CHAPTER 3

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PECTOLYTIC PATHOGENS ASSOCIATED WITH POTATO SOFT ROT IN ZIMBABWE

Abstract

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

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

3.1 INTRODUCTION

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

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

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

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

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

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

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

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

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

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

3.2 MATERIALS AND METHODS

3.2.1 Isolation of bacterial strains

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

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

3.2.2 Biochemical and physiological identification of isolates

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

3.2.3 Rep-PCR analyses

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

3.2.4 Sequencing and phylogenetic analysis

Genomic DNA was extracted from overnight broth cultures using a DNeasy™ Blood and Tissue Kit (Qiagen, Southern Cross Biotechnology). For selected bacterial strains, the *recA* and *gyrB* genes were amplified using recAF, recAR and gyrB-01F, gyrB-02F respectively (Table 2). PCR amplification was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Corp). PCR products were purified using a QIAquick™ Purification Kit (Qiagen, Southern Cross Biotechnology). The *gyrB* and *recA* genes were sequenced in both directions using primers and conditions previously described (Young and Park, 2007, Brady *et al.*, 2008). Sequencing products were cleaned using a sodium acetate precipitation step and sequenced on an ABI Prism DNA Automated Sequencer (Perkin Elmer). Incorrect basecalls were corrected using Chromas Lite v 2.01. The corrected nucleotide sequences were edited, aligned with BioEdit Sequence Alignment v 7.0.0. and both ends of each alignment were trimmed to the following final sizes: *recA,* 697 positions and *gyrB*, 740 positions. Searches were performed on each consensus sequence generated in BioEdit using the BLAST algorithm from GenBank.

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

3.2.5 Pectolytic activity

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

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

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

3.2.6 Virulence assays

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

3.2.7 Fluorescent amplified fragment length polymorphism analysis

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

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

3.3 RESULTS

3.3.1 Biochemical and physiological characterisation

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

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

3.3.2 Rep-PCR analyses

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

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

3.3.3 Sequencing and phylogenetic analysis

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

3.3.4 Pectolytic activity

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

3.3.5 Virulence assays

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

3.3.6 Fluorescent amplified fragment length polymorphism

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

3.4 DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 3.1 Continued

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

Table 3.1 Continued

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

Table 3.1 Continued

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

Table 3.1 Continued

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

Table 3.1 Continued

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

Table 3.2 List of primers used in study

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

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

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

Table 3.3 Continued

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

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

EN, Isolated from potatoes grown in Zimbabwe

JJ, Isolated from potatoes grown in South Africa

W, weak reaction

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

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

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

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

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

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

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

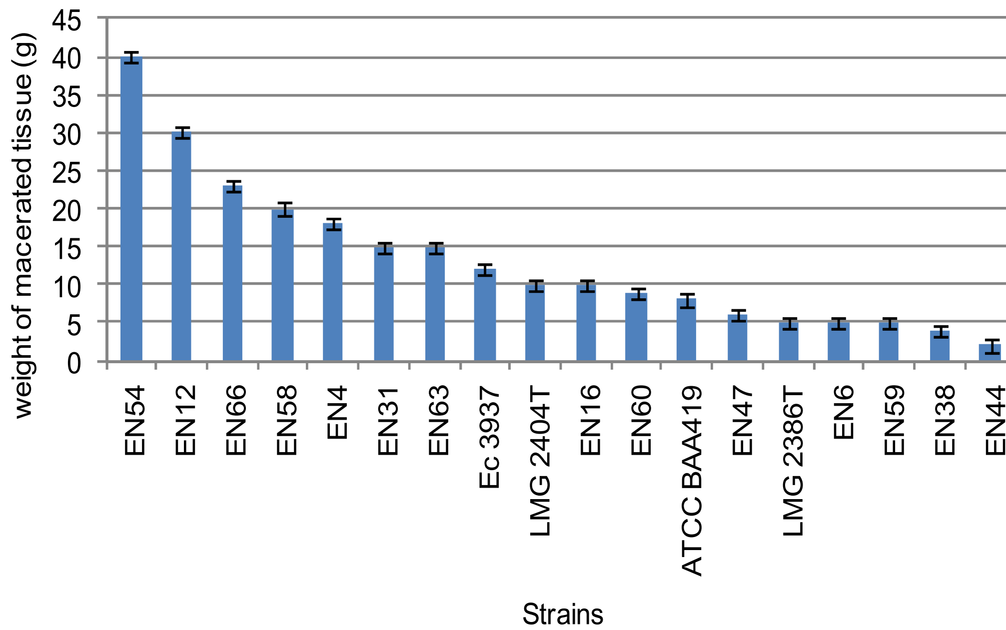


Figure 3.1 Relative virulence of atypical strains of pectolytic enterobacteria isolated from potatoes grown in Zimbabwe and the type strains of *Pectobacterium atrosepticum, Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya dadantii* subsp. *dadantii.*

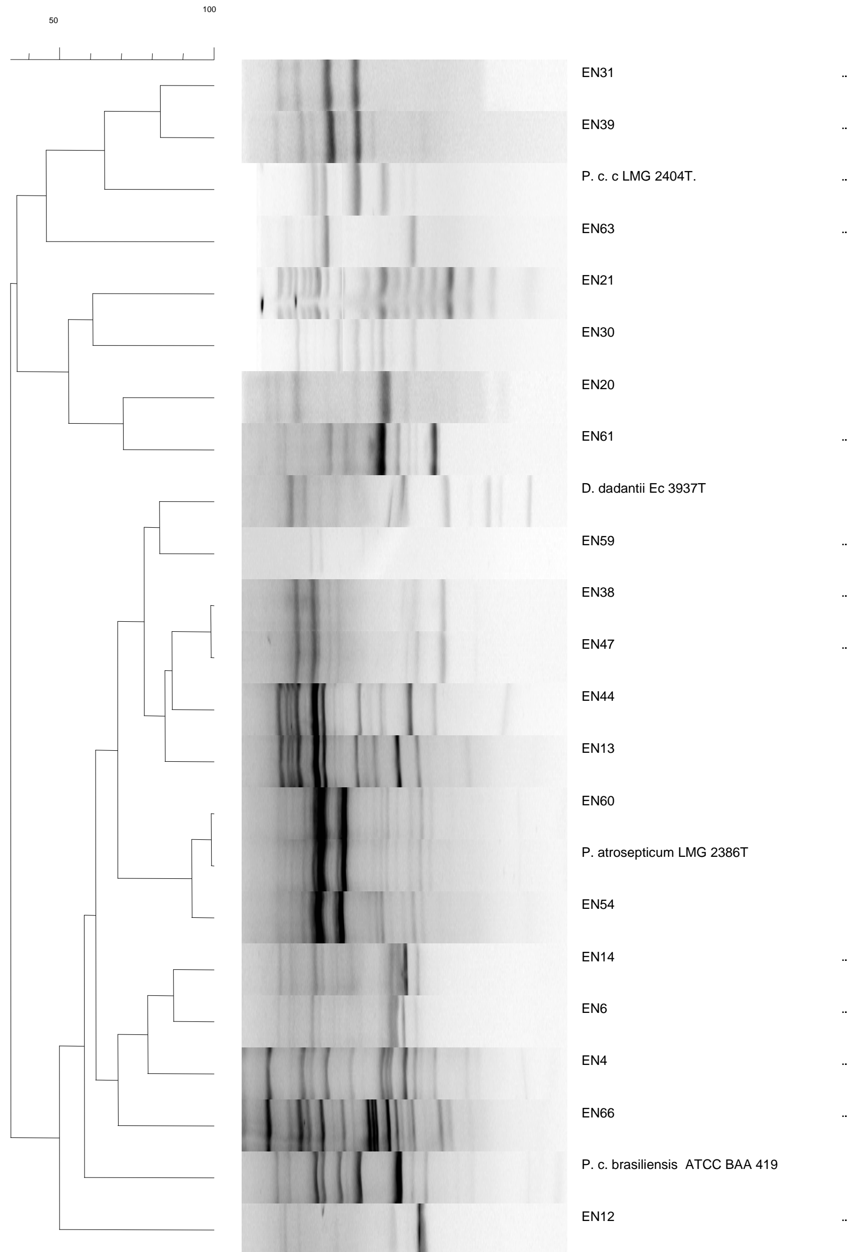


Figure 3.2 Cluster analysis of Rep-PCR fingerprint pattern generated from purified DNA of 23 *Pectobacterium* and *Dickeya dadantii* strains. The dendrogram was generated using Bionumerics with an UPGMA algorithm applied to the similarity matrix generated by Pearson's correlation coefficient from whole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

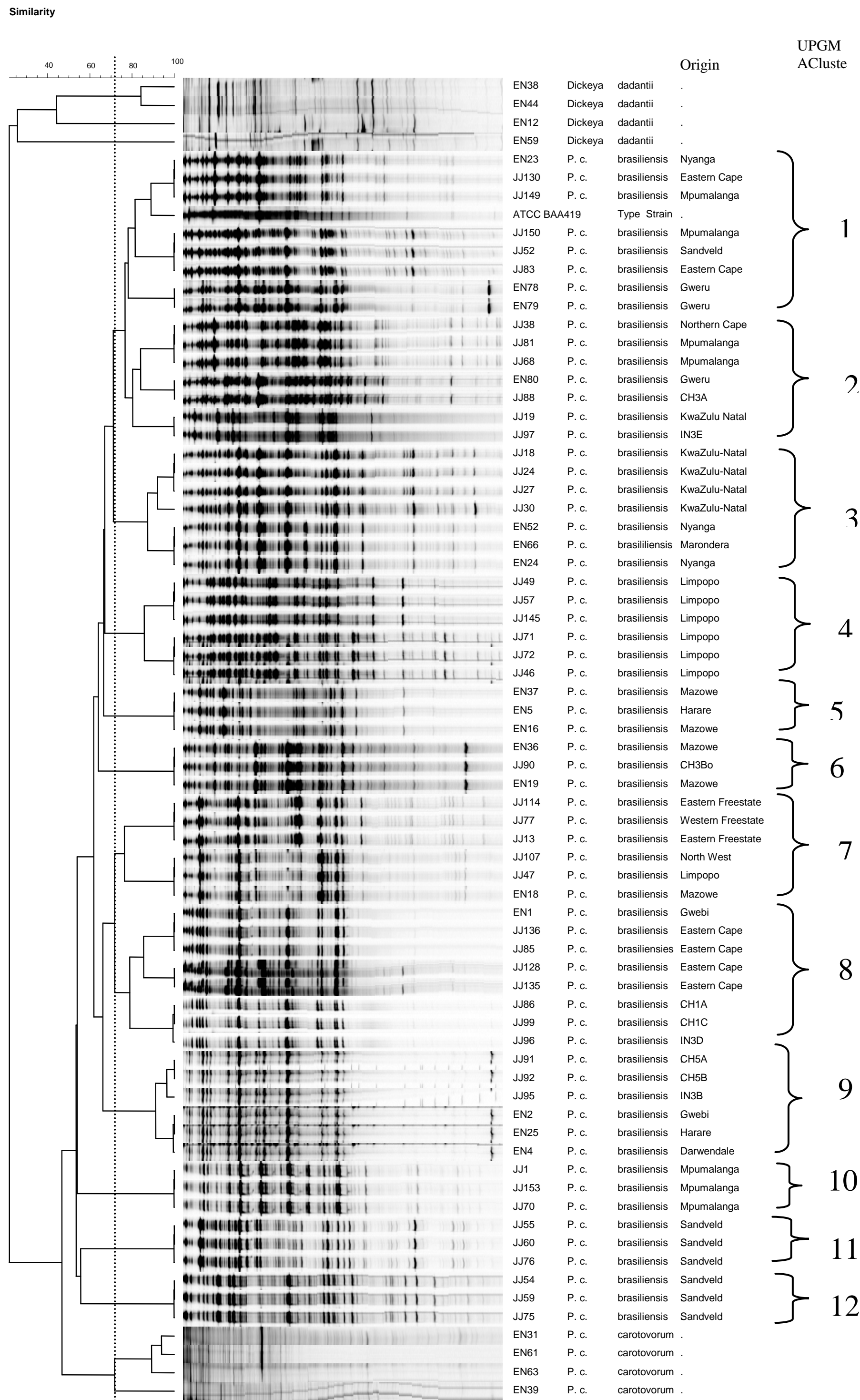
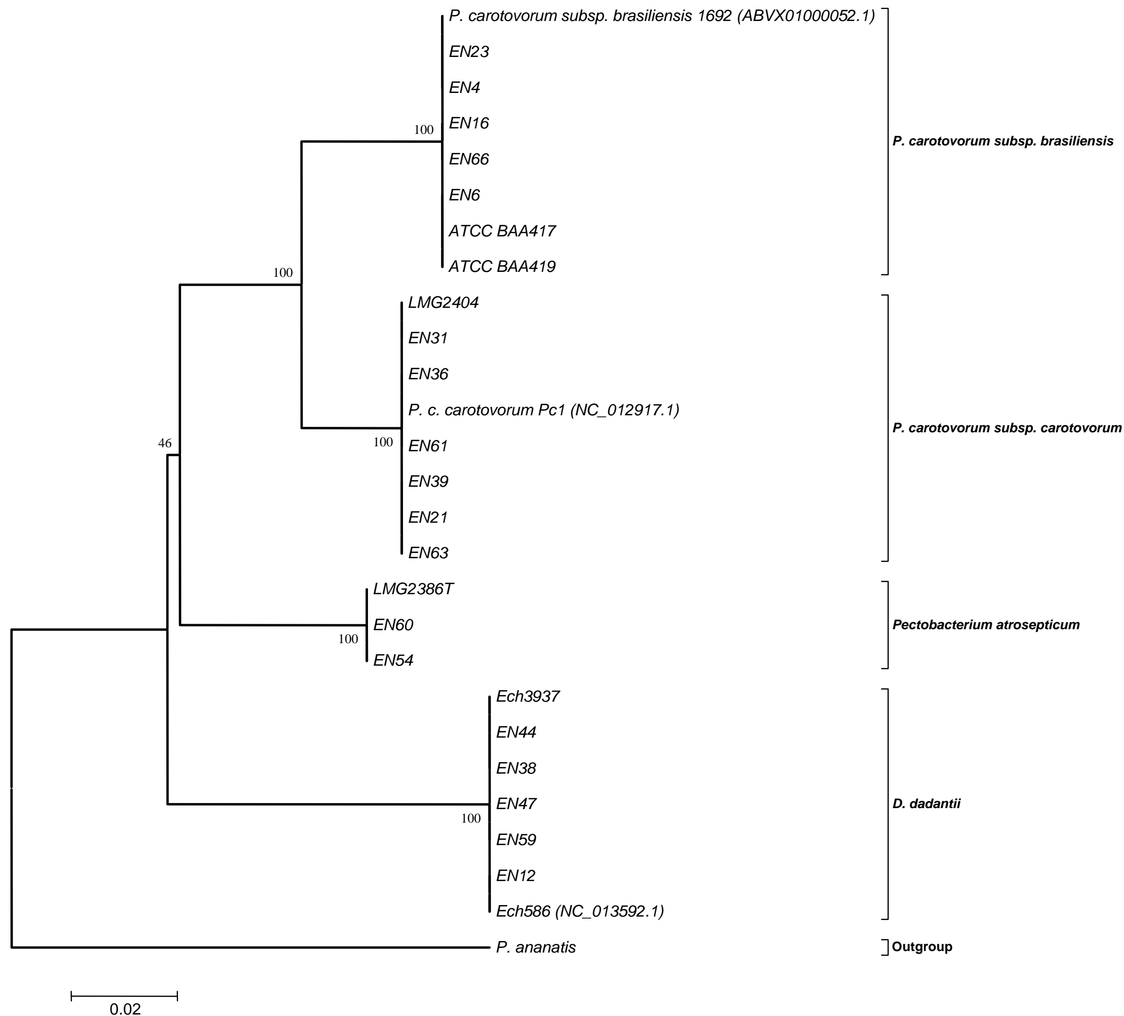


Figure 3.4 Dendrogram derived from the UPGMA linkage of correlation coefficient between AFLP profiles from strains of *P. carotovorum* subsp. *brasiliensis*, *P. carotovorum* subsp. *carotovorum* and *D. dadantii*. The levels of linkage representing the Pearson product moment correlations are expressed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



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