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Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* in naturally infected seed potatoes

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Abstract Detailed studies were conducted on the distribution of *Pectobacterium carotovorum* subsp. *carotovorum* and *Dickeya* spp. in two potato seed lots of different cultivars harvested from blackleg-diseased crops. Composite samples of six different tuber sections (peel, stolon end, and peeled potato tissue 0.5, 1.0, 2.0 and 4.0 cm from the stolon end) were analysed by enrichment PCR, and CVP plating followed by colony PCR on the resulting cavity-forming bacteria. Seed lots were contaminated with *Dickeya* spp. and *P. carotovorum* subsp. *carotovorum* (Pcc), but not with *P. atrosepticum. Dickeya* spp. and Pcc were found at high concentrations in the stolon ends, whereas relatively low densities were found in the peel and in deeper located potato tissue. Rep-PCR, 16S rDNA sequence analysis and biochemical assays, grouped all the *Dickeya* spp. isolates from the two potato seed lots as biovar 3. The implications of the results for the control of *Pectobacterium* and *Dickeya* spp., and sampling strategies in relation to seed testing, are discussed.

Keywords 16S rDNA sequencing · Biochemical assays · Blackleg · *Erwinia chrysanthemi* · *Pectobacterium atrosepticum* · Rep-PCR

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Introduction

Blackleg, a major bacterial disease of potato, is caused by bacteria belonging to *Dickeya* spp. (syn. *Erwinia chrysanthemi* or *Pectobacterium chrysanthemi* (Samson et al. 2005)), by *P. atrosepticum* (syn. *E. carotovora* subsp. *atroseptica*) (Gardan et al. 2003) (Pba), by *P. carotovorum* subsp. *carotovorum* (syn. *E. carotovora* subsp. *carotovora*) (Pcc) (De Haan et al. 2008) or by *P. c.* subsp. *brasiliensis* (Pcb) (Duarte et al. 2008). In temperate climates, *P. atrosepticum* was considered as the main causative agent of blackleg. *Dickeya* spp. was believed to be a major blackleg pathogen in tropical and subtropical regions, although 'atypical' strains of *Dickeya* spp. with a relative low growth temperature maximum were also isolated from blackleg-diseased plants in temperate regions (Janse and Ruissen 1988). Pcc is considered to play a minor role in potato blackleg in temperate zones, although it has already been proven that tuber infections with virulent Pcc strains can result in true blackleg (De Haan et al. 2008). To date, Pcb has only been found in subtropical regions (Duarte et al. 2008)

Blackleg symptoms vary depending on the initial bacterial concentrations in seed tubers, the susceptibility of the potato cultivar and environmental conditions, particularly temperature and soil moisture content (Perombelon 2002). Trials using seed potatoes vacuum-infiltrated with Pba showed that even a low concentration of 10^3 colony-forming units (cfu) ml^{-1} was sufficient to cause blackleg disease in potato (Bain

and Perombelon 1990). As well as typical blackleg symptoms, *Dickeya* spp., Pba and Pcc can cause rotting of potato tubers (soft rot) during storage (Salmond 1992, Van der Wolf and De Boer 2007).

Control of potato blackleg is hampered by the absence of effective tools and strategies. In general, knowledge on the ecology of the blackleg-causing organisms is incomplete. For example, it is unknown how *Dickeya* and *Pectobacterium* spp. are introduced in seed potatoes grown from initially pathogen-free clonal selections or from *in vitro* material. It is reported that Pcc is able to spread via surface and rain water, by aerosols and also can be transmitted by insects (Perombelon and Kelman 1980). However, for *Dickeya* spp. knowledge of the ecology and epidemiology in the potato production ecosystem is largely missing.

Selection for blackleg-resistant potato cultivars was only partially successful and never resulted in cultivars completely resistant to *Dickeya* and *Pectobacterium* spp. (Lapwood et al. 1984, Lapwood and Reed 1986). The use of physical, chemical or biological control of blackleg also resulted in a reduction, but never in an elimination of the blackleg-causing pathogens (Perombelon and Salmond 1995). Finally, there is still a need for effective seed-testing protocols to eliminate contaminated seed lots from the production system. Most detection methods still lack specificity, sensitivity or are too costly for routine application. The lack of a cost-effective sampling strategy is another constraint in seed testing programmes.

The major source of infection and the most important route of long-distance dispersal of *Dickeya* or *Pectobacterium* spp. are contaminated seed tubers. Production of pathogen-free seed lots is therefore considered as the most important strategy in controlling spread of the blackleg pathogen. Tuber contamination can occur during plant growth, but harvesting and grading are considered the most important phases (Perombelon and Van der Wolf 2002). Reduction of tuber contamination can be achieved by restricting the number of generations in the field, the application of disinfection procedures for mechanical equipment used during harvesting and grading, and disinfection of tubers (Perombelon 2002). Several methods for the reduction of pathogen populations in infected tubers were tested: hot water treatment (Robinson and Foster 1987), the use of bactericides such as streptomycin (Graham and Volcani 1961) or copper-based compounds (Aysan et al. 2003), but none resulted in an eradication of the pathogen.

Knowledge of the distribution of *Dickeya* and *Pectobacterium* spp. in and on seed tubers is required for sampling in seed testing programmes and also for the development of effective procedures for sanitation of tubers. This knowledge could also be used to understand how tuber infections occur. Hélias et al. (2000) showed that Pba was mainly present in the stolon ends of infected potato tubers although bacteria were also found in the peel. The incidence of the presence of Pba in stolon ends was always higher than in tuber peel, in which Pcc was predominantly found (De Boer 2002, Robinson and Foster 1987, Samson et al. 2005). No information is known on the distribution of *Pectobacterium* species inside tubers, and information on the distribution of *Dickeya* spp. in and on seed tubers is entirely absent.

The aim of this work was to investigate in detail the distribution and the population structure of blackleg-causing bacteria in naturally infected seed potato lots.

Materials and methods

Bacterial strains and cultivation media

Bacterial isolates of *Pectobacterium* and *Dickeya* spp. were grown at 27°C for 24–28 h on tryptic soya agar (TSA) (Oxoid) or nutrient agar (NA) (Oxoid) prior to use, unless otherwise stated. *Dickeya dianthicola* IPO1741, *P. carotovorum* subsp. *carotovorum* IPO1990 and IPO1949 and *P. atrosepticum* IPO1601 were used as reference strains in PCR amplification procedures. *Dickeya dianthicola* IPO2114, *Dickeya dadantii* IPO2120, *Dickeya* sp. IPO2222 and *Dickeya zeae* IPO2131 were used as reference strains for Rep-PCR. For long-term maintenance, strains were kept on growth factor agar (0.4 g l^{-1} KH_2PO_4, 0.05 g l^{-1} $MgSO_4.7\ H_2O$, 0.1 g l^{-1} NaCl, 0.5 g l^{-1} $NH_4H_2PO_4$, 0.01 g l^{-1} $FeCl_3$, 3 g l^{-1} yeast extract (Difco), 1 g l^{-1} glucose (Merck) and 15 g l^{-1} agar (Oxoid)) (GF) at 17°C.

Bacteria isolation from potato tubers

Two potato seed lots of cvs Arcade and Konsul, grown in different regions in the north of the Netherlands and rejected because of a high blackleg incidence in the

field, were obtained from the Dutch Plant Inspection Service for agriculture, seeds and seed potatoes (NAK).

For each seed lot, ten composite samples of ten tubers were analysed. Seed lots were washed with running tap water and dried with tissue paper. After drying, the potatoes were peeled using a hand-held kitchen vegetable peeler, excluding the stolon end part (ca. 5 mm diam). Peel (ca. 2 mm thick from the potato tuber periderm) from each lot of ten tubers (one composite sample) was collected. The peeled potato tubers were subsequently sterilised with 1% sodium hypochlorite (commercial bleach) for 5 min, washed once with tap water and subsequently sterilised with 70% ethanol for 5 min. After sterilisation, potatoes were washed twice with tap water and dried with tissue paper. A 0.5 cm-deep sample from the stolon end of each tuber was removed using a sterile cork-bore (0.5 cm diam), and the ten stolon ends were pooled. In a similar way, composite samples were made of transversely-sliced potato disks taken at 0.5, 1.0, 2.0 and 4.0 cm from the stolon end of each tuber. The knife was sterilised with 70% ethanol between each cut to minimise the possibility of cross-contamination. Ten slices taken at a specific distance from the stolon end were combined as one composite sample. All composite samples were weighed and crushed for 2 min in a food processor (CombiMax 700, Braun) after adding twice the weight of quarter-strength (1/4) Ringer's buffer (Merck) containing 0.02% diethylthiocarbamic acid (Acros Organics) as an antioxidant (Perombelon and Van der Wolf 2002). For colony counts, duplicate 100 µl of 1:1, 1:10 and 1:100 dilution of the extracts in 1/4 Ringer's buffer were spread-plated on crystal violet pectate agar (CVP) and incubated for 72 h at 28°C (Hyman et al. 2001). The cfu g^{-1} of tuber sample and cfu tuber samples were calculated for cavity-forming bacteria. These were grown to pure culture by subsequent culture on CVP and TSA (Oxoid) for further analyses.

Incidence of *Dickeya* spp. and *P. atrosepticum* presence in potato tubers

To determine the incidence of tuber infection with *Dickeya* spp. and *P. atrosepticum*, ten composite samples of ten tubers each were tested using enrichment PCR. The probability of detecting *Dickeya* spp. and *P. atrosepticum* in the composite samples of the peel, stolon end and peeled tuber disk slices at 0.5, 1.0 cm, 2.0 cm, 4.0 cm distance from the stolon end was calculated. The incidence (I) was estimated using the statistical equation: $I = ([1-(N-p)/N]^{1/n}) \times 100$ (De Boer 2002), where p is the number of composite samples that tested positive for the presence of pectinolytic bacteria, N the total number of composite samples tested and n the number of potato tubers combined together in one composite sample.

Enrichment of *Dickeya* spp. and *P. atrosepticum* in potato extracts

For enrichment, 200 µl of the potato tuber disk extracts obtained from the different distances within the tubers were added to 1,800 µl of polypectate enrichment broth (PEB) (0.3 g l^{-1} $MgSO_4.7H_2O$, 1 g l^{-1} $(NH_4)_2SO_4$, 1.31 g l^{-1} $K_2HPO_4.3H_2O$, 1.5 g l^{-1} polygalacturinic acid (Sigma), pH 7.2) (Perombelon and Van der Wolf 2002) in 2 ml Eppendorf tubes. The tubes were tightly closed to provide low oxygen conditions and incubated at 28°C for 72 h The enriched samples were used for purification of bacterial genomic DNA prior to PCR amplification.

Detection of *Dickeya* spp., *P. carotovorum* spp., virulent *P. c.* subsp. *carotovorum* and *P. atrosepticum* by colony and enrichment PCR

For characterisation of pectinolytic bacterial isolates, a colony PCR procedure was used. Cells from a suspected colony were collected from CVP or NA plates using a sterile toothpick and resuspended in 50 µl of 5 mM NaOH. Suspensions were boiled for 5 min at 95°C and immediately put on ice for 1–2 min. One or 2 µl of the cell lysate was used as a template in PCR. PCR detection of *Dickeya* spp. was performed according to Nassar et al. (1996), using ADE1/ADE2 primers (ADE1: 5' GATCAGAAAGCCCGCAGCCAGAT 3', ADE2: 5'CTGTGGCCGATCAGGATGGTTTTGTCGTGC 3'). The expected fragment length of the amplicons was 420 bp. PCR detection of *Pectobacterium* spp. was performed according to Darrasse et al. (1994), using Y1/Y2 primers (Y1: 5'TTACCGGACGCCGAGCTGTGGCGT 3', Y2: 5'CAGGAAGATGTCGTTATCGCGAGT '3). The expected fragment length of the amplicons was 434 bp. PCR detection of virulent *P. c.* subsp. *carotovorum* was performed according to De Haan et al. (2008), using

contig1R/contig1F (contig1F: 5’ CCTGCTGGCGT GGGGTATCG 3’, contig1R: 5’TTGCGGAAGATG TCGTGAGTGCG3’) primers. The expected fragment length of the amplicons was 500 bp. PCR detection of *P. atrosepticum* was performed according to Frechon et al. (1998), using Y45/Y46 (Y45: 5'TC ACCGGACGCCGAACTGTGGCGT 3', Y46: 5'TCGCCAACGTTCAGCAGAACAAGT 3') primers. The expected fragment length of the amplicons was 439 bp. In all cases, amplified DNA was detected by electrophoresis in a 1.5 % agarose gel in 0.5×TBE buffer and stained with 5 mg ml^{-1} of ethidium bromide.

For enrichment samples, bacterial DNA was extracted from 500 µl of the enrichment broth using a Genomic DNA purification Kit (Qiagen) according to manufacturer’s protocol for genomic DNA purification from Gram-negative bacteria. After extraction, approximately 100-200 ng of DNA was used in the PCR assays which were conducted as described for the colony PCR.

Detection of *Dickeya* spp. by microsphere immunoassay (MIA)

A microsphere immunoassay for characterisation of *Dickeya* spp. strains was performed as described by Peters et al. (2007) with slight modifications. Bacterial suspensions were prepared in 1/4 strength Ringer’s buffer to a final concentration of approximately 10^8 cfu ml^{-1}. Subsequently, 50 µl of the prepared suspensions were added to a well of a 96-well V-shape microtitre plate (Greiner Labor Technik) with 50 µl of magnetic beads (1,000 beads μl^{-1}) coated with IgG purified polyclonal antibodies against *D. dianthicola* (8276-01) (Prime Diagnostics, Wageningen), prepared in 2 × concentrated PBS (pH 7.4) (16 g l^{-1} NaCl, 2 g l^{-1} KH_2PO4, 29 g l^{-1} $Na_2HPO_4.12\ H_2O$) containing 0.1% Tween 20 (Acros Organics) and 1.0% of skimmed milk (Difco). Samples were incubated for 30 min in the dark, at approx. 20–24°C, with shaking (300 rpm). Plates were washed once with PBS containing 0.1% Tween 20. Per well, 50 µl of the secondary Alexa Fluor 532 (Molecular Probes) conjugated antibody solution (final concentration 40 µg ml^{-1}) in PBS was added and plates were incubated for 30 min at 20–24°C in the dark. Samples were analysed with the Luminex 100 analyser (Luminex Corporation). Analyses were finished after measuring 100 beads.

Identification of *P. carotovorum* spp. by biochemical assays

Biochemical assays, performed aseptically in test tubes, were used to differentiate *P. atrosepticum* from *P. carotovorum* subsp. *carotovorum* (Perombelon and van der Wolf 2002). Growth at 37°C was evaluated by adding 100 µl of 10^8 cfu ml^{-1} to 3 ml of nutrient broth (NB) (Oxoid) and incubating for 72 h at 37°C. The change in bacterial culture turbidity was observed visually. Acid production from maltose (Arcos Organics) and α-methyl glucosidase (Sigma) was performed as described by Perombelon and van der Wolf (2002) using minimal test medium (MTM) (10 g l^{-1} bacto-peptone (Oxoid), 0.7 ml l^{-1} 1.5% bromocresol purple solution in water, 50 ml l^{-1} 20% maltose or α-methyl glucosidase solution in water). In each case, 100 µl of 10^8 cfu ml^{-1} was added to 2.5 ml of test medium. A change in medium colour, due to the bromocresol purple serving as a pH indicator, was visually observed after 96 h. Production of reducing substances from sucrose was completed using MTM medium supplemented with 4% sucrose (Arcos Organics); 100 µl of 10^8 cfu ml^{-1} was added to 3 ml of MTM and incubated for 96 h. After adding an equal volume of Benedict’s reagent (173 g l^{-1} $Na_3C_3H_5O(COO)_3$, 100 g l^{-1} $Na_2CO_3.H_2O$, 17.3 g l^{-1} $CuSO_4.5H_2O$) to each tube, they were boiled in a water bath for 10 min before visually observing a change in medium colour. As a positive control, in each test *P. atrosepticum* IPO 1601 and *P. carotovorum* subsp. *carotovorum* IPO 1990 were used.

Identification of *Dickeya* spp. biovars by biochemical assays

Biochemical tests in 96-well microtitre plates (Greiner Labor Technik) were used for biovar determination of *Dickeya* spp. (Palacio-Bielsa et al. 2006, Samson et al. 2005). Growth of strains at 39°C was evaluated by adding 15 µl of 10^8 cfu ml^{-1} to 150 µl nutrient broth (NB) followed by incubation for 72 h. The change in turbidity was observed visually and by determining the optical density at 600 nm (OD_{600}) using a spectrophotometer. Bacterial growth at 39°C was compared with growth at 25°C under the same conditions. Utilisation of organic compounds, viz. D-tartrate, D-arabinose, D-raffinose, D-melibiose, D-mannitol, 5-keto gluconate

(all from Arcos Organics) and β-gentobiose (Sigma) was performed as described by Palacio-Bielsa et al. (2006) using Ayers medium (1 g l^{-1} $NH_4H_2PO_4$, 0.2 g l^{-1} KCl, 0.2 g l^{-1} $MgSO_4$ $.7H_2O$) with bromothymol blue (0.08 g l^{-1}) as a pH change indicator. Anaerobic hydrolysis of arginine (Arcos Organics) (arginine dihydrolase) was evaluated as described by Palacio-Bielsa et al. (2006), and inulin (Sigma) assimilation in phenol red water according to Gallois et al. (1992). Per well, 15 µl of 10^8 cfu ml^{-1} in water was added to 150 µl of Ayers medium supplemented with 0.3% of test compound (Palacio-Bielsa et al. 2006). Plates were incubated at 27°C for 72 h. A change in medium colour was visually observed after 24, 48, 72 and 96 h, and compared with control wells without bacteria. Four replicate tests were performed per isolate.

Identification of *Dickeya* spp. isolates by repetitive element PCR fingerprinting (Rep-PCR)

As all *Dickeya* spp. isolates showed the same biochemical profile in the biovar determination assay, a selection of 20 isolates randomly chosen from different tuber samples and from both seed lots were used in repetitive element PCR fingerprinting (Rep-PCR). Rep- PCR was executed according to Versalovic et al. (1991) using primers REP1R (5' IIIICGICGICATCIGGC 3') and REP2I (5' ICGICT TATCIGGCCTAC 3') with the following modifications. Genomic DNA was purified using the Qiagen Genomic DNA purification kit (Qiagen) according to the protocol provided by the manufacturer. The DNA concentration was adjusted with Millipore water (MQ) to a final concentration of approximately 100 ng $µl^{-1}$. Rep-PCR was performed in a total volume of 28 µl using 6U of Taq polymerase (Roche) per reaction and 40 PCR cycles. Amplified DNA was analysed by electrophoresis in a 1.5% agarose gel in 0.5×TBE buffer stained with 5 mg ml^{-1} of ethidium bromide. Gels were developed for 6–7 h at 100 V and at room temperature (approx. 20–24°C). A 1 kb ladder (Promega) was used as a size marker. Amplified fingerprints were compared using the Quantity One programme (Bio-Rad) according to instructions provided by the manufacturer. Cluster analyses were done with the UPGMA algorithm in order to calculate the percentage of similarity between isolates.

Identification of *Dickeya* spp. biovars by 16S rDNA sequence analysis

For purification of genomic DNA the Qiagen Genomic DNA purification Kit (Qiagen) was used. Purification was performed according to manufacturer's protocol for genomic DNA purification from Gram-negative bacteria. Amplification of a 16S rDNA fragment between 968 and 1,401 bp (numbering based on the *Escherichia coli* genome) was performed according to Heuer et al. (1999) using primers F968 (5'AACGCGAAGAACCTTAC 3') and R1401 (5'CGGTGTGTACAAGGCCCGG GAACG3'). PCR products were purified with the PCR purification kit (Qiagen) according to manufacturer's protocol. For each strain, sequencing reactions were performed with both F968 and R1401 primers using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The DNA sequences obtained were compared with available sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) for nucleotide (blastn) alignments (http://www.ncbi.nlm.nih.gov/BLAST/). Acquired 16S rDNA sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under accession numbers from EU515225 to EU515231. As a reference, GenBank sequences of *P. atrosepticum* (AY914794), *Dickeya paradisiaca* (AAF520710), *D. dianthicola* (AF520708), *D. chrysanthemi* subsp. *chrysanthemi* (Z96093), *D. chrysanthemi* subsp. *partheni* (AF520709), *D. dieffenbachia* (AF520712), and *D. dadantii* (AF520707) were used. The BioEdit Sequence Alignment Editor (Ibis Biosciences) was used for creating consensus 16S rDNA sequences from forward and reverse primers using pairwise alignment. The relationships between *Dickeya* spp. isolates were established by multiple alignment using the ClustalW2 programme accessed via the Internet (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Phylogeny studies were performed with the use of the Phylip programme (PHYLogeny Inference Package) (Felsenstein 1980). For creation of dendrograms, the Neighbour-Joining method was applied followed by calculating the *p*-distance matrix for 16S rDNA sequences with the bootstrap support fixed to 1,000 re-samplings. To root the tree, a 16S rDNA sequence from *P. atrosepticum* (GenBank AY914794) was used.

Results

Distribution of cavity-forming bacteria in potato tubers

To investigate the internal colonisation and distribution of cavity-forming bacteria in two naturally infected seed potato lots harvested from blackleg-diseased crops, potato tuber extracts from six different sample types: peel, stolon end and (peeled) tuber slices from various distances from the stolon end were analysed. For all seed lots tested, the highest numbers of cavity-forming bacteria were found in the tuber stolon ends (Fig. 1a). Relatively high densities were also found in the tuber peel and at a 0.5 cm distance from the stolon end. The densities of bacteria producing cavities on CVP (calculated as cfu g^{-1} of tuber sample) were also highest in the stolon end and decreased with increasing distance from the stolon end (Fig. 1b). In most samples no cavity-forming bacteria were found at a distance of >2 cm from the stolon end.

Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* in potato tubers

Two hundred and ninety-six cavity-forming bacteria from CVP plates, selected from different tuber parts, were grown to pure culture on TSA or GF agar; 193 strains were taken from cv. Arcade and 103 from cv. Konsul. In a PCR assay specific for *Dickeya* spp., 157 isolates (81.3%) from cv. Arcade and 73 isolates (70.8%) from cv. Konsul were positive. Fourteen isolates (7.25%) from cv. Arcade and 23 isolates (22.3%) from cv. Konsul were positive in a PCR assay specific for *Pectobacterium* spp. Twenty-nine isolates were negative in both PCRs indicating that they did not belong to *Dickeya* spp. and/or *Pectobacterium* spp.

On the basis of tuber sample weight, the densities of cavity-forming bacteria (in cfu g^{-1} of tuber sample) and the percentages of the cavity-forming bacteria positive in colony PCR for *Dickeya* spp. and *Pectobacterium* spp., the numbers of *Dickeya* spp. and Pcc bacteria per gram of tuber sample and in a tuber sample, were estimated (Fig. 2). The densities of Pcc and *Dickeya* spp. in the stolon ends were ca. 100 times higher than in the peel (Fig. 2b, d). However, the total numbers were almost equal due to the higher weight of the peel (Fig. 2a, c). The total numbers of *Dickeya* spp. and Pcc decreased with increasing distance from the stolon end.

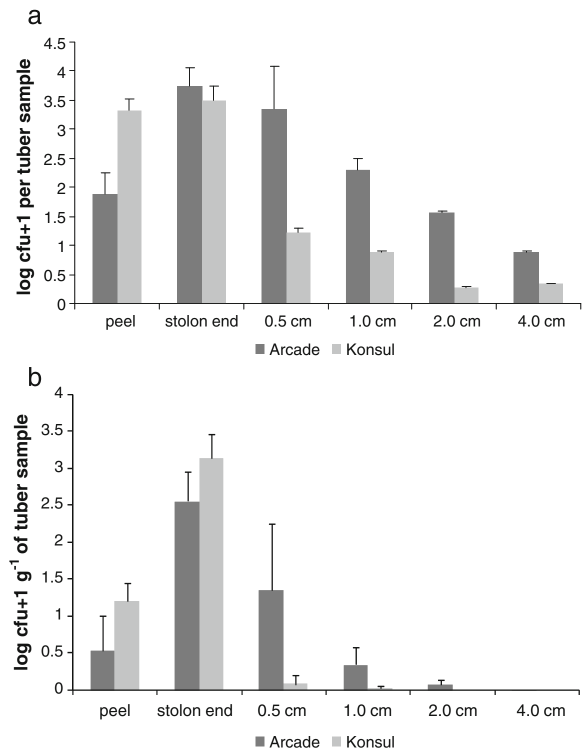


Fig. 1 Distribution of pectinolytic bacteria in tubers of two seed potato lots, shown as (a) log cfu + 1 per tuber sample and (b) as the average number of log cfu + 1 g^{-1} of tuber sample, estimated from colony counts on CVP. Six different tuber parts were sampled: the peel, the stolon end and peeled tuber slices taken at a distance of 0.5, 1, 2 and 4 cm from the stolon end

Incidence of *Dickeya* spp. and *P. atrosepticum* in potato tubers

Composite samples were analysed for *Dickeya* spp. and *P. atrosepticum* with enrichment PCR, and the incidence of bacteria present was estimated (Table. 1). None of the tested samples was positive for *P. atrosepticum.* Overall, the highest incidence of *Dickeya* spp. was found in composite extracts of stolon ends. The incidence was relatively high for peel extracts and in the peeled tuber sample extracts at 0.5 and 1 cm distance from the stolon end, and relatively low for tuber sample extracts at larger distances from the stolon end.

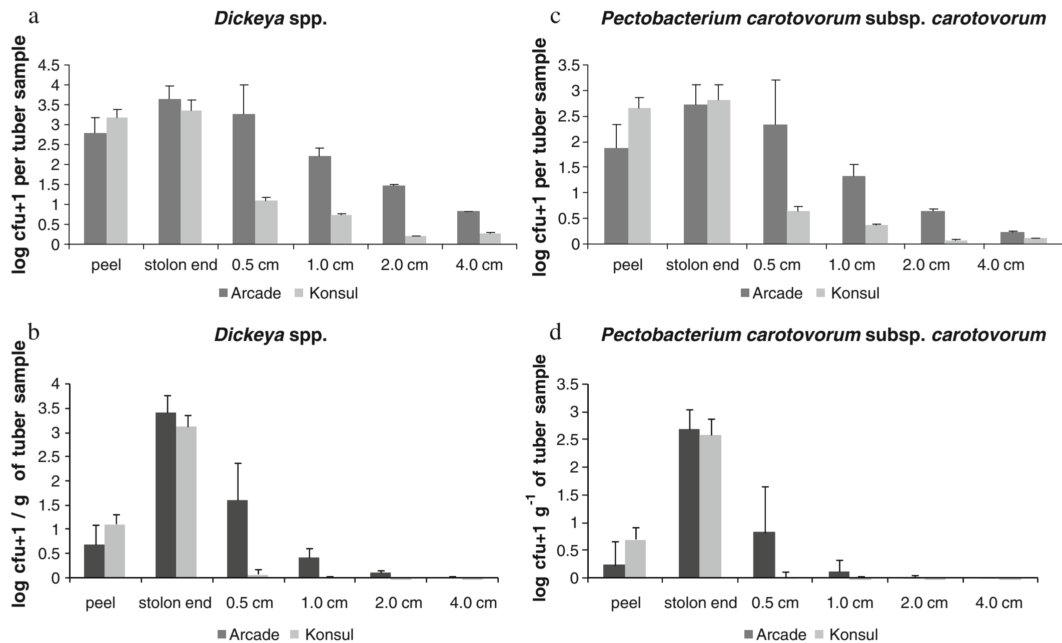


Fig. 2 Distribution of *Dickeya spp.* (a, b) and *Pectobacterium carotovorum* subsp. *carotovorum* (c, d) shown as log cfu + 1 per ml^{-1} of tuber sample (a, c) and as log cfu + 1 g^{-1} of tuber sample (b, d) for cv. Arcade and cv. Konsul. Densities were estimated on the basis of results from colony PCR analysis on cavity-forming bacteria. Six different tuber samples were taken: the peel, the stolon end and peeled tuber slices at a distance of 0.5, 1, 2 and 4 cm from the stolon end

Characterisation of *Dickeya* spp. isolates by a microsphere immunoassay (MIA)

To validate the colony PCR results for *Dickeya* spp., a selection of 38 isolates derived from different composite samples of cvs Arcade and Konsul seed lots, and selected on the basis of different colony morphology, were characterised using a microsphere immunoassay. From cv. Arcade, 27 isolates were taken: two from peel extracts, five from stolon ends, five from 0.5 cm, eight from 1.0 cm, seven from 2.0 cm and one from 4.0 cm distance from the stolon end. From cv. Konsul, 11 isolates were taken; two from peel extracts, seven from stolon end extracts, one from tuber slices taken 0.5 cm and one from 1.0 cm distance from the stolon end. All strains tested were positive in the microsphere immunoassay (data not shown).

Characterisation of *Dickeya* spp. isolates by biochemical tests

The same 38 isolates analysed in MIA were characterised with different biochemical assays for *Dickeya* spp. biovar determination. Results of the biochemical assays were identical for all tested isolates. They utilised D-raffinose, D-melibiose, and D-mannitol but were not able to use D-tartrate, L-tartrate, D-arabinose or five ketogluconate as a carbon source and were not able to grow at 39°C in NB. Strains neither assimilated inulin in peptone red water nor hydrolysed arginine under anaerobic conditions. Results showed that all 38 isolates were related closest to biovar three despite the fact that they did not grow at 39°C and did not utilise arabinose (Samson et al. 2005, Palacio-Bielsa et al. 2006).

Characterisation of *Dickeya* spp. isolates by rep-PCR

Twenty isolates, selected from the previous 38 that were characterised with the microsphere immunoassay and the biochemical tests, were analysed using rep-PCR. Two isolates were selected from each tuber sample of cv. Arcade and cv. Konsul. Fingerprints from all isolates were identical to *Dickeya* spp. IPO2222, a strain isolated from Dutch seed potatoes in 2006 and closely related to *D. zeae* IPO2131 (Fig. 3).

Table 1 Results of enrichment PCR for *Dickeya* spp. on ten composite samples of different tuber samples of two seed lots cv. Arcade and cv. Konsul

		Composite sample [a]										
Seed lot	Tuber sample [b]	1	2	3	4	5	6	7	8	9	10	Estimated incidence (%)[c]
Arcade	Peel	–[d]	+[e]	+	+	+	+	+	+	+	+	21
	Stolon end	+	+	+	+	+	-	+	+	+	+	21
	0.5 cm	+	+	+	+	+	-	+	-	-	-	9
	1.0 cm	+	−	+	−	+	−	−	−	−	−	4
	2.0 cm	+	−	+	−	+	−	−	−	−	−	4
	4.0 cm	−	−	−	−	−	−	−	−	−	+	1
Konsul												
	Peel	−	+	−	−	−	−	−	−	−	−	1
	Stolon end	−	−	+	−	+	−	+	+	−	+	7
	0.5 cm	+	−	−	−	+	−	−	−	−	−	2
	1.0 cm	−	−	−	+	+	−	−	−	−	−	2
	2.0 cm	−	−	−	−	−	−	−	−	−	−	<1
	4.0 cm	+	−	+	−	−	−	−	−	−	−	2

[a] Each composite sample represents ten potato tubers combined together

[b] Six different tuber samples were taken: the peel, the stolon end and peeled tuber slices at a distance of 0.5, 1, 2 and 4 cm from the stolon end

[c] Calculated using the equation $I=([1-(N-p)/N]^{1/n})\times 100$ (explanation provided in the text)

[d] Indicates a negative result in enrichment PCR for *Dickeya* spp.

[e] Indicates a positive result in enrichment PCR for *Dickeya* spp.

Characterisation of *Dickeya* spp. isolates by 16S rDNA

A selection of four isolates (two derived from cv. Arcade and two derived from cv. Konsul seed lots) were characterised by 16S rDNA sequencing. The sequences of the four strains were identical and according to a cluster analysis with type strains of *Dickeya* spp. deposited in the Genbank, the strains were highly similar (> 99%) to *D. dadantii* and *D. dianthicola* (data not shown).

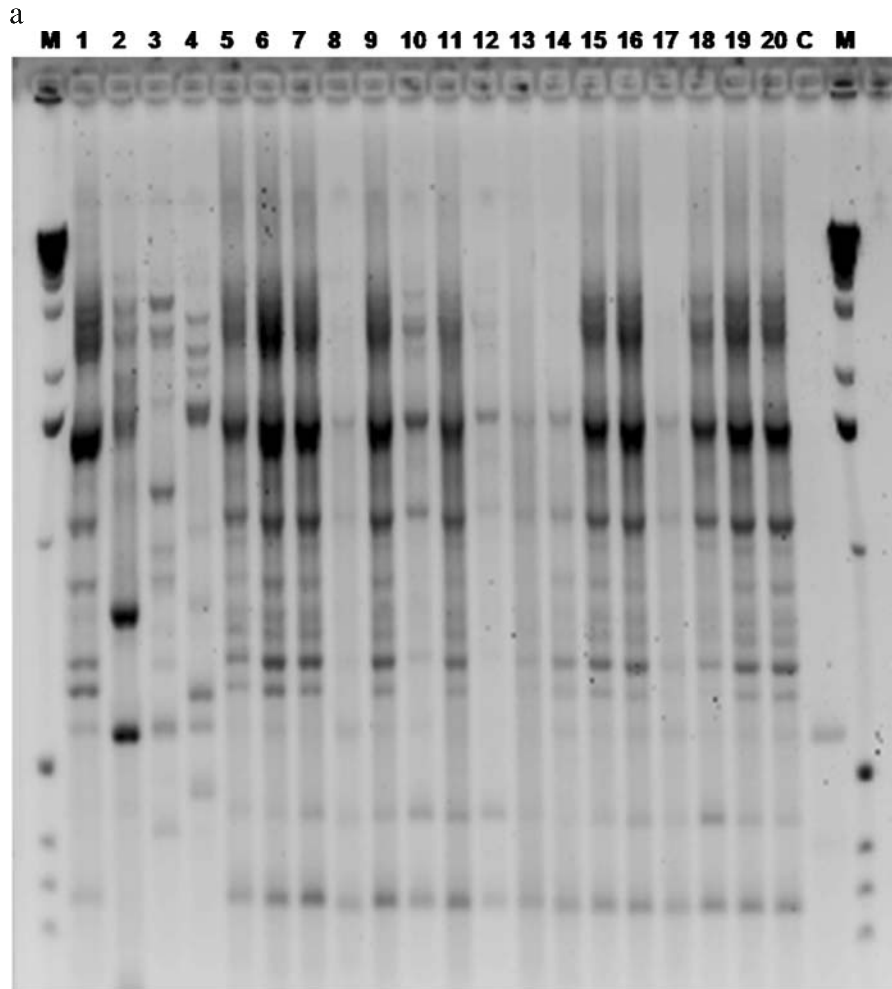
Characterisation of *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum*

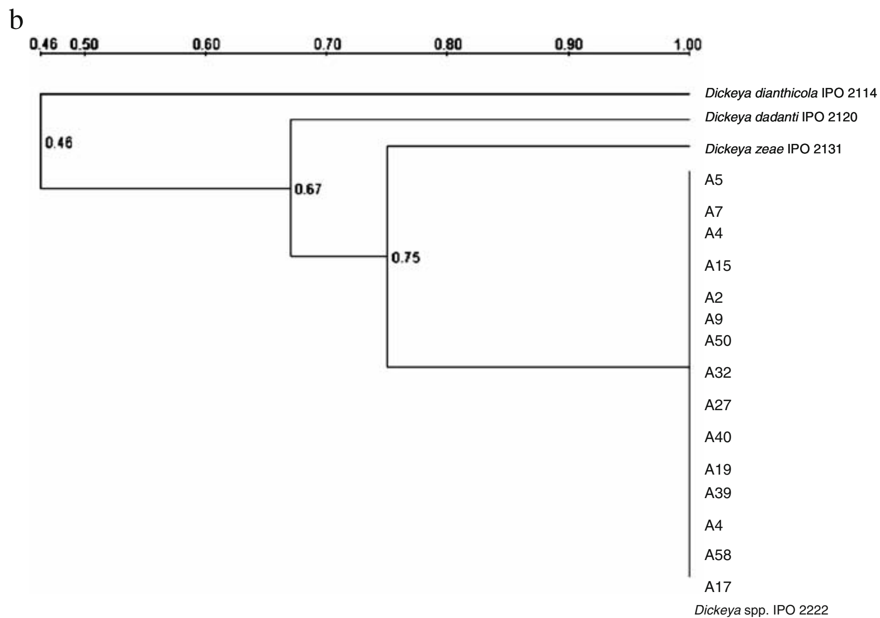
Fourteen isolates from cv. Arcade and 23 isolates from cv. Konsul positive in PCR for *Pectobacterium* spp. were all negative in the PCR specific for *P. atrosepticum* (data not shown). As there is no reliable PCR assay to characterise *Pectobacterium* spp., ten isolates per seed lot, selected from different tuber samples were tested biochemically. Isolates were able to grow at 37°C. They were not able to produce reducing substances from sucrose and were negative in acid production from maltose and α-methyl glucosidase. These characteristics are typical for *P. c.* subsp. *carotovorum*.

Characterisation of *P. c.* subsp. *carotovorum* by a PCR assay for virulent strains

Twenty isolates previously classified as *P. c.* subsp. *carotovorum* by biochemical assays were additionally

Fig. 3 Rep-PCR analysis for selected isolates of *Dickeya* spp. **a** Stained gel. M—1 kb marker, C—negative control, 1—*Dickeya* spp. IPO2222; 2—*Dickeya dianthicola* IPO2114; 3—*Dickeya dadantii* IPO2120, 4—*Dickeya zeae* IPO2131, 5—isolate A17; 6—isolate A50, 7—isolate A9; 8—isolate A21; 9—isolate A15, 10—isolate A4, 11—isolate A7, 12—isolate A5, 13—isolate A22, 14—isolate A19, 15—isolate A40, 16—isolate A27, 17—isolate A32, 18—isolate A41, 19—isolate A39, 20—isolate A58. **b** Dendrograms showing the phylogenetic relationships between isolates. The numbers adjacent to the nodes are the similarities calculated using the Neighbour-Joining method. The A-numbers are test isolates. *Dickeya dadantii* IPO2120, *Dickeya dianthicola* IPO2114, *Dickeya* spp. IPO2222 and *Dickyea zeae* IPO2131 were used as reference strains ▶


a
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 C M


b
0.46 0.50 0.60 0.70 0.80 0.90 1.00
0.46
0.67
0.75
Dickeya dianthicola IPO 2114
Dickeya dadanti IPO 2120
Dickeya zeae IPO 2131
A5
A7
A4
A15
A2
A9
A50
A32
A27
A40
A19
A39
A4
A58
A17
Dickeya spp. IPO 2222

checked by PCR to determine if they belonged to a virulent blackleg-causing subgroup of Pcc. All 20 isolates were negative in a PCR assay for virulent Pcc (data not shown).

Discussion

In this paper, the distribution of pectinolytic, blackleg-causing *Dickeya* spp. in naturally infected potato tubers is described for the first time. New information is also provided on the distribution of *P. carotovorum* subsp. *carotovorum* in seed potatoes, of which a subgroup is able to cause blackleg disease symptoms in potato in temperate climate zones (De Haan et al. 2008). The information on the distribution is important for the sampling of potatoes in seed testing programmes, to develop strategies to eliminate the pathogens in and on tubers and in general to acquire information on colonisation routes in order to develop effective management strategies.

Composite samples of tubers of two naturally-infected seed lots belonging to different cultivars and harvested from crops rejected because of the high blackleg incidences were analysed. We found that in these seed lots the highest numbers of pectinolytic bacteria were located in the stolon end and in tuber samples up to 0.5 cm distance from the stolon end, whereas only low numbers were found at larger distances from the stolon end. Frequently, the low densities of *Dickeya* spp. in deeper located tissue could only be detected after enrichment of the tuber extracts in a semi-selective broth, and not after direct plating on CVP. The estimated incidences of infection were relatively high for the peel extracts, stolon end extracts and the tissue at 0.5 cm from the stolon end, and low in deeper located tissues. It is likely that the tubers became infected via transport of bacteria through the vascular tissue from the stolon into the tuber. Also in field experiments with tubers vacuum-infiltrated with *Dickeya* spp., stolon ends became infected immediately at the formation of progeny tubers, indicating that *Dickeya* spp. readily move through vascular tissue in stems and stolons into tubers (Velvis et al. 2007). *Dickeya* spp. and *Pectobacterium* spp. seem to be less able to colonise tissues located deeper in the tuber. Relatively high numbers were also found in the peel due to lenticel infections during plant growth or contamination of tubers during harvesting and/or grading (Scott et al. 1996).

Although relatively high total numbers of pectinolytic bacteria were found in the peel, the densities (in cfu g^{-1}) were low compared to the stolon end. Due to its size and weight, the peel was found to harbour relatively large numbers of bacteria compared with the stolon end (Fig. 2). For pectinolytic bacteria, the onset of the infection process is density-dependent and regulated, among other factors, by a quorum sensing (QS) mechanism (Pirhonen et al. 1993). Synthesis of pathogenicity determinants occurs only when the bacterial population is large enough to overwhelm the plant response. Population size is sensed by the production and secretion of signal molecules called autoinducers that in high concentration can stimulate expression of genes connected with pathogenicity. From this point of view it is more likely that tuber decay is initiated from the densely populated stolon ends than from the infected peel or deeper located tissues.

So far, knowledge on the distribution and the relative incidence of pectinolytic bacteria in potato tubers is limited. Studies have been done on *P. atrosepticum* only (De Boer, 2002, Hélias et al. 2000), but never on *Dickeya* spp. or on *P. c.* subsp. *carotovorum*. Studies on *P. atrosepticum* have only been conducted to determine the relative incidence in the peel and stolon end, but never established to what depth bacteria were present in the vascular tuber sample.

For *P. atrosepticum*, De Boer (2002) found a higher incidence of infected stolon tissue than peel tissue, similar to *Dickeya* spp. Of 108 seed lots tested with enrichment ELISA for *P. atrosepticum*, 57 stolon end tissue samples were positive compared to 44 peel tissue samples (De Boer 2002). Hélias et al. (2000) also found that the stolons of *P. atrosepticum*-infected plants were more frequently infected than stems and daughter tubers, indicating the importance of transport via the vascular system in the stolons and adjacent tissues. At least these parts of the tuber should be sampled in seed testing programmes, as already advised by De Boer (2002) and practiced in inspection services in the Netherlands. Secondly, by using seed treatments, which only include superficial disinfection of tubers, a large part of the population will not be affected. Thirdly, because at harvest a large part of the contamination is already present,

hygienic measures at this time will only partly avoid infection of seed.

Enrichment PCR and dilution plating on CVP combined with characterisation of the isolates showed that seed lots were contaminated with *Dickeya* spp. and *P. c.* subsp. *carotovorum,* but not with *P. atrosepticum*. Until recently, *P. atrosepticum* was recognised as the major blackleg-causing pathogen of potato in cool and temperate climate regions. Although *D. dianthicola* had been reported to cause blackleg in northern and western Europe (Laurila et al. 2008), *Dickeya* spp. was more frequently found in regions with a higher temperature such as in Israel (Lumb et al. 1986). *Dickeya* spp. have a higher growth temperature than *P. atrosepticum* (Perombelon and Kelman 1980). The climatic change, resulting in higher temperatures during the potato growing season, may have contributed to the change in populations.

Biochemical tests, rep-PCR and 16S rDNA sequence analysis for *Dickeya* spp. proved that the test isolates were highly similar, if not identical, although they were isolated from two different potato cultivars grown at different locations in the Netherlands. They all belonged to serogroup O_1, and were similar to biovar 3, although they did not utilise arabinose and were not able to grow at 39°C. 16S rDNA sequences were identical as was the fingerprinting pattern in rep-PCR. The 16S rDNA results and biochemical data did not allow them to be designated as known *Dickeya* spp. According to 16S rDNA, the strains were closely related to *D. dianthicola* and *D. dadantii*, whereas the biochemical data suggested that they belonged to biovar 3, gathering *D. dadantii* and *D. zeae*. We were unable to identify the strains to species level, as straightforward methods for species determination are currently not available (Samson et al. 2005). Recently Tsror et al. (2008) described for the first time the presence of *Dickeya* spp. biovar 3 strains isolated from Dutch potatoes. The strains isolated in this study from two potato cvs Arcade and Konsul showed the same biochemical characteristics and rep-PCR fingerprints as presented in the work of Tsror et al. (2008). This indicates that the strains may have the same origin. It seems that this *Dickeya* spp. variant is more widely distributed in the Netherlands, suggesting that it possesses features which make it highly suitable to maintain itself as a pathogen in the potato production ecosystem. These features may include a high virulence, the production of antibacterial/antimicrobial compounds to compete with other bacteria including other *Dickeya* spp., or the ability to survive conditions unfavourable for the other blackleg-causing pathogens.

The negative results for all Pcc strains tested using PCR specific for a virulent, blackleg-causing subgroup of *P. c.* subsp. *carotovorum*, and the lack of *P. atrosepticum* present in the tested potato seed lots, indicated that the high blackleg incidence in the field was mainly due to the presence of *Dickeya* spp.

In conclusion, we have proven that tubers from blackleg-diseased crops harbour relatively high densities and high numbers of *Dickeya* spp. and *P. c.* subsp. *carotovorum* in stolon ends, whereas the peel and deeper located tuber samples are less contaminated. We have also shown that although the sampled potato tubers were taken from different cultivars and obtained from different locations, all *Dickeya* spp. isolates were identical. Characterisation of *Dickeya* spp. isolates both with biochemical assays and genetic techniques pointed to strains possessing features of biovar 3 isolates and that they were closely related to strains isolated in Israel (Tsror et al. 2008). The lack of *P. atrosepticum* and virulent Pcc strains indicated that *Dickeya* spp. was the main blackleg-causing factor in these seed lots in the field.

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