Photocatalysis: Effect of Light-Activated Nanoscale Formulations of TiO_2 on *Xanthomonas perforans* and Control of Bacterial Spot of Tomato

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ABSTRACT

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Protection of crops from bacterial diseases presents a continuing challenge, mandating the development of novel agents and approaches. Photocatalysis is a process where chemically reactive oxygen species are catalytically generated by certain minerals in the presence of light. These reactive oxygen species have the capacity to destroy organic molecular structures critical to pathogen viability. In this study, the antibacterial potential of photocatalytic nanoscale titanium dioxide (TiO_2), nanoscale TiO_2 doped (incorporation of other materials into the structure of TiO_2) with silver (TiO_2/Ag), and nanoscale TiO_2 doped with zinc (TiO_2/Zn; AgriTitan) was evaluated against *Xanthomonas perforans*, the causal agent for bacterial spot disease of tomato. In vitro experiments on photocatalytic activity and dose dependency were conducted on glass cover slips coated with the nanoscale formulations by adding a known population of *X. perforans* strain Xp-F7 and illuminating the cover slips under a visible light source. TiO_2/Ag and TiO_2/Zn had high photocatalytic activity against *X. perforans* within 10 min of exposure to 3×10^4 lux. Greenhouse studies on naturally and artificially infected transplants treated with TiO_2/Zn at ≈500 to 800 ppm significantly reduced bacterial spot severity compared with untreated and copper control. Protection was similar to the grower standard, copper + mancozeb. The use of TiO_2/Zn at ≈500 to 800 ppm significantly reduced disease incidence in three of the four trials compared with untreated and copper control, and was comparable to or better than the grower standard. The treatments did not cause any adverse effects on tomato yield in any of the field trials.

Additional keywords: bacterial disease management.

Fresh-market tomato is an important vegetable crop in the United States and is grown on >40,064 ha and valued at $1.29 billion (26). Bacterial spot caused by *Xanthomonas perforans* leads to significant reduction in crop yield, and is one of the major bacterial diseases affecting tomato in commercial fields worldwide. A recent estimate shows that the monetary losses due to bacterial spot in southwest Florida alone were $7,636/ha based on 2007–08 production costs and market values (38). Disease management using cultural practices and disease-tolerant varieties has given mediocre results in tropical and subtropical regions, where the climatic conditions favor infection and the spread of the disease. Chemical control using the antibiotic streptomycin was successfully used in the 1950s. However, strains resistant to streptomycin developed in due course that made the antibiotic ineffective (34). The disease has been managed using copper bactericides for many decades. The effectiveness of copper bactericides is enhanced when used in combination with ethylenebis-dithiocarbamate fungicides such as maneb or mancozeb due to increased availability of free copper ions. (7,19,23). Unfortunately, widespread use has led to copper tolerance and, currently, most of the strains isolated from Florida are tolerant to copper, reducing the effectiveness of this standard disease-control treatment (20).

Alternative disease-management technologies such as bacteriophages and systemic acquired resistance (SAR) inducers have been under investigation as alternatives to copper bactericides (3,17,28,29). Acibenzolar-S-methyl (ASM) is an SAR inducer that activates plant defense systems by increasing the transcription of stress-related genes. ASM has been shown to increase resistance of tomato to bacterial spot compared with untreated controls (28). Bacteriophages also have shown promise as a biological alternative to conventional copper bactericides (3,28,29). However, ensuring efficacy of a bacteriophage is challenging in field conditions due to the short time that phages remain viable and their specific environmental requirements for multiplication.

Photocatalytic materials such as crystalline titanium dioxide (TiO_2) offer a completely different approach to achieving antibacterial benefit in a range of settings. Briefly, absorption of energetic photons by TiO_2 and similar materials results in the creation of free electrons and positively charged holes in the crystal structure. These entities can migrate within the crystal to the particle surface, where they interact with water molecules to create highly chemically reactive hydroxyl and superoxide free radicals, a process termed photocatalysis. The reactive oxygen species generated by this process then oxidize any organic material in their diffusion range, with the result that microbes are killed by chemical oxidation of critical molecular structures (4,31).

Reduction of crystal particle size to the nanometer scale affords a massive increase in surface area per unit of mass compared with more familiar particle dimensions, resulting in substantial photocatalytic and antibacterial activity. Although photocatalysis is observed with a number of metal oxides, TiO_2 has emerged as a preferred material for this use due to its photocatalytic efficiency, abundance, and well-established safety (9,14).

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Photocatalysis by TiO_2 was reported in 1972 (15), with the first demonstration of an associated antibacterial effect some years later (24), when a TiO_2 surface was shown to exhibit bactericidal activity against *Escherichia coli* and *Lactobacillus acidophilus* if irradiated for 60 to 120 min with UV radiation of ≈387 nm. No inactivation of the bacteria was observed when UV was used in the absence of TiO_2. Several functional properties of photocatalytic TiO_2 nanoparticles can be altered by incorporation of various heteroatoms into the crystal structure (4,6,25), which is known as doping. Findings reported in the past decade has shown the antimicrobial potential of TiO_2 nanoparticles on a wide range of bacterial, fungal, and viral organisms (1,2,5,8,31,35,40). A recent study reported that a macro-particulate form of TiO_2 was effective against bacterial blight of geranium and bacterial leaf spot of poinsettia, caused by *X. hortorum* pv. *pelargonii* and *X. axonopodis* pv. *poinsettiicola*, respectively (27). That study also proposed that future work on the use of nanoparticle formulations of TiO_2 would be highly relevant in improving the application efficiency and reducing elemental concentration on plants.

In view of the broad antimicrobial activity of photocatalytic TiO_2, we sought to assess the potential of such materials as a technology for reducing the impact of bacterial spot on tomato. Our primary objectives included evaluation of the photocatalytic effect of TiO_2, TiO_2 doped with zinc (TiO_2/Zn), and TiO_2 doped with silver (TiO_2/Ag) nanoparticles on *X. perforans*; analysis of the effect of the nanoparticles in reducing bacterial spot in greenhouse studies; assessment of the effectiveness of TiO_2/Zn nanoparticles against bacterial spot disease in tomato field production; and evaluation of the effect of TiO_2/Zn nanoparticle treatment on tomato fruit yield.

MATERIALS AND METHODS

Nanoparticles used for in vitro, greenhouse, and field experiments. The photocatalytic nanoparticles evaluated in this work were purchased as preformulated aqueous colloidal suspensions. The undoped TiO_2 was Gens Nano C386, <8-nm average particle size, anatase crystal structure with a concentration of TiO_2 at 7,500 to 10,000 ppm in water. The TiO_2/Ag was Gens Nano C406, <8-nm average particle size, anatase crystal structure with a concentration of TiO_2 at 7,500 to 10,000 ppm containing Ag at 25 to 50 ppm in water. TiO_2 and TiO_2/Ag were obtained from Green Earth Nanoscience, Inc., Toronto Canada. The TiO_2/Zn was AgriTitan, 7-nm average particle size, anatase crystal structure with a concentration of TiO_2 at 5,000 to 8,000 ppm containing Zn at 25 to 50 ppm in water. TiO_2/Zn was supplied by EcoActive Surfaces, Inc., Pompano Beach, FL.

In vitro photocatalytic activity of TiO_2, TiO_2/Ag, and TiO_2/Zn. Glass cover slips (22 by 22 mm) (Thermo Fischer Scientific, Waltham, MA), were coated with 0.5 ml of nanoparticles, and dried in a laminar flow hood for 4 h. The photocatalytic activity was tested on copper-tolerant *X. perforans* race T4 strain Xp-F7. The bacterial strain stored in 30% glycerol at –80°C was plated on nutrient yeast nutrient agar (YNA) and incubated at 28°C for 48 h. Two to three colonies of the strain were picked from YNA, transferred to 10 ml of sterile distilled water (dH_2O) in a test tube, vortexed for 10 s, and adjusted spectrophotometrically to an optical density at 600 nm = 0.3 corresponding to 10^8 CFU/ml. The bacterial solution (100 µl) was applied to glass cover slips coated with the nanoparticles.

The glass cover slips were placed under an incandescent lamp (Cheyenne Portable Lamp with 60-W incandescent bulb) (General Electric, Fairfield, CT) for photocatalytic reaction (21,39). A light meter (model 407026; Extech Instruments, Nashua, NH) was used to measure the illumination density at the cover slips. In the photocatalytic experiment, illumination was carried out for 5, 10, 15, and 20 min at a 5-cm distance from the sample, which corresponds to an illumination density of 3×10^4 lumen/m^2 (lux). Illuminations were carried out at 4°C. Samples were also kept in the dark for 20 min as a control for this experiment to demonstrate the photocatalytic effect. In the dose-dependence experiments, illuminations were carried out for 20 min at distances of 5, 10, and 15 cm from the lamp, corresponding to illumination densities of 3×10^4, 1.2×10^3, and 3×10^2 lux, respectively. Glass cover slips not coated with the nanoparticles were used as controls in each treatment in each experiment. After illumination, the glass cover slips were placed in sterile 50-ml centrifuge tubes (Thermo Fischer Scientific). Sterile dH_2O (1 ml) was added to the tube and vortexed for 20 s for recovery of bacterial cells. The bacterial enumeration was determined by the standard plating method. The sample (100 µl) was taken to initiate a 10-fold dilution series (10^{-1} to 10^{-7}), and 100 µl of each dilution was spread on YNA and incubated at 28°C for 48 h, after which colony counts were made. Each treatment in every experiment had three replications, and all the experiments were performed twice.

Greenhouse and growth-chamber experiments. The preliminary analysis was conducted on tomato transplants of 'BHN 602' (BHN Seeds, Immokalee, FL), naturally infested with the bacterial spot disease at the three- to four-leaf stage growing in soilless potting medium (Sungro Metro-Mix 200 series; Sun Gro Horticulture Canada Ltd., Vancouver, BC, Canada) in expanded polystyrene trays of the inverted pyramid design, with cell size of 4.4 by 4.4 by 6.3 cm. Plants with a similar number of bacterial spot lesions per plant were selected for the study (data not shown). Plants were sprayed with the nanoparticles in undiluted form and diluted in sterile tap water (1:10 dilution; ≈500 to 800 ppm) until runoff. Three plants were tested for each treatment and the trial was set up in a randomized complete block design. The number of bacterial spot lesions on each plant was counted before treatment. Plants were kept in greenhouse (32°C; 70% humidity) for a period of 14 days. The number of bacterial spot lesions was counted after the period of the experiment to assess the effectiveness of each treatment.

Based on the preliminary analysis on effectiveness, TiO_2/Zn was selected for further greenhouse and field trials. The effect of nanoparticles on the *X. perforans* strain was studied on bacterial-spot-susceptible 'FL 47' tomato in a greenhouse. Tomato seedlings were grown as described above. TiO_2/Zn was added to sterile tap water at 1:10, 1:20, and 1:40 dilutions. The plants were sprayed with TiO_2/Zn until runoff from leaves. Copper (Kocide 3000), copper + mancozeb (Penncozeb 75DF), and untreated plants were kept as controls. At 3 h post treatment, plants were inoculated with *X. perforans* race T4 strain Xp-8. The greenhouse conditions were kept at 28 to 32°C and 70% humidity for a period of 14 days. Disease severity was rated at the end of the experiment using the Horsfall-Barratt scale, to assess the percentage of canopy affected by bacterial spot (16), and the ratings were converted to midpoint averages. Eight plants were tested for each treatment and the trial was set up in a randomized complete block design. The experiment was performed twice. The same experiment was also conducted once in a growth chamber at 28°C under 80 to 85% humidity. Plants were inoculated with *X. perforans* race T4 strain GCREC T4. Seedlings of FL 47 were used in this experiment and the bacterial spot disease data was recorded at 7 days after treatment.

Field experiments. Four field trials were conducted in Florida during 2010 and 2011. Three of these trials were conducted at the University of Florida North Florida Research and Education Center in Quincy during fall 2010, spring 2011, and fall 2011. Soil type was Norfolk sandy loam with pH 6.3. One trial was conducted at the University of Florida Gulf Coast Research and Education Center in Wimauma during fall 2011. Soil type was Myakka fine sand with pH 6.8. Experimental plots at Quincy locations consisted of fumigated raised beds covered with black polyethylene mulch for spring plantings and white polyethylene

mulch for fall plantings. At the Wimauma location, black polyethylene mulch was used for fall planting. Bed dimensions at both locations were 12.7 cm tall by 76.2 cm wide. Beds were spaced 1.8 and 1.2 m apart and plants were spaced 50.8 and 45.7 cm within the row at Quincy and Wimauma, respectively. Inorganic fertilizers were applied to experimental plots based on soil test results and cooperative extension recommendations (30). Seedlings of BHN 602 were transplanted on 11 August 2010, 27 March 2011, and 4 August 2011 in Quincy, FL and seedlings of 'SecuriTy 28' (Harris Moran Seed Company, Modesto, CA) on 12 September 2011 in Wimauma, FL. Each entry consisted of four replications in Quincy and six replications in Wimauma, with 18 and 14 plants, respectively, in each replication. All experiments were arranged as randomized complete block design. The treatments of TiO_2/Zn included 1:10, 1:20, 1:40, 1:60, 1:80, and 1:100 dilutions for fall 2010 and spring 2011 trials. The fall 2011 trial in Quincy and Wimauma included nanoparticles at 1:10, 1:20, and 1:40 dilutions. Copper, copper + mancozeb, and untreated plots were kept as controls in each trial at Quincy and copper + mancozeb and untreated plots at Wimauma. The plants were sprayed in the early morning at weekly intervals (eight times) starting from the second week after transplanting. Disease severity was rated at monthly intervals using the Horsfall-Barratt scale, to assess the percentage of canopy affected by bacterial spot (16). Values were converted to mid-percentages and used to generate the area under the disease progression curve (AUDPC) based on the formula $\Sigma[([x_i + x_{i-1}]/2)(t_i - t_{i-1})]$, where x_i is the rating at each evaluation time and $(t_i - t_{i-1})$ is the time between evaluations. Plots were maintained throughout the season using standard fertility, irrigation, and crop protection practices recommended for commercial tomato production in Florida (30). Twelve plants from the center of each plot were marked, and fruit was harvested from these plants two to three times during the trial at a mature green or early breaker stage typical of tomato production in Florida and graded based on United States Department of Agriculture grades (36).

Statistical analysis. In vitro, greenhouse, and field studies were set up in a randomized complete block design. The data was analyzed using analysis of variance. Mean comparisons were performed with SAS (version 9.1; SAS Institute Inc., Cary, NC).

RESULTS

Time dependence of photocatalytic activity of TiO_2, TiO_2/Zn, and TiO_2/Ag nanoparticles on *X. perforans*. TiO_2/Ag- and TiO_2/Zn-coated glass cover slips significantly reduced survival of *X. perforans* to <1 log and ≈3 log CFU/ml, respectively, within 10 min of photocatalysis compared with noncoated controls which had bacterial populations of 6 to 7 log CFU/ml (Fig. 1). Undoped TiO_2 nanoparticles did not have any effect in reducing bacterial populations at 10 min compared with the control. At 15 min, there was no bacterial survival on glass cover slips coated with TiO_2/Ag. A bacterial population of <1 log CFU/ml was recovered from TiO_2/Zn glass cover slips. At this duration of photocatalysis, the bacterial population on glass slides coated with TiO_2 dropped to ≈3.5 log CFU/ml compared with ≈7 log CFU/ml on noncoated control. At 20 min, there was no bacterial survival on glass cover slips coated with TiO_2/Ag and TiO_2/Zn. At this duration of photocatalysis, a bacterial population of ≈1 log CFU/ml was recovered from TiO_2-coated glass cover slips. There was no significant reduction in the number of bacterial cells on noncoated glass cover slips in illuminated condition compared with coated and noncoated glass cover slips under nonilluminated condition at any time period throughout the experiment.

Dose dependency of illumination on photocatalytic activity of TiO_2, TiO_2/Zn, and TiO_2/Ag nanoparticles on *X. perforans*. TiO_2/Ag-coated glass cover slips significantly reduced survival of *X. perforans* to ≈3.5 log CFU/ml compared with 6 to 7 log CFU/ml on TiO_2/Zn, TiO_2, and noncoated control when exposed to a light intensity of 3×10^2 lux (Fig. 2). At a higher light intensity of 1.2×10^3 lux, TiO_2/Ag-coated cover slips had the

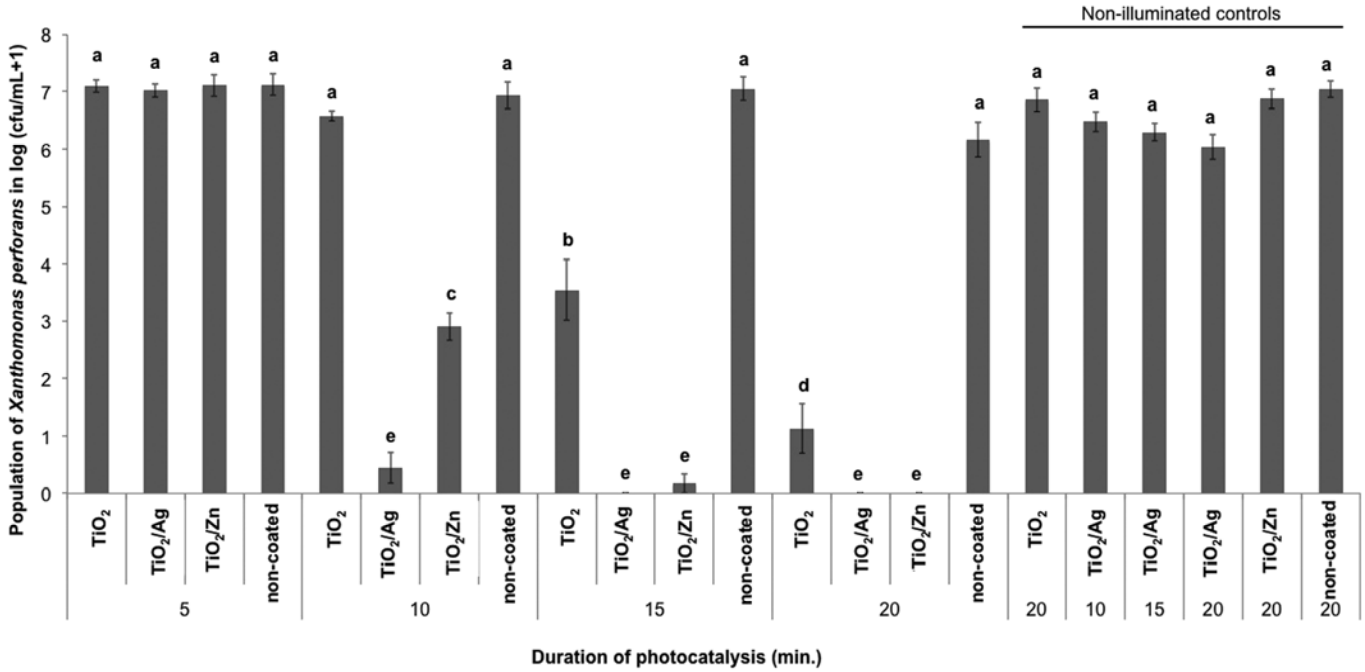

Fig. 1. Photocatalytic activity of titanium dioxide (TiO_2), TiO_2 doped with silver (TiO_2/Ag), and TiO_2 doped with zinc (TiO_2/Zn) nanoparticles on *Xanthomonas perforans* race T4 strain Xp-F7. Bacteria on nanoparticle-coated glass cover slips were exposed to visible light illumination for 5, 10, 15, and 20 min at 4°C. Illumination was carried out at a light density of 3×10^4 lumen/m^2 (lux). "Noncoated" represents glass cover slips not coated with the nanoparticles. "Nonilluminated" represents experiments conducted without illumination. For all the experiments under each illumination condition, the percentages of the surviving bacteria were determined by a standard plating method. Each treatment had three replicates, and the entire experiment was performed twice. Means followed by the same letter are not significantly different at $P \leq 0.05$ based on Student Newman Keuls' test. The error bar represents the standard error of mean.

lowest bacterial survival at ≈3 log CFU/ml, followed by TiO_2/Zn with a significantly higher survival of ≈5 log CFU/ml. There was no reduction in bacterial survival on TiO_2 and the noncoated control. There was no bacterial growth on glass slides coated with TiO_2/Ag when exposed to a light intensity of 3×10^4 lux. Bacterial survival on TiO_2/Zn-coated glass cover slips was at <1 log CFU/ml and significantly lower than that on TiO_2-coated glass cover slips at ≈3.5 log CFU/ml.

Effect of TiO_2, TiO_2/Zn, and TiO_2/Ag nanoparticles on bacterial spot lesions in the preliminary greenhouse study with plants naturally infected with *X. perforans*. Treatment with all three nanoparticle formulations significantly reduced bacterial spot lesions compared with the untreated control (Fig. 3). There was no statistical difference in the number of bacterial spot lesions between the nondiluted and 1:10 dilution (≈500 to 800 ppm) of each treatment. Doping of TiO_2 with Ag improved bactericidal activity, as indicated by the significant reduction in bacterial spot lesions in TiO_2/Ag compared with TiO_2. Although the TiO_2/Ag treatment resulted in the highest reduction in bacterial spot lesions, it was not statistically different from TiO_2/Zn. Similarly, TiO_2 and TiO_2/Zn did not exhibit a statistically significant difference in this experiment, although TiO_2/Zn was numerically better than TiO_2 in the study. Plants coated with nondiluted and 1:10 dilutions of TiO_2/Ag, TiO_2/Zn, and TiO_2 nano formulations had ≈80, ≈65, and ≈50% reduction, respectively, in the average number of bacterial spot lesions on plants compared with the untreated control.

Effect of TiO_2/Zn nanoparticles on bacterial spot disease severity in greenhouse studies. The TiO_2/Zn formulation was chosen for further studies because the Environmental Protection Agency (EPA) classifies zinc as a minimum-risk pesticide, making it exempt from registration and, thus, a more practical option for agricultural use than silver. In two independent greenhouse trials and one growth-chamber study, TiO_2/Zn at all tested levels significantly reduced bacterial spot disease severity compared with the untreated control (Table 1). Plants treated with the most concentrated TiO_2/Zn preparation (1:10 dilution) had the lowest bacterial spot severity, and control was not significantly different from the industry standard copper + mancozeb treatment in all three experiments. Although active at all application rates, less disease protection was observed on plants treated with more dilute concentrations of TiO_2/Zn.

Efficacy of TiO_2/Zn nanoparticle on bacterial spot disease severity in field trials. In the fall 2010 field trial conducted at Quincy, the application of TiO_2/Zn at 1:10 dilution caused a significant reduction of bacterial spot disease incidence compared with copper, copper + mancozeb, and the untreated control, as indicated by the AUDPC data (Table 2). This protection declined as the concentration of applied TiO_2/Zn was reduced. In this experiment, the tomato fruit yield for the TiO_2/Zn treatments of 1:10 dilution (67,191 kg/ha), 1:20 dilution (63,394 kg/ha), 1:40 dilution (60,620 kg/ha), and 1:60 dilution (65,478 kg/ha) was greater than copper (53,392 kg/ha), copper + mancozeb (57,859 kg/ha), and the untreated control (54,364 kg/ha); however, the differences were not statistically significant. None of the TiO_2/Zn treatments had any adverse effect on tomato fruit yield in this trial.

In the spring 2011 field trial conducted at Quincy, the application of TiO_2/Zn at 1:10 dilution caused a significant reduction of bacterial spot disease incidence compared with copper and the untreated control, as indicated by the AUDPC data (Table 3). There was no statistical difference between the bacterial spot incidence of TiO_2/Zn at 1:10 dilution and copper + mancozeb control. The activity of TiO_2/Zn declined with increased dilution rates. Tomato yield data from this field experiment was not collected due to a hailstorm that caused extensive damage to the crop 1 week prior to harvest.

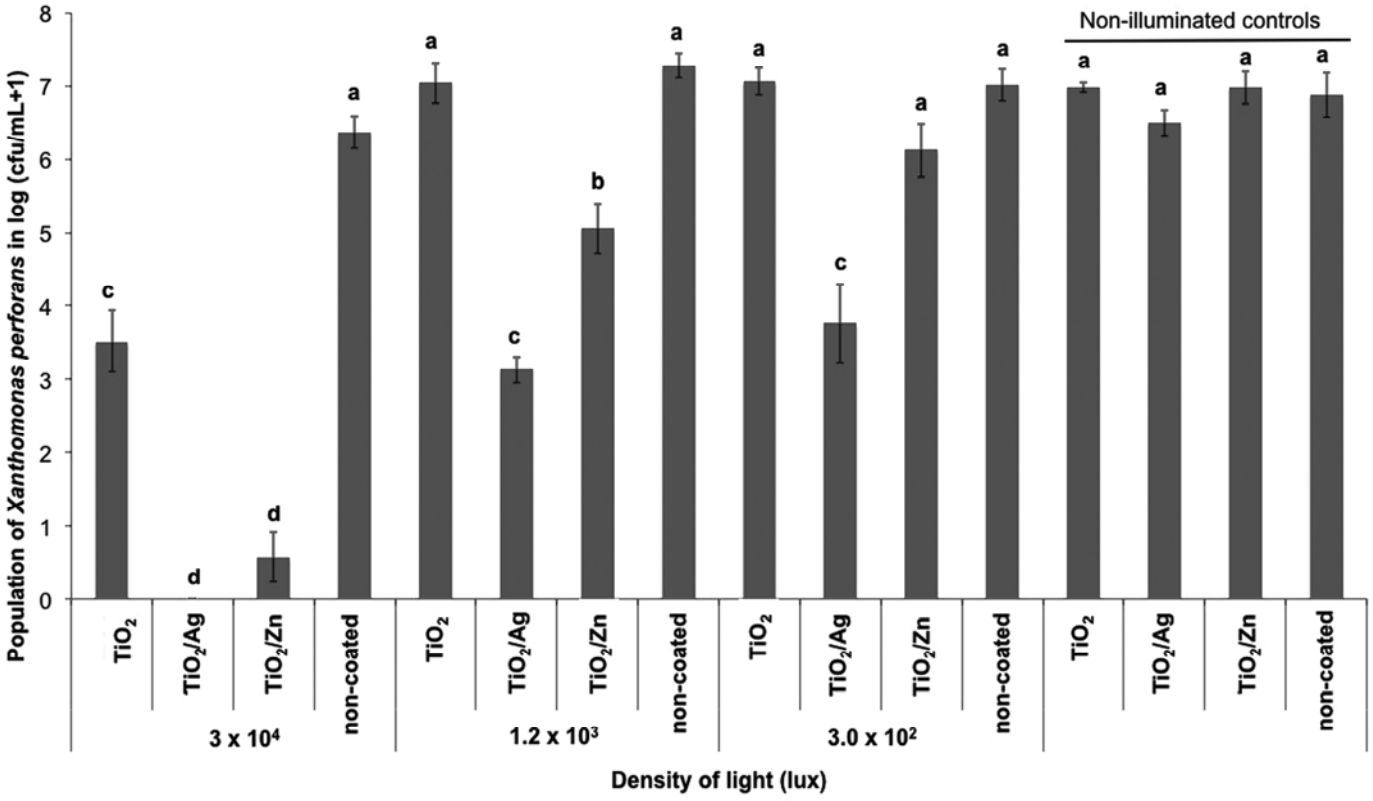

Fig. 2. Dose dependence of illumination on bactericidal action on *Xanthomonas perforans* race T4 strain Xp-F7. Bacteria on nanoparticle-coated glass cover slips were exposed to visible-light illumination at light densities of 3×10^4, 1.2×10^3, and 3×10^2 lumen/m^2 (lux) for 20 min at 4°C. "Noncoated", represents glass cover slips not coated with the nanoparticles. Each treatment had three replicates, and the entire experiment was performed twice. Means followed by the same letter are not significantly different at $P \leq 0.05$ based on Student Newman Keuls' test. The error bar represents the standard error of mean.

In the fall 2011 field trial conducted at Quincy, the application of TiO_2/Zn at 1:10 dilution caused a significant reduction of bacterial spot disease incidence compared with copper and the untreated control, as indicated by the AUDPC data (Table 4). There was no statistical difference between the bacterial spot incidence of TiO_2/Zn at 1:10 dilution and copper + mancozeb control. TiO_2/Zn at 1:20 and 1:40 dilutions had statistically similar bacterial spot incidence compared with TiO_2/Zn at 1:10 dilution but lesser bacterial spot incidence compared with the untreated control. TiO_2/Zn treatments did not have any adverse effect on tomato fruit yield.

In the fall 2011 field trial conducted at Wimauma, there was no significant difference in bacterial spot incidence between the treatments (Table 5). There were no adverse effects on tomato fruit yield from the application of TiO_2/Zn.

DISCUSSION

Bacterial spot of tomato is both a commercially important disease and also a valuable experimental system to evaluate the potential of bactericides in agriculture. Crop diseases that arise from epiphytic bacteria have a large negative impact worldwide, and their prevention with the ecologically friendly technology of photocatalysis is a new approach in plant disease management. Although this potential was suggested by a report published while our work was in progress using a macro-particulate form of TiO_2 in ornamental crops (27), the macro-particulate form of TiO_2 requires spraying a large quantity of the material. Numerous experiments conducted by our group have shown that doped versions of TiO_2 nanoparticles exhibit significant improvements in photocatalysis compared with macro forms (data not shown) and undoped nanoparticles. In our studies, TiO_2/Zn has been highly effective at low rates and, hence, ideal for field application.

Importantly, the disease protection observed for TiO_2/Zn in our initial investigations in bacterial spot of tomato were, in every case, at least comparable with those provided by conventional copper + mancozeb-based treatments. TiO_2/Zn at 1:10 dilution (≈500 to 800 ppm) did not cause any adverse effects on yield, as observed in all the field trials. Although there was significant reduction in disease incidence as shown by the AUDPC data, this did not result in a statistically significant increase in yield in these studies. However, increases in yield are seldom observed in bacterial spot trials of this scale (17,22,37). An area for our current focus is also to analyze the cost-effectiveness of using

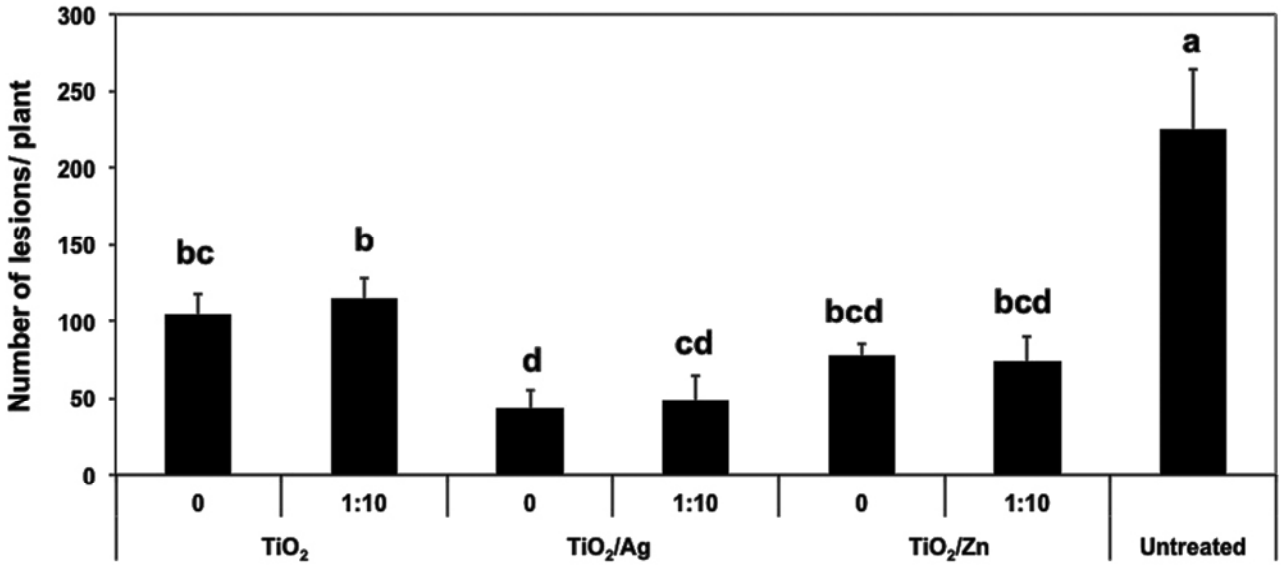

Fig. 3. Bacterial spot lesions after treatment with nanoparticles (titanium dioxide (TiO_2), TiO_2 doped with silver (TiO_2/Ag), and TiO_2 doped with zinc (TiO_2/Zn). Formulation 1 at 0 dilution (TiO_2 at 5,000 to 8,000 ppm and Zn at 25 to 50 ppm nanoparticle) and 1:10 dilution on a naturally infected seed lot of tomato 'BHN 602'. Infected plants were treated with nanoparticles at the three- to four-leaf stage and bacterial spot lesions were recorded 14 days after treatment. Three plants were tested for each treatment and the trial was set up in a randomized complete block design. Means followed by the same letter are not significantly different at $P \leq 0.05$ based on least significant difference. The error bar represents the standard error of mean.

TABLE 1. Mean bacterial spot disease severity on tomato 'FL 47' after treatment with nanoparticle titanium dioxide doped with zinc (TiO_2/Zn)

Treatment	Dilution[x]	Disease severity[y]		
		Trial 1	Trial 2	Trial 3[z]
TiO_2/Zn	1:10	5.6 c	7.3 c	18.1 cd
	1:20	12.0 bc	17.9 b	30.6 bc
	1:40	24.4 b	22.1 b	35.6 b
Copper (Kocide 3000; 2.1 g/liter)		57.9 a	22.1 b	NT
Copper (Kocide 3000; 2.1 g/liter) + Mancozeb (Penncozeb 75DF; 1.2 g/liter)	…	6.8 c	6.2 c	13.1 d
Untreated	…	64.1 a	46.9 a	63.8 a
LSD	…	13.4	9.1	14.9
$P > F$	…	<0.0001	0.0003	<0.0001

[x] Plants at the two- to three-leaf stage were treated with diluted nanoparticles 3 h before inoculation with *Xanthomonas perforans* race T4 strain Xp-8 isolated from tomato in Quincy. Experiments were conducted in greenhouse conditions.

[y] Eight plants were tested for each treatment and the trial was set up in a randomized complete block design. Disease severity was rated 2 weeks after inoculation using a nondimensional 12-point scale, to assess percentage of canopy affected by bacterial leaf spot (16). Values were converted to mid-percentages before analysis. Means followed by the same letter are not significantly different at $P \leq 0.05$ based on least significant difference (LSD). NT = not tested.

[z] Plants at the two- to three-leaf stage were treated with diluted nanoparticles three hours before inoculation with *X. perforans* race T4, strain GCREC T4 isolated from tomato in Wimauma, FL. Experiments were conducted in growth-chamber conditions.

nanoparticle formulations for field application. Studies are in progress to evaluate the effect of TiO_2/Zn at 1:10 dilution spray at 2-week intervals (a total of four sprays) in reducing disease incidence and increasing yield. In addition, field studies of an integrated package involving TiO_2/Zn with an SAR inducer are in progress. The cost-effectiveness of the nanoparticle formulation will be based upon the data generated form the trials on final dosage rate and number of applications required for effective disease control.

The strains used in the vitro and greenhouse studies were race T4 strains, which are the predominant strains of *X. perforans* present in Florida. None of the varieties currently available commercially, two of which have been used in this study, have resistance to bacterial spot race T4 strains. However, from a host cultivar perspective, nanoparticle adhesion to leaf surfaces on different cultivars may vary depending upon the number and abundance of leaf hairs. It may be a factor in the variability of effectiveness on hosts BHN 602 and SecuriTy 28 observed in this study. In addition, stickers or spreaders were not used; thus, it is possible that the material may be washed off from the leaf surfaces upon rain events. Our planned studies for 2013 include addition of spreaders and stickers to TiO_2/Zn and testing them on different cultivars for improved efficacy in reducing bacterial spot severity.

The work we report here illustrates the potential of photocatalytic materials to reduce the impact of bacterial spot on tomato. It is well established that the interaction of energetic photons with an appropriate mineral catalyst creates chemically reactive oxygen species that destroy organic molecules, damaging microbes proximal to such photocatalytic materials sufficient to reduce their viability. As indicated here by our results, photocatalysis leads to *X. perforans* cell death on surfaces, leading to reduced bacterial spot incidence.

The extent of microbial killing varies as a function of the target organism, the intensity of illumination, the efficiency of photocatalysis, and the duration of exposure. Unlike many other biocides, this effect is quite durable due to the catalytic nature of the material and the good adherence of TiO_2 to most surfaces; thus, longer intervals between applications may be feasible. We also hypothesize that the nonspecific nature of this oxidative mechanism may result in a slow rate of selection of bacterial resistance compared with agents designed to inhibit a particular biochemical pathway. Numerous studies on the mechanism of action of TiO_2 identified three possible modes of action, including (i) direct oxidation of coenzyme A, which inhibits cell respiration leading to cell death; (ii) change in cell permeability; and (iii) cell wall damage followed by cytoplasmic membrane damage (18,24).

Plant cells might also be expected to be susceptible to this chemical effect. We theorize that the physical size of plant cells and, in particular, the thickness of their cell walls may exceed the lethal radius of diffusion of the free radicals generated by TiO_2, affording selectivity compared with their pathogens. *X. perforans* has a cell wall thickness of ≈10 nm and overall cellular dimensions of roughly 500 by 1,500 nm. In contrast, a typical plant epidermal cell wall thickness is 100 to 200 nm, with overall dimensions ≈10 to 100 µm. The median radius of diffusion of hydroxyl radical in an intracellular environment was estimated to be 6 to 9 nm (32). Thus, the hydroxyl radicals formed from nanoscale TiO_2 not only will damage the outer membrane of a gram-negative organism but also have an appreciable chance of penetrating into the bacterial cytoplasm; in contrast, they are much less likely to penetrate the 10-fold thicker plant cell wall and cuticle. However, in our studies, we noticed partial leaf phytotoxicity (≈2 to 3% leaf area) in the fall 2010 trial in Quincy starting after the

TABLE 3. Effect of titanium dioxide doped with zinc (TiO_2/Zn) on the incidence of bacterial spot on tomato 'BHN 602', shown as average area under the disease progress curve (AUDPC) in a field trial conducted during spring 2011 in Quincy, FL

Treatment, dilution[y]	AUDPC[z]
TiO_2/Zn	
1:10	583.6 d
1:20	741.1 abc
1:40	724.5 bc
1:60	752.5 abc
1:80	757.8 abc
1:100	806.8 ab
Copper (Kocide 3000; 1.75 lb/A)	864.5 ab
Copper (Kocide 3000; 1.75 lb/A) + Mancozeb (Penncozeb 75DF; 1 lb/A)	642.3 cd
Untreated	892.9 a
LSD	107.5
P > *F*	<0.0001

[y] Represents the diluted formulation of TiO_2/Zn.

[z] Disease severities were rated using the Horsfall-Barratt scale, a nondimensional 12-point scale, to assess the percentage of canopy affected by bacterial spot. Values were converted to mid-percentages and used to generate AUDPC. Column means indicated with the same letters are not significantly different ($P \leq 0.05$) based on least significant difference (LSD). Yield data could not be taken due to significant hail damage on tomato plants 1 week before the first harvest date.

TABLE 2. Effect of titanium dioxide doped with zinc (TiO_2/Zn) on the incidence of bacterial spot on tomato BHN 602, shown as average area under the disease progress curve (AUDPC), and the yield of tomato[w]

	Yield (kg/ha)				
Treatment, dilution[x]	Medium	Large	Extra large	Total[y]	AUDPC[z]
TiO_2/Zn					
1:10	10,047 a	18,581 a	38,563 a	67,191 a	800.6 c
1:20	8,702 a	16,070 ab	38,622 a	63,394 a	950.3 bc
1:40	9,248 a	18,779 a	32,594 a	60,620 a	1,000.1 b
1:60	8,397 a	16,980 ab	40,102 a	65,478 a	1,033.4 ab
1:80	6,393 a	16,392 ab	32,446 a	55,231 a	933.6 bc
1:100	8,457 a	18,319 a	35,091 a	61,867 a	1,050.0 ab
Copper (Kocide 3000; 1.75 lb/A)	7,744 a	13,642 b	32,006 a	53,392 a	1,050.0 ab
Copper (Kocide 3000; 1.75 lb/A) + Mancozeb (Penncozeb 75DF; 1 lb/A)	7,653 a	14,549 b	35,656 a	57,859 a	1,033.4 ab
Untreated	6,312 a	15,293 ab	32,670 a	54,364 a	1,181.3 a
LSD	ns	3,336	ns	ns	145.5
P > *F*	0.4286	0.0343	0.6950	0.4379	0.0019

[w] The field trial was conducted during fall 2010 in Quincy, FL.

[x] Represents the diluted formulation of TiO_2/Zn.

[y] Total marketable.

[z] Disease severities were rated using the Horsfall-Barratt scale, a nondimensional 12-point scale, to assess the percentage of canopy affected by bacterial spot. Values were converted to mid-percentages and used to generate AUDPC. Column means indicated with the same letters are not significantly different ($P \leq 0.05$) based on least significant difference (LSD); ns = not significant.

eighth spray, and ≈5 to 10% leaf area in the fall 2011 trial in Wimauma starting after the sixth spray of TiO_2/Zn formulation at 1:10 dilution. This is likely due to the deposition of too much photocatalytic material on single points on the surface of the plants as a result of multiple applications using a conventional sprayer. Our most recent study (data not shown) on using an electrostatic sprayer for spraying the nanoparticles resulted in no phytotoxicity after the eighth spray of TiO_2/Zn formulation at 1:10 dilution compared with a phytotoxicity of ≈5% in plots sprayed using a conventional sprayer. We hypothesize that, with the electrostatic sprayer, the particles are evenly distributed on the plant surface, thereby limiting the chances of phytotoxicity compared with accumulation of the nanoparticles at single points on plant surfaces when a conventional sprayer is used. Further studies are in progress to evaluate the threshold for maximum application of TiO_2/Zn using small- and commercial-scale electrostatic sprayers.

TiO_2 works as a photocatalyst by absorbing radiation from sunlight or other light sources (fluorescent or incandescent lamps), leading to the production of pairs of conductive electrons and positively charged "holes" (4). The process starts with the excitation of an electron in the valence band of TiO_2 by an energetic photon present in light. The excess energy of this excited electron promotes the electron from the valence band to the conduction band of TiO_2, therefore creating the negative-electron (e^-) and positive-hole (h^+) pair, both of which are mobile within the crystal structure. If the e^- or h^+ occur at the surface of the crystal, they may interact with environmentally abundant molecules such as H_2O or O_2. The h^+ breaks apart a water molecule to form hydrogen gas and hydroxyl radical. The e^- reacts with an oxygen molecule to form superoxide anion. These reactive chemical species then destroy organic material in their diffusion range. This cycle continues so long as sufficiently energetic light is available, leading to antibacterial effects.

The energy difference between the valence band and the conduction band is known as the "band gap". The wavelength of the light necessary for photo-excitation of TiO_2 is given by the equation 1,240 (Planck's constant, h)/3.2 eV (band gap energy) = 388 nm.

Thus, for pure TiO_2, the photocatalytic effect requires photons with energies corresponding to the UV range <388 nm. The energy density of sunlight is low in the UV compared with other wavelengths due to atmospheric absorption characteristics. Importantly, the introduction of small amounts of other elements as dopants into the TiO_2 crystal structure can cause a decrease in the photon energy required to span the band gap for photocatalysis, providing access at 400 to 450 nm, which is more abundant than shorter wavelengths in most light sources. We observed results consistent with this in that the undoped TiO_2 had inferior antibacterial properties compared with the doped TiO_2 materials. Although many dopants have been reported, our results show for the first time that Zn, an ecologically friendly dopant, provides this benefit against *X. perforans*. The observed biological superiority of the doped TiO_2 formulations likely flows from superior photocatalytic activity, which arises from an improved capacity to capture useful energy from sunlight. We measured the absorbance spectra for TiO_2 and TiO_2/Zn by diffuse reflectance spectroscopy of dried material, which demonstrated that TiO_2/Zn exhibits a clear shift in its absorbance spectrum to longer wavelengths (Fig. 4). This is important because a significant increase in solar energy is available in the region of 400 to 450 nm, a region that makes only a minor contribution to photosynthesis. Thus, TiO_2/Zn is ≈25% more efficient than undoped TiO_2 in har-

TABLE 4. Effect of titanium dioxide doped with zinc (TiO_2/Zn) on the incidence of bacterial spot on tomato 'BHN 602', shown as average area under the disease progress curve (AUDPC), and the yield of tomato in a field trial conducted during fall 2011 in Quincy, FL

	Yield (kg/ha)[x]				
Treatment, dilution[w]	Medium	Large	Extra large	Total[y]	AUDPC[z]
TiO_2/Zn					
1:10	4,518 a	7,512 a	15,251 a	27,281 a	995.8 c
1:20	4,966 a	6,994 a	11,200 a	23,161 a	1,073.6 bc
1:40	4,210 a	7,900 a	17,409 a	29,519 a	1,089.4 bc
Copper (Kocide 3000; 1.75 lb/A)	4,532 a	6,986 a	11,258 a	22,776 a	1,306.4 ab
Copper (Kocide 3000; 1.75 lb/A) + Mancozeb (Penncozeb 75DF; 1 lb/A)	3,909 a	8,202 a	14,933 a	27,044 a	816.4 c
Untreated	5,449 a	7,093	15,159 a	27,700 a	1,410.5 a
LSD	ns	ns	ns	ns	277.3
P > *F*	0.7194	0.9652	0.4146	0.7845	0.0037

[w] Represents the diluted formulation of TiO_2/Zn.
[x] Column means indicated with the same letters are not significantly different ($P \leq 0.05$) based on least significant difference (LSD); ns = not significant.
[y] Total marketable.
[z] Disease severities were rated using the Horsfall-Barratt scale, a nondimensional 12-point scale, to assess the percentage of canopy affected by bacterial spot. Values were converted to mid-percentages and used to generate AUDPC.

TABLE 5. Effect of titanium dioxide doped with zinc (TiO_2/Zn) on the incidence of bacterial spot on tomato 'SecuriTy 28', shown as average area under the disease progress curve (AUDPC), and the yield of tomato in a field trial conducted during fall 2011 in Wimauma, FL

	Yield (kg/ha)[x]				
Treatment, dilution[w]	Medium	Large	Extra large	Total[y]	AUDPC[z]
TiO_2/Zn					
1:10	4,394 a	4,717 a	11,113 ab	20,223 a	754.3 a
1:20	4,264 a	4,652 a	11,049 ab	19,965 a	688.9 a
1:40	4,652 a	4,943 a	11,824 a	21,419 a	795.1 a
Copper (Kocide 3000; 1.75 lb/A) + Mancozeb (Penncozeb 75DF; 1 lb/A)	4,329 a	4,781 a	10,015 b	19,125 a	743.8 a
Untreated	4,135 a	5,104 a	11,889 a	21,128 a	733.3 a
LSD	ns	ns	1,636.3	ns	ns
P > *F*	0.8476	0.9281	0.1192	0.2914	0.6846

[w] Represents the diluted formulation of TiO_2/Zn.
[x] Column means indicated with the same letters are not significantly different ($P \leq 0.05$) based on least significant difference (LSD); ns = not significant.
[y] Total marketable.
[z] Disease severities were rated using the Horsfall-Barratt scale, a nondimensional 12-point scale, to assess the percentage of canopy affected by bacterial spot. Values were converted to mid-percentages and used to generate AUDPC.

vesting energy in a region where the solar energy flux nearly doubles compared with the region <400 nm.

Both TiO_2 and Zn have lower ecological and toxicological risks at the application rates investigated here than copper-based bactericides in normal use. TiO_2 occurs naturally in soils and in highly purified form in many commercial products over decades, and is classified as nontoxic (9,14). TiO_2 particles agglomerate at the moderate ionic strengths that exist in soils (33), reducing the large surface areas at which photocatalysis takes place. Also, once TiO_2 enters soils, it is shielded from light and, thus, photocatalytically inert. The advent of nanoparticles for commercial use in recent years has triggered many studies on the fate of TiO_2 nanoparticles in the environment, summarized in several comprehensive reviews (11–13). However, formal studies of the safety of nanoparticles for use on vegetable crops are currently lacking, due to the novelty of this approach. Consideration of the formal regulatory status of nanoscale materials as a group generally is ongoing, although the U.S. EPA indicated its intent to continue to not regard particle size as a key distinguishing characteristic under the Toxic Substances Control Act for entities having the same molecular structure (10,11). The U.S. EPA classifies Zn as a minimal-risk pesticide and it is exempt from registration. Zn does have biologic activity but the risks from this agent are relatively low. The total Zn arising from the eight applications in the field experiments described above is in the very low 10s of grams per acre. This is less than the amount recommended as a foliar treatment for Zn deficiency, and far less than the application rates of more toxic copper, which are typically in the range of 0.8 to 1.9 kg of metallic copper equivalent per acre for eight applications.

A potential limitation for using TiO_2/Zn is the lack of antibacterial activity in the absence of light. The amount of Zn in TiO_2/Zn is 25 to 50 ppm and its role in the formulation is for improving the absorbance to longer wavelengths (Fig. 4). The level of Zn in this formulation is extremely low and unlikely to cause any antibacterial effect (Fig. 1, non-illuminated control with TiO_2/Zn caused no reduction in bacterial viability). Further, in the greenhouse and field experiments, we used a 1:10 dilution of TiO_2/Zn, which contains Zn at ≈2.5 to 5 ppm, which is an even lower level. The role of the Zn in the TiO_2/Zn formulation is limited to improving the photocatalytic activity of TiO_2. A material labeled on tomato for bacterial spot suppression is mancozeb, which includes Zn as an active ingredient. Further studies will be conducted with new nanoparticles, with TiO_2 with a higher level of Zn than the currently available formulation of TiO_2/Zn, and tests of whether there is any direct antibacterial activity in the absence of light.

To the best of our knowledge, this is the first study evaluating photocatalytic nanoscale formulations of TiO_2, TiO_2/Ag, and TiO_2/Zn for bacterial disease management on tomato across a wide range of testing platforms, including in vitro, greenhouse, and field studies. The results are promising, and encourage additional work to optimize the use of nanoscale photocatalysts in plant disease management, yield improvement, and field applications.

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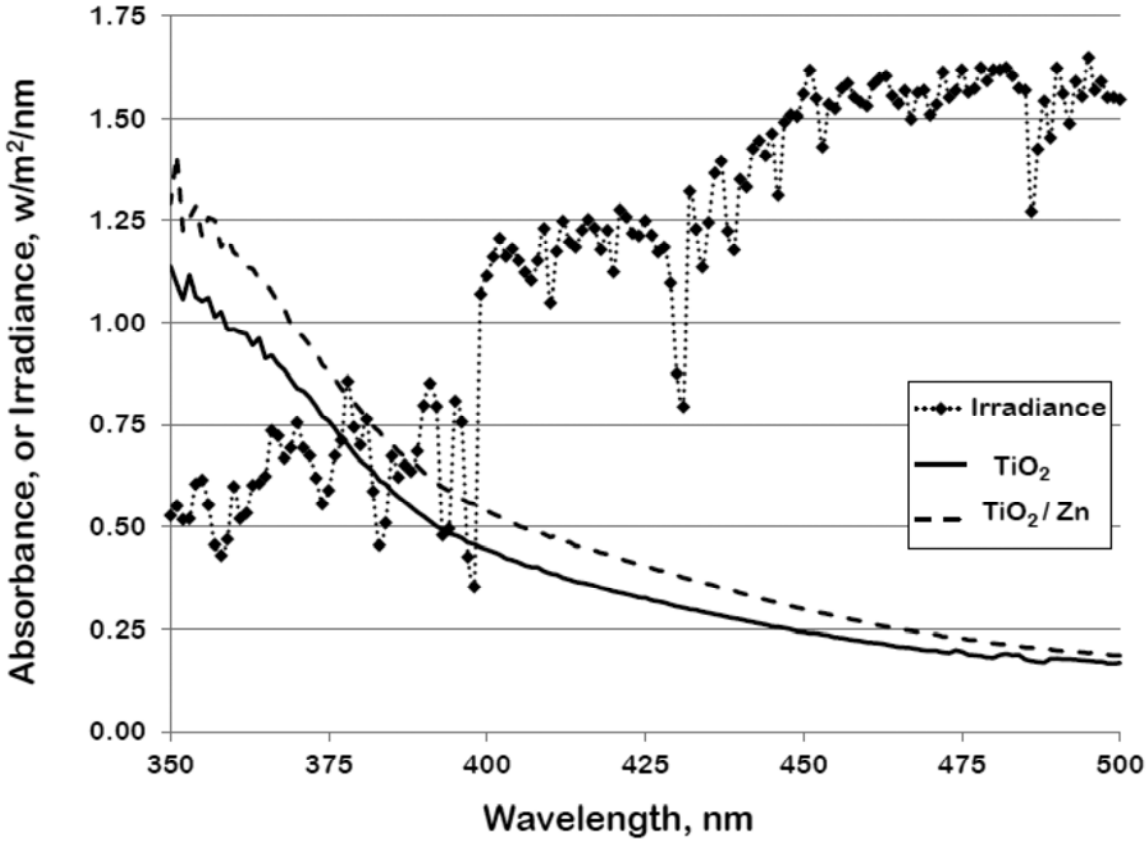

Fig. 4. Diffuse reflectance spectroscopy (DRS) of undoped titanium dioxide (TiO_2) and TiO_2 doped with Zn (TiO_2/Zn), overlaid with the solar energy density spectrum. The corresponding aqueous nanomaterial preparations used in the biological studies were dried by lyophilization to produce powders. Their absorbance spectra were then measured using a scanning spectrophotometer (Cary 500 version 8.01; software version 2.0) equipped with a DRS accessory in double-beam mode with reduced slit height and baseline correction. The solar energy density spectrum was obtained from the U.S. National Bureau of Standards and describes the radiant energy at the indicated wavelengths from hemispherical sources, thus including direct and scattered light, impinging on a plane with a 37° tilt, representing reasonable cloudless conditions at the earth's surface for the average latitude of the United States (ASTM G173-03). Solid line: undoped TiO_2; dashed line: TiO_2/Zn; dotted line with data points: irradiance.

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