Simultaneous detection of major blackleg and soft rot bacterial pathogens in potato by multiplex polymerase chain reaction†‡

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Dickeya; differentiation; identification; pectinolytic Erwinia, Pectobacterium; sampling; specific primers.

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Abstract
A multiplex polymerase chain reaction (PCR) assay for simultaneous, fast and reliable detection of the main soft rot and blackleg potato pathogens in Europe has been developed. It utilises three pairs of primers and enables detection of three groups of pectinolytic bacteria frequently found in potato, namely: Pectobacterium atrosepticum, Pectobacterium carotovorum subsp. carotovorum together with Pectobacterium wasabiae and Dickeya spp. in a multiplex PCR assay. In studies with axenic cultures of bacteria, the multiplex assay was specific as it gave positive results only with strains of the target species and negative results with 18 non-target species of bacteria that can possibly coexist with pectinolytic bacteria in a potato ecosystem. The developed assay could detect as little as 0.01 ng μL–1 of Dickeya sp. genomic DNA, and down to 0.1 ng μL–1 of P. atrosepticum and P. carotovorum subsp. carotovorum genomic DNA in vitro. In the presence of competitor genomic DNA, isolated from Pseudomonas fluorescens cells, the sensitivity of the multiplex PCR decreased tenfold for P. atrosepticum and Dickeya sp., while no change was observed for P. carotovorum subsp. carotovorum and P. wasabiae. In spiked potato haulm and tuber samples, the threshold level for target bacteria was 101 cfu mL–1 plant extract (102 cfu g–1 plant tissue), 102 cfu mL–1 plant extract (103 cfu g–1 plant tissue), 103 cfu mL–1 plant extract (104 cfu g–1 plant tissue), for Dickeya spp., P. atrosepticum and P. carotovorum subsp. carotovorum1/P. wasabiae, respectively. Most of all, this assay allowed reliable detection and identification of soft rot and blackleg pathogens in naturally infected symptomatic and asymptomatic potato stem and progeny tuber samples collected from potato fields all over Poland.

Introduction
Potato (Solanum tuberosum L.) is the world’s third most important food crop with production rate reaching 325 million tonnes annually (Birch et al., 2012). It is also one of the most important non-staple plants in agriculture. In Europe, potato yield per hectare differs largely from country to country due to the climate, national agricultural policy, differences in the manner of potato cultivation and also because of the presence of potato diseases affecting plant growth and tubers in storage (Czajkowski et al., 2012).

Pectinolytic bacteria from Dickeya genus (Dsp) (previously Erwinia chrysanthemi), Pectobacterium atrosepticum (Pba), (previously Erwinia atroseptica) and Pectobacterium carotovorum subsp. carotovorum (Pcc), (previously Erwinia carotovora subsp. carotovora) species are recognised among the most significant bacterial pathogens of potato. They are soft rot Enterobacteriaceae (SRE), causative agents of blackleg, soft rot and wilt diseases of potato and many other important arable and horticulture crops. These diseases contribute substantially to crop loss which can result in high economic damage to farmers. For example, in
Of all soft rot and blackleg causing bacteria Pcc has the broadest host range and the ability to survive in different environments both inside and on a wide range of alternate hosts (Pérombelon & Kelman, 1980; Sledz et al., 2000). Nevertheless, its contribution to blackleg and, to a lesser extent, to tuber soft rot is still disputable (Pérombelon, 2002). In contrast, bacteria belonging to Pba are restricted to potato and for a long time were the only cause of blackleg and the main reason for tuber soft rot in temperate climate (Pérombelon, 2002). Bacteria from Dickeya genus were thought to be responsible for both diseases affecting potato grown mainly in warm and tropical climates (Pérombelon & Kelman, 1980). However, since approximately 2000, Dickeya spp., especially Dickeya dianthicola and Dickeya solani (Dsol), (van der Wolf et al., 2014b), have been isolated more frequently from symptomatic potato plants in several European countries including Poland, the Netherlands, Finland, Sweden, Germany, Spain, Belgium, Denmark and Norway (Czajkowski et al., 2009b; Degefú et al., 2013; Laurila et al., 2010; Slawiak et al., 2009a; Toth et al., 2011). These findings suggest that Dickeya spp. strains can also cause disease symptoms under temperate climatic conditions. Recently, Pectobacterium wasabiae (Pwa), so far known to cause potato blackleg in New Zealand (Fitman et al., 2010), has been detected on potato in Germany, Ireland, the Netherlands, Poland and Scotland (de Boer et al., 2012; Nabhan et al., 2012; Slawiak et al., 2013; Waleron et al., 2013) but no firm data on their relative contribution to the disease are available yet. Introduction of Pwa is a potential threat to potato production in Poland and anywhere else in Europe.

Repeated attempts to breed for resistance to these bacteria in potato using wild Solanum spp. have not been successful yet (Birch et al., 2012; Lebecka et al., 2006). Moreover, the disease control under field conditions based on physical, chemical and biological methods has also failed (Czajkowski et al., 2012). Therefore, the current practical approach is based on phytosanitary measures for the production and multiplication of pathogen-free potato seed stocks (Czajkowski et al., 2012). This involves seed certification programmes to verify seed health on during field inspections and laboratory tests using reliable and sensitive molecular techniques when assessing seed tuber contamination incidence (Toth et al., 2011).

The purpose of this work was to develop a specific and sensitive multiplex polymerase chain reaction (PCR) assay for the rapid detection of Dsp, Pba and Pcc/Pwa in symptomatic and asymptomatic potato samples. It is based on the specific primers designed previously for Dickeya spp. (Laurila et al., 2010), Pba (Frechon et al., 1998) and Pcc/Pwa (Kang et al., 2003). The herein developed multiplex PCR assay for the detection of the most important bacterial pathogens of potato was evaluated for specificity and sensitivity on a large number of axenic cultures of bacteria belonging to different species in addition to assessment performed on symptomatic and asymptomatic potato tuber and plant samples.

Materials and methods

Bacterial strains, media and culture conditions

Bacterial strains used in this study are shown in Table 1. They include the reference strains of Pectobacterium atrosepticum strain SCRI 1043 (Hinton et al., 1989), P. carotovorum subsp. carotovorum strain Ecc 71 (Willis et al., 1987), P. wasabiae strain 3193 (Nykyri et al., 2012), Dickeya solani strain IFB0099 (synonyms: D. solani strain 101A9/2005 or IPO2276), (Slawiak et al., 2009b). Bacteria were grown at 28°C for 24–48 h on crystal violet pectate medium (CVP) (Hyman et al., 2001), on Luria broth agar (LA) or in Luria broth (LB) (Bertani, 1951) prior to DNA extraction, unless otherwise stated. In case of liquid preparations, bacterial cultures were grown with shaking (200 rpm).

Bacterial cell lysates and/or genomic DNA preparation from pure cultures

For the multiplex PCR assay either bacterial cell lysates or purified bacterial genomic DNA was used. For the preparation of bacterial cell lysates, cells from a single bacterial colony growing on CVP or LA were collected using a sterile toothpick and resuspended in 500 µL of sterile double distilled water. Suspensions were frozen at ~20°C for at least 30 min prior to further preparation. Before the PCR assay they were thawed and placed on ice. For purification of bacterial genomic DNA, the Genomic Mini AX Bacteria Kit (A&G Biotechnology, Gdynia, Poland) was used according to instructions provided by the manufacturer. Genomic DNA isolated from P. fluorescens strain ATCC 13525, a typical rhizosphere and plant surface inhabitant, was used as the competitor DNA in assessing the detection level of the multiplex PCR assay.

Development of the multiplex polymerase chain reaction assay

The multiplex PCR assay was developed on the basis of previously described three specific PCRs for detection of: Dsp with primers Df (AGAGTCAAAAACGCTCCTG) and Dr (TTCACCCACCCGTCAGTC) (Laurila et al., 2010),
Table 1 Characteristics of the strains used in this study

| Ordinal Number | Genomic Species | Geographic Origin, Year of Isolation | F8 Number | Other Collection Number | Multiplex PCR | Source/Reference |
|----------------|-----------------|--------------------------------------|-----------|-------------------------|---------------|-----------------|
| 1              | Dickeya chrysanthemi bv chrysanthemi | USA, 1958 | F80055 | NCPPB 402, IPO 2118 | +  -  - | Samson et al., 2005 |
| 2              | Dickeya dadantii | Comoros, 1960 | F80010 | NCPPB 898, IPO 2120 | +  -  - | Samson et al., 2005 |
| 3              | Dickeya dadantii | USA, 1957 | F80008 | IPO 1248 | +  -  - | PRI collection |
| 4              | Dickeya dadantii | Peru | F80064 | IPO 588 | +  -  - | Slawiak et al., 2009b |
| 5              | Dickeya dianthicola | Royaume-Uni, 1956, UK | F80103 | NCPPB 453, IPO 2114 | +  -  - | Samson et al., 2005 |
| 6              | Dickeya dianthicola | Netherlands | F80028 | IPO 502 | +  -  - | Slawiak et al., 2009b |
| 7              | Dickeya dianthicola | Poland, 2009 | F80157 | 27A/1/2009 | +  -  - | This study |
| 8              | Dickeya dianthicola | Netherlands, 1992 | F80188 | IPO 1741 | +  -  - | Slawiak et al., 2009b |
| 9              | Dickeya paradisiaca | Colombia, 1970 | F80117 | NCPPB 2511, IPO 2129 | +  -  - | Samson et al., 2005 |
| 10             | Dickeya solani | Poland, 2005 | F80099 | IPO 2276 | +  -  - | Slawiak et al., 2009b |
| 11             | Dickeya solani | Israel, 2008 | F80125 | IPO 1296 | +  -  - | Tuor et al., 2013 |
| 12             | Dickeya solani | Netherlands, 2007 | F80123 | IPO 2222 | +  -  - | Slawiak et al., 2009b |
| 13             | Dickeya zeae | Egypt | F80119 | NCPPB 2538, IPO 2131 | +  -  - | PRI collection |
| 14             | Dickeya zeae | USA, 1970 | F80003 | IPO 1271 | +  -  - | Samson et al., 2005 |
| 15             | Pectobacterium atrosepticum | United Kingdom | F85399 | LMG 2386 | -  +  - | Gardan et al., 2003 |
| 16             | Pectobacterium atrosepticum | Scotland, UK | F85102 | SCR1 1043 | -  +  - | Hinton et al., 1989 |
| 17             | Pectobacterium atrosepticum | Canada, 1985 | F85103 | SCR1 1086 | -  +  - | SCR1 collection |
| 18             | Pectobacterium atrosepticum | Peru, 1978 | F85007 | SCR1 85 | -  +  - | Waleron et al., 2002 |
| 19             | Pectobacterium atrosepticum | United Kingdom | F85015 | SCR1 1092 | -  -  - | Slawiak et al., 2013 |
| 20             | Pectobacterium atrosepticum | Scotland, UK, 1977 | F85014 | SCR1 1056 | -  -  - | Waleron et al., 2002 |
| 21             | Pectobacterium atrosepticum | Scotland, UK, 1985 | F85116 | SCR1 116 | -  -  - | SCR1 collection |
| 22             | Pectobacterium atrosepticum | Scotland, UK, 1955 | F85011 | SCR1 1039 | -  -  - | Waleron et al., 2002 |
| 23             | Pectobacterium atrosepticum | Scotland | F85012 | SCR1 1054 | -  -  - | Waleron et al., 2002 |
| 24             | Pectobacterium atrosepticum | Scotland | F85104 | SCR1 1088 | -  -  - | Waleron et al., 2002 |
| 25             | Pectobacterium atrosepticum | Scotland, UK, 1982 | F85106 | SCR1 1113 | -  -  - | Waleron et al., 2002 |
| 26             | Pectobacterium atrosepticum | United Kingdom | F85105 | SCR1 1091 | -  -  - | Waleron et al., 2002 |
| 27             | Pectobacterium carotovorum subsp. carotovorum | The Netherlands | F85398 | 71 | -  -  + | Willis et al., 1987 |
| 28             | Pectobacterium carotovorum subsp. carotovorum | The Netherlands, 1974 | F85391 | IPO 200 | -  -  + | Jafra et al., 2006 |
| 29             | Pectobacterium carotovorum subsp. carotovorum | Cichorium intybus | F85392 | IPO 167 | -  -  + | Jafra et al., 2006 |
| 30             | Pectobacterium carotovorum subsp. carotovorum | Brassica oleracea | F85393 | IPO 497 | -  -  + | Jafra et al., 2006 |
|Ordinal Number| Genomic Species                      | Host          | Geographic Origin, Year of Isolation | IFB Number | Source/Reference |
|--------------|--------------------------------------|---------------|--------------------------------------|------------|-----------------|
|31            | *Pectobacterium carotovorum* subsp.  | *Caulliflower*| United Kingdom                        | FB5394     | -               |
|32            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5124     | -               |
|33            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5126     | -               |
|34            | *Pectobacterium carotovorum* subsp.  | *Solanum tuberosum* | Tasmania, 1973                      | FB5127     | -               |
|35            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5124     | -               |
|36            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5125     | -               |
|37            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5120     | -               |
|38            | *Pectobacterium carotovorum* subsp.  | *Solanum tuberosum* | Tasmania, 1973                      | FB5119     | -               |
|39            | *Pectobacterium carotovorum* subsp.  | *Solanum tuberosum* | Tasmania, 1973                      | FB5111     | -               |
|40            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5123     | -               |
|41            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1973                      | FB5187     | -               |
|42            | *Pectobacterium carotovorum*         | *Solanum tuberosum* | Tasmania, 1970                      | FB5190     | -               |
|43            | *Pectobacterium wasabiae*            | *Solanum tuberosum* | Finland, 1980s                     | FB5395     | -               |
|44            | *Pectobacterium wasabiae*            | *Solanum tuberosum* | The Netherlands, 2001                | FB5396     | -               |
|45            | *Pectobacterium wasabiae*            | *Solanum tuberosum* | The Netherlands, 2002                | FB5397     | -               |
|46            | *Pectobacterium betavasculorum*      | *Beta vulgaris* | USA, 1975                           | FB5285     | -               |
|47            | *Pectobacterium odoriferum*          | *Cichorium intybus* | France, 1978                        | FB5209     | -               |

Table 1 Continued...
Table 1 Continued

| Ordinal Number | Genomic Species | Host | Geographic Origin, Year of Isolation | IFB Number | Other Collection | Source/Reference |
|----------------|-----------------|------|-------------------------------------|------------|------------------|-----------------|
| 49             | *Agrobacterium tumefaciens* | Argyranthemum | – | – | IFB9023 | – | Goodner et al., 2001 |
| 50             | *Chryseobacterium indologenes* | *Zantedeschia* sp. | Poland | – | IFB9010 | LMG 2023 | – |
| 51             | *Chryseobacterium sp.* | *Zantedeschia* sp. | Poland | 1981 | IFB9011 | 1815 | – |
| 52             | *Clavibacter michiganensis subsp. michiganensis* | *Solanum lycopersicum* | Hungary, 1981 | – | IFB9004 | LMG 2891 | Yim et al., 2012 |
| 53             | *Clavibacter michiganensis subsp. sepedonicus* | *Solanum tuberosum* | Canada, 2001 | – | IFB9020 | LMG 2891 | Bentley et al., 2008 |
| 54             | *Escherichia coli* | – | Poland | – | IFB9029 | – | Mazodier et al., 1989 |
| 55             | *Flavobacterium sp.* | *Dieffenbachia maculata* | Poland | – | IFB9005 | 1815 | Mikicinski et al., 2010a |
| 56             | *Paenibacillus polymyxa* | *Zantedeschia* sp. | Poland | – | IFB9001 | 15M | Mikicinski et al., 2010b |
| 57             | *Paenibacillus polymyxa* | *Zantedeschia* sp. | Poland | – | IFB9002 | 16M | Mikicinski et al., 2010b |
| 58             | *Pantoea agglomerans* | *Cereal* | Canada, 1977 | – | IFB9027 | M260 | – |
| 59             | *Pseudomonas fluorescens* | – | Canada, 1981 | – | IFB9028 | – | CCM |
| 60             | *Pseudomonas marginalis* | *Zantedeschia* sp. | Poland | – | IFB9013 | 7M | Mikicinski et al., 2010c |
| 61             | *Pseudomonas marginalis* | *Zantedeschia* sp. | Poland | – | IFB9014 | 8M | Mikicinski et al., 2010c |
| 62             | *Pseudomonas putida* | *Solanum lycopersicum* | Poland | – | IFB9031 | – | Golanowska et al., 2012 |
| 63             | *Pseudomonas syringae pv. syringae* | *Syringa vulgaris* | United Kingdom | – | IFB9032 | – | – |
| 64             | *Pseudomonas syringae* pv. *shahii* | *Lycopersicon esculentum* | United Kingdom, 1980 | – | IFB9033 | LMG 1247 | – |
| 65             | *Pseudomonas syringae pv. *tomato* | – | United Kingdom | – | IFB9034 | LMG 5093 | – |
| 66             | *Ralstonia solanacearum* | *Solanum tuberosum* | Poland | – | IFB9035 | – | Norman et al., 2012 |
| 67             | *Xanthomonas campestris subsp. campestris* | *Brassica* sp., leaf | Belgium, 1980 | – | IFB9036 | – | Park et al., 2004 |

*TS – type strain.

**Note:** The collection of Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk, Gdansk, Poland. PRI collection – the collection of Plant Research International, Wageningen, The Netherlands. SCRI collection – The James Hutton Institute bacteriological collection, Dundee, Scotland. IPP collection – the collection of the Department of the Virology and Bacteriology of the Institute of Plant Protection – National Research Institute, Poznan, Poland. CCM – the Czech Collection of Microorganisms, http://www.sci.muni.cz/ccm/index.html.
Pba with primers Y\textsubscript{45} (TCACCGGACCGCACTGTCGGCT) and Y\textsubscript{46} (TGGCAACGTTCAGGAAACAAGT) (Frechon \textit{et al.}, 1998) and Pcc (together with Pwa) with primers Expcc\textsubscript{F} (GAACCTGGCAGCCGGACCTTCTA) and Expcc\textsubscript{R} (GCCGTAATTGCTACCTGCTFAAG) (Kang \textit{et al.}, 2003). Extensive optimisation steps were required to achieve proper functioning of implemented primer pairs in one PCR reaction and finally simultaneous detection of all desired groups of bacteria. The optimisation procedure included establishing the concentration of magnesium chloride (from 2 to 3 mM), reaction buffer (Fermentas, Vilnius, Lithuania) used for amplification (supplemented with 50 mM KCl or with 20 mM NH\textsubscript{4}SO\textsubscript{4}), the ratio between used primers (from 1:1:1 until the optimised one) and last but not least, the protocol for amplification. It has to be stressed that the use of a well-established positive control for each target group of bacteria in a multiplex assay for each series of tested material is crucial. It excludes any non-specific but similar in size bands that might show during the analysis while testing environmental samples.

**Specificity of the multiplex polymerase chain reaction assay**

The specificity of the multiplex PCR assay was examined using axenic cultures of 71 bacterial strains, 48 of them belonging to \textit{Pectobacterium} or \textit{Dickeya} genera (Table 1). The latter 23 strains were the isolates that may potentially be present in the same environment as tested pathogen strains, i.e. in potato tubers and haulms. For the multiplex PCR assay bacterial cell lysates were used.

**Sensitivity of the multiplex polymerase chain reaction assay in bacterial culture and in plant material**

To determine the sensitivity of the multiplex PCR assay: (a) serial dilutions in sterile double-distilled water of purified bacterial genomic DNA of Pba (SCRI 1043), Pcc (Ecc 71), Pwa, and Dsol (IFB0099) in a range of 0.001 to 10 ng (amount of the stock added to the reaction mixture) and (b) serial dilutions of LB cultures of Pba, Pwa, and Dsol of OD\textsubscript{600} = 0.75 (approximately 10\textsuperscript{5} cfu mL\textsuperscript{-1}) and diluted either in 50 mM phosphate buffer pH = 7.2 (PB) or in tuber or stem extracts with densities ranging from 10\textsuperscript{3} to 10\textsuperscript{5} cfu mL\textsuperscript{-1} were tested. The prepared serial dilutions of the purified genomic DNA were directly used for the multiplex PCR (2 \(\mu\)L), while the dilutions of bacterial cultures in plant extracts were subjected to extraction of the bacterial genomic DNA from potato stems and tubers and the multiplex PCR assay. Plant extracts (homogenates) were prepared from potato stems (cv. Irys, Plant Breeding and Acclimatization Institute – IHAR, Bonin, Poland) and tubers (cv. Irga, local market place, Gdansk, Poland). The potato stems were obtained from 3-month-old potato plants grown in the mixture of 1:1 sand and compost in a growth chamber under constant temperature and light conditions (21 °C and 16/8 h day/night photoperiod) and were used directly after harvest without any further preparations. Potato tubers were washed in running water to remove soil particles, surface-sterilised in 5% sodium hypochlorite (commercial bleach) for 20 min and washed again in sterile, distilled water. For each sample, 1 g of the plant tissue was placed in an extraction bag (Bioreba, Basel, Switzerland) together with 9 mL PB and homogenised with hand homogeniser (Bioreba, Basel, Switzerland) until complete disintegration of the tissue immediately before use.

In the environmental samples, a mixture of the pathogens may be found, and that is why, different combinations of the bacterial cell suspensions (Pcc/Pwa + Pba, Pcc/Pwa + Dsol, Pba + Dsol and Pcc/Pwa + Pba + Dsol) were prepared both by using (a) serial dilutions of purified genomic DNA and (b) serial dilutions of spiked plant extracts and tested. As the PCR assay developed by Kang \textit{et al.} (2003) does not differentiate between Pcc and Pwa, only the Pwa strain 3193 or the Pcc strain Ecc 71 has been used in the presented analyses. Additionally, 100 \(\mu\)L of each serial dilution of the reference strains prepared in PB buffer was plated on CVP to determine cell density. The experiment was performed twice.

**Potato plant and tuber material preparation for multiplex assay**

Field grown plants with disease symptoms (potato haulms and tubers) were collected in different regions of Poland. One gram of the symptomatic plant tissue was placed in the extraction bag (Bioreba, Basel, Switzerland) together with 9 mL of PB and homogenised with hand homogeniser (Bioreba) until complete disintegration of the tissue immediately before use.

For detection of latent infection in asymptomatic tubers, samples of 200 potato tubers were divided into four composite samples of 50 tubers each. Stolon ends from each tuber were collected by cutting it with a knife and pooling (approximately 7 g of tissue). Composite samples were macerated in 25 mL of PB buffer as described above.

**Extraction of the bacterial genomic DNA from potato stems and tubers**

Bacterial genomic DNA was isolated from plant extracts as described earlier (Llop et al., 1999) with the following modifications: directly after maceration of the plant
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Material, the extraction bags (Bioreba) were placed still vertically for 3 min to enable all large tissue particles to sediment at the bottom of the bag (especially starch, when homogenising potato tubers). One millilitre of the plant extract from above the sediment was collected and centrifuged at 10 000 g, for 10 min. The supernatant was discarded. The pellet was resuspended in 500 μL of DNA extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP), vortexed and incubated for an hour at room temperature with continuous shaking (ca. 100 rpm). Afterwards, the samples were centrifuged at 10 000 g for 5 min to remove plant and bacterial cells debris and 450 μL of the supernatant was collected and gently mixed with 450 μL of isopropanol (Sigma, St Louis, MO, USA). The mixture was left at room temperature for 1 h for DNA precipitation. Later on, the mixture was centrifuged at 15 000 g, for 30 min at room temperature. The supernatant was discarded, and the DNA pellet dried and resuspended in 50 μL of sterile double-distilled water. For the PCR assay, bacterial genomic DNA was diluted 10 or 100 times before analysis depending on the contaminants and total genomic DNA content.

Isolation and detection of SRE in naturally infected plants and tubers using CVP medium and conventional polymerase chain reaction

Aliquots of 100 μL of plant and tuber extracts used for multiplex PCR assay were serially diluted, plated on CVP and incubated at 21°C, 28°C or 37°C. Up to 20 individual cavity forming bacterial colonies per plate were collected and purified by CVP and LA planting before incubation at the respective temperatures. Pure bacterial colonies were used for preparations of cell lysates and subsequent testing in three separate PCR reactions for identification of Dsp, Pba and Pcc isolates as described previously (Darrasse et al., 1994; Frechon et al., 1998; Nassar et al., 1996).

Results

Multiplex polymerase chain reaction assay

We developed a multiplex PCR assay that utilises three different pairs of primers (triplex) designed previously for Dsp (Laurila et al., 2010), Pba (Frechon et al., 1998) and Pcc/Pwa (Kang et al., 2003) for the detection of major SRE pathogens in potato plant samples. We propose using presented multiplex PCR assay according to the scheme shown in Fig. 1.

The optimised multiplex PCR assay was carried out in 25 μL reaction mixture containing either 2 μL of bacterial lysate, 2 μL of genomic DNA (variable DNA concentrations per reaction mixture) isolated from plant extracts or 100 ng of genomic DNA isolated from bacterial cultures.

The reaction mixture contained 1 x reaction buffer supplemented with KCl (Fermentas), 2.5 mM MgCl₂, 80 μM of each dNTPs, 0.32 μM Df and Dr primer, 0.1 μM Y₄₅ and Y₆₅ primer, 1.2 μM ExpccF and ExpccR primer and 1 U of recombinant DNA Taq Polymerase (Fermentas). Polymerase chain reactions were performed using TGradient Biometra thermocycler according to the following settings: denaturation (95°C, 4 min), 30 cycles of denaturation (94°C, 45 s), annealing (62°C, 90 s) and extension (72°C, 90 s), with a final single extension step (72°C, 3 min). The amplified products were analysed on 1.5% agarose (Prona, Madrid, Spain) gels in 0.5 x TBE buffer. Gels were run at 100 V for approximately 40 min at room temperature and at the end stained with 0.5 mg L⁻¹ of
Figure 2 Multiplex PCR assay performed for simultaneous detection of major soft rot and blackleg pathogens: P. carotovorum subsp. carotovorum, P. wasabiae, P. atrosepticum and Dickeya spp. The assay executed with different combinations of bacterial cell lysates; the size of the bands for each tested pathogen are 550 bp (Pcc/Pwa), 420 bp (Pba), 130 bp (Dsp). Pwa – P. wasabiae 3193, Pcc – P. carotovorum subsp. carotovorum Ecc71, Pba – P. atrosepticum SCRI 1043, Dsp – Dickeya spp. IFB0099, M – size marker 100 bp (Fermentas).

Specificity of the multiplex polymerase chain reaction assay

Three pairs of primers, each detecting a distinct group of bacteria namely ExpccF/ExpccR detecting Pcc and Pwa (Kang et al., 2003), Y45/Y46 detecting Pba and Df/Dr detecting Dsp (Laurila et al., 2010) were chosen for the development of the multiplex PCR. According to relevant literature, each primer set allows specific detection of the respective potato pathogen(s) with high degree of specificity and reliability. The evaluation of the specificity of the multiplex PCR was performed with cell lysates of 71 bacterial strains and is summarised in Table 1. In the multiplex PCR all strains belonging to the target species gave specific, positive results: 14 Dsp, 12 Pba, 16 Pcc and 3 Pwa (Fig. 2, Table 1). In contrast, 26 strains from other genera and species: Pectobacterium betavasculorum (1 strain), Pectobacterium carotovorum subsp. brasiliense (1), Pectobacterium carotovorum subsp. odorifera (1), Agrobacterium tumefaciens (1), Chryseobacterium spp. (3), Clavibacter michiganensis (2), Escherichia coli (1), Flavobacterium spp. (1), Paenibacillus spp. (2), Pantoea spp. (3), Pseudomonas spp. (8), Ralstonia spp. (1), Xantomonas spp. (1) gave negative results in the multiplex PCR assay (Table 1).

Sensitivity of the multiplex polymerase chain reaction assay in vitro and in plant material artificially spiked with bacteria

The level of detection of Dsol DNA was 0.01 ng μL−1 per reaction mixture whereas the sensitivity of detection of Pcc and Pba was 0.1 ng μL−1 (Fig. 3A–C). To simulate the presence of coexisting competitor genomic DNA of other microorganisms in the sample, 100 ng of genomic DNA isolated from P. fluorescens ATCC 13525 was added to each reaction mixture. In the presence of competitor DNA, the sensitivity decreased tenfold for Dsol and Pba, while no change was observed for Pcc and Pwa (data not shown). In case of combinations of the pathogens’ genomic DNA that were also tested for sensitivity, the detection limits were the same as for single pathogen reactions (Fig. 3D–G). The presented data refer to the average of two replicates.

To verify the detection sensitivity in plant extracts we used Pwa 3193 as the exemplary strain for both Pcc and Pwa. The sensitivity for the Pwa, Pba and Dsol DNA purified from plant extracts of potato stems and tubers spiked with tenfold dilutions of bacterial cultures was
Figure 3 Detection limits of *P. carotovorum* subsp. *carotovorum* Ecc71, *P. atrosepticum* SCR11043 and *Dickeya* spp. IFB0099 (template concentration: 10–0.001 ng DNA stock solution added to PCR reaction); (A) Pcc; (B) Pba; (C) Dsp; (D) Pcc + Pba + Dsp; (E) Pba + Dsp; (F) Pcc + Dsp; (G) Pcc + Pba. Pcc – *P. carotovorum* subsp. *carotovorum* Ecc71, Pba – *P. atrosepticum* SCR11043, Dsp – *Dickeya* spp. IFB0099, M – size marker 100 bp Plus (Fermentas).

10³ cfu mL⁻¹ for Pwa (10⁴ cfu g⁻¹ tissue), 10² cfu mL⁻¹ (10³ cfu g⁻¹ tissue) for Pba and 10 cfu mL⁻¹ (10² cfu g⁻¹ tissue) for Dsol in tuber extracts (Table S1). In case of the potato haulm extracts the detection levels were very similar to the ones presented above: 10³ cfu mL⁻¹ for Pw, 10² cfu mL⁻¹ for Pba and 10² cfu mL⁻¹ for Dsol. When potato extracts were spiked with more than one bacterial pathogen (combinations of Pwa + Pba, Pwa + Dsol, Pba + Dsol and Pwa + Pba + Dsol), the sensitivity decreased 10–100 times on average, but in case of Dsol the sensitivity did not decrease at all or decreased just tenfold (Table S1). According to our results, the multiplex PCR was more sensitive in detecting multiple bacterial DNA purified from potato tuber than from potato stem extracts. The data refer to the average of two replicates.

Detection of targeted bacteria in naturally infected plant samples

Total genomic DNA isolation combined with the multiplex PCR assay was used to detect SRE in naturally infected potato stems and tubers exhibiting blackleg and/or soft rot symptoms. To that end, 66 plant samples comprising 28 different potato varieties and obtained from different regions in Poland were tested (Table S2). The multiplex PCR assay was evaluated against conventional isolation of pectinolytic bacterial colonies on CVP medium followed by identification of the growing bacteria with three separate PCR reactions using three different sets of primers. In general, the same pectinolytic bacterial pathogens were found in the same plant material with both methods. Only in the case of three samples, the obtained results differed from one another (sample 29, 31 and 44), (Table S2). In these three cases, the bacterial pathogens detected via multiplex PCR were not found after plating plant extracts on CVP (Table S2).

Detection of targeted bacteria in asymptomatic plant samples

Total genomic DNA isolation combined with the multiplex PCR assay was also used to detect SRE in asymptomatic potato tubers, which could be latently infected: 48 potato tuber samples from 12 different potato cultivars (data not shown) were tested. The results obtained from the proposed multiplex PCR and conventional methods (bacterial cells isolation on CVP and separate PCR reactions performed with each pair of primers) were in accordance...
for the majority (85.5%) of the samples tested (data not shown).

Discussion

In this study, we developed and evaluated a multiplex PCR assay for specific detection and identification of SRE most frequently associated with potato blackleg and tuber soft rot in Europe. To our knowledge this is the first multiplex PCR assay developed for simultaneous detection of Pba, Pcc/Pwa and Dsp in plant samples. We showed that with the developed multiplex assay it was possible to detect bacteria from the genus Dickeya and Pectobacterium not only in infected plants and tubers but also a very low bacterial inoculum in asymptomatic, latently infected potato tubers.

The pathogen detection in the PCR may be limited in several ways. The two most important restriction factors are the presence of competitor DNA matrices and the length of the amplified PCR products (Markoulatos et al., 2002). The primers designed for the detection of Pcc together with Pwa and Pba amplify a single-copy gene, while the primers designed for detection of Dsp amplify the 16S–23S intergenic spacer region present in more than one copy in the genome. As the results, 16S–23S is more abundant and hence easier to detect.

The developed multiplex PCR assay demonstrated high specificity for detection of the bacteria from target groups: Dsp, Pba and Pcc/Pwa. It is worth to underline that no false positive results were obtained for bacteria that can possibly co-exist with the pectinolytic ones in potato ecosystem and/or belonging to the species Chryseobacterium, Paenibacillus, Flavobacterium, Pseudomonas and Xanthomonas that, according to the literature (Mikicinski et al., 2010a–c), are able to cause tissue maceration on potato and calla lily under in vitro conditions (Table 1).

The multiplex PCR assay allows much faster and at the same time more reliable detection than conventional methods used for SRE monitoring. Plating the suspected plant extracts and the analyses with standard PCR procedures require couple of days to complete and do not always result in isolation and characterisation of the causative agents (Pérombelon & van der Wolf, 2002). The recovery of soft rot coliforms on CVP semi-selective medium combined with the conventional PCR assays performed on the isolated bacterial colonies were, in most cases, in line with the results of the multiplex PCR detection. However, in few cases, these results varied probably due to the differences in the sensitivity of the techniques and bacterial abilities to grow on CVP. Such inconsistency has been observed earlier (Fraaije et al., 1996).

There is a great need for rapid and inexpensive diagnostic tools to detect common soft rot and blackleg pathogens simultaneously and directly in plant samples. Several multiplex PCR assays for the detection of Dsp and Pba had been developed previously (Diálo et al., 2009; Peters et al., 2007; Smid et al., 1995). However, our assay is the only one designed in the triplex format, allowing simultaneous detection of all major groups of soft rot and blackleg causing bacteria. The presented multiplex PCR can be used instead of three conventional, single pathogen PCR assays with the same performance level but in a triplex format. The detection limits of the proposed method are the same as in the respective conventional PCRs. It is worth to mention that the detection limit of Dickeya sp. in our multiplex PCR assay was the same as in the real time PCR assay developed by Laurila et al. (2010) or even higher for samples of potato tuber extract (10−1 cfu mL−1) after purification of total genomic DNA.

Moreover, the detection limit of our multiplex PCR assay is also approximately 100 times higher than the detection limit of multiplex assay developed by Díaz et al. (2009) and similar to the ones of Smid et al. (1995) and Peters et al. (2007). Furthermore, when using the mixture of DNAs derived from pectinolytic bacteria and P. fluorescens the detection limit was not significantly affected as for Pcc and Pwa no decrease in the sensitivity level was observed. In case of Dsp and Pba a tenfold decrease of the detection limit was noted.

A very specific, sensitive and accurate alternative for our test is the TaqMan®-based assay (BioPlex Real Time PCR). In 2009, NAK (the Netherlands General Inspection Service for Agricultural Seeds and Seed Potatoes) introduced the multiplex TaqMan®-based assay for simultaneous detection of Pectobacterium and Dickeya spp. (de Haan & van den Bovenkamp, 2009) and in 2014 van der Wolf and coworkers described development of real-time PCR assays with the use of TaqMan probes specific to six different Dickeya species (van der Wolf et al., 2014a). De Haan & van den Bovenkamp (2009) presented a fourplex assay for the detection of Dsp, Pba, virulent Pcc (identified as P. wasabiae now) and all Pectobacterium and Dickeya spp. However, they do not provide the sequence of the primers
used and implemented reaction conditions. That is why it is impossible to use this method outside NAK. What is more, the limit of detection, according to the authors, for the bacteria tested is lower than in case of our multiplex PCR (10³ cfu mL⁻¹). The presented multiplex assay serves as an efficient and inexpensive alternative to real time PCR and can be particularly advantageous for the detection of SRE. The developed method allows detection of bacteria from the genus Dickeya but not D. solani. In Europe the only species of Dickeya that cause black-leg and soft rot on potato are D. dianthicola and D. solani (Toth et al., 2011; van der Wolf et al., 2014b). Other Dickeya species were not found on potato, although they could be found in waterways (Laurila et al., 2010; Parkinson et al., 2014). Dickeya sp. but not D. solani is considered a quarantine pathogen in some countries, for example in Scotland. In order to detect and identify D. solani an additional method can be applied. We recommend application of rep-PCR, the band pattern obtained for every environmental isolate is the same (Degfel et al., 2013; Potrykus et al., 2014). The identification of D. solani could also be performed by the recA gene amplification and its specific digestion with XbaI restriction endonuclease (Waleron et al., 2013). Both mentioned methods require isolation of viable cells to perform the exact identification. There are still no PCR or real-time PCR methods available for D. solani detection in plant homogenate that would be verified and well-established. It is a new challenge to perform such studies and provide a useful technique for D. solani detection in plant samples.

It is not possible to distinguish Pcc from Pwa with the developed multiplex PCR assay. In Europe, potato tubers and plants are not tested exclusively for the presence of Pwa as, until recently, this bacterium has not been recognised as an important potato pathogen (Nabhan et al., 2012; Waleron et al., 2013). The reclassification of Pcc 3193 to Pwa 3193 initiated analysis concerning the earlier-collected Pcc strains, which were well known for their broad heterogeneity and aberrant biochemical and genetic characteristics (Gardan et al., 2003; Waleron et al., 2002). About 15% of Pcc strains were finally reclassified as Pwa (Sławiak et al., 2013; Waleron et al., 2013). For Pwa identification the application of specific primers described by de Boer et al. (2012) can be recommended. Also, the analysis of recA gene sequence could be applied (Sławiak et al., 2013; Waleron et al., 2013).

In our opinion, the presented multiplex PCR assay will be very useful to monitor the presence of pectinolytic bacteria in complex environments. SRE can contaminate river and rain water, soli, air in addition to the surface and inner tissue of potato plants (Czajkowski et al., 2009a; Laurila et al., 2010; Pérombelon & Hyman, 1989). Future studies, exploring the role of these habitats in the epidemiology of the diseases, would benefit from this and similar multiplex PCR assays. To summarise, a specific and sensitive multiplex PCR assay has been developed to detect major groups of bacteria causing soft rot and black-leg in potato on the territory of Europe. The presented multiplex PCR procedure is rapid, inexpensive and allows detection of these pathogens simultaneously in one plant sample. It has a potential for further improvement targeting D. solani and P. wasabiae detection once their relative economic importance is established. We postulate that this assay could prove extremely valuable in the routine detection of SRE for environmental studies.

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Multiplex PCR assay for detection of soft rot pathogens

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The detection level in the multiplex PCR assay performed for the potato plant homogenates spiked with different amount of bacteria

Table S2. Detection of the pathogens in symptomatic potato plant samples obtained from different regions of Poland with the use of conventional PCR and multiplex PCR