Foliar-Applied Small Molecule that Suppresses Biofilm Formation and Enhances Control of Copper-Resistant *Xanthomonas euvesicatoria* on Pepper

R. J. Worthington, S. A. Rogers, R. W. Huigens, III, and C. Melander, Department of Chemistry, and D. F. Ritchie, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695

Abstract

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We report a small molecule additive, a member of the 2-aminoimidazole (2AI) group that is an analogue of the marine sponge natural product oroidin that suppresses resistance of *Xanthomonas euvesicatoria* to copper and decreases biofilm formation in an in vitro system. In laboratory experiments, 2AI combined with copper reduced both bacterial multiplication in broth and bacterial recovery on pepper leaf discs of a copper-resistant strain of *X. euvesicatoria* to a level close to that of a copper-sensitive strain. Compound 2AI used alone exhibited minimal bactericidal activity. In 3 years of field experiments, when

combined with a copper-containing material, copper hydroxide (Kocide 3000), and other antibacterial materials, these spray mixtures resulted in decreased bacterial spot foliar disease and increased fruit yields using hybrid bell pepper (*Capsicum annuum*) cultivars and copper-resistant strains of *X. euvesicatoria*. This study demonstrates the concept for using small molecules as additives to antibacterial compounds at nonbactericidal concentrations under field conditions that, in the laboratory, were demonstrated to suppress bacterial biofilms and copper-resistant strains.

Many plants are affected by at least one major bacterial disease, causing the agricultural industry to lose hundreds of millions of dollars annually. Options for disease control and management strategies for bacterial diseases are very limited in situations in which host resistance is not effective. There are few chemicals that effectively control bacterial plant diseases (17). The most commonly used agricultural antibacterial chemicals are the many formulations of copper which are registered for use on many crops and have been used to control bacterial diseases of agricultural crops for more than 100 years (10). Although copper continues to be used to control many plant diseases, the widespread use of copper has resulted in the selection for and spread of copper-resistance genes among saprophytic and plant-pathogenic bacteria (1,4,5,10). Resistance to copper has been observed in several phytopathogenic bacterial species such as those of *Pseudomonas* and *Xanthomonas*, decreasing its efficiency (4,15,24). Tank mixing an ethylene bisdithiocarbamate fungicide such as mancozeb with a copper material enhances control of copper-resistant strains of *Xanthomonas* (15). This combination remains the current chemical management method but has limited disease-control efficacy, especially under favorable environmental conditions (26). Reduced sensitivity to copper can result from genetic mutations and from environmental factors such as pH that influence the ionic concentration and bacterial extracellular polysaccharides that can inhibit or bind the biologically active ions of copper (9,10). Given that there have been few if any new antibacterial compounds for controlling phytopathogenic bacteria introduced over the last several decades, there exists a need to develop novel strategies to combat copper-

resistant bacterial plant pathogens and to also enhance copper-resistance management.

Bacterial spot of pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) is caused by four xanthomonads: *Xanthomonas euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (11), and is an economically significant agricultural disease (19,26). Bacterial spot causes lesions on both leaves and fruit, leaf defoliation, reduction in plant growth, and a reduction in both fruit yield and quality. Bacterial spot management also is complicated because of a lack of durable host resistance in commercially acceptable cultivars (12). Thus, disease management has continued to rely on chemical sprays containing copper. A potential approach to deal with this situation is to develop compounds that strip bacteria of their resistance mechanisms, thereby allowing the reuse of conventional antibacterials that, thus far, have been reduced in effectiveness or rendered obsolete due to resistance.

We recently reported that a 2-aminoimidazole compound (2AI, MW = 387; Fig. 1), a derivative that is an analogue of the marine natural product oroidin, could suppress resistance of several drug-resistant strains of bacteria, including tetracycline-resistant *Escherichia coli*, methicillin-resistant *Staphylococcus aureus*, and carbapenem-resistant *Acinetobacter baumannii*, at concentrations at which 2AI compounds exhibited no bactericidal activity alone (28). These compounds also have been demonstrated to inhibit and disperse bacterial biofilms (29). Applying this strategy to the control of plant bacterial diseases, we aimed to provide a route for circumventing biocide resistance and allowing the reuse of typical control reagents such as copper. We chose to use pepper-bacterial spot caused by *X. euvesicatoria* as the disease system to test this approach because resistance to copper is a major problem in this production system. Preliminary data were presented in 2009 (25).

Corresponding author: D. F. Ritchie, E-mail: david_ritchie@ncsu.edu

Current address of S. A. Rogers: Department of Pharmaceutical Chemistry, University of Kansas, Lawrence 66045.

Current address of R. W. Huigens, III: American Cancer Society Postdoctoral Fellow, Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana 61801.

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Fig. 1. Structure of compound 2-aminoimidazole, MW = 387.

Materials and Methods

In vitro determination of copper minimum inhibitory concentrations against *X. euvesicatoria*. *X. euvesicatoria* strains Xcv 135 (copper sensitive, Cu^S) and Xcv 611 (copper resistant, Cu^R) were grown in a sucrose peptone broth (SPB; 2% [wt/vol] sucrose, 0.5% [wt/vol] peptone, 0.05% [wt/vol] K₂HPO₄, and 0.025% [wt/vol] MgSO₄) at 28°C with shaking for 6 h. These cultures were used to inoculate fresh SPB (5 × 10⁵ CFU/ml). The resulting bacterial suspension was aliquoted into culture tubes and CuSO₄ (Sigma-Aldrich), calculated as Cu(II) or 2AI in parts per million (micrograms per milliliter), was added at 2× the concentration to be tested. Inoculated media containing 2AI at decreasing concentrations was aliquoted (50 µl) into rows A to G of a 96-well microtiter plate, and inoculated media containing CuSO₄ at decreasing concentrations was aliquoted (50 µl) into columns 1 to 11 of the microtiter plate. All wells were mixed thoroughly. The plate was covered with a lid and incubated under stationary conditions at 28°C for 16 h. Minimum inhibitory concentration (MIC) values for each concentration of 2AI tested were recorded as the lowest concentration of copper Cu(II) in micrograms per milliliter at which no visible growth of bacteria was observed. All determinations were carried out in duplicate and repeated on at least two separate occasions.

Laboratory leaf disc assay. The effect of 2AI and copper on the cellular viability of *X. euvesicatoria* Xcv 135 Cu^S and Xcv 611 Cu^R was investigated using pepper leaf discs. Copper was obtained from Kocide 3000 at an equivalent rate of 162 g per 378 liters (Cu(II) at 128 µg/ml) or 2AI at 77.4 µg/ml [200 µM], and combinations of these compounds were individually prepared in 100 ml of tap water and misted to run-off onto individual leaves of pepper plants. Water-treated leaves served as a control. The spray deposit was allowed to dry for approximately 2 h. Using a 1.0-cm-diameter cork borer, two leaf discs were cut from each of three leaves on each treated plant. The leaf discs, adaxial side up, were placed on moist filter paper in 100-mm petri dishes. Bacteria from 48-h-old cultures on SPB with 1.5% agar (SPA) plates were used to prepare the bacterial inoculum for each strain by suspending bacteria in sterile water (SW) to approximately 5 × 10⁷ CFU/ml. From these bacterial suspensions, 25-µl aliquots were pipetted onto each leaf disc. After 24 h, each leaf disc was washed in SW and diluted in 10-fold series, and 10 µl of each dilution was spotted on SPA medium. Colonies that grew near the dilution end point were counted after 36 to 48 h of incubation at 28°C.

Evaluation of compound 2AI for potential biofilm suppression against *X. euvesicatoria*. A microtiter plate and crystal violet staining assay for detecting biofilm formation were used (28). Strain Xcv 611 Cu^R was grown in SPB on a shaker at 28°C for 15 h to a concentration of approximately 5 × 10⁸ CFU/ml. A 96-well polyvinyl chloride microtiter plate (BD Falcon; BD Biosciences) was prepared with 125 µl of fresh SPB per well. 2AI concentrations were prepared at 10× rates (1,000 and 500 µM) in SW from a stock of 100 mM solubilized in 1.0 ml of 95% EtOH. Aliquots of 15 µl each of the two concentrations of 2AI were pipetted into three wells containing 125 µl of fresh SPB. To each of these wells was added 10 µl of the 15-h-old Xcv 611 Cu^R culture. The control consisted of three wells of 125 µl of fresh SPB, 15 µl of SW, and 10 µl of Xcv 611 Cu^R culture. The microtiter plate was wrapped in plastic wrap and placed at stationary incubation at 28°C for 48 h. At the end of the incubation period, 10 µl was removed from each well, spotted at one side of a petri dish, and immediately streaked in a line toward the opposite side of the dish; then, plates were incubated at 28°C for 48 h. The remaining liquid was removed and, after drying, the wells were washed with 200 µl of SW and allowed to dry; then, 175 µl of 0.1% crystal violet was added. After 30 min, the crystal violet was removed and the wells visually observed from the side for presence and intensity of remaining crystal violet. The experiment was repeated twice.

To gain further evidence of the effect of 2AI on biofilm formation, glass cover slips, 95% ethanol sterilized and washed in SW

and dried, were incubated in SPB bacterial culture suspensions in sterile Costar 12-well polystyrene plates (Corning Incorporated). Treatments were as indicated for the 96-well microtiter plates, except that volumes were adjusted appropriately for the larger well so that half of the cover slip was submerged. The plate was incubated stationary for four days at 28°C. The cover slips were removed, gently rinsed by dipping in SW, air dried, placed in 0.1% crystal violet for 30 min, dipped in SW, air dried, and observed microscopically (×150) for bacterial attachment and aggregation.

Field efficacy experiments: plot design, cultivars, pathogen strains, inoculation, treatments, and disease assessments. Field experiments were conducted during the growing seasons of 2008, 2009, and 2010 to evaluate the efficacy of 2AI. Compound 2AI was solubilized in dimethyl sulfoxide when used in 2008 and in 95% ethanol in 2009 and 2010 before adding it to water, making the spray mixture. Field experiments were conducted using hybrid bell pepper 'X3R Camelot' (Seminis), which is resistant to pepper races 0 to 3. Because of the presence of *Tomato spotted wilt virus* (TSWV) on the research station where the experiments were conducted, in subsequent years cultivars having some level of TSWV resistance were used. These included 'Heritage VR' F1 hybrid (Harris Moran; has resistance to TSWV and pathogen races 1, 2, 3, and 5) and 'Excursion II F1' (Harris Seeds; intermediate resistance to TSWV and pathogen races 1, 2, and 3) in 2009 and 2010, respectively.

For all 3 years, *X. euvesicatoria* inoculum consisted of three strains previously isolated from diseased pepper from North Carolina fields: Xcv 376 (race 6, Cu^S), Xcv 611 (race 4, Cu^R), and Xcv 612 (race 6, Cu^R). Inoculum was prepared by growing the bacteria on plates (28°C) of SPA for 24 to 36 h, suspending in sterile, deionized water, adjusting concentration to approximately 5 × 10⁸ CFU/ml, assaying for final CFU/ml, and confirming pathogen race and copper resistance. Inoculum was transported to the field in three separate flasks in a cooler with ice for inoculation within 4 h of leaving the laboratory. At the time of inoculation, equal volumes of each strain were combined and Silwett L77 (silicone-polyether copolymer; Loveland Industries) at 0.030 ml per 100 ml (0.03%) was added. The Silwett L77 wets the pepper leaves and opens stomates, thus enhancing bacterial infection. This inoculation method consistently produces bacterial lesions in 5 to 7 days. Two plants in each plot were inoculated by dipping the top half of the plant in the inoculum suspension for 10 to 15 s. These plants served as the initial inoculum sources for the research plots. There is no natural inoculum at this research location.

The plot design used all 3 years was a randomized complete block. Transplants were grown in a greenhouse on the North Carolina State University campus and transplanted to raised beds 6 to 7 weeks after seeding. Row middles were 112 cm and space between plants within the row was 46 cm. In 2008, each treatment consisted of replications with two parallel rows of seven plants per row. Two plants adjacent the center plant in one of the rows were inoculated 6 May, 12 days after transplanting. The first spray treatment was applied approximately 4 h later. Subsequent sprays were applied 13, 20, and 27 May and 3, 10, 17, and 24 June. Treatments were applied using a backpack hand pump sprayer. The spray was directed at a 60° angle on both sides of each row of plants. Test materials were mixed in 1.5 liters of water and 350 ml was applied to each replication. This volume equates to approximately 926 liters/ha. In 2009, each plot consisted of 12 plants arranged in a single row, with five replications per treatment. The plants immediately adjacent to the end plant of each replication were inoculated 12 May as described previously. The first treatments were applied immediately following inoculation. Subsequent spray dates were 19 and 27 May and 2, 9, 16, and 19 June. A similar volume of water and application method was used in 2009 and 2010. In 2010, four 12-plant replications were used and, again, the two plants adjacent to the end plants of each replication were inoculated 11 May, 12 days after transplanting. Two days after inoculation, the first spray treatment was applied, with subsequent sprays applied on 20 and 27 May and 4, 15, and

24 June. In all 3 years, disease occurred on the inoculated plants within 7 days after inoculation.

Once symptoms were observed on the inoculated plants, disease ratings were recorded weekly until at least the first fruit harvest. Foliage of each plant was visually evaluated for symptoms and given a rating of 0 to 9 as previously described (30). Briefly, 0 = no disease observed, 1 = at least one lesion observed but <1% leaf area diseased, 3 = 11 to 20% leaf area diseased, 5 = 36 to 50% leaf area diseased, 7 = 66 to 80% leaf area diseased, and 9 = 100% diseased or defoliated. The individual plant ratings were used to calculate a plot means. These data were used to calculate the area under disease progress curves (3). At least two fruit harvests were done each year. Total fruit per replication was weighed. In 2008, disease ratings and fruit yields were recorded per plant because of the presence of TSWV-diseased plants, which were excluded from the data. Also, disease development and spread was slow because of dry weather; thus, the inoculated plants on which lesions developed were evaluated separately from the entire plot. Fruit yields in 2009 and 2010 were a total of each replication and means were calculated for each treatment. Data were statistically analyzed using analysis of variance and least significant difference procedures (SAS 9.1.2).

Results

In vitro suppression of *X. euvesicatoria* resistance to copper.

In the SPB medium, the copper-sensitive strain, Xcv 135 Cu^S, exhibited an MIC for Cu(II) of 50 µg/ml, whereas the copper-resistant strain, Xcv 611 Cu^R, exhibited an MIC for Cu(II) of 110 µg/ml under these conditions. Concurrently, the MIC was meas-

ured in the presence of increasing concentrations of 2AI (Table 1). The MIC of 2AI alone was 200 µM (77.4 µg/ml) for both strains. At a concentration of 12.5 µM (4.8 µg/ml), 2AI decreased the Cu(II) MIC against copper-resistant Xcv 611 Cu^R to 100 µg/ml, at 25 µM 2AI (9.7 µg/ml), the MIC of copper was 80 µg/ml; and at a concentration of 100 µM (38.7 µg/ml), 2AI reduced the sensitivity of the copper-resistant strain, Xcv 611 Cu^R, almost to the level of the copper-sensitive strain, Xcv 135 Cu^S, with an MIC of 60 µg/ml Cu (II).

Laboratory leaf disc assay. Treatment of the pepper leaves with 2AI alone did not result in a significant decrease in bacterial recovery for either strain compared with the control water treatment (Fig. 2). For leaf discs inoculated with the copper-sensitive strain, Xcv135 Cu^S, treatment with copper(II) (Kocide 3000) alone resulted in a 2.0 log reduction in the number of CFU compared with the water treatment, while treatment with a combination of 2AI and Kocide 3000 resulted in a 4.0 log reduction in the number of CFU compared with the water treatment. For leaf discs inoculated with the copper-resistant strain, Xcv 611 Cu^R, treatment with Kocide 3000 alone resulted in only 0.8 log reduction in the number of CFU compared with the water treatment, whereas the combination of 2AI and Kocide 3000 resulted in a 2.0 log reduction in the number of CFU.

Effect of 2AI on biofilm suppression for *X. euvesicatoria*.

Incubation of copper-resistant strain Xcv 611 Cu^R with 2AI at 50 and 100 µM decreased biofilm formation at the air-liquid interface on polyvinyl chloride surface of 96-well microtiter plate. Also, bacteria remained viable at these concentrations of 2AI because no difference in bacterial growth was observed when 10 µl was spotted and then streaked on SPA medium (Fig. 3). Microscopic observations (×150) of bacteria adhered to glass cover slips showed significant decreases in bacterial aggregation where 50 or 100 µM 2AI was included in the SPB compared with the control of SPB alone (Fig. 4).

Field experiments. Isolations from leaf lesions assayed near time of harvest confirmed the dominance of copper-resistant strain Xcv 611 Cu^R. Thus, bacterial leaf spot (BLS) control when copper was used alone was similar to the nontreated check, indicating the ineffectiveness of copper (Tables 2, 3, and 4). Significant differences among fruit yields were not always detected because BLS and weather conditions were not always favorable for infection at the time of fruit set.

In 2008, use of copper hydroxide (Kocide 3000), potassium phosphite (ProPhyt; Luxembourg-Pamol, Inc.), and 2AI alone pro-

Table 1. Cu(II) minimum inhibitory concentration (MIC) values against *Xanthomonas euvesicatoria* sensitive to copper (Xcv 135) or resistant to copper (Xcv 611) in the presence and absence of compound 2-aminoimidazole (2AI)

2AI concentration, µM (µg/ml)	Cu(II) MIC (µg/ml) ^z	
	Xcv 135	Xcv 611
100 (38.7)	10	60
50 (19.4)	20	70
25 (9.7)	30	80
12.5 (4.8)	40	100
6.25 (2.4)	40	110
0	50	110

^z MIC of compound 2AI alone was 200 µM for both strains.

Fig. 2. Bacterial recovery of copper sensitive (v135 CuS) and copper resistant (v611 CuR) strains after 24 h on pepper leaf discs cut from leaves of pepper plants sprayed with 2-aminoimidazole (2AI, compound 1), copper hydroxide [Cu(II) Kocide 3000], or a combination of 2AI (compound 1) and Kocide 3000.

vided BLS control similar to the nontreated check. The aminoglycoside antibiotic gentamicin (GWN 9350; Gowan Company), to which the bacteria were sensitive, alone provided significantly better disease control than the nontreated check (Table 2). Use of a tank-mixture of 2AI with copper hydroxide significantly improved BLS control for both the inoculated plants and for the entire plot. Adding 2AI to potassium phosphite significantly improved foliar disease control for inoculated plants but not for plot treatment means.

Bacterial spot control in 2009, using copper hydroxide (Kocide 3000) and 2AI at 50 and 100 μM alone, were not significantly different from the nontreated check (Table 3). Combining 2AI with copper hydroxide significantly improved BLS control. Although the aminoglycoside antibiotic kasugamycin (Kasumin 2L; Arysta LifeScience North America) provided disease control better than the nontreated check, combining it with 2AI significantly improved the BLS control (Table 3). Inclusion of the nonionic surfactant, Latron B-1956 (phthalic glycerol alkylid resin), improved BLS control with 2AI alone and in combination with copper hydroxide (Table 3).

In 2010, the use of copper hydroxide (Kocide 3000) and kasugamycin (Kasumin 2L) in combination with 2AI was again evaluated, as was the bio-fungicide and -bactericide, Regalia (5% extract of *Reynoutria sachalinensis*; Marrone Bio Innovations) in combination with copper hydroxide and with 2AI (Table 4). The

disease rating for plants treated with 2AI alone was comparable with that for plants treated with copper hydroxide alone. There was no significant concentration difference for copper hydroxide and 2AI used alone (Table 4). Combining 2AI with copper hydroxide provided significantly better BLS control than the nontreated check; for most combinations, better than the 0.84 kg/ha rate of copper hydroxide and comparable with the standard copper hydroxide combined with mancozeb. As in 2009, 2AI combined with kasugamycin significantly improved BLS control. There also was enhanced BLS control when 2AI was combined with the bio-fungicide and -bactericide, Regalia (Table 4). In none of the experiments was there any phytotoxicity or other injury to the pepper plants observed from spraying with the 2AI or any of the other materials evaluated.

Discussion

In laboratory experiments, 2AI combined with copper reduced both bacterial multiplication in broth and bacterial recovery on pepper leaf discs of a copper-resistant strain of *X. euvesicatoria* to a level close to that of a copper-sensitive strain. Complex media can significantly decrease the bactericidal activity of copper, which complicates the measurement of copper sensitivity (9). Yet, to allow for bacterial multiplication, such components are needed in media. Thus, the inhibitory concentrations of copper reported in Table 1 would not be expected to be the same as would occur in

Fig. 3. Biofilm formation at the air-liquid interface (arrows) after 48 h of incubation in sucrose peptone broth (SPB) for three replicated microtiter wells at 28°C. Stained with 0.1% crystal violet. Bacterial growth on SPB with 1.5% agar in petri dishes from 10- μl samples taken from each well, streaked across the agar surface.

Fig. 4. Effect of compound 1 on biofilm formation of *Xanthomonas euvesicatoria* strain Xcv 611 on glass cover slips incubated for 4 days and stained with 0.1% crystal violet. From left to right, no compound 2-aminoimidazole (2AI), compound 2AI at 50 μM , and compound 2AI at 100 μM . Magnification is $\times 150$.

Table 2. Foliar spray treatments, bacterial spot foliar area under disease progress curve (AUDPC), and fruit yields for 2008 field experiment^x

Treatments and rates ^y	Means for inoculated plants			Means per treatment		
	Foliar AUDPC ^z	Number of fruit/plant	Yield (kg)/plant	Foliar AUDPC ^z	Number of fruit/plant	Yield (kg)/plant
Nontreated check	162 a	0.00 d	0 c	45 a	6.1 ab	0.840
Compound 2AI (100 µM) 36.2 g	113 b	0.50 dc	0.057 bc	42 a	4.7 c	0.627
Copper hydroxide (Kocide 3000) 1.40 kg	123 b	0.44 dc	0.053 bc	37 ab	6.1 ab	0.886
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (100 µM) 36.2 g	62 c	1.75 abc	0.313 ab	16 c	8.2 ab	1.215
GWN-9350 (10% gentamicin) 3.92 kg + GWN-65 1.17 liters	74 c	2.11 ab	0.253 abc	15 c	8.7 a	1.171
GWN-9350 (10% gentamicin) 3.92 kg + GWN-65 1.17 liters + compound 2AI (100 µM) 36.2 g	41 c	2.69 a	0.397 a	12 c	7.4 abc	0.949
Potassium phosphite (ProPhyt 54.5%) 5.84 liters	114 b	1.13 bcd	0.139 abc	37 ab	6.7 abc	0.946
Potassium phosphite (ProPhyt 54.5%) 5.84 liters + compound 2AI (100 µM) 36.2 g	64 c	0.88 bcd	0.107 bc	25 bc	5.9 bc	0.974
Fisher's LSD, $\alpha = 0.05$	34	1.53	0.267	16	2.75	0.425
P value for F	<0.0001	0.020	0.057	0.0006	0.0466	0.483

^x Means within a column followed by the same letter do not differ significantly; Fisher's least significant difference (LSD), $\alpha = 0.05$.

^y Treatments and rates per hectare (based on water at 935 liters/ha). 2AI = 2-aminoimidazole.

^z Foliar 0-to-9 rating, with 0 = no disease, 1 = at least one diseased leaf, 2 = 1 to 5% foliage diseased or defoliated, 3 = 6 to 10%, 4 = 11 to 15%, 5 = 16 to 25%, 6 = 26 to 50%, 7 = 51 to 75%, 8 = 76 to 99%, and 9 = 100% leaves diseased or plant completely defoliated.

Table 3. Foliar spray treatments, bacterial spot foliar area under disease progress curve (AUDPC), final foliar disease rating, and fruit yield for 2009 field experiment^v

Treatments and rates per hectare	Mean AUDPC ^w	Final disease rating (0–9) ^w	Mean fruit yield (kg)
Copper hydroxide ^x			
Nontreated check	102 a	5.32 a	11
Copper hydroxide (Kocide 3000) 1.40 kg + mancozeb (Manzate Pro-Stik) 1.68 kg	53 c	2.68 d	12
Compound 2AI (50 µM) 18.1 g	96 a	5.00 ab	11
Compound 2AI (100 µM) 36.2 g	90 ab	4.90 ab	10
Copper hydroxide (Kocide 3000) 1.40 kg	87 ab	4.19 bc	11
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (50 µM) 18.1 g	64 c	3.24 d	11
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (100 µM) 36.2 g	71 bc	3.45 d	11
Fisher's LSD, $\alpha = 0.05$	21	0.60	NS
P value for F	0.0004	0.0001	0.782
Kasugamycin ^y			
Nontreated check	102 a	5.32 a	11
Copper hydroxide (Kocide 3000) 1.40 kg + mancozeb (Manzate Pro-Stik) 1.68 kg	53 d	2.68 d	12
Compound 2AI (100 µM) 36.2 g	90 b	4.90 b	10
Kasugamycin (Kasumin 2L) 4.67 liters	75 c	4.23 c	13
Kasugamycin (Kasumin 2L) 4.67 liters + compound 2AI (100 µM) 36.2 g	54 d	3.18 d	12
Fisher's LSD, $\alpha = 0.05$	14	0.60	NS
P value for F	<0.0001	<0.0001	0.134
Nonionic surfactant (Latron B-1956) ^z			
Nontreated check	102 a	5.32 a	11
Latron B-1956 0.146 liters	105 a	5.50 a	11
Compound 2AI (100 µM) 36.2 g	90 a	4.90 a	10
Compound 2AI (100 µM) 36.2 g + Latron B-1956 0.146 liters	64 b	3.78 b	12
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (100 µM) 36.2 g	70 b	3.45 bc	11
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (100 µM) 36.2 g + Latron B-1956 0.146 liters	58 b	3.11 c	12
Fisher's LSD, $\alpha = 0.05$	18	0.65	NS
P value for F	0.0002	<0.0001	0.142

^v Means within a column followed by the same letter do not differ significantly; Fisher's least significant difference (LSD), $\alpha = 0.05$; NS = not significant.

^w Foliar 0-to-9 rating, with 0 = no disease, 1 = at least one diseased leaf, 2 = 1 to 5% foliage diseased or defoliated, 3 = 6 to 10%, 4 = 11 to 15%, 5 = 16 to 25%, 6 = 26 to 50%, 7 = 51 to 75%, 8 = 76 to 99%, and 9 = 100% leaves diseased or plant completely defoliated.

^x Bacterial spot control using copper hydroxide tank-mixed with compound 2-aminoimidazole (2AI) (based on water at 935 liters/ha).

^y Bacterial spot control using the antibiotic kasugamycin tank-mixed with compound 2AI (based on water at 935 liters/ha).

^z Bacterial spot foliar disease control with addition of a nonionic surfactant (Latron B-1956) to compound 2AI (based on water at 935 liters/ha).

another medium such as water. Work by D. F. Ritchie (*unpublished data*) indicates that copper-sensitive strains exposed to copper in water can be inhibited at concentrations less than 1.0 µg/ml. In 3 years of field experiments, addition of 2AI to copper treatments resulted in decreased bacterial spot foliar disease ratings and, in some instances, increased fruit yields. The combination of 2AI with several other antibacterial compounds and plant defense-activating compounds also enhanced bacterial spot control, suggesting that 2AI has antibacterial enhancement beyond just that associated with copper. This suggests that 2AI may be affecting bacterial

fitness, possibly by suppressing bacterial biofilm formation and attachment to plants surfaces or causing bacteria to remain in a planktonic state, making them more sensitive to antibacterial agents.

Bacteria in their natural environments exist as communities attached to surfaces and other bacterial cells, usually in a structured, protective matrix composed of polysaccharides and other materials, termed a biofilm (2,22). Decreased sensitivity of bacteria to copper also can be mediated by biofilms (27).

In the laboratory, *X. euvesicatoria* formed biofilms on non-plant surfaces that 2AI suppressed. Whether 2AI suppresses or possibly

Table 4. Foliar spray treatments, bacterial spot foliar area under disease progress curve (AUDPC), and fruit yields for 2010 field experiment^x

Treatments and rates per hectare ^y	Mean AUDPC ^z	Mean fruit yield (kg)	
		Marketable	Total
Nontreated check	116 a	2.03 bcd	4.13
Copper hydroxide (Kocide 3000) 0.84 kg	98 abc	2.46 abcd	3.95
Copper hydroxide (Kocide 3000) 1.40 kg	86 bcde	2.00 bcd	3.38
Copper hydroxide (Kocide 3000) 1.40 kg + mancozeb (Manzate Pro-Stick 75DG) 0.842 kg	68 ef	2.86 abc	6.09
Compound 2AI (50 µM) 18.1 g	84 bcde	2.56 abc	4.80
Compound 2AI (75 µM) 27.2 g	83 bcde	3.90 a	5.91
Compound 2AI (100 µM) 36.2 g	81 cde	3.53 ab	5.61
Copper hydroxide (Kocide 3000) 1.40 kg + Compound 2AI (100 µM) 36.2 g	75 def	3.15 abc	4.61
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (75 µM) 27.2 g	87 bcd	2.82 abc	4.91
Copper hydroxide (Kocide 3000) 1.40 kg + compound 2AI (50 µM) 18.1 g	80 cde	1.72 cd	3.22
Copper hydroxide (Kocide 3000) 1.12 kg + compound 2AI (100 µM) 36.2 g	85 bcde	2.76 abc	4.06
Copper hydroxide (Kocide 3000) 0.84 kg + compound 2AI (100 µM) 36.2 g	79 cde	2.97 abc	4.61
Kasugamycin (Kasumin 2L) 4.67 liters	82 cde	2.80 abc	4.58
Kasugamycin (Kasumin 2L) 4.67 liters + compound 2AI (100 µM) 36.2 g	60 f	2.94 abc	5.64
Regalia 9.35 liters (5% extract <i>Reynoutria sachalinensis</i>)	95 bc	0.85 d	4.45
Regalia 4.67 liters (5% extract <i>Reynoutria sachalinensis</i>) + Copper hydroxide (Kocide 3000) 1.12 kg	101ab	0.85 d	4.49
Regalia 4.67 liters (5% extract <i>Reynoutria sachalinensis</i>) + compound 2AI (100 µM) 36.2 g	73 def	0.85 d	3.63
Fisher's LSD, $\alpha = 0.05$	18.6	1.67	2.62
P value for F	0.001	0.0025	0.622

^x Means within a column followed by the same letter do not differ significantly; Fisher's least significant difference (LSD), $\alpha = 0.05$.

^y Based on water at 935 liters/ha. 2AI = 2-aminoimidazole.

^z Foliar 0-to-9 rating, with 0 = no disease, 1 = at least one diseased leaf, 2 = 1 to 5% foliage diseased or defoliated, 3 = 6 to 10%, 4 = 11 to 15%, 5 = 16 to 25%, 6 = 26 to 50%, 7 = 51 to 75%, 8 = 76 to 99%, and 9 = 100% leaves diseased or plant completely defoliated.

disperses biofilms on plant surfaces, and whether this at least partially accounted for copper resensitization of *X. euvesicatoria* copper-resistant strains, requires further research. Phytopathogenic bacteria at some point in their life cycle must attach to their host plant in order to infect. Using genetic mutants, it has become increasingly evident that the ability of plant-pathogenic bacteria to form and to detach from biofilms is extremely important in completing disease cycles and for bacterial survival of foliar and endophytic plant-pathogenic bacteria (6–8,13, 14,16,23,33). Also, *Xylella fastidiosa* planktonic cells were as much as 13-fold more sensitive to CuSO₄ than *X. fastidiosa* cells in a biofilm (27).

The formation and dispersal of bacterial biofilms is a regulated process of chemical communications involving diffusible signal factors and quorum sensing (8,14,20,31). Additionally, two-component regulatory systems play an important role in biofilm regulation and in copper resistance for a number of bacterial species (18,21,32). In addition to the ability to suppress bacterial resistance to antibiotics, 2AI also has been shown to inhibit and disperse biofilms of a number of bacterial species, including the γ -proteobacterium *Pseudomonas aeruginosa* (29). Using quorum-sensing-deficient mutants, 2AI retained the capacity to inhibit biofilm formation (C. Melander, unpublished data). This indicates that 2AI does not, at least directly, interact with quorum-sensing signal molecules such as N-acylated L-homoserine lactone. Thus, the mechanism of 2AI differs from that of the biofilm modulation system recently reported for *Pectobacterium carotovora* and *Pseudomonas syringae* (20). Whether the mechanism of suppression of biofilm formation and resistance to copper in phytopathogenic bacteria by which 2AI operates is through a two-component regulatory system requires further investigation. Our results from field foliar-applied sprays of a small synthetic molecule (i.e., 2AI) that can modulate biofilm formation and the results previously reported with the laboratory use of synthetic quorum-sensing modulators (20) point to the practical value of pursuing this novel area of research for managing bacterial phytopathogens.

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Literature Cited

- Behlau, F., Canteros, B. I., Minsavage, G. V., Jones, J. E., and Graham, J. H., 2011. Molecular characterization of copper resistance genes from *Xanthomonas citri* subsp. *citri* and *Xanthomonas alfalfae* subsp. *citrumelonis*. *Appl. Environ. Microbiol.* 77:4089–4096.
- Branda, S. S., Vik, A., Friedman, L., and Kolter, R. 2005. Biofilms: the matrix revisited. *Trends Microbiol.* 13:20–26.
- Campbell, C. L., and Madden, L. V. 1990. *Introduction to Plant Disease Epidemiology*. Wiley and Sons, New York.
- Cooksey, D. A. 1990. Genetics of bactericide resistance in plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 28:201–219.
- Cooksey, D. A. 1994. Molecular mechanisms of copper resistance and accumulation in bacteria. *FEMS Microbiol. Rev.* 14:381–386.
- Crossman, L., and Dow, J. M. 2004. Biofilm formation and dispersal in *Xanthomonas campestris*. *Microb. Infect.* 6:623–629.
- Danhorn, T., and Fuqua, C. 2007. Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* 61:401–22.
- Dow, J. M., and Crossman, L. 2003. Biofilm formation and dispersal in *Xanthomonas campestris*. *Microb. Infect.* 6:623–629.
- Hasman, H., Bjerrum, M. J., Christiansen, L. E., Hasen, H. C. B., and Aarestrup, F. M. 2009. The effect of pH and storage on copper speciation and bacterial growth in complex media. *J. Microbiol. Method.* 78:20–24.
- Hsiao, Y., Liu, Y., Lee, P., Hsu, P., Tseng, S., and Pan, Y. 2011. Functional characterization of *copA* gene encoding multicopper oxidase in *Xanthomonas campestris* pv. *campestris*. *J. Agric. Food Chem.* 59:9290–9302.
- Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., and Schaad, N. W. 2004. Reclassification of *Xanthomonads* associated with bacterial spot disease of tomato and pepper. *Syst. Appl. Microbiol.* 27:755–762.
- Jones, J. B., Minsavage, G. V., Roberts, P. D., Johnson, R. R., Kousik, C. S., Subramanian, S., and Stall, R. E. 2002. A non-hypersensitive resistance in pepper to the bacterial spot pathogen is associated with two recessive genes. *Phytopathology* 92:273–277.
- Koczan, J. M., McGrath, M. J., Zhao, Y., and Sundin, G. W. 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Phytopathology* 99:1237–1244.
- Koutsoudis, M. D., Tsaltas, D., Minogue, T. D., and von Bodman, S. B. 2006. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc. Natl. Acad. Sci. USA* 103:5983–5988.
- Marco, G. M., and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. *Plant Dis.* 67:779–781.
- Marques, L. L. R., Ceri, H., Manfio, G. P., Reid, D. M., and Olson, M. E. 2002. Characterization of biofilm formation by *Xylella fastidiosa* in vitro. *Plant Dis.* 86:633–638.
- McManus, P. S., and Stockwell, V. O. 2001. Antibiotic use for plant disease management in the United States. *Plant Health Progress*. Online publication. doi:10.1094/PHP-2001-0327-01-RV
- Mills, S. D., Jasalavich, C. A., and Cooksey, D. A. 1993. A two-component regulatory system required for copper-inducible expression of the copper resistance operon of *Pseudomonas syringae*. *J. Bacteriol.* 175:1656–1664.

19. Moretti, C., Amatulli, M. T., and Buonauro, R., 2009. PCR-based assay for the detection of *Xanthomonas euvesicatoria* causing pepper and tomato bacterial spot. *Lett. Appl. Microbiol.* 49:466-471.
20. Palmer, A. G., Streng, E., and Blackwell, H. E. 2011. Attenuation of virulence in pathogenic bacteria using synthetic quorum-sensing modulators under native conditions on plant hosts. *ACS Chem. Biol.* 6:1348-1356.
21. Petrova, O. E., Schurr, J. R., Schurr, M. J., and Sauer, K. 2011. The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. *Mol. Microbiol.* 81:767-783.
22. Ramey, B. E., Koutsoudis, M., von Bodman, S. B., and Fuqua, C. 2004. Biofilm formation in plant-microbe associations. *Curr. Opin. Microbiol.* 7:602-609.
23. Rigano, L. A., Siciliano, F., Enrique, R., Sendin, L., Filippone, P., Torres, P. S., Questa, J., Dow, J. M., Castagnaro, A. P., Vojnov, A. A., and Marano, M. R. 2007. Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. *citri*. *Mol. Plant-Microbe Interact.* 20:1222-1230.
24. Ritchie, D. F., and Dittapongpitch, V. 1991. Copper- and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina. *Plant Dis.* 75:733-736.
25. Ritchie, D. F., Melander, C., Huigens, R. W., III, Rogers, S. A., and Cavanagh, J. 2009. Potential for a bromoageliferin analogue biofilm inhibitor-dispersant to enhance control of phytopathogenic bacterial diseases. (Abstr.) *Phytopathology* 99:S108.
26. Roberts, P. D., Momol, M. T., Ritchie, L., Olson, S. M., Jones, J. B., and Balogh, B. 2008. Evaluation of spray programs containing famoxadone plus cymoxanil, acibenzolar-S-methyl, and *Bacillus subtilis* compared to copper sprays for management of bacterial spot on tomato. *Crop Prot.* 27:1519-1526.
27. Rodrigues, C. M., Takita, M. A., Coletta-Filho, H. D., Olivato, J. C., Caserta, R., Machado, M. A., and de Souza, A. A. 2008. Copper resistance of biofilm cells of the plant pathogen *Xylella fastidiosa*. *Appl. Microbiol. Biotechnol.* 77:1145-1157.
28. Rogers, S. A., Huigens, R. W., Cavanagh, J., and Melander, C. 2010. Synergistic effects between conventional antibiotics and 2-aminoimidazole-derived antibiofilm agents. *Antimicrob. Agents Chemother.* 54:2112-2118.
29. Rogers, S. A., and Melander, C. 2008. Construction and screening of a 2-aminoimidazole library identifies a small molecule capable of inhibiting and dispersing bacterial biofilms across order, class, and phylum. *Angew. Chem. Int. Ed.* 47:5229-5231.
30. Romero, A. M., and Ritchie, D. F. 2004. Systemic acquired resistance delays race shifts to major resistance genes in bell pepper. *Phytopathology* 94:1376-1382.
31. Shepherd, R. W., and Lindow, S. E. 2009. Two dissimilar N-acyl-homoserine lactone acylases of *Pseudomonas syringae* influence colony and biofilm morphology. *Appl. Environ. Microbiol.* 75:45-53.
32. Tomaras, A. P., Flagler, M. J., Dorsey, C. W., Gaddy, J. A., and Actis, L. A. 2008. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology* 154:3398-3409.
33. Yao, J., and Allen, C. 2007. The plant pathogen *Ralstonia solanacearum* needs aerotaxis for normal biofilm formation and interactions with its tomato host. *J. Bacteriol.* 189:6415-6424.