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Chemical Compounds Effective against the Citrus Huanglongbing Bacterium, 'Candidatus Liberibacter asiaticus' in Planta

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Abstract: Citrus huanglongbing (HLB) is one of the most destructive diseases of citrus worldwide, and is threatening the survival of the Floridian citrus industry. Currently, there is no established cure for this century-old and emerging disease. As a possible control strategy for citrus HLB, therapeutic compounds were screened using a propagation test system with Las-infected periwinkle and citrus plants. The results demonstrated that the combination of penicillin and streptomycin (PS) was effective in eliminating or suppressing the Las bacterium and provided a therapeutically effective level of control for a much longer period of time than when administering either antibiotic separately. When treated with the PS, Las-infected periwinkle cuttings achieved 70% of regeneration rates vs. less than 50% with other treatments. The Las bacterial titers in the infected periwinkle plants, as measured by quantitative real-time PCR, decreased significantly following root-soaking or foliar-spraying with PS. Application of the PS via trunk injection or root soaking also eliminated or suppressed the Las bacterium in the HLB-affected citrus plants. This may provide a useful tool for the management of citrus HLB and other Liberibacter-associated diseases.

Key words: Chemical control; Penicillin G potassium; Streptomycin; Oxytetracycline; 2, 2-dibromo-3-nitrilopropionamide

Citrus huanglongbing (HLB), also known as citrus greening, is the most economically devastating disease of citrus worldwide. All commercial citrus industries that have faced the dis-

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ease suffer a decline both in production and profit (19). It has been estimated that nearly 100 million trees in 40 countries have been affected by HLB (16). In the 1960s, Reunion Island lost its entire citrus industry due to HLB (21, 32) . In Florida, Citrus HLB, first detected in August 2005 (39), is now present in all commercial citrus producing counties, and is threatening the survival of the 9 billion dollar citrus industry. The HLB bacterium, '*Candidatus* Liberibacter asiaticus' (Las) is a Gram-negative, fastidious alpha-proteobacterium. The bacterium propagates within the phloem of the host plants, resulting in die-back, yellow shoots, blotchy mottles on leaves, and off-tasting malformed fruit (23). The disease spreads relatively fast in citrus groves where control measures (such as, vector control) are not properly implemented, reaching more than 95 percent incidence in as short as 3 years after the first infection (11, 17). As the disease severity increases, the yield is significantly reduced, and thereby the lifespan for the profitable productivity of citrus trees is dramatically shortened (4, 33).

To date, there are limited control practices for preventing further spread of HLB, and all known citrus species, varieties or combinations of scion and rootstock are susceptible to HLB (2, 5, 6, 7, and 8). Control and management measures are currently limited to reducing inoculum sources by utilizing disease-free propagating stock, removing symptomatic trees, and reducing transmission of the bacterium by control of the insect vectors (4).

Antibiotics have been used to control bacterial diseases of fruit trees and to limit the contamination of micro propagation and plant tissue cultures for more than 50 years (31, 42). Nearly 40 antibiotics have been screened for plant disease control, but fewer than 10 have been used commercially, and of those, only streptomycin and tetracycline have had significant usage in fruit trees (31). Usage of streptomycin and tetracycline on plants accounted for 0.1% of all antibiotics produced annually in the USA in 1995 (3, 26). During the 1970s, tetracycline was evaluated by direct injection into the trunks of HLB-affected citrus trees in South Africa, China and Indonesia (38, 40, and 41). This work noted a significant reduction of symptoms in treated trees (14, 35 and 37). However, this control practice was later discontinued because tetracycline is only bacteriostatic, not bactericidal, requiring treatment to be repeated each year. In addition, after several trunk injections, the phytotoxicity of the antibiotic became apparent in the injected citrus trees (30, 36). Development of a bactericide or other therapeutic compounds for the control of HLB would provide an additional solution for effective disease management. However, with the exception of selected antibiotics, there is no systemic bactericide that has been registered for general use on most crops and specifically citrus (25). Here, we present a new combination of antibiotics that can effectively eliminate or suppress the HLB bacterium both in Las-infected periwinkle and HLB-affected citrus plants.

MATERIALS AND METHODS

HLB-affected citrus. Healthy 2-year old grapefruit (*Citrus paradisi* Macf.) seedlings were graft-inoculated with HLB-affected lemon [*Citrus limon* (L.) Burm. f.] scions in August 2008 and were subsequently maintained in the greenhouse. After 10 months, the typical HLB symptoms with vein corking and blotchy mottles on leaves appeared on the inoculated seedlings of grapefruit. HLB-affected citrus seedlings with typical HLB symptoms were tested for the presence of Las by using quantitative real-time PCR (qPCR) with Las-specific primers (HLBas/HLBr/HLBp) (27). For field studies, HLB-affected citrus plants at the USHRL farm at Fort Pierce, Florida were also analyzed using qPCR detection methods.

Las-infected periwinkle. Las-infected periwinkle (*Catharanthus roseus*) plants were propagated by graft transmission as described previously (43). Briefly, newly expanded branches of Las-infected periwinkle were graft-inoculated on Las-free periwinkle plants. Transmission was confirmed via qPCR using Las-specific primer sets (HLBas/HLBr/HLBp) (27). All Las-infected periwinkle plants were maintained in the greenhouse for further studies.

Antibiotic treatments using Las-infected periwinkle.

Comparison of three treatments. Three chemical treatments were evaluated for their potential to eliminate Las bacterium infection: (i) Antibiotic combination of penicillin G and streptomycin (PS) (100μg/ml penicillin G potassium salt and 10μg/ml streptomycin; Sigma-Aldrich, St. Louis, MO); (ii) the antibiotic oxytetracycline (Oxy) (100μg/ml; Sigma-Aldrich, St. Louis, MO) and (iii) Biocide agent 2, 2-dibromo-3-nitrilopropionamide (DBNPA) (200μl/liter; Dow Chemical, Midland, MI). The stems of Las-infected cuttings were individually soaked in the above solutions for 4h and then planted in 100% vermiculite using the optimized regeneration test system described before (43). The cuttings were further treated with the same solutions at 7 and 14 days post transplanting, by drenching the potting media with 10 ml per cutting. Each experiment was repeated twice using fifteen cuttings per treatment. After 2 months from the initial treatment, the plants regenerated from cuttings were recorded to determine the regeneration rate (%). In the foliar-spraying experiment, thirty Las-infected periwinkle plants were foliar-sprayed with the same three solutions (PS, Oxy and DBNPA) using ZEP 32 oz. professional sprayer, a total of three times at one week intervals. Ten plants were treated with each chemical solution. Each plant was sprayed to drip with approximately 100 ml of chemical solution.

Comparison of PS rates and adjuvants.

The effects of PS application rates and inclusion of adjuvants (DMSO at 20μl/ml and Silwet L-77 at 20μl/ml) were evaluated on the Las-infected periwinkle plants under greenhouse conditions. The factors were arranged in a split-plot design with three replicates. Adjuvant type (DMSO and Silwet L-77) was the whole plot factor, while PS at 4 different application rates and blank control (without any spraying) were considered as the subplot treatments: (i) 1× (100 mg/L penicillin and 10 mg/L streptomycin); (ii) 5× (500 mg/L penicillin and 50 mg/L streptomycin); (iii) 10× (1 g/L penicillin and 100 mg/L streptomycin); (iv) 0×: a water-treated control. Treatments were applied by spraying the Las-infected periwinkle plants using ZEP 32 oz. professional sprayer, a total of three times at one week intervals. Ten leaf samples per treatment were taken at 30, 60 and 90 days after initial treatment (DAT). Data were analyzed as a generalized linear mixed model using the SAS procedure GLIMMIX. The whole plot and subplot factors were treated as fixed effects, replication and its interaction with the whole plot factor as random effects. Differences among treatment levels were determined with the LINES option of the LSMEANS statement.

PS treatment on HLB-affected citrus. PS treatment components were compared separately and in combination. Fifteen of the 2-year old grapefruit seedlings with typical HLB symptoms were treated by root-soaking in solutions of penicillin (P) at 1.0 g/L and streptomycin (S) at 100 mg/L alone and in combination. The trial was conducted three times at one week intervals. The treated seedlings were maintained in the USHRL greenhouse under standard conditions at (25 ± 2)℃, and watered as needed for commercial citrus nursery production. Five leaf samples were taken at 30, 60, 90, and 180 DAT, respectively, and DNA was isolated for qPCR analysis.

Six HLB-affected 6-year old citrus trees (a unique hybrid (10c-5-58) which is an open pollinated seedling from the combination of Lee mandarin × Orlando tangelo) at the USHRL farm of 10-cm diameter were injected with 100 ml of each of the PS treatment (PS-5: 5 g P + 0.5 g S/tree; PS-10: 10 g P + 1 g S/tree and PS-0: water as control) using an Avo-Ject syringe injector (a catheter-tipped 60 ml syringe, Aongatete Coolstores Ltd., NZ) in June, 2009. The tapered tip was firmly fitted into a 19/64-inch (7.5mm) diameter hole, about 3cm deep, drilled into the tree. Treatments were repeated in a total of three times at once every two months (June 8, August 10, and October 6 of 2009, respectively). Before treatment, more than 30 leaf samples per tree were taken from three positions around the canopy of the treated trees for qPCR assay at two month intervals in the first 6 months, and then taken at 10 and 14 months from initial treatments.

Genomic DNA extraction and qPCR analysis. Each leaf was rinsed three times with sterile water. Midribs were separated from the leaf samples and cut into pieces of 1.0mm to 2.0mm. DNA was extracted from 0.1 g of tissue (fresh weight) of leaf midribs using Qiagen's DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed with primers and probes (HLBas/HLBr/HLBp) for the Las bacterium (27) using ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA) in a 20-μl reaction volume consisting of the following reagents: 300 nM (each) target primer (HLBas and HLBr), 150 nM target probe (HLBp), and 1 ×TaqMan qPCR Mix (Applied Biosystems). The amplification protocol was 95 ℃ for 20 s followed by 40 cycles at 95 ℃ for 3 s and 60 ℃ for 30 s. All reactions were performed in triplicate and each run contained one negative (DNA from healthy plant) and one positive (DNA from Las-infected plant) control. Data were analyzed using the ABI 7500 Fast Real-Time PCR System with SDS software. The resulting Ct values were converted to the estimated bacterial titers using the grand universal regression equation $Y = 13.82 - 0.2866X$ (Y is the estimated log concentration of templates and X is the qPCR Ct values) as described by Li et al (28). As in the previous report (43), plants were regarded as PCR-negative when there were no HLB-like symptoms in the periwinkle or citrus which had more than 36 of Ct value, equivalent to estimated bacterial titers of lower than 1 060 cells per gram plant tissue.

RESULTS

Comparison of three treatments. Antibiotics (PS and Oxy) and a biocide agent (DBNPA) were tested for potential antimicrobial activity against the Las bacterium and evaluated for promoting regeneration and growth of Las-infected periwinkle cuttings. More than 70% of the Las-infected cuttings treated with PS regenerated and grew, but less than 50% of the cuttings treated with Oxy and DBNPA successfully regenerated (Figure 1). Variance analysis showed that there were significant effects of the chemical compounds ($P = 0.000$), treatment duration ($P = 0.000$) and their interactions ($P = 0.000$) on Las bacterial titers in the fixed model. All plants regenerated from the Las-infected cuttings treated with PS showed a marked reduction in Las bacteria by 60 days after initial treatment (DAT) and tested negative for the Las bacterium via qPCR at 90 DAT, which estimated bacterial titers of lower than 500 cells per gram plant tissue. When the Las-infected cuttings were treated with DBNPA at concentration of 200μl/liter, Las bacterial titers in the regenerated plants were more than 5×10^5 (cells per gram plant tissue) at 60 DAT and 90 DAT, indicating limited suppression of Las bacterial population in the DBNPA-treated plants. However, Las bacteria of the regenerated plants from the cuttings treated with Oxy were about 8×10^5 at 60 DAT and decreased to lower than 500 at 90 DAT (Figure 2). The results indicated that the most effective chemical treatment was PS, with significant results seen at 60 DAT. Use of foliar-sprays rather than root-drenches on Las-infected periwinkle plants showed that PS and Oxy treatments eliminated the Las-bacterium, based on lower Las bacterial titers and recovered growth, while DBNPA treatment of infected periwinkle did not fully eliminate the Las bacterium, signified by higher Las bacterial titers after 90 DAT (Figure 3).

Analysis of variance showed significant effects of the PS concentrations (C, $P = 0.000$), treatments (A, $P = 0.000$) and their interactions ($A \times C$, $P = 0.000$), but no significant effects of adjuvants (B, $P = 0.420$) and its interactions to PS concentrations and treatments ($B \times C$, $P = 0.121$; $A \times B$, $P = 0.823$; $A \times B \times C$, $P = 0.164$). DMSO and Silwet L-7 had no different effects as adjuvants in the PS-treatment (Supplemental Figure 1). The Las bacterial titers in all of the PS treatments, regardless of concentration, were 1000-fold lower than those in the water and blank controls. This indicates that PS at the concentrations of 1 ×, 5 × and 10 × significantly reduced or suppressed Las bacterium in the Las-infected periwinkles (Figure 4).

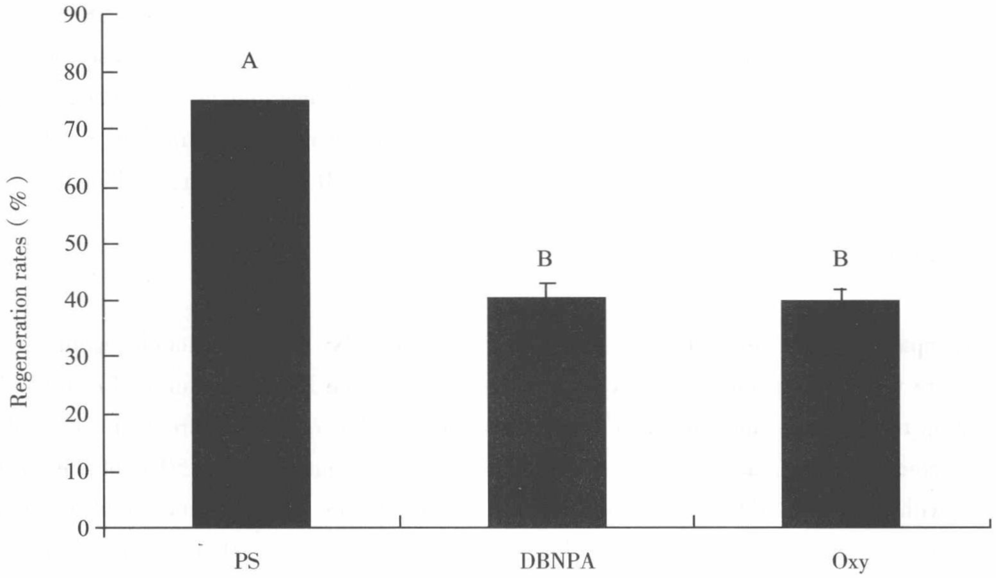

Fig. 1 Mean differences of the regeneration rates of Las-infected periwinkle cuttings treated with PS (100 mg/L of penicillin G potassium and 10 mg/L of streptomycin), Oxy (100 mg/L of oxytetracycline) and DBNPA (200μl/L of 2, 2-dibromo-3-nitrilopropionamide). Different capital letters (A and B) on the bar indicate regeneration rates are significantly different ($P < 0.01$)

HLB-affected citrus treated by root soaking with PS. The antibiotic combination, PS, effectively eliminated the Las bacterium in Las-infected citrus and provided a therapeutically effective level of control for 6 months, in contrast to treatments of penicillin (P) or streptomycin (S) alone (Figure 5). Las bacteria at 30 DAT for PS (36 733 cells per gram plant tissues) was significantly lower than those individual treatments alone (282 180 cells for P; 500 766 cells for S). Penicillin or streptomycin alone also suppressed the Las bacterial population as indicated by the lower Las bacterial titers at 90 DAT, but the Las bacterial titers returned to a relatively high population at 180 DAT (Figure 5). The results indicated that PS enhanced the overall effectiveness of penicillin and streptomycin, demonstrating a synergetic and greater prolonged activity.

HLB-affected citrus treated with PS via injections. When HLB-affected citrus trees, in the field of the USHRL farm at Fort Pierce, were injected with different dosages of the PS (PS-0: water; PS-5: 5 g P + 0.5 g S in 100 ml water/tree and PS-10: 10 g P + 1 g S in 100 ml water/tree), the resulting Las bacteria decreased from 1 080 349 (prior to treatment) to 78 346 (2 months after initial treatment with PS-5). This is approximately a 13-fold reduction in the Las bacterial titers in the treated citrus plants. The Las bacterial titers tested in Oct, 2009 were reduced to lower than 100 (undetectable in qPCR), indicating the elimination or suppression of the Las bacterium in the PS-treated plants. The Las bacterial titers in the PS-5

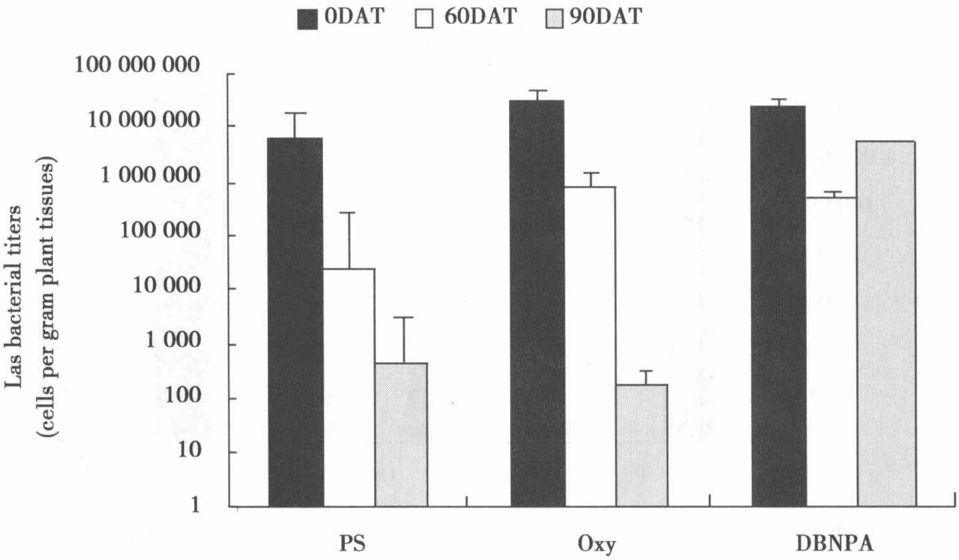

Fig. 2 Initial *Candidatus* Liberibacter asiaticus (Las) bacterial titers (cells per gram plant tissue) of Las-infected periwinkle cuttings (0 DAT) and variations of Las bacterial titers (cells per gram plant tissue) at 60 DAT (Days after treatment) and 90 DAT in the regenerated plants treated by root drench with PS (100 mg/L penicillin and 10 mg/L streptomycin), Oxy (100 mg/L oxytetracycline) and DBNPA (200μl/L of 2, 2-dibromo-3-nitrilopropionamide)

treated citrus kept at lower than 1 000 cells per gram plant tissue until 14 months after the PS treatments ceased (Figure 6). Similar results were observed when PS was applied at PS-10, except that the Las bacterial titers were kept to less than 1 000 cells per gram plant tissues. In contrast, the Las bacterial titers from the water control treated plants remained around 2×10^6 throughout the experiment (Figure 6). When compared to the water treated plants, phytotoxic effects on citrus were found at PS-10 (Supplemental Figure 2).

DISCUSSIONS

Citrus fruit is produced in 140 countries worldwide. Currently, most of citrus producing countries including the top three producers (China, Brazil, and the United States) are suffering from HLB, the most devastating disease of citrus. HLB is difficult to manage, and maintaining production of citrus has proven to be difficult and expensive in areas where HLB is widespread. HLB has not been eradicated from any region where infection has been reported (19, 32). The first step in the successful management of HLB is the production of clean nursery stock since there is little chance of developing a productive tree when the tree is already infected at planting (20). With no established curative treatments or effective biological con-

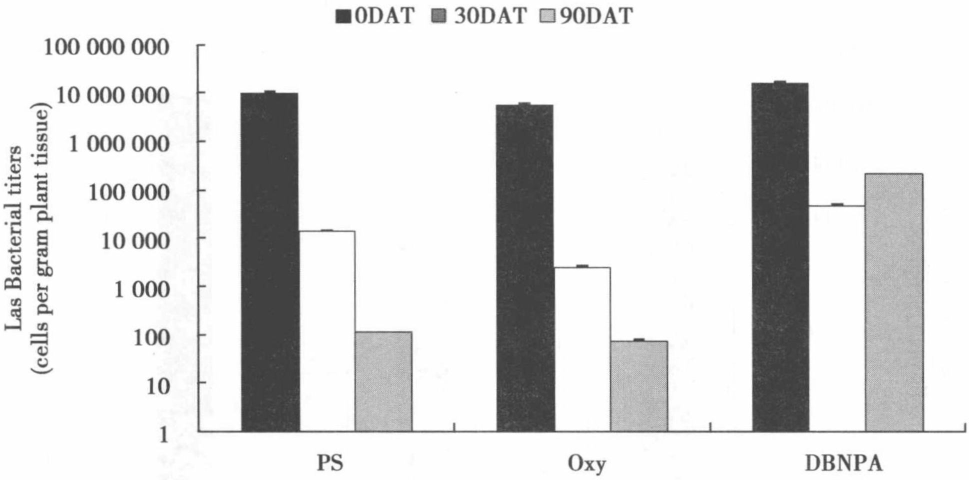

Fig. 3 ***Candidatus*** **Liberibacter asiaticus (Las) bacterial titers (cells per gram plant tissue) at 0 DAT (pre-treatment), 30 DAT (Days after treatment) and 90 DAT of Las-infected periwinkle plants treated by folial spraying with PS (100 mg/L penicillin and 10 mg/L streptomycin), Oxy (100 mg/L of oxytetracycline) and DBNPA (200μl/L of 2, 2-dibromo-3-nitrilo-propionamide)**

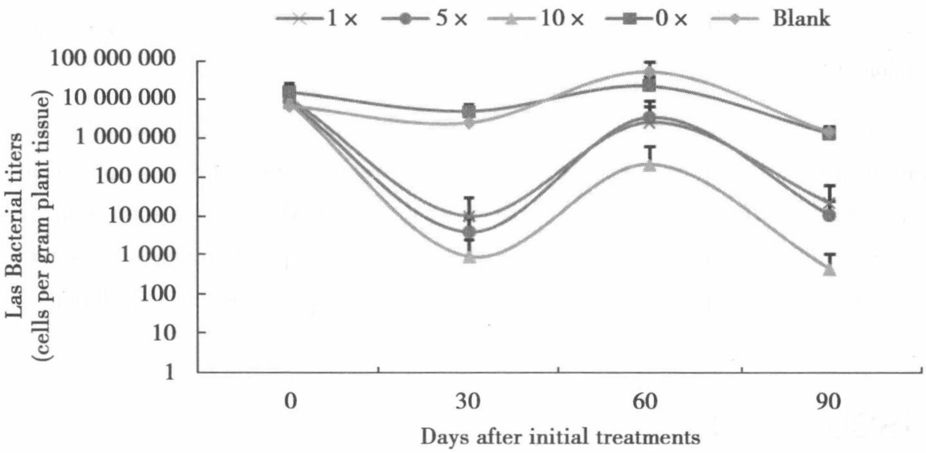

Fig. 4 Candidatus Liberibacter asiaticus (Las) bacterial titers (cells per gram plant tissue) in the Las-infected periwinkle plants by foliar-spraying with PS at concentrations of 0 × (water as control), 1 × (0.1 g/L penicillin and 10 mg/L streptomycin), 5 × (0.5 g/L penicillin and 50 mg/L streptomycin) and 10 × (1.0 g/L penicillin and 100 mg/L streptomycin). Without any treatment was setup as blank control (Blank control)

trol, disease prevention has been the only way to fight against citrus HLB. A three-pronged approach to combating HLB was put forward after its occurrence in China: (i) to eliminate the

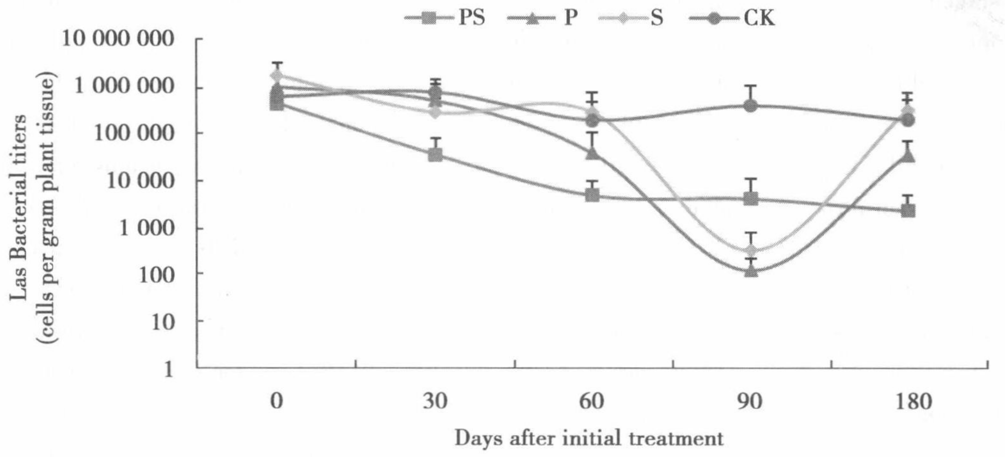

Fig. 5 Variations of Candidatus Liberibacter asiaticus (Las) bacterial titers (cells per gram plant tissue) in HLB-affected citrus seedlings soaked in PS at concentrations of 1.0 g/L penicillin G and 100 mg/L streptomycin, penicillin G at 1.0 g/L (P), streptomycin at 100 mg/L (S) and water control (CK)

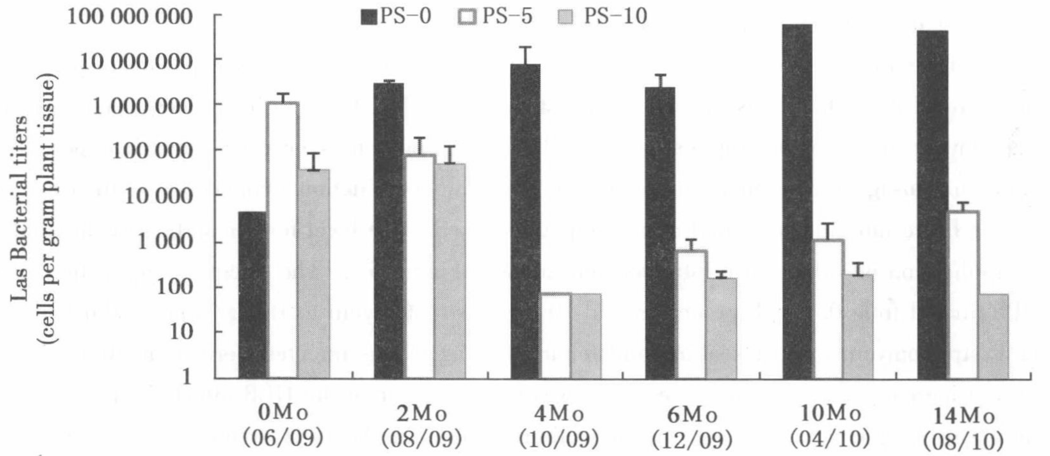

Fig. 6 Candidatus Liberibacter asiaticus (Las) bacterial titers (cells per gram plant tissue) of the HLB-affected citrus in the field treated with PS at different rates of PS-5 (5 g penicillin and 0.5 g streptomycin in 100 ml of solution) and PS-10 (10 g penicillin and 1.0 g streptomycin in 100 ml of solution) by trunk-injection. Water injection was used as a control (PS-0)

Liberibacter inoculum by removal of symptomatic trees, (ii) to keep psyllid vector populations as low as possible by insecticide treatments, and (iii) to produce uninfected citrus trees in "closed", insect-proof nurseries for new orchards as well as for replacements of removed symptomatic trees. The same three measures are currently recommended in Florida (10). In our previous reports, we optimized a regeneration test system using Las-infected periwinkle cuttings to screen antimicrobial compounds, and found that penicillin was effective in eliminating or suppressing the Las bacteria (43). In this study, the combination of penicillin G and streptomycin had higher antimicrobial activities against the Las bacterium than did the individual components alone or another tested antibiotic oxytetracycline, or the biocide agent DBNPA.

Penicillin G potassium, one of the bactericidal antibiotics, has activity against gram-positive and gram-negative aerobic and anaerobic bacteria by inhibiting bacterial cell-wall synthesis (15). It has been reported that penicillin G sodium suppresses the HLB bacterium in Las-infected periwinkle (43). Applications of antibiotics, such as water soluble penicillin G salts, as fertilizer components or as additives in irrigation water were patented for increasing the size or vigor of plants within a given period (USA Pat. US2749230) or for reducing the time required for the sugarcane to reach normal maturity (USA Pat. US3897239). Penicillin G potassium is taken up rapidly by plants, and was relatively non-phytotoxic in an earlier study (9). Streptomycin is an amino glycoside antibiotic. It was first registered as a pesticide in 1955, for use in controlling bacterial pathogens of certain agricultural and non-agricultural crops (Streptomycin, Technical Evaluation Report, January 27, 2006, Compiled by ICF Consulting for the USDA National Organic Program). The primary mechanism of streptomycin action is binding irreversibly to the bacterial 30S ribosome, changing its shape, and inhibiting protein synthesis by causing the misreading of mRNA (29).

In order to decrease the selection for antibiotic-resistant bacteria by exposure to penicillin and to avoid the side-effects caused by streptomycin (12, 30), we combined penicillin and streptomycin in a cocktail application, which has an apparent synergetic effect and permits a significant dosage reduction of streptomycin. The new combination enhanced activities against the Las bacterium and remained at a therapeutically effective level for 6 months as compared to the application of penicillin or streptomycin alone (Figure 5). The effective application rates of PS ranged from 0.1 g/L penicillin and 10 mg/L streptomycin to 1.0 g/L penicillin and 100 mg/L streptomycin by root soaking and foliar spraying of Las-infected periwinkle. More than a ten-fold increase in application rates of PS was required to treat the HLB-affected citrus by root-soaking in the greenhouse or by trunk-injection in the field. At PS-10 treatment, the PS phytotoxicity to citrus became apparent at the application rate of 100 ml/per injection (Supplemental Figure 2), which was likely induced by the streptomycin component (22, 24). The greatest concern of those opposed to antibiotic use on plants is that spraying antibiotics in the open environment and over large expanses of land might increase the emergence of antibiotic resistant bacteria. Some researchers found that streptomycin and tetracycline resistance genes were often carried on the same large plasmid in orchard bacteria, but when the plasmid was transformed into *E. coli*, the new host was only resistant to tetracycline and not to streptomycin or the other antibiotics (13, 34).

Curative effects of the new combination PS have been demonstrated on the Las-infected periwinkle by foliar-spraying, on the HLB-affected citrus seedlings in the greenhouse by root-