DETECTION OF *ERWINIA* SPP. ON POTATOES

by

ALPHEUS KGABO MATLALA

Submitted in partial fulfilment of the requirements for the degree of

M.Inst. Agrar (Plant Protection)

In the Faculty of Natural and Agricultural Sciences

Department of Microbiology and Plant Pathology

University of Pretoria

Pretoria

South Africa

`If you believe, you will
receive whatever you
ask for in prayer`

Matthew 21:22

ACKNOWLEDGEMENTS

The author would like to thank the following people:

Professor Lise Korsten, my promoter, for encouraging me throughout these studies. It was because of you that I achieved this degree.

Estelle Towsen, for always being available and giving helpful guidance. You became a pre-school teacher to a post graduate student.

Dr. Gina Swart, my co-promoter, for all her guidance, teaching and assistance with molecular work. Your excellent help will always be cherished.

Dr. Marinda Oosthuizen, for all her help concerning PAGE and analysis of the gels.

My parents for their support and advice. You were my first and best teachers on earth.

Steve and Senyane, for their inborn brotherhood. You were my sources of inspiration.

Laurenda and Lehlogonolo, my best friends, for their care and patience. I love you two.

The National Research Foundation (NRF) and the Post Graduate School of Agriculture and Rural Development for accommodation and bursaries awarded to me.

Potato South Africa for their financial support and interest in the project.

Australia-South Africa Institutional Links Program for financing my three months study at The University of Queensland (Gatton college) Australia.

CONTENTS

		Page
CHAPTER 1	**GENERAL INTRODUCTION**	1
	1.1 Literature cited	3
CHAPTER 2	**LITERATURE REVIEW**	7
	2.1 Introduction	7
	2.2 Diseases caused by *Erwinia* spp. on potatoes	10
	2.2.1 Blackleg	10
	2.2.2 Aerial stem rot	10
	2.2.3 Stem wet rot	11
	2.2.4 Early dying	11
	2.2.5 Bacterial soft rot	11
	2.2.6 Lenticel hard rot	12
	2.3 Diseases caused by *Erwinia* spp. on other crops	12
	2.4 Disease epidemiology	13
	2.4.1 Environmental factors affecting diseases expression	15
	2.4.2 Survival of *Erwinia* spp.	15
	a) Soil	16
	b) Water	16
	c) Air borne *Erwinias*	16
	d) Potato leaves	17
	e) Mechanical handling of equipment	17
	2.5 Isolation of the bacterium	18
	2.5.1 Diagnosis of the bacterium	18
	2.5.2 Traditional techniques	18
	2.5.3 Tuber incubation test	19
	2.5.4 Molecular techniques	19
	a) Polymerase Chain Reaction	19
	b) The use of serological tests	20

c) Polyacrylamide Gel Electrophoresis 21
2.6 Resistance and pathogenicity 22
2.6.1 Cultivar susceptibility 22
2.6.2 Pathogenicity 22
2.7 Disease control 23
2.7.1 Cultural practices 23
2.7.2 Clean seed 24
2.7.3 Biological control 24
2.7.4 Breeding 25
2.7.5 Chemical control 25
2.7.6 Tuber pasteurization 26
2.8 Conclusion 27
2.9 Literature cited 28

CHAPTER 3 PREVALENCE OF *ERWINIA* SPP. ON ASYMPTOMATIC POTATO TUBERS 39
3.1 Introduction 40
3.2 Materials and methods 41
3.2.1 Experimental tubers 41
3.2.2 Tuber assays 42
3.2.3 Selective media 42
3.2.4 Maintenance of cultures 43
3.2.5 Identification of *Erwinia* spp. 43
a) Microplate method 43
3.2.6 BIOLOG test 44
3.2.7 Pathogenicity tests 45
3.3 Results 45
3.3.1 Basic diagnostic tests, growth, enrichment and selective media 46
3.3.2 Biochemical tests 48
3.3.3 BIOLOG test 50

3.3.4 Pathogenicity tests 51
3.4 Discussion 52
3.5 Literature cited 54

CHAPTER 4 COMPARISON OF *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA* ISOLATES USING PCR AND SDS-PAGE 59
4.1 Introduction 60
4.2 Materials and methods 61
4.2.1 Confirmation of identity of cultures 61
4.2.2 DNA extractions 61
4.2.3 PCR amplification 62
4.2.4 Reproducibility of DNA fingerprints 62
4.2.5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis 62
4.2.6 Standard conditions for SDS-PAGE 63
4.2.7 Analysis of protein patterns 63
4.3 Results 64
4.4 Discussion 70
4.5 Literature cited 71

CHAPTER 5 GENERAL DISCUSSION 76
5.1 Literature cited 79

SUMMARY 82

SUMMARY

DETECTION OF *ERWINIA* SPP. ON POTATOES

by

ALPHEUS KGABO MATLALA

SUPERVISOR	Prof. L. Korsten
CO-SUPERVISOR	Dr. G. Swart
DEPARTMENT	Microbiology and Plant Pathology
DEGREE	M. Inst. Agrar (Plant Protection)

In South Africa, pectolytic *Erwinia* spp. cause major economic losses to the potato industry. The bacteria affect potato tubers in storage and in transit causing soft rot. They also affect the stem of the potato plant, causing blackleg, or the entire plant that can wilt and die. *Erwinia* spp. are ubiquitous and can occur on asymptomatic potato seeds. Early detection of pectolytic *Erwinias* is therefore important as a first step to control the disease. Isolations were made from potato tuber cultivars collected from various production areas in South Africa. An enrichment technique and a selective media (CVPB) were used to facilitate the ease of isolations. Three soft rot causing bacteria were differentiated from each other, using BIOLOG, biochemical and pathogenicity tests. None of these tests could differentiate between the three soft rot causing bacteria and discrepancies were observed in results. PAGE and PCR were subsequently used to further fingerprint and identify *Erwinia* spp. PAGE revealed a similarity coefficient ranging from less than 40% to 89%. RISA and BOX-PCR showed a similarity coefficient ranging from less than 60% to 100% (RISA) and less than 50% to 100% (BOX-PCR). This study highlighted the potential of using these techniques for rapid detection and identification of *Erwinia* spp.

CHAPTER 1

GENERAL INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are grown as a major food source in countries with a temperate climate (Rich, 1983). Globally, potato is the fourth most important staple food after wheat, rice and maize (Hawkes, 1992). In South Africa, potato production has increased from 55 746 ha being planted in 1995 to 56 680 ha in 1999 (Mosterd 2000, Personal conversation). Although the bulk of production is mainly for local consumption, export consignments have increased from 100 000 tons in 1996 to 120 000 tons in 1999 (Mosterd 2000, Personal conversation). Current South African exports of seed potato are mainly to Angola, Mauritius, Namibia and a lesser extent to Ruanda. Future potential markets however, include countries of the Indian Ocean Islands and certain North African states (Mosterd 2000, Personal conversation).

Consumption of potatoes in South Africa is rapidly increasing which can be attributed to the current growth in urbanization. In addition, the informal sector contributes to this trend, by making potatoes readily available on the streets, particularly in informal residential areas (Potato Producers Organization 1994-1995, Review of Industry). During the 1998 season, potatoes constituted 44, 6% of all vegetables sold on the South African fresh produce market (Crops & Markets, 2000).

In South Africa, potato production is more or less evenly distributed amongst various production areas with different climatic conditions (Potato Producers Organization 1994-1995, Review of Industry). Due to the variable environmental conditions under which potatoes are cultivated, the crop is continuously exposed to a wide variety of pathogens. One of the most important bacterial pathogens of potatoes is *Erwinia* spp. which also attack other crops such as ornamental, tropical and subtropical plants either in the field, in storage or in transit (Cother & Powell, 1983). This group of plant pathogens also causes the most damage to vegetables (Ervin & Barnes, 1968). According to De Boer (1994), *Erwinia* spp.

are amongst the most common and ubiquitous pathogens on potatoes. The different *Erwinia* spp. found on potatoes are classed under the *carotovora* or "soft rot" group (Dickey & Kelman, 1990). The species and subspecies that fall under these groups include: *Erwinia carotovora* subsp. *carotovora* (Jones, 1901) Dye; *Erwinia carotovora* subsp. *atroseptica* (Van Hall, 1902) Dye and *Erwinia chrysanthemi* (Burkholder *et al.*, 1953). The former two pathogens are also known to be dominant in the *Erwinia* disease complex on potatoes (De Boer 1994). The *Erwinia* pathogens possess an array of pectolytic enzymes used as pathogenicity factors in host pathogen interactions. *E. chrysanthemi* also produces pectolytic enzymes which differentiate it from the *carotovora* group warranting status as a separate species. Symptoms that range from blackleg, soft rot (Mackay & Shipton, 1983) and potato wilt (Serfontein *et al.*, 1988) are caused by these three *Erwinia* spp.

Blackleg is the most important of these potato diseases (Hawkins, 1990) and is recognized as a major threat to potato production world-wide (Molina & Harrison, 1977). Direct and indirect crop losses caused by *Erwinia* spp., have not been estimated, but are known to be enormous (De Boer, 1994). Perombelon & Kelman (1980), estimated that global losses caused by *Erwinia* spp. range between $50 to $100 million annually. In Southern Georgia, soft rot caused major losses of harvested and stored tubers during the early 1970's (Sherf & Macnab, 1986), while blackleg resulted in estimated losses of between 5-10% in Colorado (Molina & Harrison, 1977). *E. chrysanthemi* has been identified as causing severe losses of planted seed tubers in semi arid irrigation areas of Australia (Cother & Powell, 1983).

Although losses caused by *Erwinia* spp. in South Africa have not yet been quantified or documented, Towsen & Korsten (1998) found in a recent survey that the disease can be of major concern in certain production areas and years and that producers are aware of the disease and its economic impact.

Due to severe crop losses caused by *Erwinia* spp. on potatoes, it is important to study rapid effective detection methods, disease epidemiology and control. Detection methods are studied because correct diagnosis of any disease is a pre-requisite for effective control (Schaad, 1979). Detection of *Erwinia* spp. on seed potatoes is also an essential requirement

for production of healthy seed crops (Cother & Vruggink, 1980). According to Kelman & Dickey (1989), it is difficult to distinguish between *Erwinia carotovora* subspecies on the basis of symptoms in stems and tubers. It is therefore important to develop effective and rapid methods to identify the correct pathogen.

Different detection techniques have been used for *Erwinia* spp. but there are relatively few reports comparing these methods for consistency and effectiveness. Techniques were mainly used to study transmission of *Erwinia* spp. from mother to progeny tubers and included Polymerase Chain Reaction (PCR) (Smid *et al.*, 1995), dilution plating, Enzyme-Linked Immunosorbent Assay (ELISA) and immunofluorescence cell staining (Fraaije *et al.*, 1996). De Boer *et al.* (1987) used biochemical tests to differentiate pathogenic, pectolytic *Erwinia* spp. and subspecies, while Moline (1985) used two-dimensional Polyacrylamide Gel Electrophoresis (PAGE) to differentiate post harvest soft rotting bacteria. In this study, biochemical tests and BIOLOG were compared for their efficiency, cost effectiveness and rapidness to detect *Erwinia* spp. on healthy potato tubers for eventual commercial use by the industry. SDS PAGE and PCR-based fingerprinting methods were used for rapid identification of the isolates and to distinguish between closely related subspecies of *Erwinia* spp.

1.1 Literature cited

Cother, E. J. & Powell, V. 1983. Physiological and pathological characteristics of *Erwinia chrysanthemi* isolates from potato tubers. *Journal of Applied Bacteriology* 54: 37-43.

Cother, E. J. & Vruggink, H. 1980. Detection of viable and non-viable cells of *Erwinia carotovora* var. *atroseptica* in inoculated tubers of var. *Bintje* with enzyme-linked immunosorbent assay (ELISA). *Potato Research* 23: 133-135.

Crops & Markets. March 2000. Directorate of agricultural statistics and management information. National Department of Agriculture. South Africa, Pretoria.

De Boer, S. H. 1994. Prospects for control of potato diseases caused by pectolytic *Erwinias*. Pages 136-148 in: Zehnder, G. W., Powelson, M. L., Jansson, R. K. & Raman, K. V. (eds). *Advances in potato pest biology and management*. APS Press, St Paul, Minnesota.

De Boer, S. H., Verdonck, L., Vruggink, H., Harju, P., Bang, H. O. & De Ley, J. 1987. Serological and biochemical variation among potato strains of *Erwinia carotovora* subsp. *atroseptica* and their taxonomic relationship to other *E. carotovora* strains. *Journal of Applied Bacteriology* 63: 487-495.

Dickey, R. S. & Kelman, A. 1990. *Erwinia carotovora* or "soft rot" group Pages 44-59 in: Schaad, N. W. (ed.) *Laboratory guide for identification of plant pathogenic bacteria*. St Paul, Minnesota.

Ervin, H. & Barnes, C. 1968. *Atlas and manual of plant pathology*. Appleton-Century Crafts, New York.

Fraaije, B. A., Birnbaum, Y. & Van den Bulk, R. W. 1996. Comparison of methods for detection of *Erwinia carotovora* ssp. *atroseptica* in progeny tubers derived from inoculated tubers of *Solanum tuberosum* L. *Journal of Phytopathology* 144: 551-557.

Hawkes, J. G. 1992. History of the potato crop. Pages 1-9 in: Harais, P. (ed.) *The potato crop*. Chapman and Hall, New York.

Hawkins, J. H. 1990. Diseases of the potato crop. Pages 59-78 in: Gunn, J.S. (ed.) *Crop protection handbook - potatoes*. The Lavenham Press Limited, Lavenham, Suffolk.

Kelman, A. & Dickey, R. S. 1989. Detection of *Erwinia carotovora* and *Erwinia chrysanthemi* in potato. Pages 76-79 in: Saettler, A. W., Schaad, N. W. & Roth, D. A. (eds). *Detection of bacteria in seed and other planting materials*. APS Press, St Paul, Minnesota.

Mackay, J. M. & Shipton, P. J. 1983. Heat treatment of seed tubers for control of potato Blackleg (*Erwinia carotovora* subsp. *atroseptica*) and other diseases. *Plant Pathology* 32: 385-393.

Molina, J. J. & Harrison, M. D. 1977. The role of *Erwinia carotovora* in the epidemiology of potato blackleg 1. Relationship of *E. carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* to potato blackleg in Colorado. *American Potato Journal* 54: 587-591.

Moline, H. E. 1984. Differentiation of post harvest soft rotting bacteria with Two – dimensional polyacrylamide gel electrophoresis. *Phytopathology* 75: 549-553.

Perombelon, M. C. M. & Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology* 18: 283-285.

Rich, A. E. 1983. *Potato diseases*. Academic Press, New York.

Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. *Annual Review of Phytopathology* 17: 123-147.

Serfontein, S., Logan, A., Swanepoel, A. E., Boelema, B. H. & Theron, D. J. 1988. A potato wilt disease in South Africa caused by *Erwinia carotovora* subspecies *carotovora* and *E. chrysanthemi*. *Plant Pathology* 40: 382-386.

Sherf, A. F. & Macnab, A. A. 1986. Vegetable diseases and their control. Wiley-Interscience Publication. New York.

Smid, E. J., Jansen, A. H. J. & Gorris, L. G. M. 1995. Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using Polymerase Chain Reaction. *Plant Pathology* 44: 1058-1069.

Towsen, E. & Korsten, L. 1998. Economic importance of *Erwinia* spp. Abstract in proceedings of the 36th Southern African Society of Plant Pathology.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Potatoes (*Solanum tuberosum* L.) originate from the Andes mountains of South America (Rich, 1983; Hawkes, 1992; Thurston, 1992) and were first cultivated by the Inca Indians in 200 B.C (Burton, 1966; Rich, 1983). In 1537, the Spanish conquistadors found the Incas growing potatoes, which they called "papa" (Burton, 1966; Rich, 1983). The conquistadors soon thereafter introduced potatoes to Spain from were it was taken to Italy and later the rest of Europe. Potatoes gradually became a staple food in Europe, Russia and Ireland. In 1719 potatoes were introduced into the United States of America by Irish immigrants originating mainly from Londonderry and New Hampshire (Burton, 1966; Rich, 1983). Potatoes were subsequently introduced to most of the British Colonies and by the late seventeenth century, were grown in Japan, the West Indies and parts of Africa (Hawkes, 1992). Today, potatoes are grown as a major food source in most countries with a temperate climate (Rich, 1983). Potatoes also rank fourth in global production of important food products after wheat, rice and maize (Hawkes, 1992). In South Africa, with an estimated population of 45 000 000, the per capita use of potatoes is 38. 64 kg and the annual potato harvest is 166.6 000 000 bags. The gross value of potatoes for the 1999 season was R1127 970 (Mosterd 2000, personal communication).

Although physiological factors such as drought stress, mineral and nutrient deficiencies are responsible for appreciable economic losses, an even greater part of overall losses are due to various diseases (Burton, 1966). A wide range of fungal, viral and bacterial pathogens affects potatoes. Of these, soft rots are the most common and devastating bacterial diseases of potatoes (De Boer, 1994) and are also one of the most important problems in stored vegetables (Ervin & Barnes, 1968). One of the reasons why potatoes are easily affected by bacterial diseases is particularly due to the presence of starches and

sugars (Rich, 1983). These carbohydrates provide a good nutrient base for bacterial growth and multiplication. Actual crop losses due to bacterial soft rot has been estimated world-wide to be as high as $50 to $100 million annually (Perombelon, 1980).

This chapter will mainly deal with classification, symptomology, epidemiological aspects and detection of *Erwinia* spp. on potatoes and other crops and conclude with studies on various disease control measures.

Bacteria that cause soft rot diseases are commonly known as the "soft rot group" and include *Erwinia carotovora* subsp. *carotovora, Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* (Agrios, 1997). Bacterial soft rot was first observed on carrots in the USA by Jones in 1901 (Graham, 1964). He described the causal organism as *Bacillus carotovoras* (Rich, 1983). Almost simultaneously, Van Hall in Holland and Appel in Germany independently discovered an organism with similar morphology causing blackleg disease on potatoes (Graham, 1964). At the time, the causal organism was classified using three different schemes, which are those of Migula, those of Lehmann & Neumann and those of Smith (Graham, 1964). In all three classifications, the soft rot organisms were placed under the family *Bacteriaceae*. However, in Migula's classification they were placed under the genus *Bacillus*, while Smith's and Lehman & Neumann grouped it with the genus *Bacterium* (Graham, 1964). In 1937, Rahn suggested the introduction of a new family, *Enterobacteriaceae*, which contained one genus, *Enterobacter*. The family name and their designation as *Enterobacter* are based on the fact that the normal habitat of most members of this group is the enteric tract or intestines (Van Denmark & Batzing, 1987). However, members of the *Enterobacteriaceae* are also found in association with plants and trees and in soil and water (Van Denmark & Batzing, 1987). In 1939, the family *Enterobacteriaceae* was accepted in the fifth edition of Bergey's Manual of Determinative Bacteriology and embraces amongst others the genus *Erwinia* (Graham, 1964). Three species of *Erwiniae* may be distinguished by biochemical reaction and pathogenicity. They include: the *amylovora*, *herbicola* and *carotovora* species (Starr, 1981). The *carotovora* species has two subspecies called

atroseptica and *carotovora* that are classed together due to their DNA relatedness (Starr, 1981). *Erwinia chrysanthemi* is the last member of the *carotovora* group and has no subspecies or pathovar. All these subspecies of *Erwinia* spp. were subsequently named according to the international standards for naming pathovars of phytopathogenic bacteria as proposed by the International Society of Plant Pathology committee on Taxonomy of Phytopathogenic Bacteria (Dye *et al.*, 1980). Other three subspecies of *E. carotovora* have more recently been described: *E. carotovora* subsp. *betavasculorum* which causes soft rot of sugar beet, *E. carotovora* subsp. *wasabiae* which was isolated from Japanese horseradish and *E. carotovora* subsp. *odorifera* which was isolated from witloof-chickory, leek, allium and celery (Helias *et al.*, 1998).

Numerous disease symptoms are associated with *Erwinia* infections ranging from blackening of the stem (blackleg), other stem symptoms (aerial stem rot and stem wet rot), wilting and/or death of the entire plant (early dying), lenticel hard rot and rot of tubers. Rot, commonly known, as soft rot is the most common and devastating postharvest bacterial disease on potatoes. Symptoms of *Erwinia* spp. are usually, but not always, confined to tubers, and usually develop during storage, in the field or while in transit to and from the market place (Rich, 1983). Infected tubers typically develop a mushy soft rot accompanied by an offensive odour (Rich, 1983; Agrios, 1997). The affected area becomes soft and mushy while its surface becomes discoloured and somewhat depressed. The tissues within the affected region becomes brown to cream coloured and slimy, disintegrating into a mushy mass of disorganised cells (Agrios, 1997). A whole fruit or tuber may be converted into a soft, watery, decaying mass with a foul smell within three to five days (Agrios, 1997). According to Ervin & Barnes (1968), the most striking symptoms of soft rot are the mushy textures and rotting smell. When *Erwinia* spp. cause decay of potato seed tubers and plants, the shoots become streaked with black commonly referred to as blackleg (Ervin & Barnes, 1968). Depending on environmental conditions, the entire potato plant may die within weeks as a result thereof.

2.2 Diseases caused by *Erwinia* spp. on potatoes

Potatoes are exposed to a variety of *Erwinia* spp. that can induce diseases by attacking the tubers, stem or entire plant. These diseases are caused by various species and subspecies of *Erwinia* under different environmental conditions and will be discussed accordingly.

2.2.1 Blackleg

Blackleg is primarily caused by *E. c.* subsp. *atroseptica*. However, in warmer soils or climatic conditions *E. c.* subsp. *carotovora* may be the causal agent (Mackay & Shipton, 1983). A characteristic of this disease is the blackening of the basal portion of the stem. Normally, symptoms originate from the seed piece, spreading to the stem (Perombelon, 1992). Blackleg that occurs early in the growing season results in weak upright, chlorotic plants that easily succumb to decay. Plants that become diseased later on in the season develop a typical black tissue that often extends midway or higher up into the stem (De Boer, 1994). Affected stems may or may not turn chlorotic but eventually they wilt and decay. Potato plants are particularly susceptible to blackleg under cool, moist conditions (Mackay & Shipton, 1983; De Boer, 1994).

2.2.2 Aerial stem rot

According to Cappaert *et al.* (1988), aerial stem rot is usually caused by *E. c.* subsp. *carotovora* and affects damaged or injured vines. Symptoms do not originate from the seed piece but can be traced to damaged tissue or leaf scars that permit entry of the bacterium. The decay is usually brown in colour and may either be dry or mushy depending on field and environmental conditions. Aerial stem rot is mostly favoured by dense, succulent foliage and prolonged periods of leaf wetness (Cappaert *et al.*, 1988). Aerial stem rot develops mostly under regular overhead irrigation that causes leaf wetness (Cappaert *et al.*, 1988).

2.2.3 Stem wet rot

Stem wet rot is caused by *E. chrysanthemi* and is regarded as being of minor importance in temperate potato growing areas (Powelson & Appel, 1984). This disease can be of major importance when contaminated seed potatoes are exported from temperate to warmer areas. The symptoms of stem wet rot originates from the seed piece and are characterised by brown to black discoloration of the lower stem and decay of the pith. According to De Boer (1994), infected plants can be recognised by yellow discoloration in the apical stem region. Symptoms of stem wet rot are often difficult to distinguish from blackleg symptoms in areas where both pathogens occur.

2.2.4 Early dying

Early dying is a syndrome that involves diseases such as *Verticillium* wilt and nematode related diseases (Rowe *et al.*, 1987). Pectolytic *Erwinia* spp. mostly *E. c.* subsp. *carotovora*, are often associated with affected plants causing decay. *Erwinia* spp. may directly cause early dying symptoms in some situations or may play an important role in disease development by enhancing symptom expression (De Boer, 1994). Early dying could be a problem particularly in fields receiving overhead irrigation. High humidity and succulent foliage caused by regular overhead irrigation probably predispose the plants to infection by *Erwinia* spp. (De Boer, 1994).

2.2.5 Bacterial soft rot

Bacterial soft rot is one of the most important diseases causing post harvest losses of potatoes in storage (Wells & Butterfield, 1997). All pectolytic soft rot bacteria cause decay of tubers. When tubers are placed in storage facilities most are naturally externally contaminated with *E. c.* subsp. *carotovora* and to a lesser extent with *E. c.* subsp. *atroseptica* (Agrios, 1997). Under optimal storage conditions, these bacteria remain latent but if tubers are wet and air movement around the tubers is restricted, or if tuber

surfaces are damaged, the bacteria proliferate and cause decay. The extent of decay can be very severe causing serious economic losses (Agrios, 1997).

2.2.6 Lenticel hard rot

Lenticel hard rot occurs both in the field and in storage when conditions for multiplication of *Erwinia* spp. are favourable. During the growing season, *E. c.* subsp. *atroseptica* may invade developing lenticels of progeny tubers (De Boer, 1994). In low humidity storage environments, these bacteria cause slightly sunken, brownish-black, dry, necrotic local pockets of decay (De Boer, 1994), which result in typical lenticel hard rot. Washed tubers are particularly susceptible.

2.3 Diseases caused by *Erwinia* spp. on other crops

Although many bacteria possess the ability to produce tissue-macerating enzymes, only a few have been associated with decay of plant tissues (Perombelon & Kelman, 1980). Studies with *Erwinia* spp. showed that only a few pathogenicity characteristics are involved which are: cell wall degrading enzyme production, motility, iron uptake and cell wall structure (Perombelon, 1992). Of these, pectolytic enzyme production is believed to be the most important. Bacteria associated with decay of plant tissue include *Erwinia* spp., *Bacillus subtilis, B. megaterium*, and pectolytic strains of *Pseudomonas, Clostridium* and *Flavobacterium* spp. (Perombelon & Kelman, 1980; Lyon, 1989; Perombelon, 1992). *Salmonella* spp. have also been associated with bacterial soft rot of fresh fruits and vegetables in the market place (Wells & Butterfield, 1997). *Erwinia* spp. are naturally pathogenic to a wide range of ornamental and field crops such as maize (*Zea mays* L.), rice (*Oryza sativa* L.) and sugar beet (*Beta vulgaris* L.) (Cother & Sivasithamparam, 1983). *Erwinia* spp. also infects vegetables such as carrots (*Daucus carota* L.), potatoes (*Solanum tuberosum* L.), tomatoes (*Lycopersicon esculentum* L.), onions (*Allium cepa* L.) and beans (*Pisum sativum* L.), members of the *Cruciferae* and

Cucurbitaceae families (Table 1) and also causes diseases of stems and flowers and other above-ground parts (Ervin & Barnes, 1968; Agrios, 1997). Schuerger & Batzer (1993) found *E. c.* subsp. *carotovora* causing stem rot and vascular wilt symptoms on hydroponically grown vegetables. They subsequently inoculated stems from various hydroponically grown plants with bacterial suspensions of the pathogen to compare differences in symptom expression and to study host ranges. The different types of symptoms that have been observed on various crops are described in Table 1.

Table 1 Symptoms of *Erwinia* spp. on different plants (Schuerger & Batzer, 1993)

Peppers Capsicum annuum L.	Tobacco Nicotiana alata L.	Tomato Lycopersicon esculentum L.
Black, dry necrotic lesions at inoculation points	Black water soaked necrotic lesions at inoculation point	Similar to those of tobacco except that pith necrosis and development of hollow stalk were not observed
Stem necrosis	Black vascular bundles in dissected stems	No symptoms observed
Pith tissue degenerated resulting in hollow stalks	Some plants developed wilt and chlorosis	No symptoms observed

2.4 Disease epidemiology

When tubers are placed in storage and/or transport facilities, most are externally contaminated with *Erwinia* spp. (Perombelon & Kelman, 1980). Under optimal storage conditions, these bacteria remain latent but if tubers are wet and air movement around the tubers is restricted, or if tuber surfaces are damaged, the bacteria proliferate and cause decay (De Boer, 1994).

In order to develop effective disease control strategies, it is important to fully understand all aspects of disease development, symptomology and spread. According to Agrios

(1997), *Erwinia* spp. survive on infected fleshy organs in storage and in the field. They are also found in debris, on roots or on other plant parts of the host, in ponds and streams used for water irrigation, occasionally in the soil and in the pupae of several insects such as fruit flies and seed corn maggot flies (Allan & Kelman, 1977).

The disease may first appear in the field on plants grown from previously infected seed pieces or rotting mother tubers (Elphinstone & Perombelon, 1986; Agrios, 1997). The bacteria may also survive in association with volunteer plants or in the rhizosphere of other cultivated plants and of certain weeds (Perombelon & Kelman, 1980). Some tubers become infected through wounds or lenticels after they are formed in the soil or during mechanical harvesting and grading (Elphinstone & Perombelon, 1986; Perombelon, 1992; Agrios, 1997). When the soft rot bacteria enter wounds, they feed and multiply at first on the liquids released by broken cells within the wound surface (Agrios, 1997). They then produce large amounts of pectolytic enzymes (polygalacturonases, pectin methyl esterase, pectin lyase and several isoenzymes of pectate lyase, Perombelon, (1992), which break the pectic substances of the middle lamella and bring about maceration of the tissue (Perombelon, 1992; Agrios, 1997). *Erwinia* spp. can be airborne (insects and aerosols) (Perombelon, 1992) and can live in all stages of the insects' life cycle (Agrios, 1997).

Progeny tuber contamination occurs after rotting of the contaminated mother tubers when the bacteria are released into the soil and transmitted to the tubers by soil water (Perombelon, 1992). Because of the high osmotic pressure of the macerated tissue, water from the cells diffuses into intercellular spaces and as a result the cells plasmolyze, collapse and die (Agrios, 1997). The bacteria continue to move and multiply in the intercellular spaces, while their enzymes advance ahead of them and prepare the tissues for invasion. The invaded tissues become soft and are transformed into a slimy mass consisting of numerous bacteria swimming in the liquefied substance. The slimy mass then extrudes into the soil or on other tubers in storage providing new inoculum (Agrios, 1997).

2.4.1 Environmental factors affecting disease expression

During storage, the initiation of rotting in stored potato tubers occurs when anaerobic conditions prevail. Other factors that contribute to the initiation of rotting are free water covering tuber surfaces and prevailing temperatures above the minimum required for infection (Elphinstone & Perombelon, 1986). Rotting is more rapid under low oxygen concentrations than in air. High levels of carbon dioxide also favour the extent of decay (Perombelon & Kelman, 1980).

In the field, the highest incidence of post-emergence blackleg is correlated with moderate soil temperature and moisture conditions. Pre-emergence seed and sprout decay is high at warmer temperatures and/or high soil moisture levels (Perombelon & Kelman, 1980; Gans *et al.*, 1991). In temperate regions, a high incidence of blackleg occurs in years that are cooler than normal and have above average rainfall. In warmer regions where high soil moisture content is essential for infection, high temperature favours disease development. Disease expression is also related to treatments or conditions that damage the seed or reduce its resistance (Gans *et al.*, 1991).

2.4.2 Survival of *Erwinia* spp.

Soft rot *Erwinia* spp. are considered to represent true plant pathogens (Perombelon, 1992). However, *Erwinias* can also be soil borne, prevalent as latent infections on many host crops and have other habitats that include air, water, potato leaves, mechanical and handling equipment. It must however be noted that crops harvested early in the season are less likely to be contaminated with insect and air-borne *Erwinias* compared to late harvested crops. This is due to the widespread contamination through infected leaves and environmental conditions favouring rotting of fallen leaves. In addition, transmission of the bacteria to progeny tubers tend to occur late in the seasons that favour bacterial multiplication (Perombelon, 1992).

a) Soil

Erwinia spp. can survive in soil for short periods. High numbers of *Erwinia* spp. develop in soil when susceptible host crops such as *Brassicas* are grown (Perombelon, 1992). *Erwinia* spp. are adapted to grow in nutrient rich plant tissue and are unable to adapt their endogenous metabolism to allow successful competition with other soil micro-organisms in relatively nutrient poor soil environments, particularly at high temperatures (Perombelon, 1992). *Erwinia* spp. can however survive in microbiologically poor sub-arable soil layers (Cother, 1980).

b) Water

Survival of the bacteria in natural water sources was found in most rivers, especially *E. c.* subsp. *carotovora* (Cother & Gilbert, 1990), However, *Erwinia* spp. were not found in water from deep wells (Perombelon, 1992). Some rivers flowing through fallow fields were found to be free of *Erwinia* spp. In contrast, rivers flowing through cultivated lands became increasingly contaminated (Cappaert *et al.*, 1988). However, in some cases it is thought that *Erwinia* spp. are indigenous to certain rivers and multiply in the bottom sediments (Harrison *et al.*, 1987). *Erwinia* spp. have also been isolated from various habitats with extreme alkaline pH such as sea water and extremely low temperatures such as the snow on the Colorado mountains (Maher *et al.*, 1986). The presence of large numbers of *Erwinia* spp. in underground drain water from apparently *Erwinia*-free fields indicated that *Erwinia* can survive in both water, soil and at extreme temperature ranges (Perombelon, 1992).

c) Air borne *Erwinia* spp.

Erwinia spp. can survive in the air and have been detected on insects (Perombelon & Kelman, 1980; Perombelon, 1992; Agrios, 1997). According to Perombelon & Kelman

(1980), insect species contaminated mostly by *E. c.* subsp. *carotovora* were trapped near potato and vegetable refuse dumps and were shown to be capable of transmitting the bacteria to potato crops especially on damaged stem tissue (Harrison *et al.*, 1977). Aerosols containing *Erwinia* spp. are generated by rain impacting on blackleg affected plants. Although short-lived in air, Perombelon (1992) found that only 50% of the bacteria remained viable for 5-10 minutes and that they can also be blown away from the source of infection for several hundred metres before deposition occurs. According to Harrison *et al.* (1977), insects contaminated with *Erwinia* spp. can transmit these pathogens to damaged areas on aerial parts of potato plants.

d) Potato leaves

According to Allan & Kelman (1977), potato stems and leaves can become contaminated with *Erwinia* spp. through insects (Perombelon & Kelman, 1980), aerosols, or in irrigation water (Perombelon, 1992). Studies from Scotland showed that insects belonging to 12 genera in the Order Diptera were found contaminated with soft rot *Erwinia* spp. (Harrison *et al.*, 1977). Bacterial numbers become low on dry leaves due to unfavourable conditions for bacterial multiplication and can then only be detected through an enrichment procedure (Perombelon, 1992). Bacterial multiplication occurs only as long as the leaf surface is wet, but numbers rarely exceed 10^2 cells/cm^2 leaf area. This is due to competition with the phylloplane microflora and low levels of nutrients on leaf surfaces (Perombelon, 1992).

e) Mechanical handling of equipment

Mechanical equipment has been used more recently for ploughing, harvesting and grading of potatoes. This equipment reduces production costs but can result in the spreading of disease. Mechanical grading has been known to cause spread of *Erwinia* spp. within and between stocks. Contamination can also occur when tubers are mechanically handled at planting time and during harvest when using contaminated

implements (Perombelon, 1992). Survival of *Erwinia* spp. is related to the extent and nature of wounding. *Erwinia* spp. generally do not survive dry cold storage conditions of one month and in shallow wounds which are less than one mm deep.

2.5 Isolation of the bacterium

According to Schaad (1979), there is a real need for development of effective and rapid methods for identification and isolation of plant pathogenic bacteria. He further states that much of this increased need for rapid identification and isolation is a result of increased international trade and movement of plant propagative materials such as potato tubers.

2.5.1 Diagnosis of the bacterium

Diagnosis and identification of bacteria employs the use of a series of multiple laboratory tests. Comparing its characteristics with those of known reference strains (Van Demark & Batzing, 1987) makes the final identification of an isolate complete. Rapid and accurate diagnosis is essential for successful treatment and/or control of diseases. Diagnostic techniques of *Erwinia* spp. include the use of both traditional and modern molecular techniques.

2.5.2 Traditional techniques

Traditional techniques include mostly selective growth medium, the Gram stain, oxidase and catalase tests, fermentation of glucose, utilisation of α-methyl-D-glucoside, determination of growth at 37°C, formation of reducing substances from sucrose, phosphatase test, sensitivity to erythromycin and the indole test (De Boer & Kelman, 1975; Lelliott & Stead, 1987; Kelman & Dickey, 1989). Biochemical procedures have been developed for rapid identification. These include the use of the Analytical Profile

Index (API) and BIOLOG systems. Mergaert *et al.* (1984) used the API system to study the numerical taxonomy of *Erwinia* spp. and found it to be very successful. The BIOLOG system has also been found to be a rapid and effective method for identification of both Gram negative and Gram positive bacteria and was used for metabolic fingerprinting of *Xanthomonas campestris* pv. *citri* (Verniere *et al.*, 1993).

2.5.3 Tuber incubation test

Perombelon (1979), used the tuber incubation test to detect *E. c.* subsp. *carotovora* in potatoes. The tuber incubation method is conducted by inducing test tubers to rot under anaerobic conditions. This is followed by isolation of the bacteria on selective growth medium or by serological techniques. The efficiency of detection when using this method is influenced by the rate of decay at the time of sampling and the incubation temperature used.

2.5.4 Molecular techniques

One of the most exciting areas of basic and applied plant pathology is the use of molecular techniques to solve disease problems. Molecular techniques can be broadly defined as methods used to differentiate bacteria based on the composition of their biological molecules such as proteins, fatty acids, carbohydrates and nucleic acids (Farber, 1996).

a) Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was developed in 1983 by K. B. Mullis and F. Fallona. The reaction takes advantage of several characteristics of DNA polymerase, the enzyme that catalyses DNA replication and the availability of laboratory equipment capable of automatically synthesising short DNA molecules of any desired sequence (Wolfe, 1995). PCR is an *in vitro* method in which DNA sequences or transcripts are

amplified rapidly with very high specificity and fidelity using oligonucleotide primers in a simple automated reaction (Hadidi *et al.,* 1995). Randomly amplified polymorphic DNA (RAPD), PCR ribotyping, PCR restriction fragment length polymorphism (RFLP) and repetitive extragenic palindromic (Rep-PCR) are the various PCR based typing systems that are used to differentiate between bacteria (Farber, 1996). The PCR has great sensitivity and therefore provides a good alternative to other diagnostic methods. The rapid diagnosis and reduced sample size required, often reduce the need for using radioactive probes (Hadidi *et al.,* 1995). According to De Boer & Ward (1995), a useful PCR procedure to enhance sensitivity of detection has not yet been developed. Smid *et al.* (1995) developed a specific primer set capable of distinguishing *E. c.* subsp. *atroseptica* from other *Erwinia* spp. Darrasse *et al.* (1994), designed a PCR test based on pectate lyase-encoding pel gene sequences, but it only permits *E. c.* subsp. *atroseptica* to be identified by analysis of restriction fragment length polymorphisms of the amplified product.

b) The use of serological tests

Serology was first used to identify plant pathogenic bacteria in 1918 when Jensen showed that a strain of *Agrobacterium tumefaciens* from Denmark could be differentiated from a strain of *A. tumefaciens* from the United States of America using an immunoassay (Schaad, 1979). A variety of assays using antibodies to detect and identify seed and soil borne pathogens have been used and they include: Immuno-dot blot, Agglutination, ImmunoFluorescence (IF) and the most frequently used Enzyme Linked Immunosorbent Assay (ELISA) (Towsen, 1996). ELISA was first used in plant pathology in 1976 and has proved to be rapid and effective (Clark, 1981). Both poly- and monoclonal antibodies can be used to detect or identify microorganisms in or on field collected plant samples. However, disadvantages associated with polyclonal antibodies such as cross reactivity and high variability have led to the increased use of monoclonal antibodies (Towsen, 1996). The unique specificity of monoclonal antibodies to single epitopes also makes them an ideal tool for the quantitative and accurate diagnosis of pathogens at serogroup levels (De Boer & Mc Naughton, 1987). This specificity is not possible with polyclonal

antisera as they may fail to distinguish between strains and even species (Vernon-Shirley & Burns, 1992).

According to Jordan (1995), monoclonal antibodies have been shown to be very useful for detection and identification of plant pathogenic bacteria, as well as for determining the degree of similarity between species, strains and isolates of these different groups of pathogens. Monoclonal antibodies specific against *E. c.* subsp. *atroseptica* have been used and characterised (Gorris *et al.*, 1994). Allan & Kelman (1977), have used immunofluorescent stain procedures for detection and identification of *E. c.* subsp. *atroseptica*. However, Smid *et al.* (1995) stated that most serological methods are laborious and insensitive. De Boer & Ward (1995) supported this statement in their studies where they compared ELISA and PCR for the detection of *E. c.* subsp. *atroseptica* on potato tubers and found the later technique to be more sensitive than the former.

c) Polyacrylamide Gel Electrophoresis

The term electrophoresis describes the movement of small ions and charged macromolecules in solution within an electric field. Almost all analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of proteins into their individual polypeptide subunits that minimise aggregation (Sambrook *et al.*, 1989). Protein electrophoresis produces banding patterns that are a product of the genotype of the organism as it was functioning prior to protein extraction. For protein profile analysis, the gels are most commonly stained with Coomassie blue, which results in the band showing up dark blue. This technique is an important taxonomic tool and has many applications in plant pathology (Paul *et al.*, 1982). PAGE is used increasingly and extensively for the identification and classification of many bacteria (Kersters & De Ley, 1975; Lelliott & Stead, 1987). The use of PAGE can result in large numbers of isolates being characterised and compared in a short period of time (Bashn *et al.*, 1993). Vanderwolf *et al.* (1993) characterised fluorescent *Pseudomonas*

cross-reacting with antibodies against *E. chrysanthemi*. They further characterised cell envelopes and proteinase-k-treated cell envelopes using SDS-PAGE and western blotting.

2.6 Resistance and pathogenicity

Recent molecular genetics studies of *Erwinia* spp. showed that in contrast to many phytopathogenic bacteria, only a few pathogenicity characters are involved. They are: motility, iron uptake, cell wall structure and cell wall degrading enzymes such as: polygalacturonase, pectin methyl esterase, pectin lyase and several isoenzymes of pectate lyase (Perombelon, 1992).

2.6.1 Cultivar susceptibility

Cultivar resistance can be an important strategy towards disease control. Differences in cultivar susceptibility have been demonstrated in greenhouse plants grown from inoculated tuber pieces or from stem inoculations (Gans *et al.*, 1991). Lojkowska & Kelman (1989) tested eight potato cultivars for resistance to bacterial soft rot. He used different inoculation techniques that included: the point titration, mist chamber and slice assay. Tzeng *et al.* (1990) also tested fourteen potato cultivars for resistance to bacterial soft rot caused by *E. c.* subsp. *atroseptica*. According to Bain & Perombelon (1988), significant differences in soft rot development between cultivars are found when different inoculation techniques or test methods are used but cultivar rankings are different for each method used.

2.6.2 Pathogenicity

Lojkowska & Kelman (1989) found that the point titration method was the most effective under ambient oxygen conditions. They also found that cultivars that were relatively resistant to soft-rot under ambient oxygen levels were more susceptible under limited oxygen conditions in a nitrogen chamber. Bisht *et al.* (1993) used the petiole inoculation

method and found that length of rot lesions increased with increasing inoculum concentrations used. In their experiments they also found that the post-inoculation environment, especially humidity and temperature were critical for infection of potato plants with *Erwinia* spp. To ensure infection, inoculated seedlings should be kept in a mist chamber for several days. However when using the petiole mist chamber method, inoculation was not necessary because closed lid magenta jars provided a high enough humid environment, necessary for disease development.

It must be noted however that it is also possible that *Erwinia* spp. possess unidentified cryptic pathogenicity determinants, such as specific genes which are expressed only in the presence of plant tissue (Perombelon, 1992). Failure to compete with saprophytic pectolytic bacteria could explain why more, if not all plants in the field grown from potato seed and heavily contaminated with *E. c.* subsp. *atroseptica* or *E. chrysanthemi,* do not develop blackleg under favourable conditions. Therefore, the greater the initial inoculum of *E. chrysanthemi* or *E. c.* subsp. *atroseptica* on the seed, the more likely that the bacterium will predominate in rotting mother tubers and invade stems to cause blackleg.

2.7 Disease control

Potato blackleg caused by *E. c.* subsp. *atroseptica* is difficult to control (Gans *et al.,* 1991). However several disease management practices provide partial control (Bartz *et al.,* 1992).

2.7.1 Cultural practices

According to De Boer (1994), the traditional approach to control blackleg and seed piece decay of potatoes was through good cultural and integrated disease management practices. Since insects transmit bacteria, sanitation and insect control is of importance

(Rich, 1983; Phillips & Kelman, 1982; Perombelon, 1992; De Boer, 1994; Agrios, 1997). Minimising tuber damage during harvesting reduces the risk of bacterial soft rot due to reduction in wounding (Rich, 1983; Perombelon, 1992; 1994; Agrios, 1997). Reduction of bacterial soft rot in tubers has been associated with enhanced calcium levels in the tissue. Calcium concentrations in tubers were increased by fertilisation with gypsum. Such amendments were related to increased calcium on the periderm and medular tissues of tubers, grown in low calcium soils. Tubers with higher calcium contents had lower soft rot potentials than did tubers with low calcium content (Bartz *et al.*, 1992).

2.7.2 Clean seed

Since blackleg is a seed borne disease, the main emphasis has been on the production of clean seed (Perombelon, 1992). The use of pathogen-free seed tubers is an important strategy for controlling some diseases caused by *Erwinia* spp. Maintenance of disease free seed potatoes for several field generations is dependent on quality control that ensures each succeeding generation of progeny tubers is free from the pathogen (De Boer, 1994). According to Perombelon (1992), fewer field generations would help in reducing the risks of extensive pathogen build up under average conditions. Attempts to reduce tuber contamination more directly by chemotherapy have failed because the bacteria are well protected in the suberized lenticels and wounds. However, excellent results have been obtained using thermotherapy (Perombelon, 1992). Heat and chemical treatment with sodium hypochlorite or formaldehyde have been reported to be effective in obtaining *Erwinia*-free potatoes (De Boer, 1994). Lund & Wyatt (1979) also found dichlorophen to be bacteriostatic against *E. c.* subsp. *carotovora.*

2.7.3 Biological control

Biological control of plant diseases has been known to man since the earliest days and has more recently focused on molecular techniques (Huang, 1991). Approaches such as

genetic engineering (Cook, 1994), direct use of natural antagonistic microorganisms or genetically engineered crops (Wilson & Wisniewski, 1989) and expression of biological control mechanisms in transgenic plants (Cook, 1994). One of the best known examples of biological control is the use of *Agrobacterium radiobacter* strain K84 (Huang, 1991). The use of bacteriocins have not yet shown to be effective (De Boer, 1994). Biological control of bacterial soft rot of potatoes has been obtained by treating the seed pieces with a powdery formulation of two of fluorescent *Pseudomonas* strains (B10 and 113) before planting. Similar results were obtained with plant growth promoting *Rhizobacteria* (Rhodes & Logan, 1986). Jaynes *et al.* (1987) used antibacterial genes from the giant silk moth (*Hyalophora cecropia*) and found that bactericidal protein cecropin A had potential to control *Erwinia* spp. which cause soft rot on potatoes. *E. c.* subsp *betavasculorum* (Ecb168) produces antibiotics that suppress growth of the related bacterium *E. c.* subsp. *carotovora* in culture and in wounds of potato tubers (Costa & Loper, 1994).

2.7.4 Breeding

Long term control of bacterial diseases can be achieved through breeding for resistance. Breeding for blackleg resistance has similarly been attempted (Perombelon, 1992). Wild *Solanum* species (*Solanum brevidens)* have been crossed with the potato *S. tuberosum*, using protoplast fusion. Tubers from somatic hybrids between *S. tuberosum* and *S. brevidens* showed partial resistance to bacterial soft rots (Austin *et al.*, 1988). According to De Boer (1994), genes for specific antibiotic agents such as bacteriocins or anti-*Erwinia* metabolites from antagonistic *Pseudomonas* could be considered for construction of *Erwinia*-resistant transgenic plants (Lojkowska & Kelman, 1989).

2.7.5 Chemical control

The use of antibiotics and chemicals to control bacterial plant diseases has been known for a long time (Luxmi & Parashar, 1980). Compounds such as copper fungicides and

antibiotics have been successfully used although copper derivatives have given mixed results with the control of fire blight and certain mycoplasma-like diseases (Luxmi & Parashar, 1980). Harris (1979) experimented with five chemicals by applying them to batches of wounded potato tubers. His results showed that some chemicals, in particular, 5-nitro-8-hydroxyquinoline are able to decrease the incidence of soft rot on freshly wounded potatoes. The experiment also showed that during storage the chemicals did not significantly control soft rot and other related diseases such as blackleg caused by other *Erwinia* spp. According to Luxmi & Parashar (1980), stable bleaching powder (SBP) which contains 35% chlorine has been tested for its efficacy in controlling potato blackleg and soft rot. SBP at 1000 ppm was found to be inhibitory against *E. c.* subsp. *carotovora* both *in vitro* as well as on potato tubers.

2.7.6 Tuber pasteurisation

Heat treatment of seed tubers has been recommended as another method to control soft rot (Mackay & Shipton, 1983). Robinson & Foster (1987), determined the thermal death point of *E. c.* subsp. *carotovora, E. c.* subsp. *atroseptica* and *E. chrysanthemi* and showed that the incidence of blackleg was lower when tubers were immersed in water at 55°C for 5-10 minutes. The duration of heating required to produce 100% inactivation of *E. c.* subsp. *carotovora, E. c.* subsp. *atroseptica* and *E. chrysanthemi* are represented in Table 2. This duration of heating is in practice too long to be of commercial use.

Table 2 Time of exposure required to achieve 100% inactivation of *Erwinia* spp. (Robinson & Foster, 1987)

Pathogen	Time of exposure (minutes)		
	45°C	47°C	50°C
E. c. carotovora	>90	>55	30
E. c. atroseptica	>80	>30	15
E. chrysanthemi	>90	>55	40

2.8 Conclusion

Soft rot *Erwinia* spp. are among the most damaging and devastating pathogens that attack fruits and vegetables pre-and post harvestly causing mainly soft rots (Agrios, 1997). Rotting of potato tubers is not only caused by pectolytic *Erwinia* spp. but also *Bacillus* spp., *Pseudomonas* spp., *Corynebacterium* spp., *Clostridium* spp. and *Flavobacterium* spp. (Lyon, 1989). This makes it difficult to study the disease complexes.

It is therefore important to effectively detect these pathogens to avoid crop losses. The development of an enrichment method to detect *Erwinia* spp. (Meneley & Stanghellini, 1975) was important for the study of soft rot bacteria. However, traditional biochemical methods used to detect *Erwinia* spp. are often more time consuming and tedious (Schaad, 1979). Rapid molecular techniques are therefore proving to be more rapid and efficient. Their only disadvantage is the high cost, and a need for technical training (Clark, 1981)

Progress has been made towards better understanding the blackleg disease development, ecology and epidemiology (De Boer, 1994; Perombelon & Kelman, 1980). It must however be noted that the ecological role of soft-rot *Erwinia* spp. has mainly been studied in temperate regions. The results can therefore not be applied in the tropics because of the dominant influence of temperature and rainfall. Aspects of disease control have not yet been fully achieved except for good cultural practices (De Boer, 1994; Perombelon & Kelman, 1980). Certain aspects that still need consideration are tuber resistance under anaerobic conditions, exploitation of resistance derived from wild species, quantification of seed contamination, interaction between *Erwinia* spp. and other fungal and bacterial pathogens affecting tubers and plants.

The use of a bactericide, (Lund & Wyatt, 1979) antagonistic bacteria or tuber pasteurisation to control blackleg has been reported but none of these methods have been proven effective for commercial application (Gans *et al.*, 1991). Differences in cultivar

susceptibility have been demonstrated in green house experimental plants grown from inoculated tuber pieces (Gans *et al.*, 1991). Cultivar resistance and good cultural practices can be of importance to ensure effective control.

2.9 Literature cited

Agrios, G. N. 1997. Plant Pathology. Academic Press, California.

Allan, E. & Kelman, A. 1977. Immuno fluorescent stain procedures for detection and identification of *Erwinia carotovora var. atroseptica*. *Phytopathology* 67: 1305-1312.

Austin, S., Lojkowska, E., Ehlenfeldt, M. K., Kelman, A. & Helgeson, J. P. 1988. Fertile interspecific somatic hybrids of *Solanum*: A novel source of resistance to *Erwinia* soft rot. *Phytopathology* 78: 1216-1220.

Bain, R. A. & Perombelon, M. C. M. 1988. Methods of testing potato cultivars for resistance to soft rot of tubers caused by *Erwinia carotovora* subsp. *atroseptica*. *Plant Pathology* 37: 431-437.

Bartz, J. A., Locascio, S. J. & Weingartner, D. P. 1992. Calcium and potassium fertilisation of potatoes grown in north Florida. 11: Effect in the bacterial soft rot potential in the tubers. *American Potato Journal* 69: 39-50.

Bashan, Y., Holguin, G & Lifshits, R. 1993. Isolation and charecterization of plant growth promoting rhizobacterium. Pages 331-345 in: Glick, B. R. & Thompson, J.

E. (eds). *Methods in plant molecular biology and biotechnology*. CRC Press, London.

Bisht, V. S., Bains, P. S. & Lethal, J. R. 1993. A simple and efficient method to assess susceptibility of potato to stem rot by *Erwinia carotovora* subspecies. *American Potato Journal* 70: 611-616.

Burton, W. G. 1966. The potato. A survey of its history and of factors influencing its yield, nutritive value, quality and storage. Kent, Maidstone.

Cappaert, M. R., Powelson, M. L., Franc, G. D. & Harrison, M. D. 1988. Irrigation water as a source of inoculum of soft rot *Erwinias* for aerial stem rot of potatoes. *Phytopathology* 78: 1668-1672.

Clark, M. F. 1981. Immunosorbent assays in Plant Pathology. *Annual Review of Phytopathology* 19: 83-106.

Cook, R. J. 1994. Broad concepts and applications. Pages 1-19 in: Komanda, H., Kritani, K. & Petresen, J. (eds). *The biological control of plant diseases*. Kou Thai, Bangkok.

Costa, J. M. & Loper, J. E. 1994. Derivation of mutants of *Erwinia carotovora* subsp. *betavasculorum* deficient in export of pectolytic enzymes with potential for biological control of potato soft rot. *Applied and Environmental Microbiology* 60: 2278-2285.

Cother, E. J. 1980. Bacterial seed tuber decay in irrigated sandy soils of New South Wales. *Erwinia carotovora carotovora* and *Erwinia chrysanthemi*. *Potato Research* 23: 75-84.

Cother, E. J. & Gilbert, R. L. 1990. Presence of *Erwinia chrysanthemi* in two major river systems and their Alpine sources in Australia. *Journal of Applied Bacteriology* 54: 37-43.

Cother, E. J. & Sivasithamparam, K. 1983. *Erwinia:* The carotovora group. Pages 87-101 in: Fahy, P. C. & Persley, G. J. (eds). *Plant bacterial diseases. A diagnostic guide.* Academic Press, Sydney.

Darrase, A., Priou, S., Kotoujansky, A. & Bertheau, Y. 1994. PCR and restriction fragment length polymorphism of a pel gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. *Applied and Environmental Microbiology* 60: 1437-1443.

De Boer, S. H. 1994. Prospects for control of potato diseases caused by pectolytic *Erwinias*. Pages136-148 in: Zehnder, G. W., Powelson, M. L., Jansson, R. K. & Raman, K. V. (eds). *Advances in Potato pest biology and management*. APS Press, St Paul, Minnesota.

De Boer, S. H. & Kelman, A. 1975. Evaluation of procedures for detection of pectolytic *Erwinia* spp. on potato tubers. *American Potato Journal* 52: 117-123.

De Boer, S. H. & Mc Naughton, M. E. 1987. Monoclonal antibodies to the lipopolysacharide of *Erwinia carotovora* subsp. *atroseptica* Serogroup1. *Phytopathology* 77: 828-833.

De Boer, S. H. & Ward, L. J. 1995. PCR detection of *Erwinia carotovora subsp. atroseptica* associated with potato tissue. *Phytopathology* 85: 854-858.

Dye, D. W., Bradbury, F. F., Goto, M., Hayward, A. C., Lelliott, R. A. & Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Review of Plant Pathology* 59: 153-168.

Elphinstone, J. G. & Perombelon, M. C. M. 1986. Contamination of potatoes by *Erwinia carotovora* during grading. *Plant Pathology* 35: 25-33.

Ervin, H. & Barnes, C. 1968. Atlas and manual of plant pathology. Appleton-Century Crafts, New York.

Farber, J. M. 1996. An introduction to the Hows and Whys of molecular typing. *Journal of Food Protection* 59: 1091-1101.

Gans, P. T., Jellis, G., Little, J. G., Logan, C. & Wastie, R. L. 1991. A comparison of methods to evaluate the susceptibility of potato cultivars to blackleg in the field at different sites. *Plant Pathology* 40: 238-248.

Gorris, M. T., Alarcon, B., Lopez, M. M. & Cambra, M. 1994. Characterisation of monoclonal antibodies specific for *Erwinia carotovora* subsp. *atroseptica* and comparison of serological methods for its sensitive detection in potato tubers. *Applied and Environmental Microbiology* 60: 2076-2085.

Graham, D. C. 1964. Taxonomy of the soft rot coliform bacteria. *Annual Review of Phytopathology* 2: 13-28.

Hadidi, A., Levy, L. & Podleckis, E. V. 1995. Polymerase chain technology in Plant Pathology. Pages 124-139 in: Singh, U. S. & Singh, R. P. (eds). *Molecular Methods in Plant Pathology.* Lewis Publishers, New York.

Harris, R. I. 1979. Chemical control of bacterial soft rot of wounded potato tubers. *Potato Research* 22: 245-249.

Harrison, M. D., Franc, G. D., Maddox, D. A., Michaud, J. E. & McCater-Zoner, N. J. 1987. Presence of *Erwinia carotovora* in surface water in North America. *Journal of Applied Bacteriology* 62: 565-570.

Harrison, M. D., Quinn, C. E., Sells, A. & Graham, D. C. 1977. Waste potato dumps as a source of insects contaminated with soft rot coliform bacteria in relation to recontamination of pathogen free potato stocks. *Potato Research* 40: 238-248.

Hawkes, J. G. 1992. History of the potato crop. Pages 1-9 in: Harris, P. (ed.) *The Potato crop.* Chapman & Hall, New York.

Helias, V., Le Roux, A., Bertheau, Y., Andrivon, D., Gauthier, J & Jouan, B. 1998. Characterisation of *Erwinia carotovora* subsp. *atroseptica* in potato plants, soil and water extracts with PCR-based methods. *European Journal of Plant Pathology* 104: 685-699.

Huang, H. C. 1991. Control of soil borne pathogens by mycoparasites. Pages 103-110 in: Komanda, H., Kritani, K. & Petersen. J. (eds). *The biological control of plant diseases.* Kou Thai, Bangkok.

Jaynes, A., Xanthopoubs, K., Destefano Beltran, G. L. & Dodds J. H. 1987. Increasing bacterial disease resistance in plants using antibacterial genes from insects. *Bioassays* 6: 263-270.

Jordan, R. 1995. Production and application of monoclonal antibodies to plant pathogenic bacteria, spiroplasmas and mycoplasma-like organisms. Pages 96-106 in: Singh, U. S. & Singh, R. P. (eds). *Molecular methods in plant pathology.* Lewis Publishers, New York.

Kelman, A. & Dickey, R. S. 1989. Detection of *Erwinia carotovora* and *Erwinia chrysanthemi* in potato. Pages 76-79 in: Saettler, A. W., Schaad, N. W. & Roth, D. A. (eds). *Detection of bacteria in seed and other planting material.* APS Press, St. Paul, Minnesota.

Kersters, K. & De Ley, J. 1975. Identification and grouping of bacteria by numerical analysis of their protein electrophoretic patterns. *Journal of General Microbiology* 87: 333-342.

Lelliott, R. A. & Stead, D. E. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications. Oxford.

Lojkowska, E. & Kelman, A. 1989. Screening of seedlings of wild solanum species for resistance to bacterial stem rot caused by soft rot *Erwinias*. *American Potato Journal* 66: 379-390.

Lund, B. M. & Wyatt, K. 1979. A method for testing the effect of antibacterial compounds on bacterial soft rot of potatoes and results for preparations of Dichlorophen and Sodium hypochloride. *Potato Research* 22: 191-202.

Luxmi, C. S. & Parashar, R. D. 1980. Efficacy of stable bleaching powder in controlling soft rot and blackleg of potato. *Indian Phytopathology* 33: 409-414.

Lyon, G. D. 1989. The Biochemical basis of resistance of potatoes to soft rot *Erwinia* spp.- a review. *Plant Pathology* 38: 313-339.

Mackay, J. M. & Shipton, P. J. 1983. Heat treatment of seed tubers for control of potato Blackleg (*Erwinia carotovora* subsp. *atroseptica.)* and other diseases. *Plant Pathology* 32: 385-393.

Maher, E. A., De Boer, S. H. & Kelman, A. 1986. Serogroups of *Erwinia carotovora* involved in systemic infection of potato plants and infestation of progeny tubers. *American Potato Journal* 63: 1-11.

Menley, J. C. & Stanghellini, M. E. 1975. Isolation of soft rot *Erwinia* spp. from agricultural soils using an enrichment technique. *Review of Phytopathology* 66: 367-370.

Mergaert, J., Verdonck, L., Kersters, K., Swings, J., Boeufgras, J. M. & De Ley, J. 1984. Numerical taxonomy of *Erwinia* species using API systems. *Journal of General Microbiology* 130: 1893-1910.

Paul, G., Sealey, Y. & Southern, H. 1982. Gel electrophoresis of DNA. Pages 39-75 in: Rickwood, D. & Hames, B. D. (eds). *Gel electrophoresis of nucleic acids: A practical approach*. IRL Press, Oxford.

Perombelon, M. C. M. 1979. Factors affecting the accuracy of the tuber incubation test for the detection of contamination of potato stocks by *Erwinia carotovora*. *Potato Research* 22: 63-68.

Perombelon, M. C. M. 1992. Potato blackleg: Epidemiology, host-pathogen interaction and control. *Netherlands Journal of Plant Pathology* 98: 135-146.

Perombelon, M. C. M. & Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology* 18: 361-387.

Phillips, J. A. & Kelman, A. 1982. Direct fluorescent stain procedure applied to insect transmission of *Erwinia carotovora*. *Phytopathology* 72: 89-94.

Powelson, M. L. & Appel, J. D. 1984. Soil and seed tubers as sources of inoculum of *Erwinia carotovora* pv. *carotovora* for stem rot of potatoes. *Phytopathology* 74: 429-432.

Rhodes, D. J. & Logan, C. 1986. Effects of fluorescent Pseudomonas on the potato blackleg syndrome. *Annals of Applied Biology* 108: 511-518.

Rich, A. E. 1983. Potato diseases. Academic Press. New York.

Robinson, K. & Foster, G. 1987. Control of potato blackleg by tuber pasteurisation: The determination of time-temperature combinations for the inactivation of pectolytic *Erwinia*. *Potato Research* 30: 121-125.

Rowe, R. C., Davis, J. R., Powelson, M. L. & Rouse, D. I. 1987. Potato early dying: causal agents and management strategies. *Plant Disease* 71: 482-489.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular cloning. A laboratory manual (2nd edition). Cold Spring Harbour Laboratory Press, New York.

Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. *Annual Review of Phytopathology* 17: 123-147.

Schuerger, A. C. & Batzer, J. E. 1993. Identification and host range of an *Erwinia* pathogen causing stem rots on hydroponically grown plants. *Plant Disease* 77: 472-477.

Smid, E. J., Jansen, A. H. J. & Gorris, L. G. M. 1995. Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using Polymerase Chain Reaction. *Plant Pathology* 44: 1058-1069.

Starr, M. P. 1981. The Genus *Erwinia*. Pages 474-475 in: Starr, M. P., Stolp, H., Truper, H. G, Balows, A. & Schlegel, H. G. (eds). *The Prokaryotes: A handbook on habitats, isolation and identification of bacteria*. Springer, New York.

Thurston, H. D. 1992. Andean potato culture: 5,000 years of experience with sustainable agriculture. Pages 6-13 in: Zehnder, G. W., Powelson, M. L., Jansson, R. K. &

Raman, K. V. (eds). *Advances in potato pest biology and management*. APS Press, Minnesota.

Towsen, E. 1996. Monitoring *Bacillus subtilis* populations in preharvest biocontrol programmes on avocado. MSc thesis. University of Pretoria. Pretoria.

Tzeng, K., Mc Guire, R. G. & Kelman, A. 1990. Resistance of tubers from different potato cultivars to soft rot caused by *Erwinia carotovora* subsp. *atroseptica*. *American Potato Journal* 67: 287-305.

Van Denmark, P. J. & Batzing, B. L. 1987. The microbes: An introduction to their nature and importance. Benjamin\Cummings, New York.

Vanderwolf, J. M., Vanbeckhoven, J. R. C. M., Deboef, E. & Roozen, N. J. M. 1993. Serological characterisation of fluorescent *Pseudomonas* strains cross-reacting with antibodies against *Erwinia chrysanthemi*. *Netherlands Journal of Plant Pathology* 99: 51-60.

Verniere, C., Pruvost, O., Civerolo, E. L., Gambin, O., Jacquemoud-Collet, J. P. & Luisetti, J. 1993. Evaluation of the BIOLOG substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. *Applied and Environmental Microbiology* 59: 243-249.

Vernon-Shirley, M. & Burns, R. 1992. The development and use of monoclonal antibodies for detection of *Erwinia*. *Journal of Applied Bacteriology* 72: 97-102.

Wells, J. M. & Butterfield, J. E. 1997. *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the market place. *Plant Disease* 81: 827-867.

Wilson, C. L. & Wisniewski, M. E. 1989. Biological control of post harvest fruits and vegetables: an emerging technology. *Annual Review of Phytopathology 27:* 429-425.

Wolfe, S. L. 1995. An introduction to cell and molecular biology. Wadsworth, California.

CHAPTER 3

PREVALENCE OF *ERWINIA* SPP. ON ASYMPTOMATIC POTATO TUBERS

ABSTRACT

Soft rotting bacteria were isolated from asymptomatic potato tubers using an enrichment technique. Biochemical tests were used to identify and differentiate between the *Erwinia* spp. To further confirm identity of isolates, BIOLOG was used. Of the 85 isolations made, 45 isolates were obtained of which 15 represented *Erwinia carotovora* subsp. *carotovora*. Results from methyl red, hydrogen sulphide, Voges Proskauer and growth in 5% ethanol tests were difficult to interpret. However, BIOLOG seemed to be a more rapid and effective alternative diagnostic test compared to conventional biochemical tests. All 15 isolates were pathogenic, causing soft rot on potato tuber slices.

3.1 Introduction

The genus *Erwinia* was named after the phytobacteriologist Erwin F. Smith. It includes amongst others the soft rotting *Erwinia* spp. (Hauben *et al.*, 1998). These bacteria cause quantitative and qualitative crop losses (Peromelon & Kelman, 1980) to trees, flowers, potato tubers, fruits and vegetables (Agrios, 1997). Soft rot *Erwinia* spp. are omnipresent and often prevalent as latent infections on many host crops. They colonize and grow epiphytically on plant surfaces and may also be present as L forms (Perombelon & Kelman, 1980). *Erwinia* spp. are one of the commonest bacteria that inhabit the soil, they occur naturally on plant surfaces and can under specific environmental conditions infect plants and tubers. Infection will only occur when favourable environmental conditions such as high temperatures and humidity prevail and wounds occur simultaneously (Perombelon & Kelman, 1980).

Soft rot *Erwinia* spp. were traditionally grouped under the family *Bacteriaceae* and the genus *Bacillus* (Graham, 1964). They were latter reclassified under the genus *Bacterium* (Graham, 1964). Presently, the genus is included in the family Enterobacteriaceae according to the Fifth edition of Bergey's manual of determinative Bacteriology that includes amongst others the genus *Erwinia* (Bailey & Scott, 1974).

Standard biochemical tests were traditionally used by medical bacteriologists to characterize members of the Enterobacteriaceae (Graham, 1964). These techniques were later adopted by phytobacteriologists to differentiate the *Erwinia* soft rotting group (De Boer & Kelman, 1975; Kelman & Dickey, 1989). However, these techniques were tedious, time consuming and difficult to accurately interpret and were subsequently found inadequate to use as diagnostic tools (Schaad, 1979). Nevertheless, standard use of biochemical tests to differentiate between phenotypic characteristics of bacteria (Cother & Powell, 1983) and pathogenic species within a genus (Gillies & Dodds, 1973), remains an important diagnostic tool. Today, other biochemical, nutritional and genetic characteristics are used to differentiate between subspecies of *Erwinia* (Bailey & Scott, 1974).

During the last decade, new technologies have been developed for more rapid identification using biochemical tests (Logan, 1994). The Analytical Profile Index (API) (API, Montalieu-Vercieu) and BIOLOG (California) systems are two of the commercially available biochemical test kits now routinely used in diagnostic and medical laboratories around the world.

Thus far, *Erwinia* spp. have been identified with the API system (Mergaert, *et al.*, 1984) and more recently with the BIOLOG. Verniere, *et al.* (1993) used the BIOLOG to differentiate between the metabolic activities of various strains of *Xanthomonas campestris* pv. *citri* and Garland & Mills (1991) used it to distinguish heterotrophic microbial communities. Only limited reports exist where modern techniques are compared with traditional methods for rapid identification and differentiation of pectolytic *Erwinia* spp. Since *Erwinia* spp. are prevalent in certain potato production areas of South Africa and vary in occurrence from year to year (Towsen & Korsten, 1998), it was important to study the presence of the organism on asymptomatic potato tubers. In this study, various *Erwinia* spp. isolated from asymptomatic potato tubers were identified and compared using biochemical tests in combination with pathogenicity tests.

3.2 Materials and methods

3.2.1 Experimental tubers

Eighty-five 20kg bags of healthy potato tubers were obtained from Coen Bezuidenhout Laboratories, Pretoria. Tubers obtained represented different cultivars and included: From the Western Cape, eighteen bags of Van der Plank, five of Up to date, two of Hertha, nine of Mnandi, nine of BP1 and five of Astrid; from the Northern Province, five bags of Up to date and three of Atlantic; from Mpumalanga, two bags of Hoëvelder, four of Spuntha and five of Astrid; from Kwazulu\Natal five bags of Buffelspoort; from the North West province, four bags of Maris Pear and seven bags were of unknown origin.

3.2.2 Tuber assays

All tubers were kept at 5°C until isolations were completed which were usually within a week. Ten potato tubers were randomly selected per bag and surface disinfested by spraying with 70% ethanol, followed by two spray rinses in sterile distilled water. One air dried five grams of potato peel were removed from each of the 10 tubers using a standard flame sterilized kitchen potato peeler. The potato peeler was flame sterilized between samples. A blender jar was rinsed with 0.05% sodium hypochlorite followed by two rinses with sterile distilled water prior to and in between homogenization of potato samples (Kelman & Dickey, 1989). Five grams of peel was added to 500ml sterile distilled water in the blender jar and homogenized for 60 seconds (Maher *et al.*, 1986). Two sets of test tubes were prepared and a dilution series was made from each homogenized sample. In the first set, one ml of the homogenate was pipetted into sterile test tubes containing 9 ml of a modified enrichment medium described by Meneley & Stanghellini (1976). A standard 10-fold dilution series was prepared in duplicate in sterile Ringer's solution. With the other set, tubes were incubated in an anaerobic flask (Gaspak system) and placed in an incubator at 27°C for 48 hrs. After 48hrs incubation at 27°C, 0.1ml, of homogenate from both sets of test tubes was spread plated on either of the following media: Crystal violet pectate medium (CVPB) (Perombelon & Burnett, 1991), potato dextrose agar (PDA) (Biolab, Johannesburg), nutrient agar (NA) (Biolab), Kings B medium (KB) (Dickey & Kelman, 1990) and yeast extract-dextrose -calcium cabornate medium (YEDCC) (Dickey & Kelman, 1990). Spread plating was done using disposable sterile beijerinck rods and NA and PDA plates were incubated at 27°C for 24 hours while YEDCC and KB were incubated for 3-6 days and CVPB plates for 5-10 days. After incubation, typical colony morphology was monitored as described by Cother & Sivasithamparan (1983).

3.2.3 Selective media

Three different modified CVP media (Perombelon & Burnett, 1991) used to differentiate between *Erwinia* spp. were evaluated to distinguish between pit formation for rapid isolate grouping. The media included a new modified CVP (CVPB), double layer CVP (DLCVP)

and a CVP compiled by Cuppels & Kelman (1974). Typical *Erwinia* colonies were picked and plated on each of the modified CVP media. Plates were incubated at 27°C and pit development observed as described by Perombelon & Burnett (1991) after 72 hours.

3.2.4 Maintenance of cultures

Stock cultures were streaked on CVPB medium (Perombelon & Burnett, 1991) and purified on NA plates. Pure cultures of all test isolates that resembled *Erwinia* spp. were preserved by suspension in 3ml sterile distilled water in Bijou bottles and stored at room temperature. Two replicates of each culture were preserved.

3.2.5 Identification of *Erwinia* spp.

Selected typical *Erwinia*-like colonies were preliminarily identified using the standard sequence of identification procedures for *Erwinia* spp. described by Dickey & Kelman (1990). These included: Gram stain, catalase production, presence of cytochrome oxidase, oxidation/fermentation test, egg yolk medium for lecithinase production, utilization of α-methyl-D-glucoside, formation of indole, growth at 37°C, production of phosphatase, production of acids from maltose, lactose, trehalose and palatinose, growth in 5% ethanol, growth in 5% sodium chloride and sensitivity to erythromycin (Harrigan & McCance, 1966; Fahy & Persley, 1983).

a) Micro plate method

An adaptation of the micro plate method, described by Hayward (1995) using 96-well microtitre plates, was used for testing sugar or carbon source utilization. The sterile basal medium without any solidifying agent or agar was asceptically dispensed into a microtitre plate by pipetting 150µl of the medium into each well. The sugars that were tested included the following: α-methyl-D-glucoside, palatinose, trehalose, sucrose, maltose and lactose. Twenty four-hour-old pure cultures of all isolates were suspended in 3ml of sterile peptone water, mixed and 20µl of the suspension pippeted into each of three wells on the microtitre

plate containing the various sugars. Six wells were left as uninoculated controls. Results were recorded after two, four and six days. A colour change of the medium from green to yellow was recorded as a positive result. In all tests, both positive and negative controls were included as shown in Table 1. For comparative purposes, additional reference strains of pectolytic *Erwinia* spp. and control cultures were obtained from the Department of Microbiology and Plant Pathology (University of Pretoria) culture collection. Numerical classification of data was done using Statistica statistical analysis package. Data were subjected to Eucledian distances agglomerative hierarchical clustering method based on the sum-of-squares criteria. The resulting Euclidean distances were plotted as a dendrogram.

Table 1 List of positive and negative controls used in all biochemical and physiological tests

TESTS	POSITIVE CONTROL	NEGATIVE CONTROL
Oxidation\Fermentation	*Escherichia coli*	*Bacillus subtilis*
Lecithinase	*Bacillus subtilis*	*Escherichia coli*
Sensitivity to erythromycin	*Bacillus subtilis*	*Escherichia coli*
Indole	*Escherichia coli*	*Enterobacter cloacae*
α-methyl-D-Glucoside	*Escherichia coli*	*Pseudomonas aeruginosa*
Palatinose	*Escherichia coli*	*Pseudomonas aeruginosa*
Trehalose	*Escherichia coli*	*Pseudomonas aeruginosa*
Phosphatase	*Staphylococcus aureus*	*Staphylococcus cohnii*
Methyl Red	*Escherichia coli*	*Enterobacter cloacae*
Voges Proskauer	*Enterobacter cloacae*	*Escherichia coli*
Growth in 5% Nacl	*Bacillus subtilis*	*Bacillus polymyxa*
Growth at 37°c	*Escherichia coli*	*Pseudomonas fluorescens*

3.2.6 BIOLOG test

To further confirm identity of isolates, the BIOLOG test was conducted using BIOLOG Gram negative microplates. *Erwinia* spp. were cultured on BIOLOG Universal growth medium and incubated at 27°C. To give a transmittance of 55%, the BIOLOG

turbidimetre was used according to manufacturers instructions. An optimum cell density of 3 x 10^8cells/ml were used in all tests. Microplates were incubated at 27°C and results were first checked after four, then after 24 and finally after 48 hours

3.2.7 Pathogenicity tests

Potato tuber surfaces were surface disinfested before inoculation as described earlier with original isolates (3.2.2). Two tubers from each of the six cultivars (Table 2) were cut into 5mm thick slices with a standard flame sterilized potato peeler as described by Lojkowska & Kelman (1994). All 15 isolates (Table 2) that fitted the *Erwinia* profile were selected for pathogenicity tests. Isolates were cultured on NA plates and incubated at 27°C for 24 hours. Single colonies were transferred from NA to 50ml sterile distilled water in a 250ml Erlenmeyer flask. Sterile filter paper disks (5mm in diameter) were immersed in the prepared test isolate suspensions. Tuber slices were placed in sterile glass petri dishes and the moistened filter paper discs with the respective bacterial suspension were put in the center of the potato slice. Four slices from each cultivar were used for each test isolate. Filter paper disks with sterile distilled water were used as controls. After 48 hours incubation at 28°C the diameters of decayed tissue were measured and recorded. Data were analyzed statistically using Duncan's multiple range test and agglomerative hierarchial clustering based on the sum of squares criteria.

3.3 Results

Isolations made from all 85-potato bags that were sampled, yielded 61 potential *Erwinia* spp. based on colony formation which resembled *Erwinia* growth. Of these, the 45 most typical isolates which resembled classical *Erwinia* colony morphology and growth patterns on selective CVPB media were selected for biochemical tests. Of these, 15 isolates were further selected for BIOLOG tests (Table 3).

Table 2 List of isolates and cultivars from asymptomatic potato tubers obtained from various geographic regions of South Africa

ISOLATE	CULTIVAR	ORIGIN	PROVINCE
Ecc A13 (b)	Unknown	Unknown	Unknown
NN176\96	Buffelspoort	Natal	Kwazulu\Natal
C80(b)	Unknown	Unknown	Unknown
SS43\97	Up to date	Sandveld	Western Cape
GG71\97	BP1	George	Western Cape
GG3\97	Mnandi	George	Western Cape
SS37\97	Van der Plank	Sandveld	Western Cape
GG2\97	BP1	George	Western Cape
SRZ34	Unknown	Unknown	Unknown
1H	Unknown	Unknown	Unknown
CC60\97	Hertha	Ceres	Western Cape
Ecc A13	Unknown	Unknown	Unknown
1G	Unknown	Unknown	Unknown
C80	Unknown	Unknown	Unknown
TT7\97	Up to date	Transvaal	Northern Province

3.3.1 Basic diagnostic tests, growth, enrichment and selective media

All isolates were gram negative, producing slime in 3% potassium hydroxide, were oxidase negative showing no colour change in tetramethyl-p-phenylenediamine hydrochloride and fermented glucose. Colonies of *Erwinia* spp. on NA were creamy white in appearance. On PDA, the colonies had a "fried egg" appearance. On YEDCC medium, the colonies produced a characteristic dark-blue, insoluble pigment. Deep pits were also observed on the CVPB medium. It was noted that a higher concentration of *Erwinia* spp. were recovered from the enrichment medium after it was incubated together with the peel homogenate than when it was not. Low concentrations of *Erwinia* spp. were recovered from the set of test tubes that were incubated without the enrichment medium, i.e. peel homogenate alone.

Table 3 Number of *Erwinia* spp. recovered by cultivar

	Potato cultivars[a]													
	BP	HRT	BP1	MND	UTD	SPT	VDP	AST	MDL	MRP	ATL	HVD	UNKNOWN	TOTAL
Total number of samples	5	2	9	9	5	4	18	5	4	4	3	2	15	85
Physical examination	3	2	8	4	5	1	15	3	2	3	2	1	12	61
Biochemical tests	2	1	8	3	4	1	11	1	1	1	1	0	11	45
BIOLOG	2	1	1	1	2	0	2	0	0	0	0	0	6	15

[a] BP represents Buffelspoort, HRT, Hertha, BP1, BP1, MND, Mnandi, UTD, Up to date, SPT, Spuntha, VDP, Van Der Plank, AST, Astrid, MDL, Mondial, MRP, Marispear, ATL, Atlantic and HVD, Hoevelder.

Larger cavities were observed on CVPB compared to CVP and DLCVP medium. This signifies the higher recovery rate or selectivity of the CVPB medium. Preparation of CVPB was easier than CVP since the sodium polypectate could be added to cold water and it is not necessary to pour the medium directly after autoclaving.

3.3.2 Biochemical tests

The microplate method used for testing carbon utilization seemed more effective than the traditional test tube method based on the evaluation criteria highlighted in Table 4. Only three isolates (SS37\97, GG2\97 and SRZ34) reacted positively for the methyl red test. Other isolates such as EccA13 (b), GG71\97 and SS43\97 reacted similarly in all tests showing both negative and positive reactions (Table 5) except for only two tests which are palatinose and trehalose tests. 1G, 1H, C80, EccA13, SRZ34, NN176\96, CC60\97, C80 (b) and TT7\97 showed the same negative and positive reactions in all tests. The following three cultures GG3\97, SS37\97 and GG2\97 also reacted positively in twelve of the tests. However they reacted negative on the indole test. Results of the cluster analysis of biochemical tests revealed relatively small Euclidean distances indicating that isolates are very closely related in terms of their biochemical tests (Fig. 1).

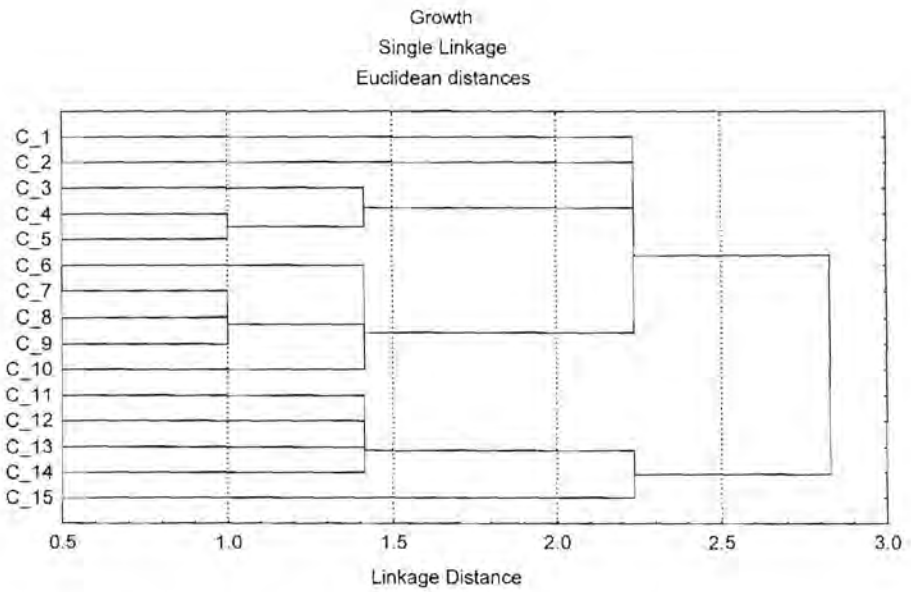
Table 4 Comparison of the micro plate and the traditional test tube methods

Microplate method	Traditional test tube method
Less basal medium required (150µl/well)	More basal medium required (9ml/test tube)
Fast to complete (24 hrs)	Slower to complete (48 hrs)
Less chance of contamination since the micro-titre plates are sterile	Greater chances of contamination if good care is not taken
Easy to incubate taking up little space	Bulky and occupy more space
Results are clear and easy to interpret	Results are sometimes difficult to interpret

Table 5 Results of biochemical tests[a]

No	KB	NA	GS	CV	OX	O/F	ERY	MDG	PAL	LEC	SUC	LAC	NaCl	MR	TRE	G37	Ind	H_2S	VP	ETH	PHOS
EccA1	-	+	-	+	-	+	-	-	-	-	-	+	+	-	-	+	+	-	+	-	+
3(b)	-	+	-	+	-	+	-	-	+	-	-	+	+	-	+	+	+	-	+	-	
NN	-	+	-	+	-	+	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+
176/96	-	+	-	+	-	+	-	+	+	+	-	+	+	-	+	+	+	-	+	+	+
C80(b)	-	+	-	+	-	+	-	+	+	-	-	+	+	-	+	+	+	+	+	+	+
SS43/97	-	+	-	+	-	+	-	+	+	-	-	+	+	-	+	+	-	-	+	+	+
GG71/97	-	+	-	+	-	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+	+
GG3/97	-	+	-	+	-	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+	+
SS37/97	-	+	-	+	-	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+	+
G2/97	-	+	-	+	-	+	-	+	+	-	+	+	+	-	+	+	-	+	+	-	+
SRZ34	-	+	-	+	-	+	-	+	+	-	-	+	+	-	-	+	+	-	+	+	+
HH	-	+	-	+	-	+	-	+	+	-	-	+	+	-	+	+	-	-	+	-	+
CC60/97	-	+	-	+	-	+	-	+	+	-	+	+	+	-	+	+	+	-	+	-	+
ECCA13	-	+	-	+	-	+	-	+	+	-	-	+	+	-	+	+	+	-	+	-	+
IG C80 TT7/97	-	+	-	+	-	+	+	+	+	-	-	+	+	-	+	+	+	+	+	-	+

[a]KB represents Fluoresence on King's B medium, NA, nutrient agar, GS, Gram staining, CV, CVPB medium, OX, oxidase test, O/F, oxidation fermentation, ERY, Sensitivity to erythromycin, MDG, α-methyl-D-glucoside, PAL, palatinose, LEC, lecithinase production, SUC, sucrose, LAC, lactose, NACL, growth in 5% Sodium chloride, TRE, trehalose, G37, growth at 37°C, IND. Indole, H_2S, hydrogen sulphide, VP, Voges Proskauer, ETH, growth in 5% ethanol and PHOS, phosphatase test.



C-values are isolate number designations assigned by statistica.

Isolates are in the same sequence as in table 2.

Figure 1. Dendogram derived from clustering of Euclidean distance coefficients.

3.3.3 BIOLOG test

Potential *Erwinia* spp. produced violet colour response patterns in the BIOLOG tests and were identified as *E. c.* subsp. *carotovora*. The BIOLOG system was rapid, effective and appeared to be more sensitive than the traditional biochemical tests (Table 6).

Table 6 Comparison of BIOLOG tests and biochemical tests

BIOLOG tests	Biochemical tests
Fast and accurate (24 hrs)	Slow and time consuming (48 hrs)
Less chances of contamination	Care must be taken to avoid possible contamination
Expensive (R85/plate)	Relatively cheap (R30/test)
Easy to perform	More labour intensive
Plates are small and easy to store	Many chemicals are involved in these tests and therefore requires a lot of storage space

3.3.4 Pathogenicity tests

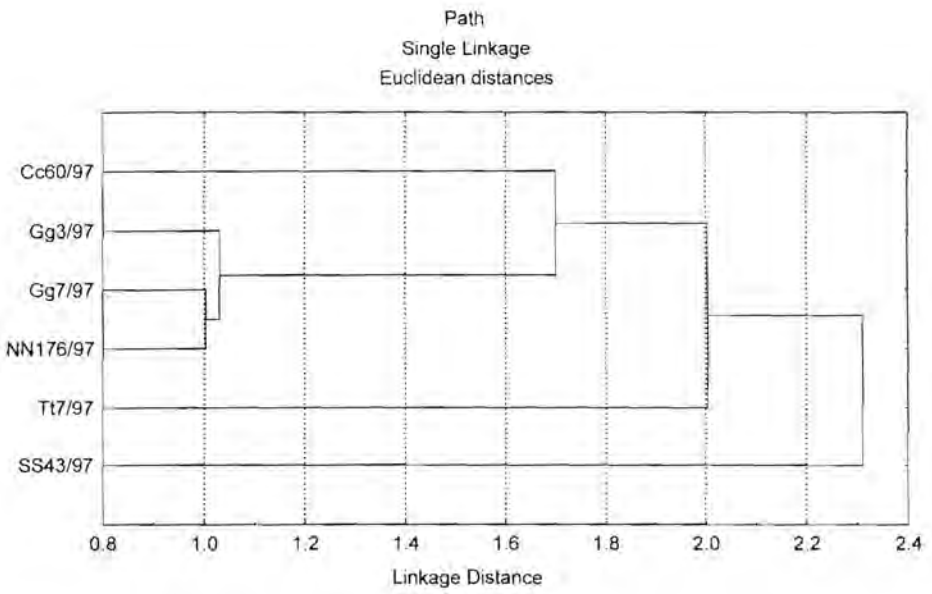
All tested strains proved to be pathogenic to inoculated potato tuber slices. Results on potato slices showed a brownish to blackish, soft and mushy rot with a foul smell. The rot was identified as a distinct characteristic of soft rot on potato tubers. The results revealed that differences in the length of rot vary significantly amongst cultivars (Table 7). Buffelspoort was the most susceptible cultivar and Van der Plank was the least susceptible cultivar followed by Mnandi. There were no significant differences in susceptibility on BP1, Hertha and UTD. Results of the cluster analysis of pathogenicity tests revealed that isolates were even more closely related in terms of pathogenicity than biochemical characters (Fig. 2).

Table 7 Average length of rot on potato slices expressed in millimeters (mm)

	MN[a]	VDP[a]	BP[a]	BP1[a]	HT[a]	UTD[a]	Avg[a]
Isolate							
Nn176/96	18	14	29	27	24	21	21.2
Ss43/97	19	13	28	25	24	21	21.2
Gg7/97	20	12	27	22	22	22	20.8
Cc60/97	19	14	25	21	23	23	21.2
Tt7/97	19	14	26	23	25	24	22.2
GG3/97	19	15	29	26	25	19	21.2
Average length	19.0c	13.7d	27.3a	22.2b	23.8b	21.7b	21.3

[a] MN represents Mnandi, VDP, Van Der Plank, BP, Buffelspoort, BP1, BP!, HT, Hertha, UTD, Up To Date and Avg represents the average length of rot.

Means were calculated for each cultivar and means in the bottom row with the same letter do not differ significantly according to Duncan's multiple range test (P=0.0001).



Cc60/97, Gg3/97, Gg7/97, NN176/97, Tt7/97 and SS43/97 represent isolates used.

Figure 2. Comparison of pathogenicity of different *Erwinia* isolates based on clustering of Euclidean distance coefficients.

3.4 Discussion

The pathogen, *E. c.* subsp. *carotovora* was successfully isolated from asymptomatic potato tubers. Results obtained in this study showed that *E. c.* subsp. *carotovora* can easily be isolated from asymptomatic potato tubers were consistent with previous studies (Dickey & Kelman, 1990). Since isolations were made from asymptomatic potato tubers, there was a low rate of recovery (15 positive isolations from 85 samples, which could be explained by the fact that asymptomatic tubers were used). The use of a modified enrichment medium (Perombelon & Burnet, 1991) facilitated the ease of isolating *Erwinia* spp. from asymptomatic potato tubers. Results of the biochemical tests obtained in this study were different from those found by other authors (Lelliott & Stead, 1987; Kelman & Dickey, 1989). Not all isolates tested, conformed to the typical *Erwinia* spp. identification profile as outlined by the standard physiological and biochemical tests described by Lelliott & Stead (1987). Investigations indicate that methyl red, Voges Proskauer and indole tests are difficult to interpret which is in correspondence with results by Mergaert *et al.* (1984).

According to Kelman & Dickey (1989), the difficulty in reading Indole production results is due to the media that contain peptones that vary in their tryptophan content. Another reason may be due to variable colour development (Lelliott & Stead, 1987). Growth in 5% alcohol was also inconsistent which is in accordance with Urquhart (1998). Variation in results were attributed by Urquart, (1998) to the volatile nature of alcohol. Such non-specific variations often hamper validity of interpretation of results. Another reason for variation in results may be that tubers originated from various geographic regions of South Africa using different farming systems. This has been shown to be the case in the Netherlands where various cultural practices such as the type of irrigation and harvesting methods used had an effect on the prevalence of *Erwinia* spp. (Perombelon, 1992). The apparent higher metabolic variation that normally occurs amongst bacterial strains could also be a reason why results varied (Verniere *et al.*, 1993).

Results of carbon source utilization obtained by Kelman & Dickey (1989) were similar to those obtained in this study. Furthermore consistent results which were obtained can be attributed to the use of the microplate method (Hayward, 1995). This technique provided more uniform test conditions for substrate utilization by *E. c.* subsp. *carotovora* strains similar to those found with the commercial BIOLOG. The faster colour response patterns of the BIOLOG (4 hours) compared to traditional test tubes, were found to be more time saving. This indicates that *E. c.* subsp. *carotovora* utilizes the substrate faster within the wells than test tubes and would therefore colour thetetrazolium violet quicker. The BIOLOG system has also been described as faster and that a single worker can do the technique on its own (one minute to inoculate plates and two to three minutes to read the plates) (Garland & Mills, 1991). BIOLOG is therefore a useful, rapid technique. However, Verniere *et al.* (1993) questioned the accuracy of identification based only on the BIOLOG technique. This he attributed to a high metabolic variation that normally occurs amongst strains. Comparing the 15 *E. c.* subsp. *carotovora* isolates using biochemical tests and the BIOLOG, confirmed that it was difficult to identify *E. c.* subsp. *carotovora* using these methods on their own as biochemical profiles and metabolic fingerprints. However, *E. c.* subsp. *carotovora* can easily be identified by the use of these standard biochemical tests compared to more sophisticated techniques using monoclonal antibodies in ELISA (Schaad, 1979) or

alternatively the PCR technique (McManus & Jones, 1996). The latter methods are expensive for application in developing countries where soft rot is often a major problem.

The phenotypic characters of strains coupled with results of pathogenicity tests have been used for the designation of *E. c.* subsp. *carotovora.* The reaction of inoculated potato tuber slices was similar to that observed in the case of natural infection i.e. all *E. c.* subsp. *carotovora* proved to be pathogenic to the original host material. It was also observed that a high concentration of bacteria (10^8 cfu/ml) was required to initiate rotting. This was in accordance with Fraaije *et al.* (1996) who were able to detect *E. c.* subsp. *carotovora* only in numbers greater that 10^8 cfu/ml in progeny tubers. This might be because soft rot *Erwinias* rely mostly on their pectic enzymes as pathogenicity determinants and characteristics of the isozymes produced when infecting potatoes (McMillan *et al.*, 1993). Obradovic & Arsinejevic (1997) stab inoculated three week old potato plants grown in the greenhouse and found that 10^7 cfu/ml was suitable to cause infection. They also found that no significant increase in infection rate occurred when the inoculum concentration was increased to more than 10^7cfu/ml. This variation in concentrations required to initiate rotting is because resistance of potato tubers to bacterial soft rot is affected by numerous factors which are: source and quality of seed tubers, physiological status and inoculum level of soft rot bacteria as well as other bacterial or fungal pathogens and favourable environmental factors (Lojkowska & Kelman, 1994).

This study did not show any relationship between *Erwinia* species and subspecies isolated from five different potato producing areas of South Africa. BIOLOG also proved to be faster than traditional biochemical tests. This is due to the fact that BIOLOG plates are readily prepared and a computer program aids interpretation of results.

3.5 Literature cited

Agrios, G. N. 1997. Plant Pathology. Academic Press, California.

Bailey, R. W. & Scott, E. G. 1974. Diagnostic Microbiology. The C.V. Mosby Company, Saint Louis.

Cother, E. J. & Sivasithamparam, K. 1983. *Erwinia*: The *carotovora* group. Pages 87-101 in: Fahy, P. C. & Persley, G. J. (eds). *Plant bacterial diseases*. A diagnostic guide. Academic Press, Sydney.

Cother, E. J. & Powell, V. 1983. Physiological and pathological characteristics of *Erwinia chrysanthemi* isolates from potato tubers. *Journal of Applied Bacteriology* 54: 37-43.

Cuppels, D. A. & Kelman, A. 1974. Evaluation of selective media for isolation of soft rot bacteria from soil and plant tissue. *Phytopathology* 64: 468-475.

De Boer, S. H. & Kelman, A. 1975. Evaluation of procedures for detection of pectolytic *Erwinia* spp. on potato tubers. *American Potato Journal* 52: 117-123.

Dickey, R. S. & Kelman, A. 1990. *Erwinia "carotovora"* or soft rot group Pages 44-59 in: Schaad, N. W. (ed.) *Laboratory guide for identification of plant pathogenic bacteria*. St Paul, Minnesota.

Fahy, P. C. & Persley, G. J. 1983. Plant bacterial diseases. A diagnostic guide. Academic Press, Sydney.

Fraaije, B. A., Birnbaum, Y. & Van den Bulk, R. W. 1996. Comparison of methods for detection of *Erwinia carotovora* ssp. *atroseptica* in progeny tubers derived from inoculated tubers of *Solanum tuberosum* L. *Journal of Phytopathology* 144: 551-557.

Garland, J. L. & Mills, A. L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* 57: 2351-2359.

Gillies, R. R. & Dodds, T. C. 1973. Bacteriology illustrated. Churchill Livingstone, London.

Graham, D. C. 1964. Taxonomy of soft rot coliform bacteria. *Annual Review of Phytopathology* 2: 13-18.

Harrigan, W. F. & McCance, M. E. 1966. Laboratory methods in microbiology. Academic Press. London.

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

Hayward, A. C. 1995. Phenotypic methods for the identification of *Pseudomonas solanacearum*: biovars and supplementary observations. Pages 27-34 in: Mehan, V. R. & McDonald, D. (eds). *Techniques for the diagnosis of Pseudomonas solanacearum and for resistance screening against groundnut bacterial wilt.* Technical manual no1.ICRISAT. Patancheru, Andhra Pradesh.

Kelman, A. & Dickey, R. S. 1989. Detection of *Erwinia carotovora* and *E. chrysanthemi* in potato. Pages 76-86 in: Saettler A. W., Schaad, N. W. & Roth, D. A. (eds). *Detection of bacteria in seed*. APS Press, St Paul, Minnesota.

Lelliott, R. A. & Stead, D. E. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific publications, London.

Logan, N. A. 1994. Bacterial systematics. Blackwell Scientific Publications, London.

Lojkowska, E. & Kelman, A. 1994. Comparison of the effectiveness of different methods of screening for bacterial soft rot resistance of potato tubers. *American Potato Journal* 71:99-113.

Maher, E. A., De Boer, S. H. & Kelman, A. 1986. Serogroups of *Erwinia carotovora* involved in systemic infection of potato plants and infestation of progeny tubers. *American Potato Journal* 63: 1-11.

McManus, P. S. & Jones, A. L. 1996. Genetic fingerprinting of *Erwinia amylovora* by repetitive sequence-PCR and PCR-ribotyping. *Acta Horticulturae* 411: 281-284.

McMillan, G. P., Hedley, D., Fyffe, L. & Perombelon M. C. M. 1993. Potato resistance to soft-rot *Erwinias* is related to cell wall pectin esterification. *Physiology and Molecular Plant Pathology* 42: 279-289.

Meneley, J. C & Stanghellini, M. E. 1976. Isolation of soft-rot *Erwinia* spp. from agricultural soils using an enrichment technique. *Phytopathology* 66: 367-370.

Mergaert, J., Verdonck, L., Kersters, K., Swinngs, J., Boeufgras, J. M. & De Ley, J. 1984. Numerical taxonomy of *Erwinia species* using API systems. *Journal of General Microbiology* 130: 1893-1910.

Obradovic, A. & Arsenijevic, M. 1997. Pathogenic differentiation of soft rot *Erwinia* potato parasites. *Acta Horticulture* 462: 345-350.

Perombelon, M. C. M. 1992. Potato blackleg: Epidemiology, host-pathogen interaction and control. *Netherlands Journal of Plant Pathology* 98: 135-146.

Perombelon, M. C. M. & Burnett, H. 1991. Two modified crystal violate pectate (CVP) media for detection, isolation and enumeration of soft rot *Erwinias*. *Potato Research* 34: 79-81.

Perombelon, M. C. M. & Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology* 18: 283-285.

Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. *Annual Review of Phytopathology* 17: 123-147.

Towsen, E. & Korsten, L. 1998. Economic importance of *Erwinia* spp. Abstract in proceedings of the 36th Southern African Society of Plant Pathology.

Urquhart, M. 1998. Isolasie van *Erwinia* spp. Unpublished material. Agricultural Research Council (ARC). Roodeplaat, South Africa.

Verniere, C., Pruvost, O., Civerolo, E. L., Gambin, O., Jacquemoud-Collet, J. P. & Luisetti., J. 1993. Evaluation of the BIOLOG substrate utilization system to identify and asses metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. *Applied and Environmental Microbiology* 59: 243-249.

CHAPTER 4

COMPARISON OF *ERWINIA CAROTOVORA* SUBSP. *CAROTOVORA* ISOLATES USING PCR AND SDS-PAGE

ABSTRACT

Genetic fingerprints of 15 *Erwinia carotovora* subsp. *carotovora* isolates obtained from various geographical regions of South Africa were made using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and two Polymerase Chain Reaction (PCR)-based methods. The SDS-PAGE was conducted according to standard procedures. For repetitive element PCR (rep-PCR), outward facing oligonucleotide primers (BOX AIR) were used, while for rRNA intergenic spacer analysis (RISA), primers directed towards amplification of the 16S-23S spacer regions were used. Gels were analyzed using Gel Compar and clustered using the unweighted pair group method of arithmetic averages. The resultant dendograms showed that SDS-PAGE, BOX-PCR and RISA are useful for identification and classification of *Erwinia* spp.

4.1 Introduction

Erwinia spp. are non-host specific pathogens which produce large amounts of pectolytic enzymes (Perombelon & Kelman 1980; Perombelon, 1992) and possess the ability to infect a wide spectrum of plant species that belong to various botanical families (Obradovic & Arsenijevic, 1997). *Erwinia carotovora* subsp. *carotovora* (*E. c.* subsp. *carotovora*) causes potato tuber soft rot (De Boer, 1994), and is the most important pathogen that causes post harvest losses of potatoes (Wells & Butterfield, 1997). Although losses caused by *Erwinia* spp. in South Africa have not yet been documented or quantified, Towsen & Korsten (1998) recently found in a soft rot survey that the disease is of major concern in certain production areas.

Due to the prevalence and economic importance of the disease, rapid, effective and accurate detection methods are required to confirm the presence of *Erwinia* spp.

Different methods to identify and compare isolates from potato tubers with soft rot *Erwinia* spp. have been studied. Classical methods based on selective isolation media (Cuppels & Kelman, 1974) and biochemical and physiological reactions (Cother & Powell, 1983; Cother & Sivasithamparam, 1983) have proved to be useful for identification and enumeration of soft rot *Erwiniae* but are laborious and time consuming for commercial purposes (Schaad, 1979). Detection methods using serological techniques such as the Enzyme-Linked Immunosorbent Assay (ELISA) or slide agglutination test, have been found to be insensitive for consistent field detection of the pathogen (De Boer & Ward, 1995). DNA probes for detection of *E. c.* subsp. *carotovora* have also been developed and found to be effective for identification purposes (Ward & De Boer, 1994). More recently, the Polymerase Chain Reaction (PCR), has been used to differentiate and detect *Erwinia* spp. on potato tubers (Maki-Valkama & Karjalainen, 1994; Smid *et al.*, 1995).

Proteins produced by different strains of bacteria provide a useful source of information for identification, characterization and classification of strains (Kersters, 1990).

Separation of bacterial proteins by means of Polyacrylamide Gel Electrophoresis (PAGE) under standardised conditions, produce patterns that can be used as "fingerprints" of bacterial strains (Kersters, 1990). This technique has been used successfully to distinguish, identify and compare plant pathogens (Moline, 1984; Serfontein & Hattingh, 1990; Van Zyl & Steyn, 1990; Guilford *et al.*, 1996; McManus & Jones, 1996). In this study, we evaluated PCR and SDS-PAGE techniques for detection and comparison of *E. c.* subsp. *carotovora* isolated from tubers collected from different potato producing areas of South Africa.

4.2 Materials and methods

4.2.1 Confirmation of identity of cultures

Erwinia spp. were isolated from asymptomatic potato tubers obtained from various potato producing areas of South Africa (Chapter 3). From the 85 original samples, only 15 isolates were positive according to the *Erwinia* identification profile. Identity of these isolates was confirmed and their purity checked before storage in sterile water at room temperature (Chapter 3). All outgroups that were included in the PCR experiment for comparative purposes, were obtained from the Agricultural Research Council (Roodeplaat, South Africa).

4.2.2 DNA extractions

A modification of the rapid lysis method described by Sambrook *et al.* (1989) was used to extract template DNA from the 15 *Erwinia* isolates described in (Chapter 3). A single colony was transferred to Luria Bertani (LB) broth and incubated for 16-18 hours at 27°C. One-milliliter cell suspension was centrifuged at 14,000 x *g*, for 10 min. The supernatant was removed and the pellet washed twice in 1ml sterile Ringers' solution (Oxoid, Johannesburg) by centrifugation (14,000 x *g*, for 10 min.). The pellet was subsequently resuspended in 100µl sterile milli Q water and heated for 10 min. at 95°C and the cell lysate immediately placed on ice.

4.2.3 PCR amplification

Primers corresponding to the 16S (5`-TTGTACACACGCCCGTCA-3`) and 23S (5`-GGTACCTTAGATGTT TCAGTTC-3`) (Fisher & Triplett, 1999) spacer regions were used for RISA. PCR assays were also carried out using oligonucleotide primers BOX A subunit of the BOX element BOXA1R (5`-CTA CGG CAA GGC GAC GCT GAC G-3) (Rosado *et al.*, 1998). Amplification in both instances was performed in a total volume of 50µl containing 20mM Tris-HCL (pH 8.4), 50mM KCL, 2.0mM $MgCL_2$ 0.1% Triton X-100. (Promega, Wisconsin) 200 µM each dATP, dCTP, dGTP and dTTP, 0.5U *Taq* polymerase (Promega). Template DNA was added and the tubes placed in a Perkin Elmer (Gene Amp PCR System 2400). PCR conditions were as follows: Denaturation was at 95°C for 3min. followed by 30 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min. and extension at 72°C for 1min., with a final extension at 72°C for 10 min. and a soak at 4°C.

4.2.4 Reproducibility of DNA fingerprints

Fingerprint profiles generated from independent DNA preparations (10µl) were separated on a 1.5% horizontal agarose gel in 0.5 x Tris–Acetate EDTA buffer (TAE). Electrophoresis was performed at 100 V for two hours. Gels were stained for 45 min. with 0.5µg/ml ethidium bromide (Merck, Johannesburg). Sizes of products were confirmed by comparison with a 100 base pair molecular mass marker. The data obtained was analysed with the Gel Compar 4.0 program (Applied Maths, Kortrijk). Dendograms were generated using the Pearson product-moment correlation coefficient (r) for calculating similarities between strains and clustered using the unweighted pair group method of arithmetic averages (UPGMA).

4.2.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Strains (Chapter3, Table 2) were streaked out on NA to obtain single colonies. Plates were incubated at 27°C for 24 hours. Whole cell extracts were prepared as described by

Dagutat (1995). Approximately 60 mg of cells were harvested from the agar surface using a clean sterile spatula and washed twice by centrifugation and resuspension in 0.2 M sodium phosphate buffer (pH 6.8). Washed cell pellets were suspended in 300µl sample treatment buffer (STB) [0.5 M Tris-HCL pH 6.8, 5% (v/v) 2-β-mercaptoethanol (BDH, Johannesburg), 10% (v/v) glycerol (Merck, Johannesburg) and 2% (m/v) SDS (Sarchem, Johannesburg)], heated to 95°C for 3 min. and disrupted with a Cole Parmer ultrasonic homogenizer (Series 4710) at 50% maximum output (40 Watts). Three hundred microlitres of STB were added to the sample, mixed and centrifuged at 15.000 x *g*, for 8 min. to remove cell debris. The clear supernatant was placed into a clean Eppendorf tube and stored at –20°C until required.

4.2.6 Standard conditions for SDS-PAGE

The original method of Laemmli (1970) as modified by Kiredjian *et al.* (1986) was used for SDS-PAGE of protein samples. Gels (1.5mm thick and 25mm long) were run on a BioRad dual cooled vertical gel slab unit. The separation gel (12%, 1.5 M Tris-HCl pH 8.6, conductivity 16.5 mS) and stacking gel (5%, 0.5 M Tris-HCl pH 6.6, conductivity 28.1 mS) were prepared from a monomer solution containing 29.2% (m/v) N^1-N^1-bismethylene acrylamide (BDH). A constant current of 30 mA through the stacking gel and 60 mA through the separation gel, was maintained while performing the electrophoresis at a constant temperature of 10°C. The gels were stained after electrophoresis with Coomassie blue solution [12.5% (v/v) Coomassie blue stock solution, 50% (v/v) methanol (Sarchem) and 10% (v/v) acetic acid (Sarchem)] was prepared from a 2% (m/v) Coomassie brilliant blue R (UniLab, Johanesburg) stock solution. After staining, gels were destained overnight in a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid.

4.2.7 Analysis of protein patterns

Gels were scanned between two glass plates with a Hoefer GS300 densitometer at a speed of 13 cm/minute. The data obtained was stored and analyzed with the GelCompar

4.0 program. A correlation (r) between the strains was computed and the result was used to cluster strains using the UPGMA. The protein pattern of *Psychrobacter immobilis* LMG 1125 was scanned, analyzed and used as reference profile on the gels (6 stacks/gels). Reproducibility of electrophoresis was determined by comparing these tracks with a *P. immobilis* protein profile selected in the Gel Compar 4.0 program as standard. A correlation (r) of 94% (where $r = r \times 100\%$) between gels was presumed acceptable for reproducible gels (Oosthuizen, 1998).

4.3 Results

Primers corresponding to conserved DNA sequences of BOX and RISA sub-units annealed to genomic DNA and generated unique genomic fingerprints for each isolate of *Erwinia* spp. The primers (BOX A1R) were chosen because the distance between them was short enough for easy amplification but not so short as to restrict polymorphism analysis in the target region.

The use of LB broth as an enrichment medium was found to be helpful to improve the level of detection since contaminating bacterial populations other than *Erwinia* spp. were unlikely to outgrow *Erwinias* (Toth *et al.*, 1999). Fingerprints that were generated produced complex banding patterns regardless of whether RISA or BOX-PCR primers were used. BOX-PCR (Fig 1) and RISA (Fig 2) fingerprints indicated genetic heterogeneity amongst *Erwinia* spp. isolated from different geographic regions of South Africa.

Approximately six DNA fragments between 100 and more than 1500 base pairs (bp) were amplified with each primer set (Fig 1 and 2). Fingerprints presented in this article were all generated from purified DNA. Reproducible differences in banding patterns were used to define fingerprint groups. The fingerprint groups defined by BOX and RISA primer assays were remarkably consistent showing almost total alignment between the different primers (Fig 1 and 2). Data from the two-primer sets were scored visually, taking into account differences in the absence or presence of major bands.

The BOX-PCR dendogram revealed two major groups joining at less than 50% similarity (Fig 1). Group A has two groups at 78% similarity with two amongst the four isolates being from the Western Cape (CC 60\97 and SS 43\97) whereas two were of unknown origin (1G and 1H). Group B has two subgroups at 52% similarity. Subgroup B1 consists of two *E. c.* subsp. *atroseptica*. Isolates that were used as outgroups and two *Erwinia* isolates (GG 2\97 and SS 73\97) from the Western Cape had an 89% similarity. Subgroup B2 was found subdivided into many smaller distinct groups. At 79% similarity, the *Erwinia* cucumber isolate was found to be related to isolate SRZ 34 from an unknown origin. The other group with an 89% similarity includes isolates C 80 (b), Ecc A13 and GG 71\97 from an unknown origin; ECC (positive control); NN 176\97 from Kwazulu\Natal and GG 71\97 and GG 3\97 from the Western Cape.

RISA protocol revealed two distinct groups with a similarity coefficient of less than 60% (Fig 2). Group A had *Erwinia* isolates C 80(b) and GG 71\97 from an unknown origin and Western Cape respectively at 93% similarity. Group B is subdivided into two groups. Subgroup B1 consists of ECA (positive control); GG 2\97 and SS 43\97 from the Western Cape; TT 7\97 from the Northern Province and NN 176\97 from Kwazulu\Natal at 91% similarity. At 70% similarity, subgroup B2 is further subdivided into distinct groups ranging from 81% to 100% similarity. *Erwinia* spp. isolates from carrot and cucumber that were used as outgroups have a 97% similarity. Other isolates with a similarity coefficient of 89% includes CC 60\97, SS 37\97, GG 3\97 and GG 71\97 from the Western Cape; ECC (positive control) and 1G, SRZ 34 Ecc A13, and C 80 from an unknown origin.

The dendogram on SDS-PAGE of proteins revealed two major groups joining at less than 40% similarity (Fig 3). Group A consists of two isolates 1G and SS 43\97 from an unknown origin and Western Cape respectively). Group B consists of TT 7\97 from the Northern Province; GG 2\97, GG 71\97, SS 37\97, GG3\97, CC 60\97 from the Western Cape; NN 176\97 from Kwazulu\Natal; SRZ 34, Ecc, C 80(b), 1H, and C(80) from an unknown origin. At 38% similarity, subgroup B1 has only one isolate from the Western

Cape (GG 7\97). Subgroup B2 is subdivided into two further groups that are subgroup B1a and subgroup B1b at 57% similarity. Subgroup B1a, which is the largest subgroup, is subdivided into other smaller subgroups starting at 65% similarity. They comprise isolates TT7/97 from the Northern Province; GG71/97, Gg2/97, SS37/97 and GG3/97f rom the Western Cape; SRZ 34 from an unknown location and Ecc (positive Control). Subgroup B1b is composed of only two isolates that are 1H and Nn176/97 from an Unknown origin and Kwazulu/Natal respectively. Further subgroups are also observed starting from 59% to 90% similarity.

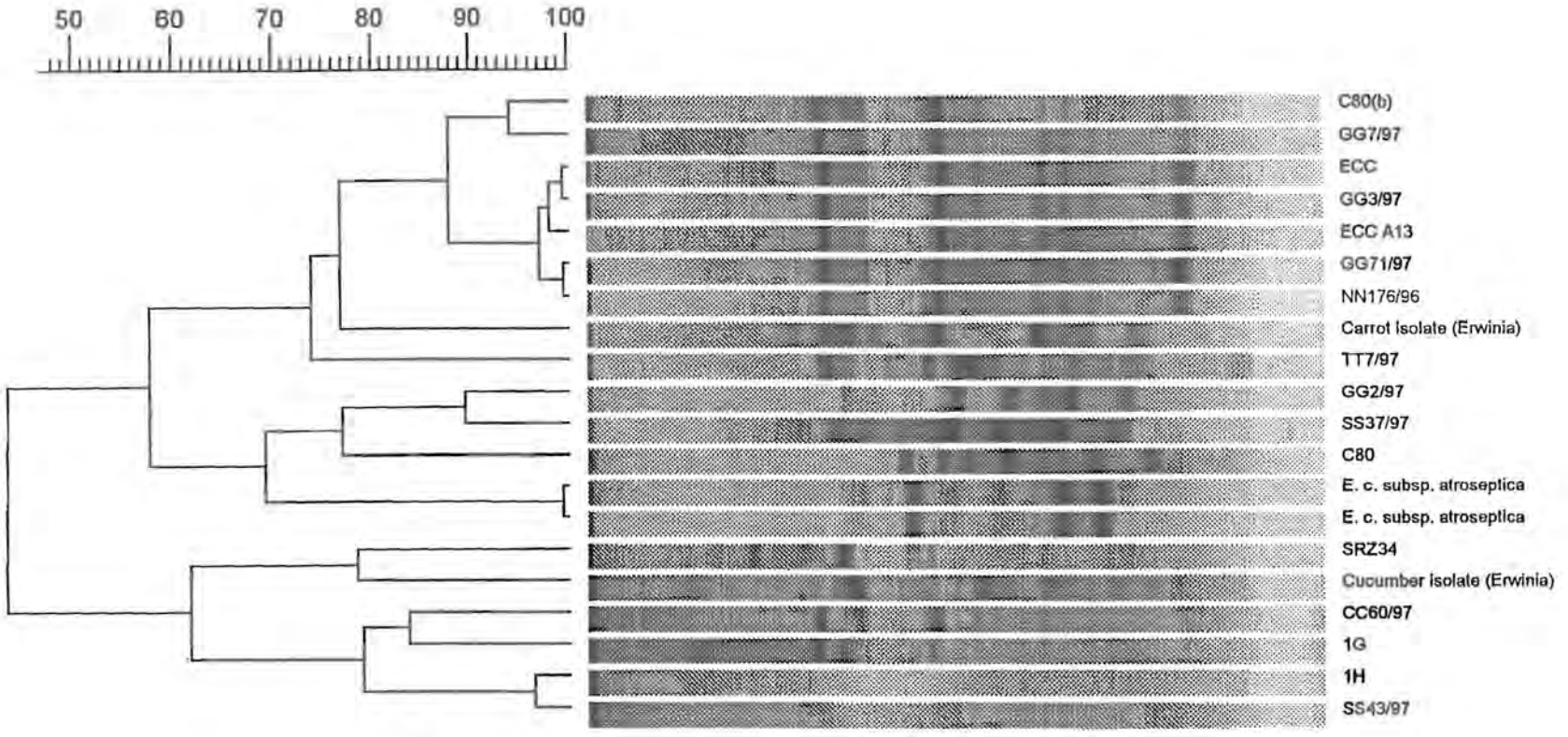


Fig 1 Box-PCR electrophoregram of *Erwinia* spp.

Cc 60/97, ss37/97, 1G, SRZ34, Ecc A13, GG3/97, GG71/97, C80, 1H, GG2/97, TT7/97, NN176/97, SS43/97, C80(b), GG7/97 represents *Erwinia* spp. Carrot and cucumber *Erwinia* isolates and *Erwinia carotovora* subsp. *atroseptica* represents outgroups

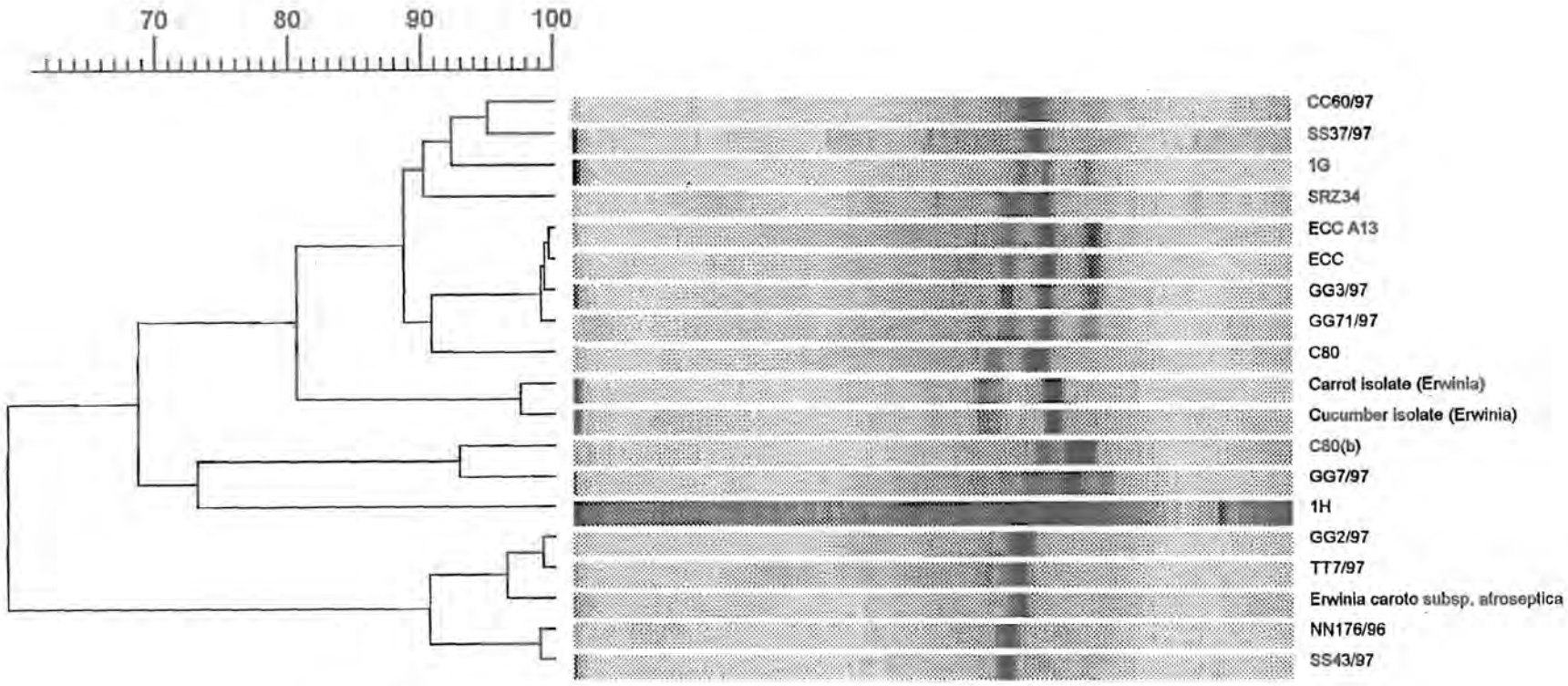


Fig 2 RISA electrophoregram of *Erwinia* spp.

Cc 60/97, ss37/97, 1G, SRZ34, Ecc A13, GG3/97, GG71/97, C80, 1H, GG2/97, TT7/97, NN176/97, SS43/97, C80(b), GG7/97 represents *Erwinia* spp. Carrot and cucumber *Erwinia* isolates and *Erwinia carotovora* subsp. *atroseptica* represents outgroups

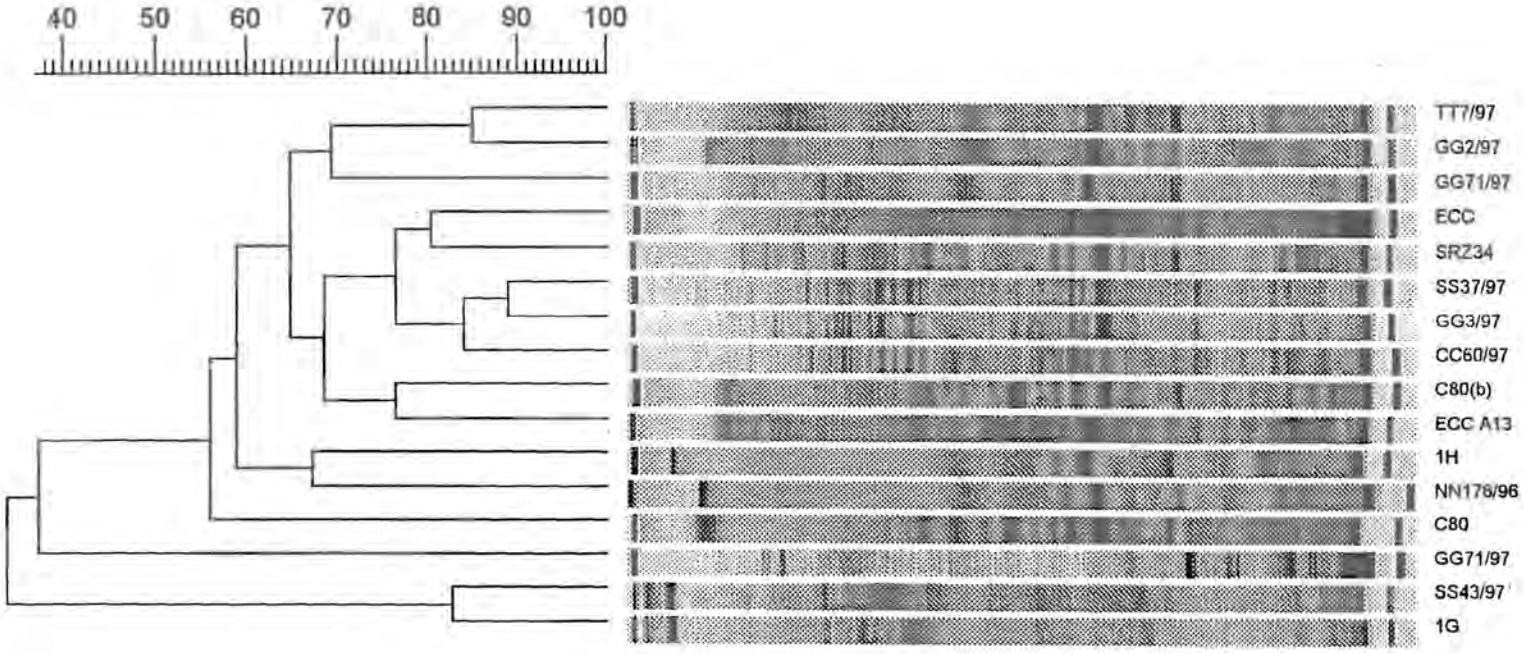


Fig 3 Protein electrophoregram of *Erwinia* spp.

Cc 60/97, ss37/97, 1G, SRZ34, Ecc A13, GG3/97, GG71/97, C80, 1H, GG2/97, TT7/97, NN176/97, SS43/97, C80(b), GG7/97 represents *Erwinia* spp. and Ecc represents a positive control

4.4 Discussion

In this study we have shown that repetitive extragenic sequences such as BOX are present in the genome of *Erwinia* spp. isolated from potato producing areas of South Africa. These results therefore supported the data presented by De Bruijn (1992) and Louws *et al.* (1994) on the ubiquitous nature of these elements in bacteria. We were also able to amplify the 16S and 23S rRNA genes of the isolated *Erwinia* spp. SDS-PAGE was found to be helpful to differentiate between various South African *Erwinia* spp. on the basis of their electrophoretic protein profiles.

The taxonomy of *Erwinia* spp. has traditionally been based on phenotypic characters such as biochemical (Lelliott & Stead, 1987) and physiological tests (Dickey & Kelman, 1990), but these approaches have been widely criticized as being too insensitive and time consuming (Schaad, 1979). Methods involving the analysis of total genomic nucleic acids such as BOX-PCR (De Bruijn, 1992) and those that involve total bacterial community DNA of the intergenic region between the small (16S) and the large (23S) subunit rRNA genes such as RISA (Fisher & Triplett, 1999) constitute a valuable complement to the classification techniques of *Erwinia* spp.

Fingerprinting patterns of both PCR and SDS-PAGE could not distinguish or separate isolates from geographic origin or cultivars from which different *Erwinia* spp. were isolated. Extensive genetic diversity was found between *Erwinia* spp. where isolates ranged in similarity from less than 40% to 89%, less than 60% to 100% and less than 50% to 100%. This genetic diversity was observed regardless of whether PCR or SDS-PAGE was used. According to Serfontein & Hattingh (1990), analysis of protein electrophoregrams can not be used to establish the geographical origin, year of isolation or virulence of strains. In South Africa, transplants and seed potatoes are distributed to growers over vast distances. Furthermore, farmers often acquire seed from each other (Serfontein & Hattingh, 1990). Hence the great diversity in results. The high diversity found on the basis of the BOX-PCR profiles reflects the variability of the total *Erwinia* spp. genome and its heterogeneous nature (Rosado *et al.*, 1998). According to Fisher &

Triplett (1999), results obtained with RISA should be cautiously interpreted since the technique relies upon total community DNA extraction and PCR amplification which is subjected to the usual systematic biases introduced by these procedures. These biases may include preferential amplification of shorter templates and biases imposed by secondary structure or DNA flanking the template region.

Fingerprinting methods have greatly increased our ability to discriminate between microorganisms and have become very common in the area of molecular epidemiology even though each method has its own advantages and disadvantages.

4.5 Literature cited

Cother, E. J. & Powell, V. 1983. Physiological and pathological characteristics of *Erwinia chrysanthemi* isolates from potato tubers. *Journal of Applied Bacteriology* 54: 37-43.

Cother, E. J. & Sivasithamparam, K. 1983. *Erwinia*: The *carotovora* group. Pages 87-101 in: Fahy, P. C. & Persley, G. J. (eds). *Plant bacterial diseases. A diagnostic guide.* Academic Press, Sydney.

Cuppels, D. A. & Kelman, A. 1974. Evaluation of selective media for isolation of soft rot bacteria from soil and plant tissue. *Phytopathology* 64: 468-475.

Dagutat, H. 1995. Identity and taxonomy of indigenous South African rhizobia. PhD thesis. University of Pretoria. Pretoria.

De Boer, S. H. 1994. Prospects for control of potato diseases caused by pectolytic *Erwinias*. Pages136-148 in: Zehnder, G.W., Powelson, M.L., Jansson, R.K. & Raman, K.V. (eds). *Advances in potato pest biology and management*. APS Press, St Paul, Minnesota.

De Boer, S. H. & Ward, L. J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* 85: 854-858.

De Bruijn, F. J. 1992 Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Applied and Environmental Microbiology* 58: 2180-2187.

Dickey, R. S. & Kelman, A. 1990. *Erwinia "carotovora"* or soft rot group Pages 44-59 in: Schaad, N. W. (ed.) *Laboratory guide for identification of plant pathogenic bacteria*. St Paul, Minnesota.

Fisher, M. M. & Triplett, E. W. 1999. Automated approach to ribosomal intergenic spacer analysis of microbial diversity and its application to fresh water bacterial communities. *Applied and Environmental Microbiology* 65: 4630-4636.

Guilford, P. J., Taylor, R. K., Clark, R. G., Hale, C. N. & Forster, R. L. S. 1996. PCR-based techniques for the detection of *Erwinia amylovora*. *Acta Horticulturae* 411: 53-60.

Kersters, K. 1990. Polyacrylamide gel electrophoresis of bacterial proteins. Pages 191-198 in: Klement, Z., Rudolph, K. & Sands, D. C. (eds). *Methods in Phytobacteriology*. Akademiai Kiao, Budapest.

Kiredjian, M., Holmes, B., Kersters, K., Guilvout, I. & De Ley, J. 1986. *Alcaligenes piechaudii*, a new species of human clinical specimens and the environment. *International Journal of Systemic Bacteriology* 36: 282-287.

Laemmli, U. K. 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) 227: 680-685.

Lelliott, R. A. & Stead, D. E. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications, Oxford.

Louws, F. J., Fulbright, D. W., Stephens, C. J. & De Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology* 60: 2286-2295.

Maki-Valkama, T. & Karjalainen, R. 1994. Differentiation of *Erwinia carotovora* subsp. *atroseptica* and *carotovora* by RAPD-PCR. *Annals of Applied Biology* 125: 301-309.

McManus, P. S. & Jones, A. L. 1996. Genetic fingerprinting of *Erwinia amylovora* by repetitive sequence-PCR and PCR-ribotyping. *Acta Horticulturae* 411: 281-284.

Moline, H. E. 1984. Differentiation of post harvest soft rotting bacteria with two – dimensional polyacrylamide gel electrophoresis. *Phytopathology* 75: 549-553.

Obradovic, A. & Arsenijevic, M. 1997. Pathogenic differentiation of soft rot *Erwinia* potato parasites. *Acta Horticulturae* 462: 345-350.

Oosthuizen, M. C. 1998. Polyphasic taxonomy and phylogeny of aerobic gram-negative heparinase producing bacteria. PhD thesis. University of Pretoria. Pretoria.

Perombelon, M. C. M. 1992. Potato blackleg: Epidemiology, host-pathogen interaction and control. *Netherlands Journal of Plant Pathology* 98: 135-146.

Perombelon, M. C. M. & Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology* 18: 283-285.

Rosado, A. S., de Azevedo, F. S., da Cruz, D. W. van Elsas, J. D. & Seldin, L. 1998. Phenotypic and genetic diversity of *Paenibacillus azotofixans* strains isolated from the rhizoplane or rhizosphere soil of different grasses. *Journal of Applied Microbiology* 84: 216-226.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 Molecular cloning. A laboratory manual (2nd edition) Cold Spring Harbor Laboratory Press.

Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. *Annual Review of Phytopathology* 17: 123-147.

Serfontein, J. J. & Hattingh, M. J. 1990. Characterization of *Pseudomonas syringae* pv. tomato by numerical analysis of phenotypic features and total soluble proteins. *Journal of Phytopathology* 128: 220-234.

Smid, E. J., Jansen, A. H. J. & Gorris, L. G. M. 1995. Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using Polymerase Chain Reaction. *Plant Pathology* 44: 1058-1069.

Toth, I. K., Hyman, L. J. & Wood, J. R. 1999. A one step PCR-based method for the detection of economically important soft rot *Erwinia* species on micropropagated potato plants. *Journal of Applied Microbiology* 87: 158-166.

Towsen, E. & Korsten, L. 1998. Economic importance of *Erwinia* spp. Abstract in proceedings of the 36th Southern African Society of Plant Pathology.

Van Zyl, E. & Steyn, P. L. 1990. Differentiation of phytopathogenic *Pseudomonas* and *Xanthomonas* species and pathovars by numerical taxonomy and protein gel electrophoregrams. *Systematic and Applied Microbiology* 13: 60-71.

Ward, I. J. & De Boer, S. H. 1994. Specific detection of *Erwinia carotovora* subsp. *atroseptica* with a digoxigenin-labeled DNA probe. *Phytopathology* 84: 180-186.

Wells, J. M. & Butterfield, J. E. 1997. *Salmonella* contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. *Plant Disease* 81: 867-872.

CHAPTER 5

GENERAL DISCUSSION

Potatoes are grown world wide as a major source of food (Rich, 1983; Hawkes, 1992). In South Africa, the potato industry is one of the most important root crop industries constituting 44.6% of all vegetables sold in the South African fresh produce market during the 1999 season (Abstract of agricultural statistics, 2000). As in the rest of the world, potatoes in South Africa are affected by a wide spectrum of diseases. The causal agents of these diseases include bacteria, fungi, mycoplasma, nematodes, viroids and viruses. One of the most important pathogens of potatoes in South Africa are *Erwinia* spp. *Erwinia* spp. causes soft rot, wilt and blackleg and are economically the most important causing subsequent loss in revenue (Towsen & Korsten, 1998).

The genus *Erwinia* has a heterogenous taxonomic structure that has not been well clarified (Kwon *et al.*, 1997). Within the *Erwinia* disease complex, we have the "carotovora " or "soft rot" group that attacks the potato crop pre-and post harvestly (Dickey & Kelman, 1990). This group is composed of different *Erwinia* species and subspecies that are found on potatoes and include the following: *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* (Dickey & Kelman, 1990).

Plant propagative material such as potato tubers and seeds are the major source of infection (Schaad, 1979). Expanding international trade and global movement of plant propagative material resulted in a heightened awareness of phytosanitary issues. In South Africa, transplants and seed potatoes are distributed to growers over vast distances and farmers often acquire seeds from each other, which can be moved between provinces (Serfontein & Hattingh, 1990). In this manner, diseases can easily be spread and introduced into new or previously unaffected areas. Knowledge of the presence and possible concentration of *Erwinia* spp. on asymptomatic mother seed tubers prior to

planting will help in forecasting possible disease outbreaks in the field (Cother & Vruggink, 1980). Current detection and identification methods of *Erwinia* spp. are by using pathogenicity, biochemical and physiological, serological tests such as ELISA and IF and molecular tests such as PAGE and PCR.

The primary objective of this study was to develop cost effective and rapid methods to detect *Erwinia* spp. on asymptomatic potato tubers obtained from different production areas of South Africa. Biochemical tests, PAGE and PCR were evaluated in this investigation to detect and distinguish between the *Erwinia* spp. isolated. Although selective media are considered effective for detection of the pathogen (Perombelon & Burnett, 1991, Perombelon, 1992), and are widely used for diagnostic purposes (Lelliott & Stead, 1987), increased accuracy can be achieved by a combination of basic and molecular diagnostic techniques.

The use of selective media (CVPB), (Perombelon & Burnett, 1991) coupled with the use of an enrichment medium (Meneley & Stanghellini, 1976) and basic biochemical tests (Lelliott & Stead, 1987) were found to be effective for detection of *Erwinia* spp. on asymptomatic potato tubers (Lojkowska & Kelman,1994). The enrichment method using selective media proved effective to isolate a range of *Erwinia* isolates. In most cases *Erwinia* spp. were isolated successfully from healthy tubers.

However, biochemical and physiological tests prepared according to standard procedures could not differentiate between soft rot causing *Erwinia* spp. In fact, discrepancies between test results and typical *E. c.* subsp. *carotovora* profiles were evident. This is in accordance with previous reports were Cother & Sivasithamparam (1983) reported similar inaccuracies.

In contrast, the BIOLOG and microplate method gave consistent results and was in agreement with previous reports (Lelliott & Stead, 1987). Hayward (1995), who described consistency in substrate utilization by *E. c.* subsp. *carotovora* used BIOLOG test experiments. BIOLOG results were more rapid and effective than traditional

biochemical tests and were found to be less laborious which was also reported by Garland & Mills (1991) and Verniere *et al.* (1993).

To confirm pathogenicity, potato tuber slices were inoculated with the different *Erwinia* isolates. All isolates proved to be pathogenic causing brownish to creamy white soft rot lesions on different cultivars after 24-48 hours followed by a watery exudate and an unpleasant odour. Obradovic & Arsenijevic (1997), reported similar results with lesion development.

PAGE was also conducted to confirm results obtained with biochemical and physiological tests. The PAGE technique proved effective to distinguish between isolates on the basis of the protein composition.

Finally, PCR based techniques were used to compare the *Erwinia* isolates and correlate them with previous methods used distinguish between isolates. PCR results correlated with PAGE and revealed that fingerprinting patterns could not be associated with geographic origin or cultivars from which *Erwinia* spp. were isolated. McManus & Jones (1996) reported similar results in their studies on *E. amylovora*. In this study, extensive genetic diversity was found within *Erwinia* spp. where many isolates ranged in similarity from less than 40% to 89% with PAGE less than 60% to 100% with BOX- PCR and less than 50% to 100% with RISA. This variation in the sequence homology values was found by Kwon *et al.* (1997) to indicate substantial intergeneric heterogeneity amongst *Erwinia* spp. Genetic diversity was observed with all molecular techniques employed. Similar findings were reported by Serfontein & Hattingh (1990) who analysed protein electrophoregrams and found the method ineffective in differentiating between strains of different geographical origin, year of isolation and virulence of strains. This study evaluated basic and molecular techniques thus providing a platform for more extensive studies on isolate variation and association with different types of symptoms.

5.1 Literature cited

Cother, E. J. & Sivasithamparam, K. 1983. *Erwinia*: The *carotovora* group. Pages 87-101 in: Fahy, P. C. & Persley, G. J. (eds). *Plant bacterial diseases. A diagnostic guide.* Academic Press, Sydney.

Cother, E. J. & Vruggink, H. 1980. Detection of viable and non-viable cells of *Erwinia carotovora* var. *atroseptica* in inoculated tubers of var. Bintje with enzyme-linked immunosorbent asay (ELISA). *Potato Research* 23: 133-135.

Dickey, R. S. & Kelman, A. 1990. *Erwinia "carotovora"* or soft rot group Pages 44-59 in: Schaad, N. W. (ed.) *Laboratory guide for identification of plant pathogenic bacteria.* St Paul, Minnesota.

Garland, J. L. & Mills, A. L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* 57: 2351-2359.

Hawkes, J. G. 1992. History of the potato crop. Pages 1-9 in: Harris, P. (ed.) *The Potato crop.* Chapman & Hall, New York.

Hayward, A. C. 1995. Phenotypic methods for the identification of *Pseudomonas solanacearum*: biovars and supplementary observations. Pages 27-34 in: Mehan, V. R. & McDonald, D. (eds). *Techniques for the diagnosis of Pseudomonas solanacearum and for resistance screening against groundnut bacterial wilt.* Technical manual no1.ICRISAT. Patancheru, Andhra Pradesh.

Kwon, S. W., Go, S., Kang, H., Ryu, Y. & Jo, J. 1997. Phylogenetic analysis of *Erwinia* species based on 16S rRNA gene sequences. *International Journal of Systemic Bacteriology* 47: 1061-1067.

Lelliott, R. A. & Stead, D. E. 1987. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications. Oxford.

Lojkowska, E. & Kelman, A. 1994. Comparison of the effectiveness of different methods of screening for bacterial soft rot resistance of potato tubers. *American Potato Journal* 71: 99-113.

Meneley, J. C. & Stanghellini, M. E. 1976. Isolation of soft-rot *Erwinia* spp. from agricultural soils using an enrichment technique. *Phytopathology* 66: 367-370.

McManus, P. S. & Jones, A. L. 1996. Genetic fingerprinting of *Erwinia amylovora* by repetitive sequence-PCR and PCR-ribotyping. *Acta Horticulturae* 411: 281-284.

Obradovic, A. & Arsenijevic, M. 1997. Pathogenic differentiation of soft rot *Erwinia* potato parasites. *Acta Horticulturae* 462: 345-350.

Perombelon, M. C. M. 1992. Potato blackleg: Epidemiology, host-pathogen interaction and control. *Netherlands Journal of Plant Pathology* 98: 135-146.

Perombelon, M. C. M. & Burnett, H. 1991. Two modified crystal violate pectate (CVP) media for detection, isolation and enumeration of soft rot *Erwinias*. *Potato Research* 34: 79-81.

Rich, A. E. 1983. Potato diseases. Academic Press. New York.

Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. *Annual Review of Phytopathology* 17: 123-147.

Serfontein, J. J. & Hattingh, M. J. 1990. Characterization of *Pseudomonas syringae* pv. tomato by numerical analysis of phenotypic features and total soluble proteins. *Journal of Phytopathology* 128: 220-234.

Towsen, E. & Korsten, L. 1998. Economic importance of *Erwinia* spp. Abstract in proceedings of the 36th Southern African Society of Plant Pathology.

Verniere, C., Pruvost, O., Civerolo, E. L., Gambin, O., Jacquemoud-Collet, J. P. & Luisetti., J. 1993. Evaluation of the BIOLOG substrate utilization system to identify and asses metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. *Applied and Environmental Microbiology* 59: 243-249.

SUMMARY

DETECTION OF *ERWINIA* SPP. ON POTATOES

by

ALPHEUS KGABO MATLALA

SUPERVISOR	Prof. L. Korsten
CO-SUPERVISOR	Dr. G. Swart
DEPARTMENT	Microbiology and Plant Pathology
DEGREE	M. Inst. Agrar (Plant Protection)

In South Africa, pectolytic *Erwinia* spp. cause major economic losses to the potato industry. The bacteria affect potato tubers in storage and in transit causing soft rot. They also affect the stem of the potato plant, causing blackleg, or the entire plant that can wilt and die. *Erwinia* spp. are ubiquitous and can occur on asymptomatic potato seeds. Early detection of pectolytic *Erwinias* is therefore important as a first step to control the disease. Isolations were made from potato tuber cultivars collected from various production areas in South Africa. An enrichment technique and a selective media (CVPB) were used to facilitate the ease of isolations. Three soft rot causing bacteria were differentiated from each other, using BIOLOG, biochemical and pathogenicity tests. None of these tests could differentiate between the three soft rot causing bacteria and discrepancies were observed in results. PAGE and PCR were subsequently used to further fingerprint and identify *Erwinia* spp. PAGE revealed a similarity coefficient ranging from less than 40% to 89%. RISA and BOX-PCR showed a similarity coefficient ranging from less than 60% to 100% (RISA) and less than 50% to 100% (BOX-PCR). This study highlighted the potential of using these techniques for rapid detection and identification of *Erwinia* spp.