Crop Protection 90 (2016) 106–116

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Effects and side effects of penicillin injection in huanglongbing affected grapefruit trees

Keumchul Shin [a, b], Marina S. Ascunce [a, b], Hossein A. Narouei-Khandan [a, b], Xiaoan Sun [c], Debra Jones [c], Oluwaseun Olawale Kolawole [a, b], Erica M. Goss [a, b], Ariena H.C. van Bruggen [a, b, *]

[a] Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611, USA
[b] Department of Plant Pathology, IFAS, University of Florida, Gainesville, FL 32610, USA
[c] Florida Department of Agriculture & Consumer Services, Division of Plant Industry, Gainesville, FL 32614, USA

ARTICLE INFO

Article history:
Received 15 July 2016
Received in revised form
22 August 2016
Accepted 24 August 2016

Keywords:
Candidatus Liberibacter asiaticus
Colony forming units (CFU)
qPCR
Penicillin resistance
Glyphosate resistance
Phytophthora nicotianae

ABSTRACT

Trunk injection with penicillin has been tested to control citrus huanglongbing (HLB), but side effects and environmental safety must be assured before approval of penicillin injection can be considered. We investigated effects of penicillin injection on densities of *Candidatus* Liberibacter asiaticus (Las) in leaves, as well as culturable bacterial populations in rhizospheres and petioles of grapefruit trees in field and greenhouse experiments. Trees were injected with penicillin G, and leaf and root concentrations were assessed in bioassays with *Bacillus subtilis*. Las densities were determined by qPCR, and bacteria were isolated on a low carbon medium from roots plus rhizosphere and surface-sterilized petioles at various times after penicillin injection. Selected bacterial isolates were tested for penicillin resistance (20 μg/mL) and glyphosate resistance (7000 μg/mL), because glyphosate is widely used and cross-resistance against antibiotics had been documented. One month after penicillin injection half of the greenhouse trees were inoculated with *Phytophthora nicotianae*. Cycle threshold (Ct) values of Las in old and young leaves significantly increased 90 days after trunk injection with penicillin. Bacterial populations in petioles and root-rhizospheres initially increased after penicillin injections, probably due to nutrient release, then returned to control levels after one week. Penicillin resistance was common in isolates from penicillin-injected and control trees (30–94%). Significantly more glyphosate resistant than sensitive isolates were penicillin resistant (81% versus 52%). Phytophthora root rot was not increased after penicillin injection. Thus, side effects of penicillin injection tested here were minimal, while Las titers were reduced after three months.

1. Introduction

Huanglongbing (HLB) also known as citrus greening is one of the most destructive diseases for Florida citrus production, which constitutes 71% of U.S. citrus production with a value of $1.4 billion in 2012 (Farnsworth et al., 2014; FDACS, 2013). The putative causal agent is the gram negative and phloem-limited α-Proteobacterium *Candidatus* Liberibacter asiaticus (Las) which is transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama and grafting (Bové, 2006; Narouei-Khandan et al., 2016; Shimwela et al., 2016; Zhang et al., 2012). While the psyllids, both nymphs and adults, feed on the phloem of infected trees, they acquire the pathogen that can then be transferred to neighboring healthy trees (Chiyaka et al., 2012). The pathogen spreads systemically in the infected tree but few or no symptoms occur for several months up to years after infection (Shen et al., 2013b). The bacterial pathogen causes root degeneration by unknown mechanisms and phloem plugging in infected trees, interfering with the transport of nutrients and eventually leading to tree decline (Bové, 2006; Johnson et al., 2014).

HLB needs to be controlled as soon as possible to avoid collapse of the citrus industry in Florida. Management for HLB has focused on reducing pathogen inoculum and insect vector populations, and production of healthy trees, which can delay disease development (Boina and Bloomquist, 2015; Bové, 2006; Shen et al., 2013a). However, these practices have not been fully successful. Foliar application of nutrients and systemic resistance-enhancing compounds have reduced titers of Las and symptom development

* Corresponding author. Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611, USA.
E-mail address: ahcvanbruggen@ufl.edu (A.H.C. van Bruggen).

http://dx.doi.org/10.1016/j.cropro.2016.08.025
0261-2194/

(Shen et al., 2013a), but again, this is insufficient for control of the disease.

Several antimicrobials have been tested for the control of Las (Bové et al., 1980; Aubert and Bové, 1980; Ke and Wang, 1991; Puttamuk et al., 2014; Zhang et al., 2010, 2011, 2012; 2013a). Irrigating citrus seedlings with a penicillin or tetracycline solution, suppressed HLB symptoms (Bové et al., 1980). Soaking cut stems in solutions of penicillin, 2,2-dibromo-3-nitrilopropionamide (DBNPA), a combination of penicillin and streptomycin or oxytetracycline was effective at controlling Las in periwinkle (Zhang et al., 2010, 2011). Soaking grapefruit cuttings for grafting in ampicillin also controlled Las in the grafted trees (Zhang et al., 2013a). Root drenching in sulfonamide antibiotics improved Las control by thermotherapy at 45 °C of HLB-infected grapefruit seedlings (Yang et al., 2016).

Streptomycin and oxytetracycline are registered and widely used as foliar sprays for the management of citrus canker in Florida (EPA, 2013; Graham et al., 2010) and are assumed to be effective in controlling Las by growers. However, spraying these antibiotics on leaf surfaces may not be effective at controlling Las because the epicuticular wax acts as a physical barrier to foliar absorption of these compounds (Buchholz et al., 1998; Buchholz and Schönherr, 2000). In addition, oxytetracycline would rapidly degrade when exposed to ultraviolet irradiation (Kumar et al., 2005; Stockwell and Duffy, 2012).

The use of other antibiotics is scrutinized due to concerns about the development and spread of antibiotic resistance among human pathogenic bacteria. Trunk injection would possibly limit the development of antibiotic resistance in the environment compared to foliar sprays (Aćimović et al., 2015). Trunk injections with streptomycin and oxytetracycline have been used successfully for the control of various bacterial diseases in landscape and orchard trees (Aćimović et al., 2015), but are not yet used in citrus groves in Florida. Trunk injections of HLB-infected citrus with penicillin or tetracycline were effective at reducing HLB symptoms, at least temporarily (Aubert and Bové, 1980; Ke and Wang, 1991; Puttamuk et al., 2014). Trunk injections of young citrus trees on their own roots with a combination of penicillin and streptomycin and of oxytetracycline and kasugamycin were tested for the control of Las in an experimental grove in Florida. These treatments reduced the Las titers and HLB symptoms in the citrus leaves several months after the injection (Zhang et al., 2011; 2013b). Similarly, Las titers were significantly reduced by trunk injection of a combination of streptomycin, penicillin and ampicillin in Malaysia (Puttamuk et al., 2014). Although tetracycline is approved for trunk injection of palm and elm trees to control phytoplasma diseases in Florida, it has not been approved for use in citrus trees partly due to its phytotoxicity to citrus (Aubert and Bové, 1980; Zhang et al., 2014).

There is concern about potential side effects of trunk injections with antibiotics on microbial communities in trees and rhizospheres. Indeed, the microbial community composition and diversity in citrus midribs were significantly affected by trunk injections with streptomycin plus penicillin or oxytetracycline plus kasugamycin (Zhang et al., 2013b). Potential effects on bacterial populations in the rhizosphere were not addressed in this study or in any other studies, as far as we know. Also, the potential effects of trunk injection with antibiotics on antibiotic resistance in rhizosphere bacteria have not been determined.

Antibiotic resistance, including penicillin resistance, is widespread in human and animal pathogens as well as agricultural soils (Chang et al., 2015; Demanèche et al., 2008; Kumar et al., 2005). This resistance can originate from the large-scale antibiotic use in animal production and manure application (Chang et al., 2015; Kumar et al., 2005), but antibiotic-resistant bacteria are abundant also in unmanured soils (Marti et al., 2013; Udikovic-Kolic et al., 2014). It has been proposed that mechanisms conferring resistance to glyphosate in bacteria can mediate resistance to various antibiotics (Kurenbach et al., 2015; Liu et al., 2013). Glyphosate is used intensively for weed control in citrus groves in Florida (FDACS, 2013; USDA NASS, 2015). Considering that penicillin is not registered for use on citrus, the question arose whether penicillin resistance could be associated with glyphosate resistance regardless of trunk injection of penicillin in citrus trees.

Another potential side effect of antibiotic injection in citrus trees could be a change in susceptibility to diseases like Phytophthora root rot, caused by *P. nicotianae* Breda de Haan (synonym – *P. parasitica* Dastur), *P. palmivora* Butler or *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian. The first two species are found in Florida, but *P. nicotianae* is more common (Graham et al., 2012). Potential changes in the microbial activity or community composition in the rhizosphere could affect the ability of *Phytophthora* spp. to colonize and infect citrus roots (Widmer et al., 1998). Moreover, HLB infection could affect the susceptibility to Phytophthora root rot (Graham et al., 2013).

In order to enable registration of penicillin injection for the control of HLB, the effect on Las concentrations, the potential risk of changing microbial populations and communities in the rhizosphere and endosphere of citrus trees, and promotion of penicillin resistance would need to be assessed. In addition, effects of penicillin injection on susceptibility of citrus trees to other diseases would need to be determined. The primary objectives of this study were to (i) estimate penicillin G concentrations in citrus trees during a one-month period after trunk injection, (ii) determine the effects of trunk injection with penicillin G on Las titers in leaves and bacterial populations in the rhizosphere and endosphere of citrus trees over the same time period, (iii) assess penicillin resistance in isolated bacteria, and (iv) determine Phytophthora root rot severity on citrus trees injected with penicillin compared to control trees. In addition, we searched for a potential reason why bacteria in untreated trees showed high levels of penicillin resistance by investigating cross-resistance to glyphosate. The effects of penicillin injection on microbial community compositions as determined by deep sequencing will be reported elsewhere.

2. Materials and methods

2.1. Experimental design

Ray Ruby grapefruit trees (*Citrus paradisi* Macf.) on Swingle root stocks, 6–7 years old, 2–3 m tall and 1.5–2 m wide, located in a commercial grove at Ft. Mead in Florida, were used for the field experiment in 2014 to evaluate the effect of trunk injection with penicillin G on Las titers in leaves, bacterial populations in the rhizosphere and petioles, and penicillin resistance in isolated bacteria post injection. All trees were naturally infected by Las and showed symptoms of HLB. The trees were sprayed regularly with a variety of insecticides to control the Asian citrus psyllid. Twenty six trees were selected in two adjacent grove sections for penicillin injection with 3 treatments (0, 1000 and 6000 μg/mL): three control trees in each section, ten trees injected with 1000 μg/mL in one section and ten with 6000 μg/mL in the adjacent section.

A greenhouse experiment was carried out in a biological safety level 2 (BSL2) greenhouse (about 7 by 3.5 m) of the Department of Plant Pathology, University of Florida in Gainesville, FL, from May to August 2015. The greenhouse was lined with plastic under the gravel on the floor to avoid seepage of any chemicals or bacteria into the soil. The inside of the greenhouse was sprayed and wiped with 10% solution of commercial bleach and 70% of ethyl alcohol before and after the experiment. For this experiment, certified healthy Ray Ruby grapefruit trees grafted on Swingle rootstocks

were grown in soil from a pasture field in black plastic pots (20 cm diameter and 60 cm high) in a greenhouse in Citra, FL, for six months in order to have sufficiently large stems for injection of penicillin. The temperatures were set at 15 °C at night and 28 °C during the day. The greenhouse was screened and the trees were sprayed about once every one or two months with insecticides (Mustang, Danitol, Dimethoate 4E, and Omni Supreme Oil) at the recommended rates to avoid psyllids and other insects. Flowers and small fruits were removed, so that no harvestable products were obtained. When the trees were moved to the BSL2 greenhouse in Gainesville after six months of growth in Citra, the average height and diameter of the stems at 10 cm above the soil surface were approximate 1.3 m and 3.1 cm, respectively. Eighteen grapefruit trees were arranged in a randomized complete block design with 6 replications containing three treatments, 2 different concentrations (1000 and 6000 μg/mL) of penicillin G and water as control. At Gainesville, insecticidal soap (M-Pede, Gowan Co., Yuma, AZ, USA) was applied once a week; no insects were observed. The greenhouse was maintained at 25 °C (±5 °C) and no artificial light was provided.

2.2. Penicillin injection

The penicillin used for tree injection was laboratory grade penicillin (Penicillin G potassium salt, Fisher Scientific, One Reagent Lane, Fair Lawn, NJ, USA). The solutions (1000 and 6000 μg/mL) were made in the Division of Plant Industry (DPI) and the Emerging Pathogens Institute (EPI), University of Florida, Gainesville, FL, for the field experiment and the greenhouse experiment, respectively.

In the field experiment, penicillin G was applied on 9/30/2014, using an application rate of 0, 1000 or 6000 μg/mL similar to the concentrations used by Ke and Wang (1991). Two holes (5.5 mm diameter) were drilled right above the bud union in the trunk of each tree to be treated. Delivery of the penicillin solution into the trunk was via a passive infusion system utilizing an IV bag via two ports inserted onto the drill holes (Fig. S1). The injection volume ranged between 800 and 1000 mL per tree depending on tree size, and entered the tree within 24 h. Control trees were injected with sterile water. In the greenhouse experiment, stem injection with 20 mL solution (0, 1000 or 6000 μg/mL penicillin) for each tree was carried out using Chemjet® tree injectors (Chemjet Trading, Queensland, Australia), syringe like devices containing a coil spring pressing the fluid into the tree (Fig. S2). One injector per tree was placed in the trunk just below the graft region, about 20 cm above the soil surface after drilling a 4.2 mm hole 25 mm deep. Sterilized distilled water was injected in control trees.

2.3. Penicillin concentration in citrus trees

The concentrations of penicillin in leaves and roots of grapefruit trees in the field were tested on 3 control trees and 10 trees injected with 1000 and 6000 μg/mL penicillin each. About five young but fully expanded leaves were collected from four branches per tree on days 1, 3, 7, 15 and 30 after injection with penicillin, and placed in four plastic bags per tree. Four root samples were collected around each tree with a shovel, about 15 cm deep, on days 1, 3 and 7 after penicillin injection, and placed in four plastic bags per tree. Samples were transported to Gainesville in a cool box with ice packs.

To determine the penicillin concentrations in the leaves and roots of the greenhouse trees, three additional trees per treatment were injected with 0, 1000 and 6000 μg/mL penicillin G. Leaf and root samples were collected at 2 and 24 h, and 7, 21 and 28 days after injection. Three leaf samples were taken from each tree in each of three directions. Roots were sampled through boring holes on four sides of each pot and 1 g of root material per tree per sampling time was submitted to DPI in Gainesville, FL, for penicillin concentration estimation using a bioassay with extracts from the citrus tissues as described below. The diameters of the clear zones in the bioassays were converted to μg/mL penicillin using the standard curves given below.

To determine penicillin concentrations in citrus tissues bioassays were carried out as follows. Luria-Bertani agar (Invitrogen, Carlsbad, CA, USA) was prepared according to label directions, and 20 mL was dispensed per plate. A suspension of a penicillin sensitive strain of *Bacillus subtilis*, grown overnight in Luria-Bertani broth at 37 °C, was diluted approximately 1:15 in sterile tap water, and 200 μL dilute suspension was spread on each plate using a sterile L-spreader. After drying, three 7 mm-diameter wells per plate were created after punching with a Grafar Auto-Gel assembly (Grafar Corp., Detroit, MI, USA). One g of citrus tissue was finely ground in 2 mL deionized water using a drill press and Agdia mesh bags (Agdia, Elkhard, IN, USA), and 100 μL of the resulting suspension was dispensed directly into each of three wells per agar plate using wide-bore pipet tips. Standard curves were prepared with known concentrations of penicillin G potassium salt. Solutions with standard concentrations penicillin (0.1, 0.5, 1.0, 1.5, 2.0, 3.0, and 5.0 μg/mL) were prepared in a 450 μL matrix of untreated root or leaf tissue from greenhouse grown citrus trees ground in a drill press and Agdia mesh as described for the experimental tissues above. One plate with 3 wells was prepared for each penicillin concentration. After 24 h incubation at 28 °C, the diameter of any inhibition zone was measured using a ruler. Three diameters were measured per well and diameters for each plate were averaged. A measurement of 0.7 cm was the minimum possible diameter and reflected no inhibition. Equations for the standard curves for leaves and roots were $y = 0.0073e^{1.6619x}$ and $y = 0.0343e^{1.3623x}$, respectively, where x = diameter in cm and y = μg/mL penicillin.

2.4. Real-time PCR assays for evaluation of Las titers in field trees

Five fully expanded mature leaves and young leaves were collected from four branches from each of the treated grapefruit trees in the field on days 0, 7, 15, 30, 60 and 90 after penicillin injection. Chopped petioles (200 mg) from each branch were used to extract total genomic DNA using the Qiagen DNeasy® Plant Mini extraction kit with some modifications (Qiagen, Valencia, CA, USA). The total DNA was suspended in 200 μL of molecular grade water and stored at -20 °C (Li et al., 2006).

TaqMan-based qPCR assay was carried out as described by Li et al. (2006) with modifications to evaluate Las titers in leaf samples. A modified HLB-forward primer (Zhou et al., 2011) and HLBr, and a TaqMan probe HLBp were used for qPCR amplification to target the 16S rRNA gene of Las. The citrus plant cytochrome oxidase (COX) gene was used as positive internal control with primers COXf, COXr and COXp. All primers and probes used were ordered from Integrated DNA Technologies Inc. (Coralville, IA, USA). The qPCR amplifications were performed using a Cepheid SmartCycler (Cepheid, Sunnyvale, CA, USA) at the Florida Department of Agriculture and Consumer Services - Division of Plant Industry. The reaction mixture was made to a final volume of 25 μL consisting of the following reagents: Platinum Taq DNA Polymerase 1 Unit, 2.5 μL 10x PCR buffer, 3.0 μL $MgCl_2$ (these 3 reagents are supplied in a set by Invitrogen, Carlsbad, CA, USA), 0.6 μL dNTP mix (Sigma-Aldrich, St. Louis, MO, USA), 240 nM each primer and 120 nM each probe. Two-step thermal profiles consisted of 95 °C for 20 s, followed by 40 cycles of 1 s at 95 °C and 40 s at 62 °C, with optical readings at 62 °C for data acquisition. Each run contained two positive and two negative control samples from citrus plants in a quarantine greenhouse at the DPI in Gainesville, FL. Data analysis to determine cycle threshold (Ct) values were performed using Smart Cycler

software version 2 (Sunnyvale, CA, USA). The mean Ct values were calculated and compared for each treatment and day after penicillin injection. Ct values lest than 34 were considered to be Las positive and Ct values above this value were considered tentatively negative (Shen et al., 2013b; Shimwela et al., 2016).

2.5. Sampling roots and petioles for bacterial isolations

In the field experiment, petiole and root samples were collected from 16 grapefruit trees (6 control trees, and 5 trees at each penicillin concentration) at the Ft. Meade field site within 2 h and on day 8 after penicillin injection. Approximately 20 leaves were collected in one plastic bag per tree, around the tree at various heights; leaves were full-grown but still young (light green and occasionally darker green). Four root samples were collected around each tree with two shovels, about 15 cm deep, and placed in one plastic bag per tree. Samples were transported in a cool box with ice packs, and stored overnight in the same cool box with fresh ice packs. Petioles were cut off from the leaves with sterilized scissors, weighed (0.8−1.0 g), and placed in one petridish per tree. Soil was shaken off the roots; small roots were cut from the larger roots, weighed (1.0 g) and placed in one petridish per tree.

For the greenhouse experiment, samples of roots with soil and petioles were collected from each tree on day 1, 7 and 28 after penicillin injection in order to determine bacterial populations and penicillin resistance. The roots (1 g) were obtained from four holes on the side of each plastic pot 15 cm above the bottom (Fig. S2). Ten petioles per tree were harvested with sterile scalpels then placed in plastic bags and stored in ice before use.

2.6. Bacterial populations on non-amended and penicillin-amended plates

S-medium (Senechkin et al., 2010; van Bruggen et al., 1988) was used to isolate bacteria from the samples. This medium is composed of $MgSO_4$ (0.4 g), KNO_3 (0.4 g), K_2HPO_4 (0.8 g), glucose (20 mg), enzymatic casein hydrolysate (40 mg), Ca $(NO_3)_2$ (0,048 g), and Bacto agar (14.4 g) dissolved in 800 mL distilled water (pH 7.2). After autoclaving at 121 °C for 30 min, penicillin solution was filtered through a 0.2 μm syringe filter and added to the luke-warm medium at 0, 2, 10 or 20 μg/mL [The minimum inhibitory concentration for resistant bacteria is considered >2 μg/mL penicillin (Macias et al., 1994)]. Well mixed media solutions were poured into 9 cm petri dishes and allowed to solidify at room temperature.

Petioles were surface sterilized in 70% alcohol and rinsed three times in sterile water. They were then added to a grinding tube with 1 mL of sterile water, and finely ground to a paste in a steel ball grinding machine (1600 MiniG, SPEX® Sample Prep, Metuchen, NJ, USA) set at 1200 rpm for 3 min. One gram of roots that were not surface sterilized was added to a grinding tube containing 1 mL of sterile water, and finely ground to a paste in the same manner as the petiole samples. The paste of petiole or root samples was added to 9 mL of sterile water in a test tube.

Serial dilutions were made up to 10^{-7} for roots and up to 10^{-4} for petioles. One hundred μL of dilutions 10^{-6} and 10^{-7} of the root suspensions and 10^{-3} and 10^{-4} of the petiole suspensions were spread evenly using a sterile spreader on petri dishes containing S-medium. For the field experiment, the dilutions were plated on the S-media amended with 0 and 2 μg/mL penicillin. Colonies isolated on medium with 2 μg/mL penicillin were then transferred to 2, 10 and 20 μg/mL penicillin after three weeks. For the greenhouse experiment, 0 and 20 μg/mL penicillin amended S-media were used for isolation. All plates were incubated at 30 °C in an incubator. Colonies on the media were counted weekly for 3 weeks after plating, and the numbers of colonies per 1 g of samples were calculated.

2.7. Penicillin G and glyphosate resistance

For the field experiment, 0 and 2 μg/mL penicillin amended S-media were used for the initial isolations, while 0 and 20 μg/mL penicillin plates were used for the greenhouse experiment (to eliminate the need to transfer from 2 μg/mL to 20 μg/mL penicillin). Isolated bacteria were counted and percent penicillin resistance was calculated relative to the number of colonies on plates with 0 μg/mL penicillin after incubation for 3 weeks. To determine the percent penicillin resistance at 10 and 20 μg/mL relative to 2 μg/mL, ten random colonies obtained from field samples on plates containing 2 μg/mL penicillin were transferred individually onto plates with 2, 10, and 20 μg/mL penicillin after three weeks.

As penicillin had never been applied directly or indirectly via manure in citrus groves or the pasture field where soil was collected for the greenhouse experiment, and many glyphosate resistant bacterial isolates are also resistant to various antibiotics (Kurenbach et al., 2015), colonies on plates with 0 or 20 μg/mL penicillin were checked for growth on glyphosate amended and penicillin amended S-medium. Roundup® Weed & Grass Killer Super Concentrate (Monsanto, St. Louis, MO, USA) which contains 50.2% glyphosate and 49.8% other ingredients was used in this study. The glyphosate solution was sterilized by filtering through a 0.2 μm syringe filter. S-media were prepared with amendment of 0 or 20 μg/mL penicillin or 7000 μg/mL glyphosate (Kurenbach et al., 2015). Bacteria isolated from root samples in the field and greenhouse experiments that were exposed or not exposed to penicillin in citrus trees and grew on 0 or 20 μg/mL penicillin plates were transferred to the non-amended S-media and S-media amended with penicillin (20 μg/mL) or glyphosate (7000 μg/mL), and incubated at 25 °C for 1 week. Fifty-one isolates obtained on 20 μg/mL penicillin plates from both the field and greenhouse experiments were tested to determine percent penicillin and glyphosate resistance. For comparison, 33 (field experiment) and 51 (greenhouse experiment) isolates on 0 μg/mL penicillin plates were similarly tested for penicillin and glyphosate resistance.

2.8. Susceptibility to Phytophthora root rot

P. nicotianae isolate 198, kindly provided by James Graham (University of Florida IFAS CREC, Lake Alfred, Florida), was used to prepare zoospore inoculum to test effects of penicillin injections on susceptibility of citrus trees to Phytophthora root rot. Fifteen mycelial plugs (5 mm in diameter) were taken from 4-day-old *P. nicotianae* cultures growing on V8 juice agar and transferred to petri dishes containing 5 mL of sterile mineral salts solution (MSS) as described by Yandoc et al. (2007). After autoclaving at 121 °C for 15 min, 1 mL of chelated iron solution that had been filtered through a 0.2-μm membrane was added in 50 mL of sterile water (Yandoc et al., 2007). Petri dishes with MSS and mycelial plugs were incubated for a total of 72 h under continuous light at 20 °C to induce the production of sporangia. Next, the plugs were rinsed three times and then covered with 5 mL of sterile distilled water and the plates were incubated at 4 °C for 20 min, after which they were left at room temperature until zoospore release. Zoospores were induced to encyst by vortexing. The number of encysted zoospores was counted with the aid of a hemacytometer. The zoospore concentration was adjusted to 1000 zoospores per 1 mL of suspension. Just prior to inoculation, a second sample of the inoculum was checked under a stereo microscope for actively swimming zoospores to make sure that the inoculum was viable.

One month after penicillin injection, three trees per treatment were inoculated with *P. nicotianae* by injecting the zoospore

suspension (10 mL per hole; 4 holes per tree; 40,000 zoospores per tree) into the same holes used for root sampling and the other three trees were water-inoculated as controls. Four weeks after *Phytophthora* inoculation, all roots were checked for disease symptoms and *P. nicotianae* was re-isolated from 10 root sections per tree on PARP media (Jeffers and Martin, 1986). The percentage of the total root length that displayed a brown, soft rot was estimated visually for each tree.

2.9. Statistical analysis

Data analysis was carried out using the GLM and MIXED procedures of SAS (version 9.4; SAS Institute Inc.) to test for significant effects of injection treatment, block, penicillin concentration in the plates, sampling time and all interactions on cycle threshold (Ct) values, colony-forming units (CFU), penicillin resistance, and Phytophthora root rot severity. PROC MIXED was used when there were missing data or an uneven design. The analyses were based on a split-plot design with injection treatment in main plots, and sampling time and medium amendment in sub-plots. As the field experiment had three control trees and five trees treated with 1000 μg/mL in one section and three control trees and five trees treated with 6000 μg/mL in the other section of the grove, analyses were carried out on all replications as well as three replications per treatment. Additional analyses were done comparing the control and treated trees (either with 1000 or 6000 μg/mL penicillin) in each section separately. All residuals were checked for normality and data were log-transformed if needed to obtain normality. All data were also analyzed for the individual sampling dates and subjected to Tukey's HSD mean separation tests. CFU on penicillin-amended versus non-amended plates were compared with paired *t* tests in Excel. A chi-square test was conducted in Excel to compare the distribution of isolates in different resistance categories (to penicillin and/or glyphosate) obtained from the greenhouse versus the field. Additionally, the isolates tested for penicillin and glyphosate resistance were separated in four groups (blocks), and *t* tests were carried out on the percentages of penicillin resistant isolates out of all isolates that were sensitive or resistant to glyphosate in each group. Statistical significance was considered at $P \leq 0.05$.

3. Results

3.1. Penicillin G concentration in citrus trees after injection

Penicillin concentrations in leaf extracts on days 1, 3, 7, 15 and 30 after penicillin injection in the grove and in root extracts on days 1, 3 and 7 after penicillin injection are presented in Table 1. In leaves the initial penicillin concentration was 2.34 μg/mL after injection with 6000 μg/mL penicillin and declined to 0.18 μg/mL within three days. In leaves from trees treated with 1000 μg/mL penicillin the initial concentration was 0.12 μg/mL and was reduced to 0.03 μg/mL within three days. Hardly any penicillin reached the roots on any of the sampling days; the maximum estimated concentration was 0.02 μg/mL.

Extracts from leaf and root tissues from the greenhouse were tested to determine the penicillin concentration at 2 and 24 h, and 7, 21 and 28 days after injection. The penicillin concentrations were highest 24 h after 6000 μg/mL penicillin injection, namely 9.43 μg/mL in the leaf samples and 2.18 μg/mL in the root samples (Table 1). After 7 days, 168 h, the penicillin concentrations had dropped to 0.41 μg/mL in the leaf samples and 0.09 μg/mL in the root samples from trees injected with 6000 μg/mL penicillin. Leaf and root samples from trees injected with 1000 μg/mL penicillin had 6.67 and 0.13 μg/mL penicillin, respectively, after 24 h and 0.39 and 0.09 μg/mL after 168 h. Thereafter, penicillin was not detectable.

3.2. Real-time PCR assays for evaluation of Las titers in field trees

The Ct values of Las in real-time PCR assays were determined on days 0, 7, 15, 30, 60 and 90 after penicillin injection. They were not significantly different among the treatments, varying between 24.07 and 26.16, during 15 days after penicillin injections. However, the Ct values significantly increased in the DNA extracts from old leaf samples of trees injected with 6000 μg/mL penicillin on day 60 and in trees injected with 1000 and 6000 μg/mL penicillin on day 90 ($P = 0.04$) (Fig. 1a). Ct values in the DNA extracts from young leaves 90 days after penicillin injection differed highly significantly among the treatments ($P = 0.004$); the mean Ct value (35.1) was highest in the DNA extracts from young leaves of trees injected with 6000 μg/mL penicillin (Fig. 1b).

3.3. Appearance of grapefruit trees in the grove

Slight symptoms of HLB were observed on the grapefruit trees before penicillin injection at the beginning of the experiment. Psyllids were not found in the grove due to intensive pesticide applications to control the insect vector. HLB symptoms were slightly reduced and canopy sizes in diameter increased noticeably half a year after penicillin injections with both 1000 and 6000 μg/mL applied in 1L (Fig. S3).

3.4. Bacterial populations after penicillin G injection

CFU per g of petiole tissue from the field experiment increased temporarily within 2 h after injection with 1000 or 6000 μg/mL penicillin (Fig. 2). In an ANOVA on log-transformed data that were normally distributed (Shapiro Wilk test, $P = 0.17$), the field treatment effects were significant ($P = 0.0003$). The interaction effect of field treatment and time since injection, as well as the time since injection itself, was also significant ($P < 0.0001$). On day 8 after penicillin injection, the bacterial populations were significantly ($P < 0.0001$) reduced in the 6000 μg/mL treatments compared to the 1000 and 0 μg/mL treatments (Fig. 2).

CFU per g of root tissue from the field experiment was not significantly ($P = 0.66$) affected by penicillin within 2 h after injection with 1000 or 6000 μg/mL penicillin (Fig. 3). In an ANOVA on non-transformed data that were normally distributed (Shapiro Wilk test, $P = 0.75$), the interaction effect of field treatment and time since injection was highly significant ($P = 0.006$), because the treatment effects were significant only on day 8 after injection. On that day, CFU per g root tissue were significantly higher after 1000 μg/mL treatment than after 0 or 6000 μg/mL penicillin ($P < 0.0001$).

Bacterial populations from petioles collected in the grove were reduced at 2 μg/mL compared to 0 μg/mL penicillin in agar plates (Fig. 2) when they were not exposed to penicillin injected in grapefruit trees on day 0 and in the control treatment on day 8 according to paired *t*-tests (Table 2). Bacterial populations were not affected by 2 μg/mL penicillin in plates after field exposure to penicillin 8 days after injection with penicillin at 1000 or 6000 μg/mL (Table 2).

In the greenhouse experiment, the CFU isolated from surface sterilized petioles were too low to be meaningful. The CFU isolated from roots were similar to those isolated from the field-grown roots (Fig. 4). Twelve hours after injection, the CFU seemed higher on/in roots of trees injected with 1000 and 6000 μg/mL penicillin compared to the water controls (Fig. 4), but the differences in log-transformed data were not significant ($P = 0.16$). On day 7, 168 h after injection, the field treatments did not result in significant differences in log CFU either ($P = 0.89$). Similarly, on day 28, 672 h after penicillin injection, the log CFU were not affected by the

Table 1
Penicillin concentrations in the leaves and roots of grapefruit trees over time after trunk injection with 0, 1000 or 6000 μg/mL solutions of penicillin G in the grove at Ft. Meade and in the greenhouse experiment.

Plant tissue	Time (h)	Field penicillin treatment (μg/mL)			Greenhouse penicillin treatment (μg/mL)		
		0	1000	6000	0	1000	6000
Leaf	2	–	–	–	0.23 ± 0.01	4.88 ± 1.29	8.24 ± 2.02
	24	0.02[a]	0.12 ± 0.03[b]	2.34 ± 0.74	0.25 ± 0.02	6.67 ± 2.00	9.43 ± 2.51
	72	0.02	0.03	0.18 ± 0.08	–	–	–
	168	0.02	0.02	0.03	0.04 ± 0.01	0.39 ± 0.16	0.41 ± 0.20
	360	0.02	0.02	0.02	–	–	–
	504	–	–	–	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
	672	–	–	–	0.04	0.05	0.05
	720	0.02	0.02	0.02	–	–	–
Root	24	0.09[c]	0.09	0.09	0.09	0.13 ± 0.04	2.18 ± 2.08
	72	0.09	0.09	0.09	–	–	–
	168	0.09	0.09	0.09	0.09	0.09	0.09
	504	–	–	–	0.09	0.09	0.09
	672	–	–	–	0.09	0.09	0.09

[a] Minimum detection level in foliage.
[b] Data are presented as mean concentration (μg/mL) ± standard error.
[c] Minimum detection level in roots.

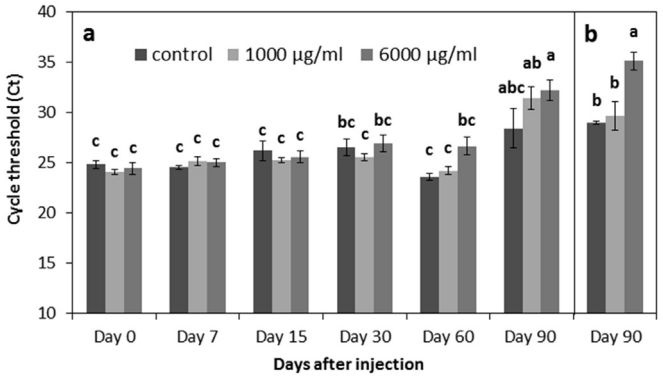

Fig. 1. Effects of penicillin concentrations on cycle threshold (Ct) values in mature leaves of grapefruit trees over time in the field (a) and young leaves of grapefruit trees 90 days after penicillin injection (b). Bars represent the standard errors of the means. Bars labeled with different letters are significantly different among the treatments at $P < 0.05$ using the Tukey's HSD test.

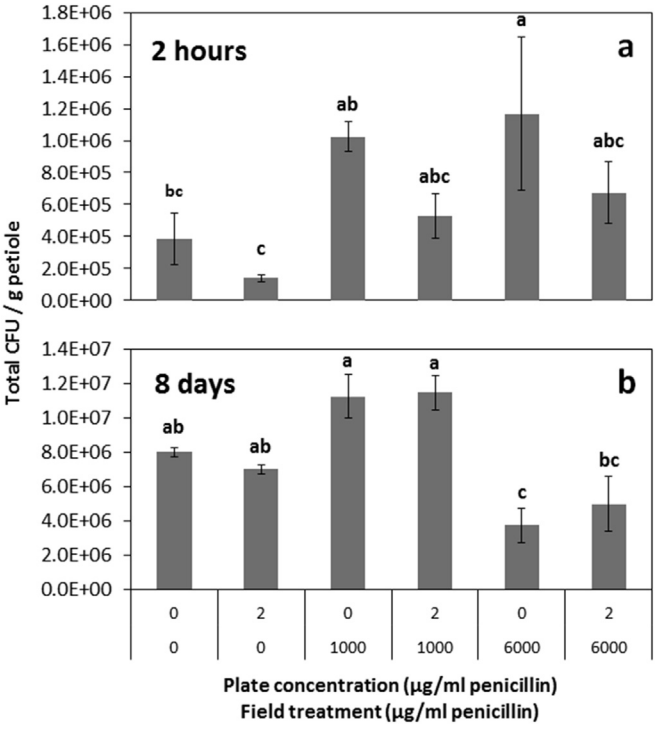

Fig. 2. Effects of penicillin concentrations in the plates and the injection solution in the field on total colony-forming units of bacteria per g of petiole of grapefruit trees at Fort Meade, 2 h after injection (a) and on day 8 after injection (b) of penicillin in the field. Bars represent the standard error of the mean. Bars labeled with different letters are significantly different among the treatments at $P < 0.05$ using the Tukey's HSD test.

injections (Fig. 4), but were significantly reduced by 20 μg/mL penicillin in the plates in some of the injection treatments ($P = 0.03$). All greenhouse data were normally distributed after log transformation (Shapiro Wilk test; $P > 0.82$).

According to paired *t*-tests (Table 3), bacterial CFU were reduced at 20 μg/mL compared to 0 μg/mL penicillin in agar plates when they had not been exposed to penicillin in grapefruit trees in the greenhouse on days 1 (12 h), 7 and 28 after tree injection treatments. Bacterial populations were not affected by 20 μg/mL penicillin in plates after exposure of the potted trees to penicillin 1 and 28 days after injection with penicillin at 1000 or 6000 μg/mL (Table 3). On day 7, the populations were reduced by 20 μg/mL penicillin in the plates even after injection of the trees with 1000 or 6000 μg/mL penicillin.

3.5. Bacterial resistance to penicillin G and glyphosate

In the field experiment, the CFU isolated from roots on S-media with 2 μg/mL penicillin amounted to 85–94% of those isolated on control plates. Ten random colonies per plate with 2 μg/mL penicillin were transferred to plates containing 0, 10 or 20 μg/mL penicillin. Almost 100% of the transferred colonies grew at 10 μg/mL and 20 μg/mL penicillin. Multiplying the percentages isolated on 2 μg/mL penicillin plates (86–94%) with the percentages grown on 20 μg/mL plates resulted in 85–94% resistance to 20 μg/mL compared to the colonies that were originally isolated on plates with 0 μg/mL penicillin (Table 4). The CFU growing on 20 μg/mL penicillin plates were not affected by penicillin injection ($P = 0.84$) or day of sampling ($P = 0.26$). In the greenhouse experiment, the numbers of CFU isolated on plates with 20 μg/mL penicillin were 30–81% of those on plates without penicillin (Table 4), but these percentages were not dependent on penicillin concentration injected in the trees ($P = 0.06$), nor on the time elapsed since injection ($P = 0.68$).

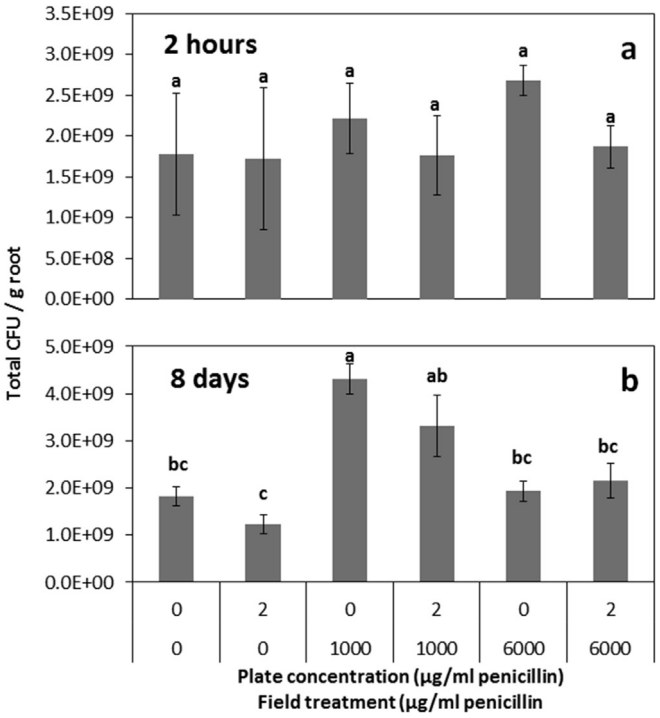

Fig. 3. Effects of penicillin concentrations in the plates and the injection solutions in the field on total colony-forming units of bacteria per g of root of grapefruit trees at Fort Meade, 2 h after injection (a) and on day 8 after injection (b) of penicillin in the field. Bars represent the standard error of the mean. Bars labeled with different letters are significantly different among the treatments at $P < 0.05$ using the Tukey's HSD test.

Table 2
Results (P-values) of one-sided, paired t-tests for the hypothesis that bacterial populations are reduced at 2 µg/mL compared to 0 µg/mL penicillin-G in agar plates before being exposed to penicillin injected in grapefruit trees in the field on day 0 or day 8 (0 µg/mL penicillin-G), and of two-sided, paired t-tests for the hypothesis that bacterial populations are not affected by 2 µg/mL penicillin in plates after field exposure to penicillin-G (day 8 after injection with penicillin-G at 1000 or 6000 µg/mL).

DAI[a]	Field treatment (µg/mL)	Penicillin on plate (µg/mL)	P-value	
			Petiole	Root
0	0, 1000 or 6000	0 vs 2	0.02	0.19
8	0	0 vs 2	0.01	0.05
	1000 or 6000	0 vs 2	0.37	0.35

[a] Days after injection.

Very few isolates (0–2%) that were obtained from plates with 20 µg/mL penicillin did grow on glyphosate-amended medium (7000 µg/mL) but not on penicillin-amended medium (20 µg/mL). More isolates (29–30%) grew on penicillin-amended medium but not on glyphosate-amended medium (Table 5). However, most isolates (54–71%) grew on both penicillin- and glyphosate-amended medium. The distribution of isolates over these three resistance categories was not significantly different between the field and greenhouse experiment according to a Chisquare test ($P > 0.05$). Combining the field and greenhouse data, the bacteria that were transferred from penicillin (20 µg/mL) amended agar and remained penicillin-resistant after this transfer had 62% cross-resistance to Roundup (7000 µg/mL glyphosate). Conversely, the average percentage of isolates that were resistant to penicillin of all isolates that grew on glyphosate-amended medium was significantly (one-sided paired *t*-test, $P = 0.05$) higher (81%) than the average percentage with penicillin resistance out of all glyphosate sensitive isolates (52%).

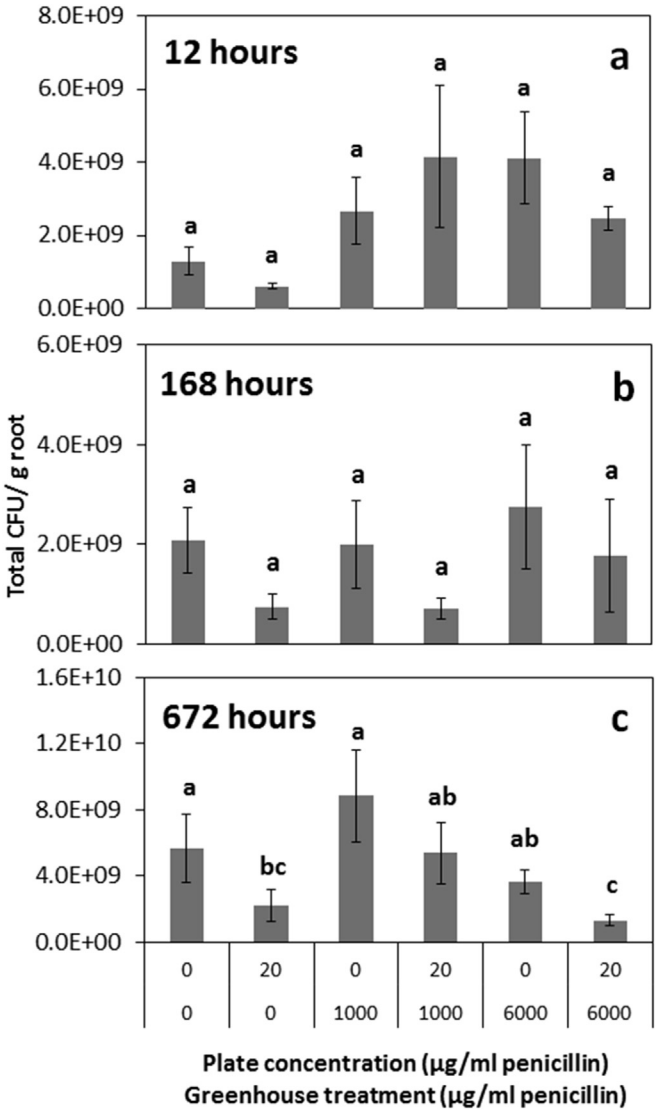

Fig. 4. Effects of penicillin concentrations in the plates and the solution injected into grapefruit trees on total colony-forming units of bacteria per g of root in the greenhouse. (a): on day 1, 12 h after injection; (b): on day 7, 168 h after injection and (c): on day 28, 672 h after injection of penicillin in the greenhouse. Bars represent the standard error of the mean. Bars labeled with different letters are significantly different among the treatments at $P < 0.05$ using the Tukey's HSD test.

3.6. Susceptibility to Phytophthora root rot

One month after inoculation of the potted grapefruit trees with *P. nicotianae*, the plants were uprooted and checked for root rot (Fig. 5). Phytophthora root rot was quite severe on inoculated trees (27–48% of the total root length affected), but was also seen on non-inoculated trees (10–15% of the root length affected). *P. nicotianae* was reisolated from all inoculated trees (Table 6), although the number of colonies per tree was low (on average 2.6 colonies from 10 root sections). The colony morphology and ovoid or obpyriform sporangia were the same for the inoculated and reisolated *P. nicotianae* isolates. When paired with *P. nicotianae* of a different mating type, oogonia were formed with amphigynous antheridia (Fig. 6c). Another *Phytophthora* species was isolated from 50% of the non-inoculated trees with symptoms, with on average 1.8 colonies from 10 root sections per tree (Table 6). This other *Phytophthora* species differed from *P. nicotianae* in colony

Table 3
Results (P-values) of one-sided, paired t-tests for the hypothesis that bacterial populations are reduced at 20 μg/mL compared to 0 μg/mL penicillin-G in agar plates without being exposed to penicillin in grapefruit trees in the greenhouse (0 μg/mL penicillin-G) on days 1, 7 and 28 after tree injection treatments and of two-sided, paired t-tests for the hypothesis that bacterial populations are not affected by 20 μg/mL penicillin in plates after greenhouse exposure to penicillin-G (1, 7 and 28 days after injection with penicillin-G at 1000 or 6000 μg/mL).

DAI[a]	Greenhouse treatment (μg/mL)	Penicillin on plate (μg/mL)	P-value
			Root
1	0	0 vs 20	0.02
	1000 or 6000	0 vs 20	0.09
7	0	0 vs 20	0.02
	1000 or 6000	0 vs 20	0.02
28	0	0 vs 20	0.03
	1000 or 6000	0 vs 20	0.12

[a] Days after injection.

Table 4
Percentages of bacterial CFU that grew on agar medium amended with 20 μg/mL penicillin compared to the same medium with 0 μg/mL penicillin, in suspensions that were isolated from roots of trees that had been injected with 0, 1000 or 6000 μg/mL penicillin on day 0 and day 8 after injection of trees with penicillin in the field and day 1, day 7 and day 28 after injection of trees with penicillin in the greenhouse.

DAI[a]	Experiment	Penicillin injection (μg/mL)		
		0	1000	6000
0	Field	85.3 a[b]	88 a	94 a
8		86 a	73 a	77 a
1	Greenhouse	45.8 a	70.8 a	37.1 a
7		36.1 a	41.1 a	44.3 a
28		41.7 a	81 a	30.4 a

[a] Days after injection.
[b] Means in the same row followed by the same letter for percentage of resistance are not significantly different from each other (P > 0.05) using the Tukey's HSD test.

morphology and sporangia shape (Fig. 6), but did not form oogonia and antheridia. Penicillin injection did not enhance the susceptibility of the trees to *P. nicotianae* (Fig. 5), nor to the other *Phytophthora* species on the non-inoculated trees (P = 0.686 for log-transformed data).

4. Discussion

An important result from this research was that penicillin injected in the trunk was distributed throughout the tree canopy (in both field and greenhouse experiments) and in the roots (in the greenhouse experiment) within a day after injection. This confirmed earlier results on the fast movement of penicillin in citrus foliage obtained in China (Ke and Wang, 1991). Although the residues declined within a week to almost undetectable levels in our experiments, the concentrations were initially apparently high enough to reduce the Las titer and symptom development after several months. It is not known for how long bioactive degradation products (Aldeek et al., 2015) remained in the tissues. In our study, the Ct values increased (and thus, the titer of Las decreased) in older leaves two or three months after injection of penicillin G at 6000 or 1000 μg/mL, respectively. The increase in Ct value was highly significant in young leaves 90 days after injection with 6000 μg/mL penicillin, while Ct values in control trees remained below 29, indicating that they were Las-positive. These results confirmed previous studies showing that Las titers were significantly reduced several weeks or months after penicillin trunk injections (Puttamuk et al., 2014; Zhang et al., 2011; 2013b).

The initial penicillin concentrations were higher in the greenhouse than in the field experiment, although only 20 mL of penicillin solution was used per tree in the greenhouse compared to 1 L in the field. However, the injection method was quite different, forcing the liquid into the trees, possibly into the phloem and xylem, in the greenhouse. The passive injection from IV bags in the field possibly resulted in movement via the xylem only. This method was initially tried in the greenhouse but was not successful due to the relatively small size (3 cm diameter) of the stems (data not shown). Despite the difference in injection method the results on bacterial populations and penicillin resistance were similar.

Total culturable bacterial populations in petioles were affected temporarily, first increasing, then returning to the usual varying population sizes after about one week (Zelenev et al., 2000, 2005). The initial increase (about ten-fold) may have been due to the activation of viable but non-culturable bacteria and multiplication of fast-growing bacteria on the nutrients released from dead bacteria killed by penicillin (Grünwald et al., 2000; Zelenev et al., 2000, 2005). CFU in/on roots responded more slowly to the penicillin injections in the grove (increased after 8 days) but responded within one day to penicillin injections in the greenhouse. The bacterial community composition is being determined by metagenomic sequencing of the same plant tissue and would likely indicate a shift in taxa composition (Ascunce et al. in prep.).

Table 5
Percentages of bacteria that originated from plates with 20 μg/mL penicillin and were able or unable to grow on plates with 20 μg/mL penicillin or 7000 μg/mL glyphosate. Data from penicillin injected and control trees were combined because there were no differences in penicillin resistance between injection treatments.

Resistance Categories	Percentages		Average of field and greenhouse
	Field	Greenhouse	
Isolates that grew on glyph but not on pen	2	0	1
Isolates that grew on pen but not on glyph	30	29.4	29.7
Isolates that grew on both glyph and pen	54	70.6	62.4

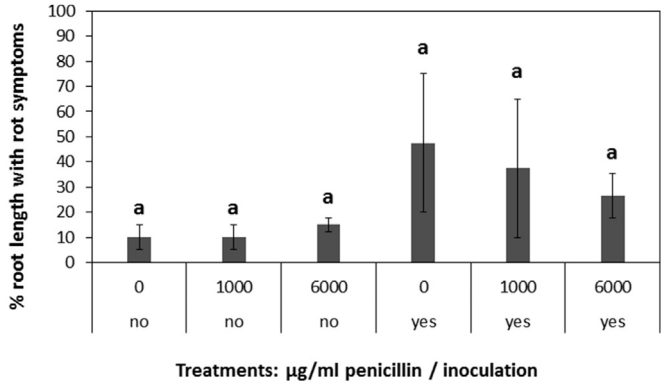

Fig. 5. Effects of penicillin concentrations (μg/mL injected) and inoculation with *Phytophthora nicotianae* on grapefruit root rot severity in a greenhouse experiment. Bars represent the standard error of the mean. Bars labeled with the same letter are not significantly different among the treatments at P < 0.05 using the Tukey's HSD test.

Table 6
Effect of inoculation with *Phytophthora nicotianae* on the number of *Phytophthora* colonies isolated from 10 root sections per tree (means and standard errors), percentages of isolates that were *P. nicotianae* and percentages that were different *Phytophthora* species.

Inoculation with *P. nicotianae*	*Phytophthora* like colonies isolated from 10 root sections per tree		% of isolates that were *P. nicotianae*	% of isolates that were other *Phytophthora*
	Mean	Standard error		
No[a]	1.78 a[b]	1.06	0	100 [c]
Yes	2.56 a	0.77	100 [d]	0

[a] Penicillin treatments combined, because there was no significant difference.
[b] Means followed by the same letter for re-isolated *Phytophthora* colonies are not significantly different form each other ($P > 0.05$) according to an unpaired two-sided *t*-test.
[c] Sporangia with one or two papillae; oogonia and lateral antheridium.
[d] Typical sporangia of *P. nicotianae*.

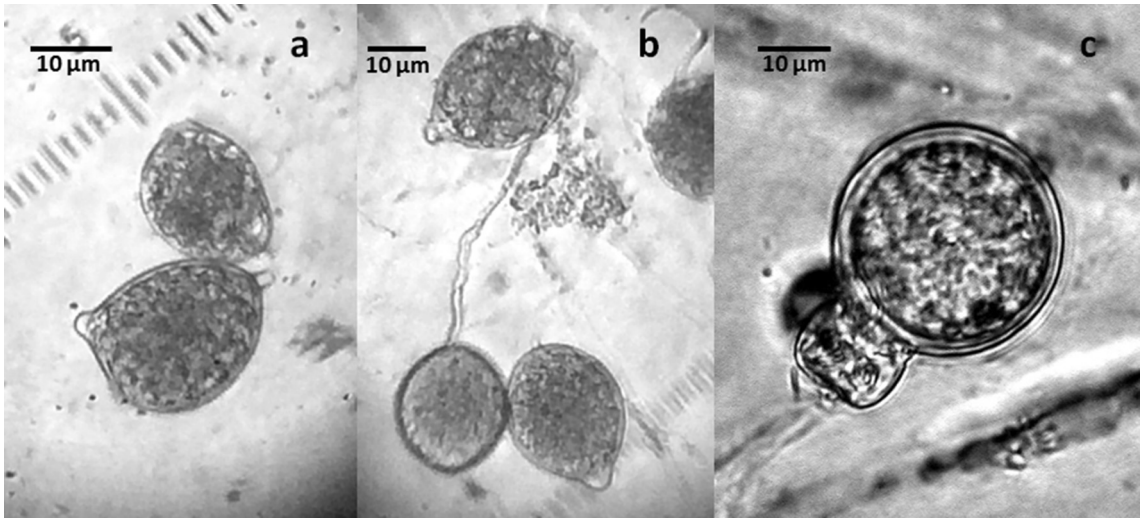

Fig. 6. Morphological characteristics of sporangia of an unknown *Phytophthora* species different from *P. nicotianae* (a), sporangia of *P. nicotianae* (b) and oogonium and amphigynous antheridium of *P. nicotianae* (c).

The number of bacterial CFU was slightly but significantly reduced on penicillin-amended media (2 or 20 μg/mL) compared to non-amended media when the bacterial communities were not exposed to penicillin injected in the trees, but not after exposure in the trees. Nevertheless, a large proportion of the bacteria isolated from control trees that had not been exposed to penicillin in the tree (85–86% in the field experiment and 36–46% in the greenhouse experiment) was resistant to 20 μg/mL penicillin, and these proportions were similar to those of the bacteria isolated from penicillin treated trees (73–94% in the field experiment and 30–81% in the greenhouse experiment). Thus, penicillin resistance seemed to be widespread in the soil used for the greenhouse experiment as well as the soil sampled from the grapefruit grove.

Penicillin was never applied in the citrus grove sampled or in the pasture that provided soil for the greenhouse experiment, as it has not been registered for use in orchards or agricultural fields. Penicillin was also not indirectly applied through manure contaminated with antibiotics or antibiotic resistant bacteria. This finding is in agreement with the notion that antibiotic resistance is very common in agricultural soils (Demanèche et al., 2008), also in soils that have not been exposed to those particular antibiotics via manure (Marti et al., 2013; Udikovic-Kolic et al., 2014). Antibiotic production by a variety of microorganisms occurs in soil and selects for resistance and the ability to degrade these compounds (Nesme and Simonet, 2015; Zhang and Dick, 2014), but the surge in antibiotic resistance encountered in recent years cannot be explained by the low concentrations of antibiotics excreted under natural conditions.

Penicillin resistance could be obtained via horizontal gene transfer even between gram positive and gram negative bacteria (Courvalin, 1994), for example of genes for the production of β-lactamases that break down penicillin (Mazodier and Davies, 1991), genes for efflux pumps extruding penicillin from the cell (Tenover, 2006), genes encoding altered penicillin binding sites (Dowson et al., 1989), or genes that limit the access of penicillin to the target site (Tenover, 2006). However, the question remains how these genes could have come to the fore in soils that have not been exposed to selection pressure by high concentrations of penicillin and that have not received antibiotic resistant bacteria in manure, for example.

The high level of cross-resistance to glyphosate and penicillin that we detected in this work, plus the reported intensive use of glyphosate in citrus orchards and pastures in Florida (USDA NASS, 2015), suggests that the selection pressure for glyphosate resistance in bacteria is high. Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is crucial to produce aromatic amino acids and secondary products in plants and many taxa of microorganisms (Staub et al., 2012). Glyphosate resistance in bacteria has been ascribed to mechanisms similar to those mentioned for penicillin resistance. Genes affecting attachment to target sites may not be similar for resistance to penicillin and glyphosate. However, overexpression of an efflux transporter gene could possibly lead to resistance to both glyphosate and penicillin (Staub et al., 2012). Cross-resistance between

glyphosate and penicillin has been documented (Kurenbach et al., 2015; Liu et al., 2013), and suggests that intensive use of glyphosate may be a driving force in the evolution of penicillin resistance among bacterial populations in fields where penicillin or penicillin-resistant bacteria have not been applied. However, additional research is needed to investigate a potential relationship between penicillin resistance and glyphosate resistance in several citrus groves that vary in the use of glyphosate. Nevertheless, high levels of penicillin resistance in the citrus groves that have not been exposed to penicillin injection, indicate that the risk of developing additional penicillin resistance as a result of trunk injection is very limited.

The initial changes observed in microbial populations in the rhizosphere could have rendered the trees more susceptible to Phytophthora diseases (Graham et al., 2013; Widmer et al., 1998; Workneh et al., 1993). However, we did not observe increased root rot severity in the trees treated with penicillin. Inoculation with *P. nicotianae* did increase root rot severity compared to non-inoculated control trees. *P. nicotianae* was only isolated from inoculated trees, while a different, unidentified *Phytophthora* species, possibly *P. cactorum*, was isolated from some non-inoculated trees. Thus from our experiment, there is no indication that the risk of Phytophthora root rot development would be enhanced by trunk injection of penicillin.

In conclusion, the potential side effects of penicillin injection in citrus trunks on the environment as tested in this study do not give any reason for concern. The benefits of penicillin injection to control HLB may outweigh the risks of changes in microbial communities and penicillin resistance. However, Las, the bacterium associated with HLB, may become resistant to penicillin when trunk injection is used on a large scale. In any case, the risks of penicillin residues in citrus fruit still need to be evaluated under field conditions before registration is possible. Recently, a sensitive LC-MS/MS method was developed to detect penicillin in citrus fruits, but these fruits were spiked with penicillin after harvest (Aldeek et al., 2015). This would probably be a better method than the bioassay used here to detect penicillin residues in citrus tissues and fruits from trees subjected to trunk injection of penicillin in groves. This precautionary work needs to be done as soon as possible to provide the necessary data for registration of trunk injection of penicillin to control HLB in Florida. Future studies will need to combine measurements of both penicillin and glyphosate concentrations in citrus roots, as well as glyphosate and penicillin resistance in bacteria isolated from those roots, in order to estimate if genetic hitchhiking of penicillin resistance on glyphosate resistance could occur in bacterial populations from citrus orchards in Florida.

Acknowledgements

This research was funded by the Florida Department of Agriculture and Consumer Services (HLB-Mac 14-8130-0469-CA). We thank Dr. Greg Hodges and Dr. Tim Schubert of the Division of Plant Industry for providing these funds to us and discussing the set-up of the experiments. Additional funding was provided by the Esther B. O'Keeffe Foundation. We are grateful to Robert H. Moore of the Pesticide Registration Review Section, Bureau of Scientific Evaluation and Technical Assistance, Division of Agricultural Environmental Services, Florida Department of Agriculture and Consumer Services, Tallahassee, FL, for providing the exemption to use penicillin in an experimental field, and in a BSL2 greenhouse and laboratory. Cody Neff, Hannah Fahsbender, Ellen Dickstein, and Yorley Nikary Bustamante participated in collecting and processing citrus samples. Mark G. Kann and Jim A. Boyer took care of the young citrus trees in the greenhouse at Citra. We thank Dr. Jeffrey B. Jones and Sujan Timilsina for reviewing an earlier draft of the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cropro.2016.08.025.

References

Aćimović, S.G., Zeng, Q., McGhee, G.C., Sundin, G.W., Wise, J.C., 2015. Control of fire blight (*Erwinia amylovora*) on apple trees with trunk-injected plant resistance inducers and antibiotics and assessment of induction of pathogenesis-related protein genes. Front. Plant Sci. 6, 16,. http://dx.doi.org/10.3389/fpls.2015.00016.

Aldeek, F., Rosana, M.R., Hamilton, Z.K., Crosswhite, M.R., Burrows, C.W., Singh, S., Gerard, G., Hammack, W., Cook, J.-M., 2015. LC-MS/MS method for the determination and quantitation of penicillin G and its metabolites in citrus fruits affected by Huanglongbing. J. Agric. Food Chem. 63, 5993–6000. http://dx.doi.org/10.1021/acs.jafc.5b02030.

Aubert, B., Bové, J.M., 1980. Effect of penicillin or tetracycline injections of citrus trees affected by greening disease in Reunion Island. In: 8th Congress of the International Society of Citriculture. Riverside, CA, USA, pp. 03–108.

Boina, D.R., Bloomquist, J.R., 2015. Chemical control of the Asian citrus psyllid and of Huanglongbing disease in citrus. Pest Manag. Sci. 71, 808–823. http://dx.doi.org/10.1002/ps.3957.

Bové, J.M., 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol. 88, 7–37 (accessed 16.08.16). http://www.sipav.org/main/jpp/volumes/0106/010602.pdf.

Bové, J.M., Bonnet, P., Garnier, M., Aubert, B., 1980. Penicillin and tetracycline treatments of greening disease-affected citrus plants in the glasshouse, and the bacterial nature of the prokaryote associated with greening. In: Eighth IOCV Conference; International Organization of Citrus Virologists. Riverside, CA, USA, pp. 102–107.

Buchholz, A., Baur, P., Schönherr, J., 1998. Differences among plant species in cuticular permeabilities and solute mobilities are not caused by differential selectivity. Planta 206, 322–328.

Buchholz, A., Schönherr, J., 2000. Thermodynamic analysis of diffusion of non-electrolytes across plant cuticles in the presence and absence of the plasticiser tributyl phosphate. Planta 212, 103–111.

Chang, Q.Z., Wang, W.K., Regev-Yochay, G., Lipsitch, M., Hanage, W.P., 2015. Antibiotics in agriculture and the risk to human health: how worried should we be? Evol. Appl. 8, 240–247. http://dx.doi.org/10.1111/eva.12185.

Chiyaka, C., Singer, B.H., Halbert, S.E., Morris, J.G., van Bruggen, A.H.C., 2012. Modeling Huanglongbing transmission within a citrus tree. Proc. Natl. Acad. Sci. U. S. A. 109, 12213–12218. http://dx.doi.org/10.1073/pnas.1208326109.

Courvalin, P., 1994. Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. Antimicrob. Agents Chemother. 38, 1447–1451.

Demanèche, S., Sanguin, H., Potè, J., Navarro, E., Bernillon, D., Mavingui, P., Wildi, W., Vogel, T.M., Simonet, P., 2008. Antibiotic-resistant soil bacteria in transgenic plant fields. Proc. Natl. Acad. Sci. U. S. A. 105, 3957–3962. http://dx.doi.org/10.1073/pnas.0800072105.

Dowson, C.G., Hutchinson, A., Brannigan, J.A., George, R.C., Hansman, D., Liñares, J., Tomasz, A., Smith, J.M., Spratt, B.G., 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. U. S. A. 86, 8842–8846.

EPA, 2013. Streptomycin; Pesticide Tolerances for Emergency Exemptions. Environmental Protection Agency 40 CFR Part 180 [EPA–HQ–OPP–2011–0852; FRL–9385–3]. Federal Register, 78, 29049 (accessed 16.08.16). https://www.gpo.gov/fdsys/pkg/FR-2013-05-17/pdf/2013-11858.pdf.

Farnsworth, D., Grogan, K.A., van Bruggen, A.H.C., Moss, C.B., 2014. The potential economic cost and response to greening in Florida citrus. Choices 29, 1–6.

FDACS, 2013. Florida citrus Statistics 2011-2012. Florida Department of Agriculture and Consumer Services, Tallahassee, Florida (accessed 16.08.16). https://www.nass.usda.gov/Statistics_by_State/Florida/Publications/Citrus/Citrus_Statistics/2011-12/fcs1112.pdf.

Graham, J.H., Colburn, G.C., Chung, K.R., Cubero, J., 2012. Protection of citrus roots against infection by *Phytophthora* spp. by hypovirulent *P. nicotianae* is not related to induction of systemic acquired resistance. Plant Soil 358, 39–49. http://dx.doi.org/10.1007/s11104-011-1119-x.

Graham, J.H., Dewdney, M.M., Myers, M.E., 2010. Streptomycin and copper formulations for control of citrus canker on grapefruit. Proc. Fla. State Hortic. Soc. 123, 92–99.

Graham, J.H., Johnson, E.G., Gottwald, T.R., Irey, M.S., 2013. Pre-symptomatic fibrous root decline in citrus trees caused by Huanglongbing and potential synergistic interaction with *Phytophthora* spp. Plant Dis. 97, 1195–1199. http://dx.doi.org/10.1094/Pdis-01-13-0024-Re.

Grünwald, N.J., Hu, S., van Bruggen, A.H.C., 2000. Short-term cover crop decomposition in organic and conventional soils: Characterization of soil C, N, microbial and plant pathogen dynamics. Eur. J. Plant Pathol. 106, 37–50. http://dx.doi.org/10.1023/A:1008720731062.

Jeffers, S.N., Martin, S.B., 1986. Comparison of two Media Selective for *Phytophthora* and *Pythium* Species. Plant Dis. 70, 1038–1043. http://dx.doi.org/10.1094/Pd-

70-1038.
Johnson, E.G., Wu, J., Bright, D.B., Graham, J.H., 2014. 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 Pathol. 63, 290–298. http://dx.doi.org/10.1111/ppa.12109.
Ke, C., Wang, Z.S., 1991. A study on the use of antibiotics to control citrus Huanglungbin. In: Proceedings of the 6th International Asia Pacific Workshop on Integrated Health Management. FAO, Rome, Italy, pp. 40–48.
Kumar, K., Gupta, S.C., Chander, Y., Singh, A.K., 2005. Antibiotic use in agriculture and its impact on the terrestrial environment. Adv. Agron. 87, 1–54.
Kurenbach, B., Marjoshi, D., Ambile-Cuevas, C.F., Ferguson, G.C., Godsoe, W., Gibson, P., 2015. Sublethal exposure to commercial formulations of the herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate cause changes in antibiotic susceptibility in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Mbio 6, e00009–15. http://dx.doi.org/10.1128/mBio.00009-15.
Li, W., Hartung, J.S., Levy, L., 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus Huanglongbing. J. Microbiol. Methods 66, 104–115.
Liu, Y.B., Long, M.X., Yin, Y.J., Si, M.R., Zhang, L., Lu, Z.Q., Wang, Y., Shen, X.H., 2013. Physiological roles of mycothiol in detoxification and tolerance to multiple poisonous chemicals in *Corynebacterium glutamicum*. Archives Microbiol. 195, 419–429. http://dx.doi.org/10.1007/s00203-013-0889-3.
Macias, E.A., Mason, E.O., Ocera, H.Y., Larocco, M.T., 1994. Comparison of e test with standard broth microdilution for determining antibiotic susceptibilities of penicillin-resistant strains of *Streptococcus pneumoniae*. J. Clin. Microbiol. 32, 430–432.
Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709. http://dx.doi.org/10.1128/Aem.01682-13.
Mazodier, P., Davies, J., 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. 25, 147–171.
Narouei-Khandan, H.A., Halbert, S.E., Worner, S.P., van Bruggen, A.H.C., 2016. Global climate suitability of citrus Huanglongbing and its vector, the Asian citrus psyllid, using two correlative species distribution modeling approaches, with emphasis on the USA. Eur. J. Plant Pathol. 144, 655–670. http://dx.doi.org/10.1007/s10658-015-0804-7.
Nesme, J., Simonet, P., 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ. Microbiol. 17, 913–930. http://dx.doi.org/10.1111/1462-2920.12631.
Puttamuk, T., Zhang, S.A., Duan, Y.P., Jantasorn, A., Thaveechai, N., 2014. Effect of chemical treatments on '*Candidatus* Liberibacter asiaticus' infected pomelo (*Citrus maxima*). Crop Prot. 65, 114–121. http://dx.doi.org/10.1016/j.cropro.2014.07.018.
Senechkin, I.V., Speksnijder, A.G.C.L., Semenov, A.M., van Bruggen, A.H.C., van Overbeek, L.S., 2010. Isolation and partial characterization of bacterial strains on low organic carbon medium from soils fertilized with different organic amendments. Microb. Ecol. 60, 829–839. http://dx.doi.org/10.1007/s00248-010-9670-1.
Shen, W., Cevallos-Cevallos, J.M., da Rocha, U.N., Arevalo, H.A., Stansly, P.A., Roberts, P.D., van Bruggen, A.H.C., 2013a. Relation between plant nutrition, hormones, insecticide applications, bacterial endophytes, and *Candidatus* Liberibacter Ct values in citrus trees infected with Huanglongbing. Eur. J. Plant Pathol. 137, 727–742. http://dx.doi.org/10.1007/s10658-013-0283-7.
Shen, W., Halbert, S.E., Dickstein, E., Manjunath, K.L., Shimwela, M.M., van Bruggen, A.H.C., 2013b. Occurrence and in-grove distribution of citrus Huanglongbing in North Central Florida. J. Plant Pathol. 95, 361–371.
Shimwela, M., Narouei-Khandan, H.A., Halbert, S.E., Keremane, M.L., Minsavage, G.V., Timilsina, S., Massawe, D.P., Jones, J.B., van Bruggen, A.H.C., 2016. First occurrence of *Diaphorina citri* in East Africa, characterization of the Ca. Liberibacter species causing Huanglongbing (HLB) in Tanzania, and potential further spread of *D. citri* and HLB in Africa and Europe. Eur. J. Plant Pathol. http://dx.doi.org/10.1007/s10658-016-0921-y.
Staub, J.M., Brand, L., Tran, M., Kong, Y., Rogers, S.G., 2012. Bacterial glyphosate resistance conferred by overexpression of an *E. coli* membrane efflux transporter. J. Ind. Microbiol. Biotechnol. 39, 641–647.
Stockwell, V.O., Duffy, B., 2012. Use of antibiotics in plant agriculture. Sci. Tech. Rev. office Int. des Epizooties 31, 199–210.
Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. Am. J. Med. 119, S3–S10.
Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl. Acad. Sci. U. S. A. 111, 15202–15207. http://dx.doi.org/10.1073/pnas.1409836111.
USDA NASS, 2015. Quick Stats 2.0. United States Department of Agriculture, National Agricultural Statistics Service (accessed 16.08.16. http://quickstats.nass.usda.gov.
van Bruggen, A.H.C., Grogan, R.G., Bogdanoff, C.P., Waters, C.M., 1988. Corky root of lettuce in California caused by a gram-negative bacterium. Phytopathology 78, 1139–1145.
Widmer, T.L., Graham, J.H., Mitchell, D.J., 1998. Composted municipal waste reduces infection of citrus seedlings by *Phytophthora nicotianae*. Plant Dis. 82, 683–688. http://dx.doi.org/10.1094/Pdis.1998.82.6.683.
Workneh, F., van Bruggen, A.H.C., Drinkwater, L.E., Shennan, C., 1993. Variables associated with corky root and Phytophthora root-rot of tomatoes in organic and conventional farms. Phytopathology 83, 581–589. http://dx.doi.org/10.1094/Phyto-83-581.
Yandoc, C.B., Rosskopf, E.N., Shah, D.A., Albano, J.P., 2007. Effect of fertilization and biopesticides on the infection of *Catharanthus roseus* by *Phytophthora nicotianae*. Plant Dis. 91, 1477–1483. http://dx.doi.org/10.1094/Pdis-91-11-1477.
Yang, C., Powell, C.A., Duan, Y., Shatters, R., Fang, J., Zhang, M., 2016. Deciphering the bacterial microbiome in huanglongbing-affected citrus treated with thermotherapy and sulfonamide antibiotics. PloS One 11 (5), e0155472. http://dx.doi.org/10.1371/journal.pone.0155472.
Zelenev, V.V., van Bruggen, A.H.C., Semenov, A.M., 2000. "BACWAVE," a spatial-temporal model for traveling waves of bacterial populations in response to a moving carbon source in soil. Microb. Ecol. 40, 260–272.
Zelenev, V.V., van Bruggen, A.H.C., Semenov, A.M., 2005. Short-term wavelike dynamics of bacterial populations in response to nutrient input from fresh plant residues. Microb. Ecol. 49, 83–93. http://dx.doi.org/10.1007/s00248-003-1054-3.
Zhang, M., Guo, Y., Powell, C.A., Doud, M.S., Yang, C., Duan, Y., 2014. Effective antibiotics against '*Candidatus* Liberibacter asiaticus' in HLB-affected citrus plants identified via the grafted-based evaluation. PloS one 9 (11), e111032,. http://dx.doi.org/10.1371/journal.pone.0111032.
Zhang, M., Powell, C.A., Benyon, L.S., Zhou, H., Duan, Y.P., 2013a. Deciphering the bacterial microbiome of citrus plants in response to '*Candidatus* Liberibacter asiaticus'-infection and antibiotic treatments. PLoS One 8 (11). http://dx.doi.org/10.1371/journal.pone.0076331.
Zhang, M., Powell, C.A., Guo, Y., Benyon, L., Duan, Y.P., 2013b. Characterization of the microbial community structure in *Candidatus* Liberibacter asiaticus-infected citrus plants treated with antibiotics in the field. BMC Microbiol. 13 http://dx.doi.org/10.1186/1471-2180-13-112.
Zhang, M., Powell, C.A., Guo, Y., Doud, M.S., Duan, Y., 2012. A graft-based chemotherapy method for screening effective molecules and rescuing huanglongbing-affected citrus plants. Phytopathology 102, 567–574.
Zhang, M., Powell, C.A., Zhou, L.J., He, Z.L., Stover, E., Duan, Y.P., 2011. Chemical compounds effective against the citrus Huanglongbing bacterium '*Candidatus* Liberibacter asiaticus' in planta. Phytopathology 101, 1097–1103. http://dx.doi.org/10.1094/Phyto-09-10-0262.
Zhang, M., Duan, Y.P., Zhou, L.J., Turechek, W.W., Stover, E., Powell, C.A., 2010. Screening molecules for control of citrus Huanglongbing using an optimized regeneration system for '*Candidatus* Liberibacter asiaticus'-infected periwinkle (*Catharanthus roseus*) cuttings. Phytopathology 100, 239–245. http://dx.doi.org/10.1094/Phyto-100-3-0239.
Zhang, Q., Dick, W.A., 2014. Growth of soil bacteria, on penicillin and neomycin, not previously exposed to these antibiotics. Sci. Total Environ. 493, 445–453. http://dx.doi.org/10.1016/j.scitotenv.2014.05.114.
Zhou, L., Powell, C.A., Hoffman, M.T., Li, W., Fan, G., Liu, B., Lin, H., Duan, Y., 2011. Diversity and plasticity of the intracellular plant pathogen and insect symbiont "*Candidatus* Liberibacter asiaticus" as revealed by hypervariable prophage genes with intragenic tandem repeats. Appl. Environ. Microbiol. 77, 6663–6673.