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RESEARCH ARTICLE

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

M. Potrykus†, W. Sledz†, M. Golanowska, M. Slawiak, A. Binek, A. Motyka, S. Zoledowska, R. Czajkowski & E. Lojkowska

Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland

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Correspondence
E. Lojkowska, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland. Email: ewa.lojkowska@biotech.ug.edu.pl

†These two authors contributed equally to this work.

‡The methods, described herein, for preparation of plant material and for detection and identification of *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum* and *Dickeya* sp. are the object of patent application P.397896, which has been filed with the Polish Patent Office by University of Gdansk, Poland.

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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 10^1 cfu mL^{-1} plant extract (10^2 cfu g^{-1} plant tissue), 10^2 cfu mL^{-1} plant extract (10^3 cfu g^{-1} plant tissue), 10^3 cfu mL^{-1} plant extract (10^4 cfu g^{-1} plant tissue), for *Dickeya* spp., *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum*/*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

the Netherlands the losses in potato production due to infection caused by *Pectobacterium* and *Dickeya* spp. may reach €30 million annually (Czajkowski *et al.*, 2012).

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; Śledź *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; Degefu *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 (Pitman *et al.*, 2010), has been detected on potato in Germany, Ireland, Norway, 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&A 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 (AGAGTCAAAAGCGTCTTG) and Dr (TTTCACCCACCGTCAGTC) (Laurila *et al.*, 2010),

Table 1 Characteristics of the strains used in this study

Ordinal Number	Genomic Species[a]	Host	Geographic Origin, Year of Isolation	IFB Number[b]	Other Collection Number	Multiplex PCR			Source/Reference[c]
						Dsp	Pba	Pcc/Pwa	
***Dickeya* spp.**									
1	***Dickeya chrysanthemi* bv *chrysanthemi*[TS]**	*Chrysanthemum morifolium*	USA, 1958	IFB0055	**NCPPB 402**, IPO 2118	+	–	–	Samson *et al.*, 2005
2	***Dickeya dadantii*[TS]**	*Pelargonium capitatum*	Comoros, 1960	IFB0010	**NCPPB 898,** IPO 2120	+	–	–	Samson *et al.*, 2005
3	*Dickeya dadantii*	*Philodendron* sp.	USA, 1957	IFB0008	IPO 1248	+	–	–	PRI collection
4	*Dickeya dadantii*	*Solanum tuberosum*	Peru	IFB0064	IPO 598	+	–	–	Slawiak *et al.*, 2009b
5	***Dickeya dianthicola*[TS]**	*Dianthus caryophyllus*	Royaume-Uni, 1956, UK	IFB0103	**NCPPB 453,** IPO 2114	+	–	–	Samson *et al.*, 2005
6	*Dickeya dianthicola*	*Solanum tuberosum*	Netherlands	IFB0028	IPO 502	+	–	–	Slawiak *et al.*, 2009b
7	*Dickeya dianticola*	*Solanum tuberosum*	Poland, 2009	IFB0157	27A/1/2009	+	–	–	This study
8	*Dickeya dianticola*	*Solanum tuberosum*	Netherlands, 1992	IFB0188	IPO 1741	+	–	–	Slawiak *et al.*, 2009b
9	***Dickeya paradisiaca*[TS]**	*Musa paradisiaca*	Colombia, 1970	IFB0117	**NCPPB 2511**, IPO 2129	+	–	–	Samson *et al.*, 2005
10	*Dickeya solani*	*Solanum tuberosum*	Poland, 2005	IFB0099	IPO 2276	+	–	–	Slawiak *et al.*, 2009b
11	*Dickeya solani*	*Solanum tuberosum*	Israel, 2008	IFB0125	IPO 3296	+	–	–	Tsror *et al.*, 2013
12	***Dickeya solani*[TS]**	*Solanum tuberosum*	Netherlands, 2007	IFB0123	IPO 2222	+	–	–	Slawiak *et al.*, 2009b
13	***Dickeya zeae*[TS]**	*Zea mays*	Egypt	IFB0119	**NCPPB 2538,** IPO 2131	+	–	–	PRI collection
14	*Dickeya zeae*	*Zea mays*	USA, 1970	IFB0003	IPO 1271	+	–	–	Samson *et al.*, 2005
Pectobacterium atrosepticum									
15	***Pectobacterium atrosepticum*[TS]**	*Solanum tuberosum*, stem	United Kingdom	IFB5399	**LMG 2386**	–	+	–	Gardan *et al.*, 2003
16	*Pectobacterium atrosepticum*	*Solanum tuberosum*	Scotland, UK	IFB5102	SCRI 1043	–	+	–	Hinton *et al.*, 1989
17	*Pectobacterium atrosepticum*	*Solanum tuberosum*	Canada, 1985	IFB5103	SCRI 1086	–	+	–	SCRI collection
18	*Pectobacterium atrosepticum*	*Solanum tuberosum*, stem	Peru, 1978	IFB5007	SCRI 85	–	+	–	Waleron *et al.*, 2002
19	*Pectobacterium atrosepticum*	unknown	United Kingdom	IFB5015	SCRI 1092	–	+	–	Slawiak *et al.*, 2013
20	*Pectobacterium atrosepticum*	*Solanum tuberosum*, tuber	Scotland, UK, 1977	IFB5014	SCRI 1056	–	+	–	Waleron *et al.*, 2002
21	*Pectobacterium atrosepticum*	*Brassica napus*	Scotland, UK, 1977	IFB5116	SCRI 116	–	+	–	SCRI collection
22	*Pectobacterium atrosepticum*	*Solanum tuberosum*	Scotland, UK, 1985	IFB5011	SCRI 1039	–	+	–	Waleron *et al.*, 2002
23	*Pectobacterium atrosepticum*	*Solanum tuberosum*	Israel, 1955	IFB5012	SCRI 1054	–	+	–	Waleron *et al.*, 2002
24	*Pectobacterium atrosepticum*	*Solanum tuberosum*	Scotland	IFB5104	SCRI 1088	–	+	–	Waleron *et al.*, 2002
25	*Pectobacterium atrosepticum*	*Solanum tuberosum*	Scotland, UK, 1982	IFB5106	SCCRI 1113	–	+	–	Waleron *et al.*, 2002
26	*Pectobacterium atrosepticum*	unknown	United Kingdom	IFB5105	SCRI 1091	–	+	–	Waleron *et al.*, 2002
Pectobacterium carotovorum* subsp. *carotovorum									
27	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	The Netherlands	IFB5398	71	–	–	+	Willis *et al.*, 1987
28	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	The Netherlands, 1974	IFB5391	IPO 200	–	–	+	Jafra *et al.*, 2006
29	*Pectobacterium carotovorum* subsp. *carotovorum*	*Cichorium intybus*	The Netherlands, 1975	IFB5392	IPO 167	–	–	+	Jafra *et al.*, 2006
30	*Pectobacterium carotovorum* subsp. *carotovorum*	*Brassica oleracea*	The Netherlands, 1978	IFB5393	IPO 497	–	–	+	Jafra *et al.*, 2006

Table 1 Continued

Ordinal Number	Genomic Species[a]	Host	Geographic Origin, Year of Isolation	IFB Number[b]	Other Collection Number	Multiplex PCR			Source/Reference[c]
						Dsp	Pba	Pcc/Pwa	
31	*Pectobacterium carotovorum* subsp. *carotovorum*	Cauliflower	United Kingdom	IFB5394	IPO 280	–	–	+	Jafra *et al.*, 2006
32	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Tasmania, 1973	IFB5122	SCRI 146	–	–	+	SCRI collection
33	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*, stem	Tasmania, 1973	IFB5126	SCRI 154	–	–	+	SCRI collection
34	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*, stem	Tasmania, 1973	IFB5127	SCRI 156	–	–	+	SCRI collection
35	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Tasmania, 1973	IFB5124	SCRI 149	–	–	+	SCRI collection
36	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*, stem	Tasmania, 1973	IFB5125	SCRI 152	–	–	+	SCRI collection
37	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Arizona, USA	IFB5120	SCRI 139	–	–	+	SCRI collection
38	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Arizona, USA	IFB5119	SCRI 138	–	–	+	SCRI collection
39	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Arizona, USA	IFB5118	SCRI 136	–	–	+	SCRI collection
40	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Tasmania, 1973	IFB5123	SCRI 147	–	–	+	SCRI collection
41	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Tasmania	IFB5187	SCRI 144	–	–	+	SCRI collection
42	*Pectobacterium carotovorum* subsp. *carotovorum*	*Solanum tuberosum*	Tasmania, 1970	IFB5190	SCRI 164	–	–	+	SCRI collection
Pectobacterium wasabiae									
43	*Pectobacterium wasabiae*	*Solanum tuberosum*	Finland, 1980s	IFB5395	SCC 3193	–	–	+	Nykyri *et al.*, 2012
44	*Pectobacterium wasabiae*	*Solanum tuberosum*	The Netherlands, 2001	IFB5396	IPO 1955	–	–	+	de Haan *et al.*, 2008
45	*Pectobacterium wasabiae*	*Solanum tuberosum*	The Netherlands, 2002	IFB5397	IPO 1949	–	–	+	de Haan *et al.*, 2008
Pectobacterium carotovorum* subsp. *brasiliense									
46	*Pectobacterium carotovorum* subsp. *brasiliense*	*Solanum tuberosum*	Brazil, 2002	IFB5390	LMG 21371	–	–	–	Duarte *et al.*, 2004
Pectobacterium carotovorum* subsp. *odorifera									
47	*Pectobacterium carotovorum* subsp. *odoriferum*	*Cichorium intybus*	France, 1978	IFB5285	CFBP 1878	–	–	–	Hauben *et al.*, 1998
Pectobacterium betavasculorum									
48	*Pectobacterium betavasculorum*	*Beta vulgaris*	USA, 1975	IFB5269	ATCC 43762	–	–	–	Gardan *et al.*, 2003
Other bacteria									

Table 1 Continued

Ordinal Number	Genomic Species[a]	Host	Geographic Origin, Year of Isolation	IFB Number[b]	Other Collection Number	Multiplex PCR			Source/Reference[c]
						Dsp	Pba	Pcc/Pwa	
49	*Agrobacterium tumefaciens*	*Argyranthemum*	–	IFB9023	c58	–	–	–	Goodner *et al.*, 2001
50	*Chryseobacterium indologenes*	*Zantedeschia* sp.	Poland	IFB9010	1M	–	–	–	Mikicinski *et al.*, 2010c
51	*Chryseobacterium* sp.	*Zantedeschia* sp.	Poland	IFB9011	5M	–	–	–	Mikicinski *et al.*, 2010c
52	*Chryseobacterium* sp.	*Dieffenbachia maculata*	Poland	IFB9004	DLO2.2	–	–	–	Mikicinski *et al.*, 2010a
53	*Clavibacter michiganensis* subsp. *michiganensis*	*Solanum lycopersicum*	Hungary, 1981	IFB9020	LMG 2891	–	–	–	Yim *et al.*, 2012
54	*Clavibacter michiganensis* subsp. *sepedonicus*	*Solanum tuberosum*	Canada, 1981	IFB9021	LMG 2889	–	–	–	Bentley *et al.*, 2008
55	*Escherichia coli*	–	–	IFB9029	S17-1	–	–	–	Mazodier *et al.*, 1989
56	*Flavobacterium* sp.	*Dieffenbachia maculata*	Poland	IFB9005	2DLO2.3	–	–	–	Mikicinski *et al.*, 2010a
57	*Paenibacillus polymyxa*	*Zantedeschia* sp.	Poland	IFB9001	15M	–	–	–	Mikicinski *et al.*, 2010b
58	*Paenibacillus polymyxa*	*Zantedeschia* sp.	Poland	IFB9002	16M	–	–	–	Mikicinski *et al.*, 2010b
59	*Pantoea agglomerans*	Cereal	Canada, 1977	IFB9026	ATCC 33243	–	–	–	Jenga *et al.*, 2001
60	*Pantoea agglomerans*	*Zea mays*	Poland, 2006	IFB9027	M260	–	–	–	IPP collection
61	*Pantoea ananatis*	*Zea mays*	Poland, 2010	IFB9025	M471	–	–	–	Krawczyk *et al.*, 2010
62	*Pseudomonas fluorescens*[TS]	unknown	unknown	IFB9020	ATCC 13525	–	–	–	CCM
63	*Pseudomonas marginalis*	*Zantedeschia* sp.	Poland	IFB9013	7M	–	–	–	Mikicinski *et al.*, 2010c
64	*Pseudomonas marginalis*	*Zantedeschia* sp.	Poland	IFB9014	8M	–	–	–	Mikicinski *et al.*, 2010c
65	*Pseudomonas putida*	*Solanum lycopersicum*	Poland, 2005	IFB9031	p487	–	–	–	Golanowska *et al.*, 2012
66	*Pseudomonas syringae* pv. *syringae*[TS]	*Syringa vulgaris*	United Kingdom	IFB9033	LMG 1247	–	–	–	Ait Tayeb, 2005
67	*Pseudomonas syringae* pv. *tomato*	*Lycopersicon esculentum*	United Kingdom, 1960	IFB9032	LMG 5093	–	–	–	Kong, 2005
68	*Pseudomonas veronii*	*Zantedeschia* sp.	Poland	IFB9012	6M	–	–	–	Mikicinski *et al.*, 2010c
69	*Pseudomonas veronii*	*Zantedeschia* sp.	Poland	IFB9015	10M	–	–	–	Mikicinski *et al.*, 2010c
70	*Ralstonia solanacearum*	*Solanum tuberosum*	Colombia, 1996	IFB9024	LMG 2294	–	–	–	Norman *et al.*, 2009
71	*Xanthomonas campestris* subsp. *campestris*	*Brassica* sp., leaf	Belgium, 1980	IFB9022	LMG 582	–	–	–	Park *et al.*, 2004

[a]TS – type strain.

[b]IFB – the collection of Intercollegiate Faculty of Biotechnology University of Gdansk and Medical University of Gdansk, Gdansk, Poland.

[c]PRI collection – the collection of Plant Research International, Wageningen, The Netherlands; SCRI collection – The James Hutton Institute bacterial collection, Dundee, Scotland; IPP collection – the collection of the Department of the Virology and Bacteriology of the Institute of Plant Protection – National Research Institute, Poznań, Poland; CCM – the Czech Collection of Microorganisms, http://www.sci.muni.cz/ccm/index.html.

Pba with primers Y_{45} (TCACCGGACGCCGAACTGTGGCGT) and Y_{46} (TCGCCAACGTTCAGCAGAACAAGT) (Frechon *et al.*, 1998) and Pcc (together with Pwa) with primers ExpccF (GAACTTCGCACCGCCGACCTTCTA) and ExpccR (GCCGTAATTGCCTACCTGCTTAAG) (Kang *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_4SO_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 *Pectobacterium* or *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 (3193) 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 $OD_{600} = 0.75$ (approximately 10^9 cfu mL^{-1}) and diluted either in 50 mM phosphate buffer pH = 7.2 (PB) or in tuber or stem extracts with densities ranging from 10^1 to 10^9 cfu mL^{-1} were tested. The prepared serial dilutions of the purified genomic DNA were directly used for the multiplex PCR (2 µL), 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 *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 µL 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

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).

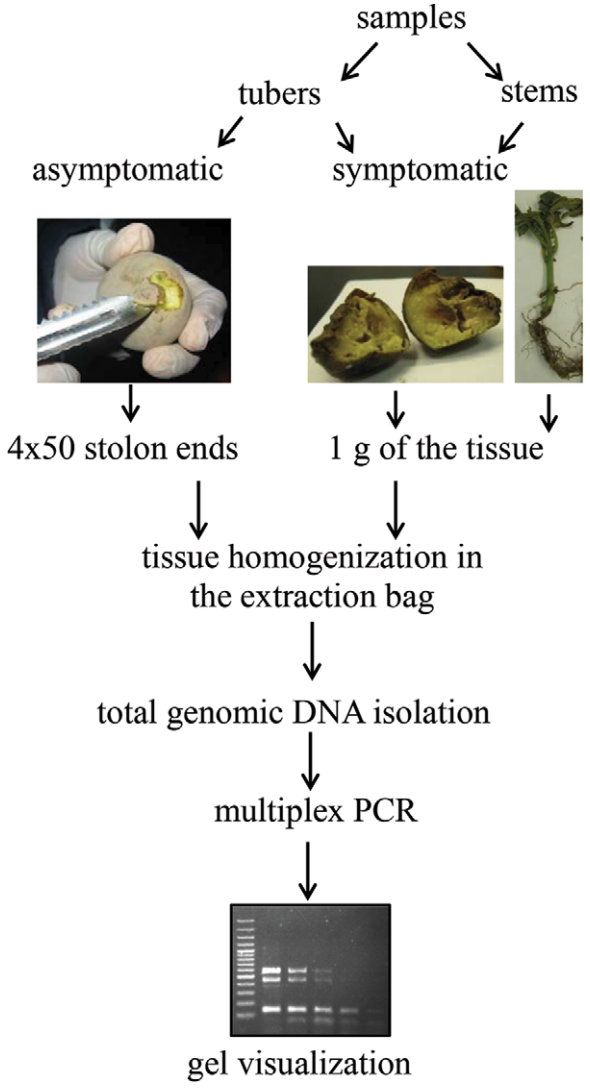

Figure 1 Scheme representing fast and simple detection of Pcc/Pwa, Pba and Dsp in environmental samples in a single step using multiplex PCR reaction.

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 1x reaction buffer supplemented with KCl (Fermentas), 2.5 mM $MgCl_2$, 80 µM of each dNTPs, 0.32 µM Df and Dr primer, 0.1 µM Y_{45} and Y_{46} 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×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

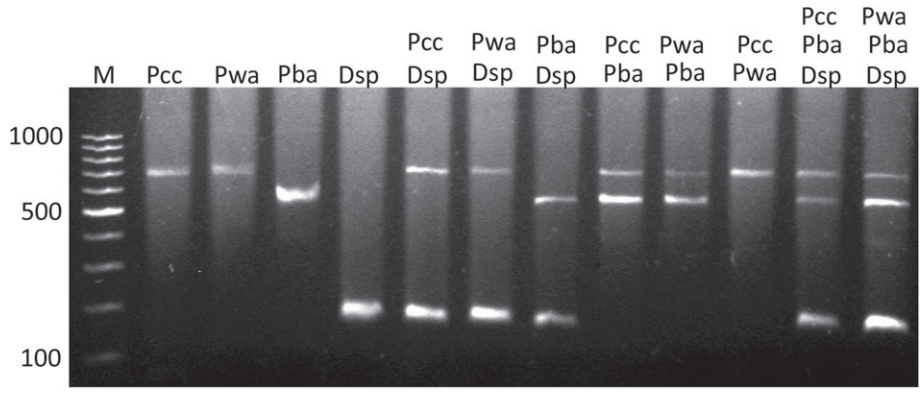

Figure 2 Multiplex PCR assay performed for simultaneous detection of major soft rot and blackleg pathogens: *P. carotovorum* subsp. *carotovorum*, *P. wasabiae*, *P. atrosepticum* and *Dickeya* spp. The assay executed with different combinations of bacterial cell lysates; the size of the bands for each 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).

ethidium bromide. A 100 bp (100 bp Gene Ruler, Fermentas) ladder was used as a size marker (Fig. 2). The obtained band sizes for each of the amplicons were as follows: 550 bp (Pcc/Pwa), 420 bp (Pba), 130 bp (Dsp). Moreover, three different polymerases were used, namely GO Taq DNA Polymerase (Promega, Madison, WI, USA), DNA Taq Polymerase (Sigma-Aldrich St. Louis, USA), and recombinant Taq Polymerase (Fermentas), to reveal that only the latter one was suitable enough for the designed PCR reaction, whereas the use of two others resulted in PCR reactions giving non-specific bands of different sizes. Additionally, to ensure maximum objectivity the multiplex PCR analysis the CVP plating followed by the PCR were performed independently by different operators in diverse conditions.

In the optimised multiplex PCR assay, the unspecific PCR products were rarely observed and appeared due to high DNA concentrations or high contamination of total genomic DNA used. Additional 10× dilution of the genomic DNA or the respective bacterial cell lysate always solved the problem of the reaction's unspecificity.

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), Y_{45}/Y_{46} 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* susbp. *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

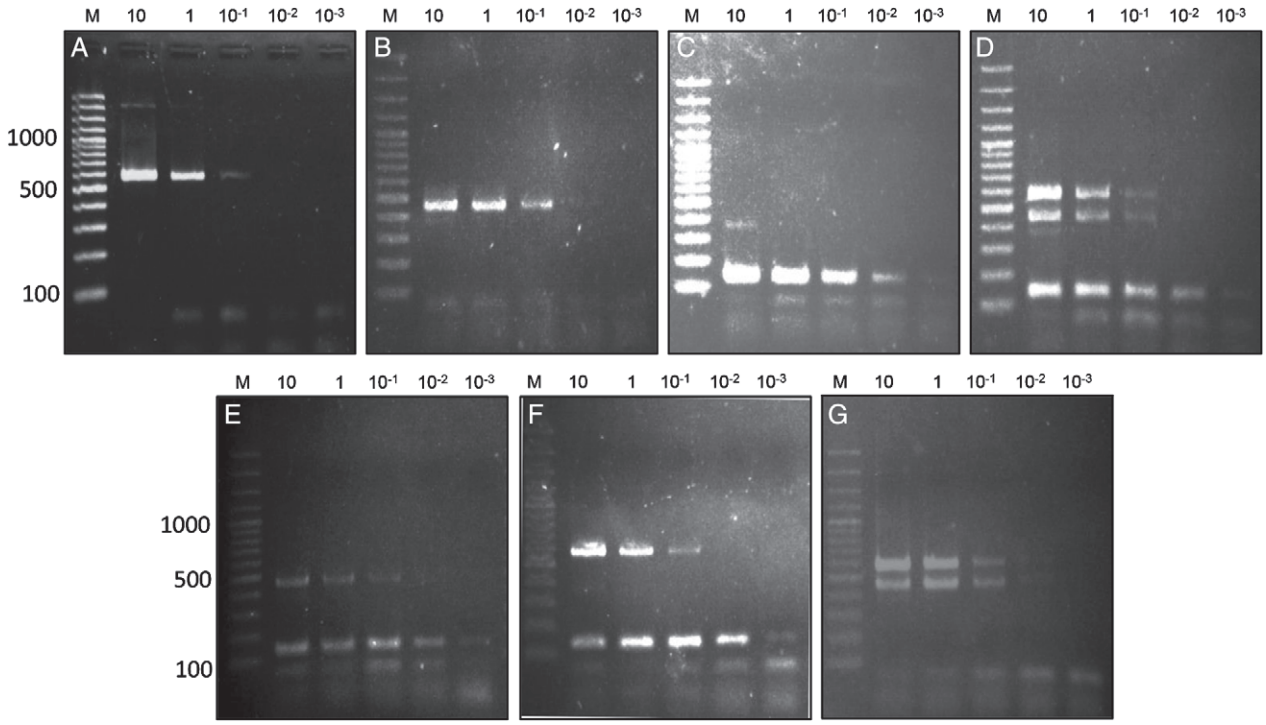

Figure 3 Detection limits of *P. carotovorum* subsp. *carotovorum* Ecc71, *P. atrosepticum* SCRI1043 and *Dickeya* spp. IFB0099 (template concentration: 10–0.001 ng DNA stock solution added to PCR reaction); (A) Pcc; (B) Pba; (C) Dsp; (D) Pcc + Pba + Dsp; (E) Pba + Dsp; (F) Pcc + Dsp; (G) Pcc + Pba. Pcc – *P. carotovorum* subsp. *carotovorum* Ecc71, Pba – *P. atrosepticum* SCRI 1043, Dsp – *Dickeya* spp. IFB0099, M – size marker 100 bp Plus (Fermentas).

10^3 cfu mL^{-1} for Pwa (10^4 cfu g^{-1} tissue), 10^2 cfu mL^{-1} (10^3 cfu g^{-1} tissue) for Pba and 10 cfu mL^{-1} (10^2 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: 10^3 cfu mL^{-1} for Pw, 10^2 cfu mL^{-1} for Pba and 10^2 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.*, 2010*a–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 may significantly differ depending on the pectate source used in the preparation of the medium (Hélias *et al.*, 2011). Furthermore, different soft rot coliforms, such as Pba or Dsp have different optimal growth temperatures and may remain unnoticed on CVP plates incubated at only one temperature of choice (de Boer, 2003). Because of these drawbacks, detection of the SRE using CVP plating techniques is becoming less popular and consequently molecular detection methods are now more routinely used (López *et al.*, 2003).

The results from plating the plant extracts on the semi-selective CVP 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 (Diallo *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 Diallo *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 (10^4 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 blackleg 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 (Degefu *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 *Xba*I 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 (Slawiak *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 (Slawiak *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.*, 2009*a*; 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 blackleg 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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References

Ait Tayeb L., Ageron E., Grimont F., Grimont P.A. (2005) Molecular phylogeny of the genus *Pseudomonas* based on rpoB sequences and application for the identification of isolates. *Research Microbiology*, **156**, 763–773.

Bashan Y., Okon Y., Henis Y. (1978) Infection studies of *Pseudomonas tomato*, causal agent of bacterial speck of tomato. *Phytoparasitica*, **6**, 135–143.

Bertani G. (1951) Studies on lysogenesis I: The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology*, **62**, 293–300.

Birch P.R.J., Bryan G., Fenton B., Gilroy E.M., Hein I., Jones J.T., Prashar A., Taylor M.A., Torrance L., Toth I.K. (2012) Crops that feed the word 8: Potato: are the trends of increased global production sustainable? *Food Security*, **4**, 477–508.

De Boer S.H. (2003) Characterization of pectolytic erwinias as highly sophisticated pathogens of plants. *European Journal of Plant Pathology*, **109**, 893–899.

De Boer S.H., Li X.Z., Ward L.J. (2012) *Pectobacterium* spp. associated with bacterial stem rot syndrome of potato in Canada. *Phytopathology*, **102**, 937–947.

Czajkowski R., Grabe G.J., van der Wolf J.M. (2009*a*) Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* in naturally infected seed potatoes. *European Journal of Plant Pathology*, **125**, 263–275.

Czajkowski R., van Veen J.A., van der Wolf J.M. (2009b) New biovar 3 *Dickeya* spp. strain (syn. *Erwinia chrysanthemi*) as a causative agent of blackleg in seed potato in Europe. *Phytopathology*, **99**, S27.

Czajkowski R., Pérombelon M.C.M., van Veen J.A., van der Wolf J.M. (2012) Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. *Plant Pathology*, **60**, 999–1013.

Darrasse A., Priou S., Kotoujansky A., Bertheau Y. (1994) PCR and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. *Applied and Environmental Microbiology*, **60**, 1437–1443.

Degefu Y., Potrykus M., Golanowska M., Virtanen E., Lojkowska E. (2013) A new clade of *Dickeya* spp. plays a major role in potato blackleg outbreaks in North Finland. *Annals of Applied Biology*, **162**, 231–241.

De Haan E.G., Dekker-Nooren T.C.E.M., van den Bovenkamp G.W., Speksnijder A.G.C.L., van der Zouwen P.S., van der Wolf J.M. (2008) *Pectobacterium carotovorum* subsp. *carotovorum* can cause potato blackleg in temperate climates. *European Journal of Plant Pathology*, **122**, 561–569.

Diallo S., Latour X., Groboillot A., Smadja B., Copin P., Orange N., Feuilloley M.G.J., Chevalier S. (2009) Simultaneous and selective detection of two major soft rot pathogens of potato: *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*) and *Dickeya* spp. (*Erwinia chrysanthemi*). *European Journal of Plant Pathology*, **125**, 349–354.

Duarte V., De Boer S.H., Ward L.J., De Oliveira A.M.R. (2004) Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *Journal of Applied Microbiology*, **96**, 535–545.

Fraaije B.A., Birnbaum Y., van den Bulk R.W. (1996) Comparison of methods for detection of *Erwinia carotovora* ssp. *atroseptica* in progeny tubers derived from inoculated tubers of *Solanum tuberosum* L. *Journal of Phytopathology*, **144**, 551–557.

Frechon D., Exbrayat P., Helias V., Hyman L.J., Jouan B., Llop P., Lopez M.M., Payet N., Pérombelon M.C.M., Toth I.K., Van Beckhoven J.R.C.M., van der Wolf J.M., Bertheau Y. (1998) Evaluation of a PCR kit for the detection of *Erwinia carotovora* subsp. *atroseptica* on potato tubers. *Potato Research*, **41**, 163–173.

Gardan L., Gouy C., Christen R., Samson R. (2003) Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 381–391.

Golanowska M., Ankiewicz H., Taraszkiewicz A., Kamysz W., Czajkowski R., Krolicka A., Jafra S. (2012) Combined effect of the antagonistic potential of selected *Pseudomonas* spp. strains and the synthetic peptide "CAMEL" on *Pseudomonas syringae* pv. *syringae* and *P. syringae* pv. *morsprunorum*. *Journal of Plant Pathology*, **94**, 69.

Goodner B., Hinkle G., Gattung S., Miller N., Blanchard M., Qurollo B., Goldman B.S., Cao Y., Askenazi M., Halling C., Mullin L., Houmiel K., Gordon J., Vaudin M., Lartchouk O., Epp A., Liu F., Wollam C., Allinger M., Doughty D., Scott C., Lappas C., Markelz B., Flanagan C., Crowell C., Gurson J., Lomo C., Sear C., Strub G., Cielo C., Slater S. (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science*, **294**, 2323–2328.

de Haan E.G., van den Bovenkamp G.W. (2009) Test development in *Erwinia* at the NAK: BioPlex real-time PCR. *Gewasbescherming*, **40**, 172–175.

Hauben L., Moore E.R.B., Vauterin L., Steenackers M., Mergaert J., Verdonck L., Swings J. (1998) Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. *Systematic and Applied Microbiology*, **21**, 384–397.

Hélias V., Hamon P., Huchet E., van der Wolf J.M., Andrivon D. (2011) Two new effective semiselective crystal violet pectate media for isolation of *Pectobacterium* and *Dickeya*. *Plant Pathology*, **61**, 339–345.

Hinton J.C.D., Sidebotham J.M., Hyman L.J., Pérombelon M.C.M., Salmond G.P.C. (1989) Isolation and characterisation of transposon-induced mutants of *Erwinia carotovora* subsp. *atroseptica* exhibiting reduced virulence. *Molecular and General Genetics*, **217**, 141–148.

Hyman L.J., Sullivan L., Toth I.K., Perombelon M.C.M. (2001) Modified crystal violet pectate medium (CVP) based on a new polypectate source (Slendid) for the detection and isolation of soft rot erwinias. *Potato Research*, **44**, 265–270.

Jafra S., Jalink H., van der Schoor R., van der Wolf J.M. (2006) *Pectobacterium carotovorum* subsp. *carotovorum* strains show diversity in production of and response to N-acyl homoserine lactones. *Journal of Plant Pathology*, **154**, 729–739.

Jeng R.S., Svircev A.M., Myers A.L., Beliaeva L., Hunter D.M., Hubbes M. (2001) The use of 16S and 16S–23S rDNA to easily detect and differentiate common Gram-negative orchard epiphytes. *Journal of Microbiological Methods*, **44**, 69–77.

Kang H.W., Kwon S.W., Go S.J. (2003) PCR-based specific and sensitive detection of *Pectobacterium carotovorum* ssp. *carotovorum* by primers generated from a URP-PCR fingerprinting-derived polymorphic band. *Plant Pathology*, **52**, 127–133.

Kong H., Blackwood C., Buyer J.S., Gulya T.J., Lydon J. (2005) The genetic characterization of *Pseudomonas syringae*

pv. tagetis based on the 16S–23S rDNA intergenic spacer regions. *Biological Control*, **32**, 356–362.

Krawczyk K., Kamasa J., Zwolinska A., Pospieszny H. (2010) First report of *Pantoea ananatis* associated with leaf spot disease of maize in Poland. *Journal of Plant Pathology*, **92**, 807–811.

Laurila J., Hannukkala A., Nykyri J., Pasanen M., Hélias V., Garlant L., Pirhonen M. (2010) Symptoms and yield reduction caused by *Dickeya* spp. strains isolated from potato and river water in Finland. *European Journal of Plant Pathology*, **126**, 249–262.

Lebecka R., Zimnoch-Guzowska E., Lojkowska E. (2006) Bacterial diseases, Chapter 10. In *Handbook of Potato Production, Improvement, and Postharvest Management*, pp. 359–386. Eds J. Gopal and S.M. Khurana. Binghamton, NY: Haworth Press.

Llop P., Caruso P., Cubero J., Morente C., López M.M. (1999) A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. *Journal of Microbiological Methods*, **37**, 23–31.

López M., Bertolini E., Olmos A., Caruso P., Gorris M., Llop P., Penyalver R., Cambra M. (2003) Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology*, **6**, 233–243.

Markoulatos P., Siafakas N., Moncany M. (2002) Multiplex polymerase chain reaction: a practical approach. *Journal of Clinical Laboratory Analysis*, **16**, 47–51.

Mazodier P., Petter R., Thompson C. (1989) Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. *Journal of Bacteriology*, **171**, 3583–3585.

Mikicinski A., Pulawska J., Sobiczewski P., Orlikowski L.B. (2010*a*) Pectolytic bacteria associated with soft rot of *Dieffenbachia* (*Dieffenbachia maculata*). *Phytopathologia*, **58**, 21–32.

Mikicinski A., Sobiczewski P., Puławska J., Treder J. (2010*b*) Involvement of *Paenibacillus polymyxa* in the etiology of bacterial soft rot of Calla Lily. *Journal of Plant Pathology*, **92**, 375–380.

Mikicinski A., Sobiczewski P., Sulikowska M., Puławska J., Treder J. (2010*c*) Pectolytic bacteria associated with soft rot of Calla Lily (*Zantedeschia* spp.) tubers. *Journal of Phytopathology*, **158**, 201–209.

Nabhan S., Wydra K., Linde M., Debener T. (2012) The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. *Plant Pathology*, **61**, 498–508.

Nassar A., Darrasse A., Lemattre M., Kotoujansky A., Dervin C., Vedel R., Bertheau Y. (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.

Norman D.J., Zapata M., Gabriel D.W., Duan Y.P., Yuen J.M.F., Mangravita-Novo A., Donahoo R.S. (2009) Genetic diversity and host range variation of *Ralstonia solanacearum* strains entering North America. *Bacteriology*, **99**, 1070–1077.

Nykyri J., Niemi O., Koskinen P., Nokso-Koivisto J., Pasanen M., Broberg M., Plyusnin I., Törönen P., Holm L., Pirhonen M., Palva E.T. (2012) Revised phylogeny and novel horizontally acquired virulence determinants of the model soft rot phytopathogen *Pectobacterium wasabiae* SCC3193. *PLoS Pathogens*, **8**, e1003013.

Park Y.J., Lee B.M., Ho-Hahn J., Lee G.B., Park D.S. (2004) Sensitive and specific detection of *Xanthomonas campestris* pv. *campestris* by PCR using species-specific primers based on *hrpF* gene sequences. *Microbiological Research*, **159**, 419–423.

Parkinson N., DeVos P., Pirhonen M., Elphinstone J. (2014) *Dickeya aquatica* sp. nov., isolated from waterways. *International Journal of Systematic and Evolutionary Microbiology*, **64**, 2264–2266.

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., Hyman L.J. (1989) Survival of soft rot coliforms, *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* in soil in Scotland. *Journal of Applied Bacteriology*, **66**, 95–106.

Pérombelon M.C.M., Kelman A. (1980) Ecology of the soft rot *Erwinias*. *Annual Review of Phytopathology*, **18**, 361–387.

Pérombelon M.C.M., van der Wolf J.M. (2002) Methods for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium carotovorum* subsp. *atrosepticum*) on potatoes: a laboratory manual. Scottish Crop Research Institute Annual Report, 10.

Peters J., Sledz W., Bergervoet J.H.W., van der Wolf J.M. (2007) An enrichment microsphere immunoassay for the detection of *Pectobacterium atrosepticum* and *Dickeya dianthicola* in potato tuber extracts. *European Journal of Plant Pathology*, **117**, 97–107.

Pitman A.R., Harrow S.A., Visnovsky S.B. (2010) Genetic characterisation of *Pectobacterium wasabiae* causing soft rot disease of potato in New Zealand. *European Journal of Plant Pathology*, **126**, 423–435.

Potrykus M., Golanowska M., Hugouvieux-Cotte-Pattat N., Lojkowska E. (2014) Regulators involved in *Dickeya solani* virulence, genetic conservation, and functional variability. *Molecular Plant–Microbe Interactions*, **27**, 700–711.

Samson R., Legendre J.B., Christen R., Fischer-Le Saux M., Achouk 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.

Slawiak M., Lojkowska E., van der Wolf J.M. (2009*a*) First report of bacterial soft rot on potato caused by *Dickeya* sp. (syn. *Erwinia chrysanthemi*) in Poland. *Plant Pathology*, **58**, 794.

Slawiak M., van Beckhoven J.R.C.M., Speksnijder A.G.C.L., Czajkowski R., Grabe G., van der Wolf J.M. (2009*b*) 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.

Slawiak M., van Doorn R., Szemes M., Speksnijder A.G.C.L., Waleron M., van der Wolf J.M., Lojkowska E., Schoen C.D. (2013) Multiplex detection and identification of bacterial pathogens causing potato blackleg and soft rot in Europe, using padlock probes. *Annals of Applied Biology*, **163**, 378–393.

Sledz W., Jafra S., Waleron M., Lojkowska E. (2000) Genetic diversity of *Erwinia carotovora* strains isolated from infected plants grown in Poland. *EPPO Bulletin*, **30**, 403–407.

Smid E.J., Jansen A.H., Gorris L.G. (1995) Detection of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* in potato tubers using polymerase chain reaction. *Plant Pathology*, **44**, 1058–1069.

Toth I.K., van der Wolf J.M., Saddler G., Lojkowska E., Hélias V., Pirhonen M., Tsror L., Elphinstone J.G. (2011) *Dickeya* species: an emerging problem for potato production in Europe. *Plant Pathology*, **60**, 385–399.

Tsror L., Ben-Daniel B., Chalupowicz L., van der Wolf J., Lebiush S., Erlich O., Dror O., Barel V., Nijhuis E., Manulis-Sasson S. (2013) Characterization of *Dickeya* strains isolated from potato grown under hot-climate conditions. *Plant Pathology*, **62**, 1097–1105.

Van der Wolf J.M., de Haas B.H., van Hoof R., de Haan E.G., van den Bovenkamp G.W. (2014a) Development and evaluation of Taqman assays for the differentiation of *Dickeya*(sub)species. *European Journal of Plant Pathology*, **138**, 695–709.

Van der Wolf J.M., Nijhuis E.H., Kowalewska M.J., Saddler G.S., Parkinson N., Elphinstone J.G., Pritchard L., Toth I.K., Lojkowska E., Potrykus M., Waleron M., de Vos P., Cleenwerck I., Pirhonen M., Garlant L., Hélias V., Pothier J.F., Pflüger V., Duffy B., Tsror L., Manulis S. (2014b) *Dickeya solani* sp. nov., a pectinolytic plant pathogenic bacterium isolated from potato (*Solanum tuberosum*). *International Journal of Systematic and Evolutionary Microbiology*, **64**, 768–774.

Waleron M., Waleron K., Lojkowska E. (2013) Occurrence of *Pectobacterium wasabiae* in potato field samples. *European Journal of Plant Pathology*, **137**, 149–158.

Waleron M., Waleron K., Podhajska A.J., Lojkowska E. (2002) Genotyping of bacteria belonging to the former *Erwinia* genus by PCR-RFLP analysis of a *recA* gene fragment. *Microbiology*, **148**, 583–595.

Willis J.W., Engwall J.K., Chatterjee A.K. (1987) Cloning of genes for *Erwinia carotovora* subsp. *carotovora* pectolytic enzymes and further characterization of the polygalacturonases. *Phytopathology*, **77**, 1199–1205.

Yim K., Lee H., Kim J., Lee S., Cho J., Cha J. (2012) Characterization of phenotypic variants of *Clavibacter michiganensis* subsp. *michiganensis* isolated from *Capsicum annuum*. *European Journal of Plant Pathology*, **133**, 559–575.

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