Phenotypic and Genetic Diversity of *Xanthomonas perforans* Populations from Tomato in North Carolina

Pragya Adhikari,¹ Tika B. Adhikari,^{2,†} Sujan Timilsina,³ Inga Meadows,²
Jeffrey B. Jones,³ Dilip R. Panthee,¹ and Frank J. Louws^{1,2,†}

¹ Department of Horticultural Science, North Carolina State University, Raleigh, NC 27695

² Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695

³ Department of Plant Pathology, University of Florida, Gainesville, FL 32611

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ABSTRACT

Bacterial spot caused by *Xanthomonas* spp. is one of the most devastating diseases of tomato in North Carolina (NC). In total, 290 strains of *Xanthomonas* spp. from tomato in NC collected over 2 years (2015 and 2016) were analyzed for phenotypic and genetic diversity. In vitro copper and streptomycin sensitivity assays revealed that >95% ($n = 290$) of the strains were copper tolerant in both years, whereas 25% ($n = 127$) and 46% ($n = 163$) were streptomycin tolerant in 2016 and 2015, respectively. Using BOX repetitive element PCR assay, fingerprint patterns showed four haplotypes (H1, H2, H3, and H4) among the strains analyzed. The multiplex real-time quantitative PCR on a subset of representative strains ($n = 45$) targeting the highly conserved *hrcN* gene identified *Xanthomonas* strains from tomato in NC that belonged to *X. perforans*. Race profiling of the representative strains ($n = 45$) on tomato and pepper differentials confirmed that ~9 and 91% of strains are tomato races T3 and T4, respectively.

Additionally, PCR assays and sequence alignments confirmed that the *copL*, *copA*, *copB* (*copLAB* copper tolerance gene cluster), and *avrXv4* genes are present in the strains analyzed. Phylogenetic and comparative sequence analyses of six genomic regions (elongation factor G [*fusA*], glyceraldehyde-3-phosphate dehydrogenase A [*gapA*], citrate synthase [*gltA*], gyrase subunit B [*gyrB*], ABC transporter sugar permease [*lacF*], and GTP binding protein [*lepA*]) suggested that 13 and 74% of *X. perforans* strains from NC were genetically similar to races T3 and T4 from Florida, respectively. Our results provide insights that bacterial spot management practices in tomato should focus on deploying resistance genes to combat emerging pathogenic races of *X. perforans* and overcome the challenges currently posed by intense use of copper-based bactericides.

Keyword: population biology

Bacterial spot (*Xanthomonas* spp.) is an economically important disease of fresh market tomato (*Solanum lycopersicum* L.) in North Carolina (NC) and several other states (Louws et al. 2001). At least four species within the genus *Xanthomonas* (*X. euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria*) have been reported to cause bacterial spot in tomato (Jones et al. 2004). *X. euvesicatoria*, *X. perforans*, and *X. vesicatoria* are favored by warm temperatures (25 to 30°C), whereas *X. gardneri* strains prefer cooler temperatures (<20°C) (Araújo et al. 2011). In addition, four pathogenic races (T1, T2, T3, and T4) of *Xanthomonas* spp. associated with differential hosts have been reported (Jones et al. 2004). The race T1 includes strains with *avrRxv* effector eliciting a hypersensitive response (HR) in a tomato differential HI 7998; all T1 strains identified so far are *X. euvesicatoria* (Scott et al. 1996; Whalen et al. 1993). T2 race differed from race T1 by the deletion of the 680-bp region of *avrRxv* (Whalen et al. 1993) and belongs to *X. vesicatoria* (Stall et al. 2009). Tomato genotype showing HR response to T2 strains has not been reported yet. The T3 race includes strains containing *avrXv3*

effector that elicits HR in any genotype with *Xv3* gene. (Jones et al. 1995, 1998). The T3 strains identified so far belong to *X. perforans*. *X. perforans* strains containing another effector *xopJ4* (previously called *avrXv4*) but lacking *avrXv3* that induces an HR in *Solanum pennellii* LA716 and any other tomato genotype with *RxopJ4* (*Xv4*)-resistant locus were designated as race T4 (Astua-Monge et al. 2000; Stall et al. 2009; Sharlach et al. 2013).

The *Xanthomonas* pathogen is introduced into tomato fields through contaminated seed, infested equipment or workers' hands, and wind-driven or rain-splashed plant material or debris (Bashan et al. 1982; Burlakoti et al. 2018; Cuppels et al. 2006; Jones et al. 1986). Integration of cultural practices and chemicals, such as copper, antibiotics (e.g., streptomycin), and plant activators, has been used to manage bacterial spot (Louws et al. 2001; Thayer and Stall 1962; Vallad and Goodman 2004). However, copper- and streptomycin-tolerant strains have been widely reported in Canada, Brazil, Tanzania, and the United States (Abbasi et al. 2015; Araújo et al. 2012; Mixon 2012; Shenge et al. 2014; Vallad et al. 2013). Although tomato growers in NC have reported a failure of copper and streptomycin to manage bacterial spot disease in tomato (F. J. Louws, unpublished data), *Xanthomonas* strains' tolerance to these chemicals has not been investigated.

The evolution of new *Xanthomonas* spp. and pathogenic races over time has hindered the development and deployment of host resistance to manage bacterial spot disease in tomato (Timilsina et al. 2016). Among the four species of the genus *Xanthomonas*, *X. euvesicatoria* and *X. vesicatoria* are distributed worldwide, whereas *X. perforans* and *X. gardneri* are mostly reported from North America and South America (Potnis et al. 2015). *X. gardneri* was reported in 1991 in the Midwest of the United States (Ma et al. 2011) and Canada on a contaminated seed lot (Cuppels et al. 2006). Although *X. vesicatoria* is widely distributed in the Midwest of the

[†]Corresponding authors: T. B. Adhikari; tbadhika@ncsu.edu, and F. J. Louws; frank_louws@ncsu.edu

Current address of P. Adhikari: Department of Crop Science, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

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United States, it was never a problem in Florida (FL) and the Southeastern United States (Louws et al. 1995). For example, *X. euvesicatoria* was the only species present as race 1 in FL until 1991 before the *X. perforans* race T3 was reported (Timilsina et al. 2015). *X. perforans* race T4 evolved in 1998 (Astua-Monge et al. 2000; Minsavage et al. 2003), and thereafter, it has been detected more frequently in FL. *X. perforans* race T4 was detected throughout FL, where races T4 and T3 were in the ratio of 3:1 in the previous surveys, indicating a major shift in the race within *X. perforans* populations (Horvath et al. 2012). It is imperative to investigate the pathogen species, race structure, and genetic diversity to develop effective disease management and breeding strategies against the disease in NC.

The antibiotic streptomycin has been extensively used since the early 1950s to manage bacterial spot of tomato and pepper (Thayer and Stall 1962). Because of the evolution of streptomycin-tolerant *Xanthomonas*, copper-based bactericides began to be used in the late 1960s (Marco and Stall 1983; Thayer and Stall 1962). To enhance the efficacy of copper bactericides, combinations of copper with ethylene bisdithiocarbamate fungicides, such as maneb or mancozeb, have been adopted to increase the availability of free copper ions (Conover and Gerhold 1981; Jones and Jones 1985; Marco and Stall 1983). However, copper-mancozeb tank mixes seemed to be ineffective to manage bacterial spot when copper-tolerant strains are prevalent (Jones and Jones 1985; Obradovic et al. 2005). In NC, most growers continue to use the mancozeb-copper tank mixes for up to five applications before harvest commences and based on extension recommendations (Kuhar et al. 2019). Although this management practice is common, the efficacy of this seems to be failing, and there is a need to characterize the *Xanthomonas* populations. We hypothesize that *X. perforans* strains in NC would possess tolerance to copper and streptomycin owing to their extensive use in the fields and greenhouses. We also predicted that *X. perforans* is the dominant bacterial spot pathogen of tomato in NC based on population genetic analysis in the Southeastern United States (Potnis et al. 2015; Timilsina et al. 2015; Vallad et al. 2013). We also tested the hypothesis that *X. perforans* strains from NC are predominantly clonal and genetically similar to strains of *X. perforans* from FL. The main objectives of this study were to

(i) evaluate strains of *Xanthomonas* species from tomato for tolerance to copper and streptomycin, (ii) assess the genetic diversity of *Xanthomonas* strains using BOX repetitive element PCR (BOX-PCR) assay and determine the species structure in NC, (iii) characterize the pathogenic races of *Xanthomonas* strains in NC, and (iv) determine and compare the phylogenetic relationships and genetic distinctness of *Xanthomonas* strains from NC with the reported *Xanthomonas* strains from other regions.

MATERIALS AND METHODS

Sample collection and pathogen isolation. The western region is the major tomato-producing region in NC, in which eight counties were surveyed using a modified random sampling scheme in 2015 and 2016 (Fig. 1). Twenty bacterial spot-infected leaves (four leaves from five sites within the field in random quadrants) were collected per field and placed in a plastic sealable bag. These bags were labeled with the date of collection, location, and tomato cultivar and brought to the laboratory for isolation. To isolate the bacterial pathogen, the infected leaves were surface sterilized with 10% Clorox (Clorox Company) for 30 s and rinsed with sterile distilled water (SDW) three times. Fresh lesions were cut at ~4 mm² with a sterile scalpel in the presence of SDW, streaked on Difco yeast dextrose agar (YDC) medium, and incubated at 27°C for 48 h. The plates were examined for the presence of yellow mucoid colonies, and only putative *Xanthomonas* colonies were subcultured on fresh YDC plates to obtain pure cultures. In total, 290 strains of *Xanthomonas* species ($n = 163$ in 2015 and $n = 127$ in 2016) were isolated from 23 fields across eight counties of NC. All pure cultures were stored in 30% glycerol at -80°C for future studies (Supplementary Table S1).

Copper and streptomycin tolerance assays. The original work was done with copper tolerance using copper sulfate (CuSO₄) at 200 µg/ml (Stall et al. 1986) and streptomycin sulfate at 100 µg/ml (Ritchie and Dittapongpitch 1991) that inhibited the growth of the strains of *X. euvesicatoria* from tomato and pepper in the United States. To assess strain tolerance to copper, we used a discriminatory dose of CuSO₄ at 200 µg/ml to distinguish between the tolerant strains using positive and negative controls as described previously (Burlakoti et al. 2018; Stall et al. 1986). Likewise, streptomycin

Fig. 1. Map of North Carolina showing counties (star symbols) from where strains of *Xanthomonas perforans* were collected and analyzed in this study.

sulfate at the recommended discriminatory dose (100 µg/ml) (Ritchie and Dittapongpich 1991) was used to discriminate between sensitive and tolerant strains. The CuSO₄ ions and streptomycin sulfate dry (Sigma-Aldrich Corporation) were obtained, and sensitivity assays for 290 strains were performed in vitro in sucrose peptone agar (SPA) medium. The SPA medium was prepared using 15.0 g of sucrose, 5.0 g of peptone, 0.50 g of dibasic potassium phosphate, 0.25 g of magnesium sulfate, and 15.0 g of Difco agar in 1,000 ml of distilled water. The pH was adjusted to 7.2 to 7.4 before autoclaving. Fresh stock solution of copper sulfate and streptomycin sulfate was prepared and filter sterilized using a 0.22-µm-pore size filter under laminar flow. The appropriate amounts of stock solutions of copper and streptomycin were added to the SPA medium after autoclaving and cooled. Bacteria were cultured in Luria–Bertani medium (Thermo Scientific) for 24 to 48 h and suspended in sterile water to adjust the concentration to 10⁸ CFU/ml. Concentrations were measured using a spectrophotometer at A₆₀₀ = 0.3 optical density (OD). A 5-µl aliquot of the suspension was pipetted and spread on the SPA medium amended with different concentrations of copper and streptomycin and incubated for 24 to 48 h at 27°C. The growth of the bacteria on amended medium was visually observed and recorded. Strains capable of growing on SPA medium containing 200 ppm of CuSO₄ or 100 ppm of streptomycin sulfate were considered as copper tolerant (Cu-T) and streptomycin tolerant (Sp-T), respectively. No bacterial growth on amended media was regarded as copper sensitive (Cu-S) and streptomycin sensitive (Sp-S). David Ritchie, Department of Entomology and Plant Pathology, North Carolina State University (Raleigh, NC) provided control strains of *Xanthomonas* spp. known to be sensitive and tolerant to copper and streptomycin, which were Xcv 611 (Cu-T, Sp-S), Xcv 43 (Cu-T, Sp-T), and Xcv 135 (Cu-S, Sp-S).

Genetic diversity analysis. BOX-PCR assay was selected for the genetic diversity analysis, because this assay is robust and yields more amplifications compared with enterobacterial repetitive intergenic consensus (ERIC)-PCR and repetitive extragenic palindromic (REP)-PCR assays (Louws and Cuppels 2001; Louws et al. 1995) in our preliminary study. Importantly, BOX-PCR (BOXA1R: 5'-CTACGGCAAGGCGACGCTGACG-3') assay has been successfully utilized for the characterization of bacterial pathogens infecting tomatoes (Araújo et al. 2017). Genomic DNA of each strain was extracted as described previously (Pitcher et al. 1989; Rademaker and de Bruijn 1997) and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Final DNA concentration was adjusted to 10 ng/µl. BOX-PCR amplification was performed in a 25-µl reaction mixture as described previously (Louws and Cuppels 2001; Louws et al. 1995) with some modifications. Briefly, one initial cycle of denaturation at 95°C for 5 min was carried out followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 48°C for 1 min, extension at 68°C for 10 min, and a final cycle of extension at 68°C for 10 min. The PCR products were separated by gel electrophoresis on 1.5% agarose (Genesee Scientific Corporation) containing ethidium bromide (10 mg/ml; Sigma-Aldrich) in 1× Tris-acetate-EDTA (Thermo Scientific) at 35 V for 10 to 12 h. The gel image was visualized using a gel documentation system (BioRad Gel Doc). Among 293 strains in our original collections, DNA quality of five strains (NC-278, NC-302, NC-312, NC-316, and NC-351) was found poor at the time of analysis. Thus, only 288 strains were analyzed with BOX-PCR assay. A hierarchical agglomerative cluster analysis was performed to generate clusters from Euclidean distance matrices using SciPy cluster package in python (Jones et al. 2014). Briefly, fingerprint profiles were scored visually: “0” for the absence of the band and “1” for the presence of the band to generate a binary matrix. The fingerprint profiles were analyzed to identify unique haplotypes. The linkage method with group average (unweighted pair group) method was used to define the hierarchical clustering by comparing data points, and cophenetic correlation coefficient

was calculated to determine how well the cluster dendrogram preserves the original distances between data points (Saraçlı et al. 2013).

Multiplex quantitative real-time PCR. To determine species structure, a subset of representative strains (*n* = 45) was selected based on BOX-PCR fingerprinting profile, copper and streptomycin sensitivity assays, and the geographical regions (Table 1). Although both *hrcN* and *hrpB7* genes are the same operon and close to each other, the correct gene name is *hrcN*. Four species-specific probes and two primer sets targeting the *hrp* operon region were designed, and a highly conserved *hrcN* gene was amplified by multiplex TaqMan quantitative PCR (qPCR) (Strayer et al. 2016). Reference strains *X. euvesicatoria* Xe 157, *X. gardneri* Xg GA2, *X. perforans* Xp 1484, and *X. vesicatoria* Xv 144 were included in the analysis (Strayer et al. 2016). For multiplex qPCR assay, the probes specific for *X. euvesicatoria*, *X. vesicatoria*, *X. gardneri*, and *X. perforans* were labeled with blue, purple, red, and green dye, respectively.

Race identification. The strains used in determining species structure were selected (Table 1), and their ability to cause disease reactions on tomato and pepper differential cultivars was evaluated as described previously (Stall et al. 2009). The tomato differentials used for virulence assay were Bonny Best (susceptible to all races), Hawaii 7998 (effective against *avrRxv*; race T1), and FL 216 (effective against *avrXv3*; race T3). The resistant genotype LA716 carrying the *RxopJ4* gene against the *XopJ4* effector of race T4 could not be used as a tomato differential because of the extreme necrosis observed in this genotype (Astua-Monge et al. 2000). In addition, four pepper differentials were included for elicitation of an HR on Early Calwonder (ECW) plants and the near-isogenic lines (ECW-10R, ECW-20R, and ECW-30R) containing resistance genes to bacterial spot (*Bs1*, *Bs2*, and *Bs3*, respectively) (Jones et al. 1998; Sahin and Miller 1998). Bacterial inoculum was prepared by suspending a culture grown on nutrient agar for 24 h at 28°C in SDW, and the concentration was adjusted to ~5 × 10⁸ CFU/ml (A₆₀₀ = 0.3 OD). Reference strains *X. euvesicatoria* E3 and *X. perforans* 91-118 (Hert et al. 2009) obtained from Jeff Jones, Department of Plant Pathology, University of Florida (Gainesville, FL) were used as controls for race T1 and race T3, respectively. Bacterial suspensions were infiltrated into the abaxial leaf surfaces with the aid of a 1-ml hypodermic syringe without a needle on 5-week-old tomato and pepper seedlings. To identify pathogenic races, the disease reactions on tomato and pepper differentials were examined for HR after 24 h and susceptible reaction up to 2 days after infiltration. HR was characterized by tissue collapse at the infiltration site followed by confluent tissue necrosis occurring up to 24 h after infiltration (Klement and Goodman 1967). Susceptible or non-HR was confirmed by necrotic spots surrounded with chlorotic halos 3 to 4 days postinfiltration (Burlakoti et al. 2018; Stall et al. 2009). Classification of tomato races (T1 to T4) was assessed according to HR or susceptible reactions on tomato and pepper differentials as described previously (Burlakoti et al. 2018; Stall et al. 2009).

Multilocus sequencing analysis. A subset of 45 representative strains of *X. perforans* as determined by BOX-PCR, geographic location, cultivar, and year of isolation was subjected to multilocus sequencing analysis (MLSA) using the six housekeeping genes as described previously (Almeida et al. 2010; Timilsina et al. 2015). These included elongation factor G (*fusA*), glyceraldehyde-3-phosphate dehydrogenase A (*gapA*), citrate synthase (*gltA*), gyrase subunit B (*gyrB*), ABC transporter sugar permease (*lacF*), and GTP binding protein (*lepA*) (Almeida et al. 2010; Timilsina et al. 2015). Briefly, the amplification was performed in a 50 µl of PCR mixture consisting of 2.5 U of Phusion high-fidelity polymerase (Biorad), 1× Taq buffer, 0.2 mM dNTP, 0.4 µM of each forward and reverse primers, and water in a Peltier element-based thermocycle. We used two-step cycling with high-fidelity Phusion polymerase, and therefore, there was no annealing step.

One initial cycle of denaturation at 98°C for 2 min was carried out followed by 35 cycles of denaturation at 98°C for 20 s and extension at 72°C for 1 min and a final cycle of extension at 72°C for 10 min. A 4-μl PCR product was confirmed by electrophoresis on a 1.0% agarose gel, and image was visualized using a gel documentation system (BioRad). Positive PCR products were submitted for Sanger platinum sequencing at the Genomic Sciences Laboratory, North Carolina State University (Raleigh, NC). In a separate study, a subset of strains ($n = 13$) used for MLSA was sequenced to confirm the presence of three principal copper tolerance genes *copL*, *copA*, and *copB* and the *avrXv3* and *avrXv4* genes in the strains as described previously (Astua-Monge et al. 2000; Behlau et al. 2013; Timilsina et al. 2016).

The sequences of six loci were phylogenetically compared with reference *Xanthomonas* strains publicly available in sequence databases of the Plant Associated and Environmental Microbes Database (PAMDB; <http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>) (Almeida et al. 2010) or the NCBI (<https://www.ncbi.nlm.nih.gov/>). Briefly, the nucleotide sequences for each gene were trimmed at both ends such that the final sizes of genes

were 591 positions for *fusA*, 444 positions for *gapA*, 501 positions for *gltA*, 411 positions for *gyrB*, 408 positions for *lacF*, and 390 positions for *lepA*. Individual gene sequences were concatenated in the alphabetical order of the six genes to give the total length of 2,745 positions (i.e., 1 to 591 for *fusA*, 592 to 1,035 for *gapA*, 1,036 to 1,536 for *gltA*, 1,537 to 1,947 for *gyrB*, 1,948 to 2,355 for *lacF*, and 2,356 to 2,745 for *lepA*). The concatenated sequences of six loci of 31 strains that had full sequences of all genes of *X. perforans* from NC and the concatenated sequences of *Xanthomonas* reference strains were aligned using MUSCLE within Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar et al. 2016). The six housekeeping gene sequences for reference strains Xp ICMP16690 (strain 767), Xv-ATCC 11551 (strain 196), Xe 85-10 (strain 162), and Xg ICMP16689 (strain 766) were imported from the PAMDB, whereas the gene sequences for reference strains Xp GEV839 (accession no. JZVK000000000), Xp 91-118 (accession no. CP019725.1), Xp 17-12 (accession no. JZVH000000000), and Xp 4-20 (accession no. JZUZ000000000) were imported from their whole-genome sequences in the NCBI database. The individual gene sequences of atypical Nigerian strain

TABLE 1. Characteristics of representative strains of *Xanthomonas perforans* collected from North Carolina from 2015 to 2016 that were selected to determine race profiles

Isolate	County	Year of collection	Tomato cultivar from which isolated ^a	Copper insensitivity ^b	Streptomycin insensitivity ^c	BOX-PCR haplotype ^d
NC-10	Henderson	2015	Plum Regal	+	+	H2
NC-11	Henderson	2015	Plum Regal	+	–	H2
NC-12	Henderson	2015	Plum Regal	+	+	H2
NC-14	Henderson	2015	Plum Regal	+	–	H2
NC-22	Henderson	2015	Plum Regal	+	+	H1
NC-28	Madison	2015	Red Defender	+	–	H1
NC-37	Madison	2015	Red Defender	+	+	H1
NC-42	Madison	2015	Red Defender	+	+	H1
NC-47	Haywood	2015	Mountain Majesty	+	–	H1
NC-67	Buncombe	2015	Biltmore	+	–	H1
NC-79	Buncombe	2015	Biltmore	+	–	H1
NC-80	Buncombe	2015	Biltmore	+	–	H1
NC-87	Henderson	2015	BHN 786	+	–	H1
NC-101	Henderson	2015	BHN 786	+	–	H2
NC-110	Madison	2015	Grafted plants	+	–	H1
NC-112	Madison	2015	Grafted plants	+	+	H3
NC-124	Madison	2015	Grafted plants	+	+	H1
NC-126	Jackson	2015	Plum Regal	+	–	H1
NC-145	Jackson	2015	Plum Regal	+	–	H1
NC-157	Haywood	2015	Roma	+	+	H1
NC-180	Swain	2015	Plum Regal	+	–	H1
NC-189	Swain	2015	Plum Regal	+	–	H1
NC-193	Haywood	2015	Roma	+	–	H1
NC-204	Haywood	2015	Roma	+	+	H1
NC-232	Henderson	2016	Mountain Majesty	+	+	H1
NC-242	Henderson	2016	Mountain Merit	+	–	H1
NC-248	Henderson	2016	Plum Regal	+	–	H1
NC-252	Macon	2016	Plum Regal	+	+	H1
NC-264	Macon	2016	Plum Regal	+	–	H1
NC-280	Macon	2016	Tasti-Lee	–	–	H2
NC-282	Macon	2016	Tasti-Lee	–	–	H4
NC-283	Macon	2016	Tasti-Lee	+	–	H2
NC-289	Madison	2016	Resolute	+	+	H1
NC-293	Madison	2016	Resolute	+	–	H1
NC-304	Rowan	2016	Red Mountain	+	–	H2
NC-309	Rowan	2016	Red Mountain	+	+	H2
NC-310	Rowan	2016	Red Mountain	+	–	H2
NC-318	Rowan	2016	Picus	+	–	H1
NC-330	Rowan	2016	Picus	+	–	H2
NC-350	Haywood	2016	Mountain Magic	+	–	H2
NC-352	Haywood	2016	Mountain Magic	+	–	H2
NC-354	Haywood	2016	Mountain Magic	+	–	H2
NC-366	Haywood	2016	Mountain Magic	+	–	H1
NC-373	Henderson	2016	Tasti-Lee	+	–	H1

^a Tomato cultivar from which *X. perforans* strains were isolated.

^b Copper insensitivity of each strain; + indicates copper insensitive, and – indicates copper sensitive.

^c Streptomycin insensitivity of each strain; + indicates streptomycin insensitive, and – indicates streptomycin sensitive.

^d Haplotype of each strain based on BOX repetitive element PCR (BOX-PCR) assay.

Xs NII (i.e., *fusA* [accession no. KJ938581.1], *gapA* [accession no. KJ938588.1], *gltA* [accession no. KJ938595.1], *gyrB* [accession no. KJ938602.1], *lacF* [accession no. KJ938629.1], and *lepA* [accession no. KJ938636.1]) were downloaded from the NCBI. All gene sequences of NC strains were deposited to GenBank (accession nos. MH887556 to MH887747). Phylogenetic analysis based on concatenated gene sequences was analyzed using the maximum likelihood method within MEGA software version 7.0 (Kumar et al. 2016). The general time-reversible model with γ -distributed invariant sites was used to construct maximum likelihood trees with 1,000 bootstrap replicates. Haplotypes for each gene were determined based on the alignment files and the presence of single-nucleotide polymorphism (SNP) variations among haplotypes. The phylogenetic relationships of the strains along with other metadata, such as geographic location, BOX-PCR profile, race profile, and copper and streptomycin sensitivity, were analyzed using T-BAS v2 (Carbone et al. 2016).

RESULTS

Copper and streptomycin sensitivity assays. More than 95% (161 of 163 in 2015 and 123 of 127 in 2016) of strains were tolerant to copper in both years, whereas 45% (73 of 163) and 25% (32 of 127) of the strains were tolerant to streptomycin in 2015 and 2016, respectively (Fig. 2A). Data suggested that the distribution of copper-tolerant strains was uniform across year and counties, and 100% of the all locations had copper-tolerant strains in both years. However, the distribution of streptomycin-tolerant strains was not as uniform throughout the counties and years of samplings compared with copper resistance. A lower percentage of streptomycin-tolerant strains was observed in 2016 compared with 2015 (Fig. 2B and C). No streptomycin-tolerant strains were observed in Haywood County in 2016, but 83% of strains were tolerant to streptomycin in the same county in the year 2015. Strains susceptible to both copper and

streptomycin were not observed in 2015, but there were five strains susceptible to both copper and streptomycin in 2016 (Fig. 2D).

Genetic diversity analysis. Despite repeated experiments, 10 strains (NC-98, NC-175, NC-176, NC-182, NC-184, NC-198, NC-205, NC-234, NC-359, and NC-368) either did not amplify or yielded faint bands with BOX-PCR primer. Thus, BOX-PCR data for only 278 strains were included in this study. BOX-PCR fingerprinting generated 13 amplicons ranging from 300 bp to 2 kb, and four haplotypes (H1, H2, H3, and H4) were detected (Table 1). Among them, nearly 93% of the strains were H1, 6.5% of strains were H2, and one strain each was as an H3 and H4 haplotype (Tables 1 and 2). The H1 was detected in all eight counties in both years. The H2 was detected in Henderson County in 2015 and Macon, Rowan, and Haywood Counties in 2016 (Table 1). The one H3 strain was found in Madison County in 2015, whereas the H4 was isolated from Macon County in 2016 (Table 1).

Multiplex real-time qPCR. The qPCR assay of representative strains targeting the highly conserved *hrcN* gene identified *Xanthomonas* strains from tomato in NC as a single species, *X. perforans*. The detection of only green fluorescence except for reference strains Xe 157, Xv 144, and Xg GA2 confirmed that all of the strains examined were *X. perforans* (Supplementary Fig. S1).

Race identification. The majority of representative strains of *X. perforans* induced HR on all four pepper differentials (Table 3), indicating that they are not pepper races. Four strains (NC-79, NC-80, NC-112, and NC312) induced HR on FL 216 and belonged to race T3 (Table 3). The remaining strains did not induce HR on both HI 7998 and FL 216, indicating that they all belong to race T4. The Xp91-118 and Xe E3 strains and water reactions were as expected.

MLSA. Phylogenetic analysis revealed that all strains of *X. perforans* from NC were grouped with the reference *X. perforans* strains from FL (Fig. 3A). No variation was observed among sequenced *X. perforans* strains in *fusA*, *lacF*, and *lepA* gene sequences, whereas two, three, and four haplotypes were observed among the *gltA*, *gyrB*,

Fig. 2. A, Overall proportions and B and C, county-based proportions of *Xanthomonas perforans* collected from diverse tomato cultivars and farms across eight counties in North Carolina during B, 2015 and C, 2016 seasons that were tolerant to copper (Cu) and streptomycin (Sp). D, Proportions of strains of *X. perforans* observed during the 2-year survey that were tolerant to copper (Cu-T), sensitive to streptomycin (Sp-S), tolerant to both copper and streptomycin (Cu-T and Sp-T), sensitive to both copper and streptomycin (Cu-S and Sp-S), and tolerant to streptomycin (Sp-T) but sensitive to copper (Cu-S).

and *gapA* gene sequences, respectively (Table 4). Strain NC-282 showed an SNP variation at nucleotide sites 27 and 78, which placed it with *X. euvesicatoria*-type strain 85-10 (isolated from FL in 1985) based on *gltA* sequences (data not shown) (Timilsina et al. 2015). The *gyrB* gene differentiated strains of *X. perforans* into two groups: 23 strains grouped with *X. perforans* reference strains GEV839 (isolated from FL in 2012), 4-20 (isolated from FL in 2006), and *X. euvesicatoria*-type strain 85-10, whereas 18 strains grouped with *X. perforans*-type strains ICMP 16690 767 (isolated from FL in 1991), 17-12 (isolated from FL in 2006), and 91-118 (isolated from FL in 1991; data not shown) (Timilsina et al. 2015). The *gapA* gene differentiated strains of *X. perforans* into three groups: 24 strains grouped with *X. perforans* reference strain GEV839 and *X. euvesicatoria* reference strain 85-10, 9 strains were grouped with *X. perforans* reference strain 17-12, and 5 strains were grouped with *X. perforans* reference strains ICMP 16690 767, 4-20, and 91-118 (data not shown). The number of segregating sites and their nucleotide positions for these genes were estimated for each locus, and *gapA* had more mutation or loss and yielded more haplotypes compared with other loci (Table 4).

Based on the concatenated sequences of six loci, strains of *X. perforans* were divided into two groups: 41% of the strains were in G1, whereas 59% strains belonged to G2. G1 was further differentiated into three subgroups: G1-A (25% of total), G1-B (13%), and G1-C (3%) (Fig. 3A). These groups corresponded to four haplotypes (H1, H2, H3, and H4) based on seven segregating sites as detected on the concatenated sequences of six loci (Table 5). H1 corresponded to group 2, whereas H2, H3, and H4 corresponded to group 1 (Table 5). The H1 in group 2 differed from H2, H3, and H4 in group 1 by four, five, and seven SNPs, respectively. In group 1, H2 differed from H3 and H4 by one and three SNPs, respectively. The H3 differed from the H4 by two SNPs. Most NC strains were similar to the *X. perforans* T4 strain GEV839 isolated from FL in 2012 (Timilsina et al. 2016) and

were grouped in G2 (Fig. 3B). NC strain G1-A was similar to the *X. perforans* T4 reference strain 17-12 isolated from FL in 2006 (Fig. 3B) (Timilsina et al. 2015). Only four strains were grouped into G1-B and similar to the reference strain *X. perforans* 91-118 from FL in 1991 (Timilsina et al. 2015) and ICMP16690-767. The strain NC-282 was placed under G1-C (Fig. 3B). Whole-genome sequencing of representative strains confirmed the presence of the *CopLAB* gene cluster and the *avrXv4* gene but the absence of the *avrXv3* gene in those strains of *X. perforans* in NC (data not shown). Partial sequences were submitted to GenBank (accession nos. for the *copLAB* gene cluster: MK616431 to MK616466 and accession nos. for the *avrXv4* gene: MK583017 to MK583029).

DISCUSSION

We have combined conventional methods and multilocus sequencing approaches to rapidly gain information on phenotypic and genetic diversity of *Xanthomonas* strains causing bacterial spot disease of tomato in NC for the first time in a systematic manner. Assay results demonstrated that there is a widespread existence of copper resistance and a modest level of streptomycin resistance in *X. perforans* in NC. The higher incidence of bacterial strains resistant to copper than streptomycin observed indicates that there has been more selection pressures for copper resistance, consistent with continuous copper use in the early field season compared with only two applications of streptomycin (typically) in the greenhouse before field setting. In planta virulence on tomato and pepper differentials indicates that the majority of *X. perforans* strains belong to race T4 followed by race T3.

Widespread existence of copper-tolerant strains had been reported in Canada (Abbasi et al. 2015), Tanzania (Shenge et al. 2014), and the United States (Mixon 2012). Both copper- and

TABLE 2. Amplicon detection in each haplotype of *Xanthomonas perforans* strains collected in North Carolina in 2015 and 2016 generated by BOX repetitive element PCR (BOX-PCR)

Haplotype	Amplicon detected in each haplotype ^a													Year ^b	County ^c	No. of strains analyzed ^d
	1	2	3	4	5	6	7	8	9	10	11	12	13			
H1	1	1	1	1	1	1	1	1	1	1	1	1	1	2015, 2016	All 8 counties	258
H2	1	1	1	0	1	1	1	1	1	1	1	1	1	2015, 2016	Henderson, Macon, Haywood, Rowan	18
H3	0	0	0	0	0	0	0	0	1	1	1	1	1	2015	Madison	1
H4	0	0	0	0	0	0	0	0	1	0	1	0	0	2016	Macon	1

^a Here, 1 represents the presence of a particular amplicon, and 0 represents the absence of a particular amplicon in the given haplotype.

^b Year of isolation of the strains belonging to each haplotype.

^c Counties from where the strains were collected.

^d Total number of strains belonging to each haplotype.

TABLE 3. Identification of pathogenic races of representative strains of *Xanthomonas perforans* from North Carolina by inoculating on tomato and pepper differential cultivars and lines

Strains	Differential lines ^a							Result of pathogenicity assay	Race score
	HI 7998	FL 216	Bonny Best	ECW 10R	ECW 20R	ECW 30R	ECW		
NC-10, NC-11, NC-12, NC-22, NC-28, NC-37, NC-42, NC-47, NC-67, NC-87, NC-101, NC-110, NC-124, NC-126, NC-145, NC-157, NC-180, NC-189, NC-193, NC-204, NC-232, NC-242, NC-248, NC-252, NC-264, NC-280, NC-282, NC-283, NC-289, NC-293, NC-304, NC-309, NC-310, NC-318, NC-330, NC-352, NC-354, NC-366, NC-373	–	–	–	+	+	+	+	Tomato	T4
NC-079, NC-80, NC-112, NC-312	–	+	–	+	+	+	+	Tomato	T3
Xp 91-118 (control)	–	+	–	+	+	+	+	Tomato	T3
Xe E3 (control)	+	–	–	+	+	–	–	Tomato, pepper	T1
Water	–	–	–	–	–	–	–		

^a The + indicates induction of hypersensitive response on specific differentials, and the – indicates absence of hypersensitive response on specific tomato and pepper differential cultivars and lines. ECW, Early Calwonder.

streptomycin-tolerant *Xanthomonas* spp. populations were reported from Brazil (Araújo et al. 2012), the Caribbean and Central America (Bouzar et al. 1999), Ethiopia (Kebede et al. 2014), and the United States (Thayer and Stall 1962; Vallad et al. 2013). Notably, all fields sampled in NC harbored *X. perforans* strains tolerant to copper, whereas streptomycin-tolerant strains were not observed in some fields. Although Ritchie and Dittapongpitch (1991) reported 63% copper-tolerant and 30% streptomycin-tolerant strains of *Xanthomonas* species in NC, their findings were focused on pepper, and collections from tomato were insignificant.

Most of the genes associated with copper tolerance in plant-pathogenic bacteria are plasmid encoded (Basim et al. 2005; Behlau et al. 2012; Cooksey 1987; Richard et al. 2017). Copper tolerance genes in *X. campestris* pv. *vesicatoria* strains were detected on 188- to 200-kb self-transmissible plasmids pXvCu and pXV10a from FL (Bender et al. 1990; Stall et al. 1986). DNA hybridization indicated that the tolerance genes on pXV10A and pXvCu plasmids share nucleotide sequence homology and may have a common origin (Bender et al. 1990). Another 100-kb nonself-transmissible plasmid containing loci for copper tolerance was also discovered in a strain

Fig. 3. A, Phylogenetic analysis of *Xanthomonas perforans* strains from North Carolina using concatenated sequences of six genes: elongation factor G (*fusA*), glyceraldehyde-3-phosphate dehydrogenase A (*gapA*), citrate synthase (*gltA*), gyrase B (*gyrB*), ABC transporter sugar permease (*lacF*), and GTP binding protein (*lepA*). **B,** Combined visualization of counties, BOX-PCR fingerprints, copper and streptomycin sensitivity assays, and race profiles using T-BAS v2. Reference strains of *Xanthomonas euvesicatoria*, *Xanthomonas gardneri*, *X. perforans*, and *Xanthomonas vesicatoria* were downloaded from the Plant Associated and Environmental Microbes Database and the NCBI database. Values indicate bootstrap values for each branch expressed as percentages. **A,** The scale bar indicates the number of substitutions per site. **B,** Branches are shaded to indicate four haplotypes generated from multilocus sequence analysis (MLSA), and rings indicate the metadata. The six housekeeping gene sequences for reference strains Xp ICMP16690 (strain id 767), Xv-ATCC 11551 (strain 196), Xe 85-10 (strain 162), and Xg ICMP16689 (strain 766) were imported from the Plant Associated and Environmental Microbes Database, whereas the gene sequences for reference strains Xp GEV839 (accession no. JZVK000000000), Xp 91-118 (accession no. CP019725.1), Xp 17-12 (accession no. JZVH000000000), and Xp 4-20 (accession no. JZUZ000000000) were imported from their whole-genome sequences in the NCBI database. The individual gene sequences of atypical Nigerian strain Xs N11 (Xs N11; i.e., *fusA* [accession no. KJ938581.1], *gapA* [accession no. KJ938588.1], *gltA* [accession no. KJ938595.1], *gyrB* [accession no. KJ938602.1], *lacF* [accession no. KJ938629.1], and *lepA* [accession no. KJ938636.1]) were downloaded from the NCBI.

TABLE 4. Sequence statistics of six housekeeping genes in the *Xanthomonas perforans* strains from North Carolina

Gene sequence ^a	Nucleotides (no.)	Haplotypes (no.)	Segregating sites (no.)	Nucleotide position of segregating sites
<i>fusA</i>	591	1	0	n/a ^b
<i>gapA</i>	444	3	4	186, 195, 283, 429
<i>gltA</i>	501	2	2	27, 78
<i>gyrB</i>	411	2	1	39
<i>lacF</i>	408	1	0	n/a
<i>lepA</i>	390	1	0	n/a
Concatenated	2,745	4	7	777, 786, 874, 1020, 1062, 1113, 1575

^a *fusA*, elongation factor G; *gapA*, glyceraldehyde-3-phosphate dehydrogenase A; *gltA*, citrate synthase; *gyrB*, gyrase B; *lacF*, ABC transporter sugar permease; *lepA*, GTP binding protein.
^b n/a = not applicable.

from California (Cooksey 1987). Chromosome-encoded copper tolerance gene clusters have also been reported in a 7,652-bp *Xba*I/*Eco*RI chromosomal fragment in *X. campestris* pv. *vesicatoria* strain XvP26 from Taiwan (Basim et al. 2005). The sequencing of six tomato and pepper copper-tolerant pathogens belonging to different species of the genus *Xanthomonas* showed that all six strains carried the previously described copper resistance gene system *copLAB* carried on a conjugative plasmid (Richard et al. 2017). The plasmid was highly conserved among five strains (from 1955 to 2010), suggesting extensive events of horizontal gene transfer at the niche level (Richard et al. 2017). The transfer of plasmids between copper-tolerant and copper-sensitive strains through conjugation has also been demonstrated under laboratory conditions (Stall et al. 1986). Similarly, the presence of a copper-tolerant *Pseudomonas putida* strain with a plasmid-borne *cop* homolog in a commercial tomato seed lot also suggests that saprophytic bacteria could contribute to the spread of resistant bacterial populations between fields and different geographical areas (Cooksey et al. 1990). The findings of this study indicate that multiple and independent introductions of copper tolerance genes have taken place for Cu^R strains of *X. perforans*. The *copLAB* gene cluster was responsible for copper tolerance in several other xanthomonads (Behlau et al. 2011, 2012). We hypothesize that different selection pressures and genetic pools providing tolerance determinants and instability of some tolerance determinants (Diancourt et al. 2010) all could contribute to explaining the severe outbreaks of bacterial spot disease in NC. Although we examined only one source of copper (i.e., CuSO₄) for *X. perforans* strains, evaluating and comparing the efficacy of newly developed copper composites, such as core-shell copper, multivalent copper, and fixed quaternary ammonium copper FQ-Cu (Strayer-Scherer et al. 2018), and understanding mechanisms of copper tolerance in *X. perforans* strains from NC would be potentially useful to manage copper-tolerant strains and reduce bacterial spot incidence of tomato.

Evidence for both chromosomal and plasmid-mediated streptomycin tolerance in plant-pathogenic bacteria has been reported (Burr et al. 1988; Minsavage et al. 1990; Springer et al. 2001). Furthermore, streptomycin tolerance might occur at the cellular level either by an alternation of binding affinity of ribosomal proteins for the antibiotic by chromosomal mutations or by modification of the antibiotic by periplasmic enzymes (Davies 1986; Gale et al. 1972). We found that some strains are tolerant or insensitive to streptomycin. Thus, streptomycin tolerance was not as prevalent as copper tolerance. Although the frequency of copper and streptomycin tolerance genes was correlated with repeated loading of the selective chemical agents into the environment (Burr et al. 1988; Canteros 1999; Stall and Thayer 1962), reasons for the existence of inconsistent streptomycin tolerance over years, fields, and counties in *X. perforans* populations in NC are uncertain. We hypothesize that tolerance plasmid was introduced in seed sources, and the incidence of contaminated seed may vary by seed lot and year. In addition, tomato growers in NC usually purchase transplants from nurseries instead of growing seedlings themselves. It is likely

that copper- and streptomycin-tolerant strains have been introduced into different tomato-growing areas in NC through common transplant nurseries. The prevalence of bacterial spot strains and the incidence of tolerance to copper and streptomycin suggest that future research is needed to determine if the strains can persist in the tomato ecosystem from one year to the next or if seed and/or transplants are the primary sources of inoculum and the main carriers for long-distance movement.

Both BOX-PCR fingerprinting and MLSA assays revealed low genetic diversity within *X. perforans* in NC. The MLSA of *X. perforans* populations from NC based on six genes showed the presence of two distinct groups and seemed to be clonal within the lineage, which is consistent with the groups observed in *X. perforans* in FL (Timilsina et al. 2015). The largest group in the phylogenetic tree that contained 59% of *X. perforans* strains of NC included the T4 strain GEV839 isolated from FL in 2012, suggesting that the majority of *X. perforans* strains in NC are in the same phylogenetic group as that of *X. perforans* strains in FL (Timilsina et al. 2016). Similarly, 25% of *X. perforans* strains in NC were grouped with *X. perforans* T4 strain Xp17-12 isolated from FL in 2006. Few *X. perforans* strains in NC (13%) were grouped with *X. perforans* T3 strain 91-118 and ICMP166690-767. The strains NC-12, NC-42, and NC-204, which were detected as race T4 based on HR assay, were grouped with race T3-type strains in the phylogenetic tree. In addition to strain-level variation, allelic diversification in type III effectors might resolve their race profile (Abrahamiana et al. 2018). However, both results clearly indicate the presence of race T3 strains in NC. The strain NC-282 with Ha4 haplotype based on BOX-PCR was also grouped separately from the rest of the strains in the phylogenetic tree constructed using concatenated sequences of six housekeeping genes in this study. This strain had *gltA* gene sequence similar to that of *X. euvesicatoria*, whereas the other five gene sequences were similar to that of *X. perforans*. Intriguingly, the strain NC-282 was sensitive to both copper and streptomycin. Previously, one atypical Nigerian strain had also been reported that had *fusA* and *gyrB* genes identical with *X. perforans* but *gltA* and *lacF* genes identical to *X. euvesicatoria* (Timilsina et al. 2015). Overall, MLSA revealed that *X. perforans* strains in NC were similar to *X. perforans* strains in FL, confirming that *X. perforans* strains from NC might have a common origin to that of FL strains. Results in this study show that genomic fingerprints generated by BOX-PCR give a high and rapid level of taxonomic resolution currently achievable by PCR methods (Louws and Cuppels 2001; Louws et al. 1995). It is a tool to rapidly “bin” strains into genetic groups, and then, representative strains can be selected for additional in-depth work. Thus, the BOX-PCR method allows us to differentiate the strains even at the intraspecies level, and this technique is more suitable for rapid discrimination of a high number of newly isolated strains of plant pathogens. MLSA indicates the sequencing and concatenation of the housekeeping genes and provides a more robust tree topology and an improved understanding of speciation events in comparison with a tree based only on

TABLE 5. Haplotypes and single-nucleotide polymorphism variation among *Xanthomonas perforans* strains from North Carolina based on concatenated sequences of six housekeeping genes: elongation factor G (*fusA*), glyceraldehyde-3-phosphate dehydrogenase A (*gapA*), citrate synthase (*gltA*), gyrase B (*gyrB*), ABC transporter sugar permease (*lacF*), and GTP binding protein (*lepA*)^a

Haplotypes	Nucleotide position (bp)							Group	Strains
	777	786	874	1020	1062	1113	1575		
Ha1	T	G	A	G	A	T	T	G2	MRS-30P-11, NC-28, NC-47, NC-67, NC-87, NC-126, NC-145, NC-189, NC-193, NC-232, NC-242, NC-248, NC-252, NC-289, NC-309, NC-318, NC-352, NC-354, NC-373, Xp-GEV839
Ha2	G	C	G	G	A	T	C	G1-A	NC-10, NC-11, NC-14, NC-22, NC-101, NC-304, NC-330, NC-350, Xp 17-12
Ha3	G	C	G	C	A	T	C	G1-B	NC-12, NC-42, NC-112, NC-204, Xp-91-118, Xp ICMP16690-767
Ha4	G	C	G	C	C	C	C	G1-C	NC-282

^a Haplotypes are based on multilocus sequencing of six housekeeping genes: *fusA* (nucleotide positions 1 to 591), *gapA* (592 to 1035), *gltA* (1036 to 1536), *gyrB* (1537 to 1974), *lacF* (1948 to 2355), and *lepA* (2356 to 2745).

BOX-PCR; therefore, we focus on the MLSA data and not on the BOX-PCR data.

Given that we are working solely with *X. perforans* strains, our main interest was strictly in tomato as a host. Thus, one of our goals of this research was to gain insight into the current race profiles of *X. perforans* and their distributions in major tomato-producing areas in NC. Based on the HR host assay, 91 and 9% of them belonged to races T4 and T3, respectively, which indicate that race T3 has not been completely replaced by race T4 in NC as observed in FL (Vallad et al. 2013). *X. perforans* race T3 was identified first in 1991 in FL (Hert et al. 2005; Tudor-Nelson et al. 2003). Later, strain *Xp4B*, a tomato race T4, was identified in 1998 from FL, which carries a mutation in the *avrXv3* gene. Studies conducted between 2006 and 2012 identified that the majority of race T4 strains carried either frameshift mutations or transposon insertions in *avrXv3* (Horvath et al. 2012). We hypothesize that the emergence of *X. perforans* race T4 in NC could be owing to the mutations in the *avrXv3* gene (Astua-Monge et al. 2000; Horvath et al. 2012). In another study, whole-genome sequencing of the strains used in this study was completed. To genetically confirm tomato race T4, a subset of strains used for MLSA was present in the *avrXv4* gene, which also confirmed the absence of the *avrXv3* gene in those strains. The absence of diversity in NC strains suggested that the *X. perforans* tomato race T4 is an established population in the Southeastern United States. This similar observation was found in Taiwan (Burlakoti et al. 2018), where the majority of the characterized *X. perforans* strains were identified as race T4. However, only one T4 strain was reported from Ethiopia in 2014 (Kebede et al. 2014). The shifts to new strains and the rapid increase of clonal strains in diverse geographic regions over the years are likely owing to strain movement via global trade. Although our study demonstrated the absence of diversity in *X. perforans* populations from tomato, monitoring bacterial populations over time and space is imperative to generate timely information about the genetic structure of pathogen populations that is relevant to breeding programs and resistance deployment.

In summary, we found that *X. perforans* race T3 is rarely present, whereas race T4 is widely distributed in the major tomato-growing regions of western NC. The majority of *X. perforans* strains were tolerant to copper-based bactericide. These results suggest that the use of copper products to manage bacterial spot in tomato would be ineffective in NC and the Southeastern United States. Our study demonstrated low phenotypic and genetic diversity of *X. perforans* populations in NC. Race T4 was common in several tomato-growing areas in NC, and thus, short-term control strategies should target this population. Tomato growers should implement best-management practices, such as the use of plant activators that induce systemic-acquired resistance (e.g., Acibenzolar-S-methyl or Actigard 50WG; Syngenta Crop Protection), use of biopesticides, use of *X. perforans*-free seed, and host plant resistance (Bhattarai et al. 2017; Louws 2018; McGrath and LaMarsh 2014; Miller et al. 2014; Vallad and Goodman 2004). Effector-based breeding strategies (Timilsina et al. 2016) supported by new genomic tools for deploying multiple *R* genes in adapted tomato varieties should be exploited to combat emerging races of *X. perforans* in NC and the Southeastern United States as a long-term strategy. Moreover, knowledge of the effector load in the *X. perforans* populations will also guide strategies for achieving durable resistance to *X. perforans* (Schwartz et al. 2015).

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