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Magnesium Oxide Nanomaterial, an Alternative for Commercial Copper Bactericides: Field-Scale Tomato Bacterial Spot Disease Management and Total and Bioavailable Metal Accumulation in Soil

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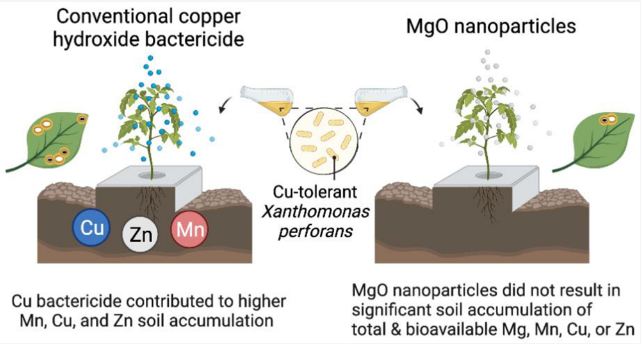

ABSTRACT: Copper (Cu) is the most extensively used bactericide worldwide in many agricultural production systems. However, intensive application of Cu bactericide have increased the selection pressure toward Cu-tolerant pathogens, including *Xanthomonas perforans*, the causal agent of tomato bacterial spot. However, alternatives for Cu bactericides are limited and have many drawbacks including plant damage and inconsistent effectiveness under field conditions. Also, potential ecological risk on nontarget organisms exposed to field runoff containing Cu is high. However, due to lack of alternatives for Cu, it is still widely used in tomato and other crops around the world in both conventional and organic production systems. In this study, a Cu-tolerant *X. perforans* strain GEV485, which can tolerate eight tested commercial Cu bactericides, was used in all the field trials to evaluate the efficacy of MgO nanomaterial. Four field experiments were conducted to evaluate the impact of intensive application of MgO nanomaterial on tomato bacterial spot disease severity, and one field experiment was conducted to study the impact of soil accumulation of total and bioavailable Cu, Mg, Mn, and Zn. In the first two field experiments, twice-weekly applications of 200 μg/mL MgO significantly reduced disease severity by 29−38% less in comparison to a conventional Cu bactericide Kocide 3000 and 19−30% less in comparison to the water control applied at the same frequency ($p = 0.05$). The disease severity on MgO twice-weekly was 12−32% less than Kocide 3000 + Mancozeb treatment. Single weekly applications of MgO had 13−19% higher disease severity than twice weekly application of MgO. In the second set of two field trials, twice-weekly applications of MgO at 1000 μg/mL significantly reduced disease severity by 32−40% in comparison to water control applied at the same frequency ($p = 0.05$). There was no negative yield impact in any of the trials. The third field experiment demonstrated that application of MgO did not result in significant accumulation of total and bioavailable Mg, Mn, Cu, or Zn in the root-associated soil and in soil farther away from the production bed compared to the water control. However, Cu bactericide contributed to significantly higher Mn, Cu, and Zn accumulation in the soil compared to water control ($p = 0.05$). This study demonstrates that MgO nanomaterial could be an alternative for Cu bactericide and have potential in reducing risks associated with development of tolerant strains and for reducing Cu load in the environment.

KEYWORDS: *Metal oxide, nanoparticles, nanomaterials, soil health, metal accumulation, pesticide, nanotechnology*

■ INTRODUCTION

The History of the Use of Cu Fungicides Globally and in Florida Tomato Production. Due to its low cost, ease of use, and broad-spectrum activity, copper (Cu) is the most widely and heavily used bactericide/fungicide in the world. For example, farmers in Florida typically apply copper up to 15 times in a season and twice a week especially during environmental conditions that favor bacterial and fungal disease development and spread. The first use of Cu as a fungicide dates back to 1807 when bluestone (copper sulfate) was used as a seed treatment to manage smut spores on cereals.[1] Subsequently, a limewater bath containing Cu was shown to be an improved fungicide.[2] However, the first official recommendation of Cu as a fungicide was described in 1845, when it was used in a mixture of copper sulfate, lime, and

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marine salt to treat potatoes infected by the oomycete pathogen *Phytophthora infestans* causing late blight, one of the major factors contributing to the Great Potato Famine in Ireland.[3] Next came the Bordeaux mixture, a combination of copper sulfate pentahydrate and lime, as an effective fungicide against grape downy mildew caused by the oomycete pathogen *Plasmopara viticola* in 1885.[4] This further led to widespread use of Cu globally in many crops and against many pathogens.[5] It is estimated that a combined ~200,000 tonnes of copper sulfate and smaller levels of copper oxide and copper oxychloride are used in plant disease management annually.[6]

A high-value crop with a long history of Cu fungicides use is tomato (*Solanum lycopersicum*). In 2019, tomatoes were harvested from 5,030,545 ha worldwide.[7] In tomato production fields in Florida, the largest fresh market tomato producing state (26,000 acres harvested in 2019) in the U.S,[8] use of Cu fungicides can vary depending on the season, environmental conditions, varietal tolerance, and presence of bacterial spot disease and fungal/oomycete diseases.

Among the many bacterial diseases affecting tomato in commercial open field production, bacterial spot disease caused by *Xanthomonas perforans* is the most important disease economically (Supporting Information Figure 1). An estimate shows that the financial losses due to tomato bacterial spot disease in southwest Florida were at $3,090/acre.[9] Cultural practices as well as disease tolerant varieties have been minimally effective in tropical and subtropical regions, due to the warm and wet climatic conditions that favor infection and the spread of the pathogen. The antibiotic streptomycin was successfully used in the 1950s, but resistance developed in bacterial strains that made it ineffective.[10] During the past decades, the disease has been managed using Cu bactericides. The effectiveness of Cu bactericides generally is enhanced when used in combination with ethylenebisdithiocarbamate (EBDC) fungicides such as maneb or mancozeb. This is due to the increased availability of free Cu ions, which provide improved bactericidal activity in comparison to sole use of conventional Cu bactericides.[11−13] Similar to the streptomycin examples presented above, continuous and widespread use led to the development of Cu resistant strains of the bacterium making Cu bactericides ineffective.[14] A recent study in which 585 *X. perforans* strains were isolated from tomatoes across many farms in Florida showed that 99.8% of all *X. perforans* strains are Cu-tolerant.[15] Thus, there is an urgent need to find effective alternatives for Cu-based bactericides.

In recent years, a variety of nanomaterials have been developed/discovered to have antibacterial properties both *in vitro* and in planta against Cu-tolerant strains of *X. perforans*. This includes copper-core−shell silica, multivalent copper, and copper-fixed quat,[16−18] DNA-graphene oxide-Ag,[19,20] TiO_2 doped with Zn or Ag,[21] MgO, ZnO, and Cu_2O,[22,23] and Cu−Zn hybrid.[24] Among these MgO is potentially a more sustainable treatment compared to Cu, and it is not on the list of the United State Environmental Protection Agency (EPA) Toxic Release Inventory (TRI) Program or in the Integrated Risk Information System (https://www.epa.gov/toxics-release-inventory-tri-program; https://www.epa.gov/iris). The United States Food and Drug Administration (FDA) also views MgO as a generally recognized as safe (GRAS) compound.[25] A wide range of metallic nanomaterials have been studied on a range of pathogens affecting crops and other plant-based applications around the world. Use of materials which are GRAS is of top priority for future directions.[26,27]

Liao et al. 2019a and 2019b studied the antibacterial ability of nonformulated MgO (20 nm) against a Cu-tolerant *X. perforans* strain in greenhouse and field conditions. Compared to the untreated water control, nano MgO at 200 μg/mL significantly reduced the disease severity of tomato bacterial spot by 37−49% in comparison to Kocide 3000, and MgO had no negative impact on yield. Non-nanoscale MgO was also studied which showed that while they had activity *in vitro* with 100% cell death, in field it did not provide adequate disease management.[22] However, all these studies utilized single weekly application of MgO and did not compare twice weekly applications which is a typical program for Cu bactericides in places with wet weather conditions like Florida. In the above studies fruits treated with MgO did not differentially accumulate Mg, Cu, Ca, K, Mn, P, and S, even when compared to the untreated water control.[22] However, leaching or runoff of MgO from tomato field soil along with the risk of total and bioavailable elemental accumulation in the soil is largely unknown.

The objectives of this study were to compare single and twice weekly applications of MgO to a conventional Cu fungicide with and without EBDC and/or water control on the progression of bacterial spot disease severity under field conditions, and to understand the fate of the particles at the root-associated soil and movement in soil farther out of the production bed. The hypothesis is that MgO at twice weekly applications will perform better than the same application regime of Cu in managing Cu-tolerant *X. perforans* under field conditions but will be like Cu-EBDC. We also hypothesize that applications of MgO may result in an increase in level of Mg compared to plots sprayed with water only.

MATERIAL AND METHODS

In vitro Experiments for Assessing Tolerance of _Xanthomonas perforans_ to Commercial Cu Bactericides. *X. perforans* strain GEV485 (Cu-tolerant) isolated from tomatoes in Florida was used in this study to evaluate antibacterial activity of a selected group of labeled commercial Cu agrochemicals on tomato in Florida. The bacterial strain was streaked on nutrient agar medium (NA, DifcoTM Sparks, MD) and incubated at 28 °C for 24 h prior to use. For culturing of GEV485 strain, NA was amended with copper(II) sulfate pentahydrate ($CuSO_4$ $5H_2O$) at 20 μg/mL (Fisher Scientific − Hampton, NH) to serve as a Cu based selective media. Bacterial cells were collected from NA plates, resuspended in 0.02 μL/mg $MgSO_4$, and adjusted to A600 = 0.3 at λ = 600 nm (~5 x10^8 CFU/mL) and the final suspension was adjusted to 10^5 CFU/mL. The Cu agrochemicals used in the experiment were as follows: (1) Nordox (Nordox As, 50% cuprous oxide), (b) Kocide 3000 (DuPont, Wilmington DE, 30% copper hydroxide), (c) Kentan DF (Isagro, Durhman NC, 40% copper hydroxide), (d) Camelot-O (SePRO, Carmel, IL, 1.8 copper octanoate), (e) Previsto (Gowan Company, Yuma AR, 3.3% copper hydroxide), (f) Badge SC (Gowan Company, Yuma AR, 20% copper oxychloride and copper hydroxide), and (g) Basic Copper 50W HB (Albaugh LLC, 50% copper sulfate). A 20 μL aliquot of the bacterial suspension, prepared as described above, was added to tubes containing 2 mL of agrochemicals at 200 μg/mL of its respective Cu equivalent. Tubes containing 2 mL of $MgSO_4$ at 0.02 μg/mL served as the control. The tubes were incubated in an orbital shaker at 150 rpm for 1, 2, 4, and 8 h in 28 °C. After incubation, 50 μL of the solution were plated in NA and the plates were again incubated at 28 °C for 48 h. Each treatment consisted of three replicates, and the experiment was performed twice. MgO was not used as a control in this study, as its antibacterial properties have been demonstrated in prior studies.[22,23]

Field Trials To Study Intensive Application of MgO on Bacterial Spot Disease Severity. Our prior studies showed that MgO nanomaterials have bactericidal properties and completely inhibited growth of *X. perforans* strain GEV485 (Cu-tolerant) *in vitro* by 4 h at 50 μg/mL concentration,[22] and hence we used the same strain in this study. Bacterial cells from pure cultures were suspended in sterile 30% glycerol solution and stored at −80 °C. For experiments, bacteria were grown on NA medium (BBL, Becton Dickinson and Co., Cockeysville, MD) at 28 °C and were transferred every 24 to 48 h. Bacterial cells were collected from cultures grown on NA for 24 h and suspended in 0.01 M $MgSO_4$, and the suspensions were adjusted to $A_{600} = 0.3$ at $\lambda = 600$ nm which corresponds to ~5×10^8 CFU/mL.

Magnesium oxide (MgO, 99+%, 20 nm) was purchased in powder form from US Research Nanomaterials, Inc. (Houston, TX, USA). The powder was suspended in autoclaved deionized water (DI) and sonicated in Branson B-22-4 Ultrasonic Cleaner (Danbury, CT, USA) for 10 min in autoclaved DI. The suspension was adjusted to 1 mg/mL and used as a stock suspension. The size of MgO in DI water was measured using the dynamic light scattering (DLS) technique (PDDLS/Cool/Bath 40T Precision Detector) (Supplemental Figure 2). The surface charge of MgO in DI water was measured by ZetaPlus Zeta Potential Analyzer (Malvern Instruments) (Supplemental Table 1). The electron microscopic image of MgO was provided by U.S. Nano (Supplemental Figure 3). The Resonicated MgO was used for field-testing against bacterial spot disease of tomato in two trials during fall 2016 (Location 1: Aug 13 - Oct 18, Quincy, FL, Location 2: Aug 8 - Oct 26, Wimauma, FL), each treatment including four replications consisting of 15 Quincy tomato plants in Quincy, FL and 10 Tycoon tomato plants in Wimauma, FL. For the 2019 Fall trial (Aug 14 - Oct 25, Quincy, FL), and the 2020 Fall trial (Aug 28 − Oct 30, Quincy, FL), each treatment including four replications consisting of 15 Grand Marshall (Sakata America, Immokalee, FL) tomato plants. The plots were arranged in a randomized complete block design in 2016 and 2020 and an incomplete block design in 2019. 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. Fertilizers were applied to plots based on soil type and cooperative extension recommendations.[28] The soils for the Quincy trials were Tifton loamy fine sand (Fine-Loamy, Kaolinitic, Thermic Plinthic Kandiudults) and Norfolk loamy fine sand (Fine-Loamy, Kaolinitic, Thermic Typic Kandiudults) with a soil pH of 6.3. The Wimauma trials included Myakka fine sand (Sandy, Siliceous, Hyperthermic Oxyaquic Alorthod) with a pH of 6.0. Fertilizers used were 196−112−168 kg/ha $N-P_2O_5-K_2O$, 22 kg/ha Mg, 2.8 kg/ha Mn, 1 kg/ha Zn, and Ca content is maintained at a minimum of 650 ppm with lime applications. The fumigant used was 280 kg/ha 1,3-dichloropropene plus chloropicrin 40:60 (w:w). Insecticides used include dinotefuran 0.7 l/ha, and 3 applications of bifenthrin 0.37 l/ha as needed. The planting beds were raised with plastic mulch (black for spring and white for fall trials). Tomato transplants were grown in the greenhouse in 128-cell containers with peat vermiculite mixture (Metro Mix; Sun Gro Horticulture Canada Ltd., Agawam, MA) and grown for 5 weeks. The plants were fertilized weekly with 200 ppm nitrogen. The plants had 4-to-5 true leaves at the time of transplanting. After transplanting, the treatments were sprayed on foliar parts of the tomato plant at the rate of 1.2 L per four plots 1 week prior to bacterial inoculation. The treatments consisted of 100, 200, and/or 1000 μg/mL of MgO (20 nm) suspension, sonicated in Branson B-22-4 Ultrasonic Cleaner (Danbury, CT, USA) for 10 min, with constant shaking while applying. The controls were Kocide 3000 (2.1 g/L), the grower standard Kocide 3000 (2.1 g/L) in combination with Penncozeb 75DF (1.2 g/L) (Cu-EBDC) and an untreated water control. No adjuvants were used in the treatments. To ensure adequate disease development in field plots, a suspension of Cu-tolerant *X. perforans* strain GEV485 was adjusted to 5×10^8 CFU/mL in DI was applied to the foliage in the field by spraying the first, middle, and last plant in each plot in 2016 and 2020 trials at both locations 4 days after first application of treatments. Natural infection happened before artificial inoculation in Quincy in 2019; therefore, artificial inoculation was not used in that trial. Each test treatment was applied to each plot weekly/twice a week with a backpack sprayer pressurized with a CO_2 tank providing full coverage to plants until 1 week before harvesting fruit.

The plants were assessed for disease severity and phytotoxicity indicating percentage of leaf area affected weekly using the Horsfall-Barratt disease severity scale[29] every week after inoculation until harvest. The midpoint averages of the disease severity scale were used to calculate the Area Under the Disease Progress Curve (AUDPC),[30] a method to evaluate cumulative disease progression over time. There were four replications per treatment in all of the field experiments. In two trials, fall 2016 (artificially infected) and fall 2019 (naturally infected) at Quincy, FL, all plants, excluding the two toward the two ends of plots, were harvested for assessing yield. Fruits were harvested at green/early breaker stage and then graded by United States Department of Agriculture (USDA) standards.[31] At least two harvests were made for each field experiment, which is common for fresh-market tomato production in Florida. None of the trials were in the same field location. in all the trials at weekly intervals. A fungicide program included chlorothalonil 2 pt/A and Scala 7 oz/A applied in the field trial sites for management of early blight and target spot, the two fungal diseases of importance and hence these diseases were in excellent control and hence no external impact of these fungal diseases was noted in the studies.

Field Trial to Determine Total and Bioavailable Metal Concentration in Soil. A field trial was conducted in Quincy, FL during spring 2018 from Apr 2 to June 12. The tomato variety cv. Quincy was used, and treatments with only weekly applications listed above were used to study the impact of MgO and Cu on total and bioavailable concentrations of Mg, Cu, Mn, and Zn in soil. The spray boom is specifically focused on plants and not on soil, and each treatment plot is separated by a wide area to minimize the risk of cross contamination between plots. After the final treatment application, soil samples (100 g) were collected on Jun 12 from (1) the root-associated soil collected from the planting hole where direct deposition of MgO and Cu treatments would have occurred, (2) soil at the center of the width of one side of the bed under the plastic at 25.4 cm from the planting hole, and (3) the edge of the plot not covered by the plastic of the raised bed at 50.8 cm from the base of the plant where most of the runoff from the leaves and drift would have occurred. Soil was collected using a deluxe soil probe (Nasco, Fort Atkinson, WI) inserted 15 cm into the ground at the locations indicated above while carefully removing any roots to obtain a sufficient amount of sample for analysis (total 100 g). The sampling approach is shown in Supplemental Figure 4.

Both total and bioavailable metal contents in soil samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS, 7900 Agilent Technology, Santa Clara, CA) (Supplemental Table 2). The total Mg, Cu, Mn, and Zn concentrations of each soil sample were measured by digesting ~0.3 g soil samples in 10 mL 1:3 HNO_3/HCl at 200 °C for 1.5 h in a microwave digestion system (Multiwave Eco, Anton Paar), followed by analysis via ICP-MS. Soil samples (~1 g soil) were placed in 15 mL conical test tubes, mixed with 10 mL of DI water on an end-overend shaker (Dayton-6Z412A Parallel Shaft roller mixer, USA) with a speed of 70 rpm at room temperature for 7 days to ensure sufficient leaching time. Then, the tubes were centrifuged at 10,000 rpm for 20 min to separate soil and water, and the supernatant was collected for leached bioavailable Mg, Cu, Mn, Zn concentrations determined by ICP-MS. Even though this study was also artificially inoculated as described in the methods listed above, the overall bacterial spot disease severity was low and hence not presented.

Statistical Analysis. The data collected from the experiments were evaluated for normal distribution and statistical significance using analysis of variance followed by pairwise comparisons using Student−Newman−Keuls test for *in vitro* studies and least significant difference method for field experiments in IBM SPSS Statistics, version 22. A p value of 0.05 was used to evaluate significance. To rule out the effect of natural infection in 2019 Quincy trial, the fit model was run to ensure normality of data. In this trial data, we ran a linear mixed model to account for the blocking effect of row and effect, and the residuals of the model were visually inspected for normality and homogeneity of variance.

RESULTS

Tolerance of *Xanthomonas perforans* to Commercial Cu Bactericides *in Vitro*. Cu-tolerant *X. perforans* strain GEV85 showed tolerance to all Cu bactericides tested in this study (Figure 1). Commercially available Cu bactericides Kocide 3000, Kentan, and Badge at 200 μg/mL had significantly lower efficacy when compared to the other tested Cu bactericides ($p = 0.05$), in other words, significantly higher population of Cu tolerant *X. perforans* can be recovered after treated with Kocide 3000, Kentan, and Badge ($p = 0.05$). Nordox consistently had the highest efficacy among all the treatments at all incubation durations. Nordox 200 μg/mL consistently provided 100 fold reduction in bacterial population when compared to untreated control; however, none of the Cu fungicides completely inhibited the bacterial growth. Together, these data indicates that Cu-tolerant *X. perforans* strains could tolerate a wide range of Cu bactericides with different Cu actives.

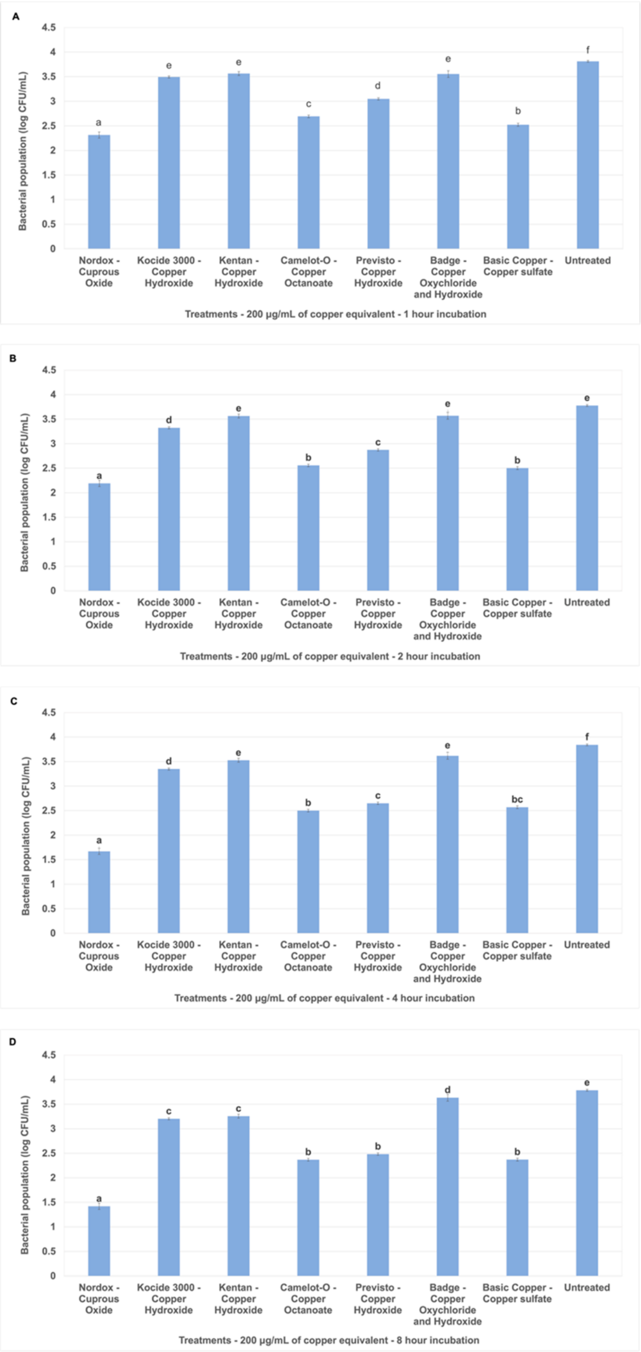

Figure 1. Effect of commercial Cu-based materials (Nordox, Kocide 3000, Kentan, Camelot-O, Previsto, Badge and Basic Copper) in vitro at 200 μg/mL Cu equivalent rate against Cu tolerant *Xanthomonas perforans* GEV485 at (a) 1 h, (b) 2 h, (c) 4 h, and (d) 8 h of incubation. The error bars show standard error of mean. Different letters on the top of the bars show statistical differences at $p = 0.05$ using pairwise comparisons with Student−Newman−Keuls test.

Efficacy of Intensive Application of MgO for Disease Control in the Field. MgO nanomaterial at 200 μg/mL were applied once or twice (intensive) weekly in comparison with the conventional Cu bactericide Kocide 3000 and the grower's standard Cu-EBDC. In the first field experiment, conducted during fall 2016 in Quincy, FL (Figure 2A), MgO at 200 μg/mL applied twice a week had significantly reduced disease severity compared with Cu bactericide Kocide 3000, Cu-EBDC, and water treatments ($p = 0.05$). Cu-EBDC applied twice a week did not statistically reduce disease severity compared to the water treated plots ($p = 0.05$). Weekly applications of MgO did not differ from Kocide 3000 but was

Figure 2. Bacterial spot disease severity as indicated by Area Under Disease Progress Curve (AUDPC) on tomato in fields treated with once or twice weekly MgO nanomaterial (20 nm) at 200 μg/mL in comparison to Cu-based bactericide (Kocide 3000, 2100 μg/mL), the grower standard (Cu-EBDC, Cu-mancozeb), and the untreated water control in (A) 2016 Fall, Quincy, FL and (B) 2016 Fall, Wimauma, FL. Cu-EBDC is composed of Cu (Kocide 3000, 2,100 μg/mL) and mancozeb (Penncozeb 75DF, 1,200 μg/mL). Numbers with different letters in the same column show significant differences (p = 0.05) based on Least Significant Difference analysis. The error bars show standard error of mean.

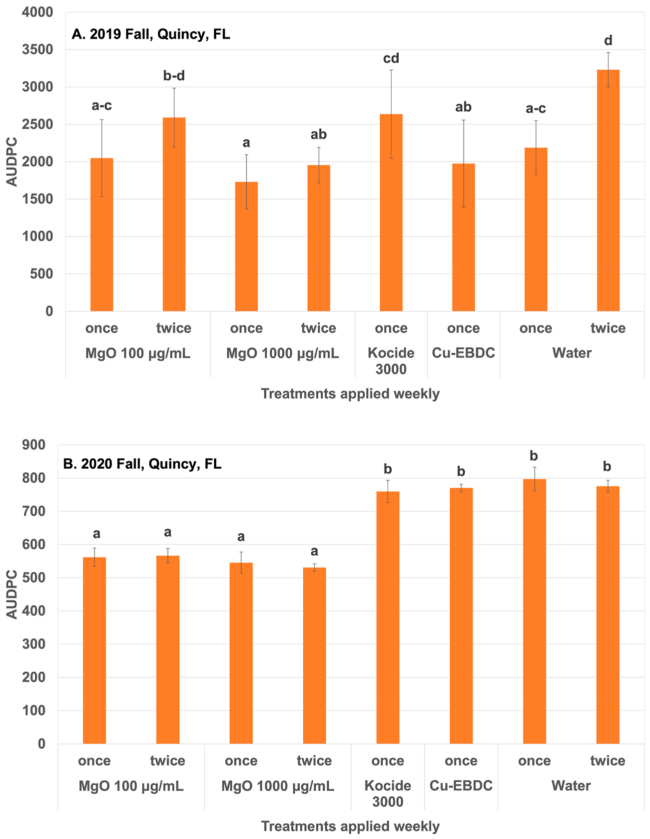

Figure 3. Bacterial spot disease severity as indicated by Area Under Disease Progress Curve (AUDPC) on tomato in fields treated with once or twice weekly MgO nanomaterial (20 nm) at 100 and 1000 μg/mL in comparison to Cu-based bactericide (Kocide 3000, 2100 μg/mL), the grower standard (Cu-EBDC, Cu-mancozeb), and the untreated water control in (A) 2019 Fall, Quincy, FL and (B) 2020 Fall, Quincy, FL. Cu-EBDC is composed of Cu (Kocide 3000, 2,100 μg/mL) and mancozeb (Penncozeb 75DF, 1,200 μg/mL). Numbers with different letters in the same column show significant differences (p = 0.05) based on Least Significant Difference analysis. The error bars show standard error of mean.

different from controls. In the second field trial in fall 2016 Wimauma, FL, MgO at 200 μg/mL applied twice a week significantly reduced disease severity compared to the Cu bactericide Kocide 3000 and water treatments (p = 0.05) (Figure 2B). MgO at 100 μg/mL and 1000 μg/mL were applied once or twice weekly in comparison to the Cu bactericide Kocide 3000 and the grower standard Cu-EBDC in trials conducted in fall 2019 and 2020 in Quincy, FL. In the 2019 trial, none of the treatments including MgO and the Cu bactericides when applied once per week significantly reduced disease severity compared to plots sprayed with water once a week (p = 0.05). MgO at 1000 μg/mL applied once/twice a week and grower's standard Cu-EBDC significantly reduced disease severity compared to plots treated with Kocide 3000 and water treatments applied twice a week (p = 0.05) (Figure 3A). The 2019 field experiment was repeated in fall 2020 in Quincy, FL. All MgO treatments significantly reduced disease compared to the Cu bactericide Kocide 3000, grower's standard Cu-EBDC, and water treated control (p = 0.05) (Figure 3B). In the two experiments where yield was analyzed no significant differences were noted for plots treated with MgO compared to water control (Table 1). However, during the fall 2016 experiment a significant reduction in yield with use of once-a-week application of Cu-EBDC compared to the water treated control was observed (p = 0.05). In fall 2019 no significant differences in yield were observed for any of the treatments.

Elemental Accumulation in Tomato Field Soil. *Root-Associated Soil.* Soil from plots treated with MgO had no significant differences for total and bioavailable concentrations of any of the metals analyzed when compared to the water control (Table 2). Plots treated with Cu bactericide Kocide 3000 and Cu-EBDC had significantly higher bioavailable Mn, Cu, and Zn ions in root-associated soil compared to the soil collected from the water treated plots (p = 0.05). No significant differences in total metal concentration were noted for Cu bactericide Kocide 3000 and Cu-EBDC compared to water control.

Soil at the Center of the Width of One Side of the Bed under the Plastic. There were no statistical differences between any of the treatments for total and bioavailable metal accumulation. However, a numerical increase was observed for bioavailable Cu and Zn from plots treated with Cu bactericide Kocide 3000 and Cu-EBDC compared to the water control.

Table 1. Marketable Yield of Tomatoes Treated with MgO Nanomaterial (20 nm) in Comparison to Cu Bactericide (Kocide 3000), the Grower Standard (Cu-EBDC), and Water Control during Fall 2016 and Fall 2019 Trials in Quincy, FL

Treatment	Weekly application frequency	Yield (kg/ha)
Fall 2016 Quincy, FL		
MgO 200 μg/mL	Once	47,204 ab[a]
MgO 200 μg/mL	Twice	40,525 ab
Kocide 3000 2100 μg/mL	Once	58,496 b
Kocide 3000 2100 μg/mL	Twice	42,853 ab
Cu-EBDC[b]	Once	35,220 a
Cu-EBDC[b]	Twice	51,649 ab
Water (Untreated)	Once	56,408 b
Water (Untreated)	Twice	53,092 ab
Fall 2019, Quincy, FL		
MgO 100 μg/mL	Once	52,511 a
MgO 100 μg/mL	Twice	64,151 a
MgO 1000 μg/mL	Once	58,642 a
MgO 1000 μg/mL	Twice	61,507 a
Kocide 3000 2100 μg/mL	Once	67,260 a
Cu-EBDC[b]	Once	65,039 a
Water (Untreated)	Once	65,955 a
Water (Untreated)	Twice	54,599 a

[a] Number with different letters in the same column has significant difference (p value of = 0.05) based on Least Significant Difference statistical analysis. [b] Cu-EBDC is composed of Cu (Kocide 3000, 2100 μg/mL) and mancozeb (Penncozeb 75DF, 1200 μg/mL).

Soil at the Edge of the Plot Not Covered with Plastic. Soil from plots treated with MgO had no significant differences for total and bioavailable concentrations of any of the metals analyzed when compared to the water control (Table 2). Soil collected from plots treated with Cu-EBDC had significantly higher concentration of bioavailable Mg, Mn, Cu, Zn ion at the edge of the plot compared to the water treated plots (p = 0.05). Plots treated with Cu bactericide Kocide 3000 by itself showed a similar trend except in the case of bioavailable Cu, even though the levels were numerically higher than in the case of the water control.

DISCUSSION

A wide range of Cu bactericides are labeled for use on tomatoes in Florida and other places in the U.S. Use of Cu fungicide in Florida tomatoes alone on tomatoes can range from 7 to 15 applications in a season. This could be up to a maximum Cu use range of 8.75−18.75 lb/A based on current labeled medium rate for Cu (e.g Kocide 3000 at 1.25 lb/A/application) on tomato.[32] Converted to metallic Cu use this corresponds to 2.625−5.625 lb/A (30% metallic Cu in present in Kocide 3000). If a conservative estimate is considered, with only one-half of the expected maximum use by tomato farms in 26,000 acres which equates to 34,125−73,125 lb of metallic Cu annually used in Florida tomatoes. While application of Cu could vary between crops under different climatic growing conditions, Cu is one of the most used active ingredients in both conventional and organic production globally. Implications of this widespread use of Cu based agrochemicals could mean increased evolutionary pressure on the development of Cu-tolerant *Xanthomonas* strains in tomato growing regions. This is well-documented and is a growing concern for tomato producers and scientists working in this area.[13,15,33−38] Thus, identifying alternatives to Cu compounds that can manage Cu tolerant strains of *Xanthomonas* spp. and would also have minimal impact in soil accumulation is critical.

In a prior study, our group demonstrated that a single application in a week with 200 μg/mL concentration of MgO significantly reduced disease severity compared to the water treated plots.[23] However, tomato growers may apply Cu-EBDC multiple times in a week in the commercial field during favorable conditions for disease development.[12,39] When MgO as an alternative for Cu was contemplated, it was a clear consideration to compare single and twice weekly applications with Cu-EBDC application to mimic typical grower practices. In this study conducted at multiple field experiments at two field sites in Florida, tomato plants treated with MgO applications provided higher efficacy compared to the grower's standard and untreated control in three out of four field trials. In the 2016 fall study in Quincy, MgO twice a week application was the only treatment that was statistically better in AUDPC than twice a week application of Cu-EBDC. However, no clear statistical separation was found in the 2016 fall study in Wimauma. Interestingly, MgO twice a week application separated out statistically from water control in both trials, while once a week only separated out only in one trial. Even though in 2019 and 2020 studies we did not see the impact of MgO twice a week compared to once a week application on AUDPC, the 2016 studies suggest that twice a week application may have utility under field conditions, but not always in comparison to once a week application. Thus, this study is novel and is seminal to our understanding how performances of nanomaterials in direct comparison to grower practices. Regarding yield, in the case of bacterial spot studies at a scale of a maximum trial area of ∼1 acre for all treatments in a research setting, it would be rare to see significant yield increases as noted in many prior studies.[16,22,23,39] The important aspect we observe in this case is whether there are any negative yield impacts, which we have not seen in this study or any of our previous studies of MgO.

Additional field trials in different locations and different seasons might be required to validate whether MgO is more effective than grower's standard Cu-EBDC in managing the bacterial spot disease of tomato, not merely providing the same level of efficacy. However, with the concerns of ecological risks posed by potential runoff of Cu, the EPA has proposed that Cu applications should be limited for certain crops.[40] According to the field study conducted and presented in this work, none of the soil samples collected from the tomato production field treated with MgO had significantly higher total or bioavailable Mg, Mn, Zn, and Cu concentration compared to the soil collected from the water-treated plot. To our best knowledge, this is the first time an assessment of nano MgO and soil elemental accumulation in tomato production system has been studied and presented that demonstrates that MgO did not lead to accumulation of the elements tested. On the other hand, soil collected from Cu bactericide Kocide 3000 and grower's standard Cu-EBDC treated plots had higher bioavailable Cu or/and total Cu concentration compared to the soil collected from the water treated plots. This result indicated that Cu bactericide application might result in Cu, Zn, and Mg runoff to the edge of the plot and potentially lead to increased overall metal accumulations. However, this study only looked at immediate accumulation and did not look into long-term accumulation. A long-term study on the use of mancozeb showed accumulation of Mn and metabolite

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Table 2. Total and Bioavailable Metal Concentrations (ng/g-soil) from Soil[a] Collected from Field Experiment Following Treatment of Tomato Plants with MgO Nanomaterial (20 nm, 100 and 200 μg/mL) in Comparison to Cu Bactericide (Kocide 3000), the Grower Standard (Cu-EBDC, Composed of Cu (Kocide 3000, 2100 μg/mL) and Mancozeb (Penncozeb 75DF, 1200 μg/mL) and Water Control during Spring 2018 in Quincy, FL

Root-associated soil[b]																
	Mg (ng/g-soil)				Mn (ng/g-soil)				Cu (ng/g-soil)				Zn (ng/g-soil)			
Treatments	Total		Bioavailable ion		Total		Bioavailable ion		Total		Bioavailable ion		Total		Bioavailable ion	
MgO 100 μg/mL	1,15,472.35	a[e]	14,080.31	a	59,767.46	a	894.22	ab	1,336.50	a	43.80	ab	7,339.09	a	295.85	ab
MgO 200 μg/mL	1,28,722.46	a	12,160.32	a	81,022.83	a	458.69	a	2,418.96	a	32.16	a	8,546.67	a	207.23	a
Kocide 3000	1,19,710.50	a	17,794.16	a	64,783.64	a	1,331.61	**b**	1,821.61	a	74.25	**b**	8,615.15	a	422.88	**b**
Cu-EBDC	2,29,114.60	a	21,218.59	a	67,406.20	a	1,161.11	**b**	2,263.88	a	73.20	**b**	14,369.38	a	419.42	**b**
Water	1,08,594.79	a	13,028.50	a	59,970.86	a	480.34	a	1,275.18	a	28.25	a	14,334.86	a	215.72	a
Significance (p = 0.05)	No		No		No		Yes		No		Yes		No		Yes	

Soil at the center of the width of one side of the bed under the plastic[c]																
	Mg (ng/g-soil)				Mn (ng/g-soil)				Cu (ng/g-soil)				Zn (ng/g-soil)			
Treatments	Total		Bioavailable ion		Total		Bioavailable ion		Total		Bioavailable ion		Total		Bioavailable ion	
MgO 100 μg/mL	1,69,939.58	a	38,185.31	a	65,528.06	a	369.49	a	1,339.96	a	12.39	a	6,440.96	a	94.43	a
MgO 200 μg/mL	1,87,040.01	a	33,151.67	a	62,162.11	a	143.96	a	1,314.84	a	6.54	a	7,089.65	a	47.06	a
Kocide 3000	1,62,055.83	a	48,985.13	a	60,757.40	a	355.12	a	1,348.08	a	18.49	a	7,576.36	a	135.37	a
Cu-EBDC	3,19,919.28	a	43,625.65	a	65,690.46	a	376.15	a	1,442.11	a	23.58	a	10,779.57	a	156.48	a
Water	1,90,888.59	a	33,615.40	a	64,647.81	a	235.19	a	1,168.70	a	11.15	a	5,880.51	a	64.05	a
Significance (p = 0.05)	No		No		No		No		No		No		No		No	

Soil at the edge of the plot not covered by the plastic[d]																
	Mg (ng/g-soil)				Mn (ng/g-soil)				Cu (ng/g-soil)				Zn (ng/g-soil)			
Treatments	Total		Bioavailable ion		Total		Bioavailable ion		Total		Bioavailable ion		Total		Bioavailable ion	
MgO 100 μg/mL	96,465.74	a	9,562.70	ab	60,647.55	a	1,253.02	ab	1,616.09	a	66.71	a	6,303.49	a	338.44	ab
MgO 200 μg/mL	1,43,049.26	a	10,528.68	ab	60,906.32	a	869.40	a	1,774.09	a	55.33	a	7,787.03	a	302.37	a
Kocide 3000	1,04,903.77	a	13,052.95	**b**	65,042.20	a	1,585.97	**b**	4,053.86	b	136.86	a	8,513.52	ab	485.91	**b**
Cu-EBDC	1,24,854.68	a	14,287.96	**b**	74,040.60	a	1,680.88	**b**	7,678.61	**c**	259.57	**b**	11,356.67	**b**	495.01	**b**
Water	94,139.37	a	7,307.20	a	64,276.22	a	783.40	a	2,320.48	ab	52.41	a	6,295.28	a	226.40	a
Significance (p = 0.05)	No		Yes		No		Yes		Yes		Yes		Yes		Yes	

[a] Soil was collected from tomato field after harvest, and the field was applied with treatments weekly for 8 weeks. [b] At the planting hole. [c] 25.4 cm from the planting hole. [d] 50.8 cm from the planting hole. [e] Number with different letter in the same column has significant difference (p value of = 0.05) based on Least Significant Difference statistical analysis using the IBM SPSS program.

Ethylenethiourea in banana plantations.[41] Similar studies in Florida fields with a long history of use of Cu fungicides would be needed to address the impact of long-term use of Cu fungicides in soil as well as Cu and other metal accumulation. Another aspect for consideration is that different types of Cu bactericides (conventional and nano) with different properties including but not limited to rainfastness and particle size could affect the potential for leaching into soil which has been shown in a study on citrus leaves exposed to nine different Cu bactericides.[42] The authors observed in this study that retention of a nano CuO on leaves was very high (>97%) and suggested potential absorption of Cu particles or retention in leaves. This may explain that even after multiple MgO applications, we did not see a significant increase in total and bioavailable Mg in soil. Prior studies have looked at elemental accumulation in fruits after field applications of MgO and found that there is no significant increase of levels of Mg in the fruits.[22,23] However, in this study or in prior studies, we have not conducted leaf elemental analysis and hence further studies would be required to understand the nature of adhesion/absorption of Mg by tomato leaves. A key aspect to note for this study is that the total amount of Cu applied on plants for twice-weekly application of Kocide 3000 during the entire period of the experiment was ∼10 kg/acre and the total amount MgO at 200 μg/mL for the same application frequency was ∼2 kg/acre based on 2016 trial rates.

Application of MgO at 100 and 200 μg/mL did not result in accumulation of total Mg, Mn, Cu, or Zn in the root-associated soil, and more importantly it did not result in an increase in bioavailable ions (Mg, Mn, Cu, Zn) in this region. These MgO treatments also did not result in accumulation of these metals or the bioavailable ions at the edge of the plot. In contrast, the Cu-based treatments (Kocide 3000 and Cu-EBDC) resulted in an increase in bioavailable Cu, Zn, and Mn ions in the root-associated soil and bioavailable Cu, Mg, Mn, and Zn at the edge of the plot. The Cu treatments also resulted in increased total Cu and Zn at the edge of the plot. The increase in total Cu from 2.32 mg/kg to 4.05 to 7.68 mg/kg at the edge of the plot indicates that even a single year of treatment can result in a significant increase in Cu concentrations. While these levels are still relatively low compared to soils that have received Cu pesticides repeatedly, it is important to consider the impact on the soil microbial community.[43−45] Several studies have shown that bioavailable Cu can be toxic to microbes, as well as a number of aquatic and terrestrial organisms at concentrations around 1 mg/L, indicating that even at current application rates of Cu, they may be affecting soil microbes.[46] To put these concentrations into context, agricultural soils in Europe with permanent crops have concentrations of 36.6 mg Cu/kg, with a median of 22.6 mg Cu/kg. European vineyards, which receive a significant amount of Cu pesticides, have a mean concentration of 49.3 mg Cu/kg, after years of treatment.[47] Similarly, agricultural soils in Chile where Cu pesticides have been applied can have up to 21 mg Cu/kg, with bioavailable (exchangeable Cu) concentrations ranging from 0.1 to 2.0 mg Cu/kg, depending on soil type.[43] A national assessment of Cu contaminated agricultural soils in China, based on 1,731 sites, found that around 21% of the sites exceeded 50 mg Cu/kg. A clear linear correlation was observed between the amount of fungicide applied and the concentrations of Cu in the soils.[48] From the standpoint of mechanism of action, nano MgO led to pronounced changes in cell morphology *X. perforans* cells and are bactericidal in nature.[22,23] Reactive oxygen species (ROS) accumulation could also play an important role for the antibacterial efficacy of MgO, causing DNA damage, against *Ralstonia solanacearum*, another devastating bacterial pathogen of tomato.[49] Also, studies on formulating antibacterial materials by Mg coating has been conducted. For example, Mg hydroxide nanoparticles were synthesized followed by coating with water-soluble capping agents, trisodium citrate or betaine which showed high bactericidal properties and no phytotoxicity on tomatoes.[50] Electron microscopic studies confirmed the formation of ∼10 nm-sized cubical NPs with citrate and ∼100-nm-sized lamellar NPs with betaine. These approaches provide unique possibilities for coating of magnesium-based nanomaterials for new formulations.

This study concludes that intensive application of MgO nanomaterial is effective against the Cu-tolerant strain of *X. perforans* and is thus an effective alternative to conventional Cu bactericide Kocide 3000, without significantly increasing total and bioavailable metal accumulation in the soil. Use of MgO nanomaterial as a bactericide alternative to Cu could have a significant benefit to the soil microbial communities, compared to continued application of Cu-based pesticides, whether ionic, bulk, or nano, and such studies need to be conducted in Florida production systems to further validate the hypothesis. A recent review highlighted the relevance of transitioning from lab to field scale studies and demonstration of effectiveness of nanomaterials and safety as a way to overcome some of the existing barriers in sustainably implementing plant nanotechnology including regulatory approval.[27] This study design specifically tackles those existing barriers by building more scientific data that provides further information in generating an early stage technology readiness level (TRL) assessment. In this study field experiments have been conducted across seasons, years, and locations which add strength to the findings presented. Further studies on the impact of MgO on soil and phyllosphere microbiomes, industry partnership, regulatory framework, and consumer acceptance assessment are needed to expand the scope of this study. The data from this study and our prior studies[22,23] suggest that MgO nanomaterials may be at the crossroads of moving from a well identified nanomaterial alternative to Cu fungicides into consideration for development of formulated agricultural grade Mg-based fungicides. A set of comprehensive reviews have been presented in recent years highlighting the critical possibilities in agricultural nanotechnology on plant diseases and field-level management.[26,27,51] This study further adds critical new information on near-to-field scale practice for disease management for a critical bacterial disease affecting tomatoes worldwide.

■ ASSOCIATED CONTENT

Supporting Information

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Supplemental figures and tables (PDF)

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Author Contributions

M.L.P., A.K., J.B.J., J.F., and G.V. conceptualized the project and received USDA and NSF grants to conduct the research. Y.L., Y.H., R.C., M.C., A.H., and S.D. conducted the experiments, analyzed the data, and wrote the manuscript. J.C. reviewed and conducted statistical analysis. M.L.P., Y.L., Y.H., and A.K. completed the final review and submission.

Notes

The authors declare no competing financial interest.

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