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

E.R. Araújo\textsuperscript{1,2}, J.R. Costa\textsuperscript{2}, M.A.S.V. Ferreira\textsuperscript{1} and A.M. Quezado-Duval\textsuperscript{2}

\textsuperscript{1} Departamento de Fitopatologia, Instituto de Biologia, Universidade de Brasília, Brasília, DF Brazil
\textsuperscript{2} Laboratório de Fitopatologia, Embrapa Hortaliças, Brasília, DF Brazil

\textbf{Abstract}

\textbf{Aims:} To establish protocols for the simultaneous detection and identification of \textit{Xanthomonas} species causing tomato bacterial spot.

\textbf{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 \textit{Xanthomonas euvesicatoria}, 30 of \textit{X. vesicatoria}, 50 of \textit{X. perforans} and 50 of \textit{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 \(\mu\text{l}^{-1}\) for purified DNA and ranged from \(5 \times 10^2\) to \(5 \times 10^4\) 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.

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

\textbf{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.

\textbf{Introduction}

A complex of species of the genus \textit{Xanthomonas} are causal agents of tomato bacterial spot, namely \textit{X. euvesicatoria}, \textit{X. vesicatoria}, \textit{X. perforans} and \textit{X. gardneri} (Jones \textit{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 \textit{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 \textit{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 \textit{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 agent 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). Koenraad 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. 2002; Pulauskas 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 phytopathobacteria [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 avrRpt2 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 KH₂PO₄; 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
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 ml⁻¹ and kept at −20°C until used.

Conventional PCR

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

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

<table>
<thead>
<tr>
<th>Species/ Strains</th>
<th>Host</th>
<th>Origin†</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthomonas euvesicatoria</td>
<td>Tomato/Pepper</td>
<td>PE; MG; AM; SP; RR; DF; GO; ES; CE; BA</td>
<td>1996; 1998; 1999; 2002; 2009; 2010; 2011</td>
</tr>
<tr>
<td>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 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*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthomonas vesicatoria</td>
<td>Tomato</td>
<td>GO; SC; RJ; MG</td>
<td>1995; 1996; 2007; 2008; 2010</td>
</tr>
<tr>
<td>Xanthomonas perforans</td>
<td>Tomato</td>
<td>GO; BA; MG; SC; SP</td>
<td>2005; 2006; 2007; 2008; 2009; 2010; 2011</td>
</tr>
<tr>
<td>Xanthomonas gardneri</td>
<td>Tomato</td>
<td>GO; PR; RS; ES;SC; MG</td>
<td>2006; 2007;2008; 2009,2010</td>
</tr>
<tr>
<td>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*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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.
GTATCG-3’) and BS-XeR (5’-GTGGACATGTTGAC-ACATAC-3’), 173 bp for *X. euvesicatoria*; BS-XvF (5’-CCATGTTGCGGTTGAATCTCC-3’) and BS-XvR (5’-AGAGATGTGGTTATGATTTC-3’), 138 bp for *X. vesicatoria*; BS-XpF (5’-GTGCGTTGAGCAGGTGTCG-3’) and BS-XpR (5’-GTGCGGAGTGCAATTACGAATGTTG-3’), 197 bp for *X. perforans*; and BS-XgF (5’-TGCGGACATGTTGAC-ACATAC-3’) and BS-XgR (5’-TGACCGTAAAGACTGCGAAAG-3’), 154-bp amplicon for *X. gardneri*. PCR was performed in a thermocycler–My Cycler.

### Table 2

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

<table>
<thead>
<tr>
<th>DNA Samples</th>
<th>Host</th>
<th>Species-specific primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthomonas euvesicatoria</td>
<td>Tomato/pepper</td>
<td>BS-XeF/XeR BS-XeV/XeR BS-XpF/XpR BS-XgF/XgR</td>
</tr>
<tr>
<td>EH 36P; EH 37P; EH 38P;</td>
<td></td>
<td>+ – – –</td>
</tr>
<tr>
<td>Xanthomonas vesicatoria</td>
<td>Tomato</td>
<td>BS-XvF/XvR BS-XpF/XpR</td>
</tr>
<tr>
<td>Xanthomonas perforans</td>
<td>Tomato</td>
<td>BS-XpF/XpR</td>
</tr>
<tr>
<td>EH 2005-54; EH 2006-44; EH 2009-106; EH 2009-107</td>
<td></td>
<td>– –</td>
</tr>
<tr>
<td>EH 2009-143; EH 2009-148; EH 2009-149; EH 2010-09; EH 2010-60; EH 2010-61; EH 2010-93; IBSBF 2370</td>
<td></td>
<td>+ –</td>
</tr>
<tr>
<td>Xanthomonas gardneri</td>
<td>Tomato</td>
<td>BS-XgF/XgR</td>
</tr>
<tr>
<td>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</td>
<td></td>
<td>– –</td>
</tr>
<tr>
<td>Xanthomonas raphani, IAPAR 11300; IBSBF 1590</td>
<td>Brassica sp.</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas campestris pv. campestris, UnB 828</td>
<td>Brassica sp.</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas axonopodis pv. manihotis, UnB 1159</td>
<td>Cassava</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas campestris pv. vitians, UnB 1079; UnB 110; UnB 830</td>
<td>Lettuce</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas citri pv. anacardi, IBSBF 2579</td>
<td>Cashew</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas campestris pv. viticola, UnB 1318</td>
<td>Grape</td>
<td></td>
</tr>
<tr>
<td>Xanthomonas citri pv. mangiferaeindicae, IBSBF 2586</td>
<td>Mango</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas marginalis, IBSBF 1240</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas cichorii, IBSBF 402; IBSBF 1748; UnB 1142</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas syringae pv. syringae, IBSBF 451; IBSBF 375; IBSBF 281</td>
<td>Tomato/Lilac</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas syringae pv. tomato, IBSBF 836; IBSBF 432; EH 75</td>
<td>Tomato</td>
<td>No amplification</td>
</tr>
<tr>
<td>Pseudomonas viridiflava, IBSBF 1464</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Agrobacterium tumefaciens, UnB 1138</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Clavibacter michiganensis subsp. michiganensis, UnB 1151</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Raistonia solancearum, UnB 1273</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Acidovorax avenae subsp. citrulli, 646-2</td>
<td>Melon</td>
<td></td>
</tr>
<tr>
<td>Erwinia chrysanthemi, UnB 336</td>
<td>Chard</td>
<td></td>
</tr>
<tr>
<td>Erwinia psidii, IBSBF 1347; IBSBF 453</td>
<td>Guava</td>
<td></td>
</tr>
<tr>
<td>Pectobacterium carotovorum, UnB 1036</td>
<td>Turnip</td>
<td></td>
</tr>
<tr>
<td>Alternaria solani, IBSBF 1940</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Corynespora cassiicola, IBSBF 1828</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Stemphylium sp., EH 502</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>Unknown epiphytic and/or endophytic bacteria (13)</td>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>DNA tomato</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

+, amplification; – no amplification.
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 (1.26–11 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 pg μl⁻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 × 10² 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 × 10⁴ 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

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection limit</th>
<th>X. euvesicatoria</th>
<th>X. vesicatoria</th>
<th>X. perforans</th>
<th>X. gardneri</th>
<th>All species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional PCR</td>
<td>Bacterial suspension</td>
<td>5 × 10²</td>
<td>5 × 10⁴</td>
<td>5 × 10²</td>
<td>5 × 10⁴</td>
<td>–</td>
</tr>
<tr>
<td>Purified DNA</td>
<td>(μg μl⁻¹)</td>
<td>5 × 10³</td>
<td>5 × 10⁴</td>
<td>5 × 10³</td>
<td>5 × 10⁴</td>
<td>5 × 10⁷</td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Initial concentration of bacterial suspensions that were detected after an enrichment step on NA medium for 48 h (Bio-PCR).
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 pg μl⁻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 × 10² to 5 × 10⁴ 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
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 \textit{X. euvesicatoria} strongly amylolytic, a unique characteristic of \textit{X. vesicatoria} and \textit{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 \textit{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 \textit{X. perforans} and \textit{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 \textit{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, but also for other \textit{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 \textit{X. perforans}. It is worth mentioning the primer pairs designed by Moretti et al. (2009), specific for \textit{X. euvesicatoria} from tomato and pepper and those by Astua-Monge et al. (2000) for \textit{X. perforans}. The primers designed by Koenraadt et al. (2009) and used in the present study had been validated with \textit{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 \textit{Xanthomonas} species, originating from different states in the country. \textit{Xanthomonas} gardneri and \textit{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 \textit{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 \textit{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 \textit{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 (Robêne-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.

Acknowledgements

We acknowledges National Research Council (CNPq)/Ministry of Agriculture for financial support (grant no. 578-775/2008-5).

References


