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Specific primers for *Xanthomonas vesicatoria*, a tomato bacterial spot causal agent

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Abstract *Xanthomonas vesicatoria* is a member of the species complex associated with tomato bacterial spot. New and specific primers for *X. vesicatoria* were developed and validated. The primers were highly specific and detection was positive using purified bacterial DNA, bacterial suspensions and foliar lesions. These primers represent an additional tool for detection and identification of one of the species involved in this important disease complex.

Keywords *Solanum lycopersicum* · *Xanthomonas vesicatoria* · Molecular detection

Bacterial spot has a worldwide occurrence and causes serious economic losses (Jones et al. 1991). *Xanthomonas vesicatoria* is one of the species that compose the complex causing bacterial spot of tomato. Before the proposal of reclassification by Jones et al. (2004), the causal agents of the disease were classified as two distinct groups A and B, namely *Xanthomonas axonopodis* pv. *vesicatoria* and *X. vesicatoria* (Vauterin et al. 1995). Thus, the diversity within these two groups was still not well understood. Currently, four species are recognized as causal agents of tomato bacterial spot: *X. vesicatoria*, X. *euvesicatoria*, *X. perforans*, and *X. gardneri* (Jones et al. 2004). The most recent reports concerning *X. vesicatoria* were in the Russian Federation (Ignatov et al. 2009), the Indian Ocean region (Hamza et al. 2010), Tanzania (Mbega et al. 2012), and Brazil (Pereira et al. 2011; Costa et al. 2012). *Xanthomonas campestris* pv. *raphani* (Vicente et al. 2006), and *X. arboricola* (Mbega et al. 2012) may also be associated with lesions on tomato leaves, but biological and epidemiological aspects of these agents need to be better elucidated.

Specific primers are often used as molecular tools for the detection of various plant pathogens (Robène-Soustrade et al. 2010; López et al. 2012), including the tomato bacterial spot species complex (Koenraadt et al. 2009). However the genetic diversity of microorganisms can be generated and/or maintained by punctual deletions or insertions in the genome or by horizontal gene transfer (Arber 2000). Thus, to avoid false negatives in the identification process it is advisable to use more than one specific primer, whenever available. To overcome this constraint, primers designed to amplify conservative genome areas are being applied, such as the *gyrB* gene, which is rarely transmitted horizontally and is distributed ubiquitously among bacterial species (Kasai et al. 1998). This study aimed to develop specific primers from the partial DNA sequence of *gyrB* gene for *Xanthomonas*

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vesicatoria, and to validate them using a collection of *X. vesicatoria* from Brazil in assays with purified DNA, bacterial suspensions and symptomatic tomato leaves.

Fifteen strains of *X. vesicatoria* from three Brazilian regions (Midwest, Southeast and South) were used for the primers validation. The following strains were used as references: *X. vesicatoria*, IBSBF 2364 (Instituto Biológico de Campinas, São Paulo=XV1111, from New Zealand); *X. euvesicatoria*, IBSBF 2363 (Instituto Biológico de Campinas, São Paulo=Xvp197, from the USA), and EH 1996–212; *X. perforans*, IBSBF 2370 (Instituto Biológico de Campinas, São Paulo=ATCC BAA-983, from the USA), and EH 2009–111; *X. gardneri*, IBSBF 2373 (Instituto Biológico de Campinas, São Paulo=XCGA2, from Yugoslavia), and

Table 1 DNA samples used in this study and Xv-gyrB-F/Xv-gyrB-R-based PCR assay specificity

DNA Samples	Host	PCR
Xanthomonas vesicatoria		
EH 1995–95; EH 1995–99; EH 1995–101; EH 1994–102; EH 1995–103; EH 1995–104; EH 1995–105; EH 1995–107; EH 1995–108; EH 2009–42; EH 2010–08; EH 2010–24; EH 2010–25; EH 2010–41; EH 2010–59; IBSBF 2364[a]	Tomato	+
X. euvesicatoria	Tomato	–
EH 1996–212; IBSBF 2363 [a]		
X. perforans	Tomato	–
EH 2009–111; IBSBF 2370 [a]		
X. gardneri	Tomato	–
EH 2010–64; IBSBF 2373 [a]		
X. raphani, IAPAR 11300; IBSBF 1590	*Brassica* sp.	–
X. campestris pv. *campestris*, UnB 828	*Brassica* sp.	–
X. axonopodis pv. *manihotis*, UnB 1159	Cassava	–
X. campestris pv. *vitians*, UnB 110; UnB 830	Lettuce	–
X. citri pv. *anacardii*, IBSBF 2579	Cashew	–
X. campestris pv. *armoraciae*, IBSBF 1102	*Iberis* sp.	–
X. campestris pv. *viticola*, UnB 1318	Grape	–
X. citri pv. *mangiferaeindicae*, IBSBF 2586	Mango	–
Pseudomonas marginalis, IBSBF 1240	Tomato	–
P. cichorii, IBSBF 402; IBSBF 1748	Tomato	–
P. syringae pv. *syringae,* IBSBF 451; IBSBF 375; IBSBF 281	Tomato/Lilac	–
P. syringae pv. *tomato,* IBSBF 836; IBSBF 432; EH 75	Tomato	–
Pseudomonas viridiflava, IBSBF 1464	Tomato	–
Clavibacter michiganensis subsp. *michiganensis*, UnB 1151	Tomato	–
Ralstonia solanacearum, UnB 1273	Tomato	–
Acidovorax avenae subsp. *citrulli*, 646-2	Melon	–
Erwinia chrysanthemi, UnB 336	Chard	–
Erwinia psidii, IBSBF 1347; IBSBF 453	Guava	–
Pectobacterium carotovorum subsp. c*arotovorum,* IBSBF 776	Tomato	–
Alternaria solani, IBSBF 1940	Tomato	–
Corynespora cassiicola, IBSBF 1828	Tomato	–
Stemphylium sp., EH 502	Tomato	–
Unknown epiphytic and/or endophytic bacteria (10)	Tomato	–
DNA tomato	–	–

+, amplification; – no amplification

[a]*Xanthomonas* species reference strains associated with tomato bacterial spot, IBSBF 2363 originated from the USA, IBSBF 2364 from New Zealand, IBSBF 2370 from the USA and IBSBF 2373 from Yugoslavia

EH 2010–64. These strains are maintained in the work collection of the Laboratory of Plant Pathology of the Brazilian Vegetable Research Center (Embrapa Hortaliças), Brasília, DF, Brazil. Brazilian isolates were identified by: i) cultural characteristics in NA – nutrient agar medium (Schaad et al. 2001), ii) comparison of genomic profiles generated by BOX-PCR (Versalovic et al. 1991) with reference strain IBSBF 2364, and iii) pathogenicity tests on tomato (var. Yuba). Additionally, in order to attest the specificity of the new primers, 32 bacterial and fungal strains pathogenic to tomato or other hosts, from different collections (Embrapa Hortaliças – EH; Instituto Agronômico do Paraná – IAPAR, Instituto Biológico de Campinas – IBSBF and Universidade de Brasília – UnB), and 10 bacterial isolates representative of epiphytic and/or endophytic populations on tomato leaves were used (Table 1). Finally, the primers were evaluated with the DNA of tomato plants (vars. Floradade and Ponderosa).

DNA extraction was performed according to Wilson (1999) and/or Mahuku (2004). DNA samples were diluted up to the approximately concentration of 50 ng/μl and kept at – 20 °C until used. For amplification reactions with bacterial suspensions, we recovered the colonies grown on NA for 48 h at 28 °C. Bacterial suspensions were prepared in sterile distilled water, and its concentration was adjusted in spectrophotometer (OD_{600}=0.3) to approximately 5×10^8 CFU/ml (Jones et al. 2000). We used both purified DNA and bacterial suspensions (2 μl) for PCR reaction.

The primers were designed with Primer-BLAST Primer designing tool (http://www.ncbi.nlm.nih.gov), to amplify a 104-bp fragment from the partial sequence of the gyrase beta subunit (*gyrB*) gene of strain LMG 920 of *X. vesicatoria*, available at GenBank (access number EU015399.1). By using BLAST bases similarity (http://www.ncbi.nlm.nih.gov), 100 % query cover was obtained only for *X. vesicatoria* strains. The oligonucleotide sequences were: Xv-gyrB-F (5′-ATACGCGT TGGGCGAGCCT-3′) and Xv-gyrB-R (5′-CATCGCTGAAGATGGCCACG GCT-3′). PCR was performed in a thermocycler My Cycler™ (BIO RAD) with the following program: 95 °C for 4 min followed by 30 cycles of 95 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 3 min. The reactions consisted of: 1.62 mM of $MgCl_2$; 0.2 mM of each dNTPs; 1 μM of each primer; 1.26 U of *Taq* DNA polymerase; approximately 50 ng/μl of DNA; and Milli-Q® water to a final volume of 12 μl.

PCR products were analyzed by agarose gel (1.5 %) electrophoresis in 0.5X TBE buffer conducted at 5 V/cm for 2 h and scanned using the imaging system L-PIX ST (Loccus Biotecnologia, São Paulo, Brazil).

Bacterial suspensions (≈5×10^8 CFU/ml) were prepared in magnesium sulphate solution (10 mM) and homogeneously sprayed over leaf surfaces (two plants

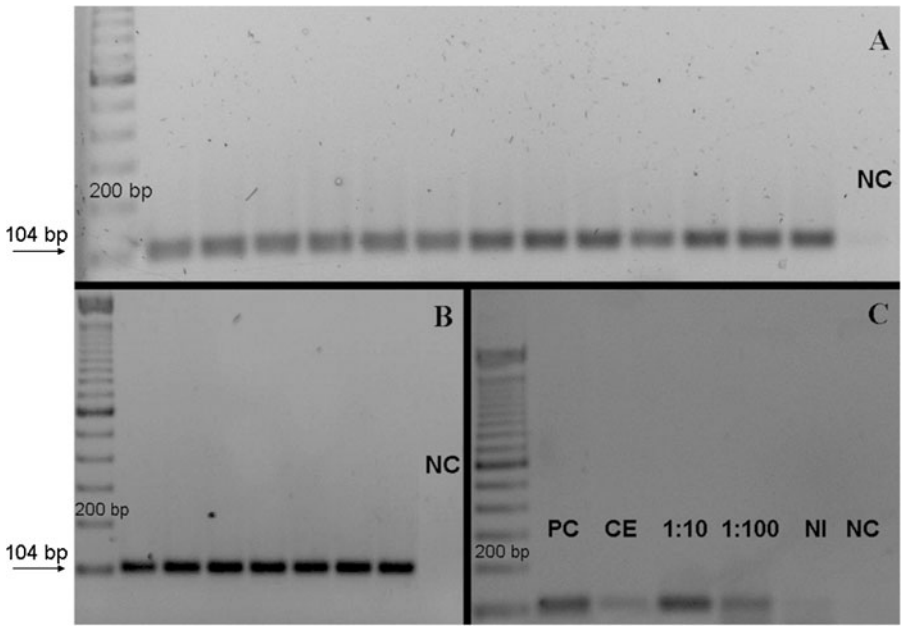

Fig. 1 Specificity of Xv-gyrB-F/Xv-gyrB-R-based PCR assay for the detection of *Xanthomonas vesicatoria* in A: purified DNA (≈ 50 μl/ml); B: Bacterial suspension (≈ 5×10^8 CFU/ml); C: Symptomatic leaves of tomato. PC: Positive control (IBSBF 2364); CE: Crude extract of symptomatic leaves of tomato; 1:10: 10-fold dilution of the crude extract of symptomatic leaves; 1:100: 100-fold dilution of the crude extract of symptomatic leaves; NI: Uninoculated leaves; NC: Negative control; M: molecular marker (100 bp DNA Ladder, Invitrogen)

with strain EH 2010–25) up to the run-off point. Plants were then incubated for 48 h in moist chambers consisting of plastic bags previously moistened with tap water. After symptom development, a circular section with a diameter of 10 mm contained one individual leaf spot was macerated in 200 μl of distilled sterilized water 10 days after the inoculation. For PCR reaction, 2 μl of the undiluted leaf crude extract and its dilutions of 1:10 and 1:100 were used. The visualization of the fragments was as previously described. For all assays, each reaction was performed at least twice.

The primers were specific, amplifying the purified DNA and bacterial suspensions of all strains of *X. vesicatoria* used (Fig. 1) but not DNA from the other species associated with tomato bacterial spot (Table 1). Similarly, there was no DNA amplification from other plant pathogens tested, or from tomato leaves.

The development of specific primers for *Xanthomonas* species associated with tomato bacterial spot has been reported in some studies. All four species can be identified and distinguished by the primers designed by Koenraadt et al. (2009). *Xanthomonas perforans* can be identified indirectly with primers (RST 88/89) developed by Astua-Monge et al. (2000) to amplify avirulence gene *avrXv3*. In the same way, *X. euvesicatoria* can be identified indirectly with primers (RST 27/28) designed by Bouzar et al. (1994) to amplify the gene *avrRxv* and also with specific primers (Xeu2.4/Xeu2.5) developed by Moretti et al. (2009). For *Xanthomonas vesicatoria*, besides the pair specific primers of Koenraadt et al. (2009), new specific primers were designed by Beran and Mráz (2013). These primers were developed from partial DNA sequences of *atpD*, and were validated with purified bacterial DNA. Primers Xv-gyrB-F/Xv-gyrB-R, here reported, are an additional tool for confirming *X. vesicatoria* identification, that can be also used for direct detection of the pathogen on its host tissue were, speeding up the process of diagnosis (Fig. 1).

The crude extracts samples inhibited PCR, but from a 1:10 dilution it was possible to detect the pathogen through a positive PCR signal. Inhibition of PCR when using crude extracts as templates is often observed because plant polyphenols can form complexes with nucleic acids (Koonjul et al. 1999). The studies of Moretti et al. (2009) for *X. euvesicatoria*, and of Araújo et al. (2012), for the four tomato bacterial spot *Xanthomonas* have also shown the applicability of specific primers directly in symptomatic plants.

The primers developed in this study need to be further evaluated for their sensitivity and reproducibility for routine use. However, their specificity for *X. vesicatoria* and a detection limit that allows the amplification of the pathogens DNA in a diluted crude extract from a lesion, reveal their potential to be also used in diagnostic assays with infected seeds and seedlings.

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