ANTIBACTERIAL POTENTIAL OF METALLIC OXIDE NANOMATERIAL AGAINST XANTHOMONAS PERFORANS CAUSING BACTERIAL SPOT OF TOMATO

By

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To my family for their unconditional love, support and faith in me.
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ANTIBACTERIAL POTENTIAL OF METALLIC OXIDE NANOMATERIAL AGAINST XANTHOMONAS PERFORANS CAUSING BACTERIAL SPOT OF TOMATO

By

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Bacterial spot disease of tomato, incited by four species of Xanthomonas, causes significant disease when environmental conditions are optimal and can lead to major yield loss and fruit quality reduction. Due to the limited availability of effective disease management options, copper(Cu)-based bactericides are frequently used by growers in Florida. Consistent use of Cu bactericides over many decades has led to the development of copper-tolerant strains. Due to the presence of copper (Cu)-tolerant strains of X. perforans, which is the dominant species in Florida, copper-bactericides are of limited use; thus, there is a critical need for finding effective alternatives. In this study the antibacterial activity of magnesium oxide (MgO) and other metallic oxide nanomaterials were evaluated against Cu-tolerant and sensitive X. perforans strains. In vitro assays demonstrated that MgO have high antibacterial ability compared to the equivalent concentration of a Cu-based bactericide against both strains. In three greenhouse experiments disease severity was significantly reduced by MgO at 200 µg/ml compared to copper-mancozeb, the grower standard and the untreated control (p = 0.05). In three field experiment, MgO at 200 µg/ml significantly reduced disease severity compared to the untreated control (p = 0.05). There was no significant difference in the yield between the treatments. In order to determine if
treatment with various bactericides affected accumulation of various elements in the fruit, fruit samples were collected and analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-OES). Fruits treated with MgO did not have significant accumulation of Mg, Cu, Ca, K, Mn, P and S compared to the untreated. (p = 0.05). This study shows the antibacterial potential of MgO nanomaterials against *X. perforans* and its potential use against bacterial spot of tomato.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Tomato is an economically valuable crop widely planted in the United States. According to the 2015 annual report, 97,500 acres of fresh market tomatoes were planted in the U.S with a harvest of 1.2 million metric tons, valued at 1.24 billion dollars (USDA, 2015). Florida is the largest fresh market producer in the U.S and contributed 36% of the annual tomato production value (USDA, 2015). However, with the high humidity and high temperature during the growing seasons in Florida, tomato has been challenged with a variety of plant pathogens. Bacterial spot disease, which can lead to up to 50% yield loss and fruit quality reduction, is one of the most critical bacterial diseases in fresh market tomato production. The management strategies applied presently are not effective in controlling bacterial spot disease. Development of novel disease management strategies is highly critical for sustainable production of tomatoes in the future.

**Bacterial Spot of Tomato**

**History**

Bacterial spot of tomato (*Solanum lycopersicum*) was originally described in South Africa in 1914. It was not until 1920 that a detailed description of the disease and causal agent was published. Doidge (Doidge, 1921) named the pathogen *Bacterium vesicatorium* and described the infected tomato fruit as disfigured with scabby lesions. At almost the same time, Gardner and Kendrick (Gardner & Kendrick, 1923) published a similar article on the same disease, in which they were prepared to name the causal organism *Bacterium exitiosum* but deferred to the name proposed by Doidge despite the two organisms varying in amylolytic ability (Stall et al., 2009, Gardner & Kendrick, 1921, Jones et al., 2004). Gardner and Kendrick (Gardner & Kendrick, 1923) discovered that *B. vesicatorium* also caused a leaf spot on pepper.
The organism associated with pepper and tomato then was classified as *Xanthomonas vesicatoria* and later as *X. campestris* pv. *vesicatoria* (Young, 1978).

Originally the organism was considered as a single species, *Xanthomonas vesicatoria*, infecting two hosts: pepper and tomato. The bacterium has been reclassified several times. It was transferred to *X. campestris* pv. *vesicatoria* by Dye et al. in 1978. Later it was transferred into two separate species, *X. vesicatoria* and *X. axonopodis* pv. *vesicatoria* (Vauterin et al., 1995). Currently, the causal agents of bacterial spot of pepper and tomato consist of four distinct species: *X. euvesicatoria* (Xe), *X. vesicatoria* (Xv), *X. perforans* (Xp), and *X. gardneri* (Xg) (Jones et al., 2004). Bacterial spot is now present everywhere in the world where tomatoes are grown and is caused by Xe and Xv on tomato and pepper, and Xp and Xg on tomato. Due to its wide diversity within the bacterial spot disease complex, this pathogen could tolerate wide temperatures. This ability makes this pathogen group a threat to tomato and pepper production worldwide (Potnis et al., 2015). However, each group of pathogens was distributed differently. The disease is favored by warm, humid climates, and in the United States it is most widely present in the Southeastern and Midwestern regions (Obradovic, 2008).

**Host Range**

The host range of *Xanthomonas* spp. causing bacterial spot includes a wide range of plants in the Solanaceae family. The main hosts are tomato (*Solanum lycopersicum*), cherry tomato (*Solanum lycopersicum* var. *cerasiforme*), currant tomato (*L. pimpinellifolium*), pepper (*Capsicum annuum*), and chili peppers (*Capsicum frutescens, C. baccatum, C. anomalum, C. chinensis and C. pubescens*) (Baker et al., 2014, Potnis et al., 2015, Sahin & Miller, 1998). It was not until the 1970s that Cook’s group demonstrated that host specificity was associated with hypersensitive responses (Cook, 1973, Potnis et al., 2015). Pathogenic races were identified based on differential plant reactions following inoculation. To date, researchers have identified
four tomato races and 11 pepper races based on the presence and absence of avirulence (avr) genes including *avrBs1*, *avrBs2*, *avrBs3*, *avrBs4*, *avrBsT*, *avrRxv*, *avrXv3*, *avrXv4* and *avrBs4* (Stall et al., 2009).

**Symptoms**

Bacterial spot is a description of a distinctive set of symptoms on pepper and tomato. The symptoms of bacterial spot of tomato are observed on the above ground parts of the plant including leaf, stem and fruit on susceptible tomato plants (Ritchie, 1991). The symptoms start with small water-soaked circular lesions, which usually are observed on the abaxial leaf surface. When conditions of high humidity and high rainfall exist, lesions expand rapidly and change from a dark green to brown color. The lesions enlarge and dry out eventually; however, they generally do not expand to more than 3 mm in diameter. In addition, bacterial spot disease may cause scabby lesions on fruits, which are larger than foliar lesions. Fruit marketability can be seriously affected due to the presence of unsightly fruit (Obradovic, 2008). Subtle symptom differences are observed with the different pathogens. More specifically, Xp develops a distinct shot-hole lesion on the leaves and large fruit lesions compared to other species (Stall et al., 2009).

**Management**

Bacterial spot of tomato is difficult to control, especially in Florida due to the tropical and subtropical environmental conditions which favor the disease. In order to control the disease, various approaches have been tested including using bactericides and a plant defense activator, biological control agents and resistant cultivars. Among these disease management strategies evaluated for management of bacterial spot disease, there are only limited effective options. Currently, the management strategies rely mainly on a variety of chemical control strategies and
sanitary methods to minimize introduction of the pathogen on seed and minimize spread of the pathogen (Potnis et al., 2015).

Introgression of plant resistance to bacterial spot of tomato has been the focus of much research (Scott et al., 1995, Hert et al., 2005, Sharlach et al., 2013, Stall et al., 2009). Conventional resistance (R), a gene-mediated resistance that relies on avirulence genes in the pathogen and resistance genes in the plant that result in plant-microbe interactions (Flor, 1971) has been the focus of many breeding programs. However, durability of resistant cultivars is hard to maintain due to the rapid shift of the genome composition of the Xanthomonas spp. populations (Stall et al., 2009). Techniques such as using quantitative trait loci (QTLs) by connecting markers in the plant with resistance to bacterial spot have been developed. T1, T2 and T3, three races of Xanthomonas spp., were identified based on their reaction toward the three tomato cultivars Hawaii 7998 (H7998), Hawaii 7981 (H7981) (Scott & Jones, 1986, Jones & Scott, 1986) and Bonny Best (Jones et al., 1995, Jones et al., 1998). Rx3 gene was used as a source of resistance against T1 Xe strains (Yang & Francis, 2005). Different host-specific resistant genes against xanthomonads were selected subsequent to the appearance of new races (Scott et al., 1995, Yang et al., 2005, Robbins et al., 2009, Hutton et al., 2010). On indicator cultivars, T2 strains are not able to induce hypersensitive response (HR). However, T3 strains, which carry the avirulence gene $avrXv3$, induce an HR on the tomato genotype H7981, which has the $Xv3$ gene, and on all pepper cultivars tested (Minsavage et al., 1996). Due to mutations and interdiction of new strains, resistance in commercial cultivars is hard to maintain for long-term management of the disease (Stall et al., 2009).

Biological control strategies have been used in the past with variable results. For example, bacteriophage use is one of the strategies that offers some degree of control when
compared with the grower standard Kocide 2000-Mancozeb (Cu-mancozeb) treatment (Balogh et al., 2003, Obradovic et al., 2008, Flaherty et al., 2000, Momol et al., 2002). However, environmental factors including UV irradiation, humidity and temperature negatively affect the efficiency of bacteriophage application. In addition, the combination of copper bactericides and phage could cause a reduction of phage population on leaf surfaces (Iriarte et al., 2007). On the other hand, commercially available bacteriophages are a relatively expensive control strategy compared to copper bactericides. Besides bacteriophages, other biological agents have been tested in multiple studies. However, efficacy is limited when using biological agents alone (Fravel, 2005). Plant growth promoting rhizobacteria (PGPRs), *Pseudomonas fluorescens* 89B-61 and *Bacillus pumilis* SE34 are three biocontrol agents which have shown significant control ability in some field trials (Ji et al., 2006). The study by Ji et al. (2006) suggested that some PGPR strains might provide bacterial spot disease management ability by inducing plant resistance under field conditions. Biocontrol is a promising area in agricultural management; however, the overall use of biocontrol reagents for plant disease control was reported to be relatively small and represented about 1% of agricultural chemical sales (Cook, 2000).

Chemical control is commonly used in bacterial spot disease management. The plant activator, Acibenzolar-S-methyl (ASM) (Actigard 50WG, Syngenta Crop Protection, Greensboro, NC, USA), which is a systemic acquired resistance (SAR) triggering material (plant defense activator), has been shown to reduce disease symptoms during the early phase of treatment. However, it did not provide disease control through the whole season (Roberts et al., 2008). In addition, ASM is considered to be associated with decreased yield (Graves & Alexander, 2002, Romero et al., 2001). In another study ASM was effective in disease control but it did not result in a yield increase (Louws et al., 2001, Obradovic et al., 2005).
Antibiotics were used for field control of bacterial spot disease on tomato in the 1950s. An example is streptomycin, which has been used in the greenhouse and field. However, streptomycin-resistant Xanthomonas strains were common in the fields in the early 1960s (Thayer & Stall, 1961, Stall & Thayer, 1962). After the discovery of streptomycin-resistant xanthomonads, growers relied heavily on copper-based bactericides. However, not much later, copper-tolerant strains were present in grower fields in the 1960s (Marco & Stall, 1983). With the presence of copper-tolerant strains, growers changed from applying copper to applying Cu-mancozeb combinations, which reduced bacterial spot disease severity in the field.

Routinely, Cu-mancozeb was used to control Cu-tolerant strains (Conover & Gerhold, 1981, Marco & Stall, 1983). Cu-mancozeb was shown to release more soluble copper than a copper suspension of Kocide 101 (cupric hydroxide; Griffin Corporation, London, England) alone (Marco and Stall, 1983). This ability allowed Cu-mancozeb to have better disease control ability than copper by itself (Conover & Gerhold, 1981). However, Cu-mancozeb still could not effectively control copper-tolerant strains in a disease favored condition (Jones & Jones, 1985). In addition, yield responses have not been commonly observed with the use of a combination of Cu-mancozeb (Jones & Jones, 1985). Xp strains when first detected in Florida in 1991 were copper sensitive but in 2006 all strains of Xp present in Florida were copper tolerant (Horvath et al., 2012). Cu-mancozeb provided mediocre control for copper-tolerant Xp strains (Conover & Gerhold, 1981). Another chemical control option investigated by researchers is 2AI, which is an analogue of the marine sponge natural product oroidin. 2AI has been evaluated in combination with copper for the control of bacterial spot and was shown to efficiently control bacterial spot disease caused by Xe on pepper with yield response by biofilm formation disturbing ability (Worthington et al., 2012).
Due to the limited availability of effective disease management options, copper-based bactericides are frequently used by growers in Florida. Consistent use of copper bactericides over many decades has led to the development of copper-tolerant strains, which makes Cu ineffective. In addition, the heavy use of copper-based bactericides has led to heavy metal accumulation in soil (Ninot et al., 2002, Radix & Seigle-Murandi, 1993, Kaplan, 1999, Pietrzak & McPhail, 2004). Therefore, developing compounds with lower Cu concentrations that are effective would be considered as promising materials.

**History of Nanoparticles for Bacterial Spot Disease Management**

In recent years, nanotechnology has provided novel antimicrobials for the management of pathogenic bacteria affecting agricultural crops and mammals. Recently, nanomaterials have been the focus of a number of studies for the control for bacterial spot of tomato. Photocatalytic crystalline titanium dioxide (TiO$_2$) is one of them. TiO$_2$ is energized by absorbing photons; this reaction leads to the release of free electrons, which creates positively charged holes in the crystal structure (Page et al., 2009, Chen & Mao, 2007). These electrons can migrate from the crystal to the nanoparticle surface. Those charged-nanoparticles then interact with water molecules to create superoxide free radicals. Those reactive oxygen species (ROS) oxidize localized organic material including bacterial pathogens (Paret et al., 2013). Nanoscaled crystallized TiO$_2$ has a massively increased surface area compared to its micron-crystal form and was expected to provide better antibacterial ability then the micron-crystal form (Paret et al., 2013, Hagfeldt & Graetzel, 1995, Henglein, 1993). Paret et al. (2013) tested three different TiO$_2$ nanomaterials: nanoscale TiO$_2$, nanoscale TiO$_2$ doped (incorporation of other materials into the structure of TiO$_2$) with silver (TiO$_2$/Ag) and nanoscale TiO$_2$ doped with zinc (TiO$_2$/Zn) (Averett & Averett, 2015) against Xp. In the field study, all three chemicals showed similar protection ability to the grower standard, copper-mancozeb. TiO$_2$/Zn at $\approx$500 to 800 $\mu$g/ml showed the best
protection ability among the three nanoscale treatments. (Paret et al., 2013) However, TiO$_2$/Zn had no antibacterial effect without light exposure, and tomato plants exhibited phytotoxicity following the sixth application at approximately 500 µg/ml in field trials in Florida (Paret et al., 2013). These two limitations are potential problems associated with TiO$_2$/Zn applications in the field.

A nano form of silver (Ag) was used as an antibacterial agent toward animal pathogens in several studies (Elechiguerra et al., 2005, Kim et al., 2007, Kim et al., 2009, Galdiero et al., 2011). However, unformulated silver nanomaterial has the tendency to aggregate, which leads to contact surface reduction between pathogen and the nanomaterials (Bae et al., 2010). Based on this observation, a Ag-dsDNA-GO material, which has controlled size and aggregation prevention, was designed to solve this difficulty (Ocsoy et al., 2013a, Ocsoy et al., 2013b). With the use of a reducing agent, Ag nanoparticles (AgNP) were imbedded on double-stranded DNA (dsDNA). Then this combination was attached to graphene oxide (GO) sheets during the manufacture. dsDNA functions as a connection bridge between AgNP and GO. Ocsoy et al. (2013) describe GO as single carbon formed sheet with active surface hydroxyl, epoxy and carboxyl groups. The GO sheet increased adhesive forces between the AgNP and the bacterial cell; this phenomenon increased the antibacterial ability of AgNP. GO also helped provide a more uniform distribution of AgNP (Ocsoy et al., 2013a, Strayer et al., 2015).

Strayer et al. (2016) demonstrated the antibacterial ability of Ag-dsDNA-GO against copper-tolerant and copper-sensitive Xp strains compared with standard copper and Cu-mancozeb use rates in vitro and in planta. The risk of phytotoxicity was also evaluated. The in vitro assays revealed that Ag-dsDNA-GO at concentrations as low as 10 µg/ml killed all copper-tolerant and copper-sensitive Xp strains in suspensions containing approximately $10^3$ CFU/ml.
within 15 minutes of exposure. Ag-dsDNA-GO at 10 µg/ml contained Ag at less than 2 µg/ml. In contrast, the same copper concentrations had no effect on the copper-tolerant Xp even after 24 h of exposure compared to the non-treated. For copper-sensitive strains, the lowest copper concentrations inhibited bacterial growth after 1 h. Ag-dsDNA-GO not only had antibacterial ability toward Xp but Xe, Xg and Xv. In greenhouse experiments, all Ag-dsDNA-GO (100, 200, 500 µg/ml) concentrations significantly reduced bacterial spot disease severity compared to copper-mancozeb. However, two of the concentrations (200 and 500 µg/ml) resulted in phytotoxicity. In addition, the 100 µg/ml concentration of the Ag-dsDNA-GO performed the best among the three in all three greenhouse experiments. This novel material is effective at the concentration of 100 µg/ml rate which is equivalent to 16 µg/ml of Ag. Even though not a direct comparison with Ag, the level of copper currently sprayed to control bacterial spot in transplant houses or tomato fields is approximately 540 µg/ml (Strayer et al., 2015). The usage of Ag-dsDNA-GO could reduce heavy metals accumulating in the soil deposited in the soil by about 30 times (Strayer et al., 2015). Based on this study, Ag-dsDNA-GO might be a potential alternative to copper. However, the material is currently unavailable in large quantities and is costly. Additionally, after the treatment, the fate of Ag-dsDNA-GO in the environment needs to be evaluated, including accumulation in soil and fruit.

Cu nano-materials have been applied to various crops. For example, (Cu)–Silica Nanocomposite Containing Valence-Engineered Cu was tested against Xanthomonas alfalfae on citrus (Young & Santra, 2014). This material shows antibacterial ability. However, this material caused phytotoxicity on Vinca sp. and Hamlin orange in greenhouse (Young & Santra, 2014) and needs further research.
Nanotechnology is thus a developing field, which has a potential role in plant pathology for use in plant disease management. Many of the previous studies in this area have demonstrated improved antibacterial ability of nanoparticles compared to their bulk counterparts; using reduced sized particles may lead to greater antibacterial ability toward copper-resistant bacterial strains.

**Toxicity for Nano-Material**

Nanoparticles could be possibly applied in the agro-food chain using several different methods. With each method the possibility exists for free nanoparticles being consumed by humans. In agricultural production, one of the methods was applying nanosensors (Ghormade et al., 2011) in order to bind to organic contaminants for detection. Using nanoparticle-based pesticides also have high likelihood of free nanoparticles exposure to consumers and introduction in the food chain (Baruah & Dutta, 2009). Other methods including water purification and soil contamination removal (Gehrke et al., 2015) are also considered as resources contributing to nanoparticles in human consumption. For food processing and food production, refrigerators, storage containers and food preparation equipment could be treated with nanoparticles including incorporated nano-sized particles for antibacterial purposes (Bouwmeester et al., 2009). Nanoparticles have also been used in food preservation and packaging for better oxygen scavenging to prevent the growth of pathogens (Bouwmeester et al., 2009). As a result of the potential uses of nanoparticles, toxicologists have concerns with regard to the use of free and insoluble nanoparticles including agglomerates that are thought to be the highest risk to consumer health.

In several studies, nanoparticles were evaluated for their exposure to humans via food (Chen et al., 2006, Graveland-Bikker & De Kruiif, 2006, Mozafari et al., 2006). The detection of nanoparticles in food is difficult because of the lack of adequate methods for detection in food.
(Govers et al., 1994, Borm & Kreyling, 2004). Additionally, the presence of nanoparticles in the food may increase the bioavailability of other substances including both nutrients and contaminants already contained in food (Chen et al., 2006, Graveland-Bikker & De Kruif, 2006, Mozafari et al., 2006). In both scenarios including bioavailability and exposure assessment, there are three major aspects of food safety and toxicology of nano-materials that needs to be considered. First, how to determine quantitatively the presence of the nanoparticle in individual foods and diets, including its fate during the processes within the food production chain; second, how to determine the consumption activity and patterns of the individual foods containing the relevant nanomaterials; and third, how to link between the largest amount of approximate consumer intake level and the relevant nanomaterial being present in these foods at high levels (Kroes et al., 2002).

**Hypotheses and Objectives**

The goals of this project are to evaluate novel disease management strategies for bacterial spot of tomato and to gain a better understanding of this disease. Based on the former study in which Ag-based nanomaterials controlled copper-tolerant strains of Xp in planta (Strayer et al., 2015, Ocsoy et al., 2013b), we hypothesized that reducing the particle size of metallic oxide to a nanoparticle form will improve antibacterial properties when compared to its micron size counterparts and more effectively control bacterial spot of tomato.

In this study, the objectives are to: I) evaluate the antibacterial properties of metallic oxide nanomaterials on the growth of copper-tolerant and sensitive strains of Xp in vitro, in the greenhouse and in the field; and II) determine whether metallic oxide nanomaterials lead to elemental accumulation in fruits.
CHAPTER 2
EVALUATING THE POTENTIAL OF METALLIC OXIDE NANOPARTICLES FOR MANAGEMENT OF BACTERIAL SPOT OF TOMATO

Bacterial spot disease of tomato, caused by four species of *Xanthomonas* (*X. euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria*) (Jones et al., 2004) causes significant damage when environmental conditions are optimal. The disease can lead to major yield losses and fruit quality reduction (Louws et al., 2001). Since 2006, *X. perforans* (Xp) has been the dominant species on tomato in Florida (Horvath et al., 2012).

Due to the limited availability of effective disease management options, copper (Cu)-based bactericides are frequently used by growers (Ritchie & Dittapongpitch, 1991, Marco & Stall, 1983, Vallad et al., 2010). Consistent use of Cu bactericides over many decades has led to the development of copper-tolerant strains, which makes Cu ineffective. In addition, since the heavy use of copper-based bactericides might lead to heavy metal accumulation in soil (Pietrzak & McPhail, 2004), a lower metal concentration with same effect would be considered as a promising material. A developing area of research is nanotechnology for use in bacterial disease management.

In the past decade, small sized MgO including micron-sized and nano-sized particles were shown to have potential as an antibacterial agent against bacterial pathogens (Sawai, 2003, Aruoja et al., 2009, Huang et al., 2005). Sawai et al. (2003) treated the mammalian pathogens *Staphylococcus aureus* and *Escherichia coli* with MgO powder (3.7 μm in sterile saline) and quantitatively evaluated the change in electrical conductivity as a measurement of bacterial metabolism. Concentrations as low as 0.2 μg/ml MgO showed antibacterial ability after 15 h. In this study, the authors observed that the contact between MgO powder and bacterial cells is an important bactericidal factor. Generation of oxygen radicals was suggested as one of the possibilities for the antibacterial mechanism (Sawai et al., 2000). For plant pathogens, Wani and
Shah (Wani & Shah, 2012) reported a high inhibition rate in the germination of fungal spores of *Alternaria alternata*, *Fusarium oxysporum*, *Rhizopus stolonifer*, and *Mucor plumbeus* upon exposure to MgO (~50 ±10nms) at concentrations as low as 100 mg/L. In that study, the authors suggested that nanoparticles may inhibit the release of extracellular enzymes and metabolites that are essential for fungal survival against toxins or environmental changes (Pérez-de-Luque & Rubiales, 2009). It was also observed that nanoparticles suppress enzymes and toxins used by the fungal pathogens for pathogenicity (Bhainsa & D'souza, 2006, Vahabi et al., 2011).

Huang et al. (2005) evaluated the possible mechanism of MgO antibacterial activity. The study showed that the bactericidal efficacy of nano-MgO increased with decreasing particle size against *Bacillus subtilis* var. *niger* spores and *Staphylococcus aureus* bacteria. For particles in the size range of ~45–70 nm, the bactericidal efficacy of nano-MgO increased slowly with decreasing particle size. The threshold was 45 nm; a strong improvement of bactericidal efficacy was observed below that particle size. To compare with nano-TiO$_2$ (29 nm, light activated), MgO at size 26 nm showed similar bactericidal activity against *S. aureus* and a much higher bactericidal efficiency against *B. subtilis* without light activation. As Sawai et al. (2003) discovered, Huang et al. (Huang et al., 2005) also confirmed that the mechanism of bactericidal activity of nano-MgO is the release of high concentrations of highly active superoxide ions generated on the surface of the nano-MgO particles. Those reactive oxygen species (ROS) can break the peptide linkages in the cell wall of bacteria or spores (Huang et al., 2005).

The goal of this project was to evaluate novel disease management strategies for bacterial spot of tomato. We hypothesized that nanoparticle metallic oxides will have better antibacterial properties compared to commercial micron size copper and provide more effective control of bacterial spot of tomato. The objectives of the study were to 1) evaluate the antibacterial
properties of metallic oxide nanomaterials on the growth of copper-tolerant *X. perforans* in vitro, 2) assess its ability in reducing bacterial spot disease severity under greenhouse and field conditions, and 3) study the potential of the metallic oxide nanoparticles in causing phytoxicity on tomatoes.

**Materials and Methods**

**Bacterial Strains and Storage**

Two Xp strains GEV485 (copper tolerant) and 91-118 (copper sensitive) were used in this study. The strains were originally isolated from tomatoes in Florida. The pure culture of the strains were suspended in sterile 30% glycerol solution and stored at -80°C. For working stock, bacteria were grown on nutrient agar (NA) medium (BBL, Becton Dickinson and Co., Cockeysville, MD) at 28°C and transferred every 24 to 48 hr. Bacterial cells were collected from 24 hr NA plates, suspended in 0.01 M MgSO₄, and the suspension was adjusted to A₆₀₀=0.3-0.33 at λ = 600 nm (~5 x10⁸ CFU/mL). The final concentration of bacteria in the test-tube was 10⁸ CFU/ml.

**Metal and Metallic Oxide Nanomaterials**

Nanomaterials: silver nanoparticles (Nano-Ag) (Ag, 99.99%, 20nm, metal basis); copper(I) oxide nanoparticles (Cu₂O) (Cu₂O, 18nm, Super fine 99.86%); zinc oxide nanoparticles (ZnO) (ZnO, 99+%, 10-30 nm); magnesium oxide nanoparticles (MgO) (MgO, 99+%, 20 nm); manganese oxide nanoparticles (Mn₂O₃) (Mn₂O₃, 99.2%, 30 nm); iron oxide nanoparticles (Fe₂O₃) (Fe₂O₃, gamma, 99%, 20-40 nm) were purchased in powder form from US Research Nanomaterials, Inc. (Houston, TX, USA). Each nanomaterial was suspended in autoclaved deionized water and adjusted to 10,000 µg/ml stock suspension.
In vitro Assays

In the in vitro assay, two Xp strains, GEV485 and 91-118 were used. The bacterial strains were transferred from long storage stock to nutrient agar (NA) plates than incubated for 24 hr at 28°C. After 24 hr, the bacterial colonies were transferred to copper NA plates (20 μg/ml of copper (II) sulfate pentahydrate) (CuSO₄·5H₂O) (Sigma-Aldrich®, St. Louis, MO) and incubated for 24 hr at 28°C. Bacterial cells were collected from NA plates and suspended in sterile 0.01 M solution of MgSO₄ (2.46 g/L) in deionized water. Suspensions were diluted to 10⁵ CFU/mL and 20 μL of the bacterial suspension were transferred to 2 mL of each treatment in a sterile glass tube: Nano-Ag, Cu₂O, ZnO, Fe₂O₃, MgO, Mn₂O₃ and the copper bactericide, Kocide® 3000 (DuPont), were prepared at 100 and 1000 μg/ml. Kocide 3000 contains 30% metallic copper in the form of copper hydroxide (Cu(OH)₂). One gram per liter of Kocide 3000 contains ~300 μg/ml of copper. Each treatment consisted of three replications for each bacterial strain. For the control group, the glass tube only contained 2 mL of sterile tap water and 20 μL of the bacterial working suspension. The tubes were incubated at 28°C on a shaker. At 15 min, 1 hr, 4 hr, and 24 hr, 50 μL were sampled from each tube and placed on nutrient agar. Bacterial colonies were counted on each plate and converted to colony forming units (CFU)/mL. The in vitro assay was repeated two times.

Greenhouse Experiments

Cu₂O, ZnO and MgO were selected for further testing as potential bactericides for control of bacterial spot disease of tomato since they showed better antibacterial activity than the other compounds in the in vitro assays. Two-hundred milliliters of the following suspensions were prepared in sterile tap water: 100, 200, 500 and 1000 μg/ml of Cu₂O, ZnO and MgO; or a combination of Kocide 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L). Sterile tap water served as the control. The materials were sprayed on the foliage of three- to four-week old Bonnie Best
tomato plants. The sprays were allowed to air dry before being inoculated with a suspension of the copper tolerant Xp strain, GEV485, adjusted to $5 \times 10^8$ CFU/mL. The inoculated plants were then placed in plastic bags tightened around the base of the pot with a rubber band and placed in a growth chamber at 28°C. After 48 hr, the bags were removed and the plants transferred to the greenhouse. The plants were assessed for disease severity and phytotoxicity using the Horsfall-Barratt disease severity scale (Barratt & Horsfall, 1945) by rating every other day beginning at 2 days post-inoculation with the last rating at 14 days post-inoculation. The area under disease progress curve (AUDPC) was then calculated using the midpoint values (Campbell & Madden, 1990). There were four replications per treatment and the experiment was repeated twice.

**Field Experiments**

In the greenhouse experiments, two nanoparticle treatments, Cu$_2$O and MgO, showed better disease management ability than the other compounds, and hence were selected for field testing against bacterial spot disease of tomato in three trials (8/4/2015-9/11/2015 Quincy, FL; 4/18/2016-6/13/16 Quincy, FL; 3/31/16-5/26/16 Balm, FL). Each treatment had four replications consisting of 15 BHN 602 tomato plants (BHN Seeds, Immokalee, FL). The plots were arranged in a completely randomized block design. The treatments consisted of 200 and 1000 µg/ml of Cu$_2$O or MgO, Kocide 3000 (2.1 g/L), the grower standard Kocide 3000 (2.1 g/L) in combination with Penncozeb 75DF (1.2 g/L) and an untreated control. The treatments were sprayed on foliar parts of transplanted tomatoes at the rate of 1.2 L for four plots one week prior to bacterial inoculation. A suspension of copper tolerant Xp strain GEV485 bacterial strain adjusted to $5 \times 10^8$ CFU/ml in DI water was applied to the foliage in the field by spraying the 1$^{\text{st}}$, 8$^{\text{th}}$, and 15$^{\text{th}}$ plants in each plot. Treatments were applied weekly until one week before fruit harvesting. The plants were assessed for disease severity and phytotoxicity using the Horsfall-Barratt disease severity scale (Barratt & Horsfall, 1945) every week after inoculation until
harvest. The area under disease progress curve (AUDPC) was then calculated using the midpoint values (Campbell & Madden, 1990). There were four plot replications per treatment and the experiment was done three times.

**Statistical Analysis**

The data collected from the in vitro assays, greenhouse and field experiments were evaluated for statistical significance using ANOVA followed by comparisons using either the Least Significant Difference (LSD) or the Student Newman-Keuls (SNK) method in IBM® SPSS® Statistics Version 22 and a p-value of 0.05 to evaluate significance.

**Results**

**Effect of Metallic Oxide Nanomaterials on in vitro Growth of Xp**

In the first experiment (Fig.2-1A-D), several metallic oxide nanomaterials and the silver nanomaterial (Nano-Ag) were compared with Kocide 3000 for toxicity to the copper-tolerant strain, GEV485. Nano-Ag is an antibacterial material (Lok et al., 2007, Pal et al., 2007, Marambio-Jones & Hoek, 2010) and the active ingredient in the formulated silver-based nanomaterial Ag-dsDNA-GO (Strayer et al., 2015), and hence was used in this experiment as a comparison to other metallic oxide nanomaterials. Nano-Ag showed the most significant antibacterial activity (Fig. 2-1A), killing the copper-tolerant strain within 1 h (Fig.2-1B), whereas ZnO and MgO at 100 µg/ml showed significant antibacterial ability after 4 hr (Fig.2-1C). Bacterial cells could not be recovered from all concentrations of ZnO and MgO treated samples after 24 hr (Fig.2-1D). Cu₂O and Mn₃O₅ showed significant antibacterial ability after 24 hr (Fig.2-1D) with bacterial cells not being recovered from Cu₂O treated samples.

In the second experiment (Fig. 2-2 A-D) several metallic oxide nanomaterials and the silver nanomaterial Nano-Ag were compared with Kocide 3000 for toxicity to the copper-tolerant strain GEV485 and copper-sensitive strain 91-118. Nano-Ag, ZnO and MgO had high
antimicrobial activity at all concentrations against the copper-tolerant strain, GEV485. Both concentrations of Nano-Ag (1000 and 100 µg/ml) and ZnO at 1000 µg/ml completely inhibited bacterial growth within 15 min (Fig. 2-2A). Both MgO and ZnO at 100 µg/ml reduced bacterial populations at 1 hr and 4 hr (Fig. 2-2B & Fig. 2-2C) compared to the untreated control. Cu$_2$O at 1000 µg/ml inhibited bacterial growth within 24 hr (Fig. 2-2D). In contrast, neither Kocide concentration significantly reduced the bacterial populations when compared to the untreated control. The experiments were repeated once with Xp copper-tolerant strains. Nano-Ag, ZnO, and MgO compared to the equivalent concentration of the Cu-based bactericide showed significant antibacterial activity towards both a copper-tolerant and copper-sensitive Xp strain.

For the copper-sensitive strain 91-118 (Fig. 2-3 A-D), similar results were observed as with the copper tolerant strain except that antibacterial activity was also observed with both of the copper compounds (Cu$_2$O and Kocide 3000). Viable bacterial cells could not be recovered after 15 min (Fig 2-3A) following treatment with the lower concentration (100 µg/ml) of copper. Based on the in vitro test, besides the high antibacterial ability of Ag nanomaterial, which was shown in the previous study (Strayer et al., 2015), ZnO, MgO and Cu$_2$O showed greater antibacterial activity against both the copper-sensitive and copper-tolerant strain compared to the copper based bactericide Kocide 3000.

**The Effect of Metallic Oxide Nanomaterials on Bacterial Spot Disease Severity under Greenhouse Conditions**

ZnO, MgO and Cu$_2$O at several concentrations (100 µg/ml, 200 µg/ml, 500 µg/ml and 1000 µg/ml) were compared to the commercial copper bactericide for control of bacterial spot in greenhouse experiments. In the first greenhouse experiment (Fig. 2-4A), after foliar nanomaterial applications and inoculation of the copper-tolerant strain GEV485, all Cu$_2$O concentrations and the two MgO concentrations (1000 µg/ml and 200 µg/ml) significantly decreased disease
severity compared to the grower standard (Cu-mancozeb) and the untreated control. On day 13, phytotoxicity was assessed based on percentage defoliation (Fig. 2-5A). Only Cu$_2$O at 100 µg/ml was significantly different from the untreated control. The other treatments showed no phytotoxicity.

In the second greenhouse experiment (Fig. 2-4B), all treatments significantly reduced disease severity compared to the untreated control. Significant phytotoxicity was observed at 500 µg/ml Cu$_2$O and 1000 µg/ml ZnO compared to the untreated control, but no significant differences were observed between the two treatments. (Fig. 2-5B)

In the third greenhouse experiment (Fig. 2-4C), all of the treatments showed a dose effect of the different concentrations. For both Cu$_2$O and MgO nanomaterial, concentrations as low as 200 µg/ml showed a significant reduction in disease severity compared to the untreated control. Compared with the grower’s standard Cu-mancozeb and Kocide 3000 alone, Cu$_2$O at 1000 and 500 µg/ml and MgO at 1000 µg/ml showed reduced diseased severity. ZnO at both concentrations (1000 and 200 µg/ml) showed significantly reduced diseased severity compared to the untreated control. However, this was not significant compared to the Cu-mancozeb and the Kocide 3000 treatments. Only 1000 µg/ml and 500 µg/ml of Cu$_2$O nanomaterial showed phytotoxicity compared to the untreated control(Fig. 2-5C).

**The Effect of Metallic Oxide Nanomaterials on Bacterial Spot Disease Severity under Field Conditions**

Based on the results of greenhouse experiments (Fig. 2-4), 200 and 1000 µg/ml of MgO and Cu$_2$O were selected for field studies (Fig. 2-6, Fig. 2-8, Fig. 2-10) and compared with Cu-mancozeb, the grower standard. In the first field experiment in fall 2015 in Quincy, FL (Fig. 2-6) plants receiving either concentration of MgO (1000 µg/ml or 200 µg/ml) had significantly less disease severity compared to the untreated control, but were not different from the other
treatments. Both concentrations of CuO and Cu-mancozeb were not significantly different than the control. No phytotoxicity was observed for any of the treatments in this experiment. In the 2016 spring trial in Balm, FL (Fig. 2-8), 200 µg/ml MgO treated plants showed significantly reduced disease severity compare to the untreated as in the 2015 Fall Quincy trial. However, 1000 µg/ml MgO treatment did not reduce disease severity compared to the untreated control. The 1000 µg/ml CuO treatment significantly reduced disease severity compared to the untreated control; however, this result was not observed in the 2015 Fall Quincy trial. In the 2016 spring trial in Quincy (Fig. 2-10), both concentrations (1000 and 200 µg/ml) of the MgO and CuO nanomaterial treatments significantly reduced disease compared to the untreated control while the grower’s standard Cu-mancozeb did not significantly reduce disease in the three field trials compared to the untreated control. However, despite disease control, there were no significant marketable yield differences between the treatments (Fig. 2-7). This experiment was repeated two additional times with no marketable yield response (Fig. 2-9 & Fig. 2-11).

Discussion

In this study, MgO was shown to have potential as an alternative to copper-based bactericides for bacterial spot management in tomato production because it showed consistent disease control ability while other metallic oxide nanomaterials including CuO did not. Based on this research, the MgO nanomaterial (99+%, 20 nm, US; US Research Nanomaterials, Inc.) at 100 µg/ml and 1000 µg/ml controlled copper–tolerant and copper-sensitive Xp strains in the in vitro experiments. These results indicate that compared to Kocide 3000, which does not have efficient antibacterial ability against the copper-resistant strain of bacterial spot disease of tomato, MgO nanomaterial provided greater disease control against bacterial spot disease of tomato. In vitro experiments demonstrated that MgO was more effective than the Kocide 3000 for killing the copper-tolerant strain. After 4 hr MgO at 100 µg/ml killed the copper tolerant
strain, while Kocide 3000 did not kill the bacterium even at 1000 µg/ml after 4 hr. Cu₂O also showed significant antibacterial ability toward the Cu-tolerant strain in vitro. In addition, ZnO also had significant antibacterial ability against both of the strains. Although Cu₂O did not have the same level of activity as MgO, it is interesting that the nanoform of copper showed better antibacterial activity against the copper-tolerant strain compared to the copper based bactericide. Neither bacterial strain could be recovered from tubes containing 1000 µg/ml of ZnO after 15 minutes and 100 µg/ml of ZnO after 4 hr; however, ZnO did not provide significant disease control in the greenhouse. From the greenhouse experiment, ZnO showed good disease control ability on the early stage of the disease. However, symptoms developed rapidly afterward. ZnO nanomaterials might be a potential antibacterial material to control bacterial spot disease when combined with other bactericides; however this needs to be studied further. Researchers demonstrated that ZnO nano-materials have antibacterial (Jayaseelan et al., 2012, Liu et al., 2009) and antifungal activity (Dimkpa et al., 2013, He et al., 2011), significantly increased biomass/yield on pearl millet (Pennisetum americanum) (Tarafdar et al., 2014) and significantly increased plant growth for peanuts (Prasad et al., 2012). However, several studies showed that ZnO nano-material may cause phytotoxicity. Burman et al. (2013) showed that a foliar application on chickpea (Cicer arietinum L var. HC-1) of 10 mg/L ZnO negatively impacted root biomass (Burman et al., 2013), while a negative effect was observed in corn for inhibition of germination after treating the seed with 2000 mg/L ZnO (Lin & Xing, 2007). As a matter of fact, both particle concentrations and plant species will be major factors controlling the efficiency of ZnO nano-material. ZnO showed good antibacterial ability in vitro; however, according to the results in greenhouse, ZnO might not be an efficient alternative for controlling bacterial spot disease on tomato.
In the greenhouse experiments, application of MgO and Cu₂O at 200 µg/ml to tomato plants significantly reduced bacterial spot disease severity compared to Cu-mancozeb and the untreated controls (p = 0.05). However, in the two field experiments in Quincy, FL and Balm, FL, application of Cu₂O did not significantly reduce disease severity compared to the untreated control. MgO at 200 µg/ml provided a significant reduction in bacterial spot severity compared to the untreated control (p = 0.05) with no sign of phytotoxicity in all the field experiments. 200 µg/ml MgO nano-material was comparable to Cu-mancozeb. There was no significant difference in the yield between any of the treatments.

MgO at 200 and 1000 µg/ml significantly decrease disease severity in the field compared to the untreated control, although MgO treatments were not significantly different than the grower’s standard Cu-mancozeb. The copper concentration currently sprayed to control bacterial spot in tomato fields is around 540 µg/ml. That is about two times higher than the level of Mg from the MgO 200 µg/ml treatment. In addition, MgO application would help to reduce heavy metal accumulation in the field. Using MgO nano-material as an alternative could help to relieve the pressure of potential copper accumulation for copper-tolerance strains to appear.

Based on this study, MgO might be a good chemical alternative as part of an IPM program and potentially could be combined with other bactericides. There are two important approaches to control bacterial spot of tomato in the field: reducing inoculum and minimizing plant susceptibility (Momol et al., 2002). The MgO nano-material was considered as a more environmental friendly chemical, which can be potentially applied as an alternative to copper in an IPM program to reduce inoculum. In this scenario, it would be less of a risk for developing more copper tolerant bacteria in the field.
This study shows the potential for developing MgO-based nanomaterials for management of bacterial spot of tomato. However, the material in this study is crude and not a non-formulated suspension, which can affect its homogeneity. Because the nano-material tends to be aggregated, the pathogen might not be able to come into contact with antibacterial agent efficiently. This may explain why there was high variation in the greenhouse and in the field experiment with the higher concentration tending to have greater standard deviation than the lower concentration. This phenomenon of aggregation fits the observation in the previous study by Sawai et al. (Sawai, 2003) that the antibacterial ability is contact dependent between MgO nanoparticle and the target bacteria. This suggested the importance of development of a formulated MgO as a more homogeneous suspension for further research on managing the disease. Future formulated MgO nano-materials with better homogenizing ability could result in an antibacterial agent that could be used as an alternative to currently available bactericide strategies.
Figure 2-1. In vitro inhibition of copper tolerant *X. perforans* strain GEV485 following exposure to various chemicals over time (15 min, 1 hr, 4 hr, and 24 hr). The treatments were as follows: Ag, Cu$_2$O, ZnO, Fe$_2$O$_3$, MgO and Mn$_2$O$_3$ at 100 and 1000 µg/ml; copper prepared from Kocide® 3000 (K3000) at 1000 (3.3 g/L), 500 (1.67 g/L), 200 (0.668 g/L) and 100 µg/ml (0.334 g/L); and a water control (UT). A) after 15 minutes B) after 1 hr. C) after 4hr. D) after 24 hrs incubations. Error bars= Standard Error. p-value of 0.05 was used in the IBM® SPSS® using SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-1. Continued
Figure 2-2. Repeated in vitro inhibition of copper tolerant *X. perforans* strain GEV485 following exposure to various chemicals over time (15 min, 1 hr, 4 hr, and 24 hr). The treatments were as follows: Ag, Cu₂O, ZnO, Fe₂O₃, MgO and Mn₂O₃ at 1000 and 100 µg/ml effect on GEV485 (Cu tolerant *X. perforans* strain) growth over time (15 min, 1 hr, 4 hr, and 24 hr). The treatments were as follows: Ag, Cu₂O, ZnO, Fe₂O₃, MgO and Mn₂O₃ at 100 and 1000 µg/ml; copper prepared from Kocide® 3000(K3000) at 1000 (3.3 g/L), 500 (1.67 g/L), 200 (0.668 g/L) and 100 µg/ml (0.334 g/L). The treatments were compared with water control (UT). A) after 15 minutes B) after 1 hr. C) after 4hr. D) after 24 hrs incubations. Error bars= Standard Error. p-value of 0.05 was used in the IBM® SPSS® using SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-2. Continued
Figure 2-3. Repeated in vitro inhibition of copper tolerant X. perforans strain GEV485 following exposure to various chemicals over time (15 min, 1 hr, 4 hr, and 24 hr). The treatments were as follows: Ag, Cu₂O, ZnO, Fe₂O₃, MgO and Mn₂O₃ at 1000 and 100 µg/ml effect on 91-118 (Cu sensitive X. perforans strain) growth over time (15 min, 1 hr, 4 hr, and 24 hr). The treatments were as follows: Ag, Cu₂O, ZnO, Fe₂O₃, MgO and Mn₂O₃ at 100 and 1000 µg/ml; copper prepared from Kocide® 3000(K3000) at 1000 (3.3 g/L), 500 (1.67 g/L), 200 (0.668 g/L) and 100 µg/ml (0.334 g/L). The treatments were compared with water control (UT). A) after 15 minutes B) after 1 hr. C) after 4hr. D) after 24 hrs incubations. Error bars= Standard Error. p-value of 0.05 was used in the IBM® SPSS® using SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-3. Continued
Figure 2-4. Control of bacterial spot of tomato in the greenhouse. Bonny Best tomato plants were sprayed with 100, 200, 500 or 1000 µg/ml of Cu$_2$O or MgO; a combination of DuPont™ Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M); or sterile tap water prior to inoculating with a copper tolerant strain, GEV 485 of Xanthomonas perforans. The experiments were conducted in A) fall 2015, B) late spring 2016 and C) summer 2016. Disease severity of bacterial spot of tomato in greenhouse conditions is expressed as area under disease progress curve (AUDPC). Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-4. Continued
Figure 2-5. Phytotoxicity percentage on the 13th day after application of the test compounds in greenhouse conditions. A) First greenhouse completed in fall 2015 evaluated the following treatments: 100, 200, 500 and 1000 µg/ml of Cu₂O, MgO; a combination of Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M); and sterile tap water. Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® LSD (Least Significant Difference) statistical analysis. B) Second greenhouse completed in late spring 2016 with the same treatments as in A. C) Third greenhouse completed in summer 2016 with same treatments as in A and B.
Figure 2-5. Continued

Figure 2-6. Comparison of various treatments for control of bacterial spot of tomato in field conditions in Quincy in fall 2015. Disease severity was expressed as area under disease progress curve (AUDPC). The following treatments were compared: 200 and 1000 µg/ml for Cu$_2$O or MgO; DuPont™ Kocide® 3000 (2.1 g/L); a combination of Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M); and sterile tap water. Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® using SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-7. Yield from 2015 Fall Quincy, FL bacterial spot of tomato trial. Marketable yield harvested from plots treated with 200 and 1000 µg/ml for Cu₂O or MgO; DuPont™ Kocide® 3000 (2.1 g/L); a combination of Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M); and sterile tap water. Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® SNK (Student-Newman-Keuls) statistical analysis.

Figure 2-8. Comparison of various treatments for control of bacterial spot of tomato in field conditions in Balm in Spring 2016. The following treatments were evaluated: 200 and 1000 µg/ml for Cu₂O or MgO; DuPont™ Kocide® 3000 (2.1 g/L); a combination of DuPont™ Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (C+M); and sterile tap water. Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-9. The effect of various treatments on yield in Balm 2016 Spring bacterial spot of tomato trial. Total harvest from each treatment. Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® SNK (Student-Newman-Keuls) statistical analysis.

Figure 2-10. Disease severity of bacterial spot of tomato in field conditions. Completed in Quincy Spring 2016 evaluated the following treatments: 200 and 1000 µg/ml for Cu₂O or MgO; DuPont™ Kocide® 3000 (2.1 g/L); a combination of Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M); and sterile tap water. Error bars= Standard Error. P-value of 0.05 was used in the IBM® SPSS® SNK (Student-Newman-Keuls) statistical analysis.
Figure 2-11. Yield from 2016 Spring Quincy bacterial spot of tomato trial. Total harvest from each treatment. P-value of 0.05 was used in the IBM® SPSS® SNK (Student-Newman-Keuls) statistical analysis.
Nano-materials for different purposes have been introduced into food and agro-products industry in the recent years (Bouwmeester et al., 2009). They are widely used in diverse areas including: medical treatments, food packing and food safety, new technologies for pathogen detection, drug delivery systems and in cosmetics and sunscreen. A nano-material is defined as; “a discrete entity that has three dimensions of the order of 100 nm or less” (SCENIHR, 2007, Donaldson et al., 2001). The small size gives nano-materials unique features and physicochemical properties including different chemical composition (purity, crystallinity, electronic properties, etc.), surface structure (surface reactivity, surface groups, inorganic or organic coatings, etc.), solubility, shape, and aggregation ability (Dowling et al., 2004). Due to the different physical properties, nano-materials thus have unique features and have huge potential for various applications (Bouwmeester et al., 2009). Due to the widespread use of novel engineered nano-materials, safety of these materials is a concern for consumers (Maynard et al., 2006, Weiss et al., 2006). In addition, given that people in general are not aware of the impact and toxicity of nano-materials, a better understanding of the environmental impact of nano-materials is needed.

Nano-materials could be possibly used in the agro-food chain in several applications (Dowling et al., 2004). Nano-material based pesticides are possible, and have the potential of introducing free particles into the food chain. Nano-materials are used in water purification and reducing soil contamination by binding micro-organisms for the purpose of pathogen and contaminant removal (Baruah & Dutta, 2009). This is another activity that might introduce nano-materials into human consumption (Sekhon, 2014). Food processing and food production, refrigerators, storage containers and food preparation equipment could be treated with nano-
materials including incorporated nano-sized materials for antibacterial purposes (García et al., 2010). Nano-materials have also been used in food preservation and packaging for better oxygen scavenging to prevent the growth of pathogens (Bouwmeester et al., 2009, García et al., 2010). As a result of the potential uses of nano-materials, toxicologists have concerns regarding the use of free and insoluble nano-materials, including agglomerates that are thought to be the highest risk to consumer health (Ray et al., 2009).

Several studies in the last decade suggest that different environmental conditions may affect the ion concentration in crops (Netondo et al., 2004, Ashraf & Orooj, 2006, Isla & Aragüés, 2010). High ionic concentration of essential and non-essential elements absorbed by plants can lead to toxicity (Ke et al., 2007). Also, toxic elements with no known function in plant metabolism can be accumulated in plant tissue (Bondada et al., 2004, Lopez et al., 2009, Peralta-Videa et al., 2009, Arias et al., 2010). Low concentrations of toxic elements in plants could be transferred to consumers. For example, arsenic (As), which is known to promote cancer of the bladder, lung and skin (Hughes, 2002, Salgado et al., 2006), is a great concern in Bangladesh, China, Hungary and India (Chen et al., 2006). Besides its natural presence in soils (Luong et al., 2007), As is also released into the environment from smelting and mining processes, agricultural practices, wood preservation and food additives (Aldrich et al., 2007). Several plant species such as ferns have been studied for their ability to accumulate As in their fronds (Kertulis et al., 2005). Worldwide, rice is known for its high risk of accumulating As due to its tendency to be watered with groundwater containing high As concentrations (Zhu et al., 2008, Peralta-Videa et al., 2009).

As the possibility of nano-material exposure to crops increases due to the many potential applications (Khot et al., 2012), some studies have focused on the toxicity of nano-materials on
edible crops including rape (*Brassica napus*), radish (*Raphanus sativus*), lettuce (*Lactuca sativa*), corn (*Zea mays*) and cucumber (*Cucumis sativus*) (Lin & Xing, 2007, Lee et al., 2008, Barrena et al., 2009). The most studied metallic nano-materials are TiO$_2$, CeO$_2$, Fe$_3$O$_4$ and ZnO. In a review by Rico et al. (2011) uptake of Au, Ag, Cu and Fe nanoparticles by plants was discussed and provided a basic understanding for how nano-materials might act in plants. Current studies suggest that the accumulation of the nano-materials depend on the species and size of the plants and the types of the nano-materials. However, published studies on nanomaterial uptake by plants is inconclusive (Parsons et al., 2010, Lee et al., 2008). Several possible models for nano-material uptake by plants have been introduced (Rico et al., 2011, Hall & Williams, 2003). Some studies suggest that nanomaterials can enter plant cells by binding to carrier proteins, through aquaporins, ion channels, or endocytosis, by creating new pores (preferably for CNTs), or by binding to organic chemicals in the environment (Rico et al., 2011). Most of the metallic nano-materials were reported to be taken up by plants with ion transporters (Hall & Williams, 2003).

With the limited studies, there is no comprehensive information on the toxicity of Fe$_3$O$_4$, CeO$_2$, SiO$_2$, TiO$_2$ and ZnO to crops, people and the environment; however, with the very limited information, a new field of nanoecotoxicology has emerged to address the effects of nano-materials on the living organisms of ecosystems (Baun et al., 2008, Rico et al., 2011). We hypothesize that application of nanomaterials as a bactericide to tomato fruit does not cause excessive elemental accumulation in the harvested fruits. As the fresh market tomato is the major product in Florida, the objective is to evaluate if application of metallic oxide nano-materials on tomatoes results in accumulation of heavy metals in fresh tomato fruits.
Materials and Methods

Experimental Design

The experimental design was a completely randomized block design. Each treatment consisted of four plots with each plot consisting of 15 BHN602 (BHN Seed, Immokalee, FL) tomato plants. Treatments were 200 and 1000 µg/ml of Cu$_2$O, 200 and 1000 µg/ml of MgO; DuPont™ Kocide® 3000 at 1.75 lb/a; the grower’s standard of Kocide 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L); and an untreated control. The treatments were sprayed on foliage one week prior to bacterial inoculation. Treatments were applied weekly until one week before harvesting fruit. At harvest, five mature-green stage fruits exposed to the treatments were collected from each of four plots for each of the treatments. Tomato fruit size is medium with diameters between 5.72 and 6.43 cm according to USDA (Agriculture, 1997, Kelley et al., 2010) were collected from the outside of the canopy from the 2015 Fall Quincy trial. Hand-washed fruits were collected twice for analysis 7 and 20 days after final application.

Vegetation Matrix

Fruit were analyzed for the following elements: Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, S and Zn. Four to eight grams of fresh tomato fruit, which had been separated into peel and flesh, and the whole fruit were dried in an electric oven at 70°C for 48 h. Dried samples were pre-digested overnight with 2 ml of concentrated nitric acid and 2 ml of H$_2$O$_2$. After the pre-digestion, these samples were digested at 115°C for 45 min and then cooled to room temperature. After cooling to room temperature, the samples were filtered through cotton plugs and the volume adjusted to 50 ml. The samples were stored at room temperature until analysis.

Analysis for Heavy Metals

Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn concentrations were determined by Inductively Coupled Plasma Emission Spectroscopy (ICP-OES) using the Atom Scan 16
(Thermo-Jarrell Ash, Franklin, MA, USA). Analysis was performed following the methods described in previous studies (Stilwell & Graetz, 2001, Mattina et al., 2003).

**Results**

The fruits harvested 7 days after the final nanomaterial application in the 2015 Fall field test in Quincy were evaluated with ICP-OES to determine whether different treatments influenced Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, S and Zn elemental accumulation in fruit. Treatments were compared to the untreated control (p=0.05) with IBM® SPSS® SNK (Student-Newman-Keuls), which was used for statistical analysis. There were no significant differences for any of the elements when comparing the elemental concentration in dry fruit with different treatments (data not shown). As for fresh fruit (Fig. 3-1A), both concentrations of MgO nanomaterial and the grower standard, Cu-mancozeb, showed significantly lower Al concentrations than the untreated control, with Al accumulation twice as high in untreated fruit than in the other three treatments. In addition, only the grower standard, copper-mancozeb, showed significantly higher P content in fruit compared to the control fruit (Fig. 3-1C). The P content was around 100 mg/kg more than in untreated fresh weight. Interestingly, only Cu-mancozeb showed significant Cu accumulation in whole fruit, and was 0.2 mg/kg more compared to the untreated control, while MgO and Cu₂O at both concentrations did not differ from the untreated control (Fig. 3-1B).

For the fruits collected on the 20\textsuperscript{th} day after last application of test materials, Cu-mancozeb showed significantly higher Al content in the whole fruit which is 0.04 mg/kg more than in the untreated fruit (Fig. 3-2A). However, Al did not significantly accumulate in either peel or flesh for the Cu-mancozeb treatment. For other treatments, Cu₂O at 1,000 µg/ml showed significantly lower Al content, almost half of that accumulated in the untreated fruit (Fig. 3-2B). For Cu accumulation (Fig. 3-3), fruit receiving Cu-mancozeb applications showed significantly
greater Cu accumulation in whole fruit (+0.22 mg/kg more) (Fig. 3-3A), peel (+0.31 mg/kg) (Fig. 3-3B) and flesh (+0.16 mg/kg) (Fig. 3-3C) compared to the untreated control. Fruits receiving Kocide showed significant Cu accumulation in the peel (+0.27 mg/kg).

**Discussion**

In this study we measured elemental accumulation in fruit following application of metal nanomaterials to leaf and fruit tissue. Analysis of fresh fruit from the first harvest revealed that only Cu-mancozeb had significant Cu accumulation in whole fruit, and was 0.2 mg/kg more than the untreated control. For the fruit collected 7 d after the last application, treatments with Cu-mancozeb had significant Cu accumulation in whole fruit and flesh compared to the untreated control. Fruit receiving Kocide showed significant Cu accumulation in the peel compared to the untreated control. None of the other treatments, including MgO and Cu₂O nanomaterials, had significantly greater Cu accumulation in fruit compared to the untreated control.

Copper (Cu) is an essential element for mammals and plants; however, elevated Cu levels may be toxic (Gaetke & Chow, 2003). In one study, Cu was found in small amounts in a variety of tissues and organs, with the liver having the highest concentration in the human body (Turnlund et al., 1998). The only way copper normally enters the digestive system of mammals living on land is via the alimentary tract (Linder, 2013). Cu consumption is dependent on food choices and diet. Generally, ground water contains 4-10 μg /L of Cu bound with organic material (Barceloux, 1999). US EPA limits a maximum Cu concentration in drinking water to 1.3 mg Cu/L (Fitzgerald, 1998). However, other than water, mammals including humans, usually intake Cu by food (Sandstead, 1995). Around 30–50% of ingested Cu, mostly in the form of Cu²⁺, is absorbed in the small intestine and stomach (Turnlund et al., 1997). Following absorption in the small intestine, Cu is transported in the blood then bound predominantly to albumin or transcuprein (Turnlund et al., 1998).
The amount of Cu ingested in food and water is relatively low. Cu levels can be adjusted in the body by most humans and animals by either decreased absorption or increased excretion (Gaetke & Chow, 2003). However, Cu toxicity resulting from exposure to excess Cu does occur. Chronic Cu toxicity effects can occur in the liver, which is the primary organ for Cu accumulation once Cu enters into the blood stream. Cu toxicity typically will develop into liver cirrhosis with episodes of hemolysis and damage to renal tubules, the brain, and other organs. The symptoms have potential to progress into coma or death (Winge & Mehra, 1990). Chronic Cu toxicity has been reported in patients receiving dialysis via Cu tubing (Klein et al., 1972) and in infants receiving intravenous total parenteral nutrition (Beshgetoor & Hambidge, 1998). Acute Cu toxicity occurred while consuming Cu-contaminated water or food (Knobeloch et al., 1998, Spitalny et al., 1984). The symptoms of acute Cu poisoning include weakness, lethargy, and anorexia (Semple et al., 1960, Winge & Mehra, 1990). More advanced symptoms include erosion of the epithelial lining of the gastrointestinal tract, hepatocellular necrosis in the liver and acute tubular necrosis in the kidney (Barceloux, 1999). Animals vary in their sensitivity toward Cu. In humans the estimated lethal dose of Cu in an untreated adult is about 10–20 g (Winge & Mehra, 1990, Gaetke & Chow, 2003).

In our experiment, 0.2 mg more of copper per kilogram fresh weight tomato fruit accumulated in fruit in which the grower standard Cu-mancozeb was applied compared to the untreated control. On the other hand, there was no significant Cu accumulation of Cu due to use of Cu₂O and MgO treatments at all concentrations. In this scenario, Cu-mancozeb application might lead to more Cu exposure for customers in which the current disease control strategy of applying copper-based bactericides is used.
Although copper accumulation is a concern, aluminum (Al) accumulation might be a bigger risk. According to our results, in the first week of harvest (Fig. 3-1A), both concentrations of MgO nanomaterial and the grower’s standard Cu-mancozeb showed significantly lower Al concentration than the untreated control. For the fruit from the first collection, those treated with Cu-mancozeb showed significantly higher Al content in the whole fruit compared to the untreated (Fig. 3-2A). However, Al did not significantly accumulate in either peel or flesh for the Cu-mancozeb treatment. For other treatments, Cu₂O at 1,000 µg/ml showed significantly lower Al content (approximately half the concentration) than in the untreated control (Fig. 3-2B).

Al has been shown to cause dialysis encephalopathy and Al-induced osteomalacia. This metal has been implicated with Alzheimer disease, but the issue is at present controversial after a century of debate (Campbell et al., 2001, Tomljenovic, 2011, Perl & Brody, 1980). The disease was first described as neurofibrillary tangles (NFT) in 1907 (Alzheimer, 1907), and is described as progressive dementia that occurs in middle or late life (McKhann et al., 1984). In 1973, Crapper et al. (Crapper et al., 1973) reported aluminum accumulation in brains of four patients with Alzheimer symptoms. Two studies were conducted in which there was no direct evidence linking Al accumulation with Alzheimer disease with Al absorption varying largely between individuals. In general, Al absorption increases with aging but researchers observed that young Alzheimer patients tend to have more Al absorption compared to other individuals (Mjöberg et al., 1997, Taylor et al., 1992). However, there is still no direct evidence linking Al accumulation with Alzheimer disease. On the other hand, most of the resorbed Al is discharged, while the non-discharged Al accumulated in bone. As the Al is slowly released into cells from bone, this reaction might interfere with DNA transcription in cells (Lukiw et al., 1992) and result in an increase in beta-amyloid production (Clauberg & Joshi, 1993, Zatta et al., 1993). To sum up,
although there is no direct evidence for Al toxicity in food, higher Al accumulation in tomato might still potentially be a risk to the public (Becaria et al., 2002).

Magnesium (Mg) is the fourth most abundant metal in the body and serves as the second most common intracellular electrolyte (Greene et al., 1988). In a study, Mg was mostly eliminated by the urinary system but about 90–95% of filtrated Mg was reabsorbed in the kidney (De Baaij et al., 2012). Excessive Mg primarily occurs in patients with serious renal insufficiency. The excess Mg tends to block neuromuscular transmission because of the diminution in endplate potential (ENGBAEK, 1952, Prasad, 2013). High concentration of Mg may cause hypermagnesemia and decrease deep tendon reflexes after Mg level reaches 4 meq/L (Prasad, 2013). However, Mg insufficiency is a much more common situation and is associated with a wide spectrum of diseases, including Type 2 diabetes, hypertension, osteoporosis, tetany, seizures and depression (Musso, 2009, Quamme, 2008, Dimke et al., 2010, De Baaij et al., 2012). Various studies indicate that dietary intake of magnesium is inadequate in the U.S. population as well as in other countries (Pennington & Schoen, 1995, Galan et al., 1997, Wilson et al., 1997, Ford, 1997, Ford & Mokdad, 2003). In the current research, using MgO nano-material did not cause a significantly higher or lower Mg concentration in fruit compared to the untreated.
Figure 3-1 Elemental accumulation of A) aluminum (Al) B) copper (Cu) and C) phosphorus (P) in fruits (fresh weight; mg/kg) from the first harvest (7 days from the last application) in fall 2015 trial following treatment with: 200 or 1000 µg/ml of Cu₂O or MgO, Kocide® 3000, a combination of Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M), and untreated control. Error bar= standard error. P-value = 0.05. IBM® SPSS® SNK (Student-Newman-Keuls) was used for the statistical analysis.
Figure 3-1. Continued
Figure 3-2. Al accumulation in fruits (fresh weight; mg/kg) collected from the second harvest (20 days from the last application) in fall 2015 trial following treatment with: 200 or 1000 µg/ml of Cu₂O, MgO and Kocide® 3000; a combination of Kocide® 3000 (2.1 g/L) and Penncrozeb® 75DF (1.2 g/L) (K+M); and untreated control A) From whole fruit B) From peel only C) From flesh only. Error bar= standard error. P-value =0.05.

IBM® SPSS® SNK (Student-Newman-Keuls) was used for the statistical analysis.
Figure 3-3. Cu accumulation in fruits (fresh weight; mg/kg) collected from the second harvest (20 days from the last application) in fall 2015 trial following treatment with: 200 or 1000 µg/ml of Cu₂O, MgO and Kocide® 3000; a combination of Kocide® 3000 (2.1 g/L) and Penncozeb® 75DF (1.2 g/L) (K+M); and untreated control A) From whole fruit B) From peel only C) From flesh only. Error bar= standard error. P-value of 0.05. IBM® SPSS® SNK (Student-Newman-Keuls) was used for the statistical analysis.
Figure 3-3. Continued
Bacterial spot caused by *Xanthomonas* spp. is one of the most detrimental diseases of tomato (Ritchie, 1991). The disease is caused by four distinct species of *Xanthomonas* (*X. euvesicatoria (Xe), X. gardneri (Xg), X. perforans (Xp), and X. vesicatoria (Xv)) (Jones et al., 2004). The disease can cause yield losses of >50% (Jones & Jones, 1985). Since 2006, *X. perforans* (Xp) has been the dominant species on tomato in Florida (Horvath et al., 2012).

Continuous use of Copper (Cu) bactericides over many decades has led to the development of Cu-tolerant strains, which renders Cu ineffective. Cu-tolerant strains of *Xanthomonas* was first detected in Florida in the late 1960’s (Marco & Stall, 1983). Due to the limited availability of effective alternative management options, Cu is still used by growers in Florida. Growers use copper-mancozeb, which was used in this study as the grower’s standard, for managing bacterial spot of tomato in transplant and field production. Nanotechnological approaches are a new and active area of research for management of bacterial diseases. Various studies have recently demonstrated that nanomaterials improve antibacterial properties compared to their bulk counterparts (Paret et al., 2013, Hagfeldt & Graetzel, 1995, Henglein, 1993). We hypothesize that (1) using reduced sized particles of metallic oxides may lead to greater antibacterial activity with both copper- sensitive and Cu-tolerant strains, (2) Using those nano-materials for bacterial spot disease management with foliar application would not lead to elemental accumulation in fruits. In this study the antibacterial activity of metallic oxide nanoparticles copper oxide (*Cu_2O*), magnesium oxide (*MgO*), zinc oxide (*ZnO*), manganese oxide (*Mn_2O_3*), iron oxide (*Fe_2O_3*) and silver (*Ag*) were evaluated against Cu-tolerant and sensitive *X. perforans*. The in vitro assay demonstrated that *Ag, ZnO, Cu_2O* and *MgO* nanomaterials have significantly high antibacterial properties compared to the equivalent concentration of the Cu-based bactericide (Kocide 3000).
towards Cu-tolerant and sensitive *X. perforans* strains. In a greenhouse experiment, application of MgO and Cu$_2$O at 200 µg/ml to tomato plants significantly reduced bacterial spot disease severity compared with copper-mancozeb and untreated controls (p = 0.05). In field experiments in Quincy, FL and Balm, FL applications of MgO at 200 and 1000 µg/ml provided a significant reduction in bacterial spot disease severity compared to the untreated control (p = 0.05). The effect was comparable to Kocide 3000+Mancozeb, the grower standard. There was no significant difference in the yield between any of the treatments.

Inductively Coupled Plasma Emission Spectroscopy (ICP-OES) was used to determine if the nanomaterials applications led to elemental accumulation in the fruits. This demonstrated that Cu, Mg and other metals including Al, B, Ca, Fe, K, Mn, Mo, Na, P, S and Zn did not significantly accumulate in the fruits due to application of Cu$_2$O and MgO nanomaterials. The only treatment with significant accumulation of Cu compared to the untreated control was for Kocide 3000+Mancozeb treatment (p = 0.05). This study shows the potential for MgO-based nanomaterial as an excellent candidate for further studies on development of a commercial grade formulation for management of bacterial spot of tomato.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Ying-Yu Liao was born in 1990, Taipei, Taiwan. She graduated from high school in 2009 from Taipei First Girls’ High School, Taiwan while doing researches in Institute of Plant and Microbial Biology at Academia Sinica. She completed her undergraduate degree in Agricultural Chemistry from National Taiwan University in Taiwan. Her undergraduate research was focused on “Identification of genes involved in interbacterial competition ability and regulation of type VI secretion system (T6SS)-related genes using a random mutagenesis approach.” under guidance of Dr. Nai-Chun Lin during August, 2011 to June 2013. In August 2013, she joined the Department Plant Pathology, University of Florida as a Master student and research assistant under the supervision of Dr. Mathews Paret and Dr. Jeffery B. Jones. She conducted research on “Evaluating the potential for development of metallic oxide nanoparticles for management of bacterial spot of tomato” and “Understanding the relationship between Type VI secretion system and Bacteriocin A in Xanthomonas perforans” in her Master program.