This is to certify that the

thesis entitled

GENOMIC VARIATION AND CONTROL OF CLAVIBACTER MICHIGANENSIS SUBSP. MICHIGANENSIS IN MICHIGAN

presented by

Nicole A. Werner

has been accepted towards fulfillment of the requirements for

M.S. degree in Botany and Plant Pathology

Mary K Hausbeck
Major professor

Date 1/4/01

GENOMIC VARIATION AND CONTROL OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* IN MICHIGAN

By

Nicole A. Werner

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

2001

ABSTRACT

GENOMIC VARIATION AND CONTROL OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* IN MICHIGAN.

By

Nicole A. Werner

Strains of *Clavibacter michiganensis* subsp. m*ichiganensis* were isolated from infected tomato plants collected from 14 and 9 Michigan commercial fields in 1997 and 1998, respectively, and subjected to rep-PCR fingerprinting. The most commonly observed *C. m.* subsp. m*ichiganensis* strain from the southwest region was type C (302). Avirulent type C strains (16%) were detected in two southwest fields. All 85 strains from a northeast farm were of type D. In 1997, strains isolated from two fields of one north-central grower were primarily type C strains (58) with few type D (2). In 1998, two north-central fields of the same grower contained 35 type D and 9 type C strains. In 1997 and 1998, the tomato fields sampled in the southeast region of Michigan contained 66 A, 63 B and 78 C type strains.

In 1996, copper hydroxide alone and with mancozeb or streptomycin reduced pathogen populations relative to acibenzolar-S-methyl (ASM), ASM/copper hydroxide and avirulent *C. m.* subsp. *michiganensis* strains. In 1997 only, copper hydroxide/ mancozeb limited spread compared to copper hydroxide/streptomycin. In the field, inoculated control plants produced yields that were 61% (1996), 93% (1997) and 98% (1998) of those produced by the uninoculated controls. Fruit spotting occurred regardless of treatment and was highly variable.

DEDICATION

In loving memory of my grandfather, who instilled in me an interest and respect for the environment that has remained with me to adulthood. His love for the outdoors and the living things around him has inspired me to choose a career in biology.

ACKNOWLEDGMENTS

I would like to thank my co-advisor, Dr. Mary Hausbeck, for giving me the opportunity to study under her guidance and for the work-related as well as moral support she has afforded me over the years. I would also like to thank my co-advisor, Dr. Dennis Fulbright, for his guidance and questions he challenged me with over the course of my studies. In addition, Dr. Andy Jarosz has been invaluable with advice on statistical issues as well as with insightful comments and suggestions.

I couldn't have achieved my objectives without the assistance of the technical staff and graduate students of Dr. Hausbeck's and Dr. Fulbright's labs. The countless hours of spray applications, plantings, harvests and data collection could not have been done without them. In addition, Dr. Robert Podolsky's statistical consulting was critical for the completion of this project.

I thank my parents for investing in my undergraduate studies and for being there for me under any circumstance. And last, but not least, I would like to thank my good friend, Craig Skoczylas, for supporting me in my studies in addition to uprooting from his life-long home to accompany me during my graduate career.

TABLE OF CONTENTS

LIST OF TABLES

Table | Page

LIST OF FIGURES

Figure Page

LITERATURE REVIEW

INTRODUCTION

In 1999, the Michigan fresh market tomato (*Lycopersicon esculentum* Mill.) industry comprised 2,700 of the 111,750 harvested vegetable acreage in Michigan and generated nearly 21 million dollars at the point of sale (4). In Michigan, tomato seedlings are grown in local greenhouses and transplanted into fields for fruit production. Large-scale producers may grow a single or a few cultivars in their fields, while smaller road-side market growers may plant several cultivars to satisfy the needs of varied clientele. Common cultural practices include methyl-bromide fumigation of raised beds covered with black plastic. The plants are supported by tying stems to wooden stakes. A customary method for disease management is rotation; the practice of refraining from growing the same crop in a particular field over consecutive years. Some growers in Michigan may move tomato production miles away from a previous year's field while other growers, having limited options, may rotate to a field within yards of a previous year's planting. The number of growing seasons a field is kept out of tomato production also varies depending on the grower's situation.

Bacterial canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al., is an important disease of tomato in Michigan. A significant outbreak occurred in 1969 in the north-central and northeast United States (16). A more recent epidemic occurred in 1984 in the mid-western United States and Canada (20). Over the years, the disease has caused significant financial loss for both the

processing and fresh market tomato industries. In Ontario, Canada yield losses averaged 5-10% annually from 1965-1971 (32).

Transplants grown in greenhouses are subjected to humid conditions and overhead watering conducive to bacterial growth and spread (23). An important source of inoculum is infested seed (7, 17, 21, 41, 47). Seedlings may become systemically infected yet remain asymptomatic (18) and as a consequence serve as primary inoculum for field outbreaks. In addition, bacteria are spread throughout the field by various means of dispersal and mechanical damage resulting in secondary infections.

THE BACTERIAL CANKER PATHOGEN

Clavibacter michiganensis subsp. *michiganensis* was first reported in 1909 by Erwin F. Smith following detection on greenhouse tomatoes in Grand Rapids, Michigan (41). Today this organism causes disease in all regions of the world where tomatoes are grown, and is important in the seed production regions of Asia, Mexico and South America (41).

Plants associated with the pathogen. The pathogen affects not only tomato, but other members of the *Solanaceae* as well. Sweet pepper (*Capsicum annuum*) (51) and bell pepper (*C. frutescens*) (26) are also hosts of *C. michiganensis* subsp. *michiganensis*, although tomato remains the only economically important host (21). The spiny Puerto Rican weed (*Solanum mammosum*) (40), common perennial nightshade (*S. douglasii*) (2), black nightshade (*S. nigrum*) (1) and hairy nightshade (*S. triflorum*) (1) are all hosts of *C. michiganensis* subsp. *michiganensis*, having been found naturally infected near tomato fields. Further study indicated that an isolate from tomato was pathogenic to tomato, yet

not to *S. douglasii* (2). Additional *Solanaceae* species and genera have shown some response only through seed, root, stem, or cotyledon inoculations (1, 22, 40, 46).

Symptoms on tomato. Symptoms caused by the pathogen can be classified into two groups: localized, resulting from surface infection; and systemic, resulting from vascular infection (41). Marginal leaf necrosis, often referred to as the "firing" stage, is a common symptom in the field. This necrosis tends to be limited to the leaf margins and may border chlorotic tissue. Local fruit lesions, commonly called bird's eye spots, develop under conditions of high relative humidity (41). The lesions first appear as white, superficial spots that eventually develop a necrotic center and are generally 3-6 mm in diameter. Bird's eye spots are a distinctive symptom of the disease, therefore, are useful in diagnostics. A recent study found that when inoculated, flowers were most susceptible to infection two-days post anthesis (34). In addition, small green fruit developed bird's eye lesions when inoculated with *C. michiganensis* subsp. *michiganensis*.

One of the first systemic symptoms observed in field-grown plants is wilting, which generally begins on the basal leaves and continues up the plant. Unique to bacterial canker, leaves may wilt unilaterally until eventually the entire leaf withers, although the petiole holds its turgidity for some time. When severely infected, the plant may die. More often, however, the plant survives, yet yield is notably reduced. Yellow to brown discoloration may occur along stems and petioles, which may split resulting in cankers. Less commonly, fruits may be systemically infected through the vascular system and become stunted, mottled, and malformed (27). Symptom development is likely

affected by a wide range of factors including pathogen variability, environmental conditions, age of plants when infected, and infection site (41).

In the early stages of systemic infection, bacteria invade the xylem vessels of the plant. At this time, only the invaded cells are affected, suggesting cellulolytic and hemicellulolytic enzyme activity (52). Prior to vessel wall degradation, swelling, and shredding of the walls and middle lamella may occur, suggested to be caused by increased acidity or cation complexing organic acids (5).

During later stages of infection, Wallis (52) found that the contiguous walls and middle lamella adjacent to infected vessels are susceptible to degradation in the presence or absence of bacteria suggesting that the production and translocation of extracellular bacterial enzymes through conductive tissues. Furthermore, primary vessel walls, middle lamella, parenchymatous ground tissue, and phloem cells may be invaded and collapse.

Three explanations were proposed for the wilting symptoms caused by bacterial canker; vessel plugging, toxic action theory (41), and enzyme activity (52). Possible substances that may block vessels include extracellular polysaccharides (EPS), bacterial cells, or degraded tissue (13). However, Wallis (52) found little EPS, low concentrations of bacterial cells, and a minimal amount of degraded tissue in infected stems. A related pathogen, *C. michiganensis* subsp. *sepidonicus*, produces a toxin which causes cell walls to degrade in a layering pattern (43). Wallis (52) reported that in the presence of *C. michiganensis* subsp. *michiganensis* the vessel walls do not exhibit this layering pattern and are completely dissolved, suggesting that an enzyme, rather than a toxin, is at work.

Epidemiology of bacterial populations on greenhouse grown transplants. *Clavibacter michiganensis* subsp. *michiganensis* survives on the surface and in the layers of the seed coat (7, 41) and can be transmitted to emerging seedlings (7, 10, 47). Chang et al. (10) investigated the transmission of *C. michiganensis* subsp. *michiganensis* from seed to transplant by growing seed from systemically infected plants and observed a seed transmission rate of 0 to 0.9%. Bryan (7) found 1 to 5% seed transmission from naturally infected seeds and 21 to 40% with artificially infested seed. As seedlings emerge, epiphytic bacteria thrive in the humid greenhouse (47) and may spread to adjacent, uninfected plants via overhead watering (Hausbeck, unpublished data). In a study performed by Hausbeck, et al. (23), populations on greenhouse plants reached levels greater than 10^8 CFU/g fresh weight. The study determined that pathogen populations on greenhouse grown transplants must reach a threshold level of 1.0×10^7 CFU/g of tissue if severe symptoms and yield losses are to occur in the field.

Spread of pathogen populations among transplants can occur during handling of both infected and healthy seedlings at the time of transplanting (32). In addition, seedlings are often pruned prior to transplanting, resulting in the dissemination of the organism from infected to healthy plants (9). Populations were first detected on pruned, asymptomatic seedlings 9 to 13 days after clipping. Concentrations of the pathogen ranged from non-detectable levels to 10^7 CFU/g fresh weight throughout the study.

Epidemiology of bacterial populations on tomato plants in the field. Bryan (7) reported that a 1% seed transmission rate resulted in 54.5% infection of field plants. Field sources of inoculum include infected transplants, surface pathogen populations on

tomato and alternative weed hosts, and overwintered tomato debris (10). Epiphytic populations on field plants may spread by water splashing, pesticide spraying, pruning, tying, sand blasting, and harvesting (20). The highest incidence of disease in the field occurred when seedlings were clipped prior to transplanting (10). Leaf surface populations may play a larger part in causing systemic infection of fresh market tomatoes which are more susceptible to wounding during staking and removal of axillary leaves (20, 25).

Gleason et al. (19) reported that epiphytic populations on fruit and foliage in an Iowa research field increased and stabilized at 10^6 to10^8 and 10^5 to10^6 CFU/g, respectively. In a study by Chang, et al. (10), bacteria remained on uninoculated tomato plants for 7 weeks after infected neighboring plants were removed. However, population sizes were significantly smaller than those treatments where infected plants were not removed, indicating the potential of secondary spread. Secondary infection was not observed until populations reached 10^6 and 10^7 CFU/g fresh weight and appeared 56 to 70 days after transplanting, both depending on susceptibility of the cultivar (10). Ricker and Reidel (37) determined that secondary spread did not reduce yield of processing tomatoes. In another study, systemically infected plants exhibited a 5-7% decrease in yield for each 10% of systemically infected plant incidence (11).

Alternative host plants may also harbor surface populations (see previous section "Host Range"). Chang, et al. (10) found that surface populations on tomato plants (10^6-10^9 cfu/g) were higher than on alternative host plants (0-10^8 cfu/g), and were subsequently higher than on non-host plants (0-10^3 cfu/g). Leaf surface populations on alternative host

plants did not increase throughout the season as they did on tomato.

The pathogen overwinters in tomato debris as found in Georgia (8), Utah (6), Italy (12), California (21), North Carolina (42) and the Midwest United States (15, 19). Experiments with greenhouse soils (54) and outdoor soil tubes in Washington, D. C. and New York (7) determined that survival of the pathogen occurred from a few to 11 months, respectively. Basu (3) found that without debris the organism survives only 3-4 weeks in the soil at 5 to 35°C, yet survives up to 36 weeks in soil at -20°C. Both Chang et al. (10) and Gleason et al. (20) reported a higher rate of bacterial population decline when *C. michiganensis* subsp. *michiganensis* infested tomato debris was buried as opposed to being left on the soil surface. In an experiment performed by Gleason (20), populations were detected on foliage of plants after one week of transplanting into a debris infested field. After 5-7 weeks, field populations had reached 10^6-10^8 CFU/leaflet. The pathogen has the potential to overwinter for 2 years in the north-central US (19). There is also evidence that the pathogen overwinters on the weed host *S. douglasii* (2).

Detection and growth in culture. D2 media selects for *C. michiganensis* subsp. m*ichiganensis* by altering the surface components of bacterial membranes to select for a specific genus (24). D2 media was efficient in detecting population levels up to 10^7 cfu/g infected tissue and successfully selected for *C. michiganensis* subsp. *michiganensis* in a mixture of other important phytobacteria. Fatmi and Schaad (17) developed semi selective media for *C. michiganensis* subsp. *michiganensis* (SCM) that was successful in detecting one infested seed in 10,000. More importantly, *C. michiganensis* subsp. *michiganensis* colonies were morphologically distinct from most saprophytic

contaminants. Researchers modified SCM to enhance the distinction of *C. michiganensis* subsp. *michiganensis* colonies and reduce the size of saprophytic colony size (53).

MOLECULAR TECHNIQUES

Bacterial genomes typically include a variety of low copy number repeated sequences including interspersed repetitive DNA sequences (IRSs) that range from 15 to several hundred base pairs (49). They are widely dispersed throughout prokaryotic genomes, non-coding, and intercistronic. Examples of known conserved DNA elements include repetitive extragenic palindrome (REP), enterobacterial repetitive intergenic consensus (ERIC), and interspersed, repetitive BOX sequences (BOX) (49). The presence, widespread distribution and highly conserved nature of these sequences suggest that these repetitive sequences are important to the structure and evolution of the bacterial genome, yet their function(s) is not yet known (31). These elements are thought to be present in both orientations within bacterial genomes (38) and contain highly conserved regions that have been utilized as primer binding sites in PCR to amplify DNA sequences between the elements (49). BOX elements, in particular, are modular in nature, consist of three differentially conserved subunits, and were the first IRSs found in a Gram + bacterium (*Streptococcus pneumoniae*) (49).

Repetitive-sequence-based (rep)-PCR is a method used to identify and differentiate microbes. The technique utilizes outwardly facing primers complementary to IRSs within the target genome resulting in the amplification of differently sized DNA fragments between the elements. When the PCR products are fractionated utilizing gel electrophoresis, PCR-DNA fingerprints are generated (28, 29, 30, 38, 49, 50).

Rep-PCR differs from other PCR procedures in that it allows the simultaneous amplification of varied sizes of DNA fragments. Smaller fragments amplify more efficiently than larger ones, therefore, conditions must be adjusted to allow the amplification of all fragments (49). In order to accomplish this, excess dNTP's, primer, and initial template DNA are added (49). In addition, a longer elongation time during each cycle is needed. It was later found that reproducibility of REP-fingerprints was possible using whole-cell PCR (38). Because of possible contamination and amplicon carryover between cycles, a negative control must be run each time (49).

PCR amplification of Gram + bacterial DNA can be challenging because of the presence of a single lipid bilayer with a thick peptidoglycan layer and an additional carbohydrate polymer, lipoteichoic acid (35). In order to lyse the Gram positive cells, detergent and enzymes are included in the PCR reaction mixture to dissolve the lipid membrane and to cleave the glycosidic linkages, respectively (49). If the cells are lysed in the reaction tube, the presence of cell wall constituents, cell proteases, and cell nucleases may interfere with the reaction (49). Versalovic, et al. (49) suggested that by reducing the preparation time, the loss of Taq DNA polymerase and the original target DNA may be avoided. In addition, immediately freezing the amplified products may reduce any loss of amplified product.

Subdivisions within *C. michiganensis* subsp. *michiganensis* distinguished using BOX-PCR. Louws, et al. (29) reported that using rep-PCR with the BOX primer (BOX-PCR) *C. michiganensis* subsp. *michiganensis* is distinguished into four goups (A, B, C, D). Utilization of this finding expedites diagnosis and may have useful

implications to epidemiological studies. Epidemiological studies have traditionally utilized antibiotic resistance as a method to mark strains. Rep-PCR may be employed to mark strains in epidemiological studies as well as in plant-microbe interactions (28). Another use of rep-PCR may be to determine the origin of an infection or epidemic (33).

Louws, et al. reported that 30% of the A type strains included in their study were non-pathogenic (29). Additionally, the type A strains were found to be associated more frequently with processing-type cultivars than any other PCR type. The majority of the strains representing Michigan field locations in this study were of the C and B fingerprint types. The type D was the least represented type and was detected in only two Michigan fields in the study.

CONTROL METHODS

Bactericidal sprays. Traditionally, growers apply bactericides to field plants after disease symptoms have appeared. Typically, the products used to control *C. michiganensis* subsp. *michiganensis* are copper based products alone or mixed with mancozeb. A North Carolina study conducted on a fresh market cultivar indicated significant control of foliar marginal necrosis and a decrease in yield loss with weekly field applications of copper (39). These treatments did not, however, impact fruit spotting.

A recent study focused on greenhouse applications of copper alone or with mancozeb and found the treatments reduced pathogen population size and spread among greenhouse seedlings and reduced subsequent yield loss in the field (23). They also determined that bacterial populations on the greenhouse transplants were suppressed

below the treshold level (10^7 CFU/g fresh weight) needed for severe symptoms to occur in the field.

Resistant cultivars. Resistance to bacterial canker has been reported in two wild species of tomato, *L. hirsutum* (22) and *L. pinpinefollium* (44, 45). Accessions of *L. esculentum* with similar phenotypes to the wild species have also been noted (44, 45). Processing cultivars developed by H. J. Heinz Co., Pittsburgh, PA have held promise in resistance screenings (14). Tomato cultivars moderately resistant to bacterial canker have been developed that exhibit reduced foliar blight and yield losses compared to a susceptible cultivar (36). No tomato species have been found to be immune to the pathogen. Somaclonal variation was explored by van den Bulk, et al. (48) with limited resistance potential.

LITERATURE CITED

1. Ark, P. A., and Thompson, J. P. 1960. Additional hosts for tomato canker organism, *Corynebacterium michiganense*. Plant Dis. Rep. 44:98-99.

2. Baines, R. C. 1947. Perennial nightshade, a host for *Corynebacterium michiganense*. Report of the twenty-eighth annual meeting of the pacific division of the American Phytopathological Society. 37:359.

3. Basu, P. K. 1970. Temperature, an important factor determining survival of *Corynebacterium michiganense* in soil. Phytopathology 60:825-827.

4. Bay, D. M., and Wyant, D. (eds). 1999. Michigan Agricultural Statistics. MDA, Michigan pp. 42-45.

5. Beckman, C. H. 1964. Host responses to vascular infection. Ann. Rev. of Phytopathol. 2:231-252.

6. Blood, H. L., and Wann, F. B. 1932. Botany and Plant Pathology. Pages 49-54 In: Biennial Rept. Utah Agr. Ext. Sta. 1931/1932. Bull. 235.

7. Bryan, M. K. 1930. Studies on bacterial canker of tomato. J. Agr. 41:825-851.

8. Bryan, M. K., and Boyd, O. C. 1930. Control of bacterial canker of tomatoes. (Abstr.). Phytopathology 20:127.

9. Chang, R. J., Ries, S. M., and Pataky, J. K. 1991. Dissemination of *Clavibacter michiganensis* subsp. *michiganensis* by practices used to produce tomato transplants. Phytopathology 81:1276-1281.

10. Chang, R. J., Ries, S. M., and Pataky, J. K. 1992. Local sources of *Clavibacter michiganesis* subsp. *michiganensis* in the development of bacterial canker on

tomatoes. Phytopathology 82:553-560.

11. Chang, R. J., Ries, S. M., and Pataky, J. K. 1992. Reductions in yield of processing tomatoes and incidence of bacterial canker. Plant Dis. 76:805-809.

12. Ciccarone, A., and Carill, A. 1948. Field observations on *Corynebacterium michignense* (Smith) Jensen and consideration on a possible instance of its survival in the soil. Boll. Staz. Pat. Veg. Roma Ser. 3. 6:177-179.

13. Dimond, A. E., and Waggoner, P. E. 1953. On the nature and role of vivitoxins in plant diseases. Phytopathology 43:229-235.

14. Emmatty, D. A., and John, C. A. 1973. Evaluation of resistance to bacterial canker of H2990, a new tomato variety. Plant Dis. Rep. 57:584-586.

15. Farley, J. D. 1971. Recovery of *Corynebacterium michiganense* from overwintered tomato stems by the excised-petiole inoculation method. Plant Dis. Rep. 55:654-656.

16. Farley, J. D., and Miller, T. D. 1972. Spread and control of bacterial canker in tomato transplants during clipping. Ohio Agr. Res. Develop. Center Res. Sum. 65:23-25.

17. Fatmi, M., and Schaad, N. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology 78:121-126.

18. Gitaitis, R. D., Beaver, R. W., and Voloudakis, A. E. 1991. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato transplants. Plant Dis. 75:834-838.

19. Gleason, M. L., Braun, E. J., Carlton, W. M., and Peterson, R. H. 1991. Survival and dissemination of *Clavibacter michiganensis* subsp. *michiganensis* in tomatoes. Phytopathology 81:1519-1523.

20. Gleason, M. L., Gitaitis, R. D., and Ricker, M. D. 1993. Recent progress in understanding and controlling bacterial canker of tomato in eastern North America. Plant Dis. 77:1069-1076.

21. Grogan, R. G., and Kendrick, J. B. 1953. Seed transmission, mode of overwintering and spread of bacterial canker of tomato caused by *Corynebacterium michiganense.* (Abstr.). Phytopathology 43:473.

22. Hassan, A. A., D. L. Strider, and Konsler, T. R. 1968. Application of cotyledonary symptoms in screening for resistance to tomato bacterial canker and in host range studies. Phytopathology 58:233-239.

23. Hausbeck, M. K., Bell, J., Medina-Mora, C., Podolsky, R., and Fulbright, D. W. 2000. Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganenesis* on tomatoes in the greenhouse and on disease development and crop yields in the field. Phytopathology 90:38-44.

24. Kado, C. I., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. Phytopathology 60:969-976.

25. Kontaxis, D. G. 1962. Leaf trichomes as avenues for infection by *Corynebacterium michiganense*. Phytopathology 52:1306-1307.

26. Lai, M. 1976. Bacterial canker of bell pepper caused by *Corynebacterium*

michiganense. Plant Dis. Rep. 60:339-342.

27. Layne, R. E. C. 1967. Foliar trichomes and their importance as infection sites for *Corynebacterium michiganense* on tomato. Phytopathology 57:981-985.

28. Louws, F. J., Bell, J., Medina Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., deBruijn, F. J., and Fulbright, D. W. 1998. rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis.* Phytopathology 88:862-868.

29. Louws, F. J., Fulbright, D. W., Stephens, C. T., and Bruijn, F. J. d. 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria.* Phytopathology 85:528-536.

30. Louws, F. J., Fulbright, D. W., Stephens, C. T., and De Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.

31. Lupski, J. R., and Weinstock, G. M. 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. J. Bacteriol. 174:4525-4529.

32. McKeen, C. D. 1973. Occurrence, epidemiology, and control of bacterial canker of tomato in southwestern Ontario. Can. Plant Dis. Surv. 53:127-130.

33. McManus, P. S., and Jones, A. L. 1995. Genetic fingerprinting of *Erwinia amylovora* strains isolated from tree-fruit crops and *Rubus* spp. Phytopathology 85:1547-1553.

34. Medina-Mora, C. M., Hausbeck, M. K., and Fulbright, D. W. 2000. Bird's eye

lesions of tomato fruit produced by aerosol and direct application of *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. (In press).

35. Prescott, L. M., J. P. Harley, and Klein, D. A. 1993. Microbiology, 2nd ed. in K. Kane, Ed. Wm. C. Brown Publishers, Dubuque, Iowa.

36. Ricker, M. D., Emmatty, D. A., Schott, M. D., and Ozminkowski, R. H. 1995. Heinz tomato varieties resistant to bacterial canker. Pages 37-39 in: 10th Annual Tomato Disease Workshop, Windsor, Ontario Canada.

37. Ricker, M. D., and Riedel, R. M. 1993. Effect of secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* on yield of northern processing tomatoes. Plant Dis. 77:364-366.

38. Schneider, M., and De Bruijn, F. J. 1996. Rep-PCR mediated genomic fingerprinting of rhizobia and computer-assisted phylogenetic pattern analysis. World J. Microbiol. Biotechnol. 12:163-174.

39. Shoemaker, P. B. 1992. Evaluation of different materials for tomato bacterial canker, 1991. Fung. Nematicide Tests 47:155.

40. Smith, E. F. 1914. The Grand Rapids tomato disease. Carnegie Institution, Washington.

41. Strider, D. L. 1969. Bacterial canker of tomato caused by *Corynebacterium michiganense*; a literature review and bibliography. North Carolina Experiment Station, Technical Bulletin 193:110 pages.

42. Strider, D. L., and Konsler, T. R. 1965. Cotyledonary symptoms of bacterial canker of tomatoes. Plant Dis. Rep. 49:634-635.

43. Strobel, G. A., and Hess, W. M. 1968. Biological activity of a phytotoxic glycopeptide produced by *Corynebacterium sepedonicum*. Plant Physiol. 43:1673-1688.

44. Thyr, B. D. 1968. Resistance to bacterial canker in tomato, and its evaluation. Phytopathology 58:279-281.

45. Thyr, B. D. 1969. Additional sources of resistance to bacterial canker of tomato (*Corynebacterium michiganense*). Plant Dis. Rep. 53:234-237.

46. Thyr, B. D., Samuel, M. J., and Brown, P. G. 1975. New solanaceous host records for *Corynebacterium michiganense*. Plant. Dis. Rep. 59:595-598.

47. Tsiantos, J. 1987. Transmission of bacterium *Corynebacterium michiganense* pv. *michiganense* by seeds. J. Phytopathology 119:142-146.

48. van den Bulk, R. W., Jansen, J., Lindhout, W. H., and Loffler, H. J. M. 1991. Screening of tomato somaclones for resistance to bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*). Plant Breed Z. Pflanzenzucht 107:190-196.

49. Versalovic, J., Schneider, M., De Bruijn, F. J., and Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. and Cell. Biol. 5:25-40.

50. Versalovic, J., T. Koeuth, A., and Lupski, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nuc. Acids Res. 19:6823-6831.

51. Volcani, Z., Zutra, D., and Cohn, R. 1970. A new leaf and fruit spot disease of pepper caused by *Corynebacterium michiganense*. Plant Dis. Rep. 54:804-806.

52. Wallis, F. M. 1977. Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense*. Physiol. Plant Pathol. 11:333-342.

53. Waters, C. M., and, and Bolkan, H. A. 1992. An improved semi selective medium and method of extraction for detecting *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. (Abstr.) Phytopathology 82:1072.

54. Williams, C. C. 1928. The Grand Rapids disease of tomatoes. Ohio Agr. Expt. Sta. Bull. 117:37-38.

SECTION I

GENOMIC VARIATION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* IN MICHIGAN

ABSTRACT

Strains of *Clavibacter michiganensis* subsp. m*ichiganensis* were isolated from infected foliage and fruit collected from 14 and 9 Michigan commercial tomato fields in 1997 and 1998, respectively, and subjected to rep-PCR fingerprinting. The cultivar Mountain Spring dominated the plantings in southwest Michigan during 1997 and 1998 and the most commonly observed *C. michiganensis* subsp. m*ichiganensis* strain was type C (302 out of 319). Avirulent type C strains (16%) were detected from two southwest fields. All 85 strains from one northeast farm where several cultivars were sampled in 1997 and 1998 were of type D. In 1997, strains isolated from two fields of one north-central grower were primarily type C (58) with 2 type D strains. Conversely, in 1998, two north-central fields of the same grower contained mostly type D (35) and a few type C strains (9). In 1997 and 1998, the tomato fields sampled in the southeast region of Michigan contained 66 A, 63 B and 78 type C strains.

INTRODUCTION

In 1999, the Michigan tomato (*Lycopersicon esculentum* Mill.) industry generated over 27 million dollars (1). Bacterial diseases, including bacterial canker of tomato (causal agent: *Clavibacter michiganensis* subsp. *michiganensis*), are a yearly concern for growers. Infection by this pathogen can result in plant wilting, stunting, reduced yields, and plant death. Less severe disease symptoms include marginal leaf necrosis that may be bordered by chlorosis, and fruit lesions appearing as superficial white spots (3-6 mm) that develop a necrotic center.

Field sources of inoculum include infected transplants, surface pathogen populations on tomato and alternative weed hosts, and overwintered tomato debris (4). The pathogen may be seed borne and can overwinter in fields of north-central United States for up to 2 years (8). *Clavibacter michiganensis* subsp. *michiganensis* survives on the surface and in the layers of the seed coat (2, 15) and can be transmitted to emerging seedlings (2, 4, 16). Epiphytic bacteria thrive in the humid greenhouse (16) and may spread to uninfected plants via overhead watering (Hausbeck, unpublished data). In the Midwest, tomato transplants are grown in local greenhouses for use in establishing production fields. Greenhouse transplants can appear healthy, yet harbor high bacterial populations that may lead to severe disease symptoms in the field (10). Epiphytic populations on field plants may spread by water splashing, pesticide spraying, pruning, tying, sand blasting, and harvesting (9).

Bacterial genomes typically include a variety of low copy number repeated sequences including interspersed repetitive DNA sequences (IRSs) that range from 15 to

several hundred base pairs (17). They are widely dispersed throughout prokaryotic genomes, non-coding, and intercistronic; one example being interspersed, repetitive BOX sequences (BOX) (17). The presence, widespread distribution and highly conserved nature of these sequences suggest that these repetitive sequences are important to the structure and evolution of the bacterial genome, yet their function(s) is not yet known (17). Repetitive-sequence-based (rep)-PCR is a method used to identify and differentiate microbes. The technique utilizes outwardly facing primers complementary to IRSs within the target genome resulting in the amplification of differently sized DNA fragments between the elements. When *C. michiganensis* subsp. *michiganensis* is subjected to rep-PCR with BOX as a primer (BOX-PCR) and PCR products are electrophoretically size fractionated in agarose gels the pathogen is distinguished into four fingerprint types (A, B, C, D) (11).

Prior to this study, little was known concerning the genomic variation of the pathogen in commercial fields. Characterization of the variation within a pathogen population may be beneficial in addressing epidemiological questions. The objective of this study was to characterize the variation in populations of *C. michiganensis* subsp. *michiganensis* within and among Michigan commercial tomato fields using BOX-PCR fingerprinting.

MATERIALS AND METHODS

Sample collection. Foliar and fruit samples were collected from 14 and 9 commercial fields from four regions of Michigan in 1997 and 1998, respectively (Table 1; Figure 1). Samples were haphazardly collected according to cultivar or location in a

Table 1. Strains of *Clavibacter michiganensis* subsp. *michiganensis* isolated from fruit and foliage collected from Michigan commercial fresh market and processing tomato fields in 1997 and 1998.

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
Southwest (SW)			
SW/1/1/97-July	B	Mountain Spring	91-2[c]
	C	Mountain Spring	2-2[c], 8-2[d], 11-2, 17-1[c], 27-1, 28-1, 47-2, 50-1, 63-1, 70-2, 74-2, 78-1, 83-1, 86-2, 98-2, 101-2, 103-1[c], 104-1, 108-2, 109-2[d], 112-2, 119-2[c], 130-1, 131-2, 136-2, 137-2[c], 146-2, 152-2, 154-2[c]
SW1/1/97-August	B	Mountain Spring	2-2, 17-2, 91-1
	C	Mountain Spring	8-1, 11-2[c], 27-2, 28-2, 47-2[c], 50-2, 74-1, 78-1, 83-1[c], 86-1, 98-2[c], 101-1, 103-1, 104-1, 108-1, 109-1, 112-1[c], 119-1, 124-2, 130-1[c], 131-2, 136-2, 137-2, 146-2, 152-1, 154-1[c]
SW/1/1/97-October	C	Mountain Spring	2-1[c], 8-2, 11-2, 17-2[c], 28-2, 47-1, 50-1[c], 63-1, 74-1, 78-1, 83-2, 86-2[d], 91-1, 98-2, 103-1, 104-2, 108-2[d], 109-1, 119-1[c], 124-2, 130-1, 131-2, 136-2, 137-1[c], 146-2, 152-2, 154-1
SW/1/2/97-September	B	Mountain Spring	m4-2
	C	Mountain Spring	e1-1, e2-2[c], e3-2, e4-1, e6-2, e9-1[c], e10-1[c], d1-2, d2-2[c], d4-1[c], nd1-1[c], nd2-2, nd3-2[c], nd4-1, m1-2, m2-1, m3-1[d], m5-1[c], m7-2[c], m8-2, m10-2, f1-2, f3-1[d], f4-1, f5-2, f7-1[c], f9-2, f10-1, H2-4

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
SW/1/3/97-September	C	Mountain Spring	e2-3, e2-4[c], e3-1, e3-2, e3-3, e4-1, e4-2, e5-1, e5-2, e5-3[c], f1-4, f2-2, f3-5, f4-1, f4-3, f4-4-1, f4-5, f5-3, m1-2, m1-3, m1-4-1, m1-4-2, m1-5-3, m3-3, m3-4, m4-2, m4-3, m5-1, m5-2, m5-3[c]
SW/2/1/97-September	C	Mountain Spring	e1-1[c], e2-1, e2-2, e3-1, e4-1, e4-2, e5-1, e5-2[d], e6-2, e7-2, e8-1, e8-2, e9-1, e9-2, e10-2, m1-2[c], m2-1, m2-2, m3-1, m4-1, m4-2, m5-2, m5-3s, m6-2, m7-2[c], m8-2, m9-1,m9-2, m10-2[d]
SW/2/2/97-September	B	Mountain Spring	m4, m6-2, NO8
	C	Mountain Spring	OL1-3, OL3, OL4, OL5-1, OL6-1, OL7, OL8-2, OL9, OL10-2, e1-2, e2-2, e3-1, e4-2, m2-1, m3-1, m5-2, m7-2, m8-1, m9, NO1-1, NO3, NO4, NO5, NO6-2, NO7, NO9, NO10
SW/3/1/97-September	B	roma type	r2-2
	C	Mountain Spring	f1-1, f1-2, f2-1, f3-1, f4-3, f5-1, f5-2, f8-1, f9-1, m1-1, m1-2, m2-2, m3-2, m4-2, m5-1, m6-1, m6-2, m7-1, m8-1, m9-1, m9-2, m10-1
		roma type	r1-1, r3-2[c], r4-2, r4-3, r5-1, r7-1, r8-1[c]
SW/4/1/97-September	A	mixed fresh market	NE2-2, NW1-1[d], NW2-2, NW1g-1, NW3g-1[c]
		roma type	Rw2-2, Rw8-1, Rw8-3
	C	mixed fresh market	NE1-2, NE2-2, NW1-2

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
		roma type	Rw1-1, Rw2-1, Rw5-1, Rw7-1, Rw9-1, Rw10-2, Re1-1, Re1-2, Re7-1, Rm1g-2, Rm4g-3
		Stokes fresh market	SE1-1, SE1-2
		Mountain Spring	SW1-1, SW1-2, SW2-2, SW3-2
SW/1/2/98-August	C	Mountain Spring	BL1, BL2[c], BC1, BC2, BR1, BR2, CL1, CL2, CR1, CR2[c], f1-1[c], f2-2[c], f3-2[c], f4-1, f5-1[d], f6-1, m1-1, m2-2[c], m4-1, m5-1, n3-1[c], n5-1[c], r2-2, r3-2[d], r4-1[d], r5-2[d]
SW/2/3/98-August	C	Mountain Spring	f1-1[d], f2-1[d], f3-2, f4-2[d], f5-1, f6-2[d], f7-1, f8-1, f9-2, f10-1, m1-1, m2-1[d], m3-2, m5-1, m6-1[d], m7-1, m8-2, m9-1, m10-2, r1-1, r2-2[d], r3-2, r4-2[d], r5-1, r6-1[d], r8-2, r9-2, r10-1
Northeast (NE)			
NE/1/1/97-September	D	mixed fresh market	Aeh2, Aeh3, Aeh4, Aeh5, Aet1
		Aztec	AZ1, AZ4, AZ5
		mixed fresh market	Beh1, Beh2, Beh3, Bet1-5-1, Bet3-5-1
		Flavormore	FM1-4-1, FM2
		Mountain Fresh	MF1-5-1
		pear type	P1
		roma type	R1-5-2, R3-5-1
		Red Cherry	RC2-5-1, RC3-4-1
		Red Rider	RR1-5-1, RR3-5-1

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
		Springfield	SP1
		Asgrow 145	145-1, 145-2
NE/1/1/98-June	D	mixed fresh market [g]	GH1f[h], GH2-4[c], GH3-5[c]
		Majesty	M1-1, M1-4
		Mountain Fresh	MF4-2[c]
		Mountain Spring	MS6-2
		Mountain Supreme	MSu4-1[c], MSu5-2
		Red Rider	4d-2[d], 5a-1, 5c-2
		roma type	R1-2[c], R4-3
		Springfield	SP2-1
		Super Beef	Sbf1-2, Sbf6-1[c], SBf6-5
		Sunbrite	Sbrt1-1, SB2-1[c]
		Sun Gem	SG2-1[c], SG3-1[d]
		Sun Start	SSt1f4[h], SSt2f1[h], SS5-2[b]
		Trust	T1-2[b], T1-3, T2f1
NE/1/1/98-July	D	Beef King	BK2-1, BK5-2[c]
		heirloom type	H1-1, H5-2
		Majesty	M5-2
		Mountain Fresh	MF5-2
		Mountain Spring	MS1-1[c], MS3-2

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
		Mountain Supreme	MSU1-1, MSU3-1
		Plum Dandy	R2-1, R4-1[c]
		Red Rider	RR1-2, RR4-2[c]
		Springfield	SP4-2[c]
		Sunbrite	SBR2-1[d], Sbrt2-2[c], SBR5-1[c], Sbrt5f[ch]
		Sun Gem	SG2-1, SG2-2[d], SG3-1, SG4-1
		Sunstart	SS4-1[c], 5-2-1[c], 6-4-1[c]
		Super Beef	SBF1-1, SBF4-1
		Trust	T2-1[c], T3-1, T4-2
North-Central (NC)			
NC/1/1/97-August	C	Jet Star	44-2, 45-1, 46-2, 48-1, 52-2, 56-2, 58-1, 59-1, 60-1
		roma type	26-2, 27-2, 30-2, 33-2, 34-1, 35-2[c], 37-1, 39-1, 40-1[c]
		Sun Gem	2-2, 6-2, 9-2, 10-2, 14-2, 15-2, 16-1, 18-2, 20-2, 22-2
	D	Jet Star	53-1
		roma type	36-1
NC/1/2/97-August	C	Jet Star	1-1[c], 2-1, 5-1, 6-2, 8-2[c], 9-1, 10-2, 11-1, 12-2[c], 14-2, 18-1, 19-2[c]
		Sun Start or Red Rider	21-1, 25-1[c], 31-1, 32-1, 35-2[c], 38-1, 41-1, 43-2, 44-2, 46-2, 48-2, 49-1, 50-1, 56-2, 57-1, 58-1, 59-2, 60-1

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
NC/1/3/98-September	C	Mountain Supreme	MSu2-2, MSu10-2
		Sun Start	SSt1-1, SSt2-2, SSt3-1, SSt4, SSt6-2, SSt7-1, SSt9-1
	D	Mountain Supreme	MSu1-2, MSu3-1, MSu4-2, MSu5-1, MSu6, MSu7-2, MSu8, MSu9-2
		Red Rider	RR1-1, RR2-1, RR3-1, RR4, RR5-2, RR6-1, RR7, RR8-2, RR9-2, RR10-1
		Sun Start	SSt5-1, SSt8-2, SSt10-1
NC/1/4/98-September	D	Roma	R1-1, R1-2, R2-1[c], R3-2[c], R4-1, R4-2, R5-1[c], R6-1[c], R7-1, R7-2, R8-2[c], R9-1, R9-2, R10-1[c]
Southeast (SE)			
SE/1/1/97-September	A	orange cherry type	Ech1-2[b], Ech1-3, Ech2-1[b], Ech2-3
		red cherry type	Er1-3
		"Spring" cv.	Esp1e-1[c], Esp2-2, Esp2-3, Esp3-2
		yellow plum type	EYpl2-2, EYpl3-2
	B	red cherry type	Er1-2
		"Spring" type	Esp4-1, Esp2e-3
		yellow plum type	EYpl1-1
	C	"Spring" cv.	Esp1-1, Esp2e-2[c], Esp3e-1[c], Esp3e-3

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
SE/1/2/97-September	A	Cherry Pink	CP1-2, CP2-2[d], CP3-2, CP4-2, CP5-2
		Golden Nugget	GN1-2, GN2-1[b], GN3-1, GN4-2[c], GN5-1
	B	yellow pear type	YP1-1[c]
	C	yellow plum type	YPl1-1, YPl2-1
SE/2/1/97-September	A	Sunrise	BbA4-1
	B	Doubletake	D1-2, D2-1[c], D4-2
		Mountain Spring	C2-2[c], C5-1, C6, CbB5-1
		Nickelow	E1-1, E4-1, E7-1[c]
		Sunrise	B4-1, B8-2[c], BbA2-1[c], BbC5-2, BbC6f[ch]
		Sunstart	A4f[h], A5
	C	Doubletake	D3-1
		Sunrise	B7-1, BbA1-1, BbC2-1, BbC1f[h], BbC4-1[c]
		Sunstart	A1-2, A3f[h], AbB1f[h], AbB5(1)6f[h]
SE/3/1/97-September	A	Mountain Fresh	D3-2
		Sunrise	A1.1-1, A3.1-1, A4.1-2
	B	Mountain Fresh	D1-1, D2.1-1
		Sunrise	C3.2-2
	C	Mountain Fresh	D4-2, D5-1
		Springfresh	B1.1-1, B4-2

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
		Sunrise	A2.1-2,C1.2-1[c],C11(1)1f[h]
SE/1/2/98-August	A	Asgrow 6000	A9-2, A10-2
		Golden Nugget	GN2-1, GN4-2[c]
		Italian Gold	IG4-1
		Market Pride	MP2-1
		Mountain Fresh	MF3-2, MF6-1
		Sun Gold	SG2-2[c], SG3-1
		yellow pear type	YP1-2, YP2-1[c]
	B	Asgrow 6000	A4-2
		Enterprise	E2-2[d]
		heirloom type	H1-1, H2-2[c], H3-1
		Italian Gold	IG2-2[c], IG3-1[d]
		Market Pride	MP1-2, MP8-2
		Mountain Fresh	MF1-2, MF2-1, MF4-1
		Mountain Gold	MG1-1, MG2-2[d]
		roma type	R2-2[c]
		Sun Gold	SG1-1[d]
SE/1/3/98-August	A	Red Rider	RR1, RR3, RR6, RR10
		Sun Brite	SB(GA)2[e], SB(GA)4-2[e]

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
		Sun Gem	SG1, SG(GA)2[d], SG3-1, SG(GA)4-2[e], SG(GA)7[e], SG10, SG(GA)10-1[ce]
		Sun Start	SS1, SS(GA)1-2[ce], SS2, SS4, SS(GA)6[e], SS8
	B	Sun Gem	SG(GA)6-2[e]
		Sun Start	SS(GA)5[e]
	C	Red Rider	RR2
		Sun Brite	SB(GA)7[e]
		Sun Gem	SG2-2, SG4, SG(GA)5[e], SG(GA)8[e], SG(GA)9[de]
		Sun Start	SS3
SE/4/1/98-August	A	Asgrow 3[f]	A3-9-1[h]
		Glamour	G7-1[h], G10-1[h]
	B	Asgrow-2[f]	A2-2[h], A2-6-1, A2-10-2
		Asgrow-3[f]	A3-2[h], A3-7-1[h]
		Glamour	G8-1
		Golden Jubilee	GJ10[h]
		Mountain Fresh	MF1[h], MF3[h], MF8-1
		Mountain Spring	MS3[h], MS4[h], MS8[h]
		Sunbrite	SB1[h], SB6-2
	C	Asgrow-2[f]	A2-3[h], A2-4[h], A2-6-2, A2-10-1
		Asgrow-3[f]	A3-1[h], A3-4-2, A3-6-1, A3-6-2, A3-9-2
		Glamour	GL1[h], GL2[h], GL3[h], GL4[h], GL7-2, GL8-2, GL10-2

Table 1. continued

Region/Grower/ Field/Date Collected	PCR type[a]	Cultivar	Strain(s)[b]
		Golden Jubilee	GJ7[h]
		Mountain Fresh	MF4[h], MF6[h], MF9-2, MF10-1
		Mountain Spring	MS8-2, MS9[h]
		Springfield	SP7-2, SP8[h], SP9[h]
		Sunbrite	SB3[h], SB5[h], SB6-1[h]
		Sun Gem[e]	A1-6[h], A1-7[h], A1-10[h]
SE/5/1/98-August	A	SO12	I-1, I-2, I-5-1[h]
		401	III-2
		9701	IV-3, IV-6-1[h]
	B	696	II-1, II-5
		401	III-5[h]
		9701	IV-1, IV-2, IV-7-2[h]
	C	SO12	I-3[h], I-4, I-5-2[h], I-6[h], I-8[h], I-9[h]
		696	II-1, II-2, II-4, II-7, II-9[h]
		401	III-3, III-7
		9701	IV-5[h], IV-7-1[h], IV-8[h], IV-10[h]

[a] Polymerase chain reaction designated type using BOX primer (Louws et al., 1998).
[b] Strains were named with respect to a particular cultivar or location in a field followed by a dash and the colony number the strain was isolated from; i.e. cultivar Mountain Spring-colony 2 = MS-2.
[c]BOX-PCR fingerprints identical in duplicate PCR reactions.
[d]BOX-PCR fingerprints of same PCR type yet differ in polymorphic bands when replicated.
[e] Transplants grown in Georgia.
[f] Transplants grown in Florida.
[g] Strains isolated from greenhouse grown plants.
[h] Strains isolated from a fruit lesion; all other strains were isolated from foliage.

Figure 1. Four regions of Michigan from which *Clavibacter michiganensis* subp. *michiganensis* strains were isolated from tomato plants and fruit collected from processing and fresh market commercial fields in 1997 and 1998.

field. One exception was field SW1/1/97, which was sampled July, August and October, 1997 along diagonal transects. In this field only, plants to be sampled were labeled and sampled each month. Foliar samples consisted of 2 to 3 basal leaves exhibiting bacterial canker foliar symptoms. Fruit exhibiting bird's eye spots were also collected. All samples were placed in plastic bags, transported in ice chests and stored at 4°C for a maximum of 3 days.

Bacterial isolation from foliage and fruit. Stems and leaves were chopped and homogenized for approximately 60 seconds in a Lab-Blender 400 stomacher (Tekmar Co., Cincinnati, OK) using phosphate buffer (0.05 M, ph 7.4; 2 ml/g of plant tissue) amended with Tween-20 detergent (0.02%). Each sample was either undiluted or subjected to a 10-fold serial dilution and grown on nutrient broth yeast extract (NBY) (2) agar modified by omitting glucose (MNBY) and/or semi-selective media for *C. michiganensis* subsp. *michiganensis* (SCM) (5) both amended with nalidixic acid (0.03g/L) and cycloheximide (0.1g/L). One ml of the plant extract was vortexed with 0.5 ml of 40% glycerol and stored at -20°C. After a pure culture was obtained, each isolate was handled in one of two methods: 1) inoculated into 5 mL of MNBY broth and placed on a rotary shaker at 175 rpm for 48 hours, after which 1 ml was stored in 0.5 mL 40% glycerol; 2) inoculated into a sterile cryo-storage solution containing distilled water (19% by volume), tryptic soy broth (TSB; 78% by volume) and glycerol (3% by volume). The isolates were then stored at -20°C for later PCR.

Fruit lesions resembling bird's eye spots were probed using sterilized toothpicks directly under the surface of the lesion. The toothpicks were streaked on

MNBY and colonies resembling *C. michiganensis* subsp. *michiganensis* were stored as described above.

Whole-cell PCR amplification and electrophoresis. Strains were isolated from approximately 30 individual plant and/or fruit samples from each field and analyzed using the whole-cell method of rep-polymerase chain reaction (rep-PCR) as described by Louws et al. with the following modifications (11). Colonies grown for PCR amplification were grown on either MNBY or SCM agar. Amplification was performed in a thermal cycler (Genemate-Techne, Princeton, NJ) equipped with a heated lid, therefore, eliminating the need for mineral oil. Each 25 :l reaction included DNA AmpliTaq polymerase (0.4:l; Perkin Elmer, Norwalk, CT). The primer, BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (12), was synthesized at the Macromolecular Facility, Department of Biochemistry, Michigan State University, East Lansing. DNA fragments within the PCR products (6:l) were separated at 4°C on 1.5% agarose gels in 0.5x Tris-acetate-EDTA (TAE; 0.4 M Tris-acetate and 1 mM EDTA, ph8.0) buffer at 83 volts for 13 hours. The gels were stained with ethidium bromide (0.5 :g/ml), soaked in 5x TAE and photographed under UV illumination using Polaroid Type 55 or 57 film. Genomic DNA isolated from strain 936 (11) of PCR type A was included as a positive control with each PCR run. A negative control consisting of the reaction mixture with the omission of bacterial cells was also included with each run. Gel photographs were analyzed by eye to determine the designation of PCR fingerprint types with the corresponding strains. Twenty-one percent of the total strains were amplified twice using different reaction mixtures and performed on different days (Table 1). When the

fingerprints of the replicated strains were compared, all of the isolates were the same PCR fingerprint type, yet 24% of the replicated strains differed in some polymorphic bands (Table 1).

Hypersensitivity response (HR) assays. Four o'clock (*Mirabilis jalapa*) foliage has been shown to develop hypersensitive reaction lesions when inoculated with *C. michiganensis* subsp. *michiganensis* (6) and is a method used as a standard pathogenicity test for the bacterial canker pathogen in Georgia and other seed-testing facilities (9). Broth cultures of *C. michiganensis* subsp. *michiganensis* were grown in liquid MNBY broth for 48 hours with shaking at room temperature. Cells were harvested by spinning on high in a micro-centrifuge for 3 minutes and washed two times with sterile distilled water. The suspensions were adjusted with sterile distilled water to 50% transmittance at a wavelength of 600 nm measured with a spectrometer 20 and kept on ice. The bacterial concentration of suspensions prepared in this way contain approximately 10^8 CFU/ml (6). Four o'clock plants were grown in clay pots in a commercial potting mixture in a research greenhouse. A needleless hypodermic syringe was used to infiltrate the leaves with the prepared bacterial suspensions. Sterile water and a known HR-positive strain (strain 68) (11) were infiltrated on a leaf of each plant as negative and positive controls, respectively. The presence or absence of local lesions were observed and recorded two days after infiltration. All tests were performed in duplicate on separate plants.

RESULTS

The cultivar Mountain Spring dominated commercial plantings in southwest Michigan during 1997 and 1998 and the most commonly observed *C. michiganensis*

subsp. *michiganensis* strain was type C (302 out of 319 strains) (Table 2). In 1997, the southwest fields containing only 'Mountain Spring' tomatoes (SW/1/1, SW/1/2, SW/2/1) or 'Mountain Spring' and roma-type tomatoes (SW/3/1) harbored *C. michiganensis* subsp. *michiganensis* strains representing primarily the type C strains (27 to 30 strains/field) and few type B strains (0 to 3 strains/field) (Table 2). Field SW/4/1/97 contained at least four cultivars and isolates were mostly type C strains (20) and some type A strains (8) while two plants had a mix of type A and C strains (Table 2). In 1998, two fields (SW/1/2, SW/2/3) each containing only 'Mountain Spring' harbored only type C strains (Table 2).

When 'Mountain Spring' tomatoes were planted in the same fumigated field in consecutive years (SW/1/2/97-98), the C fingerprint type was dominant in 1997 (29 strains) and 1998 (26 strains) with one type B strain found only in 1997 (Table 3). When a commercial 'Mountain Spring' tomato field (SW/1/1/97) was sampled three times over the course of a growing season, the *C. michiganensis* subsp. *michiganensis* PCR types remained relatively stable consisting primarily of type C strains (26 to 29 strains/sample time) and few type B strains (0 to 3 strains/sample time) (Table 4). Three of the 31 plants sampled harbored both type B and C strains over the time sequence (Table 4).

At the beginning of the1997 growing season (July) 8 virulent and 6 avirulent *C. michiganensis* subsp. *michiganensis* strains were detected in field SW/1/1/97 (data not shown). During mid- to late season (August) virulent strains increased nearly three-fold (21) while avirulent strains were decreased by half (3). By the end of the season

Table 2. BOX-PCR fingerprint types of *Clavibacter michiganensis* subsp. *michiganensis* and isolated from fruit and foliage collected from Michigan commercial fresh market and processing tomato fields in 1997 and 1998 according to region.

Region/Grower/ Field/Date Collected	No. of Cultivars	PCR fingerprint types				Totals
		A	B	C	D	
Southwest (SW)						
SW/1/1/97-July	1	0	1	29	0	30
SW/1/1/97-August	1	0	3	26	0	29
SW/1/1/97-October	1	0	0	29	0	29
SW/1/2/97-September	1	0	1	29	0	30
SW/1/3/97-September	1	0	0	30	0	30
SW/2/1/97-September	1	0	0	29	0	29
SW/2/2/97-September	1	0	3	27	0	30
SW/3/1/97-September	2	0	1	29	0	30
SW/4/1/97-September	4	8	0	20	0	28
SW/1/2/98-August	1	0	0	26	0	26
SW/1/3/98-August	1	0	0	28	0	28
Region Total		8	9	302	0	319
Northeast (NE)						
NE/1/1/97-September	9	0	0	0	26	26
NE/1/1/98-June	12	0	0	0	28[a]	28[a]
NE/1/1/98-July	14	0	0	0	31	31
Region Total		0	0	0	85	85

Table 2. continued

Region/Grower/ Field/Date Collected	No. of Cultivars	PCR fingerprint types A	B	C	D	Totals
North-central (NC)						
NC/1/1/97-August	3	0	0	28	2	30
NC/1/2/97-August	3	0	0	30	0	30
NC/1/3/98-September	3	0	0	9	21	30
NC/1/4/98-September	1	0	0	0	14	14
Region Total		0	0	67	37	104
Southeast (SE)						
SE/1/1/97-September	4	11	4	4	0	19
SE/1/2/97-September	4	10	1	2	0	13
SE/2/1/97-September	5	1	16	10	0	27
SE/3/1/97-September	4	4	3	7	0	14
SE/1/2/98-August	11	12	16	0	0	28
SE/1/3/98-August	4	19	2	8	0	29
SE/4/1/98-August	9	3	15	33	0	51
SE/5/1/98-August	4	6	6	17	0	29
Region Total		66	63	81	0	210

[a] Include three type D strains isolated from mature greenhouse grown tomato plants.

Table 3. BOX-PCR fingerprint types of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from foliage collected in consecutive years (1997 and 1998) from Michigan commercial fresh market tomato fields.

Region/Grower/ Field/Date Collected	No. of Cultivars	BOX-PCR Fingerprint Types				Totals
		A	B	C	D	
SW/1/2/97-September	1	0	1	29	0	30
SW/1/2/98-September	1	0	0	26	0	26
NE/1/1/97-September	9	0	0	0	26	26
NE/1/1/98-June	12	0	0	0	28[a]	28
NE/1/1/98-July	14	0	0	0	31	31
SE/1/2/97-September	4	10	1	2	0	13
SE/1/2/98-September	11	12	16	0	0	28

[a] Includes three type D strains isolated from mature greenhouse grown tomato plants.

Table 4. BOX-PCR fingerprint types of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from foliage collected at three times from a Michigan commercial 'Mountain Spring' tomato field in 1997.

Region/Grower/ Field/Date Collected	Summary of BOX-PCR Fingerprint Types				Totals
	A	B	C	D	
SW[a]/1/1/97-July	0	1	29	0	30
SW/1/1/97-August	0	3	26	0	29
SW/1/1/97-October	0	0	28	0	28

Sample Month	Individual Plant Sample Number and Corresponding BOX-PCR Fingerprint Type															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
July	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	C
August	B	C	C	B	C	C	C	C	...	...	C	C	C	C	B	C
October	C	C	C	C	...	C	C	C	C	C	C	C	C	C	C	C

Sample Month	Individual Plant Sample Number and Corresponding BOX-PCR Fingerprint Type														
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
July	C	C	C	C	C	C	C	...	C	C	C	C	C	C	C
August	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
October	...	C	C	C	C	...	C	C	C	C	C	C	C	C	C

[a] SW = Southwest

(October) 13 virulent and 6 avirulent strains were detected. In another field (SW/1/2/97), a greater number of virulent strains (17) than avirulent strains (2) were detected. Similar results were found when this field was sampled the following year, resulting in the detection of 18 virulent and 1 avirulent strain(s).

All strains from the one northeast farm (NE/1/1) where several cultivars (9 to 14) were sampled in 1997 and 1998 were of type D (Table 3). In 1998, some fruit produced in a greenhouse on this grower's farm had symptoms of bird's eye spots from which *C. michiganensis* subsp. *michiganensis* type D strains were isolated (Table 1; strains GH1f, GH2-4, GH3-5).

In 1997, the strains isolated from two fields (NC/1/1, NC/1/2) of one grower in the north-central region were primarily type C strains (28 and 30 strains, respectively) with few type D strains (2 and 0 strains, respectively) (Table 2). Conversely, in 1998, fields NC/1/3 and NC/1/4 contained mostly type D strains (21 and 14, respectively) and few type C strains (9 and 0, respectively) (Table 2).

The plants sampled from fields in southeast Michigan contained 66 type A , 63 type B and 78 type C strains (Table 2). In field SE/1/2 the type A fingerprint was dominant (10 out of 13) with type B and C strains detected in low numbers (1 and 2, respectively) in 1997 (Table 3). When the same field was sampled the following year, type A strains (12) and an increased number of type B strains (16) were detected (Table 3). Two plants in field SE/1/1/97 harbored mixed PCR type strains; one with A and B and another with B and C. Field SE/3/1/97 contained two plants with mixed fingerprint types of A with C and B with C, respectively. In a third field (SE/5/1/98), isolated strains

obtained from eight plants were identified as mixed type strains; three with A and C and the other five with B and C, respectively.

DISCUSSION

When recommendations to control bacterial canker on tomato are considered, a major focus has been on the source of inoculum. It has been well documented that the pathogen can be seed borne (3, 7) or transmitted via infested tomato debris overwintered from a previous planting (8). Historically, Michigan growers utilized transplants that had been grown in southern outdoor seedbeds. After a severe bacterial canker outbreak in the mid-western United States and Canada was traced back to infected transplants from Georgia (7), infected transplants were recognized as an additional source of inoculum. Although Michigan growers now rely on local greenhouse-grown tomato transplants to avoid bacterial canker outbreaks, this change has not eliminated the disease. The warm and humid environment of the greenhouse in addition to frequent overhead watering creates favorable growing conditions for bacterial populations, therefore, infected transplants continue to be a concern as a source of inoculum in Michigan. Inferences may be made between some of the bacterial canker outbreaks observed in this study and the three sources of inoculum mentioned above.

Southwest Michigan is a major fresh market growing area and is unique because of the dominance of 'Mountain Spring.' This cultivar was present in every field sampled and was the only cultivar in six of the eight fields included in our study. The dominant (95%) fingerprint type found in this region was type C. In a *C. michiganensis* subsp. *michiganensis* collection spanning from 1981 to 1994 (11), type C strains (11) detected in

southwest Michigan fields were present at a higher incidence than type A (5) and B (3) strains. In our study, A and B strains comprised 5% of our isolates from this region. In one southwest field (SW/1/2) that was sampled in 1997 and 1998, symptom severity varied between years. This field had been called to our attention in 1997 because of young, newly established seedlings that were exhibiting severe wilting and eventually died. It is our experience in Michigan that such severe symptoms are the result of high *C. michiganensis* subsp. *michiganensis* populations on the seedling at the time of transplanting (10). Whether the *C. michiganensis* subsp. *michiganensis* originated from a contaminated greenhouse or from infected seed is not known. However, in 1998, disease symptoms in that same field were minor, consisting of marginal leaf necrosis on an insignificant number of plants. This grower compensates for a shortened of lack of roatation by fumigating the plant beds yearly. Although overwintered host debris was not readily observed, the soil between the plant beds was not fumigated leaving a possible source of inoculum. It is also possible that seedlings with populations far below the threshold required for more severe symptoms to occur were used to establish the field in 1998.

While northeast Michigan has only one significant tomato grower, isolates collected from this farm offer a unique perspective on the potential role of greenhouse and field as potential sources of *C. michiganensis* subsp. *michiganensis* inoculum. Although multiple cultivars (≤ 14) were grown, only one fingerprint type D was observed among the 85 isolates collected during 1997 and 1998. The grower in the northeast region produced tomatoes within a 5 acre plot that was rotated within a 20 acre field and

did not fumigate. Transplants used in the production fields were grown in greenhouses located on the farm but not adjacent to the production field. After the transplants were moved to the field, the grower used the greenhouse to produce full-sized plants and fruit from which *C. michiganensis* subsp. *michiganensis* strains were isolated in 1998. The greenhouse-grown plants did not show foliar symptoms, yet did show minimal fruit spotting. A recent study reported that plants are most susceptible to fruit at the flowering stage and small fruit stage of plant development (13). This suggests that the pathogen populations were high enough at the time of flowering to cause minimal fruit spotting, yet not high enough to cause severe symptoms on the mature plants. Sanitation practices in this greenhouse had not been closely adhered to. The routine of extended greenhouse production together with the lack of field rotation create a situation in which bacterial populations may have overfallowed on buried debris and/or alternative weed hosts in the field and/or the greenhouse, thereby providing a reservoir of *C. michiganensis* subsp. *michiganensis* from 1997 to 1998.

Type D strains were not found in the major fresh market tomato growing regions of the southwest or southeast but were found on another Michigan farm located in the north-central region approximately 80 miles away from the northeast grower. The grower from the north-central region purchased transplants from the grower in the northeast region, although the year of the original connection between these growers is not clear. The north-central grower practiced short rotation (>2 years), did not fumigate the plant beds and typically grew multiple cultivars (≤ 3). Infected transplants that were grown in greenhouses of the northeast grower likely harbored type D strains that were then

transmitted to north-central field. Louws et al. (11) identified three type D strains from this farm from samples collected in 1994. The north-central grower did not have type D strains exclusively as did the northeast grower, but also had type C strains. A shift in the incidence of the type D strains was observed from 1997 (3%) to 1998 (80%). Without knowing the number of seedlings and cultivars provided by the northeast grower, it is difficult to form a hypothesis regarding the reason for this change.

Another primer, $(GTG)_5$ has been shown to distinguish BOX-PCR type D into three groups when subjected to rep-PCR (Medina-Mora, thesis). Further analysis of the type D strains from the north-central and northeast fields using this primer may provide additional information on the variation between the type D strains found on these farms.

Typical cultural practices of the southeast region included long rotation (>3 years), no fumigation and a diversity of cultivars within and between farms. Unique to any other field in this study, field SE/4/1/98, containing 3 type A, 15 type B and 33 type C strains, had never been planted to tomatoes and was the first year this grower had ever grown this crop (Table 2). With the exception of three Florida-grown cultivars, all of the seedlings used to establish this field were grown in the grower's greenhouses that previously had been used only for production of flowering ornamentals and melon transplants. With the elimination of overwintered debris and greenhouse transplants as likely inoculum sources, seed remains the most likely source of inoclum for the disease outbreak in this field. Disease symptoms were severe within some cultivars (wilting and stunting), yet minor within others (marginal necrosis). The variation of symptom severity between cultivars strengthens the possibility of seed contamination. Seed from one or

more cultivars that were contaminated would likely exhibit severe symptoms in the field as pathogen populations had time enough to reach threshold levels. Spread from infected plants to uninfected plants in the greenhouse and/or in the field is suggested by the presence of bird's eye fruit spotting on all cultivars. Ricker and Reidel (14) determined that secondary spread causes less severe symptoms of marginal necrosis and fruit spotting, yet does not lead to yield loss.

Type A strains have been described as being most frequently associated with processing cultivars (11) and in our study were only found in one southwestern Michigan field (SW/4/1/97) that included a roma-type cultivar considered to be a processing type. However, in this field, the type A strains were detected on both the roma-type and the fresh market cultivars. Louws et al. (11) included four *C. michiganensis* subsp. *michiganensis* strains obtained in 1994 from a southwest Michigan field (SW/4) that contained roma-type tomatoes and also detected a type A strain: the only type A strain detected in commercial fields in that region that year.

Louws, et al. (11) found a significant number (30%) of avirulent strains associated with the type A strain. In our study 16% of the type C strains were avirulent, while type C strains were found by Louws et al. (11) to be predominantly virulent. This study is the first to report a substantial number of avirulent type C strains in field populations.

Although 24% of the strains replicated in two BOX-PCR runs exhibited some differing polymorphic bands, some polymorphisms have been shown to be stable (Fulbright, personal communication). These stable polymorphisms have been used in epidemiological studies as a means of tracing a particular strain throughout the

experiments.

Prior to the technology of BOX-PCR fingerprinting, it would have been impossible to make inferences concerning the transmission of bacterial strains from the three type of inoculum sources discussed above. Further research is needed to test the theories stated in this study. Any biological significance of the four fingerprint types is not known and it is of interest to determine if one fingerprint type has a biological advantage over another. This type of information may help to explain the presence of dominant fingerprint types in some of the fields examined in this study.

LITERATURE CITED

1. Bay, D. M., and Wyant, D. (eds). 1999. Michigan Agricultural Statistics. MDA, Michigan pp. 42-45.

2. Bryan, M. K. 1930. Studies on bacterial canker of tomato. J. Agr. 41:825-851.

3. Chang, R. J., Ries, S. M., and Pataky, J. K. 1991. Dissemination of *Clavibacter michiganensis* subsp. *michiganensis* by practices used to produce tomato transplants. Phytopathology 81:1276-1281.

4. Chang, R. J., Ries, S. M., and Pataky, J. K. 1992. Local sources of *Clavibacter michiganesis* subsp. *michiganensis* in the development of bacterial canker on tomatoes. Phytopathology 82:553-560.

5. Fatmi, M., and Schaad, N. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology 78:121-126.

6. Gitaitis, R. D. 1990. Induction of a hypersensitivelike reaction in four-o'clock by *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. 74:58-60.

7. Gitaitis, R. D., Beaver, R. W., and Voloudakis, A. E. 1991. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato transplants. Plant Dis. 75:834-838.

8. Gleason, M. L., Braun, E. J., Carlton, W. M., and Peterson, R. H. 1991. Survival and dissemination of *Clavibacter michiganensis* subsp. *michiganensis* in tomatoes. Phytopathology 81:1519-1523.

9. Gleason, M. L., Gitaitis, R. D., and Ricker, M. D. 1993. Recent progress in understanding and controlling bacterial canker of tomato in eastern North America. Plant Dis. 77:1069-1076.

10. Hausbeck, M. K., Bell, J., Medina-Mora, C., Podolsky, R., and Fulbright, D. W. 2000. Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganenesis* on tomatoes in the greenhouse and on disease development and crop yields in the field. Phytopathology 90:38-44.

11. Louws, F. J., Bell, J., Medina Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., deBruijn, F. J., and Fulbright, D. W. 1998. rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis.* Phytopathology 88:862-868.

12. Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenbeck, R., Morrison, D. A., Bounois G. J., Glaverys, J-P. 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. 20:3479-3483.

13. Medina-Mora, C. M., Hausbeck, M. K., and Fulbright, D. W. 2000. Bird's eye lesions of tomato fruit produced by aerosol and direct application of *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. (In press).

14. Ricker, M. D., and Riedel, R. M. 1993. Effect of secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* on yield of northern processing tomatoes. Plant Dis. 77:364-366.

15. Strider, D. L. 1969. Bacterial canker of tomato caused by *Corynebacterium michiganense*; a literature review and bibliography. North Carolina Experiment Station, Technical Bulletin 193:110 pages.

16. Tsiantos, J. 1987. Transmission of bacterium *Corynebacterium michiganense* pv. *michiganense* by seeds. J. Phytopathology 119:142-146.

17. Versalovic, J., Schneider, M., De Bruijn, F. J., and Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Meth. Mol. and Cell. Biol. 5:25-40.

SECTION II

EFFECT OF BACTERICIDES, RESISTANT CULTIVARS, AN SAR-INDUCING COMPOUND AND AVIRULENT STRAINS ON POPULATION SIZE AND SPREAD OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* ON SEEDLING TOMATOES IN THE GREENHOUSE

ABSTRACT

Treatments reduced population sizes of *Clavibacter michiganensis* subsp. *michiganensis* among tomato seedlings in the greenhouse compared to the untreated inoculated control in 1996 and 1998. In 1996, copper hydroxide alone or in combination with mancozeb or streptomycin reduced pathogen populations relative to acibenzolar-S-methyl, acibenzolar-S-methyl/copper hydroxide and avirulent *C. michiganensis* subsp. *michiganensis* strains. Copper hydroxide/streptomycin reduced populations in comparison to copper hydroxide/mancozeb. In 1998, treatments did not differ significantly in affecting population sizes. In 1997 only, treatments differed from each other in affecting pathogen spread. Copper hydroxide mixed with mancozeb limited spread compared to copper hydroxide mixed with streptomycin. Streptomycin sprays were less effective than using resistant cultivars to limit pathogen spread. In the field, inoculated control plants produced yields that were 61% (1996), 93% (1997) and 98% (1998) of those produced by the uninoculated controls. With the exception of the inoculated control in 1996, which resulted in yield loss, pathogen populations on transplants used to establish field plots did not reach threshold levels of 10^7 CFU/g fresh weight needed for severe symptom development or yield loss. Fruit spotting occurred regardless of treatment and was highly variable.

INTRODUCTION

In 1999, the Michigan tomato (*Lycopersicon esculentum* Mill.) industry generated over 27 million dollars (1). Bacterial diseases, including bacterial canker of tomato (causal agent: *Clavibacter michiganensis* subsp. *michiganensis*), are a yearly concern for growers in the Midwest. Infection by *C. michiganensis* subsp. *michiganensis* can result in plant wilting, stunting, reduced yields, and plant death. Less severe disease symptoms include marginal leaf necrosis that may be bordered by chlorosis, and fruit lesions appearing as superficial white spots (3-6 mm) that develop a necrotic center.

In the Midwest, tomato transplants are grown in local greenhouses for use in production fields. Greenhouse transplants can appear to be healthy, yet harbor high bacterial populations that may lead to severe disease symptoms in the field. Applications of copper bactericides to transplants in the greenhouse can limit *C. michiganensis* subsp. *michiganensis* population size and spread and subsequently decrease yield loss (11). The pathogen population must be suppressed below 10^7 CFU/g at the time of transplanting to prevent yield loss in the field (11). Therefore, bactericide applications should begin in the greenhouse rather than in the field. There are few strategies to manage bacterial canker after symptoms appear, but growers can apply copper bactericides in the field.

Long term and continuous use of copper bactericides may lead to copper resistant bacteria (5). While resistance to copper has not been noted to date for *Clavibacter* spp. (5), we desired to develop a disease management program that is not wholly reliant on copper bactericides. Novel products for management of bacterial diseases include those that activate systemic acquired resistance (SAR) in plants (10) such as acibenzolar-S-

methyl (Actigard, Novartis Crop Protection, Inc., Greensboro, NC) (13). Tomato cultivars moderately resistant to bacterial canker have been developed that exhibit reduced foliar blight and yield losses compared to a susceptible cultivar (19). Attempts have been made to use close relatives of plant pathogens as biological control agents. In a study by Frey et al. (7), avirulent mutants of *Pseudomonas solanacerum* (causal agent of wilt in tomato) were able to establish themselves and persist in host tissue, and when challenged with virulent strains, reduced disease symptoms. The objective of this study was to compare the effectiveness of different management schemes including the use of resistant cultivars, an SAR-inducing compound, and avirulent *C. michiganensis* subsp. *michiganensis* strains along with standard bactericides in reducing pathogen populations and spread among greenhouse tomato seedlings. *C. michiganensis* subsp.. *michiganensis* populations in the field and resulting yields were also of interest.

MATERIALS AND METHODS

Culture of virulent and avirulent strains. The virulent *C. michiganensis* subsp. *michiganensis* strain used was a rifampicin-resistant mutant of a rep-polymerase chain reaction (PCR) fingerprint type B strain isolated in 1987 from tomato fruit from a farm in northeastern Ohio. This strain has a BOX-PCR DNA fingerprint that is different from *C. michiganensis michiganensis* strains commonly encountered in Michigan (15). The spontaneous rifampicin-resistant mutation was selected by plating cells from a nutrient broth yeast extract (NBY) broth culture modified by omitting glucose (MNBY) (15) on MNBY agar containing 50 μg of rifampicin per ml. The strain was tested for pathogenicity to tomato by clipping the petioles of 10- to 14-day-old seedlings (cultivar

H8704; H. J. Heinz Co., Pittsburgh, PA) with scissors dipped in a suspension of the strain, which had been grown in MNBY broth, centrifuging at 1,564 x g for 10 min at room temperature in a GSA rotor in a Sorvall RC5C centrifuge (DuPont Co., Wilmington, DE), resuspending in sterile distilled water, and adjusting spectrophotometrically to 3 x 10^8 CFU/ml. In all years, inoculated test plants developed severe disease symptoms including stunting, wilting, and death.

The avirulent *C. michiganensis* subsp. *michiganensis* strains used as avirulent strain treatments were streptomycin-resistant mutants of BOX-PCR DNA fingerprint type A. Strain 40S was isolated from fresh market tomato stem tissue in 1988, and strain 302S was isolated from a processing tomato fruit lesion in 1994; both samples were obtained in southeastern Michigan. The spontaneous streptomycin-resistant mutation was selected by plating cells from MNBY broth culture on MNBY containing 50 µg of streptomycin per ml.

The virulent *C. michiganensis* subsp. *michiganensis* strain used in the greenhouse experiments was prepared by inoculating two 5-ml broth culture tubes of MNBY containing 100 µg of rifampicin per ml and incubating them for 48 h at room temperature with shaking at 190 rpm. These cultures were used to inoculate 1 liter of MNBY that was incubated for 48 h at 25°C with shaking at 75 rpm. The culture was centrifuged and the pellets were resuspended and combined in sterile distilled water to a final volume of 1 liter; the final concentration was 5 x 10^8 CFU/ml. The avirulent strains were prepared as described above, except MNBY broth contained 100 µg of streptomycin per ml. The suspensions were kept on ice and applied within three hours using a CO_2 backpack

sprayer with two flat-fan 8002 nozzles (Teejet, Chicago) (1996, 1997) or a single nozzle (1998) which were operated at 2.8 kg/cm^2 delivering approximately 748 liter/ha.

Experimental design and treatments. On 13 and 14 May 1996, 24 March 1997, and 23 March 1998, plastic plug sheets (52.5 cm x 26.5 cm x 4.0 cm) each containing 288 cells filled with soilless medium were individually seeded with the tomato cultivars H8704, H9144, and H70214 (H. J. Heinz Co., Pittsburgh, PA; 1996, 1997) and cultivar Mountain Spring (Novartis Seeds, Inc., Gilroy, CA; 1998) and germinated for 3 days in a walk-in germination chamber in a commercial greenhouse in southwestern Michigan. Data collected in 1996 and 1997 are included in this study to strengthen the importance of this research, although were not a part of the work performed to complete the masters degree. Plug sheets were then transported to a commercial polyethylene greenhouse (approximately 12.2 m x 29.3 m) in Stockbridge, Michigan, where they were placed on overturned plastic flats on the earthen greenhouse floor, that was covered with a black woven polyethylene groundcover. Flats were arranged in blocks, each consisting of 12 plug sheets (1996, 1997) or 15 plug sheets (1998) (Fig. 1). Seedlings were watered overhead as required, and irrigated with 100- or 200-ppm N and K_2O fertilizer solution (Peter's 20-20-20; Grace-Sierra Horticultural Products Co., Milpitas, CA) as needed. Greenhouse temperatures were monitored and average maximum and minimum temperatures of 25.9°C (day) and 11.6°C (night), respectively were recorded. The average relative humidity was 95.4%.

Each year the plug sheets were arranged in a block design, with four replicates per

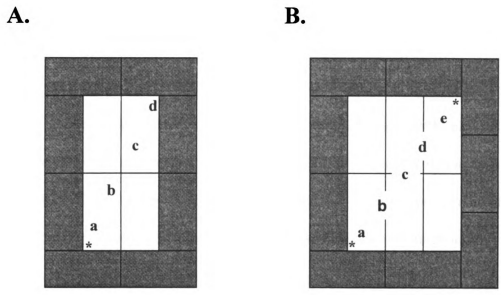

Figure 1. Diagrams of treatment blocks with shaded areas serving as a buffer between blocks and each rectangle representing a 52.5 x 26.5 cm tray containing 288 tomato seedlings. In 1996-1997 (A), seedlings were sampled from each block following the diagonal (regions a to d) from the *Clavibacter michiganensis* subsp. *michiganensis* inoculum focus (*). In 1998 (B), seedlings were sampled from each block following the diagonal (regions a to e) between the *C. michiganensis* subsp. *michiganensis* inoculum foci (*).

treatment. The inner four (2×2) plug sheets (1996, 1997), and the inner six (2×3) plug sheets (1998) of each block were targeted for treatment, with the surrounding flats considered as buffers between blocks (Fig. 1). Fungicides were applied as needed to manage nontarget pathogens including *Botrytis cinerea* (1997, 1998), *Alternaria solani* (1998), *Sclerotinia sclerotiorum* (1998) and *Oidiopsis sicula* (1998).

The following treatments were investigated: (i) copper hydroxide (Kocide 40DF, 3.0 g a.i./liter; Griffin LLC, Valdosta, GA); (ii) copper hydroxide (Kocide 40DF, 3.0 g a.i./liter) mixed with mancozeb (Dithane F-45, 2.3 ml a.i./liter; Rohm and Haas Co., Philadelphia); (iii) copper hydroxide (Kocide 40DF, 3.0 g a.i./liter) mixed with streptomycin (Agri-mycin 17, 0.25 g a.i./liter; Novartis Crop Protection, Inc. Ag. Products, Greensboro, NC) (1996 and 1997 only); (iv) SAR-inducing compound acibenzolar-S-methyl (Actigard 50WDG, 0.1 g a.i./liter; Novartis Crop Protection, Inc. Ag. Products, Greensboro, NC); (v) acibenzolar-S-methyl (Actigard 50WDG, 0.1 g a.i./liter) mixed with copper hydroxide (Kocide 40DF, 3.0 g a.i./liter) (1996 only); (vi) resistant cultivar H70214 (H. J. Heinz Co.; 1996 and 1997 only); (vii) resistant cultivar H9144 (H. J. Heinz Co.; 1996 and 1997 only); (viii) avirulent streptomycin-resistant *C. michiganensis* subsp. *michiganensis* strain 302S (1996 and 1997 only); (ix) avirulent streptomycin-resistant *C. michiganensis* subsp. *michiganensis* strain 40S (1996 and 1997 only); (x) streptomycin (Agri-mycin 17, 0.25 g a.i./liter) (1997 and 1998 only); and (xi) no treatment (control). Untreated, uninoculated plants of each cultivar were grown in a separate greenhouse under environmental and cultural conditions similar to those of the inoculated treatments. In 1998, three sets of four replicates of untreated, inoculated

controls were included to provide sufficient plants for subsequent field treatments.

Treatment sprays were applied using a CO_2 backpack sprayer with two flat-fan 8002 nozzles (Teejet, Chicago) (1996, 1997) or a single nozzle (1998) which were operated at 2.8 kg/cm^2 delivering approximately 748 liter/ha. Sprays were initiated when the first true leaves of the seedlings were visible and 1 to 2 days prior to inoculation with the virulent rifampicin-resistant *C. michiganensis* subsp. *michiganensis*. Subsequent sprays were applied every 5 days (10 June to 5 July 1996; 22 April to 17 May 1997; 25 April to 4 June 1998) until the seedlings were removed from the greenhouse and planted in the field.

Inoculation. On 11 June 1996, 23 April 1997, and 27 April 1998, seedlings (cultivar H8704) were inoculated by misting the leaves with a virulent rifampicin-resistant *C. michiganensis* subsp. *michiganensis* suspension (prepared as described above) using a Preval pressurized sprayer (Precision Valve Corporation, Yonkers, NY). The first true leaf of each individual seedling was then removed by clipping the petiole close to the stem with scissors dipped in the same *C. michiganensis* subsp. *michiganensis* suspension. Inoculated seedlings were incubated overnight on a laboratory bench in loosely closed plastic bags to maintain high humidity. To initiate infection in the commercial greenhouse, 16 seedlings with their soilless plugs and roots intact were removed from the corner closest to the 'a' site (1996, 1997), or from each of the two opposite corners of each treatment block (1998) and replaced with the same number of inoculated seedlings to establish an inoculum focus(i) (Fig. 1).

Sampling of greenhouse seedlings. At 28 days (1996), 29 days (1997), and 42

days (1998) after inoculation, five to eight seedlings were removed from four regions (a to d; 1996, 1997) or five regions (a to e; 1998) within each block along the diagonal from the inoculum focus(i) (Fig. 1). The inoculated plants used to initiate disease within each treatment block were not included in the foliar samples; they were dead by the time of sampling. Samples from each region were stored individually in plastic bags in a cooler at 4°C for a maximum of 3 days; each sample was individually processed. Shoots and leaves were chopped with a sterile straight razor and homogenized for 2 min in a Lab-Blender 400 stomacher (Tekmar Co., Cincinnati, OH) using sterile phosphate buffer (0.05 M, ph 7.4; 2 ml/g of plant tissue) amended with Tween-20 detergent (0.02%). One mL of the plant extract was then stored in 0.5 ml of 40% glycerol and frozen at -20°C until further processing.

Each sample was thawed on ice and population sizes of the virulent pathogen were estimated from 10-fold serial dilutions spread on MNBY agar containing 100 μg of rifampicin per ml. Colonies were counted after 4 to 7 days. The lower limit of detection was 30 CFU/g of tissue. To verify that these isolates had the same BOX-PCR DNA fingerprint type as the inoculum, 18 (1996), 10 (1997), and 23 colonies (1998), were chosen for BOX-PCR DNA fingerprinting. In 1996, 15 colonies were the rifampicin-resistant B type, while the others were not *C. michiganensis* subsp. *michiganensis*. In 1997, none of the chosen colonies were *C. michiganensis* subsp. *michiganensis*. In 1998, all colonies were the rifampicin-resistant B type.

Untreated, uninoculated plants were sampled by haphazardly removing four seedlings from one flat (1996, 1997) or six seedlings from each cultivar (1998) and

processing as previously described. Serially diluted homogenates were plated on MNBY agar containing 100 µl of rifampicin per ml. There were no colonies resembling *C. michiganensis* subsp. *michiganensis* in any year.

Statistical analysis of greenhouse populations. Population data were analyzed by ANOVA as a completely randomized split-plot design with the main plots as the block, which differed by treatment, and position as sub-plots. The 1996 and 1997 data were transformed using Y=ln(CFU+1) to achieve normality, as determined by analyzing residuals using the SAS procedure, Proc Univariate (SAS Institute, Cary, NC). The assumption of equality of variances was examined using Levene's Test (22). Because the 1998 variances were not equal, an ANOVA of ranked population sizes was conducted (4) (Proc Rank and Proc GLM [SAS Institute, Cary, NC]). This analysis is similar to other non-parametric procedures for less complex designs (4). Although the analyses is affected by unequal variances, this effect should be minimal, especially compared with a parametric analysis. The following linear model was used for all ANOVA procedures: mean + treatment + position + treatment × position + block nested within treatment + error; block nested within treatment was used as the error term for treatment.

For all years, linear contrasts were used to examine the differences between treatments and the effects of treatment on the relationship of populations with position in the experimental blocks. The following contrasts were used to examine the differences in treatments for 1996: (i) inoculated susceptible control versus all other treatments; (ii) inoculated resistant cultivars versus all other treatments; (iii) copper hydroxide alone or mixed with streptomycin or mancozeb versus acibenzolar-S-methyl alone or with copper

hydroxide, and avirulent strain treatments; (iv) acibenzolar-S-methyl alone or with copper hydroxide versus avirulent strain treatments; (v) copper hydroxide alone versus copper hydroxide mixed with streptomycin or mancozeb; (vi) copper hydroxide/streptomycin versus copper hydroxide/mancozeb; (vii) acibenzolar-S-methyl versus acibenzolar-S-methyl/copper hydroxide (1996 only); (viii) avirulent strains 40S versus 302S. The effects of treatment on the relationship of populations with position in the experimental blocks (interaction of treatment and position) was examined using contrasts similar to those above for 1997, except the streptomycin treatment was considered a positive control.

The following contrasts were used to examine the differences in treatments for 1998: (i) copper hydroxide alone or mixed with mancozeb versus the inoculated control; (ii) copper hydroxide alone versus copper hydroxide/mancozeb; (iii) acibenzolar-S-methyl versus inoculated control; (iv) streptomycin versus inoculated control; (v) copper hydroxide versus acibenzolar-S-methyl and streptomycin; (vi) acibenzolar-S-methyl versus streptomycin. Because these contrasts were not independent, and not entirely *a-priori* comparisons, Bonferonni's correction was used to adjust α to 0.008 (0.05/6); only contrasts with a p-value less than 0.008 were considered significant.

Field Study. On 16 and 17 July 1996, 28 May 1997, and 10 June 1998, 36 seedlings were randomly selected from the center (Fig. 1, regions b and c; 1996, 1997) or (Fig. 1, region c; 1998) of each treatment block and planted in a randomized complete block design with 4 replications at the Botany and Plant Pathology Research Farm, East Lansing, Mich, in sandy loam (1996, 1998) or clay loam (1997). The fields had been

previously planted to sweet corn (1996), or rye (1997, 1998). Each treatment plot consisted of 12 seedlings planted into each of three 3.6-m-long rows spaced 1.5 m apart with 30.5 cm between plants within rows. The experimental sites were fertilized, and weeds were managed according to standard commercial practices.

In 1996 and 1997, to prevent spread of the pathogen from nearby inoculated plants, uninoculated control plants received streptomycin (Agri-mycin 17, 0.187 kg a.i./ha) applications weekly using a pneumatic handsprayer with a single nozzle operated at 2.8 kg/cm^2 delivering 748 liters/ha. In 1998, streptomycin was applied to a plot of uninoculated plants just outside the experimental field using a CO_2 backpack sprayer with a 8003LP nozzle operated at 2.8 kg/cm^2, delivering 467 liters/ha. In this year the uninoculated plants randomized within the experimental field did not receive streptomycin applications. Each year, all treatments were sprayed, as needed, using the same method as described above, with the fungicide chlorothalonil (Bravo Weather Stik, 2.5 kg a.i./ha; Zeneca Ag Products, Wilmington, DE) to prevent disease caused by fungal pathogens.

Field sampling of fruit and foliage. On 2 October 1996, 8 September 1997, and 23 September 1998, plant stand counts were recorded for the middle 3.6-m-long row, and total yield, and incidence of fruit spotting were recorded in a single harvest for the five innermost plants of the middle row. Foliage from plants was sampled on 26 September 1996, 4 September 1997, and 21 September 1998 at random from five to six central plants in the center row of each treatment plot and processed as previously described to determine the pathogen population sizes. To verify that the field isolates were identical to

the one used to inoculate the seedlings in the greenhouse, 44 (1996), and 106 (1998) colonies resembling *C. michiganensis* subsp. *michiganensis* were chosen for BOX-PCR DNA fingerprinting. In 1996, 21 colonies were rifampicin-resistant B types, 3 were rifampicin-resistant C types, and 20 were not the pathogen. In 1998, 103 colonies were the rifampicin-resistant B type, 1 was a rifampicin-resistant C type, and 2 were not *C. michiganensis* subsp. *michiganensis*.

In 1998, diseased fruit were surface sterilized with 70% ethanol (v/v), and a sterile toothpick inserted just below the surface of bird's eye lesions and streaked onto MNBY containing 100 µl of rifampicin per ml. Fourty-seven colonies resembling *C. michiganensis* subsp. *michiganensis* were subjected to BOX-PCR DNA fingerprinting and determined to be the rifampicin-resistant B type, except for one colony that was a rifampicin-resistant C type.

Statistical analysis of field measurements. The 1996 and 1997 field data were analyzed as a randomized complete block design. Total fruit weight was transformed using $Y = (X+1)^{1/4}$. The transformed data were normally distributed with equal variances. Stand count data were not normally distributed, the variances were not equal, and the data could not be transformed to meet these assumptions. Therefore, ranks of this variable were used in all subsequent analyses. The following contrasts were used to examine the differences in treatments for 1996: (i) susceptible and resistant inoculated versus susceptible and resistant uninoculated controls; (ii) susceptible control, inoculated or not versus all other treatments; (iii) resistant cultivars, inoculated or not versus all other treatments; (iv) inoculated susceptible control versus all other chemical, acibenzolar-S-

methyl alone or with copper hydroxide and avirulent strain treatments; (v) uninoculated susceptible control versus all other chemical, acibenzolar-S-methyl alone or with copper hydroxide and avirulent strain treatments; (vi) inoculated resistant cultivars versus all chemical, acibenzolar-S-methyl alone or with copper hydroxide and avirulent strain treatments; (vii) uninoculated resistant cultivars versus all chemical, acibenzolar-S-methyl alone or with copper hydroxide and avirulent strain treatments; (viii) the interaction between inoculation and control (positive vs. negative); (ix) copper hydroxide alone or mixed with streptomycin or mancozeb versus acibenzolar-S-methyl alone or with copper hydroxide and avirulent strain treatments; (x) acibenzolar-S-methyl alone or with copper hydroxide versus avirulent strain treatments; (xi) copper hydroxide versus copper hydroxide mixed with streptomycin or mancozeb; (xii) copper hydroxide/streptomycin versus copper hydroxide/mancozeb; (xiii) acibenzolar-S-methyl vs. acibenzolar-S-methyl/copper hydroxide (1996 only); (xiv) avirulent strain 40S versus 302S. In 1997, similar contrasts were used, except the streptomycin treatment was considered a positive control, and used as such for the following contrasts: (i) susceptible and resistant inoculated versus susceptible and resistant uninoculated controls; (ii) resistant cultivars, inoculated or not versus all other treatments.

Plant stand and yield data for the 1998 field plot were not statistically analyzed due to additional treatments applied in the field that were not included in the 1996 and 1997 studies and therefore not reported in this paper.

RESULTS

Impact of bactericides, resistant cultivars, an SAR-inducing compound, and avirulent strains on pathogen populations and spread among transplants in the greenhouse. Symptoms observed on seedlings that were directly inoculated with bacteria and introduced into the treatment blocks included cankers, firing, wilt, and death. In all years, *C. michiganensis* subsp. *michiganensis* populations were detected in all inoculated treatment blocks but not in the uninoculated controls.

In 1996, treatments significantly ($P < 0.001$) reduced pathogen populations compared to the inoculated susceptible control (Table 1). However, pathogen population sizes on treated plants were the same as on the inoculated resistant culitvars ($P = 0.409$). Copper hydroxide alone or mixed with mancozeb or streptomycin significantly reduced pathogen populations compared to acibenzolar-S-methyl alone or with copper hydroxide and avirulent strain treatments ($P = 0.054$). The addition of streptomycin or mancozeb to copper hydroxide did not enhance control ($P = 0.377$), yet copper hydroxide/streptomycin significantly reduced population sizes compared to copper hydroxide/mancozeb ($P = 0.035$). There was no apparent advantage in adding copper hydroxide to acibenzolar-S-methyl since pathogen populations were comparable ($P = 0.084$). Acibenzolar-S-methyl alone or mixed with copper hydroxide reduced pathogen population sizes compared to the avirulent strain treatments ($P = 0.019$). The avirulent strain treatment 40S did not differ from 302S in limiting pathogen populations ($P = 0.953$). In 1997, treatments did not affect pathogen population sizes ($P = 0.333$).

Table 1. Summary of contrast results comparing treatments applied to tomato seedlings in the greenhouse when inoculated with *Clavibacter michiganensis* subsp. *michiganensis* in relation to pathogen population size in 1996.

	1996	
Contrast	***F***	***P***
Inoculated susceptible control vs. all other treatments[a]	19.77	<0.001*[b]
Inoculated resistant cultivars vs. all chemical, SAR-inducing compound[c] and avirulent strain treatments	0.70	0.409
Copper hydroxide alone or mixed with streptomycin or mancozeb vs. SAR-inducing compound and avirulent strain treatments	4.03	0.054*
SAR-inducing compound vs. avirulent strain treatments	6.12	0.019*
Copper hydroxide alone vs. copper hydroxide mixed with streptomycin or mancozeb	0.80	0.377
Copper hydroxide/streptomycin vs. copper hydroxide/mancozeb	4.88	0.035*
Acibenzolar-S-methyl vs. acibenzolar-S-methyl/copper hydroxide	3.19	0.084
Avirulent strain treatments (40S vs. 302S)	0.00	0.953

[a] Copper hydroxide (Kocide 40DF, 3.0 g a.i./liter); mancozeb (Dithane F-45, 2.3 ml a.i./liter); streptomycin (Agri-mycin 17, 0.25 g a.i./liter); acibenzolar-S-methyl (Actigard 50WDG, 0.1 a.i./liter); resistant cultivars H70214 and H9144 (H. J. Heinz Co., Pittsburgh, PA); avirulent streptomycin-resistant *C. michiganensis* subsp. *michiganensis* strains 40S and 302S. Number of applications (if pertinent) were 1x/5 days = five sprays.

[b] * Indicates the contrast is significant at $P \leq 0.05$.

[c] Acibenzolar-S-methyl and acibenzolar-S-methyl/copper hydroxide.

In 1998, copper hydroxide alone or mixed with mancozeb significantly reduced populations relative to the inoculated control ($P < 0.001$) as did acibenzolar-S-methyl ($P < 0.001$) and streptomycin ($P < 0.001$) treatments (Table 2). However, adding mancozeb to copper hydroxide did not offer a significant benefit compared to copper hydroxide alone ($P = 0.265$). Copper hydroxide was comparable to acibenzolar-S-methyl and streptomycin ($P = 0.109$) and acibenzolar-S-methyl was comparable to streptomycin ($P = 0.509$) in limiting population sizes.

Treatments significantly affected pathogen spread ($P = 0.001$) in 1997 only. Copper hydroxide mixed with mancozeb limited spread compared to copper hydroxide mixed with streptomycin ($P < 0.001$), yet there was no difference in spread between copper hydroxide alone versus copper hydroxide mixed with mancozeb or streptomycin ($P = 0.074$). Streptomycin was less effective than the inoculated resistant cultivars in limiting pathogen spread ($P = 0.004$).

Impact of bactericides, resistant cultivars, an SAR-inducing compound, and avirulent strains on field productivity. At the time of transplanting, the maximum bacterial populations in the untreated susceptible controls used to establish field plots at regions b and c (1996, 1997; Fig. 1), or at region c (1998; Fig. 1) were 4.1×10^7 (1996), 6.1×10^5 (1997), and 1.4×10^5 (1998) (Fig. 2). All treatments limited pathogen populations at regions b and c to $<8.2 \times 10^6$ CFU/g (1996) and $<4.2 \times 10^5$ CFU/g (1997) of tissue. In 1998, all treatments had undetectable pathogen populations at region c (Fig. 3).

In all years, plants showed foliar symptoms of bacterial canker at the end of the

Table 2. Summary of contrast results comparing treatments applied to tomato seedlings in the greenhouse when inoculated with *Clavibacter michiganensis* subsp. *michiganensis* in relation to pathogen size in 1998.

Contrast	***F***	***P***
Copper hydroxide[a] alone or mixed with mancozeb[b] vs. inoculated control	74.95	<0.001*[c]
Copper hydroxide vs. copper hydroxide/mancozeb	1.31	0.265
Acibenzolar-S-methyl[d] vs. inoculated control	30.88	<0.001*
Streptomycin[e] vs. inoculated control	37.07	<0.001*
Copper hydroxide vs. acibenzolar-S-methyl and streptomycin	2.81	0.109
Acibenzolar-S-methyl vs. streptomycin	0.45	0.509

[a] Copper hydroxide (Kocide 40DF, 3.0 g a.i./liter). Number of applications for all treatments were 1x/5 days = nine sprays.

[b] Copper hydroxide (Kocide 40DF, 3.0 g a.i./liter) mixed with mancozeb (Dithane F-45, 2.3 ml a.i./liter).

[c] * Indicates the contrast is significant at $P < 0.05$.

[d] Acibenzolar-S-methyl (Actigard 50WDG, 0.1 g a.i./liter).

[e] Streptomycin (Agri-mycin 17, 0.25 g a.i./liter).

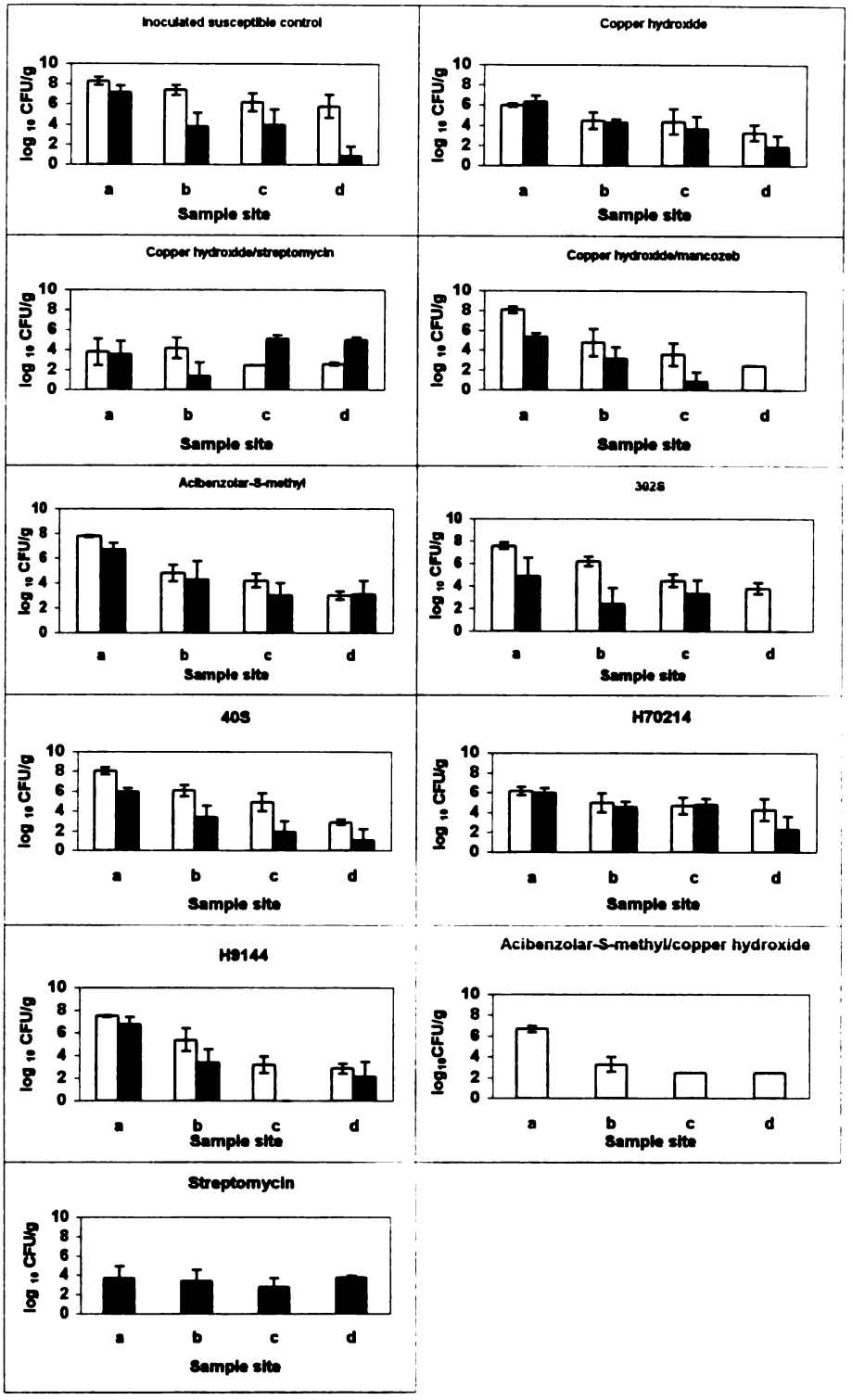

Figure 2. *Clavibacter michiganensis* subsp. *michiganensis* populations (CFU/g) on treated or untreated tomato seedlings in the greenhouse prior to transplanting in the field in 1996 (□) and 1997 (■) when sampled from sites adjacent to *C. michiganensis* subsp. *michiganensis* inoculum focus (a), the center of the treatment block (b,c), and the point farthest from the inoculum focus (d). Error bars represent standard error.

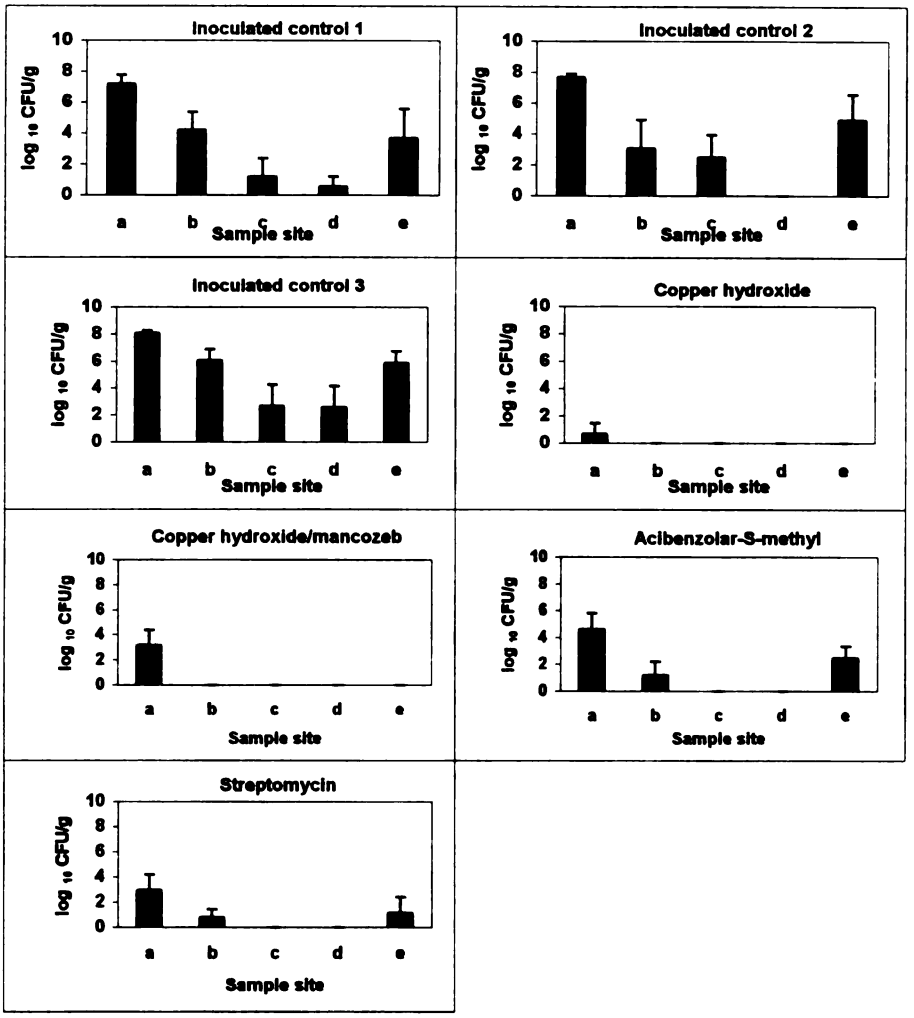

Figure 3. *Clavibacter michiganensis* subsp. *michiganensis* populations (CFU/g) on treated or untreated tomato seedlings in the greenhouse prior to transplanting in the field in 1998 when sampled from sites adjacent to *C. michiganensis* subsp. *michiganensis* inoculum foci (a, e), the center of the block (c), and the points in between (b, d). Three untreated controls were included in the study to supply enough untreated plants for subsequent field treatments. Error bars represent standard error.

growing season including necrosis of the outer leaf edges, or "firing", and brown to tan lesions on peduncles. Final foliar populations ranged from 9.3×10^7 to 1.8×10^9 (1996), 1.4×10^7 to 3.9×10^8 (1997), and 1.6×10^6 to 3.1×10^8 (1998) CFU/g of tissue. The uninoculated untreated plants, with applications of streptomycin in 1996 and 1997 only, had populations of 7.1×10^5 to 9.1×10^6 (1996), 1.7×10^7 to 3.6×10^7 (1997), and 4.2×10^4 to 5.0×10^6 CFU/g of tissue (1998).

In 1996, fewer plants survived in the inoculated untreated susceptible (9.5 out of a maximum of 12) compared to the other treatments (11.0) ($P = 0.005$). In 1997, plant stand was reduced in all treatments due to hot, dry conditions during establishment of transplants in the field. Plant survival in the inoculated susceptible control (8 out of a maxiumum of 12) did not differ significantly from the other treatments (7.3 to 10.3 plants) ($P = 0.411$). In 1998, all plants survived in the inoculated susceptible control. Plant survival ranged from 8.5 to 12 in the other treatments.

Inoculated control plants produced yields that were 61% (1996), 93% (1997) (Table 3), and 98% (1998) (data not shown) of those produced by the uninoculated controls. In 1996, the yield from the inoculated susceptible control was significantly reduced compared to the treatments included in this study ($P = 0.021$). In 1997, the yield from the inoculated susceptible control did not differ significantly from the other treatments ($P = 0.457$). However, the yield from plants treated with copper hydroxide alone was significantly reduced compared to copper hydroxide mixed with streptomycin or mancozeb ($P = 0.005$). The yields of the resistant cultivars (inoculated and uninoculated) were significantly less than the other treatments in 1996 ($P = 0.019$) but not

Table 3. Fruit yield and summary of contrast results comparing treatments on tomato plants; seedlings inoculated or not inoculated with *Clavibacter michiganensis* subsp. *michiganensis* and subjected to spray treatments in the greenhouse in 1996 and 1997.

	Yield (kg/5 plants)	
Treatment[a]	**1996**	**1997**
Uninoculated susceptible control[b]	9.5	17.6
Inoculated susceptible control	5.8	16.4
Copper hydroxide	9.3	11.6
Copper hydroxide/mancozeb	9.1	16.0
Copper hydroxide/streptomycin	9.0	19.1
Streptomycin	...[c]	20.6
Acibenzolar-S-methyl	8.6	15.4
Acibenzolar-S-methyl/copper hydroxide	9.4	...
Avirulent strain 302S	9.8	19.1
Avirulent strain 40S	8.7	19.4
Inoculated resistant cultivar (H70214)	4.9	19.8
Inoculated resistant cultivar (H9144)	8.5	17.8
Uninoculated resistant cultivar (H70214)[b]	6.8	14.9
Uninoculated resistant cultivar (H9144)[b]	8.3	17.8

Table 3. continued

Contrast	Yield (kg/5 plants) 1996 F	1996 P	1997 F	1997 P
Inoculated resistant, susceptible and susceptible treated with streptomycin (1997 only) cultivars vs. uninoculated resistant and susceptible cultivars	3.81	0.059	1.99	0.167
Inoculated susceptible control and uninoculated susceptible control vs. all other treatments	1.17	0.287	0.14	0.711
Inoculated and uninoculated resistant cultivars and inoculated susceptible cultivar treated with streptomycin (1997 only) vs. all chemical, SAR-inducing compound[d], and avirulent strain treatments	6.02	0.019*[c]	1.84	0.184
Inoculated susceptible control vs. all chemical, SAR-inducing compound, and avirulent strain treatments	5.82	0.021*	0.57	0.457
Uninoculated susceptible control vs. all chemical, SAR-inducing compound, and avirulent strain treatments	0.35	0.557	0.00	0.961
Inoculated resistant cultivars vs. all chemical, SAR-inducing compound, and avirulent strain treatments	5.86	0.021*	4.65	0.038*
Uninoculated resistant cultivars vs. all chemical, SAR-inducing compound, and avirulent strain treatments	2.00	0.166	0.46	0.503
Interaction between inoculation and control (+ vs. -)	1.86	0.181	1.60	0.213
Copper hydroxide alone or mixed with streptomycin or mancozeb vs. SAR-inducing compounds and avirulent strains	0.02	0.882	2.84	0.101

Table 3. continued

Contrast	Yield (kg/5 plants)			
	1996		1997	
	F	*P*	*F*	*P*
SAR-inducing compound vs. avirulent strain treatments	0.03	0.858	3.43	0.072
Copper hydroxide vs. copper hydroxide mixed with streptomycin or mancozeb	0.00	0.979	9.08	0.005*
Copper hydroxide/streptomycin vs. copper hydroxide/mancozeb	0.00	0.964	1.33	0.256
Acibenzolar-S-methyl vs. acibenzolar-S-methyl/copper hydroxide[f]	0.19	0.662	...	...
Avirulent strains (40S vs. 302S)	0.40	0.533	0.00	0.959
Streptomycin vs. inoculated resistant cultivars	...	...	0.57	0.454

[a] Copper hydroxide (Kocide 40DF, 3.0 g a.i./liter); mancozeb (Dithane F-45, 2.3 ml a.i./liter); streptomycin (Agri-mycin 17, 0.25 g a.i./liter); acibenzolar-S-methyl (Actigard 50WDG, 0.1 a.i./liter); resistant cultivars H70214 and H9144 (H. J. Heinz Co., Pittsburgh, PA); avirulent streptomycin-resistant *C. michiganensis* subsp. *michiganensis* strains 302S and 40S. Number of applications (if pertinent) were 1x/5 days = five sprays in 1996 and 1997.

[b] Transplants grown in a separate greenhouse.

[c] Treatment not included in that particular year.

[d] Acibenzolar-S-methyl and acibenzolar-S-methyl/copper hydroxide.

[e] * Indicates contrast is significant at $P<0.05$.

[f] Copper hydroxide was mixed with acibenzolar-S-methyl in 1996 only.

(Table 4). In 1996, there were no diseased fruits in the uninoculated resistant cultivars or in 1997 ($P = 0.184$). However, the yield of the inoculated resistant control was significantly less ($P = 0.021$) in 1996 and significantly greater ($P = 0.038$) in 1997 than all other treatments. In 1998, the yield from the uninoculated control was 26.9 kg/5 plants and the yield from the treatments ranged from 26.4 to 32.7 kg/5 plants.

The number of fruit with spots was highly variable among years with incidence much lower in 1996 (2.8%) (Table 4) and 1998 (3.3%) (data not shown) than in 1997 (47.9%) the susceptible control compared to ≤ 1.6% when these controls were inoculated. In most treatments, <1% of the fruit were diseased. However, in 1997, the overall incidence of fruit with spots was high (47.9%). Incidence of fruit spots was 30.8% versus ≤16.2% in the inoculated susceptible control and resistant cultivars, respectively. In the uninoculated resistant cultivars and the susceptible control, the incidence of fruit with spots was ≤8.9%. While treatment with copper hydroxide resulted in fruit spotting of 47.9%, all other treatments resulted in fruit spotting incidence of 11.7% to 22.4%. In 1998, no fruit were diseased in the uninoculated untreated plot compared to 2.4% in the inoculated untreated plot (data not shown). The incidence of fruit with spots was ≤ 0.3% in all treatments with the exception of copper hydroxide/mancozeb (3.3%).

DISCUSSION

A *C. michiganensis* subsp. *michiganensis* population of 10^7 CFU/g of tissue or higher occurred on the inoculated untreated susceptible seedlings at the end of the greenhouse growing cycle (just prior to planting in the field) in 1996 and was associated with development of severe disease symptoms in the field such as plant stunting and yield

Table 4. Incidence of fruit spotting on tomato plants in the field; seedlings inoculated or not inoculated with *Clavibacter michiganensis* subsp. *michiganensis* and subjected to spray treatments in the greenhouse in 1996 and 1997.

	Diseased fruit (%)[b]	
Treatment[a]	**1996**	**1997**
Uninoculated control[c]	0.0	8.9
Inoculated control	1.6	30.8
Copper hydroxide	0.2	47.9
Copper hydroxide/mancozeb	2.8	22.4
Copper hydroxide/streptomycin	2.0	11.7
Streptomycin	¼[d]	17.1
Acibenzolar-S-methyl	0.8	20.2
Acibenzolar-S-methyl/copper hydroxide	0.3	¼
Avirulent strain 302S	0.0	17.2
Avirulent strain 40S	1.8	19.1
Inoculated resistant cultivar (H70214)	0.3	16.2
Inoculated resistant cultivar (H9144)	0.6	5.3
Uninoculated resistant cultivar (H70214)[c]	0.0	7.9
Uninoculated resistant cultivar (H9144)[c]	0.0	6.5

[a] Copper hydroxide (Kocide 40DF, 3.0 g a.i./liter); mancozeb (Dithane F-45, 2.3 ml a.i./liter); streptomycin (Agri-mycin 17, 0.25 g a.i./liter); acibenzolar-S-methyl (Actigard 50WDG, 0.1 a.i./liter); resistant cultivars H70214 and H9144 (H. J. Heinz Co., Pittsburgh, PA); avirulent streptomycin-resistant *C. michiganensis* subsp. *michiganensis* strains 302S and 40S. Number of applications (if pertinent) were 1x/5 days = five sprays in 1996 and 1997.

[b] By weight.

[c] Transplants grown in a separate greenhouse.

[d] Treatment was not included in that particular year.

loss (39%). A previous study indicated that susceptible seedlings with *C. michiganensis* subsp. *michiganensis* populations of 10^7 CFU/g of tissue or higher were associated with systemic infection symptoms in the field including plant stunting, yield loss, and death (11). Treatments included in this study suppressed pathogen populations on susceptible transplants below the threshold level (10^7 CFU/g of tissue) and yield losses were not observed.

In 1997 and 1998, pathogen populations at the end of the greenhouse growing cycle did not exceed 6.1×10^5 CFU/g of tissue, even in the inoculated untreated susceptible seedlings. Consequently, yield losses in the field were not observed among treatments or the controls, verifying our theory that transplants placed in the field with low pathogen populations will produce commercially acceptable yields under Michigan growing conditions. One exception occurred in 1997 with copper hydroxide that had a very low plant stand count (3 out of 12 plants) in the field shortly after transplanting in one of the four blocks due to unfavorable field conditions and likely contributed to the observed decreased yield.

Treatments significantly reduced pathogen population sizes among tomato seedlings in the greenhouse compared to the untreated control in 1996 and 1998. In 1996 only, the conventional bactericides including copper hydroxide alone or mixed with mancozeb or streptomycin reduced pathogen populations among seedlings in the greenhouse compared to acibenzolar-S-methyl alone or mixed with copper hydroxide. However, acibenzolar-S-methyl alone or mixed with copper hydroxide was more effective than the avirulent strains. Streptomycin was included in our trial as a standard

but cannot be recommended for use in Michigan because the label does not specify greenhouse as an application site.

In 1996 only, yields from the inoculated and uninoculated resistant cultivars were significantly reduced compared to the other treatments. These plants did not exhibit significant disease symptoms and the pathogen population did not exceed the 10^7 CFU/g of tissue threshold. The resistant cultivars used were late maturing and may have been negatively affected by the shortened growing season in 1996 (66 days) compared to that of 1997 (99 days). In 1997, these same inoculated cultivars yielded more than the chemical, acibenzolar-S-methyl and avirulent strain treatments.

In our study, it was not beneficial to add mancozeb to copper hydroxide to reduce pathogen spread in the greenhouse, as suggested in a previous study (11). In other studies, copper hydroxide/mancozeb has been shown to enhance control of bacterial diseases and reduce epiphytic bacterial populations compared with using copper alone (12, 17). In 1997 only, pathogen spread was limited among transplants treated with copper hydroxide/mancozeb compared to plants treated with copper hydroxide/streptomycin and among inoculated resistant plants compared to plants treated with streptomycin.

Other studies have shown positive results with acibenzolar-S-methyl for management of bacterial diseases. In a field study, acibenzolar-S-methyl provided better control of bacterial spot (causal agent: *Xanthomonas campestris* pv. *vesicatoria*) (14), and bacterial speck (causal agent: *Pseudomonas syringae* pv. *tomato*) (16) on tomato than copper hydroxide mixed with either maneb or mancozeb, respectively. In a greenhouse

tobacco study, acibenzolar-S-methyl alone, or mixed with copper oxychloride was more effective than copper oxychloride alone in reducing chlorosis and necrosis caused by *Pseudomonas syringae* pv. *tabaci* tox + (3). In tobacco fields, disease (causal agent, *P. syringae* pv. *tabaci* tox +) symptoms were reduced when seedlings were treated with copper oxychloride only in the seed bed (9.3%), or treated with acibenzolar-S-methyl alone (2.7%) or in combination with copper oxychloride in the seed bed and once in the field (4.7%), compared to the untreated control (72%) (3).

Final foliar field populations at the end of the growing seasons ranged from 1.4×10^7 to 1.8×10^9; the uninoculated controls had slightly lower pathogen populations (4.2×10^4 to 3.6×10^7). All treatments, including the uninoculated control, exhibited leaf margin necrosis. Chang et al. determined that relatively high (10^7 to 10^9 CFU/g fresh weight) leaf surface populations of *C. michiganensis* subsp. *michiganensis* can occur at least 2.7 m from the focus of infection resulting in secondary spread and relatively minor disease symptom development (2). The field populations observed in our study exceeded the leaf surface population of *C. michiganensis* subsp. *michiganensis* of 10^6 to 10^7 CFU/g fresh weight that Chang et al. determined is necessary before symptoms of secondary infection, including spotted fruit and firing of leaflets, occur (2). In Michigan, bacterial canker symptoms such as necrosis of the leaf margin occur in mid to late season but are seldom associated with economically significant losses (20, 21).

"Bird's-eye" fruit spotting occurred regardless of treatment, including the uninoculated control plants that received field applications of streptomycin, indicating pathogen spread within the field. Generally, "bird's-eye" spotting does not affect quality

of processing tomatoes grown for paste but causes problems in tomatoes processed for whole pack (21) and for tomatoes grown for the fresh market. Research on the epidemiology of fruit spot formation is lacking, yet it is known that bird's eye spots are a result of flower (18) and young fruit infection (6). A recent study found that when inoculated, flowers were most susceptible to infection two-days post anthesis (18). In addition, small green fruit developed bird eye lesions when inoculated with *C. michiganensis* subsp. *michiganensis*. Similarly, a study by Getz, et al. determined that spots on tomato fruit caused by *Pseudomonas syringae* pv. *tomato* occurred during the time when flowers were past anthesis and until fruit were a maximum of 3 cm in diameter (9). Concurrent scanning electron microscopy studies revealed that the bacterial populations inhabited trichomes prior to anthesis and populated the openings left after the trichomes were shed indicating the source of infection sites on fruit (8).

This study verifies findings of Hausbeck, et al. (11), that to prevent severe bacterial canker in the field, growers should initiate and sustain bactericide applications in the greenhouse to suppress pathogen populations. Oftentimes, treatments are made in response to disease symptoms occurring in the field. By focusing control strategies in the greenhouse rather than in the field, growers will have better control of the pathogen and avoid economic losses resulting from yield losses caused by this disease. Since acibenzolar-S-methyl (13) has no direct affect on the pathogen and depends on the onset of SAR in the plant, this product should be applied preventively. This study identifies novel disease management strategies that upon further testing should ease reliance on copper for control of bacterial canker.

LITERATURE CITED

1. Bay, D. M., and Wyant, D. (eds). 1999. Michigan Agricultural Statistics. MDA, Michigan pp. 42-45.

2. Chang, R. J., Ries, S. M., and Pataky, J. K. 1992. Local sources of *Clavibacter michiganesis* subsp. *michiganensis* in the development of bacterial canker on tomatoes. Phytopathology 82:553-560.

3. Cole, D. L. 1999. The efficacy of acibenzolar-S-methyl, an inducer of systemic acquired resistance, against bacterial and fungal diseases of tobacco. Crop Prot. 18:267-273.

4. Conover, W. J. 1980. Practical Nonparametric Statistics, 2nd ed. Wiley, New York.

5. Cooksey, D. A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. Annu. Rev. Phytopathol. 28:201-19.

6. Ehrig, F., and Grisbach, E. 1985. Development of bird eye spots on tomato fruits-Investigations by scanning electron microscopy. Arch. Phytopathol. Pflanzenschutz 21:419-423.

7. Frey, P., Smith, J. J., Albar, L., Prior, P., Saddler, G. S., Trigalet Demery, D., and Trigalet, A. 1996. Bacteriocin typing of *Burkholderia* (*Pseudomonas*) *solanacearum* race 1 of the French West Indies and correlation with genomic variation of the pathogen. Appl. Environ. Microbiol. 62:473-479.

8. Getz, S., Fulbright, D. W., and Stephens, C. T. 1983. Scanning electron microscopy of infection sites and lesion development on tomato fruit infected

with *Pseudomonas syringae* pv. *tomato*. Phytopathology 73:39-43.

9. Getz, S., Stephens, C. T., and Fulbright, D. W. 1983. Influence of developmental stage on susceptibility of tomato fruit to *Pseudomonas syringae* pv. *tomato*. Phytopathology 73:36-38.

10. Hammerschmidt, R., and Becker, J. S. 1997. Acquired resistance to disease in plants. Pages 247-289 in : Horticultural Reviews, vol. 18. Janick, J. (ed.) John Wiley & Sons, Inc., New York.

11. Hausbeck, M. K., Bell, J., Medina-Mora, C., Podolsky, R., and Fulbright, D. W. 2000. Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganenesis* on tomatoes in the greenhouse and on disease development and crop yields in the field. Phytopathology 90:38-44.

12. Jones, J. B., Woltz, S. S., Jones, J. P., and Portier, K. L. 1991. Population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets treated with copper bactericides. Phytopathology 81:714-719.

13. Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heaney, S. P., Murray, D. C., Powell, K. A., Schultz, M. A., Spinks, C. A., Worthington, P. A., and, and Youle, D. 1997. Rationale and perspectives on the development of fungicides. Annu. Rev. Phytopathol. 35:349-72.

14. Louws, F. J., Ritchie, D. F., Kousik, C. S., Romero, A. M., Pollard, D. W. 1996. Evaluation of the plant-activator CGA-245704 50WG for control of bacterial spot of tomato. Pages 18-19 in: 12th Annual Tomato Disease Workshop Columbus, Ohio.

15. Louws, F. J., Bell, J., Medina Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., deBruijn, F. J., and Fulbright, D. W. 1998. rep-PCR-mediated genomic fingerprinting: a rapid and effective method to identify *Clavibacter michiganensis.* Phytopathology 88:862-868.

16. Manley, B. S., Bassi, Jr., A. B., Tally, A. H., Young, T. R., Payan, L., Coud, G., and Reuss, W. 1997. Actigard (CGA 245704), a novel approach to bacterial spot and bacterial speck control in tomato. Pages 48-49 in: 13th Annual Tomato Disease Workshop, Indianapolis, IN.

17. Marco, G. M. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity of copper. Plant Dis. 67:779-781.

18. Medina-Mora, C. M., Hausbeck, M. K., and Fulbright, D. W. 2000. Bird's eye lesions of tomato fruit produced by aerosol and direct application of *Clavibacter michiganensis* subsp. *michiganensis*. Plant Dis. (Accepted pending revision).

19. Ricker, M. D., Emmatty, D. A., Schott, M. D., and Ozminkowski, R. H. 1997. Heinz tomato varieties resistant to bacterial canker. Pages 37-39 in: 10th Annual Tomato Disease Workshop Windsor, Ontario Canada.

20. Ricker, M. D., and Riedel, R. M. 1993. Effect of secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* on yield of northern processing tomatoes. Plant Dis. 77:364-366.

21. Ricker, M. D., and Riedel., R. M. 1988. Economic importance of secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* in northern-grown

processing tomatoes. (Abstr.). Phytopathology 78:1570.

22. Zar, J. H. 1996. Biostatistical Analysis 3rd ed. Prentice Hall, Upper Saddle River, NJ.