Seed Transmission of *Clavibacter michiganensis* subsp. *michiganensis* and Development of Strategies to Control the Pathogen in Seed

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Xiulan Xu, M.S.

Graduate Program in Plant Pathology

The Ohio State University

2010

Dissertation Committee:

Sally A. Miller, Advisor

Gireesh Rajashekaraa

Mark A. Bennett

Pierce A. Paul

David L. Coplin
Abstract

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is a Gram-positive bacterium that causes wilting and cankers, leading to severe economic losses in commercial tomato production worldwide. The initial means of bacterial canker entry into a crop is through *Cmm*-infected seeds or transplants. In order to study the biology of *Cmm* seed transmission and seedling infection, bioluminescent *Cmm* strains were constructed by transformation of the reporter gene, *lux*-operon, into *Cmm*. A promoterless *lux*-operon, originally derived from *Photorhabdus luminescens*, was linked downstream of a chloramphenicol exporter in a *Cmm* transposon mutagenesis vector, pKGT452Cβ. Electroporation of the constructed vector pXX2 carrying the *Cmx':luxABCDE::Tn1409* cassette resulted in insertion of *lux*-operon into the *Cmm* chromosome. Among 19 bioluminescent *Cmm* mutants obtained, the virulent, stable, constitutively bioluminescent strain BL-Cmm17 that grew similarly to its parent strain C290 was selected to study seed transmission and seedling infection. Healthy tomato seeds were inoculated with BL-Cmm17 and the dynamics of bacterial colonization on germinating seeds were monitored in real-time using an *in vivo* imaging system (IVIS). Our results showed that *Cmm* colonized the hypocotyl and the cotyledon at an early stage of germination. Visualization of seedling systemic infection by BL-Cmm17 through wound showed that *Cmm* moved towards both root than shoot under both low (45%) and high (83%) relative humidity (RH) regimes.
High RH accelerated the systemic movement of *Cmm* in stem. Luminescent signals were also observed in tomato seedling roots over time. Root development was reduced in inoculated plants maintained under both humidity regimes. The strong positive correlation between light intensity and bacterial population *in planta* suggested that bioluminescent *Cmm* strains have additional potential applications such as evaluating the efficacy of bactericides and resistant cultivars.

Because of the importance of seed transmission, seed must be the first point of focus in developing an integrated program to manage this disease. In this study, the effects of seed sanitation treatments combined with post-sanitation treatments of beneficial bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* were evaluated. Seed sanitation treatments eliminated the pathogen in seed and reduced disease incidence in seedlings in laboratory and greenhouse grow-out tests. However, beneficial bacteria applied after seed sanitation treatments did not reduce disease incidence or influence seedling growth. Among all sanitation treatments, commercial disinfectants Kleengrow and Virkon most effectively eliminated *Cmm* from seed and reduced the pathogen populations in greenhouse seedlings. The plant essential oil thymol was phytotoxic at the concentration evaluated, increasing the rate of production of abnormal seedlings. Seed vigor analysis determined using the Seed Vigor Imaging System (SVIS) suggested that thymol, kasugamycin and dry heat treatments decreased seed vigor over long-term storage.
Dedication

Dedicated to My Grandparents
Acknowledgements

First I would like to express sincerest gratitude to my advisor, Dr. Sally A. Miller, whose guidance and support were crucial for me to accomplish this research. I am thankful for the excellent example she has set as a successful female plant pathologist. I gratefully acknowledge my student advisory committee members Dr. Gireesh Rajashekar, Dr. Mark A. Bennett, Dr. David L. Coplin and Dr. Pierce A. Paul for their insightful comments and encouragement. I would like to thank Dr. Brain B. McSpadden Gardener and Dr. Larry V. Madden for their academic help and support.

It has been a pleasure to work with Melanie L. Ivey, Fulya Baysal-Gurel, Jhony Mera in the vegetable pathology lab and I thank them for their valuable suggestions for my research and technical support in lab and field work during these four years. I would like to thank Nagendra Subedi, Freddy Cruz and the farm crew supervised by Bill Bardall for assistance in production of the tomato seeds for my study. I would like to acknowledge all members in the seed biology lab for assisting in the seed vigor testing. I am very grateful to Dr. Tea Meulia in MCIC for helping me search for bioluminescence imaging equipment.

Finally, I thank my parents Heping Lin and Baojian Xu, my husband Linjian Jiang and my daughter Ziqi Jiang, whose love encouraged me to move forward.
Vita

2003........................................B.S. Agronomy, China Agricultural University

2006........................................M.S. Plant Pathology, China Agricultural University

2006 to present.......................Graduate Research Associate, Department of Plant Pathology, The Ohio State University

Publications


Fields of Study

Major Field: Plant Pathology
# Table of Contents

Abstract ................................................................................................................................. ii
Dedication ............................................................................................................................. iv
Acknowledgments ............................................................................................................... v
Vita ........................................................................................................................................ vi
List of Tables ....................................................................................................................... ix
List of Figures ...................................................................................................................... xi
Chapter 1: Introduction ...................................................................................................... 1

Chapter 2: Bioluminescent Imaging of *Clavibacter michiganensis* subsp.
*michiganensis* Colonization of Germinating Tomato Seeds ........................................... 25
  Introduction ....................................................................................................................... 27
  Materials and Methods .................................................................................................... 30
  Results ............................................................................................................................... 39
  Discussion ......................................................................................................................... 45
  References ......................................................................................................................... 52

Chapter 3 Colonization of tomato seedlings by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* under different humidity regimes ..................... 68
  Introduction ....................................................................................................................... 69
  Materials and Methods .................................................................................................... 72
List of Tables

Table 2.1 Bacterial strains and plasmids used in this study ........................................ 59
Table 2.2 Oligonucleotides used in this study ............................................................ 60
Table 2.3 Induction of the hypersensitive response (HR) in four o’clock plant and virulence to tomato plants by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* strains ................................................................................................. 61
Table 3.1 Comparison of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato compound leaves under low (45%) and high (83%) relative humidity (RH) regimes. ........................................... 90
Table 3.2 Comparison of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 movement in tomato stems under low (45%) and high (83%) relative humidity (RH) regimes by tissue imprinting. ......................... 91
Table 4.1 Growth of *Clavibacter michiganensis* subsp. *michiganensis* strains C290 and A300 on yeast dextrose carbonate agar medium (YDC) amended with selected inhibitors at different concentrations. ........................................................................ 133
Table 4.2 Determination of conditions for seed treatment with selected inhibitors and dry heat on eliminating *Clavibacter michiganensis* subsp. *michiganensis* from seed and influence on germination ..................................................................................... 134
Table 4.3 Optimized conditions for seed sanitation treatments. ............................... 135
Table 4.4 Efficacy of sanitation treatments in eliminating *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) from tomato seed. .......................................................136

Table 4.5 Statistical analysis of tomato seed sanitation and beneficial bacteria treatments on tomato bacterial canker control and seedling establishment, Experiment 1. .............................................................................................................137

Table 4.6 Effects of seed sanitation treatments on tomato bacterial canker incidence, seed germination and seedling growth, Experiment 1. .........................................................138

Table 4.7 Effects of seed sanitation treatments on tomato bacterial canker incidence, seed germination and seedling growth, Experiment 3. .............................................................139

Table 4.8 Statistical analysis of effects of sanitation and beneficial bacterial treatments on tomato seed vigor over long-term storage, Experiment 1. ..................140

Table 4.9 Statistical analysis of effects of sanitation and beneficial bacterial treatments on tomato seed over long-term storage, Experiment 2. ............................141

Table 4.10 Influence of seed treatments on seed vigor before and after long-term storage, Experiment 1. ...............................................................................................142

Table 4.11 Influence of seed treatments on seed vigor before and after long-term storage, Experiment 2. ...............................................................................................143
Lists of Figures

Figure 2.1 Physical maps of constructed vector pXX1(A), pXX2(B) and pXX3(C) carrying the lux-operon. Abbreviations: Amp, ampicillin resistance; cmx, chloramphenicol resistance; ME, mosaic end; Kan, kanamycin resistance; tnpA, transposase IS1409; IRR, right inverted repeat; LRR, left inverted repeat. Arrows indicate location of primers used for sequencing ......................................................62

Figure 2.2 (A) Representative image of bioluminescent Clavibacter michiganensis subsp. michiganensis mutants recovered on a SB plate screened by IVIS. (B) PCR analysis with CmxF/CmxR and LuxCF/R primers of the colonies recovered after pXX2 electroporation. Lane 1, 1 kb plus ladder; Lanes 2 and 7, wild-type C290 as a negative control; Lanes 3 and 8, pXX2 plasmid as a positive control; Lanes 4-6 and 9-11, bioluminescent transposon mutants. Only results from representative strains are shown. .....................................................................................................................................63

Figure 2.3 Comparison of bioluminescent Clavibacter michiganensis subsp. michiganensis mutant strains in light production. Bacterial culture growth in NBY medium was to late log phase (OD about 0.8~1.0) and 100 µl bacterial suspension for each strain was used for light emission assessment. Relative light unit (RLU) is the log transformed value of total flux (photon/s). Data shown are means of three replicates, error bars are standard error of the mean..........................................................64
Figure 2.4 (A) Hypersensitive response in a four o’clock leaf induced by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* strain BL-Cmm17. Bacterial suspensions (10^8 CFU/ml) were injected into fully expanded leaves of four o’clock plants using needle-less syringes and HR reactions were recorded after 24 hours. (B) Virulence test on 4-week-old tomato seedlings. Tomato seedlings were monitored for wilting for 3 weeks after inoculation. .................................65

Figure 2.5 (A) Bioluminescence and growth curves of *Clavibacter michiganensis* subsp. *michiganensis* wild-type C290 and BL-Cmm17 strains. Both strains were inoculated into NBY broth, and the optical density at 600 nm and photon emissions were measured from 4 h to 48 h. Relative Light Unit (RLU) is log transformed values of the average radiance (photons/sec); the error bars indicate standard deviations. (B) Growth of BL-Cmm17 and C290 in Cmm minimal medium. The optical density at 600 nm and photon amounts were measured from 8 h to 58 h. Data points are means of three replicates with standard deviations. .................................66

Figure 2.6 (A) Bioluminescence of strain BL-Cmm17 inoculated onto tomato seed. Images were acquired daily by using an *in vivo* imaging system. Bacteria were observed on seed coats throughout the germination period and colonized other seedling components as they emerged. The number at the bottom of each image indicates the day of germination. The rainbow scale represents photon counts (photons/sec). (B) Population dynamics of bioluminescent BL-Cmm17 strain on
tomato seed coats during germination. Bacterial counts were estimated from serial dilution plating and the total CFU were log transformed. Each point represents the mean bacterial count for 12 seeds (days 1-3) and seed coat (days 4-5) isolations; bars indicate standard deviation. (C) Distribution of bioluminescent BL-Cmm17 strain on tomato seedlings on the fifth day of germination. Each point represents the mean bacterial count (n=12); error bars indicate standard deviations. (D) Regression analysis of light intensity vs. populations of BL-Cmm17 strain on tomato seed coats during days 1, 2 and 3 days of germination.  

Figure 3.1 Bioluminescence imaging of \textit{Clavibacter michiganensis} subsp. \textit{michiganensis} bioluminescent strain BL-Cmm17 colonization of tomato seedlings under low (45%) and high (83%) relative humidity (RH) regimes in real time. Images of the same seedlings were acquired 5, 10, 15 and 20 days post inoculation (DPI, indicated at bottom of the images) using an \textit{in vivo} imaging system (IVIS, Xenogen). The upper images are of a seedling held under low relative humidity and the lower images are of a seedling held under high relative humidity. Arrows point to the strong luminescence signals at nodes. 

Figure 3.2 Comparison of bacterial populations (log CFU/g) of \textit{Clavibacter michiganensis} subsp. \textit{michiganensis} bioluminescent strain BL-Cmm17 recovered from stems of tomato seedlings under low (45%) and high (83%) relative humidity (RH) regimes 5, 10, 15 and 20 days post inoculation (DPI). Each point represents the
mean of bacterial populations (n=6), bars indicate the standard deviations. ..........93

Figure 3.3 Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato leaves from a tomato seedling under high relative humidity (83%) 20 days post inoculation. Numbers at the top from left to right indicates the leaf position from the youngest (7) to the oldest leaf (1). Images of leaves 1-3 and 5-7 were taken on the adaxial surface; the image of leaf 4 was taken on the abaxial surface. The rainbow scale represents light intensity. .................................................................94

Figure 3.4 Recovery and identification of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 from tomato stem tissue imprinted on D2ANX semi-selective medium using an *in vivo* imaging system (IVIS, Xenogen). This image shows a representative plate of stem tissue imprints of three tomato seedlings under high relative humidity (83%) 10 days after inoculation. Each square within the grid was imprinted with one cut section of stem tissue above and below the cotyledon node. Numbers in the grid squares indicate the distance (cm) from the cotyledon nodes (>0 is above cotyledons, <0 is below). Columns a and b, c and d, and e and f were imprints from three samples. .................................................................95

Figure 3.5 Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato seedling roots under low (45%) and high (83%) relative humidity (RH) regimes. Images were
acquired at four time points (5, 10, 15 and 20 days post inoculation (DPI), indicated at the bottom of images) using the in vivo imaging system (IVIS, Xenogen). The upper images were roots of seedlings under low relative humidity and the lower images were under high relative humidity. .................................................................96

Figure 3.6 Comparison of populations of Clavibacter michiganensis subsp. michiganensis recovered from roots (A) and fresh weight of roots (B) under low (45%) and high (83%) relative humidity (RH) regimes. CK represents the root weight of the water-inoculated control under high relative humidity. Each bar represents the mean (n=3) of bacterial population (log CFU/g) or fresh weight (g) and error bars indicate standard deviations. .................................................................97

Figure 3.7 Regression analysis of light intensity versus population of bioluminescent Clavibacter michiganensis subsp. michiganensis strain BL-Cmm17 recovered from tomato seeding stems. Data points were bacterial populations and light intensities from 1 cm above the cotyledons in stems (n=20). .........................................................98

Figure 4.1 Antagonism of Bacillus subtilis (strain MBI 600, left) and Pseudomonas fluorescens (strain Wayne 1R, right) on Clavibacter michiganensis subsp. michiganensis (strain C290) growth on YDC medium. The YDC medium was spread with a suspension of C290, then beneficial bacterial strains were immediately streaked on each side. .................................................................................................144
Figure 4.2 (A) and (B) Morphology of abnormal seedlings 2 weeks after seed were sown. (C) Water-soaked spots (arrows), typical symptoms of tomato bacterial canker on a cotyledon and first true leaf of a 5 week-old seedling. .........................................145

Figure 4.3 Detection of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouse tomato seedlings by terminal dilution PCR (TDPCR) before and after bio-enrichment in NBY broth. Abbreviations: M, DNA ladder of 1 Kb plus; 3^n, n=the number of three fold dilutions. ..................................................................................146

Figure 4.4 Relationship between the population estimates of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in greenhouse tomato seedlings after seed treatment determined by serial dilution plating and by bio-enriched terminal dilution polymerase chain reaction (TDPCR). The X-axis (TDPCR) indicates the number of the last dilution that was positive by PCR assay; the Y-axis indicates log_{10} *Cmm* populations assessed by serial dilution plating. .................................................................147

Figure 4.5 Box plots showing the relative *Clavibacter michiganensis* subsp. *michiganensis* population levels determined by bio-enriched terminal dilution PCR (TDPCR) in tomato seedlings after various seed treatments, Experiment 1 (A, B and C) and Experiment 3 (D). The X-axis indicates the sanitation treatments and CK represents *Cmm*-infested seeds not subjected to a sanitizing treatment. The Y-axis (TDPCR) indicates the number of the terminal dilution that was positive by the PCR assay. Beneficial bacteria *Bacillus subtilis* MBI 600 (BS) or
*Pseudomonas fluorescens* Wayne1R (PF) were applied after sanitation treatments in (B) and (C) .................................................................148
Tomato (Solanum lycopersicum [syn. Lycopersicon esculentum]) is one of the most important vegetable crops cultivated worldwide. In 2009, the production of tomato worldwide reached 140 million metric tons (FAO 2010). The United States ranks second in tomato production with 178,911 hectares of planted area that had a total value of over 2.5 billion dollars in 2009. In the United States, Ohio ranks third for both processing and fresh market tomato production. There were 2,670 hectares of processing tomatoes and 1,982 hectares of fresh tomatoes with a total value of 89 million dollars in Ohio in 2009 (USDA 2010).

Tomato bacterial canker, caused by Clavibacter michiganensis subsp. michiganensis (Smith) Davis et al. (Cmm), is considered one of the most important diseases of tomato in temperate zones and greenhouses worldwide. Early symptoms are marginal necrosis and spots on the leaves, and brown or silver streaks or spots on the stems or peduncles. Sometimes, leaflets on one side of the rachis will wilt, while those on the other side remain healthy. The streaks usually crack open at intervals and form cankers. The vascular tissues of systemically infected plants appear tan to light brown in color initially and may darken as the infections progress. Systemic
infection through vascular tissue causes stunting and gradual or rapid wilting of the plant, depending on the growth stage of the plant infected. Vascular discoloration (tan to light brown) in the main stem is conspicuous of late stages of infection. The most typical symptoms on tomato fruit in the field are bird’s-eye lesions, which are small, round, necrotic spots surrounded by white halos. Bird’s-eye lesions are observed on both unripe and ripe fruit (Bryan, 1930; Gleason et al, 1993). However, they are not typically seen in greenhouse tomatoes. Infected fruit in greenhouse production may be symptomless or appear netted or marbled (ASTA, 2010).

Yield losses from tomato bacterial canker are due to plant wilting and death, fruit lesions (field) or netting (greenhouse), and reduced fruit set and size. Severe epidemics can cause up to 80% yield loss (Strider, 1969). The disease was first discovered in a Michigan greenhouse in 1909 and quickly spread to other states in the USA (Bryan, 1930). Severe epidemics have occurred in the 1930s, 1960s and 1980s in North America. Other early documented occurrences of tomato bacterial canker date from 1922 to 1965 in European countries and 1942 in Africa (Strider, 1969; Gleason et al, 1993). Currently, tomato bacterial canker has been reported in almost all tomato production areas around the world (EPPO, 2005). However, it does not appear to be a serious problem for field production in tropical or sub-tropical areas (Sally Miller, Jeffrey Jones, personal communications). Long-distance spread of the disease is mainly through infected seeds and transplants. To prevent its spread to disease-free areas, Cmm is subject to quarantine by the European and Mediterranean
Plant Protection Organization and many other countries (EPPO, 2005; Eichenlaub et al, 2006).

The causal agent was first cultured and named *Bacterium michiganense* by E. F. Smith in 1910 (Bryan, 1930). The pathogen was later renamed *Corynebacterium michiganense* in 1934, on the basis of its morphology and Gram reaction (Strider, 1969). It is an aerobic, rod-shaped, non-motile, Gram-positive bacterium. The name *Corynebacterium michiganense* was accepted for decades until it was more recently placed into a new genus *Clavibacter*, based on the cell wall composition in 1984 (Davis et al., 1984). The development of molecular genetics in the last several decades has significantly influenced bacterial taxonomy. *Clavibacter* is currently classified as a member of the family *Microbacteriaceae* according to sequence analysis of 5S rRNA (Park et al., 1993). *Clavibacter michiganensis* is the only species in the genus *Clavibacter* and it is divided into five subspecies according to host specificity. These subspecies include: subsp. *michiganensis*, which infects tomato; subsp. *sepedonicus*, which causes ring rot of potato; subsp. *nebraskensis*, which is responsible for wilt and blight of maize; subsp. *tessellarius*, which induces leaf freckles and spots in wheat; and subsp. *insidiosus*, which is the pathogen causing wilting and stunting in alfalfa. These bacteria are all vascular pathogens and seed transmitted. Besides Cmm, *C. michiganensis* subsp. *sepedonicus* and subsp. *insidiosus* are also quarantine pathogens in many countries (Jahr et al., 1999; Eichenlaub et al, 2006).
Like other plant pathogenic bacteria, *Cmm* infects the plant through wounds and natural openings, such as stomata and hydathodes. Bacteria multiply quickly in the large intercellular spaces of the hydathodes, move into the vessels of the large marginal fimbriate veins, then are distributed to the leaf margins, causing marginal necrosis (Carlton et al., 1998). Agricultural practices, such as grafting and pruning, facilitate the spread of disease and increase the chances of direct induction of the pathogen into vascular tissue and rapid wilting. Fruit are also systemically infected via the vascular system (Strider, 1969; Gleason et al. 1993). Seed infection may be through the calyx scar and vascular bundle of the fruit (Uematsu et al. 1977). After the growing season, the bacteria persist in plant debris. While *Cmm* is not a soil-inhabiting bacterium, it can survive in plant debris in soil for more than two years in Ohio (Fatmi and Schaad, 2002). The pathogen can also infect pepper in nature or other solanaceous plants by artificial inoculation, but *Cmm* does not cause severe systemic infection in hosts other than tomato. However, it is suspected that other solanaceous plants may serve as natural reservoirs of *Cmm* (Thyr et al., 1975; Ivey and Miller, 2000).

There are several hypotheses for the mechanisms by which *Cmm* causes wilting. Early reports considered extracellular polysaccharides (EPS) as a very important virulence factor, since accumulation of high molecular weight EPS was known to hinder water transport in the xylem (Rai and Strobel, 1968). Furthermore, purified EPS from *Cmm* proved to be phytotoxic in an *in vitro* test on tomato seedlings (Van den Bulk et al., 1989). However, a mutant strain that produced only 10% of the wild-
type amount of EPS was not altered in virulence. Interestingly, *Cmm* strains that produced EPS composed of fucose, glucose and galactose in a molar ratio of 2:1:1 colonized plants efficiently, while another group of strains producing a small amount of EPS of a different composition failed to propagate in tomato. Thus, EPS may not be a crucial factor in pathogenicity but the chemical structure of EPS may influence early host recognition (Bermpohl et al., 1996).

In recent years, molecular genetic studies of *Cmm* better elucidated the mechanism of pathogenicity. The genome of *Cmm* strain NCPPB382 is 3.298 Mb in size, with a high G+C content of 72.6% (Gartemann et al., 2008). Two large plasmids, pCM1 (27.5 Kb) and pCM2 (70 Kb), carry genes that are essential for virulence. Loss of either plasmid delayed the onset of wilting and a *Cmm* strain without any plasmids was unable to cause symptoms although it colonized tomato plants (Meletzus et al., 1993). The first virulence gene identified in *Cmm* was *pat*-1, located on plasmid pCM2 (Dreier et al. 1997). The protein encoded by the *pat*-1 ORF is a 29.7kDa serine protease. Several genes homologous to *pat*-1, which are involved in tomato-*Cmm* interactions, are found in pCM1 and the chromosome (Burger et al., 2005; Stork et al., 2008). The *celA* gene, located on pCM1 and required for pathogenicity, encodes a 78Kda protein, CelA, which is similar to endo-β-glucanases of the cellulase family A1. CelA may directly contribute to pathogenicity by degrading xylem vessel walls, allowing the spread of bacteria to adjacent tissue (Jahr et al., 2000). Several other extracellular enzymes produced by *Cmm*, including pectin methylesterase, polygalacturonase and xylanase, are also
involved in cell wall degradation (Strider, 1969; Beimen et al. 1992). However, the genes encoding these enzymes and regulating their expression have not been identified.

Traditional bacterial disease management measures, such as application of antibiotics and copper bactericides, have not been successful against tomato bacterial canker and canker-resistant tomato cultivars are not available. Consequently, seed testing and maintaining pathogen-free seeds and transplants are currently the most appropriate tactics to minimize the introduction and spread of the disease (Jahr et al. 1999). *Clavibacter michiganensis* subsp. *michiganensis* can be present as a surface contaminant on seed as well as under the seed coat (Bryan 1930; EPPO, 2005). From an early report, the seed transmission rate from naturally infested seed varies from 1 to 5% based on symptom inspection (Bryan, 1930). However, even 0.01% transmission from seed to seedling can initiate a disease epidemic under favorable conditions (Chang et al. 1991).

Currently, for both fresh market and processing tomato production, seedlings are usually produced in greenhouses for 4-6 weeks before transplanting. Seeds infected with *Cmm* can give rise to symptomless seedlings, which may have latent infections or carry the pathogen epiphytically (Tsiantos, 1987; Gitaitis et al., 1991; Shirakawa et al., 1991). An early study showed that seedlings grown from artificially-infected seeds were contaminated with $10^4$-$10^8$ CFU/seedling (Tsiantos, 1987). Seedlings grown from naturally infected seeds can also harbor $10^3$-$10^4$ CFU/g of the pathogen.
in shoots (Shirakawa et al. 1991). As the planting density of seedlings is very high, any epiphytic pathogens are easily spread through irrigation and leaf-to-leaf contact. Despite its apparent significance in Cmm epidemiology, the mechanism of seed to seedling transmission of Cmm is not well understood.

It is known that Cmm is a good endophyte and moves into xylem after its entrance through wounds or nature openings (Carlton et al., 1998; Gartemann et al., 2003). Mechanical wounds, which are easily made during clipping, harvesting, shipping and transplanting, promote the dissemination of Cmm directly into vascular tissue (Chang et al., 1991). In addition, environmental conditions in seedling production greenhouses favor bacterial infection. Varying of temperatures (15-28°C) had no effect on canker development, but high relative humidity (87-97%) enhanced the symptoms in 2-3 week-old tomato seedlings (Basu, 1966). Previous studies have focused on Cmm spread by cultural practices and the development of symptoms; however, few studies have addressed Cmm movement in planta during latent infection and how the environment influences Cmm growth as an endophyte in tomato transplants.

An approach to better understand the dynamics of seed to seedling transmission and seedling infection of Cmm is the use of genetically altered bioluminescent bacteria, which allow monitoring of plant-pathogen interactions in real-time. The natural light-emitting reactions encoded by the luxCDABE genes of bioluminescent bacteria have been used to assess gene expression and monitor internalization and
distribution of bacteria in plant hosts (Chabot et al., 1996; Cirvilleri and Lindow, 1994; Cirvilleri et al., 2000 and 2008; De Weger et al., 1991; Fan et al., 2008; Fukui et al., 1996; Kamoun and Kado, 1990; Paynter et al., 2006; Shaw et al., 1988; Shaw et al., 1992). Particularly, bioluminescent phytopathogenic *Xanthomonas campestris* pvs. and *Pseudomonas* spp. have been used to track bacterial movement and distribution in host plants (Cirvilleri and Lindow, 1994; Cirvilleri et al., 2008; Fukui et al., 1996; Paynter et al., 2006; Shaw et al., 1992), and to assess host susceptibility quantitatively (Fukui et al., 1996). Likewise, the *lux* genes have been also transferred to beneficial bacteria such as *Rhizobium leguminosarum* and *Pseudomonas* spp. to visualize colonization patterns in rhizospheres (Chabot et al., 1996; De Weger et al., 1991).

The genes that determine bioluminescence are the *luxAB* genes, which express luciferase enzymes that catalyze the luminescent reaction, and the *luxCDE* genes, which encode enzymes required for biosynthesis of the fatty aldehyde substrate necessary for the reaction (Meighen, 1993; Szittner and Meighen, 1990). Bioluminescence involves the intracellular oxidation of the reduced form of a flavin mononucleotide (FMNH$_2$) and a fatty aldehyde (RCHO) by luciferase in the presence of molecular oxygen (Stewart and Williams, 1992).

\[
FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light}
\]

Cells that express the *lux* operon spontaneously emit photons that can be captured by a sensitive charge-coupled device (CCD) camera, enabling imaging and
visualization of bacterial cells (Hutchens and Luker, 2007) in situ. Luciferase activity depends on the metabolic integrity of the cell, while the amount of photons emitted correlates with biomass of living bacteria (Fan et al., 2008; Paynter et al., 2006). Furthermore, since the half-life of luciferase binding to its substrate is several seconds (Meighen, 1993), captured light events reflect processes in real time and are not artifacts of accumulated signals. Consequently, live imaging of bioluminescence provides a sensitive means for visualizing bacterial colonization and invasion of hosts and allows real-time representation and examination of pathogen-plant interactions (Kamoun and Kado, 1990; Shaw et al., 1992).

The first objective of my research is to understand Cnm seed transmission and seedling infection and the role of relative humidity in these processes. A bioluminescent Cnm strain was developed as a tool to visualize epiphytic and endophytic colonization. My hypotheses are that 1) seeds are colonized rapidly during germination and 2) that relative humidity significantly influences the Cnm infection process in tomato seedlings. Previous studies on Cnm seed transmission have been based on symptoms or determining bacterial populations by viable cell counts; no study has yet been conducted to monitor Cnm seed to seedling transmission and seedling infection in planta. Incorporation of a bioluminescent marker into Cnm is a novel approach to elucidate the interaction of plants with this economically important pathogen. Bioluminescent Cnm coupled with CCD camera imaging system provides a rapid, non-destructive way to monitor the pathogen distribution, growth and infection in tomato seedlings in real-time. Better
understanding of the biology of *Cmm* seed transmission and transplant infection will be helpful in improving sampling methods and control strategies for this disease.

The management of tomato canker is a major challenge once the disease is established in a field or greenhouse. Seeds must be the first point of focus in developing an integrated program to manage this disease. Treatments to reduce or eliminate populations of bacterial pathogens on or in seeds have been developed over many years. These include both chemical and heat treatments. Hot water treatment (50°C), performed using standard protocols, is effective in eliminating bacterial pathogens from tomato seed, but it may delay seed germination for some seed lots (Ivey and Miller, 2005). Seed can also be heated by hot air in an oven instead of soaking in hot water. Dry heat treatment (80°C) has been evaluated for eliminating *Pepino mosaic virus* (PepMV) on tomato seed (Córdoba-Sellés et al., 2007) and it may serve as an alternative means of heat treatment to control *Cmm* without influencing seed vigor.

Traditional chemical treatments may present a lower risk of seed damage than heat treatments, but do not kill *Cmm* under the seed coat. For instance, sodium hypochlorite and hydrochloric acid treatments of tomato seed significantly reduced bacterial canker development in the field, but did not eliminate the disease completely (Dhanvantari, 1989; Dhanvantari and Brown, 1993). Germination was also negatively influenced by soaking seed in 0.6 M hydrochloric acid (Dhanvantari, 1989; Fatmi et al., 1991).
Chemical disinfectants are widely used for plant disease management in greenhouses. One example is Virkon, which mainly contains potassium peroxymonosulfate. Virkon is marketed for veterinary use for sanitation of bacterial and viral pathogens and has also been used for human health care in recent years (Hernández et al., 2000). Virkon has been found to be effective in disinfecting tools contaminated with plant viruses, including *Pepper mild mosaic virus* (PMMV) and *Tobacco mosaic virus* (TMV) (Stijger, 1999; Lewandowski and Lu et al., 2010). Additionally, treatment of lettuce seed with Virkon reduced the severity of bacterial leaf spot, caused by *Xanthomonas campestris* pv. *vitiants*, without influencing germination (Carisse et al, 2000). Kleengrow is another commercial disinfectant with wide spectrum fungicidal and bactericidal properties. The active intergradient is didecyl dimethyl ammonium chloride. Foliar application of Kleengrow in a greenhouse significantly reduced leaf spots and incidence of dead plants caused by *Cmm* in tomato (Ingram and Lu, 2009). Therefore, both Virkon and Kleengrow have great potential for seed treatment to eliminate bacterial canker in seed.

Other potential seed sanitation treatments are plant essential oils, which are considered alternative organic pesticides and more environmental friendly than synthetic chemicals. Thymol is composed of phenolic compounds isolated from species of *Origanum* and *Thymus*. The antifungal, antibacterial effect of thymol against several plant pathogens, including *Cmm* was demonstrated by *in vitro* testing (Daferera et al., 2003). In practice, fumigation using thymol has reduced bacterial
wilt caused by *Ralstonia solanacearum* in tomato transplants under field conditions (Ji et al., 2005).

The use of antibiotics to control bacterial diseases in plants has received less attention than the use of other products, primarily due to the lack of commercialized antibiotics to use against plant bacterial pathogens and for the concern that overuse of antibiotics will select for antibiotic-resistant mutants amongst bacteria in the environment and compromise the use of related antibiotics in medicine (Cooskey, 1990). Only two antibiotics have been tested for tomato bacterial canker control. One is streptomycin (Agri-mycin 17, Ag streptomycin), which has limited labeling for use in vegetable transplants and fruit trees for bacterial disease control. Streptomycin was reported to reduce *Cmm* populations on tomato seedlings in a greenhouse study (Hausbeck et al. 2000). The antibiotic kasugamycin (Kasumin; newly marketed by Arysta Life Sciences) has been shown to reduce *Cmm* growth *in vitro* (Theodoro and Maringoni, 2000) and disease severity in a field study (Miller et al., 2007). Both streptomycin and kasugamycin have the potential to be applied as seed treatments to control bacterial canker in tomato seedlings.

Seed sanitation treatments not only eliminate pathogens, but also reduce populations of saprophytic microorganism on seed (Fatmi et al., 1991; Bülow et al., 1994). This may create a “biological vacuum” that may enhance the ability of beneficial bacteria, applied post-sanitation, to colonize and survive on seeds. Beneficial bacteria include plant growth-promoting rhizobacteria (PGPR), which
promote plant health by production of allelochemicals inhibitory to a broad spectrum of pathogens or by inducing systemic resistance (ISR) in host plants against certain pathogens (Compant et al., 2005). *Bacillus* species are well studied PGPR for their effectiveness as biological control agents against many plant pathogens. *Bacillus* spp. elicit ISR in plants and produce antibiotics, including iturin and surfactin, to inhibit soil-borne pathogens (McSpadden Gardener, 2004, Kloeper et al., 2004). *Bacillus subtilis* prevents bacterial canker development in greenhouse tomato seedlings by foliar spray before inoculation (Utkhede and Koch, 2004). *Pseudomonas* species that produce 2, 4-diacetylphloroglucinol (DAPG) can also suppress plant pathogens by a variety of mechanisms. Many oomycete and fungal plant pathogens are sensitive to low concentrations of the antibiotic DAPG as are some bacteria (Raaijmakers et al., 2002; Isnansetyo et al., 2003). Furthermore, DAPG has also been found to be an inducer of plant host defense (Iavicoli et al., 2003). Indeed, tomato seed treatment with *Pseudomonas fluorescens* improved seedling establishment against *Pythium ultimum* in greenhouse test (Warren and Bennett, 1999) and reduced tomato bacterial canker incidence in field test (Umesha, 2006).

Seed companies and growers often resist the use of sanitizing and biological seed treatments due to the concern that such treatments may reduce seed and seedling vigor, especially after months of seed storage. Therefore, it is critical that any seed treatment be assessed for its effects on seed vigor over time. The standard germination test to assess seed quality, by manual germination counts and measurements of seedling length, is both labor- and time-consuming. The Seed Vigor
Imaging System (SVIS) is an automated vigor assessment process developed in the OSU seed biology laboratory (Sako et al., 2001). The system comprises digital imaging of seedlings, germinated under standardized conditions, and imaging processing software. The software provides accurate and standardized measurements that are normalized and combined into a 0 to 1000 vigor index. Our preliminary work with tomato seed indicates that this system is highly amenable to rapid and objective tomato seed vigor assessments.

The current tomato seed treatments with traditional chemicals or hot water have shown flaws in either influence on seed vigor or failure to eradicate the pathogen. The second objective of my research was to develop an approach to seed health that combines sanitizing treatments with post-sanitation application of beneficial bacteria to eliminate *Cmm* on and in seed without hindering seed vigor, further reduce *Cmm* populations on seedlings and control the disease in the greenhouse. My hypotheses are: 1) that seed sanitation treatment (chemical, heat, antibiotics) will kill *Cmm* on and in seed; 2) that post-sanitation treatments with PGPR bacteria on tomato seed will enhance tomato canker control in seedlings and improve plant growth in the greenhouse; and 3) that there will be effective treatments that do not influence seed vigor over time. The outcome of this work will not only provide tomato growers with inexpensive, practical and effective methods to manage bacterial canker disease using seed treatments but also have broader implications towards seed health in many crop species.
References

American Seed Trade Association (ASTA),


Accumulation of phenolic compounds in leaves of tomato plants after infection with Clavibacter michiganense subsp. michiganense strains differing in virulence. Z. Naturforsch. 47: 898-909.


Antonie Leeuwenhoek W307-313.


United States Department of Agriculture (USDA) National Agricultural Statistic Service,


Chapter 2

Bioluminescent Imaging of *Clavibacter michiganensis* subsp. *michiganensis* Colonization of Germinating Tomato Seeds

**ABSTRACT**

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is a Gram-positive bacterium that causes wilting and cankers leading to severe economic losses in commercial tomato production worldwide. An important means of spreading disease is through infected seeds and seedlings. Due to the difficulty of, and lack of tools for genetic manipulation, very little is known regarding the mechanisms of seed to seedling transmission of *Cmm*. To facilitate studies of *Cmm* transmission in germinating seeds, we constructed bioluminescent *Cmm* strains by transforming the *lux* reporter gene into this bacterium. A vector, pXX2, was constructed by inserting a modified promoterless *lux*-operon into a *Cmm* transposon mutagenesis vector, pKGT452Cβ. After electroporation of pXX2 carrying the *Cmxr*::*luxABCDE*::Tn1409 cassette, the *lux*-operon was inserted into the *Cmm* chromosome. In total, 19 bioluminescent *Cmm* mutants were obtained. The mutants varied in light production, but all tested strains induced a hypersensitive response on *Mirabilis jalapa* (four o’clock) and caused wilting in tomatoes. The virulent, stable, constitutively bioluminescent strain BL-Cmm17 was selected for further characterization of growth properties and inoculation on tomato seeds. Using this bioluminescent strain, the
dynamics of *Cmm* colonization dynamics of germinating seeds were monitored in real-time using an *in vivo* imaging system (IVIS). Our results showed that *Cmm* colonized the hypocotyl and the cotyledon at an early stage of germination. These results emphasize the potential use of bioluminescent *Cmm* to better elucidate *Cmm*-tomato plant interactions. Furthermore, we have demonstrated the broader applicability of this tool by successful transformation of *C. michiganensis* subsp. *nebraskensis* (*Cmn*) with Tn1409::lux. Thus, our approach should be highly useful to understand pathogenesis of diseases caused by other subspecies of agriculturally important *C. michiganensis*. 
INTRODUCTION

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is a Gram-positive, aerobic bacterium that belongs to a group of plant pathogenic actinomycetes (Stackebrandt et al., 1997). Infections by *Cmm* cause bacterial canker and wilt of tomato, one of the most destructive and economically significant diseases of this crop. Severe epidemics can cause up to 80% yield loss (Strider, 1969), mainly due to wilting and death of plants and lesions on fruit. Bacterial canker was first discovered in Michigan (USA) greenhouses in 1909, and has now been reported to occur in most tomato production areas around the world (EPPO, 2005).

Plant wounds facilitate but are not required for infection by *Cmm*, which invades the xylem vessels, and causes vascular disease with high titers (10⁹ bacteria/g of plant tissue) (Carlton et al., 1998; Meletzus et al., 1993), impairing water transport and leading to plant wilting, canker stem lesions, and death (Gartemann et al., 2003, Jahr et al., 1999). Alternatively, asymptomatic infections can be induced by *Cmm* during late stages of plant development, resulting in the production of contaminated seeds, a major source of outbreaks of *Cmm* infections in tomato production (Fatmi et al., 1991; Ricker and Riedel, 1993). Traditional bacterial disease management measures, such as applications of antibiotics and copper bactericides, have not been successful against this disease, and canker-resistant tomato cultivars are not available. As a result, *Cmm* has been included under international quarantine regulation (Eichenlaub et al., 2006, EPPO, 2005). Consequently, seed testing and maintaining pathogen-free seeds and transplants is currently the most appropriate approach to minimize the
introduction and spread of the disease (Jahr et al., 1999). Even a low Cnm transmission rate (0.01%) from seed to seedling can cause a disease epidemic under favorable conditions (Chang et al., 1991). Seeds infected with Cnm give rise to contaminated seedlings, which may be latent infected or carrying the pathogen epiphytically (Tsiantos, 1987; Gitaitis, 1991; Shirakawa et al. 1991). An early study showed that seedlings grown from artificially infected seed were contaminated with $10^4$-$10^8$ CFU/seedling (Tsiantos, 1987). Seedlings grown from naturally infected seed can also carry $10^3$-$10^4$ CFU/g of the pathogen in shoots (Shirakawa et al. 1991). Due to overcrowding of seedlings during transplant production, the pathogen can easily spread through splashing irrigation water and leaf contact. Despite its apparent significance in Cnm epidemiology, the mechanism of seed to seedling transmission of Cnm is not well understood.

Developing adequate control measures for bacterial canker is complicated by the complexity of genetic manipulation of Gram-positive bacteria, which impairs analysis and characterization of pathogenesis mechanisms (Jahr et al., 1999). Consequently, there is a need for developing molecular techniques that will allow a better understanding of Cnm infections. One method of interest is using engineered bioluminescent bacteria to monitor plant-pathogen interactions in real-time. By exploiting natural light-emitting reactions that are encoded by the *luxCDABE* genes, bioluminescent bacteria have been used to assess gene expression and monitor internalization and distribution of bacteria in hosts (Chabot et al., 1996; Cirvilleri and Lindow 1994; Cirvilleri et al., 2000; Cirvilleri et al., 2008; De Weger et al., 1991;
Fan et al., 2008; Fukui et al., 1996; Kamoun et al., 1990; Paynter et al., 2006; Shaw et al., 1988; Shaw et al., 1992). In particular, bioluminescent phytopathogenic *Xanthomonas campestris* pathovars and *Pseudomonas* spp. have been used to track bacterial movement and distribution in host plants (Cirvilleri and Lindow 1994; Cirvilleri et al., 2008; Fukui et al., 1996; Paynter et al., 2006; Shaw et al., 1992), and to assess host susceptibility quantitatively (Fukui et al., 1996). Likewise, the *lux* genes have been also transferred to beneficial bacteria including *Rhizobium leguminosarum* and *Pseudomonas* spp. to visualize colonization pattern in rhizospheres (Chabot et al., 1996; De Weger et al., 1991).

The genes that carry the function of light emission are: *lux*AB, which express luciferase enzymes that catalyze the bioluminescent reaction, and the *lux*CDE encode the enzymes required for biosynthesis of a fatty aldehyde substrate necessary for the reaction (Meighen, 1993; Szittner and Meighen, 1990). Bioluminescence involves an intracellular oxidation of the reduced form of flavin mononucleotide and the fatty aldehyde, by luciferase in the presence of molecular oxygen; therefore, bacterial bioluminescence also requires oxygen, a source of energy (Stewart and Williams, 1992). Cells that express the *lux* operon spontaneously emit photons that can be captured by a sensitive charge-coupled device (CCD) camera, enabling imaging and visualization of bacterial cells (Hutchens and Luker, 2007). Luciferase activity depends on the metabolic integrity of the cell, while the amount of photons emitted correlates with biomass of living bacteria (Fan et al., 2008; Paynter et al., 2006). Furthermore, since the half-life of luciferase binding to its substrate is several seconds
(Meighen, 1993), captured light events reflect processes in real time and are not artifacts of accumulated signals. Consequently, live imaging of bioluminescence provides a sensitive means for visualizing bacterial colonization and invasion of hosts and allows real-time representation and examination of pathogen-plant interactions (Kamoun and Kado, 1990; Shaw et al., 1992).

Very little information is available about the mechanisms of Cmm pathogenesis and its colonization of seeds and subsequent transmission to seedlings. This is largely attributed to lack of tools and difficulties in genetically manipulating this Gram-positive bacterium (Metzler et al., 1997). However, recent development of an insertion sequence element IS1409 (Tn1409)-based efficient transposon mutagenesis system for Cmm has increased our knowledge of pathogenesis by this bacterium (Gartemann et al., 2001, Kirchner et al., 2001). To better understand the dynamics of seed to seedling transmission of Cmm, we constructed a bioluminescent Cmm strain using the Tn1409 transposon mutagenesis system. Our results demonstrated the utility of using a bioluminescent Cmm strain as a novel approach to elucidate the interaction of plants with this economically important pathogen.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 2.1. C. michiganensis subsp. michiganensis strains A300 and C290 were isolated from infected tomato plants in Ohio and belong to BOX-PCR fingerprint type A and C, respectively (Louws et al., 1998). Clavibacter
*michiganensis* subsp. *nebraskensis* strain LexIIC was obtained from Dr. David L. Coplin, The Ohio State University. Depending on the assay, *Clavibacter* strains were grown in yeast dextrose calcium carbonate medium (YDC: yeast extract at 10 g/L, CaCO₃ at 20 g/L, glucose at 10 g/L, agar at 15 g/L; Wilson et al., 1967), nutrient-broth yeast extract broth (NBY: nutrient broth at 8 g/L, yeast extract at 5 g/L, K₂PO₄ at 2 g/L, KH₂PO₄ at 0.5 g/L, glucose 5 g/L, MgSO₄ 7H₂O at 0.5 g/L; Vidaver, 1967), tryptone broth with yeast (TBY: tryptone at 10 g/L, yeast extract at 5 g/L, NaCl at 5 g/L, pH7.5) or sorbitol broth media (SB: tryptone at 10 g/L, yeast extract at 5 g/L, NaCl at 4 g/L, sorbitol 91.1 g/L, MgCl₂ 0.4 g/L, CaCl₂ 0.3 g/L, pH 7.5; Kirchner et al., 2001). *Escherichia coli* strains (DH5α and ER2925) were grown on Luria-Bertani medium (LB: tryptone at 10 g/L, yeast at 5 g/L, NaCl at 10 g/L; Bertani, 1951) at 37° C. Growth medium was supplemented with the antibiotics chloramphenicol (20 µg/ml for *E. coli* and 10 µg/ml for *Cmm* and *Cmn*), kanamycin (50 µg/ml for *Cmm*) and ampicillin (150 µg/ml for *E. coli*), where necessary.

**Construction of plasmids pXX1, pXX2 and pXX3.** Both pXen5 and pXen13 (Xenogen Corporation, Alameda, CA) carry the promoterless *lux*-operon originally isolated from *Photorhabdus luminescens*. The *lux*-operon (*luxABCDE*) in pXen5 had been modified by adding a Gram-positive bacterial ribosome binding site (AGGAGG) upstream of each *lux* gene for optimal expression in Gram-positive bacteria (Francis et al., 2000).

To construct pXX1, the *lux*-operon and kanamycin resistance gene from pXen5
were amplified by long range, high fidelity PCR using Herculase Polymerase (Stratagene, La Jolla, CA) with LuxpXen5F/LuxpXen5R primers. Oligonucleotides were designed using Vector NTI® software (Invitrogen) and commercially synthesized by Integrated DNA Technologies (Skokie, IL). All the oligonucleotides used in this study are listed in Table 2.2. Appropriate restriction sites were included in the primers to facilitate cloning. The PCR program was as follows: 2 min 95 °C, 30 cycles of 30 s 95 °C, 30 s 55 °C and 7 min 68 °C, final extension 10 min 68 °C. The amplified PCR product (6.8 Kb) was purified from an agarose gel (1%) using a Qiagen PCR product purification kit (Qiagen, Valencia, CA), then digested with EcoRI and SalI and ligated to a similarly digested Tn5 transposon construction vector pMod 3<R6Kgori/MCS> (Epicentre, Madison, WI). The recombinant plasmid pXX1 (9.3Kb) carries the EZ::TN<lux-kan> cassette, which is flanked by the Tn5 transposon mosaic ends.

pXX2 was constructed by amplifying the lux-operon from pXen-5 with primers LuxpXen5F2/LuxpXen5R2. Inverse PCR was performed on the Tn1409 transposon vector pKGT452Cβ (Kirchner et al., 2001) with pKGT1F/pKGT1R primers that were designed to amplify all but the region of lux insertion. The PCR program was the same as described above. The restriction sites NotI and XhoI were added to the PCR primers to facilitate cloning. The inverse PCR product was digested and ligated to the similarly digested lux operon from pXen5, generating pXX2 (cmxr::luxABCDE::Tn1409, 12.2Kb).
To construct pXX3, the *lux*-operon (*luxCDABE*) was obtained by digesting pXen13 with *NotI* and *XhoI* and ligated to the similarly digested inverse PCR product of the transposon vector pKGT452Cβ amplified using the pKGT2F/pKGT2R primers. pXX3 (12.3Kb) carries *cmx':: luxCDABE::Tn1409*.

The pUWGR4 plasmid was constructed in a manner similar to that used to make pXX1 by modifying the EZ::TN pMod-3 transposon in a previous study (Rajashekara, et al. 2003). The difference was that the *lux*-operon carried by pUWGR4 was obtained from pXen 13.

Recombinant plasmids pXX1, pXX2, pXX3 and pUWGR4 were transformed to *E. coli* DH5α and subsequently propagated in the *dam* and *dcm* deficient *E. coli* strain ER2925, since the use of unmethylated DNA improves transformation efficiency in *Cmm* (Kirchner et al., 2001).

**Mutagenesis and isolation of bioluminescent Cmm.** Bioluminescent Cmm strains were generated either by electroporation of the modified EZ::TN transposomes from pXX1 or pUWGR4 or by directly electroporating the suicide vectors containing the modified transposon Tn1409 (pXX2 and pXX3). Before electroporation, pUWGR4 and pXX1 were digested with *PvuII* and the resulting approximately 7 kb linear DNA fragments containing the EZ::TN-*lux*, and kanR gene were gel purified using a Qiagen gel purification kit. The EZ::TN transposome was prepared as described by the manufacturer (Epicentre). Briefly, 2 μl of each purified DNA (100 ng/μl) were mixed
separately with 4 μl of EZ::TN transposase (Epicentre) and 2 μl of 100% glycerol in an 8 μl reaction. Following incubation for 30 min at room temperature, the transposome complexes were stored at –20°C until further use.

Electrocompetent *Cmm* aliquots were prepared as described previously (Kirchner et al., 2001). Briefly, bacteria were grown to OD$_{600}$ 0.5-0.7 in TBY broth at 25°C. Cells were then pretreated with 2.5% glycine for 2 h and harvested by centrifugation at 9,000 × g for 10 min, followed by washing with sterile ice cold distilled water three times and 10% glycerol twice. The cell pellet was then resuspended in 15% glycerol to 1/250 of the original volume. Transformation was achieved by mixing 100 μl of electrocompetent *Cmm* cells with 2 μl of the EZ::TN transposome complex or 1 μl of suicide plasmids pXX2 or pXX3 (1 μg each) and electroporation was performed in a 0.2-cm cuvette, using a Gene Pulser Xcell electroporator (BioRad, Hercules, CA) with the following settings: voltage, 2.5 kV; capacitance, 25 μF; resistance, 600 Ω. Immediately after electroporation, the cells were mixed with 0.4 ml of SB medium, and transferred into a 10 ml sterile disposable tube and incubated with shaking (140 rpm) for 3 h at 28°C. Finally, the cells were spread on SB agar plates containing kanamycin or chloramphenicol and incubated for 4 to 7 days at 28°C. The Kan$^\text{r}$ or Cmx$^\text{r}$ colonies were recovered and streaked onto NBY plates containing appropriate antibiotics and screened for bioluminescence using a CCD camera in an *in vivo* imaging system (IVIS; Xenogen). The bioluminescent colonies were purified and stored at -80°C for further analysis.
For electroporation of *C. michiganensis* subsp. *nebraskensis*, electrocompetent cells were prepared and transformed with 1 μg of pXX2 as described above. Transformants were selected on SB agar containing chloramphenicol and screened for bioluminescence using a CCD camera after 4 days of incubation at 28° C.

**PCR analysis of bioluminescent Cmm strains.** To confirm the integration of the *lux* operon into the *Cmm* chromosome, PCR analysis was performed on all recovered colonies. After electroporation with pXX1, colonies were tested using the KanF/KanR primers that target part of the kanamycin resistance gene. Additionally, colonies recovered after electroporation with pXX2 or pXX3 were tested for the presence of the chloramphenicol resistance (*Cmx*) and *luxC* genes using primers CmxF/CmXR and LuxCF/LuxCR, respectively. PCR was performed on genomic DNA obtained from bioluminescent strains using a *Taq* PCR Master Mix kit (Qiagen).

**Analysis of bioluminescence.** To quantify the amount of bioluminescence emitted by the transformed *Cmm*, transformants were grown aerobically in NBY broth containing the appropriate antibiotics 28° C for 48 h. Three replicates of 100 μl of each culture (1 x 10⁹ CFU) were then transferred to a black 96-well plate and bioluminescence was quantified using IVIS. The photon output (radiance) of each well (photons/s) was normalized for background luminescence by subtracting the value of a control well containing NBY broth only. The OD₆₀₀ was measured and the radiance was divided by the OD to normalize for culture cell density. Highly bioluminescent clones were selected for further studies.
Hypersensitive response (HR) and pathogenicity assays. The bioluminescent
*Cmm* strains were tested in four o’clock plants (*Mirabilis jalapa*) for HR and by
tomato seedling inoculation for pathogenicity. For the HR test, four o’clock plants
were grown in autoclaved soil (50% silt loam:50% muck was mixed and sieved, then
30.3 L of peat was added to every 151.5 L of soil mix and supplemented with 350 g
of lime and 150 g of Peters 4-45-15 fertilizer). Bacterial suspensions (10^8 CFU/ml)
were prepared in sterile water and injected into fully expanded leaves of four o’clock
plants using needle-less syringes, ensuring that the bacteria would infiltrate into
intercellular spaces. Typically, the wild-type virulent *Cmm* induces HR-characteristic
necrosis in the infiltrated areas within 36-48 hours (Gitaitis et al., 1990). For each
strain, four injections were applied on one leaf and inoculated plants were placed for
up to 48 hours in a greenhouse with a 14 hour light/10 hour dark photoperiod. The
wild-type *Cmm* strain C290 and sterile water were used as positive and negative
controls, respectively.

For pathogenicity assay, the inoculation of tomato seedlings bioluminescent *C.
michiganensis* subsp. *michiganensis* strains was performed as previously described
(Poysa et al., 1993). The cotyledons of tomato seedlings (4-weeks old) were clipped
using dissecting scissors that had been dipped in a *Cmm* suspension (10^8 CFU/ml).
Tomato seedlings (n=3) were inoculated with each strain and monitored for
symptoms for 3 weeks. Furthermore, virulence was assessed by determining the
number of plants that exhibited wilting symptoms within 3 weeks after inoculation.
To quantify the bacterial populations in infected tomato plants, stem samples from three plants were taken from 3 cm above the cotyledons and weighed (0.2-0.3 g). Samples were ground in 3 ml phosphate buffer (pH=7.4, 10mM) and centrifuged at 400 × g for 3 min. The supernatant was serially diluted and plated onto NBY agar plates that were supplemented with chloramphenicol. For samples inoculated with C290, serial dilutions were spread onto D2ANX (a semi-selective medium for Cmm; Chun, 1982). Colony counts were recorded 7 days later. The number of bacteria present in tomato tissue (CFU/g) was calculated as number of colonies * dilution factor * 3ml / weight of sample tissue. The HR and pathogenicity assays were done twice.

To further compare the virulence of the selected bioluminescent strain BL-Cmm17 against its parent strain C290, 50 tomato seedlings were clip-inoculated and virulence was rated using a wilting index. The wilting index is defined as the number of days required for 50% of the inoculated plants to show wilting symptoms on leaves (Meletzus et al., 1993). This virulence test was done twice.

**Assessing *in vitro* growth, constitutive expression of bioluminescence, and stability of Tn1409-lux insertion in a virulent bioluminescent Cmm strain.** The *in vitro* growth of BL-Cmm17 was compared to that of its parent strain C290 in a rich medium, NBY broth, as well as in Cmm minimal medium (MgSO4 7H2O at 0.25 g/liter, KH2PO4 at 0.5 g/liter, K2HPO4 at 2 g/liter, sucrose at10 g/liter, and yeast extract at 0.1 g/liter; Alarcón et al., 1998). BL-Cmm17 growing on NBY plates for
48 h at 28 °C was inoculated into a 200 ml flask containing 50 ml NBY broth or the Cmm minimal medium and incubated at 28°C with shaking at 160 rpm for 50 h (three replications per strain). Bacterial growth was monitored by measuring the optical density (OD$_{600}$) from 1 ml culture every 4 hours. To assess constitutive lux expression, at each time point light intensity from 100 μl of culture was determined using IVIS. Relative light units were calculated by log transformation of average radiance.

Stability of the transposon insertion was verified by growing BL-Cmm17 without antibiotic selection in 5 ml NBY broth at 28°C for 72 h, for a total of five transfers (with 5 μl for each re-inoculation). After the fifth round of growth, the culture was diluted in NBY broth and plated on NBY agar plates without antibiotics and incubated at 28°C for 72 h. One hundred and fifty colonies were randomly selected and plated onto NBY agar medium with chloramphenicol. The growth on the antibiotic plate and bioluminescence of each colony was assessed.

Mapping the Tn1409 insertion site. Genomic DNA was extracted from BL-Cmm17 using a MasterPure Gram-positive DNA Purification Kit (Epicentre) and the insertion site was determined by bi-directional sequencing using the primers Xu3F/Xu3R (Table 2.2). Sequencing was performed using dye terminators at a DNA sequencing core facility (The Plant-Microbe Genomic Facility, The Ohio State University). Sequences were compared to the Cmm genome to determine the site of insertion.
Seed inoculation with bioluminescent Cmm. Healthy tomato seeds (cv. OH9242) were inoculated by soaking in BL-Cmm17 suspension ($10^8$ CFU/ml) in a sterilized 100 ml beaker. The beaker was placed in a Nucerite Dessicator (Nalge Sybron Corporation, Rochester, NY) and vacuum was applied for 5 min using an Air Cadet pump (Barnant, Barrington, IL) with maximum 18 psi pressure. Seeds inoculated with sterilized water were used as controls. After inoculation, seeds were air dried and placed on water agar (1 %) in a square petri dish ($10 \times 10$ cm with 13 mm grid) and incubated at 25° C in the dark. Bioluminescent Cmm colonization of germinating seeds was monitored daily using the IVIS. Six seeds were sampled each day for 5 days to estimate the bacterial population on each seed. On the first and second day of germination, seeds were ground individually in 0.5 ml potassium phosphate buffer and serial dilutions were plated on YDC medium supplemented with chloramphenicol (10 $\mu$g/ml). From the third to fifth day, seedlings were carefully dissected and seed coat, cotyledons, hypocotyls and radicals were collected aseptically with sterile forceps and bacteria were isolated by serial dilution plating as described above. The experiment was done twice.

RESULTS

Isolation of bioluminescent Cmm strains. In order to generate a bioluminescent Cmm strain, we desired stable chromosomal insertion of the lux operon as well as constitutive expression of bioluminescence. Unlike the expression of lux from a plasmid, chromosomal integration will lead to stable expression of lux even in the absence of antibiotic selection that is necessary for real time studies in plants, where
there is no antibiotic selection. Because of the lack of tools for genetic manipulation of *Cmm*, we tried several approaches to integrate *lux* genes into the *Cmm* chromosome. Since the *lux*-operon in pXen13 was originally derived from the Gram-negative bacterium *P. luminescens*, it might not be expressed efficiently in *Cmm*, a high (G+C) Gram-positive bacterium. Consequently, the ribosome binding sites of each *lux* gene in the *luxABCDE* operon in pXen5 were optimized for expression in Gram-positive bacteria. Initially, we tried the Tn5-based transposon mutagenesis approach that has been successfully used in *Corynebacterium matruchotii*, an actinomycete related to *Cmm* (Wang et al., 2006). After electroporation of the *Tn5:*lux-transposome complexes prepared from pUWGR4 or pXX1 (Figure 2.1 A) into *Cmm* strains A300 and C290, a total of 84 kanamycin resistant colonies were obtained; however none were bioluminescent. PCR analysis targeting the kanamycin gene indicated that the strains lacked the *EZ::TN* insertion. Further, *Cmm* electroporated without an *EZ::TN<lux-Kan>* cassette (negative control), also resulted in kanamycin resistant colonies, which suggested that *Cmm* strains used in this study can inherently develop kanamycin resistance or Tn5 might not work in *Cmm*.

Kirchner et al. (2001) developed an efficient mutagenesis system for *Cmm* using Tn1409. Therefore, we modified the Tn1409-containing vector pKGT452 Cβ to contain either a *lux* operon optimal for expression in Gram-positive bacteria (pXX2) (Figure 2.1B) or a *lux* operon from a Gram-negative bacterium (pXX3) (Figure 2.1C). After electroporation of pXX3 into *Cmm* strains, three colonies were recovered, which were found to harbor the chloramphenicol resistance gene analyzed by PCR.
(data not shown), indicating the presence of the transposon in their genome.

However, none of these strains were bioluminescent, which is likely due to the lack of expression of Gram-negative \textit{lux} in Gram-positive bacteria as previously suggested (Francis et al., 2000). When transposon vector pXX2 was electroporated into \textit{Cmm}, bioluminescent colonies were obtained successfully (Figure 2.2A). The transformation efficiency of \textit{Cmm} with pXX2 was about an average of two transformants/\mu g of vector DNA, which was similar when using either \textit{Cmm} C290 or A300. Twenty-six chloramphenicol resistant colonies were obtained; however, only 19 colonies (BL-Cmm1 to 19) were bioluminescent. Upon further analysis using PCR, both the Cmx resistance gene and \textit{luxC} genes were detected in the genomes of the bioluminescent strains (Figure 2.2B). In addition, all seven non-bioluminescent strains also contained the Cmx resistance gene gene.

Strain BL-Cmm17 exhibited significantly higher bioluminescence compared to other strains (P \leq 0.05), which differed by up to two log units (Figure 2.3). Since integration of Tn1409 in the \textit{Cmm} genome is non-specific (Kirchner et al., 2001), the \textit{lux} operon may be integrated downstream of promoters that are expressed at different levels. Consequently, variations in the \textit{lux} operon expression are expected, leading to the observed differences in bioluminescence emitted by different bioluminescent strains.

The impact of the \textit{lux} insertion on the virulence of bioluminescent \textit{Cmm} strains.

Since Tn1409 is inserted non-specifically into the \textit{Cmm} genome (Kirchner et al.,
2001), the *lux*-operon might affect down-stream genes that are essential for virulence. Nine bioluminescent strains (BL-Cmm strains 1 to 8, and strain 17) that emitted a different range of light intensities were selected for the HR assay. All strains induced strong HR in four o’clock plants, indicating that the bioluminescent strains should be pathogenic to tomato (Table 2.3, Figure 2.4). The same nine BL-Cmm strains were inoculated into tomato seedlings and all strains caused plant wilting in a similar manner as wild-type *Cmm* C290. In addition, the number of bacteria retrieved from the stem tissue of plants inoculated with any of the bioluminescent strains or the wild-type strain was similar, suggesting that the ability of the bioluminescent strains to colonize the vascular tissue was not affected by the *lux*-insertion. Since the bacteria recovered from the stem tissues were strongly bioluminescent, it was concluded that the *lux*-insert was stable in these strains. Further comparison of the virulence of BL-Cmm17 with that of C290 showed very similar wilting indices, 13.5±0.7 days and 12.0±1.4 days, respectively.

**Characterization of a bioluminescent *Cmm* strain.** Since BL-Cmm17 exhibited strong bioluminescence and was virulent in both HR and tomato pathogenicity assays, similar to its parental strain C290, we used this strain for future studies. The *in vitro* growth of BL-Cmm17 in nutrient medium NBY was similar to that of C290, both displaying a generation time of 4 h. Bioluminescence increased as BL-Cmm17 grew in log phase and started to decrease when its growth reached the stationary phase suggesting a constitutive expression of Lux (Figure 2.5 A). Furthermore, the similar growth patterns of BL-Cmm17 and C290 were observed in minimal medium
indicating that the *lux* insertion in BL-Cmm17 had no auxotrophic effect on the growth and survival of BL-Cmm17 (Figure 2.5 B). The *lux* insertion was stable in BL-Cmm17; after five rounds of non-selective growth for approximately 50 generations, 150/150 independent BL-Cmm17 colonies from three replicate experiments were resistant to chloramphenicol and were bioluminescent.

Sequence analysis using bidirectional outward primers Xu3F/Xu3R (see Figure 2.1B for primer location) suggested a whole vector integration since the nucleotide sequences obtained highly aligned (90% identity) with pXX2 sequence flanking the inverted repeats. However, this is not surprising and has been observed previously when using this mutagenesis system in *Cmm* (K. H. Gartemann, personal communication). The plasmid profile from BL-Cmm17 was identical to that of wild-type C290 further confirming the plasmid integration. In addition, restriction analysis of plasmids extracted from BL-Cmm17 and C290 with *Xho*I/*Not*I enzymes suggested a chromosomal integration of the Tn1409::*lux* (data not shown). Further analysis is needed to identify the precise insertion site. Since the insertion of the *lux*-operon did not influence the growth of BL-Cmm17 and the expression of the *lux*-operon was stable and constitutive, it can be assumed that the *lux*-operon was inserted downstream of a strong promoter without disturbing any housekeeping or essential virulence genes in the BL-Cmm17 genome.

**Monitoring the colonization of bioluminescent *Cmm* in germinating tomato seeds.**

In total, 100 seeds were imaged using IVIS and representative results are shown in
Figure 2.6 A. Luminescent signals were detected on the seed coat throughout 5 days of germination. On the third day, the radicle punctured the seed coat and luminescence was also observed on some of the radicles. On the fourth and fifth day, cotyledons emerged from the seed coat and hypocotyls and radicles elongated. Luminescent signals were usually stronger on the hypocotyls, cotyledons and seed coats than on the radicles. Consistent with the bioluminescence imaging, viable cell counts indicated up to 6 logs units of bacteria were present on the seed coat during the first 3 days post infection and up to 5 logs of bacteria were detected on day 5 post infection (Figure 2.6 B). Further, hypocotyls, cotyledons and radicles contained on average 4.5 to 5.4 logs of bacteria at day 5 (Figure 2.6 C). Light intensity was positively correlated with the number of bacteria on individual seedling components with the highest determination coefficient ($R^2 = 60.5\%$) for seed coats tested from days 1 to 3 (Figure 2.6 D).

**Isolation of bioluminescent *C. michiganensis* subsp. *nebraskensis* strains.** Tn1409 transposon mutagenesis is effective in other subsp. of *C. michiganensis* (Kirchner et al., 2001). Therefore, to demonstrate the utility of our modified Tn1409-lux for the generation of bioluminescent bacteria in a closely related subsp. of *C. michiganensis*, we transformed pXX2 into *C. michiganensis* subsp. *nebraskensis*, the causal agent of wilt and leaf blight in maize. Electroporation of pXX2 resulted in a total of 22 chloramphenicol-resistant colonies, of which eight were bioluminescent. The transformation frequency was very similar to that observed with *Cmm*. These results highlight the usefulness of our approach to elucidate, in real-time, the plant-pathogen
interactions in diseases caused by other agriculturally important subsp. of *C. michiganensis*.

**DISCUSSION**

Despite the importance of *Cmm* as a debilitating crop pathogen with a significant economic impact on tomato production, little is known about its ecology in and on tomato plants under modern propagation and production practices. This lack of knowledge is partly attributed to the difficulty in genetically manipulating this Gram-positive bacterium. Consequently, molecular approaches that would facilitate the study of *Cmm* interactions with plant hosts, colonization of seeds, transmission to seedlings and the spread of this pathogen among tomato crops are needed. Of particular interest is the *lux*-operon, which has been successfully used as a bioluminescent reporter in bacterial pathogens, facilitating the characterization of bacterial distribution, growth and infection in plant hosts, and allowing the assessment of plant responses to bacterial infection (Cirvilleri and Lindow, 1994; Cirvilleri et al., 2000; De Weger et al., 1991; Fan et al., 2008; Fukui et al., 1996; Paynter et al., 2006). Therefore, we generated a bioluminescent *Cmm* strain that retained its virulence and growth properties and enabled visualization of colonization dynamics of this pathogen *in planta* in real-time.

The construction of bioluminescent *Cmm* strains required testing different mutagenesis approaches, among which the use of a modified Tn1409 vector, pKGT452Cβ, carrying the *lux*-operon optimized for expression in Gram-positive
bacteria proved successful. Insertion of the lux operon allowed the isolation of a strain that constitutively expressed strong bioluminescence. We desired a promoter that was active both in planta and in vitro, and a bioluminescent strain that was not compromised in virulence to use for in planta real-time infection. Initially, we constructed a Tn5-based transposon containing lux operon; however, we were not successful in generating bioluminescent Cmm strains using this approach, although Tn5 transposon mutagenesis has been used successfully in the related Corynebacterium matruchottii (Wang et al., 2006). Alternatively, use of vectors carrying the Tn1409 transposon (pKGT452Cβ) effectively resulted in transformed bioluminescent Cmm that harbored the lux genes. The pKGT452Cβ vector was of particular interest, since transposon Tn1409 was shown to integrate non-specifically in the Arthrobacter chromosome (a genus closely related to Clavibacter), and later proved to be efficient in Cmm mutagenesis (Gartemann et al., 2001; Kirchner et al., 2001). Specifically, electroporation with the suicide vector-carrying transposon Tn1409 yielded approximately $1 \times 10^3$ mutants per μg of DNA, and resulted in random insertion of the transposon in the Cmm chromosome (Kirchner et al., 2001). However, Cmm electroporation with pXX3 carrying the Tn1409::lux resulted in a very low number of transformants, which carried the lux-operon but were not bioluminescent. Although the low transformation efficiency was likely due to the large size of the lux insert, the lack of bioluminescence by the transposon mutants suggests a lack of expression of the Gram-negative bacteria-derived lux operon in Cmm. The luxCDABE operon originated from the Gram-negative bacterium, P. luminescens, which is relatively low in GC (42%) (NCBI genome database). The
Cmm genome has a high GC content (~73%), which possibly explains the lack or inefficient expression of lux genes. Therefore, we used pXX2 that has a lux operon optimized for expression in Gram-positive bacteria. Similar to pXX3, the transformation frequency with pXX2 was very low, which again could be attributed to the large size of the lux operon; however, we can not exclude the possibility of genetic variation among Cmm strains contributing to observed differences in the transformation frequency (Kirchner et al., 2001).

For the bioluminescent Cmm strains to constitute a suitable tool for studying colonization and plant invasion, it was important to test if the lux insertion affected their growth and virulence. Our results showed that strain BL-Cmm17, which was strongly bioluminescent, exhibited growth similar to that of the wild-type strain (Figure 2.5), carried a stable lux operon that was not lost after successive growth cycles in non-selective medium, and possessed virulence properties comparable to the wild type (Figure 2.4, Table 2.3). These observations showed that although the precise lux operon insertion site within BL-Cmm17 could not be determined, insertion of the lux operon did not impede essential functions involved in bacterial growth and virulence. Furthermore, the bioluminescence could be detected in BL-Cmm17 at different growth stages. Specifically, the bioluminescence of BL-Cmm17 reached its highest level in late stages of exponential growth. This can be the result of an efficient activation of the promoter controlling the lux expression or a reflection of bacterial numbers during this stage of growth. The decrease in BL-Cmm17 bioluminescence after entering the stationary growth phase might have been the result
of down-regulation of cell metabolism, as it has been shown that bioluminescence requires energy derived from cell metabolism (Hakkiala et al., 2002). These observations suggest that this strain is suitable for experiments that mimic the plant colonization processes by wild-type bacteria.

Few studies have addressed *Cmm* seedling infections initiated by contaminated seeds in general, and data are almost completely lacking concerning *Cmm* transmission from seeds, a major route for long-distance spread of this pathogen. Since naturally deposited *Cmm* on the surface of seeds during seed extraction precedes colonization (EPPO, 2005), it was assumed that tomato seeds artificially inoculated with BL-Cmm17 simulated naturally infected seeds. Subsequently, real-time imaging showed that BL-Cmm17 aggregated on the hypocotyls and cotyledons (Figure 2.6) of the seedlings, possibly advancing colonization and prompting the onset of future infections. These observations and conclusions were supported by previous reports showing that early canker symptoms appeared on the cotyledons and stems and epiphytic *Cmm* populations were associated with tomato leaves (Gleason et al., 1993; Strider, 1969). Perhaps one of the most important features of using a bioluminescent *Cmm* is the ability to quantify the number of pathogens colonizing the plant in real time. Emitted light can be correlated to bacterial abundance using predetermined growth curves. Therefore, we were able to monitor *Cmm* colonization and assess the abundance of the population over time (Figure 2.6). Our seed inoculation experiments were primarily designed to highlight the value of using bioluminescence in studying *Cmm* interactions with the plant host. Our results
corroborated this value and shed a new light on early *Cmm* colonization of tomato seedlings. Correlation between radiance and bacterial colony counts were highest for the seed coat, and lowest for radicles. It is possible that *Cmm* was less aggregated on radicles than the seed coat, resulting in lower photon emissions per unit area. Additionally, electrolytes diffused through the seed coat may promote bacterial growth at the early stages of germination (Chachalis et al., 2008). Further analysis and longer observation intervals are needed to fully characterize the epiphytic stage of *Cmm* on seeds, and its transmission to seedlings and mature plants.

In summary, we have developed a novel approach to characterize *Cmm* plant infection and colonization. Incorporation of a bioluminescent marker in the *Cmm* chromosome has allowed us to monitor the infection temporally. Significant to *Cmm* pathogenesis, our results reveal the colonization dynamics of *Cmm* infection of hypocotyls and cotyledons of the seedlings that promote onset of future infections. Therefore, our approach provides a novel visual representation of the disease process and accelerates understanding of *Cmm* ecology. Furthermore, these bioluminescent strains will have a wide scope of application, including determining plant factors that affect *Cmm* growth and metabolism, and screening antibiotics and bactericides for canker control in an effective real-time approach. As importantly, our results indicated that Tn1409-lux can be used to generate bioluminescent *C. michiganensis* subsp. *nebraskensis*. Since Tn1409 is also an effective mutagenesis system for other important subspecies of *C. michiganensis*, including subsp. *insidiosus* (causes wilt and stunt on alfalfa), and subsp. *sepedonicus* (causes ring rot on potato) (Kirchner et
al., 2001), the vector pXX2 constructed in this study could also be used for
transformation of the lux operon into these important pathogens, facilitating real time
transmission, control, and plant interaction studies.
ACKNOWLEDGEMENTS

We thank Dr. Larry V. Madden and Dr. Pierce A. Paul for their assistance with statistical analyses, Dr. David L. Coplin for providing the *C. michiganensis* subsp. *nebraskensis* strain and critical comments on virulence tests and growth assays, and Dr. Issmat Kassem for critical review of the manuscript. The research in Dr. Rajashekara’s and Dr. Miller’s labs is supported by a USDA-NRI grant and by the Ohio Agricultural Research and Development Center, The Ohio State University.
REFERENCES


Chun W.C.C. 1982. Identification and detection of *Corynebacterium michiganense* in tomato seed using the indirect enzyme-linked immunosorbent assay. MSc Thesis. Honolulu, HI, USA: University of Hawaii.


Meighen, E. A. 1993. Bacterial bioluminescence: organization, regulation, and
application of the *lux* genes. The FASEB Journal 7:1016-1022.


Table 2.1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Description $^a$</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> C290</td>
<td>Wild-type virulent <em>Cmm</em> strain isolated in Ohio</td>
<td>Lab collection</td>
</tr>
<tr>
<td><em>C. michiganensis</em> subsp. <em>michiganensis</em> A300</td>
<td>Wild-type virulent <em>Cmm</em> strain isolated in Ohio</td>
<td>Lab collection</td>
</tr>
<tr>
<td><em>C. michiganensis</em> subsp. <em>nebraskensis</em> LexIIC</td>
<td>Wild-type virulent <em>Cmn</em> strain isolated in Nebraska</td>
<td>Dr. David L. Coplin</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Used in cloning experiments</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> ER2925</td>
<td>Dam/dcm methylation negative strain</td>
<td>NEB</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pXen5</td>
<td>Source for promoterless <em>lux</em> operon optimized for expression in Gram-positive bacteria; Ery, Kan, 18.3Kb</td>
<td>Xenogen</td>
</tr>
<tr>
<td>pXen13</td>
<td>Source for Gram-negative <em>lux</em>-operon from <em>P. luminescence</em>; Amp, 8.7Kb</td>
<td>Xenogen</td>
</tr>
<tr>
<td>pMod3</td>
<td>Containing EZ::TN transposon; 2.8Kb</td>
<td>Epicentre</td>
</tr>
<tr>
<td>pKGT452Cβ</td>
<td>Tn1409 Cβ; tnpA and cmx; Amp, Cmx, 6.4Kb</td>
<td>Kirchener et al., 2001</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source of kanamycin resistance marker, Kan, Amp</td>
<td>Amersham</td>
</tr>
<tr>
<td>pUWGR4</td>
<td>pMod3 containing EZ::TN transposon and <em>lux</em> operon from pXen13; Kan, 10Kb</td>
<td>Rajashekara et al., 2005</td>
</tr>
<tr>
<td>pXX1</td>
<td>pMod3 containing promoterless <em>lux</em> operon from pXen5; Kan, 9.3Kb</td>
<td>This study</td>
</tr>
<tr>
<td>pXX2</td>
<td>pKGT452Cβ containing promoterless <em>lux</em> operon from pXen5; Amp, Cmx, 12.2Kb</td>
<td>This study</td>
</tr>
<tr>
<td>pXX3</td>
<td>pKGT452Cβ containing promoterless <em>lux</em> operon from pXen13; Amp, Cmx; 12.3Kb</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$Kan, kanamycin resistance; Amp, ampicillin resistance; Cmx, chloramphenicol resistance; Ery, erythromycin resistance.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence $^a$</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>LuxpXen5F</td>
<td>5’ ATAAAAGAATTCGACTCCTGTGAAATGATC</td>
<td>Amplification of the lux-operon and kanamycin gene from pXen5</td>
</tr>
<tr>
<td>LuxpXen5R</td>
<td>5’ AAAAAACTCGACCGGATGTACTTCAGAGAAAGA</td>
<td></td>
</tr>
<tr>
<td>pKGT1F</td>
<td>5’ AAAAAAGCGGCGCACATCAGCGGCTTAGAGCTT</td>
<td>Inverse PCR amplification of pKGT452Cβ to clone lux operon from pXen5</td>
</tr>
<tr>
<td>pKGT1R</td>
<td>5’ AAAAAACTCGAGCTAGACCTGGGGA</td>
<td></td>
</tr>
<tr>
<td>KanF</td>
<td>5’ GAAGCGTTTAGATGTTAAGT</td>
<td>Confirmation of EZ::TN&lt;lux-kan&gt; integration into chromosome</td>
</tr>
<tr>
<td>KanR</td>
<td>5’ GGTACTAAACAATCTCC</td>
<td></td>
</tr>
<tr>
<td>LuxpXen5F2</td>
<td>5’ ATAAAAGCGGCGCGAAACAGCTATGACCATGAT</td>
<td>Amplification of the lux-operon from pXen5</td>
</tr>
<tr>
<td>LuxpXen5R2</td>
<td>5’ AAAAAACTCGACCTTCCTCTCTCAGAGAAGG</td>
<td></td>
</tr>
<tr>
<td>pKGT2F</td>
<td>5’ AAAAAACTCGAGATCCGAGCGCTAGACCTGG</td>
<td>Inverse PCR amplification of pKGT452Cβ to clone lux operon from pXen13</td>
</tr>
<tr>
<td>pKGT2R</td>
<td>5’ AAAAAAGCGGCGCGCTACAGACAGATGGT</td>
<td></td>
</tr>
<tr>
<td>CmxF</td>
<td>5’ AGAGTACTGCGAGCGGCA</td>
<td>Confirmation of Tn1409-lux integration into chromosome</td>
</tr>
<tr>
<td>CmxR</td>
<td>5’ ACTGTCGATCTCTCCTC</td>
<td></td>
</tr>
<tr>
<td>LuxCF</td>
<td>5’ GTTGATGAAATATCCACCTCT</td>
<td>Confirmation of Tn1409-lux integration into chromosome</td>
</tr>
<tr>
<td>LuxCR</td>
<td>5’ GGACATACATTGCGAGCTTA</td>
<td></td>
</tr>
<tr>
<td>Xu3F</td>
<td>5’ GGGTTTGCTGGGTTGGTTCTG</td>
<td>Mapping of insertion site of Tn1409-lux in Cmm genome</td>
</tr>
<tr>
<td>Xu3R</td>
<td>5’ CGGCCCCACAGGAGCAATT</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Restriction sites added to 5’ end of each primers are underlined.
Table 2.3 Induction of the hypersensitive response (HR) in four o’clock plant and virulence to tomato plants by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>HR</th>
<th>Virulence</th>
<th>Log CFU/g in planta&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL-Cmm1</td>
<td>+</td>
<td>6/6</td>
<td>9.57±0.18</td>
</tr>
<tr>
<td>BL-Cmm2</td>
<td>+</td>
<td>5/6</td>
<td>10.63±0.14</td>
</tr>
<tr>
<td>BL-Cmm3</td>
<td>+</td>
<td>6/6</td>
<td>10.55±0.18</td>
</tr>
<tr>
<td>BL-Cmm4</td>
<td>+</td>
<td>6/6</td>
<td>8.77±1.03</td>
</tr>
<tr>
<td>BL-Cmm5</td>
<td>+</td>
<td>5/6</td>
<td>9.88±1.04</td>
</tr>
<tr>
<td>BL-Cmm6</td>
<td>+</td>
<td>6/6</td>
<td>9.11±0.57</td>
</tr>
<tr>
<td>BL-Cmm7</td>
<td>+</td>
<td>6/6</td>
<td>9.23±0.9</td>
</tr>
<tr>
<td>BL-Cmm8</td>
<td>+</td>
<td>6/6</td>
<td>8.77±0.94</td>
</tr>
<tr>
<td>BL-Cmm17</td>
<td>+</td>
<td>6/6</td>
<td>9.43±0.28</td>
</tr>
<tr>
<td>C290</td>
<td>+</td>
<td>6/6</td>
<td>9.08±0.60</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>0/6</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>, positive for HR reaction; -, negative for HR reaction.

<sup>b</sup> Number of wilting plants/total inoculated plants.

<sup>c</sup> Mean population of bacteria ± SD recovered from BL-Cmm-inoculated stem tissue; no significant differences in population counts from inoculated plants were observed (P≤0.05).
Figure 2.1 Physical maps of constructed vector pXX1(A), pXX2(B) and pXX3(C) carrying the lux-operon. Abbreviations: Amp, ampicillin resistance; cmx, chloramphenicol resistance; ME, mosaic end; Kan, kanamycin resistance; tnpA, transposase IS1409; IRR, right inverted repeat; LRR, left inverted repeat. Arrows indicate location of primers used for sequencing.
Figure 2.2  (A) Representative image of bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* mutants recovered on a SB plate screened by IVIS. (B) PCR analysis with CmxF/CmxR and LuxCF/R primers of the colonies recovered after pXX2 electroporation. Lane 1, 1 kb plus ladder; Lanes 2 and 7, wild-type C290 as a negative control; Lanes 3 and 8, pXX2 plasmid as a positive control; Lanes 4-6 and 9-11, bioluminescent transposon mutants. Only results from representative strains are shown.
Figure 2.3 Comparison of bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* mutant strains in light production. Bacterial culture growth in NBY medium was to late log phase (OD about 0.8~1.0) and 100 μl bacterial suspension for each strain was used for light emission assessment. Relative light unit (RLU) is the log transformed value of total flux (photon/s). Data shown are means of three replicates, error bars are standard error of the mean.
Figure 2.4 (A) Hypersensitive response in a four o’clock leaf induced by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* strain BL-Cmm17 and wild-type C290. Bacterial suspensions (10⁸ CFU/ml) were injected into fully expanded leaves of four o’clock plants using needle-less syringes and HR reactions were recorded after 24 hours. (B) Virulence test on 4-week-old tomato seedlings. Tomato seedlings were monitored for wilting for 3 weeks after inoculation.
Figure 2.5  

(A) Bioluminescence and growth curves of *Clavibacter michiganensis* subsp. *michiganensis* wild-type C290 and BL-Cmm17 strains. Both strains were inoculated into NBY broth, and the optical density at 600 nm and photon emissions were measured from 4 h to 48 h. Relative Light Units (RLU) are log transformed values of the average radiance (photons/sec); the error bars indicate standard deviations.  

(B) Growth of BL-Cmm17 and C290 in *Cmm* minimal medium. The optical density at 600 nm and photon amounts were measured from 8 h to 58 h. Data points are means of three replicates with standard deviations.
Figure 2.6 (A) Bioluminescence of strain BL-Cmm17 inoculated onto tomato seed. Images were acquired daily by using an *in vivo* imaging system. Bacteria were observed on seed coats throughout the germination period and colonized other seedling components as they emerged. The number at the bottom of each image indicates the day of germination. The rainbow scale represents photon counts (photons/sec). (B) Population dynamics of bioluminescent BL-Cmm17 strain on tomato seed coats during germination. Bacterial counts were estimated from serial dilution plating and the total CFU were log transformed. Each point represents the mean bacterial count for 12 seeds (days 1-3) and seed coat (days 4-5) isolations; bars indicate standard deviation. (C) Distribution of bioluminescent BL-Cmm17 strain on tomato seedlings on the fifth day of germination. Each point represents the mean bacterial count (n=12); error bars indicate standard deviations. (D) Regression analysis of light intensity vs. populations of BL-Cmm17 strain on tomato seed coats during days 1, 2 and 3 days of germination.
Chapter 3

Colonization of tomato seedlings by bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* under different humidity regimes

**ABSTRACT**

Tomato bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), is transmitted by infected seeds and transplants. Mechanical wounds, which are easily made during greenhouse transplant production, facilitate the dissemination of the pathogen. However, little is known regarding translocation of *Cmm* in tomato seedlings through wound infection or the influence of environmental factors on *Cmm* growth as an endophyte. A virulent, stable, constitutively bioluminescent *Cmm* strain BL-Cmm 17 coupled with an *in vivo* imaging system (IVIS, Xenogen), a quantitative low-light device, allowed visualization of the *Cmm* colonization process in tomato seedlings in real-time.

The dynamics of bacterial infection in seedlings through wounds were compared under low (45%) and high (83%) relative humidity. Bacteria multiplied rapidly in cotyledon petioles remaining after clip inoculation and moved in the stem towards both root and shoot. The high humidity accelerated bacterial systemic movement in the stem.

Luminescent signals were also observed in tomato seedling roots over time. Root development was reduced in inoculated plants maintained under both humidity regimes.

A strong positive correlation between light intensity with bacterial population *in planta*
suggested that bioluminescent Cmm strains may have additional potential research applications, such as evaluating the efficacy of bactericides and resistant cultivars.

INTRODUCTION

Tomato bacterial canker, one of the most destructive bacterial diseases of tomato, causes serious economic losses worldwide (Strider, 1969; Gartemann et al., 2003). The pathogen, *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), is a rod-shaped, Gram-positive bacterium that belongs to the family *Microbacteriaceae* (Park et al., 1993). Yield loss from tomato canker is due to plant wilting and death, reduced photosynthetic capacity due to foliar lesions, fruit lesions (field) or netting (greenhouse), and reduced fruit set and size (ASTA, 2010). Severe epidemics can cause up to 80% yield loss (Strider, 1969).

The pathogen *Cmm* is seed-borne and infected seeds or transplants are considered the source of primary inoculum in a disease-free field or greenhouse (Strider, 1969; Gitaitis et al., 1991). Occurrence of tomato canker in a production greenhouse can be an economic disaster due to the difficulty of eradication of the pathogen, additional cost of sanitation and yield reduction. Economic loss caused by bacterial canker is as high as 25-30% in greenhouse tomato production nationally (M. Bledsoe, personal communication). The management of tomato bacterial canker is primarily dependent on maintaining pathogen-free seed and transplants, since commercial tomato cultivars highly resistant to canker and effective foliar or root-applied bactericides are not available (Jahr et al., 1999). Although tomato seed is routinely tested for *Cmm* and production of healthy transplants
is an industry priority, epidemics of tomato canker continue to occur. For example, a severe field outbreak of tomato canker in the US Midwest and Ontario in 1984 was initiated with certified transplants produced in Georgia (Gitaitis et al., 1991). Several outbreaks of bacterial canker in tomato propagation and production greenhouses in the US, Canada, Mexico and Guatemala in the last decade have caused serious losses (S. Miller, personal communication).

For both fresh market and processing field tomato production, seedlings are usually produced in a greenhouse for 4-6 weeks before transplanting. Seeds infected with Cmm give rise to symptomless seedlings, which may be latent infected or carrying the pathogen epiphytically (Tsiantos, 1987; Gitaitis et al., 1991; Shirakawa et al., 1991). An early study showed that seedlings grown from artificially-infested seed were contaminated with $10^4$-$10^8$ CFU/seedling (Tsiantos, 1987). Seedlings grown from naturally infected seed can also carry $10^3$-$10^4$ CFU/g of the pathogen in shoots (Shirakawa et al., 1991). In greenhouse transplant production, as the density of seedlings is very high, epiphytic Cmm are easily spread through irrigation and leaf-to-leaf contact. It is known that Cmm is a good endophyte and moves into the xylem after its entrance through wounds or natural openings (Carlton et al., 1998; Gartemann et al., 2003). Mechanical wounds, which are easily made during clipping, harvesting, shipping and transplanting, promote the dissemination of Cmm directly into vascular tissue (Chang et al., 1991). In addition, environmental conditions in seedling production greenhouses favor bacterial infection. It has been reported that optimum conditions for tomato bacterial canker symptom development are warm (23-28°C) temperature and high relative humidity (greater than
80%) (Basu, 1966). Previous studies focused on Cnm spread by cultural practices and the development of symptoms; however, few studies have addressed Cnm movement in planta during latent infection and the influence of environmental conditions on Cnm growth as an endophyte in tomato transplants. We propose that systemic infection by Cnm develops rapidly in young tomato plants, within days after the pathogen is introduced via wounding, and that Cnm endophytic growth and translocation are influenced by humidity.

To facilitate monitoring the dynamics of Cnm infection in tomato seedlings, we have constructed bioluminescent Cnm strains by transformation of a lux-operon originally from Photobacterium luminescens into the Cnm genome (Xu et al., 2010; Chapter 2). The natural light-emitting reactions encoded by the luxCDABE genes have also been transferred to other phytopathogenic Xanthomonas comperstris pvs. and Pseudomonas spp. to track bacterial movement and distribution in host plants (Cirvilleri and Lindow, 1994; Cirvilleri et al., 2000; Paynter et al., 2006; Shaw et al., 1992), and to assess host susceptibility quantitatively (Fukui et al., 1996). In particular, lux-marked Ralstonia solanacearum has been used to study bacterial translocation in single or double grafted tomatoes with combinations of resistant and susceptible cultivars (Hikichi et al., 1999).

In this study, we used a bioluminescent Cnm strain coupled with a sensitive imaging system to successively monitor bacterial systemic infection of tomato seedlings under different humidity regimes in real time. A better understanding of the biology of Cnm infection of seedlings will be useful in developing sampling methods and strategies to control this disease.
MATERIALS AND METHODS

**Bacterial strain and growth conditions.** Strain BL-Cmm17, which is virulent, stable, and constitutively bioluminescent, was selected for this study (Xu et al., 2010; Chapter 2). BL-Cmm17 was cultured from glycerol stock maintained at -80°C. Bacteria were grown on NBY medium (nutrient broth at 8 g/L, yeast extract at 5 g/L, K2PO4 at 2 g/L, KH2PO4 at 0.5 g/l, glucose at 5 g/L, MgSO4 7H2O at 0.5 g/L, agar 15 g/L; Vidaver, 1967) containing chloramphenicol at 10μg/ml at 28°C. The strain cultured on NBY agar medium was imaged with 30 seconds exposure time to test luminescence using an *in vivo* imaging system (IVIS, Xenogen Corporation, Alameda, CA).

**Tomato seedling inoculation and incubation under different relative humidity regimes.** Tomato seeds (cv. OH9242) were sown in Fafard superfine germination soil mix (Conrad Fafard Inc., Agawam, MA) in 50-well trays and maintained in the greenhouse. General fertilizer (Peters 20-20-20, J.R. Peters Inc., Allentown, PA) was applied weekly. Tomato seedlings were inoculated by clipping the cotyledons with *Cmm*-contaminated scissors when they were 4 weeks old. Briefly, cultures of BL-Cmm17 were collected from NBY plates after incubation at 28°C for 4 days and suspended in sterilized water to an optical density (OD) of 0.25 (ca. 3.6×10^8 CFU/ml). Petioles of both cotyledons were cut using a pair of sterilized dissecting scissors that had been dipped in the bacterial suspension. In total, 30 seedlings were inoculated with BL-Cmm17 and 30 seedlings were controls cut with scissors dipped in sterilized water. After cutting the cotyledons, all seedlings were placed in a growth chamber (Conviron Model...
EF7 (Conviron company; Manitoba, Canada)) with high relative humidity (80% RH) at 25 °C for 6 hours. Half of the inoculated (n=15) and control seedlings (n=15) were then transferred to a low humidity growth chamber. Both chambers were set up with a 12 hr photoperiod, 20°C/25°C dark/light. HOBO U14 LCD loggers (Onset Computer Corporation, Bourne, MA) placed in each growth chamber showed similar temperature data for both chambers, whereas the RH was 83±5% (high RH chamber) and 45±12% (low RH chambers).

**Bioluminescent imaging of BL-Cmm17 colonization of tomato seedlings.** Tomato seedlings were imaged to monitor the movement of bacteria every 5 days post inoculation (DPI) using the IVIS for four time points (5, 10, 15, and 20 DPI). Tomato seedlings were placed in the image chamber, which is light tight with a sample stage temperature of 25°C. Due to the high sensitivity of the IVIS thermoelectrically cooled CCD camera, plant phosphorescence was detected immediately after the plants were placed in the image chamber, resulting in strong background. A preliminary study in which plants were placed in the dark on the sample stage and imaged with 3 minutes of exposure time every 5 minutes with dark incubation of 20 minutes for 4 images, indicated that the background phosphorescence was quenched after 10 minutes in the dark. Therefore all images were taken after 10 minutes of dark incubation. Images of individual leaves were taken 20 days post inoculation. Tomato seedlings roots were also imaged every 5 days post inoculation. Tomato roots were washed in water gently to remove the attached soil and dried at room temperature for 5 minutes before taking bioluminescent images. Incubation in the dark was not necessary for roots, which lacked background
phosphorescence. All images were saved and analyzed using the Living Image software (Xenogen) at a PC workstation. Total flux (photons/s) from the region 1 cm above the cotyledons (the tissue sampled for isolation) was measured using Living Image software to determine the relationship of light intensity with bioluminescent bacterial populations in stems.

Quantification of BL-Cmm17 in planta and imprinting of stem tissue. Three petioles were randomly sampled immediately after clipping and ground in a 1.2 ml library tube with 0.6 ml potassium phosphate buffer (pH=7.4, 10mM). Ten-fold serial dilutions of each sample were made and 0.1 ml aliquots were plated on NBY containing chloramphenicol at 10 μg/ml. After each bioluminescent imaging (5, 10, 15 and 20 DPI), three seedlings were selected at random from low and high RH for Cmm enumeration. Each tomato compound leaf was sampled separately using sterilized forceps and surface sterilized by spraying with 70% ethanol and wiped with tissue paper. Stem samples were taken from the inoculation point to 1 cm above using a sterilized scalpel. Leaf and stem samples were weighed and ground in whirl-pack sample bags (VWR International LLC., West Chester, PA) with 3 ml potassium phosphate buffer using a roller/tissue homogenizer (Agdia Inc., Elkhart, IN). Root samples were taken for bacterial isolation after imaging as described above. Roots were weighed and surface sterilized by dipping quickly in 70% ethanol. Roots were ground in mesh extraction bags (Agdia Inc.) with 5 ml potassium phosphate buffer. The supernatant was serially diluted and 0.1 ml of each diluted sample was plated onto NBY agar medium supplemented with chloramphenicol (10μg/ml). For root samples, serial dilutions were spread onto an improved Cmm semi-
selective medium D2ANX (Alveraz, et al. 2005). Colonies were checked for bioluminescence using the IVIS and counted on the fourth (on NBY) or seventh day (on D2ANX) after plating. The number of bacteria present in tomato tissue (CFU/g) was calculated as (the number of colonies * dilution factor * 3 or 5 ml) / fresh weight of sample tissue. Bioluminescence imaging and isolation from seedlings leaves and stems was done twice, and root imaging and isolation was done once.

The movement of the bioluminescent bacteria up and down the stem from the inoculated cotyledons was determined by stem tissue imprinting (Gitaitis, et al., 1991). Three of the tomato seedlings that were sampled for leaf isolation were tested by stem tissue imprinting. The stem was cut every centimeter above, below and including the cotyledon node using a sterilized scalpel and the cut surface was imprinted on D2ANX medium in a square plate (10 ×10 cm with 13 mm grid). The colonies on the plates were imaged using the IVIS after 7 days of incubation at 28°C. This experiment was done twice.

**Data analysis.** Bacterial population data (CFU/g) in planta were log transformed (log CFU/g). Data for leaf and stem bacterial populations from two experimental runs were pooled. Comparison of fresh weight of roots, and bacterial populations in leaves, stems and roots were subjected to analysis with SAS version 9.1 (SAS Institute, Cary, NC.) using a MIXED model; means were separated by an LSD test at the 0.05 confidence level. Regression analysis of light intensity versus bacterial population in stems was performed by using Minitab/Regression/Fitted Line plot.
RESULTS

Bioluminescent *Clavibacter michiganensis subsp. michiganensis* colonization of tomato seedlings. Representative images of bioluminescent *Cmm* colonization of tomato seedlings under high and low relative humidity (RH) are presented in Figure 3.1. The inoculum load on the cotyledon petioles was log 3.75±0.40 CFU/wound after clipping cotyledons with contaminated dissecting scissors. At 5 days post inoculation (DPI), strong luminescence appeared in the cotyledon petioles, indicating rapid multiplication of BL-Cmm 17, and populations reached log 7.23±0.20 and 7.43±0.15 in low and high RH, respectively. At this time, no significant difference was found in bacterial populations in cotyledon petioles of seedlings kept under different humidity regimes. The first and second true leaves were infected with low titers of bacteria under low RH (Table 3.1). In contrast, four leaves were infected under high RH. However, the titers were too low to be detected by the imaging system. The *Cmm* population in leaf 3 was significantly lower under low RH than under high RH. Luminescence signals observed in stems indicated the initiation of stem colonization. Bacterial populations in stems sampled 1 cm above the cotyledon node were not significantly different and reached log 7.37±0.30 and log 7.60±0.40 in low and high RH, respectively (Figure 3.2).

Obvious luminescence signals in stems were observed at 10 DPI in seedlings maintained in both low and high RH. The bacteria propagated in the stem quickly and moved towards both roots and shoots. The petioles of cotyledons wilted and fell from the seedlings prior to imaging at 10 DPI. Light signals were detected in the petioles of the first and second true leaves at 10 DPI in tomato seedlings under high RH (Figure 3.1).
Isolation of bacteria from leaves showed that all leaves were infected in seedlings under both low and high RH without significant differences in \textit{Cmm} populations between humidity regimes (Table 3.1). However, there was a dramatic increase in \textit{Cmm} populations in all leaves at 10 DPI compared to 5 DPI.

At 15 DPI, bioluminescence was observed in lower leaves of tomato seedlings under low RH and in all leaves of seedlings under high RH. The first true leaf of seedlings maintained under high RH wilted slightly within 15 DPI; leaves of seedlings under both low and high humidity were obviously wilted by 20 DPI. Significantly higher \textit{Cmm} populations were detected in leaves 1, 5 and 6 at 15 DPI (Table 3.1) from seedlings maintained under high RH than from those under low RH. Light signals were observed throughout the entire seedling under both RH regimes at 20 DPI. Higher amounts of light were emitted from the nodes in stems than from the internodes. In individual leaves, the strongest signals appeared in the petiole nearest the main stem at 20 DPI (Figure 3.3). Additionally, \textit{Cmm} moved along the rachis of tomato leaves.

The comparative increase of bacterial population in stems under high and low RH is shown in Figure 3.2. There was no significant difference in the average log CFU/g in stems of plants maintained under low and high RH over time. At 20 DPI, the bacterial population in stems under both low and high RH regimes reached log 10.89 ±0.38 and log 11.08 ±0.42 in low and high RH, respectively.
**Monitoring bioluminescent Clavibacter michiganensis subsp. michiganensis movement in stems by tissue imprinting.** A representative image of BL-Cmm17 movement in stems determined by tissue imprinting is shown in Figure 3.4. Bioluminescent Cmm was detected at 5 DPI up to 3 or 4 cm above the cotyledons in seedlings held under low or high RH, and 4 cm below the cotyledons in both (Table 3.2). Similar results were obtained when the experiment was repeated (data not shown). In general, the translocation of bacteria in stems was more rapid in seedlings under high RH than in seedlings under low RH, and bacteria moved faster towards roots than shoots under both humidity regimes. In addition, bacteria were detected in the top of the stems but not in the middle in one seedling under high RH at 10 DPI. The absence of bacteria in the middle stem was also observed in two more samples under both low and high RH regimes in the repeat experiment (data not shown).

**Bioluminescent Clavibacter michiganensis subsp. michiganensis colonization of roots.** Tomato roots were also colonized when seedlings were inoculated by clipping the cotyledons (Figure 3.5). Very few signals were found at the juncture of roots and stems at 5 DPI, when obvious signals were observed in the tap roots at 10 DPI. Luminescent signals continued to increase in roots at 15 DPI and strong signals in lateral roots were observed at 20 DPI. Bacterial populations in roots at 5 DPI reached log 5.09±0.04 and 5.45±0.07 CFU/g under low and high RH, respectively (Figure 3.6 A). The Cmm populations reached over log 8 CFU/g at 10 DPI. In general, there were no significant differences in Cmm populations in roots of seedlings under low and high RH. No bioluminescence was observed in and no bioluminescent bacteria were isolated from
roots of water-inoculated control seedlings. The fresh weight of roots from inoculated seedlings was significantly reduced at 20 DPI compared to the water inoculated control under high RH (Figure 3.6 B).

**Relationship between bioluminescence and bacterial population in planta.**

Regression analysis showed a significant positive relationship between bioluminescent light intensity and bacterial population in stems (P<0.0001, r=0.94), indicating that stronger light signals were corresponding with higher bacterial populations in planta.

**DISCUSSION**

This is the first study in which *Cmm* colonization of tomato plants has been visualized in planta in real-time. The bioluminescent strain BL-Cmm17, with *lux*-genes encoding enzymes for light production, was detected in plants using a highly sensitive CCD camera, which allowed noninvasive monitoring of this vascular pathogen in individual plants over a period of 20 DPI. Green fluorescent protein (GFP) is much more commonly used as a marker in the study of plant-pathogen interactions than bioluminescence. Previously, Chalupowicz et al. (2010) constructed a GFP-labeled *Cmm* strain and used it to study *Cmm* colonization of the xylem, as well as *Cmm* epiphytic fitness and biofilm formation on leaves. However, GFP was carried on a plasmid that was not stable in planta for more than one month. Additional problems with the use of GFP are that fluorescence imaging requires UV excitation light and dissection of plant tissues. In contrast, the chromosome insertion of *lux*-operon in *Cmm* strain was stable and bioluminescence imaging allows visualization of bacteria in planta without tissue dissection or excitation light. Therefore,
bioluminescence imaging is a more suitable approach than fluorescence imaging for real-time, non-invasive evaluation of Cmm colonization and latent infection as described in this study.

Most of the previous studies of bioluminescence imaging to monitor lux-marked bacterial infection in plant hosts involved exposure of the inoculated leaf, stem or root to X-ray film (Kaumon and Kado, 1990; De Weger et al., 1991; Fukui et al., 1996; Cirvilleri et al., 2000). Although the use of X-ray film is inexpensive, this indirect imaging technique is relatively insensitive and time-consuming. Only a few studies have demonstrated luminescence directly in infected plants using low-light detecting cameras. This was done by taking two photos, one with light to show the plant tissue and another in the dark to detect the emitted light and locate the bioluminescent pathogen (Shaw et al., 1992; Chabot et al., 1996). The imaging system (IVIS, Xenogen) used in this study takes two images with a highly sensitive thermoelectrically cooled (-90°C) CCD camera capable of detecting 100 photons/s/cm². In addition, an overlay image of the object in light and luminescence in dark is generated automatically by the Living Image® software and light intensity is represented by pseudocolor. The software enables quantitative analysis on light emission, which is very useful to correlate luminescence with the bacterial population in the host.

Although it is recognized that Cmm is a good endophyte that colonizes the vascular tissue of tomato, little is known regarding systemic movement and translocation of this pathogen in seedlings. An early study done by staining inoculated tomato tissues
demonstrated that \textit{Cmm} infects vascular elements of the leaf traces, moving into the main axis of the stem and then extensive movement occurred in spiral vessel elements of the primary xylem (Pine et al., 1954). Later microscopic observations, including a recent preliminary report, of both wild-type and GFP-labeled \textit{Cmm} strains further demonstrated that \textit{Cmm} attaches to spiral rings in protoxylem vessels at an early stage of infection and then colonizes the whole xylem vessels extensively at later stages of infection (Carlton et al., 1998; Chalupowicz et al., 2010). Pine and coworkers (1954) also used the cotyledon petiole clip inoculation method and reported that they found \textit{Cmm} up to 1.32 cm above and 3.25 cm below the inoculation point at 5 days after inoculation. Our results are consistent with their observation that bacteria move faster towards roots than shoots, although we found \textit{Cmm} up to 3 cm (low RH) or 4 cm (high RH) above and 4 cm below the inoculation point in seedlings at 5 DPI (Table 3.2). Interestingly, we noticed the accumulation of \textit{Cmm} in nodes, which might be due to a higher percentage of vascular tissue in this part of the plant than others, allowing for more extensive bacterial multiplication. Bacterial populations were high in roots at an early stage of infection, as well as in the newest leaf at late stages of infection. The rapid migration and accumulation of \textit{Cmm} in systemically infected seedlings suggests that if the tools used for pruning and grafting in greenhouse transplant production are contaminated \textit{Cmm} can quickly and easily spread the pathogen through wounds. Thorough sanitation during the grafting process is therefore necessary to prevent spread of bacterial canker. In addition, when screening for \textit{Cmm} latent infections in seedlings in the greenhouse, sampling of the nodes in stems and roots will increase the opportunity of successful detection of the pathogen.

81
In an early study of environmental conditions necessary for tomato canker symptom development by Basu (1966), tomato seedlings were inoculated by spraying a Cmm suspension on plants that were then maintained under different temperature and humidity conditions. Their results suggested that humidity plays a more important role in symptom development than temperature. Symptoms on leaves developed similarly under various temperatures (15-28°C), but high relative humidity (87-97%) aggravated the symptoms. In another study (Tsiantos, 1987), the percentage of diseased seedlings arising from artificially-infected seed was higher under high RH than low RH. High humidity generates free water on the leaf surface, thereby enhancing bacterial multiplication and spread. It is interesting that Cmm systemic movement in stems was also accelerated by high humidity in this study.

The mechanism of Cmm movement in tomato xylem is not clearly understood. The genus Clavibacter has no flagella and is non-motile (Davis et al., 1984). It is reasonable to propose that bacterial migration from root to shoot is facilitated by water transport in the xylem. However, we demonstrated downward translocation of Cmm in tomato stems under both RH regimes and accelerated movement under higher RH, proving that Cmm can also move against the direction of transpiration stream in the xylem. Microscopic observations have shown that Cmm degrades the walls of xylem vessels by extracellular enzymes, which leads to collapse of plant cells and creation of large intercellular spaces (Pine et al., 1954; Carlton et al., 1998; Jahr et al., 1999; Gartemann et al., 2003). It appears that formation of bacterial pockets facilitates intercellular expansion (Pine et al.
Xylella fastidiosa, which is xylem-limited, migrates against the transpiration stream through a type IV pilus-driven twitching motility (Meng et al., 2005). Twitching motility is also found in the tomato wilt pathogen Ralstonia solanacearum, and is required for full virulence (Liu et al., 2001). Twitching motility appears to be largely restricted to Gram-negative bacteria with only one report on Gram-positive bacterium Streptococcus sanguis (Henriksen and Henrichsen, 1975). Though putative pilin genes are found in the Cnm genome (Gartemann et al., 2008), whether these genes are involved in motility is unknown. Further study on the motility of Cnm in planta is needed to reveal the mechanisms of environmental influences on Cnm systemic colonization.

The intensity of light produced by bioluminescent Cnm in infected tomato tissues was highly correlated with bacterial populations in planta. As luciferase activity depends on the metabolic integrity of the cell, the amount of photons emitted correlates with biomass of living bacteria (Fan et al., 2008; Paynter et al., 2006). The sensitivity of the current system is about $10^7$ CFU Cnm/g fresh tissue, which is less than reported in other host-pathogen systems with lux-tagged Gram-negative bacteria. For example, bioluminescent P. syringae was detected at ca. $10^4$ CFU/ml of bean leaf or $10^4$ CFU/leaf disc of Arabidopsis thaliana (Paynter et al., 2006; Fan et al., 2008). In this case, light measurements by luminometry of inoculated leaf discs or ground tissue in buffer may have increased the detection limit of bioluminescent bacteria or the lux-genes are expressed more efficiently in Gram-negative than Gram-positive bacteria. Since the AT-rich (>69%) lux-operon is originally from Gram-negative P. luminescens, low expression in high-GC (>70%), Gram-positive Cnm would not be surprising. Future work on
optimizing expression of the lux-genes in Cmm may improve the limit of detection.

Microscopic observation of naturally bioluminescent bacterial cells or lux-labeled phytopathogenic bacteria in plant tissues has been shown using appropriate equipment (Sternberg et al., 1997; Hikichi et al., 1998). Thus, future visualization of bioluminescent Cmm in plant cells at the microscopic level together with successive observation of pathogen growth and temporal spread in vivo will accelerate our understanding of Cmm-tomato interactions. Currently there are no tomato varieties resistant to Cmm and bactericides are ineffective once the pathogen has entered the plant. Bioluminescent Cmm strains coupled with a sensitive imaging system, as demonstrated in this study, may prove to be very useful in developing and/or screening disease management tools such as canker-resistant cultivars and effective bactericides.
REFERENCES


Table 3.1 Comparison of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato compound leaves under low (45%) and high (83%) relative humidity (RH) regimes.

<table>
<thead>
<tr>
<th>Leaf Position</th>
<th>5 DPI Y</th>
<th>10 DPI</th>
<th>15 DPI</th>
<th>20 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low RH</td>
<td>High RH</td>
<td>Low RH</td>
<td>High RH</td>
</tr>
<tr>
<td>Leaf 7</td>
<td>NA Z</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Leaf 6</td>
<td>NA</td>
<td>NA</td>
<td>1.98±1.73 b</td>
<td>6.55±0.62 a</td>
</tr>
<tr>
<td>Leaf 5</td>
<td>0 a</td>
<td>0 a</td>
<td>3.68±0.91 a</td>
<td>3.22±2.04 a</td>
</tr>
<tr>
<td>Leaf 4</td>
<td>0 a</td>
<td>0.84±1.88 a</td>
<td>4.95±1.94 a</td>
<td>4.83±1.34 a</td>
</tr>
<tr>
<td>Leaf 3</td>
<td>0 b</td>
<td>1.35±1.66 a</td>
<td>5.42±2.37 a</td>
<td>5.25±1.94 a</td>
</tr>
<tr>
<td>Leaf 2</td>
<td>1.88±2.16 a</td>
<td>2.57±1.84 a</td>
<td>7.25±1.13 a</td>
<td>7.71±0.43 a</td>
</tr>
<tr>
<td>Leaf 1</td>
<td>2.93±1.66 a</td>
<td>3.89±0.56 a</td>
<td>7.99±0.55 a</td>
<td>8.50±0.24 a</td>
</tr>
</tbody>
</table>

X Leaf position listed from the newest leaf (leaf 7) to oldest leaf (leaf 1).

Y Bacterial populations (log CFU/g) were compared in same leaf position between low and high RH 5, 10, 15 and 20 days post inoculation (DPI). Means (n=6) followed by the same letter were not significantly different at P<0.05.

Z NA, not applicable as the leaf had not yet been produced at the indicated sampling time.
Table 3.2 Comparison of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 movement in tomato stems under low (45%) and high (83%) relative humidity (RH) regimes by tissue imprinting.

<table>
<thead>
<tr>
<th>Distance (cm) from inoculated cotyledons in stem</th>
<th>5 DPI</th>
<th>10 DPI</th>
<th>15 DPI</th>
<th>20 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low RH</td>
<td>High RH</td>
<td>Low RH</td>
<td>High RH</td>
</tr>
<tr>
<td>Above cotyledon node</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td>+++</td>
<td>+++</td>
<td>x -</td>
<td>- + x</td>
</tr>
<tr>
<td>5</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>+ -</td>
</tr>
<tr>
<td>4</td>
<td>+ -</td>
<td>+++</td>
<td>++ -</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Cotyledon node</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>-1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>-2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>-3</td>
<td>++ -</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>-4</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Three plants were sampled 5, 10, 15 and 20 days post inoculation (DPI). The symbol “+” indicates the presence of BL-Cmm17 in each sample by culturing on D2ANX semi-selective medium and identification by luminescence imaging, “-” = not present, “×” = data not available because the seedling had not reached that height at the indicated sampling time.
Figure 3.1 Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato seedlings under low (45%) and high (83%) relative humidity (RH) regimes in real time.

Images of the same seedlings were acquired 5, 10, 15 and 20 days post inoculation (DPI, indicated at bottom of the images) using an *in vivo* imaging system (IVIS, Xenogen). The upper images are of a seedling held under low relative humidity and the lower images are of a seedling held under high relative humidity. Arrows point to the strong luminescence signals at nodes. The rainbow scale represents light intensity.
Figure 3.2 Comparison of bacterial populations (log CFU/g) of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 recovered from stems of tomato seedlings under low (45%) and high (83%) relative humidity (RH) regimes 5, 10, 15 and 20 days post inoculation (DPI). Each point represents the mean of bacterial populations (n=6), bars indicate the standard deviations.
Figure 3.3 Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato leaves from a tomato seedling under high relative humidity (83%) 20 days post inoculation. Numbers at the top from left to right indicates the leaf position from the youngest (7) to the oldest leaf (1). Images of leaves 1-3 and 5-7 were taken on the adaxial surface; the image of leaf 4 was taken on the abaxial surface. The rainbow scale represents light intensity.
Figure 3.4 Recovery and identification of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 from tomato stem tissue imprinted on D2ANX semi-selective medium and visualized using an *in vivo* imaging system (IVIS, Xenogen). This image shows a representative plate of stem tissue imprints from three tomato seedlings under high relative humidity (83%) at 10 days after inoculation. Each square within the grid was imprinted with one cut section of stem tissue above and below the cotyledon node. Numbers in the grid squares indicate the distance (cm) from the cotyledon nodes (>0 is above cotyledons, <0 is below). Columns a and b, c and d, and e and f were imprints from three samples.
Figure 3.5 Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* bioluminescent strain BL-Cmm17 colonization of tomato seedling roots under low (45%) and high (83%) relative humidity (RH) regimes. Images were acquired at four time points (5, 10, 15 and 20 days post inoculation (DPI, indicated at the bottom of images) using the *in vivo* imaging system (IVIS, Xenogen). The upper images were roots of seedlings under low relative humidity and the lower images were under high relative humidity. The rainbow scale represents light intensity.
Figure 3.6 Comparison of populations of *Clavibacter michiganensis* subsp. *michiganensis* recovered from roots (A) and fresh weight of roots (B) under low (45%) and high (83%) relative humidity (RH) regimes. CK represents the root weight of the water-inoculated control under high relative humidity. Each bar represents the mean (n=3) of bacterial population (log CFU/g) or fresh weight (g) and error bars indicate standard deviations.
Figure 3.7 Regression analysis of light intensity versus population of bioluminescent *Clavibacter michiganensis* subsp. *michiganensis* strain BL-Cmm17 recovered from tomato seeding stems. Data points were bacterial populations and light intensities from 1 cm above the cotyledons in stems (n=20).
Chapter 4
Efficacy of Sanitizing and Biological Seed Treatments to Control Bacterial Canker Tomato

ABSTRACT

Seed treatment is the most effective and economical means of preventing seed to seedling transmission of Clavibacter michiganensis subsp. michiganensis, the pathogen causing tomato bacterial canker. In this study, the effects of seed sanitation treatments combined with post-sanitation applications of the beneficial bacteria Bacillus subtilis and Pseudomonas fluorescens were evaluated. Seed sanitation treatments eliminated the pathogen from seed and reduced disease incidence in seedlings in laboratory and greenhouse grow-out tests. However, beneficial bacteria applied after seed sanitation treatments did not reduce disease incidence or influence seedling growth or establishment. Among all sanitation treatments, the commercial disinfectants Kleengrow and Virkon most effectively killed Cmm from seed and reduced pathogen populations in greenhouse seedlings. The plant essential oil thymol was phytotoxic at the concentration evaluated, increasing the rate of production of abnormal seedlings. Seed vigor analysis, determined using the Seed Vigor Imaging System (SVIS), suggested that thymol, kasugamycin and dry heat treatments decreased seed vigor over long-term storage.
INTRODUCTION

Since first reported in Grand Rapids, Michigan in 1909, tomato bacterial canker has become one of the most serious tomato diseases worldwide. Tomato bacterial canker can cause up to 80% yield loss due to plant wilting and death, reduced photosynthetic capacity, fruit lesions (field) or netting (greenhouse), and reduced fruit set and size (Strider, 1969; EPPO, 2005; ASTA, 2010). The pathogen *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is an aerobic, rod-shaped, Gram-positive bacterium. Although *Cmm* can be spread by irrigation or propagation practices, infected seeds and transplants are considered the primary inoculum source (Strider, 1969; Chang et al., 1991; Fatmi et al., 1991). The pathogen can be present both externally and internally in tomato seed. Direct deposition of *Cmm* on the seed coat may occur during seed harvesting (EPPO, 2005), while internal invasion of *Cmm* in seed might be through the vascular bundle of fruit (Bryan, 1930; Uematsu et al., 1977). Early research showed that naturally infested seed resulted from 1 to 5% of the diseased plants in the greenhouse, whereas artificially inoculated seed had a higher rate (21 to 40%) of infection (Bryan, 1930). However, even 0.01% transmission from seed to seedling can initiate a disease epidemic under favorable conditions (Chang et al., 1991). Therefore, seed must be the first point of focus in developing an integrated program to manage this disease.

Seed treatments to reduce or eliminate populations of bacterial pathogens in seed have been developed over many years. These include both chemical and heat treatments. Hot water treatment, performed using standard protocols, is effective in
eliminating bacterial pathogens from tomato seed, but seed germination may be delayed for some seed lots (Ivey and Miller, 2005). Traditional chemical treatments may present a lower risk of seed damage than heat treatments, but may not kill Cmm under the seed coat (Dhanvantari, 1989; Dhanvantari and Brown, 1993).

Chemical disinfectants are widely used for plant disease management in greenhouses and some of them have been tested for seed sanitation. For example, seed treated with Virkon (potassium peroxymonosulfate) reduced the severity of bacterial leaf spot caused by *Xanthomonas campestris pv. vitians* in lettuce without influencing germination (Carisse et al., 2000). Kleengrow (didecyl dimethyl ammonium chloride) is another commercialized disinfectant with wide spectrum fungicidal and bactericidal properties. A recent report showed that foliar application of Kleengrow in a greenhouse significantly reduced leaf spots and incidence of dead plants caused by *Cmm* in tomato (Ingram et al., 2009). Other chemicals potentially useful for seed sanitation are the antibiotics streptomycin and kasugamycin, since both reduced tomato canker disease development in greenhouse and field studies (Hausbeck et al., 2000; Miller et al., 2007). Thymol, which is a plant essential oil extracted from species of *Origanum* and *Thymus*, inhibited *Cmm* growth *in vitro* (Daferera et al., 2003). However, thymol, antibiotics and most chemical disinfectants with potential to prevent *Cmm* seed to seedling transmission have not been tested for this purpose.
Seed sanitation treatments not only eliminate pathogens, but also largely reduce populations of saprophytic microorganisms on seed (Fatmi et al., 1991; Bülow et al., 1994). This may create a “biological vacuum” that could enhance the ability of beneficial bacteria, applied post-sanitation, to colonize and survive on seeds. Beneficial bacteria include plant growth-promoting rhizobacteria (PGPR), which promote plant health by production of allelochemicals inhibitory to a broad spectrum of pathogens and induce systemic resistance (ISR) in host plants against certain pathogens (Compant et al., 2005). *Bacillus* spp. elicit ISR in plants and produce antibiotics, including iturin and surfactin, to inhibit soil-born pathogens (McSpadden Gardener, 2004; Kloeper et al., 2004). Particularly, *Bacillus subtilis* prevented bacterial canker development in greenhouse tomato seedlings by foliar spray before artificial inoculation with *Cmm* (Utkhede and Koch, 2004). *Pseudomonas* species produce 2, 4-diacetylphloroglucinol (DAPG) that in low concentrations can suppress many oomycete and fungal plant pathogens (Raaijmakers et al., 2002; Isnansetyo et al., 2003). Furthermore, DAPG is an inducer of plant host defense (Iavicoli et al., 2003). Indeed, *Pseudomonas fluorescens* applied as a seed treatment reduced tomato bacterial canker incidence in the field as well as improved seed quality in lab tests (Umesha, 2006).

The current tomato seed treatments with traditional chemicals or hot water have shown flaws by either decreasing seed vigor or failing to eradicate the pathogen. The purpose of this study was to improve seed health through the combination of sanitizing treatments and post-sanitation applications of beneficial bacteria. We
hypothesized that the sanitizing treatments would effectively eliminate *Cmm* in seed without affecting seed vigor over long-term storage and the combination with beneficial bacteria might further suppress *Cmm* populations and enhance seedling growth.

**MATERIALS AND METHODS**

**Bacterial strains.** *Clavibacter michiganensis* subsp. *michiganensis* strains A300 and C290 (Francis et al., 2001) were used to produce naturally infected/infested seed and for *in vitro* testing. The beneficial bacterium *Pseudomonas fluorescens* strain Wayne 1R (McSpadden Gardener, et al. 2005) was provided by Dr. Brian B. McSpadden Gardener (Plant Pathology, OSU). *Bacillus subtilis* strain MBI 600 (Fiddaman and Rossall, 1994) is a commercial product (Subtilex; Becker Underwood Inc., Ames, IA). Strains of *Cmm* and *P. fluorescens* were cultured from glycerol stock maintained at -80 °C and *B. subtilis* was cultured from spores in powder formulation (5×10^{10}CFU/g) stored at 4°C.

**Source of seed infested with Clavibacter michiganensis subsp. michiganensis.**

**Source 1.** Tomato (*Solanum lycopersicum*) seedlings (4-6 week-old, mixed varieties) were inoculated 7 days prior to transplanting with a bacterial suspension of *Cmm* C290 (10^8 CFU/ml) using a Preval Spray Gun (Preval Sprayer Division, Yonkers, NY). Inoculated seedlings were transplanted to experimental field plots at the Ohio Agricultural Research and Development Center (OARDC), Wooster, OH and mature symptomatic fruits were collected in late September 2003. Seeds from infected fruits
were collected using an enzymatic seed extraction protocol described by Dhanvantari (1989). Briefly, fruits were cleaned with tap water and crushed with a tomato seed extractor (Department of Food, Agricultural and Engineering, The Ohio State University). Seeds with pulp were collected in plastic containers and incubated in warm water (ca. 30 °C) with pectinase (M. Ricker, Nunhems Seed Co., Parma, ID.) (0.25% v/v) for 2 hours. After incubation, seeds were pressure-washed three times with tap water and collected on sieves. Seeds were dried on an air bench for 2-3 days and stored in paper bags at 10°C before use.

Source 2. Tomato seedlings of the inbred line OH9242 (8 weeks old) were transplanted to two field plots at Snyder Farm, OARDC, Wooster, OH. Each plot consisted of 600 plants arranged in rows spaced 1ft apart with 5 ft between rows. The plants in one plot were inoculated with a bacterial suspension (mixture of Cmm C290 and A300, 10^8 CFU/ml) using a CO2-pressurized sprayer (20 psi) 6 weeks after transplanting. The plants in the second plot were not inoculated and used to collect fruit for clean seed. Weeds and fungal diseases were managed by applications of appropriate herbicides and manual weeding, as well as preventative fungicides on a 7-10 day schedule (Miller et al., 2007) in both plots. Mature fruits were collected and seeds were harvested using the enzymatic seed extraction protocol described above. The infestation frequency of seed by Cmm was determined by plating 100 seeds on an improved Cmm semi-selective medium D2ANX (Alvarez et al., 2005) for 7 days at 28°C. Colonies surrounding the seeds and resembling Cmm were tested by polymerase chain reaction (PCR) with Cmm-specific primers as described below.
The infestation level was approximately 50% and 20% in the 2003 and 2007 seed lots, respectively. Infested seeds from 2007 were diluted with clean seeds (1:1 w:w) to achieve a final infestation frequency of approximately 10% and stored in paper bags at 10°C before use.

**Source 3.** Artificially infested seed were inoculated by vacuum infiltration. Briefly, clean seed (40 g, harvested in 2007) were soaked in a bacterial suspension (mixture of *Cmm* strain C290 and A300, $10^8$CFU/ml) in a 500ml baker. The beaker was placed in a Nucerite dessicator (Nalge Sybron Corporation, Rochester, NY) and a vacuum was applied for 5 min using an Air Cadet pump (Barnant, Barrington, IL) with maximum -18 psi pressure. After inoculation, seeds were air dried and mixed with naturally infested seeds (400 g, harvested in 2007).

**Sensitivity of *Clavibacter michiganensis* subsp. *michiganensis* to antibiotics, thymol and beneficial bacteria in vitro.** Test concentrations of thymol (EMD Chemicals Inc., Gibbstown, NJ), dissolved in 70% ethanol, streptomycin (Agrimycin 17, Nufarm Americas Inc., Burr Ridge, IL) and kasugamycin (Kasumin, Arysta LifeScience North America LLC., Cary, NC), as well as ethanol (100 µg/L) (Table 4.1) were added individually to autoclaved, cooled (ca. 50° C) yeast dextrose calcium carbonate (YDC) agar medium. Strains of *Cmm* C290 and A300 were tested separately by plating the bacterial suspensions (100 µl at $10^3$ CFU/ml) on medium amended with one the test compounds. Three replications for each compound at each concentration were tested. Control cultures of *Cmm* A300 and C290 were plated as
described on non-amended medium. Cultures were incubated at 28°C and bacterial growth was assessed by the formation of colonies 3 days later.

Antagonism of *P. fluorescens* and *B. subtilis* against *Cmm* was determined by streaking each strain from a suspension of $10^8$CFU/ml on each side of the YDC plate that has been spread with 100 μl *Cmm* C290 or A300 suspension ($10^8$CFU/ml). Three replications for each *Cmm* strain were tested. Cultures were incubated at 28°C and inhibition of *Cmm* growth was determined 3 days later.

**Optimization of conditions for sanitizing seed treatments.** Seeds infested with *Cmm* harvested in 2003 were treated with sanitizing agents at different concentrations and exposure times (Table 4.2). Twenty-five seeds in each of three replicates were treated individually with 2 μl/seed of thymol (dissolved in 70% ethanol), streptomycin (dissolved in water), or kasugamycin (liquid formulation) at different concentrations and tested for infestation frequency by plating seed on D2ANX medium. Infested seeds (n=75) were treated with thymol at 5.0 μg/seed, streptomycin at 0.5 μg/seed or kasugamycin at 4.6 μg/seed and tested for germination by incubating them on filter paper moistened with sterilized water at 28°C for 5 days. Seed germination rate was calculated as the number of seed germinated/number of total seeds tested × 100%. To test dry heat treatment, three bags, each containing 1g of seeds, were loosely wrapped in cheesecloth bags and three bags of seeds were incubated in a Precision Thelco Oven Model 28 (Thermo Fisher Scientific Inc., Rockford, IL) with pre-warming at 50°C for 4 hour, then heating at 80°C for 24, 48
107

and 72 hours, respectively. Kleengrow (Pace49 Inc., Burnaby, NC, Canada) and Virkon (DuPont, Wilmington, DE) were diluted or dissolved in water at different concentrations (Table 4.2) in a volume of 500 ml in a 1 L beaker. Seeds (1g) were loosely wrapped in cheesecloth and soaked in the solutions for 30 min. After soaking, seeds were washed under tap water for 3 min, then air dried in a laminar hood for 8 hours. Seeds (100) were tested for infestation level immediately after treatment by direct plating on D2ANX medium and another 100 seeds were tested for germination as described above. The same number of untreated seeds was tested as a control for both infestation level and germination.

**Seed treatments.**

**Application of disinfectants.** In addition to Virkon and Kleengrow, disinfectant treatments included hydrochloric acid (VWR International LLC., West Chester, PA) and sodium hypochlorite (Bleach, Cul-Mac Industries Inc., Wayne, MI). Treatment conditions for hydrochloric acid (0.6M, 1h) and sodium hypochlorite (0.6%, at 50°C for 15 min) were as described in previous studies (Dhanvantari, 1989; Dhanvantari and Brown, 1993). The *Cmm*-infested seeds (10 g) were wrapped loosely in a cheesecloth bag, soaked in 500 ml disinfectant solution for the time indicated in Table 4.3, then washed under tap water for 3 min. Seeds were dried in a laminar flow hood for 6-8 hours, then collected in clean Petri dishes for further treatment and evaluation.

**Application of antibiotics and thymol.** Streptomycin, kasugamycin and thymol were applied using a liquid seed treater, Hege 11 (Wintersteiger Inc., Salt Lake City, UT)
with a volume of 0.5 ml to 10 g seed at the various concentrations given in Table 4.3.

**Application of heat treatments.** The hot water treatment was applied by soaking seed as described previously (Miller and Ivey, 2005). Dry heat treatment included pre-warming at 50°C for 4 h and heating at 80°C for 24 h as described above with 10 g seed in cheesecloth bags.

**Application of beneficial bacteria.** Beneficial bacteria were applied after seed sanitizing treatments. *Pseudomonas fluorescens* (Wayne 1R) was inoculated into 100 ml Trypticase Soy Broth (TSB) and incubated at 28°C with shaking at 200 rpm for 18 hours. The bacteria were harvested by centrifugation at 10,377×g for 5 minutes, and then re-suspended in sterile distilled water. The concentration was adjusted to $2 \times 10^{10}$ CFU/ml. *Bacillus subtilis* MBI 600 spores (2.5 g Subtilex ($5 \times 10^{10}$CFU/g)) were suspended in 50 ml sterilized water for a final concentration of $2.5 \times 10^{9}$ CFU/ml. The beneficial bacteria suspensions were applied to seed with the Hege 11 seed treater in a volume of 0.5 ml per 10 g seeds.

Each treatment consisted of four replications of 10 g seeds each. Sanitizing treatments combined with beneficial bacteria were tested twice (Experiment 1 and 2) and the sanitizing treatments alone were tested three times in total (Experiment 1, 2 and 3). In Experiments 2 and 3, seeds were soaked directly in disinfectant solutions or hot water and stirred intermittently using glass rods while soaking. Other treatments were applied as described above. Seeds after treatments were stored in
seed envelopes (5.7×8.9 cm, Staples Inc., Framingham, MA), which were placed in plastic containers with desiccant beads at 10°C for long-term storage. Desiccant beads were replaced every 2 months or as needed.

**Efficacy of seed sanitation treatments in eliminating *Clavibacter michiganensis* subsp. *michiganensis* from seed.** Four replications of 108 randomly selected seeds per sanitizing treatment were plated directly onto the Cmm D2ANX semi-selective medium within 2 weeks after treatment. Colonies resembling Cmm recovered from individual seeds were tested by polymerase chain reaction (PCR) assay with primers Cmm5/6 as described below and the seed infestation frequency was calculated (number of infested seeds/number of total seeds tested).

Internal infection of Cmm in seeds after various treatments was tested by culturing the pathogen from germinated seeds. Briefly, 100 seeds per treatment were placed on filter paper moistened with sterilized water. Germinated seeds from each replicate treatment were collected individually 3 days later in 150 ml sterilized flasks and washed in 30 ml of potassium phosphate buffer (KPB, pH=7.4). The flasks were shaken at 100 rpm/min for 30 minutes at room temperature, then the washing buffer was transferred to 50 ml centrifuge tubes. After 10 minutes centrifugation at 10, 377 × g the supernatant was discarded and the pellet re-suspended in 1 ml KPB buffer. Ten-fold serial dilutions were prepared and 100 µl of each were plated on D2ANX agar. Putative Cmm colonies were tested by PCR with the Cmm5/6 primers as described below. The population of Cmm in seed (CFU/100 seed) was calculated as
the number of confirmed \( Cmm \) colonies \( \times \) dilution factor \( \times 10 \).

**Bacterial canker disease incidence in tomato seedlings and treatment effects on seedling growth.** Grow-out evaluations of seed treatments were conducted in a greenhouse with an automatic overhead irrigation system set to water three times daily with about 400 ml water per flat at each watering. The greenhouse temperature range was set to 25-30°C. A HOBO U14 LCD logger (Onset Computer Corporation, Bourne, MA) was placed in the greenhouse to record temperature and relative humidity hourly. The average temperature and relative humidity were 22±6°C and 64±25% in Experiment 1, 25±4°C and 63±11% in Experiment 2, 24±5°C and 50±20% in Experiment 3, respectively.

Seed was sown in 128-cell flats. Each treatment and the non-infested control consisted of four replications of 128 seeds each. The number of normal and abnormal seedlings that emerged 2 weeks after sowing was recorded. The number of the seedlings with water-soaked lesions, typical symptoms of \( Cmm \) infection, was determined 5 weeks after sowing; disease incidence was calculated as (the number of symptomatic seedlings/total number of seedlings) \( \times 100\% \). Ten randomly selected seedlings per replicate treatment were sampled for height and dry weight at 6 weeks after sowing.
Bio-enriched terminal dilution polymerase chain reaction (TDPCR) assessment of tomato seedling populations of *Clavibacter michiganensis* subsp. *michiganensis*.

Twenty-five cotyledons (one cotyledon/seedling) from symptomatic and randomly selected asymptomatic seedlings were sampled from each replicate treatment. Cotyledons were combined, weighed and placed in a plastic sample bag (Whirl-Pak sample bag, size: 15.2×22.9 cm, Nasco Co., Fort Atkinson, WI). Tissues were macerated by hand using a roller, then 30 ml KPB buffer was added to each sample, which was then shaken in a Pulsifier (Microbiology International, Frederick, MD) for 1 minute. The suspension was filtered through two layers of autoclaved cheesecloth into a 50 ml centrifuge tube and centrifuged at 10,377 ×g for 10 min. The supernatant was discarded, and the pellet re-suspended in 1 ml KPB buffer. The suspension was diluted 1:3 in NBY liquid medium (200 μl into 400 μl) and eight (1:3) serial dilutions in 1.2 ml sterile Library Tubes (VWR International LLC.) were prepared. The tubes were incubated with shaking at 200 rpm for 3 days at 28°C. Bio-enriched cultures were frozen at -20°C overnight. The PCR assay was then done using 1 μl thawed culture sample with *Cmm* specific primers Cmm5/6 as described below. The last (terminal) dilution that was positive by PCR for *Cmm* was recorded. If no dilution was positive by PCR, the result was recorded as 0.

**Evaluation of sensitivity and accuracy of bio-enriched terminal dilution polymerase chain reaction.** The frozen cotyledon extract from an untreated control sample was thawed and serially diluted 1:3 in water, then tested by PCR. The *Cmm* population in this sample was also assessed by serial dilution plating on D2ANX agar.
as described below. *Cmm* populations in randomly selected cotyledon extract samples (n=15, including at least two untreated controls; the rest were randomly selected) in Experiment 1 and Experiment 2, and all samples (n= 44) in Experiment 3 were enumerated by serial dilution plating (0.1ml) on D2ANX agar. Colonies resembling *Cmm* on D2ANX medium after 7 days incubation at 28°C were tested by *Cmm*-specific PCR as described below. Bacterial population from each cotyledon sample was calculated as the number of confirmed *Cmm* colonies × dilution factor.

**Polymerase chain reaction to detect Clavibacter michiganensis subsp. michiganensis.** Polymerase chain reaction (PCR) assays were carried out using *Cmm*-specific primers CMM5 (5’-GCG AAT AAG CCC ATA TCA A- 3’) and CMM6 (5’-CGT CAG GAG GTC GCT AAT A-3’) (Dreier et al., 1995). Amplification was performed in a total volume of 25 μl containing 12 μl GoTaq Green Master Mix (Promega, Madison, WI), 10 μl nuclease free water, 1 μl of each primer (1 μM) and 1 μl template. The PCR program was set for initial denaturation at 95°C for 3 min, 30 cycles of amplification at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, then final elongation at 72°C for 10 min. PCR was performed in a MJR PTC-100 thermocycler (MJ Research Inc., Waltham, MA). The PCR product (10 μl) was separated by agarose gel (1.2%) electrophoresis in 0.5X TBE buffer at 100 V for 50 minutes. Gels were stained in ethidium bromide (2 μl/ml) and visualized under UV light using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak Company, New Haven, CT).
Evaluation of seed vigor using the Seed Vigor Imaging System (SVIS). One hundred seeds per replicate treatment were placed on moistened blue blotter papers (14×23 cm, Anchor Paper company., St. Paul, MN) in a plastic box (15×23×4 cm, Model 600-C, Pioneer Packaging, Dixon, KY) and germinated in a growth chamber at 20°C/30°C, with 16/8 hour of light/dark (AOSA, 1987). Seedlings were scanned on the blotter paper 5 days later using an Epson GT-15000 scanner (Epson American Inc., Long Beach, CA) with a resolution of 150 dpi. Images were saved in jpeg format and analyzed using Seed Vigor Imaging System (SVIS) software (Sako et al., 2001). Seed vigor was assessed within a month after treatment and after 6, 12 and 18 months of post-treatment storage at 10°C in Experiment 1 and after 1, 6, and 12 months storage in Experiment 2.

Experimental design and statistical analysis. The greenhouse experiment for evaluation of the effect of seed treatments on control of bacterial canker and seedling establishment was a randomized block design with two factors: seed sanitation treatment (10 levels, including four disinfectants, two heat treatments, two antibiotics, one essential plant oil and an untreated control), and beneficial bacterial treatments (three levels, including *P. fluorescens*, *B. subtilis* and an untreated control). The full factorial experiment was conducted twice (Experiments 1 and 2). The experiment was conducted a third time (Experiment 3) with only the seed sanitation treatments.

Data were appropriately transformed to normalize variance before analysis: Bacterial populations on seeds (CFU/100 seeds) and in cotyledons (CFU/ml) were log
transformed; percentage data were square root transformed, and terminal dilution
PCR data were ranked. Data were analyzed using Statistical Analysis Software 9.1
(SAS institute, Cary, NC) MIXED model and means were separated by a LSD test at
a confidence level of 0.05. Regression analysis was performed by using a Fitted Line
plot in Minitab.

RESULTS

Sensitivity of *Clavibacter michiganensis* subsp. *michiganensis* to antibiotics,
beneficial bacteria and thymol *in vitro*. Growth of *Cmm* was fully inhibited by
thymol at 100 μg/ml, streptomycin at 0.1 μg/ml, and kasugamycin at 50 μg/ml *in vitro*
(Table 4.1). Both *P. fluorescens* and *B. subtilis* were antagonistic to *Cmm* on YDC
medium (Figure 4.1).

Optimization of conditions for sanitizing seed treatments. Untreated control seeds
were highly infested with *Cmm* at a frequency of 48% (Table 4.2). The pathogen was
eliminated from seed by thymol at 5 μg/seed, streptomycin at 0.5 μg/seed and
kasugamycin at 4.6 μg/seed. The seed germination rate after these treatments was at
least 90%. The dry heat treatment and disinfectants Kleengrow and Virkon all killed
*Cmm* on seed, except for Virkon at 0.5%. However, delayed germination (data not
shown) was observed in all dry heat treatments at 80°C for 24 and 48 h. The seed
germination rate was reduced to 16% with the longer incubation time of 72 h at 80°C.
Seedlings with shorter radicals were observed after soaking seed in Kleengrow at a
concentration of 1.12%. Optimal seed conditions selected for further evaluation were
thymol at 5 μg/seed, streptomycin at 0.5 μg/seed, kasugamycin at 4.6 μg/seed, 0.56% Kleengrow for 30 min, and 2% Virkon for 20 min. Although dry heat treatment at 80°C for 24 hours slowed seed germination, the germination rate was not reduced and therefore dry heat treatment was included in the study. As 1 g seed consists of approximately 300 seeds, the selected concentrations of streptomycin, kasugamycin and thymol applied to 1 g seed were converted by a multiplication factor of 300 (Table 4.3).

**Efficacy of seed sanitation treatments in eliminating* Clavibacter michiganensis* subsp. *michiganensis* from seed.** Untreated seeds were infested with *Cmm* at a frequency of 11.8% and a population of $10^5$ CFU/100 seeds in Experiment 1 (Table 4.4). Among all of the seed sanitation treatments, the disinfectants hydrochloric acid, sodium hypochlorite, Kleengrow and Virkon were most effective in killing *Cmm* both on and inside seed. Seed treatment with hot water, dry heat, streptomycin and thymol also reduced *Cmm* infestation compared with the untreated control, but the pathogen was not eliminated completely. The *Cmm* infestation frequency in untreated control was 5.2% in Experiment 2 and 16.3% in Experiment 3, with populations of approximately $10^4$ and $10^5$ CFU/100 seed, respectively. Results obtained in Experiments 2 and 3 were consistent with those of Experiment 1 with the exception of the hot water treatment. Although hot water did not eradicate the pathogen from seed in Experiment 1, it reduced the infestation frequency to 0% and the *Cmm* population to 0 CFU/100 seeds in Experiments 2 and 3.
Bacterial canker disease incidence in tomato seedlings and treatment effects on seedling growth. In Experiment 1, the main effect of beneficial bacteria (*B. subtilis* MBI 600 or *P. fluorescens* Wayne 1R) on incidence of *Cmm*-symptomatic seedlings and seedling vigor indicators (emergence rate, rate of abnormal seedlings, root and shoot growth) was not significant (Table 4.5). In addition, interactions between beneficial bacteria and sanitation treatments were not significant. Therefore, the main effects of seed sanitation treatments on disease development and seed vigor were compared by means across the beneficial bacteria.

Bacterial canker incidence reached 2.3% in the untreated control, with typical water-soaked lesions observed primarily on the cotyledons and first true leaves (Figure 4.2). All treatments reduced the incidence of symptomatic seedlings compared with the untreated control in Experiment 1 (Table 4.6). Hydrochloric acid, sodium hypochlorite, Kleengrow, Virkon, hot water and thymol decreased disease incidence to less than 1%. There were no symptomatic seedlings observed from seed treated with Virkon or Kleengrow. The thymol treatment significantly reduced the seedling emergence rate and increased the percentage of abnormal seedlings (Figure 4.2). The other treatments did not influence emergence of seed, seedling height or dry weight of shoots and roots.

Bacterial canker incidence was very low in tomato seedlings in Experiment 2. A low number of symptomatic seedlings were observed in one replication of the untreated control and one treatment with *P. fluorescens*; none were observed in the
other treatments (data not shown). Results of Experiment 3 with only the sanitation treatments were consistent with those of Experiment 1 (Table 4.7), except that hot water treatment effectively suppressed canker incidence in Experiment 3 but not Experiment 1. All treatments except kasugamycin significantly reduced disease incidence and no symptomatic seedlings were observed from seeds treated with hot water, hydrochloric acid, sodium hypochlorite, streptomycin, Kleengrow, or Virkon. There were no significant effects of seed treatments on emergence rate, dry weight of shoots and roots or shoot height. The thymol treatment resulted in a higher percentage of abnormal seedlings than all other treatments and the untreated control in Experiment 3 as in Experiment 1.

**Sensitivity and accuracy of bio-enriched terminal dilution PCR in evaluation of *Clavibacter michiganensis* subsp. *michiganensis* populations in seedlings.** A positive PCR result was obtained for an untreated control cotyledon sample with $10^6$ CFU Cmm/ml at the $3^{-2}$ dilution before bio-enrichment compared to the $3^{-5}$ dilution after bio-enrichment in NBY medium (Figure 4.3). Traditional serial dilution plating enabled detection of Cmm in 5/15, 3/15, and 7/44 of the samples in the three experiments, respectively. Bio-enriched TDPCR detected Cmm in 5/15, 4/15 and 12/44 samples in the same three experiments. In total, 15 samples with Cmm were detected by both methods and six more samples were detected by bio-enriched TDPCR than serial dilution plating. There was a strong positive regression ($P<0.001$) of Cmm populations determined by serial dilution versus the bio-enriched TDPCR value (Figure 4.4). The detection threshold of TDPCR was about $10^2$ CFU Cmm/ml.
in cotyledon extraction buffer.

**Effect of seed treatments on tomato seedling populations of *Clavibacter michiganensis subsp. michiganensis*** populations. The results of bio-enriched TDPCR in Experiment 1 and 3 are presented in box-plots (Figure 4.5). In Experiment 1, the main effects of seed sanitation treatment and beneficial bacteria on *Cmm* populations from tomato seedlings were both significant (P<0.05) and interactions were not significant (P=0.26). The median TDPCR value for the untreated control was higher than that the values for all sanitation treatments, and *Cmm* was not detected in thymol, Kleengrow and Virkon treatments (Figure 4.5 A). The TDPCR results for seed sanitation treatments combined with *B. subtilis* were similar to those of seed sanitation treatments alone. Additionally, *Cmm* was not detected from cotyledons from seeds treated with hydrochloric acid or sodium hypochlorite (Figure 4.5 B). In contrast, *Cmm* was not detected in any treatments combined with *P. fluorescens*, with the exception of the kasugamycin treatment (Figure 4.5 C). In Experiment 2, the *Cmm* population was low and the pathogen was only detected from cotyledons from one replication each of untreated control, the *P. fluorescens* treatment, and the kasugamycin treatment combined with *P. fluorescens* (data not shown). In Experiment 3, TDPCR results for the dry heat, kasugamycin and streptomycin treatments were consistent with those observed in Experiment 1(Figure 4.5 D). However, *Cmm* populations were detected from cotyledons in the thymol treatment, which was not observed in Experiment 1. The pathogen was not detected from cotyledons produced from seed treated with hydrochloric acid, hot water,
sodium chloride, Kleengrow or Virkon in Experiment 3.

**Effect of seed treatments on seed vigor.** The statistical analyses presented in Table 4.8 and 4.9 indicated that the main effect of seed treatment on seed vigor immediately after treatment and over long-term storage was significant, but beneficial bacteria did not influence seed vigor in either Experiment 1 or 2. The interaction of sanitation treatment and beneficial bacteria was not significant.

There were no significant differences in the vigor of seeds treated with hydrochloric acid, Kleengrow, Virkon, hot water, sodium hypochlorite, or streptomycin immediately after treatment and after long-term storage compared with the untreated, *Cmm*-infested control. However, the thymol, kasugamycin and the dry heat treatments reduced the seed vigor index significantly (Table 4.10). Reductions of seed vigor in these treatments were also observed during long-term storage 6, 12 and 18 months after the later treatments. In Experiment 2, seed vigor was significantly decreased after treatment with kasugamycin and dry heat. The influence of these two treatments on seed vigor was maintained after 6 and 12 month in storage (Table 4.11).

**DISCUSSION**

Seed treatment can be an effective and economic means to manage seed transmitted pathogens with minimal application of pesticides. In this study, the effects of seed sanitation treatments combined with beneficial bacteria were evaluated
for control of bacterial canker in tomato seedlings. Soaking seed in hot water, acid and disinfectants was effective in eliminating *Cmm* from seed and reducing *Cmm* populations in seedlings.

Results of *in vitro* tests of thymol, streptomycin and kasugamycin against *Cmm* were consistent with those of previous studies (Daferera et al., 2003; Theodoro et al., 2000). However, these compounds were relatively ineffective as *Cmm*-sanitizing seed treatments compared with other sanitation treatments. Particularly, thymol was phytotoxic to tomato seeds, resulting in increased production of abnormal seedlings. The phytotoxicity of thymol to weed seed was reported in a previous study (Kordali et al., 2008). In contrast, thymol applied as biofumigant against bacterial wilt in tomato caused by *Ralstonia solanacearum* increased yield compared to the untreated control under field conditions (Ji et al., 2005). Thus, the phytotoxic effect of thymol is likely related to the application method, dose, and age of the plant. The potential of thymol as a biofumigant to reduce *Cmm* in soil and plant debris should be investigated further in future studies.

The thermal death point for *Cmm* in pure culture varies between 50 to 56°C with different strains (Bryan, 1930; Wakimoto et al., 1969). It is intriguing that dry heat at 80°C for 24 hours was less effective than hot water at 50°C for 25 minutes in eliminating *Cmm* from tomato seeds. The same dry heat treatment did not eradicate *Pepino mosaic virus* (PepMV) from tomato seed completely (Córdoba-Sellés et al., 2007). The location of *Cmm* inside seed might be related to the failure of thermal
treatment. Bryan (1930) first demonstrated that Cmm exists beneath the hilum under the seed coat. *Clavibacter michiganensis* subsp. *michiganensis* is able to survive in naturally infected seed over months, even years (S. Miller, unpublished data). However, the mechanisms of *Cmm* survival inside seeds and resistance to heat stress are unknown. It is possible that bacteria do not exist inside seeds independently, but in combination with seed components that impart greater stability. Further, imbibition of water leads to rapid leakage, protein hydration and structural perturbation, particularly in membranes (Haigh and Barlow, 1987; Bewley, 1997). Consequently, the bacteria are challenged by both heat stress and physiological changes in seed, which may increase the efficiency of eliminating the pathogen. We observed incomplete elimination of *Cmm* from seed in hot water treatment in Experiment 1, but not in Experiments 2 and 3. The reduced efficacy of hot water treatment was probably the result of tight wrapping of the seeds in cheesecloth during soaking in the first experiment. Hot water treatment was more effective in eliminating *Cmm* from seed in Experiments 2 and 3, in which seeds were not wrapped but directly soaked and stirred in 50ºC water.

*Clavibacter michiganensis* subsp. *michiganensis* was not detected from tomato seed or 3-day-old germinated seeds in the disinfectant treatments including hydrochloric acid, sodium hypochlorite, Virkon and Kleengrow. However, in Experiment 1, low *Cmm* populations were detected from seedlings produced from seeds treated with hydrochloric acid and sodium hypochlorite. Previous studies have also shown that *Cmm* was not detected from seed with these treatments in lab tests,
but low canker incidences were found after seedlings were transplanted to the field (Dhanvantari, 1989; Dhanvantari and Brown, 1993). In contrast, no Cmm was detected in seedlings produced from seed treated with Virkon and Kleengrow in all three of our experiments. The effectiveness of soaking seed with these disinfectants might be enhanced by imbibition and permeability of the seed coat (Salanenka and Taylor, 2008). However, more work is needed to determine the mechanism of Cmm eradication from tomato seeds by these disinfectants and the feasibility of their use as a routine seed sanitation treatment.

Results of previous studies suggest that P. fluorescens applied as a seed treatment (Umesha, 2006) and B. subtilis as a foliar application (Utkhede and Koch, 2004) may be effective against Cmm. However, even though our tests showed that both bacteria were antagonistic towards Cmm in vitro, the combination of seed sanitation treatments with post treatments of B. subtilis and P. fluorescens did not reduce disease incidence or promote tomato seedling growth. Since colonization and antagonistic activity of B. subtilis and P. fluorescens are greatly influenced by soil temperature and matric potential (Schmidt et al., 2004), the results of this study may reflect unfavorable environmental conditions for the growth and survival of the beneficial bacteria, either while on seed or during or after seed germination. In addition, kasugamycin was inhibitory to P. fluorescens and B. subtilis, and streptomycin to B. subtilis in our in vitro tests (data not shown). Therefore it is likely that, when seeds treated with either antibiotic, colonization by these beneficial bacteria would be greatly reduced.
Seeds infested with *Cmm* often give rise to symptomless seedlings, which may have latent infections or carry the pathogen epiphytically (Tsiantos, 1987; Gitaitis et al., 1991; Shirakawa et al., 1991). Therefore, it is important to evaluate the efficacy of seed treatment in controlling *Cmm* populations in or on tomato seedlings. For samples with very low populations of *Cmm* but high populations of saprophytes, the more competitive saprophytes may out grow or form more colonies on non-selective culture media, so that conventional serial dilution plating on normal media may not detect slower-growing *Cmm*. In addition, serial dilution plating requires a large amount of medium and suspect colonies must be confirmed by PCR, which is both time- and labor-consuming. Bio-enriched TDPCR was generally as sensitive and accurate as serial dilution plating in assessing *Cmm* populations on seedlings, but also may be subject to competition between *Cmm* and saprophytes during bio-enrichment in NBY. This may be why *Cmm* was not detected in most sanitation treatments combined with *P. fluorescens* by bio-enriched TDPCR, except where the seeds were treated with both kasugamycin and *P. fluorescens*. Thus, our negative PCR results were probably due to the presence of *P. fluorescens*, which grows faster than *Cmm* in NBY broth, and it likely inhibited *Cmm* growth during bio-enrichment. However, kasugamycin, which killed *P. fluorescens in vitro*, may have greatly reduced *P. fluorescens* colonization in seed. Consequently, kasugamycin increased the probability of *Cmm* detection by bio-enriched TDPCR in combined treatment with kasugamycin with *P. fluorescens*. Whereas in other sanitation treatments combined with *P. fluorescens*, it is suspected that the *Cmm* population was not suppressed by *P.
fluorescens, but that *P. fluorescens* interfered with detection of *Cmm* by TDPCR.

Seed companies and growers often have concerns regarding the application of sanitizing and biological seed treatments due to a widely held belief that such treatments may reduce seed and seedling vigor, especially after months of seed storage. The traditional way to address this issue is to use the standard germination test, which is labor- and time-consuming, requiring manual germination counts and measurements of seedling length. The Seed Vigor Imaging System (SVIS), an automated vigor assessment system, provides rapid and accurate parameters of seed quality (Sako et al. 2001). The consistency of SVIS was proven in two independent tests in this study. The disinfectant treatments that eliminated tomato bacterial canker from tomato seeds did not affect seed vigor during long-term storage, suggesting that these seed sanitization treatments are applicable for use by individual growers and seed companies.

In summary, besides traditional hydrochloric acid, sodium hypochlorite and hot water treatments, we found that the disinfectants Virkon and Kleengrow effectively controlled seed to seedling transmission of bacterial canker without risking reducing seed germination and vigor, even after 12-18 months of storage. Virkon and Kleengrow are relatively new disinfectants that have been used for general sanitation in greenhouses, but few studies have evaluated the potential of these disinfectants as a seed treatment. The outcome of this study not only provides more options for growers and seed companies to incorporate seed treatment in integrated management
of tomato bacterial canker, but also reveals the optimal concentrations and timing for future tests on seed-transmitted pathogens in other crops using these disinfectants.
ACKNOWLEDGEMENTS

This research was founded by the Ohio Agricultural Research and Development Center and the British Columbia Greenhouse Grower Association. The author thanks the Seed Biology Lab in the Department of Horticulture and Crop Science, The Ohio State University, for assistance in testing seed vigor, and Dr. Pierce Paul and Dr. Brian McSpadden Gardener, Department of Plant Pathology, The Ohio State University, for guidance on statistical analysis, and for providing the *Pseudomonas fluorescens* strain and valuable suggestions on terminal dilution PCR, respectively.
REFERENCES

American Seed Trade Association (ASTA),


derived from crosses to *L. esculentum*. Plant Dis. 85:1171-1176.


of thymol as biofumigant for control of bacterial wilt of tomato under field conditions. Plant Dis. 89:497-500.


Sako, Y., M. B. McDonald, K. Fujimura, A. F. Evans, and M. A. Bennett. 2001. A

Influence of soil temperature and matric potential on sugar beet seedling
colonization and suppression of Pythium damping-off by the antagonistic bacteria
_Pseudomonas fluorescens_ and _Bacillus subtilis_. Phytopathology 4: 351-363.


Theodoro, G. F. and A. C. Maringoni. 2000. _In vitro_ and _in vivo_ action of chemicals
on _Clavibacter michiganensis_ subsp. _michiganensis_, causal agent of the bacterial

michiganense by seeds. J. Phytopathology 119:142-146.

Uematsu, T., H. Fuji, and K. Ohata. 1977. Relationship between inoculation methods

Umesha, S. 2006. Occurrence of bacterial canker in tomato fields of Karnataka and
effect of biological seed treatment on disease incidence. Crop Prot. 25:375-381.

Utkhede, R. and C. Koch. 2004. Biological treatment to control bacterial canker of

Wakimoto, S., T. Uematsu, and T. Mizukami. 1969. Bacterial canker disease of
Table 4.1 Growth of *Clavibacter michiganensis* subsp. *michiganensis* strains C290 and A300 on yeast dextrose carbonate agar medium (YDC) amended with selected inhibitors at different concentrations.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Concentration (ug/ml)</th>
<th>Cmm strains</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C290</td>
<td>A300</td>
<td></td>
</tr>
<tr>
<td>Thymol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100</td>
<td>-&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>+&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>600</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> - = no colonies; + = colonies of Cmm.

<sup>b</sup> Dissolved in 70% ethanol.

<sup>c</sup> Tested on non-amended medium.
Table 4.2 Determination of conditions for seed treatment with selected inhibitors and dry heat on eliminating *Clavibacter michiganensis* subsp. *michiganensis* from seed and influence on germination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Test Conditions (concentrations, temperature, exposure time)</th>
<th>Infestation frequency (%)</th>
<th>Germination rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymol d</td>
<td>0.5 ug/seed</td>
<td>35</td>
<td>NT e</td>
</tr>
<tr>
<td></td>
<td>1.0 ug/seed</td>
<td>15</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>5.0 ug/seed</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.005 ug/seed</td>
<td>25</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>0.050 ug/seed</td>
<td>10</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>0.500 ug/seed</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>0.23 ug/seed</td>
<td>35</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>0.46 ug/seed</td>
<td>30</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>4.6 ug/seed</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Dry Heat</td>
<td>80°C, 24 h</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>80°C, 48 h</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>80°C, 72 h</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Kleengrow</td>
<td>0.28% (v/v), 30 min</td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>0.56% (v/v), 30 min</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>1.12% (v/v), 30 min</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>Virkon</td>
<td>0.5% (w/v), 20 min</td>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>1.0% (w/v), 20 min</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>2.0% (w/v), 20 min</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>Control f</td>
<td>-</td>
<td>48</td>
<td>93</td>
</tr>
</tbody>
</table>

**d** Dissolved in 70% ethanol.

**e** Not tested.

**f** Untreated seeds were tested as a control. Data presented are averages from two tests.
Table 4.3 Optimized conditions for seed sanitation treatments.

<table>
<thead>
<tr>
<th>Name of Treatment</th>
<th>Treatment Conditions (Temperature, concentration, timing and equipment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat treatment</td>
<td>Pre-warm at 50°C for 4 hours, then 80°C for 24 hours; Precision Thelco Oven Model 28</td>
</tr>
<tr>
<td></td>
<td>Pre-warm at 37°C for 10 minutes, then 50°C for 25 minutes; seed soaking</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>0.15 mg/g (^x), Hege 11 liquid seed treater</td>
</tr>
<tr>
<td></td>
<td>1.15 mg/g, Hege 11 liquid seed treater</td>
</tr>
<tr>
<td>Essential plant oil</td>
<td>1.5 mg/g, Hege 11 liquid seed treater</td>
</tr>
<tr>
<td>Disinfectants</td>
<td>0.6 M, soaking seed for 1 hour</td>
</tr>
<tr>
<td></td>
<td>0.6%, 50°C; soaking seed for 15 minutes</td>
</tr>
<tr>
<td></td>
<td>0.56%, soaking seed for 30 minutes</td>
</tr>
<tr>
<td></td>
<td>2%, soaking seed for 20 minutes</td>
</tr>
</tbody>
</table>

\(^x\) weight of active ingredient/weight of seed.
Table 4.4 Efficacy of sanitation treatments in eliminating *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) from tomato seed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
<th>Experiment 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Cmm</em> Infestation frequency (%)</td>
<td><em>Cmm</em> population (log CFU/100 seeds)</td>
<td><em>Cmm</em> Infestation frequency (%)</td>
<td><em>Cmm</em> population (log CFU/100 seeds)</td>
<td><em>Cmm</em> Infestation frequency (%)</td>
<td><em>Cmm</em> population (log CFU/100 seeds)</td>
</tr>
<tr>
<td>Control</td>
<td>11.8 a$</td>
<td>5.3 a</td>
<td>5.2 a</td>
<td>3.8 a</td>
<td>16.3 a</td>
<td>5.1 a</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>10.1 ab</td>
<td>5.0 a</td>
<td>1.4 b</td>
<td>1.2 bc</td>
<td>4.2 b</td>
<td>2.4 b</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>6.3 b</td>
<td>3.9 b</td>
<td>0.7 bc</td>
<td>1.3 bc</td>
<td>4.6 b</td>
<td>2.3 bc</td>
</tr>
<tr>
<td>Thymol</td>
<td>2.3 c</td>
<td>3.9 b</td>
<td>1.6 b</td>
<td>2.2 b</td>
<td>2.6 c</td>
<td>2.3 bc</td>
</tr>
<tr>
<td>Dry heat</td>
<td>7.6 b</td>
<td>3.7 b</td>
<td>0.5 bc</td>
<td>0.9 bc</td>
<td>3.0 bc</td>
<td>1.2 cd</td>
</tr>
<tr>
<td>Hot water</td>
<td>2.5 c</td>
<td>- #</td>
<td>0 c</td>
<td>0 c</td>
<td>0 d</td>
<td>0 d</td>
</tr>
<tr>
<td>HCl</td>
<td>0 d</td>
<td>0 c</td>
<td>0 c</td>
<td>0 c</td>
<td>0 d</td>
<td>0 d</td>
</tr>
<tr>
<td>NaClO/hot water</td>
<td>0 d</td>
<td>0 c</td>
<td>0 c</td>
<td>0 c</td>
<td>0 d</td>
<td>0 d</td>
</tr>
<tr>
<td>Kleengrow</td>
<td>0 d</td>
<td>0 c</td>
<td>0 c</td>
<td>0 c</td>
<td>0 d</td>
<td>0 d</td>
</tr>
<tr>
<td>Virkon</td>
<td>0 d</td>
<td>0 c</td>
<td>0 c</td>
<td>0 c</td>
<td>0 d</td>
<td>0 d</td>
</tr>
</tbody>
</table>

$^5$ Means (n=4) followed by the same letter within a column are not significantly different at P≤0.05.

# Data not available due to overgrowth of *Cmm* by saprophytes on D2ANX medium.
Table 4.5 Statistical analysis of the effects of tomato seed sanitation and beneficial bacteria treatments on tomato bacterial canker control and seedling establishment, Experiment 1.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Incidence of symptomatic seedlings (%)</th>
<th>Emergence rate (%)</th>
<th>Abnormal rate (%)</th>
<th>Dry weight of shoot (g)</th>
<th>Dry weight of root (g)</th>
<th>Height of shoot (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanitation Treatment</td>
<td>&lt;0.0001 *</td>
<td>&lt;0.0001 *</td>
<td>0.0001 *</td>
<td>0.0281 *</td>
<td>0.6114</td>
<td>0.6754</td>
</tr>
<tr>
<td>Beneficial Bacteria</td>
<td>0.2149</td>
<td>0.1905</td>
<td>0.4037</td>
<td>0.1778</td>
<td>0.5349</td>
<td>0.7464</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.6235</td>
<td>0.5781</td>
<td>0.4727</td>
<td>0.5699</td>
<td>0.1811</td>
<td>0.3529</td>
</tr>
</tbody>
</table>

Data presented are P values and * indicates a significant effect at P<0.05.
Table 4.6 Effects of seed sanitation treatments on tomato bacterial canker incidence, seed germination and seedling growth, Experiment 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of symptomatic seedlings (%)</th>
<th>Emergence rate (%)</th>
<th>Abnormal rate (%)</th>
<th>Dry weight of shoot (g)</th>
<th>Dry weight of root (g)</th>
<th>Height of shoot (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.3 a&lt;sup&gt;5&lt;/sup&gt;</td>
<td>94.5 abc</td>
<td>1.5 bc</td>
<td>0.365 ab</td>
<td>0.138 a</td>
<td>10.1 a</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>1.5 b</td>
<td>92.9 bc</td>
<td>2.0 abc</td>
<td>0.353 ab</td>
<td>0.136 a</td>
<td>10.5 a</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1.1 bc</td>
<td>93.2 bc</td>
<td>2.2 ab</td>
<td>0.391 a</td>
<td>0.138 a</td>
<td>10.8 a</td>
</tr>
<tr>
<td>Thymol</td>
<td>0.9 bc</td>
<td>88.9 d</td>
<td>2.8 a</td>
<td>0.345 ab</td>
<td>0.144 a</td>
<td>10.3 a</td>
</tr>
<tr>
<td>Dry heat</td>
<td>0.9 bc</td>
<td>92.4 c</td>
<td>1.5 bc</td>
<td>0.325 b</td>
<td>0.120 a</td>
<td>10.6 a</td>
</tr>
<tr>
<td>Hot water</td>
<td>0.8 bc</td>
<td>94.9 ab</td>
<td>1.0 c</td>
<td>0.377 ab</td>
<td>0.150 a</td>
<td>11.0 a</td>
</tr>
<tr>
<td>HCl</td>
<td>0.5 c</td>
<td>95.4 a</td>
<td>2.1 ab</td>
<td>0.380 ab</td>
<td>0.140 a</td>
<td>10.8 a</td>
</tr>
<tr>
<td>NaClO/hot water</td>
<td>0.2 cd</td>
<td>95.9 a</td>
<td>1.6 bc</td>
<td>0.363 ab</td>
<td>0.139 a</td>
<td>10.3 a</td>
</tr>
<tr>
<td>Kleengrow</td>
<td>0 d</td>
<td>95.8 a</td>
<td>1.6 bc</td>
<td>0.397 a</td>
<td>0.129 a</td>
<td>10.7 a</td>
</tr>
<tr>
<td>Virkon</td>
<td>0 d</td>
<td>95.7 a</td>
<td>1.5 bc</td>
<td>0.394 a</td>
<td>0.140 a</td>
<td>11.1 a</td>
</tr>
</tbody>
</table>

<sup>5</sup>Means (n=12) followed by the same letter within a column are not significantly different at P$\leq0.05$. 

138
Table 4.7 Effects of seed sanitation treatments on tomato bacterial canker incidence, seed germination and seedling growth, Experiment 3.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence of symptomatic seedling (%)</th>
<th>Emergence rate (%)</th>
<th>Abnormal rate (%)</th>
<th>Dry weight of shoot (g)</th>
<th>Dry weight of root (g)</th>
<th>Height of shoot (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 a&lt;sup&gt;x&lt;/sup&gt;</td>
<td>95.3 a</td>
<td>0.6 b</td>
<td>0.781 a</td>
<td>0.585 a</td>
<td>15.9 a</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>1.4 a</td>
<td>95.5 a</td>
<td>0.2 b</td>
<td>0.723 a</td>
<td>0.558 a</td>
<td>15.0 a</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0 b</td>
<td>95.3 a</td>
<td>0 b</td>
<td>0.788 a</td>
<td>0.618 a</td>
<td>16.5 a</td>
</tr>
<tr>
<td>Thymol</td>
<td>0.4 b</td>
<td>93.7 a</td>
<td>2.1 a</td>
<td>0.756 a</td>
<td>0.527 a</td>
<td>16.0 a</td>
</tr>
<tr>
<td>Dry heat</td>
<td>0.2 b</td>
<td>91.6 a</td>
<td>0.2 b</td>
<td>0.680 a</td>
<td>0.503 a</td>
<td>15.5 a</td>
</tr>
<tr>
<td>Hot water</td>
<td>0 b</td>
<td>95.1 a</td>
<td>0 b</td>
<td>0.720 a</td>
<td>0.642 a</td>
<td>15.6 a</td>
</tr>
<tr>
<td>HCl</td>
<td>0 b</td>
<td>96.8 a</td>
<td>0.2 b</td>
<td>0.672 a</td>
<td>0.637 a</td>
<td>15.8 a</td>
</tr>
<tr>
<td>NaClO/hot water</td>
<td>0 b</td>
<td>93.6 a</td>
<td>0.4 b</td>
<td>0.690 a</td>
<td>0.622 a</td>
<td>15.8 a</td>
</tr>
<tr>
<td>Kleengrow</td>
<td>0 b</td>
<td>94.5 a</td>
<td>0 b</td>
<td>0.703 a</td>
<td>0.610 a</td>
<td>15.3 a</td>
</tr>
<tr>
<td>Virkon</td>
<td>0 b</td>
<td>96.2 a</td>
<td>0.4 b</td>
<td>0.770 a</td>
<td>0.654 a</td>
<td>16.1 a</td>
</tr>
</tbody>
</table>

<sup>x</sup> Means (n=4) followed by the same letter within a column are not significantly different at P≤0.05.
Table 4.8 Statistical analysis of the effects of sanitation and beneficial bacterial treatments on tomato seed vigor before and after long-term storage, Experiment 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test before storage</td>
</tr>
<tr>
<td>Sanitation treatment</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Beneficial bacterial</td>
<td></td>
</tr>
<tr>
<td>treatment</td>
<td>0.60</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* Significant at P ≤ 0.05.
Table 4.9 Statistical analysis of the effects of sanitation and beneficial bacterial treatments on tomato seed vigor before and after long-term storage, Experiment 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P value</th>
<th>Test before storage</th>
<th>Test after 6 months storage</th>
<th>Test after 12 months storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanitation treatment</td>
<td>&lt;0.001*</td>
<td>&lt;0.001*</td>
<td>0.002*</td>
<td></td>
</tr>
<tr>
<td>Beneficial bacterial treatment</td>
<td>0.12</td>
<td>0.21</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>0.98</td>
<td>0.42</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at P≤0.05.
Table 4.10 Influence of seed treatments on seed vigor before and after long-term storage, Experiment 1.

<table>
<thead>
<tr>
<th>Seed Treatment</th>
<th>Seed vigor index</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test before</td>
<td>Test</td>
<td>Test</td>
<td>Test</td>
</tr>
<tr>
<td></td>
<td>storage</td>
<td>after 6</td>
<td>after 12</td>
<td>after 18</td>
</tr>
<tr>
<td>Control</td>
<td>801 ab</td>
<td>727 a</td>
<td>769 a</td>
<td>739 a</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>603 cd</td>
<td>492 d</td>
<td>660 bc</td>
<td>570 d</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>766 bc</td>
<td>684 ab</td>
<td>597 bc</td>
<td>726 a</td>
</tr>
<tr>
<td>Thymol</td>
<td>747 c</td>
<td>594 c</td>
<td>629 bc</td>
<td>621 cd</td>
</tr>
<tr>
<td>Dry heat</td>
<td>536 d</td>
<td>487 d</td>
<td>562 c</td>
<td>640 bc</td>
</tr>
<tr>
<td>Hot water</td>
<td>785 b</td>
<td>645 bc</td>
<td>612 bc</td>
<td>707 ab</td>
</tr>
<tr>
<td>HCl</td>
<td>833 a</td>
<td>624 bc</td>
<td>621 bc</td>
<td>710 ab</td>
</tr>
<tr>
<td>NaClO/hot water</td>
<td>778 b</td>
<td>664 ab</td>
<td>703 ab</td>
<td>717 a</td>
</tr>
<tr>
<td>Kleengrow</td>
<td>797 ab</td>
<td>663 ab</td>
<td>715 ab</td>
<td>708 ab</td>
</tr>
<tr>
<td>Virkon</td>
<td>789 b</td>
<td>681 ab</td>
<td>661 abc</td>
<td>717 a</td>
</tr>
</tbody>
</table>

* Means (n=12) followed by the same letter within a column are not significantly different at P ≤ 0.05.
Table 4.11 Influence of seed treatments on seed vigor before and after long-term storage, Experiment 2.

<table>
<thead>
<tr>
<th>Seed Treatment</th>
<th>Seed vigor index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test before storage</td>
</tr>
<tr>
<td>Control</td>
<td>803 abc&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kasugamycin</td>
<td>662 d</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>823 ab</td>
</tr>
<tr>
<td>Thymol</td>
<td>757 c</td>
</tr>
<tr>
<td>Dry heat</td>
<td>621 d</td>
</tr>
<tr>
<td>Hot water</td>
<td>811 abc</td>
</tr>
<tr>
<td>HCl</td>
<td>804 abc</td>
</tr>
<tr>
<td>NaClO/Hot water</td>
<td>832 a</td>
</tr>
<tr>
<td>KleenGrow</td>
<td>789 bc</td>
</tr>
<tr>
<td>Virkon</td>
<td>824 ab</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means (n=12) followed by the same letter within a column are not significantly different at P≤0.05.
Figure 4.1 Antagonism of *Bacillus subtilis* (strain MBI 600, left) and *Pseudomonas fluorescens* (strain Wayne 1R, right) on *Clavibacter michiganensis* subsp. *michiganensis* (strain C290) growth on YDC medium. The YDC medium was spread with a suspension of C290, then beneficial bacterial strains were immediately streaked on each side.
Figure 4.2 (A and B) Morphology of abnormal seedlings 2 weeks after seed were sown. (C) Water-soaked spots (arrows), typical symptoms of tomato bacterial canker on a cotyledon and first true leaf of a 5 week-old seedling.
Figure 4.3 Detection of *Clavibacter michiganensis* subsp. *michiganensis* in greenhouse tomato seedlings by terminal dilution PCR (TDPCR) before and after bio-enrichment in NBY broth. Abbreviations: M, DNA ladder of 1 Kb plus; \(3^n\), \(n=\)the number of three fold dilutions.
Figure 4.4 Relationship between the population estimates of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in greenhouse tomato seedlings after seed treatment determined by serial dilution plating and by bio-enriched terminal dilution polymerase chain reaction (TDPCR). The X-axis (TDPCR) indicates the number of the last dilution that was positive by PCR assay; the Y-axis indicates log$_{10}$ *Cmm* populations assessed by serial dilution plating.
Figure 4.5 Box plots showing the relative *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) population levels determined by bio-enriched terminal dilution PCR (TDPCR) in tomato seedlings after various seed treatments, Experiment 1 (A, B and C) and Experiment 3 (D). The X-axis indicates the sanitation treatments and CK represents *Cmm*-infested seeds not subjected to a sanitizing treatment. The Y-axis (TDPCR) indicates the number of the terminal dilution that was positive by the PCR assay. Beneficial bacteria *Bacillus subtilis* MBI 600 (BS) or *Pseudomonas fluorescens* Wayne1R (PF) were applied after sanitation treatments in (B) and (C). Continued
Figure 4.5 continued.
Bibliography


American Seed Trade Association (ASTA),


Chachalis, D., E. M. Khah, and M. K. Darawsheh. 2008. Effects of initial seed moisture content, imbibition temperature and seed vigour on germination, electrolyte leakage and seedling growth in plum tomatoes. J. Food Agri. and


Chun, W.C.C. 1982. Identification and detection of *Corynebacterium michiganense* in tomato seed using the indirect enzyme-linked immunosorbent assay. MSc Thesis. Honolulu, HI, USA: University of Hawaii.


Gartemann, K.-H., O. Kirchner, J. Engemann, I. Gräfen, R. Eichenlaub, and A. Burger. 2003. Clavibacter michiganensis subsp. michiganensis: first steps in the


Hernández, A., E. Martró, L. Matas, M. Martín and V. Ausina. 2000. Assessment of


control of bacterial leaf spot and bacterial canker on fresh market and processing

Jahr, H., J. Dreier, D. Meletzus, R. Bahro, and R. Eichenlaub. 2000. The Endo-β-1,4-
glucanase CelA of Clavibacter michiganensis subsp. michiganensis is a
pathogenicity determinant required for induction of bacterial wilt of tomato. Mol.
Plant-Microbe Interact. 1:703-714.

between Clavibacter michiganensis and its host plants. Environ. Microbiol.
1:113-118.

of thymol as biofumigant for control of bacterial wilt of tomato under field

campestris pv. campestris encodes an exocellular component required for growth

Kirchner, O., K.-H. Gartemann, E.-M. Zellermann, R. Eichenlaub, and A. Burger.
2001. A highly efficient transposon mutagenesis system for the tomato pathogen
14:1312-1318.

Kloepper, J. W., C-M. Ryu, and S. Zhang. 2004. Induced systemic resistance and
promotion of plant growth by Bacillus spp. Phytopathology 94:1259-1266.

Antifungal, phytotoxic and insecticidal properties of essential oil isolated from


Rai, P. V. and G. A. Strobel. 1968. Phytotoxic glycopetides produce by
*Corynebacterium michiganense*. II. Biological properties. Phytopathology 59:53-57.

analysis of pathogenic events in virulent and avirulent *Brucella melitensis*

Ricker, M. D., and R. M. Riedel. 1993. Effect of secondary spread of *Clavibacter*
michiganensis subsp. michiganensis on yield of northern processing tomatoes.

Sako, Y., M. B. McDonald, K. Fujimura, A. F. Evans, and M. A. Bennett. 2001. A

Salanenka, Y. A. and A. G. Taylor. 2008. Seed coat permeability and uptake of

Influence of soil temperature and matric potential on sugar beet seedling
colonization and suppression of *Pythium* damping-off by the antagonistic bacteria
*Pseudomonas fluorescens* and *Bacillus subtilis*. Phytopathology 4: 351-363.

for detection of genetically engineered microorganisms released into the

Shaw, J. J., L. G. Settles, and C. I. Kado. 1988. Transposon Tn4431 mutagenesis of
*Xanthomonas campestris* pv. campestris: characterization of a non pathogenic


Theodoro, G. F., and A. C. Maringoni. 2000. *In vitro* and *in vivo* action of chemicals


United States Department of Agriculture (USDA) National Agricultural Statistic Service,


Wakimoto, S., T. Uematsu, and T. Mizukami. 1969. Bacterial canker disease of

mutagenesis of an integral membrane transporter in Corynebacterium matruchotii.

27: 489-499.

Wilson, E. E., F. M. Zeitoun, and D. L. Fredrickson. 1967. Bacterial phloem canker, a

Xu, X., S. A. Miller, F. Baysal-Gurel, K.-H. Gartemann, R. Eichenlaub, and G.
76:3978-3988.