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ORIGINAL RESEARCH

Pectobacterium carotovorum subsp. *brasiliensis* causing blackleg on potatoes in South Africa

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Abstract In South Africa during the 2006/2007 potato growing season, outbreaks of blackleg occurred, causing severe economic losses in commercial potato production fields. Symptoms were initially observed on only one stem per plant, on which the top leaves rolled upwards, wilted and became necrotic. As symptoms progressed to the lower leaves with subsequent leaf desiccation, a light to dark brown discolouration of the vascular system at the stem base developed, followed by external darkening. Under prevailing wet and humid conditions stems became slimy and pale. In the stems, the pith became necrotic and hollow. These symptoms were similar to those described in Brazil, where the causal agent was identified as a new subspecies, *Pectobacterium carotovorum* subsp. *brasiliensis* (*Pbcb*). Isolations from plants showing typical blackleg symptoms were made on CVP medium. Sequences and phylogenetic analysis of the partial 16S–23S rDNA intergenic spacer region indicated that the isolates were *Pbcb.* Comparison of PCR-RFLP patterns of the 16S–23S rDNA of isolates to reference cultures confirmed the identity of the South African blackleg strains as *Pbcb*, identical to strain 8 isolated in Brazil. This is the first report of *Pbcb* in South Africa and it appears to be the most important causal agent of blackleg in South Africa. The disease poses a major potential threat to the South African potato industry especially in terms of seed exports, tuber quality and yield.

Keywords Blackleg-like symptoms · Soft rot · Economic yield losses

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Introduction

Potato blackleg is a seed-borne disease causing severe economic losses to the potato seed and commercial production industries. Blackleg is mainly caused by *Pectobacterium atrosepticum* (*Pba*) but under certain conditions *Pectobacterium carotovorum* (*Pbc*) and *Dickeya* spp. can also cause similar symptoms

(Pérombelon et al. 1987, Oliveira et al. 2003, De Haan et al. 2008). These species were formerly known as the soft rot *Erwinia* spp., but they were recently reclassified into two new genera, namely *Pectobacterium* and *Dickeya,* on the basis of 16S rDNA sequences, their ability to grow at 39°C and host ranges (Hauben et al. 1998, Garden et al. 2003, Samson et al. 2005, Laurila et al. 2008). *Dickeya* spp., formerly placed in a single species *Erwinia chrysanthemi* (*Ech*), were reclassified into six species (Samson et al. 2005). The important *Dickeya* spp. causing disease on potatoes are *Dickeya dadantii* (formerly *Ech* 3937), *D. zeae* (formerly *Ech* biovar 3) and *D. dianthicola* (formerly *Echr* biovars 1 and 7) (Samson et al. 2005, Elphinstone and Toth 2007, Tsror et al. 2009). Recently, a new subspecies from Brazil, *Pectobacterium carotovorum* ssp. *brasiliensis* (*Pbcb*), was described, which also causes blackleg-like symptoms on potatoes (Duarte et al. 2004).

In Canada, USA and Europe typical blackleg symptoms i.e. wilted appearance with yellowish foliage are caused by *Pba.* Similar symptoms accompanied by a brownish, sticky discolouration at the stem base (external) with brown staining of the vascular tissues (internal), which can be followed by necrosis and stem hollowing (Pérombelon et al. 1987), appeared in Europe (Netherlands, England, France, Hungary, Jersey, Spain and Switzerland) and Israel but are caused by *Dickeya* spp. (Palacio-Bielsa et al. 2006, Elphinstone and Toth 2007, Tsror et al. 2009). The primary difference between the epidemiological development of *Pba* and *Dickeya* spp. is weather conditions that determine successful establishment of infection. *Pectobacterium atrosepticum* causes blackleg under cool, wet conditions, whereas *Dickeya* spp. are pathogenic under warm or warm-dry conditions (Elphinstone and Toth 2007). In Brazil, *Pbcb* causes disease in areas with a humid subtropical climate, but relatively cool temperatures (17–20˚C) during the growing season (Duarte et al. 2004). De Haan et al. (2008) stated that *Pbc* can cause typical blackleg symptoms in a temperate climate.

According to De Boer (2004) preliminary results suggest that *Pba* does not occur on potatoes if *Pbcb* is present. This suggests that *Pbcb* is more virulent than *Pba*. The incidence and severity of blackleg in the field is affected by specific climatic factors, in particular temperature, soil moisture content and soil nutrient status.

The most important determining factor for symptom expression in potatoes infected with *Pectobacterium* and *Dickeya* spp. is the titre of the bacterial population present in contaminated seed tubers at the time of planting (Bain et al. 1990, Pérombelon 2002). Seed tubers are commonly contaminated with various soft rot bacteria in the latent state in the lenticels and wounds. To control the maceration processes these bacteria communicate with each other by means of a quorum-sensing process that relies on the production of *N*-acylhomoserine lactones (Smadja et al. 2004, Toth et al. 2004). The bacteria multiply until a critical cell density between 10^7 and 10^8 cfu ml^{-1} is reached; the rotting process is initiated in the seed tubers, which subsequently moves up into the stem through the vascular system. Here the bacteria remain quiescent until environmental conditions are favourable, after which they become active and cause disease expression in the above-ground plant parts. Simultaneously, bacteria can move downwards in the vascular bundles, infecting progeny tubers. Depending on the bacterial concentration and rate of multiplication, symptoms will be expressed immediately or during the next growing season. Therefore, the most important means of dissemination of inoculum is movement of latently-infected seed tubers (Pérombelon and Kelman 1987, Laurila et al. 2008, Czajkowski et al. 2009).

In South Africa, *Pbc* and *Dickeya* spp. were detected on potatoes for the first time during the 1988 growing season, causing typical wilting symptoms. *Dickeya* spp. were detected in Mpumalanga, Northern Cape and Gauteng with *Dickeya zeae* occurring in the Northern Cape and *Dickeya dianthicola* mainly in Mpumalanga and Gauteng. *Pectobacterium carotovorum* was detected in Mpumalanga, Northern Cape, Eastern Free State, Gauteng, Ceres and Eastern Cape (Serfontein et al. 1991). Symptoms initially developed on only one stem of the plant, as a one-sided wilt of the upper leaves that tended to roll upwards and wilt without chlorosis. These symptoms spread to the lower leaves, which became desiccated and subsequently the whole plant wilted. In addition, vascular discolouration at the stem base developed (Serfontein et al. 1991). During severe infection, the pith rotted, became hollow and eventually desiccated. Wilted plants were mostly associated with prevailing wet and humid conditions (Serfontein et al. 1991).

Serfontein et al. (1991) identified isolates using standard bacteriological methods and confirmed their

identity using fatty acid profiling. Results indicated that the causal agents in South Africa were *Pectobacterium carotovorum* (*Erwinia carotovora* ssp. *carotovora*), *Dickeya zeae* (*Erwinia chrysanthemi* biotype IV, pathovar *zeae*) and *Dickeya dianthicola* (*Erwinia chrysanthemi* biotype V, pathovar *dianthicola*). Interestingly, none of the isolates were identified as *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*).

The objective of this research was to isolate and identify the soft rot bacteria associated with recent outbreaks of blackleg on potatoes in South Africa.

Materials and methods

Sampling and isolation of causal agents

The stems and tubers of potato plants with typical blackleg symptoms (soft rot, wilting, internal and external darkening) were collected throughout the 2006 and 2007 growing seasons from seven production regions where blackleg outbreaks occurred in South Africa. Severe outbreaks occurred in Limpopo, Mpumalanga and the Sandveld. Pieces of the infected stems and tubers were macerated in 0.01 M magnesium sulphate ($MgSO_2$). Isolations were performed on the selective medium, Crystal Violet Pectate (CVP) (Hyman et al. 2001) and plates were incubated at 26°C for 48 h. Isolates that tested positive for pectolytic cavity formation were purified on the same medium and transferred to nutrient agar (NA) (Merck, Darmstadt, Germany), on which they were maintained. A total of 142 isolates were obtained and subjected to IGS-PCR, which identified 77% of these isolates as *Pbcb*. All isolates were stored in sterile water at room temperature and in 15% glycerol medium at −80°C and maintained in the Potato Pathology Programme culture collection at the University of Pretoria, Pretoria, South Africa.

Three isolates (JJ74, JJ145, and JJ147) obtained from plants with typical blackleg symptoms were selected for further study. These isolates are representative of the Sandveld, Limpopo and Mpumalanga growing regions, respectively, which had the worst blackleg outbreaks during the 2007 growing season. Type strains of the various species and subspecies were also included in this study (Table 1).

Single colonies were used for biochemical tests. These tests included Gram testing (3% KOH) and the Hugh and Leifson`s oxidation/fermentation test (Merck 2005). Representative *Pbcb* (JJ54) and *Pbc* (JJ68) strains were grown on Tryptone Soya Agar (TSA) for 7–8 h. Nutritional requirements and the use of specific carbon sources for growth were tested with GN2 Microplates™ (Biolog Inc., Hayward, CA). The optical density of the suspension was adjusted as recommended by the manufacturer. Microplates™ were inoculated with 150 µl of suspension per well, incubated at 28°C overnight and read visually.

Pathogenicity

All three strains were tested for pathogenicity on potato tubers (*Solanum tuberosum* cv. Mondial), green peppers and 6 week-old potato plants grown in 20 cm diam pots in a 24°C regulated greenhouse. Inoculations were done by dipping sterile toothpicks into bacterial colonies grown for 48 h on NA at 26°C.

Two stems per potato plant, 5 cm above the stem base, were immediately pierced with the contaminated toothpick after which the inoculated point was covered with Vaseline. Control potato plants were pierced with sterile toothpicks and the wounds covered with Vaseline. Potato plants were observed daily for visible blackleg symptoms.

Potato tubers and green peppers were inoculated to test isolate pectolytic ability. The inoculation process was the same as for the potato stems but tubers and peppers were pierced three times each. Control tubers and peppers were inoculated with toothpicks dipped in sterile NB. The inoculated tubers and green peppers were placed in plastic bags and sealed to maintain high humidity at 27°C in the dark. After 24 h, tubers were evaluated daily for the presence of symptoms.

DNA extractions

DNA was extracted from 48-h-old pure cultures grown on NA using a DNeasy™ Blood & Tissue Kit (Qiagen, Southern Cross Biotechnology). RNA was removed with Ribonuclease A from bovine pancreas (Sigma-Aldrich) by incubating overnight at room temperature. Extracted DNA was stored at −20°C.

Table 1 Reference cultures used in this study

Name	Code	Obtained from
Pectobacterium carotovorum subsp. *carotovorum*	LMG 2404^T	Belgian Coordinated Collections of Microorganisms (BCCM™)
Pectobacterium atrosepticum	LMG 2386^T	Belgian Coordinated Collections of Microorganisms (BCCM™)
Pectobacterium carotovorum subsp. *brasiliensis* strain 8	ATCC BAA-417	American Type Culture Collection (ATCC)
Dickeya dadantii (*Erwinia chrysanthemi* 3937)		Scottish Crop Research Institute (SCRI)

Partial 16S–23S rDNA sequencing

The partial 16S–23S rDNA intergenic spacer (IGS) region of selected strains JJ74, JJ142 and JJ145 was amplified using species-specific primers BR1f (5′-GCGTGCCGGGTTTATGACCT-3′) and L1r (5′-CARGGCATCCACCGT-3′) (Integrated DNA Technologies, Whitehead Scientific). BR1f was designed from the IGS region based on the restriction enzyme site of SexAl and L1r from the 5′ end of the 23S rRNA gene (Duarte et al. 2004). The amplified products of the isolates were then compared to those from the type strain, *Pbcb* 371 (ATCC BAA-419). Each 25 µl pre-amplification reaction contained 1 × NH_4 reaction buffer, 2 mM $MgCl_2$ solution, 100 µM dNTP mix, 30 pmol of each primer and 1.25 U BIOTAQ™ (Whitehead Scientific (Pty) Ltd.). PCR reactions were carried out with the following thermal profile: 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 90 s and a final extension step of 72°C for 7 min. Amplified products were electrophoresed through a 2% agarose gel in 1 × TBE buffer at 75 V for 2 h.

PCR products were purified using the QIAquick™ Purification Kit (Qiagen, Southern Cross Biotechnology). The partial 16S–23S rDNA was sequenced in

Fig. 1 Typical blackleg symptoms without chlorosis on potatoes infected with *Pbcb* in a commercial field under wet, cool conditions

Fig. 2 During prevailing wet and humid conditions stems become slimy and pale and leaves become chlorotic

Fig. 3 Severely infected potato plants dry out and die

both directions using the forward primer BR1f and reverse primer L1r (Duarte et al. 2004). Each 10 µl sequencing reaction contained 2 µl Big Dye Sequencing Reaction Mix, 1 × sequencing buffer, 30 pmol primer and 3 µl purified DNA template. Sequencing PCR conditions included denaturation at 96°C for 1 min, 25 cycles of denaturation at 96°C for 10 s, annealing of primer at 50°C for 5 s and elongation at 60°C for 4 min. PCR 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 sequences were edited, aligned and trimmed using BioEdit Sequence Alignment Editor v 7.0.0. Searches were performed on each consensus sequence generated on BioEdit using the BLAST programme from the GenBank database.

All the selected sequences were aligned using MAFFT. A phylogenetic tree for the data set was inferred by using the neighbour-joining programme of MEGA v 4 (Tamura et al. 2007). The stability of the phylogenetic tree was assessed by performing bootstrap analysis of the neighbour-joining data based on 1000 bootstrap replicates (Kwon et al. 1997, Fessehaie et al. 2002).

PCR-RFLP of the 16S–23S rDNA region

After DNA extractions of isolates, JJ74, JJ145 and JJ147, PCR-RFLP of the amplified 16S–23S rDNA region was performed and reference strains, *Pbc*, *Pba*, *Dickeya dadantii* and *Pbcb* strain 8 were used for amplification. The amplification of DNA from the intergenic spacer (IGS) between the 3′ end of the 16 S and the 5′ end of the 23 S rDNA genes was performed with primers 1491f (5′-GAAGTCGTAACAAGGTA-3′) and L1r (5′-CA(A/G)GGCATCCACCGT-3′) (Integrated DNA Technologies, Whitehead Scientific) (Fessehaie et al. 2002; Duarte et al. 2004). Each 25 µl pre-amplification reaction contained 1 × NH_4 reaction buffer, 2 mM $MgCl_2$ solution, 100 µM dNTP mix, 50 pmol of each primer and 1.25 U BIOTAQ™ (Whitehead Scientific (Pty) Ltd.). PCR amplification was carried out using the following thermal profile: 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 90 s and a final extension step of 72°C for 7 min. A negative water control was included to monitor contamination. Amplified products were electrophoresed through a 2% agarose gel in 1 × TBE buffer at 75 V. Subsequently, the PCR amplified products were digested with *RsaI*, *TaaI* (*Tsp4CI*) and *Hin6I* (*HhaI**) (Fermentas Life Science, Inqaba). The restriction digestion was performed at 37°C for 3 h and left overnight at room temperature in a 30 µl final volume, according to the manufacturer's recommendations. DNA fragments were separated by gel electrophoresis on 3% agarose gels in the presence of ethidium bromide at 75 V for 5 h in 1 × TBE buffer. RFLP patterns were visualised under UV light and compared with reference cultures.

Fig. 4 Black soft rot can develop during storage, under excessive moisture and high temperatures

Table 2 Physiological characteristics of the South African *Pectobacterium carotovorum* subsp. *brasiliensis* and *Pectobacterium carotovorum* isolates

Characteristic	JJ54 *Pbcb*	JJ74 *Pbcb*	JJ145 *Pbcb*	JJ147 *Pbcb*	JJ68 *Pbc*	*Pbcb*[b] (*n*=16)[c]	*Pbc*[b] (*n*=5)
Gram testing (3% KOH)	–[a]	–	–	–	–		
Hugh and Leifson`s oxidation/fermentation test	+	+	+	+	+		
α-Cyclodextrin	–	–	–	–	–	–	–
Dextrin	–	–	–	–	–	–	40[d]
Tween 40	–	–	–	–	–	–	20
Tween 80	–	–	–	–	–	-	60
N-Acetyl-D-Glucosamine	w+	w+	+	w+	+	–	–
L-Arabinose	+	w+	+	w+	+		
D-Arabitol	–	–	–	–	–	–	–
D-Cellobiose	+	w+	+	w+	+	+	+
D-Fructose	w+	w+	+	+	+		
D-Galactose	w+	w+	+	–	+		
Gentiobiose	+	–	+	w+	+	94	100
α-D-Glucose	+	w+	+	+	+		
m-Inositol	+	–	w+	w+	+		
Lactulose	–	–	–	–	+	6	–
Maltose	–	–	–	–	–	19	–
D-Melibiose	+	+	+	+	+	+	+
β-Methyl-D-Glucoside	+	w+	+	w+	+		
D-Psicose	–	–	–	–	+	6	100
D-Raffinose	+	w+	+	w+	+	–	20
L-Rhamnose	+	+	+	w+	+		
D-Sorbitol	–	–	–	–	–	100	100
Sucrose	+	w+	+	+	+	50	80
D-Trehalose	+	–	+	–	+		
Pyruvic Acid Methyl Ester	w+	+	+	+	+		
Acetic Acid	–	–	–	–	–	12	80
Citric Acid	w+	–	+	w+	w–	75	80
Formic Acid	w+	+	+	w+	–	19	80
D-Galactonic Acid Lactone	w+	–	+	w+	–	–	–
D-Galacturonic Acid	+	–	w+	–	+	–	–
D-Gluconic Acid	w+	–	–	–	–	69	20
D-Glucosaminic Acid	–	–	–	–	–	–	–
D-Glucuronic Acid	–	–	–	–	–		
D,L-Lactic Acid	–	–	–	–	w+		
Malonic Acid	–	–	–	–	–	100	100
Succinic Acid	–	–	w+	–	+	–	–
Bromosuccinic Acid	w+	+	+	+	w+	31	100
Succinamic Acid	–	–	–	–	–	38	100
L-Proline	–	–	–	–	–		
Inosine	–	–	w+	–	–		
Uridine	–	–	w+	–	–	–	60
Thymidine	–	–	w+	–	w+		

Table 2 (continued)

Characteristic	JJ54 *Pbcb*	JJ74 *Pbcb*	JJ145 *Pbcb*	JJ147 *Pbcb*	JJ68 *Pbc*	*Pbcb*[b] (*n*=16)[c]	*Pbc*[b] (*n*=5)
Glycerol	w+	w+	+	w+	+		
D,L,α-Glycerol Phosphate	–	w+	+	w+	+		
α-D-Glucose-1-Phosphate	–	–	–	–	–		
D-Glucose-6-Phosphate	+	+	+	w+	+		

[a] –, negative reaction; w–, weak negative; + , positive reaction; w+, weak positive

[b] Compared to *Pbcb* and *Pbc* strains from Duarte et al. (2004)

[c] Number of strains tested

[d] Percentage of strains showing a positive reaction

Results

Disease description

Field symptoms were often observed on only one stem per plant. Initially the upper leaves (sometimes only one side of the upper leaves) began to roll upwards at the margins and wilted, later becoming necrotic (Fig. 1). Most of the leaves showed no sign of chlorosis. However, in a few cases leaves developed marginal chlorosis (Fig. 2). Symptoms progressed to the lower leaves, which subsequently wilted. As the leaves wilted, a light to dark brown discolouration of the vascular system at the stem base developed from the rotting mother tuber, followed by the upward movement of external darkening (Fig. 1). During prevailing wet and humid conditions stems became slimy and pale (Fig. 2).

Inside the stem at the point of infection the rotten pith became necrotic and hollow. Severely infected plants dried out and died (Fig. 3).

In storage, tubers developed sunken, darkened lenticels which either remained latent or developed into tuber soft rot under excessive moisture and humidity. Generally, when seed tubers were suddenly exposed to higher temperatures a black soft rot developed (Fig. 4). Black soft rot of seed tubers resulted in non-emergence, blanking or blackleg development during the early stages of vegetative growth.

Biochemical characterisation

Biochemical tests could not definitively identify the South African blackleg isolate as *Pectobacterium carotovorum* subsp. *brasiliensis*, since differences between the South African *Pbcb* and the Brazilian *Pbcb* were observed in the oxidation of N-acetyl-D-glucosamine and succinamic acid (Table 2). However, numerous atypical isolates and strains are commonly detected, which do not show typical biochemical reactions (Yap et al. 2004).

Pathogenicity

Typical blackleg symptoms on potato stems appeared 3–4 days after stem inoculation. Even though JJ145 and JJ147 were more virulent than JJ74, the same symptoms were observed on all inoculated plants. Initial symptoms

Fig. 5 Blackleg symptoms in potato cv. Mondial after inoculation with JJ145

appeared at the inoculation site on the stem base as external darkening, extending up and downwards on the stem (Fig. 5). Symptoms such as wilting of the leaves, chlorosis and desiccation as well as the hollowing of the stems followed (Fig. 5). JJ74, JJ145 and JJ147 strains consistently developed typical soft rot symptoms in all three inoculation points on potato tubers and green peppers within 24 to 48 h after inoculation.

Although JJ145 and JJ147 were more virulent than JJ74, after 24 h all the green peppers were completely rotten and all the potato tubers showed symptoms. The control potato stems, tubers and green peppers treated with sterile NB did not develop any symptoms.

Partial 16S–23S rRNA sequencing

The phylogenetic tree constructed using partial 16 S–23 S rDNA sequences placed the South African strains in the same cluster as *Pbcb* strains (Fig. 6). These results confirmed that the South African blackleg strains were different from *Pba* as well as *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium carotovorum* subsp. *betavasculorum* (*Pbcv*) (Duarte et al. 2004).

PCR-RFLP of the 16S–23S rDNA region

After PCR amplification of the IGS region, two fragments were generated, which allowed differentiation of *Pectobacterium* spp. and *Dickeya* spp. The *Pectobacterium* spp. including subsp. *brasiliensis* and subsp. *betavasculorum* yielded the expected amplified bands of small (~440 bp) and large (~490) IGS regions, respectively. In comparison the *Dickeya* spp. yielded two different DNA fragments of small (~354 bp) and large (~486 bp) IGS regions (Fessehaie et al. 2002).

Restriction enzymes *RsaI*, *TaaI* (*Tsp4CI*) and *Hin6I* (*HhaI**) used individually, produced different restriction patterns that allowed identification and differentiation between the reference cultures *Pbc*, *Pba*, *Dd* and *Pbcb*. When digested with all three enzymes the representative South African blackleg strains (JJ74, J145 and JJ147) produced characteristic banding patterns that were identical to the patterns of *Pbcb* strain 8 (Figs. 7, 8 and 9). Based on the banding patterns generated, the South African blackleg strains were thus positively identified as *Pbcb*.

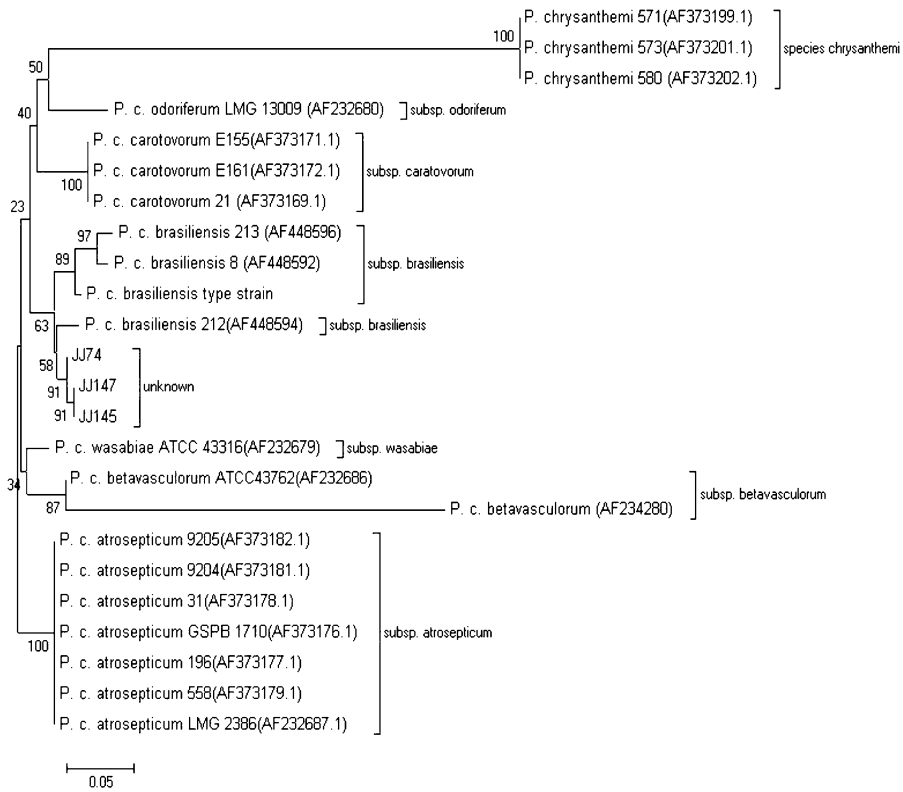

Fig. 6 Phylogenetic tree based on partial 16 S–23 S IGS gene sequences showing the phylogenetic relationships among South African strains and different *Pectobacterium* spp. The phylogram was produced by the neighbour-joining programme (Tamura et al. 2007). The numbers on the branches indicate bootstrap value support based on neighbour-joining analyses of 1000 bootstrap replications. Accession numbers of reference strains in GenBank are in parenthesis

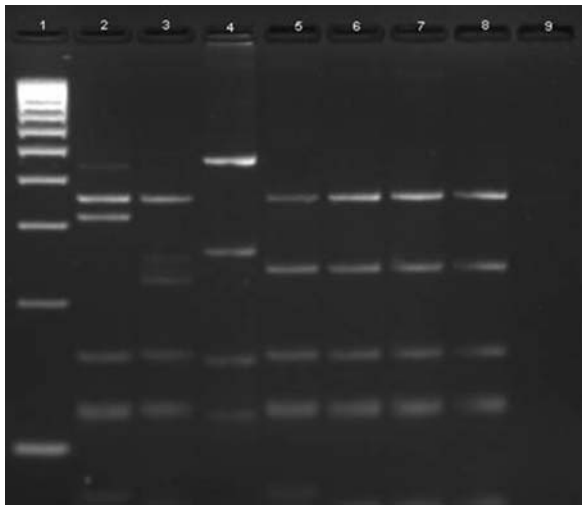

Fig. 7 Agarose gel electrophoresis of the PCR-RFLP of the IGS region digested with *RsaI* to differentiate between subspecies *Pectobacterium* spp. and *Dickeya dadantii*. Lanes: 1, 100 bp DNA ladder; 2, *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T); 3, *Pectobacterium carotovorum* subsp. *atrosepticum* (LMG 2386^T); 4, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 5, *Pectobacterium carotovorum* subsp. *brasiliensis* strain 8 (ATCC BAA-417); 6, JJ74; 7, JJ145; 8, JJ147; 9, Negative control (water)

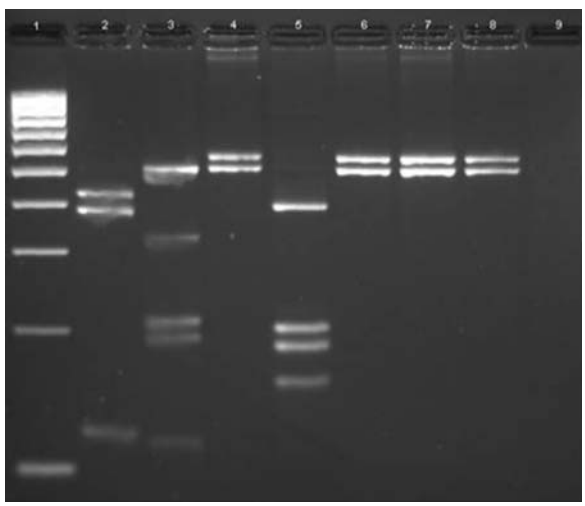

Fig. 8 Agarose gel electrophoresis of the PCR-RFLP of the IGS region digested with *TaaI* (*Tsp4CI*) to differentiate between subspecies *Pectobacterium* spp. and *Dickeya dadantii*. Lanes: 1, 100 bp DNA ladder; 2, *Pectobacterium carotovorum* subsp. *carotovorum* (LMG 2404^T); 3, *Pectobacterium carotovorum* subsp. *atrosepticum* (LMG 2386^T); 4, *Pectobacterium carotovorum* subsp. *brasiliensis* strain 8 (ATCC BAA-417); 5, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 6, JJ74; 7, JJ145; 8, JJ147; 9, Negative control (water)

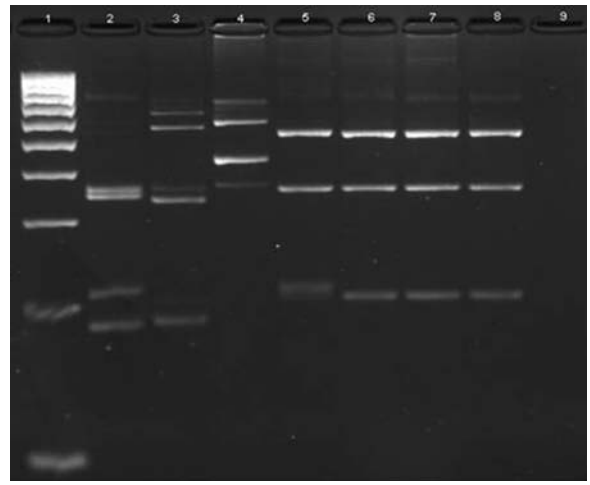

Fig. 9 Agarose gel electrophoresis of the PCR-RFLP of the IGS region digested with *Hin6I* (*HhaI**) to differentiate between subspecies *Pectobacterium* spp. and *Dickeya dadantii*. Lanes: 1, 100 bp DNA ladder; 2, *carotovorum* subsp. *carotovorum* (LMG 2404^T); 3, *carotovorum* subsp. *atrosepticum* (LMG 2386^T); 4, *Dickeya dadantii* (*Erwinia chrysanthemi* 3937); 5, *Pectobacterium carotovorum* subsp. *brasiliensis* strain 8 (ATCC BAA-417); 6, JJ74; 7; 8, JJ147; 9, Negative control (water)

Discussion

Biochemical tests, partial 16 S–23 S rDNA sequence analysis and PCR-RFLP analysis proved that these isolates are closely related to *Pbcb* strains isolated in Brazil. The phylogenetic tree constructed using partial 16 S–23 S rDNA sequences clearly showed that the South African strains are grouped in the same cluster as *Pbcb* strains. These results confirmed that the South African blackleg strains were different from *Pba, Pbc* and *Pbcv* (Duarte et al. 2004). To clearly differentiate between the *Pectobacterium* spp. further identification was necessary which focused on the analysis of PCR-RFLP banding patterns (Toth et al. 2001). The unique banding patterns of the three South African blackleg strains were compared to those of the reference isolates. The three isolates generated the same characteristic banding patterns as *Pbcb* strain 8 isolated in Brazil (Duarte et al. 2004). This is, therefore, the first report of *Pectobacterium carotovorum* subsp. *brasiliensis* causing typical blackleg symptoms in South Africa. *Pectobacterium carotovorum* subsp. *brasiliensis* is the most important causal agent of blackleg in South Africa, since 77% of the 142 isolates were shown to be *Pbcb*, and it is widely

spread throughout the potato production regions. Further research is necessary since it would appear if the etiology of blackleg locally differs from blackleg in Canada, USA and Europe.

It is interesting to note that the symptoms described by Serfontein et al. (1991) are similar to the symptoms recently observed in this study. However, the causal agents identified are different. This could be the result of misidentification of the causal blackleg bacterium in the early stages of potato pathology in South Africa or due to climatic changes, leading to selection for *Pbcb*. *Pectobacterium carotovorum* subsp. *brasiliensis* is virulent in both cool and warm temperatures and high disease incidence occurs when cool and warm temperatures alternate. Ma et al. (2007) also stated that the *Pbcb* clade is most likely widely distributed and has also been found in USA and Israel.

The bacterium could have entered the country through import of contaminated seed tubers a few years ago and subsequently spread throughout the country (Czajkowski et al. 2009, Tsror et al. 2009). Another hypothesis is that the recent increase in plantings (Potatoes South Africa 2008) of a highly blackleg susceptible potato cultivar from the Netherlands, Mondial, has led to the proliferation and spread of *Pbcb* in the South African potato industry. Tsror et al. (2009) stated that imported seed tubers (cvs Nicola and Mondial) from the Netherlands into Israel were the cause of blackleg-diseased plants. However the causal organisms were identified as *Dickeya* spp.

Pectobacterium carotovorum subsp. *brasiliensis* is an extremely aggressive pathogen, causing severe wilting in the field, which results in major economical losses. Latent infection of seed tubers may result in significant disease levels in the following growing season and therefore increase the economic risk of repetitive potato production especially when retaining tubers for the subsequent seasons. These findings are highly significant for the South African potato industry, in particular the fact that South African climatic conditions in many growing areas are favourable for *Pbcb*. The presence of this new species adds to the complexity of the soft-rot / blackleg disease complex on potatoes in South Africa and thus warrants further research to develop strategies to eliminate and manage these pathogens effectively.

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