Etiology of Soft Rot and Blackleg on Potatoes in South Africa

by

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DECLARATION

I hereby declare that this dissertation submitted to the University of Pretoria for the degree of MSc (Agric) Plant pathology has not previously been submitted by me in respect of a degree at any other University.

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ETIOLOGY OF SOFT ROT AND BLACKLEG ON POTATOES IN SOUTH AFRICA

by

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ABSTRACT

Pectobacterium carotovorum (*Pbc*), *Dickeya* spp., *Pectobacterium atrosepticum* (*Pba*), and a new, atypical strain, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) can cause potato blackleg, soft rot and aerial stem rot. To determine the impact and extent of these pathogens on the South African potato industry, samples were collected from 72 potato fields in 10 potato production regions during the 2006/7 production seasons. During these seasons, blackleg outbreaks occurred in commercial production fields causing severe economic losses.

Bacteria isolated from diseased material that were Gram-negative, facultative anaerobic with pectolytic ability were identified using a Multiplex PCR targeting the 16S-23S region. Isolates were subjected to partial sequencing of the 16S-23S rDNA and a subsequent PCR-RFLP of the 16S-23S rDNA region. Comparison of RFLP patterns of isolates to reference cultures confirmed the identity of the South African blackleg strains as *Pbcb*. This is the first report of *Pbcb* in South Africa. A total of 128 isolates were obtained from 10 production regions. Of these 77% were shown to be *Pbcb,* 17% *Pbc* and 6% unkown. *Dickeya* spp. and *Pba* were not detected in South Africa. From imported tubers 14 isolates were obtained, 13 of which were identified as *Pbcb* and one isolate as *Pbc*.

Thus *Pbcb* was shown to be the most important causal agent of blackleg and soft rot in South Africa and poses a threat to the South African potato industry.

Through a questionnaire survey the farmers` knowledge of soft rot / blackleg disease complex and the need for research on these potato diseases in South Africa was determined. A total of 41 questionnaires were collected from potato growers and analysed. Estimates of economic losses experienced by growers due to soft rot / blackleg disease complex, ranged from 1 to 70%. It appears that in South Africa disease symptoms are mainly prevalent at temperate (10 – 20°C) to warm climates with prolonged wet or humid conditions. To reduce disease incidence, low generation seed tubers need to be planted and good crop rotation systems need to be followed.

Blackleg is mainly a seed-borne disease and is therefore difficult to control. This prompted the search for a possible management strategy to increase plant / tuber resistance to blackleg and soft rot and to minimise losses. The study also focussed on the effect of a calcium silicate slag soil amendment on phenolic formation in cell walls of potato peels, and subsequent tuber resistance to *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*). Pot trials were conducted with the following treatments: Control with and without the pathogen; slag (30% Si) with and without the pathogen; lime treatment ($CaCO_3$) as pH control with and without the pathogen. Results from pot trials show that phenol production in potato stems and tubers increased in both lime and slag treatments, compared to the controls. This suggests that soil pH may play a role in phenol production. This could, however be due to increased silicon absorption by plants at higher pH values. Results also indicate that the best Si source to use is Calmasil, since Si and Ca combined have a synergistic effect in enhancing tuber resistance. This is, however, the first study on the effect of Si on defence responses of potatoes and further research is required to elucidate the modes-of-action of Si in the potato plant.

This study has enhanced the understanding of the etiology of soft rot / blackleg diseases in South Africa and opened up new possibilities for the use of Si in commercial production to improve plant health status. However the need for future research on this disease complex has been highlighted in this thesis.

Keywords: *Pectobacterium carotovorum* subsp. *brasiliensis*, soft rot, blackleg, *Solanum tuberosum*, survey, silicon

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PAGE

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND AND MOTIVATION OF THE STUDY

Potatoes (*Solanum tuberosum* L.) are a major food crop worldwide. Every year an average of 322 million tonnes of potatoes are produced worldwide, planted on 19 million hectares. Of this Africa produces 5% of the world's potatoes. In South Africa potato production for the 2006/2007 season exceeded 1,92 million tonnes, cultivated on 54 037 ha. In South Africa potatoes are produced throughout the year in sixteen main areas, namely Mpumalanga, Eastern Free State, KwaZulu-Natal, Western Free State, Northern Cape, North-Eastern Cape, Eastern Cape, South-Western Cape, Southern Cape, Sandveld, Ceres, Gauteng, North-West, Limpopo, South-Western Freestate and Loskopvalley (Potatoes South Africa, 2008).

Potatoes are very susceptible to bacterial and fungal diseases due to their high starch and sugar content (Pérombelon, 2002). Furthermore, they are clonally propagated by planting whole seed tubers or seed pieces and this promotes the spreading of causal pathogens from one area to another (De Boer, 2002; Hélias *et al,* 2000). Pathogenic bacteria overwinter in stored potatoes thus requiring an effective certification system to minimize disease infestations. However certification systems are based on a set of tolerance levels encountered during visual inspections of growing crops and harvested seed tubers. Therefore certification systems need to incorporate the use of accurate and sensitive post-harvest diagnostic testing, to prevent the spread of the disease and ultimately reduce the risk of economic losses (Elphinestone & Toth, 2007).

Some of the most important bacterial diseases of potatoes are seed piece decay, blackleg and aerial stem rot in the field as well as tuber soft rot in storage. These diseases are caused by *Pectobacterium carotovorum* (Jones) (*Pc*), *Pectobacterium atrosepticum* (Van Hall) Dye (*Pa*), *Dickeya dadantii* (Burkholder) (*Dd*) (Kang *et al.,*

2003; Garden *et al*., 2003; Samson *et al*., 2005; Grenier *et al*., 2006) and a newly-described, atypical strain, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) (Duarte *et al*., 2004). These pectolytic strains, formerly known as *Erwinia* spp., have been researched intensively because of their economic importance and were recently classified into separate genera, *Pectobacterium* and *Dickeya* (Garden *et al.*, 2003).

Disease development is initiated when the temperature is optimal, oxygen concentration is low, humidity is high and enough available free water is present (Smadja *et al*., 2004; Toth *et al*., 2003). Soft rot bacteria can cause systemic and vascular disorders (Toth *et al*., 1999) and therefore elicit a range of symptoms on stems of potato plants and tubers. These symptoms are not strain specific but are dependent on climatic conditions (De Haan *et al*., 2008).

During the 1988 growing season in South Africa, a *Pectobacterium* wilt disease was detected on potatoes (Serfontein *et al.,* 1991). The importance of this disease in southern Africa was vastly underestimated (Gouws, 2003). Aspects such as etiology, epidemiology, population biology, economic impact and control have therefore not yet received adequate attention. However, over the past decade blackleg, *Pectobacterium* wilt and soft rot have become a serious problem in the South African potato industry, especially for seed growers. It is for this reason that an epidemiological study was conducted, to improve tuber quality and reduce the risk of economic losses.

1.2 FUNDAMENTAL OBJECTIVE

The aim of this study is to investigate epidemiological factors of blackleg, soft rot, and *Pectobacterium* wilt on potatoes in South Africa, focusing on the farmers`knowledge of soft rot and blackleg diseases in South Africa; determination of the effect of Si soil amendments on disease development and elucidation of the current disease situation in South Africa.

1.3 SPECIFIC OBJECTIVES

- To investigate the current situation and distribution of *Pectobacterium* and *Dickeya* spp. in South Africa
- To isolate and identify the soft rot bacteria associated with recent outbreaks of blackleg on potatoes in South Africa
- To determine and document the impact and extent of soft rot and blackleg on the South African potato industry
- To investigate possible management strategies based on silicon-amended soil which can enhance plants' resistance to blackleg and tuber soft rot diseases.

1.4 CHAPTER OUTLINE

Chapter 2 The literature review focuses on the morphology, pathogenicity and diagnostics of the soft rot and blackleg causal agents, as well as symptoms, etiology, epidemiology, host range, syngerism, natural defence mechanisms, host nutrition and management strategies of these diseases.

Chapter 3 Isolates of *Pectobacterium* spp. were collected from randomly selected potato fields in different potato production regions in the country. These isolates were identified using a Multiplex PCR together with the amplification of the 16S-23S rDNA region in order to determine the current situation of the blackleg / soft rot disease complex in SA.

Chapter 4 Recently, a new subspecies from Brazil, *Pectobacterium carotovorum* ssp. *brasiliensis* (*Pbcb*), was described, which causes blackleg-like symptoms on potatoes. This prompted a study to identify if soft rot bacteria is associated with recent outbreaks of blackleg in South Africa. Three representative isolates obtained from plants with typical blackleg symptoms were selected.

Isolates were identified using partial 16S-23S rDNA sequencing in concert with the PCR-RFLP of the16S-23S rDNA region. (Submitted to *European Journal of Plant Pathology* for publication)

Chapter 5 A possible management strategy was investigated based on the effect of silicon on defense responses of potatoes to *Pectobacterium* early dying, blackleg and tuber soft rot. Silicon is known to promote the production of phenolics and phytoalexins in response to pathogen infection.

Chapter 6 A survey was conducted among South African potato growers, which highlighted the distribution and impact of blackleg and soft rot diseases on the South African potato industry

Chapter 7 General discussion and final conclusion which includes the impact of soft rot diseases on the South African potato industry and suggestions for future research.

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CHAPTER 2

A REVIEW OF BLACKLEG, AERIAL STEM ROT AND SOFT ROT DISEASES ON POTATOES

ABSTRACT

Some of the most important bacterial diseases of potatoes are tuber soft rot, blackleg and aerial stem rot in the field as well as tuber soft rot in storage. These diseases are caused by *Pectobacterium carotovorum, P. atrosepticum* and *Dickeya dadantii.* The pectolytic bacteria are ubiquitous and can be found in debris, on roots or other parts of the host, in alternative hosts, in water, in soil and in the pupae of several insects. These opportunistic pathogens enter the plant through wounds, lenticels and stolon ends where they can cause symptoms or remain symptomless. They are present extracellularly in the lenticels and suberized wounds on the periderm in the latent phase. The bacteria remain quiescent until ecological factors are favourable to initiate symptom development. They secrete numerous extracellular enzymes in order to exploit a wide range of living or dead plant substrates. Disease development is initiated when the temperature is optimal, oxygen concentration is low, humidity is high and enough available free water is present. Symptoms are not strain specific but are dependant on climatic conditions. Control measures include planting clean seed, planting in well-drained soil, avoiding over irrigation, good sanitation and cultural practices.

2.1 INTRODUCTION

Globally, potatoes (*Solanum tuberosum* L.) are a major food crop. Every year an average of 322 million tonnes of potatoes planted on 19 million hectares, are produced worldwide (Potatoes South Africa, 2008). Africa produces 5% of the world's potatoes. In South Africa potato production for the 2006/2007 season exceeded 1,92 million tonnes, cultivated on 54 037 hectares.

There are 681 active commercial potato growers in South Africa of which ± 200 produce seed potatoes throughout the year. The three most popular cultivars planted during 2006/2007 were Mondial, BP1 and Up-to-date, respectively (Potatoes South Africa, 2008).

Potatoes are very susceptible to pests, diseases and quality problems. To address these problems the cost of production increases and therefore increases the grower economic risks, making potato production less profitable due to variable yields, quality and market prices. Potatoes are vegetatively propagated; therefore the most important source of disease inoculum is latently infected potato seed tubers (Hélias *et al,* 2000b; De Boer, 2002). Therefore, certification systems have been developed to minimise disease dissemination. In most countries the seed potato classification schemes are based on set tolerance levels encountered during visual inspections of growing crops and harvested tubers (Elphinstone & Toth, 2007). Thus certification systems are only partially effective and work only to the extent that disease symptoms can be observed.

The most important bacterial diseases of potatoes are seed piece decay, blackleg and aerial stem rot in the field as well as tuber soft rot in storage. These symptoms are caused by *Pectobacterium carotovorum* (Jones) (*Pbc*), *Pectobacterium atrosepticum* (Van Hall) Dye (*Pba*) and *Dickeya* spp. (Dickey), particularly *Dickeya dadantii* (*Dd*) (Dadant), *Dickeya dianthicola* (Hellmers) and *Dickeya zeae* (Sabet) (Hauben *et al.,* 1999; Pérombelon, 2002; Euzéby, 2003; Samson *et al*., 2005; Grenier *et al*., 2006; Ma *et al.*, 2007) and a newly-described, atypical strain, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) (Duarte *et al*., 2004). These pectolytic strains, formerly known as *Erwinia* spp., were recently reclassified as separate genera, *Pectobacterium* and *Dickeya* (Garden *et al.*, 2003). The genera names were changed based on the production of large quantities of pectolytic enzymes in plant pathogenesis, their related 16S ribosomal DNA homology groups and host range (De Boer, 2003; Garden *et al*., 2003; Ma *et al*., 2007).

Pectobacterium carotovorum, *Pba* and *Dickeya* spp. are known to have a wide geographical distribution, which is dependent on a specific temperature and host range (Oliveira *et al*., 2003; Tsror *et al*., 2009).

Soft rot pectobacteria can be found on plant surfaces, in water and in soil (Hélias *et al.,* 2000a). They are causal agents of systemic and vascular disorders and therefore elicit a range of symptoms on stems of potato plants and tubers. They enter the plant through wounds, lenticels and stolon ends, where they can cause symptoms or remain symptomless and survive in the lenticels (De Boer, 2002; Schroeder, 2003; Toth *et al.,* 2003b). These symptoms are not strain specific but are dependent on climatic conditions (De Haan *et al*., 2008). Disease development is initiated when the temperature is optimal, oxygen concentration is low, humidity is high and enough available free water is present (Toth *et al*., 2003a; Smadja *et al*., 2004b).

In South Africa *Pectobacterium* wilt as well as *Dickeya* wilt diseases were observed for the first time on potatoes during the 1988 growing season (Serfontein *et al.,* 1991). *Pectobacterium* wilt is part of a disease complex and has been economically underestimated in South Africa (Gouws, 2003). Recently, blackleg, *Pectobacterium* wilt and soft rot incidence in South Africa has been increasing, becoming a significant problem, reducing yield, resulting in downgrading or rejection of potatoes especially seed potatoes in the South African potato industry. It is for this reason that this study was conducted.

2.2 THE PATHOGENS

2.2.1 Taxonomy, morphology and biochemical characteristics

Pectobacterium carotovorum (*Pbc*), *Pectobacterium atrosepticum* (*Pba*) and the *Dickeya* spp. complex are commonly known as soft rot pathogens (Kado, 2006). They are all members of the family, *Enterobacteriaceae* (Hauben *et al.,* 1998). Limited diversity is found in *Pba* and, therefore, it is considered a distinct, unique species, restricted to potato (Bell *et al*., 2004; De Boer, 2003). In comparison, *Pbc* and *Dickeya* strains show great diversity and a wide host range (Oliveira *et al.*, 2003; Ma *et al*., 2007). *Erwinia chrysanthemi (*Burkholder) (*Ech*) was also recently placed in a new genus, *Dickeya,* based on DNA-DNA hybridization, phenotypic tests and 16S rRNA gene sequences (Samson *et al.*, 2005; Charkowski, 2006).

Pectobacterium subspecies can be differentiated from each other and distinguished from *Dickeya* spp. based on polymorphism in the *recA* gene (for recombination), the *pel* gene (for pectate lyase) and the intergenic spacer ribosomal (*rrn*) gene cluster (De Boer, 2003). However, identification of *Dickeya* spp. is still very difficult since only a limited number of strains of each species have been characterised and sequenced completely (Palacio-Bielsa *et al*., 2007).

Further classification of subgroups is based on host preference and their ability to survive in different geographic environments (De Boer, 2003). *Pectobacterium carotovorum* is classified into subspecies, *betavasculorum*, *odorifera* and *wasabiae* that are host specific for beet, chicory, and horse radish, respectively (Toth *et al.*, 1999a; Fessehaie *et al*., 2002; Kang *et al.*, 2003). Recently, Duarte *et al.* (2004) described a new subspecies from Brazil, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*), which also causes blackleg-like symptoms on potatoes.

Nasser *et al*. (1996) discovered that species in tropical areas on Solanaceous crops have a greater diversity and thus proposed that South and Central America might be the centre of diversification of *Dickeya* spp. The most important *Dickeya* spp. causing diseases on potatoes are: *Dickeya dadantii* (formerly *Ech* 3937) (*Dd*) and *D. zeae* (formerly *Ech* biovar 3) which are pathogenic in warm climates. However the *D. dianthicola* (formerly *Echr* biovar 1 and 7) stains are more temperate-adapted and recently become a problem in Europe (Samson *et al*., 2005; Elphinstone & Toth, 2007; Tsror *et al*., 2009).

Pectobacterium carotovorum, Pba and *Dickeya* spp. are plant pathogenic bacteria that are facultative anaerobic, peritrichously flagellated, gram-negative rods, 0.5 – 1.0 by 1.0 – 3.0 µm in size and non-spore forming (Agrios, 1997; Charkowski, 2006). *Pectobacterium carotovorum* and *Dickeya* spp. have fimbriae or pili present on their cells but these do not occur on *Pba* cells (Pérombelon & Kelman, 1980). All species are catalase positive and oxidase negative. Soft rot pectobacteria can be distinguished from other plant pathogenic enterobacteria by their pectolytic activity and their colony characteristics on Crystal Violet Pectate (CVP) medium (Kelman & Dickey, 1988; Charkowski, 2006).

Garden *et al*. (2003) described the different characteristics of soft-rot *Pectobacterium* spp. The main characteristics to distinguish between species are that *P. carotovorum* can grow at 36°C and cause soft rot on various hosts, whereas *P. atrosepticum* cannot grow at 36°C and causes blackleg and soft rot on potatoes tubers and on tomato plants (Darrasse *et al*., 1994). *Pectobacterium carotovorum* subsp. *brasiliensis,* the new strain detected in Brazil, can grow at temperatures > 37°C (Duarte *et al*., 2004).

2.2.2 Pathogenicity and secretion systems

Soft rot bacteria are pectolytic-saprophytic and therefore have a complex metabolism to utilize living or dead plants (Pérombelon, 2002). Toth & Birch (2005) described these bacteria as hemi-biotrophic due to their ability to change during infection from a biotrophic to a necrotrophic existence. Initially, soft rot bacteria are present in the latent phase in lenticels and wounds where they multiply aggressively until a critical population density (between 10^7 and 10^8 cfu/ml) is reached. They colonise and multiply in the apoplast before plant cell death. Once a critical population density is reached the production of enzymes is initiated and causes degradation of the plant cell wall and maceration of the plant tissue around the infection area (Smadja *et al.,* 2004a; Smadja *et al.*, 2004b).

Pathogenicity relies primarily on the production of extracellular plant cell wall degrading enzymes (PCWDE) including pectinases (pectate lyase, pectin lyase, polygalacturonases, petin methylesterases), proteases, cellulases and β-glucosidases which collectively participate in virulence (Whitehead *et al.,* 2002; Bell *et al*., 2004; Faure & Dessaux, 2007). Further degradation of polysaccharides is promoted by the existence of enzymes in multiple isozyme forms (Byers *et al.*, 2002; An *et al*., 2005).

The most important exoenzyme responsible for tissue maceration and indirectly cell death is pectinase (Toth *et al.*, 2003a). Pectinase degrades pectins and pectates in the middle lamella between cells and in plant cell walls, causing tissue to collapse, which ultimately results in cell damage and cell content leakage. Four types of pectinase are produced, namely pectate lyase (Pel), pectin lyase (Pnl), pectin methyl esterase (Pme) and polygalacturonase (Peh) (Pérombelon, 2002; De Boer, 2003).

Generally, primary and secondary Pels have a low enzyme activity and play a major role in host specificity and infection (Pérombelon, 2002).

The expression of pathogenicity factors (the production of PCWDE, production of carbepenem, other virulence factors such as harpin production and symbiosis) in soft rot pectobacteria is under tight control of cell density-dependent regulation (quorum sensing) (Von Bodman *et al.,* 2003; Faure & Dessaux, 2007). To ensure successful infection, quorum sensing allows bacterial multiplication in host tissue without triggering host resistance (no maceration of plant tissue occurs), until a certain threshold level is reached to overcome it (Miller & Bassler., 2001). Toth *et al.* (2004) suggested that PCWDE regulation later in the infection stages is probably due to an increase in demand for nutrients, rather than avoiding plant defences, as PCWDE generate plant cell-wall products that are recognised by the plant defence mechanisms.

Quorum sensing (QS) in soft rot pectobacteria relies upon *N*-acylhomoserine lactones (AHL) -based systems (Smadja *et al.*, 2004a; Zhu *et al.*, 2006; Barnard *et al.,* 2007). Each species produces its own unique AHL molecules to ensure successful infection (Whitehead *et al.*, 2001; Von Bodman *et al*., 2003; Chatterjee *et al*., 2005*).* *N*-acylhomoserine lactone production in soft rot pectobacteria is regulated by a complex set of interactions between different genes (*carI*, *expI*, *expR*) (Corbett *et al*., 2005; McGowan *et al*., 2005; Burr *et al.* 2006; Barnard *et al*., 2007).

The production of AHL plays an important role in modulating the secretion of exoenzymes as well as in the biosynthesis of a β-lactam antibiotic, carbapen-2-em-3-carboxylic acid (carbepenem) (Smadja *et al.*, 2004; Toth *et al.*, 2004; McGowan *et al.*, 2005). The success of pathogenicity is dependent on the expression of exoenzymes and the antibiotic, carbepenem. When genes encoding for exoenzyme and carbepenem are interrupted, it can directly influence virulence negatively *in planta* (Whitehead *et al.,* 2001). Soft rot pectobacteria*, in planta,* produce AHL in small quantities that accumulate directly as bacterial density increases. As the AHL concentration increases, both the onset and the severity of disease increase due to the massive secretion of PCWDE (Toth *et al.*, 2004).

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Quorum sensing determines the production of PCWDE. At the early infection stage bacterial populations are low (10^2 cfu/ml), AHL are below QS levels and no PCWDE are secreted. As the bacterial density increases, AHL accumulate. At higher concentrations (>10^6 cfu/ml) QS is activated and PCWDE are produced. During this stage plant defences are induced, however they are insufficient to prevent disease development (Toth *et al.*, 2004).

Antibiotic production confers a fitness advantage to eliminate a number of competitor bacterial species from colonising the nutrient-rich niche of infection (Miller & Bassler, 2001; Byers *et al*., 2002; Pérombelon, 2002; Faure & Dessaux, 2007). *Pectobacterium* spp. produce different forms of carotovoricin that kills closely related stains and species. Carotovoricin production is initiated when DNA is damaged and temperature is between 23 - 26ºC (Charkowski, 2006).

In soft rot bacteria, pathogenicity requires a number of protein secretion systems to transport pathogenic determinants to the extracellular environment to physically attack host cells (De Boer, 2003). Recently, genome sequences of *Pbc*, *Pbcb* and *Pba* (SCRI1043) suggested that these bacteria use similar tools for plant cell maceration but diverse mechanisms for initial host-pathogen interactions. *Pectobacterium atrosepticum* (SCRI1043) is the only bacterium identified that requires type I and II systems together with types III, IV and V (autotransporter), two-partner secretion and additional putative type I secretion system for full virulence. The type IV secretion system is also present in *Pbcb*. Genes required for PCWDE, type II secretion system and homologues of type III were conserved in all three species, although *hrpK* was absent in *Pba* (Bell *et al*., 2004; Marquez-Villavicencio *et al*., 2007).

The type I secretion system (T1SS) is based on the secretion of protease and plays a minor role in pathogenicity (Toth *et al*., 2003a; Toth *et al*., 2006; Barnard *et al*., 2007). The type II secretion system (T2SS) is essential for soft rot disease development and is responsible for the delivering of PCWDEs mainly Pels, Peh, Cel (pectate lyase, pectate hydrolases and cellulases) to the plant cell wall (Toth *et al*., 2003a; Corbett *et al*., 2005; Toth & Birch, 2005; Toth *et al*., 2006).

In *Pbc* and *Pba* an additional necrotrophic mechanism, Nip (necrosis-inducing virulence proteins) may be required for virulence on potato and necrosis on tobacco (Toth & Birch, 2005).

The type III secretion system (T3SS) plays a major role in host-pathogen interactions by using the *hrp* (hypersensitive response and pathogenicity) system and *hrc* (hypersensitive response conserved) genes (Yap *et al*., 2005; Toth *et al*., 2006). The effector proteins (*DspE*/*A*) and the harpin proteins (HrpN and HrpW) manipulate or suppress plant defence responses and play a role in translocation processes and in aggregation, respectively (De Boer, 2003; Yap *et al.*, 2005; Toth *et al*., 2006; Faure & Dessaux, 2007). Harpin N protein (HrpN) expression is under the control of QS (Barnard *et al*., 2007) and can be induced in non-host (resistant) plants (Smadja *et al*., 2004a; Toth & Birch, 2005). The type IV secretion system (T4SS) transports macromolecules between bacteria and from bacteria into their hosts using the *virB* gene cluster (Toth *et al*., 2006).

Pathogenicity, development of disease symptoms and expression of PCWDE are environment-dependent. Temperature determines the level of pectate lyase synthesis. *Pectobacterium atrosepticum* produces larges quantities of enzyme at 15ºC but the enzyme is undetectable at 30ºC. In contrast, *Pbc* produces large quantities of enzymes at both temperatures (Pérombelon & Kelman, 1980). The optimum temperature for pectate lyase synthesis in *Pbc* and *Pba* is <20ºC whereas in *Dickeya* spp., production at 25ºC is 20 times higher than at 37ºC. Iron deficiency can also promote pectate lyase synthesis, which is higher under anaerobic than under aerobic conditions (Faure & Dessaux, 2007).

2.2.3 Diagnostics

To ensure that a correct diagnosis is made, identification systems for *Pectobacterium* spp. and *Dickeya* spp. are required and important to reduce pathogen spread as the majority of pathogens are transmitted through mother tubers (Toth *et al*., 2001; Degefu, 2006). Accurate species identification and pathogen detection is a prerequisite for studying the pathogen epidemiology in order to set effective control strategies (Bell *et al*., 2004, Henz *et al.*, 2006).

Soft rot pectobacteria species are closely related however great variability exists within species. Many of the species are pathogenic on common hosts, causing similar disease symptoms, therefore making correct identification of the causal agent very difficult (Nasser *et al*., 1996; Avrova *et al*., 2002). Ma *et al*. (2007) found considerable diversity between previous methods and work recently done on potatoes. This increases the demand for a simple, accurate, routine detection method that is rapid and has a high level of specificity and sensitivity (Degefu, 2006).

Commonly used identification methods for isolation and characterisation of soft rot bacteria are time-consuming, laborious, imprecise and insensitive. Isolates are usually purified on a selective medium, crystal violet pectate (CVP), and further characterised using various biochemical tests. However, identification is limited to species level although groupings are questionable (Darrasse *et al*., 1994; Avrova *et al*., 2002). Elphinstone & Toth (2007) observed that *Dickeya dianthicola* grows poorly on CVP medium.

Serological tests depend on the specificity of monoclonal and polyclonal antibodies but due to their complexity within (especially *Pbc*) and between subspecies (high number of serogroups) these tests have been found to be insensitive. Fatty acid profiling has also been used to identify *Pectobacterium* spp. but was unsuccessful in distinguishing between subspecies (Toth *et al*., 2001). Currently the focus is based on development of new molecular tools for use in diagnosis (Darrasse *et al*., 1994; Toth *et al*., 2001; Mahmoudi *et al*., 2007).

Another limitation in accurate identification of soft rot bacteria is the amplification of non-pathogenic strains on potato, though they are not commonly found in the environment. The great variability in the sensitivity level *in planta* among strains, cultivars and replications has been reduced by using an enrichment step in either Luria broth (at axenic situations) or pectate enrichment medium (at non-axenic situations) prior to PCR (Toth *et al*.,1999b).

In order to study phylogenetic relationships between species, 16 rRNA sequencing is used. Unfortunately, for routine identification it is unrealistic and has a limitation in sensitivity at subspecies level (Toth *et al*., 2001).

Toth *et al.* (1999b) developed a one-step 16S rRNA PCR-based detection system for screening potato plants and tubers for the existence of soft rot *Erwinia*, to test the health status during seed certification. Universal primers, SR3F and SR1cR, have been used and therefore enabled the differentiation of species and subspecies. DNA-DNA hybridization is used in DNA-relatedness studies and is accurate, sensitive but time-consuming, expensive and unsuitable for routine use (Toth *et al*., 2001; Kang *et al*., 2003).

Effective approaches are amplified fragment length polymorphism (AFLP) and repetitive sequencing but use is limited due to the need of specialised computer analysis for identification (Toth *et al*., 2001). Amplified fragment length polymorphism is used to study taxonomic classification and phylogenetic relationships, to distinguish between species, subspecies and the different serogroups. However, interspecific relationships cannot be determined using this technique (Avrova *et al*., 2002).

The randomly amplified polymorphic DNA (RAPD)-PCR method is inexpensive, quick and simple, however analysis of complex patterns can be difficult and lack reproducibility. RAPD-PCR analysis was used to differentiate between *Pbc*, *Pba* and *Pseudomonas* spp.. DNA fragments can be easily generated of which selected bands can be cloned and sequenced for the development of SCAR markers that can be useful in PCR-based detection of *Pba* isolates (Mäki-Valkama & Karjalainen, 1994; Parent *et al*., 1996; Toth *et al*., 2001).

The universal rice primers (URPs) have been used to fingerprint genomes of different organisms. URP-PCR uses long primers (20 nucleotides) and highly stringent PCR conditions in contrast to RAPD-PCR. The nucleotide sequence of the *Pbc*-specific primer was generated from the URP-PCR product, a specific DNA probe that detects *Pcc* and *Pectobacterium carotovorum* subsp. *wasabiae* (*Pcw*) species. The specific primer set, EXPCCF/EXPCCR was designed and used in specie-specific PCR. *Pbc* and *Pcw* can be differentiated using biochemical tests, pathogenicity, host range and G + C content (51-53 mol %).

For higher sensitivity of detection (as few as 2 - 4 cfu per reaction) a nested PCR is conducted with primers INPCCR/INPCCF selected from the sequence bases downstream at the 3'-ends of primers EXPCCF/EXPCCR (Kang *et al.,* 2003).

Restriction fragment length polymorphism (PCR-RFLP) analyses applied to pectate lyase genes (especially *pel* genes from the *Y* family) have been used for diagnosis, but are unsuccessful in identifying all the subspecies in the soft rot *Erwinia* group. A subsequent RFLP is necessary to differentiate between species. The primers Y_1 and Y_2 were designed specifically for *Pbc, Pba, Pectobacterium carotovorum* subsp. *odorifera* and subsp. *wasabiae* but could not amplify *Dickeya* spp. or *Pectobacterum* subsp. *betavasculorum* (Frechon *et al.*, 1998).

It is important to apply diagnostic tests in conjunction with pathogenicity studies (Darrasse *et al*., 1994). Duarte *et al*. (2004) found that PCR amplification with primers, Y_1/Y_2 targeting the *pel* genes, amplified the DNA of *Pbcb* successfully, however *Pba* and some *Pbcb* strains were not amplified with the Y_1/Y_2 primers. A PCR kit for specific detection of *Pba* was evaluated based on the *pel* genes. A specie-specific primer set, Y45/Y46 was used together with a capture probe to target sequences, in contrast to gel electrophoresis which detects non-specific amplified DNA (Frechon *et al.*, 1998).

In order to detect *Dickeya* spp., a species-specific PCR was developed based on the sequences of the open reading frames of the *pelADE* gene cluster which are conserved regions (about 420bp long). Primers ADE1 and ADE2, amplified the *pel* fragment in *Dickeya* spp. and were compared with RFLP analysis to differentiate within and between strains (pathovars and biovars) (Nasser *et al*., 1996).

Ward & De Boer (1994) selected a DNA probe specific for all four serogroups of *Pba* from an *EcoRI* digest of a cloned library constructed with the combined genomic DNA. This isolated fragment of *Pba* DNA can be used as a specific probe for *Pba* alone. The nucleotide sequence of the *Pba*-specific DNA probe was determine using T3 and T7 plasmid primers. Primers ECA1f and ECA2r were highly conserved for the typical blackleg pathogen in comparison with the *pel* gene primer sequences (De Boer & Ward (1995).

Recently in Brazil, blackleg-like symptoms were shown to be caused by a new more virulent strain, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) that produces no PCR product with *Pba*-specific primers (Duarte *et al*., 2004). Therefore, a new primer, BR1f was developed from the 16S – 23S intergenic spacer region (IGS) based on the restriction site of SexAI together with primer L1r to amplify DNA from *Pbcb* (Duarte *et al*., 2004).

The 16 - 23S intergenic transcribed spacer (ITS) exhibits a greater sequence and length variation that can be exploited in a simple PCR-RFLP- based test (ITS-PCR in combination with ITS-RFLP) and is suitable for differentiation between species and subspecies. Incidences did however occur where *Pbc* and *Pectobacterium carotovorum* subsp. *oderifera* (*Pbco*) could not be differentiated as their ITS regions are very closely related. To differentiate between them, an α-methyl glucoside test was done; which is positive for *Pbco* and negative for *Pbc*. Different banding patterns within *Pbc* and *Dickeya* spp. reflect the high level of diversity within species /subspecies. This PCR-RFLP-based test is a rapid, accurate and simple method for the identification of soft rot bacteria to species / subspecies level (Toth *et al*., 2001). Fessehaie *et al.* (2002) used sequencing of the 16S rDNA and the 16S – 23S rDNA IGS with primers, 27f/L1r and 1491f/L1r respectively to study the relatedness among subspecies of soft rot pectobacteria. The 16S rRNA gene contained a small number of unique nucleotide positions but very few strains were misplaced. The small 16S - 23S rDNA IGS regions were very diverse in size and nucleotide sequence except in *Pba,* where they were highly conserved. The large IGS did not provide phylogenetic information but did group related taxa together successfully (Fessehaie *et al*., 2002).

The RecA protein is multifunctional and involved in recombination, DNA repair and SOS response. Analysis of restriction fragment length polymorphism of the *recA* gene fragment amplified by PCR (*recA* PCR-RFLP) can be used to rapidly study species and subspecies diversity of soft rot *Pectobacterium* spp. and identify all species belonging to the *Pectobacterium* and *Pantoea* genera (Waleron *et al*., 2002).

A specific PCR using crude plant extracts to detect *Pcb* and *Dickeya* spp. was developed, based on the combination of two specific primers (ATROREV/CHRREV) with the *Pectobacterium*-specific primer (ERWFOR), to detect *Pca* and *Dickeya* spp. simultaneously. However, the shared-primer PCR assay is less specific than the primer sets individually; therefore, faint bands were visible due to the amplification of non-target DNA of *Pbc* (Smid *et al*., 1995). Furthermore, extracted bacterial DNA from tuber tissue inhibited DNA amplification due to the presence of plant-derived compounds. In order to obtain bacterial DNA free from plant-derived compounds, a more specific extraction method was developed using low-speed centrifugation of crude extract, high-speed centrifugation of low-speed supernatant followed by lysis of the extracted bacteria. This PCR assay is highly sensitive and can detect a single infected tuber in a batch of 30 tubers. The PCR assay can be adjusted on the basis of viable counts in pure cultures (Smid *et al.,* 1995).

2.3 THE DISEASE COMPLEX

2.3.1 Symptoms

Blackleg and aerial stem rot (aerial blackleg) diseases develop on potato stems under favourable environmental conditions during the production season. Tuber soft rot affects tubers as seed piece decay (stolon-end rot and early dying) and soft rot of daughter tubers (lenticel rot) in the field and storage (Darrasse *et al*., 1994; Hélias *et al*., 2000a; Roberts *et al*., 2007). These symptoms vary with climate and are not dependent on a specific strain (Lumb *et al*., 1986; Rowe *et al*., 1995). *Dickeya* and *Pectobacterium* spp. cause similar disease symptoms on potato plants. These include wilting, stunting, chlorosis and a brown to black vascular browning at the stem base that extends upwards and causes the whole plant to collapse (Elphinstone & Toth, 2007). Incidences have occurred where *Pbc* and *Pba* were found in mixed populations. In such cases the prevalent climate determines which strain will be pathogenic (Darrasse *et al*., 1994).

Blackleg

Blackleg is an economically important disease that can cause a great reduction in yield and downgrading or rejection of seed tubers during certification (Pérombelon, 2000). Blackleg symptoms are not specie-specific but dependent on the prevailing climate in a specific geographic area. For instance the causal agent of blackleg in Europe is *Pba* and in Arizona/Colorado is *Pbc* (Darrasse *et al*., 1994).

Blackleg symptoms develop at various times during the vegetative growing season and generally only on one stem per plant. Blackleg originates from a completely rotted mother tuber that progress, externally or internally, upwards via the vascular system into the stem (Lumb *et al*., 1986; Allefs *et al*., 1996; Pérombelon, 2000; Elphinstone & Toth, 2007). Light brown to inky-black lesions become visible (Fig. 2.1). Stem discolouration, darkening, progress upwards due to the ability of the soft rot bacteria to migrate into the vascular tissue and macerate the parenchymatous tissue, resulting in necrosis of the pith and an internal cavity (Abenthum *et al*., 1995; Hélias *et al*., 2000a; Rowe *et al*., 1995; Elphinstone & Toth, 2007).

Fig 2.1 Inky-black discolorations on a blackleg stem originating from a rotten mother tuber.

Infected plants are stunted and symptoms initially appear as a wilt on the top leaves (sometimes on one side of the upper leaves) that have the tendency to roll upwards at the margins. Subsequent desiccation around the margins develops, until the whole leaf area is affected. Leaves may also become chlorotic.

Symptoms progress gradually to the lower leaves (Lumb *et al*., 1986; Elphinstone & Toth, 2007). Under humid weather, affected stems are soft and water soaked, while under drier conditions lesions shrivel and harden (Roberts *et al*., 2007). In advanced cases of infection plants die and dry out.

***Pectobacterium* wilt**

First symptoms were a one-sided wilt of the top leaves and subsequent desiccation around the margins until the entire leaf area was affected. These symptoms gradually spread to the lower leaves, until the entire plant wilt. The stem usually remains green until leaf desiccation is complete. Severely wilted plants dried out and die. Wilting is normally confined to a single stem of the infected plant. Plants with mild infections may recover overnight or during cool weather. Infected plants showed occasionally rotting of the stem base, or stem hollowing due to the necrosis of the pith. Wilted plants were associated with a discoloration of the vascular system at the stem base without external darkening of the stem. Symptom development was usually associated with a soft rot of the mother tuber (Lumb *et al*., 1986; Serfontein *et al*., 1991; Niederwieser, 2003)

Tuber soft rot

Tuber soft rot can occur in the field and in storage, beginning at lenticels (point of entry and where the pathogen remains latent) and wounds. The most common symptoms caused by soft rot bacteria on potatoes in South Africa are tuber soft rot and wilting. Under conditions of high relative humidity, wilting of stems can develop and result in tuber soft rot (Serfontein *et al.,* 1991). Tuber rot ranges from a slight vascular discolouration at stolon ends to complete decay. In the field mother tubers are the main source of contamination, causing seed piece and sprout decay in the soil pre- or post emergence, which results in poor stands (Elphinstone & Toth, 2007).

Usually, soft rot begins in lenticels (especially near the apical end of the tuber, where lenticels are the most abundant) or wounds as small, circular, uniform, water-soaked areas that are sometimes brown with a sunken pox-effect on the surface. Infection spots coalesce and enlarge until the whole tuber is softened and granular (Davidson, 1948).

The macerated tissue is cream- to tan-coloured and delineated by a brown or black margin separating diseased tissue from healthy tissue (Morgan & Wicks, 1998; Roberts *et al*., 2007) (Fig. 2.2). In some cases lenticel infections dry up and form chalky, white spots (Roberts *et al*., 2007). In the early stages of infection decaying tissue is odourless but during advanced stages decay becomes stringy or slimy with a foul odour due to secondary infection. The whole tuber disintegrates leaving pieces of the peel in the soil (Gouws, 2003; Rowe *et al*., 1995).

Fig. 2.2 Bacterial soft rot on potatoes with the characteristic brown / black margin separating diseased tissue from healthy tissue.

Plants that show blackleg-like symptoms in the field often develop a characteristic black soft rot during storage of tubers. Symptoms associated with soft rot of the mother tuber depend on the level of infection (Abenthum *et al*., 1995; Hélias *et al.,* 2000a; Rowe *et* al., 1995; Roberts *et al*., 2007). Thus, when tubers of crops that showed high blackleg incidence are stored, soft rot can be severe (Pérombelon, 2000).

Aerial stem rot (Aerial blackleg)

Aerial stem rot or aerial blackleg is a secondary soft rot of stems and petioles which does not originate from rotting mother tubers (Roberts *et al*., 2007). Infections on the stems are initiated in wounds caused by hail, windblown sand, insect feeding, cultivation practices or natural openings like leaf scars. Secondary spread of bacteria is in rain and irrigation water. Disease development is intensified by dense foliage, high humidity and wet conditions (Pérombelon, 1992).

Initially symptoms on the stem start as a soft green decay that develops quickly into brownish to inky-black spots that enlarge to a soft, watery rot and progresses until the entire stem wilts and dies (Rowe *et al*., 1995; Roberts *et al*., 2007). Infestation can spread through the soil from contaminated seed tubers to daughter tubers which develop rot either in the field or during storage (Toth *et al*., 2003b).

2.3.2 Etiology

Soft rot bacteria remain extracellular on host tissue in the latent phase, in lenticels, vascular system and wounds of tubers until favourable environmental conditions are present to initiate disease development (De Boer, 1987; Pérombelon, 1992; 2000; Toth *et al*., 2003b). Symptoms of wilting and desiccation are most common under dry conditions and soft rot under wet conditions (Pérombelon, 1992). During the early stages of *Pectobacterium*-host interactions, reduced oxygen levels, high amounts of available free water, optimal temperature and the production of factors involved in host recognition are required for disease development (Toth *et al.,* 2004).

The level of tuber contamination directly influences the probability of blackleg occurring in the field, thereby reflecting the health status of the seed tuber (Smid *et al*., 1995). In the field a high percentage of seed tubers can be infected with a low concentration of soft rot bacteria (Hélias *et al*., 2000a). The threshold level for disease development is 10^2 – 10^3 colony forming units (cfu)/ml (Pérombelon, 2002).

Pathogenicity of the different pectobacteria species is temperature-dependent and will therefore determine which strain has the greatest chance of causing infection. In temperate, cool (<15 ºC), moist climates *Pba* is the primary causal agent of blackleg disease however *Pbc* can also cause disease (Pérombelon & Kelman, 1980; Hélias *et al*., 2000a; De Haan *et al*., 2008). At high temperatures (>37 ºC) *Pbc*, *Pbcb* and *Dd* can cause blackleg-like symptoms and rotting of tubers (Duarte *et al*., 2004). *Pectobacterium atrosepticum* and *Dickeya* spp. can also cause blackleg at cool temperatures in the early stages of infection. With an increase in temperature later in the growing season, disease incidence also increases (Pérombelon, 1992). All soft rot bacterial species can cause disease at relatively similar rates when the temperature is close to 22 ºC (Pérombelon & Kelman, 1980).

However, during the harvesting period tuber soft rot occurs commonly when soil temperatures are higher than 20 °C, especially in the Northern Hemisphere (Morgan & Wicks, 1998).

The importance of relative humidity (RH) in symptom expression during field trails was emphasised by Lumb *et al.* (1986). At a low RH more leaf desiccation and less rotting occurred but at a high RH a reduction in leaf desiccation occurred, favouring soft rot (Davidson, 1948; Wicks *et al.*, 2007).

Soft rot symptoms in the field are initiated during heavy rainfall periods when soil is at field capacity, forcing the lenticels to stay open (Davidson, 1948; Wicks *et al.*, 2007). The lenticels absorb water and cause high turgidity, which promotes lenticel breakage. The existence of a low oxygen concentration affects the cell membrane integrity and leads to solute leakage. A liquid state develops between the cortex and lenticels and makes it possible for the bacteria to penetrate into the cortex tissue causing the tuber to rot. Another penetration method is through breaks and wounds in the periderm caused by wind, mechanical, insect or fungal damage (Pérombelon & Kelman, 1980).

In the field the incidence and severity of blackleg is affected by the soil moisture content, oxygen levels, temperature, soil nutrient content, cultivar resistance and the synergistic and antagonistic effect of other pathogens (Pérombelon *et al*., 1990). The highest incidence of post emergence blackleg is found when moderate soil temperature, wet and humid conditions prevail (Pérombelon & Kelman, 1980; Serfontein *et al.,* 1991).

The incidence of bacterial soft rot on stored tubers increases by favourable physiological factors such as prevailing anaerobic conditions (low oxygen levels) caused by the presence of a continuous film of free water covering the tuber surface, free moisture and optimum temperature. Conversely, these environmental conditions are adversative for potato tuber resistance against bacterial infection (Maher & Kelman, 1987). Interestingly, although these soft rot bacteria are facultative anaerobic, *in vitro* they grow better under aerobic than anaerobic conditions (Pérombelon & Kelman, 1980).

2.3.3 Epidemiology

Inoculum in plant material

The most important inoculum source of soft rot bacteria is progeny tubers contaminated by mother tuber decay. Bacteria can be spread and transmitted by contaminated mechanical equipment, through cracks and wounds in the periderm and cortex (De Boer, 1987). During harvest and grading, bacteria are easily spread from contaminated tubers to fresh wounds on other tubers (Pérombelon, 2000).
Soft rot bacteria can overwinter in small amounts in crop residues and rotten tubers in cool soil after harvest for approximately 6 months. On leaf surfaces *Pbc* and *Pba* can only grow and survive when surfaces stay wet (Pérombelon & Kelman, 1980). *Pectobacterium* spp. can successfully survive for a full growing season on volunteer plants and agricultural weeds. *Pectobacterium carotovorum* is found as part of the natural rhizosphere microflora of several cruciferous plants, lupine plants and certain weeds (Burr & Schroth, 1977).

Potatoes are vegetatively propagated and therefore exposed to a high risk of soft rot bacterial infection. The major source of contamination with *Pbc, Pba* and *Dickeya* spp. is commercial seed stocks. The level of tuber contamination on a tuber surface can be from 0 – 10^2 cfu per lenticel. In winter storage after 6 – 7 months a great reduction in surface contamination occurs, but the bacterial population in the lenticels is maintained or only slightly reduced (Pérombelon & Kelman, 1980).

During seed-stock multiplication from microplants or mini-tubers the incidence of tuber contamination is low early in the production season. In the following generations affected stocks lead to a higher incidence in the field due to the build up of pathogen inoculum levels. Blackleg incidence is proportional to the level of seed contamination (Pérombelon *et al.,* 1998). Prevailing environmental and cultural conditions directly influence the variation in contamination level between and within potato seed stocks (Pérombelon, 2000).

Inoculum in soil

Soil, and blackleg-affected plant debris, are not important sources of inoculum in long crop-rotation systems (>4 years) and in well rogued crops (Pérombelon & Hyman, 1992; Pérombelon, 2000). In fields where crop rotation is not applied, bacteria can survive in the soil in low numbers. In south-western Australia *Dickeya* spp. have been reported to survive in the soil whereas in Israel the bacteria were not detected in the soil or in irrigation water (Lumb *et al*., 1986). In Wisconsin, in soils of previous potato fields, *Pba* and *Pbc* could not be detected prior to planting of crops for the following season (De Boer *et al*., 1979). Thus soft rot pectobacteria are not able to survive in soil for more than one year except when they survive in diseased tubers or plant debris (Rowe *et al*., 1995).

Nutrient availability and additional nutrients in the soil extend the bacterial survival rate, whereas a low nutrient supply will result in a reduction in bacterial numbers (Pérombelon & Kelman, 1980). The soil population can also be reduced by the presence of antagonistic bacteria, actinomycetes and fungi producing antibiotics. *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 produces antimicrobial substances that reduce potato blackleg and soft rot development (Sharga & Lyon, 1998). Another antagonistic bacterial strain is *Pseudomonas putida* (Trevisan 1889) Migula 1895, which can reduce *Pectobacterium carotovorum* populations (Sturz & Matheson, 1996). In fallow soil a reduction of soft rot bacteria can occur over time (Burr & Schroth, 1977).

Inoculum in rain, irrigation water and in post harvest water

Windblown rain can transmit soft rot bacteria over a relatively long distance. A certain level of soft rot bacteria can be present in irrigation- and borehole water (Serfontein *et al*., 1991). Aerosols contaminated with bacteria are generated by rain impaction on diseased and pulverized stems (Pérombelon & Kelman, 1980). *Dickeya* spp. are commonly present in rivers (Laurila *et al*., 2007).

The presence of a continuous film of free water covering the tuber surface promotes rapid oxygen depletion in the tuber especially during the washing process. High inoculum concentrations are found in water baths and water flumes during the washing process (Pérombelon & Kelman, 1980; Wicks *et al*., 2007).

This is due to the hydrostatic pressure that forces bacterial infiltration into lenticels and eases lenticel opening. The highest number of rotted tubers and the highest level of rot development on tubers were found after passage through the tumbler (Pérombelon & Kelman, 1980; Morgan & Wicks, 1998; Wicks *et al*., 2007).

Dissemination by insects

Inoculum dissemination between plants by insects increases disease dispersal (Hélias *et al*., 2000a). Airborne insects are vectors for the dispersal of soft rot bacteria. Their bodies become contaminated during the larval stage when they come into contact with the rotting seed pieces. The bacteria are then carried to healthy plants (Agrios, 1997).

Potato tissue infected with *Pbc* and *Pba* attracts fruit flies (*Drosophila melanogaster* (Meigen, 1830)) (Rich, 1983). Insects in the order *Diptera* generally present near potato and vegetable fields and often transmit *Pbc* and *Pba*. Insects damage the stem tissue, and deposit bacteria into injured tissue, which results in the development of aerial blackleg symptoms under favourable conditions (Pérombelon & Kelman, 1980). Seed corn maggots (*Hylemyia* spp.) become infested after feeding on contaminated tubers and then carry the bacteria in the intestinal tract. When healthy seed tubers are wounded the bacteria are transmitted (Rich, 1983).

2.3.4 Host range

Pectobacterium spp. infects a relatively wide range of tuber-, corn-, and bulb-crops and ultimately cause great economic losses in vegetable production. The alternative hosts and causal agents are found to be different from country to country. *Pectobacterium atrosepticum* is more specifically associated with potato blackleg and soft rot. However, recently post harvest soft rot of pepper (*Capsicum annuum* L.) fruits and tomatoes was caused by *Pba* as well as *Pbc* (Hadas *et al*., 2001; El-Hendawy *et al*., 2002).

Pectobacterium carotovorum has the widest host range and causes losses in crops grown in subtropical and temperate regions, such as lettuce, garlic, sweet potato, eggplant, zucchini, onion, cauliflower, arracacha, melon, sweet-pepper, okra, cabbage, tomato, collard, brussels sprout, carrot, celery, cucumber, capsicum, turnip, chicory, sugar beet, potato, crown imperial (*Fritillaria imperialis* L.*)*, tobacco and mulberries (*Morus* spp.) (Seo *et al*., 2003; Fiori & Schiaffino, 2004; Mahmoudi *et al*., 2007; Xia & Mo, 2007).

Dickeya spp. has a wide host range and can affect carnations, Leopold lily, maize, sunflower, pineapple, potato and the important model host African violet [*Saintpaulia ionantha* (Wendl)], *Aloe vera* (L.) (Burm), carrots and onions (Fraaije *et al*., 1997 ; Hélias *et al.,* 1998; El-Hendawy *et al*., 2002 ; Toth *et al.,* 2003a; Mandal & Maiti, 2005; Henz *et al*., 2006 ; Palacio-Bielsa *et al*., 2007).

2.3.5 Synergism

In the field pathogens exist in a diverse ecological environment with other organisms which may or may not compete to inhabit a particular niche. Certain bacterial species can live in close association with fungal pathogens and therefore help one another in successfully infecting hosts, even when the bacteria are not pathogenic to the specific host or when environmental conditions are adverse for disease development (Newton *et al*., 2004).

The synergistic interaction between *Pba* and fungal mycelium (certain fungal pathogens such as *Verticillium dahliae*) can cause secondary infection and subsequently increase blackleg incidence (Pérombelon, 1987). When *Pba* and *Fusarium* spp. exist in a relationship it can cause non-emergence in a great number of crops. Another interaction was found between *Phytophthora infestans* ((Montagne) de Bary, 1876) and *Pba* in rotting tubers. *Phytophthora infestans* stimulated *Pba* pathogenicity by the production of oligogalacturonide during the enzymatic degradation process of pectin. When plant tissue is infected with *P. infestans* and *Fusarium coeruleum* (Libert ex Saccardo, 1886) the pH remains neutral. This promotes *Pba* pathogenicity by maintaining a pH closer to the optimum for polygalcturonase activity (Sicilia *et al.,* 2002).

2.3.6 Natural defence and bacterial infection

During the early stages of tuber formation, before lenticel suberization, the lenticels are very susceptible to penetration by pathogens (Yahiaoui-Zaidi *et al*., 2003). Lenticels of stored tubers are normally closed and suberized. The formation of the suberin layer is inhibited by a large amount of free water and in the presence of high bacterial concentration, which promotes an oxygen deficiency in tuber tissues. These conditions are conducive for soft rot development (Morgan & Wicks, 1998). Oxygen is essential for tuber resistance to soft rot, therefore when artificially inoculated tubers are incubated under aerobic conditions, soft rot does not develop (Charkowski, 2006).

Potato tubers protect themselves against bacterial invasion by a physical barrier, the Casparian band, which is produced during maturation (Morgan & Wicks, 1998). Thus, immature tubers are more susceptible to soft rot than mature tubers (De Boer, 1987). The Casparian band is a layer composed mainly of suberin. Suberin is a hydrophobic lipid complex attached to a phenolic, aromatic or lignin-like domain that is attached to the cell wall (Lulai & Corsini, 1998; Hopkins, 1999).

When seed tubers are cut, processes such as deposition of suberin, phenolics, aliphatic components and soluble waxes are initiated, which play a major role in disease resistance. Resistance to infection can be promoted by the optimisation of environmental conditions that enhance suberization (Lulai & Corsini, 1998). Normally, infection is prevented by the thickening of the lipid, lignin and periderm layers. The amount and morphology of lignin at the stem base can influence the mobility of soft rot bacteria from the seed tuber upwards in the stem (Abenthum *et al*., 1995).

Generally, the phenolic composition of potato peels among different potato varieties is similar and not influenced by the colour of the potato peels. Chlorogenic acid, caffeic acid and protocatechuic acid are major phenolic acids in potato peels whereas minor phenolic acids include ferulic acid, *p*-hydroxybenzoic acid, gallic acid, vanillic acid and *p*-coumaric acid (Onyeneho & Hettiarachchy, 1993). The phenolic concentrations detected in tuber peels are much higher than in the tuber flesh (Lewis *et al*., 1998a).

The resistance of potatoes to soft rot bacteria during lenticel infection and wound-healing is mainly associated with the formation of phenolic acids. Lewis *et al*. (1998b) showed that the effect of diseased peels and flesh greatly enhanced the concentrations of total phenolic acids. The amount of phenolics found in tubers is correlated to the degree of resistance to soft rot pectobacteria. Thus tubers susceptible to soft rot have the least amount of phenolics in comparison to resistant tubers. High levels of phenolic compounds improved the quality and shelf-life of tubers.

Kumar *et al*. (1991) found that the antibacterial activities of caffeic acid, gallic acid, catechol and p-hydroxybenzoic did not differ significantly. However Ghanekar *et al*., (1984) found that caffeic acid and ferulic acid have a more inhibitory effect on *Pectobacterium* spp. growth than chlorogenic acid. Chlorogenic acid, caffeic acid and ferulic acid in combination exerted antibacterial activities more effectively even at lower concentrations than individually (Ghanekar *et al*., 1984).

Another important defence mechanism is the accumulation of phytoalexins in diseased potato stems and tubers. Phytoalexins were not detected in healthy tuber tissue (Ghanekar *et al.,* 1984). High concentrations of solavetivone, followed by rishitin and phytuberin accumulated in stem tissue infected with *Pectobacterium* spp.. Smaller amounts of phytuberol, hydroxysolavetivone, stereoisomer and solanascone were also detected (Abenthum *et al.*, 1995). Rishitin and phytuberin have antibiotic properties, which result in structural modifications of the outer membranes of the bacteria (Ghanekar *et al*., 1984).

Potato cultivars show different levels of resistance to post-harvest tuber soft rot. Tubers with relatively high dry matter content are less susceptible to *P. carotovorum* than tubers with a lower dry matter content. In the field only partial resistance exists against potato blackleg, however it is very difficult to measure. The rate of the rotting process during various growth phases of the plant will indicate the level of resistance, taking into account that resistance is directly influenced by environmental conditions (Allefs *et al*., 1996).

Three wild potato species *Solanum boliviense*, *Solanum chacoense* and *Solanum sancta-rosae* have been identified as highly resistant to potato blackleg and tuber soft rot and are used in breeding programs (Pérombelon, 1992; Charkowski, 2006).

2.3.7 Host Nutrition

Plant nutrition is known to affect incidence and severity of important phytopathogens. Krištufek *et al*. (2000) found that the soil exchangeable Ca influences the incidence and severity of potato common scab (*Steptomyces scabies*). High levels of nitrogen fertilizer decrease phenolic content in tubers, thereby enhancing the susceptibility of cell walls to pectolytic enzyme degradation (Wegener & Jansen, 1996).

Calcium (Ca) is a macro-nutrient in soil that binds to pectate in the middle lamella, which provides stability and strengthens the cells walls (Gunter, 2002).
High Ca concentrations in cell walls can reduce the rate of soft rot development by enhancing the structural integrity of cells walls and membranes. Thus high levels of soil Ca can increase the resistance of potato stems and tubers to pectolytic enzyme degradation. An increased extracellular Ca concentration can also suppress the expression of endopolygalacturonase (PehA) which is the main virulence determinant of *Pectobacterium carotovorum* in the early stages of infection (Flego *et al*., 1997).

Gypsum ($CaSO_4$) soil amendments pre-plant have shown to result in a significant reduction in blackleg incidence the growing season. The effect however decreased towards the end of the season. Thus, the stem resistance appears to be related to Ca and Mg concentrations in plant tissue (Bain *et al*., 1996). The increase in Ca concentrations in the tuber periderm is, however, associated with a decrease in the Mg concentrations (Simmons & Kelliing, 1987). Lambert *et al*. (2005) found that the application of Mg can also suppress tuber soft rot, but to a lesser extent in combination with Ca.

Calcium can also be applied in the form of calcium nitrate but is limited by the nitrogen requirement of the potato plants (Lambert *et al*., 2005).

Khalil *et al*. (1999) found that Ca and Boron and play an important role in maintaining membrane integrity and cell permeability of the host, but also have an effect on the production of bacterial metabolites (Khalil *et al*., 1999).

2.3.8 Control

In order to prevent rotting of the mother tuber and subsequent blackleg outbreaks, the planting of pathogen-free seed potatoes is critical. However, the production of infected seed is often unavoidable, due to the high risk of contamination as a result of the complexity of different infection pathways (Pérombelon & Hyman, 1992; Pérombelon *et al.,* 1998). Critical points at which to apply effective control measures are based an in-depth understanding of disease epidemiology (Oliveira *et al*., 2003).

The best control strategy is to plant seed tubers with a reduced tuber contamination level ($<10^2 – 10^3$ cfu/ml), rather than trying to treat blackleg symptoms on the crop during the growing season (Pérombelon *et al.,* 1998).
Thus effective control of blackleg is based on the detection and quantification of the causal agent in seed tubers, in conjunction with the elimination of severely contaminated seed lots (Pérombelon & Hyman, 1992; Mäki-Valkama & Karjalainen, 1994). Presently, certification schemes fail to eliminate blackleg because they are based on visual inspections and rogueing to a given set of tolerance levels, and cannot detect latent infection in the progeny tubers. However, visual blackleg inspections and rogueing can help by reducing the risk of spread from contaminated tubers and can eliminate crops with an abnormally high blackleg incidence (Pérombelon, 2000). Currently, in South Africa no official post-harvest testing programme exits and the use of diagnostics is on a voluntary basis only.

Blackleg control could also be obtained by breeding for resistant cultivars. Resistant cultivars can be effectively produced by conventional breeding and molecular engineering. Sources of high resistance to soft rot pectobacteria have been found in wild *Solanum* spp. such as *Solanum brevidens* (Pérombelon & Hyman, 1992).

Good sanitation at all stages of production combined with good crop management practices, including a 2 to 3 year rotation programme with non-host crops can minimise infection (Osborn, 1995; Sharga & Lyon, 1998; Oliveira *et al*., 2003) and improve the health status of seed tubers (Pérombelon, 2000). Seed tubers need to be planted relatively shallow in well-drained soil when the soil temperature is moderate. Excessive irrigation during the growing season should be avoided (Sturz & Clark, 2005). Early harvesting can prevent tuber contamination (Pérombelon, 1992). Tubers should be harvested when fully matures and soil temperatures are lower than 20 °C (Sturz & Clark, 2005). Various biological control agents, especially *Bacillus subtilis* BS 107, can be applied to the soil or seed to reduce potato blackleg and soft rot development (Sharga & Lyon, 1998).

During the washing process at harvest, sanitising agents such as Oxine, Nylate, Klorman, Liquid pool chlorine, Sporekill and Proxitane reduce levels of soft rot bacteria in wash water. It is important to prevent build up of bacteria in the wash water. It is therefore necessary that the water in the wash and tumbler areas is replaced frequently with clean or treated recycled water (Morgan & Wicks, 1998; Wicks *et al*., 2006).

The bacteria in contaminated seed tubers die rapidly when seed tubers dry out (Pérombelon & Kelman, 1980). Therefore, tubers should be handled with care and should be very dry before going into storage (Osborn, 1995). Cool (5 – 10°C), dry, well ventilated conditions in storage need to be maintained to reduce the development of tuber soft rot (Morgan & Wicks, 1998; Lambert *et al*., 2005). In order to avoid condensation due to high metabolic activity associated with sprout lengthening it is essential to use forced air ventilation in comparison to naturally ventilated boxes (Pringle & Robinson, 1996).

2.4 CONCLUSION

The potato crop is very susceptible to pests, diseases and quality problems that result in increased input cost to prevent economical losses.

Due to the difficulty to reduce risk and control disease development it is necessary to identify high-risk areas and predict times of soft rot or wilt outbreaks. To improve disease management strategies it is also necessary to investigate the population biology, disease etiology, the host–pathogen interactions and host resistance.

Soft rot bacteria cause enormous losses in agricultural crops due to their population dynamics, complexity of the group of pathogens, ubiquity, virulence, variation in symptom expression and their ability to multiply rapidly. In the field the most important inoculum source of soft rot bacteria is progeny tubers contaminated by mother tuber decay. Contaminated water and insects can also be a source of inoculum whereas contaminated soil is not an important source of bacteria.

Blackleg, aerial stem rot (aerial blackleg) and tuber soft rot can develop during the production season and in storage. Symptoms are not specie specific but are dependent on climatic conditions. Therefore, *Dickeya* and *Pectobacterium* spp. can cause similar disease symptoms on potato. In the field the incidence and severity of blackleg is affected by soil conditions, oxygen levels, temperature, soil nutrient content, cultivar resistance and the synergistic and antagonistic effect of other pathogens. In storage, optimal conditions for tuber soft rot are low oxygen levels, high amounts of available free water and high temperatures.

Pathogenicity of soft rot pectobacteria relies primarily on the production of extracellular plant cell wall-degrading enzymes including pectinases, proteases, cellulases and β-glucosidases. To ensure successful infection without triggering host resistance, the expression of these pathogenicity factors is under tight control of cell density-dependent regulation (quorum sensing). In soft rot bacteria pathogenicity also requires a number of protein secretion systems to transport pathogenic determinants to the extracellular environment to physically attack host cells.

Resistance of potatoes to soft rot bacteria is mainly associated with the formation of phenolic acids with antibacterial action. Another important defence mechanism is the accumulation of phytoalexins in diseased potato stems and tubers. Host resistance is increased by optimal plant nutritional.

High concentrations of Ca, B and to a lesser extent Mg can reduce the incidence and severity of soft rot and blackleg.

Since there are no effective chemical control measures for this disease complex and no resistant cultivars available, management of the disease is primarily based on the production of healthy seed. Therefore sensitive, accurate diagnostic and identification methods are required to detect and quantify *Pectobacterium* spp. and *Dickeya* spp. in order to develop effective integrated control measures (Degefu, 2006).

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CHAPTER 3

PECTOBACTERIUM AND *DICKEYA SPP*. ON POTATOES IN SOUTH AFRICA

ABSTRACT

Pectobacterium carotovorum (*Pbc*), *Dickeya* spp., *Pectobacterium atrosepticum* (*Pba*) and a new, atypical strain, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*), can cause potato blackleg, soft rot and associated symptoms. To determine the impact and extent of these pathogens in the potato industry in South Africa, a countrywide survey was conducted. Stem and tuber samples were collected from 72 potato fields in 10 potato production regions from the beginning of 2006 until the end of 2007. Bacteria were isolated on a selective medium, Crystal Violet Polypectate (CVP), and maintained on Nutrient Agar (NA). Isolates that were Gram-Negative, facultatively anaerobic with pectolytic ability were identified using a Multiplex PCR and amplification of the 16S-23S rDNA intergenic spacer region. One hundred and twenty eight isolates were identified. Of these 77% were shown to be *Pbcb* and 17% *Pbc*. *Dickeya* spp. or *Pba* were not detected in South Africa. Some isolates (8%) could not be identified. Isolations were also made from imported tubers and 14 isolates were obtained, 13 of which were identified as *Pbcb* and one isolate as *Pbc*. This research demonstrates that *Pbcb* is a problem in potato production regions in South Africa and represent an important early step towards the understanding of the epidemiology of soft rot pectobacteria.

3.1 INTRODUCTION

Globally potatoes (*Solanum tuberosum* L.) are a major food crop cultivated in most parts of the world. In South Africa potato production for the 2006/2007 season exceeded 1,92 million tonnes, cultivated on 54 037 hectares (Potatoes South Africa, 2008).

The South African potato production areas are divided into sixteen main regions namely Limpopo, North-West, Gauteng, Mpumalanga, Northern Cape, Western Free State, Eastern Free State, KwaZulu-Natal, Sandveld, Ceres, South-Western Cape, Southern Cape, Eastern Cape, North-Eastern Cape, South-Western Freestate and Loskopvalley (Niederwieser, 2003; Potatoes South Africa, 2008).

Worldwide, one of the biggest problems in the production of seed tubers is the contamination thereof with one or a combination of soft rotting pectolytic bacteria. These bacteria can cause tuber soft rot in storage and infections in seed tubers that result in symptoms such as poor emergence, chlorosis, *Pectobacterium* wilt, tuber and stem rot, blackleg, haulm desiccation and plant death (De Haan *et al.,* 2008). Commonly, when blackleg in the field and tuber soft rot in storage occurs, seed lots are discarded (Laurila *et al*., 2008). Symptoms are not species specific and can therefore differ within and between specific regions (Pérombelon *et al*., 1987). When a mixture of strains is present, one strain usually predominates and initiates symptom development under specific climatic conditions (Lumb *et al*., 1986; Pérombelon *et al.*, 1987).

During the 1988 growing season typical wilting symptoms associated with stem rot appeared for the first time on potatoes in South Africa (Serfontein *et al*., 1991). *Pectobacterium carotovorum* (Jones) (*Pbc*) (formerly known as *Erwinia carotovora* subsp. *carotovora*) and *Dickeya* spp. (Burkholder) (formerly known as *Erwinia chrysanthemi*) were found to be the causal agents (Serfontein *et al*., 1991; Garden *et al*., 2003; Samson *et al*., 2005). No isolates were identified as *Pectobacterium atrosepticum* (Van Hall) Dye (*Pba*) (formerly known as *Erwinia carotovora* subsp. *atroseptica*) (Serfontein *et al*., 1991; Garden *et al.*, 2003). However, two isolates were identified by the National Collection of Plant Pathogenic Bacteria (NCPPB) using fatty acid profiling as *Dickeya zeae* (formerly known as *Erwinia chrysanthemi* biotype IV (pathovar *zeae*)) and *Dickeya dianthicola* (formerly known as *Erwinia chrysanthemi* biotype V (pathovar *dianthicola*)) respectively (Serfontein *et al*., 1991; Samson *et al*., 2005; Ma *et al*., 2007).

Blackleg is an economically important disease and is characterised by the blackening of the stem base of potato plants, which originates from the rotting mother tuber (Pérombelon *et al*., 1987). Blackleg appears to be unpredictable in the field due to the ability of soft rot pectobacteria to stay latent in seed tubers (Laurila *et al*., 2008). *Pectobacterium atrosepticum* is an important causal agent of blackleg in various geographical areas of Canada, US and Europe (Duarte *et al.*, 2004). However, *Dickeya* spp. can also cause blackleg-like symptoms. *Pectobacterium carotovorum* can also be virulent in a temperate climate causing blackleg symptoms (De Haan *et al*., 2008). Recently a new, atypical strain, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) was described in Brazil, also causing blackleg-like symptoms on potatoes. *Pbcb* was found to be more virulent than *Pba*, the typical causal agent of potato blackleg. Results also suggested that there is a possibility that when the newly-described subspecies is present on potatoes, *Pba* will not be detected (Duarte *et al*., 2004).

The pathogens, *Pbc, Pba* and *Dickeya* spp., have a typical geographical distribution that reflects their pathogenicity, which is dependent on prevailing climatic conditions and the variation in local weather conditions (Lumb *et al*., 1986). Factors such as temperature changes, soil moisture content and pathogen host ranges (Bain *et al*., 1990), have been found to correlate with the incidence and severity of soft rot and blackleg in a specific season as well as with its survival and dissemination worldwide (Smadja *et al*., 2004). Incidence is, however, primarily dependent on the population density of the pathogen in planted seed tubers (Pérombelon *et al.*, 1987; Pérombelon & Kelman, 1987).

Pectobacterium carotovorum and *Dickeya* spp. are phenotypically and genetically far more diverse than *Pba*, with the greatest variability being within *Pbc*. *Pectobacterium carotovorum* and *Dickeya* spp. are widely distributed within a wide host diversity that plays a major role in survival (Pérombelon & Kelman, 1987; Toth *et al*., 2003). The survival of *Pbc* and *Dickeya* spp. in plant-free soil is less than one year, therefore over-wintering is unlikely (Rowe *et al*., 1995). Soft rot bacteria can also be present in irrigation- and borehole water (Serfontein *et al*., 1991). *Dickeya* spp. are commonly found in river water (Laurila *et al*., 2008).

The most important source of inoculum, however, is contaminated seed tubers and the pathogen can thus be spread over long distances via infected vegetative material (Elphinstone & Toth, 2007). The means of dissemination of *Pbcb* are unknown and need to be investigated in order to understand the epidemiology of the disease (Duarte *et al*., 2004).

The objective of this study was to determine the causal agents of blackleg and soft rot of potatoes and their distribution in South Africa.

3.2 MATERIALS AND METHODS

3.2.1 Sampling of diseased plant material

Stems and tubers of plants with typical symptoms (soft rot, wilting, internal and external darkening), as well as symptomless seed tubers were collected throughout the 2006/2007 growing seasons from both potato fields and storage facilities. Samples were randomly collected from 72 potato fields in 10 production regions in South Africa: Mpumalanga (13); Eastern Free state (6); KwaZulu-Natal (9); Western Free State (6); Northern Cape (6); Northern Province (8); Sandveld (10); Ceres (4); Eastern Cape (6) and North-West (4) (Figures in parentheses indicate number of farms visited) (Fig. 3.1). Imported techni-tubers from two foreign countries were also sampled. Random samples of ten techni-tubers were collected from three seed lots from one country and five seed lots from the other country. The number of samples chosen was relative to the size of the consignment.

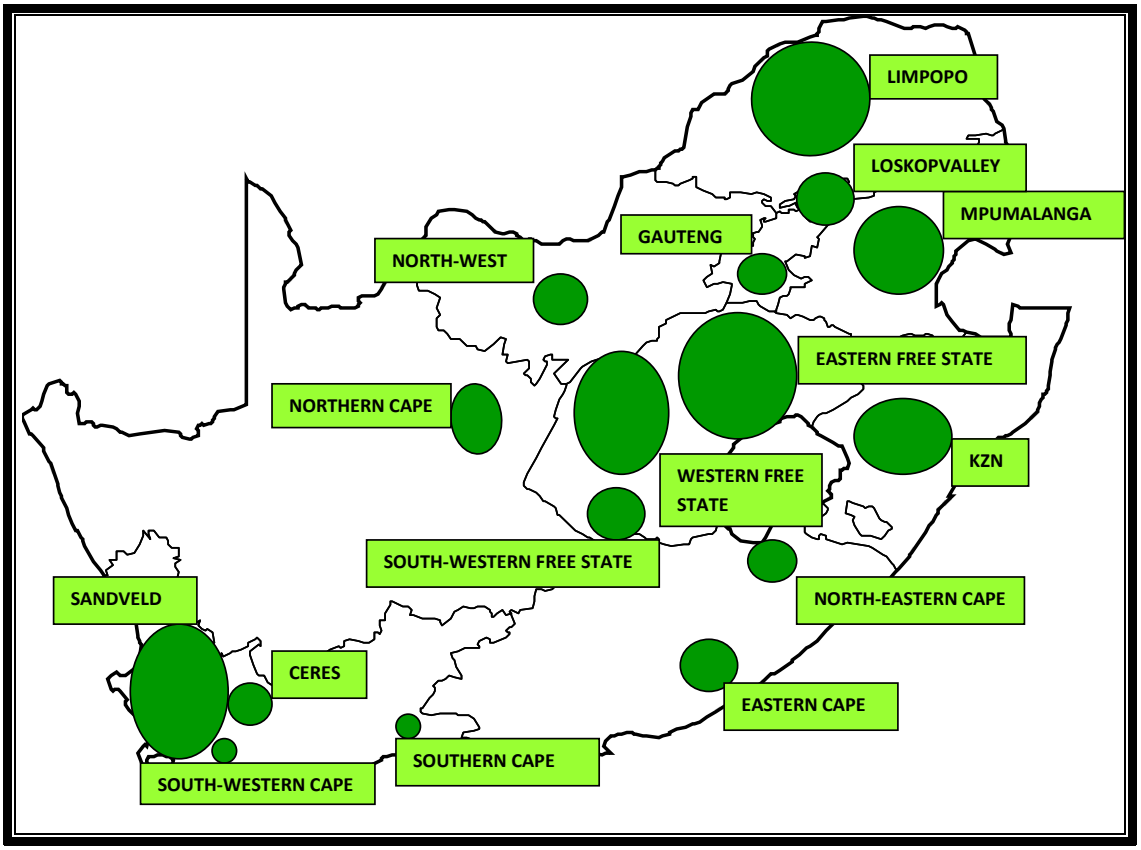

Fig. 3.1 Hectares cultivated per potato production regions in South Africa

3.2.2 Isolations

Samples were transported in brown paper bags and stored at 4°C. Isolations were done immediately or stored at room temperature not longer than five days. Plant material was washed under running tap water. Infected stem parts 10 cm in length and tuber peels from the margin of infected tissue were placed in Bioreba bags and macerated in 1ml of 0.01M Magnesium sulphate. Loopfulls of the suspension were streaked on a modified selective medium, Crystal Violet Pectate (CVP) (Hyman *et al*., 2001) and incubated at 26°C for 48h. Isolates that produced characteristic pectolytic cavities were purified by sub-culturing from single discrete colonies on CVP and then transferred to Nutrient Agar (NA) (Merck).

3.2.3 Phenotypic identification

For differentiation and classification of Gram-negative bacteria, single colonies were purified and subjected to biochemical tests. These biochemical tests included Gram staining (3% KOH) and the Hugh and Leifson`s oxidation/fermentation test (Merck, 2005).

Isolates that utilised glucose both oxidatively and fermentatively were stored in sterile water at room temperature and in 15% glycerol : nutrient broth solutions at -80°C. All isolates are maintained in the Potatoes@UP culture collection, University of Pretoria, South Africa. Representative isolates (JJ54 & JJ68) were also deposited in the NCPPB (National Collection of Plant pathogenic Bacteria) library. Their assigned codes are 20712982 and 20712983, respectively (Appendics B1 & B2).

3.2.4 Type strains

Two of the type cultures used in the study were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM™), namely *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T) and *Pectobacterium atrosepticum* (LMG 2386^T). Type cultures were also obtained from the American Type Culture Collection (ATCC), for *Pectobacterium carotovorum* subsp. *brasiliensis* strains 8 and 371 (ATCC BAA-417 and 419, respectively) (Duarte *et al*. 2004). The *Dickeya dadantii* (*Erwinia chrysanthemi* 3937) type strain was obtained from the Scottish Crops Research Institute (SCRI).

3.2.5 DNA extractions

DNA was extracted from 48-hour-old cultures grown on NA using a DNeasy™ Blood & Tissue Kit (Qiagen). RNA was removed with Ribonuclease A from bovine pancrease (Sigma-Aldrich) (1mg/ml) by incubating overnight at room temperature. Extracted DNA was stored at -20°C.

3.2.6 Multiplex PCR

A Multiplex PCR as described by De Boer, S.H., Personal Communication, 2007 was optimised and used to identify and differentiate between *Pbcb*, *Pbc*, *Pba* and *Dickeya* spp. Purified DNA was amplified using species-specific primers BR1f/L1r (Duarte *et al.,* 2004); ECA1f /ECA2r (De Boer & Ward, 1995); EXPCCF/EXPCCR (Kang *et al*., 2003) and ADE1/ADE2 (Nasser *et al*., 1996) (Whitehead Scientific (Pty) Ltd.) for *Pbcb*, *Pba, Pbc* and *Dickeya* spp., respectively (Table 3.1).

Table 3.1 Oligonucleotide primer pairs used in the Multiplex PCR and the expected amplified product sizes of *Pbcb*, *Pbc*, *Dickeya* spp. and *Pba*, used in identification of bacterial isolates.

Specie	Primer	Sequences (5''3', base pairs)	Amplified product size (bp)	References	Region amplified
Pbcb	BR1f	GCGTGCCGGGTTTATGACCT	322	Duarte *et al* ., 2004	Partial 16S - 23S rDNA region
	L1r	CARGGCATCCACCGT			
Pbc	EXPCCF	GAACTTCGCACCGCCGACCTTCTA	550	Kang *et al* ., 2003	Region in pECC2F nucleotide
	EXPCCR	GCCGTAATTGCCTACCTGCTTAAG			
Dickeya spp.	ADE1	GATCAGAAAGCCCGCAGCCAGAT	420	Nasser *et al* ., 1996	Conserved region of the *pelADE*
	ADE2	CTGTGGCCGATCAGGATGGTTTTGTCGTGC			
Pba	ECA1f	CGGCATCATAAAAACACG	690	De Boer & Ward, 1995	*Pba* genomic DNA (gDNA)
	ECA2r	GCACACTTCATCCAGCGA			

The primer concentrations were optimised and subsequently a gradient PCR was performed to optimise annealing temperatures. Thus each 25µl pre-amplification reaction contained 1 x NH_4 Reaction Buffer; 2mM $MgCl_2$ solution; 100µM dNTP mix; 20 - 30pmol of each primer and 1.25U BIOTAQ^{TM} (Whitehead Scientific (Pty) Ltd.). PCR amplification was carried out using a thermal profile: 95°C for 2min, followed by 30 cycles of 94°C for 45s, 62°C for 45s, 72°C for 90s and a final extension step of 72°C for 10min. A negative water control was included in each PCR reaction to check for contamination, as well as positive controls using reference cultures of each species. The amplified products were run on a 2% agarose gel and visualised under UV light.

Due to primer non-specificity and therefore some misidentification, primer complementary was tested using DNAMAN version 4.13, Lunnon Biosoft. Primer interference was also tested. Therefore, to distinguish between *Pbcb* and *Dickeya* spp., the amplification of the 16S-23S rRNA region was done

3.2.7 Amplification of the 16S-23S rDNA region

To distinguish between *Pbcb* and *Dickeya* spp., the 16S-23S rDNA intergenic spacer (IGS) region was amplified using primers 1419F and L1R from the conserved sequences flanking the 3` end of the 16S rRNA gene and the 5` end of the 23S rRNA gene, respectively (Fessehaie *et al*., 2002). Each 25µl pre-amplification reation contained 1 x NH_4 Reaction Buffer; 2mM $MgCl_2$ solution; 100µM dNTP mix; 50pmol of each primer and 1.25U BIOTAQ^{TM} (Whitehead Scientific (Pty) Ltd.).

PCR reactions were carried out with the following thermal profile: 95°C for 2min, followed by 30 cycles of 94°C for 30s, 62°C for 45s, 72°C for 90s and a final extension step of 72°C for 7min. Amplified products were electrophoresed through a 2% agarose gel in 1 x TBE Buffer at 75V.

3.3 RESULTS

During the field surveys, 128 isolates from healthy and diseased stems and tubers were collected from 80% of the farms (Appendix A). Fourteen isolates were obtained from imported techni-tubers. Pectolytic bacteria that were Gram negative and scored positively in the Hugh and Leifson`s oxidation/fermentation test were used for further identification. In total, 142 isolates from stems and tubers with and without symptoms were identified using the optimised multiplex PCR (Fig 3.2).

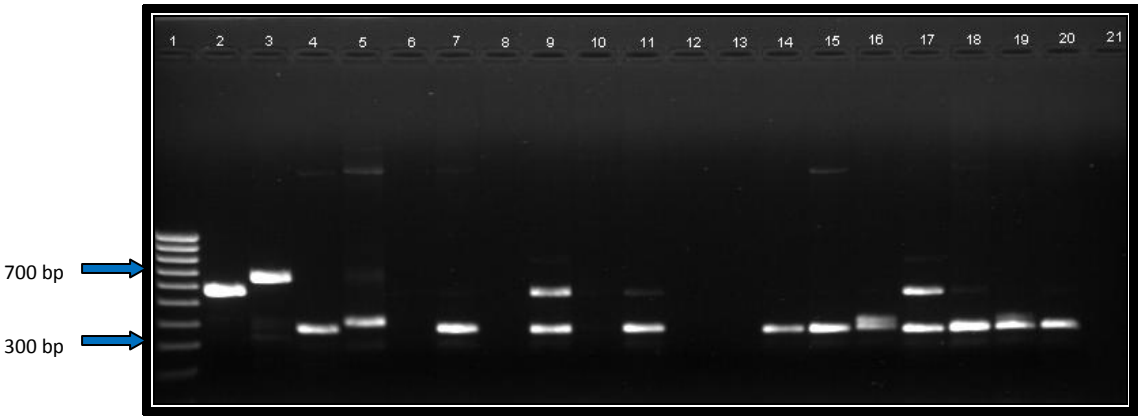

Fig. 3.2 Agarose gel electrophoresis of Multiplex PCR - amplified DNA products to test for the presence of four different bacterial strains. Lanes: 1, 100bp DNA ladder; 2, *Pectobacterium carotovorum* (LMG 2404^T) (550bp amplified product); 3, *Pectobacterium atrosepticum* (LMG 2386^T) (690bp amplified product); 4, *Pectobacterium carotovorum* subsp. *brasiliensis* 371 (ATCC BAA-419) (322bp amplified product); 5, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937) (420bp amplified product); 6 - 20, Isolates from potatoes in SA; 21, Negative control (water). Amplified products were run on a 2% agarose gel.

Results obtained from the Multiplex PCR (Fig. 3.2) suggested that of the isolates obtained in South African potato fields, 75% were *Pbcb*; 17% were *Pbc*; 6% were *Dickeya* spp. and 4% were unknown.

These results take into consideration that five isolates showed amplification of two bands (*Pbcb* and *Pbc)* using the Multiplex PCR, which was indicative of mixed populations of *Pbcb* and *Pbc* (Fig. 3.2). Of the isolates obtained from imported techni-tubers, 13 were identified as *Pbcb* and only one isolate as *Pbc.* By using specie-specific primer sets in the multiplex PCR, expected DNA fragments were obtained from isolates and reference cultures to identify strains of *Pbc*, *Pba* and *Dickeya* spp. However, the primers specifically used for *Pbcb* showed non-specific banding as well as amplified *Dickeya* spp. (Fig. 3.3).

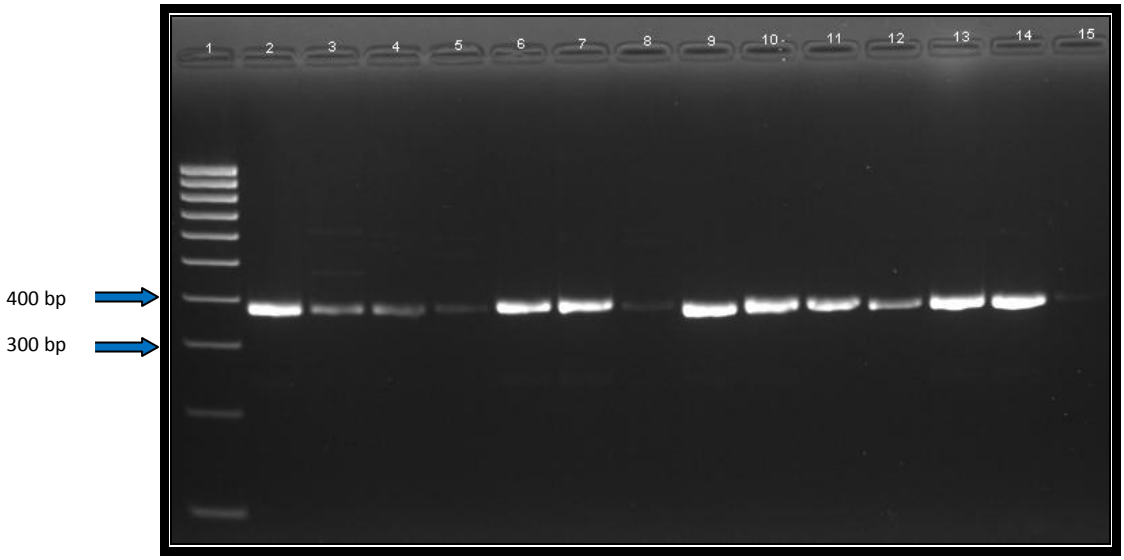

Fig. 3.3 Agarose gel electrophoresis of specie-specific PCR for *Pbcb* to test specificity of primers between *Pbcb* and *Dickeya* spp. isolates. Lanes: 1, 100bp DNA ladder; 2, *Pectobacterium carotovorum* subsp. *brasiliensis* 371 (ATCC BAA-419) (322bp amplified product); 3, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937) (420bp amplified product); 4 - 14, Isolates from potatoes in SA preliminary identified as *Dickeya* ssp.; 15, Negative control (water). Amplified products were run on a 2% agarose gel

In the Multiplex-PCR primer pairs, the DNA fragments amplified by primer sets ADE1/ADE2 and BR1f/L1r had only a small difference (98kb) in band size and it was therefore difficult to differentiate between *Pbcb* and *Dickeya* spp. For this reason primer specificity was tested and it was found that BR1f/L1r is not only specific for *Pbcb* but also amplifies DNA fragments of *Dickeya* spp.

Non-specfic amplification could be due to the fact that the reverse primer, L1r, has a short length of 15bp and can therefore attach to more than one specific binding site within a genome (Dieffenbach *et al*., 1993; Edwards & Gibbs, 1994). When primer complementarity was tested, it was shown that between 7 and 13 bases were complementary in the two primer sets, therefore enhancing primer-dimer formation. Thus, this multiplex PCR cannot distinguish between *Pbcb* and *Dickeya* spp..

To distinguish between *Pectobacterium* spp. and *Dickeya* spp. amplification of the IGS was done, which generated two PCR fragments as reported by Fessehaie *et al*. (2002). One hundred and twenty five isolates preliminarily identified as *Pbcb* or *Dickeya* spp. were subjected to IGS-PCR. The results showed that in South Africa *Pbcb* strains are dominant (77%) and that none of the isolates obtained in this study were *Dickeya* spp. (Fig. 3.4). However, 6% of the isolates were not amplified and two isolates showed similar bands to *Dickeya* spp. but these bands were very faint. *Pbcb* was detected in nine of the 10 production regions visited. The exception was in Ceres, where only *Pbc* was detected. *Pectobacterium carotovorum* was detected in seven production regions, but not in North-West, Northern Cape or KwaZulu Natal.

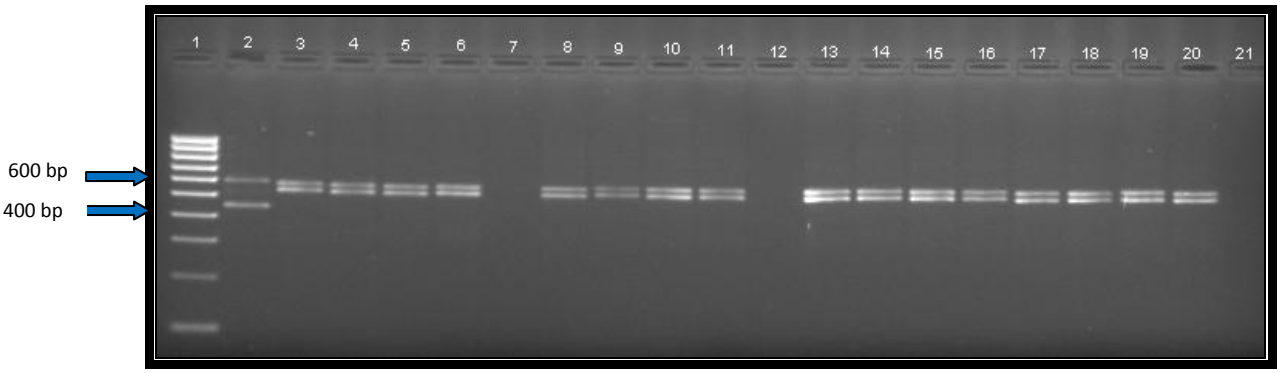

Fig. 3.4 Agarose gel electrophoresis of IGS PCR - amplified DNA products to test for the presence of *Pectobacterium carotovorum* subsp. *brasiliensis* and *Dickeya dadantii*. Lanes: 1, 100bp DNA ladder; 2, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 3, *Pectobacterium carotovorum* subsp. *brasiliensis* 371 (ATCC BAA-419); 3 - 20, samples previously identified as either *Pectobacterium carotovorum* subsp. *brasiliensis* or Dickeya spp. using the multiplex PCR; 21, Negative control (water). Amplified products were run on a 2% agarose gel.

Some of the isolates identified as *Pectobacterium* spp. by IGS-PCR were, however, not amplified using the multiplex PCR.

These isolates could be totally different species or might correspond to *Pectobacterium carotovorum* subsp. *betavasculorum,* as described by Thomson *et al*. (1981) (cited by Hélias *et al*., 1998). This can be explained by the fact that all the *Pectobacterium* spp. including subsp. *brasiliensis* and subsp. *betavasculorum* yielded the expected amplified bands of small (~440bp) and large (~490) IGS regions. In comparison to the *Dickeya* spp. yielded two different DNA fragments of small (~354bp) and large (~486bp) IGS regions (Fessehaie *et al*., 2002), thus differentiating *Pectobacterium* spp. from *Dickeya* spp.

3.4 DISCUSSION

The data generated in this study revealed that 77% of the isolates obtained were *Pbcb*, 17% were *Pbc* and 6% of the isolates obtained could not be identified. It is interesting to note that isolates obtained from imported techni-tubers were also contaminated with *Pbcb* (13 isolates identified) and *Pbc* (one isolate identified).

In South Africa *Pbcb* appears to be most virulent and better adapted to the South African conditions than the other soft rot bacteria species. This could possibly be due to climatic changes and an increased demand for imported seed tubers.

This research has also supported the findings of Duarte *et al*. (2004) that *Pbcb* has the ability to cause blackleg-like symptoms at higher temperatures than *Pba*. Disease severity and incidence of blackleg is dependent primarily on initial inoculum in seed tubers and the prevailing climatic conditions such as optimal temperatures, humidity and excessive rainfall in poorly drained soils. Accordingly when seed tubers are contaminated with pectobacteria it may reflect the region where the seed was produced rather than where the crop was sampled.

Although Serfontein *et al*. (1991) isolated *Dickeya* spp. in South Africa, none of the isolates obtained in this study were found to be *Dickeya dianthicola* and *Dickeya zeae*. There is a possibility, though that *Dickeya* spp. could exist in tubers and plants in very low numbers but due to the prevailing climatic conditions during the season, it could not be detected.

The low bacterial numbers also could be due to competition with more virulent species. It is however important to note that *D*. *dianthicola* has recently also been reported from Israel and the Netherlands as causing similar symptoms as *Pbcb* (Elphinestone & Toth, 2007), and the type strains of this species should be included in future molecular studies in South Africa.

Pectobacterium carotovorum subsp. *brasiliensis* was found in nine potato production regions, whereas *Pbc* was found in seven production regions (Appendix A). However the distribution of *Pbcb* and *Pbc* has no apparent relation to geographic regions, temperature or rainfall, thus other epidemiological factors are involved in dissemination. The possibility of tubers and plants being contaminated through soil is very low due to the application of good crop rotation systems (4 - 6 year rotation systems with maize, corn, wheat, oats, grass, cauliflower and canola). Therefore potato seeds especially of susceptible cultivars, insects, irrigation water and host plants, are primarily responsible for the survival and dissemination pattern of these pathogens.

The multiplex PCR together with the IGS-PCR used in this study is a convenient tool for identifying and differentiating between *Pbc*, *Pba* and *Pbcb* or *Dickeya dadantii*. Although *Pbcb* and *Dickeya dadantii* could not be differentiated using the multiplex, IGS-PCR was used to accurately distinguish between the two species. This research also demonstrates that *Pbcb* is a problem in the potato production industry in South Africa and represents an important early step towards the understanding of the epidemiology of the soft rot / blackleg disease complex in South Africa.

The best strategies to control bacterial diseases are based on resistance breeding, good cultural practices (crop rotation and elimination of plant debris) and the use of healthy seeds (Pérombelon & Hyman, 1992). Therefore management and control strategies will benefit from tools allowing rapid and specific pathogen detection at a minimal level of infection (Kang *et al*., 2003). Bacteria in seed tubers can remain latent until the tubers are planted and weather conditions become optimal for the bacteria to become pathogenic (Pérombelon *et al.*, 1987). Contaminated seed tubers often escape detection and increase the risk of major economic losses caused by the soft rot disease complex.

Another concern is the significant effect of growing conditions on disease development which makes it impossible to accurately predict blackleg incidence. This is because when seed tubers are assessed, the expected climatic conditions during the following growing season are unknown (Bain *et al*. 1990).

3.5 CONCLUSION

This study has shown that *Pbcb* (77%) is the predominant causal agent of the soft-rot / blackleg disease complex on potatoes in South Africa. This pathogen is highly contagious and aggressive. Low tuber inoculum levels can lead to high blackleg incidence under environmental conditions favourable for bacterial multiplication and disease development. Losses due to potential infections by *Pbc* (17%) are not expected to be significant under South African growing conditions, although seed infections may result in significant disease levels if environmental conditions favour disease development. To date, in this study, no isolates have been identified as *Dickeya* spp. The survey supports previous findings (Serfontein *et al*., 1991) that *Pba* is not known to be present on potatoes in South Africa.

Besides the previous report (Serfontein *et al*., 1991), this is the first broad survey conducted on the occurrence of soft rot pectobacteria in South Africa. The abundance of the new pathogenic strain, *Pbcb* in South Africa indicates that a better understanding of the epidemiological factors is essential to effectively manage this subspecie in or on seed potatoes under prevailing local conditions. A study of the pathogenicity of *Pbcb* in different climates is crucial for the seed potato industry, since certification systems in South Africa are based on a set of tolerance levels encountered during visual inspections of growing crops and harvested seed tubers. The use of accurate and sensitive diagnostic testing post-harvest may also be a way to reduce the risk of economic losses.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

FIRST REPORT OF *PECTOBACTERIUM CAROTOVORUM* SUBSP. *BRASILIENSIS* CAUSING BLACKLEG ON POTATOES IN SOUTH AFRICA

ABSTRACT

In South Africa during the 2006/2007 potato growing season, outbreaks of blackleg occurred, causing severe economic losses in commercial potato production fields. Symptoms were initially observed on only one stem per plant, on which the top leaves rolled upwards, wilted and became necrotic. As symptoms progressed to the lower leaves with subsequent leaf desiccation, a light to dark brown discolouration of the vascular system at the stem base developed, followed by external darkening. Under prevailing wet and humid conditions stems become slimy and pale. In the stems, the pith became necrotic and hollow. These symptoms were similar to those described in Brazil, where the causal agent was identified as a new subspecie, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*). Isolations from plants showing typical blackleg symptoms were made on CVP medium. Sequences and phylogenetic analysis of the partial 16S-23S rDNA intergenic spacer region indicated that the isolates were *Pectobacterium carotovorum* subsp. *brasiliensis*. However PCR-RFLP of the 16S-23S rDNA region differentiated the South African blackleg strains from subspecies of *Pectobacterium* spp. and *Dickeya* spp. Comparison of RFLP patterns of isolates to reference cultures confirmed the identity of the South African blackleg strains as *Pbcb*, identical to strain 8 isolated in Brazil. This is the first report of *Pbcb* in South Africa and it appears to be the most important causal agent of blackleg in South Africa. The disease poses a major potential threat to the South African potato industry especially in terms of seed exports, tuber quality and yield.

4.1 INTRODUCTION

Potato blackleg is a seed-borne disease causing severe economic losses to the potato seed and commercial production industries. Blackleg is mainly caused by *Pectobacterium atrosepticum* (*Pba*) but under certain conditions *Pectobacterium carotovorum* (*Pbc*) and *Dickeya* spp. can also cause similar symptoms (Pérombelon *et al*., 1987; Oliveira *et al*., 2003; De Haan *et al*., 2008). These species were formerly known as the soft rot *Erwinia* spp., but they were recently reclassified into two new genera, namely *Pectobacterium* and *Dickeya,* on the basis of 16S rDNA sequences, their pectolytic ability and host ranges (Hauben *et al*., 1998; Ma *et al*., 1998; Garden *et al*., 2003; Samson *et al*., 2005; Laurila *et al*., 2008). *Dickeya* spp., formerly placed in a single species *Erwinia chrysanthemi,* (*Ech*) were reclassified in six species (Samson *et al*., 2005). The important *Dickeya* spp. causing disease on potato are *Dickeya dadantii* (formerly *Ech* 3937), *D. zeae* (formerly *Ech* biovar 3) and *D. dianthicola* (formerly *Echr* biovars 1 and 7) (Samson *et al*., 2005; Elphinstone and Toth, 2007; Tsror *et al*., 2009). Recently, a new subspecies from Brazil, *Pectobacterium carotovorum* ssp. *brasiliensis* (*Pbcb*), was described, which also causes blackleg-like symptoms on potatoes (Duarte *et al*., 2004).

In Canada, USA and Europe typical blackleg symptoms i.e. wilted appearance with yellowish foliage are caused by *Pba.* Similar symptoms accompanied by a brownish, sticky discolouration at the stem base (external) with brown staining of the vascular tissues (internal), which can be followed by necrosis and stem hollowing (Pérombelon *et al*., 1987), appeared in Europe (Netherlands, England, France, Hungary, Jersey, Spain and Switzerland) and Israel but are caused by *Dickeya* spp. (Palacio-Bielsa *et al*., 2006; Elphinstone and Toth, 2007 & Tsror *et al.,* 2009). The primary difference between the epidemiological development of *Pba* and *Dickeya* spp. is weather conditions that determine successful establishment of infection. *Pectobacterium atrosepticum* causes blackleg under cool, wet conditions, whereas *Dickeya* spp. are pathogenic under warm or warm-dry conditions (Elphinstone and Toth, 2007). In Brazil, *Pbcb* causes disease in areas with a humid subtropical climate, but relatively cool temperatures (17 - 20˚C) during the growing season (Duarte *et al*., 2004).

De Haan *et al*. (2008) stated that *Pbc* can cause typical blackleg symptoms in a temperate climate.

According to De Boer (2004) preliminary results suggest that *Pba* does not occur on potatoes if *Pbcb* is present. This suggests that *Pbcb* is more virulent than *Pba*. The incidence and severity of blackleg in the field is affected by specific climatic factors, in particular temperature, soil moisture content and soil nutrient status. However, the most important determining factor for symptom expression is the titre of the bacterial population present in contaminated seed tubers at the time of planting (Bain *et al*., 1990). Seed tubers are commonly contaminated with various soft rot bacteria in the latent state in the lenticles and wounds. To control the maceration processes these bacteria communicate with each other by means of a quorum-sensing process that relies on the production of *N*-acylhomoserine lactones (Smadja *et al*., 2004; Toth *et al*., 2004). The bacteria multiply until a critical cell density between 10^7 and 10^8 cfu/ml is reached, the rotting process is initiated in the seed tubers, which subsequently moves up into the stem through the vascular system. Here the bacteria remain quiescent until environmental conditions are favourable, after which they become active and cause disease expression in the above-ground plant parts. Simultaneously, bacteria can move downwards in the vascular bundles, infecting progeny tubers. Depending on the bacteria concentration and rate of multiplication, symptoms will be expressed immediately or during the next growing season. Therefore, the most important means of dissemination of inoculum is movement of latently infected seed tubers (Pérombelon and Kelman, 1987; Laurila *et al*., 2008).

In South Africa, *Pbc* and *Dickeya* spp. were detected on potatoes for the first time during the 1988 growing season, causing typical wilting symptoms. *Dickeya* spp. were detected in Mpumalanga, Northern Cape and Gauteng whereas *Dickeya zeae* was found in Northern Cape and *Dickeya dianthicola* was found mainly in Mpumalanga as well as Gauteng. *Pectobacterium carotovorum* was detected in Mpumalanga, Northern Cape, Eastern Free State, Gauteng, Ceres and Eastern Cape (Serfontein *et al*., 1991). Symptoms initially developed on only one stem of the plant, as a one-sided wilt of the upper leaves that tended to roll upwards and wilt without chlorosis.

These symptoms spread to the lower leaves, which became desiccated and subsequently the whole plant wilted. In addition, vascular discolouration at the stem base developed. During severe infection, the pith rotted, became hollow and eventually desiccated. Wilted plants were mostly associated with prevailing wet and humid conditions (Serfontein *et al*., 1991). Serfontein *et al*. (1991) identified isolates using standard bacteriological methods and confirmed their identity using fatty acid profiling. Results indicated that the causal agents in South Africa were *Pectobacterium carotovorum* (*Erwinia carotovora* ssp. *carotovora*), *Dickeya zeae* (*Erwinia chrysanthemi* biotype IV, pathovar *zeae*) and *Dickeya dianthicola* (*Erwinia chrysanthemi* biotype V, pathovar *dianthicola*). Interestingly, none of the isolates were identified as *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*).

The objective of this research was to isolate and identify the soft rot bacteria associated with recent outbreaks of blackleg on potatoes in South Africa.

4.2 MATERIALS AND METHODS

4.2.1 Sampling and isolation of causal agents

The stems and tubers of potato plants with typical blackleg symptoms (soft rot, wilting, internal and external darkening) were collected throughout the 2006 and 2007 growing seasons from seven production regions where mainly blackleg outbreaks occurred in South Africa. Severe outbreaks occurred in Limpopo, Mpumalanga and the Sandveld. Pieces of the infected stems and tubers were macerated in 0.01M magnesium sulphate ($MgSO_2$). Isolations were performed on the selective medium, Crystal Violet Pectate (CVP) (Hyman *et al*., 2001) and plates were incubated at 26ºC for 48h. Isolates that tested positive for pectolytic cavity formation were purified on the same medium and transferred to Nutrient Agar (NA), on which they were maintained. A total of 142 isolates were obtained and subjected to IGS-PCR which identified 77% of these isolates as *Pbcb*. All isolates were stored in sterile water at room temperature and in 15% glycerol medium at -80°C and maintained in the potatoresearch@up culture collection, University of Pretoria, Pretoria, South Africa.

Three isolates (JJ74, JJ145, and JJ147) obtained from plants with typical blackleg symptoms were selected for further study. These isolates are representative of the Sandveld, Limpopo and Mpumalanga growing regions, respectively, which had the worst blackleg outbreaks during the 2007 growing seasons. Type strains of the various species and subspecies were also included in this study (Table 4.1).

Single colonies were used for biochemical tests. These tests included Gram testing (3% KOH) and the Hugh and Leifson`s oxidation/fermentation test (Merck 2005). Representative *Pbcb* (JJ54) and *Pbc* (JJ68) strains were grown on Tryptone Soya Agar (TSA) for 7 – 8 hours. Nutritional requirements and the use of specific carbon sources for growth were tested with GN2 Microplates™ (Biolog Inc., Hayward, CA). The optical density of the suspension was adjusted as recommended by the manufacturer. Microplates™ were inoculated with 150µl of suspension per well, incubated at 28°C overnight and read visually.

Table 4.1 Reference cultures used in this study.

Name	Code	Obtained from
Pectobacterium carotovorum* subsp. *carotovorum	LMG 2404^T	Belgian Coordinated Collections of Microorganisms (BCCM™)
Pectobacterium atrosepticum	LMG 2386^T	Belgian Coordinated Collections of Microorganisms (BCCM™)
***Pectobacterium carotovorum* subsp. *brasiliensis* strain 8**	ATCC BAA-417	American Type Culture Collection (ATCC)
***Dickeya dadantii* (*Erwinia chrysanthemi* 3937)**		Scottish Crop Research Institute (SCRI)

4.2.2 Pathogenicity

All three strains were tested for pathogenicity on potato tubers (*Solanum tuberosum* cv. Mondial), green peppers and potato plants (6 weeks-old) grown in 20 cm diameter pots in a 24°C regulated greenhouse. Inoculations were done by dipping sterile toothpicks into bacterial colonies grown for 48 hours on Nutrient Agar (Merck) at 26°C.

Two stems per potato plant, 5 cm above the stem base, were immediately pierced with the contaminated toothpick after which the inoculated point was covered with Vaseline. Control potato plants were pierced with sterile toothpicks. Potato plants were observed daily for visible blackleg symptoms.

Potato tubers and green peppers were inoculated to test the isolates' pectolytic ability. The inoculation process was the same as for the potato stems but tubers and peppers were pierced three times each. The inoculated tubers and green peppers were placed in plastic bags and sealed to maintain high humidity at 27°C in the dark. After 24 hours, tubers were evaluated daily for the presence of symptoms.

4.2.3 DNA extractions

DNA was extracted from 48-hour-old pure cultures grown on Nutrient Agar (NA) using a DNeasy™ Blood & Tissue Kit (Qiagen, Southern Cross Biotechnology). RNA was removed with Ribonuclease A from bovine pancreas (Sigma-Aldrich) by incubating overnight at room temperature. Extracted DNA was stored at -20ºC.

4.2.4 Partial 16S-23S rDNA sequencing

The partial 16S-23S rDNA intergenic spacer (IGS) region of selected strains JJ74, JJ142 and JJ145 was amplified using species-specific primers BR1f (5'-GCGTGCCGGGTTTATGACCT-3') and L1r (5'-CARGGCATCCACCGT-3') (Integrated DNA Technologies, Whitehead Scientific). BR1f was designed from the IGS region based on the restriction enzyme site of SexAI and L1r from the 5' end of the 23S rRNA gene (Duarte *et al*., 2004). The amplified products of the isolates were then compared to those from the type strain, *Pbcb* 371 (ATCC BAA-419). Each 25µl pre-amplification reaction contained 1 x NH_4 Reaction Buffer, 2mM $MgCl_2$ solution, 100µM dNTP mix, 30pmol of each primer and 1.25U BIOTAQ™ (Whitehead Scientific (Pty) Ltd.). PCR reactions were carried out with the following thermal profile: 95°C for 2min, followed by 30 cycles of 94°C for 30s, 62°C for 45s, 72°C for 90s and a final extension step of 72°C for 7min. Amplified products were electrophoresed through a 2% agarose gel in 1 x TBE Buffer at 75V for 2h. PCR products were purified using the QIAquick™ Purification Kit (Qiagen, Southern Cross Biotechnology).

The partial 16S – 23S rDNA was sequenced in both directions using the forward primer BR1f and reverse primer L1r (Duarte *et al*., 2004). Each 10 µl sequencing reaction contained 2µl Big Dye Sequencing Reaction Mix, 1 x Sequencing buffer, 30pmol primer and 3µl purified DNA template. Sequencing PCR conditions included denaturation at 96°C for 1min, 25 cycles of denaturation at 96°C for 10s, annealing of primer at 50°C for 5s and elongation at 60°C for 4min. PCR products were cleaned using a sodium acetate precipitation step and sequenced on an ABI Prism DNA Automated Sequencer (Perkin-Elmer). Incorrect basecalls were corrected using Chromas lite v 2.01. The corrected sequences were edited, aligned and trimmed using BioEdit Sequence Alignment Editor v 7.0.0. Searches were performed on each consensus sequence generated on BioEdit using the BLAST programme from the GenBank database. All the selected sequences were aligned using MAFFT. Phylogenetic tree for the data set was inferred by using the neighbour-joining program of MEGA v 4 (Tamura *et al*., 2007). The stability of the phylogenetic tree was assessed by performing bootstrap analysis of the neighbour-joining data based on 1000 bootstrap replicates (Kwon *et al*., 1997; Fessehaie *et al*., 2002).

4.2.5 PCR-RFLP of the 16S-23S rDNA region

After DNA extractions of isolates, JJ74, JJ145 and JJ147, PCR-RFLP of the amplified 16S-23S rDNA region was performed and reference strains, *Pbc*, *Pba*, *Dickeya dadantii* and *Pbcb* strain 8 were used for amplification. The amplification of DNA from the intergenic spacer (IGS) between the 3' end of the 16S and the 5' end of the 23S rDNA genes was performed with primers 1491f (5'-GAAGTCGTAACAAGGTA-3') and L1r (5'-CA(A/G)GGCATCCACCGT-3') (Integrated DNA Technologies, Whitehead Scientific) (Fessehaie *et al*., 2002; Duarte *et al*., 2004). Each 25µl pre-amplification reaction contained 1 x NH_4 Reaction Buffer, 2mM $MgCl_2$ solution, 100µM dNTP mix, 50pmol of each primer and 1.25U BIOTAQ^{TM} (Whitehead Scientific (Pty) Ltd.). PCR amplification was carried out using the following thermal profile: 95°C for 2min, followed by 30 cycles of 94°C for 30s, 62°C for 45s, 72°C for 90s and a final extension step of 72°C for 7min. A negative water control was included to monitor contamination. Amplified products were electrophoresed through a 2% agarose gel in 1 x TBE Buffer at 75V.

Subsequently, the PCR amplified products were digested with *RsaI*, *TaaI* (*Tsp4CI*) and *Hin6I* (*HhaI**) (Fermentas Life Science, Inqaba). The restriction digestion was performed at 37°C for 3h and left overnight at room temperature in a 30µl final volume, according to the manufacturer's recommendations. DNA fragments were resolved by gel electrophoresis on 3% agarose gels in the presence of ethidium bromide at 75V for 5h in 1 x TBE Buffer. RFLP patterns were visualised under UV light and compared with reference cultures.

4.3 RESULTS

4.3.1 Disease description

Field symptoms were often observed on only one stem per plant. Initially the upper leaves (sometimes only one side of the upper leaves) began to roll upwards at the margins and wilted, later becoming necrotic (Fig. 4.1). Most of the leaves showed no sign of chlorosis. However, in a few cases leaves developed marginal chlorosis (Fig. 4.2). Symptoms progressed to the lower leaves, which subsequently wilted. As the leaves wilted, a light to dark brown discolouration of the vascular system at the stem base developed from the rotting mother tuber, followed by the upward movement of external darkening (Fig. 4.1). During prevailing wet and humid conditions stems became slimy and pale (Fig. 4.2). Inside the stem at the point of infection the rotten pith became necrotic and hollow. Severely infected plants dried out and died (Fig. 4.3).

In storage, table tubers developed sunken, darkened lenticels which either stayed latent or developed into tuber soft rot under excessive moisture and humidity. Generally, when seed tubers were suddenly exposed to higher temperatures a black soft rot developed (Fig. 4.4). Black soft rot of seed tubers resulted in non-emergence, blanking or blackleg development during the early stages of vegetative growth.

Fig. 4.1 Typical blackleg symptoms without chlorosis on potatoes infected with *Pbcb* in a commercial field under wet, cool conditions.

Fig. 4.2 During prevailing wet and humid conditions stems become slimy and pale and leaves become chlorotic.

Fig. 4.3 Severely infected potato plants dry out and die.

Fig. 4.4 Black soft rot can develop during storage, under excessive moisture and high temperatures.

4.3.2 Biochemical characterisation

Biochemical identification could not definitively identify the South African blackleg isolate as *Pecotobacterium carotovorum* subsp. *brasiliensis*, since striking differences between the South African *Pbcb* and the Brazilian *Pbcb* were observed in the oxidation of N-Acetyl-D-Glucosamine and Succinamic Acid. However, there are numerous atypical isolates and strains commonly detected that do not have typical biochemical reactions (Yap *et al*., 2004; Charkowski, 2006).

Table 4.2 Physiological characteristics of the South African *Pectobacterium carotovorum* subsp. *brasiliensis* and *Pectobacterium carotovorum.*

Characteristic	JJ54 *Pbcb*	JJ68 *Pbc*	*Pbcb*[b] (n=16)[c]	*Pbc*[b] (n=5)
Gram testing (3% KOH)	-[a]	-		
Hugh and Leifson`s oxidation/fermentation test	+	+		
α-Cyclodextrin	-	-	-	-
Dextrin	-	-	-	40[d]
Tween 40	-	-	-	20
Tween 80	-	-	-	60
N-Acetyl-D-Glucosamine	w+	+	-	-
L-Arabinose	+	+		
D-Arabitol	-	-	-	-
D-Cellobiose	+	+	+	+
D-Fructose	w+	+		
D-Galactose	w+	+		

Gentiobiose	+	+	94	100
α-D-Glucose	+	+		
m-Inositol	+	+		
Lactulose	-	+	6	-
Maltose	-	-	19	-
D-Melibiose	+	+	+	+
β-Methyl-D-Glucoside	+	+		
D-Psicose	-	+	6	100
D-Sorbitol	-	-	-	20
Sucrose	+	+		
D-Trehalose	+	+	100	100
Acetic Acid	-	-	50	80
Citric Acid	w+	w-		
Formic Acid	w+	-		
D-Galactonic Acid Lactone	w+	-	12	80
D-Galacturonic Acid	+	+	75	80
D-Gluconic Acid	w+	-	19	80
D-Glucosaminic Acid	-	-	-	-
D-Glucuronic Acid	-	-	-	-
D,L-Lactic Acid	-	w+	69	20
Malonic Acid	-	-	-	-
Succinic Acid	-	+		
Bromosuccinic Acid	w+	w+		
Succinamic Acid	-	-	100	100
L-Proline	-	-	-	-
Uridine	-	-	31	100
Thymidine	-	w+	38	100
Glycerol	w+	+		
D,L,α-Glycerol Phosphate	-	+		
α-D-Glucose-1-Phosphate	-	-	-	60
D-Glucose-6-Phosphate	+	+		

[a], negative reaction; w-, weak negative; +, positive reaction; w+, weak positive

[b,] Compared to *Pbcb* and *Pbc* strains from Duarte *et al*. (2004)

[c] Number of strains tested [d] Percentage of strains showing a positive reaction

4.3.3 Pathogenicity

Typical blackleg symptoms on potato stems appeared 3 - 4 days after stem inoculation. JJ145 and JJ147 were more virulent than JJ74. However, the same symptoms were observed on inoculated plants. Initial symptoms appeared at the inoculation site on the stem base as external darkening, up and downwards on the stem (Fig. 4.5). Symptoms such as wilting of the leaves, chlorosis and desiccation as well as the hollowing of the stems followed (Fig. 4.5). JJ74, JJ145 and JJ147 strains consistently developed typical soft rot symptoms in all three inoculation points on potato tubers and green peppers within 24 to 48 hours after inoculation.

JJ145 and JJ147 were more virulent than JJ74; however after 24 hours all the green peppers were completely rotten and all the potato tubers showed symptoms. The development of symptoms was more rapid on tubers inoculated with JJ145 and JJ147. The control plants treated with sterile NB/Ringers solution did not develop any symptoms on potato stems, tubers and green peppers.

Fig. 4.5 Blackleg symptoms in potato cv. Mondial after inoculation with JJ145

4.3.4 Partial 16S-23S rRNA sequencing

The phylogenetic tree constructed using partial 16S – 23 S rDNA sequences placed the South African strains in the same cluster as *Pbcb* strains (Fig. 4.6). These results confirmed that the South African blackleg strains were different from *Pba* as well as *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *betavasculorum* (Duarte *et al*., 2004).

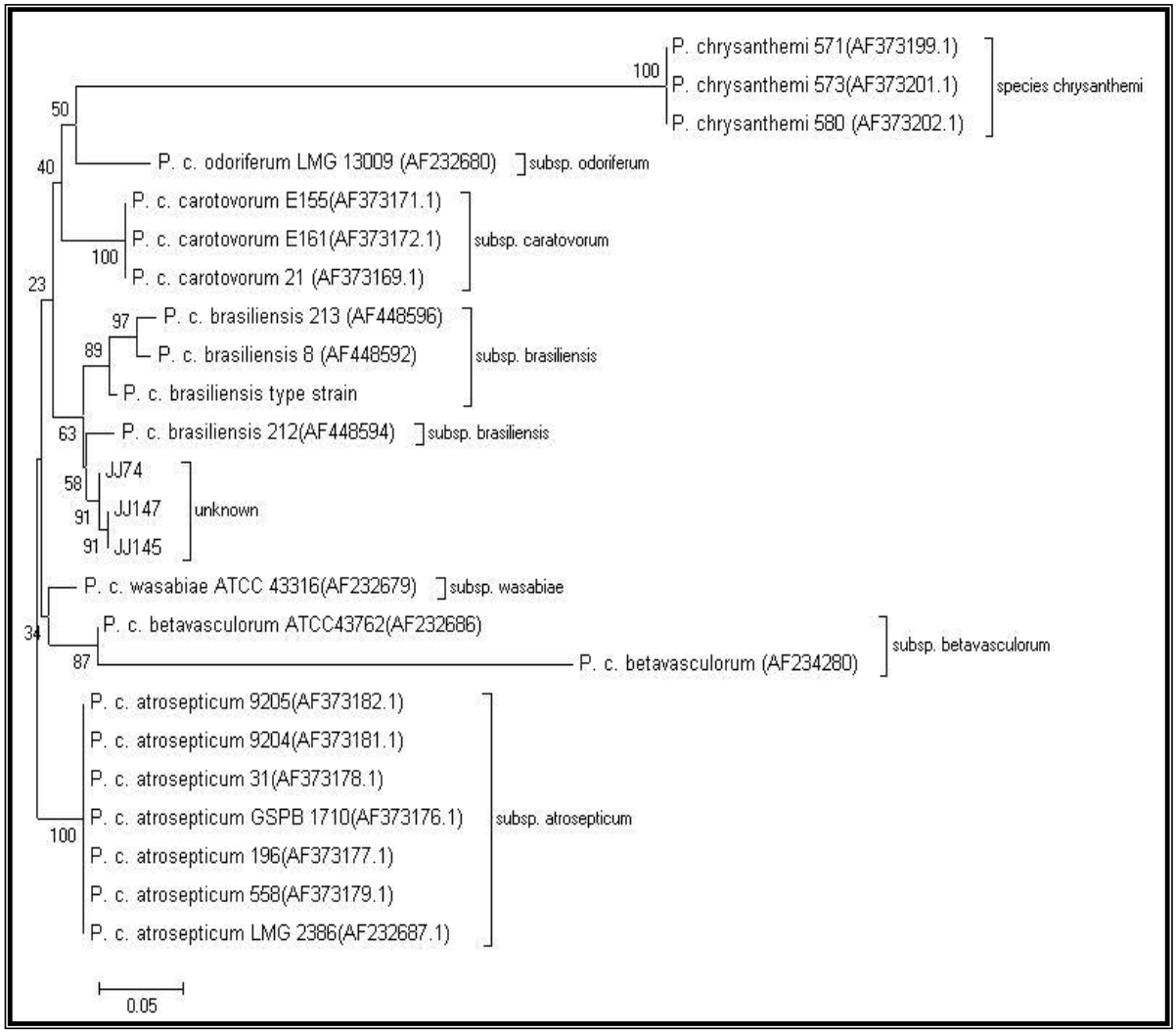

Fig. 4.6 Phylogenetic tree based on partial 16S – 23S IGS gene sequences showing the phylogenetic relationships among South African strains and different *Pectobacterium* spp. The phylogram was produced by the neighbour-joining program (Tamura *et al*., 2007). The numbers on the branches indicate bootstrap value support based on neighbour-joining analyses of 1000 bootstrap replications. Accession numbers of reference strains in Genbank are in parenthesis.

4.3.5 PCR-RFLP of the 16S-23S rDNA region

After PCR amplification of the IGS region, two fragments were generated, which allowed differentiation of *Pectobacterium* spp. and *Dickeya* spp. The *Pectobacterium* spp. including subsp. *brasiliensis* and subsp. *betavasculorum* yielded the expected amplified bands of small (~440bp) and large (~490) IGS regions.

In comparison the *Dickeya* spp. yielded two different DNA fragments of small (~354bp) and large (~486bp) IGS regions (Fessehaie *et al*., 2002).

Restriction enzymes *RsaI*, *TaaI* (*Tsp4CI*) and *Hin6I* (*HhaI**) used individually, produced different restriction patterns that allowed identification and differentiation between the reference cultures *Pbc*, *Pba*, *Dd* and *Pbcb*. When digested with all three enzymes the representative South African blackleg strains (JJ74, J145 and JJ147) produced characteristic banding patterns that were identical to the patterns of *Pbcb* strain 8 (Figs 4.7, 4.8 and 4.9). Based on the banding patterns generated, the South African blackleg strains were thus positively identified as *Pbcb*.

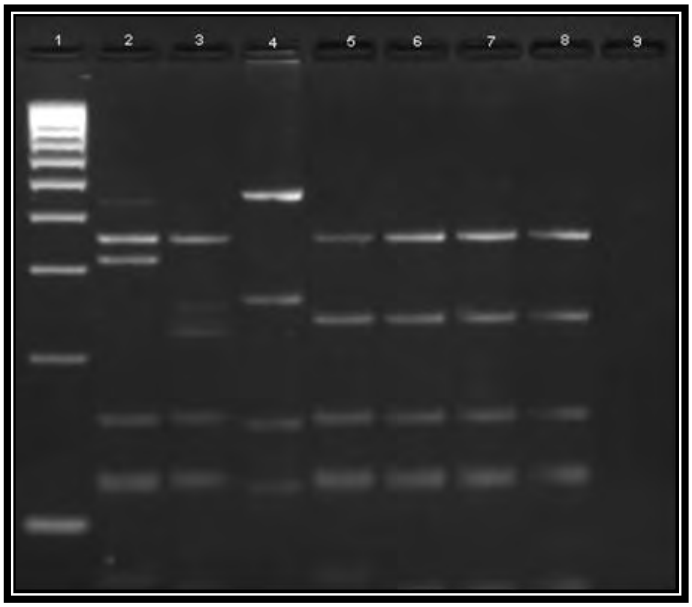

Fig. 4.7 Agarose gel electrophoresis of the PCR-RFLP of the IGS region digested with *RsaI* to differentiate between subspecies *Pectobacterium* spp. and *Dickeya dadantii*. Lanes: 1, 100bp DNA ladder; 2, *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T); 3, *Pectobacterium carotovorum* subsp. *atrosepticum* (LMG 2386^T); 4, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 5, *Pectobacterium carotovorum* subsp. *brasiliensis* strain 8 (ATCC BAA-417); 6, JJ74; 7, JJ145; 8, JJ147; 9, Negative control (water).

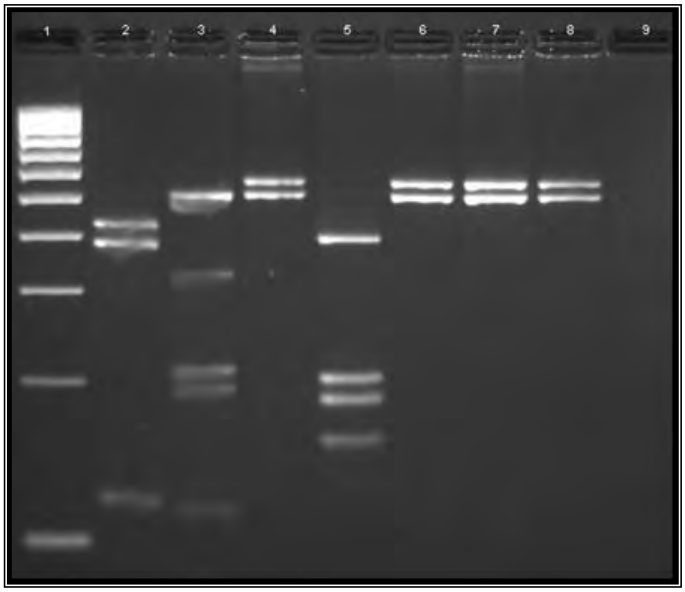

Fig. 4.8 Agarose gel electrophoresis of the PCR-RFLPof the IGS region digested with *TaaI* (*Tsp4CI*) to differentiate between subspecies *Pectobacterium* spp. and *Dickeya dadantii*. Lanes: 1, 100bp DNA ladder; 2, *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T); 3, *Pectobacterium carotovorum* subsp. *atrosepticum* (LMG 2386^T); 4, *Pectobacterium carotovorum* subsp. *brasiliensis* strain 8 (ATCC BAA-417); 5, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 6, JJ74; 7, JJ145; 8, JJ147; 9, Negative control (water).

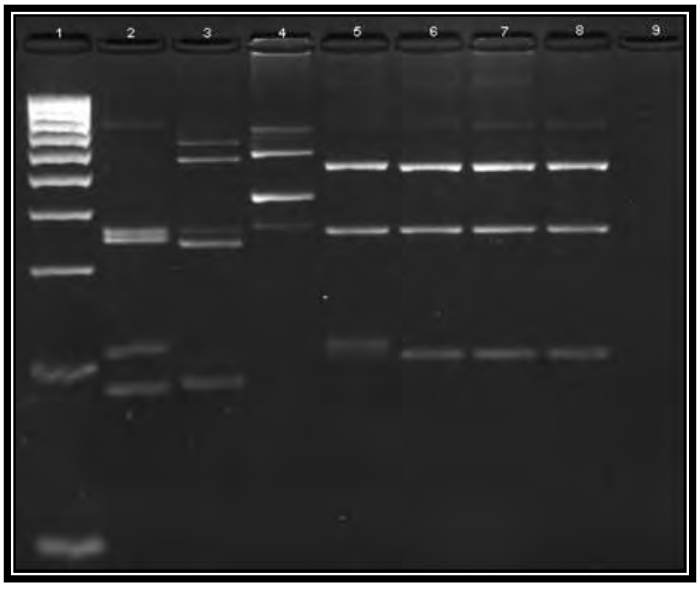

Fig. 4.9 Agarose gel electrophoresis of the PCR-RFLP of the IGS region digested with *Hin6I* (*HhaI**) to differentiate between subspecies *Pectobacterium* spp. and *Dickeya dadantii*. Lanes: 1, 100bp DNAladder; 2, *Pectobacterium*

carotovorum subsp. *carotovorum* (LMG 2404^T); 3, *Pectobacterium carotovorum* subsp. *atrosepticum* (LMG 2386^T); 4, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 5, *Pectobacterium carotovorum* subsp. *brasiliensis* strain 8 (ATCC BAA-417); 6, JJ74; 7, JJ145; 8, JJ147; 9, Negative control (water).

4.4 DISCUSSION

The phylogenetic tree constructed using partial 16S – 23S rDNA sequences clearly showed that the South African strains are grouped in the same cluster as *Pbcb* strains. These results confirmed that the South African blackleg strains were different from *Pba, Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *betavasculorum* (Duarte *et al*., 2004). To clearly differentiate between the *Pectobacterium* spp. further identification was necessary which focused on the analysis of PCR–RFLP banding patterns (Toth *et al*., 2001). The unique banding patterns of the three South African blackleg strains were compared to those of the reference isolates. The three isolates generated the same characteristic banding patterns as *Pbcb* strain 8 isolated in Brazil (Duarte *et al*., 2004). This is therefore the first report of *Pectobacterium carotovorum* subsp. *brasiliensis* causing typical blackleg symptoms in South Africa. *Pbcb* is the most important causal agent of blackleg in South Africa and is widely spread throughout the potato production regions. Furthermore research is necessary since it seem as the etiology of blackleg in SA can be significantly different than the blackleg pathogen in Canada, US and Europe.

It is interesting to note that the symptoms described by Serfontein *et al.* (1991) are similar to the symptoms recently observed in this study. However, the causal agents identified are different. This could be the result of misidentification of the causal blackleg bacterium in the early stages of potato pathology in South Africa or due to climatic changes, leading to selection for *Pbcb*. *Pectobacterium carotovorum* subsp. *brasiliensis* is virulent in both cool and warm temperatures and high disease incidence occurs when cool and warm temperatures alternate.

Ma *et al*. (2007) also stated that the *Pbcb* clade is most likely widely distributed and has also been found in USA and Israel.

The bacterium could also have entered the country through import of contaminated seed tubers a few years ago and subsequently spread throughout the country. Another hypothesis is that the recent increase in plantings (33% of total hectares planted in 2007) of a highly blackleg susceptible potato cultivar from the Netherlands, Mondial, countrywide has lead to the proliferation and spread of *Pbcb* in the South African potato industry (Potatoes South Africa, 2008). Tsror *et al*. (2009) stated that seed imported tubers (cvs Nicola and Mondial) from the Netherlands into Israel was the cause of blackleg diseased plants. However the causal organisims were identified as *Dickeya* spp.

Pectobacterium carotovorum subsp. *brasiliensis* is an extremely aggressive pathogen, causing severe wilting in the field which results in major economical losses. Latent infection of seed tubers may result in significant disease levels in the following growing season and therefore increase the economic risk of repetitive potato production especially when retaining tubers for the subsequent seasons.

These findings are highly significant for the South African potato industry, in particular the fact that South African climatic conditions in many growing areas are favourable for *Pbcb*. Therefore this new strain adds additional complexity to the soft-rot / blackleg disease complex on potatoes in South Africa and thus warrants further research.

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CHAPTER 5

EFFECT OF SILICON SOIL AMENDMENTS ON RESISTANCE OF POTATOES TO SOFT ROT BACTERIA

ABSTRACT

Blackleg of potatoes, caused primarily by *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) in South Africa, is a seed borne disease and therefore difficult to control. This prompted the search for a possible management strategy to increase resistance to blackleg and soft rot to minimise losses. Therefore the aim of this study was to investigate the effect of silicon-amended soil on phenolic formation in cell walls of potato peels, and the effect thereof on tuber resistance to *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*). Pot trials were conducted using; untreated control and inoculated positive control; a slag (30% Si) with pathogen and without pathogen and a lime treatment ($CaCO_3$) (as a pH control) with pathogen (LiP) and without pathogen (LiN). At harvest potatoes were peeled and the total phenolics were extracted from mature (first trial) and immature (second trial) tubers. Phenolics were identified and quantified as chlorogenic, caffeic and ferulic acids using HPLC analysis. In the first pot trial it was found that the plants treated with silicon had significantly higher levels of total phenolic compounds than the control plants. However, in the second pot trial plants treated with lime had higher levels of total phenolic compounds than silicon treated plants. The concentration of caffeic acid was higher than chlorogenic acid in the first trial compared with the second. Results suggest that pH could play a role in phenol production and that the calcium (Ca) content in the soil appears to affect the concentration of phenolics in the tubers. An *in vivo* pilot study on progeny tubers produced in silicon-amended soil showed that the rate of development of blackleg was significantly lower in Calmasil (30% Si) than Durapozz treated tubers. These results indicate that the best Si source to use is Calmasil, since Si and Ca combined have a synergistic effect in enhancing tuber resistance. This is, however, the first such study on the effect of Si on defence responses of potatoes to *Pbcb.*

Further studies need to be done to elucidate the effect of Si on the phenolic content of potato stems, and the role thereof in resistance to soft rot bacteria. Additional studies should focus on how much Si is accumulated in potato plants. Preliminary data showed positive results and opened up new possibilities for the use of Si in commercial production to improve plant health status and disease resistance, thereby potentially reducing the use of chemicals.

5.1 INTRODUCTION

Silicon (Si) is the second most common element in the soil and exists as silica or silicates, which are combined with various metals. Therefore, most forms of Si are insoluble and unavailable for the plant to absorb (Richmond & Sussman, 2003; Ma & Yamaji, 2006; Gunes *et al*., 2007). Roots absorb Si by diffusion and mass flow (transpiration-induced root absorption) in the form of amorphous silica (SiO_2 nH_2O) and soluble silicic acid [$Si(OH)_4$] (Epstein, 1994; Fawe *et al*., 1998). However, Si accumulation in the shoots differs significantly among plant species and can range from 1% to greater than 10%. Generally dicotyle plants are poor accumulators of Si, whereas plant families such as *Poaceae, Equisetaceae* and *Cyperaceae* are hyperaccumulators. The differences in Si accumulation are mainly due to the Si absorption ability of the roots (Mitani & Ma, 2005; Rodrigues & Datnoff, 2005).

Three main Si-sources are available in South Africa for agricultural use. Silicon is often applied as a commercial liming agent, slag, which is a by-product of the steel and iron industries. The primary form of Si in slag is calcium silicate (Ca_2SiO_3). Another commercially available Si source is fly ash (a slag), which contains mainly potassium silicate, a slow-release fertilizer. The third source is silicon fume, which contains approximately 99 % Si in the form of a calcium silicate hydrate (Ma & Takahashi, 2002).

According to Fawe *et al.* (1998), the positive physiological effect of Si nutrition in plants has been extensively studied, but the means by which Si influences the different physiological processes is poorly understood and results are controversial.

The benefits of Si amendments include enhanced resistance against pathogens, pests and drought, heavy metal tolerance, increased quality and yield of agricultural crops and improved physiological properties. These effects are primarily associated with substantial deposition of phenolics and phytoalexins in cell walls, which leads to mechanical strengthening and rigidity (Fauteux *et al*., 2005; Richmond & Sussman, 2003; Ma & Yamaji, 2006). Silicon can also act as a physical barrier against penetration by strengthening the cell wall through impregnation beneath the cuticle layer. The beneficial effects of Si are mostly evident under conditions of biotic or abiotic stress (Richmond & Sussman, 2003; Fauteux *et al*., 2005).

Soluble Si plays a direct role in stimulating host defense responses. It can promote the production of phenolics and phytoalexins in response to pathogen infection and enhance the activity of defense-associated enzymes (Hammerschmidt, 2005; Ma & Yamaji, 2006). It is also well known that infected plants have an increased polyphenoloxidase activity and phenol concentrations (Lovrekovich *et al*., 1967). There are, however, currently no comprehensive reports available on the effect of Si on disease resistance of potato plants.

Flego *et al*. (1997) found that soil calcium (Ca) increases the resistance of potato stems and tubers to degradation of pectolytic enzymes such as pectate lyase and polygalacturonase. Generally, Ca is known to increase the resistance of plant tissue by enhancing the structural integrity of cell walls and membranes. When soil was treated with gypsum ($CaSO_4$) pre-planting, a significant reduction in blackleg incidence was found during the growing season; however the effect decreased towards the end of the season (Bain *et al*., 1996). Lambert *et al*. (2005) found that the application of Mg can also suppress tuber soft rot, but to a lesser extent when used in combination with Ca.

Phenols and phytoalexins play an important role in defence mechanisms to soft rot bacteria (Ghanekar *et al*., 1984), but potato tubers commonly contain relatively low levels of phenolic acids (Ramamurthy *et al.*, 1992). Wegener & Jansen (2007) found that total soluble phenolics and anthocyanins are responsible for resistance expression in tuber tissue.

Therefore, the amount of total soluble phenolics (chlorogenic and caffeic acid) was considerably higher in tuber peel than in tuber flesh. Ghanekar *et al.* (1984) found that the three main phenolic compounds in potato tubers, viz. chlorogenic, caffeic and ferulic acid, possess antibacterial activity against soft rot bacteria and were more effective in combination than individually, even at low combination concentrations. The total phenol content was directly proportional to the inhibition of the soft rot bacteria (Kumar *et al.*, 1991). The oxidation of these phenolic acids can therefore be directly involved in reducing soft rot development (Hammerschmidt, 2005). These facts support the viewpoint that the abundance of phenolic compounds in tubers positively correlates with the level of resistance to soft rot pectobacteria (Lovrekovich *et al*., 1967; Andreu *et al.*, 2001).

During the 2006/2007 South African potato growing seasons there were numerous blackleg outbreaks countrywide, causing major economic losses. The primary causal agent was identified as *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) and to a lesser extent *Pectobacterium carotovorum* (*Pbc*) (Chapter 3). *Pectobacterium* spp. have the ability to macerate plant tissue by the massive secretion of extracellular, degrading, pectinolytic enzymes (Flego *et al*., 1997). During infection, *Pectobacterium* spp. secrete pectinase, which induces polyphenoloxidase activity that subsequently oxidizes phenols. Visually it forms a black margin that serves as an infection barrier (Lovrekovich *et al*. 1967).

Blackleg is mainly a seed-borne disease but can also be soil-borne and therefore difficult to control. Seed tubers are almost always contaminated with soft rot pectobacteria. The best management method is therefore to maintain the bacterial populations at levels below the disease threshold levels. This prompted the search for a possible management strategy to enhance resistance to *Pbcb* the causal agent of blackleg and tuber soft rot diseases. The aim of this study was therefore to investigate the effect of Si on the formation of phenolic compounds in the cells of potato plants, thereby enhancing resistance to *Pbcb*.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strain

Strain, JJ54 was isolated from potato tubers showing black soft rot symptoms in South Africa in 2006. This isolate was identified using Gram staining (3% KOH), Hugh and Leifson`s oxidation/fermentation test (Merck, 2005) and the ability to degrade pectate on a modified selective medium, Crystal Violet Pectate (CVP) (Hyman *et al*., 2001). Further identification was done using a Multiplex PCR and amplification of the 16S-23S region (Chapter 3). The isolate was stored in 15% glycerol : nutrient broth solutions at -7 °C and -80 °C.

Confirmation of identification was done by the Central Science Laboratory, York. JJ54 was deposited in the NCPPB library as Ref No. 20712982. It was confirmed that JJ54 appears to be closely related to (but not identical to) *Pectobacterium carotovorum* subsp. *brasiliensis* (strains 213 and 371) (Appendices B1 & B2).

5.2.2 Pathogenicity

Isolate JJ54 was tested for pathogenicity on potato tubers (*Solanum tuberosum* cv. Mondial). Bacterial cultures grown for 48 hours on Nutrient Agar (Merck) at 26°C were suspended in Nutrient broth (Merck) and shaken at 150 rpm at 25 °C for 8 hours to reach a concentration of 10^5 cfu/ml. The bacterial suspension was diluted to $1x10^4$ cfu/ml by adding Ringers solution and was then used for inoculation.

Tubers were immersed in the bacterial suspension and held under a vacuum of 1 bar for 20 min (Helias et al., 2000). Control tubers were vacuum infiltrated with a sterile nutrient broth solution. Inoculated tubers were placed in plastic bags and sealed to maintain high humidity at 27°C in the dark. After 48 hours tubers were evaluated for symptoms.

5.2.3 Preliminary pot trial

A preliminary pot trial was conducted to determine if Si soil amendments will have any effect on the incidence and severity of blackleg on potato stems. The pot trial consisted of 45 plants. Red topsoil was treated and mixed five days before planting.

Generation 4, Up-to-Date seed potatoes were used. Tubers were dipped for 30min in a bacterial suspension of *Pectobacterium carotovorum* subsp. *brasiliensis* (JJ54) at a concentration of 10^4 cfu/ml.

All treatments (five replicates each) were either treated with the pathogen or not and included the following; untreated control (CN) and inoculated positive control (CP); treated Camasil slag (30% Si) (SIP) or untreated Camasil slag (SlN) ; treated (VGP) or untreated fly ash (50% Si) (VGN) and silica ash (~99% Si) treated (ASP) or untreated (ASN). As a pH control a lime treatment ($CaCO_3$) with pathogen (LiP) was used. Tubers were left to dry overnight and planted the next day at a depth of 10cm. The experimental layout was a complete randomised block design. During the experiment, water was applied according to plant requirements and temperature was maintained at 24±1 °C.

5.2.4 Extraction of total phenolic compounds from preliminary pot trial

Plants were harvested 17 weeks after planting. Incidence of blackleg was determined by visual observations, rating the presence of typical blackleg-like symptoms. Isolations were made from all plants per treatment to determine if the causal agent was present. The tuber yield was very low because of poor quality soil and very long day lengths, therefore phenolic compounds were not extracted from tubers in this study. However, the stems of four randomly chosen plants per treatment were used for phenolic extractions. Stems were cut into 30cm pieces and freeze dried for four days. The dried material was ground to a fine powder and 0.05g of the powder was placed in 1.5ml Eppendorf tubes. One ml of a cold mixture of methanol: acetone: water (7:7:1, v:v:v) was added, ultrasonified for 5min, shaken for 20min at 150rpm while kept on ice, followed by centrifugation for 1min. Extractions were performed in triplicate and the supernatants pooled. These were then evaporated in a laminar flow until dry and dissolved in 1ml distilled water. The crude extracts were stored at 4°C.

5.2.5 Identification and quantification of phenolics

Preliminary identification and quantification of total phenolic compounds was performed using Thin-Layer Chromatography (TLC) and the Folin-Ciocalteau reagent.

Thin-Layer Chromatography was conducted on Si gel plates (Merck no. 1.05721) using the following solvent: Butan-1-ol, acetic acid, water (4:1:2.2). The dry TLC plates were observed under UV light. Preliminary identifications were made from comparison with known standards (caffeic acid and ferulic acid) (Lewis *et al.*, 1998). The concentration of phenolic compounds was determined spectrophotometrically in 96-well ELISA plates after reaction with 175µl distilled water, 5µl standard or crude extract, 25µl Folin-Ciocalteau reagent and 50µl 20% (v/v) Na_2CO_3 (Bray & Thorpe, 1954). ELISA plates with the extract and Folin-Ciocalteau mixture were incubated at 40°C for 30min. The absorbance was measured at 690nm at 25°C using a Multiskan Ascent V1.24, Version 1.3.1. The total phenolic content of each sample was determined from a standard curve (y=1.3527x + 0.019) using gallic acid, which was expressed as mg gallic acid equivalent per gram of dry weight.

5.2.6 Pot trials I and II

Results from the preliminary trial I suggested that Calmasil slag is the best Si source to use, thus only Calmasil was used in further trials. To determine whether Si had any effect on the production of phenolics in potato peels against *Pbcb*, phenolics were extracted from both inoculated and uninoculated Si-treated tubers. Identification and quantification of phenolics from tubers were performed using HPLC. Silicon treatments with and without pathogen were compared with control treatments.

Treatments, with five replicates, consisted of a control with (CP) and without pathogen (CN) and a slag (30% Si) with pathogen (SIP) and without pathogen (SIN). As a pH control a lime treatment ($CaCO_3$) with pathogen (LiP) and without pathogen (LiN) was used. Virgin topsoil (from S25°47' 34.6", E28°32'16.8", South Africa) with a pH of 5.4 was treated and mixed five days before planting.

Generation 5, Caren (pot trial I) and Generation 4, BP1 (pot trial II) certified seed potatoes were used. *Pectobacterium carotovorum* subsp. *brasiliensis* (JJ54) was grown in nutrient broth while shaken at 150rpm for 17 - 18h at 25°C. Concentration was determined using the spread - plate method and was adjusted with Ringers solution to give a final concentration of 1 x 10^7 cfu/ml.

This was done to increase disease incidence during the growing season since pot trial I showed very few disease symptoms. Tubers were soaked for 10 min in bacterial suspension and left to dry overnight on paper at room temperature. Tubers were planted in the premixed moist, sandy soil at a depth of 10cm in 4L plastic pots. Fertilizer was applied in a solution of 4:3:4 (33) at planting. Each pot trial consisted of 30 plants, planted in a greenhouse at 24±1 °C on a rotating table (pot trial I) and in a phytotron with day/night temperatures of 25°C/15°C and day/night length of 14h/10h (pot trial II). During the experimental trials, water was applied according to plant requirements and 500dpm Supafeed (Plaaskem, South Africa) fertilizer was applied every second week, until flowering.

5.2.7 Extraction of phenolic compounds

For pot trials I and II plants were harvested 17 weeks after planting. Mature and immature tubers respectively were washed under running tap water and peeled with a potato peeler. Tuber peels were freeze-dried for five days after which it was ground to a fine powder. For each sample, 200mg fine powder was put through a 1mm sieve and placed in a 1.5ml Eppendorf tube for extractions. Aliquots of 1ml of a cold mixture of methanol: acetone: ultra-pure water (7:7:1, v:v:v) were added, vortexed and ultrasonified for 5min. After sonification, samples were shaken for 20min at 160rpm while on ice. Samples were centrifuged for 5min and the supernatant of each sample was transferred to a 20ml centrifuge tube. This process was repeated in triplicate and supernatants finally evaporated in a laminar flow cabinet at room temperature. The residue was dissolved in 1ml sterile, ultra-pure water. Finally, samples were filtered through 0.45µm, 25mm, Ascrodise, GHP, syringe filters (Separations, South Africa). Samples were stored at 4 °C until analysis using reverse phase – high performance liquid chromatography (RP-HPLC).

5.2.8 Reverse Phase – High Performance Liquid Chromatography

For identification and quantitative analysis of samples, 10µl of purified extract per sample was analysed using RP-HPLC (Hewlett Packard Agilent 1100 series) with a UV diode array detector, at 325 and 340nm. Separation was achieved on a Gemini 3µ, C18, 110A (Phenomenex®) reverse phase column (250mm length, 5µm particle size, 4.6mm inner diameter).

A gradient elution was performed with water (pH 2.6 adjusted with H_3PO_4) and acetonitrile (ACN) and consisted of 0min, 7% ACN; 0 – 20min, 20% ACN; 20 – 28min, 23% ACN; 28 – 40min, 27% ACN; 40 – 45min, 29% ACN; 45 – 47min, 33% ACN; 47 – 50min, 80% ACN. Solvent flow rate was 0.7ml.min^{-1}. Identification of the phenolic compounds was done by comparing their retention times and UV apex spectrum to those of standards, chlorogenic acid, caffeic acid and ferulic acid. The column was re-equilibrated with initial conditions for 10min, after each run. Peaks were detected at 280nm, although this wavelength is not optimal for ferulic acid (Zhou *et al*., 2004).

5.2.9 Initial field trial: Screening for stem-base resistance

Seed tubers of cultivar Mondial were planted in a field trial, under sprinkle irrigation, during the 2006 summer production season in the Western Free State region. Soil was amended before planting with either Dura-Pozz® (fly ash ≈50% Si, 0.5t / ha) or Calmasil (Slag ≈30% Si, 1t / ha). The field trial was conducted under natural environmental conditions. Tubers were harvested in 2007 and left at room temperature until etiolated sprouts were 3 to 5 cm long. Stem-base resistance of tubers from this trial was evaluated using a modification of the technique described by Allefs *et al*. (1996). One cylinder from each tuber was cut (0.5 – 1cm long) containing more than one sprout at the centre. Inoculation was carried out by placing the cylinder on 1.5g cottonwool that was previously soaked in 5ml of a 2x10^7 cfu/ml bacterial suspension of *Pbcb* (JJ54). Control cylinders were placed on cottonwool soaked in nutrient broth solution. Four replications per bacterial specie per treatment together with associated controls were tested in a completely randomized block design. The experiment was carried out in closed jars and placed at 24 - 27°C in the dark. The level of resistance (sprout symptom latency period) was measured in time comparing date of initial blackleg symptom appearance at the point where the sprout is attached to the tuber (Allefs *et al*., 1996).

5.2.10 Statistical analysis

The experiment was designed as a completely randomized block design. The results of the concentrations of the total phenolics were analysed using the statistical program GenStat® (Payne, 2003).

The differences between treatment effects were tested on 9 treatments (preliminary pot trial); 6 treatments (pot trial I and II) and 4 treatments (initial field trial) by analysis of variance (ANOVA). The data was sufficiently normal, but with heterogeneous treatment variances. Therefore, treatment means were compared at the 5% level of significance using Fishers' protected t-test least significant difference (LSD) (Snedecor & Cochran, 1980).

5.3 RESULTS

5.3.1 Pathogenicity

Symptoms developed within one to three days after inoculation, beginning at the lenticels. Although the severity of symptoms varied between tubers, the virulence of the isolate was confirmed. Differences between the rates of symptom development could be due to climatic conditions such as high humidity or physiological differences between inoculated tubers.

5.3.2 Preliminary trial

All the plants showed a certain degree of blackleg symptoms, indicating that all the seed tubers were infected. This could be possible due to the well known fact that soft rot bacteria are generally present on most tubers. Identification of phenolic compounds in stems using TLC plates showed similar patterns.

The control treatments had significantly lower levels of total phenolics than Si treated plants (Table 5.1). Although crude phenolic concentrations were heterogeneous within treatments, no clear assumption can be made concerning the effect of Si on the phenolic content of potato stems in the presence of soft rot bacteria. However, it is clear that Si has a significant effect on the concentration of phenolic compounds in potato stems.

Table 5.1 Mean (± SD) of total soluble phenolic compounds (mg) of stems per gram dry weight.

TREATMENTS	STEMS
UNTREATED SILICA ASH (~99% SI)	5.156 ± 0.780 a
TREATED FLY ASH (50% SI)	4.654 ± 0.677 ab
TREATED CAMASIL SLAG (30% SI)	4.288 ± 0.988 abc
UNTREATED CAMASIL SLAG (30% SI)	4.148 ± 0.660 bcd
TREATED SILICA ASH (~99% SI)	3.639 ±1.311 cde
TREATED FLY ASH (50% SI)	3.245 ± 0.470 def
LIME TREATMENT WITH PATHOGEN	2.782 ± 0.795 ef
INOCULATED POSITIVE CONTROL	2.467 ± 0.674 f
UNTREATED CONTROL	2.436 ± 1.005 f
SEM	0.2456
PROBABILITY	<0.001
LSD (1%)	0.9121
CV%	23.3

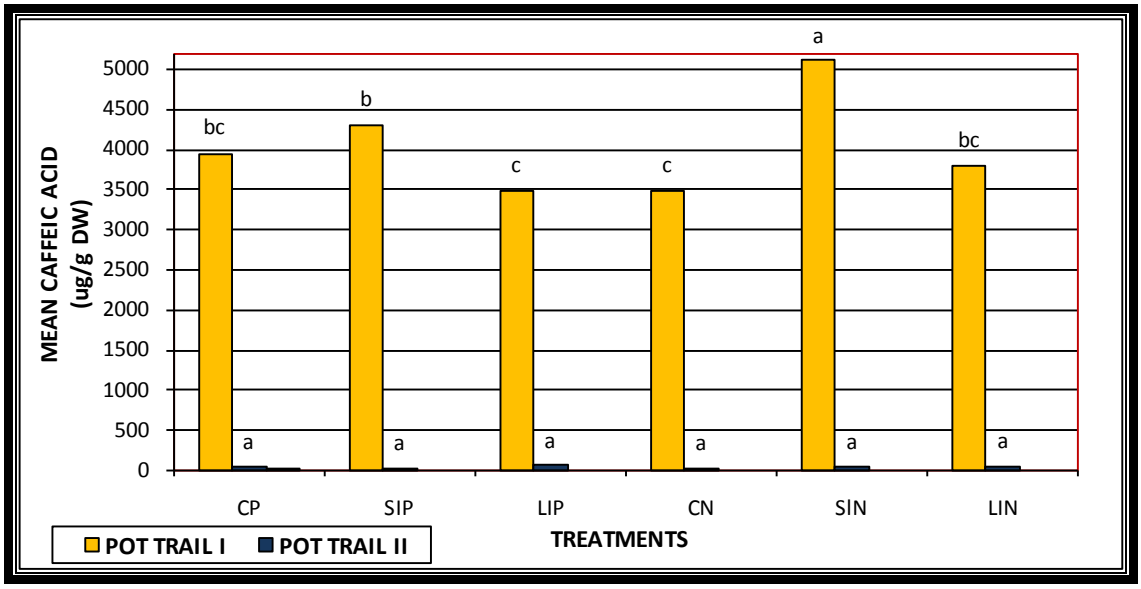
SD is the standard deviation of the means
SEM is the standard error of the means
LSD is the Fisher's protected t-test least significant difference at the 1% level
Means per column followed by a different letter are significantly different at the 1% level
CV% is the percentage coefficient of variation

5.3.3 Pot trials I and II

During pot trial I, the incidence of the disease symptoms was very low (~6%). However, in pot trial II the plants died at tuber initiation before any disease symptoms were visible, due to an unexpected heat shock in the phytotron. Therefore, symptom expression could not be measured. Potato peels from pot trial I had very high concentrations of caffeic acid in comparison to chlorogenic acid, which was expected to be highest. In contrast, the concentration of ferulic acid was very low or not detectable in samples, and was therefore not included in the results. However, potato peels from pot trial II showed higher concentrations of chlorogenic acid in comparison to caffeic acid. Although symptom development on progeny tubers was not severe, differences between tubers treated with different treatment could be observed (Fig. 5.1).

Fig. 5.1 Effect of Si-amended soil on resistance of progeny tubers to soft rot.

In pot trial I Si-treated plants without pathogen (SIN) showed the highest concentration of caffeic acid, followed by Si-treated plants with pathogen which were not significantly different form control with pathogen (CP) or lime treatment without pathogen (LiN). Tuber peels from lime treatments and controls showed low levels of caffeic acid and were not significantly different from each other. In pot trial II the levels of caffeic acid in all treatments were low and not significantly different. Figure 5.2 clearly indicates that mature tubers from Si-treated plants (pot trial I) have a higher phenolic concentration than those from the control treatments. However, the caffeic acid concentrations were very low in immature tubers (pot trial II).



CP- Control with *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers

SIP- *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers planted in Calmasil amended soil

LIP- pH control with *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers

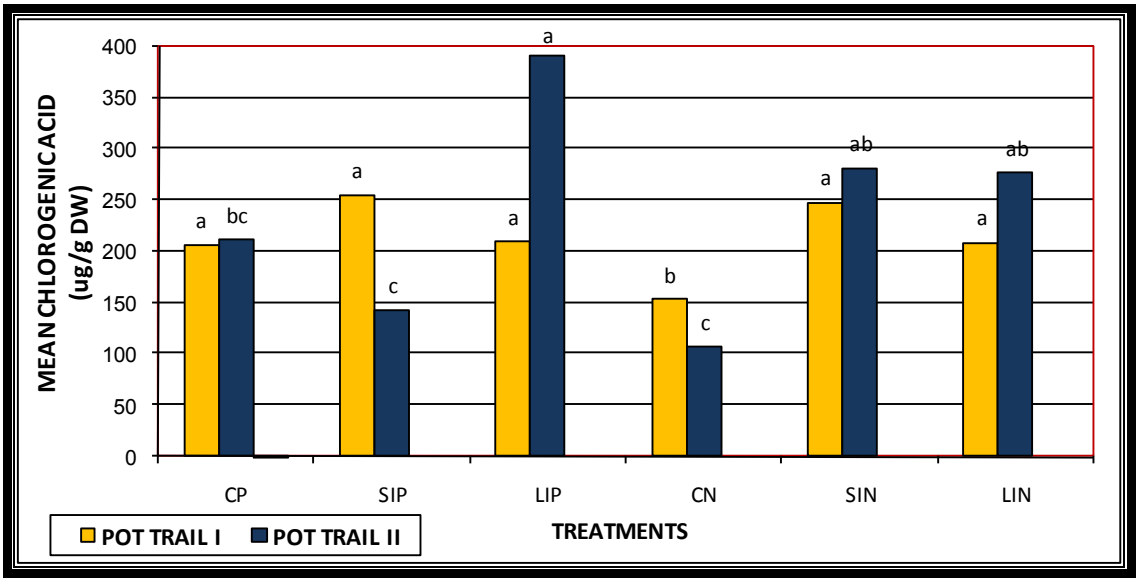
CN- Control without pathogen

SIN- Uninfected tubers planted in Calmasil amended soil

LIN-pH control without pathogen

Fig. 5.2 Effect of silicon-amended soil (Calmasil) on the concentration of caffeic acid in tuber peels from inoculated / uninoculated plants from two pot trials. Bars represent the mean value of five replications per treatment. Pot trial I values in table with different symbols indicate significant differences at 5% level of significance. The coefficients of variation between treatments were 13.4%. Data of pot trial II was not significant at the 5% level of significance.

In pot trial I phenolics extracted from all tubers sampled contained lower concentrations of chlorogenic acid (153 – 255 $\mu g.g^{-1}$ dry weight) than found in other studies (Onyeneho & Hettiarachchy, 1993; Lewis *et al*., 1998; Nara *et al.,* 2006). The relative amounts of chlorogenic acid among treatments were not significantly different at the 5% level, although Si-treated plants had the highest concentration in comparison to the non-inoculated control. In pot trial II the highest concentration of chlorogenic acid was found in the lime treatments, followed by Si-treated tubers without pathogen. Chlorogenic acid levels in Si-treated tubers with pathogen were unexplainably low and significantly lower than the lime treatments at the 5% level of significance (Fig. 5.3).



CP- Control with *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers

SIP- *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers planted in Calmasil amended soil

LIP- pH control with *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers

CN- Control without pathogen

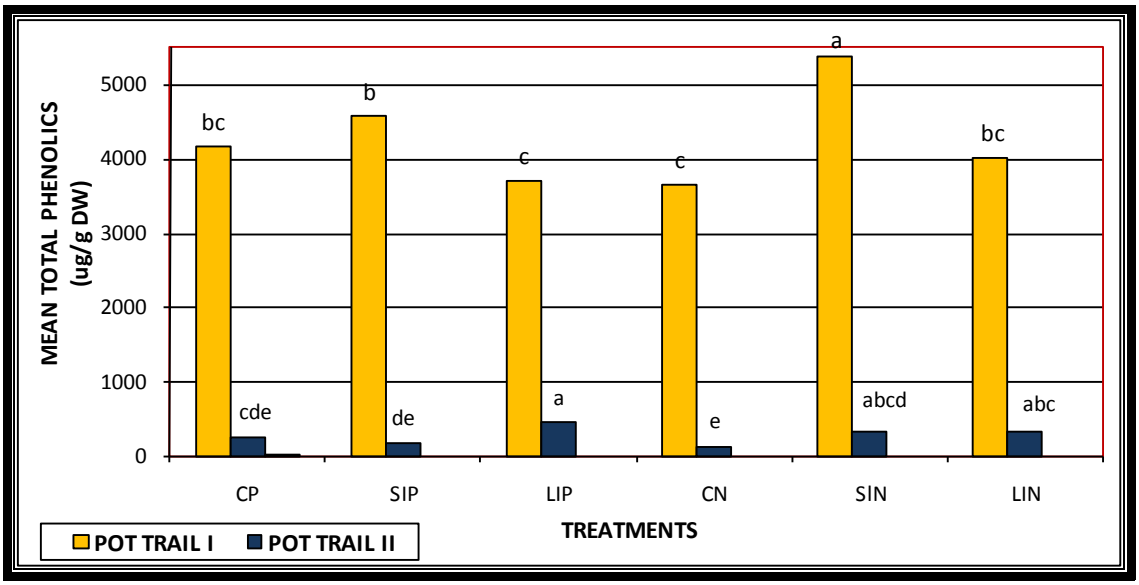
SIN- Uninfected tubers planted in Calmasil amended soil

LIN-pH control without pathogen

Fig. 5.3 Effect of silicon amended soil (Calmasil) on the concentration of chlorogenic acid in tuber peels from inoculated / uninoculated plants of two pot trials. Bars represent the mean value of five replications per treatment. Pot trial I and Pot trial II - values in table with different symbols indicate significant differences at 5% level of significance. The coefficients of variation between treatments were 17.6% and 29.1%, respectively.

In pot trial I the analysis of total phenolics indicated that control and lime treatments had the lowest concentration of total phenolics, whilst Si treatments had the highest concentration (Fig. 5.4). Silicon treated tubers without pathogen had the highest concentration of total phenolics in comparison to Si treated tubers with pathogen. The lime treatment and controls in pot trial I was not significant different. In pot trial II the lime treatment with pathogen had the highest concentrations of total phenolics. However, this lime treatment was not significantly different from the lime and Si treatments without pathogen, shows that pH could have an effect on the phenolic concentrations in tubers.

Levels of total phenolics in the tubers from the Si-treatment with pathogen were not significantly different from those of the control treatments, at the 5% level of significance (Fig. 5.4).



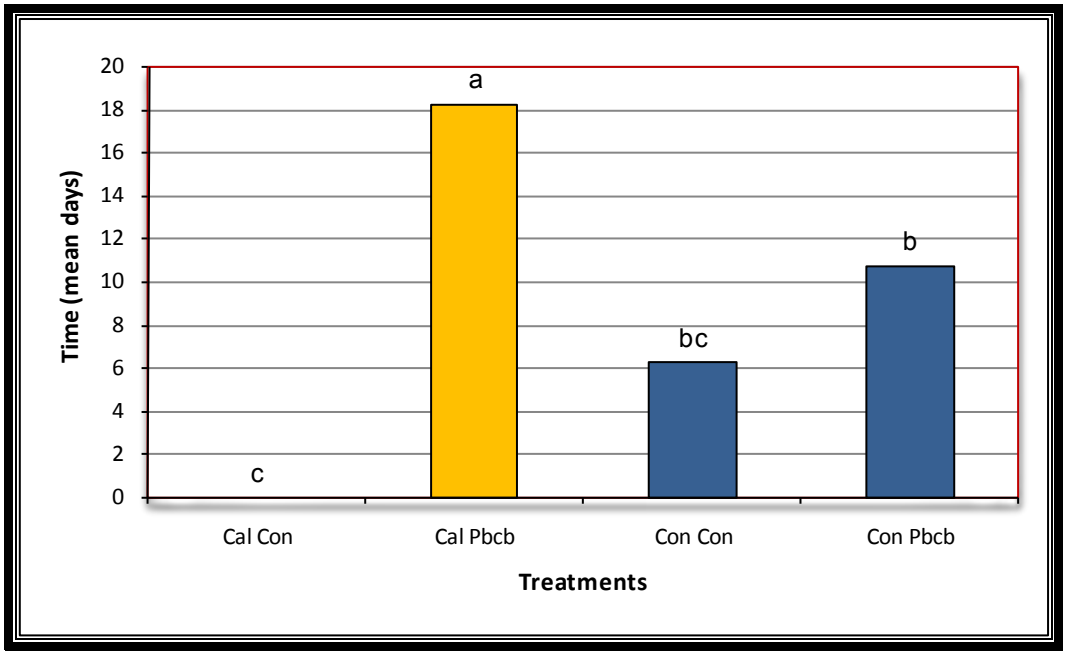
CP- Control with *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers
SIP- *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers planted in Calmasil amended soil
LIP- pH control with *Pectobacterium carotovorum* subsp. *brasiliensis* inoculated tubers
CN- Control without pathogen
SIN- Uninfected tubers planted in Calmasil amended soil
LIN-pH control without pathogen

Fig. 5.4 Effect of silicon amended soil (Calmasil) on total phenolic concentrations in tuber peels from inoculated / uninoculated plants in two pot trials. Bars represent the mean value of five replications per treatment. Pot trial I and Pot trial II - values in table with different symbols indicate significant differences at 5% level of significance. The coefficients of variation between treatments were 13.0% and 30.2%, respectively.

5.3.4 Initial field trial – Screening of stem-base resistance

In this experiment initial blackleg symptoms were visible as a discolouration of the stem tissue associated with bacterial rotting that was initiated from a completely rotten mother tuber. Thus, the speed of the rotting process of the tuber tissue could be considered as a putative component of resistance (Allefs *et al*., 1996).

For progeny tubers from plants grown in Calmasil-treated soil, the mean sprout symptom latency period was 18 days, in comparison to progeny tubers from Dura-Pozz®-treated soils, which had a mean sprout symptom latency period of 10 days (Fig. 5.5). The experiment was carried out in conditions optimal for disease initiation and reduction of tuber resistance. Calmasil treated tubers had a significantly higher resistance than Dura-Pozz® treated tubers at the 5% level of significance. Control tuber cylinders from the Calmasil treatment showed no blackleg symptoms. However, the control tuber cylinders from the Dura-Pozz® treatment resulted in a few diseased sprouts. There is a possibility that tuber cylinders of controls could be naturally infected. This could, however, not be confirmed as the trial was discarded after 3 – 4 weeks due to interference by secondary pathogens and saprophytes.



Cal Con – Calmasil Control (Nutrient broth solution)
Cal Pcbc – Calmasil (*Pectobacterium carotovorum* subsp. *brasiliensis* nutrient broth solution)
Con Con – Durapozz Control (Nutrient broth solution)
Con Pcbc – Durapozz (*Pectobacterium carotovorum* subsp. *brasiliensis* nutrient broth solution)

Fig. 5.5 Effect of Calmasil-amended soil on resistance of etiolated progeny tubers against blackleg. Bars represent the mean value (mean days after inoculation of tuber cylinders when blackleg symptoms were visible) of two independent treatments, with five replications per treatment. Values in table with different symbols indicate significant differences at a 5% level of significance.

5.4 DISCUSSION

The pre-planting broadcast application of calcium silicate slag has various positive effects in biological systems but its mode of action in plants as a factor contributing to disease resistance remains a matter of speculation (Fawe *et al*., 1998; Fauteux *et al*., 2005). Preliminary results obtained support the fact that Si-amended soil increases the resistance of potato stems to soft rot bacteria by increasing the production of phenolic compounds, as in rice (Bélanger *et al*., 1995; Rodrigues & Datnoff, 2005). The heterogeneous data within treatments could be due to high levels of nitrogen in the organic fertilizers used in the pot trials. This could result in an increase in soft rot incidence with a proportional decrease in the phenolic content (Kumar *et al.*, 1991). An overall assumption could be made, though, that the pH of the soil could play a role in phenolic formation. De Camargo *et al*. (2007) found that calcium silicate increases the pH and therefore increases silicon absorption by plants which is associated with the accumulation of phenolics and phytoalexins together with the activation of some PR-genes (Rodrigues & Datnoff, 2005).

In these experiments slag (Calmasil, calcium silicate) was shown to be the best form of Si to apply to the soil. Further studies should therefore focus primarily on the role of Calmasil in increasing the inherent resistance of potatoes to pathogens. Results from pot trial I indicated that Si soil-amendments could have a positive effect on the production of phenolics in mature potato tubers, which enhances the protection and resistance against soft rot (Yildrim & Tokusoglu, 2005).

The blackleg incidence in all treatments remained very low, since favourable environmental conditions (soil type, temperature and moisture) are critical for disease development in the field and in pots. The assumption could also be made that the concentration of bacteria in the stem remained relatively low in these trials due to the low incidences of blackleg symptoms, although most of the mother tubers were completely rotten. Charkowski (2006) stated that many scientists have struggled to obtain blackleg disease development in pot trials, and that despite high bacterial concentrations in the inoculated seed tubers, the final disease incidence may be very low.

This is because of specific environmental conditions (such as temperature, moisture and soil type) being critical for blackleg development. Another reason for low disease incidence could be the resistance associated with a high Ca concentration located in the cell walls due to the pre-planting application of calcium, as a calcium silicate or gypsum ($CaSO_4$) (Bain *et al*., 1996; Flego *et al.*, 1997). Surprisingly, the concentration of caffeic acid was higher in tuber peels than the more commonly found chlorogenic acid. This could be explained by the fact that chlorogenic acid is the storage form of caffeic acid, which can be converted during stress conditions to caffeic acid (Ghanekar *et al*., 1984).

In pot trial II, however, the effect of Si was inconsistent in progeny tubers. The caffeic acid concentrations are not significantly different between treatments. Thus could be the effect of the heat shock, an abiotic stress that is known to enhance certain defence responses (Fauteux *et al*., 2006). In addition, the caffeic acid concentrations in pot trial I (mature tubers) were much higher than in pot trial II (immature tubers). These results are indicative of a higher resistance to soft rot in mature tubers than progeny tubers.

In pot trial II, tubers from lime-amended soil had the highest levels of chlorogenic acid, however, the levels between treatments were not significantly different. It seems that available Ca has a greater effect on immature progeny tubers than on mature tubers, but this effect is not consistent through the growing season. Bain *et al*. (1996) found that pre-plant, soil treatment with gypsum ($CaSO_4$) resulted in a significant reduction of blackleg incidence at the beginning and in the middle of the growing season, but near the end of the season the effect decreased. Simmons & Kelling (1987) found that daughter tubers treated with gypsum (soil calcium concentration between 650 – 950 ppm depending on the soil type) contained high Ca concentrations in their cortex and tuber periderm, which significantly reduced the severity of tuber soft rot (Bain *et al*., 1996).

The results obtained in this study showed that caffeic acid concentrations (pot trial I) were higher in uninoculated mature tubers treated with Si, than in inoculated mature tubers treated with Si. However, in pot trial II caffeic acid concentrations were very low and not significantly different between treatments.

The highest concentrations of chlorogenic acid were detected in pot trial II. However, in pot trial ll it seems that Ca had a greater effect on the resistance of progeny tubers than Si. These results indicate that chlorogenic acid is formed as a defence mechanism in potatoes against pathogen infection or injury (Ghanekar *et al*., 1984). Caffeic acid, however, is known to have antibacterial activity against soft rot bacteria by inhibiting growth (Kumar *et al*., 1991). Therefore, when caffeic acid and chlorogenic acid are present together it may significantly inhibit infection by soft rot bacteria (Ghanekar *et* al., 1984; Kumar *et al*., 1991).

The trends in results obtained in the various experiments did not show significant differences between treatments. However, in pot trial I the levels of the total phenolics in tubers from Si treatments were higher than in treatments without Si. The concentrations of total phenolics in tubers were significantly higher in uninoculated Si treatments than in inoculated Si treatments. However, the phenolic concentration measured at harvest may not reflect conditions present during the infection period. Another explanation could be due to prevalent physiological conditions rather than pathogen infection, and environmental conditions that were not optimal for disease development.

In vivo studies on Si treated tubers showed that the rate of development of blackleg (Allefs *et al.*, 1996) was significantly slower in Calmasil (pH effect) treated tubers than Durapozz (no pH effect) treated tubers. This could be due to the fact that Si becomes more plant-available in soils with a higher pH.
Maximum Si absorption occurs within the 4.7 - 7.6 pH range (De Camargo *et al*., 2007). However it is still unclear whether the increased Si uptake is due to an increase in pH or the Si-amendment added to the soil.

These results suggest that the stem base forms a defence barrier by preventing the movement of a mass of bacteria from the rotten tuber tissue to the stem. This indicates that Calmasil, a calcium silicate, is a better Si source to use than DuraPozz, to increase phenolics in the plant cell walls and thus enhance resistance to soft rot bacteria via a physical barrier. According to Allefs *et al.* (1996) the most important factor in determining resistance to soft rot bacteria is stem-base resistance rather than tuber tissue resistance, stem tissue resistance or mother tuber decay.

Results obtained need to be confirmed by follow-up trials using a larger number of progeny tubers.

Several other studies have confirmed the beneficial role of Si in increasing baseline resistance of different crops to various pathogens (Bélanger *et al.*, 1995; Fauteux *et al*., 2005). However, this is the first study on the effect of Si on defence responses of potatoes to soft rot and blackleg. It therefore opens new possibilities for the use of Si in commercial production to improve plant health status and disease resistance, thereby reducing the use of chemicals.

Further investigations are underway to determine the effect (mode of action studies) of Si and Ca, either individually or in combination on the production of phenolic compounds in the potato tubers, as well as the effect of Si soil amendments on other potato pathogens.

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CHAPTER 6

FARMERS` KNOWLEDGE OF SOFT ROT AND BLACKLEG DISEASES ON POTATOES IN SOUTH AFRICA

ABSTRACT

A survey was conducted to determine the level of knowledge, that farmers have regarding soft rot / blackleg disease complex, their perception of the prevalence of the disease, its control and need for research in South Africa. Questionnaires were distributed through farmers' visits to 14 production regions and a total of 41 were completed from 10 areas. Estimates of economic loss due to soft rot (seedpiece decay, post planting), blackleg (*Pectobacterium* wilt) and post harvest soft rot ranged from >1 to 70%. However, average yield losses were estimated at approximately 11%. The most popular cultivars planted in all the regions are Mondial, BP1 and Up-to-date, which were rated as susceptible by more than 50% of the growers' respondents. It appears that in South Africa disease symptoms are prevalent under temperate (10 - 20°C) or warm climates with prolonged wet or humid conditions. To reduce disease incidence, low generation seed tubers need to be planted and good crop rotation systems should be followed. Research in South Africa should focus mainly on effective disease management measures integrated with the development of resistant cultivars and low generation seed tubers, to produce high-quality and long shelf-life tubers.

6.1 INTRODUCTION

In South Africa potato production for the 2006/2007 season exceeded 1,92 million tonnes, cultivated on 54 037 hectares (Potatoes South Africa, 2008). The most important potato production areas in South Africa are Limpopo, Mpumalanga, Western Free State, Eastern Free State, KwaZulu-Natal and Sandveld (Niederwieser, 2003).

In the past, the soft-rot / blackleg disease complex was vastly underestimated in South Africa. During the 2006/2007 growing season outbreaks of blackleg occurred causing severe economic losses in commercial potato production fields, necessitating research on the epidemiology of this disease complex.

Some of the most important bacterial diseases of potatoes are tuber soft rot prior to planting, blackleg and aerial stem rot in the field as well as tuber soft rot in storage. These symptoms are caused by *Pectobacterium carotovorum* (Jones) (*Pbc*), *Pectobacterium atrosepticum* (Van Hall) Dye (*Pba*) and *Dickeya* spp. (Dickey), particularly *Dickeya dadantii* (*Dd*) (Dadant), *Dickeya dianthicola* (Hellmers) and *Dickeya zeae* (Sabet) (Hauben *et al.,* 1998; Pérombelon, 2002; Euzéby, 2003; Samson *et al*., 2005; Ma *et al.*, 2007). These three pectolytic strains were formerly known as *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi,* respectively (Garden *et al.*, 2003). Recently, Duarte *et al.* (2004) described a new virulent subspecies from Brazil, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*), which also causes blackleg-like symptoms on potatoes.

In South Africa blackleg symptoms are usually noted on only one stem per plant, on which the top leaves roll upwards, wilt and become necrotic. As symptoms progress to the lower leaves with subsequent leaf desiccation, a light to dark brown discolouration of the vascular system at the stem base develops, followed by external darkening. Under prevailing wet and humid conditions stems become slimy and pale. In the stem the rotten pith becomes necrotic and hollow. In storage, tubers develop deep sunken darkened lenticels which can stay latent or develop into a tuber soft rot under excessive moisture and humidity. When seed tubers are suddenly exposed to higher temperatures a black soft rot can develop. Black soft rot of seed tubers results in non-emergence, blanking or blackleg development during the early stages of vegetative growth (Serfontein *et al*., 1991).

Blackleg and soft rot symptoms are not species specific but are dependent on climatic conditions (Gudmestad *et al*., 1988, Hélias *et al*., 2000, De Haan *et al*., 2008).

Disease development is initiated when the temperature is optimal, oxygen concentration is low, humidity is high and enough available free water is present (Table 6.1) (Smadja *et al*., 2004; Toth *et al*., 2003). Soft rot pectobacteria can be found on plant surfaces, in water, but are not able to survive for longer than about 1 year in soil (Rowe *et al*., 1995; Hélias *et al.,* 2000).

Table 6.1 Optimal environmental conditions for each soft rot species for disease expression.

Pathogen	Environmental conditions	References
Pectobacterium atrosepticum	Cool (<25°C), moist, temperate climates	(Smadja *et al*., 2004)
Pectobacterium carotovorum	Temperate and tropical areas under low and high temperatures	
Dickeya dadantii *Dickeya zeae*	Warmer, humid tropical and sub-tropical environments	(Samson *et al*., 2005; Elphinstone & Toth, 2007; Fraaije *et al.*, 1997).
Dickeya dianthicola	Warm, dry or warm, wet growing conditions as well as cool temperate climates	
Pectobacterium carotovorum subsp. *brasiliensis*	Humid subtropical climate with cool temperatures (17-20°C) (Duarte *et al*., 2004)	(Duarte *et al*., 2004)

Management practices are based on farmers' levels of knowledge and ability to make disease control decisions to effectively implement and maintain Integrated Pest Management (IPM) strategies on a farm basis (McRoberts *et al*., 2003). Thus perceptions of diseases involve both scientific and practical frameworks (Kiros-Meles & Abang, 2008). The success of IPM systems is driven by regular transfer of knowledge between scientists, agronomists and growers to ensure early disease prediction, effective implementation of sustainable practices and to continually improving best management practices (Ingram, 2008).

Therefore the purpose of this study was to investigate the farmers` knowledge of soft rot and blackleg diseases, the importance of research to effectively control the disease and the perceptions and comments of potato growers regarding the disease in South Africa.

6.2 MATERIALS AND METHODS

A structured questionnaire (Appendix C) was distributed to potato growers in South Africa during the 2006/2007 growing season. The questionnaire was completed by commercial potato growers and seed potato growers in each region while diseased potato plants were collected. The questionnaires were drawn up to determine (i) the prevailing temperatures and average rainfall of each region; (ii) crop production practices such as irrigation and cultivar selection (iii)the impact of blackleg and pre- and post-harvest soft rot on estimated yield losses and (iv) general crop production practices. Growers were asked to rate potatoes cultivars to pre– and post harvest soft rot and blackleg susceptibility, on a scale of 1 to 3; with 1, being resistant; 2, moderately susceptible/resistant and 3, susceptible. Growers were also requested to make suggestions for research and general comments about their perceptions of the disease.

Answers were summarised in table format for statistical analysis. Due to the low number of responses and uncomplete questions, chi-square one-sample test could not be used. Therefore the data collected were just summarised. A one-way ANOVA was used to test the differences in mean yield losses, temperature and rainfall data for each region using the statistical program GenStat® (Payne, 2003).

6.3 RESULTS

A total of 41 questionnaires were collected from 10 out of the 14 potato growing regions in South Africa. Questionnaires were collected from: Mpumalanga (4); Eastern Free State (2); KwaZulu-Natal (6); Western Free State (8); North-West Province (1); Northern Cape (4); Limpopo (4); Sandveld (3); Eastern Cape (5) and Southern Cape (4) (figures in parentheses indicate number of farms visited).

Questionnaires were not collected from Gauteng, North-Eastern Cape, South-Western Cape or Ceres. Only one response was received from North-West Province and was therefore combined with those from Limpopo, which has similar climatic conditions.

Ninety percent of the potato fields represented in the survey is under irrigation; of which 32% are irrigated on a weekly basis, 29% irrigated twice a week and 35% follow other irrigation programmes according to field capacity. All the respondents use certified seed. However, 3% retain and replant a small percentage of their own seed. The best control measures implemented by growers are those based on good crop rotation systems. Crop rotation systems are applied within 1-2 year, 4-5 year or 6-8 year cycles with maize, sweet corn, wheat, sunflower, canola, oats, beans, soybeans, ryegrass, supergrass, *Eragrostis curvula*, onions, cauliflower, carrots, broccoli or sometimes left fallow.

According to Potatoes South Africa, (2008) 33% of total hectares planted in 2007 was Mondial, followed by 20% BP1 and 9% Up-to-date. Most of the growers, 80%, planted Mondial whereas BP1 and Up-to-date planted by 50% and 40%, respectively. Seventy percent of the growers rated Mondial as susceptible to pre- and postharvest soft rot as well as blackleg. BP1 and Up-to-date were rated as susceptible to the soft rot disease complex by 50% of the growers` respondents. Estimates of economic losses due to soft rot (seedpiece decay, post planting), blackleg (*Pectobacerium* wilt) and post harvest soft rot ranged from >1 to 70% (Table 6.2).

Table 6.2 Mean estimated yield losses due to pre- and post harvest soft rot and blackleg (*Pectobacterium* wilt) in potato growing regions in South Africa.

Potato growing Regions	**Soft rot (seedpiece decay, post planting)**	**Blackleg (*Pectobacterium* wilt)**	**Soft rot (post harvest)**
Mpumalanga	11%	7%	11%
Eastern Free State	4%	<1%	4%
KwaZulu Natal	10%	14%	10%

Western Free State	9%	15%	10%
Northern Cape	8%	<1%	1%
Limpopo	14%	13%	18%
Sandveld	33%	40%	34%
Eastern Cape	7%	8%	5%
Southern Cape	0	<1%	0

Some of the growers requested research focussed on:

- Disease forecasting based on favourable environmental conditions
- Preventative measures such as good crop rotation systems (alternation with non-host crops)
- Effective evaluation and selection of seed tubers
- Disease difference between cut seed tubers and uncut tubers
- Influence of pre-planting factors on disease severity, such as soil temperature, planting depth and the plant-available nutritional composition of the soil.
- Role of poor water quality, water pH and *Pectobacterium* contamination in irrigation water in disease development
- Possibility of effective chemical control measures
- Effect of washing water processes on disease spread and development
- Survival of the causal bacteria in the soil and effective soil fumigation
- Breeding resistant cultivars

The general comments of growers were summarised as follows:

- An average of 15% of crop stand (emergence) is lost due to *Pectobacterium* and most growers thus consider research on the disease complex of significant value to the South African potato industry
- Disease outbreaks are more prevalent when early rainfall occurs before emergence and are associated with rainfall incidents
- Disease outbreaks occur during very wet periods, followed by warm, hot temperatures with a lack of oxygen in soils (moist soil conditions). Potatoes in sandy soils are more prone to rot in hot conditions than cool conditions
- Plant generations G2 – G4 are known to have low disease incidence

- The importance of the transportation of seed tubers from cold storage to the farm prior to planting. Seed tubers should be acclimatised first to prevent condensation and subsequent rotting
- Infection originates from infected seed tubers and initiates disease in alternating warm and cool temperatures with associated wet conditions
- Ridging when temperatures are high enhances disease development
- Sometimes very little tuber decay is noted in storage but after germination plants start to show symptoms of wilting
- Mondial / Dutch varieties according to some growers appear to be more susceptible to the disease
- To prevent disease, early detection, such as during seed certification is necessary. This should include testing for latently infected tubers.

6.4 DISCUSSION AND CONCLUSION

Although the growers` responses were very low, the questionnaires received were found to be representative of Chapter 3 & 4. Responses in the questionnaires showed that most potato growers in South Africa are aware of soft rot, aerial stem rot and blackleg. The greatest yield losses are caused by blackleg and associated with post-planting and post harvest soft rot. Although the majority of growers use certified seed, the causal agent of blackleg in South Africa, *Pectobacterium carotovorum* subsp. *brasiliensis*, is widely distributed throughout the country (van der Merwe *et al*., In Press). This is because the seed potato classification schemes are set on tolerance levels encountered during visual inspections of growing crops and harvested tubers and there is no official post-harvest testing programme. It is interesting to note that Mondial, a popular cultivar from the Netherlands, is most widely planted in South Africa. Although rated as a very susceptible cultivar by growers, popularity remains due to a very high yield potential (Niederwieser, 2003). Therefore to decrease blackleg and soft rot incidence on Mondial, the number of generations needs to be limited, thus reducing inoculum levels in seed. Interesting to note that although both BP1 and Up-to-date were rated by the growers as susceptible, these potato cultivars remain popular choices.

Based on the questionnaire responses, the region with the highest yield losses due to the soft-rot / blackleg disease complex is the Sandveld, followed by Limpopo, KwaZulu Natal and Western Free State. Sandveld and Western Free State have a maximum summer temperature of between 26 - 30°C and a maximum winter temperature between 16 - 20°C. Limpopo has the same winter temperatures but maximum summer temperatures are higher than 30°C. Average annual rainfall in these regions is between 250 – 500mm but due to the variable South African conditions, some seasons may have heavier rainfalls causing very wet periods. The maximum summer temperatures in KwaZulu Natal are 21- 25°C with maximum winter temperatures of 10 - 15°C, with an average rainfall of more than 1000mm p.a.. The average yield losses in these regions range from 9% to 40%. Countrywide the average yield losses are approximately 11%. Thus, the incidence and severity of soft rot is dependent on environmental conditions of each specific region, especially temperature and available free water. The climatic conditions optimal for blackleg and soft rot development in South Africa are temperate (10 - 20°C) to warm conditions with prevailing wet, humid conditions.

Research in South Africa should focus mainly on pre- and post harvest preventative and curative control measures (such as chlorine), integrated with the development of resistant cultivars and low generation seed tubers in order to increase tuber quality and prolong shelf-life.

Soft rot diseases originate from infected seed tubers, even if the seed tubers appear healthy. Therefore early detection of contaminated seed tubers is necessary to prevent disease development. To develop effective local disease management strategies, it is necessary to understand the epidemiological factors associated with the incidence and severity of pre- and post-harvest soft rot and related blackleg.

Our findings stress the importance of knowledge exchange and collaboration between researchers and farmers. It is important that researchers understand farmers` knowledge, perceptions and management strategies of soft rot diseases.

Knowledge transfer has been shown to develop new opportunities for improving and implementing sustainable, integrated disease management practices (Ingram, 2008; Kiros-Meles & Abang, 2008), thus making farming with potatoes more economically viable.

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CHAPTER 7

GENERAL DISCUSSION

Pectobacterium diseases on potatoes were reported for the first time in South Africa during the 1988 growing season (Serfontein *et al.,* 1991). Since then, the importance of soft rot diseases in Southern Africa has been vastly underestimated. Recently, blackleg / soft rot diseases have become a significant problem in the South African potato industry, especially for seed growers. Therefore, there was a drastic need to collect information on the epidemiology, population biology, economic status and control of the blackleg / soft rot disease complex in South Africa. The research in this study will help to reduce the economic risk of producing potatoes, enhance the production of high-quality and long shelf-life tubers, and provide growers with possible management strategies.

In order to determine the real status of *Pbcb* and the occurrence of other causal agents of blackleg and soft rot diseases on potatoes in South Africa, blackleg samples were collected from 72 potato fields in 10 potato production regions (Chapter 3). A multiplex PCR together with an IGS-PCR was used to identify and differentiate between *Pectobacterium carotovorum* (*Pbc*), *Pectobacterium atrosepticum* (*Pba*), *Pbcb* and *Dickeya dadantii*. Although *Pbcb* and *Dickeya dadantii* could not be differentiated using the multiplex, IGS-PCR was used to distinguish between the two species. Of 128 isolates, 77% were shown to be *Pbcb* and 17% *Pbc*. *Dickeya* spp. were not detected in South Africa, although Serfontein *et al*. (1991) had classified some isolates as *Dickeya*. *Pbcb* is a problem in the South African potato industry and these results represent an important early step towards the understanding of the epidemiology especially the pathogenicity of soft rot pectobacteria in South Africa. Furthermore this information is essential to effectively manage this subspecie in or on seed potatoes under prevailing local conditions.

The distribution of *Pbcb* and *Pbc* in South Africa was not correlated with the geographic regions, temperature or rainfall.

However the research supported the findings of Duarte *et al*. (2004) that *Pbcb* has the ability to cause blackleg-like symptoms at higher temperatures than *Pba*. We could further conclude that blackleg incidence and severity is primarily dependent on initial inoculum levels in seed tubers. Accordingly when seed tubers are contaminated with pectobacteria it may be indicative of the region where the seed was produced rather than where the crop was sampled.

Imported techni-tubers were also tested. Fourteen isolates were obtained, 13 of which were identified as *Pbcb* and 1 isolate as *Pbc*. This confirmed the importance of phytosanitary requirements for imported plant material as some lots can be contaminated with latent bacterial infections. The presence of latent infections increases the risk of introducing a new pathogen into South Africa. Consequently the pathogen will spread countywide as the progeny seed tubers produced from the imported tubers will be planted the following season at a different location Furthermore, Elphinstone & Toth (2007) highlighted the risk of importing or exporting seed tubers to different climates. They found that the same contaminated seed stock produced locally can give rise to significantly more soft rot / blackleg diseases when planted in a foreign country. These findings supported the fact that a post harvest diagnostic programme need to be included in the South African certification system since it is currently only based on a set of tolerance levels encountered during visual inspections of growing crops and harvested seed tubers. However field inspections are only based on the recognition of the disease symptoms without discriminating between the true causal organisms and are therefore often unreliable (De Haan *et al*., 2008). The use of accurate and sensitive diagnostic testing of post-harvest pathogens may therefore be the best approach to reduce the risk of introducing new pathogens into South Africa with the resultant economic losses.

During the 2006/2007 potato growing season in South Africa, blackleg outbreaks occurred in commercial potato fields causing severe economic losses (Chapter 4). Typical blackleg symptoms were initially observed as the top leaves showed symptoms of rolling upwards, wilting and becoming necrotic. As symptoms progressed to the lower leaves a light to dark brown discolouration of the vascular system at the stem base developed, followed by external darkening.

It was interesting to note that these symptoms were similar to those described in Brazil, where the causal agent was identified as a new subspecie, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*) (Daurte *et al*., 2004). In South Africa severe outbreaks was recorded in Limpopo and Mpumalanga Provinces and the Sandveld region. Strains isolated from typical blackleg symptoms from each region were selected for identification. A phylogenetic tree was constructed using partial 16S – 23S rDNA sequences, clearly showed that the South African strains are grouped in the same cluster as *Pbcb* strains. These results confirmed that the South African blackleg strains were different from *Pba, Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *betavasculorum* (Duarte *et al*., 2004). Comparison of RFLP patterns of isolates to reference cultures confirmed the identity of the South African blackleg strains as *Pbcb*, identical to strain 8 isolated in Brazil. This is the first report of *Pbcb* in South Africa which poses a threat to the South African potato industry especially in seed exports and ultimate food quality.

Kiros-Meles & Abang (2008) emphasised that the perceptions of disease involve both scientific and practical frameworks. In order to support the findings presented in Chapters 3 & 4, a countrywide questionnaire survey was conducted among growers, and were discussed in Chapter 6. This was done because of the importance of incorporating farmers` knowledge, perceptions and cultural practices of crop diseases, when implementing the Integrated Pest Management (IPM) strategies on a farm basis (McRoberts *et al*., 2003). The success of IPM systems is driven by a regular transfer of knowledge between scientists, agronomists and growers to ensure early disease prediction, implementation of sustainable practices and to improve a range of best management practices (Ingram, 2008).

Responses in questionnaires distributed countrywide showed that the greatest yield losses are caused by blackleg, associated with post-planting and post harvest soft rot. Additionally, losses vary from region to region and are influenced by climatic conditions of growth and storage. Estimates of economic losses due to soft rot (seedpiece decay, post planting), blackleg and post harvest soft rot ranged from >1 to 70%, with an average of 11%.

The estimated yield losses were the highest in the Sandveld region, Limpopo and Mpumalanga Provinces, which showed a correlation between the results from field surveys of diseased plants and causal agents (Chapter 3) and what the growers experience in the field (Chapter 6). In South Africa disease is prevalent under temperate conditions of between 10 - 20°C to warmer climates (25 - 36 °C) with prolonged wet or humid conditions (Chapter 6).

It is interesting to note that Mondial, a popular cultivar from the Netherlands, is most widely planted in South Africa (Chapter 3 & 6). Although rated as a very susceptible cultivar by growers, popularity remains due to a high yield potential (Niederwieser, 2003). Therefore to decrease blackleg and soft rot incidence on Mondial, the number of generations needs to be limited, thus reducing inoculum levels in seed.

Blackleg is a seed-borne disease and therefore difficult to control. Practical chemical control is not available and the cultural measures are only partially effective (Elphinstone & Toth, 2007). This prompted the search for a possible management strategy to increase resistance to blackleg and tuber soft rot to minimise losses (Chapter 5). The study was based on the fact that calcium silicate slag applied pre-planting has various positive effects in biological systems, including increasing disease resistance (Fawe *et al*., 1998; Fauteux *et al*., 2005). Preliminary results obtained support the fact that Si-amended soil increases the resistance of potato stems and tubers to soft rot bacteria by increasing the production of phenolic compounds, as has been demonstrated in rice (Bélanger *et al*., 1995; Rodrigues & Datnoff, 2005; Yildrim & Tokusoglu, 2005). Although the data was heterogeneous, an overall assumption could be made that the pH of the soil could play a role in phenolic formation. De Camargo *et al*. (2007) stated that calcium silicate increases the pH and therefore increases silicon absorption by plants, which is in turn associated with the accumulation of phenolic compounds (Chapter 5).

Results from Si pot trials indicated that the best Si source to use is Calmasil slag (calcium silicate), since Si and Ca combined have a synergistic effect in enhancing tuber resistance. This is, however, the first such study on the effect of Si on defence responses of potatoes to soft rot and blackleg.

Further studies thus need to be done to elucidate the effect of Si and Ca either individually or in combination on the production of phenolics in potato stems and tubers, and the role thereof in resistance to soft rot bacteria. These findings showed positive results and opened up new possibilities for the use of Si in commercial production to improve plant health status and disease resistance, thereby reducing the use of chemicals.

This research supported the fact that the blackleg/soft rot disease complex is becoming an increasing problem in the South African potato industry. Currently the only way to reduce losses is to integrate management measures such as:

- Plant healthy seed tubers
- An effective crop rotation system
- Well-drained soil
- Seed certification
- Avoid tuber damage post harvest
- Optimal storage conditions:
    - Dry tubers before storage
    - Store seed tubers on crates (palette)
    - Good ventilation during storage and transport
    - Avoid temperature fluctuations to reduce condensation
    - Store potatoes in diffused light rather than in the dark.

This study therefore brought together a broad spectrum of information on the soft rot/blackleg disease complex in South Africa. This will allow growers to make informed decisions regarding problems they face in the field during the production season. It also stresses the importance of knowledge transfer between researches and growers for improving and implementing sustainable, integrated disease management practices (Ingram, 2008; Kiros-Meles & Abang, 2008), thus making farming with potatoes more economically viable.

This thesis opens up new possibilities for future research in South Africa. The best way to prevent economic losses due to this disease complex is to incorporate an accurate, sensitive and routine diagnostic testing programme in the certification system, especially on imported seed tubers and seed tubers locally used for planting. Phylogenetic analysis can also contribute to a better understanding of the epidemiology and thus management of the soft rot / blackleg diseases and therefore a better chance of controlling the disease. Further research in South Africa should be based on effective disease management measures integrated with the development of resistant cultivars and low generation seed tubers, to produce high-quality and long shelf-life tubers. Additional studies should focus on the possibility of enhancing disease resistance to soft rot pectobacteria by the application of soil-amendments such as Calcium silicate.

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APPENDIX A

A LIST OF *PECTOBACTERIUM* STRAINS ISOLATED FROM POTATO (*SOLANUM TUBEROSUM*) PLANTS USED IN THE STUDY

Code	Number	Region	Potato Field	Isolated from	Multiplex-PCR	IGS-PCR
MP_1TB	**JJ1o**	Mpumalanga	1	Harvested tubers	Pbcb	Pectobacterium spp
MP_1TBo	**JJ1**	Mpumalanga	1	Harvested tubers	Pbcb	Pectobacterium spp
MP_2TB	**JJ2**	Mpumalanga	2	Harvested tubers	Pbcb ; Pbc	Pectobacterium spp
MP_3TB	**JJ4**	Mpumalanga	3	Harvested tubers	Pbcb	Pectobacterium spp
MP_4TA	**JJ5**	Mpumalanga	4	Harvested tubers	Pbcb	Pectobacterium spp
MP_5TB	**JJ6**	Mpumalanga	5	Harvested tubers	Pbcb ; Pbc	Pectobacterium spp
EFS_1TB	**JJ7**	Eastern Freestste	1	Harvested tubers	-	Pectobacterium spp
EFS_1TC	**JJ8**	Eastern Freestate	1	Harvested tubers	-	Pectobacterium spp
EFS_2TA	**JJ9**	Eastern Freestate	2	Harvested tubers	Pbcb	Pectobacterium spp
EFS_2TAo	**JJ10**	Eastern Freestate	2	Harvested tubers	Pbcb	
EFS_4TB	**JJ11**	Eastern Freestate	4	Harvested tubers	Pbcb ; Pbc	Pectobacterium spp
EFS_4TE	**JJ12**	Eastern Freestate	4	Harvested tubers	Pbcb	Pectobacterium spp
EFS_5TA	**JJ13**	Eastern Freestate	5	Harvested tubers	Pbcb	-
EFS_5TD	**JJ14**	Eastern Freestate	5	Harvested tubers	-	-
KZN_1TDo	**JJ15**	KwaZulu-Natal	1	Harvested tubers	Pbcb	Pectobacterium spp
KZN_1TD	**JJ16**	KwaZulu-Natal	1	Harvested tubers	Dd	Pectobacterium spp
KZN_2TBo	**JJ17**	KwaZulu-Natal	2	Harvested tubers	Pbcb	Pectobacterium spp
KZN_2TB	**JJ18**	KwaZulu-Natal	2	Harvested tubers	Pbcb	Pectobacterium spp
KZN_2TE	**JJ19**	KwaZulu-Natal	2	Harvested tubers	Pbcb	Pectobacterium spp
KZNU	**JJ20**	KwaZulu-Natal	9	Harvested tubers	Pbcb	-
KZN_4TB	**JJ21**	KwaZulu-Natal	4	Harvested tubers	Pbcb	-
KZN_5TE	**JJ23**	KwaZulu-Natal	5	Harvested tubers	Pbcb	Pectobacterium spp
KZN_6TC	**JJ24**	KwaZulu-Natal	6	Harvested tubers	Pbcb	Pectobacterium spp
KZN_6TE	**JJ25**	KwaZulu-Natal	6	Harvested tubers	Pbcb	-
KZNU	**JJ27**	KwaZulu-Natal	9	Harvested tubers	Pbcb	Pectobacterium spp
KZN_7KV_3TA	**JJ28**	KwaZulu-Natal	7	Seed tubers	Pbcb	Pectobacterium spp
KZN_8TC	**JJ30**	KwaZulu-Natal	8	Harvested tubers	Pbcb	Pectobacterium spp
WFS_1TA	**JJ31**	Western Freestate	1	Harvested tubers	Pbcb	-

WFS_1TD	**JJ32**	Freestate	1	Harvested tubers	Pbcb	Pectobacterium spp
WFS_2TB	**JJ33**	Western Freestate	2	Harvested tubers	Pbcb	Pectobacterium spp
WFS_2TC	**JJ34**	Western Freestate	2	Harvested tubers	Pbcb	-
WFS_4TD	**JJ36**	Western Freestate	4	Harvested tubers	Pbcb	Pectobacterium spp
WFS_5TE	**JJ37**	Western Freestate	5	Harvested tubers	Pbcb	Pectobacterium spp
NC_1TA	**JJ38**	Northern Cape	1	Harvested tubers	Dd	Pectobacterium spp
NC_1TB	**JJ39**	Northern Cape	1	Harvested tubers	Dd	Pectobacterium spp
S_1TA	**JJ41**	Sandveld	1	Seed tubers	Dd	Pectobacterium spp
N_2TA	**JJ45**	Limpopo	2	Harvested tubers	Pbcb	Pectobacterium spp
N_3SB	**JJ46**	Limpopo	3	Harvested tubers	Pbcb ; Pbc	Pectobacterium spp
N_6SA	**JJ47**	Limpopo	6	Harvested tubers	Pbcb	Pectobacterium spp
N_6SB	**JJ48**	Limpopo	6	Harvested tubers	Pbcb	Pectobacterium spp
N_6TA	**JJ49**	Limpopo	6	Harvested tubers	Pbcb	Pectobacterium spp
N_7TB	**JJ50**	Limpopo	7	Harvested tubers	Pbcb ; Pbc	Pectobacterium spp
N_5TB	**JJ51**	Limpopo	5	Harvested tubers	Pbc	Pectobacterium spp
S_4TAo	**JJ52**	Sandveld	4	Seed tubers	Pbc	Pectobacterium spp
S_4TA	**JJ53**	Sandveld	4	Seed tubers	Pbc	Pectobacterium
S_4TD	**JJ54**	Sandveld	4	Seed tubers	Pbcb	Pectobacterium spp
S_4TE	**JJ55**	Sandveld	4	Seed tubers	Pbcb	Pectobacterium spp
N_4SC	**JJ56**	Limpopo	4	Stems	Pbcb	Pectobacterium spp
N_5TC	**JJ57**	Limpopo	5	Harvested tubers	Pbc	Pectobacterium spp
N_5SC	**JJ58**	Limpopo	5	Stems	Pbcb	Pectobacterium spp
S_6TB	**JJ59**	Sandveld	6	Seed tubers	Pbcb	-
S_7TC	**JJ60**	Sandveld	7	Seed tubers	Pbcb	Pectobacterium spp
C_2TA	**JJ61**	Ceres	2	Seed tubers	Pbc	Pectobacterium spp
C_4TD	**JJ62**	Ceres	4	Seed tubers	Pbc	Pectobacterium spp
C_4TA	**JJ63**	Ceres	4	Seed tubers	Pbc	Pectobacterium spp
WFS_4TA	**JJ64**	Western Freestate	4	Seed tubers	Pbcb	Pectobacterium spp
WFS_4TC	**JJ65**	Western Freestate	4	Seed tubers	Pbcb	Pectobacterium spp
WFS_4TE	**JJ66**	Western Freestate	4	Seed tubers	Pbcb	Pectobacterium spp
WFS_5TB	**JJ67**	Western Freestate	5	Seed tubers	Pbcb ; Pbc	-
MP_7SA	**JJ68**	Mpumalanga	7	Stems	Pbc	Pectobacterium spp

MP_7TA	**JJ69**	Mpumalanga	7	Tubers	Pbcb	Pectobacterium spp
MP_7SD	**JJ70**	Mpumalanga	7	Stems	Pbcb	Pectobacterium spp
N_5SC	**JJ71**	Limpopo	5	Stems	Pbcb	Pectobacterium spp
N_3TC	**JJ72**	Limpopo	3	Harvested tubers	Pbcb	Pectobacterium spp
N_6TB	**JJ73**	Limpopo	6	Harvested tubers	Pbcb	Pectobacterium spp
S_6TD	**JJ74**	Sandveld	6	Seed tubers	Pbcb	Pectobacterium spp
S_7TB	**JJ75**	Sandveld	7	Seed tubers	Pbcb	Pectobacterium spp
S_7TC	**JJ76**	Sandveld	7	Seed tubers	Pbcb	Pectobacterium spp
WFS_5TD	**JJ77**	Western Freesate	5	Seed tubers	Pbcb	Pectobacterium spp
MP_8SA	**JJ78**	Mpumalanga	8	Stems	Pbcb	Pectobacterium spp
C	**JJ79**	Mpumalanga		CBS	Pbcb	Pectobacterium spp
D	**JJ80**	Sandveld		CBS	Pbcb	Pectobacterium spp
E	**JJ81**	Mpumalanga		CBS	-	Pectobacterium spp
EC_1TA	**JJ82**	Eastern Cape	1	Seed tubers	Pbcb	Pectobacterium spp
EC_1TB	**JJ83**	Eastern Cape	1	Seed tubers	Pbcb	Pectobacterium spp
EC_1TC	**JJ84**	Eastern Cape	1	Seed tubers	Pbcb	Pectobacterium spp
EC_1TF	**JJ85**	Eastern Cape	1	Seed tubers	Pbcb	Pectobacterium spp
CH_1A	**JJ86**	CH1A	1	Techni-tubers	Pbcb	-
CH_1B	**JJ87**	CH1B	1	Techni-tubers	Pbcb	-
CH_3A	**JJ88**	CH3A	3	Techni-tubers	Pbcb	Pectobacterium spp
CH_3B	**JJ89**	CH3B	3	Techni-tubers	Pbcb	Pectobacterium spp
CH_3Bo	**JJ90**	CH3Bo	3	Techni-tubers	Pbcb	-
CH_5A	**JJ91**	CH5A	5	Techni-tubers	Pbcb	Pectobacterium spp
CH_5B	**JJ92**	CH5B	5	Techni-tubers	Pbcb	Pectobacterium spp
IN_3B	**JJ94**	IN3B	3	Techni-tubers	Pbcb	Pectobacterium spp
IN_3C	**JJ95**	IN3C	3	Techni-tubers	Pbc	Pectobacterium spp
IN_3D	**JJ96**	IN3D	3	Techni-tubers	Pbcb	Pectobacterium spp
IN_3E	**JJ97**	IN3E	3	Techni-tubers	Pbcb	Pectobacterium spp
CH_3C	**JJ98**	CH3C	3	Techni-tubers	Pbcb	Pectobacterium spp
CH_1C	**JJ99**	CH1C	1	Techni-tubers	Pbcb	Pectobacterium spp
CH_1Co	**JJ103**	CH1Co	1	Techni-tubers	Pbcb	Pectobacterium spp
NW_1TA	**JJ105**	North west	1	Harvested tubers	Pbcb	Pectobacterium spp
NW_1TB	**JJ106**	North West	1	Harvested tubers	Pbcb	Pectobacterium spp

NW_2TB	**JJ107**	North West	2	Harvested tubers	Pbcb	Pectobacterium spp
NC_4TA	**JJ108**	Northern Cape	4	Harvested tubers	Pbcb	Pectobacterium spp
NC_4TB	**JJ109**	Northern Cape	4	Harvested tubers	Pbcb	Pectobacterium spp
MP_8TA	**JJ110**	Mpumalnga	8	Harvested tubers	Pbcb	Pectobacterium spp
MP_8TAo	**JJ111**	Mpumalanga	8	Harvested tubers	Pbcb	Pectobacterium spp
MP_8TB	**JJ112**	Mpumalanga	8	Harvested tubers	Pbcb	Pectobacterium spp
MP_8TBo	**JJ113**	Mpumalanga	8	Harvested tubers	Pbcb	Pectobacterium spp
S_9TA	**JJ114**	Sandveld	9	Seed tubers	Pbcb	Dickeya spp ?
S_9TAo	**JJ115**	Sandveld	9	Seed tubers	Pbcb	-
NC_2	**JJ116**	Northern Cape	2	Stems	Dd	Pectobacterium spp
EFS_6TA	**JJ119**	Eastern Freestate	6	Harvested tubers	Pbcb ; Pbc	Pectobacterium spp
EFS_6TB	**JJ120**	Eastern Freestate	6	Harvested tubers	Pbcb	Pectobacterium spp
EFS_6TBo	**JJ121**	Eastern Freestate	6	Harvested tubers	Pbcb	Pectobacterium spp
EFS_6TC	**JJ122**	Eastern Freestate	6	Harvested tubers	Pbcb	Pectobacterium spp
EC_4TAs	**JJ123**	Eastern Cape	4	Harvested tubers	Pbc	Pectobacterium spp
EC_4TBT	**JJ124**	Eastern Cape	4	Harvested tubers	Pbc	Pectobacterium spp
EC_2TEs	**JJ125**	Eastern Cape	2	Stems	Pbcb	Pectobacterium spp
EC_4TBs	**JJ126**	Eastern Cape	4	Stems	-	Pectobacterium spp
EC_2TEs*	**JJ127**	Eastern Cape	2	Stems	Pbcb	Pectobacterium spp
EC_4TAs	**JJ128**	Eastern Cape	4	Stems	Pbcb	Pectobacterium spp
EC_2TDt	**JJ129**	Eastern Cape	2	Harvested tubers	Pbcb	Pectobacterium spp
EC_2TDt*	**JJ130**	Eastern Cape	2	Harvested tubers	Pbcb	Pectobacterium spp
EC_2TDt”	**JJ131**	Eastern Cape	2	Harvested tubers	Pbcb	Pectobacterium spp
EC_2TEs”	**JJ132**	Eastern Cape	2	Stems	Pbcb	-
$EC2_2TEs$^	**JJ133**	Eastern Cape	2	Stems	Pbcb	Pectobacterium spp
$EC2_2TDs$	**JJ134**	Eastern Cape	2	Stems	Pbcb	Pectobacterium spp
EC_2TDs	**JJ135**	Eastern Cape	2	Stems	Pbcb	Pectobacterium spp
EC_5TAs	**JJ136**	Eastern Cape	5	Stems	Pbcb	Pectobacterium spp
EC_4TAs	**JJ137**	Eastern Cape	4	Stems	Pbc	Pectobacterium spp
EC_4TBs	**JJ138**	Eastern Cape	4	Stems	Pbc	Pectobacterium spp
EC_4TBs	**JJ139**	Eastern Cape	4	Stems	Pbc	Pectobacterium spp
EC_4TAt*	**JJ140**	Eastern Cape	4	Progeny tubers	Pbc	Pectobacterium spp
EC_4TBt*	**JJ141**	Eastern Cape	4	Progeny tubers	Pbcb	Pectobacterium spp

N_8SC	**JJ142**	Limpopo	8	Stems	-	Pectobacterium spp
$N_8SD\times$	**JJ143**	Limpopo	8	Stems	Pbcb	Pectobacterium spp
N_8SD	**JJ144**	Limpopo	8	Stems	Pbcb	Pectobacterium spp
N_8SA	**JJ145**	Limpopo	8	Stems	Pbcb	Pectobacterium spp
MP_9SA	**JJ146**	Mpumalanga	9	Stems	Pbcb	Pectobacterium spp
MP_9SA*	**JJ147**	Mpumalanga	9	Stems	Pbcb	Pectobacterium spp
MP_9SAo	**JJ148**	Mpumalanga	9	Stems	Pbcb	Pectobacterium spp
MP_9SB	**JJ149**	Mpumalanga	9	Stems	Pbcb	Pectobacterium spp
MP_9SB*	**JJ150**	Mpumalanga	9	Stems	Pbcb	Pectobacterium spp
MP_9SC	**JJ151**	Mpumalanga	9	Stems	Pbcb	Pectobacterium spp
MP_9SC*	**JJ152**	Mpumalanga	9	Stems	Pbcb	-
$MP_{10}SA$	**JJ153**	Mpumalanga	10	Stems	Pbcb	Pectobacterium spp
$MP_{10}SA$*	**JJ154**	Mpumalanga	10	Stems	Pbcb	Pectobacterium spp
NW_4TB	**JJ155**	North-West	4	Harvested tubers	Pbcb	Pectobacterium spp
$MP_{11}SC$	**JJ156**	Mpumalanga	11	Stems	Pbcb	Pectobacterium spp
NW_4TA	**JJ157**	North-West	4	Harvested tubers	Pbcb	Pectobacterium spp

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APPENDIX B1

REPORT - IDENTIFACTION OF SOUTH AFRICAN ISOLATES AT THE CENTRAL SCIENCE LABORATORY, YORK

Dr. Emma Douglas
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Scottish Crop Research Institute
Invergowrie
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DD2 5DA

Customer email: emma.douglas@scri.ac.uk
Customer ref: JJ54 & JJ68
CSL reference: 20712982-3
No. of pages: 1
Date: 30/07/07

Culture Identification Samples - Interim Report

Your samples were tested using Fatty Acid Profiling*. Thank you for sending us these cultures which we received safely 18/07/07. Both cultures were checked for purity before testing by Fatty Acid Profiling (FAP) commenced. Please see the following results: Fatty Acid profiling against our NCPPB library gave the following matches:

JJ54 (20712982)
0.943 *Pectobacterium carotovorum* subsp *odoriferum*
0.934 *Pectobacterium carotovorum* subsp *carotovorum*
JJ68 (20712983)
0.927 *Pectobacterium carotovorum* subsp *carotovorum*
0.867 *Pectobacterium carotovorum* subsp *odoriferum*

For both **JJ54** & **JJ68** This confirms the cultures as *Pectobacterium carotovorum* but FAP is not sufficiently accurate to differentiate between the two closest matches. We can identify the cultures to sub-species using molecular methods for a further cost of £35 per sample. (Current test costs are £35 per sample ex. VAT.) Please contact me to let me know if you would/would not require the identification to sub-species. I will hold the culture for 14 days after which time an invoice will be raised and the cultures disposed of if I have not heard otherwise. I hope this information is of use to you. If you have any queries regarding this report please contact me on the number below.

Yours sincerely

Brian Carter
Bacteriologist
Tel: 01904 462000
Fax: 01904 462147
E-mail: b.carter@csl.gov.uk

CSL Diagnostics

*Pest and Disease Diagnostic Services have been accredited by the United Kingdom Accreditation Service to BS/EN/ISO/IEC 17025 *General Requirements for the competence of testing and calibration laboratories* for tests detailed in the schedule of accreditation number 1642 (available at www.ukas.org).

UKAS TESTING 1642

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APPENDIX B2

FINAL REPORT - IDENTIFICATION OF SOUTH AFRICAN ISOLATES AT THE SENTRAL SCIENCE LABORATORY, YORK

Diagnostics

Dr. Emma Douglas
PPI
Scottish Crop Research Institute
Invergowrie
Dundee
DD2 5DA

Customer email: emma.douglas@scri.ac.uk
Customer ref: JJ54 & JJ68
CSL reference: 20712982-3
No. of pages: 1
Date: 30/07/07

Culture Identification Samples - Final Report

Your samples were tested using Fatty Acid Profiling*.

Both cultures were checked for purity before testing by Fatty Acid Profiling (FAP) commenced. These results were provided in the Interim report (30/07/07).

As requested we have carried out further identification with the following results from partial 16S sequence (>600 bases):

JJ54 (20712982) appears to be closely related to (but not identical to) *Pectobacterium carotovorum* subsp. *brasiliensis* (strains 213 and 371 from Duarte et al. J. Appl. Microbiol. 96 (3), 535-545 (2004).

JJ68 (20712983) is more closely related to an isolate of *Pectobacterium carotovorum* subsp. *carotovorum* isolated recently in New Zealand by Andy Pitman et al. (unpublished) than the reference strains of *Pectobacterium carotovorum* subsp. *brasiliensis*.

I hope this information is of use to you. If you have any queries regarding this report please contact me on the number below.

Yours sincerely,

Brian Carter

Bacteriologist
Tel: 01904 462000
Fax: 01904 462147
E-mail: b.carter@csl.gov.uk

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APPENDIX C

SOFT ROT/BLACKLEG QUESTIONNAIRE SURVEY IN SOUTH AFRICA

Erwinia POTATO RESEARCH PROJECT QUESTIONNAIRE
Erwinia AARTAPPEL NAVORSINGSPROJEK VRAELYS

Potatoes South Africa & Department of Microbiology and Plant Pathology, University of Pretoria.
Aartappels Suid-Afrika & Departement van Mikrobiologie en Plantpatologie, Universiteit van Pretoria

Producer / Produsent: .....
Tel. No./ Tel. Nr.: .....
Fax. No. / Faks. Nr.: .....
Email address / Epos adres: .....
Region / Streek: .....
Name of farm / Naam van plaas: .....
Postal address / Posadres .....
.....
.....

1. Average maximum temperatures? / Gemiddelde maksimum temperature?

Summer (day) / Somer (dag):		15-20°C	21-25°C	26-30°C	>30°C
Winter (day/ dag):	<10°C	10-15°C	16-20°C	21-25°C	>25°C

2. Average rainfall per year? (mm) / Gemiddelde reënval per jaar? (mm)

<250	250-500	501-750	751-1000	>1000

3. Are the potatoes irrigated? / Word aartappels besproei?

Yes / Ja	No / Nee

How often? / Hoe gereeld?

Weekly / Weekliks	2X per week	Other / Ander

4. Do you use certified seed? Gebruik u gesertifiseerde saad?

Yes / Ja	No / Nee

5. Are you familiar with: (Yes / No) / Is u bekend met: (Ja / Nee)

Soft rot – seed piece deca Sagte vrot – saadstuk verrotting	Blackleg (*Erwinia* wilt) Swartstam (*Erwinia* verwelk)	*Erwinia* soft rot (post harvest) *Erwinia* sagtevrot (na-oes)

6. What measures are implemented on the farm to control *Erwinia*? / Watter maatreëls word op die plaas gebruik om *Erwinia* te beheer?

None / Geen	Crop rotation / Wisselbou Which crop(s)? Watter gewas(se)?	Removal of infected plants (roging Verwydering van geinfekteerde plante (suiwering)	Other / Ander

7. Please evaluate the cultivars that you are familiar with for their susceptibility to *Erwinia* diseases. Evalueer asseblief die kultivars waarmee u vertroud is vir hulle vatbaarheid vir *Erwinia* siektes.

1 = resistant; 2 = moderately susceptible; 3 = susceptible
1 = weerstandbiedend; 2 = matig vatbaar; 3= vatbaar

Cultivar / Kultivar	Soft rot (post planting) / Sagtevrot (na plant)			Blackleg / Swartstam			Soft rot (post harvest) / Sagtevrot (na-oes)		
	1	2	3	1	2	3	1	2	3

8. What are the estimated yield losses (in R or %) of potatoes due to *Erwinia* diseases? / Wat is die beraamde oes verliese (in R of %) van aartappels a.g.v. *Erwinia* siektes?

- Soft rot (seedpiece decay, post planting) / Sagtevrot (saadstuk verrotting, na plant)
  ……
- Blackleg (*Erwinia* wilt) / Swartstam (*Erwinia* verwelk)
  ……
- Soft rot (post harvest) / Sagtevrot (na-oes)
  ……

9. What aspects do you think are important for future *Erwinia* research? / Watter aspekte beskou u as belangrik vir toekomstige *Erwinia* navorsing?

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10. Other comments or observations regarding *Erwinia*. / Ander komentaar of opmerkings oor *Erwinia*.

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