were able to demonstrate that the mode of action was likely to be the induction of systemic resistance. *Streptomyces longisporus* can inhibit *Helminthosporium oryzae* and *Alternaria solani* (Chattopadhyay & Nandi, 1982), whereas *S. griseus* and *S. hygroscopicus* are antagonistic against *R. solani* (Merriman *et al*., 1974; Rothrock & Gottlieb, 1984). Non-pathogenic *Streptomyces* is also well known for their role in suppression of common scab, which will be discussed in more detail in the following section.

Common scab suppressive soils. The first indications of saprophytic *Streptomyces* spp. reducing common scab incidence was reported by Millard & Taylor (1926). They found a decrease in scab incidence when saprophytic *Streptomyces* were added to scab conducive soil under greenhouse conditions. Menzies (1959) later reported that a biological factor in an “old scab land” was responsible for disease suppression. Lorang *et al*. (1989) came to the same conclusion by studying a continuously planted potato field from 1942 to 1972, which was used for screening potato breeding lines for common scab tolerance. The field started showing a decline in common scab disease incidence from 1960 onwards, even though efforts were made to re-introduce the disease, it was not successful.

Non-pathogenic *Streptomycetes* play a role in the development of soil suppressiveness towards common scab after prolonged cultivation of potatoes. Liu (1992) isolated non-pathogenic *Streptomyces* spp. from potato tubers planted in a continuously potato cropped scab-suppressive field. The *Streptomyces* spp. were shown to produce antibiotics *in-vitro,* inhibit virulent *S. scabiei* species, and were subsequently utilized in biological control studies for the suppression of common scab in disease conducive fields (Lorang *et al*., 1995). Liu *et al*. (1996) also isolated several non-pathogenic *Streptomyces* spp. from potato tubers grown in common scab conducive and suppressive fields. When the antagonistic *Streptomyces* spp. were inoculated into common scab infected fields, significant disease reduction was observed. Interestingly, all of the virulent and 54 % of the suppressive *Streptomyces* species was classified as *S. scabiei* (Liu *et al.*, 1996).

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Antibiosis was one of the first and most important mechanisms that were studied for its contribution to common scab suppression by non-pathogenic *Streptomyces*, but it is not the only mechanism involved. Neeno-Eckwall & Schottel (1999) studied the development of antibiotic resistant, pathogenic *Streptomyces* species that could circumvent this control measure. They found that antibiotic resistant mutants of a pathogenic *S. scabies* species arose spontaneously at a frequency of 10^{-4}, when exposed to antibiotics produced by suppressive *Streptomyces* species. However, the resistant mutants were less virulent than the pathogenic *Streptomyces* parental species (Neeno-Eckwall & Schottel, 1999). Schottel *et al.* (2001) later demonstrated that spontaneous mutants of two scab suppressive streptomycetes that were defective in *in-vitro* pathogen inhibition, demonstrated significant inhibition of pathogenic *S. scabies* species when potato plants were used. The results indicated that *in-vitro* pathogen inhibition as assessed by antibiotic and co-plate assays, is a poor predictor of antagonism as a successful control measure for common scab. They also found that there was a high degree of specificity between pathogenic and antagonistic *Streptomyces* spp. interactions.

In addition to antibiosis, competition is also an important factor in disease suppression by non-pathogenic *Streptomycete* spp., especially in a system in which antibiotic resistant pathogenic mutants arise from pathogenic parent species (Neeno-Eckwall *et al.*, 2001). The total number of characteristic grey and white sporulating streptomycete colonies was measured in both the treated and non-treated pots and soil colonization differences were determined by comparison of population densities between treatments. The greenhouse assays demonstrated that when inoculated into soil individually a suppressive strain always resulted in higher total streptomycete densities than when this strain was co-inoculated with a pathogen. A lower disease incidence was, however, observed that was positively correlated with a higher total streptomycete population density (Neeno-Eckwall *et al.*, 2001).

Several other mechanisms are also important in common scab suppression by non-pathogenic *Streptomyces* spp. Vigorous growth (Liu *et al.*, 1996) as well as interspecies communication (Becker *et al.*, 1997) between the non-pathogenic *Streptomyces* species are also important factors. Interspecies communication was studied by growing suppressive and pathogenic *Streptomyces* species individually in liquid medium and determining whether broth of these species could induce antibiotic production by a pathogen-suppressive *S. diastatochromogenes* PonSSII isolate. Conditioned broth from one of the suppressive strains

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and one of the pathogenic strains (RB4), triggered antibiotic production by the PonSSII isolate at an earlier stage of culture growth and enhanced antibiotic production levels in comparison to the control suggesting that interspecies communication was occurring between the *Streptomyces* spp. that may contribute to pathogen suppression (Becker *et al*., 1997).

Rhizosphere soil is the primary source of suppressive *Streptomyces* species for biological control of common scab on potato tubers (Ryan & Kinkel, 1997). When the effect of inoculum density and population dynamics of a pathogenic and suppressive *Streptomyces* spp. on potato roots and in the potato rhizosphere was studied, a positive correlation was identified between disease severity and population densities of the pathogen in roots and soil. In contrast, a negative correlation was found between population densities of the non-pathogenic *Streptomyces* spp. and disease severity. In soils that are suppressive to other plant diseases, several soil microbial genera are known to be important. In common scab suppressive soils, none of these other microbial genera e.g. fluorescent *Pseudomonas* etc. have been studied.

Managing soil disease suppression. Managing soil disease suppression has proven to be a very difficult task (Kinkel *et al*., 2011; 2012). Several studies have used the approach of amending soil with single microbial isolates that are known to suppress the disease of interest. Although several of these studies have yielded positive results, many of them were conducted in the greenhouse. The establishment of introduced biological control agents into large commercial fields remains difficult and yields variable results, due to environmental factors that cannot be managed (Mazzola, 2004). A more feasible alternative to using introduced microbial species is the use of crop management practices that indirectly alter existing microbial communities. These crop management practices include crop rotation, crop production systems, tillage practices, fertilization programs, amendment management and cultivation practices (Mazzola, 2002; Mazzola, 2004). All of these practices have a direct or indirect effect on the native microbial community in the soil and the changes brought about can influence the suppressive potential of the soil. These influences can be positive or negative since it may enhance the suppressive nature of the soil or it may lead to selective enhancement of specific populations that can result in increased disease incidence (Mazzola, 2007).

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CONCLUSION

Potatoes are the most important non-cereal food crop in the world. Most of the economically important potato diseases are caused by soilborne pathogens. Currently, common scab is one of the major soilborne factors limiting successful production of potatoes in South Africa. The percentage of bags containing scab infected seed tubers averages 32 %, with a corresponding rejection or decertification of the seed. The disease also reduces the cosmetic value of ware potatoes, and with the growing consumer-demand for blemish-free produce, this is causing an upward trend towards downgrading of consignments on the ware market.

Streptomyces scabiei is regarded as the main causal agent of common scab on potatoes. However, world-wide several studies have shown that the disease can be caused by a complex of *Streptomyces* species, with the dominant species varying in different regions. The identification of pathogenic streptomycetes was initially only based on phenotypic characteristics, and even though it proved to be very useful, it has since been enhanced by more accurate cellular fatty acid analysis and DNA-based techniques, e.g. DDH and 16S rRNA sequence analysis. 16S rRNA data has been used to develop species-specific PCR primers that target unique regions of the 16s rDNA gene in different *Streptomyces* species, thus allowing rapid, high throughput and cost effective identification of known scab pathogens (Wanner, 2004). More recent taxonomic studies have highlighted the inability of the 16S rRNA region to differentiate between closely related Streptomycete spp. Therefore, MLSA using concatenated gene trees including three to five genes (*atpD*, *gyrB, recA, rpoB* and *trpB*) are becoming increasingly important for identifying species.

Effective management strategies for common scab are limited. The only chemical that is presently registered for the control of soilborne inoculum of the pathogen is quintozene, whereas flusulfamide and mancozeb are registered as tuber treatments. However, lack of consistent efficacy of these chemicals requires the implementation of an integrated management strategy. Such a tactic may include the use of tolerant/resistant cultivars, long-term crop rotations and cultural practices that involve incorporation of organic amendments. The use of certain crop residues and soil amendments that cause a decrease in disease incidence has been investigated, such as the application of plant residues to yield a

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biofumigation response. Disease suppression by means of biofumigation is based on the presence of ß-D-thioglucosidic compounds known as glucosinolates (GSLs) in the *Brassicaceae* and other families of the order Capparales (Brown & Morra, 1997). Another mechanism that might be involved in disease suppression through biofumigation is a shift in native microbial populations towards populations that can suppress plant pathogens (Mazzola & Manici, 2012). Several potato pests and diseases are reduced and/or inhibited by *Brassica* amendments. There have also been a few reports on common scab reduction on potatoes by means of *Brassica* amendments (Larkin & Griffen, 2007; Larkin *et al.*, 2011).

In South Africa, some information is available on the *Streptomyces* spp. causing common scab, and the effect of *Brassicaceae* amendments on common scab suppression. Gouws (2006) identified *S. scabiei* as the main causal agent of common scab pathogen in South Africa using morphological and physiological characteristics. However, there were two other phenotypically distinct species identified with characteristics that varied from *S. scabiei* suggesting that this may not be the only species of importance in South Africa. Recently, a novel *Streptomyces* sp. causing fissure scab has also been reported from South Africa (Gouws and McLeod, 2012). Under South African conditions, greenhouse and tunnel trials have shown that *Brassicaceae* residue amendments (fresh and dry) can cause a reduction in common scab incidence on potatoes (Gouws & Wehner, 2004; Gouws, 2006). However, the mechanism of common scab control by means of *Brassica* amendments has not been elucidated and requires investigation in order to optimize the management strategy and reduce disease incidence. The aims of the study were therefore to i) characterize *Streptomyces* isolates obtained from common scab lesions on field grown potato tubers cultivated in South Africa ii) to determine if common scab of potato can be managed with cabbage residues and if volatiles from various *Brassica* species had any effect on *Streptomyces* spp. and iii) to determine if induced resistance is a mechanism for suppression of potato common scab in cabbage and mustard amended soil.

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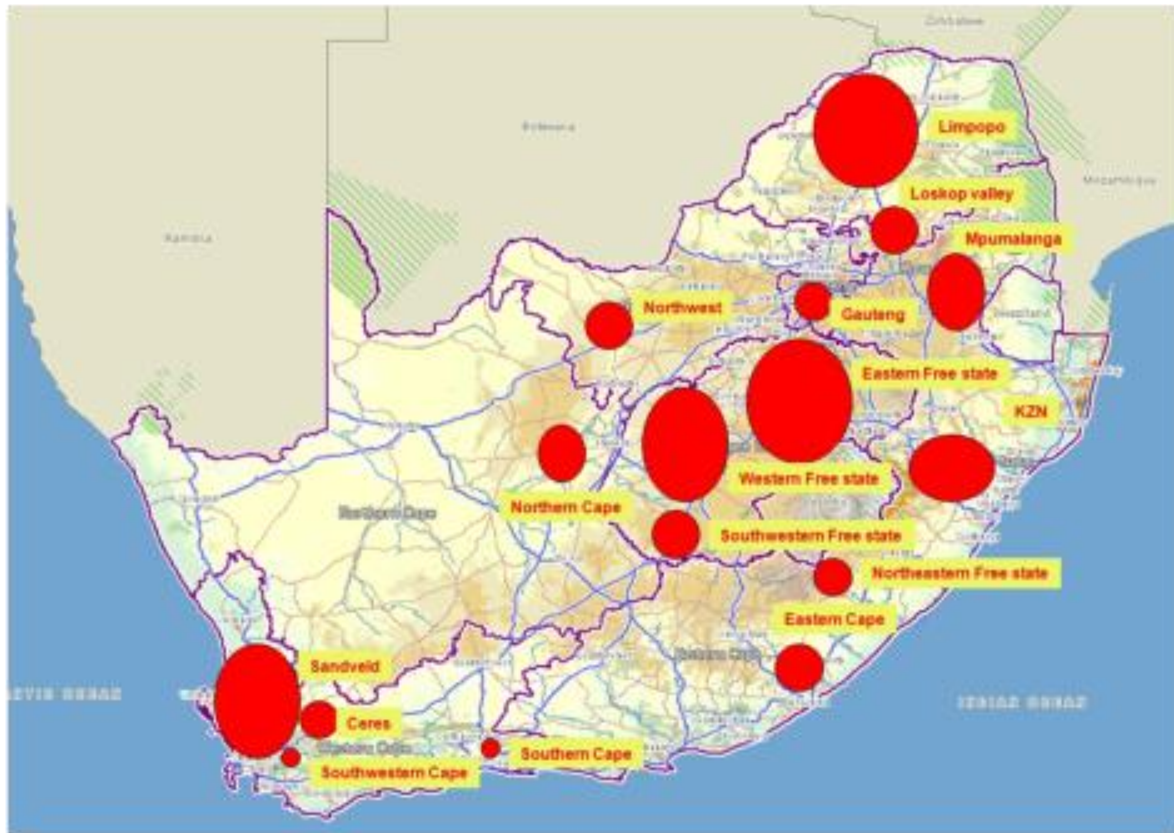

Figure 1. Map of South Africa showing the 16 potato production areas of the South African potato industry**.**

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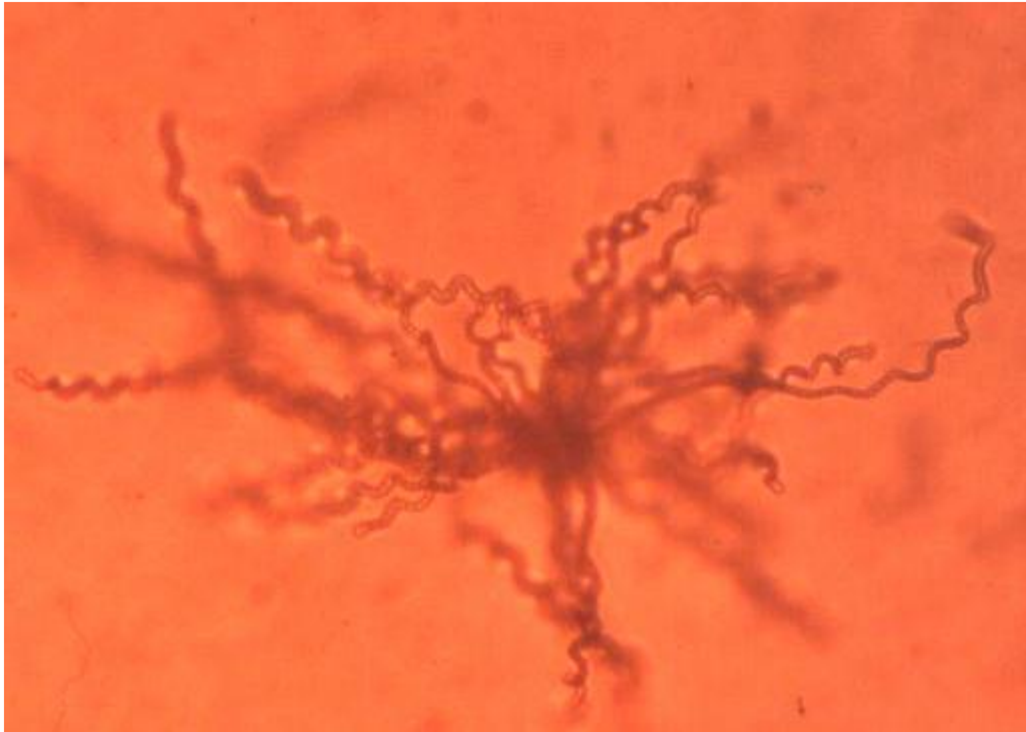

Figure 2. Typical sporulation of *Streptomyces* showing the non-fragmented substrate mycelium.

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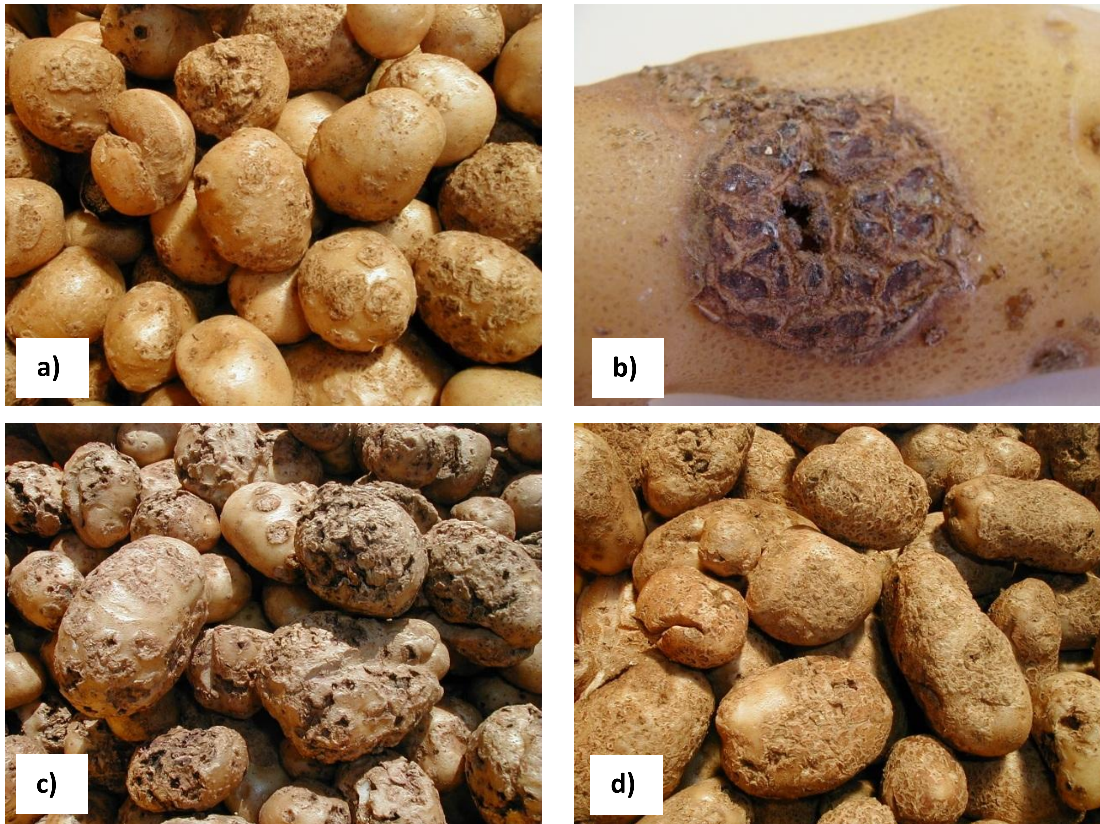

Figure 3. Common scab symptoms caused by phytopathogenic *Streptomyces* isolates that have been identified in South Africa include a) raised scab lesions, b) superficial scab lesions, c) deep-pitted scab lesions and d) netted/russet scab lesions.

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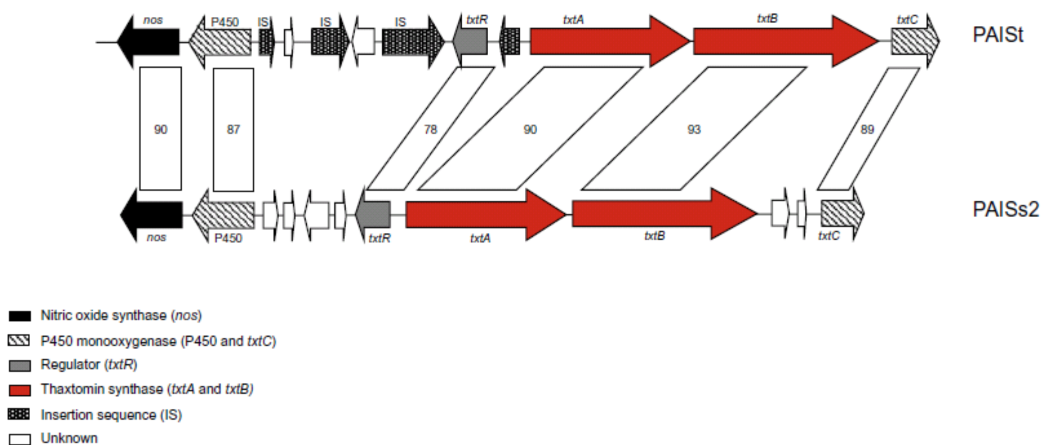

Figure 4. Syntenic regions between the PAIs of *Streptomyces scabiei* (PAISs2) and *S. turgidiscabies* (PAISt), which also show important genes within thaxtomin biosynthetic pathway including *nos, txtA, txtB, txtC* and the regulator gene of the thaxtomin pathway *txtR*. White bars indicate homology and percentage identity at the amino acid level. The putative functions of genes in the depicted regions are provided in the legend (Huguet-Tapia *et al.*, 2011).

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2. CHARACTERIZATION OF *STREPTOMYCES* ISOLATES CAUSING COMMON SCAB ON POTATO IN SOUTH AFRICA

ABSTRACT

In South Africa, *Streptomyces scabiei* is still regarded as the main causal agent of common scab. However, world-wide, the disease is caused by a complex of *Streptomyces* species, with the dominant species varying in different regions. Therefore, a total of 132 *Streptomyces* isolates collected from six South African potato production regions were characterized. Fifty three percent (70 isolates) of the isolates were pathogenic towards potato under tunnel conditions. Evaluation of a radish- and potato micro-tuber pathogenicity assay showed that these assays could replace the time consuming potato pot trials, since there was a positive correlation (P = 0.05) between the results from the three assays. All isolates were evaluated with species-specific primers targeting the 16S rRNA region, and a subset using phylogenetic analyses [16S rRNA phylogeny and multilocus phylogeny (beta sub unit, recombinase A, RNA polymerase beta subunit and tryptophan synthase genes)]. This showed that *S. scabiei* was the most prominent species in South Africa comprising 51.4 % of the pathogenic isolates, followed by *S. europaeiscabiei* (30 %) *S. cavisabies* (synonym *S. fimicarius*) (5.7 %), and *S. stelliscabies* (1.45 %). The remaining 11.45 % of the pathogenic isolates comprised three related but distinct taxa, which fit within phylogenetic clades that do not contain common scab isolates from any country other than South Africa, and are named here *Streptomyces* strains RSA1 (5.7 %), RSA2 (4.3 %) and RSA3 (1.45 %). The species status of these isolates is difficult to determine due to low species resolution provided by the 16S rRNA phylogeny, and the limited number of type strains for which multilocus protein sequence data are available for. In the pathogenicity assays, disease symptoms mostly consisted of superficial lesions, except for *S. caviscabies* that caused deep-pitted lesions and *Streptomyces* strain RSA1 that resulted in fissure scab symptoms. For most species, isolates within the species varied in virulence, except for *S. europascabiei*. PCR analyses targeting three marker pathogenicity island (PAI) genes [thaxtomin biosynthesis gene (*txtAB*), necrosis gene (*nec1*) and tomatinase gene (*tomA*)] showed that among the pathogenic isolates *nec1* occurred in 89 % of the isolates, *tomA* in 81 % and *txtAB* in 89 % of the isolates. The isolates that did not contain the *txtAB* gene belonged to *S. caviscabies* and *Streptomyces* strains RSA2 and RSA3. These isolates also did not produce thaxtomin in an oat bran agar- and a potato tuber slice assay. *Streptomyces* strain RSA1 contained the *txtAB* and *nec1* genes, but not the *tomA* gene. The *S. scabiei* and *S. stelliscabies* isolates contained all three PAI genes.

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INTRODUCTION

Common scab of potato is one of the oldest known plant diseases and was first described in 1890 (Thaxter, 1891). The disease is currently almost as widely distributed as the host itself (Wanner, 2006). It is present in all the potato-growing areas of Canada, North America, Europe, the Far East, India, Australia, Korea, Japan and the United Kingdom (Elesaway & Szabo, 1979; Bouchek-Mechiche, *et al.*, 2000; Bencheikh & Setti, 2007; Flores-Gonzalez, *et al.,* 2008). In South Africa, the disease is present in all 16 potato production regions, albeit at varying levels (Theron, 2003). Most South African potato cultivars are susceptible to the disease, with disease symptoms varying from superficial to netted ordeep-pitted necrotic lesions on the surface of potato tubers. The specific lesion type and surface area covered often lead to economic losses in the ware market due to a reduction in the cosmetic value of produce (Theron, 2003). The seed potato and processing industries are also affected by the disease, which causes a reduction in the amount of potato seed available for planting, and the availability of good quality product for processing purposes.

Common scab is caused by several species within the genus *Streptomyces. Streptomyces scabiei* is the most common species and has been reported worldwide (Faucher *et al.*, 1992; Loria *et al.*, 1997; Miyajima *et al.*, 1998; Gouws, 2006). Other well-known pathogenic species include *S. acidiscabies* (Eastern North America; Japan, Korea) and *S. turgidiscabies* (Japan, Korea, Scandinavia). Species that are less well-known include *S. diastatochromogenes, S. atroolivaceus, S. lydicus* and *S. resistomyficus* that were all reported as causing deep-pitted scab in Washington state, *S. botropensis* (Egypt and North America), *S. caviscabies* (Canada) and *S. aureofaciens* (Finland) (Archuleta & Easton, 1981; Goyer *et al.*, 1996). *Streptomyces aureofaciens* has been reported to cause netted / russet scab in Finland and Canada, but the taxonomic status of this species and its association with common scab is not clear (Kreuze *et al.*, 1999; Goth *et al.*, 2003; Faucher *et al.*, 1993). In Europe, several new pathogenic species, which were subsequently also reported in North America, were recently described including *S. reticuliscabiei* (European netted scab), *S. europaeiscabiei* and *S. stelliscabiei* (Bouchek-Mechiche *et al.*, 2000; Wanner, 2006). Other newly described species from Korea include *S. luridiscabiei, S. puniscabiei* and *S. niveiscabiei* (Park *et al.*, 2003). Three undescribed pathogenic species that may represent new species have also been published including *Streptomyces* sp. IdahoX (United States)

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Streptomyces sp. DS3024 (Korea) and *Streptomyces* strain NRRL 24085 (Wanner, 2007; Hao *et al.*, 2009; Labeda, 2011).

The identification of *Streptomyces* species is difficult, with much confusion and controversy still existing at the taxonomic level for the 615 currently described species that is increasing each year (Labeda *et al.*, 2012). To start addressing this problem, the International *Streptomyces* Project committee (ISP) was established in 1963. The committee instituted a set of standardised tests and procedures for the identification of *Streptomyces* spp., and a large number of detailed species descriptions were produced for the identification of *Streptomyces* spp. (Gyllenburg, 1976). The descriptions from the ISP classification system are based on phenotypic characteristics including spore chain morphology, colony colour, reverse side colony colour, melanoid pigment formation and carbon utilization (L-Arabinose, D-Fructose, D-Glucose, D-Mannitol, Raffinose, Rhamnose, Sucrose, D-Xylose, *meso*-Inositol) (Shirling & Gottlieb, 1966). Williams *et al.* (1983) was the first to publish a comprehensive numerical taxonomic study on the genus *Streptomyces*, which resulted in the assignment of species to various clusters, followed by an even more extensive numerical taxonomic study by Kämpfer *et al.* (1991).

Even though the ISP system has proven to be very useful, more accurate species identifications can be obtained through DNA-based techniques such as DNA-DNA hybridisation, 16S rRNA sequence analyses and multilocus sequence analyses (MLSA). DNA-DNA hybridization is a very reliable technique for species identification. However, the technique is very labour intensive and also requires substantial expertise (Bowers, *et al.*, 1995; Ndwora, *et al.*, 1995; Takeuchi, *et al.*, 1996; Song *et al.*, 2004; Bignell *et al.*, 2010). In contrast, 16S rRNA sequence data are routinely used for *Streptomyces* species identification due to the routine based nature of the technique. The most extensive 16S rRNA phylogeny for almost all described *Streptomyces* species (~ 600 taxa) was published by Labeda *et al.* (2012). The phylogeny identified 130 statistically supported clades and many unsupported and single member clades. Common scab-causing species are also routinely identified with 16S rRNA data, and species-specific primers based on this region have been published for application in the identification of *S. scabiei, S. europaeiscabiei, S. stelliscabiei, S. bottropensis, S. acidiscabies, S. turgidiscabies,* and *S. aurofaciens* (Wanner, 2006).

Recently, use of MLSA was shown to provide better taxonomic resolution of *Streptomyces* species than the 16S rRNA gene, especially for some species that show high similarity at the 16S rRNA level. MLSA data also correlated with DNA-DNA hybridization studies (Rong *et al.*, 2009; Rong & Huang, 2010; Labeda, 2011; Rong & Huang, 2012). Most MLSA studies have been conducted on non-pathogenic *Streptomyces* species of industrial importance and investigated five protein coding housekeeping genes [beta sub unit (*atpD*), DNA gyrase B subunit (*gyrB*), recombinase A (*recA*), RNA polymerase, beta subunit (*rpoB*), tryptophan synthase, beta sububit (*trpB*)] (Guo *et al.,* 2008; Rong *et al.*, 2009; Rong & Huang, 2010; Han *et al.*, 2012; Rong & Huang, 2012). Since none of these single genes contain sufficient phylogenetic information to reliably discriminate all species, concatenation of the multigene sequences are required for phylogenetic inference. Only two studies have used MLSA for investigating a robust number of the phytopathogenic *Streptomyces* spp. Weon *et al.* (2011) used a combined RNase P RNA (*rnpB*) and 16S RNA gene tree to investigate relantionships among 41 scab-causing *Streptomyces* isolates. The combined gene tree had a topology similar to the 16S rRNA tree, but showed more divergent phylogenetic clades. Labeda (2011) conducted a MLSA analyses with four protein coding genes (*atpD, recA, rpoB* and *trpB*) on 62 *Streptomyces* species, which included the type strains of ten known phytopathogenic species and six uncharacterized phytopathogenic isolates. This study did not analyse the *gyrB* gene since the *S. scabiei* RL87.22 genome sequence contain two copies of the *gyrB* operon (one degenerate) and the *gyrB* alignments of previous studies (Guo *et al.*, 2008; Rong *et al.,* 2009; Rong & Huang, 2010) contained some evidence that more than one locus was amplified and sequenced in *Streptomyces* (Labeda, 2011). The concatenated four-gene tree of Labeda (2011) showed that the phytopathogenic species are taxonomically distinct from each other despite high 16S rRNA gene sequence similarities. The four-gene tree provided higher bootstrap support for clades than the 16S rRNA tree and also clearly separated *S. scabiei* from *S. europascabiei* (Labeda, 2011).

Streptomyces scabiei, S. acidiscabies and *S. turgidiscabies* were the first gram positive bacterial species in which a pathogenicity island (PAI) was reported (Kers *et al.,* 2005). PAIs are discrete genome regions that contain clusters of pathogenicity and virulence genes that can be mobilized into other genetic backgrounds (Alfano *et al*., 2000). In common scab pathogens, the PAI resides within a 325-660kb region that contains three marker genes, of which only some are known to be involved in pathogenicity and virulence. The marker

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genes include the thaxtomin biosynthesis genes (*txtAB*), a necrosis gene (*nec1*) and a tomatinase gene (*tomA*). Thaxtomin, which includes two main active compounds designated thaxtomin A and B, is a phytotoxin involved in symptom development, and was first isolated and identified in 1989 (King *et al*., 1989; King, *et al*., 1991; King & Lawrence, 1996; Healy & Lambert, 1991). Thaxtomin is considered to be a pathogenicity factor since a positive correlation exists between thaxtomin synthesis and pathogenicity in *Streptomyces* strains, and with the quantity of thaxtomin produced and strain virulence (Kinkel, *et al*., 1998). Unlike the *txtAB* gene, the *nec1* and *tomA* genes are not essential for pathogenicity (Wanner, 2006; Florez-Gonzales *et al*., 2008). The *nec*1 gene is known to be involved in virulence, but not pathogenicity since although it has necrogenic activity on potato tuber tissue it is absent in several pathogenic isolates (Bukhalid *et al* 1998; Kreuze *et al*., 1999; Park *et al*., 2003; Wanner, 2004; Joshi *et al*., 2007; Wanner, 2007). The product of the tomatinase gene, α-Tomatin, is a well-characterized enzyme from plant pathogenic fungi (*Septoria lycopersici* and *Fusarium oxysporum* f. sp. *lycopersici*) that assists plant pathogenic fungi in suppressing plant defence responses (Bouarab *et al*., 2002; Seipke & Loria, 2008). A tomatinase homologue has also been identified in the plant pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Kaup *et al*., 2005). The importance of the *tomA* gene in common scab pathogenicity has not been established, although the gene is functional in *S. scabiei* (Wanner, 2007). Only a few studies have investigated the presence of *tomA* in large collections of pathogenic *Streptomyces* isolates, where the gene was shown to be present in most pathogenic *Streptomyces* isolates, but several isolates lacked the *tomA* gene (Wanner, 2006; Hao & Meng, 2009). Since several *Streptomyces* isolates have been discovered that contain different subsets of the three PAI marker genes, variability in the presence of these genes on the PAI seems common (Wanner 2006).

There exists minimal information concerning the species of *Streptomyces* causing common scab in South Africa. To date, there are only two studies that have investigated the identity, characteristics and prevalence of scab-causing species in South Africa (Gouws, 2006; Slabbert *et al*., 1994). Gouws (2006) found, using only phenotypic identifications, that *S. scabiei* was the major causal agent of common scab on potato and that it occurred in all the potato production regions of South Africa. The study did, however, report that there were phenotypic variations among the *S. scabiei* isolates and that 14 % of the pathogenic isolates did not produce thaxtomin A. Slabbert *et al*. (1994) observed a positive correlation between

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thaxtomin A production and the pathogenicity of *Streptomyces* isolates, but did not identify pathogenic isolates that did not produce thaxtomin. The aims of the current study were to determine (i) the *Streptomyces* spp. causing common scab of potato in South Africa and whether isolates and species varied in virulence and thaxtomin production, (ii) whether there is a correlation in results obtained from three pathogenicity assays; the potato double pot technique, radish and micro-tuber assays, and (iii) the prevalence of three PAI marker genes (*tomA, nec1* and *txtAB*) in pathogenic and non-pathogenic isolates. The characterized isolates were collected from six potato production regions over a four year period.

MATERIALS AND METHODS

Sample collection

Diseased tubers displaying common scab lesions were collected from six of the main potato production regions in South Africa from 2008 to 2011 (Fig. 1). Sites within each of the production regions were chosen for sampling based on the common scab history of each field, as well as the disease incidence on the potato tubers at harvest. One to ten tubers were sampled per field and used in the isolation studies. Common scab-diseased samples were also sent to the Agricultural Research Council (ARC) at Roodeplaat by field agents of Potato South Africa (PSA) or potato producers from the various regions.

Streptomyces isolation and culturing

Isolations from symptomatic tubers were made according to a modified method described by Loria & Davis (1989). Scab lesions were aseptically removed with a scalpel and the underlying tissue excised and surface disinfested in 1.5 % bleach for 1-2 min. Tissue was rinsed several times with sterile distilled water and ground in a sterile mortar and pestle containing 1 ml of sterile distilled water. A droplet from the ground solution was transferred and streaked onto Inorganic Salt Solution Agar (ISSA, ISP medium 4) plates. Plates were incubated at 28 °C for 7-15 days after which individual characteristic *Streptomyces* colonies were picked and serially transferred until a pure culture was obtained. Oatmeal agar (OA, ISP medium 3) and Yeast Malt Extract (YME, ISP medium 2) media were used for routine

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culturing of *Streptomyces* isolates. Isolates were maintained as spore suspensions in 20 % glycerol at -70 °C.

Phenotypic characterization of *Streptomyces* isolates

Phenotypic characterization was conducted using a subset of the methods developed during the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966). Each *Streptomyces* isolate was plated onto fresh YME agar and incubated at 28 °C for 3-4 weeks. Spore-chain type, mycelium and spore colour was observed.

Pathogenicity assays

Three methods were used for assessing the pathogenicity (ability to cause disease) and virulence (severity of disease) of the *Streptomyces* isolates. The methods included the potato double pot technique, an *in-vitro* micro-tuber assay and an *in planta* assay with radish seedlings. The results of the different methods were compared to determine whether the methods yielded similar results with regards to pathogenicity.

Potato double pot technique. All the *Streptomyces* isolates collected in the study (Table 1) were evaluated using the double pot technique described by Marais & Vorster (1988). Two reference strains were included as positive controls; strain O2 (pathogenic), and strain O9 (non-pathogenic), which were identified and characterized previously (Chapter 2). Inoculum was produced by first growing the isolates on YME agar plates for 14 days at 30 °C. *Streptomyces* spore suspensions were prepared by flooding the plates with sterile distilled water and rubbing with a bent glass rod to harvest the spores. The suspensions were adjusted to a final concentration of 1.2 x 10^6 cfu/ml.

Disease-free potato tubers of the cultivar BP1 (common scab susceptible) were obtained from RSA Seed potato producers, surface disinfested with 1% sodium hypochloride (NaOH) solution and planted in plastic pots (15 cm diameter) filled with 600 cm^3 of silica sand (0.6 mm x 1.5 mm). One potato tuber was planted into each of the 15 cm pots, which

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were all placed on top of a rectangular planting box (15 m x 1.5 m x 0.4 m) filled with natural Hutton soil. The 15 cm pots were irrigated until the roots grew through the holes at the bottom of the plastic pot, into the Hutton soil. Subsequently, irrigation was only applied to the Hutton soil, thus creating dry conditions in the tuber development area (15 cm pot), which is conducive for common scab development. The potato plants were inoculated in the 15 cm pots during the tuber initiation stage by adding 10 ml inoculum per plant, six replicates per isolate. A negative control treatment receiving a water suspension made by flooding an oat meal agar plate was also included. The experiment was conducted in a tunnel that was maintained at an average of 25 °C day and 8 °C night conditions. Each isolate was evaluated for pathogenicity in two independent experiments.

Potatoes were harvested after 90 days and common scab lesions were assessed according to a scab disease index that included both tuber surface area covered and lesion type. For surface area covered the scale was: 0 = no scab lesions; 1 = 1-12 % surface area covered; 2 = 13-25 % surface area covered; 3 = 26-50 % surface area covered 4 = 51-75 % and 5 = 76-100 % surface area covered. For lesion type the scale was: 6 = superficial lesion; 7 = netted/russet scab lesion and 11 = deep-pitted scab lesion. The final disease index was assigned on a scale of 0-55, which was calculated by multiplying the surface area covered with the lesion type. Isolates producing ratings of ≥12 were scored as pathogenic.

In-vitro micro tuber assay. A subset of 22 isolates from four potato production regions were evaluated, and included 2 isolates from Ceres, ten Northern Cape isolates, six Sandveld and five Limpopo isolates (Table 1). A *S. scabiei* reference culture (O65) (Chapter 2) was included as positive control in all the assays. Inoculum was prepared in a similar manner as that described for the potato double pot technique, except that oat meal agar was used for growing the isolates.

Potato micro-tubers (cv BP1) were produced and maintained *in-vitro* by aseptically cutting 2 cm sterile sections from 3-week-old dark-grown sprouts and inserting the sprouts in White's amended medium containing 8 % sucrose (King *et al*., 1991) within small glass jars (60 mm diameter). The jars were incubated at 20 °C in the dark. A drop of inoculum was

transferred to a 5-mm-diameter Whatman no.1 filter paper disc that was placed on the surface of each micro tuber in a sterile moist chamber (90 mm Petri dish) at 25 °C. Each isolate was tested on eight micro-tubers. The development of visible scab lesions was evaluated and recorded 4-6 days after inoculation. On day 4-6, the filter paper discs were removed to allow the potato tissue to dry. Tubers were then re-evaluated on day 14. An isolate was considered to be pathogenic when at least three of the eight tubers developed scab symptoms. Isolations were made from all lesions to confirm Koch's postulates. Pathogenicity testing was conducted twice using this assay.

Radish pathogenicity assay. The same subset of 22 isolates and positive control used in the micro-tuber assay were used in the radish assay. The radish assay was conducted using the methodology of Wanner (2004). Inoculum for the assay was produced by growing the *Streptomyces* cultures in oatmeal broth for 2 days (oatmeal agar prepared without agar), and diluting the suspension to a 1.2 x 10^6 cfu/ml spore suspension.

Radish seedlings were produced from seed (cv STAR 1651, Sakata Seed, South Africa) sown in seed trays, filled with Hygromix (Hygrotech, South Africa). The seedlings where transferred from seed trays to 15 cm pots filled with Hygromix, four seedlings per pot, and placed at 21 °C in a greenhouse for 7 weeks. The seedlings were then inoculated with 10 ml inoculum that was added to the soil surface of each seedling in the vicinity of the seedling roots. There were six replicates for each isolate, and a negative control that received no inoculum was also included. After 21-28 days, the plants were removed and evaluated for visual disease symptoms. Isolations were made from all lesions to confirm Koch's postulates. Pathogenicity testing was conducted twice.

Statistical analysis. Results of the potato double pot technique were evaluated using analyses of variance (ANOVA). The common scab disease index frequencies observed in the 15 classes were subjected to a generalized linear model (GLM) technique with a logistic link function. The 15 ordinal classes included; 0, 6, 7, 12, 14, 18, 21, 22, 24, 28, 30, 33, 35, 44 and 55. A class of 0 indicated no disease and a class of 55 indicated 100 % tuber coverage with deep-pitted scab symptoms. The maximum likelihood estimators (XBeta's) were calculated on an underlying scale (McCullagh & Nelder, 1989). These estimators (location values), that

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are on an interval scale were subjected to a randomized block design analysis of variance using SAS version 9.2 (SAS, 1999). The standardized residuals were subjected to the Shapiro-Wilk's test for non-normality (Shapiro & Wilk, 1965). Student's t-LSD (Least Significant Differences) was calculated at a 5 % significance level to compare means of significant effects.

The statistical analysis of the correlation between the pathogenicity assays were analysed by determining the average measurement values of the three methods and calculating Pearson's correlation coefficient (alpha = 0.05) to determine if the methods could be correlated (Snedecor & Cochran, 1967).

Thaxtomin production

Oat bran agar assay. All the isolates collected in the study were evaluated (Table 1) for thaxtomin production using the methodology developed by Goyer *et al*. (1998). Each *Streptomyces* isolate was plated onto YME media and grown for 5 days at 30 °C. Developing colonies were then transferred to oat bran agar and incubated at 30 °C for 6 days. Isolates that produced colonies surrounded by a yellowish halo were scored positive for thaxtomin production.

Tuber slice assay. Eight of the pathogenic isolates that did not produce thaxtomin on oat meal agar (Table 1), were also evaluated for thaxtomin production using the modified method of Hao *et al*. (2009). A *S. scabiei* reference culture known to produce thaxtomin was included as a positive control. Inoculum was prepared from 5-7-day-old oatmeal agar cultures, by adding 20 µl of sterile distilled water to each culture plate to produce a spore suspension.

Disease-free potato tubers (cv BP1) were washed and surface disinfested with 10 % NaClO solution for 1 min. A tuber tissue disc (20 mm diameter x 7 mm height) was excised from the sterilized tuber and placed on moist filter paper in a 90 mm diameter plastic Petri

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dish, and 10 µl inoculum was dropped onto the centre of each tuber disk. The disks were incubated in a closed container lined with moist filter paper, at 22-24 °C, in the dark. The necrotic area of the tuber tissue was evaluated from 36h–7 days by subtracting the final necrotic area from the area of inoculation. A negative water control was included.

Molecular species identification of *Streptomyces* isolates

DNA extraction from Streptomyces cultures. DNA was extracted from 5-day old *Streptomyces* cultures grown on YME agar. For each isolate, approximately 100 mg of mycelia was transferred to a 2.2 ml Eppendorf tube containing 200 µl of nuclease-free water. Total genomic DNA was extracted from the mycelial solution, using the Zymo Research (ZR) Fungal/Bacterial DNA MiniPrep Kit (Inqaba Biotech™, Pretoria, South Africa). Two hundred microliter of buffer was added to the extracted suspension before the entire content of the tube was transferred to the ZR bashing bead lysis tube together with 750 µl of lysis solution. The tubes were placed in a bead beater (TS-100C Thermo shaker; Heyns Lab Supplies, Johannesburg, South Africa) and processed at maximum speed for 5 min. for optimal lysis. Subsequent steps were according to manufacturer's instruction. The DNA quality and quantity were checked by gel electrophoresis and UV spectrophotometry (A_{260}/A_{280}) using a Nanodrop® ND-1000 spectrophotometer (Inqaba Biotech™). All DNA extractions were diluted to a concentration of 2 ng/µl for use in PCR reactions.

Streptomyces species identification using species-specific primers. Isolates were assigned to *Streptomyces* species based on the results of PCR amplification with three published species-specific primer pairs targeting the 16s rRNA gene in *S. turgidiscabies, S. acidiscabies* and *S. scabiei / S. europaeiscabiei* (as a group) (Wanner, 2006, Table 2). The species-specificity of all three primer pairs (Table 2) was validated using DNA from several *Streptomyces* isolates, including *Streptomyces* sp., *S. scabiei* (ATCC 49173), *S. turgidiscabies* (ATCC 700248) and *S. acidiscabies* (ATCC 49003).

Suitability of all DNA extractions for PCR amplification with the species-specific primers was first verified by conducting PCR amplification with universal primers (16s1F

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and 16s1R; Table 2) that target the 16S rRNA gene. All primers used in this study were purchased from IDT® (Integrated DNA Technologies, Coralville, Iowa, USA). Amplification was conducted in a total reaction volume of 40 µl, which contained 200 µM of each dNTP, 0.2 µM of each primer, 0.65 units of BIOTAQ™ DNA polymerase (Bioline, Biotaq Incorporated, Gaithersburg, USA), 1 x PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.2 mg bovine serum albumin (BSA) and 8ng genomic DNA. PCR amplification conditions consisted of an initial denaturation step at 95 °C for 5 min., followed by 35 reaction cycles of 30 sec denaturation at 95 °C, 45 sec annealing at 55 °C, and 2 min. extension at 72 °C. The final amplification step consisted of one cycle of extension at 72 °C for 10 min. All amplifications were conducted in a Veriti 96 well Fast Thermal Cycler (Applied Biosystems, Johannesburg SA). Amplified DNA fragments were separated on 1.5 % agarose gels, stained with ethidium bromide and visualised under UV light.

All *Streptomyces* isolates listed in Table 1 were included in the species-specific PCR analyses. The PCR conditions used for amplification of each primer pair were similar to those described for primer pair 16s1F /16s1R, except that each primer pair was amplified using a specific $MgCl_2$ concentration in the PCR reaction and annealing temperature during amplification, as specified in Table 2. The expected amplicon sizes for each species-specific primer pair are shown in Table 2.

Differentiation of S. scabiei and S. europaeiscabiei. All isolates that tested positive with the *S. scabiei / S. europaeiscabiei* primer pair were evaluated further using PCR-RFLP (restriction fragment length polymorphism) analyses, in order to differentiate the two species. The 16S-23S rDNA ITS region was amplified from all isolates that tested positive with the *S. scabiei / S. europaeiscabiei* species-specific primers, using primers ITSL and ITSR (Song *et al.*, 2004). The reaction conditions were similar to those described for the 16s1F/16s1R primer pair. PCR amplification conditions consisted of denaturation for 5 min. at 95 °C, followed by 35 cycles of 95 °C for 30 s, annealing at 58 °C for 45 s and extension for 1 min. at 72 °C, followed by a final extension step at 72 °C for 10 min. The PCR product was digested using the restriction enzyme *Hpy*991 (Flores-Gonzalez *et al.*, 2008). The *Hpy*991 digest reaction consisted of 1x enzyme buffer (NEB, Ipswich, USA), 1 µL *Hpy*991 (NEB)

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and 8 µL PCR product in a total volume of 25 µL. The digest was incubated overnight at 37 °C, and 15 µl was run on a 1 % agarose gel along with a 100 bp DNA ladder.

Phylogenetic analysis of 16S rRNA gene. A few of the pathogenic isolates from South Africa did not yield amplicons with the species-specific primers (see Results section). Therefore, these isolates were analysed phylogenetically to determine their species identity and relatedness to known species. The species identity of a few pathogenic isolates that were identified with the species-specific primers was also confirmed through sequence analyses (Table 2, Fig. 3). The 16S rRNA region was amplified using primers 16S-1F and 16S-1R as previously described. The PCR products were sequenced directly by Inquaba Biotech (Pretoria, South Africa), using primers 16S-1F, 16S-1R, 16S455-435 and 16S1346-1366 (Table 2). Chromatograms were edited and consensus sequences were constructed using Geneious Pro v5.5 (Drummond *et al*. 2010).

In order to identify the most important sequences to be included in the phylogeny, the sequences of the unidentified pathogenic South African isolates were subjected to GenBank Blast analyses to (i) identify sequences that had the highest similarity to these sequences and (ii) identify the *Streptomyces* clade number (Labeda *et al.*, 2012) to which the isolates most likely belonged to. The phylogeny included all known phytopathogenic species and putative new species, the unknown species isolates from South Africa and the sequences to which they had the highest similarity, and *Streptomyces* species that fitted into the Ladeda *et al.* (2012) clades to which the South African isolates most likely belonged to. Sequence alignment (ClustalW) and phylogenetic analyses were conducted with version 5 of the MEGA software package (Tamura *et al..*, 2011). The tree was rooted with *Streptoalloteichus tenebrarius,* which was the species also used by Labdea *et al.* (2012) to root their 16S rRNA phylogeny. The data set was analysed using the neighbour-joining (NJ) algorithm with the Tamura 3-parameter distance coefficient, which MEGA identified as the best model. The topologyof the tree was evaluated with bootstrap analyses of a 1000 re-samplings in MEGA.

Phylogenetic analyses of multi-locus sequence data. The South African isolates that were not identified as a known phytopathogenic species, were further analysed using three

protein coding gene regions (*rpoB, trpB* and *atpD*). The genes were amplified using the primers (Table 2) of Guo *et al.* (2008). The PCR reaction for *rpoB* and *atpD* consisted of 200 µM of each dNTP, 0.4 µM of each primer, 1x PCR buffer (Bioline), 0.65 units of BIOTAQ™ DNA polymerase (Bioline), 3 mM $MgCl_2$ and 30 ng genomic DNA in a total reaction volume of 30 µl. The PCR reactions for *trpB* consisted of 200 µM of each dNTP, 1 µM of each primer, 1x PCR buffer (Bioline), 2 µl DMSO (Sigma-Aldrich, Kempton Park, South Africa, catalogue nr. D8418), 3 mM $MgCl_2$ and 30 ng genomic DNA in a total reaction volume of 30 µl. PCR amplification for all four genes consisted of an initial denaturation step of 95°C for 5 min, followed by 34 cycles of 95°C for 30s, annealing at temperatures as indicated in Table 2 for 30s, and extension at 72°C for 90s. The final extension step consisted of 10 min. at 72°C. The PCR products were sequenced using the PCR amplification primers, and consensus sequences were constructed as previously described (McLeod *et al.*, 2009).

The sequences that were included in the phylogeny consisted of protein coding gene sequences to which the South African isolates had the highest similarity in Blast analyses. Additional sequences that were included consisted of other *Streptomyces* species with high similarity to these gene Bank sequences, and all the phytopathogenic species. All the aforementioned sequences were also used in the publications of Labeda (2011) and Han *et al.* (2012). The tree was rooted using *S. cinereorectus*, which was also used by Labeda (2011) for rooting their phylogeny. Alignment of the concatenated three gene sequences were conducted in BioEdit sequence alignment Editor (T. Hall, Abbott Laboratories, Ibis Biosiences, CA), and phylogenetic analyses were conducted with version 5 of the MEGA software package (Tamura *et al.*, 2011). The tree-making algortithm that was used consisted of the NJ algorithm (Tamura 3- parameter model). The topology of the tree was evaluated with bootstrap analyses of a 1000 re-samplings in MEGA.

PCR amplification of marker genes in the PAI.

All isolates were analysed for the presence of the *txtAB, tomA* and *nec1* genes. Published primers were used for PCR amplification of all three genes (Wanner, 2006). The genes were amplified using the PCR reaction and amplification conditions that were described for the 16s1F/16s1R primer pair. The primer pairs used, the expected PCR product

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sizes as well as the annealing temperature and $MgCl_2$ concentrations used in PCR reactions are listed in Table 2. In each group of reactions reference isolates *S. scabiei*, *S. acidiscabies* and *S. turgidiscabies* were included as positive controls, which each contain all three marker genes.

RESULTS

Streptomyces isolation and culturing

A total of 132 *Streptomyces* isolates were recovered from symptomatic tuber tissues and included isolates from six major potato production areas in South Africa (Fig. 1). The isolates were mainly obtained from potato cultivars such as BP1 and a few other cultivars (Up to Date, Buffelspoort) that are known to be susceptible to common scab in South Africa. However, a few isolates were also obtained from the cultivar Mondial, which is known to be tolerant to common scab in South Africa. The largest number of isolates were obtained from the Gauteng Province (43 %), followed by the Northern Cape (21 %), Limpopo (18 %), Sandveld (11 %), Free State (6 %) and Kwazulu-Natal (1 %). The number of isolates that were obtained from the production areas correlated with the amount of samples that were collected and received for each location. The largest number of samples were received from Gauteng (50) followed by Northern Cape (29), Limpopo (25), Sandveld (17), Free State (6) and Kwazulu Natal (1).

Phenotypic characterization of *Streptomyces* isolates

Most of the pathogenic *Streptomyces* isolates produced brown mycelia with grey spores borne on spiral spore chains, except for the *S. caviscabies* isolates that produced gold-brown mycelium with white spores borne on flexuous spore chains. The phenotypic morphology of the non-pathogenic *Streptomyces* isolates varied from cream to light brown mycelia, with grey to green spores borne on spiral and flexuous spore chains (Table 1).

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Pathogenicity assays

Double pot technique. Levene's variance ratio test (Levene, 1960) showed that the variance for the data from the two repeat trials differed significantly (P< 0.05), therefore, weighted analysis was conducted in order to combine the data from the two trials.

The 132 evaluated isolates differed significantly in their pathogenicity (P<0.0001; Table 3). Seventy isolates were identified as pathogens since they had a significantly higher (P <0.05) scab index value than the control treatment, whereas the scab index of the remaining 62 isolates did not differ significantly (P <0.05) from the control treatment, and these isolates were thus identified as non-pathogenic (data not shown). Isolates that were the most virulent had a scab index of 24.43 and those that had the lowest virulence had a scab index of 12.19. At the species level (see Molecular identification of species section), isolates that were capable of inciting common scab were dominated by *S. scabiei* (51.4 %) followed by *S. europaeiscabiei* (30 %), *Streptomyces* strain RSA1 (5.7 %), *S. caviscabies* (5.7 %), *Streptomyces* strain RSA2 (4.3 %), *Streptomyces* strain RSA3 (1.45%) and *S. stelliscabiei* (1.45 %). Disease symptoms mainly consisted of superficial lesions but in the case of *S. caviscabies* isolates, deep-pitted lesions were prevalent on potato tubers. The *Streptomyces* strain RSA1 isolates (N8, N38, N55, N92) caused a symptom that was distinct from the most prevalent common scab symptoms. This symptom corresponded to the fissure scab symptoms recently reported by Gouws & McLeod (2012) (Fig. 2).

Significant differences in virulence were evident among isolates belonging to *S. scabiei* (P < 0.0001), *S. caviscabies* (P < 0.001) and *Streptomyces* strain RSA1 (P = 0.02), but not isolates belonging to *S. europaeiscabiei* (P = 0.82) (Table 3). The ten most virulent isolates included five *S. scabiei* isolates (O38, O10, O36, N28, N44), three *S. europaeiscabiei* isolates (O8, N43, O1) and two *S. caviscabies* isolates (O69, O47). *Streptomyces* strain RSA1 (N38, N92, N8) and RSA3 (N2) isolates were among the twenty most virulent isolates. The ten isolates that had the lowest virulence, and that also differed significantly in virulence (P < 0.05) from the most virulent isolates, consisted of one *S. stelliscabiei* isolate (O68) four *S. europaeiscabiei* isolates (O5, N35, N20, N26) and five *S. scabiei* isolates (O23, O66, O7, O11, N52) (data not shown).

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In-vitro micro tuber and radish pathogenicity assays. All the isolates that tested positive for pathogenicity in the double pot and micro-tuber assay tested positive in the radish assay. There was a positive correlation (Pearson's test; alpha = 0.05) between the three techniques, with non-pathogens testing as non-pathogenic in all assays and pathogens as pathogenic in all assays (Table 4).

Thaxtomin production

Oat bran agar assay. None of the 62 non-pathogenic isolates produced thaxtomin. Among the 70 evaluated pathogenic isolates, 89 % (62 isolates) produced thaxtomin. All the pathogenic isolates that were identified as thaxtomin producing isolates using the oat bran agar assay, also contained the *txt*AB gene (see under "PCR amplification of marker genes in the PAI" section). Eleven percent (8 isolates) of the pathogenic isolates did not produce thaxtomin and also did not contain the *txt*AB gene and included isolates O15, O17, O31, O47, O52, O56, O69 and N2 that represented *S. caviscabies* and the *Streptomyces* strains RSA2 and RSA3 isolates (Table 1).

Tuber slice assay. The tuber slice assay showed that none of the pathogenic isolates that tested negative in the oat bran assay, including the *S. caviscabies* and the *Streptomyces* strains RSA2 and RSA3 isolates were able to cause necrosis on the potato tuber slice tissue. The positive control *S. scabiei* isolate produced on average a 10 mm necrotic zone around the inoculation point.

Molecular identification of *Streptomyces* isolates

Streptomyces species identification using species-specific primers and differentiation of S. scabiei and *S. europaeiscabiei isolates.* All 62 non-pathogenic isolates failed to yield amplicons in reactions that utilized primers specific for *S. turgidiscabies, S. acidiscabies* and *S. scabiei / S. europaeiscabiei.* Among the 70 pathogenic isolates, a total of 57 isolates tested positive with the *S. scabiei / S. europaeiscabiei* primer pair. These isolates were analysed

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further using PCR-RFLP analyses, which showed that twenty one of the isolates (O1, O2, O5, O8, O13, O21, 024, 032, 038, 057, 058, 065, N4, N7, N19, N20, N23, N26, N31, N35, N43) could be classified as *S. europaeiscabiei,* with the remaining isolates being *S. scabiei.* Nineteen percent (13 isolates) of the pathogenic isolates did not yield positive amplicons in reactions that employed the species-specific primers. The majority of the pathogenic isolates were isolated from BP1 potatoes showing superficial lesions on the tuber surface. However, a few of the isolates (N8, N38, N55, N92) that did not yield amplicons with the species-specific primers were isolated from the cultivar Mondial.

Phylogenetic analysis of 16S rRNA gene. There was low bootstrap support for the backbone of the NJ tree (Fig. 3). The overall topology of the NJ tree agreed with the NJ tree published by Labeda *et al.* (2012), except that differences were observed in the bootstrap support obtained for some nodes. The South African isolates O69 and O52 were identified as *S. caviscabies,* since the sequences clustered with the type strain of *S. caviscabies.* Two other South Africa isolates (O31 and O47) had 100% sequence similarity to these isolates and were thus also identified as *S. caviscabies.* All the *S. caviscabies* sequences grouped within a large well-supported clade (100 % bootstrap) that is known as the *S. griseus* 16S rRNA clade, and had high similarity to the sequences of several other isolates including *S. fimicarius*. The *S. griseus* 16S rRNA clade also included *S. luridiscabiei* and the undescribed phytopathogenic species *Streptomyces* sp. DS3024 of Hao *et al.* (2009). The South African isolate O68 was identified as *S. stelliscabiei* since it clustered (99 % bootstrap support) with the *S. stelliscabiei* type sequence in clade 24 of Labeda *et al.* (2012). All the *S. scabiei* isolates, including the South African *S. scabiei* isolates (N31, N39 & O65) grouped in a clade with low bootstrap support (66 %), whereas the *S. europascabiei* South African isolate (N26) and the type strain isolate were unresolved in the phylogeny. In the study of Labeda *et al.* (2012) these isolates all fitted into clade 25 along with *S. diastochromogenes*, which differed from the current study where there was no bootstrap support for this clade. The *Streptomyces* sp. IdahoX (Wanner, 2007) sequence was unresolved in the phylogeny. Other clades from Labeda *et al.,* (2012) that included phytopathogenic species were clades 68 (*S. lydicus*), 55 (*S. aureofaciens*), 22 (*S. reticuliscabiei* and *S. turgidiscabies*), 10 (*S. niveiscabiei* and *S. puniciscabiei*) and 5 (*S. acidiscabies*).

The remaining pathogenic South African isolates (N38, N92, N8, O56, O17 and N2) grouped into a large well supported clade (88 % bootstrap) that formed part of Labeda *et al.* (2012) clades 86, 100 and 112 (Fig. 3). This large clade did not contain any known previously reported common scab causing species. Within the large clade, the South African isolates grouped into two well supported sub-clades. Isolates N38, N92 and N8, which some were isolated from the cultivar Mondial, grouped with an isolate (RG01) that has been reported as causing fissure scab in South Africa (Gouws and McLeod, 2012) into a well supported clade (99 %) that corresponded to Labeda *et al.* (2012) clade 86. These isolates were thus all identified as fissure scab isolates, and are designated here as *Streptomyces* strain RSA1. The *Streptomyces* strain RSA1 isolates may belong to *S. gancidicus, S. werranensis, S. cyaneus* or *S. pseudogriseolus* since these were the type strains with which the isolates clustered (91 % bootstrap) and had the highest sequence similarity (99 %). The South African isolates N2, O56 and O17 grouped in a clade terminal to clade 100 of Labeda *et al.* (2012), with their nearest neighbour being *S. flaveolus.* Isolate N2 was present on a long branch and most likely represent a species separate from isolates O56 and O17. Isolate N2 is hereafter designated as *Streptomyces* strain RSA2, and isolates O17 and O56 as RSA3.

Phylogenetic analyses of multi-locus sequence data. The topology of the three-gene-concatenated phylogeny (Fig. 4) in general agreed with the trees of Labeda (2011) and Han *et al.* (2012). The multigene phylogeny was also characterized by long branch lengths, revealing the greater sequence variation that is present in the protein coding genes than in the 16S rRNA gene. It was also notable that the relationships between species identified in the multigene phylogeny sometimes differed from those identified in the 16S rRNA phylogeny. For example, in the multigene phylogeny *S. acidiscabies* and *S. niveiscabiei* were related (73 % bootstrap), whereas in the 16S rRNA phylogeny *S. niveiscabiei* was most closely related to *S. puniciscabiei* (80 % bootstrap). These contradictions were also evident when comparing phylogenies from Labeda (2011) and Labeda *et al.* (2012). Unlike the 16S rRNA tree, the multilocus tree was able to show the high relatedness of *S. stelliscabiei, S. botropensis, S. scabiei* and *S. europaiscabiei* since these isolates all grouped into a clade with high bootstrap support (99 %). Another important grouping (100 % boostrap support) was that of *S. caviscabies* with *S. fimicarius*, which suggest that these species are synonyms (Fig. 4).

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The *Streptomyces* isolates RSA1, RSA2 and RSA 3 grouped within a clade that had moderate bootstrap support (73 %). Within this clade, the *Streptomyces* isolates RSA1 (N8, N38, N92), which produced fissure scab symptoms, grouped within a sub-clade with high bootstrap support (100 %), with their nearest neighbours being *S. speibonae, S. labedae* and *S. erythrogriseus*. These isolates are all related but distinct based on long branch lengths. *Streptomyces* strain RSA2 and RSA3 clustered into a different sub-clade with high bootstrap support (100 %), with their nearest neighbours being *S. coelicolor* (isolate from which the genome has been sequenced), *Streptomyces* sp. FXJ6.295 and *Streptomyces* sp. FXJ6.033.

The pathogenic *Streptomyces* species that were identified were differentially distributed among the different potato production regions. However, this result may be a function of sampling bias since unequal numbers of isolates were evaluated from each province. *Streptomyces scabiei* and *S. europaeiscabiei* were identified in the Northern Cape, Limpopo, Sandveld, Free State and Gauteng, *S. caviscabies* in Sandveld and *S. stelliscabiei* in Limpopo. The pathogenic *Streptomyces* strains RSA1, 2 and 3 originated from Gauteng and Free State (*S.* sp. RSA1), Northern Cape (*S.* sp. RSA2) and Kwazulu Natal (*S.* sp. RSA3).

PCR amplification of marker genes in the PAI

Analyses for the three marker PAI genes (*txtAB, nec1* and *tomA*) showed that most of the pathogenic isolates possessed some of the genes, whereas one or more of these genes were detected in only a few of the non-pathogenic isolates. The *txtAB* and *nec1* virulence factors were the most commonly detected PAI marker genes among the pathogenic *Streptomyces* isolates (89 %), followed by *tomA* (81 %) (Table 1). Among the non-pathogenic *Streptomyces* isolates there were two isolate, O3 and O29, which contained the *nec1* and *tomA* genes respectively. The *txtAB* pathogenicity factor was not detected in any of the non-pathogenic *Streptomyces* isolates. The *S. caviscabies, Streptomyces* strain RSA3 and *Streptomyces* strain RSA 2 isolates did not contain the *nec1, tomA* or *txtAB* genes, and also did not produce thaxtomin based upon the oatmeal agar and tuber slice assay. All three genes (*nec1*, *txtAB* and *tomA*) were present in the South African *S. europaeiscabiei* and *S. scabiei* isolates. The *S. stelliscabiei* and *Streptomyces* strain RSA1 isolates lacked the *tomA* gene (Table 1).

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DISCUSSION

In South Africa, four known *Streptomyces* species and three putative new phytopathogenic species were identified as the causative agents of potato scab. A large percentage (47 %) of the *Streptomyces* isolated from scab lesionswere non-pathogenic. This finding concurs with that of Wanner (2007) who also reported a high proportion of non-pathogenic *Streptomyces* isolates recovered from scab tuber lesions. Among the pathogenic strains, *S. scabiei* was the most prevalent species in South Africa and was detected in five of the six surveyed potato production regions. This concurs with several other studies in which *S. scabiei* was identified as the primary causal agent of common scab (Faucher *et al.*, 1992; Loria *et al.*, 1997; Miyajima *et al.*, 1998; Gouws, 2006). The other three known phytopathogenic species from South Africa each comprised 37 % of the pathogenic isolates identified in this study, and included *S. caviscabies, S. europaeiscabiei* and *S. stelliscabiei. S. caviscabies and S. stelliscabies* were each identified in one of the production regions, whereas *S. europaeiscabiei* was identified in five of the six potato production regions. This is the first report of *S. caviscabies, S. europascabiei* and *S. stelliscabiei* causing common scab in South Africa. *Streptomyces caviscabies* has been reported as a common scab pathogen in the USA and Canada (Goyer *et al.*, 1996) and *S. europaeiscabiei* and *S. stelliscabiei* occur in Europe and North America (Bouchek-Mechiche *et al.*, 2000). It should, however, be noted that *S. caviscabies* was recently shown by Rong & Huang (2010) to be a later heterotypic synonym of *S. fimicarius*, which was also supported by the multigene phylogeny from the current study. The three putative new phytopathogenic species, which in total comprised 11 % of the pathogenic isolates recovered in this study, included *Streptomyces* strain RSA1, which occurred in two production regions and *Streptomyces* strains RSA2 and RSA3 that each occurred in one production region. The *Streptomyces* strain RSA1 isolates corresponded to a recently reported undescribed *Streptomyces* species from South Africa that causes fissure scab (Gouws & McLeod, 2012).

Identification of the putative new phytopathogenic species was aided by phylogenetic analyses of the 16S rRNA region and a multilocus phylogeny. As expected, the 16S rRNA phylogeny provided less resolution than the multilocus phylogeny, but was useful since the 16S rRNA sequences of many *Streptomyces* species have been published including the extensive phylogeny of Labeda *et al.* (2012). In contrast, the published multigene

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phylogenies have focused only on a few species with emphasis on clades containing *S. griseus* (Guo *et al.,* 2008; Rong & Huang, 2010), *S. hygroscopicus* (Rong & Huang, 2012), *S. albidoflavus* (Rong *et al.*, 2009) and the study of Han *et al.* (2012) that only investigated a few species within the family *Streptomycetaceae*. The study of Labeda (2011) focused on a multilocus phylogeny of phytopathogenic *Streptomyces* species. The 16S rRNA phylogeny from the current study suggested that *Streptomyces* strain RSA1 could belong to *S. gancidicus, S. werranensis, S. cyaneus* or *S. pseudogriseolus*. Unfortunately, there is no protein coding gene sequences available in GenBank for the aforementioned species. Therefore, in future studies the protein coding gene sequences of these type strain isolates will have to be obtained and included in analyses to determine whether the South African isolates belong to these species. *Streptomyces* RSA2 and RSA 3 isolates were closely related to each other but distinct, and had *S. flaveolus* as their nearest neighbour in the 16S rRNA phylogeny. In the multigene phylogeny *Streptomyces* RSA2 and RSA3 isolates seemed related and may represent the same species.

In the 16S rRNA phylogeny and multigene phylogeny, the South African *Streptomyces* strains RSA1, RSA2 and RSA3 were shown to be somewhat related since they grouped together in very large clade with relative good bootstrap support (73 % in the multilocus phylogeny), which did not contain any known scab pathogens from a country other than South Africa. The relatedness of *Streptomyces* strains RSA1, RSA2 and RSA3 to known phytopathogenic species, i.e. to which species it might be ancestral, could not be determined due to the backbone of the 16S rRNA phylogeny and the multigene phylogeny not containing bootstrap support at most of the nodes. The fact that the putative new phytopathogenic species from South Africa were phylogenetically distinct from other known phytopathogens, could suggest that these pathogens developed from local soil *Streptomyces* populations in South Africa and independent from other global populations. This is possible since it is known that phytopathogenic *Streptomyces* isolates evolve through horizontal gene transfer of a PAI that contain virulence and pathogenicity genes (Kers *et al.*, 2005).

Of notable consequence was the failure to detect important PAI-associated marker genes in *Streptomyces* strains RSA1, RSA 2 and RSA3. For the *Streptomyces* strains RSA2 and RSA3 isolates this included the absence of the *txt*AB gene that is involved in thaxtomin

production despite their capacities to produce disease symptoms on potato. Thaxtomin production is thought to be essential for pathogenicity since the presence of *txtAB* has been consistently associated with the pathogenicity of common scab isolates (King, *et al.,*1991; Healy & Lambert, 1991; Loria *et al.,* 1995; Wanner, 2006). *Streptomyces* strains RSA2 and RSA3 also did not contain the other two PAI marker genes (*nec1* and *tomA*). Another species from South Africa that apparently lacked all three of the marker genes yet caused symptoms was *S. caviscabies. Streptomyces* strain RSA1 contained the *nec1* and *txtAB* genes but lacked the *tomA* gene. The apparent absence of the PAI marker genes, especially the *txtAB* gene, in some isolates, however, will have to be confirmed through Southern analyses, since the isolates may have mutations in the *txtAB* primer annealing sites and other marker genes. Whole genome sequencing can also be conducted to determine whether the apparent lack of the PAI in these isolates is an artefact of divergent marker gene sequences. However, it will be important to re-test the pathogenicity of the isolates that lacked the *txtAB* gene before conducting these analyses, since these isolates were collected and stored more than two years ago, and a period of 6 months elapsed from the first pathogenicity screening to the subsequent PCR analysis. It is thus possible that these isolates might have lost their pathogenicity, since it is not uncommon for phytyopathogens, including *Streptomyces*, to lose their pathogenicity when cultured away from the plant host (Dasgupta, 1988). This may account for the lack of the *txtAB* gene is these isolates. If the isolates, however, truly lack the *txtAB* gene, this will be an important factor to consider when using this gene for PCR detection and quantification of common scab pathogens in South Africa, as previously described by Qu *et al.* (2008). The South African *S. stelliscabies* isolates were more typical of pathogenic scab species and contained two (*txtAB* and *nec1*) of the three marker genes, whereas the *S. scabiei* and *S. europaeiscabiei* isolates contained all three marker genes.

The isolates from South Africa that lacked the *txtAB* gene, and that comprised 11 % of the pathogenic isolates, were also shown to lack thaxtomin production based on an oatmeal agar assay and a tuber slice assay. A previous study from South Africa, using a thin layer chromatography (TLC) method for analyzing thaxtomin production, identified an even higher percentage (14 %) of pathogenic isolates that did not produce thaxtomin (Gouws, 2006). The lack of thaxtomin production by pathogenic *Streptomyces* isolates previously has been reported (Wanner, 2004; Cao *et al*., 2012). Cao *et al.* (2012) reported a new pathogenic *Streptomyces* strain (GK18) that does not produce thaxtomin and did not hybridize with its

biosynthetic probe. The authors hypothesized that a different phytotoxin, borrelidin, could function as a pathogenicity factor in this strain. In Canada, Goyer *et al*. (1996) also reported that their *S. caviscabies* isolates lacked *nec1* and did not produce thaxtomins in oatmeal media. However, when they tested the same isolates on media composed of potato or sweet potato peel, they produced thaxtomin. For this reason, the South African *S. caviscabies* isolates and *Streptomyces* strains RSA2 and RSA3 that did not produce thaxtomin on oatmeal agar, were also evaluated using a tuber slice assay, an assay that has been used extensively as a diagnostic method to evaluate thaxtomin production (Loria *et al*., 1995; Kim *et al*., 1999). The South African isolates also did not produce thaxtomin using this alternative assay method.

The phytopathogenic *Streptomyces* species from South Africa varied somewhat in the type of symptom induced on the host and in virulence (combination of lesion type and tuber surface coverage, which is expressed as the common scab disease index). The *S. caviscabies* isolates produced deep-pitted scab lesions. Goyer *et al.* (1996) also found that their *S. caviscabies* isolates were mostly associated with deep-pitted scab lesions isolated from potatoes sampled in Saint-Ambriose, Quebec. The symptom caused by the *Streptomyces* strain RSA1 isolates was notably different from the other common scab symptoms and consisted of fissure lesions that cause severe cosmetic damage to tubers, as was first reported by Gouws & McLeod (2012) in South Africa. This symptom has not been reported from any other region of the world and is of concern in South Africa since it occurs on the cultivar Mondial that is tolerant to typical common scab. Isolates within *S. scabiei, S. caviscabies* and *Streptomyces* strain RSA1 differed in virulence, and high virulence could thus not be linked to a specific species. The variability in virulence within *S. scabiei* was evidenced by the fact that when all pathogenic isolates were compared, isolates that were among the ten most virulent and ten least virulent isolates included *S. scabiei* isolates. Isolates from *S. caviscabies* were also among the ten most virulent isolates, whereas isolates from *Streptomyces* strain RSA1 and RSA3 were among the twenty most virulent isolates. Variability in virulence amongst common scab-causing *Streptomyces* isolates has been reported in various other studies (McKee, 1958; Bramwell *et al*., 1998; Wanner, 2004; Pasco *et al*., 2005; Wanner & Haynes, 2009).

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The genetic diversity observed in phytopathogenic *Streptomyces* isolates recovered from symptomatic field-grown potato tubers sampled from six production regions in South Africa suggests that different populations of scab-causing species could contribute to differences in the incidence and severity of common scab on potato. Seven pathogenic *Streptomyces* species were identified, four known and three putative new phytopathogenic species, compared to the identification of only *S. scabiei* in a previous study (Gouws, 2006). All the putative new phytopathogenic species are related taxa, but phylogenetically distinct from all other known phytopathogenic *Streptomyces* isolates. Further investigations should be conducted to evaluate the relative prevalence of distinct taxa in South African production areas that were not sampled in this study. Several of the putative new phytopathogenic species and *S. caviscabies* did not produce thaxtomin and also appeared to lack all three PAI marker genes, suggesting that other factors might function as determinants in their pathogenicity or that the isolates lost these factors in prolonged storage. The *Streptomyces* isolates from South Africa varied in virulence, and the identification of highly virulent isolates among the *Streptomyces* strain RSA1 isolates that causes fissure scab is of major concern, especially since some of these isolates were obtained from the cultivar Mondial that is tolerant to typical common scab in South Africa. This poses a serious threat to the South African potato industry including the seed, ware and processing industries.

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Table 1. Characteristics and origin of *Streptomyces* isolates from South Africa

Species[a]	Isolate codes[b]	Location[c]	Phenotypic traits[d]			PAI genes[e]			Thaxtomin production[f]
			Spore type	Spore colour	Mycelium colour	*nec1*	*tomA*	*txtAB*	
Pathogenic isolates									
S. scabiei	07, 010, 011, 012*, 023, 033, 036, 038#, 041, 046, 055*, 059#*, 061, 062, 063*, 065#*, 066#*,, N5, N6, N11, N14, N16, N23#, N28, N30, N32, N36, N37, N39, N42, N44#, N50, N51, N52, N54, N56	N Cape, Limpopo, Sandveld, Free State, Gauteng	Sp	Gr	Br	+	+	+	+
S. europaeiscabiei	01, 02, 05, 08, 013, 021, 024, 032, 038, 057, 058, 065, N4, N7, N19, N20, N23, N26#, N31#, N35, N43	N Cape, Limpopo, Sandveld, Free State, Gauteng	Sp	Gr	Br	+	+	+	+
S. caviscabies	031#*, 047#*, 052#, 069#	Sandveld	Fl	Wh	GBr	-	-	-	-
S. stelliscabiei	068#*	Limpopo	Sp	Gr	Br	+	-	+	+
Streptomyces strain RSA1	N8#, N38#, N55#, N92#	Free State, Gauteng	Sp	Gr	Br	+	-	+	+
Streptomyces strain RSA2	015#*, 017, 056	N Cape	Sp	Gr (R)	Br	-	-	-	-
Streptomyces strain RSA3	N2#	KZN	Sp	Gr	Gr	-	-	-	-

Species[a]	Isolate codes[b]	Location[c]	Phenotypic traits[d]			PAI genes[e]			Thaxtomin production[f]
			Spore type	Spore colour	Mycelium colour	*nec1*	*tomA*	*txtAB*	
Non-pathogenic isolates									
Unknown	016, 026*, 030, O42, N42	Limpopo N Cape Gauteng	Sp	Gr	LBr	-	-	-	-
Unknown	029	N Cape	Sp	Gr	Br	-	+	-	-
Unknown	034, 039*, N22	N Cape Gauteng Limpopo	Sp	Gr	Gr	-	-	-	-
Unknown	045, 070,	Limpopo	Fl	Gr	Cream	-	-	-	-
Unknown	03	Sandveld	Sp	Gr	Br	+	-	-	-
Unknown	04, 06, 018, 019*, 022, 025, 027, 037, 040, 043, 044, 048, 049, 050, 051, 054*, 060, 067*, N1, N3, N12, N13, N15, N17, N18, N21, N24, N25, N27, N29, N33, N34, N41, N45, N46, N47, N53, N57, N58	Limpopo, N Cape, Free State, Gauteng	Sp	Gr	Br	-	-	-	-
Unknown	09, N40, N48, N49	N Cape, Gauteng Free State	Sp	Gr	LBr	-	-	-	-

Species[a]	Isolate codes[b]	Location[c]	Phenotypic traits[d]			PAI genes[e]			Thaxtomin production[f]
			Spore type	Spore colour	Mycelium colour	*nec1*	*tomA*	*txtAB*	
Unknown	014, 071*	Limpopo N Cape	Sp	Gr (R)	Br	-	-	-	-
Unknown	028*	N Cape	Sp	GrWh	LBr	-	-	-	-
Unknown	053*	N Cape	Fl	Gr	LBr	-	-	-	-
Unknown	N59	OFS	Fl	Gr	Br	-	-	-	-
Unknown	N9	Gauteng	Fl	Green	Br	-	-	-	-
Unknown	N10	Gauteng	Sp	GrGre	LBr	-	-	-	-

[a] The species to which each isolate belonged to were determined using species-specific primers that are able to identify *S. scabiei / europascabiei* (as a group), *S. acidiscabies* and *S. turgidiscabies* (Wanner, 2006). The species identity of a few of the pathogenic isolates that did not yield an amplicon with the species-specific primers was determined through phylogenetic analyses of the 16S RNA gene and multi locus sequence analyses.

[b] Isolates that were sequenced to determine, or confirm their species identity are indicated by a "#". The isolates indicated with "*" were evaluated with three different pathogencity tests including the potato double pot technique, the radish- and micro-tuber assay.

[c] N Cape = Northern Cape; OFS = Orange Free State.

[d] Spore chain morphology: Sp = Spiral; Fl = Flexuous; Spore colour: Gr = Grey; GrWh = Grey white; Gr(R) = Grey with red lining; GrGre = Grey green, Wh = white; Mycelium colour: Br = Brown mycelium; GBr = Goldenbrown, LBr = Light brown mycelium.

[e] The presence of the thaxtomin A and B gene (*txtAB*), a necrosis gene (*nec1*) and a tomatinase gene (*tomA*) on the pathogenicity island (PAI) was evaluated using PCR analyses with published primers (Wanner, 2006). In each group of PCR reactions, reference isolates of *S. scabiei*, *S. acidiscabies* and *S. turgidiscabies* were included as positive controls since these isolates each contain all three marker genes. The positive identification of each gene in an isolate is indicated by "+" and the absence by "–".

[f] The oat bran agar assay developed by Goyer *et al*. (1998) was used to screen *Streptomyces* isolates for thaxtomin production, where colonies that produce thaxtomin are indicated by the production of a yellowish halo. Isolates that tested positive for thaxtomin production are indicated by "+" and those that tested negative are indicated by "–".

Table 2. Primer pairs and polymerase chain reaction (PCR) amplification conditions that were used in the study to characterize *Streptomyces* isolates from South Africa.

Gene [a]	Primer pair	Ann. temp (°C)	$MgCl_2$ (mM)	Product size (bp)	Reference
Marker genes for the PAI					
nec1	Nf:5'-ATGAGCGCGAACGGAAGCCCCGGA-3'				Bukhalid *et al.*,
	Nr:5'-GCAGGTCGTCACGAAGGATCG-3'	60	2.0	700	1998
txtAB	T+tAB1:5'-CCACCAGGACCTGCTCTTC-3'				
	T+tAB2:5'-TCGAGTGGACCTCACAGATG-3'	48	1.0	385	Wanner, 2006
tomA	Tom3:5'-GAGGCGTTGGTGGAGTTCTA-3'				
	Tom4:5'-TTGGGGTTGTACTCCTCGTC-3'	55	1.5	392	Wanner, 2006
***Streptomyces* species-specific primers**					
S. scabies & *europaeiscabiei* [b]	scab1m:5'-CGACACTCTCGGGCATCCGA-3'	60	1.0	1278	Wanner, 2006
	scab2m:5'-TTCGACAGCTCCCTCCCTTAC-3'				
S. acidiscabies	Aci1:5'-TCACTCCTGCCTGCATGGGCG-3'				
	Aci2:5'-CGACAGCTCCCTCCCACAAG-3'	60	1.5	1278	Wanner, 2006
S. turgidiscabies	Turg1m:5'-CCCTCGCATGGGGGTGGGTTC-3'				
	Turg2m:5'-CGACAGCTCCCTCCCCGTGAG-3'	60	1.5	1273	Wanner, 2006

Gene [a]	Primer pair	Ann. temp (°C)	$MgCl_2$ (mM)	Product size (bp)	Reference
Primers used for amplifying and sequencing 16s rDNA genes					
	16s 1F: 5’-CATTCACGGAGAGTTTGATCC-3’				Bukhalid *et al.*,
	16s 1R: 5’-AGAAAGGAGGTGATCCAGCC-3’	55	1.5	1531	2002
	16s 455-435:5’ –ACTTTCGCTTCTTCCCTGCT-3’				Wanner, 2006
	16s 1346-1366:5’ –ATTGCTGCGGTGAATACGTT-3’				Wanner, 2006
Primers used for amplification and sequencing of protein coding genes for the multilocus phylogeny					
rpoB	rpoBPF: 5’-GAGCGCATGACCACCCAGGACGTCGAGGC-3’	65	3	994	Guo *et al.,* 2008
	rpoBPR: 5’- CCTCGTAGTTGTGACCCTCCCACGGCATGA-3’				
trpB	trpBPF: 5’ – GCGCGAGGACCTGAACCACACCGGCTCACACAAGATCAACA-3’	65	3	822	Guo *et al.,* 2008
	trpBPR: 5’ – TCGATGGCCGGGATGATGCCCTCGGTGCGCGACAGCAGGC-3’				
recA	recAPF: 5’-CCGCRCTCGCACAGATTGAACGSCAATTC- 3’	53	3	913	Guo *et al.,* 2008
	recAR: 5’- GCSAGGTCGGGGTTGTCCTTSAGGAAGTTGCG-3’				
atpD	atpDPF: 5’- GTCGGCGACTTCACCAAGGGCAAGGTGTTCAACACC-3’	63	3	998	Guo *et al.,* 2008
	atpDPR: 5’- GTGAACTGCTTGGCGACGTGGGTGTTCTGGGACAGGAA-3’				

[a] Genes that were analysed included the thaxtomin A and B gene (*txtAB*), a necrosis gene (*nec1*), a tomatinase gene (*tomA*), ATP synthase F1, beta sub unit (*atpD*), recombinase A (*recA*), RNA polymerase, beta subunit (*rpoB*) and tryptophan synthase, beta subunit (*trpB*) genes.

[b] The primers identify *S. scabies* and *S. europaeiscabiei* as a group and do not differentiate between the species.

Table 3. Analysis of variance for the effect of 126 isolates from *Streptomyces* species on mean common scab incidence on potato tubers in a tunnel trial.

Source	**DF**		**Sum of Squares**	**Mean Square**	**F Value**	**Pr>F**
Experiments	1		875.9	875.88	9.86	0.0017
Reps within experiments	10		1092.1	109.21	1.23	0.2659
Isolates	126		774737.4	6148.71	69.23	<0.0001
Isolates within *Streptomyces caviscabies*		3	1394.1	464.70	8.19	0.0000
Isolates within *Streptomyces europaeiscabiei*		1	2.9	2.94	0.05	0.8199
Isolates within *Streptomyces scabiei*		54	46180.6	855.20	15.08	<0.0001
Isolates within *Streptomyces* strain.RSA1		3	548.7	182.92	3.23	0.0219
Isolates within *Streptomyces* strain RSA2		0	0.0	0.00	0.00	1.0000
Isolates within *Streptomyces* strain RSA3		0	0.0	0.00	0.00	1.0000
Isolates within non-pathogenic *Streptomyces*		58	0.0	0.00	0.00	1.0000
Experiments x Isolates	124		2676.8	21.59	0.24	1.0000
Experiment x Strain		7	546.0	78.00	1.38	0.2118
Strain x Group		1	217.8	217.76	3.84	0.0503
Between *Streptomyces* strains		7	726161.6	1829.35		<0.0001
Experimental Error	1216		68956.0	56.7073		
Sample Error	9352		830651.2	88.821		
Corrected Total	10829		1678989.401	1040803.64		

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Table 4. Correlation matrix (Pearson test) for the evaluation of three pathogenicity methods; Double pot technique, Micro tuber assay, Radish assay for identifying common scab pathogens.

Variables	Scab index	Micro-tuber assay	Radish assay
Scab index	1	0.438	0.442
Pathogenicity A (%)	0.438	1	0.905
Pathogenicity B (%)	0.442	0.905	1

*Values in bold are different from 0 with a significance level alpha = 0.05

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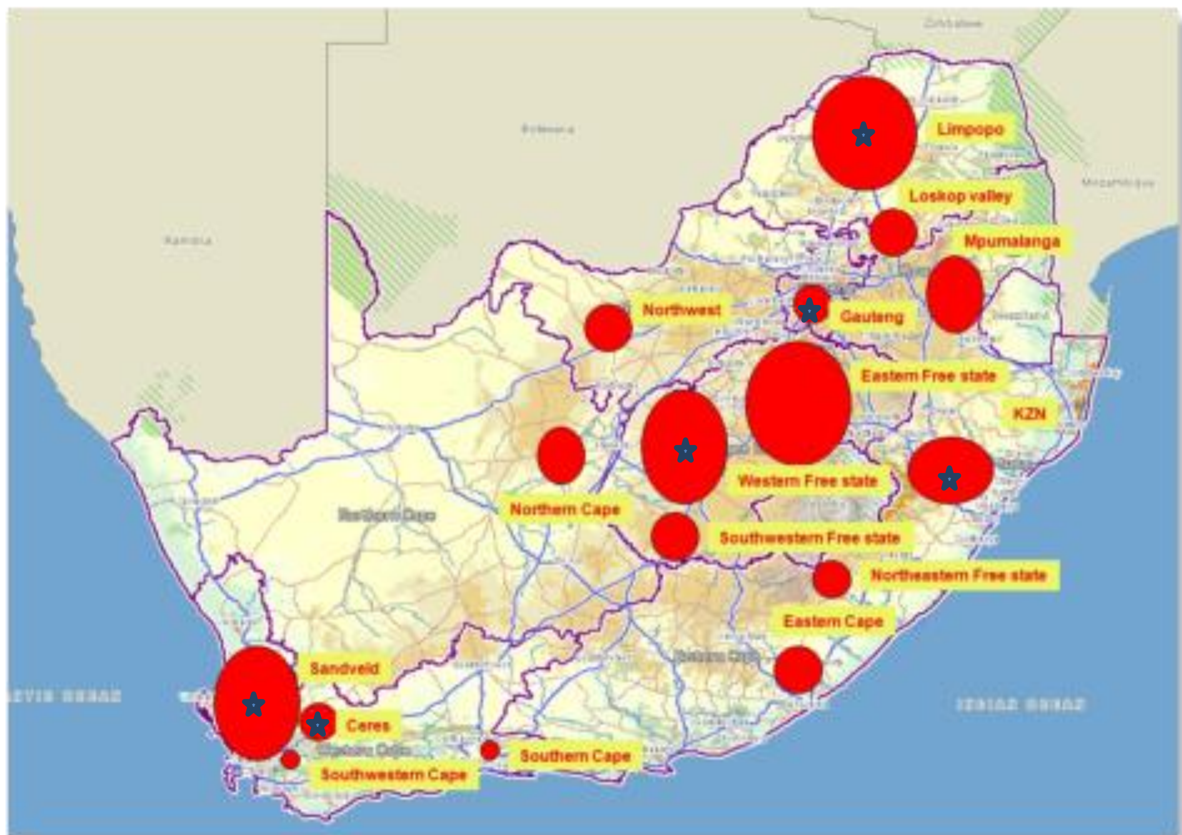

Figure 1. Map of South Africa showing the potato production regions, indicated by "★", from which potato common scab tubers were sampled. The red circles indicate all 16 potato production regions, with the size of the circle representing the relative size of the production regions. Regional map provided courtesy of Potato South Africa.

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Figure 2. Symptoms, known as fissure scab, caused by *Streptomyces* strain RSA1. a) Potato tubers collected from fields during the common scab survey from which *Streptomyces* strain RSA1 isolates were obtained and b) symptoms (top) produced by *Streptomyces* strain RSA1 in pathogenicity assays using the potato double pot inoculation method. Koch's postulate was fulfilled by re-isolating the pathogen. Tubers from the un-inoculated control are shown at the bottom.

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Figure 3. Phylogenetic tree based on the 16S rRNA gene of *Streptomyces* isolates. The tree was constructed using the neighbour-joining algorithm and the Tamura 3-parameter evolutionary distance model. Percentages at the nodes represent levels of bootstrap support from 1000 re-sampled datasets, with bootstrap values above 60 % being shown. Scale bar represents 0.005 substitutions per nucleotide position. Phytopathogenic *Streptomyces* species or isolates are shown in bold, with those originating from South Africa in the current study shown in blue. *Streptomyces* sequences that were obtained from type strains are indicated by "^{T}". The GenBank accession numbers are shown to the right of the isolate names. The cluster designation of Labeda *et al.* (2012) is shown to the right, with the major clades with high bootstrap support (>85 %) shown in orange.

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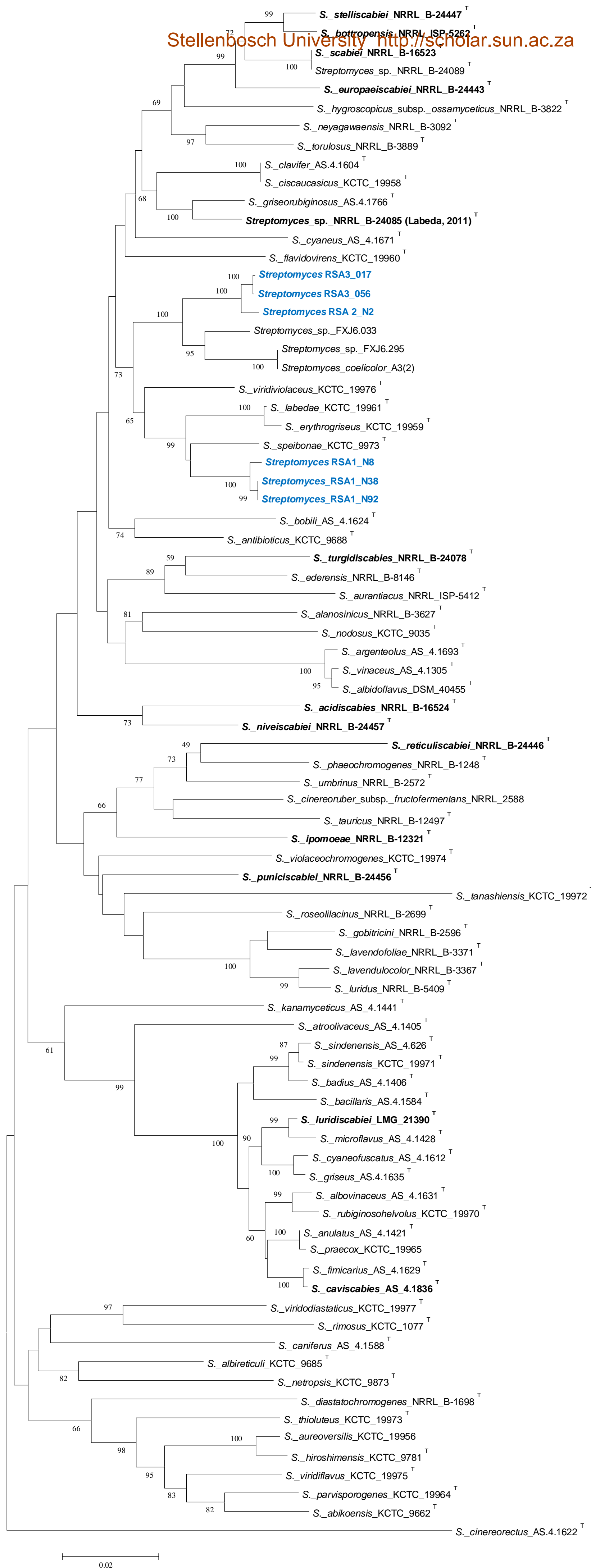

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Figure 4. Phylogenetic tree based on concatenation of three protein coding genes [ATP synthase F1, beta sub unit (*atpD*), RNA polymerase, beta subunit (*rpoB*) and tryptophan synthase, beta subunit (*trpB*)] of the genus *Streptomyces*. The tree was constructed using the neighbour-joining algorithm and the Tamura 3-parameter evolutionary distance model. Percentages at the nodes represent levels of bootstrap support from 1000 re-sampled datasets, with bootstrap values above 60 % being shown. Scale bar represents 0.01 substitutions per nucleotide position. Phytopathogenic *Streptomyces* species or isolates are shown in bold, with those originating from South Africa in the current study shown in blue. *Streptomyces* sequences that were obtained from type strains are indicated by "^{T}". The *Streptomyces* isolate codes are shown to the right of the species names.

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3. MANAGING COMMON SCAB OF POTATO WITH CABBAGE RESIDUES, AND THE EFFECT OF VOLATILES FROM VARIOUS *BRASSICA* SPECIES ON *STREPTOMYCES*

ABSTRACT

Common scab of potato is an economically important soilborne disease that is caused by various *Streptomyces* species. The disease is very difficult to control, but biofumigation has recently shown some potential. Biofumigation consists of the incorporation of *Brassica* spp. crop residues containing glucosinolates that upon cell disruption are hydrolysed by the enzyme myrosinase to yield a diversity of biologically-active hydrolysis products, of which volatile isothiocyanates are the most toxic to soil microbes. Common scab was significantly reduced through incorporation of fresh and air-dried residues of *Brassica oleracea* var. *capitata* (cabbage) when applied prior to two consecutive potato plantings, but not in a subsequent third potato planting. In contrast, the incorporation of only cabbage root tissue was never effective in suppressing disease incidence. The *in-vitro* effect of volatile emissions from various *Brassica* species on pathogenic and non-pathogenic *Streptomyces* isolates was also evaluated using two bioassay methods. An *in-vitro* agar plate bioassay showed that, in general, volatile emissions from water activated freeze-dried tissue of *B. juncea/S. alba* mix and *B. napus* were superior to those from *B. oleracea* var *italica* and *B. oleracea* var *capitata* for suppression of growth and sporulation of the 14 *Streptomyces* isolates examined. Volatiles generated from root tissue were more effective than shoot tissue in limiting growth of *Streptomyces*. In a chamber bioassay that used freshly macerated *Brassica* tissue, *B. oleracea* var *capitata* and *B. juncea/S. alba* mix suppressed sporulation but not hyphal growth of the 78 evaluated *Streptomyces* isolates. Unlike the agar plate bioassay, *B. oleracea* var *capitata* and *B. juncea/S. alba* mix were equally effective at suppressing sporulation of the *Streptomyces* isolates. The chamber bioassay also showed that the *Brassica* tissue volatiles were bacteriostatic, since isolates re-grew when removed from the chamber and transferred to fresh media. Significant components of both the pathogenic (50 %) and non-pathogenic (20 %) *Streptomyces* population examined were unaffected by the *Brassica* tissue derived volatiles. This suggests that soil biofumigation could significantly influence composition of the *Streptomyces* community in agricultural soils.

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INTRODUCTION

All potato markets are negatively affected by common scab, which is caused by various soil-inhabiting *Streptomyces* species (Theron, 2003). Common scab reduces the cosmetic value of ware and seed potatoes due to the production of circular, raised, tan to brown corky lesions on the surface of tubers. The disease has resulted in downgrading of consignments on the market because of the growing consumer demand for blemish-free produce and planting material. In the 2006/7 season, 32 % of all bulk potato bags produced were discarded as a result of consignment downgrading due to common scab symptoms. When taking into consideration that average potato production per annum is 180 million 10 kg bags, then an estimated 57.6 million 10 kg bags were downgraded or rejected as a result of this disease. This amounts to a total loss of R1.7 billion per year for the potato industry in South Africa.

It is very difficult to control common scab since it has a long soil survival period and chemicals to control the soilborne inoculum often do not reach their target due to the complex structure and characteristics of different soils. Therefore, an integrated disease management approach is required. Management strategies that can be considered include irrigation scheduling, cultivar tolerance, agro-chemical applications, crop rotation, green manuring, *Brassica* crop amendments and other organic amendments (Larkin, 2008). Among these strategies, *Brassica* crop amendment is an important consideration, since it is an environmentally-friendly approach that can enhance soil health, and can also be cost effective (Snapp & Borden, 2005; Snapp *et al*., 2007). The incorporation of *Brassica* crop residues into soil to suppress soilborne pathogens has received considerable attention as a disease control strategy due to the potential for release of anti-microbial substances during residue decomposition. The process has been termed biofumigation due to the fact that certain compounds released in this practice, isothiocyantes (ITCs), are related to the active compound derived from certain commercial chemical fumigants (Kirkegaard *et al*., 1993). A wide range of soilborne pathogens can be suppressed by biofumigation, with most studies focusing on fungal- and oomycete pathogens or plant parasitic nematodes (Mayton *et al*., 1996; Stapleton & Duncan, 1998; Harding & Wicks, 2000). Only a few studies have investigated the potential of biofumigation for the suppression of bacterial pathogens, including studies demonstrating its potential for managing common scab (Larkin *et al*., 2006; Larkin & Griffen, 2007) and a study by Akiew *et al*. (1996) that investigated the effect of decaying residues of *Brassica* plants on bacterial wilt, caused by *Ralstonia solanacearum*.

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An important mechanism of disease suppression through *Brassica* amendments is the natural emission of sulphur-containing volatile gasses from *Brassica* tissue upon incorporation into soil (Lewis & Papavizas, 1970; Kirkegaard *et al*., 1996; Angus *et al*., 1994). *Brassica* tissues contain ß-D-thioglucosidic compounds known as glucosinolates (Brown & Morra, 1997; Kirkegaard & Sarwar, 1998). Glucosinolates are hydrolysed by the enzyme myrosinase in the presence of water resulting in the release of various biologically active compounds of which volatile isothiocyanates are considered the most toxic (Brown & Morra, 1997). Glucosinolates are present at varying levels in different *Brassica* species, plant parts (roots and shoots) and growth stages (Brown & Morra, 1997), which can all influence the efficacy of biofumigation. For example certain cultivars of *B. juncea* and *S. alba* possess a higher glucosinolate concentration than *B. oleraceae* var *capitata* and *B. oleraceae* var *italica* (Kirkegaard, 1996). Efficacy of *Brassica* amendments as a biofumigant can also be affected by temperature (Borek *et al*., 1995), soil type (Lehman, 1942; Mason-Sedun *et al*., 1986; Matthiesson *et al*., 1996), moisture and organic matter content (Borek *et al*., 1995) and various other factors that are not yet well understood (Motisi *et al*., 2010).

An additional factor that can influence the efficacy of biofumigation is whether the plant residues utilized are fresh or air-dried. In general, most studies have used freshly incorporated *Brassica* residues (Muelchen, 1990; Akiew *et al*., 1996; Keinath, 1996; Mayton *et al*., 1996), whereas only a few have investigated air-dried residues (Lewis & Papavizas, 1971; 1974; Chan & Close, 1987) or seed meals that are by-products of oil extraction from various *Brassica* species (Mazzola *et al*., 2001, 2007; Cohen *et al*., 2005; Weerakoon *et al*., 2012). Few studies have conducted comparative analyses of the disease control efficacy attained when using air-dried versus fresh *Brassica* residues. Ramirez-Villapudua & Munnecke (1988) found that air-dried tissue of *B. oleracea* var *capitata* was superior to fresh residues in reducing *B. oleracea* var *capitata* yellows caused by *Fusarium oxysporum* f. sp. *conglutinans*. The response of *F. oxysporum* f. sp. *conglutinans* to fresh and air-dried *Brassica* residue soil amendments differed with time of exposure, wherein fresh residues required 10 days longer to achieve the same level of efficacy as air-dried residues. Angus *et al.* (1994) also found that air-dried roots of *B. juncea* and *B. napus* was more effective than fresh roots in suppressing take all of wheat caused by *Gaeumannomyces graminis* var *tritici*. However, Rhaman *et al.* (2011) investigated the efficacy of *B. juncea* cv Nemfix incorporated as fresh shoots or as seed meal, and did not

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observe any significant difference in root knot nematode (*Meloidogyne javanica*) suppression on vines (*Vitis vinifera*) between the two treatments.

In South Africa, common scab is of importance for commercial producers and is especially problematic for subsistence farmers that have limited resources and funding. Therefore, the first aim of this study was to determine the capacity of *Brassica oleraceae* var. *capitata* (cabbage) incorporated into soil as fresh or air-dried residues to suppress common scab under field conditions over three consecutive seasons. *Brassica oleracea* var *capitata* was specifically selected since subsistence farmers can sell part of the crop and still benefit from incorporating only a section of the crop. A treatment was also included where the whole crop was removed, thus effectively only evaluating the efficacy of the root biomass. The second aim of the study was to evaluate the effect of volatiles derived from fresh or freeze dried (with or without water activation) root and shoot tissue from various *Brassica* crops [cabbage, broccoli (*B. oleraceae* L. var *italica* L), canola (*B. napus*) and mustard mix (*B. juncea/S. alba*)] on the *in-vitro* growth and sporulation of pathogenic and non-pathogenic *Streptomyces* isolates.

MATERIALS AND METHODS

Efficacy of fresh and air-dried *B. oleracea* var. *capitata* residues in suppressing common scab under field conditions

Trial site. The trial was conducted at Dendron in the Limpopo Province, South Africa, from January 2003 to December 2005. The trial site was naturally infested with a high incidence of common scab [90 % disease incidence on the preceding potato planting (cultivar BP1); superficial lesion type and 50-75 % coverage of tuber surface; scab disease index = 18].

Experimental design and treatments. The trial was a completely randomized block design, with each treatment represented by six replicates (8 m x 3.2 m plots). The trial was repeated over 3 planting seasons (three years). After each harvest, and prior to establishment

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of the *Brassica* crops, the soil was ploughed and treatments were applied on exactly the same blocks as previously planted i.e. treatment blocks remained on the same location each year.

Trial treatments consisted of (1) control or fallow plots, (2) incorporation of air-dried *B. oleracea* var *capitata* residue, (3) incorporation of fresh *B. oleracea* var *capitata* residue and (4) growing *B. oleracea* var *capitata* but removing the heads and residue without incorporation into the soil.

Brassica cultivation and incorporation. Brassica oleracea var. *capitata* (cultivar 'Conquestador') seedlings were planted in January of each year and drip-irrigated. The crops were maintained from January to May, when mature heads were harvested and sold. The remaining residues (±800 g outer leaves and roots per plant) were incorporated at a rate of 2 % w/w directly after harvest (fresh) or after 10 days on the field (air-dried) by means of a rotovator. Fertilizer and irrigation recommendations were based on soil sampling before each planting and accurate monitoring of precipitation that was maintained throughout each planting season.

Potato crop establishment and trial evaluation. The highly susceptible potato cultivar BP1 was planted in June of each year approximately 14 days after soil incorporation of *Brassica* tissue. The trial was dripper irrigated and maintained until harvest in September. Fertilization and irrigation were conducted as described for the *B. oleracea* crop.

Potatoes were cultivated for 90 days, and progeny tubers were evaluated for common scab incidence according to the scale developed by Marais & Vorster (1988), which includes the tuber surface area covered and lesions type. For surface area covered the scale was: 0 = no scab lesions; 1 = 1-12 % surface area covered; 2 = 13-25 % surface area covered; 3 = 26-50 % surface area covered 4 = 51-75 % and 5 = 76-100 % surface area covered. For lesion type the scale was: 6 = superficial lesion; 7 = netted/russet scab lesion and 11 = deep-pitted scab lesion. The final disease index was assigned on a scale of 0-55, which was calculated by multiplying the surface area covered with the lesion type.

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The common scab disease index frequencies were observed in 15 ordinal classes; 0, 6, 7, 12, 14, 18, 21, 22, 24, 28, 30, 33, 35, 44 and 55. A class of 0 indicated no disease and a class of 55 indicated 100 % tuber coverage with deep-pitted scab symptoms. The disease index frequencies were subjected to a generalized linear model (GLM) technique with a logistic link function. The maximum likelihood estimators (XBeta's) were calculated on an underlying scale (McCullagh & Nelder, 1989). These estimators (location values), that are on an interval scale were subjected to a randomized block design analysis of variance using SAS version 9.2 (SAS, 1999). The repeated measurements over years were included as a sub-plot factor (Table 1). The standardized residuals were subjected to the Shapiro-Wilk's test for non-normality (Shapiro & Wilk, 1965). Student's t-LSD (Least Significant Differences) was calculated at a 5 % significance level to compare means of significant effects.

In-vitro efficacy of _Brassica_ tissue against growth and sporulation of pathogenic and non-pathogenic _Streptomyces_ isolates

Brassica crop production. Brassica crops that were grown in the greenhouse were utilized in the *in-vitro* bioassays. *Brassica oleraceae* var *capitata*, *B. oleraceae* var *italica, B. napus* and *B. juncea/S. alba* mix seed (Hygrotech, Pretoria, South African) was surface-disinfested with 1.5 % sodium hypochlorite for 3 minutes and sown in standard polystyrene seedling trays (67 cm x 33.5 cm x 6 cm) filled with Hygromix© seedling medium (Hygrotech, Pretoria, South Africa). Seedlings were maintained for approximately 2-4 weeks, and were then transplanted to 25-cm-diameter plastic pots filled with a riversand:vermiculite (1:1) mixture containing river sand (with a pH of 7.53 and coarseness of 0.6 to 1.5 mm) and vermiculite (Hygrotech, Pretoria, South Africa) that were heat-sterilised in an industrial oven at 200 °C overnight. Twenty pots containing two seedlings each were planted for each crop. The pots were arranged in a complete randomized design in a greenhouse with a night/day temperature of 8/25 °C. The pots received regular irrigation through a mini-sprinkler system, as well as 100 ml of a 0.1 % Multifeed P ® nutrient solution (Plaaskem Pty. Ltd., Lilianton, South Africa) per pot every third week or as required.

Streptomyces isolates used in bioassays and inoculum preparation. The *Streptomyces* isolates were obtained from a previous study (Chapter 2) and are all maintained at the ARC-VOPI culture collection at Roodeplaat, Pretoria, South Africa. The isolates were previously

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characterized for pathogenicity, and the species identity was also determined where possible (Chapter 2). Isolates were recovered from the -70 °C storage unit and plated onto oatmeal agar (OA, ISP medium 3) and yeast malt extract (YME, ISP medium 2) media, and incubated at 30 °C for 5-7 days. *Streptomyces* spore suspensions that were used in the bioassays were obtained by growing isolates for 14 days on YME media at 30 °C in the dark. Spore suspensions were prepared by flooding the plates with sterile distilled water and rubbing with a bent glass rod to harvest the spores. The suspensions were adjusted to a final concentration of 1.2 x 10^6 cfu/ml.

Evaluation of freeze-dried Brassica tissue using an agar plate bioassay. The disease control potential of freeze-dried *Brassica* tissue from four species, *B. oleraceae* var *capitata*, *B. oleraceae* var *italica, B. napus* and *B. juncea/S. alba* mix, was evaluated using a modified bioassay developed by Kirkegaard *et al*. (1996). *Brassica* root and shoot material was harvested from the greenhouse plantings at 50 % flowering, a point at which maximum glucosinolate concentration is characteristically attained (Malik *et al*., 2010). The plant material was freeze-dried directly after harvesting in a Dura Dry UP microprocessor control freeze-dryer (FTS Systems, Stone Ridge, New York) to preserve intact glucosinolates and prevent hydrolysis by myrosinase during storage. The *Brassica* tissue was then ground in a Wiley mill with a 1 mm screen.

Assays were conducted using freeze dried *Brassica* tissue, with or without the addition of water in order to evaluate the effect of glucosinolate hydrolysis products on bacterial growth. A total of 14 *Streptomyces* isolates were used, six pathogenic and eight non-pathogenic isolates. YME agar plates were inoculated with a *Streptomyces* spore suspension (1.2 x 10^6 cfu/ml) at a rate of 0.01 ml/plate. Four plates were prepared per *Streptomyces* isolate and for each *Brassica* tissue type (shoot or roots) for each of the treatments, where water or no water was added to the freeze dried tissue. Once prepared, two to four holes were made into each inoculated agar plate with a sterile 50 mm cork borer. Each of the wells was filled with 50 mg of freeze-dried *Brassica* tissue, wetted with 60 µl sterile distilled water, or with no water addition. The plates were sealed immediately with two layers of parafilm and incubated for 5-7 days at 30 ºC before evaluation. *Streptomyces* isolate inhibition was recorded as the formation of an inhibition zone relative to the non-treated control. Colony

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growth and sporulation (or lack of sporulation) of the *Streptomyces* isolates were recorded. The experiment was conducted twice.

Evaluation of fresh Brassica tissue using a gas chamber bioassay. Two *Brassica* species, i.e. *B. oleraceae* var *capitata* (low glucosinolate producer) and *B. juncea/S. alba* mix (high glucosinolate producer), were used in the gas chamber bioassay. The *in-vitro* gas chamber bioassay is a modified version of the method developed by Ramirez-Villapudua & Munnecke (1988). A total of 77 *Streptomyces* isolates were examined including 50 pathogenic and 27 non-pathogenic isolates. YME media in 90 mm petri plates were inoculated with a *Streptomyces* spore suspension (1.2 x 10^6 cfu/ml) at a rate of 10 µl/plate, with three plates per isolate. Each pre-inoculated *Streptomyces* plate was inversely fitted to the underside of the screw top lid of a glass Consol© bottle (Consol, Germiston, South Africa). Freshly harvested *Brassica* tissue (*B. oleraceae* var *capitata* cv Conquestador or *B. juncea/S. alba* mix cv Caliente 119) was prepared in a Waring Blender and incorporated into a natural sandy loam soil at a rate of 2 % (w/w). The Consol© bottles were filled with 3.5 kg of the *Brassica* amended soil and 700 ml water (field water capacity) was added to the soil to activate the enzymatic release of the volatile emissions. The screw top lids containing the inoculated cultures were fastened to the bottles and sealed.

The experiment included three replicates per isolate, and the trial was conducted twice. Bottles were incubated at room temperature for 7 days after which the *Streptomyces* plates were removed and scored using the following criteria: (i) overgrown by fungi that originated from fungal spores that were released from the soil in the bottom of the bottle, (ii) *Streptomyces* growth but no sporulation and (iii) *Streptomyces* growth and sporulation. Subsequently, an agar plug from the inhibition zone was sub-cultured to fresh culture media, and the isolates were scored again for growth and sporulation after 7 days of growth. An agar plug from a plate taken from an empty bottle, which served as the control, was also plated onto fresh agar as a control. Empty bottles without soil, but with a culture plate in the lid were included as control treatments in all experiments.

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RESULTS

Efficacy of fresh and air-dried *B. oleracea* var. *capitata* residues in suppressing common scab under field conditions

The common scab index in the control plots increased from 21 in the first planting, to 24 in the second and third plantings, indicating that the disease pressure was severe. ANOVA showed that there were significant differences for treatments and years (Table 1). In the first (2003) and second (2004) plantings, the air-dried and fresh *B. oleraceae* var *capitata* amendments showed a significant decrease in common scab disease index when compared to the non-treated control (Table 2). These two treatments from the first and second planting did not differ significantly from each other in reducing the scab index. Incorporation of *Brassica* root biomass did not significantly reduce the scab index in either the 2003 or 2004 planting. In the third and final planting (2005) none, of the treatments were effective in reducing scab; (Table 2), the disease index ranged between 22 and 24 in all treatments.

In-vitro efficacy of *Brassica* tissue against growth and sporulation of pathogenic and non-pathogenic *Streptomyces* isolates

Evaluation of freeze-dried Brassica tissue using an agar plate bioassay. There was a significant shoot × root and *Brassica* crop × *Streptomyces* isolate interactions (Table 3) in the effects of *Brassica* on *Streptomyces* growth.

Water was required for myrosinase-activated glucosinolate hydrolysis in the agar plate bioassay. Consequently, in treatments where volatile emission was not generated due to the absence of water, inhibition of growth and sporulation for any of the 14 *Streptomyces* isolates was not observed regardless of the *Brassica* species (data not shown). In contrast, when volatiles were produced in response to the addition of sterile distilled water, growth of some of the *Streptomyces* isolates was inhibited by the *Brassica* species shoot tissue and a clear inhibition zone was evident (Fig. 1). Volatiles derived from root tissues induced an inhibition zone in which both mycelial growth and sporulation were inhibited (Fig. 2). Inhibition was calculated by measuring the total size of the two inhibition zones.

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Both *Brassica* species and plant tissue type influenced *Streptomyces* inhibition. The freeze-dried root tissue of all the *Brassica* species was significantly more effective in reducing *Streptomyces* growth than shoot tissue, although this effect was more pronounced for *B. napus* and the *B. juncea/S. alba* mix. For the shoot tissue, the *B. napus* and *B. juncea/S. alba* mix treatments in general caused a significant two to three fold larger inhibition zone than the two *B. oleracea* varieties (Fig. 3). This effect was even more pronounced for the root tissue, where inhibition zones induced by volatiles derived from *B. napus* and *B. juncea/S. alba* mix were in general more than ten times larger than the zones caused by the two *B. oleracea* varieties The two *B. oleracea* varieties did not differ significantly from each other in suppressing *Streptomyces* growth for root and shoot tissue (Fig. 3). The root and shoot tissue of the *B. juncea/S. alba* mix and *B. napus* freeze-dried tissue inhibited the growth of all 14 isolates. However, this was not true for the *B. oleracea* varieties, since two of the isolates (O5; pathogenic and N4; pathogenic) were not inhibited by the shoots or roots of the two *B. oleracea* varieties, whereas another three isolates (O1; pathogenic, O2; pathogenic and O6; non-pathogenic) were only inhibited by the root tissue of the two varieties (Table 4).

Evaluation of fresh Brassica tissue using a gas chamber bioassay. The gas chamber bioassay showed that the volatiles emitted from the *Brassica* species did not affect the growth of the *Streptomyces* isolates, but that the sporulation of most isolates was negatively affected (Table 5, Fig. 4). The response of the *Streptomyces* culture plates exposed to *B. oleraceae* and *B. juncea/S. alba* mix volatiles can be divided into five categories (A to E) based on; their response to the two *Brassica* species, overgrowth of plates by fungi, and whether isolates sporulated after exposure of the plates to volatiles and after re-culturing of the isolates onto fresh media (Table 5). The growth of all the *Streptomyces* isolates was unaffected by the volatiles emitted by the *B. oleracea* var *capitata* and *B. juncea/S. alba* mix treatments. However, sporulation of most isolates was suppressed by *B. oleracea* (77 % of isolates; including the overgrown cultures) and *B. juncea/S. alba* mix (77 % of isolates) (Table 5, categories A to C). There were, however, a few isolates (21 %) for which sporulation was unaffected by *B. oleracea* and *B. juncea/S. alba* mix, with most (13 %) of these isolates being pathogenic (Table 5, category D). In general, the suppression of sporulation by the volatiles was only temporarily since 94 % (74/79) of the *Streptomyces* isolates exhibited abundant colony growth and sporulation when sub-cultured to fresh media

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(Table 5, categories A, B and D). In contrast, a group of isolates (5 %), that included an equal number of pathogenic and non-pathogenic isolates, were severely affected by the volatiles since both *Brassica* species inhibited their sporulation after volatile exposure and the isolates did not sporulate after sub-culturing to fresh media (Table 5, category C). An interesting observation during the bioassay was that 46 % of the isolate plates from the *B. oleraceae* var *capitata* treatments were overgrown with fungi (Table 5, category A), whereas the *B. juncea/S. alba* mix plates were rarely (3 % of isolates) overgrown by fungi (Table 5, category E).

DISCUSSION

The study showed that *B. oleracea* var. *capitata* has potential as a rotation crop for subsistence farmers, since it can suppress common scab in systems that employ successive potato plantings. Although the efficacy of the *B. oleracea* var *capitata* biofumigation was marginal, with the scab index only being reduced by a maximum of 14 %, this may be attributed to the high disease pressure at the experimental site. This could have also contributed to the observation that the annual *Brassica* biofumigation only demonstrated efficacy during the first two potato plantings, but not the third. Subsistence farmers might therefore attain better disease suppression with *B. oleracea* var *capitata* amendments in fields that have lower disease pressures. Farmers will further benefit from the *Brassica* management method since they can sell most of the cabbage crop, with disease suppression only requiring the incorporation of a small fraction of the above ground dried or fresh plant residue along with the remaining roots. However, soil incorporation of some of the aboveground leaves is required to achieve disease suppression, as the use of only the roots provided no disease control.

The incorporation of dried and fresh *B. oleracea* var *capitata* residues were equally effective in suppressing common scab in the first and second field plantings. Gouws & Wehner (2004) also found that oven dried and fresh *B. oleracea* var *capitata* residues suppressed common scab under glasshouse and tunnel conditions. However, in both studies the efficacy of the dried residues might have been overestimated somewhat, since the weight of the added fresh and dry tissue was similar, which effectively resulted in more "active" compounds

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being applied with the application of the dried tissue. For subsistence farmers, from a practical point of view, the incorporation of dried material is more feasible since they first need to transport and sell their crop before time can be spent on the incorporation of residues and field cultivation. Stapleton & Duncan (1998) observed no significant difference in the capacity of fresh and dried *Brassica* residues of numerous species (*B. nigra; B. oleracea* var *chinensis, B. oleracea* var. *italiensis, B. oleracea* var. *capitata, B. oleracea* var. *compacta* and *Raphanus sativus*) for reducing the incidence of soilborne fungal phytopathogens (*Pythium ultimum* and *Sclerotium rolfsii*). They suggested that no clear advantage is gained through drying of crop residue before incorporation into soil. However, in a study conducted on fungal pathogens, Ramirez-Villapudua & Munnecke (1988) showed that air-dried *B. oleracea* var *capitata* residues were more effective than fresh residues.

Two bioassays, an agar plate- and gas chamber bioassay, were used to investigate the *Streptomyces* suppressive potential of other *Brassica* species relative to *B. oleracea* var *capitata*. In the agar plate bioassay it was shown that enzymatic hydrolysis of the glucosinolates is required to attain a reduction in *Streptomyces* growth. Kirkegaard *et al.* (1996) also found that disease suppression in response to the incorporation of *Brassica* residues was only obtained with the addition of water. These authors were also able to show that the degree of fungal suppression by various freeze-dried *Brassica* species (*B. juncea* and *B. napus*) differed in their bioassays (Kirkgaard *et al.*, 1996). This concurs with the results of the current agar plate bioassay where *Brassica* species differed in efficacy towards suppressing *Streptomyces* isolates. Broccoli (*B. olearacea* var *italica*), which could also serve as a cash crop for farmers, was ineffective in suppressing *Streptomyces* isolates. The *B. juncea/S. alba* mix and *B. napus* were more effective than *B. oleracea* var *capitata* and *B. oleracea* var *italica* in suppressing *Streptomyces* in the plate assay. This is in contrast with results obtained in the gas chamber bioassay where the two *Brassica* species were equally effective at suppressing *Streptomyces*. Factors contributing to these differences could be that the addition of water in the agar plate bioassay resulted in the diffusion of water soluble compounds into the agar that can further affect *Streptomyces* growth, aside from volatiles. Furthermore, some volatiles might be more effective at the short distance that was present between the point of volatile release and *Streptomyces* growth within the agar assay, compared to the larger spatial separation between isolate growth and the point of volatile release within the gas chamber bioassay. The findings of the bioassay should be evaluated

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further under glasshouse and field conditions before recommendations can be made to subsistence farmers on whether the *B. juncea/S. alba* mix*, B. napus* and *B. oleracea* var *italica* could be useful rotation crops to include along with *B. oleracea* var *capitata* in potato production systems. The fact that Larkin *et al.* (2006) found *B. juncea* to be effective for suppressing common scab under field conditions, shows promise for the use of this species.

Tissue type was important for the suppression of *Streptomyces*, since volatile emissions from the root tissue were more effective at reducing *Streptomyces* growth than the volatiles from shoot tissue. This finding is in accordance with research conducted by Matthiesson *et al*. (1996) where *in-vitro* exposure to freeze-dried *Brassica* root tissue was more toxic to white-fringed weevil larvae than the same rate of shoot tissue. Kirkegaard *et al.* (1996) also found tissue type to be important and showed that differences in pest suppression efficacy were related to the concentration and type of isothiocyanate released. Although in the agar plate bioassay, the root tissue was much more effective than shoot tissue, this result was not reflected in the field study where the incorporation of only root biomass did not result in a significant decrease in disease incidence. This could be due to the small quantity of root biomass being incorporated into the soil of the field trial, relative to the larger quantity of shoot biomass that was incorporated, and effectively reduced the disease under field conditions. The root biomass would thus have yielded only low levels of the active volatiles, thus being unable to suppress the disease. In contrast, in the agar bioassay equal quantities of the two tissue types were used.

The gas chamber bioassay showed that the *B. juncea/S. alba* mix and *B. oleracea* var *capitata* were equally effective at suppressing *Streptomyces* sporulation, but neither of the *Brassica* species could suppress *Streptomyces* growth. The fact that the *Brassica* species were equally effective at suppressing sporulation was unexpected, since it has been reported that the *B. juncea/S. alba* blend can produce much higher quantities of glucosinolates [13.3 µmols/g (comprising of mostly sinigrin; ±154.28 mg/g)] at 50 % flowering (Cerealtoscana, Livorno, Italy) than the *B. oleraceae* var *capitata* that is expected to only produce an average of 0.04-0.09 mg/g total glucosinolates per fresh weight of which 0.02 – 0.023 mg/g is aliphatic (mainly sinigrin) and 0.012 – 0.0472 mg/g fw indole (composed of a variety of glucobrasiccin compounds) (Verkerk *et al.*, 2009). The ability of *Brassica* volatiles to selectively affect only specific developmental stages, of pathogens has not been reported

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previously. However, since the 'hyphae' of the *Streptomyces* isolates were not investigated under high resolution microscropy, some morphological damage might have occurred and thus affected this growth stages. Lewis & Papavizas (1971) reported that for the oomycete pea root pathogen, *Aphanomyces euteiches*, air-dried root and shoot tissue of *B. oleracea* var *capitata* adversely affected the morphology of several developmental stages, including oöspores and mycelia.

An important observation from the gas chamber bioassay was that volatiles and substances from *Brassica* species were only bacteriostatic, that pathogens and non-pathogens were equally affected, and that not all *Streptomyces* isolates were adversely affected. The bacteriostatic effect was evident in the gas chamber bioassay, since most isolates including pathogenic isolates when sub-cultured to fresh media after exposure to *B. oleracea* var *capitata* or the *B. juncea/S. alba* mix, resumed growth. Twenty-one percent of the isolates, including 13 % of the pathogenic isolates, were not affected at all by *B. oleracea* var. *capitata* or *B. juncea/S. alba* mix in the gas chamber bioassay. A similar finding was made for the *B. oleracea* varieties in the agar plate bioassay where 14 % of the *Streptomyces* isolates were not inhibited by the shoot or root tissue, and 21 % were not inhibited by the root tissue. These findings have important implications for the management of common scab under field conditions and may contribute to the variability in common scab disease suppression in the field (unpublished data). The bacteriostatic affect suggests that biofumigation only temporarily disturbed and inhibited streptomycete communities (pathogens and non-pathogens), and that they may recover when the selective agents have completely volatilized. In the soil environment, where *Streptomyces* populations form part of a large and complex microbial community, pathogen populations could be further affected by other microbes that might also only be temporarily disrupted by the biofumigation.

An interesting observation in the gas chamber bioassay was that the plates of the *B. oleraceae* treatment were overgrown by fungi released from the soil mixture in the bottom of the jar for almost 50 % of the isolates, whereas this was not true for *B. juncea/S. alba* mix. This is most likely due to the fact that the dominant volatile released by *B. juncea/S. alba* mix, allyl isothiocyanate, is more effective against fungi in general than those from *B. oleracea* var *capitata* (Brown & Morra, 1997). The fact that half of the isolates that received the *B. oleracea* var *capitata* treatment were not overgrown by fungi, could suggest that these

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Streptomyces isolates produce antibiotics and other secondary metabolites that effectively inhibit a wide range of soil fungi. This was evident for both pathogenic and non-pathogenic streptomycetes, which could, if they survive the biofumigation, further alter the microbial community structure through antibiotic production. The addition of the *Brassica* tissue that serve as a rich and abundant food source for microbes could indeed contribute to this, since antibiotics are secondary metabolites that are only expressed under certain environmental conditions including an abundant food source (Osman *et al*., 2011).

In conclusion, incorporating *Brassica* residues (root and shoot biomass) as fresh or air-dried amendments is a relatively easy-to-implement control method that could have considerable impact on reducing common scab incidence for subsistence farmers. It may also enable farmers to secure an income by producing *Brassica* cash crops (e.g. *B. oleracea* var *capitata*) that they can sell, since only a fraction of the crop has to be incorporated to attain common scab suppression. Currently, recommendations cannot be made on whether the *B. juncea/S. alba* mix, which is not a cash crop, will be more effective than *B. oleracea* var *capitata* at suppressing common scab, since the two bioassays yielded contrasting results with regards to the efficacy of these two *Brassica* species. The bioassays revealed an important differential effect of *Brassica* volatiles towards different *Streptomyces* isolates, since several isolates were unaffected by the volatiles including those that produced antibiotics that were effective against soil fungi. The toxic effect of the volatiles was also only bacteriostatic. Altogether this suggests that the "biofumigation" effect of *Brassica* amendments only forms part of the disease suppressive response, and that shifts in microbial communities could also be an important factor contributing to disease suppression.

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Table 1. Analysis of variance table of the location values of the common scab disease index

Source of variation	**DF**	**MS**	***P***
Replicate	4	0.269	0.2023
Treatment	3	1.656	0.0010
Error	12	0.153	
Year	2	6.109	<0.001
Treatment x Year	6	0.226	0.318
Experimetal error	32	0.184	
Corrected total	59		

Table 2. Means on interval scale, with scab disease index in brackets, of the effect of *Brassica oleracea* var *capitata* amendments (fresh and air-dried) and *B. oleracea* var *capitata* root biomass on the common scab disease index on potatoes during three consecutive potato plantings from 2003 – 2005

	Year[a]		
Treatment	**2003**	**2004**	**2005**
Control	-0.060 (21) cd	0.711 (24) a	0.500 (24) ab
Root biomass	-0.360 (18) d	0.329 (24) abc	0.495 (24) ab
Fresh cabbage	-0.940 (18) e	0.095 (21) cd	0.302 (24) abc
Dry Cabbage	-1.164 (18) e	-0.202 (21) cd	0.296 (24) abc

[a]Means in columns and rows with the same letter/s do not differ significantly at the 5 % significance level.

Table 3. Analysis of variance for the effect of four *Brassica* species on eight pathogenic and six non-pathogenic *Streptomyces* spp. in an *in-vitro* agar plate bioassay

Source of variation	**DF**	**MS**	***P***
Isolate	14	61.8738	< .001
Species	3	738.0333	< .001
Plantpart	1	1372.8167	< .001
Isolate × species	42	9.3190	< .001
Isolate × plantpart	14	24.1917	< .001
Species × plantpart	3	282.0056	< .001
Isolate × species × plantpart	42	6.0591	< .001
Residual	120	0.4417	
Total	239		

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Table 4. Inhibition zones (mm) formed in an agar plate bioassay when pathogenic and non-pathogenic *Streptomyces* spp. were exposed to freeze-dryed *Brassica* tissue dissolved in sterile distilled water.

***Streptomyces* isolate**[a]	***B oleracea* var *capitata***		***B. oleraceae* var *italica***		***B. napus***		***B. juncea/S. alba***	
	Shoot	***Root***	***Shoot***	***Root***	***Shoot***	***Root***	***Shoot***	***Root***
Control	0	0	0	0	0	0	0	0
O1*	0	1	0	2	3	6	4	10
O2*	0	2	0	1	2	14	3	12
O3	1	2	1	2	4	20	4	18
O4	2	1	2	2	2	11	4	12
O5	0	0	0	0	2	6	3	9
O6	0	1	0	2	3	9	4	10
O7	1	2	2	3	3	10	3	13
N1	1	3	1	2	2	15	4	18
N2*	2	4	2	4	4	18	5	22
N3	2	3	1	2	2	16	3	17
N4*	0	0	0	0	2	3	2	4
N5*	0	2	0	2	4	11	4	12
N6*	1	2	1	2	3	14	4	15
N7	0	1	0	2	3	9	3	11

[a] Pathogenic *Streptomyces* isolates are indicated by a "*".

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Table 5. Effect of volatile emissions from high and low glucosinolate containing *Brassica* tissue on pathogenic and non-pathogenic *Streptomyces* spp.

% Isolates (nr)[a]	Pathogenicity [b]	*B. oleraceae* var *capitata* [c]	*B. juncea / S. alba* [c]	Re-culturing [c]
Category A: For *B. oleracea* var *capitata* volatiles there was positive *Streptomyces* colony growth and plates were overgrown by fungi, whereas for *B. juncea/S. alba* mix volatiles there was positive *Streptomyces* colony growth but no sporulation or fungal growth. Sporulation was observed after exposure to both volatiles when re-cultured.				
4 % (3)	-	+ OVG	+ NSP	+ SP
35 % (28)	+	+ OVG	+ NSP	+ SP
5 % (4)	+/-	+ OVG	+ NSP	+ SP
Total: 44 % (35)				
Category B: For *B. oleracea* var *capitata* and *B. juncea/S. alba* mix there were positive *Streptomyces* colony growth but no sporulation. Sporulation by isolates for both volatiles after re-culturing.				
9 % (7)	+	+ NSP	+ NSP	+ SP
6 % (5)	-	+ NSP	+ NSP	+ SP
13 % (10)	+/-	+ NSP	+ NSP	+ SP
Total: 28 % (22)				
Category C: For *B. oleracea* var *capitata* and *B. juncea/S. alba* mix volatiles there were positive *Streptomyces* colony growth and no sporulation, and also no sporulation after re-culturing for both volatiles.				
2.5 % (2)	+	+ NSP	+ NSP	+ NSP
2.5 % (2)	-	+ NSP	+ NSP	+ NSP
Total: 5 % (4)				
Category D: For *B. oleracea* var *capitata* and *B. juncea/S. alba* mix volatiles there were positive *Streptomyces* colony growth and sporulation, and also sporulation after re-culturing.				
16 % (13)	+	+ SP	+ SP	+ SP
4 % (3)	+/-	+ SP	+ SP	+ SP
Total: 20 % (16)				
Category E: For *B. oleracea* var *capitata* and *B. juncea/S. alba* mix volatiles there were positive *Streptomyces* colony growth and plates were overgrown by fungi. Sporulation for both volatiles after re-culturing.				
3 % (2)	+	+ OVG	+ OVG	+ SP
Total: 3 % (2)				

[a] The percentage of isolates that had the specific response to the treatments. The number of isolates is indicated in brackets.

[b] Pathogenicity of isolates was determined using the double pot method (Marais & Visser, 1988), where + = pathogenic isolate, - = non-pathogenic isolate and +/- = isolate that was pathogenic is some experiments but non-pathogenic in other experiments.

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[c] Positive *Streptomyces* colony growth is indicated by “+” in front of the abbreviations. Plates that were overgrown by fungi (OVG), isolates that did not sporulate on the plates (NSP) and isolates that sporulated (SP).

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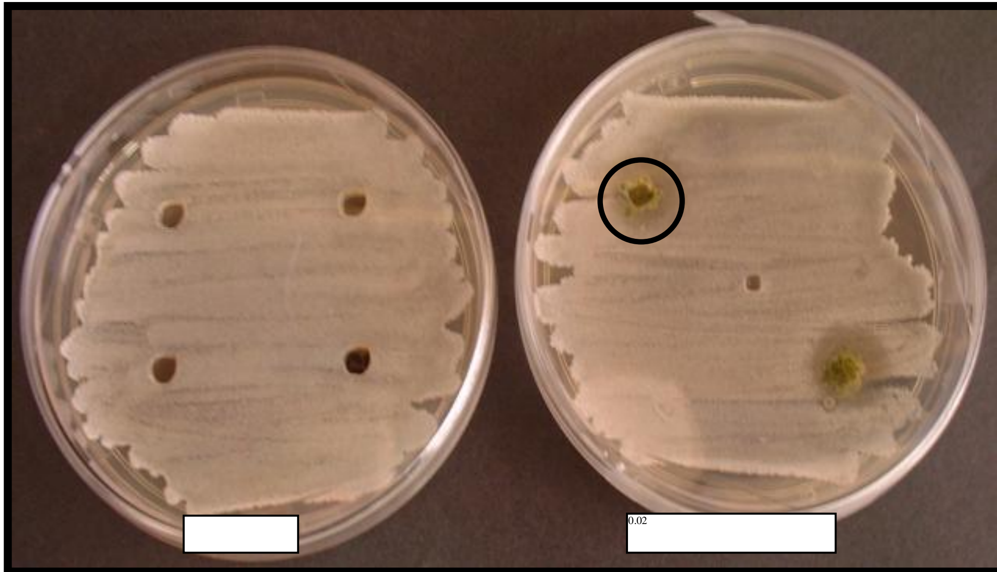

Figure 1. Effect of *Brassica* shoot tissue on growth of *Streptomyces*. Freeze-dried *Brassica* shoot tissue was added to cavities in Petri dishes containing pre-inoculated *Streptomyces* cultures. Reactions were activated by adding sterile water to the freeze-dried *Brassica* tissue. Inhibition of *Streptomyces* growth was assessed by measuring inhibition zones formed in response to activated *Brassica* shoot tissue. Negative control (no amendment; left), *Brassica* shoot tissue amendment (right).

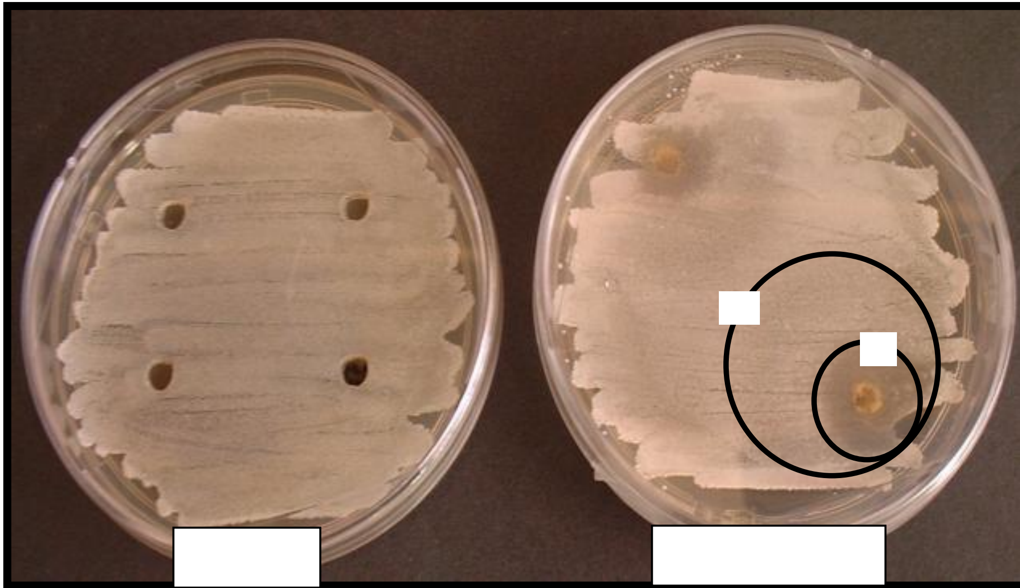

Figure 2. Effect of *Brassica* root tissue on growth and sporulation of *Streptomyces*. Freeze-dried *Brassica* root tissue was added to cavities in Petri dishes containing pre-inoculated *Streptomyces* cultures. Reactions were activated by adding sterile water to the freeze-dried *Brassica* tissue. Inhibition of *Streptomyces* growth was assessed by measuring inhibition zones formed in response to activated *Brassica* shoot tissue. Negative control (no amendment; left), *Brassica* root tissue amendment (right).Two layers of zoning (a, b) were present, with zone a) showing a reduction in growth and zone b) showing a reduction in sporulation. For data analyses purposes the total inhibition (a + b) was calculated for each *Streptomyces* isolate.

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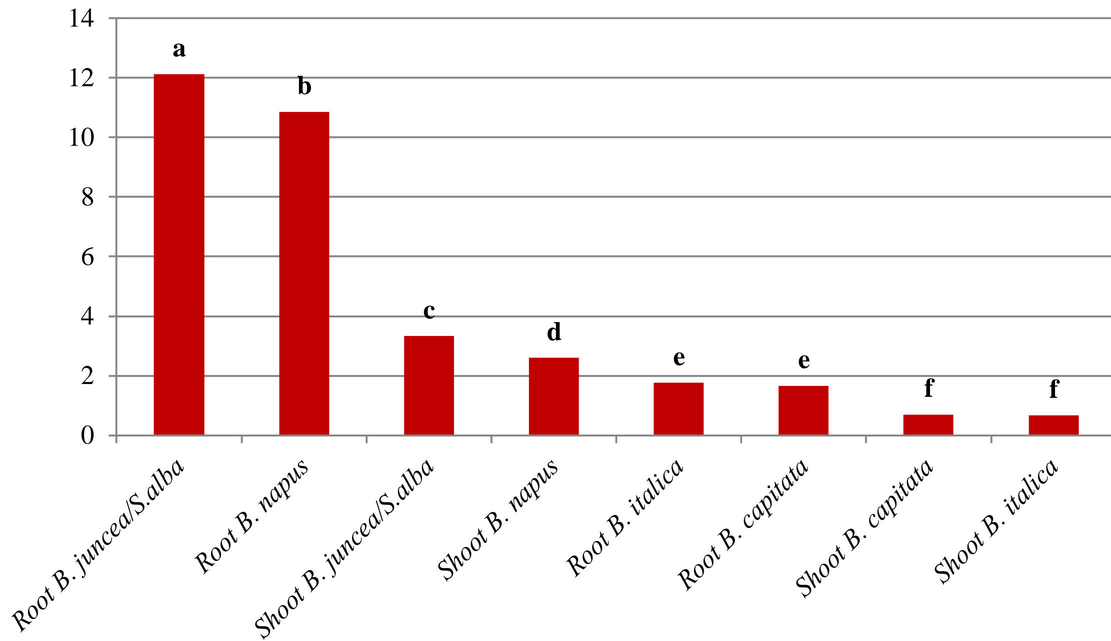

Figure 3. Mean inhibition of *Streptomyces* isolates (n = 14) by freeze-dried *Brassica* root and shoot tissue from four *Brassica* species (*B. juncea/S. alba* mix, *B. napus, B. oleraceae* var *capitata* and *B. oleraceae* var *italica*) in an agar plate bioassay where *Brasscia* volatile emissions were activated through water addition. Bars containing the same letter are not significantly different from each other according to Fischer's protected LSD (P<0.05).

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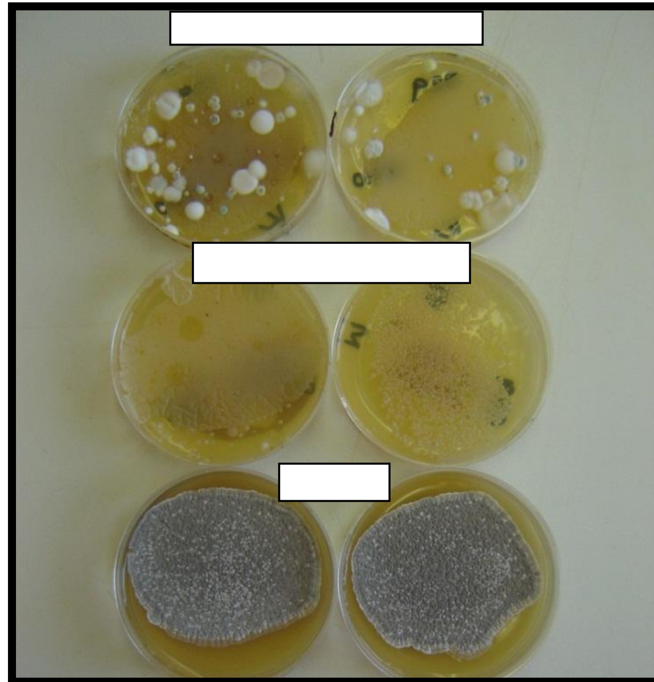

Figure 4. Inhibition of the sporulation of a *Streptomyces* isolate by volatile emissions from *Brassica oleracea* var *capitata* and *B. juncea/S. alba* mix in a gas chamber bioassay. Control plates are at the bottom. Abundant fungal growth is present on plates that were exposed to *B. oleracea* var *capitata* emissions (top plates).

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4. INDUCED RESISTANCE AS A MECHANISM FOR SUPPRESSION OF POTATO COMMON SCAB IN CABBAGE- AND MUSTARD-AMENDED SOIL

ABSTRACT

Brassica crops used as green manures and organic amendments have been associated with a reduction in soilborne diseases including potato common scab. Mechanisms of disease reduction have been attributed to the effect of toxic volatile emissions from the hydrolysis of naturally occurring glucosinolates (GLNs) in *Brassica* tissue, changes in the structure of microbial communities involved in induced resistance (IR) and/or general microbial suppression. A potato split-root experiment that spatially separated the progeny tubers and roots of *Brassica juncea/ Sinapus alba* (mustard mix) and *Brassica oleracea* var *oleracea* (cabbage) amended soil sub-units from non-amended soil sub-units, showed that IR was involved in common scab suppression. For the individual mother plant progeny tubers that developed in the non-amended units showed a significant reduction in common scab. For the same mother plant, a similar finding was made for progeny tubers that grew in the *Brassica*-amended units, with mustard and cabbage being equally effective in the amended and non-amended units. The role of toxic GLN hydrolysis products was ruled out in IR-mediated disease suppression, since volatiles were released from *Brassica*-amended soil prior to initiating the experiment. Increased microbial activity in the *Brassica*-amended units was evidenced by significant increases in ß-glucosidase, urease and acid phospatase (only cabbage-amended soil) activity. Principal component analyses revealed some trends in the overall soil, tuber and root-associated microbial genera (*Trichoderma, Pseudomonas, Streptomyces,* total bacteria and *Fusarium*) in the *Brassica*-amended and non-amended units. The mustard-amended units were microbiologically most distinct from the inoculated and uninoculated controls, followed by the mustard and cabbage non-amended units. These treatments, and to a lesser extent the cabbage-amended units, showed trends towards increases in soil *Fusarium* and *Trichoderma* and root *Trichoderma* populations, and decreases in total bacterial and *Streptomyces* populations in soil and tubers and *Streptomyces* in roots. However, when considering the relative abundance of each of the specific genera, differences were mostly not significant for any treatment. The exceptions were *Streptomyces* populations in the tubers and roots (except the mustard non-amended sub-unit), and total bacteria in the tubers and soil of the *Brassica*-amended and non-amended sub-units that were all significantly lower than the *Streptomyces* inoculated controls. The study could not identify

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a specific microbial genus associated with roots that was involved in IR, but some evidence was found for direct general microbial suppression playing a role in disease suppression in the *Brassica*-amended sub-units.

INTRODUCTION

Successful management of common scab of potato, a tuber-borne disease that reduces the cosmetic value of potatoes in the ware, seed and processing industries, is a difficult proposition (Theron, 2003; Dees & Wanner, 2012). The disease occurs worldwide and is caused by a variety of *Streptomyces* species of which *S. scabiei*, *S. acidiscabiei* and *S. turgidiscabies* are the best characterized (Loria *et al.*, 1997). Several management options for these pathogens and the symptoms that they cause have been examined, but when applied individually the methods have proven to be ineffective. Therefore, an integrated management approach is required and may include chemical treatments (seed tuber and soil treatments), irrigation management during the tuber initiation phase, use of tolerant cultivars, long-term crop rotations with non-hosts, and cultural practices that involve the incorporation of organic amendments (Gouws, 2006; Larkin, 2008). The use of organic amendments, due to a drive towards more sustainable crop production, has received renewed attention world-wide for managing plant diseases, with a particular emphasis on the use of residues from members of the plant family *Brassicaceae* (crucifer and mustard family) (Larking & Griffen, 2007; Bonanomi *et al.*, 2007; Lu *et al.*, 2010; Bonanomi *et al.*, 2010; Nunez-Zofio *et al.*, 2011). *Brassica* residues also have the added benefit of causing an increase in mineralization of carbon in the soil, which has a positive effect on soil fertility and microbial activity within agricultural systems (Wang *et al.*, 2012).

Two primary mechanisms, toxic volatiles and changes in microbial communities, have been proposed for suppression of soilborne plant pathogens through *Brassica* soil amendments. The toxic volatile mechanism involves the concept of biofumigation, where compounds toxic to plant pathogens and other microbes are released from macerated *Brassica* tissue. The toxic compounds originate from ß-D-thioglucosidic compounds, known as glucosinolates (GSLs), which are found in *Brassica* root and shoot tissues (Brown & Morra, 1997; Kirkegaard & Sarwar, 1998). The GSLs are hydrolysed in the presence of water

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and myrosinase (endogenous thioglucoside glucohydrolase) to produce compounds such as organic cyanides, ionic cyanate, oxazolidinethiones and isothiocyanates (ITCs), with the latter considered to be the most toxic compounds to a variety of soilborne pathogens and nematodes, ultimately resulting in a reduction in disease. The second microbial mechanism involves a shift towards a soil microbial community that is suppressive towards soilborne pathogens upon soil incorporation of *Brassica* tissue, through various direct and/or indirectt microbial actions (Davis *et al*., 1996; Smolinska, 2000; Mazzola *et al*., 2001; Larkin, 2003; Cohen *et al*., 2005; Weerakoon *et al*., 2012). The two proposed disease suppressive mechanisms, of course, are most likely not mutually exclusive, with the dominant mechanism varying in a temporal manner (Mazzola *et al*., 2007).

Suppression of plant disease by soil microbial communities through various functional mechanisms is well-known and has been studied extensively in naturally occurring disease suppressive agricultural soils. Disease suppressive soils can be classified as operating via a mechanism of general suppression or specific suppression. In soils exhibiting general suppression, disease suppression is most commonly attributed to a consortium of microbes that usually have a large biomass that leads to competitive pressure on the pathogen. In contrast, the phenomenon of specific suppression is attributed to specific microbial groups (Weller *et al*., 2002; Van Loon, 2007; Mendes *et al*., 2011). The major microbial genera that have been identified as having a functional role in a soil demonstrating specific soil suppressiveness include *Trichoderma, Fusarium, Pseudomonas* and actinomycetes such as non-pathogenic *Streptomyces* (Liu & Baker, 1980; Mazzola, 2002; Weller *et al*., 2002). These microbes suppress pathogens through various direct and indirect mechanisms that can be strain specific. In general, direct mechanisms include antagonism, parasitism, antibiotics and competition for resources. An indirect mechanism of pathogen suppression occurs when microbes induce host plant resistance against pathogens through a mechanism known as induced resistance. Initially, studies showed that there are two clearly defined forms of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Loon *et al*., 1998). The two resistance mechanisms were distinguished from each other by the nature of the elicitor and the regulatory pathways involved. SAR can be triggered when resistance in plants are elicited by abiotic factors, virulent, avirulent or non-pathogenic microbes. Establishment of SAR is mediated via the salicylic acid regulatoray pathway wherein salicylic acid and pathogenesis-related proteins (chitinase and glucanase)

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accumulate. ISR, on the other hand, is potentiated by plant growth promoting rhizobacteria (PGPR) and does not involve the accumulation of salicylic acid, chitinase and glucanase (Pieterse *et al*., 2002). ISR was first demonstrated in *Pseudomonas* spp. but have since also been identified in *Trichoderma, Fusarium* and *Streptomyces* (Weller, 1988; Haas & Defago, 2005; Hopkins *et al*., 1987; Larkin *et al*., 1993; Vinale *et al*., 2008; Liu *et al*., 1996; Mazzola, 1999; Wiggins & Kinkel 2005b; Cohen & Mazzola, 2006). Rhizobacteria-mediated ISR is regulated by jasmonic acid and ethylene signalling in plants (Van Loon *et al*., 1998). However, as more studies were conducted, it became clear that the delineation of ISR and SAR based on the nature of the elicitor and the regulatory pathways is not clear cut. For example, ISR induced by some *Bacillus* strains requires salicylic acid and not jasmonic acid (Ryu *et al*., 2003; Bostock, 2005). Therefore, for the purpose of the current study differentiation between ISR and SAR will not be made, and reference will only be made to induced resistance (IR) that refers to ISR and/or SAR. Furthermore, the IR will be defined in a similar manner than ISR, in that it is the process where treatment of plants with microbes elicits host defences, which results in a reduction in disease severity caused by pathogens that are spatially separated from the inducing agent/s (Leeman *et al*., 1995; Kloepper *et al*., 2004).

The overall aim of the study was to determine whether induced resistance is involved in the suppression of common scab of potato in *B. oleracea* var *capitata* (low glucosinolate producer) and *B. juncea/S. alba* mix (high glucosinolate producer) amended soils, using a potato split-root tunnel assay. In this assay, a component of the mother plant root system was grown in *Brassica*-amended sub-units and the other half was grown in non-amended soil that was spatially separated from the amended soil. All soils were inoculated with *S. scabiei.* The second aim was to investigate whether general or specific suppression and which microbial genera were potentially involved in disease suppression in the *Brassica*-amended and non-amended subunits by studying (i) soil densities of *Streptomyces, Fusarium,* fluorescent *Pseudomonas, Trichoderma* and total bacteria and (ii) enzymatic soil activities (ß-glucosidase, urease and acid phosphatase) that are all indicative of microbial biomass activity.

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MATERIALS AND METHODS

Brassica crop production.

Brassica crops were cultivated in the greenhouse for use in these studies and included *B. oleraceae* var *capitata* (cabbage) and *B. juncea/S. alba* (mustard mix). Cabbage (cv Conquestador) and mustard (cv Caliente 119) seed (Hygrotech, Pretoria, South African) were used to grow seedlings and mature plants in 25-cm-diameter plastic pots, with two seedlings per pot, as previously described (Chapter 3). The plants were harvested at 50 % flowering (the optimum physiological glucosinolate production period) and foliage and shoots were dried overnight at 50 °C in a 240 L Manual Oven, Series 2000, Model 296, (Scientific Engineering Pty. Ltd., Industria, Johannesburg, South Africa). The resulting biomass was ground to a powdered form and used as a soil amendment in the split-root experiments.The *Brassica* tissue was then ground to a powder in a Wiley mill with a 1 mm screen and stored for further use.

Inoculum preparation

A pathogenic *Streptomyces scabiei* isolate (N23, Chapter 2), which has been deposited in the ARC-VOPI culture collection at Roodeplaat, Pretoria, South Africa, was used as inoculum. The isolate was recovered from the -70 °C glycerol storage unit and plated onto oatmeal agar (OA, ISP medium 3) and yeast malt extract (YME, ISP medium 2) media, and incubated at 30 °C for 7 to 10 days. The *S. scabiei* spore suspension that served as inoculum was obtained by growing the isolate for 14 days on YME media at 30 °C in the dark, and flooding the plates with sterile distilled water to harvest spores. The suspensions were adjusted to a final concentration of 1.2 x 10^6 cfu/ml.

Potato split-root experiment

The efficacy of the cabbage and mustard residues for disease suppression was assessed using a custom made potato split-root unit (designed and manufactured by C.J. van Dyk, Pretoria, South Africa), which ensured spatial separation of the roots and progeny tubers from a mother potato seed tuber into two sub-units (Fig. 1). Soil used in all treatments was a

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natural sandy loam soil with pH 7.22 and 18 % clay. The soil was amended with a 2 % (w/w) cabbage or mustard powder. To ensure that the involvement of toxic volatiles, such as allyl isothiocyanate, could be ruled out as a potential mechanism of disease control in this experimental setup, treated soil was watered to activate myrosinase activity and corresponding glucosinolates. Soil was subsequently air-dried for 4 weeks prior to commencement of the experiments. The split-root sub-units were filled with 600 cm^3 of either cabbage- or mustard powder amended soil in sub-unit A (left hand sub-unit; Fig. 1), or non-amended soil in sub-unit B (right hand sub-unit; Fig. 1). The middle section of the splitter unit was filled with the same natural sandy loam soil that was used for the sub-units. For the inoculated control and un-inoculated control, both sub-units were each filled with 600 cm^3 of the natural sandy loam non-amended soil. Disease free BP1 tubers were obtained from RSA Seed potato producers (Silver Lakes, Pretoria, South Africa), surface disinfested with 1 % sodium hypochloride (NaOH) solution, and planted in the middle section of the splitter unit. The splitter units were only watered in the middle section until the roots were well established in the sub-unit containers (A & B), after which only the sub-units (A & B) were watered periodically using a slow dripper line. This prevented flooding of the sub-units and ensured dry conditions during tuber initiation.

The trial consisted of a completely randomized block design. There were six split-root units for each treatment and the trial was conducted twice. The treatments consisted of (1) un-inoculated control (no treatment), (2) *S. scabiei*-inoculated control, (3) soil treated with mustard powder in sub-unit A of the split-root and no soil amendment to sub-unit B of the splitter and (4) soil treated with cabbage powder in sub-unit A of the split-root and no soil amendment to sub-unit B of the splitter. The middle sections of the split-root unit except for the un-inoculated control, were each inoculated with 10 ml of a *Streptomyces* 1 x 10^6 spores/ml suspensions. The trial was conducted in a clear polypropylene tunnel, covered with 50 % green shade cloth. The tunnel was equipped with a pad and fan cooling system that regulates ambient temperature to average values of 25 °C in the day and 8 °C at night. The trial was terminated after 90 days and the soil, roots and tubers were evaluated as described below.

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Evaluation of split-root experiments

Common scab disease index determination. After 90 days of cultivation, progeny tubers were evaluated for common scab incidence according to the scale developed by Marais & Vorster (1988), which include the tuber surface area covered and lesion type. For surface area covered the scale was: 0 = no scab lesions; 1 = 1-12 % surface area covered; 2 = 13-25 % surface area covered; 3 = 26-50 % surface area covered 4 = 51-75 % and 5 = 76-100 % surface area covered. For lesion type the scale was: 6 = superficial lesion; 7 = netted/russet scab lesion and 11 = deep-pitted scab lesion. The final disease index was assigned on a scale of 0 to 55, which was calculated by multiplying surface area covered value by lesion type value. The data was statistically analysed by Analysis of Variance using the common scab disease index means for all tubers within a replicate (GENSTAT 14.1). All pairwise comparisons were tested with Fisher's Protected Least Significant Difference test (P = 0.05).

Microbial communities assayed by dilution plating. Soil, roots and tubers were assayed for the presence of total bacteria, fluorescent *Pseudomonas, Streptomyces, Fusarium* and *Trichoderma.* Soil samples (approximately 500 g) were collected from each split-root sub-unit for all treatments. Ten grams of each soil sample was suspended in 90 ml of sterilized water in 125 ml Erlenmeyer flasks and shaken for 20 min at 300 rpm. For root and tuber sampling, potato roots and tubers were collected from each sub-unit, and shaken loosely so that the attached soil was removed. Ten grams of roots were suspended in 10 ml of sterile distilled water and blended in a Waring Blender for 2 min and samples were utilized to characterize rhizosphere/endophyte populations. Potato tubers were peeled and tuber peels were suspended in 10 ml of sterile distilled water and blended in a Waring Blender for 2 min. The tuber data thus comprised microbial communities colonizing the soil attached to the tuber surface and those colonizing the potato tuber ectodermis (outer peel layer). Root, soil and tuber suspensions were serially diluted and 100 µl of each dilution was spread onto three replicate plates of four different semi-selective agar media. The media used and the target microbial communities were (i) one-tenth-strength tryptic soy agar (TSA, Biolab) for total bacteria, (ii) King's B+ [King's B base medium amended with cycloheximide (75-100 ug/ml) ampicillin (100 ug/ml) and chloramphenicol for fluorescent *Pseudomonas*, (iii) *Streptomyces* selective medium (Inorganic Salt Solution Agar, ISSA; ISP medium 5) for streptomycetes and (iv) *Trichoderma* selective medium (TSM) for recovery of both *Trichoderma* and *Fusarium* (Elad *et al*., 1981).

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Enzymatic activities in soil. β-glucosidase (EC 3.2.1.21) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 11.0) activities were assessed based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl glucosub-unit and *p*-nitrophenyl phosphate, respectively) (Dick *et al.*, 1996). For the β-glucosidase assay, 1.0 g soil (air dried) from each sub-unit for each of the replicates was placed separately in 100 ml screw-cap Scott bottle and incubated for 1 h at 37 °C with 0.25 ml toluene, 4 ml modified universal buffer (pH 6.0) and 1 ml *p*-nitrophenyl-β-D-glucosidase (PNG). The reaction was terminated by the addition of 1 ml 0.5 M calcium chloride ($CaCl_2$) and 4 ml 0.1 M Tris (hydroxy methyl)-aminomethane buffer (pH 12.0). Controls were performed by adding the substrate immediately after incubation, before the addition of $CaCl_2$ and Tris-buffer. The soil suspension was immediately filtered through Whatman no. 2 filter paper and the absorbance of the filtrate was measured at 410 nm. β-glucosidase activity was expressed as mg *p*-nitrophenol/g dry weight/h. Alkaline (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, pH 1 1.0) phosphatase activity was assayed using the method described of Alef & Nannipieri (1995). Modified universal buffer pH 11.0 was used to stabilize the soil pH to determine alkaline phosphomonoesterase activity. Phosphatase activity was expressed as mg *p*-nitrophenol/g dry weight/h for each of the two sub-units of each replicate using one gram of soil.

Urease (urea amidohydrolase, EC 3.5.1.5) activity was assayed using the procedure of Alef & Nannipieri (1995). Air-dried soil (5.0 g) from each sub-unit for each replicate was incubated separately in 2.5 ml urea solution at 37 °C for 2 h. After incubation, 50 ml of 1.0 M potassium chloride (KCl) solution was added, and the flasks were shaken for 30 min. The soil suspensions were filtered through Whatman no. 2 filter paper and the absorbance of the filtrate measured at 600 nm. Controls were prepared with 2.5 ml distilled water and the urea solution was added at the end of the incubation, immediately before the addition of the KCl solution. Urease activity was expressed as µg NH_4-N/g dry weight/2 h.

Statistical analyses. The enzymatic, microbial and disease index data were subjected to parametric statistical analyses using STATISTICA 6.1 (StatSoft, Inc., Tulsa, Oklahoma, USA, 2004) and Fisher's Least Significant Difference (LSD) test at $P < 0.05$. Principal component analysis (PCA) was also conducted on the microbial data and enzyme data

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separately. The microbial data was log (x + 1) transformed, before performing PCA analyses. For a few replicates of some treatments no tubers formed, and these replicates were thus removed, resulting in some treatments having less than six replicates. All multivariate analyses were conducted using the ADE-4 software (Thioulouse *et al.*, 1997). For all data, only the first two axes (*F*1 and *F*2) were analyzed. The factorial values of each replicate were also projected onto factorial maps for the microbial data, where no disease was indicated by squares, and disease severity was indicated by circles. Specific differences between sub-units A and B of the same replicate treatments were further investigated using a two table comparison in the program ADE-4. The two table comparison uses normed values (Y [i,j] – X[I,j]) and is very usefull for comparing the change between two sets of data collected at the sample place, and measured for the same parameters. The technique involves conducting a PCA on the differences between two sets of data, in this case the microbial differences between associated sub-units A and B of each replicate treatment. The two tables consisted of the microbial count data where columns represented the colony forming units measured for each of the different microbial genera/groups in soil, tubers and roots, and rows represented the replicates of the different treatments.

RESULTS

Common scab disease index determination

The post-hoc Fisher's Protected LSD test ($P = 0.05$) showed that the common scab disease index in the positive inoculated control sub-units (IA & IB) was significantly higher than the disease indexes of all other treatments. All tubers cultivated in a split-root unit to which *Brassica* residue was added to soil, had a significantly lower disease index value relative to the inoculated controls (IA & IB), irrespective of whether the tubers were recovered from the amended cabbage (CA) and mustard (MA) sub-units, or from the non-amended cabbage (CB) and mustard (MB) soil sub-units. However, tubers from the amended soil sub-units (CA & MA) had significantly lower disease incidence when compared to their associated non-amended sub-units (CB & MB) (Table 1).

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Microbial communities assayed by dilution plating

The number of *Streptomyces* in the tubers and roots (except for the non-amended mustard sub-unit), and total bacteria in the soil and tubers were significantly higher in the inoculated control treatments (Fischer's Protected LSD test, P <0.05; Table 1). The *Fusarium, Pseudomonas* and *Trichoderma* numbers were not significantly different between any of the treatments (data not shown).

For PCA analyses the results of the two independent experiments were combined, since the analyses showed that the two sub-units (A and B) of each of the control treatments (no treatment and *S. scabiei* inoculated) from the two repeat experiments nearly possessed the same location on the factorial map (data not shown). PCA revealed that the microbial data from the soil, roots and tubers did not have a strong structure (combined data did not explain a lot of the variability) since the first two factors of the PCA described only 40.82 % ($F1$ = 27.98 and $F2$ = 12.84) of the variability.

PCA analyses showed that each of the treatments and their sub-units were located at a specific region of the factorial plan (Fig. 2). Located in the middle of the factorial plan, the negative controls did not have a tendency towards dominance by any of the microbial groups (Fig. 2 a, b). Sub-units A and B of each of the control treatments (IA, IB, NCA & NCB) almost had the same position on the factorial map (Fig. 2 b), confirming that no positional effects were present between the two sub-units of each split-root unit. The inoculated controls had the highest correlation with the negative part of $F1$, and were associated with high *Streptomycete* numbers in the roots (StrepRoot), soil (StrepSoil) and tubers (StrepTuber) and bacteria in the soil (BactSoil) and tubers (BactTuber), and low numbers of *Trichoderma* in the roots (TrichRoot) and soil (TrichSoil), and *Fusarium* in the soil (FusSoil) (Fig. 2 a, b). It is important to note that the total bacterial numbers also included *Streptomyces* colonies. CA, CB, MB and MA were all located in the positive part of $F1$, with MA and CA having the highest and lowest correlation respectively with the positive part of $F1$. These four treatments (MA, MB, CA, CB) were all associated with higher *Trichoderma* numbers in the roots (TrichRoot) and soil (TrichSoil) and *Fusarium* in the soil (FusSoil), and lower *Streptomyces*

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and total bacteria in the soil (StrepSoil, BactSoil), *Streptomyces* in the roots (StrepRoot) and tubers (StrepTuber), and bacteria in tubers (BactTuber). MB had the highest correlation with the negative part of *F*2 and had a tendency towards higher *Pseudomonas* numbers in the soil (PseuSoil), roots (PseuRoot) and tubers (PseuTuber) and less *Streptomyces* in the soil (StrepSoil), *Fusarium* in the roots (FusRoot) and *Trichoderma* in the tubers (TrichTuber) (Fig. 2a, b). However, projection of the disease severity data onto the factorial map showed that replicates from MB that did have disease, had a tendency towards higher *Pseudomonas* numbers in the soil (PseuSoil) and tubers (PseuTuber) and lower *Streptomyces* (StrepSoil) in the soil and *Fusarium* in roots (FusRoot), with the exception of one replicate (Fig. 2 c).

The two-table comparison that investigated the microbial differences between associated subunits A and B, revealed some important relationships between the A and B sub-units of each specific treatment (Fig. 3). On the factorial map TrichoRoot, StrepSoil, FusRoot and FusTubers were strongly correlated with the positive values of *F*1, whereas PseuSoil and PseuRoots were strongly correlated with the negative values of *F*1. The differences between MA and MB were due to MB having increased *Pseudomonas* numbers in the soil (PseuSoil), roots (PseuRoot) and tubers (PseuTuber) and a decrease in *Trichoderma* in roots (TrichRoot), *Streptomyces* in soil (StrepSoil) and *Fusarium* in roots (FusRoot) and tubers (FusTuber). Furthermore, when tubers were diseased (30 scab index) in the mustard non-amended sub-unit (MB), differences in microbial groups between sub-units A and B became larger as indicated by the length of the projections (Fig. 3b). This would suggest that in MB, tubers that were diseased were associated with very high *Pseudomonas* numbers, low *Streptomyces* numbers in the soil (StrepSoil), low *Trichoderma* in roots (TrichRoot) and low *Fusarium* in the roots (FusRoot) and tubers (FusTuber). The two table comparison further indicated that for the cabbage sub-units, the differences between sub-units A and B varied between replicates and in general were not large (shorter projections), suggesting that the differences were not constant between replicates (Fig. 3b). It was furthermore confirmed that the inoculated control and negative control sub-unit B's did not differ much from their sub-units A, since the projections were in general short, although this was more evident for the inoculated control (Fig. 3b).

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Enzymatic activities in soil

The amended sub-units (CA, MA) had significantly higher β-glucosidase and urease activities than the inoculated controls (Fischer's Protected LSD test, $P < 0.05$). The cabbage (CA) and mustard (MA) amended soil also had significantly higher β-glucosidase activity levels that their non-amended sub-units (CB, MB). The acid phosphatase analysis showed that only CA had significantly higher values than the inoculated controls (Table 1).

As conducted for the microbial genera data, for the PCA analyses of the enzymatic data, the results of the two independent experiments were combined since the two sub-units of each of the two control treatments (inoculated and uninoculated) were located at the same position on the factorial plan (data not shown). There was strong structure for the enzyme data, since the first two factors described 93.20 % ($F1$ = 86.24 %, $F2$ = 7.68 %) of the variability. A size effect was evident for the enzyme data in the first factorial plan, since all the variables had strong positive F1 values (Fig. 4a). As expected, the mustard-amended sub-unit A (MA) and cabbage-amended sub-unit A (CA), which received the organic matter, were both correlated with high positive $F1$ values. In contrast, all the other treatments correlated with low negative $F1$ values (Fig. 4b).

DISCUSSION

The study investigated whether IR was involved in suppression of common scab of potato when soil was amended with cabbage and mustard residues. Using a potato split-root assay, it was apparent that IR that developed in response to *Brassica* amendments contributed to the reduction in common scab. The assay enabled the spatial separation of progeny tubers and roots in mustard- (MA) and cabbage (CA) amended treatments from those grown in non-amended mustard- (MB) and cabbage (CB) sub-units. The function of IR in the observed disease control was evidenced by the fact that progeny tubers derived from the same mother plant, but from the non-amended sub-units (CB & MB), had significantly less disease than the inoculated controls (IA & IB). Mustard and cabbage were equally effective at inducing IR and reducing common scab. The involvement of ITC derived directly from the introduced plant residues was ruled out in the non-amended sub-units (CB & MB), since the *Brassica*-

amended soils were thoroughly wetted several weeks prior to commencement of the trials to ensure complete hydrolysis of GLSs and dissipation of the associated toxic volatiles. Depending upon the tissues utilized, production and emission of the dominant volatile generated in response to soil incorporation of *B. juncea* residues has been reported as several hours (Mazzola *et al.,* 2007) to a few days (Morra & Kirkegaard, 2002). The current study has increased our knowledge of the occurrence of IR in potato, which has previously only been reported for the potato cyst nematode (*Globodera pallida*). For this system, IR was mediated by *Agrobacterium radiobacter, Bacillus sphaericus* or *Rhizobium etli* (Hasky-Gunther *et al.*, 1998).

Trichoderma, Streptomyces, Fusarium, Pseudomonas and several other bacteria are known as important rhizosphere genera that can elicit and mediate IR (Larkin *et al*., 1993; Van Loon *et al*., 1998; Van Loon, 2007; Vinale *et al*., 2008). The density of these microbes were thus investigated in the *Brassica*-amended sub-units (CA & MA), to determine whether any of these genera might contribute to IR signalling toward the associated non-amended sub-units (CB & MB) where disease suppression was observed. In this analyses, the roots were blended resulting in the inclusion of both endophytic and rhizosphere associated microbes. Therefore, reference will be made to these microbes as rhizosphere/endophytic populations. There were no significant increases in the density of any of the investigated rhizosphere/endophytic genera recovered from roots growing in the amended *Brassica* sub-units (CA & MA), but a significant decrease was found in *Streptomyces* numbers relative to the inoculated control (IA and IB) rhizosphere/endophytic populations. The reduced endophytic/rhizosphere *Streptomyces* populations could be indicative of suppression of the pathogen population in roots in the *Brassica*-amended soils (CA & MA). However, this response will require confirmation through further analyses that differentiate between *S. scabiei* and non-pathogenic *Streptomyces* that were indigenous in the sandy loam soil.

Several factors may have contributed to the lack of increase in density of specific microbial genera in the rhizosphere/endophytic populations. First of all, the presence of endophytes may have masked the true rhizosphere populations. Secondly, the fact that the total number of rhizoshere/endophytic bacteria did not increase in the *Brassica*-amended sub-units (CA & MA) does not rule out the role of rhizobacteria, since specific bacterial genera

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and strains respond differentially in response to *Brassica* amendments and differ in their ISR ability (Van Loon *et al*., 1998). For instance, populations of fluorescent *Pseudomonas* spp. declined in response to *Brassica napus* seed meal soil amendment but populations of *Streptomyces* spp. increased by two orders of magnitude (Cohen *et al*, 2005). Therefore, a shift towards genera and strains that are effective ISR inducers will not always be reflected in total counts. A similar situation might be true for the lack of significant increases in the *Trichoderma, Fusarium* and *Pseudomonas* rhizosphere/endophytic populations, since in these genera only specific strains are also effective ISR inducers (Woo *et al*, 2006; Larkin, 2008; *W*eerakoon *et al*., 2012). Thirdly, there may have been an increase in non-pathogenic *Streptomyces* in the amended sub-units (CA & MA) relative to the inoculated control (IA & IB), which would not be evident in the current study since *S. scabiei* was not differentiated from non-pathogenic *Streptomyces.* This is particularly relevant as the sandy loam soil used in the study contained a large population of indigenous non-pathogenic *Streptomyces.* A reduction in pathogen populations would be expected, since this is in agreement with the fact that in ISR a reduction in disease severity is usually associated with a decrease in pathogen growth and colonization of induced plant tissues (Choudhary *et al*., 2007). A fourth reason for the lack of significant increases in the density of specific microbial groups could be the serial dilution plating method that was employed to estimate microbial densities. With this method, some *in-vitro* antagonistic relationships between species and isolates can develop on the plates that can result in a negative or positive effect on densities for certain genera. The use of molecular methods such as real-time PCR quantification of the 16S rRNA region for quantification of total bacteria could circumvent this problem.

In the mustard and cabbage non-amended IR sub-units (MB & CB) there were some trends, as revealed through principal component analyses (PCA), towards differences in the relative microbial community composition in the soil, roots and tubers of these treatments when compared to the inoculated controls (IA & IB). The cabbage associated sub-unit (CB) had a tendency towards higher numbers of *Trichoderma* (roots and soil) and *Fusarium* (soil) and lower numbers of *Streptomycetes* (soil, tubers and roots) and total bacteria (soil and tubers) than the inoculated control (IA & IB). This may suggest that signalling from the associated amended sub-unit (CA) induced a change in root exudation and host responses that not only reduced pathogen populations but also affected other microbial genera. It is also interesting that the mustard associated sub-unit (MB) was microbiologically distinct from the

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inoculated control (IA & IB) and the cabbage associated sub-unit (CB), suggesting that the mustard amendment resulted in root exudate and host responses that were different from those induced by cabbage. The mustard associated sub-unit (MB) was associated with increased *Pseudomonas* (roots, soil and tubers) and lower densities of *Streptomyces* and *Fusarium* in the soil. However, when the *Pseudomonas* numbers were too high and the *Streptomycetes* in soil were low, there was a tendency for increased disease of tubers. This could suggest that a balance between microbial groups is important in disease suppression.

Disease suppression was significantly more effective in the split-root sub-units that received the *Brassica* amendments themselves (CA & MA), than in the IR associated sub-units (CB & MB). This is most likely due to the fact that the direct addition of organic material resulted in altered microbial community composition and microbial biomass. Therefore, the relative microbial population densities were analysed through PCA that considered all the evaluated genera, in an attempt to discern whether certain shifts (positive or negative) could be associated with relative disease development. The population densities of the microbial genera did not describe a high amount of the variability between treatments (40.82 % for the first two factors). This may be due to large variability in the data set of microbial colony forming units and the fact that not all the microbial groups that reside in these soils were evaluated, for example various zygomycetes numbers (e.g. *Mortierella, Mucor*) that are dramatically elevated in response to *Brassica* amendments (Cohen & Mazzola, 2006). The mustard amendment (MA) induced the most significant changes among the genera considered in this study as this treatment was located most distantly from the inoculated (IA & IB) and uninoculated controls (NCA & NCB) on the factorial map, and also had the largest factorial value on the positive F1 axis. This could be due to the fact the mustard had a very high potential to produce ITC that would have resulted in a large disruption of microbial communities at the start of the experiment when the mustard and soil mix was watered to release ITC before being placed in the split-root units. Cabbage contains much lower levels of glucosinolate and will produce less ITC (Brown & Morra, 1997; Kushad *et al*., 1999), thus also likely causing less of a disturbance in microbial communities. It was indeed found that the cabbage amendment (CA) was more similar tothe controls than the mustard treatment (MA). Both of these *Brassica* amendments (CA & MA) were associated with higher numbers of *Trichoderma* in the roots and soil, *Fusarium* in the soil and lower densities of *Streptomycetes* in the soil, tubers and roots, and fewer total bacteria in the

soil and tubers. The tendency towards increased *Fusarium* and *Trichoderma* populations could result in resource competition and niche (bulk soil, rhizosphere or tubers) exclusion of the pathogen. Elevated *Trichoderma* densities in response to *B. juncea* seed meal amendment was associated with long-term suppression of root infection by the pathogen *Pythium abapressorium* (Weerakoon *et al*., 2012). *Trichoderma* is known to produce a large number of secondary metabolites, including antibiotics with biological activity that might also be involved. However, little is known about the effect of *Trichoderma* on *Streptomyces* and other bacteria, with most work being conducted on fungi (Harman 2006; Woo *et al*., 2006; Vinale *et al*., 2008).

Effective disease suppression in the *Brassica*-amended sub-units (CA & MA) could be attributed to an overall increase in microbial activity and general suppression in response to the added organic matter. In general suppression, direct microbial mediated disease suppressive mechanisms function such as competition, parasitism and antibiosis, which is usually, associated with the bulk soil microbial communities (Cook & Baker, 1983). Evidence for general suppression was found in the measurement of the activities of three soil enzymes that are all indicative of increased microbial activity (Weller *et al*., 2002)). The soil enzymatic activities described 93.20 % of the variability (the first two factors) between treatments. The mustard (MA) and cabbage (CA) amended sub-units contained significantly higher ß-glucosidase and urease activities than the inoculated controls (IA & IB), indicating increased microbial activity. Only the cabbage (CA) treatment showed a significant increase in acid phosphatase. Nunez-Zofio *et al*. (2011) also reported a significant increase in ß-glucosidase, urease and acid phosphatase activities when soil was amended with various organic amendments, including *Brassica* pellets and green manure. An increase in total microbial activity with the addition of *Brassica* residues has also been reported by Larkin (2008) and Larkin *et al.* (2011), where microbial activity was measured using soil dilution plating, substrate utilization profiles and fatty acid methyl ester profiles (Larkin, 2008; Larkin *et al*., 2011).

In conclusion, this research indicated that the application of ground residues of mustard and cabbage could be used as a soil amendment to decrease the incidence of common scab on potato tubers. The disease control outcome in response to these amendments appears to function through the mechanisms of IR and general microbial suppression. IR

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suppression in itself is less effective than the two mechanisms combined since the IR response detected in the non-amended sub-units (CB & MB) had a significantly lower level of disease control relative to the amended sub-units (CA & MA). The mustard and cabbage amendments were equally effective at reducing disease incidence, which was expected since the biologically active volatiles, including allyl isothiocyanate, were released from the *Brassica*-amended soils prior to initiation of the experiment. The *Brassica* amendments had different effects on the establishment and persistence of the soil, root and tuber microbial communities (*Trichoderma, Fusarium, Streptomyces* and total bacteria) in the amended (CA & MA) and non-amended IR sub-units (CB & MB). The mustard and cabbage-amended sub-units (CA & MA) supported similar microbial community densities based on the investigated microbial groups, but the change in microbial community densities was more evident for the mustard treatment (MA). Similarly in the non-amended IR sub-units (CB & MB) the community in the mustard sub-unit (MB) was more distinct from those in the cabbage sub-units (CB) and controls. This suggests that the IR initiated in the mustard-amended sub-units (MA) resulted in different host responses, root exudates and associated microbial communities in the non-amended mustard sub-units (MB), than those observed in the cabbage non-amended sub-unit (CB). Although all the mustard (MA) and cabbage (CA) amended sub-units showed reduced pathogen populations, a specific microbial genus that might be involved in IR signaling, could not be identified. Active management of soil microbial communities and the associated mechanisms involved in disease suppression through *Brassica* amendment has significant potential as a disease control alternative. In future studies further investigation into the interactions and effects among the microbial communities are required, as well as practical implementation of this management system.

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Table 1. Effect of cabbage and mustard amendments on common scab incidence, microbial densities in soil, roots and tubers and soil enzymatic analyses obtained from potato split-root growth units.

Parameter measured	Cabbage [b]		Mustard [b]		Inoculated Control [b]		Un-inoculated control [b]	
	Amended A	Non-amended B	Amended A	Non-amended B	A	B	A	B
Common scab disease index determination[a]								
Scab index	0 a	8 b	0 a	12 b	30 c	30 c	0 a	0 a
Microbial colony forming units[a]								
Streptomyces soil	4×10^7 a	9×10^6 a	6×10^6 a	4.3×10^6 a	1×10^9 a	5.4×10^9 a	7×10^6 a	7×10^6 a
Streptomyces roots	1.6×10^4 a	5.2×10^4 a	8.4×10^5 a	1.7×10^6 ab	6.7×10^6 b	1.6×10^7 b	2.2×10^6 ab	2.1×10^6 ab
Streptomyces tubers	5.2×10^6 a	1.7×10^5 a	9.5×10^6 a	1.7×10^4 a	2.5×10^8 b	2.3×10^8 b	184 a	166 a
Total bacteria soil	2×10^9 a	9×10^8 a	4×10^8 a	1×10^9 a	1×10^{10} b	1.2×10^{10} b	1×10^9 a	1.1×10^9 a
Total bacteria roots	1×10^{10} a	2×10^9 a	4×10^9 a	4×10^9 a	3×10^9 a	2.1×10^9 a	3×10^9 a	2.8×10^9 a
Total bacteria tubers	4×10^9 a	2×10^9 a	4×10^9 a	6×10^9 a	4×10^{10} b	3×10^{10} b	4×10^9 a	4.6×10^9 a
Enzymatic activities in soil[a]								
B-glucosidase	546.1201 d	356.0469 bc	483.7596 cd	309.2596 ab	229.4668 ab	219.5476 ab	171.3105 a	169.2314 a
Acid phosphatase	809.5405 c	376.6339 ab	701.8402 bc	360.1738 ab	359.4215 ab	361.3167 ab	177.8253 a	178.3765 a
Urease	51.66279 b	15.22697 a	50.95297 b	16.81527 a	11.67668 a	11.6248 a	10.49392 a	10.4856 a

[a] Values within a row followed by the same letter, do not differ significantly from each other ($P = 0.05$)

[b] Potato split-root units consisted of two sub-units (A and B). In the cabbage and mustard treatments, sub-unit A was amended with *Brassica* tissue and sub-unit B was not amended.

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Figure 1. Potato split-root unit used to evaluate whether induced resistance is involved in common scab disease suppression when a sandy loam soil was amended with cabbage or mustard residues. In the mustard and cabbage treatments, sub-unit A soil was amended with *Brassica* powder (mustard or cabbage) whereas sub-unit B only contained non-amended soil.

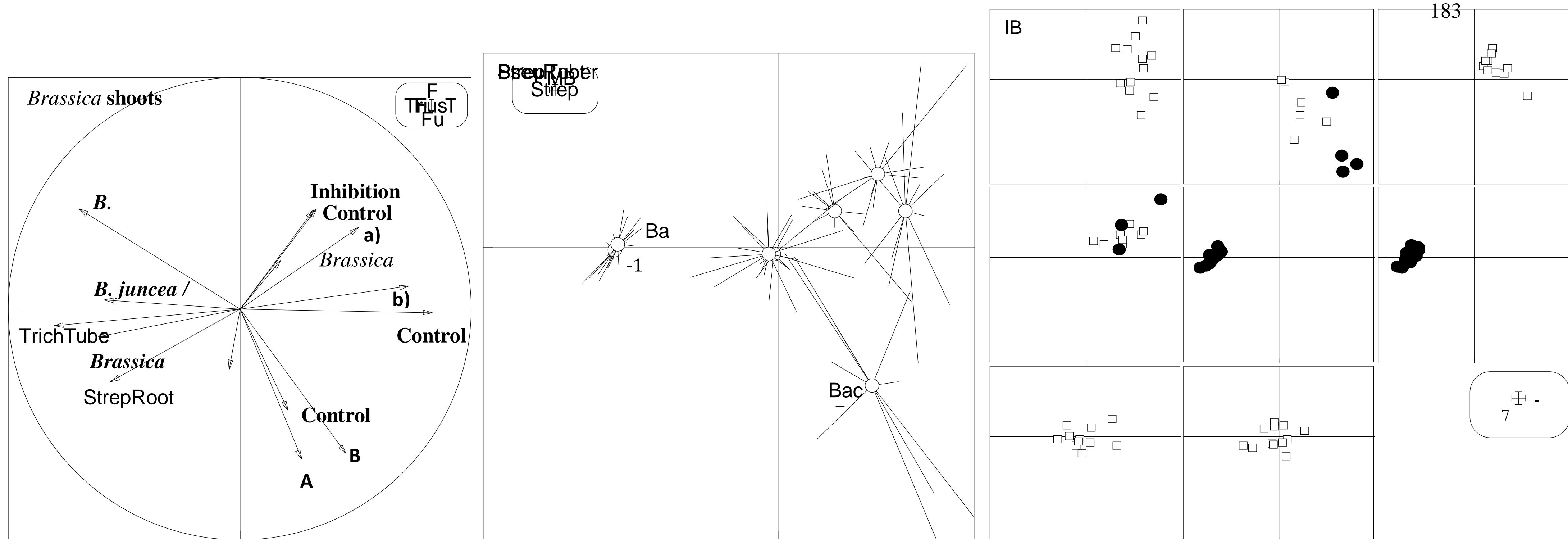
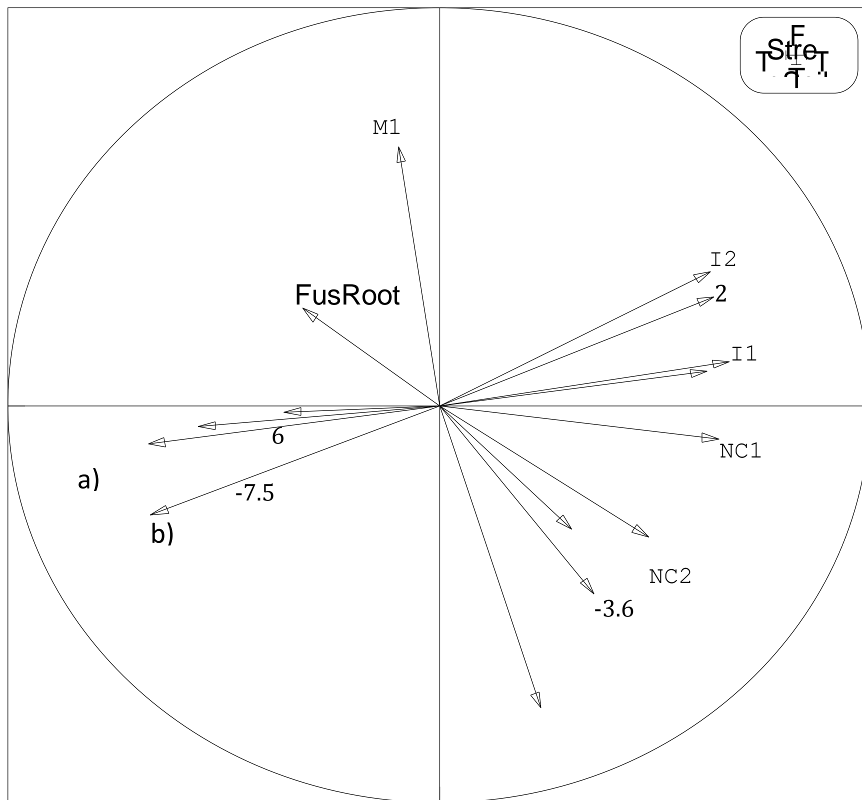


Figure 2. Principal component analyses of the microbial count data as influenced by mustard and cabbage amendments that were evaluated using potato split-root units that contained two sub-units (A and B). a) $F1 \times F2$ factorial plan of the microbial variables [bacterial soil (BactSoil), root (BactRoot), and tuber (BactTuber), *Streptomyces* soil (StrepSoil), root (StrepSoil) and tuber (StrepTuber), *Fusarium* soil (FusSoil), root (FusRoot) and tuber (FusTuber); *Pseudomonas* soil (PseuSoil), root (PseuRoot) and tuber (PseuTuber) colony forming units] and b) the corresponding plan showing the position of the different treatments [mustard-amended soil in sub-unit A (MA), non-amended soil in sub-unit B (MB); cabbage-amended soil in sub-unit A (CA), non-amended soil in sub-unit B (CB), inoculated control in sub-units A and B (IA and IB) and un-inoculated controls in sub-units A and B (NCA and NCB)] on the factorial plan. Each branch of a star joins one replicate of a specific treatment to the gravity centre of the treatment. c) Projection of the disease severity (no disease = square and disease = circle) onto the factorial plan of the different treatments, with each circle or square representing one replicate.



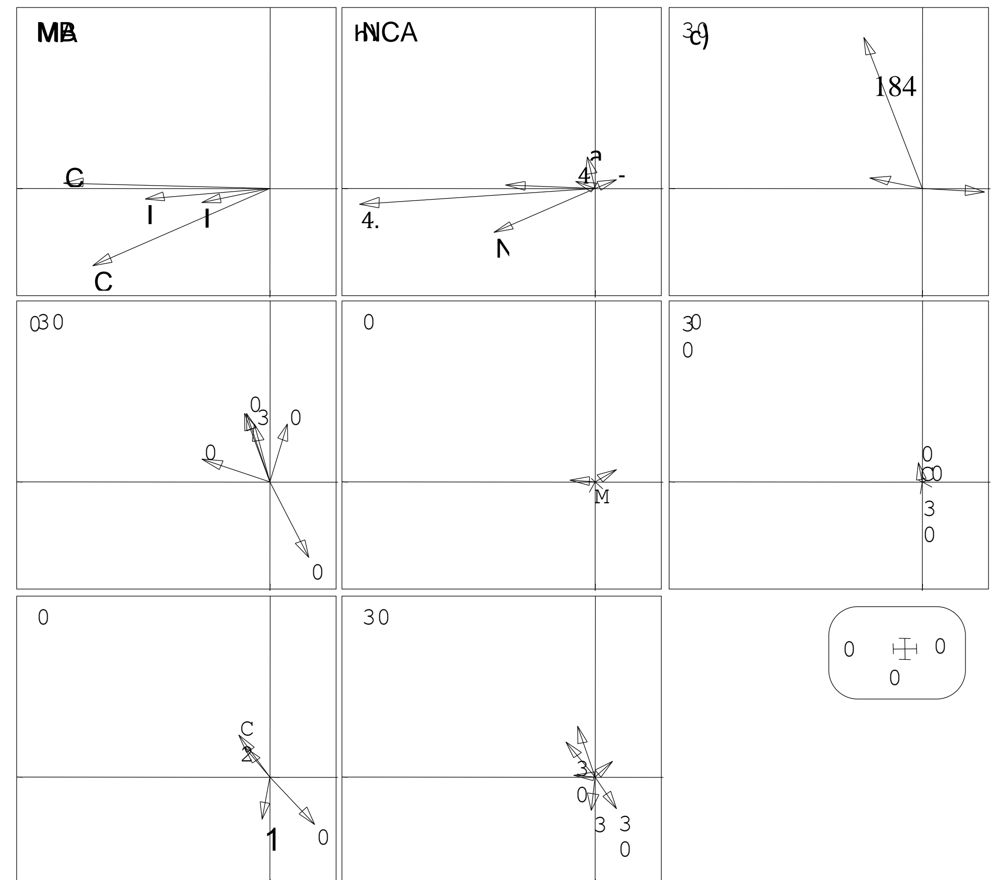


Figure 3. Two table comparison analyses that investigated differences between microbial groups in sub-unit A and sub-unit B of potato split-root units that received different treatment. a) Factorial plan of the microbial variables [bacterial soil (BactSoil), root (BactRoot), and tuber (BactTuber); *Streptomyces* soil (StrepSoil), root (StrepSoil) and tuber (StrepTuber); *Fusarium* soil (FusSoil), root (FusRoot) and tuber (FusTuber); *Pseudomonas* soil (PseuSoil), root (PseuRoot) and tuber (PseuTuber)] and b) factorial plan of the individuals corresponding to the different replicates grouped per treatment (cabbage [C1 & C2], mustard [M1 & M2], inoculated control [I1 & I2] and negative control [NC1 & NC2]). Both experiments were represented separately for clarity. The centre of the plan represent sub-unit A and the extremity of the arrow indicates the bacterial parameters that increase or decrease in sub-unit B according to the factorial map in (a). Arrows followed by a “0” had no disease and arrows followed by a “3” had a scab disease index of 30.

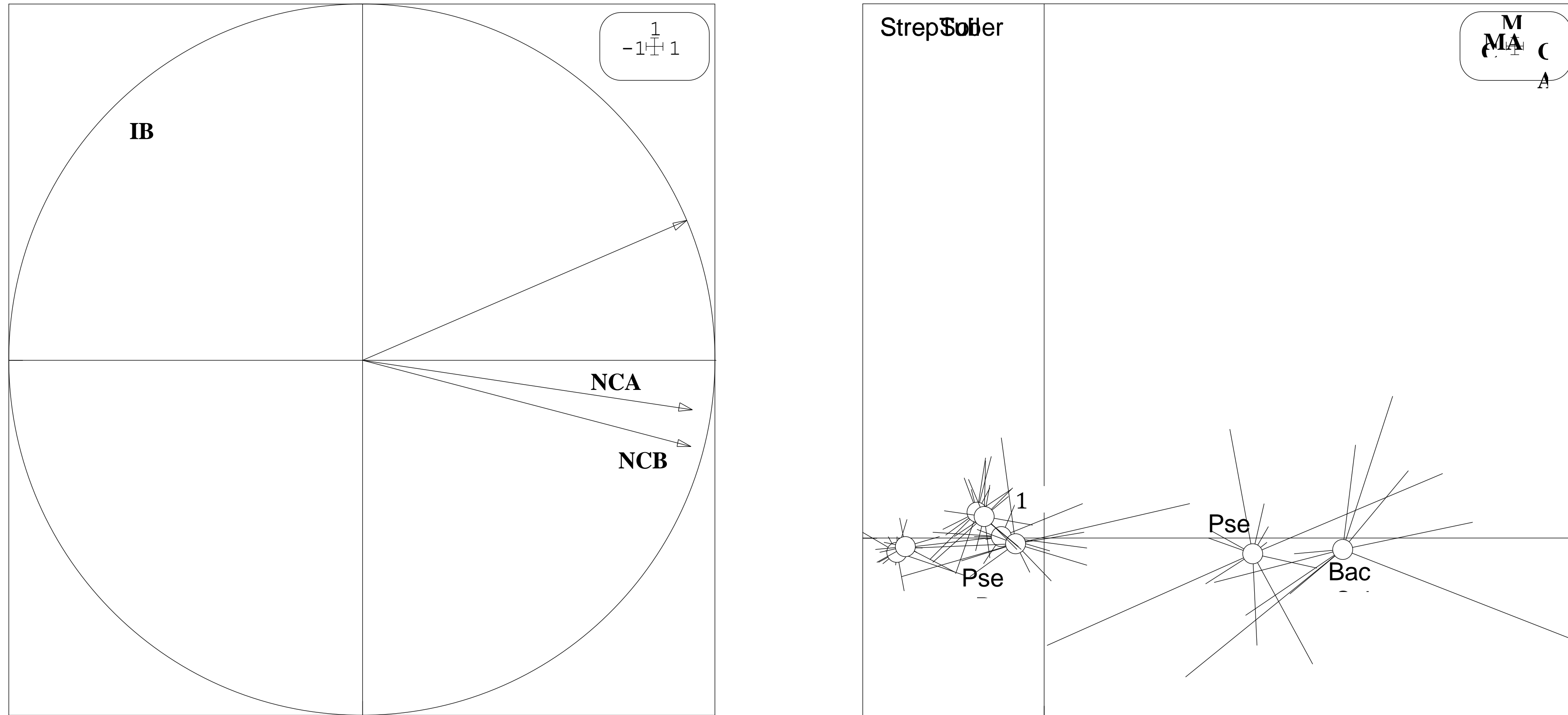


Fig. 4. Principal component analyses (PCA) of soil enzyme data as influenced by mustard and cabbage amendments that were evaluated in potato split-root units that contained two sub-units (A and B). a) $F1 \times F2$ factorial plan of the enzyme variables $F1 \times F2$ (phosphatase, B-glucosidase and urease), b) the corresponding plan showing the position of the different treatments [mustard-amended soil in sub-unit A (MA), non-amended soil in sub-unit B (MB); cabbage-amended soil in sub-unit A (CA), non-amended soil in sub-unit B (CB), inoculated control in sub-units A and B (IA and IB) and un-inoculated controls in sub-units A and B (NCA and NCB)] on the factorial plan.

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