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Detection of *Xanthomonas campestris* pv. *campestris* through a real-time PCR assay targeting the *Zur* gene and comparison with detection targeting the *hrpF* gene

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Abstract *Xanthomonas campestris* pv. *campestris* (Xcc) is a seedborne bacterium that causes black rot of crucifers. A real-time PCR assay based on a dual-labeled hydrolysis TaqMan® probe has been developed for the rapid and sensitive detection of Xcc and related pathovars that affect mainly *Brassicaceae* crops and ornamentals. Primers were designed to specifically amplify a 152 bp fragment of the *Zur* gene from *X. campestris*. To confirm the specificity of the detection, primers targeting the *Zur* and *hrpF* genes were used for standard and real-time PCR with DNA samples from 13 Xcc strains, seven *Xanthomonas* species and pathovars and five different bacterial endophytes including *Bacillus*, *Erwinia*, *Klebsiella*, *Pantoea* and *Pseudomonas,* previously isolated from tissues of crucifers. PCR products amplified with *Zur* and *hrpF* primers were sequenced to assess the genetic diversity of these genes in the tested isolates. The real-time PCR protocol was optimized to allow the detection at the level of ten copies of *Zur* PCR fragment per one microliter of DNA. Although the real-time based on detection of *Zur* also detected *X. campestris* pvs *raphani*, *armoraciae*, *incanae* and a strain of *X. hortorum* pv. *carotae*, it improved the specificity in relation to the previously published *hrpF* based real-time method. A multiplex assay for *Zur* and *hrpF* genes further improved the specificity by excluding *X. hortorum* pv. *carotae*. Tests of brassica tissues and seeds artificially inoculated with Xcc showed that the real-time PCR based on detection of *Zur* is an efficient and robust assay.

Keywords Real-time PCR · TaqMan® probe · Crucifers · Sequencing · *Zur* · *hrpF*

Introduction

Xanthomonas campestris pv. *campestris* (Xcc), the causal agent of black rot of crucifers, is generally considered to be the most important disease of cruciferous plants worldwide (Williams 1980, Vicente and Holub, 2012). One contaminated seed among 10,000 healthy seeds may be enough to initiate an outbreak of disease after planting (Cook et al. 1952; Schaad et al. 1980). This gram-negative bacterium causes typical V-shaped lesions on mature leaves that expand towards the base of the leaf and eventually become necrotic. The presence of black veins is also diagnostic of Xcc infection (Cook et al. 1952; Alvarez et al. 1994; Assis et al. 1999). The widespread dissemination of Xcc is mostly caused by

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infected seeds and insufficient use of agronomic control practices such as crop rotation and elimination of infected plant debris.

Successful infection and multiplication of bacteria in host tissues rely on different virulence factors. Molecular genetic approaches have allowed the identification and characterization of more than 100 genes contributing to the virulence of Xcc and approximately 250 genes have been predicted to be linked with pathogenesis (da Silva et al. 2002; Qian et al. 2005). This creates a broad scope for identification of genes that are conserved in the genus *Xanthomonas*, but contain variability between its different species and possibly pathovars. The development of a rapid and sensitive detection method for Xcc in brassica seeds and plants is crucial for disease management, breeding programs and certification purposes.

The system of detection of Xcc on *Brassica* spp. seeds currently recommended by ISTA (International Seed Testing Association) consists of plating diluted seed extracts on semi-selective media followed by pathogenicity and/or PCR tests. For the specific detection, the combination of primer pairs DLH 120–125 (Berg et al. 2005) and ZUP 2309–2310 (Rijlaarsdam et al. 2004) are usually used. These primers were validated by Fargier and Manceau (2007), but ZUP primers were not considered as pathovar-specific on a broader spectrum of isolates.

The standard PCR assay developed by Berg et al. (2005) targets the *hrpF* (hypersensitive response and pathogenicity gene) of *X. campestris*. It detects four pathovars that affect brassica plants: *X. campestris* pvs. *aberrans*, *armoraciae*, *campestris*, *raphani* and the closely related pathovars, *X. campestris* pv. *barbarae* and *incanae*. Based on these results, a real-time PCR assay was developed (Berg et al. 2006); this assay was performed as a multiplex reaction in which the first pair of primers targeted the *hrpF* gene and the second primer pair the segment of the *Brassica* spp., the 18S–25S internal transcribed spacer (ITS) region used as the internal control (Berg et al. 2006). The *hrpF* gene was also used by Park et al. (2004) in a conventional PCR assay. Likewise, Zaccardelli et al. (2007) supported its usability on genes with similar characteristics for detection of *X. campestris* pathovars; the *hrcC* gene (secretin-like chromosomal gene) showed specificity in detection of Xcc from pure cultures and plant material and the system developed was considered equivalent to the protocol of Berg et al. (2005). The protocol of Rijlaarsdam et al. (2004) used the primer pair ZUP 2309–2310, but this is not recommended for real-time assay because of the resulting fragment length of 370 bp is longer than the recommended size.

Other genes associated with the virulence and pathogenicity such as the *rpf*, *gum*, *wxc* genes (Vicente and Holub 2012) and the *Zur* gene (Tang et al. 2005) might be suitable targets for detection assays. In particular, the *Zur* gene (zinc uptake regulator gene) is essential for the full virulence of Xcc and therefore seems to be appropriate for specific detection (Eichmeier et al. 2015; Tang et al. 2005). Moreover, *Zur* of *X. campestris* is involved in hypersensitive response and directly regulates the expression of the *hrp* cluster (Huang et al. 2009). Based on these facts, detection of *Zur* has the potential to be more reliable and sensitive than detection based on the genes of the *hrp* cluster.

The aims of this study were to: (i) compare a standard PCR assay based on the *Zur* gene with standard assay based on *hrpF* gene, (ii) design a real-time PCR approach targeting the *Zur* gene based on TaqMan® probe and compare it with real-time PCR targeting the *hrpF* gene, and (iii) verify if the real-time PCR method developed is suitable, sufficiently specific and sensitive for detection of Xcc in cabbage plant samples and seeds.

Materials and methods

Bacterial isolates

Bacterial isolates were obtained from various culture collections and natural conditions. Isolates of *X. campestris* pv. *campestris* (Xcc) WHRI 3811 (race 1), 3971A (race 1) and 1279A (race 4) were obtained from the collection of the University of Warwick, School of Life Sciences, Wellesbourne Campus, UK, and isolates NZ and Ked were obtained from the Central Institute for Supervising and Testing in Agriculture (CISTA, Czech Republic). In addition, four SU isolates were obtained from field conditions, from naturally infected cabbage heads and kohlrabi leaves growing in the Czech Republic (Svijanský Újezd, 50°35′14.13″N, 15°2′37.42″E), the isolate Led from Lednice (48°79′28.136″N, 16°80′26.908″E), the isolates Sum from Šumperk (49°96′52.839″N, 16°97′06.086″E) and the isolate Mal2 from Malešovice (49°02′35.942″N, 16°50′12.881″E) (Table 1).

Table 1 Results of standard and real-time PCR for detection of *Zur* and *hrpF* genes. Twenty isolates of *Xanthomonas* species and pathovars, five other bacterial isolates and negative controls were tested

Sample*	Standard PCR for detection of *Zur** (Eichmeier et al. 2015)	Standard PCR for detection of *hrpF** (Berg et al. 2005)	Real-time PCR for detection of *Zur*		Real-time PCR for detection of *hrpF* (Berg et al. 2006)
			Rep. Ct**	Rep. Calc. Conc. (copies μl^{-1})	Rep. Ct**
No Template Control	–	–	–	0	–
Negative Control	–	–	–	0	–
Xcc 3811	+	+	9.77	7.99E+06	14.72
Xcc 3971A	+	+	9.89	7.41E+06	14.75
Xcc 1279A	+	+	8.06	2.23E+07	12.76
Xcc SU	+	+	8.89	1.35E+07	14.00
Xcc SU2	+	+	9.02	1.25E+07	8.25
Xcc SU3	+	+	7.93	2.40E+07	8.52
Xcc SU4	+	+	9.31	1.05E+07	10.05
Xcc Mal2	+	+	5.83	8.51E+07	8.08
Xcc NZ	+	+	12.70	1.37E+06	17.63
Xcc Ked	+	+	11.19	3.39E+06	16.33
Xcc Led3	+	+	7.97	2.35E+07	7.95
Xcc Sum1	+	+	7.13	3.82E+07	9.62
Xcc Sum2	+	+	8.2	2.05E+07	10.23
Xc pv. *raphani*	+	+	8.13	2.13E+07	7.40
Xc pv. *incanae*	+	+	9.07	1.21E+07	8.16
Xc pv. *armoraciae*	+	+	10.59	1.38E+06	10.83
Xh pv. *carotae*	+	–	12.91	1.21E+06	–
X. cucurbitae	–	–	–	0	23.44
Xa pv. *phaseoli*	–	–	–	0	23.42
X. vesicatoria	–	–	–	0	18.10
Bacillus sp.	–	–	–	0	23.49
Erwinia sp.	–	–	–	0	24.12
Klebsiella sp.	–	–	–	0	22.23
Pantoea sp.	–	–	–	0	19.62
Ps. fluorescens	–	–	–	0	19.98

*-, negative reaction; +, positive reaction

**Rep. Ct, the average Ct of all samples with the same name as sample, Rep. Calc. Conc., the calculated concentration for all the samples with the same name, this is not simple average but rather geometric mean

For the comparison of the newly designed detection assay specificity, four pathovars/species of *X. campestris* occurring on different horticultural crops were included. An isolate received as *X. campestris* pv. *armoraciae* (Xca, B.01281) and isolates of *X. hortorum* pv. *carotae* (Xhca, B.01586), *X. cucurbitae* (Xcu, B.01397), *X. axonopodis* pv. *phaseoli* (Xaph, B.01695) and *Xanthomonas vesicatoria* (Xv, B.01859) were obtained from the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Hungary). Isolates of *X. campestris* pv. *raphani* (Xcr, WHRI 8503) and *X. campestris* pv. *incanae* (Xci, WHRI 6377) were obtained from the University of Warwick, School of Life Sciences, Wellesbourne Campus, UK.

Bacteria isolated from stored cabbage heads were used as negative controls including *Bacillus* sp.

(GenBank/NCBI Acc. No. KX160113), *Erwinia* sp. (KX160104), *Klebsiella* sp. (KX160125), *Pantoea* sp. (KX160105) and *Pseudomonas fluorescens* (KX160121) (Eichmeier et al. 2017a). All isolates are described in Supplementary Table 1.

DNA isolation and standard PCR

All isolates were cultured on Petri dishes on standard meat-peptone agar (MPA) (HiMedia, Mumbai, India) at 25 °C in the dark for 48 h. The bacterial cultures were collected into physiological solution (autoclaved 0.9% solution of NaCl) and the DNA was isolated by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to Peňázová et al. (2015). DNA from leaf tissues was isolated with the same kit. The concentration of DNA was adjusted to approximately 50 ng ml^{-1} according to fluorometry (Peňázová et al. 2015). Firstly, the specificity of standard PCR systems targeting the *Zur* gene (Eichmeier et al. 2015) and the *hrpF* gene (Berg et al. 2005) of *X. campestris* were compared. The standard PCR were performed as described in Eichmeier et al. (2015) using primer pair and ZUR1 EAC-CAE (Eichmeier et al. 2015) and DLH 120–125 (Berg et al. 2005) with all *X. campestris* isolates listed in Supplementary Table 1. Products of expected sizes (305 bp for *Zur* gene and 619 bp for *hrpF* gene) were gel purified and subjected to nucleotide sequencing as described by Eichmeier et al. (2010).

Design of a new real-time PCR assay based on *Zur* gene detection

The forward primer Zur2-EAC-fwd (5`-CAAACCGG TCAAGGCCTA-3`) and TaqMan® probe Zur1-TP (5`-HEX-CGCTGGATTTTTTGATGG-BHQ-3`) were designed in this study using CLC Genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The reverse primer Zur1-CAE-rev (5`-AGGCGACGAAGGCATTGA-3`) described by Eichmeier et al. (2015) was used. For verification of the newly designed method, an in silico specificity test was performed (Table 2). The analysis was based on 100% identity of available nucleotide sequences in GenBank/NCBI database with the designed primers and probe sequences. For the real-time PCR, the 2 × HoTaq Real-time PCR kit (MCLAB, San Francisco, USA) was used. The reaction mix consisted of 10 μl of 2 × HoTaq Real-time PCR kit, 6 μl of nuclease free water (Ambion, Foster City, USA), 0.8 μl of each primer (10 μM) Zur2-EAC-fwd and Zur1-CAE-rev, 0.4 μl of the TaqMan® probe (10 μM) and 2 μl of DNA template (approx. Concentration of 50 $ng.ml^{-1}$ from pure bacterial cultures and 200 ng ml^{-1} from plant tissues). All samples were tested in triplicate. For quantification, the cloning standards from range 10^7 to 10^1 copies μl^{-1} were used. The reaction was carried out on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) using the following temperature profile: denaturation at 95 °C for 3 min, 35 repeats of 95 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s and final extension at 72 °C for 7 min. The PCR products were checked on 1.2% agarose gels by electrophoresis.

Table 2 Species of *Xanthomonas* genus from GenBank/NCBI database with 100% nucleotide identity with primers or probes (in silico test) used in this study

Zur real-time assay (developed in this study)		
Zur2-EAC-fwd	Zur1-CAE-rev	Zur1-TP
X. campestris pv. *campestris*	*X. campestris* pv. *campestris*	*X. campestris* pv. *campestris*
X. campestris pv. *raphani*	*X. citri* pv. *glycines*	*X. campestris* pv. *raphani*
X. citri pv. *glycines*	*X. citri* pv. *vignicola*	*X. vesicatoria*
X. oryzae pv. *oryzicola*	*X. fuscans* pv. *aurantifolii*	–
–	*X. fuscans* pv. *fuscans*	–
–	*X. vesicatoria*	–
hrpF real-time assay (Berg et al. 2006)		
DLH153	DLH154	P7
X. campestris pv. *campestris*	*X. campestris* pv. *campestris*	*X. campestris* pv. *campestris*
X. campestris pv. *raphani*	*X. campestris* pv. *raphani*	*X. campestris* pv. *raphani*
–	*Aspergillus fumigatus*	–

Comparison of real-time PCR assays for detection of *Zur*, detection of *hrpF* and detection of both *hrpF* and *Zur* genes simultaneously

The newly designed real-time PCR assay based on *Zur* gene detection was compared with the ISTA recommended and approved real-time PCR assay designed Berg et al. (2006) targeting the *hrpF* gene. Each sample was tested following the method of Berg et al. (2006) using a 2 × HoTaq Real-time PCR kit (MCLAB, San Francisco, USA).

A combination or multiplexing of both assays, *hrpF* and *Zur*, was tested. The mastermix was based on 2 × HoTaq Real-time PCR kit (MCLAB, San Francisco, USA), including: 10 μl of the kit; 6 μl of nuclease free water (Ambion, Foster City, USA); 0.4 μl of each primer (10 μM) Zur2-EAC-fwd, Zur1-CAE-rev, DLH153, DLH154; 0.2 μl of the TaqMan® probes (10 μM) Zur1 TP and P7 and 2 μl of DNA template (approx. concentration of 50 ng.ml^{-1} from pure bacterial cultures and 200 ng ml^{-1} from plant tissues).

In planta and seed tests

These tests were done to verify the detection limits of the newly designed real-time PCR system. Plants of *B. rapa* var. *pekinensis* (cvs. Orange Heart and Beijing Spring Mini No. 2), *Brassica rapa subsp. chinensis* (cvs. Zi Guan No. 1 and Dwarf Milk) and *Brassica oleracea* convar. *capitata* (cvs. Albatros and Avak from MoravoSeedCZ) were grown a greenhouse in 2015 and 2016 at the Department of Mendeleum (MENDELU), in three replicates, and the real-time PCR assay was carried out in triplicates per plant. The first sampling was done after 1 week, the second after 1 weeks and the third after 3 weeks. The plants were inoculated in phenophase of four leaves by spraying and three Xcc strains were used (3971A, 1279A and SU). The spraying of inoculum was performed by hand atomizer Bosch PFS 55 (Robert Bosch GmbH, Gerlingen, Germany). Sprayed plants were covered by polyethylene bags to increase humidity and placed into the climabox with 28 °C and 16/8 h photoperiod. After 48 h, they were returned to greenhouse conditions. Control variants for each cultivar and strain were included. In total, 54 isolated total DNAs were used for the planta test, DNA was isolated as described lower from 50 mg of plant tissue. Then, the sample of cv. Zi Guan No. 1 from the third sampling (Ct 8.36, 3.24E+08) inoculated with the strain 3971A was diluted serially to see the detection limit.

The *Zur* based real-time PCR assay was used to detect Xcc in batches of Brassica seeds. Seeds of *Brassica oleracea* var. *sabellica* cv. Scarlet obtained from the same seed company were used. Five different batches of seeds (OS1-OS5) were artificially inoculated in a suspension of 10E+07-10E+08 cells per ml according to Roberts et al. (1999); a non-infected batch of seeds (NIS) was used as control. The seeds were placed on standard meat-peptone agar (MPA) (HiMedia, Mumbai, India) enriched with 0.05 g l^{-1} of cyclohexamide (Biosynth, Staad, Switzerland). Thirty seeds were plated in each petri dish and were kept at 25 °C in the dark for 48 h, two replicates per one batch. Then, the isolation was carried out from the growing bacterial cultures and also from the germinating seeds. The mix was frozen at -80 °C and grinded in friction bowl also frozen at -80 °C. Subsequently, 50 mg of the homogenized mix obtained from two replicates was used for DNA isolation as described below.

Results

Standard PCR systems targeting *Zur* and *hrpF* genes

Results of standard PCR targeting the *Zur* and the *hrpF* genes are presented in Table 1. PCR targeting the *Zur* gene was positive for *Xanthomonas campestris* pv. *campestris* (Xcc) and for strains received as *X. campestris* pvs. *Armoraciae* (Xca), *incanae* (Xci), *raphani* (Xcr) and also for *X. hortorum* pv. *carotae* (Xhca, syn. *X. campestris* pv. *carotae*). The PCR targeting *hrpF* gene was positive for all Xcc samples and for Xca, Xci, Xcr and *Bacillus* sp., *Erwinia* sp., *Klebsiella* sp., *Pantoea* sp. and *Pseudomonas fluorescens*.

Sequences of amplified fragments were compared with other available sequences in the GenBank/NCBI database. The isolate obtained as *X. campestris* pv. *armoraciae* (Xca) showed 99% identity of nucleotide sequences with Xci and Xcc (strain Sum2) in *Zur* gene and 99% identity with Xcc strains in the case of the *hrpF* fragment. For the Xhca, a product of *Zur* primers with length close to the target size was also amplified and sequenced; the highest similarity according to blastX (100% identity) in NCBI (Altschul et al. 1990) was to "cation:proton antiporter [*Xanthomonas hortorum* pv.

carotae str. M081]". Therefore, the sequence of this isolate was assigned to Xhca.

The obtained sequences were submitted into GenBank/NCBI under Acc. Nos. MG907588-MG907604 for *Zur* gene and MG907572-MG907587 for the *hrpF* gene. A molecular phylogenetic analysis of *Zur* (Supplementary Fig. 1) and *hrpF* (Supplementary Fig. 2) gene fragments using a maximum likelihood method showed close relationships between Xcc, Xcr, Xci and Xca. Moreover, the results of the phylogenetic analysis revealed three main clusters based on *Zur* gene where the first contains 10 Xcc and one Xcr strains, the second contains only the Xhc strain and the third very closely related Xci with Xca and three Xcc isolates. Supplementary Fig. 2 showed two clusters with no significant differences between Xcc, Xcr, Xca and Xci pathovars.

Real-time PCR assay based on *Zur* gene

The in silico test of newly designed primers and probe sequences proved 100% identity with available sequences of Xcc isolates (Table 2). The PCR efficiency of real-time *Zur* detection regarding the standard curve reached 82% (Fig. 1). R^2-value reached 0.99973, indicating a reliable relation between PCR-signal and standards concentrations. The calculation of concentration of samples was set by the regression equation $y = -3.835x + 36.23$ where $y = Ct$ value and $x = Log$ Qty. Ct values obtained from standard curve are indicated through Table 3.

The accuracy of the real-time PCR assay for Xcc detection was assessed on DNA isolated from pure bacterial cultures (Table 1) including 13 Xcc strains from different geographic origins, four bacterial strains representing other *X. campestris* pathovars, three members of *Xanthomonas* genus and five different cabbage bacterial endophytes. All Xcc isolates provided clearly positive results. Positive results were also obtained for Xca, Xci and Xcr and also *X. hortorum* pv. *carotae*. Other *Xanthomonas* species and pathovars and bacteria associated with cabbage health provided negative results (Table 1).

Comparison of real-time PCR assays for detection of *Zur*, detection of *hrpF* gene and detection of *hrpF* and *Zur* genes simultaneously

Using the real-time PCR developed by Berg et al. (2006) for detection of the *hrpF* gene, the same spectra of bacteria was detected as using the newly designed assay based on *Zur* gene including Xcc, Xca, Xci, with the exception of X. *hortorum* pv. *carotae*. In addition,

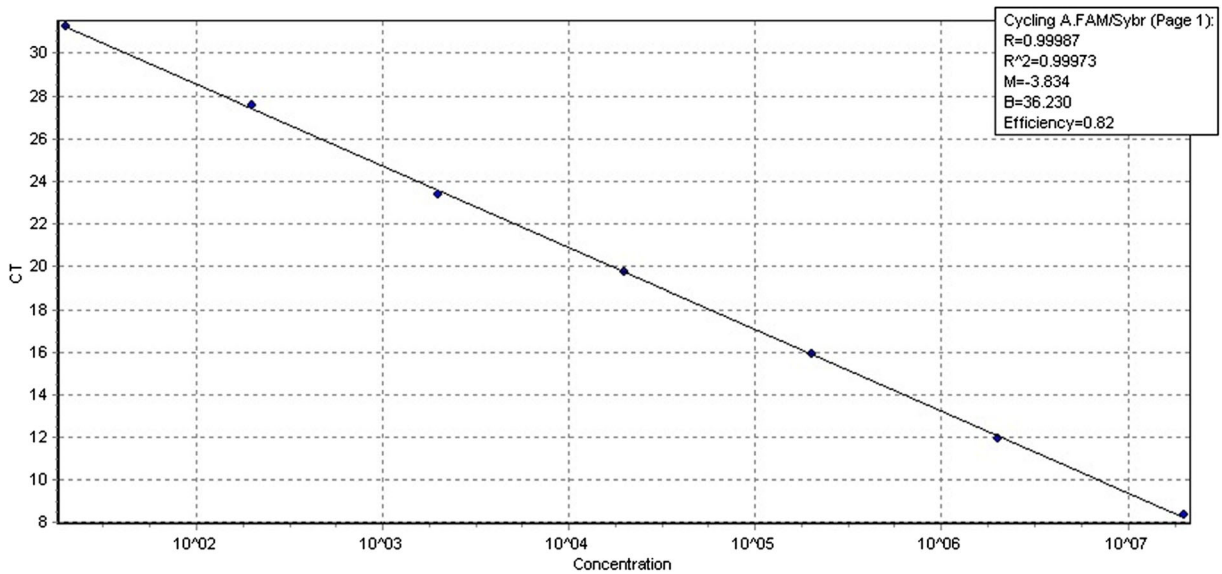

Fig. 1 Standard curve of real-time simplex *Zur* assay for *Xanthomonas campestris* pv. *campestris*. There are depicted the triplicates of the cloned standard prepared by Generi-Biotech (Hradec Králové, Czech Republic). The graph was constructed with Rotor-gene v. 6.0 (Corbett Research, Sydney, Australia)

Table 3 Diluted standards for determination of standard curve for *Zur* assay. Standard was prepared by cloning (Generi-Biotech, Hradec Králové, Czech Republic) a 152 bp fragment of the *Zur* gene of sample SU1 into plasmid pCR4 with known number of copies

Dilution	Real-time PCR for detection of *Zur*	
	Rep. Ct	Rep. Calc. Conc. (copies μl^{-1})
Standard 10^7	8.40	1.82E+07
Standard 10^6	11.91	2.20E+06
Standard 10^5	15.93	1.97E+05
Standard 10^4	19.73	2.01E+04
Standard 10^3	23.39	2.24E+03
Standard 10^2	27.56	1.82E+02
Standard 10^1	31.26	1.98E+01

detection using *hrpF* gene (Berg et al. 2006) was positive for a range of other bacteria including X. *cucurbitae*, Xa pv. *phaseoli*, X. *vesicatoria*, *Bacillus* sp., *Klebsiella* sp., *Pantoea* sp. and *Pseudomonas fluorescens* (Table 1).

The results based on multiplex real-time PCR detection of both genes, *Zur* and *hrpF*, are presented in Supplementary Table 2. Thirteen samples of Xcc were evaluated with both assays as positive. The isolate received as Xca from *Iberis* was also positive and the isolate of Xci was positive with lower values than all the Xcc isolates. Multiplex assay corresponded to simplex assays for Xcc isolates detection, but Ct (Threshold Cycle) values were mostly higher; in case of *Zur* the Ct mean increased from 8.91 to 16.48 and *hrpF* from 11.76 to 17.3.

Simplex (Table 1) and multiplex *hrpF* assay (Supplementary Table 2) did not detect *X. hortorum* pv. *carotae* whereas *Zur* assay detected Xhc.

Table 5 shows absolute quantification of *Zur* gene based on dilution of a standard (10E+07 copies.μl^{-1} prepared by Generi-Biotech company). For the cross-reaction test was selected the strain 3971A inoculated on *Brassica rapa subsp. chinensis* cv. Zi Guan No. 1 (total DNA extracted from the plant after 3 weeks). The multiplex *Zur* / *hrp* assay results are included in Supplementary Table 2. The Ct values match between simplex and multiplex assays. *Zur* assay in multiplex reaction shows Ct values from 9.36 (no dilution) to 21.42 (10,000 times diluted), multiplex *hrpF* assay shows from 14.51 (no dilution) to 25.92 (10,000 times diluted) and simplex *hrpF* assay shows from 12.44 (no dilution) to 28.06 (10,000 times diluted).

In planta and seed tests

Detection limits of *Zur* gene test were examined in planta tests. Six cultivars of brassicas were inoculated with three Xcc bacterial isolates (Table 4) and one of the randomly selected isolates was diluted as standard to test the differences in sensitivity between simplex and multiplex assays (Supplementary Material 1).

In planta tests verified the Ct variability of assay for determination of Xcc presence in *Brassica* tissues (Table 4) and also in seed samples (Table 5) which was based on detection of Xcc in extracted total DNA from infected plant tissues. The developed assay detected three strains of Xcc in tissues with the range of Ct values from 8.6 (1,16E+06) to 33.08 (1,36E+01) (Table 4) The seed test confirmed Xcc in the five batches of the seeds with the range of Ct values from 15.15 (3.14E+05) isolate OS5 and 23.48 (2.12E+03) isolate OS4 and the non-infected seeds were negative (Table 5).

Discussion

The detection of plant pathogens can be relatively simple when typical symptoms occur, but the identification of pathogens is complicated in asymptomatic plants or seeds as it requires sensitive and reliable systems that allow detection of pathogens in field samples. For this purpose, real-time PCR seems to be a suitable technique for testing asymptomatic plants and seeds. Real-time detection systems for the genus *Xanthomonas* are commonly used and specific reactions using labelled probes were previously published for *X. arboricola* pv. *pruni* (Palacio-Bielsa et al. 2011), *X. fragariae* (Vandroemme et al. 2008) and for *Xanthomonas* species associated with bacterial spot of tomato (Strayer et al. 2016). Protocols for Xcc were previously published by Rijlaarsdam et al. (2004) and Berg et al. (2006) based on *conserved hypothetical protein* and *hrpF* genes respectively. The assay of Rijlaarsdam et al. (2004) for standard PCR with amplicons of 370 and 445 bp and the assay of Berg et al. (2006) was used for real-time PCR detection. Companies providing real-time PCR components generally recommend the amplicon size in the range of 50 to 200 bp (50–150 bp, Applied BioSystems 2005; 75–200 bp, Bio-Rad Laboratories 2006; bellow 300 bp,

Table 4 *In planta* test for verification of the variability of the real-time *Zur* assay for determination of Xcc presence in *Brassica* tissues based on detection of Xcc in extracted total DNA from infected plant tissues. Six cultivars of brassicas inoculated (spraying by hand atomizer Bosch PFS 55) with the three isolates (SU, 1279A race 4, 3971A race 1) and the sampling was carried out three times. The mentioned values are the averages of triplicates. Each variant of cv./isolate inoculation was proved with the control which was not inoculated plant. All control plants were Xcc negative

Cultivar of brassica	Isolate	Sampling 1 (1 week after inoculation)			Sampling 2 (2 weeks after inoculation)			Sampling 3 (3 weeks after inoculation)		
		Rep. Ct	Rep. Ct Std. Dev.	Rep. Calc. Conc.	Rep. Ct	Rep. Ct Std. Dev.	Rep. Calc. Conc.	Rep. Ct	Rep. Ct Std. Dev.	Rep. Calc. Conc.
Orange Heart	SU	12.65	1.16	1.68E+04	26.07	1.68	7.10E+04	22.42	0.57	2.09E+04
Beijing Spring Mini No. 2	SU	12.83	0.89	1.87E+04	26.16	0.29	6.79E+02	26.78	1.06	8.24E+03
Zi Guan No. 1	SU	30.82	1.51	6.43E+03	28.73	0.46	8.29E+03	28.36	0.45	2.04E+03
Dwarf Milk	SU	29.89	0.86	4.40E+04	20.35	0.44	4.88E+04	20.02	0.63	3.14E+05
Albatros	SU	25.57	0.66	4.40E+04	10.23	0.06	1.07E+01	33.08	0.95	1.36E+01
Avak	SU	26.37	1.35	4.12E+04	31.19	1.16	1.35E+02	31.52	1.15	3.86E+01
Orange Heart	1279A (race 4)	8.60	1.47	1.16E+06	17.22	0.65	3.42E+06	19.40	1.59	1.56E+05
Beijing Spring Mini No. 2	1279A (race 4)	11.14	0.87	6.63E+04	23.79	0.37	1.37E+05	19.22	0.37	1.16E+05
Zi Guan No. 1	1279A (race 4)	25.65	5.47	1.53E+05	20.02	1.11	8.10E+05	20.30	0.97	6.42E+04
Dwarf Milk	1279A (race 4)	20.82	0.58	1.42E+05	21.75	0.75	2.57E+04	16.86	0.30	7.71E+05
Albatros	1279A (race 4)	19.50	0.72	1.15E+06	20.90	1.27	3.94E+04	22.44	0.65	4.07E+04
Avak	1279A (race 4)	20.42	1.24	7.28E+05	20.10	0.16	7.35E+04	21.53	0.31	4.74E+04
Orange Heart	3971A (race 1)	20.32	0.39	9.18E+06	17.08	0.21	5.53E+07	9.10	0.74	1.93E+08
Beijing Spring Mini No. 2	3971A (race 1)	22.11	0.20	3.02E+06	19.27	0.31	7.45E+06	9.22	0.81	2.01E+08
Zi Guan No. 1	3971A (race 1)	20.91	0.45	5.68E+06	18.33	0.20	1.79E+07	8.68	0.35	2.83E+08
Dwarf Milk	3971A (race 1)	18.45	0.23	4.39E+07	18.51	0.47	1.59E+07	10.03	0.17	1.29E+08
Albatros	3971A (race 1)	20.03	0.16	1.51E+07	19.28	0.40	7.94E+06	10.14	1.01	9.85E+07
Avak	3971A (race 1)	20.19	0.42	9.91E+06	19.66	0.51	5.59E+06	9.09	0.46	1.94E+08

Illumina 2011; 70–200 bp, Qiagen 2014). Although the amplicon length is more important in case of double-dye hydrolysis probes as TaqMan®, the SYBR Green based system also showed higher efficiency for smaller targets (Debode et al. 2017).

Standard PCR methods generally do not have the level of sensitivity required for seed testing and do not allow the quantification of pathogen particles in the samples. Therefore, we have adapted a standard PCR targeting the *Zur* gene (Eichmeier et al. 2015) by designing a sensitive real-time PCR assay for detection of Xcc. There is a higher probability of sensitive detection targeting *Zur* sequences (Huang et al. 2009; Tang et al. 2005) when comparing to the previous methods based on detection of genes belonging to the *hrp* cluster. The designed simplex real-time PCR system with TaqMan® probe was mainly specific for X. *campestris* as it detected 13 Xcc isolates from different origins, an isolate received as *Xc* pv. *armoraciae* (Xca) from *Iberis*, isolates of *Xc* pv. *raphani* (Xcr) from *B. oleracea*, *Xc* pv. *incanae* (Xci) from *Matthiola* and a X. *hortorum* pv. *carotae* (syn. X. *campestris* pv. *carotae*) (Xhca) isolate.

Table 5 Seed test based on *Zur* gene quantification. The seed test was based on isolated DNA from cultivated seeds (two replicates of 30 seeds, 50 mg of homogenized mix)

Isolate	Rep. Ct - aver. of triplicate	Rep. Ct Std. Dev. - average of triplicate	Rep. Calc. Conc. - average of triplicate
OS1	22.83	0.25	3.12E+03
OS2	9.64	1.16	2.12E+04
OS3	18.41	0.8	4.45E+04
OS4	23.48	0.22	2.12E+03
OS5	15.15	1.45	3.14E+05
NIS	0	0	0

NIS – non-infected batch of seeds

The same isolates were positive in standard PCR targeting the *Zur* gene (Eichmeier et al. 2015) and standard PCR targeting the *hrpF* gene (Berg et al. 2005) with the exception of X. *hortorum* pv. *carotae* that was not amplified in the latter test. We found out that the real-time system designed by Berg et al. (2006) is not working with high sensitivity for Xcc detection as it detects a range of other organisms that can occur in brassica samples. Detection of *Zur* gene is very useful as it can rule out some of these organisms. A multiplex test based on the detection of the two genes is more reliable than detection of one gene; although the price of multiplex assay is lower than two simplex reactions, the simplex *Zur* detection might be reliable and more straight-forward.

Sequencing of the *Zur* and *hrpF* regions showed that the Xca isolate from candytuft (*Iberis*) is closely related pvs. *campestris* and *raphani* for the *Zur* region and closely related to pv. *campestris* in case of *hrpF* gene. This isolate is identified in culture collections NCAIM (B.01281), NCPPB (347), CFBP (3838), ICMP (7), LMG (535) or VdM (13) as Xca. Vicente et al. (2001) expressed doubts about the identification of this isolate because no symptoms were found on horseradish after inoculation and rep-PCR showed that the isolate was closely related to Xcc (Vicente et al. 2006). Fargier and Manceau (2007) confirmed this and included this isolate in Xcc. The pathovar status of this isolate is still debatable as it does not cause disease in brassicas, but it causes a vascular disease in the host of origin, candytuft (Vicente et al. 2001; Vicente et al. 2006).

For the Xhca isolate from carrot, a product was amplified with *Zur* primers with length close to the target size; the sequence of this product is only 87% similar to Xcc, but the annealing positions of the primers were compatible enough to amplify the *Zur* fragment in conventional PCR described by Eichmeier et al. (2015) and in the *Zur* real-time assay.

The multiplex *Zur* and *hrpF* (Berg et al. 2006) assay generally confirmed the results obtained by simplex real-time assays differing in the Ct values that were higher in multiplex assay. The multiplex assay provides a robust detection method as it reliably and sensitively detects Xcc and only three other closely related Xc pathovars (Xcr, Xci and Xca). The multiplex assay was considered negative for Xhca as one of the tests was negative whereas the positive reaction was obtained only for the target gene. Cross reactions that happen using *hrpF* assay with bacteria naturally present in cabbage as *Pseudomonas*, *Bacillus*, *Erwinia* or *Pantoea* sp. (Eichmeier et al. 2017a) can be solved by the *Zur* assay (in simplex or multiplex real-time PCR). The fact that the multiplex real-time PCR increased the specificity of the detection is important. Samples of X. *cucurbitae* and Xa pv. *phaseoli* were not detected by multiplex assay and even in simplex *hrpF* assay they provided very high Ct values. Higher Ct values could mean that the signal could be provided by background of PCR (Ahmad and Ghasemi 2007) and simplex assays generally have higher amount of chemistry therefore generating more positives (Operator Manual, Rotor-Gene™3000).

With recent taxonomy, there were commonly expressed doubts about *Xanthomonas* classification (Schaad et al. 2000; Vauterin et al. 2000). In case of Xc pathovars, the distinction based on induced symptoms and the host from which they were firstly isolated was found as insufficient by Fargier and Manceau (2007). The disputes over the exact classification and also determination of isolates in culture collections can be solved by genetic analysis and pathogenicity tests (Khodakaramian and Swings 2002). For this purpose, more experimental data are necessary and methods involving whole genome comparisons should be used.

There is strong requirement to solve the genetic diversity according to molecular data and determine how many genes should be sequenced and to unravel the level of similarity typical for different groups of *Xanthomonas* strains. Whole genome sequencing and comparison data will possibly provide the most precise information for *Xanthomonas* taxonomy and provide information for the design of specific detection systems. At the moment, the distinction on the basis of

reaction of selected cultivars to the infection is still the main method for distinguishing pathovars and races of X. *campestris*. The determination by sequencing needs deeper knowledge about pathovars and their characterization. Molecular tools such as 16S rRNA gene sequencing provided a major improvement in the taxonomic approach at the genus level; nevertheless, this method is very limited at the species level for *Xanthomonas* because of the low polymorphism (Devulder et al. 2005). The sequencing of housekeeping or other genes is recommended by ad hoc committee (Stackebrandt et al. 2002; Drancourt and Raoult 2005). They advise the protein-coding gene sequence analysis to genomically circumscribe the taxon species and differentiating it from neighbouring species detected by rDNA sequences. On the basis of these recommendations, the *Zur* gene was chosen and successfully evaluated for its suitability for Xcc detection and for inclusion in the genes used for multi-genomic determination of phylogenetic relationships of Xcc isolates.

The *Zur* real-time assay is useful for Xcc detection from tissue samples of different *Brassica oleracea* species and seeds and is able to detect latent infection in tissues (Eichmeier et al. 2017b) (Supplementary Table 1). This study shows that a detection system based on the *Zur* gene is a sensitive, suitable method for testing *Brassica* sp. plant material and multiplexing the *Zur* and *hrpF* assays improves the specificity. Multiplex real-time assay provides a very robust real-time PCR detection for *X. campestris* pv. *campestris* as only two other isolates were positive - an isolate received as Xca that has been included in Xcc by Fargier and Manceau (2007) and an isolate of Xci that causes a vascular disease in ornamental stock (*Matthiola incana* L.). For *Brassica* seed testing and field surveys, the designed simplex Zur assay or the multiplex real-time PCR should be suitable to detect *Xanthomonas campestris* pathovars that cause vascular diseases that growers and seed producers consider a real threat. The multiplex assay saves money because one dose of the real-time kit is used for two gene detections. The methods developed in this study are much more specific than the real-time *hrpF* assay previously described by Berg et al. (2006) and currently recommended by ISTA. The additional *Zur* gene detection works well in the multiplex real-time PCR with *hrpF* and increases specificity; therefore the *Zur* simplex or the *hrpF/Zur* multiplex method can be used as alternatives to the recommended method.

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