Proc. Fla. State Hort. Soc. 132:68-71. 2019.

Translocation of Oxytetracycline in Citrus Plants after Root Drench and Stem Delivery

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Aditional index words. antibiotic translocation, citrus, ELISA, HPLC, oxytetracycline

Huanglongbing (HLB) is the most destructive disease to the citrus industry. In North America, HLB is caused by *Candidatus* Liberibacter asiaticus (*C*Las) and is transmitted by the Asian citrus psyllid, *Diaphorina citri*. Recent studies showed that antibiotics such as oxytetracycline and streptomycin were effective against the *C*Las pathogen *in planta*. The objectives of this study were to investigate the uptake, translocation, and stability of oxytetracycline in citrus seedlings. Oxytetracycline was delivered via root or stem. The level of oxytetracycline in treated plants was monitored using high-performance liquid chromatography (HPLC) and/or enzyme-linked immunosorbent assay (ELISA). The HPLC and the ELISA methods showed similar results at high concentrations; however, the ELISA was more sensitive than the HPLC method. The highest level of oxytetracycline after root incubation was found in roots, followed by stem-xylem, stem-phloem, and in leaves. On the other hand, the level of oxytetracycline in the xylem and phloem was higher than that found in the root when delivered via stem. Oxytetracycline was still detectable in all tested tissues thirty-five days after treatment, indicating that oxytetracycline was relatively stable in citrus plants and could inhibit *C*Las growth for a few months in the field.

Citrus greening disease, which is also known as Huanglongbing (HLB), is currently threatening the citrus industry in Florida. HLB is caused by *Candidatus* Liberibacter asiaticus (*C*Las) and is transmitted by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Grafton-Cardwell et al., 2013), while feeding on citrus phloem sap. Currently, HLB is widespread throughout the state of Florida and has significantly reduced citrus production.

Management of HLB mainly depends on the control of the *D. citri* using a wide range of insecticides. However, the heavy use of insecticides can negatively affect human health, beneficial insects, environment, and induce insecticide resistance in *D. citri* (Tiwari et al., 2013). Enhanced nutritional programs (ENPs) were also proposed to alleviate HLB symptoms and maintain the productivity of infected plants. However, ENPs were not effective in the field and can enhance the spread of HLB by increasing the lifespan of infected trees (Gottwald et al., 2012). Removal of infected trees was also suggested to reduce *C*Las inoculum; however, this strategy is difficult to implement in heavily infected regions such as Florida. Greenhouse studies showed that thermotherapy was effective against *C*Las, however this technology did not show any promising results in the field (Blaustein et al., 2017). Consequently, due to the difficulty of HLB management, the use of antibiotics is immediately needed for the control of this destructive disease.

The use of antibiotics for the control of HLB was initiated in the 1970s (Blaustein et al., 2017). Early studies showed that injection of tetracycline into the trunks of *C*Las-infected trees significantly reduced HLB symptoms (Martinez et al., 1970; Schwarz and van Vuuren, 1970). Early studies also showed that stem delivery of antibiotics was more efficient than foliar sprays and application under the bark (Capoor and Thirumal, 1973). Unfortunately, the use of antibiotics for the control of HLB was discontinued after a short period. However, in the last few years, the use of antibiotics for the control of HLB has regained a great interest by many scientists due to significant losses in the citrus industry. Recent studies showed that several antibiotics such as penicillin, streptomycin, ampicillin, and carbenicillin, were effective in eliminating the *C*Las titer and rescuing HLB-infected plants (Zhang et al., 2012; Zhang et al., 2015; Shin et al., 2016). In addition, several antimicrobials such as aluminum hydroxide, nicotine, D, L-buthionine sulfoximine, and surfactin were found to be effective against *C*Las (Doud et al., 2018).

Recently, oxytetracycline and streptomycin sulfate have been approved for the treatment of *C*Las-infected trees via foliar spray in Florida (Wang et al., 2017). In this study, we investigated the uptake, distribution, and stability of oxytetracycline in citrus plants.

Materials and Methods

Plant materials. Healthy Mexican lime (*Citrus aurantifolia*) seedlings were used in this study. Plants were kept in a greenhouse (28 ± 1 ºC, 60 ± 5% relative humidity, L16:D8 h photoperiod) at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, FL. All seedlings were about three months old and about 15 ± 5 cm tall.

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Uptake of oxytetracycline by root drench. Oxytetracycline was purchased from Fisher Scientific (Pittsburgh, PA.). To study the uptake of oxytetracycline by citrus seedlings, the roots were cleaned from soil by washing under tap water, blot-dried on paper towels and immersed in 200 μg·mL^{-1} oxytetracycline solution. Control plants were incubated in distilled water. Five seedlings were used for each treatment. At the end of incubation time (16 h), the plants were washed with distilled water. The level of oxytetracycline in treated plants was determined by high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA).

Stem versus root delivery. To study the translocation of oxytetracycline through the stem application, several shallow cuts were made in the stem of each seedling using a scalpel and a large pipette tip cut lengthwise, placed around the cut area of the stem, and sealed with Parafilm. The space between the stem and the pipette tip was filled with 5 mL oxytetracycline solution (200 μg mL^{-1}) and incubated for 72 h with this antibiotic solution, while the roots were placed back into the soil. To compare stem delivery of antibiotics to root delivery, root delivery was conducted again, but for same time as stem delivery, 72 h. Control plants were incubated in distilled water without oxytetracycline. Five seedlings were used for each treatment in this experiment. At the end of incubation time, all seedlings were dissected and the antibiotic concentration was determined by HPLC.

Stability of oxytetracycline in plant tissues. To study the persistence of oxytetracycline in citrus seedlings, twenty-five citrus seedlings were incubated (via root delivery) in 200 μg mL^{-1} oxytetracycline solution for 72 h. At the end of incubation time, the roots were washed with distilled water to remove any adsorbed oxytetracycline, and the plants were returned to their original pots and left for different time intervals (0, 3, 7, 14, and 35 d). Control plants were incubated in distilled water. Oxytetracycline levels were measured using HPLC.

Extraction of oxytetracycline in plant tissues. Plant tissues were ground with liquid nitrogen using a mortar and pestle and 0.1 g of the homogenous sample was transferred to a 2-mL centrifuge tube. One mL aliquot of 0.1M HCl/0.01M EDTA solution was added to each tube and the sample was vortexed for 1 min followed by sonication for 30 min. The vortex and sonication procedures were repeated twice, and the samples were centrifuged at 12,000 rpm for 10 min at 20 ºC. The supernatant was transferred into a new tube and kept at –20 ºC until analysis using either ELISA or HPLC. To determine the efficiency of our extraction method, 100 mg of citrus leaves (control plant) was spiked with 100 μL of oxytetracycline solution of 1000 μg.mL^{-1}. Control samples were spiked with 100 μL distilled water. Oxytetracycline was extracted as described above and analyzed by HPLC and ELISA.

Analysis of oxytetracycline. *ELISA assay*. Oxytetracycline ACCEL ELISA kit was purchased from Plexense, Inc., (Davis, CA) and was used according to manufacturer recommendations.

HPLC assay. Oxytetracycline standard was purchased from Alfa Aesar (Ward Hill, MA). A stock solution of oxytetracycline (1000 μg·mL^{-1}) was prepared in methanol. A set of dilutions was made in water and were injected into the HPLC to construct the standard curve.

The detection of oxytetracycline was accomplished with an Agilent 1200 system coupled to a diode array detector (HPLC-DAD) and a Luna C18 column (150 × 4.6 mm, Phenomenex, Torrance, CA) with isocratic mobile phase of buffer solution and methanol (70:30, v:v). The pH of the phosphate buffer was adjusted to 3.0 using phosphoric acid. The flow rate of the mobile phase was 0.8 mL/min, injection volume was 20 mL, the column temperature was set at 28 °C, and the detector wavelength was set at 355 nm.

Statistical analysis. Data were analyzed using JMP 9.0 software (SAS, Cary, N.C.). The *t*-test ($P < 0.05$) was used to compare between the level of oxytetracycline obtained by HPLC and that measured by ELISA kit. The *t*-test ($P < 0.05$) was also used to compare between the levels of oxytetracycline measured after stem and root delivery. Analysis of variance (ANOVA) followed by post hoc pairwise comparisons using Tukey-Kramer honestly significant different test (Tukey HSD) were used to compare levels of oxytetracycline among 0, 3, 7, and 14 dpt ($P < 0.05$).

Results

Percentage recovery of oxytetracycline. The recovery of oxytetracycline from spiked leaf tissues using the ELISA kit and HPLC method was 85.6 ± 8.7% and 87.4 ± 9.4%, respectively. No oxytetracycline was detected in controls or blank samples. The high percentage recovery indicates that our extraction method was efficient for the detection of oxytetracycline. No significant

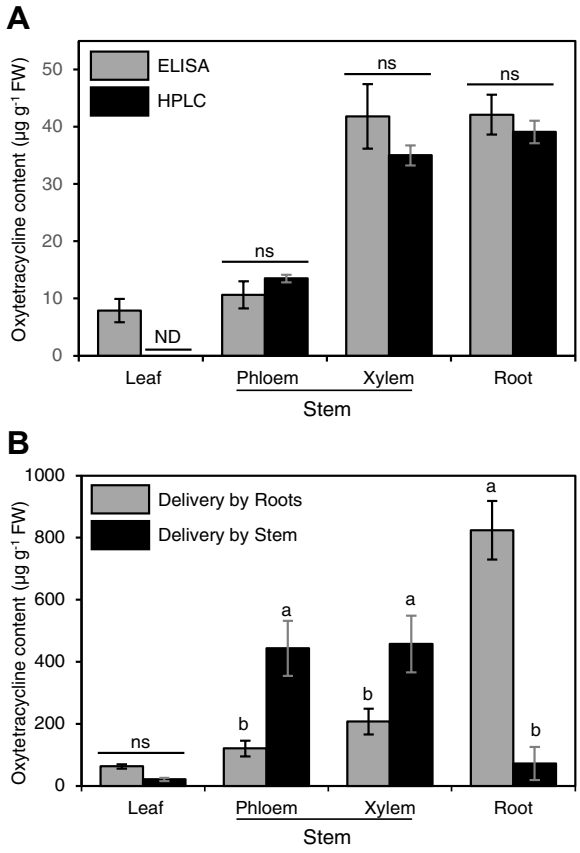

Fig. 1. Oxytetracycline content (μg g^{-1}) in fresh weight (FW) found in different Mexican lime (*Citrus aurantifolia*) seedling tissues after 16 h incubation of the roots in 200 μg·mL^{-1} oxytetracycline solution, and analyzed by HPLC and ELISA kit (A). (B) HPLC analysis of oxytetracycline content in various tissues in citrus plants treated with 200 μg·mL^{-1} oxytetracycline solution for 72 h via root or stem delivery methods. For these analyses, the stem bark was dissected into the outer bark tissue (representing the phloem) and the inner bark (representing the xylem). Data are the means ± SD of five biological replicates. Columns with different letters indicate statistically significant differences by Tukey HSD ($P < 0.05$). ND: not detected; ns: not a significant difference.

difference in detecting oxytetracycline levels was observed between HPLC and ELISA methods.

Translocation of oxytetracycline in citrus seedlings after root drenches (16 h). Oxytetracycline was detected in all parts of the plants (root, stem xylem, stem phloem, and leaves), indicating that oxytetracycline was translocated throughout the plant (Fig. 1A). The highest concentration of oxytetracycline was found in roots and xylem, followed by phloem and leaves (Fig. 1A). The concentration of oxytetracycline in leaves after 16 h of incubation was below the limit of quantification (LOQ) of the HPLC method, and therefore, only the ELISA results were reported for leaves (Fig. 1A). Nevertheless, no significant differences in levels of oxytetracycline detected in other tissues (root, xylem, and phloem) were observed between the HPLC and ELISA results (Fig. 1A).

Translocation of oxytetracycline in citrus seedlings after stem and root delivery (72 h). In this experiment, we assessed translocation dynamics of oxytetracycline by comparing between the stem and root delivery. The HPLC results showed that the levels of oxytetracycline in the xylem and phloem after stem delivery were significantly higher than those obtained after root delivery (Fig. 1B). On the other hand, the levels of oxytetracycline in the root after stem delivery were significantly lower than those obtained after root delivery (Fig. 1B). No significant differences were observed in the levels of oxytetracycline in the leaves via root or stem delivery (Fig. 1B).

The highest concentration of oxytetracycline was detected in the root followed by the xylem, phloem, and leaves via root delivery (Fig. 2A). The UV-visible spectra of oxytetracycline in oxytetracycline-treated treated plants were similar to that of oxytetracycline standard (Fig. 2A).

Stability of oxytetracycline in citrus plants. The level of oxytetracycline in the root and xylem showed a continuous decline with time (Fig. 2B). The level of oxytetracycline in the leaves and phloem followed a different trend than those that were observed in root and the xylem; it peaked after seven and fourteen days and declined thereafter (Fig. 2B).

Discussion

Evaluation of oxytetracycline extraction and analysis methods. The extraction solvent showed high recovery of oxytetracycline from plant tissues. We compared the HPLC

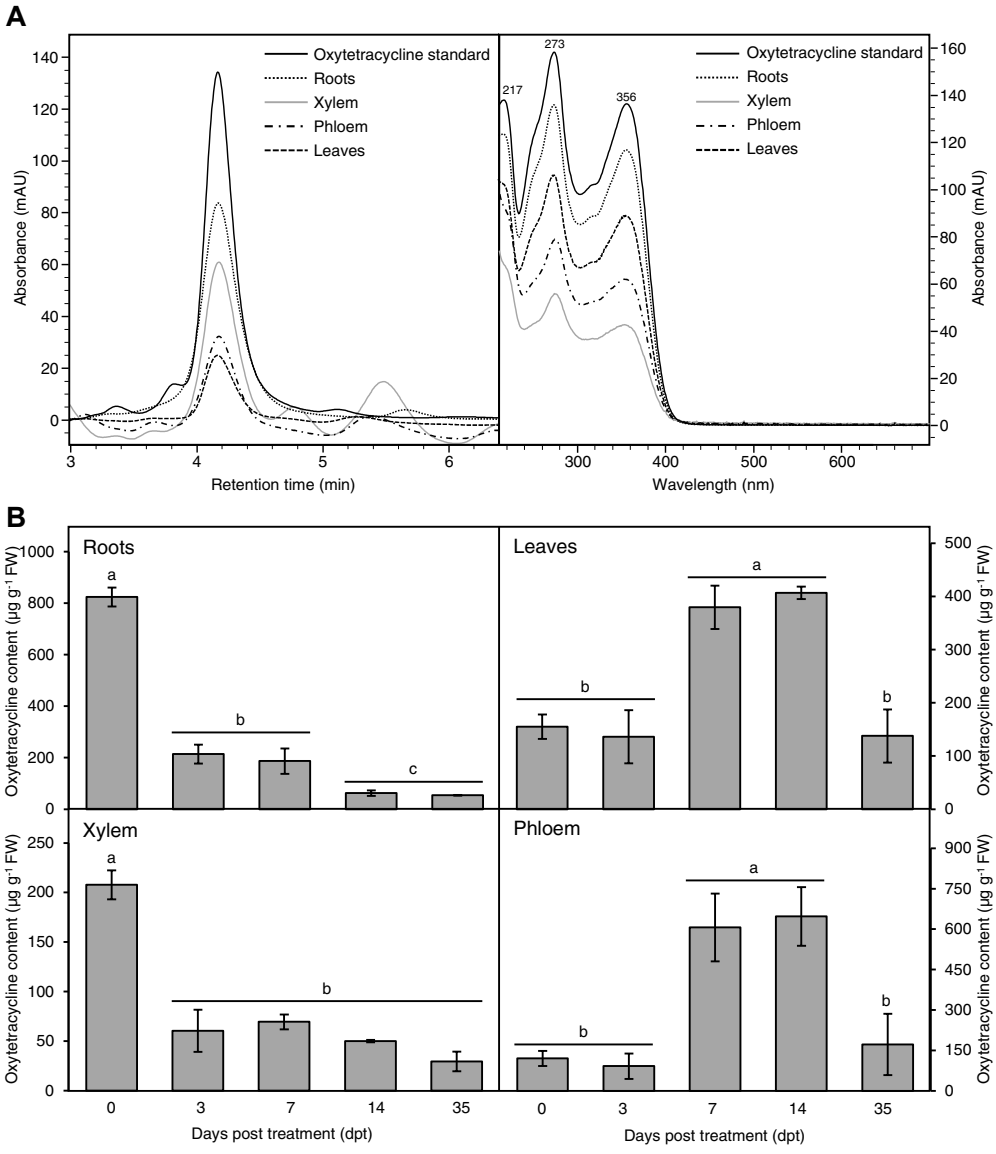

Fig. 2. HPLC results of oxytetracycline (μg g^{-1}) fresh weight (FW) detected in the root, xylem, phloem, and leaf of treated Mexican lime (*Citrus aurantifolia*) seedlings. (A) HPLC chromatogram (left) and UV-visible spectra (right) of standard and oxytetracycline in plants incubated in 200 μg·mL^{-1} oxytetracycline solution for 72 h via root drench method. (B) The level of oxytetracycline at 0, 3, 6, 14, and 35 d post-treatment in different plant tissues as measured by HPLC after incubation in 200 μg.mL^{-1} oxytetracycline solution for 72 h via root drench method. Plant roots were washed with distilled water after treatments and the plants returned to their original soil until analysis. For these analyses, the stem bark was dissected into the outer bark tissue (representing the phloem) and the inner bark (representing the xylem). Data are the means ± SD of five biological replicates. Columns with different letters indicate statistically significant differences by Tukey HSD ($P < 0.05$).

method to the ELISA kit, and our comparison indicated that both methods were suitable for the detection of oxytetracycline. However, each method has its own advantages. The HPLC has a high dynamic range from 5–200 $\mu g \cdot mL^{-1}$, and it provides extra confirmation of oxytetracycline (UV-spectra and retention time). On the other hand, the ELISA was more sensitive (1.56 $ng \cdot mL^{-1}$) than HPLC; however, it had a very narrow linear dynamic range (1.56-50 $ng \cdot mL^{-1}$).

Translocation of oxytetracycline. Oxytetracycline was detected in the xylem, phloem, and leaves of citrus seedlings by ELISA after their roots were incubated in oxytetracycline solution for 16 h. These results indicated that oxytetracycline was taken up by the roots and translocated through the xylem. The highest concentration was found in the roots, followed by the xylem, phloem, and leaves. The level of oxytetracycline in the leaves was not detected by HPLC (below LOQ), and therefore, was measured using ELISA. The presence of oxytetracycline at high concentration in the phloem indicated that oxytetracycline could be effective against *C*Las, since it resides in the phloem. Previous research indicated that high efficiency of antibiotics against plant pathogens in vitro, does not guarantee high efficiency in planta. For example, several antibiotics were effective against spiroplasmas and phytoplasmas in vitro; however, only oxytetracycline was effective against these agents in planta, indicating that it has the potential for a high translocation rate in the phloem (Daniels, 1982).

Stem versus root delivery. Our results showed that the levels of oxytetracycline in the phloem and xylem of citrus seedlings after stem delivery were higher than those obtained after root delivery. On the other hand, the level of oxytetracycline in the root treated after root delivery was higher than that measured in the root after stem delivery. This result showed that low amounts of oxytetracycline were translocated from the stem to the root after stem delivery. In agreement with our results, no antibiotic activity was detected in the small roots of sweet orange citrus trees that were injected with 10–30 g of oxytetracycline/tree via trunk injection, indicating limited downward movement (Timmer et al., 1982). Twigs and leaves showed high antibiotic activity, indicating higher upward movement of oxytetracycline most probably via xylem vessels (Timmer et al., 1982). Lee et al. (1982) also showed that oxytetracycline activity was high in the canopy following trunk injection, whereas roots showed low or no activity. On the other hand, high antibiotic activity was observed in the roots after drench treatment (Lee et al., 1982). The combination of drench and trunk injection resulted in high activity in the root and canopy of treated plants (Lee et al., 1982).

Stability and persistence of oxytetracycline in citrus seedlings. Oxytetracycline was still detectable in all tissues tested including in the phloem, xylem, leaves, and roots up to thirty-five days after treatment, indicating that oxytetracycline was relatively stable in citrus plants. In agreement with our results, the activity of oxytetracycline was still detected (2-3 $\mu g \cdot g^{-1}$) in leaves thirty-six days after injection of 6.0 g into the trunk of coconut trees (McCoy, 1976). The activity of oxytetracycline also persisted in the twigs and leaves of sweet oranges 3–8 months after trunk injection of 10–30 g/tree (Timmer et al., 1982). The observed half-life of oxytetracycline in coconut palm trees was estimated to be 2–3 weeks (Hunt et al., 1974; McCoy, 1976).

Conclusion

Our results showed that oxytetracycline was translocated in citrus plants after root and stem delivery methods. The presence of oxytetracycline at high concentrations in the phloem suggested that it could be effective against *C*Las. Oxytetracycline was detectable in all tested tissues 35-day posttreatment, indicating that it was relatively stable in citrus plants. Future studies should evaluate the efficacy of oxytetracycline against the *C*Las pathogen in the field.

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