

ORGANIC SEED TREATMENTS FOR THE REDUCTION OF *Xanthomonas*
euvesicatoria ON TOMATO SEED

by

JUDITH McFARQUHAR

(Under the Direction of Elizabeth Little)

ABSTRACT

The effects of OxiDate 2.0, sodium hypochlorite, thyme oil, rosemary stem infusion and ginkgo leaf infusion were tested *in vitro* on the bacterial cells of *Xanthomonas euvesicatoria*, *Clavibacter michiganensis* subsp. *michiganensis*, and *Pseudomonas syringae* pv. *tomato*. All treatments significantly reduced bacterial populations except for the infusions. Tomato seed artificially inoculated with *X. euvesicatoria* was treated with OxiDate 2.0, NaOCl, thyme oil or hot water and assayed by dilution plating of seed washes. All treatments gave significant reductions. Germination was not affected. Seedlings grown for 14 days from treated seed were assayed for bacterial populations using dilution plating and real-time PCR. Bacterial transmission to seedlings occurred in all treatments although seedlings from treated seeds had significantly lower populations than the control. NaOCl and hot water were the most effective seed treatments for reducing pathogen populations.

INDEX WORDS: Bacterial spot, *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas euvesicatoria*, Organic seed treatments, Tomato disease, Sodium hypochlorite, OxiDate 2.0, Thyme oil, Hot water seed treatment

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A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2015

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DEDICATION

This work is dedicated to the memory of my maternal grandmother, Mrs. Vioris Bent-Elliott, who passed away on March 25, 2013 when this work was yet in its embryonic stages. She was a woman of the soil, having been a small farmer in the fertile village of Top Hill, St. Elizabeth, Jamaica, for most of her life. She understood firsthand the input of labor and materials to produce a crop and the risk of yield loss. Grandma would appreciate and respect the work of plant pathologists, for though she led a simple, uncluttered life she admired "the increase of man's knowledge" and endeavors that improved the lives of hard-working people.

ACKNOWLEDGEMENTS

Recognition and gratitude are here extended to the faculty, staff and graduate students who all contributed to the completion of this project and a successful journey. I have been taught, guided and helped by persons who were generous with their knowledge and expertise. In particular, I am grateful for the guidance and editorial skills of my major professor Dr. Elizabeth Little and committee members Dr. Ron Walcott and Dr. David Langston Jr. Special recognition is due to Dr. Bhabesh Dutta, Dr. Tim Denny, Dr. Mike Deom, Dr. Harald Scherm, Matthew Seader, Kevin Besler, Gong Chen, Na Jiang, Dr. Dave Andrews, Judy Gray, Hunt Sanders for their direct help with the progress of this work.

Tuition, other fees and living expenses were funded for the first two years of the program by the Fulbright Program administered by LASPAU. Had it not been for the scholarship, I may never have ventured into this rewarding and edifying experience.

Immeasurable practical and emotional support was constantly given by my dear husband, Roger McFarquhar, in so many ways. My home team from Jamaica – family, friends, colleagues at The Mico University College, The Papine Wesleyan Holiness Church and fellow Fulbrighters – were unflinching in letting me know that I was not taking this journey for myself only or by myself.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Planting material, such as seeds, bulbs and cuttings can be a significant source of primary inoculum for many pathogens, and are often the only source of primary inoculum (Baker and Smith 1966; Elmer 2001). The textured or pubescent surfaces of seeds facilitate contamination with pathogen propagules. Not only can seed surfaces be contaminated, but many pathogens can get under the seed coat or enter seed tissues through the vascular system or floral parts (Maude 1996). The primary recommendation for control of seedborne diseases is to use seeds that are pathogen-free, especially for diseases that have the potential to cause significant losses (Gitaitis and Walcott 2007; Maude 1996; McGee 1995). Producing pathogen-free (clean) seed is challenging, in part because phytopathogens may infest or infect seeds without producing symptoms on the host or seed tissues (Bashan et al. 1982; Darrasse et al. 2007; Dutta et al. 2014) and field inspections of seed crops may not be sufficient for certifying seed as pathogen-free (Schultz and Gabrielson 1986). Testing for pathogen inoculum in seed samples is therefore necessary to prevent the spread of pathogens but there are challenges in sampling and detection of pathogens on or in seed. The distribution of infested seed within a seed lot is not homogenous so samples must include several thousand seeds in an attempt to be statistically representative of the level of contamination in a seed lot (Gitaitis and Walcott 2007; Schaad 1982; Schaad 1988). Even then, the test results are only reflective of the sample tested (Morrison 1999). The level of inoculum in a sample

may be low enough to escape detection by seed health testing procedures unless enrichment techniques are used (Jones et al. 1986; McGee 1995) but sufficient to initiate a disease outbreak (Stall et al. 2009). Hence, seed treatments may be needed even if a seed lot has been certified pathogen-free.

Bacterial plant diseases are difficult to manage once introduced into a field, partly because there are few effective chemistries (Jones et al. 1986; Lo Cantore et al. 2009) and host resistance is not universally available or effective (Stall et al. 2009). In greenhouse production, the close proximity of plants, high moisture conditions and handling of plants facilitates the rapid spread of bacteria and disease outbreaks (Hausbeck et al. 2000). In addition to the use of clean seed, other commonly recommended disease management strategies for bacterial diseases involve the use of copper bactericidal sprays (Jones et al. 2006) and cultural practices, including sanitation and crop rotation (Goode and Sasser 1980; Hausbeck et al. 2000; Jones et al. 1986; Ritchie and Averre 1996; Sun et al. 2002). The challenges are greater for organic agriculture because certification regulations prevent or restrict use of many antimicrobial compounds.

In the European Union, organic growers must use seed produced using organic methods (Groot et al. 2004; Lammerts van Bueren et al. 2003). The USDA's National Organic Program (NOP) regulations for seeds and planting stock production specify that certified organic operations must use organic seed whenever possible (US GPO 2015). The use of nonorganic seed is allowed if the unavailability of suitable organic seed can be clearly demonstrated. In such instances, the seed must be untreated or treated with a substance allowed for organic agriculture (USDA NOP 2013). According to Groot et al. (2004), because of this derogation of the organic seed regulations, seed companies have

little incentive to invest in organic seed production. The current restrictions on the use of nonorganic seed may increase the risk of seedborne disease due to a sometimes higher disease incidence in organic seed production fields (Kühne et al. 2005). Conventional seed production is dominated by a few companies and the Organic Seed Alliance (2011) has observed that these large seed companies have not adequately addressed the needs for organic seed in the organic sector. Therefore, organic seed production may largely be the responsibility of smaller seed companies that specialize in organic seed.

Seed treatments are important in an integrated approach to prevent disease outbreaks, but because seed treatments often reduce the shelf life of the seed, growers are often responsible for treating the seed. Heat treatment (especially hot water) of seeds can be effective (Fatmi et al. 1991; Jahn et al. 2006) but does not always eliminate the pathogen (Schaad 1982). Organic production is dominated by small-scale growers who often do not have access to specialized equipment for heat treatments. Treatments using NOP-approved materials in relatively simple formulations and applications are therefore most desirable to obtain consistent and reliable results for small-scale growers.

Lammerts van Bueren et al. (2003) identified the need for more research on the production of high quality organic planting material. In reference to NOP's regulations, du Toit (2004) echoed this opinion, noting the increased demand for organically produced seed and for research into suitable seed treatments that ensure clean seed for organic growers. This project investigates the efficacy of organic seed treatments in reducing seed contamination with the bacterial spot of tomato pathogen, *Xanthomonas euvesicatoria*. Although the treatments were tested against *X. euvesicatoria*, they could be useful against other seedborne bacterial pathogens.

Literature Review

Tomato production statistics. Tomato (*Solanum lycopersicum* L.) is native to South America and is currently the most widely grown vegetable in the world. The United States is the second largest producer in the world and fresh market tomato is produced in every state. In 2013, the United States produced 12.6 billion kg (27.8 billion pounds) of tomatoes for the fresh and processing markets, valued at over \$2 billion. Georgia was the tenth largest fresh market tomato-producing state in 2014 in terms of acreage harvested, with yield valued at \$17.48 million representing 1.5% of the national total for fresh market tomatoes (USDA NASS Quickstats). Although it still represents a small percentage of total acreage and production, market demand for organic produce has been increasing, evidenced by the rise in acreage devoted to organic agriculture over the past several years. In 2011, the total acreage for organic tomato production in the US was more than three times what it was in 2006 (USDA ERS 2013).

Bacterial Spot of Tomato: Disease Development and Biology. The genus *Xanthomonas* contains almost 30 species and all are exclusively plant-associated. Xanthomonads are strictly aerobic, gram negative, rod-shaped, yellow-pigmented bacteria with a high GC content (Aysan and Sahin 2003; Stall et al. 2009; Thieme et al. 2005). The genus contains saprophytes and epiphytes but most *Xanthomonas* species are pathogens of approximately 400 angiosperm hosts (Leyns et al. 1984; Ryan et al. 2011; Vauterin et al. 1990).

Bacterial spot, caused by either *X. euvesicatoria* or *X. vesicatoria*, is a disease of worldwide importance in tomatoes as well as peppers (*Capsicum spp.*) (Aysan and Sahin 2003; Black et al. 2001; Hamza et al. 2010; Kavitha and Umesha 2007; Ward and

O'Garro 1992). The disease can be found in all tomato and pepper producing areas.

Bacterial spot is often the most damaging disease in the major tomato production states of Florida (Jones 1991; Pernezny and Collins 1997; Sun et al. 2002) and California (Davis et al. 2014).

Bacterial spot was first observed on tomato in the United States in 1912 and in Pretoria, South Africa in 1914 (Doidge 1921). A similar disease was identified in pepper in the United States in 1918 (Jones et al. 1998). The tomato pathogen was identified and described in South Africa in 1921 by Ethel M. Doidge who named it *Bacterium vesicatorium* (Doidge 1921). In a later publication that same year, M. Gardner and J. Kendrick described the pathogen in the United States. The pathogen was renamed *Pseudomonas vesicatoria* in 1925, then *Phytomonas vesicatoria* in 1930, *Xanthomonas vesicatoria* in 1939 and later *X. campestris* pv. *vesicatoria*. *X. campestris* is the most complex of the xanthomonad species (Vauterin et al. 1990) having over 140 pathovars (Jones et al. 1998; Jones et al. 2004; Vauterin et al. 1990). In 1998, Jones et al. divided bacterial spot strains into four groups, A, B, C and D. Strains of *X. campestris* pv. *vesicatoria* were placed into groups A and B based on amylolytic and pectolytic activity as well as the expression of unique heat-stable proteins, reaction patterns with monoclonal antibodies and DNA:DNA hybridization (Jones et al. 1998). Vauterin et al. (1995), using DNA:DNA hybridization and carbon utilization assays, determined that groups A and B were two distinct species, which were renamed *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*, respectively. Almost ten years later, a reclassification of *X. campestris* pv. *vesicatoria* was proposed, dividing the diverse group of strains into four species, namely *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri* on the

basis of DNA:DNA hybridization (Jones et al. 2004). *X. campestris* pv. *vesicatoria* was found to have five races, T1, T2, T3, T4 and T5 (Yang et al. 2005) based on pathogenic reactions with different tomato genotypes (Bouzar et al. 1994) but with reclassification, these tomato races no longer belong to the same species (Hert et al. 2009). The etiological agent of interest in this project is *Xanthomonas euvesicatoria*, the most widespread bacterial spot pathogen worldwide (Moretti et al. 2009). *X. euvesicatoria* strains are host specific and may be pathogenic on tomato only, pathogenic on pepper only or pathogenic on both hosts (Bonas et al. 1991).

Bacterial spot of tomato and pepper is most severe in warm (24°C to 30°C) tropical and subtropical regions where moisture levels are moderate to high (Jones et al. 2000) from early in the growing season. In a growth chamber, pathogen populations do not increase on the leaf surface at a relative humidity of less than 40% (Bashan et al. 1982). The pathogen is usually introduced on infested seeds or transplants (Bashan and Okon 1986; Langston 2014; Ryan et al. 2011) and populations can increase to high numbers on asymptomatic hosts before the appearance of disease symptoms (Bashan et al. 1982). Economic losses from bacterial spot are due to reduced yield, leaf abscission, sunscald of fruit, and unmarketable fruit (Davis et al. 2014; Ritchie 2000).

Symptoms typically first appear on the leaves, sometimes exclusively, accounting for earlier references to this disease as bacterial leaf spot. Epiphytic populations increase on the leaf surface before bacteria enter the leaves through stomata, hydathodes or damaged epidermal cells. As the populations increase in the intercellular spaces (Ryan et al. 2011; Sharon et al. 1982) leaf spots appear as small water-soaked areas on the underside of the leaf that penetrate to the upper surface and become chlorotic. The

centers of these irregularly-shaped lesions become somewhat raised and necrotic as they expand and with persistent moist conditions the spots coalesce resulting in large dead areas on the leaves (Ritchie and Averre 1996; Sun et al. 2002). Leaf damage and abscission (especially in peppers) result from the production of host-derived ethylene, which is stimulated by pathogen-derived ethylene in susceptible cultivars (Ben-David et al. 1986). Ethylene causes expansion of chlorosis in leaf lesions and may limit growth of necrotic areas on tomato leaves so that the symptoms are spots rather than blights (Bashan et al. 1985; Ciardi et al. 2000; Lund et al. 1998; Stall and Hall 1984). Spots do not always occur on the fruit although contaminated seeds can be produced by asymptomatic fruit. Fruit spots develop only on immature fruit and are initially small, irregular and either blister-like or depressed, with yellowish or white water-soaked halos. Spots later become dark and wart-like, and the halo disappears. Although the spots are superficial this makes the fruit undesirable for both fresh and processing markets because of reduced aesthetic quality, misshapen appearance, interference with peeling during processing, and the facilitation of secondary infections leading to rot (Goode and Sasser 1980; Ritchie and Averre 1996; Sun et al. 2002).

Seed contamination. Infested seed are an important source of initial inoculum and the pathogen can survive on seed stored for up to 10 years (Bashan et al. 1982). Bacterial spot is not a systemic disease and *X. euvesicatoria* populations are established between host cells in the mesophyll layer of leaves rather than inside vascular tissue (Ryan et al. 2011). Contamination of seeds through the vascular system is unlikely. Warm, wet conditions facilitate the spread of epiphytic populations to the flowers and fruit where the pathogen may gain access to seeds (Bashan and Okon 1986). The stigmas

are a favorable site for colonization. The bacteria pass through the style, enter the ovaries and establish populations that contaminate the seed (Dutta et al. 2014). Contamination may also occur during the seed extraction process (Agrios 2005; Zitter 1985).

Pathogen populations on seed contaminate seedlings during germination. The pathogen is readily spread to other plants in the greenhouse or field by water, such as splash from irrigation and wind-driven rain, by airborne aerosols and by mechanical means from worker and equipment activities (McInnes et al. 1988; Pohronezny et al. 1990; Ryan et al. 2011; Sun et al. 2002). Other sources of initial inoculum include volunteer tomatoes, tomato debris, diseased leaves, soil, and the rhizosphere of host and some nonhost plants such as solanaceous weeds (Bashan et al. 1982; Jones et al. 1986; Ritchie 2000).

Management strategies. Transplants are the preferred planting material in both conventional and organic systems as this avoids the challenges of direct-seeding and allows the crop to establish quickly and uniformly in the field (Boyhan and Kelley 2014). To reduce the risk of infested planting material, seed and transplants are ideally produced in cool, arid areas certified disease-free (du Toit 2004; Langston 2014) but this strategy is not always successful (Darrasse 2007; Gitaitis and Walcott 2007; Schultz and Gabrielson 1986), especially with a pathogen that can colonize the host and contaminate seed without producing symptoms (Bashan et al. 1982; Dutta et al. 2014; Stall et al. 2009).

Cultural practices can create less favorable conditions for disease development. Sanitation, crop rotation, and drip, furrow or trickle irrigation are recommended for management of bacterial spot. Sanitation removes sources of initial inoculum that could lead to disease the following season. *Xanthomonas* survives only 16 days in sandy soil

with no viable host material, but several months in host-free loam soil, up to several months on crop residues depending on the rate of decomposition, and up to twelve months on volunteer tomatoes (Bashan et al. 1982; Jones et al. 1986; Ryan et al. 2011; Stall et al. 2009). Solanaceous weeds may serve as hosts providing inoculum for the next season although they may not serve as hosts for long-term survival of the pathogen (Gitaitis et al. 1992). Therefore, weed control and the disking or removal of postharvest debris and volunteers are important. Cull piles should not be established near the crop (Langston 2014). The use of disinfectants to clean tools and work surfaces and the use of skin-safe disinfectant washes reduce the risk of transmission by workers and their equipment (Pohronezny et al. 1990). The best sanitation efforts may not, however, remove all sources of inoculum. Considering that the pathogen can survive several months on crop residues, a minimum of one year crop rotation with nonsolanaceous crops (Davis et al. 2013; Langston 2014) is advised but may not be economically feasible.

Fixed copper compounds have broad-spectrum bactericidal activity and foliar sprays are effective in reducing the impact of bacterial spot on tomato (Dougherty 1978) and other bacterial diseases. They are effective as protectants and must contact the pathogens before infection. Therefore, coppers must be used early, usually at the first sign of disease or emergence of the first true leaves (Hausbeck et al. 2000; Sun et al. 2002). Copper compounds are more effective when tank-mixed with ethylene bisdithiocarbamate (EBDC) fungicides. The mode of action that increases its efficacy is not clearly understood. Marco and Stall (1983) suggested that it increases the amount of available copper but Hausbeck et al. (2000) disagreed, postulating a synergistic effect, although they did not observe any bactericidal effects of the fungicide.

Coverage of the entire plant is important because bacterial populations on leaf surfaces are nonuniform. Higher numbers are usually found on the abaxial surface (Beattie and Lindow 1999; Sharon et al. 1982). Epiphytic bacteria may enter substomatal spaces or colonize other protected areas such as buds where they evade contact by copper sprays (Hugouvieux et al. 1998; Pernezny and Collins 1997). The challenge of using this chemical tool is compounded by the risk of copper phytotoxicity (Lalancette and McFarland 2007; Mazhoudi et al. 1997; Ouariti et al. 1997) and the development of tolerance to copper in some strains of the pathogen (Jones et al. 1991; Ritchie and Dittapongpitch 1991; Ward and O'Garro 1992). Copper resistance genes are located on plasmids that can be transmitted to other bacteria (Behlau et al. 2013; Bender et al. 1990). Public health concerns have been raised about the use of EBDCs (Yang et al. 2005) and they are no longer allowed in some states for use on processing tomatoes (Hausbeck et al. 2000). EBDC compounds are not allowed in organic production.

Antibiotics have been tried for bacterial spot management but are not presently in common usage. Streptomycin is the most common antibiotic used in plant production, but oxytetracycline and kasugamycin have also been studied in greenhouse and field applications (Ritchie and Averre 1996; Sun et al. 2002; Vallad et al. 2010). Antibiotic resistance develops relatively quickly in *Xanthomonas* (Lai et al. 1977; Minsavage et al. 1990) and antibiotics may have phytotoxic effects (Humaydan et al. 1980; McManus et al. 2002). Resistance to antibiotics has been reported among a number of bacteria, including strains of bacterial spot pathogen (Ritchie and Dittapongpitch 1991; Ward and O'Garro 1992). Antibiotic use is not advisable in the field (Ritchie and Averre 1996) except for high value crops because of the high cost and the short time for which the

antibiotic is effective (Stockwell and Duffy 2012). Concerns have been raised about the spread of antibiotic resistance among clinically important bacteria resulting from use of antibiotics in agriculture (Stockwell and Duffy 2012). Antibiotics have not been recommended for bacterial spot management for over forty years (Stall et al. 2009).

Host resistance against bacterial spot is the most desirable method of disease management. Tomato cultivars resistant to races of tomato and pepper bacterial spot pathogens have been identified (Bonshtien et al. 2005; Gibly et al. 2004; Horvath et al. 2012; Scott et al. 1995; Yang et al. 2005). However, these cultivars have not gained widespread use (Langston 2014) possibly because the resistance is not durable (Clarke et al. 2014; Stall et al. 2009), the available resistant cultivars are not resistant to all strains of the pathogen and they lack the horticultural characteristics demanded by the fresh and processing tomato markets (Hartz et al. 2008; Jones et al. 2006; Le Strange 2000). Recent research has determined that eggplant (*Solanum melongena*) and related species may be a promising genetic resource for resistance to *X. euvesicatoria* and *Pseudomonas syringae* pv. *tomato* (Clarke et al. 2014).

Biological control organisms such as antagonistic bacteria and fungi as well as plant growth-promoting rhizosphere (PGPR) bacteria have been used as seed treatments and in foliar applications (El Hendawy et al. 2005; Fontenelle et al. 2011; Hert et al. 2009; Kavitha and Umesha 2007). The effectiveness of biocontrol agents is influenced by interactions with other microorganisms as well as the chemical and physical components of the phyllosphere and rhizosphere. Such interactions cannot be predicted and biocontrol organisms usually exhibit a low to moderate level of control when compared to chemical bactericides. Only some strains of a biocontrol agent may be useful against a particular

strain of the pathogen and even with the appropriate biocontrol strain, the effectiveness at reducing disease is variable (Byrne et al. 2005). Even the most effective biocontrol microorganisms cannot prevent disease outbreaks when environmental conditions are conducive. Biocontrol is not intended to be a substitute for chemical bactericides but can be part of an integrated disease management plan.

Bacteriophages (or phages) have demonstrated effective control of their bacterial targets and in 1995 the EPA registered a phage-containing product for use against bacterial spot and speck in tomatoes (US EPA 2011). Phages have been identified that are specific to *X. euvesicatoria* but have no effect on other bacterial spot pathogens (Gašić et al. 2011). The effectiveness of phages is limited by their sensitivity to UV light (Silverman et al. 2013), high temperatures, pH changes and other environmental conditions, and they require free moisture in which to diffuse to their host cells. Bacteriophages degrade relatively quickly (Jones et al. 2007) necessitating frequent spray applications. Phage preparations are more effective when a mixture of bacteriophages is used and protective compounds are added to extend the period for which they are active on leaf surfaces (Balogh et al. 2003; Flaherty et al. 2000; Obradovic et al. 2004).

Acibenzolar-s-methyl (ASM) is a synthetic plant activator shown to induce systemic acquired resistance that is as effective as copper-EBDC sprays in controlling bacterial spot on both peppers and tomatoes (Huang et al. 2012; Louws et al. 2001; Obradovic et al. 2005; Romero et al. 2001). Efficacy is observed whether ASM is applied before (Huang et al. 2012) or after (Cavalcanti et al. 2007) the plants are inoculated with the pathogen. Host defense mechanisms against bacterial spot are also induced by foliar applications of chitosan (Coqueiro et al. 2011).

Essential oils have been used for centuries for their putative antiseptic properties. They are formed in aromatic plants as volatile, secondary metabolites. Oil extracts have a strong odor, and can have a protective function against herbivory (Bakkali et al. 2008). The activity of essential oils against insects (Choi et al. 2003; Lale 1992; Waliwitiya et al. 2005) fungi (Kordali et al. 2008; Marinelli et al. 2012; Omidbeygi et al. 2006) and bacteria (Daferera et al. 2003; Fisher and Phillips 2009; Friedman et al. 2002; Rasooli et al. 2006) is well documented. Essential oils or their components have also been used against microorganisms in seed disinfection (Lo Cantore et al. 2009) and in food preservation and safety (Holley and Patel 2005; Mishra and Dubey 1994; Solomakos et al. 2008; Tzortzakis and Economakis 2007). The antimicrobial effects of essential oils are attributed to nonspecific modes of action that affect cellular membranes, macromolecules (including enzymes and ion channels) and energy production (Isman 2000; Janssen et al. 1987). Gram negative bacteria appear to be less susceptible to the damaging effects of essential oils than gram positive bacteria due to cell wall structure (Abozid and Asker 2013; Bakkali et al. 2008; Iacobellis et al. 2005; Ziani et al. 2011).

Investigations into the efficacy of essential oils against phytopathogens are often limited to *in vitro* studies that suggest the potential for their use in disease management and against storage fungi (Daferera et al. 2003; Omidbeygi et al. 2006; Riccioni and Orzali 2011; Suwitchayanon and Kunasakdakul 2009; Tzortzakis and Economakis 2007). Effective use as foliar sprays has been recorded by da Silva et al. (2014) in which essential oils of thyme, clove, eucalyptus, cinnamon, citronella, tea tree and lemongrass reduced populations of *Pseudomonas syringae* pv. *tomato*, causal agent of bacterial speck of tomato, in greenhouse trials. Pre-infection treatments provided better control

than post-infection sprays. de Lira Guerra et al. (2014) used foliar sprays of essential oils from eleven plants and saw reduction of soft rot of Chinese cabbage caused by *Pectobacterium carotovorum* ssp. *carotovorum*. Foliar applications and seed treatment using thyme oil suspensions have been effective against bacterial spot of tomato (Altundag and Aslim 2011). Six of the eleven essential oils tested by Mbega et al. (2012a) were effective against *X. perforans*, another bacterial spot pathogen. These six oils were subsequently used as seed treatments. Tomato seeds treated with essential oils of clove, thyme and lemongrass did not emerge when planted, possibly because of the relatively high concentrations used (2%), or the long time for which they were soaked in the treatment (overnight). The remaining three oils of eucalyptus, rosemary and niaouli (*Melaleuca viridiflora*) were effective in reducing incidence and severity of the disease (Mbega et al. 2012a). Bean seeds infested with *X. campestris* pv. *phaseoli* var. *fuscans* were treated by Lo Cantore et al. (2009) with various components of essential oils. The phenol and alcohol components of the oils were the most effective in reducing bacterial populations on the seeds. Microorganisms are not all affected in the same way by a particular concentration of an essential oil (Riccioni and Orzali 2011; Tzortzakis and Economakis 2007). Determining the lowest effective concentration of essential oil is important because of phytotoxicity risks, including inhibition of germination and seedling growth (Kordali et al. 2008). Other plant extracts have been suggested as seed treatments (Mbega et al. 2012b; van der Wolf et al. 2008) but there is too much variation in the sources, preparation and concentrations of the active compounds to allow for reliable recommendations. Some of these studies did not obtain similar results when repeated (van der Wolf et al. 2008).

Compost mixtures and their water extracts have been investigated for bacterial spot management. Used as soil amendments, foliar sprays and seed treatments (Abbasi et al. 2002; Al-Dahmani et al. 2003; Al-Dahmani et al. 2005; Reddy et al. 2012), compost and compost extracts are generally effective against bacterial spot only when disease pressure is high. Overall yield does not always increase, but there may be a higher yield of marketable fruit because of reduced spot symptoms on the fruit.

Seed treatments. The desire for clean seed has led to decades of research on seed treatments (Carisse et al. 2000; Mbega et al. 2012a, 2012b; Walker 1948). Seed treatments may be chemical or physical, such as the use of heat. Chemical treatments, such as with hypochlorite (in chlorine bleach) or inorganic and organic acids, sanitize the seed surface while hot water treatments can also reduce bacterial populations within the seed. Hot water seed treatments are usually effective (McGee 1981; Mtui et al. 2010) especially if the infestation in the seed lot is relatively low (du Toit and Hernandez-Perez 2005). Such treatments can be difficult to conduct because of the precise temperature required to kill the pathogen without impacting the germination potential, seedling vigor and shelf life of the seed (Fatmi et al. 1991; Goode and Sasser 1980; Hopkins et al. 2003; Kordali et al. 2008; Lo Cantore et al. 2009; Nega et al. 2003; van der Wolf et al. 2008). Treating small numbers of seeds may be impractical for large-scale production and there is no guarantee that all the seeds in a large treatment batch will attain the treatment temperature for the recommended duration (du Toit and Hernandez-Perez 2005). In addition, Humaydan et al. (1980) demonstrated that hot water treatments are not always more effective than chemical disinfection.

Chlorine compounds are widely used disinfectants, especially sodium hypochlorite (NaOCl) which is the active ingredient in chlorine bleach. Clorox® brand contains 5.25% NaOCl but lower and higher concentrations are present in other brands. Sodium (or calcium) hypochlorite is also frequently used in laboratory investigations to sanitize seed or seedling surfaces in preparation for experimental work. The recommendations for the use of hypochlorite in seed treatments can vary from 0.5% to 20% in a water mixture and the duration of soaking from 2 to 40 min (Bashan 1986; Juhnke et al. 1989; Kordali et al. 2008; Ritchie and Averre 1996). In all cases, the seeds were thoroughly rinsed in water after soaking in order to minimize any negative impact on seed vitality (Khah and Passam 1992). Hypochlorite concentrations equivalent to less than 1% bleach can kill some bacterial spores within 5 to 10 minutes (Rutala et al. 2008). Organic matter (Ivancev-Tumbas et al. 1999), bacterial biofilms (Jaglic et al. 2012; Wirtanen and Salo 2003) and possibly the xanthans produced by *Xanthomonas* species (Brown et al. 1993; Maude 1996) can quickly neutralize chlorine compounds and reduce the efficacy of disinfectants. In heavily infested seeds, especially where pathogens may be clumped on the surface, the biofilm may protect some pathogens from exposure to the treatment. Therefore, hypochlorite concentrations need to be high enough to compensate for these challenges but without damaging the seed (Khah and Passam 1992).

Inorganic and organic acids have also shown effectiveness as seed treatments. The most commonly used inorganic acid is hydrochloric (Fatmi et al. 1991; Hopkins et al. 2003). Acetic acid (Borgen and Nielsen 2001; van der Wolf et al. 2008) and peracetic (peroxyacetic) acid (Hopkins et al. 2003) are the most commonly used organic acids but lactic acid and ascorbic acid also showed promising results (van der Wolf et al. 2008).

Each of the management tools discussed is only partially effective against bacterial spot. Some are not permitted in organic agriculture. Therefore, in both organic and conventional production, disease management requires an integrated approach of which seed treatments are an essential part.

Objectives

1. Investigate the *in vitro* efficacy of proposed seed treatments in reducing populations of the bacterial pathogens *Xanthomonas euvesicatoria*, *Clavibacter michiganensis* subsp. *michiganensis* and *Pseudomonas syringae* pv. *tomato*.
2. Determine the efficacy of seed treatments in eliminating *Xanthomonas euvesicatoria* populations from infested seed.
3. Determine the efficacy of seed treatments in reducing transmission of *Xanthomonas euvesicatoria* populations to seedlings.

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CHAPTER 2

**AN *IN VITRO* ASSESSMENT OF THE EFFECT OF PROPOSED SEED
TREATMENTS ON POPULATIONS OF SEEDBORNE PHYTOPATHOGENIC
BACTERIA¹**

¹ McFarquhar, J. A., and Little, E. L. To be submitted to Plant Disease.

Abstract

Two *in vitro* methods were used to determine the effects of five antimicrobial compounds, sodium hypochlorite, OxiDate 2.0, thyme oil and infusions of rosemary stem and ginkgo leaf, on populations of phytopathogenic bacteria. Bioscreen C, an automated turbidimetric system, was used to measure the population growth of three seedborne pathogens, *Xanthomonas euvesicatoria*, causal agent of bacterial spot of tomato and pepper, *Clavibacter michiganensis* subsp. *michiganensis*, causal agent of bacterial canker of tomato, and *Pseudomonas syringae* pv. *tomato*, causal agent of bacterial speck of tomato, in nutrient broth with or without the treatments. A second method exposed *X. euvesicatoria* cells to the treatments followed by a rinse in buffer and plating onto culture media. This method distinguished bacteriostatic effects of the treatments from bactericidal effects. Sodium hypochlorite killed all bacterial cells regardless of method used. No bacterial growth was observed with OxiDate 2.0 in the Bioscreen C but with the plate method, OxiDate 2.0 significantly ($P < 0.0001$) reduced bacterial populations by 52.3% but did not kill all *X. euvesicatoria* cells. The thyme oil treatment resulted in significant reductions in the growth of *X. euvesicatoria* and *C. michiganensis* subsp. *michiganensis*, but not *P. syringae* pv. *tomato*, using the Bioscreen C. Some *X. euvesicatoria* cells survived the thyme oil treatment using the plate method although there was a significant ($P < 0.0001$) reduction in population by 56.5%. The rosemary stem infusion inhibited the growth of *C. michiganensis* subsp. *michiganensis* in the Bioscreen C. *X. euvesicatoria* growth was not inhibited by rosemary stem infusion regardless of the method used. The ginkgo leaf infusion inhibited the growth of *C. michiganensis* subsp. *michiganensis* and *X. euvesicatoria* in the Bioscreen C, but there

was no inhibition of the growth of *X. euvesicatoria* in the plating method. In the case of *P. syringae* pv. *tomato*, both infusions resulted in a significant ($P < 0.0001$) increase in growth using the Bioscreen C.

Introduction

Planting material, such as seed, is often the source of primary inoculum (Baker and Smith 1966) for many bacterial diseases. Seed may carry bacterial pathogens even though the seed crop displayed no symptoms. Additionally, transplants grown from contaminated seed may also be asymptomatic (Dutta et al. 2014; Gitaitis and Walcott 2007). Seed health assays, though essential, cannot guarantee that an entire seed lot is free of pathogens (Morrison 1999; Schaad 1982). Techniques for disinfecting seeds are therefore necessary for disease management when seed is the most important primary source of inoculum. Hot water seed treatments are effective and commonly used but may be difficult for small-scale growers to conduct because of the precision of exposure time and temperature required to achieve disinfection without negatively affecting seed physiology (Fatmi et al. 1991; Goode and Sasser 1980). In addition, some studies have shown that hot water treatment may not always be 100% effective (Schaad 1982) and that chemical seed treatments may be more effective (Humaydan et al. 1980). Heat may also reduce the shelf life, germination percentage and vigor of seed (McGee 1995). Chemical treatments are often more convenient to use, although care must be taken to use the minimum concentration required for the minimum exposure time as these can also decrease seed vitality (van der Wolf et al. 2008). While the specific time and temperature of each hot water treatment is prescribed for seed from a particular crop, chemical seed

treatments may be effective against a range of pathogens (Feng et al. 2012) on different seeds. Each crop is threatened by more than one pathogen (Bajpai et al. 2011), any number of which may be carried on its seeds. Seeds may even be contaminated with microorganisms that are pathogens of another crop (Dutta et al. 2014) and this is a potential problem in rotated crops. Hence, broad-spectrum seed treatments are desirable if they are effective.

For organic systems, the use of clean seed may be even more important (Lammerts van Bueren et al. 2003). Fewer effective in-season treatments are available for organic growers and therefore prevention of disease is critical both in the seed production fields and in crop production. Organic growers should use organically produced seed when available but in cases where suitable organic seed is unavailable, organic growers are permitted to use nonorganic seed, provided it is untreated or has been treated with a method or material approved for organic use (USDA NOP 2013). Large seed companies do not appear to be meeting the needs of organic producers for a number of reasons, as discussed in the State of Organic Seed Report (OSA 2011). Organic growers also are more likely to save, trade and sell seed, and as a result, the responsibility of seed treatment for disease control is often left to the grower. Treatments that are relatively easy to prepare and apply without special equipment and that are effective against more than one pathogen are preferred. In this way, seeds can be treated with a minimum number of products thus reducing any risk of deterioration associated with seed treatments (Kordali et al. 2008; Mbega et al. 2012a).

Seed is known to be an important source of primary inoculum for important bacterial pathogens of solanaceous crops, including *Xanthomonas euvesicatoria*, causal agent of bacterial spot of tomato and pepper (Leite et al. 1995), *Clavibacter michiganensis* subsp. *michiganensis* causal agent of bacterial canker of tomato (Fatmi et al. 1991), and *Pseudomonas syringae* pv. *tomato*, the causal agent of bacterial speck of tomato (McCarter et al. 1983). In-season management of bacterial diseases is difficult (Jones et al. 2007; Lo Cantore et al. 2009). Bactericidal preparations of fixed copper are widely used to suppress bacterial disease but the effectiveness of copper compounds is limited and the repeated use of copper compounds can lead to copper accumulation in the soil (Pietrzak and McPhail 2004). Antibiotics can be effective in managing some bacterial diseases but antibiotics are expensive. Additionally, the use of antibiotics, such as streptomycin and oxytetracycline, is usually restricted to a few bacterial diseases on high-value crops (McManus et al. 2002; Stockwell and Duffy 2012). Finally, bacterial resistance to both copper and antibiotics commonly develops with repeated use (Behlau et al. 2013; Cazorla et al. 2002; Jones et al. 1991). The primary management strategy for bacterial diseases is therefore to prevent the introduction of the pathogens through the use of clean planting material (Gitaitis and Walcott 2007; McGee 1995).

Sodium hypochlorite (NaOCl) is a commonly used seed disinfectant (Benhamou et al. 1994; Carisse et al. 2000; Pernezny et al. 2002). Sodium hypochlorite is an effective broad-spectrum disinfectant because of its toxic secondary products, such as hypochlorous acid, which causes nonspecific damage to proteins (Urano and Fukuzaki 2005) and other cell components. While NaOCl can cause lethal DNA damage, pathogens on the seed surface are probably killed through cell surface damage rather than

DNA denaturation (Miché and Balandreau 2001). Sodium hypochlorite seed treatments are commonly used due to the low effective concentrations (0.52%, Sahin and Miller 1997 to 2%, Mtui et al. 2010), low cost, common availability and ease of application.

OxiDate 2.0 is labeled as a treatment for *Pythium*, *Phytophthora*, *Rhizoctonia*, *Fusarium* and *Thielaviopsis* and unspecified bacterial pathogens on or in seed. Although it is not labeled for any specific bacterial diseases and studies on the use of this product as a seed treatment were not found, its active ingredients, hydrogen dioxide (27.1%) and peroxyacetic acid (2.0%), have both been effective on phytopathogenic bacteria (Hopkins et al. 2003; Pernezny et al. 2002). At least one other commercial product with the same active ingredients (in different proportions) has been shown to be effective as a seed treatment for another bacterium (Feng et al. 2009).

Thyme oil from common thyme, *Thymus vulgaris* L., is a frequently investigated essential oil that is used mainly in flavorings and pharmaceuticals (Abozid and Asker 2013; Janssen et al. 1987; van der Wolf et al. 2008). The putative main active ingredient in thyme oil, thymol, constitutes almost 40% of the oil but other components may also have antimicrobial activity (Abozid and Asker 2013; Friedman et al. 2002). Thyme oil has a relatively high solubility in water (Ziani et al. 2011) making it possible to make mixtures that do not require emulsifiers. Various *in vitro* methods using essential oils extracted from different species of *Thymus* demonstrated effective inhibition of human and plant bacterial pathogens (Daferera et al. 2003; Lucas et al. 2012; Rasooli et al. 2006). Aqueous plant extracts have also been tested for their efficacy against phyto-bacteria (Feng et al. 2012), including their usefulness as seed treatments (Mbega et al. 2012b; van der Wolf et al. 2008). Extraction is usually made from plant parts, such as

leaves, stems, bark and rhizomes, rather than whole plants. Generally, plant extracts that significantly reduced pathogen populations had higher minimum inhibitory concentrations than effective essential oils (van der Wolf et al. 2008) but did not have a negative effect on germination (Mbega et al. 2012b; van der Wolf et al. 2008).

In vitro assays can be used to test the antimicrobial effectiveness of chemicals including plant extracts and oils (Bisignano et al. 1999), disinfectants (Gomes et al. 2001), synthetic antibiotics (Murakawa et al. 1980) and fungicides (Fenn and Coffey 1984; Hardy et al. 2001), as well as the compatibility of treatments that will be applied simultaneously (Constantinescu et al. 2014). Testing of potential seed treatments begins with *in vitro* assays of the effects of the treatments on pure cultures of the pathogen (Feng et al. 2012). Effective treatment concentrations and exposure times are then tested on contaminated seeds or susceptible plants (Weller et al. 1985).

The Bioscreen C turbidimetric system is an automated system for measuring growth using optical density (OD) and provides a reliable method of comparing treatment effects on pathogen growth over time. Like most *in vitro* assays (Janssen et al. 1987), Bioscreen C keeps the bacterial cells in constant contact with the compound, hence, this system does not distinguish between cells being killed by the treatment (bactericidal effect) and cells being temporarily suppressed in growth (bacteriostatic effect). The Bioscreen C can assay 200 samples simultaneously, allowing for a number of replicates of a range of treatments to be assayed efficiently. Bioscreen C uses optical density as a measure of growth, so although replicates may be compared to each other, the results do not provide estimates of population sizes. The color and turbidity of the treatments will influence the Bioscreen C results and, therefore, appropriate controls must be used.

Some *in vitro* methods for assessing treatment efficacy use agar plates amended with the treatment but this method may only suppress cell growth temporarily. Exposing a suspension of bacterial cells to the treatment followed by rinsing and plating cells onto culture media allows only viable cells to grow, providing a more accurate assessment of the ability of the treatment to kill the cells. The objective of this study was to ascertain the effects of proposed seed treatments (1.05% NaOCl, 0.33% thyme oil, 0.99% OxiDate 2.0, 10% (w/v) rosemary stem infusion, 10% (w/v) ginkgo leaf infusion) on the *in vitro* growth of *X. euvesicatoria*, *C. michiganensis* subsp. *michiganensis*, and *P. syringae* pv. *tomato* using the Bioscreen C turbidmetric system. A plating method developed for this study was used to determine the effects of these treatments on *X. euvesicatoria*.

Materials and Methods

Bacterial strains. *X. euvesicatoria* strain XCV 04-100 was isolated from pepper in Georgia and is pathogenic on both pepper and tomato. *C. michiganensis* subsp. *michiganensis* and *P. syringae* pv. *tomato* strain 88-84 were both isolated from tomato in Georgia (Table 2.1). The bacteria were maintained in 15% glycerol at -80°C and subcultured on nutrient yeast dextrose agar (NYDA, 8 g nutrient broth, 3 g yeast extract, 5 g dextrose, 17 g agar per liter distilled water). For each assay, bacterial cell suspensions in 10 mM MgSO₄ were prepared from three-day-old NYDA cultures of *X. euvesicatoria* and *P. syringae* pv. *tomato*, and four-day-old NYDA cultures of *C. michiganensis* subsp. *michiganensis*. The suspensions were adjusted spectrophotometrically (Spectronic 20, Bausch and Lomb, Bridgewater, NJ, USA) to 10⁸ CFU/ml. OD values had been previously determined by dilution plating onto NYDA.

Preparation of treatments. Treatments were prepared and diluted using sterile distilled water (SDW). Chlorine bleach was diluted to 1.05% sodium hypochlorite (NaOCl) (Fatmi et al. 1991; Miller and Lewis Ivey 2005). OxiDate 2.0 (BioSafe Systems, Hartford, CT, USA) is a commercial product for plant disease control labeled for seed treatment and was used at the recommended 0.99% (1:100) dilution. Thyme oil (white, Sigma-Aldrich, St. Louis, MO, USA) was diluted without an emulsifier to a concentration of 0.33% (van der Wolf et al. 2008). The infusions were prepared using rosemary stems (RS) or ginkgo leaves (GL) cut into pieces approximately 2 cm long. Ten grams of plant material was added to 100 ml distilled water in a 250 ml beaker to obtain a 10% (w/v) concentration (Mbega et al. 2012b). This mixture was heated to boiling, immediately removed from heat and allowed to cool for 10 min. Plant material was removed by filtering through two layers of cheesecloth and the filtrate sterilized by autoclaving. The infusions were stored at 4°C and used within four days of preparation.

Bioscreen C method. Suspensions of each bacterium were prepared for use in the Bioscreen C (Growth Curves USA, Piscataway, NJ, USA) by diluting to 10^5 CFU/ml using 10 mM MgSO₄. Each well of the Bioscreen C 100-well plate contained a total volume of 400 µl, of which 10% consisted of the bacterial suspension (10^3 CFU/well), 50% was nutrient broth (8 g/l) and 40% was each of the following treatments: 1.05% NaOCl, 0.99% OxiDate 2.0, 0.33% thyme oil, 10% (w/v) RS infusion or 10% (w/v) GL infusion. There were 5 wells with nutrient broth (NB) (negative control) in order to ensure that there was no contamination of the medium. Negative controls (5 wells per pathogen) included bacterial suspension (40 µl, 10^5 CFU/ml) and sterile distilled water (SDW) (360 µl) only. Positive control (10 wells per pathogen) each included bacterial

suspension (40 μ l, 10^5 CFU/ml), NB (200 μ l) and SDW (160 μ l). Wells with the treatments (8 wells per treatment per pathogen) included bacterial suspension (40 μ l, 10^5 CFU/ml), NB (200 μ l) and treatment (160 μ l). Plates were incubated in the Bioscreen C machine at 28°C with shaking at medium amplitude. Optical density measurements were taken every 30 min at 600 nm for 60 h (2.5 days).

Dilution plating method. The effective duration of exposure of the bacterium to each treatment was determined using a 10^8 CFU/ml suspension of *X. euvesicatoria* in 10 mM MgSO₄ buffer. Sodium hypochlorite was tested at 10, 20, 30 and 40 min. OxiDate 2.0, labeled for a 2 min treatment, was tested at 2 min and 10 min. The RS and GL infusions were tested at 10, 30 and 60 min. Each replicate investigated one treatment at a time with treatment duration as the only manipulated variable. Thyme oil was not included because previous studies had determined that a 30-min soak was the effective exposure time without compromising seed germination percentage (Gomah 2008; van der Wolf et al. 2008).

For each of the treatments, 1 ml of bacterial suspension (10^8 CFU/ml) in a 1.5 ml microcentrifuge tube was centrifuged at 13,000 rpm for 5 min (Hermle Z180M, Labnet, Edison, NJ, USA). The pellet was resuspended in 500 μ l of the treatment and incubated at room temperature (\approx 23°C) for the relevant duration. Timing of the application was done so that all the treatments were rinsed from the cells at the same time. Bacterial cells were then separated from the treatment by centrifugation, and the pellet was rinsed by resuspending in 500 μ l of 10 mM MgSO₄ buffer and incubating for 10 min at room temperature. The suspension was centrifuged and the pellet was again resuspended in 500 μ l of 10 mM MgSO₄ buffer. For the thyme oil treatment, the tube was mixed vigorously

by a vortexer for a few seconds every three to five minutes during the treatment and rinse to prevent separation of the mixture. Tenfold dilutions using 10 mM MgSO₄ buffer were made from each of the suspensions of rinsed cells and three 20 µl aliquots were spotted on each of four quadrants on plates of NYDA (Fig. 2.5). Plates were incubated for 24 h at 28°C (Percival Intellus environmental controller) and colonies were counted using a dissecting microscope at 10X to 15X magnification. Bacterial cells treated with SDW for 30 min served as a negative control. The experiment was repeated three times.

Data analysis. For the Bioscreen C results, the area under the growth curve (AUGC) for each replicate (each well) was computed (SigmaPlot version 13, Systat Software Inc., San José, CA, USA) using the OD readings as a relative measure of growth. An analysis of variance (ANOVA) on the AUGC values was conducted using Proc GLM in SAS version 9.2 (SAS Institute, Cary, NC, USA). The plate counts were log₁₀ transformed and used for ANOVA. Mean separation was determined by Student's t test (least significant difference, LSD).

Results

Bioscreen C. NaOCl and OxiDate 2.0 were the only treatments that completely inhibited the growth of all three pathogens based on the AUGC (Figs. 2.1 to 2.3). Thyme oil significantly suppressed the growth of *X. euvesicatoria* and *C. michiganensis* subsp. *michiganensis*, but the growth of *P. syringae* pv. *tomato* was not significantly different from that of the untreated (SDW) control ($P < 0.0001$). The AUGC was significantly higher than the positive control (NB) for both RS and GL treatments for all three bacteria except for *X. euvesicatoria* which was significantly suppressed in growth by the GL

treatment. The growth curves (Fig. 2.4) indicated inhibition of the growth of *C. michiganensis* subsp. *michiganensis* by RS infusion but no inhibition of *P. syringae* pv. *tomato* and *X. euvesicatoria*. Growth curves also showed that GL infusion inhibited the growth of *C. michiganensis* subsp. *michiganensis* and *X. euvesicatoria* while the growth of *P. syringae* pv. *tomato* was not inhibited by GL infusion.

Dilution plating assay. In preliminary results, the 10 min treatment with NaOCl was as effective as the 20, 30 and 40 min exposures. No *X. euvesicatoria* colonies were seen on the plates after 24 h for any of the NaOCl treatment times. Treatment with OxiDate 2.0 for 10 min was as effective as the labeled 2 min soak (Table 2.2). There were no significant differences in bacterial populations among the treatment times for RS and GL infusions. The longest treatment time was chosen for simultaneous treatments to give maximum exposure of the cells to the infusions.

When tested simultaneously (Fig. 2.6), NaOCl was the most effective treatment with no viable bacterial cells detected on the NYDA plates. OxiDate 2.0 reduced bacterial populations by 52.3% and thyme oil by 56.5% (Table 2.3). Infusions of RS and GL did not provide a significant reduction in bacterial cell numbers, according to Student's t test ($P < 0.0001$).

Discussion

Most *in vitro* assays for determining the antimicrobial activity of a compound use solid media amended with the compound (Mishra and Dubey 1994) or an agar overlay technique (Janssen et al. 1987) that measures zones of inhibition (Feng et al. 2012) or mycelial growth diameter (Fenn and Coffey 1984). This approach is appropriate in testing for antagonism between living organisms (Rajendiran et al. 2010), but such assays

keep the pathogen constantly exposed to the treatment and do not distinguish between bactericidal and bacteriostatic effects. Compounds for use in seed treatments must be bactericidal. Hernández et al. (2000) described a method in which the antimicrobial agent was neutralized before the treated pathogens were mixed with growth medium. In the current study, a method was developed in which the treatment compounds were rinsed from the bacterial cells before the suspension was plated onto solid medium.

In *in vitro* tests against various pathogenic bacteria 1% NaOCl was among the effective treatments with varying minimum exposure times required to kill the bacteria (Vianna et al. 2004). A gram positive bacterial species known to be resistant to NaOCl in low concentrations required 20 min exposure but gram negative species were all killed when exposed to 1% NaOCl for 10 min or less (Gomes et al. 2001; Saejung et al. 2014; Vianna et al. 2004). *X. euvesicatoria* is gram negative, so the results of this study are consistent with other *in vitro* assays using gram negative bacteria.

The highly inhibitory effects of OxiDate 2.0 observed on bacterial cells in suspension using both Bioscreen C and plating methods were not surprising given the known antibacterial effects of this product. However, the plating method indicated that OxiDate 2.0 did not completely kill all bacterial cells and may not be a good seed treatment as only a few bacterial cells left on the seeds could result in a disease outbreak (Hausbeck et al. 2000).

Preliminary investigations in this study used 0.25% thyme oil because it was the minimum inhibitory concentration for a number of bacteria (Janssen et al. 1987). This concentration only reduced *X. euvesicatoria* populations tenfold. A concentration of 0.33% was effective as a seed treatment without adverse effects on seed germination (van

der Wolf et al. 2008). This higher concentration (0.33%) increased the effectiveness of the treatment in the current study by reducing populations from 10^8 CFU/ml to 10^3 CFU/ml. Some preparations of thyme oil have enhanced solubility by using ethanol (Altundag and Aslim 2011; Tanović et al. 2013), but ethanol would also be expected to have antimicrobial effects on the pathogens. Thyme oil was therefore used without emulsifier so that any antimicrobial effects of the emulsifier could be avoided.

Mbega et al. (2012b) used RS and GL infusions to treat tomato seed infested with *X. perforans* and reported 100% reduction of the pathogen population on seeds treated with RS infusion and 94% reduction on seeds treated with GL infusion. Based on their findings, the RS and GL treatments were expected to inhibit growth significantly when used on pure cultures of the bacteria. However, this was not observed. A plant extract may be effective against a number of genetically unrelated microorganisms (Elgayyar et al. 2001; Feng et al. 2012) so the difference in bacterial species is not likely to be the reason for this inconsistency. The infusions are expected to vary in composition among preparations and different studies because of the use of different species or cultivars of the plant. Even if the same cultivar is used, the concentration of active ingredient may be different each time the infusions are prepared (Janssen et al. 1987).

RS infusion appeared to have a suppressive effect on *X. euvesicatoria* in preliminary investigations using amended media in which water in the NYDA mixture was replaced with the infusion before the medium was autoclaved (results not shown). Bioscreen C results contradicted these observations using the amended media, as the growth of *X. euvesicatoria* was not affected by the RS infusion (Fig. 2.4). The inhibition of growth of *X. euvesicatoria* by GL infusion observed with Bioscreen C appeared to be

bacteriostatic rather than bactericidal as there was no inhibition of growth observed with the plating method. Most of the AUGC values for RS and GL infusions were higher than that of the NB positive control for the same bacterium. While this suggests that the infusions enhanced the growth of the bacteria, this does not appear to always be the case (Fig. 2.4). Optical density is used by Bioscreen C as a measure of growth and is influenced by the color of the cell suspension. The greater AUGC values for the infusions, even when there was no apparent growth, appeared to be due to the higher baseline OD of the infusion mixtures, perhaps due to the brown pigment in the infusions. The infusions are poor candidates for use as seed treatments since *P. syringae* pv. *tomato* grew in both infusions and *X. euvesicatoria* grew in the RS infusion (Fig. 2.4). In addition, no inhibition of growth was observed for the infusions in the plating method (Fig. 2.6). The reason for the enhanced growth may be due to substances in the infusions that act as a food source for some bacteria.

As expected, the SDW control had the lowest AUGC values for all three bacteria. NaOCl, OxiDate 2.0 and thyme oil treatments were all colorless. Adding the colored NB resulted in higher AUGC values than the control, even if the pathogens did not grow. For more meaningful comparison among AUGC values, controls of the nutrient broth and nutrient broth with the treatments in the same concentrations as in the samples but without the bacterium should be included. The OD values from the controls could then be subtracted from the OD of the test samples to obtain more accurate AUGC values.

Dilution plating often involves using a bent glass rod to spread the bacterial suspension on the surface of a plate of agar medium. Large numbers of plates may be used with this method of bacterial cell enumeration. For *X. euvesicatoria*, colonies are not

usually visible to the unaided eye for at least three days when plates are incubated at 28°C. With the spot plate method, more than one dilution can be tested on one agar plate (Fig 2.5), which conserves media, and results can be obtained earlier than the spread plate method by using a dissecting microscope to count colonies after 24 h incubation.

Bioscreen C demonstrated the effects of treatments on bacterial growth over time and allowed comparison of all the treatments growth curves on the same graph. The shape of the growth curves may also be useful for determining the minimum treatment exposure time of the bacterium. The plating method showed the efficacy of the treatments in killing bacterial populations in a given amount of time. Plating determined whether the observed growth suppression by the treatments with Bioscreen C were bacteriostatic, only inhibiting bacterial growth, or if the effects were bactericidal, killing the cells. In addition, the plating method detected only viable cells, while the OD measurements of the Bioscreen C may have detected dead cells remaining in the mixture. Therefore, there is merit in using both Bioscreen C and plating methods for *in vitro* assays.

The results of this study indicate that sodium hypochlorite, OxiDate 2.0 and thyme oil are effective in eliminating or significantly reducing *X. euvesicatoria* populations and warrant further study as seed treatments. Treatments with RS and GL infusions were not significantly different from the untreated controls in some of the results from the Bioscreen C assay and in the plating assay. The infusions should not be further investigated as seed treatments.

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Tables and Figures**Table 2.1.** Bacterial strains used in this study.

Strain	Host	Geographic Region
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> ^a	Tomato	Georgia
<i>Pseudomonas syringae</i> pv. <i>tomato</i> 88-84 ^a	Tomato	Georgia
<i>Xanthomonas euvesicatoria</i> XCV 04-100 ^b	Pepper	Georgia

^a Strains provided by R. Walcott, University of Georgia

^b Strain provided by B. Dutta, University of Georgia

Table 2.2. Results of preliminary investigations to optimize treatment duration for *in vitro* assays on *Xanthomonas euvesicatoria* (10^8 CFU/ml in 10 mM MgSO₄). Bacterial cells were incubated in the treatments at room temperature ($\approx 23^\circ\text{C}$), rinsed in 10 mM MgSO₄ then spot plated onto NYDA. Plates were incubated for 24 h at 28°C and colonies were counted using a dissecting microscope.

Treatment	<i>Xeu</i> population (log ₁₀ CFU/ml) ^y					
	2 ^z	10	20	30	40	60
SDW (control)	-	8.58 a	-	8.45 ab	-	8.60 a
1.05% NaOCl	-	0 f	0 f	0 f	0 f	-
0.99% OxiDate 2.0	4.09 de	4.66 d	-	-	-	-
0.33% Thyme oil	-	-	-	3.72 e	-	-
10% (w/v) Rosemary stem infusion	-	7.79 abc	-	8.42 ab	-	8.24 ab
10% (w/v) Ginkgo leaf infusion	-	7.54 bc	-	7.12 c	-	7.83 abc

^y Values with the same letter are not significantly different, according to Student's t test, $P < 0.0001$.

^z Treatment duration in minutes.

Table 2.3 Effects of treatments on reduction of *Xanthomonas euvesicatoria* populations after soaking, rinsing the cells in 10mM MgSO₄ and spot plating on NYDA. Plates were incubated for 24 h at 28°C and colonies were counted using a dissecting microscope.

Treatment	log ₁₀ CFU/ml ^y	Percent reduction ^z
SDW (control)	8.58 a	-
1.05% NaOCl	0 c	100
0.99% OxiDate 2.0	4.09 b	52.3
0.33% Thyme oil	3.73 b	56.5
10% (w/v) Rosemary stem infusion	8.24 a	4.0
10% (w/v) Ginkgo leaf infusion	7.83 a	8.7

^yValues with the same letter are not significantly different according to Student's t test, P < 0.0001.

^zPercent reduction calculated by $100 - (Tc/Cc \times 100)$, where Cc is log₁₀CFU/ml of control and Tc is log₁₀CFU/ml of treatment.

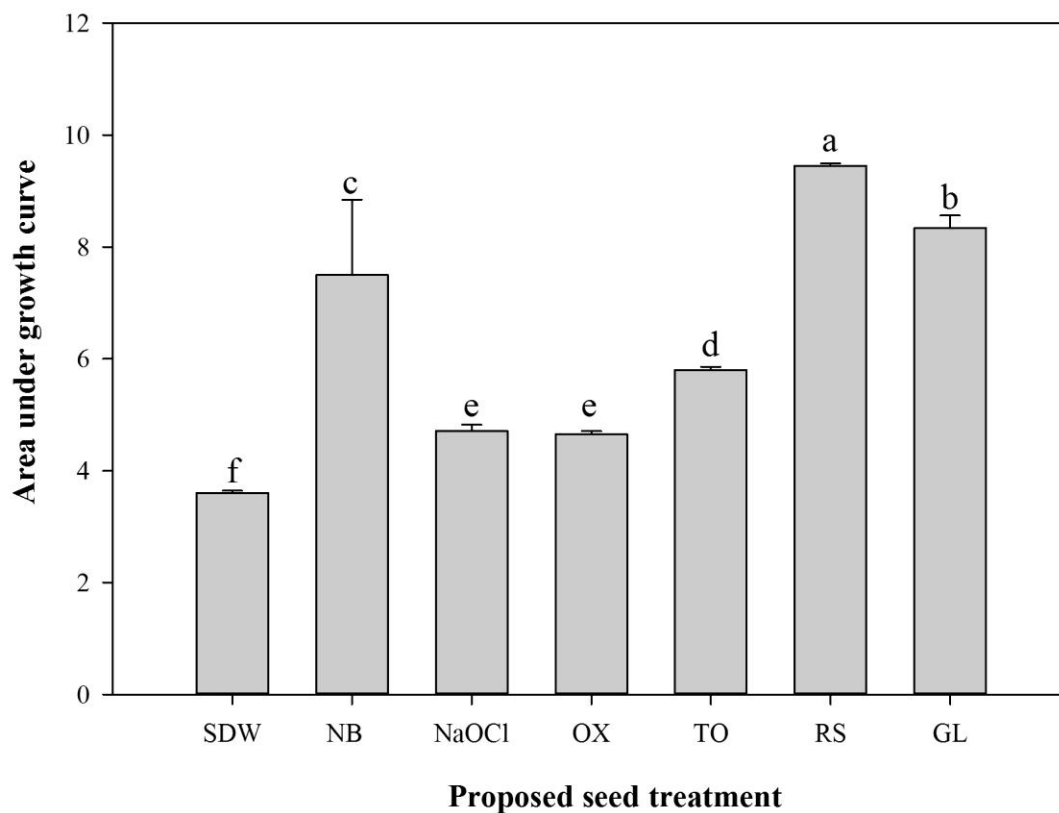


Fig 2.1. Effect of seed treatments on the growth of *Clavibacter michiganensis* subsp. *michiganensis* after incubation for 60 h using the Bioscreen C turbidimetric system. SDW: sterile distilled water (negative control), NB: nutrient broth (positive control), NaOCl: 1.05% sodium hypochlorite, OX: 0.99% OxiDate 2.0, TO: 0.33% thyme oil, RS: 10% (w/v) rosemary stem infusion, GL: 10% (w/v) ginkgo leaf infusion. Comparisons of the AUGC values here are as estimates because growth was measured as optical density and was therefore affected by the color of the NB and treatments, especially RS and GL. Bars with the same letter are not significantly different according to Student's t test, $P < 0.0001$.

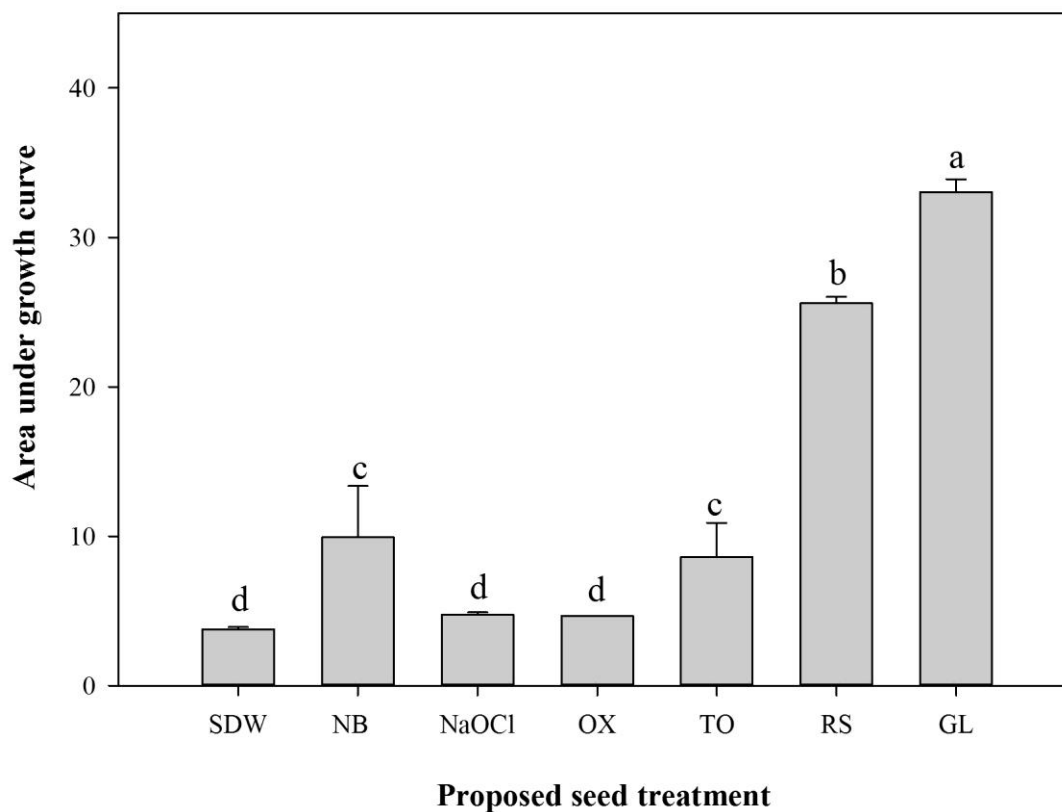


Fig. 2.2. Effect of seed treatments on the growth of *Pseudomonas syringae* pv. *tomato* after incubation for 60 h using the Bioscreen C turbidimetric system. SDW: sterile distilled water (negative control), NB: nutrient broth (positive control), NaOCl: 1.05% sodium hypochlorite, OX: 0.99% OxiDate 2.0, TO: 0.33% thyme oil, RS: 10% (w/v) rosemary stem infusion, GL: 10% (w/v) ginkgo leaf infusion. Comparisons of the AUGC values here are as estimates because growth was measured as optical density and was therefore affected by the color of the NB and treatments, especially RS and GL. Bars with the same letter are not significantly different according to Student's t test, $P < 0.0001$.

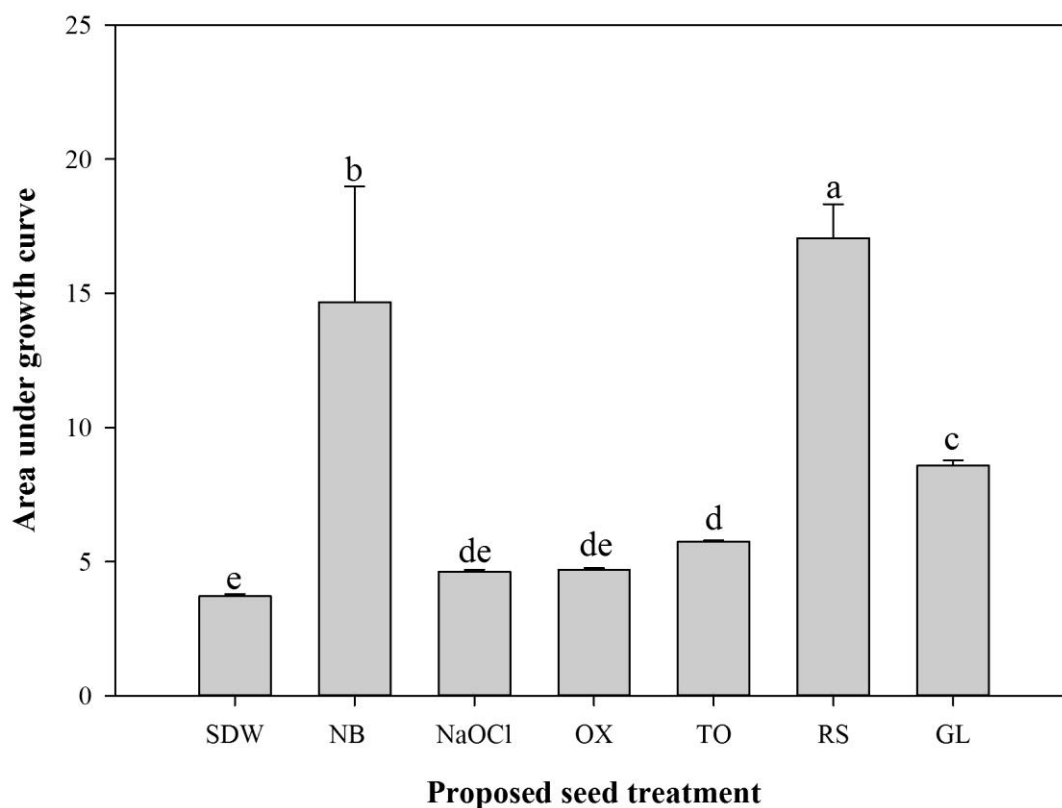


Fig. 2.3. Effect of seed treatments on the growth of *Xanthomonas euvesicatoria* after incubation for 60 h using the Bioscreen C turbidimetric system. SDW: sterile distilled water (negative control), NB: nutrient broth (positive control), NaOCl: 1.05% sodium hypochlorite, OX: 0.99% OxiDate 2.0, TO: 0.33% thyme oil, RS: 10% (w/v) rosemary stem infusion, GL: 10% (w/v) ginkgo leaf infusion. Comparisons of the AUGC values here are as estimates because growth was measured as optical density and was therefore affected by the color of the NB and treatments, especially RS and GL. Bars with the same letter are not significantly different according to Student's t test, $P < 0.0001$.

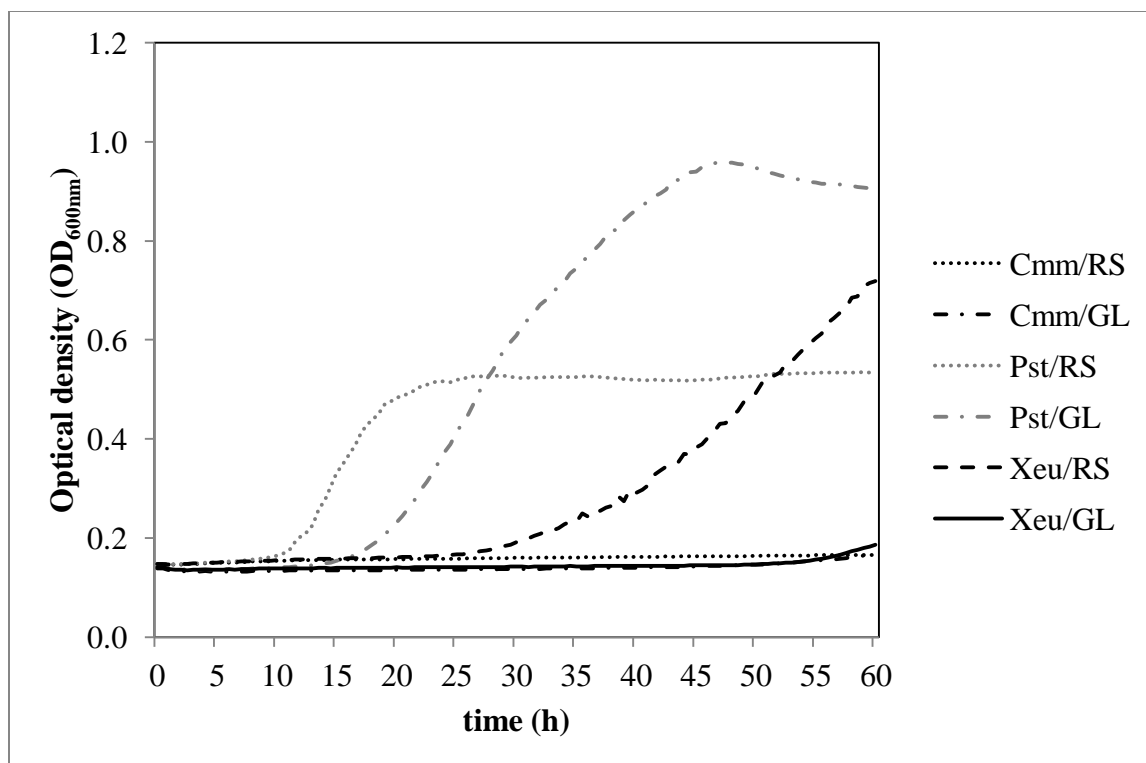


Fig 2.4. Effect of rosemary stem (RS) and ginkgo leaf (GL) infusions on the growth curves of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Pseudomonas syringae* pv. *tomato* (Pst) and *Xanthomonas euvesicatoria* (Xeu) after incubation for 60 h using the Bioscreen C turbidimetric system.

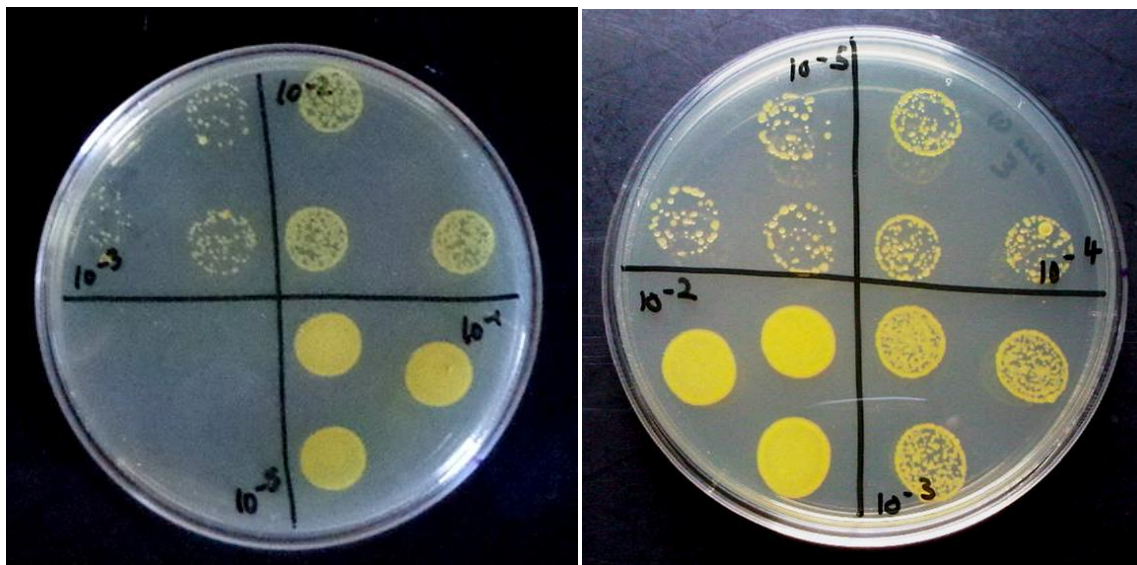


Fig 2.5. Spot plate technique used to assess the bactericidal effects of chemicals. Each spot is 20 μ l and there are three spots per dilution and four dilutions on each plate. Colonies were counted using a dissecting microscope after 24 h incubation at 28°C. Plates were left at room temperature (\approx 24°C) for an additional 3 days until colonies were visible to the unaided eye to confirm identity of the bacterium.

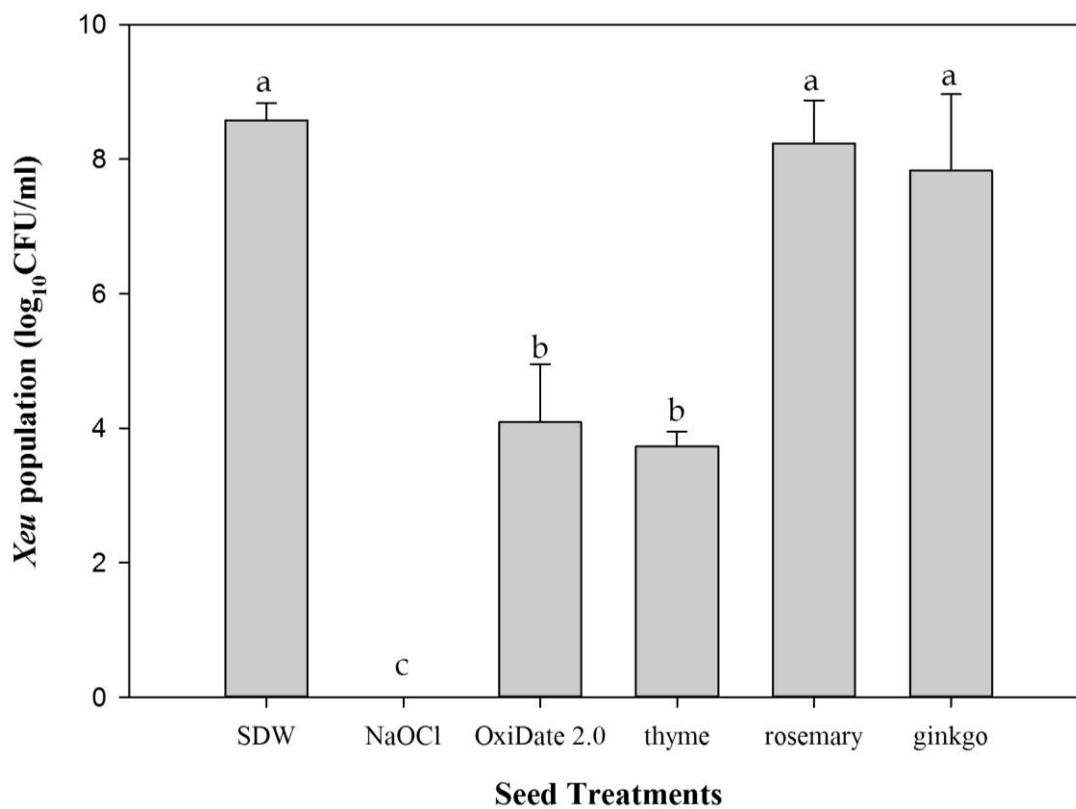


Fig. 2.6. *Xanthomonas euvesicatoria* CFU recovered after treatment using dilution plating. Cells were soaked in the proposed seed treatments, rinsed in 10mM MgSO₄ and dilutions spotted (20 μ l) onto NYDA. Plates were incubated for 24 h at 28°C. Bars with the same letters are not significantly different according to Student's t test, $P < 0.0001$.

CHAPTER 3

THE EFFECT OF ORGANIC SEED TREATMENTS ON POPULATIONS OF

***Xanthomonas euvesicatoria* ON TOMATO SEEDS²**

² McFarquhar, J. A., and Little. E. L. To be submitted to Plant Disease.

Abstract

Tomato seed was artificially infested with *Xanthomonas euvesicatoria* and subjected to one of the following seed treatments: hot water at 50°C for 25 min, 1.05% NaOCl for 10 min, 0.99% OxiDate 2.0 for 2 min or 0.33% thyme oil for 30 min. The treated seed was assayed for bacterial populations by seed wash and dilution plating. Seeds were also assayed for the effect of the seed treatment on germination. Compared to the sterile distilled water control, the hot water and NaOCl treatments reduced *X. euvesicatoria* populations on the seed to undetectable levels. OxiDate 2.0 reduced the populations of *X. euvesicatoria* by 49.8 to 80.3% and thyme oil gave 80 to 93.9% reduction. There was no significant difference in the percentage or rate of seed germination among the treatments and the controls.

Introduction

Bacterial plant diseases are very difficult to manage once they become established in the field. Resistant plant host cultivars are available for only a few bacterial diseases and major gene resistance often breaks down in the field (Clarke et al. 2014; Stall et al. 2009). Fixed copper, sometimes mixed with ethylene bisdithiocarbamate (EBDC) fungicides, is the most widely used effective chemical treatment available to manage bacterial diseases but bacterial populations readily acquire resistance to copper with repeated use (Jones et al. 1991; Spotts and Cervantes 1995). Concerns about copper accumulation in the soil and phytotoxicity on sensitive plants also restrict the use of copper sprays (Lalancette and McFarland 2007). Antibiotics such as streptomycin are

used against certain bacterial diseases in high value crops. However, the use of antibiotics is limited by the cost, concerns about the use of human antibiotics in agriculture, and antibiotic resistance development in the target bacterial populations (McManus et al. 2002; Popowska et al. 2010; Stockwell and Duffy 2012).

The initial inoculum for many bacterial diseases is often seedborne and the use of clean seed is an important part of integrated disease management (Hausbeck et al. 2000; Jones et al. 2007). Chemical seed treatments are effective and commonly used for seedborne fungal diseases (Maude 1996), but for bacterial diseases, the most commonly used seed treatment methods are hot water (Mtui et al. 2010), sodium hypochlorite (Carisse et al. 2000; Chun et al. 1997), and acid (Fatmi et al. 1991; Thyr et al. 1973). Certified organic agriculture requires the use of seed that is produced and treated in ways that are consistent with the National Organic Program (NOP) rules. Synthetic chemicals are restricted or prohibited (USDA NOP 2013). Even when conventionally produced seed is allowed in organic production, it must be untreated seed or seed treated with permitted chemicals (Lammerts van Bueren et al. 2003; US GPO 2015). Commercial vegetable seed, whether organically produced or not, is usually not treated for bacterial diseases and in many cases is not assayed for the presence of phyto-bacteria. Therefore, the grower must make the choice of whether to treat the seed. Hot water treatments, though effective, must be conducted using precise instruments to avoid damage to the seed from (Jahn et al. 2006), and small-scale growers generally lack a precise temperature-controlled water bath to administer hot water treatments.

Sodium hypochlorite (NaOCl) is often used as a seed surface disinfectant.

Effective concentrations and exposure durations need to be determined for each type of seed to minimize adverse effects on seed germination and shelf life. Studies have shown that 1% NaOCl effectively reduced seedborne fungi (du Toit and Hernandez-Perez 2005; Sauer and Burroughs 1986) and bacteria (Carisse et al. 2000; Pernezny et al. 2002). Generally, the effectiveness increased with increased soak time up to 40 min but the pathogen was not completely eliminated. A 40-min treatment could negatively affect germination (Khah and Passam 1992). Miller and Lewis Ivey (2005) recommend soaking tomato seeds in a 1.05% NaOCl mixture with surfactant for only 1 minute to control bacterial spot. The hairs and crevices on the tomato seed coat may provide areas in which the pathogen may evade treatment (Sauer and Burroughs 1986), so tomato seed may require a longer soak to ensure exposure of any pathogens in these protected areas. A 10-min soak in 1.05% NaOCl (without surfactant) was therefore used in this study. When following the NOP rules, the use of NaOCl as a seed treatment is not clearly stated and may not be allowed in some cases. Certified organic growers should get the approval of the certifying agency before using NaOCl as a seed treatment (USDA NOP 2011).

Tóbiás et al. (2007) found that acidic seed treatments were more effective than alkaline and that some essential oils successfully inhibited bacterial tomato pathogens. OxiDate 2.0 is a commercial product labeled for use as a seed treatment and is permitted under the NOP rules (OMRI 2015). Although it is not labeled for control of specific bacterial diseases, the active ingredients in OxiDate 2.0, hydrogen dioxide (peroxide) (27.1%) and peroxyacetic acid (2.0%), are effective as seed treatments for pathogenic bacteria (Buchholz and Matthews 2010; Hopkins et al. 2003; Pernezny et al. 2002).

Thyme oil from common thyme, *Thymus vulgaris* L., is a frequently investigated essential oil that is used mainly in flavorings and pharmaceuticals (Abozid and Asker 2013; Janssen et al. 1987; van der Wolf et al. 2008). The active ingredient in thyme oil, thymol, constitutes almost 40% of the oil (Abozid and Asker 2013; Friedman et al. 2002). Thyme oil has a relatively high solubility in water (Ziani et al. 2011) making it possible to make mixtures that do not require emulsifiers. Preparations of thyme oil have demonstrated antimicrobial activity *in vitro* (Abozid and Asker 2013; Riccioni and Orzali 2011), and have been effective in reducing bacterial populations when used as foliar applications (Altundag and Aslim 2011) and as seed treatments (Tóbías et al. 2007; van der Wolf et al. 2008).

Bacterial spot caused by the bacterium *Xanthomonas euvesicatoria* continues to be of the most common bacterial diseases of tomato and pepper, and seed is considered an important source of initial inoculum (Bashan and Okon 1986; Jones et al. 1986). In Georgia, bacterial spot continues to cause crop losses in tomato and pepper crops (Williams-Woodward 2013). Similar to many other bacterial phytopathogens, *X. euvesicatoria* will establish epiphytic populations on the host plant before infection (Beattie and Lindow 1995; Hirano and Upper 1983; Zhang et al. 2009). The bacterium may colonize host tissues, including seed, without producing disease symptoms (Dutta et al. 2014b; McGuire et al. 1991; Sharon et al. 1982). Therefore, scouting for the disease symptoms in seed production fields may not guarantee pathogen-free seed (Schultz and Gabrielson 1986). Since tomato and pepper seed are considered important sources of initial inoculum for outbreaks of bacterial spot in the field, the use of tomato seeds contaminated with *X. euvesicatoria* to test seed treatments would provide useful

information for growers. *X. euvesicatoria* is easily isolated and cultured on bacterial media. The pathogen can be identified using species-specific PCR primers (Moretti et al. 2009) making this bacterium a good candidate for this study.

Naturally infested seed is preferred for seed treatment investigations since the level of seed contamination would be reflective of the type of seed that growers would be purchasing and treating. However, the bacterial populations on naturally infested seed are often low (Gitaitis and Walcott 2007; Morrison 1999) and the distribution of infested seed is heterogenous within seed lots (Hadas et al. 2005) so replicated tests on subsamples may not produce comparable results. Artificially infested seed is often used to test the efficacy of seed treatments due to the ability to produce a seed lot with a consistently high level of the target bacterium (Hopkins et al. 2003; Kritzman 1991; Pernezny et al. 2002).

The objective of this study was to produce tomato seed infested with *X. euvesicatoria* and test the efficacy of seed treatments (hot water, 1.05% NaOCl, 0.99% OxiDate 2.0, 0.33% thyme oil) in reducing *X. euvesicatoria* populations on the seed.

Materials and Methods

Bacterial strain and inoculum preparation. *X. euvesicatoria* XCV 04-100 was obtained from Dr. Bhabesh Dutta at the University of Georgia, Tifton, GA and was originally isolated from pepper leaves (Dutta et al. 2014a). The bacterium was stored in 15% glycerol at -80°C and sub-cultured on nutrient yeast dextrose agar (NYDA, 8 g nutrient broth, 3 g yeast extract, 5 g dextrose, 17 g agar per liter distilled water).

The inoculum was prepared by placing a single bacterial colony from a 3-day NYDA culture in 50 ml of nutrient broth (8 g/liter, Difco, Becton, Dickson and Company, Sparks, MD, USA) in a 125 ml Erlenmeyer flask. The culture was incubated in a rotary shaker (Model G25 Incubator Shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at $29^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 200 rpm for 14 to 16 h. The bacterial cells were harvested by centrifugation for 5 min at 5000 rpm (Allegra 25R Centrifuge, Beckman Coulter, Indianapolis, IN, USA) and the pellet was resuspended in 1X PBS (phosphate buffered saline), which was prepared by a ten-fold dilution of 10X PBS (80 g NaCl, 2 g KCl, 11.5 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 2 g KH_2PO_4 per liter distilled water). The cell concentration was adjusted to 10^8 CFU/ml ($\text{OD}_{600\text{nm}} = 0.3\text{A}$) (Spectronic 20, Bausch and Lomb, Bridgewater, NJ, USA).

Seed production. In early spring 2013, tomato plants (cv. Celebrity, Johnny's Seeds, Winslow, ME, USA) were established in the greenhouse. Inoculation of the flowers began approximately 10 weeks after planting. *X. euvesicatoria* inoculum was applied to open flowers with an atomizer (DeVilbiss, Somerset, PA, USA) sanitized with 70% ethanol and rinsed with 1X PBS before filling with inoculum. The flowers were sprayed to run off each day, for a maximum of three applications per flower. This was repeated on all open flowers for four weeks. Inoculation was repeated in the fall of 2013 on greenhouse-grown tomatoes. Flowers were inoculated daily for only one week as most of the fruit harvested from the spring planting were from flowers inoculated during the first week. A third lot of tomato seeds was produced from field-inoculated tomato plants at the Tifton Vegetable Park, Tifton, GA. Transplants of tomato cultivar BHN 602 were obtained from Lewis Taylor Farms (Tifton, GA, USA) and placed in the field in mid May

2014. The plants were inoculated at flowering with a 10^6 CFU/ml suspension of *X. euvesicatoria* using a CO₂ backpack sprayer calibrated to deliver 40 gal/acre. Tomatoes were harvested 46 days after planting and transported to Athens, GA for seed extraction.

Seed extraction. Seeds from all three lots were extracted by fermentation as follows (McCormack 2004). The tomatoes were surface-disinfested with 70% ethanol (spring 2013) or 0.5% NaOCl (fall 2013), crushed by hand and left to ferment for 3 to 7 days, stirring twice daily. The fruit produced in summer 2014 were rinsed but not surface-disinfested before crushing as surface microbes may have been necessary for more efficient fermentation. Seeds were separated from the pulp and spread to air-dry for two days at room temperature. To test the effect of fermentation on *X. euvesicatoria* populations, some of the seed was not fermented. The locular jelly from the unfermented seed was removed by rubbing with a bleach-sanitized microfiber cloth. Seed was rinsed and air-dried for two days at room temperature.

Seed inoculation. The seeds from field-grown cultivar BHN 602 were vacuum infiltrated (Hadas et al. 2005; Kritzman 1991; Mbega et al. 2012) with a 10^8 CFU/ml suspension of *X. euvesicatoria* to obtain a consistently high level of detectable bacterial populations in the seed lot. A nutrient broth culture from a single colony of XCV 04-100 was grown for 14 to 16 h at 29°C then centrifuged at 5000 rpm for 10 min (Allegra 25R Centrifuge, Beckman Coulter, Indianapolis, IN, USA) and the supernatant discarded. The pellet was resuspended in sterile distilled water and the concentration was adjusted to 10^8 CFU/ml ($OD_{600nm} = 0.3A$) (Spectronic 20, Bausch and Lomb, Bridgewater, NJ, USA). The OD value had been previously determined by dilution plating onto NYDA. The tomato seeds were immersed in the bacterial suspension in a sterile 1000 ml beaker in a

vacuum chamber. Vacuum was applied to the seeds and disrupted at 15 min intervals. After each disruption, the seeds were stirred with a sanitized glass rod. This was repeated three times for a total vacuum time of 45 min. The seeds were stored at 4°C and used within one month of inoculation.

Preparation of seed treatment mixtures. Two hundred milliliters of each mixture were prepared immediately before treatment in sterile 250 ml Erlenmeyer flasks. Dilutions were prepared using sterile distilled water (SDW). Chlorine bleach was diluted to 1.05% sodium hypochlorite (NaOCl) (Fatmi et al. 1991; Miller and Lewis Ivey 2005). Thyme oil (white, Sigma-Aldrich, St. Louis, MO, USA) was diluted without emulsifier to a concentration of 0.33% (van der Wolf et al. 2008). OxiDate 2.0 (BioSafe Systems, Hartford, CT, USA) was used at the label recommended 0.99% (1:100) dilution.

Seed treatments. The concentrations of the seed treatments used were based on the results of *in vitro* assays in an earlier study (chapter 2). For each treatment, 5 g of seed was suspended in a sterile drawstring cheesecloth pouch in a sterile 400 ml beaker. All treatments, except the hot water treatment, were done at room temperature ($\approx 23^{\circ}\text{C}$) using 200 ml of each treatment to completely immerse the seeds. Seeds were soaked in SDW for 30 min (control), 1.05% NaOCl for 10 min and 0.99% OxiDate 2.0 for 2 min on a rotary shaker at 85 rpm (Innova 2100 platform shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA). The seeds were exposed to the thyme oil for 30 min on a stir plate (setting 8, Thermolyne Nuova II) to prevent the separation of the thyme oil and water suspension and maintain contact of the treatment mixture with the seeds. In compliance with label directions, seeds treated with OxiDate 2.0 treatment were not rinsed. Seeds treated with SDW, NaOCl, and thyme oil were rinsed three times in

distilled water in a 600 ml beaker with stirring (setting 8, Thermolyne Nuova II) for 12 to 15 min. Hot water treated seeds were pre-warmed in distilled water at 37°C for 10 min then immediately transferred to 50°C SDW in a shaking water bath for 25 min (Mtui et al. 2010; Miller and Lewis Ivey 2005). The seeds were immersed in SDW at 10°C to 15°C for 10 min immediately after the treatment period ended.

The seeds were spread in their loosened cheesecloth pouches on sterile 90 mm plastic petri dishes lined with sterile Whatman no. 1 filter paper and air-dried for 48 h at room temperature ($\approx 24^\circ\text{C}$). Experiments were conducted twice in a completely randomized design with four replicates.

Pathogen detection on seeds. A subsample of 3 g of seed (≈ 820 seeds) was placed in a 250 ml Erlenmeyer flask containing 100 ml peptone buffer (5.3 g KH_2PO_4 , 8.61 g Na_2HPO_4 , 1 g bactopectone per liter distilled water). The mixture was shaken at 150 rpm (Gyrotory shaker model G2, New Brunswick Scientific Co., Inc., Edison, NJ, USA) for 3.5 h at $4^\circ\text{C} \pm 1^\circ\text{C}$ followed by shaking at room temperature ($\approx 23^\circ\text{C}$) for 2 h. The wash was separated from the seed by filtering through three layers of sterile cheesecloth. The filtrate was centrifuged at 8000 rpm for 30 min (Beckman J2-21 Floor Model Centrifuge, Beckman Coulter, Indianapolis, IN, USA) and the pellet resuspended in 2 ml peptone buffer containing 20 μl Tween 80. Aliquots of 100 μl of this undiluted suspension as well as 10-fold dilutions to 10^{-4} in peptone buffer were spread onto Tween Medium B (TMB, McGuire et al. 1986) plates in triplicate and incubated at 28°C (Percival Intellus environmental controller) for 4 days. Yellow, round, glossy, raised colonies were often visible by the third day. Two to three representative colonies were isolated onto NYDA from each replicate and tested by PCR to confirm *X. euvesicatoria*.

PCR protocol. A bacterial colony from a 3-day-old NYDA plate was suspended in 500 μ l 1X PBS and heated at 95°C in a dry heat block for 10 min during which the tubes were vigorously mixed twice. PCR was carried out in a final volume of 25 μ l containing 12.5 μ l MasterMix (Qiagen), 1.25 μ l Xeu2.4, 1.25 μ l Xeu2.5, 8.5 μ l RNase free water and 1.5 μ l of the lysed cells. The PCR primers Xeu2.4 (5'-CTGGGAAACTC-ATTCGCAGT-3') and Xeu2.5 (5'-TTGTGGCGCTCTTATTTTCCT-3') primer set (Moretti et al. 2009) were diluted to 10 mM before use. The temperature profile had an initial denaturing step of 94°C for 3 min, followed by 35 cycles of a denaturing step of 94°C for 45 s, an annealing step at 64°C for 50 s, an elongation step at 72°C for 50 s, and a final elongation step of 72°C for 10 min. The samples were held at 4°C until used. A 10 μ l aliquot of each amplified PCR product was electrophoresed on a 1.5% agarose gel, at 46V for 5 min then 95V for 100 min in 1X TBE buffer, stained with ethidium bromide, and visualized on a UV transilluminator.

Germination tests. Three subsamples of 100 seeds each from the four treatment replicates were germinated on wet blotter paper in transparent boxes. Seeds were kept at 22°C \pm 1°C with 16 h light per day for 14 days (Mastouri et al. 2010). Seedlings with emerged radicles were counted and removed daily.

Data analysis. Colony counts were log₁₀ transformed before analysis. Data were analyzed as a completely randomized block. An analysis of variance (ANOVA) was conducted using Proc GLM in SAS version 9.3 (SAS Institute, Cary, NC, USA). Mean separation (t grouping) was determined by Student's t test.

Results

Determination of *X. euvesicatoria* populations in seed lots. The spring 2013 seed lot had an infestation level of 19 to 155 CFU/g of seed. Preliminary investigations assessed *X. euvesicatoria* populations on fermented seed and unfermented seed from the fall 2013 seed lot. More bacteria were recovered from the unfermented seed. The fermented seed lot had an infestation level of 2.34×10^2 CFU/g of seed and the unfermented seed had 5.3×10^3 CFU/g of seed. The level of *X. euvesicatoria* populations on the vacuum-infiltrated seed was approximately 10^7 CFU/g of seed as determined by dilution plating of seed wash onto NYDA.

Seed Wash. The populations of *X. euvesicatoria* on the untreated seed (SDW) were 1.25×10^7 CFU/g of seed in repetition 1 of the experiment and 6.5×10^6 CFU/g of seed in repetition 2. Two slightly different types of yellow colonies were seen on the SDW plates (Fig 3.1). Isolates of both types were tested by PCR and were all positive as *X. euvesicatoria* (Table 3.1). All treatments significantly reduced the *X. euvesicatoria* populations on the seeds ($P < 0.0001$) (Fig. 3.2). Hot water or NaOCl eliminated detectable populations of *X. euvesicatoria* from the seed. OxiDate 2.0 and thyme oil significantly reduced but did not eliminate *X. euvesicatoria* populations on the seeds. OxiDate 2.0 reduced bacteria on the seeds by 49.8% to 3.93×10^3 CFU/g of seed in the first repetition, while the thyme oil was significantly more effective than OxiDate 2.0 with an 80% reduction to 7.25×10^1 CFU/g of seed. In the second repetition, however, the reduction by OxiDate 2.0 and thyme oil were not statistically different, giving reductions of 80.3% to 7.83×10^1 CFU/g and 93.9% to 1.13×10^1 CFU/g respectively (Tables 3.2a, 3.2b).

Germination. Percent germination ranged from 69.5% to 77% in repetition 1 and from 71.3% to 74.5% in repetition 2. The NaOCl-treated seeds had the highest germination percentage in both experiments, but there was no significant difference between the germination percent of the control (SDW) and the treatments in both experiments according to Student's t test, $P = 0.05$ (Fig. 3.3). Federal standards require a minimum germination percentage of 75% for tomato seeds (US GPO 2011) so most of the replicates fell below the standard. This may have been because the temperature (21°C to 23°C) was below 25°C (Mastouri et al. 2010).

Discussion

Seed infestation by pathogenic phyto bacteria has been shown to be a passive process in which bacteria can colonize host surfaces, including the blossoms, and move onto the developing seed without causing disease symptoms (Dutta et al. 2014a, 2014b; van der Wolf and van der Zouwen 2010). Blossom inoculation with *X. euvesicatoria* was attempted with tomatoes in the greenhouse. However, the resulting naturally-infested seed lots were not useful to test seed treatments due to a low level of detection of *X. euvesicatoria* on the seed. The fermentation process reduces seedborne populations of *Clavibacter michiganensis* subsp. *michiganensis* (Dhanvantari 1989). Preliminary investigations comparing *X. euvesicatoria* populations on fermented seed with unfermented seed revealed more bacteria from the unfermented seed, suggesting that fermentation may also reduce populations of *X. euvesicatoria*. Leite et al. (1995) were unsuccessful in recovering plant-pathogenic xanthomonads from naturally infested tomato and pepper seeds using seed washes and plating onto yeast extract nutrient agar or

TMB. By vacuum infiltrating tomato seeds with *Xanthomonas perforans*, Mbega et al. (2012) were able to see significant effects on disease incidence and severity of the essential oils tested. Working with *Clavibacter michiganensis* subsp. *michiganensis* on tomato seeds, Hadas et al. (2005) used naturally infested seeds and seeds vacuum infiltrated with various concentrations of bacterial suspensions. They tested for infestation levels using individual seeds from each sample and found a much wider range of infestation levels in the naturally infested seed sample than the infiltrated samples. For the current study, the production of naturally infested seed was attempted by inoculation of host plant flowers with the bacterium but seed wash assays did not indicate high inoculum levels. To increase infestation homogeneity and measurability of the treatment effect, seed artificially infested by vacuum infiltration was used to test the efficacy of NOP-acceptable seed treatments in reducing seedborne populations of *X. euvesicatoria*.

The dilution plates from seed washes of NaOCl treated seeds had no detectable pathogen populations. *In vitro* assays (see chapter 2) in this study found 1.05% NaOCl for 10 min exposure to be just as effective as a 40 min soak in killing the pathogen. Studies using 1% NaOCl to disinfect vegetable seeds contaminated with bacterial pathogens, demonstrated significant reduction, but not elimination, of pathogen populations (Carisse et al. 2000; Pernezny et al. 2002). A higher concentration (2%) was used by Mtui et al. (2000) for bacterial spot pathogen on tomato seed but the exposure time was 5 min. Though there was significant reduction, the treatment did not eliminate the pathogen from the seeds. The elimination of *X. euvesicatoria* from tomato seeds in the current study supports the use of NaOCl to disinfect seeds. The relatively short treatment time means that several batches of seed can be treated per day.

The thyme oil treatment significantly reduced pathogen populations but there were still detectable levels of *X. euvesicatoria*. A number of studies report antimicrobial activity of thyme oil against fungal and bacterial pathogens (Marinelli et al. 2012; Schmitt et al. 2004) including the use of thyme oil as a seed treatment (Singh et al. 2003; van der Wolf et al. 2008). Although there were no studies found using the same protocol as in this study, the results are consistent with other seed treatment studies, which found that a treatment time of 30 min is not detrimental to germination percentage (Gomah 2008; Tinivella et al. 2009) although the pathogen may not be completely eliminated from the seed (van der Wolf et al. 2008). Thyme oil concentrations lower than 0.3% provide little control (Singh et al. 2003; van der Wolf et al. 2008) unless treatment temperature was raised (Tinivella et al. 2009). Higher concentrations and longer soak times eliminated the pathogen from the seed but may reduce germination (Mbega et al. 2012).

Overall, the OxiDate 2.0 treatment was the least effective. As a foliar treatment for bacterial or fungal disease, the product gave moderate control in field trials (Gubler et al. 2014; Howard et al. 2006). The effectiveness of seed sanitation with OxiDate 2.0 is variable. Seed treatments using either or both of the components of OxiDate 2.0 eliminated pathogens when used at concentrations higher than the label recommendation (Pernezny et al. 2002) but could be detrimental to seed vitality (Feng et al. 2009). Methods using hydrogen peroxide or a mixture containing hydrogen peroxide were observed by Montville and Schaffner (2004) to be among the most effective for seed sanitization. They concluded that the variability in treatments containing hydrogen peroxide is influenced by soak time and organism more than by concentration of the

treatments. Although OxiDate 2.0 is labelled for 2 min soak in 1:100 dilution, a variety of soak times and concentrations should be investigated for *X. euvesicatoria* in tomato to determine the best combination that reduces pathogen numbers without decreasing germination percentage.

Further investigation of the treatments tested here is warranted as they may be useful as disinfestants against bacterial phytopathogens on seed, especially NaOCl and thyme oil, as many of the dilution plates from seed washes from these two treatments had very little bacterial growth. Preparation of the treatment mixtures is simple and requires no special equipment. While thyme oil is more expensive than NaOCl, 0.33% thyme oil is only 1 teaspoonful (5 ml) thyme oil in 1515 ml water. At these low concentrations, the treatment is not costly. For pathogens that are known to occur beneath the seed coat, however, disinfestants will be insufficient and physical treatments such as hot water soak may be the best option (Fatmi et al. 1991).

The effectiveness of a seed treatment for disease management cannot be measured solely by the reduction or elimination of pathogens from the seed surface. Studies that investigate transmission from seed to seedling, the incidence and severity of disease in the field and impact on yield will help to determine seed inoculum thresholds in each pathosystem. Such investigations often involve the planting of seeds with known levels of infestation, then assessing the plants for disease symptoms and pathogen populations (Dutta et al. 2014b; Tsiantos 1987). In this study, bacteria were able to infest seeds of host and nonhost plants through blossom-inoculation and these bacteria were transmitted to the seedlings (Dutta et al. 2014b). Even within a single bacterial species that has different hosts there can be variation in the host-pathogen interactions (Jones et al. 1998).

The features of a particular host-pathogen system should not, therefore, be used to make generalizations regarding disease management of another host crop. Transmission assays are needed for specific host-pathogen systems in order to make decisions regarding the need for seed treatments based on seed health assays and more realistically determine the effectiveness of a seed treatment.

Hot water and NaOCl treatments are effective seed surface disinfestants against *X. euvesicatoria* and may be recommended for the management of bacterial spot. Although thyme oil and OxiDate 2.0 did not eliminate the pathogen from the seed surface, they significantly reduced the levels of inoculum and may help to reduce disease incidence resulting from infested seed lots. Additional management strategies such as sanitation, crop rotation and avoiding overhead irrigation should still be used. *X. euvesicatoria* is not a zero tolerance pathogen, therefore elimination of the pathogen from the seeds may not be required for a seed treatment to be considered effective.

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Tables and Figures

Table 3.1. PCR confirmation of suspected *Xanthomonas euvesicatoria* colonies isolated from seed wash spread plates. Colonies were isolated onto NYDA then stored at -80°C in 15% glycerol until grown out to be checked by PCR using species-specific primers.

Seed treatment	No. of yellow strains checked by PCR (type A + type B) ^y	Percent of strains confirmed as <i>X. euvesicatoria</i> ^z
SDW (control)	22 + 19	100 (41)
Hot water	0	0 (0)
NaOCl	2	0 (0)
OxiDate 2.0	25	100 (25)
Thyme oil	4	100 (4)

^y Fig. 3.1 shows the morphology of the colony types. The differences were noticeable only in the SDW plates. Colonies in plates from all other treatments appeared to be of type A.

^z Numbers in parentheses are the number of isolates that were PCR-positive.

Table 3.2a. *Xanthomonas euvesicatoria* populations recovered from artificially inoculated tomato seeds after treatment in experiment repetition 1. Populations were determined by seed wash and dilution plating on semiselective Tween Medium B. Values with the same letter in the same column are not statistically different, according to Student's t test, $P < 0.0001$.

Treatment	Population recovered (log ₁₀ CFU/g seed)	Percent reduction of bacterial populations
SDW (control)	7.04 a	-
Hot Water	0 d	100
NaOCl	0 d	100
OxiDate 2.0	3.53 b	49.8
Thyme oil	1.41 c	80

Table 3.2b. *Xanthomonas euvesicatoria* populations recovered from artificially inoculated tomato seeds after treatment in experiment repetition 2. Populations were determined by seed wash and dilution plating on semiselective Tween Medium B. Values with the same letter in the same column are not statistically different, according to Student's t test, $P < 0.0001$.

Treatment	Population recovered (log ₁₀ CFU/g seed)	Percent reduction of bacterial populations
SDW (control)	6.79 a	-
Hot Water	0 c	100
NaOCl	0 c	100
OxiDate 2.0	1.34 bc	80.3
Thyme oil	0.42 bc	93.9

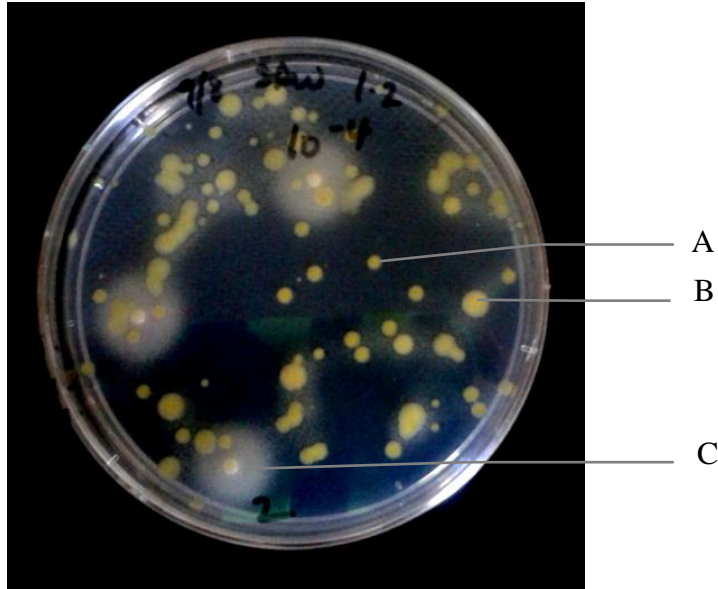


Fig 3.1. Dilution plating of seed wash from control (SDW) seeds on Tween Medium B. Counts were taken after incubation at 28°C for 3 days then the plate left at room temperature ($\approx 24^\circ\text{C}$) for 2 days so that morphologies could be seen more clearly for photographing. Three colony morphologies are shown. The yellow colonies are of two types, one (B) larger and more mucoid than the other (A), both with small, thin zones of white crystals surrounding the colonies. PCR confirmed both yellow colony types as *Xanthomonas euvesicatoria*. The third colony type (C) has a large dense zone of white crystals surrounding a creamy-white colony. Colony type C has the characteristic colony morphology described by McGuire et al. (1986) for *X. euvesicatoria* but were not yellow when isolated onto NYDA media and grew noticeably faster than *X. euvesicatoria*.

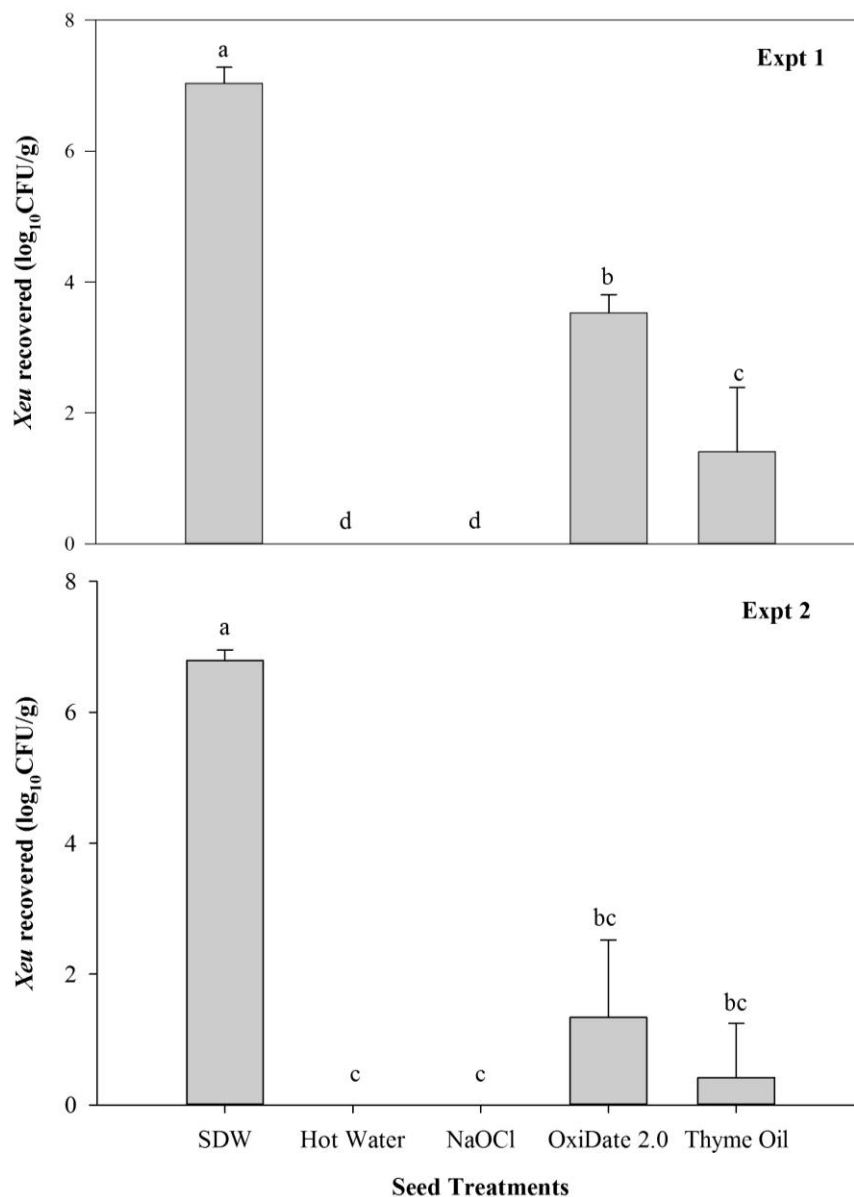


Figure 3.2. Effects of seed treatments on *Xanthomonas euvesicatoria* populations on the seed as determined by seed wash and spread plating on Tween Medium B. No viable cells were recovered from seed treated with hot water or 1.05% NaOCl. Means with the same letter are not significantly different according to Student's t test, $P < 0.0001$.

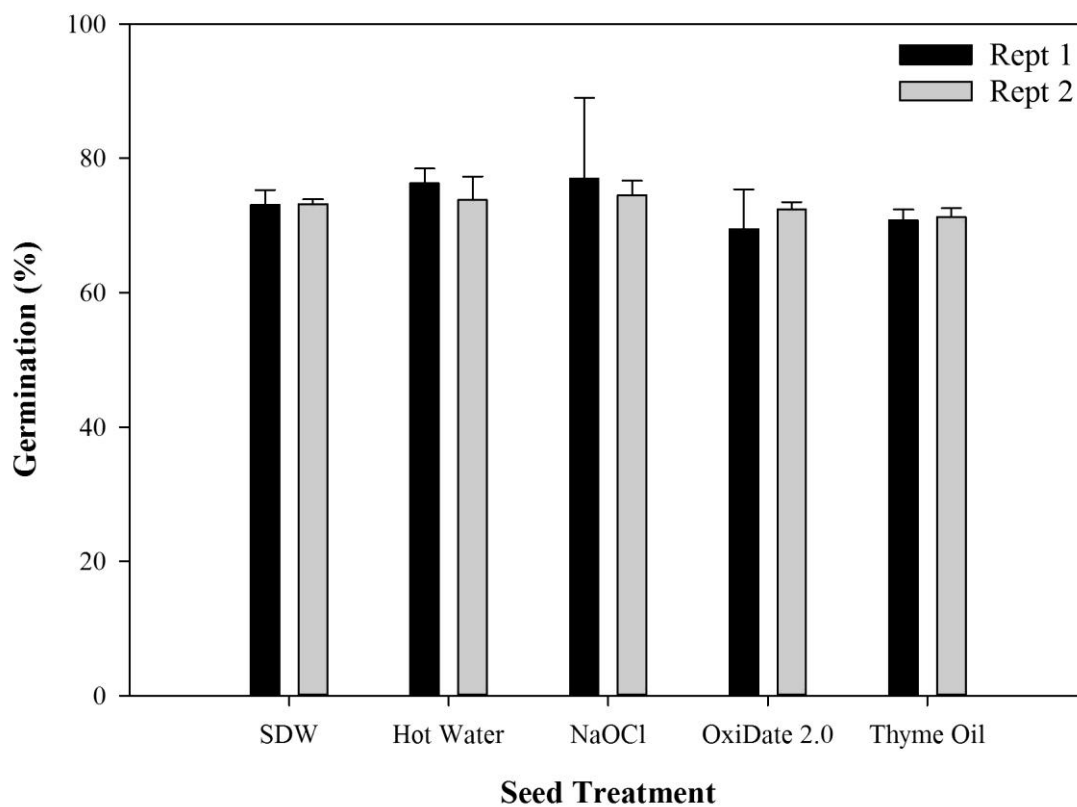


Figure 3.3. Effect of seed treatments on percent germination of tomato seed. For each treatment, three replicates of 100 tomato seeds each were kept on moist blue blotter paper for 14 days. Seeds with radicles emerged were removed and counted daily. Percent germination was calculated as (no. germinated seeds ÷ total no. of seeds) x 100. There is no statistically significant difference between the germination percent of the control (SDW) and the treatments or among the treatments according to Student's t test, $P = 0.37$ (Rept. 1), $P = 0.25$ (Rept. 2).

CHAPTER 4

**THE EFFECT OF ORGANIC SEED TREATMENTS ON THE SEED-TO-
SEEDLING TRANSMISSION OF BACTERIAL SPOT OF TOMATO³**

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Abstract

Bacterial spot transmission was assessed on shoots of 14 day-old tomato seedlings grown from *Xanthomonas euvesicatoria* infested seed that had been subjected to seed treatments prior to planting. The seedlings were grown in sterilized Magenta GA-7 vessels on water agar. The seedlings were washed in buffer and the wash buffer was assayed for bacterial populations by dilution plating on Tween Medium B and by real-time PCR. Real-time PCR detected *X. euvesicatoria* in seedlings grown from the hot water- and NaOCl-treated seed although the dilution plating did not detect any pathogen populations. Seedlings grown from OxiDate 2.0- and thyme oil-treated seed had detectable populations of the pathogen using both methods but had significantly fewer bacterial cells than the control (sterile distilled water). Comparison was also made between seed wash assays performed on the seed prior to planting and the seedling wash results. Seeds that were heavily infested gave rise to relatively large populations on the seedlings. Seeds that showed no infestation using the dilution plating method had no viable cells detected on the seedlings and had the smallest bacterial populations detected by real-time PCR.

Introduction

Seedborne diseases result from the transfer of inoculum from infected or infested seed to the germinating seedlings (Maude 1996) and under conducive environmental conditions will lead to outbreaks in the field. Bacterial plant diseases are difficult to control once established in the field (Jones et al. 2007) and initial inoculum sources must

be reduced or eliminated to prevent disease outbreaks (Gitaitis and Walcott 2007). Seed treatments are an important tool in the exclusion of seedborne pathogens. For seedborne pathogens, disease management considers not only the presence of pathogens on the seed but also the dynamics of transmission to the seedling in the particular pathosystem (McGee 1995). Transmission tests are important in determining the effectiveness of seed treatments because even if seed assays detect viable pathogens on the seed, this does not always result in disease transmission (Baker and Smith 1966). Also, pathogens may escape treatment if they are within the seed (Thyr et al. 1973) or in protected areas (Maude 1996) such as grooves on the seed surface. Bacteria often form a protective coating of adhesive exopolysaccharides (EPS) that make the cells resistant to seed washes used for pathogen detection (Danhorn and Fuqua 2007; Salcedo et al. 1992). Results of seed assays could indicate pathogen-free seed even when inoculum is present on the seed, giving false negative results.

Seedling grow out (SGO) tests are common seed health assays that are based on the expression of disease symptoms on the seedlings (Gitaitis and Walcott 2007). Symptom development in SGO tests is not reliable for assessing transmission of many bacterial pathogens because plants can be asymptomatic even when epiphytic pathogen populations are present (Bashan et al. 1985; Pernezny and Collins 1997; Rigano et al. 2007; Sharon et al. 1982). In some cases, symptoms may take several weeks to develop (Grondeau and Samson 2009) or may develop only when conducive conditions, such as high humidity, are created (McCarter et al. 1983).

Wash buffers are often used to remove pathogens from the surfaces of seedlings or leaflets for subsequent estimation of pathogen populations (Lindemann et al. 1984). Epiphytic bacteria are usually rinsed easily from the surface of leaves (Lindow and Brandl 2003) but the xanthomonad EPS, xanthan, which enhances epiphytic survival, increases adhesion to the leaf surface (Dunger et al. 2007). To increase the efficiency of bacterial removal from the shoots, a surfactant such as 0.02% Tween 80 is usually added (Hausbeck et al. 2000; Leite et al. 1995; Pillay and Nowak 1996).

Dilution plating on semiselective medium is a common method of quantifying viable cells (Jones et al. 1991; Leite et al. 1995). The target organism may be difficult to quantify when nontarget microorganisms are growing on the plates. The semiselective medium for the identification of colonies of *Xanthomonas euvesicatoria*, Tween Medium B (TMB, McGuire et al. 1986), reduces the variety of microorganisms that grow on the plates through the addition of antifungal and antibacterial chemicals. Colonies of *X. euvesicatoria* form a characteristic zone of crystals surrounding the colonies as the bacteria metabolize the fatty acids in the Tween and form calcium salts (McGuire et al. 1986). The exclusion of some microorganisms and the production of a visible metabolite make TMB useful in detecting and isolating viable *X. euvesicatoria* cells from samples containing a variety of microorganisms. Unfortunately, TMB allows growth of other xanthomonads, including nonpathogenic species and saprophytes (Sijam et al. 1991). Checking every colony by PCR to confirm its identity is not feasible and an over-estimation of pathogen populations may occur if some of these nontarget xanthomonads are included. Therefore, representative colony types must be tested for identity.

PCR amplification of the seedling wash is highly specific to the target bacterium and can be more sensitive than plating (Gitaitis and Walcott 2007; Herrera-Vasquez et al. 2009). Real-time PCR, also called quantitative PCR (qPCR), provides results in threshold cycle (C_t) values that allow for relative quantification of samples. The higher the C_t value, the lower the quantity of the target nucleic acid in the sample. A standard curve of C_t values for various known concentrations of bacterial cells can be determined and used to convert C_t values into population sizes (CFU/ml). However, qPCR may amplify DNA from nonviable target cells. Using a plating method that recovers only viable cells along with qPCR of the seedling wash provides a more comprehensive understanding of the pathogen populations on seedlings (Leite et al. 1995).

The objective of this study was to determine the efficacy of the seed treatments in reducing seed-to-seedling transmission through the assessment of epiphytic populations on the seedlings. Tomato seedlings were grown from seeds that had been previously subjected to various seed treatments to reduce populations of *X. euvesicatoria*. Seedlings were washed in buffer and the wash buffer analysed for bacterial populations using dilution plating on TMB. The wash buffer was also analysed using real-time PCR.

Materials and Methods

Seed treatments. The tomato seeds (cv. BHN 602) used to produce the seedlings in this experiment were infiltrated with a suspension of *X. euvesicatoria* and treated as described previously (see chapter 3) (Table 4.1).

Seedling growth and seedling wash. Thirty to thirty-five seeds from each treatment replicate were placed on the surface of approximately 100 ml of water agar (8 g per liter distilled water, Difco bactoagar, Becton, Dickson and Company, Sparks, MD, USA) in sterile Magenta GA-7 vessels (Sigma-Aldrich, St. Louis, MO, USA) and grown for 14 days at 21°C to 23°C with 16 h light per day (Mastouri et al. 2010) (Fig. 4.1). At day 14, a maximum of 25 seedlings per treatment replicate were assayed as follows. The shoots of the seedlings were removed aseptically, weighed in sterile 125 ml flasks and washed in sterile peptone buffer (5.3 g KH_2PO_4 , 8.61 g Na_2HPO_4 , 1.0 g bacto-peptone per liter distilled water) with 200 μl Tween 80 (BioXtra, Sigma-Aldrich, St. Louis, MO, USA) per liter added to give a final concentration of 0.02% Tween 80 (Leite et al. 1995; Pillay and Nowak 1997). Ten milliliters of buffer per gram of seedling was added to the seedling shoots (Jones et al. 1991). Seedlings were washed at room temperature ($\approx 24^\circ\text{C}$) for 30 min at 150 rpm on a platform shaker (Innova 2300 platform shaker, New Brunswick Scientific Co., Ltd., Edison, NJ, USA) (Jones et al. 1991), after which the wash buffer was decanted into sterile tubes. Serial ten-fold dilutions were made in peptone buffer without Tween 80. One hundred microliter aliquots of each dilution were spread on plates of Tween Medium B (TMB, McGuire et al. 1986) in triplicate. The plates were incubated at 28°C (Percival Intellus environmental controller) for five days and, beginning on day three, they were checked daily for the appearance of round, raised, yellow colonies with smooth margins. These were counted and one to four representative colonies from each treatment were subcultured on nutrient yeast dextrose agar (NYDA, 8 g nutrient broth, 3 g yeast extract, 5 g dextrose, 17 g agar per liter distilled water).

Conventional PCR protocol. Single colonies from 3-day-old NYDA plates of bacterial isolates taken from seedling wash plates were suspended in 500 μ l 1X PBS and heated at 95°C in a dry heat block for 10 min during which time the tubes were vigorously mixed twice. PCR amplification was carried out in a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using the *X. euvesicatoria*-specific primer set Xeu2.4 (5'-CTGGGAAACTCATTCGCAGT-3') and Xeu2.5 (5'-TTGTGG-CGCTCTTATTT-CCT-3') (Moretti et al. 2009) each diluted to 10mM before use. The reaction volume was 25 μ l containing 12.5 μ l PCR Master Mix (Qiagen), 1.25 μ l Xeu2.4, 1.25 μ l Xeu2.5, 8.5 μ l RNase free water and 1.5 μ l lysed cells. The temperature profile had an initial denaturing step of 94°C for 3 min, followed by 35 cycles of a denaturing step of 94°C for 45 s, an annealing step at 64°C for 50 s, and an elongation step at 72°C for 50 s. A final elongation step of 72°C for 10 min was run, and samples were held at 4°C until used. A 10 μ l aliquot of each amplified PCR product was electrophoresed on a 1.5% agarose gel at 46V for 5 min then 95V for 100 min in 1X TBE buffer, stained with ethidium bromide, and visualized on a UV transilluminator.

Quantitative PCR. SYBR Green-based qPCR was used for relative estimates of bacterial populations in the seedling washes. Heat-lysed cells were used because preliminary investigations showed there was no PCR inhibition by the seedling washes. A subsample of 1.5 ml was taken from each seedling wash and heated at 95°C for 10 min, during which the subsamples were vigorously mixed twice. After cooling on ice, the subsamples were concentrated 30-fold by centrifugation of at 13,000 rpm for 6 min (Hermle Z180M, Labnet, Edison, NJ, USA), and the pellet resuspended in 50 μ l of sterile Millipore water. Each PCR reaction of 20 μ l contained 10 μ l Power SYBR Green PCR

master mix (Applied Biosystems by Life Technologies, Warrington WA1 4SR, UK), 2 μ l each of primers Xeu2.4 and Xeu2.5, 4 μ l sterile Millipore water and 2 μ l of the concentrated seedling wash sample. Real-time PCR was carried out in 96-well plates in StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following thermal profile: denaturation at 94°C for 3 min and 35 cycles each of denaturation at 94°C for 45 s, annealing at 64°C for 50 s, and extension at 72°C for 50 s (Dutta et al. 2014; Moretti et al. 2009). A standard curve for the estimation of *X. euvesicatoria* populations was constructed using 10-fold dilutions of *X. euvesicatoria*, strain XCV 04-100. The dilutions were prepared from a single colony of *X. euvesicatoria* from a 3-day-old NYDA culture, grown for 14 to 16 h in 50 ml nutrient broth at 29°C at 200 rpm. The broth culture was centrifuged at 5,000 rpm for 5 min, the pellet was resuspended in sterile Millipore water and the suspension adjusted to 10^8 CFU/ml ($OD_{600nm} = 0.3A$) spectrophotometrically (Spectronic 20, Bausch and Lomb, Bridgewater, NJ, USA). Serial 10-fold dilutions to 10^2 CFU/ml were made using sterile double distilled water. Real-time PCR analysis of each dilution was completed using the reagents, quantities and thermal profile as described above for the seedling wash samples.

Data analysis. Data from dilution plates and qPCR were \log_{10} transformed before analysis and expressed as CFU/g shoot tissue. An analysis of variance (ANOVA) was conducted using Proc GLM in SAS version 9.3 (SAS Institute, Cary, NC, USA). Mean separation was determined by Student's t test (least significant difference, LSD).

Results

Shoot weight. The fresh weight of seedling shoots ranged from 25 to 33 mg with averages from 26 to 30 mg. The differences in the fresh weights among the treatments were not statistically significant according to Student's t test, $P > 0.11$.

Dilution plating. All treatments significantly reduced pathogen populations on the seedlings compared to the controls (Fig. 4.2). No viable cells were recovered from the seedlings of hot water- or NaOCl-treated seeds in both repetitions of the experiment. The thyme oil treatment eliminated all detectable bacterial populations on the seedlings in the second repetition but bacterial populations (27.6 CFU/g) were recovered in the first repetition (Table. 4.2). Bacterial populations were recovered from the OxiDate 2.0 treatment in both repetitions (1.35×10^3 CFU/g in rept. 1, 1.35×10^2 CFU/g in rept. 2) (Table 4.2, Fig. 4.2). The population numbers recovered from the OxiDate 2.0 treatment were not significantly different from the numbers recovered from the thyme oil treatment.

Samples of yellow colonies with smooth margins were isolated from each treatment onto NYDA. Except for the SDW control where 5 of the 39 strains tested were PCR negative, and the NaOCl treatment where neither of the two strains were positive, all colonies checked by PCR were confirmed as *X. euvesicatoria* (Table 4.3). Most of the plates from the NaOCl treatments had little bacterial growth. The plates from the SDW and hot water treatments had several colonies with the "fried-egg" appearance described by McGuire et al. (1986) as characteristic of *X. euvesicatoria* growing on TMB. Sijam et al. (1991) noted that other bacteria may produce colony morphology similar to *X. euvesicatoria* on TMB. These strains grew faster than *X. euvesicatoria* on both TMB and NYDA, were not yellow on either medium, and all 11 strains were PCR negative.

Real-time PCR. Populations estimates were calculated from the equation for the standard curve ($y = -3.1376x + 40.552$) generated by known concentrations (10^2 to 10^8 CFU/ml) of *X. euvesicatoria* ($R^2 = 0.9934$, efficiency = 1.0831). In the first repetition, the mean number of *X. euvesicatoria* cells detected ranged from 1.41 CFU/g shoot tissue (NaOCl treatment) to 27.5 CFU/g shoot tissue (control) but these differences were not significant at $P = 0.05$ (Table 4.2). Hot water treatment seedlings had populations (2.88 CFU/g) slightly higher than the NaOCl treatment seedlings, and the OxiDate 2.0 (9.0 CFU/g) and thyme oil (8.27 CFU/g) treatments had the highest populations of all the treatments, not including SDW. In the second repetition, hot water was the most effective treatment with 1.29 CFU/g shoot tissue, compared to 135 CFU/g shoot tissue in the control. Seedlings from NaOCl and OxiDate 2.0 treatment had similar populations (7.18 CFU/g and 7.16 CFU/g, respectively). Except for the SDW control, seedlings from thyme oil treatment had the largest populations (15.3 CFU/g).

In samples from both repetitions, qPCR detected *X. euvesicatoria* on seedlings from seed treated with hot water and NaOCl in approximately half the replicates (Table 4.4) even though *X. euvesicatoria* was not detected in these treatments using the plating method (Fig 4.2). For the control, OxiDate 2.0 and thyme oil treatments, the numbers of bacterial cells detected using qPCR were lower than in the plating assay (Table 4.4). In both repetitions of the experiment, bacterial cells recovered by plating the wash buffer from the SDW (control) treatment was 10^7 CFU/g shoot tissue while qPCR only estimated 10^1 to 10^2 CFU/g shoot tissue (Figs. 4.4a, 4.4b). In the OxiDate 2.0 treatment, the plating assay detected about 100 times the number of cells per gram than were detected by qPCR. In the first repetition of the thyme oil treatment, plating detected 27.6

CFU/g while qPCR detected 8.27 CFU/g, and in repetition 2 plating did not yield any viable *X. euvesicatoria* cells while qPCR detected 15.3 CFU/g shoot tissue (Table 4.4).

In both plating and qPCR assays of the seedling washes, seedlings from treated seed had smaller *X. euvesicatoria* populations than the control seeds, but this was not always statistically significant (Table 4.4).

Discussion

Seed to seedling transmission of phytopathogenic bacteria often results in the establishment of epiphytic populations before the pathogen colonizes internal tissues and produces disease symptoms (Sharon et al. 1982). Therefore, quantifying epiphytic populations may serve as an indicator of the risk of disease incidence or severity (Lindemann et al. 1984; Stromberg et al. 1999; Umesh et al. 1998) provided that the impact of environmental variables (Hirano and Upper 1983; Schaad 1988) and risk for dispersal from infected plants (Darrasse et al. 2007; McInnes et al. 1988; Schaad et al. 1980) are taken into account. Seeds with the highest *X. euvesicatoria* populations gave rise to the seedlings with the highest epiphytic populations. Seed lots treated with hot water and NaOCl had no viable cells of *X. euvesicatoria* detected by plating and, in most cases, gave rise to the lowest qPCR-detectable populations. According to Moretti et al. (2009) the detection limit of the PCR protocol was 2×10^2 CFU/ml. The results obtained to construct the standard curve supported this, as in 35 cycles some replicates of the 10^2 CFU/ml samples had threshold cycle (C_t) values of 33 or above. The lowest pathogen level detected was from a hot water treatment that contained 6.82×10^1 CFU/ml of

concentrated seed wash. Based on the detection limits of the qPCR protocol, the pathogen would not have been detected by qPCR in this sample had it not been concentrated.

Concentration of the samples, therefore, increased the chances of detecting the pathogen.

Comparing dilution plating and qPCR results for hot water and NaOCl treatments in both repetitions and the thyme oil treatment in repetition 2, the qPCR method was more sensitive. Also, qPCR detected cells in a larger proportion of samples than dilution plating. Unexpected results were seen in the control (SDW) and OxiDate 2.0 treatments of both repetitions and the thyme oil treatment in repetition 1, in that the qPCR results gave lower pathogen numbers than the plating assays. This difference was greatest in the controls in repetition 2, with plating assays yielding 10^7 CFU/g shoot tissue and qPCR detecting 10^2 CFU/g. Cells may have been lost in the removal of the supernatant after centrifuging in the process of concentrating the samples, but this would have affected all samples as they were concentrated in the same manner. The dilution plating may have overestimated the populations. Although the majority of isolates suspected to be *X. euvesicatoria* tested positive by conventional PCR, the two yellow colonies isolated from the NaOCl treatments were PCR negative. Other yellow colonies may have been counted that were not *X. euvesicatoria*.

The qPCR method detected *X. euvesicatoria* on seedlings that were grown from hot water- and NaOCl-treated seeds even though no viable cells were detected when the seeds were assayed following treatment (data not shown, see chapter 3). One explanation may be that qPCR detected the DNA from nonviable cells, i.e. false positives.

Alternatively, some bacterial cells may have been located internally thus escaping a seed soak treatment. Vacuum infiltration of the seeds with *X. euvesicatoria* may have

deposited cells under the seed coat. The NaOCl treatment would only affect pathogens on the surface of the seed. Sauer and Burroughs (1986) found that NaOCl treatments were sometimes ineffective because hairs, cracks and bubbles on the seed surface protected the pathogen from the treatment. In addition, wash assays may not remove all the cells from the seed surface (Schaad 1982) due to hairs on the surface of tomato seeds and the protective xanthan exopolysaccharide produced by xanthomonads (Dunger et al. 2007). Combined, these factors allow pathogen cells to escape the NaOCl seed treatment, and to remain on the seed after washing, later colonizing the seedlings (McGuire et al. 1991). Hot water treatments are effective because they can kill bacteria within the seed but they do not always eradicate the pathogen from the seed (Schaad 1982). Some bacterial cells may have remained alive in the hot water treatment if some of the seeds were not exposed to the correct temperature for the duration of the treatment. Slightly more than half of the replicates had no detectable pathogens using qPCR but this does not mean that half the seeds were infested. Since the seedlings from each vessel were assayed in bulk, the bacterial cells detected in the positive replicates could have originated from only one or a few seeds in each Magenta vessel.

Despite the addition of cycloheximide and antibiotics to the medium (TMB), background growth of fungi and non-xanthomonad-like bacteria was observed. In the plates from the control (SDW) seedling wash, there were a variety of bacteria-like organisms. The plates from the hot water and OxiDate 2.0 treatments had an abundance of one common bacterium-like growth. The OxiDate 2.0 treatment had significant fungal growth on many of the plates. In a number of plates, the nontarget microorganisms prevented the isolation of suspected *X. euvesicatoria* colonies for PCR confirmation. This

could explain why the pathogen was not detected on plates from the hot water-treated seed or seedlings, yet was detected by qPCR. The plates from the NaOCl and thyme oil treatments had the lowest diversity of microorganisms, with some of the plates having no visible growth.

Further assays are needed to determine if the low population numbers estimated using qPCR in the samples from hot water- and NaOCl-treated seed will result in unacceptable levels of bacterial spot in the field. Enumeration of epiphytic populations on individual seedlings grown in isolation (to prevent seedling to seedling transfer) before planting in the field would determine if epiphytic population numbers are a useful predictor of disease incidence and severity in the field (Lindemann et al. 1984; Stromberg et al. 1999; Sudisha et al. 2006). Under conducive environmental conditions, epiphytic populations can increase rapidly resulting in disease outbreaks. On spray-inoculated pepper leaves, for example, epiphytic populations of the bacterial spot pathogen increased from 10^3 CFU/g leaf tissue to almost 10^8 CFU/g in 3 days (Sharon et al. 1982). The results of seed to seedling transmission studies should be correlated with environmental factors such as temperature and moisture (Schaad 1988; Umesh et al. 1998) so that reliable seed inoculum thresholds can be determined.

The seed inoculum threshold for preventing significant disease outbreaks has not been determined for *X. euvesicatoria*. Jones et al. (1986) reported a detection limit of one infested seed in 1,000 (0.1%) but there was no indication that this was the inoculum threshold for bacterial spot of tomato. A threshold of one infested seed in 10,000 (0.01%) was determined by Schaad et al. (1980) to be the maximum level of contamination to prevent black rot of crucifers caused by *X. campestris* pv. *campestris*. Grondeau and

Samson (2009) found the threshold for *Acidovorax valerianellae* in corn-salad seeds to be 0.1% to 0.5% infected seed with between 10^2 and 10^4 CFU/g seed required for severe disease outbreaks. Umesh et al. (1998) found that 10^4 CFU/g seed was the contamination level that produced bacterial blight (*Xanthomonas campestris* pv. *carotae*) in carrots but considered this a high threshold, noting that the climatic conditions were not conducive to disease development. For *Clavibacter michiganensis* subsp. *michiganensis*, a transmission level of one seed in 10,000 (0.01%) could lead to a serious epidemic (Hausbeck et al. 2000). Darrasse et al. (2007) showed that for *X. axonopodis* pv. *phaseoli* transmission did not occur in naturally infested seed with inoculum levels below 10^3 CFU/seed and transmission rates as high as 0.1% produced plants that were asymptomatic although contaminated with epiphytic populations. Schaad (1982) reported that a bean seed certification scheme in Michigan for *X. axonopodis* pv. *phaseoli* established 0.005% or fewer symptomatic seed plants as an acceptable level during field inspections but there was a zero tolerance for the pathogen in seed test assays. In the seed assays, surface-disinfested beans were soaked in water for 24 h and the leachate injected into healthy seedlings, which were later observed for disease symptoms.

Fewer than 600 seedlings were assayed per treatment in the current study and *X. euvesicatoria* was detected by qPCR in all treatments, though not in all replicates. If the inoculum in each Magenta vessel of seedlings came from a single seed, then at least 1.8% of the seeds were infested (11 infested seeds in 600) in the hot water treatment. This is higher than any of the thresholds outlined above, indicating that although the treatments reduced pathogen populations on the treated seed, they may be inadequate on their own for preventing transmission and the subsequent development of disease.

Due to the important of seedborne inoculum in disease development, research on seed treatments to prevent bacterial disease needs to continue. In this study, NaOCl seed treatment was as effective as hot water treatment and warrants further investigation. Thyme oil and OxiDate 2.0 were less effective at eliminating the pathogen from seeds and are probably not useful as seed treatments. Future research could vary treatment concentrations and soak durations or use vacuum infiltration of treatments. Pretreatment of the seeds to decrease the hydrophobic surfaces increased the contact of the NaOCl treatment with the seed surface (Sauer and Burroughs 1986) and improved seed treatment effectiveness. Seed sanitization with NaOCl followed by application of a biological control as seed treatment (Kavitha and Umesha 2007) may also improve disease control, as well as combining seed treatments simultaneously or sequentially. Kritzman (1993) showed that a chemi-thermal treatment reduced the treatment time of tomato seeds from 25 min to 10 min and the temperature from 50°C to 45°C and eliminated the bacterial spot pathogen. Even with improvements to seed treatments, cultural practices and sanitation are still needed to reduce additional sources of initial inoculum and pathogen spread that may lead to disease outbreaks.

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Tables and Figures

Table 4.1. Seed treatments applied to tomato seeds used for the seedling grow out tests. Each treatment had four replicates and the experiment was conducted twice. For each of these replicates, three subsamples of 30 to 35 seeds each were grown in 0.8% water agar in sterile Magenta vessels for 14 days.

Seed treatment	Duration (min)
Sterile distilled water (SDW) (control)	30
Hot water (50°C) ^a	25
1.05% NaOCl	10
0.99% OxiDate 2.0	2
0.33% Thyme oil	30

^a Seeds were preheated at 37°C for 10 min before treatment and cooled at 13°C ± 2°C immediately after treatment duration.

Table 4.2. Effect of seed treatments on the transmission of *Xanthomonas euvesicatoria* from seed to seedling. The number of pathogens detected by plating and real-time PCR from seedling wash samples is given as CFU/g shoot tissue.

Seed treatment	<i>Xeu</i> detected by dilution plating (CFU/g) ^a		<i>Xeu</i> detected by real-time PCR ^b (CFU/g) ^a	
	Repetition 1	Repetition 2	Repetition 1	Repetition 2
SDW	2.15 x 10 ⁷	1.23 x 10 ⁷	2.75 x 10 ¹	1.35 x 10 ²
Hot water	0	0	2.88	1.29
NaOCl	0	0	1.41	7.18
OxiDate 2.0	1.35 x 10 ³	1.35 x 10 ²	9.0	7.16
Thyme oil	2.76 x 10 ¹	0	8.27	1.53 x 10 ¹

^aCFU/g calculated by converting log₁₀ averages back to numbers.

^bSamples were concentrated 30-fold before PCR amplification.

Table 4.3. PCR confirmation of suspected *Xanthomonas euvesicatoria* colonies isolated from seedling wash spread plates. Colonies were stored at -80°C in 15% glycerol until suncultured to be checked by PCR.

Seed treatment	No. of yellow colonies checked by PCR	Percent of strains confirmed as <i>X. euvesicatoria</i>
SDW	39	87.2 ^a
Hot water	1	0
NaOCl	2	0
OxiDate 2.0	22	100
Thyme oil	3	100

^a 5 yellow colonies did not have *X. euvesicatoria* morphology when isolated but were checked. None of these were PCR-positive.

Table 4.4. Percentage of seedling wash replicates in which *Xanthomonas euvesicatoria* was detected by dilution plating and real-time PCR. Each of the 5 treatments had 4 replicates. For each of these replicates, 3 subsamples of seedlings were assayed, giving 24 replicates for each treatment across both repetitions of the experiment.

Seed treatment	Percent replicates with <i>Xeu</i> detected by plating	Percent replicates with <i>Xeu</i> detected by real-time PCR
SDW	87.5 (21 of 24) ^a	95.8 (23 of 24)
Hot water	0 (0 of 24)	45.8 (11 of 24)
NaOCl	0 (0 of 24)	54.2 (13 of 24)
OxiDate 2.0	25 (6 of 24)	79.2 (19 of 24)
Thyme oil	4 (1 of 24)	62.5 (15 of 24)

^a Numbers in parentheses are the number of replicates in which *Xeu* was detected.



Fig 4.1. Tomato seedlings growing in a Magenta vessel. Thirty to thirty-five seeds were placed on 0.8% water agar in each vessel and grown in the closed vessels for 14 days at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with 16 h of light per day. Shoots from a maximum of 25 seedlings from each vessel were washed with peptone buffer with 0.02% Tween 80 added. The seedling washes were assayed by dilution plating and real-time PCR.

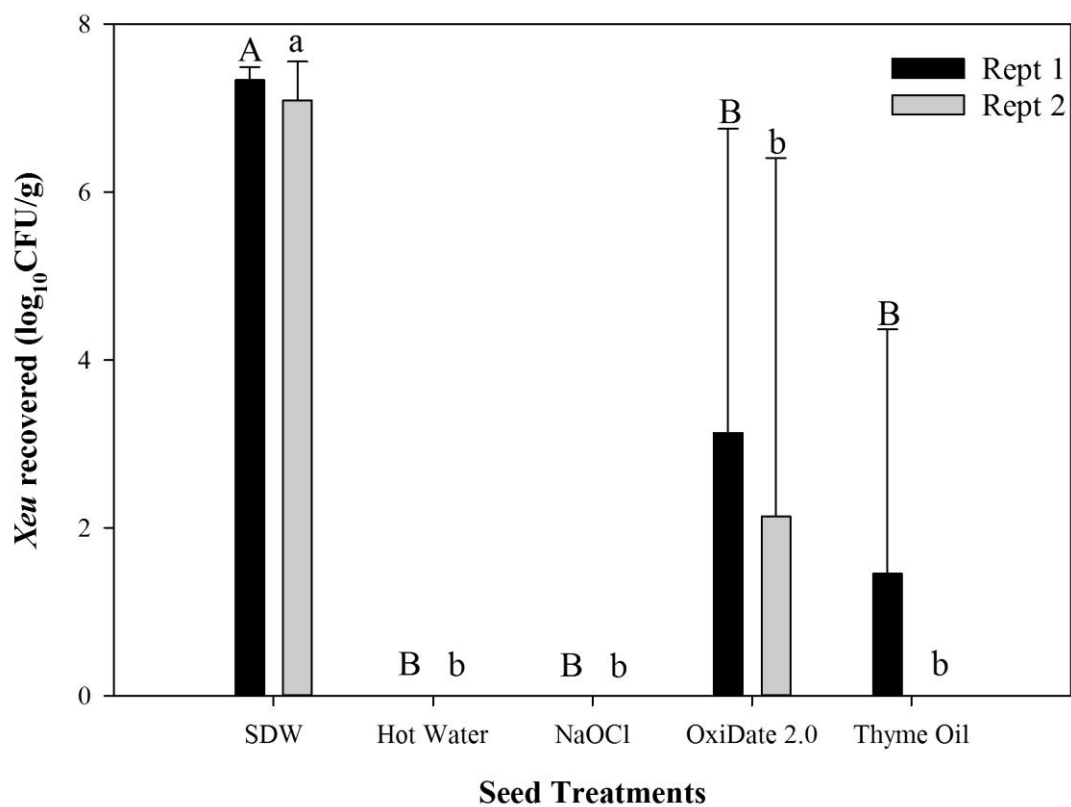


Figure 4.2. Populations of *Xanthomonas euvesicatoria* on 14-day-old tomato seedlings grown from treated seeds, determined by dilution plating of seedling washes onto Tween Medium B. Bars with the same letters are not significantly different, according to Student's t test, $P = 0.0008$ (Rept. 1), $P = 0.0003$ (Rept. 2).

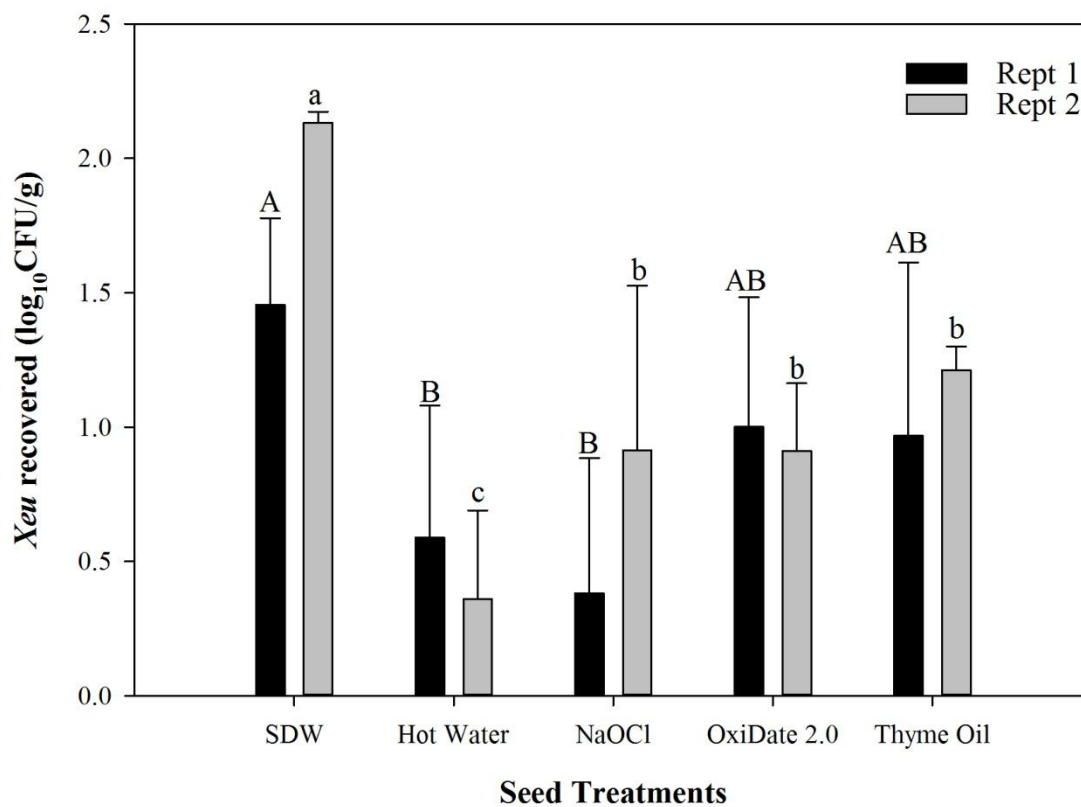


Figure 4.3. Populations of *Xanthomonas euvesicatoria* on 14-day-old tomato seedlings grown from treated seeds, determined by real-time PCR of concentrated seedling washes. Bars with the same letters are not significantly different, according to Student's t test, $P = 0.07$ (Rept. 1), $P = 0.0001$ (Rept. 2).

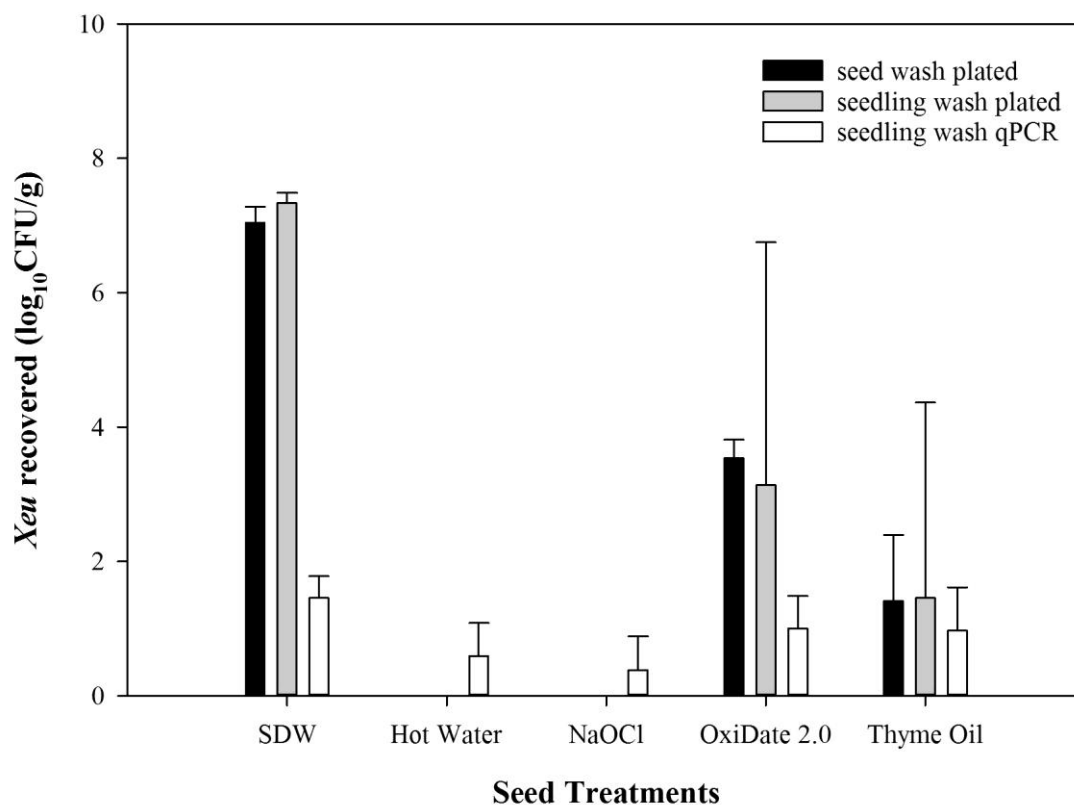


Figure 4.4a. Comparison of seed wash and seedling wash assays from repetition 1. Seed treatments were applied to tomato seed that were vacuum infiltrated with *Xanthomonas euvesicatoria*. Dilution plating of seed wash (black bars) was used to estimate bacterial populations. Treated seed were grown for 14 days and seedling shoots washed in buffer. Seedling wash buffer was assayed for *X. euvesicatoria* by dilution plating (gray bars) and quantitative PCR (qPCR) (white bars). Plating of the seed and seedling washes was done on Tween Medium B (TMB).

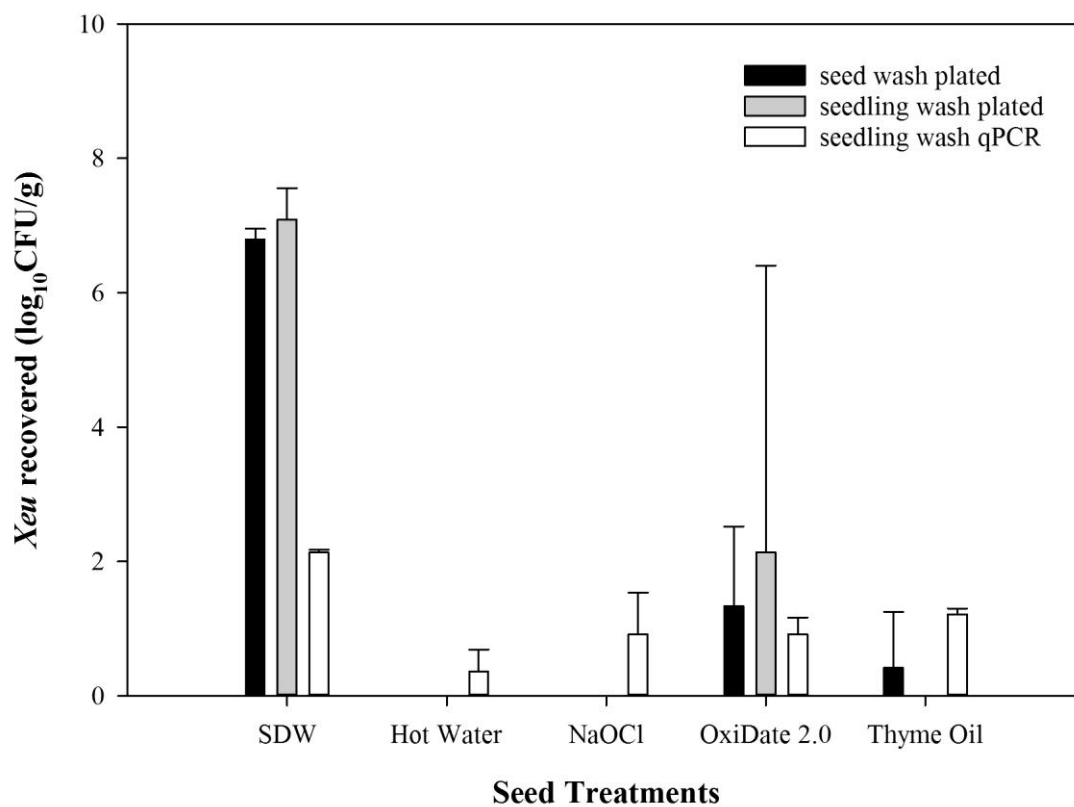


Figure 4.4b. Comparison of seed wash and seedling wash assays from repetition 2. Seed treatments were applied to tomato seed that were vacuum infiltrated with *Xanthomonas euvesicatoria*. Dilution plating of seed wash (black bars) was used to estimate bacterial populations. Treated seed were grown for 14 days and seedling shoots washed in buffer. Seedling wash buffer was assayed for *X. euvesicatoria* by dilution plating (gray bars) and quantitative PCR (qPCR) (white bars). Plating of the seed and seedling washes was done on Tween Medium B (TMB).

CHAPTER 5

SUMMARY AND CONCLUSION

Organic agriculture is a steadily growing sector fuelled by the desire for sustainable food production, ecological health and healthful foods. The USDA National Organic Program (NOP) and other regulatory bodies worldwide have specified that organic growers use organic seed (Le Buanec 2004; USDA NOP 2013). Where organic seed is unavailable, the NOP allows certified growers to use nonorganic seed that is untreated or has been treated with materials or processes permitted for organic systems (USDA NOP 2013). Seed treatments are necessary to prevent seedborne diseases (Lammerts van Bueren et al. 2003). Bacterial phytopathogens in particular are difficult to control and preventing the introduction of inoculum into the field is the primary approach to disease management (Gitaitis and Walcott 2007).

Seed treatment for bacterial phytopathogens can be physical, usually using heat, biological, or chemical, for which organic acids and essential oils are the most popular in organic systems (Le Buanec 2004). Hypochlorites such as sodium hypochlorite (NaOCl), the active ingredient in chlorine bleach, are allowed with restrictions (USDA NOP 2011). Heat treatments are effective, especially if the pathogen is inside the seed, but require precision and special equipment to prevent heat damage to the seed. Seed treatments using organic acids and essential oils are usually easy to prepare and apply without special equipment but care must be taken to use the lowest effective concentration and exposure time to avoid phytotoxic effects (Mbega et al. 2012).

This study showed that hot water, NaOCl, OxiDate 2.0 and thyme oil were effective in reducing the population of *Xanthomonas euvesicatoria* on tomato seed. The bacterium was not recovered from the hot water- and NaOCl-treated seed by dilution plating. Populations were recovered from the OxiDate 2.0 and thyme oil treatments.

Seedlings were grown from the treated seed to determine if seedling transmission of *X. euvesicatoria* from the seed was reduced. No viable cells of *X. euvesicatoria* were recovered by the dilution plating methods on the seedlings grown from the hot water- and NaOCl-treated seed but real-time PCR detected the pathogen in the seedling wash subsamples of both treatments. Inoculum in a seed lot does not always result in disease and significant yield loss in a crop (Baker and Smith 1966; Langerak et al. 2004; van der Wolf et al. 2013) especially when the levels of infestation are low. Thresholds of contamination need to be determined for bacterial spot pathogens on tomato seed so that the usefulness of the seed treatments can be determined. This work contributes to the knowledge on the most promising preparations suitable for use as organic seed treatments to control bacterial phytopathogens.

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