Characterisation of *Pectobacterium carotovorum* subsp. *brasiliense* isolates causing blackleg and soft rot diseases of potato in South Africa

by

MPHARISENI LEONARD MASHAVHA

Submitted in partial fulfilment of requirements

for the degree of

Master Scientae (Biotechnology)

In the Faculty of Natural and Agricultural Sciences

Department of Microbiology and Plant Pathology

University of Pretoria

March 2013

DECLARATION

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

Mphariseni Leonard Mashavha

March 2013

ACKNOWLEDGEMENTS

I would like to acknowledge God Almighty, through Jesus Christ, without whom I would not be where I am today.

I am very grateful and wholly indebted to the most invaluable help and guidance of my supervisor, Dr Lucy N. Moleleki. Her kind and enduring patience, support and suggestions guided my research to reach this pinnacle today.

Prof. Teresa Coutihno my co-supervisor is hereby gratefully acknowledged for the motivation and guidance throughout.

Professor Mike Wingfield and the FABI family are acknowledged for offering us the infrastructure and warm fellowship in the beginning of this work.

Prof. Ian Toth from James Hutton Insititute is kindly acknowledged for his guidance and motivation throughout my research.

Gratitude is hereby extended to my sponsors for financial assistance. The National Research Foundation (NRF) and the Technology Innovation Agency of South Africa (TIA).

I would also like to thank and acknowledge my friends and colleagues who were always there to offer support and encouragement throughout. Mr Daniel Montsho (Greenhouse), Mr Edward M. Onkendi, Mr Michael Mbenoun, Mr Divine Shyntum, Dr Gilbert Kamkang, Mr Charles K. Wairuri, Mr Aobakwe “Buxx” Mongae, Ms Gugulethu C. Kubheka and Mr. Rudi Pretorius.

Not least of all, I would also like thank my family (Parents and brothers) for their patience, understanding, love and encouragement throughout the course of this project.

Characterisation of *Pectobacterium carotovorum* subsp. *brasiliense* isolates causing blackleg and soft rot diseases of potato in South Africa

by

Mphariseni Leonard Mashavha

Supervisor:	Dr L.N Moleleki
Co-Supervisor:	Prof T.A. Coutinho
Department:	Plant Pathology and Microbiology
Degree:	University of Pretoria
	MSc. Biotechnology

ABSTRACT

Pectobacterium carotovorum subsp. *brasiliense* (*Pcb*) is a plant pathogenic bacterium that causes blackleg and tuber soft rot disease of potato worldwide. *Pectobacterium* spp. are characterized by the secretion of large quantities of plant cell wall degrading enzymes. As the name indicates, *Pectobacteria* are pectinolytic pathogens, producing enzymes such as pectate lyase, polygalacturonase, and many others that are used to catalyse the breakdown of pectin, the main plant cell wall component. Consequently, virulence of *Pectobacteria* is highly reliant upon the production and secretion of macerating enzymes. Hence these bacteria are also referred to as "brute-force" pathogens. Infection and disease symptoms on plants commonly result in the development of blackleg disease, a characteristic black-like decay extending on the stems of infected potato plants. Furthermore, the infection of tubers results in the development of soft rot disease. *Pcb* is of particular interest in that among *Pectobacterium* spp. such as *Pectobacterium atrosepticum (Pa), P. carotovorum* subsp. *carotovorum (Pcc),* and *P. wasabiae*, *Pcb* strains are reported to be the most aggressive and virulent pathogens causing blackleg and soft rot disease of potato in many growing regions across the world. The fact that strains of *Pcb* were recently reported and isolated in South Africa has necessitated that this work be undertaken in order to characterise this newly described important pathogen of potato in regard to its phenotypic,

genetic diversity, virulence and host range traits. Therefore in this work *Pcb* strains were subjected to multilocus phylogenetic analyses (MLSA) in order to investigate and determine whether there is any interspecies and intraspecies genetic diversity among the South African *Pcb* isolates. It was thus established that there is a significant genetic diversity that exists both on an interspecies and intraspecies level among *Pcb* isolates. As a result we sought to investigate further if the level of genetic diversity observed can be reflected in terms of the pathogen's virulence, biochemical, phenotypic as well as host range characteristics. The results of virulence assays on potato tubers and stems indicated that *Pcb* strains are significantly much more virulent on potato compared to closely related *Pectobacterium* spp. such as *Pa* and *Pcc*. Moreover, the level of intraspecies diversity observed through phylogeny was also evident and reflected on the phenotypic, virulence and host range characteristics of the pathogen. This study also focused on investigating virulence factors employed by *Pectobacterium* spp. during infection. Such factors include the ability to produce and secrete of various extracellular macerating enzymes, as well as screening for the presence of virulence associated effectors and phytotoxin genes. It was of interest to observe that *Pcb* strains have the ability to grow and produce substrate-degrading enzymes much more rapidly compared to *Pa* and *Pcc*. This phenomenon was also observed in virulence assays where *Pcb* strains were noted to cause more rapid and most severe maceration symptoms on potato tubers and stems. Thus in agreement with other studies, our results suggests that *Pcb* is a uniquely sophisticated but diverse plant pathogen which can be considered to be one of the most aggressive causal agents of blackleg and soft rot disease of potato in South Africa.

Key words: *Pectobacterium carotovorum* subsp. *brasiliense (Pcb),* Multilocus sequence analyses (MLSA), blackleg, soft rot.

TABLE OF CONTENTS

CHAPTER ONE

A review of *Pectobacterium* species causing soft rot and blackleg disease of potato in South Africa.

CHAPTER TWO

Multilocus sequence analysis of *Pectobacterium carotovorum* subsp. *brasiliense* isolates from potatoes and other hosts in South Africa

CHAPTER THREE

Phenotypic diversity, host range and virulence of *Pectobacterium carotovorum* subsp. *brasiliense* isolates in South Africa

CHAPTER FOUR

General summary and conclusion

LIST OF FIGURES

CHAPTER TWO:

CHAPTER THREE

LIST OF TABLES

CHAPTER TWO:

CHAPTER THREE

APPENDICES

CHAPTER TWO

ABBREVIATIONS

% - percent

icdA- isocitrate dehydrogenase

+ - positive-sense

A260 - absorbance at 260 nanometre

A280 - absorbance at 280 nanometre

acnA- aconitate hydratase 1

AFLP- amplified fragment length polymorphism

AHL- *N*-acyl-homoserine lactone

ANOVA- Analysis of variance

ATs- autotransporters secretion

bp - base pairs

cfa-coronafacic acid gene

CFA-coronafacic acid protein

cfa6- coronafacic acid gene fragment-6

cfa7- coronafacic acid gene fragment-7

cfl-coronafacic ligase

-CFU-colony forming unit

CMA-coronamic acid

COR-coronatine

DNA - deoxyribonucleic acid

dNTP - 2' -deoxynucleoside- 5' -triphosphate

EDTA - ethylene diamine tetra-acetic acid

et al. - et alia (and others)

expI-exoprotein inducer

ExpR1-exoprotein receptor1

ExpR2-exoprotein receptor2

Fig. - figure

g - gram

gapA- glyceraldehyde-3-phosphate dehydrogenase A

h - hour

HAI2- horizontally acquired island2

Hcp-hemolysin co-regulated protein

HR- hypersensitivity response

Hrc- hypersensitivity response conserved

Hrp- hypersensitivity response and pathogenicity

HrpK- hypersensitivity response factor K

HrpN- hypersensitivity response factor N

HrpW- hypersensitivity response factor W

ITS-intergenic transcribed spacer region

kb - kilobase pairs

kDa - kilodalton

KOH- Potassium hydroxide

l - litre

LB-Luria-Bertani

M - molar

m/v - mass per volume

MAFFT-multiple sequence alignment

mdh-malate dehydrogenase

MEGA- molecular evolutionary genetics analysis

$MgSO_4$-Magnesium sulphate

min - minutes

ML- maximum likelihood

ml - millilitre

MLSA-multilocus sequence analyses

mm - millimetres

mM - millimolar

mtlD- mannitol-1-phosphate 5-dehydrogenase

MW - molecular weight

NaCl-sodium chloride

NaOCl-sodium hypochlorite

NCBI-national center for biotechnology information

ND-nanodrop

Nm-nanometres

nt - nucleotide

^{O}C - degrees Celsius

OD-optical density

Pa-Pectobacterium atrosepticum

PAUP-phylogenetic analysis using parsimony

Pcb-Pectobacterium carotovorum subsp. *brasiliense*

Pcc-Pectobacterium carotovorum subsp. *carotovorum*

PCR - polymerase chain reaction

PCWDEs-plant cell wall degrading enzymes

Pgi- glucose-6-phosphate isomerase

pH - negative logarithm of the hydrogen ion concentration

PHT-partition homogeneity test

PHYML-phylogenetic estimation using maximum likelihood

proA- c-glutamyl phosphate reductase

QS-quorum sensing

RNA-ribonucleic acid

rpm - revolutions per minute

rRNA-ribosomal RNA

RT - room temperature

s - second

SCRI-Scottish Crop Research Institute

spp.- species

T1SS-type 1 secretion system

T2SS- type 2 secretion system

T3SS- type 3 secretion system

T4SS- type 4 secretion system

T5SS- type 5 secretion system

T6SS- type 6 secretion system

TAE- Tris base, acetic acid and EDTA

Temed - N, N, N', N' -tetramethylethylenediamine

T_{m}-annealing temperature

TPS-two partner system

USA-United states of America

UV - ultraviolet

v/v - volume per volume

vas-virulence associated

VgrG-valine glycine repeat protein G

w/v - weight per volume

µ (prefix) - micro (10^{-6})

µl - microlitre

µm- micrometer

.

CHAPTER ONE

A review of *Pectobacterium* species causing soft rot and blackleg disease of potato in South Africa

1.1 Introduction

The soft rot *Enterobacteriaceae* (SRE) consist of two key genera, namely *Dickeya* and *Pectobacterium* spp. (Charkowski *et al.*, 2012) cause destructive soft rot diseases that affect nearly all plant families ranging from crops, fruits, vegetables and ornamental plants worldwide (Toth *et al.*, 2003a). These plant pathogenic enterobacteria are associated with important diseases of potato (Pérombelon, 1992). The SRE cause soft rot disease of potato tubers during storage as well as blackleg disease on potato stems in the field (Pérombelon, 2002; Costa *et al.*, 2006). The SRE cause severe economic losses within the potato industry and as a result these pathogens are considered to be of economic importance worldwide (Toth *et al.*, 2003a). Temperature and humidity are two factors that play an important role in the development of tuber soft rot and blackleg disease of potato (Pérombelon, 2002). Due to the influence of high temperature on the development of tuber soft rot diseases, the SRE are considered to be an even bigger problem in poor or developing countries where there are no adequate refrigeration facilities during storage (Charkowski, 2009). Since this work focuses mainly on the one genus of the SRE, namely *Pectobacterium* spp, this review will pay more attention towards members of this genus than *Dickeya*, although where relevant *Dickeya* spp. will be discussed.

Pectobacterium spp. are Gram-negative pectinolytic bacteria, with rod-shaped cells of about 0.5-1.0 by 1.0-3.0 µm in size (Pérombelon and Kelman, 1980). These bacteria are facultative anaerobes that do not form spores and are motile by means of peritrichous flagella (Charkowski, 2006). As pectinolytic bacteria, *Pectobacteria* are simply characterized by their ability to synthesize and secrete large quantities of plant cell-wall degrading enzymes (PCWDEs) (Barras *et al.*, 1994; De Boer, 2002; Pérombelon, 2002). The *Pectobacterium* group was formerly referred to as *Erwinia* spp. (Hauben *et al.*, 1998). Currently the *Pectobacterium* genus consists of

five distinct species and subspecies namely; *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) [formerly known as *E. carotovora* subsp. *carotovorum*], *Pectobacterium atrosepticum* (*Pa*) [formerly known as *E. carotovorum* subsp. *atrosepticum*], *Pectobacterium wasabiae*, *Pectobacterium betavasculorum* and *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb*) (Gardan *et al.*, 2003; de Haan *et al.*, 2008; Nabhan *et al*., 2012).

Several studies have in the past focused extensively on investigating and unraveling various aspects of blackleg-tuber soft rot disease complex caused by *Pectobacterium* and *Dickeya* spp. (Pérombelon, 2002; Toth *et al.,* 2003a). To date there is a wealth of knowledge in literature pertaining to the taxonomy, ecology and epidemiological characteristics of SRE (Pérombelon and Kelman, 1980; Pérombelon, 2002; Hauben *et al.*, 1998; Costa *et al.*, 2006; Charkowski, 2006; Ma *et al.*, 2007; Toth *et al.*, 2011). *Pcb* strains were recently described as economically important soft rot pathogens of potato in South Africa and are causal agents of tuber soft rot and blackleg disease in potato (van der Merwe *et al.*, 2010). Therefore this review primarily focus on comparing the ecological, virulence and taxonomic characteristics of *Pcb* strains with those of closely *Pectobacterium* spp. namely; *Pa* and *Pcc* (Ma *et al.*, 2007; Glasner *et al.*, 2008; Nabhan *et al.*, 2011).

1.1.1. Ecological and epidemiological characteristics of *Pectobacterium* spp.

The distribution and host range of the SRE can be determined by ecological conditions under which they thrive. An environmental factor such as temperature plays an important role in the distribution of these pathogens (Pérombelon, 1992). Of the three *Pectobacterium* spp., strains from *P. carotovorum* group have the broadest host range and a wider geographical distribution. The wide host range of *P. carotovorum* and *Dickeya spp.* (formerly known as the soft rotting *Erwinia*) is reported to cover more than 35% of angiosperm plant orders (Ma *et al.*, 2007). Strains of *P. carotovorum* can survive in a diverse range of environments, thus contributing to the high genetic diversity of this pathogen (Toth *et al.*, 2003a). In contrast, *Pa* is considered to be a narrow-host range pathogen which is mainly restricted to potato. Recently, Marquez-Villavicencio *et al.* (2011), reported that under laboratory conditions strains of *Pa* can cause

maceration in several other plant species in addition to potato. However, *Pa* is still widely considered a strict narrow-host range pathogen of potato (Czajkowski *et al.*, 2011).

To date *Pectobacterium* spp. have not been reported to infect grain crops. Nonetheless, closely related species from the genus *Dickeya* are reported to infect and cause disease in a number of field crops such as maize and dahlia in addition to causing blackleg of potato (Pérombelon, 1992; Helias *et al.*, 2000; Pérombelon, 2002). In recent years, the *P. carotovorum* group has been expanded to include two newly reported subspecies in the form of *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb*) and *P. wasabiae* (Duarte *et al*., 2004; Pitman *et al*., 2010; Nabhan *et al.*, 2012). Strains of *Pcb* and *P. wasabiae* are associated with incidences of blackleg disease and tuber soft rot of potato. Recently in our laboratory, Moleleki *et al*. (2012) published a first report of *P. wasabiae* strains causing blackleg and soft rot of potato in South Africa. Strains of *P. wasabiae* were previously reported to cause blackleg disease of potato in New Zealand (Pitman *et al.*, 2010). Although currently little is known about the general ecology, genetics and distribution of *Pcb* strains, reports indicate that *Pcb* strains have a wider geographical and host range distribution resembling that of *Pcc* (Ma *et al.*, 2007). To date strains of *Pcb* have been reported in USA, Israel and South Africa after they were isolated and described for the first time in Brazil in 2004 (Duarte *et al.*, 2004; Ma *et al.*, 2007; Kim *et al.*, 2009). Fairly recently, there have been new updates in respect to the occurrence of blackleg-causing *Pcb* strains across the globe. De Boer *et al*. (2012), recently reported strains of *Pcb* in Canada detected on potato stems with blackleg symptoms using a species- and subspecies specific polymerase chain reaction (PCR). In the same manner, Panda *et al*. (2012), also published a first report of *Pcb* strains causing blackleg and soft rot on potato in New Zealand. These past and present reports indicate that *Pcb* is indeed a geographically well distributed pathogen of potato worldwide.

With regard to environmental factors, temperature has long been correlated with the epidemiology of soft rot pathogens (Pérombelon and Kelman, 1980). In this regard, *Pectobacterium* spp. can be characterized according to the climatic conditions under which they thrive or infect their hosts (Pérombelon, 1992). *P. carotovorum* strains are more widespread in temperate tropical zones whereas the closely related *Dickeya* spp. are commonly found to affect plant species in warmer (above 25°C) subtropical and tropical regions (Henz *et al.*, 2006). *Pa* is

primarily prevalent in cooler temperate regions such as in Europe and Canada where it causes blackleg disease at temperatures lower than 25°C (Toth *et al.*, 2003a). When strains of *Pcb* were first identified in Brazil, they were isolated from infected plants under humid subtropical and tropical climatic conditions (Duarte *et al.*, 2004). Although the association of temperature with the occurrence and distribution of *Pcb* strains remain to be investigated, these pathogens are reported to occur in the USA, Israel and South Africa under varying climatic conditions thus indicating that *Pcb* strains are adaptable plant pathogens (Ma *et al.*, 2007; van der Merwe *et al.*, 2010). According to Duarte *et al.* (2004), even in temperate conditions under which *Pa* is known to persist and dominate; *Pcb* strains were found to be more virulent than *Pa* strains on potatoes.

Factors that determine niche and host specificity among *Pectobacterium* spp. are not very clear. As a result, it is likely that diversity in gene regulatory mechanisms among the three *Pectobacterium* spp. (*Pa*, *Pcc* and *Pcb)* may reflect adaptation to specific ecological niches (Glasner *et al.*, 2008). The extent of genetic diversity among *Pectobacterium* species may determine the type of environmental stimuli such as varying temperatures, humidity and stressful environmental conditions under which a specific bacterium may thrive and be able to cause soft rot disease (Pérombelon, 2002; Glasner *et al.*, 2008).

1.1.2 The Soft Rot Disease Syndrome

The soft rot disease complex is comprised of tuber soft rot, blackleg, and aerial stem rot, each of which can develop under different environmental conditions during the potato production season or during postharvest in storage (Helias *et al.*, 2000). Phytopathogens such as *Pectobacterium* and *Dickeya* spp. rely on the suppression of host defense mechanisms to initiate disease development, and as a result they are commonly referred to as opportunistic pathogens (Faqua *et al.*, 2001; Pérombelon, 2002). Prior to the emergence of symptoms, *Pectobacterium* spp. tend to remain latent for long periods within lenticels of infected host tissues (Toth *et al.*, 2003b). Symptom development is mainly dependent on the susceptibility of the potato cultivar used as well as prevailing environmental conditions (De Boer, 2002). Conditions associated with the suppression of host resistance mechanisms include excess free water in the host tissues which leads to anaerobiosis (Maher and Kelman, 1983; Pérombelon, 2002). Typical symptoms that

signify the occurrence of *Pectobacterium* spp. infection on potato plants include non-emergence of plants, chlorosis, wilting, haulm desiccation and complete collapse of plants (Helias *et al.*, 2000; Pérombelon, 2002).

Tuber soft rot refers to the decaying of the tuber tissue which is caused by pectolytic enzymes that are produced and secreted by *Pectobacterium* spp. upon infection. The rotting of tubers is normally initiated from infected mother tubers after the pathogen has entered and colonized the tissues through natural openings (lenticels) or through wounds on the tuber surface (Pérombelon, 2002). The macerated tissue starts as a cream coloured slimy decay, turning into gray to black decay when exposed to air. Thereafter, rotting tubers develop an accompanying characteristic foul smell as a result of invasion by secondary saprophytic organisms (Pérombelon, 2002). Tuber soft rot symptoms are more severe under humid climatic conditions and reports indicate that in South Africa, high relative humidity results in wilting of stems which subsequently leads to tuber soft rot development (van der Merwe *et al.*, 2010). All three *Pectobacterium* spp. can cause tuber soft rot disease where symptoms are not species-specific. With regard to soft rot disease severity, *Pcc* and *Pcb* are considered to be highly aggressive pathogens compared to *Pa* which is only reported to play a minor role (De Boer, 2002; Pérombelon, 2002).

Blackleg disease refers to basal stem rot or the black rot lesion which extends from the rotting mother tuber up the stems of growing potato plants (Helias *et al.*, 2000). Blackleg is considered as an economically important disease of potato accounting for major crop yield losses worldwide (Pérombelon, 2002). The inky black-like lesion on potato stems is the characteristic symptom and first indication of host infection by *Pectobacterium* and *Dickeya* spp. (Elphinstone, 1987; Pérombelon and Kelman, 1980). Progression of rot from a completely decayed mother tuber extends via the vascular system into the stem. This results in the development of blackleg's characteristic symptoms such as stem discoularation or darkening, as well as necrosis of the pith and internal cavity (Helias *et al.*, 2000; De Boer, 2002). Development of blackleg symptoms on infected plants is largely dependent on the prevailing environmental conditions such as temperature and humidity and similar to soft rot, blackleg symptoms are not species-specific (Pérombelon, 1992; Helias *et al.*, 2000; De Boer, 2002). Although blackleg symptoms on potato plants are mainly associated with *Pa*, it is common to isolate multiple *Pectobacterium* and *Dickeya* spp. co-existing and infecting the same plant host (Pérombelon, 1992). Although *Pa* has

long been associated with incidences of blackleg in Europe, *Pcb* and *P. wasabiae* have recently emerged as highly aggressive strains responsible for the majority of blackleg incidences in Brazil and South Africa, respectively (Duarte *et al.*, 2004; van der Merwe *et al.*, 2010; Pitman *et al.*, 2010; Moleleki *et al*., 2012). Of the three *Pectobacterium* spp., *Pcc* is regarded as a secondary invader, only causing symptoms post-infection by other pathogens such as *Pa* and *Dickeya* (De Boer, 2002). According to De Haan *et al.* (2008), *Pcc* has since been established as a true causal agent of blackleg in temperate climates such as in Scotland and Canada where strains of *Pcc* can result to up to 50% of blackleg diseased plants.

Aerial stem rot is defined as the secondary soft rot of stems and petioles. It differs from blackleg in that aerial stem rot does not originate from the rotting mother tuber. It occurs as a result of stem rot originating from wounds on the aerial parts of the plant caused either by air- or water-borne *Pectobacterium* spp. (Pérombelon, 1992). Thus a clear distinction between blackleg and aerial stem rot is on the basis of origin of inoculum (Pérombelon, 1992). As a result, aerial stem rot symptoms starts from the upper parts of the plant and progress downwards to the base of the stem. Of the three *Pectobacterium* spp., *Pcc* is strongly associated with incidences of aerial stem rot under persistently excessive rainy conditions (Pérombelon, 2002). Recent reports indicate that aerial stem rot can also be caused by the transmission of soft rot pathogens by airborne insects from diseased plants to disease free crops (Czajkowski *et al.*, 2009). Although *Pcb* is considered to be a highly aggressive causal agent of blackleg, cases of aerial stem rot associated with this pathogen have not yet been reported (Czajkowski *et al.*, 2009).

As has already been established, *Pectobacterium* and *Dickeya* spp. are causal agents of economically important soft rot diseases of potato. In order to understand disease processes among the *Pectobacterium* spp., it is of great importance to first understand the pathogenicity factors and virulence mechanisms associated with these pathogens. In the following subsections of this study we review comparatively pathogencity factors among the three *Pectobacterium* spp., namely *Pa*, *Pcc* and *Pcb.*

1.2 . Comparison of Pathogenicity Factors amongst Strains of *Pa, Pcc* and *Pcb*.

Pathogenicity refers to the ability of the pathogen to cause disease in a host organism (Yishay *et al.,* 2008). For the pathogen to express its virulence on the targeted host, it requires genetic, biochemical and structural components that determine the success or degree of pathogenicity inferred onto the host. Bacterial pathogens such as *Pectobacterium* spp. rely on the use of various pathogenicity factors that aid the pathogen during colonization, infection and disease development (Toth *et al.,* 2003a).

Important determinants of pathogenicity in *Pectobacterium* spp. include PCWDEs, secretion systems, quorum sensing, as well as secreted phytotoxins and antibiotics (Mattinen *et al.,* 2008). We hereby compare and highlight important similarities and variations in pathogenicity factors by referring to the genomes of the three sequenced *Pectobacterium* spp.; *Pa* SCRI 1043, *Pcc* WPP14 and *Pcb* 1692 (Glasner *et al.,* 2008).

1.2.1.1 Plant cell wall degrading enzymes

The production and secretion of large quantities of extracellular PCWDEs by soft rot bacteria disrupt host cell integrity and cause tissue maceration thus promoting rotting and subsequent development of soft rot disease (Pérombelon, 2002; Toth *et al.,* 2003a). As a result of the role of PCWDEs in the virulence process, *Pectobacterium* spp. are often referred to as brute-force pathogens (Toth and Birch, 2005). Extracellular enzymes produced by *Pectobacterium* spp. include pectin- and pectate lyases as well as polygalacturonases. These are the main enzymes directly involved in the maceration of plant tissues by actively digesting the pectin that cement plant cells together (De Boer, 2003; Toth *et al.,* 2003a; Mattinen *et al.,* 2007). Pectinases generally include those produced at a higher optimum pH (~8.0); these include, pectate/pectate lyase, pectin and methyl esterase, whereas polygalacturonase is produced at a lower optimum pH (~6.0). Each of these enzymes may exist in multiple forms as isoenzymes encoded by independent genes (Pérombelon, 2002; Toth *et al.,* 2003a).

The activity of pectinolytic enzymes is reported to be significantly augmented by proteases and cellulases although these proteins are reported to only play minor roles in pathogenicity (Barrass *et al.*, 1994). The production of cellulases is mainly associated with the endoglucanase activity which is exhibited during the breakdown of cellulose in the primary and secondary cell walls of the host plant. The breakdown of cellulose through cellulases in turn provides nutrients for bacterial growth (Barras *et al.*, 1994; Pérombelon and van der Wolf, 1998; Toth *et al.*, 2003a). The role of proteases in pathogenicity is to provide amino acids for the biosynthesis of microbial proteins and/or the degradation of host resistance associated proteins. Altogether, cellulases and proteases significantly enhance the potency of the main pectolytic enzymes directly involved in the maceration of host tissues (Barras *et al.*, 1994; Pérombelon, 2002; Toth *et al.*, 2003a).

The recent availability of complete whole-genome sequences for *Pectobacterium* spp. such as *Pa* SCRI 1043 has made it possible and easier for researchers to study and compare the production, regulation and secretion of pectolytic enzymes among closely related pathogens (Mattinen *et al.*, 2007; Glasner *et al.*, 2008). In a comparative genomics study by Glasner *et al,* (2008), it was revealed that the genomes of *Pcc* and *Pcb* appear to encode more PCWDEs than *Pa*. Differences in the number of PCWDEs genes harbored within the genomes are likely to account for differences with regard to virulence among the three *Pectobacterium* spp. In this regard, *Pectobacterium* strains that produce and secrete more PCWDEs are likely to cause more aggressive maceration symptoms on host tissues (Salmond, 1994; Toth *et al.*, 2003a). *Pcb* and *Pcc* strains isolated from diseased potato stems and tubers are reported to exhibit more aggressive symptoms when compared to *Pa* strains infecting the same host (van der Merwe *et al.*, 2010; Marquez-Villavicencio *et al.*, 2011).

All three *Pectobacterium* spp. are reported to cause similar soft-rot and stem-rot symptoms due to the conservation of PCWDEs among them. Both *Pcb* and *Pcc* are reported to encode putative cell-wall degrading enzymes that are not present in *Pa*. According to Glasner *et al.* 2008, additional enzymes that are absent in *Pa* but present in *Pcb* and *Pcc* include a putative permease that could import the digested polymer, a gene encoding a putative polysaccharide deacetylase, as well as an Asp/Gluracemase capable of degrading host polymer. All three *Pectobacterium* spp. appear to control the synthesis of PCWDEs in a similar regulatory system through a number

of conserved regulatory factors such as KdgR, ExpRI, RexZ, Crp and H-NS (Bell *et al.*, 2004; Toth *et al.*, 2006).

PCWDEs are likely to play an important role in determining host range among the three *Pectobacterium* spp. It is not known whether differences in the production of PCWDEs play a role in determining the narrow-host range of *Pa.* Some researchers suggest that the restriction of *Pa* to potato might be as a result of secreted effector proteins that are easily detected by host defenses in many plant species. However, it is not known whether the detectable secreted proteins are strictly PCWDEs or other proteins (Pérombelon, 2002; Charkowski, 2006; Marquez-Villavicencio *et al.*, 2011).

1.2.1.2 Secretion systems

To date, six secretion systems (type I to type VI) have been identified in Gram-negative bacteria (Bingle *et al.*, 2008). Secretion systems are classified in terms of the secretion structure and type of secreted proteins they transport (Bingle *et al.*, 2008). The secretion of PCWDEs is distinctly associated with the type II secretion system (T2SS). All three *Pectobacterium* spp. are reported to encode nearly identical T2SS and similar sets of PCWDEs. All three *Pectobacterium* spp. can encode the type I secretion system (T1SS) through which proteases are secreted. However, the T1SS is considered to play only a minor role in virulence (Gerlach and Hensel, 2007). The minor role of T1SS in virulence is believed to be due to the fact that many plant species can easily detect and defend against proteases by producing numerous enzyme inhibitors (Quilis *et al.*, 2007). The role of proteases in virulence of *Pectobacterium* strains is still not clearly known. However, there is an extracellular protease known as PrtW, this protein has been shown to be important and required for virulence in *Pectobacteria*. Its mechanism of action appears to be to catalyze non-specific cleavage of other proteins (Marits, 1999). Work by Marits *et al. (*1999), demonstrated that protease activity in *Pcc* was necessary for normal progression of disease symptoms on potato tubers. *Pectobacterium* strains defective in protease gene (*prtW*) are reported to exhibit reduced virulence and symptom severity on plant hosts.

Many Gram-negative bacteria possess a cluster of genes that encode the type III secretion system

(T3SS). In plant-pathogenic bacteria, the T3SS is encoded by the *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response conserved) genes. Hypersensitivity response (HR) refers to a rapid, localized, defense-associated, programmed cell death within host tissues. The HR is triggered by the recognition of a plant pathogen (López-Solanilla *et al.,* 2004; Holeva *et al.,* 2004). Thus the *hrp* system plays a major role in host-pathogen interactions. It encodes effectors associated with the elicitation of HR in an incompatible interaction and pathogenesis in a compatible interaction (López-Solanilla *et al.,* 2004). The T3SS *hrp* cluster as it is known, is present in the genomes of the three *Pectobacterium* spp., but only *Pcc* and *Pa* are known to elicit the T3SS-dependent HR on tobacco plants (Kim *et al.,* 2009). Furthermore, all three *Pectobacterium* spp. are reported to encode homologous T3SS clusters with all genes known to be required for a functional T3SS. Some of the T3SS effector proteins include DspE and the harpin/helper proteins HrpN and HrpW that are involved in the suppression of plant defenses (Holeva *et al.,* 2004; Kim *et al.,* 2009). However, the main difference is that the T3SS of *Pa* lacks the HrpK protein that was reported to be involved in the translocation of effectors across the plant cell wall in *Pseudomonas syringae*. Other studies have since established that the lack of *hrpK* in *Pa* is not associated with the ability of the pathogen to elicit HR in tobacco (Glasner *et al.,* 2008). It is interesting to note that all strains of *P. wasabiae* isolated to date lack a T3SS and are as a result unable to elicit HR on tobacco plants (Kim *et al.,* 2009; Pitman *et al*., 2010; Moleleki *et al.,* 2012).

The type IV secretion system (T4SS) also contributes to virulence through the secretion of protein effectors. T4SS is characterized by the ability to transport protein complexes or macromolecules such as single stranded DNA between bacteria and from bacteria into their eukaryotic hosts (Gerlach and Hensel, 2007; Toth *et al.,* 2006). The T4SS has been well studied in *Agrobacterium tumefaciens* and is encoded by the *virB* gene family. Although it is reported that T4SS is only partially present in *Pcc*, *virB* gene clusters are reported to be present in *Pa* and *Pcb* in different locations. It still remains to be explored whether proteins secreted via the T4SS are conserved among *Pa* and *Pcb* (Glasner *et al.,* 2008). Work published by Bell and co-authors (2004) reported that a *virB* mutant of *Pa* SCRI1043 showed a significant reduction in virulence on potato. This indicates that T4SS is likely to be involved in the translocation of effectors that

may play an important role in the pathogenesis of *Pectobacterium* spp. on potato (Bell *et al.*, 2004).

The type V secretion system (T5SS) has the simplest protein secretion mechanism and is reported to be generally dedicated to the secretion of single specific bacterial effectors (Zheng and Leung, 2007). The T5SS includes several mechanisms such as the autotransporters secretion (ATs), and two-partner system (TPS) (Gerlach and Hensel, 2007). Both the ATs and TPS systems are present in a wide variety of Gram-negative bacteria including *Pa*. These systems are involved in the transport of large proteins across the inner and outer membrane in a *sec*-dependent pathway (Toth *et al.*, 2006; Gerlach and Hensel, 2007). The T5SS is reported to play an important role particularly in the pathogenicity of *Dickeya* spp.

The Type VI secretion system (T6SS) is the latest secretion system machinery that was recently identified in several plant pathogenic Gram-negative bacteria including *Pa* SCRI 1043 (Shrivastava *et al.*, 2008; Leiman *et al.*, 2009). Bacteria that possess the T6SS are known to maintain pathogenic or symbiotic interactions with eukaryotic host organisms (Pukatzi *et al.*, 2008). Although the role of the T6SS in pathogenicity is not known, the highly conserved virulence associated (*vas*) gene clusters are reported to be present in one or more copies in many pathogenic Gram-negative bacteria. In some pathogenic bacteria, the T6SS gene cluster was implicated to contribute to virulence-related processes (Pukatzi *et al.*, 2006; Saurez *et al.*, 2007; Mattinen *et al.*, 2008).

The T6SS is similar to other three types of secretion systems, T1SS, T3SS, and T4SS in that they all use a sec-independent mechanism of effector translocation (Leiman *et al.*, 2009). However, the T6SS is characterized by secretion of two effector proteins; VgrG (valine-glycine repeat protein G) and Hcp (hemolysin co-regulated protein). It remains to be elucidated whether these proteins are truly secreted as effector proteins or whether they form part of the structural components of the T6SS (Filloux *et al.*, 2008; Shrivastava *et al.*, 2008). A study undertaken by Mattinen *et al.* (2007) revealed that mutant strains of *Pa* lacking one of the *hcp* genes was not affected in virulence. However, an overexpression of the same gene was shown to have an increased virulence. Therefore, this report indicates and suggests that the T6SS is likely to play an important role as a virulence determinant of *Pa*. Much work regarding the presence of T6SS

genes in *Pectobacterium* spp. has so far been focused on *Pa*. As a result, it remains to be explored whether the same gene clusters are present in the genomes of *Pcc* and *Pcb*. Furthermore, it remains to be investigated whether these virulence determinants play a meaningful role in the pathogenicity of *Pectobacterium* spp. such as *Pcb* (Bell *et al.,* 2004; Mattinen *et al.,* 2007).

1.2.1.3 Phytotoxins

Although *Pectobacterium* spp. primarily relies on the secretion of pectolytic enzymes for pathogenesis in potato, there are other virulence factors such as phytotoxins that are associated with pathogenesis (Brooks *et al.,* 2005). Toth *et al.* (2006), reported that the genome of *Pa* SCRI 1043 contains at least 17 major putative horizontally acquired islands (HAIs). However, comparative genomics by Glasner *et al,* (2008) has revealed that some of the HAIs which were previously identified in *Pa* are completely lacking from the genomes of both *Pcc* and *Pcb*. Such genomic islands include the HAI2, HAI3, HAI4, and HAI9. The HAI2 is particularly of great importance in that it includes genes encoding the *cfa* cluster (Toth *et al.,* 2006; Glasner *et al.,* 2008). It has since been established that the *cfa*-like polyketide phytotoxin clusters (HAI2) found in *Pa* play a significant role and contributes to the virulence of the pathogen (Bell *et al.,* 2004). Bell and colleagues established that there is a correlation between the presence of the *cfa*-cluster in *Pa* SCRI1043 and the induction of blackleg disease on potatoes (Bell *et al.,* 2004).

There has not been much work done on the role of the *cfa* genes in *Pectobacterium* spp., however, the best studied *cfa*-system close to that of *Pectobacterium* spp. in found in *Pseudomonas syringae* (Bender *et al.,* 1999). The genome of *Pa* harbors an entire *cfa* operon reported to be similar in structure and organization with that found in *P. syringae* (Toth *et al.,* 2006). In this regard an understanding of the *cfa*-system from *P. syringae* is likely to shed light to the understanding of virulence mechanisms associated with the *cfa*-cluster in *Pectobacterium* spp. (Bender *et al.,* 1999). The successful suppression of host defenses has commonly been associated with virulence factors secreted via the T3SS. In recent times it has been revealed from *P. syringae*, that a synthesized phytotoxin coronatine (COR) encoded by the *cfa* cluster is one of the major virulence factors which contribute to the pathogenesis of the bacteria (Bell *et al.,* 2004; Slawiak and Lojkowska 2009). COR is hypothesized to be involved in the suppression of host

defense mechanisms by mimicking the host signaling pathways (Bender *et al.,* 1999; Bell *et al.,* 2004; Brooks*et al.,* 2005). In *P. syringae,* COR is formed by the conjugation of two intermediates, the polyketide coronafacic acid (CFA), and coronamic acid (CMA). Nonetheless, a variety of coronafacoyl conjugates can be formed through the coupling of CFA to other amino acids. The CFA and CMA conjugates are synthesized independently from the *cfa* gene cluster and cma operon. Therefore, the conjugation process of CFA to CMA to form COR is facilitated and enabled by coronafacic ligase (cfl) (Bender *et al.,* 1999).

It is well established that during host-pathogen interactions, the success of a pathogen to cause disease is dependent on the pathogen's ability to enter the host tissue, suppress and/or evade the host defenses (Pérombelon and, van der Wolf, 1998). The invading pathogen needs to be able to survive within the plant environment without triggering the host defenses. The survival of the pathogen within the plant host is dependant on its ability to obtain nutrients and water (Pérombelon and, van der Wolf, 1998). Furthermore, the pathogen's ability to multiply to as high enough population density as possible so as to induce host tissue damage is crucial for survival (Brooks *et al.,* 2005). The critical factor in plant colonization by the pathogen lies in the ability of the pathogen to manipulate and successfully suppress the plant's basal antimicrobial host defense responses (Faqua *et al.,* 2001). Such defense responses include microbial attack through rapid oxidative burst, accumulation of endogenous signaling molecules such as salicylic acid (SA), jasmonates and ethylene. As well as induction of pathogen related genes and other antimicrobial factors such as the release of phytoalexins and lytic enzymes (Bender *et al.,* 1999; Brooks *et al.,* 2005).

In studies associated with the pathogenesis of *P. syringae,* it is reported that the overproduction of CFA was found to be correlated with a delay in the induction of hypersensitive response (HR). This suggests that COR is likely to be involved in the suppression of host defenses. Thus, the role of COR in pathogenesis of *P. syringae* is the suppression of SA-dependent plant defenses and the promotion of disease symptom development through the SA-independent mechanism. To date the role of COR as a virulence factor in *Pectobacterium* spp. has been reported in *Pa* strains. Accordingly, Bell *et al.,* (2004), reported that *Pa* SCRI1043 possesses an HAI2 containing genes highly similar to the *cfa* gene cluster in *P. syringae* (Bell *et al.,* 2004). The *Pa* SCRI1043 strain

was reported to possess the *cfl* gene, but lacks genes similar to those of the cma operon (Toth *at el.,* 2006). These reports suggest that *Pa* SCRI1043 is likely to have acquired the *cfa*-like polyketide phytotoxin clusters through horizontal gene transfer. The *cfa*-gene cluster is likely to contribute to the pathogenesis of *Pa* SCRI 1043 on potato as was shown in virulence assays on potato stems (Bell *et al.,* 2004) Pathogenicity of *Pa* on potato was found to be significantly reduced when knockout mutations were performed on the genes *cfa6* and *cfa7*, thus suggesting that CFA plays an important role as a virulent determinant factor in this bacterium (Bell *et al.,* 2004, Toth *et al.,* 2006) However, comparative genomics revealed that unlike *Pa*, the genomes of *Pcb* and *Pcc* were found to lack the *cfa* cluster, but were rather encoding a polyketide or peptide synthetase system which was completely absent in *Pa* (Glasner *et al.,* 2008).

1.2.1.4 Quorum Sensing

The production of virulence factors such as PCWDEs, phytotoxins and antibiotics in Gram-negative bacteria is regulated through a density-dependent regulatory mechanism of quorum sensing (QS) (Pirhonen *et al.,* 1993). This mechanism allows the population of bacteria to communicate via the secretion and detection of small, diffusible signal molecules called *N*-acyl-homoserine lactone (AHL) (von Bodman *et al.,* 2003; Miller and Bassler, 2001, Charkowski, 2009). In *Pectobacterium* spp. the AHL quorum sensing system is believed to control a number of key virulence phenotypes such as pectinase, cellulase, and protease activities. In addition, production of HrpN which is secreted via T3SS, biofilm formation, motility and production of phytotoxins, and antibiotics are some of the traits reported to be under the regulation of QS (von Bodman *et al.,* 2003; Liu *et al.,* 2008). Genes that encode secreted virulence proteins are collectively referred to as exoproteins, in *Pectobacterium* there are two AHL exoprotein receptors namely ExpR1 and ExpR2. Additionally, there is also an exoprotein inducer known as ExpI (Pirhonen *et al.,* 1993). AHL molecules are produced in a cell density-dependent manner whereby the signal accumulates to a threshold level at which point cognate receptor proteins are activated (Miller and Bassler, 2001). At signal threshold, AHL binds to ExpR1 and ExpR2 receptors, causing them to lose affinity for their target, *rsmA*. RsmA is known to repress the production of extracellular proteins and the AHL synthase *expI* through the degradation of messenger RNAs that encode them (Charkowski, 2009). Therefore, the inhibition of *rsmA* leads

to an increased production and accumulation of extracellular proteins such as pectate lyase, polygalacturonase, cellulases and proteases (Pirhonen *et al.,* 1993; Chatterjee *et al.,* 1995; Cuy *et al.,* 2005, Charkowski, 2009).

Quorum sensing is an important virulence determinant factor in *Pectobacterium* spp., and as a result, the development of maceration symptoms can be explained in terms of this cell-density dependent regulatory system (Pérombelon, 2002). The QS regulatory system allows the secretion of extracellular enzymes at a high bacterial cell-density ensuring that the bacteria evade triggering host defenses. In this manner, at a high cell-density there are adequate bacterial cell numbers capable of producing high concentration of enzymes sufficient to overcome and overwhelm host defenses and consequently mount a successful infection (Miller and Bassler, 2001; Faqua *et al.,* 2001; von Bodman *et al.,* 2003). The biosynthesis of antibiotics such as carbapenem is also reported to be regulated through QS and both extracellular enzymes and antibiotics are released simultaneously during infection (Faqua *et al.,* 2001; Miller and Bassler, 2001). The role of QS as a virulent determinant has been studied in many Gram-negative phytopathogens particularly in *Pcc* strains from the *Pectobacterium* genus (Charkowski, 2009; Põllumaa *et al.,* 2012). Reports indicate that *Pcc-expI* mutants are unable to produce AHL and as a result lose their virulence implying that QS is involved in the pathogenesis of *Pcc* (Pirhonen *et al.,*, 1993; Liu *et al.,* 2008). It therefore remains to be elucidated whether QS is important and required for the pathogenesis of *Pcb* strains during colonization and infection of potato (von Bodman *et al.,* 2003; Liu *et al.,* 2008).

1.3 Dissemination of *Pectobacterium* spp.

Soft rot pathogens can be spread and transmitted between infected and healthy tissues during storage or in transit (Helias *et al.,* 2000; Toth *et al.,* 2003b). Potato tubers stored under conditions of relatively high temperature and humidity are more susceptible to infection by soft rot pathogens (Toth *et al.,* 2003b). In the field, it is reported that when contaminated mother tubers rot, the bacteria can be disseminated by means of soil water to the lenticels of neighboring progeny tubers (Pérombelon, 2002). Insects feeding on or moving through decayed tissues have also been implicated to spread the pathogens to uninfected host tissues (Czajkowski *et al.,* 2011).

1.4. The use of phylogeny to classify *Pectobacterium* spp.

1.4.1 Bacterial classification methods

It is of great importance to acquire an understanding of the diversity and relationships that may exist among plant pathogenic bacteria. This knowledge allows for accurate classification, identification and characterization of the economically important strains (Pitman *et al.*, 2008). According to Stackebrandt, and Goebel (1994), previous studies relied on two techniques that served as the 'gold standards' for bacterial species classification; namely 16S rRNA gene sequencing and genomic DNA-DNA re-association. The DNA-DNA hybridization method involves a pair-wise comparison of two complete genomes thereby reflecting the overall sequence similarity between the two genomes. The drawback with the DNA-DNA hybridization method is that it is complicated, technically challenging, time consuming and labour-intensive. Furthermore, this method cannot be rapidly and readily used by many small laboratories as it requires a great level of expertise (Martens *et al.*, 2008).

The 16S rRNA gene sequencing method is reported to be effective in resolving the degree of relatedness between organisms above the species level (Stackebrandt *et al.*, 2002; Martens *et al.*, 2008). Several studies have demonstrated that the classification of bacterial species through the DNA-DNA reassociation method only reveals genomic relatedness of above 70%. However, 16S rRNA gene sequencing offers a better resolving power in that it offers up to 97% sequence similarity between the species making it the better method of choice (Stackebrandt and Goebel, 1994).Over the years 16S rRNA genes have become a standard for determining phylogenetic relationships among bacterial species. Although the 16S rRNA gene sequencing method provides high resolving power in contrast to the DNA-DNA reassociation, there are some associated limitations. For example, according to Acinas (2004), the weakness with the 16S rRNA sequences is that many bacteria harbour multiple, heterogenous rRNA operons. In this manner, multiple copies of *rrn* operons occurring within rRNA sequences can result in variations in strains of the same bacterial species therefore inferring inaccurate phylogenetic relationships. As a result, rRNA sequence heterogeneity within single genomes can lead to significant

problems and severe overestimation of bacterial diversity and classifications. Therefore these limitations make the 16S rRNA technique a less accurate method for bacterial classification (Crosby *et al.*, 2003; Acinas *et al.*, 2004).

Recently the availability of whole-genome sequences such as *Pa* SCRI 1043, *Pcc* WPP14 and *Pcb* 1692 makes it easier to accurately study and classify bacterial species on the basis of multiple housekeeping genes (Acinas *et al.*, 2004; Ma *et al.*, 2007; Martens *et al.*, 2008). For this reason, newer and more effective classification techniques such as the multilocus sequence analysis are routinely employed to unravel phylogenetic relationships among bacterial species. In the subsequent sections we review and highlight some of the advantages of using multilocus sequence analysis to classify bacterial species such as enterobacteria.

1.4.2 Multilocus sequence analyses (MLSA)

Techniques such as 16S rRNA sequencing analysis and DNA-DNA reassociation have in the past contributed greatly to the delineation of bacterial species through the use of nucleotide sequences (Palys *et al.*, 1997, Stackebrandt *et al.*, 2002). However, recently the multilocus sequence analyses (MLSA) has become a popular and widely used tool for the delineation of bacterial species (Nørskov-Lauritsen *et al.*, 2005; Ma *et al.*, 2007; Brady *et al.*, 2008; Martens *et al.*, 2008; Pitman *et al.*, 2010; Nabhan *et al.*, 2011). The MLSA technique advances bacterial delineation efforts in that, through the use of partial DNA sequences derived from house-keeping genes, the elucidation of genomic relatedness is measured both at the inter- and intraspecific level (Stackebrandt *et al.*, 2002; Ma *et al.*, 2007).

There are several advantages associated with the use of MLSA as an effective technique for the delineation of bacterial species and the investigation of taxonomic relationships. According to Martens *et al.* (2008), the MLSA technique is a better alternative to DNA-DNA re-association in that the use of multiple house-keeping genes allows for the prediction of taxonomic relationships amongst many closely related organisms. Furthermore, the MLSA technique is useful in studying bacterial relationships at a wide range of evolutionary distances (Zeigler, 2003; Martens *et al.*, 2008). In contrast to 16S rRNA, an added advantage of the MLSA technique is that information

derived from the comparison and combination of multiple genes has the potential to give a global and reliable overview of inter-relatedness among species (Martens *et al.*, 2008). Moreover, when compared to the 16S rRNA technique, the MLSA method provides a higher degree of sequence divergence. MLSA is also capable of yielding sequence clusters at a wide range of taxonomic levels more than 16S rRNA or DNA-DNA re-association technique (Martens *et al.*, 2008). Recently, the MLSA method has widely been employed to identify and characterize many bacterial species including *Enterobacteria* (Ma *et al.*, 2007; Nabhan *et al.*, 2011). In these studies MLSA has demonstrated a greater level of accuracy in inferring phylogenetic and genomic diversity among the strains than can otherwise be achieved using traditional characterization techniques such as biochemical and physiological phenotypes (Avrora *et al.*, 2002; Yap *et al.*, 2004; Pitman *et al.*, 2010).

The concept of MLSA involves the selection of house-keeping genes to represent a chromosome. The availability of complete chromosome sequences has propelled this technique forward as a method of choice which can now be widely applied in systematic sequence studies (Stackebrandt *et al.*, 2002; Young *et al.*, 2007; Ma *et al.*, 2007). In the past MLSA was primarily used for the study of mammalian bacterial pathogens (Naser *et al.*, 2005; Martens *et al.*, 2008). It is now a practicable technique that can be used to delineate an increasing number of bacterial taxa such as *Pectobacterium* spp. (Ma *et al.*, 2007). For example, in many recent studies this technique has been extensively used to delineate species of *Pantoea, Pectobacterium, Dickeya* and many other plant bacterial pathogens (Ma *et al.*, 2007; Young *et al.*, 2007; Brady *et al.*, 2008; Pitman *et al.*, 2010; Nabhan *et al.*, 2011).

In *Enterobacteria* a number of protein-encoding gene sequences have been identified that are suitable to serve as phylogenetic markers in a multilocus phylogenetic analysis. The advantage of using sequences from more than one gene in MLSA is that this technique reduces the possibility of ambiguities caused by genetic recombination or specific selection often associated with single gene phylogenies (Ma *et al.*, 2007; Brady *et al.*, 2008). In a study by Ma *et al.*, 2007, phylogenetic relationships of the soft rot *Enterobacteriaceae* genera, *Pectobacterium* and *Dickeya* spp. were investigated using seven house-keeping genes. Partial fragments of housekeeping genes; aconitate hydratase 1 (*acnA*), glyceraldehyde-3-phosphate dehydrogenase

A (*gapA*), isocitrate dehydrogenase (*icdA*), malate dehydrogenase (*mdh*), glucose-6-phosphate isomerase (*pgi*), mannitol-1-phosphate 5-dehydrogenase (*mtlD*) and c-glutamyl phosphate reductase (*proA*) are now used as a standard in phylogenetic analysis of enterobacteria (Ma *et al.*, 2007; Nabhan *et al.*, 2011).

Housekeeping genes are chosen on the basis of several characteristics. For example genes suitable for MLSA should be housekeeping genes whose products are involved in diverse aspects of bacterial metabolism (Cohan *et al.*, 2007). Furthermore, these genes should provide enough sequence diversity to allow for the reconstruction of phylogenies and, the genes should not be clustered in the genome but be located at diverse chromosomal loci and widely distributed among taxa (Stackebrandt *et al.*, 2002, Zeigler, 2003; Ma *et al.*, 2007, Nabhan *et al.*, 2011). Therefore, through the use of MLSA phylogenetic approaches there is a need to explore the diversity of *Pectobacterium* and *Dickeya* spp. infecting potatoes in South Africa (van der Merwe *et al.*, 2010). As a bacterial classification tool, MLSA has been demonstrated to be useful in identifying several atypical pectolytic strains infecting potato in other countries (Pitman *et al.*, 2010; Palacio-Bielsa *et al.*, 2010; Nabhan *et al.*, 2011).

1.5. References

Acinas, S.G., Marcelino, L.A., Klepac-Ceraj, V., Polz, M.F. (**2004**). Divergence and Redundancy of 16S rRNA Sequences in Genomes with Multiple *rrn* Operons. Journal of Bacteriology. 186 (9), 2629-2635

Avrora, A.O., Hyman, L.J., Toth, R. L & Toth, I.K. (**2002**). Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. Applied and Environmental Microbiology. 68:1499-1508

Barrass, F., Van Gijsegem, F., and Chatterjee, A.K., (**1994**). Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annual Reviews of Phytopathology. 32:201-234

Bell, K.S., Sebaihia, M., Pritchard, L., Holden, M.T.G., Hyman, L.J., Holeva, M.C., Thomson, N.R., Bentley, S.D., Churcher, L.J.C., Mungall, K., Atkin, R., Bason, N., Brooks, K., Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail,, M.A., Sanders, M., Walker, D., Whitehead, S., Salmond, G.P.C., Birch, P.R.J., Parkhill, J., and Toth, I.K. (**2004**). Genome sequence of the

enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *PNAS*. 101 (30):11105–11110

Bender, C.L., Chaidez, F.A.N., and Gross, D.C. (**1999**). *Pseudomonas syringae* Phytotoxins: Mode of Action, Regulation, and Biosynthesis by Peptide and Polyketide Synthetases. Microbiology and Molecular Biology Reviews, 63 (2): 266-292

Bingle, L. E.H., Bailey C.M., Pallen, M.J., (**2008**), Type VI secretion: a beginner's guide. Current Opinion in Microbiology. 11:3–8

Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., Coutinho, T., (**2008**). Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Systematic and Applied Microbiology. 3, 447–460

Brooks, D.M., Bender, C.L., Kunkel, B.N. (**2005**). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. *Molecular Plant Pathology*. 6 (6): 629-639

Charkowski, A.O., (**2006**). The soft rot *Erwinia*. Pp. 423-505 In: Gnananmanickam, S.S (Ed) Plant-associated Bacteria, Netherlands

Charkowski, A.O., (**2009**) Decaying signals: will understanding bacterial-plant communications leads to control of soft rot? Current Opinion in Biotechnology. 20: 178-184

Charkowski, A.O., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., Hugouvieux-Cotte-Pattat, N., Lopez-Solanilla, E., Low, D., Moleleki, L., Pirhonen, M., Pitman, A., Perna, N., Reverchon, S., Palenzuela, P.R., Toth, I., Tsuyumu, S., vna der Waals, J., van der Wolf, J., Van Gijsegem, F., Yang, C.H., and Yedidia, I. (**2012**) The Role of Secretion Systems and Small Molecules in Soft-Rot Enterobacteriaceae Pathogenicity. Annual Review of Phytopathology. 50:425–49

Chatterjee A., Cui Y., Liu. Y., Dumenyo C.K., Chatterjee A.K. (**1995**). Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulose, and protease in *Erwinia carotovora* subsp. *carotovora* in the absence of starvation/cell density-sensing signal, *N*-(3-oxohexanoyl)-l-homoserine lactone. Applied and Environmental Microbiology. 61:1959-1967

Cohan, F.M., Perry, E.B. (**2007**). A systematics for discovering the fundamental units of bacterial diversity. Current biology. 17, R373-R386

Costa, A.B., Eloy, M., Cruz., L., Janse J.D., Oliveira, H., (**2006**). Studies on *Pectobacteria* spp. in Portugal reveal unusual strains of *E. carotovora* subsp. *atroseptica*. Journal of Plant Pathology, 88 (2), 161-169

Crosby, L. D., and C. S. Criddle. (**2003**). Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. Biotechniques 34:790–802

Cuy Y.,Chatterjee A., Hasegawa S., Dixit V., Leigh N., Chatterjee A.K., (**2005**). ExpR, a LuxR homolog of *Erwinia carotovora* subsp. *carotovora*, activates transcription of *rsmA*, which specifies a global regulatory RNA-binding protein. Journal of Bacteriology. 187:4792-4803.

Czajkowski, R., Grabe, G.J., van der Wolf, J.M., (**2009**) Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* in naturally infected seed potatoes. *European Journal of Plant Pathology*. 125:263–275

Czajkowski, R., Pérombelon, M.C.M., van Veen, J.A., van der Wolf, J.M., (**2011**) Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. Plant Pathology.60, 999–1013

De Boer S.H. (**2003**). Characterization of *Pectobacteri as* highly sophisticated pathogens of plants. *European Journal* of *Plant Pathology* 109: 893-899

De Boer S.H., (**2002**). Relative incidence of *Erwinia carotovorum* subsp. *atroseptica* in stolon end, and peridermal tissue of potato tubers in Canada. Plant Disease. 86:960-964

De Boer, S. H., Li, X., and Ward, L. J. **(2012**). *Pectobacterium* spp. associated with bacterial stem rot syndrome of potato in Canada. Phytopathology 102:937-947

De Haan, E.G., Dekker-Nooren, T.C.E.M, Van den Bovenkamp, G.W., Speksnijder, A.G.C.L., van der Zouwen, P.S., and van der Wolf, J.M. (**2008**). *Pectobacterium carotovorum* subsp. *carotovorum* can cause potato blackleg in temperate climatic. European Journal of Plant Pathology. 122:561-569

Duarte, V.J., De Boer S.H., Ward, L.J., and De Oliveira, A.M.R (**2004**) Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. Journal of Applied Microbiology. 96: 535-545

Elphinstone, J.G., (**1987**). Soft rot and blackleg of potato; *Erwinia* spp., *Technical Information Bulletin 21*, International Potato Center, Lima, Peru.18 pp.

Faqua, C., Parsek, M.R., Greenberg, E.P. (**2001**). Regulation of gene expression by cell-to-cell communication: Acyl-Homoserine Lactone Quorum Sensing. Annu. Rev. Genet. 35:439–68

Filloux, A., Hachani, A., Bleves, S., (**2008**). The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiology, 154:1570-1583

Gardan, L.J., Gouy, C., Christen, R., and Samson, R. (**2003**). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 53: 381-391

Gerlach R.G., and Hensel, M., (**2007**). Protein secretion systems and adhesins: The molecular armoury of Gram-negative pathogens. International Journal of Medical Microbiology. 297: 401-415

Glasner J.D., Marquez-Villavicencio, M, Kim, H.S., Jahn, C.E., Ma, B., Biehl, B. S., Rissman, A.I., Mole, B., Yi, X., Yang, C.H., Dangl, J.L., Grant, S.R., Perna, N.T., Charkowski, A.O. (**2008**) Niche-Specificity and the Variable Fraction of the *Pectobacterium* Pan-Genome. MPMI. 21, (12):1549-1560

Hauben, L. Moore, E.R., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., and Swings, J., (**1998**). Phylogenetic position of Phytopathogens within the Enterobacteriaceae. Systematic and Applied Microbiology. 21:384-397

Helias, V., Andrivon, D., and Jouan, B., (**2000**). Development of symptoms caused by *Erwinia carotovora* ssp. *atroseptica* under field conditions and their effects on yield of individual potato plants. Plant Pathology. 49:23-32

Henz, G.P., Relfschneider, F.J.B., and Duarte, V., (**2006**). *Erwinia chrysanthemi*: Pectolytic bacterium causing soft rot outbreaks of aracacha in Brazil. Pesquisa Agropecuaria Brasileria. 41: 1567-1571

Holeva, M.C., Bell, K.S., Hyman, L.J., Avrova, A.O., Whisson, S.C., Paul R. J. Birch, P.R.J., and Toth, I.K. (**2004**) Use of a Pooled Transposon Mutation Grid to Demonstrate Roles in Disease Development for *Erwinia carotovora* subsp. a*troseptica*, Putative Type III Secreted Effector (DspE/A) and Helper (HrpN) Proteins. MPMI 17, (9), 943–950

Kim, H.S., Ma, B., Perna, N.T., Charkowski, A.O., (**2009**). Phylogeny and virulence of naturally occurring type III secretion system deficient *Pectobacterium* strains. Applied Environmental Microbiology. 75(13):4539–4549

Leiman, P.G., Basler, M., Ramagopal, U.A., Bonanno, J.B., Sauder, J.M., Pukatzki, S., Burley, S.K., Almo, S.C., Mekalanos, J.J.(**2009**) Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. PNAS.106. (11): 4154-4159

Liu, H., Coulthurst, S.J., Pritchard, L., Hedley, P.E., Ravensdale, M., S. Humphris., Burr, T., Takle, G., Brurberf, M.B., Birch, P.R.J., Salmond, G.P.C., Toth, I.K. (**2008**). Quorum Sensing Coordinates Brute Force and Stealth Modes of Infection in the Plant Pathogen *Pectobacterium atrosepticum*. *PLoS Pathog* 4(6): e1000093. doi:10.1371/journal.ppat.1000093

López-Solanilla, E., Bronstein, P.A., Schneider, A.R., and Collmer, A., (**2004**), HopPtoN is a *Pseudomonas syringae* Hrp (type IIIsecretion system) cysteine protease effector that suppresses pathogen-induced necrosis associated with both compatible and incompatible plant interactions. Molecular Microbiology, 54(2): 353–365

Ma, B., Hibbing, M.E., Kim, H., Reedy, R.M., Yedidia, I., Breuer, J., Glasner, J.D., Perna, N.T., Kelman, A., and Charkowski, A.B., (**2007**). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacteria* and *Dickeya*. Phytopathology. 97:1156-1163

Maher, E. A., and Kelman, A. (**1983**).Oxygen status of potato tuber tissue in relation to maceration by pectic enzymes of *Erwinia carotovora*. Phytopathology. 73:536-539.

Marits, R., Koiv, V., Laasik, E., and Mae, A. (1999). Isolation of an extracellular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity. Microbiology. 145, 1959-1966.

Marquez-Villavicencio, M. D. P., Groves, R. L., and Charkowski, A. O. (**2011**). Soft rot disease severity is affected by potato physiology and *Pectobacterium* taxa. Plant Disease. 95:232-241

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A. (**2008**). Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). International Journal of Systematic and Evolutionary Microbiology. 58, 200–214.

Mattinen, L., Nissinen, R., Riipi, T., Kallinen, M., and Pirhonen, M., (**2007**).Host-extract induced changes in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*. Proteomics, 7, 3527–3537

Mattinen, L., Somervuo, P., Nykri, J., Kouvonen, P., Corthals, G., Auvinen, P., Aittamaa, M., Valkonen, J.P.T., and Pirhonen, M., (**2008**).Microarray profiling of host-extract-induced genes and characterization of the type VI secretion cluster in the potato pathogen *Pectobacterium atrosepticum*. Microbiology. 154: 2387-2396

Miller, M.B., and **Bassler**, B.L. (**2001**). Quorum sensing in bacteria. Annual Review of Microbiology. 55: 165-199

Moleleki. L.N., Onkendi, E.M., Mongae. A., Kubheka. G.C., (**2012**) Characterization of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa. European Journal of Plant Pathology.DOI 10.1007/s10658-012-0084-4

Nabhan, S., De Boer, S.H., Maiss, E., and Wydra, K., (**2012**). Taxonomic relatedness between *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum and Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. Journal of Applied Microbiology. 113: 904-913

Nabhan, S., Wydrab, K, Lindec, M, Debene, T. (**2011**). The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. Plant Pathology. Doi: 10.1111/j.1365-3059.2011.02546.x

Naser S.M., Thompson F.L, Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., Swings, J., (**2005**).Application of multilocus sequence analysis(MLSA) for rapid identification of *Enterococcus species* based on *rpoA* and *pheS* genes. Microbiology. 51: 2141–2150

Nørskov-Lauritsen., N., Bruun, B., Kilian, M. (**2005**).Multilocus sequence phylogenetic study of the genus *Haemophilus* with description of *Haemophilus pittmaniae* sp. nov**.** International Journal of Systematic and Evolutionary Microbiology. 55: 449–456

Oliveira, A.M.R., Duarte, V., Silveira, J.R.P., and Moraes, M.G., (**2002**). Incidence of pectolytic *Erwinias* associated with blackleg of potato in Rio Grande do Sul. Fitopatologia Brasileira. 28:49-53

Palacio-Bielsa, A., Mosquera, M.E.R., Alvarez, M.A.C., Rodriguez, I.M.B., Lopez-Solanilla, E., and Rodriguez-Palenzuela. (**2010**). Phenotypic diversity, host range and molecular phylogeny of *Dickeya* isolates from Spain. European Journal of Plant Pathology. 127:311-324

Palys, T., Nakamura, L.K., Cohan, F.M., (**1997**). Discovery and Classification of Ecological Diversity in the Bacterial World: the Role of DNA Sequence Data. International Journal of Systematic Bacteriology. 47 (4), 1145-1156.

Panda P, Fiers M.A,W.J, Armstrong K, Pitman AR, (**2012**). First report of blackleg and soft rot of potato caused by *Pectobacterium carotovorum* subsp. *brasiliensis* in New Zealand. New Disease Reports. 26, 15.

Pérombelon, M.C.M. and, **van der Wolf**, J.M., (**1998**). Methods for the detection and quantification of *Erwinia carotovora* subsp. a*troseptica* (*Pectobacterium carotovorum* subsp.

atrosepticum) on potatoes: a laboratory manual. Scottish Crop Research Institute Occasional Publication No. 10, 1998.

Pérombelon, M.C.M., (**1992**). Potato blackleg, epidemiology, host-pathogen interaction and control. Netherlands Journal of Plant Pathology. 98:135-146

Pérombelon, M.C.M., (**2002**). Potato disease caused by soft rot *Erwinias*: an overview of pathogenesis. Plant Pathology. 51: 1-12

Pérombelon, M.C.M., and **Kelman,** A. (**1980**). Ecology of the soft rot *Erwinias*. Phytopathology 18: 361-387

Pirhonen, M., Flagon, D., Heikinheimo R., and Palva, E.T., (**1993**) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora.* The EMBO Journal, 12(6):2467-2476

Pitman, A.R., Harrow, S.A., Visnovsky S.B., (**2010**).Genetic characterization of *Pectobacterium wasabiae* causing soft rot disease of potato in New Zealand. European Journal of Plant Pathology. 126:423–435

Pitman, A.R., Wright, P.J., Galbraith, M.D., Harrow, S.A. (**2008**). Biochemical and genetic diversity of pectolytic enterobacteria causing soft rot disease of potatoes in New Zealand. Australasian Plant Pathology. 37, 559–568.

Põllumaa, L., Alamäe T., and Mäe A., (**2012**) Quorum Sensing and Expression of Virulence in *Pectobacteria.* Sensors, 12: 3327-3349

Pukatzi, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., Heidelberg, J.F., and Mekalanos, J.J. (**2006)**. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *PNAS* 103(5):1528-1533

Pukatzi, S., McAuley, S.B., Miyata, S.T. (**2008**). The type VI secretion system: translocation of effectors and effector-domains. Current Opinion in Microbiology. 12:1-7

Pukatzki, S., Ma, A.T., Revel, A.T., Sturtevant, D., and Mekalanos, J.J. (**2007**). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. PNAS. 104 (39): 15508-15513

Quilis, J., Meynard, D., Vila, L., Aviles, F. X., Guiderdoni, E., and SanSegundo, B. (**2007**). A potato carboxypeptidase inhibitor gene provides pathogen resistance in transgenic rice. Plant Biotechnol. J. 5(4):537-553.

Salmond, G.P.C., (**1994**). Secretion of extracellular virulence factors by plant pathogenic bacteria. Annual Review of Phylopathology. 32:181-200

Saurez, G., Sierra, J.C., Sha, J, Wang, S., Erova, T.E., Fadl, A.A., Foltz, S.M., Horneman, A.J., Chopra, A.K. (**2007**). Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila.* Microbial Pathogenesis. Doi:10.1016/j.micpath.2007.10.005

Shrivastava S., Mande, S.S. (**2008**). Identification and functional characterization of Gene components of type VI secretion system in bacterial genomes. PLoS ONE. 3(8): e2955

Slawiak, M.**,** and **Lojkowska**, E., (**2009**). Genes responsible for coronatine synthesis in *Pseudomonas syringae* present in the genome of soft rot bacteria. European Journal of Plant Pathology. 124:353-361

Stackebrandt, E., and Goebel, M., (**1994**). Taxonomic Note: A place for DNA-DNA reassociation and 16s rRNA sequence analysis in the present species definition in Bacteriology. 44 (4), 846-849.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A. D., Kampfer, P., Maiden, M.C.J, Nesme, X.,Rossello-Mora, R., Swings, J., Truper, H.G., Vauterin, L., Ward, A.C., Whitman, W.B. (**2002**). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. International Journal of Systematic and Evolutionary Microbiology, 52, 1043–1047.

Toth, I.K., Bell, K.S., Holeva, M.C., Birch, P.R.J., (**2003a**). Soft rot *Erwiniae*: from genes to genomes. Molecular Plant Pathology.4 (1),17–30

Toth, I.K., Sullivan, L., Brierley, J.L., Avrova, A.O., Hyman, L.J., Holeva, M., Broadfoot, L., Pérombelon, M.C.M., McNicol, J., (**2003b**). Relationship between potato seed tuber contamination by *Erwinia carotovora ssp. atroseptica,* blackleg disease development and progeny tuber contamination. Plant Pathology. 52: 119–126

Toth, I.K., and **Birch**, P.R.J., (**2005**). Rotting softly and stealthily. Current Opinion in Plant Biology. 8:424–429

Toth, I.K., Pritchard, L., and Birch, P.R.J (**2006**). Comparative genomics reveals what makes an enterobacterial plant pathogen. Annual Review of Phytopathology. 44: 305-336

Toth I.K., van der Wolf, J.M., Saddler, G., Lojkowska, E., Helias, V., Pirhonen, M., Tsor., L., Elphinstone, J.G. (**2011**). *Dickeya* species: an emerging problem for potato production in Europe. Plant Pathology.60, 385–399

van der Merwe, J.J., Coutinho, T.A., Korsten, L., and van der Waals, J., (**2010**). *Pectobacterium carotovorum* subsp. *brasiliensis* causing blackleg on potatoes in South Africa. European Journal of Plant Pathology. 126:175-185

von Bodman, S.B., Dietz Bauer, W., Coplin, D.L., (**2003**). Quorum sensing in plant-pathogenic bacteria. Annula Review of Phytopathology 41:455-482

Yap, M.N., Barak, J.D., Charkowski, A.O., (**2004**). Genomic Diversity of *Erwinia carotovora* subsp. *carotovora* and Its Correlation with Virulence. Applied and Environmental Microbiology. 70(5):3013–3023.

Yap, M.N., Barak, J.D., Courtney, E.J., Charkowski, A.O. (**2005**). The *Erwinia chrysanthemi* Type III Secretion System is Required for Multicellular Behaviour. Journal of Bacteriology. 187: 639-648

Yishay, M., Burdman, S., Valverde, A., Luzzatto, T., Ophir, R., Ydidia, I, (**2008**). Differential pathogenicity and genetic diversity among *Pectobacterium carotovorum* ssp. *carotovorum* isolates from monocot and dicot hosts support early genomic divergence within this taxon. Environmental Microbiology. 10: (10), 2759-2759

Young, J.M., and **Park**, D.C. (**2007**). Relationships of plant pathogenic enterobacteria based on partial *atpD*, *carA*, and *recA* as individual and concatenated nucleotide and peptide sequences. Systematic and Applied Microbiology. 30: 343–354

Zeigler, D.R., (**2003**). Gene sequences useful for predicting relatedness of whole genomes in bacteria. International Journal of Systematic and Evolutionary Microbiology. 53: 1893–1900.

Zheng, J., Leung, K.Y., (**2007**). Dissection of a type VI secretion system in *Edwardsiella tarda*. Molecular Microbiology. 66(5), 1192–1206

CHAPTER TWO

Multilocus sequence analysis of *Pectobacterium carotovorum* subsp. *brasiliense* isolates from potatoes and other hosts in South Africa

2.1.Introduction

Two genera of the *Enterobacteriaceae* are associated with soft rot of potatoes, namely, *Pectobacterium* and *Dickeya* (Charkowski *et al*., 2012). There are three subspecies of *P. carotovorum* classified as *Pc* subsp. *odoriferum* (Gallois *et al*., 1992), *Pc* subsp. *brasiliensis* (Duarte *et al*., 2004) and *Pc* subsp. *carotovorum* (Gardan *et al*., 2003). *P. wasabiae* (Moleleki *et al*., 2012) and *P. atrosepticum* (Gardan *et al.,* 2003), as well as the latter two subspecies of *P. carotovorum* have been isolated from potatoes (Gardan *et al.,* 2003; de Haan *et al.,* 2008; Pitman *et al*., 2010; Moleleki *et al*., 2012; Nabhan *et al*., 2012). *P.c* subsp. *odoriferum* and *P.c* subsp. *brasiliense* consist of strains that have been described as "atypical" of *P. atrosepticum* because of their biochemical characteristics (Nabhan et al., 2012). Strains of *Pc subsp. brasiliense*, are different from *P. atrosepticum* in that although they both produce reducing substances from sucrose and acid from α-methylglucoside, *Pc* subsp. *brasiliense* strains can grow at 37°C whereas *P. atrosepticum* cannot (Duarte et al., 2004). Environmental conditions, particularly temperature and humidity are critical in the ability of *Pectobacteria* to cause soft rot disease (Pérombelon, 2002). Thus the role of temperature in *Pcb* pathogenicity will be examined in the subsequent chapter.

Pectobacterium carotovorum subsp. *brasiliense* (*Pcb*) was first isolated from potato plants showing typical blackleg symptoms (Duarte *et al*., 2004). Subsequent to this first report, *Pcb* has now become an economically important pathogen of potatoes in Brazil, USA, Israel, South Africa and Zimbabwe (Ma *et al*., 2007; van der Merwe *et al*., 2010; Nabhan *et al*., 2011; Ngadze *et al*., 2012). In the study by van der Merwe *et al*. (2010), only four representative South African isolates were identified using 16S rRNA sequence analysis whereas the others were identified

using a multiplex PCR. The limitation with the use of 16S rRNA to characterize bacteria has been noted in a number of studies. According to Young and Park (2007), the 16S rRNA gene is limited in that it provides only coarse resolution of enterobacterial phytopathogens due to its relatively conserved rates of mutation. However, with the advent of fully sequenced genomes, the multilocus sequence analyses (MLSA) of housekeeping genes has emerged as an invaluable and robust method which increases the accuracy of identifying and differentiation of *Pectobacterium* and *Dickeya* strains (Yap *et al.*, 2004; Ma *et al*., 2007; Brady *et al*. 2008; Martens *et al*., 2008; Nabhan *et al*., 2011).

Reports indicate that *P. atrosepticum* (*Pa*) strains show a low level of genetic diversity and this is reflected in their restricted host range and specificity to potato (Toth *et al*., 2003a; Toth and Birch, 2005). Strains of *Pcc* and *Pcb* on the other hand are reported to have a broad-host range and wider geographical distribution which can also be reflected by their broad genetic diversity (Ma *et al*., 2007; Nabhan *et al*., 2012). In fact, there has been a suggestion that *Pcc* constitutes a complex of species from which some new novel species could be identified (Nabhan *et al*., 2012).

To date *Pcb* has only been isolated from diseased potato plants, from carrots in Japan (Nabhan *et al*., 2012), from the stem of *Ornithogalum* spp. and peppers from Israel (Ma *et al*., 2007). In this study, the identity of *Pectobacterium* strains isolated from lettuce and cucumber was determined using a MLSA approach. In addition reference *Pectobacterium* strains from potato plants were included in order to confirm their identity as *Pcb* and determine their diversity.

2.2. Materials and methods

2.2.1. Bacterial strains used

Bacterial strains used in the multilocus sequence analyses are listed in Table 1. Suspected South African *Pcb* isolates were obtained from the Potato Pathology Program culture collection at the University of Pretoria, South Africa. Type strains that used were *P. carotovorum* subsp. *carotovorum* (LMG 2404^{T}), *P. carotovorum*. subsp. *brasiliense* 212 (LMG 21371^{T}), as well as *P. atrosepticum* (ATCC BAA-672). Type strains LMG 21371^{T} (*Pcb* 1692) and ATCC BAA-672 (*Pa* SCRI 1043) were requested and obtained from A. O., Charkowski (Wisconsin-Madison,

USA) and I. Toth (SCRI, Scotland) respectively. All bacterial strains were streaked and grown on Luria-Bertani (LB) agar; liquid cultures were prepared from single colonies by inoculation into LB broth. In all experiments *Pectobacterium* strains were incubated at 28°C with the exception of *Pa* strains which were grown at 24°C.

2.2.2. Isolations from diseased lettuce and cucumber

Diseased cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*) were collected from the Tshwane Fresh Produce Market in South Africa. Bacterial isolations from diseased cucumber and lettuce vegetables were conducted as follows. Pieces of the infected vegetables were macerated in 10 mM magnesium sulphate ($MgS0_4$). Isolations were performed on the selective medium, Crystal Violet Pectate (CVP) as described by Hyman et al. 2001. Plates were subsequently incubated at 28°C for 48 h. Isolates that tested positive for pectolytic cavity formation (through formation of pith on the media) were purified and transferred to and maintained on nutrient agar (NA) (Merck, Darmstadt, Germany). Isolates that were obtained from cucumber and lettuce were subsequently subjected to intergenic transcribed spacer region (ITS)-PCR, as well as PCR amplification using species-specific primers BR1f (5'-GCGTGCCGGGTTTATGACCT-3') and L1r (5'-CARGGCATCCACCGT-3') (Duarte *et al*., 2004). All isolates were stored in sterile water at room temperature and in 15% glycerol medium at -80°C.

2.2.3. Pathogenicity on potatoes

The pathogenicity of *Pcb* isolates resulting from cucumber and lettuce were tested on potato tubers (*Solanum tuberosum* cv. Mondial). For each treatment, potato tubers were inoculated by placing 10 µl of a 10^8-CFU/ml bacterial suspension into 1.5-cm-deep holes poked into the tubers with a pipette tip. Tuber wounds were sealed with Vaseline jelly and tubers were then placed into airtight plastic containers to maintain humidity before incubation for 3 days at 28°C. Sterile $MgSO_4$ (10 mM) was used to inoculate negative controls. After incubation, the tubers were cut open, and the macerated tissue was scooped from the tubers and weighed.

2.2.4. DNA extractions

Genomic DNA was directly extracted from 48-hour old bacterial colonies using standard DNA extraction methods (Sambrook and Russel, 2001). A single bacterial colony was suspended into 200 µl of nuclease-free water, followed by boiling at 100 ºC for 10 min. Samples were centrifuged at 14 000 rpm for 30 s and suspensions were pelleted and the supernatant containing the DNA was decanted into a clean microcentrifuge tube. Extracted genomic DNA was quantified on 1% agarose gels, with the O'GeneRuler™ 1 Kb DNA ladder (Fermentas) serving as reference. DNA purity was further assessed by NanoDrop ND®-1000 photometry at a ratio of 260/280 nm. Genomic DNA samples were stored at -20°C until use.

2.2.5. PCR amplifications and Sequencing

All synthetic oligonucleotides for primers were synthesized at Inqaba Biotech™, Pretoria, South Africa. PCR reactions were carried out using a minimum of 50 ng DNA, 10x DreamTaq™ buffer, 100 µM dNTP mix (Promega), 40 pmol of each primer and 1.25 U DreamTaq™ DNA polymerase (Fermentas) and purified distilled H_2O in a total volume of 25 µL reaction mix. PCR amplifications were carried out in a Biometra T1 96 well thermocycler with the amplification cycle as follows: initial denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, all reactions were performed for 35 cycles and a final extension at 72°C for 10 min. PCR products were separated on a 1% agarose gels in 1x TAE buffer at 80 V for 45 min, and PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions. Primers used in this study are listed in Table 2 below.

Sequencing of the samples was performed on an ABI3730 automated sequencer in the DNA sequence laboratory at the University of Pretoria sequencing facility using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Mix v3.1 (ABI). Sequencing PCR was set as follows: Each 12 µl pre- amplification reaction mixture contained 5 x sequencing buffer, 0.5 µl ABI BigDye® Terminator v3.1 cycle sequencing Ready mix, 0.3 µl undiluted primer (40 pmol), 4 µl DNA template (50 ng), and 4.7 µl autoclaved nuclease-free water. PCR amplifications were carried out in a Biometra T1 96 well thermocycler with the amplification cycle as follows: initial

denaturation at 96°C for 10 s, annealing at 50°C for 5 s, extension at 60°C for 4 min, all reactions were performed for 25 cycles and a final extension at 72°C for 5 min.Unincorporated dye terminators were subsequently removed from sequencing PCR products by ethanol precipitation as follows: In a fresh 0.5 µl microcentrifugetube, 12 µl of PCR product was added to 2 µl of 3M Sodium Acetate (pH 4.6), together with 25 µl of 99% (v/v) ice cold ethanol. The mixture was centrifuged at 14 000 rpm for 30 min. The supernatant was decanted and the pellet was washed twice in 150 µl of 70% (v/v) ice cold ethanol and then centrifuged for 5 min at room temperature. To allow for complete ethanol evaporation, microcentrifuge tubes containing the pellet were air dried under a laminar flow.

2.2.6. Sequence analysis

Sequence chromatogram output files for individual housekeeping genes were edited, assembled, and aligned into consensus sequences using CLC Main Workbench 6 (CLCBio, Aarhus, Denmark). Multiple alignments were generated for the individual sequences of *acnA*, *gapA*, *icdA*, *mdh*, *mtlD* and *proA* using CLUSTAL W in MEGA version 5.0 with a gap penalty of 15 (Tamura *et al*. 2011). Prior to phylogenetic analysis, sequence alignments were also carried out using the online version of MAFFT (multiple sequence alignment program v.6.0). All reference strains and other supplementary data that aided in the reconstruction of phylogenies were obtained electronically from NCBI GenBank. Partial sequences of *acnA*, *gapA*, *mdh*, *icdA*, *proA* and *mltD* housekeeping genes corresponding to other enterobacteria spp. were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers EF550599 to EF550964 (Ma *et al*. 2007).

Phylogenetic analysis was performed using PAUP* 4.0b10 (Swofford, 2001) and MEGA 5.0 (Tamura *et al*. 2011) based on sequences of 49 bacterial strains including reference sequences from GenBank. Strains of *Yersinia* spp. were used as outgroup in all phylogenetic reconstructions (Ma *et al*. 2007). The selection of the best-fit model of nucleotide substitution for individual genes and concatenated data set were determined in jModelTest 0.1.1 (Posada, 2008). Maximum likelihood (ML) trees were constructed in PHYML (Guindon and Gascuel, 2003). Bootstrap values were estimated in order to evaluate the support for each clade using 1000 replicates for ML and with sampling limited to parsimony-informative characters and a cut-off value of 50 (Kim *et al*. 2009). Prior to the construction of a concatenated phylogenetic tree for

the six gene fragments, the congruency of the genes was evaluated using a partition homogeneity test (PHT) as determined in PAUP* 4.0b10.

2.3. Results

2.3.1. Isolations from diseased lettuce and cucumber

Four *Pcb* isolates (Lett01, Lett02, Cucu01, and Cucu02) obtained from diseased lettuce and cucumber vegetables were selected for further study. In Fig. 2.1 below the results of a PCR amplification of the isolates using *Pcb* specific primers (Br1f and L1r) are shown. The type strain *Pcb* 1692 was used as a positive control in all amplifications. Subsequent to PCR amplifications, isolates were subjected to sequencing and BLAST searches in NCBI GenBank database. Sequences of the four isolates were found to match those of other *Pcb* reference strains such as type strain *Pcb* 1692 in GenBank thus confirming their identity as *Pcb* strains.

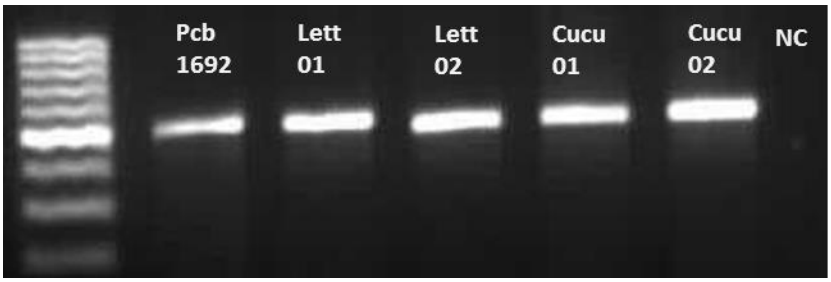

Fig. 2.1 PCR amplification of cucumber and lettuce isolates using *Pcb* specific-primers (Br1f and L1r). 1, 100 bp DNA ladder; 2, *Pectobacterium carotovorum* subsp. *brasiliense* (LMG 21371^{T}); 3, and 4, *Pcb* isolates from lettuce. 4, and 5, *Pcb* isolates from cucumber. NC: negative control (water).

All four isolates tested were able to cause maceration symptoms on potato tubers. The cucumber and lettuce isolates were observed to be slightly less virulent with regard to symptom severity compared to type strain *Pcb* 1692. However, within 48 h post-inoculation all four isolates were able to cause complete decay of potato tubers. Control tubers treated with 10 mM $MgSO_4$ did not develop any symptoms.

2.3.2. Phylogenetic analyses (MLSA)

The multi-locus sequence analysis of 49 isolates yielded six individual ML phylogenetic trees representing each gene. The results of the partition homogeneity test allowed for the data set to be combined (P = 0.34), thus indicating significant homogeneity among the six housekeeping genes and allowing for the reconstruction of a concatenated ML tree. The results for the best substitution model were determined in jModelTest 0.1. These parameters were subsequently used in PHYML 3.0 were ML trees for individual and concatenated data sets were constructed (Fig. 2).

Phylogenetic reconstruction using single gene DNA sequences and concatenated sequences resolved the relationships between the South African isolates and reference strains of *Pectobacterium* and *Dickeya* spp. In all phylogenetic reconstructions the *Pectobacterium* genus was divided into five distinct clades. Accordingly, clade I represented *P. carotovorum* subsp. *brasiliense*, clade II included *P. carotovorum* subsp. *carotovorum*, clade III strains included *P. atrosepticum* and clade IV were *P. betavasculorum* and clade V represented *P. wasabiae* strains respectively. As shown in the concatenated ML tree in Fig. 2 all South African isolates formed a monophyletic group in clade I with all other *Pcb* reference strains obtained from GenBank.

The ML tree constructed from a concatenated data set shown in Fig. 2 indicates a high level of intraspecies variation among the South African isolates. All the South African strains used in the analyses grouped accurately with clade I strains identified as *Pc.* subsp. *brasiliense*. Overall clade I is a strong monophyletic group distinguished from other *Pectobacterium* clades and supported by bootstrap value of 96%. Although clade I represent only *Pc. subsp. brasiliense* strains it is, however, evident that within this clade there is a significant level of intraspecies diversity that was not observed in any other clade.

It was observed that in all the six individual ML trees the topology only varied slightly with regard to the subclustering and phylogenetic positions of SA isolates in relation to other *Pectobacterium* strains. That is, the phylogenetic tree topology observed from single gene phylogenies resembled that drawn when all data sets were concatenated. Overall, the high bootstrap support among the *Pectobacterium* clades in give high levels of confidence in resolving the phylogenetic relationships of SA isolates compared with other closely related

Pectobacterium strains. As shown in Fig. 2, the South African *Pc. subsp. brasiliense* isolates are clustered together in clade I with similar strains isolated from USA, Brazil and Israel such as WPP 165 and *Pcb* Echbr 1692.

South African isolates exhibit a high level of intraspecies diversity in regard to the geographical location and potato tissue from which the individual strains were isolated. Two subclusters were observed within clade I. As shown in Fig. 2, most of the strains isolated in SA such as lettuce and cucumber seem to form a subcluster which includes the type strain *Pcb* Ecbr 1692 supported by a bootstrap value of 98%, whereas the other isolates form a separate subcluster with a bootstrap support of 88%. In addition, it is also observed that isolates such as *Pcb* 21, *Pcb* 09 and *Pcb* 41 seem to be closely related to the type strain *Pcb* Ecbr 1692. The remaining South African isolates are dispersed randomly within clade I strains indicating a degree of genetic diversity among the isolates. Interestingly, we observed that in all of our phylogenetic reconstructions two particular SA isolates, *Pcb* 147 and *Pcb* 148, which were isolated from stems in Mpumalanga appear to group consistently with a Brazilian strain WPP165. It is likely that these two particular strains may be the same isolate or may only differ in few nucleotide positions in their sequences. In contrast to what was observed for the two isolates from Mpumalanga, we noted that surprisingly a significant level of intraspecies genetic diversity is evident among isolates from the same geographical location, isolated from the same diseased host tissue. For example, isolates *Pcb* 41 and *Pcb* 55 isolated from diseased seed tubers from Sandveld appear to be diverse in that *Pcb* 41 clusters closely with *Pcb* Ecbr 1692 whereas *Pcb* 55 consistently groups within a distinct subcluster with other *P. brasiliense* strains. Interestingly, as observed in Fig. 2.2 the three *Pcb* strains isolated from cucumbers in South Africa (*P. bras* cucu) were also found to group with other *Pcb* strains isolated from potato, thus further highlighting the broad host-range nature of soft rot *Pectobacteria*. Overall our results show that clade I exhibits a level of high intraspecies and interspecies genetic diversity that cannot be found in any of the other four clades. This is possibly corroborated by the fact that *P. atrosepticum* is a narrow-host range pathogen compared to *P. brasiliense* and *P. carotovorum* strains which are known to cause disease in a wide range of other plant species. *P. carotovorum* subsp. *Odoriferum* SCRI482 was placed within clade II with other *P. carotovora* strains as was also reported in similar studies. Other groups such as *Dickeya* and *Yersinia* were distinctly separated from the *Pectobacterium*

species and were thus used as broad outgroups in this analysis. Overall, the phylogenetic tree constructed from concatenated multilocus gene fragments provided evidence supporting identification of South African *Pectobacterium* isolates as true *Pcb* strains.

2.4. Discussion

Pectobacterium strains isolated from diseased cucumber and lettuce vegetables were confirmed to be *Pcb* strains through PCR amplifications using *Pcb*-specific primers and BLAST searches. Subsequent work will focus on the biochemical characterizations of these isolates in order to ascertain whether there are any inter- or intraspecies variations among the isolates when compared to other closely related *Pectobacterium* species. A multilocus phylogenetic analysis (MLSA) of soft rot *Enterobacteriaceae* is valuable in determining the identity and diversity that may exist among closely related pathogens as was shown in similar studies (Yap *et al.*, 2004; Ma et al., 2007; Pitman *et al.*, 2008; Kim *et al.*, 2009; Pitman *et al*. 2010; Nabhan *et al.*, 2011). In this study the MLSA phylogenetic approach was employed to determine the identity of *Pcb* strains isolated from blackleg diseased plants in South Africa, as well as those isolated from diseased cucumber and lettuce. Furthermore this work was aimed at examining the genetic diversity that may exist among these isolates both at an interspecies and intraspecies level. The use of multiple housekeeping genes to reconstruct phylogenetic relationships is advantageous in that it provides reliable and unambiguous species identification (Kim *et al*. 2009; Pitman *et al*. 2010; Nabhan *et al*. 2011). The ML phylogenetic tree constructed using six housekeeping genes (*acnA, mdh, gapA, mtlD, proA* and *icdA*) sequences noticeably showed that the South African strains are grouped in the same cluster as *Pcb* strains. In addition, similar to results obtained from other MLSA studies, the results of our concatenated ML tree clearly differentiated the *Pectobacterium* genus into five distinct clades. Hence the topology of our ML tree was similar to that published by other researchers with regard to clade differentiation (Ma *et al.*, 2007; Kim *et al*. 2009; Pitman *et al*. 2010; Nabhan *et al*. 2011; Moleleki et al., 2012)

From the concatenated ML tree we were able to answer the two questions that prompted this work; firstly we sought to employ the multilocus sequence approach to determine and establish the identity of South African *Pcb* isolates. Previously, only four South African *Pcb* isolates were identified using the 16S rRNA sequence approach (van der Merwe et al., 2010). In this regard our results confirm that South African *Pcb* isolates are undeniably similar to *Pcb* strains isolated

from other potato growing regions across the world. This finding was supported by the fact that from the concatenated phylogenetic tree in Fig. 2.2, all of the isolates that were identified by species specific primers as *P. carotovorum* subsp. *brasiliense* clustered closely with those of *P. carotovorum* subsp. *brasiliense* isolated elsewhere across the globe (as shown from clade I in Fig. 2.2). Clade I which also include *Pcb* isolates from cucumber and lettuce is supported by a high bootstrap value of 96 %. Accordingly the high bootstrap value also indicates the high levels of confidence for this specific clade being distinct from other *Pectobacterium* clades. Therefore, the phylogenetic tree constructed provided evidence supporting the identification of all South African isolates as *Pcb.*

The *Pectobacterium* genus formed a 100% bootstrap value-supported supergroup which comprised of diverse subgroups making up five genetically distinct *Pectobacterium* clades namely: *Pc.* Subsp. *brasiliense*, *Pc.* subsp *carotovorum*, *P. wasabiae*, *P. betavasculorum* and *P. atrospeticum*. The five clades formed from the *Pectobacterium* genus were firstly reported in a study by Ma et al. 2007, as well as in other subsequent MLSA phylogenetic analysis of soft rot *Enterobacteria* (Pitman *et al.*, 2008; Pitman *et al*., 2010; Nabhan *et al*. 2011; Moleleki *et al*., 2012). Closely related *Enterobacteria* species such as *Dickeya* and *Yersinia* were also shown to be clearly distinct from the *Pectobacterium* genus. However, within the *Pectobacterium* genus we observed that there is a high level of intraspecies diversity among clade I and Clade II strains. The high level of intraspecies diversity was also noted among South African isolates. For example, strains *Pcb* 41 and *Pcb* 55 were both isolated from seed tubers in the Sandveld potato growing region of South Africa, but even though isolation factors such as environmental conditions, geographical location and host tissue were the same for these respective strains, according to phylogenetic analysis they still appear to be genetically distinct. Although the two *Pcb* strains clearly cluster within clade I strains, they do not belong to the same subcluster, thus demonstrating a level of intraspecies genetic diversity. As noted earlier, *Pcb* strains isolated from cucumber and lettuce in South Africa are phylogenetically similar to strains of *Pcb* isolated from potatoes. Hence, this finding may indicate that the same *Pcb* strains that were previously isolated from potato are also infecting other crops such as lettuce and cucumbers. If this hypothesis is true, it may indicate that South African *Pcb* isolates have a much broader host-range in addition to potato. According to Yap *et al.*, 2004, in some occasions, genetic diversity among *Pectobacterium* strains can be correlated with virulence. As far as we know a correlation

between the genetic diversity of *Pcb* strains and relative virulence on potato remains to be determined. So far it has only been established that there is a lot of diversity among the *Pcb* isolates tested. In this regard in the next chapter, the endeavor will be to determine if the level of diversity observed from the phylogenetic analysis can also be attributed to or expressed in the phenotypic, virulence, as well as host range characteristics of *Pcb*.

Finally from the phylogenetic tree it was also observed that two specific isolates, *Pcb* 147 and *Pcb* 148 formed a strongly supported subcluster within clade I strains. Further analysis of these isolates revealed that they were isolated from the same geographical location and host tissue. This observation was in contrast to an earlier observation made concerning other *Pcb* isolates which were all shown to exhibit intraspecies diversity. This finding therefore demonstrates that whereas genetic elements among many of the South African isolates may be diverse, in respect to these two isolates, their genetic characteristics seem to be shared. As a result, it appears that these two isolates share a highly conserved genetic homogeneity. This result is not surprising as it has been reported that *Pectobacterium* species sharing the same ecological niche have a high likelihood of sharing genetic traits through co-evolution and horizontal gene transfer (Glasner et al., 2008).

Overall, from this study it has been established and confirmed that all South African isolates derived from diseased potato plants as well as cucumber and lettuce are indeed *Pcb* strains. Secondly, it has been established that a high level of inter- an intraspecies diversity exists among *Pcb* strains compared to other closely related soft rot *Enterobacteriaceae*.

2.5. References

Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., Coutinho, T., (**2008**). Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Systematic and Applied Microbiology. 31, 447–460

Charkowski, A.O., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., Hugouvieux-Cotte-Pattat, N., Lopez-Solanilla, E., Low, D., Moleleki, L., Pirhonen, M., Pitman, A., Perna, N., Reverchon, S., Palenzuela, P.R., Toth, I., Tsuyumu, S., van der Waals, J., van der Wolf, J., Van Gijsegem, F., Yang, C.H., and Yedidia, I. (**2012**) The Role of Secretion Systems and Small Molecules in Soft-Rot Enterobacteriaceae Pathogenicity. Annual Review of Phytopathology. 50:425–49

De Haan, E.G., Dekker-Nooren, T.C.E.M, Van den Bovenkamp, G.W., Speksnijder, A.G.C.L., van der Zouwen, P.S., and van der Wolf, J.M. (**2008**). *Pectobacterium carotovorum* subsp. *carotovorum* can cause potato blackleg in temperate climatic. European Journal of Plant Pathology. 122:561-569

Duarte, V.J., De Boer S.H., Ward, L.J., and De Oliveira, A.M.R (**2004**) Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. Journal of Applied Microbiology. 96: 535-545

Gardan, L.J., Gouy, C., Christen, R., and Samson, R. (**2003**). Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 53: 381-391

Gallois, A., Samson, R., Ageron, E. and Grimont, P. A. D. (**1992**). *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory (Cichorium intybus L.). International journal of systemic Bacteriology. 42, 582–588.

Glasner J.D., Marquez-Villavicencio, M. Kim, H.S., Jahn, C.E., Ma, B., Biehl, B. S., Rissman, A.I., Mole, B., Yi, X., Yang, C.H., Dangl, J.L., Grant, S.R., Perna, N.T., Charkowski, A.O. (**2008**). Niche-Specificity and the Variable Fraction of the *Pectobacterium* Pan-Genome. MPMI. 21(12)12:1549-1560

Guindon, S. and Gascuel, O. (**2003**). A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704

Hyman, L. J., Sullivan, L., Toth, I. K., & Pérombelon, M. C. M. (**2001**). Modified crystal violet pectate medium (CVP) based on a new polypectate source (Slendid) for the detection and isolation of soft rot erwinias. Potato Research, 44, 265–270.

Kim, H.S., Ma, B., Perna, N.T., Charkowski, A.O., (**2009**). Phylogeny and virulence of naturally occurring type III secretion system deficient *Pectobacterium* strains. Applied Environmental Microbiology. 75(13): 4539–4549

Ma, B., Hibbing, M.E., Kim, H., Reedy, R.M., Yedidia, I., Breuer, J., Glasner, J.D., Perna, N.T., Kelman, A., and Charkowski, A.B., (**2007**). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacteria* and *Dickeya*. Phytopathology. 97:1156-1163

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A. (**2008**). Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*). International Journal of Systematic and Evolutionary Microbiology. 58: 200–214.

Moleleki, L.N., Onkendi, E.M., Mongae. A., Kubheka. G.C., (**2012**) Characterization of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa. European Journal of Plant Pathology.DOI 10.1007/s10658-012-0084-4

Nabhan, S., Wydrab, K, Lindec, M, Debene, T. (**2011**). The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. Plant Pathology.Doi: 10.1111/j.1365-3059.2011.02546.x

Nabhan, S., De Boer, S.H., Maiss, E., and Wydra, K., (**2012**). Taxonomic relatedness between *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum and Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. Journal of Applied Microbiology. 113: 904-913

Ngadze, E., Brady, C.L., Coutinho, T.A., and van der Waals, J. (**2012**). Pectinolytic bacteria associated with potato soft rot and blackleg in South Africa and Zimbabwe. European Journal of Plant Pathology 134 (3): 533-549

Pérombelon, M.C.M., (**2002**). Potato disease caused by soft rot *Erwinias*: an overview of pathogenesis. Plant Pathology. 51: 1-12

Pitman, A.R., Harrow, S.A., Visnovsky, S.B. (**2010**). Genetic characterization of *Pectobacterium wasabiae* causing soft rot disease of potato in New Zealand. European Journal of Plant Pathology. 126:423–435

Pitman, A.R., Wright, P.J., Galbraith, M.D., Harrow, S.A. (**2008**). Biochemical and genetic diversity of pectolytic enterobacteria causing soft rot disease of potatoes in New Zealand. Australasian Plant Pathology. 37: 559–568.

Posada, D. (**2008**). jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution.

Sambrook, J., and D. W. **Russell**. (**2001**). Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y

Swofford, D.L. (**2001**) 'PAUP*: Phylogenetic analysis using parsimony (*andother methods) version 4.0.' (Sinauer Associates: Sunderland, MA)

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (**2011**) MEGA 5.0: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution

Toth, I.K., Bell, K.S., Holeva, M.C., Birch, P.R.J., (**2003a**). Soft rot *Erwiniae*: from genes to genomes. Molecular Plant Pathology. 4 (1), 17–30

Toth, I.K., and **Birch**, P.R.J., (**2005**). Rotting softly and stealthily. Current Opinion in Plant Biology. 8:424–429

van der Merwe, J.J., Coutinho, T.A., Korsten, L., and van der Waals, J., (**2010**). *Pectobacterium carotovorum* subsp. *brasiliensis* causing blackleg on potatoes in South Africa. European Journal of Plant Pathology. 126:175-185

Yap, M.N., Barak, J.D., Charkowski, A.O. (**2004**). Genomic Diversity of *Erwinia carotovora* subsp. *carotovora* and Its Correlation with Virulence. Applied and Environmental Microbiology, 70(5): 53013–3023.

Young, J.M., Park, D.C. (**2007**). Relationships of plant pathogenic enterobacteria based on partial *atpD*, *carA*, and *recA* as individual and concatenated nucleotide and peptide sequences. Systematic and Applied Microbiology 30: 343–354

Appendix 2.A.

Tables and Figures.

Table 2.1 Bacterial strains used in the multilocus sequence analyses

***Pectobacterium* isolates**	**Host plant**	**Origin/Location**	**Source**
Pcb cucu01	*Cucumis sativus/Cucumbers*	SA, Gauteng	UP
Pcb cucu02	*Cucumis sativus/Cucumbers*	SA, Gauteng	UP
Pcb cucu03	*Cucumis sativus/Cucumbers*	SA, Gauteng	UP
Pcb lett01	*Lactuca sativa /Lettuce*	SA, Gauteng	UP
Pcb lett02	*Lactuca sativa /Lettuce*	SA, Gauteng	UP
Pcb 02	*S. tuberosum* (Harvested tubers)	SA, Mpumalanga	UP
Pcb 09	*S. tuberosum* (Harvested tubers)	SA, Eastern Freestate	UP
Pcb 21	*S. tuberosum* (Harvested tubers)	SA, KwaZulu-Natal	UP
Pcb 41	*S. tuberosum* (Seed tubers)	SA, Sandveld	UP

Pcb 49	*S. tuberosum* (Harvested tubers)	SA, Limpopo	UP
Pcb 55	*S. tuberosum* (seed tubers)	SA, Sandveld	UP
Pcb 38	*S. tuberosum* (Stems)	SA, Limpopo	UP
Pcb 60	*S. tuberosum* (Seed tubers)	SA, Limpopo	UP
Pcb 64	*S. tuberosum* (Seed tubers)	SA, Western Freestate	UP
Pcb 108	*S. tuberosum* (harvested tubers)	SA, Northern Cape	UP
Pcb 116	*S. tuberosum* (stems)	SA, Northern Cape	UP
Pcb 147	*S. tuberosum* (stems)	SA, Mpumalanga	UP
Pcb 148	*S. tuberosum* (stems)	SA, Mpumalanga	UP
Pcc (LMG 2404T)	*S. tuberosum*	Brazil	UP
Pcb 371 (LMG 21373^{T})	*S. tuberosum*	Brazil	UP
Pa SCRI 1043 (ATCC BAA-672)	*S. tuberosum*	Scotland	I. Toth
Pcb 1692 (LMG 21371^{T})	*S. tuberosum*	Brazil (V. Duarte)	A.O Charkowski

Table 2.2 Primers used for the amplification of housekeeping genes

Gene	Primer sequence	Origin
*acnA*_F	GCCTCGCCGCCGCTGGTGGT	Ma *et al*., 2007
*acnA*_R	CCGCGCATCATCACTTCATG	
*gapA*_F	ATCTTCCTGACCGACGAAACTGC	Ma *et al*., 2007
*gapA*_R	ACGTCATCTTCGGTGTAACCCAG	
*icdA*_F	GGTGGTATCCGTTCTCTGAAGG	Ma *et al*., 2007
*icdA*_R	TAGTCGCCGGTTCAGGTTCATACA	
*mdh*_F	GCGCGTAAGCCGGGTATGGA	Ma *et al*., 2007
*mdh*_R	CGCGGCAGCCTGGCCCATAG	
*mtlD*_F	GGCCGGTAATATCGGCCGTGG	Ma *et al*., 2007
*mtlD*_R	CATTCGCTGAAGGTTTCCACCGT	
*proA*_F	CGGYAATGCGGTGATTCTGCG	Ma *et al*., 2007
*proA*_R	GGGTACTGACCGCCACTTC	

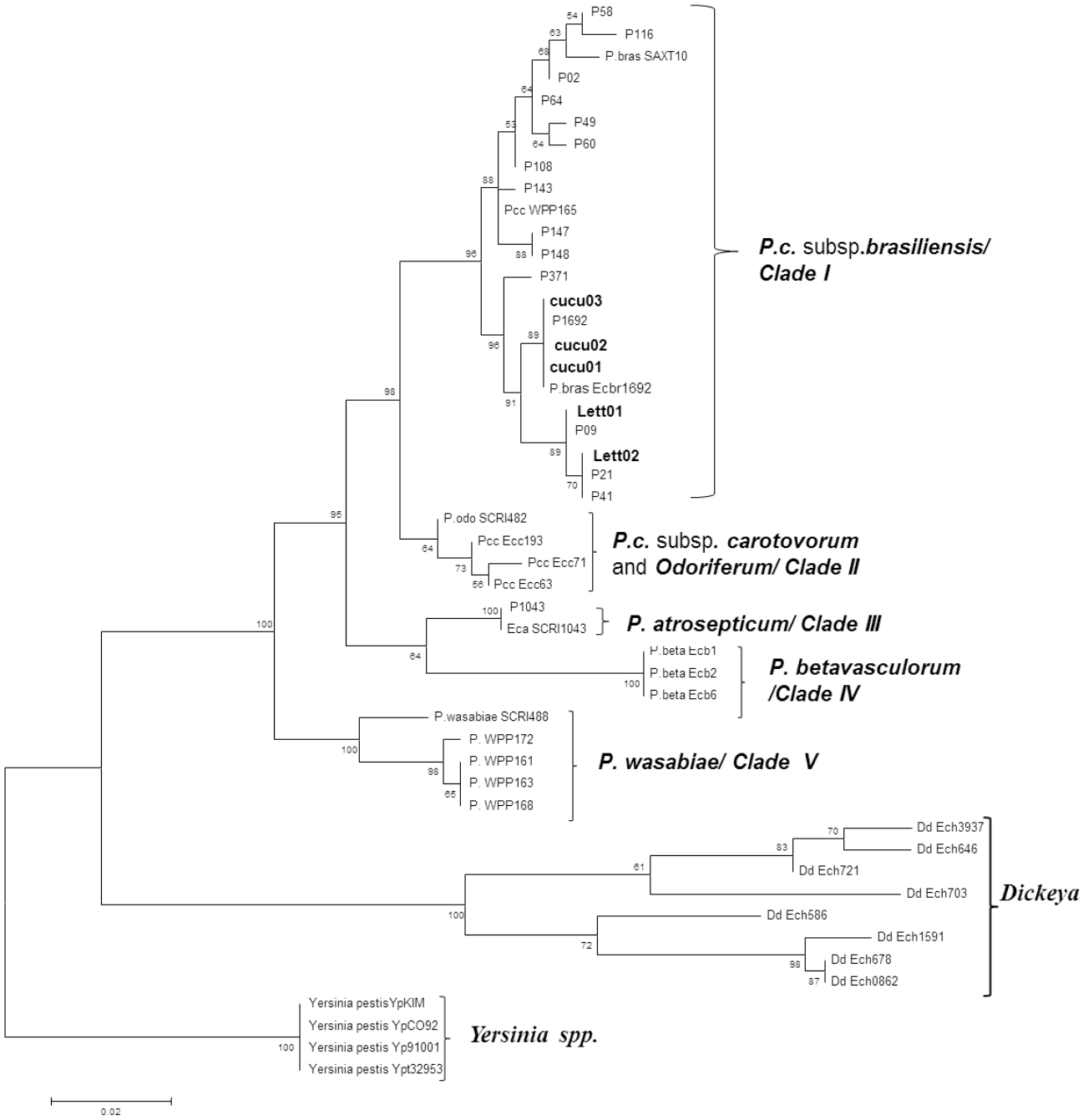

Fig. 2.2 A concatenated maximum likelihood (ML) phylogenetic analysis of *Pectobacterium* spp. phylogenetic relationships between strains from South Africa and reference *Pectobacterium*

strains isolated elsewhere were inferred based on six partial gene sequences (*acnA*, *mdh*, *mtlD*, *proA*, *gapA* and *icdA*). The *Pectobacterium* genus was divided into five distinct clades (I: *P. brasiliense*, II: *P. carotovorum*, III: *P. atrosepticum* IV: *P. betavasculorum*, and V: *P. wasabiae*. Numbers at the nodes represent bootstrap support values > 50 % from 1 000 replicates using heuristic searches based on parsimony with substitutions weighted according to the instantaneous rate matrix. *Yersinia* spp. were used as outgroups.

CHAPTER THREE

Phenotypic diversity, host range and virulence of *Pectobacterium carotovorum* subsp. *brasiliense* isolates from South Africa

3.1. Introduction

Pectobacteria are Gram-negative soft rot pathogens mainly characterized by the production of large quantities of extracellular pectolytic enzymes (Pérombelon, 2002). Pathogenesis in *Pectobacteria* is mediated through various virulence determinant factors which result in the development of soft rot and blackleg disease in infected host plants. Virulence-associated factors in *Pectobacterium* spp. include the secretion of a wide range of plant cell wall degrading enzymes (PCWDEs) that are used to disrupt and metabolize plant cells. Additionally, there are several secreted effectors that contribute to bacterial invasion, establishment, multiplication and host resistance impairment (Pérombelon, 2002; Toth *et al.*, 2003a). The use of solid media supplemented with appropriate substrates to test for enzymatic activity permits for the rapid screening of large populations of bacteria for the presence or absence of specific secreted enzymes (Hankin and Anagnostakis, 1975). As 'brute-force' pathogens relying mainly on the secretion of pectolytic enzymes to cause tissue maceration and disease, differences in the presence and absence of specific enzymes are likely to play important roles in pathogenicity of *Pectobacterium* spp.

Apart from PCWDEs, other virulence associated factors that play important roles during infection and disease development include putative phytotoxins, quorum sensing systems and secretion systems such as the type III secretion system (T3SS) (Pérombelon, 2002; Toth *et al.*, 2003a, Yap *et al.*, 2005). The T3SS is encoded by the hypersensitive response and pathogenicity (*hrp*) and *hrp* conserved (*hrc*) genes. In phytopathogens, the *hrp* gene cluster has been implicated in the establishment of disease during compatible interactions and the elicitation of plant defences, leading to resistance in incompatible interactions (Yang *et al.*, 2002; Holeva *et al.*, 2004). Effectors secreted by the T3SS are associated with the suppression of host defense mechanisms and include proteins such as HrpN, HrpW and DspE/A. HrpN and HrpW proteins

are implicated in the elicitation of hypersensitivity response, while HrpN and disease specific effector DspE/A are associated with pathogenicity (Holeva *et al.*, 2004; Yap *et al.*, 2005).

With regard to secreted phytotoxins associated with *Pectobacterium* spp., coronatine has been implicated to contribute to virulence of *Pa* SCRI 1043 (Bell *et al.*, 2004; Toth *et al.*, 2006). Coronatine is a toxin formed from the ligation of a polypeptide coronafacic acid (Cfa) and coronamic acid (Cma), a biochemical reaction which is catalysed by coronafacic ligase (Cfl). Although the biosynthesis of coronatine has been well studied in *Pseudomonas syringae* where it is reported to play an important role in virulence of the bacterium, recent reports indicate that the genome of *Pa* SCRI 1043 possesses genes coding for Cfa and Cfl (Bender *et al*. 1999; Glasner *et al*., 2008). It has been established that mutations in the genes *cfa6* and *cfa7* in *Pa* SCRI 1043 significantly reduce pathogenicity in potato. This suggests that Cfa does play an important role in the pathogenicity of *Pa* SCRI 1043 *(*Bell *et al*., 2004*)* Therefore, considering that coronatine is an important virulence determinant factor in *Pa* SCRI 1043, it remains to be determined whether strains of *Pcb* also possess genes for the *cfa*-cluster and *cfl* and whether, if present, these genes are important for virulence of *Pcb (*Bell *et al*., 2004*).*

In addition to bacterial virulence-associated factors, environmental factors play a critical role in the initiation of infection and subsequent disease development in host tissues. Environmental factors such as water availability, low oxygen levels and optimal temperatures are important and required for bacterial growth and disease development (Pérombelon, 2002).

The *Pectobacterium* genus has been divided into four species namely; *P. carotovorum (Pcc), P. atrosepticum (Pa* SCRI 1043*), P. wasabiae,* and *P. betavasculorum*. Although all these species are associated with soft rot and blackleg disease in many crop and ornamental plants, mainly *Pa* SCRI 1043, *Pcc*, and *P. wasabiae* are commonly found on potato and are considered as economically important pathogens thereof (Toth *et al.*, 2006). Of the three *Pectobacterium* spp. commonly found on potato, *Pa* is considered to be the main causal agent of blackleg disease in countries where potatoes are cultivated under cool temperate climatic conditions (Toth *et al*., 2006). Recently, a new subspecies of *Pectobacterium carotovorum* namely; *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb*) was identified and reported as a causal agent of blackleg of potato (Duarte *et al.* 2004). Since their first report in Brazil (2004), virulent strains of *Pcb* associated with blackleg of potato have been isolated in other countries under different climatic

conditions (Ma *et al.*, 2007). As a result, *Pcb* is increasingly being recognized as an important soft rot pathogen of potato worldwide (Marquez-Villavicencio *et al*,. 2011).

After its first report in Brazil (2004), the taxon, *P. carotovorum* subsp. *brasiliensis,* was yet to be validated. Consequently a recent report by Nabhan *et al*. 2012, has since confirmed this taxon's subspecies status using novel and standard microbial taxonomic approaches. Accordingly the taxon name has now been proposed to be *P. carotovorum* subsp. *brasiliense (*Nabhan *et al*., 2012). To date *Pcb* strains are reported to be the most aggressive causal agents of blackleg disease in Brazil, USA, Israel, and recently in South Africa (Ma *et al*. 2007; van der Merwe *et al.* 2010).

The overall genetic diversity that exists among *Pectobacterium* spp. has been investigated and reported in various studies were techniques such as PCR amplification and sequencing of 16S-23S rDNA intergenic transcribed spacer region (ITS), and restriction fragment length polymorphism (RFLP) were used (Avrora *et al.,* 2002; Yap *et al.,* 2004; Pitman *et al.,* 2010; Nabhan *et al.* 2011). Recently, the use of the multilocus sequence analysis (MLSA) with the aid of housekeeping genes has further validated previous results by revealing that there exists significant genetic diversity among *Pectobacterium carotovorum* species (Ma *et al.,* 2007; Kim *et al.,* 2009; Nabhan *et al.,* 2011). In the previous chapter we investigated the diversity among South African *Pcb* isolates and found that there is high interspecies and intraspecies variability within South African *Pcb* isolates in relation to other potato infecting *Pectobacterium* reference strains. Therefore, the goal of this chapter is to investigate the diversity of South African *Pcb* isolates with regards to their phenotype, pathogenicity as well as determination of their host range characteristics by assessing their maceration ability on other crops in addition to potato. Furthermore, we aim to screen for the presence of virulence associated factors such as specific secreted pectolytic enzymes and a putative phytotoxin, coronatine, which is associated with the suppression of host resistance in other pathogens.

3.2. Materials and methods

3.2.1. Bacterial strains

All South African *Pcb* isolates and reference strains used in this section of the study were similar to those listed in Table 2.1 (refer to Chapter 2). Bacterial strains were streaked and grown on

Luria-Bertani (LB) agar and liquid cultures were prepared from single colonies by inoculation into LB broth. All *Pectobacterium* strains were incubated at 28°C with the exception of *Pa SCRI* 1043 strains which were grown at 24°C.

3.2.2. DNA extractions

Genomic DNA was directly extracted from 48-hour old bacterial colonies using standard DNA extraction methods (Sambrook and Russel, 2001). A single bacterial colony was suspended into 200 µl of nuclease-free water, followed by boiling at 100 ºC for 10 min. Samples were centrifuged at 14 000 rpm for 30 s and suspensions were pelleted and the supernatant containing the DNA was decanted into a clean microcentrifuge tube. Extracted genomic DNA was quantified on 1% agarose gel, with the O'GeneRuler™ 1 Kb DNA ladder (Fermentas) serving as reference. DNA purity was further assessed by NanoDrop ND®-1000 photometry at a ratio of 260/280 nm. Genomic DNA samples were stored at -20°C until use.

3.2.3. PCR amplifications and sequencing

All synthetic oligonucleotides for primers were synthesized at Inqaba Biotech™, Pretoria, South Africa. PCR reactions were carried out using a minimum of 50 ng DNA, 10x DreamTaq™ buffer, 100 µM dNTP mix (Promega), 40 pmol of each primer, 1.25 U DreamTaq™ DNA polymerase (Fermentas) and purified distilled H_2O in a total volume of 25 µL reaction mix. PCR amplifications were carried out in a Biometra T1 96 well thermocycler with the amplification cycle as follows: initial denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min. All reactions were performed for 35 cycles and a final extension at 72°C for 10 min. PCR products were separated on a 1% (w/v) agarose gels in 1 x TAE buffer at 80 V for 45 min and PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions.

3.2.4. Biochemical characterization of isolates

Strains were assessed by standard bacteriological methods using the following tests as described by Cother and Sivasithamparam (1983): gram staining using 3% KOH, oxidative-fermentation of glucose, production of phosphatase and test for oxidase and catalase activity. The strains were further assayed for salt tolerance by growing them in LB medium containing 5% NaCl. Strains

were subjected to growth at various temperatures ranging from 20, 25, 30, and 35 up to 45°C. Furthermore, growth-rate curves were also conducted in order to investigate and compare the growth rate of *Pcb* isolates compared with other *Pectobacterium* species specifically *Pcc* and *Pa* SCRI 1043. Representative strains from each species were grown overnight in LB-media. Overnight cultures were subsequently adjusted to an optical density (OD_{600} = 1.000) using an absorbance spectrophotometer. Cultures were then grown for 16 hours while absorbance readings were taken at 30 min time intervals in order to determine and compare the rate of growth as a function of time among the strains.

3.2.5. Enzymatic assays

Strains were assayed for pectolytic activity on solid media supplemented with appropriate substrates as indicated in appendix 3A. The presence or absence of extracellular enzymes such as pectate lyase, cellulase, protease and lipase was assessed according to the methods described by Hankin and Anagnostakis (1975) and Chatterjee *et al.* (1995). Inocula were prepared by growing selected bacterial strains overnight on LB medium until an optical density (OD_{600} = 1.000) was reached. For each treatment single 5 µl drop of bacterial suspension was spot plated on solid media plates containing appropriate substrates. Negative controls were inoculated using sterile 10 mM $MgSO_4$.

3.2.6. Virulence assays on potato

Virulence assays were conducted with potato tubers purchased from a local market. Additionally assays were also performed on stems of 6-week old potato plants *(Solanum tuberosum* cv. Mondial). The relative virulence of the *Pcb* strains on potato tubers was evaluated by weight-measuring the amount of macerated tissue. Prior to inoculations, tubers were surface-sterilized in 5% NaOCl, rinsed thoroughly in sterile water and allowed to dry in the laminar flow. Inocula were prepared by growing bacterial strains overnight on LB medium until an optical density (OD_{600} = 1.000) was reached. Bacterial cells were then suspended in sterile 10 mM $MgSO_4$. For each treatment, potato tubers were inoculated by placing 10 µl of a 10^8-CFU/ml bacterial suspension into 1.5-cm-deep holes poked into the tubers with a pipette tip. Tuber wounds were sealed with Vaseline and tubers were then placed into airtight plastic containers to maintain humidity before incubation for 3 days at 28°C. Sterile $MgSO_4$ (10 mM) was used to inoculate negative controls. After incubation, the tubers were cut open, and the macerated tissue was

scooped from the tubers and weighed. For assays in potato stems, 6-weeks old *Solanum tuberosum* cv. Mondial plants were used. Stem inoculations were conducted using inocula consisting of 1 x10^8 CFU/ml and 20 µl of the bacterial suspension was used per injection site. Virulence on stems was expressed by the relative longitudinal lesion sizes as well as the wilting of plant leaves 3 days post inoculation. Plants were incubated in the glass house at room temperature. The experiment was repeated three times and data was analyzed statistically using one-way analysis of variance.

3.2.7. Pathogenicity in other plant hosts

To determine the maceration ability and host range of *Pcb* isolates, four other plant species (vegetables) were used as test hosts. These included onion (*Allium cepa*), green pepper (*Capsicum annuum*), baby marrow (*Zucchini species*) and eggplant (*Solanum melongena L.*). Bacterial inocula for all the test hosts was prepared as described for potato tubers above. Inoculations were done by introducing 10 µl (1x 10^5 CFU/ml) of bacterial suspensions into the onion bulbs, green pepper, baby marrow and eggplant vegetables, respectively. The different plant species were all incubated for 3 days in relative humidity at 28°C. Maceration damage was evaluated by measuring and calculating the lesion size (mm) around the stab wound area. Similar to potato assays, sterile $MgSO_4$ was also used to inoculate stems as a negative control.

3.2.8. T3SS and HR assays on tobacco

HR assays were performed by infiltrating bacterial suspensions of *Pcb* strains into tobacco leaves (*Nicotiana benthamiana*). *Pcb* strains were grown in LB broth at 28°C until mid-log phase and bacterial cells were washed with sterile deionized water and adjusted to a concentration of 2×10^8 CFU/ml of bacterial suspension in 10 mM $MgSO_4$. Fully expanded tobacco leaves were infiltrated with bacteria and were incubated at room temperature. Negative controls were inoculated with sterile 10 mM $MgSO_4$. Visible necrosis was determined 2 days post inoculation.

3.2.9. Identification of *cfa* genes in *Pcb* isolates

The presence of *cfa* genes in *Pcb* strains were investigated using PCR. In Table 3.1 below primer characteristics used for the amplification of representative gene fragments are indicated.

Table 3.1 Characteristics of PCR primers used to amplify *Cfa* genes in *Pcb*

Gene	primers	Sequences (5-'3')	Origin
Cfl	*Cfl_F*	AATCCAGCGAATAGCCACAG	Slawiak and Lojkowska, (2009)
	Cfl_R	TGAAGGTGTTCCTGCAATCC	
Cfa6	*Cfa_6F*	AACGGGCATAACCTCAACTG	Slawiak and Lojkowska, (2009)
	Cfa_6R	TGCAGTACGGTATCGAGCAG	
Cfa7	*Cfa_7F*	GCTGCCTACCTATCCCTTCC	Slawiak and Lojkowska, (2009)
	Cfa_7R	CTCCCAGTTCCGCATGAC	

PCR conditions for the amplification of *cfa* genes where as follows: PCR reactions were carried out using a minimum of 50 ng DNA, 10x DreamTaq™ buffer, 100 µM dNTP mix (Promega), 40 pmol of each primer, 1.25 U DreamTaq™ DNA polymerase (Fermentas) and purified distilled H_2O in a total volume of 25 µL reaction mix. PCR amplifications were carried out in a Biometra T1 96 well thermocycler with the amplification cycle as follows: initial denaturation at 95°C for 3 min, followed by subsequent denaturation at 94°C, and annealing for 1 min at an optimal temperature (T_m) for each primer set as shown in Table 3.1. Extension was at 72°C for 2 min. All reactions from denaturation at 94°C were performed for 32 cycles, with a final extension at 72°C for 5 min, and stored at 4°C until used. PCR amplicons were separated on a 1% (w/v) agarose gels in 1X TAE buffer at 80 V for 45 min and PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions.

3.2.10. Bacterial Growth curve

Bacterial growth characteristics were examined by using a growth curve. Inocula were prepared by growing selected bacterial strains overnight on LB medium until an optical density (OD_{600} = 1.000) was reached. Absorbance was adjusted to OD_{600} = 0.005 and absorbance readings were taken every 30 minutes for a total time interval of 16 hours.

3.2.11. Statistical analysis

Statistical analysis of data was performed in GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Three independent biological replicates were conducted in each experiment. One-way analysis of variance (ANOVA) and T-Test were used where applicable to determine statistical significance. Means were compared and separated using Turkey's multiple comparison test (P = 0.05). Error bars presented on bar charts indicates standard error of means. Letters on some of the error bars on bar charts were used to indicate significant difference between the means in a multiple comparison analysis.

3.3 Results

3.3.1 Biochemical characterization of isolates

Biochemical assays were applied to characterize a total number of 18 *Pectobacterium* strains including *Pcb* strains isolated in South Africa. On the basis of these biochemical assays, all strains were confirmed as Gram negative when tested on 3% KOH. Furthermore, all strains were negative for phosphatase and oxidase activity but were shown to be positive for catalase activity. We also established that although majority of the strains were able to grow on media containing 5% NaCl, only a few of our isolates were salt intolerant and could not grow at all at this salt concentration.

All of the strains were able to ferment glucose and all strains were able to grow across all temperature ranges from 20°C up to 45°C, with the exception of *Pa* SCRI 1043, *Pcb* 108, *Pcb* 21, *Pcb* 41 and *Pcb* 02. Although most of the isolates could grow at 45°C, it was observed that growth at this elevated temperature could not be sustained for long periods. Interestingly, we observed that all strains tested were able to grow at 35°C, except for strain *Pa* SCRI 1043 that could only grow at 25°C and only weakly at 30°C. A summary of biochemical assay results are listed in Table 3.2 below.

As indicated in Table 3.2, we observed very little variability among the isolates with regard to biochemical reactions tested on different media. Phenotypic characteristics were scored based on the ability of the bacterial strains to grow on different media. That is, + was assigned for strains that showed rapid or aggressive growth, whereas +w was assigned for isolates that showed

delayed, weak or partial growth. Finally a – negative score was assigned for isolates that failed to grow altogether.

3.3.2. Enzymatic assays

The solid media approach was used to evaluate bacterial enzyme production and thus screen for the production or lack of specific enzymes. Phenotypic variations in enzyme production are likely to indicate genetic variation among the individual *Pcb* isolates. We chose 9 representative isolates to investigate and compare enzymatic production and results are shown in Fig. 3.1 and Fig. 3.2, respectively. We observed that the representative isolates exhibited greater lipolytic activity as measured by the formation of crystals around the colonies as shown in Fig. 3.2 (A). Crystal formation on lipolytic activity indicates the degradation of calcium from the media.

The production of pectate lyase was also evident bythe formation of clear zones (halos) around the colonies, shown in Fig. 3.2 (B). We observed that protease activity Fig. 3.2 (C) was absent from strains of *Pa* SCRI 1043 and *Pcc* used (see also results in Fig. 3.1 (D)), but in all the representative *Pcb* strains we could detect variation among the isolates with regard to the size of halos formed on the plates. The size of halos formed around the colonies was used as a measure of enzyme production and activity. This was tested on solid media supplemented with the appropriate plant substrate to induce the production of that particular enzyme. Although halos formed for cellulase activity were not clearly visible as was the case in other tests, we were still able to measure the halos formed on the media as shown in Fig. 3.2 (D).

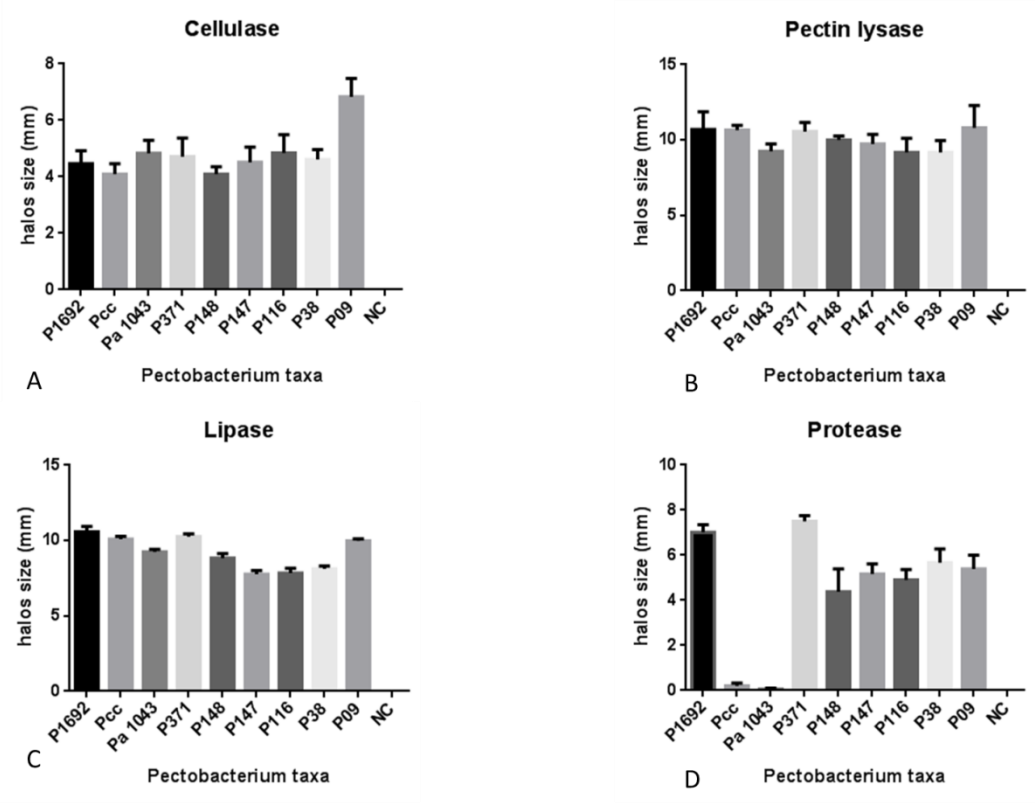

Fig. 3.1 Assessment of *Pectobacterium* strains able to produce enzymes and to degrade substrates on solid media. Enzymes tested were Cellulase (A), Pectin lysase (B), Lipase (C) and Protease (D), respectively. Results were quantified by measuring the size of halos around the colonies as shown in Fig. 3.2 below. Error bars represent standard error which is a measure of the means of individual *Pectobacterium* strains based on their ability to produce enzymes and degrade substrates on the media. Significant differences between the means were determined according to Tukey's multiple comparisons test ($P = 0.05$). NC: negative control.

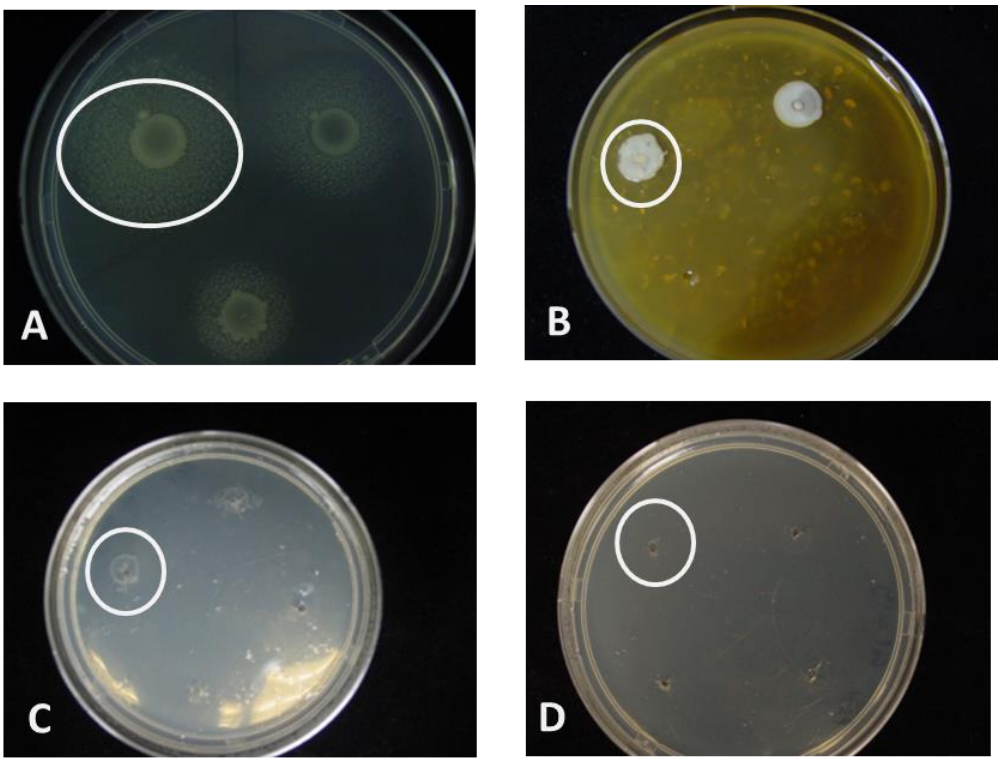

Fig. 3.2 Production of extracellular enzymatic assays of on solid media supplemented with appropriate substrate to induce enzyme production. White rings are drawn to illustrate the size of the halos around the colonies. (A) Lipolytic activity was tested on media supplemented with calcium. (B) Pectolytic activity was tested on media supplemented with pectin. (C) Protease activity was tested on media supplemented with gelatin. (D) Cellulase activity was tested on media supplemented with cellulose.

3.3.3. Virulence of *Pectobacterium* strains on potato tubers

The relative virulence of each of the *Pcb* strains was quantified using a potato tuber assay. From our results we observed variation in symptom severity among the strains. Our observations were that South African *Pcb* isolates are significantly more aggressive compared to *Pa* SCRI 1043, but appear to share similar virulence traits with *Pcc* (see Fig. 3.3). However, even among the *Pcb* isolates we still observed significant intraspecies variation according to Tukey's multiple comparisons test (P = 0.05). Strains such as *Pcb* 1692 and *Pcb* 09 were shown to exhibit the most aggressive maceration behavior as measured in terms of tissue decay. Although strain *Pcb*

116 showed the least maceration ability compared to all other *Pcb* isolates (Fig. 3.3), this isolate was still more aggressive than *Pa* SCRI 1043. Therefore, overall we observed that in all the replicates, *Pa* SCRI 1043 was shown to be the least aggressive strain to cause tuber maceration. As expected, negative controls did not show tuber soft rot symptoms in any of the treatments.

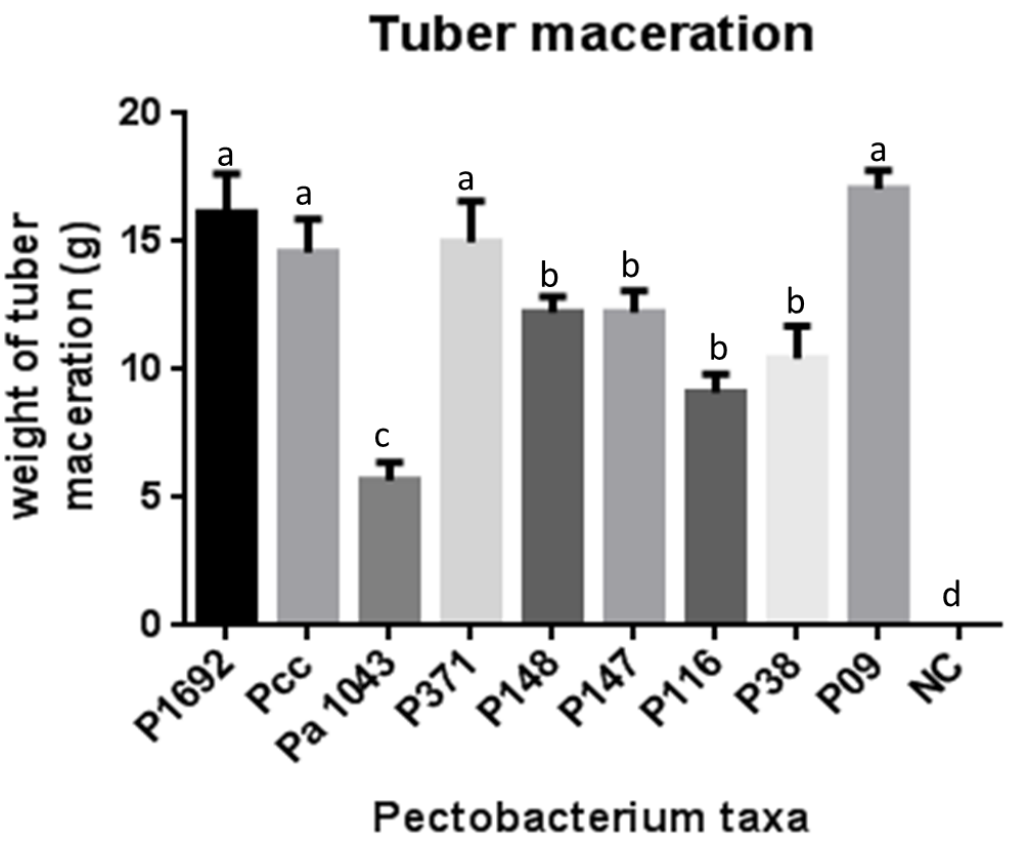

Fig. 3.3 Relative virulence of *Pectobacterium* strains on potato tubers. Tubers were inoculated separately with 10^5 CFU/ml of bacterial suspension. Decayed tissue was weighed after 3 days of incubation at 28°C for all other strains but incubation was at 24°C for *Pa*. Bars represent the standard error of means of macerated tissue recovered from inoculated tubers. Means with the same letter are not significantly different according to Tukey's multiple comparisons test ($P = 0.05$). NC: negative control.

Variation in symptom severity was also observed on the maceration of stems. Symptoms appeared on plants 3 to 4 days post inoculation and were observed as stem decay from the point

of inoculation, spreading both upwards and downwards along the stem length. Wilting of the leaves, followed by desiccation and complete collapse of the stems were characteristic symptoms that were observed as shown in Fig. 3.5.

In contrast to tuber maceration results, we observed that *Pa* SCRI 1043 was able to cause more aggressive stem decay symptoms. Similar stem decay severity was observed for *Pcb* 1692. Overall there was no significant variability among *Pcc* and other *Pcb* strains isolates with regard to stem maceration. Moreover, we observed that although strains such as *Pcb* 371 and *Pcb* 148 appear to cause similar maceration symptoms on stems, in all experimental repetitions, the South African strain *Pcb* 09 was shown to be the most aggressive of isolates used (see Fig. 3.4).

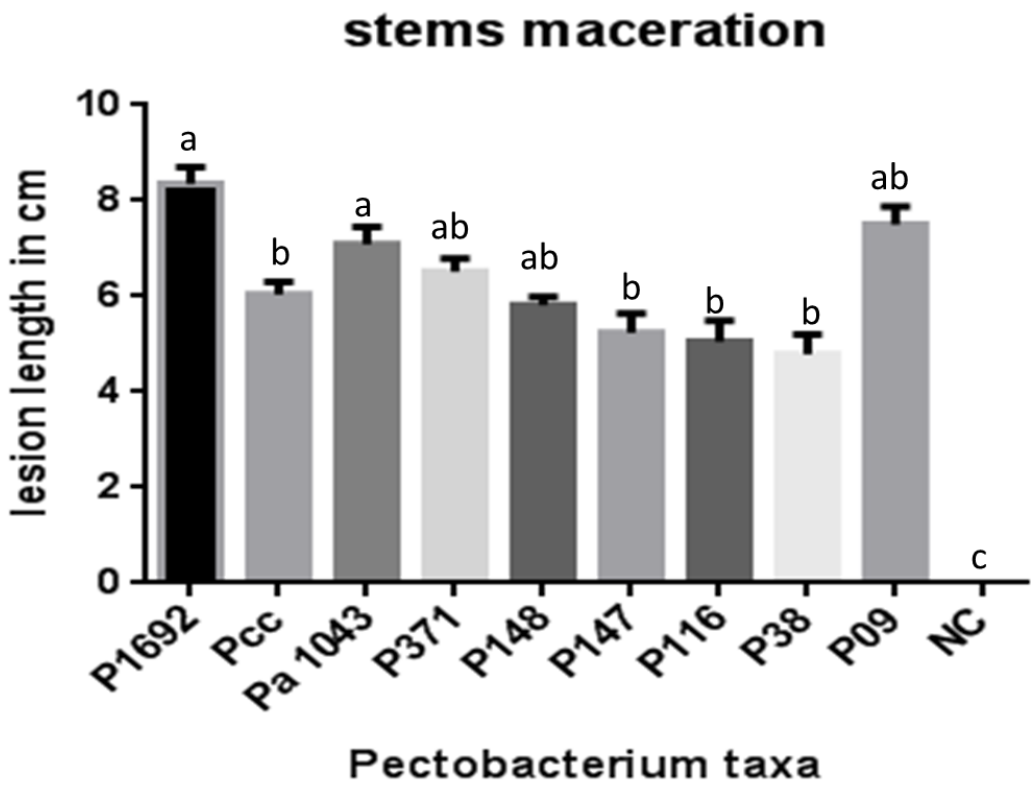

Fig. 3.4 Relative virulence of *Pectobacterium* strains on potato stems. Six-week old potato plant stems were inoculated separately with 10^8 CFU/ml of different strains. Lesion lengths on stems were measured

after 4 days of incubation at 28°C. Error bars represent standard error. Means with the same letter are not significantly different according to Tukey's multiple comparisons test (P = 0.05). NC: negative control.

Fig. 3.5 Progression of rot in potato plants (*Solanum tuberosum* cv. Mondial) inoculated with bacterial suspensions (1 x10^8 CFU/ml). (A) Healthy plants from first day of inoculation. (B) Infected plants three days post inoculation and incubation in the glasshouse. (C) Diseased plants showing wilting and total collapse seven days post inoculation.

3.3.4. Virulence on other plant hosts

To determine host range and the maceration ability of *Pcb* strains in plant species other than potato, additional vegetables were used for this assay. These included onion, green pepper, baby marrow and eggplant. In our results we observed that *Pcb* strains were able to cause soft rot symptoms on all plant species tested as shown in Fig. 3.7 below. We also observed that green peppers and baby marrows were the most susceptible hosts to tissue maceration. For both green pepper and baby marrow, rapid and severe symptoms were observed 16 hours post inoculation

whereas for the other two vegetables (eggplant and onions), symptoms were only observed 3 days post inoculation. These observations were made even though the incubation conditions for all the above vegetables remained the same. With regards to *Pcb* strains, we observed a similar pattern of symptom severity in the four vegetables using all strains. That is, we did not observe any significant intraspecies differences in symptom severity among the South African *Pcb* strains. Nonetheless strain *Pcb* 09 was consistently observed to be the most aggressive *Pcb* isolate, second only to type strain *Pcb* 1692 as shown in the bar chart in Fig. 3.6 below.

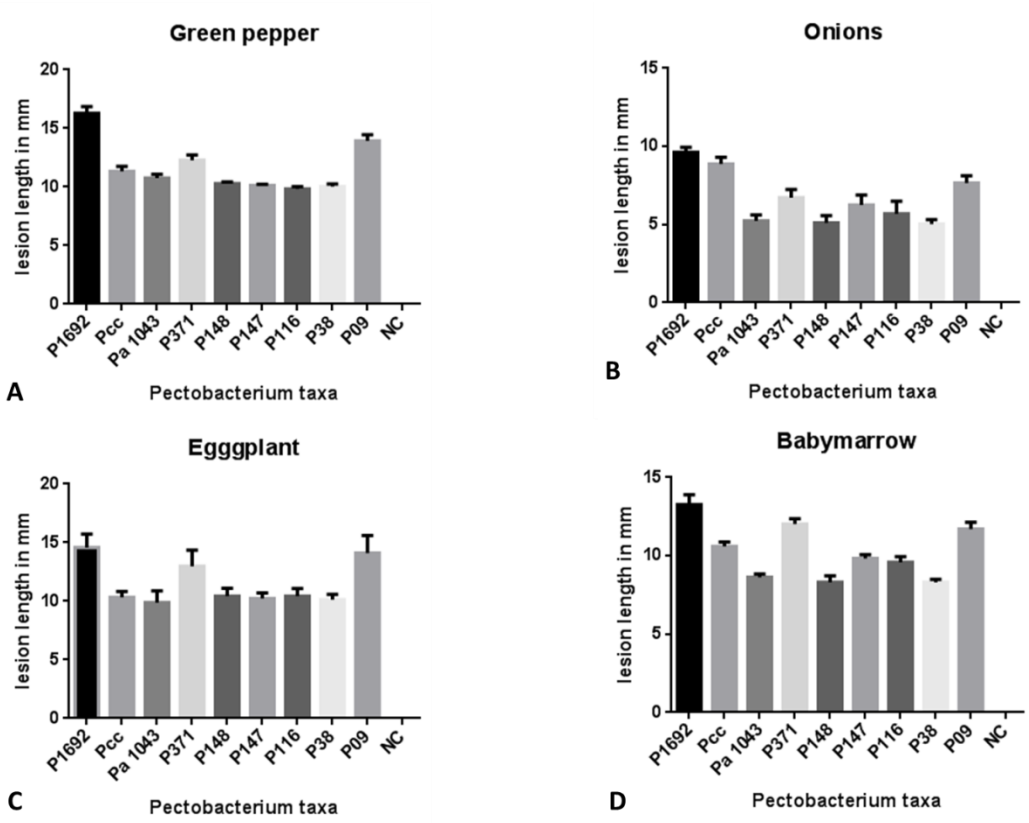

Fig. 3.6 Relative virulence of *Pectobacterium* strains on other plant hosts. Plant species (vegetables) were inoculated with 10^8 CFU/ml of bacterial suspension. Maceration symptoms on plants were measured after 16 hours to 3 days post incubation. (A) Green pepper. (B) Onions. (C) Eggplant. (D) Baby marrow. Error bars represent standard error of means. Significant differences were determined according to Tukey's multiple comparisons test (P = 0.05). NC: negative control.

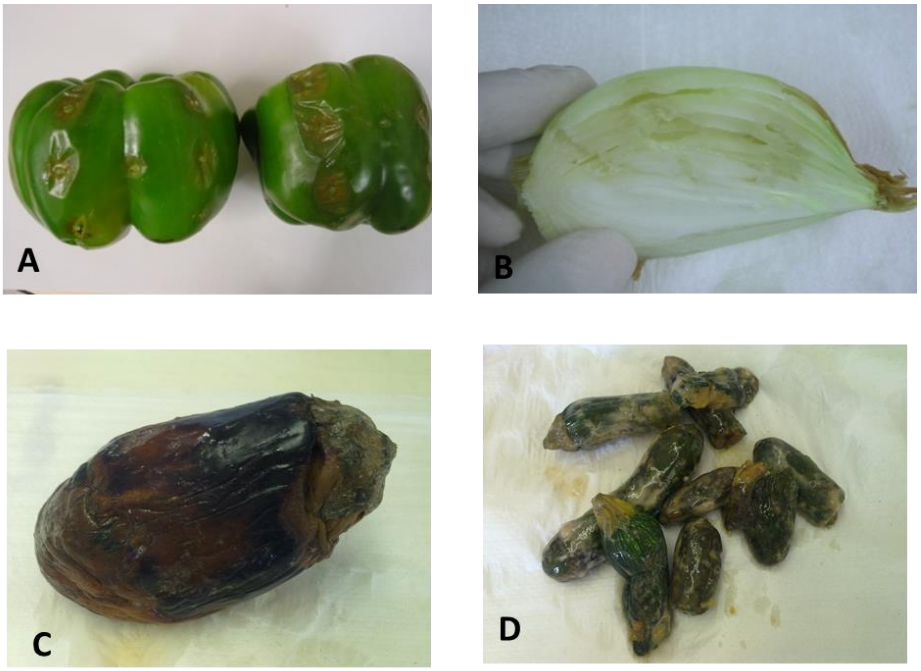

Fig. 3.7 Maceration assays of *Pcb* strains on multiple plant species (vegetables). (A) Green pepper (B) Onion (C) Eggplant (D) Baby marrow. Vegetables were inoculated with bacterial suspensions at 1 x 10^5 CFU/ml and were incubated at 28°C and lesion lengths were measured 3 days post inoculation.

3.3.5. T3SS and HR assays on tobacco

All *Pectobacterium* strains were tested on tobacco leaves for HR elicitation. From the results we observed a collapse of plant cells at the point of infection indicating successful HR elicitation on non-host tobacco leaves (Fig.3.8). We observed that *Pa* SCRI 1043 were unable to elicit a HR on tobacco leaves when compared to *Pcb* and *Pcc* strains. Among the *Pcb* strains there was a significant intraspecies variability in regard to the strength of HR elicitation. Strength of HR elicitation varied significantly between strains, for example, isolates *Pcb* 147 and *Pcb* 09 showed the strongest HR elicitation, whereas *Pcb* 116 showed the least aggressive HR elicitation on tobacco leaves. These results were consistent with what was previously observed in virulence assays on potatoes for these specific isolates. We can thus correlate the strength of HR elicitation on tobacco leaves with the severity of tuber maceration on potatoes. Tissue collapse induced by a HR is a characteristic of the T3SS as effectors translocated through the T3SS are associated with the elicitation of HR on non-hosts. So, these results also indicate the likelihood of a functional

virulence associated- T3SS *hrp* cluster harbored within the genomes of *Pcb* strains. Interestingly, *Pa* which was noted to be the least virulent strain in regard to of tuber maceration was unable to elicit a HR on tobacco leaves.

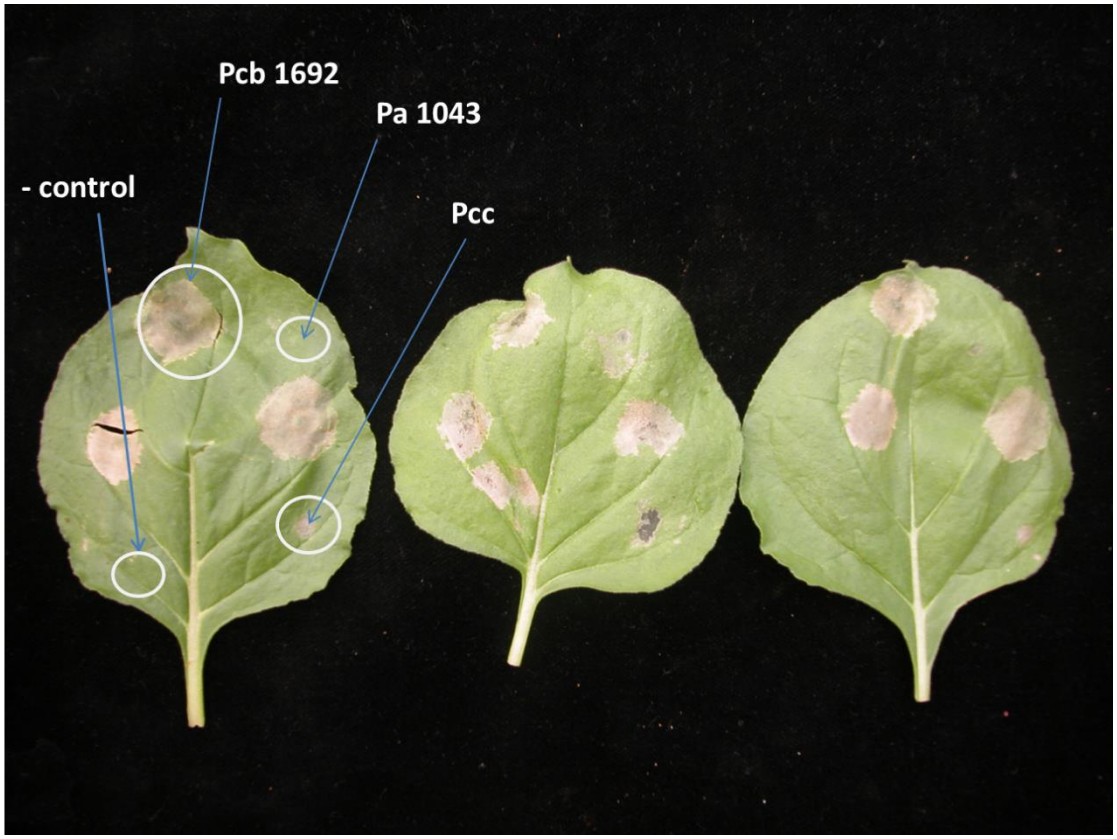

Fig. 3.8 HR assays results with tobacco (*Nicotiana. benthamiana*). *Pectobacterium* spp. were infiltrated into tobacco leaves at a level of 2 x 10^8 CFU/ml. Sterile $MgSO^4$ (10 mM) was used as a negative control. The white rings on the leaves indicate the point of infiltration and tissue collapse as a result of HR elicitation.

3.3.6. Identification of *cfa* genes in *Pcb* isolates

The presence of the *cfa* and *cfl* gene fragments was investigated in *Pcb* strains. In other studies, the *cfa* locus was only reported in *Pa* SCRI 1043 but absent in *Pcc* WPP14 and *Pcb* 1692 (Glasner *et al*., 2008). Recently, Slawiak and Lojkowska, (2009), reported the presence of *cfa* fragments in the genomes of *Pcc* strains. The results of this study through PCR amplifications and sequencing of *Pcb* strains shows that *cfa*-7 and *cfl* gene fragments are present in some of the *Pcb* isolates tested. In Fig. 3.9 we show successful PCR amplification of gene fragments, *cfa-7*

(A) and *cfl* (B) respectively. Successful PCR amplicons were confirmed by subsequent sequencing and BLAST searches on GenBank. Although it appears that *Pcb* strains do harbor fragments of the *cfa*-operon, the role of the phytotoxin coronatine in virulence of *Pcb* remains to be elucidated.

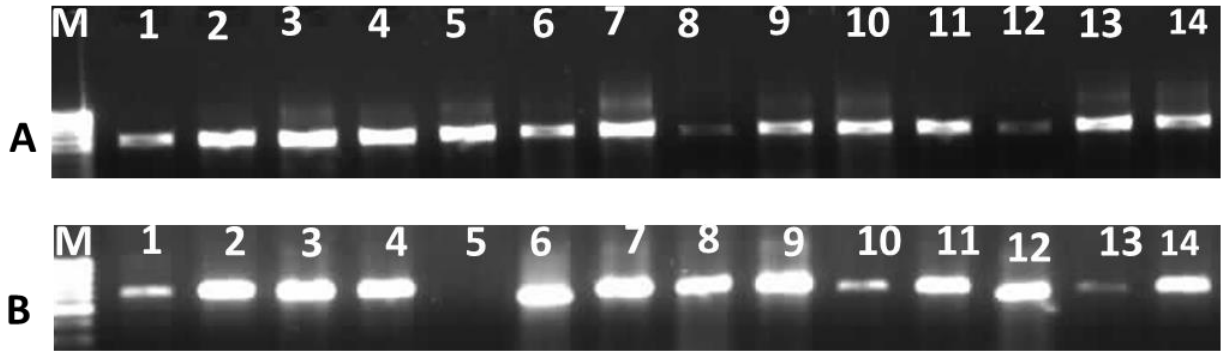

Fig. 3.9 PCR screening *of cfa-7* (A) and *cfl* (B) gene fragments from *Pectobacterium* isolates. M: DNA ladder (1Kb), 1: *Pa* 1043, 2: *Pcb* 1692, 3*: Pcb* 02, 4: *Pcb* 09, 5: *Pcb* 21, 6: *Pcb* 38, 7: *Pcb* 41, 8: *Pcb* 49, 9: *Pcb* 55, 10: *Pcb* 58, 11: *Pcb* 371, 12*: Pcb* 147, 13: *Pcb* 148, 14: *Pcb* 116.

3.3.7. Growth rate characteristics

In all our experimental analysis we observed that specific *Pectobacterium* strains consistently yielded similar results. Type strain *Pcb* 1692 and South African isolate *Pcb* 09 were shown to exhibit highly aggressive virulent traits, whereas strain *Pcb* 116 was shown to be the least virulent of South African *Pcb* isolates in all experiments. For this reason, a growth experiment was undertaken in order to compare the growth characteristics of selected strains in comparison with *Pa* SCRI 1043 and *Pcc* type strains. Results are shown in Fig. 3.10 below.

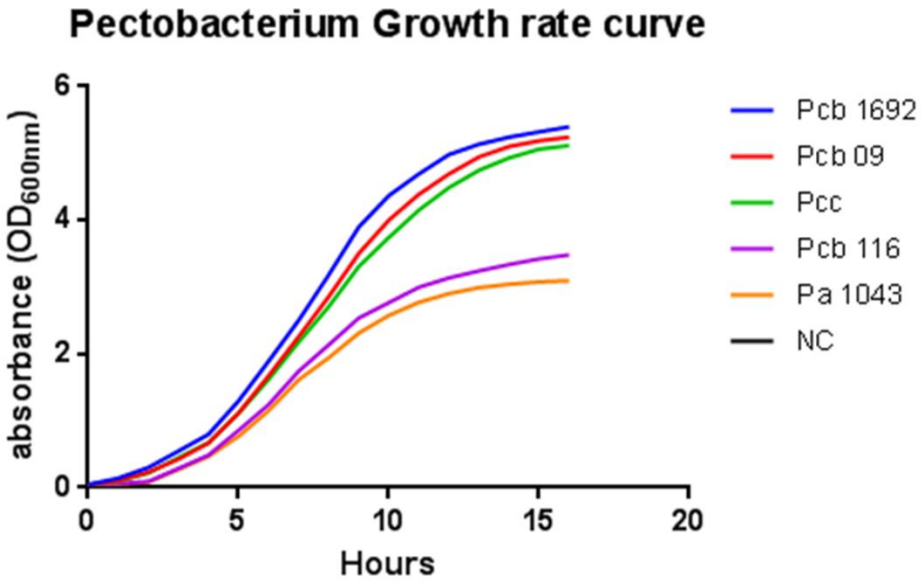

Fig. 3.10 Bacterial growth curve of five selected *Pectobacterium* strains grown on LB-media. Strains were incubated at 28°C and absorbance readings (OD_{600nm}) were taken every 30 minutes time intervals for a period of 16 hours. As determined by the T-Test analysis-, *Pa* SCRI 1043 is significantly different from *Pcb* 1692 and *Pcc*; but *Pa* SCRI 1043 is not significantly different from *Pcb 116.*

Results of the growth curve showed there were significant differences with regard to the rate of hrowth amomg the tested isolates. Strains *Pcb* 1692, *Pcc* and *Pcb* 09 were observed to grow much more rapidly compared to all other strains. In contrast, strains *Pa* and *Pcb* 116 were shown to grow much slower. This observation was also in agreement with the results noted on the relative virulence of Pectobacterium spp. were more virulent strains (*Pcb* 1692 and *Pcb* 09) appear to grow much quicker and cause most severe symptoms compared to least virulent strains (*Pa* and *Pcb* 116).

3.4. Discussion

Pcb is a recently reported subspecies of *Pectobacterium carotovorum* . which is increasingly being recognized as an important pathogen of potato (Duarte *et al.*, 2004; Ma *et al.*, 2007). Strains of *Pcb* were recently reported to be the causal agents of blackleg disease of potato in South Africa (van der Merwe *et al*. 2010).

The genomes of three main *Pectobacterium* spp. (*Pa* SCRI 1043, *Pcc* WWP14 and *Pcb* 1692) associated with soft rot diseases of potato have recently been sequenced. From the genomic analysis it appears that there is still a lot that remains to be explored with regards to the biological characteristics and virulence mechanisms of these pathogens. In view of the fact that *Pcb* strains were recently identified as blackleg-causing pathogens of potato in South Africa, the focus of this study was to characterize the biological traits and virulence mechanisms of this particular pathogen. As outlined in Table 3.2 earlier, the results of our biochemical assays on *Pectobacterium* strains were in agreement with those reported in other studies (De haan *et al.*, 2008, Palacio-Bielsa *et al.*, 2010; van der Merwe *et al.*, 2010). In this regard, all *Pcb* strains tested negative for phosphatase and oxidase activity but positive for catalase activity (Duarte *et al*. 2004). Most of the *Pcb* strains were also observed to be salt tolerant at 5% NaCl. Furthermore, *Pcb* strains were able to ferment glucose and grow at elevated temperatures of 37 to 45°C. Biochemical and physiological tests revealed some slight intraspecies variations among the isolates. However, in comparison with other studies, our findings confirmed that South African *Pcb* isolates were phenotypically similar to those reported and characterized elsewhere (Oliveira *et al*. 2002; Duarte *et al*. 2004).

Pectobacterium spp. are referred to as 'brute force' pathogens owing to their virulence associated factors such as the secretion of large quantities of plant macerating enzymes (Pérombelon, 2002; Toth *et al.*, 2003a). In this work we focused on investigating and comparing the production and activity of specific secreted enzymes among *Pcb* strains. In our results we observed that under conditions where strains were induced to produce extracellular enzymes, all *Pcb* isolates tested were able to actively produce specific enzymes and degrade the substrate. These secreted extracellular enzymes (pectate lyase, cellulase, protease and lipase) are required for the maceration and disruption of plant cell wall components during infection (Barras *et al*., 1994). It has been documented that at least one cellulase produced by *Pectobacteria* causes virulence symptoms in host plants (Mäe *et al.*, 1995). Our findings therefore reinforce the view that the secretion of extracellular enzymes is one of the primary virulence mechanisms employed by *Pcb* strains as brute-force pathogens (Pérombelon, 2002, Toth *et al.*, 2003a). This conclusion is drawn from the fact that all strains tested were able to produce enzymes on solid media supplemented with substrates that mimicked typical plant cell wall components such as pectin,

and cellulose (Perez *et al.*, 2003). Proteases secreted by *Pectobacterium* spp. also contribute to plant cell wall degradation (Marits *et al.*, 1999). However, from the results of our enzymatic assays we observed that strains such as *Pcc* and *Pa* SCRI 1043 showed a significantly reduced production of the protease enzyme and were subsequently unable to degrade gelatin compared to *Pcb* strains. Although the role of proteases in virulence of *Pectobacterium* strains is not clearly defined, reports by Marits *et al. (*1999), demonstrated that at least in *Pcc* protease activity was necessary for normal progression of disease symptoms on potato tubers. *Pectobacterium* strains defective in protease gene are reported to exhibit reduced virulence and symptom severity on plant hosts. An extracellular protease known as PrtW has been shown to be important and required for virulence. Its mechanism of action appears to be to catalyze non-specific cleavage of proteins (Marits, 1999). Furthermore, there is a hypothesis that extracellular proteases such as PrtW are produced by plant pathogens in order to aid in the provision of either amino acids for the biosynthesis of microbial proteins or in the degradation of host proteins associated with the resistance mechanism (Heilbronn *et al.*, 1995). However the role of proteases in the genome of *Pcb* still remains to be elucidated. Overall it was noted that enzyme production among the strains tested was observed to be the highest among *Pcb* and *Pcc* strains. Therefore, this observation may corroborate findings by Glasner *et al*, (2008) who reported that genes that vary among the genomes of the three *Pectobacterium* spp. include those encoding enzyme production and secretion proteins. Furthermore, *Pcb* and *Pcc* were reported to harbor more genes coding for enzyme production than was found in *Pa* SCRI 1043 (Glasner *et al.*, 2008). For that reason, it remains to be clarified whether the aggressiveness or severity of *Pcb* strains on potato plants can be correlated to the production and quantity of extracellular enzymes secreted. In agreement to findings by Yap *et al.*, (2005), we also noted significant intraspecies variation among *Pcb* strains in regard to enzyme production. For example, *Pcb* isolates such as *Pcb* 147 and *Pcb*148 were observed to display varying phenotypes even though the two strains were isolated from the same plant host and geographical region and were also shown to form a strong subcluster on the phylogenetic tree.

The diversity of *Pcb* strains observed in regard to phylogenetic relationships (Chapter 2) among the strains was also evident and reflected in terms of virulence on potato and other plant species. In our virulence assays on both potato stems and tubers we found that the aggressiveness of *Pcb*

strains was significantly diverse. The relative virulence of *Pcb* strains on potato tubers was high compared to *Pcc* and *Pa* SCRI 1043. We observed that given the same experimental conditions, *Pcb* strains were able to cause more severe soft rot symptoms than *Pa* SCRI 1043 and *Pcc* strains. Although both *Pcb* and *Pcc* strains were fairly aggressive on stems, we observed that *Pa* SCRI 1043 was much more aggressive on stems compared to most *Pcb* strains except for *Pcb* 1962 and *Pcb* 09. This finding was not surprising as *Pa* strains are commonly associated with the blackleg disease of potato more other than tuber soft rot (Pérombelon, 2002; Toth *et al*., 2003a). Our results were also in agreement with those reported by Marquez-Villavicencio *et al*. (2011), who also noted that under the same laboratory conditions, *Pa* strains were more aggressive on stems compared to tubers. Surprisingly, although in our results we observed that *Pa* was able to cause severe stem necrosis under laboratory conditions, there is no field report of blackleg incidences of potato caused by *Pa* strains in South Africa.

Pcb strains were inoculated into the bulb/fruits of multiple plant species to determine whether *Pcb* has a wide host range resembling that of *Pcc*. We observed that all *Pectobacterium* strains tested were able to cause soft rot symptoms on onions, green pepper, eggplant and baby marrows. Here too, our results were in agreement with those reported by Marquez-Villavicencio *et al.* (2011). In their work, Marquez-Villavicencio *et al.*, (2011), also noted that under laboratory conditions *Pa* SCRI 1043 strain in particular was able to macerate other plant species in addition to potato. This was an unexpected finding since *Pa* SCRI 1043 is considered to have a narrow host range restricted mainly to potato (Toth *et al.*, 2003a). Nevertheless, from our laboratory based experiments we have established that *Pcb* strains have a wide host range similar to that observed for *Pcc* strains. This finding was expected as *Pcb* is a known to be a subspecies of *Pectobacterium carotovorum* and therefore it is probable that several phenotypic traits will be shared among the two closely related pathogens.

Secretion systems play an important role in the translocation of effectors during host-pathogen interactions (Gerlach and Hensel, 2007). As such, the T3SS has been implicated in the pathogenicity of many Gram-negative bacteria including *Pectobacterium* spp. such as *Pa* SCRI 1043 and *Pcc* (Holeva *et al.*, 2004). Previously, we investigated the presence of the T3SS in *Pcb* strains by amplifying genes within the *hrp*-cluster of T3SS (unpublished date- results not

included). Hence, as a means of determining the functional role of T3SS in *Pcb*, each strain was tested on tobacco leaves to assess whether any of the isolates could elicit a HR. Results show that South African *Pcb* isolates can elicit a HR on tobacco, thus indicating a functional T3SS cluster. These results were in agreement with those reported in other studies screening for the presence and functionality of T3SS in *Pectobacterium* species (Pitman *et al.*, 2010). Nonetheless, the role of T3SS in the virulence of *Pcb* remains to be elucidated as it was reported in other studies that the absence of T3SS can be correlated with reduced virulence on potato (Holeva *et al.*, 2004). Although genes associated with the functional T3SS are reported to be present in the genomes of sequenced *Pectobacterium* species; yet to date only *Pcc* and *Pcb* are reported to elicit HR on tobacco (Glasner *et al.*, 2008). In this regard we also observed in our results and confirm that strains of *Pa* SCRI 1043 were unable to elicit a HR on tobacco leaves.

The *cfa*-locus which encodes a phytotoxin coronatine is reported to be missing from the genomes of *Pcc* and *Pcb* but present in *Pa* SCRI 1043 (Glasner *et al.*, 2008). Coronatine production has been previously linked with blackleg symptom development in potato stems. For example, *Pa* wild type causes blackleg whereas *cfa* mutants do not (Bell *et al.*, 2004). In a study by Pitman *et al*. (2008), they showed that a few of their isolates that lack *cfa* genes were also unable to elicit blackleg in potato stems. Furthermore, Slawiak and Lojkowska (2009), demonstrated a direct link between blackleg and *cfa* cluster. In this study we aimed to determine whether *Pcb* strains possess genes homologous to genes encoding Cfa and Cfl. Therefore, the successful amplification of *cfa* gene fragments from *Pcb* strains suggests that the *cfa*-locus may actually be present in *Pcb*. We were able to PCR amplify and sequence two gene fragments, *cfa*-7 and *cfl* from most of the *Pcb* strains tested. However, in some of the *Pcb* strains tested the results were negative. This is likely due to the absence of the *cfa*-locus in the respective strains as subsequent PCR screenings yielded no results, thus demonstrating that the gene cluster was absent. Our results need further analysis with regards to the functionality of the *cfa*-cluster within *Pcb* strains. However, in a study by Slawiak and Lojkowska, (2009), it was also reported that several *Pcc* strains tested also harbored *cfa* genes. Consequently, it is possible that some of the *Pectobacterium* strains may simply lack the genes in question as the *cfa*-locus in *Pa* SCRI 1043 is located within a horizontally acquired island (HAI2) (Bell *et al.*, 2004).

It appears that temperature plays an important role in the growth of *Pectobacterium* strains (Hasegawa *et al.*, 2005). Consequently the rapid rate of growth at elevated temperatures seems to be correlated with virulence (Nguyen et al., 2002). In our results we noted that growth rate was positively correlated with virulence traits of the strains tested. For example, as shown in the growth curve (Fig. 3.10), the most virulent strains as tested on potato tubers and stems were *Pcb* 1692 and *Pcb* 09. These strains were observed to grow much faster compared to the least virulent strains, *Pa* 1043 and *Pcb* 116, respectively. As a result, it can be concluded that temperature does play a role in the virulence of *Pcb* strains. According to reports in other studies, the role of temperature in virulence might be due to the activation and expression of genes encoding important virulence associated enzymes such as pectate lyases, as well as carotovoricin which are responsible for cell lysis in plant pathogenic bacteria such as *Pcc* (Nguyen *et al.*, 2002, Hasegawa *et al*., 2005).

The aim of this current work was to examine the intraspecies variation that may exist among South African *Pcb* isolates in regard to phenotypic, host range and virulence mechanisms. Overall, we have established that there is a high level of genetic diversity among the *Pcb* strains isolated from the same region in the same season and same host species in South Africa. The level of genetic diversity observed here is therefore reflected in all the phenotypic, pathogenic and host range characteristics of the pathogen. *Pcb* is increasingly becoming an important pathogen of potato, as such, it is imperative that knowledge pertaining to the genetic, phenotypic, as well as virulence-associated characteristics of this pathogen are well understood. Such knowledge will possibly aid in the control and eradication strategies of the soft rot and blackleg disease of potato perpetuated by *Pcb.* For this reason, we believe that the findings of this present study will contribute positively to the knowledge of *Pcb* strains in South Africa and that together with other related projects on this subject, *Pcb* which is an important pathogen of potato will eventually be brought under control. Future studies will be focused on characterizing *Pcb* strains on a molecular genetic level, in order to screen for, identify and describe important virulence associated genes harbored within the genome of this pathogen.

3.5. References

Avrora, A.O., Hyman, L.J., Toth, R. L & Toth, I.K. (**2002**). Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. Applied and Environmental Microbiology. 68:1499-1508

Barrass, F., Van Gijsegem, F., and Chatterjee, A.K., (**1994**). Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annual Reviews of Phytopathology. 32:201-234

Bell, K.S., Sebaihia, M., Pritchard, L., Holden, M.T.G., Hyman, L.J., Holeva, M.C., Thomson, N.R., Bentley, S.D., Churcher, L.J.C., Mungall, K., Atkin, R., Bason, N., Brooks, K., Chillingworth, T., Clark, K., Doggett, J., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Norbertczak, H., Ormond, D., Price, C., Quail,, M.A., Sanders, M., Walker, D., Whitehead, S., Salmond, G.P.C., Birch, P.R.J., Parkhill, J., and Toth, I.K. (**2004**). Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. PNAS. 101 (30):11105–11110

Bender, C.L., Chaidez, F.A.N., and Gross, D.C. (**1999**). *Pseudomonas syringae* Phytotoxins: Mode of Action, Regulation, and Biosynthesis by Peptide and Polyketide Synthetases. Microbiology and Molecular Biology Reviews, 63 (2): 266-292

Chatterjee A., Cui Y., Liu. Y., Dumenyo C.K., Chatterjee A.K. (**1995**). Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulose, and protease in *Erwinia carotovora* subsp. *carotovora* in the absence of starvation/cell density-sensing signal, *N*-(3-oxohexanoyl)-l-homoserine lactone. Applied and Environmental Microbiology. 61:1959-1967

Cother, E. J., and, **Sivasithamparam**, K. (**1983**). Erwinia: the "carotovora" group. In G. J. Fahy & P. C. Persley (Eds.), Plant bacterial diseases. A diagnostic guide (pp. 87–101).
Sydney: Academic.

De Haan, E.G., Dekker-Nooren, T.C.E.M, Van den Bovenkamp, G.W., Speksnijder, A.G.C.L., van der Zouwen, P.S., and van der Wolf, J.M. (**2008**). *Pectobacterium carotovorum* subsp. *carotovorum* can cause potato blackleg in temperate climates. European Journal of Plant Pathology. 122:561-569

Duarte, V.J., De Boer S.H., Ward, L.J., and De Oliveira, A.M.R (**2004**) Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *Journal of Applied Microbiology*. 96: 535-545

Gerlach R.G., and **Hensel**, M., (**2007**). Protein secretion systems and adhesins: The molecular armoury of Gram-negative pathogens. International Journal of Medical Microbiology. 297: 401-415

Glasner J.D., Marquez-Villavicencio, M. Kim, H.S., Jahn, C.E., Ma, B., Biehl, B. S., Rissman, A.I., Mole, B., Yi, X., Yang, C.H., Dangl, J.L., Grant, S.R., Perna, N.T., Charkowski, A.O. (**2008**) Niche-Specificity and the Variable Fraction of the *Pectobacterium* Pan-Genome. MPMI. 21(12):1549-1560

Hankin, L. and **Anagnostakis**, S.L. (**1975**) Mycologia, 67, 597-607. Henikoff,S. (1984) Gene, 28, 351-359.

Hasegawa, H., Chatterjee, A., Cui, Y., and Chaterjee, A.K., (**2005**). Elevated Temperature Enhances Virulence of *Erwinia carotovora* subsp. *carotovora* Strain EC153 to Plants and Stimulates Production of the Quorum Sensing Signal, *N*-Acyl Homoserine Lactone, and Extracellular Proteins. Applied and Environmental Microbiology, 71 (8): 4655–4663

Heilbronn, J., Johnston D.J., Dunbar, B., Lyon G.D. (**1995**). Purification of a metalloprotease produced by *Erwinia carotovora ssp. carotovora* and the degradation of potato lectin *in vitro***.** Physiological and Molecular Plant Pathology. 47 (5): 285-292

Holeva, M.C., Bell, K.S., Hyman, L.J., Avrova, A.O., Whisson, S.C., Paul R. J. Birch, P.R.J., and Toth, I.K. (**2004**) Use of a Pooled Transposon Mutation Grid to Demonstrate Roles in Disease Development for *Erwinia carotovora* subsp. a*troseptica*, Putative Type III Secreted Effector (DspE/A) and Helper (HrpN) Proteins. MPMI 17 :(9), 943–950

Kim, H.S., Ma, B., Perna, N.T., Charkowski, A.O., (**2009**). Phylogeny and virulence of naturally occurring type III secretion system deficient *Pectobacterium* strains. Applied Environmental Microbiology. 75(13): 4539–4549

Ma, B., Hibbing, M.E., Kim, H., Reedy, R.M., Yedidia, I., Breuer, J., Glasner, J.D., Perna, N.T., Kelman, A., and Charkowski, A.B., (**2007**). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacteria* and *Dickeya*. Phytopathology. 97:1156-1163

Mäe A, Heikinheimo R., and Palva ET (**1995**) Structure and regulation of the Erwinia *Carotovora* subspecies carotovora SCC3193 cellulase gene celV1 and the role of Cellulase in phytopathogenicity. Mol Gen Genetics 247, 17–26.

Marits, R., Koiv, V., Laasik, E., and Mae, A. (**1999**). Isolation of an extracellular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity. Microbiology 145, 1959-1966.

Marquez-Villavicencio, M. D. P., Groves, R. L., and Charkowski, A. O. (**2011**). Soft rot disease severity is affected by potato physiology and *Pectobacterium* taxa. Plant Disease. 95:232-241

Moleleki, L.N. Onkendi, E.M., Mongae. A., Kubheka. G.C., (**2012**) Characterization of *Pectobacterium wasabiae* causing blackleg and soft rot diseases in South Africa. European Journal of Plant Pathology.DOI 10.1007/s10658-012-0084-4

Nabhan, S., Wydrab, K, Lindec, M, Debene, T. (**2011**). The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. Plant Pathology. Doi: 10.1111/j.1365-3059.2011.02546.x

Nabhan, S., , De Boer, S.H., , Maiss, E., and Wydra, K. (**2012**). Taxonomic relatedness between *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum and Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. Journal of Applied Microbiology. 113, 904-913

Nguyen, H.A., Kaneko, J., Kamio, Y. (**2002**). Temperature-dependant production of carotovoricin and pectate lyase in phytopathogenic *Erwinia carotovorum* subsp. *carotovora* Er. Biosci. Biotechnol. 66 (4): 444-447

Oliveira, A.M.R., Duarte, V., Silveira, J.R.P., and Moraes, M.G. (**2002**). Incidence of pectolytic *Erwinias* associated with blackleg of potato in Rio Grande do Sul. Fitopatologia Brasileira. 28:49-53

Palacio-Bielsa, A., Mosquera, M.E.R., Alvarez, M.A.C., Rodriguez, I.M.B., Lopez-Solanilla, E., and Rodriguez-Palenzuela. (**2010**). Phenotypic diversity, host range and molecular phylogeny of *Dickeya* isolates from Spain. European Journal of Plant Pathology. 127:311-324

Pérombelon, M.C.M. (**2002**). Potato disease caused by soft rot *Erwinias*: an overview of pathogenesis. Plant Pathology. 51: 1-12

Pérez S., Rodríguez-Carvajal, M.A., and Doco, T. (**2003**). A complex plant cell wall polysaccharide: rhamnogalacturonanII. A structure in quest of a function. Biochimie 85:109–121

Pitman, A.R, Wright P., Galbraith M., Harrow S., Marshall J. (**2008**). Biochemical and molecular diversity among pectolytic Erwinia from potato in New Zealand. Australasian Plant Pathology 37, 559-568.

Pitman, A.R., Harrow, S.A., Visnovsky S.B. (**2010**). Genetic characterization of *Pectobacterium wasabiae* causing soft rot disease of potato in New Zealand. European Journal of Plant Pathology. 126:423–435

Sambrook, J., and D. W. **Russell**. (**2001**). Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y

Slawiak, M.**,** and **Lojkowska**, E. (**2009**). Genes responsible for coronatine synthesis in *Pseudomonas syringae* present in the genome of soft rot bacteria. European Journal of Plant Pathology. 124:353-361

Toth, I.K., Bell, K.S., Holeva, M.C., Birch, P.R.J. (**2003a**). Soft rot *Erwiniae*: from genes to genomes. Molecular Plant Pathology. 4 (1):17–30

Toth, I.K., Pritchard, L., and Birch, P.R.J. (**2006**). Comparative genomics reveals what makes an enterobacterial plant pathogen. Annual Review of Phytopathology. 44: 305-336

van der Merwe, J.J., Coutinho, T.A., Korsten, L., and van der Waals, J. (**2010**) *Pectobacterium carotovorum* subsp. *brasiliensis* causing blackleg on potatoes in South Africa. European Journal of Plant Pathology. 126:175-185

Yang C.J., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., Chen, J. W., Perna, N.T., and Keen, N.T. (**2002**). hrp Genes of Erwinia chrysanthemi 3937 are Important Virulence Factors. MPMI: 15 (5,): 472–480

Yap, M.N., Barak, J.D., Charkowski, A.O., (**2004**). Genomic Diversity of *Erwinia carotovora* subsp. *carotovora* and Its Correlation with Virulence. Applied and Environmental Microbiology. 70 (5):3013–3023.

Yap, M.N., Barak, J.D., Courtney, E.J., Charkowski, A.O. (**2005**). The *Erwinia chrysanthemi* Type III Secretion System is required for Multicellular Behaviour. Journal of Bacteriology. 187: 639-648

Appendix 3A.

Enzymatic assay Materials and Methods

Pectolytic activity media was used to detect pectate lyase production and the media contained 500 ml mineral salts solution, 1 g yeast extract, 15 g of agar, 5 g of pectin, 500 ml of deionized

water; pH 7.0. Plates were incubated under room temperature for 3 days and were then flooded with 1% aqueous solution of hexadecyltrimethylammonium bromide (CTAB).

Cellulase activity media was used to detect cellulolytic activity. The media contained carboxymethylcellulose (CMC) agar (0.2% NaNO3, 0.1% K2HPO4, 0.05% MgSO4,0.05% KCl, 0.2% carboxymethylcellulose (CMC) sodium salt, 0.02% peptone, and 1.7% agar), ; pH 6.0. Plates were then flooded with 1% aqueous CTAB solution.

Proteolytic activity media was used to test for production of proteolytic enzymes and ability to degrade gelatin. The media contained NA agar plus 0.4% gelatin; *pH* 6.0. Plates were then flooded a saturated solution of ammonium sulphate.

Lipolytic activity media was used to test for production of lipolytic enzymes. The media contained peptone, 10 g, NA agar 20g, NaCl 5 g, $CaCl_2.2H_2O$, 0.1 g; pH 6.0.

Table 3.2 Results of biochemical assays with *Pectobacterium* spp.

Strains	Growth 20°C	Growth 25°C	Growth 30°C	Growth 35°C	Growth 45°C	5% NaCl	Oxidase activity	Phosphatase activity	Catalase activity	Fermentation from glucose
Pcb cucu01	+	+	+	+	+w	+	-	-	+	+
Pcb 02	+w	+	+	+	-	+	-	-	+	+
Pcb 09	+	+	+	+	+	+	-	-	+	+
Pcb 21	+	+	+	+	-	+	-	-	+	+
Pcb 38	+	+	+	+	+	+	-	-	+	+
Pcb 41	+w	+	+	+	-	+	-	-	+w	+
Pcb 49	+	+	+	+	+	+w	-	-	+	+
Pcb 55	+	+	+	+	+w	+	-	-	+	+
Pcb 58	-	+	+	+	+w	-	-	-	+	+
Pcb 60	+	+	+	+	+	+w	-	-	+	+
Pcb 64	+	+	+	+	+w	+	-	-	+	+
Pcb 108	-	+	+w	+	+w	-	-	-	+	+
Pcb 116	+	+	+	+	+w	+	-	-	+w	+
Pcb 147	+w	+	+	+	+w	+	-	-	+	+
Pcb 148	+	+	+	+	+w	+	-	-	+	+
*Pcb*371	+	+	+	+	+w	+	-	-	+	+
Pa SCRI 1043	+	+	+w	-	-	+	-	-	+	+
Pcb 1692	+	+	+	+	+w	+	-	-	+	+

CHAPTER FOUR

General summary and conclusions

4.1. General summary and Conclusion

It appears that temperature plays an important role in the growth of *Pectobacterium* strains (Hasegawa *et al.*, 2005). Consequently the rapid rate of growth at elevated temperatures seems to be correlated with virulence (Nguyen et al., 2002). In our results we noted that growth rate was positively correlated with virulence traits of the strains tested. For example, as shown in the growth curve (Fig. 3.10), the most virulent strains as tested on potato tubers and stems were *Pcb* 1692 and *Pcb* 09. These strains were observed to grow much faster compared to the least virulent strains, *Pa* 1043 and *Pcb* 116, respectively. As a result, it can be concluded that temperature does play a role in the virulence of *Pcb* strains. According to reports in other studies, the role of temperature in virulence might be due to the activation and expression of genes encoding important virulence associated enzymes such as pectate lyases, as well as carotovoricin which are responsible for cell lysis in plant pathogenic bacteria such as *Pcc* (Nguyen *et al.*, 2002, Hasegawa *et al*., 2005).

The aim of this current work was to examine the intraspecies variation that may exist among South African *Pcb* isolates in regard to phenotypic, host range and virulence mechanisms. Overall, we have established that there is a high level of genetic diversity among the *Pcb* strains isolated from the same region in the same season and same host species in South Africa. The level of genetic diversity observed here is therefore reflected in all the phenotypic, pathogenic and host range characteristics of the pathogen. *Pcb* is increasingly becoming an important pathogen of potato, as such, it is imperative that knowledge pertaining to the genetic, phenotypic, as well as virulence-associated characteristics of this pathogen are well understood. Such knowledge will possibly aid in the control and eradication strategies of the soft rot and blackleg disease of potato perpetuated by *Pcb*. For this reason, we believe that the findings of this present study will contribute positively to the knowledge of *Pcb* strains in South Africa and that together with other related projects on this subject, *Pcb* which is an important pathogen of potato will

eventually be brought under control. Future studies will be focused on characterizing *Pcb* strains on a molecular genetic level, in order to screen for, identify and describe important virulence associated genes harbored within the genome of this pathogen.

4.2. References

Hasegawa, H., Chatterjee, A., Cui, Y., and Chaterjee, A.K., (**2005**). Elevated Temperature Enhances Virulence of *Erwinia carotovora* subsp. *carotovora* Strain EC153 to Plants and Stimulates Production of the Quorum Sensing Signal, *N*-Acyl Homoserine Lactone, and Extracellular Proteins. Applied and Environmental Microbiology, 71 (8): 4655–4663

Nguyen, H.A., Kaneko, J., Kamio, Y., (**2002**) Temperature-dependant production of carotovoricin and pectate lyase in phytopathogenic *Erwinia carotovorum* subsp. *carotovora* Er. Biosci. Biotechnol. 66 (4): 444-447