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SHORT COMMUNICATION

# Detection and identification of the crucifer pathogen, *Xanthomonas campestris* pv. *campestris*, by PCR amplification of the conserved Hrp/type III secretion system gene *hrcC*

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**Abstract** The development of a rapid detection method for *Xanthomonas campestris* pv. *campestris* (Xcc) in crucifer seeds and plants is essential for high-throughput certification purposes. Here we describe a diagnostic protocol for the identification/detection of Xcc by PCR amplification of fragments from the pathogenicity-associated gene *hrcC*. Under stringent conditions of amplification, a PCR product of 519 bp from *hrcC* was obtained from a collection of 46 isolates of Xcc, with the exception of two isolates from radish. No amplicons were obtained from 39 pure cultures of the phytopathogenic bacteria *Xanthomonas campestris* pv. *cerealicola*, *X. campestris* pv. *juglandis*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *vitians*, *X. arboricola* pv. *pruni*, *X. axonopodis* pv. *phaseoli*, *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria*, *Pseudomonas syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *P. fluorescens*, *P. marginalis*, *Pectobacterium atrosepticum*, *P. carotovorum* subsp. *carotovorum*. In addition, PCR reactions were negative for fifty unidentified environmental isolates purified from the surface of crucifers. The PCR fragment was obtained from four strains previously classified as *X. campestris* pv. *aberrans*, *X. campestris* pv. *armorociae*, *X. campestris* pv. *barbarae* and *X. campestris* pv. *incanae* using pathogenicity assays. Our PCR protocol specifically detected Xcc in inoculated leaves, seeds and naturally infected leaves of crucifers.

**Keywords** Phytobacteria · Crucifers · Pathogenicity genes · *Hrp* · Polymerase chain reaction · Vascular blackening

*Xanthomonas campestris* pv. *campestris* (Xcc) is the causal agent of vascular blackening, or black rot, of crucifers. Symptoms consist of blackening of the vascular tissue and marginal chlorosis of the leaves followed by necrosis (Mazzucchi, 1968). The bacterium is seed-transmissible and the use of certified materials is a key practice for disease management (Janse & Wenneker, 2002). Detection of Xcc is currently performed by plating differentially centrifuged seed extracts on Fieldhouse-Sasser (FS) or on mCS20ABN media (Roberts & Koenraadt, 2003). After incubation at 28–30°C for 4–5 days, colonies tentatively identified as *Xanthomonas* spp. based on morphology are subcultured on Yeast Dextrose Calcium Carbonate Agar (YDCA) (Stolp & Starr, 1964). Identification is confirmed by classical bacteriological tests (morphology of the colonies on YDCA, hydrolysis

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of amylose in BS or BSCAA-agar, hydrolysis of esculin and casein, growth at 35°C, acidity from glucose, arabinose and mannose) (Schaad, Jones, & Lacy, 2002) and inoculation on susceptible Brassica seedlings (Roberts & Koenraadt, 2003).

These methods are time-consuming (~2 weeks) and not well suited for rapid and high-throughput screening of seed samples. Molecular methods based on DNA technology, especially those based on PCR, are very powerful in plant disease diagnosis (Henson & French, 1993) and have the greatest potential for high-throughput applications. A molecular method for detection of Xcc based on a DNA probe is described in the literature but it was not effective for detecting pathogens in seeds due to assay sensitivity limitations (Shih, Lin, Huang, Tzeng, & Hsu, 2000). Application of PCR to Xcc detection has been delayed by insufficiency of genomic information and by the large variability observed using arbitrary primers, such as M13-PCR, BOX-PCR and ERIC-PCR (Zaccardelli, 2001; Zaccardelli, Del Galdo, Cagliati, & Buonaurio, 2002). Here we report on the development of a new PCR-based diagnostic protocol optimized for pure cultures and plant materials (cabbage seeds and leaves). This new procedure targets a genetic locus associated with pathogenicity and includes a modification of the official method of the International Seed Testing Association (ISTA) (Roberts & Koenraadt, 2003) which enabled us to obtain diagnostic results from plant materials within a few hours.

An ideal molecular target for diagnostic purposes must be genetically stable, linked to pathogenicity, and specific for the species/pathovar of interest. After publication of the *X. campestris* genome (da Silva et al., 2002), we decided to focus on chromosomal targets genetically linked to the *hrp*/type III secretion pathogenicity island. A similar approach has been used successfully by our group for other phytobacteria (Zaccardelli, Spasiano, Bazzi, & Merighi, 2005). More recently a PCR-based detection method for *X. campestris* pathovars was described using *hrpF* gene from *X. campestris* (Berg, Tesoriero, & Hailstones, 2005) as the molecular target. In all the necrogenic phytobacteria, including the genus *Xanthomonas*, studied so far, *hrp* genes have been shown to be essential for *in planta* growth and symptom development (Alfano & Collmer, 1997). *Hrp* genes encoding for conserved components of the structural apparatus seem ideal candidates for molecular diagnostics. These gene products are essential components of the secretion machinery and they are absolutely required for virulence. Even though this genetic region is quite conserved across different species, the latter contain regions with sufficient divergence to allow the design of primers and oligonucleotide probes for molecular detection.

After analyzing several candidates, we selected the *hrcC* secretin-like chromosomal gene as a potential target. HrcC is an outer membrane protein paralog of the *E. coli* type II secretion protein PulD. Orthologs of HrcC are present in every functional type III secretion system, representing the basal component of the injectisome (He, 1998). $HrcC_{xcc}$ gene is more conserved than *hrpF*, the target of a similar study (Berg et al., 2005). In fact, *hrpF* is one of the least conserved genes of the *hrp* cluster (Büttner, Nennstiel, Klüsener, & Bonas, 2002).

The DNA sequences were obtained from Genbank database at the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov). By alignment of the DNA sequences of several *hrcC* genes available using Clustal X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997), several pairs of primers were manually designed on regions of the gene specific to xanthomonads and Xcc (Fig. 1). After preliminary amplification experiments (data not shown), two primers, HrcCF2 (5′-CGTGTGGATGT GCAGACC-3′) and HrcCR2 (5′-CAGATCTGTCT GATCGGTGTCG-3′), amplifying an internal fragment of 519 bp of *hrcC* were selected for further investigation.

Forty-six Xcc isolates, 39 phytopathogenic bacteria of various species and genera, and fifty unidentified bacterial isolates from crucifer plants were used for in vitro experiments (Table 1). *Xanthomonas* spp. were routinely grown on YDCA (Stolp & Starr, 1964); *Pseudomonas* spp. were grown on King's Medium B Agar (King, Ward, & Raney, 1954); *Erwinia* spp., *Pectobacterium* spp. and unidentified bacteria were grown on Nutrient Glucose Agar (NGA) (Civerolo, 1990). Bacterial suspensions were prepared in sterile double distilled water at $OD_{600\ nm}$ = 0.1 (corresponding to ~$4.3 \times 10^7$ CFU $ml^{-1}$ for Xcc) after growth at 27°C for 48 h. Alternatively, total genomic DNA was extracted and purified from pellets obtained from bacterial cultures, using the CTAB-method (Wilson, 1989); DNA


**Fig. 1** Multiple sequence alignments and the positions of the forward and reverse primers used in this work. Numeration of the coordinates starts at the first base of the ORF obtained from Genbank. Legend: Pa = *Pectobacterium atrosepticum*; Pcc = *P. carotovorum* subsp. *carotovorum*; Ecc = *Erwinia chrysanthemi* pv. *chrysamthemi*; Ep = *E. pyrofolia*; Pstw = *Pantoea stewartii*; Psp = *Pseudomonas syringae* pv. *phaseolicola*; Psav = *P. savastanoi*; Pst = *P. syringae*; Pfl = *P. fluorescens*; Xac = *Xanthomonas axonopodis* pv. *citri*; Xag = *X. axonopodis* pv. *glycinea*; Xoo = *X. oryzae* pv. *oryzae*; Xcc = *X. campestris* pv. *campestris*; Rsol = *Ralstonia solanacearum*; Aac = *Acidovorax avenae* subsp. *citrulli*; Yent = *Yersinia enterocolitica*

concentration was measured spectrophotometrically in a Bio-photometer (Eppendorf, Germany). PCR was performed in 25 µl of reaction mixture containing 0.2 µM of each HrcCF2 and HrcCR2 primer, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1 U of *Taq*-DNA polymerase (Sigma, Germany), and 1 µl of bacterial suspension or 5 ng of DNA as template. The amplification programme consisted of an initial denaturation at 95°C for 5 min, 15 cycles of denaturation at 95°C for 1 min and annealing-extension at 72°C for 1 min and 30 s, followed by 15 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 30 s, extension at 72°C for 1 min, final extension at 72°C for 15 min and stored at 4°C until used. Amplicons were visualized by standard agarose gel electrophoresis and ethidium bromide staining. Gel concentration was 1.5% and electrophoresis was run in TAE buffer. Step Ladder 50 bp (Sigma-Aldrich, Germany) or DNA Ladder Mix (M-Medical-Genenco, Italy) were used as molecular markers.

An amplicon of 519 bp was obtained from all Xcc isolates, except two isolated from radish in New Zealand, and no amplicons were obtained from the isolates of related and unrelated phytopathogenic bacteria including *X. campestris* pv. *cerealicola*, *X. campestris* pv. *juglandis*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *vitians*, *X. arboricola* pv. *pruni, X. axonopodis* pv. *phaseoli, X. axonopodis* pv. *vesicatoria*, *X. vesicatoria*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *P. fluorescens*, *P. marginalis*, *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum*. No amplicons were obtained from 50 unidentified bacteria isolated from crucifer crops (Fig. 2).

We unexpectedly obtained the 519-bp amplicon typical of Xcc from *X. campestris* pv. *aberrans*, *X. campestris* pv. *armoraciae*, *X. campestris* pv.

**Table 1** Bacterial collection used in this study

| Strains | Host (Country) | Strains | Host (Country) |
|---|---|---|---|
| *Xanthomonas campestris* pv. *campestris* | | *Xanthomonas campestris* pv. *incanae* | |
| LMG 568 | Bruxelles sprouts (UK) | DAR 75959 | Stock (USA) |
| LMG 8001 | Garden-cabbage (UK) | *Xanthomonas campestris* pv. *cerealicola* | |
| LMG 8030 | Cauliflower (France) | NCPPB 1944 | Bromus (USA) |
| LMG 8058 | Cauliflower (Holland) | NCPPB 3212 | Weath (USA) |
| NCPPB 1025 | Cauliflower (Malawi) | *Xanthomonas campestris* pv. *juglandis* | |
| NCPPB 1143 | Cauliflower (UK) | IPV-BO 796 | Black walnut (Italy) |
| NCPPB 1147 | Garden-cabbage (UK) | *Xanthomonas campestris* pv. *pelargonii* | |
| ISPaVe 1032 | Garden-cabbage (Italy) | IPV-BO 2535 | Geranium (Italy) |
| OMP-BO 588/90 | Garden-cabbage (Italy) | *Xanthomonas campestris* pv. *vitians* | |
| IPV-NA 1′ | Cauliflower (Italy) | ISCI 95a and 95 b | Cos lettuce (Italy) |
| IPV.CT 62.4 | Kohl-rabi (Italy) | *Xanthomonas arboricola* pv. *pruni* | |
| IPV.CT 66.1 | Broccoli (Italy) | NCPPB 1607 | Peach (Australia) |
| IPV.CT 67.2 | Kohl-rabi (Italy) | NCPPB 416 | Japanese plum (New Zealand) |
| ISCI 1 | Kohl-rabi (Italy) | NCPPB 419 | Peach (New Zealand) |
| ISCI 2 | Kohl-rabi (Italy) | NCPPB 923 | Plum (South Africa) |
| ISCI 5 | Kohl-rabi (Italy) | 69 VR | Peach tree (Italy) |
| ISCI 6 | Kohl-rabi (Italy) | *Xanthomonas axonopodis* pv. *phaseoli* | |
| ISCI 12 | Cauliflower (Italy) | NCPPB 1420 | Common bean (Italy) |
| ISCI 14 | Cauliflower (Italy) | IPV-BO 1921 | Common bean (Hungary) |
| ISCI 16 | Kohl-rabi (Italy) | *Xanthomonas axonopodis* pv. *vesicatoria* | |
| ISCI 18 | Kohl-rabi (Italy) | DAPP-PG 137 | Tomato (Italy) |
| ISCI 22 | Kohl-rabi (Italy) | DAPP-PG 148 | Tomato (Italy) |
| ISCI 31 | Broccoli (Italy) | DAPP-PG 418 | Tomato (Italy) |
| ISCI 41 | Kale (Italy) | *Xanthomonas vesicatoria* | |
| ISCI 44 | Kale (Italy) | OMP-BO 790 A/94 | Tomato (Italy) |
| ISCI 45 | Savoy-cabbage (Italy) | IVIA 1779-2 | Pepper (Spain) |
| ISCI 50 | Cauliflower (Italy) | DAPP-PG 419 | Tomato (Italy) |
| ISCI 67 | Cauliflower (Italy) | *Pseudomonas syringae* pv. *phaseolicola* | |
| ISCI 70 | Crambe (Italy) | GSPB 1549 | Common bean (Germany) |
| ISCI 88 | Garden-cabbage (Italy) | *Pseudomonas syringae* pv. *syringae* | |
| ISCI 120 | *Brassica* sp. (Italy) | DAPP-PG 53 | Pepper (Italy) |
| ISCI 129 | Kale (Italy) | LMG 1247 | Lilac (UK) |
| DAPP-PG 247 | Garden-cabbage (Italy) | IPV-CT $45S_21$ | *Citrus* sp. (Italy) |
| DAPP-PG 249 | Garden-cabbage (Italy) | Y 37 | Common bean (Italy) |
| DAPP-PG 341 | Cauliflower (Italy) | B 366 | Sugarbeet (Italy) |
| DAPP-PG 362 | Cauliflower (Italy) | *Pseudomonas syringae* pv. *tomato* | |
| DAPP-PG 353 | Cauliflower (Italy) | IVIA 177.4 | Tomato (Spain) |
| DAPP-PG 355 | Cauliflower (Italy) | DC 3000 | Tomato (UK) |
| DAPP-PG 357 | Cauliflower (Italy) | OMP-BO 443 and 748 | Tomato (Italy) |
| DAPP-PG 370 | Cauliflower (Italy) | ISCI 204 | Tomato (Italy) |
| DAPP-PG 399 | Rutabaga (Italy) | *Pseudomonas fluorescens* | |
| DAPP-PG 400 | Rutabaga (Italy) | IPV-CT $65_1$ and $47_1$ | Citrus sp. (Italy) |
| DAPP-PG 405 | Kale (Italy) | *Pseudomonas marginalis* | |
| DAPP-PG 410 | Garden-cabbage (Italy) | OMP-BO 232/96 | Lettuce (Italy) |

**Table 1** continued

| Strains | Host (Country) | Strains | Host (Country) |
|---|---|---|---|
| DAR 75543 and 75975 | Radish (New Zealand) | ES 4326 | Turnip |
| *Xanthomonas campestris* pv. *aberrans* | | *Pectobacterium carotov.* subsp. *atrosepticum* | |
| DAR 75944 | Cabbage (Australia) | ISPaVe 1156 | Potato (Italy) |
| *Xanthomonas campestris* pv. *armorociae* | | *Pectobacterium carotov.* subsp. *carotovorum* | |
| DAR 75942 | Candytuft (Tanzania) | ISPaVe 1062 | Artichoke (Italy) |
| *Xanthomonas campestris* pv. *barbarae* | | Unidentified bacteria | |
| DAR 75945 | Upland cress (USA) | Fifty ISCI isolates | Crucifer crops (Italy) |

DAPP-PG: Dipartimento di Arboricoltura e Protezione delle Piante, Perugia, Italy; GSPB: Göttinger Sammlung Phytopathogener Bakterien, Göttingen, Germany; DAR: Australian Collection of Plant Pathogenic Bacteria, Australia; IPV-BO: Istituto di Patologia Vegetale, Bologna, Italy; IPV-CT: Istituto di Patologia Vegetale, Catania, Italy; IPV-NA: Istituto di Patologia Vegetale, Portici, Italy; ISCI: Istituto Sperimentale per le Colture Industriali, Battipaglia, Italy; ISPaVe: Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; IVIA: Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; LMG: Culture Collection, Rijksuniversitseit, Belgium; NCPPB: National Collection of Plant Pathogenic Bacteria, York, Great Britain; OMP-BO: Osservatorio per le Malattie delle Piante, Bologna, Italy; OMP-VR: Osservatorio per le Malattie delle Piante, Verona, Italy; B and Y: strains provided from Prof. N.S. Jacobellis

*barbarae* and *X. campestris* pv. *incanae*, while the two New Zealand Xcc strains from radish gave no PCR product. These PCR results were in agreement with the results obtained with the same isolates using primers for the *hrpF* gene of *X. campestris* (Berg et al., 2005). Identity of the amplicons obtained from five Xcc strains was confirmed by DNA sequencing at MWG (Germany), followed by database searches with BLAST (Genbank database at www.ncbi.nlm.nih.gov).

Titration experiments using suspensions from pure cultures showed that the detection threshold was 4.3–43 CFU/25 µl PCR reaction. This detection limit was lower than reported in the literature ($6 \times 10^4$ CFU) for a DNA dot blot assays (Shih et al., 2000) but higher than the detection limit of 0.1–1 CFU/reaction tube described by Berg et al. (2005).

To assess the robustness of the PCR assay when analyzing field samples, we performed experiments with artificially inoculated leaves and seeds of healthy cabbage and symptomatic leaves of naturally infected cabbage plants. Infected materials (total of twenty samples) were collected during autumn 2003 from crops located in Salerno Province (southern Italy). Artificial inoculations of the leaves were performed by adding ten-fold dilutions of a suspension of Xcc to obtain concentrations from $8.6 \times 10^8$ to 8.6 CFU $g^{-1}$ of leaves. Artificial inoculation of the seeds was performed with Xcc suspension to obtain a concentration of about $4.5 \times 10^7$ CFU $g^{-1}$ of seeds. We prepared and analyzed samples with decreasing percentages of contaminated seeds (from 100 to 0.39%) by mixing healthy and inoculated seeds in different proportions. Infected/inoculated plant samples (0.05 g of leaves or 0.25 g of seeds) were ground in a pestle and mortar upon addition of 2 ml of antioxidant extraction buffer (Gorris et al., 1996). Homogenates were filtered through Whatman No. 1 and, from the pellet obtained by centrifugation at 13,000 rpm for 10 min, DNA was extracted by the CTAB-method (Wilson, 1989).

Another protocol described in the official methods of the International Seed Testing Association (ISTA) (Roberts & Koenraadt, 2003) and integrated with extraction of DNA, was also applied to seed samples. Samples of 1000 seeds each were shaken for 5 min at 125 rpm in 10 ml of sterile saline solution plus 0.02% of Tween 20. Two samples (1 ml each) were aliquoted in Eppendorf tubes and used either for direct isolation or for DNA extraction by the CTAB-method (Wilson, 1989). The remaining washing fluid was shaken for 2.5 h and then 1 ml was used for direct isolation and 1 ml for DNA extraction. Direct isolation was performed by plating ten-fold dilutions of extract on mCS20ABN plates. After incubation at 28°C for five/seven days, colonies with typical translucid halos were counted and selected ones collected and identified using the PCR protocol procedure developed in this study.

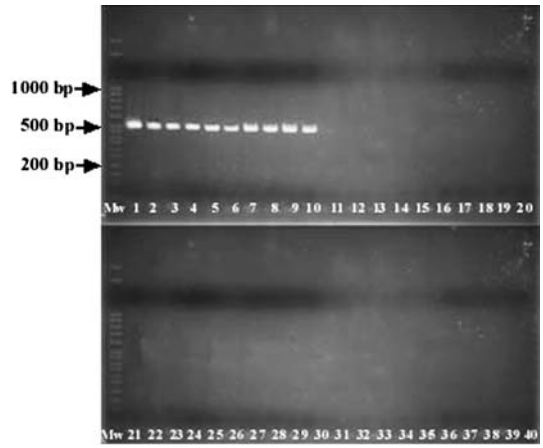


**Fig. 2** Gel electrophoresis analysis of PCR products amplified from DNA or bacterial suspension of *X. campestris* pv. *campestris* strains (lanes 1 to 10), related and unrelated phytopathogenic bacteria (lanes 11 to 25), and unidentified bacterial isolates obtained from crucifer plants (lanes 26 to 39). Reactions were performed using primers $hrcF_2$ and $hrcR_2$. Lane Mw: molecular weight marker Step Ladder 50 bp (Sigma-Aldrich, Germany); lane 1: IsPaVe 1032; lane 2: OMP-BO 588/90; lane 3: ISCI 2; Lane 4: ISCI 6; lane 5: ISCI 16; lane 6: ISCI 45; lane 7: ISCI 67; lane 8: DAPP-PG 247; lane 9: DAPP-PG 341; lane 10: DAPP-PG 362; lane 11: *Xanthomonas vesicatoria* strain DAPPG 419; lane 12: *X. axonopodis* pv. *vesicatoria* strain DAPP-PG 148; lane 13: *X. axonopodis* pv. *vitians* strain ISCI 95a; lane 14: *X. axonopodis* pv. *phaseoli* strain NCPPB 1420; lane 15: *X. arboricola* pv. *pruni* strains NCPPB 923; lane 16: *X. arboricola* pv. *juglandis* strain IPV-BO 796; lane 17: *X. translucens* pv. *cerealis* strain NCPPB 1944; lane 18: *X. hortorum* pv. *pelargonii* strain IPV-BO 2535; lane 19: *Pectobacterium carotovorum* subsp. *carotovorum* strain ISPaVe 1062; lane 20: *Pseudomonas syringae* pv. *syringae* strain LMG 1247; lane 21: *P. syringae* pv. *phaseolicola* strain GSPB 1549; lane 22 and 23: *P. syringae* pv. *tomato* strains OMP-BO 443 and ISCI 204; lane 24: *P. fluorescens* strain IPV-CT $65_1$; lane 25: *P. marginalis* strain OMP-BO 232/96; lanes from 26 to 39: unidentified bacterial isolates from cabbage plants; lane 40: no template (water)

PCR conditions for plant materials were the same as used for bacterial cultures except for $MgCl_2$ (2.5 mM) and *Taq*-DNA polymerase (Triple Master PCR System Kit, Eppendorf, Germany). An aliquot of 1 µl out of 100 µl of total DNA extracted from either pellets of inoculated/infected leaves, seeds or washing fluid was used as the template. The same procedures that were applied to inoculated/infected plant materials were used for healthy leaves or seeds (negative controls).

The 519-bp amplicon was generated from all the DNA samples from the twenty naturally infected

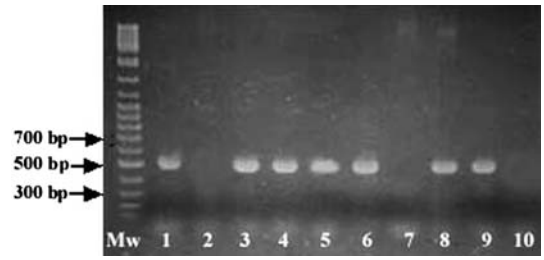


**Fig. 3** Gel electrophoresis analysis of PCR products amplified from DNA extracted from artificially contaminated or naturally infected (symptomatic) leaves of crucifers and from artificially contaminated cabbage seed. Lane Mw: molecular weight marker DNA Ladder Mix (M-Medical–Genenko, Italy); lane 1: DNA of *Xanthomonas campestris* pv. *campestris* strain ISCI 1 (positive control); lane 2: water (negative control); lanes 3 and 4: DNA of artificially contaminated leaves of crucifers; lanes 5 and 6 : DNA of naturally infected leaves of crucifers; lane 7: healthy leaves of cabbage; lanes 8 and 9: DNA of artificially contaminated cabbage seeds; lane 10: uncontaminated cabbage seeds

symptomatic cabbage leaf samples. Detection thresholds of $8.6 \times 10^5$ CFU $g^{-1}$ of leaves and $3.5 \times 10^5$ CFU $g^{-1}$ of seeds, respectively, were obtained when using DNA extracted from artificially inoculated leaves and mortar-ground seeds. No amplicons were ever obtained from DNA samples of healthy leaves or seeds (Fig. 3). By using DNA extracted from washing fluid samples of artificially inoculated seeds, Xcc was detected at $3.4 \times 10^5$ CFU $g^{-1}$ of seeds (0.78% of seed contamination) for washing fluid samples collected after 5 min or 2.5 h (Table 2). Nevertheless, in 8 out 24 cases, no amplicons were obtained from DNA of washing fluid samples collected after 2.5 h, whereas the corresponding samples of washing fluid collected after 5 min always yielded amplicons. These results suggest that washing seeds a long time probably increases extraction of substances that inhibit the PCR reaction. By using the PCR protocol described by Berg et al. (2005), the detection threshold for plant material was one artificially infected seed ($5 \times 10^4$ CFU) among 10,000 healthy seeds; assuming 1.5 g to be the weight of 10,000 crucifer seeds, the detection limit was $3.3 \times 10^4$ CFU $g^{-1}$ of seeds. The detection threshold for seeds by our protocol was ten times higher than that of Berg et al. (2005), but in principle, detection of the amplicons by methods other than ethidium bromide staining, can be employed for achieving even higher sensitivity (Merighi et al., 2000).

**Table 2** Response analysis about isolation and detection of Xanthomonas campestris pv. campestris from different contaminated seeds. Legend: + = positive result; − = negative result

| Percentage of seed contamination | Replicate | Isolation on plate from wash fluid | | PCR on DNA from wash fluid | | PCR on DNA from mortar extract |
|---|---|---|---|---|---|---|
| | | after 5 min. | after 2.5 h | after 5 min. | after 2.5 h | |
| 100 | I | + | + | + | − | + |
| | II | + | + | + | + | + |
| | III | + | + | + | + | + |
| 50 | I | + | + | + | − | + |
| | II | + | + | + | + | + |
| | III | − | + | + | + | + |
| 25 | I | + | − | + | − | + |
| | II | + | + | + | + | + |
| | III | + | + | + | + | + |
| 12.5 | I | − | − | + | − | + |
| | II | + | + | + | + | + |
| | III | + | + | + | + | + |
| 6.25 | I | − | − | + | − | + |
| | II | + | + | + | + | + |
| | III | + | + | + | + | + |
| 3.12 | I | + | + | + | + | + |
| | II | + | + | + | − | + |
| | III | + | + | + | − | + |
| 1.56 | I | + | + | + | + | + |
| | II | + | + | + | + | + |
| | III | + | − | + | − | + |
| 0.78 | I | + | + | + | + | + |
| | II | + | + | + | + | + |
| | III | + | − | + | + | + |
| 0.39 | I | − | − | − | − | − |
| | II | − | − | − | − | − |
| | III | − | − | − | − | − |
| Ctrl | I | − | − | − | − | − |
| | II | − | − | − | − | − |
| | III | − | − | − | − | − |

The detection threshold in washing fluids from artificially contaminated seeds was $3.4 \times 10^5$ CFU $g^{-1}$of seeds (0.78% of seed contamination) when using direct isolation on mCS20ABN plates. Nevertheless, 3 out of 24 and 5 out of 24 washing fluid samples collected after 5 min and 2.5 h, respectively, did not yield any colonies after incubation on mCS20ABN (Table 2). All the suspected colonies collected from the other plates after incubation, were confirmed by PCR using the HrcCF2 + HrcCR2 primer pair.

Our *hrcC* PCR protocol is suitable for rapid and specific identification of Xcc from pure cultures during the confirmatory phase of diagnosis when pure cultures of the bacteria are available, and is equivalent to the protocol based on primers designed on basis of the *hrpF* gene of *X. campestris* (Berg et al., 2005). The procedure is also suitable for detection of Xcc in symptomatic and asymptomatic plant materials because in all cases our results confirmed the presence of Xcc cells in infected samples. In contrast to the PCR protocol of Berg et al. (2005), our PCR procedure does not require multiplex PCR (e.g. the use of DLH138 and DLH139 primers for the amplification of the ITS region of *Brassica* spp.)

because the use of Triple Master PCR System Kit seems sufficient to avoid false negative results.

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## References

Alfano, J. R., & Collmer, A. (1997). The type III (Hrp) secretion pathway of plant pathogenic bacteria: Trafficking: Harpins, Avr proteins, and death. *Journal of Bacteriology, 179*, 5655–5662.

Berg, T., Tesoriero, L., & Hailstones, D. L. (2005). PCR-based detection of *Xanthomonas campestris* pathovars in *Brassica* seed. *Plant Pathology, 54*, 416–427.

Büttner, D., Nennstiel, D., Klüsener, B., & Bonas, U. (2002). Functional analysis of HrpF, a putative type III translocon protein from *Xanthomonas campestris* pv. *vesicatoria*. *Journal of Bacteriology, 184*, 2389–2398.

Civerolo, E. L. (1990). *Xanthomonas campestris* pv. *citri*. Quarantine procedure. *EPPO Bulletin, 20*, 263–272.

da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Stuys, M. A., Almeida, N. F., Alves, L. M. C., do Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M. B., Coutinho, L. L., Cursino-Santos, J. R., El-Dorry, H., Farla, J. B., Ferreira, A. J. S., Ferreira, R. C. C., Ferro, M. I. T., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G. M., Lemos, M. V. F., Locali, E. C., Machado, M. A., Madeira, A. M. B. N., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F. M., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T. M., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A. D. Silva, C., de Souza, R. F., Spinola, L. A. F., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I. D., Trindade dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C., & Kitajima, J. P. (2002). Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. *Nature 417*, 459–462.

Gorris, M. T., Cambra, M., López, M. M., Lecomte, P., Chartier, R., & Paulin, J. P. (1996). A sensitive and specific detection of *Erwinia amylovora* based on ELISA-DASI enrichment method with monoclonal antibodies. *Acta Horticulture, 411*, 41–45.

He, S. Y. (1998). Type III protein secretion systems in plant and animal pathogenic bacteria. *Annual Review of Phytopathology, 36*, 363–392.

Henson, J. M., & French, R. (1993). The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology, 31*, 81–109.

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.

King, E. O., Ward, M. K., & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine, 44*, 301–307.

Mazzucchi, U. (1968). Annerimento vascolare delle Crucifere. *Informatore Fitopatologico, 18*, 399–402.

Merighi, M., Sandrini, A., Landini, S., Ghini, S., Girotti, S., Malaguti, S., & Bazzi, C. (2000). Chemiluminescent and colorimetric detection of *Erwinia amylovora* by immunoenzymatic determination of PCR amplicons from plasmid pEA29. *Plant Disease, 84*, 49–54.

Roberts, S. J., & Koenraadt, H. (2003). ISTA-PDC technical report: revised method for detection of *Xanthomonas campestris* pv. *campestris* in brassica seed. ISTA Method Validation Report 1, 1–9.

Schaad, N. W., Jones, J. B., & Lacy, G. H. (2002). *Xanthomonas*. In: N. W. Schaad, J. B. Jones, & W. Chun (Eds.), *Laboratory guide for the identification of plant pathogenic bacteria*, (175–200). St Paul: American Phytopathological Society.

Shih, H. D., Lin, Y. C., Huang, H. C., Tzeng, K. C., & Hsu, S. T. (2000). A DNA probe for identification of *Xanthomonas campestris* pv. *campestris*, the causal organism of black rot of crucifers in Taiwan. *Botanical Bulletin of Academic Sinica, 41*, 113–120.

Stolp, H., & Starr, M. P. (1964). Bacteriophage reactions and speciation of phytopathogenic xanthomonads. *Phytopatologische Zeitschrift, 51*, 442–478.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Wilson, K. (1989). Preparation of genomic DNA from bacteria. In: F. M. Ausubel, R. Brent, & R. E. Kinston (Eds.), *Current Protocols in Molecular Biology*, Vol. 1 (pp 2.4.1–2.4.5) New York: John Wiley & Sons.

Zaccardelli, M. (2001). Molecular characterization of *Xanthomonas campestris* pv. *campestris* isolates. *Journal of Plant Pathology, 83*, 245.

Zaccardelli, M., Del Galdo, A., Cagliati, C., & Buonaurio, R. (2002). Genetic variability of *Xanthomonas campestris* pv *campestris* populations: first results. *Journal of Plant Pathology, 84*, 199.

Zaccardelli, M., Spasiano, A., Bazzi, C., & Merighi, M. (2005). Identification and *in planta* detection of *Pseudomonas syringae* pv. *tomato* using PCR amplification of hrp$Z_{pst}$. *European Journal of Plant Pathology, 111*, 85–90.