A Multiplex Real-Time PCR Assay Differentiates Four *Xanthomonas* Species Associated with Bacterial Spot of Tomato

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Abstract

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Bacterial spot of tomato, a major problem in many tomato production areas, is caused by *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*. In order to detect and identify the bacterial spot pathogens, we evaluated a region of *hrpB* operon as a source for primers and probes for real-time polymerase chain reaction (PCR). A 420-bp fragment of the *hrpB7* gene was amplified by PCR from 75 strains representing the four species. The PCR products were sequenced and phylogenetic analysis revealed that *hrpB7* is highly conserved within each species, with a single-nucleotide polymorphism (SNP) among the *X. vesicatoria* strains. *X. euvesicatoria*

and *X. perforans* varied by two SNP. Four probes and two primer sets were designed to target the four bacterial spot pathogens based on their *hrpB7* gene sequences. In order to simultaneously detect the four bacterial spot pathogens, the four probes and two primer sets were optimized for a multiplex real-time TaqMan PCR assay. The optimized multiplex assay was determined to be highly specific to the four bacterial spot pathogens. Because the optimized multiplex assay facilitated the identification of each bacterial spot pathogen from pure cultures and infected plant tissue, it holds great potential as a diagnostic tool.

Bacterial spot, one of the most detrimental diseases of tomato, has been reported to cause yield losses of up to 50% (Pohronezny and Volin 1983; Scott et al. 1989). The disease occurs worldwide but is most prevalent in warm and humid tomato-growing regions. Bacterial spot was first observed in South Africa in 1914 (Doidge 1921). Currently, it is caused by four distinct species of *Xanthomonas* (*Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. gardneri*, and *X. perforans*) (Jones et al. 2004). Historically, *X. euvesicatoria* and *X. vesicatoria* have been worldwide in distribution, with the other two species being less prevalent; however, *X. perforans* and *X. gardneri* have been isolated more frequently in recent years from diverse geographic locations (Potnis et al. 2015). Infection can result in leaf and fruit lesions, defoliation, and yield loss of marketable fruit (Louws et al. 2001). Leaf symptoms are the most prominent but can vary depending on environmental conditions (Ritchie 2000). Generally, bacterial spot lesions are initially circular and water-soaked in appearance but turn dark brown to black, with a wet or greasy appearance (Vallad et al. 2004). Although *X. perforans* leaf spot lesions can develop shot holes, it can be difficult to differentiate the four bacterial spot pathogens by symptoms alone (Stall et al. 2009).

Various techniques such as carbon utilization, fatty acid profiles, 16S rRNA sequences, and DNA-DNA hybridization have been used to identify plant-pathogenic xanthomonads (Bouzar et al. 1994; Cho and Tiedje 2001; Jones et al. 2004; Vauterin et al. 1995; Yang et al. 1993). However, our ability to identify xanthomonads to species has greatly improved due to advances in more recent DNA-based techniques. Koenraadt et al. (2007) designed oligonucleotide primers based on amplified fragment length polymorphism (AFLP) analysis that distinguished between the four pathogens. However, the

specificity of this assay needed further validation because the number of *X. perforans* and *X. gardneri* strains tested was limited. Araújo et al. (2012) validated Koenraadt's assay by testing more strains and creating a multiplex polymerase chain reaction (PCR) to simultaneously detect all four species. This method was used to effectively detect and differentiate all of the strains tested representing the four bacterial spot pathogens but is gel based and labor intensive, especially when processing large samples. Under our laboratory conditions, this identification method has yielded false negatives and variable results with DNA extracted from multiple *X. perforans* strains. It is unclear how conserved the regions of DNA are that are targeted by these primers. Based on whole-genome sequencing, there are two bacterial spot strains (*X. euvesicatoria* strain NI1 and *X. perforans* strain 1712) that do not contain the genome sequences that these primers are intended to amplify and, thus, they would be misidentified (N. Potnis, unpublished). NI1, which was isolated from tomato in Nigeria, behaves like *X. perforans* in terms of host differential reactions on tomato. However, it is more closely related to *X. euvesicatoria* based on multilocus sequence analysis (MLSA) (Timilsina et al. 2015) and was determined to be a hybrid between *X. perforans* and *X. euvesicatoria* based on whole-genome sequencing (N. Potnis, unpublished). Thus, the focus of this study was to develop a new diagnostic method for bacterial spot that does not require gel electrophoresis and is based on conserved gene sequences.

Real-time PCR assays are gaining interest in diagnostic laboratories because they are rapid, sensitive, and easily reproducible and do not require a timely electrophoresis step (Li et al. 2006; Mackay et al. 2002). Several real-time PCR assays have been developed to detect bacterial plant pathogens, including *Clavibacter michiganensis* subsp. *sepedonicus*, *Ralstonia solanacearum*, *Agrobacterium* sp., *Xylella fastidiosa*, 'Candidatus Liberibacter asiaticus', and *Xanthomonas citri* pv. *citri* (Golmohammadi et al. 2007; Li et al. 2006; Schaad et al. 1999, 2002; Weller and Stead 2002; Weller et al. 2000). Real-time PCR is especially beneficial in diagnostics for detecting prokaryotes in plant tissue. The speed and sensitivity of real-time PCR has aided regulatory agencies in detecting and managing the potential spread of quarantined pathogenic phytopathogens (Li et al. 2006). Additionally, a multiplex real-time PCR assay would allow researchers to track shifts in the prevalence of one bacterial spot pathogen over

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another in time and space. One can easily be developed to detect up to four plant-pathogenic species simultaneously (Schena et al. 2006).

The *hrpB7* gene sequence was evaluated as a source of primers and probes for multiplex real-time TaqMan PCR assays for the four bacterial spot species. The *hrpB* gene cluster belongs to the hypersensitive response and pathogenicity (*hrp*) gene cluster and is essential for type III protein secretion and pathogenicity (Rossier et al. 2000). Most plant-pathogenic xanthomonads have a type III secretion system that delivers effector proteins into the host cell to downregulate defense responses (Alfano and Collmer 2004; Bonas et al. 1991; Cornelis 2006; Noël et al. 2002). The *hrpB7* gene has been explored in the past for identifying the causal agents of bacterial spot and appears to be conserved in species of *Xanthomonas* (Leite et al. 1994; Obradovic et al. 2004). Leite et al. (1994) evaluated the use of *hrpB7*-based PCR primers and restriction enzyme analysis for differentiating between 28 different *X. campestris* pathovars. This assay resulted in unique and distinctive banding patterns for different pathovars of *Xanthomonas*. Obradovic et al. (2004) used the *hrpB7*-based PCR primers to amplify DNA from 39 strains representing the four bacterial spot pathogens. The PCR products were digested with three restriction enzymes, which resulted in distinct banding patterns for the four pathogens.

The goal of this study was to develop and evaluate a multiplex real-time TaqMan PCR assay using *hrpB7* gene primers and probes to simultaneously detect and differentiate the four bacterial spot of tomato pathogens. It was hypothesized that the *hrpB7* would be conserved among strains within the same species of *Xanthomonas*. Therefore, it could be used to accurately detect *Xanthomonas* spp. associated with bacterial spot. The specific objectives of this study were to (i) determine the genetic variation in the *hrpB7* gene among strains of the four bacterial spot pathogens from diverse locations, (ii) construct TaqMan PCR probes that target *hrpB7* gene variation identified in objective 1 among the four pathogens and develop a multiplex real-time PCR assay, and (iii) test the sensitivity and accuracy of the multiplex real-time PCR assay using pure cultures and naturally and artificially infected samples.

Materials and Methods

Isolation and storage of bacterial strains. Bacterial strains used in this study are listed in Table 1. Bacteria were grown on nutrient agar (NA) (BBL, Becton Dickinson and Co., Cockeysville, MD) at 28°C and subcultured every 24 to 48 h. For long-term storage, purified cultures were stored in sterile 30% glycerol solution at -80°C. Bacteria were isolated from artificially and naturally infected plant tissue for real-time PCR testing. Individual lesions (approximately 1 to 2 mm in size) were aseptically cut from tomato. The diseased tissue was transferred to 25 µl of sterile tap water in a petri dish and macerated using a sterile scalpel. In order to obtain pure cultures, a small amount of this suspension was streaked with a sterile inoculating loop for individual colonies on NA. To confirm pathogenicity of the isolated strains, inoculum was prepared by suspending 24-h cultures grown on NA in sterile tap water and adjusting the suspension to absorbance at 600 nm (A_{600}) = 0.3 (approximately 5×10^8 CFU/ml). The bacterial suspensions were then infiltrated into tomato leaves and incubated until disease symptoms developed. The strains in Table 1 were also characterized using MLSA, race differentiation, or sequence analysis of part of the *hrpB7* (420 nucleotides) amplified with *hrpB7* primers (Kebede et al. 2014; Obradovic et al. 2004; Timilsina et al. 2015).

Bacterial DNA extraction from pure cultures and infected leaf tissue. Bacterial DNA was extracted from pure cultures using either the cetyltrimethylammonium bromide (CTAB) method (Wilson 1987) or by boiling fresh bacterial cells. Using the end of a sterile toothpick, a small amount of bacterial cells was transferred from a pure culture to a sterile, 1.5-ml capped microcentrifuge tube containing 400 µl of sterile tap water. These tubes were incubated at 100°C for 20 min, transferred to ice for 5 min, and centrifuged ($16,872 \times g$) for 5 min. The supernatant was removed without disturbing the pellet and transferred to a new 1.5-ml capped microcentrifuge tube. DNA was extracted from infected plant tissue as follows. Three to four individual bacterial spot lesions per sample

(approximately 1 to 2 mm in size) were aseptically cut from artificially or naturally infected tomato leaves and fruit. Individual lesions were then rinsed with sterile tap water. The rinsed lesions were transferred to sterile 1.5-ml capped microcentrifuge tubes containing 500 µl of sterile tap water and macerated using a sterile pestle. Plant debris was allowed to settle to the bottom of the tube for 1 h. Approximately 300 µl of the supernatant containing the bacterial cells was transferred to a new sterile 1.5-ml capped microcentrifuge tube, which was then incubated at 100°C for 20 min, transferred to ice for 5 min, and centrifuged ($16,872 \times g$) for 5 min. The supernatant was collected without disturbing the pellet and transferred to a new 1.5-ml capped microcentrifuge tube for real-time PCR analysis.

PCR assay, sequencing and phylogenetic analysis. As described by Obradovic et al. (2004), *hrpB7*-specific primers (RST65 [5'-GTC GTCGTTACGCAAGGTGGTCG-3'] and RST69 [5'-TCGCCCA GCGTCATCAGGCCATC-3']) were used to amplify a 420-bp fragment from strains of the four bacterial spot pathogens (75 strains in total; Table 1). The following protocol was used to amplify the DNA: (i) 95.0°C for 5 min (1 cycle); (ii) 95.0°C for 30 s, 63.0°C for 30 s, and 72.0°C for 45 s (29 cycles); and (iii) 72.0°C for 5 min and 4.0°C ∞ (1 cycle). Amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA), then submitted to the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville) for Sanger sequencing. Additional *hrpB7* gene sequences representing a range of plant-pathogenic *Xanthomonas* spp. were obtained from the National Center of Biotechnology Information (NCBI) database. All sequences were aligned and trimmed using the ClustalW feature in MEGA6 (Tamura et al. 2013). The maximum-likelihood tree, with 1,000 bootstrap samples, based on the aligned sequences was created in MEGA6.

Real-time TaqMan PCR probe and primer design. The *hrpB7* sequences obtained in this study and from the NCBI database were used to design TaqMan probes and primers to target each of the four bacterial spot pathogens (Table 2). Four probes were designed manually to have the following characteristics: 60 to 80% GC content, runs of identical nucleotides were avoided, a guanine was not placed at the 3' or 5' end, and the melting temperature (T_m) was kept at 69°C for potential multiplexing. The 5' end of each probe was labeled with 5' 6-FAM (*X. perforans* probe), 5' TET (*X. euvesicatoria* probe), 5' Texas Red-X (NHS Ester) (*X. gardneri* probe), or 5' Cy5 (*X. vesicatoria* probe) to allow for the simultaneous detection of the four pathogens (Integrated DNA Technologies Inc. [IDT], Coralville, IA). A black hole quencher—3' Iowa Black FQ (*X. perforans* probe), 3' Black Hole Quencher 2 (*X. euvesicatoria* probe), 3' Iowa Red RQ-Sp (*X. gardneri* probe), or 3' Iowa Black RQ-Sp (*X. vesicatoria* probe)—was also attached to the 3' end of each probe (IDT). Black hole quenchers convert energy received from a fluorophore to heat, which prevents any interference among fluorescence emission spectra of fluorophores (Schena et al. 2006). The two forward and two reverse primers were developed manually to have the following characteristics: primers were designed as close as possible to the binding site of the probe without overlapping those sequences; the primer was 60 to 80% GC content, in which long runs of identical nucleotides were avoided; and the T_m for each primer was kept at 59°C. Primer sets were designed to amplify more than one bacterial spot pathogen and to minimize the quantity of reagents in the reaction mixture.

Simplex real-time TaqMan PCR assay optimization. DNA from *X. vesicatoria* strain Xv144, *X. euvesicatoria* strain Xe157, *X. perforans* strain Xp1484, and *X. gardneri* strain XgGA2 was extracted using the CTAB method (Wilson 1987). DNA concentration and quality were measured via Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). Each DNA sample was adjusted to 80 ng/µl and used to optimize the multiplex real-time TaqMan PCR assay. Cycling protocols varying in T_m , which ranged from 57 to 69°C, were programmed in a Cepheid SmartCycler (Cepheid, Sunnyvale, CA). The real-time PCR cycling protocol was as follows: stage 1 = 95°C for 30 s, optics off; and stage 2 = (i) 95°C for 3 s, optics off; (ii) T_m for 30 s, optics on; and (iii) 72°C for 30 s, optics off. Each probe and primer set was optimized individually

prior to multiplexing. The simplex master mix contained 8.2 µl of nuclease-free water; 2.5 µl of 10× PCR buffer; 3.0 µl of 50 mM MgCl₂; 0.6 µl of dNTP; 3.0 µl each of two primers at 100, 200, 400, 800, or 1,200 nM; 2.5 µl of a single probe; and 1 U of Platinum *Taq* (Invitrogen, Carlsbad, CA) per sample. The simplex master mix (23 µl) was

used in each assay, with 2 µl of the 80-ng DNA samples as template. The final DNA concentration in the reaction mixture was 12.8 ng/µl.

Multiplex real-time TaqMan PCR assay optimization and validation. The results from the simplex optimization studies were used to determine the optimal cycling protocol and the most efficient

Table 1. Bacterial strains used in this study for the phylogenetic analysis, validation of the multiplex real-time TaqMan polymerase chain reaction (PCR) assay, or both

Species, strain	Origin	<i>hrpB7</i> sequenced ^s	TaqMan ^t
<i>Xanthomonas perforans</i>			
621 ^u	Florida	NT	+
938 ^v	Florida	+	+
1220 ^v	Thailand	+	+
Xp1484 ^{v,w}	Mexico	+	+
1712 ^v	Florida	NT	+
2010 ^v	Florida	+	+
GEV839 ^{v,w} , 872 ^v , 893 ^v , 904 ^v , 909 ^v , 915 ^v , 917 ^v , 936 ^v , 940 ^v , 968 ^v , 993 ^v , 1001 ^v , 1026 ^v , 1044 ^v , 1054 ^v , and 1063 ^v	Florida	+	NT
ETH5 ^{v,x} , 11 ^v , 13 ^v , 14 ^v , 19 ^v , 21 ^v , 22 ^v , 25 ^v , 26 ^v , 28 ^v , 29 ^v , 31 ^v , 32 ^v , and 33 ^v	Ethiopia	+	+
<i>X. euvesicatoria</i>			
11 ^y	China	NT	+
153 ^v	Florida	+	+
155 ^v	Florida	+	+
Xe157 ^{v,w}	Australia	+	+
330 ^v	Barbados	+	+
338 ^v	Barbados	+	+
9060 ^y	Barbados	+	+
1085 ^v	Mexico	+	+
1520 ^y	Sudan	NT	+
1605 ^v	Ohio	+	+
IP ^y	Vietnam	NT	+
XV881 ^y	Spain	NT	+
<i>X. vesicatoria</i>			
56 ^v	Brazil	+	+
141 ^v and 1111 ^v	New Zealand	+	+
Xv144 ^{v,w}	Argentina	+	+
ETH1 ^{v,x} , 2, 3 ^x , 4, 17 ^v , 18, and 20	Ethiopia	+	+
MME ^y	Ohio	NT	+
UF1 ^z	California	+	+
UF2 ^z	California	NT	+
UF3 ^z	Italy	+	+
UF4 ^z	California	NT	+
UF6 ^z	California	+	+
UF7 ^z	Brazil	+	+
UF8 ^z	Brazil	NT	+
<i>X. gardneri</i>			
444 ^v	Costa Rica	+	+
451 ^v	Costa Rica	+	+
1782 ^v	Brazil	+	NT
1783 ^v	Brazil	+	NT
OOT12B ^v	Ohio	+	NT
ETH7 ^x , 8 ^v , 9 ^v , 10 ^v , 15 ^v , 16, 23, 24, and 30 ^v	Ethiopia	+	+
FURMAN-3 ^v	Pennsylvania	+	NT
XgGA2 ^{v,w}	Hungary	+	+
UF5 ^z	Canada	+	+
UF9 ^z	Brazil	+	+
XG51 ^z	Canada	NT	+

^s Symbols: += DNA extracted from the pure culture of the strain was amplified using the *hrpB7* primers (RST65 and RST69) and sequenced by the Interdisciplinary Center for Biotechnology Research and NT = DNA from this strain was not tested and *hrpB7* sequence was not obtained for that strain.

^t Symbols: + = DNA extracted from the pure culture of the strain was amplified only by its corresponding species-specific probe and primer set and NT = the strain was not tested using the multiplex real-time TaqMan PCR.

^u Strain was characterized using race differentiation (Obradovic et al. 2004).

^v Strain was characterized using multilocus sequence analysis involving the sequencing of six housekeeping genes (*gyrB*, *lepA*, *lacF*, *gltA*, *fusA*, and *gapA*) (Kebede et al. 2014).

^w Strain designation was changed in order to aid the reader.

^x “Type” strain sequence used in the condensed phylogenetic tree based on the *hrpB7*.

^y Strain was characterized by race differentiation, *hrpB*-PCR, and multiplex PCR using amplified length polymorphism primers (Araújo et al. 2012; Obradovic et al. 2004).

^z Strain was characterized by race differentiation and *hrpB*-PCR (Obradovic et al. 2004).

master mix formulation for the multiplex real-time TaqMan PCR assay. The DNA from representative strains (Xv144, Xe157, Xp1484, and XgGA2) of the four *Xanthomonas* spp. that cause bacterial spot were used to optimize the multiplex real-time PCR assay and as controls during the validation phase. The final optimized real-time PCR cycling protocol contained a T_m of 69°C. The optimized multiplex master mix is as follows: 5.7 µl of molecular-grade water, 2.5 µl of 10× PCR buffer, 3.0 µl of 50 nM MgCl₂, 0.6 µl of dNTP, 3.0 µl of each forward or reverse primer at 6 µM, 1.25 µl of each probe at 3 µM, and 1 U of platinum Taq per sample. Pure cultures of each species (66 strains in total) were tested (two replicates) to verify the accuracy of the multiplex assay. During validation, nuclease-free water (Ambion, Austin, TX) was used as a negative control and DNA from strains Xv141, Xv144, Xe157, Xp1484, and XgGA2 were used as positive controls for each real-time PCR assay. Furthermore, a sample that had a cycle threshold (Ct) value <35 was considered to be positive and a sample that had a Ct value ≥37 was considered to be negative. If the Ct value was between 35 and 37, the result was considered to be inconclusive and the sample was retested.

Multiplex real-time TaqMan PCR assay sensitivity and specificity. One strain of each of the four *Xanthomonas* spp. that causes bacterial spot (Xp1484, Xv144, Xe157, and XgGA2) was incubated at 28°C on NA for 24 h. Bacterial suspensions were prepared in sterilized tap water and adjusted to $A_{600} = 0.3$ (approximately 5×10^8 CFU/ml). The bacterial suspensions were serially diluted to 10^1 CFU/ml. DNA was extracted from the suspensions as follows: 400 µl of each diluted suspension (10^1 to 10^8 CFU/ml) was transferred to a sterile 1.5-ml capped microcentrifuge tube, which then was incubated at 100°C for 20 min, transferred to ice for 5 min, and then centrifuged at $16,872 \times g$ for 5 min. The supernatant was transferred without disturbing the pellet to a new 1.5-ml capped microcentrifuge tube. Each DNA extract (2 µl) was tested using the optimized multiplex real-time TaqMan PCR assay.

In order to evaluate the specificity of this multiplex real-time PCR assay, the *hrpB7* gene sequences of the four bacterial spot pathogens were compared with other *hrpB7* gene sequences and additional sequenced genes in the NCBI database using the Standard Nucleotide BLAST[®] (blastn suite) tool. Each probe and primer sequence was located in the aligned *hrpB7* gene sequence, and these regions in the xanthomonads identified in the NCBI BLAST were scanned for SNP.

Verification of real-time TaqMan PCR assay for detection of bacteria in infected plant tissue. In all, 120 tomato leaflets and two tomato fruit expressing bacterial spot symptoms were utilized in this study. Symptomatic leaflets ($n = 100$) were collected from tomato plants that were artificially inoculated with *X. euvesicatoria* (strain Xe157), *X. gardneri* (strain XgGA2), *X. perforans* (strain Xp1484), or *X. vesicatoria* (strain Xv144) as described below. The strains were grown on NA plates for 24 h at 28°C. Bacterial suspensions were prepared and adjusted to $A_{600} = 0.3$ (approximately 5×10^8 CFU/ml). Suspensions were sprayed onto both sides of the tomato leaves. The inoculated plants were placed in clear plastic bags to maintain high humidity and placed in a growth chamber at 28°C for 48 h. The bags were then removed and leaf samples with obvious

lesions were collected approximately 10 days postinoculation. In addition, 20 tomato leaflets and two tomato fruit were collected from naturally infected tomato plants in a production field in Immokalee, FL.

Results

***HrpB7* PCR, sequencing, and phylogenetic analysis.** Amplified PCR products and *hrpB7* gene sequences were obtained from all strains listed in Table 1. Additional *hrpB7* sequences from type strains of *Xanthomonas*—*X. euvesicatoria*_8510 (AM039952), *X. gardneri* (AEQX00000000), *X. perforans*_91-118 (AEQW00000000), *X. vesicatoria* (AEQV00000000), *X. axonopodis* pv. *citrumelo*_F1 (CP002914), *X. oryzae* pv. *oryzae* (AP008229), *X. oryzae* pv. *oryzicola* (CP003057), *X. axonopodis* pv. *syngonii* (AY576625), *X. axonopodis* pv. *glycines* (AF499777), *X. fuscans* subsp. *fuscans* (EU215387), and *X. campestris* pv. *campestris* (AE008922)—were obtained from the online NCBI database. No nucleotide variation was seen among strains belonging to either *X. euvesicatoria*, *X. gardneri*, or *X. perforans*. However, there was one single nucleotide difference that was identical in 7 of the 19 *X. vesicatoria* strains tested at position 331. Therefore, strains ETH1, -2, and -4; UF3 and -6; 141; and 1111 that contained the single nucleotide difference were designated as *X. vesicatoria* group 1 and strains ETH3, -17, -18, and -20; UF1 and -7; 56; and Xv144 without a nucleotide difference were designated as *X. vesicatoria* group 2. Additionally, *X. euvesicatoria* and *X. perforans* only varied by two nucleotides, at positions 257 and 260. The aligned *hrpB7* gene sequences were used to create a phylogenetic tree (Fig. 1). The phylogenetic tree confirmed that the *hrpB7* gene is highly conserved among strains within the same *Xanthomonas* sp.

Real-time TaqMan PCR probe and primer design. The aligned *hrpB7* sequences were used to create four species-specific probes and two corresponding primer sets for detection of all four *Xanthomonas* spp. causing bacterial spot (Table 2). The *X. gardneri*-specific probe was predicted to form a hairpin or secondary structure at the recommended T_m (64.7°C) by OligoAnalyzer 3.1 (IDT), which may decrease amplification efficiency.

Simplex real-time TaqMan PCR assay optimization. The master mix containing 800-nM *X. perforans* probe and primer set yielded the most consistent Ct values with the lowest standard deviation for Xp1484 (Table 3). However, there was also amplification of the DNA from Xe157 (Table 3). The DNA from Xp1484 consistently crossed the threshold approximately one cycle before the DNA from Xe157. The T_m was increased to 61 and 63°C in the qPCR protocol but this did not increase the specificity (data not shown). Additionally, increasing the concentration of the *X. perforans* probe and primer set to 1,200 nM did not improve the specificity or the Ct values (Table 3). DNA from Xe157 and Xp1484 were amplified using the *X. euvesicatoria* probe and primer set at all concentrations (400, 800, and 1,200 nM) at a T_m of 59°C but the probe and primer concentrations of 800 nM yielded the greatest difference in Ct values (5.36; Table 4). These two probe and primer sets did not bind to DNA extracted from either XgGA2 or Xv144.

There was no amplification of DNA from XgGA2 or any of the other samples using the *X. gardneri* probe and primer set at a concentration of 800 nM with a T_m of 59°C (data not shown). Therefore, different protocols were tested with a T_m of 57, 62, 63, 65, 67, or 69°C.

Table 2. Real-time TaqMan polymerase chain reaction probes (with the target single-nucleotide polymorphism in bold) and primers used in this study

Probe, primer ^y	Sequence	G + C (%)	T_m (°C) ^z
<i>Xanthomonas perforans</i>	5'/56-FAM/CGGGCAAGGAGCCATCGCCTGT/31ABkFQ/-3'	68.1	66.6
<i>X. euvesicatoria</i>	5'/5TET/CGGGCAAGGCGCAATCGCCTGT/3BHQ_2/-3'	68.1	67.5
FP1	5'-CGTCGACGGCCTGGGCGA-3'	77.7	66.1
RP1	5'-CCGGTGCCTGCGCCTGGA-3'	77.7	66.7
<i>X. gardneri</i>	5'-/5TexRd-XN/TGCGCCAGCGT G ACGGCACGC/3IAbRQSp/-3'	76.2	70.4
<i>X. vesicatoria</i> 1	5'-/5Cy5/TGCGCCAGCGATGGCACGC/3IAbRQSp/-3'	76.2	70.8
FP2	5'-AGGTCAGCCTGGGCGAGGT-3'	68.4	64.0
RP2	5'-TGAAGCCACCACTCGGC-3'	68.4	63.0

^y Forward primer (FP) 1 and reverse primer (RP) 1 are specific for *X. perforans* and *X. euvesicatoria*, and FP2 and RP2 are specific for *X. vesicatoria* and *X. gardneri*.

^z Melting temperature.

Target amplification was not observed until the T_m reached 67°C and was optimal at 69°C (data not shown). Additionally, the *X. gardneri* probe was utilized to only amplify XgGA2 DNA and not DNA from the other control strains. The *X. gardneri* probe and primer set was validated by testing DNA extracted from strains UF1 to UF9 (two *X. gardneri* and seven *X. vesicatoria* strains) in addition to Xv141, Xv144, Xe157, Xp1484, and XgGA2. The assay using the *X. gardneri* probe and primer set only amplified from the *X. gardneri* strains UF5 and UF9 and the positive control XgGA2, but not from strains of *X. vesicatoria*, *X. perforans*, or *X. euvesicatoria* (data not shown). The *X. vesicatoria* probe and primer set only bound to DNA from *X. vesicatoria* strains (data not shown).

Multiplex real-time TaqMan PCR assay optimization and validation. Three different multiplex master mixes were prepared by varying the probe and primer concentrations (1 and 2, 2 and 4, and 3 and 6 μ M, respectively). Real-time PCR with the multiplex master mix that contained the 3- μ M probe and 6- μ M primer resulted

in the lowest Ct values and curves for all samples (data not shown). When the T_m of 59°C was used, the use of the *X. perforans* probe and primer set resulted in the amplification of DNA from Xp1484 (mean Ct value of 27.05 ± 0.5194 [$\pm$ standard deviation]), which crossed the threshold 4.23 cycles before Xe157 (mean Ct value of 31.28 ± 0.5069). On average, the *X. euvesicatoria* probe resulted in the amplification of DNA from Xe157 (mean Ct value of 28.86 ± 0.8013), which crossed the threshold 9.71 cycles before Xp1484 (mean Ct value of 38.57 ± 0.4554). However, when a T_m of 69°C was used, the use of the *X. euvesicatoria* and *X. perforans* probes only resulted in the amplification of DNA from Xe157 (mean Ct value of 27.57 ± 0.9582) and Xp1484 (mean Ct value of 25.49 ± 0.9582), respectively. DNA from two *X. vesicatoria* (MME and 1111), six *X. euvesicatoria* (11, 330, 1520, 9060, XV881, and IP), three *X. gardneri* (XG51, 444, and 451), and two *X. perforans* (ETH21 and ETH31) strains were targeted only by their corresponding probes and primers at the 3- and 6- μ M concentrations, respectively, and using a T_m of 69°C (Table 5). All

Fig. 1. Condensed maximum-likelihood phylogenetic tree with bootstrap values based on the *hrpB7*. The scale bar indicates the number of substitutions per site and was created using 1,000 bootstrap samples.

66 strains gave the expected results with the multiplex real-time TaqMan PCR assay (Table 1).

Multiplex real-time TaqMan PCR assay sensitivity and accuracy.

The multiplex assay using the four probes and two primer sets was able to detect DNA extracted from bacterial suspensions of 10^5 to 10^8 CFU/ml (Table 6). There was a difference of approximately three cycles between values for each 10-fold dilution of the DNA template for each species-specific probe and corresponding primer set. The detection limit for all four *Xanthomonas* spp. causing bacterial spot was 5×10^5 CFU/ml (Table 6), corresponding to approximately 200 bacterial cells in the PCR mixture (10^5 CFU/ml $\times$ 2 μ l $\times$ 1 ml/1,000 μ l).

The NCBI blast results and the phylogenetic analysis of the *hrpB7* gene sequences indicated that this multiplex real-time assay could potentially amplify plant-pathogenic xanthomonads other than the four bacterial spot pathogens. Based on the blast results, the *hrpB7* gene sequence for *X. axonopodis* pv. *citrumelo* F1 strain shared a 98% identity with the *X. euvesicatoria* and *X. perforans* strains. For the *X. axonopodis* pv. *citrumelo* *hrpB7* gene sequence, three SNP were observed in the *X. euvesicatoria* probe sequence at positions 257, 260, and 269 and two SNP were observed in the *X. perforans* probe sequence at positions 257 and 269. The *hrpB7* gene sequence of *X. citri* pv. *citri* and *X. axonopodis* pv. *glycines* shared a 96% identity with the *X. euvesicatoria* and *X. perforans* strains. Additionally, the *hrpB7* sequence of *X. oryzae* pv. *oryzae* shared a 97% identity with *X. euvesicatoria* and *X. perforans*. For *X. citri* pv. *citri*, *X. axonopodis* pv. *glycines*, *X. fuscans* subsp. *fuscans*, and *X. oryzae* pv. *oryzae* *hrpB7* gene sequences, two SNP were observed in the *X. euvesicatoria*

probe sequence at positions 257 and 269, two SNP were observed in the *X. perforans* probe sequence at positions 260 and 269, and one SNP in the forward primer's sequence at position 230. The *hrpB7* gene sequence for *X. campestris* pv. *campestris* shared a 90% identity with the *X. vesicatoria* and a 94% identity with *X. gardneri* strains. For *X. campestris* pv. *campestris* *hrpB7* gene sequence, one SNP was observed in the *X. vesicatoria* probe sequence at position 62 and one SNP was observed in the *X. gardneri* probe sequence at position 59. The *X. campestris* pv. *campestris* strains were not amplified with any of the probes and primers. The *X. perforans* probe and primer set amplified *X. axonopodis* pv. *citrumelo* strain F1 (mean Ct value of 24.32 ± 0.8335). Although *X. citri* pv. *citri* strain A306 had a mean Ct value of 39.40 ± 0.5577 with the *X. perforans* probe and primer set, it was considered negative because its mean Ct value was greater than 37.

Evaluation of multiplex real-time TaqMan PCR analysis from plant tissue.

All of the DNA extracted directly from 122 infected leaf and fruit lesions were positive for one of the four bacterial spot pathogens (Table 7). DNA samples that were extracted from pure cultures isolated from the same bacterial spot lesions confirmed the real-time PCR results of the DNA extracted directly from the infected tissue (Table 7). For all of the tested samples, the Ct values were lower for DNA extracted from pure cultures than from infected plant tissue.

Discussion

We developed a multiplex real-time TaqMan PCR assay for identification of the four bacterial spot pathogens. This assay includes

Table 3. Simplex optimization results for *Xanthomonas perforans* probe (XP) and primers (FP1 and RP1) only^z

Strain	Species	Probe and primer concentration (nM)	Probe and primers	Mean Ct value
Xp1484	<i>X. perforans</i>	1,200	XP, FP1, and RP1	29.22 $\pm$ 0.900
Xp1484	<i>X. perforans</i>	800	XP, FP1, and RP1	29.38 $\pm$ 0.650
Xp1484	<i>X. perforans</i>	400	XP, FP1, and RP1	31.39 $\pm$ 0.777
Xp1484	<i>X. perforans</i>	200	XP, FP1, and RP1	39.30 $\pm$ 0.280
Xp1484	<i>X. perforans</i>	100	XP, FP1, and RP1	No amplification
Xe157	<i>X. euvesicatoria</i>	1,200	XP, FP1, and RP1	30.96 $\pm$ 0.619
Xe157	<i>X. euvesicatoria</i>	800	XP, FP1, and RP1	30.76 $\pm$ 0.318
Xe157	<i>X. euvesicatoria</i>	400	XP, FP1, and RP1	32.39 $\pm$ 1.118
Xe157	<i>X. euvesicatoria</i>	200	XP, FP1, and RP1	39.35 $\pm$ 0.276
Xe157	<i>X. euvesicatoria</i>	100	XP, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	1,200	XP, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	800	XP, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	400	XP, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	200	XP, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	100	XP, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	1,200	XP, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	800	XP, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	400	XP, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	200	XP, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	100	XP, FP1, and RP1	No amplification

^z FP = forward primer, RP = reverse primer, and $\pm$ = standard deviation of the cycle threshold (Ct) values of the three replicates.

Table 4. Simplex optimization results for *Xanthomonas euvesicatoria* probe (XE) and primers (FP1 and RP1) only^z

Strain	Species	Probe and primer concentration (nM)	Probe and primers	Mean Ct value
Xp1484	<i>X. perforans</i>	1,200	XE, FP1, and RP1	31.34 $\pm$ 0.509
Xp1484	<i>X. perforans</i>	800	XE, FP1, and RP1	36.32 $\pm$ 0.329
Xp1484	<i>X. perforans</i>	400	XE, FP1, and RP1	35.01 $\pm$ 0.665
Xe157	<i>X. euvesicatoria</i>	1,200	XE, FP1, and RP1	30.41 $\pm$ 0.832
Xe157	<i>X. euvesicatoria</i>	800	XE, FP1, and RP1	30.36 $\pm$ 0.681
Xe157	<i>X. euvesicatoria</i>	400	XE, FP1, and RP1	33.20 $\pm$ 0.821
Xv144	<i>X. vesicatoria</i>	1,200	XE, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	800	XE, FP1, and RP1	No amplification
Xv144	<i>X. vesicatoria</i>	400	XE, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	1,200	XE, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	800	XE, FP1, and RP1	No amplification
XgGA2	<i>X. gardneri</i>	400	XE, FP1, and RP1	No amplification

^z FP = forward primer, RP = reverse primer, and $\pm$ = standard deviation of the cycle threshold (Ct) values of the three replicates.

four species-specific probes and two primer sets based on the *hrpB7* gene sequence. As predicted, the sequence alignment revealed that the *hrpB7* gene is highly conserved among strains from the same species of *Xanthomonas* and is an ideal target for diagnostic purposes. All of the tested *X. perforans*, *X. euvesicatoria*, and *X. gardneri* strains were 100% identical within their given species. *X. perforans* and *X. euvesicatoria* were the two most closely related species based on the *hrpB7* gene sequence, in which they only varied by two SNP. There was an SNP at position 331 of the *hrpB7* gene that distinguished two groups of *X. vesicatoria* strains. Additionally, the

phylogenetic tree confirmed that the *hrpB7* gene is highly conserved among strains of the same bacterial spot pathogen. It also demonstrated that the *hrpB7* gene sequences for each bacterial spot species are different from the *hrpB7* sequences of most of the other plant-pathogenic xanthomonads. Therefore, each bacterial spot pathogen differs from each other and from other xanthomonads. Additionally, the *X. vesicatoria* strains formed two distinct branches.

During optimization, it was revealed that the *X. gardneri*-specific probe was unable to amplify its target until a T_m of 67 or 69°C was used. The *X. gardneri*-specific probe was predicted to form a hairpin

Table 5. Multiplex optimization results for the *Xanthomonas perforans* probe (XP) and *X. euvesicatoria* probe (XE) and their corresponding primers (FP1 and RP1), and the *X. gardneri* probe (XG) and *X. vesicatoria* probe (XV) and their corresponding primers (FP2 and RP2)^z

Strain	Species	Probe, primer concentration (μM)	Probe and primers	Mean Ct value
MME	<i>X. vesicatoria</i>	3, 6	XV, FP2, and RP2	32.02 ± 0.570
1111	<i>X. vesicatoria</i>	3, 6	XV, FP2, and RP2	29.87 ± 0.742
11	<i>X. euvesicatoria</i>	3, 6	XE, FP1, and RP1	24.72 ± 0.649
330	<i>X. euvesicatoria</i>	3, 6	XE, FP1, and RP1	23.21 ± 0.945
1520	<i>X. euvesicatoria</i>	3, 6	XE, FP1, and RP1	25.70 ± 0.813
9060	<i>X. euvesicatoria</i>	3, 6	XE, FP1, and RP1	24.49 ± 0.588
XV881	<i>X. euvesicatoria</i>	3, 6	XE, FP1, and RP1	25.91 ± 0.620
IP	<i>X. euvesicatoria</i>	3, 6	XE, FP1, and RP1	26.51 ± 0.532
ETH21	<i>X. perforans</i>	3, 6	XP, FP1, and RP1	24.21 ± 0.606
ETH31	<i>X. perforans</i>	3, 6	XP, FP1, and RP1	25.75 ± 0.480
Xg51	<i>X. gardneri</i>	3, 6	XG, FP2, and RP2	29.07 ± 0.850
444	<i>X. gardneri</i>	3, 6	XG, FP2, and RP2	28.43 ± 0.654
451	<i>X. gardneri</i>	3, 6	XG, FP2, and RP2	27.37 ± 0.612

^z Each strain was only amplified by its corresponding probe and primer. FP = forward primer, RP = reverse primer, and ± = standard deviation of the cycle threshold (Ct) values of the three replicates.

Table 6. Sensitivity results for the multiplex real-time polymerase chain reaction (PCR) assay using the *Xanthomonas perforans* probe (XP) and *X. euvesicatoria* probe (XE) and their corresponding primers (FP1 and RP1), and the *X. gardneri* probe (XG) and *X. vesicatoria* probe (XV) and their corresponding primers (FP2 and RP2)^y

Strain	Species	Probe and primers	Bacterial concentration (CFU/ml) ^z	Mean Ct value
Xp1484	<i>X. perforans</i>	XP, FP1, and RP1	5×10^8	27.03 ± 0.559
			5×10^7	30.87 ± 0.907
			5×10^6	34.80 ± 0.236
			5×10^5	37.91 ± 0.308
			5×10^4	No amplification
			5×10^3	No amplification
			5×10^2	No amplification
Xe157	<i>X. euvesicatoria</i>	XE, FP1, and RP1	5×10^1	No amplification
			5×10^8	23.80 ± 0.7705
			5×10^7	27.32 ± 0.4250
			5×10^6	33.14 ± 0.6768
			5×10^5	39.12 ± 0.3502
			5×10^4	No amplification
			5×10^3	No amplification
Xv144	<i>X. vesicatoria</i>	XV, FP2, and RP2	5×10^2	No amplification
			5×10^1	No amplification
			5×10^8	26.25 ± 0.5645
			5×10^7	29.43 ± 0.4979
			5×10^6	34.81 ± 0.5173
			5×10^5	39.35 ± 0.7068
			5×10^4	No amplification
XgGA2	<i>X. gardneri</i>	XG, FP2, and RP2	5×10^3	No amplification
			5×10^2	No amplification
			5×10^1	No amplification
			5×10^8	27.39 ± 0.5450
			5×10^7	30.29 ± 0.6107
			5×10^6	34.86 ± 0.7055
			5×10^5	39.08 ± 0.5359
			5×10^4	No amplification
			5×10^3	No amplification
			5×10^2	No amplification
			5×10^1	No amplification

^y Probe and primer concentrations were 3 and 6 μM, respectively. DNA of each strain was only amplified by its corresponding probe and primer. FP = forward primer, RP = reverse primer, and ± = standard deviation of the cycle threshold (Ct) values of the three replicates.

^z When the real-time multiplex PCR is detecting a starting bacterial suspension of 5×10^5 CFU/ml, it is actually detecting 200 bacterial cells in the PCR mixture.

at 64.7°C, which can affect its ability to amplify the target sequence. This could explain why the higher T_m of 69°C is required for the *X. gardneri*-specific probe to bind and amplify the target DNA from *X. gardneri* strains. When compared with the multiplex real-time PCR results, the *X. perforans* probe and *X. euvesicatoria* probe were not as specific to their targets in the simplex real-time PCR assays. In the simplex real-time PCR assays, the *X. perforans* probe and *X. euvesicatoria* probe bound to DNA extracted from both *X. perforans* and *X. euvesicatoria*. However, the *X. perforans* probe and *X. euvesicatoria* probe bound to DNA samples extracted from Xp1484 and Xe157, respectively, more efficiently than they did to nontargeted DNA. When the *X. perforans* probe and primer set were used, DNA from Xp1484 would cross the threshold approximately one cycle before DNA from Xe157. Because probe and primer efficiency can be inferred from Ct values, the *X. perforans* probe and primer set bound to Xp1484 DNA more efficiently than it did to Xe157 DNA. This difference in Ct values led to the hypothesis that multiplexing the probes and increasing the T_m to 69°C would cause them to compete with one another for their targets, which would increase the specificity of the probes. Thus, the *X. perforans* probe would show preferential binding to *X. perforans* DNA, which may prevent the *X. euvesicatoria* probe from binding to the *X. perforans* DNA. The data presented in this study support this hypothesis.

After optimization, the multiplex real-time TaqMan PCR assay was specific to the four causal agents of bacterial spot. It correctly identified DNA extracted from pure cultures of 66 strains representative of the four species associated with bacterial spot of tomato. This assay correctly detected all four bacterial spot pathogens in all of the DNA samples extracted from lesions on naturally and artificially infected tomato tissue. The Ct values for DNA extracted from infected plant tissue were higher than the Ct values of DNA extracted from pure cultures (Table 7). This difference in Ct values can be expected when comparing pure culture DNA and infected plant tissue DNA. In pure culture DNA, there are no inhibitors or other sources of DNA present in the sample that can interfere with assay efficiency. Due to the presence of inhibitors such as complex polysaccharides and humic acid, the amplification of bacterial DNA from plant tissue can be problematic by directly effecting the *Taq* DNA polymerase or by binding to the targeted DNA (Bessettii 2007; Palacio-Bielsa et al. 2011). Additionally, the predominant plant DNA (99%) present mixed in with pathogen DNA (<1%) is known to interfere with *Taq* DNA Polymerase. Plant DNA has a strong DNA-binding domain, which removes *Taq* DNA polymerase from the reaction and reduces its activity (Hoy et al. 2001). Because some trace *Taq* polymerase will be able to bind to the pathogen DNA, it could take several more cycles before the *Taq* DNA polymerase can find the target, which would result in a higher Ct value. The direct detection of the bacterial spot pathogens in infected tissue allows growers to receive a more rapid diagnosis and researchers to monitor changes in population structure. Unfortunately, this multiplex real-time PCR assay amplified the *X. axonopodis* pv. *citrumelo* strain with the *X. perforans* primer-probe combination. Although it is closely related to *X. euvesicatoria* and *X. perforans* based on the *hrpB7* gene, *X. axonopodis* pv. *citrumelo* causes a bacterial spot disease

of citrus but it has a completely different host range (Jalan et al. 2011). Because *X. axonopodis* pv. *citrumelo* only infects a few citrus varieties (Graham et al. 1990), it is unlikely that it would be isolated from tomato and is not a major concern for this assay. In terms of sensitivity, the multiplex real-time TaqMan PCR assay was able to detect DNA extracted from 5×10^5 CFU/ml when bacterial suspensions were analyzed. The Ct values of DNA extracted from 122 bacterial spot lesions ranged from 26.37 to 32.00. When compared with the Ct values for the pure culture assay, the results indicate that the sensitivity was 200 bacterial cells in the reaction mixture. Thus, sensitivity was not an issue in this study because all DNA extracted from infected plant tissue and pure cultures were correctly amplified.

Real-time PCR has gained popularity in plant disease diagnostics because it is a rapid and reproducible technique that reduces the risk of carryover contamination (Li et al. 2006; Mackay et al. 2002). There are additional advantages to using multiplex real-time PCR for the bacterial spot of tomato pathogens. The main advantage involves time. Obradovic et al. (2004) used PCR *hrpB7* gene-based primers to amplify DNA from the four bacterial spot species. The PCR products were then digested with three separate restriction enzymes overnight and the fragments were separated by electrophoresis to yield distinct banding patterns for the four pathogens. Additionally, Araújo et al. (2012) developed a multiplex PCR assay to simultaneously detect all four species. These primers were designed based on distinct banding patterns that were observed using AFLP analysis. The PCR products were then separated on a 3% agarose gel. For pure cultures, both of these methods can take 8 to 16 h for a single sample. However, our multiplex real-time TaqMan PCR assay can identify the bacterial spot pathogen within 4 h. A positive attribute of this method is that it does not require gel electrophoresis. The elimination of the electrophoresis step saves time and materials, especially when analyzing large sample numbers. Additionally, our multiplex real-time PCR assay was able to detect the two bacterial spot strains (NII and *X. perforans* strain 1712) that would not be amplified using the multiplex method of Araújo et al. (2012). NII is a hybrid between *X. perforans* and *X. euvesicatoria* and was detected as a bacterial spot of tomato pathogen by the *X. perforans* probe. These strains cannot be amplified using the multiplex PCR assay because they lack the targeted gene sequence (N. Potnis, unpublished). The primers of the multiplex PCR described by Araújo et al. (2012) are based on non-coding regions, which are not as conserved as some coding regions of DNA.

In summary, the outlined multiplex real-time TaqMan PCR assay is rapid, accurate, and reproducible. To our knowledge, only one other multiplex real-time TaqMan PCR assay has been developed to simultaneously detect four different plant-pathogenic species (Schena et al. 2006). Our novel assay will allow diagnosticians to easily and quickly identify the bacterial spot pathogens from pure cultures or infected tissue. These results will also allow researchers to monitor changes in bacterial spot species in a population over time and space. This multiplex real-time TaqMan PCR assay has the potential to aid plant pathologists and is an improvement over the current identification techniques available for these four pathogens.

Table 7. Multiplex real-time TaqMan polymerase chain reaction results for DNA extracted from infected plant tissue and pure cultures following isolation

Tissue type	Species	Source ^y	N ^z	Mean Ct value for ^x	
				Lesion DNA	Culture DNA
Tomato	<i>Xanthomonas perforans</i>	Field	20	32.00 ± 2.434	24.56 ± 1.404
Leaves	<i>X. perforans</i>	GH	40	28.51 ± 2.80	25.26 ± 1.369
	<i>X. euvesicatoria</i>	GH	20	26.37 ± 1.490	25.17 ± 2.236
	<i>X. gardneri</i>	GH	20	30.10 ± 2.002	27.63 ± 1.508
	<i>X. vesicatoria</i>	GH	20	30.35 ± 1.712	28.52 ± 1.961
	<i>X. perforans</i>	Field	2	28.63 ± 1.054	24.79 ± 0.717

^x Pure cultures were isolated from the same bacterial spot lesions that were analyzed; ± = standard deviation of the cycle threshold (Ct) values of the three replicates

^y GH = greenhouse.

^z Number of samples.

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In the article “A Multiplex Real-Time PCR Assay Differentiates Four *Xanthomonas* Species Associated with Bacterial Spot of Tomato” by A. L. Strayer, A. Jeyaprakash, G. V. Minsavage, S. Timilsina, G. E. Vallad, J. B. Jones, and M. L. Paret, the gene name *hrpB2* is incorrectly used. The correct gene name is *hrpB7*, which should be used throughout the article. Also, the reference to Hartmann et al. 2012 (mentioned on the second page of the article) has been replaced by references to Leite et al. (1994) and Obradovic et al. (2004), and Hartmann et al. 2012 has been removed from the list of literature citations.