Nano-Magnesium Oxide: A Novel Bactericide Against Copper-Tolerant *Xanthomonas perforans* Causing Tomato Bacterial Spot

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ABSTRACT

Bacterial spot caused by *Xanthomonas perforans* causes significant damage on tomato in Florida. Due to the presence of copper (Cu)-tolerant *X. perforans* strains, Cu bactericides are not effective in disease management. Hence, there is a critical need to find alternatives for Cu. Antibacterial activity of magnesium oxide (Nano-MgO), and other metal oxide nanoparticles, were evaluated against a Cu-tolerant and -sensitive *X. perforans* strain. In vitro experiments demonstrated high antibacterial activity of Nano-MgO against both strains compared with the commercial Cu. The minimum inhibitory concentration of Nano-MgO is 25 µg/ml and the minimum bactericidal concentration is 100 µg/ml against a Cu-tolerant *X. perforans* strain after 4 h of exposure. Structural changes in the bacterial

membrane following exposure to Nano-MgO treatments compared with the controls were observed using transmission electron microscopy. In two greenhouse experiments with a Cu-tolerant strain, bacterial spot severity was significantly reduced by Nano-MgO at 200 µg/ml compared with Cu-ethylene bis-dithiocarbamate (grower standard), and the untreated control ($P = 0.05$). In three field experiments, Nano-MgO at 200 µg/ml significantly reduced disease severity with no negative impact on yield compared with the untreated control. Inductively coupled plasma mass spectrometric analysis of the fruit confirmed that Nano-MgO application did not lead to the accumulation of Mg, Cu, Ca, K, Mn, P, and S. This study is the first to demonstrate the potential of Nano-MgO against bacterial spot of tomato.

Tomato (*Solanum lycopersicum*) is an economically important specialty crop in the United States. In 2017, the total production was 12.5 million metric tons. The value of the crop totaled \$1.67 billion (USDA 2018). Florida, which accounted for 36% of the annual tomato production value (USDA 2018), is the largest fresh-market producer in the United States. Bacterial spot of tomato, caused by four *Xanthomonas* spp. (i.e., *Xanthomonas euvesicatoria*, *X. gardneri*, *X. perforans*, and *X. vesicatoria*) (Jones et al. 2004), is difficult to manage in Florida due to environmental conditions that favor the disease throughout the year. Although bacterial spot disease is a major bacterial disease in fresh-market tomato production, leading to as much as 50% yield losses and reduced fruit quality (Vallad et al. 2010), there are only a few treatment options available for effective disease management.

In the 1950s, the antibiotic streptomycin was used extensively for field management of bacterial spot on tomato. Shortly thereafter, streptomycin-resistant *Xanthomonas* strains became common in the field (Stall and Thayer 1962; Thayer and Stall 1961). After the discovery of streptomycin-resistant xanthomonads, growers began to rely heavily on copper (Cu) bactericides. Continuous use of Cu bactericides over many decades has provided high selection pressure, which favors Cu-tolerant strains. As a result, Cu-tolerant strains were detected as early as the 1960s and were later characterized in 1983 (Cooksey et al. 1990; Marco and Stall 1983).

With the presence of Cu-tolerant strains, growers moved to applying combinations of Cu and mancozeb, an ethylene-bis-dithiocarbamate (EBDC) fungicide, to reduce foliar bacterial populations. However, Cu-EBDC combinations could not effectively manage Cu-tolerant strains when environmental conditions favored the disease (Jones and Jones 1985).

Given the limited availability of effective disease management options, Cu bactericides have continued to be frequently used by growers (Marco and Stall 1983; Ritchie and Dittapongpitch 1991; Vallad et al. 2010). The heavy use of Cu bactericides might lead to metal accumulation in the soil (Pietrzak and McPhail 2004). Considering the negative aspects of Cu use and accumulation, identifying sustainable and effective alternative management strategies is critical. Other currently available treatment options have significant limitations as well; a commercial plant defense activator can reduce yield (Obradovic et al. 2005) and the efficacy of bacteriophages is adversely affected by low humidity, UV light, and high temperature (Jones et al. 2012; Obradovic et al. 2004). Florida tomato growers continue to use a combination of Cu bactericides, plant defense activators, biofungicides, and cultural practices, including use of healthy seed and transplants (Potnis et al. 2015).

Recently, nanomaterials have been the focus of a number of studies for management of plant diseases (Servin et al. 2015). Bacterial spot of tomato has been one of the targeted diseases for studies on the use of nanomaterials for disease control due to the limitations of Cu bactericides and other management options. In one study, three different photocatalytic crystalline nanoscale titanium dioxide (Nano-TiO₂) compounds were compared for disease control (Paret et al. 2013). One of the TiO₂ nanomaterials, nanoscale TiO₂ doped (incorporation of other materials into the structure of Nano-TiO₂) with zinc (TiO₂/Zn) (Averett and Averett 2015) at approximately 500 to 800 µg/ml showed greater ability to control bacterial spot in the field in comparison with the grower

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standard, Cu-EBDC (Paret et al. 2013). However, TiO₂/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). Another hybrid nanomaterial that was recently designed and assessed for controlling bacterial spot on tomato transplants is Ag-dsDNA-graphene oxide (GO), a material which has minimal aggregation properties, unlike commercial silver (Ag) nanoparticles, as a result of being formulated with GO and double-stranded (ds)DNA (Ocsoy et al. 2013a,b). This nanomaterial was effective in reducing disease severity when applied at a concentration of 100 µg/ml, which is equivalent to Ag at approximately 16 µg/ml (Strayer et al. 2016). In comparison, Cu routinely sprayed on tomato in transplant houses or in the field to control bacterial spot may reach approximately 540 µg/ml. Although Ag-dsDNA-GO may be a potential alternative to Cu, the material is currently unavailable in large quantities and is costly. Additionally, after treatment, the fate of Ag-dsDNA-GO in the environment needs to be evaluated, including Ag accumulation in the soil and fruit.

Another nanomaterial of interest as a new bactericide is magnesium oxide nanoparticles (Nano-MgO). In the past decade, micron-sized and nano-sized MgO particles were shown to have antibacterial activity against several mammalian pathogens (Aruoja et al. 2009; Huang et al. 2005; Sawai 2003). Sawai (2003) treated mammalian pathogens *Staphylococcus aureus* and *Escherichia coli* with micron-sized MgO powder (3.7 µm in sterile saline) and quantitatively evaluated the change in electrical conductivity as a measurement of bacterial metabolism. Antibacterial activity was observed after 15 h of exposure to micron-sized MgO concentrations as low as 0.2 µg/ml. In that study, generation of oxygen radicals was suggested as one of the possibilities for antibacterial activity (Sawai et al. 2000). For plant pathogens, high inhibition rates in germination of fungal spores of *Alternaria alternata*, *Fusarium oxysporum*, *Rhizopus stolonifer*, and *Mucor plumbeus* upon exposure to Nano-MgO (approximately 50 ± 10 nm) at concentrations as low as 100 mg/liter were reported (Wani and Shah 2012). 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 and Rubiales 2009). It was also observed that nanoparticles suppress enzymes and toxins used for pathogenicity by the fungal pathogens (Bhainsa and D'Souza 2006; Vahabi et al. 2011).

Huang et al. (2005) evaluated the possible mechanism of Nano-MgO antibacterial activity and observed that activity against *Bacillus subtilis* var. *niger* spores and *S. aureus* cells increased with decreasing particle size. For particles in the size range of 45 to 70 nm, the bactericidal efficacy of Nano-MgO increased slowly with decreasing particle size. A strong improvement of bactericidal efficacy was observed below 45 nm. To compare with Nano-TiO₂ (29 nm, light activated), Nano-MgO at 26 nm showed similar bactericidal activity against *S. aureus* and much higher bactericidal efficiency against *B. subtilis* without light activation. Huang et al. (2005) also confirmed that the mechanism of bactericidal activity of Nano-MgO is the release of high concentrations of highly reactive superoxide ions generated on the surface of the Nano-MgO particles. Those reactive oxygen species can break the peptide linkages in the bacterial cell wall or endospores (Huang et al. 2005). Because other metal oxide nanoparticles, including Cu(I) oxide (Nano-Cu₂O), zinc oxide (Nano-ZnO), manganese oxide (Nano-Mn₂O₃), and iron oxide (Nano-Fe₂O₃), showed bactericidal activity in other studies (Azam et al. 2012; Hoseinzadeh et al. 2017; Servin et al. 2015), we examined the antibacterial potentials of these metallic oxide nanoparticles along with Nano-MgO.

The goal of this study was to evaluate novel disease management strategies for bacterial spot of tomato. We hypothesized that the nanoparticle MgO and other metal oxides will have greater antibacterial properties compared with commercial micron-size

Cu and provide more effective control of bacterial spot of tomato. The objectives of this study were to (i) evaluate the in vitro antibacterial activity of metal oxide nanoparticles against Cu-tolerant *X. perforans*, (ii) assess the ability of nanoparticles to reduce bacterial spot severity under greenhouse and field conditions, (iii) study potential phytotoxic effects of the metallic oxide nanoparticles on tomato, and (iv) evaluate whether application of metal oxide nanoparticles on tomato results in the increased accumulation of elements in fresh tomato fruit.

MATERIALS AND METHODS

Bacterial strains and storage. Two *X. perforans* strains, GEV485 (Cu-tolerant) and 91-118 (Cu-sensitive), were isolated from tomato in Florida and used in this study. Bacterial cells from pure cultures of these strains were suspended in sterile 30% glycerol solution and stored at -80°C. For each experiment, bacteria were streaked from the glycerol stock onto nutrient agar (NA) medium (BBL, Becton Dickinson and Co., Cockeysville, MD), incubated at 28°C, and transferred every 24 to 48 h until used for streaking on NA plates for inoculum. Bacterial cells were collected from cultures grown on NA for 24 h and suspended in 0.01 M MgSO₄, and the suspensions were adjusted to absorbance at 600 nm = 0.3 (approximately 5 × 10⁸ CFU/ml). The final concentrations of bacterial suspensions were adjusted to 10⁸ CFU/ml.

Metal oxide nanoparticles. Ag nanoparticles (Nano-Ag) (Ag, 99.99%, 20 nm, metal basis), Cu(I) oxide nanoparticles (Nano-Cu₂O) (Cu₂O, 18 nm, super fine 99.86%), Zn oxide nanoparticles (Nano-ZnO) (ZnO, 99+%, 10 to 30 nm), Nano-MgO (MgO, 99+%, 20 nm), Mn oxide nanoparticles (Nano-Mn₂O₃) (Mn₂O₃, 99.2%, 30 nm), and Fe oxide nanoparticles (Nano-Fe₂O₃) (Fe₂O₃, γ, 99%, 20 to 40 nm) were purchased in powder form from U.S. Research Nanomaterials, Inc. (Houston, TX). Each nanoparticle was suspended and sonicated in autoclaved deionized water, adjusted to 10,000 µg/ml, and used as a stock suspension.

In vitro assays. In order to select effective nanoparticles for controlling bacterial spot disease of tomato, several metallic oxide nanoparticles and Nano-Ag were compared with Kocide 3000 (a micron-sized commercial Cu bactericide and fungicide; DuPont, Wilmington, DE) for antibacterial activity against the Cu-tolerant strain GEV485 and Cu-sensitive strain 91-118. Nano-Ag is an antibacterial material (Lok et al. 2007; Marambio-Jones and Hoek 2010; Pal et al. 2007) and is the active ingredient in the formulated silver-based nanoparticle Ag-dsDNA-GO (Strayer et al. 2016); hence, it was used in this experiment as a comparison with other metal oxide nanoparticles. The bacterial cells were grown on NA plates for 24 h at 28°C and bacterial cells were then transferred to NA plates containing Cu at 20 µg/ml; that is, Cu (II) sulfate pentahydrate (CuSO₄·5H₂O) (Sigma-Aldrich, St. Louis), and incubated for 24 h at 28°C. Bacterial cells were collected from NA plates and suspended in sterile 0.01 M solution of MgSO₄ (2.46 g/liter) in deionized water. Suspensions were diluted to 10⁵ CFU/ml and 20 µl of the bacterial suspension was transferred to 2 ml of each treatment in a sterile glass tube: Nano-Ag, Nano-Cu₂O, Nano-ZnO, Nano-Fe₂O₃, Nano-MgO, Nano-Mn₂O₃, and the Cu bactericide, Kocide 3000 (DuPont), were prepared at 100 and 1,000 µg/ml. Kocide 3000 contains 30% metallic Cu in the form of copper hydroxide (Cu (OH)₂). Kocide 3000 at 1 g/liter contains Cu at approximately 300 µg/ml. 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 an orbital shaker (150 rpm). At 1, 4, and 24 h, 50 µl was sampled from each tube and plated on NA. NA plates were incubated at 28°C for 48h. Bacterial colonies were counted on each plate and converted to CFU/ml. The in vitro assay was repeated twice.

Determination of minimum inhibitory concentrations and minimum bactericidal concentrations. To further understand the bactericidal ability of Nano-MgO, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined using the in vitro assays described above. MIC was defined as the lowest concentration with significant decrease in pathogen viability, MBC was defined as the lowest concentration at which 99.9% or more of the pathogen is killed (Cosentino et al. 1999). As previously described, Cu-tolerant strain GEV485 suspensions were diluted to 10^5 CFU/ml and 20 μ l of the bacterial suspension were transferred to tubes containing 2-ml suspensions of Nano-MgO at 12.5, 25, 50, or 100 μ g/ml. Each treatment consisted of three replications for each bacterial strain. For the control, the glass tube only contained 2 ml of sterile tap water and 20 μ l of the bacterial working suspension. The tubes were incubated at 28°C on an orbital shaker (150 rpm). After 4 h, 50 μ l were sampled from each tube and plated on NA. Bacterial colonies were counted on each plate and converted to CFU/ml. The in vitro assay was repeated once.

Transmission electron microscopy. An in vitro assay was used for treating *X. perforans* cells with Nano-MgO or the Cu bactericide, Kocide 3000 (DuPont), for morphological analysis of the bacterial cells using transmission electron microscopy (TEM). Bacterial suspension (1 ml of 10^8 CFU/ml) of Cu-tolerant strain GEV485 was transferred to 1 ml of Nano-MgO at 100 μ g/ml or Kocide 3000 at 100 μ g/ml. For the control, the glass tube only contained 1 ml of sterile tap water and 1 ml of the bacterial suspension. The glass tubes were incubated for 4 h at 28°C. Untreated controls were incubated for the same duration. One drop of 2% paraformaldehyde-fixed Nano-MgO, Kocide 3000-treated, and untreated *X. perforans* cell suspensions were trapped on carbon-coated copper grids and allowed to dry. The cells were negatively stained by 2% uranyl acetate for 30 s, and the excess was removed with filter paper. The samples were washed with one drop of sterile deionized H₂O. The cells were observed using FEI Spirit TEM, 120 kV (FEI Company, Hillsboro, OR).

Greenhouse experiments. Nano-Cu₂O, Nano-ZnO, and Nano-MgO, which showed greater antibacterial activity than the other compounds in the in vitro assays, were selected for further testing in the greenhouse experiments. The following suspensions (200 ml each) were prepared in sterile tap water: Nano-Cu₂O, Nano-ZnO, and Nano-MgO at 100, 200, 500, and 1,000 μ g/ml; or a combination of Kocide 3000 (2.1 g/liter) and Penncozeb 75DF (Cu-EBDC, 1.2 g/liter; United Phosphorus, Inc., King of Prussia, PA). Sterile tap water served as the control. Approximately 30 ml of the material was sprayed on the foliage of 3- to 4-week-old Bonny Best tomato plants. The leaf surfaces receiving the spray materials were allowed to air dry before spraying the leaf surfaces with a suspension of the Cu-tolerant *X. perforans* strain, GEV485, adjusted to 5×10^8 CFU/ml. The inoculated plants were then placed in plastic bags that were tightened around the base of the pot with a rubber band and placed in a growth chamber at 28°C. After 48 h, 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 and Horsfall 1945) by rating every other day beginning at 2 days postinoculation, with the last rating at 14 days postinoculation. The disease rating assessed the overall affected area based on symptoms that included lesions, foliar blighting, and discoloration. The area under the disease progress curve (AUDPC) was then calculated using the midpoint values (Campbell and Madden 1990). There were four replications per treatment and the experiment was repeated twice.

Field experiments. Based on performance in greenhouse experiments, Nano-Cu₂O and Nano-MgO were selected for field testing against bacterial spot disease of tomato in three trials (4 August to 11 September 2015, Quincy, FL; 18 April to 13 June 2016, Quincy, FL; and 31 March to 26 May 2016, Wimauma, FL).

Each treatment had four replications consisting of 15 BHN 602 tomato plants in Quincy and HM1823 in Wimauma. The plots were arranged in a completely randomized block design. In Quincy, bed dimensions were 20.3 cm tall and 76.2 cm wide. Beds were spaced 1.8 m apart and plants were spaced 50.8 cm within the row (McAvoy et al. 2012). In Wimauma, beds were spaced 1.5 m apart and plants were spaced 60.96 cm within the row. Fertilizers were applied to plots based on soil type and cooperative extension recommendations (Freeman et al. 2014). Tomato transplants were grown in the greenhouse in 128-cell containers before transplant. After transplanting, the treatments were sprayed on foliar parts of the tomato plant at the rate of 1.2 liters for four plots 1 week prior to bacterial inoculation. The treatments consisted of Nano-Cu₂O or Nano-MgO sonicated suspension at 200 and 1,000 μ g/ml with constant shaking while applying, Kocide 3000 (2.1 g/liter), the grower standard Kocide 3000 (2.1 g/liter) in combination with Penncozeb 75DF (Cu-EBDC, 1.2 g/liter), and an untreated control. To insure adequate disease development in the field plots, a suspension of Cu-tolerant *X. perforans* strain GEV485 bacterial strain adjusted to 5×10^8 CFU/ml in deionized water was applied to the foliage in the field by spraying the 1st, 8th, and 15th plant in each plot. One liter of each antibacterial treatment was applied to each plot weekly with CO₂ tanks and a u-shaped sprayer until 1 week before fruit harvesting. The plants were assessed for disease severity and phytotoxicity using the Horsfall-Barratt disease severity scale (Barratt and Horsfall 1945) every week after inoculation until harvest. The AUDPC was then calculated as above (Campbell and Madden 1990). There were four replications per treatment and the experiment was done three times. In all, 12 of 15 plants, excluding the 2 toward the two ends of plots, were harvested for assessing the yield. The fruit were harvested at green/early breaker stage and then graded by United States Department of Agriculture (USDA) standards. At least two harvests were made for each field experiment, which is common for fresh-market tomato production in Florida.

Elemental analysis of the fruit. At harvest, five medium-sized mature-green stage fruit, with diameters between 5.72 and 6.43 cm according to the USDA standards (Kelley et al. 2010; USDA 1997) were collected from each of the treatments. The fruit were harvested from outside of the canopy from the first and the last plants of each of four plots from the 2015 fall Quincy trial. Hand-washed fruit were collected twice for analysis at 7 and 20 days after final application. In total, 4 to 8 g of fresh tomato fruit with peel and flesh and 4 to 8 g of peel-only and flesh-only samples were dried in an electric oven at 70°C for 48 h. Dried samples were predigested overnight with 2 ml of concentrated nitric acid and 2 ml of H₂O₂. After the predigestion step, these samples were digested at 115°C for 45 min, then cooled to room temperature. The samples were filtered through cotton plugs and the volume was adjusted to 50 ml. The samples were stored at room temperature until analysis. Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, S, and Zn concentrations were determined by inductively coupled plasma optical emission spectroscopy (Thermo-Jarrell Ash, Franklin, MA). Multielemental standard solution (100 mg/liter) was obtained from SPEX CertiPred (Metuchen, NJ). Analysis was performed following the methods described in previous studies (Mattina et al. 2003; Musante and White 2012; Stilwell and Graetz 2001). The digests were quantified against a four-point calibration curve that had been previously evaluated for linearity and accuracy. Analytical blanks, matrix blanks, and calibration verification samples were included in each sequence.

Statistical analysis. The data collected from the in vitro assays and greenhouse and field experiments were evaluated for statistical significance using analysis of variance followed by pairwise comparisons using either the least significant difference or the Student-Newman-Keuls method in IBM SPSS Statistics, version 22. A *P* value of 0.05 was used to evaluate significance.

RESULTS

Effect of metallic oxide nanoparticles on in vitro growth of *X. perforans*. Nano-Ag, Nano-ZnO, and Nano-MgO had significant antimicrobial activity at all concentrations against the Cu-tolerant strain, *X. perforans* GEV485 (Fig. 1). Both concentrations of Nano-Ag (1,000 and 100 µg/ml) and Nano-ZnO at 1,000

µg/ml completely inhibited bacterial growth (100% reduction) within 1 h (Fig. 1A). Both Nano-MgO and Nano-ZnO at 100 µg/ml reduced bacterial populations at 1 h (approximately 70% reduction for Nano-ZnO and approximately 20% reduction for Nano-MgO) and 4 h (100% reduction) (Fig. 1A and B) compared with the untreated control. Nano-Cu₂O at 1,000 µg/ml inhibited bacterial growth within 24 h (100% reduction) (Fig. 1C). In contrast, the Cu

Fig. 1. In vitro inhibition of Cu-tolerant *Xanthomonas perforans* strain GEV485 following exposure to nanomaterials for 1, 4, and 24 h. Treatments were as follows: Ag, Cu₂O, ZnO, Fe₂O₃, MgO, and Mn₂O₃ nanoparticles at 1,000 and 100 µg/ml; or Cu prepared from Kocide 3000 (K3000) at 1,000 (3.3 g/liter), and 100 µg/ml (0.334 g/liter). The treatments were compared with water control (UT) at incubation times of **A**, 1 h; **B**, 4 h; and **C**, 24 h. Error bars indicate standard error of the mean. *P* value of 0.05 was used in the IBM SPSS using Student-Newman-Keuls statistical analysis.

bactericide (Kocide 3000), Nano-Mn₂O₃, or Nano-Fe₂O₃ at either concentration had minimal or no effect on bacterial populations as compared with the untreated control. The MIC and the MBC were 25 and 100 µg/ml, respectively, after exposure to Nano-MgO for 4 h (Fig. 2).

For the Cu-sensitive strain *X. perforans* 91-118 (Fig. 3A to C), similar results were observed as with the Cu-tolerant strain, except that significant antibacterial activity was also observed with both of the Cu compounds (Nano-Cu₂O and Kocide 3000). Viable bacterial cells could not be recovered after 1 h (100% reduction) (Fig. 3A) following treatment with the lower concentration (100 µg/ml) of Nano-Cu₂O, Kocide 3000, Nano-Ag, and Nano-ZnO. Again, Nano-Mn₂O₃ or Nano-Fe₂O₃ had no effect on the bacterial populations. Based on the in vitro test with both strains, Nano-Ag, Nano-ZnO, Nano-MgO, and Nano-Cu₂O all showed greater antibacterial activity than the Cu bactericide Kocide 3000.

Morphological comparisons of Cu-tolerant *X. perforans* GEV485 following exposure to Nano-MgO and controls.

Untreated *X. perforans* cells that were negatively stained with uranyl acetate were observed using TEM (Fig. 4A1 to A4). The surface of the healthy *X. perforans* was smooth and showed typical characteristics of a rod shape, with an undamaged structure of the inner membrane and an intact, slightly wavy outer membrane (Fig. 4A2 and A3). The bacterial cells treated with Nano-MgO at 100 µg/ml (Fig. 4B1 to B4) were severely damaged, with a rough and folded surface (Fig. 4B2 and B3). The membrane was misshapen, with rough edges. The cells treated with the Cu bactericide, Kocide 3000 (DuPont), at 100 µg/ml (Fig. 4C1 to C4) were smooth and resembled the untreated healthy cells (Fig. 4C).

The effect of metallic oxide nanoparticles on bacterial spot disease severity under greenhouse conditions. In the first greenhouse experiment with the Cu-tolerant strain GEV485 (Fig. 5A), all Nano-Cu₂O concentrations and the two Nano-MgO concentrations (1,000 and 200 µg/ml) significantly decreased disease severity compared with the grower standard (Cu-EBDC) and the untreated control. For phytotoxicity, only Nano-Cu₂O at 100 µg/ml was significantly different from the untreated control. The other treatments produced no overt signs of phytotoxicity (data not shown).

In the second greenhouse experiment (Fig. 5B), both Nano-Cu₂O and Nano-MgO concentrations as low as 200 µg/ml showed a

significant reduction in disease severity compared with the untreated control. Compared with the grower's standard Cu-EBDC and Kocide 3000 alone, Nano-Cu₂O at 1,000 and 500 µg/ml and Nano-MgO at 1,000 µg/ml showed reduced disease severity. Nano-ZnO at both concentrations (1,000 and 200 µg/ml) showed significantly reduced disease severity compared with the untreated control. However, this was not significant compared with the Cu-EBDC and Kocide 3000 treatments. None of the treatments except for Cu₂O at 1,000 and 500 µg/ml showed phytotoxicity compared with the untreated control (data not shown).

Comparison of metallic nanoparticles with Cu and Cu-EBDC under field conditions for management of bacterial spot.

Based on the results of greenhouse experiments (Fig. 5), Nano-MgO and Nano-Cu₂O at 200 and 1,000 µg/ml were selected for field studies (Table 1) and were compared with Cu-EBDC, the grower standard. In the first field experiment in fall 2015 in Quincy, FL, plants receiving either concentration of Nano-MgO (1,000 or 200 µg/ml) had significantly less disease compared with the untreated control but were not different from the other treatments (Table 1). Both concentrations of Nano-Cu₂O and Cu-EBDC 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 Wimauma, FL, plants treated with Nano-MgO at 200 µg/ml showed significantly reduced disease severity compared with the untreated control (Table 1), whereas the 1,000-µg/ml Nano-MgO treatment did not reduce disease severity compared with the untreated control. However, the 1,000-µg/ml Nano-Cu₂O treatment significantly reduced disease severity compared with the untreated control.

In the 2016 spring trial in Quincy, both concentrations (1,000 and 200 µg/ml) of the Nano-MgO and Nano-Cu₂O treatments significantly reduced disease compared with the untreated control, whereas the grower's standard Cu-EBDC did not significantly reduce disease in the three field trials compared with the untreated control (Table 1). There were no significant negative impacts on marketable yield due to Nano-MgO treatments in any of the field trials (Table 2).

Accumulation of metals in harvested tomato fruit treated with metallic oxide nanoparticles. Fruit were collected 7 days after last application of test materials and analyzed for elemental composition. There were no significant differences

Fig. 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Nano-MgO on Cu-tolerant *Xanthomonas perforans* strain GEV485 for 4h. Treatment concentrations tested were 100, 50, 25, and 12.5 µg/ml. Treatments were compared with water control (UT). Error bars indicate standard error of the mean. *P* value of 0.05 was used in the IBM SPSS using Student-Newman-Keuls statistical analysis.

for any of the elements when comparing the elemental concentration in dry fruit with different treatments (data not shown). As for fresh fruit (Table 3), both concentrations of Nano-MgO nanoparticle and the grower standard, Cu-EBDC, showed significantly lower Al concentrations relative to the untreated control, with Al content twice as high in untreated fruit as in the other three treatments. In addition, only the grower standard, Cu-EBDC, showed significantly higher P content in fruit compared with the controls. The P content was approximately 100 mg/kg higher than in untreated fresh weight. Interestingly, only Cu-EBDC showed significant Cu accumulation in whole fruit, and was 0.2 mg/kg more compared with the untreated control, whereas Nano-MgO and Nano-Cu₂O at both concentrations did not differ from the untreated

control. For the fruit collected 20 days after the last application of test particles (Table 4), Cu-EBDC showed significantly higher Al content in the whole fruit, with approximately 0.04 mg/kg more Al than in the untreated fruit (Table 4). However, Al did not significantly accumulate in either peel or flesh for the Cu-EBDC treatment (Table 4). For other treatments, Cu₂O at 1,000 µg/ml showed significantly lower Al content, to nearly half of that accumulated in the untreated fruit (Table 4). For Cu accumulation, fruit receiving Cu-EBDC applications showed significantly greater Cu accumulation in whole fruit (+0.22 mg/kg more), peel (+0.31 mg/kg), and flesh (+0.16 mg/kg) (Table 4) compared with the untreated control. Fruit receiving Kocide 3000 showed significant Cu accumulation in the peel (+0.27 mg/kg).

Fig. 3. In vitro inhibition of Cu-sensitive *Xanthomonas perforans* strain 91-118 following exposure to various chemicals over time (1, 4, and 24 h). Treatments were as follows: Ag, Cu₂O, ZnO, Fe₂O₃, MgO, and Mn₂O₃ nanoparticles at 1,000 and 100 µg/ml; or Cu prepared from Kocide 3000 (K3000) at 1,000 (3.3 g/liter), and 100 µg/ml (0.334 g/liter). Treatments were compared with water control (UT) at incubation times of **A**, 1 h; **B**, 4 h; and **C**, 24 h. Error bars indicate standard error of the mean. *P* value of 0.05 was used in the IBM SPSS using Student-Newman-Keuls statistical analysis.

DISCUSSION

In this study, for the first time, Nano-MgO was shown to have potential as an alternative to a Cu bactericide for bacterial spot management in tomato production because it yielded consistent reductions in disease severity, while other metal oxide nanoparticles, including Nano-Cu₂O, did not provide the same efficacy. Based on this research, Nano-MgO at 100 and 1,000 µg/ml

controlled Cu-tolerant and Cu-sensitive *X. perforans* strains in the in vitro experiments. Nano-MgO compared with the conventional copper bactericide, Kocide 3000, was highly toxic to the Cu-tolerant strain. Specifically, after 4 h, Nano-MgO at 100 µg/ml significantly inhibited growth of the Cu-tolerant strain, whereas Kocide 3000 had no impact at 1,000 µg/ml during the same exposure period. Nano-Cu₂O also showed significant antibacterial ability toward the Cu-tolerant strain in vitro. In addition, Nano-ZnO

Fig. 4. Morphological analysis of Cu-tolerant *Xanthomonas perforans* GEV485 treated with 100 µg/ml Nano-MgO with transmission electron microscopy. Untreated and the Cu bactericide Kocide 3000 were used as the controls. **A**, Untreated cells; **B**, Nano-MgO-treated cells; and **C**, Kocide 3000 (DuPont)-treated cells.

also had significant antibacterial ability against both the strains used in the study. Although Nano-Cu₂O did not have the same level of activity as Nano-MgO, it is interesting that the nanoform of Cu showed greater antibacterial activity than the conventional Cu bactericide. Cu nanoparticles are known to be more toxic than bulk particles to a range of species; however, the mechanism is unclear.

Nano-ZnO was interesting in that neither bacterial strain was recovered following exposure to Nano-ZnO at 1,000 µg/ml after 1 h and Nano-ZnO at 100 µg/ml after 4 h of incubation in the in vitro

assay but the compound provided little disease control in the greenhouse study. Initially, Nano-ZnO showed some disease management potential in the early stages of the disease but symptoms developed rapidly afterward (data not shown). Nano-ZnO may still be a potential antibacterial material to manage bacterial spot disease when combined with other bactericides; however, this needs to be studied further. Nano-ZnO does not always show consistent disease management and sometimes caused phytotoxicity to the plants according to the former studies (Burman et al. 2013; Lin and Xing

Fig. 5. Management of bacterial spot of tomato in the greenhouse. Bonny Best tomato plants were sprayed with Nano-Cu₂O, Nano-MgO or Nano-ZnO at 1,000, 500, 200, or 100 µg/ml; Cu prepared from Kocide 3000 (K3000) at 1,000 (3.3 g/liter), 500 (1.67 g/liter), 200 (0.668 g/liter), and 100 µg/ml (0.334 g/liter); a combination of Kocide 3000 (2.1 g/liter) (K3000) and Penncozeb 75DF (1.2 g/liter) (Cu-ethylene-bis-dithiocarbamate [K+M]); or sterile tap water prior to inoculating with Cu-tolerant strain GEV 485 of *Xanthomonas perforans*. Experiments were conducted in **A**, fall 2015 and **B**, summer 2016. Disease severity of bacterial spot of tomato in greenhouse conditions is expressed as area under disease progress curve (AUDPC). Error bars represent the standard error of the mean. *P* value of 0.05 was used in the IBM SPSS Student-Newman-Keuls statistical analysis.

TABLE 1. Comparison of magnesium oxide (Nano-MgO), and copper oxide (Nano-Cu₂O) nanomaterials with the grower standard (Cu-ethylene-bis-dithiocarbamate [EBDC]) for control of bacterial spot disease severity (area under disease progress curve [AUDPC]) on tomato in three field experiments in Quincy and Wimauma, FL^x

Treatment	Rate (µg/ml)	AUDPC in different locations and seasons ^y		
		Quincy 2015 fall	Wimauma 2016 spring	Quincy 2016 spring
Nano-Cu ₂ O	1,000	987.4 ab	669.6 a	1,063.8 a
Nano-Cu ₂ O	200	930.4 ab	761.4 ab	877.7 a
Nano-MgO	1,000	805.0 a	866.4 ab	913.5 a
Nano-MgO	200	836.9 a	580.1 a	853.6 a
Kocide 3000	2,100	1,196.4 ab	972.1 ab	1,135.4 ab
Cu-EBDC ^z	...	1,092.9 ab	773.4 ab	1,188.0 ab
Water (untreated)	...	1,330.9 b	1,136.8 b	1,402.1 b

^x Tomato cultivars were BHN602 in Quincy and HM1823 in Wimauma.

^y Field trials were conducted in two locations and two seasons. AUDPC was calculated using the midpoint values of Horsfall-Barratt disease severity scale (Barratt and Horsfall 1945; Campbell and Madden 1990). Numbers followed by different letters in the same column had a significant difference ($P = 0.05$) based on Student-Newman-Keuls statistical analysis using the IBM SPSS program.

^z Cu-EBDC is composed of Kocide 3000 (2,100 µg/ml) and Penncozeb 75DF (1,200 µg/ml).

TABLE 2. Total marketable yield in three field experiments following treatment of tomato plants with magnesium oxide (Nano-MgO) and copper oxide (Nano-Cu₂O) nanomaterials, the grower standard (Cu-ethylene-bis-dithiocarbamate [EBDC]), and the untreated control in Quincy and Wimauma, FL^w

Treatment	Rate (µg/ml)	Marketable yield (kg/ha) in different locations and seasons ^x		
		Quincy 2015 fall	Wimauma 2016 spring	Quincy 2016 spring
Nano-Cu ₂ O	1,000	50,368.2	26,228.5	24,647.5
Nano-Cu ₂ O	200	41,070.7	32,926.8	9,192.6
Nano-MgO	1,000	34,099.8	32,061.9	17,793.3
Nano-MgO	200	46,912.8	30,296.9	11,335.5
Kocide 3000	2,100	44,932.9	32,256.1	20,357.2
Cu-EBDC ^y	...	37,601.9	32,132.5	17,648.1
Water (untreated)	...	42,709.1	28,708.4	18,986.0
Significance ($P = 0.05$) ^z	...	NS	NS	NS

^w Tomato cultivars were BHN602 in Quincy and HM1823 in Wimauma.

^x Field trials took place in two locations and two seasons.

^y Cu-EBDC is composed of Kocide 3000 (2,100 µg/ml) and Penncozeb 75DF (1,200 µg/ml).

^z None of the treatments had significant yield impact compared with the water treatment ($P = 0.05$) based on Student-Newman-Keuls statistical analysis using the IBM SPSS program; NS = not significant.

TABLE 3. Elemental accumulation in tomato fruit (BHN 602, fresh weight; mg/kg) collected from fields treated with magnesium oxide (Nano-MgO), and copper oxide (Nano-Cu₂O) nanomaterials in comparison with copper (Kocide 3000), the grower standard (Cu-ethylene-bis-dithiocarbamate [EBDC]) and the untreated control in Quincy from the first harvest (7 days from the last application) in fall 2015 trial^x

	Rate	Elemental accumulation in fruit (mg/kg FW) ^y												
Treatment	(µg/ml)	Al	B	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn
Nano-Cu ₂ O	1,000	0.12	0.82	84.65	0.72	2.58	2,712.5	129.2	3.01	0.03	18.27	286.9*	135.8	1.76
Nano-Cu ₂ O	200	0.11	0.83	71.35	0.66	2.68	2,677.5	134.3	2.67	0.03	21.60	295.9*	129.4	1.74
Nano-MgO	1,000	0.07*	0.75	74.62	0.64	2.42	2,653.2	133.3	2.78	0.02	20.27	262.1	128.8	1.66
Nano-MgO	200	0.07*	0.75	75.23	0.67	2.68	2,565.4	129.4	2.47	0.03	19.65	276.9	128.5	1.86
Kocide 3000	2,100	0.07*	0.85	67.23	0.83*	3.00	2,815.3	145.5	2.99	0.03	24.41	309.7*	141.9	1.95
Cu-EBDC ^z	...	0.09*	0.77	72.58	0.77	2.78	2,620.6	126.7	2.89	0.03	24.87	272.9	128.7	1.81
Water (untreated)	...	0.15	0.76	73.22	0.58	2.59	2,523.8	120.2	2.11	0.03	18.60	212.0	116.3	1.48
Significance (<i>P</i> = 0.05)	...	Yes	NS	NS	Yes	NS	NS	NS	NS	NS	NS	Yes	NS	NS

^x All of the samples were evaluated with inductively coupled plasma emission spectroscopy for elemental accumulation.

^y The concentration of 1 mg of copper in 1 kg of fresh weight tomato = 1 mg/kg FW Cu. An asterisk (*) indicates significant difference compared with the water treatment ($P = 0.05$) based on Student-Newman-Keuls statistical analysis using the IBM SPSS program; NS = not significant.

^z Cu-EBDC is composed of Kocide 3000 (2,100 µg/ml) and Penncozeb 75DF (1,200 µg/ml).

2007). The particle concentration, rate of ion release or particle dissolution, and plant species could be major factors controlling the efficacy of Nano-ZnO.

TEM was useful in demonstrating that exposure of bacterial cells of a Cu-tolerant bacterium to a suspension of Nano-MgO compared with exposure to a Cu bactericide suspension resulted in structural changes in the bacterial cells. Following exposure to Nano-MgO at 100 µg/ml, the bacterial cells were distorted compared with healthy-looking cells following exposure to tap water or the Cu bactericide suspension. Some of the cells treated with Nano-MgO showed gentle leakage and fragments around the cells (Bode et al. 1993; Li et al. 2010; Paret et al. 2012). Thus, exposure of *X. perforans* cells to a Nano-MgO suspension led to pronounced changes in cell morphology. Based on the 50 micrographs analyzed in our study, approximately 90% of the cells showed morphological changes after exposure to Nano-MgO at 100 µg/ml compared with nearly all cells appearing healthy in controls (Kocide 3000-treated cells and untreated control) (data not shown). The in vitro MIC and MBC studies confirmed these observations of the antibacterial activity of Nano-MgO. Further experiments are needed to understand the mode of action of Nano-MgO compared with other metallic nanoparticles.

In the greenhouse experiments, application of Nano-MgO and Nano-Cu₂O at 200 µg/ml to tomato plants significantly reduced bacterial spot disease severity compared with Cu-EBDC and the untreated controls ($P = 0.05$). Although greenhouse experiments provide reproducible results under controlled conditions, they do not take into account the diverse environmental factors that could influence bactericide efficacy under field conditions. Therefore, field experiments were conducted in this study. In the two field experiments in Quincy, FL and Wimauma, FL, application of Nano-Cu₂O did not significantly reduce disease compared with the untreated control. Nano-MgO at 200 µg/ml provided a significant reduction in bacterial spot severity compared with the untreated control ($P = 0.05$), with no signs of phytotoxicity in all field experiments. In addition, the 200-µg/ml rate of Nano-MgO nanoparticles was comparable with Cu-EBDC for disease management. There was no impact on the yield between any of the treatments.

The metallic Cu concentration currently used for controlling bacterial spot in tomato fields is approximately 540 µg/ml. That is approximately two times higher than the level of Mg from the Nano-MgO 200-µg/ml treatment (metallic MgO concentration = 120 µg/ml). In addition, Nano-MgO application would help to moderate metal accumulation in the field. If commercialized, using Nano-MgO as an alternative could help to relieve the pressure of potential Cu accumulation in the soil (Wightwick et al. 2008) and further development of Cu-tolerant pathogens.

This study indicates that Nano-MgO could be a good chemical alternative to Cu bactericides. There are two important approaches for controlling bacterial spot of tomato in the field: reducing inoculum

TABLE 4. Elemental accumulation in fruit collected from tomato BHN602 in the fields treated with magnesium oxide (Nano-MgO) and copper oxide (Nano-Cu₂O) nanomaterials in comparison with Cu (Kocide 3000), the grower standard (Cu-ethylene-bis-dithiocarbamate [EBDC]) and the untreated control (Water) in Quincy from the second harvest (20 days from the last application) in the fall 2015 trial^w

	Rate	Elemental accumulation in fruit (mg/kg FW) ^x												
Treatment	(µg/ml)	Al	B	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn
Whole fruit														
Nano-Cu ₂ O	1,000	0.05	0.68	66.26	0.64	2.35	2,371.04	120.54	1.84	0.02	15.99	232.60	116.66	1.71
Nano-Cu ₂ O	200	0.07	0.66	72.26	0.60	2.38	2,253.44	118.40	2.13	0.03	18.05	241.52	118.78	1.49
Nano-MgO	1,000	0.08	0.66	70.19	0.58	2.26	2,053.04	114.03	2.72	0.02	15.63	238.99	110.77	1.57
Nano-MgO	200	0.06	0.65	73.88	0.51	2.10	2,031.10	107.87	2.31	0.02	15.66	218.88	108.01	1.54
Kocide 3000	2,100	0.12*	0.77	58.82	0.90	2.76	2,279.24	123.88	2.65	0.02	23.54	268.36	123.90	1.98
Cu-EBDC ^y	...	0.07	0.72	59.20	0.79	2.55	2,277.98	115.19	2.69	0.03	21.41	264.63	118.44	1.68
Water	...	0.08	0.69	67.17	0.68	2.58	2,109.24	121.03	2.43	0.03	14.60	239.91	116.92	1.64
Significance ^z	...	Yes	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Peel														
Nano-Cu ₂ O	1,000	0.04*	0.72	81.78	0.64	2.42	2,353.60	124.66	1.73	0.02	15.83	232.50	116.95	1.66
Nano-Cu ₂ O	200	0.06	0.66	75.54	0.52	2.32	2,193.72	118.52	1.72	0.03	16.71	234.83	117.43	1.46
Nano-MgO	1,000	0.06	0.66	76.07	0.50	2.25	2,072.64	112.03	2.29	0.02	14.86	230.96	113.79	1.51
Nano-MgO	200	0.05	0.62	69.43	0.43	2.15	2,094.00	94.37	1.68	0.02	14.29	205.72	106.84	1.46
Kocide 3000	2,100	0.10	0.83	71.90	0.86*	2.56	2,284.95	120.08	2.38	0.02	20.24	249.71	123.46	1.88
Cu-EBDC ^y	...	0.09	0.74	67.98	0.82*	2.36	2,165.51	113.95	2.21	0.03	19.18	251.01	116.91	1.65
Water	...	0.08	0.76	73.71	0.55	2.58	2,079.83	114.61	1.85	0.02	13.10	215.72	123.28	1.64
Significance ^z	...	Yes	NS	NS	Yes	NS	NS	NS	NS	NS	NS	NS	NS	NS
Flesh														
Nano-Cu ₂ O	1,000	0.03	0.64	42.52	0.92	2.90	1,984.82	131.16	2.97	0.02	17.16	316.11	123.66	1.88
Nano-Cu ₂ O	200	0.03	0.63	45.74	0.72	2.40	1,951.79	115.65	2.45	0.03	19.63	276.48	113.78	1.53
Nano-MgO	1,000	0.05	0.64	50.87	0.73	2.45	1,818.84	113.47	3.17	0.02	15.92	273.97	107.05	1.66
Nano-MgO	200	0.04	0.63	55.98	0.69	2.51	1,902.14	123.72	2.75	0.02	17.46	252.12	113.87	1.62
Kocide 3000	2,100	0.08	0.70	43.65	1.04	3.14	2,091.54	134.95	3.49	0.02	24.98	329.50	129.64	1.96
Cu-EBDC ^y	...	0.05	0.70	52.05	0.90	2.82	2,075.94	137.34	3.90	0.03	24.12	322.98	123.64	1.80
Water	...	0.10	0.66	56.01	0.88	2.63	1,978.23	132.40	2.97	0.02	16.53	268.30	113.52	1.73
Significance ^z	...	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

^w In addition to whole-fruit samples, flesh, and peel samples were also collected separately. All of the samples were evaluated with inductively coupled plasma emission spectroscopy for elemental accumulation.

^x The concentration of 1 mg of copper in 1 kg of fresh weight tomato = 1 mg/kg FW Cu. An asterisk (*) indicates significant difference compared with water treatment ($P = 0.05$) based on Student-Newman-Keuls statistical analysis using the IBM SPSS program; NS = not significant.

^y Cu-EBDC is composed of Kocide 3000 (2,100 µg/ml) and Penncozeb 75DF (1,200 µg/ml).

^z Significance at $P = 0.05$.

and minimizing plant susceptibility (Momol et al. 2002). Nano-MgO could reduce inoculum in the field with its antibacterial ability. In addition, MgO would be a more sustainable treatment option in that MgO, unlike copper, is not on the list of Environmental Protection Agency Toxic Release Inventory Program or in the Integrated Risk Information System. The Food and Drug Administration also views magnesium oxide as a generally recognized as safe compound (Clydesdale 1997). As a result, if commercialized, Nano-MgO can be potentially be applied as an alternative to Cu in Integrated Pest Management programs to reduce inoculum. Also, potentially, it could be combined with a plant defense activator or with biocontrol agents; however, this needs to be studied further. In this scenario, selective pressure on the development of Cu-tolerant *X. perforans* in the field would also be reduced.

In the elemental accumulation study, analysis of fresh fruit from the first harvest revealed that only Cu-EBDC had significantly greater Cu accumulation in whole fruit, and was 0.2 mg/kg (36%) more than the untreated control. For the fruit collected 7 days after the last application, treatments with Cu-EBDC had significantly greater Cu accumulation in whole fruit and flesh compared with the untreated control. In addition, fruit receiving Kocide 3000 showed significant Cu accumulation in the peel compared with the untreated control. None of the other treatments, including MgO and Cu₂O nanoparticles, had significantly greater Cu or Mg accumulation in fruit compared with the untreated control. Cu is an essential element for mammals and plants; however, elevated Cu levels may be toxic (Gaetke and Chow 2003). In humans, the estimated lethal dose of Cu in an untreated adult is 10 to 20 g (Gaetke and Chow 2003; Winge and Mehra 1990). In our experiment, 0.2 mg more Cu per kilogram fresh weight of tomato fruit accumulated in fruit in which the grower standard Cu-EBDC was applied compared with the untreated control. In this scenario, consumption of tomato treated with Cu-EBDC might

lead to more Cu exposure for customers but the level is still well within the daily dietary limits. In other words, one would need to consume 10 to 20 kg of Cu bactericide-treated tomato in a day to achieve a toxic dose.

This study is the first study that shows the potential for developing MgO-based nanoparticles for management of bacterial spot of tomato with no significant increase in elemental accumulation in fruit. Importantly, significant antibacterial activity was achieved in this study in spite of the fact that the material was present in an uncharacterized, nondisperse, and heterogeneous solution. Because the nanoparticles were likely aggregated, the antibacterial agent might have actually limited contact with the pathogen. This may explain why there was variation in the greenhouse and in field experiments, with the higher concentration tending to have greater standard deviations than the lower concentration. This phenomenon of aggregation fit the observation in the previous study by Sawai (2003) that the antibacterial ability is contact dependent between MgO nanoparticles and the target bacteria. This highlights the importance of developing MgO formulations as a more homogeneous suspension, possibly through the addition of surface coatings, functional groups, or polymers to control particle dissolution. This type of formulated MgO nanoparticle would likely result in an effective antibacterial agent that could be used as an alternative to currently available bactericide strategies.

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