CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND AND MOTIVATION OF THE STUDY

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

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

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

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

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

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

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

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

1.2 FUNDAMENTAL OBJECTIVE

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

1.3 SPECIFIC OBJECTIVES

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

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

1.4 CHAPTER OUTLINE

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

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

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

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

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

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

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CHAPTER 2

A REVIEW OF BLACKLEG AND SOFT ROT DISEASES IN POTATOES

Abstract

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

2.1 INTRODUCTION

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

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

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

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

2.2 THE PATHOGENS

2.2.1 Morphology of pectolytic bacteria

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

2.2.2 Occurrence and host range of soft rot enterobacteria

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

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

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

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

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

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

2.2.3 Disease symptoms in potatoes

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

Blackleg

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

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

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

Tuber soft rot

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

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

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

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

Aerial stem rot (Aerial blackleg)

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

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

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

2.2.4 Epidemiology

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

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

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

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

2.3 THE DISEASE PROCESS

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

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

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

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

2.3.1 Pectolytic enzymes

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

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

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

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

2.3.2 Regulation and secretion of exoenzymes

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

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

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

2.3.3 Global virulence regulation network

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

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

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

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

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

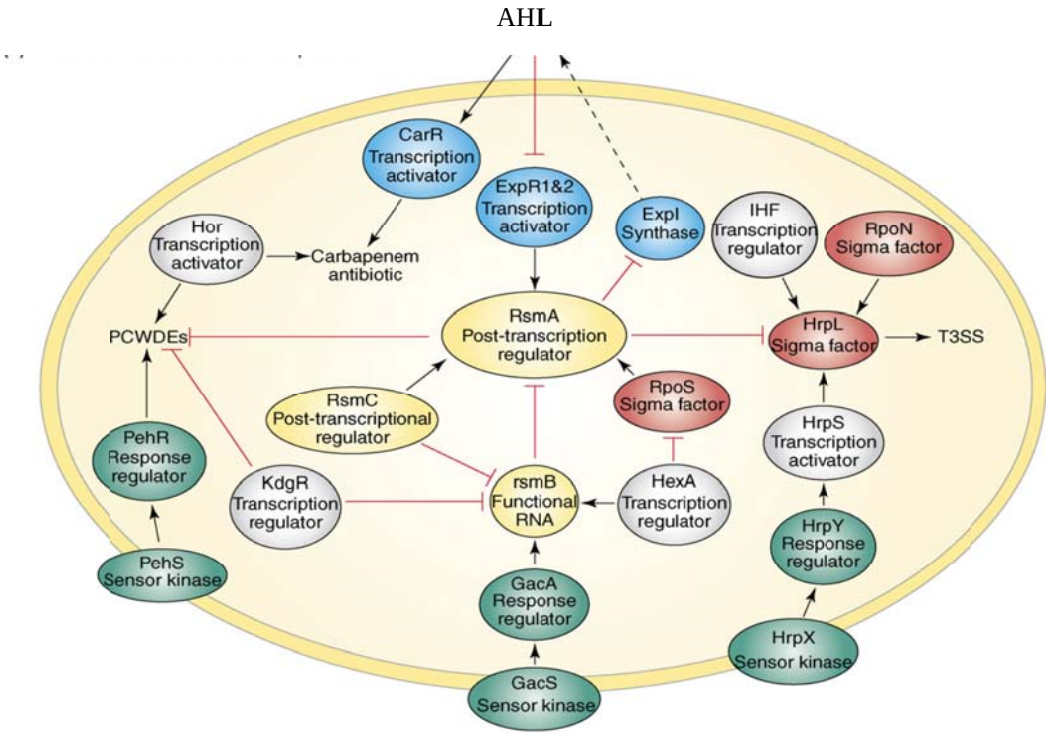

Fig 2.4 The global virulence regulation of *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). **Color code**: blue, associated with cell-to-cell communication; green, TCST systems; red, sigma factors; yellow, part of the Rsm system; grey, other members that do not fit into major systems discussed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

2.4 DIAGNOSTICS AND FINGERPRINTING

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.5 DEFENSE SYSTEMS IN PLANTS

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

2.5.1 Phenolic compounds

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

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

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

2.5.2 Polyphenol oxidase (PPO)

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

2.5.3 Phenylammonia lyase (PAL)

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

2.5.4 Effect of soil calcium amendment in disease resistance

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

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

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

2.6 CONTROL

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

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

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

2.7 CONCLUSION

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

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

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

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