CHARACTERISATION OF *PECTOBACTERIUM WASABIAE* CAUSING BLACKLEG AND SOFT ROT DISEASES IN SOUTH AFRICA

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Abstract

Pectolytic bacteria were isolated from potato tubers and stems showing tuber soft rot and blackleg symptoms. Approximately half (52%) of the isolates could grow at both 27 and 37 °C while another half (48%) failed to grow at 37 °C. All isolates could be amplified with primers specific to the pectate lyase (*pel*) gene. Carbon utilisation profiles could not conclusively identify these isolates. PCR amplification using primers specific for *Pectobacterium carotovorum* subsp. *brasiliensis* was positive for all isolates that grew at 37°C. However, the group that did not grow at 37 °C failed to amplify with *P. atrosepticum* specific primers. To characterise this group of isolates, the intergenic transcribed spacer region (ITS) was amplified and PCR products digested with two restriction enzymes (RsaI and CfoI) to generate ITS-PCR-RFLP profiles. The profiles of these new isolates were compared to those of the type strains of other pectolytic bacteria. Profiles of five of the selected atypical strains generated with the enzyme CfoI appeared to be most similar to those of *P. wasabiae* type strain. Phylogenetic analysis using concatenated partial gene sequences of housekeeping genes *mdh* and *gapA* clustered these isolates together with those of *P. wasabiae* reference strains thus confirming their identity. These strains were virulent on potato tubers and stems but did not elicit hypersensitive response on tobacco plants. This is the first report of *P. wasabiae* causing soft rot and blackleg of potatoes in South Africa.

Key words: T3SS; soft rot *Erwinia*; Emerging soft rot *Enterobacteriaceae*

Introduction

Pectobacterium and *Dickeya* species are members of the family *Enterobacteriaceae.* Because of their pectolytic nature, they are generally known as soft rot *Erwinia* or more recently *Enterobacteriaceae* (SRE) (Charkowski *et al*., 2012). The SRE have undergone several taxonomic, classification and nomenclature changes and as a result, the former *Erwinia carotovora* and *E*. *chyrsanthemi* species were subdivided into the genus *Pectobacterium* and *Dickeya* respectively based on biochemical, molecular and host range differences (Hauben *et al*., 1998; Gardan *et al*., 2003; Samson *et al*., 2005).

The genus *Pectobacterium* has been divided into four species and these are *Pectobacterium atrosepticum* (*Pa*), *P. wasabiae* (*Pw*), *P*. *betavasculorum* (*Pb*) and *P*. *carotovorum* (*Pc*) which has been further divided into three subspecies; *P*. *carotovorum* subsp. *carotovorum* (*Pcc*), *P*. *carotovorum* subsp. *oderiferum* (*Pco*) and *P*. *carotovorum* subsp. *brasiliensis* (*Pcb*). In general, *Pectobacterium spp*. are broad host range pathogens infecting crop and ornamental plants including both monocots and dicots spanning over 35% angiosperms plant species (Ma *et al*., 2007). However, *P*. *betavasculorum* and *P*. *atrosepticum* are an exception as they appear to have a much narrower host range almost exclusively restricted to sugar beet and potato, respectively.

Geographically, *Pectobacterium carotovorum* subspecies *carotovorum* and *P. atrosepticum* are important pathogens of potatoes in temperate regions causing tuber soft rot and blackleg diseases. *Pectobacterium carotovorum* subsp. *carotovorum* has been previously isolated from potatoes in South Africa but to date no *P. atrosepticum* has been identified (Serfontein *et al*., 1991). *Pectobacterium carotovorum* subsp. *brasiliensis* was first reported in Brazil in 2004 and later shown to occur in other regions including Israel and USA (Duarte *et al*., 2004; Ma *et al*., 2007). A recent survey of pectolytic bacteria infecting potatoes in

South Africa also identified presence of *Pectobacterium carotovorum* subsp. *brasiliensis* (van der Merwe *et al*., 2010). *Pectobacterium wasabiae*, originally isolated from horse radish, has been shown to cause diseases in potatoes and ornamental plants in the USA, New Zealand and Iran (Goto and Matsumoto, 1987; Kim *et al*., 2009; Pitman *et al*., 2008; 2010; Baghaee-Ravari *et al*., 2011). However, there has been no previous report of *P. wasabiae* in South Africa.

Pectobacterium species that have been shown to occur in potatoes are *P. atrosepticum*, *P. wasabiae*, *P. carotovorum* subspp. *carotovorum* and *brasiliensis* causing blackleg, tuber soft rot, stem wilt and stem rot symptoms (Kim *et al*., 2009; Pitman *et al*., 2010; van der Merwe *et al*., 2010; Baghaee-Ravari *et al*., 2011). Because there is an overlap in symptoms caused by the SRE, it is nearly impossible to identify the causal agent by looking at symptoms. Hence biochemical and genetic methods of identification are required in order to accurately identify the relevant causal agent. The SRE are typically seed potato borne pathogens and transmission is mainly through the movement of latently infected seed tubers (Pérombelon and Kelman 1980; Tsror *et al*., 2009). This movement often leads to the spread of pathogens occurring over long distances as well as across national borders (Toth *et al*., 2011). As a result, new atypical and highly pathogenic *Pectobacterium spp*. strains have been identified and these are associated with new potato disease outbreaks in different countries (Pitman *et al*., 2008; van der Merwe *et al*., 2010; Baghaee-Baghari *et al*., 2011). For this reason, it is important that new potentially invasive species are identified and characterised to minimise the impact of the SRE on potato productivity worldwide.

In this study, new, highly invasive, atypical strains of *Pectobacterium* isolated from potato fields causing potato soft rot and blackleg were identified as *P. wasabiae*. This is the first report of *P. wasabiae* occurring in potatoes in South Africa.

Materials and Methods

Bacteria strains

During the 2010/2011 potato growing seasons, samples of plants showing blackleg and potato tubers showing soft rot symptoms as well as those without symptoms were obtained from the Free State potato growing region of South Africa. Bacterial strains were isolated from tubers and plants with or without symptoms. Type strains were obtained from the International Collection of Belgium and that of the University of Pretoria. The type strains were *P. carotovorum* subsp. *carotovorum* (LMG 2404^T), *P. atrosepticum* (LMG 2386^T), *P. carotovorum* subsp. *brasiliensis* 371 (LMG 21373), *P. carotovorum.* subsp. *brasiliensis* 212 (LMG 21371), *P. betavasculorum* (LMG 2466^T), *P. wasabiae* (LMG 8444 ^{T}) and *Dickeya spp*. (LMG 2804^T).

Media and culture conditions

Bacteria were isolated from samples showing blackleg and soft rot symptoms by blending plant tissue in dH_2O using a blender. Crushed infected material was homogenised and serial dilutions were made with 10 mM $MgSO_4$. Serial dilutions were plated on Crystal Violet Pectate (CVP) (Hyman *et al*., 2001) and plates were routinely incubated at 27 and 37 °C for 24 – 48 h. Single pit forming colonies were transferred to nutrient agar in order to obtain pure colonies for further characterisation. A total of 92 isolates were obtained. All isolates were stored at -80 °C in 10% glycerol.

Phenotypic and Biochemical characterisation of isolates

The carbon utilisation profiles of isolated pectolytic bacterial strains were determined using Biolog GN microplates (Biolog, Haywood, CA, USA). Single colonies of each isolate were transferred from nutrient agar and streaked onto tryptone soy agar (TSA) at 27 °C for 48 h. Colonies were then scraped off TSA plates and suspended in sterile saline as described by Toth *et al*. (1999) and the OD adjusted to 0.3. Thereafter, 150 µl bacterial suspension of each isolate was dispensed into each of the 96 well-microplate and incubated at 27 °C for 16 h. Readings were taken at OD (595nm) with a Multiscan GO (Thermoscientific) microtitre plate reader.

Molecular identification

DNA was extracted using standard DNA extraction methods (Sambrook and Russell, 2001). All the primers used in this study were obtained from Inqaba Biotechnologies (South Africa) and are listed in Table 1. All cavity-forming isolates were PCR-amplified with primers Y1 and Y2 specific for the *pel* gene. Specific PCR assays were carried out to differentiate *Pcb* and *Pa* isolates using primers Eca1f and Eca2r and Br1f and L1r, respectively (Table 1). All strains that were unable to grow at 37 °C were PCR amplified with *P. atrosepticum* specific primers while all strains that were able to grow at 37 °C were amplified with *P. carotovorum* subsp. *brasiliensis* specific primers (Duarte *et al.* 2004). The PCR reaction was performed in a total volume of 25 µl consisting of 10x DreamTaq buffer (supplemented with 20 mM $MgCl_2$), 2.5 mM dNTPs, 10 µM each forward and reverse primer (Br1f and L1r), 0.5 U DreamTaq Polymerase (Fermentas) and 100 ng DNA template. PCR amplification was conducted using a Biometra Thermocycler (Germany) with the following thermal regime; initial denaturing for 2 min at 95 °C, 30 cycles of denaturing at 94 °C for 30 sec, followed by

annealing at 62 °C for 45 sec, and elongation at 72 °C for 90 sec followed by a final extension step at 72 °C for 7 min. Amplification products were stained with GelRed (Biotium, Hayward, California, USA) and separated by electrophoresis on 1 % (w/v) agarose gel (Lonza, USA).

The 16S-23S intergenic transcribed spacer (ITS) region was PCR amplified using primers L1 and G1 according to Toth *et al*., 2001. Each reaction was made to a final volume of 50 µl using 50 ng DNA template, 2U Taq Polymerase (Fermentas), 5.0µl of 10x Taq DNA polymerase reaction buffer (Fermentas), 200 µM dNTPs and 0.4 µM each forward and reverse primer. The amplification conditions were as follows: denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2 min. PCR products were electrophesed on a 2 % (w/v) agarose gel.

Sequencing and sequence analysis

Primers designed to anneal to conserved regions of the *malate dehydrogenase* (*mdh*) and *glyceraldehyde-3- phosphate dehydrogenase A* (*gapA*) genes were used for PCR amplification of representative strains. The PCR reactions were performed in a total volume of 50 µl using 50 ng DNA template, and 2U Taq Polymerase (Fermentas), 5 µl of 10x Taq DNA polymerase reaction buffer (Fermentas), 200 µM dNTPs and 0.4 µM forward and reverse primers each. The PCR reaction was carried out using Biometra, Analytik Jena thermocycler and reaction conditions were as follows: initial denaturation at 94 °C for 30 sec, 35 cycles of denaturation at 94 °C for 30 sec annealing at 52 °C for 30 sec and extension at 72 °C for 60 sec followed by a terminal extension at 72°C for 7 min. All amplified PCR products were stained with Gelred® and confirmed by gel electrophoresis on a 1% agarose gel.

All PCR amplicons were purified using Wizard® SV Cleanup System (Promega) and sequenced using ABI3500xl at the University of Pretoria. DNA sequences were manually edited using Bioedit v7.1.3 and aligned using ClustalW with a gap opening penalty of 10 and gap extension penalty of 0.2 for multiple alignments. For comparison, reference sequences of *Pectobacterium, Dickeya* and outgroup sequences of *Yersinia* species were obtained from the GenBank database (Ma *et. al*., 2007). Phylogenetic analysis of aligned gene sequences was conducted using MEGAv5.0 (Tamura *et al*., 2011) and PAUP* through maximum likelihood and maximum parsimony methods on individual and combined data sets. A 1000 parsimony bootstrap replicates were used to assess the stability of clusters. Sequences obtained in this study were deposited in GenBank and accession numbers were obtained (JX312158 – JX312169).

Pathogenicity assays

The virulence of South African isolates was analysed alongside that of the type strains on both potato tubers and stems of potato (*Solanum tuberosum*) cultivar Mondial. Potato tubers were surface sterilised in 10 % (v/v) sodium hypochlorite and Tween 20 for 20 min. Each tuber was then inoculated with 10 µl (1 x 10^8 cfu.ml^{-1}) overnight culture of each strain grown on Luria Bertani broth for 16 h at 27 °C for *P. wasabiae* and *P. atrosepticum* strains and 37 °C for *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliensis* strains. Using a sterile pipette tip, holes of a uniform depths were stabbed into potato tubers and 10 µl inoculum injected. The inoculum was sealed with Vaseline (Petroleum Jelly) and inoculated tubers were incubated at 25 °C. After 72 h, rotting tissue was scraped from the tubers and weighed to determine the extent of tissue maceration. This experiment was performed three

independent times using a total of seven tubers per trial. Statistical significance was analysed by T-Test or ANOVA (JMP v5).

To evaluate stem rot and blackleg, five week old potato plants *Solanum tuberosum* (cv Mondial) were inoculated with 10 µl (1 x 10^8 cfu.ml^{-1}) of overnight bacterial cultures (using the same strains used for tuber soft rot assays) suspended in 10 mM $MgSO_4$. Plants were inoculated by piercing the stem with a micropipette approximately 10 cm above the soil level. Inoculation sites were immediately wrapped with parafilm to prevent desiccation. Plants were incubated in a glasshouse at 25 °C at high humidity and blackleg lesions were evaluated daily for 21 days post inoculation (dpi). Three plants each consisting of 2-3 stems were inoculated per strain.

Hypersensitive response

Hypersensitive response of the type strains of *P. carotovorum* subsp. *brasiliensis* (positive control), *P. atrosepticum* (negative control), *P. wasabiae* and selected South African strains was evaluated on tobacco leaves (*Nicotiana benthamiana* and *N. tabaccum* [Xanthi]). A negative control of 10 mM $MgSO_4$ was included for each experiment. Overnight cultures (1 x 10^8 cfu.ml^{-1}) were washed twice and re-suspended in 10 mM $MgSO_4$. Tobacco leaves were then infiltrated with bacterial suspension and inoculated plants were maintained at room temperature for 24 – 48 h. Hypersensitive response was recorded at 48 hours post inoculation (hpi). Three leaves of two plants each *N. tabaccum* and *N. benthamiana* were infiltrated and two independent biological replicates were performed.

RESULTS

Identification of pectolytic bacteria

Potato stems and tubers with or without typical symptoms of blackleg and tuber soft rot were obtained from the Free State potato growing region of South Africa. Isolation of pectolytic bacterial strains was carried out on CVP and in total, 92 isolates of cavity forming bacteria were obtained. Of the samples tested, pectolytic bacteria were isolated from 8 sample batches consisting of either rotting seed potato tubers from generations 4 – 6 (G4 – G6) of production or stems with blackleg symptoms. On average, approximately 12 pectolytic bacteria were isolated per sample. All isolates were further identified as pectolytic bacteria by PCR amplification of the *pel* gene using Y1 and Y2 primers. The expected amplicon of 434 bp was obtained for all isolates. Nearly half (52%) of the isolates were able to grow at both 27 °C and 37 °C while another half (48%) were not able to grow at 37 °C. The type strains of *P. atrosepticum* and *P. wasabiae* were also not able to grow at this elevated temperature. Carbon utilisation profiles of these isolates could not be used to conclusively differentiate them from those of *P. carotovorum* subsp. *carotovorum* (results not shown). Hence, further characterisation of these isolates using other techniques was deemed necessary.

Molecular typing of *Pectobacterium spp*. isolates

The newly isolated *Pectobacterium spp*. were further characterised using species and subspecies specific primers (Eca1f and Eca2r and Br1 and L1R) for *P. atrosepticum* and *P. carotovorum* subsp. *brasiliensis* respectively. The expected amplicon of 322 bp was obtained from the type strains of *P. carotovorum* subsp. *brasiliensis* as well as all 48 isolates that were able to grow at both 27 °C and 37 °C. Thus this group of isolates could positively be identified as *P. carotovorum* subsp. *brasiliensis* using species specific primers. To characterise the remaining group of isolates, these were subjected to PCR amplification using

P. atrosepticum specific primers. None of these strains could amplify the expected amplicon of 690 bp which was observed only for the type strain of *P. atrosepticum* (results not shown). Given that carbon profiles of these isolates were similar to those of *P. carotovorum* subsp. *carotovorum* but they could not grow at 37 ºC and failed to amplify with *P. atrosepticum* specific primers, these isolates were labelled as atypical. Five of these atypical isolates, G63 and G67 representing isolates from stem tissue and GT6, GT13 and GT9 representing isolates from infected seed tubers were selected for further characterisation.

The ITS regions of the five selected atypical strains from South Africa including the type strains for *P. carotovorum carotovorum*, *P. betavasculorum*, *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis, P. wasabiae* and *D. dadantii* were PCR-amplified using general primers L1 and G1. The ITS-PCR profiles of the type strains of *P. atrosepticum* and *D. dadantii* were clearly different from all other strains (results not included). However, there was no clear difference between ITS-PCR profiles of atypical South African strains and those of *P. carotovorum* subsp. *carotovorum, P. betavasculorum* and *P. carotovorum* subsp. *brasiliensis* type strains. To enable us to distinguish atypical South African strains from the type strains of *P. carotovorum* subsp. *carotovorum, P. betavasculorum* and *P. carotovorum* subsp. *brasiliensis* strains, ITS-PCR products were digested with two different restriction digest enzymes (CfoI or RsaI) and the resulting PCR-RFLP profiles were compared. The PCR-RFLP banding profiles generated with RsaI could clearly differentiate between the five selected atypical strains (Figure 1A lanes 1 – 5) and the type strains of *D. dadantii, P. atrosepticum, P. betavasculorum, P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliences* strain 357. However, the profiles of the atypical strains generated using RsaI could not be used to conclusively distinguish between our strains and those of *P. wasabiae* type strain and *P. carotovorum* subsp. *brasiliensis* strain 358 (Figure 1A). Thus, to further identify these isolates, ITS-PCR-RFLP profiles were generated using a second

restriction digest enzyme, CfoI. Using this enzyme, the five atypical strains could clearly be distinguished from other *Pectobacteriums spp*. type strains (Figure 1B 1-2 and 4-6) and were identified as being most similar to those of *P. wasabiae* type strain (Figure 1B).

Phylogenetic analysis of atypical South African strains

The use of partial housekeeping genes such as the *malate dehydrogenase* (*mdh*) and *gluteraldehyde-3-phosphate A* (*gapA*) genes has been widely used for phylogenetic characterisation of the SRE (Ma *et al*. 2007; Pitman *et al*., 2010). Thus, these genes were amplified from representative South African strains identified as *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae* together with representative type strains, sequenced and aligned using clustalW. Maximum likelihood trees were constructed using individual genes and concatenated sequences of the two genes, *mdh* and *gapA* (Figure 2). In the concatenated phylogentic tree in Figure 2, all of the isolates that were identified by species specific primers as *P. carotovorum* subsp. *brasiliensis* clustered closely with those of *P. carotovorum* subsp. *brasiliensis* isolated elsewhere in clade I. The clade was supported with a high bootstrap value of 97%. On the other hand, all the South African atypical strains identified as *P. wasabiae* using ITS-PCR-RFLP clustered closely together with those of *P. wasabiae* type and reference strains in clade IV. The bootstrap support value of this clade was 94% indicating high levels of confidence for the clade. The phylogenetic tree constructed provided evidence supporting identification of these isolates as *P. wasabiae.* This further substantiated PCR-RFLP identification of these isolates as *P. wasabiae*. This is the first report of *P. wasabiae* in potatoes in South Africa. Previous reports of *P. wasabiae* strains infecting potatoes were in the USA, New Zealand and Iran (Kim *et al*., 2009; Pitman *et al*., 2008; Baghaee-Ravari *et al*., 2011).

Virulence and hypersensitive response

The relative virulence of South African strains identified as *P. wasabiae* was compared to those of the type strains of *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *P. wasabiae* and *P. carotovorum* subsp. *brasiliensis* by inoculation on potato tubers and stems (*Solanum tuberosum* cv Mondial). Tuber maceration was quantified by scooping and weighing rotting tissue. As expected, there was no rotting observed where potato tubers were inoculated with the negative control, $MgSO_4$. The highest average tissue maceration was observed for *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliensis* type strains including one South African *P. wasabiae* strain SA G620 (Figure 3). The other five South African *P. wasabiae* strains showed variable maceration ability which was significantly higher than the type strains of *P. wasabiae* and *P. atrosepticum*. In stem assays, mock inoculated stems remained without symptoms for the entire duration of the experiment (Figure 4A). Typical blackleg symptoms appeared on inoculated stems within 3 dpi in the form of black lesions extending upwards and downwards from the point of inoculation (Figure 4B and C). In some cases, severe wilting and complete collapse of the plant was observed (Figure 4C).

The type III secretion system (T3SS) is an important pathogenicity factor of many Gram negative bacteria. Effector proteins of the T3SS when injected into non host plants elicit a hypersensitive response. Atypical *P. wasabiae* and *Pseudomonas syringae* strains lacking a functional T3SS or with a highly evolved T3SS have been isolated previously (Kim *et al*., 2009; Clarke *et al*., 2010; Pitman *et al*., 2010). Typically, such strains lack the ability to elicit HR when inoculated into tobacco plants. Thus, the ability of five *P. wasabiae* strains isolated from South Africa to elicit hypersensitive response on tobacco plants was

investigated. Six weeks old *N. tabaccum* (cv Xanthi) and *N. benthamiana* plants were infiltrated with 1 x 10^8 cfu.ml^{-1} cells of the type strains of *P. wasabiae* and *P. carotovorum* subsp. *brasiliensis* (used as a positive control), *P. atrosepticum* and $MgSO_4$ (included as negative controls) together with the South African *P. wasabiae* strains G63, G67, GT6, GT13 and GT19. At 24 hpi, typical tissue collapse indicative of HR, could be detected in areas infiltrated with *P. carotovorum* subsp. *brasiliensis* but as expected, no signs of tissue collapse were visible in areas infiltrated with $MgSO_4$ or *P. atrosepticum* (Figure 5). Furthermore, no HR was observed in areas infiltrated with *P. wasabiae* type strain nor any of the South African *P. wasabiae* strains.

DISCUSSION

In this study, potato tubers and stems showing symptoms of blackleg and potato soft rot were collected and the causal agent identified. Using a combination of physiological, biochemical and molecular diagnostic techniques, we were able to show that 52% of the strains were *Pectobacterium carotovorum* subsp. *brasiliensis* which has previously been reported as the main cause of potato blackleg and tuber soft in potato fields in South Africa (van der Merwe *et al*., 2010). However, we identified a second group of isolates whose identity could not be resolved by carbon utilisation profiles generated using biolog assays. These isolates were initially described as atypical *P. carotorovorum* subsp. *carotovorum* because, unlike typical *P. carotovorum* subsp. *carotovorum* strains, they could not grow at 37 ºC. Several studies have shown that physiological and biochemical methods on their own are often unable to accurately discriminate between SRE species, particularly those closely related to *P. carotovorum* subsp. *carotovorum* (Toth *et al*., 1999; Pitman *et al*., 2008). It has also been shown that although biolog has been used widely for identification of other bacteria, it cannot

clearly distinguish between related members of the *Pectobacterium spp*. (Toth *et al*., 1999). Considering that *P. atrosepticum* isolates like our isolates, cannot grow at the elevated temperature of 37 ºC, PCR amplification of these isolates using species specific primers for *P. atrosepticum* was attempted. However, none of these isolates amplified with these primers. Thus, other molecular diagnostic tools were selected for use to further characterise atypical South African isolates.

Molecular diagnostic tools such as species specific primers, ITS-PCR and PCR-RFLP have been successfully used to resolve identities of the SRE that could not be resolved using biochemical and physiological characteristics (Toth *et al*., 2001). Thus ITS-PCR and PCR-RFLP were used to further resolve the identities of these strains. For this purpose, five of these isolates were selected. The profiles generated using the restriction enzyme RsaI could discriminate between our isolates and those of *Dickeya spp*., *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum* and one *P. carotovorum brasiliensis* strain. However, we could not discriminate between our isolates and another strain of *P. carotovorum* subsp. *brasiliensis* or *P. wasabiae*. The use of CfoI was better able to identify our isolates as *P. wasabiae*. This was further confirmed using maximum likelihood phylogenetic analysis which showed that the atypical South African strains clustered closely to those of *P. wasabiae* strains isolated from other geographic locations. Strains that were positively identified as *P. carotovorum* subsp. *brasiliensis* using species specific primers clustered closely within the *P. carorotovorum* subsp. *brasiliensis* clade. Both *P. wasabiae* and *P. carorotovorum* subsp. *brasiliensis* were often isolated together as a mixed population from a single batch of samples, and from our observations, there was no clear 'preference' for stem or tuber by either strain.

Pectobacterium wasabiae was originally isolated from horse radish. However, there have been several reports of its occurrence in potatoes (Ma *et al*., 2007; Pitman *et al*., 2008; Kim *et al*., 2009; Baghaee-Ravari *et al*., 2011). Interestingly, Ma *et al.* (2007) previously

identified several *P. wasabiae* strains lacking a type III secretion system (T3SS). Pitman *et al* (2010) also isolated *P. wasabiae* strains which elicited a mild form of hypersensitive response in *Nicotiana tabaccum* (cv Xanthi). They showed that these strains also lacked key effector-encoding genes such as *dspE* and *hrpN*. Thus we sought to determine whether the South African strains could elicit HR. None of the South African *P. wasabiae* isolates were able to elicit hypersensitive response in *N. benthamiana* or *N. tabaccum*. The same observation was made for *P. wasabiae* and *P. atrosepticum* type strains. However, the type strain and all South African *P. carotovorum* subsp. *brasiliensis* isolates were able to elicit a form of tissue collapse within 24 hpi in both *N. benthamiana* and *N. tabaccum* (results not included). These results are in keeping with earlier observations by Glasner *et al*. (2008) who previously reported that both *P. carotovorum* subspp. *carotovorum* and *brasiliensis* were able to elicit HR while *P. atrosepticum* (SCRI 1043) could not. Similar to Ma *et al*. (2007), our study indicated a lack of HR elicitation by all five South African *P. wasabiae* isolates that were selected for HR assays, suggesting a possible lack of some key genes of T3SS if not the entire cluster. A PCR screening of at least two genes (*hrpN* and *dspE*) with primers designed based on conserved gene regions could not detect presence of these genes in the South African *P. wasabiae* isolates (results not included). It is important to note that the absence of PCR amplicons cannot be used as conclusive evidence that these isolates lack a T3SS. Further work is required to verify this. Although *P. wasabiae* strains isolated in South Africa did not elicit HR in *Nicotiana* plants, they were highly virulent with one strain causing rot equivalent to that of *P. carotovorum* subp. *carotovorum* and *P. carotovorum* subsp. *brasiliensis*, both of which are often reported as being the more aggressive *Pectobacterium spp*. on potatoes (Marquez-Villavicencio *et al*., 2011). Furthermore, all the selected strains tested were virulent on potato plant stems causing mild to aggressive forms of blackleg and wilting leading in some cases to complete collapse of the plant. Thus, it appears that there was no

correlation between the presence or absence of a T3SS in these strains and the ability to cause disease. These observations are consistent with those of other researchers (Ma *et al*. 2007; Kim *et al*., 2009; Pitman *et al*. 2010; Baghaee-ravari *et al*. 2011).

In conclusion, our study identified *P. wasabiae* strains causing potato blackleg and soft rot symptoms in South African potatoes using a combination of molecular and phylogenetic analyses. These strains, although not able to elicit HR in tobacco plants remained aggressive on potato stems and tubers. Survival and virulence mechanisms used by these T3SS-deficient *Pectobacterium spp*. in comparison to other *Pectobacterium spp*. warrants further research. To our knowledge, this is the first report of *P. wasabiae* in South Africa. Occurrence of this pathogen in other potato growing regions will need to be investigated.

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APPENDICES

Table 1: Primers used in this study

Figure 1 : ITS-PCR-RFLP profiles of *Pectobacterium spp* isolates using A. RsaI and B. CfoI restriction restriction enzyme. Ladder is a standard molecular ladder. The South African *Pectobacterium* strains SA G63; SA G67; SA GT6; SA GT13; SA GT19; were compared to type strains *P. carotovorum* subsp. *brasiliensis* 357 (*Pcb* 357); *P. wasabiae (Pw*^{T}*)*; *P. atrosepticum* (*Pa*^{T}*)*; *P. betavasculorum* (*Pb*^{T}); *P. carotovorum* subsp. *carotovorum* (*Pcc*^{T}*)*, *P. carotovorum* subsp. *brasiliensis* 358 (*Pcb 358*^{T}) and *Dickeya dadantii* (*Dda*^{T}).

Figure 2: A concatenated maximum likelihood phylogenetic analysis of *Pectobacterium spp* strains from South Africa based on *mdh* and *gapA* partial gene sequences. Numbers represent bootstrap support values greater than 50% from 1 000 replicates using heuristic searches. *Yersinia* spp. were used as outgroups.

Figure 3: Relative virulence of South African *P. wasabiae* strains. The relative virulence of South Africa *P. wasabiae* strains was evaluated by inoculating the following strains into potato (*Solanum tuberosum* cv Mondial) tubers: *P. carotovorum brasiliensis* 357; *P. wasabiae* (*Pw*^{T}); *P. atrosepticum* (*Pa*^{T}); *P. carotovorum* subsp. *carotovorum* (*Pcc*^{T}); South African *P. wasabiae* strains SA GT6, 2; SA GT13, SA GT19, SA G63; SA G67; SA G620 and $MgSO_4$ control.

Figure 4: Potato blackleg symptoms on *Solanum tuberosum* (cv Mondial) after stem inoculations with A. $MgSO_4$ control; B. SA GT19 and C. SA G620

Figure 5: Hypersensitive response elicitation in tobacco plants. A. *Nicotiana tabaccum* (cv Xanthi) plants were infiltrated with 1. *P. carotovorum* subsp. *brasiliesis*, 2. *P. wasabiae* and 3. *P. atrosepticum* type strains, 4. $MgSO_4$ and South African. *P. wasabiae* strains 5. SA G63; 6. SA G67; 7. SA GT19; 8. SA G620 B. *Nicotiana benthaminana* plants were infiltrated with 1. *P. carotovorum* subsp. *brasiliesis*, 2. *P. wasabiae* type strains 3. $MgSO_4$, and South African *P. wasabiae* strains 4. SA G63; 5. SA G67 and 6. SA GT19

Table 1: Primers used in this study

Primer	Sequence (5' – 3')
Eca1f	CGGCATCATAAAAACACG
Eca2r	GCACACTTCATCCAGCGA
Y1	TTACCGGACGCGAGCTGTGGCGT
Y2	CAGGAAGATGTCGTTATCGCGAGT
Br1f	GCGTGCCGGGTTTATGACCT
L1r	CARGGCATCCACCGT
L1	CAAGGCATCCACCGT
G1	GAAGTCGTAACAAGG
mdh2	GCGCGTAAGCCGGGTATGGA
mdh4	CGCGGCAGCCTGGCCCATAG
gapA	ATCTTCCTGACCGACGAAACTGC
gapA	ACGTCATCTTCGGTGTAACCCAG

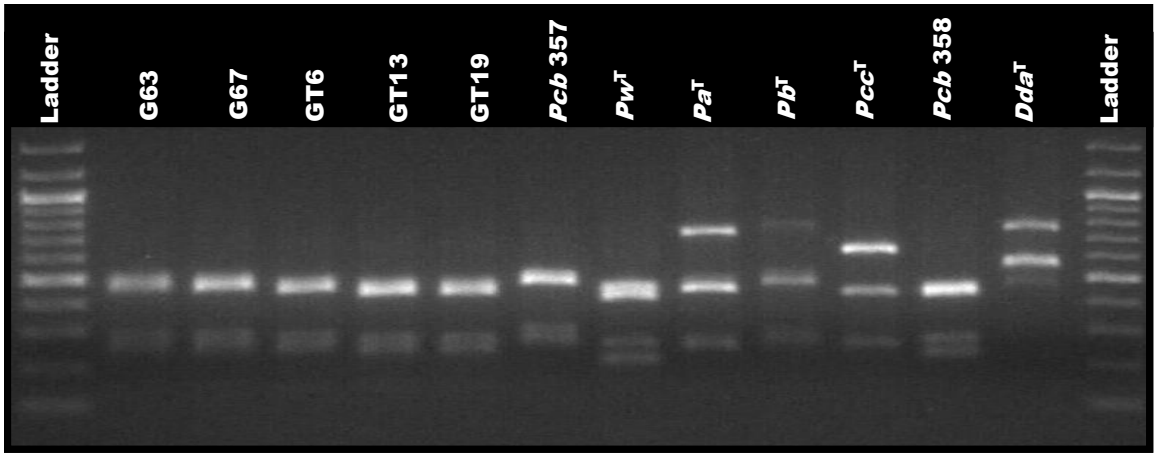


Figure 1A

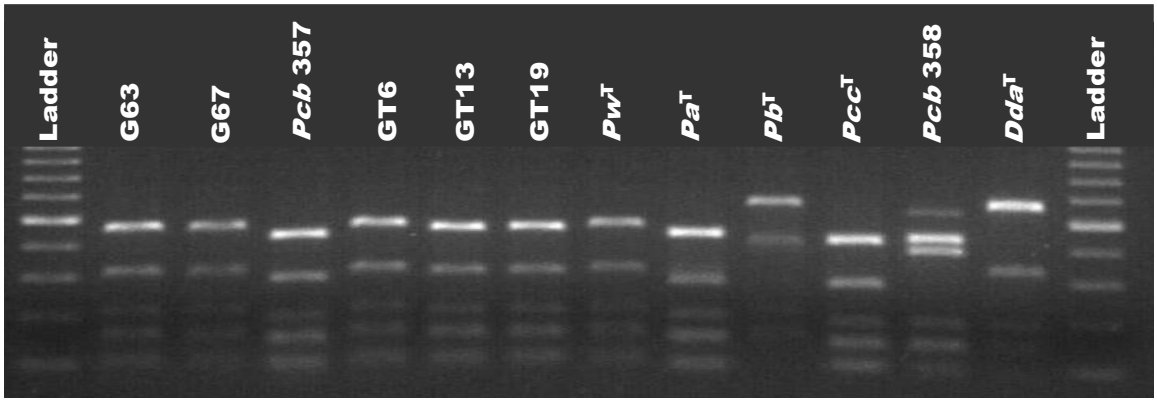
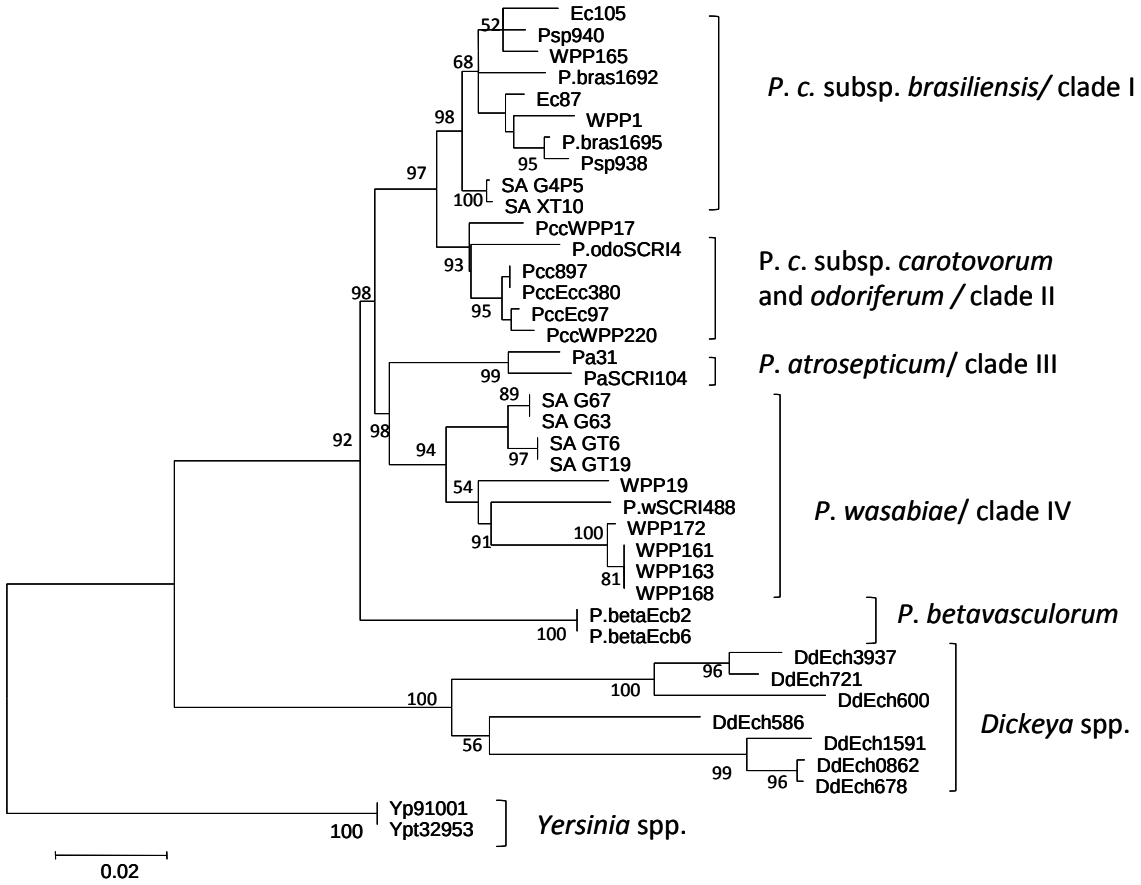


Figure 1B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WPP165
P.bras1692
Ec87
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Psp938
SA G4P5
SA XT10
PccWPP17
P.odoSCRI4
Pcc897
PccEcc380
PccEc97
PccWPP220
Pa31
PaSCRI104
SA G67
SA G63
SA GT6
SA GT19
WPP19
P.wSCRI488
WPP172
WPP161
WPP163
WPP168
P.betaEcb2
P.betaEcb6
DdEch3937
DdEch721
DdEch600
DdEch586
DdEch1591
DdEch0862
DdEch678
Yp91001
Ypt32953
0.02
P. c. subsp. brasiliensis/ clade I
P. c. subsp. carotovorum and odoriferum / clade II
P. atrosepticum/ clade III
P. wasabiae/ clade IV
P. betavasculorum
Dickeya spp.
Yersinia spp.

Figure 2

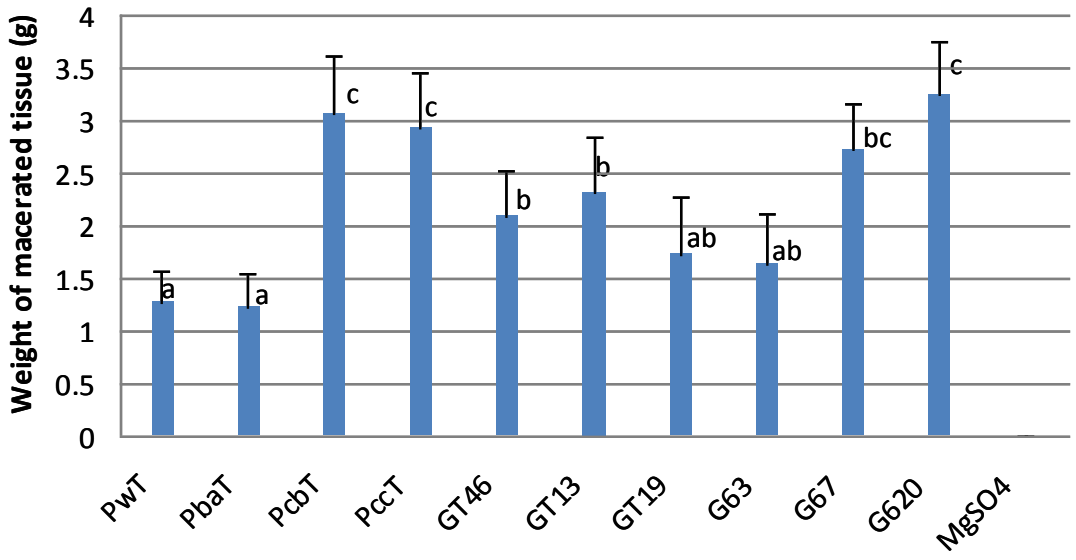
Weight of macerated tissue (g)
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
c
c
b
b
ab
ab
bc
c
PwT
PbaT
PcbT
PccT
GT46
GT13
GT19
G63
G67
G620
MgSO4

Figure 3

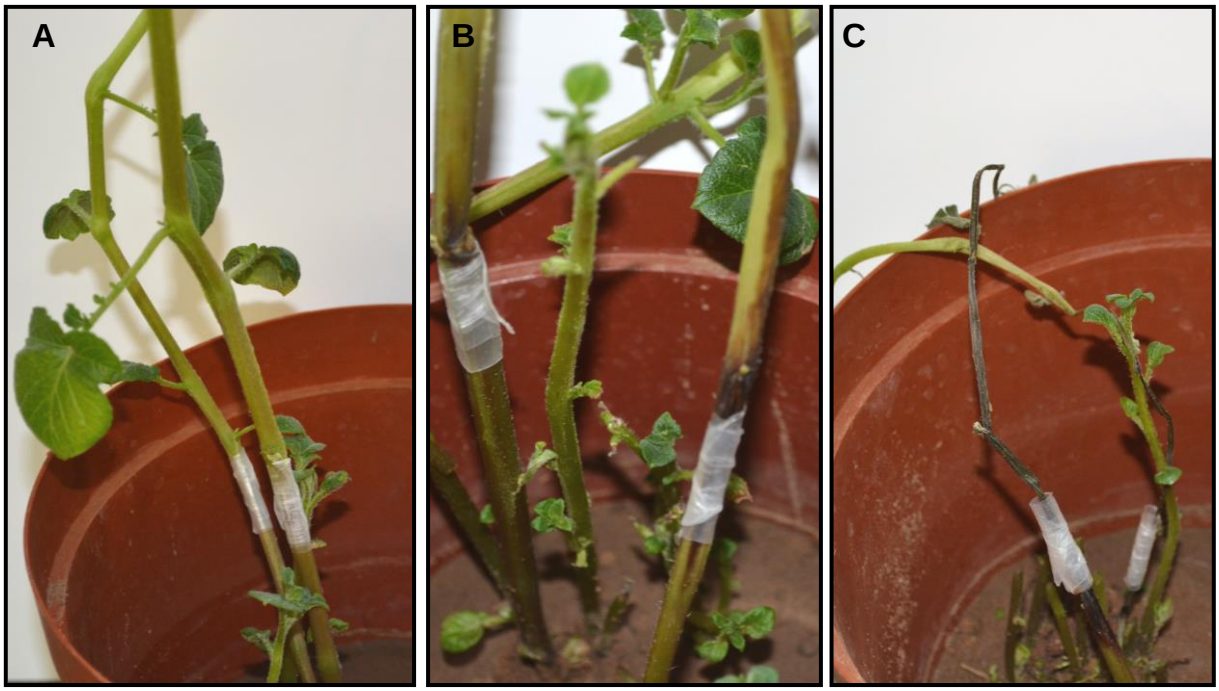

Figure 4

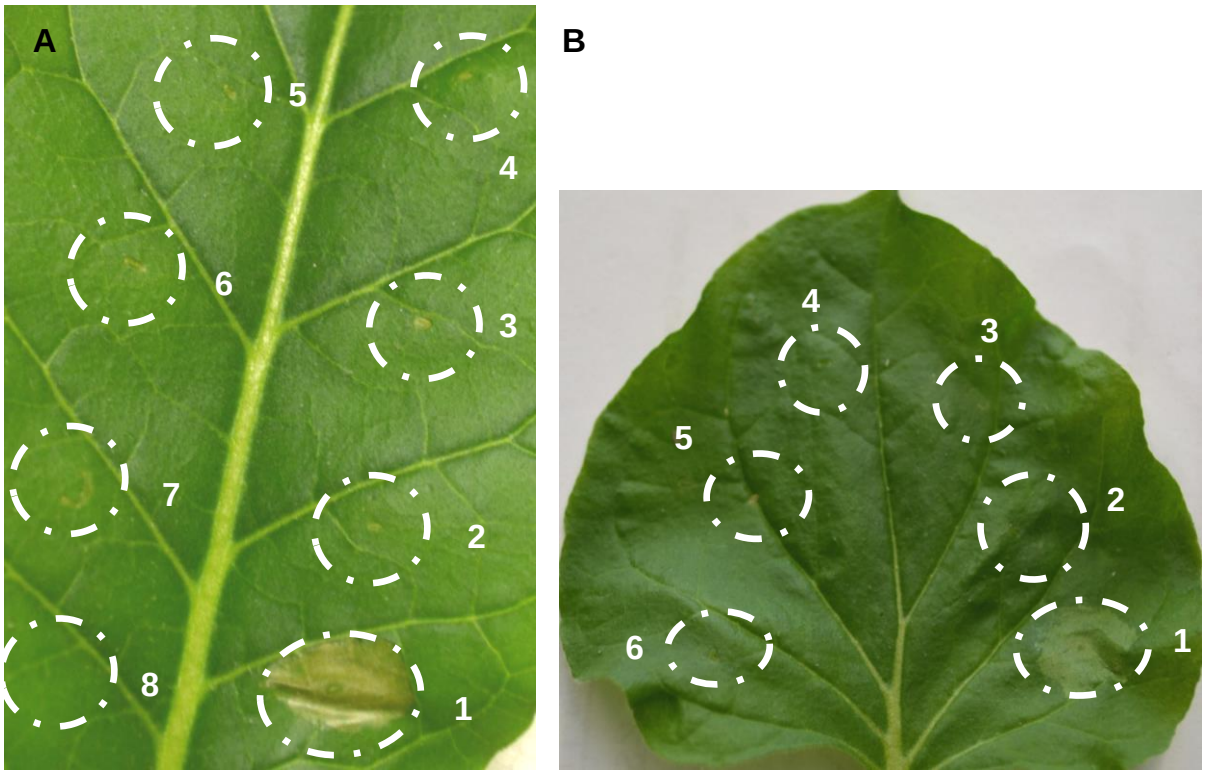

Figure 5