Elucidating the role of microbial factors and water stress in causing corky crack blemishes on potato tubers in South Africa

by

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Declaration

I, Sasha-Lee Gush, declare that this thesis/dissertation, which I hereby submit for the degree MSc in Plant Pathology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: …….

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Abstract

Potato (*Solanum tuberosum* L.) is the most consumed and economically important vegetable crop in the world and is a staple crop in many communities. However, the potato crop is susceptible to devastation by various diseases that reduce the yield and quality of fresh produce. Blemishes, rots and deformation are some of the tuber symptoms caused by fungi, bacteria, viruses and nematodes. Abiotic factors such as water stress (dry conditions and thereafter high moisture conditions), has also been associated as causal agents of tuber blemishes.

Potato tuber blemishes constitute a persistent quality problem in the production of potato throughout the world and can be a result of known causes (typical blemishes), which are manageable; or unknown causes (atypical blemishes), which are difficult to manage. Atypical corky crack blemishes are an emerging problem of potatoes in South Africa. There has been a large debate in the South African potato industry regarding the causal agents of corky cracks.

This study investigated the role of microbial factors and water stress in causing corky crack blemishes in South Africa. In a pilot trial, *R. solani* AG2-2IIIB and various *Streptomyces* species were isolated from corky crack blemishes on potatoes in South Africa. However, none of these microorganisms, alone or in combination resulted in the development of corky cracks when tested in pot trials. Therefore, in an effort to confirm the causal agent(s) of corky crack blemishes, additional tubers with symptoms of either growth cracks or corky cracks were sampled from different potato growing regions of South Africa and further isolations of bacteria and fungi were made from the different symptoms. Water stress and the detection of Potato Virus Y (PVY) was also studied to describe the association with corky crack blemishes in South Africa.

Binucleate *Rhizoctonia* (BNR) AG-A and BNR AG-R as well as *Fusarium oxysporum* were the most predominant fungal species isolated from the corky crack symptoms, while no fungal species were isolated from growth cracks. *Streptomyces collinus*, *S. yaanensis*, *S. corchorus*si, *S. viridochromogenes* and *S. griseorubens* were the most predominant bacterial species isolated from growth cracks and no *Streptomyces* species were isolated from corky cracks. The *Streptomyces* species identified are not known to be pathogenic to potatoes and are, therefore, not likely causal agents of the corky cracks. PVY was not amplified from corky crack tissue

using molecular techniques and it was, therefore, assumed that PVY is not associated with corky cracks on the tested samples of potato tubers in South Africa.

Greenhouse pathogenicity trials using the isolated microorganisms alone and in combination were done to confirm Koch's postulates. Corky crack blemishes were not observed on progeny tubers in single inoculations for fungal or bacterial isolates; however, corky crack blemishes were observed on the progeny tubers inoculated with a combination of *Rhizoctonia* species. This suggests a synergistic interaction between the three *Rhizoctonia* species tested in this study. The findings of this study are crucial in the development of effective integrated strategies for the management of corky crack disease and to improve the tuber quality in the South African market and globally.

Keywords: Binucleate *Rhizoctonia*, *Streptomyces* species, PVY, water stress, corky crack blemishes

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Chapter 1

General Introduction

1.1 Background and motivation for the study

A major challenge facing the world today is the need to produce enough food to feed the ever-growing human population. Global statistics show that, out of the total 7633 million people, 2718 million are food insecure (FAO 2019). The attainment of food security in many communities is constrained by several factors, among them; adverse environmental conditions, pests and diseases.

Potato (*Solanum tuberosum* L.) is the most consumed and economically important vegetable crop in the world (FAOSTAT 2017). Potato is a staple crop in many communities in Asia, Africa, South America, the United States of America (USA) and Northern Europe (Lutaladio and Castaldi 2009). Globally, approximately 383 million metric tons of potatoes were produced worldwide in 2016 and approximately 2.5 million tons in 2017 in South Africa (FAOSTAT 2019). Nutritionally, potato is ideal for human consumption and provides a balanced source of starch, high-quality proteins, vitamins, trace elements and dietary fibre (Black 2008). This makes it an ideal crop to feed many communities and ensure food security.

To increase potato production, research is focused on disease control, soil health, good farm management practices, seed quality and breeding of high-yielding varieties (Veeman and Veeman 2004). The potato crop, as with many other agricultural crops, is susceptible to devastation by various diseases (Fiers et al. 2012). Potato diseases reduce the yield and quality of fresh produce and therefore pose a threat to food security (Black 2008). The potato crop is subject to attack by more than 100 pathogens, of which 40 are soil-borne pathogens (Fiers et al. 2010). Such diseases are caused by fungi, bacteria, viruses and nematodes (Agrios 2005).

Potato tuber blemishes constitute a persistent quality problem in the production of potato throughout the world (Fiers et al. 2010). Tuber blemishes, where the causal agent(s) are known and Koch's postulates have been fulfilled, are commonly referred to as typical blemishes (Fiers

et al. 2010; Zimudzi et al. 2017). Fungal and bacterial pathogens causing typical tuber blemishes include *Rhizoctonia solani*, *Colletotrichum coccodes*, *Spongospora subterranea* f. sp. *subterranea*, *Helminthosporium solani*, *Fusarium* species and *Streptomyces* species (Nærstad et al. 2012). These pathogens cause various symptoms on potato skin, including black scurf, black dot, powdery scab, silver scurf, dry rot and common scab, respectively. Tuber blemishes are also caused by viruses (Carnegie and McCreath 2010) and nematodes (Holgado et al. 2009). In contrast, atypical blemishes are due to unknown causes and there are contradictory reports on the causal agent, thus are difficult to manage (Fiers et al. 2010; Zimudzi et al. 2017). Atypical blemishes include corky cracks, star-like corky lesions, corky spots or 'rhizoscab' and 'elephant hide' (Fiers et al. 2010).

Pathogens causing typical and atypical blemishes on seed tubers are of great concern to the potato seed industry as they can re-infect progeny tubers of plants produced by the contaminated seed. They can cause severe losses in yield, rejection of seed batches, the downgrading of potatoes on the market or rejection of potatoes for processing (Tsror 2010). The demand for washed potatoes by South African consumers increases the problem of blemished tubers. Potato blemishes therefore pose an economic threat to farmers and cause downgrading of potato tubers in the fresh produce market in South Africa. Knowledge of the causative agents of atypical blemishes is important for farmers to optimize their disease management strategies to avoid yield losses and downgrading of tubers in the market (Jordaan and van der Waals 2015).

In South Africa, there has been an increase in the incidence of various atypical blemishes, such as the so-called 'growth cracks' and 'corky cracks' on the surface of the tubers (Muzhinji et al. 2014). The occurrence and causal agents of potato tuber blemishes are one of the most contentious issues in the potato industry in South Africa and other parts of the world. Studies have suggested that these corky cracks have been associated with *R solani* (Muzhinji et al. 2014), binucleate *Rhizoctonia* species (Muzhinji et al. 2015; Zimudzi et al. 2017) and *Streptomyces* species (Gouws and McLeod 2012). In addition, viruses such as Potato Mop Top Virus (PMTV) and Potato Virus Y (PVY) have also been implicated in causing tuber corky cracks (Fiers et al. 2010; Brierley et al. 2016), although this assertion lacks full direct empirical scientific evidence and symptom expression is highly dependent on the virus-cultivar interaction (Visser et al. 2012).

On the other hand, abiotic factors such as water stress (dry conditions and thereafter high moisture conditions), temperature, humidity, mechanical damage, nutrient deficiency, chemical damage and light can cause tuber cracks, skin discolouration, bruising or enlarged lenticels (Fiers et al. 2010). Some studies have suggested that irregular polygonal lesions on potato tubers, similar to the corky crack symptom, are associated with high moisture content or excess organic matter in the soil (Hart 1971; Sexton 2003). Fiers et al. (2010) reported that high moisture and a physiological reaction of the plant could be the cause of the irregular polygonal symptom on potato tubers and not the pathogens themselves. Tuber growth cracking is in fact due to water stress and can be characterized by the bursting of the periderm to form deep cracks that extend lengthwise on the tuber (Hiller et al. 1985; Begum et al. 2018). Begum et al. (2018), reported that the plant experiences water stress when the soil tension exceeds 20 kPa. Furthermore, atypical blemishes offer entry points for pathogens that may lead to more severe disease symptoms (Nærstad et al. 2012). This suggests that abiotic factors play a role in symptom development. Under such circumstances, it is prudent to speculate that corky cracks may have more than one causal agent from both microbial and abiotic origin.

1.2 Objectives of this research

The present study was aimed at determining the role of microbial factors and water stress in causing corky crack blemishes on potato tubers from different potato growing regions in South Africa. Knowledge about the microbial and abiotic factors causing potato tuber blemishes in South Africa is important in assisting the development of science-based management decisions to improve potato tuber quality and grower's profitability, livelihoods and ultimately food security.

Specifically, the aims were:

(i) To isolate micro-organisms associated with corky crack blemishes on potato tubers in South Africa

(ii) To determine the pathogenicity of isolated micro-organisms on potato tubers, to fulfil Koch's postulates

(iii) To determine if PVY is associated with corky cracks on potato tubers

(iv) To investigate the association between water stress factors and the occurrence of corky crack blemishes on potato tubers

1.3 Chapter outline

Research in this dissertation is presented in the following chapters:

Chapter 1: An introduction and background related to the topic of study, presenting the research aims

Chapter 2: A review of corky crack blemishes on potato tubers. This review outlines the theoretical framework and identifies gaps in research areas. The literature review discusses the host, origin, potato production and distribution. Furthermore, potato diseases, synergistic interactions, tuber blemishes, and specifically, corky crack blemishes were reviewed.

Chapter 3. Characterization of two microbial causal agents *Rhizoctonia solani* and *Streptomyces* species associated with corky crack blemishes on potato tubers in South Africa. Experiments were carried out on the *Rhizoctonia* species and *Streptomyces* species associated with corky crack blemishes on potato tubers. Pathogenicity tests were conducted to fulfil Koch's postulates.

Chapter 4: Characterization of the all microorganisms isolated from corky crack blemishes on potato tubers in South Africa, using morphological and molecular methods. Furthermore, the detection of PVY was also conducted to describe the association of the virus with the corky crack symptom in South Africa. Description of the isolated causative agents of corky crack blemishes on potato tubers in South Africa. Experiments were carried out on the microorganisms associated with corky crack blemishes on potato tubers. Pathogenicity tests were conducted to fulfil Koch's postulates.

Chapter 5: A general discussion, concluding the investigations and studies done on corky crack blemishes on potato tubers in South Africa, with suggestions for future research and implications for the potato industry.

1.4 Conclusion

In summary, the investigations conducted in this dissertation will provide considerable information regarding corky crack blemishes in South Africa. The findings of this work will contribute to an increased understanding of potato disease management. Results will also have implications for seed certification schemes and the broader potato industry, as currently there is uncertainty regarding the causes of this symptom. Therefore, this work will contribute significantly to the potato tuber blemish discourse and prevent severe economic losses and ultimately ensure food security.

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Chapter 2

A review of corky crack blemishes on potato tubers

2.1 The Host: *Solanum tuberosum*

Potato (*Solanum tuberosum* L.) is a crop of economic importance (Lutaladio and Castaldi 2009; Nærstad et al. 2012) and is considered the most important dicotyledonous source of human food globally (Hooker 1981). Potato is the world's fourth most consumed food crop after wheat (*Triticum aestivum* Linn.), maize (*Zea mays* Schrader) and rice (*Oryza sativa* Köhler) (Rowe 1993; FAOSTAT 2017). Nutritionally, potato provides a balanced source of starch, high-quality proteins, vitamins, trace elements and dietary fibre to many communities globally (Black 2008). Not only are potatoes used for human consumption, but they are also used for animal feed as well as the production of alcohol and starch (Black 2008; FAOSTAT 2017).

2.2 Origin

The origin of wild potatoes lies in the Andes Mountains of South America, which includes Columbia, Peru, Venezuela, Argentina, Bolivia, Ecuador and central Mexico. However, these wild potatoes are also widely distributed in areas of Chile and the United States of America (USA) (Stevenson et al. 2001). Cultivated species of potato can also be found within these areas (FAO 2008). In the mountains of South America, potatoes served as a staple food for the native people for thousands of years (FAO 2008; Spooner et al. 2014). Around 8000 BC to 5000 BC, the Inca Indians from Peru were the first to cultivate potatoes (Spooner et al. 2014). Due to the Spanish invasion of the Inca Empire in 1532, potatoes were introduced to the rest of Europe and have since been spread around the world to become a staple crop in many countries worldwide (Black 2008; FAOSTAT 2017). The potato crop was introduced into East Africa in 1880 by German and British colonists and was used in North Africa to feed soldiers

during World War I (Black 2008). It was first grown in South Africa in 1830, however, it is not known how the crop was introduced into the country (NAMCCT and Trust 2017).

2.3 Potato production and distribution

The world is currently facing climate change, higher demands on natural resources and a food crisis. Food is a basic human need (Rowe 1993), which makes potato a priority crop to feed the increasing human population. The crop also plays an important role in ensuring food security in South Africa. According to the FAOSTAT (2017), 383 million metric tons of potatoes were produced worldwide in 2016. In South Africa, potatoes are rated as one of the most important staple foods (Potatoes South Africa 2018). Compared to grain crops, potatoes produce two to four times more food per hectare and use seven times less water (International Potato Center 2019).

In South Africa, potatoes are produced throughout the year and production is spread over 16 different geographical regions (Figure 2.1) with different climatic conditions and a wide range of soil types (Potatoes South Africa 2018). The potato growing regions are Limpopo, Eastern Free State, Western Free State, Sandveld, North West, Northern Cape, KwaZulu-Natal, Mpumalanga, South Western Free State, North Eastern Cape, Gauteng, Ceres, Loskop Valley, Eastern Cape, Southern Cape and South Western Cape (Potatoes South Africa 2018). Potatoes are mainly produced under irrigation. Dryland production still occurs and is successful in some regions such as the Eastern Free State (Potatoes South Africa 2018).

Figure 2.1. Map showing the potato producing regions and approximate size of each in South Africa (Van der Waals et al. 2016).

Potatoes are grown globally under tropical, subtropical and temperate conditions (Black 2008). The crop is frost sensitive and therefore prefers temperate regions. It grows best in cool weather conditions, yielding optimally at temperatures between 18 °C and 20 °C (Steyn 2016). Temperatures above 30 °C and below 10 °C inhibit tuber growth, making temperature the main limiting factor to production. Different temperatures are required at various stages of the plant's lifecycle to ensure optimal production. The high yielding and significantly nutritious crop makes it a favourable crop in ensuring food security for the increasing population. In South Africa, the climate and topography are not ideal for producing potatoes, therefore, potatoes are produced under temperature stress due to long days and the high-intensity photoperiods in the country (Franke et al. 2013). This stress makes the plant more susceptible to plant pathogens, thus leading to yield losses. However, due to the diversity of microclimates and soils across the country, farmers are able to produce potatoes throughout the year (Franke et al. 2013; Steyn 2016).

Potatoes grow best in soils that are at least 600 mm deep and that are well-drained, such as sandy soils, with a pH of 5.2-6.4 (Black 2008). Compacted soil with poor aeration can negatively affect tuber development as the tubers will not have access to enough oxygen. Potatoes have a poorly developed and shallow root system, making it necessary to provide adequate amounts of water throughout the growth stages. Tuber yields are affected and malformation can occur even with short periods of water stress (Steyn 2014).

The warm climate and water scarcity in South Africa also make potato production in the country sub-optimal. However, commercial potato farmers in the country are able to compensate for water scarcity through the use of irrigation (Steyn 2014). Potatoes are planted from August to January in frost-prone areas and from February to June in frost-free areas (Potatoes South Africa 2018).

Different potato cultivars have different growing periods. Cultivars with short growing periods are harvested approximately 90 days after planting, medium growing cultivars are harvested after approximately 110 days after planting and long growing cultivars are harvested approximately 150 days after planting (Black 2008). Harvesting occurs when the above-ground parts of the plant appear to be yellow, which indicates that the plant has reached its full maturity. The potato vines are killed prior to harvesting to facilitate the maturation process of the tubers.

Once potatoes are harvested, they can be used for various purposes, including use as a home-cooked vegetable, processing for potato chips and French fries, livestock feed for chicken, pigs and cattle (FAO 2008), processed for starch where, for example, the fermentable sugars are used in the distillation of alcoholic beverages such as akvavit, poitin and vodka (OECD 2018) or retained as seed potatoes. Potato starch, which is 100% biodegradable, can be used for glue, fuel-grade ethanol, paper, wood and in textile industries as a texture agent, adhesive binder and filler (FAO 2008; OECD 2018).

2.4 Potato diseases

The potato crop, as with many other agricultural crops, is susceptible to devastation by various diseases caused by bacteria, fungi, viruses, nematodes and insects, which can cause damage to all parts of the plant (Stevenson et al. 2001; Black 2008). The potato industry faces several challenges concerning disease pressure that negatively affects the quality of tubers. Potato is known to be susceptible to more than 100 pathogens, of which more than 40 are soilborne pathogens (Fiers et al. 2010; Gherbawy and Gashgari 2013). Soil-borne diseases primarily affect the tubers, which are the most economically important part of the plant. Blemishes, rots and deformation are some of the tuber symptoms caused by soilborne pathogens (Fiers et al. 2012). Other symptoms of soil-borne diseases include leaf rolling, wilting, chloroses and necroses that appear on the aerial parts of the plant. Light brown lesions, stem piths and watery lesions can appear on the stems and roots.

Fungi are considered to be the most important group of plant pathogens (Agrios 2005; Fiers et al. 2012) and they cause the greatest damage on potatoes (Fiers et al. 2012). There are many different genera of fungi that attack potato and at least 14 fungal diseases have been identified on potatoes thus far (Stevenson et al. 2001; Steyn 2014). There are several economically important fungal diseases causing various symptoms on potato. These include powdery scab caused by *Spongospora subterranea* (Wallr.) Lagerh. f. sp. *subterranea* Toml. (Merz 2008), black scurf and stem canker caused by *Rhizoctonia solani* (Kühn) (Woodhall et al. 2008), *Fusarium* dry rot and wilt caused by *Fusarium* species (Link) (Peters et al. 2008), brown spot and black pit caused by *Alternaria alternata* (Fr.) Keissl. (Van der Waals et al. 2011), early blight caused by *Alternaria solani* Sorauer (Van der Waals et al. 2004), late blight caused by *Phytophthora infestans* (Mont.) de Bary (Nowicki et al. 2012), potato early die caused by *Verticillium dahliae* Kleb (Rowe 1987), black dot caused by *Colletotrichum coccodes* (Wallr.) Hughes (Tsror 2004) and silver scurf caused by *Helminthosporium solani* Durieu & Mont. (Denner et al. 1997).

There are some bacteria that cause various diseases on potatoes, such as rotting of stems and tubers as well as wilting of leaves and stems both in storage and in the field (Black 2008). These include blackleg and soft rot caused by *Pectobacterium* and/or *Dickeya* species (Czajkowski et al. 2011), bacterial wilt caused by *Ralstonia solanacearum* Smith (van Elsas et

al. 2000) as well as common scab and netted scab caused by *Streptomyces* species (Lambert and Loria 1989).

Potato diseases caused by viruses generally cause foliar symptoms such as mosaic, dwarfing, leaf distortion, leaf and vein necrosis, leaf rolling and crinkling. However, viruses such as potato virus Y (PVY), potato mop-top virus (PMTV), tobacco necrosis virus (TNV) and tobacco rattle virus (TRV) can cause blemishes and internal discolouration of the tubers (Black 2008; Fiers et al. 2012). There are nine significant potato viruses that are found in South Africa (Black 2008). With regard to crop losses in the country, the most severe viruses, monitored by the potato certification scheme are PVY, potato leafroll virus (PLRV) and tomato spotted wilt virus (TSWV) (Thompson and Strydom 2003). These viruses spread via infected host plants and tubers that remain in the soil or by insects, such as aphids that puncture the plant tissue and transmit the virus (Black 2008). Aphids are known to be the main source of dissemination of viruses and can lead to the spread of viruses to seed and table potatoes, therefore, reducing yield and quality (Black 2008). The use of infected propagation material in cultivation, incorrect farming methods and lack of vector control can introduce viruses into potato plants (Visser and Bellstedt 2009). Thus, regular monitoring and control of vector populations are necessary to avoid serious damage to foliage caused by viruses and prevent further yield losses.

PVY is an important potato pathogen in South Africa (Visser and Bellstedt 2009). It is a member of the *Potyvirus* genus and *Potyviridae* family and is the most widespread virus infecting potatoes and other crops such as tomato, pepper and tobacco. It was first classified into three strains namely PVY^{C}, PVY^{O} and PVY^{N} with different degrees of pathogenicity (Cuevas et al. 2012; Visser et al. 2012). The most economically important strains are known to be recombinant and are, therefore, likely to impact the evolution of the PVY population. These include PVY^{NTN} and PVY^{N-W} (Cuevas et al. 2012). Visser and Bellstedt (2009), established that PVY^{O}, PVY^{C}, PVY^{N}, recombinant PVY^{N}/PVY^{O}, PVY^{N-Wi} and PVY^{NTN} strains infect South African potatoes.

2.5 Synergistic interactions and disease complexes

Plant diseases are often thought to be caused by one pathogenic species or by a specific strain (Gest 2004). However, in nature, microbes form complex multispecies communities that have also been reported to cause diseases on plants (Lamichhane and Venturi 2015). Pasteur in the 1800s demonstrated that diseases can be due to the synergistic interactions of different microbes (Gest 2004; Le May et al. 2009). Nonetheless, most studies have focused on single microbial strains capable of growing in pure culture in artificial culture media. This has seemingly excluded other pathogens that cannot or are extremely difficult to culture. Hence, full information and knowledge of possible inter-kingdom or inter-species interactions of pathogens in nature are obscured. Several human and animal diseases have now been recognized to be due to synergistic interactions of different pathogens, increasing the complexity of the disease and diagnosis as well as the development of new control measures (Peters et al. 2008; Singer 2010). However, in plants, there are only a few reports of synergistic infections and most disease epidemics are still considered to be caused by a single microbial pathogen (Fitt et al. 2006). The mechanisms of synergistic interactions are largely unknown.

In recent years, there has been some evidence of the synergistic interactions between different pathogens in causing plant diseases and the understanding of multispecies synergistic interactions is an emerging area of research (Short et al. 2014). Until recently, only a few studies reported that plant species can be infected by more than one pathogen concurrently (Martin et al. 2013; Dung et al. 2014). In such cases, interestingly, the inoculation of a single microbe was not successful in inducing the disease, whereas, co-infection with a different microbe led to disease development (Fitt et al. 2006). In pathogenicity studies, mono-species are usually used to evaluate disease occurrence, because knowledge of synergism between multiple pathogens in causing disease is poor. It is possible that pathogen interactions can lead to more severe disease symptoms (Begon et al. 1986; Le May et al. 2009) and it is, therefore, important to include all pathogens involved in causing disease in pathogenicity tests to be able to develop effective control measures.

According to Lamichhane and Venturi (2015), there are two possible ways in which plant pathogens can interact. The interaction can be between multiple plant pathogens in the same phylum or species or different phyla. It is hypothesised that synergism between multiple

pathogens increases the disease severity leading to more serious disease symptoms (Begon et al. 1986; Fitt et al. 2006; Liu et al. 2019). The study of synergistic interactions of potato pathogens in causing disease may lead to a better understanding of the disease epidemiology and in turn, suggest solutions to better disease management.

2.5.1 Fungi-fungi

Synergistic interactions between various fungal pathogens have partially been studied by microbiologists and plant pathologists. A number of fungal disease complexes have been described, however, not much of this research has been done on potatoes. An example is the genus *Alternaria* that consists of several species complexes (Woudenberg et al. 2015). It is known that *A. alternata* and *A. solani* are the two most predominant *Alternaria* species infecting potato and have a significant impact on potato quality and tuber yield (Woudenberg et al. 2015). The known *Alternaria* disease complex includes *Alternaria tenuissima* (Nees) Wiltshire, *Alternaria dumosa* E.G. Simmons, *Alternaria arborescens* E.G. Simmons, *Alternaria infectoria* E.G. Simmons, *Alternaria arbusti* E.G. Simmons and *Alternaria interrupta* E.G. Simmons, which cause leaf blight on potatoes in Iran (Ardestani et al. 2010), Russia (Orina et al. 2012), the USA (Tymon et al. 2016) and China (Zhao et al. 2016). Recently, *Alternaria grandis* E.G. Simmons (Rodrigues et al. 2010) and *Alternaria protenta* E.G. Simmons (Woudenberg et al. 2015), as well as the *Alternaria arborescens* species complex (AASC), including *Alternaria geophila* Dasz., *A. arborescens, Alternaria cerealis* E.G. Simmons & C.F. Hill and *Alternaria senecionicola* E.G. Simmons & C.F. Hill have also been connected to the *Alternaria* complex affecting potatoes (Woudenberg et al. 2015).

2.5.2 Bacteria-bacteria

In bacteria-bacteria interactions in potatoes, it has been reported that *Candidatus* Liberibacter solanacearum (Lso) and *Candidatus* Liberibacter psyllaurous are associated with zebra chip disease as a complex in North America, where it was originally thought that only Lso was the causal pathogen (Wen et al. 2009). Tomato (*Solanum lycopersicum* L.), in the same family as potato, has eight bacterial species known to cause tomato pith necrosis. The bacterial pathogens, *Pseudomonas cichorii* (Paula Wilkie and Dye 1974), *Pectobacterium carotovorum*

(Dhanvantari and Dirks 1987), *Pectobacterium atrosepticum* (Gardan et al. 2003), *P. viridiflava, Dickeya dadantii* (Alivizatos 1986; Samson et al. 2005), *Pseudomonas mediterranea, P. corrugata* (Moura et al. 2004) and *P. fluorescens* (Saygili et al. 2008), can cause tomato pith necrosis either alone or with other bacterial species, thus making this disease complex a good example of synergistic interactions and co-infection among multiple bacterial pathogens. It has also been reported that *Pseudomonas corrugata* and *P. marginalis* or *P. corrugata* and *P. mediterranea* cause severe infection together but not individually (Moura et al. 2004; Kůdela et al. 2010).

2.5.3 Bacteria-fungi

There are a few reports of mixed interactions between fungi and bacteria related to plant disease complexes, involving more than one pathogenic microbial phylum (Lamichhane and Venturi 2015). Such interactions involve synergism between fungal and bacterial pathogens. Bacteria and fungi co-inhabit a wide variety of environments. Their interaction with one another can influence their survival, pathogenesis and colonization. It is also known that bacteria can provide fungi with compounds that enhance the production of fungal virulence determinants (Wargo and Hogan 2006). Such an example is the interaction between the bacterium, *R. solanacearum* causing bacterial wilt in a number of hosts including potato, and 34 species of fungi across three diverse taxa (Ascomycetes, Basidiomycetes and Zygomycetes). This bacterium was found to produce chlamydospores that induced morphological differentiation amongst the fungal taxa, which were previously not known to produce chlamydospores. A lipopeptide of *R. solanacearum* induces chlamydospore development in fungi and facilitates bacterial entry into fungal tissues, which may, in turn, cause disease (Spraker et al. 2016). These chlamydospores have thickened cell walls that enhance the persistence of soil-borne fungi, providing survival benefits (Crone et al. 2013). It was speculated that the endofungal colonization benefits *R. solanacearum* survival in the soil in the absence of a plant host and that the bacterium holds the accumulated lipids in chlamydospores (Spraker et al. 2016).

Dung et al. (2014) conducted a study to examine the role of co-infection of potatoes by *Verticillium dahliae* Kleb. and *Pectobacterium* species, a disease complex, which causes potato early dying disease (PED). The disease is known to cause PED symptoms as well as aerial stem rot of potato. No significant synergistic effects were observed following co-inoculation

between the fungus and bacteria; however, infection rates and premature senescence were greater in co-inoculated host plants compared to single pathogen inoculated plants. It has been noted that when these two pathogens are present together, their destructive potential increases compared to their independent effects (Zink and Secor 1982; Rowe et al. 1987). These results indicate that even though co-infection by *V. dahliae* and *Pectobacterium* species does not always result in significant synergistic interactions on potato, the co-infection still increases disease severity (Dung et al. 2014). Other reports demonstrate the association of bacteria and fungi, however, the mechanisms of interaction that result in communication and synergism of the pathogens in these disease complexes are not well described (Lamichhane and Venturi 2015).

2.5.4 Virus-virus

It is known that when two independent viruses infect a host plant, both viruses can produce an infection whereby neither virus is negatively affected by the other (Scheets 1998; Zhou et al. 2017). Synergisms between multiple plant pathogenic viruses are known to increase the severity of the disease. No potato virus complexes have been reported; however, the best-studied virus synergistic interaction involves PVY and potato virus X (PVX) in tobacco (Goodman and Ross 1974; Scheets 1998; González-Jara et al. 2004). The mechanism of infection has been reported by Bance (1991) and Pruss et al. (1997), whereby the infection of tobacco plants by both PVY and PVX produces venal necrosis disease in the leaves. The study demonstrated that the PVX accumulation in leaves co-inoculated with PVY increased up to 10-fold compared to plants infected with a single virus, while PVY levels remained the same. The PVX (-) strand RNA concentration increased disproportionally, three times higher, in infected cells than the PVX (+) RNA strand. The experiment indicated that the levels of both viruses increased in co-infected plants to the same extent as the level of PVX particles, explaining that PVY induced a change in the regulation of PVX replication. Furthermore, the PVY/PVX synergism was observed to resemble the synergistic interaction between three other members of the potyvirus group: tobacco vein mottling virus (TVMV), pepper mottle virus (PepMoV) and tobacco etch virus (TEV), for both the increase in disease severity as well as the change in PVX replication (Vance et al. 1995).

It was later found that the increase and accumulation of PVX is mediated by the potyviral 5' proximal sequences encoding P1 and helper component proteinase (HC-Pro), which also functions as a pathogenicity enhancer and activator of replication of other independent viruses, such as the Cucumber mosaic virus (CMV) and Tobacco mosaic virus (TMV). The PVY/PVX synergism, therefore, indicates that the expression of the P1/HC-Pro gene product, excluding the RNA sequence itself, is sufficient to induce the increase of PVX pathogenicity and suggests a mechanism of transactivation of viral replication (Pruss et al. 1997).

Despite these examples of synergisms between multiple plant viruses, the role of vectors in transmitting the viral complexes is unknown. Further research should be done to identify whether different viruses are transmitted by the same vector and whether they are transmitted at the same time to the host plant. This would facilitate the development of effective control measures of these virus complexes.

2.6 Tuber blemishes

Potato tuber blemishes constitute a persistent and quality problem in the production of potatoes throughout the world (Fiers et al. 2010). Tuber blemishes are known not only to be caused by pathogens and pests but also abiotic factors (Bouchek-Mechiche et al. 2013). They affect the tuber skin and can cause severe loss in yield, rejection of seed batches, the downgrading of potatoes on the market or rejection of potatoes for processing (Tsror 2010; Fiers et al. 2012). The demand for washed potatoes by South African consumers increases the problem of revealing blemishes on the tuber surface. Potato blemishes, therefore, pose an economic threat to farmers and cause downgrading of potato tubers on the fresh produce market or rejection of seed lots in South Africa. Farmers must optimize their disease management strategies to avoid the loss of yield and downgrading of tubers on the market or as seed (Jordaan and van der Waals 2015).

Skin blemishes that occur on potato can be characterized into two groups, namely: Typical blemishes, where the causal agent(s) is known; and atypical blemishes, where the causal agent(s) is unknown (Fiers et al. 2010). Typical blemishes are manageable in contrast to atypical blemishes (Zimudzi et al. 2017). Although considerable research has been done on

determining the causative agents of typical potato tuber blemishes, the proposed work will focus on an atypical blemish and contribute significantly to the potato industry. It is important to prevent severe economic losses by studying and determining the responsible causal agent(s) of the occurrence of specific atypical blemishes on potatoes in South Africa.

2.6.1 Typical blemishes

Potato blemishes that result from a pathogenic origin or an unfavourable environment, where the causal agent has been identified, the symptom nomenclature is in agreement and Koch's postulates fulfilled are known as typical blemishes. Typical blemishes resulting from biotic factors are caused by fungal, bacterial, viral or nematode pathogens (Fiers et al. 2010). Fungal and bacterial pathogens causing typical blemishes include *R. solani*, *C. coccodes*, *S. subterranea* f. sp. *subterranea*, *H. solani*, *Fusarium* species and *Streptomyces* species (Nærstad et al. 2012). These pathogens cause various symptoms on potato skin, including black scurf, black dot, powdery scab, silver scurf, dry rot and common scab respectively. These blemishes are well studied and contribute to a major reduction in quality and economic losses (Fiers et al. 2010; Nærstad et al. 2012). Tuber blemishes can also be caused by nematodes and viruses (Fiers et al. 2010; Yellareddygari et al. 2018). Rapid diagnosis of causal agents of blemishes is easier when they have previously been associated with well-documented symptoms (Zimudzi et al. 2017).

2.6.2 Atypical blemishes

Potato tuber blemishes for which the causal agent has not been identified are referred to as atypical blemishes (Fiers et al. 2010). Correct identification of the causal agent of a disease is crucial to successfully control the disease. Incorrect identification of a causal agent of a disease can lead to incorrect control recommendations and further yield losses (Wang et al. 2012). Atypical blemishes include corky cracks, star-like corky lesions, corky spots or 'rhizoscab' and irregular polygonal sunken corky lesions (Fiers et al. 2010; Gush et al. 2019). Recent studies have suggested that the most frequently isolated pathogens from these atypical blemishes fall in the genera *Rhizoctonia*, *Fusarium*, *Alternaria*, *Penicillium*, *Clonostachys* and *Streptomyces*

(Fiers et al. 2010; Gouws and McLeod 2012; Muzhinji et al. 2015; Zimudzi et al. 2017). In one study, Fiers et al. (2010) found that inoculation of potatoes with these pathogens singly did not result in the reproduction of atypical blemishes, whereas control tubers produced atypical blemishes. It is, therefore, hypothesised in the present study, assuming the causal agent is of biological origin, that a consortium of pathogens or a single pathogen aided by certain environmental factors is responsible for causing atypical blemishes instead of a single pathogen as previously proposed.

2.7 Corky crack blemishes

In South Africa, there has been an increase in the incidence of various atypical blemishes, in particular, 'growth cracks' and 'corky cracks' on the surface of potato tubers (Muzhinji et al. 2014). The occurrence and causal agents of these tuber blemishes on potato have been one of the most contentious issues in the potato industry in South Africa (Gouws and McLeod 2012; Muzhinji et al. 2014) and other parts of the world (Fiers et al. 2010). However, studies about the causal agents of tuber blemishes are starting to accumulate, with various *R. solani* anastomosis groups (AGs) predominantly implicated as the causal agents of corky cracks on potato tubers (Figure 2.2.a and 2.2.b) in South Africa (Muzhinji et al. 2014; Zimudzi et al. 2017) and elsewhere (Fiers et al. 2010; Bouchek-Mechiche et al. 2016)

Gouws and McLeod (2012), identified *Streptomyces* species from 'fissure scab' symptoms on potato tubers (Figure 2.2.c), symptoms similar to corky cracks caused by *R. solani* AG-3PT (Figure 2.2a.); however, Koch's postulates were not fulfilled. It is known that *Streptomyces* species cause common scab on potatoes worldwide with variable symptoms of brown, circular, corky lesions. Interestingly, these symptoms were observed on the potato cultivar Mondial, which is known to be tolerant to common scab (Jordaan and Van der Waals 2016). Gush (2019) isolated *R. solani* AG 2.2IIIB and non-pathogenic *Streptomyces* species from corky cracks. On the other hand, Campion et al. (2003) and Fiers et al. (2010) observed corky crack symptoms in both their experimental and control treatments. Under such circumstances of an unknown causal agent, it is plausible to speculate that corky cracks may have more than one causal agent. Therefore, further studies should be done to consider all pathogens isolated from corky cracks as possible causal agent(s) of these atypical corky crack blemishes. Due to the high demand for

healthy unblemished potatoes, it is important to determine the causal agents of these atypical blemishes on tubers, to advise growers on the correct management strategies.

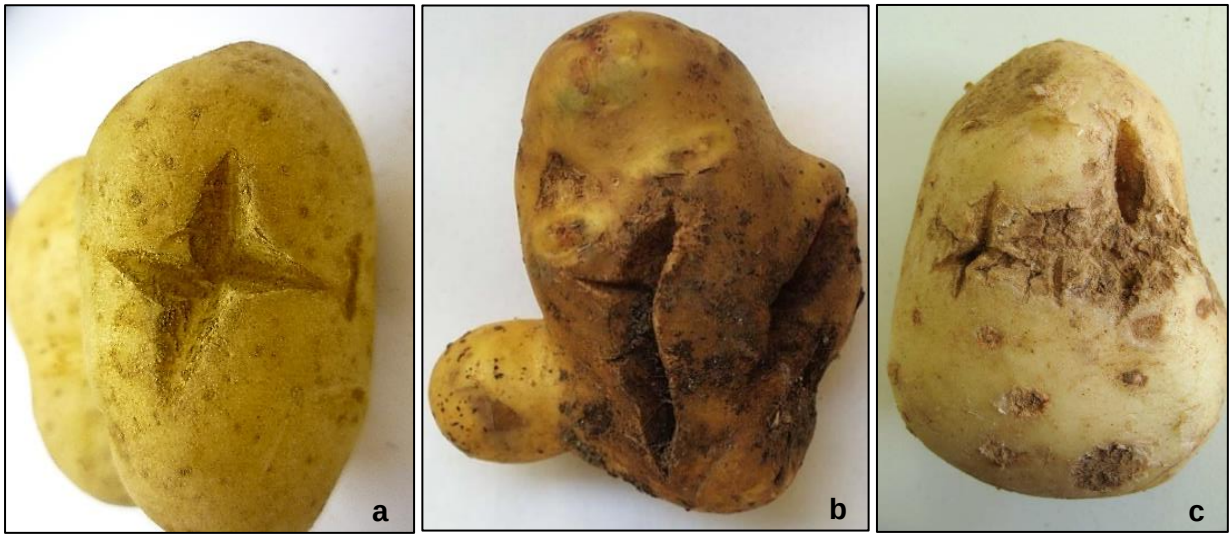

Figure 2.2. Potato tubers showing symptoms of (a) elephant hide and growth cracking caused by *Rhizoctonia solani* AG 3-PT (Muzhinji et al. 2014), (b) deformation and growth cracking caused by *R. solani* (Bouchek-Mechiche et al. 2016) and (c) fissure scab caused by an unknown *Streptomyces* species (Gouws and McLeod 2012).

Figure 2.3. Potato tubers sampled in different regions in South Africa showing symptoms of corky cracks (Pictures: S. Gush).

2.7.1 Biotic causes associated with corky crack blemishes

2.7.1.1 *Rhizoctonia* species

Rhizoctonia spp. (Basidiomycota) are saprophytes, pathogens and mycorrhizal symbionts of many plant species and cause various plant diseases (Sneh et al. 1991). On potatoes, *R. solani* is responsible for causing black scurf and stem canker (Woodhall and Lees 2004). *Rhizoctonia solani* develops sclerotia on the progeny tubers (black scurf) as well as brown sunken lesions (cankers) on the stolons, stems and roots of the plant (Banville 1989). Furthermore, severe symptoms of infection on tubers include deformation, cracking, elephant hide and pitting, leading to poor quality and downgraded tubers (Campion et al. 2003; Muzhinji et al. 2014).

Rhizoctonia solani is a soil-borne and tuber-borne pathogen and is therefore difficult to manage in the production of potatoes (Muzhinji et al. 2015). The fungus is transmitted via infected seed tubers, providing a long-distance dispersal mechanism. Sclerotia and mycelia are produced by the pathogen once established in the soil, providing another source of primary inoculum. It is taxonomically complex, consisting of 13 anastomosis groups (AGs), based on their hyphal interaction, and differ morphologically, ecologically, in pathogenicity and host range (Sneh et al. 1991; Sharon et al. 2006). It is commonly known that *R. solani* AG-3 subgroup PT is the main AG infecting potatoes (Banville 1989) and is accepted that *R. solani* AG-3PT is commonly associated with tubers infected with black scurf. However, various other AGs have also been found to cause disease on potatoes, albeit at lower frequencies namely AG2-1 causing stem canker and black scurf (Carling and Leiner 1990; Woodhall et al. 2008), AG-4 causing root, stolon and tip burning (Anguiz and Martin 1989; Balali et al. 1995), AG-5 causing black scurf and stem canker (Anguiz and Martin 1989; Campion et al. 2003; Truter and Wehner 2004; Woodhall et al. 2008) and AG-7 infecting roots and shoots (Carling et al. 1998; Abd-Elsalam et al. 2009) and AG-8 infecting roots (Woodhall et al. 2008). These AG groups are isolated from atypical tuber blemishes referred to as corky or scabby deformed lesions (Campion et al. 2003). *R. solani* AG-7 and AG-9 are rarely found on potatoes (Carling and Leiner 1990). In addition to *R. solani* AG-3PT, *R. solani* AG 2-2IIIB, AG 4HG-I, AG 4HG-III and AG 5 have recently been isolated from different *Rhizoctonia* disease symptoms on potatoes in South Africa (Muzhinji et al. 2015).

The *Rhizoctonia* genus is difficult to identify into AGs, due to the similar morphological characteristics and can lead to confusing nomenclature. Culture-based methods for identification are difficult and time-consuming; therefore, taxonomists now rely on molecular techniques such as Polymerase Chain Reaction (PCR) that use specific primers for amplification of the DNA and sequencing to effectively identify the pathogen of interest (Flores-González et al. 2008; Sharon et al. 2008).

2.7.1.2 Binucleate *Rhizoctonia* species

The *Rhizoctonia* genus is classified into three groups namely uninucleate, binucleate and multinucleate based on the number of nuclei per cell (Ogoshi et al. 1983). Binucleate *Rhizoctonia* (BNR) differ morphologically from *R. solani* by the presence of non-sporulating, binucleate somatic hyphae, *Rhizoctonia*-like anamorphic phases and parasitic teleomorphic phases. BNR produces fruiting bodies with basidia produced from the basal hyphae (Sharon et al. 2008). BNR are grouped into 21 anastomosis groups and subgroups (Sharon et al. 2008), where AG-A (Muzhinji et al. 2015), AG-R (Muzhinji et al. 2015), AG-W (Yang et al. 2014) and an unknown BNR (Woodhall et al. 2011) have been identified on potatoes. BNR has recently been associated with several diseases on various plants. These include, BNR AG-A, AG-K, AG-U and AG-F being pathogens of several crops in China (Yang et al. 2014; Yang et al. 2015), unknown BNR causing elephant hide on potato tubers in Great Britain (Woodhall et al. 2011) and BNR AG-A causing disease in potato stems and sugar beetroots in the Pacific Northwest (Miles et al. 2013). BNR AG-A and AG-R were reported to be causal pathogens of black scurf and stem canker on potato in South Africa (Muzhinji et al. 2015) and in a recent study, Zimudzi et al. (2017) described the first report of BNR AG-A causing tuber malformations, which include linear and circular defects.

2.7.1.3 *Streptomyces* species

Streptomyces species (Actinomycetes) are saprophytic, filamentous, pathogenic soil-borne bacteria and are pathogens on many plant species. *Streptomyces scabies* and other pathogenic *Streptomyces* species cause common scab, an economically important disease on potato tubers (Jordaan and van der Waals 2015). However, several hundred *Streptomyces* species can infect

growing potato tubers as well as various other underground developing plants (Braun et al. 2017). According to Labeda (2016) *S. scabiei*, *S. acidiscabies* and *S. turgidiscabies* are the best-classified scab-causing species in the genus. Jordaan and van der Waals (2015) also include *S. europaeiscabiei* as a scab-causing species.

Due to genetic differences between potato varieties, commercially grown varieties differ in susceptibility towards common scab (Goth et al. 1993; Haynes et al. 1997; Wanner 2006, 2009). The interactions between *Streptomyces* species, the host and environmental factors are complex, resulting in unpredictable disease symptoms, severity and incidence, making the disease complicated to study (Wanner 2009). Common scab is therefore caused by a *Streptomyces* species complex rather than a single species (Loria et al. 1997; Wanner 2009). Some reports suggest that the different *Streptomyces* species can be found within the same field and the same scab lesions have been studied to determine whether there is species relatedness towards the specific symptoms observed as well as the sensitivity of the species to various environmental factors such as soil pH and moisture (Bouchek-Mechiche et al. 2000; Aittamaa et al. 2008; Wiechel and Crump 2010; Jordaan and van der Waals 2015). The pathogenic ability of *Streptomyces* species involves the horizontal gene transfer of its virulence genes including *txtAB*, *nec1* and *tomA*, located on the pathogenicity island. Over the years, there has been an increase in the number of identified pathogenic *Streptomyces* species, making the disease difficult to understand (Kers et al. 2005; Loria et al. 2006; Wanner 2009). In South Africa, there are no cultivars resistant to common scab, making disease management difficult (Jordaan and van der Waals 2015).

2.7.1.4 Abiotic causes associated with corky crack blemishes

The effects of environmental factors and climate change on plant diseases have been the subject of debate for many years (Das et al. 2017). Numerous plant diseases are likely to increase due to the effect of climate change, therefore posing a risk to sustainable crop production (Visser and Bellstedt 2009; Mahato 2014). Factors such as temperature, humidity, nutrient deficiency, chemical products, irrigation/rainfall, soil type, soil moisture, fertilizer and soil pH are some of the most important factors that have an impact on crop production and the development of plant disease. These factors can cause the enlargement of tuber lenticels, tuber cracking, skin discolouration and bruising (Fiers et al. 2010). Various research studies have been done on the

effects of the environment on the development of plant diseases. However, more information is needed on specific plant pathogens and their associated diseases on host crops to understand their adaptive mechanisms against the changing environmental conditions (Mahato 2014).

It is known that water stress conditions cause potato tuber splitting, which is similar to growth crack symptoms (Agrios 2005). Fiers et al. (2010), postulated that irregular polygonal sunken corky lesions could be due to the effect of environmental stress conditions on the plant. In their study, it was found that single isolates of *Fusarium*, *Alternaria*, *Rhizoctonia*, *Penicillium*, *Clonostachys* species and various strains of *Streptomyces* did not allow the reproduction of atypical blemishes in single inoculations, whereas control tubers produced atypical blemishes. This suggests that environmental factors could play a role in symptom development, where the atypical blemish is a reaction to unfavourable environmental conditions. The complexity of the interactions between a pathogen and its host, influenced by biotic and abiotic factors of the environment, makes it difficult to decipher causal agents responsible for the occurrence of disease, such as corky cracks.

Viruses like PMTV and PVY have also been implicated in causing tuber corky cracks (Fiers et al. 2010), although this assertion lacks full direct empirical scientific evidence and symptom expression is highly dependent on the virus-cultivar interaction (Romancer et al. 1994; Visser et al. 2012).

2.8 Conclusion

Knowledge about the biotic and abiotic factors causing potato tuber blemishes in South Africa is important in assisting the development of science-based management decisions to improve potato tuber quality, grower's profitability, livelihoods, food security and reduce economic losses. The present study aims at determining the biotic causes and if water stress is a contributing factor to corky crack symptoms on potato tubers from different potato growing regions in South Africa. It is also taken into consideration that it is possible that a consortium of pathogens can cause disease, as a complex or as a result of unfavourable environmental conditions that lead to the respective atypical blemishes of corky cracks. The soil and environment can affect the interaction between a pathogen and its host and even between the

pathogens themselves (Fiers et al. 2012). It is therefore important to understand the complex interactions between pathogens, host and environment in order to develop effective control strategies to minimize losses due to diseases. The results from the proposed work may also have implications for the potato market and seed certification schemes, as currently there is uncertainty regarding the causes of the corky crack disease.

2.9 References

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Chapter 3

Consideration of two microbial causal agents of corky crack blemishes on potato tubers in South Africa

Abstract

Potato tuber blemishes are of economic importance in potato production worldwide. They reduce the quality of potato tubers destined for seed and fresh produce markets. The incidence of potato tuber blemishes such as 'corky cracks' and 'growth cracks' is on the increase in South Africa and other potato producing areas globally. The occurrence of corky cracks has been often attributed to *Rhizoctonia solani* and *Streptomyces* species. In an effort to confirm the causal agent of corky crack blemishes, fungal and bacterial species were isolated from potato tubers with corky crack symptoms sampled from different potato growing regions of South Africa. The isolated *Rhizoctonia* species and *Streptomyces* species were identified by morphological and molecular techniques. *Rhizoctonia solani* AG 2-2IIIB and *Streptomyces* species including *S. chrestomyceticus, S. flavoviridis* and *S. fradiae* were the most predominant species isolated from the corky crack symptoms. Pot trials with potatoes planted in soil inoculated with *R. solani* AG 2-2IIIB, the *Streptomyces* species complex alone and a combination of the two were done to confirm Koch's postulates. No corky crack symptoms were observed from any of the treatments; however, elephant hide symptoms were observed on progeny tubers from plants inoculated with *R. solani* AG 2-2IIIB. This is the first report of *R. solani* AG 2-2IIIB causing elephant hide symptoms on potatoes in South Africa. Higher disease severity of elephant hide was observed for co-inoculated plants, suggesting a possible synergistic interaction between pathogens. It is therefore important to focus on all pathogens involved in causing disease in pathogenicity tests to develop effective control measures.

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3.1 Introduction

Potato (*Solanum tuberosum* L.) is the world's most important non-grain food crop and is central to global food security. Plant pathogens cause quantitative and qualitative yield losses on potato (Fiers et al. 2012). The qualitative yield losses occur due to a decrease in the quality of potato tubers caused by tuber blemishes (Muzhinji 2014). Most of the causative agents of potato tuber blemishes are well known, for example, black scurf caused by *Rhizoctonia solani* (Kühn) (Woodhall et al. 2008), powdery scab caused by *Spongospora subterranea* (Wallr.) Lagerh. f. sp. *subterranea* Toml. (Merz 2008), black dot caused by *Colletotrichum coccodes* (Wallr.) Hughes (Tsror 2004) silver scurf caused by *Helminthosporium solani* Durieu & Mont. (Denner et al. 1997) and common scab caused by *Streptomyces* species (Lambert and Loria 1989).

However, there are atypical tuber blemishes on the surface of potato tubers that have been attributed to various causes and their occurrence is still a topic of extensive investigations. These potato tuber blemishes are often referred to by various descriptive names. Some studies have attributed corky crack blemishes on potato tubers to environmental conditions such as temperature and fluctuating moisture content of soil and further studies to other abiotic factors including high organic matter, high soil moisture content or fertilization (Fiers et al. 2010).

Recently in South Africa, there has been a rise in the occurrence of corky crack blemishes on potato tubers across the potato growing regions. Comprehensive investigations about the causes of these tuber blemishes have been initiated and are continuing (Muzhinji 2014; Zimudzi et al. 2017). Knowledge about the causative agents of tuber blemishes such as corky cracks is important to assist in the development of efficient control strategies to improve potato quality, production and profit for growers. The occurrence and causal agents of blemishes on potato are one of the most contentious issues in the potato industry in South Africa and other parts of the world. Nonetheless, studies about the occurrence and causal agents thereof in South Africa are starting to accumulate with *R. solani* AG 3-PT being the predominant pathogen isolated from corky cracks (Figure 1) on potato tubers (Muzhinji 2014; Zimudzi et al. 2017). However, in some instances, atypical blemishes have been observed on potato tubers with no *Rhizoctonia* infestation (Banville 1989; Campion et al. 2003; Fiers et al. 2010). Such atypical blemishes may have more than one causal agent, which makes it challenging to determine the pathogens causing the symptoms (Banville et al. 1996). It has been stated that these corky

cracks may not only be due to *R. solani* infection, but they have also been associated with *Streptomyces* species (Gouws and McLeod 2012), and more recently with Potato Mop Top Virus and Potato Virus Y infection (Brierley et al. 2016).

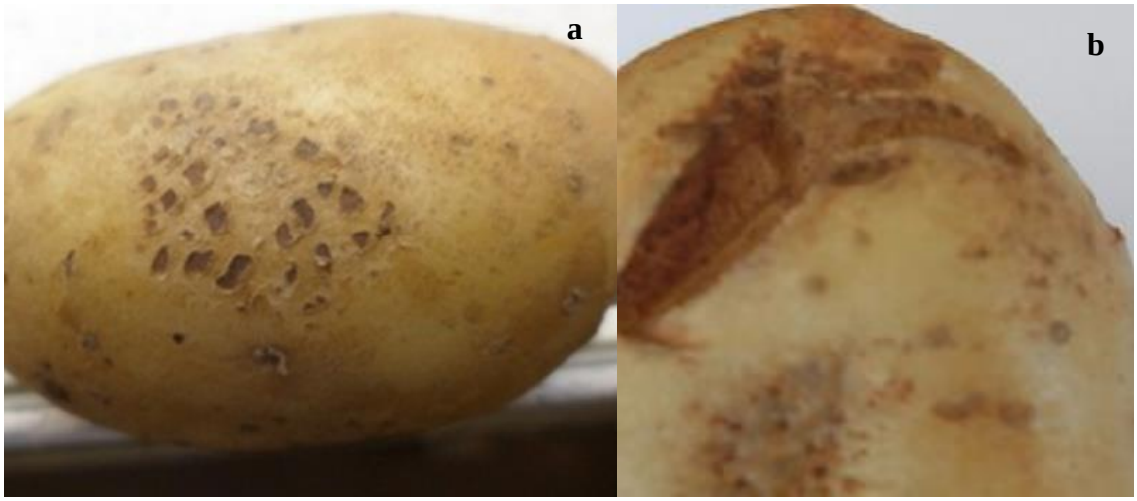

Figure 3.1. Potato tubers from the study conducted by Muzhinji (2014), showing different blemishes (a) elephant hide and (b) elephant hide and growth cracking caused by *Rhizoctonia solani* AG 3-PT.

A study by Fiers et al. (2010), also suggests that atypical blemishes could be due to the reaction of the plant to environmental stress conditions. In their study, they could only fulfil Koch's postulates for *R. solani* AG 3. No symptoms were obtained for other fungal or bacterial isolates. These included *Fusarium*, *Alternaria*, *Rhizoctonia*, *Penicillium*, *Clonostachys* species and various strains of *Streptomyces*. However, irregular polygonal sunken corky lesions were observed on non-inoculated tubers which suggest environmental factors also play a role in symptom development.

In another study, deep longitudinal cracks and corky-like lesions, referred to as fissure scab, were observed on tubers in South Africa and were reported to be due to an identified *Streptomyces* species (Gouws and McLeod 2012). It is known that *Streptomyces* species cause common scab on potatoes worldwide with variable symptoms of brown, circular, corky lesions. Interestingly, these symptoms were observed on the potato cultivar Mondial, which is tolerant to common scab. These symptoms appeared to be very similar to the elephant hide symptoms caused by *R. solani* AG 3-PT (Muzhinji 2014). Koch's postulates were proven for *R. solani*

AG 3-PT from the elephant hide symptom (Muzhinji 2014) but not *Streptomyces* species from the fissure scab lesions (Gouws and McLeod 2012) in South Africa. Therefore, further studies should be done to confirm the causal pathogen of the atypical corky crack blemishes.

Figure 3.2. A potato tuber showing a deep fissure scab lesion, caused by an unknown pathogenic *Streptomyces* species (Gouws and McLeod 2012).

The objective of this study was therefore to investigate the causative agents of corky cracks on potato tubers in South Africa with particular consideration of two biotic causal agents; *Rhizoctonia solani* and *Streptomyces* species. Since literature has associated corky crack blemishes with *R. solani* and *Streptomyces* species, this study focused on isolating these pathogens for further pathogenicity trials to confirm the causative agent.

3.2 Materials and methods

The fungal and bacterial species present on potato tuber blemishes were determined by systematic isolation and identified by morphological and molecular techniques. In order to confirm Koch's postulates, the pathogenicity of the isolates was investigated via pot trials and re-isolations. The pathogen optimal growth conditions, potato cultivar and environmental conditions were taken into consideration when performing pot trials. The environmental conditions experienced by the tubers in the field were simulated in the greenhouse. Single

inoculations, as well as co-inoculations, were performed to examine the synergistic interactions that occur.

3.2.1 Sample collection

Potato tubers showing corky crack blemishes were collected from three potato production regions of South Africa. Each batch of potato tuber samples was labelled with their specific geographic location and cultivar name (Table 3.1).

3.2.2 Isolation of pathogens from blemished tubers

3.2.2.1 Fungal isolation and morphological identification

Infected tubers were washed with tap water and dried. Small lesions of 5 mm in diameter and 5 mm deep were excised from infected tissue using a sterile scalpel and tweezers. The excised lesions were surface-sterilized for 2 minutes with 1% NaOCl and rinsed twice with sterile distilled water for 2 minutes each. The lesions were plated onto a 90 mm diameter Petri dish containing 1.5% water agar (WA, Biolab), modified with 50 mg/L streptomycin sulphate (Sigma-Aldrich). The agar plates were incubated for 48 hours at 25 °C. With the use of a compound microscope (100 X magnification), hyphal characteristics such as hyphae branching, hyphae diameter and septa at branches were examined, to preliminarily identify the fungal cultures based on morphological characteristics. Hyphal tips of cultures that conformed to fungal morphology of *Rhizoctonia* species were transferred to 90 mm diameter plates containing potato dextrose agar (PDA, Biolab), also amended with 50 mg/L streptomycin sulphate (Sigma-Aldrich) (Carling and Leiner 1990). The presence or absence of sclerotia and colony colour was examined after 14 days of incubation on PDA

Table 3.1. Potato tuber samples showing corky crack symptoms collected from three potato production regions of South Africa.

Region	Cultivar	Photo of symptoms
Eastern Free State	Mondial	
Marquard, Western Free State	Mondial	
Limpopo	Mondial	

3.2.2.2 Bacteria isolation and morphological identification

Infected tubers were washed with tap water and dried. Lesions of 100 mm^3 were excised together with the yellow coloured flesh of the potato tuber. Each lesion was macerated in a Bioreba bag (Labretoria) using a pestle. A volume of 2 ml of sterile distilled water was added to each bag and 200 µl of the extractant was added to 90 mm diameter plates containing WA (Biolab) and spread using a hockey stick (Loria et al. 2001). The plates were incubated in the dark for 3 weeks at 28 °C. Examination of the plates was performed at regular intervals during the 3 weeks of incubation until clear *Streptomyces* colonies were visible. Spores were identified with the use of a light microscope (400 X magnification). Colonies were transferred to plates containing yeast malt extract agar (YMEA, Biolab) and pure cultures were obtained.

3.2.3 DNA extraction and identification by Polymerase Chain Reaction (PCR)

3.2.3.1 Genomic DNA extraction

Isolates were sub-cultured on PDA for fungal cultures and YMEA for bacterial cultures and incubated for 7 days at 25 °C. Mycelia and bacterial colonies were scraped from plates and transferred to 2 ml Eppendorf tubes, respectively. Using the ZR Fungal/Bacterial DNA Miniprep extraction kit (Zymo Research Corporation), total genomic DNA was extracted according to the manufacturer's protocol.

3.2.3.2 PCR amplification and sequencing

Using the universal primers, ITS1-F (5'-CTTGGTCATTTAGAGGAAG-3') (Gardes and Bruns 1993) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) the ITS region of each *R. solani* isolate was amplified. These annealed to the flanking 18S and 28S rRNA genes. Amplification was conducted in 50 µl reaction mixtures that contained 4 ng of template DNA; 250 µM of dATP, dTTP, dGTP and dCTP (Bioline) each; 10 x NH_4 reaction buffer, containing 160 mM $(NH_4)_2$ SO_4, 670 mM Tris-HCL at pH 8.8 and 100 mM KCl (Bioline); 0.25 U of BIOTaq DNA polymerase (Bioline); 3 mM $MgCl_2$; and 0.2 µM of each primer. Amplification was carried out in a thermal cycler (Applied Biosystems, 2720) with an initial denaturation step at 95 °C for 3 min; followed by 35 cycles at 95 °C for 30 secs, 55 °C for 30 sec and 72 °C for 45 sec; and a final extension of 72 °C for 5 min.

For bacterial isolates, 16S rRNA gene amplification was performed by amplifying the 16S RNA gene region using primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach et al. 1992). The PCR was conducted in 25 µl reaction mixtures containing 5 u/µl Taq DNA polymerase (recombinant), 10 x NH_4 reaction buffer (Bioline), 2.5 mM dNTP mix (Bioline), 50 mM $MgCl_2$ (Bioline) and 0.5 µl template DNA. Amplification was carried out in a Thermal Cycler with an initial denaturation at 95 °C for 5 min; followed by 35 cycles at 95 °C for 20 sec, annealing for 30 sec and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min and holding at 4 °C. The annealing temperature for primer pair TxtAtB1/TxtAtB2, for the amplification of the phytotoxin thaxtomin and primer pair Tom3/Tom4, for the amplification of the enzyme

tomatinase was set at 50 °C and for the forward and reverse primers the annealing temperature was set at 64 °C.

A 5 µl aliquot of each PCR product was separated by electrophoresis on 1.5% agarose stained with ethidium bromide solution (0.1 mg/liter) and observed under a UV gel imager. The PCR product was then purified using Sephadex spin column (5 g of SephadexG-50 powder dissolved in 75 ml of sterile water) and sequenced in both directions using the primers and BigDye Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems) (Jordaan and van der Waals 2015; Muzhinji et al. 2015).

The DNA sequences obtained were manually edited and consensus sequences created using BioEdit v 7.1.3 (Hall 1999). Bacterial and fungal isolates were identified based on sequence similarities to homologous 16S rRNA and ITS gene fragments, respectively, in the nucleotide database GenBank, available through the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm. The resulting fungal and bacterial sequences were deposited in GenBank (Table 3.3 and 3.4).

3.2.4 Inoculum preparation and disease rating

3.2.4.1 Fungal isolates

The pathogenicity of the most prevalent isolate, *R. solani* AG 2-2IIIB, was tested on potato plants, cultivar Mondial. Inoculum of the fungal isolate was prepared following the protocol described by Muzhinji (2014). The fungal isolate was first cultured on PDA. Five PDA plugs, 10 mm in diameter, of each isolate, were added to 10 g of barley grains in conical flasks sterilized by autoclaving at 121 °C for 20 min. The fungal isolate was incubated for 14 days, shaking at 2-day intervals, until barley grains were completely colonized by visible fungal mycelia. Each flask was observed for possible contamination and contaminated flasks were discarded. In the greenhouse, visually disease-free sprouted Mondial mini-tubers were planted in 5 L pots which contained a pasteurized 5:2 sand-coir mix. Each pot was planted with a single seed tuber (experimental unit) at a depth of 10 cm and covered with a 1 cm layer of sand-coir and 10 g of inoculum spread evenly on the layer. Control pots were inoculated with sterile

barley grains only. Plants were grown for 92 days in the greenhouse at 26 ± 2 °C with light for 12 h a day.

3.2.4.2 Bacterial isolates

Using two three-week-old cultures of each representative *Streptomyces* species on YMEA, a volume of 5 ml of sterile distilled water was added to each isolate and the spores were scraped off and mixed with 750 ml of Say's solution (20 g sucrose, 1.2 g L-asparagine, 0.6 g K_2HP0_4, 10 g yeast extract, in 1 L sterile distilled water: adjusted to pH 7.2) and 150 g vermiculite (Wilson et al. 2009). A volume of 5 ml was added to sterile YMEA plates and used as controls. Inoculated vermiculite was incubated at 28 °C for three weeks. In the greenhouse, visually disease-free sprouted Mondial mini-tubers were planted in 5 L pots containing pasteurized sand-coir. Each pot was planted with a single seed tuber (experimental unit) at a depth of 10 cm and filled two-thirds with 10 % vol/vol inoculated vermiculite-soil mix. The control pots were inoculated with sterile vermiculite-soil mix only. Plants were grown for 92 days in the greenhouse at 26 ± 2 °C with light for 12 h a day.

3.2.5 Co-inoculations

To evaluate the possibility of pathogen complexes causing corky crack blemishes on potato tubers, all possible combinations of micro-organisms isolated from these blemishes were tested for Koch's postulates. The combinations are presented in Table 3.2.

Table 3.2. Pathogen combinations of micro-organisms used in this study.

Treatment	Pathogen combination
1	*Rhizoctonia solani* AG 2-2IIIB
2	*Streptomyces* species complex (*S. chrestomyceticus, S. flavoviridis* and *S. fradiae*)
3	*Rhizoctonia solani* AG 2-2IIIB + *Streptomyces* species complex
4	Normal watering uninoculated control
5	*Rhizoctonia solani* AG 2-2IIIB with water stress
6	*Streptomyces* species complex with water stress
7	*Rhizoctonia solani* AG 2-2IIIB + *Streptomyces* species complex with water stress
8	Water stress uninoculated control

3.2.6 Water stress conditions

Potato plants in the normal watering treatment were watered every second day for 92 days to field capacity. Potato plants in the water stress treatment were watered every day to field capacity until the plant was well established (approximately 2 weeks after planting). Thereafter, plants were not watered until early wilting was observed (approximately 3 days) and were then watered to field capacity. This process was repeated until the end of the 92-day period.

3.2.7 Experimental design and layout

A greenhouse trial was conducted under normal watering and water stress conditions. The greenhouse experiment was repeated for both watering conditions. Each experiment was laid out as a randomized complete block design with eight treatments. Each treatment consisted of three replicates. The treatments are shown in Table 3.3. Data analysis was done in a factorial analysis with the primary factor of water conditions and secondary factor the different

pathogens. Analysis of variance (ANOVA) was used to test for the differences between the eight treatments. Treatment means were separated using Fisher's Protected Least Significant Difference (LSD) at the 5% level of significance. Disease severity and index were tested, using ANOVA on ranks with the Tukey's studentized range test for separation of the means at the 0.05 and 0.1 level of significance, respectively.

Table 3.3. The greenhouse layout of the randomized complete block design for the experimental layout of this pot trial.

Block	Treatment	Block	Treatment	Block	Treatment
1	T1	2	T6	3	T8
	T2		T5		T7
	T3		T2		T6
	T4		T1		T5
	T5		T3		T4
	T6		T4		T3
	T7		T7		T2
	T8		T8		T1

*Block indicates the replicate number and T represents the treatment number.

The potatoes were harvested at the end of the 92-day period and re-isolations were made from the diseased potato tubers to confirm Koch's postulates. With modifications of Carling and Leiner (1990), disease severity was estimated using a 0-5 scale, where 0 = no blemishes observed, 1 = less than 1% of the tuber surface is covered with blemishes, 2 = between 1 and 10% of the tuber surface is covered with blemishes, 3 = between 11 and 20% of the tuber surface is covered with blemishes, 4 = between 21 and 50% of the tuber surface is covered with blemishes and 5 = 51% or more of the tuber surface is covered with blemishes. Disease index was calculated using the following formula: Disease Index= $[(n_1+n_2+n_3+n_4+n_5)/N_{total}]$ x 100 (Jordaan and van der Waals 2015; Muzhinji et al. 2015), where n_x is the number of tubers in the x rating category per replicate and N is the total number of tubers rated per replicate.

3.3 Results

3.3.1 Fungal identification

Sequencing results revealed that the most prevalent fungal species obtained from the three potato production regions in South Africa were identified as *Rhizoctonia solani* AG 2-2IIIB (Figure 3.3). Genetic characterization and GenBank accession numbers are shown in Table 3.4. All these isolates had brown mycelia and produced dark brown sclerotia on PDA. Typical right-angle branches were observed at the distal septae of cells, as observed under the microscope. Most of the isolates from all three potato production regions were identified as *R. solani* AG 2-2IIIB and one isolate from the Eastern Free State was identified as AG 2-2IV.

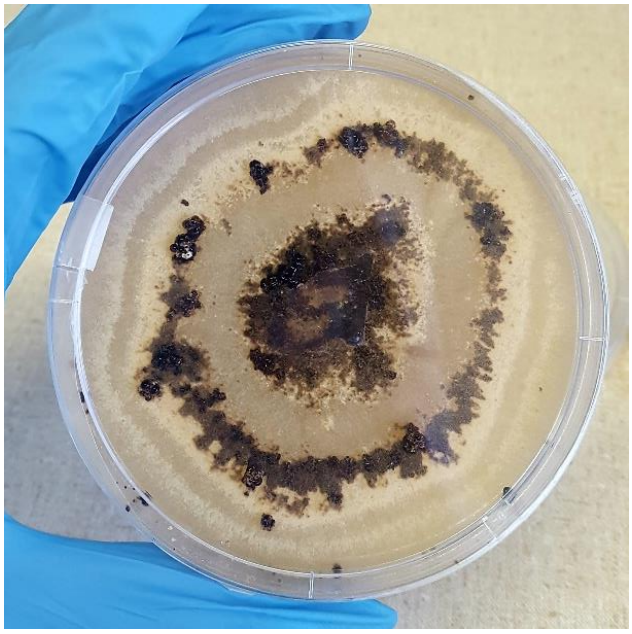

Figure 3.3. A four-week-old pure culture of *Rhizoctonia solani* AG 2-2IIIB on Potato Dextrose Agar (S. Gush).

Table 3.4. The most prevalent *Rhizoctonia solani* AGs collected from three potato production regions in South Africa.

Isolate number	Potato production region	Potato variety	AG group	GenBank accession number
R3Ar	Eastern Free State	Mondial	AG 2-2IIIB	FJ492113.1
R3Bf	Eastern Free State	Mondial	AG 2-2IV	AB911320.1
R3Br	Eastern Free State	Mondial	AG 2-2IIIB	FJ492110.3
R1Ar	Marquard, Western Free State	Mondial	AG 2-2IIIB	FJ492113.3
R1Af	Marquard, Western Free State	Mondial	AG 2-2IIIB	GU811673.1
R8Bf	Limpopo	Mondial	AG 2-2IIIB	FJ492113.3
R8Br	Limpopo	Mondial	AG 2-2IIIB	FJ492113.3

3.3.2 Bacterial identification

The most prevalent bacterial species obtained were *Streptomyces chrestomyceticus* isolated from tubers from the Eastern and Western Free State, *Streptomyces flavoviridis* from the Eastern and Western Free State and *Streptomyces fradiae* from Limpopo. Genetic characterization and GenBank accession numbers are shown in Table 3.5. The chains of spores had the typical appearance of corkscrews and spore chain structures when observed under the microscope. *Streptomyces chrestomyceticus* colonies appeared white in colour (Figure 3.4), while *Streptomyces flavoviridis* and *Streptomyces fradiae* were grey in colour.

Figure 3.4. Two-week-old pure cultures of *Streptomyces chrestomyceticus* on Yeast Malt Extract Agar (S. Gush).

Table 3.5. The most prevalent *Streptomyces* species isolates collected from three potato production regions in South Africa.

Isolate number	Potato production region	Potato variety	Identification	GenBank accession number
S5A2r	Eastern Free State	Mondial	*S. chrestomyceticus*	KF668652.1
S5A2f	Eastern Free State	Mondial	*S. chrestomyceticus*	KF668652.1
S5B2r	Eastern Free State	Mondial	*S. flavoviridis*	KF815976.1
S5Af	Marquard, Western Free State	Mondial	*S. chrestomyceticus*	KF668652.1
S5Ar	Marquard, Western Free State	Mondial	*S. flavoviridis*	KF815976.1
S5Bf	Limpopo	Mondial	*S. fradiae*	KF147894.1
S5Br	Limpopo	Mondial	*S. fradiae*	KF147894.1

3.3.3 Greenhouse trial

No corky crack symptoms were observed at harvest after the 92-day growth period. Possible symptoms of common scab caused by *Streptomyces* were observed (Figure 3.6). However, typical stem canker lesions and elephant hide symptoms were observed on the potato tubers in pots inoculated with *Rhizoctonia solani* AG 2-2IIIB (Figure 3.5 and Figure 3.7) and those co-inoculated with *R. solani* AG 2-2IIIB and the *Streptomyces* species complex. Potential common scab symptoms were also observed on progeny tubers inoculated with the *Streptomyces* species complex (Figure 3.6). Re-isolations were made from the observed elephant hide symptoms in this study since no corky crack symptoms were observed.

3.3.4 Statistical analysis

When comparing the tubers inoculated with *Rhizoctonia solani* AG 2-2IIIB there were significant differences ($P < 0.1$) with regards to the disease index (Table 3.6). All isolates of *R. solani* AG 2-2IIIB caused elephant hide on progeny tubers (Figure 3.7), with exception of treatment 5 replicate 1 (*R. solani* AG 2-2IIIB, under water stress conditions), which did not produce any progeny tubers and, therefore, no observable disease symptoms.

Disease index values were higher ($P < 0.1$) in treatments under water stress compared to treatments under normal watering conditions (Table 3.6). This was observed for *R. solani* AG 2-2IIIB single inoculated plants, with a mean of 77.56 under normal water conditions and 113.08 under water stress conditions. Disease index values also were higher ($P < 0.1$) in co-inoculated plants under water stress, with a mean of 121.50, as compared to co-inoculated plants under normal watering conditions, with a mean of 107.94. It was observed that the disease index values were higher in the *Streptomyces* species complex inoculated plants, with a mean of 12.35 under normal water conditions and 20.65 under water stress conditions; however, it was not significant.

Dark brown stem cankers were observed as a typical symptom of *R. solani* infection on plants inoculated with *R. solani* AG 2-2IIIB alone as well as potato plants co-inoculated with *R. solani* AG 2-2IIIB and the *Streptomyces* species complex. Stem cankers were more severe ($P < 0.1$) in co-inoculated plants under water stress conditions as compared to normal watering conditions (Figure 3.5).

The different pathogen treatments had a significant effect on the disease index values ($P < 0.05$), whereas the interaction between the different pathogen treatments and the different watering conditions had no effect (Table 3.8).

A significant difference ($P < 0.01$) in disease severity in co-inoculated potato plants was observed under the different watering conditions (Table 3.9). Disease severity values of co-inoculated plants (treatment 3) were higher as compared to single inoculated plants (treatment 1 and 2) under normal watering conditions, but not significantly so (Table 3.6). Higher disease severity ($P < 0.05$) and index ($P < 0.1$) values for the elephant hide symptoms were also observed under the water stress conditions for the co-inoculated plants (treatment 7) as compared to the single inoculated plants (treatment 5 and 6) (Table 3.6).

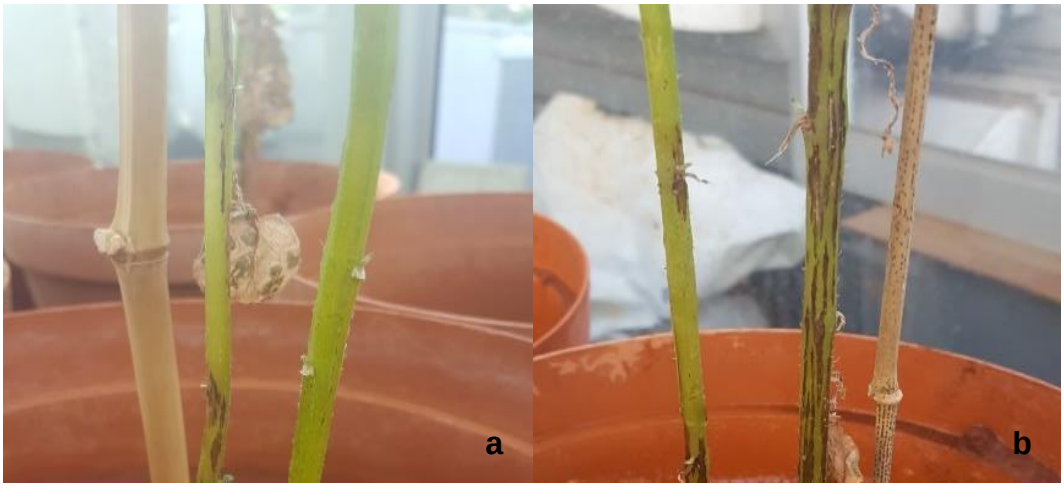

Figure 3.5. Dark brown stem cankers observed on (a) plants inoculated with *Rhizoctonia solani* AG2-2IIIB alone (treatment 1) and (b) potato plants co-inoculated with *R. solani* AG 2-2IIIB and the *Streptomyces* species complex (treatment 3) (S. Gush)

Figure 3.6. Progeny potato tuber showing potential common scab symptoms caused by the *Streptomyces* species complex (treatment 2). (S. Gush).

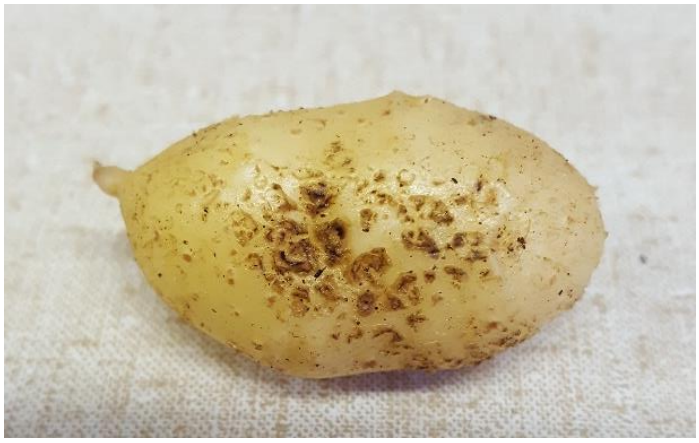

Figure 3.7. Progeny potato tuber showing typical elephant hide symptoms caused by *R. solani* AG 2-2IIIB (S. Gush).

Table 3.6. Disease severity and index means for *Rhizoctonia solani* AG 2-2IIIB and the *Streptomyces* species complex.

Treatment	Disease severity	Disease index
Rhizoctonia solani AG 2-2IIIB	1.26 abcd	77.56 ab
Streptomyces species complex (*S. chrestomyceticus, S. flavoviridis* and *S. fradiae*)	0.37 ab	12.35 ab
Rhizoctonia solani AG 2-2IIIB + *Streptomyces* species complex	1.68 cd	107.94 ab
Normal watering uninoculated control	0 a	0 a
Rhizoctonia solani AG 2-2IIIB with water stress	1.40 bcd	113.08 bc
Streptomyces species complex with water stress	0.42 abc	20.65 ab
Rhizoctonia solani AG 2-2IIIB + *Streptomyces* species complex with water stress	1.84 d	121.50 bc
Water stress uninoculated control	0 a	0a

*Numbers within the same column which share the same letters are not significantly different at P = 0.05 for disease severity and P =0.1 for disease index.

Table 3.7. Two-way ANOVA showing effect of *Rhizoctonia solani* AG 2-2IIIB (R) and the *Streptomyces* species complex (S) on disease index values.

	df	SS	MS	F score	F table (0,01)	P value	Conclusion
Total	23	152429,3					
R	1	56187,57	56187,57	11,91727	8,1	p<0.01	Reject H0, significant
S	1	1933,036	1933,036	0,409993	8,1	p>0.01	Do not reject H0
RxS	1	12,65854	12,65854	0,002685	8,1	p>0.01	Do not reject H0
Error	20	94296,07	4714,803				

Table 3.8. Two-way ANOVA showing effect of pathogens (P) and watering conditions (W), and P x W on disease index values.

	df	SS	MS	F score	F table (0,05)	P value	Conclusion
Total	23	152429,3					
Pathogen	3	58133,26	19377,75	3,369163	3,24	p<0.05	Reject H0, significant
Water	1	1234,818	1234,818	0,214695	4,49	p>0.05	Do not reject
PXW	3	1037,189	345,7295	0,060111	3,24	p>0.05	Do not reject
Error	16	92024,06	5751,504				

Table 3.9. One-way ANOVA showing effect of *Rhizoctonia solani* AG 2-2IIIB and the *Streptomyces* species complex on disease severity values.

	df	SS	MS	F score	F table (0.01)	P value	Conclusion
Between	1	11,84	11,84	20,17661	7,95	p<0.01	Reject H0, significant
Within	22	12,91	0,586818				
Total	23	23,15	1,006522				

3.4 Discussion and conclusion

In this study, the causative agents of corky crack symptoms on potato tubers in South Africa were investigated. Two biotic causal agents namely, *Rhizoctonia solani* and *Streptomyces* species were considered in this study due to the uncertainty of the main causal pathogen in literature. Water stress was also considered as an abiotic factor, which may play a role in disease development.

Previously, Muzhinji (2014) characterized *R. solani* AG 3-PT as the causal pathogen causing cracking on potato tubers. In the same study, *R. solani* AG 3-PT was also found to cause elephant hide symptoms on potato tubers, confirming Koch's postulates. The elephant hide symptom has also been reported in France (Fiers et al. 2010); however, Koch's postulates were not proven. It is known that *R. solani* AG 3-PT is the most predominant AG group in potatoes, typically associated with qualitative and quantitative yield losses (Woodhall et al. 2013; Muzhinji 2014), also causes atypical tuber blemishes such as elephant hide consisting of corky cracks and lesions on the tuber surface (Fiers et al. 2010).

In this study, *R. solani* AG2-2IIIB was found to be the most prevalent AG group on potato tubers sampled from three of the sixteen potato production regions in South Africa namely

Limpopo, Eastern and Western Free State. Muzhinji et al. (2015) described the predominance of *R. solani* AG 3-PT associated with potato disease in South Africa but also showed that AG 2-2IIIB, AG 4HG-I, AG HG-III, and AG 5 are pathogenic to potato in the country, at lower frequencies. Muzhinji et al. (2015) also reported *R. solani* AG 4HG-1 and AG 2-2IIIB to cause stolon and stem canker on potatoes in South Africa; however, AG 3-PT still causes more severe stolon and stem canker symptoms.

No corky crack symptoms were reproduced on progeny tubers at harvest. No current literature refers to *R. solani* AG 2-2IIIB causing cracks on potato tubers. Nonetheless, elephant hide symptoms were observed on potato tubers inoculated with *R. solani* AG 2-2IIIB as well as on progeny tubers co-inoculated with *R. solani* AG 2-2IIIB and the *Streptomyces* species complex (*S. chrestomyceticus, S. flavoviridis* and *S. fradiae*). Thereforre, is the first report of *R. solani* AG 2-2IIIB causing elephant hide on potato tubers in South Africa.

It has previously been reported that *R. solani* AG 2-2IIIB is the causal pathogen of root and crown rot in maize, soybean (Liu and Sinclair 1991) and sugar beet (Engelkes and Windels 1996; Kluth et al. 2010). Engelkes and Windels (1996), demonstrated in their study that *R. solani* AG 2-2IIIB and AG 2-2IV, both found in this study on potato, cause root and crown rot in sugar beet in the USA. Muzhinji et al. (2015) also found one *R. solani* AG 2-2IIIB isolate (GenBank accession number KJ777580) from potato in the North-West province of South Africa, cultivar Mondial, with the known preceding crop of sugar beet. This suggests that *R. solani* AG 2-2IIIB still proliferates in the soil and is a potential pathogen of potato in the North-West potato production region. Therefore, the localization of the AG groups in specific potato production regions in South Africa could be due to a previous crop susceptible to the pathogen.

The fact that neither *R. solani* AG 2-2IIIB nor the *Streptomyces* species complex in this study caused corky crack symptoms on potato tubers in this study could be due to various factors. The growth period of 92 days may have been too short to be able to observe typical corky crack symptoms on the cultivar Mondial. Previous studies have allowed longer growth periods from 120 to 180 days (Wanner 2006; Fiers et al. 2010; Muzhinji 2014; Zimudzi et al. 2017). There is a possibility that no corky crack symptoms were observed due to the fact that this study used cultivar Mondial mini-tubers whereas cultivar BP1 was used in studies of both Gouws and McLeod (2012) and Muzhinji (2014).

The *Streptomyces* species complex in this study included *S. chrestomyceticus, S. flavoviridis* and *S. fradiae,* which naturally occur in the soil and are not considered to be plant pathogenic bacteria (Janssen et al. 1989). Even though possible disease symptoms caused by *Streptomyces* species were observed on potato tubers after harvest, these species were not re-isolated from the tubers, confirming that they are non-pathogenic to potato tubers and therefore do not cause disease. Likewise, Fiers et al. (2010), isolated several unknown *Streptomyces* species from blemished potato tubers, however, none of these species were related to corky crack symptoms. The currently known *Streptomyces* species causing scab symptoms on potato are *S. scabiei* (Loria et al. 2006), *S. stelliscabiei* (Bouchek-Mechiche et al. 1998), *S. acidiscabies* (Loria et al. 2006), *S. turgidiscabies* (Miyajima et al. 1998), *S. europeiscabiei* (Bouchek-Mechiche et al. 2000; Jordaan and van der Waals 2015), *S. ipomoeae* (Loria et al. 2008), *S. luridiscabiei, S. puniciscabiei,* S. niveiscabiei (Park et al. 2003), *S. bottropensis, S. reticuliscabiei,* (Bouchek-Mechiche et al. 2000), *S. caviscabiei* (Goyer et al. 1996), *S. aureofaciens* (Faucher et al. 1992), *S. griseus* (Corbaz 1964) and *S. sp. IdahoX* (Wanner 2007).

Results from this study showed that plants co-inoculated with both *R. solani* AG 2-2IIIB and *Streptomyces* species developed more severe disease symptoms on tubers as well as more severe stem cankers compared to single inoculated plants. Statistical analysis further confirmed that disease severity was higher for co-inoculated plants than single inoculated plants. This suggests that there could be a possible synergistic interaction between *R. solani* AG 2-2IIIB and *Streptomyces* species, resulting in more severe elephant hide symptoms on potato tubers. It was demonstrated that even though co-infection by the fungus, *Verticillium dahliae* and bacteria from the *Pectobacterium* genus does not always result in significant synergistic interactions on potato, co-inoculation still increases the disease severity (Dung et al. 2014). The same result was observed in this study between the fungus, *R. solani* AG 2-2IIIB and bacterium, *Streptomyces* species, where no significant synergistic interactions were observed between the two pathogens on potato. However, the co-inoculation increased the disease severity. Further studies should be done to understand the mechanism of interaction between pathogens in causing disease as not much research has been done on potato disease complexes.

In this study, it was found that water stress does not play a role in causing corky crack symptoms on potato. However, it is possible that the growth period of 92 days, may have been too short to be able to see a significant effect of water stress on disease development. Water

stress did; however, result in smaller potato tubers, compared to potatoes grown under normal watering conditions. On the other hand, it is known that some soil-borne pathogens such as *Rhizoctonia solani*, *Phytophthora* species, *Sclerotium rolfsii* and *Pythium* species. infect and reproduce successfully under high soil moisture conditions, thereby resulting in increased disease incidence and severity (Olson et al. 1990; Clover et al. 1999; Agrios 2005). It has been demonstrated that *Streptomyces* species tend to remain active under water stress conditions (Zenova et al. 2007) and since they are Gram-positive bacteria, they are more tolerant than Gram-negative bacteria to drought conditions (Griffin 1981; Fierer et al. 2003; Manzoni et al. 2012). Moreover, fungi are known to be more tolerant than bacteria to water stress conditions, since they can accumulate osmoregulatory solutes that do not impair their metabolism (Braun et al. 2017) and filamentous structure which enables the pathogen to accumulate substrates at low water levels (Griffin 1981; Magan and Lynch 1986; Manzoni et al. 2012). This explains why the disease index and values in this study were higher in water stress trials for the *Streptomyces* species complex. Surprisingly, the disease index values were higher for *R. solani* AG 2-2IIIB as compared to the *Streptomyces* species complex. The *Rhizoctonia* species is known to require high moisture soil environments for infection to take place (Jeger et al. 1996). However, it is also known that at high soil moisture contents, lenticels on the tuber open, thus allowing for infection by a pathogen to take place (Helias 2008). For example, *R. solani* requires at least a low of 45 % soil moisture for infection to take place (Fiers et al. 2012).

Future research should consider all factors including biotic and environmental conditions when determining the causal agents of disease, such as corky cracks on potato. Firstly, all pathogenic microorganisms isolated from corky crack symptoms should be identified and inoculated as a complex of microorganisms in pot trials to conclude whether this symptom is caused by pathogenic microorganisms. Secondly, if corky crack symptoms are observed after pot trials, a further study could be done to investigate possible synergistic interactions between causal pathogens or if a potential disease complex is involved in causing corky cracks on tubers. If no corky crack symptoms are observed after these studies, it could be assumed that abiotic and environmental factors may be the cause of the symptom and further investigations should be carried out considering these factors. Thirdly, corky crack symptoms may be due to a sequence of events, during which potato plants experienced water stress, resulting in cracking of the tuber periderm and therefore allow the entry of potential pathogenic microorganisms via the wound, causing the corky crack symptom. Lastly, potato viruses should also be taken into

consideration. A study by McCreath and Carnegie (2008), demonstrated that Potato Virus A (PVA) and Potato Virus Y (PVY), known to cause mosaic symptoms on leaflets, also caused growth cracking on potato tubers. Their study also suggested more severe growth crack symptoms in the co-infection of both viruses as compared to single virus infections, hence, a synergistic interaction may be involved. However, only certain cultivars are known to be prone to growth cracking when infected with a virus. For example, with the infection of PVY, it was revealed that the cultivar Chieftain was more prone to growth cracking than the cultivar Maris Piper (McCreath 2016). It has also been shown that no growth cracking occurred in the cultivar Désirée infected with PVA, which may suggest the susceptibility to the virus (McCreath and Carnegie 2008).

This study aimed to determine the cause of corky crack symptoms on potato tubers from three different potato production regions in South Africa, with specific consideration of two microbial causal agents, namely *Rhizoctonia solani* AG 2-2IIIB and the *Streptomyces* species complex isolated from blemished tubers. No corky crack symptoms were observed on progeny tubers from pathogenicity trials; however, typical elephant hide symptoms were observed on progeny tubers from plants inoculated with *R. solani* AG 2-2IIIB. Although potato tubers grown under water stress produced smaller tubers and more severe disease symptoms, water stress conditions were not found to be a significant factor in causing the corky crack disease on potatoes. Higher disease severity for elephant hide symptoms was observed for co-inoculated plants, compared to single inoculated plants, suggesting a possible synergistic interaction between the pathogens.

Further studies should be conducted to determine the possible synergistic mechanisms involved between the pathogens isolated in this study. It is important to focus on all pathogens involved in causing disease in pathogenicity tests and in future research to develop effective control strategies to improve potato quality, production and profit for growers in South Africa. A conclusive study should, therefore, be performed considering all potential microbial and abiotic factors involved in causing disease. Determining the exact causes of the corky crack symptom can save the industry from severe economic losses.

3.5 References

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Chapter 4

Role of isolated microbial agents, water stress and PVY in causing corky crack blemishes on potato tubers in South Africa.

Abstract

Potato tuber blemishes can be a result of known causes (typical blemishes), which are manageable; or unknown causes (atypical blemishes), which are difficult to manage. In a previous pilot trial, *R. solani* AG2-2IIIB, and various *Streptomyces* species were isolated from corky crack blemishes on potatoes in South Africa. However, none of these microorganisms, alone or in combination resulted in the development of corky cracks when tested in pot trials. Therefore, in an effort to confirm the causal agent(s) of corky crack blemishes, additional tubers, with symptoms of either growth cracks or corky cracks were sampled from different potato growing regions of South Africa and further isolations of bacteria and fungi were made from the different symptoms. Bacterial and fungal species were identified by morphological and molecular techniques. Binucleate *Rhizoctonia* (BNR) AG-A and BNR AG-R as well as *Fusarium oxysporum* were the most predominant fungal species isolated from the corky crack symptoms, while no fungal species were isolated from growth cracks. *Streptomyces collinus*, *S. yaanensis*, *S. corchorus*si, *S. viridochromogenes* and *S. griseorubens* were the most predominant bacterial species isolated from growth cracks and no *Streptomyces* species were isolated from corky cracks. The *Streptomyces* species identified are not known to be pathogenic to potatoes and are therefore not likely causal agents of the corky cracks. PVY was not amplified from corky crack tissue using molecular techniques and it was therefore assumed that PVY is not associated with corky cracks on the tested sample of potato tubers in South Africa. Greenhouse pathogenicity trials using the isolated microorganisms alone and in combination were done to confirm Koch's postulates. Corky crack symptoms were not observed in single inoculations for fungal or bacterial isolates; however, corky crack symptoms were observed on the progeny tubers inoculated with a combination of *Rhizoctonia* species. This suggests a synergistic interaction between the three *Rhizoctonia* species tested in this study. Disease index of the *Rhizoctonia* species complex in this study was significantly ($P < 0.05$) higher for progeny tubers under normal (field capacity) watering conditions compared to

potatoes grown under drought stress conditions, suggesting the fungal preference for soil moisture for infection to take place. It was found that no significant differences ($P > 0.05$) were observed in water stress with regards to disease incidence and severity between the between water stress and pathogen treatments. The findings of this study are crucial in the development of effective integrated strategies for the management of corky crack disease and to improve the tuber quality in the South African market and globally.

4.1 Introduction

Potato is the world's most important non-grain food crop and is the fourth main food crop after maize, rice and wheat (FAO 2019). However, the crop is susceptible to various pests and pathogens, such as fungi, bacteria, viruses, nematodes and insects (Fiers et al. 2012). Potato is a crop of importance globally, therefore, it is vital to control diseases affecting the crop to meet the increasing demand for food and to reduce yield losses due to downgrading of diseased tubers (Fiers et al. 2012). Pathogens that attack potatoes, can cause damage to all parts of the plant, including the tubers which are the most important organ used for human and animal consumption. It is essential to reliably and correctly identify disease-causing pathogens in order to successfully control diseases (Srinivasa et al. 2012). Misidentification can lead to incorrect control recommendations and further crop loss (Wang et al. 2012).

Blemishes on potato tubers contribute to major economic losses in South Africa (Zimudzi et al. 2017) and globally (Gherbawy and Gashgari 2013). The unfavourable visual appearance of the tubers makes it difficult to compensate for the consumer demand of fresh, high-quality washed tubers (Jordaan and van der Waals 2015). Therefore, the production of blemish-free potato tubers is of utmost importance to meet customers' quality requirements and prevent severe economic losses.

Potato tuber blemishes are also reported to be caused by viruses such as Potato Mop Top Virus (PMTV), Potato Virus Y (PVY) and Potato Spindle Tuber Viroid (PSTVd), as well as nematodes (Fiers et al. 2010; Bouchek-Mechiche et al. 2013). However, viruses or viroids are also known to cause foliar symptoms such as mosaic, leaf distortion, crinkling, dwarfing, leaf rolling and leaf and vein necrosis (Black 2008; Fiers et al. 2012). The majority of viruses are

tuber-borne and are spread from infected tubers remaining in the soil or in seed-tubers. Viruses are also transmitted by vectors such as fungi, e.g. *Spongosphora subterranea* f. sp. *suberranea* (Potato Mop Top Virus), aphids (PVY, Potato Leaf Roll Virus, (PLRV)) and nematodes, such as *Trichodorus* species and *Paratrichodorus* species (Tobacco Rattle Virus) (Thompson and Strydom 2003). In South Africa, the most important viruses, monitored by the potato certification scheme are PVY, PLRV and tomato spotted wilt virus (Thompson and Strydom 2003). This study investigated the role of PVY in causing corky crack blemishes, as it is an important plant pathogen that infects potatoes and significantly reduces yield under warm conditions in South Africa. Most importantly, PVY has been speculated to cause potato tuber blemishes (Visser and Bellstedt 2009; Visser et al. 2012).

In contrast to typical blemishes, atypical blemishes are those with unknown causes and for which Koch's postulates have not been fulfilled (Zimudzi et al. 2017). The diagnosis of atypical blemishes is not as rapid and simple compared to that of typical blemishes. Some atypical blemishes include corky cracks, star-like corky lesions, corky spots or 'rhizoscab' and 'elephant hide' (Fiers et al. 2010).

In South Africa, there has been an increase in the prevalence of atypical blemishes such as corky cracks (corky skin inside the crack) and growth cracks (smooth skin inside the crack) on potato tubers (Muzhinji et al. 2014). Some studies have suggested that the causal agents of corky cracks in South Africa are *Rhizoctonia* and/or *Streptomyces* species (Gouws and McLeod 2012; Muzhinji et al. 2014). However, studies specifically focusing on the occurrence and causal agents in South Africa are starting to accumulate with *R. solani* species being the predominant pathogen isolated from corky cracks on potato tubers in South Africa (Muzhinji et al. 2014; Zimudzi et al. 2017) and elsewhere (Fiers et al. 2010; Woodhall et al. 2011). Gouws and McLeod (2012) identified *Streptomyces* species from 'fissure scab' symptoms on potato tubers (Gouws and McLeod 2012). The fissure scab symptom is similar to the cracks caused by *R. solani* AG-3PT (Muzhinji 2014). It is, however, known that *Streptomyces* species cause common scab on potatoes worldwide with variable symptoms of brown, circular, corky lesions. Interestingly, these symptoms were observed on the potato cultivar Mondial, which is known to be tolerant to common scab (Jordaan and van der Waals, 2015). Gush et al. (2017) (Chapter 3) isolated *Rhizoctonia* AG 2.2IIIB and *Streptomyces* species from corky cracks, but Koch's postulates were not fulfilled. Under such circumstances, it is prudent to hypothesise that corky cracks may have more than one causal agent.

Growth cracks are known to be caused by water stress or variation in soil moisture content during early tuber bulking (Begum et al. 2018). Growth cracks are similar to corky cracks; however, the growth crack has a smooth texture instead of a corky texture. It has been reported that potato plants experience water stress when the soil water tension exceeds 20 kPa and this results in a reduction in grade and yield (Epstein and Grant 1973; Begum et al. 2018). The effects of environmental factors and climate change on plant diseases have been the subject of debate for many years (Das et al. 2017). Numerous plant diseases are likely to increase due to climate change, therefore, posing a risk to the sustainability of crop production (Mahato 2014). Factors such as temperature, irrigation/rainfall, soil type, soil moisture, plant nutrition and pH are some of the most important factors that have an impact on crop production and the development of plant disease (Agrios 2005; Fiers et al. 2012). Various research studies have been done on the effects of the environment on plant diseases; however, more information is needed on specific plant pathogens and their associated diseases on host crops in order to understand their adaptive mechanisms against the changing environmental conditions (Mahato 2014). By understanding the field responses of plant pathogens under the stress of changing environmental conditions over time, specific agricultural measures can be developed to overcome or prevent the development of diseases, such as corky cracks, and limit further production losses.

Rapid and accurate detection and identification of plant pathogens is important to develop effective control measures and avoid disease spread, which can lead to severe yield losses (Gherbawy and Gashgari 2013). Morphological identification of fungi, such as *Rhizoctonia* species is based on form, colour, size and elevation of hyphae in specific growth media. The hyphal structure can be examined under the microscope (Zimudzi et al. 2017). Morphological characteristics of many *Rhizoctonia* species overlap, therefore it is difficult to identify *R. solani* species using morphological techniques alone. The hyphal fusion and 'bridging' (fusion of more than one anastomosis group) method is helpful; however, there are uncertainties regarding the accuracy thereof (Ogoshi 1987; Carling et al. 1994). For example, previous studies could not identify different AGs using the hyphal fusion method, possibly due to the presence of new and unknown AGs (Carling and Leiner 1986; Anguiz and Martin 1989). Therefore, molecular techniques are important to characterize and correctly identify the different AGs of *Rhizoctonia solani* species.

Streptomyces species are characterized by colony colour and shape when grown on media as well as the spore structure, which can be examined under the microscope (Tashiro et al. 2012). Molecular studies of *Streptomyces* species have shown a great diversity within the strains previously identified as *S. scabies* (Healy and Lambert 1991; Paradis et al. 1994). This highlights the importance of further characterization of *Streptomyces* isolates at species level using molecular methods.

Presently, due to the difficulties in morphologically based identification methods, taxonomists now rely on unique patterns or sequences present in the genome of the pathogen studied. The most adapted molecular technique is based on polymerase chain reaction (PCR), which amplifies specific DNA regions of the pathogen. Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP) is best suited to study differences or diversity within a species, whether it is a fungus, bacterium or nematode (White et al. 1990; Reysenbach et al. 1992; Cullen et al. 2007; Flores-González et al. 2008). A widely used approach is to use oligonucleotide primers specific for the conserved flanking regions of the internal transcribed spacers (ITS) of the fungal rRNA gene (White et al. 1990; Gardes and Bruns 1993) or 16S for the bacterial rRNA gene, which is more accurate than AFLP and RFLP (Reysenbach et al. 1992). The ITS and 16S coding regions play a critical role in functional rRNA development and are promising regions for molecular assays (Reysenbach et al. 1992; Gardes and Bruns 1993).

Plant diseases occur as a result of interactions between a pathogen, susceptible host plant and optimal environment (Fiers et al. 2012). These diseases are often thought to be caused by one pathogenic species or by a specific strain (Gest 2004). In nature, microbes form complex multispecies communities that have been reported to cause diseases on plants (Lamichhane and Venturi 2015). Pasteur in the 1800s demonstrated that diseases can be due to the synergistic interactions of different microbes (Gest 2004; Le May et al. 2009). However, most studies have focused on single microbial strains capable of growing in pure culture in artificial culture media. In plants, there are only a few reports of synergistic interactions and the mechanisms are still unknown (Fitt et al. 2006).

Complex interactions can occur between a pathogen and a host plant and can be influenced by biotic and abiotic factors, which make it extremely difficult to control the diseases (Fiers et al. 2012). Soilborne diseases are caused by pathogens found in the soil environment. Interestingly,

the soil can interfere with the interactions between pathogens, microorganisms and even between the microorganisms or pathogens themselves (Fiers et al. 2012).

Until now, only a few studies have been published showing that plants can be infected by more than one pathogen concurrently (Martin et al. 2013; Dung et al. 2014). In such cases, interestingly, inoculation by a single microbe was not successful in inducing the disease, whereas, co-infection with a different microbe lead to disease development (Fitt et al. 2006). In pathogenicity studies, mono-species are usually used to evaluate disease development, because our knowledge of synergism between multiple pathogens in causing disease is poor. It is possible that interactions between pathogens can lead to more severe disease symptoms (Begon et al. 1986; Le May et al. 2009) and it is, therefore, important to include all pathogens involved in causing disease in pathogenicity tests to be able to develop effective control measures.

Assuming that corky cracks are due to a synergistic interaction between pathogens, this study involved greenhouse trials of single inoculations of all pathogens isolated and identified as well as co-inoculation with combinations of the different pathogens. An abiotic factor, water stress, was also included in the study to test whether it plays a role in causing corky cracks alone, or together with the pathogen(s).

Therefore, the objective of this study was to determine all pathogenic microorganisms associated with corky crack blemishes on potato tubers in South Africa, using morphological and molecular identification methods and pathogenicity tests; and furthermore, to determine whether PVY is associated with the corky crack symptom in South Africa. Although considerable research has been done on determining the causative agents of typical potato tuber blemishes, the results from this work will contribute significantly to the potato tuber industry by determining the causal agent(s) of corky crack blemishes on potatoes in South Africa and thus helping to prevent severe economic losses due to these blemishes.

4.2 Materials and methods

4.2.1 Sample collection

Potato tubers showing corky crack blemishes were collected from potato growing regions with different agroecological climates in South Africa. Potato tubers submitted by potato growers, and farmer advisers to the Potato Pathology Diagnostic Clinic @ UP were also included in the study.

4.2.2 Isolation of pathogens from blemished tubers

4.2.2.1 Fungal and bacterial isolation

Infected tubers were washed with distilled water and air-dried. Small lesions of 5 mm in diameter and 5 mm deep were excised from infected tissue using a sterile scalpel blade and tweezers. The excised lesions were surface-sterilized for 3 minutes with 1% NaOCl and rinsed twice with sterile distilled water. The lesions were then plated onto a 90 mm diameter Petri dish containing 1.5% water agar (WA, Biolab), amended with 50 mg/L streptomycin sulphate (Sigma-Aldrich). The agar plates were incubated for 48 hours at 25 °C. Fungal hyphae were observed under a compound microscope (100 X and 400 X magnification) and transferred to 90 mm diameter plates containing potato dextrose agar (PDA, Biolab), amended with 50 mg/L streptomycin sulphate (Sigma- Aldrich) (Carling and Leiner 1990).

4.2.2.2 Bacterial isolation

For bacteria, potato tuber lesions of 5 mm in diameter were excised together with the yellow coloured flesh of the potato tuber. Each lesion was macerated in a Bioreba bag (Labretoria) using a pestle. Two ml of sterile distilled water were added to each bag, 200 µl of the extractant pipetted onto 90 mm diameter plates containing WA (Biolab) and spread using a glass hockey stick (Loria et al. 1997). The plates were incubated in the dark for 3 weeks at 28 °C. Examination of the plates was performed at regular intervals during the 3 weeks of incubation

until clear *Streptomyces* colonies were visible. Spores were identified with the use of a light microscope (100 X magnification). Colonies were transferred to plates containing yeast malt extract agar (YMEA, Biolab).

4.2.3 DNA extraction and identification by Polymerase Chain Reaction (PCR)

4.2.3.1 Genomic DNA extraction

Isolates were sub-cultured on PDA for fungal cultures and Yeast Malt Extract Agar for bacterial cultures and incubated 25 °C for 7 days at. Mycelia of fungal cultures and bacterial colonies were scraped off and transferred to 2 ml Eppendorf tubes. Using the ZR Fungal/Bacterial DNA Mini-prep extraction kit (Zymo Research Corporation), total genomic DNA was extracted following the manufacturer's recommended protocol.

4.2.3.2 PCR amplification and sequencing

DNA extracted from fungal cultures was amplified using universal primers, ITS1-F (5'-CTTGGTCATTTAGAGGAAG-3') (Gardes and Bruns 1993) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) that target the ITS region. Amplification was conducted in 50 µl PCR reaction mixtures containing 4 ng of template DNA; 250 µM of dATP, dTTP, dGTP and dCTP (Bioline) each; 10 x NH_4 reaction buffer containing 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCL at pH 8.8 and 100 mM KCl (Bioline); 0.25 U of BIO*Taq* DNA polymerase (Bioline); 3 mM $MgCl_2$; and 0.2 µM of each primer. Amplification was carried out in a thermal cycler (Thermo Scientific) with an initial denaturation step of 95 °C for 3 min; followed by 35 cycles at 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec; and a final extension of 72 °C for 5 min. A 5 µl aliquot of each PCR product was separated by electrophoresis on 1.5% (wt/vol) agarose (Lonza) stained with ethidium bromide solution (0.1 mg/litre) and visualized using a UV transilluminator. When the expected size bands (400 bp - 700 bp) were observed, the remaining PCR product was purified using Sephadex spin column (Sigma Aldrich Co.) (5 g of Sephadex G- 50 powder dissolved in 75 ml of sterile water) and the resulting amplicons were sequenced in both directions using the same primers and the

BigDye Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems) at the University of Pretoria's Bioinformatics Sequencing Unit.

For bacterial DNA, the 16S rRNA gene amplification was performed by amplifying the 16S RNA gene region using primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach et al. 1992). Amplification was conducted in a 25 µl PCR reaction mixture containing 4 ng of template DNA; 250 mM each dATP, dTTP, dGTP, and dCTP (Fermentas); 10 × PCR reaction buffer, consisting of 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl at pH 8.8, and 100 mM KCl; 0.25 U of Bio*Taq* DNA polymerase (Bioline) and 0.2 mM each primer (Inqaba Biotechnical Industries, South Africa). Amplification was carried out in a thermal cycler (Thermo Scientific) with the following conditions: an initial step of 95 °C for 3 min; followed by 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s; and a final extension of 72 °C for 5 min. A 5 µl aliquot of each PCR product was separated by electrophoresis on 1.5% (wt/vol) agarose (Lonza) stained with ethidium bromide solution (0.1 mg/litre) and visualized using a UV transilluminator. When the bands of the appropriate size (620 bp - 700 bp) were observed the amplicons were sequenced as described for fungal isolates.

The DNA sequences obtained were manually edited and consensus sequences created using BioEdit v 7.1.3 (Hall 1999). Bacterial and fungal isolates were identified based on sequence similarities to homologous 16S rRNA and ITS gene fragments, respectively, in the nucleotide database GenBank, available through the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm. The resulting fungal and bacterial sequences were deposited in GenBank (Table 4.1 and 4.2).

4.2.4 PVY detection from potato tubers

4.2.4.1 RNA extraction

Potato tubers with corky crack symptoms were collected from the Tshwane Fresh Produce Market and were used for RNA extraction. From each tuber, three sections were excised at different areas along the corky crack. A sub-sample of 0.1 g of blemished tissue was excised from each of the three sections per tuber, ground and RNA extracted using an RNA extraction

kit (ZR viral RNA kit). Three positive controls were used, namely + PVY, + leaf and + tuber. The + PVY sample was a positively identified freeze-dried leaf sample of PVY obtained from the Agricultural Research Centre (ARC). The + leaf and + tuber samples were collected from the Sandveld potato growing region. The + tuber sample showed necrotic ringspot symptoms on the tuber, typical to PVY.

4.2.4.2 Reverse Transcription-Polymerase Chain Reaction

Reverse Transcription (RT)-PCR was carried out using 4 µl of the extracted RNA, 2 µl of $d(T)_{23}$ VN, 10 µl of ProtoScript II Reaction Mix (2X), 2 µl of ProtoScript II Enzyme Mix (10X) and 2 µl of Nuclease-free water to make a total volume of 20 µl (New England Biolabs) according to the manufacturer's instructions and utilizing oligo (dT)18 primer (Thermo Scientific) at a final concentration of 10 µM. The RT-PCR reaction was incubated at 42 °C for 60 min, followed by 80 °C for 5 min for terminating the reaction. PVY1 (5'-GATGGTTGCCTTGGATGATG-3') and PVY 2 (5'-TAAAAGTAGTACAGGAAAAGCCA-3') (Fuentes et al. 2019) as well as PVY 2F (5'-CACGATTGCTCAAGCAAGAA- 3') and PVY 2R (5'-GGCGGAGTATGCACCAAGTA-3') (Fuentes et al. 2019) primers were used. Amplification was conducted in 25 µl reaction mixtures containing 1 µl of template DNA, 0.25 µl of each forward and reverse primer and 12.5 µl of My Taq Mix (Bioline). Amplification for PVY 1 and PVY 2 primers was carried out in a thermal cycler (Thermoscientific) with the following conditions: an initial step at 95 °C for 1 min; followed by 35 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 10 s; and a final extension of 72 °C for 7 min. Amplification for PVY 2F and PVY 2R primers was carried out in a thermal cycler (Thermoscientific) with the following conditions: an initial step at 95 °C for 1 min; followed by 35 cycles at 95 °C for 15 s, 61 °C for 15 s, and 72 °C for 10 s; and a final extension of 72 °C for 7 min.

A 5 µl aliquot of each PCR product was separated by electrophoresis on 1.5% (wt/vol) agarose (Lonza) in TAE buffer (40 mM Tris acetate, 1 mM EDTA) stained with ethidium bromide solution (0.1 mg/litre). The gel was visualized using a UV transilluminator (UVitec UK). An amplicon size of 807 bp is expected for PVY.

4.2.5 Inoculum preparation

4.2.5.1 Fungal isolates

Inoculum of fungal isolates (Table 4.1) was prepared following the protocol of Muzhinji et al. (2014). Briefly, fungal isolates were first cultured on PDA for 2 weeks in the dark at 25 °C. Barley grains, 10 g per pot and moistened with distilled water, were added to an Erlenmeyer flask and autoclaved twice at 121 °C for 30 min with a 24 h interval. Five PDA plugs of an actively growing culture of each isolate, 10 mm in diameter, were then added to the representative Erlenmeyer flask. The fungal isolates were incubated on the barley grains at room temperature for 14 days, shaking at 2-day intervals, until the barley grains were fully colonized by visible fungal mycelia. Each flask was checked each day for visible signs of contamination and contents of contaminated flasks were discarded.

Table 4.1. Fungal isolates obtained from corky crack blemishes in South Africa and used for inoculum preparation and pathogenicity testing.

Isolate Number	Potato Growing Region	Isolate Identity	Accession Number
Rh6	Mpumalanga	*Rhizoctonia solani* AG 3-PT	KF234144
4	KwaZulu-Natal	Binucleate *Rhizoctonia* AG-A	MK828401
10	Eastern Free State	Binucleate *Rhizoctonia* AG-R	MK828399
P2mus	Limpopo	*Fusarium oxysporum*	MN720640

4.2.5.2 Bacterial isolates

Using two Petri dishes of three-week-old bacterial cultures (Table 4.2) on YMEA, a volume of 5 ml of sterile distilled water was added to each isolate and the spores were scraped off and mixed with 750 ml of Say's solution (20 g sucrose, 1.2 g L-asparagine, 0.6 g K_2HP0_4, 10 g yeast extract, in 1 L sterile distilled water: adjusted to pH 7.2) and 150 g vermiculite per pot (Wilson et al 2009). A volume of 5 ml was added to sterile YMEA plates and used as a negative control. Colonized vermiculite was used as inoculum and incubated at 28°C for three weeks.

Table 4.2. Bacterial isolates obtained from corky crack blemishes in South Africa and used for inoculum preparation and pathogenicity testing.

Isolate Number	Potato Growing Region	Isolate Identity	Accession Number
B9ii	Northern Cape	*Streptomyces viridochromogenes*	MK828372
B12	Northern Cape	*Streptomyces griseorubens*	MK828374
B9iii	Northern Cape	*Streptomyces corchorussi*	MK828374
B8iii	Eastern Free State	*Streptomyces yaanensis*	MK828370
B14	Northern Cape	*Streptomyces collinus*	MK828375

4.2.5.3 Co-inoculations

To evaluate the possibility of pathogen complexes causing corky crack blemishes on potato tubers, Koch's postulates were carried out with all possible combinations of micro-organisms isolated from these blemishes. The pathogen treatments used in pot trials are presented in Table 4.3.

Table 4.3. Pathogen treatments used in pot trials

Treatment	Pathogen(s)
1	*Rhizoctonia solani* AG 3-PT
2	Binucleate *Rhizoctonia* AG A
3	Binucleate *Rhizoctonia* AG R
4	*Streptomyces* species complex (*S. collinus*, *S. yaanensis*, *S. corchorussi*, *S. viridochromogenes* and *S. griseorubens*)
5	*Fusarium oxysporum*
6	*R. solani* AG 3-PT, binucleate *Rhizoctonia* AG-A, binucleate *Rhizoctonia* AG-R
7	*R. solani* AG 3-PT, binucleate *Rhizoctonia* AG-A, binucleate *Rhizoctonia* AG-R and *Streptomyces* species complex
8	*R. solani* AG 3-PT, binucleate *Rhizoctonia* AG-A, binucleate *Rhizoctonia* AG-R, *Streptomyces* species complex, *Fusarium oxysporum*
9	Uninoculated

4.2.6 Experimental design and layout

A greenhouse trial was conducted under normal watering (field capacity) and water stress conditions (just above permanent wilting point). The greenhouse experiment was repeated for both watering conditions. Each experiment was laid out using a randomized complete block design with nine treatments. Each treatment consisted of five replicates (Table 4.3). Data was analysed as a factorial design with the primary factor of water conditions and secondary factor the different pathogens.

Table 4.4. Standard soil analyses for plaster sand medium used in the greenhouse experiments.

Soil properties	Result
pH (H_2O)	6.74
pH (KCl)	5.42
EC (mS/cm) at 25 °C	20.3
% Carbon	0.02
% Sand	87.8
% Silt	2.2
% Clay	10
P Bray (mg/kg)	1.24
Ammonium acetate (mg/kg)	
K	12.59
Mg	67.41
Na	2.40
Ca	107.14
EDTA (mg/kg)	
Cu	0.41
Fe	10.71
Mn	32.65
Zu	1.59
Al	14.55

4.2.7 Environmental conditions

In the greenhouse, plants experienced 12 h natural light per day, with an average temperature of 26.5 ± 2 °C. The first trial was conducted from July 2018 to November 2018. The second trial was conducted from April 2019 to August 2019.

Disease-free mini-tubers (cv. Mondial) were planted in 5 L pots filled with steam-sterilized plaster sand. The physical and chemical properties of the plaster sand are provided in Table 4.4. Each pot was planted with a single seed tuber (experimental unit) at a depth of 10 cm. Ten grams of fungal inoculum was spread evenly on the sand layer above the tuber and then covered

with moist sand. Sterile barley grains were added to the soil in the non-inoculated treatments (negative controls). For bacterial inoculum, 150 g of fully colonized vermiculite was spread evenly on the sand layer above the tuber and then covered with moist sand. For co-inoculations, inoculum was prepared separately for each isolate and then mixed into the soil of each respective pot. Plants were fertilized with 1 g Multifeed nutrient solution (Nulandis) (3:1:6) per pot, once at planting and again at 2 months after emergence. Calcium sulphate ($CaSO_4$), 1 g per pot, was applied once at planting due to the calcium content of the plaster sand (Table 4.4).

The role of soil moisture in causing corky crack blemishes on potato tubers was assessed in pot trials. Soil moisture content is affected by the soil texture and also depends on the climate and cultural practices applied (Fiers et al. 2012). For moisture content, two different treatments were used, field capacity (normal watering conditions) and just above permanent wilting point (water stress conditions). To induce water stress plants were watered only once the first sign of wilting was observed (soil moisture therefore fluctuated between wilting point and field capacity). Plants in the normal watering condition treatments were not subjected to water stress and were watered every second day with approximately 400 ml of tap water (field capacity).

4.2.8 Pathogenicity testing and disease evaluation

To confirm Koch's postulates for microorganisms isolated from corky crack blemishes on potato tubers, pathogenicity tests were carried out in a greenhouse experiment. Tuber count and yield were measured for every pot and the incidence and severity of blemishes on harvested progeny tubers were evaluated 120 days after planting. Re-isolations were made from diseased progeny tubers as described previously to confirm pathogen identity and thus Koch's postulates. With modifications of Carling and Leiner (1990), disease severity was estimated using a 0-5 scale. 0 = no blemishes observed, 1 = less than 1% of the tuber surface is covered with blemishes, 2 = between 1 and 10% of the tuber surface is covered with blemishes, 3 = between 11 and 20% of the tuber surface is covered with blemishes, 4 = between 21 and 50% of the tuber surface is covered with blemishes and 5 = 51% or more of the tuber surface is covered with blemishes. To calculate the disease index, the following formula was used: Disease Index= $[(n_1+n_2+n_3+n_4+n_5)/N_{total}]$ x 100 where n_x is the number of tubers in the x severity rating category per replicate and N is the total number of tubers rated per replicate.

4.2.9 Statistical analysis

Data was analysed using the statistical analysis software ARM version 2019.8. The treatment means were separated using the least significant difference (LSD) calculated with Student-Newman-Keuls test at a 5% level of significance. Disease incidence and disease severity were scored and disease index was calculated.

4.3 Results

4.3.1 Fungal identification

The most prevalent fungal isolates were chosen as representative cultures for sequencing and identification. Sequencing results revealed that the most prevalent fungal species obtained from the three potato production regions in South Africa were Binucleate *Rhizoctonia* (BNR) AG-R, BNR AG-A and *Fusarium oxysporum* (Table 4.5). Genetic identification of isolates and GenBank accession numbers are shown in Table 4.5. BNR AG-R had dark brown mycelia and produced dark brown sclerotia on PDA (Figure 4.1a). BNR AG-A had light brown mycelia and produced light brown sclerotia on PDA (Figure 4.1b). Typical right-angle branches of the *Rhizoctonia* isolates were observed at the distal septae of cells (Figure 4.2). Binucleate *Rhizoctonia* isolates were obtained from Mpumalanga, Limpopo, KwaZulu-Natal and the Free State. *Fusarium oxysporum* had pink mycelium on PDA (Figure 4.1c) and microconidia were observed under the microscope (Figure 4.3). Most of the *F. oxysporum* isolates were obtained from Limpopo, Free State and KwaZulu-Natal.

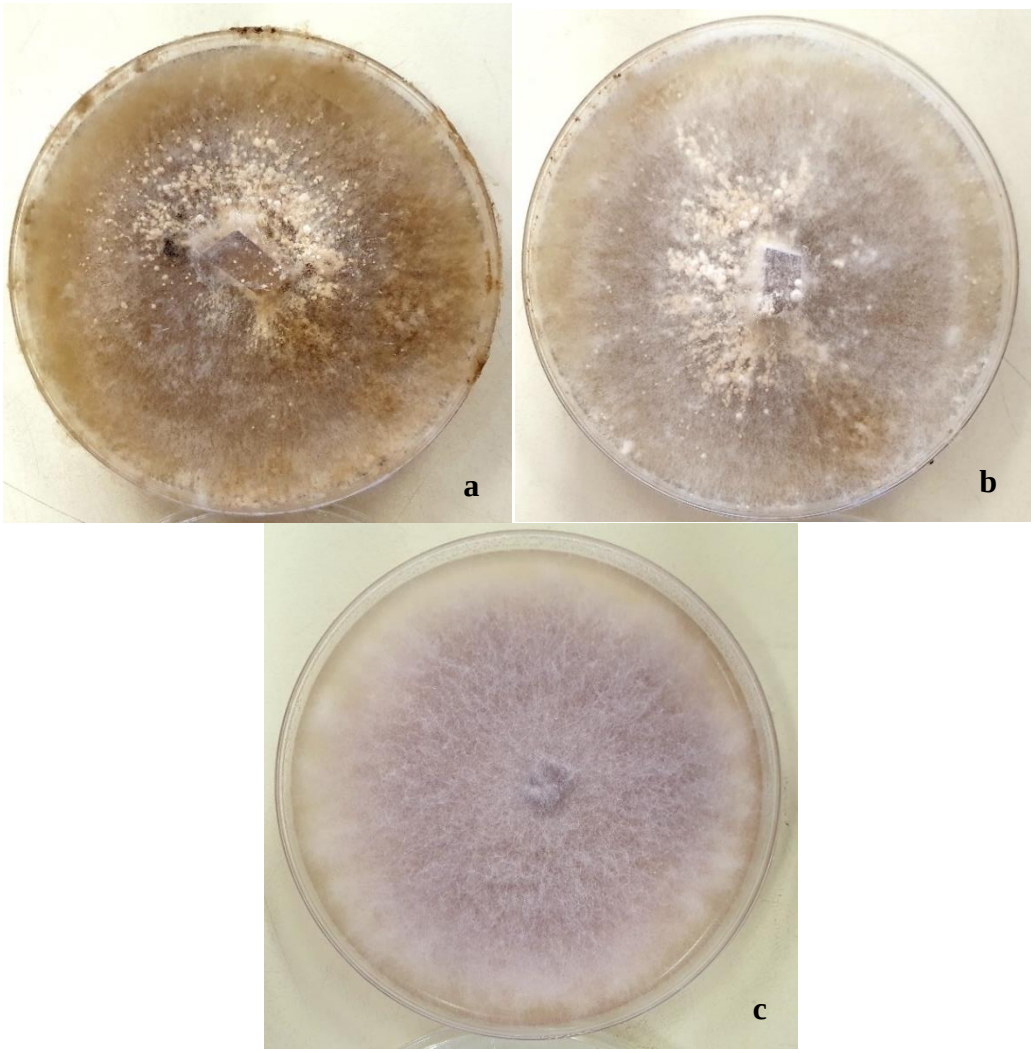


Figure 4.1. Four-week-old pure cultures of Binucleate *Rhizoctonia* AG-R (a), Binucleate *Rhizoctonia* AG-A (b) and *Fusarium oxysporum* (c) showing differences in hyphal colour on Potato Dextrose Agar (S. Gush).

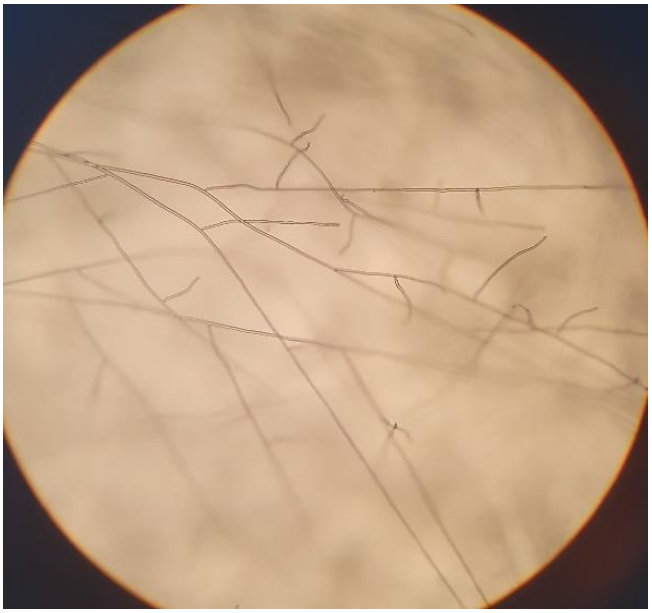


Figure 4.2. Binucleate *Rhizoctonia* showing typical right-angled branching of mycelium, observed under the microscope at 100 X magnification (S. Gush).

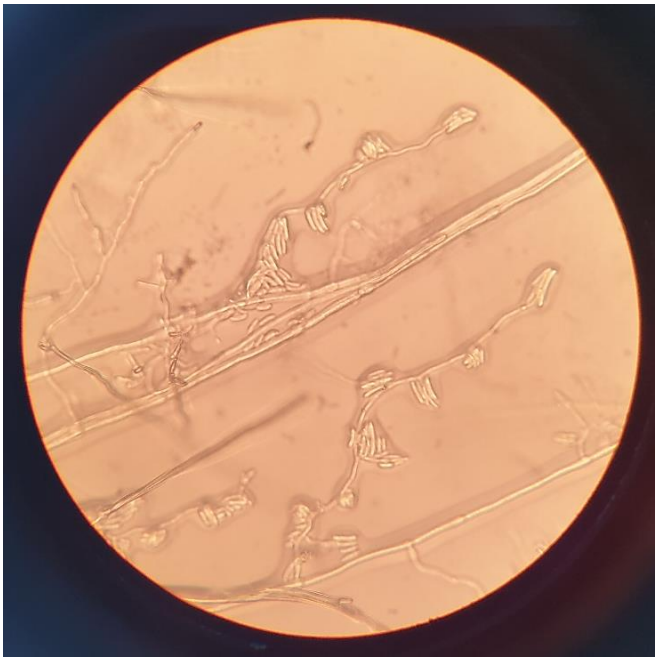
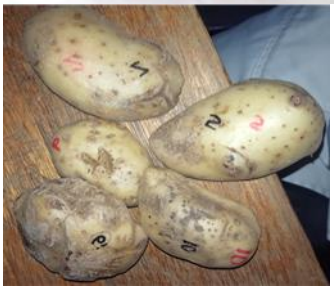


Figure 4.3. *Fusarium oxysporum* showing typical microconidia observed under the microscope at 400 X magnification (S. Gush).

Table 4.5. Most prevalent fungal isolates isolated from corky crack blemishes on tubers in South Africa

Isolate Number	Potato Growing Region	Cultivar	Symptoms	Identified Organism	GenBank Accession Number
P3i	Middleburg	Mondial		*Fusarium oxysporum*	MN720641
10	Eastern Free State	Mondial		Binucleate *Rhizoctonia* AG-R	MK828399
P2mus	Limpopo	Mondial		*Fusarium oxysporum*	MN720640
F2C8	KwaZulu-Natal	Mondial		Binucleate *Rhizoctonia* AG-A	MK828400
P6	Warden, Free State	Mondial		*Fusarium oxysporum*	MN720642

P15	KwaZulu-Natal	Mondial		*Fusarium oxysporum*	MN720643
P16	KwaZulu-Natal	Mondial		*Fusarium oxysporum*	MN720644
4	KwaZulu-Natal	Mondial		Binucleate *Rhizoctonia* AG-A	MK828401
10	Eastern Free state	Mondial		Binucleate *Rhizoctonia* AG-R	MK828399
6	Eastern Free State	Mondial		Binucleate *Rhizoctonia* AG-A	MK828398

4.3.2 Bacterial identification

The *Streptomyces* colonies ranged from light grey to dark grey/blue-green in colour on the YME agar (Figure 4.4). The *Streptomyces* species confirmed by the appearance of corkscrews and spore cain structures when observed under the microscope (Figure 4.5). Sequencing results showed the most prevalent bacterial species were *Streptomyces yaanensis*, *S. viridochromogenes*, *S. corchorussi*, *S. griseorubens* and *S. collinus* (Table 4.6). Genetic characterization and GenBank accession numbers are shown in Table 4.6. Most of the *Streptomyces* isolates were obtained from the Northern Cape and Free State.

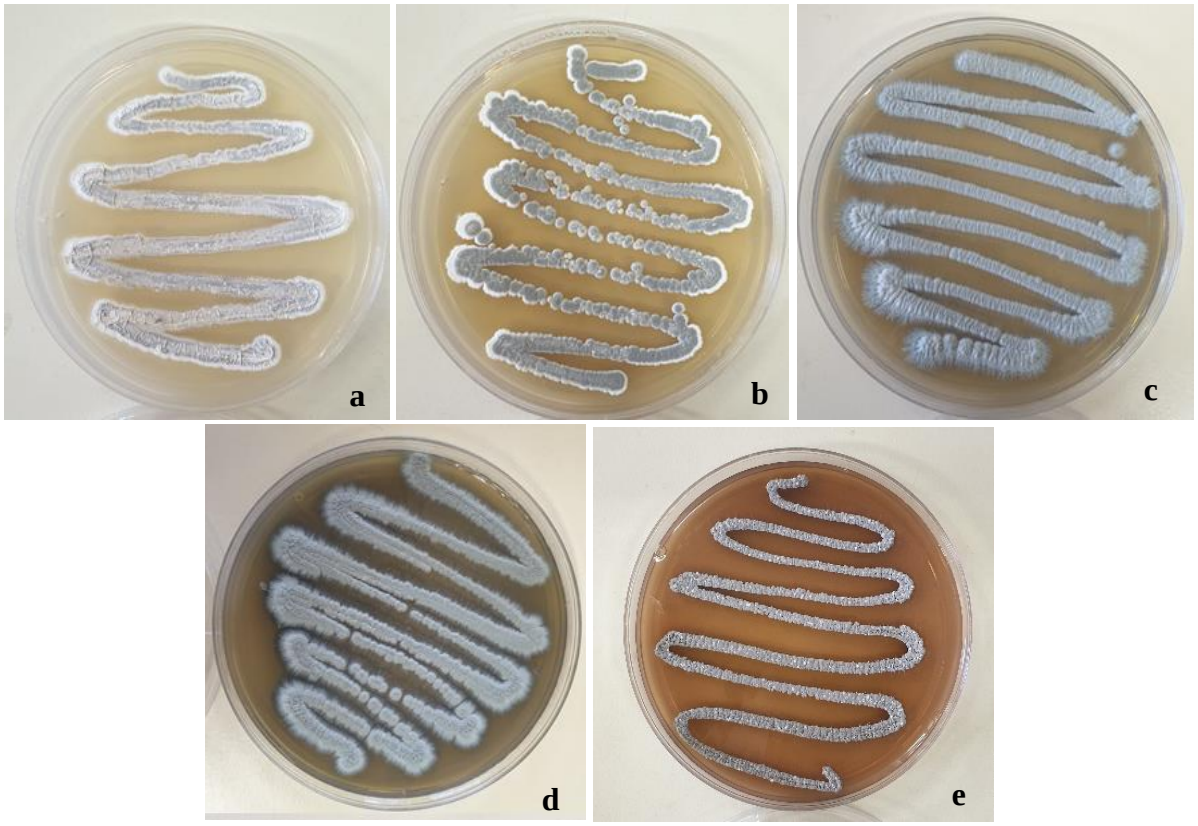


Figure 4.4. Two-week-old pure cultures of *S. collinus* (a), *S. yaanensis* (b), *S. corchorussi* (c), *S. viridochromogenes* (d) and *S. griseorubens* (e) showing different colony size, colony colour and changes in colour of Yeast Malt Extract Agar (S. Gush).

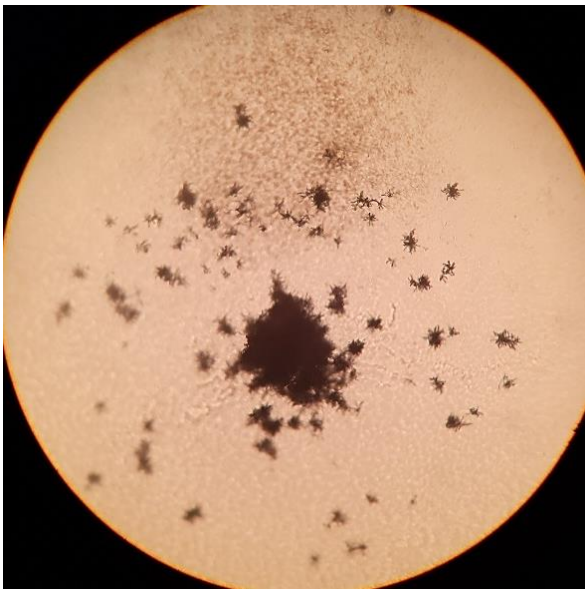


Figure 4.5. *Streptomyces* species with typical corkscrew-like structures observed under the microscope at 100 X magnification (S. Gush).

Table 4.6. *Streptomyces* species isolated from corky crack blemishes on tubers in South Africa

Isolate Number	Potato Growing Region	Cultivar	Symptoms	Identified Organism	GenBank Accession Number
B9ii	Northern Cape	Mondial		*Streptomyces viridochromogenes*	MK828372
B12	Northern Cape	Mondial		*Streptomyces griseorubens*	MK828374

B9iii	Northern Cape	Mondial		*Streptomyces corchorussi*	MK828374
B8iii	Eastern Free State	Mondial		*Streptomyces yaanensis*	MK828370
B14	Northern Cape	Mondial		*Streptomyces collinus*	MK828375

4.3.3 PVY detection

RNA extraction and cDNA synthesis were performed on the blemished samples and three positive controls. The agarose gel electrophoresis showed amplification in only one of the three positive controls, + PVY (Figure 4.6). The leaf (+L) and tuber (+T) samples did not amplify since the tissue of these samples may have already been degraded. A total of 24 samples were tested, but none of the samples amplified. These results are shown on the agarose gel electrophoresis after RT-PCR using primers PVY 1 and PVY 2 (Figure 4.6) as well as PVY 2F and PVY 2R (Figure 4.7). In both gels, + PVY was used as a positive control.

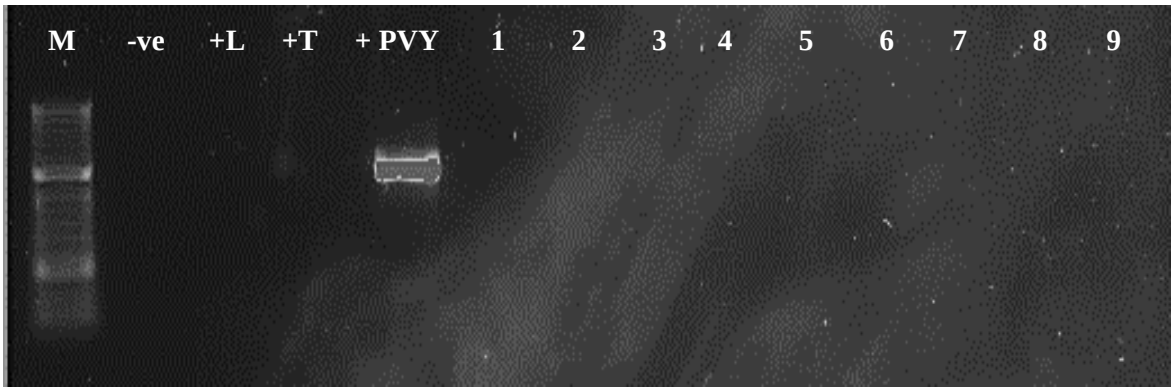


Figure 4.6. Gel electrophoresis image showing a 50 bp marker (M), leaf (+ L), tuber (+ T), positive freeze-dried leaf sample (+ PVY) and samples 1 to 9 subjected to RT-PCR using primers PVY 1 and PVY 2 (S. Gush).

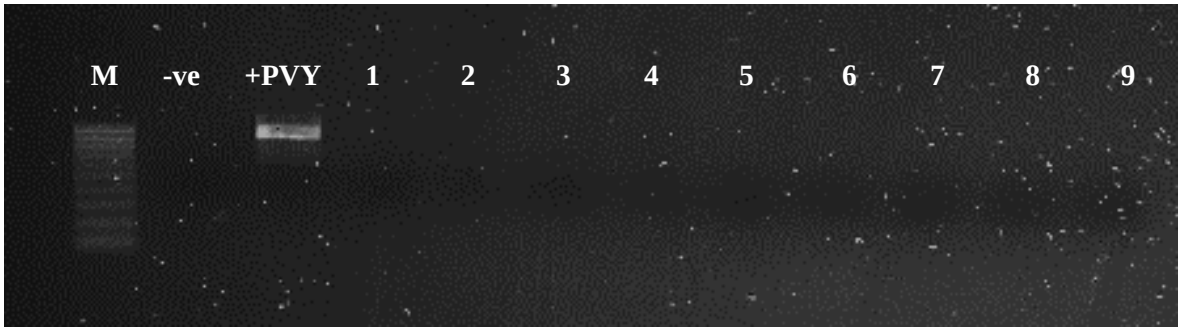


Figure 4.7. Gel electrophoresis image showing a 100 bp marker (M), positive freeze-dried leaf sample (+ PVY) and samples 1 to 9 subjected to RT-PCT using primers PVY 2F and PVY 2R (S. Gush).

4.3.4 Greenhouse trials

No corky crack symptoms were reproduced on progeny tubers from single inoculations of *R. solani* AG 3-PT (treatment 1), BNR AG-A (treatment 2), BNR AG-R (treatment 3), the *Streptomyces* species complex (treatment 4) or *F. oxysporum* (treatment 5) in either of the pot trials. However, in the single inoculations each isolate produced disease symptoms characteristic to the specific species. Inoculation with *R. solani* AG 3-PT, BNR AG-A and BNR AG-R resulted in symptoms typically caused by *Rhizoctonia* species on potatoes, such as

elephant hide, black scurf and tuber deformation (Figure 4.8). *Fusarium oxysporum* resulted in stolon end rot (Figure 4.9) and the *Streptomyces* species complex resulted in common scab symptoms (Figure 4.10) on the progeny tubers.

In the first pot trial, corky crack blemishes were observed on progeny tubers in the co-inoculation treatments of the *Rhizoctonia* species complex (AG 3-PT, BNR AG-A and BNR AG-R) (treatment 6) (Figure 4.11) under normal watering and water stress conditions as well as the co-inoculation of the *Rhizoctonia* species complex and the *Streptomyces* species complex (*S. collinus*, *S. yaanensis*, *S. corchorus*si, *S. viridochromogenes* and *S. griseorubens*) (treatment 7) (Figure 4.10) under normal watering conditions. In the second pot trial, corky crack blemishes were reproduced in the all the co-inoculation treatments (treatments 6, 7 and 8) under normal watering and water stress conditions. *R. solani* AG 3-PT, BNR AG-A and BNR AG-R were successfully re-isolated from corky crack lesions on progeny tubers from treatments 6, 7 and 8. However, the *Streptomyces* species and *F. oxysporum* could not be re-isolated from corky crack lesions on progeny tubers from treatments 7 and 8, respectively. No disease symptoms were observed on control tubers (treatment 9) under normal watering or water stress conditions, in either pot trial.

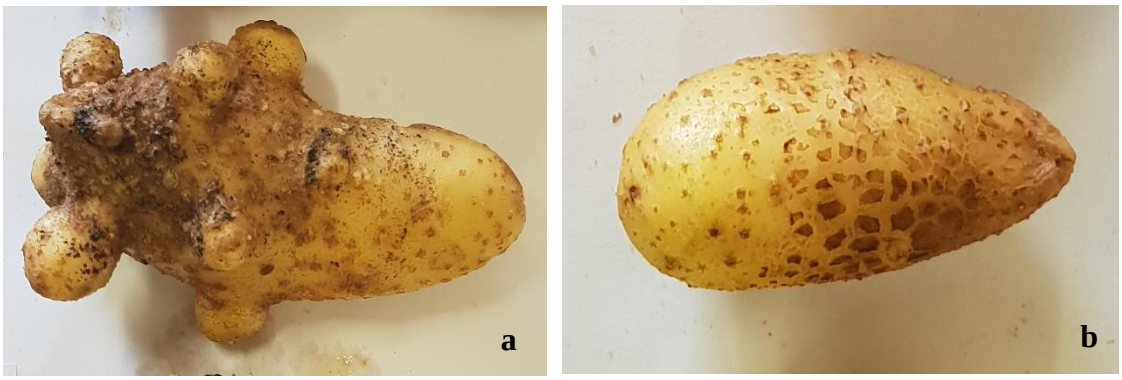


Figure 4.8. (a) Progeny tubers from the greenhouse experiment showing symptoms of black scurf and deformation (treatment 2) and (b) elephant hide (treatment 2).

Figure 4.9. Progeny tuber from the greenhouse experiment showing symptoms of stolon end rot (treatment 7)

Figure 4.10. Progeny tuber from the greenhouse experiment showing symptoms of common scab (treatment 5).

Figure 4.11. Progeny tubers from the greenhouse experiment showing corky crack blemishes (treatment 6).

4.3.5 Yield and disease evaluation

According to the univariate procedure and normality plots, the data was normally distributed with homogenous treatment variance.

When comparing the two watering control treatments (normal watering and water stress conditions), there was no significance difference in terms of the number of tubers and yield at $P = 0.05$ (Table A1, A2, A3 and A4, appendix). Significant differences in yield ($P < 0.05$) were observed between the normal watering and water stress conditions for the *Rhizoctonia* species complex (treatment 6). Significantly lower yield ($P < 0.05$) was observed in the treatment receiving the *Rhizoctonia* species complex under water stress conditions compared to the same treatment under normal watering conditions. Progeny tubers from plants in the *Rhizoctonia* species complex treatment had significantly ($P < 0.05$) higher disease index under normal watering conditions compared to those from plants under water stress conditions.

When comparing the *Rhizoctonia* species complex treatment (treatment 6) to the single inoculations of *R. solani* AG 3-PT (treatment 1), BNR AG-A (treatment 2) and BNR AG-R (treatment 3) under normal watering conditions alone, significant differences ($P < 0.05$) were observed in terms of disease index. No corky cracks were observed in treatment 1, 2 and 3 as compared to a high disease index of corky cracks in treatment 6. The same differences ($P < 0.05$) were observed under water stress conditions.

When comparing the *Rhizoctonia* species complex treatment (treatment 6) to the *Rhizoctonia* species complex + *Streptomyces* species complex treatment (treatment 7) under normal watering conditions, there were significant differences ($P < 0.05$) in terms of tuber yield and disease index. treatment 6 had higher disease index as compared to treatment 7. Under water stress conditions, no corky cracks were observed in treatment 7 as compared to a high disease index of corky cracks in treatment 6.

No significant differences ($P > 0.05$) were observed with regards to tuber count, for the *Rhizoctonia* species complex + *Streptomyces* species complex under normal watering conditions or under water stress conditions.

Overall, the same trend in disease index values were observed in the first and second pot trial, except for the *Rhizoctonia* species complex + *Streptomyces* species complex + *F. oxysporum* treatment (treatment 8) in the second pot trial. Corky cracks were reproduced in treatment 8

under normal watering and water stress conditions in the second pot trial, whereas no corky cracks were reproduced in treatment 8 in the first pot trial. The disease incidence, severity and index means for both pot trials are shown in table 4.7, 4.8, 4.9 and 4.10).

Table 4.7. Disease incidence, severity and index means of corky crack blemishes on the different treatments in the first pot trial under normal watering conditions

Treatment	Disease incidence	Disease severity	Disease index
R. solani 3-PT	0 c	0 c	0 c
BNR AG-A	0 c	0 c	0 c
BNR AG-R	0 c	0 c	0 c
Streptomyces species complex	0 c	0 c	0 c
F. oxysporum	0 c	0 c	0 c
R. solani complex	55.71 a	1.68 a	104.21 a
R. solani complex + *Streptomyces* species complex	33.71 b	0.95 b	37.40 b
R. solani complex + *Streptomyces* species complex + *F. oxysporum*	0 c	0 c	0 c
Uninoculated control	0 c	0 c	0 c

*Numbers in the same column that share the same letters are not significantly different at P = 0.05.

Table 4.8. Disease incidence, severity and index means of corky crack blemishes on the different treatments in the second pot trial under normal watering conditions

Treatment	Disease incidence	Disease severity	Disease index
R. solani 3-PT	0 c	0 c	0 c
BNR AG-A	0 c	0 c	0 c
BNR AG-R	0 c	0 c	0 c
Streptomyces species complex	0 c	0 c	0 c
F. oxysporum	0 c	0 c	0 c
R. solani complex	58.50 a	2.04 a	136.46 a
R. solani complex + *Streptomyces* species complex	21.38 b	0.51 b	14.14 b
R. solani complex + *Streptomyces* species complex + *F. oxysporum*	8.57 c	0.14 c	6.12 c
Uninoculated control	0 c	0 c	0 c

*Numbers in the same column that share the same letters are not significantly different at P = 0.05.

Table 4.9. Disease incidence, severity and index means of corky crack blemishes on the different treatments in the first pot trial under water stress conditions

Treatment	Disease incidence	Disease severity	Disease index
R. solani 3-PT	0 b	0 b	0 b
BNR AG-A	0 b	0 b	0 b
BNR AG-R	0 b	0 b	0 b
Streptomyces species complex	0 b	0 b	0 b
F. oxysporum	0 b	0 b	0 b
R. solani complex	30.33 a	0.77 a	34.12 a
R. solani complex + *Streptomyces* species complex	0 b	0 b	0 b
R. solani complex + *Streptomyces* species complex + *F. oxysporum*	0 b	0 b	0 b
Uninoculated control	0 b	0 b	0 b

*Numbers in the same row which share the same letters are not significantly different at P = 0.05.

Table 4.10. Disease incidence, severity and index means of corky crack blemishes on the different treatments in the second pot trial water stress conditions

Treatment	Disease incidence	Disease severity	Disease index
R. solani 3-PT	0 c	0 c	0 c
BNR AG-A	0 c	0 c	0 c
BNR AG-R	0 c	0 c	0 c
Streptomyces species complex	0 c	0 c	0 c
F. oxysporum	0 c	0 c	0 c
R. solani complex	16.60 a	0.50 a	12.23 a
R. solani complex + *Streptomyces* species complex	2.50 b	0.03 b	0.31 b
R. solani complex + *Streptomyces* species complex + *F. oxysporum*	0 c	0 c	0 c
Uninoculated control	0 c	0 c	0 c

*Numbers in the same column that share the same letters are not significantly different at P = 0.05.

4.4 Discussion and conclusion

Atypical tuber blemishes were first described by Fiers et al. (2010), as potato skin blemished due to unknown causes. In South Africa, there has been an increase in the incidence of various atypical blemishes, such as ‘growth cracks’ and ‘corky cracks’ on potato tubers (Muzhinji et al. 2014). The occurrence and causal agents of these tuber blemishes on potato have been one of the most contentious issues in the potato industry in South Africa (Gouws and McLeod 2012; Muzhinji et al. 2014). Similar blemishes have been found in Zimbabwe (Zimudzi et al. 2017), Asia (Gherbawy and Gashgari 2013) and Europe (Fiers et al. 2010; Woodhall et al. 2011; Fiers et al. 2012).

Corky crack blemishes were collected from different growing regions in South Africa, namely Limpopo, KwaZulu-Natal, Eastern Free State and Mpumalanga. Tubers with growth cracks were observed on tubers collected from the Northern Cape region. The corky crack blemishes in the present study showed corky crack blemishes similar to those that have previously been observed in South Africa (Muzhinji et al. 2014; Zimudzi et al. 2017) and in other parts of the world such as Asia (Gherbawy and Gashgari 2013) and Europe (Fiers et al. 2010; Fiers et al. 2012).

Tuber blemishes may result from biotic or abiotic factors. The objective of this study was to investigate the microbial origin of corky crack blemishes on potato tubers in South Africa. The most prevalent fungal species isolated from the corky crack symptoms in the present study included binucleate *Rhizoctonia* (BNR) and *Fusarium* species. The same results were observed in France and Saudi Arabia where *Fusarium* species were isolated most frequently from blemished tubers (Fiers et al. 2010; Gherbawy and Gashgari 2013). However, it is known that *Fusarium* and *Rhizoctonia* species are present on the surface of potato tubers as well as in the rhizosphere of the potato plants (Cwalina-Ambroziak 2002; Pieta and Patkowska 2003). It has been reported that *Fusarium* species can be saprophytes or opportunistic pathogens on the surface of plants, (Chehri et al. 2011; Zimudzi et al. 2017).

In this study, fungal species isolated from corky cracks were identified through PCR and sequencing as binucleate *Rhizoctonia* (BNR) AG-A, binucleate *Rhizoctonia* AG-R and *Fusarium oxysporum*. *R. solani* AG 3-PT was also included as a treatment, although it was not isolated from the initial symptoms. However, various studies in South Africa and Zimbabwe have isolated *R. solani* AG 3-PT from corky crack symptoms. In a study conducted by Muzhinji et al. (2014), tubers with symptoms of elephant hide and cracking (similar to the corky crack blemish symptom) were collected from nine potato growing regions in South Africa, where four of the regions were the same as in the present study. Results from the study indicated that *Rhizoctonia solani* AG 3-PT was the most frequently isolated pathogen from diseased potatoes and it was shown to be the causal agent of elephant hide and cracking on potatoes in South Africa. Later, Zimudzi et al. (2017) confirmed that *R. solani* AG-3PT causes tuber cracking and elephant hide in Zimbabwe.

In the present study, single inoculations of the fungal and bacterial isolates did not reproduce corky cracks and therefore Koch's postulates were not fulfilled for *R. solani* AG 3-PT, BNR AG-A, BNR AG-R and *Fusarium oxysporum*. However, well-documented symptoms of each

pathogen were observed. For example, *R. solani* AG 3-PT, BNR AG-A and BNR AG-R produced symptoms of black scurf, elephant hide and deformation on the progeny tubers, typical to the Rhizoctonia disease on potatoes (Banville 1989; Campion et al. 2003; Muzhinji et al. 2014). Previously, Muzhinji (2014) characterized *R. solani* AG 3-PT as the causal pathogen of cracking on potato tubers. However, Koch's postulates were not fulfilled for *R. solani* AG 3-PT to cause corky cracks in the present study. This could be due to the fact that the *R. solani* AG 3-PT isolate had lost its pathogenicity over time from re-plating after storage (Jinks 1952). This study confirmed that *R. solani* AG 3-PT causes elephant hide on potato tubers in South Africa, as reported by Muzhinji et al. (2014). The elephant hide symptom has also been reported in France (Fiers et al. 2010); however, the causal agent was not confirmed. Progeny tubers of potato plants in this study inoculated with *F. oxysporum* developed stem-end rot disease. In South Africa, stem-end and dry rot disease of potato, caused by *F. oxysporum*, *F. solani* still commonly occurs throughout the country (Theron 1989). *F. oxysporum* has been found to cause stem-end rot and dry rot elsewhere (Pont 1976; Peters et al. 2008; Aktaruzzaman et al. 2014).

There are 16 known AGs in the binucleate group of *Rhizoctonia* (Sharon et al. 2008). BNR have been thought to be saprophytic and some have been confirmed to be biological control agents against plant pathogens (Carling and Leiner 1986). However, BNR have recently been associated with several diseases on various plants. These include BNR AG-A, AG-K, AG-U and AG-F being pathogens of several crops in China (Yang et al. 2014; Yang et al. 2015), unknown BNR causing elephant hide on potato tubers in Great Britain (Woodhall et al. 2011) and BNR AG-A causing disease on potato stems and sugar beetroots in the Pacific Northwest (Miles et al. 2013). In South Africa, BNR AG-A and AG-R were reported to be the causal pathogens of black scurf and stem canker on potato (Muzhinji et al. 2015) and in a recent study, Zimudzi et al. (2017) described the first report of BNR AG-A causing tuber malformations, which include linear and circular defects. BNR has also been found on apples in South Africa (Tewoldemedhin et al. 2011). With the past and present frequent isolations of pathogenic BNR from tuber blemishes, it is prudent to investigate the role of BNR in causing disease on potatoes. The present study supports the increasing importance of pathogenic BNR in causing potato tuber blemishes.

Host range, pathogenicity, ecology, mycelium colour and physiology vary within a given AG of a BNR. More work on the classification and taxonomy of BNR is still needed to identify

additional AGs and subgroups and determine the diversities within and between AGs, using molecular methods. Various unidentified BNRs have been reported to cause severe damage on potato plants (Lehtonen et al. 2008; Muzhinji et al. 2015; Woodhall et al. 2011; Zimudzi et al. 2017), therefore it is important for additional research to be conducted to elucidate possible existence of new AGs and subgroups within BNR.

This study showed that co-inoculation of plants with *R. solani* AG3 -PT, BNR AG-A and BNR AG-R resulted in the development of corky crack blemishes on potato tubers in South Africa. This suggests the possibility of a synergistic interaction between these *Rhizoctonia* species, where their combined effect was greater than the sum of their separate effects and lead to the development of corky crack blemishes on the progeny tubers. The present study took into consideration disease complexes and various combinations of all microbial pathogens isolated from the original diseased tubers. Most studies on pathogenicity tests have focused on inoculation of plants with single microbial strains; however, microbes occur in complex multispecies communities in nature, many of which have been reported to cause diseases on plants (Lamichhane and Venturi 2015).

This study confirms the role of favourable environmental conditions for a plant pathogen to cause more severe disease symptoms. According to Jeger et al. (1996), it is important to avoid cold, moist and poorly drained soils when planting as these conditions favour the rapid growth of *Rhizoctonia* species in the soil. The high disease severity of the *Rhizoctonia* species complex (treatment 6) can, therefore, be explained by the fact that *Rhizoctonia* species require moist soil environments for infection to take place as demonstrated in the current study. Various other studies indicate that temperature is another important factor together with a moist soil environment in infection of plants by *Rhizoctonia* species (Harrison 1978; Hide and Firmager 1989; Jeger et al. 1996). The penetration of plants by *Rhizoctonia* species is through intact cuticle and epidermis or through natural openings such as lenticels, stomata and wounds (Dijst 1990). It is also known that at high soil moisture contents, lenticels on the tuber open, thus allowing for infection by a pathogen to take place (Helias 2008). For example, *R. solani* requires at least 45 % soil moisture for infection to take place (Fiers et al. 2012).

Greenhouse trials in this study were performed in warmer conditions (25 °C) than other similar studies (18 °C). It has been reported that various AG groups of *Rhizoctonia*, including BNR AG-A and AG-R, are able to infect at warmer temperatures. It is possible that *Rhizoctonia* populations in South African growing regions have adapted to the warm South African climate

(Muzhinji et al. 2015; Zimudzi et al. 2017). This is an indication that the introduction of pathogens into new environments due to changes in pathogen dispersal, land use or climate change will result in new pathogen-pathogen or pathogen-host interactions (Rosenzweig et al. 2001). These changes allow the introduced pathogens to adapt and cause severe infection in native host populations. The pathogens do not only affect the host plants directly but also the interactions with their respective pathogens, therefore, can result in more severe disease or the development of new diseases (McDonald 1997; Chakraborty and Newton 2011). Host plants adapt much slower to altered environmental conditions than pathogens, as pathogens have short lifecycles and more effective dispersal mechanisms (Garrett et al. 2006). Pathogens with short lifecycles, high dispersal mechanisms and efficient reproduction rates tend to respond fast to climate change and will in turn adapt faster to the change of environmental conditions (Coakley et al. 1999; Yáñez-López et al. 2012). It is important to understand the potential effects of climate change on plant pathogens and future risks of crop diseases to develop effective control strategies (Juroszek et al. 2019).

No fungal species were isolated from the initial growth crack symptoms in the present study. It has been speculated that these growth crack symptoms are due to severe infection of stolons by microbial pathogens including Potato Tuber Spindle Viroid (PSTVd), Potato Mop-Top virus (PMTV), or from and abiotic factors such as water stress, or herbicide damage (Fiers et al. 2010; Bouchek-Mechiche et al. 2013). The abiotic factors can cause tuber cracking, bruising or enlargement of lenticels, thus creating wounds on the tuber and an entry point for pathogens (Fiers et al. 2012). The present study included water stress in the greenhouse experiment to test if water stress will create a corky crack symptom or whether normal growth cracks will develop from the water stress treatment. However, no growth cracks on progeny tubers were observed after harvest in the normal watering or water stress control treatments.

Streptomyces species were not isolated from corky crack blemishes in the present study. Similarly, Fiers et al. (2010), did not isolate *Streptomyces* species from corky blemished tubers. However, *Streptomyces* species were isolated from the tuber samples that showed growth crack symptoms in the present study. All these isolates showed differences in colony size, colony colour on yeast malt extract agar as well as changes in the colour of the media itself. PCR and sequencing were performed to identify the *Streptomyces* isolates to species level. The most prevalent *Streptomyces* species isolated from the growth cracks were *S. viridochromogenes, S. griseorubens, S. corchorussi, S. yaanensis, S. cinerochromogenes* and *S. collinus*. *S.*

corchorussi and *S. viridochromogenes* showed similarities in colony size, colour and changes in the colour of the media. However, sequencing results showed that they are two separate species. This highlights the importance of molecular identification when identifying microbes. These *Streptomyces* species are not considered to be plant pathogens (Janssen et al. 1989). Most of these species are known to be natural inhabitants in the soil (Weitnauer et al. 2001; Rückert et al. 2013; Zheng et al. 2013; Al-Askar et al. 2015). However, it has been reported that *S. corchorussi* may cause deep-pitted scab on potatoes (Archuleta and Easton 1981). Koch's postulates were not fulfilled in terms of reproducing corky crack blemishes on the progeny tubers in this study and, thus, it can be confirmed that these *Streptomyces* species are not likely to be the causal agents of the corky crack disease on potato tubers in South Africa. The potato plants inoculated with the *Streptomyces* species complex did; however, result in progeny tubers with common scab symptoms.

The *Streptomyces* genus is very large containing mostly saprophytic bacteria that are ubiquitous and very abundant in the soil (Janssen 2006; Wanner 2009). They are well-known for their antimicrobial, anti-inflammatory and antibiotic compounds (Hopwood 2006) and several species contain virulence genes encoding enzymes for the biosynthesis of thaxtomin, which are clustered on the conserved and mobile pathogenicity island (PAI). These genes include *txtAB, tomA* and *nec 1* (Loria et al. 2006; Jordaan and Van der Waals 2016). The genes on the PAI are capable of being transferred from bacterium to bacterium, as well as to different species through the horizontal gene transfer mechanism, creating new pathogenic species and a concomitant increase in the number of pathogenic *Streptomyces* identified (Loria et al. 2006). The currently known *Streptomyces* species causing scab symptoms on potato are *S. scabiei* (Loria et al. 2006), *S. stelliscabiei* (Bouchek-Mechiche et al. 1998), *S. acidiscabies* (Loria et al. 2006), *S. turgidiscabies* (Miyajima et al. 1998), *S. europeiscabiei* (Bouchek-Mechiche et al. 2000; Jordaan and van der Waals 2015), *S. ipomoeae* (Loria et al. 2008), *S. luridiscabiei, S. puniciscabiei, S. niveiscabiei* (Park et al. 2003), *S. bottropensis, S. reticuliscabiei,* (Bouchek-Mechiche et al. 2000), *S. caviscabiei* (Goyer et al. 1996), *S. aureofaciens* (Faucher et al. 1992), *S. griseus* (Corbaz 1964) and *S. sp. IdahoX* (Wanner 2007). Furthermore, various common scab symptoms have been described on potato tubers in South Africa; however, they were not been associated with one specific pathogenic *Streptomyces* species (Jordaan and Van der Waals 2016). The *Streptomyces* species in the present study were re-isolated from the progeny tubers showing typical common scab symptoms after harvest. Thus, further studies should consider

screening the non-pathogenic *Streptomyces* species found in this study to confirm whether they carry the virulence genes located on the pathogenicity island. The pathogenicity of the strains could be confirmed by the presence and expression of *txtAB, tomA* and *nec 1* genes in future studies (Lambert and Loria 1989; Lehtonen et al. 2004).

Symptoms of common scab disease can vary from erumpent, pitted or superficial and the severity depends on the virulence of the bacterial strain and susceptibility of the potato cultivar (Lambert and Loria 1989). For example, *S. turgidiscabies* symptoms were denoted as severe, rough, erumpent, corky lesions in Japan (Takeuchi et al. 1996) and were later classified as deep-pitted lesions in Finland (Kreuze et al. 1999). The technique of isolating the microflora at the surface of the blemished tissue of the tubers may have been ineffective in isolating all the pathogenic *Streptomyces* strains present. Extracting DNA directly from the symptoms or using a metagenomics approach may result in an even larger spectrum of microorganisms, which enables the consideration of all possible causal agents (Fiers et al. 2010). Thus, it is unclear if the *Streptomyces* species isolated from the symptoms in this study represent the causal agents of the growth cracks. Further research is required to study the role of non-pathogenic *Streptomyces* in the development of growth crack disease.

When the *Streptomyces* species complex was combined with the *Rhizoctonia* species complex in co-inoculations, disease severity was significantly lowered compared to the *Rhizoctonia* species complex alone. This suggests a possible antagonistic effect between the two pathogen groups, competing for the same host and nutrients. Schrey and Tarkka (2008), described the complex interactions that occur in the rhizospheres of plants between *Streptomyces* species, plant roots and other microbes present in the soil. It was suggested that *Streptomyces* species can act as a direct antagonist or as a growth promoter of soil microorganisms due to their ability to produce various secondary metabolites. The *Rhizoctonia* fungus colonizes below ground plant surfaces in response to root and shoot exudates (Jeger et al. 1996).

Biocontrol involves the use of organisms (bioagents) that are antagonistic to target pathogens, such as *R. solani*. The mechanisms of biocontrol involve the production of antibiotics from the biocontrol agent, the biocontrol agent becomes endophytic in the host plant or parasitizes the target pathogen for resources. Antagonistic pathogens against *R. solani* have been reported with various biocontrol agents including some fungi and bacteria. These include *Pseudomonas fluorescens* (Howell and Stipanovic 1979), *Bacillus* spp. (Pleban et al. 1995) *Trichoderma* spp.

(Tsror et al. 2001), *Gliocladium* spp. (Lewis and Lumsden 2001), *Verticillium biguttatum* (Van den Boogert and Velvis 1992), BNR and nematodes (Escande and Echandi 1991).

Further research should be done to study the interaction of *Streptomyces* species in the soil with pathogenic *Rhizoctonia* species on potato plants, to develop possible biocontrol strategies against diseases caused by *Rhizoctonia* species. It has been suggested that *Streptomyces* species are effective as antagonists and biocontrol agents against fungi (Handelsman and Stabb 1996). Proposed mechanisms include hyperparasitism, synthesis of hydrolytic enzymes and toxic compounds, such as antibiotics or in competition with one another (Viterbo et al. 2007). In a study conducted by Al-Askar et al. (2015), it was demonstrated that *Streptomyces griseorubens*, also found in the present study, inhibited the mycelial growth of *R. solani.*

In this study, the disease index decreased the more pathogens co-inoculated with one another. This contradicts what some previous research has stated, that higher disease index values can be observed under co-inoculation with different pathogens (Begon et al. 1986; Le May et al. 2009). This is most often observed between a bacterial and a fungal interaction (Dung et al. 2014). However, the highest disease index was observed in the co-inoculation of the *Rhizoctonia* species complex in this study. When *Streptomyces* species and *F. oxysporum* were added to this combination, the overall disease index decreased. This suggests that the interaction between the *Rhizoctonia* species in this study is a synergistic interaction and not an additive effect.

In South Africa, PVY is one of the most devastating viruses and is monitored by the potato certification scheme, together with PLRV and Tomato Spotted Wilt Virus (Thompson and Strydom 2003). Potato Virus Y has been described as the causal agent of potato tuber blemishes (Black 2008; Visser and Bellstedt 2009; Fiers et al. 2012). Previous studies on PVY used potato leaf tissue for the isolation of the virus rather than tuber tissue (Karasev et al. 2008; Kamangar et al. 2014; Lindner et al. 2015). In this study, tissue from the corky crack symptom on the tuber was used for the detection of PVY as it was the focus symptom in this study, and no leaves from the diseased plants were reported to show viral symptoms. All the tuber samples used for the detection of the PVY virus in this study tested negative. It is possible that tuber samples used, as well as the leaf and tuber positive control samples, had degraded over time before RNA was extracted. Therefore, this emphasizes the importance of using fresh tissue or well-preserved tissue for the rapid extraction of RNA and further detection of the presence of the virus. However, with the tuber samples used in this study for the detection of PVY, it can

be provisionally confirmed that PVY is not associated with the corky crack disease in South Africa. PVY is one of the most important viruses causing potato crop losses globally and symptoms vary on leaves and tubers depending on the potato cultivar (Nie et al. 2012; Kamangar et al. 2014). Therefore, it is important that future research should consider the detection of PVY in atypical blemishes of potato. For instance, studies should consider using fresh leaf samples of plants showing corky crack symptoms on the tubers or a metagenomic approach to detect whether PVY is present in the plant with the corky crack disease.

To conclude, the results of this study indicate that the corky crack disease is due to the co-infection of potatoes by a *Rhizoctonia* species complex, which include *R. solani* AG 3-PT, BNR AG-A and BNR AG-R. These results highlight the importance of taking into account all possible interactions between plants and pathogens when studying diseases, rather than focusing on a single pathogen. Future studies should also consider all the possible environmental factors that could play a role in causing the disease as pathogens are adapting to climate change. Therefore, it is important to understand their adaptive and survival mechanisms in order to develop effective and efficient control strategies for the future.

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Chapter 5

General Conclusion

Potato (*Solanum tuberosum* L.) is one of the most important food crops in the world and most economically important vegetable in South Africa and globally (Lutaladio and Castaldi 2009). It is the fourth most highly produced food crop after rice, wheat and maize and is produced in more than 150 countries in the world (FAOSTAT 2017). Global potato production is limited due to diseases caused by various pathogens, which include fungi, bacteria, viruses and nematodes (Fiers et al. 2012). Potato is a staple crop in South Africa and it is therefore vital to safeguard the crop against these diseases to compensate for the increasing human population.

Potato tuber blemishes affect the quality and quantity of potato yield. They can cause severe loss in yield, rejection of seed batches, the downgrading of potatoes on the market or rejection of potatoes for processing (Tsror 2010). The demand for washed potatoes by South African consumers, raises the problem of blemished tubers. Potato blemishes pose an economic threat to farmers and therefore it is important to optimize their disease management strategies to avoid the loss of yield and downgrading of tubers in the market (Jordaan and van der Waals 2015).

Atypical corky crack blemishes are an emerging problem of potatoes in South Africa (Muzhinji et al. 2014). There has been a large debate in the South African potato industry regarding the causal agents of corky cracks. These atypical tuber blemishes have also been referred to as elephant hide with corky lesions and are known to be associated with *Rhizoctonia solani* (Banville et al. 1996; Campion et al. 2003; Fiers et al. 2010). Such atypical blemishes may have more than one causal agent, which makes it challenging to determine the pathogens responsible for causing the symptoms (Banville et al. 1996). It has been speculated that these corky cracks may not only be due to *R. solani* infection, but they have also been associated with *Streptomyces* species (Gouws and McLeod 2012), the Potato mop-top virus and Potato Virus Y (PVY) infection (Brierley et al. 2016) as well as abiotic factors, such as water stress (Fiers et al. 2010).

A study by Fiers et al. (2010), suggests that atypical blemishes could be due to the reaction of the plant to abiotic stress conditions. In their study, they could only fulfil Koch's postulates for

R. solani AG-3. No symptoms were obtained for other fungal or bacterial isolates. These included *Fusarium, Alternaria, Rhizoctonia, Penicillium, Clonostachys* species and various strains of *Streptomyces*. However, irregular polygonal sunken corky lesions were observed on non-inoculated tubers, which suggests abiotic factors also play a role in symptom development.

It is important to conduct a conclusive study to determine the pathogen(s) and/or abiotic factors responsible for the corky cracks on potato tubers in South Africa. The results may have implications for the potato market and seed certification scheme, as there is uncertainty regarding the causes of this symptom. Determining the causes of these symptoms can save the industry from severe economic losses.

The main objectives of this study were: (i) to isolate and identify the *Rhizoctonia solani* and *Streptomyces* species associated with corky crack blemishes in South Africa, using morphological and molecular techniques; (ii) to further isolate and identify other microorganisms associated with corky crack blemishes in South Africa, using morphological and molecular techniques; (iii) to determine the pathogenicity of the isolated pathogens on the potato tubers, thereby fulfilling Koch's postulates; (iv) to determine if Potato Virus Y (PVY) is associated with corky cracks on potato tubers in South Africa and (v) to determine if water stress is associated with corky crack blemishes on potato tubers in South Africa.

For the first part of the study, two groups of microorganisms, *R. solani* AG2-2IIIB and *Streptomyces* species namely *S. chrestomyceticus, S. flavoviridis* and *S. fradiae* were successfully isolated and identified using morphological and molecular techniques. A greenhouse study was conducted with these isolates in single inoculations and in combination. No corky crack symptoms were observed on progeny tubers; however, elephant hide symptoms were observed on progeny tubers from plants inoculated with *R. solani* AG2-2IIIB. This is the first report of *R. solani* AG 2-2 IIIB causing elephant hide on potato tubers in South Africa. Higher disease severity was observed on plants inoculated with a combination of *R. solani* AG2-2IIIB, *S. chrestomyceticus, S. flavoviridis* and *S. fradiae*. No significant synergistic interactions were observed between *R. solani* AG2-2IIIB and the *Streptomyces* species complex in this study (*S. chrestomyceticus, S. flavoviridis* and *S. fradiae*) in terms of disease index. However, the co-inoculation of the two different pathogens resulted in higher disease severity. It has been explained that even though co-infection does not always result in

significant synergistic interactions on potato, co-inoculation may still increase disease severity (Dung et al. 2014).

For the second part of the study, all microbial pathogens isolated from corky crack blemishes were identified and used in pot trials, since no corky crack blemishes resulted after the first greenhouse experiment with representative *R. solani* AG2-2IIIB and *Streptomyces* species (*S. chrestomyceticus, S. flavoviridis* and *S. fradiae*). After successful isolation and identification, using morphological and molecular techniques, the most prevalent fungal species isolated from corky cracks included binucleate *Rhizoctonia* (BNR) AG-A, BNR AG-R and *Fusarium oxysporum* while no fungal species were isolated from growth cracks. *Streptomyces collinus, S. yaanensis, S. corchorussi, S. viridochromogenes* and *S. griseorubens* were the most predominant bacterial species isolated from the growth cracks and no *Streptomyces* species were isolated from corky cracks.

Greenhouse pathogenicity trials using the isolated microorganisms alone and in combination were done to confirm Koch's postulates. *Rhizoctonia solani* AG 3-PT (isolate Rh6, University of Pretoria culture collection) was also incorporated into the greenhouse experiment to ascertain whether this fungal pathogen causes corky cracks on potato tubers in South Africa. Corky crack symptoms were not observed on progeny tubers at harvest in single inoculations for either fungal or bacterial isolates; however, corky crack symptoms were observed on progeny tubers inoculated with a combination of the *Rhizoctonia* species complex in this study (binucleate *Rhizoctonia* (BNR) AG-A, BNR AG-R and *R. solani* AG 3-PT). A significant synergistic interaction was observed between the three *Rhizoctonia* species in the complex, including *R. solani* AG 3-PT, BNR AG-A and BNR AG-R complex in this study. This study also confirms that co-inoculation of plants with more than one fungal pathogen can increase severity of disease symptoms (Whitelaw-Weckert et al. 2013). Furthermore, disease index of corky cracks caused by *Rhizoctonia* species complex was significantly higher under normal watering conditions (field capacity) than under water stress, confirming the fungal preference for soil moisture for infection to take place (Jeger et al. 1996).

In contrast, the co-inoculation of the *Rhizoctonia* species complex (binucleate *Rhizoctonia* (BNR) AG-A, BNR AG-R and *R. solani* AG 3-PT) and the *Streptomyces* species complex (*S. collinus, S. yaanensis, S. corchorussi, S. viridochromogenes* and *S. griseorubens*) did not result in higher disease severity with regards to corky crack blemishes. Rather, a decrease in disease

index was observed the more pathogens added into combination i.e. *Rhizoctonia* species complex, *Streptomyces* species complex and *Fusarium oxysporum*. This contradicts what some previous research has stated, that a higher disease index can be observed under co-inoculation with different pathogens (Begon et al. 1986; Le May et al. 2009). This is most often observed between a bacterial and a fungal interaction (Dung et al. 2014).

The *Streptomyces* species identified in both sections of the study are not known to be pathogenic to potatoes (Janssen et al. 1989) and are, therefore, not likely causal agents of the corky crack symptoms. *S. chrestomyceticus, S. flavoviridis, S. fradiae, S. collinus*, *S. yaanensis*, *S. corchorussi*, *S. viridochromogenes* and *S. griseorubens* are all known to be natural soil inhabitants and could be possible saprophytes. This could explain why these species were isolated from growth cracks and not corky cracks. Growth cracks on potato tubers usually as a result of water stress (Agrios 2005) and not pathogen infection (Weitnauer et al. 2001; Rückert et al. 2013; Zheng et al. 2013; Al-Askar et al. 2015). However, it has been reported that *S. corchorussi* may cause deep-pitted scab on potatoes (Archuleta and Easton 1981). In this study *S. chrestomyceticus, S. flavoviridis* and *S. fradiae* did; however, cause common scab symptoms in combination as well as *S. collinus, S. yaanensis*, *S. corchorussi*, *S. viridochromogenes* and *S. griseorubens* in combination. Therefore, further studies should be conducted to screen whether the *Streptomyces* species found in this study carry the virulence genes *txtAB*, *tomA* and *nec1*.

In South Africa, Potato Virus Y (PVY) is one of the most devastating viruses and is monitored by the potato certification scheme, together with Potato Leaf Roll Virus (PLRV) and tomato Spotted Wilt Virus (Thompson and Strydom 2003). PVY has been described as the causal agent of potato tuber blemishes (Visser and Bellstedt 2009; Black 2008; Fiers et al. 2012). However, PVY was not detected in this study through species-specific RT-PCR and it was, therefore, assumed that it is not associated with corky cracks on the tested samples of potato tubers in South Africa. Further studies should consider the detection of PVY using fresh leaf samples of the potato plants which resulted in progeny tubers with corky crack blemishes. A metagenomics approach can also be used to conclude whether PVY is present in corky cracks in South Africa.

Different watering conditions were tested in this study. It is known that water stress conditions cause potato tuber splitting, which is similar to growth crack symptoms (Agrios 2005).

However, when considering the control treatments in this study, water stress conditions (soil moisture fluctuating between wilting point and field capacity) did not result in corky cracks or growth cracks. Normal watering conditions did provide favourable conditions for the *Rhizoctonia* species in this study to infect potato plants and eventually cause various symptoms of the Rhizoctonia disease (black scurf, elephant hide and deformation) as well as corky crack blemishes in co-inoculated plants. Factors such as temperature, irrigation/rainfall, soil type, soil moisture, plant nutrition and soil pH are some of the most important factors that have an impact on crop production and abiotic factors should always be considered when studying the development of plant diseases. Various research studies have been done on the effects of the environment on plant diseases, however, more information is needed on the corky crack disease. With the knowledge of field responses of pathogens causing corky crack blemishes, particularly in the light of climate change, specific agricultural measures can be developed to overcome or prevent the development of corky cracks and growth cracks and limit further production losses.

The complexity of the interactions between a pathogen and its host, influenced by biotic and abiotic factors, makes it difficult to develop effective control strategies against plant diseases. Most chemical control strategies applied in agriculture target a specific plant pathogen, and therefore do not result in successful disease management of multiple pathogens that may also contribute to the disease (Lamichhane and Venturi 2015). The first step in controlling a plant disease is to perform a diagnosis that correctly identifies the pathogens related to the disease. Once this has been achieved, suitable control measures can be implemented. However, establishing these measures is more complicated when studying disease complexes rather than a single pathogen (Willocquet et al. 2008; Adams et al. 2013). It is, therefore, necessary to study the pathogen-pathogen synergistic interactions associated with plant disease complexes, as well as the other mechanisms involved in causing a disease to be able to identify the important links in pathogen-pathogen interaction that can be changed to ensure crop health and food security (Willocquet et al. 2002). Willocquet et al. (2002) for instance, developed models that have been able to simulate yield losses due to multiple pathogens under different conditions. They successfully demonstrated the impact of different pathogens in causing disease on different crops; however, they did not consider the possible pathogen-pathogen interactions amongst the pathogens. Future research should, therefore, focus on understanding pathogen-pathogen interactions in order to design a suitable model to simulate synergism

between pathogens to predict future outcomes of disease, such as corky crack blemishes on potatoes.

For bacteria and fungi, the use of traditional isolation and culturing techniques provide a platform for studying new emerging diseases, however, may not yield all of the causal pathogens related to the disease (Adams et al. 2013). In the present study, isolations were performed to identify the culturable pathogens associated with corky crack blemishes on potato tubers in South Africa. Thereafter, morphological (microscopic) and molecular techniques (PCR) were conducted for the identification of causal pathogens associated with corky crack blemishes. For viruses, methods included the use of species-specific identification (RT-PCR). These traditional methods of the diagnosis of causal pathogens and management related to complex diseases are lengthy and can result in loss of yield (Nikolaki and Tsiamis 2013). More recently, newer methods and techniques based on next-generation sequencing (NGS) technology may aid in the understanding of multiple pathogenic species involved in causing plant diseases. The next-generation sequencing technology provides a powerful generic screen well suited for viruses and provides a high-throughput analysis of complex microbial populations (Adams et al. 2013; Van Dijk et al. 2014). It is proposed that future research should involve a metagenomic study, as an effective tool to understand the role of multiple plant pathogens in causing the corky crack disease on potato; however, pathogenicity tests are still necessary to confirm Koch's postulates.

In summary, considering both parts of this study, no corky crack blemishes were reproduced in progeny tubers at harvest with single inoculations of fungal or bacterial isolates. However, significant corky crack blemishes were observed on progeny tubers inoculated in the *Rhizoctonia* species complex including binucleate *Rhizoctonia* (BNR) AG-A, BNR AG-R and *R. solani* AG 3-PT in the second part of the study, suggesting a synergistic interaction between these *Rhizoctonia* species. Both parts of the study confirm that the co-inoculation of more than one pathogen causes more severe disease symptoms. However, in the second part of the study, this is only true with regards to the fungal combination.

Plant pathologists should take into account the various possible interactions between plant pathogens when studying diseases, rather than focusing on a single pathogen. It is important to focus on all pathogens involved in causing disease in pathogenicity tests and in future research studies to develop effective control strategies. Future studies should involve the possible synergistic, antagonistic and mutualistic interactions between plant pathogens occurring in a

disease as well as the role of the multiple pathogens involved. A conclusive study should, therefore, be performed, considering all potential biotic and abiotic factors involved in causing the corky crack disease. Determining the exact causes of corky crack blemishes can save the industry from severe economic losses.

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Appendix

Table A1. Disease ratings of corky crack blemishes on the different treatments in the first pot trial under normal watering conditions

Treatment	Replicate	Disease Score						Total number of tubers	Disease Incidence (%)	mean DI/trt	Disease Severity	mean DS/trt	Disease Index	mean DI/trt
		0	1	2	3	4	5							
1	1	2	0	0	0	0	0	2	0		0		0	
1	2	3	0	0	0	0	0	3	0		0		0	
1	3	6	0	0	0	0	0	6	0		0		0	
1	4	7	0	0	0	0	0	7	0		0		0	
1	5	13	0	0	0	0	0	13	0	0	0	0	0	0
2	1	4	0	0	0	0	0	4	0		0		0	
2	2	5	0	0	0	0	0	5	0		0		0	
2	3	10	0	0	0	0	0	10	0		0		0	
2	4	6	0	0	0	0	0	6	0		0		0	
2	5	5	0	0	0	0	0	5	0	0	0	0	0	0
3	1	7	0	0	0	0	0	7	0		0		0	
3	2	6	0	0	0	0	0	6	0		0		0	
3	3	8	0	0	0	0	0	8	0		0		0	
3	4	9	0	0	0	0	0	9	0		0		0	
3	5	8	0	0	0	0	0	8	0	0	0	0	0	0

4	1	6	0	0	0	0	0	6	0		0		0	
4	2	5	0	0	0	0	0	5	0		0		0	
4	3	8	0	0	0	0	0	8	0		0		0	
4	4	5	0	0	0	0	0	5	0		0		0	
4	5	5	0	0	0	0	0	5	0	0	0	0	0	0
5	1	3	0	0	0	0	0	3	0		0		0	
5	2	4	0	0	0	0	0	4	0		0		0	
5	3	5	0	0	0	0	0	5	0		0		0	
5	4	7	0	0	0	0	0	7	0		0		0	
5	5	4	0	0	0	0	0	4	0	0	0	0	0	0
6	1	3	0	0	1	2	0	6	50,00		1,83		91,67	
6	2	2	0	1	1	2	0	6	66,67		2,17		144,44	
6	3	4	0	1	1	0	0	6	33,33		0,83		27,78	
6	4	1	0	3	1	2	0	7	85,71		2,43		208,16	
6	5	4	0	1	2	0	0	7	42,86	55,71	1,14	1,68	48,98	104,21
7	1	3	0	0	0	0	0	3	0,00		0,00		0,00	
7	2	2	2	0	0	0	0	3	66,67		0,67		44,44	
7	3	3	0	0	0	2	0	5	40,00		1,60		64,00	
7	4	5	0	0	1	1	0	7	28,57		1,00		28,57	
7	5	4	0	0	0	1	1	6	33,33	33,71	1,50	0,95	50,00	37,40
8	1	4	0	0	0	0	0	4	0		0		0	
8	2	6	0	0	0	0	0	6	0		0		0	
8	3	6	0	0	0	0	0	6	0		0		0	

8	4	8	0	0	0	0	0	8	0		0		0	
8	5	5	0	0	0	0	0	5	0	0	0	0	0	0
9	1	6	0	0	0	0	0	6	0		0		0	
9	2	3	0	0	0	0	0	3	0		0		0	
9	3	4	0	0	0	0	0	4	0		0		0	
9	4	6	0	0	0	0	0	6	0		0		0	
9	5	4	0	0	0	0	0	4	0	0	0	0	0	0

Table A2. Disease ratings of corky crack blemishes on the different treatments in the second pot trial under normal watering conditions

Treatment	Replicate	Disease Score						Total number of tubers	Disease Incidence (%)	mean DI/trt	Disease Severity	mean DS/trt	Disease Index	mean DI/trt
		0	1	2	3	4	5							
1	1	12	0	0	0	0	0	12	0		0		0	
1	2	5	0	0	0	0	0	5	0		0		0	
1	3	6	0	0	0	0	0	6	0		0		0	
1	4	6	0	0	0	0	0	6	0		0		0	
1	5	8	0	0	0	0	0	8	0	0	0	0	0	0
2	1	12	0	0	0	0	0	12	0		0		0	
2	2	9	0	0	0	0	0	9	0		0		0	
2	3	7	0	0	0	0	0	7	0		0		0	
2	4	5	0	0	0	0	0	5	0		0		0	
2	5	7	0	0	0	0	0	7	0	0	0	0	0	0
3	1	11	0	0	0	0	0	11	0		0		0	
3	2	10	0	0	0	0	0	10	0		0		0	
3	3	6	0	0	0	0	0	6	0		0		0	
3	4	6	0	0	0	0	0	6	0		0		0	
3	5	6	0	0	0	0	0	6	0	0	0	0	0	0
4	1	7	0	0	0	0	0	7	0		0		0	
4	2	11	0	0	0	0	0	11	0		0		0	
4	3	8	0	0	0	0	0	8	0		0		0	
4	4	7	0	0	0	0	0	7	0		0		0	

4	5	11	0	0	0	0	0	11	0	0	0	0	0	0
5	1	8	0	0	0	0	0	8	0		0		0	
5	2	6	0	0	0	0	0	6	0		0		0	
5	3	8	0	0	0	0	0	8	0		0		0	
5	4	6	0	0	0	0	0	6	0		0		0	
5	5	4	0	0	0	0	0	4	0	0	0	0	0	0
6	1	5	0	1	2	1	0	9	44,44		1,33		59,26	
6	2	5	0	1	1	3	0	5	100,00		3,40		340,00	
6	3	7	0	0	0	1	2	10	30,00		1,40		42,00	
6	4	3	0	1	1	1	2	8	62,50		2,38		148,44	
6	5	4	0	2	1	2	0	9	55,56	58,50	1,67	2,04	92,59	136,46
7	1	8	0	1	1	0	0	10	20,00		0,50		10,00	
7	2	5	0	1	1	0	0	7	28,57		0,71		20,41	
7	3	6	1	0	1	0	0	8	25,00		0,50		12,50	
7	4	4	0	1	1	0	0	6	33,33		0,83		27,78	
7	5	11	0	0	0	0	0	11	0,00	21,38	0,00	0,51	0,00	14,14
8	1	10	0	0	0	0	0	10	0,00		0,00		0,00	
8	2	5	0	0	0	0	0	5	0,00		0,00		0,00	
8	3	6	0	0	0	0	0	6	0,00		0,00		0,00	
8	4	4	1	2	0	0	0	7	42,86		0,71		30,61	
8	5	8	0	0	0	0	0	8	0,00	8,57	0,00	0,14	0,00	6,12
9	1	6	0	0	0	0	0	6	0		0		0	
9	2	5	0	0	0	0	0	5	0		0		0	
9	3	10	0	0	0	0	0	10	0		0		0	
9	4	10	0	0	0	0	0	10	0		0		0	

9	5	9	0	0	0	0	0	9	0	0	0	0	0	0

Table A3. Disease ratings of corky crack blemishes on the different treatments in the first pot trial under water stress conditions

Treatment	Replicate	Disease Score						Total number of tubers Disease	Incidence (%) mean DI/trt		Disease Severity mean DS/trt		Disease Index mean DI/trt	
		0	1	2	3	4	5							
1	1	4	0	0	0	0	0	4	0		0		0	
1	2	4	0	0	0	0	0	4	0		0		0	
1	3	4	0	0	0	0	0	4	0		0		0	
1	4	3	0	0	0	0	0	3	0		0		0	
1	5	5	0	0	0	0	0	5	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	0		0		0	
2	2	4	0	0	0	0	0	4	0		0		0	
2	3	4	0	0	0	0	0	4	0		0		0	
2	4	3	0	0	0	0	0	3	0		0		0	
2	5	5	0	0	0	0	0	5	0	0	0	0	0	0
3	1	5	0	0	0	0	0	5	0		0		0	
3	2	5	0	0	0	0	0	5	0		0		0	
3	3	4	0	0	0	0	0	4	0		0		0	
3	4	4	0	0	0	0	0	4	0		0		0	
3	5	5	0	0	0	0	0	5	0	0	0	0	0	0
4	1	5	0	0	0	0	0	5	0		0		0	
4	2	6	0	0	0	0	0	6	0		0		0	
4	3	4	0	0	0	0	0	4	0		0		0	
4	4	2	0	0	0	0	0	2	0		0		0	
4	5	5	0	0	0	0	0	5	0	0	0	0	0	0
5	1	4	0	0	0	0	0	4	0		0		0	
5	2	3	0	0	0	0	0	3	0		0		0	
5	3	2	0	0	0	0	0	2	0		0		0	
5	4	3	0	0	0	0	0	3	0		0		0	

5	5	3	0	0	0	0	0	3	0	0	0	0	0	0
6	1	3	0	1	1	1	0	6	50,00		1,50		75,00	
6	2	2	0	3	0	0	0	5	60,00		1,20		72,00	
6	3	0	0	0	0	0	0	6	0,00		0,00		0,00	
6	4	3	0	1	0	0	0	4	25,00		0,50		12,50	
6	5	5	0	0	0	1	0	6	16,67	30,33	0,67	0,77	11,11	34,12
7	1	5	0	0	0	0	0	5	0		0		0	
7	2	5	0	0	0	0	0	5	0		0		0	
7	3	5	0	0	0	0	0	5	0		0		0	
7	4	5	0	0	0	0	0	5	0		0		0	
7	5	6	0	0	0	0	0	6	0	0	0	0	0	0
8	1	5	0	0	0	0	0	5	0		0		0	
8	2	3	0	0	0	0	0	3	0		0		0	
8	3	4	0	0	0	0	0	4	0		0		0	
8	4	6	0	0	0	0	0	6	0		0		0	
8	5	4	0	0	0	0	0	4	0	0	0	0	0	0
9	1	4	0	0	0	0	0	4	0		0		0	
9	2	5	0	0	0	0	0	5	0		0		0	
9	3	5	0	0	0	0	0	5	0		0		0	
9	4	6	0	0	0	0	0	6	0		0		0	
9	5	4	0	0	0	0	0	4	0	0	0	0	0	0

Table A4. Disease ratings of corky crack blemishes on the different treatments in the second pot trial under water stress conditions

Treatment	Replicate	Disease Score						Total number of tubers Disease	Incidence (%)	mean DI/trt	Disease Severity	mean DS/trt	Disease Index	mean DI/trt
		0	1	2	3	4	5							
1	1	6	0	0	0	0	0	6	0		0		0	
1	2	5	0	0	0	0	0	5	0		0		0	
1	3	7	0	0	0	0	0	7	0		0		0	
1	4	5	0	0	0	0	0	5	0		0		0	
1	5	6	0	0	0	0	0	6	0	0	0	0	0	0
2	1	2	0	0	0	0	0	2	0		0		0	
2	2	5	0	0	0	0	0	5	0		0		0	
2	3	7	0	0	0	0	0	7	0		0		0	
2	4	10	0	0	0	0	0	10	0		0		0	
2	5	7	0	0	0	0	0	7	0	0	0	0	0	0
3	1	6	0	0	0	0	0	6	0		0		0	
3	2	7	0	0	0	0	0	7	0		0		0	
3	3	9	0	0	0	0	0	9	0		0		0	
3	4	7	0	0	0	0	0	7	0		0		0	
3	5	7	0	0	0	0	0	7	0	0	0	0	0	0
4	1	7	0	0	0	0	0	7	0		0		0	
4	2	6	0	0	0	0	0	6	0		0		0	
4	3	4	0	0	0	0	0	4	0		0		0	
4	4	9	0	0	0	0	0	9	0		0		0	
4	5	9	0	0	0	0	0	9	0	0	0	0	0	0
5	1	6	0	0	0	0	0	6	0		0		0	
5	2	4	0	0	0	0	0	4	0		0		0	
5	3	7	0	0	0	0	0	7	0		0		0	

5	4	6	0	0	0	0	0	6	0		0		0	
5	5	5	0	0	0	0	0	5	0	0	0	0	0	0
6	1	5	0	0	0	2	0	7	28,57		1,14		32,65	
6	2	4	0	1	1	0	0	6	33,33		0,83		27,78	
6	3	8	0	0	1	0	0	9	11,11		0,33		3,70	
6	4	9	0	1	0	0	0	10	10,00		0,20		2,00	
6	5	10	0	0	0	0	0	10	0,00	16,60	0,00	0,50	0,00	13,23
7	1	7	0	0	0	0	0	7	0,00		0,00		0,00	
7	2	7	1	0	0	0	0	8	12,50		0,13		1,56	
7	3	0	0	0	0	0	0	0	0,00		0,00		0,00	
7	4	6	0	0	0	0	0	6	0,00		0,00		0,00	
7	5	6	0	0	0	0	0	6	0,00	2,50	0,00	0,03	0,00	0,31
8	1	5	0	0	0	0	0	5	0		0		0	
8	2	5	0	0	0	0	0	5	0		0		0	
8	3	10	0	0	0	0	0	10	0		0		0	
8	4	7	0	0	0	0	0	7	0		0		0	
8	5	7	0	0	0	0	0	7	0	0	0	0	0	0
9	1	11	0	0	0	0	0	11	0		0		0	
9	2	4	0	0	0	0	0	4	0		0		0	
9	3	5	0	0	0	0	0	5	0		0		0	
9	4	4	0	0	0	0	0	4	0		0		0	
9	5	7	0	0	0	0	0	7	0	0	0	0	0	0