Eur J Plant Pathol (2009) 125:349–354
DOI 10.1007/s10658-009-9477-4

Simultaneous and selective detection of two major soft rot pathogens of potato: *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atrosepticum*) and *Dickeya* spp. (*Erwinia chrysanthemi*)

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Received: 24 October 2008 / Accepted: 16 April 2009 / Published online: 3 May 2009

Abstract *Dickeya* spp. and *Pectobacterium atrosepticum* are major pathogens of potato. Current methods to detect these soft-rotting bacteria require separate identification steps. Here we describe a simple method allowing simultaneous detection of both pathogens based on multiplex PCR. The sensitivity of the primer sets was first examined on purified genomic DNA of the type strains *Dickeya chrysanthemi* 2048^T and *P. atrosepticum* 1526^T. The specificity and detection limits of the primer sets were successfully tested on 61 strains belonging to various *Dickeya* and *Pectobacterium* species, on artificially inoculated and on naturally contaminated potato plants. This new method provides a gain in time and materials, the main advantages for large-scale processes such as pathogen-free seed certification.

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Keywords Pathogen detection · Multiplex PCR · Blackleg

Pectobacterium atrosepticum and various *Dickeya* spp. (formerly belonging to *Erwinia chrysanthemi*) are major pathogens of potato, involved in similar soft-rotting symptoms during storage, and blackleg symptoms in the field (Pérombelon 2002). *Pectobacterium atrosepticum* is classically found under cool temperate climates (Smadja et al. 2004b), but occurrence of mesophile *Dickeya* spp. in blackleg-infected plants has recently increased (Pérombelon 2002; Van der Wolf, personal communication). They represent a major bacterial threat in temperate regions where potatoes are usually grown.

As seed tubers are among the main source and vector of inoculum (Hélias et al. 2000; Pérombelon 1992), and since no chemical control of the pathogen exists, disease prevention methods are limited to sanitary measures and the use of certified pathogen-free seed tubers (Janse and Wenneker 2002). Certification necessitates large-scale and extensive detection techniques. Molecular detection methods -immunological and PCR based techniques- are the most rapid and accurate approaches (Ward et al. 2004; Alvarez 2004). However, immunological assays for *P. atrosepticum* display high detection levels but allow recognition of only two of nine serogroups (Pérombelon 2002), and use of the specific anti-*Dickeya* antibody produced by Singh et al. (2000) does not result in good detection levels. PCR-based methods allow selective, rapid and

Table 1 Bacterial strains used to test specificity of multiplex PCR assay

CFBP	Host	Origin	Collection depositor	Multiplex	
				Y45/46	Ech1/1'
Dickeya sp.					
2268 to 2274	*S. t.*	Australia	Cother E.J.	–	+
2467	*nd*[a]	France	Jouan B.	–	+
2468	*S. t.*	France	Chauveau J.F.	–	+
2469	*S. t.*	France	Jouan B.	–	+
2488	*S. t.*	France	Jouan B.	–	+
2593	*S. t.*	Peru	French E.R.	–	+
2594	*S. t.*	Peru	French E.R.	–	+
2596	*S. t.*	CH	Cazelles O.	–	+
2711	*S. t.*	Australia	Cother E.J.	–	+
3890	*S. t.*	NL	Janse J.D.	–	+
3891	*S. t.*	NL	Janse J.D.	–	+
Dickeya dadantii					
4151	*P. s.*	USA	Dye D.W.	–	+
Dickeya dianthicola					
2288	*S. t.*	France	Jouan B.	–	+
2592	*S. t.*	Brazil	Graham D.C.	–	+
3705	*S. t.*	CH	Samson R.	–	+
4155	*K. b.*	NL	Janse J.D.	–	+
Dickeya chrysanthemi bv. *chrysanthemi*					
2048[T]	*C.m.*	USA W.H.	Burkholder	–	+
Pectobacterium sp.					
194	*S. t.*	Morocco	Prunier J.P.	–	–
1336 to 1342	*S. t.*	UK	Perombelon M.C.M.	–	–
1349	*S. t.*	Italy	Mazzucchi U.	–	–
Pectobacterium atrosepticum					
511	*S. t.*	France	Prunier J.P.	+	–
1329 to 1335	*S. t.*	UK	Perombelon M.C.M.	+	–
1453	*L. e.*	France	Barzic M.R.	+	–
1525	*S. t.*	USA	Kelman A.	+	–
1526[T]	*S. t.*	UK	Graham D.C.	+	–
1527	*S. t.*	USA	Allan E.	+	–
3139	soil	UK	Logan C.	+	–
Pectobacterium betavasculorum					
1539[T]	*B. v.*	USA	Schroth M.	+	–
3291	*S. t.*	Romania	Lazar I.	+	–
Pectobacterium carotovorum subsp. *carotovorum*					
2046[T]	*S. t.*	Denmark	Hellmers E.	–	–
2136 to 2141	*S. t.*	France	Samson R.	–	–
Pectobacterium carotovorum subsp. *odoriferum*					
1878	*C.i*	France	Samson R.	–	–
2281	*C.i*	France	Samson R.	–	–

Table 1 (continued)

CFBP	Host	Origin	Collection depositor	Multiplex	
				Y45/46	Ech1/1'
Pectobacterium cypripedii					
3613^T	*Cy. sp.*	USA	*nd*	–	–
Pectobacterium wasabiae					
3304	*E.w*	Japan	Goto M.	–	–
3306	*E.w*	Japan	Goto M.	–	–
Pseudomonas marginalis pv. *marginalis*					
1538	*S. t.*	USA	Cuppels D.	–	–
4033	*S. t.*	UK	Paton A.M.	–	–

CFBP: French Collection of phytopathogenic bacteria identification number, *B.v.: Beta vulgaris; C.i: Cichorium intybus C.m.*: *Chrysanthemum morifolium; Cy.*: *Cypripedium; E.w:Eutrema wasabi; K.b.*: *Kalanchoe blossfeldiana*; *L.e.*: *Lycopersicon esculentum; P.s.*: *Philodendron scandens S.t.*: *Solanum tuberosum*

[a] *nd*: not determined

sensitive detection of *Pectobacterium* spp. (Hyman et al. 2000; Hélias et al. 1998; Darrasse et al. 1994) and the *Dickeya* genus (Smid et al. 1995; Nassar et al. 1996) but separately. In order to reduce time and material costs for the detection of these major pathogens, we developed a multiplex PCR method for simultaneous detection and distinction of these bacterial species showing high genome similarities.

In a first step, we tested the specificity of the most useful primers Y45/46 and ADE previously described for *P. atrosepticum* and *Dickeya* spp. by Fréchon et al. (1998) and Nassar et al. (1996), respectively. Specificity was assessed by testing one colony of 61 strains from a wide collection as follow: 23 *Dickeya*, 9 *Pectobacterium* sp., 13 *P. atrosepticum*, 2 *P. betavasculorum*, 9 *P. carotovorum,* 1 *P. cypredii,* 2 *P. wasabiae* and 2 *Pseudomonas marginalis* strains (Table 1). Primers Y45/46 allowed amplification of a unique 420 bp fragment (Fig. 1a) from all of the 13 *P. atrosepticum* strains (Table 1). DNA from none of the other tested strains was amplified, excepted the *P. betavasculorum* strains. This result could be explained by the high similarity of these two species as established by Avrova et al. (2002). However, meso-

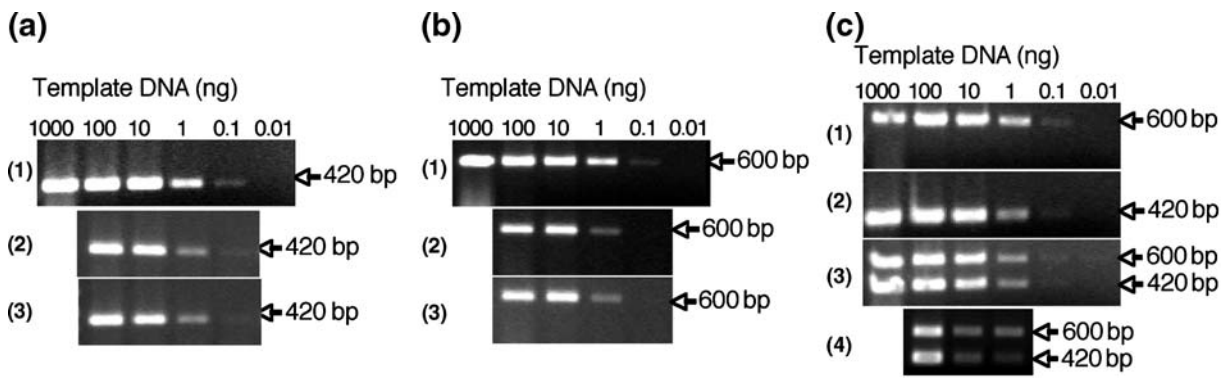

Fig. 1 Sensitivity and competitive assay of uniplex and multiplex PCR assays. Uniplex PCR with Y45/46 primers (**a**) on various amounts (0.01 to 1000 ng) of *P. atrosepticum* template DNA, (1) pure DNA template, (2) and (3) addition of 100 ng of competitor DNA: *P. carotovorum* subsp. *carotovorum* and *Dickeya* respectively. Uniplex PCR with Ech1/1' primers (**b**) on *Dickeya* template DNA (1) pure DNA, (2) and (3) addition of 100 ng competitor DNA: *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum* respectively. Multiplex PCR with primers Ech1/1' and Y45/46 (**c**) on *P. atrosepticum* template (1) and *Dickeya* template (2) or both (3 and 4) DNA varying from 0.01 to 1000 ng. Presence of *P. carotovorum* subsp. *carotovorum* competitor DNA (100 ng) was tested in lane 4

Table 2 Sequence and melting temperature (Tm) of the PCR primers used in the study

Primer name	primer sequences	Tm	References
Y45/46	5'-TCACCGGACGCCGAACTGTGGCGT-3' 5'-TCGCCAACG TTCAGCAGAACAAGT-3'	64°C 57°C	Fréchon et al. (1998)
ADE	5'-GATCAGAAAGCCCGCAGCCAGAT-3' 5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3'	59°C 66°C	Nassar et al. (1996)
Ech1/1'	5'-TGGCGCGTCAGGAAGTTTAT-3' 5'-TCACCGGTCAGGGTGAAGTT-3'	52°C 54°C	This work

phile *P. betavasculorum* is commonly associated with sugar beet, and is rarely found on potato (Ma et al. 2007). Consequently, the risk of obtaining a false-positive result is low and can be easily controlled by discriminating these species on the basis of physiologic traits (Thomson et al. 1981). ADE primers also led to specific PCR amplification of the 23 *Dickeya* tested. However, the size of the fragments obtained with both primers was similar, preventing the simultaneous use of both primer sets for distinction of the two pathogens. Since more data were available with Y45/46, we conserved it and searched for new specific primers allowing the detection of *Dickeya* spp.

Because of their taxonomic proximity, we chose to discriminate these bacteria on the basis of their pectinolytic enzyme genes rather than on their ribosomal genes. Consequently, we selected 17 primers sets specific for genes characterising soft-rotting bacteria species after multiple alignment using ClustalW software (http://www. infobiogen.fr, Thompson et al. 1994). We focused on the few sequence dissimilarities between *Dickeya chrysanthemi* 2048^T and *P. atrosepticum* 1526^T based on genome comparison and chose primers which were able to work in the conditions imposed by the Y45/Y46 primer set. All of these primers were then tested for their specificity using chromosomal purified DNA. Some of them led to amplification of several-non-specific PCR fragments. However, the primer set Ech1/1' (Table 2), located in the *pelI* ORF of *D. chrysanthemi* 2048^T amplified a unique 600 bp fragment (Fig. 1b). Subsequent testing of this new primer set on individual bacterial colonies showed exclusive amplification of all of the *Dickeya* strains tested (Table 1).

Sensitivity of the primers was first evaluated on purified genomic DNA as described by Grimberg et al. (1989). In these conditions, Y45/46 allowed detection of 0.1 ng of DNA in agreement with the results of Smid et al. (1995) and de Boer and Ward (1995). Specificity was checked by adding competitor DNA to the PCR mix (Fig. 1). Presence of competitor DNA (extracted from *D. chrysanthemi* 2048^T or *P. carotovotum* subsp. *carotovorum* 2046^T) together with *P. atrosepticum* DNA affected neither recognition nor detection limit of the target DNA (Fig. 1b). The second set of primers, designed to recognise *D. chrysanthemi,* allowed the detection of 0.1 ng of purified DNA (Fig. 1b). Presence of a high amount of competitor DNA in the PCR mix (*P. carotovotum* subsp. *carotovorum* 2046^T or *P. atrosepticum* 1526^T) also allowed specific amplification of *Dickeya* DNA but reduced the limit of sensitivity to 1 ng.

Thereafter, we assayed primers Ech1/1' and Y45/56 together in a single multiplex PCR procedure *in vitro*. We first evaluated the efficiency of detection for each bacterial strain. As expected, amplification of *P. atrosepticum* 1526^T and *D. chrysanthemi* 2048^T DNA produced a 420 and a 600 bp PCR product, respectively. No amplification of *P. carotovorum* DNA was observed (data not shown). When amplifying *P. atrosepticum* and *Dickeya* together, the two PCR products were concomitantly produced and easily distinguished by agarose gel electrophoresis (Fig. 1c). Adding *P. carotovorum* subsp. *carotovorum* competitor DNA in the multiplex mix with the target DNAs did not affect the specificity of recognition. The multiplex PCR assay was then tested on individual colonies of the complete bacterial collection resulting in specific amplification of DNA from all *Dickeya* and *P. atrosepticum* strains (Table 1). The detection limit of the multiplex PCR assay was similar to that of the uniplex PCR *i.e.* 0.1 ng of target DNA. As in the case in the uniplex reaction for *Dickeya*, competitor DNA reduced the detection limit to 1 ng (Fig. 1c). Consequently, *in vitro* assessment of the multiplex PCR assay showed a good specificity and a high sensitivity compared to the two uniplex PCR assays.

Potato plants display great variability in secondary metabolites, notably phenolic compounds, like anthocyanin pigments, which are known to interact with the PCR reaction. The specificity of the multiplex method was therefore estimated *in planta* on artificially infected asymptomatic potato tubers exhibiting different pigmentations (red and yellow cultivars) and its efficiency was tested on 172 naturally infected symptomatic potatoes belonging to 18 different cultivars. First, various *D. chrysanthemi* 2048^T and *P. atrosepticum* 1526^T concentrations, ranging from 10^0 to 10^6 cfu, were inoculated on sterilised tubers as described by Smadja et al. (2004a). After 7 days of infection, 500 mg of potato tissue were resuspended in 1 ml of NaCl 0.9% and vortexed for 1 min. Numeration on solidified LB plates and DNA extraction were achieved on 100 µl and 500 µl of bacterial suspension, respectively. For DNA extraction, the bacterial suspension was centrifuged at 13,000*g* for 15 min, and the pellet mixed in 500 µl of TEN buffer (Tris-HCl 10 mM pH 8.3, EDTA 0.1 mM, NaCl 1 M) containing 4 mg ml^{-1} lysozyme for 1 h at room temperature to destabilise the bacterial wall. Cells were disrupted at 37°C overnight with 30 µl of SDS 10% and 10 µl of proteinase K (20 mg ml^{-1}). Cell disruption was completed with 100 µl NaCl (5 M) and 80 µl CTAB at 65°C for 10 min. DNA was then extracted according to Grimberg et al. (1989) and resuspended in 50 µl of TE buffer (Tris-HCl 10 mM pH 8.3, EDTA 0.1 mM). Finally, DNA was diluted 10 times prior testing by PCR (Le Roux A. C., personal communication).

In these conditions, both *P. atrosepticum* and *Dickeya* were detected by the multiplex system whereas no maceration of the potato tissues was observed. Assays on yellow cultivars allowed an average detection of 10^4 and 4×10^4 cfu ml^{-1} tuber extract on *P. atrosepticum* and *Dickeya* respectively, after final numeration. Assays on the red cultivar slightly reduced the sensitivity of the multiplex system allowing an average detection of 10^4 and 3×10^5 cfu ml^{-1} tuber extract of *P. atrosepticum* and *Dickeya,* respectively. In the present experimental conditions, both uniplex systems allowed detection of about 10 times less bacteria than the multiplex system. In all cases (uniplex and multiplex systems), standard deviations were lower than 10%. The detection limit of *P. atrosepticum* we obtained, was consistent with the previously published results on yellow potato cultivars (Smid et al. 1995; Fréchon et al. 1998). To our knowledge, the efficiency of a PCR assay on *Dickeya* and *P. atrosepticum* was not investigated previously on red cultivars.

We finally tested the multiplex PCR assay on 172 symptomatic naturally infected potato plants belonging to 18 cultivars. Recognition of soft-rotting bacteria by the multiplex PCR assay was successful for 97% of the infected plants, confirming the efficiency of this system for rapid detection and pathogen discrimination.

We propose here an efficient method allowing simultaneous detection of the major pectinolytic bacteria causing soft rot on potato tubers, i.e *Dickeya* spp. and *P. atrosepticum*. Use of PCR for discriminating between these bacteria is a real challenge as extensive genome similarity between the species did not allow distinction in previous trials. The PCR primers developed in this study led to an assay with good detection limits and the presence of a competitor did not affect the specificity of the PCR reactions. The multiplex PCR assay was effective in discriminating among bacteria in a collection of 61 strains from diverse ecological origins, confirming the assay's usefulness for taxonomic distinction of the pectinolytic bacteria in epidemiological studies. The multiplex PCR assay was also applied efficiently on both artificially and naturally contaminated tissues confirming that this method can provide a gain in time and materials, the main advantages for large-scale processes such as pathogen-free seed certification.

Acknowledgments The authors wish to thank the Plate Forme Technologique d'Evreux, the Region of Haute-Normandie, and the Comité Nord for financial support. We wish to thank V. Hélias, N. Chaabi, A. Chapalain, G. Hemery and M. Vincent for technical assistance.

References

Alvarez, A. M. (2004). Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial disease. *Annual Review of Phytopathology, 42*, 339–366.

Avrova, A. O., Hyman, L. J., Toth, R. L., & Toth, I. K. (2002). Application of amplified fragment length polymorphism fingerprinting for taxonomy and identification of the soft rot bacteria *Erwinia carotovora* and *Erwinia chrysanthemi*. *Applied and Environmental Microbiology, 68*, 1499–1508.

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.

de Boer, S. H., & Ward, L. J. (1995). PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology, 85*, 854–858.

Fréchon, D., Exbrayat, P., Hélias, V., Hyman, L. J., Jouan, B., Llop, P., et al. (1998). Evaluation of a PCR kit for the detection of *Erwinia carotovora* subsp. *atroseptica* on potato tubers. *Potato Research, 41*, 163–173.

Grimberg, J., Maguire, S., & Belluscio, L. (1989). A simple method for the preparation of plasmid and chromosomal *E. coli* DNA. *Nucleic Acid Research, 17*, 8893.

Hélias, V., Le Roux, A. C., Bertheau, Y., Andrivon, D., Gauthier, J. P., & Jouan, B. (1998). Characterisation of *Erwinia carotovora* subspecies and detection of *Erwinia carotovora* subsp. *atroseptica* in potato plants, soil and water extracts with PCR-based methods. *European Journal of Plant Pathology, 104*, 685–699.

Hélias, V., Andrivon, D., & Jouan, B. (2000). Internal colonization pathways of potato plants by Erwinia carotovora subsp. atroseptica. *Plant Pathology, 49*, 33–42.

Hyman, L. J., Birch, P. R. J., Dellagi, A., Avrova, A. O., & Toth, I. K. (2000). A competitive PCR-based method for the detection and quantification of *Erwinia carotovora* subsp. *atroseptica* on potato tubers. *Letters in Applied Microbiology, 30*, 330–335.

Janse, J. D., & Wenneker, M. (2002). Possibilities of avoidance and control of bacterial plant diseases when using pathogen-tested (certified) or -treated planting material. *Plant Pathology, 51*, 523–536.

Ma, B., Hibbing, M. E., Kil, H. S., Reedy, R. M., Yedidia, I., Breuer, J. J. B., et al. (2007). Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*. *Phytopathology, 97*, 1150–1663.

Nassar, A., Darrasse, A., Lemattre, D., Kotoujansky, A., Dervin, C., Vedel, R., et al. (1996). Characterization of *Erwinia chrysanthemi* by pectinolytic isozyme polymorphism and restriction fragment length polymorphism analysis of PCR-amplified fragments of *pel* genes. *Applied and Environmental Microbiology, 62*, 2228–2240.

Pérombelon, M. C. M. (1992). Potato blackleg: epidemiology, host-pathogen interaction and control. *Netherlands Journal of Plant Pathology, 98*, 135–146.

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

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

Smadja, B., Latour, X., Faure, D., Chevalier, S., Dessaux, Y., & Orange, N. (2004a). Involvement of N-acylhomoserine lactones throughout plant infection by *Erwinia carotovora* subsp. *atroseptica* (*Pectobacterium atrospecticum*). *Molecular Plant Microbe Interaction, 17*, 1269–1278.

Smadja, B., Latour, X., Trigui, S., Burini, J.-F., Chevalier, S., & Orange, N. (2004b). Thermodependence of growth and enzymatic activities implicated in pathogenicity of two *Erwinia carotovora* subspecies (*Pectobacterium* spp.). *Canadian Journal of Microbiology, 50*, 19–27.

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

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Research, 22*, 4673–4680.

Thomson, S. V., Hildebrand, D. C., & Schroth, M. N. (1981). Identification and differentiation of the *Erwinia* sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi*. *Phytopathology, 71*, 1037–1042.

Ward, E., Foster, J. S., Fraaije, B. A., & McCartney, H. A. (2004). Plant pathogen diagnostic: immunological and nucleic acid-based approaches. *Annals of Applied Biology, 145*, 1–16.