

ORIGINAL ARTICLE

Simultaneous detection and identification of the *Xanthomonas* species complex associated with tomato bacterial spot using species-specific primers and multiplex PCR

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multiplex PCR, PCR, sensibility, specificity, *Xanthomonas euvesicatoria*, *X. gardneri*, *X. perforans*, *X. vesicatoria*.

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Abstract

Aims: To establish protocols for the simultaneous detection and identification of *Xanthomonas* species causing tomato bacterial spot.

Methods and Results: We verified the specificity and sensitivity of the previously reported sets of primers designed for strains of the four species of Brazilian tomato bacterial spot xanthomonads, consisting of 30 of *Xanthomonas euvesicatoria*, 30 of *X. vesicatoria*, 50 of *X. perforans* and 50 of *X. gardneri*. Furthermore, we tested a multiplex PCR protocol for the purpose of concurrent species identification. The possibility of direct detection of the pathogens in diseased leaf samples was also verified. The primers were highly specific, amplifying only target DNA. The sensitivity of the primers in conventional PCR was 50 pg μl^{-1} for purified DNA and ranged from 5×10^2 to 5×10^4 CFU ml^{-1} when bacterial suspensions were analysed. The multiplex PCR was suitable for the detection of all four species and showed similar sensitivity to conventional PCR when tested on purified DNA. When using bacterial suspensions, its sensitivity was similar to conventional PCR only when a biological amplification step (Bio-PCR) was included. Both methods were able to detect the pathogens in symptomatic tomato leaves.

Conclusions: Brazilian *Xanthomonas* strains causing tomato bacterial spot can be differentiated and identified at species level by a PCR-based method and by a multiplex PCR.

Significance and Impact of the Study: This protocol may be a feasible alternative tool for the identification and detection of these pathogens in plant material and may be used for routine diagnostic purposes in plant pathology laboratories.

Introduction

A complex of species of the genus *Xanthomonas* are causal agents of tomato bacterial spot, namely *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri* (Jones *et al.* 2004). Bacterial spot has a worldwide occurrence and can be found on all aerial parts of the plant. Yield reduction because of the disease is a result of the direct effect on the photosynthetic leaf area, the drop of buds

and flowers and the reduction in commercial fruit value (Jones *et al.* 1991). Profits also decrease with the cost of chemical control (Quezado-Duval and Lopes 2010). Infected seeds, volunteer crop plants and diseased plant debris may serve as inoculum sources of the disease (Jones *et al.* 1991; Quezado-Duval and Lopes 2010). The organism can be disseminated by rain and/or sprinkler irrigation droplets driven by the wind (Jones *et al.* 1991; Quezado-Duval and Lopes 2010) within fields and from

nearby fields. The four species associated with bacterial spot have been reported in Brazilian commercial fields of both the processing and fresh market tomato segments (Quezado-Duval *et al.* 2005; Pereira *et al.* 2011). Therefore, the establishment of a methodology for the rapid and efficient diagnosis of the disease and detection of the pathogen(s) is necessary for both routine work and research related to this pathosystem.

Molecular identification of *Xanthomonas* species including the tomato bacterial spot agents has been performed by PCR followed by restriction enzyme analyses (Leite Júnior *et al.* 1994, 1995) and by rep-PCR (Louws *et al.* 1995). Species-specific primers have been designed recently allowing the identification of a single or several, but not all, species of the *Xanthomonas* complex associated with bacterial spot (Cuppels *et al.* 2006; Moretti *et al.* 2009). Koenraadt *et al.* (2009), on the other hand, had designed primers based on AFLP analysis for all four species, emphasizing that a broader spectrum of strains should be tested for primer validation, mainly for *X. vesicatoria*, *X. perforans* and *X. gardneri*. These specific primers seemed to be interesting for use in Brazil, where all four species have been reported as occurring in tomato-producing areas (Quezado-Duval *et al.* 2005). Therefore, it is necessary to evaluate their specificity and sensitivity with Brazilian strains, so that they can be used routinely for the detection and identification of bacteria in different parts of the plant.

In addition, these specific primers could be used in a multiplex format where the species complex of bacterial spot could be simultaneously detected and identified. The detection of plant pathogens by multiplex PCR has been an increasingly common practice in diagnostic procedures (Berg *et al.* 2006; Pulawska *et al.* 2006; Robène-Soustrade *et al.* 2010). The methods or protocols described so far for tomato bacterial spot have not been able to accomplish this simultaneous detection and identification of the different species causing the disease.

A multiplex PCR protocol for the simultaneous detection of three seed-borne tomato phyto-bacteria [*Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas axonopodis* pv. *vesicatoria*, presently known as *X. euvesicatoria* (Jones *et al.* 2004; Young *et al.* 2008)] was developed by Özdemir (2005, 2009). Using this technique, it was possible to simultaneously identify the different species that may be associated epiphytically or surviving in host tissues. This shows the potential of multiplex PCR for identification of the four tomato bacterial spot xanthomonads.

Thus, this study aimed to validate more broadly the specific primers previously developed for these species with representative Brazilian strains, verifying its

specificity and sensitivity, and also to establish a multiplex PCR protocol for simultaneous detection.

Materials and methods

Strain collection and DNA extraction

The strains were maintained at the work collection of the Laboratory of Plant Pathology of the Brazilian Vegetable Research Center (Embrapa Hortaliças), Brasília, DF, Brazil, and had been previously identified at the species level by pathogenicity tests on susceptible tomatoes (var. Yuba or Bonny Best), rep-PCR and presence of avirulence genes *avrRxv* in *X. euvesicatoria* and *avrXv3* in *X. perforans*, verified by PCR with primers RST27/28 and RST 88/89, respectively (Bouzar *et al.* 1994; Astua-Monge *et al.* 2000). The majority had been obtained from tomato (*Solanum lycopersicum* L.) leaves and fruits showing typical symptoms of bacterial spot, collected in fourteen states of Brazil. However, a few strains of *X. euvesicatoria* were also isolated from pepper (*Capsicum* spp.) fields as this species was not frequently found in tomato (Table 1). The isolation was performed in nutrient agar (NA) culture medium (Schaad *et al.* 2001), and the strains were preserved in phosphate buffer (8.5 mmol l⁻¹ K₂HPO₄; 7.5 mmol l⁻¹ KH₂PO₄, pH 7.0) at room temperature and/or in nutrient broth amended with 30% glycerol at -80°C. The strains preserved on phosphate buffer were recovered every 6–12 months, and all rep-PCR haplotypes and strains representing different geographical origins were also maintained at -80°C. Thirty strains of *X. euvesicatoria*, 30 of *X. vesicatoria*, 50 of *X. perforans* and 50 of *X. gardneri* were used (Table 1). Thirty-two bacterial and fungal strains pathogenic to tomato or other hosts, from different collections (Table 2), and 13 bacterial strains representative of epiphytic and/or endophytic populations on tomato leaves were also used. These strains were obtained by macerating leaves of plants taken from the field and isolation in NA. These strains were selected for differences in their colony morphology.

In addition, the following strains from the reference collection of plant pathogenic bacteria of the Biological Institute of São Paulo were used: IBSBF 2363 = Xvp197 (*X. euvesicatoria*); IBSBF 2364 = XV1111 (*X. vesicatoria*); IBSBF 2370 = ATCC BAA-983 (*X. perforans*); and IBSBF 2373 = XCGA2 (*X. gardneri*).

All bacterial strains were recovered and grown on NA for 48 h at 28°C. Whenever necessary, bacterial suspensions were prepared in sterile distilled water, and its concentration was adjusted in spectrophotometer (OD₆₀₀ = 0.3) to 5 × 10⁸ CFU ml⁻¹ (Jones *et al.* 2000).

DNA extraction was performed according to Wilson (1999) and/or Mahuku (2004) and both yielded DNA of

Table 1 *Xanthomonas* spp. causing bacterial spot of tomato used in this study

Species/ Strains	Host	Origin†	Year of isolation
<i>Xanthomonas euvesicatoria</i> EH 36P; EH 37P; EH 38P; EH 39P; EH 40P; EH 41P; EH 44P; EH 83P; EH 84P; EH 85P; EH 92P; EH 96P; EH 97P; EH 100P; EH 101P; EH 110P; EH 116P; EH 118P; EH 1996-209; EH 1996-210; EH 1996-211; EH 1996-212; EH 1996-213; EH 1996-214; EH 1996-216; EH 1996-217; EH 1996-219; EH 1996-220; EH 2009-46; EH 2009-47; IBSBF 2363*	Tomato/Pepper	PE; MG; AM; SP; RR; DF; GO; ES; CE; BA	1996; 1998; 1999; 2002; 2009; 2010; 2011
<i>Xanthomonas vesicatoria</i> EH 1995-95; EH 1995-96; EH 1995-99; EH 1995-101; EH 1995-102; EH 1995-103; 1995-104; 1995-105; EH 1995-107; EH 1995-108; EH 1995-110; EH 1995-111; EH 1995-112; EH 1995-113; EH 1995-122; EH 1996 202; EH 2007-08; EH 2008-32; 2008-167; EH 2008-168; EH 2008-169; EH 2008-170; EH 89T; EH 2010-01; EH 2010-08; EH 2010-24; EH 2010-25; EH 2010-41; EH 2009-42; EH 2010-59; IBSBF 2364*	Tomato	GO; SC; RJ; MG	1995; 1996; 2007; 2008; 2010
<i>Xanthomonas perforans</i> EH 2005-54; EH 2006-44; EH 2007-26; EH 2008-13; EH 2008-16; EH 2009-11; EH 2009-12; EH 2009-13; EH 2009-104; EH 2009-105; EH 2009-106; EH 2009-107; EH 2009-110; EH 2009-112; EH 2009-133; EH 2009-134; EH 2009-136; EH 2009-143; EH 2009-148; EH 2009-149; EH 2009-156; EH 2009-172; EH 2009-212; EH 2009-229; EH 2010-18; EH 2010-19; EH 2010-20; EH 2010-33; EH 2010-49; EH 2010-60; EH 2010-61; EH 2010-93; EH 2010-94; EH 2010-107; EH 2010-111; EH 2010-121; EH 2010-122; EH 2010-133; EH 2010-134; EH 2010-142; EH 2010-155; EH 2010-161; EH 2010-162; EH 2010-163; EH 2010-185; EH 2011-01; EH 2011-02; EH 2011-03; EH 2011-04; EH 2011-05; IBSBF 2370*	Tomato	GO; BA; MG; SC; SP	2005; 2006; 2007; 2008; 2009; 2010; 2011
<i>Xanthomonas gardneri</i> EH 2006-17; EH 2006-21; EH 2006-52; EH 2007-12; EH 2007-13; EH 2007-17; EH 2007-19; EH 2007-20; EH 2007-22; EH 2007-34; EH 2007-39; EH 2007-41; EH 2008-22; EH 2009-37; EH 2010-02; EH 2010-03; EH 2010-04; EH 2010-06; EH 2010-07; EH 2010-09; EH 2010-10; EH 2010-12; EH 2010-13; EH 2010-14; EH 2010-15; EH 2010-17; EH 2010-21; EH 2010-22; EH 2010-23; EH 2010-31; EH 2010-32; EH 2010-37; EH 2010-38; EH 2010-42; EH 2010-44; EH 2010-45; EH 2010-46; EH 2010-52; EH 2010-53; EH 2010-56; EH 2010-57; EH 2010-62; EH 2010-63; EH 2010-64; EH 2010-65; EH 2010-67; EH 2010-68; EH 2010-70; EH 2010-73; EH 2010-74; IBSBF 2373*	Tomato	GO; PR; RS; ES; SC; MG	2006; 2007; 2008; 2009; 2010

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

†The strains originated from different Brazilian states. The abbreviations represent the names of the states.

similar quality. Quantification of DNA was performed on agarose gels by visual comparison with the marker DNA High Mass Ladder (Invitrogen, Carlsbad, CA). DNA samples were diluted to approximately 50 ng μl^{-1} and kept at -20°C until used.

Conventional PCR

The primers used were those designed by Koenraad *et al.* (2009). Sequences, target species and expected PCR products were as follows: BS-XeF (5'-CATGAAGAACTCGGC-

Table 2 Specificity of primers to *Xanthomonas* spp. that cause bacterial spot of tomato

DNA Samples	Host	Species-specific primers			
		Bs-XeF/XeR	Bs-XvF/XvR	Bs-XpF/XpR	Bs-XgF/XgR
<i>Xanthomonas euvesicatoria</i> EH 36P; EH 37P; EH 38P; EH 1996-210; EH 1996-211; EH 1996-212; EH 1996-213; EH 1996-220; EH 2009-46; EH 2009-47; IBSBF 2363	Tomato/pepper	+	–	–	–
<i>Xanthomonas vesicatoria</i> EH 1995-95; EH 1995-96; EH 1995-99; EH 1995-101; EH 2008-169; EH 2008-170; EH 2010-01; EH 2010-41; EH 2009-42; EH 2010-59; IBSBF 2364	Tomato	–	+	–	–
<i>Xanthomonas perforans</i> EH 2005-54; EH 2006-44; EH 2009-136; EH 2009-143; EH 2009-148; EH 2009-149; EH 2010-49; EH 2010-60; EH 2010-61; EH 2010-93; IBSBF 2370	Tomato	–	–	+	–
<i>Xanthomonas gardneri</i> EH 2006-17; EH 2006-21; EH 2006-52; EH 2010-38; EH 2010-42; EH 2010-44; EH 2010-65; EH 2010-67; EH 2010-73; EH 2010-74; IBSBF 2373	Tomato	–	–	–	+
<i>Xanthomonas raphani</i> , IAPAR 11300; IBSBF 1590	<i>Brassica</i> sp.				
<i>Xanthomonas campestris</i> pv. <i>campestris</i> , UnB 828	<i>Brassica</i> sp.				
<i>Xanthomonas axonopodis</i> pv. <i>manihotis</i> , UnB 1159	Cassava				
<i>Xanthomonas campestris</i> pv. <i>vitians</i> , UnB 1079; UnB 110; UnB 830	Lettuce				
<i>Xanthomonas citri</i> pv. <i>anacardii</i> , IBSBF 2579	Cashew				
<i>Xanthomonas campestris</i> pv. <i>viticola</i> , UnB 1318	Grape				
<i>Xanthomonas citri</i> pv. <i>mangiferaeindicae</i> , IBSBF 2586	Mango				
<i>Pseudomonas marginalis</i> , IBSBF 1240	Tomato				
<i>Pseudomonas cichorii</i> , IBSBF 402; IBSBF 1748; UnB 1142	Tomato				
<i>Pseudomonas syringae</i> pv. <i>syringae</i> , IBSBF 451; IBSBF 375; IBSBF 281	Tomato/Lilac				
<i>Pseudomonas syringae</i> pv. <i>tomato</i> , IBSBF 836; IBSBF 432; EH 75	Tomato		No amplification		
<i>Pseudomonas viridiflava</i> , IBSBF 1464	Tomato				
<i>Agrobacterium tumefaciens</i> , UnB 1138	Tomato				
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> , UnB 1151	Tomato				
<i>Ralstonia solanacearum</i> , UnB 1273	Tomato				
<i>Acidovorax avenae</i> subsp. <i>citrulli</i> , 646-2	Melon				
<i>Erwinia chrysanthemi</i> , UnB 336	Chard				
<i>Erwinia psidii</i> , IBSBF 1347; IBSBF 453	Guava				
<i>Pectobacterium carotovorum</i> , UnB 1036	Turnip				
<i>Alternaria solani</i> , IBSBF 1940	Tomato				
<i>Corynespora cassicola</i> , IBSBF 1828	Tomato				
<i>Stemphylium</i> sp., EH 502	Tomato				
Unknown epiphytic and/or endophytic bacteria (13)	Tomato				
DNA tomato	–				

+, amplification; – no amplification.

GTATCG-3') and BS-XeR (5'-GTCCGACATAGTGGAC-ACATAC-3'), 173 bp for *X. euvesicatoria*; BS-XvF (5'-CCATGTGCCGTTGAAATACTTG-3') and BS-XvR (5'-ACAAGAGATGTTGCTATGATTTGC-3'), 138 bp for *X. vesicatoria*; BS-XpF (5'-GTCGTGTTGATGGAGCGTTC-3')

and BS-XpR (5'-GTGCGAGTCAATTAT-CAGAATGTGG-3') 197 bp for *X. perforans*; and BS-XgF (5'-TCAGTGCTTAGTT-CCTCATTTGTC-3') and BS-XgR (5'-TGACCGATAAAGACTGCGAAAG-3'), 154-bp amplicon for *X. gardneri*. PCR was performed in a thermocycler—My Cycler

(Bio-Rad) with the following program: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 66°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 7 min. The reactions consisted of: 1.5 mmol l⁻¹ of MgCl₂; 0.2 mmol l⁻¹ of each dNTPs; 2 μmol l⁻¹ for all primers; 1.26 U of *Taq* DNA polymerase (Invitrogen); approximately 50 ng μl⁻¹ of DNA; and Milli-Q[®] water to a final volume of 12 μl. The reactions using bacterial suspensions were carried out with the same concentrations of the reagents, adding 2 μl of each suspension (5 × 10⁸ CFU ml⁻¹) per template to a final volume of 12 μl. PCR products from different species were analysed by agarose gel (1.5%) electrophoresis in 0.5X TBE buffer conducted at 100 V for 2 h and scanned, photographed using the imaging system L-PIX ST (Loccus Biotecnologia, Cotia, São Paulo, Brazil). For each strain, each reaction was performed at least twice.

Specificity of primers in conventional PCR

The study was carried out using 209 strains (Tables 1 and 2). For the specificity, assays primers were tested with strains of their respective species, as well as with strains from other phytopathogenic bacteria and fungi that cause disease on tomato or other hosts (Table 2). In the case of bacterial spot xanthomonads, each primer pair was challenged with 10 strains of each of the three nonrespective species. The primers were also tested with DNA of epiphytic and/or endophytic bacterial strains originated from tomato leaves. Finally, all primers were also evaluated with DNA extracted from healthy tomato leaves (varieties Yuba, Floradade and Ponderosa).

Sensitivity of specific primers in conventional PCR

For determination of the sensitivity of the PCR primers, the following strains were used: *Xanthomonas euvesicatoria* (IBSBF 2363, EH 2009-46), *X. vesicatoria* (IBSBF 2364, EH 2010-41), *X. perforans* (IBSBF 2370, EH 2008-13) and *X. gardneri* (IBSBF 2373, EH 2010-42). The sensitivity of each primer pair was evaluated using both purified DNA and bacterial suspensions (2 μl). A concentration gradient from 50 ng μl⁻¹ to 0.5 pg μl⁻¹ was obtained by 10-fold serial dilutions of a DNA aliquot of each species. For bacterial suspensions, the gradient was obtained by a 10-fold serial dilution, from 5 × 10⁸ CFU ml⁻¹ up to 5 CFU ml⁻¹. Each dilution was tested in three subsamples per strain (two of each species). The PCR was repeated twice.

Multiplex PCR protocol

As for the conventional PCR, both purified DNA and bacterial suspensions in a gradient of concentrations were applied in multiplex PCR assays.

The final volumes of PCR varied according to the combination of primer–DNA from different species, ranging from 46 to 52 μl. Thus, each reaction contained 4.8 μl of buffer 1X; 1.44 μl of MgCl₂ (1.5–1.33 mmol l⁻¹); 3.84 μl of dNTPs (0.2–0.18 mmol l⁻¹ of each dNTPs); 19.2 μl of a mix containing the four primer pairs (2.0–1.77 μmol l⁻¹ for all primers); 1 μl of *Taq* DNA polymerase (1.26–1.11 U); 13.72 μl of Milli-Q[®] water; and aliquots of 2 μl template of each species.

Bio-PCR (Schaad *et al.* 1995) was employed in an attempt to increase the sensitivity of the multiplex assay (Guo *et al.* 2000). Enrichment was accomplished by streaking the bacterial suspension at different concentrations on NA. Plates were then incubated at 28°C for 48 h, and the colonies were washed with 1 ml of sterilized water. A sample of 2 μl was used for PCR. The amplicons were separated on agarose gels (3.0%) by electrophoresis in 0.5X TBE buffer at 100 V for 2 h and 30 min and scanned, photographed as previously described.

Detection of xanthomonads in symptomatic leaves

The diagnostic potential of the PCR methods to directly detect bacterial spot *Xanthomonas* species was evaluated in artificially inoculated tomato plants (var. Yuba) with four to five true leaves. Species representative strains were used for inoculation (*X. euvesicatoria*, EH 2009-46; *X. vesicatoria*, EH 2010-41; *X. perforans*, EH 2008-13 and *X. gardneri*, EH 2010-42). Bacterial suspensions were adjusted to 5 × 10⁸ CFU ml⁻¹ in magnesium sulphate solution (10 mmol l⁻¹) and homogeneously sprayed over leaf surfaces (two plants for each strain separately) 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. The onset of symptoms occurred from 7 up to 10 days, when the symptomatic leaves were sampled for PCR procedures.

Individual lesions were macerated in 200 μl of distilled sterilized water. The undiluted crude extract and dilutions 1 : 10, 1 : 100 and 1 : 1000 were used for both single and multiplex PCR. The amplicons were analysed on agarose gels 1.5 or 3.0%, respectively, depending on the PCR method. For each sample, each reaction was repeated at least twice.

Results

Specificity of primers in conventional PCR

The four primer pairs tested amplified DNA only from their respective target species. A total of 164 strains (31 of *Xanthomonas euvesicatoria*; 31 of *X. vesicatoria*; 51 of

X. perforans and 51 of *X. gardneri*), including Brazilian strains and references (Table 1; Fig. 1), were tested. No other pathogens from tomato or representatives of epiphytic and/or endophytic bacterial populations of tomato leaves were amplified by any of the evaluated primer pairs (Table 2). In the same way, DNA samples and crude extracts of healthy tomato leaves were not PCR amplified by any of these primer sets.

Sensitivity of the specific primers in conventional PCR

When all primers were individually tested with DNA from their respective target species, the detection threshold was $50 \text{ pg } \mu\text{l}^{-1}$ (Table 3). However, when bacterial suspensions were used directly in the reactions,

differences in sensitivity were observed. Primer pair Bs-XeF/Bs-XeR specific for *X. euvesicatoria* and primer pair Bs-XpF/Bs-XpR for *X. perforans* were able to detect the target bacteria in a cell suspension of up to $5 \times 10^2 \text{ CFU ml}^{-1}$, which corresponds to one bacterial cell per reaction. Primers pairs for *X. vesicatoria* and *X. gardneri* (namely Bs-XvF/Bs-XvR and Bs-XgF/Bs-XgR, respectively) were less sensitive, amplifying target DNA in suspensions with a minimum of $5 \times 10^4 \text{ CFU ml}^{-1}$ or the equivalent of 100 bacterial cells per reaction (Fig. 2).

Multiplex PCR

It was possible to perform the detection and identification of individual species of *Xanthomonas* involved in the

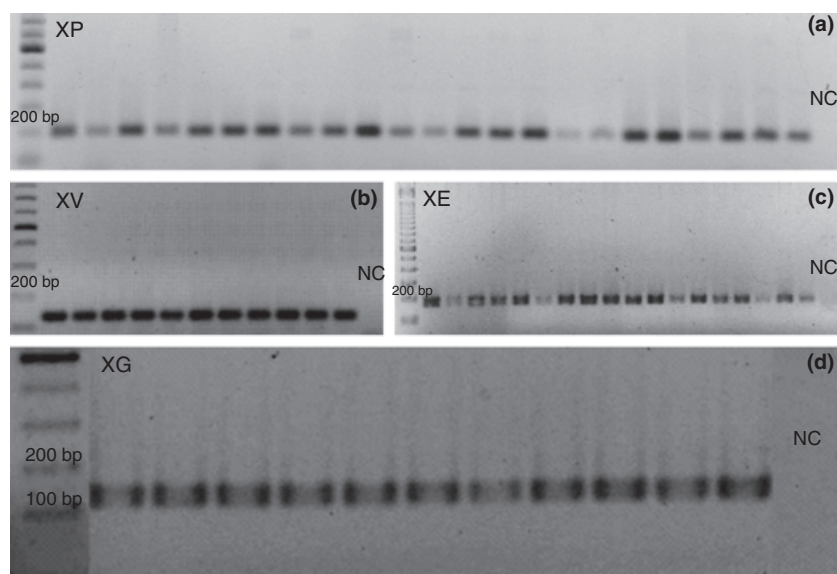


Figure 1 Amplicons from DNA of different *Xanthomonas* species causing bacterial spot of tomato obtained with species-specific primers. (a) *X. perforans* with primer pair Bs-XpF/Bs-XpR, (b) *X. vesicatoria* with primer pair Bs-XvF/Bs-XvR, (c) *X. euvesicatoria* with primer pairs Bs-XeF/Bs-XeR (d) *X. gardneri* with primer pair Bs-XgF/Bs-XgR. NC, negative control. M, molecular markers [100-bp DNA Ladder, Invitrogen, in (b) and (d); 1 kb Plus DNA Ladder, Invitrogen, in (a) and (c)].

Table 3 Sensitivity of species-specific primers to *Xanthomonas* spp. associated with tomato and pepper bacterial spot using different PCR methods

Method	Detection limit				
	<i>Xanthomonas euvesicatoria</i>	<i>Xanthomonas vesicatoria</i>	<i>Xanthomonas perforans</i>	<i>Xanthomonas gardneri</i>	All species
	Bacterial suspension (CFU ml^{-1})				
Conventional PCR	5×10^2	5×10^4	5×10^2	5×10^4	–
Multiplex PCR*	5×10^2	5×10^4	5×10^2	5×10^4	5×10^7
	Purified DNA ($\mu\text{g } \mu\text{l}^{-1}$)				
Conventional PCR	50	50	50	50	–
Multiplex PCR	50	50	50	50	50 000

–, not applicable.

*Initial concentration of bacterial suspensions that were detected after an enrichment step on NA medium for 48 h (Bio-PCR).

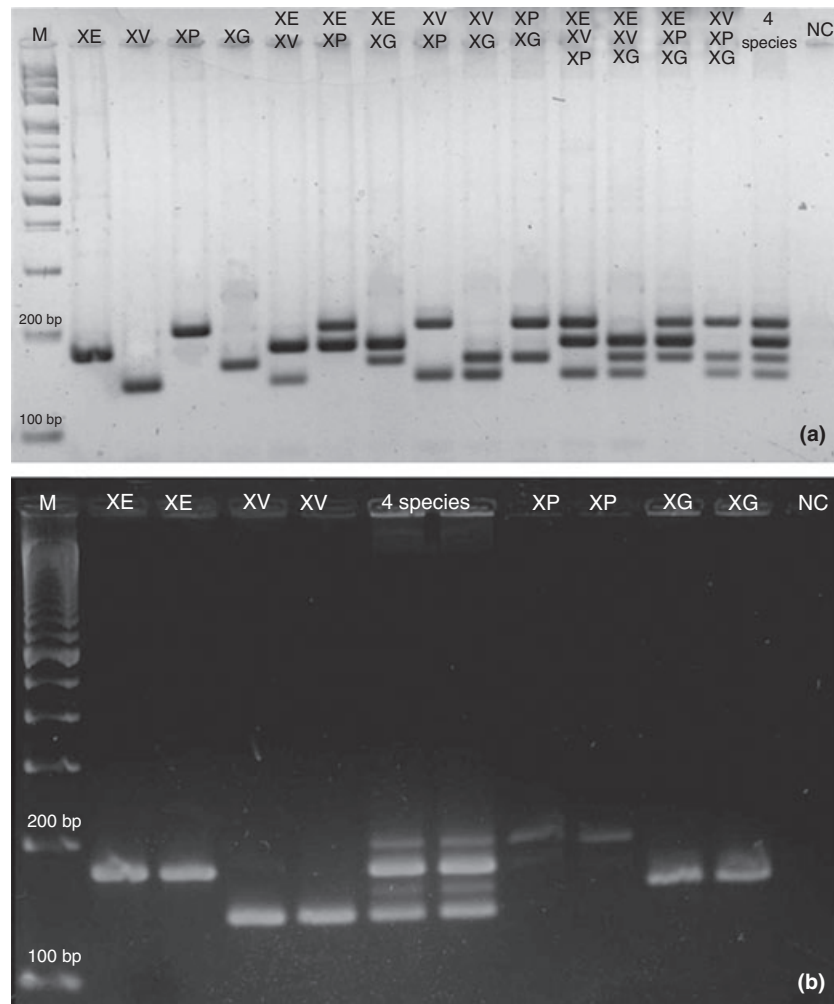


Figure 2 Sensitivity of species-specific primers of *Xanthomonas* spp. causing tomato bacterial spot in conventional and multiplex PCR. (a) Sensitivity of primer pairs Bs-XeF/Bs-XeR to *X. euvesicatoria* using bacterial suspensions by multiplex PCR, (b) sensitivity of primer pairs Bs-XpF/Bs-XpR to *X. perforans* using purified DNA by conventional PCR, (c) sensitivity of primer pairs Bs-XeF/Bs-XeR to *X. euvesicatoria* using bacterial suspensions by conventional PCR, (d) sensitivity of primer pairs Bs-XvF/Bs-XvR to *X. vesicatoria* using bacterial suspensions by conventional PCR. NC, negative control.

species complex of tomato bacterial spot using multiplex PCR, employing the same PCR conditions used for the conventional PCR (Fig. 3). Depending on the combinations used, the expected amplicons were obtained for both purified DNA and bacterial suspension using the multiplex PCR protocol.

Regarding the sensitivity of multiplex PCR, the detection threshold for purified DNA was $50 \text{ pg } \mu\text{l}^{-1}$ of each individual species. However, when using bacterial cell suspensions directly into the reactions, we observed lower levels of sensitivity. To overcome this reduced sensitivity, an enrichment step (Bio-PCR) was used, and the detection thresholds of the four species separately were similar to those found in conventional PCR (Table 3). It was possible to detect the pathogens in bacterial suspensions with initial concentrations from 5×10^2 to $5 \times 10^4 \text{ CFU ml}^{-1}$, 48 h after the enrichment step, depending on the *Xanthomonas* species. When we used all species in a single PCR, we observed reduced sensitivity in

simultaneous pathogen detection using both purified DNA and bacterial suspensions as sources of templates.

Detection of xanthomonads in symptomatic leaves of tomato

It was possible to detect and identify *Xanthomonas* species inoculated in tomato plants using both conventional PCR and multiplex PCR (Fig. 4). Overall, the crude extracts from symptomatic tomato leaves inhibited PCR, but successful amplification was achieved after diluting the extract at least tenfold (1 : 10) using both the conventional and multiplex PCR.

Discussion

Since the recognition of genetic diversity among xanthomonads associated with tomato bacterial spot (Lazo and

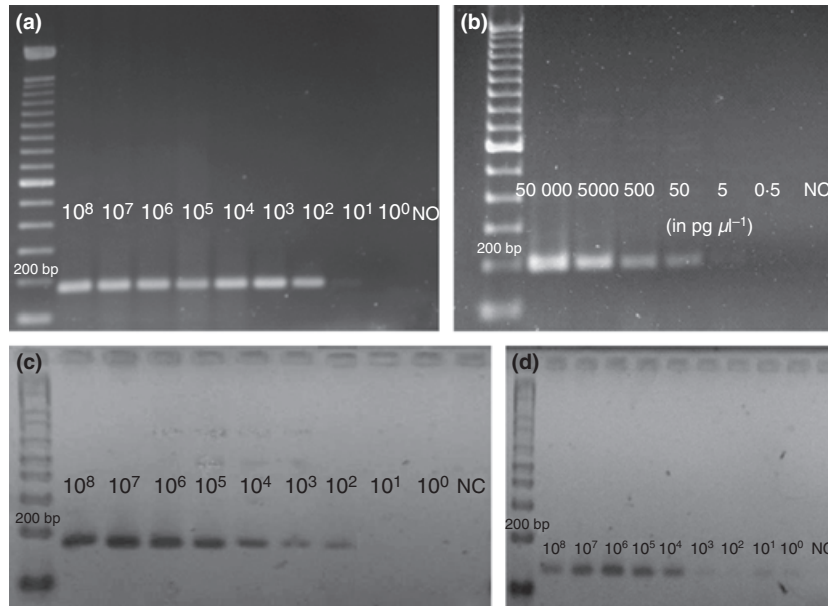


Figure 3 Amplicons of different *Xanthomonas* species by multiplex PCR. (a) Different combinations of purified DNA (50 ng ml^{-1}) of the four species, (b) amplicons obtained using bacterial suspensions ($5 \times 10^8 \text{ CFU ml}^{-1}$). XE, *Xanthomonas euvesicatoria*; XV, *X. vesicatoria*; XP, *X. perforans*; XG, *X. gardneri*. NC, negative control; M, molecular marker (100 bp DNA Ladder, Invitrogen).

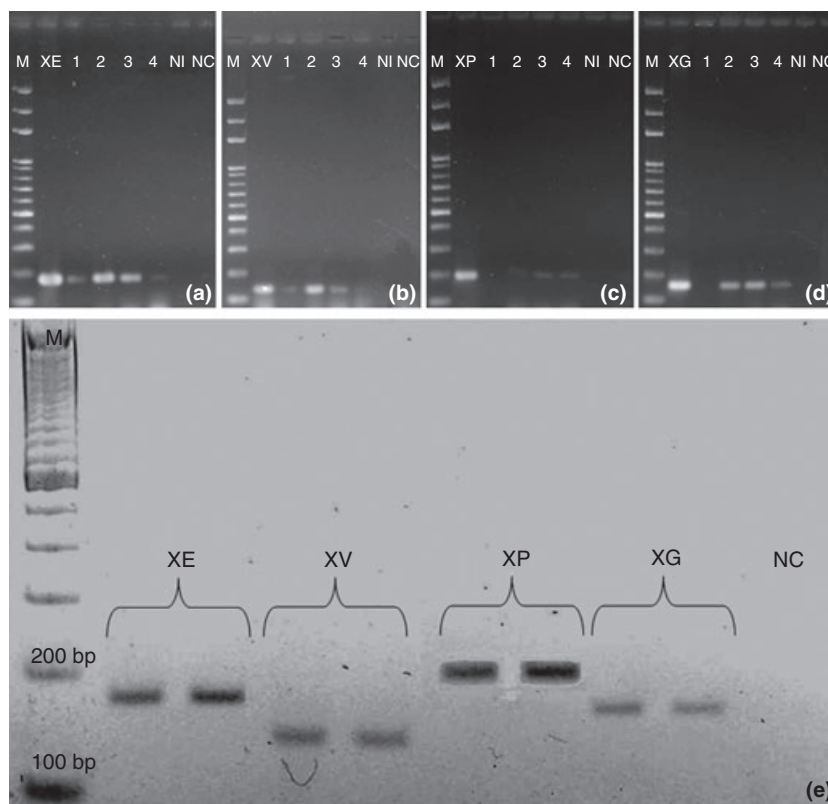


Figure 4 *Xanthomonas* species detection in symptomatic tomato leaves 8 days after inoculation. (a), (b), (c), (d) conventional PCR using species-specific primers for each species. 1. crude extract of symptomatic tomato leaves; 2. dilution (1 : 10) of the crude extract of symptomatic leaves, 3. dilution (1 : 100) of the crude extract of symptomatic leaves; 4. dilution (1 : 1000) of the crude extract of symptomatic leaves, (e) multiplex PCR from crude extract dilution (1 : 10) of symptomatic tomato leaves; NI. Uninoculated leaves; NC, Negative control; XE, *Xanthomonas euvesicatoria*; XV, *X. vesicatoria*; XP, *X. perforans*; XG, *X. gardneri*. M, Molecular marker (100 bp DNA Ladder, Invitrogen).

Gabriel 1987; Vauterin *et al.* 2000), biochemical and molecular tools have been established to properly differentiate the species for a multitude of purposes. Identification and differentiation of the complex of *Xanthomonas* species

which cause tomato bacterial spot have been accomplished by both molecular and biochemical methods.

The differentiation between species based on biochemical tests has been used (Jones *et al.* 1998), but the

appearance of new distinct strains may prevent proper species identification. Recently, as an example, Hamza *et al.* (2010) reported a new type of *X. euvesicatoria* strongly amyolytic, a unique characteristic of *X. vesicatoria* and *X. perforans*.

Restriction enzyme analyses (Leite Júnior *et al.* 1994, 1995) and rep-PCR (Bouzar *et al.* 1994, 1999; Pereira *et al.* 2011) have been used to characterize *Xanthomonas* strains causing bacterial spot, but these methods often require purified DNA of good quality and gel electrophoresis to generate fingerprints with high resolution and reproducibility (Ishii and Sadowsky 2009).

Young *et al.* (2008), using multilocus sequence analysis (MLSA), proposed that the species *X. perforans* and *X. euvesicatoria* were treated as synonyms. MLSA along with rep-PCR seems to be interesting for epidemiological studies (López *et al.* 2006; Feng *et al.* 2009). More recently, Potnis *et al.* (2011) presented and compared the complete genome sequences of all four species causing tomato bacterial spot and confirmed their separation in four entities. DNA sequencing is a powerful technique for new species descriptions and proposals of reclassifications but requires high-cost, more specialized equipment and is not always suitable for routine diagnosis.

Species-specific primers have been used successfully for diagnosis of plant bacteriosis caused by *Xanthomonas* spp. (Pan *et al.* 1999; Park *et al.* 2006). The RST 65/69 primers developed by Leite Júnior *et al.* (1995) amplified DNA from the four species (which corresponds to the former genetic groups A, B, C and D) associated with bacterial spot. However, an amplicon of the same size (420 bp) was produced not only for the tomato bacterial spot xanthomonads, but also for other *Xanthomonas* spp. Moreover, the PCR step has to be followed by restriction analysis with a set of enzymes (*CfoI*, *HaeIII* or *TaqI*), which is more laborious than a specific PCR. Furthermore, single-species isolates have to be tested in fingerprinting methods.

The first specific primer pair developed by Cuppels *et al.* (2006), namely BSX 1/2, is able to detect three of the four species involved in the complex, producing amplicons of 579 bp, but was unable to detect group C, presently *X. perforans*. It is worth mentioning the primer pairs designed by Moretti *et al.* (2009), specific for *X. euvesicatoria* from tomato and pepper and those by Astua-Monge *et al.* (2000) for *X. perforans*. The primers designed by Koenraadt *et al.* (2009) and used in the present study had been validated with *X. euvesicatoria* strains, as this species used to be prevalent in the United States (Jones *et al.* 1998). In this case, the four sets of primers (BS-XeR/F, BS-XvR/F, BS-XpR/F and BS-XgR/F) were successfully validated with a wide range of representative

Brazilian strains of bacterial spot *Xanthomonas* species, originating from different states in the country. *Xanthomonas gardneri* and *X. perforans* were represented by a higher number of strains in our study because of their prevalence in tomato fields in Brazil (Quezado-Duval *et al.* 2004; Araújo *et al.* 2011; Pereira *et al.* 2011).

The use of these primer pairs has enabled the establishment of an additional protocol, such as multiplex PCR that could be employed in bacterial spot diagnosis procedures. However, it is important to note that optimization processes of multiplex PCR must be elaborated for better efficiency of the method for this pathosystem. The correct identification of the species causing bacterial spot from symptomatic field samples directly speeds up diagnostic procedures leading to allowing more effective control measures.

The detection threshold of each individual primer pair in conventional PCR was equivalent to some reported thresholds for detection of bacterial plant pathogens. Some examples are the primers designed for other *Xanthomonas* (Hartung *et al.* 1993; Manulis *et al.* 1994; Pothier *et al.* 2011) with sensitivities for bacterial suspensions similar or better than those found here. On the other hand, when using purified DNA, the sensitivity of other published primers (TXT/TXT4R; RST2/Xcv3R) for *Xanthomonas* spp. (Sakthivel *et al.* 2001; Trindade *et al.* 2007) was higher, with threshold up to 55 fg.

Using multiplex PCR followed by separation of PCR products in 3.0% agarose gels, with reference strains as positive controls, made it possible to identify with greater ease up to the four bacterial spot *Xanthomonas* species present in a sample. The main purpose of the multiplex method is the detection and identification in a single step of one or more of the causal agents of bacterial spot. A negative result with PCR multiplex could thus rule out the presence of any of the species associated with bacterial spot in that sample, giving agility to the diagnostic process. Furthermore, depending on the presence of one or more species in one particular region or field, the protocol could be adjusted to different formats, such as a duplex or triplex PCR.

The sensitivity of the multiplex PCR was reduced when using a combination of species compared with one single species. It is known that factors such as concentrations of PCR components may favour the amplification of a species over another (Henegariu *et al.* 1997). In the present work, PCR component concentrations varied slightly, depending on the species combinations. Thus, further tests would be desirable to find out PCR component concentrations that lead to an equal sensitivity limit for the four xanthomonads. However, when there was a step of enrichment in NA medium, the detection threshold could be increased. It is common knowledge that Bio-PCR

improves the detection threshold of specific primers, as exemplarily reported by Wang *et al.* (1999) for *Xanthomonas albilineans*. Thus, the Bio-multiplex PCR could be used as a method for detection of viable bacterial cells and simultaneously identify all bacterial spot species known so far associated with asymptomatic greenhouse-grown transplants and seed extracts. It is still worth to mention that low concentration of bacterial cells is expected on those kinds of samples. Also, depending on their origin, for example, previously unknown bacterial spot xanthomonads could be involved in the sample infection (Pernezny and Collins 1997).

The sensitivity of multiplex PCR in the presence of the four species altogether was considered low. The use of PCR with a hot start (Chou *et al.* 1992) or using nested PCR (Robène-Soustrade *et al.* 2010) has been shown to improve the efficiency and sensitivity of multiplex PCR. These processes have been effective in reducing PCR inhibition factors as the formation of primer dimers that may occur before the start of thermocycling (4–25°C). Adjustments related to concentrations of reagents such as primers, magnesium and *Taq* DNA polymerase must be optimized, because it has a strong influence on the quality and reproducibility of the multiplex PCR (Henegariu *et al.* 1997). However, it does not seem to be a common situation the co-occurrence of all species in the complex in the same field (Quezado-Duval *et al.* 2004, 2005). Therefore, the multiplex protocol still seems to be suitable for the detection of a single species causing bacterial spot in a sample. Also, the direct detection from symptomatic leaves can be easily accomplished with a 1 : 10 dilution of the plant extract for both conventional and multiplex PCR, as the crude extract of leaves may contain PCR inhibitors (Xin *et al.* 2003). However, the here presented multiplex PCR still has to be further optimized for better sensitivity results.

Here we described a method to be further evaluated for routine and large-scale applications, by comparisons with standard methods such as isolation on semi-selective/selective media as Tween B and CKTM (McGuire *et al.* 1986; Sijam *et al.* 1991) followed by biochemical and/or DNA fingerprinting methods. Currently, the use of multiplex PCR is being used to investigate the occurrence of epiphytic bacterial spot xanthomonads on greenhouse-grown tomato transplants, which may serve as primary inoculum sources for field infections.

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