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"Prevalence of Copper Resistance among Foliar Bacterial Pathogens of Tomato in Tennessee"

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To the Graduate Council:

I am submitting herewith a thesis written by Jonathon Thomas Mixon entitled ""Prevalence of Copper Resistance among Foliar Bacterial Pathogens of Tomato in Tennessee"." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Entomology and Plant Pathology.

Steven C. Bost, Major Professor

We have read this thesis and recommend its acceptance:

Bonnie Ownley, Carl Sams, Kimberly Gwinn

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

"Prevalence of Copper Resistance among Foliar Bacterial Pathogens of Tomato in Tennessee"

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Jonathon Thomas Mixon

May 2012

DEDICATION

This thesis is dedicated to everyone who has offered a kind word, a generous hand, and a giving heart to me while I have been a graduate student. My family and friends have made all the difference throughout this stage of my life. Without those people I would never have made it this far.

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I am grateful to Dupont, Chemtura, Dow, Sygenta, Altria, IR-4, Tennessee AgResearch, the Rehabilitation Corporation of Tennessee, and the Entomology and Plant Pathology department of the University of Tennessee for providing funding of my graduate research project and education.

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ABSTRACT

Foliar bacterial diseases are among the most important diseases of tomato. Bacterial spot is caused by four species: *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*, hereafter referred to as *Xanthomonas* spp. Bacterial speck is caused by *Pseudomonas syringae* pv. *tomato* and bacterial canker is caused by *Clavibacter michiganensis* subsp. *michiganensis*. Fixed copper products are relied upon extensively for control, due to a lack of effective and economical alternatives. Copper resistance in bacterial spot and speck pathogens has been reported worldwide. Copper resistance quite likely exists in Tennessee, but the extent has never been determined. The objectives of this study were to (i) optimize the conditions for in-vitro determinations of the level of copper sensitivity in *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* cultures in a protocol that would be practical for processing large sample numbers, and validation with bioassays; (ii) determine the incidence and distribution of copper resistance in Tennessee populations of these pathogens. Parameters selected for *in-vitro* copper resistance testing were sucrose peptone agar adjusted to pH 6.8 (*Xanthomonas* spp. and *C. michiganensis* subsp. *michiganensis*) or 6.2 (*P. syringae* pv. *tomato*), amended with 2-(*N*-morpholino)ethanesulfonic acid (20 mM) and $CuSO_4 \cdot 5H_2O$ (200 µ[micro]g/ml), and amended with 200 µ[micro]g/ml $CuSO_4 \cdot 5H_2O$. After solidifying, media were promptly streaked with cells produced on solid agar, and incubated for three days. Bacterial growth was visually assessed on a 0 to 10 scale. For rapid identification, a multiplex PCR protocol was developed and used successfully to detect the three pathogens from symptomatic plants. When plants were co-inoculated with *P. syringae* pv. *tomato* and *C. michiganensis* subsp. *michiganensis*, the latter could not be detected. As a result, alternative methods of pathogen identification were employed in the field survey. A survey of 19 fields representing 195 acres in six Tennessee counties during the 2010 and 2011 growing seasons, 162 were identified as *Xanthomonas* spp., none as *P. syringae* pv. *tomato*, and four as *C. michiganensis* subsp. m*ichiganensis.* Copper resistance was

detected in 95.1% of *Xanthomonas* spp. isolates and 4.9% were sensitive. All *C. michiganensis* subsp. *michiganensis* isolates were sensitive.

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CHAPTER 1 : LITERATURE REVIEW

Solanum lycopersicum and its Production in Tennessee

Tomato production in Tennessee consists primarily of trellised, fresh market culture. From 2000 to 2009, the statewide average harvested acreage was 3,950, with an average value of over 38.3 million dollars per year (24). Currently, the state of Tennessee ranks sixth in the nation for fresh market tomato production (24), following California, Florida, Virginia, Georgia, and Ohio. Major production areas are in eastern (Unicoi, Cocke, Washington, Greene, Hamblen, and Grainger counties), east-central (Bledsoe, Rhea, and Sequatchie counties), and western (Lauderdale county) Tennessee. Greenhouse tomato production is also important to the state's economy, and tomatoes are an important home garden plant in Tennessee.

Bacterial Spot

Bacterial spot occurs wherever tomatoes or peppers are grown, and is a problem in warm, moist climates such as in Tennessee. Symptoms can occur on leaves, stems, and fruit and can vary considerably in appearance, depending on environmental conditions. Leaf lesions may be dark green and water-soaked to brown and dry. Lesions are usually less than 3 mm in diameter, but may be larger on fruit. The range of bacterial spot symptoms can overlap considerably with that of bacterial speck.

Bacterial spot of tomato was first observed in 1914 in South Africa by Doidge (5), who designated the causal agent *Bacterium vesicatorium*. For years, a single bacterial species, classified as *Xanthomonas vesicatoria* (6) and later as *X. campestris* pv. *vesicatoria* (1), was considered the cause of bacterial spot of both pepper and tomato. Considerable variation within the species led to a division into four groups, referred to as A, B, C, and D (11; 22; 25). Distinctions among the groups were based on starch utilization, fatty acid profile, carbon utilization pattern, and DNA relatedness (3). Vauterin (25) placed Groups A and C in a new species, *X. axonopodis* (syn. *campestris*) pv. *vesicatoria* and gave species status to Group B, *X. vesicatoria*. Jones et al. (10) proposed a division of *X. a.* pv. *vesicatoria* into two species, *Xanthomonas euvesicatoria* (5) sp. nov., sp. rev. (Group A) and *X. perforans* (10) sp. nov. (Group C), retained *X. vesicatoria* (25), and recognized strain D as a distinct species, *X. gardneri* (23) sp. nov.

Xanthomonas spp. can overwinter in plant debris (9). Infested seeds, from which volunteer tomatoes, transplants, and weeds arise, can introduce the bacteria into a new crop. Infested tools and equipment can also be a source of inoculum. Spread occurs during rains, especially wind-driven, and during production practices. Infection occurs primarily through wounds such as abrasions.

Control methods include field rotation, use of disease-free transplants, seed disinfestation, sanitation practices, and application of recommended crop protection products (9). Effective resistant cultivars are not available because of an abundance of variability in the pathogen. Copper products comprise a major component of the spray programs used in Tennessee to control bacterial spot.

Bacterial Speck

Bacterial speck has worldwide distribution and is particularly problematic in mild climatic areas. Symptoms of bacterial speck can be found on the stems, leaves, and fruit of tomato plants. Lesions are generally smaller than those of bacterial spot. On leaves, they measure approximately 1-2 mm in diameter, and are often surrounded by a yellow halo. Similar to bacterial spot, lesions may coalesce to form larger necrotic portions. Bacterial speck and bacterial spot lesions can vary in size, texture, and color and are easily confused with each other, and with symptoms of other diseases.

Pseudomonas syringae pv. *tomato*, the causal agent of bacterial speck, was described and identified in Taiwan as *Bacterium tomato* (14). In 1948, it was transferred to the genus *Pseudomonas* by Burkholder (19). The disease received little attention until widespread losses, thought to be caused by seed-borne transmission, occurred in 1978 (8). Tomatoes are the only major cultivated host. The bacterium favors temperatures cooler than *Xanthomonas* spp. (18-24°C), and high humidity. Pathogen survival, mode of introduction into a crop, and spread are similar to that for bacterial spot. Infection does not require wounds, as the bacteria can enter plant tissue through stomata.

Control of bacterial speck is similar to that recommended for bacterial spot. Commercially available cultivars that are resistant to Race 0 contain the *pto* gene (18). There is no resistance to Race 1 in commercial cultivars.

Bacterial Canker

Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis*, is found in most tomato-producing areas of the world. Early symptoms can include turning down of lower leaves, turning up of leaflet edges, and V-shaped chlorotic areas at leaf margins. The systemic phase is characterized by wilt (sometimes resulting in plant death) and stem cankers. Local infections cause marginal necrosis of leaves and raised, brown, scabby lesions, occasionally with white haloes, on the fruit (9).

The causal agent was first described as *Corynebacterium michiganense* subsp. *michiganense* in 1909 by Smith (21). The name was changed when Davis et al. erected the genus *Clavibacter* (4). Four haplotypes of *C. michiganensis* subsp. *michiganensis*, namely A, B, C, and D, have been defined using molecular markers (12), but there are no recognized host races.

Pathogen survival, mode of introduction into a crop, and spread are similar to that for bacterial spot. Entry into leaves through hydathodes is the primary method of local infections, which can lead to systemic infections when the bacteria enter the vascular system. Systemic infections can also result from the entry of bacteria into roots. Control practices are similar to those for bacterial spot and speck. There are no commercially available tomato cultivars with resistance to bacterial canker.

Copper Resistance Testing Methods

For successful control of foliar bacterial diseases, spray programs must be designed to accommodate the likely copper sensitivity status of the bacterial population. Field surveys may be conducted to determine the likelihood that growers will encounter resistant strains (1; 13; 16; 20). The ability of the collected strains to tolerate copper in artificial growth media is used to measure copper resistance in the field. Thus, effective spray program design depends upon the use of *in-vitro* copper resistance assessments that accurately reflect the degree of control likely to be encountered in the field.

Copper is toxic to bacteria in the free ionic state (26) and toxicity is reduced by culture medium conditions that promote ion complexation with medium components. High pH levels, in

particular, are known to promote complexation. The choice of culture medium can also influence results of copper resistance assessments. For example, casitone yeast extract agar is a low-complexation medium (26) and may represent a suitable medium for such studies. Other experimental conditions and practices that can affect the outcome of copper resistance assessments include the type and concentration of buffer (2), the method of culture application to the test medium, the length of culture incubation, and the freshness of the medium. Test accuracy also depends on appropriate interpretation of the results by relating them to disease control efficacy by copper in the field (17).

Identification Methods

Xanthomonas spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* can cause similar symptoms and can occur together in diseased plant tissue. As a result, diagnostic methods must be used that provide simultaneous identification of multiple pathogens or separate the pathogens to allow individual identifications. Methods used in an extensive study such as a survey should be selected according to economy and efficiency.

Diagnostic tests. Identification procedures for bacterial pathogens of tomato can be facilitated by consideration of plant symptoms, bacterial colony morphology, and the frequency of the colony type. Based on these characteristics, appropriate semi-selective media, biochemical and physiological tests, enzyme-linked immunosorbent assays, polymerase chain reaction (PCR), and pathogenicity tests may be selected and conducted.

Multiplex PCR. PCR is used often in pathogen research and is a valuable identification tool. Simultaneous detection, generally termed multiplexing, of *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis*, from tissue samples could reduce costs and time needed for identification, and facilitate population studies for large production areas. A study on multiplex PCR for detection of these three tomato pathogens has been documented with another set of species specific primers using pure cultures (15). There is a need for a protocol allowing simultaneous detection of these pathogens in mixed populations in plant tissue.

The objectives of this thesis were: 1) to develop a protocol for *in-vitro* determinations of the level of copper sensitivity in *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* cultures in a protocol that would be practical for processing

large sample numbers, and to validate the results with *in-planta* assays; and 2) to determine the incidence and distribution of copper resistance in Tennessee populations of these pathogens.

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CHAPTER 2 : OPTIMIZATION OF COPPER RESISTANCE TESTING METHODS FOR FOLIAR BACTERIAL PATHOGENS OF TOMATO

Abstract

Foliar bacterial diseases are among the most important diseases of tomato. Bacterial spot is caused by four species: *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*, hereafter referred to as *Xanthomonas* spp. Bacterial speck is caused by *Pseudomonas syringae* pv. *tomato* and bacterial canker is caused by *Clavibacter michiganensis* subsp. *michiganensis*. Fixed copper products are relied upon extensively for bacterial control, despite reports of resistance to copper in the bacterial spot and bacterial speck pathogens worldwide. The prevalence of copper resistance in these pathogens in Tennessee is not known. An efficient, time-saving protocol for copper resistance determination in these pathogens is required for such a study. The objectives of this study were to develop a protocol for *in-vitro* determinations of the level of copper sensitivity in *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* cultures that would be practical for processing large sample numbers, and to validate the results with bioassays. The accuracy of the *in-vitro* testing parameters were measured against greenhouse bioassays which, in turn, were validated with field plot results for four isolates of *Xanthomonas* spp. and one isolate of *P. syringae* pv. *tomato*. There was good correspondence between greenhouse and field data for *Xanthomonas* spp. isolates. The *P. syringae* pv. *tomato* isolate tested moderately resistant in the field and sensitive in the greenhouse bioassay. The former reaction was used as the standard against which *in-vitro* results for this pathogen were compared. The field plots with bacterial canker did not produce usable data, precluding a comparison with those obtained in the greenhouse. Since there is no documented evidence of copper resistance in *C. michiganensis* subsp. *michiganensis,* complete sensitivity was the standard against which *in-vitro* results for this pathogen were compared. Parameters selected for *in-vitro* copper resistance testing were sucrose peptone agar adjusted to pH 6.8 (*Xanthomonas* spp. and *C. michiganensis* subsp. *michiganensis*) or 6.2 (*P. syringae* pv. *tomato*), amended with 2-(*N*-morpholino)ethanesulfonic acid (20 mM) and $CuSO_4 \cdot 5H_2O$ (200 µg/ml). After solidifying, media were promptly streaked with cells produced on solid agar, and incubated for three days. Bacterial growth was visually assessed on a 0 to 10 scale. Copper-sensitive isolates consistently tested highly sensitive. *Xanthomonas* spp. and *P. syringae* pv. *tomato* isolates with reduced sensitivity produced varying levels of sensitivity among repetitions of the trials. Since these isolates tended to react as moderately resistant to highly resistant in greenhouse bioassays, isolates showing any reduced sensitivity under this test protocol were

considered resistant. All *C. michiganensis* subsp. *michiganensis* isolates were highly sensitive to copper *in-vitro*, agreeing with the sensitive standard. In greenhouse bioassay, moderate levels of infection occurred on copper-treated plants, a reaction consistent with field reports of sub-optimal control of bacterial canker. No field data was available in this study. The relatively poor field control by copper of a sensitive pathogen warrants additional investigation.

Introduction

Foliar bacterial diseases of tomato include bacterial spot, caused by *Xanthomonas euvesicatoria*, *X. vesicatoria, X. perforans*, and *X. gardneri*; bacterial speck, caused by *Pseudomonas syringae* pv. *tomato*; and bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis*. Copper products have been relied upon extensively for control of all three diseases (12; 16; 33). Such excessive usage has resulted in occurrences of resistance to copper in *Xanthomonas* spp. (1; 21; 33) and *P. syringae* pv. *tomato* (10; 14; 29). Although copper resistance has not been reported in *C. michiganensis* subsp. *michiganensis* (13), efforts to control bacterial canker with copper products are sometimes unsuccessful (20; 26; 27; 36; 37). The systemic, most damaging, phase of bacterial canker is particularly difficult to prevent with copper sprays (37).

For successful control of foliar bacterial diseases, spray programs must be designed to accommodate the copper sensitivity status of the bacterial population. Field surveys may be conducted to determine the incidence of resistant strains (1; 22; 29; 35). The ability of isolated field strains to tolerate copper in artificial growth media is used as a basis for conclusions regarding the likely efficacy of copper applications in the field (15; 28; 29). Thus, effective spray program design depends on the use of *in-vitro* copper resistance assessments that reflect the degree of control most likely to be encountered in the field.

Methods of copper resistance determination, such as minimum inhibitory concentration are not practical for large-scale surveys because of the resources required. Methods that are less complex and require fewer resources, such as assessing the amount of growth on solid media, are usually used for this purpose. Criteria for determining copper sensitivity designations have included growth (22; 31; 33; 38), confluent growth (2; 6; 15; 28; 39), or growth equal to a copper-free control (9; 29; 30) in the presence of differential concentrations of copper. There is

little consistency among the studies regarding the concentrations of copper used to distinguish the classes of sensitivity. Pernezny et al. (29) defined sensitivity classes by quantifying the growth on a single concentration of copper and expressing it as a percentage of the growth on a copper-free control medium. Most survey analyses involve application of cultures to the agar by streaking cells obtained from solid media. Aqueous suspensions of cells, followed by enumeration of colony-forming units, have also been used (35).

Media used to conduct copper resistance assays for *Xanthomonas* spp. include nutrient agar (NA) (1; 28; 38), glucose nutrient agar (GNA) (29; 30), casitone yeast extract (CYE) (22; 30) and sucrose peptone agar (SPA) (34), and mannitol-glutamate yeast extract agar (MGY) (5) while CYE (2; 39), GNA (29), King's medium B (KB) (35), and MGY (6; 15; 31) have been used for cultures of *P. syringae* pathovars. The choice of medium can influence copper resistance ratings through its effect on the bactericidal activity of copper. For example, CYE has a low affinity for complexing with copper ions, rendering them more available for biological activity (30; 41). However, Pernezny et al. (30) indicated this trait does not necessarily produce results that correspond with field results and suggested that an optimum medium may be species specific.

The availability of free copper ions is also influenced by pH and is maximized at low pH levels (18; 30). Few studies have been conducted to determine whether media favoring ionic forms of copper represent a more accurate measure of copper sensitivity in plant pathogenic bacteria (24). In some studies of copper resistance in *Xanthomonas* spp. (29; 33; 34) and *P. syringae* pv. *tomato* (39) pH levels have been reported, but there has been little reference to buffer selection and use. To be suitable for use in tests of copper sensitivity in bacteria, buffers must not complex with copper, and the chosen rates must maintain pH and not affect bacterial growth.

In *in-vitro* studies of bacterial resistance to copper, decisions regarding the level of bacterial growth needed to distinguish resistant and sensitive strains are often arbitrarily derived. Such non-validated sensitivity classifications encounter the risk of having low correspondence with the performance of copper in control of bacterial diseases in the field. Cooksey (13) reported poor field control of an isolate of *P. syringae* pv. *tomato* deemed sensitive based on *in-vitro* results. The success of an attempt to validate an *in-vitro* sensitivity classification can depend on the validation method chosen. Greenhouse bioassay results indicated sensitive

reactions for a pepper strain of *X. campestris* pv. *vesicatoria* resistant to copper based on field and *in-vitro* observations (1) and for a strain of *X. perforans* resistant to copper *in-vitro* (30). *In-vitro* experimental methods must be manipulated to produce results that reflect those that could be expected by the grower.

The objectives of this study were to develop a protocol for *in-vitro* determinations of the level of copper sensitivity in *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* cultures that would be practical for processing large numbers of samples, and to validate the results with bioassays.

Materials and Methods

Bacterial isolates used in developing the protocol are shown in the appendix, Table 2-10.

Bioassays

Outdoor mini-plots. Conventional small plot trials were conducted in which protective treatments were applied to trellised tomatoes on a conventional weekly schedule. The trials, located at the Highland Rim Research and Education Center in Springfield, TN, were arranged in a randomized complete block with four replications. Disease severity was assessed visually once per week. In the 2010 trial, treatments consisted of two, 3.4m-long rows on 1.1m spacing. Spacing between treatments was 2.1m, and alleys between blocks were 1.8m. Seven plants were set in each row, at 45cm spacing. One plant that had been inoculated with *Xanthomonas* spp., isolate 943, one week earlier and maintained in a greenhouse was planted at each end of each row. These plants served as a source of inoculum for test plants and were excluded from the analysis. Treatments were applied with a CO_2-pressurized (275.8 kPa) backpack sprayer at 187.1 to 655 L/ha. Products were applied initially at lowest labeled rates, increasing to highest labeled rates as plants grew. In 2011, plots consisted of one, 4.3 m-long row containing nine plants and inoculum consisted of *Xanthomonas* spp. isolate BL10. Treatments were Kocide 3000 (0.84 - 1.96 kg/ha), Kocide 3000 (0.84 - 1.96 kg/ha) + Manzate Pro-Stik (1.68 - 3.36 kg/ha), or Kocide 3000 (0.84 - 1.96 kg/ha) + Tanos (0.56 kg/ha) alternated with Kocide 3000 (0.84 - 1.96 kg/ha) + Manzate Pro-Stik (1.68 - 3.36 kg/ha).

Outdoor micro-plots, 2011. An experiment was designed to allow natural spread of the bacterium of interest to test plants, while allowing maintenance of the copper residue. All

plants were grown in a greenhouse for 4 to 6 weeks prior to field transplanting and hardened off daily by moving outside the greenhouse for several hours per day. Single-plant treatments were arranged in pairs (copper-treated or non-treated) in a single row, with pairs separated by a plant infected with the pathogen of interest. An infected plant was also planted at each end of the row. The plants were spaced 70cm apart and trellised. Treatments were arranged in a randomized complete block with four replications. Infected plants were produced as described for the greenhouse bioassays. Treated plants were sprayed with the labeled rate of Kocide 3000 (1.5 g/L) every 7 to10 days or after each significant rain event, whichever occurred sooner. Trials were conducted for two isolates of *Xanthomonas* spp., and one isolate each of *P. syringae* pv. *tomato* and *C. michiganensis* subsp. *michiganensis*. The trials were separated by space or time. Disease severity was visually assessed one to two times per week using percent leaf area affected.

Greenhouse. Plants of the cultivar 'Bonnie Best' were grown from seed in 10-cm-diameter pots containing soilless media for 4 to 6 wk. The plants were sprayed with a solution of copper hydroxide (Kocide 3000, 1.5 g/L) plus surfactant (Latron CS-7, 0.002%) or a surfactant control. After allowing plants to dry for at least 2 h, they were misted with a suspension of bacteria (ca. 10^8 CFU/ml) incubated in sucrose peptone broth (SPB) on a rotary shaker for 24 h. Plants were kept at a high humidity in plastic boxes for 36 h at 25°C to allow infection to occur. After 2 wk, disease severity was recorded according to percent leaf area, with both necrotic and chlorotic tissue considered affected. Treatments were replicated three times in a completely random design. For *C. michiganensis* subsp. *michiganensis*, four methods of inoculation were used to evaluate copper tolerance: (i)foliar-spray method following application of treatments and allowing to dry; (ii) foliar-spray method following application of light finger pressure to the stem to break the trichomes, application of treatments, and allowing to dry; (iii) using scissors dipped in inoculum to cut the petiole of the first true leaf of 12-day-old seedlings , following application of treatments and allowing to dry (11); (iv) lightly rubbing leaflets with sterile cotton balls dipped in inoculum and immediately spraying with the treatment.

Optimization of *in-vitro* resistance determination parameters

Medium composition and pH, buffer type and concentration, copper concentration, and test procedures were investigated to determine which combinations would give results that best approximated *in-planta* results for copper sensitivity determinations. Unless otherwise noted, the

following procedures were used in all of the tests. Following autoclaving, the media were cooled to 55°C, and filter-sterilized 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer and $CuSO_4 \cdot 5H_2O$ solutions were added to produce 20 mM and 200 µg/ml concentrations, respectively. Non-copper-amended media adjusted to pH 6.5 were used as controls. Media were poured into 100mm-diameter petri dishes and inoculated within three days. Bacteria were streaked with a loop onto the solidified medium, in triplicate. Treatments were arranged in a completely randomized design. Bacterial growth and pH were evaluated after three days of incubation at 25-26° C on a laboratory bench. The pH was measured on solidified, non-inoculated media with a pH Spear® (Oakton Instruments, Vernon Hills, IL).

Medium composition. GNA, NBY, CYE, and SPA were compared for their correspondence with the standard of sensitivity for *C. michiganensis* subsp. *michiganensis* before *in-vitro* test parameters were refined. Based on those findings, CYE and SPA were considered to have the best potential for reproducing *in-planta* results. All subsequent experiments with various experimental parameters were intended to maximize the correspondence between copper sensitivity ratings obtained on CYE or SPA and those obtained *in planta*.

Buffer optimization. *Complexing activity.* MES was selected for its known low complexing activity (19; 23). Attempts to confirm this trait were conducted by testing increasing concentrations of copper in the presence of MES for a linear effect on bacterial growth. After autoclaving and cooling to 55°C, SPA or CYE were amended with copper (0, 125, 250, or 375 µg/ml) and pH 6.8 MES buffer (10, 20, or 30 mM). A moderately resistant (1254) or a highly resistant (451b) isolate of *Xanthomonas* spp. was streaked onto the agar.
Buffering ability and growth effects. After autoclaving and cooling to 55°C, copper-free SPA was amended with pH 6.8 MES buffer (0, 12, or 20 mM). A culture of *Xanthomonas* spp. (six isolates), *P. syringae* pv. *tomato* (one isolate), or *C. michiganensis* subsp. *michiganensis* (one isolate) was streaked onto the medium. The possible effects of autoclaving on buffer performance were determined by comparing pH and bacterial growth on SPA to which MES had been added pre-autoclaving or post-autoclaving.

Optimum pH level. The pH level of SPA or CYE was adjusted to 6.2, 6.5, 6.8, or 7.0, using 0.1 N NaOH. After autoclaving, the media were cooled to 55°C and amended with MES buffer (20 mM). Cultures of *Xanthomonas* spp. (four isolates) or *C. michiganensis* subsp. *michiganensis* (two isolates) were streaked onto the medium

Effects of storage on prepared media. Effect of storage on the activity of MES and copper on bacteria was assessed using *Xanthomonas* spp. (seven isolates) or *C. michiganensis* subsp. *michiganensis* (one isolate). Copper-free SPA amended with MES (0, 12, or 20 mM) was inoculated simultaneously on 14-day-old and freshly-prepared medium. Effect of storage on the bacteriotoxic effect of copper was assessed by inoculating simultaneously 14-day-old and freshly-prepared copper-amended SPA (pH 6.2, 6.5, or 6.8). Growth was expressed as a percentage of that on copper-free controls.

Method of application of bacteria to medium. Two methods of applying bacteria to the medium were compared for their effect on copper sensitivity ratings: cell suspension and bacteria grown from an agar plate. Bacteria were grown in SPB (50 ml) shaken for 20 h at 100 rpm. The suspension (30 µl) was pipetted onto the agar and spread with a bent glass rod. Bacteria from solid agar were added by touching the edge of a sterile disposable loop one time to the bacteria and making four streaks onto the medium. Three isolates were used to inoculate copper-amended or copper-free SPA plates.

Culture incubation time. Test evaluations were conducted at three and four days after inoculation, to determine the most appropriate incubation time, based on correspondence with *in-planta* results.

Statistical analysis. Data obtained from field and greenhouse trials was analyzed with ARM 7 (Gylling Systems, Inc., Brookings, SD). For *in-vitro* copper resistance data, the PROC MIXED program of SAS (SAS, Inc., Cary, NC) was used.

Results

Bioassays

Mini-plots and micro-plots. Growing seasons in 2010 and 2011 were very dry, resulting in limited disease spread. Moderate disease severity occurred in the 2010 trial with *Xanthomonas* spp. isolate 943. The isolate displayed considerable sensitivity to copper, as evidenced by the significant control by the non-amended Kocide 3000 and only slight improvement in that control by the addition of the copper enhancement products Manzate Pro-Stik or Tanos (Table 2-1). Despite frequent sprinkler irrigation, the dry weather of 2011 limited disease development by *Xanthomonas* spp. isolate BL10. Absence of differences among treatments (Table 2-1) could

indicate a high level of resistance in the isolate, or more likely a reflection of low disease activity as evidenced by the control treatment. In micro-plots in 2011 (Table 2-2), copper sprays did not reduce the severity of disease caused by *Xanthomonas* spp. isolate 451b; whereas, disease caused by *Xanthomonas* spp. isolate MA11-1 was effectively prevented by copper sprays. Disease severity of *P. syringae* pv. *tomato* isolate 1318 was reduced by copper sprays. Disease was not successfully established in the bacterial canker trial.

Table 2-1. Copper sensitivity of two *Xanthomonas* spp. isolates as determined by degree of control by Kocide sprays with and without amendments in separate outdoor mini-plot field trials.

		2010 Isolate 943		2011 Isolate BL10
Treatment	Rate/ha[y]	Leaf area affected (%)	Total yield (kg/plot)	Leaf area affected (%)
Control		22.5 a[z]	32.4	2.0
Kocide 3000	0.84 - 1.96 kg	9.5 b	34.4	2.3
Kocide 3000 + Manzate Pro-Stik	0.84 - 1.96 kg 1.68 - 3.36 kg	6.8 bc	36.9	2.5
Kocide 3000 + Tanos alt Kocide 3000 + Manzate Pro-Stik	0.84 - 1.96 kg 0.56 kg 0.84 - 1.96 kg 1.68 kg - 3.36 kg	6.0 c	41.0	2.1
P > F		0.0001	0.577	0.989

[y] Applications made every 7 to10 days beginning two wk after transplanting. Rates were proportional to plant size.

[z] Column means followed by the same letter are not significantly different (LSD, *P*=0.05)

Greenhouse. The isolates of *Xanthomonas* spp. tested in greenhouse bioassays ranged from sensitive to highly resistant to copper (Figure 2-1). There was good correspondence between these results and those obtained in field plots (Table 2-3). The isolate of *P. syringae* pv. *tomato* produced infection severities on copper-sprayed plants of 34.0 and 12.7% of that on the non-sprayed control in the outdoor and greenhouse trials, respectively (data not shown). Isolates of *C. michiganensis* subsp. *michiganensis* tested in greenhouse bioassays produced moderately high disease ratings on copper-treated plants, for all four inoculation methods (data not shown). Data were not available from the outdoor microplot trial due to lack of infection.

Table 2-2. Copper sensitivity of two isolates of *Xanthomonas* spp. and one isolate of *Pseudomonas syringae* pv. *tomato* determined in separate outdoor micro-plots in 2011[y].

Treatment	*Xanthomonas* spp. isolate 451b Leaf area affected (%)	*Xanthomonas* spp. isolate MA11-1 No. lesions per leaflet	*P. syringae* pv. *tomato* isolate 1318 Leaf area affected (%)
Control	14.5	3.3	5.3
Kocide 3000[z]	14.3	0.1	1.8
P > F	0.703	0.045	0.018

[y] Values represent the mean of four, single-plant replications.
[z] Kocide 3000 was sprayed until runoff every 7 to 10 days at 1.52 g/L.

Table 2-3. Correspondence between greenhouse bioassay and field plot copper sensitivity determinations[y] for *Xanthomonas* spp. isolates.

Isolate	Greenhouse bioassay[z]	Field plots[z]
MA11-1	S	S
943	MR	MR
451b	HR	HR
BL10	HR	HR

[y] S – sensitive (Disease severity on copper-sprayed plants 0-30% of that on copper-free control); MR – moderately resistant (31-60%); HR – highly resistant (>60%)
[z] Greenhouse bioassays: Copper-treated and nontreated plants were inoculated with sprays of bacterial suspensions. Disease ratings were obtained after two weeks. Field bioassays: Plots were sprayed with copper products every 7 to 10 days and evaluated weekly over the course of a growing season. The source of inoculum for miniplots was an infected plant at each end of each 14-ft-long plot. For microplots, each plot consisted of one artificially infected plant, one plant sprayed with copper, and one nontreated control.

Optimization of *in-vitro* resistance determination parameters

Preliminary medium selection. Preliminary evidence for the suitability of four media for copper resistance determinations was obtained using isolates of *C. michiganensis* subsp. *michiganensis*, for which there is no documented case of resistance to copper. On CYE and SPA, all isolates appeared sensitive (Table 2-4); whereas, certain isolates appeared moderately resistant or highly resistant on GNA or NBY. Based on these results, CYE and SPA were considered potentially suitable for copper resistance determinations. Media GNA and NBY were reserved for additional evaluation after an optimized protocol was developed for SPA and CYE.

Table 2-4. Reactions of selected isolates of *Clavibacter michiganensis* subsp. *michiganensis* to copper on four media, determined with published *in-vitro* techniques[y].

Isolate	Glucose Nutrient Agar	Nutrient Broth Yeast Extract Agar	Casitone Yeast Extract Agar	Sucrose Peptone Agar
DA10	S[z]	HR	S	S
GR08	S	HR	S	S
NC7	S	MR	S	S
NC55	S	HR	S	S
931	HR	MR	S	S
451	HR	MR	S	S
411	MR	HR	S	S
611	MR	HR	S	S

[y] Ratings represent results of some commonly used experimental parameters for test media, such as concentration of copper sulfate (200 µg/ml), pH level (7.0), and absence of a buffer.

[z] S = sensitive (growth on copper-amended medium of 0-30% of non-amended control); MR = moderately resistant (31-60%); HR = highly resistant (>60%).

Buffer optimization. In preliminary tests, the buffer capacity of a 9 mM concentration MES was not sufficient to prevent pH depression caused by autoclaving and the addition of CuSO4·5H2O (200 µg/ml) to the media. A MES concentration of 20 mM is needed to maintain pH within approximately 0.2 unit (Figure 2-2), a range considered acceptable. Suppression of bacterial growth at increasing concentrations of copper was linear at all concentrations of buffer, indicating that there was no problem with copper ion chelation (Figure 2-3). Range of concentrations of MES tested did not affect growth of any of the isolates (Table 2-5). Autoclaving did not adversely affect the pH buffering ability or the innocuous nature of MES toward bacteria (data not shown).

Optimum pH level. Medium pH had a significant effect on copper toxicity to all isolates of *Xanthomonas* spp. that had appreciable levels of resistance (Figure 2-1). Resistance ratings for higher pH levels corresponded well with greenhouse bioassay resistance ratings (Table 2-6). pH 6.8 and 7.0 produced an equal number of resistance ratings matching those of greenhouse bioassays. The resistance ratings for *P. syringae* pv. *tomato* were 33.3, 100, 100, and 100 for pH=6.2, 6.5, 6.8, and 7.0, respectively. pH 6.2 was the most appropriate for this isolate, based on the infection level obtained on copper-sprayed plants (34% of non-sprayed) in a field micro-plot test. Isolates of *C. michiganensis* subsp. *michiganensis* were sensitive at all pH levels, but appeared moderately resistant in greenhouse bioassays.

Table 2-5. Effect of buffer on bacterial growth on sucrose peptone agar.

Isolate	Buffer[x] concentration (mM)		
	0	12	20
Xanthomonas spp.	--Bacterial growth[y]--		
451b	7.7[z]	7.3	7.7
MA11-2	7.7	7.3	7.0
HA11	7.7	7.3	7.0
MA11-1	7.0	6.3	6.3
BL10	7.3	7.3	7.7
1254	5.7	6.3	6.0
Pseudomonas syringae pv. *tomato*			
1318	5.3	6.0	5.7
Clavibacter michiganensis subsp. *michiganensis*			
NC7	3.7	4.0	4.7

[x] 2-(*N*-morpholino)ethanesulfonic acid, pH 6.8
[y] Scale of 0 to 10; 0=no growth, 10=abundant growth.
[z] Means within a row with a letter in common do not differ (LSD P=0.05). Absence of letters indicates no significant difference.

Medium selection. The four media (GNA, NBY, CYE, and SPA) previously evaluated using published techniques (Table 2-4) were compared using parameters optimized for SPA. Results for isolates of *Xanthomonas* spp. indicated poor correspondence between greenhouse bioassay results and those obtained on GNA or NBY (data not shown). There was a higher correlation (r = 0.83) with greenhouse data for isolates of *Xanthomonas* spp. on SPA than on CYE (r = 0.76).

Table 2-6. Effect of medium pH on accuracy of *in-vitro*[y] copper resistance determinations for isolates of *Xanthomonas* spp., as determined by comparisons with greenhouse bioassays[z].

Correspondence parameters for *Xanthomonas* spp. isolates 451b, 943, 1254, BL10, MA11-1, MA11-2, HA11, FL87-2, 3-3, and 3-20a	pH level			
	6.2	6.5	6.8	7.0
Correlation coefficient (r)	0.43	0.60	0.57	0.67
Percent isolates matching (by resistance rating)	10.0	40.0	60.0	60.0

[y] *In-vitro* ratings were determined with sucrose peptone agar amended with $CuSO_4 \cdot 5H_20$ (200 µg/ml) and 2-(*N*-morpholino)ethanesulfonic acid buffer (20 mM).
[z] Bioassay ratings were obtained in a greenhouse by inoculation of copper-sprayed tomato plants.

Isolates of *C. michiganensis* subsp. *michiganensis* were highly sensitive to copper on SPA and CYE (Table 2-7), differing from the moderately resistant reactions in greenhouse bioassays. An isolate of *P. syringae* pv. *tomato* that tested moderately resistant in a field microplot was highly resistant on SPA and CYE. SPA was selected for further study.

Method of bacterial application. Colony growth on agar was more difficult to quantify visually when cells were applied by pipetting suspensions than by streaking growth from a solid medium.

Culture incubation time. Resistance ratings increased between the third and fourth day after medium inoculation, for isolates of *Xanthomonas* spp. with intermediate ratings. Correlation with greenhouse bioassay ratings for the four isolates tested was slightly higher for three days ($r = 0.95$) than for four days ($r = 0.88$).

Effects of storage on prepared media. Media stored for 14 days produced higher resistance ratings than fresh media, with a greater difference at lower pH levels (Table 2-8). Storage did not affect resistance ratings for the sensitive isolate, MA11-1. A very slight decline in medium pH occurred during storage, but degree of change for the copper-amended media was similar to that for the copper-free control (data not shown). Aging of prepared medium did not affect the innocuous nature of MES toward bacteria (data not shown).

Table 2-7. Effect of media on *in-vitro* copper resistance ratings of selected bacterial isolates.

Isolate	Bacterial growth on copper-amended media[z] (percent of non- amended control)	
	Casitone Yeast Extract Agar	Sucrose Peptone Agar
Xanthomonas spp.		
451b	58.8[z]	83.3
943	83.3	100.0
1254	42.1	54.5
BL10	80.0	95.2
MA11-1	0.0	0.0
MA11-2	68.8	95.2
HA11	64.7	82.6
FL87-2	33.3	41.7
8-5	0.0	0.0
6-3	0.0	0.0
3-3	33.3	57.7
3-20 a	27.3	58.3
Pseudomonas syringae pv. *tomato*		
1318	80.0	88.9
Clavibacter michiganensis subsp. *michiganensis*		
611	0.0	0.0
DA10	0.0	0.0
411	0.0	0.0
NC7	0.0	0.0

[z] Media contained $CuSO_4 \cdot 5H_20$ (200 µg/ml) and 2-(*N*-morpholino) ethanesulfonic acid buffer (20 mM; pH 6.8).

Table 2-8. Comparison of bacterial growth at 0 and 14 days after medium preparation.

	Bacterial growth on copper-amended medium[y] (percent of non-amended control)					
	pH 6.2		pH 6.5		pH 6.8	
Isolate	0 d	14 d	0 d	14 d	0 d	14 d
Xanthomonas spp.						
451b	24.3 a[z]	85.7 b	71.4 a	114.3 b	85.7 a	114.3 b
MA11-2	13.7 a	85.7 b	41.1 a	114.3 b	86.3 a	114.3 b
HA11	12.5 a	86.3 b	66.3 a	109.6 b	87.5 a	100.0 b
MA11-1	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
BL10	51.8 a	95.9 b	75.9 a	100.0 b	84.3 a	95.9 a

[y] Media contained $CuSO_4 \cdot 5H_20$ (200 µg/ml) and 2-(*N*-morpholino) ethanesulfonic acid buffer (20mM; at varying pH levels).

[z] Means within column pairs within a row with a letter in common do not differ (LSD P=0.05)

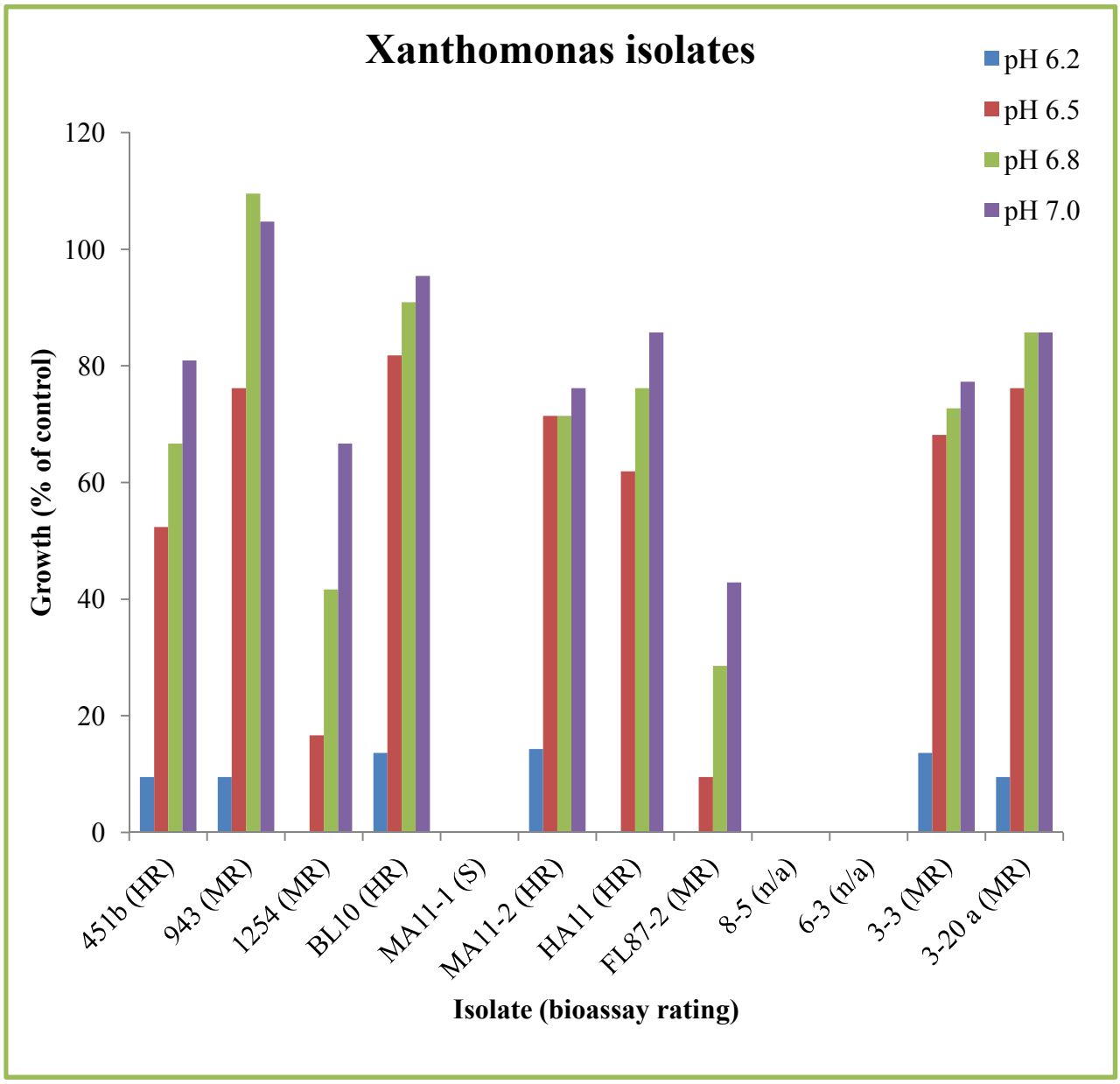

Figure 2-1. Effect of medium pH on copper sensitivity ratings for several *Xanthomonas* spp. isolates. *In-vitro* ratings were obtained from sucrose peptone agar amended with $CuSO_4 \cdot 5H_20$ (200 µg/ml). Bioassay ratings were obtained in a greenhouse by inoculation of copper-sprayed tomato plants.

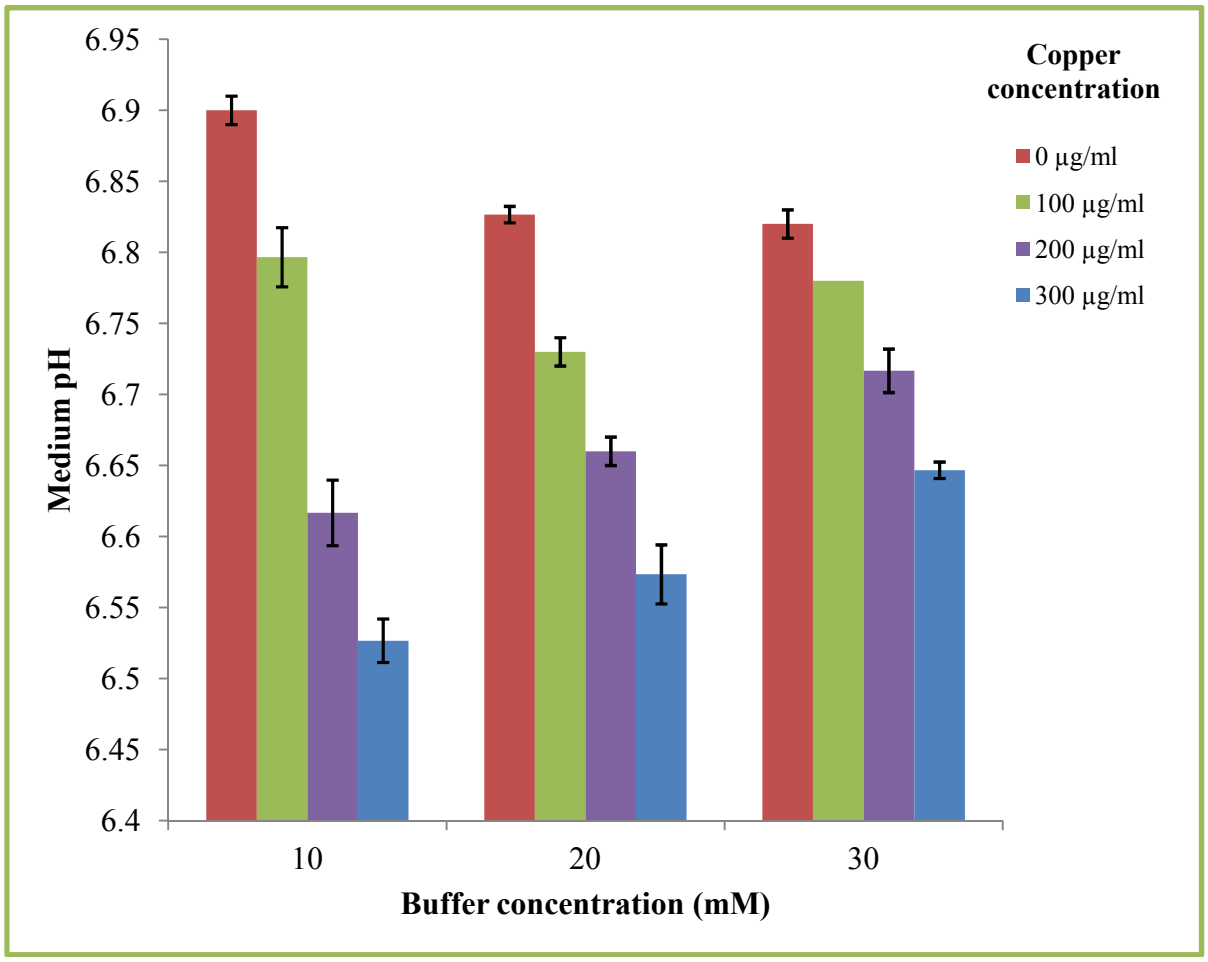

Figure 2-2. Effects of increasing concentrations of 2-(N-morpholino)ethanesulfonic acid, pH 6.8 buffer and increasing copper sulfate amendments on the post-autoclave pH of sucrose peptone agar.

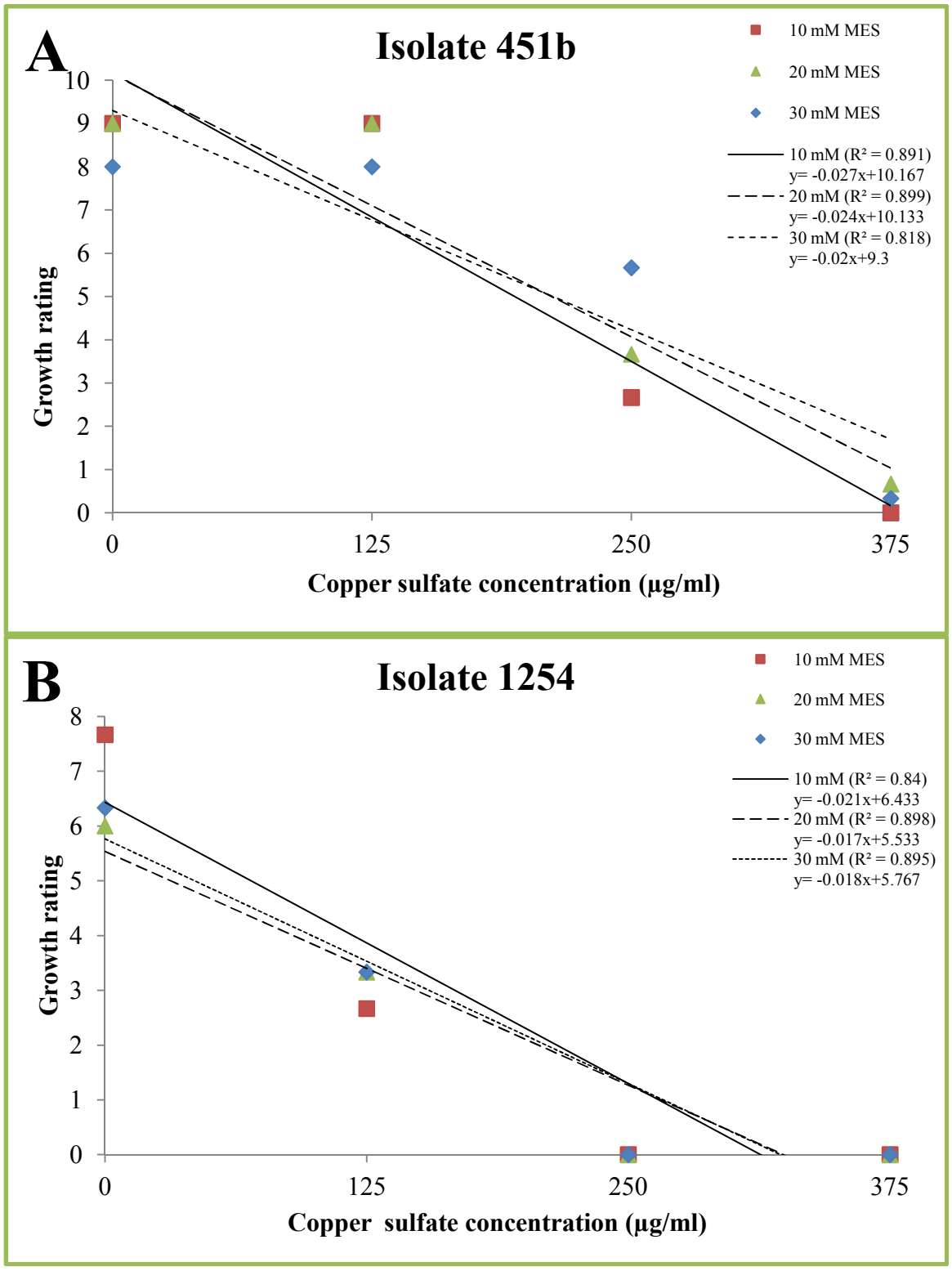

Figure 2-3. Effects of increasing rates of copper on growth of *Xanthomonas* spp. A, isolate 451b; and B, isolate 1254 on sucrose peptone agar at three concentrations of 2-(*N*-morpholino) ethanesulfonic acid, pH 6.8 buffer. Growth was assessed on a 0 (no growth) to 10 (abundant growth) scale.

Discussion

Conducting tests for microbial resistance to copper in culture media requires maintaining the copper in a bioavailable form while providing the nutrients needed for microbial growth. The variability encountered among repetitions of the tests in this study (Table 2-9) illustrates the difficulty in maintaining this balance and consistently producing accurate copper sensitivity results. Greater precision in determining the level of copper resistance for a bacterial population may be possible with procedures such as minimum inhibitory concentration, but the goal of this study was to develop a rapid procedure that would be practical to implement in large-scale projects such as surveys. The free ionic state (Cu^{2+}) is thought to be the only form of copper that is toxic to bacteria (24; 41). Since copper is highly reactive and a strong chelation agent, the challenge is to maintain the element at the concentration that produces results that reflect those that may be found in nature. Producing those results requires a certain combination of growth medium type, pH, buffer type and concentration, and experimental technique. This study attempted to develop such a protocol.

Culture media vary in content of organic and inorganic molecules that chelate copper, rendering it unavailable (17; 41). Selection of culture medium, a major factor in the accuracy of copper resistance studies, has received little attention (24; 30). In this study, GNA and NBY, containing high levels of metal-complexing nutrients (32), were quickly eliminated from consideration. Medium SPA (pH 6.8) was selected for *Xanthomonas* spp. tests because of its higher correlation with greenhouse bioassays than that for CYE. It may be possible to obtain better correlation data for CYE by reducing the copper concentration to compensate for the greater reactivity caused by the low-complexing nature of this medium. For *P. syringae* pv. *tomato*, pH 6.2 (SPA) was chosen, based on the correspondence of results with those of the greenhouse bioassay for the only isolate tested. The five isolates of *C. michiganensis* subsp. *michiganensis* tested were uniformly sensitive on all media at all pH levels, matching the reactions expected for this pathogen.

Medium pH influences the availability of free copper ions through its effect on the solubility of particulate copper (7) and on the availability of the components of the medium that serve as potential ligands for copper complexes (4). Thus, pH is crucial to the availability of free copper ions and proper interpretation of the relationship of total copper concentration to colony growth. This study found that dissolution of the copper sulfate molecule reduces medium pH, as

does the autoclaving process. Buffers, essential to countering the tendency for medium pH to fall, must be chosen carefully so as to avoid those that may precipitate copper or reduce bacterial growth. Those requirements were fulfilled by MES, which provides a buffering range that includes the desired pH. Correspondence between copper resistance ratings (low, moderate, or high) produced *in-vitro* with those produced in greenhouse bioassays was greatest for medium pH levels of 6.8 and 7.0. Since the range of optimum buffering activity for MES is pH 5.5 to 6.9 (40), pH 6.8 was selected. The slight decline in pH caused by autoclaving and the addition of copper is not beyond the pH range (6.5 to 7.0) in which the highest correlations with greenhouse bioassays were obtained.

Storage of prepared medium for 14 days reduced the bacteriotoxic effect of copper, possibly because of differential rates of reaction of copper-complexing ligands in the medium. The amount of copper added to the medium saturated the more-reactive chelators and left an excess of free copper ions available for biological activity (24). The free copper ions may be removed over time as the less-reactive chelators complex with them.

Application of cell cultures to test media by streaking cell growth from solid culture produced more quantifiable cell growth than that produced by pipetting cell suspensions. The latter produced superior results, however, for isolates incapable of growth on copper-amended media. When streaked, these isolates often produced a thin film of non-viable cells that could be mistaken for growth. These films were not sufficiently robust to change a sensitivity rating, however, and do not invalidate the streak method for routine applications.

The purpose of the greenhouse bioassay in this study was to validate the parameters under consideration for inclusion in the *in-vitro* testing protocol. However, greenhouse bioassays have occasionally been reported to be inaccurate (1; 13). The greenhouse methods used in this study for *Xanthomonas* spp. and *P. syringae* pv. *tomato* were validated by comparison with the results of field plot tests, which are assumed to fairly represent farm conditions. By extension, *in-vitro* results that corresponded well with greenhouse bioassays were considered to be realistic in terms of what a grower could expect from efforts to control the disease with copper sprays. Correlation analysis was difficult to conduct because of variability among repetitions of *in-vitro* assays. Isolates of *P. syringae* pv. *tomato* and *Xanthomonas* spp. having reduced sensitivity tended to vary in sensitivity from trial to trial. The changes in copper sensitivity could have been related to experimental conditions. The cultures used in developing the protocol were maintained

through serial transfers on GNA. Copper sensitivity for certain serially transferred isolates did not significantly differ from that of their counterparts stored in water when compared simultaneously, indicating that no genetic changes had taken place. A greenhouse trial conducted concurrently with an *in-vitro* trial using the same isolate indicated a moderately resistant reaction in the former and a highly resistant reaction in the latter. Extracellular polysaccharide production (8) is a possible explanation for increased resistance on agar plates; polysaccharide production *in-vitro* may be heavier than in nature. Sensitive isolates remained completely sensitive, even under culture conditions that mitigated the toxic effect of copper, such as high pH and medium aging. Considering the consistent nature of the sensitive reaction and the fact that isolates showing reduced sensitivity *in-vitro* tended to react as moderately resistant to highly resistant in greenhouse bioassays, it is suggested that isolates showing any reduced sensitivity under this test protocol be considered resistant.

All isolates of *C. michiganensis* subsp. *michiganensis* were highly sensitive to copper *in-vitro.* However, the copper sensitivity level of these isolates, as measured by severity of plant infections in the greenhouse, was only moderate, for all methods of inoculation evaluated in this study. The greenhouse bioassay results were consistent with the difficulties often encountered with attempts to control bacterial canker with copper spray programs (20; 26; 27; 36; 37), despite the absence of documented cases of resistance. The reduced ability of copper to kill sensitive cells in the phyllosphere could be related to the mode of cell ingress into the plant. However, results obtained with inoculation methods that involve wounding were similar to those that allow entry through hydathodes. An alternative explanation could be the copper chelation of organic compounds found in plant exudates (3; 25). However, reduced kill of *Xanthomonas* spp. and *P. syringae* pv. *tomato* by copper on plant surfaces did not occur. On the contrary, bactericidal effect was greater *in-planta* than *in-vitro*, for certain *Xanthomonas* spp. isolates. Despite the disparity between the *in-vitro* and *in-planta* results for *C. michiganensis* subsp. *michiganensis* isolates, the *in-vitro* experimental technique produced results that agree with a standard of copper sensitivity commonly accepted for this pathogen. Assuming this standard is accurate, additional investigation into the cause of mediocre control of bacterial canker with copper is warranted.

Table 2-9. Variation in isolate resistance to copper among repetitions of copper resistance assays.

Isolate	Number of trials[y]	Resistance rating (% of copper-free control)		
		Mean of means	Minimum and maximum means	Standard error
Xanthomonas spp.				
451b	7	74.6	23, 100	9.4
943	7	85.7	10, 110	12.9
1254	8	59.1	31, 93	7.7
BL10	7	82.1	67, 91	2.8
MA11-1	6	0.0	0, 0	0.0
MA11-2	6	82.3	37, 105	10.3
HA11	6	80.0	33, 100	10.2
FL87-2	6	44.2	10, 95	11.6
Pseudomonas syringae pv. *tomato*				
1318	3	45.7	33, 71	12.7
Clavibacter michiganensis subsp. *michiganensis*[z]				
611	3	1.7	0, 5	1.7
DA10	3	4.0	0, 12	4.0
411	3	1.3	0, 4	1.3
NC7	4	4.3	0, 17	4.3

[y] The data represent trials that included sucrose peptone agar at pH 6.8 (*Xanthomonas* spp. and *C. michiganensis* subsp. *michiganensis*) or pH 6.2 (*P. syringae* pv. *tomato*).

[z] The ratings for *C. michiganensis* subsp. *michiganensis* cultures represent apparent colony growth on copper-amended medium that was determined to be non-viable cells.

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Appendix

Table 2-10. Origin of isolates used in development of copper resistance testing protocol.

Isolate	Origin
Xanthomonas spp.	
451b	Bledsoe County, TN 2010
943	Bledsoe County, TN 2010
1254	Bledsoe County, TN 2010
BL10	Bledsoe County, TN 2010
MA11-1	Madison County, TN 2011
MA11-2	Madison County, TN 2011
HA11	Hardeman County, TN 2011
FL87-2	Florida 1987
8-5	Lauderdale County, TN 2011
6-3	Lauderdale County, TN 2011
3-3	Greene County, TN 2010
3-20 a	Greene County, TN 2010
Pseudomonas syringae pv. *tomato*	
1318	Tennessee 1991
Clavibacter michiganensis subsp. *michiganensis*	
DA10	Davidson County, TN 2010
NC7	North Carolina 2010
NC55	North Carolina 2010
GR08	Grainger County, TN 2008
931	Bledsoe County, TN 2010
451	Bledsoe County, TN 2010
411	Bledsoe County, TN 2010
611	Bledsoe County, TN 2010

CHAPTER 3 : SURVEY OF COPPER RESISTANCE IN TENNESSEE TOMATO PRODUCTION

Abstract

Foliar bacterial diseases are among the most important diseases of tomato in Tennessee. Bacterial spot is caused by four species: *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*, hereafter referred to as *Xanthomonas* spp. Bacterial speck is caused by *Pseudomonas syringae* pv. *tomato* and bacterial canker is caused by *Clavibacter michiganensis* subsp. *michiganensis.* Fixed copper products are relied upon extensively for control, due to a lack of effective and economical alternatives. Resistance to copper in tomato and pepper bacterial spot pathogens has been reported worldwide. Resistance to copper in *P. syringae* pv. *tomato* has been reported in many areas of the world also. Excessive reliance on copper has undoubtedly provided selection pressure that resulted in the development of resistant strains, and movement of infested plant materials has aided distribution of the strains. Resistance to copper quite likely exists in Tennessee, but the extent has never been determined. Knowledge of the degree and location of resistance in the state would lead to more prudent use of control products. The objectives of this study were to determine the incidence and distribution of copper resistance in Tennessee populations of these pathogens. Leaf samples were collected from 19 fields representing 195 acres in six Tennessee counties during 2010 and 2011. Bacterial colonies were isolated from single lesions or small portions of marginal necrosis from one leaf per acre. Isolates were identified by use of semi-selective media, enzyme-linked immunosorbent assay, Biolog (carbon utilization and chemical tolerance), pathogenicity tests, and physiological tests, in concert with field symptoms and bacterial colony appearance. Colonies were subjected to copper resistance testing on sucrose peptone agar adjusted to pH 6.8 with 2(*N*-morpholino) ethanesulfonic acid buffer (20 mM), and amended with 200 µg/ml $CuSO_4 \cdot 5H_2O$. Solidified media were promptly streaked with cells produced on solid agar, and incubated for three days. Bacterial growth was visually assessed on a 0 to 10 scale. The discriminatory reaction for resistance was any growth on copper-amended agar. One hundred sixty two isolates were identified as *Xanthomonas* spp., four as *C. michiganensis* subsp. m*ichiganensis,* and no *P. syringae* pv. *tomato* isolates were recovered. Copper resistance was detected in 95.1% of the *Xanthomonas* spp. isolates and 4.9% were sensitive. All *C. michiganensis* subsp. *michiganensis* isolates were sensitive.

Introduction

Tomato production in Tennessee is valued at 38.3 million dollars per year, placing the state in the top ten in the nation for tomato production (39). Bacterial spot, caused by as many as four *Xanthomonas* species: *X. euvesicatoria* (11) sp. nov., sp. rev.; *X. vesicatoria* (40), *X. perforans* (19) sp. nov.; and *X. gardneri* (38) sp. nov. as described by Jones et al. (19), hereafter referred to as *Xanthomonas* spp.; bacterial speck, caused by *Pseudomonas syringae* pv. *tomato* (25); and bacterial canker, *Clavibacter michiganensis* subsp. *michiganensis* (36), are among the most economically important diseases of tomatoes in Tennessee. In addition to yield and quality losses caused by the diseases, chemical control costs are high due to the rigor of spray programs needed to obtain satisfactory control.

Fixed copper products are relied upon extensively for control, due to lack of effective and economical alternatives (5). Resistance to copper in the tomato and pepper bacterial spot pathogens has been reported in Florida (37), California (8), Oklahoma (4), North Carolina (29), Georgia (14), Ohio (30), Mexico (1), Barbados (41), U.S. Virgin Islands, Costa Rica, Guadeloupe, Guatemala, Nicaragua, Puerto Rico (6), Australia (21), Korea (20), India (35), and Brazil (28). Resistance to copper in *P. syringae* pv. *tomato* has been reported in California (3), Virginia (2), Czech and Slovach Republics (26), Ontario, Greece (9), and Tanzania (33). Copper resistance has not been reported for *C. michiganensis* subsp. *michiganensis*, but control is often less than desirable (18; 23; 34). Excessive reliance on copper has undoubtedly provided selection pressure that resulted in the development of resistant strains, and movement of infested plant materials has aided distribution of the strains. Resistance to copper quite likely exists in Tennessee, but the extent has never been determined. Knowledge of the degree and location of resistance in the state would lead to more prudent use of control products. Alternative control product use would be justified and producers would spend less on ineffective products. The need for new product development by industry would be reinforced, and the data would provide support for alternative product special labels.

Proper pathogen identification is a prerequisite for a test of resistance to a pesticide. Accurate identification of bacterial pathogens causing multiple diseases of a host may be complicated by the presence of common symptoms, as is the case for *Xanthomonas* spp., *P. syringae* pv. *tomato,* and *C. michiganensis* subsp. *michiganensis* (15; 16). Many identification methods have been developed for these pathogens of tomato (31), but few accommodate the

throughput needed for the number of samples needed in a large study. A consideration of plant symptoms in concert with bacterial colony morphology can narrow the choice of colonies retained for testing with other diagnostic procedures.

Many different approaches to determination of copper resistance in these pathogens have been employed. There are few accounts of validation of experimental techniques, and assumptions regarding the accuracy of these techniques may be misplaced (22; 27). *In-vitro* protocols specific to *Xanthomonas* spp., *P. syringae* pv. *tomato,* and *C. michiganensis* subsp. *michiganensis* have been developed and validated (Chapter 2).

The objective of this study was to determine the incidence and distribution of resistance to copper in Tennessee populations of the bacterial spot, bacterial speck, and bacterial canker pathogens of tomato.

Materials and Methods

Field survey collection. Samples of leaves exhibiting symptoms of bacterial diseases were collected from Tennessee tomato fields during 2010 and 2011. Single-leaf samples, each representing one acre, were collected in a systematic fashion from equally-spaced locations. Samples were transported to the laboratory in an ice chest, stored in a cooler, and processed promptly.

Isolation and purification of pathogens. Several discrete lesions or small pieces of necrotic tissue taken from the margin of a leaf were disinfested, macerated in one drop of sterile, distilled water (SDW), and allowed to soak for 2 min. A loopful of the bacterial suspension from the leaf macerate was streaked onto glucose nutrient agar (GNA) and incubated at 25°C for 48 to 72 h. Colonies that appeared consistent with *Xanthomonas* spp. (convex, mucoid, yellow), *P. syringae* pv. *tomato* (thin, cream colored or translucent), or *C. michiganensis* subsp. *michiganensis* (mucoid, yellow, orange/yellow, or cream colored) were purified by repeated streaking of single colonies on GNA and stored in SDW at 25°C.

Confirmation of pathogen identity. *Semi-selective media.* Cell numbers were increased on GNA and streaked on media appropriate to colony appearance and field plant symptoms for *Xanthomonas* spp. (12; 29), *P. syringae* pv. *tomato* (24), and *C. michiganensis* subsp. *michiganensis* (13; 15; 42) (Table 3-1).

Nutritional, biochemical, and serological testing. In addition to the procedures for suspected *Xanthomonas* spp. isolates shown in Table 3-1, one isolate per field was randomly selected for testing with the Biolog microbial identification system, using the MLM software and the Gen III database (Biolog Inc., Hayward, CA). In preliminary trials, attempts to validate the system for identification of a known isolate of *P. syringae* pv. *tomato* was unsuccessful. Suspected isolates of *P. syringae* pv. *tomato* were confirmed by levan production, utilization of sucrose as a carbon source, inability to use erythritol as a carbon source, and inability to cause pectolysis according to Schaad et al. (32). Confirmation of suspected *C. michiganensis* subsp. *michiganensis* isolates was attempted with an enzyme linked immunosorbent assay (ELISA) ImmunoStrip test (Agdia, Elkhart, IN, USA) and with Biolog.

Pathogenicity testing. Pathogenicity tests were conducted on one isolate per field for suspected *Xanthomonas* isolates. Plants of the cultivar 'Fletcher' were grown from seed in 10-cm-diameter pots containing soilless media for 4 to 6 wk. The plants were misted with a suspension of bacteria (10^8 CFU/ml) that had been shaken in sucrose peptone broth for 18 h, followed by incubation at high humidity in plastic boxes for 36 h at 25-26°C. The plants were removed to greenhouse benches and observed for the development of bacterial spot symptoms. Similar tests were conducted for certain fluorescent pseudomonad isolates. For suspected *C. michiganensis* subsp. *michiganensis* isolates, 2-wk-old seedlings were inoculated by cutting the first true leaf at the point of attachment with scissors dipped in a broth suspension of cells (10^8 CFU/ml bacterial) (7). Seedlings were kept in a moisture chamber for 36 h after inoculation. Disease severity was rated at 16 to 17 days after inoculation using a disease assessment key according to Chang et al. (7).

***In-vitro* copper resistance assays.** Purified isolates were streaked onto sucrose peptone agar that was adjusted to pH 6.8 prior to autoclaving. After cooling to 55°C, the medium was amended with filter sterilized (0.22 µm pore size) 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (20 mM; pH 6.8) and 200 µg/ml $CuSO_4 \cdot 5H_2O$ or with MES (20 mM; pH 6.8) only (control). A small amount of colony growth was used to make four, 5-cm streaks per replicate plate, using a disposable loop. Each isolate was replicated three times and completely randomized. Cultures were incubated for 72 h at 30°C (*Xanthomonas* spp.) or 25°C (*P. syringae* pv. *tomato* or *C. michiganensis* subsp. *michiganensis*) and rated on a scale of 0 to 10, with 0=no growth and 10=robust growth.

Table 3-1. Process used for identification of plant pathogenic bacteria in a survey of 19 Tennessee tomato fields in 2010 and 2011.

Colony appearance on glucose nutrient agar	Diagnostic medium[w]	Preliminary conclusion based on colony characteristics	Additional tests
Mucoid and yellow color	YDC	*Xanthomonas* spp., if convex, mucoid, yellow	Pathogenicity tests
	SCM[x]	*C. michiganensis* subsp. *michiganensis*, if gray flecks in clear, mucoid growth	ELISA[y] and pathogenicity tests
	mSCM[x]	*C. michiganensis* subsp. *michiganensis*, if yellow flecks in clear, mucoid growth	
Cream color	KBC	*P. syringae pv. tomato*, if fluorescent	Biochemical, nutritional[z], and pathogenicity tests
	SCM[x]	*C. michiganensis* subsp. *michiganensis*, if gray flecks in clear, mucoid growth	ELISA and pathogenicity tests
	mSCM[x]	*C. michiganensis* subsp. *michiganensis*, if yellow flecks in clear, mucoid growth	
Orange-yellow color	SCM[x]	*C. michiganensis* subsp. *michiganensis*, if gray flecks in clear, mucoid growth	ELISA and pathogenicity tests
	mSCM[x]	*C. michiganensis* subsp. *michiganensis*, if yellow flecks in clear, mucoid growth	
Thin and translucent	KBC	*P. syringae pv. tomato*, if fluorescent	Biochemical, nutritional, and pathogenicity tests

[w] YDC= yeast dextrose carbonate agar; KBC= King's medium B amended with cyclohexamide and cephalexin; SCM= semiselective medium for *C. michiganense*; mSCM= modified semiselective medium for *C. michiganense.*

[x] Conducted only if bacterial canker symptoms present in field. SCM and mSCM used simultaneously (15).

[y] Enzyme linked immunosorbent assay, ELISA ImmunoStrip test (Agdia, Elkhart, IN, USA).

[z] Refer to text for materials and methods.

Growth on the copper-amended medium was expressed as a percent of the growth on the non-amended control. Isolates were considered sensitive if there was no growth on copper-

amended plates and abundant growth on the control plates. Resistance was indicated if there was growth on the copper-amended plates (Chapter 2).

Results

Leaf samples were collected from 19 fields in six counties representing 195 acres of commercial tomato production (Table 3-2). The 11 farms in the survey included 113 acres from northeastern Tennessee, 21 acres from central-eastern Tennessee, 14 acres from central Tennessee, and 47 acres from western Tennessee. Copper products had been applied in all fields. Symptoms typical of bacterial spot or speck (discrete lesions) were present in all fields. Leaf marginal necrosis was observed in certain fields in Bledsoe, Grainger, and Washington counties. Fruit lesions were sparse in most fields.

One hundred sixty-two bacterial isolates, representing all sampled fields, were identified as *Xanthomonas* spp., based on colony characteristics on YDC. A representative number of identifications was confirmed by Biolog and pathogenicity tests. Bacterial speck symptoms and conditions conducive to the disease were present in some locations. However, pseudomonad colonies fluorescent on KBC were identified as saprophytic or weakly pathogenic species, according to nutritional and physiological reactions for utilization of sucrose and erythritol, levan production, and pectolytic activity. *C. michiganensis* subsp. *michiganensis* was found only in a Bledsoe County field. Four isolates tentatively identified on GNA were confirmed with ELISA and pathogenicity tests. Suspect isolates based on reactions on SCM and mSCM failed confirmation. Copper tolerance was prevalent among the *Xanthomonas* spp. populations sampled (Table 3-2). According to *in-vitro* tests, 92.6% of the isolates were highly resistant, with colony growth on copper-amended medium greater than 60% of that on the control medium. A high level of sensitivity was found in only 4.9% of the isolates. Lauderdale County, where copper use tended to be less intense, was the only area of the state in which sensitive isolates were found. Few isolates in the survey (2.5%) showed intermediate levels of resistance. Isolates in this category tend to produce variable results *in-vitro* (Chapter 2) and, unless confirmed with bioassays, should be considered highly resistant. All four isolates of *C. michiganensis* subsp. *michiganensis* were characterized as sensitive.

Table 3-2. Field information and copper sensitivity status of foliar bacterial pathogens collected from a survey of Tennessee tomato fields.

Year and county	Plant origin[x]	Copper application (no.)	Field size (acres)	Number of isolates: *Xanthomonas* spp., by copper sensitivity reaction[y] S	R	Number of isolates: *Clavibacter michiganensis* subsp. *michiganensis*[z]
2010						
Bledsoe	Southern Georgia	14	14	0	8	4
Greene	Western North Carolina	10-12	11	0	2	0
Greene	Western North Carolina	10-12	23	0	14	0
Grainger	Grainger County	10-12	8	0	3	0
2011						
Lauderdale	on-farm	5-6	7	0	4	0
Lauderdale	on-farm	5-6	7	3	1	0
Lauderdale	on-farm	2	9	4	4	0
Lauderdale	on-farm	2	6	1	5	0
Lauderdale	on-farm	2	10	0	8	0
Lauderdale	on-farm	2	8	0	8	0
Grainger	on-farm	10	5	0	6	0
Grainger	on-farm	10	4	0	4	0
Grainger	on-farm	11	4	0	1	0
Washington	on-farm	7	14	0	15	0
Washington	Southern Georgia	10	8	0	8	0
Washington	on-farm	12	27	0	33	0
Washington	on-farm	12	14	0	15	0
Greene	Western North Carolina	10-12	6	0	5	0
Cocke	Western North Carolina	10-12	10	0	10	0

[x] Transplants were imported to the farm unless the source was indicated as "on-farm".
[y] R = resistant, S = sensitive.
[z] All *C. michiganensis* subsp. *michiganensis* isolates were characterized as sensitive *in-vitro.*

Ten of the 162 isolates of *Xanthomonas* spp. were obtained from leaf marginal necrosis, a symptom usually associated with bacterial canker. All 10 xanthomonads collected from marginal necrosis were characterized as highly resistant to copper.

In addition to the field survey, samples were collected from four home gardens. *Xanthomonas* spp. isolates, two of which were highly resistant to copper and one of which was sensitive, were recovered from three of the gardens. From the remaining garden, a copper-sensitive isolate of *C. michiganensis* subsp. *michiganensis* was recovered from plants displaying symptoms of bacterial canker.

Discussion

The tomato-pathogenic *Xanthomonas* spp. group was found in every field surveyed. Bacterial spot is typically more prevalent in Tennessee than bacterial speck or bacterial canker in summers with above-average temperatures, such as occurred in 2010 and 2011. *P. syringae* pv. *tomato* was not recovered and *C. michiganensis* subsp. *michiganensis* was found in only one field. Typical foliar symptoms of bacterial spot, discrete lesions with confluence in advanced cases, were observed in each field. In addition, the Bledsoe County field displayed symptoms of bacterial canker (marginal necrosis). Ten samples of *Xanthomonas* spp. were obtained from marginally necrotic areas of leaf tissue in the absence of *C. michiganensis* subsp. *michiganensis*. Although the necrotic area was thinner than that typical of bacterial canker, this symptom is not usually associated with bacterial spot.

Fewer pathogenic species were successfully isolated than expected. Plant samples were handled carefully and bacterial extractions were conducted expeditiously, but the growth of non-target bacteria on the culture plates complicated efforts to visually identify and isolate the three pathogens of interest. While most of the non-target bacteria were saprophytic, etiology determinations can also be complicated by the fact that other pseudomonads can induce lesions on tomato leaves (17). *P. viridiflava* was isolated from a Bledsoe County sample and caused mild symptoms in a greenhouse test. Cultures from some fields were stored in sterile water (10) for as long as one year, and some pathogen loss may have occurred. The differential media (SCM, mSCM, and KBC) employed in the survey did not effectively contribute toward isolation of the three pathogens from mixed cultures. The Biolog system correctly identified known cultures of *Xanthomonas* spp. (as *X. campestris* pv. *vesicatoria*) and *C. michiganensis* subsp. *michiganensis*, but not *P. syringae* pv. *tomato*. PCR-based methods would enhance isolation efforts by identifying pathogens present in non-purified cultures. Multiplex PCR would reduce

the cost of conducting large studies such as this, by simultaneously identifying all three pathogens

Copper resistance was widespread among *Xanthomonas* spp. populations, occurring in all surveyed areas of the state. Few *Xanthomonas* spp. isolates were sensitive to copper, occurring in only three of 19 fields. The fact that highly resistant isolates occurred in fields containing highly sensitive isolates suggests multiple sources of field infestation or that the transition from copper sensitivity to resistance is a rapid process. The latter is supported by the fact that few isolates in the survey (2.5%) showed intermediate levels of resistance in the single performance of the test. Since there was no relationship between the incidence of resistance and the source of the transplants, no conclusion can be made regarding the source of the infestations. Based on the results of this survey, there appears to be no safety in on-farm production of transplants. However, transplants are not the only possible source of field infestation.

The *in-vitro* copper resistance assay used in this study was effective in rapidly characterizing bacterial isolates allowing the processing of a large number of samples in a relatively short amount of time. All isolates showing reduced sensitivity were considered resistant because of a tendency to vary in the degree of resistance *in-vitro* (Chapter 2). Nonetheless, the resistant reactions obtained with this experimental protocol represent biologically accurate designations, applicable to field situations (Chapter 2).

Resistance to copper products is widespread in Tennessee and the use of copper bactericides may be delivering far less than satisfactory results to producers. Anecdotal evidence exists for poor control of bacterial diseases during periods of high rainfall, even with frequent applications of copper products. Tank mixing copper products with ethylene bis-dithiocarbamate fungicides, obtaining complete coverage of vines, following a preventative spray schedule, and using recommended alternative products such as acibenzolar-S-methyl are important practices for managing foliar diseases caused by copper-resistant bacteria. There is a need for alternative product labeling. New products that are both economical and effective would alleviate the problem of bacterial resistance and allow copper to remain useful in a rotation of bactericides. The negative impact of chemical resistance places more importance on seed certification and plant origin. Growers should be aware of seedborne pathogen issues with transplants, temperature and moisture conditions conducive to pathogen development, and transportation conditions that would promote disease spread before plants arrive at the field. Awareness of

potential disease threats could prompt plant suppliers to pursue healthier transplant culture and producers who grow their own transplants to make appropriate changes in their own operations. To remain useful to commercial producers, a rotation that encourages reduced use of copper while incorporating existing and new products should be recommended.

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CHAPTER 4 : DEVELOPMENT OF MULTIPLEX PCR TO DETECT THREE BACTERIAL PATHOGENS OF TOMATO FROM SYMPTOMATIC PLANTS

Abstract

Diagnosis of foliar bacterial diseases of tomato has been facilitated the use of polymerase chain reaction (PCR) and primers for the detection of pathogens. Furthermore, species-specific primers have been developed to detect specific pathogens in mixed bacterial populations. Previous work in other laboratories has evaluated multiplex PCR reactions for simultaneous detection of multiple species of bacterial pathogens using pure cultures. The objective of this study was to optimize a multiplex PCR protocol for specific detection of *Xanthomonas* species, *Pseudomonas syringae* pv. *tomato*, and *Clavibacter michiganensis* subsp. *michiganensis* in field samples of symptomatic tomato foliage. The primers selected were RST65 and RST69 (specific for *Xanthomonas* spp.), MM5F and MM5R (specific for *Pseudomonas syringae* pv. *tomato*), and CMM5 and CMM6 (specific for *Clavibacter michiganensis* subsp. *michiganensis*). Temperature parameters and concentrations of reaction components for single PCR reactions with these pathogens and their respective primers were harmonized to allow for DNA amplification of the three pathogens in one reaction. The resulting multiplex PCR gave optimal results for all three primer pairs with a touchdown protocol ranging from 63 to 57°C for 12 cycles, then 24 more cycles with an annealing temperature of 57°C. Pure cultures were used to develop the protocol, and samples of plant tissue with single and mixed infections caused by the three pathogens were used to test the ability of the multiplex PCR reaction to detect these pathogens. A sensitive multiplex PCR protocol for evaluation of symptomatic field samples will provide a valuable diagnostic tool that will allow for rapid pathogen identification and facilitate pathogen population studies.

Introduction

Bacterial spot, caused by *Xanthomonas* species, occurs in warm, moist climates. Lesions occur on leaves, stems, and fruit and vary in appearance, depending on environment. Lesions are usually < 3 mm in diameter, but may be larger on fruit (5). Bacterial spot symptoms can be confused with bacterial speck, which is caused by *Pseudomonas syringae* pv. *tomato,* and is problematic in cooler climates. Speck lesions are generally smaller than those of bacterial spot and can also be found on stems, leaves, and fruit. Lesions of both diseases may coalesce to form large necrotic areas (5). A third disease, bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis*, is potentially the most damaging because infections can be systemic. Symptoms include wilt, stem cankers, turning down of lower leaves, and turning up of young leaflets. Local infections cause marginal necrosis of leaves and raised, brown, scabby lesions, occasionally with white haloes, on the fruit (5). These three diseases cause substantial losses to tomato producers through increased defoliation, reduced fruit quality and yield, and increased costs of disease control.

Several *in-vitro* techniques and assays, such as isolation on semi-selective or differential media (3; 4; 7; 11) coupled with physical characteristics, carbon utilization patterns, enzyme linked immunosorbent assays (ELISA), denaturing gradient gel electrophoresis (DGGE), and polymerase chain reaction (PCR) have gained popularity and are used for detection and identification of plant bacterial pathogens. In particular, PCR identification of nucleic acids has been developed for use with universal or species-specific primers. The use of PCR for simultaneous detection of *Xanthomonas* spp., *P. syringae* pv. *tomato*, and, *C. michiganensis* subsp. *michiganensis*, from leaf samples could reduce costs and time needed for identification, and facilitate population studies for large production areas. Currently, recommended controls (copper compounds) for foliar bacterial diseases of tomato are similar, regardless of species. However, specific identification would be useful for determining the geographic distribution of the three pathogens in a tomato production region, as well as evidence of copper resistance in *Xanthomonas* and *Pseudomonas*. A study on multiplex PCR for detection of these three tomato pathogens has been documented with a different set of species-specific primers using pure cultures of the pathogens only (9). However, for such a technique to be valuable to disease diagnostics for tomato production, detection of pathogens from mixed bacterial communities on symptomatic plant tissue is desired.

In order to detect DNA of the three tomato pathogens of interest, multiplex PCR has to include three different species-specific primer pairs and a harmonized reaction annealing temperature for those primers. This would allow all three reactions to occur with minimal competitive interference. Consideration needs to be given to the decreased sensitivity of the assay compared to single PCR and proper primer selection is needed to eliminate primer/DNA cross reaction. A well-known primer set for detection of *C. michiganensis* subsp. *michiganensis,* CMM5 and CMM6, developed in 1995 (2), was derived from the *pat-1* gene, which is involved in pathogenicity and produces a 614-bp PCR product. The primer set has been tested against other Gram-positive plant pathogens including other subspecies of *Clavibacter*, all which yielded no band in gel electrophoresis (2). Modifications were published to amend the original sequence due to a base pair mismatch; this report also discussed changing the annealing temperature for improved performance (6). *Pseudomonas syringae* pv. *tomato* can be detected with the primer set, MM5F and MM5R developed in 2005 (12), which was derived from the $HrpZ_{Pst}$ gene found on the *hrp/hrc* pathogenicity island. The PCR amplicon produced is 532 bp. The MM5F and MM5R primer set was also tested against several different pathovars and other Gram negative, plant pathogenic bacteria, including *Pseudomonas syringae* pv. *syringae*, which often produces similar symptoms. None produced a band in gel electrophoresis (12). The *Xanthomonas spp*. the primer set, RST 65 and RST 69, was developed in 2004 (8). This primer set was derived from the *hrpB* operon from the *hrp* gene cluster that is involved with pathogenicity, and produces a 420-bp product (8). This primer set was tested against various Gram negative plant pathogenic bacteria. The product was produced with 36 of 40 strains of *Xanthomonas* tested. It did not produce a band with any other genera of bacteria that were tested. To differentiate the four bacterial spot species outlined by Jones in 2004, restriction enzyme analysis was conducted (8).

Other primer sets have been produced for all of these three pathogens, but these are of particular interest because the size differences of the three PCR amplicons allow them to be differentiated in agarose gel electrophoresis. Once the multiplex reaction parameters for detection of the three pathogens is established, sequencing is no longer needed due to the specificity of the primer and confidence in the lack of cross reaction taking place; therefore the appearance of bands of the expected amplicon sizes in agarose electrophoresis gels can be considered positive for detection of the pathogens.

The objective of this study was to develop a multiplex PCR protocol that facilitates detection of three bacterial pathogens of tomato, namely *Xanthomonas* spp., *P. syringae* pv. *tomato*, and, *C. michiganensis* subsp. *michiganensis*, in mixed bacterial populations from symptomatic plants.

Materials and Methods

Bacterial Pathogens. Isolates of *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis*, originally isolated from tomato, were used from stored collections as known standards. Identity was confirmed with published PCR protocols and species-specific primers that map to pathogenicity related loci (Table 4-1), and comparison of sequences obtained with sequences in GenBank.

Isolation of Bacteria from Symptomatic Field Plants. Lesions were excised from tomato leaflets, surface-sterilized in 10% bleach (1 min) and 95% ethanol (1 min), and rinsed with 3 ml of deionized (DI) water. Tissue was macerated in DI water, streaked onto nutrient broth yeast extract agar (NBY), and incubated for 3 to 4 days. A loopful of bacterial lawn was stored in sterile DI water until needed. Prior to DNA extraction, water culture (100 µl) was plated onto tryptic soy agar (TSA) for 3 days to increase the number of bacterial cells for extraction of target DNA, a method termed Bio-PCR (10).

DNA Extraction from field samples and standards. TSA plates were washed with 1 ml of TE buffer, and a 500-µl aliquot of suspension was added to a 1.5-ml Eppendorf tube. DNA was extracted with a Wizard Genomic DNA extraction kit (Promega, Madison, WI). Extracted DNA was stored at 4°C for 3 to 5 days to allow dissolution of DNA in TE + 0.02% Triton X rehydration solution.

DNA Quantification and Dilution. In general, the DNA concentration of samples was adjusted to 5 ng/µl (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad, CA) for PCR.

Primers. Primer pairs for detection of *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato*, and *Xanthomonas* spp. were selected that were specific for pathogenicity loci (Table 1). For *Xanthomonas* spp., RST65 and RST69 (derived from the *hrp B* operon), were used. The primer pair MM5F and MM5R (derived from an internal fragment corresponding to the $hrpZ_{Pst}$ gene that maps on a pathogenicity-associated operon of the *hrp/hrc* pathogenicity

island), was chosen for detection of *P. syringae* pv. *tomato*. To detect *C. michiganensis* subsp. *michiganensis*, the primers CMM5 and CMM6 (derived from partial nucleotide sequence of the *pat*-1 plasmid-borne gene) were utilized. PCR amplicons were 420-, 519-, and 609-bp, for *Xanthomonas* spp., *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis*, respectively, and could be visualized as individual bands in an agarose gel following amplification of DNA from mixed cultures.

Gel Electrophoresis and Visualization. Amplicons were separated in 1 to 2% agarose gels with 8 µl of SYBR Safe (Invitrogen, Carlsbad, CA, USA) added. Electrophoresis was conducted at 80 to 85 volts for 3 to 3.5 h. Gels were visualized with Carestream Molecular Imaging Software (Woodbridge, CT) and a Fisher Scientific UV documentation system (Pittsburgh, PA).

Development of Multiplex PCR Protocol.

1. DNA was extracted from pure cultures of each pathogen separately, and amplified with published protocols (Table 4-1).
2. Thermal cycler conditions and reaction components of simplex PCR protocols for each pathogen were harmonized so that all three pathogens could be detected with a single protocol.
3. Pure cultures of the three pathogens were mixed and composite DNA samples were extracted and amplified with the new protocol. Gradient temperature experiments (57 to 65°C) were conducted to determine the optimal annealing temperature for multiplex PCR.
4. DNA from naturally occurring bacteria (extracted separately) from leaves of field-grown plants was added to each pathogen DNA individually, and each pathogen DNA was amplified.
5. DNA was extracted from a composite culture of *C. michiganensis* subsp. *michiganensis, P. syringae* pv. *tomato,* and *Xanthomonas* spp., and naturally occurring bacteria on field grown leaves to simulate field samples. To further enhance DNA amplification of target pathogens, a touchdown protocol with annealing temperature ranging from 63 to 57°C was developed.
6. Volumes and concentrations of reaction components for the multiplex PCR were varied to optimize detection of the three pathogens.

Table 4-1. Initial parameters of simplex PCR for identification of *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato,* and *Xanthomonas* spp. from DNA extractions of bacteria in pure culture

Bacterial pathogen	Primers pairs	Protocol	Reference
Clavibacter michiganensis subsp. *michiganensis*	CMM5: 5`-GCGAATACGCCCATATCAA-3`* CMM6: 5`-CGTCAGGAGGTCGCTAATA-3`	Initial: 5 min, 95°C 30 cycles Denaturation: 45 s, 94°C* Annealing: 30 s, 65°C Extension: 30 s, 72°C	Dreier *et al.* 1995 Kokošková *et al.* 2010 noted a mismatch in the CMM5 primer and changed the original protocol.
Pseudomonas syringae pv. *tomato*	MM5F: 5`-GAACGAGCTGAAGGAAGACA-3` MM5R: 5`-CAGCCTGGTTAGTCTGGTTA-3`	Initial: 5 min, 94°C 36 cycles Denaturation: 1 min, 94°C Annealing: 1 min, 57°C Extension: 1 min, 72°C Final: 10 min, 72°C	Zaccardelli *et al.* 2005
Xanthomonas spp.	RST65: 5`-GTCGTCGTTACGGCAAGGTGGTCG-3` RST69: 5`-TCGCCCAGCGTCATCAGGCCATC-3`	Initial: 2 min, 95°C 40 cycles Denaturation: 40 s, 95°C Annealing: 40 s, 63°C Extension: 50 s, 72°C Final: 5 min, 72°C	Obradovic *et al.* 2004 Cuppels *et al.* 2006 (1). This paper is the source of protocol used.

Plant assay. To simulate extraction of DNA from symptomatic plants when mixed pathogen populations were present, a plant assay was conducted. Terminal leaflets of 8-week-old 'Mountain Fresh' tomato plants were inoculated with *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato*, and *Xanthomonas* spp., singly or in combination. Treatments were: sterile water (control), *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato*, *Xanthomonas* spp., *C. michiganensis* subsp. *michiganensis* + *P. syringae* pv. *tomato*, *C. michiganensis* subsp. *michiganensis* + *Xanthomonas* spp., *P. syringae* pv. *tomato* + *Xanthomonas* spp., and *C. michiganensis* subsp. *michiganensis* + *P. syringae* pv. *tomato* + *Xanthomonas* spp.. Leaflets were pricked (30×) with a sterile needle and treatments were swabbed onto the wounded leaflet. Bacterial concentration was 10^8 CFU/ml of sterile DI water. Leaves with a terminal inoculated leaflet were enclosed in a plastic bag. After 11 days, leaves were removed, surface-sterilized, and macerated as described earlier. One ml of macerate was used to inoculate NBY plates for Bio-PCR with a Wizard Genomic DNA extraction kit (Promega, Madison, WI), and 0.5 ml was used for immediate DNA extraction with a PowerSoil DNA isolation kit (MOBIO, Carlsbad, CA). Extracted DNA was amplified with the final multiplex protocol.

Results

Optimization of multiplex PCR parameters. Thermal cycler conditions and reaction components of simplex PCR protocols for each pathogen were harmonized so that all three pathogens could be detected with a single protocol. A gradient temperature experiment (57 to 65°C) was conducted to determine the best annealing temperature for the multiplex PCR; an annealing temperature of 57°C was selected. To further enhance DNA amplification, a touchdown protocol with annealing temperature ranging from 63 to 57°C for 12 cycles, followed by 24 cycles at 57°C was developed (Table 4-2).

Volumes and concentrations of reaction components for the multiplex reaction are listed in Table 4-3. A primer ratio of 6.67:1.67:1 was the most effective for amplification of the three target DNAs from pure cultures, mixed cultures, and symptomatic tomato

plants. Additionally, dimethyl sulfoxide (DMSO) was added for detection of *Xanthomonas* spp. Additional *Taq* was added to ensure sufficient polymerase was available for competing reactions (Table 4-3). About 2 to 6 µl of template DNA was added to a total concentration of 5-10 ng per 50-µl reaction.

Table 4-2. Parameters for multiplex PCR for simultaneous detection of *C. michiganensis* subsp. *michiganensis, P. syringae* pv. *tomato,* and *Xanthomonas* spp.

Cycles	Parameters
Initial	10 min at 94°C
12	30 sec at 95°C TD[z]: 30 sec at 63°C (-0.5 °C) 45 sec at 72°C
24	30 sec at 95°C 30 sec at 57°C 45 sec at 72°C
Final	5 min at 72°C

[z] The touchdown (TD) program decreased annealing temperature by 0.5°C every cycle for 12 consecutive cycles; after 12 cycles, annealing temperature was 57°C.

Detection of *C. michiganensis* subsp. *michiganensis*, *P. syringae* pv. *tomato*, and *Xanthomonas* spp. with multiplex PCR from symptomatic plants. All three pathogens were detected singly with Bio-PCR (population of bacterial cells increased prior to DNA extraction) (Fig. 1A, Lanes 5-7) and direct DNA extraction from infected leaflets (Fig. 1B, Lanes 5-7). In mixed inoculations of *P. syringae* pv. *tomato* + *Xanthomonas* spp., both pathogens were detected with both Bio-PCR and direct extraction methods (Fig. 1A-B, Lane 9). However, in mixed inoculations with *C. michiganensis* subsp. *michiganensis* + *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* + *P. syringae* pv. *tomato* + *Xanthomonas* spp., *C. michiganensis* subsp. *michiganensis* could not be detected (Fig. 1A-B, Lanes 8 and 11, respectively). On

singly inoculated plants we observed lesions with *P. syringae* pv. *tomato* and *Xanthomonas* spp. at 2 and 4 days post-inoculation, respectively. However, lesions from *C. michiganensis* subsp. *michiganensis* were not evident until 10 days post-inoculation. In mixed inoculations with *C. michiganensis* subsp. *michiganensis* and *Xanthomonas* spp., *C. michiganensis* subsp. *michiganensis* detection was enhanced with Bio-PCR (Fig. 1A, Lane 10).

Table 4-3. Reaction components of multiplex PCR for detection of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, and *Xanthomonas* spp.

Component	Volume/Concentration	Source
Takara ExTaq Perfect Shot:	25 µl total volume	Takara, Otsu, Shiga, Japan
Taq	1.25 U	
Mg^{2+}	4 mM	
dNTPs	0.2 mM	
Loading Dye: Orange G/Bromophenol Blue		
Primers:		
CMM5	5 µl/5 µM	Eurofin, Luxemburg, Germany
CMM6	5 µl/5 µM	
MM5F	1.25 µl/5 µM	
MM5R	1.25 µl/5 µM	
RST65	0.75 µl/5 µM	
RST69	0.75 µl/5 µM	
DMSO	1 µl	Acros Organics, Geel, Belgium
AmpliTaq Gold	0.3 µl of 5 U/µl stock	Applied Biosystems, Carlsbad, CA
DNA grade Water	7-10 µl	Fisher Scientific, Waltham, MA
Template DNA	2-6 µl, 5-10 ng	
TOTAL	50 µl	

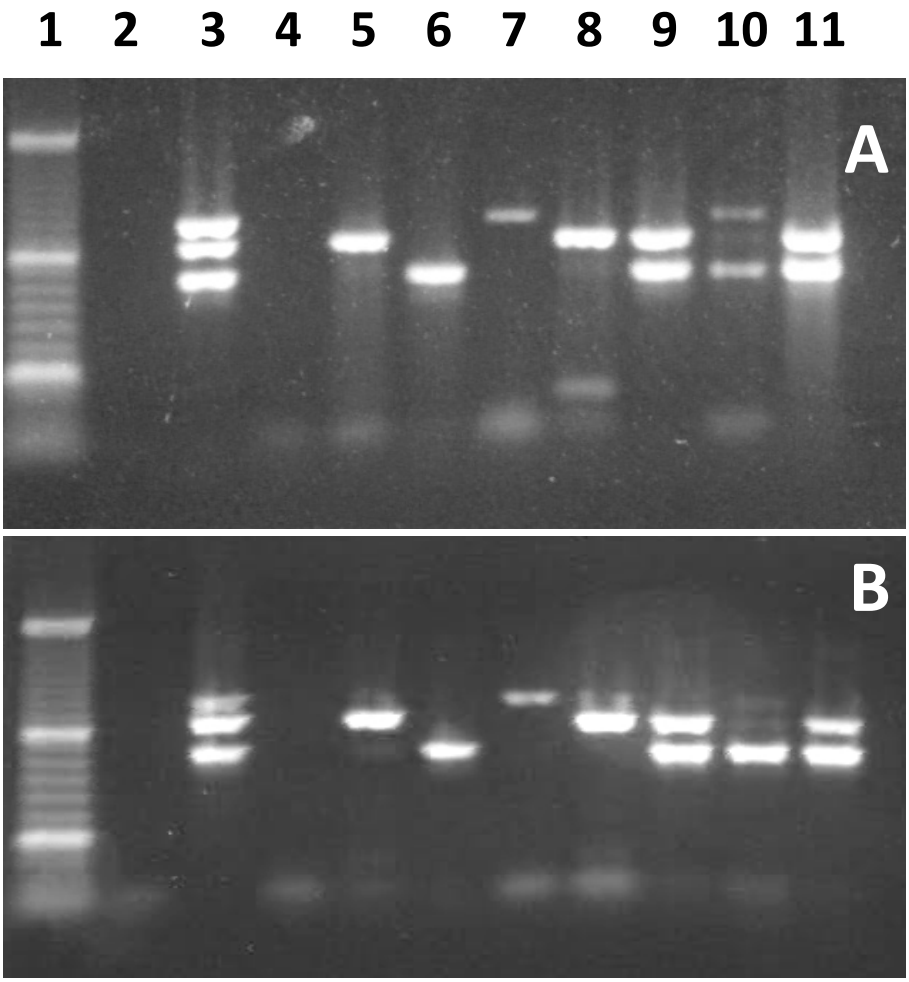

Figure 4-1. Detection of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, and *Xanthomonas spp.* with multiplex PCR following DNA extraction from symptomatic tomato leaves after inoculation with a single pathogen, or combinations of the three pathogens: (A) Multiplex Bio-PCR, (B) Multiplex PCR with DNA extraction directly from leaf macerate. Lane **1** = 50 bp DNA Ladder, *Controls***:** Lane **2** = Negative Control, **3** = Positive Control (DNA from all three pathogens extracted with DNA from the phyllosphere community of a healthy plant); *Plant inoculations*: **4** = Sterile water, **5** = *P. syringae* pv. *tomato*, **6** = *Xanthomonas* spp., **7** = *C. michiganensis* subsp. *michiganensis*, **8** = *P. syringae* pv. *tomato* + *C. michiganensis* subsp. *michiganensis*, **9** = *P. syringae* pv. *tomato* + *Xanthomonas* spp., **10** = *C. michiganensis* subsp. *michiganensis* + *Xanthomonas* spp., **11** = *C. michiganensis* subsp. *michiganensis* + *P. syringae* pv. *tomato* + *Xanthomonas* spp.

Discussion

In a previous study (9), using DNA from pure cultures of each pathogen extracted separately, and different primers pairs, a primer ratio of 3:1:2.5 for *C. michiganensis* subsp. *michiganensis, P. syringae* pv. *tomato*, and *Xanthomonas* spp., respectively, was used. A specific ratio was needed to guarantee equal opportunity for amplification of the target DNA. In our study, a primer ratio of 6.67:1.67:1 was the most effective for amplification of the three target DNAs from pure cultures, mixed cultures, and symptomatic tomato plants. Dimethyl sulfoxide (DMSO) was added to relax *Xanthomonas* spp. DNA; however, amounts greater than 1 µl per 50-µl PCR reaction inhibited detection of *C. michiganensis* subsp. *michiganensis*. The amount of *Taq* from the Takara Perfect Shot tubes was found to be insufficient for a multiplex reaction, warranting the addition of extra *Taq*. The total concentration for each reaction was intentional due to the increased stringency needed for the protocol. Higher DNA concentrations typically interfered with DNA amplification of *C. michiganensis* subsp. *michiganensis*.

A multiplex PCR protocol was developed and used successfully to detect *C. michiganensis* subsp. *michiganensis, P. syringae* pv. *tomato* and *Xanthomonas* spp. from symptomatic plants infected with single pathogens, with both Bio-PCR, and PCR of DNA extracted directly from infected tomato leaves. Likewise, in mixed infections, *P. syringae* pv. *tomato* and *Xanthomonas* spp. could be detected simultaneously, as could *Xanthomonas* spp. and *C. michiganensis* subsp. *michiganensis*. However, Bio-PCR was needed for detection of *C. michiganensis* subsp. *michiganensis* in leaves co-infected with *Xanthomonas* spp. When plants were co-inoculated with *P. syringae* pv. *tomato* and *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *michiganensis* could not be detected. This may be due to lack of infection by *C. michiganensis* subsp. *michiganensis* or a more rapid disease reaction with *P. syringae* pv. *tomato* and *Xanthomonas* spp.

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CHAPTER 5 : THESIS CONCLUSIONS AND SUMMARY

Reliance on copper as a control for bacterial pathogens of tomato has long been a standard practice in the United States and in many other countries. This study sought methods that would give an accurate representation of the prevalence and incidence of copper resistance in the foliar bacterial pathogens in the state of Tennessee. The accuracy of the *in-vitro* testing parameters under consideration was determined by comparison with greenhouse bioassays which, in turn, were validated by comparisons with field plot results. Parameters selected for *in-vitro* copper resistance testing were sucrose peptone agar adjusted to pH 6.8 (*Xanthomonas* spp. and *C. michiganensis* subsp. *michiganensis*) or 6.2 (*P. syringae* pv. *tomato*), buffered with 2-(*N*-morpholino)ethanesulfonic acid (20 mM), and amended with 200 µg/ml $CuSO_4 \cdot 5H_2O$. After solidifying, media were promptly streaked with cells produced on solid agar, and incubated for three days. Bacterial growth was visually assessed on a 0-10 scale. Isolates having reduced sensitivity *in-vitro* tended to vary greatly among repetitions of the trials. Since these isolates tended to react as moderately resistant to highly resistant in greenhouse bioassays, it is suggested that all isolates appearing to have a reduced sensitivity to copper be considered resistant. *In-vitro* testing methods for *C. michiganensis* subsp. *michiganensis* were compared with a standard of sensitivity, since there have been no reports of copper resistance for this pathogen. Greenhouse results showing a moderate resistance is more typical of the control obtained in the field. Additional studies are needed to investigate the cause of mediocre control of a sensitive pathogen in commercial fields.

A survey of 19 Tennessee tomato fields found bacterial spot pathogens in each field. Most isolates of *Xanthomonas* spp. were resistant to copper, with only 4.9% classified as sensitive. The loss of efficacy against bacterial spot pathogens indicates the need for the use of alternative products for control of this disease. *C. michiganensis* subsp. *michiganensis* was found in only one field. All isolates were sensitive to copper. No *P. syringae* pv. *tomato* was found. The isolation and identification methods used in the survey were considered inefficient and may have adversely affected recovery of the three pathogens. Methods of identification such as multiplex PCR would aid in

identification of pathogens rapidly, as it is designed to facilitate large studies with multiple pathogens.

The results from the multiplex PCR work support the fact that multiple pathogens and primer sets can be combined and used to detect pathogen presence or absence using symptomatic plant tissue. This may prove to be a reliable diagnostic tool that could have uses in future studies where pathogen identity may determine bactericidal treatment, geographic pathogen presence is sought, or even in facilitation of large surveys. Pathogen co-existence in phyllosphere bacterial communities may provide extended understanding of disease physiology and multiplex PCR would provide the means to study those aspects.

The end result of this work attempts to produce consistent copper resistance testing and methods of identification that can be replicated in a rapid manner to allow use in sizable field surveys. Research efforts providing consistent measurements of increasing resistance would aid industry in proper development of new control products. For producers to remain economically competitive and continue to provide quality produce, they must adapt to growing resistance in bacterial pathogens and push for more effective and economical control products from industry.

VITA

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