Low Concentrations of a Silver-Based Nanocomposite to Manage Bacterial Spot of Tomato in the Greenhouse

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Abstract

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Bacterial spot, caused by four *Xanthomonas* spp., is one of the most damaging diseases of tomato worldwide. Due to limited disease management options, growers rely heavily on copper-based bactericides, which are often ineffective due to the presence of copper-resistant *Xanthomonas* strains. This study was undertaken to characterize the antibacterial activity of a silver-based nanocomposite, Ag-dsDNA-GO, and its potential as an alternative to copper. Ag-dsDNA-GO at rates as low as 10 µg/ml killed all bacterial cells of copper-tolerant and -sensitive *Xanthomonas perforans* strains in suspensions containing approximately 10³ CFU/ml within 15 min of exposure in vitro, whereas equivalent rates of copper (10, 25, and 50 µg/ml)

were unable to significantly reduce populations compared with the untreated control after 24 h of exposure ($P = 0.05$). All copper concentrations killed the copper-sensitive *X. perforans* strain but required exposure for ≥ 1 h. Ag-dsDNA-GO also exhibited antibacterial activity against copper-tolerant *X. vesicatoria*, *X. euvesicatoria*, and *X. gardneri* strains. In greenhouse studies, tomato plants treated with Ag-dsDNA-GO at either 75 or 100 µg/ml prior to artificial inoculation significantly reduced disease severity when compared with copper-mancozeb and negative controls ($P = 0.05$). This study highlights the potential of Ag-dsDNA-GO as an alternative to copper in tomato transplant production.

Bacterial spot of tomato is caused by four species of *Xanthomonas* (*Xanthomonas euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria*) (Jones et al. 2004). It is one of the most detrimental diseases of tomato worldwide, having a high negative impact on yield of tomato plants grown in warm, humid regions. Infection typically results in leaf and fruit lesions, defoliation, and yield loss of marketable fruit (Louws et al. 2001; Ritchie 2000). If weather conditions are optimal for disease development, bacterial spot can cause yield losses of up to 50% (Louws et al. 2001; Pohronezny and Volin 1983; Scott et al. 1989). Therefore, an effective management strategy for bacterial spot of tomato is crucial for maintaining the tomato industry in the United States.

Antibiotics and copper-based bactericides have been used extensively to manage bacterial spot. Although copper bactericides have been used since the mid-1900s, streptomycin was the dominant bactericide for bacterial spot management during the 1950s and early 1960s (Marco and Stall 1983; Thayer and Stall 1962). The pathogen quickly developed resistance to streptomycin and the antibiotic became ineffective, even when applied at high rates such as 400 µg/ml (Thayer and Stall 1962). As a result, copper-based bactericides became the chemical of choice for bacterial spot management. Due to continuous use, copper-resistant bacterial populations became prevalent in the late 1960s in Florida (Marco and Stall 1983). The addition of ethylene-bis-dithiocarbamates such as mancozeb to copper-based bactericides resulted in improved disease control (Conover and Gerhold 1981; Marco and Stall 1983). Copper-tolerant *Xanthomonas* spp. were sensitive to this mixture, in part due to the release of more soluble copper than in a copper suspension alone (Marco and Stall 1983). Unfortunately, during optimal disease conditions, copper-mancozeb fails to adequately control bacterial spot when copper-tolerant strains are present (Jones and Jones 1985; Obradovic et al.

2004). Currently, copper-mancozeb is used in combination with cultural practices such as using disease-free transplants to reduce bacterial spot severity. Exclusion of the pathogen from transplant production systems is a major component of an effective disease management program; however, the lack of effective bactericides drastically reduces the ability to produce disease-free transplants (Cooksey 1990; Gitaitis et al. 1992; Thayer and Stall 1962).

Other chemical approaches for managing bacterial spot of tomato include systemic acquired resistance (SAR) inducers, biofilm inhibitors, and light-activated nanomaterials. Synthetic SAR inducers such as acibenzolar S-methyl (ASM; Actigard 50W, Syngenta Crop Protection, Greensboro, NC) trigger the plant's natural defenses, increasing resistance to bacterial spot in tomato plants (Obradovic et al. 2005). Additionally, Worthington et al. (2012) reported that the small molecule additive 2-aminoimidazole (2AI), in combination with copper, suppressed copper resistance and decreased biofilm formation of *X. euvesicatoria* in vitro. Furthermore, pepper plants treated with 2AI and copper had less bacterial spot severity and increased fruit yields when compared with the untreated control. Paret et al. (2013) evaluated the use of a light-activated nanoscale form of titanium dioxide (TiO₂) alone and in combination with silver (Ag) or zinc (Zn) for managing bacterial spot. Greenhouse and field trials revealed that TiO₂/Zn significantly reduced disease incidence when compared with copper-based bactericides. However, TiO₂/Zn had no antibacterial effect in the absence of light, and tomato plants exhibited phytotoxicity following the sixth application at approximately 500 µg/ml in field trials in Florida. This is a potential limiting factor in commercialization of this technology.

Another potential alternative to copper-based bactericides is silver nanoparticles (AgNP). AgNP have antimicrobial activity against many animal pathogens, such as *Escherichia coli*, *Candida albicans*, *Hepatitis B virus*, and human immunodeficiency virus type 1 (Elechiguerra et al. 2005; Kim et al. 2007; Kim et al. 2009; Lu et al. 2008). Unfortunately, AgNP are prone to aggregation in the environment, which reduces their antibacterial activity due to the reduction in surface area covered (Bae et al. 2010). To prevent aggregation and increase antibacterial activity, Ochoy et al. (2013a,b) designed an Ag-based nanocomposite termed Ag-dsDNA-GO. To control

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nanoparticle size and prevent aggregation, AgNP were grown on double-stranded (ds)DNA using a reducing agent. The dsDNA was attached to graphene oxide (GO) and functioned as a bridge between the AgNP and GO. GO is a carbon-based sheet that is composed of a single layer of carbon atoms with active surface hydroxyl, epoxy, and carboxyl groups. GO increases the antibacterial activity of AgNP by increasing the adhesive force between the nanocomposite and bacterial cell membranes, and by allowing for a more uniform distribution of the AgNP to increase the total surface area. Ocoy et al. (2013b) demonstrated that Ag-dsDNA-GO decreased *X. perforans* cell viability in vitro and in a preliminary in planta experiment. In vitro, Ag-dsDNA-GO had improved stability and exhibited enhanced antibacterial activity when compared with AgNP alone and Ag-GO. For example, Ag-dsDNA-GO at 20 µg/ml killed more than 95% of *X. perforans* cells, while AgNP alone did not significantly reduce the number of *X. perforans* cells until a concentration of 50 µg/ml or higher was used. Although the study by Ocoy et al. (2013b) demonstrated that AgNP could effectively kill *X. perforans* in vitro, it did not compare the efficacy of Ag-dsDNA-GO on a copper-tolerant strain to a copper-sensitive strain or to equivalent levels of copper. Furthermore, in this study, only one preliminary greenhouse experiment was conducted to test the ability of Ag-dsDNA-GO to reduce bacterial spot severity. Because the greenhouse experiment was not repeated in this study, the results have not been validated. Additionally, the highest concentration tested in the greenhouse was 100 µg/ml, which does not address the potential risk of phytotoxicity. Thus, further greenhouse experiments are needed to assess the potential of Ag-dsDNA-GO to manage bacterial spot and the risk of phytotoxicity in planta. Additionally, this study only evaluated the nanocomposites antibacterial activity against *X. perforans* and not the other three bacterial spot pathogens.

Thus, the goal of this study was to compare the bactericidal effects of Ag-dsDNA-GO to copper and assess the risk of phytotoxicity. The specific objectives of this study were to (i) compare the in vitro activity of Ag-dsDNA-GO and copper on the survival of copper-tolerant and copper-sensitive *X. perforans* strains; (ii) determine the effect of Ag-dsDNA-GO on the survival of copper-tolerant *X. euvesicatoria*, *X. gardneri*, and *X. vesicatoria* in comparison with field applied rates of copper in vitro; (iii) compare the effects of Ag-dsDNA-GO and copper-mancozeb on bacterial spot severity caused by a copper-tolerant *X. perforans* strain under greenhouse conditions; and (iv) assess the risk of phytotoxicity of Ag-dsDNA-GO on the tomato foliage.

Materials and Methods

Bacterial strains and storage. As of 2006, the dominant species causing bacterial spot of tomato in Florida has been *X. perforans* (Horvath et al. 2012). Hence, two *X. perforans* strains, GEV485 (copper-tolerant) and 91-118 (copper-sensitive) were used in this study. The other bacterial spot of tomato pathogens also pose a threat to tomato production in several other states such as Ohio, Michigan, California, Georgia, and Indiana (Potnis et al. 2015). Thus, three additional copper-tolerant *Xanthomonas* strains—444 (*X. gardneri*), E3 (*X. euvesicatoria*), and Bv54a (*X. vesicatoria*)—representing the other bacterial spot pathogens were used in an additional in vitro assay. For short-term storage and experiments, bacteria were grown on nutrient agar (NA) medium (BBL, Becton Dickinson and Co., Cockeysville, MD) at 28°C and transferred every 24 to 48 h. For long-term storage, purified cultures were stored in sterile 30% glycerol solution at -80°C.

Synthesis of DNA. The nucleotides and controlled pore glass were provided by Glen Research (Sterling, VA). DNA oligonucleotides DNA-1: 5'-AAT GTG CTC CCC CAGCGCGCTT-FITC-3' and DNA-2: 3'-TTA CAC GAG GGG GT-5' were synthesized on an ABI 3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). FITC dye modification on DNA-1 was done to confirm the binding of DNA-1 to the surface of GO and accumulation of AgNP in the major grooves of dsDNA. After deprotection of DNA1 and DNA2, they were purified with reversed-phase high-performance liquid chromatography (ProStar, Varian, Walnut Creek, CA) using

a C18 column (Econosil, 5 µm, 250 by 4.6 mm) from Alltech (Deerfield, IL). After purification, DNA solutions were dried in a centriVap centrifugal vacuum concentrator (Labconco, Kansas City, MO), and the DNA was dissolved in water. The concentration of each DNA solution was calculated using the absorbance value of DNA from UV-Vis spectrum and extinction coefficient of DNA obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

Synthesis of Ag-dsDNA-GO. The concentration and size of the single layer of GO (Graphene Supermarket, Ronkonkoma, NY) were 5 g/liter and 0.5 to 3 µm, respectively. Silver nitrate (AgNO₃; 99%), sodium borohydride (NaBH₄; 98%), and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (St. Louis). Sodium nitrate (NaNO₃) was obtained from Alfa-Aesar (Ward Hill, MA) and 18.2 MΩ Ultrapure water (Millipore Co., Billerica, MA) was used in all the experiments. Both DNA-1: 5'-AAT GTG CTC CCC CA GCGCGCTT FITC-3' and DNA-2: 5'-TGG GGG AGC ACA TT-3' (1 µm each) were hybridized by mixing in 10 mM HEPES buffer (pH 7.5) for 30 min. The underlined bases (GCGCGTT) indicate the extension bases of DNA1, which creates the major groove of dsDNA where the AgNP can accumulate and allows for the dsDNA to be absorbed onto the surface of GO. The hybridized DNA solution was incubated with GO suspension at 50 µg/ml for 30 min to adsorb dsDNA to the GO surface. AgNO₃ solution was added to the dsDNA-adsorbed GO suspension to achieve a final concentration of 100 µM Ag⁺ ions. After stirring for 10 min, freshly prepared NaBH₄ was added to the mixture slowly to reach a final concentration of NaBH₄ of 500 µM. The resulting mixture was stirred for 1 h. The final mixture was then centrifuged to stop the formation of the AgNP. The precipitate was suspended in HEPES buffer and centrifuged at 12,000 rpm for 15 min. The washing process was repeated three times. Growth of the AgNP on dsDNA occurs during the precipitation step.

In vitro assays. Two *X. perforans* strains, GEV485 and 91-118, were grown on NA plates for 24 h at 28°C. The bacterial strains were then transferred to NA plates containing copper (II) sulfate pentahydrate (CuSO₄ · 5H₂O) (Sigma-Aldrich) at 0.08 µmol/liter and incubated for 24 h at 28°C. Bacterial suspensions were prepared in sterilized tap water, adjusted to absorbance at 600 nm = 0.3 (approximately 5 × 10⁸ CFU/ml), then diluted to 10⁵ CFU/ml. For each strain, 20 µl of each bacterial suspension was transferred to sterile glass tubes containing 2 ml each of the following: Ag-dsDNA-GO at 10, 25, and 50 µg/ml (Ag at approximately 1.6, 4, and 8 µg/ml, respectively) and copper prepared from Kocide 3000 (DuPont, Wilmington, DE) at 10 µg/ml (0.0333 g/liter), 25 µg/ml (0.0667 g/liter), and 50 µg/ml (0.167 g/liter). Kocide 3000 contains 30% metallic copper (Kocide 3000 at 1 g/liter contains copper at approximately 300 µg/ml) in the form of copper hydroxide (Cu(OH)₂). Additionally, three sterilized glass tubes containing 2 ml of sterilized tap water and 20 µl of each bacterial suspension served as controls. The tubes were incubated on a shaker (150 rpm) at 28°C. Bacterial populations were measured by plating 50 µl from each tube on NA after 15 min, 1 h, 4 h, and 24 h of incubation. The plates were incubated for 48 h at 28°C; then, the number of colonies formed on each plate was counted to calculate the CFU per milliliter for each time point. Each treatment consisted of three replicates and each in vitro assay was conducted two times. Furthermore, the assay described above was used to evaluate the efficacy of Ag-dsDNA-GO on three copper-tolerant *Xanthomonas* strains (444, E3, and Bv5-4a) representing the three remaining bacterial spot pathogens. The in vitro assay protocol described above was utilized but the bacterial populations were only sampled at one time point (15 min). The treatment list was as follows: Ag-dsDNA-GO at 10 µg/ml (approximately 1.6 of Ag) and copper prepared from Kocide 3000 at 500 µg/ml (1.67 g/liter). GEV485 was used as a control strain for this experiment. Each treatment consisted of three replicates and each in vitro assay was conducted two times.

Greenhouse experiments. The following treatments (200 ml) were prepared in sterile tap water for the first experiment: Ag-dsDNA-GO at 100, 200, and 500 µg/ml; a combination of Kocide 3000 (2.1 g/liter) and Penncozeb 75DF (1.2 g/liter; United Phosphorus, Inc., King of Prussia, PA); and sterile tap water. For the second and third greenhouse experiments, a 75 µg/ml concentration of

Ag-dsDNA-GO was substituted for the 500 µg/ml concentration of Ag-dsDNA-GO. There were four plants per treatment in each experiment. The chemical suspensions were applied as foliar sprays onto the abaxial and adaxial leaf surfaces of 2- to 3-week-old 'Bonnie Best'

Fig. 1. In vitro activity of various concentrations of Ag-dsDNA-GO and copper (Kocide 3000) on *Xanthomonas perforans* survival over time (black bars = 15 min, dark-gray bars = 1 h, white bars = 4 h, and light-gray bars = 24 h). There were three replicates per treatment and the treatments were as follows: Ag-dsDNA-GO at 10, 25, and 50 µg/ml or copper prepared from DuPont Kocide 3000 at 10 µg/ml (0.033 g/liter), 25 µg/ml (0.0667 g/liter), and 50 µg/ml (0.167 g/liter). **A**, GEV485 (copper-tolerant *X. perforans* strain); **B**, 91-118 (copper-sensitive *X. perforans* strain). Error bars = standard deviation. A *P* value of 0.05 was used in the IBM SPSS least significant difference statistical analysis. In vitro experiments were conducted twice and the effect of experiment was not significant. Therefore, the data were pooled for analysis.

Fig. 2. In vitro activity of Ag-dsDNA-GO at 10 µg/ml (white bars) and copper at 500 µg/ml prepared from DuPont Kocide 3000 (1.67 g/liter) (gray bars) compared with the untreated control (black bars) against copper-tolerant strains of *Xanthomonas euvesicatoria* (E3), *X. gardneri* (444), *X. perforans* (GEV485), and *X. vesicatoria* (Bv5-4a). There were three replicates per treatment. Error bars = standard deviation. A *P* value of 0.05 was used in the IBM SPSS least significant difference statistical analysis. In vitro experiments were conducted twice and the effect of experiment was not significant. Therefore, the data were pooled for analysis.

tomato plants until run-off. Four plants were sprayed with sterile tap water, which served as the negative control. The chemical suspensions were allowed to air dry on the leaf surface completely before inoculation, which usually took between 2 and 6 h. The copper-tolerant *X. perforans* strain, GEV485, was used in the greenhouse experiments. GEV485 was grown on NA plates for 24 h at 28°C. A bacterial suspension (5×10^8 CFU/ml) was sprayed onto both sides of the tomato leaves. The inoculated plants were placed in plastic bags to maintain high humidity and incubated in a growth chamber at 28°C for 48 h. The bags were then carefully removed and the plants were incubated in the growth chamber for 5 additional days before being transferred to the greenhouse. In the greenhouse, tomato plants were subjected to natural light conditions (approximately 12 h of light per day), average nightly temperatures

Fig. 3. Effect of various Ag-dsDNA-GO concentrations and copper-mancozeb on control of bacterial spot of tomato under greenhouse conditions. AUDPC = area under the disease progress curve. **A**, Experiment 1 consisted of the following treatments: Ag-dsDNA-GO at 100, 200, and 500 µg/ml; a combination of DuPont Kocide 3000 (2.1 g/liter) and Penncozeb 75DF (1.2 g/liter) (Cu-Mancozeb); and sterile tap water as a control. **B**, Experiment 2 consisted of the following treatments: Ag-dsDNA-GO at 200, 100, and 75 µg/ml; a combination of DuPont Kocide 3000 (2.1 g/liter) and Penncozeb 75DF (1.2 g/liter) (Cu-Mancozeb); and sterilized tap water as a control. **C**, Experiment 3 consisted of the following treatments: Ag-dsDNA-GO at 200, 100, and 75 µg/ml; a combination of DuPont Kocide 3000 (2.1 g/liter) and Penncozeb 75DF (1.2 g/liter) (Cu-Mancozeb); and sterilized tap water as a control. Error bars = standard deviation. *P* value of 0.05 was used in the IBM SPSS Student-Newman-Keuls statistical analysis. There were four plants per treatment in each experiment.

of 21°C, and average daytime temperatures of 32°C. Disease was assessed using the Horsfall-Barratt disease severity scale every other day beginning 10 days postinoculation and ending 17 days postinoculation (Horsfall and Barratt 1945). The area under the disease progress curve (AUDPC) was then calculated, as previously described (Madden et al. 2007).

Statistics. All statistical analyses were completed using IBM SPSS Statistics Version 22. There was no significant effect of the trial on bacterial recovery of a single strain, as shown by analysis of variance with a P value of 0.05 in the in vitro experiments (data not shown). Thus, the data were combined and analyzed for statistical significance using least significant difference with a P value of 0.05. The data collected from each greenhouse experiment were analyzed for statistical significance using the Student-Newman-Keuls method and a P value of 0.05.

Results

The effect of Ag-dsDNA-GO on in vitro *Xanthomonas* spp. growth. In vitro, all concentrations of Ag-dsDNA-GO demonstrated antimicrobial efficacy against the copper-tolerant *X. perforans* strain, GEV485 (Fig. 1A). All concentrations of Ag-dsDNA-GO (10, 25, and 50 µg/ml) completely inhibited bacterial growth within 15 min. In contrast, none of the copper concentrations (10, 25, and 50 µg/ml) significantly ($P = 0.05$) reduced the bacterial population when compared with the untreated control. As expected, all copper concentrations adversely affected growth of the copper-sensitive strain 91-118 (Fig. 1B). All copper concentrations significantly ($P = 0.05$) reduced the bacterial population within 15 min. The 50 µg/ml concentration of copper eliminated recovery of the bacterium after 1 h. However, the 10 and 25 µg/ml copper concentrations required exposure for more than 4 h to completely eliminate bacterial recovery. Additionally, Ag-dsDNA-GO had the same effect on 91-118 as it did on GEV485. Ag-dsDNA-GO had greater antibacterial activity on the copper-sensitive and copper-tolerant strains than copper. Additionally, Ag-dsDNA-GO at 10 µg/ml inhibited the growth of all four copper-tolerant *Xanthomonas* strains representing the four bacterial spot pathogens within 15 min in vitro (Fig. 2). In comparison, copper at 500 µg/ml was unable to significantly reduce copper-tolerant bacterial populations when compared with the untreated control (Fig. 2).

Effect of Ag-dsDNA-GO on bacterial spot severity under greenhouse conditions. In the first greenhouse experiment (Fig. 3A), all concentrations (100, 200, and 500 µg/ml) of Ag-dsDNA-GO provided significant ($P = 0.05$) suppression of bacterial spot when compared with copper-mancozeb and the negative control. Copper-mancozeb also significantly reduced bacterial spot severity when compared with the negative control. Furthermore, no visible adverse effects were observed on plants treated with Ag-dsDNA-GO at 100 µg/ml. However, the 500 µg/ml concentration of Ag-dsDNA-GO caused phytotoxicity on approximately 30 to 50% of the leaf area (Fig. 4E and F). The 200 µg/ml concentration caused phytotoxicity on approximately 6 to 15% of the leaf area (Fig. 4D). Therefore, the 500 µg/ml concentration was eliminated from future experiments.

In the second greenhouse experiment (Fig. 3B), all concentrations (75, 100, and 200 µg/ml) of Ag-dsDNA-GO provided significant ($P = 0.05$) suppression of bacterial spot when compared with copper-mancozeb and the negative control. The 75 µg/ml concentration of Ag-dsDNA-GO provided better disease control than the copper-mancozeb treatment but it was less effective than the other two concentrations of the nanocomposite. The AUDPC for the 75 µg/ml treatment was almost twice that of the 100 and 200 µg/ml treatments. However, this difference in disease severity was not statistically significant. Phytotoxicity was not observed for any of the treatments in this experiment.

In the third greenhouse experiment (Fig. 3C), all concentrations (75, 100, and 200 µg/ml) of Ag-dsDNA-GO significantly ($P = 0.05$) reduced bacterial spot. Of the three Ag-dsDNA-GO treatments, the 75 µg/ml concentration had the highest disease severity. The AUDPC for the lowest rate of Ag-dsDNA-GO was approximately twice as high as the values for the 100 and 200 µg/ml treatments; however, bacterial spot severity was significantly less than that observed for treatments with copper-mancozeb. In the third greenhouse experiment, no phytotoxicity was observed.

Discussion

In this study, Ag-dsDNA-GO showed potential as an alternative to copper-based bactericides for managing bacterial spot of tomato on transplants. Although the mode of action has not been determined

Fig. 4. Deposition of various concentrations of Ag-dsDNA-GO on tomato leaf surfaces: **A**, 500 µg/ml 2 h after treatment; **B**, 200 µg/ml 2 h after treatment; **C**, 100 µg/ml 2 h after treatment; **D**, phytotoxicity with 200 µg/ml 14 days after treatment; **E**, phytotoxicity observed with 500 µg/ml 14 days after treatment; and **F**, phytotoxicity observed with 500 µg/ml 14 days after treatment.

for AgNP, these particles can cause structural deformation of the bacterial cell membrane by interacting with surface proteins (Ocosoy et al. 2013b; Panáček et al. 2006). In our study, the in vitro assays revealed the bactericidal activity of Ag-dsDNA-GO at concentrations as low as 10 µg/ml. In order to make a direct comparison with copper, it should be noted that Ag-dsDNA-GO at 50 µg/ml contains Ag at approximately 8 µg/ml. The lowest nanocomposite concentration contained Ag at less than 2 µg/ml and completely killed all bacterial cells within 15 min. On the other hand, the copper concentrations had no effect on the copper-tolerant *X. perforans* strain even after exposure for 24 h. All copper concentrations inhibited bacterial growth of the copper-sensitive strain but required at least 1 h or more to do so. Thus, the in vitro assays indicated that Ag-dsDNA-GO is effective against both copper-sensitive and copper-tolerant strains of *X. perforans*. Additional in vitro assays revealed that this composite is highly effective against copper-tolerant strains representing *X. euvesicatoria*, *X. gardneri*, and *X. vesicatoria* in addition to *X. perforans*. Furthermore, this composite is also effective against a copper-tolerant *Pseudomonas syringae* pv. *tomato* strain (data not shown). This demonstrates the broad activity of the Ag-dsDNA-GO against many copper-tolerant bacterial strains.

All Ag-dsDNA-GO concentrations tested significantly reduced bacterial spot severity relative to copper-mancozeb. However, phytotoxicity was observed for two concentrations of Ag-dsDNA-GO. As a result, the 500 µg/ml concentration was eliminated. The 100 µg/ml concentration of the Ag-based nanocomposite consistently performed the best in all three greenhouse experiments. An advantage of using the 100 µg/ml concentration is that the actual amount of Ag in the nanocomposite, approximately 16 µg/ml, is low. In comparison, the level of copper that is currently sprayed to control bacterial spot in transplant houses or tomato fields is approximately 540 µg/ml. Hence, the use of the Ag nanocomposite could reduce the amount of heavy metals accumulating in the soil.

Another advantage of the Ag nanocomposite is that it uses GO to minimize the aggregation of AgNP. GO is a versatile material that is being explored for uses in electronics, live cell imaging, and as a carrier for drug delivery (Sanchez et al. 2012; Sun et al. 2008). The versatility of GO is also evident in our composite as it increases stability by protecting dsDNA from being cleaved by DNases that occurs naturally in the environment (Cui et al. 2013). As researchers are gaining interest in the biomedical applications of GO, the research on environmental health and safety is also expanding (Sanchez et al. 2012). Although environmental health and safety studies are limited, Carpio et al. (2012) revealed that GO-nanoparticles have antibacterial activity against *E. coli*, *Cupriavidus metallidurans*, *Bacillus subtilis*, and *Rhodococcus opacus* but are not significantly toxic to mammalian fibroblast cells at levels as high as 1 mg/ml. Thus, the amount of GO in the 100 µg/ml concentration of Ag-dsDNA-GO should have a minimal impact on the environment. Although GO is not a primary concern for toxicity, it is unclear at this time how the Ag in this composite will accumulate over time and affect the environment. With respect to durability, this composite will be stable in storage for several months due to the strong interactions between AgNP and dsDNA, and AgNP and the functional groups of GO (Ocosoy et al. 2013a).

In general, the nanocomposite did not distribute evenly over the entire leaf surface but aggregated to form heavy, thick droplets, which varied in color depending on the concentration (Fig. 3). This aggregation was likely caused by the use of a conventional hand-held spray bottle, which has limitations in delivering fine droplet sizes at higher concentrations of Ag-dsDNA-GO. This issue could be eliminated by the use of fine mist overhead spraying systems used by commercial transplant houses or an electrostatic sprayer, which needs to be studied prior to commercialization. By far, the 500 µg/ml concentration created the darkest and thickest droplets, which may have caused the observed phytotoxicity. The 75 and 100 µg/ml concentrations resulted in lightly colored and thinner droplets, which did not cause visible adverse effects on the tomato plants. The greenhouse experiments corroborated the results seen in vitro and in the study conducted by Ocosoy et al. (2013b) that Ag-dsDNA-GO is toxic to copper-tolerant *X. perforans* strains.

Copper-tolerant bacterial spot *Xanthomonas* strains have been isolated since the 1960s and, currently, all of the *X. perforans* strains present in Florida are copper-tolerant (Horvath et al. 2012; Marco and Stall 1983). When copper-tolerant strains are present in warm, humid environments, bacterial spot is difficult to control with copper-based bactericides. Therefore, researchers continue to look for alternatives to copper and have had some success with ASM, 2AI, and light-activated nanomaterials (Obradovic et al. 2005; Paret et al. 2013; Worthington et al. 2012). Based on this study, Ag-dsDNA-GO appears to be a promising alternative to copper. However, more research is needed before it can be used to manage bacterial spot in the field or commercial transplant production facilities. Additionally, the fate of Ag-dsDNA-GO in the environment needs to be evaluated because it is unknown how long the nanocomposite can persist in soil or on plant tissue and how it would affect tomato fruit.

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Literature Cited

- Bae, E., Park, H. J., Lee, J., Kim, Y., Yoon, J., Park, K., Choi, K., and Yi, J. 2010. Bacterial cytotoxicity of the silver nanoparticle related to physicochemical metrics and agglomeration properties. *Environ. Toxicol. Chem.* 29:2154-2160.
- Carpio, I. E. M., Santos, C. M., Wei, X., and Rodrigues, D. F. 2012. Toxicity of a polymer-graphene oxide composite against bacterial planktonic cells, biofilms, and mammalian cells. *Nanoscale* 4:4746-4756.
- Conover, R. A., and Gerhold, N. R. 1981. Mixtures of copper and mancozeb for control of bacterial spot of tomato and their compatibility for control of fungus diseases (*Phytophthora infestans*, *Stemphylium solani*, *Xanthomonas campestris* pv. *vesicatoria*, Florida). *Proc. Annu. Meet. Fla. State Hortic. Soc.* 94:194-196.
- Cooksey, D. A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 28:201-219.
- Cui, L., Song, Y., Ke, G., Guan, Z., Zhang, H., Lin, Y., Huang, Y., Chu, Z., and Yang, C. J. 2013. Graphene oxide protected nucleic acid probes for bioanalysis and biomedicine. *Chem. Eur. J.* 19:10442-10451.
- Elechiguerra, J. L., Burt, J. L., Morones, J. R., Camacho-Bragado, A., Gao, X., Lara, H. H., and Yacaman, M. J. 2005. Interaction of silver nanoparticles with HIV-1. *J. Nanobiotechnol.* 3:1-10.
- Gitaitis, R., McCarter, S., and Jones, J. 1992. Disease control in tomato transplants produced in Georgia and Florida. *Plant Dis.* 76:651-656.
- Horsfall, J. G., and Barratt, R. W. 1945. An improved grading system for measuring plant diseases. (Abstr.) *Phytopathology* 35:655.
- Horvath, D. M., Stall, R. E., Jones, J. B., Pauly, M. H., Vallad, G. E., Dahlbeck, D., Staskawicz, B. J., and Scott, J. W. 2012. Transgenic resistance confers effective field level control of bacterial spot disease in tomato. *PLoS One* 7:e42036.
- Jones, J. B., and Jones, J. P. 1985. The effect of bactericides, tank mixing time and spray schedule on bacterial leaf spot of tomato. *Proc. Fla. State Hortic. Soc.* 98:244-247.
- Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., and Schaad, N. W. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Syst. Appl. Microbiol.* 27:755-762.
- Kim, J. S., Kuk, E., Yu, K. N., Kim, J. H., Park, S. J., Lee, H. J., Kim, S. H., Park, Y. K., Park, H., Hwang, C. Y., Kim, Y. K., Lee, Y., Jeong, D. H., and Cho, M. H. 2007. Antimicrobial effects of silver nanoparticles. *Nanomedicine* 3:95-101.
- Kim, K. J., Sung, W. S., Suh, B. K., Moon, S. K., Choi, J. S., Kim, J. G., and Lee, D. G. 2009. Antifungal activity and mode of action of silver nano-particles on *Candida albicans*. *Biomaterials* 22:235-242.
- Louws, F. J., Wilson, M., Campbell, H. L., Cuppels, D. A., Jones, J. B., Shoemaker, P. B., Sahin, F., and Miller, S. A. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. *Plant Dis.* 85:481-488.
- Lu, L., Sun, R. W., Chen, R., Hui, C. K., Ho, C. M., Luk, J. M., Lou, G. K., and Che, C. M. 2008. Silver nanoparticles inhibit hepatitis B virus replication. *Antivir. Ther.* 13:253-262.
- Madden, L. V., Hughes, G., and van den Bosch, F. 2007. The Study of Plant Disease Epidemics. American Phytopathological Society, St. Paul, MN.
- Marco, G. M., and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. *Plant Dis.* 67:779-781.
- Obradovic, A., Jones, J. B., Momol, M. T., Balogh, B., and Olson, S. M. 2004. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant Dis.* 88:736-740.
- Obradovic, A., Jones, J. B., Momol, M. T., Olson, S. M., Jackson, L. E., Balogh, B., Guven, K., and Iriarte, F. B. 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Dis.* 89:712-716.

- Ocsoy, I., Gulbakan, B., Chen, T., Zhu, G., Chen, Z., Sari, M. M., Peng, L., Xiong, X., Fang, X., and Tan, W. 2013a. DNA-guided metal-nanoparticle formation on graphene oxide surface. *Adv. Mater.* 25:2319-2325.
- Ocsoy, I., Paret, M. L., Ocsoy, M. A., Kunwar, S., Chen, T., You, M., and Tan, W. 2013b. Nanotechnology in plant disease management: DNA-directed silver nanoparticles on graphene oxide as an antibacterial against *Xanthomonas perforans*. *ACS Nano* 7:8972-8980.
- Panáček, A., Kvitek, L., Prucek, R., Kolář, M., Večeřová, R., Pizúrová, N., Sharma, V. K., Nevěčná, T., and Zbořil, R. 2006. Silver colloid nanoparticles: Synthesis, characterization, and their antibacterial activity. *J. Phys. Chem. B* 110:16248-16253.
- Paret, M. L., Vallad, G. E., Averett, D. R., Jones, J. B., and Olson, S. M. 2013. Photocatalysis: Effect of light-activated nanoscale formulations of TiO₂ on *Xanthomonas perforans* and control of bacterial spot of tomato. *Phytopathology* 103:228-236.
- Pohronezny, K., and Volin, R. B. 1983. The effect of bacterial spot on yield and quality of fresh market tomatoes. *HortScience* 18:69-70.
- Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., Vallad, G. E., and Jones, J. B. 2015. Bacterial spot of tomato and pepper: Diverse *Xanthomonas* species with a wide variety of virulence factors posing a worldwide challenge. *Mol. Plant Pathol.* 16:907-920.
- Ritchie, D. F. 2000. Bacterial spot of pepper and tomato. Online publication. Plant Health Inst. <https://www.apsnet.org/edcenter/intropp/lessons/prokaryotes/Pages/Bacterialspt.aspx> doi:10.1094/PHI-I-2000-1027-01
- Sanchez, V. C., Jachak, A., Hurt, R. H., and Kane, A. B. 2012. Biological interactions of graphene-family nanomaterials: An interdisciplinary review. *Chem. Res. Toxicol.* 25:15-34.
- Scott, J. W., Somodi, G. C., and Jones, J. B. 1989. Resistance to bacterial spot fruit infection in tomato. *HortScience* 24:825-827.
- Sun, X., Liu, Z., Welsher, K., Robinson, J. T., Goodwin, A., Zaric, S., and Dai, H. 2008. Nano-graphene oxide for cellular imaging and drug delivery. *Nano Res.* 1:203-212.
- Thayer, P. L., and Stall, R. E. 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. *Plant Dis.* 46:389-392.
- Worthington, R. J., Rogers, S. A., Huigens, R. W., III, Melander, C., and Ritchie, D. F. 2012. Foliar-applied small molecule that suppresses biofilm formation and enhances control of copper-resistant *Xanthomonas euvesicatoria* on pepper. *Plant Dis.* 96:1638-1644.