Identification and control of potato soft rot and blackleg pathogens in Zimbabwe

by

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Declaration

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

SIGNATURE: ……

DATE: ……..

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LIST OF ABBREVIATIONS

AFLP	-	Amplified fragment length polymorphism
ATCC	-	American Type Culture Collection
BCC	-	Belgian Coordinated Collection of Microorganisms
Bp	-	base pair
˚C	-	degrees Celsius
Cfu	-	colony forming units
Dd	-	*Dickeya dadantii*
DNA	-	deoxyribonucleic acid
dNTP's	-	deoxynucleotide triphosphate
Fig.	-	figure
gyrB	-	gene encoding DNA gyrase
LMG	-	Laboratory of Microbiology Ghent University
µl	-	microlitre
µm	-	micromolar
Ml		militre
NCPPB	-	National collection of Plant Pathogenic Bacteria
NRF	-	National Research Foundation
Pa		*Pectobacterium atrosepticum*
PAL	-	Phenylalanine ammonia lyase
Pcb		*Pectobacterium carotovurum* subsp. *brasiliensis*
Pcc		*Pectobacterium carotovurum* subsp. *carotovorum*
PCR	-	Polymerase chain reaction
POD	-	Peroxidases
PPO	-	poly phenol oxidase
recA	-	gene encoding recombinase A
rep-PCR	-	repetitive extragenic palindromic – PCR
subsp.	-	subspecies
sp	-	species
UPGMA	-	unweighted pair groups method using arithmetic average
V	-	volts

PREFACE

Potato (*Solanum tuberosum*) is one of the most popular food crops grown as a substitute staple in Zimbabwe. Its production is constrained by pest and diseases which reduce the yield drastically. The blackleg / soft rot disease complex caused by *Pectobacterium* and *Dickeya* species (formerly known as *Erwinia*) has been identified as a potential threat to potato production. The soft rot pathogens can cause systemic and vascular infections in potatoes, which result in the development of various symptoms on the stem and tubers. The symptoms that develop on the plant are not species specific but depend on climatic conditions prevailing at the infection stage. The pathogens affect the crop at all stages of production, which include in the field, storage and in transit.

The primary objectives of this study were to identify the pathogens which cause blackleg and soft rot on potatoes in Zimbabwe; document the grower's knowledge of blackleg and soft rot diseases in Zimbabwe; evaluate cultivars grown in Zimbabwe and South Africa for tolerance to *Pectobacterium* and *Dickeya* species; determine the role of calcium in blackleg and soft rot development; and determine the genetic diversity of *Pectobacterium carotovorum* subsp. *brasiliensis* isolates from South Africa, Zimbabwe and mini-tubers imported from China.

The first chapter introduces the research highlighting the importance of potatoes globally and in Zimbabwe. It discusses production constraints caused by soft rot pathogens, as well as the pathogenesis of *Pectobacterium* and *Dickeya* spp. It also summarises the research objectives and thesis outline. Chapter 2 provides an overview of published literature on morphology, pathogenicity and identification of blackleg and soft rot pathogens, epidemiology, host range, defense mechanisms in plants, host nutrition and disease management strategies.

Taxonomic revisions have led to the reclassification of pectolytic *Erwinia* into several genera. Strains formerly described as *Erwinia carotovora* have been incorporated into the genus *Pectobacterium*, and

strains classified as *Erwinia chrysanthemi* are now assigned to the genus *Dickeya.* Several species and subspecies of *Pectobacterium* and *Dickeya* have isolated from infected potatoes. In Zimbabwe only *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *carotovorum* have until now been identified as the causal agents of blackleg and soft rot diseases, respectively. Although other species and subspecies of *Pectobacterium* and *Dickeya* have been isolated from infected potato plants in Zimbabwe, their potential to cause diseases on potatoes should not be underestimated.

In chapter 3 bacterial isolates collected from infected plants showing typical blackleg / soft rot disease symptoms were identified using biochemical and physiological methods, as well as rep-PCR, Amplified Fragment Length Polymorphism (AFLP) and single gene sequencing using two genes, viz. *gyrB* and *recA.* Amplified Fragment Length Polymorphisms (AFLPs) were used to study the genetic diversity among potato isolates from Zimbabwe, South Africa and mini-tubers imported from China.

Pectobacterium and *Dickeya* species enter the plant through wounds and natural openings such as lenticels. When they invade the plant they colonise the vascular tissue and spaces between the thin walled parenchyma cells. They remain inside these cells as latent infections and disease symptoms develop when host resistance is impaired or when environmental conditions become conducive for disease development. Chapter 4 investigates the role of defense related enzymes, polyphenol oxidase, phenylalanine ammonia lyase, peroxidases, chlorogenic acid and total soluble phenols in host resistance to potato soft rot. The potato varieties were assayed for activity of polyphenol oxidase and phenylalanine ammonia lyase, peroxidases and concentration of chlorogenic acid and total soluble phenols in tuber tissue. The role of these four components in imparting resistance against the soft rot pathogens was also investigated.

Blackleg and soft rot diseases are seed-borne and difficult to control using chemical, physical and cultural methods. Several approaches aimed at controlling blackleg and tuber rot have been studied but the degree of success has been variable. Plant nutrition is an important component of natural

disease resistance. The effect of calcium soil amendments in enhancing resistance to potato soft rot pathogens was investigated in Chapter 5. A management strategy based on calcium-induced defense responses of potato to *Pectobacterium* and *Dickeya* spp. was investigated. Calcium promotes production of phenolics and strengthens the cell wall, making the plants resistant to maceration by pectolytic enzymes.

In Chapter 6 a survey was conducted in nine potato growing regions of Zimbabwe using an informal structured questionnaire. The findings highlighted the distribution and impact of blackleg and soft rot diseases on the Zimbabwean potato industry. A broad spectrum of information on blackleg / soft rot disease complex was gathered and this information can help growers to make informed decisions about control strategies to apply.

Each chapter in this thesis has been treated as an independent entity. Thus redundancy between chapters could not be avoided. It is my hope that the results of these studies on soft rot pathogens in Zimbabwe will contribute to a better understanding of the blackleg / soft rot disease complex that they cause. I also hope that these studies will form the basis of detailed and future investigations in epidemiology and disease control strategies.

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND AND MOTIVATION OF THE STUDY

Potato (*Solanum tuberosum*) is the only vegetable listed among the five principal world food crops (FAOSTAT, 2010) and is also one of the most popular food crops grown in Zimbabwe (Chigumira wa Ngwerume, 2002) as a substitute staple third after maize and rice. Potato is also popular due to its varied uses which include making chips, crisps, vegetable relish/salad, canning and livestock feed (Manzira, 2010). In Zimbabwe potatoes have become a common household food since they can be consumed as they are in most marginalized homes, or as a relish with *sadza* [thick porridge prepared from maize meal]. They are a common crop in most backyard gardens because their peels are not thrown into dustbins, but are thrown into the gardens where they sprout and produce plants which can be harvested and utilized. Potato is also the fourth highest yielding crop plant in the world after wheat, rice and maize (FAOSTAT 2010).

Although potatoes have great potential to produce high yields per unit area (Dean, 1994; Manzira, 2010), they are prone to a wide range of diseases which drastically reduce yield and quality. Of particular importance are pectolytic enterobacteria which cause stem and tuber rot in potato. The soft rot bacteria can cause systemic and vascular infections which result in the development of a range of symptoms on the stem and tubers. The symptoms that develop on the plant are not strain specific but are dependent on the climatic conditions prevailing at the infection stage (De Haan *et al.*, 2008). The pathogens infect the crop in the field, in transit and in storage. Seed piece decay, blackleg, and aerial stem rot occur in the field while soft rot affects the crop in the field, in transit and in storage.

The soft rot bacteria were until recently classified within the genus *Erwinia*, which was divided into several species and subspecies on the basis of molecular, biochemical and host range differences (Lelliot and Dickey 1984; Gallois *et al.*, 1992; Hélias *et al.*, 1998). Strains formally classified as *Erwinia carotovora* have been incorporated into the genus *Pectobacterium* while those classified as

Erwinia chrysanthemi are now assigned to the genus *Dickeya* (Gardan *et al*., 2003; Samson *et al*., 2005). *Pectobacterium carotovorum* subsp. *carotovorum*(Jones) (*Pcc*) and *Pectobacterium atrosepticum*(Van Hall) (*Pa*) are the most common cause of tuber and stem rot of potatoes grown in many regions. Both species have been listed as the major causes of potato blackleg and soft rot diseases in Zimbabwe (Masuka *et al*., 1998). *P. atrosepticum* almost exclusively infects potato, causing blackleg of the stem and tuber soft rot (Pérombelon 2002). By contrast, *P. carotovorum* subsp. *carotovorum* has a broad host range, causing soft rot disease in various crops including potato, carrot, capsicum and calla lily (Toth *et al*., 2001). The other pathogens which also affect potato are *Dickeya dianthicola* (Gardan *et al*., 2003; Samson *et al*., 2005) and *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) (Duarte *et al*., 2004, van der Merwe *et al*., 2010).

The main attribute separating *Pectobacterium* and *Dickeya* from other enterobacteria is their ability to produce an array of pectolytic enzymes including pectin lyase (Pnl), pectate lyase (Pel), polygalacturonase (Peh) and pectin methyl esterase (Pme). The enzymes exist as isoforms and are encoded by different genes. Pel is produced in higher amounts than Pme by *Pectobacterium* spp. (Moran and Starr, 1969; Gardner and Kado, 1976; Chatterjee *et al*., 1979; Favey *et al*., 1992). Pectic lyase (PL) causes a rapid decrease in the viscosity of polygalacturonic acid (Glenn, 1976; Braun and Schmitz, 1980; Favey *et al*., 1992) but not pectin. Pectin methyl esterase is correlated with the pathogenicity of the pathogen. Pectolytic enzymes enable the bacterial species to macerate parenchymatous tissue of many plant species resulting in tissue collapse and decay.

Pectobacterium and *Dickeya* spp. are considered broad host range pathogens for two reasons. Firstly they have been isolated from many different plant species and secondly, single strains can be pathogenic to numerous plant species under experimental conditions (Ma *et al*., 2007). Exceptions to the broad host range nature of *Pectobacterium* spp. are the strains *Pectobacterium atrosepticum* (*Pa)* (Gardan *et al*., 2003), which was reported almost exclusively on potato (*Solanum tuberosum* L.), and

P. betavasculorum (Gardan *et al*., 2003) which has always been associated with infections in sugar beet.

During the 2007/8 growing season a major outbreak of soft rot disease occurred in potato growing areas around Chinhoyi, Darwendale, Gweru, Harare, Marondera, Mazowe, Nyanga, Shamva and Shurugwi. The main reason for embarking on this study was to identify and control the pathogens affecting potatoes in Zimbabwe so as to reduce the economic losses caused by these pathogens.

1.2 FUNDAMENTAL OBJECTIVE

The aims of this study are to: identify the pathogens which cause blackleg and soft rot on potatoes in Zimbabwe; document the grower's knowledge of blackleg and soft rot diseases in Zimbabwe; evaluate cultivars grown in Zimbabwe and South Africa for tolerance to *Pectobacterium* and *Dickeya* species; determine the role of calcium on blackleg and soft rot development; determine the genetic diversity of *Pectobacterium carotovorum* subsp. *brasiliensis* isolates from South Africa and Zimbabwe.

1.3 SPECIFIC OBJECTIVES

- To isolate and identify the bacteria associated with outbreaks of soft rot and blackleg on potato in Zimbabwe.
- To assess the prevalence and distribution of *Pectobacterium* and *Dickeya* spp. in Zimbabwe.
- To evaluate potato varieties grown in South Africa and Zimbabwe for resistance to *Pectobacterium* and *Dickeya* spp. and determine the role of Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL), Peroxidases (PO), chlorogenic acid and total soluble phenols in disease tolerance.
- To investigate possible control strategies based on calcium amended soil which can enhance plant's resistance to blackleg and tuber soft rot diseases.

- To determine the genetic diversity among South African and Zimbabwean *Pectobacterium* carotovorum subsp. *brasiliensis* isolates.

1.4 CHAPTER OUTLINE

Chapter 2 The literature review focuses on the morphology, pathogenicity and diagnosis of the blackleg and soft rot causal agents, epidemiology, host range, defense mechanisms in plants, host nutrition and control strategies for these diseases.

Chapter 3 Pectolytic pathogens associated with potato soft rot in Zimbabwe. Isolates of *Pectobacterium* and *Dickeya* spp. were collected from selected potato fields in the different potato production regions in the country. These isolates were identified using Rep-PCR, Amplified Fragment Length Polymorphism (AFLP) and *gyrB* and *recA* sequencing to differentiate strains of *Pectobacterium* and *Dickeya* spp.

Chapter 4 Role of polyphenol oxidase, phenylalanine ammonia lyase, peroxidases, chlorogenic acid and total soluble phenols in host resistance to potato soft rot. The potato varieties were assayed for activity of polyphenol oxidase and phenylalanine ammonia lyase, peroxidases and concentration of chlorogenic acid and total soluble phenols in tuber tissue. The role of these four components in imparting resistance against the soft rot pathogens was also investigated.

Chapter 5 Effect of calcium soil amendment in increasing resistance of potato to soft rot pathogens. A management strategy based on calcium-induced defense responses of potato to infection by *Pectobacterium* and *Dickeya* spp. Calcium promotes production of phenolics and strengthens the cell wall thereby reducing blackleg and soft rot disease incidence and severity.

Chapter 6 A survey was conducted in nine potato growing regions of Zimbabwe. The findings highlighted the distribution and impact of blackleg and soft rot diseases on the Zimbabwean potato industry.

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nov., Dickeya dianthicola sp. nov., Dickeya diefferenbachiae sp. nov. and Dickeya zeae sp. nov. *Int. J. Syst. Evol. Microbiol.* ***55:*** *1415 - 1427*.

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CHAPTER 2

A REVIEW OF BLACKLEG AND SOFT ROT DISEASES IN POTATOES

Abstract

Potato production globally is constrained by factors which cause substantial economic losses. These can be in the form of biotic and abiotic factors. The greatest losses are due to diseases and some of the important bacterial diseases are tuber soft rot, blackleg and aerial stem rot in the field. Tuber soft rot also causes high economic losses in transit and in storage. These diseases are caused by *Pectobacterium* and *Dickeya* spp. These pectolytic bacteria have various overwintering sites such as plant debris, water and pupae of several insect species. The pathogens enter plant tissues through wounds, lenticels and stolon ends where they remain latent, or cause symptoms if conditions are conducive for disease development. In latent infections, the bacteria remain dormant until climatic conditions are favourable for symptom development. The pathogens secrete different isoforms of extracellular enzymes which can break down living and dead plant tissues. Conditions which favour disease development are optimal temperature, low oxygen concentration, high humidity and availability of free water. Symptoms are not strain specific but are dependent on climatic conditions. Chemicals are not effective in controlling soft rot pathogens; control strategies rely on the use of resistant cultivars, good agronomic practices such as planting certified disease-free seed, planting in well-drained soil and good sanitation.

2.1 INTRODUCTION

Plant pathogens which macerate and decay plant tissues, sometimes referred to as pectolytic erwinias (formerly called *Erwinia*) were named after plant pathologist, Erwin F. Smith (De Boer, 2003). *Erwina carotovora* and *Erwinia chrysanthemi* were important plant pathogens found in this genus, but have now been placed into two new genera, viz. *Pectobacterium* and *Dickeya* spp. respectively (Gardan *et al*., 2003; Samson *et al*., 2005). Pectolytic bacteria are ubiquitous in environments that support plant growth, can also be found in association with asymptomatic plants and have been viewed as opportunistic pathogens (De Boer, 2003).

Pectobacterium carotovorum subsp. *carotovorum* and *Pectobacterium atrosepticum* are the primary causes of soft rot in temperate climates. *Pectobacterium carotovorum* subsp. *carotovorum* causes soft rot disease in many plant species. This may be attributed to its survival in many environments (Avrova *et al*., 2002), whereas *Pectobacterium atrosepticum* is largely restricted to potato, usually associated with the blackleg disease (Pèrombelon, 2002). Another more virulent strain, *Pectobacterium carotovorum* subsp. br*asiliensis,* has been identified as the major cause of blackleg and soft rot diseases in Brazil (Duarte *et al*., 2004) and in South Africa (van der Merwe *et al*., 2010). *Erwinia chrysanthemi* has been reported as the causal agent of aerial stem rot and wilt disease on potatoes. Taxonomic alteration has separated *E. chrysanthemi* into six species which all fall in the genus *Dickeya* and they are *Dickeya dadantii*, *Dickeya zea*, *Dickeya dianthicola*, *Dickeya paradisiaca*, *Dickeya dieffenbachiae* and *Dickeya chrysanthemi.* The strains which infect potato have been classified into several of these six species (Samson *et al*., 2005).

In Zimbabwe *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subspecies *carotovorum* have been listed as the major pathogens which cause blackleg and tuber soft rot diseases respectively (Masuka *et al*., 1998) and recently *D. dadantii* has been isolated from potato tubers with typical soft rot symptoms (Ngadze *et al*., 2010). Soft rot causes huge economic losses estimated to be between 40 to 80% depending on climatic conditions (Chigumira wa Ngwerume, 2002; Manzira 2010). Although some of the enterobacterial soft rot pathogens have not been identified in Zimbabwe on potato, their importance as disease agents in the crop should not be underestimated, as typical soft rot symptoms have been observed in experiments involving artificial inoculation of potato tubers (unpublished data). Misdiagnosis of enterobacterial strains in Zimbabwe may also be due to limitations in diagnostic techniques to differentiate bacterial species. Detection and differentiation of strains are important aspects in disease management strategies. Several identification techniques have been developed and they rely on the molecular and biochemical properties of microbes for the detection of the pathogens (De Boer and McNaughton, 1987; De Boer and Ward, 1995; Kang *et al*., 2003).The techniques have proved to be consistent, accurate and fast in the identification of many

microorganisms. Some of the methods used are PCR restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP), which have been used in genetic diversity studies (Toth *et al.*, 2001; Avrova *et al.*, 2002). This study focuses on understanding the epidemiology of the blackleg and soft rot diseases as a way of finding solutions which can reduce the economic losses caused by the pathogens.

2.2 THE PATHOGENS

2.2.1 Morphology of pectolytic bacteria

Soft rot bacteria are peritrichously flagellated rods, pathogenic to plants, gram-negative, facultative anaerobic, between 0.5 – 1.0 by 1.0 -3.0 µm and do not produce spores (Agrios, 1997; Charkowski *et al.*, 2006). The bacteria reduce nitrate to nitrite and ferment many carbohydrates to acids (Graham, 1964). Most strains produce only small amounts of extracellular polysaccharides when cultured on media containing high sugar content. Fimbriae or pilli are present on cells of many strains of *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Dickeya* spp., but are absent on cells of *Pectobacterium atrosepticum (Pa)* (Pèrombelon and Kelman, 1980). Many strains of *Pcc* and *Dickeya* spp. are bacteriocinogenic (Itoh *et al.*, 1978) and strains of *Pa* do not produce bacteriocins. All species are catalase positive and oxidase negative. Soft rot bacteria can be selected from other plant pathogenic enterobacteria by their pectolytic activity and colony characteristics on Crystal Violet Pectate (CVP) medium (Kelman and Dickey, 1988; Charkowski *et al.*, 2006, Hélias *et al.*, 2011).

2.2.2 Occurrence and host range of soft rot enterobacteria

Soft rot *Pectobacteria* are pathogens with a worldwide distribution, but with divergent host ranges and host specificities. These characteristics are shown by their serological reactions and temperature requirements (Pérombelon and Kelman, 1980). *Pa* is a pathogen that mainly affects potato grown in cool climatic conditions, but strains similar to *Pa* have been identified on other crops (Dickey, 1979). Most *Pa* strains irrespective of origin form a serologically homogenous group (De Boer *et al.*, 1979)

and do not grow at temperatures above 30˚C. *Pcc* and *Dd* on the other hand, prefer higher temperatures and have wide host ranges. *Pcc* strains cause soft rots in many crops in the temperate and tropical regions and cause diseases in many plant species than *Dd* (Graham, 1964).

Pectobacterium and *Dickeya* species have been reported as pathogens in one half of the angiosperm plant orders and these host ranges overlap but are not identical (Ma *et al*., 2007). *Pectobacterium* spp. were reported as pathogens of 16 dicot families in 6 orders. *Dickeya* spp. were reported to cause disease in 11 dicot families in 10 plant orders and in 10 monocot families in 5 orders. In many cases, only one report of one species per family and order could be found. Although *Pectobacterium* and *Dickeya* spp. cause disease in many identical host species, their reported host ranges do not completely overlap. Of all these reported plant family hosts of *Pectobacterium* and *Dickeya* spp., only 6 dicot and 4 monocot families have been reported as hosts for both genera (Ma *et al*., 2007). This lack of host overlap extends to higher levels of classification, for example, *Pectobacterium* has been reported on avocado (*Persea americana* Miller) (Volcani, 1957) which is in the magnolis clade, but there are no reports on *Dickeya* spp. causing disease on any species in the same clade. Ma *et al*. (2007) found no reports of soft rot enterobacteria causing disease under natural conditions in non-angiosperms, the basal angiosperm orders, or the eudicots clade in their review.

Like many plant pathogens, soft rot enterobacteria have been reported on healthy plants from numerous species including weeds (Meneley and Stangellini, 1975; McCarter-Zorner *et al*., 1985). In 1942, a *Pectobacterium* sp was reported on Saguaro cacti (*Carnegiea gigantean*) (Lightle *et al*., 1942), but the type culture was lost and the name has been rejected (Alcorn and Orum, 1988). Cactus pathogens later isolated by Alcorn *et al*. (1991) and a recent sequencing of the 16s rRNA gene suggested that the cactus pathogen was most likely a *Pectobacterium* species.

Bremer *et al*. (2003) noted some striking apparent specialization in the hosts of *Pectobacterium* and *Dickeya* spp. For example, they observed that only *Pectobacterium* and not *Dickeya* was isolated from all the 3 hosts of plant orders in the eurosids II clade, including cabbage (Brassicales), cotton

(*Gossypium hirsutum*, Malvales) and mango (*Mangifera indica* L., sapindales). The same researchers also noted that *Dickeya* spp. have been reported on several agriculturally important *Poales* spp. ranging from rice to maize.

2.2.3 Disease symptoms in potatoes

Soft rot pathogens cause maceration and decaying of parenchymatous tissue in the infected tissues (Pérombelon and Kelman, 1980). Decaying of the seed tuber before emergence or infection of emerging sprouts, results in non emergence, poor stand, stunting and missing hill. All these symptoms are common manifestations of the disease (Pérombelon and Kelman, 1980; Shroeder, 2003).

Blackleg

Blackleg symptoms range from seed piece decay to lesions which extend up the stem from the soil line (Shroeder, 2003). Infection originates from a completely rotted mother tuber and it progresses, externally or internally via the vascular system into the stem, causing blackleg disease (Allefs *et al*., 1996; Pèrombelon, 2002; Elphinstone and Toth, 2007). A decaying seed piece is not the only cause of blackleg and in some cases this type of infection does not always result in development of blackleg (Pérombelon and Kelman, 1980). A light brown or inky-black lesion develops on infected stems (Fig 2.1) and it can spread upwards as the pathogen migrates into the vascular tissue. Pectolytic enzymes macerate the parenchymatous tissue resulting in necrosis and formation of an internal cavity (Elphinstone and Toth, 2007). A cross section of the stem shows discolouration of the vascular system in infected plant or tuber. It is an economically important disease that can cause huge yield reductions in potato production.

Fig 2.1 Inky black lesion caused by blackleg infection on a potato stem (infection from mother tuber). Photo taken by Jacquie van der Waals.

Blackleg infection in many crops including potatoes causes wilting and chlorosis in the foliage especially in the early stages of attack even when water is available. Initially it was believed that only *Pa* caused typical blackleg symptoms, but Meneley and Stanghellini (1975) reported that all *Pectobacterium* and *Dickeya* species can cause blackleg and that it was difficult to distinguish symptoms caused by different strains under field conditions optimal for pathogenesis.

Tuber soft rot

The disease can occur in the field, in storage or transit and it normally begins at the point of entry which can be either a lenticel or a wound. The symptoms range from a slight vascular discolouration at the stolon end to complete decay. Under field conditions, the mother tubers are the main sources of contamination, causing seed piece and sprout decay in the soil pre- or post-emergence, which results in poor crop stands (Elphinstone and Toth, 2007). In potatoes, the pathogens usually enter the tuber

through the lenticels. Rotting starts wherever the pathogen has penetrated the tuber tissue. The other entry points can be the stolon end of the tuber, wounds or bruises (Pérombelon and Kelman, 1980).

Fig 2.2 Tuber soft rot on potatoes with characteristic brown / black margin separating diseased tissue from healthy tissue. Photo taken by Jacquie van der Waals.

Tuber soft rot is characterised by symptoms which range from light vascular discolouration to complete seed piece decay. The infected tuber tissue is often cream coloured and soft to the touch (Shroeder, 2003).

Aerial stem rot (Aerial blackleg)

Aerial stem rot is characterized by water soaking of the aerial portions of the plant and is a secondary soft rot of the stem or petiole which does not originate from seed piece infection (Roberts *et al.*, 2007). The water soaked tissue is very soft to the touch and can range from light green, yellow to light brown in colour. The infections are initiated in wounds caused by mechanical damage, insect feeding or

natural ruptures such as leaf scars. Disease development is intensified by dense foliage, high humidity and wet conditions (Pèrombelon, 1992).

Fig 2.3 Aerial stem rot on potato stem. Photo taken by Jacquie van der Waals.

2.2.4 Epidemiology

Soft rot bacteria do not overwinter in soil, survival is restricted to periods ranging from 1 week to 6 months, depending on environmental conditions such as soil temperature, moisture and pH. The pathogens can survive for longer periods on plant debris or volunteers. The bacteria cannot survive in soil in a crop rotation system of 3 – 8 years (Pērombelon and Hyman, 1988).

The major source of blackleg infection is latently infected seed mother tubers (Pērombelon, 1992). When the mother tubers rot, the bacteria are released into the soil and can be transmitted by soil water to contaminate neighbouring progeny tubers. The bacteria in the soil can also colonize potato roots and subsequently move via the vascular system into progeny tubers. In the stem the bacteria can survive in the latent form if conditions are not conducive for pathogen multiplication (Czajkowski *et al*., 2010).

Crop contamination can also arise from airborne sources (Pērombelon, 1992). Insect vectors can transmit the bacteria over long distances and can contaminate other potato crops. Aerosols can be generated by rain impact on blackleg infected plants and the bacteria can be blown away for several hundred metres before deposition on plant surfaces (Czajkowski *et al.*, 2011). Contamination of crops can also occur during harvesting, handling (grading) and in store. Tissue from rotting tubers can contaminate machinery and enter into the wounds inflicted during handling (Pērombelon and van der Wolf, 2002)

River and irrigation water can also be infected by the soft rot pathogens. Surface water in USA and Scotland was found to be contaminated with *Pcc* and *Pa* (McCarter-Zorner *et al.*, 1985). Surface water used for irrigation can be a source of the pathogens.

2.3 THE DISEASE PROCESS

The soft rot pathogens can survive in living plants as latent infections, epiphytes on plant surfaces of either hosts or non hosts and saprophytes in the plant debris and in water (Pérombelon and Kelman, 1980; Pérombelon and Salmond, 1995). These pathogens enter the plant through wounds and natural openings such as the lenticels. When they invade the plant they colonise the vascular tissue and spaces between thin walled cells. They remain inside these cells until environmental conditions become conducive for disease development. Some of the requirements for disease development are free water, adequate oxygen supply and appropriate temperature (Pérombelon and Kelman, 1980; Pérombelon and Salmond, 1995).

Free water allows movement of bacterial cells from cell to cell within the host plant. Free water also reduces the amount of oxygen within the host cells thereby creating anaerobic conditions required by the bacteria for multiplication. The anaerobic condition within the plant affects oxygen-dependent defense mechanisms inside the host (Bolwell and Wojtaszek, 1997). Free water can also increase the

turgidity of plant cells while a shortage of oxygen on the other hand affects cell membrane integrity. These two factors can lead to leakage of solutes from the cells and this increases susceptibility of the host to rotting (Pérombelon and Lowe, 1975).

Temperature also plays a crucial role in the disease cycle and it determines the pathogen that survives at the prevailing conditions. Pèrombelon *et al*. (1987a) showed that *Pa* was not pathogenic at temperatures above 20°C while *D. dianthicola* did not cause disease at soil temperatures below 20°C. The ability of the soft rot pathogens to grow at diverse temperatures can be shown clearly in vitro, and this parameter can be used in the identification of pathogens. All three pathogens grow at 27°C, *Pcc* grows at 33.5°C only and *D. dianthicola* can grow at 37°C (Pérombelon *et al*., 1987b). Production of pectolytic enzymes (exoenzymes) was shown by Lanham *et al*. (1991) and Nguyen *et al*. (2002) to be under tight thermal regulation. These enzymes are produced by the pathogen when it is growing at the optimal temperature conducive for enzyme production.

2.3.1 Pectolytic enzymes

The major attribute of *Pectobacterium* and *Dickeya* species is the coordinated production of high concentrations of extracellular plant cell wall degrading enzymes (PCWDE) which include pectinases (pectate lyase, pectin lyase, polygalacturonases, pectin methyl estarases), cellulases, proteases and β- gucosidases. The enzymes break down pectin, a major component of plant cell walls and release nutrients essential for the growth of microbes (Pérombelon, 2002). They also participate collectively in virulence expression (Bell *et al*., 2002; Whitehead *et al*., 2002).

Pectinases are the major enzymes required for pathogenesis. They break down and utilize pectins which are components of the middle lamella and plant cell walls. The effect of these enzymes results in tissue disintegration, cell damage and seepage of cell contents (Barras *et al*., 1994; Pérombelon, 2002). Most of the pectinases - pectate lyase (Pel), pectin lyase (Pnl), pectin methyl esterase (Pme)

and polygalacturonase (Peh) are produced in different types or forms (isoenzymes). Production of the isoforms of these enzymes is controlled by separate genes that are clustered on the genome (Barras *et al.*, 1987; McMillan *et al.*, 1994). Pectate lyases (Pels) are the major enzymes involved in the expression of disease symptoms and the number of pectinases produced by the pathogens differs from one species to the other. *Dickeya dadantii* produces five major types of Pels which fall in two families (Pel A,D,E and Pel B,C) and four secondary Pels (Pel I, L, Z and X), while *Pcc* produces four key Pels (Pel A, B, C and D) and other minor Pels. *Pa* only produces three main Pels (Pel A, B and C) (Barras *et al.*, 1987; Kelemu and Collmer, 1993; McMillan *et al.*, 1994). The secondary Pels are only produced in planta, have low enzymatic activity and are important in host specificity (Barras *et al.*, 1987). Although pectinases play a crucial role in disease symptom expression, all isoenzymes are not required in all situations.

Cellulases are not required in disease development and do not assist the other exoenzymes when the pathogens invade the plant (Boyer *et al.*, 1984; 1987; Boccara *et al.*, 1994). The major role of this enzyme is in the breakdown of cellulose to compounds which can be utilised by the pathogen for growth. Cellulose breakdown occurs after the pathogen has already invaded the host. Several proteases have been identified in *Dd* and at least one in *Pcc* and they breakdown proteins into amino acids required for microbial growth. They also break down host proteins related to resistance (Heilbronn and Lyon, 1990). Proteases play a small role in disease development (Marits *et al.*, 1999).

2.3.2 Regulation and secretion of exoenzymes

The ability of soft rot pathogens to produce large quantities of pectolytic enzymes and release them at crucial stages of disease development makes them formidable pathogens. This is made possible by complex regulatory and secretory networks found in the pathogen (Toth *et al.*, 2003). The regulatory networks are controlled by positive or negative feedback mechanisms which can work on either one (targeted regulation) or several (global regulation) systems. The regulatory and secretion systems are

activated by several conditions which can be availability of oxygen and nitrogen, appropriate temperature, osmolarity, lack of iron, growth stage of bacteria, catabolite repression, plant breakdown intermediates and plant exudates (Cui *et al.*, 2001; Nguyen *et al.*, 2002).

Breakdown products formed when pectinases degrade pectins act as positive feedback mechanisms and accelerate exoenzyme production in the host (Collmer and Bateman, 1981; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1987). The breakdown intermediates can be in the form of 5-keto-4-deoxyuronate (DKI), 2,5-diketo-3-deoxygluconate (DKII) and 2-keto-3-deoxygluconate (KDG) which interact with *KdgR*, a transcriptional repressor (Chatterjee *et al.*, 1985; Condemine *et al.*, 1986). In healthy plants, *KdgR* is bound in a sealed binding site which controls genes implicated in pectinolysis. The genes code for a number of functions such as the production of pectate lyases, other exoenzymes (cellulases and proteases), Type II and Type III secretion systems (Condemine and Robert-Baudouy, 1987; Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1989; Condemine *et al.*, 1992). After infection, the breakdown intermediates formed activate *KdgR* and cause its detachment from the binding site, leading to production of pathogenicity factors. These factors must be produced at a specific stage in the disease cycle in order to avoid host defences. This process leads to development of the soft rot disease (Hugouvieux-Cotte-Pattat *et al.*, 1996; Thomson *et al.*, 1999).

2.3.3 Global virulence regulation network

Phytopathogens are ubiquitous and have several survival strategies and modes of nutrition to suit the various environments they live in (Mole *et al.*, 2007). Expression of virulence factors is controlled in order to save energy required for functions like disease development in a suitable host, avoidance of host defense systems and dissemination of the pathogen to other hosts. The survival of a pathogen depends on the ability to control the global virulence network. *Pcc* and *Dd* are necrotrophs which cause diseases in a wide range of plants (Barnard and Salmond, 2007). The pathogens can live in many varied environments before they encounter susceptible hosts. The global regulatory networks

focus on controlling the Type III secretion systems, production of toxins and extracellular enzymes (Mole *et al*., 2007). The type III secretion system releases effector proteins into host cells and these effector proteins interfere with host defense mechanisms (Jones and Dangl, 2006).

The infection strategies of pectolytic bacteria require rapid adjustments to environmental signals and this can affect the gene expression and physiology of the pathogen. These infection strategies rely on communication between cells to control critical processes in disease development (Mole *et al*., 2007). Quorum sensing, a density dependent phenomenon is one such communication system used by these pathogens. Quorum sensing regulates virulence expression, the Type III secretion system (T3SS), production of plant cell wall degrading enzymes and also regulates antibiotic production.

Quorum sensing in *Pectobacterium* species employs up to 3 transcription stimulators that respond to N-acylhomoserine lactones (AHL) molecules that are encoded by synthase (Fig 2.4). Each species produces a unique AHL molecule to ensure successful infection (Whitehead *et al*., 2002; Chatterjee *et al*., 2005). Once the AHL signalling molecules build up, they activate different genes (*CarR*, *ExpR1* and *ExpR2*) (Fig 2.4). One of the genes, *CarR* binds 3-oxo-C6-HSL and later binds the *CarA* promoter, which regulates the *car* operon. The *car* operon controls the production of the antibiotic carbapenem (Welch, 2000). *Hor,* a transcription regulator, controls the *car* operon. *ExpR1* and *ExpR2*, another set of genes directly inhibit virulence when AHL threshold levels are not reached, by controlling the functions of *rsmA* (Sjöblom *et al*., 2006). *RsmA* is found in the post transcriptional Rsm system and its function is to disrupt mRNA transcription involved in the production of PCWDEs. The Rsm system controls production of virulence factors such as cellulose, pectate lyase and protease. The components of the Rsm system are *RsmA*, *RmsC* (proteins) and *RsmB,* the regulatory RNA (Cui *et al*., 2005). *RsmB* binds to the protein *RsmA* and this result in the production of RsmA-targetted mRNAs. High concentrations of 3-oxo-C8-HSL attach to *ExpR1* while 3-oxo-C6-HSL binds to *ExpR2*. Attachment of AHL inactivates *ExpR1* and *ExpR2*, and this stops the expression of *rsmA.* The mRNA transcripts that encode for plant cell wall degrading enzymes (PCWDEs) is released (Sjöblom *et al*.,

2006; Cui *et al.*, 2005). Mutant *Pcc* isolates lacking Exp1 do not produce AHL and PCWDEs. This makes the strains avirulent (Pirhonen *et al.*, 1993). Quorum sensing in *Pectobacterium* controls the production of virulence factors which ensures successful disease development through negative regulation by *ExpR1* and *ExpR2* (Mole *et al.*, 2007).

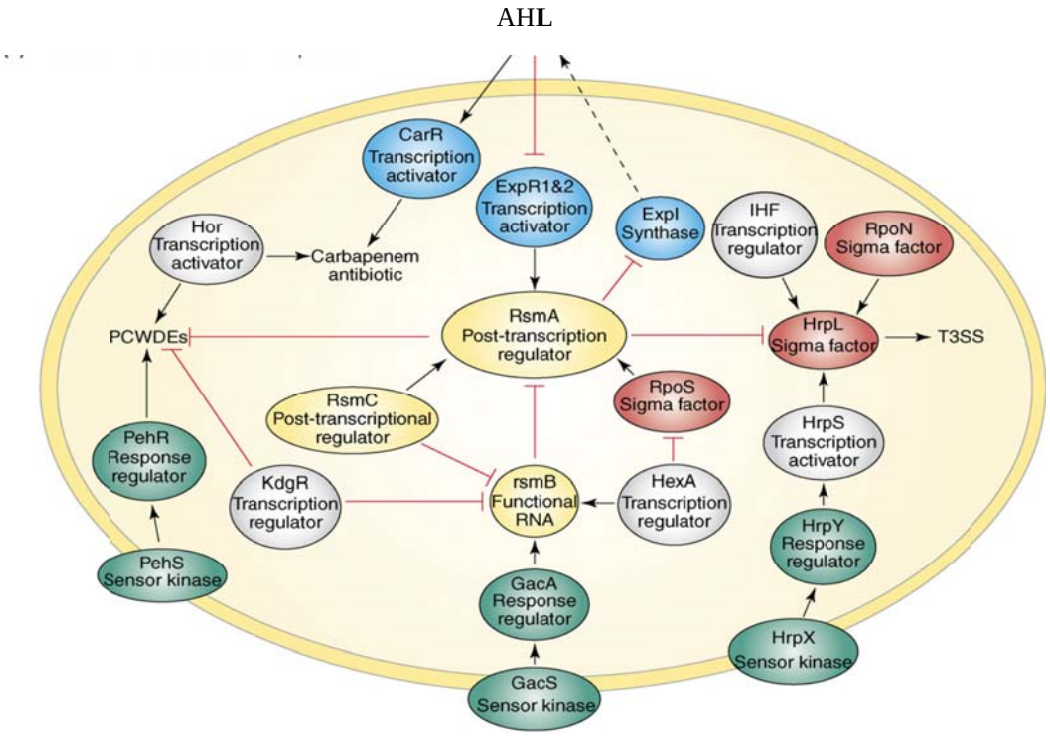

Fig 2.4 The global virulence regulation of *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). **Color code**: blue, associated with cell-to-cell communication; green, TCST systems; red, sigma factors; yellow, part of the Rsm system; grey, other members that do not fit into major systems discussed in detail here. Extracellular enzymes and EPS regulation are found on the left, T3SS regulation is found on the right, and global regulators are situated in the centre. Arrows represent positive regulation, barred arrows represent negative regulation, and broken arrows represent synthesis (Adapted from Mole *et al.*, 2007).

2.4 DIAGNOSTICS AND FINGERPRINTING

Detection and identification methods based on biochemical tests on isolated bacteria, serology and more recently, molecular biology have been developed to detect and characterize *Pectobacterium* and *Dickeya* spp. Not all methods can be used routinely for analyses on potato tubers, or allow specific detection of each *Pectobacterium* species. Biochemical tests (Lelliot and Dickey, 1984) permit the differentiation of all subspecies but can only be applied to purified cultures. The selective medium, crystal violet pectate (CVP) (Cuppels and Kelman, 1974) avoids the purification stage but does not discriminate between *Pcc*, *Pc* subsp. *betavasculorum*, *Pc* subsp. *odorifera* and *Pc* subsp. *wasabiae*. Both methods are time consuming and are not sensitive enough for identification purposes (Hèlias *et al*., 1998), as the pathogens are identified to the species level and the groupings are questionable (Avrova *et al*., 2002); furthermore D. dianthicola grows poorly on CVP medium (Elphinestone and Toth, 2007). Slow growth of several *Dickeya* isolates on CVP or rapid growth of opportunists can mask the pathogen leading to improper diagnosis (Jones *et al*., 1994). Methods based on the use of selective media have been useful in the detection and quantification of soft rot pathogens (Stewart, 1962; Cuppels and Kelman, 1974; Pierce and McCain, 1992). These methods can be tedious and a lot of time is wasted in processing samples. Some of the techniques are serological, for example Enzyme Linked Immuno-Sorbent Assay (ELISA) (Vernon-Shirley and Burns, 1992; Jones *et al*., 1993) and slide agglutination tests (McLead and Pèrombelon, 1992).

Serological techniques do not require isolation of the bacteria, but are usually regarded as being inaccurate because efficiency depends on the specificity of antibodies used. Monoclonal antibodies are more specific than polyclonal antisera (Gorris *et al*., 1994) but have a lower affinity for the antigens (De Boer and MacNaughton, 1987). These techniques are not sensitive enough to detect low, but epidemiologically significant bacterial populations. Serological tests depend on the specificity of monoclonal and polyclonal antibodies; sensitivity of the tests is affected by the high number of serogroups within and between subspecies. Conductimetric assays have been used in the detection

of *Pa* and *Dickeya* spp. in tuber peel extracts (Fraaije *et al*., 1997) but the technique relies on the growth rate of strains on selective media and accuracy depends on detection limits. For example the detection limit for *Pa* is between 10^2 – 10^3 colony forming units (cfu) ml^{-1}, while that of Dickeya is is 10^3 – 10^4 cfu ml^{-1}.

Molecular sequences that act in unrelated ways in bacterial metabolic processes can be selected as identification tools in molecular diagnosis. Some of the genes that have been used for the classification of microorganisms are *rec*A and *gyr*B. The gene *rec*A (recombinase A) is a multifunctional protein involved in the S.O.S. mechanism of DNA repair. *Gyr*B gene encodes for the subunit B protein of DNA gyrase (topoisomerase type II). This gene can be used as a target of highly specific probes useful in identification of microorganisms. DNA gyrase is an enzyme required for the replication of DNA and controls supercoiling of double-stranded DNA. This enzyme is found in all bacterial species.

16S rRNA sequencing can be used in phylogenetic studies but cannot be used for routine identification because the technique has limited sensitivity at subspecies level (Toth *et al*., 2001). Toth *et al*. (1999) developed a one-step 16S rRNA PCR-based detection method for screening potato plants and tubers for soft rot pathogens during seed certification. The method uses universal primers SR3F and SRIcR which can differentiate species and subspecies. DNA-DNA hybridization is an accurate and sensitive method which can be used in DNA-related studies. This method cannot be used in routine identification because it is time consuming and expensive (Toth *et al*., 2001; Kang *et al*., 2003).

Genomes of most microbes contain many types of repetitive DNA sequences which are randomly distributed throughout the genome and their specific location on the gene differ from species to species and among strains. The repetitive elements make up 5% of the genome and are localized on the regions within and outside the microbial genome (Ishii and Sadowsky, 2009). The repetitive

elements can be as short as 15 or as long as hundreds of base pairs (bp) in length depending on the species of the microorganism (Versalovic and Lupski, 1998). Repetitive extragenic palindromic (REP) elements occur in all microorganisms although some repetitive elements can only be found in certain microbial species. (Tobes and Ramos, 2005; Sadowsky and Hur, 1998; Versalovic and Lupski, 1998).

The sequences of these repetitive elements can be used as primers in PCR, since each microbial strain or isolate has repetitive sequences situated in different regions of the genome. Identification can be based on the amplification of these elements and total genomic DNA as the template. The amplified fragments generated are of different sizes and this is directly proportional to the genomic distance between adjacent repetitive elements (Ishii and Sadowsky, 2009). After electrophoresis, the distribution of the amplified fragments form a genomic DNA fingerprint specific for each bacterial species (Rademaker and de Bruijn, 2008). Rep-PCR can be used in the detection, tracking and examination of many bacterial species of medical importance (Rademaker and de Bruijn, 2008; Sadowsky and Hur, 1998; Versalovic and Lupski, 1998). Short polytrinucleotides have been used as priming sites for PCR and they include elements such as (GTG)5, REP sequences 35 – 40 bp, enterobacterial repetitive intergenic consensus (ERIC) sequences 124 – 127 bp or the 154bp BOX elements (Rademaker and de Bruijn, 2008; Versalovic *et al.*, 1991).

The amplified fragments can be separated using electrophoresis to yield a banding pattern which is the rep-PCR genomic fingerprint and studied using a computer assisted pattern analysis (Ishii and Sadowsky, 2009; Versalovic *et al.*, 1994). One of the advantages of the rep-PCR genomic fingerprinting method is that reproducible fingerprints can be generated from purified genomic DNA, crude lysates, whole cells from liquid cultures or colonies on plates (Versalovic *et al.*, 1994; Woods *et al.*, 1993) and from infected plant tissues such as root nodules or plant lesions (Robinson *et al.*, 1995). The rep-PCR genomic fingerprints have been used in the identification of bacteria to the species, subspecies and strain level. This represents the most discriminatory and reproducible

genomic fingerprinting technique for rapid examination of multiple samples (Versalovic and Lupski 1998).

Amplified Fragment Length Polymorphism (AFLP) is a genomic fingerprinting method based on discriminatory PCR amplification of DNA pieces cut from complete digestion of genomic DNA. DNA is cut with two restriction enzymes and this can be applied to DNA from any source or complexity (Vos *et al.*, 1995). Double stranded adapters are joined to the terminal ends of the DNA fragments to produce template DNA for amplification. The primers bind to the adapter and adjacent DNA fragment and the DNA fragment is amplified with specific primers (Vos *et al.*, 1995). AFLP is used in taxonomic classification and phylogenetic relationships to distinguish between species, subspecies and different serogroups.

Prior knowledge of the genome sequence is not required when identifying microorganisms. AFLP can be used for detection and characterization of microorganisms, as it can distinguish to species or subspecies level (Tanksley *et al.*, 1989). The method is divided into three stages: i) cutting of genomic DNA with two restriction enzymes ii) joining of double-stranded adaptors to the cut fragments, preamplification and selective amplification of restriction fragments with two sets of primers complimentary to the ligated adaptors iii) separation of the amplified products using electrophoresis. The AFLP technique can be used in detection, taxonomy and epidemiology of enterobacteria (Avrova *et al.*, 2002).

Universal rice primers (URPs) have been used in fingerprinting genomes of different organisms. The *Pcc* specific primer URP-PCR EXPCCF/EXPCCR which was designed from the URP-PCR product is used in the detection of *Pcc* and *P. c.* subsp. *wasabiae* (*Pcw). Pcb* and *Pcw* can be differentiated on the basis of biochemical reactions, host range and G+C content (51 – 53 mol %). Sensitivity can be increased by using a nested PCR with primers INPCCR/INPCCF (Kang *et al.*, 2003).

Restriction fragment length polymorphism (RFLP) analysis applied to the pectate lyase genes has been used for diagnosis but cannot identify all the soft rot pathogens. Y1 and Y2 primers, which were specifically, designed for *Pcc*, *Pa*, *P. c.* subspecies *odorifera* and *wasabiae*, could not amplify *Dickeya* spp. or *P. betavasculorum* (Frechon *et al.*, 1998). The Y1/Y2 primers successfully amplified *Pcc* but could not amplify *Pa* and some *Pcc* strains. A PCR kit for specific detection of *Pa* was evaluated based on the *Pel* genes. Primer set Y45/Y46 was used in the evaluation and capture probe was used in place of gel electrophoresis in order to avoid detection of amplified non-species DNA (Frechon *et al.*, 1998)

2.5 DEFENSE SYSTEMS IN PLANTS

Plants respond to wounding and pathogen invasion by activating an array of defense systems. The defense responses restrict spread and growth of pathogens inside the host cells. Programmed cell death is one of the defense strategies used by plants to stop the pathogen from advancing to healthy cells (Dangl *et al.*, 1996; Li and Steffens, 2002). The other processes involve activation of defense or defense-related genes (Dixon and Harison 1990), cross linking and strengthening of cell walls, production of phytoalexins and metabolism of phenolic compounds (Nicholson and Hammerschmidt, 1992) and release of highly reactive oxygen species (singlet oxygen, superoxide, hydrogen peroxide) and hydroxyl radicals (Bolwell and Wojtaszek, 1997). Necrotic spots that appear at the site of infection are due to a hypersensitive response which occurs in plants and this result in the death of host cells. Systemic acquired resistance develops in healthy parts of the plant and provides long term resistance against pathogens.

2.5.1 Phenolic compounds

Phenolics are ubiquitous in all plant parts and their concentration in tissues increases after pathogen invasion (Matern *et al.*, 1995). Plant phenols can be divided into two classes: 1) produced during the normal growth and development of the plant and these are preformed inhibitors (phytoanticipins)

associated with non-host resistance; they are also referred to as innate phenols and 2) those that are produced in response to injury, infection or other stress (Nicholson and Hammerschmidt, 1992). The chemical composition of phenols is diverse and may differ considerably from plant to plant and from tissue to tissue within a species. Phenols are produced by specialised cells and stored in the vacuoles during the normal process of differentiation. These specialized phenolic storing cells can be uniformly distributed in all cells of tissues or can be randomly scattered. In some plant species, these specialized cells can be situated at potential entry points (Beckman, 2000).

Phenols are synthesized in thylakoids of plastids and transported to the cell vacuoles for storage (Beckman, 2000). The phenols are stored in the vacuole in an inactive form, but can be rapidly oxidized as soon as they are released. The release of phenols can be activated by biotic and abiotic stresses (Beckman, 2000). Swift build up of toxic phenols restrict the pathogen at the point of entry (Matern and Kneusel, 1988; Fernandez and Heath, 1989). Phenols are stored as glycosides, a form which is not toxic to the plants but can be activated by glycosidase when the need arises. The glycosidase is located in the plant cell near the vacuoles (Esen, 1993).

2.5.2 Polyphenol oxidase (PPO)

PPO is a nuclear encoded enzyme found in all parts of a plant (Mayer and Harel 1979; Mayer, 1987; Thipyapong and Steffens, 1997). PPO catalyzes the oxidation of phenols to quinines which are highly reactive molecules. This process results in the alteration of many cellular nucleophiles through a 1,4 addition mechanism. Melanin-like black or brown compounds are formed by polymerization. PPO derived quinones may make use of an anti-nutritive defense mechanism against pests (Zhang *et al.*, 2008). PPO also has direct antibiotic and cytotoxic effects on pathogens (Mayer and Harel, 1979). The enzyme which is produced when plants are wounded or invaded by pathogens prevents further attack by the pathogens and insects (Thipyapong *et al.*, 1995; Thipyapong and Steffens, 1997; Stout *et al.*, 1999).

2.5.3 Phenylammonia lyase (PAL)

Phenolic compounds come from the phenylpropanoid pathway (PPP) and play an important role in plant defenses against pathogens. Phenylalanine ammonia lyase is the principal enzyme in PPP biosynthesis which serves as a precursor of various secondary metabolites. It leads to the conversion of L-phenylalanine into trans-cinnamic acid with the elimination of ammonia. Production of PAL is controlled by several factors which include mechanical wounding, infection by pathogens and processes which occur during plant growth (Dixon and Paiva, 1995). PAL activity has been associated with increases in both phenols and lignin deposition (Bhattacharyya and Ward, 1988; Cahill and McComb, 1992; Whetten and Sederoff, 1995; Hemm *et al.*, 2004) and production of phytoalexins (Graham, 1995).

2.5.4 Effect of soil calcium amendment in disease resistance

A high calcium content in host plant has been linked with disease resistance in many plants (Bateman and Lumsden, 1965; Bateman and Millar, 1966; Forster and Echandi, 1975; McGuire and Kelman, 1983, 1984). Plants grown in high Ca^{2+} conditions have shown resistance to soft rot. Increased resistance in high-calcium tissues has been attributed to decreased maceration owing to calcium deposition in the cell wall as calcium pectate, leading to structural enhancement of cell wall integrity (McGuire and Kelman, 1984; 1986; Carpita and Gibeaut, 1993). The ability of soft rot pathogens to macerate plant tissue is dependent on the massive production and secretion of plant cell wall-degrading enzymes, especially pectinolytic enzymes like polygalacturonase (Peh), pectin lyase (Pnl) and isoforms of pectate lyase (Pel) (Collmer and Keen, 1986). These enzymes are crucial for the virulence of *Pectobacterium.* Mutations that affect production and secretion of these enzymes lead to reduced virulence (Pirhonen *et al.*, 1991; 1993). Inactivation of a single gene encoding a particular pectic enzyme can reduce virulence drastically (Saarilahti *et al.*, 1990; 1992). Bateman and Lumsden (1965) suggested that excess calcium combines with pectin to form calcium pectate which cannot be macerated by polygalacturonase (PG).

Calcium plays a role in cell metabolism and its effect on disease resistance is related to the cellular responses. The responses involving calcium occur within cell walls and on the surface of the plasmalemma. The processes make use of the exchangeable Ca^{2+} ions. In some cases the responses occur in the cytoplasm and only very low concentrations are used (Kirkby and Pilbeam, 1984). Calcium stabilizes the cell wall and is also involved in controlling acidic growth and ion-exchange processes (Dermarty *et al.*, 1984).

Most processes which occur inside cells are affected by Ca^{2+} concentration in the cells. These processes involve specific proteins which act as intermediates in the transport of calcium ions. Calmodulin, a Ca^{2+} binding protein, activates and regulates a number of key enzymes, regulates Ca^{2+} transport within the cell and mediates transport of compounds to the vacuoles. Ca^{2+} is an important secondary messenger involved in the transport of phytoalexins (Zook *et al.*, 1987). It also affects availability and uptake of other ions.

2.6 CONTROL

Control of blackleg and soft rot pathogens is constrained by lack of appropriate ways and mechanisms when dealing with the pathogens. Information on the survival strategies of these pathogens in various environments is limited. *Dickeya* and *Pectobacterium* spp. have been detected in seed tubers grown in pathogen free environments and on tissue culture plants. Researchers have failed to identify the sources of inoculum in such circumstances (Czajkowski *et al.*, 2009). Pérombelon and Kelman (1980) reported that *Pcc* can be spread through surface and rain water, by aerosols and by insect vectors. Czajkowski *et al.* (2009) feel that information on the ecosystem and epidemiology of *Dickeya* spp. in potato production is largely missing.

Use of resistant cultivars as a way of controlling blackleg and soft rot disease can be a crucial option, although resistant cultivars have not yet been identified. Lapwood *et al.* (1984) failed to identify

cultivars which could resist the maceration effect of *Pectobacterium* and *Dickeya* spp. The use of other methods like physical, chemical and biological control methods merely reduced the levels of the inoculum but could not eliminate the pathogen completely (Pérombelon and Salmond, 1995). Czajkowski *et al.* (2009) feel there is a need to establish methods for detection of latent infections in seed tubers in order to eradicate the spread of the pathogens. Most detection methods currently in use are not specific or sensitive and are too expensive for routine application (Czajkowski *et al.*, 2009). Lack of cheap, sensitive and specific detection methods is a major problem in seed certification programmes. Contaminated seed tubers are the main source of infection and it is also the most important means by which *Dickeya* and *Pectobacterium* spp. are disseminated over short and long distances.

2.7 CONCLUSION

Soft rot bacteria cause extensive economic losses in potato production due to complexity of the pathogens, ubiquity, virulence, variation in symptom expression and their ability to multiply fast. The most important source of inoculum in the field is contamination from the mother tubers, which can spread to progeny tubers. Contaminated water and insects can also be sources of the pathogens in the field.

The diseases caused by pectolytic bacteria are blackleg, aerial stem rot and tuber soft rot. Tuber soft rot is the only disease which can develop in the field, in storage and in transit. *Pectobacterium* and *Dickeya* spp cause similar disease symptoms in the field. The symptoms are not species specific but are dependent on climatic conditions. Blackleg incidence and severity is affected by soil conditions, oxygen levels, temperature, cultivar resistance, host nutrient status and interaction of pathogen with other pathogens. Optimal conditions for tuber soft rot in storage are low oxygen levels, high humidity and high temperatures. The use of certified disease-free seed is the most practical way of reducing *Pectobacterium* and *Dickeya* infection in potato production schemes. Contamination of tubers can take place during the various stages of plant growth, harvesting and grading, which are vital steps in

potato production (Pérombelon and van der Wolf, 2002). Contamination of tubers may be reduced by limiting the number of generations in the field, proper sanitation and hygiene during handling of tubers (Pérombelon, 2002). Methods that can be used for reducing pathogen concentrations in infected tubers are hot water treatment (Robinson and Forster, 1987), dipping in antibiotic solution (streptomycin) (Graham and Volcani, 1961) and copper based fungicides (Aysan *et al.*, 2003). However, none of these methods completely destroy the pathogen.

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CHAPTER 3

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PECTOLYTIC PATHOGENS ASSOCIATED WITH POTATO SOFT ROT IN ZIMBABWE

Abstract

Blackleg / Soft rot disease complex causes economic losses in potato production in Zimbabwe, estimated to be between 20 and 60%, depending on climatic conditions. The aim of the study was to identify the pathogens which cause potato blackleg / soft rot disease complex in this country. Infected samples, comprising of stems and tubers from potato plants with blackleg / soft rot disease complex symptoms (soft rot, wilting, internal and external darkening on stems), were collected throughout the 2008/9 growing season from nine production areas where disease outbreaks occurred. The isolates from these plants and tubers yielded pectolytic bacteria on crystal violet pectate medium, and colonies were characterized after purification on nutrient agar. All isolates were Gram negative rods producing soft rot symptoms on inoculated tubers. Identification was based on biochemical and phenotypic characteristics and sequences of *gyrB* and *recA* genes. *Dickeya dadantii* subsp. *dadantii* (*Dd*) was the dominant pathogen (35%) and was isolated from samples collected from all the regions. *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), *Pectobacterium atrosepticum* (*Pa*), and *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) were also isolated from infected plants and tubers. *P. atrosepticum* was the only pathogen isolated from samples collected in Nyanga. Genetic diversity of *Pcb* isolates from Zimbabwe, South Africa and mini-tubers imported from China was determined using AFLP analysis. The South African isolates and isolates from the Chinese minitubers used in the study were obtained from the Potato Pathology Programme at University of Pretoria, South Africa culture collection. AFLP analysis separated the *Pcb* strains into 12 clusters, reflecting subdivision in terms of geographic origin, and *Pcc* isolates were clearly differentiated from *Pcb* isolates which displayed clearer and more intense banding profiles. Four of the 12 clusters contained isolates from both South Africa and Zimbabwe; four contained isolates only from South Africa; 2 contained isolates from Zimbabwe and China; one cluster was made up of isolates from South Africa and China and one cluster contained isolates only from Zimbabwe. A large degree of DNA polymorphism was evident between these 12 clusters, suggesting that several different *Pcb* populations, derived from a common ancestor, are causing the blackleg symptoms seen in these two

countries. This is the first report of *D. dadantii* subsp. *dadantii* and *P.c. brasiliensis* on potato in Zimbabwe.

3.1 INTRODUCTION

The major source of tuber and stem rot in potatoes in tropical and subtropical climates arise from infection by one or a combination of soft rotting pectolytic enterobacteria. These bacteria were, until recently, classified within the genus *Erwinia*, which was divided into numerous species and subspecies on the basis of molecular, biochemical and host range differences (Lelliot and Dickey, 1984; Gallois *et al*., 1992; Hèlias *et al*., 1998). Subsequent revisions have led to the taxonomic reclassification of pectolytic *Erwinia* into several genera. Strains formally described as *Erwinia carotovora* have been incorporated into the genus *Pectobacterium*, and strains classified as *Erwinia chrysanthemi* are now assigned to the genus *Dickeya* (Hauben *et al*., 1998; Gardan *et al*., 2003; Samson *et al*., 2005).

Pectobacterium atrosepticum almost exclusively infects potato, causing blackleg of the stem and tuber soft rot. Blackleg is an economically important disease, which originates from the rotting mother tuber and is characterised by the blackening of the stem base of potato plants (Pèrombelon, 2002). Blackleg is unpredictable in the field due to the ability of the pathogen to remain latent in the seed tuber (Laurila *et al*., 2008). *Pa* is the causal agent of blackleg disease in many geographical areas (Duarte *et al*., 2004).

By contrast *Pectobacterium carotovorum* subsp. *carotovorum* has a broad host range, causing soft rot disease in various crops including potato, carrot, capsicum and calla lily (Wright 1998; Toth *et al*., 2003). *P. carotovorum* can also be virulent in temperate climates causing blackleg symptoms (De Haan *et al*., 2008). Another atypical, highly virulent enterobacterial subspecies, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*) was described in Brazil (Duarte *et al*., 2004) and South Africa

(van der Merwe *et al*., 2010). The pathogen causes severe soft rotting of tubers and blackleg of the stems.

The genus *Dickeya* is extremely diverse and comprised of species that are pathogenic to many important crops and ornamental plants, mostly in tropical and subtropical climates. Species belonging to this genus cause soft rot and blackleg on potato in temperate regions (Pèrombelon 2002). The species structure of *Dickeya* is not well defined and there is considerable overlap in host range. Isolates infecting potato are now distributed among the six described species of *Dickeya* (Samson *et al*., 2005) and it remains unclear whether these strains produce the same symptoms during disease development, or whether only some are responsible for blackleg-like symptoms. The discovery of distinct species of *Pectobacterium* and *Dickeya* associated with certain hosts supports the idea that identifiable groups of pectolytic enterobacteria are found in specific ecological niches and may be responsible for distinct disease symptoms on potato (De Boer, 2003).

Pectobacterium atrosepticum and *Pectobacterium carotovorum* subsp. c*arotovorum* have been identified as the causal agents of potato blackleg and soft rot diseases in Zimbabwe (Masuka *et al*., 1998; Manzira, 2010). Recently *D. dadantii* subsp. *dadantii* was isolated from plants with typical wilting symptoms and rotting tubers (Ngadze *et al*., 2010). Although the other *Pectobacterium* subspecies have not as yet been isolated from potato in Zimbabwe, their potential to cause diseases on potatoes should not be underestimated.

Misidentification of enterobacterial strains in Zimbabwe may be due to lack of reliable diagnostic techniques to differentiate them. Detection and differentiation of pathogen strains are important aspects of disease management strategies and several molecular and biochemical tools have been developed for the identification of pectolytic enterobacteria (De Boer and McNaughton, 1987; De Boer and Ward, 1995; Kang *et al*., 2003). PCR restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) techniques have also been used in the study of

genetic diversity (Toth *et al*., 2001; Avrova *et al*., 2002). AFLP analysis has demonstrated that a greater diversity of pectolytic enterobacteria infect potato than previously thought (Avrova *et al*., 2002; Pitman *et al*., 2010). Repetitive extragenic palindromic-PCR (rep-PCR), which can differentiate between closely related strains of bacteria, is considered as one of the most effective methods to differentiate between bacterial species (Rademaker *et al*., 2004). In rep-PCR DNA fingerprinting, PCR amplification of the DNA between adjacent repetitive extragenic elements, is used to obtain strain-specific DNA fingerprints which can be easily analysed with pattern recognition computer software (Rademaker *et al.,* 2004).

Pcb was recently identified in South Africa and Zimbabwe as causal agents of the blackleg / soft rot disease complex on potatoes. This was the first report of the pathogen affecting potato in both countries. As Zimbabwe has been importing potato seed from South Africa for almost a decade, it is of great interest to examine the genetic diversity of *Pcb* isolates from these two countries.

The objectives of the study were (i) to isolate and identify the causal agents of potato soft rot and blackleg in Zimbabwe by rep-PCR and sequencing two genes involved in bacterial metabolic processes, *recA* (encoding recombinase A) and *gyrB* (encoding DNA gyrase), and (ii) determine and compare the genetic diversity of *Pcb* isolates from South Africa and minitubers imported from China to those occurring in Zimbabwe using AFLP analysis.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of bacterial strains

The stems and tubers of potato plants with soft rot and blackleg symptoms (wilting, internal and external darkening on stems) were collected throughout the 2008/9 growing season from nine production areas where disease outbreaks occurred in Zimbabwe. Severe outbreaks occurred in Chinhoyi, Darwendale, Gwebi, Harare, Marondera, Mazowe, Nyanga, Shamva, and Shurugwi. Pieces of infected stems and tubers were macerated in 0.01 M magnesium sulphate ($MgSO_2$). Isolations were made on the selective medium, Crystal Violet Pectate (CVP) (Hyman *et al*., 2001) and plates

were incubated at 25ºC for 48 h. Isolates that tested positive for pectolytic cavity formation (formed pits on CVP medium) were purified on the same medium and then transferred to nutrient agar (NA) (Merck, Darmstadt, Germany), on which they were maintained. All isolates were stored in sterile water at room temperature and in 15% (v/v) Nutrient Agar glycerol medium at -80ºC and maintained in the Potato Pathology Programme culture collection at the University of Pretoria, Pretoria, South Africa. A list of isolates and type strains of the various species and subspecies of *Pectobacterium* and *Dickeya* used in this study is shown in Table 1.

3.2.2 Biochemical and physiological identification of isolates

Single colonies of each isolate were used for biochemical tests. The isolates were identified by standard bacterial methods based on Cother and Sivasithamparam (1983). The tests performed were Gram reaction, oxidase activity, glucose metabolism, pectate degradation in Sutton's medium, production of phosphatase, indole production from tryptophan, gelatine hydrolysis, production of reducing substances from sucrose, production of acid from α-methylglucoside and trehalose, malonate utilisation, sensitivity to erythromycin (15 µg), growth at 37ºC which was determined after 24 h in nutrient broth (NB, Difco) and salt tolerance which was checked after 48 h growth in NB with 5 g l^{-1} NaCl.

3.2.3 Rep-PCR analyses

Whole genome rep-PCR fingerprints were generated from all strains (including type strains and reference strains) according to Rademaker *et al*. (2004). Cluster analysis of rep-PCR profiles was performed in BioNumerics 4.0 (Applied Maths) using Pearson's correlation indices of similarity with the unweighted pair-group method using arithmetic averages (UPGMA). The patterns of each of the independently processed strains analyzed on independent gels always shared > 70% similarity. Thus, the cut-off used to define rep types was 70%.

3.2.4 Sequencing and phylogenetic analysis

Genomic DNA was extracted from overnight broth cultures using a DNeasy™ Blood and Tissue Kit (Qiagen, Southern Cross Biotechnology). For selected bacterial strains, the *recA* and *gyrB* genes were amplified using recAF, recAR and gyrB-01F, gyrB-02F respectively (Table 2). PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Corp). PCR products were purified using a QIAquick™ Purification Kit (Qiagen, Southern Cross Biotechnology). The *gyrB* and *recA* genes were sequenced in both directions using primers and conditions previously described (Young and Park, 2007, Brady *et al*., 2008). Sequencing 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 nucleotide sequences were edited, aligned with BioEdit Sequence Alignment v 7.0.0. and both ends of each alignment were trimmed to the following final sizes: *recA,* 697 positions and *gyrB*, 740 positions. Searches were performed on each consensus sequence generated in BioEdit using the BLAST algorithm from GenBank.

All the selected sequences were aligned using MAFFT. Phylogenetic trees for the individual gene sets were inferred by using the neighbour-joining programme of MEGA v 4 (Tamura *et al*., 2007). The stability of the phylogenetic trees were assessed by performing bootstrap analysis of the neighbour-joining data based on 1 000 bootstrap replicates (Kwon *et al*., 1997).

3.2.5 Pectolytic activity

(I) Preliminary test on potato tuber slices Fourteen representative samples were selected from six growing regions and used in this assay. The isolates used were: EN4, EN6, EN16, EN31, EN38, EN44, EN47, EN54, EN58, EN59, EN60, EN63 and EN66. Type strains of the various species and subspecies of *Pectobacterium* and *Dickeya* were also included in this assay (Table 1). All the bacterial isolates were tested for pectolytic activity on potato tubers (*Solanum tuberosum* cv Amethyst). The tubers were washed under running tap water, air dried and then dipped in 96%

ethanol, flamed and cut into 10 mm thick slices. The slices were placed on wet sterile filter papers in Petri dishes. Two loops of each bacterial isolate grown on Nutrient Agar were uniformly spread on the upper surface of 20 slices. Development of rot on the slices was examined 24 – 48 h after incubation at 25ºC. The number of rotten slices was expressed as a percent. Control tubers were treated in the same manner but sterile distilled water was used in place of the bacterial isolate.

(ii) Quantitative test on potato tuber slices using filter paper discs This test was performed as described by Wegener (2002). A filter paper disc 10 mm in diameter, previously dipped in a water suspension of each bacterial isolate containing 10^8 cfu ml^{-1}, was placed at the centre of the upper surface of each potato slice. Each isolate was tested on 20 potato slices. After incubation at 25ºC for 2 days, the rotting zone diameter on each tissue slice was measured. For the control, tubers were inoculated with filter paper discs soaked in sterile distilled water.

3.2.6 Virulence assays

Virulence assays were performed on potato tubers. For tuber maceration assays, potatoes (cv. Amethyst) were surface sterilised by submersion in 10% sodium hypochlorite for 10 min and each potato inoculated with 10 µl of a 48 h culture of each strain grown in Luria-Bertani broth. Holes of a fixed depth of 20 mm were stabbed into the potatoes using a sterile pipette tip filled with inoculum and the holes then sealed with Vaseline. The inoculated potato was then wrapped in a plastic bag and incubated at 25ºC for 72 h. Rotting tissue was scraped from the potato and weighed to establish the degree of potato maceration.

3.2.7 Fluorescent amplified fragment length polymorphism analysis

The diversity of *Pcb* isolates causing soft rot of potatoes in Zimbabwe, South Africa and minitubers imported from China (isolates from South Africa and mini-tubers imported from China came from Potato Pathology Programme at University of Pretoria, South Africa culture collection) was evaluated using AFLP fingerprinting. Sixty-four *Pcb*, 4 *Ddd* and 4 *Pcc* isolates from 16 different regions of South Africa and Zimbabwe as well as those from imported minitubers were used in the analysis.

Fluorescent AFLP analysis was performed according to the method described by Pitman *et al*., (2010). AFLP products were visualised by a PRISM 310 Genetic Analyser (Applied Biosystems) using the GeneScan 500 size standard (Applied Biosystems). All primers used in AFLP reactions are listed in Table 2. Band patterns were analysed with BioNumerics 4.0 (Applied Maths) and compared with a database containing profiles of reference strains of all *Pectobacterium* and *Dickeya* species with validly published names. A UPGMA dendrogram was constructed using Pearson's correlation coefficient.

3.3 RESULTS

3.3.1 Biochemical and physiological characterisation

The analysis of the biochemical properties of the enterobacterial isolates from potatoes in Zimbabwe indicated that the majority were *D. dadantii* subsp. *dadantii* (*Ddd*) (*Table* 3). Four isolates were identified as *Pectobacterium atrosepticum* and 21 as *Pectobacterium carotovorum* subsp. *carotovorum*. Based on the biochemical properties examined, *Pcb* could not be differentiated from *Pcc* and *D. dadantii*. The results indicated that additional, more accurate approaches were required for the precise identification of the isolates.

Most of the isolates, except the putative *Pectobacterium atrosepticum* and the reference sample of the same species, grew at 37°C supporting the provisional classification of the majority as *Dickeya dadantii* subsp. *dadantii* and *Pcc*.

3.3.2 Rep-PCR analyses

The rep-PCR fingerprint patterns consisted of 10 to 45 PCR products ranging in size from about 200 to 10 000 bp. When analyzed using the Pearson's correlation coefficient, the rep-PCR fingerprints had an average similarity of 86%. Cluster analysis of the profiles revealed four groups, which separated the isolates into *Pa, Pcc, Pcb* and *Dd* (Fig. 1) The reference isolates for each of these species (LMG

2404^T, LMG 2386^T, Ech 3937 and ATCC BAA-417) fell into their respective clusters and grouped with the appropriate isolates. Polymorphic patterns sharing multiple bands of equal mobility could be distinguished for some of the isolates.

3.3.3 Sequencing and phylogenetic analysis

Partial *recA* and *gyrB* gene sequences of the potato isolates confirmed their identity as belonging to species of *Pectobacterium* and *Dickeya*. Phylogenetic analyses grouped the strains and reference samples into four distinct clusters in both trees (Fig.2, Suppl. Fig. 1). In the *gyrB* phylogenetic tree (Fig. 2), all strains identified biochemically as *Dickeya dadantii* subsp. *dadantii* clustered in a single clade, containing the type strain and reference strains, which was supported by a bootstrap value of 86 %. Fifteen strains formed a stable cluster with reference strains of *Pectobacterium carotovorum* subsp. *brasiliensis* (ATCC BAA-417, ATCC BAA-419, LMG 21370 and LMG 21371) with a bootstrap value of 82 %. *RecA* and *gyrB* sequencing also confirmed the identity of both *P. atrosepticum* and *P. c.* subsp. *carotovorum* strains, as the isolates fell into separate distinct clusters with their respective type and reference strains supported by strong bootstrap values in both trees.

3.3.4 Pectolytic activity

All isolates evaluated showed pectolytic activity on potato tuber slices. *Pa* (LMG 2386^T, EN54), *Pcb* (ATCC BAA-417, EN16, EN66), *Pcc* (EN31) and *Dd* (EN47, EN58) caused rots which penetrated the entire thickness of potato slices within 24 h whereas isolates LMG 2404^T (*Pcc*), *E. c* 3937^T, EN38, EN44, EN59 (*Dd*), EN4, EN6 (*Pcb*) and EN60 (*Pa*) caused the same extent of rotting in 48 h (Table 4). Evaluation of rotting zone diameters after 4 days showed variable responses. The largest rotting zone diameter of 36mm was observed in potato slices inoculated with LMG 2386^T, EN54 (*Pa*), ATCC BAA-417, EN66 (*Pcb*), EN 12 (*Dd*) and EN63 (*Pcc*). The reference sample LMG 2404^T (*Pcc*) and EN6 (*Pcb*) recorded the smallest rotting zone diameters (Table 4).

3.3.5 Virulence assays

The relative virulence of strains was quantified using tuber assays. The amount of tissue macerated was notably higher for EN12 (*Dd*) and EN54 (*Pa*). These strains also showed greater levels of maceration than the reference samples used in the study (Fig. 3). Virulence was not consistent within groups of genetically and biochemically similar strains, for example, EN54 and EN60 which are *Pa* isolates, exhibited different levels of maceration on the potato tubers (Fig. 3). The tuber maceration results were supported by the results of the quantitative pectolytic activity in which the rotting zone diameters were significantly ($P < 0.05$) larger on potato slices inoculated with EN12 and EN54 (Table 4). The reference samples were not as virulent as strains EN12 (*Dd*) and EN54 (*Pa*). EN44 was the least virulent strain, as it showed very little maceration of tubers in the virulence assays and a relatively small rotting zone diameter for the quantification of pectolytic activity.

3.3.6 Fluorescent amplified fragment length polymorphism

The restriction enzymes *Eco*RI and *Mse*I together with selective primers *E00* and *MseI-C* generated between 40 and 60 distinguishable bands within a range of 40 – 550bp for each isolate tested (Fig. 4). Following numerical analysis of the AFLP banding profiles by UPGMA, the isolates clustered according to identity as determined by rep-PCR and *gyrB*- and *recA*-gene sequencing. *D. dadantii* subsp. *dadantii*, *P. c.* subsp. *brasiliensis* and *P. c.* subsp. *carotovorum* isolates clustered separately in different clades. Within the *P. c. brasiliensis* clade, isolates grouped into 12 distinct clusters, several according to geographical origin, at a similarity level of 70 %. The reference strain ATCC BAA419 grouped with the isolates in cluster 1. In clusters 1, 2, 3, 6 and 7 isolates from South Africa clustered with those from Zimbabwe. Clusters 4 and 10 contained isolates from Limpopo and Mpumalanga provinces respectively (South Africa), while clusters 11 and 12 were exclusively made up of isolates from the Sandveld region, also in South Africa. Isolates from Zimbabwe (Marondera and Harare) grouped together in cluster 5, while clusters 6 and 9 contained isolates from Zimbabwe and China. Cluster 8 included isolates from South Africa, Zimbabwe and China. *Pcc* isolates clustered at a similarity level of 75%, while *Dd* isolates clustered at a relatively low similarity level of 30%.

3.4 DISCUSSION

The results of biochemical, physiological and genetic analyses revealed the identity of pectolytic enterobacteria infecting potatoes in Zimbabwe. The majority of isolates in the collection were shown to be *Pectobacterium carotovorum* subsp. *brasiliensis* (36 %), followed by *D. dadantii* subsp. *dadantii* (32 % of isolates). Fewer isolates were identified as *Pectobacterium carotovorum* subsp. *carotovorum* or *Pectobacterium atrosepticum*.

Dickeya chrysanthemi (formerly *Erwinia chrysanthemi*) is the causal agent of tuber soft rot and blackleg-like symptoms in many potato growing areas of the world (Young *et al*., 1992). However, our study found *D. dadantii* subsp. *dadantii* to be one of the most predominant species in Zimbabwe. This finding was not surprising because *D. dadantii* subsp. *dadantii* is a warm climate pathogen (Pèromberlon, 2002) and Ngadze *et al*. (2010), also isolated and identified *D. dadantii* subsp. *dadantii* isolates from plants with typical wilting symptoms and rotting tubers in Zimbabwe. The strains isolated in this study shared biochemical and genetic features with the species *D. dadantii*. Phylogenetic analysis of the *recA* and *gyrB* gene sequences demonstrated high genetic homogeneity between the potato isolates from Zimbabwe and *D. dadantii* subsp. *dadantii* reference strains, with sequence similarity ranging from 86 to 97 %. The origin of this pathogen cannot be confirmed although Zimbabwe imported seed potato from Europe during the period of 1993 to 1995 (K. Simango, Personal communication) and it is an important pathogen in the European potato industry.

Pectobacterium carotovorum subsp. *carotovorum* is prevalent on potatoes worldwide (Laurila *et al*., 2008). This pathogen has been identified and reported as a causal agent of potato soft rot in Zimbabwe (Masuka *et al*. 1998), and the present study confirms this although *Pcc* was isolated less frequently than *Pcb*. The ubiquitous nature of *Pcc* may be due to an earlier divergence, wider geographical distribution or broader host range and it is thought to have resulted in the extensive genetic variability of this species (Avrova *et al*., 2002). These observations are supported by genetic analysis of *Pcc* isolates in the present study. Phylogenetic analysis of partial *gyrB* sequences

demonstrated a high genetic divergence between potato isolates from Zimbabwe and reference strains of *Pcc*. The type strain of *Pcc* (LMG 2404T) clustered with a reference strain (LMG 2407) on the border of *Pcb*, while the Zimbabwe potato isolates formed a strongly supported cluster with a second reference strain on a separate branch at a lower level. The polyphyletic arrangement of *Pcc* suggests that another subspecies may exist within *Pectobacterium carotovorum*, although a greater number of strains would have to be examined to provide further proof.

Several studies have shown the usefulness of the rep-PCR technique to fingerprint a large variety of bacteria as well as its ability to differentiate at the species, pathovar or strain level (Versalovic *et al*., 1991; Rademaker *et al.,* 2004). Therefore selected isolates from potatoes in Zimbabwe were characterized by rep-PCR fingerprinting of genomic DNA. The technique clustered the isolates in this study into four groups, each containing a reference strain. Isolates identified as either *Dd* or *Pa* produced fingerprints which were nearly identical to those of their respective reference strains, suggesting a high level of homogeneity. In contrast, several visually different fingerprints were observed in both the *Pcc* and *Pcb* clusters providing further evidence of the genetic divergence of this species.

All potato isolates tested were pectolytic on potato tuber slices and CVP medium, showing that they produce cell wall degrading enzymes. These enzymes break down pectin and release nutrients which are essential for microbial growth. The effect of these enzymes results in tissue disintegration, cell damage and seepage of cell contents (Pèrombelon, 2002) which produces the disease symptoms seen on plants and tubers. The isolates exhibited differences in virulence which can be attributed to their origin. The isolates are from different geographical locations and were isolated from different potato cultivars. Other studies have also reported a link between differences in virulence and taxonomic diversity based on location and host (Pitman *et al*., 2010).

AFLP analysis separated the *Pcb* strains into 12 clusters, reflecting subdivision in terms of geographic origin, and *Pcc* isolates were clearly differentiated from *Pcb* isolates which displayed clearer and more intense banding profiles. Such intraspecies diversity has been noted in *Pcc* isolates analyzed with PCR-RFLP (Darrasse *et al.*, 1994; Hèlias *et al.*, 1998) and this may reflect molecular diversity within subspecies. Four clusters contained isolates from both South Africa and Zimbabwe, four clusters contained isolates only from South Africa, two clusters contained isolates from Zimbabwe and China, one cluster was made up of isolates from South Africa and China and one cluster contained isolates only from Zimbabwe. A large degree of DNA polymorphism was evident between these 12 clusters, suggesting that several different *Pcb* populations, derived from a common ancestor, are causing the blackleg symptoms seen in these two countries. It also appears that there are two different lineages of *Pcb* in the Sandveld region (South Africa) as clusters 11 and 12 contain isolates from this region that are genotypically different from each other. As Zimbabwe has been importing potato seed from South Africa for many years, it is possible that *Pcb* was introduced to Zimbabwe on infected seed. This would explain the similar banding patterns of South African and Zimbabwean isolates within clusters 1, 2, 3 and 7.

AFLP analysis provides a basis for an evolutionary analysis of *Pcb* pathotypes. Most strains collected from the same geographical area clustered together in the UPGMA tree, showing that *Pcb* populations from each area represent a distinct phenetic group. However in some cases, where numerous phenetic groups exist, it is possible that different founder effects occurred, probably because different potato cultivars are grown in diverse geographical areas in South Africa and Zimbabwe. The inconsistencies within some clusters (isolates from different geographical areas grouping together) may also reflect genetic exchanges that have occurred between groups.

Despite the genetic diversity evident in *Pcb*, a characteristic pattern of 2 to 3 bands was clearly visible in all isolates, providing potential molecular markers for identification and diagnosis. In most cases the subspecies-specific bands appeared more intense than others. This could be attributed to their higher

genomic copy numbers (Avrova *et al*., 2002) and it has been suggested that rDNA genes could be the source of these bands (Keim *et al*., 1997).

In conclusion, *D. dadantii* subsp. *dadantii* was found to be the most frequently isolated soft rot pathogen from potatoes in Zimbabwe, followed by *P. carotovorum* subsp. *brasiliensis*. *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum* were also isolated but in lower frequencies. Both *gyrB* and *recA* sequencing could reliably identify isolates to the subspecies level, although *gyrB* demonstrated a greater intraspecies diversity. Selected isolates belonging to the four taxa could also be differentiated by rep-PCR and a high degree of genetic diversity was evident for *Pcc* and *Pcb*. The genetic diversity in *Pcb* was also observed by AFLP analysis which showed great discriminatory power by separating isolates into 12 different clusters. The rep-PCR and AFLP results were not correlated in the present study and more strains must be analyzed by rep-PCR to determine the existence or lack of correlation between both techniques.

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Table 3.1 *Dickeya* spp., *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *brasiliensis* and *Pectobacterium carotovorum* subsp. *carotovorum* used in this study

Strain	Host	Origin
Pectobacterium atrosepticum LMG 2386^T	Solanum tuberosum	Belgium Coordinated Collections of Microorganisms (BCCM™)
EN53	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN54	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN55	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN60	*Solanum tuberosum*	Nyanga (Zimbabwe)
Pectobacterium carotovorum subsp. *brasiliensis* strain 8 ATCC BAA-417	*Solanum tuberosum*	American Type Culture Collection (ATCC) – originated from Brazil
Pectobacterium carotovorum subsp. *brasiliensis* strain 371 ATCC BAA-419	*Solanum tuberosum*	American Type Culture Collection (ATCC) – originated from Brazil
JJ1	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ13	*Solanum tuberosum*	Eastern Freestate (South Africa)
JJ18	*Solanum tuberosum*	KwaZulu-Natal (South Africa)
JJ19	*Solanum tuberosum*	KwaZulu-Natal (South Africa)
JJ24	*Solanum tuberosum*	KwaZulu-Natal (South Africa)
JJ27	*Solanum tuberosum*	KwaZulu-Natal (South Africa)
JJ30	*Solanum tuberosum*	KwaZulu-Natal (South Africa)
JJ38	*Solanum tuberosum*	Northern Cape (South Africa)
JJ46	*Solanum tuberosum*	Limpopo (South Africa)
JJ47	*Solanum tuberosum*	Limpopo (South Africa)

Table 3.1 Continued

Strain	Host	Origin
JJ49	*Solanum tuberosum*	Limpopo (South Africa)
JJ52	*Solanum tuberosum*	Sandveld (South Africa)
JJ54	*Solanum tuberosum*	Sandveld (South Africa)
JJ55	*Solanum tuberosum*	Sandveld (South Africa)
JJ57	*Solanum tuberosum*	Limpopo (South Africa)
JJ59	*Solanum tuberosum*	Sandveld (South Africa)
JJ60	*Solanum tuberosum*	Sandveld (South Africa)
JJ64	*Solanum tuberosum*	Western Freestate (South Africa)
JJ68	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ70	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ71	*Solanum tuberosum*	Limpopo (South Africa)
JJ72	*Solanum tuberosum*	Limpopo (South Africa)
JJ74	*Solanum tuberosum*	Sandveld (South Africa)
JJ75	*Solanum tuberosum*	Sandveld (South Africa)
JJ76	*Solanum tuberosum*	Sandveld (South Africa)
JJ77	*Solanum tuberosum*	Western Free State (South Africa)
JJ81	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ83	*Solanum tuberosum*	Eastern Cape (South Africa)
JJ85	*Solanum tuberosum*	Eastern Cape (South Africa)
JJ86	*Solanum tuberosum*	CH1A (China)
JJ88	*Solanum tuberosum*	CH3A (China)
JJ90	*Solanum tuberosum*	CH3Bo (China)
JJ91	*Solanum tuberosum*	CH5A (China)

Table 3.1 Continued

Strain	Host	Origin
JJ92	*Solanum tuberosum*	CH5B (China)
JJ95	*Solanum tuberosum*	IN3C (china)
JJ96	*Solanum tuberosum*	IN3D (China)
JJ97	*Solanum tuberosum*	IN3E (China)
JJ99	*Solanum tuberosum*	CH1C (China)
JJ107	*Solanum tuberosum*	North West (South Africa)
JJ114	*Solanum tuberosum*	Sandveld (South Africa)
JJ128	*Solanum tuberosum*	Eastern Cape (South Africa)
JJ130	*Solanum tuberosum*	Eastern Cape (South Africa)
JJ135	*Solanum tuberosum*	Eastern Cape (South Africa)
JJ136	*Solanum tuberosum*	Eastern Cape (South Africa)
JJ145	*Solanum tuberosum*	Limpopo South Africa
JJ147	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ149	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ150	*Solanum tuberosum*	Mpumalanga (South Africa)
JJ153	*Solanum tuberosum*	Mpumalanga (South Africa)
EN1	*Solanum tuberosum*	Gwebi (Zimbabwe)
EN2	*Solanum tuberosum*	Gwebi (Zimbabwe)
EN4	*Solanum tuberosum*	Darwendale (Zimbabwe)
EN5	*Solanum tuberosum*	Harare (Zimbabwe)
EN6	*Solanum tuberosum*	Harare (Zimbabwe)
EN16	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN18	*Solanum tuberosum*	Mazowe (Zimbabwe)

Table 3.1 Continued

Strain	Host	Origin
EN19	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN23	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN24	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN25	*Solanum tuberosum*	Harare (Zimbabwe)
EN26	*Solanum tuberosum*	Harare (Zimbabwe)
EN36	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN37	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN51	*Solanum tuberosum*	Shurugwi (Zimbabwe)
EN52	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN66	*Solanum tuberosum*	Marondera (Zimbabwe)
EN67	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN68	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN78	*Solanum tuberosum*	Gweru (Zimbabwe)
EN79	*Solanum tuberosum*	Gweru (Zimbabwe)
Pectobacterium carotovorum subsp. *carotovorum* LMG 2404^{T}	Solanum tuberosum	Belgium Coordinated Collections of Microorganisms (BCCM™)
EN7	*Solanum tuberosum*	Harare (Zimbabwe)
EN20	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN21	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN28	*Solanum tuberosum*	Harare (Zimbabwe)
EN30	*Solanum tuberosum*	Nyanga (Zimbabwe)

Table 3.1 Continued

EN31	*Solanum tuberosum*	Darwendale (Zimbabwe)
EN32	*Solanum tuberosum*	Darwendale (Zimbabwe)
EN35	*Solanum tuberosum*	Gweru (Zimbabwe)
EN39	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN58	*Solanum tuberosum*	Shamva (Zimbabwe)
EN61	*Solanum tuberosum*	Gweru (Zimbabwe)
EN63	*Solanum tuberosum*	Harare (Zimbabwe)
EN69	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN70	*Solanum tuberosum*	Darwendale (Zimbabwe)
EN71	*Solanum tuberosum*	Harare (Zimbabwe)
EN75	*Solanum tuberosum*	Mazowe (Zimbabwe)
Dickeya dadantii subsp. *dadantii* (Erwinia chrysanthemi 3937)	*Saintpaulia ionantha*	Scottish Crop Research Institute (SCRI)
EN3	*Solanum tuberosum*	Gwebi (Zimbabwe)
EN10	*Solanum tuberosum*	Marondera (Zimbabwe)
EN11	*Solanum tuberosum*	Marondera (Zimbabwe)
EN12	*Solanum tuberosum*	Marondera (Zimbabwe)
EN13	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN14	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN17	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN29	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN38	*Solanum tuberosum*	Mazowe (Zimbabwe)
EN40	*Solanum tuberosum*	Gweru (Zimbabwe)
EN44	*Solanum tuberosum*	Harare (Zimbabwe)

Table 3.1 Continued

EN45	*Solanum tuberosum*	Harare (Zimbabwe)
EN47	*Solanum tuberosum*	Darwendale (Zimbabwe)
EN48	*Solanum tuberosum*	Gwebi (Zimbabwe)
EN50	*Solanum tuberosum*	Gwebi (Zimbabwe)
EN59	*Solanum tuberosum*	Nyanga (Zimbabwe)
EN72	*Solanum tuberosum*	Harare(Zimbabwe)
EN74	*Solanum tuberosum*	Gweru (Zimbabwe)
EN76	*Solanum tuberosum*	Harare (Zimbabwe)
EN77	*Solanum tuberosum*	Harare (Zimbabwe)

Table 3.2 List of primers used in study

Primer Code	Sequence	Source
*gyr*B 01-F	TAA RTT YGA YGA YAA CTC YTA YAA AGT	Brady *et al.*, 2008
*gyr*B 02-R	CMC CYT CCA CCA RGT AMA GTT	Brady *et al.*, 2008
*gyr*B 07-F	GTV CGT TTC TGG CCV AG	Brady *et al.*, 2008
*gyr*B 08-R	CTT TAC GRC GKG TCA TWT CAC	Brady *et al.*, 2008
*rec*AF	GAR KCB TCN GGT AAA ACV AC	Young & Park 2007
*rec*AR	TTC GCY TTR CCC TGR CCR ATC	Young & Park 2007
*rec*AR2	RTT GAT RCC TTC GCC GTA SA	Young & Park 2007
REP IR	III ICG ICG ICA TCI GGC	Versalovic *et al.,* 1991
REP 2IR	ICG ICT TAT CIG GCC TAC	Versalovic *et al.,* 1991
E00	GAC TGC GTA CCA ATT C	Pitman *et al.*, 2010
M00	GAT GAG TCC TGA GTA A	Pitman *et al.*, 2010
MseI-C	GAT GAG TCC TGA GTA AC	Pitman *et al.*, 2010

Table 3.3 Biochemical and physiological properties of selected *Dickeya dadantii*, *Pectobacterium atrosepticum*, *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliensis* strains used in this study

Strain[a]	Phosphatase activity	Indole from tryptophan	Gelatin hydrolysis	Reduced substances from sucrose[c]	Acid from α-methylglucoside	Acid from threalose	Malonate utilization	Erythromycin sensitivity	Growth 37ºC	Growth 5 g^{l-1} NaCl
P. atrosepticum LMG 2386^T (BCCM™	-	-	+	+	+	+	-	-	-	+
EN54[b]	+	-	+	+	+	+	-	-	-	+
EN60[b]	-	-	+	+	+	+	-	-	-	+
P. c. subsp *brasiliensis* strain8 ATCC BAA-417	-	-	+	-	-	+	-	-	+	+
P. c. subsp *brasiliensis* strain371 ATCC BAA-419	-	-	+	-	-	+	-	-	+	+
JJ38	+	-	+	-	-	-	+	+	+	-
JJ54	+	-	+	-	-	-	+	+	+	-
JJ68	-	-	+	-	-	+	-	-	+	+
JJ74	+	-	+	-	-	-	+	+	+	-
JJ145	+	-	+	-	-	-	+	+	+	-
JJ147	+	-	+	-	-	-	+	+	+	-
EN4[b]	-	-	+	-	-	+	-	-	+	+
EN6[b]	-	-	+	-	-	+	-	-	+	+
EN16[b]	-	-	+	-	-	+	-	-	+	+
EN66[b]	-	-	+	-	-	+	-	-	+	+

Table 3.3 Continued

Strain[a]	Phosphatase activity	Indole from tryptophan	Gelatin hydrolysis	Reduced substances from sucrose[c]	Acid from α-methylglucoside	Acid from threalose	Malonate utilization	Erythromycin sensitivity	Growth 37ºC	Growth 5 g^{l-1} NaCl
P. c subsp *carotovorum* LMG 2404^T (BCM™)	-	-	+	-	-	+	-	-	+	+
EN31[b]	-	-	+	-	-	+	-	-	+	+
EN39[b]	-	-	+	-	-	+	-	-	+	-
EN63[b]	-	-	+	-	-	+	-	-	+	+
D. dadantii subsp. *dadantii* (*E. chrysanthemi* 3937) (SCRI)	+	+	+	-	-	-	+	+	+	-
EN12[b]	+	-	+	-	-	-	+	+	+	-
EN38[b]	+		+	-	-	-	+	+	+	-
EN44[b]	+	+	+	-	-	-	+	+	+	W+
EN47[b]	+		+	-	-	-	+	+	+	W+
EN59[b]	+		+	-	-	-	+	+	+	-

ATCC American Type Culture Collection, BCCM Belgian Coordinated Collections of Microorganisms, SCRI Scottish Crop Research Institute, Dundee, Scotland, UK

EN, Isolated from potatoes grown in Zimbabwe

JJ, Isolated from potatoes grown in South Africa

W, weak reaction

[a]Gram, Oxidase, glucose metabolism and pectate degradation in Sutton's medium test results of all the strains agreed with those expected for *Dickeya dadantii*, *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *brasiliensis*, according to Cother and Sivasithaparam (1983)

[b]Strains considered as representative of bacteria obtained from different potato growing regions in Zimbabwe and selected for further analysis

[c]Orange zones developing in the culture medium after 5 min boiling when treated with Benedict's reagent

Table 3.4 Soft rot thickness and diameter on potato slices after inoculation with bacterial isolates from potato tubers

Symbol of isolate/ strain	Penetration of potato slice tissue by soft rot (% of slice's thickness affected)[a]		Diameter of Soft rot (mm) After 48 h of incubation[b]
	24 h	48 h	
Control – water	0	0	0a
LMG 2404^T (Pcc)	30	100	24b
EN31	100	100	30cde
EN63	100	100	36e
LMG 2386^T (Pa)	100	100	36e
EN54	100	100	36e
EN60	50	100	27bcd
ATCC BAA – 417 (Pcb)	100	100	36e
ATCC BAA-419 (Pcb)	100	100	31cde
EN4	50	100	30de
EN6	50	100	25b
EN16	100	100	31cde
EN66	100	100	36e
Ech 3937 (Dd)	50	100	28bcd
EN12	100	100	36e
EN38	50	100	28bcd
EN44	50	100	27bcd
EN47	100	100	27bcd
EN58	100	100	32cde
EN59	50	100	25b

[a] Preliminary test (average value of 20 slices per isolate)

[b] Quantitative test; values with the same letter in a column are not significantly different at $P< 0.05$

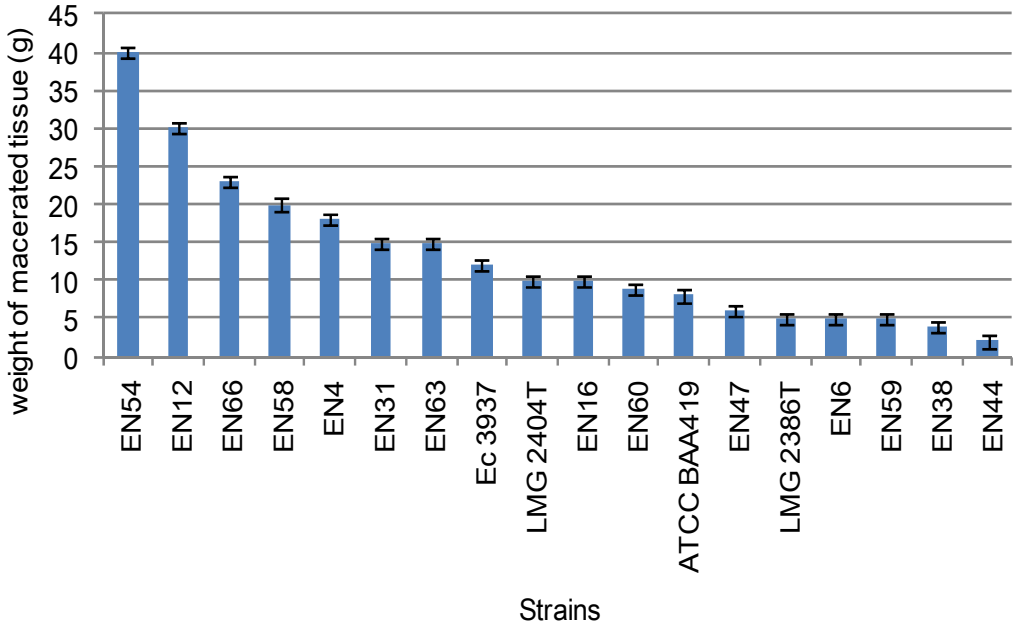


Figure 3.1 Relative virulence of atypical strains of pectolytic enterobacteria isolated from potatoes grown in Zimbabwe and the type strains of *Pectobacterium atrosepticum, Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dadantii* subsp. *dadantii.*

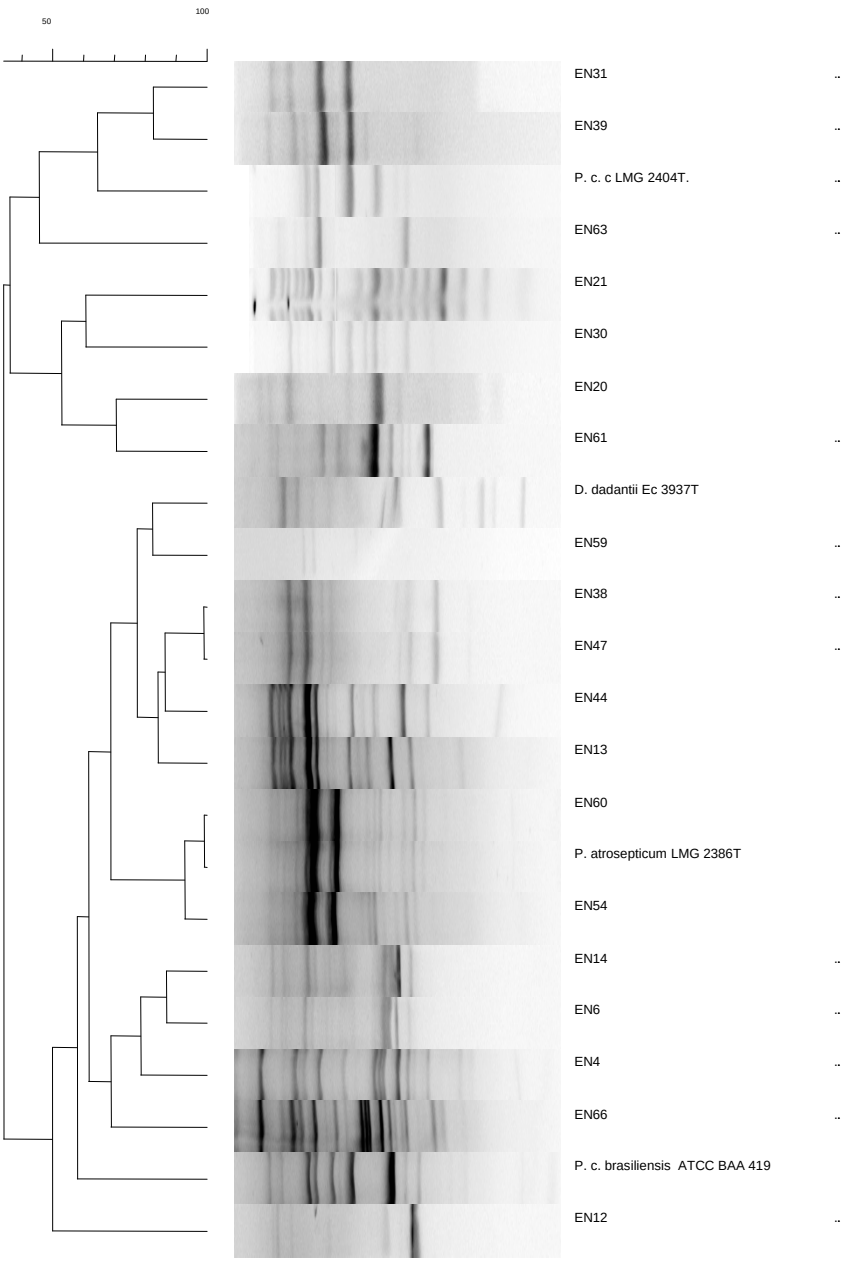


Figure 3.2 Cluster analysis of Rep-PCR fingerprint pattern generated from purified DNA of 23 *Pectobacterium* and *Dickeya dadantii* strains. The dendrogram was generated using Bionumerics with an UPGMA algorithm applied to the similarity matrix generated by Pearson's correlation coefficient from whole patterns of individual gel tracks.

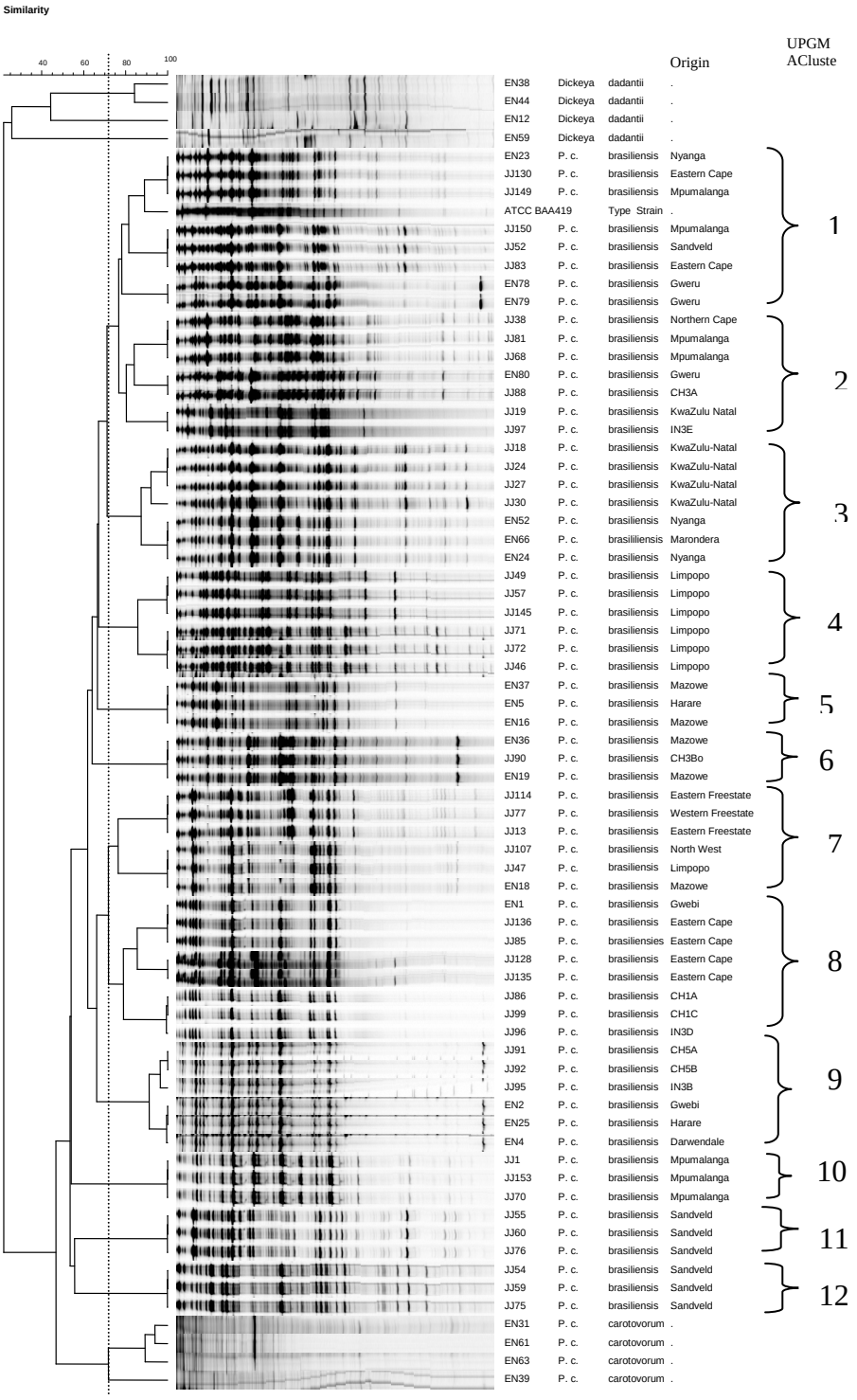
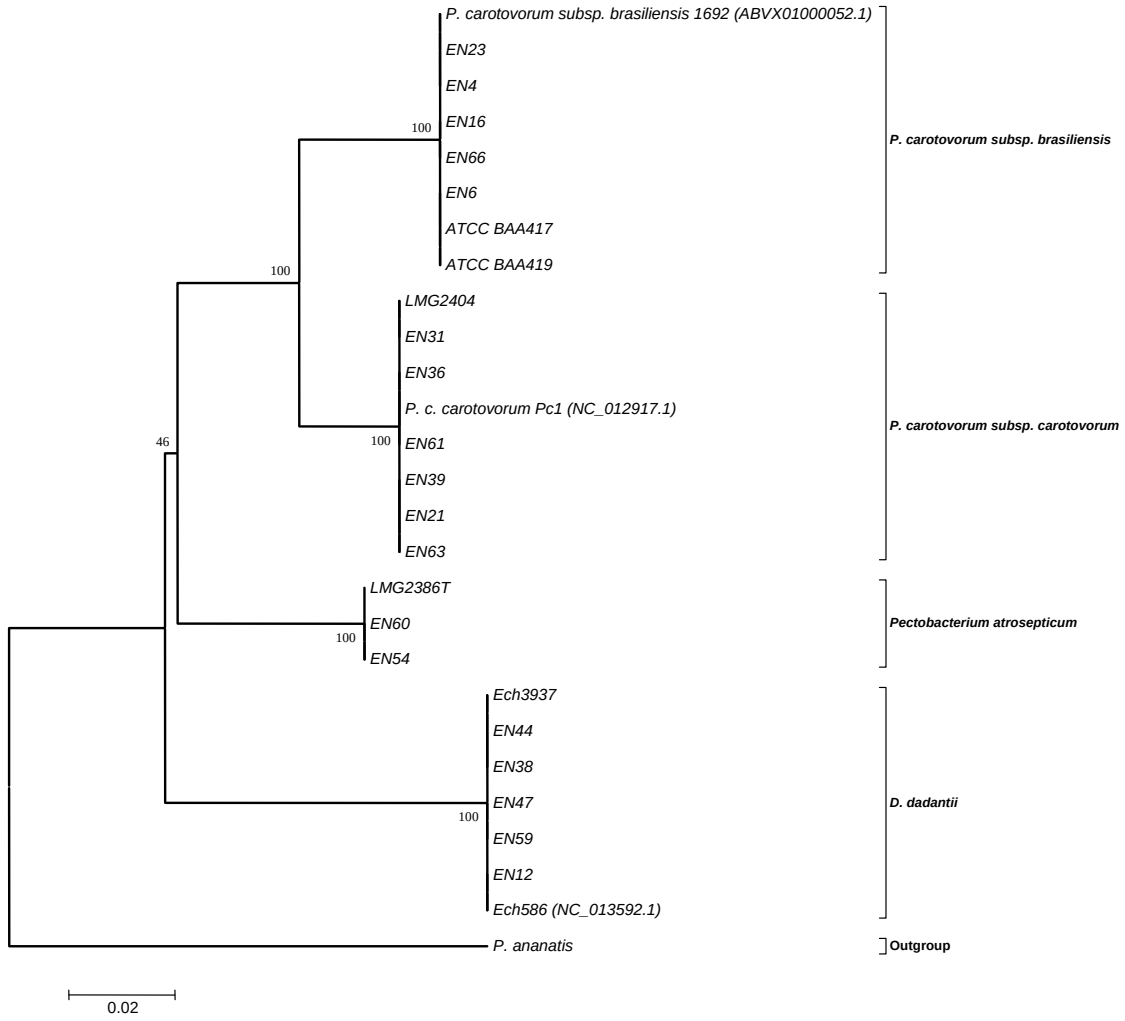


Figure 3.4 Dendrogram derived from the UPGMA linkage of correlation coefficient between AFLP profiles from strains of *P. carotovorum* subsp. *brasiliensis*, *P. carotovorum* subsp. *carotovorum* and *D. dadantii*. The levels of linkage representing the Pearson product moment correlations are expressed as percentages for convenience. The banding profiles against each branch each branch represent normalized and background-subtracted digitized gel strips processed in Bionumerics



Supplementary 3.1 Phylogenetic tree based on *recA* gene sequence showing the phylogenetic relationship among Zimbabwean strains, *D. dadantii* and different *Pectobacterium* spp. The phylogram was produced by the neighbour-joining programme (Tamura *et al*. 2007). The numbers on the branches indicate bootstrap value support based on neighbour-joining analyses of 1000 bootstrap replication. Accession numbers of references strains in GenBank are in parenthesis. T, type strain

CHAPTER 4

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Ngadze et al 2012 Role of Polyphenol Oxidase, Peroxidase, Phenylalanine Ammonia Lyase, Chlorogenic Acid and Total Soluble Phenols in Resistance of Potatoes to Soft Rot. Doi ;10.1094/PDIS-02-11-0149

ROLE OF POLYPHENOL OXIDASE, PEROXIDASE, PHENYLALANINE AMMONIA LYASE, CHLOROGENIC ACID AND TOTAL SOLUBLE PHENOLS IN RESISTANCE OF POTATOES TO SOFT ROT

Abstract

Pectobacterium atrosepticum (*Pa*), *P. carotovorum* subsp. *brasiliensis* (*Pcb*) and *Dickeya* spp. cause soft rot of potato (*Solanum tuberosum*) worldwide. Plants respond to bacterial invasion by activating defense responses associated with accumulation of several enzymes and inhibitors, which prevent pathogen infection. This study focused on the role of polyphenol oxidase (PPO), Peroxidase (POD), phenylalanine ammonia lyase (PAL), chlorogenic acid and total soluble phenols in imparting resistance to soft rot pathogens. Seven and 11 varieties grown by farmers in South Africa and Zimbabwe, respectively, were used in the study. The results showed significantly higher ($P < 0.001$) enzyme activity of PPO and PAL as well as higher concentrations of chlorogenic acid and total soluble phenols in Vanderplank, Pentland Dell, M69/11, Romano, M59/20 and Mondial$_{Zw}$. PAL activity increased significantly with time in all varieties and the highest activity was recorded 8 h after cutting. The resistance of the varieties was correlated with high PPO and PAL enzyme activity as well as increased concentrations of chlorogenic acid and total soluble phenols. PPO, POD and PAL activities increased significantly in wounded and inoculated tubers. These findings show that PAL, PPO, POD, chlorogenic acid and total soluble phenols play a role in imparting resistance to potato soft rot infection.

4.1 INTRODUCTION

Tuber soft rot of potatoes (*Solanum tuberosum* L.) caused by *Pectobacterium atrosepticum,* subspecies of *P. carotovorum* and *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) (Samson *et al.*, 2005) leads to significant economic losses in agriculture worldwide (Stevenson *et al.*, 2001). The disease affects the crop both in the field and in storage. After invading host tissue, the bacterial population increases to a critical density, above which a large amount of cell wall degrading pectolytic

enzymes are produced resulting in tissue maceration and granular rot of infected tubers. Rotted tissue is initially white or cream coloured, but brown pigments subsequently appear due to the oxidation of diseased tissue (Thipathi and Verma, 1975). The major virulence factors of *Pectobacterium* and *Dickeya* spp. are isoforms of pectate lyase (PL) enzymes that degrade plant cell wall pectin into unsaturated oligogalacturonates (OG) eliciting plant defense responses (Ryan, 1988). The virulence of soft rot bacteria appears to be influenced by temperature and humidity in conjunction with other factors. These other factors include intrinsic differences in the resistance of individual potato varieties to the pathogen, ability of the pathogen to multiply at the site of inoculation and differences in aggressiveness of bacterial isolates (Peltzer and Sivasithaparam 1985).

Disease resistance in plants is associated with activation of a wide array of defense responses that slow down or halt infection at certain stages of the host-pathogen interaction. The defense mechanisms include preexisting physical and chemical barriers that interfere with pathogen establishment. Other methods of protection rely on inducible defense responses in the form of enzymes that are activated upon infection (Vanitha *et al.*, 2009). The interaction between the pathogen and the host plant induces some changes in cell metabolism; primarily activity of enzymes, particularly phenylalanine ammonia lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO), lipoxygenase (LOX), superoxide dismutase (SOD) and β-1,3 glucanase (Fukasawa-Akada *et al.*, 1996; Hammerschmidt *et al.*, 1982; Mauch *et al.*, 1988, Ohta *et al.*, 1991; Thipathi and Verma1975; Thipyapong and Steffens 1992). PAL is the primary enzyme in the phenylpropanoid pathway, which leads to the conversion of L-phenylalanine to trans-cinnamic acid with the elimination of ammonia. It is the key enzyme in the synthesis of several defense-related secondary compounds such as phenols and lignin (Hemm *et al.*, 2004). PPO is a nuclear encoded enzyme that catalyzes the oxygen-dependent oxidation of phenols to quinones. PPO levels in a plant increase when a plant is wounded or infected (Vanitha *et al.*, 2009).

Although tuber soft rot causes considerable economic losses in Zimbabwe and South Africa, potato varieties grown in the two countries have not been assessed for resistance to the pathogens that cause the disease. The objectives of this study were (i) to determine the concentrations of PPO, PAL, POD, chlorogenic acid and total soluble phenols in potato tubers and (ii) to establish the relationship between the defense-related enzymes PPO, PAL, chlorogenic acid and total soluble phenols and resistance in potato tuber tissue to *P. atrosepticum (Pa), P. carotovorum* subsp. *brasiliensis (Pcb)* and *Dickeya dadantii* (*Dd*).

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Experiments were carried out to evaluate potato varieties for tolerance to soft rot pathogens. The seed was sourced from various potato seed companies. The seed of eleven varieties from Zimbabwe and seven from South Africa (Table 1) were evaluated in the experiment. The seed was harvested and stored for a month at 4˚C prior to the experiments. Seed tubers from each of the 18 varieties were used for the PPO, POD, PAL, chlorogenic acid and total soluble phenol assays. Each experiment was repeated twice.

4.2.2 Bacterial cultures

Bacterial strains of *P. atrosepticum* (LMG 2386^T, Belgian Coordinated Collections of Microorganisms), *Pectobacterium carotovorum*. subsp. *brasiliensis* (ATCC BAA-419 *Pcb* Strain 371, American Type Culture Collection) and *D. dadantii* (*Erwinia chrysanthemi* 3937, Scottish Crop Research Institute) were used in the study. *Pcb* and *Dd* were used as they have been shown to be the dominant soft-rotting bacteria of potatoes in South Africa (van der Merwe *et al*., 2010) and Zimbabwe (Ngadze *et al*., 2010), respectively. It has always been assumed that *P. atrosepticum* is the only causal agent of potato blackleg in Zimbabwe (unpublished data) and this is the reason for its inclusion in this study.

The bacterial cultures were grown for 24 h at 25˚C in a shaken culture of 25 ml Luria Bertani (LB) broth (pH 7.0) supplemented with 0.1 % pectin from citrus fruits (Sigma) and 0.1 % lyophilized potato cell sap. After centrifugation at 5,000 rpm for 5 min (4˚C) the bacteria were washed with sterile water, centrifuged again and re-suspended in sterile water before adjusting for the appropriate density of 6 x 10^8 CFU mL^{-1} (OD_{600} – 0.6).

4.2.3 Maceration of potato tuber tissue by bacteria and viability of cells after inoculation

Eight randomly selected seed tubers from each variety were washed thoroughly under running tap water and surface-disinfected by dipping in 70% ethanol for 5 min, before being cut longitudinally into halves (giving 16 halves). Each tuber half was inoculated with 10 mm diameter filter paper discs (Whatman's No. 1), that had been soaked for 10 min in a bacterial suspension of *P. atrosepticum, P. carotovorum* subsp. *brasiliensis* and *D. dadantii,* respectively. For the control, filter paper discs were soaked in sterile distilled water for 10 min and placed on tuber halves. One filter paper disc was placed in the pith and four around the edges of each tuber half. The experimental design was a Randomized Complete Block Design (RCBD) with four replicates per variety per pathogen (4 half tubers per variety per pathogen). After inoculation and incubation at 25˚C for 24 h the filter paper discs were removed and the diameter of each rotting zone was measured. The tubers halves were incubated for a further 24 h and the rotting zone diameter was measured again.

Cell viability of the inoculated half tubers was determined after measuring the rotting zone diameters at 48 h. Each tuber half was shaken in 49 ml sterile distilled water for 1 h at 20˚C in order to generate osmotic stress, and then stained for 90 min in a solution containing 50 mg $litre^{-1}$ neutral red dissolved in 0.2 M Na-phosphate buffer (pH 7.5) containing 0.8 M KNO_3. The tuber halves were rinsed in distilled water, after which the absorbed dye was extracted twice from the tuber tissue in 96 % ethanol for 10 min each. The final volume of dye from each tuber half was adjusted to 50 ml by adding 0.01 M sulphuric acid, before determination of optical density at OD_{535} according to the method by Wegener

(2002). Cell viability was determined by comparing the amount of neutral red retained by the inoculated tubers to that retained by the control tubers and the experiment was repeated.

4.2.4 Inoculation of tubers

Tubers of selected cultivars were surface sterilized by submersion in 1% sodium hypochlorite for 10 min and each potato inoculated with 10 µl of a 48 h culture of *P. carotovorum* subsp. *brasiliensis* grown in Luria-Bertani broth. Holes of a fixed depth were stabbed into the potatoes using a sterile pipette tip, filled with inoculum and sealed with Vaseline. Three tubers of each cultivar were inoculated and wounded before the enzyme assays. The inoculated potato was then wrapped in a plastic bag and incubated at 25ºC for 8 h. The inoculated tubers were assayed for PPO (Jockusch, 1966), POD (Zhang *et al.*, 2008) and PAL (Okey *et al.*, 1997) activity at 8 h after inoculation.

4.2.5 Polyphenol oxidase assay

PPO activity was measured according to the method described by Jockusch (1966) with minor modifications and 10 tubers of each variety were used for the assay. One cylinder, about 20 mm long was cut from each of the potato tubers using a 5 mm diameter cork borer. The peel was excised from each end of the cylinder using a scalpel and discarded. The tissue cylinders were pooled and ground in liquid nitrogen using a mortar and pestle, and 2.5 g of homogenate was mixed with 5 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 5 % polyvinylpolypyrrolidone (wt/vol). The homogenate was filtered through 4 layers of muslin cloth and the filtrate was centrifuged at 13,000 rpm for 5 min at 4˚C. One ml of supernatant was transferred to a new tube and mixed with 2.9 ml of 0.05 M sodium phosphate buffer and 1 ml of 0.1 M catechol (Sigma). The mixture was aliquoted into three portions for measurement of PPO activity. In the control the tuber extract was replaced with 1 ml of sodium phosphate buffer. The absorbance at 546 nm was measured for 4 min at 20 s intervals and the values per minute calculated. The results were presented as U μl^{-1} min^{-1} and the experiment was repeated.

4.2.6 Peroxidase assay

POD activity was measured according to the method described by Zhang *et al*., (2008) with minor modifications and 10 tubers of each variety were used for the assay. One cylinder, about 20 mm long was cut from each of the potato tubers using a 5 mm diameter cork borer. The peel was excised from each end of the cylinder using a scalpel and discarded. The tissue cylinders were pooled and ground in liquid nitrogen using a mortar and pestle, and 2.5 g of homogenate was mixed with 5 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 5 % polyvinylpolypyrrolidone (wt/vol). The homogenate was filtered through 4 layers of muslin cloth and the filtrate was centrifuged at 13,000 rpm for 5 min at 4˚C. One ml of supernatant was transferred to a new tube and mixed with 2.9 ml of 0.05 M sodium phosphate buffer, 1 ml guaiacol (Sigma), and 1 ml of 2 % H_2O_2 (vol/vol). The mixture was aliquoted into three portions for measurement of POD activity. In the control the tuber extract was replaced with 1 ml of sodium phosphate buffer. The absorbance at 470 nm was measured for 4 min at 20 s intervals and the values per minute calculated. The results were presented as U μl^{-1} min^{-1} and the experiment was repeated.

4.2.7 Phenylalanine ammonia lyase assay

PAL activity was assayed following the method of Okey *et al*. (1997) with minor modifications. Three 20 mm long cylinders were cut longitudinally from each of 10 tubers per variety using a 5 mm diameter cork borer. The peel was excised from each end of the cylinder and discarded. PAL activity was measured at 3 time intervals of 0, 2 and 8 h after sectioning and one cylinder per tuber was used at each time interval. The cylinders for each time interval were pooled and homogenized in liquid nitrogen using a mortar and pestle, and 2.5 g of homogenate was mixed with 5 ml of a buffer containing 50 mM Tris, 15 mM 2-mercaptoethanol and 5 % polyvinylpolypyrrolidone (Sigma) and filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 13,000 rpm for 5 min at 4˚C. One ml of supernatant was mixed with 2 ml 0.05 M borate buffer (pH 8.8) and 1 ml 0.02 M L-phenylalanine. The samples were incubated at 30˚C for 1 h. In the control the extract was replaced

with 1 ml borate buffer. The reaction was stopped by adding 0.2 ml of 6 M trichloroacetic acid (TCA). The supernatant was aliquoted into 3 portions for measurement. One activity unit was defined as a change in absorbance of 0.01 at 290 nm h^{-1} g^{-1} protein. The results are presented as µg PAL g^{-1} tissue and the experiment was repeated.

4.2.8 Total soluble phenols determination

A 5 mm diameter cork borer was used to excise two 20 mm long tissue samples from each of 10 potato tubers per variety. The potato cylinders were left at room temperature for 24 h. After 24 h, the cylinders were pooled to make a composite sample for each variety. The cylinders were ground in liquid nitrogen using a mortar and pestle, and 1 g of the tissue homogenate was mixed with 4 ml of methanol. The suspension was shaken for 1 h and centrifuged at 5,000 rpm for 10 min. The supernatant was removed, and the extraction procedure was repeated. The amount of total soluble phenols present in the extracts was determined using Folin-Ciocalteau reagent (Sigma) according to Cahill and McComb (1992). The supernatant was aliquoted into 3 portions for measurement. The concentration of total soluble phenols was calculated as g p-coumaric acid per kg tissue (Sigma) and the experiment was repeated.

4.2.9 Chlorogenic acid assay

Two 5 mm diameter cores, 20 mm long, were excised from 10 tubers per variety. The tissue samples were pooled and freeze-dried. The freeze-dried samples were assayed for chlorogenic acid, using the sodium nitrite method according to Griffiths *et al.* (1992). Standards were prepared from commercially available chlorogenic acid (Sigma). The procedure was repeated three times. The concentration of chlorogenic acid was expressed as g kg^{-1} fresh weight.

4.2.10 Statistical analyses

Data were explored for normality using the Anderson-Darling test ($P < 0.05$) and plotted for residuals using Minitab Release 12.22 (1998) to check assumptions for the Analysis of Variance (ANOVA) (the assumptions checked were independence, homogeneity and normality). The ANOVAs and Standard Errors of Difference (SED) were generated using GenStat Discovery Edition 2 (2006). All significant means were separated using the Least Significant Difference ($P < 0.05$). The error bars on the figures represent SED. The dendrogram was created using the software, Minitab Release 12.22, 1998. Hierarchical cluster analysis was performed using means of rotting zone diameters, PAL, POD, PPO, chlorogenic acid, total soluble phenols and cell viability per variety across the three pathogens evaluated. These values were used in generating the clusters using the complete linkage method and Squared Euclidian distance. The data are expressed as the average of two independent experiments.

4.3 RESULTS

4.3.1 Maceration of potato tuber tissue by bacteria and viability of cells after inoculation

There was a significant interaction ($P \leq 0.001$) of variety x subspecies at both 24 and 48 h of incubation with *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis* and *D. dadantii* (Table 2). The largest rotting zone diameter for *D. dadantii*, 24 h after inoculation was 15.8 mm, recorded in BP1. The smallest rotting zones for the same pathogen and time of incubation were recorded in M69/11, M59/20, $Mondial_{Zw}$ and Up-to-Date. After 48 h of incubation with *D. dadantii*, significantly smaller rotting zone diameters ($P < 0.05$) were recorded in Vanderplank, M69/11 and $Mondial_{Zw}$. Viability of cells 48 h after incubating tubers with *D. dadantii* ranged from 1.4% recorded for Up-to-Date and M62/51 to 81.4% recorded in M69/11 (Table 2).

Caren recorded the largest rotting zone diameter of 14.85 mm for *P. atrosepticum* at 24 h, while BP1 recorded the smallest diameter of 8.6 mm for the same pathogen and incubation time. The largest rotting zone diameter 48 h after inoculation with *P. atrosepticum* was 29.17 mm, recorded in both

Caren and Amethyst. The highest percentage of viable cells after inoculation with *P. atrosepticum* was recorded in Romano (92.6%) and the lowest value of 1.7% was recorded in M62/51.

The largest rotting zone diameter for *P. carotovorum* subsp. *brasiliensis*, 24 h after inoculation was 15.36 mm, recorded in Maradona. The smallest rotting zone for the same pathogen was recorded in BP1, which was 8.6 mm. After 48 h of incubation with *P. carotovorum* subsp. *brasiliensis*, Montclare had the largest rotting zone diameter of 28.2 mm. The smallest rotting zone was recorded in M69/11 (13.88 mm). The viability of cells inoculated with *P. carotovorum* subsp. *brasiliensis* ranged from 1.7% in Up-to-Date and Montclare to 82.3% in M69/11. *Dickeya dadantii* was the most virulent pathogen because 10 out of the 18 varieties tested recorded a cell viability of less than 10 % for *D. dadantii*, compared to 5 out of 18 and 4 out of 18 recorded for *P. atrosepicum* and *P. carotovorum* subsp. *brasiliensis,* respectively (Table 2).

4.3.2 Polyphenol oxidase assay

Significant differences in PPO enzyme activity ($P < 0.05$) were recorded across the varieties. PPO activity ranged from 1.111 U μl^{-1} min^{-1} for variety M62/51 to 1.94 U μl^{-1} min^{-1} for Vanderplank. Varieties that recorded significantly high concentrations of PPO ($P < 0.05$) were Pentland Dell, Vanderplank, M69/11, Romano, $Mondial_{Zw}$ and M59/20 (Fig. 1). PPO increased significantly ($P < 0.05$) in wounded and inoculated tubers of M69/11, Romano, Amethyst and M62/51 compared to the healthy (Table 3). PPO significantly correlated with rotting zone diameters at 24 h ($r = -0.624$, $P < 0.05$), 48 h ($r = -0.75$, $P < 0.01$) and viability of cell ($r = -.081$, $P < 0.05$) (Table 4). In addition PPO also significantly correlated with chlorogenic acid ($r = -0.654$, $P < 0.01$) and total soluble phenols ($r = -0.855$, $P < 0.01$). However PPO did not significantly correlate with PAL and POD.

4.3.3 Peroxidase assay

There was no significant difference in POD activity in healthy tubers of all cultivars. However POD activity increased significantly ($P < 0.05$) in wounded and inoculated tubers of Romano, Amethyst, M69/11 and M62/51 (Table 3). POD was significantly ($P < 0.05$) correlated to viability of cell ($r = -0.534$, $P < 0.05$ (Table 4).

4.3.4 Phenylalanine ammonia lyase assay

Enzyme activity of PAL increased significantly ($P < 0.05$) with time and the highest enzyme concentrations in all varieties were recorded at 8 hours after sectioning (Fig. 2). At the initial stage (0 h after sectioning), M59/20 recorded the highest enzyme activity of 1.716 µg g^{-1}. This value remained unchanged 2 hours after cutting the tubers. The lowest value recorded immediately after cutting the tubers was 1.115 µg g^{-1} in Amethyst. At 2 h after sectioning the tubers, the highest significant enzyme activity ($P < 0.05$) was recorded in $Mondial_{Zw}$ and Montclare, and the lowest in Up-to-Date, Amethyst and BP1. At 8 hours the highest enzyme activity was recorded in Montclare, which also showed the highest increase in enzyme activity over time (Fig. 2). PAL increased significantly ($P < 0.05$) in wounded and inoculated tubers of M69/11, Romano, Amethyst and M62/51 compared to the healthy (Table 4). Neither PAL at 0 h nor PAL at 2 h was significantly correlated with rotting zone diameters after 24 and 48 h after inoculation. However PAL at 8 h was significantly correlated with rotting zone diameter after 48 h ($r = -0.536$, $P < 0.05$ and viability ($r = -0.694$, $P < 0.05$) (Table 3).

4.3.5 Total soluble phenols and chlorogenic acid assays

The highest concentrations of total soluble phenols in the Zimbabwean varieties were recorded in M62/51, M69/18 and Montclare while Amethyst and Maradona recorded the lowest (Fig. 3A). A significantly high ($P < 0.05$) concentration of chlorogenic acid was recorded in Up-to-Date and the lowest was recorded in Buffelspoort, BP1 and Caren, for varieties grown in South Africa. The highest concentration of total soluble phenols was recorded in Up-to-Date, while BP1 contained the lowest

concentration of the same compound (Fig. 3A). Total soluble phenols significantly correlated with rotting zone diameters after 24 h ($r = -0.791$, $P < 0.05$), after 48 h ($r = -0.648$, $P < 0.05$) and viability ($r = -0.947$, $P < 0.01$) (Table 4).

4.3.6 Chlorogenic acid

For the varieties grown in Zimbabwe, the highest concentrations of chlorogenic acid were recorded in M59/20, M69/11, Mondial and Romano while the lowest were recorded in Amethyst and Maradona. As for the cultivars grown in South Africa, Vanderplank and Pentland Dell recorded significantly high concentrations of chlorogenic acid and the lowest was recorded in BP1. Chlorogenic acid significantly correlated with rotting zone diameter after 24 h ($r = -0.68$, $P < 0.01$), 48 h ($r = -0.600$, $P < 0.01$) and viability ($r = -0.579$, $P < 0.05$) (Table 4).

4.3.7 Cluster analysis

The varieties were divided into 5 clusters according to tolerance and susceptibility to the three pathogens. The resistance / susceptibility levels were based on the rotting zone diameters that developed after 24 and 48 h after inoculation and cell viability 48 h after inoculation. The varieties were divided into 5 clusters. Vanderplank, M69/11, Romano, M59/20, $Mondial_{Zw}$ and Pentland Dell were grouped in cluster 1, which is composed of resistant varieties (Fig. 4). These varieties recorded rotting zone diameters of less than 20 mm for the three pathogens 48 h after incubation and viability of cells was more than 70% (Table 2). The members of cluster 1 also recorded significantly higher concentrations of PPO, PAL, chlorogenic acid and total soluble phenols (Figs. 1, 2, and 3A and B). $Mondial_{SA}$, Maradona and Buffelspoort fell in cluster 2, while M69/18, Montclare, 157/91 and 157/79/91 were grouped in cluster 3. Clusters 2 and 3 contained varieties with intermediate resistance. BP1 and Amethyst fell into cluster 4. Cluster 5 contained Caren, Up-to-Date and M62/51. Varieties in clusters 4 and 5, with the exception of Up-to-Date, also recorded significantly lower levels of PPO, PAL, chlorogenic acid and total soluble phenols (Figs. 1, 2, and 3A and B) and were highly

susceptible to the three pathogens. These varieties recorded significantly the largest ($P < 0.05$) rotting zone diameters and the lowest viability (less than 10 %) (Table 2, Fig. 4). Mondial, a variety grown in both countries fell into two different classes. $Mondial_{Zw}$ fell into cluster 1 resistant varieties), while $Mondial_{SA}$ fell into cluster 2 (intermediate resistance). $Mondial_{Zw}$ recorded significantly higher ($P < 0.05$) PPO and PAL activity than $Mondial_{SA}$. Viability of cells as well as concentrations of chlorogenic acid and total soluble phenols was notably higher in $Mondial_{Zw}$.

4.4 DISCUSSION

In our study tuber resistance, expressed as rotting zone diameter and viability of cells after inoculating tubers with *Dd, Pa* and *Pcb*, was determined in 7 and 11 potato varieties grown in South Africa and Zimbabwe, respectively. Our results clearly show that there is a wide range of susceptibility to soft rot in potato varieties currently grown in both countries. These measurements do, however, have some limitations, since the inoculation method challenges the pith directly and bypasses the periderm, which is a physical barrier to entry of pathogens. However, other researchers found that immersing mechanically harvested tubers in a suspension of *P. atrosepticum* and injecting bacterial suspension into the tubers had no effect on the ranking of clones for susceptibility (Tzeng *et al.*, 1990). This may suggest that the periderm does not play a major role in determining resistance to tuber soft rot (Peltzer & Sivasithaparam 1985).

Laboratory assessment for tolerance must be linked with field data and the use of varieties of known susceptibility is therefore essential. In the field varieties such as Up-to-Date and Amethyst are considered susceptible to many diseases and in storage they also tend to rot at a faster rate than others (unpublished data) this is the main reason why they were included in the study. The mean rotting zone diameters of these varieties was more than 20 mm and the viability of cells after inoculation was less than 10 %, compared to no rotting and 100% cell viability recorded for the controls, thus confirming these field observations. Romano and M59/20, considered by growers to be

tolerant to most diseases, had mean rotting zone diameters of less than 20 mm and cell viability above 70 % compared to no rotting and cell viability of 100 % recorded for the controls. However, clear cut classes of resistance and susceptibility could not be established, confirming findings of other researchers who observed similar trends in their evaluation of susceptibility of different potato varieties to soft rot (Allefs *et al.*, 1995; Lapwood and Read 1985). The virulence of the three pathogens evaluated was different. *D. dadantii* was the most virulent pathogen and this could be explained by the fact that it produces five major and at least four secondary pectate lyase (Pels), whereas *P.carotovorum* subsp. *brasiliensis* and *P. atrosepticum* secrete four and three major Pels respectively. The secondary Pels are only produced *in planta*, have low enzymatic activity and are important in host specificity (Kelemu and Collmer, 1993). Although the production of pectinases is essential for pathogenicity, not all isoenzymes are required in all situations. This phenomenon could also explain the response of BP1 to the different pathogens evaluated in the experiment. *D. dadantii* was more virulent on BP1 compared to the other pathogens.

The difference in the behaviour of Mondial grown in the two countries could be attributed to macro- and micro-environmental variation and different agronomic practices between the two countries. The significant effect of the environment and agronomic practices on tuber tolerance to soft rots was reported by Wegener (2002) and Kumar *et al.* (1991), respectively. Contradictory results on ranking resistance of tubers to soft rot were also reported by Bourne *et al.* (1981) and Lapwood and Read (1985) when they evaluated the variety Desiree grown under different environmental conditions.

Metabolic changes in plants resulting from wounding or pathogen invasion have been reported in numerous host-pathogen systems (Okey *et al.*, 1997). In this study, enzyme activities of PPO, POD and PAL as well as concentrations of chlorogenic acid and total soluble phenols were found to differ significantly in resistant and susceptible varieties. Activities of these three enzymes also increased significantly in wounded and inoculated tubers. The potato varieties differed in three major aspects.

First, PPO activity was notably higher in the varieties that also exhibited high PAL activity (except Montclare). Secondly, PAL activity in all varieties increased significantly 8 h after cutting the tubers and after inoculating with *Pcb*. Other researchers have also recorded peaks in enzyme activity around 4 and 8 h after wounding or inoculating plants (Pereira *et al.*, 1999; Zhang *et al.*, 2008) and this was followed by a sharp drop in enzyme activity around 10 h (Ryan, 1988). This is because the accumulation of phenolic compounds occurs before polymerization thereof by peroxidase into lignin (Pereira *et al.*, 1999). Thirdly, concentrations of total soluble phenols and chlorogenic acid were considerably higher in some of the varieties. The results confirm the findings of Lewis *et al.* (1998), who recorded different concentrations of chlorogenic acid in different varieties. They also reported that chlorogenic acid was more concentrated in the tuber tissues than in the rest of the plant.

It was interesting to note that potato varieties that showed enhanced PPO activity also recorded a higher concentration of total soluble phenols and chlorogenic acid. These varieties (except Up-to-Date) exhibited on average higher tolerance to soft rot. After 48 h the extent of rotting was less in some of the varieties tested, particularly those with higher PPO activity. Of the 18 varieties tested, Pentland Dell, Mondial$_{ZW}$, Romano, Vanderplank, M59/20 and M69/11, with high concentrations of PPO, chlorogenic acid and total soluble phenols in tuber tissue were most resistant. These resistant varieties also had the highest number of viable cells and lowest extent of decay at 24 and 48 h after inoculation with the three pathogens. It appears that these resistant varieties produce more secondary metabolites involved in plant defense mechanisms than the other varieties tested. However, there are numerous components involved in tolerance to soft rot pathogens (Lyon and McGill, 1989) and each disease interaction can rarely be generalized as representative of host defense response in diverse plant species (Hammerschmidt *et al.*, 1982; Nicholson and Hammerschmidt, 1992). This was demonstrated by Up-to-Date, which was susceptible and prone to maceration by pectolytic enzymes despite having a high concentration of total soluble phenols and chlorogenic acid.

Total soluble phenols together with PPO appear to play a role in tolerance to soft rot since these compounds were present in considerably higher levels in tuber tissue of resistant varieties. This is supported by the work of Li and Steffens (2002), who reported that an over-expression of PPO coincided with a 10-fold increase in PPO enzyme activity, which led to enhanced bacterial disease resistance in transgenic tomatoes. Thipathi and Verma (1975) and Kumar *et al*. (1991) also observed that resistant potato cultivars demonstrated a high PPO activity and contained large amounts of phenolic compounds. PPO is important in the initial stage of plant defense where membrane damage causes release of phenols, such as chlorogenic acid. Phenol lyases catalyse the oxidation of these compounds. Phenols and their oxidative products were shown to inhibit *P. carotovorum* (Ghaneka *et al*., 1984; Lyon and McGill, 1989; Weber *et al*., 1996) and their cell wall-degrading enzyme activity of the bacteria (Lyon *et al*., 1992; Lyon and McGill, 1989). Phenolic compounds also function as precursors in the synthesis of lignin (Lyon *et al*., 1992) and suberin (Eslelie *et al*., 1986; Riley and Kolattukudy, 1975), which are involved in the formation of physical barriers that can block the spread of *Pectobacterium* spp. (Fox *et al*., 1971). In addition, PPO is known to catalyze the oxidation of phenolics to free radicals, which can react with biological molecules, thus creating an unfavorable environment for pathogen development (Jockusch, 1966). Phenolic compounds and PPO alone do not guarantee resistance against *Pectobacterium*, as can be seen in Montclare, which was severely macerated by the pathogen despite having a high concentration of total soluble phenols. This result supports the findings of Lojkowska and Holubowska (1992), who reported a low level of tolerance in Polish potato cultivars that had a high content of phenolic compounds and PPO. High densities of pectobacteria can inhibit and reverse phenol oxidation (Lovrekovich *et al*., 1967).

PPO, POD and PAL activity increased significantly in wounded and inoculated potato tubers. This is supported by the findings of Okey *et al*., (1997), who reported significantly high enzyme activities in inoculated and wounded cocoa clones. The researchers also reported that clones with higher enzyme activities exhibited resistance to *Phytophthora palmivora* infection. The increase in POD activity was not surprising because POD is involved in the oxidative polymerization of hydroxycinnamyl alcohols to

yield lignin (Vance and Sherwood, 1980) and crosslinking isodityrosine bridges in cell walls (Fry, 1982). These compounds act as barriers against pathogen invasion and hence constitute part of host resistance mechanisms.

PAL through its formation of cinnamic acid, an important compound in the biosynthesis of phenolics and lignin, also plays a role in plant defense systems (Fukasawa-Akada *et al.*, 1996). For PAL to be effective it must be available in potato tuber tissues in high concentrations at the initial stages of infection. This was shown in Montclare, which was severely macerated by pectolytic enzymes despite the fact that it had the highest increase in enzyme activity 8 h after cutting the tuber. The slow buildup of PAL allows the bacteria to multiply within host tissues, and reach the critical population density required for the production of pectic enzymes.

The significant correlation of viability with PPO, POD, PAL after 8 h of wounding, chlorogenic acid and total soluble phenols reflects the importance of the enzyme in resistance expression. However these findings contradict the findings of Lojkowska and Holubowska (1992), who reported that there was no correlation between soft rot resistance and enzyme activities. The difference in response to soft rot resistance between Polish and Zimbabwean or South African cultivars could be due to differences in the conditions under which the experiments were carried out. Factors such physiological state, size and maturity of the tubers can affect the response of the tubers to pathogen infection (Marquez-Villavicencio et al., 2011). These researchers also reported that soft rot susceptibility could be correlated with tuber weight, small tubers being more resistant to soft rot. They also observed differences in responses among tubers of the same cultivar harvested from different fields.

The importance of PPO, POD, chlorogenic acid and total soluble phenols in defense mechanisms against *Pectobacterium* cannot be underestimated. The cluster analysis performed on all tested variables grouped the varieties into three categories namely resistant, intermediate and susceptible, confirming field observations. All the varieties that fell in the resistant group had high enzyme activity,

while those in the susceptible category had low activity. These experiments clearly show a positive correlation between amounts of PPO, POD, PAL and the level of degradation by pectic enzymes.

The findings indicate that PPO, POD, PAL, chlorogenic acid and total soluble phenols play a role in disease resistance against *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis* and *D. dadantii.* Potato varieties with a high content of these compounds in tuber tissue can exhibit tolerance to pathogen attack. Of the varieties tested, Vanderplank and Pentland Dell are the South African varieties that are most resistant to soft rot bacteria, while Caren and Up-to-Date are the most susceptible. Zimbabwean varieties M69/11, Romano and M59/20 were identified as the most resistant, while Montclare and M62/51 were the most susceptible.

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Table 4.1. Potato varieties used in the study

Variety	Country where grown
Bp[x]	South Africa
Buffespoort[x]	South Africa
Caren[x]	South Africa
Mondial[x]	South Africa
Pentland Dell[x]	South Africa
Up-to-Date[x]	South Africa
Vanderplank[x]	South Africa
157/79/91[y]	Zimbabwe
157/91[y]	Zimbabwe
Amethyst[x]	Zimbabwe
M59/20[y]	Zimbabwe
M6/18[y]	Zimbabwe
M62/51[y]	Zimbabwe
M69/11[y]	Zimbabwe
Maradona[x]	Zimbabwe
Mondial[z]	Zimbabwe
Montclare[x]	Zimbabwe
Romano[x]	Zimbabwe

[x] Cultivated commercially, originated from Holland

[y] New release, bred in Zimbabwe

[z] Experimental, imported from South Africa

Table 4.2. Viability of cells and rotting zone diameter (mm) after incubating at 20˚Cpotato half tubers inoculated with *Pectobacterium* species and *Dickeya dadanti*

		Dickeya dadantii			*Pectobacterium atrosepticum*			*P. carotovorum subsp. brasiliensis*		
Country	Variety	Extent of Decay (mm)		Viability	Extent of Decay (mm)		Viability	Extent of Decay (mm)		Viability
		24h	48h	(%)	24h	48h	(%)	24h	48h	(%)
South African varieties	BP 1	15.80e	25.47d	2.00a	8.60a	16.73b	44.00b	8.60a	19.03c	58.50b
	Buffelspoort	14.13d	24.93c	3.10a	14.05e	19.00c	55.10c	14.05e	16.57b	43.90b
	Caren	14.60d	28.83d	1.60a	14.85f	29.17f	7.10a	14.85f	25.07e	2.90a
	MondialSA	12.05b	17.23b	32.50b	12.40d	18.50c	54.30c	12.40e	12.65d	68.20c
	Pentland Dell	12.27b	18.23d	75.50b	12.65d	18.50c	75.50d	12.65d	16.67b	79.80d
	Up-to-Date	10.80a	28.67d	1.40a	11.95c	28.43d	2.90a	11.95c	22.40e	1.70a
	Vanderplank	13.70c	15.93a	79.80c	10.10b	18.93c	76.40d	10.10b	15.67a	76.10d
	157/79/91	13.47d	26.43c	5.80a	11.88c	20.47d	29.70a	12.81d	22.51d	50.40b
	157/91	13.47d	26.43c	5.80a	11.88c	20.47d	28.80a	12.18d	21.08d	46.80b
	Amethyst	14.60e	28.83d	1.80a	14.00e	29.17f	9.40b	14.85f	10.07a	2.80a
	M59/20	8.96a	17.60b	79.50c	8.80a	11.24a	87.30d	8.96a	14.00a	70.60d
	M62/51	10.80b	28.67d	1.40a	12.40d	28.43f	1.70a	11.95c	22.40d	12.50a
Zimbabwean varieties	M69/11	9.36a	14.72a	81.40c	9.36a	12.44a	90.90d	10.92b	13.88a	82.30d
	M69/18	12.05c	28.23d	4.10a	11.40c	18.50c	33.80b	11.40b	15.65b	62.60c
	Maradona	13.84d	17.60b	1.50a	10.20b	12.68a	52.10c	15.36f	20.44c	52.10b
	MondialZw	9.08a	13.68a	79.20b	10.12b	12.68a	88.60d	9.32a	13.93a	73.20d
	Montclare	13.25d	29.80d	1.50a	12.50d	23.52e	8.10a	13.20c	28.20e	1.70a
	Romano	10.36a	17.08b	81.00c	10.36b	12.32a	92.60d	12.36d	16.08b	80.90d
	P Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	SED	0.286	1.174	15.22	0.386	0.678	15.22	0.289	0.479	15.22
	LSD (0.05)	0.579	2.348	30.59	0.772	1.346	30.59	0.590	0.952	30.59

[y] Data are expressed as the average of two independent experiments

[z] Means followed by the same letter in a column are not significantly different at LSD (0.05)

Table 4.3. Polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and peroxidise (POD) activity in health, wounded and inoculated tuber tissues[z]

Variety	PPO activity (A_{546}) nm min^{-1} g^{-1} fresh mass			POD activity (A_{470}) nm U min^{-1} μl^{-1}			PAL activity (A_{290}) nm cinnamic acid min^{-1} g^{-1} fresh mass		
	Healthy	Wounded	Inoculated	Healthy	Wounded	Inoculated	Healthy	Wounded	Inoculated
M69/11	1.852	2.154	2.209	0.451	0.979	1.200	1.521	1.975	2.133
Romano	1.810	2.218	2.553	0.481	0.832	0.895	1.450	1.785	1.906
Amethyst	1.120	1.500	1.556	0.342	0.773	0.772	1.115	1.612	1.632
M62/51	1.113	1.497	1.624	0.265	0.567	0.662	1.162	1.599	1.674

LSD ($P < 0.05$) cultivar x treatment 0.380. Values are means of 3 replicates. Assessments were made 8 h after wounding or inoculation

Table 4.4. Correleration coefficients for the enzyme activities and the extension of rotting on tuber halves (first two rows) and viability of cells in the tuber slices (third row)[y]

Assay	PAL 0 h	PAL 2 h	PAL 8 h	PPO	POD	Chlorogenic acid	Total soluble phenols
After 24 h	-0.261^{ns}	-0.351^{ns}	-0.391^{ns}	-0.75^{**}	-0.197^{ns}	-0.68^{**}	-0.791^{*}
After 48 h	-0.201^{ns}	-0.291^{ns}	-0.536^{*}	-0.624^{*}	-0.435^{ns}	-0.600^{**}	-0.648^{*}
Cell viability	-0.283^{ns}	-0.54^{ns}	-0.694^{*}	-0.817^{*}	-0.534^{*}	-0.579^{*}	-0.947^{**}

[y]PAL = phenylalanine ammonia lyase; PPO = polyphenol oxidase; POD = peroxidase

[z] * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; ns = not significant

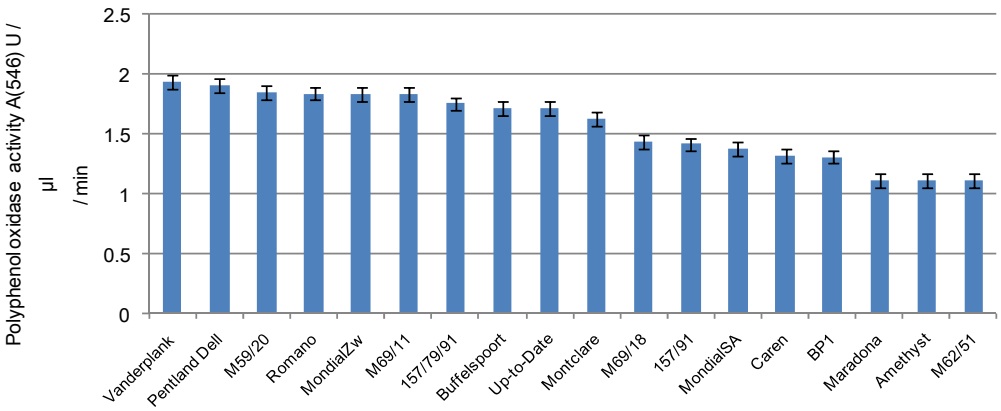


Figure 4.1. PPO activity measured in tuber tissue of the various cultivars. The data are expressed as the average of two independent experiments with three replicates. Error bars indicate SED.

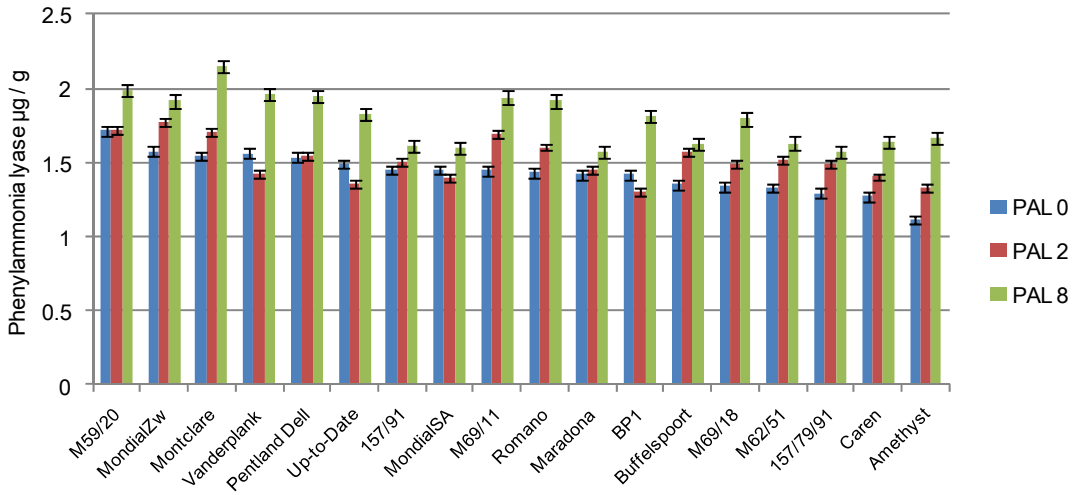
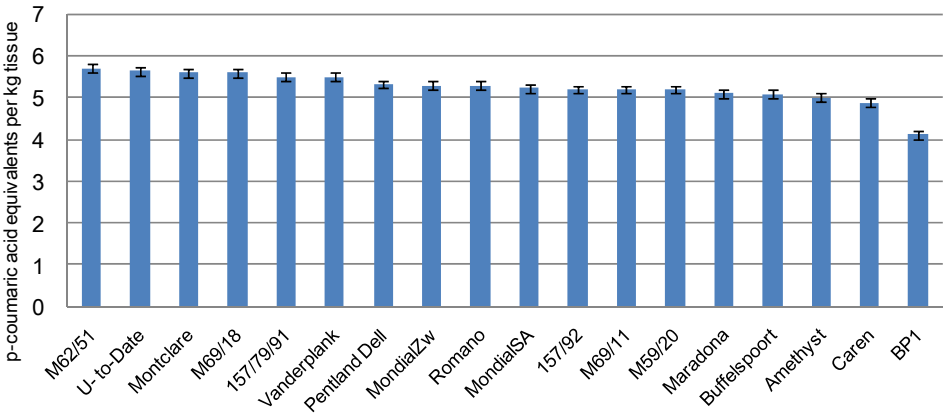
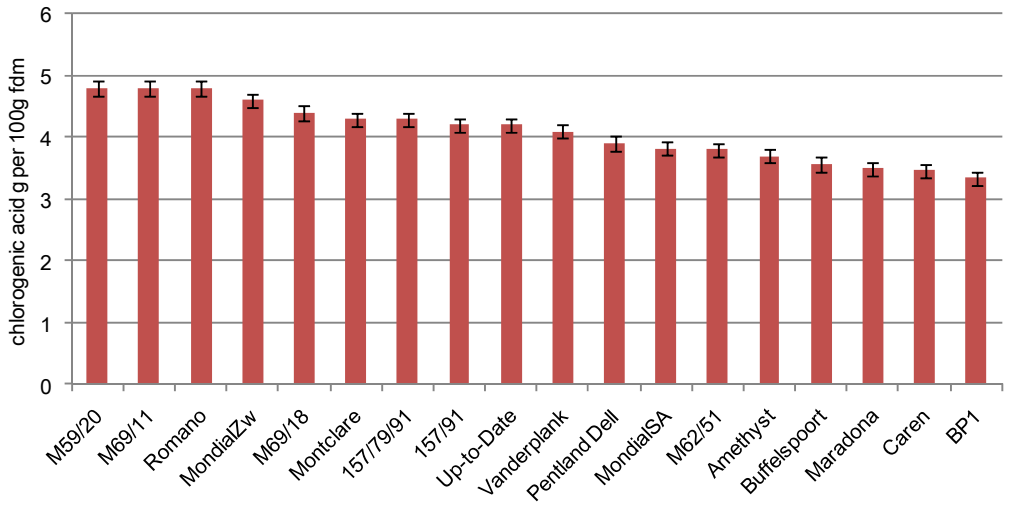


Figure 4.2. PAL activity measured in tuber tissue of the various cultivars at 0, 2 and 8 h after sectioning the tubers. The data are expressed as the average of two independent experiments with three replicates. Error bars indicate SED.



A



B

Figure 4.3. Concentration of total soluble phenols presented as p-coumaric acid equivalents per kg tissue (A) chlorogenic acid (B) 24 hrs after sectioning the tubers of the different cultivars ($P < 0.05$). The data are expressed as the average of two independent experiments with three replicates. Error bars indicate SED.

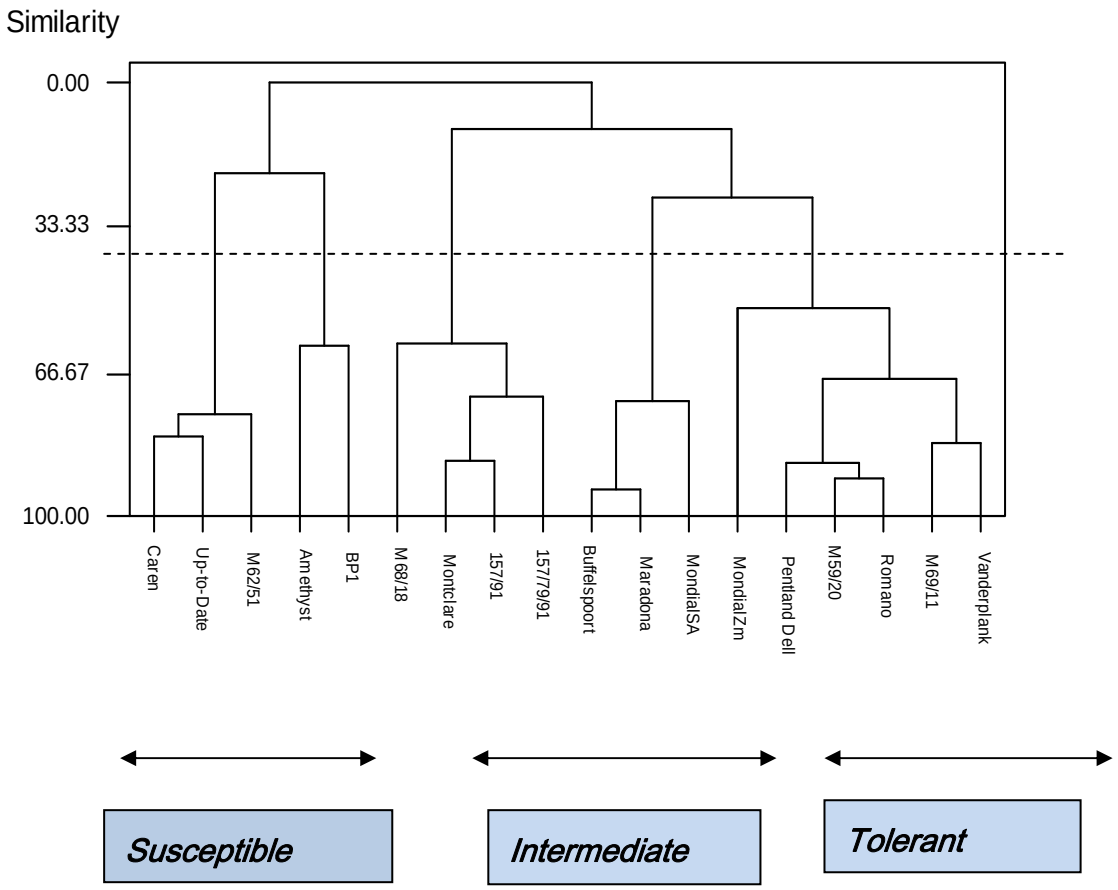


Figure 4.4. Dendrogram for hierarchical clustering of 18 potato cultivars. The dendrogram was created using the software, Minitab Release 12.22, 1998. The dendrogram was cut at the 57.05 % similarity level, producing five subgroups. The data from means of rotting zone diameters, phenylalanine ammonia lyase, peroxidise, polyphenol oxidase, chlorogenic acid, total soluble phenols and cell viability were used in the generation of the dendrogram. The data are expressed as the average of two independent experiments with three replicates.

CHAPTER 5

EFFECT OF CALCIUM SOIL AMENDMENTS IN INCREASING RESISTANCE OF POTATO TO SOFT ROT PATHOGENS

Abstract

Dickeya and *Pectobacterium* species cause blackleg / soft rot disease complex on potato in Zimbabwe. The disease is seedborne and difficult to control. This research focuses on ways of increasing the inherent resistance of potato plants and tubers to blackleg and soft rot. The effect of calcium soil amendment on phenolic compound formation in potato peels and ensuing tuber resistance to the pathogens was investigated. Two field experiments were conducted at the University of Zimbabwe campus plots in 2008 and 2009 summer seasons. Sprouted tubers of cv. BP1 were inoculated with a mixture of *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *brasiliensis* and *Dickeya dadantii*. The inoculated tubers were planted in plots treated with different fertilizer combinations. The treatments were: 1) compound S (7N: 21P: 8K) + ammonium nitrate 2) compound D (7N: 14P: 7K) + calcium nitrate 3) compound S + calcium nitrate and 4) compound D + ammonium nitrate. Blackleg incidence and severity were significantly lower ($P < 0.05$) in the plots where calcium nitrate was applied in both experiments. Soft rot incidence in the progeny tubers was also significantly reduced by the calcium treatment. In addition, calcium significantly reduced ($P < 0.05$) soft rot losses of tubers in storage. Total soluble phenols and calcium content were measured in leaves and tuber peels at physiological maturity. Phenolic compounds were identified as chlorogenic, caffeic and ferulic acid using HPLC analysis. Chlorogenic acid and calcium content were significantly higher ($P < 0.05$) in plants grown in calcium treated plots. Calcium reduced maceration effect of the bacteria, significantly smaller ($P < 0.05$) rotting zone diameters were recorded on tubers harvested from the calcium treated plots. This study shows that soil amendments of calcium reduce blackleg and soft rot diseases in potato and increases concentration of calcium and chlorogenic acid in tuber peels.

5.1 INTRODUCTION

Bacterial soft rots caused by *Pectobacterium atrosepticum* (*Pa*) (Van Hall) Dye and *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (Jones) Bergy *et al.* are a major cause of potato tuber loss in the field, storage and transit in many parts of the world (Pérombelon and Kelman, 1980). Potato blackleg is a seedborne disease caused mainly by *Pectobacterium atrosepticum* under cool environments. *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*), *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya* spp. can also cause blackleg and soft rot diseases under different environmental conditions.

In Zimbabwe, potato growers face the challenge of significant post harvest losses of potato tubers (20 – 60%) due to soft rot (Manzira, 2010). Severe blackleg / soft rot disease complex outbreaks occurred in the 2008 / 9 growing season, causing huge economic losses. The causal agents were *Pcb*, *Pcc*, *Pa* and *Dickeya dadantii* (Chapter 3, this study). The pathogens macerate plant tissues by the production of large quantities of pectolytic enzymes (Flego *et al.*, 1997). *Pectobacterium* spp. secrete pectinases that induce polyphenoloxidase activity in the hosts, which subsequently oxidises phenols, forming a black margin around the infection site. The margin restricts pathogen from spreading (Lovrekovich *et al.*, 1967) Most of the potato cultivars grown in Zimbabwe have some level of susceptibility to soft pathogens (Ngadze *et al.*, 2012 in press).

Seed tubers almost always carry latent infections of soft rot pectobacteria in the lenticels and wounds (Pérombelon, 2002) where they multiply aggressively under favourable environmental conditions until they reach a critical population density of between 10^7 and 10^8 cfu ml^{-1}. They colonize and multiply in the apoplast before plant cell death. A number of factors trigger *Pectobacterium* and *Dickeya* spp. to produce pectolytic enzymes when the tubers are in storage. These factors can be changes in water potential (Kelman *et al.*, 1978),

membrane permeability, intracellular concentrations of reducing sugars (Otazu and Secor. 1981), polyphenol oxidase (Lovrekovich *et al.*, 1967; Tripathi and Verma, 1975) or the phytoalexin rishitin (Lyon *et al.*, 1975). The concentration of oxygen within the tuber tissue is of paramount importance and can decrease rapidly if a film of water is maintained on the tuber surface for several hours (Burton and Wigginton, 1970). At low oxygen conditions the tuber becomes more susceptible to attack by even small numbers of bacteria (De Boer and Kelman, 1978). The best management strategy of blackleg and soft rot disease of potatoes is to suppress or prevent multiplication of soft rot bacteria (Pérombelon, 2002).

Calcium is an essential nutrient and considered one of the most important nutrients associated with plant defense (Datnoff *et al.*, 2007). It has been implicated in the interaction between plant pathogenic bacteria and their host plants. Calcium confers some resistance to pests and diseases in plants via its influence on growth pattern, anatomy, morphology and chemical composition of the plant. Increased plant calcium has been shown to enhance resistance to plant tissue macerating bacterial phytopathogens (Perombelon and Kelman, 1980; McGuire and Kelman, 1984; 1986). Calcium is involved in eliciting signal transduction pathways and in membrane and cell wall integrity (McGuire and Kelman, 1984; Busse and Palta, 2006; Datnoff *et al.*, 2007). Calcium increases the resistance of potato stems and tubers to maceration by pectolytic enzymes such as pectate lyase and polygalacturonase (Flego *et al.*, 1997). Bain *et al.* (1996) reported a significant reduction in blackleg incidence during the growing season after preplant application of gypsum ($CaSO_4$), although the effect decreased towards the end of the season. It also enhances the structural integrity of cell walls and membranes. Adjustments in mineral nutrition could reduce disease severity (Mashner, 1995; Datnoff *et al.*, 2007).

Some nutrients, especially silicon stimulate host defenses in plants by stimulating the production of phenolic compounds and phytoalexins in response to pathogen infection. This

can enhance the activity of defense-associated enzymes (Hammerschmidt, 2005). Infected plants have increased polyphenol oxidase activity and phenol concentration. (Lovrekovich *et al.,* 1967). Phenols and phytoalexins play an important role in defense mechanisms to soft rot bacteria (Ghanekar *et al.*,1984). Potato tubers contain relatively low levels of phenolic compounds (Ramamurky *et al.,* 1992). Wegener and Jansen (2007) reported that the phenolics and anthocyanins were responsible for resistance expression in tuber tissues. Ganekar *et al.,* 1984 found that chlorogenic, caffeic and ferulic acid possess antibacterial activity against soft rot pathogens. The total soluble content was directly proportional to the inhibition of soft rot bacteria (Kumar *et al.,* 1991).

Although calcium has been reported to be effective in disease management in different crops (Benson, 2009), including blackleg and soft rot in potato (McGuire and Kelman, 1984; 1986), the effect of this nutrient on disease resistance in potatoes has not been investigated in Zimbabwe. In addition and most Zimbabwean potato farmers do not apply calcium fertilizers to crops. Some of the soils in Zimbabwe have acidic low CEC and base saturation, possibly leading to calcium and / or magnesium deficiencies in the potato growing regions where growers are do not commonly apply these nutrients. The objective of this study was to determine the effect of calcium on resistance of potato to blackleg and soft rot pathogens and to measure the concentration of phenolic compounds in plants grown in calcium amended soils.

5.2 MATERIALS AND METHODS

5.2.1 Experimental site

Two experiments were conducted at the University of Zimbabwe (UZ) campus in the 2008 (experiment 1) and 2009 (experiment 2) summer seasons (August - December). The UZ campus is situated in Harare (17º50′ South and 31º30′ East) at an altitude of 1500m above

sea level. The area is characterized by fersiallitic red clay soils with more than 40% clay and receives an annual rainfall of between 800 to 1000mm. Average temperatures during the growing season ranged from 20 to 25ºC. The fields were planted to Brassicae prior to the experiment. The crop was hoe weeded when necessary and pests were controlled with carbaryl used at the recommended rate.

5.2.2 Experimental design

The experiment was laid out as a randomized complete block design with four treatments. The treatments were: 1) compound S [7N: 21P: 8K] + ammonium nitrate; 2) compound D [7N: 14P: 7K] + calcium nitrate (19Ca : 15.5N); 3) compound S + calcium nitrate and 4) compound D + ammonium nitrate. The different fertilizer treatments were applied to the appropriate plots. Three blocks were used in the experiment and the treatments were replicated three times in each block. Certified potato seed of cultivar BP1 was used in the experiment.

5.2.3 Agronomic practices

Compounds D (7N: 14P: 7K) and S (7N: 21P: 8K) fertilizers were applied as basal fertilizers at a rate of 1000 kg ha^{-1} in the relevant treatments. Calcium nitrate at a rate of 250 kg ha^{-1} was mixed with basal fertilizer for the relevant treatments. The first application was applied at the planting stations after opening the furrows, and then slightly covered with the soil before planting the tubers at a depth of 10cm. Ammonium nitrate and the second calcium nitrate application were applied as top dressing at a rate of 250 kg ha^{-1} and 250 kg ha^{-1}, respectively, 6 weeks after crop emergence. The fertilizer was placed about 5 cm away from the plants to avoid scorching. The fields were irrigated when necessary and 300 mm of water was applied to the field as a supplement for the whole season.

5.2.4 Chemical properties of soil

Samples were obtained from the two field trial sites on 11 August 2008 and on 10 August 2009, respectively, prior to planting the potatoes. Soil was sampled from 5 random locations (20 cm deep) in each field. Soil chemistry analysis was conducted at the Soil and Plant Analysis Laboratory, University of Zimbabwe using a method described by Baysal *et al.* (2008) with minor modification. The soil samples were dried at room temperature for a week, ground and passed through a 2 mm sieve. Soil pH was analyzed in a 1:25 (wt:wt) water suspension with a pH meter (model Hanna 2210, Sigma-Aldrich). pH was measured before the application of the various fertilizers only. Cation exchange capacity (CEC) and exchangeable cations (CaO, MgO, K_2O) were measured by the shaking extraction method (Page *et al.*, 1982).

5.2.5 Bacterial cultures

Bacterial strains of *P. atrosepticum* (LMG 2386^T, Belgian Coordinated Collections of Microorganisms), *P. c.* subsp. *brasiliensis* (ATCC BAA-419 *Pcb* Strain 371, American Type Culture Collection) and *D. dadantii* (*Erwinia chrysanthemi* 3937, Scottish Crop Research Institute) were used in the study. They were grown for 24 hours at 25˚C in a shaken culture of 25 ml Luria Bertani (LB) broth (pH 7.0) supplemented with 0.1 % pectin from citrus fruits (Sigma) and 0.1 % lyophilized potato cell sap. After centrifugation at 5000 rpm for 5 minutes (4˚C) the bacteria were washed with sterile water, centrifuged again and re-suspended in sterile water before adjusting to the appropriate density (OD_{600} – 0.1).

5.2.6 Inoculation method

The seed tubers were sprouted and inoculated with a mixture of the three pathogens using the method described by Hélias *et al.* (2000) with minor modifications. Three pathogens were used in the study as opposed to the one pathogen used by Helias *et al.* (2000). The sprouted

seed potato tubers were dipped in the inoculum for 15 minutes and dried overnight before planting.

5.3 MEASUREMENTS

5.3.1 Disease incidence

Diseased plants were defined as those showing at least one stem with blackleg or blackleg associated symptoms (soft rot, wilting, internal and external darkening on stems), excluding non-emergence. The plants showing symptoms were then expressed as the percentage of the total number of plants that emerged (Hélias *et al.*, 2000) per treatment per block.

5.3.2 Disease severity

Disease severity assessment was based on a scale developed by Hélias *et al.* (2000) and was carried out fortnightly per plant until the plants reached physiological maturity: The scale was defined as:

i) Wilting / chlorosis (Wlg / chl),

ii) Blackleg (Bl)

iii) Haulm desiccation - corresponding to death of the stems of the plant

iv) Plant death - (Dth) – complete desiccation of all stems

v) Symptomless plants, recorded as healthy (Hth).

5.3.3 Plant mineral analysis

The mineral content of the various tissues of the potato plant was determined by inductively coupled plasma-optical emission spectrometry by the Soil and Plant Analysis Laboratory, University of Zimbabwe. For determination of calcium in the leaf tissue, the top fully expanded leaf was collected from each of 10 plants / block at the flowering stage and these were bulked. For tuber analysis, five tubers were randomly selected at harvest from each treatment

per block and taken as the representative sample. Calcium content in tubers was determined using the method described by McGuire and Kelman (1984) with minor modifications; only the peel was used.

5.3.4 Extraction of phenolic compounds

Five tubers randomly selected from harvested tubers of each treatment were washed under running water and peeled with a potato peeler. Tuber peels were freeze-dried for five days and then ground to a fine powder. Two hundred milligrams of fine powder was passed through a 1mm sieve and placed in a 1.5 ml micro centrifuge tube for extractions. Aliquots of 1 ml of a cold mixture of methanol: acetone: ultra-pure water (7:7:1, v:v:v) were added, vortexed and ultrasonified for 5 min. After sonification, samples were shaken for 20 min at 160 rpm while on ice. Samples were centrifuged for 5 min and the supernatant of each sample was transferred to a 20 ml 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 0.45 µm, 25 mm, Ascrodise, GHP, syringe filters (Separations, South Africa). Samples were stored at 4 ºC until analysis using reverse phase – high performance liquid chromatography (RP – HPLC).

5.3.5 Reverse Phase – High Performance Liquid Chromatography

For identification and quantitative analysis of samples, 10 µl of purified extract per sample was analyzed using RP - HPLC (Hewlett Packard Agilent 1100 series) with a UV diode array detector, at 325 and 340 nm. Separation was achieved on a Gemini 3µ, C18, 110A (Phenomenex®) reverse phase column (250 mm length, 5 µm particle size, 4.6 mm inner diameter). A gradient elution was performed with water (pH 2.6 adjusted with H_3PO_4) and acetonitrile (ACN) and consisted of 0 min, 7% ACN; 0 – 20 min, 20% ACN; 20 – 28 min, 23%

ACN; 28 – 40 min, 27% ACN; 40 – 45 min, 29% ACN; 45 – 47 min, 33% ACN; 47 – 50 min, 80% ACN. Solvent flow rate was 0.7 ml 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 10 min, after each run.

5.3.6 Yield assessment and yield losses

At harvest tubers from each plant were examined for soft rot disease. Healthy tubers were collected in sacks. The weight of tubers was recorded at harvest using a digital weighing scale (model DS410, Teraoka South Africa). Rotten progeny tubers were also weighed and mass converted to percentage yield loss. The mother tubers were inspected for rotting and number of rotten seed tubers was also expressed as a percentage of number of tubers planted per treatment.

5.3.7 Losses of tubers in storage

Forty progeny tubers were randomly selected from each treatment. The tubers from the various treatments were placed in 10 kg pockets of meshed black polythene material, and then stored at 25 ˚C and 60 % relative humidity. The potato tubers were stored for eight weeks and assessed fortnightly for rotting. The number of rotten tubers in each pocket was noted and converted to a percentage.

5.3.8 Maceration of potato tuber tissue by bacteria

Eight Randomly selected seed tubers from each treatment were washed thoroughly under running tap water and surface-disinfected by dipping in 70% ethanol for 5 min, before being cut longitudinally into halves (giving 16 halves). Each tuber half was inoculated with 10 mm diameter filter paper discs (Whatman's No. 1), that had been soaked for 10 min in a bacterial

suspension of *Pcb* (only Pcb was for this test). For the control, filter paper discs were soaked in sterile distilled water for 10 min and placed on tuber halves. One filter paper disc was placed in the pith and four around the edges of each tuber half. The experimental design was a Randomized Complete Block Design (RCBD) with four replicates per treatment (4 half tubers per treatment). After inoculation and incubation at 25˚C for 24 h the filter paper discs were removed and the diameter of each rotting zone was measured. The tuber halves were incubated for a further 24 h and the rotting zone diameter was measured again.

5.3.9 Statistical analysis

The disease progress curves were constructed from severity scores and area under the disease progress curve (AUDPC) was calculated for each treatment using trapezoidal integration (Sigma Plot, 2000).

The trapezoidal integration formula used is:

Trapezoidal integration = yi[(xi=1)-xi] + (1/2)[(yi+1)-yi][xi+1)-xi]

Where xi = time of scoring

Yi = severity score

All disease severity data were square root (x +0.5) transformed before analysis. Analysis of variance (ANOVA) was carried out using a Minitab Version 12 (2001) of Statistical Package. Disease incidence was analysed using repeat measures analysis.The standard error of difference (SED) calculated was used for mean separation when $P<0.05$. The rest of the data was subjected to analysis of variance (ANOVA) using the GenStat statistical package (GenStat, 2002 Release 6.1, Lawes Agricultural Trust, Rothamstead) (Payne, 2003). Means were separated using Fisher's protected Least Significant Difference (LSD at $P < 0.05$).

5.4 RESULTS

5.4.1 Blackleg incidence

Calcium nitrate significantly reduced ($P < 0.05$) blackleg incidence by more than 20% in experiments 1 and 2 at 14 Weeks After Crop Emergence (WACE). The lowest disease incidence was recorded from 2 – 14 WACE in calcium treated plots and the highest disease incidence of 50% was recorded in the plots treated with compound D + ammonium nitrate in both experiments (Figures 1A and B). In experiment 2 no disease was observed in calcium treated plots at 2 WACE (Figure 1B). There was no significant difference in disease incidence in plants grown in plots treated with compound D or compound S top dressed with calcium nitrate in experiments 1 and 2 (Figures 1A and B).

5.4.2 Blackleg severity

Blackleg severity was significantly higher ($P < 0.05$) from 2 to 12 WACE in the plots top dressed with ammonium nitrate in both experiments 1 and 2. The highest disease severity score of more than 6.5 was recorded in the plots treated with compound D + ammonium nitrate in both experiments 1 and 2. This was not significantly different from the disease score recorded for compound D + ammonium nitrate in experiment 2 (Figures 2A and B). Calcium nitrate in combination with either compound D or S significantly reduced ($P < 0.05$) blackleg severity in both experiments. No disease symptom was observed in calcium treated plots in either experiment at 2 WACE.

5.4.3 Soft rot incidence

There were no significant differences in the percentage of rotten mother tubers between treatments (Table 3). The lowest percentage of rotten progeny tubers was recorded in plots treated with calcium nitrate. There was no significant difference in the percentage of rotten progeny tubers between compound D + calcium nitrate and compound S + calcium nitrate in

both experiments 1 and 2. The highest percentage of rotten progeny tubers was recorded in plots treated with compound D + ammonium nitrate in both experiments (Table 3).

5.4.4 Soil and plant nutrients

In 2008 and 2009 preplant soil values were 6.0, 6.7 pH; 193.4, 207.8 ppm CaO; 32.4, 45.8 ppm Mg and 24.3, 29.4 ppm K respectively. CEC in these soils ranged from 14.9 to 15.6 Me/100g. The concentration of calcium in both the leaves and tuber peels was significantly higher ($P < 0.001$) in plants grown in the plots amended with calcium nitrate. The amount of calcium in the leaves was almost double that which was recorded for the tubers in both experiment 1 and 2 (Table 3).

5.4.5 Phenolic compounds

Ferulic acid was not detected at the set parameters in all samples and the results are therefore not included. Tuber peels from all treatments showed low levels of caffeic acid and there were no significant differences between the treatments. However chlorogenic acid concentration was significantly higher ($P < 0.05$) in tuber peels from the calcium treatments. Significantly higher ($P < 0.05$) concentrations of chlorogenic acid were recorded in plots where $Ca(NO_3)_2$ was applied in combination with either compound D or S for both experiments 1 and 2 (Table 2).

5.4.6 Yield assessment and yield losses

The highest yields of 33.87 ton ha^{-1} and 39.27 ton ha^{-1} were recorded in the plots treated with compounds S + ammonium nitrate, for experiments 1 and 2 respectively, while the lowest yields were for the compound D + $Ca(NO_3)_2$ treatments. Significantly higher ($P < 0.05$) yield losses (progeny tubers rotting before harvesting) of 16.53% and 39.27% were recorded in 2008 and 2009 in the plots treated with compound D + ammonium nitrate than in other plots.

5.4.7 Losses in storage

The highest number of rotten tubers was recorded at 4, 6 and 8 weeks after harvesting for the ammonium nitrate top dressing treatments. Calcium significantly ($P < 0.05$) reduced losses in storage. At 2 weeks after harvesting, the percentage of tubers which rotted in storage ranged from 0 % recorded for tubers harvested from plots treated with compound S + calcium nitrate to 5 % recorded for plots treated with compound D + ammonium nitrate for both experiments 1 and 2 (Figures 5 A and B). At 6 weeks, the highest number of rotting tubers was recorded in harvested from plots treated with compound D + ammonium nitrate in experiment 1 and for both compound D + ammonium nitrate and compound S + ammonium nitrate for experiment 2. At 8 weeks tubers from plots top dressed with ammonium nitrate recorded a significantly higher percentage of rotting, more than 20 % (Figures 5 A and B). Calcium significantly reduced the number of tubers which rotted in storage for both experiments 1 and 2, but the total percentage of rotten tubers was lower in experiment 1 than in experiment 2.

5.4.8 Maceration of potato tuber tissue by bacteria

Addition of calcium nitrate to the soil significantly reduced ($P < 0.05$) the maceration effect of *Pectobacterium carotovorum* subsp. *brasiliensis*. Significantly smaller ($P < 0.05$) rotting zone diameters were recorded on tubers harvested from plots treated with $Ca(NO_{3)2}$ in both experiments (Table 5.2).

5.5 DISCUSSION

Calcium is an essential mineral that has been shown to be important in many physiological processes, such as plant defense. A deficiency of calcium in the plant can create conditions favourable for pathogen infection (Rahman and Punja, 2007). Calcium has been implicated in the interaction between plant pathogenic bacteria and their hosts. Increased plant calcium

has been shown to enhance resistance to plant tissue macerating bacterial phytopathogens (Perombelon and Kelman, 1980; McGuire and Kelman, 1984; 1986).

In this study the concentration of calcium was higher in the leaves than in the tubers, which was not surprising because stems and leaves are richer in calcium compounds than underground storage organs. This is related to the inability of phloem to transport calcium from the leaves to the root system (Demarty *et al.*, 1984).

Addition of calcium to the soil resulted in increased levels of calcium in the plant. The plants with higher concentrations of calcium recorded low disease incidence and severity showing that calcium is beneficial in increasing the resistance of potatoes to blackleg disease. This supports the findings of McGuire and Kelman (1984), who reported that more susceptible potato varieties have low calcium levels in the tubers, although the concentration of calcium recorded for the plants in this study were higher than those recorded by McGuire and Kelman (1984). The differences in calcium concentrations might be due to the soil types used, in this study the researchers grew the potatoes in red fersiallitic soils with high CEC whereas McGuire and Kelman used sandy soils with low CEC. Furthermore different calcium formulations were used in the two studies. McGuire and Kelman (1984) used $CaSO_4$ as a source of calcium and in this study the source of calcium was calcium nitrate. Environmental conditions might have also contributed to the differences in calcium concentrations recorded in plant tissues of the two studies. Residual calcium recorded in the soil before planting the two experiments might have contributed to the high concentrations of calcium recorded in all treatments, including those planted in plots not treated with calcium.

Low blackleg and soft rot incidences could also be attributed to resistance associated with a high calcium concentration in the cell walls due to the application of calcium nitrate as a basal fertilizer. This is supported by the findings of Bain *et al.* (1996) and Flego *et al.* (1997) who

reported that pre-planting application of calcium as calcium silicate or gypsum ($CaSO_4$) increased calcium concentration in the cells making the plants more resistant to blackleg development. Calcium also increases cell wall integrity, promotes thicker skin netting and ensures proper cell signaling of pathways involving calmodulin; thus reducing disease incidence and severity (Rahman and Punja, 2007). Calmodulin, a Ca^{2+} binding protein, activates and regulates a number of key enzymes, regulates Ca^{2+} transport within the cell and mediates transfer thereof to the vacuoles. Ca^{2+} is also regarded as an important secondary messenger in the elicitation of phytoalexins, which also play an important role in host defense mechanisms (Zook *et al.*, 1987). There was no correlation between soft rot incidence and blackleg incidence.

A high calcium content in host tissues has been correlated with increased resistance to several diseases (Bateman and Lumsden, 1965; Bateman and Millar, 1966; Forster and Echandi, 1975; McGuire and Kelman, 1983; 1984). Plants grown in nutrient conditions of high Ca^{2+} content showed resistance to soft rot diseases. Calcium nitrate significantly reduced the rotting zone diameters in inoculated tuber halves. The increased resistance in tissues with high levels of calcium has been attributed to decreased maceration owing to calcium deposition in the cell wall pectate and structural enhancement of cell wall integrity (McGuire and Kelman, 1984; 1986; Carpita and Gibeaut, 1993). Bateman and Lumsden (1965) suggested that the excess calcium combines with pectin to form calcium pectate, which is resistant to the action of polygalacturonase (PG).

The ability of *Pc.* subsp. *carotovorum* and other soft rot species to macerate plant tissue is dependent on the massive production and secretion of plant cell wall-degrading enzymes, especially pectinolytic enzymes such as polygalacturonase (Peh), pectin lyase (Pnl) and isoforms of pectate lyase (Pel) (Collmer and Keen, 1986). These enzymes are crucial for the

virulence of *Pectobacterium* subsp. and mutations which affect production and secretion of these enzymes lead to reduced virulence (Reeves *et al*., 1994; Pirhonen *et al*., 1991; 1993). The reduced rotting zone diameters and reduced postharvest losses noted in this experiment could also be attributed to calcium interfering with the production of endopolygalacturonase, an enzyme required in the early stages of infection, which could also in turn lead to reduced virulence. Increased extracellular calcium inactivates the gene which codes for endopolygalacturonase production but does not affect the production of other cell wall degrading enzymes (Flego *et al*., 1997). Inactivation of a single gene encoding a particular pectic enzyme can drastically reduce virulence (Saarilahti *et al*., 1992). Reduced maceration recorded in tubers harvested from the calcium treated plots could have been due to high concentrations of chlorogenic acid and caffeic acid in these tubers. Chlorogenic acid is formed as a defense in potatoes in response to infection or injury (Ghanekar *et al*., 1984). Caffeic acid is known to have antibacterial activity against soft rot bacteria by inhibiting growth (Kumar *et al*., 1991). The combination of caffeic acid and chlorogenic acid may significantly inhibit infection by soft rot pathogens (Ghanekar *et al*., 1984; Kumar *et al*., 1991).

The yields recorded for the plants grown in compound S + ammonium nitrate were in line with the average yields recorded by farmers in Zimbabwe who grow the same variety. The average yield for BP1is estimated to be at 30 t/ha (Manzira, 2010). The lower yield losses recorded in the calcium treated plots could be attributed to the lower nitrogen content in calcium nitrate which was used for top dressing the crop. Ammonium nitrate contains 34 % nitrogen while calcium nitrate contains 15.5 % nitrogen. Lower yields were recorded for plants treated with compound D compared to those grown in plots treated with compound S. These findings support the findings of Manzira (2010), who recommended the use of compound S as a basal fertilizer for potato and compound S also contains a higher content of potash which is required by potatoes.

Chlorogenic, caffeic and ferulic acid are the three main phenolic compounds found in potato tubers and they have antibacterial effects and inhibit growth of soft rot bacteria (Ghanekar *et al.*, 1984; Kumar *et al.*, 1991). Ferulic acid was not detected in all treatments. These results were consistent with the findings of van der Merwe (2009) who did not detect Ferulic acid in tubers grown in silicon amended soil. Although there was no significant difference in the concentration of caffeic acid between treatments, the concentration of caffeic acid was lower than that of chlorogenic acid. This was expected because chlorogenic acid is the storage form of caffeic acid and can be converted to caffeic acid during stress conditions (Ghanekar *et al.*, 1984). Chlorogenic acid was significantly higher in tubers harvested from the calcium amended plots. The results show that calcium has a positive effect on the production of chlorogenic acid which enhances the protection and resistance against soft rot pathogens. Chlorogenic acid is formed in potato tissues in response to infection or injury (Ghanekar *et al.*, 1984). Caffeic acid and chlorogenic acid significantly inhibit soft rot infection when present at the same time (Ghanekar *et al.*, 1984; Kumar *et al.*, 1991).

Several researchers have reported the beneficial effects of calcium in increasing potato resistance against soft rot pathogens (McGuire and Kelman, 1984; 1986; Bain *et al.*, 1996; Flego *et al.*, 1997). This study has confirmed these findings under Zimbabwean conditions. It will be beneficial for the potato growers to supplement calcium in the field in order to reduce the blackleg / soft rot disease complex since calcium improves tuber resistance against the pathogens.

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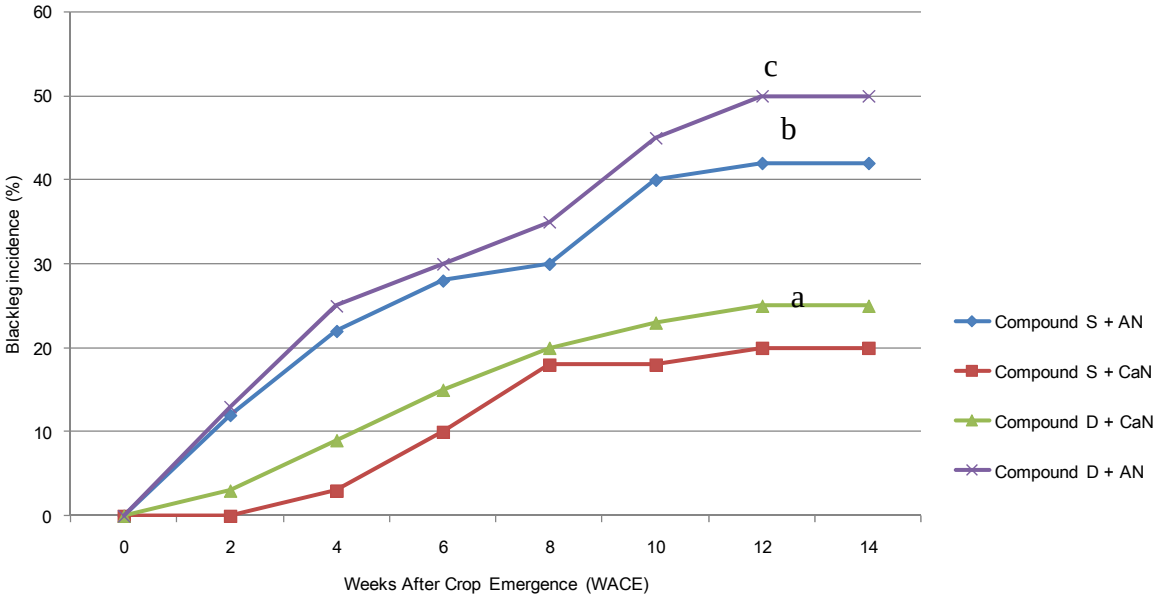
Table 5.1: Chemical composition of soils in the two experimental fields at the UZ campus taken in 2008 and 2009 prior to the experiment.

Year	Field	pH	CEC Me/100g	CaO (ppm)	MgO (ppm)	K_2O (ppm)
2008	Block 4	6.0	15.6	193.4	32.4	24.3
2009	Block 2	6.7	14.9	207.8	45.8	29.4

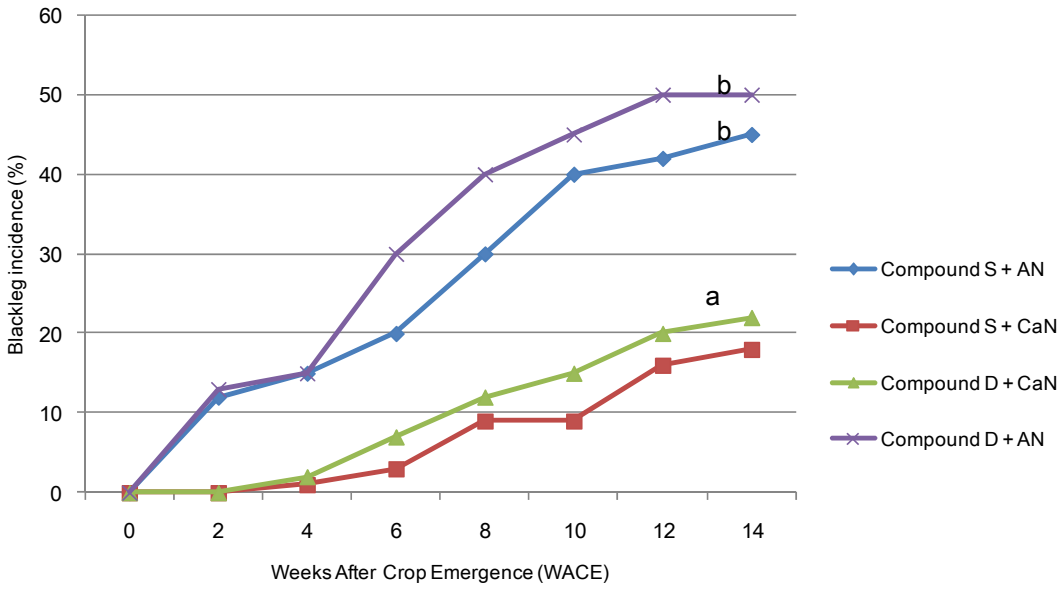
Table 5.2: Soft rot incidence in mother and progeny tubers of BP1 at harvest for experiments 1 (2008) and 2 (2009)

Treatment	Experiment 1 (2008)				Experiment 2 (2009)			
	Yield (tons ha^{-1})	Rotten Mother Tubers (%)	Rotten Progeny Tubers (%)	Rotting zone diameter (mm)	Yield (tons ha^{-1})	Rotten Mother Tubers (%)	Rotten Progeny Tubers (%)	Rotting zone diameter (mm)
CompD+AN	21.52b	41.1	16.53c	29.90b	26.00b	39.7	30.13c	30.00b
CompS+CaN	27.42c	36.1	6.53a	20.10a	32.67c	36.1	3.50a	16.90a
CompD+CaN	16.58a	37.2	5.21a	19.70a	23.73a	35.0	6.44a	18.20a
CompS+AN	33.87d	38.9	9.44b	26.30b	39.27d	38.9	19.55b	28.50b
P-Value	0.003	0.370	<0.001	<0.001	<0.001	0.120	< 0.001	<0.001
SED	1.459	2.830	1.833	0.479	0.827	2.080	3.925	1.174
LSD (0.05)	2.918	NS	3.663	0.954	1.654	NS	7.850	2.348

Means followed by the same letter in a column are not significantly different at LSD 0.05

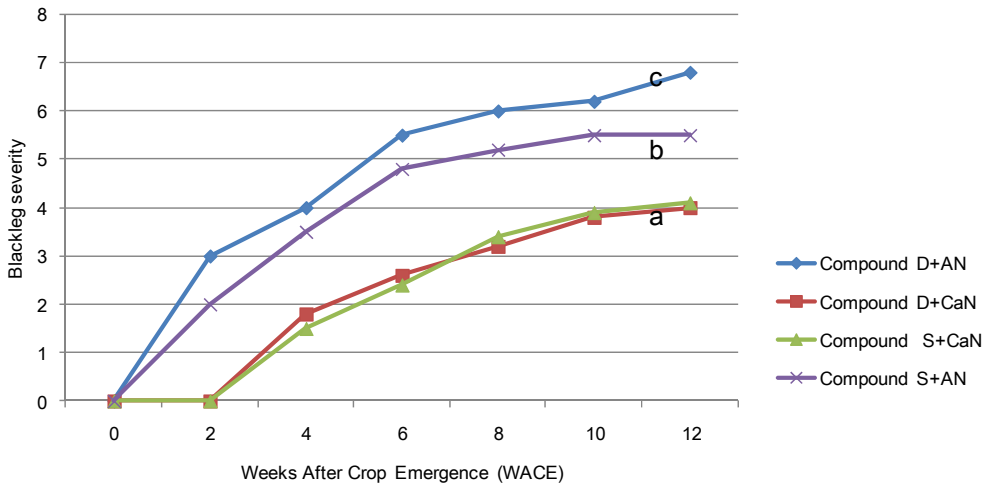


A

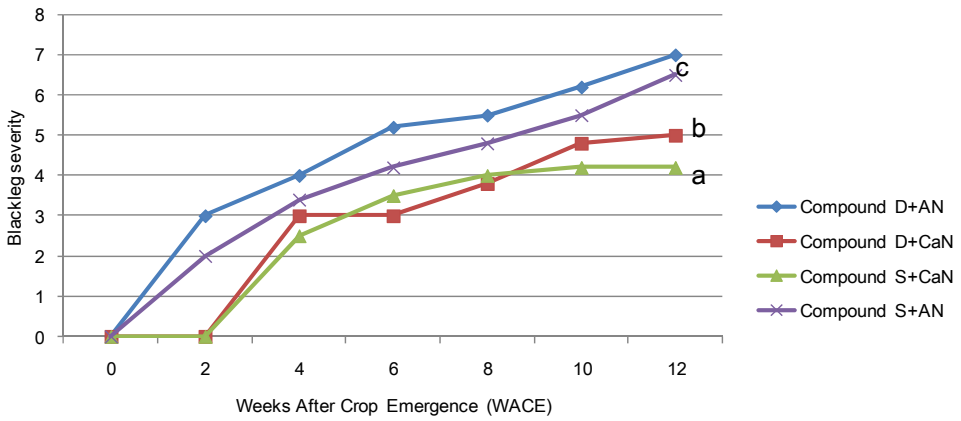


B

Figure 5.1 Field experiments evaluating the effect of calcium application on the incidence of potato blackleg and soft rot in two experiments **A**) 2008 **B**) 2009. Data analysed using repeat measures analysis Different letters denote significant difference according to ANOVA and least significant difference ($P < 0.05$)



A



B

Figure 5.2 Area Under Disease Progress curves calculated for blackleg severity in two experiments **A**) 2008 **B**) 2009. Different letters denote significant difference according to least significant difference ($P < 0.05$).

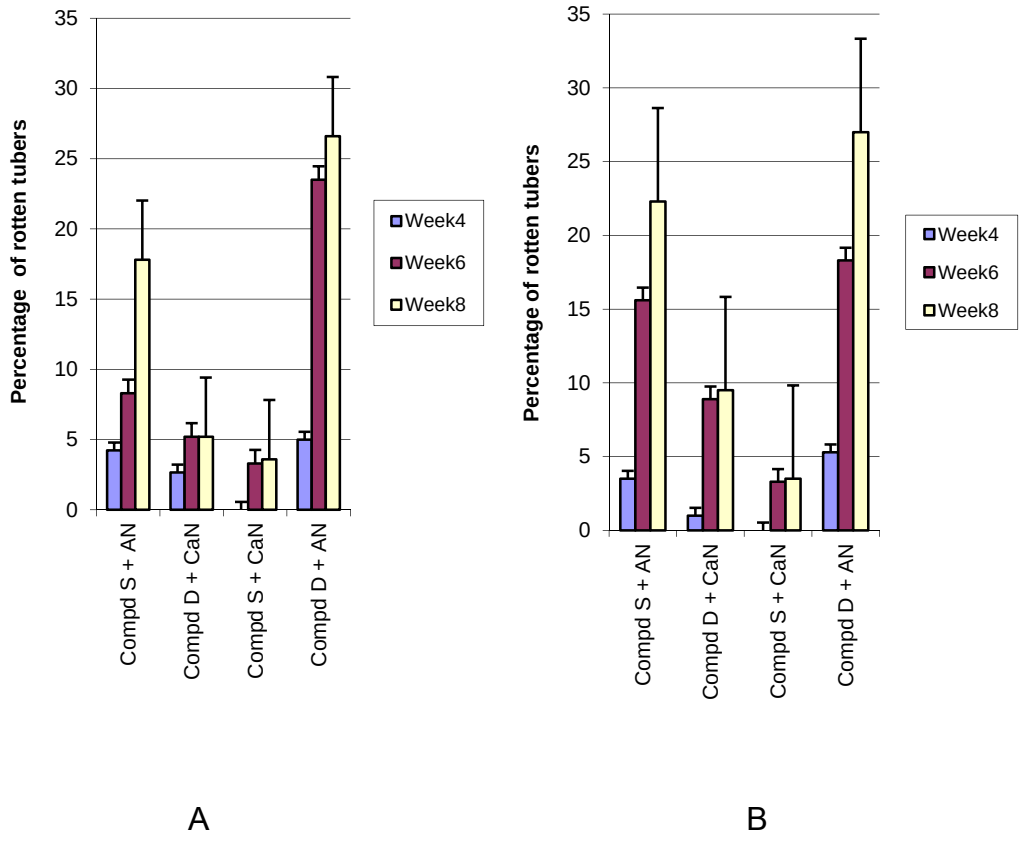


Figure 5.3 Number of tubers which rotted in storage at 4, 6 and 8 weeks after harvesting for experiment 1 (2008) **(A)** experiment 2 (2009) **(B)** ($P < 0.05$). The data represent the mean of three replicates and results were analyzed separately. Error bars on figures represent SED.

CHAPTER 6

SURVEY: TO ASSESS THE DISTRIBUTION AND IMPACT OF POTATO BLACKLEG AND SOFT ROT DISEASES IN ZIMBABWE

Abstract

A survey to assess the distribution and impact of the potato blackleg / soft rot disease complex in Zimbabwe was conducted from September 2009 to June 2010 using an informally structured questionnaire. Sixty-four questionnaires were collected from 9 potato growing areas in Zimbabwe. The farmers are aware of the existence of soft rot post-harvest decay, seed piece decay and blackleg disease. The most predominant management method for the 3 diseases was the use of rotation cycles with various crops. Estimates of economic losses caused by the diseases ranged from <1 to 65 % with the average being approximately 23%. The most popular cultivars in Zimbabwe are Amethyst and BP1, which were ranked as susceptible by more than 50% of the growers. The disease symptoms are more prevalent at temperatures between 15 - 20 ˚C with moist or humid conditions.

6.1 INTRODUCTION

Potato is one of the most popular food crops grown in Zimbabwe as a substitute staple, third after maize and rice (Chigumira wa Ngwerume, 2002). It is widely grown because of its varied uses which include chips, crisps, vegetable relish/salad, canning and livestock feed (Manzira, 2010). In Zimbabwe potatoes have become a common household food because they do not require processing and they are consumed as relish with *sadza* (thick porridge prepared from maize meal) in many marginalized homes. In most backyard gardens, potatoes are a common crop because peels are thrown into the gardens where they sprout and grow into plants which can be harvested. Potato is the fourth largest yielding crop in the world after wheat, rice and maize (FAOSTAT, 2010).

Although potatoes have the potential to produce high yields per area, they are prone to a wide range of pathogens that drastically reduce yield and quality. Pathogens of major importance are

Pectobacterium and *Dickeya* species, which cause the soft rot / blackleg disease complex. Soft rot bacteria produce large quantities of pectolytic enzymes such as pectinases, pectate lyases, cellulases and proteases which cause tissue maceration (Collmer and Keen, 1986; Pèrombelon, 2002). In Zimbabwe potato growers face the challenge of significant post-harvest losses of tubers ranging from 20 to 100 % (Chigumira wa Ngwerume, 2002; Manzira, 2010; Ngadze *et al*., 2010) leading to significant financial losses. A severe outbreak of potato soft rot disease occurred in some of the potato growing regions of Zimbabwe in the 2008/9 season. A survey was thus carried out in the 2009/10 growing season in order to determine the prevalence and impact of the disease complex in Zimbabwe.

Blackleg and soft rot diseases are caused by soft rot bacteria formerly known as *Erwinia* species. Subsequent revisions have led to the taxonomic reclassification of pectolytic *Erwinia* into several genera. Strains formally described as *Erwinia carotovora* have been incorporated into the genus *Pectobacterium*, and strains classified as *Erwinia chrysanthemi* are now assigned to the genus *Dickeya* (Hauben *et al*., 1998; Gardan *et al*., 2003; Samson *et al*., 2005). The bacteria are Gram-negative, non-spore forming, facultative anaerobes, characterized by the production of large quantities of cell wall degrading enzymes which cause disease (Collmer and Keen, 1986).

Pathogenesis of soft rot pathogens is temperature dependent. *Pectobacterium atrosepticum* tends to cause blackleg at temperatures <25◦C and *Dickeya* spp. at higher temperatures (Pèrombelon, 2002). Some cold tolerant strains have been found which can cause blackleg symptoms in cool, temperate climates. *Pectobacterium atrosepticum* tends to cause blackleg at temperatures <25◦C and *Dickeya* spp. at higher temperatures (Pèrombelon, 2002). Some cold tolerant strains have been found which can cause blackleg symptoms in cool, temperate climates.

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa*) are the primary causes of soft rot in temperate climates. *Pectobacterium carotovorum* subsp. *carotovorum*

causes soft rot disease in many plant species and has been isolated from plants with blackleg and aerial stem rot symptoms (Powelson and Apple 1984). This may be attributed to its survival in many environments (Avrova *et al*., 2002), whereas *Pectobacterium atrosepticum* is largely restricted to potato, usually associated with the blackleg disease (Pèrombelon, 2002). Another more virulent strain, *Pectobacterium carotovorum* subsp *brasiliensis* (*Pcb*), has been identified as the major cause of blackleg and soft rot diseases in Brazil (Duarte *et al*., 2004) and in South Africa (van der Merwe *et al*., 2010).

Erwinia chrysanthemi has been reported to be the causal agent of aerial stem rot and wilt disease on potatoes. Taxonomic alteration has separated *E. chrysanthemi* into six species which all fall in the genus *Dickeya.* The strains which infect potato have been classified into several of these six species (Samson *et al., 2005*). These include *Dickeya dadantii*, *Dickeya zeae*, *Dickeya paradisiaca*, *Dickeya chrysanthemi*, *Dickeya dianthicola* and *Dickeya diffienbachiae*.

Latent infection of potato tubers by the soft rot pathogens is widespread and disease tends to develop when host resistance is impaired (Pèrombelon and Kelman, 1980) or when environmental conditions are favourable (Pèrombelon, 2002). Bacterial cells remain dormant but numbers can fluctuate depending on tuber storage conditions, increasing under moist and decreasing under dry conditions (Pèrombelon, 2002). The bacteria can survive for several months in the soil, long enough to bridge the gap between cropping seasons (Pèrombelon, 2002). The main environmental factor for a shift from latency to disease development is the presence of water on tubers, which triggers disease development. The film of water on the tubers leads to anaerobiosis and tissue maceration.

In Zimbabwe *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subspecies *carotovorum* have been listed as the major pathogens causing blackleg and tuber soft rot diseases respectively (Masuka *et al*., 1998) and recently *D. dadantii* has been isolated from potato tubers with typical soft rot symptoms (Ngadze *et al*., 2010). Although *Pectobacterium wasabiae*, *Dickeya* zeae, *Dickeya*

chrysanthemi and *Dickeya dianthicola* have not been identified in Zimbabwe as causal agents of the blackleg / soft rot complex, their existence in the country should not be ignored.

Zimbabwe is divided into five natural regions or agro-ecological zones with rainfall as the main criterion of division. Agricultural production potential of any area in Zimbabwe is dependent on its agro-ecological classification (Table 6.1, Fig. 6.1). Potato seed is mostly produced in Nyanga (Region I) and Harare (Region II) whereas table potato is grown throughout the country as long as there is adequate water for supplementary irrigation. The objective of the study was investigate the farmer's knowledge of soft rot and blackleg diseases, the importance of research to effectively control the disease complex and the perceptions and comments of potato growers regarding the disease in Zimbabwe.

6.2 MATERIALS AND METHODS

A survey was conducted from September 2009 to June 2010 in three of the five Natural Regions (NR) of Zimbabwe. The survey was carried out in nine potato growing areas, namely, Chinhoyi, Darewndale, Gweru, Harare, Marondera, Mazowe, Nyanga, Shamva and Shurugwi. These areas are representative of the 3 Natural regions selected for the survey. An informal structured questionnaire was distributed to seed and table potato growers. Table potato farmers were comprised of large scale and small scale resettled farmers. A random sample of growers in each region completed the questionnaire and the number of participants from each region ranged from 15 to 30. The questionnaire was divided into four main sections drawn up to determine (i) environmental conditions (average daily maximum temperatures in summer and winter and rainfall) in each region (ii) crop production practices (irrigation, type of seed and cultivars planted) (iii) crop protection including control method; application method for the different fungicides used to control various diseases; impact of blackleg and soft rot on yield of potato; pre- and post-harvest yield losses and (iv) general crop production practices. Farmers were asked to rank potato cultivars for blackleg, pre- and post-

harvest soft rot susceptibility on a scale of 1 to 3 with 3 being susceptible, 2 moderately susceptible and 1 resistant. Farmers were also requested to make suggestions about research priorities and general comments about their perceptions of the disease.

Responses were summarized in a table format for statistical analysis and frequencies for the various parameters were calculated using the SPSS statistical package. The frequencies of answers pertaining to irrigation frequency, control practices and susceptibility of cultivars were tested using the chi-square one-sample test. A one way ANOVA was used to test the difference in mean yield losses, temperature and rainfall data for each region using the Minitab Statistical program Minitab Release 12.22 (1998).

6.3 RESULTS

Sixty-four questionnaires were collected from 3 Natural Regions surveyed. The numbers of completed questionnaires from each region are shown in Table 6.2.

6.3.1 Crop production.

Sixty-eight percent of the respondents irrigate their potato crop; of these 53.1% irrigate once a week, 15.6% twice a week and the remaining 31.3% follow other irrigation schedules. These frequencies were not significantly different (X^2 =3.050, p=0.218, df=2). The other irrigation schedules mentioned were 2-3 times per week, only in the early part of the season and when necessary. Ninety-two percent of the respondents use certified seed while the remainder retained their own seed.

6.3.2 Control practices.

The predominant method for controlling blackleg and soft rot diseases was a 1-2 or 4-5 year crop rotation cycle with *Katambora Rhodes* grass, maize, beans, onion, ryegrass or left fallow. With regard to chemical control, 45.3% of the farmers use different fungicides in the field and in storage to control

various diseases in seed tubers. Twenty-five percent of the farmers dust the tubers in storage, 5.4% dip the tubers in fungicide solution prior to planting and 14.9% spray the plants in the field in order to reduce incidences of fungal diseases. The frequencies for the chemical control methods differed significantly from one another (X^2=10.449; P=0.005; df=2).

6.3.3 Major potato cultivars grown in Zimbabwe.

The majority of the growers (81%) planted Amethyst with more than 75% of these respondents planting in excess of 10 ha to Amethyst. BP1 and Montclare were also popular, planted by 58% and 32% of the respondents, respectively. A small proportion of the farmers, 6% and 2% grow Pimpernel and Garnet respectively. The frequencies of respondents who planted Amethyst, BP1 and Montclare were significantly different (X=31.461; P=0.000; df=5). The other cultivars Mondial, Hertha, KY20 and Mnandi were grown by between 1% and 3% of the farmers.

6.3.4 Estimated economic losses.

Economic losses due to blackleg, soft rot and see piece decay ranged from <1% to 65%, with the average being 23% (Table 6.3). Only 56% of 56% of the respondents answered this question.

6.3.5 Ranked susceptibility of cultivars.

Amethyst, the most widely planted cultivar, was rated as the most susceptible variety. Eighty-one percent of the respondents answered this question; 70% ranked Amethyst as the most susceptible, 33.8% as moderately susceptible and only 6.2% as resistant. BP1, the second most popular variety, was ranked as susceptible by 60.5%, moderately susceptible by 24.8% while 4.7% ranked it as resistant. Montclare, which is grown by 15 % of the respondents, was ranked as resistant by 6.2% while the remaining 9.4% ranked it as moderately susceptible. Pimpernel and Garnet were ranked as resistant by all the respondents.

6.3.6 Areas of research requested by farmers.

The section was completed by 85% of the respondents and 75% of them highlighted that there was a need for research on the disease in Zimbabwe. Some of the respondents cited more than one research area. The areas of research requested by growers were summarized as follows (numbers of respondents citing area of research is shown in parenthesis):

- Effective control methods (45)
- Evaluation / selection of resistant cultivars (20)
- Breeding for resistance (15)
- Survival period of the causal pathogens in soil and alternate hosts (30)

6.3.7 General comments of respondents.

Comments were summarized as follows:-

- Soft rot and blackleg disease prevalence increasing especially during wet periods followed by warm to dry temperatures.
- Soft rot incidence higher in potatoes harvested from wet soil.
- Enclosed storerooms result in higher soft rot incidence than open sheds
- Few tubers rot in storage but after emergence plants show symptoms of wilting
- A high proportion of tubers rot during transportation especially when covered

6.4 DISCUSSION

Although the majority of growers plant certified seed, this is not likely to affect the disease situation in the country as the potato seed certification scheme relies solely on visual inspection of the crop in the field and the tubers after harvesting. Many commercial seed grade stocks are contaminated (or latently infected) with bacteria found mainly in the lenticels and suberized wounds on tubers (Pérombelon and Kelman, 1980). Tuber contamination can occur before, during or after harvest and several sources of contamination have been identified which include tubers, irrigation water, machinery and insects. Detection and identification of soft rot pathogens in all potato growing regions

is hampered by lack of reliable and sensitive diagnostic tools to detect the latent infections in seed (Czajwoski *et al*., 2009). The yield losses caused by blackleg and soft rot will not be reduced until the latent infections can be detected in seed lots.

It was interesting to note that both cultivars Amethyst and BP1, ranked by the majority of farmers and by Ngadze *et al*. (2012) as susceptible are still widely grown. The choice of these two cultivars might be attributed to physiological characteristics and yield. The yield for these cultivars has been reported to be in excess of 30 tonnes ha^{-1} under good management practices (Manzira, 2010). Pimpernel and Garnet which were ranked as resistant cultivars are grown by a few farmers who are contracted by the companies which use them for making crisps. These two cultivars are not preferred by consumers because of their poor culinary properties. They are not as high yielding as Amethyst and BP1 (Manzira, 2010).

From the responses, it was evident that the farmers were aware of blackleg and soft rot diseases and this survey has confirmed reports of yield losses as high as 90% if tubers are not handled properly (Chigumira wa Ngwerume, 2002, Manzira, 2010). The differences in estimated yield losses between natural regions could be attributed to a number of factors such control practices, cultivars grown, different perceptions about the disease and different climatic conditions. According to the questionnaire, the highest yield losses due to the soft rot/blackleg complex were recorded in Darwendale followed by Mazowe and Harare. All these areas fall in Natural Region II, characterised by average maximum summer temperatures of 21 –25 ˚C and average maximum winter temperatures of 10 – 15 ˚C. *Pectobacterium carotovorum* subspecies *carotovorum*, *P. carotovorum* subspecies *brasiliensis* and *Dickeya dadantii* were identified as the causal agents of the blackleg / soft rot disease complex. The region receives an average annual rainfall of 750 – 1000 mm and 70% of the farmers irrigate their crop. Marondera, an area found in the same natural region, recorded lower disease incidences because it is much colder than the other areas in the same region. The average yield losses in Natural Region II ranged from 9 to 40% and countrywide the average yield losses are

around 23 %. Yield losses were also reported to be high in Chinhoyi and Shamva (Natural Region III). The rainfall received in this region is not adequate for potato production and all the farmers irrigate their crop. Nyanga, found in Natural Region I has the lowest disease incidence. Disease incidence and severity of blackleg and soft rot diseases depend on temperature and free water (Pérombelon, 2002). Conditions optimal for blackleg and soft rot development are between 15 and 25 ˚C with prevailing wet conditions.

Some of the research areas requested by farmers covered information already known by scientists, for example, the issue pertaining to survival of the pathogen in the soil and alternate hosts. This shows that there is a knowledge gap that can be bridged by scientists networking and collaborating with the farmers. Farmers should participate in some of the trials dealing with evaluation of cultivars for resistance to the pathogens and selection of effective control strategies.

Research in Zimbabwe should focus on identification of effective control strategies and epidemiological studies in order to understand the disease development process in the country. Since blackleg and soft rot diseases originate from infected seed tubers, research should focus on the development of reliable and sensitive pathogen detection techniques which can be used in the screening of seed tubers.

6.5 ACKNOWLEDGEMENTS

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Table 6.1: Rainfall characteristics of the five agro-ecological zones of Zimbabwe and suitable agricultural activities (adapted from FAO sub-regional office for East and Southern Africa, 2000).

Agro-ecological zone	Area (km^2)	% of total	Rainfall Characteristics	Agricultural activities
I	7,000	2	More than 1050mm per annum with some rain in all months	Specialized and diversified farming region. Suitable for forestry, temperate fruit and intensive livestock production
II	58,600	15	700-1050mm confined to summer. Infrequent heavy rainfall. Subject to seasonal droughts	Flue-cured tobacco, maize, soybean, cotton, sugar beans and coffee can be grown. Sorghum, groundnuts, seed maize, wheat and barley are also grown. Wheat and barley grown in winter under irrigation. Mixed cropping with poultry, beef and dairy production common.
III	72,900	18	500-700mm per annum. Infrequent heavy rainfall. Subject to periodic seasonal droughts, prolonged mid season dry spells and unreliable starts of the season.	A semi intensive farming area. Smallholder farmers occupied 39% of this area and most of the land was used for extensive ranching before resettlement in 2000. Maize production dominated commercial production. Irrigation played an important role in sustaining crop production in commercial farming areas.
IV	147,800	38	450-600mm per annum	Suitable for extensive ranching and wildlife management. Too dry for successful crop production of most crops suitable for sorghum and millets and other drought tolerant crops. Maize is commonly grown by smallholder farmers. Sugar cane and cotton are produced under irrigation in large estates.
V	104,400	27	Normally less than 500mm per annum	Extensive ranching and wildlife management are the most suitable activities.
Total	390,700	100		

Table 6.2: The number of completed questionnaires received from the different areas surveyed.

Natural Region	Area surveyed	Number of questionnaires
I	Nyanga	20
II	Harare	10
	Marondera	5
	Mazowe	7
	Darwendale	4
III	Shamva	7
	Chinhoyi	3
	Shurugwi	3
	Gweru	5

Table 6.3: Mean estimated yield losses due to pre- and post-harvest soft rot and blackleg losses in potato growing regions of Zimbabwe.

Potato growing Region	Soft rot seed piece decay post planting (%)	Blackleg (Pectobacterium wilting) (%)	Soft rot postharvest (%)
Nyanga	2	5	10
Harare	21	10	20
Marondera	8	3	5
Mazowe	20	8	35
Darwendale	40	10	65
Shamva	8	15	20
Chinhoyi	10	<1	20
Gweru	<1	2	10
Shurugwi	0	1	5

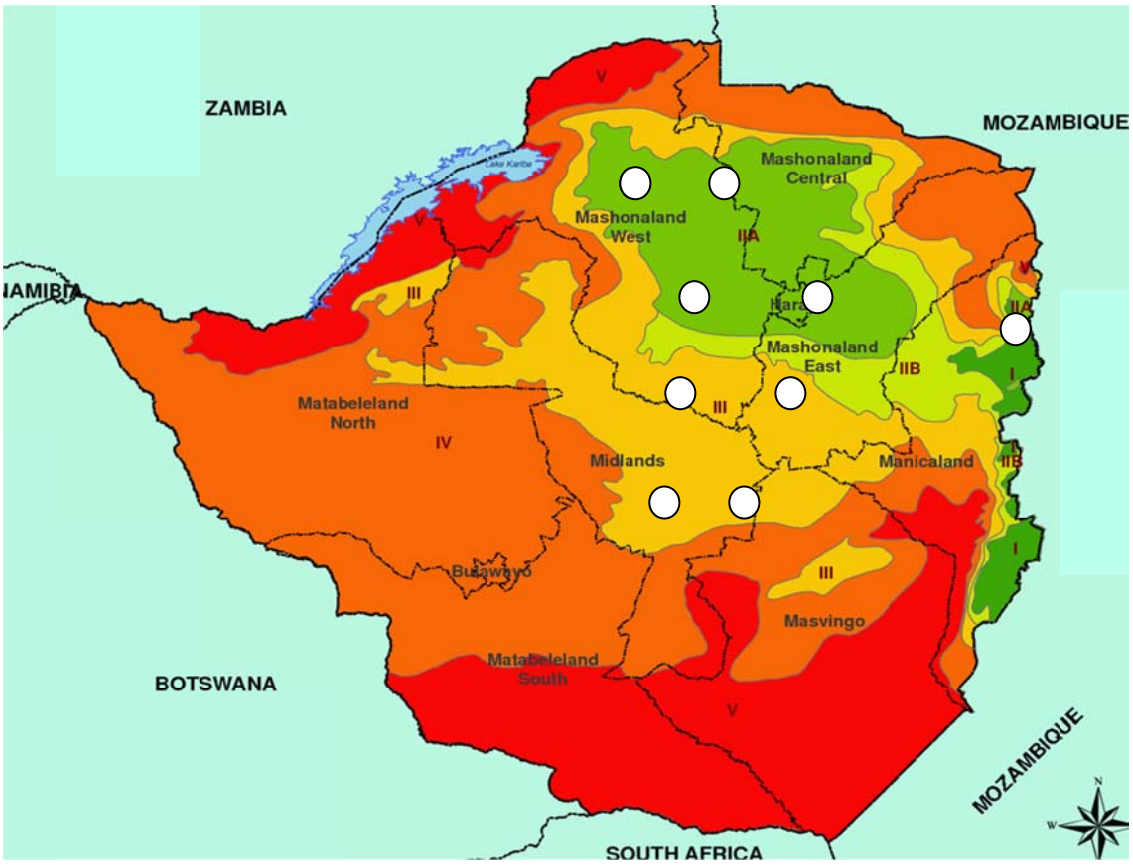

Figure 6.1 Map showing the natural regions of Zimbabwe. Areas surveyed are shown by white circles. (Adapted from http://relifweb.int/sites)

SUMMARY

Potato production in Zimbabwe is severely constrained by soft rot pathogens, namely *Pectobacterium* and *Dickeya* species which can cause yield losses estimated to be between 20 and 60 % depending on environmental conditions. The bacteria produce cell wall degrading enzymes which break down pectin and release nutrients which are essential for microbial growth.

The objectives of this study were to identify the pathogens associated with blackleg and soft rot on potatoes in Zimbabwe; assess the impact and prevalence of blackleg and soft rot diseases in Zimbabwe; evaluate cultivars grown in Zimbabwe and South Africa for tolerance to *Pectobacterium* and *Dickeya* spp.; assess the role of calcium soil amendments in increasing resistance against blackleg and soft rot pathogens and to determine the genetic diversity of *Pectobacterium carotovorum* subsp. *brasiliensis* isolates from South Africa, Zimbabwe and from mini-tubers imported from China.

In chapter 3 bacterial isolates from infected plants were identified using biochemical and phenotypic characteristics, rep-PCR, Amplified Fragment Length Polymorphisms (AFLP) and sequences of *gyrB* and *recA* genes. The results revealed the identity of pectolytic enterobacteria infecting potatoes in Zimbabwe. *Dickeya dadantii* subsp. *dadantii* (*Dd*) was the most dominant pathogen followed by *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pcb*). *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa)* were also isolated from infected plants. Phylogenetic analysis of the *recA* and *gyrB* gene sequences and rep-PCR fingerprinting of genomic DNA demonstrated high genetic homogeneity between the potato isolates from Zimbabwe and reference strains. This is the first report of *Dd* and *Pcb* on potato in Zimbabwe. Misdiagnosis of enterobacterial strains in Zimbabwe may have been due to limitations in diagnostic techniques to differentiate bacterial strains as earlier identification was solely based on biochemical and physiological assays.

AFLP analysis provides a basis for an evolutionary analysis of *Pcb* pathotypes. Genetic diversity of *Pcb* isolates from South Africa and Zimbabwe, and from mini-tubers imported from China was analysed using AFLP analysis. The analysis separated the strains into 12 clusters, reflecting subdivision in terms of geographic origin. A large degree of DNA polymorphism was evident between these 12 clusters, most strains collected from the same geographical area clustered together in the UPGMA tree, showing that *Pcb* populations from each area represent a distinct phenetic group. However in some cases, where numerous phenetic groups exist, it is possible that different founder effects occurred, probably because different potato cultivars are grown in diverse geographical areas in South Africa and Zimbabwe. Despite the genetic diversity evident in *Pcb*, a characteristic pattern of 2 to 3 bands was clearly visible in all isolates, providing potential molecular markers for identification and diagnosis.

The main feature distinguishing *Pectobacterium* and *Dickeya* species from othe enerobacteria is the production of large quantities of pectolytic enzymes. Pectinases, one of the enzymes produced, induces polyphenol oxidase (PPO) activity in the host. PPO subsequently oxidises phenols, forming a black margin around the infection foci and the margin restricts the pathogen from spreading. In chapter 4 the role of polyphenol oxidase, phenylalanine ammonia lyase, chlorogenic acid and total soluble phenols in imparting tolerance in potato to the soft rot pathogens was investigated. Potato varieties grown by farmers in South Africa and Zimbabwe were evaluated. Significantly higher enzyme activities of PPO and PAL as well as higher concentrations of chlorogenic acid and total soluble phenols were recorded in resistant cultivars. The resistance coincided with high PPO and PAL enzyme activity as well as higher concentrations of chlorogenic acid and total soluble phenols. The rotting zone diameter was significantly smaller and the viability of cell was relatively higher in varieties which recorded higher concentrations of these four compounds. The study showed that these compounds play a role in imparting resistance in potato to pectolytic pathogens.

Some of the soils in Zimbabwe have acidic low CEC and base saturation, possibly leading to calcium and / or magnesium deficiencies in the potato crop because most farmers do not fertilise with these nutrients. Calcium deficient potato plants are more susceptible to soft rot pathogens. Chapter 5 focussed on ways of increasing inherent resistance of potato plants and tubers to blackleg and soft rot pathogens by increasing the amount of calcium in the soil. The effect of calcium soil amendments on formation of phenolic compounds in the peel and ensuing tuber resistance to the pathogens was investigated. The results showed that calcium soil amendments increase concentrations of calcium in the plant, which result in increased chlorogenic acid concentrations and subsequently in improved resistance to soft rot pathogens. In addition, calcium significantly reduced the maceration effect of soft rot pathogens and soft rot losses of tubers in storage. Pre-plant application of calcium to the soil reduced blackleg and soft rot diseases in potato.

A major outbreak of the blackleg / soft rot disease complex occurred in the 2007/8 growing season in several potato growing seasons in Zimbabwe. A survey was carried out in the following year and samples of infected plants and tubers were also collected at the same time so that the pathogens responsible for the disease could be identified. The survey carried out in chapter 6 provided information which can be useful for researchers, growers and the potato industry. The information collected gave an insight of the epidemiology, population biology, economic status and control strategy for blackleg / soft rot disease complex in Zimbabwe.

The study has shown that the blackleg / soft rot disease complex in Zimbabwe is caused by several pathogens, namely *Dd*, *Pa*, *Pcb* and *cc*. The findings have indicated that PPO, POD, PAL, chlorogenic acid and total soluble phenols play a role in disease resistance against *P. atrosepticum*, *P. carotovorum*. subsp. *brasiliensis* and *D. dadantii*. Potato varieties with a high concentration of these compounds in tuber tissue can exhibit tolerance to pathogen attack.

Several researchers have reported the beneficial effects of calcium in increasing potato resistance against soft rot pathogens. This study has confirmed these findings under Zimbabwean conditions. It will be beneficial for growers to supplement with calcium in the field so as to reduce the intensity of the blackleg / soft rot complex since calcium improves tuber resistance against the pathogens. Useful information was gathered during the survey. Research in Zimbabwe should focus on development of effective control strategies and epidemiological studies in order to understand the disease development process in the country. The project enhanced the understanding of the disease complex and possible management strategies have been highlighted.

APPENDICES

Appendix A

A LIST OF BACTERIAL ISOLATES FROM POTATO (*SOLANUM TUBEROSUM*) PLANTS USED IN STUDY

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
1	*P.c. subsp. brasilinsis*	15/12/2007	Potato	Gwebi
2	*P.c. subsp. brasilinsis*	15/12/2007	Potato	Gwebi
3	*Dickeya dadantii*	15/12/2007	Potato	Gwebi
4	*P. carotovorum subsp brasiliensis*	15/12/2007	Potato	Darwendale
5	*P.c. subsps brasilinsis*	15/12/2007	Potato	Harare
6	*P. carotovorum subsp brasiliensis*	10/02/2008	Potato	Harare
7	*P. carotovorum subsp carotovorum*	10/02/2008	Potato	Harare
8	*Not Identified*	10/02/2008	Potato	Harare

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
9	*Not Identified*	10/02/2008	Potato	Harare
10	*Dickeya dadantii*	22/04/2008	Potato	Marondera
11	*Dickeya dadantii*	22/04/2008	Potato	Marondera
12	*Dickeya dadantii*	22/04/2008	Potato	Marondera
13	*Dickeya dadantii*	22/04/2008	Potato	Mazowe
14	*Dickeya dadantii*	22/04/2008	Potato	Mazowe
15	*Not Identified*	22/04/2008	Potato	Mazowe
16	*P.c. subsps brasilinsis*	6/5/2010	Potato	Mazowe
17	*Dickeya dadantii*	6/5/2008	Potato	Mazowe
18	*P.c. subsps brasilinsis*	6/05/200	Potato	Mazowe
19	*P.c. subsps brasilinsis*	6/5/2008	Potato	Mazowe

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
20	*P. carotovorum subsp carotovorum*	6/5/2008	Potato	Mazowe
21	*P. carotovorum subsp carotovorum*	12/5/2008	Potato	Nyanga
22	*Not Identified*	12/5/2008	Potato	Nyanga
23	*P.c. subsps brasilinsis*	12/5/2008	Potato	Nyanga
24	*P.c. subsps brasilinsis*	12/5/2008	Potato	Nyanga
25	*P.c. subsps brasilinsis*	12/5/2008	Potato	Harare
26	*P.c. subsps brasilinsis*	12/5/2008	Potato	Harare
27	*Not Identified*	12/5/2008	Potato	Harare
28	*P. carotovorum subsp carotovorum*	12/5/2008	Potato	Harare
29	*Dickeya dadantii*	12/5/2008	Potato	Nyanga
30	*P. carotovorum subsp carotovorum*	12/5/2008	Potato	Nyanga
31	*P. carotovorum subsp carotovorum*	11/11/2008	Potato	Darwendale

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
32	*P. carotovorum subsp carotovorum*	11/11/2008	Potato	Darwendale
33	*Not Identified*	11/11/2008	Potato	Gweru
34	*Not Identified*	11/11/2008	Potato	Gweru
35	*P. carotovorum subsp carotovorum*	9/12/2008	Potato	Gweru
36	*P.c. subsps brasilinsis*	9/12/2008	Potato	Mazowe
37	*P.c. subsps brasilinsis*	9/12/2008	Potato	Mazowe
38	*Dickeya dadantii*	9/12/2008	Potato	Mazowe
39	*P. carotovorum subsp carotovorum*	9/12/2008	Potato	Mazowe
40	*Dickeya dadantii*	28/01/2009	Potato	Gweru
41	*Not Identified*	28/01/2009	Potato	Gweru
42	*Not Identified*	28/01/2009	Potato	Gweru

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
43	*Not Identified*	28/01/2009	Potato	Harare
44	*Dickeya dadantii*	28/01/2009	Potato	Harare
45	*Dickeya dadantii*	28/01/2009	Potato	Harare
46	*Dickeya dadantii*	28/01/2009	Potato	Harare
47	*Dickeya dadantii*	28/01/2009	Potato	Darwendale
48	*Dickeya dadantii*	18/02/2009	Potato	Gwebi
49	*Not Identified*	18/02/2009	Potato	Gwebi
50	*Dickeya dadantii*	18/02/2009	Potato	Gwebi
51	*P. carotovorum subsp brasiliensis*	18/02/2009	Potato	Shurugwi
52	*P. carotovorum subsp brasiliensis*	18/02/2009	Potato	Nyanga
53	*Pectobacterium atrosepticum*	18/02/2009	potato	Nyanga
54	*Pectobacterium atrosepticum*	18/02/2009	Potato	Nyanga

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
55	*Pectobacterium atrosepticum*	4/03/2009	Potato	Nyanga
56	*P. carotovorum subsp carotovorum*	4/03/2009	potato	Nyanga
57	*P. carotovorum subsp brasiliensis*	4/03/2009	Potato	Shamva
58	*P. carotovorum subsp carotovorum*	4/03/2009	Potato	Shamva
59	*Dickeya dadantii*	4/03/2009	Potato	Nyanga
60	*Pectobacterium atrosepticum*	4/03/2009	potato	Nyanga
61	*P. carotovorum subsp carotovorum*	23/10/ 2009	Potato	Harare
63	*P. carotovorum subsp carotovorum*	23/10/2009	Potato	Harare
66	*P. carotovorum subsp brasiliensis*	28/10/2009	Potato	Marondera
67	*P. carotovorum subsp brasiliensis*	5/12/2009	Potato	Nyanga
68	*P. carotovorum subsp brasiliensis*	5/12/2009	Potato	Nyanga
69	*P. carotovorum subsp carotovorum*	5/12/2009	Potato	Nyanga

Isolate no.	Scientific name	Isolation date	Host plant	Region isolated from
70	*P. carotovorum subsp carotovorum*	18/12/2009	Potato	Darwendale
71	*P. carotovorum subsp carotovorum*	18/12/2009	Potato	Harare
72	*Dickeya dadantii*	18/12/2009	Potato	Harare
73	*Dickeya dadantii*	15/01/2010	Potato	Grweru
74	*Dickeya dadantii*	15/01/2010	Potato	Gweru
75	*P. carotovorum subsp carotovorum*	15/01/2010	Potato	Mazowe
76	*Dickeya dadantii*	17/03/2010	Potato	Harare
77	*Dickeya dadantii*	17/03/2010	Potato	Harare
78	*P. carotovorum subsp brasiliensis*	23/05/2010	Potato	Gweru
79	*P. carotovorum subsp brasiliensis*	23/05/2010	Potato	Gweru
80	*P. carotovorum subsp brasiliensis*	23/05/2010	Potato	Gweru
81	*P. carotovorum subsp brasiliensis*	23/05/2010	Potato	Shurugwi

Appendix B

ERWINIA POTATO RESEARCH PROJECT QUESTIONNAIRE

Crop Science Department: University of Zimbabwe

Producer ……

Tel No. ……

Fax No. ……

Email address ……

Region ……

Name of Farm ……

Postal address ……

1. Average maximum temperature

Summer/Winter

	15 – 20^{0}C	21 – 25^{0}C	26 – 30^{0}C	> 30^{0}C
<10^{0}C	10 – 15^{0}C	16 – 20^{0}C	21 – 25^{0}C	> 25^{0}C

2. Average rainfall per year? (mm)

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

3. Are the potatoes irrigated?

Yes	No

How often?

Weekly	2x per week	Other

4. Do you use certified seed?

Yes	No

5. Are you familiar with: (Yes/No)

Soft rot – seed piece decay	Blackleg (Erwinia wilt)	Other

6. What measures are implemented on the farm to control *Erwinia*?

None	Crop rotation Which crops?	Removal of infected plants (rouging)	Other

7. Are potatoes treated with any chemicals to control *Erwinia*?

Yes	No

If yes: Chemicals used

……

Dosage

……..

Method of application

Dip	Spray	Dusting	Other

8. Please evaluate the cultivars that you are familiar with for their susceptibility to *Erwinia* diseases.

1 = resistant; 2 = moderately susceptible; 3 = susceptible

Cultivar	Soft rot (Post planting)			Blackleg			Soft rot (Post harvest)		

9. What are the estimated yield losses (in $ or %) of potatoes due to *Erwinia* diseases?

- Soft rot (seed piece decay, post planting)

……

- Blackleg (*Erwinia* wilt)

……

- Soft rot (post harvest)

……

10. What aspects do you think are important for future *Erwinia* research?

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