BIOLOGY AND MANAGEMENT OF BACTERIAL SPOT OF TOMATO IN OKLAHOMA

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Abstract: In surveys of tomato fields during 2018 and 2019, bacterial spot was the most frequent foliar disease (72% of fields) followed by Septoria leaf spot (7%), and early blight (7%). Isolates of *Xanthomonas* spp. from 1998-2014 and those from 2018-2019 were identified to species using sequences of a 420 bp region of the *hrp* gene cluster amplified by the RST65/RST69 primers. The 1998-2014 isolates were *X. perforans* (n=17), *X. gardneri* (n=1), and *X. vesicatoria* (n=1); while the 2018-2019 isolates were also mostly *X. perforans* (n=42) followed by *X. euvesicatoria* (n=7) and *X. gardneri* (n=2). Races of *X. perforans* isolates included race T3 (n=15) and race T4 (n=6). Applications of bacteriophage, *Bacillus amyloliquefaciens*, acibenzolar-s-methyl, and copper bactericides were evaluated in field trials in 2018 and 2019. All treatments except *B. amyloliquefaciens* reduced disease levels, but none were as effective as copper hydroxide alone and a copper-mancozeb tank-mix. Alternatives to copper programs generally provided variable control with bacteriophage being the most consistent. However, none of the bactericide treatments increased yield compared to the non-treated control. In the absence of highly effective bactericide treatments and resistant cultivars, other preventive strategies such as sanitation, volunteer management, and crop rotation are critical to limit pathogen carryover and prevent early disease development. Race structure is diverse within the state. Host plant resistance should combine multiple resistance genes to be of greatest benefit to producers in the region.

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CHAPTER I

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop grown extensively in the U.S. for both processing and fresh market. Annual production of fresh market tomato in the U.S. is valued at over 1 billion dollars (USDA-NAS, 2019). In Oklahoma, fresh-market tomato is an important crop for direct market vegetable farmers and gardeners (Brandenberger et al., 2014; Hillock & Rebek, 2013). The lengthy growing season in central and eastern Oklahoma of between 200 and 230 frost-free days provides tomato producers the opportunity for a long harvest season that typically begins in late June and continues into September (Brandenberger et al., 2014). This extended harvest season comes with the challenge of managing foliar disease. Several common foliar diseases of tomato in Oklahoma are Septoria leaf spot (*Septoria lycopersici*), early blight of tomato (*Alternaria solani*), bacterial speck (*Pseudomonas syringe* pv. *tomato*), and bacterial spot (*Xanthomonas* spp.) (Damicone & Brandenberger 2016; Damicone & Brandenberger 2017).

Effective control of foliar disease hinges on an accurate diagnosis of the causal agent(s) involved. This is because bacteria that cause foliar disease in tomato are

managed differently than those caused by fungi (Jones et al., 1991). In recent years foliar disease of tomato in Oklahoma has been diagnosed as early blight in most instances. A survey of foliar disease of tomato in Oklahoma is needed to better understand the risk that each disease poses to producer's tomato crops. Bacterial spot of tomato is known to occur in Oklahoma but since being reclassified from *X. campestris* pv. *vesicatoria* in 2004, the cause of bacterial spot of tomato in Oklahoma has been unknown (Bender et al., 1990; Jones et al., 2004). Identifying causal agents based on their species and race should inform breeding efforts to develop genetic resistance to manage bacterial spot of tomato in Oklahoma. The objectives of the work described in chapter III are as follows: i) determine the incidence of bacterial spot relative to other foliar diseases of tomato in Oklahoma; ii) Identify the reclassified species of *Xanthomonas* causing bacterial spot of tomato in Oklahoma; iii) characterize race structure of *Xanthomonas* causing bacterial spot of tomato in Oklahoma.

The application of copper bactericides in a preventative program has been used extensively since the early 1800's to control bacterial plant pathogens (Obradovic et al., 2008). Unfortunately, some strains of the xanthomonads causing bacterial spot became tolerant to copper in the 1980's, which reduced bactericidal efficacy and prompted a search for alternative control measures (Marco & Stall, 1983; Obradovic et al., 2008). Isolates of *X. campestris* pv. *vesicatoria*, from North Carolina in 1986-1990 had a high frequency of copper and streptomycin resistance (Ritchie & Dittapongpitch, 1991). Improved bactericide efficacy through the combination of copper bactericide and ethylene-bis-dithiocarbamate fungicide has been demonstrated (Conover & Gerhold, 1981; Marco & Stall, 1983). However the efficacy of copper-mancozeb applications may

be inadequate when conditions favor severe disease caused by copper tolerant strain of *X. perforans* (Strayer-Scherer et al., 2018). In addition to copper bactericides, antibiotics have been used for control of bacterial diseases (Sundin & Wang, 2018). Observations of antibiotic resistance developing in plant pathogenic bacteria, fears over resistance being transferred to human pathogenic bacteria, and the implications of such a transfer to human medicine have resulted in the limited usage of antibiotics in U.S. crop production. However, the antibiotic streptomycin is registered for use in control of bacterial spot in the greenhouse production of tomato and pepper transplants (McManus et al., 2002).

Novel approaches in the control of bacterial diseases include application of viral bacteriophages that kill bacteria and competitive displacement by biocontrol agents (Obradovic et al., 2008). The efficacy of biological control agents can be impacted by environmental conditions, necessitating climate and region specific evaluation of commercially available products. Another alternative management tactic is the use of a chemical that induces systemic resistance to disease in the plant. Acibenzolar-s-methyl is a widely used systemic acquired resistance inducer that has been shown to be effective at preventing diseases caused by bacteria, fungi, and viruses (Oostendorp et al., 2001). Copper-resistant strains of *X. campestris* pv. *vesicatoria* have been isolated from tomato trial plots in eastern Oklahoma (Bender et al., 1990). Both the challenge of controlling copper-tolerant *Xanthomonas* strains, and the need to integrate control measures to prevent the selection of tolerant strains, warrant the evaluation of alternative control measures. The objective of the research found in Chapter IV was to determine the efficacy of bactericides that utilize various modes of action in the management of bacterial spot of tomato.

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CHAPTER II

LITERATURE REVIEW

Taxonomy of tomato

The cultivated tomato (*Solanum lycopersicon* L.) is an herbaceous perennial that is grown as an annual crop (Peralta & Spooner, 2007). Tomato is a member of the plant family, *Solanaceae*, which is shared by several other plant species of horticultural importance including potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.), tobacco (*Nicotiana tabacum* L.), and petunia (*Petunia* spp. Juss.). The wild ancestors of the cultivated tomato are native to modern day Ecuador, Peru, and northern Chile, and have been adapted to diverse climactic conditions from arid coastal climates near sea level to Andean highlands at elevations as great as 3,300 m (Peralta & Spooner, 2000). Until recently, a lack of consensus over the taxonomic classification of tomato left ambiguity around whether it belonged in the genus *Solanum* or *Lycopersicon*. Linnaeus originally placed tomato in *Solanum* on the grounds of botanical similarity with other members of the genus, particularly potato. A recent

molecular analysis of chloroplast DNA restriction site data has provided support for a common phylogeny between tomato and potato and has reinforced Linnaeus original assertion that the cultivated tomato belongs in the genus *Solanum* (Peralta & Spooner, 2007).

Cultivation of tomato

A well-drained soil with a texture of sandy to sandy-loam and a pH of 6.0 to 6.8 is ideal for optimum plant health in tomato (Brandenberger et al., 2014). Use of transplants in commercial field production of tomato is standard practice. Ideal tomato transplants are immature, 6 to 8 inches in height, and preconditioned to the prevalent environmental conditions of the production environment at the time of transplanting (Kelley & Boyhan, 2017). Transplanting rather than direct seeding can help plants reach maturity earlier in the spring when temperatures are more favorable for efficient pollination and fruit set. Tomato is primarily self-pollinated in nature (Kelley & Boyhan, 2017). Wind currents ensure effective pollination in field grown tomato, while tomatoes grown in protected culture (greenhouse, unheated tunnel, etc.) benefit from the activity of bumblebees (*Bombus impatiens* Cresson) ensuring optimal pollination and fruit set (Morandin et al., 2001; Kelley & Boyhan, 2017). Tomato plants growing in environments with day temperatures that exceed 34 °C or night temperatures that exceed 21 °C can experience a sharp reduction in fruit set due to blossom drop (Brandenberger et al., 2014). Tomato varieties exhibit one of the following growth habits, determinate, semideterminate, or indeterminate (Elkind et al., 1991). Determinate tomato varieties terminate growth of the shoot apical meristem in the production of an inflorescence. Determinate varieties have

been described as a plant with five or less inflorescences on the main stem and a height that is genetically determined (Elkind et al., 1991; Macarthur, 1932) Semideterminate plants also exhibit a height that is genetically predetermined but in contrast to growth of a determinate variety, semideterminates produce six or more inflorescences on the main stem (Elkind et al., 1991). Indeterminate varieties demonstrate continuous shoot growth and have no genetically predetermined height. Indeterminate varieties require staking and pruning of shoots, while determinate varieties are staked and pruned at the choice of the producer (Brandenberger et al., 2014). In general, pruning is used to balance the vegetative and reproductive growth of tomato. Pruning can be an effective method for increasing fruit size and can reduce disease by improving air movement through the canopy (Brandenberger et al., 2014; Kelley & Boyhan, 2017).

Tomato production

In 2018, the United States produced a total of 12.5 million tonnes of tomatoes (USDA-NAS, 2019). In the United States tomatoes are produced commercially for both fresh market and processing uses (USDA-NAS, 2019). According to the Food and Agriculture Organization of the United Nations (FAO) between 2000 and 2018 the average national production in the top three tomato producing countries of China, the United States, and India, was 42.1, 13.3, and 13.0 million tonnes, respectively (FAO-STAT, 2020). In the United States tomatoes are grown extensively for processing, which accounts for over 90% of total production. California produces nearly all of the United States processing tomatoes with more than 11.1 million tonnes produced in 2018 (USDA-NAS, 2019). Florida produced nearly 362,000 tonnes of fresh-market tomatoes in 2017

while California produced nearly 332,000 tonnes the same year (USDA-NAS, 2019). In Oklahoma, commercial tomato production is primarily for local markets and fruit are often marketed directly to consumers (Brandenberger et al., 2014).

Systematics of bacterial spot of tomato

Doidge (1921) described a 'canker' disease of tomato that affected the fruit surface with scab-like lesions at a market in Pretoria, South Africa, and named the causal agent, *Bacterium vesicatorium*. At around the same time in Indiana, U.S.A. the tomato canning crops of 1918 and 1919 were impacted by what Gardner and Kendrick described as a disease causing lesions or 'spots' on the foliage as well as scab-like lesions on the fruit (Gardner & Kendrick, 1921) Gardner and Kendrick inoculated the yellow bacteria isolated from the tomato crop in Indiana to pepper foliage and observed symptoms consistent with what had previously been described as 'bacterial spot of pepper'. They attributed the causal organism of 'bacterial spot of pepper' to also be the cause of the observed disease of tomato, and named the disease 'bacterial spot of tomato' (Gardner & Kendrick, 1921). The cause of bacterial spot of tomato and pepper was briefly classified as *Pseudomonas vesicatorium* by Stevens (1925), and then reclassified as *Phytomonas vesicatoria* by Bergey et al. (1930).

When proposing the genus *Xanthomonas*, Dowson (1939) reclassified the causal agent of bacterial spot of tomato and pepper as *Xanthomonas vesicatoria*, which would remain the classification for decades. Dye and Lelliot (1974) reduced the number of species in the genus *Xanthomonas* and in the process reclassified *X. vesicatoria* to *X. campestris*. Young et al. (1978) proposed that the species *Xanthomonas campestris* be

further classified into pathovars and after subsequent acceptance by Dye et al. (1980) the causal agent of bacterial spot of tomato and pepper became classified as *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. Within *X. campestris* pv. *vesicatoria* Stall et al. (1994) and Vauterin et al. (1995) described two distinct groups distinguished by the strongly amylolytic and pectolytic characteristics of group B, which contrasted with the weak capacity of group A to hydrolyze starch and pectin. Group B was reclassified by Vauterin et al. (1995) as *X. axonopodis* pv. *vesicatoria*.

There are currently four known causal agents of bacterial spot of tomato (*Xanthomonas euvesicatoria, X. vesicatoria, X. perforans* and *X. gardneri*) (Jones et al., 2004). Jones et al. (2004) reclassified the strains into four species using a combination of phenotypic and DNA homology data. Jones et al. (2004) retained *X. vesicatoria*, which had been previously described by Vauterin et al. (1995) on the basis of its carbon source utilization pattern. Jones et al. (2004) proposed *X. euvesicatoria* to classify the strain originally isolated by Doidge in 1921. This organism is described as weakly amylolytic and pectolytic, and unlike other bacterial spot pathogens, utilized cis-aconitic acid in all strains tested. The strain reclassified by Jones et al. (2000) as *X. gardneri* was first isolated by Šutic (1957) in Yugoslavia and named *Pseudomonas gardneri*. This organism was considered by Dye (1966) to be '*Xanthomonas vesicatoria'* on the grounds of morphological and biochemical tests. Jones et al. (2004) later found *X. gardneri* to be distinguishable from other tomato and pepper pathogenic species by its non-utilization of certain carbon sources, especially dextrin, which is utilized by all other bacterial spot

pathogens. Phenotypically, *X. gardneri* is weakly amylolytic and pectolytic, which distinguishes this species from *X. vesicatoria* and *X. perforans*.

Jones et al. (2004) describes *X. perforans* as producing lesions that rapidly become necrotic and whose centers fall out leaving a shot hole symptom on infected leaves. *X. perforans* was first identified from tomato fields in Florida in the 1990's (Jones et al., 1995; Jones et al., 2004). Its original classification as a T3 strain of *X. campestris* pv. *vesicatoria* was based on differential reaction to resistant tomato cultivars and the strong amylolytic and pectolytic activity of the strain (Jones et al., 1995). Each of the four *Xanthomonas* spp. causing bacterial spot have been shown to have less than 70 % DNA relatedness with one another based on DNA:DNA hybridization (Jones et al., 2004).

Biology of bacterial spot of tomato

Increase and within field spread of bacterial spot occurs when conducive environmental factors are present, such as high humidity, temperatures ranging from 24 to 30 °C, and wind-driven rain events (Momol et al., 2002). Upon contact with the plant surface, the pathogen enters through wounds as well as natural openings, such as stomata (Momol et al., 2002). *Xanthomonas* species pathogenic to tomato produce symptoms, which include circular necrotic lesions or 'spots', on the foliage, stem, sepals and fruit (Jones et al., 2004; Jones et al., 1991). Leaf lesions can develop to 3 mm in diameter and coalesce until the foliage becomes entirely necrotic (Jones et al., 1991). Fruit lesions, which may or may not be present, appear as blisters or scabs that are sunken on green fruit and slightly raised on ripening fruit (Jones et al., 1991). The bacteria can be readily

cultured from lesions on nutrient agar, on which they produce circular, mucoid, yellow colonies (Jones et al., 1991).

Variation in symptoms and aggressiveness among the bacterial spot pathogens has been observed (Potnis et al., 2015). In 2010, an outbreak of bacterial spot affecting commercial fields of canning tomatoes in Ohio and Michigan was primarily caused by *X. gardneri*, which produced significant fruit spotting symptoms (Ma & Miller, 2011). *X. gardneri* has also been identified as a primary cause of bacterial spot of tomato in Pennsylvania in 1995, and has since reoccurred there in 2001, and every year between 2003 and 2009 (Kim et al., 2010). *X. gardneri* regularly causes large scab-like lesions on tomato fruit as well as causing greater disease severity at lower temperatures (20 °C) compared to the other bacterial spot pathogens (Araujo et al., 2010; Ma & Miller, 2011). By contrast, *X. perforans* is most aggressive at warmer temperatures (30 °C) (Araujo et al., 2010). A virulent isolate of *X. perforans* race 4 was recovered from commercial tomato fields in southern Louisiana in 2013 and 2014 (Lewis Ivey et al., 2016). Tomato race 4 of *X. perforans* has been reported to be the dominant pathogen causing bacterial spot of tomato in Florida and North Carolina (Adhikari et al., 2019; Horvath et al., 2012). In 2017, seventeen copper tolerant strains of *X. perforans* were isolated from commercial tomato fields in central Mississippi (Abrahamian et al., 2018). Survey data suggests that *X. perforans* has become a common causal agent of bacterial spot of tomato throughout the southern United States (Abrahamian et al., 2018; Adhikari et al., 2019; Horvath et al., 2012; Lewis Ivey et al., 2016).

Management of bacterial spot of tomato

Chemical control measures

Control measures have historically depended on streptomycin or copper-based bactericidal compounds, both of which have decreased in efficacy with the emergence of resistant strains of *Xanthomonas* spp. (Marco & Stall, 1983; Potnis *et al*., 2015; Stall & Thayer, 1962). The tank-mixing of copper formulations with mancozeb or maneb fungicide has been shown to improve the chemical control of bacterial spot and is a standard treatment for commercial tomato growers (Conover & Gerhold, 1981). However, results of a trial conducted in eastern Oklahoma in 1987 reported that mancozeb-cupric hydroxide applications to control a Cu tolerant strain of *X. campestris* pv. *vesicatoria* were unable to improve yield or reduce disease incidence compared to the untreated control (Bender et al., 1990). Chemical protectants may fail to provide a level of control that is adequate to prevent losses when environmental conditions are optimal for bacterial spot development (Momol et al., 2002). Additionally, the extensive use of copper compounds as bactericides can have deleterious effects on the production environment because copper ions may build up to phytotoxic levels in the soil (Momol et al., 2002).

Biological control agents

Ji et al. (2006) tested foliar applications of *Pseudomonas syringae* and *P. putida* strains in various combinations with the plant growth promoting rhizobacteria (PGPR) *P. fluorescens* and *Bacilus pumilus* for control of *Xanthomonas campestris* pv. *vesicatoria* (Ji et al., 2006). The biological control agent, *P. syringae* strain Cit7 was found to

significantly decrease bacterial spot severity compared to the non-treated control and provided control that was statistically equivalent to a copper and mancozeb spray program (Ji et al., 2006). Interestingly, combinations of PGPR and biological control agents showed no consistent improvement across locations in controlling the disease when compared with biological control agent alone (Ji et al., 2006).

Bacterial viruses or bacteriophages specifically targeting the bacterial spot pathogen have been tested alone and in combination with the systemic acquired resistance inducer, acibenzolar-s-methyl for bacterial spot control (Balogh et al., 2003; Flaherty et al., 2000; Obradovic et al., 2004). Flaherty et al. reported a 17% reduction in disease severity of plants treated with bacteriophage verses an 11% reduction in plants treated with copper-mancozeb (Flaherty et al., 2000). Bacterial spot control with bacteriophage was improved by formulating them in skim milk, casecrete, or pregelatinized corn flour, and by timing applications to occur in the evening, rather than during the morning hours (Balogh et al., 2003).

Systemic acquired resistance inducers

Louws et al. (2001) evaluated the effect of the systemic acquired resistance inducer, acibenzolar-s-methyl on fruit yield and disease severity of tomato in field experiments carried out in Alabama, Florida, North Carolina, Ohio, and Ontario. In 13 of 15 experiments, acibenzolar-s-methyl reduced bacterial spot severity on tomato foliage compared with the non-treated control. However, the average total fruit yield across all 15 experiments was not significantly different between the acibenzolar-s-methyl treated plots, the standard bactericide program (copper hydroxide + mancozeb or maneb) treated

plots, and the non-treated control. The amount of extra large fruit harvested was reportedly less in the acibenzolar-s-methyl treated plots, which suggests a potential impact to fruit quality from treatment with acibenzolar-s-methyl. Additionally, acibenzolar-s-methyl was used in combination with a standard bactericide program to determine if an additive effect exists. Acibenzolar-s-methyl applied alone was as effective at reducing bacterial spot disease as acibenzolar-s-methyl combined with the standard bactericide program.

Host plant resistance

Host plant resistance to bacterial spot in tomato has been extensively studied but has not been developed in commercial tomato varieties (Timilsina et al., 2016). In contrast, resistance to bacterial spot has become widely available in improved varieties of pepper (*Capsicum annuum*) (Keinath, 2017). Several sources of monogenic resistance to bacterial spot have been identified in tomato, which has provided the ability to characterize the bacterial spot pathogen(s) by race (Astua-Monge et al., 2000; Jones, Stall, & Bouzar, 1998).

The pathogens that cause bacterial spot of tomato can be differentiated into one of four pathogen races, T1 to T4 (Astua-Monge et al., 2000; Jones, Stall, & Bouzar 1998). Race differentiation is dependent on the presence of functional avirulence gene or effector in the pathogen, which interacts with a resistance gene found in the plant to produce a resistant hypersensitive reaction (Astua-Monge et al., 2000; Jones, Stall & Bouzar, 1998). Jones and Scott (1986) characterized the tomato line, Hawaii 7998, as the first cultivar with resistance to bacterial spot of tomato being caused by a hypersensitive

response (Jones & Scott 1986; Vanderplank, 1963). Strains of *Xanthomonas* spp. capable of initiating a hypersensitive response on Hawaii 7998 were classified as race T1 (Jones et al., 1998). A second source of major gene resistance was identified in the cultivar Hawaii 7981 (Jones et al., 1995). Strains capable of initiating a hypersensitive response in Hawaii 7981 have been designated as race T3 (Jones et al., 1995; Jones et al., 1998). Strains causing a compatible reaction on the cultivars Bonny Best, Hawaii 7998, and Hawaii 7981 are designated as race T2. Most recently, race T4 was identified that elicited a hypersensitive response by the resistance gene *Xv4* in LA716 a wild relative of tomato, *Lycopersicon pennellii* (Astua-Monge et al., 2000).

Table 2.1 Differential reactions of races of xanthomonads on tomato resistance genes

Effector					
Gene	Bonny best none	Hawaii 7998 (*rx1*,*rx2*,*rx3*)	Florida 216 (*Xv3*)	LA 716 (*Xv4*)	Race
AvrRxv	+	HR	+	+	T1
None	+	+	+	+	T2
AvrXv3, *AvrXv4*	+	+	HR	HR	T3
AvrXv4	+	+	+	HR	T4

Adapted from tables and data reviewed by Stall, Jones, and Minsavage (2009)

The shifting of races within the pathogen populations to overcome major resistance genes has posed a challenge to breeders working to develop host plant resistance that is widely adapted and stable (Bhattarei et al., 2017; Jones et al., 1998). Bhattarai et al. (2017) identified sources of resistance to *X. perforans* race T4, the most prevalent race in North Carolina, by screening tomato genotypes that included breeding lines developed at North Carolina State University and described the common parentage of these bacterial spot resistant lines as including *Solanum pimpinellifolium* L3707 in

their pedigrees suggesting that multigenic resistance may be found in this genotype. Stall et al. (2009) suggested that future development and deployment of resistance to bacterial spot focus on combining multiple major resistance genes into one genotype, combining minor or quantitative resistance genes into one genotype, and growing mixtures of tomato genotypes with different resistance genes, called multilines, to reduce selection pressure on bacterial spot pathogens.

Cultural control practices

Cultural practices such as disposing of culled fruit, removal or tillage of plant debris, rotation to a non-host, and elimination of volunteer tomato seedlings play an important role in breaking the disease cycle (Momol et al., 2002). The potential of crop residues, weedy hosts, volunteer seedlings, and contaminated seed to act as primary inoculum for bacterial spot in Florida has been explored (Jones et al., 1986). It was determined that residue from a previous winter crop is unlikely to provide sufficient inoculum to cause disease in the following winter production cycle (Jones et al., 1986). Conversely it was demonstrated that overwintering of *Xanthomonas vesicatoria* in tomato residue in Indiana did serve as primary inoculum for infection in the subsequent spring crop, which suggests that lower temperatures increase pathogen carryover (Peterson, 1963). On Florida, weedy host species are likely not a primary contributor to bacterial spot epidemics and contamination levels are very low in tomato seed lots (Jones et al., 1986). Good field sanitation and use of disease free transplants are among the most important tools to prevent the introduction of bacterial spot into the production environment (Momol et al., 2002).

Bacterial spot of tomato in Oklahoma

Bacterial spot is a common foliar disease of tomato in Oklahoma (Damicone & Brandenberger, 2017). Tomatoes are grown in Oklahoma during the warmest summer months of July and August when daytime high temperature averages exceed 90 °F (32 °C). High relative humidity and rainfall often occur during May and June in central Oklahoma, which coincides with crop establishment and early harvest. Abundant moisture in the early cropping period coupled with consistently high temperatures later in the growing season provides a highly conducive environment for the growth and spread of bacterial spot (Jones et al., 1991). A study evaluating spray programs for control of bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) and bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato in Oklahoma has shown that yield of marketable fruit increased with the use of weekly applications of copper or copper + mancozeb (Damicone & Trent, 2003). However, results of an earlier trial in Oklahoma showed that mancozeb-cupric hydroxide applied to control a copper-tolerant strain of *X. campestris* pv. *vesicatoria* did not improve yield or reduce disease incidence compared to the untreated control (Bender et al., 1990). Xanthomonads that cause bacterial spot are genetically diverse and capable of overcoming chemical control and host plant resistance (Bender et al., 1990; Stall et al., 2009; Sundin & Bender, 1995). In order to effectively address this challenge for Oklahoma tomato producers more needs to be known about the biology of the bacterial spot of tomato pathogens found in Oklahoma and the efficacy of management tactics employing diverse modes of action against the pathogens.

This research seeks to understand the biology of bacterial spot of tomato, its distribution in Oklahoma, and the efficacy of various management strategies.

The objectives of the research in Chapter III were: i) determine the incidence of bacterial spot of tomato relative to other major foliar diseases of tomato in Oklahoma; ii) identify the reclassified species of *Xanthomonas* causing bacterial spot of tomato in Oklahoma; iii) characterize the local races of xanthomonads that cause bacterial spot of tomato. The objective of the research in Chapter IV was: i) describe the efficacy of bactericides that utilize various modes of action in the management of bacterial spot.

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CHAPTER III

DISTRIBUTION AND BIOLOGY OF BACTERIAL SPOT OF TOMATO IN OKLAHOMA

ABSTRACT

A survey of foliar diseases of tomato was conducted in Oklahoma during 2018 and 2019. Bacterial spot (*Xanthomomnas* spp.) occurred most frequently (72 % of sites) followed by early blight (*Alternaria solani*) and Septoria leaf spot (*Septoria lycopersici*), which both occurred at 7 % of sites. Isolates of *Xanthomonas* spp. from 1998-2014 and those from 2018-2019 were identified to species using sequences of a 420 bp region of the *hrp* gene cluster amplified by the RST65/RST69 primers. Isolates from 2018 were also identified as *X. euvesicatoria* by amplification of a 173 bp DNA fragment by Bs-XeF/Bs-XeR primers in a PCR-assay. Isolates were infiltrated in resistant cultivars to identify race. The 1998-2014 isolates were *X. perforans* (n=17), *X. gardneri* (n-1) and *X. vesicatoria* (n=1); while the 2018-2019 isolates were also mostly *X. perforans* (n=42) followed by *X. euvesicatoria* (n=7) and *X. gardneri* (n=2). *X. perforans* isolates were race T3 (n=15) and T4 (n=6). Bacterial spot is the most important foliar disease of tomatoes in Oklahoma and is primarily caused by *X. perforans*. Host plant resistance should combine multiple resistance genes to be of greatest benefit to producers in the region.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop in the United States that is worth over 1 billion dollars annually (USDA-NAS, 2019). In Oklahoma, tomatoes are produced primarily for local fresh markets and in residential gardens (Brandenberger et al., 2014). The sub-tropical climactic conditions in the state include warm temperatures, high humidity, and periodic rain events conducive to the development of foliar diseases. Foliar diseases caused by both fungal and bacterial plant pathogens such as bacterial spot, Septoria leaf spot, and early blight are known to commonly affect tomatoes in Oklahoma (Damicone & Brandenberger, 2016; Damicone & Brandenberger, 2017). However, disease identification by producers and extension professionals is difficult because symptoms of the various foliar diseases appear similar. Yield reductions due to foliar diseases of tomato occur indirectly through plant defoliation, and with some diseases (e.g. bacterial spot, early blight) occur directly through fruit spotting (Jones et al., 1991). Fruit losses due to early blight of between 30 and 50 % of fruit may occur.

Septoria leaf spot, caused by the fungus *Septoria lycopersici* Speg, overwinters in plant debris as the source of primary inoculum the following season (Jones et al., 1991). Symptoms first appear as yellow areas on leaves that later become small, circular gray lesions with a dark border, often surrounded by a yellow halo (Damicone & Brandenberger, 2016). Pyncidia develop in the lesions, and produce conidia, which are spread by rain splash and re-infect the host (Jones et al., 1991). Infection by *S. lycopersici* occurs most readily at moderate temperatures (20 to 25 °C). Septoria leaf spot

development is most prolific during periods of high relative humidity and leaf wetness.

Early blight caused by the fungus *Alternaria solani* (Ell. & Mart.) is favored by warm temperatures (28-30 °C) frequent rainfall, and high relative humidity (Jones et al., 1991). Primary inoculum is from soilborne and residue borne spores from the previous crop (Damicone & Brandenberger, 2016). Airborne conidia from leaf spots serve as secondary inoculum (Jones et al., 1991). Lesions on foliage are >6 mm in diameter, dark brown with concentric rings, often surrounded by leaf chlorosis. Fruit lesions occur at the calyx end, are dark, and may show concentric rings.

Bacterial spot is a damaging foliar disease that typically develops in climates that are warm (24-30 °C) and humid, except when caused by *Xanthomonas gardneri* which can develop at 20 °C (Araujo et al., 2010; Momol et al., 2002). The four *Xanthomonas* species known to cause bacterial spot include *X. euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria* (Jones et al., 2004). The causal agents of bacterial spot were long classified as *X. campestris* pv. *vesicatoria* (Dye et al., 1980). However strains were identified that varied in metabolic traits such as amylolytic and pectolytic activity (Stall et al., 1994; Vauterin et al., 1995). Strains in Group A display strong amylolytic and pectolytic activity, while B strains are less capable of hydrolyzing starch and pectin. Primary inoculum may be introduced into the production environment by infected seed or seedlings or from infected crop residue (Jones et al., 1991). Secondary inoculum is dispersed by wind driven rain, aerosols, and mechanical transmission.

The pathogens that cause bacterial spot of tomato can be differentiated into one of four races, T1 to T4 (Astua-Monge et al., 2000; Jones, Stall, & Bouzar, 1998). Race differentiation is dependent on the presence of an avirulence gene in the pathogen, which corresponds with a resistance gene found in one of a differential set of tomato cultivars (Astua-Monge et al., 2000; Jones, Stall, & Bouzar, 1998). The cultivar, Hawaii 7998, was first found to exhibit a hypersensitive resistant response to inoculation (Jones & Scott, 1986). Strains of *Xanthomonas* that elicit hypersensitive resistant response in Hawaii 7998 have been classified as race T1 (Whalen et al., 1993). Race T2 strains have been described as lacking the *avrRxv* effector, which is responsible for induction of HR in Hawaii 7998 by T1 strains. Race T2 causes a compatible or susceptible reaction on Hawaii 7998. Race T3 was first reported in 1995 causing a resistant response on the differentials Hawaii 7981, PI 126932, and PI 128216; but a susceptible response on Hawaii 7998 (Jones et al., 1995; Obradovic et al., 2008). The avirulence gene (*avrRxv3*) in T3 strains of *X. campestris* pv. *vesicatoria* induces a resistant response in PI 128216 (Minsavage et al., 1996). Race T4 induces hypersensitive resistant response on the differential cultivar LA716 of *Lycopersicon pennellii* that carries the *Xv4* resistance gene (Astua-Monge et al., 2000). Race T4 induces hypersensitive response on *Xv4* carrying host differential cultigens, but does not induce hypersensitive response on *Xv3* carrying host plants. *X. perforans* race T4 is capable of overcoming multiple known sources of host plant resistance in tomato (*rx1*, *rx2*, *rx3*, *Xv3*) and has become a major impediment in the deployment of resistance to manage bacterial spot (Stall, Jones, & Minsavage, 2009).

Table 3.1 Differential reactions of races of xanthomonads on tomato resistance genes

Effector Gene	Bonny best none	Hawaii 7998 (*rx1*,*rx2*,*rx3*)	Florida 216 (*Xv3*)	LA 716 (*Xv4*)	Race
AvrRxv	+	HR	+	+	T1
None	+	+	+	+	T2
AvrXv3, *AvrXv4*	+	+	HR	HR	T3
AvrXv4	+	+	+	HR	T4

Adapted from tables and data reviewed by Stall, Jones and Minsavage (2009)

The distribution of *Xanthomonas* species causing bacterial spot varies by continent possibly influenced by mean annual temperature (Araujo et al., 2010; Obradovic et al., 2008). In Taiwan, *X. euvesicatoria* and *X. vesicatoria* have been displaced by *X. perforans* in recent years (Burlakoti et al., 2018). *X. gardneri* was not found in Taiwan. In North America all four species occur (Potnis et al., 2015). In Ohio and Michigan, *X. gardneri* was the primary causal agent and caused extensive fruit spotting (Ma et al., 2011). Before 2010, the primary causal agent there was *X. euvesicatoria,* which was detected along with *X. vesicatoria* in 2010. Strains collected in eastern Pennsylvania from 1995 to 2009 were identified as *X. gardneri* (Kim et al., 2010). The regular occurrence of *X. gardneri* in northern states is consistent with experimental data showing an increased competitiveness at lower temperatures of 20 °C (Abbasi et al., 2015; Araujo et al., 2010; Kim et al., 2010; Ma et al., 2011). In North Carolina *X. perforans* was the predominant pathogen and race T4 strains far outnumbered T3 strains (Adhikari et al., 2019). In Ontario, *X. perforans* was the most common pathogen (Abbasi et al., 2015). *X. gardneri* was also found in Ontario, as well as *X. euvesicatoria* and *X. vesicatoria* which were detected at low levels. In Florida where *X. perforans* race T3 was first discovered, this pathogen together with *X. perforans* race T4 are the only major

causal agents of bacterial spot (Horvath et al., 2012; Jones et al., 1995). *X. perforans* was also found in southern Louisiana in 2013 and 2014 and Mississippi in 2017 (Abrahamian et al., 2019; Lewis-Ivey et al., 2016).

Foliar diseases of tomato are a major challenge for producers in Oklahoma due to the regions climate (Damicone & Brandenberger, 2016; Damicone & Brandenberger, 2017). Effective control of foliar disease hinges on an accurate diagnosis of the causal agent(s) involved. A survey of foliar disease of tomato in Oklahoma is needed to better understand the risk that each disease poses to producers. Bacterial spot of tomato has been previously diagnosed in Oklahoma but since being reclassified from *X. campestris* pv. *vesicatoria* in 2004 the cause of bacterial spot of tomato in Oklahoma has been unknown (Jones et al., 2004). Identifying the causal species and race is important in developing management strategies and genetic resistance to manage the disease. The objectives of this work are: i) determine the distribution and severity of bacterial spot relative to other foliar diseases of tomato in Oklahoma; ii) Identify the reclassified species of *Xanthomonas* causing bacterial spot of tomato in Oklahoma; iii) characterize the race structure of the pathogens identified.

MATERIALS & METHODS

Foliar disease survey

In 2018 and 2019 a survey of 29 field sites was conducted to determine the occurrence and severity of foliar diseases of tomato in Oklahoma (Figure 3.1). Sites were selected based on reports by growers or extension personnel of unidentified foliar disease

in field-grown tomato. In 2018, 15 sites in 11 counties were surveyed. In 2019, 14 sites in seven counties were surveyed. Three of the sites were surveyed both years. Sites visited between 15 June and 12 July in 2018 and between 10 June and 16 July in 2019 were evaluated for disease incidence and severity. The Logan county site visited on 3 August 2018 evaluated disease occurrence but not severity because of concerns over the time of evaluation biasing results. Disease was visually assessed on 3 typical plants per site. Disease incidence, the percentage of leaves with symptoms of foliar disease including defoliation, and defoliation alone were assessed on each plant. Samples of diseased leaves and/or fruit were taken to the lab for diagnosis. Plant disease incidence was determined by the number of symptomatic plants per field divided by the total number of plants assessed.

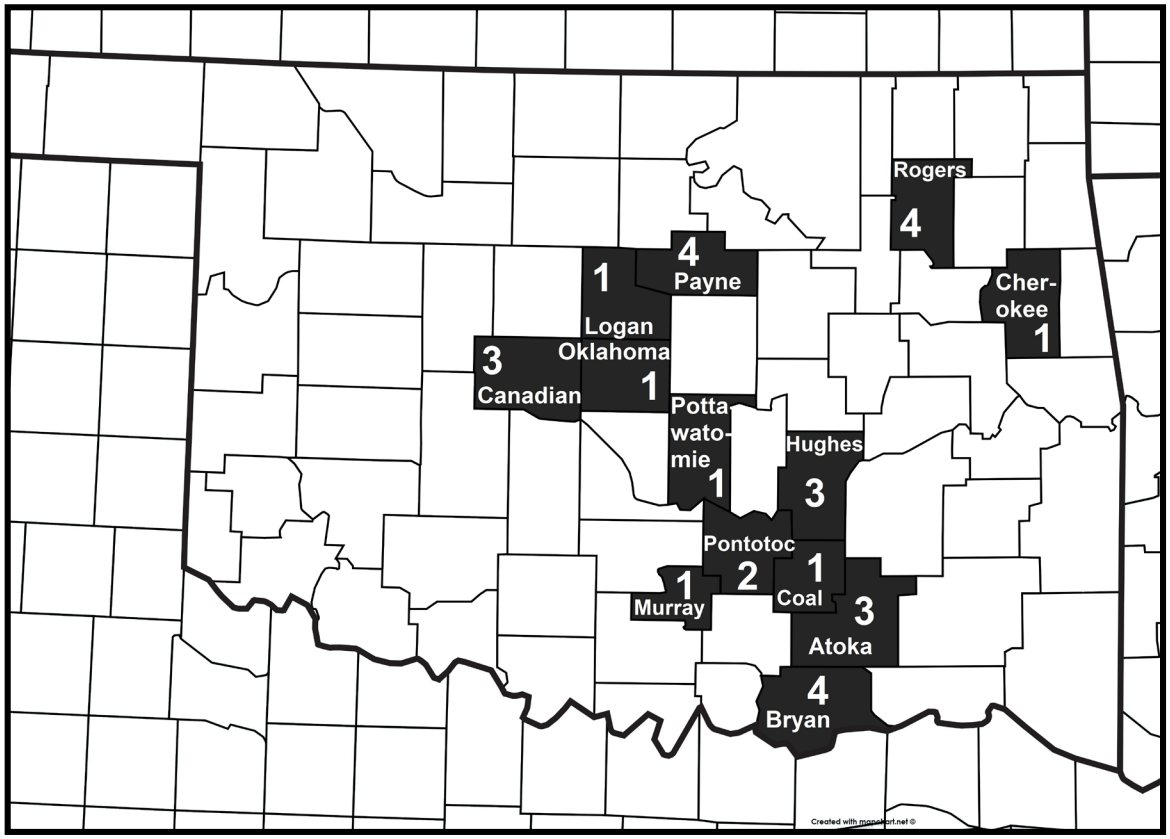

Figure 3.1 Counties in Oklahoma surveyed for foliar disease of field-grown tomatoes in 2018 and 2019. Number of field sites surveyed in each county is shown (n=29 sites).

Disease diagnosis

Lesions characteristic of fungal disease symptoms were selected. Two-sided tape was used to capture exterior fungal structures from the surface of the leaf material. Tape was mounted to standard microscope slide with the sample facing away from the slide. Sterile distilled water was placed on the surface of the sample and a standard cover slip was placed over the suspension. Samples were mounted on a compound light microscope and viewed at 400X magnification. Early blight was diagnosed by observation of large numbers of conidia with cross and longitudinal septa and characteristic beak morphology that is diagnostic for *Alternaria solani* (Barnett & Hunter, 1998; Kemmit, 2002). Septoria leaf spot was diagnosed by observation of large numbers of narrowly elongate, hyaline, several celled conidia that were diagnostic of *Septoria lycopersici* (Barnett & Hunter, 1998). Bacterial spot was diagnosed by culturing of yellow-mucoid colonies typical of *Xanthomonas* spp. from symptomatic leaves on nutrient agar and detection of DNA fragment conserved among *Xanthomonas* by PCR-assay.

Bacterial identification, isolation, and storage

Bacteria were isolated from lesions with symptoms typical of bacterial spot. Lesions (1 per plant, 3 per site in 2018 and 2 per plant, 6 per site in 2019) were excised into 2 mm^2 sections, and surface sterilized in 0.05 % sodium hypochlorite and 10 % ethyl alcohol for 30 s. Sections were rinsed for 60 s in sterile distilled water, cut into smaller pieces in a drop of sterile distilled water, incubated for 30 min, and streaked to nutrient agar. Cultures were incubated at 28 °C for 48 h and yellow-mucoid colonies typical of *Xanthomonas* spp. were re-streaked twice for purification and stored in 15% glycerol at -

70 °C. A total of 24 isolates that represented 24 field samples were collected in 2018. A total of 42 isolates that represented 24 field samples were collected in 2019. A set of 21 strains collected from tomato between 1998 and 2014 were also included in this study. Reference strains of *X. perforans* (91-118), *X. euvesicatoria* (75-3), *X. gardneri* (444), and *X. vesicatoria* (56) were obtained from Dr. Jeff Jones, University of Florida and were used as controls in species identification.

Pathogenicity of bacterial isolates

Isolates were assessed for pathogenicity to tomato cv. Moskvich, Bonny Best, or Red Bounty by spray inoculation with a bacterial suspension adjusted to a concentration of 6 x 10^8 cfu/ml (0.3 Absorbance at 600 nm), and/or by leaf infiltration at 10^7 to 10^8 cfu/ml (0.2 Absorbance at 600 nm). Pathogenicity for leaf infiltrations and spray inoculations was assessed at 6 d and 10 – 12 d after inoculation, respectively. Isolates were determined to be pathogenic by observation of necrotic lesions with surrounding chlorosis.

Identification of bacterial isolates to genus

A set of 44 isolates collected between 1998 and 2018 were identified to genus using primers targeting the chromosomal replication initiation factor (dnaA) gene in a PCR-assay (Dhakal et al., 2019). Isolates were grown on nutrient agar for 48 hours at 28 °C. Bacterial cells were lysed using a dry bath incubation (Boekel Scientific, Boekel Industries, PA, USA) set to 97 °C for 10 minutes according to a quick DNA extraction method described by Moore et al. (2004). PCR master mixes consisted of 2 uL of DNA,

5.5 uL of sterile nuclease free water, 2.5 uL of each of two primers (dnaA-F, dnaA-R), and 12.5 uL of EconoTaq® Green Master Mix (Lucigen Corporation, Middleton, WI, USA). PCR amplification was performed with a DyNAcycler DCR-96 thermocycler, (Dynalab Corporation, Rochester, NY) under the conditions of: 1 cycle of 94 °C for 30 seconds, 35 cycles of 94 °C for 30 seconds, 60 °C for 1 minute, 72 °C for 30 seconds, followed by 1 cycle 72 °C for 10 minutes. The PCR products were visualized in a 15% agarose gel electrophoresis in 70 mL 1X TAE buffer + 1.05 g + 9.5 uL Ethidium Bromide at 136 Volts for 35 m and viewed under ultraviolet light. The expected 928 bp amplicon was referenced to a 100 bp ladder (Invitrogen™ Track-It™, Thermo-Fisher Scientific, Waltham, MA).

Identification of *Xanthomonas euvesicatoria* by polymerase chain reaction (PCR)-assay

Set of 18 isolates collected in 2018 that were identified to genus by PCR-assay amplifying a 928 bp dnaA gene fragment as previously described were identified as *X. euvesicatoria* by PCR-assay with the Bs-XeF/Bs-XeR primers that amplify a 173 bp DNA fragment (Koenraadt et al., 2007). DNA was extracted from pure cultures as described previously. Known isolates for each species were included as reference strains. The PCR master mix consisted of 2 uL of DNA, 5.5 uL of sterile nuclease free water, 2.5 uL of each of two primers (Bs-XeF, Bs-XeR), and 12.5 ul of Taq polymerase PCR amplification was conducted in the thermocycler under the conditions of: 1 cycle of 94 °C for 5 m, 25 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s, followed by 1

cycle 72 °C for 7 m. PCR products were visualized by gel electrophoresis as described previously.

Identification of bacterial isolates to genus by polymerase chain reaction (PCR)-assay and to species by sequencing of *hrp* gene

A set of 74 isolates collected from 1998 – 2019 and 4 reference isolates previously identified as pathogenic on tomato were identified to genus using RST65/RST69 PCR primers that amplify a 420 bp fragment of the *hrp* gene conserved among *Xanthomonas* spp. (Obradovic et al., 2004). Isolates were grown on nutrient agar for 48 h at 28 °C. DNA was extracted using DNeasy Ultra Clean Microbial Kit (QIAGEN, Germantown, MD) and suspended in TE buffer. DNA samples were evaluated for quantity (ng/uL) and quality using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). All DNA samples were diluted to a concentration of 20 ng/uL for use in PCR reactions. The PCR master mix consisted of 2 uL of DNA, 5.5 uL of sterile nuclease free water, 2.5 uL of each of two primers (RST65, RST69), and 12.5 uL of Taq polymerase. PCR amplification was conducted in the thermocycler under the conditions of: 1 cycle of 95 °C for 5 m, 29 cycles of 95 °C for 30 s, 63 °C for 1 m, 72 °C for 45 s, followed by 1 cycle 72 °C for 5 m. PCR products were visualized by gel electrophoresis using methods described previously. PCR product of isolates identified to genus by PCR-assay were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Life Sciences, Marlborough, MA). Amplicons were Sanger sequenced in the Core Facility of the Department of Biochemistry and Molecular Biology at Oklahoma State University. Database sequences were compared to sequences tested

using BLAST function of the National Center for Biotechnology Information (NCBI). Known isolates for each species were sequenced as described above and used as comparison for sequence homology and percent identity of survey isolates.

Race determinations

Seedlings of the differential tomato cultivars Bonny Best, 216, and H7998, containing no resistance gene, *Xv3*, and *rx1, rx2, rx3,* respectively, were used to determine the frequency of corresponding avirulence genes found in the isolates collected in Oklahoma (Stall, Jones and Minsavage 2009). Seed of differential cultivars was obtained from Dr. Jeff Jones, University of Florida. A fourth differential used in the race test was the *Solanum pennellii* var. *pennellii* ascension PI 246502 identified as LA 716, which contains the resistance gene *Xv4* and was obtained from the USDA-ARS Germplasm Resource Information Network (GRIN) (Stall, Jones and Minsavage 2009). Differential cultivars were grown in SunGro soilless media (Professional Growers Mix, SunGro, Agawam, MA) in a greenhouse at 25 °C for between 28 and 64 d before inoculation. Isolates were grown on nutrient agar for approximately 48 h at 28 °C. The reference strains described above were used as controls. Bacteria were suspended in sterile water and adjusted to 6 x 10^8 cfu/ml at (0.3 absorbance at 600 nm) using a spectrophotometer (Spectronic 20+, Milton-Roy, Ivyland, PA). Tomato leaves were infiltrated on the abaxial leaf surface with approx. 1 ml of bacterial suspension using a needleless syringe. Plants were evaluated for hypersensitive response at 48 h after inoculation. A hypersensitive response consisted of collapse and a rapid necrosis as

evidenced by a rapid collapse and necrosis at infiltration site. Compatible reactions were assessed at 6 d after inoculation necrosis at injection site surrounded by chlorosis.

RESULTS

Foliar disease survey

In 2018, bacterial spot was diagnosed in 11 of the 15 fields surveyed compared to 2 with septoria leaf spot (Table 3.2). In 2019, 10 of 14 fields had bacterial spot and two had early blight caused by *A. solani*. Over the 2 years, bacterial spot was found in 72 % of fields surveyed. Septoria leaf spot and early blight of tomato each caused disease in only 7 % of fields surveyed. Disease incidence, defoliation, and field incidence, of bacterial spot were numerically higher in 2019.

In the 2018 and 2019 site visits, Septoria leaf spot and early blight were diagnosed in 2 sites each. Disease severity, as measured by diseases incidence and defoliation on plants, and plant incidence, was lower for bacterial spot compared to Septoria leaf spot and early blight. Other foliar disease symptoms observed in field sites were bacterial canker and bacterial speck. However, isolation from symptomatic leaves recovered yellow-mucoid colonies characteristic of *Xanthomonas*.

Pathogenicity of isolates

In the set of isolates collected between 1998 and 2014, 20 of 21 isolates were pathogenic on tomato. In the 2018 isolates, 22 of 24 isolates were pathogenic. In the 2019 isolates, 41 of 42 isolates were pathogenic.

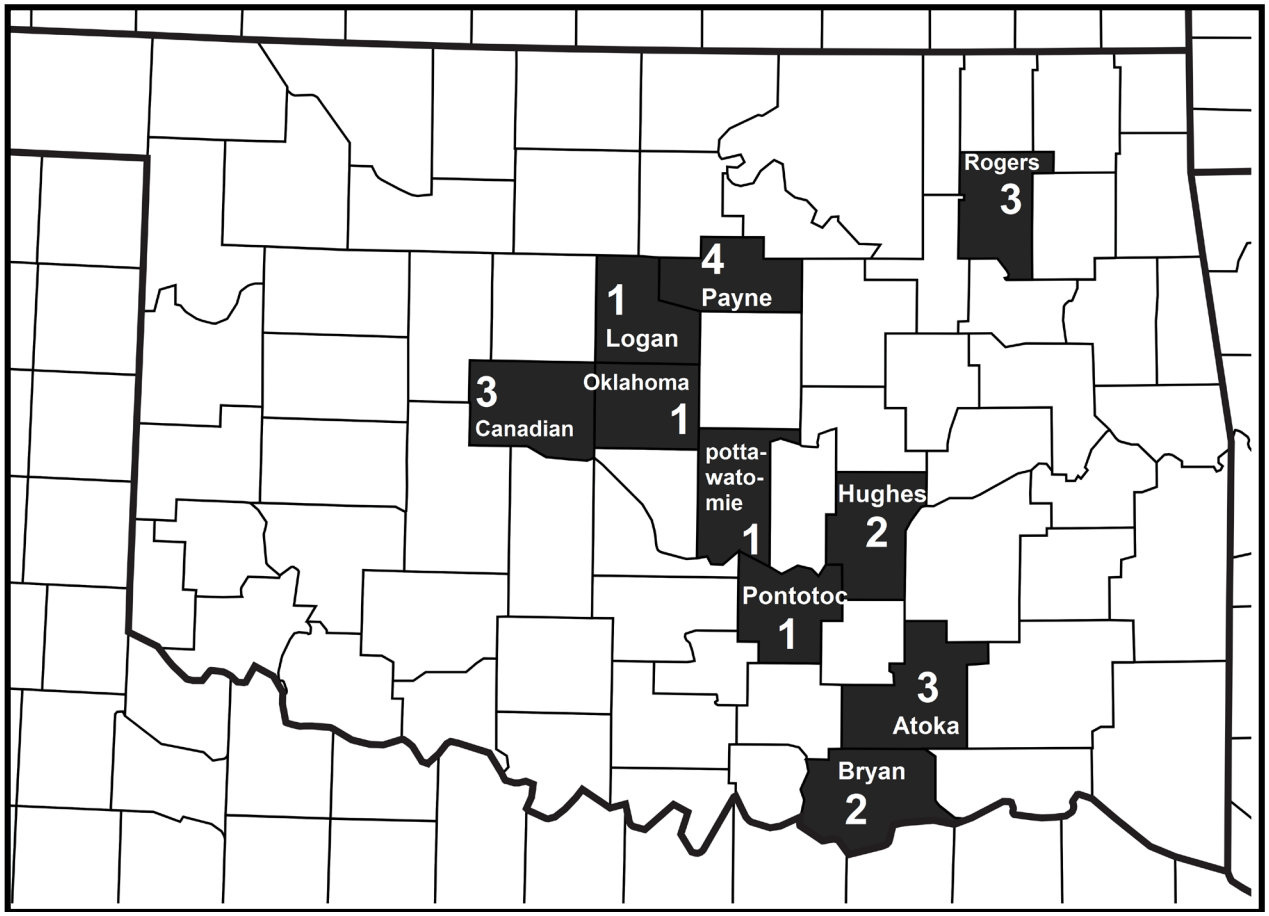

Figure 3.2 Counties in Oklahoma where field site had confirmed bacterial spot in 2018 (n=11 sites) and 2019 (n=10 sites).

Table 3.2 Occurrence and severity of foliar diseases of tomato in Oklahoma.

County[a]	Disease	Year	Disease (%)		
			[d]DI leaves	[e]DEF leaves	[f]DI leaves
Payne (n=2)	Bacterial spot[b]	2018	31.7	6.7	85
Oklahoma (n=1)	Bacterial spot	2018	26.7	10	60
Rogers (n=1)	Bacterial spot	2018	3.3	0.0	10
Canadian (n=3)	Bacterial spot	2018	46.7	15.6	63.3
Pottawatomie (n=1)	Bacterial spot	2018	13.3	6.7	10
Atoka (n=1)	Bacterial spot	2018	10	0	10
Hughes (n=1)	Bacterial spot	2018	46.7	13.3	90
Logan (n=1)	Bacterial spot	2018	-	-	-
Mean			25.5	7.5	46.9
Payne (n=2)	Bacterial spot	2019	20.8	3.3	22
Rogers (n=2)	Bacterial spot	2019	56.7	29.2	100
Hughes (n=1)	Bacterial spot	2019	40	3.3	100
Atoka (n=2)	Bacterial spot	2019	81.7	36.7	90.5
Bryan (n=2)	Bacterial spot	2019	43.3	28.3	54
Pontotoc (n=1)	Bacterial spot	2019	63.3	6.7	90
Mean			51	17.9	76.1
Rogers	Septoria leafspot[c]	2018	86.7	73.3	90
Cherokee	Septoria leafspot	2018	16.7	6.7	15
Mean			51.7	40	52.5
Bryan (n=2)	Early blight[c]	2019	33.3	22.5	13

Mean	33.3	22.5	13

[a] County where foliar disease was diagnosed.
[b] Disease diagnosed by culturing of yellow-mucoid colonies typical of *Xanthomonas* spp. from symptomatic leaves on nutrient agar and detection of DNA fragment conserved among *Xanthomonas* by PCR-assay.
[c] Disease diagnosed by microscopy.
[d] Disease Incidence (DI), defined as the percentage of leaves with symptoms of bacterial spot including defoliated leaves, visually assessed on three plants / site.
[e] Defoliation (DEF) was visually assessed on three plants / site.
[f] Plant Incidence (DI), percentage of plants in the field showing symptoms of specified foliar disease.

Bacterial spot of tomato identification of *Xanthomonas* to species

Between 1998 and 2014, 90 % of the isolates were *X. perforans* and only 5 % were *X. vesicatoria* and *X. gardneri* (Table 3.3). In 2018, *X. perforans* (53 %), *X. euvesicatoria* (41 %), and *X. gardneri* (6 %) were identified (Table 3.4). In 2019, 97% of the isolates were *X. perforans* and 3 % were *X. gardneri* (Table 3.5). From 1998 to 2019 the majority (84 %) of isolates were *Xanthomonas perforans* followed by *X. euvesicatoria* at 10%, *X. gardneri* at 4 % and *X. vesicatoria* at 1%. Reference strains for each of the four known species causing bacterial spot were identified correctly based on sequences of 420 bp *hrp* gene fragment (Table 3.6).

Table 3.3 Identification of *Xanthomonas* isolates from tomatoes in Oklahoma collected between 1998 and 2014

Isolate	County[a]	Year	Species identification[b]	BLASTn Results[c]			
				Total Score	Query Coverage	E value	Percent Identity
XCVT1	Tulsa	1998	*X. gardneri*	569	96	3e-158	93.49
XCVT2	Tulsa	2001	*X. perforans*	701	96	0.0	99.74
XCVT3	Oklahoma	2001	*X. perforans*	702	96	0.0	99.74
XCVT4	Oklahoma	2002	*X. perforans*	702	96	0.0	99.74
XCVT5	Oklahoma	2002	*X. perforans*	701	96	0.0	99.74
XCVT6	Oklahoma	2002	*X. perforans*	701	96	0.0	99.74
XCVT7	Payne	2002	*X. perforans*	701	96	0.0	99.74
XCVT8	Payne	2002	*X. perforans*	702	97	0.0	99.74
XCVT9	Tulsa	2002	*X. perforans*	702	97	0.0	99.74
XCVT11	Canadian	2002	*X. perforans*	702	96	0.0	99.74
XCVT14	Tulsa	2002	*X. vesicatoria*	564	96	1e-156	99.68
XCVT15	Tulsa	2002	*X. perforans*	701	96	0.0	99.74
XCVT17	Tulsa	2002	*X. perforans*	701	96	0.0	99.74
XCVT18	Oklahoma	2004	*X. perforans*	701	96	0.0	99.74
XCVT19	Oklahoma	2004	*X. perforans*	702	97	0.0	99.74
XCVT20	Oklahoma	2004	*X. perforans*	701	97	0.0	99.74
XCV14-T1	Payne	2014	*X. perforans*	701	96	0.0	99.74
XCV14-T3	Payne	2014	*X. perforans*	701	96	0.0	99.74
XCV14-T4	Payne	2014	*X. perforans*	695	96	0.0	99.48

[a] County in Oklahoma where *Xanthomonas* was isolated from tomato.

[b] Causal agent of disease as determined by Sanger sequencing of RST65/RST69 amplified fragment of *hrp* gene region (Obradovic et al., 2004).

[c] BLASTn result of *hrp* fragment sequence comparison of known bacterial spot of tomato causal agent in National Center for Biotechnology Information (NCBI) database.

Table 3.4 Identification of *Xanthomonas* isolates from tomatoes in Oklahoma collected in 2018

Isolate	County[a]	Species Identification[c]	BLASTn Results[d]			
			Total Score	Query Coverage	E value	Percent Identity
XCVT18-1	Payne	*X. euvesicatoria*	689	80	0.0	99.22
XCVT18-2	Payne	*X. euvesicatoria*	689	79	0.0	99.22
XCVT18-6	Oklahoma	*X. perforans*	691	61	0.0	99.22
XCVT18-7	Rogers	*X. perforans*	686	61	0.0	98.96
XCVT18-8	Rogers	*X. perforans*	682	97	0.0	99.47
XCVT18-9	Rogers	*X. perforans*	688	97	0.0	99.22
XCVT18-10	Payne	*X. perforans*	682	97	0.0	98.70
XCVT18-11	Logan	*X. euvesicatoria*[b]	-	-	-	-
XCVT18-12	Canadian	*X. euvesicatoria*	693	98	0.0	98.72
XCVT18-13	Canadian	*X. perforans*	699	96	0.0	99.48
XCVT18-14	Canadian	*X. perforans*	691	97	0.0	99.22
XCVT18-15	Canadian	*X. perforans*	686	96	0.0	98.96
XCVT18-16	Pottawatomie	*X. perforans*	691	97	0.0	99.22
XCVT18-18	Atoka	*X. euvesicatoria*	691	97	0.0	99.22
XCVT18-21	Hughes	*X. euvesicatoria*[b]	-	-	-	-
XCVT18-23	Logan	*X. gardneri*	532	96	3e-147	91.88
XCVT18-24	Logan	*X. euvesicatoria*	691	97	0.0	99.22

[a] County in Oklahoma where *Xanthomonas* was isolated from tomato.

[b] Causal agent of disease identified as *X. euvesicatoria* by PCR-assay using BS-XeF/BS-XeR amplified fragment (Koenraadt et al., 2007).

[c] Causal agent of disease identified by Sanger sequencing of RST65/RST69 amplified fragment of *hrp* gene region (Obradovic et al., 2004).

[d] BLASTn result of *hrp* fragment sequence comparison of known bacterial spot of tomato causal agent in National Center for Biotechnology Information (NCBI) database.

Table 3.5 Identification of *Xanthomonas* isolates from tomatoes in Oklahoma collected in 2019

Isolate	County[a]	Species Identification[b]	BLASTn Results[c]			
			Total Score	Query Coverage	E value	Percent Identity
XCVT19-1	Payne	*X. gardneri*	529	94	5e-146	92.04
XCVT19-5	Payne	*X. perforans*	702	96	0.0	99.74
XCVT19-6	Payne	*X. perforans*	702	96	0.0	99.74
XCVT19-7	Payne	*X. perforans*	701	96	0.0	99.74
XCVT19-8	Payne	*X. perforans*	702	96	0.0	99.74
XCVT19-9	Payne	*X. perforans*	702	96	0.0	99.74
XCVT19-10	Payne	*X. perforans*	702	97	0.0	99.74
XCVT19-11	Rogers	*X. perforans*	658	96	0.0	97.66
XCVT19-12	Rogers	*X. perforans*	658	96	0.0	97.66
XCVT19-13	Rogers	*X. perforans*	658	96	0.0	97.66
XCVT19-14	Rogers	*X. perforans*	647	95	0.0	97.38
XCVT19-15	Rogers	*X. perforans*	656	96	0.0	97.65
XCVT19-16	Rogers	*X. perforans*	656	97	0.0	97.18
XCVT19-18	Rogers	*X. perforans*	702	96	0.0	99.74
XCVT19-19	Rogers	*X. perforans*	704	96	0.0	99.74
XCVT19-20	Rogers	*X. perforans*	701	96	0.0	99.74
XCVT19-21	Rogers	*X. perforans*	702	96	0.0	99.74
XCVT19-23	Hughes	*X. perforans*	697	97	0.0	99.48
XCVT19-24	Atoka	*X. perforans*	702	96	0.0	99.74
XCVT19-26	Atoka	*X. perforans*	702	96	0.0	99.74
XCVT19-27	Atoka	*X. perforans*	701	96	0.0	99.74
XCVT19-28	Atoka	*X. perforans*	702	96	0.0	99.74
XCVT19-29	Atoka	*X. perforans*	702	96	0.0	99.74
XCVT19-30	Atoka	*X. perforans*	701	96	0.0	99.74
XCVT19-31	Atoka	*X. perforans*	701	96	0.0	99.74
XCVT19-34	Bryan	*X. perforans*	702	96	0.0	99.74
XCVT19-35	Bryan	*X. perforans*	697	97	0.0	99.48
XCVT19-36	Bryan	*X. perforans*	704	96	0.0	99.74

XCVT19-37	Bryan	*X. perforans*	702	96	0.0	99.74
XCVT19-38	Pontotoc	*X. perforans*	701	96	0.0	99.74
XCVT19-39	Pontotoc	*X. perforans*	702	96	0.0	99.74
XCVT19-40	Pontotoc	*X. perforans*	702	96	0.0	99.74
XCVT19-41	Pontotoc	*X. perforans*	697	97	0.0	99.48
XCVT19-42	Pontotoc	*X. perforans*	702	96	0.0	99.74

[a] County in Oklahoma where *Xanthomonas* was isolated from tomato.
[b] Causal agent of disease as determined by Sanger sequencing of RST65/RST69 amplified fragment of *hrp* gene region (Obradovic et al., 2004).
[c] BLASTn result of *hrp* fragment sequence comparison of known bacterial spot of tomato causal agent in National Center for Biotechnology Information (NCBI) database.

Table 3.6 *Xanthomonas* spp. reference strains.

Isolate	Species Identification[a]	BLASTn Results[b]			
		Total Score	Query Coverage	E value	Percent Identity
Xp (91-118)	*X. perforans*	701	96	0.0	99.74
Xv (56)	*X. vesicatoria*	695	96	0.0	99.48
Xe (75-3)	*X. euvesicatoria*	702	97	0.0	99.74
Xg (444)	*X. gardneri*	704	96	0.0	99.74

[a] Causal agent of disease as determined by Sanger sequencing of RST65/RST69 amplified fragment of *hrp* gene region (Obradovic et al., 2004).
[b] BLASTn result of *hrp* fragment sequence comparison of known bacterial spot of tomato causal agent in National Center for Biotechnology Information (NCBI) database.

Avirulence genes and races of *Xanthomonas* spp.

X. perforans race T3 and T4 were identified as 15 and 6 of the isolates, respectively (Table 3.7). The most common race of *X. euvesicatoria* was T1 (n=3). The single *X. vesicatoria* isolate was identified as race T4, which differed from the *X. vesicatoria* reference isolate, Xv56, that was identified as race T2. The *X. gardneri* reference isolate, Xg444, identified as race T2. Xg444 differed from the two survey isolates identified as *X. gardneri*, which both caused hypersensitive reaction on all resistant cultivars tested (Table 3.7, Table 3.8). The *X. perforans* reference isolate Xp91-118 did not group with race T3 or T4 isolates producing a compatible reaction on LA716. Forty reactions by differential resistant cultivars to *Xanthomonas* spp. were atypical and did not group with one of the four previously described pathogenic races (T1,T2,T3,T4) of bacterial spot of tomato (Table 3.8). Reactions included all possible combinations of hypersensitive response to the three resistant cultivars except the race T2 response.

Table 3.7 Identification of races of *Xanthomonas* isolates from tomatoes in Oklahoma

Isolate	Species (*hrp* region)	Bonny Best	FL 216	HI 7998	LA 716	Race score
XCVT11, XCVT17, XCVT18-7, XCVT18-14, XCVT18-15, XCVT19-7, XCVT19-21, XCVT19-27, XCVT19-30, XCVT19-31, XCVT19-35, XCVT19-36, XCVT19-38, XCVT19-39, XCVT19-40,	*X. perforans*	+[b]	HR[a]	+	HR	T3
XCVT19-5, XCVT19-6, XCVT19-26, XCVT19-28, XCVT19-29, XCVT19-41,	*X. perforans*	+	+	+	HR	T4
XCVT18-2, XCVT18-11, XCVT18-24, Xe75-3	*X. euvesicatoria*	+	+	HR	+	T1
Xg444	*X. gardneri*	+	+	+	+	T2
XCVT14	*X. vesicatoria*	+	+	+	HR	T4
Xv56	*X. vesicatoria*	+	+	+	+	T2

[a] HR = hypersensitive reaction, presence of avirulence (*avr*) gene in pathogen eliciting the response.
[b] + = compatible or susceptible reaction

Table 3.8 Identification of novel reactions of *Xanthomonas* isolated from tomatoes in Oklahoma to differentially resistant tomato cultivars

Isolate	Species (*hrp* region)	Bonny Best	FL 216	HI 7998	LA 716
XCVT2, XCVT5, XCV14-T1, XCVT18-6, XCVT19-8, XCVT19-11, XCVT19-12, XCVT19-13, XCVT19-14, XCVT19-15, XCVT19-16, XCVT19-20, XCVT19-34	*X. perforans*	+[b]	HR[a]	HR	HR
XCVT18-23, XCVT19-1	*X. gardneri*				
XCVT18-21	*X. euvesicatoria*				
XCVT9, XCVT15, XCVT19, XCVT18-9, XCVT19-18, XCVT19-19, XCVT19-42, Xp91-118	*X. perforans*	+	HR	+	+
XCVT3, XCVT4, XCVT6, XCVT7, XCVT8, XCVT18, XCVT20	*X. perforans*	+	HR	HR	+
XCVT18-1, XCVT18-12	*X. euvesicatoria*				
XCVT18-10, XCVT18-16, XCVT19-9, XCVT19-10, XCVT19-23, XCVT19-24, XCVT19-37	*X. perforans*	+	+	HR	HR
XCVT18-18	*X. euvesicatoria*				

[a] HR = hypersensitive reaction, presence of avirulence (*avr*) gene in pathogen eliciting the response.
[b] + = compatible or susceptible reaction

DISCUSSION

A majority (72 %) of fields surveyed were infected with bacterial spot of tomato, which was an incidence above that of other foliar diseases detected during the survey. By comparison, symptoms of foliar diseases caused by fungal pathogens were observed less frequently in the fields surveyed but they caused more defoliation. This survey is the only systematic account of the distribution and severity of foliar diseases of tomato in Oklahoma. Preston reported previously that these diseases occurred in several counties in Oklahoma although the bacterial spot bacterium was named *Xanthomonas vesicatoria* (Preston, 1945). This survey increases the reported range by county of bacterial spot of tomato, as well as, early blight of tomato and Septoria leaf spot.

The widespread occurrence of bacterial spot in tomato poses a major challenge to producers in Oklahoma. Once fields become infested intensive management is often required. Rotation of tomato and/or pepper producing fields into a non-host crop, incorporation of crop residues, and strict field sanitation to eliminate weedy reservoirs and volunteer tomato seedlings, are all strategies used to reduce inoculum (Damicone & Brandenberger, 2017; Jones et al., 1986). Preventative weekly applications of bactericides such as copper tank mixed with the fungicide, mancozeb, or bacteriophage formulations have been used successfully to reduce disease severity and increase yield (Damicone & Trent, 2003; Flaherty et al., 2000). However, this study showed only marginal control by bacteriophage with no yield effect, and better control by copper-mancozeb but also with no yield effect.

The frequency of *X. perforans* in the 1998 to 2014 collection of bacterial spot of tomato isolates (89 %) provides evidence suggesting that *X. perforans* has been the

dominant causal agent of bacterial spot of tomato in Oklahoma for decades. In 2018, *X. euvesicatoria* was an important cause of bacterial spot in Oklahoma but was less frequent than *X. perforans*. In 2019, *X. perforans* caused 97 % of cases of bacterial spot surveyed, which suggests that *X. perforans* is at a competitive advantage over other species infecting tomato in the climate of central and eastern Oklahoma. *X. euvesicatoria* has been reported as a common causal agent of bacterial spot of pepper in Oklahoma (Cevallos, et al. 2015). The increased incidence of *X. euvesicatoria* in 2018 surveys could be the result of local transmission from intercropped pepper plantings at trial locations. However, the importance of pepper in field transmission of bacterial spot to tomato in Oklahoma is unknown at this time. *Xanthomonas gardneri* is known to be less competitive than *X. perforans* at warmer temperatures (Araujo et al., 2010), which likely reduces its ability to thrive in Oklahoma's climate. *X. vesicatoria* was not isolated in 2018 or 2019 in Oklahoma. *X. gardneri* and *X. vesicatoria* are unlikely to be major causal agents of bacterial spot of tomato in Oklahoma, and their presence could be the result of infrequent introductions through contaminated seed or seedlings.

All four *Xanthomas* spp. that cause bacterial spot have been reported in the United States (Potnis et al., 2015). However, in Florida and Georgia all of the strains isolated in 2006 were *Xanthomonas perforans* (Horvath et al., 2012). Our results indicate a similar species composition in Oklahoma, consistent with the wide distribution of *X. perforans* in the southern United States.

Of the *X. perforans* isolates from this survey tested on differential cultivars the majority were race T3 (Table 3.7, Table 3.8). These results suggest that race T3 strains have some competitive advantage over T1 and T4 strains in Oklahoma. In Indiana, the

majority of isolates were identified as *X. perforans* race T4 (Egel et al., 2018). In the Indiana survey *X. gardneri* race T2 and *X. perforans* race T3 were also isolated from tomato, but at lower frequencies than *X. perforans* race T4 (Egel et al., 2018). *X. gardneri* was isolated largely from field locations in the northern half of Indiana, which is further evidence of the pathogens competitiveness at lower temperatures (20 °C) (Araujo et al., 2010; Egel et al., 2018). In North Carolina, *X. perforans* race T4 strains now far outnumber *X. perforans* T3 strains (Adhikari et al., 2019). In the southern U.S bacterial spot of tomato is mostly being caused by *X. perforans* race T4 (Adhikari et al., 2019; Horvath et al., 2012; Lewis Ivey et al., 2016). Race structure in Oklahoma is diverse. Development of host plant resistance should combine multiple resistance genes (*rx1, rx2, rx3, Xv3, Xv4*) to be of greatest benefit to producers in the region. Knowing which races of *Xanthomonas* predominate in our region will inform plant breeders and producers about the expected durability of different combinations of genetic resistance when resistant cultivars are deployed for control.

In summary, bacterial spot is a common challenge facing tomato producers in Oklahoma. It is important that bacterial spot be distinguished from early blight or Septoria leaf spot because the use of specific fungicides as a control tactic is ineffective for bacterial disease. Integrated strategies that combine use of disease-free seed and seedlings, with crop rotation, and field sanitation should be emphasized until commercially available cultivars with resistance to bacterial spot are developed (Damicone & Brandenberger, 2017; Damicone & Trent, 2003; Flaherty et al., 2000).

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CHAPTER IV

BIOLOGICAL CONTROL AGENTS AND CHEMICAL BACTERICIDES FOR MANAGEMENT OF BACTERIAL SPOT OF TOMATO IN OKLAHOMA

ABSTRACT

Bacterial spot is an important disease of tomato in Oklahoma and other warm, humid, and rain-fed climates. The bacterial spot pathogen *Xanthomonas perforans* poses a particular challenge to commercial producers of field-grown tomato who crop tomatoes continuously in the same field. Chemical controls, such as copper formulations and the antibiotic streptomycin, have been heavily relied upon for management of bacterial spot. Development of tolerance to copper and resistance to antibiotics has necessitated the need for development and evaluation of control measures based on novel modes-of-action. The efficacy of bacteriophage, a bacteriocin producing bacterial antagonist, a systemic acquired resistance inducer, and copper formulations were evaluated in a two-year field study. Disease incidence, defined as the percentage of symptomatic leaves including defoliated leaves, and defoliation alone, were assessed at approximately 30 and 60-d after inoculation. In both years, the resistance inducer acibenzolar-s-methyl in rotation with

bacteriophage or copper hydroxide reduced disease at 30 d post-inoculation. In 2019, a reduction in disease at 30 and 60 d after inoculation was observed for acibenzolar-s-methyl in rotation with copper hydroxide, bacteriophage applied alone, copper hydroxide applied alone, and copper hydroxide plus mancozeb. The bacteriocin producing antagonist *Bacillus amyloliquefaciens* strain D747 did not reduce disease during either year. Copper hydroxide alone or applied with mancozeb resulted in greater disease control compared to bacteriophage and *B. amyloliquefaciens*. None of the treatments significantly increased yield compared to the non-treated control in 2018 or 2019. Copper-mancozeb was the most effective treatment for tomato growers in Oklahoma. However bacteriophage may be beneficial where copper tolerant strains develop.

INTRODUCTION

Bacterial spot is a damaging disease of tomato that has been reported to cause up to 50% yield reduction in field-grown tomato (*Solanum lycopersicum* L.) (Pohronezny & Volin, 1983). Yield reductions caused by bacterial spot occur through plant defoliation and, in certain cases, through lesion development on tomato fruit (Potnis et al., 2015). Bacterial spot is caused by four species in the genus *Xanthomonas*, which include: *X. perforans*, *X. gardneri*, *X. euvesicatoria*, and *X. vesicatoria* (Potnis et al., 2015). Pepper (*Capsicum* spp.) is also an important host of *X. gardneri*, *X. euvesicatoria*, and *X. vesicatoria* (Ritchie, 2000). The four species causing bacterial spot of tomato and pepper were previously classified as *X. campestris* pv. *vesicatoria* (Dye et al., 1980).

Xanthomonas perforans is now the dominant causal agent of bacterial spot on tomato in Florida and North Carolina (Adhikari et al., 2019; Horvath et al., 2012).

Environmental conditions that favor the development of bacterial spot include, warm temperatures of 24 - 30° C, high humidity, and consistent moisture (Momol et al., 2002). Bacterial spot can be introduced into production systems through the use of contaminated seed or infected transplants (Jones et al., 1986). Once established in the field, the pathogens survive on living plants or infested crop debris. Tillage and/or crop rotation with a non-host crop have both been used successfully to reduce inoculum during subsequent tomato crops (Jones et al., 1986; Moura et al., 2020). Survival of the pathogen on plant debris in the field is likely influenced by climate with cooler climates favoring longer durations of survival (Jones et al., 1986; Peterson, 1963). In Indiana, *X. vesicatoria* was capable of survival from one season to the next by overwintering in tomato crop residues (Peterson, 1963). Volunteer tomato seedlings are an important source of primary inoculum (Jones et al., 1986). In commercial fields where tomatoes or peppers are produced continuously or in short rotations of less than 2 years, bacterial spot can be a yearly problem and must be managed by preventative applications of chemical bactericides, biological control agents, or plant resistance inducers (Momol et al., 2002).

The application of copper bactericides in a preventative program has been used extensively since the early 1800's to control bacterial plant pathogens (Obradovic et al., 2008). Unfortunately, some strains of the xanthomonads causing bacterial spot became tolerant to copper in the 1980's, which reduced bactericidal efficacy and prompted a search for alternative control measures (Obradovic et al., 2008). Isolates of *X. campestris* pv. *vesicatoria*, from North Carolina in 1986-1990 had a high frequency of copper and

streptomycin resistance (Ritchie & Dittapongpitch, 1991). Improved bactericide efficacy through the combination of copper bactericide and ethylene-bis-dithiocarbamate fungicide has been demonstrated (Conover & Gerhold, 1981). However the efficacy of copper-mancozeb applications may be inadequate when conditions favor severe disease caused by copper-tolerant strains of *X. perforans* (Strayer-Scherer et al., 2018). In addition to copper bactericides, antibiotics have been used for control of bacterial diseases (Sundin & Wang, 2018). Observations of antibiotic resistance developing in plant pathogenic bacteria, fears over resistance being transferred to human pathogenic bacteria, and the implications of such a transfer to human medicine have resulted in the limited usage of antibiotics in U.S. crop production (Sundin & Wang, 2018). However, the antibiotic streptomycin is registered for use to control of bacterial spot in the greenhouse production of tomato and pepper transplants (McManus et al., 2002).

Novel approaches in the control of bacterial diseases include application of viral bacteriophages that kill bacteria and competitive displacement by biocontrol agents. The efficacy of biological control agents can be impacted by environmental conditions, necessitating climate and region specific evaluation of commercially available products (Obradovic et al., 2008). An alternative management tactic is the use of a chemical that induces localized or systemic resistance to disease in the plant. Acibenzolar-s-methyl is a widely used systemic acquired resistance inducer that has been shown to be effective at preventing diseases caused by bacteria, fungi, and viruses (Oostendorp et al., 2001). Acibenzolar-s-methyl is a functional analog of salicylic acid, which acts as a signal molecule in the pathway and initiates gene expression of proteins related to pathogenesis (Oostendorp et al., 2001). β-1,3-glucanase (PR-2), is a protein shown to be associated

with decreased bacterial disease through cleavage of β-1,3-glucosidic bonds in β-1,3-glucan, a molecule known to function in plant defense suppression by *Xanthomonas campestris* pv. *campestris* (Itako et al., 2015; Jain & Khurana, 2018; Rigano et al., 2007). Acibenzolar-s-methyl and bacteriophage in North Florida had good efficacy in controlling bacterial spot caused by a copper-sensitive isolate of *X. campestris* pv. *vesicatoria* which was comparable to the standard copper-mancozeb program (Obradovic et al., 2004).

Spray programs containing combinations of copper, mancozeb, and acibenzolar-s-methyl evaluating control of bacterial spot and bacterial speck of tomato (*Pseudomonas syringae* pv. *tomato*) in 2002 in Oklahoma found yield of marketable fruit was increased by spray programs that included weekly applications of copper or copper + mancozeb (Damicone & Trent, 2003). However, copper-resistant strains of *X. campestris* pv. *vesicatoria* have been isolated from tomatoes in eastern Oklahoma (Bender et al., 1990). Should copper-tolerant strains of *Xanthomonas* spp. become widespread it is important to evaluate alternative treatments. The objective of this trial was to evaluate the efficacy of commercially available biocontrol products, acibenzolar-s-methyl, and two different copper bactericide formulations for control of bacterial spot of tomato in Oklahoma.

MATERIALS & METHODS

Pathogen identification and inoculum preparation

An isolate of *Xanthomonas perforans*, XCVT11, from tomato in 2002 in Canadian County, Oklahoma, was used to inoculate field plots. The isolate was found to be pathogenic by leaf infiltration or spray inoculation of tomato plants using a bacterial suspension at 10^8 CFU / mL (0.3 Absorbance at 600 nm). The isolate was identified to genus using the *Xanthomonas*-specific dnaA primer set in a polymerase chain reaction (PCR) and as *Xanthomonas perforans* by Sanger sequencing of a 420 bp region of the *hrp* gene amplified using the RST65/RST69 primer set (Arif, personal communication; Obradovic et al., 2004). Suspensions of XCVT11 in sterile tap water were adjusted to a concentration 6 x 10^8 CFU per mL and used to inoculate plots.

Transplant production and cultural practices

Transplants of the cultivar Red Mountain were grown in a greenhouse for 6 weeks at 21-28° C in soil-less potting mix (SunGro Professional Mix, Sun Gro Horticulture, Agawam, MA). Transplants were fertilized twice weekly with 26 g per liter of a soluble fertilizer 24-8-16 N-P-K. In 2018 and 2019, tomato plants were transplanted into formed beds covered with black plastic on 15 May and 17 May, respectively. Plants were drip irrigated and trellised by the stake-and-weave method. In 2018, the trial was conducted in a field of Easpur Loam that was previously fallowed. Prior to transplanting the herbicides trifluralin (Treflan, Corteva Agrisciences) and S-metolachlor (Dual II Magnum, Syngenta) were applied at rates of 305 and 585 g a.i./ha, respectively, and incorporated into the soil. Granular fertilizer at 52-0-0 kg/ha N-P-K was incorporated into the soil

prior to bed forming and transplanting. Additional fertilizer at 25-25-25 kg/ha N-P-K was delivered by irrigation on 1 June and 29 June. Chlorantraniliprole (Prevathon, Corteva Agrisciences) was applied at a rate of 74 g a.i./ha on 30 June to control insect pests. In 2019, the trial was conducted in a field of Norge loam that has been previously fallowed. Granular fertilizer at 87-156-0 kg/ha N-P-K and trifluralin at 305 g a.i./ha were incorporated into the soil prior to bed forming and transplanting. The insecticide chlorantraniliprole was applied as described above on 19 June.

Treatments and experimental design

Treatments consisted of acibenzolar-s-methyl (Actigard 50WG, Syngenta Inc, Greensboro, NC), bacteriophage (Agriphage, Omnilytics, Sandy, UT), copper hydroxide (Kocide 3000DF, Certis LLC, Columbia MD), *Bacillus amyloliquefaciens* strain D747 (Double Nickel LC, Certis LLC), copper octanoate (Cueva FL, Certis LLC), and mancozeb (Dithane 75 DF, Corteva Agriscience, Wilmington, DE). In 2018, acibenzolar-s-methyl was applied at 26 g a.i./ha in alternation with copper hydroxide at 0.65 kg a.i./ha on 7-d intervals. *Bacillus amyloliquefaciens* was applied alone at 6.9 kg a.i./ha on 7-d intervals. Copper octanoate at 0.47 kg a.i./ha was alternated every 7-d with two applications of bacteriophage applied at 0.12 g a.i./ha within a 7-d period. Acibenzolar-s-methyl was applied at 26 g a.i./ha in alternation with two applications of bacteriophage applied at 0.12 g a.i./ha within a 7-d period. In 2019, all treatments tested in 2018 were repeated with the addition of copper hydroxide at 0.91 kg a.i./ha applied on 7-d intervals, bacteriophage at a rate of 0.12 g a.i./ha applied twice per week, and copper hydroxide at 0.91 kg a.i./ha plus mancozeb at 1.70 kg a.i./ha applied on 7-d intervals.

The experimental design was a randomized complete block with four repetitions. Each plot consisted of 6 plants spaced 2-ft-apart with an 8-ft row spacing. 58-ft long border rows were placed along the periphery of trial plots and 4-ft borders with a single plant were placed between plots within the row. Isolate suspension was applied at a rate of 50 mL per plant in border rows. In 2018, plants were inoculated with *X. perforans* after the third bactericide application on 27 June. In 2019, tomato plants in the border rows were inoculated with *X. perforans* on 11 July. In 2019, a second inoculation at 90 ml / plant was made to border plants within rows on 22 July due to unfavorable environmental conditions and low disease pressure throughout July.

In 2018, treatments were applied as directed sprays through two 8005vs flat-fan nozzles per row using a CO2-pressurized wheelbarrow sprayer calibrated to deliver 284 l/ha at 276 kPa. In 2018, treatments were applied beginning on 12 June. In 2019, treatments were applied as described above but at 265 l/ha at 276 kPa using two nozzles per row when plants were small and 512 l/ha at 276 kPa using four nozzles per row when plants were large. In 2019, treatments were applied beginning on 19 June.

Data collection & analysis

Disease incidence, defined as the percentage of leaves with symptoms of bacterial spot including defoliated leaves, and defoliation alone, were visually assessed on three plants per plot approximately 30 and 60 d after inoculation. In 2018 fruit was harvested, weighed, and graded as marketable or unmarketable, eight times over a 41-d period. In 2019 fruit was harvested, weighed, and graded as marketable or unmarketable ten times over a 66 d period. In both years, unmarketable yield was further divided into two

categories, unmarketable as a direct result of disease symptoms or unmarketable for any other reason. The effect of treatment on disease and yield was tested using the GLM procedure of the software SAS 9.4 (SAS Institute Inc.). Means were separated by Fisher's least significant difference test at P=0.05.

RESULTS

In 2018, average monthly temperatures in Stillwater were 24° C for May, 26.6° C for June, 27.8° C for July, and 26.2° C for August within the range conducive (24 - 30° C) for bacterial spot development (Momol et al., 2002). Monthly rainfall totals in 2018 during the trial period months were 98.55 mm for May, 151.64 mm for June, 79.25 mm for July, and 141.99 mm for August. Major rain events (>25.4 mm in a 24 hr period) occurred four times during the cropping period. In 2018 acibenzolar-s-methyl applied in rotation with bacteriophage provided a 16% reduction in disease incidence compared with non-treated control at 30 d after inoculation (Table 4.1). Acibenzolar-s-methyl applied in rotation with copper hydroxide provided a 15% reduction in disease incidence compared with the non-treated control at 30 d after inoculation. Defoliation was reduced by 18% by treatment with acibenzolar-s-methyl in rotation with bacteriophage at 30 d after inoculation. In 2018 disease incidence and defoliation levels did not differ between treatments at 60 d after inoculation. In 2018, marketable yield averages by treatment ranged from 4.4 to 5.6 t/ha (Table 4.1). Yield averages by treatment ranged from 5.7 to 7.4 t/ha. Fruit spotting and sunscald of fruit caused by bacterial spot occurred in all

treatments. Unmarketable fruit as a result of fruit spotting and sunscald by treatment ranged from 0.8 to 1.0 t/ha. Yield did not differ among treatments in 2018.

In 2019, average monthly temperatures were 19.6° C for May, 24.3° C for June, 27.4° C for July, 27.2° C for August, and 26.2° C for September. Average temperatures were 0.3° C below normal in May and June, 0.1° C below normal in July, near normal in August, and 3.8° C above normal in September. Average monthly temperatures from June to September were conducive for bacterial spot. Monthly rainfall totals were 439.42 mm for May, 106.93 mm for June, 19.30 mm for July, 209.80 mm for August, and 165.35 mm for September. Rainfall in May was 304 mm above average followed by rainfall totals in June and July that were 25 and 59 mm below average, respectively.

In 2019, the disease incidence was reduced at 30 d after inoculation by all treatments, except the biological control agent, *B. amyloliquefaciens* strain D747 (Table 4.2). At 60 d after inoculation the disease incidence was reduced by acibenzolar-s-methyl rotated with copper hydroxide, bacteriophage alone, copper hydroxide alone, and copper hydroxide applied together with mancozeb. The greatest reduction (54%) in disease incidence at 60 d post-inoculation was observed in plots treated with copper hydroxide applied together with mancozeb. Defoliation at 30 d post-inoculation was low (<10%) in all treatments including the non-treated control and was not significantly reduced by any treatment. Defoliation was reduced at 60 d by acibenzolar-s-methyl in rotation with copper hydroxide, bacteriophage alone, copper hydroxide alone, and copper hydroxide tank-mixed with mancozeb. The greatest reduction (36.7%) in defoliation at 60 d post-

inoculation was observed in plots treated with copper hydroxide tank-mixed with mancozeb. In 2019, yield, marketable yield, and diseased yield, were not significantly different among treatments (Table 4.2). Marketable yield averages by treatment ranged from 9.4 to 12.6 t/ha. Fruit spotting and sunscald of fruit caused by bacterial spot occurred in all treatments. Unmarketable fruit as a result of fruit spotting and sunscald was between 1.3 to 3.6 t/ha.

The combined analysis of treatments that were the same in 2018 and 2019 both showed that disease incidence at 30 d post-inoculation was significantly reduced by acibenzolar-s-methyl in rotation with either copper hydroxide or bacteriophage, and copper octanoate in rotation with bacteriophage formulation (Table 4.3). Defoliation at 30 d was decreased only by acibenzolar-s-methyl in rotation with bacteriophage formulation. However, disease incidence at 60 d was not significantly different between treatments. Defoliation at 60 d was decreased by acibenzolar-s-methyl in rotation with copper hydroxide.

Table 4.1 Evaluation of chemical bactericides and biological control agents for control of bacterial spot on 'Red Mountain' tomato in 2018.

Treatment rate a.i./ha (timing)[z]	Days after inoculation				Yield (t/ha)		
	26 d		58 d		Total	Marketable	Diseased[t]
	DI[v]	DEF[u]	DI[v]	DEF[u]			
Non-treated check	87.5 ab[y]	41.7 a	96.7 a	75.9 a	7.1 a	5.6 a	1.0 a
Acibenzolar-s-methyl 26 g (1,4,7,10,13) Bacteriophage 0.12 g (2,3,5,6,8,9,11,12,14,15)	71.2 c	23.3 b	91.7 a	68.3 a	5.7 a	4.4 a	0.8 a
Acibenzolar-s-methyl 26 g (1,4,7,10,13) Copper hydroxide 0.65 kg (2,5,8,11,14)	72.9 c	30.9 ab	93.3 a	74.5 a	6.3 a	5.0 a	0.8 a
Bacillus amyloliquefaciens strain D747 6.9 kg (1,2,4,5,7,8,10,11,13,14)	89.2 a	45.0 a	89.2 a	70.8 a	6.7 a	5.0 a	1.0 a
Copper octanoate 0.47 kg (1,4,7,10,13) Bacteriophage 0.12 g (2,3,5,6,8,9,11,12,14,15)	79.2 bc	30.4 ab	91.7 a	72.5 a	7.4 a	5.5 a	0.9 a
P>F[x]	<0.01	0.05	0.23	0.60	0.86	0.90	0.92
LSD (P=0.05)	9.0	15.3	NS[w]	NS	NS	NS	NS

[z] Timings 1 to 15 correspond to the spray dates of 1=12 Jun, 2=19 Jun, 3=25 Jun, 4=26 Jun, 5=3 Jul, 6=6 Jul, 7=10 Jul, 8=17 Jul, 9=20 Jul, 10=24 Jul, 11=31 Jul, 12=3 Aug, 13=7 Aug, 14=14 Aug, and 15=17 Aug.

[y] Values in a column followed by the same letter are not different at P=0.05 by Fisher's least significant difference test

[x] Probability of a significant treatment effect.

[w] NS=Treatment effect not significant at P=0.05.

[v] DI=disease incidence, the percentage of leaves with symptoms of bacterial spot including defoliated leaves.

[u] DEF=percentage of leaves defoliated.
[t] Disease=Unmarketable fruit caused by fruit spotting and sunscald of fruit as a result of defoliation.

Table 4.2 Evaluation of chemical bactericides and biological control agents for control of bacterial spot on 'Red Mountain' tomato in 2019.

Treatment rate a.i./ha (timing)[z]	Days after inoculation				Yield (t/ha)		
	30 d		60 d		Total	Marketable	Diseased[t]
	DI[v]	DEF[u]	DI[v]	DEF[u]			
Non-treated check	24.2 a[y]	6.7 b	81.3 a	44.2 bc	27.1 a	12.6 a	3.6 a
Acibenzolar-s-methyl 26 g (1,5,9,13,17) Bacteriophage 0.12 g (3,4,7,8,11,12,15,16,19,20)	16.3 b	7.5 ab	75.4 ab	49.2 ab	22.2 a	11.1 a	2.2 a
Acibenzolar-s-methyl 26 g (1,5,9,13,17) Copper hydroxide 0.65 kg (3,7,11,15,19)	8.8 c	5.0 b	55.8 c	19.6 ef	24.8 a	13.3 a	3.0 a
Bacillus amyloliquefaciens strain D747 6.9 kg (1,3,5,7,9,11,13,15,17,19)	25.8 a	10.0 a	82.1 a	59.6 a	23.5 a	10.7 a	3.0 a
Copper octanoate 0.47 kg (1,5,9,13,17) Bacteriophage 0.12 g (3,4,7,8,11,12,15,16,19,20)	12.1 bc	5.4 b	78.8 ab	36.3 cd	25.2 a	11.8 a	2.8 a
Bacteriophage 0.12 g (1-20)	12.5 bc[y]	6.3 b	68.8 b	25.4 de	21.9 a	9.4 a	2.9 a
Copper hydroxide 0.91 kg (1,3,5,7,9,11,13,15,17,19)	5.8 c	5.0 b	39.2 d	10.8 fg	18.4 a	11.3 a	1.4 a

Copper hydroxide 0.91 kg + Mancozeb 1.7 kg (1,3,5,7,9,11,13,15,17,19)	5.8 c	5.4 b	27.1 e	7.5 g	21.9 a	11.8 a	1.3 a
P>F[x]	<0.01	<0.01	<0.01	<0.01	0.12	0.93	0.34
LSD (P=0.05)	7.2	2.5	11.8	11.6	NS[w]	NS	NS

[z] Timings 1 to 20 correspond to the spray dates of 1=19 Jun, 2=21 Jun, 3=26 Jun, 4=28 Jun, 5=2 Jul, 6=5 Jul, 7=10 Jul, 8=12 Jul, 9=17 Jul, 10=19 Jul, 11=24 Jul, 12=26 Jul, 13=31 Jul, 14=2 Aug, 15=7 Aug, 16=9 Aug, 17=14 Aug, 18=16 Aug, 19=21 Aug, 20=23 Aug

[y] Values in a column followed by the same letter are not different at P=0.05 by Fisher's least significant difference test

[x] Probability of a significant treatment effect.

[w] NS=Treatment effect not significant at P=0.05.

[v] DI=disease Incidence, the percentage of leaves with symptoms of bacterial spot including defoliated leaves.

[u] DEF=defoliation.

[t] Disease=Unmarketable fruit caused by fruit spotting and sunscald of fruit as a result of defoliation.

Table 4.3 Evaluation of chemical bactericides and biological control agents for control of bacterial spot on 'Red Mountain' tomato averaged over 2018 and 2019.

Treatment rate a.i./ha	Days after inoculation				Yield (t/ha)		
	30 d		60 d		Total	Market-able	Disease[s]
	DI[u]	DEF[t]	DI[u]	DEF[t]			
Non-treated check	55.8 a[y]	24.6 b	89.0 a	60.0 a	17.1 a	9.0 a	2.3 a
Acibenzolar-s-methyl 26 g Bacteriophage 0.12 g	43.7 b	15.4 c	83.5 a	58.8 a	13.9 a	7.7 a	1.5 a
Acibenzolar-s-methyl 26 g Copper hydroxide 0.65 kg	40.8 b	17.9 bc	74.6 a	47.1 b	15.5 a	9.1 a	1.9 a
Bacillus amyloliquefaciens strain D747 6.9 kg	57.5 a	27.5 a	85.7 a	65.2 a	15.1 a	7.8 a	2.0 a
Copper octanoate 0.47 kg Bacteriophage 0.12 g	45.6 b	17.9 bc	81.9 a	54.4 ab	16.3 a	8.9 a	1.8 a
P>F[x]	<0.01	<0.01	0.06	0.03	0.76	0.92	0.67
LSD (P=0.05)	9.75	4.39	NS[v]	3.25	NS	NS	NS
P>F[w]	0.48	0.07	0.05	0.01	0.98	0.97	0.85
LSD (P=0.05)	0.89	NS	2.67	3.97	NS	NS	NS

[y] Values in a column followed by the same letter are not different at P=0.05 by Fisher's least significant difference test
[x] Probability of a significant treatment effect.
[w] Probability of a significant year by treatment effect.
[v] NS=Treatment effect not significant at P=0.05.
[u] DI=disease Incidence, the percentage of leaves with symptoms of bacterial spot including defoliated leaves.
[t] DEF=defoliation.
[s] Disease=Unmarketable fruit caused by fruit spotting and sunscald of fruit as a result of defoliation.

DISCUSSION

Bacteriophage applied twice-weekly resulted in a season-long reduction in disease incidence and late season reduction in defoliation compared with the non-treated control. In Florida reported better control was achieved by treatment with acibenzolar-s-methyl in combination with bacteriophage compared to bacteriophage alone (Obradovic et al., 2004). However in this study, bacteriophage was applied in rotation with acibenzolar-s-methyl, which differs from the Florida experiment where bacteriophage was applied twice per week with the addition of acibenzolar-s-methyl every 14 d (Obradovic et al., 2004). Results suggest that the application of bacteriophage could be a more important contributor to disease control than acibenzolar-s-methyl, but it is also possible that effects of combining bacteriophage application with intermittent applications of acibenzolar-s-methyl are additive (Obradovic et al., 2004). In 2019, bacteriophage applied in rotation with either copper octanoate or acibenzolar-s-methyl was less effective than weekly applications of copper hydroxide alone or in combination with mancozeb. In 2019 when bacteriophage was applied twice per week efficacy was improved but was still less effective than weekly copper hydroxide application. In Florida, disease control from bacteriophage applied twice per week was comparable to superior to a standard copper-mancozeb program (Flaherty et al., 2000). Bacteriophage applied twice per week may be a viable alternative to copper for control of bacterial spot, especially when confronted with copper-tolerant pathogens.

While only tested in 2019, the most effective control of bacterial spot resulted from treatment with copper hydroxide in combination with mancozeb. This result suggests a lack of tolerance to copper in the local *Xanthomonas perforans* isolate,

XCVT11, and demonstrated the continued efficacy of a widely utilized bactericide treatment (copper hydroxide-mancozeb) for control of bacterial spot of tomato in Oklahoma. Treatment with copper hydroxide alone was also highly effective, but had 12% greater disease incidence at 60 d after inoculation than copper hydroxide-mancozeb. Results are consistent with early studies evaluating the use of the ethylene bisdithiocarbamate (EBDC) fungicides mancozeb and maneb in combination with copper hydroxide for improved bacterial spot control in Florida tomato fields (Conover & Gerhold, 1981). In Oklahoma, the evaluation of spray programs for control of bacterial spot (*X. campestris* pv. *vesicatoria*) and bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato resulted in increased yield of marketable fruit with weekly applications of copper or copper + mancozeb (Damicone & Trent, 2003). However, an earlier trial in Oklahoma showed that applications of copper hydroxide + mancozeb to control a copper-tolerant strain of *X. campestris* pv. *vesicatoria* did not improve yield or reduce disease incidence (Bender et al., 1990).

In 2018 environmental conditions conducive for disease development throughout cropping period contributed to inadequate control and reduced yield. Overall, yield was well below the 25.5 t/ha that can be expected for field-grown, fresh-market tomato production in Oklahoma (Brandenberger et al., 2017). In 2019, environmental conditions were less favorable for the rapid development of bacterial spot. Although disease was less severe in 2019 and control was much improved this did not result in improved yield. The lack of a yield effect was probably due to the delayed development of the disease in the month of July while weekly harvests had commenced. Yield of combined trial treatments ranged from between 13.9 t/ha to 17.1 t/ha. Differences in the yield of combined trial

treatment means were not statistically significant (Table 4.3). The lack of a yield response to copper hydroxide with or without mancozeb may have been affected by the duration and time of harvest. In 2019 July harvests were made in the absence of disease symptoms. In the previous study in Oklahoma, yield was taken within a more narrow time frame of approximately 30 d, compared to the present study where yield was taken over approximately 60 d (Damicone & Trent, 2003). In the present study plots were inoculated late in the season allowing the plants to become established before infection, which may have had an equalizing effect on yield among trial plots.

The only treatment that was not effective in managing bacterial spot was the biological control agent *Bacillus amyloliquefaciens* strain D747. *B. amyloliquefaciens* strain D747 is one of several species in the genera *Bacillus* known to produce antagonistic compounds, such as antibiotics, that have suppressive effects on the growth of some fungal and bacterial plant pathogens (Wulff et al., 2002). One such case of fungicidal activity by *B. amyloliquefaciens* strain D747 was reported in the control of Cercospora leaf spot (*Cercospora beticola*) of table beet (*Beta vulgaris* ssp. *vulgaris*) in upstate New York (Pethybridge et al., 2017). The effectiveness of *B. amyloliquefaciens* strain D747 in combination with copper octanoate for control of *Xanthomonas cucurbitae,* the causal agent of bacterial spot of pumpkin, was evaluated in field trials in Illinois (Thapa & Babadoost, 2016). The authors observed a reduction in disease severity comparable to plots treated with copper hydroxide-mancozeb, but concluded that no single treatment evaluated was highly effective in controlling *X. cucurbitae*. Field trials conducted in Florida evaluated the effectiveness of *B. amyloliquefaciens* strain D747 in combination with copper octanoate to manage bacterial spot on tomato and found that the

reduction in disease severity was statistically equivalent to the reduction observed in plots treated with copper octanoate alone (Abrahamian et al., 2019). The present results corroborate those of the Florida study and suggest that the effect of copper octanoate might be necessary to reduce disease severity. *B. amyloliquefaciens* strain D747 appears to be poorly suited to control bacterial pathogens in the genus *Xanthomonas*.

Tomato growers in Oklahoma face a challenge in controlling bacterial spot given the regions climate and environmental conditions, which often favor disease. In lieu of a superior alternative to copper formulations the use of copper-mancozeb is still the most effective chemical control measure for tomato growers in Oklahoma. In the event that growers see a precipitous decline in the efficacy of copper-mancozeb due to copper-tolerant strains of *Xanthomonas* that cause bacterial spot the use of bacteriophage applied twice per week is recommended. However, the most successful control strategy will integrate chemical or biological protectants with cultural measures such as the use of disease-free seed and transplants, crop rotation to a non-host, use of genetic resistance when available, and removal or tillage of crop residues at crop termination.

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