Potential of Nano-Formulated Zinc Oxide for Control of Citrus Canker on Grapefruit Trees

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Abstract

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Antimicrobial activity of experimental formulations of two structurally different nano-zinc oxide materials, plate-like Zinkicide SG4 and particulate Zinkicide SG6, was evaluated against *Xanthomonas citri* subsp. *citri*, the cause of citrus canker. In vitro assay demonstrated Zinkicide SG4 had a twofold lower minimum inhibitory concentration (MIC) against *Escherichia coli* and *X. alfalfae* subsp. *citrumelonis* (62.5 to 250 µg/ml) compared with copper sulfate (250 µg/ml), copper hydroxide (250 to 500 µg/ml), or cuprous oxide/zinc oxide (125 to 250 µg/ml). Zinkicide SG6 had a sevenfold to eightfold lower MIC against *Escherichia coli* and *X. alfalfae* subsp. *citrumelonis* (31 to 250 µg/ml). Leaves of sweet orange (*Citrus sinensis*) and fruit of 'Ruby Red' grapefruit (*C. paradisi*) were evaluated for citrus canker disease control. A greenhouse assay with foliage

demonstrated that spray treatment with Zinkicide reduced citrus canker lesion development after injection-infiltration of *X. citri* subsp. *citri* into the leaf intercellular space. In field trials conducted in Southeast Florida in 2014 and 2015, Zinkicide SG4 and SG6 reduction of grapefruit canker incidence exceeded that of cuprous oxide and cuprous oxide/zinc oxide bactericides. Zinkicide formulations were also effective against the fungal diseases, citrus scab (*Elsinoe fawcetti*) and melanose (*Diaporthe citri*), on grapefruit. No sign of phytotoxicity to the fruit rind was observed during either season. Antimicrobial activity of Zinkicide for protection of leaves and fruit against *X. citri* subsp. *citri* was comparable or exceeded that for commercial copper and zinc oxide formulations which may be attributed to translaminal movement of Zinkicide.

In Florida, Asiatic citrus canker, caused by the bacterial pathogen *Xanthomonas citri* subsp. *citri* (syn. *X. axonopodis* pv. *citri*), is a serious disease of commercial citrus cultivars. Grapefruit (*Citrus paradisi* Macf.), the most important fresh fruit citrus grown, and early-midseason oranges (*C. sinensis* (L.) Osb) that are processed for juice, develop necrotic, erumpent lesions on leaves, stems, and fruits that induce a range of symptoms including defoliation, premature fruit drop, twig dieback, and general tree decline (Graham et al. 2004).

Canker suppression on susceptible citrus cultivars is challenging when the crop is grown in wet tropical or subtropical areas (Behlau et al. 2010; Leite and Mohan 1990; Stein et al. 2007; Timmer and Zitko 1996). Control with copper bactericides is reduced due to wind-blown rain that drives bacteria directly into stomata, bypassing the protective copper film on the plant surface (Bock et al. 2010; Graham and Myers 2013). Recently in Florida, windbreaks have been deployed at the perimeter in 5 to 10 ha blocks of the most susceptible grapefruit cultivars to minimize the wind-driven inoculum (Bock et al. 2010; Graham and Myers 2013). Even with windbreaks, frequent re-applications of copper are still required to protect fruit tissue that is continuously expanding over 90 to 120 days of development depending on the citrus cultivar (Behlau et al. 2010; Dewdney et al. 2012; Stall et al. 1982; Stein et al. 2007). Aggressive foliar applications of copper to control epidemic citrus canker began after the Florida state-federal eradication program ended in 2006 (Dewdney and Graham 2016). The amount of metallic copper used and the frequency of spray applications per season can depend upon weather conditions and the desired period of protection (Dewdney and Graham 2016). Although the copper spray program in Florida has yet to be fully optimized, about 0.54 to 1.12 kg of metallic copper per ha

is applied every 3 weeks to protect spring flush leaves and fruit surfaces.

Prolonged use of copper fungicides has led to the accumulation of copper in citrus soils with potential adverse environmental effects (Alva et al. 1995). Excessive copper use causes phytotoxicity in the fruit peel (Dewdney and Graham 2016; Graham et al. 2010) and accumulates to toxic levels in citrus roots (Alva et al. 1995). In addition, excessive dependence on copper bactericides has resulted in reduced disease control due to copper resistance in several phytopathogenic bacteria, including *X. citri* subsp. *citri* and *X. alfalfae* subsp. *citrumelonis* (Behlau et al. 2013). Potential alternatives such as antibiotics (Behlau et al. 2012) and systemic acquired resistance (SAR) inducing neonicotinoids (Graham and Myers 2013) are less attractive, as these compounds have potential nontarget impacts (Graham and Vallad 2011). Hence, there is compelling need for a robust, environmentally acceptable bactericide especially for control of copper resistant bacterial pathogens.

Zinc oxide (ZnO) is an inorganic oxide of zinc that exhibits exceptional optical, electrical, photocatalytic, and magnetic properties (Wang 2004). Zinc oxide is used in a wide range of industrial applications including ceramics, lubricants, pharmaceuticals, animal feed, and rubber manufacturing (Djurisic et al. 2012; Gomez and Tigli 2013; Kolodziejczak-Radzimska and Jesionowski 2014; Moezzi et al. 2012). The U.S. Food and Drug Administration lists zinc oxide as a "generally recognized as safe" (GRAS) (U.S. Food and Drug Administration 2015) for use as a food additive (Espitia et al. 2012). Zinc oxide nano-formulations have been used in personal care products including sunscreens (Keller et al. 2014; Morabito et al. 2011).

In agriculture, zinc oxide is primarily used as a fertilizer source but zinc has antimicrobial properties as well (Dizaj et al. 2014). The agricultural biocide market is dominated by copper formulations due to the higher antimicrobial potency of copper compared with zinc ions. Zinc oxide dissolution rate is directly proportional to the particle's surface area, i.e., the smaller the particle, the higher the zinc ion release rate (David et al. 2012). Antimicrobial activity of nano-zinc oxide may also be related to zinc induction of reactive oxygen species (Sawai et al. 1998).

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The purpose of this study was to evaluate the antimicrobial activity of experimental formulations of two structurally different nano-zinc oxide materials, plate-like Zinkicide SG4 and particulate Zinkicide SG6, for control of citrus canker in comparison with cuprous oxide (Cu₂O) and zinc oxide bactericides registered for citrus disease control.

Materials and Methods

Bacterial culture and inoculum preparation. *X. citri* subsp. *citri* strain X2004-0056 used in this study was isolated in 2004 from sweet orange in Dade County, Florida. The strain was stored in glycerol at -80°C. To produce inoculum, *X. citri* subsp. *citri* was grown overnight in nutrient broth, the suspension centrifuged at 10,000 × g for 20 min and the pellet resuspended in sterile phosphate buffer saline (PBS; 40 mM Na₂HPO₄ + 25 mM KH₂PO₄). The bacterial suspension was adjusted to 0.1 OD (at 620 nm), equivalent to 10⁸ colony forming units (CFU) per ml and diluted with PBS to 10⁴ CFU per ml for inoculation trials (see below).

Properties of Zinkicide nano-formulations. Two structurally different forms of Zinkicide, SG4 and SG6, were formulated at the NanoScience Technology Center at the University of Central Florida. Zinkicide SG4 has a two-dimensional plate-like structure in the 0.2 to 0.5 µm range with a thickness of ~10.0 nm. Zinkicide SG6 has a gel-like structure consisting of interconnecting particles in the 4 to 6 nm range. Zinkicide was synthesized in a controlled chemical environment using a soluble zinc source and other ingredients (including inerts) to control the materials' structure (Santra and Berroth 2015). Both Zinkicide SG4 and SG6 were formulated in de-ionized water adjusted to pH 7.5.

Assay of Zinkicide antimicrobial activity. The antimicrobial activity of Zinkicide SG4 and SG6 formulations was compared with reagent grade copper sulfate (CQ Concepts, Ringwood, IL) and two bactericides registered for citrus disease control, copper hydroxide

(Kocide 3000; DuPont, Wilmington, DE) and cuprous oxide/zinc oxide (Nordox 30/30; Brandt USA, Springfield, IL) (Table 1). Bacterial assay strains were *X. alfalfae* subsp. *citrumelonis* strain F1 isolated in 1984 from *Citrus* sp. located in a citrus nursery in Polk County, Florida and *E. coli* (ATCC 10536).

A minimum inhibitory concentration (MIC) assay for a range of concentrations (31 to 500 µg/ml) for each antimicrobial was conducted according to the broth microdilution method following the protocol from the Clinical and Laboratory Standards Institute (CLSI 2012). Briefly, 100 µl of nutrient broth for *X. alfalfae* subsp. *citrumelonis* and Luria Bertani (LB) broth for *E. coli* was pipetted into a set of wells in a microtiter plate. Then 100 µl of a 4,000 µg/ml stock for each test compound was added to the second set of wells and serially diluted across the set of wells to concentrations of 2,000, 1,000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 1.96 µg/ml. The first column of wells was used as growth and sterility checks. A 10-µl aliquot of 5 × 10⁶ CFU/ml bacteria suspension was added to each well except for the sterility check, resulting in a bacterial concentration of ~5 × 10⁵ CFU/ml at the start of incubation. Each bacterial species was tested on separate microtiter plates and incubated at 37°C for *E. coli* and 26°C for *X. alfalfae* subsp. *citrumelonis*. Each test compound was tested in triplicate. The accuracy of the MIC determination was improved by adding 10 µl of resazurin dye (0.0125%, wt/vol) per 100 µl well volume and observing color changes (blue to pink for live cells). This metabolic staining step reduced error associated with the turbidity in the broth caused by the colloidal Zinkicide suspension. Quantification of activity against the bacterial strains for each antimicrobial compound was also determined by a CFU assay. The same range of concentrations (31 to 500 µg/ml) for each compound in the MIC assay was tested for the CFU assay. Samples were serially diluted in PBS and select dilutions (10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷) were spread on LB agar for *E. coli* assay or spread on nutrient agar for *X. alfalfae* subsp. *citrumelonis* assay. Dilutions were plated by placing a 10-µl aliquot on the agar surface, allowing the drop to air dry in a biosafety cabinet and then inverting the plate. After 24 h incubation at 37°C for *E. coli* or 48 h incubation at 26°C for *X. alfalfae* subsp. *citrumelonis*, individual colonies were counted and expressed as log CFU/ml. The CFU assay was performed in triplicate.

Greenhouse evaluation of Zinkicide. Activity against *X. citri* subsp. *citri* of Zinkicide SG6 was compared with the locally systemic antibiotic, streptomycin sulfate (Firewall 17WP; Agrosources, Inc., Tequesta, FL), and the SAR inducer, acibenzolar-*S*-methyl (Actigard 50WP; Syngenta Crop Protection, Greensboro, NC). Seeds of 'Pineapple' sweet orange (*C. sinensis*) were sown in 150-cm³ containers (Stuewe & Sons Inc., Corvallis, OR) containing Metro Mix 500 (Hummert International, Earth City, MO) soilless medium and seedlings were fertilized weekly with 50 ml of Jack's Pro 20-20-20 (N-P-K liquid) (A.M. Leonard, Inc., Piqua, OH). The 9-month-old seedlings

Table 1. Minimal inhibitory concentration (MIC) of antimicrobial compounds against selected gram negative bacteria^z

Antimicrobial compound	MIC (µg/ml)	
	<i>Xanthomonas alfalfa</i> subsp. <i>citrumelonis</i>	<i>Escherichia coli</i>
Zinkicide SG6	62.5	31
Zinkicide SG4	62.5–125	125–250
Copper sulfate	250	250
Copper hydroxide	250–500	250–500
Cuprous oxide/zinc oxide	125–250	125–250

^z MIC is defined as the lowest concentration of an antimicrobial compound that inhibits the visible growth of a microorganism after overnight incubation in an in vitro assay. MIC values are based on three replicate assays.

Table 2. Rates of zinc and copper formulations tested on 6- and 7-year-old 'Ray Ruby' grapefruit trees at Vero Beach, FL in 2014 and 2015^z

Trial year Treatment – rate	Cuprous oxide (%)	Zinc oxide (%)	Rate of cuprous oxide or zinc oxide (kg/ha)	Metallic copper or zinc (kg/ha)
2014				
Untreated check	–	–	–	–
Cuprous oxide	75	–	1.49	1.12
Cuprous oxide/zinc oxide – 0.56	30	30	1.49	0.56
Zinkicide SG4 – 0.56	–	30	1.86	0.56
Zinkicide SG6 – 0.56	–	30	1.86	0.56
2015				
Untreated check	–	–	–	–
Cuprous oxide	75	–	1.49	1.12
Cuprous oxide/zinc oxide – 0.56	30	30	1.49	0.56
Cuprous oxide/zinc oxide – 0.28	30	30	0.93	0.28
Zinkicide SG4 – 0.56	–	30	1.86	0.56
Zinkicide SG6 – 0.56	–	30	1.86	0.56
Zinkicide SG4 – 0.28	–	30	0.93	0.28
Zinkicide SG6 – 0.28	–	30	0.93	0.28

^z Source/supplier of the formulations are provided in Materials and Methods section.

were transplanted into 4,000 cm³ containers and supplemented with 10 g of controlled release granular fertilizer (18-5-10; Harrell's, Lakeland, FL). Seedlings of uniform vigor and stem caliper (approximately 1 cm diameter) were cut back to allow two dominant shoots to develop. When three to four leaves on each shoot reached three-fourths to full expansion, the entire plant was sprayed with test compounds using an airbrush (Crown Spra-Tool, Aervoe Industries, Inc., Gardnerville, NV) at 10 ml per seedling. Run-off into the soilless medium was minimized to avoid root exposure of test compounds. Two days after treatment, five seedlings per treatment were inoculated with 10⁴ CFU/ml of *X. citri* subsp. *citri* strain X2002-0014 prepared as described above. The suspension of *X. citri* subsp. *citri* in PBS was injection-infiltrated in three areas on each side of the midrib to produce six distinct inoculation sites per leaf on three to four leaves per replicate seedling for a total of 15 inoculated leaves per treatment. Seedlings were randomly located on a bench in an air conditioned greenhouse with day/night temperatures of 28 and 25°C, respectively.

Inoculated shoots were covered with plastic bags for 1 day to maintain high humidity conducive for bacterial infection. At 14 days after inoculation, lesions were counted at each inoculation site and summed as total lesions per leaf. The greenhouse assay was performed twice in 2014. In trial 1, seedlings were inoculated on 18 June 2014 and lesions counted on 9 July 2014; in trial 2, seedlings were inoculated on 29 September 2014 and lesions counted on 13 October 2014.

Field evaluation of Zinkicide. Antimicrobial activity of Zinkicide SG4 and SG6 formulations was compared with cuprous oxide (Nordox 75WG; Brandt USA) and cuprous oxide/zinc oxide (Nordox 30/30; Brandt USA) (Table 2). In 2014, an orchard of 6-year-old bearing 'Ruby Red' grapefruit trees was located in Vero Beach, Indian River County, Florida (27°33'59.59"N, 80°35'06.02"W). The block was 4.5 ha in area with a perimeter windbreak of *Corymbia torelliana* approximately 6 m in height. Citrus tree spacing was 3.65 m × 7.62 m (360 trees per ha). The experiment was a completely randomized design with five plots of five trees per plot for each treatment. Treatment materials were mixed with well-water and applied as foliar spray at 3.0 liters per tree (1,078.6 liters per ha) with a handgun sprayer at 1,380 kPa of air pressure. Treatments were initiated after the spring foliar flush in April 2014. Ten foliar sprays were applied in 2014 on 1 April, 22 April, 12 May, 2 June, 23 June, 14 July, 4 August, 25 August, 15 September, and 6 October. To protect fruits, sprays were initiated after spring flush and before the period of maximum fruit susceptibility in late April to early May. All trees received a rotation of foliar sprays of insecticides (abamectin, fenpropathrin, and dimethoate) coincident with leaf flush production for control of citrus leafminer (*Phyllocnistis citrella*) to minimize leaf damage and the interaction of damage with canker incidence and severity (Stein et al. 2007).

In 2015, the trial was conducted with the same 7-year-old trees. The treatments were similar to those in 2014 with additional rates of the treatment materials (Table 2). Ten foliar sprays were applied in 2015 on 24 March, 14 April, 5 May, 26 May, 16 June, 7 July, 28 July, 18 August, 8 September, and 28 September. Otherwise, application methods were the same as in 2014.

Monthly rainfall in 2014 and 2015 was recorded at the Indian River, Florida Agricultural Weather Network (FAWN, <http://fawn.ifas.ufl.edu/>) weather station near Vero Beach and compared with the average from 2000 to 2010 at the FAWN weather station at the University of Florida, Indian River Research and Education Center at Ft. Pierce (Table 3).

Table 3. Monthly rainfall at Indian River Florida Agricultural Weather Network (FAWN) station^z

Month/rainfall (mm)	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.
Trial year								
2014	61	56	76	231	172	147	292	38
2015	41	124	19	239	193	152	254	51
Ft. Pierce average	64	58	84	168	173	165	150	97

^z Average monthly rainfall from 2000 to 2010 obtained from FAWN at University of Florida, Indian River Research and Education Center.

On 20 October 2014 and 2 November 2015, incidence of fruit with canker lesions and the age of the lesions were assessed on 100 fruit collected arbitrarily from the middle three trees in each five tree plot. Lesions were classified as old if they were greater than 0.6 cm in diameter, coalescing with surrounding lesions, black, exuding gum, or had a prominent yellow halo; and young if lesions were less than 0.6 cm in diameter, brown, and not coalescing with surrounding lesions. A fruit with both lesion ages present was considered to have old lesions for calculation of the incidence of lesioned fruit. Incidence of phytotoxicity and lesions of citrus scab (*Elsinoe fawcetti*) and melanose (*Diaporthe citri*) were visually identified and assessed for the same set of fruit.

Statistical analysis. For each experiment, data were subjected to analysis of variance in PROC GLM (SAS Institute, Cary, NC). Means were separated using Student-Newman-Keuls multiple range test at $\alpha = 0.05$.

Results

Zinkicide antimicrobial activity. In vitro assay demonstrated Zinkicide SG4 had a twofold lower MIC against *E. coli* (MIC 62.5 µg/ml) and *X. alfalfae* subsp. *citrumelonis* (MIC 250 µg/ml) compared with copper sulfate (MIC 250 µg/ml) and bactericides containing copper hydroxide (MIC 250 to 500 µg/ml) or cuprous oxide/zinc oxide (MIC 125 to 250 µg/ml) (Table 1; Fig. 1). Zinkicide SG6 had a sevenfold to eightfold lower MIC against *E. coli* (MIC 31 µg/ml) and *X. alfalfae* subsp. *citrumelonis* (MIC 62.5 µg/ml) compared with the same bactericides (Table 1; Fig. 1).

Fig. 1. Colony forming unit (CFU) assay of antimicrobial compounds against **A**, *Escherichia coli* and **B**, *Xanthomonas alfalfa* subsp. *citrumelonis*. Kanamycin (50 µg/ml) was included as antimicrobial check. Values are the mean of three replicate assays. Bars with different letters are significantly different according to Student-Newman-Keuls multiple range test at $\alpha = 0.05$.

Greenhouse evaluation of Zinkicide. In trials 1 and 2, Zinkicide SG-6, sprayed to runoff at a concentration equivalent to a field spray of zinc at 0.56 kg/ha, reduced the number of canker lesions on Pineapple orange leaves by 38 and 42%, respectively (Fig. 2). Reduction in lesion number by acibenzolar-S-methyl was 63 and 68% and by streptomycin sulfate was 21 and 9% in trials 1 and 2, respectively. Compared with the untreated check, all treatments in both trials with the exception of streptomycin sulfate in trial 2 significantly reduced incidence of canker lesions.

Field evaluation of Zinkicide. Incidence of old lesions (i.e., fruit infections before July) was higher than young lesions probably due to above average rainfall in June (Tables 3 and 4). When the total incidence of fruit with canker lesions in the untreated check (63%) was compared with cuprous oxide at the rate of 1.12 kg of copper per ha per application the incidence on fruit was significantly reduced to 21%. Cuprous oxide/zinc oxide applied at 0.56 kg of copper per ha and 0.56 kg of zinc per ha reduced disease incidence from 63 to 25%. The two Zinkicide formulations applied at the same zinc concentration as cuprous oxide/zinc oxide reduced fruit canker incidence from 63% to 7.0 and 9.0%, respectively, which was significantly lower than cuprous oxide and cuprous oxide/zinc oxide. Signs of spray phytotoxicity were not observed on the fruit rind in any of the treatments. Fruit in the untreated check had 18 and 9% incidence of citrus scab and melanose, respectively (Table 5). The incidence was significantly reduced by the cuprous oxide and cuprous oxide/zinc oxide bactericides and by both Zinkicide formulations. Zinkicide SG6 was slightly more active than Zinkicide SG4 against citrus canker, scab, and melanose.

Incidence of young lesions (i.e., fruit infections after July) was higher than old lesions (Tables 3 and 4). Compared with the untreated check with 60% fruit canker incidence, cuprous oxide at 1.12 kg of copper significantly reduced incidence to 29% (Table 4). Zinkicide SG4 at 0.28 kg of zinc per ha and Zinkicide SG6 at 0.56 kg of zinc per ha reduced fruit canker incidence to 14 and 17%, respectively, significantly lower than the treatments with cuprous oxide at 1.12 kg of copper per ha and the cuprous oxide/zinc oxide at 0.28 kg of copper and zinc per ha. Zinkicide SG6 at 0.28 kg of zinc per ha resulted in a fruit canker incidence of 24%, significantly lower compared with the cuprous oxide treatment. Copper toxicity or other signs of phytotoxicity on the

fruit rind were not observed in any of the treatments. The incidence of citrus scab and melanose on the untreated check was 25 and 12%, respectively (Table 5). Disease incidence was significantly reduced by all the standard and experimental copper and zinc materials. Cuprous oxide and cuprous oxide/zinc oxide resulted in the lowest incidence of melanose, which was matched by the Zinkicide SG6 formulation at 0.56 kg of zinc per ha.

Discussion

Two structurally different nano-zinc oxide formulations, Zinkicide SG4 and SG6, were evaluated for efficacy against citrus canker on

Table 4. Effect of foliar applications at 21-day intervals for cuprous (Cu) oxide, cuprous oxide/zinc (Zn) oxide, and Zinkicide formulations on incidence of citrus canker on fruit of 6- and 7-year-old 'Ray Ruby' grapefruit trees at Vero Beach, FL in October 2014 and 2015^z

Trial year Treatment – rate	Incidence of old lesions (%)	Incidence of young lesions (%)	Total incidence (%)
2014			
Untreated check	45 a	18 a	63 a
Cu oxide – 1.12	17 b	4.4 b	21 b
Cu oxide/Zn oxide – 0.56	16 b	8.8 b	25 b
Zinkicide SG4 – 0.56	3.0 c	6.2 b	9.2 c
Zinkicide SG6 – 0.56	4.6 c	2.4 b	7.0 c
2015			
Untreated check	23 a	37 a	60 a
Cu oxide – 1.12	10 bc	20 b	29 b
Cu oxide/Zn oxide – 0.56	8.2 bcd	13 cd	21 cd
Cu oxide/Zn oxide – 0.28	9.0 bd	14 c	23 c
Zinkicide SG4 – 0.56	6.2 bd	11 cd	17 de
Zinkicide SG6 – 0.56	5.6 d	10 cd	16 e
Zinkicide SG4 – 0.28	5.2 d	8.4 c	14 e
Zinkicide SG6 – 0.28	11 b	13 cd	24 c

^z Source/supplier of the formulations are provided in the Materials and Methods section. Means ($n = 5$) within each trial year and column followed by unlike letters are significantly different at $\alpha = 0.05$ according to Student-Newman-Keuls multiple range test.

Fig. 2. Greenhouse evaluation of foliar application of Zinkicide SG6 (applied at 4.14 mg per plant) compared with streptomycin sulfate (SS, Firewall 17WP, 17% streptomycin) applied at 18 mg per plant, and the systemic acquired resistance inducer, acibenzolar-S-methyl (ASM, Actigard 50WP, 50% ASM) applied at 400 μ g per plant, sprayed 2 days before injection-infiltration with *Xanthomonas citri* subsp. *citri* at 1×10^4 CFU/ml into leaves of 'Pineapple' sweet orange. Bars represent the mean of the percent reduction of canker lesion formation at 14 days after inoculation compared with the untreated check. Within each trial, bars with different letters are significantly different according to Student-Newman-Keuls multiple range test at $\alpha = 0.05$.

highly susceptible grapefruit. In field trials in 2014 and 2015, control of citrus canker by Zinkicide SG4 and SG6 exceeded that of cuprous oxide and cuprous oxide/zinc oxide bactericides registered for control in Florida citrus. In the 2014 season, both formulations of Zinkicide achieved the lowest fruit disease incidence recorded for grapefruit in Florida trials conducted since 2008 (Graham et al. 2010). Zinkicide formulations were also effective against the fungal diseases citrus scab and melanose indicating that nano-formulated Zinkicide has broad spectrum antimicrobial activity. No sign of phytotoxicity was observed throughout the season, which suggests that release of free zinc ions from nano-zinc oxide at neutral pH conditions was limited (Zhang et al. 2015).

In vitro assay demonstrated that Zinkicide SG4 and SG6 had two-fold to eightfold lower MIC against *Escherichia coli* and *X. alfalfae* subsp. *citrumelonis* compared with copper formulations. The greenhouse assay of sweet orange leaves established that Zinkicide SG6 apparently moved translaminal in that it reduced lesion development after injection-infiltration of *X. citri* subsp. *citri* into the leaf intercellular space. By comparison, streptomycin sulfate that has local systemic activity against bacterial pathogen infection (Crosse and Garrett 1958), was somewhat effective against *X. citri* subsp. *citri*. Acibenzolar-S-methyl, a SAR inducer with fully systemic activity against *X. citri* subsp. *citri* (Francis et al. 2009) was more effective than Zinkicide SG6. Effectiveness of Zinkicide SG6 in leaves and fruit as an antimicrobial is probably associated with translaminal movement.

Regarding potential concerns about the fate of nano-particle zinc, Zinkicide is designed to be metabolized by the plant. A comparison of zinc oxide-nanoparticles and soluble zinc chloride and cowpea growing in soil detected no differences in plant growth and accumulation or speciation of zinc in planta or soil (Wang et al. 2013). Similarly, root uptake and toxicity of zinc from zinc oxide-nanoparticles and soluble zinc from soil to cowpea indicated no specific risk of the zinc nanoparticles.

Due to the hydrophilic properties of Zinkicide SG4 and SG6, the formulations mix readily with water facilitating spray application. In the Zinkicide formulation process, metallic zinc loading can be as high as 50,000 mg/liter and remain fairly stable for at least one year in storage without loss of efficacy (S. Santra, unpublished data). These attributes are considered favorable for formulating a commercially viable bactericide for plant disease control.

The detailed mechanism of Zinkicide mode of action is yet to be fully understood. Previous reports on zinc oxide nanoparticles

suggest that there is a good correlation between antimicrobial efficacy and surface area of the material (Raghupathi et al. 2011; Xie et al. 2011). Greater efficacy of Zinkicide could be therefore linked to the increased bioavailability of metallic zinc due to high surface area of nano-size oxide material. However, the possibility that Zinkicide releases reactive oxygen species locally due to their surface defects at the nanoscale cannot be ruled out (Sirelkhatim et al. 2015). We have not seen any major difference in efficacy of two nano-formulations of Zinkicide, suggesting that material structure has minimal impact on antimicrobial activity. In future studies, we plan to elucidate the mode of action of Zinkicide.

In conclusion, Zinkicide controlled bacterial canker and fungal diseases on fruit and foliage as well or better than copper and copper/zinc formulations. Formulation of nano-zinc oxide as a bactericide/fungicide has at least three major advantages: (i) low toxicity compared with copper, (ii) high surface area and antimicrobial activity, and (iii) ability to move translaminal. As a replacement for copper bactericides, Zinkicide could prevent further loading of copper into soils and provide a rotation product that reduces the risk for development of bacterial resistance to copper.

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Table 5. Effect of foliar applications at 21-day intervals for cuprous oxide, cuprous (Cu) oxide/zinc (Zn) oxide, and Zinkicide formulations on incidence of citrus scab and melanose on fruit of 6- and 7-year-old 'Ray Ruby' grapefruit trees at Vero Beach, FL in October 2014 and 2015^z

Trial year Treatment – rate	Scab incidence (%)	Melanose incidence (%)
2014		
Untreated check	18 a	9.0 a
Cu oxide – 1.12	3.0 b	1.8 b
Cu oxide/Zn oxide – 0.56	4.0 b	2.4 b
Zinkicide SG4 – 0.56	2.2 b	0.6 b
Zinkicide SG6 – 0.56	1.2 b	0.4 b
2015		
Untreated check	25 a	12 a
Cu oxide – 1.12	2.4 b	1.0 b
Cu oxide/Zn oxide – 0.56	2.4 b	1.6 b
Cu oxide/Zn oxide – 0.28	2.2 b	1.0 b
Zinkicide SG4 – 0.56	5.4 b	4.6 b
Zinkicide SG6 – 0.56	4.2 b	1.8 b
Zinkicide SG4 – 0.28	3.2 b	2.2 b
Zinkicide SG6 – 0.28	4.2 b	2.6 b

^z Source/supplier of the formulations are provided in the Materials and Methods section. Means ($n = 5$) within each trial year and column followed by unlike letters are significantly different at $\alpha = 0.05$ according to Student-Newman-Keuls multiple range test.

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