Cardinal Temperature Differences, Determined *in vitro,* Between Closely Related Species and Subspecies Of Pectinolytic Bacteria Responsible For Blackleg And Soft Rot On Potatoes

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Abstract:

Potato blackleg and soft rot cause major losses and are caused by two bacterial genera, *Pectobacterium* and *Dickeya*. Species affecting potatoes are *Pectobacterium atrosepticum* (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pectobacterium carotovorum* subsp. *brasiliense* (Pcb), *Pectobacterium wasabiae* (Pwa), *Dickeya dadantii* (Dda) and *Dickeya solani* (Dso). Pathogenicity of these species is dependent on

temperature, with each species having its own optimal temperature and temperature range for growth, leading to varying degrees of losses. *Pectobacterium atrosepticum*, a temperature sensitive species, mainly occurs in temperate climates, Pcc in temperate to tropical and *Dickeya* spp. in subtropical environments. The aim of this study was to determine the cardinal growth temperatures for the species responsible for blackleg and soft rot *in vitro*. Bacterial isolates were incubated in a temperature gradient shaking incubator at 30 different temperatures ranging from ±5°C to ±56°C, and growth measured at two set time intervals. Results were statistically analysed using the Gaussian function. The optimal temperature of 31°C and temperature range of 20°C to 38°C for *Pectobacterium carotovorum* subsp. *brasiliense*, was similar to those recorded for Pcc. *Pectobacterium wasabiae* grew at an optimal temperature of 29°C and range of 20°C to 34°C. Higher optimal temperatures of 32°C and 34°C, with ranges of 21°C to 38°C and 23°C to 41°C were recorded for Dda and Dso, respectively. The minimal variation in optimal temperatures between different species might be an indication that temperature ranges, rather than optimal temperature, play an important role in disease development. Results for Dso, which has not yet been reported in South Africa, are especially important in light of prevailing temperatures in South African potato production regions.

Keywords:

Pectobacterium; *Dickeya*; Growth; Optimal temperature; Temperature range, Pectinolytic bacteria

Introduction:

Temperature is regarded as one of the most important factors affecting growth and survival of organisms. Each microorganism has its own range of cardinal temperatures at which life persists. Optimal temperatures are those at which growth is at its best; the minimum temperature is that below which no active growth occurs, and maximum temperature designates a temperature above which cell death occurs (Madigan and Martinko 2006). These cardinal temperatures also play an important role in the pathogenicity of members of the Enterobacteriaceae, which are responsible for soft rot and blackleg in potato (*Solanum tuberosum* L.). The pathogenicity of *Pectobacterium atrosepticum* (van Hall 1902) Hauben et al. 1999 (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Jones 1901) Hauben et al. 1999 (Pcc) (Gardan et al. 2003), *Pectobacterium carotovorum* subsp. *brasiliense* (Pcb) (Nabhan et al. 2012), *Pectobacterium wasabiae* (Goto and Matsumoto 1987) Hauben et al. 1999 (Pwa) (Gardan et al. 2003), *Dickeya dadantii* (Dda) (Samson et al. 2005) and *Dickeya solani* (Dso) (van der Wolf et al. 2014) has been linked to temperature.

Temperature plays an important role in pathogenicity differentiation of soft rot and blackleg pathogens (Pérombelon and Kelman 1980; Pérombelon et al. 1987; Pérombelon 1992). It is generally accepted that *Pectobacterium* spp. grow better and are more pathogenic at lower temperatures (<25^{o}C), compared to *Dickeya* spp. (>25^{o}C) (Pérombelon and Kelman 1980; Elphinstone 1987; Pérombelon et al. 1987; Pérombelon 1992; Tsror et al. 2009, 2013). Each of these blackleg and soft rot bacterial species, however, prefer specific temperatures at which they cause disease.

Pectobacterium atrosepticum grows optimally in cool to temperate (15°C to 24°C) and wet environmental conditions, although some isolates have been shown to survive at temperatures of up to 37°C (Pérombelon et al. 1979; Pérombelon 1992; Pérombelon and Salmond 1995; Oliveira et al. 2003; Duarte et al. 2004; Smadja et al. 2004; Elphinstone and Toth 2007). *Pectobacterium carotovorum* subsp. *carotovorum* prefers temperate and tropical environments, with optimal temperatures between 25°C and 28°C (Pérombelon 1992; Pérombelon and Salmond 1995; Smadja et al. 2004). *Pectobacterium carotovorum* subsp. *brasiliense* prefers humid subtropical conditions, making it extremely virulent at both cool and warm temperatures and unlike Pba, Pcb has the ability to grow at temperatures above 37°C (Duarte et al. 2004; van der Merwe et al. 2010). On the other hand *Dickeya* spp. are known to grow at temperatures higher than *Pectobacterium* spp. and prefer warmer, drier, tropical and sub-tropical conditions (Pérombelon 1992; Smadja et al. 2004; Elphinstone and Toth 2007). It has been reported that *Dickeya* spp. can survive at elevated temperatures, growing weakly at 39°C, up to a maximum of 41°C (Tsror et al. 2009).

Temperature does not only control survival and rate of development of bacteria (Molina and Harrison 1977; Pérombelon and Kelman 1980; Elphinstone 1987), but also determines species presence and controls the expression of pathogenicity factors (Laurent et al. 2000, 2001; Smadja et al. 2004). Various field studies have demonstrated the effect of temperature on species selection and disease development (Molina and Harrison 1977; Pérombelon et al. 1979; Molina and Harrison 1980; Pérombelon and Kelman 1980; Pérombelon et al. 1987; Ali et al. 2012). *Pectobacterium atrosepticum* is generally responsible for blackleg at temperatures lower than 25°C while *Dickeya* spp. are more prevalent at temperatures above 25°C (Elphinstone and Toth 2007). Research undertaken by Serfontein et al. (1991) showed that Pcc could not cause any symptoms at temperatures between 28°C and 32°C, whereas *Erwinia chrysanthemi* (now *Dickeya* spp.) was unable to cause symptoms at temperatures between 20°C and 25°C.

Literature available to date for these pathogens is contradictory with regards to optimal temperatures and temperature ranges for their survival and pathogenicity. New species have been reported and reclassification of

the old *Erwinia chrysanthemi* group has introduced more confusion into the basic knowledge on the effect of temperature on the growth and virulence of this group of bacteria. In order to better understand the effect of temperature on these destructive pathogens, this study aimed at determining the effect of different temperatures on the growth of the most important pectinolytic potato pathogens, namely Pba, Pcb, Pcc, Pwa, Dda and Dso *in vitro*. These bacteria are the most important blackleg- and soft rot causing species in potato. The optimal temperature and temperature range for growth of each of the species was determined *in vitro*.

Materials and methods:

Isolates of the six most important pectinolytic bacterial species and subspecies on potatoes, viz. *Pectobacterium atrosepticum* (Pba), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pectobacterium carotovorum* subsp. *brasiliense* (Pcb), *Pectobacterium wasabiae* (Pwa), *Dickeya dadantii* (Dda) and *Dickeya solani* (Dso) (Isolate number MK14; obtained from G. Saddler, SASA) were obtained from South African and international culture collections. Reference strains included Pcc (LMG 2404^{T}) from the Belgian Coordinated Collection of Microorganisms, Pcb (ATCC 8T) from the American Type Culture Collection and Dda 3937 from the James Hutton Institute. Three Pba isolates were received from the Bacterial Culture Collection located in FABI at the University of Pretoria, which were collected from different hosts causing soft rot in the United States of America (USA) and United Kingdom (UK). Only one South African (SA) isolate of each of Pwa (Isolate number G620; Moleleki et al. 2013) and Dda (Isolate number JJ16; Van der Merwe 2009) were available. South African Pcb and Pcc isolates used in this study were sourced from the Potato Pathology Program at the University of Pretoria culture collection.

The growth of each isolate was tested over a range of 30 temperatures from $\pm 5^{\circ}C$ to $\pm 56^{\circ}C$, in a temperature gradient shaking incubator (Scientific Industries Inc., Model TGI). A randomized complete block design was used for the experiment. Four replications were done with every isolate and the entire experiment was done twice. Specially designed test tubes were filled with 10ml nutrient broth (NB) (Merck), autoclaved and left to cool. An aliquot of 10µl of a bacterial suspension grown overnight in NB and adjusted to 10^{8} CFU ml^{-1} (OD_{600}= 0.1) was added to each test tube, one species per cycle. The test tubes were slotted into the incubator after which growth of bacteria was measured at 14 and 24 hours post inoculation using a spectrophotometer (OD_{600}) (LKB BIOCHRON Ultraspec 4050). The Pba isolates, however, were measured at 24 and 30 hours due to their slower growth *in vitro* (Marquez-Villavicencio et al. 2011). The temperature in each test tube was measured after each

cycle using a Fluke wire probe (51K/J Thermometer). Data was statistically prosessed using the Gaussian function. ANOVA was done on the data and means separated with Tuckey's test.

Results:

Results indicated that Pba grew much slower at all temperatures compared to the other *Pectobacterium* and *Dickeya* spp.. Growth for Pba was only observed after 24 hours compared to 12 hours for the other species. Significant differences were found in this study between Pba isolates; Pba_Bcc10 showed a higher optimal growth temperature compared to Pba_Bcc396 and Pba_Bcc872.

All South African Pcc isolates tested differed significantly from the reference strain, growing optimally at temperatures $\pm 2^{\circ}$C lower than the reference strain, which grew optimally at 34°C. South African isolate SdR19, isolated from a cool region, grew at a significantly lower optimal temperature compared to JJ95 and JJ63.

High variability with regards to temperature range was seen between isolates within the species Pba, Pcc, Dda (Table 1). *Pectobacterium wasabiae* showed an optimal temperature similar to Pcb, Pcc and Dda, but with a slightly cooler growth range, comparable to that of Pba. *Dickeya solani* had a noticeably higher optimal temperature and a very wide growth range (Tables 1 and 2).

Discussion:

Results of this study indicate that there are significant differences between the soft rot-causing Enterobacteriaceae species, Pba, Pcc, Pcb, Pwa, Dda and Dso, as well as between isolates within a species with regard to optimal temperatures and growth ranges *in vitro*.

Pectobacterium atrosepticum, a species not yet reported in South Africa, has been identified in the neighbouring country Zimbabwe (Ngadze et al. 2012). The Pba isolates grew at significantly lower optimal temperatures and growth ranges than the other species tested. Optimal temperatures of 26°C to 27°C with a temperature growth range of 18°C to 31°C found in this study, are similar to results found by other researchers. Pérombelon and Salmond (1995) recorded optimal disease development at 25°C, while Smadja et al. (2004) reported an optimal

Table 1: Cardinal temperatures for different isolates of pathogenic Enterobacteriaceae responsible for blackleg and soft rot on potatoes.

Species	Isolate	Reference / SA / Other	Minimum Temperature (˚C)	Maximum Temperature (˚C)	Optimal Temperature* (˚C)
Pectobacterium atrosepticum	Bcc396	UK[iii]	18.1	30.8	25.7 a
	Bcc872	USA[iv]	18.4	31.0	25.8 a
	Bcc10	UK	20.0	31.2	26.7 b
Pectobacterium carotovorum subsp. *brasiliense*	ATTC 8T	Reference	20.6	37.8	30.8 c
	JJ1	SA[v]	20.2	38.6	32.1 d
	JJ12	SA	20.9	38.2	31.8 d
	JJ83	SA	20.3	38.6	31.6 d
Pectobacterium carotovorum subsp. *carotovorum*	LMG 2404^T	Reference	21.6	39.9	33.6 e
	JJ63	SA	21.3	38.1	32.1 d
	JJ95	SA	20.7	38.7	31.7 d
	SdR19	SA	21.3	35.7	30.5 c
Pectobacterium wasabiae	G620[i]	SA	20.6	33.8	30.2 c
Dickeya dadantii	3937	Reference	23.8	38.8	34.4 f
	JJ16	SA	20.1	36.5	30.7 c
Dickeya solani	MK14[ii]	Reference	23.8	42.0	35.3 g

[i] Moleleki *et al.* (2013); [ii] Obtained from G. Saddler, SASA; [iii] United Kingdom; [iv] United States of America; [v] South Africa; * Tuckey's 95% Confidence interval; CV% = 0.9; SE = 0.28

Table 2: Comparison of optimal temperatures and temperature growth ranges of different *Pectobacterium* and *Dickeya* spp.

Species	**Optimal Temperature**	**Temperature Growth Range**
Pectobacterium atrosepticum	**26-27°C**	**18-31°C**
Pectobacterium carotovorum* subsp. *brasiliense	**31-32°C**	**20-39°C**
Pectobacterium carotovorum* subsp. *carotovorum	**31-34°C**	**20-40°C**
Pectobacterium wasabiae	**30°C**	**20-34°C**
Dickeya dadantii	**31-34°C**	**20-39°C**
Dickeya solani	**35°C**	**24-42°C**

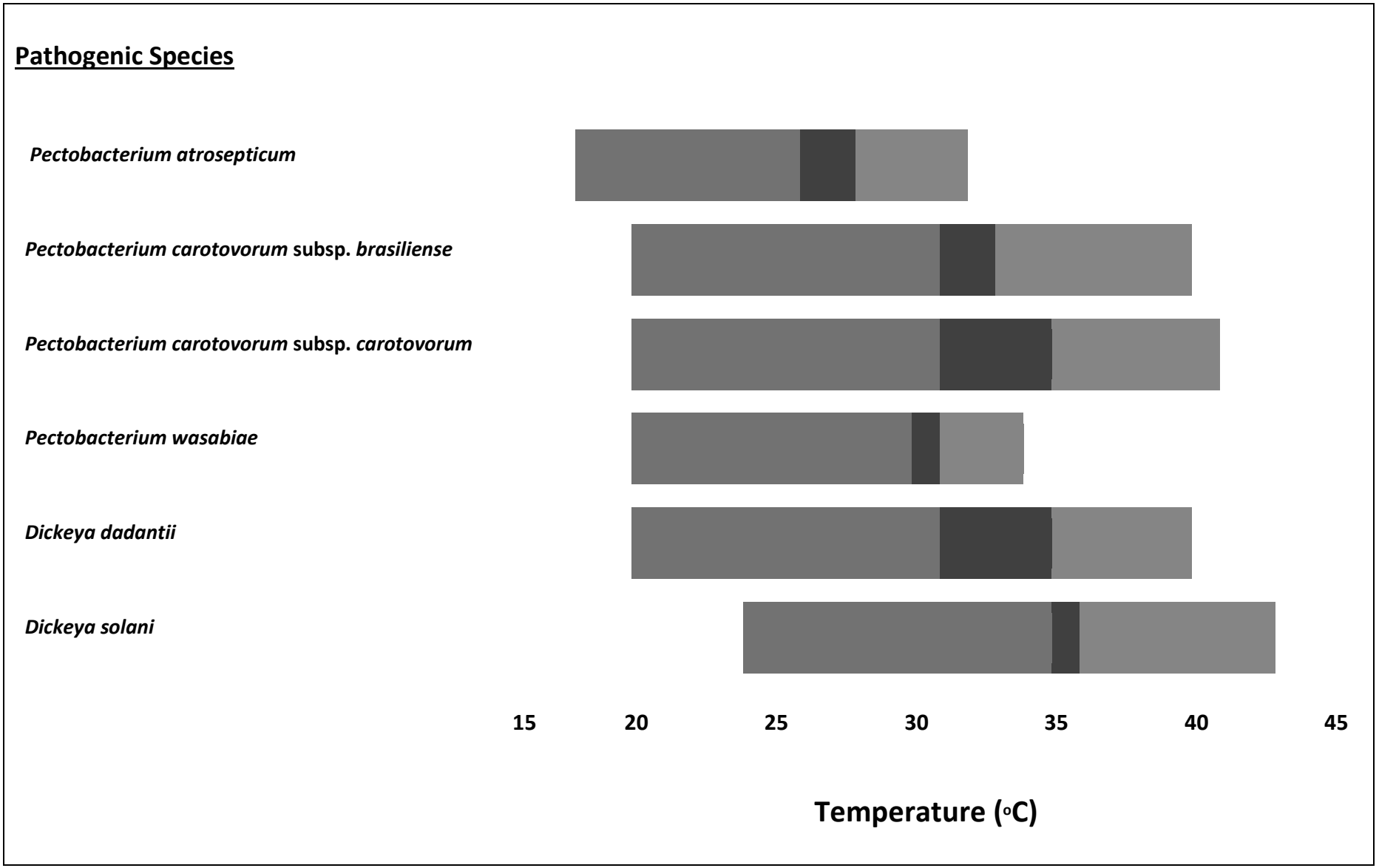

Figure 1 Comparison of cardinal temperature range for six different Enterobacteriaceae potato pathogens responsible for blackleg and sot rot

growth temperature of 24°C for Pba *in vitro*. Various greenhouse studies, field trials and surveys confirmed the circumstantial predominant association of Pba with disease occurrence in cooler environmental conditions (Molina and Harrison 1977; Pérombelon et al. 1979; Molina and Harrison 1980; Pérombelon and Salmond 1995; Smadja et al. 2004; Ali et al. 2012). In this study no growth was observed at 37°C as was also found by Duarte et al. (2004). This unique characteristic could be used to distinguish Pba from other pathogenic species causing soft rot and blackleg in potato, as proposed by De Boer and Kelman (2001). However, up to 10% of Pba isolated are atypical and able to grow at temperatures above 37°C (Pérombelon and Hyman 1986; Oliveira et al. 2003). Using temperature to characterise species might therefore result in incorrect characterisation in cases where atypical isolates are encountered.

Temperature differences for *in vitro* growth of isolates Pba_Bcc396 and Pba_Bcc872 isolated from potatoes and Pba_Bcc10 isolated from celery indicate that these differences may be related to host rather than geographical origin. Higher optimal temperatures for Pba isolates that do not originate from potatoes have been reported by Pérombelon and Hyman (1986). This might be an indication that differences within the species might occur due to host specificity. Results from this study thus indicate a versatile adaptation of this species to be able to grow and possibly remain pathogenic in new temperature niches.

Temperature growth patterns in this study grouped the *Pectobacterium carotovorum* subspecies, Pcb and Pcc, together and were unable to separate these two species on either their optimal growth temperatures or growth ranges. However, within each subspecies significant differences were seen between isolates with regards to optimal temperatures and growth ranges. *Pectobacterium carotovorum* subsp. *carotovorum* isolates were much more variable compared to Pcb isolates, which showed statistically closer temperature clustering of isolates.

Pectobacterium carotovorum subsp. *brasiliense* was first reported in SA in 2010 and has been identified as the most prevalent species in South African potato growing regions (Van der Merwe et al. 2010, Ngadze et al. 2012). The South African Pcb isolates showed no significant differences in their optimal growth temperatures (31°C to 32°C) or temperature ranges (20°C to 39°C). The reference strain Pcb ATCC-8T from Brazil, however, grew optimally at a significantly lower optimal temperature compared to the South African isolates and exhibited a slightly lower maximum temperature. This suggests that the SA isolate could to be different to those found in Brazil, indicative of broad geographical adaptation as was similarly reported by Nabhan et al. (2012), although differences may be due to isolate variability. The absence of differences between South African Pcb isolates indicates minimal differentiation or adaptation of these isolates within a region such as South Africa.

Studies undertaken by Ngadze et al. (2012), found Pcb populations of various lineages in South Africa grouping into distinct phenetic groups according to their environmental origins.

Variation in optimal growth temperatures (31°C to 34°C) but similar growth ranges were found in the Pcc isolates tested. These variations are in agreement with other studies where a low optimal temperature of 25°C was reported by Pérombelon and Salmond (1995), while Smadja et al. (2004) found optimal growth temperatures above 28°C. Although the optimal temperatures reported in literature are lower than the optimal temperatures found in this study, it could be explained by isolate variation or the fact that Pérombelon and Salmond (1995) were investigating disease development and not *in vitro* growth. Results from this study also do not concur entirely with prediction models which indicate optimal temperatures for Pcc to be 20°C to 25°C, although these models were based on tuber slice assays and not *in vitro* tests (Kushalappa and Zulfiqar 2001).

The temperature growth range of Pcc was found to be 20°C to 40°C, which did not differ extensively from Pcb, but was much more variable between isolates. This is contradictory to the results of Pérombelon and Hyman (1986) which indicated a lack of growth of Pcc at 37°C, but confirms results of Oliveira et al. (2003) who found Pcc to grow at 37°C. The differences in optimal temperatures for growth can have important consequences in industry as a slight decrease in temperature could result in Pcb becoming dominant in the field, and this subspecies has been reported to be more virulent than other *Pectobacterium* spp. (Duarte et al. 2004).

Pectobacterium wasabiae was reported for the first time in SA in 2013 (Moleleki et al. 2013). An optimal temperature of ±30°C grouped Pwa together with the *Pectobacterium carotovorum* group, but the limited growth range and maximum growth temperature of ±34°C, it appears that Pwa is relatively temperature sensitive *in vitro*, similar to Pba. *Pectobacterium wasabiae* is able to grow at temperatures higher than Pba, but not as high as the *Pectobacterium carotovorum* subspecies. Waleron et al (2013) noted that approximately 50% of their Pw potato isolates were able to grow at 37°C while the other half failed to do so. Similar results were found by Nykyri et al. (2012). This might therefore be an indication of possible variation within the species. Differentiation of Pw from Pcc or Pcb based on optimal growth temperature *in vitro* is not reliable (Waleron et al. 2013).

The preference of *Dickeya* spp. to tropical environments and warm temperatures (Tsror et al. 2009), was confirmed in this study. Significant differences were noted between *Dickeya* spp. which have not been reported previously. The reference isolate Dda_3937 grew at a significantly higher optimal temperature (34.4°C) compared to the South African isolate JJ16 (30.7°C), which showed a wider, but lower, temperature growth range. The temperature profile of the South African Dda isolate groups it together with the *Pectobacterium*

carotovorum group in terms of optimal temperatures. This can contribute to the formation of species complexes with the *Pectobacterium carotovorum* group. The species Dso presented an extremely broad temperature range with a high optimal temperature of $\pm 35^{o}$C. These temperatures place Dso in its own group. This is contradictory to the results of Tsror et al. (2009), which indicated that *Dickeya* spp. isolated from potatoes in Israel were not able to grow at temperatures above 41^{o}C *in vitro*. Nonetheless, results from this study and others have shown that the broad temperature range in which Dso flourishes is notable and might explain the increase in recent reports of this species in many countries in Europe (Slawiak et al. 2009; Tsror et al. 2009; Laurila et al. 2010; Toth et al. 2011).

Results of this study indicate the imperative role played by environmental temperature on growth and therefore possibly also on species selection in the field. Each species has its own cardinal temperature ranges in which growth is sustained. The species have a definite maximum temperature above which they cannot survive; but the minimum growth temperature is more difficult to determine, since growth slows down significantly at temperatures below 5˚C. It is, however, not yet known how pathogenicity and symptom development is influenced by these extreme low or high temperatures. The effect of temperature over time has been proven to significantly influence disease development (Kushalappa and Zulfiqar 2001). Optimal temperatures will result in high bacterial concentrations in a short time period, while at lower temperatures bacterial growth will be inhibited and can even cause the bacteria to convert to a dormant state (Dickey 1979, Pérombelon and Kelman 1980; Pérombelon and Salmond 1995). Maximum temperatures conversely result in death due to protein denaturation, collapse of cytoplasmic membranes and thermal lysis (Madigan and Martinko 2006).

Climate change studies done by van der Waals et al. (2013), predict a 1.6˚C temperature increase in South Africa by 2050. This temperature increase will bring along longer growing seasons and faster pathogen replication, which will cause an increase of blackleg and soft rot incidence and severity in most South African potato production regions. Increased disease occurrence can be worsened by significant changes in species presence and selection driven by temperature as was described by Pérombelon and Lowe (1975) and results presented in this study. Survival of these pathogens in soil and environments outside the plant will also be greatly affected by temperature. Climate change might result in environmental temperatures rising above some of the species' maximum growth temperatures. This temperature shift can lead to the exclusion of the cooler growing species, which will be replaced by temperate and warm growing species resulting in a species shift in certain regions. A temperature change can lead to the establishment of some of these pectinolytic bacteria in new niches. Increased temperature might result in the exclusion of soft rot and blackleg pathogens from now

favourable environments and a move to environments previously unfavourable for the sustainability of these bacteria, resulting in pathogen movement.

Temperature influences several of the pathogenicity factors that also in turn affect each other. Although temperature plays an important role in species selection and pathogenicity, adequate free soil water is required for motility, growth and spread of the bacteria (Pérombelon and Lowe 1975; Pérombelon 1992; Toth et al. 2003). Temperature directly affects enzyme expression which is regarded as the most important virulence factor (De Boer 2004). It has been shown that the various enzymes responsible for tissue maceration, namely pectate lyase, pectin lyase, pectin methyl esterase, polygalacturonase and endopolygalacturonic transamilase are influenced differently by temperature in the various species (Pérombelon and Ghanekar 1979 as cited by Pérombelon and Kelman 1980; Lanham et al. 1991; Laurent et al. 2000, 2001; Smadja et al. 2004). Simultaneously this enzyme activation depends on quorum sensing, cell-density-dependant regulation (Pérombelon 2002; Toth et al. 2003), which is again modulated by reaching the critical cell density *in planta* (10^7-10^8 CFU ml^{-1}) (Pérombelon and Salmond 1995; Pérombelon 2002). Thus the species exposed to its optimal temperature will have the advantage of maximum growth rate allowing it to reach critical numbers first and to activate enzyme production. Bacterial growth and multiplication can thus be reasoned to be an important virulence factor, which is highly dependent on temperature.

Comparing the results from this study with survey data from a previous study (van der Merwe et al. 2010), there do not appear to be any correlations between cardinal temperatures obtained in this experiment and species prevalence in different climatic regions. This raises the question of why Pcc and Pcb are not equally prevalent in diseased potatoes in SA. Neither does this explain why there is not more of the warmer growing species, Dda, present in South Africa. The data also emphasizes the importance of quarantine practices to ensure that species such as Dso which have not yet been reported in South Africa do not enter the country. The broad temperature growth range and high optimal temperature of Dso make the warm South African climate ideal for establishment of this species. Tsror et al. (2013) found Dso to be more virulent in the warmer environments of Israel, compared to cooler European environments. South Africa has climatic conditions similar to Israel implying that Dso might also be more virulent in South Africa than in Europe should it enter the country.

The data generated in this study can be useful in understanding the epidemiology of these species, in the development of prediction models and can be used in integrated management programmes. The possibility of host or environmental adaptation for these pectinolytic bacterial pathogens cannot not be excluded and highlights the need for more elaborate and in-depth research. Significant differences in the temperature margins

and optima of the South African isolates compared to reference strains from other countries point to possible geographical and climatic adaptations of these subspecies in SA. Future research should thus repeat this study using a wider range of isolates and species from various locations across the world and from various hosts. *In planta* trials should also be done in order to determine how these temperatures not only influence growth of these pathogens, but also disease development.

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