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Symptoms and yield reduction caused by *Dickeya* spp. strains isolated from potato and river water in Finland

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Abstract Biochemical characterisation of *Dickeya* strains isolated from potato plants and river water samples in Finland showed that the majority of the strains were biovar 3. They thus resembled the strains recently isolated from potato in the Netherlands, Poland and Israel and form a new clade within the *Dickeya* genus. About half of the Finnish isolates resembling strains within this new clade were virulent and caused wilting, necrotic lesions and rotting of leaves and stems. Similar symptoms were caused by *D. dianthicola* strains isolated from one potato sample and from several river water samples. Frequently, the rotting caused by the *Dickeya* strains was visible in the upper parts of the stem, while the stem base was necrotic from the pith but hard and green on the outside, resulting in symptoms quite different from the blackleg caused by *Pectobacterium atrosepticum*. The presence of *Dickeya* in the symptomatic plants in the field assay was verified with a conventional PCR and with a real-time PCR test developed for the purpose. The virulent *Dickeya* strains reduced the yield of individual plants by up to 50% and caused rotting of the daughter tubers in the field and in storage. Management of *Dickeya* spp. in the potato production chain requires awareness of the symptoms and extensive knowledge about the epidemiology of the disease.

Keywords *Dickeya* · *Erwinia chrysanthemi* · Stem rot · Symptoms · Yield

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Introduction

Several species of plant pathogenic Gram-negative enterobacteria in the genus *Dickeya* cause rotting in numerous crop plants (Samson et al. 2005; Ma et al. 2007). These bacterial species were previously included in *Erwinia chrysanthemi* and considered pathogenic in warm and tropical climates, where several of them caused soft rot and stem rot in potato (Pérombelon 2002). Accordingly, *Dickeya* spp. have been isolated from potatoes cultivated in the warm

regions in Europe (Palacio-Bielsa et al. 2006). Recently, *Dickeya* strains were isolated from potato plants in the Netherlands and Poland, and from diseased potato plants and river water in Finland, suggesting that *Dickeya* strains are no longer restricted to warm areas in Europe but are common also in temperate and even in cool regions in Europe (Sławiak et al. 2009; Laurila et al. 2008). These pathogens are also common in greenhouse-cultivated ornamentals, such as carnation and chrysanthemums, on which some of the strains are considered to be quarantine pathogens in Europe (http://www.eppo.org/QUARANTINE/listA2.htm). Furthermore, in the Netherlands *Dickeya* has been reported to cause soft rot in hyacinth under field conditions (Jafra et al. 2009).

Bacterial strains in the *Dickeya* genus are divided into six species, some of which correspond to the former biovars of *E. chrysanthemi* (Samson et al. 2005). Within these new species, *D. dianthicola* consisting of some strains in biovars 1, 7 and 9, and *D. zeae* corresponding to biovars 3 and 8, cause diseases in potato stems and tubers (Samson et al. 2005). *Dickeya dianthicola* has also been found to cause disease on potato in Europe and in other countries with temperate climates, whereas *D. zeae* has been associated with potato diseases in warm and tropical countries (Boccara et al. 1991; Nassar et al. 1994; Singh et al. 2000). Recently, strains corresponding to several biovars were isolated from diseased potato plants in Spain (Palacio-Bielsa et al. 2006), which suggests that the *Dickeya* population in Europe might be more variable than earlier anticipated. This was also suggested by characterisation of 16 S and 16 S–23 S rDNA intergenic spacer sequences of the Finnish isolates, which showed that the strains isolated from diseased potato or river water samples could be grouped into three clusters, two of which were not identical with the previously isolated strains (Laurila et al. 2008). In the Netherlands, recently isolated potato strains were characterised as biovar 3 and shown to differ from previously characterised *Dickeya* strains isolated from potato and to resemble a Dutch strain isolated from hyacinth. (Sławiak et al. 2009). Similar strains were isolated in Israel from plants grown from Dutch seed potatoes (Tsror et al. 2009).

Dickeya strains cause either wilting, stem rot or both in diseased potato plants. In tropical areas with high relative humidity (RH), the *Dickeya* infection has been reported to cause extensive stem rot, whereas wilting alone or in combination with limited stem rot has been observed in temperate regions in Japan and Switzerland (Tanii and Baba 1971; Jaggi and Winiger 1979, reviewed by Palacio-Bielsa et al. 2006), and only wilting was seen in dry areas in Israel (Lumb et al. 1986).

The objective of this study was to identify the Finnish *Dickeya* strains to biovar level and to verify whether some of the newly isolated strains resemble the new strains isolated in the Netherlands. A further objective was to characterise the symptoms caused by the Finnish *Dickeya* strains in potato under field conditions and to examine whether the *Dickeya* strains cause yield loss. Preliminary results of some of these experiments have been published earlier (Laurila et al. 2008). In addition, the development of a real-time PCR test for the identification of potato-infecting *Dickeya* spp. in symptomatic plant tissue is reported.

Materials and methods

Biovar determination

Dickeya strains were characterised by biochemical tests according to Samson et al. (1987) and Ngwira and Samson (1990). Briefly, the bacteria were grown in King's B medium for 24–48 h, collected by centrifugation and resuspended in sterile water. The cultures were adjusted to 0.1–0.2 OD_{600} and 500 µl of the bacterial solution was used to inoculate 2 ml of Ayers medium (Ayers et al. 1919) containing either 0.5% D-arabinose, D-raffinose, melibiose, mannitol or D-tartrate (Sigma T2032). The utilisation of carbohydrates was seen as colour change to yellow and utilisation of tartrate as medium alcalinisation, which produced a blue colour. Utilisation of inulin was assessed with the phenol red method of Gallois et al. (1992) and anaerobic utilisation of arginin with Moeller broth (Decarboxylase Medium Base, Difco). Growth at 39°C was studied in liquid nutrient broth and on nutrient agar plates (Difco). Positive and negative reactions were scored by comparing to *Dickeya* reference strains CFBP1200, CFBP1269, CFBP1270, CFBP2051, CFBP2052 and CFBP3477.

Field assay

A field trial to assess symptoms and yield losses caused by different *Dickeya* strains was carried out in 2006 at the Potato Research Institute, Lammi (67.76676° N, 25.54389° E). *Pectobacterium atrosepticum* strain Pa s0350 was used as a reference for bacterial contamination and water inoculum as a reference for healthy seed. The design of the field test has been previously described (Laurila et al. 2008). The potato tubers of cv. Nicola were vacuum-infiltrated with bacterial suspension containing ~5×10^5–10^6 cfu ml^{-1}. The inoculated tubers were planted in the field in a randomised complete block trial design with seven replicates. Within each block, 11 tubers inoculated with each bacterial strain and a water control were individually randomised. The basic trial unit for symptom assessment was an individual plant resulting in 77 true replicates for each treatment.

The ridges for planting were made by a trans-planting machine and simultaneously the soil was fertilised with a compound fertiliser: N 79 kg ha^{-1}, P 53 kg ha^{-1} and K 150 kg ha^{-1}. The seed potatoes were planted manually into the ridges on 22nd of May. Plant distance was 40 cm and row distance 80 cm. The soil type was fine sand containing 4% organic matter. Weeds were controlled by one application of linuron containing herbicide before emergence and one application of rimsulfuron containing herbicide two weeks after emergence. Due to a very dry season the plot was sprinkler-irrigated on 7, 10, and 20 July., and 2 and 8 August, corresponding to 20 mm, 20 mm, 14 mm, 14 mm and 14 mm of rain, respectively. The crop was protected against potato late blight by fungicide applications at 7 to 10 days intervals from 14th of July onwards. First application was made with a mixture of famoxadone and cymoxanil, second with a mixture of dimethmorf and mancozeb, and three final applications with fluazinam.

All disease symptoms on each plant were recorded weekly from 7th of July until 31st of August. The final assessment was done on 11th of September. The crop was harvested manually 18th of September. Five of the seven blocks were harvested and tuber yield of the 11 plants in each treatment was combined resulting in five replicates for yield analyses. The healthy and rotten tubers were weighed two days after harvest. The healthy tubers were stored and inspected for soft rot during the first weeks after harvest and tubers that rotted after harvest were weighed 6th of October. Thereafter no more rotten tubers were found.

Statistical analysis

For the statistical analyses the symptoms of *Dickeya* infection were classified into six groups: non-emergence; delayed growth; stem rot; wilted or dried stems; necrotic or hollow vascular tissue; rotten petioles or leaves. The probability of the appearance of different symptoms after inoculation of tubers with different *Dickeya* strains in general and with each strain individually was studied by estimating odds ratios for each predictor value. The ‘odds’ of an event are defined as the probability of the outcome event occurring divided by the probability of the event not occurring. In general, the ‘odds ratio’ is one set of odds divided by another. An odds ratio of 1 indicates that the event under study is equally likely in both values of the predictor. An odds ratio >1 indicates that the event is more likely in the first value, whilst an odds ratio <1 indicates that the event is less likely in the first value. The confidence intervals are related to the *P*-values such that the odds ratio will not be statistically significant if the confidence interval contains 1. Logistic regression was used to analyse the symptom frequency data because it was considered more appropriate than other statistical methods, which assume that the residuals, or errors, are drawn from a normal distribution. Logistic regression has the additional advantage that all of the predictors can be binary, a mixture of categorical and continuous or just continuous. The disadvantage of the method is that predictors cannot be calculated if all observations in a certain category have a value of 0 or all are rated as 1 (Allison 1999). The use of logistic regression to analyse similar frequency data has been described in detail by Lehtinen et al. (2007). These analyses were performed by the SAS/ logistic procedure in SAS/ STAT version 9 (SAS Institute Inc., Cary, NC, USA).

Modelling of the effects of infection by the *Dickeya* isolates on yield was done by analysis of variance (ANOVA), while multiple comparisons between treatments were done by Tukey’s Studentised range (HSD) test. Linear regression models were used to study the relationships between different types of symptoms and the yield parameters. Prior to model-

ling the data was thoroughly analysed for its suitability for the comparisons based on least square approaches. The normality and other crucial assumptions of applicability of the ANOVA were studied using PROC UNIVARIATE procedure available in SAS/STAT version 9 (SAS Institute Inc., Cary, NC, USA). The modelling was done by PROC GLM procedure for general linear models in SAS/STAT version 9 (SAS Institute Inc., Cary, NC, USA) (Littell et al. 1991). In the model *Dickeya* treatments and block (replicate) were explanatory class variables and yield measurements were the response variables to be modelled. In regression models types of symptoms were explanatory variables and yield measurements response variables.

Design of a real-time PCR test for *Dickeya* spp

16S–23S intergenic region sequences of the characterised *Dickeya* strains and homologous *Dickeya* sequences from databases (Laurila et al. 2008) along with 25 partly homologous 16 S–23 S sequences of various strains in genera *Klebsiella*, *Enterobacter*, *Xanthomonas, Paenibacillus*, *Bacillus*, *Clavibacter*, *Ralstonia*, and *Pseudomonas* obtained from databanks were used to design real-time primers specific for *D. dianthicola* and the biovar 3 strains. A primer pair recognising *Dickeya* strains but excluding all the other sequences were identified with AlleleID programme at TATAA Biocentre (http://www.tataa.com/) in Gothenburg, Sweden. The real-time PCR reactions were analysed using DyNAmo HS SYBR Green qPCR Kit (Finnzymes) in 25 μl reaction volumes using 0.6 μM final concentration of the primers and 5 μl sample volume. The samples were analysed with ABI Prism 7700 Sequence Detection System (Applied Biosystems) with a standard programme having 95°C dissociation and 60°C annealing temperatures. The cycle was repeated 40 times. The specificity of the primers was assessed by melting curve analysis and sequencing of the PCR products and the sensitivity of the assay was tested by assaying 10× dilutions of the bacteria cells in culture grown in Luria-Bertani (LB) medium. The bacterial concentration in samples was estimated by comparing the test results with a standard curve from a 10-fold dilution series of bacteria grown in vitro. The bacterial concentration of each dilution was determined by plating and colony counting.

Preparation of conventional and real-time PCR samples

For conventional PCR tests, potato stem samples were plated on CVP-S2 plates according to Pérombelon and van der Wolf (2002) and bacterial cells from the mixed cultures were used as template in conventional PCR without prior purification. For real-time PCR, bacterial cultures were grown at 28°C in LB medium until stationary phase, diluted 1/10 in water and adjusted with water to 0.25 OD_{600}. Standard curves were made from dilution series of *in vitro* cultures grown in LB at 28°C. Plant samples were prepared from frozen stems by cutting 1 cm long segments from stems and vortexing in 1 ml water in Eppendorf tubes for 5 min prior to using the liquid as template for real-time PCR. All the plant samples were diluted 1/100 before assaying by real-time PCR.

Results

Phenotypic characterisation of the *Dickeya* strains isolated in Finland

In our previous publication, the phylogenetic analysis of 16 S and 16 S–23 S rDNA sequences of Finnish *Dickeya* isolates suggested that the strains fell into three groups, one group resembling *D. dianthicola* and two groups not resembling any of the previously characterised *Dickeya* strains (Laurila et al. 2008). Characterisation of the same 22 strains by biochemical tests showed that the groups differed from each other (Table 1). The strains in group I frequently isolated from diseased potato plants grew on arabinose, melibiose, raffinose and mannitol but did not utilise tartrate and were negative in the inulin test and were unable to hydrolyse arginine under anaerobic conditions. They did not grow in 39°C in liquid medium but formed small colonies at 39°C on solid nutrient agar medium, possibly because bacterial cells grown on solid media are more stress tolerant. By comparison to published biovar characteristics, these strains were identified as biovar 3 (Samson et al. 1987; Ngwira and Samson 1990). They thus resemble the Dutch *Dickeya* strains that are thought to form a new species within the *Dickeya* genus (Sławiak et al. 2009). Strain D s0411 that had been found to have very low virulence (Laurila et al. 2008), was found to

Table 1 Phenotypic characterisation of Finnish *Dickeya* isolates by biochemical tests

	Group I[a] 12 strains	Group II 5 strains	Group III 4 strains
(-)-D-Arabinose	+	−	−
(+)-D-Melibiose	+	+/−[b]	+
(+)-D-Raffinose	+	+/−[b]	+
Mannitol	+	+	−
Inuline	−	+	−
ADH Arginine	−	+	−
(-)-D-Tartrate	−	+	−
Growth at 39°C[c]	+	−	+

[a] The slow-growing strain D s0411 has not been included in the table

[b] Two of the strains, D w04L and D w04K, were negative in these tests

[c] Growth at 39°C was assessed on nutrient agar plates. Group I strains grew slowly and formed small colonies, whereas group III strains grew fast and formed large colonies on the plates

differ from all the others and from previously characterised biovars possibly because it grew very poorly in all test media (data not shown). It was not further characterised.

The strains in group II that resembled *D. dianthicola* in the phylogenetic analysis, did not grow at 39°C, and were unable to utilise arabinose. Three of the strains, D s053-3, D s053-6 and D w04M, were positive in all the other tests, being thus identical with biovar 1. The strains D w04L and D w04K resembled the other strains but did not grow on melibiose and raffinose, being thus identified as biovar 7. In the new taxonomy biovars 1 and 7 are both included in phenon 5 containing *D. dianthicola* (Samson et al. 2005).

The strains in group III, which were isolated only from river water and which had low virulence on potato (Laurila et al. 2008), were positive in melibiose and raffinose tests and negative in the other assays, and grew very well at 39°C even in liquid nutrient broth. The strains were not identical to any of the characterised biovars. They resembled biovar 6, the only difference being that they did not utilise mannitol as do the typical biovar 6 strains (Samson et al. 1987; Ngwira and Samson 1990). They also resembled biovar 4, from which they differed by their negative results in arabinose and tartrate tests.

Symptoms of *Dickeya* infection in potato plants

The virulence of *Dickeya* strains from all three groups was studied in a field trial with plants grown from vacuum-inoculated seed tubers. Each plant was inspected and assessed for disease symptoms from 6 weeks after planting until September, when the plants started to senescence. Especially, some of the biovar 3 strains in group I and *D. dianthicola* strains in group II were found to cause symptoms already at the first inspection, and the incidence of symptoms increased towards the end of the growing season (Fig. 1). The strains causing least symptoms were also the ones showing most symptoms late during the growing season.

Until 23rd of August, three months after the planting, there was a statistically significant difference in the incidence of symptoms between most of the strains and the water-treated control, whereas after that comparison with symptoms appearing in water-treated controls was less clear because of senescence of the plants. Therefore, only the symptoms detected 23rd of August or earlier were interpreted as being caused by the inoculated bacteria. (Fig. 2). The first symptom was wilting and in some wilted plants also blackleg-like rotting of the stem base was observed (Fig. 3a and b). Later during the growing season the stem base did not rot but the infection seemed to spread up in the pith of the stem (Fig. 3c) and produce symptoms in the upper parts of the plant, including decay in the petioles and leaves (Fig. 3d and e). Especially, the *D. dianthicola* strains caused dark dry necrotic spots on the stems (Fig. 3f). Later during the growing season, the bacteria caused decay of the upper parts of the infected stems manifested as a very dark brown rot in most plants. In many plants the stem base stayed green very long and was the last part of the plant to rot (Fig. 4). There was no difference in the appearances of the rotted plants between plants inoculated with the different groups of *Dickeya* strains.

There was considerable variation in disease progress measured as apparent infection rate (AIR) or area under disease progress curve (AUDPC) between plants inoculated with different *Dickeya* strains. The most virulent strains caused symptoms in almost all plants while the prevalence of symptoms in plants inoculated with the least virulent strains was at the same level as plants inoculated with water. The

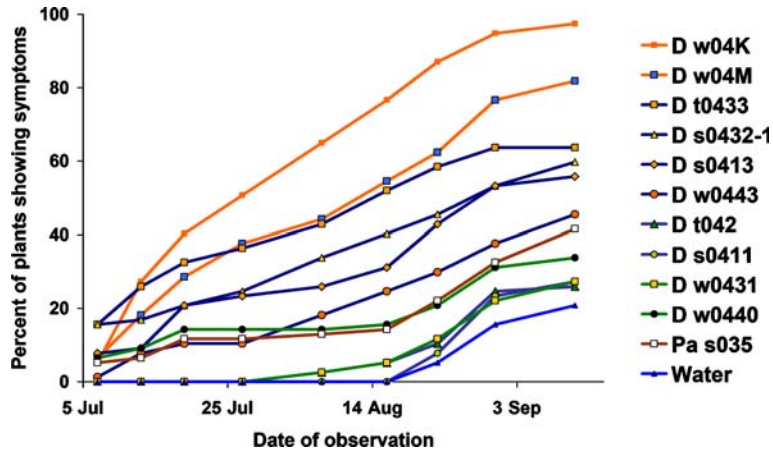

Fig. 1 Progress of wilting and decay symptoms in *Dickeya*-inoculated potato plants in the field trial during growing season 2006. The seed tubers were vacuum-inoculated with *Dickeya* strains prior to planting and the disease symptoms in the plants were assessed throughout the growing season. The *Dickeya dianthicola* strains are marked with an orange line, biovar 3 strains with a dark blue line, the unidentified strains in the group III with a green line, *Pectobacterium atrosepticum* control with a brown line and water-treated mock control with a clear blue line. The field inspection dates are marked under the x axis

disease progress was fastest in plants inoculated with the two *D. dianthicola* strains D w04K and D w04M. Three biovar 3 strains, D s0413, D s0432-1 and D t0433 in group I were relatively virulent, whereas two biovar 3 strains D s0411 and D t042 and both strains in group III, D w0431 and D w0440 proved to be weak pathogens in comparison to other strains.

One half of the strains in each group caused pre-emergence death of plants (Fig. 2) although there was no statistically significant difference between strains

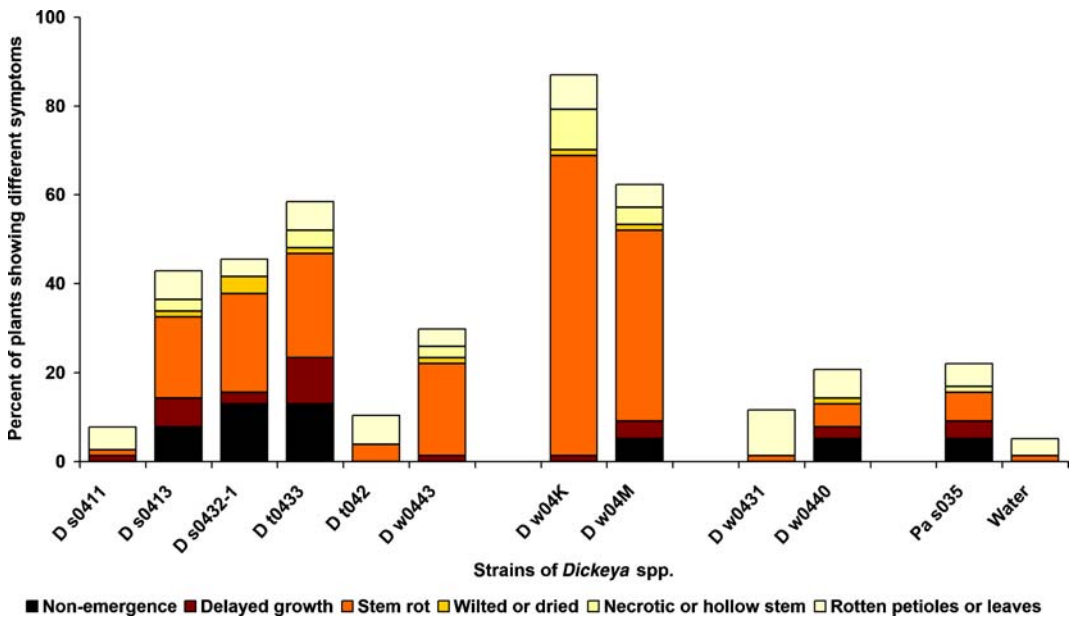

Fig. 2 Percentage of non-emerged potatoes and different symptom categories on potato grown in the field from seed inoculated with *Dickeya* strains. The cumulative symptoms observed in the inoculated plants during three months after planting were divided into different symptom categories. Strains D s0411–D w0443 were identified as biovar 3, D w04K and D w04M as *D. dianthicola*, strains D w0431 and Dw0440 represent the unidentified strains in group III, Pa s035 is a *Pectobacterium atrosepticum* strain used as a control and water denotes mock inoculation with tap water

Fig. 3 *Dickeya* symptoms in potato plants. Wilting and blackleg-like stem rot symptoms early in the growing season in plants inoculated with **a** biovar 3 strain D t0433 and **b** *Dickeya dianthicola* D w04M. Necrosis of the pith (**c**) in plants inoculated by *D. dianthicola* D w04K and rotting of petioles (**d**) and leaves (**e**) in plants inoculated by D w04M. Dry necrotic lesions in the stems (**f**) of a plant grown from tuber inoculated by D w04K

in their ability to cause non-emergence. Most strains delayed growth of a few potato plants. Only strain D t0433 showed statistically significantly higher ability than the other isolates for delaying growth (Table 2). All strains were able to cause stem rot. The *D. dianthicola* strains w04K and Dw04M in group II had the highest proportion of plants with stem rot symptoms (Fig. 2). The probability of these two strains to cause stem rot was statistically significantly higher in comparison to any other strain. In addition, the risk of stem rot was statistically significantly higher for four strains in group I in comparison to the rest of the strains (Table 2). Certain strains in group I and II caused wilting, and necrotic or hollow stems. None of these symptoms could be statistically connected to any specific strain. Rotten petioles or leaves were present at the end of the season regardless of which strain had been used for inoculation.

Effect of *Dickeya* infection on potato yield

At the end of the growing season, the potato plants in five blocks were harvested manually and the weight of the harvest was measured (Fig. 5). From several plants rotten tubers were also obtained. They were removed from the harvest and weighed separately. Also during the first weeks of storage, rotten tubers were observed and removed from the harvest.

Fig. 4 Typical stem rot caused by *Dickeya* infection at the end of the growing season, caused by biovar 3 strain D t0433. The symptoms can be seen as rotting and wilting of leaves and dark brown rotting of the stems

The *Dickeya* strains causing severe symptoms also caused considerable yield losses. The most virulent *D. dianthicola* and biovar 3 strains (D w04M, D w04K, D t0433, D s 0413 and D s0432-1) reduced both total and healthy yield by 50 to 30% in comparison to water control (Total yield: *F*-value 23.26, $P<0.0001$, R^2 0.86; Healthy yield: *F*-value 26.34, $P<0.0001$, R^2 0.87). They also increased the proportion of rotten tubers at harvest and in storage (*F*-value 2.61, $P=0.0128$, R^2 0.43). On the other hand, some strains (D s0411, D t042, D w0443 and D w031) had no adverse effect on total yield in comparison to the water control. These isolates also did not increase the proportion of rotten tubers with the exception of strain D w0443. Strain D w0443 increased the incidence of tuber rot to a similar level with more virulent strains D s0413, D s0432-1 and D w0440.

The severity of yield loss was clearly related to the total prevalence of diseased plants (*F*-value 7.36, $P<0.0001$, R^2 0.46) and even more clearly to the incidence of certain symptom categories (model: *total yield = 27.07 −1.50 * N of stem rot symptoms − 1.86 * N of non-emerged plants − 3.33 * N of plants with delayed growth − 1.11 * N of necrotic or hollow stems + 0.28 * N of rotten petioles and leaves + 0.01 * N of wilted or dead plants*; *F*-value 12.64, $P<0.0001$, R^2 0.59) . The increase in stem rot symptoms accounted for most of the yield reduction in the model (*F*-value 29.41, $P<0.0001$). One unit increase in stem rot reduced the total yield by 7%. In addition, increase of plants with delayed growth (*F*-value 12.41, $P=0.0009$) and increase in non-emerged plants (*F*-value 3.73, $P=0.0583$) decreased total yield. Incidence of plants with necrotic or hollow vascular tissue (*F*-value 0.95, $P=0.3353$), plants with wilted or dried stems (*F*-value 0.00, $P=0.9947$) or plants with rotten petioles or leaves (*F*-value 0.26, $P=0.6150$) had no effect on total yield.

Among different symptom categories only the incidence of stem rot had an influence on the total proportion of rotten tubers at harvest and in storage. The influence of stem rot on rotting of tubers was pronounced. One unit increase in number of plants with stem rot increased the proportion of rotten tubers by almost 7 units (estimate for the slope of regression line 0.66, *F*-value 33.74, $P<0.0001$).

Detection of *Dickeya* from symptomatic potato tissue with conventional and real-time PCR

To verify that the stem rot symptoms observed in the inoculated plants were caused by *Dickeya* infection, stem samples were collected from some of the diseases plants in the field trial. The bacteria present in the plant tissues were grown as mixed cultures on CVP-S2 plates and the bacterial cells from the mixed cultures were used as template in a conventional PCR test (Nassar et al. 1996). The presence of *Dickeya* could be verified in most of the samples taken from plants inoculated with biovar 3 strains or with *D. dianthicola*. Also one of the samples from control plants treated with water but showing symptoms of infection during the last inspection on 11th of September was positive (Table 3).

Because the cultivation of bacteria from the samples on plates followed by conventional PCR was rather a laborious method for the identification of bacteria from large numbers of samples, a real-time PCR test was developed based on the 16 S–23 S sequence (Laurila et al. 2008). The PCR primers were selected to detect all the *Dickeya* strains, but primers could not be found within available sequences that

Table 2 Pair-wise statistical comparison of *Dickeya* strains in their ability to delay growth or cause stem rot

Symptom type	Pair-wise comparison of *Dickeya* strains[a]	Odds ratio estimate[b]	*P*-value
Delayed growth			
	D t0433 vs. D s0411	8.8	0.0427
	D t0433 vs. D w0443	8.8	0.0427
Stem rot			
	D w04K vs. D s0411	166.6	<.0001
	D w04K vs. D w0431	157.9	<.0001
	D w04K vs. D t042	52.9	<.0001
	D w04K vs. D w0440	38.0	<.0001
	D w04K vs. D s0413	9.3	<.0001
	D w04K vs. D w0443	7.9	<.0001
	D w04K vs. D s0432-1	7.2	<.0001
	D w04K vs. D t0433	6.8	<.0001
	D w04K vs. D w04M	2.8	0.0023
	D w04M vs. D w0431	56.9	<.0001
	D w04M vs. D s0411	55.6	<.0001
	D w04M vs. D t042	18.5	<.0001
	D w04M vs. D w0440	13.7	<.0001
	D w04M vs. D s0413	3.4	0.0012
	D w04M vs. D w0443	2.9	0.0038
	D w04M vs. D s0432-1	2.6	0.0066
	D w04M vs. D t0433	2.5	0.0111
	D t0433 vs. D s0411	23.3	0.0026
	D t0433 vs. D w0431	23.2	0.0025
	D t0433 vs. D t042	7.5	0.0018
	D t0433 vs. D w0440	5.6	0.0031
	D s0432-1 vs. D s0411	21.7	0.0033
	D s0432-1 vs. D w0431	21.5	0.0033
	D s0432-1 vs. D w0440	5.2	0.0048
	D s0433-1 vs. D t042	7.0	0.0028
	D w0443 vs. D w0431	19.9	0.0042
	D w0443 vs. D s0411	20.0	0.0042
	D w0443 vs. D t042	6.5	0.0042
	D w0443 vs. D w0440	4.8	0.0075
	D s0413 vs. D w0431	16.9	0.0070
	D s0413 vs. D t042	5.5	0.0098
	D s0413 vs. D w0440	4.1	0.0181

[a] Only comparisons with statistically significant difference ($P<0.05$) are presented of the total 65 different comparisons made for each symptom type

[b] Odds ratio estimate shows how many times more probable the occurrence of the symptom is for the first strain vs. the second strain

detected all the *Dickeya* strains and excluded *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum* strains. Because the strains in group III were isolated only from river water and because they did not appear to be particularly virulent, at least on potato, the real-time PCR primers were designed based on the sequences of the strains in groups I and II. These primers, Df (AGAGTCAAAAGCGTCTTG) and Dr (TTTCACCCACCGTCAGTC), amplified a 133 bp fragment from biovar 3 strains in group I, *D. dianthicola* strains in group II, and type strains of the different *Dickeya* species, but they did not recognise the *Pectobacteria* strains (Fig. 6a).

The standard curve prepared from cells grown in laboratory media showed that the real-time PCR assay could detect bacterial cells without DNA isolation,

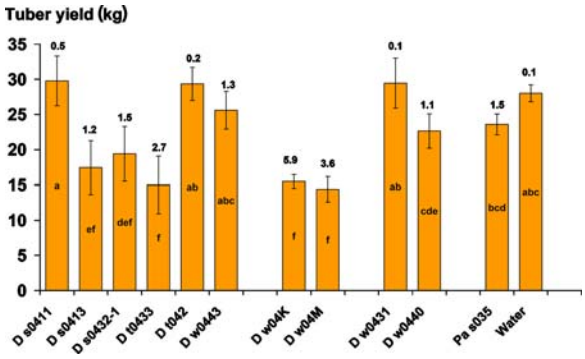

Fig. 5 The average tuber yield kg/block containing eleven plants inoculated with each *Dickeya* strain, *Pectobacterium atrosepticum* or treated with water in the mock treatment. Strains D s0411–D w0443 correspond to biovar 3 strains, D w04K and D w04M are *D. dianthicola*, D w0431 and Dw0440 represent the unidentified strains in group III, Pa s035 is a *P. atrosepticum* used as control and water denotes mock inoculation with tap water. The error bars show standard deviation between the yields of five replicates. The strains producing yield that do not differ statistically from each other are marked with the same letter inside the bars and the amount of rotten tubers is reported as percentage on the top of the bars

even if only a low number of target cells were present (Fig. 6b). The frozen stem samples, which had been previously analysed with conventional PCR, were re-analysed with the real-time test along with additional samples (Table 3). Samples with Ct values corresponding to 10^2 bacteria or more ml^{-1} were considered clearly positive and samples with lower Ct values were classified as negative. Most of the samples collected from plants inoculated with virulent biovar 3 strains or with *D. dianthicola* strains scored positive, whereas most of the other samples did not give a positive reaction. The real-time test results agreed with the conventional PCR test results for all the other samples, except for one sample taken from the water-treated negative control plant (Table 3). For this sample the positive result obtained with conventional PCR could not be repeated with real-time PCR, suggesting that the initial result might have been caused by contamination.

Discussion

Phenotypic characterisation of *Dickeya* strains isolated in a survey in 2004–2005 in Finland showed that most of the strains isolated from potato plants were similar to biovar 3. It was suggested that similar *Dickeya* strains isolated recently in the Netherlands form a new, previously unidentified clade within the *Dickeya* genus. The similarity between Finnish and Dutch strains is evident from the identical 16 S rRNA and *recA* genes (Sławiak et al. 2009; Pirhonen, unpublished). Previously, biovar 3 *Dickeya* strains have been isolated from potato in warm climates such as in Australia and Peru, whereas in Europe biovar 3 strains have been identified from other plants such as ornamentals or corn (Cother and Powell 1983; Nassar et al. 1994; Ferreira-Pinto et al. 1994). It has been suggested previously that biovar 3 *Dickeya* strains typically infect potato in tropical climates (Boccara et al. 1991). However, the phylogenetic analysis of the 16 S, 16S–23S and *recA* sequences suggested that the new European biovar 3 strains are different from the earlier biovar 3 isolates now included in the species *D. dadantii* and *D. zeae* (Laurila et al. 2008; Sławiak et al. 2009). It has been suggested that the source of the potato-infecting biovar 3 strains is ornamentals, from which the bacteria have spread to potato fields and are now spreading with commercial seed potatoes (Tsror et al. 2009; Sławiak et al. 2009). This idea is supported by the isolation of biovar 3 strains in Finland from newly released varieties for which seed potato was recently imported. The incidence of biovar 3 strains in two rivers in southern Finland and the

Table 3 Identification of *Dickeya* sp. from diseased stems with conventional and real-time PCR, shown as number of positive samples out of the number of analysed samples

Strain	Conventional PCR[a]	Real-time PCR
D s0411	0/1	0/1
D s0413	2/2	5/5
D s0432-1	2/2	4/5
D t0433	2/3	4/5
D t042	0/1	0/1
D w0443	2/2	5/5
D w04K	2/2	5/5
D w04M	2/2	4/4
D w0431	0/2	0/3
D w0440	0/2	0/2
Pa s035	0/2	0/2
Water	1/5	0/5

[a] The samples that were analysed with conventional PCR were re-analysed with real-time PCR and the results were found to be identical. Only exception was the positive sample from the water-treated control plant; this was negative in real-time PCR

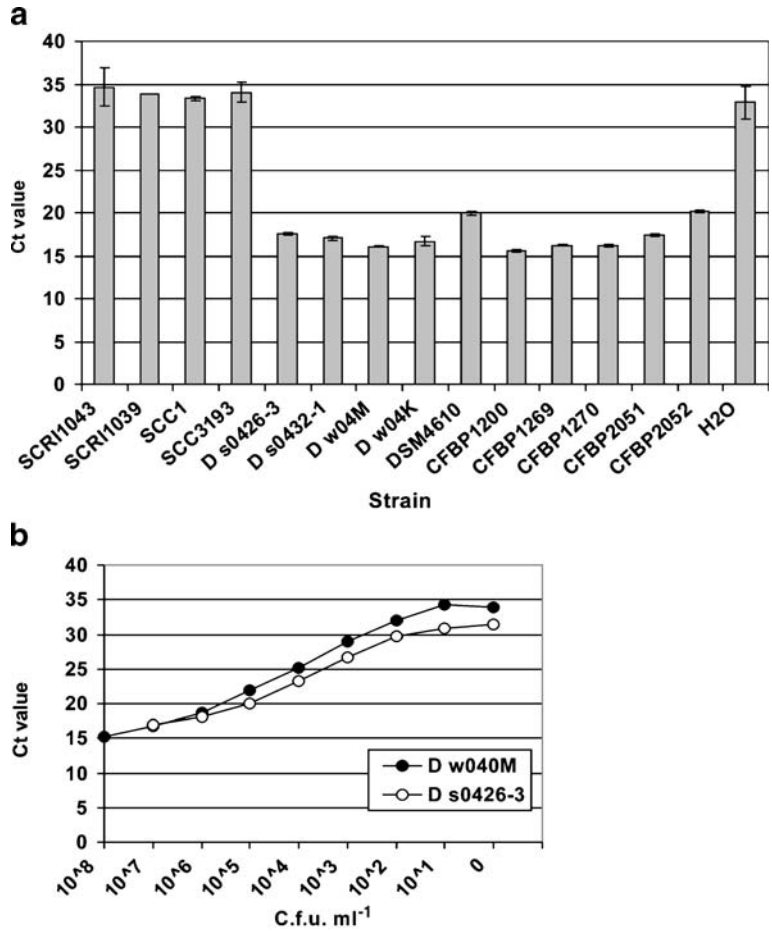

Fig. 6 Detection of *Dickeya* with real-time PCR. Identification of *Dickeya* species with real-time PCR from *in vitro*-grown bacterial cells without DNA isolation. **a** Bacteria were grown in LB overnight and adjusted with water to 0.25 OD_{600} and 5 µl of the bacterial solution was used as a template in real-time PCR. The test detected the Finnish *D. dianthicola* (D w04M and D w04K) and biovar 3 strains (D s0426-3 and D s0432-1) as well as all the type strains corresponding to previously characterised five *Dickeya* species, but did not detect *P. atrosepticum* (SCRI1043 and SCRI1039) or *P. carotovorum* subsp. *carotovorum* strains (SCC1 and SCC3193). **b** Standard curve for determination of bacterial concentration in samples. Serial 10-fold dilutions were prepared in water of overnight cultures grown in LB and 5 µl of the dilutions were used as template in real-time PCR

possible spread of the bacteria in the field suggest that they may be able to survive and spread in the environment.

The virulence varied among the Finnish biovar 3 strains. Some of the strains were more virulent than others both in stem and tuber assays (Laurila et al. 2008) as well as in the field trial. Besides virulence, no other differences between strains having high and low virulence could be detected. Similar variation in virulence among the strains in this clade has not been reported elsewhere.

The strains in group II that resembled *D. dianthicola* in phylogenetic analyses of 16 S sequences (Laurila et al. 2008) were characterised as biovar 1 and biovar 7, which supports the conclusion that these strains are *D. dianthicola* (Samson et al. 2005). Bacterial strains identified as biovar 1 and biovar 7 have been isolated from potato in Europe in several studies (Palacio-Bielsa et al. 2006; Hélias, unpublished). It has been suggested that *D. dianthicola* is adapted to potato in temperate climates (Boccara et al. 1991; Nassar et al. 1994; Singh et al. 2000). *Dickeya dianthicola* was found in Finland only in one potato sample obtained from a northern potato production area (Laurila et al. 2008), which also supports its prevalence in cool climates. This is the first time that

biovars 1 and 7 have been identified from river water samples. In spite of the fact that it was present in rivers, it seemed not to be present or cause symptoms in potato in southern Finland in 2004–2005, possibly as a result of high field temperatures during the growing season.

The third group of strains that were found only in river water samples resembled biovars 6 and 4. According to Samson et al. (2005) biovar 6 strains included in the *D. chrysanthemi* bv. *parthenii* and biovar 4 strains are currently called *D. paradisiaca*. These strains and also the *D. dianthicola* strains found in rivers in southern Finland may originate from imported ornamentals, fruit and other products. Alternatively, the atypical, weakly pathogenic strains may be natural free-living inhabitants of water, as suggested earlier by Cother et al. (1992).

The results of the field experiment suggested that about half of the biovar 3 strains and *D. dianthicola* can cause symptoms with high frequency in potato stems and tubers. All the *Dickeya* strains caused similar symptoms in potato plants, and the symptoms were different when compared to the blackleg caused by *P. atrosepticum*. Instead of the rotting in the stem base, the symptoms often appeared in stems higher up in the plant while the stem base remained green and hard. Internal necrosis of the pith was detected inside these stems and even in stems showing no external rotting. The symptoms caused by the *Dickeya* strains in Finland resemble the symptoms of *Dickeya* infection described earlier in warmer climates. Previously, wilting and desiccation of the leaves was observed in plants infected with *Dickeya* in Israel, South Africa and Spain, and these symptoms were associated with low RH (Lumb and Pérombelon 1984; Lumb et al. 1986; Serfontein et al. 1991; Palacio-Bielsa et al. 2006). In the tropical climate zones of Peru and in the temperate regions of Japan and Switzerland, extensive stem rot has been reported, possibly due to high RH in these production areas (Tanii and Baba 1971; Jaggi and Winiger 1979, reviewed by Lumb et al. 1986 and in Serfontein et al. 1991). The weather in 2006 when the field trial was carried out was exceptionally dry and warm, and the fields were regularly irrigated to ensure symptom development. Different symptoms might be observed in non-irrigated fields or during cooler summers.

Plants affected by *Dickeya* rarely had yellow or chlorotic foliage clearly distinguishable from the distance, water-soaked lesions or dark brown decay at the lower parts of stem base, all typical signs of *Pectobacterium* blackleg. The symptoms caused by *Dickeya* could be confused with other diseases, such as *Verticillium* wilt, or natural senescence, as has been previously suggested (Lumb et al. 1986). It was often observed that the potato plants or tubers decayed by *Dickeya* had milder and less distinct odour than in plants with *Pectobacterium* decay (Jaana Laurila and Asko Hannukkala, unpublished). There is a considerable risk that the incidence of *Dickeya* symptoms is ignored or underestimated in field inspections during the seed certification procedure, where crop health assessment for bacterial rots is based on visible symptoms. In inspections, stems of all plants with suspicious symptoms should be split in half to reveal possible internal *Dickeya* decay. It is probable that the recently observed transmission of *Dickeya* in the potato production chain is due to the fact that its symptoms are not recognised in field inspections.

Several of the *Dickeya* strains caused considerable yield loss due to lower yield and rotting of harvested tubers, which suggests that contamination of seed tubers with *Dickeya* may lead to economic losses at all levels of the potato production chain. Increased stem rot incidence in the field reduced yield and increased the incidence of rotted tubers observed during harvest and in storage. The reason why the other symptoms, such as dry, necrotic vascular tissue or rotting of leaves did not affect yield is not known. It could be speculated that only the rotting of the stems affects the transport of nutrients into the tubers, thus affecting the yield. The multiplication of the bacteria in the rotten stems may increase the entry of the bacteria into the daughter tubers either through vascular tissue or through lenticels from the soil, thus causing rotting of tubers after harvest. The dry necrotic tissue may contain lower numbers of bacteria and the rotten leaves may dry too fast for the bacteria to spread to the rest of the plant.

In this project, a real-time PCR assay was developed for the identification of Finnish *Dickeya* strains from diseased plants. Because the optimal amplicon size in real-time PCR should not exceed 150 base pairs (http://www.uic.edu/depts/rrc/cgf/realtime/primer.html), which is much shorter than the amplicon size obtained with the primers used in conventional PCR, a new primer pair was designed

for the real-time PCR assay. Besides biovar 3 strains, the primers also recognised control strains representing all known *Dickeya* species, and could thus be useful in identification of *Dickeya* strains from symptomatic plants. Comparison of the test results with the conventional PCR test from media-grown bacteria showed that these two tests are equivalent for detecting the bacteria from symptomatic stem tissue, but the real-time protocol is faster and allows determination of the bacterial concentration in the diseased tissue. However, the test should be improved to include also the group III strains.

Conventional and real-time PCR analysis of bacteria present in the inoculated plants showed that *Dickeya* was present in the samples collected from plants inoculated with the strains having high virulence, whereas no positive identification was observed with the tests from the plants corresponding to the less virulent biovar 3 strains or most of the samples collected from the control treatments. The high incidence of disease among the water-treated negative control plants late during the growing season suggests that the seed potatoes had been latently infected. That is also supported by the fact that *Dickeya* could not be identified from the plants inoculated with the weakly pathogenic biovar 3 strains and from two plants inoculated with the virulent strains, and thus even in these plants the disease might be caused by other soft rot bacteria.

Our results show that both *D. dianthicola* and some of the newly identified biovar 3 strains are highly virulent and severely reduce the yield of individual plants, suggesting that both of these pathogens are economically important potato pathogens. Therefore, more information about their distribution, dispersal, survival and other epidemiological characters as well as alternative hosts are needed for the estimation of their economical impact and development of management strategies for potato production.

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References

Allison, P. D. (1999). *Logistic regression using the SAS system: Theory and application* (p. 304). Cary: SAS Institute Inc.

Ayers, S. H., Rupp, P., & Johnson, W. T. (1919). *A study of alkali forming bacteria found in milk*. Washington: USDA; Bulletin of the US Department of Agriculture 782.

Boccara, M., Vedel, R., Lalo, D., Lebrun, M. H., & Lafay, J. F. (1991). Genetic diversity and host range in strains of *Erwinia chrysanthemi*. *Molecular Plant-Microbe Interactions, 4*, 293–299.

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

Cother, E. J., Bradley, J. K., Gillings, M. R., & Fahy, P. C. (1992). Characterization of *Erwinia chrysanthemi* biovars in alpine water sources by biochemical properties, GLC fatty acid analysis and genomic DNA fingerprinting. *Journal of Applied Bacteriology, 73*, 99–107.

Ferreira-Pinto, M. M., Cruz, M. L., & Oliveira, H. (1994). The occurrence in Portugal of the bacterial disease of maize crops caused by *Erwinia chrysanthemi*. *Plant Pathology, 43*, 1050–1054.

Gallois, A., Samson, R., Ageron, E., & Grimont, P. A. D. (1992). *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory (*Cichorium intybus* L.). *International Journal of Systematic Bacteriology, 42*, 582–588.

Jafra, S., Przysowa, J., Gwizdek-Wiśniewska, A., & van der Wolf, J. M. (2009). Potential of bulb-associated bacteria for biocontrol of hyacinth soft rot caused by *Dickeya zeae*. *Journal of Applied Microbiology, 106*, 268–277.

Jaggi, W., & Winiger, F. A. (1979). Schwarzbeinigkeit und bakterielle Welke bei Kartoffeln. *Mitteilungen fur die Schweizerische Landwirtschaft, 27*, 207–216.

Laurila, J., Ahola, V., Lehtinen, A., Joutsjoki, T., Hannukkala, A., Rahkonen, A., et al. (2008). Characterization of *Dickeya* strains isolated from potato and river water samples in Finland. *European Journal of Plant Pathology, 122*, 213–225.

Lehtinen, A., Hannukkala, A., Rantanen, T., & Jauhiainen, L. (2007). Phenotypic and genetic variation in Finnish potato-late blight populations, 1997–2000. *Plant Pathology, 56*, 480–491.

Littell, R. C., Freund, R. J., & Spector, P. C. (1991). *SAS® system for linear models* (3rd ed., p. 329). Cary: SAS Institute Inc.

Lumb, V. M., & Pérombelon, M. C. M. (1984). A comparative study of blackleg epidemiology in Scotland and Israel. Report of the Scottish Crop Research Institute for 1983, pp. 100–101.

Lumb, V. M., Pérombelon, M. C. M., & Zutra, D. (1986). Studies of a wilt disease of the potato plant in Israel caused by *Erwinia chrysanthemi*. *Plant Pathology, 35*, 196–202.

Ma, B., Hibbing, M. E., Kim, H. S., Reedy, R. M., Yedidia, I., Breuer, J., et al. (2007). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. *Phytobacteriology, 97*, 1150–1163.

Nassar, A., Bertheau, Y., Dervin, C., Narcy, J. P., & Lemattre, M. (1994). Ribotyping of *Erwinia chrysanthemi* strains in relation to their pathogenic and geographic distribution. *Applied and Environmental Microbiology, 60*, 3781–3789.

Nassar, A., Darrase, A., Lematre, M., Kotoujansky, A., Dervin, C., Vedel, R., et al. (1996). Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR amplified fragments of pel genes. *Applied and Environmental Microbiology, 62*, 2228–2235.

Ngwira, N., & Samson, R. (1990). *Erwinia chrysanthemi*: description of two new biovars (bv 8 and 9) isolated from kalanchoe and maize host plants. *Agronomie, 10*, 341–345.

Palacio-Bielsa, A., Cambra, M. A., & López, M. M. (2006). Characterisation of potato isolates of *Dickeya chrysanthemi* in Spain by a microtitre system for biovar determination. *Annals of Applied Biology, 148*, 157–164.

Pérombelon, M. C. M. (2002). Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathology, 51*, 1–12.

Pérombelon, M. C. M., & van der Wolf, J. M. (eds). (2002). *Methods for the detection and quantification of Erwinia carotovora subsp. atroseptica (Pectobacterium carotovorum subsp. atrosepticum) on potatoes: A laboratory manual*. Invergowrie: Scottish Crop Research Institute.

Samson, R., Poutier, F., Sailly, M., & Jouan, B. (1987). Caractérisation des *Erwinia chrysanthemi* isolées de *Solanum tuberosum* et d'autres plantes-hôtes selon les biovars et sérogroupes. *Bulletin OEPP, 17*, 11–16.

Samson, R., Legendre, J. B., Christen, R., Fischer le Saux, M., Achouak, W., & Gardan, L. (2005). Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 55*, 1415–1427.

Serfontein, S., Logan, C., Swanepoel, A. E., Boelema, B. H., & Theron, D. J. (1991). A potato wilt disease in South Africa caused by *Erwinia carotovora* subspecies *carotovora* and *E. chrysanthemi*. *Plant Pathology, 40*, 382–386.

Singh, U., Trevors, C. M., & De Boer, S. H. (2000). Fimbrial-specific monoclonal antibody-based ELISA for European potato strains of *Erwinia chrysanthemi* and comparison to PCR. *Plant Disease, 84*, 443–448.

Sławiak, M., van Beckhoven, J. R. C. M., Speksnijder, A. G. C. L., Czajkowski, R., Grabe, G., & van der Wolf, J. M. (2009). Biochemical and genetical analysis reveal a new clade of biovar 3 *Dickeya* spp. strains isolated from potato in Europe. *European Journal of Plant Pathology, 125*, 245–261.

Tanii, A., & Baba, T. (1971). Bacterial plant diseases in Hokkaido. II. Bacterial stem rot of potato plant caused by *Erwinia chrysanthemi* Burkholder et al. (*Pectobacterium carotovorum* var. *chrysanthemi*). Hokkaido Project. Agricultural Experiment Station Bulletin No. 24, pp. 1–10.

Tsror, L. (Lahkim), Erlich, O., Lebiush, S., Hazanovsky, M., Zig, U., Sławiak, M., et al. (2009). Assessment of recent outbreaks of *Dickeya* sp. (syn. *Erwinia chrysanthemi*) slow wilt in potato crops in Israel. *European Journal of Plant Pathology, 123*, 311–320.