A novel Zn chelate (TSOL) that moves systemically in citrus plants inhibits growth and biofilm formation of bacterial pathogens

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Abstract

Ternary solution (TSOL) is a novel Zn chelate-based systemic antimicrobial formulation designed for treating citrus bacterial pathogens ‘Candidatus Liberibacter asiaticus’ and Xanthomonas citri subsp. citri. TSOL is a component of MS3T, a novel multifunctional surface/sub-surface/systemic therapeutic formulation. Antimicrobial activity of TSOL was compared with the antimicrobial compound ZnO against X. citri subsp. citri and ‘Ca. L. asiaticus’ surrogate Liberibacter crescens in batch cultures. X. citri subsp. citri and L. crescens were also introduced into microfluidic chambers, and the inhibitory action of TSOL against biofilm formation was evaluated. The minimum inhibitory concentration of TSOL for both X. citri subsp. citri and L. crescens was 40ppm. TSOL was bactericidal to X. citri subsp. citri and L. crescens above 150 ppm and 200 ppm, respectively. On the contrary, ZnO was more effective as a bactericidal agent against L. crescens than X. citri subsp. citri. TSOL was more effective in controlling growth and biofilm formation of X. citri subsp. citri in batch cultures compared to ZnO. Time-lapse video imaging microscopy showed that biofilm formation of X. citri subsp. citri was inhibited in microfluidic chambers treated with 60 ppm TSOL. TSOL also inhibited further growth of already formed X. citri subsp. citri and L. crescens biofilms in microfluidic chambers. Leaf spraying of TSOL showed higher plant uptake and systemic movement in citrus (Citrus reshni) plants compared to that of ZnO, suggesting that TSOL is a promising antimicrobial compound to control vascular plant pathogens such as ‘Ca. L. asiaticus’.
Introduction

Huanglongbing (HLB) is the most devastating disease of citrus worldwide [1], and it is associated with Gram-negative, phloem-restricted and non-cultured bacteria 'Candidatus Liberibacter asiaticus' (CLas), 'Candidatus Liberibacter africanus' (CLaf) and 'Candidatus Liberibacter americanus' (CLam) [2]. HLB can be transmitted from tree to tree by grafting and through citrus psyllid vectors Trioza erytreae in Africa and Asian citrus psyllid (ACP) Diaphorina citri in Asia and the Americas [2]. HLB associated with CLas is found in Asian countries, as well as in Brazil and USA [2]. HLB symptoms include yellowing of shoots, stunting and declining of trees, deformed (lop-sided) and poorly colored (greening) fruits, aborted seeds in fruits and weak root systems with relatively few fibrous roots [3]. Progression of symptoms can be very fast and severe symptoms have been observed within one to five years after symptoms appear, depending on the age of the tree at the time of infection and the number of infections per tree. Yield reduction and a decrease in fruit quality occur with the increase in disease severity [1, 4–7]. Yield reduction in HLB infected trees occur at a faster rate compared to the development of symptoms [3, 8].

There are three management practices recommended for HLB. These include use of insecticides to control ACP to minimize the spread of inoculum, removal of HLB-infected trees to reduce the sources of inoculum, and planting HLB-free trees to prevent introduction of new inoculum [9, 10]. Insecticides such as Imidacloprid, Fenpropathrin, Chlorpyrifos, Thiamethoxam and Dimethoate are considered effective against ACP [9, 11]. Effective control of ACP using insecticides largely depends on environmental conditions, ACP control in the surrounding areas and removal of diseased trees to reduce inoculum loads [9]. Therefore, control of HLB through insecticides by controlling ACP is difficult in areas endemic to HLB [9]. A study in Brazil has shown that there was no relationship between the number of insecticide sprays and HLB incidence; and systemic insecticide applications in localized areas were not efficient in preventing CLas transmission by infectious psyllids from external sources [12]. Another study done by Hall et al. (2013) concluded that none of the ACP management programs they investigated prevented introduction and spread of HLB [9]. Berhamin-Filho et al. (2009) reported that eradication of symptomatic trees is more effective in controlling HLB incidence than use of insecticides [9, 12]. However, eradication of symptomatic trees may not be a viable solution to control HLB, since CLas has a long and highly variable incubation time. Approximately 1 to 2.5 years of incubation time is estimated for 7 to 10 years old trees, whereas it could be only 6 to 12 months for younger plants. Therefore, CLas-infected but asymptomatic trees may act as a source of infection [1]. Within an infected plant, bacterial populations vary in different parts of the tree and may be below the threshold of PCR detection limit, but grafting experiments still can result in new infected trees [1, 13–15]. PCR detection in the field is generally negative or inconclusive for 9 to 12 months, or longer, after trees become infected [16]. One of the solutions to HLB is to develop resistant cultivars through breeding programs. However, it would require many years of field testing to determine horticultural suitability of a new cultivar [16]. Even the most promising resistant cultivars could become susceptible as a result of the pathogen acquiring resistance through mutations [16]. There are reports of new varieties with enhanced resistance to HLB through the introduction of resistance genes [17, 18]. Consumer acceptance of such technologies could play an important role in commercialization of the end product.

Xanthomonas citri subsp. citri (Xcc) is another important pathogen that causes citrus cancer in Florida [19]. It is a serious disease in commercial citrus cultivars such as grapefruit (Citrus paradisi Macf.) and sweet orange (Citrus sinensis (L.) Osbeck) [20]. Xcc is an epiphyte that infects host plants through stomata and wounds [19]. Raised corky lesions on twigs and fruit,
defoliation, twig dieback, premature fruit drop and general decline symptoms are associated with citrus canker [19, 20]. Control of citrus canker in susceptible citrus cultivars is difficult when the plants are grown in tropical and subtropical areas [21, 22]. Xcc can infect new host plants from lesions on infected trees when there is moisture on the lesion surface [23]. Rain water splashes can disperse Xcc at short ranges whereas windblown rain droplets can disseminate Xcc at medium to long ranges [19]. Copper bactericides have been successful in controlling citrus canker but there are several disadvantages to using copper fungicides, including development of copper resistance in Xcc populations and phytotoxicity in citrus soils as a result of accumulation of copper [24–26]. Therefore, Zn-based bactericides could provide a better alternative to copper-based bactericides in the future [20].

In this study, we evaluated the antimicrobial properties of a novel Zn-chelate, ternary solution (TSOL), compared to the known antimicrobial compound ZnO. TSOL has been designed as a systemic antimicrobial compound [27] and is one of the main active ingredients in the experimental formulation MS3T (Multifunctional, surface/subsurface/systemic therapeutic) [27]. Zn-chelates have been used as a fertilizer in agriculture [28] and foliar application of Zn-chelates has been found to be more effective than application of ZnSO$_4$ to alleviate Zn deficiency in cereals [29]. Antimicrobial properties of many Zn-chelates have been reported previously [30]. Wang et al. (2004) found that Zn-chelates have 4–16 times lower MIC compared to that of ZnSO$_4$. The effectiveness of Zn-chelates was consistent with both Gram-positive and Gram-negative bacteria [30]. ZnO, the most common form of Zn that has been used as an antimicrobial compound, has antimicrobial activity against Gram-positive bacteria such as Bacillus subtilis and Staphylococcus aureus [31–33] and Gram-negative bacteria such as Pseudomonas aeruginosa, Campylobacter jejuni and Escherichia coli [34–38]. Release of reactive oxygen species and Zn-ions are responsible for the antimicrobial properties of ZnO [39, 40]. However, bulk ZnO may have limited uptake and systemic movement in plants as a result of its particle size and is not effective against systemic plant pathogens such as CLas.

The objective of this work was to evaluate effectiveness of the Zn-chelate TSOL to control growth and biofilm formation of Liberibacter crescens (Lcr) and Xcc in vitro in batch cultures and under constant nutrient flow conditions in microfluidic chambers (MC) mimicking the plant vascular system. Since CLas could not be maintained in culture, we used Lcr as a surrogate for CLas since it is the only member of the genus Liberibacter that has been cultured [41, 42]. We demonstrate that TSOL inhibits growth and biofilm formation of Xcc and Lcr in batch cultures and under flow conditions in MC. Furthermore, TSOL moves systemically in plants and can reach concentrations above MIC of Xcc and Lcr in leaves, stems and roots.

Materials and methods

Bacterial strains and growth conditions

*Xanthomonas citri* subsp. citri (Xcc) strain 306 was grown at 28˚C with shaking at 150 rpm in Silva Buddenhagen (SB) medium or on SB agar plates [43]. *Liberibacter crescens* BT-1 (Lcr) [41] was grown at 28˚C with shaking at 150 rpm in BM7 medium or on BM7 agar plates [41]. Stocks of the strains in 20% glycerol were kept frozen at -80˚C.

Synthesis and major properties of TSOL

TSOL is a Zn-based antimicrobial ternary complex consisting of zinc metal, salicylic acid, and hydrogen peroxide components synthesized according to Santra et al. [27]. A manuscript describing the formulation and molecular aspects of TSOL has been recently published by our group [44]. Salicylic acid can chelate metals with 2+ oxidation states and form a stable complex primarily through carboxyl and alcohol functional groups [45]. The stock solution of TSOL
retains 4.4% (wt/wt) metallic Zn making it suitable for industrial-scale applications. As synthesized, TSOL solution was observed to be a white colloidal solution having a specific gravity of 1.14 g/cm³. The pH of TSOL stock solution was 3.4 and water (pH 6.5)-diluted TSOL solutions at 800 µg/mL and 1,600 µg/mL increased the pH to 5.0 and 4.8, respectively.

**Evaluation of growth inhibition and minimum inhibitory concentration (MIC) of Xcc and Lcr treated with TSOL and ZnO**

Xcc cells were obtained from frozen stocks and re-streaked once on SB agar (SBA) plates incubated at 28˚C before use. Liquid cultures of Xcc were started by collecting cells from a re-streaked SBA plate using 10 ml of SB medium. Xcc was inoculated to a final concentration of optical density (OD₆₀₀) of 0.05 (approximately 5x10⁵ cells) in 2 ml of SB medium containing a TSOL or ZnO concentration gradient ranging from 0–150 ppm in 24-well tissue culture plates (VWR International, LLC). Two ml of uninoculated SB medium containing a TSOL or ZnO concentration gradient ranging from 0–150 ppm metallic Zn were used as blanks. The 24-well plates were sealed with Parafilm (Bemis Company, Inc) and surgical tapes (Durapore™, 3M Healthcare) to prevent evaporation and incubated at 28˚C with shaking at 150 rpm for 3 days. Total growth of Xcc was measured by OD₆₀₀ using a plate reader (CYTATION 3 imaging reader, BioTek Instruments, Inc.). Planktonic growth was determined by transferring SB medium with TSOL or ZnO from inoculated and non-inoculated plates into new 24-well plates and measuring OD₆₀₀. Biofilm growth was measured following the protocol described by O’Toole and Kolter (1998) using the original plate after removing liquid media for planktonic growth [46]. MIC was determined as described by Wang et al. [47].

A BM7 plate was inoculated with Lcr from a frozen stock culture and incubated at 28˚C for 7–10 days. A liquid culture of Lcr was obtained by pouring 10 ml of BM7 medium into a 7-10-day old plate of Lcr. Lcr was inoculated to a final concentration of OD₆₀₀ of 0.1 (approximately 5x10⁵ cells) and total, planktonic and biofilm growth were measured as described above following TSOL or ZnO treatment. Lcr batch cultures were incubated at 28˚C with shaking at 150 rpm for 7 days.

**Determination of viability inhibition using alamarBlue assay**

Xcc and Lcr were grown in 24-well plates in SB or BM7 medium, respectively, with a TSOL or ZnO concentration gradient ranging from 0–150 ppm as described above for 3 days. Two ml of uninoculated SB medium containing a TSOL or ZnO concentration gradient ranging from 0–150 ppm metallic Zn were used as blanks. An aliquot of 200 µl alamarBlue reagent (Serotec Inc, MS) was added to each well and incubated for 18 hours at 28ºC with shaking at 150 rpm. Absorbances at 570 nm and 600 nm were measured using the Cytation 3 plate reader. Xcc viability was assessed as the Percentage Difference in Reduction of alamarBlue (PDRAB), as described by the manufacturer. Briefly, the equation PDRAB = [(O₂xA₁)–(O₁xA₂)]/[(O₂xP₁)–(O₁xP₂)] × 100 was used, where O₁ = molar extinction coefficient (E) of oxidized alamarBlue at 570 nm; O₂ = E of oxidized alamarBlue at 600 nm; P₁ = absorbance of positive growth control at 570 nm; P₂ = absorbance of positive growth control at 600 nm; A₁ = absorbance of test wells at 570 nm; A₂ = absorbance of test wells at 600 nm. Percentage of viability inhibition, used for graphs, was calculated by this equation: Viability inhibition (%) = 100 –PDRAB.

**Determination of culturability by plating**

Xcc and Lcr were grown in 24-well plates as described in the alamarBlue assay. An aliquot of 100 µl was taken from each well and plated onto SB agar or BM7 agar plates, respectively. SB
plates with Xcc were incubated at 28˚C for 6 days, and BM7 agar plates with Lcr were incubated at 28˚C for 4 weeks.

Evaluation of biofilm formation in microfluidic chambers (MC)

The fabrication of microfluidic chambers (MC) was performed as previously described by De La Fuente et al. (2007) [48]. A two parallel-channel MC design was used for all the MC experiments. The MC were 80 μm wide, 3.7 cm long and 50 μm deep. Each channel in the MC has two separate inlets to introduce bacterial growth media and bacteria and one outlet to collect flowing bacteria growth media. Inlets and outlets in MC were connected to tubing, syringes and syringe pumps (Pico Plus; Harvard Apparatus, Holliston, MA, USA) as described in De La Fuente et al. (2007). In separate experiments, either Xcc or Lcr bacterial suspensions obtained from SB or BM7 plates, respectively, were introduced to both MC channels through one of the two inlets and the media flow in the MC was set to 0.05 μl/min. Syringe pumps connected to bacterial inlets were stopped after 2–3 hours when bacteria were attached to the MC walls. Attached bacteria was allowed to form biofilm in the MC for 3 days (Xcc) or 7 days (Lcr) or until the bacteria attach to MC and form abundant biofilm in both channels. Lcr forms abundant biofilm in a modified BM7 medium [(with addition of 0.75 g per L of methyl-β-cyclodextrin and without fetal bovine serum (FBS)] named bBM7+0.75mβc [49]. Therefore, this medium was used for MC experiments with Lcr. A growth medium containing 60 ppm TSOL was introduced in one channel after biofilm formed in both channels, and changes in biofilm formation before and after TSOL treatment compared to biofilm from the untreated channel were observed with a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY) with phase-contrast and Normarski differential interference contrast (DIC) optics. Time-lapse images were recorded every 1 min (Xcc) or 2 min (Lcr) with a Nikon DS-Q1 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) connected to the microscope and controlled by NIS-Elements imaging software version 3.0 [48].

Evaluation of TSOL uptake by citrus plants

A plant uptake study was performed using Citrus reshni (Cleopatra mandarin) seedlings in growth chambers (Panasonic Environmental Test Chamber, Model MLR-352H, Kadoma, Japan). Temperature and relative humidity were maintained at 35˚C and 85%. Eleven-month old seedlings were foliar-sprayed using a hand-pump garden sprayer with the following treatments: 300 ml of TSOL, ZnO (ZnO grade 400, Zinc Oxide LLC) either at 800 μg Zn/mL or 1600 μg Zn/mL concentrations, and deionized water as a control. Foliar-spray concentrations were selected based on the field application rate of MS3T to control citrus canker. Leaf spraying was carried out in the morning to promote plant uptake through stomata and the pots were covered with Parafilm to prevent treatment solutions coming into contact with soil and seedling roots. Seedlings were returned to the growth chamber for two days prior to the analysis of Zn content. Plants were carefully removed from the soil and surface-bound material was removed as follows: whole plants were dipped in 40 ml of 1% detergent (Alconox, Alconox Inc.) in 2 L and gently rinsed for 10 minutes, and the rinsed twice with 2 L of DI water for 2 minutes. Afterwards, plants were rinsed in 2 L of 0.1% HCL (aq) for 30 seconds and 2 L of DI water for 2 minutes in order to remove surface deposited mineral elements including Zn [50]. Leaves, roots and stem sections were separated after washing and left in a drying oven (Cabela’s Inc, Sydney, NE) at 45˚C for 48h. The dried leaves, stems and roots were pulverized separately using mortars and pestles prior to acid digestion. One gram of dry powder of leaves and stems, and 0.5 grams of roots were acid digested following EPA method 3050B "Acid
Digestion of Sediments, Sludge, and Soils” [51]. Zn concentrations in leaves, stems and roots were quantified using Atomic Absorption Spectroscopy (AAS).

**Statistical analysis**

Treatment effects were analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks and significant differences between treatments and the non-treated control were analyzed using Dunn’s Method (tested at \( P < 0.05 \)) using the SigmaPlot Software, Version 13.0 (Systat Software, Inc., San Jose California USA).

**Results**

**Inhibition of growth and biofilm formation by TSOL**

Xcc grows well and forms robust biofilm in SB medium compared to Nutrient Broth (NB) in MC (data not shown). Therefore, we selected SB medium to test growth and biofilm formation in batch cultures and under flow conditions in MC. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of TSOL resulting in no growth of Xcc after 3 days [47, 52]. Total and planktonic growth of Xcc was significantly lower \(( P < 0.001)\) compared to the untreated control when Xcc was treated with TSOL concentrations of 20 ppm or higher (Fig 1A). Treatment of TSOL at concentrations of 40 ppm or higher completely inhibited the growth of Xcc, therefore 40 ppm was considered as the MIC of TSOL for Xcc. Biofilm formation of Xcc was significantly reduced using 20 ppm \(( P = 0.019)\) or higher \(( P < 0.001)\) concentrations of TSOL (Fig 1B).

In the case of Lcr total and planktonic growth was significantly lower \(( P < 0.001)\) compared to the untreated control when treated with 30 ppm or higher concentrations of TSOL (Fig 1C). Treatment of TSOL at a concentration of 40 ppm or higher inhibited the growth of Lcr (Fig 1C), therefore 40 ppm was considered as the MIC of TSOL for Lcr. Biofilm formation of Lcr was low in BM7 medium and the effects of biofilm formation in response to TSOL was highly variable (Fig 1D). Nevertheless inhibition of biofilm formation was observed at TSOL concentrations higher than 30ppm.

**Inhibition of growth and biofilm formation by ZnO**

Antimicrobial properties of ZnO were tested on Xcc and Lcr (Fig 2) to compare its effectiveness compared to TSOL on Xcc and Lcr. Total growth and planktonic growth of Xcc was significantly lower \(( P < 0.001)\) compared to the untreated control when treated with 35 ppm ZnO (Fig 2A). However, there was a slight increase in both total growth and planktonic growth of Xcc when treated with 40 ppm and 45 ppm, respectively, but still the growth was significantly lower \(( P = 0.001, P = 0.004; \text{and planktonic growth } P = 0.001, P = 0.013 \text{ for 40 ppm and 45 ppm, respectively})\) compared to the untreated control. Total and planktonic growth of Xcc was significantly lower \(( P < 0.001)\) for all the treatments at ZnO concentrations of 50 ppm or higher. However, ZnO was not bacteriostatic to Xcc in the range of ZnO concentrations tested \(( < 200 \text{ ppm})\) (Table 1). Therefore, the MIC of ZnO was considered to be greater than 200 ppm for Xcc. Biofilm formation of Xcc showed a similar pattern to that of total and planktonic growth. Xcc biofilm formation was inhibited at ZnO concentrations of 50 ppm or higher (Fig 2B).

Total and planktonic growth of Lcr was significantly lower \(( P < 0.001)\) compared to the untreated control when Lcr was treated with 30 ppm ZnO (Fig 2C). There was no increase of growth in Lcr treated with ZnO concentrations of 30 ppm or higher. Therefore the MIC of ZnO on Lcr was established at 30 ppm. Biofilm formation of Lcr was minimal in BM7 medium and the effects of biofilm formation in response to ZnO were highly variable as observed with TSOL treatment (Fig 2D).
Effect on cell viability of TSOL and ZnO

The percentage reduction in cell viability of Xcc and Lcr after TSOL treatment was measured by alamarBlue assay (Fig 3) \[53, 54\]. Both Xcc and Lcr treated with TSOL showed 80% or higher reduction in cell viability at TSOL concentrations of 40 ppm or higher (Fig 3A and 3B). Conversely, reduction in Xcc viability by ZnO was much less severe. The highest inhibition of viability (40%), was observed with 200 ppm ZnO concentration (Fig 3C). Reduction in Lcr viability by ZnO treatment was much higher compared to TSOL as Lcr treated with ZnO showed 90% or higher inhibition of viability at ZnO concentrations of 30 ppm or higher (Fig 3D).

Effects of TSOL and ZnO on culturability of bacteria

TSOL inhibited the growth (as measured by OD\(_{600}\), see above) of Xcc at TSOL concentrations of 40 ppm or higher. However, when TSOL-treated (10–120 ppm) Xcc cells were plated on SBA, colonies were observed 3–6 days after plating (Table 1). Therefore, TSOL was considered bacteriostatic to Xcc from 40 ppm up to 120 ppm \[55\]. However, no Xcc colonies were...
observed when Xcc was treated with 150 ppm of TSOL followed by plating on SBA. Therefore, TSOL was considered bactericidal to Xcc at 150 ppm [55]. When Xcc was treated with ZnO and plated on SBA plates, Xcc colonies were observed after 3–6 days even with 200 ppm ZnO treatment (Table 1). Therefore no bactericidal effect of ZnO on Xcc was observed in the concentration range tested (< 200 ppm), although a bacteriostatic growth inhibition was observed at ~35 ppm (see above).

For the case of Lcr, colonies were observed on BM7 agar plates when Lcr was treated with up to 150 ppm TSOL followed by plating. Therefore, TSOL is bacteriostatic to Lcr between 30 and 150 ppm. There were no Lcr colonies on BM7 agar plates when Lcr was treated with 200 ppm TSOL followed by plating. Therefore, TSOL was considered bactericidal to Lcr at TSOL concentrations of 200 ppm or higher (Table 1). There were no Lcr colonies on BM7 agar plates when Lcr was treated with 20 ppm or higher ZnO concentrations followed by plating. Therefore, ZnO is bactericidal to Lcr at 20 ppm or higher concentrations (Table 1).

Fig 2. Inhibition of growth and biofilm formation by ZnO. Xanthomonas citri subsp. citri (Xcc) (A, B) and Liberibacter crescens (Lcr) (C, D) batch cultures in 24-well plates were treated with a ZnO concentration gradient ranging from 0–200 ppm. Total and planktonic growth (A, C) and biofilm formation (B, D) were measured by spectrophotometry (see materials and methods). Values on each graph represent means from 3 independent experiments (n = 18, with n = 6 for each treatment at each experimental repetition), and the error bars show standard error (SE) of the mean. Asterisks (‘) denote statistically significant differences (P < 0.05) of the treatment compared to untreated control. 

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TSOL activity under flow conditions

CLas is a phloem-restricted pathogen [2]. Therefore, in vitro evaluation of any antimicrobial compound to control CLas has to be tested in a comparable system to plant vascular tissues. Evaluation of TSOL on Xcc batch cultures (Fig 1B) and in MC (S1 Fig) showed that TSOL prevented initial biofilm formation of Xcc at TSOL concentrations of 40 ppm or higher in batch cultures and 60 ppm TSOL concentration in MC. Biofilms exhibit elevated antimicrobial tolerance as a result of their structural and physiological adaptations [56, 57]. Therefore, we evaluated effectiveness of TSOL to stop further growth of Xcc (Fig 4) and Lcr (Fig 5) biofilms after the initial biofilm formation using concentrations slightly higher than the determined MIC. Xcc forms biofilm in MC channels when SB medium is flowing through both MC channels at a rate of 0.05 μl/min (Fig 4A). The biofilm mass increased with time as Xcc cells divide in the biofilm. Further growth of biofilm was inhibited in the MC channels treated with SB medium containing 60 ppm TSOL at a flow rate of 0.05 μl/min, although biofilm was not disrupted. On the contrary a steady growth of Xcc biofilm was observed in the MC control channel containing only SB medium (Fig 4B).

Effects of TSOL on biofilm formation of Lcr were analyzed as described above. Lcr grows very slowly compared to Xcc in batch cultures. Furthermore, the biofilm formation and attachment of cells to the MC walls was very low in Lcr compared to that of Xcc. Therefore, Lcr was grown in bBM7+0.75mβc medium in which Lcr shows more cell attachment to MC and forms robust biofilm [49]. Lcr formed biofilm in MC channels when bBM7+0.75mβc medium flows through both MC channels (Fig 5). Lcr cells attached to the MC channel containing only the bBM7+0.75mβc medium showed an increase in the amount of biofilm over time, whereas Lcr cells attached to the MC channel treated with 60 ppm TSOL in bBM7+0.75mβc medium did not divide or show any increase in biofilm (Fig 5).

Plant uptake and movement of TSOL

TSOL should have better plant uptake by leaves and systemic movement in the phloem to control systemic plant pathogens such as CLas. Therefore, we evaluated Zn uptake and movement in citrus plants by spraying leaves with TSOL and bulk ZnO. According to our results, 800 μg Zn/mL and 1600 μg Zn/mL treatments of TSOL had significantly higher uptake in leaves

Table 1. Culturability of Xcc and Lcr treated with TSOL or ZnO.

| Treatment (ppm) | Culturability of Xcc | Culturability of Lcr |
|----------------|----------------------|----------------------|
| 0              | + + + +              | + + + +              |
| 10             | + + + +              | + + + +              |
| 20             | + + + +              | + + + +              |
| 30             | + + + +              | + + + +              |
| 40             | + + + +              | + + + +              |
| 50             | + + + +              | + + + +              |
| 60             | + + + +              | + + + +              |
| 90             | + + + +              | + + + +              |
| 120            | + + + +              | + + + +              |
| 150            | - + + +              | - + + +              |
| 200            | - + + +              | - + + +              |

(+) colonies present; (-) colonies absent.

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(P = 0.002 and P < 0.001, respectively) compared to the same concentrations of ZnO and also the untreated control (Fig 6). The 1600 μg Zn/mL TSOL treatment had significantly higher (P = 0.05) Zn uptake by leaves than 800 μg Zn/mL TSOL treatment. Significantly higher Zn content was found in stems of the citrus plants treated with 1600 μg Zn/mL TSOL and ZnO treatments (P < 0.001). However, there was no significant difference in Zn content between the stems treated with 1600 μg Zn/mL of TSOL and ZnO (Fig 6). There was significantly higher (P < 0.001) Zn content in the roots of citrus plants treated with 1600 μg Zn/mL TSOL (Fig 6). According to our results TSOL has significantly higher leaf uptake and movement to roots compared to that of ZnO.

**Discussion**

Currently, no viable economical treatment exists to reduce CLas populations in HLB-affected trees or to reverse the decline of HLB-affected trees [58]. Thermal therapy of HLB-affected plants has been tested previously, but lack of treatment effects in the root system where CLas is found is a problem [58]. Elimination of CLas from the entire citrus tree through thermal
therapy may be impossible without a chemical treatment [58]. Trunk injection of plant defense activators and antibiotics has been tested and showed reduction in CLas populations and HLB symptom expression [59–61]. However, trunk injection is labor intensive and is not an economically sustainable strategy. There are several effective ACP repellants including sulfur volatiles and essential oils that have been shown to be successful in the field, but they are not available to citrus growers since there are no commercially available application methods for these compounds [62–64]. The Kaolin clay-based ACP repellant SurroundWP has shown good efficacy in altering ACP feeding behavior and is commercially available [65, 66]. However, without an antimicrobial treatment against CLas, ACP repellant alone will not be enough to control the spread of HLB. Therefore, a comprehensive HLB management solution that is industrially viable, affordable to growers and sustainable and also has minimal negative impact on the environment and human health is required.

Fig 4. Control of biofilm growth of Xcc by TSOL. Xanthomonas citri subsp. citri (Xcc) cells were introduced to the upper and lower channels of microfluidic chambers (MC) containing SB medium (control) under constant flow of 0.05 μL/min. Sixty ppm TSOL in SB medium was introduced to the lower channel after biofilm was formed in both upper and lower channels. Growth of biofilm in treated and non-treated channels was observed by time-lapse video imaging microscopy. A) t1 = 0h, t2 = 4h. B) t1 = 0 h, t2 = 3–4 h, t3 = 4–7 h, t4 = 7–8 h. Scale bar = 50 μm. See also S1 and S2 File.
MS3T technology has been developed by combining several products customized to deliver comprehensive HLB and citrus canker control [27]. MS3T is made of chemicals which are either naturally occurring, “Generally Recognized as Safe” (GRAS) by the USA Food and Drug Administration (FDA) or the USA Environmental Protection Agency (EPA) approved for “Food Use Only” [67, 68]. The current MS3T formulation consists of Kaolin clay, a quaternary ammonium compound (Fixed-Quat) and TSOL. A naturally-occurring aluminosilicate, Kaolin clay is an ACP repellent and is used as a delivery system for TSOL [27, 69, 70]. Fixed-Quat is an antimicrobial compound and was designed to control surface and subsurface restricted pathogens such as Xcc. TSOL is a water soluble Zn-chelate [27] and is designed to be a plant tissue permeable systemic bactericide [27]. As we describe here, TSOL can be absorbed through leaves and transported to stems and root systems most likely through phloem flow. Therefore, it is expected to be effective against systemic plant pathogens such as CLAs.

There are many reports of antimicrobial properties of ZnO [31–38], the most common Zn-based chemical commercially available that was used here for comparison. Antimicrobial
properties of ZnO are based on the catalysis of formation of ROS from water and oxygen and the release of Zn ions [35, 39, 40, 71–73]. These properties are directly dependent on the particle size of the ZnO. Bulk ZnO is not easily taken up by plants and has limited systemic movement in plants as a result of its bigger particle size. Therefore, bulk ZnO as an antimicrobial compound is ineffective in controlling systemic plant pathogens. Nano-ZnO formulations such as Zinkicide has better antimicrobial activity as well as proposed systemic movement in plants [20]. However, Zinkicide is not commercially available to citrus farmers at this time. Therefore, Zn-chelates such as TSOL could offer antimicrobial activity similar to Nano-ZnO formulations with better plant uptake and could be fast-tracked for EPA registration.

According to studies of antimicrobial activity of ligands and its complexes, metal chelates have higher antimicrobial activity than their free ligand [74, 75]. Polarity of a metal ion is reduced as a result of overlapping ligand orbital and partial sharing of the positive charge with donor groups [74]. Therefore, metal chelates are more lipophilic than free ligands. Higher liposolubility of metal chelates could be partly responsible for higher antimicrobial activity of metal chelates compared to that of free ligands. Therefore, metal chelates can penetrate lipid membranes and block metal binding sites on the enzymes of bacteria [74]. We observed a higher antimicrobial activity in TSOL compared to bulk ZnO against Xcc. However, that was not the case against Lcr. Since, TSOL is designed to be a systemic antimicrobial compound, TSOL is more effective than bulk ZnO against systemic pathogens such as CLAs by reaching Zn concentrations higher than MIC in planta [27]. Our results from plant uptake and movement of Zn in citrus plants treated with TSOL and ZnO show significantly higher amounts of TSOL taken up by citrus leaves and this leads to significantly higher Zn concentrations in the roots of citrus plants compared to those of ZnO treated plants. Therefore, our plant Zn uptake study shows that even though ZnO has slightly better antimicrobial activity against Lcr, its limited uptake and movement in plants may hinder its effectiveness as a systemic bactericide.
Uptake of TSOL by the leaves, basipetal movement and the resulting high TSOL concentrations in the roots within two days after treatment application all support the movement of TSOL through the phloem. \textit{C. las} infects phloem and moves from infected leaves to roots and back to young leaves through the phloem \cite{76}. Therefore, higher TSOL concentrations in the leaves, stems and roots should help to lower \textit{C. las} populations in planta.

The MIC of an antimicrobial agent is the lowest concentration of the antimicrobial compound that can kill (bactericidal activity) or inhibit (bacteriostatic activity) the growth of bacteria \cite{52}. Therefore, the MIC values do not indicate whether a particular antimicrobial compound is bacteriostatic or bactericidal. Plating of treated bacteria in respective growth medium helps to determine whether the antimicrobial compound is bactericidal or bacteriostatic. However, some pathogenic and non-pathogenic bacteria can enter a viable but non-culturable (VBNC) state when they are exposed to stress \cite{77}. Processes that are normally bactericidal to bacteria could also lead to a VBNC state in bacteria \cite{79}. Del Campo et al. (2009) reported that Xcc can enter a VBNC state after copper sulfate treatment for a short time \cite{80}. Several methods have been described to determine viable counts of bacteria in a VBNC state \cite{81–83}. We evaluated the viability of Xcc and Lcr by the alamarBlue assay (Fig 3) \cite{84–86}. However, we did not observe Xcc or Lcr entering a VBNC state after TSOL or ZnO treatments according to an oxidation-reduction indicator. According to Del Campo et al. (2009) the VBNC state in Xcc was induced by a 10 min treatment of 135 $\mu$M CuSO$_4$ but a 30 min treatment did not induce a VBNC state in Xcc \cite{80}. VBNC state in Lcr after a treatment with an antimicrobial compound has not been previously recorded. Since MS3T is designed to form a layer on citrus leaves and fruits, and to release TSOL over time \cite{27}, the probability of Xcc exposure to a high dose of TSOL is very low in field conditions. Therefore, our results suggest it is unlikely that Xcc or Lcr will enter the VBNC state under field conditions.

Culturability of Xcc after TSOL treatment indicates that TSOL is bacteriostatic to Xcc at concentrations between 40–120 ppm, and bactericidal at 150 ppm or higher. ZnO is not bactericidal to Xcc up to 200 ppm. According to culturability data after TSOL treatment, TSOL is bacteriostatic to Lcr from 40–150 ppm and bactericidal to Lcr at 200 ppm. ZnO is bactericidal to Lcr at 20 ppm or higher ZnO concentrations. Previous studies on antimicrobial activity of ZnO have shown that ZnO is more effective against Gram-positive bacteria than Gram-negative bacteria \cite{33, 35, 38, 87}. The differences in antimicrobial activity of ZnO within Gram-negative bacteria may result from antioxidant cellular content and resistance to oxidative stress in different bacteria species \cite{73}. We observed such differences in antimicrobial activity of ZnO against Xcc and Lcr. However, there was no such difference in antimicrobial activity of TSOL against Xcc and Lcr. Graham et al (2016) reported that a ZnO nano formulation with 62.5 ppm MIC against \textit{Xanthomonas alfalfae} was effective in reducing citrus canker lesion development in citrus leaves \cite{20}. We have determined the MIC of TSOL against Xcc to be 40 ppm. Therefore, we can expect similar efficacy of TSOL against citrus canker.

Many bacteria in the natural environment form biofilm when they are associated with solid surfaces \cite{56, 57, 88, 89}. Bacteria cells in biofilm are physiologically different from those of planktonic cells \cite{90, 91}. Biofilms have higher tolerance to antimicrobial compounds as a result of their distinct physiology and biofilm structure \cite{57}. Plant pathogenic bacteria form various multicellular aggregations on plant surfaces. A few other plant pathogens form multicellular aggregates in phloem and xylem and they are generally dependent on insect vectors or wounding for infection \cite{92–95}. \textit{C. las} form extensive biofilm in ACP alimentary canals whereas biofilm formation of \textit{C. las} in planta has not been reported \cite{76, 96, 97}. We evaluated effectiveness of TSOL to control biofilm formation of Xcc and Lcr in MC under similar conditions found in
vascular tissues of plants. Studies of biofilm formation of vascular plant pathogens such as *Xylella fastidiosa* in MC have provided insights of pathogenicity and possible treatments options [48, 98, 99]. TSOL was effective against at controlling growth and biofilm formation of Xcc and Lcr in batch cultures as well as in MC. Control of biofilm formation of Xcc required slightly higher TSOL concentrations (60 ppm) than MIC (40 ppm) for Xcc in batch cultures. A 60 ppm TSOL concentration prevented the growth of already formed Xcc and Lcr biofilm. However, it did not disrupt already formed Xcc and Lcr biofilms in MC. MIC values, investigation of viability with plating and alamarBlue assays, experiments under flow conditions (MC), TSOL uptake by citrus leaves and systemic movement of TSOL show that it has potential as an antimicrobial treatment to control growth and biofilm formation of plant surface pathogens such as Xcc and systemic plant pathogens such as CLAs. We could not investigate effectiveness of TSOL against CLAs in vitro since CLAs is not cultivable. Therefore, ongoing field efficacy testing of TSOL on CLAs affected plants will provide a better understanding of the effectiveness of TSOL against CLAs. We did not observe any signs of phytotoxicity in citrus leaves after TSOL treatment (S2 Fig). Hippler et al (2015) have studied uptake and distribution of Zn by citrus plants after Zn treatment and found that citrus fruits are not sinks of zinc [100]. Application of the $^{68}$Zn isotope showed significant increases of Zn levels in roots, trunks, branches, leaves and flowers. However, there was no significant increase in Zn levels in fruits [100]. Furthermore, oral LD50 of Zn(NO$_3$)$_2$ is 1190 mg/kg which is two-fold higher than that of commonly used antimicrobial compound CuSO$_4$ [101, 102]. Efficacy of TSOL is currently being tested in the field to control HLB and citrus canker.

Supporting information

S1 Fig. Evaluation of effects of TSOL on biofilm formation of Xcc in microfluidic chambers. *Xanthomonas citri* subsp. *citri* (Xcc) was introduced to upper and lower microfluidic chamber (MC) channels while SB medium was flowing through the upper channel with the flow rate of 0.05 $\mu$L/min and the lower channel was treated with SB medium containing 60 ppm with a flow rate of 0.05 $\mu$L/min. t1 = 0 h, t2 = 2–3 h, t3 = 5–6 h, t4 = 8–9 h, t5 = 10 h. Scale bar = 80 $\mu$m.

(TIFF)

S2 Fig. Evaluation of phytotoxicity of TSOL, ZnO and copper sulfate after foliar spraying. a) DI water. (b) TSOL at 800 $\mu$g Zn/mL, (c) TSOL at 1,600 $\mu$g Zn/mL, (d) ZnO 400 at 800 $\mu$g Zn/mL, (e) ZnO 400 at 1,600 $\mu$g Zn/mL, (f) Copper sulfate at 1,600 $\mu$g Cu/mL. No injury on plants was observed for all materials at tested concentrations. Images were taken 72 hours after leaf spraying.

(TIFF)

S1 File. Growth of *Xanthomonas citri* subsp. *citri* in microfluidic chambers. *Xanthomonas citri* subsp. *citri* (Xcc) cells were introduced in both channels of microfluidic chambers (MC) containing SB medium (control) under constant flow of 0.05 $\mu$L/min. Movie shows growth and biofilm formation observed by time-lapse video imaging microscopy. Scale bar = 50 $\mu$m. Movie corresponds to images shown in Fig 4A.

(MP4)

S2 File. Effect of TSOL on *Xanthomonas citri* subsp. *citri* growth and biofilm formation in microfluidic chambers. *Xanthomonas citri* subsp. *citri* (Xcc) cells were introduced to the upper and lower channels of microfluidic chambers (MC) containing SB medium under constant flow of 0.05 $\mu$L/min. 60 ppm TSOL in SB medium was introduced to the lower channel after biofilm was formed in both upper and lower channels. Growth of biofilm in treated and
non-treated channels was observed by time-lapse video imaging microscopy. Scale bar = 50 μm. Movie corresponds to images shown in Fig 4B. (MP4)

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References

1. Gottwald TR. Current epidemiologic understanding of citrus huanglongbing. Annual review of phytopathology. 2010; 48:119–39. https://doi.org/10.1146/annurev-phyto-073009-114418 PMID: 20415578

2. Bové JM. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of plant pathology. 2006:7–37.

3. Bassanezi RB, Montesino LH, Gasparoto MCG, Bergamin Filho A, Amorim L. Yield loss caused by huanglongbing in different sweet orange cultivars in São Paulo, Brazil. European journal of plant pathology. 2011; 130(4):577–86.

4. Aubert B. Citrus greening disease, a serious limiting factor for citriculture in Asia and Africa. Proc Intern Soc Citricult. 1992; 1:817–20.

5. Gottwald T, Aubert B, Zhao X-Y. Preliminary analysis of Citrus greening(huanglungbin) epidemics in the People’s Republic of China and French Reunion Island. Phytopathology. 1989; 79(6):687–93.

6. Lin C. Notes on citrus yellow shoot disease. Acta Phytophylact Sin. 1963; 2:243–51.

7. Schwarz R, Knorr L, Prommintara M. Presence of citrus greening and its psylla vector in Thailand. FAO Plant Protection Bulletin. 1973; 21:132–8.

8. Johnson E, Wu J, Bright D, Graham J. Association of ‘Candidatus Liberibacter asiaticus’ root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. Plant pathology. 2014; 63(2):290–8.

9. Hall DG, Gottwald TR, Stover E, Beattie GAC. Evaluation of management programs for protecting young citrus plantings from huanglongbing. HortScience. 2013; 48(3):330–7.

10. Gottwald TR. Citrus canker and citrus huanglongbing, two exotic bacterial diseases threatening the citrus industries of the Western Hemisphere. Outlooks on Pest Management. 2007; 18(6):274.
11. Rogers ME, Stanisly PA, Stelinski LL. Florida citrus pest management guide: Asian citrus psyllid and citrus leafminer. IFAS Extension Publication ENY-734 University of Florida, Gainesville, FL, USA. 2012.

12. Berhamin-Filho A, Gasparoto M, Bassanezi R, Amorim L, editors. Relationship between insecticide sprays and huanglongbing progress in a citrus orchard in Sao Paulo, Brazil. Proceedings of the International Research Conference on Huanglongbing, Orlando, Florida, USA, December; 2008.

13. Tatineni S, Sagaram US, Gowda S, Robertson CJ, Dawson WO, Iwanami T, et al. In planta distribution of ‘Candidatus Liberibacter asiaticus’ as revealed by polymerase chain reaction (PCR) and real-time PCR. Phytopathology. 2008; 98(5):592–9. https://doi.org/10.1094/PHYTO-98-5-0592 PMID: 18943228

14. Teixeira DC, Saillard C, Couture C, Martins EC, Wulff NA, Eveillard-Jagoueix S, et al. Distribution and quantification of Candidatus Liberibacter americanus, agent of huanglongbing disease of citrus in Sao Paulo State, Brasil, in leaves of an affected sweet orange tree as determined by PCR. Molecular and Cellular Probes. 2008; 22(3):139–50. https://doi.org/10.1016/j.mcp.2007.12.006 PMID: 18400468

15. Gottwald TR, McCollum TG. Huanglongbing solutions and the need for anti-conventional thought. Journal of Citrus Pathology. 2017; 4(1):1.

16. Dutt M, Barthe G, Irey M, Grosser J. Transgenic citrus expressing an Arabidopsis NPR1 gene exhibit enhanced resistance against Huanglongbing (HLB; Citrus Greening). PloS one. 2015; 10(9): e0137134. https://doi.org/10.1371/journal.pone.0137134 PMID: 26398891

17. Hao G, Stover E, Gupta G. Overexpression of a modified plant thionin enhances disease resistance to citrus canker and huanglongbing (HLB). Frontiers in plant science. 2016; 7:1078. https://doi.org/10.3389/fpls.2016.01076 PMID: 27499757

18. Graham JH, Gottwald TR, Cubero J, Achor DS. Xanthomonas axonopodis pv. citri: factors affecting successful eradication of citrus canker. Molecular plant pathology. 2004; 5(1):1–15. https://doi.org/10.1046/j.1364-3703.2004.00197.x PMID: 20565577

19. Graham J, Johnson E, Myers M, Young M, Rajasekaran P, Das S, et al. Potential of nano-formulated zinc oxide for control of citrus canker on grapefruit trees. Plant disease. 2016; 100(12):2442–7. https://doi.org/10.1094/PDIS-05-16-0598-RE PMID: 28832140

20. Alva A, Graham J, Anderson C. Soil pH and copper effects on young ‘Hamlin’ orange trees. Soil Science Society of America Journal. 1995; 59(2):481–7.

21. Adams LK, Lyon DY, Alvarez PJ. Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. Water research. 2006; 40(19):3527–32. https://doi.org/10.1016/j.watres.2006.06.004 PMID: 17011915
Kasemets K, Ivask A, Dubourguier H-C, Kahru A. Toxicity of nanoparticles of ZnO, CuO and TiO2 to bacteria Vibrio fischeri and crustaceans Daphnia magna and Thamnocephalus platyurus. Toxicology in vitro. 2009; 23(6):1116–22. https://doi.org/10.1016/j.toxive.2009.05.015 PMID: 19486936

Heinlaan M, Ivask A, Blinova I, Dubourguier H-C, Kahru A. Toxicity of nanosized and bulk ZnO, CuO and TiO2 to yeast Saccharomyces cerevisiae. Toxicology in vitro. 2009; 23(6):1116–22. https://doi.org/10.1016/j.toxive.2009.05.015 PMID: 19486936

Premanathan M, Karthikeyan K, Jeyasubramanian K, Manivannan G. Selective toxicity of ZnO nanoparticles toward Gram-positive bacteria and cancer cells by apoptosis through lipid peroxidation. Nanomedicine: Nanotechnology, Biology and Medicine. 2011; 7(2):184–92.

Kasemets K, Ivask A, Dubourguier H-C, Kahru A. Toxicity of nanoparticles of ZnO, CuO and TiO2 to yeast Saccharomyces cerevisiae. Toxicology in vitro. 2009; 23(6):1116–22. https://doi.org/10.1016/j.toxive.2009.05.015 PMID: 19486936

Sawai J, Shoji S, Igarashi H, Hashimoto A, Kokugan T, Shimizu M, et al. Hydrogen peroxide as an antibacterial factor in zinc oxide powder slurry. Journal of Fermentation and Bioengineering. 1998; 86(5):521–2.

Brayner R, Ferrari-Ililiou R, Brivois N, Djediat S, Benedetti MF, Fievé F. Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano letters. 2006; 6(4):866–70. https://doi.org/10.1021/nl052326h PMID: 16608300

Ohtsu T, Yamamoto O, Iida Y, Nakagawa Z-e. Antibacterial activity of ZnO powder with crystallographic orientation. Journal of Materials Science: Materials in Medicine. 2008; 19(3):1407–12. https://doi.org/10.1007/s10856-007-3246-8 PMID: 17914627

Landt MT, Fagen JR, Davis-Richardson AG, Davis MJ, Trippett EW. Complete genome sequence of Liberibacter crescens BT-1. Standards in genomic sciences. 2012; 7(2):271. https://doi.org/10.4056/sigs.3326772 PMID: 23408754

Fagen JR, Leonard MT, McCullough CM, Edirisinghe JN, Henry CS, Davis MJ, et al. Comparative genomics of cultured and uncultured strains suggests genes essential for free-living growth of Liberibacter. PLoS One. 2014; 9(1):e84469. https://doi.org/10.1371/journal.pone.0084469 PMID: 24416233

Mongkolsuk S, Loprasert S, Whangsuk W, Fuangthong M, Atichartpongkul S. Characterization of transcription organization and analysis of unique expression patterns of an alkyl hydroperoxide reductase C gene (ahpC) and the peroxide regulator operon ahpF-foxY-orfX from Xanthomonas campestris pv. phaseoli. Journal of bacteriology. 1997; 179(12):3950–5. https://doi.org/10.1128/jb.179.12.3950-3955.1997 PMID: 9190811

Liu SH, Rawal TB, Soliman M, Lee B, Maxwell T, Rajasekarar P, et al. Antimicrobial Zn-Based “TSOL” for Citrus Greening Management: Insights from Spectroscopy and Molecular Simulation. Journal of agricultural and food chemistry. 2019. Epub 2019/06/01. https://doi.org/10.1021/acs.jafc.9b02466 PMID: 31150237.

Anjaneyulu Y, Rao RP. Preparation, characterization and antimicrobial activity studies on some ternary complexes of Cu(II) with acetylacetone and various salicylic acids. Synthesis and Reactivity in Inorganic and Metal-Organic Chemistry. 1986; 16(2):257–72.

O’toole GA, Kolter R. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Molecular microbiology. 1998; 28(3):449–61. PMID: 9632250

Li J, Wang N. Foliar Application of Biofilm Formation-Inhibiting Compounds Enhances Control of Citrus Canker Caused by Xanthomonas citri subsp. citri. Phytopathology. 2014; 104(2):134–42. https://doi.org/10.1094/PHYTO-04-13-0100-R PMID: 23901828

De La Fuente L, Montanes E, Meng Y, Li Y, Burr TJ, Hoch H, et al. Assessing adhesion forces of type I and type IV pili of Xylella fastidiosa bacteria by use of a microfluidic flow chamber. Applied and environmental microbiology. 2007; 73(8):2690–6. https://doi.org/10.1128/AEM.02649-06 PMID: 17293518

Naranjo E, Merfa MV, Ferreira V, Jain M, Davis MJ, Bahar O, et al. Liberibacter crescens biofilm formation in vitro: establishment of a model system for pathogenic ‘Candidatus Liberibacter spp.’. Scientific Reports. 2019;9. https://doi.org/10.1038/s41598-018-36956-2 WOS:000462298600037.

Smith M, Storey J. Zinc concentration of pecan leaflets and yield as influenced by zinc source and adjuvants. J Amer Soc Hort Sci. 1979; 104(4):474–7.
51. "Method 3050B: Acid Digestion of Sediments, Sludges, and Soils," US EPA Washington, DC, 1996.

52. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature protocols. 2008; 3(2):163. https://doi.org/10.1038/nprot.2007.521 PMID: 18274517

53. Yajko DM, Madej JJ, Lancaster MV, Sanders CA, Cawthon VL, Gee B, et al. Colorimetric method for determining MICs of antimicrobial agents for Mycobacterium tuberculosis. Journal of clinical microbiology. 1995; 33(9):2324–7. PMID: 7494021

54. Rampersad SN. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors. 2012; 12(9):12347–60. https://doi.org/10.3390/s120912347 PMID: 23112716

55. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nature Reviews Microbiology. 2016; 14(5):320. https://doi.org/10.1038/nrmicro.2016.34 PMID: 27080241

56. Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C. Biofilm formation in plant–microbe associations. Current opinion in microbiology. 2004; 7(6):602–9. https://doi.org/10.1016/j.mib.2004.10.014 PMID: 15556032

57. Stoodley P, Sauer K, Davies DG, Costerton JW. Biofilms as complex differentiated communities. Annual Reviews in Microbiology. 2002; 56(1):187–209.

58. Hoffman MT, Doud MS, Williams L, Zhang M-Q, Ding F, Stover E, et al. Heat treatment eliminates ‘Candidatus Liberibacter asiaticus’ from infected citrus trees under controlled conditions. Phytopathology. 2013; 103(1):15–22. https://doi.org/10.1094/PHYTO-06-12-0138-R PMID: 23035631

59. Hu J, Wang N. Evaluation of the spatiotemporal dynamics of oxytetracycline and its control effect against citrus Huanglongbing via trunk injection. Phytopathology. 2016; 106(12):1495–503. https://doi.org/10.1094/PHYTO-02-16-0114-R PMID: 27482624

60. Zhang M, Powell CA, Zhou L, He Z, Stover E, Duan Y. Chemical compounds effective against the citrus Huanglongbing bacterium ‘Candidatus Liberibacter asiaticus’ in planta. Phytopathology. 2011; 101(9):1097–103. https://doi.org/10.1094/PHYTO-09-10-0262 PMID: 21834727

61. Hu J, Jiang J, Wang N. Control of Citrus Huanglongbing via Trunk Injection of Plant Defense Activators and Antibiotics. Phytopathology. 2017; 108(2):186–95. https://doi.org/10.1094/PHYTO-05-17-0175-R PMID: 28945516

62. Mann R, Tiwari S, Smoot J, Rouseff R, Stelinski L. Repellency and toxicity of plant-based essential oils and their constituents against Diaphorina citri Kuwayama (Hemiptera: Psyllidae). Journal of applied entomology. 2012; 136(1-2):87–96.

63. Onagbola E, Rouseff R, Smoot J, Stelinski L. Guava leaf volatiles and dimethyl disulphide inhibit response of Diaphorina citri Kuwayama to host plant volatiles. Journal of Applied Entomology. 2011; 135(6):404–14.

64. Leong SCT, Abang F, Beattie A, Kueh RJH, Wong SK. Impacts of horticultural mineral oils and two insecticide practices on population fluctuation of Diaphorina citri and spread of huanglongbing in a citrus orchard in Sarawak. The Scientific World Journal. 2012; 2012:651416. https://doi.org/10.1100/2012/651416 PMID: 22629178

65. McKenzie C, Lapointe SL, Hunter W, Puterka GJ. Efficacy of Surround for control of Asian citrus psyllid on citrus, 2000. Arthropod Management Tests. 2002; 27(1).

66. Hall DG, Lapointe SL, Wenninger EJ. Effects of a particle film on biology and behavior of Diaphorina citri (Hemiptera: Psyllidae) and its infestations in citrus. Journal of Economic Entomology. 2007; 100(3):847–54. https://doi.org/10.1603/0022-0493(2007)100[847:eopfn2.0.co;2] PMID: 17598547

67. FDA. PART 182—SUBSTANCES GENERALLY RECOGNIZED AS SAFE 2018. Available from: https://www.ecfr.gov/cgi-bin/text-idx?SID=e956d645a8b4e6b3e34e4e5d1b690209&m= true&node=pt21.3.182

68. Espitia PJP, Soares NdFF, dos Reis Coimbra JS, de Andrade NJ, Cruz RS, Medeiros EAA. Zinc oxide nanoparticles: synthesis, antimicrobial activity and food packaging applications. Food and Bioprocess Technology. 2012; 5(5):1447–64.

69. Quarles W. IPM for Asian citrus psyllid and huanglongbing disease. IPM Practitioner, XXXIV (1/2). 2013.

70. Santra S, Young M. Non-phytotoxic composite polymer film barrier as a repellent for controlling infection. Google Patents; 2018.

71. Yamamoto O. Influence of particle size on the antibacterial activity of zinc oxide. International Journal of Inorganic Materials. 2001; 3(7):643–6.

72. Zhang L, Jiang Y, Ding Y, Povey M, York D. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). Journal of Nanoparticle Research. 2007; 9(3):479–89.
73. Applerot G, Lipovsky A, Dror R, Perkas N, Nitzan Y, Lubart R, et al. Enhanced antibacterial activity of nanocrystalline ZnO due to increased ROS-mediated cell injury. Advanced Functional Materials. 2009; 19(6):842–52.

74. Raman N, Muthuraj V, Ravichandran S, Kulandaisamy A. Synthesis, characterisation and electrochemical behaviour of Cu (II), Co (II), Ni (II) and Zn (II) complexes derived from acetylacetone and p-anisidine and their antimicrobial activity. Journal of Chemical sciences. 2003; 115(3):161–7.

75. Raman N, Raja JD, Sakthivel A. Synthesis, spectral characterisation of Schiff base transition metal complexes: DNA cleavage and antimicrobial activity studies. Journal of Chemical sciences. 2007; 119(4):303–10.

76. Wang N, Pierson EA, Setubal JC, Xu J, Levy JG, Zhang Y, et al. The Candidatus Liberibacter–host interface: insights into pathogenesis mechanisms and disease control. Annual review of phytopathology. 2017; 55:451–82. https://doi.org/10.1146/annurev-phvoy-080516-035513 PMID: 28637377

77. Oliver JD. The viable but nonculturable state in bacteria. The Journal of Microbiology. 2005; 43(1):93–100.

78. Oliver JD. The public health significance of viable but nonculturable bacteria. Nonculturable microorganisms in the environment: Springer; 2000. p. 277–300.

79. Gunasekera TS, Sørensen A, Attfield PV, Sørensen SJ, Veal DA. Inducible gene expression by nonculturable bacteria in milk after pasteurization. Applied and environmental microbiology. 2002; 68(4):1988–93. https://doi.org/10.1128/AEM.68.4.1988-1993.2002 PMID: 11916722

80. Del Campo R, Russi P, Mara P, Mara H, Peyrou M, De León IP, et al. Xanthomonas axonopodis pv. citri enters the VBNC state after copper treatment and retains its virulence. FEMS microbiology letters. 2009; 298(2):143–8. https://doi.org/10.1111/j.1574-6968.2009.01709.x PMID: 19624747

81. Kogure K, Simidu U, Taga N. A tentative direct microscopic method for counting living marine bacteria. Canadian Journal of Microbiology. 1979; 25(3):415–20. PMID: 378340

82. Zimmermann R, Iturriaga R, Becker-Birck J. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Applied and Environmental microbiology. 1978; 36(6):926–35. PMID: 367268

83. Rodriguez G, Phipps D, Ishiguro K, Ridgway H. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Applied and Environmental microbiology. 1992; 58(6):1801–8. PMID: 1622256

84. Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, et al. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. Journal of clinical microbiology. 1998; 36(2):362–6. PMID: 9466742

85. Bueno C, Villegas M, Bertolotti S, Previtali C, Neumann M, Encinas aV. The excited-state interaction of resazurin and resorufin with aminesin aqueous solutions. Photophysica and photochemical reaction. Photochemistry and photobiology. 2002; 76(4):385–90. PMID: 12405144

86. Collins L, Franzblau SG. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrobial agents and chemotherapy. 1997; 41(5):1004–9. PMID: 9145860

87. Sawai J, Igarashi H, Hashimoto A, Kokugan T, Shimizu M. Evaluation of growth inhibitory effect of ceramics powder slurry on bacteria by conductance method. Journal of Chemical Engineering of Japan. 1995; 28(3):288–93.

88. Webb JS, Givskov M, Kjelleberg S. Bacterial biofilms: prokaryotic adventures in multicellularity. Current opinion in microbiology. 2003; 6(6):578–85. PMID: 14662353

89. Parsek MR, Fuqua C. Biofilms 2003: emerging themes and challenges in studies of surface-associated microbial life. Journal of bacteriology. 2004; 186(14):4427–40. https://doi.org/10.1128/JB.186.14.4427-4440.2004 PMID: 15231774

90. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. Journal of bacteriology. 2002; 184(4):1140–54. https://doi.org/10.1128/JB.184.4.11401154.2002 PMID: 11807075

91. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in Pseudomonas aeruginosa biofilms. Nature. 2001; 413(6858):860. https://doi.org/10.1038/35101627 PMID: 11677611

92. Monier J-M, Lindow S. Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. Applied and environmental microbiology. 2004; 70(1):346–55. https://doi.org/10.1128/AEM.70.1.346355.2004 PMID: 14711662

93. Morris CE, Monier J-M. The ecological significance of biofilm formation by plant-associated bacteria. Annual review of phytopathology. 2003; 41(1):429–53.
94. Bloemberg GV, Lugtenberg BJ. Bacterial biofilms on plants: relevance and phenotypic aspects. Microbial biofilms: American Society of Microbiology; 2004. p. 141–59.
95. Chatterjee S, Almeida RP, Lindow S. Living in two worlds: the plant and insect lifestyles of Xylella fastidiosa. Annual review of phytopathology. 2008;46.
96. Vyas M, Fisher TW, He R, Nelson W, Yin G, Cicero JM, et al. Asian citrus psyllid expression profiles suggest Candidatus Liberibacter asiaticus-mediated alteration of adult nutrition and metabolism, and of nymphal development and immunity. PLoS One. 2015; 10(6):e0130328. https://doi.org/10.1371/journal.pone.0130328 PMID: 26091106
97. Wang N, Trivedi P. Citrus huanglongbing: a newly relevant disease presents unprecedented challenges. Phytopathology. 2013; 103(7):652–65. https://doi.org/10.1094/PHYTO-12-12-0331-RVW PMID: 23441969
98. Kandel PP, Lopez SM, Almeida RP, De La Fuente L. Natural competence of Xylella fastidiosa occurs at a high frequency inside microfluidic chambers mimicking the bacterium’s natural habitats. Applied and environmental microbiology. 2016; 82(17):5269–77. https://doi.org/10.1128/AEM.01412-16 PMID: 27316962
99. Whidden M, Cogan N, Donahue M, Navarrete F, De La Fuente L. A Two-Dimensional Multiphase Model of Biofilm Formation in Microfluidic Chambers. Bulletin of mathematical biology. 2015; 77(12):2161–79. https://doi.org/10.1007/s11538-015-0115-3 PMID: 26621357
100. Hippler FWR, Boaretto RM, Quaggio JA, Boaretto AE, Abreu-Junior CH, Mattos D Jr. Uptake and distribution of soil applied zinc by citrus trees—addressing fertilizer use efficiency with 68Zn labeling. PLoS One. 2015; 10(3):e0116903. https://doi.org/10.1371/journal.pone.0116903 PMID: 25751056
101. Zink nitrate; MSDS No. S25901 [Online]; AquaPhoenix Scientific, Hanover, PA; 10.24.2014; https://beta-static.fishersci.com/content/dam/fishersci/en_US/documents/programs/education/regulatory-documents/sds/chemicals/chemicals-z/S25901.pdf; (Accessed 4.9.2019).
102. Copper(II) sulfate; MSDS No. AD422870050 [Online]; Fisher Scientific, Fair Lawn, NJ; 4.9.2010; https://www.fishersci.com/msds?productName=AC422870050; (Accessed 4.9.2019).