Foliar Application of Biofilm Formation–Inhibiting Compounds Enhances Control of Citrus Canker Caused by *Xanthomonas citri* subsp. *citri*

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ABSTRACT

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Citrus canker caused by the bacterium *Xanthomonas citri* subsp. *citri* is an economically important disease of citrus worldwide. Biofilm formation plays an important role in early infection of *X. citri* subsp. *citri* on host leaves. In this study, we assessed the hypothesis that small molecules inhibiting biofilm formation reduce *X. citri* subsp. *citri* infection and enhance the control of citrus canker disease. D-leucine and 3-indolylacetonitrile (IAN) were found to prevent biofilm formation by *X. citri* subsp. *citri* on different abiotic surfaces and host leaves at a

concentration lower than the minimum inhibitory concentration (MIC). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis indicated that IAN repressed expression of chemotaxis/motility-related genes in *X. citri* subsp. *citri*. In laboratory experiments, planktonic and biofilm cells of *X. citri* subsp. *citri* treated with D-leucine and IAN, either alone or in combination, were more susceptible to copper (CuSO₄) than those untreated. In greenhouse assays, D-leucine and IAN applied alone or combined with copper reduced both the number of canker lesions and bacterial populations of *X. citri* subsp. *citri* on citrus host leaves. This study provides the basis for the use of foliar-applied biofilm inhibitors for the control of citrus canker alone or combined with copper-based bactericides.

Citrus canker, caused by the gram-negative bacterial pathogen *Xanthomonas citri* subsp. *citri* (syn. *X. citri*, *X. campestris* pv. *citri*, and *X. axonopodis* pv. *citri*) (6,30,34), is one of the major constraints to citrus production worldwide (10,12). Citrus canker affects most commercial citrus varieties and is widely distributed in many tropical and subtropical citrus growing regions (10,12). Characteristic citrus canker symptoms include raised necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings on leaves, stems, and fruit of infected trees; and when conditions are highly favorable for disease, it also causes defoliation, twig dieback, general tree decline, blemished fruit, and premature fruit drop (10,12). Wind-blown rain, which facilitates invasion via stomata or wounds (12), is the primary short-distance (i.e., within the same tree or between neighboring trees) spread mechanism for citrus canker (9). Long-distance dissemination of the disease often occurs with the transportation of diseased propagating materials and infected fruit (9). Direct canker-related losses are attributed to the decrease of fruit quality and yield. Moreover, a serious consequence is the significant impact on commerce resulting from restrictions to national and international citrus trade from canker-affected areas (10,12,31).

Copper-based bactericides are currently the primary control measure for citrus canker worldwide, as limited strategies exist for suppression of citrus canker on susceptible cultivars once the pathogen has been established (10,12). For effective disease control, multiple applications of copper-based bactericides are needed throughout the year due to their partial effectiveness under wind-

blown rain conditions (2,11). As a consequence, long-term use of copper bactericides led to resistance to copper in xanthomonad populations (3,26) and the accumulation of copper metal in soils or ground water affecting the environment and plant health (1,39). Thus, it is desired to reduce the usage of copper bactericides. A potential approach is to develop compounds that reduce bacterial resistance to copper bactericides and thereby enhancing the bactericidal effect and reducing the application of copper.

Much effort has been made to understand the biology and molecular basis of *X. citri* subsp. *citri* pathogenesis over the past decade (5,7,18,38). The canker bacterium, considered to be a hemibiotrophic pathogen, initially grows epiphytically (on leaf surfaces) and then enters into the host through stomata or wounds to colonize the mesophyll parenchyma and multiply in the apoplast (intercellular spaces). *X. citri* subsp. *citri*, like many other plant-pathogenic bacteria, has evolved multiple virulence factors to promote infection and establish themselves successfully in host plants (18,38). Among the virulence factors important for *X. citri* subsp. *citri* infection, biofilms have been suggested to play an important role in the early stages of infection by enhancing epiphytic persistence on host leaves. Importantly, multiple mutants of *X. citri* subsp. *citri* impaired in biofilm formation consistently exhibit a decrease in bacterial growth in planta and have reduced ability to elicit canker symptoms in susceptible citrus leaves (8,9,13,20,25,37). This led us to hypothesize that compounds inhibiting biofilm formation by *X. citri* subsp. *citri* may reduce its infection and enhance the control of canker disease. The goal of this study was thus twofold. First, we intended to identify small molecules that inhibit *X. citri* subsp. *citri* biofilm formation. Second, we aimed to determine if the selected biofilm formation inhibitors could increase the control effect of copper bactericides or reduce the usage of copper bactericides.

Here, we investigated the activity against *X. citri* subsp. *citri* biofilm formation of two sets of compounds, D-amino acids and

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indole derivatives, which have been shown to inhibit biofilm formation and influence virulence in human bacterial pathogens such as *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (17,19). We provided evidence that D-leucine and 3-indolylacetonitrile (IAN) are active in inhibiting biofilm formation by *X. citri* subsp. *citri* in nutrient broth (NB) liquid medium at a concentration lower than the minimum inhibitory concentration (MIC). Moreover, we found that D-leucine and IAN increased the sensitivity of *X. citri* subsp. *citri* to copper and reduced symptom development and bacterial populations on citrus leaves in greenhouse. These results provided the basis for the use of foliar-applied biofilm inhibitors for the control of citrus canker alone or combined with copper-based bactericides.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *X. citri* subsp. *citri* strain 306 (rifamycin [Rif] resistant [29]; copper sensitive, Cu^S [4]) was used throughout this study. *X. citri* subsp. *citri* strain A44 (copper resistant, Cu^R) (4) was also used in the examination of bacterial resistance to copper with or without biofilm inhibitors. Nutrient agar (NA) and NB were used as the media for the growth of *X. citri* subsp. *citri*. The bacterium was initially streaked from -80°C glycerol stock on an NA plate and a fresh single colony was inoculated in NB (40 ml) in 150 ml flasks and cultured at 28°C with agitation at 200 rpm. The overnight cultures were diluted in NB to standardize the cultures to obtain an optical density at 600 nm (OD₆₀₀) of 1.0 prior to setting up the biofilm assay and cell growth measurements. When necessary, rifamycin was added in the medium at a final concentration of 50 µg/ml.

Preparation of test compounds. D-amino acids (D-alanine, D-leucine, D-methionine, D-serine, D-tryptophan, and D-tyrosine) and indole derivatives (3-indoleacetic acid [IAA], IAN, and indole-3-propioninic acid [I3PA]) from plant sources were purchased from Sigma-Aldrich Co. (St. Louis, MO). The other chemicals including crystal violet (CV), ethyl alcohol, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific Co. (Pittsburg, PA). Stock solutions (500 mM in sterilized distilled water [SDW] for D-amino acids; 5 mg/ml in 1% DMSO for indole derivatives) were filter sterilized and stored at -20°C and diluted in SDW or DMSO for the initial test concentrations.

Evaluation of D-amino acids and indole derivatives for potential biofilm inhibition ability. A static biofilm formation assay in 96-well polystyrene plates coupled with CV staining was performed as previously reported with modifications (20). Briefly, a 96-well polystyrene plate (Nunc) surface, Nunclon, Denmark) was prepared with 170 µl of NB plus 1.0% glucose per well. Aliquots of 20 µl each of the different concentrations of the tested compound were pipetted into eight wells containing 170 µl of NB plus 1.0% glucose. To each of these wells, 10 µl of the standardized overnight *X. citri* subsp. *citri* strain 306 culture (OD₆₀₀ = 1.0) was added. Control wells contained NB plus 1.0% glucose and SDW or 0.1% DMSO as a sterility control, or standardized overnight *X. citri* subsp. *citri* strain 306 culture as a growth control. The plate was covered by a MicroWell lid (Nunc), sealed with parafilm to prevent evaporation, and incubated at 28°C for 48 h without shaking. After incubation, the planktonic growth was measured at OD₆₀₀. To quantify the amount of biofilms formed on the surfaces of the wells, the culture was removed from the wells. After drying, the wells were washed twice with 200 µl of SDW for 5 min, allowed to dry, and then 200 µl of 0.1% CV was added. After 30 min, the CV was removed. The wells were washed with excess SDW to remove unbound CV and air dried in an inverted position for 2 h. Afterward, 200 µl of 95% ethanol was added to the wells and incubated for 30 min at room temperature to elute bound CV. The eluted CV was twofold diluted in double distilled water and the absorbance at 590 nm was measured. Each data point was averaged from eight replicate wells.

To gain further evidence of the effect of selected compounds on biofilm formation, biofilm formation in glass tubes and on leaf surfaces was examined as described previously (21). Briefly, the standardized overnight cultures of *X. citri* subsp. *citri* strain 306 (OD₆₀₀ = 1.0) were diluted 1:10 in fresh NB containing 1.0% glucose and the selected compound. For biofilm formation assay in glass tubes (Fischer Scientific), 1 ml of the diluted bacterial suspension was transferred into each sterilized borosilicate glass tube and incubated at 28°C without shaking for 48 h. The planktonic growth was then discarded and the tubes were gently washed three times with SDW. The biofilm formed on the tubes was visualized by staining with 0.1% CV. The stain remaining in cells on glass tubes was dissolved in 95% ethanol and quantified by measuring the optical density at 590 nm. For biofilm formation assay on leaf surfaces, 20 µl of the diluted bacterial suspension was dropped onto the abaxial surface of citrus leaves. The leaves were kept in a humidified chamber at 28°C for 24 h without shaking. The biofilm formed on the leaf surfaces was visualized by staining with 0.1% CV. The biofilm assays were repeated three times with four replicates each time.

Determination of MICs. *X. citri* subsp. *citri* was grown in NB at 28°C with shaking at 200 rpm for 7 h. The cultures were standardized to an OD₆₀₀ of 0.03 (5 × 10⁷ colony forming unit [CFU]/ml) in NB and then aliquoted into wells of a 96-well plate, 190 µl per well. The initial test concentrations of the compounds were diluted (1:20) in the culture (10 µl of compound in 190 µl of culture) and incubated at 28°C under stationary conditions. The cultures were monitored at 24 and 48 h at OD₆₀₀, and the lowest concentration resulting in no growth after 48 h compared with the control samples was defined as the MIC for *X. citri* subsp. *citri*. All determinations were conducted in eight replicate wells and repeated three times.

Evaluation of resistance of *X. citri* subsp. *citri* biofilm cells to copper. The level of copper resistance of biofilms was evaluated using a cell viability assay. Briefly, *X. citri* subsp. *citri* biofilms were prepared using NB with stationary incubation in glass tubes as described above. After 48 h incubation, the cultures were removed and bacterial cells attached to the tubes were gently washed three times with SDW. One milliliter of fresh NB with CuSO₄ (1.0 mM), D-leucine (10 mM), or IAN (100 µg/ml), or a combination of these compounds was added to each tube. NB alone was used as control. Tubes were kept at room temperature for 24 h followed by shaken vigorously for 5 min. The suspensions were diluted in 10-fold series, and 10 µl of each dilution was spotted in triplicate on NA plate. Plates were incubated at 28°C for 48 to 72 h prior to assessing bacterial growth. Colonies that grew near the dilution end-point were counted and bacterial populations in the initial suspensions prior to dilution were calculated. Each treatment comprised four replicates and the experiment was repeated three times.

RNA preparation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). *X. citri* subsp. *citri* was cultured in NB medium with or without biofilm inhibitor (IAN [100 µg/ml] or D-leucine [10 mM]) at 28°C without shaking. Cells were collected after 48 h of incubation by centrifugation at 12,000 × g for 5 min at 4°C and used for RNA extraction. For the analysis of gene expression in *X. citri* subsp. *citri* planktonic and biofilm cells treated with sub-MICs concentrations of CuSO₄ (0.1 mM for *X. citri* subsp. *citri* strain 306; 0.5 mM for *X. citri* subsp. *citri* strain A44), we followed the same procedure described for evaluation of resistance of *X. citri* subsp. *citri* biofilm cells to copper in glass tubes. Both biofilm cells attached to the glass tube at the medium-air interface and planktonic cells in culture were collected. Cells collected after 48 h of incubation in the presence of CuSO₄ were washed by centrifugation at 12,000 × g for 5 min at 4°C with diethylpyrocatechol-treated water. Cells collected from five tubes were combined and served as one biological replicate. The pellet was stored at -80°C until RNA

extraction. Total RNA of *X. citri* subsp. *citri* cells was isolated using RNA protect bacterial reagent (Qiagen, Valencia, CA) and RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Genomic DNA was removed using a TURBO DNA-free kit (Ambion, Austin, TX). RNA purity and quality were evaluated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

A one-step qRT-PCR was conducted using a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) with a QuantiTect SYBR green RT-PCR kit (Qiagen) following the manufacturer's instructions. The gene specific primers (Table 1) were designed based on the genome sequence of *X. citri* subsp. *citri* strain 306 (7) and nucleotide sequence for copper resistance genes from *X. citri* subsp. *citri* strain A44 (4), respectively. In the biofilm inhibition mechanism studies, those primers targeted 15 genes that were previously identified to be related to biofilm formation in *X. citri* subsp. *citri* strain 306 (20). In the copper resistance analysis, those primers targeted the gum genes *gumB* and *gumD*, polysaccharides-related gene *galU*, lipopolysaccharide (LPS) O-antigen biosynthesis gene *rfbC*, and copper resistance-related genes *copA* and *copB* (3,33). The DNA gyrase subunit A encoding gene *gyrA* was used as endogenous control. The relative fold change in gene expression was calculated using the formula $2^{-\Delta\Delta CT}$ (23). qRT-PCR was repeated twice with four independent biological replicates each time.

Plant test in greenhouse. The effect of selected biofilm inhibitors on *X. citri* subsp. *citri* infection/virulence was investigated using 20-week-old potted grapefruit (*Citrus paradise* 'Duncan' grapefruit) plants in a quarantine greenhouse at the Citrus Research and Education Center, Lake Alfred, FL. Copper (CuSO_4 , 100 $\mu\text{g/ml}$), D-leucine (10 mM), IAN (100 $\mu\text{g/ml}$), and a combination of these compounds were individually prepared in 100 ml of SDW. Bacterial inoculum was prepared by growing *X. citri* subsp. *citri* strain 306 on NA plates at 28°C for 48 h, suspending in SDW, and adjusting concentration to approximately $\text{OD}_{600} = 0.3$ (5×10^8 CFU/ml). The inoculation was performed by a spray method as described previously (21) with modifications. Briefly, the abaxial surfaces of fully expanded, immature leaves of each plant were sprayed with the following treatments: SDW, *X. citri* subsp. *citri* strain 306, and *X. citri* subsp. *citri* strain 306 combined with CuSO_4 (100 $\mu\text{g/ml}$), D-leucine (10 mM), IAN (100 $\mu\text{g/ml}$), or a combination of these compounds. Silwett-L77 (silicone-polyether copolymer, Fisher Scientific) was added to each treatment at a 0.03% (vol/vol) final concentration. After inoculation, the plants were covered with plastic bags for 24 h to maintain a high (>90%) relative humidity and then kept in a greenhouse (approximately 60% relative humidity) for symptom development. All inoculations included a minimum of three immature leaves at a similar developmental stage from each plant, and each treatment comprised four plants. The experiments were

TABLE 1. Genes and corresponding primers used in quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Gene	Locus_tag	Function of protein product	Primer sequence (5'-3')
<i>Xanthomonas citri</i> subsp. <i>citri</i> strain 306			
<i>gumB</i>	XAC2585	EPS xanthan biosynthesis	Forward: agaacggccatatttcgttg Reverse: tgcagataaccgttgcgata
<i>gumD</i>	XAC2583	EPS xanthan biosynthesis	Forward: tccgtaccaccatacagacatt Reverse: taccagcttgacgttgatcg
<i>galU</i>	XAC2292	Polysaccharides biosynthesis	Forward: acagtgcggaagaaatgct Reverse: agctcataggccttgcgaa
<i>rfbC</i>	XAC3598	LPS O-antigen biosynthesis	Forward: atcatcccggtctgcaatac Reverse: ggaatgcgcttctgaactc
<i>cheA</i>	XAC1930	Chemotaxis protein	Forward: gacgatattgctgccgattt Reverse: gagctgtcggcttctct
<i>cheY</i>	XAC1904	Bacterial chemotaxis regulator	Forward: tacaagttacccccatgct Reverse: gcgatcagctgttctgatt
<i>mcpA</i>	XAC1746	Chemotaxis protein	Forward: cctatcgaaactgcttgac Reverse: cactcgtccagtcgatacc
<i>motB</i>	XAC1908	Flagellar motor protein	Forward: atctgtggtcaggtggag Reverse: gttcccagttggaggaaat
<i>fleN</i>	XAC1934	Flagellar biosynthesis	Forward: agtctcgttcttcgcttga Reverse: gttggtcagctggcgatt
<i>fliC</i>	XAC1975	Flagellar biosynthesis	Forward: cagcgtattcgtgagctgtc Reverse: ccgttgaaagttggtctggt
<i>pilB</i>	XAC3239	Pilus biogenesis	Forward: caagtgtaccgctgttcaa Reverse: gcgacggatctgatctcat
<i>clp</i>	XAC0483	cAMP regulatory protein	Forward: gaactaccatgagcccagga Reverse: gccgctgatcagctagtaga
<i>colR</i>	XAC3250	Response regulator	Forward: ttggcgattacctgaagac Reverse: gttgaggtcgagcacgatg
<i>rpfG</i>	XAC1877	Response regulator	Forward: ggatctgggattgaacatcg Reverse: agtccagcaacagcagatcc
<i>rpoN</i>	XAC1969	RNA polymerase sigma-54 factor	Forward: gcttccatgaagacgacat Reverse: gctctccaactccagcaac
<i>copA</i>	XAC3630	Copper resistance protein	Forward: cgaatgtttcaggagcagtca Reverse: tgttcaaacgacggaacag
<i>copB</i>	XAC3631	Copper resistance protein	Forward: ctacccagacacgactaa Reverse: ccgatcgagcaggacataat
<i>gyrA</i>	XAC1631	DNA gyrase subunit A	Forward: cgtcacgttgatccgtgt Reverse: gcttgcttggtccactccct
<i>X. citri</i> subsp. <i>citri</i> strain A44			
<i>copA</i>		Copper resistance protein	Forward: ctacttgatgaacggcagca Reverse: aaccggatggacatacaagc
<i>copB</i>		Copper resistance protein	Forward: ctttacggccgagtagtctc Reverse: gcggacacttcgaactcat
<i>copL</i>		Transcriptional repressor	Forward: gatcgtcatgctcgtgctta Reverse: ctttcaatcgaggtggcacc

repeated three times independently. SDW and *X. citri* subsp. *citri* strain 306 mixed with SDW were used as controls. To determine whether the timing of application of biofilm inhibitors in relation to *X. citri* subsp. *citri* strain 306 affected canker symptom suppression, the biofilm inhibitors were inoculated on leaves 6 h prior to or after inoculation with *X. citri* subsp. *citri* strain 306.

For bacterial population assays, the leaves of grapefruit plants were inoculated as described above. Two leaf discs randomly selected from each of three inoculated leaves were cut with a cork borer (0.8 cm in diameter) and then ground in 1 ml of SDW. The suspensions were serially diluted and plated on NA plates containing rifamycin. After incubation at 28°C for 48 h, bacterial colonies were counted and the number of CFU per square centimeter of leaf tissue was calculated. The assays were repeated three times independently.

RESULTS

D-leucine and IAN are potent biofilm inhibitors of *X. citri* subsp. *citri*. Six D-amino acids and three indole derivatives derived from plant sources were tested for their ability to inhibit

biofilm formation of *X. citri* subsp. *citri* strain 306 in 96-well plate biofilm assays. In the absence of D-amino acid or indole derivative, *X. citri* subsp. *citri* strain 306 formed robust biofilms after 48 h of incubation (Fig. 1A). The addition of 10 mM of D-leucine, D-serine, and 100 µg/ml IAN significantly reduced *X. citri* subsp. *citri* strain 306 biofilm formation by 45 to 68%, whereas the other D-amino acids or indole derivatives tested did not affect *X. citri* subsp. *citri* strain 306 biofilm formation at the tested concentrations (Fig. 1A). Furthermore, the compounds D-leucine, D-serine, and IAN decreased *X. citri* subsp. *citri* strain 306 biofilm in a dose-dependent manner from 0 to 15 mM for D-leucine and D-serine (Fig. 1B) and 0 to 150 µg/ml for IAN (Fig. 1C).

To test the toxicity of the three biofilm inhibitors, the MICs were determined in NB medium. The results showed that D-serine had the lowest MIC of 8.0 mM, followed by D-leucine and IAN with MICs of 16.0 mM and 200 µg/ml, respectively (Table 2). D-leucine and IAN had relatively high MICs but paradoxically demonstrated the potent decrease in biofilm mass (58% decrease for D-leucine; 65% decrease for IAN) at lower concentrations than MICs (10 mM for D-leucine and 100 µg/ml for IAN) (Fig. 1A). Due to its low toxicity to *X. citri* subsp. *citri* strain 306 and

Fig. 1. Effect of D-amino acids and indole derivatives on biofilm formation of *Xanthomonas citri* subsp. *citri* strain 306. **A**, Effect of D-amino acids at 10 mM and indole derivatives (3-indoleacetic acid [IAA], 3-indolylacetonitrile [IAN] and indole-3-propioninc acid [I3PA]) at 100 µg/ml on normalized (optical density at 590 nm [OD₅₉₀]/OD₆₀₀) biofilm formation of *X. citri* subsp. *citri* strain 306 in nutrient broth (NB) medium at 28°C after 48 h in 96-well plates. All of the indole derivatives were dissolved in 0.1% dimethyl sulfoxide (DMSO). DMSO and NB were used as the negative controls (normal biofilm formation) and no bacterial inoculation (CK+) was used as the positive control (no biofilm formation), respectively. Dose-dependent effect of **B**, D-leucine and D-serine (0, 5, 10, and 15 mM) and **C**, IAN (0, 25, 50, 100, and 150 µg/ml) on *X. citri* subsp. *citri* strain 306 normalized (OD₅₉₀/OD₆₀₀) biofilm formation was observed. The experiments were repeated three times with similar results with eight replicates (eight wells), and the error bars indicated standard deviations.

apparent potency against biofilm formation, D-leucine and IAN were selected for further studies.

D-leucine and IAN reduce biofilm formation on abiotic surfaces and host leaves at sub-MIC concentrations. To further evaluate and confirm the antibiofilm properties of D-leucine and IAN, biofilm formation of *X. citri* subsp. *citri* strain 306 with the compounds at sub-MIC (10 mM for D-leucine and 100 µg/ml for IAN), a concentration that does not significantly decrease planktonic cell density (data not shown), was examined on three different kinds of surfaces: polystyrene, glass, and host leaves. The D-leucine- or IAN-treated cultures exhibited a significant reduction in biofilm formation both on polystyrene surface and in glass tubes compared with the untreated control, where the levels of biofilm formation were reduced to 50 and 60% of control, respectively (Fig. 2A and B). Similar to the observations on polystyrene surface and in glass tubes, the D-leucine- or IAN-treated cultures showed declined biofilm formation on citrus leaf surfaces (Fig. 2C), suggesting that D-leucine and IAN reduced biofilm formation of *X. citri* subsp. *citri* strain 306 on citrus leaves. These findings confirmed that D-leucine and IAN had

specific activity inhibiting biofilm formation by *X. citri* subsp. *citri* strain 306.

Differential gene expression of *X. citri* subsp. *citri* strain 306 cells with IAN. To obtain insight into the mechanisms by which D-leucine and IAN inhibit biofilm formation, we evaluated the effect of these two compounds on expression of genes important for biofilm formation in *X. citri* subsp. *citri* strain 306 using qRT-PCR. The selected genes included the gum genes *gumB* and *gumD*, polysaccharides-related gene *galU*, O-antigen biosynthesis gene *rfbC*, chemotaxis and motility genes *cheA*, *cheY*, *mcpA*, and *motB*, type IV twitching motility gene *pilB*, flagellar biosynthesis genes *fleN* and *fliC*, and regulator genes *colR*, *clp*, *rpfG*, and *rpoN*. In our previous studies, these genes were identified to be important for biofilm formation in *X. citri* subsp. *citri* strain 306 (20). The results showed that IAN significantly ($P < 0.05$, Student *t* test) repressed expression of chemotaxis and motility genes *cheY*, *motB*, and *pilB*, whereas D-leucine had no effect on expression of the tested genes (Fig. 3).

Suppression of *X. citri* subsp. *citri* resistance to copper by D-leucine and IAN. The copper resistance of planktonic cells of *X. citri* subsp. *citri* strain 306 was evaluated with and without biofilm inhibitor. In the NB medium, planktonic cells exhibited a MIC of 0.50 mM CuSO₄ without biofilm inhibitor. In the presence of D-leucine, IAN or a combination of the two compounds at sub-MIC concentrations (10 mM for D-leucine and 100 µg/ml for IAN), the MICs of CuSO₄ against *X. citri* subsp. *citri* strain 306 planktonic cells were decreased to 0.25 mM (Table 2). Similar results were obtained with the Cu^R strain *X. citri* subsp. *citri* strain A44. The MIC of CuSO₄ alone against planktonic cells of A44 was 2.5 mM in NB medium. In the presence of D-leucine, IAN or a combination of the two compounds at sub-MIC concentrations (10 mM for D-leucine and 100 µg/ml for IAN), the MICs were decreased to 1.25 mM (Table 2). These results suggested that D-leucine and IAN increased the susceptibility of *X. citri* subsp. *citri* planktonic cells to copper under the applied conditions.

In the cell viability assays, biofilms treated with D-leucine, IAN, or a combination of the two compounds at sub-MIC concentrations (10 mM for D-leucine and 100 µg/ml for IAN) were about 10 times more susceptible to CuSO₄ than the untreated

TABLE 2. Minimum inhibitory concentrations (MICs) of copper (CuSO₄) against *Xanthomonas citri* subsp. *citri* strains 306 and A44 in nutrient broth medium with or without biofilm inhibitor^a

Treatment	MIC (mM)	
	Xac 306	Xac A44
CuSO ₄	0.5	2.5
D-leucine	16.0	20.0
D-serine	8.0	ND
IAN	200 (µg/ml)	250 (µg/ml)
CuSO ₄ /D-leucine (10 mM)	0.25	1.25
CuSO ₄ /IAN (100 µg/ml)	0.25	1.25
CuSO ₄ /D-leucine (10 mM)/IAN (100 µg/ml)	0.25	1.25

^a The MICs were examined using a 96-well plate assay at 28°C under stationary conditions as described in the Materials and Methods. The MIC was defined as the lowest concentration resulting in no bacterial growth measured at optical density at 600 nm after a 48 h incubation compared with the control samples. ND: not determined. The determinations were repeated three times with eight replicate wells each time.

Fig. 2. Biofilm formation by *Xanthomonas citri* subsp. *citri* strain 306 on different surfaces. Biofilm formation in **A**, polystyrene 96-well plates, **B**, glass tubes, and **C**, on citrus abaxial leaf surfaces was visualized using crystal violet staining. Biofilm formations in glass tubes were quantified by measuring the optical density at 590 nm after dissolution in 95% ethanol. NB: nutrient broth medium; D-leu: D-leucine (10 mM); IAN: 3-indolylacetonitrile (100 µg/ml); CK-: NB medium without inoculation of *X. citri* subsp. *citri* strain 306. All experiments were performed in four replicates and repeated three times with similar results, and only one representative result is presented. Means $\pm$ standard deviations are shown.

control. The cell viability of *X. citri* subsp. *citri* biofilms exposed to CuSO₄ (1.0 mM for *X. citri* subsp. *citri* strain 306; 2.0 mM for *X. citri* subsp. *citri* strain A44) was significantly reduced by D-Leucine or IAN. Biofilms exposed to CuSO₄ alone for 24 h contained an average of 3.0×10^7 CFU/ml for *X. citri* subsp. *citri* strain 306 and 1.3×10^8 CFU/ml for *X. citri* subsp. *citri* strain A44, respectively; while the biofilms treated by D-leucine (10.0 mM), IAN (100 µg/ml), or a combination of the two compounds contained an average of 1.1×10^6 , 4.3×10^6 , and 2.5×10^6 CFU/ml (for *X. citri* subsp. *citri* strain 306) and 2.4×10^7 , 2.9×10^7 , and 1.6×10^7 CFU/ml (for *X. citri* subsp. *citri* strain A44), respectively (Fig. 4). These findings indicated that D-leucine and IAN increased the susceptibility of *X. citri* subsp. *citri* biofilm cells to copper under the applied conditions.

To obtain insight into the mechanisms by which D-leucine and IAN increase the susceptibility of *X. citri* subsp. *citri* cells to copper, we evaluated the effect of the two compounds on expression of the polysaccharides-related gene *galU*, gum genes *gumB* and *gumD*, LPS O-antigen biosynthesis gene *rfbC*, and copper resistance related genes *copA* and *copB* in *X. citri* subsp. *citri* strain 306 planktonic and biofilm cells and expression of the copper resistance genes *copA*, *copB*, and *copL* in *X. citri* subsp. *citri* strain A44 planktonic and biofilm cells, respectively. The qRT-PCR results showed that either D-leucine or IAN did not affect the expression of any of the genes tested in *X. citri* subsp. *citri* planktonic or biofilm cells in the presence of CuSO₄ (Supplemental Figure 1).

D-leucine and IAN reduce canker symptom production and bacterial populations in planta. Plant inoculation by spray showed that both D-leucine (10 mM) and IAN (100 µg/ml) were able to reduce canker symptom development on grapefruit leaves when applied along with or prior to the pathogen inoculation, as evidenced by decreased lesion numbers compared with the positive control (pathogen inoculation alone) (Fig. 5A). Differences in lesion numbers were notable by 14 days postinoculation and more distinctive over the remainder of the experiment. When applied

after the pathogen inoculation, either D-leucine or IAN did not affect the development of canker symptoms (Fig. 5A). Both D-leucine and IAN could reduce the level of canker lesions to that of copper spray (CuSO₄, 100 µg/ml). The leaves sprayed with D-leucine (10 mM), IAN (100 µg/ml), or CuSO₄ (100 µg/ml) displayed a similar level of canker lesions, less than those sprayed

Fig. 4. Effect of D-leucine and 3-indolylacetonitrile (IAN) on cell viability of *Xanthomonas citri* subsp. *citri* strains 306 (Xac 306) and (Xac A44) in biofilms exposed to copper. Viable cells in biofilms exposed to the agent alone or in combination were quantified using the dilution-plating method. Cu: CuSO₄ (1.0 mM for strain 306; 2.0 mM for strain A44); D-leu: D-leucine (10 mM); IAN: 3-indolylacetonitrile (100 µg/ml); NB: nutrient broth medium alone. The experiments were repeated three times with similar results with four replicates. The error bars indicated standard deviations.

Fig. 3. Differential gene expression in *Xanthomonas citri* subsp. *citri* strain 306 cells in the presence of 3-indolylacetonitrile (IAN) at 100 µg/ml as revealed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Data were presented as the ratio of transcript number in the treatment of IAN or D-leucine (D-Leu) compared with that in the untreated control (CK). *gyrA* was used as an endogenous control. The asterisk denotes $P < 0.05$ (t test) compared with the untreated control. qRT-PCR was repeated twice with similar results with four independent biological replicates each time.

with the pathogen alone (Fig. 5A). The combination of D-leucine (10 mM), IAN (100 µg/ml), and CuSO₄ (100 µg/ml) was more effective in suppressing the development of canker symptoms than CuSO₄ alone, with fewer lesions produced on the leaves (Fig. 5A). These visual observations correlated with bacterial populations recovered from the inoculated leaves after inoculation that revealed an approximately 10- to 100-fold decrease in CFU per square centimeter of leaf tissue for treatment with D-leucine, IAN, or CuSO₄, alone or in combination, compared with the untreated control (Fig. 5B). Treatment of the grapefruit leaves with D-leucine, IAN, or CuSO₄ alone resulted in an approximate 1.0 log reduction in the number of CFU compared with the untreated control, while treatment with a combination of D-leucine or/and IAN and CuSO₄ resulted in an approximate 2.0 log reduction in the number of CFU per square centimeter compared with the untreated control from 7 to 28 days after inoculation (Fig. 5B).

DISCUSSION

In this study, we demonstrated that D-leucine and IAN reduced biofilm formation by *X. citri* subsp. *citri* and increased the susceptibility of *X. citri* subsp. *citri* to copper at a concentration without affecting its growth. Canker control activity was evaluated as the ability to reduce the number of lesions and populations of *X. citri* subsp. *citri* on citrus leaves after applications. The results of these greenhouse assays support the use of foliar-applied biofilm inhibitors alone or combined with copper-based bactericides for the control of canker disease on citrus trees. These observations were similar to those described by Worthington et al. (35), which showed that a derivative from the small molecule 2-aminoimidazole (2AI) at nonbactericidal concentrations exhibited the antibiofilm property and enhanced control of copper-resistant *X. euvesicatoria* on peppers by decreasing the resistance of *X. euvesicatoria* to copper. Interestingly, Palmer and colleagues

Fig. 5. Effect of D-leucine and 3-indolylacetoneitrile (IAN) on *Xanthomonas citri* subsp. *citri* strain 306 (Xac 306) infection on citrus leaves. **A**, D-leucine and IAN reduced canker symptom development on grapefruit leaves spray-inoculated by *X. citri* subsp. *citri* strain 306 (approximately 10⁸ CFU/ml). Images are representative of four independent replicates at 28 days postinoculation. Xac306: strain 306 alone; D-leu/Xac306: D-leucine (10 mM) inoculated simultaneously with *X. citri* subsp. *citri* strain 306; IAN/Xac306: 3-indolylacetoneitrile (100 µg/ml) inoculated simultaneously with *X. citri* subsp. *citri* strain 306; D-leu/Xac306 (6 h): D-leucine (10 mM) inoculated 6 h before *X. citri* subsp. *citri* strain 306 inoculation; IAN/Xac306 (6 h): 3-indolylacetoneitrile (100 µg/ml) inoculated 6 h before *X. citri* subsp. *citri* strain 306 inoculation; H₂O: water only; Cu/Xac306: CuSO₄ (100 µg/ml) inoculated simultaneously with *X. citri* subsp. *citri* strain 306; D-leu/Cu/Xac306: D-leucine (10 mM) and CuSO₄ (100 µg/ml) inoculated simultaneously with *X. citri* subsp. *citri* strain 306; IAN/Cu/Xac306: 3-indolylacetoneitrile (100 µg/ml) and CuSO₄ (100 µg/ml) inoculated simultaneously with *X. citri* subsp. *citri* strain 306; D-leu/IAN/Cu/Xac306: D-leucine (10 mM), 3-indolylacetoneitrile (100 µg/ml) and CuSO₄ (100 µg/ml) inoculated simultaneously with *X. citri* subsp. *citri* strain 306; Xac306/D-leu (6 h): D-leucine (10 mM) inoculated 6 h after *X. citri* subsp. *citri* strain 306; Xac306/IAN (6 h): 3-indolylacetoneitrile (100 µg/ml) inoculated 6 h after *X. citri* subsp. *citri* strain 306. **B**, D-leucine and IAN affected the growth of *X. citri* subsp. *citri* strain 306 populations on grapefruit leaves following spray inoculation (10⁸ CFU/ml). Bacterial cells were extracted from the leaves at different time points after inoculation and quantified using the standard serial diluting-plating method. The values shown are means of three repeats and standard deviations. All the assays were repeated three times with similar results.

(24) recently evaluated small molecule modulators of quorum sensing (QS) for inhibitors of QS-related bacterial virulence and found that four synthetic *N*-acylated L-homoserine lactone (AHL) analogues could attenuate virulence both in *Pectobacterium carotovora* and *Pseudomonas syringae* on their hosts potato and green bean. Altogether, our observations along with these reports indicate practical value of using small molecule biofilm formation and/or QS modulators to manage bacterial plant pathogens under field conditions.

D-leucine and IAN appear to have distinct mechanisms of action in reducing biofilm formation by *X. citri* subsp. *citri*. In this study, we have found that both D-leucine and IAN inhibit the biofilm formation of *X. citri* subsp. *citri* on different abiotic surfaces and host leaves at sub-MIC concentrations (Fig. 2), while only IAN repressed expression of several biofilm formation-related genes (chemotaxis/motility-related genes) in *X. citri* subsp. *citri* (Fig. 3). Flagellar-mediated chemotaxis/motility and type IV pili protein are necessary for *X. citri* subsp. *citri* biofilm formation (20). The observation of IAN repressing expression of genes related to bacterial motility was consistent with previous reports for IAN effect on biofilm formation in the human bacterial pathogen *Pseudomonas aeruginosa* (19). Hence, IAN probably reduces *X. citri* subsp. *citri* biofilm formation by repressing these chemotaxis/motility-related genes (*cheY*, *motB*, and *pilB*) (Fig. 3) and thus reducing its chemotaxis and motility even though we could not rule out other possibilities. Interestingly, it has been documented that the small molecule 2AI could inhibit biofilm formation in the human dental caries pathogen *Streptococcus mutans* by down-regulating the expression of six biofilm-associated genes (22), while whether 2AI suppresses biofilm formation in *X. euvesicatoria* using similar mechanism(s) requires further investigation (35). Intriguingly, it has been found that several D-amino acids including D-leucine inhibit biofilm formation in *Bacillus subtilis* and *Staphylococcus aureus* by preventing protein localization at the cell surface (15,17,28). Whether D-leucine prevents protein localization at the cell surface of *X. citri* subsp. *citri* remains to be characterized.

Reduced sensitivity to copper in bacteria may result from genetic mutations and from the change of environmental factors influencing the ionic concentration or production of bacterial extracellular polysaccharides (EPS) that can bind the biologically active ions of copper (14,16). LPS is also known to be a key component of the outer membrane of gram-negative bacteria and a protective barrier against harsh environmental conditions including copper stress (36). It has been shown that decreased sensitivity of bacteria to copper can be mediated by biofilms (27). A synergistic effect was suggested between diffusion barriers to antimicrobial compounds and other mechanisms associated with bacterial resistance including cation/multidrug efflux of the cells in biofilms (27). In our experimental conditions, the results showed that both D-leucine and IAN increased the susceptibility of the Cu^S (*X. citri* subsp. *citri* strain 306) and Cu^R (*X. citri* subsp. *citri* strain A44) *X. citri* subsp. *citri* planktonic and biofilm cells to copper (Fig. 4), but did not affect expression of genes responsible for gum EPS biosynthesis, LPS O-antigen production, or genes related to copper resistance. It is possible that D-leucine and IAN may affect *X. citri* subsp. *citri* resistance by suppressing biofilm formation or causing bacteria to remain in a planktonic state making them more sensitive to copper. Similarly, the small molecule 2AI was suggested to decrease the resistance of *X. euvesicatoria* to copper by inhibiting biofilm formation or causing bacteria to remain in a planktonic state making them more sensitive to antibacterial agents (35). Importantly, this is the case in the copper resistance of *Pseudomonas aeruginosa* and *Xylella fastidiosa*, where the planktonic cells were more susceptible than biofilm cells (27,32).

Preventive effects of D-leucine and IAN have been demonstrated on *X. citri* subsp. *citri* spray-inoculated young citrus plants

by foliar spraying. In greenhouse assays, foliar spray applications of D-leucine and IAN, 6 h pre-inoculation or co-inoculation, reduced the number of lesions produced by *X. citri* subsp. *citri* and the bacterial populations on grapefruit leaves (Fig. 5). The data obtained suggests that D-leucine and IAN suppress biofilm formation of *X. citri* subsp. *citri* on host plant surfaces, which may cause impaired infection. Furthermore, the combined application of D-leucine, IAN, and copper enhanced activities against the *X. citri* subsp. *citri* bacterium compared with the application of copper alone, suggesting an apparent additive effect that may permit a dosage reduction of copper. It would be of great value to decrease the copper usage in the open environment as it reduces the potential for side effects to the environment. Overall, this work validated the potential of pursuing small molecule biofilm inhibitors for managing citrus canker disease.

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