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The Use of Laser Light to Enhance Penetration of Antimicrobials into Citrus Leaves

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Penetration of externally applied agricultural solutions into citrus trees is hindered by the natural protective layers covering plant surfaces. At the present time, the battle against or Huanglongbing (or citrus greening) focuses on the possible application of antimicrobial substances. A possible solution to overcome the barrier imposed by the waxy cuticle is the use of laser technology. Laser light is capable of "melting" away microscopic "pin holes" on plant surfaces allowing for the penetration of applied substances. Using fluorescent molecules such as deoxyglucose and carboxyfluorescin, our investigation demonstrated the effectiveness of laser light technology in enhancing penetration of foliarly applied aqueous substances. We observed the movement of fluorescent deoxyglucose and carboxyfluorescin down the leaf in petiole within 4 hours and down the stem after 24 hours. The data presented in this communication demonstrated the usefulness of laser light in enhancing penetration of test substances into the leaf and movement through the tree.

Efforts to curb huanglogbing (HLB or citrus greening) in Florida have been hampered by numerous roadblocks ranging from failure to culture the bacteria *Candidatus* Liberibacter asiaticus (*C*Las) to the excessive number of abandoned trees statewide. An emerging approach to extend the productive life of trees until a permanent solution can be formulated is the use of antimicrobial substances. However, antimicrobial efficacy is intimately dependent on the degree of penetration into the plant and on the percent uptake by phloem cells.

Given the phloem-limited nature of *C*Las (Md-Sajedul et al., 2012) and the basipetal direction of phloem flow, foliar application of antimicrobial substances should provide a more complete coverage as substances picked up by the phloem in leaves should be transported throughout the plant. However, penetration of foliarly applied substances on citrus trees is severely hindered by the presence of protective layers such as the thick cuticle (made up of wax/cutin) on leaves, and of cork (made up of suberized cells) on stems. Although the primary functions of these protective covers are to guard against invading pests and to minimize water loss, the cuticle and bark also make formidable protective shields rendering penetration of externally supplied substances virtually impossible. Penetration into leaves is mostly feasible through the stomata openings (found almost exclusively on the abaxial side in citrus leaves) and through occasional cracks on the cuticle itself. Nevertheless, the collective surface area of stomatal openings that would allow for penetration of externally supplied solutions into the leaf is minimal, even under optimum circumstances, since stomata often close under a variety of biotic and abiotic conditions. The cork, a complex tissue made up of dead suberized cork cells to the exterior, has slightly better permeability than the cuticle through the cell wall fibrous material.

To overcome the obstacles imposed by the cuticle in order to increase penetration of externally supplied substances, we propose the use of laser light. Laser light technology involves the use of low level light energy to disperse the cuticle creating microscopic and superficial indentations of approximately 250 μm (Etxeberria et al., 2009) that would allow penetration of applied substances. This technology has been approved by FDA for labeling fruits and vegetables (Patent #5,660,747 and Patent #5,897,797; FDA Docket # FDA-2007-F-0390). To test for the possible use in antimicrobial treatments, we used several phloem-mobile fluorescent substances, and demonstrated that laser light facilitates penetration, uptake and transport of substances into the phloem and throughout the tree.

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Materials and Methods

Plant material. Two-year-old 'Valencia' orange (*Citrus sinensis* L. Osbeck) trees on Swingle (*Citrus paradisi* x *Poncirus trifoliata*) rootstock were kept in a greenhouse with natural lighting. When needed for experimental treatment, trees were brought to the laboratory.

Laser treatment. Leaves were lasered using a low-energy carbon dioxide laser etching machine (model XY Mark-10; GPD Technologies, Peachtree City, Georgia) located at the University of Florida's Citrus Research and Education Center in Lake Alfred, FL. The lasered area consisted of two successive rectangles (25 rows of alternating 13 and 12 depressions each for a total of 313 depressions per rectangle; Fig. 1). Each leaf was lasered once on each side of the mid-vein. Immediately after lasering, 10 μL of test solutions were manually smeared on each lasered area. After the solution dried off initially, water mist was applied and each leaf was covered with a zip-lock bag containing a piece of small, wet gauze.

All leaves were rinsed before observation in the microscope to rid of any background fluorescence produced by unabsorbed substances.

Test solutions. To test for penetration into the leaf, movement through the cell wall, and uptake into the phloem, we used the following solutions: 1. 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose), a fluorescent analog of glucose (Ex/Em: 465/540); 2. Carboxyfluorescin, a cell permeant

fluorescent compound (Ex/Em : 492/517); 3. Dextran-Texas Red, a fluorescent dextran with a mw of 3,000 (Ex/Em: 595/615); 4. Q-dots, CdSe/ZnS crystals of an approximate diameter of 15 nm (Ex/Em: 405-525/565); 5. Alexa-488, a membrane impermeable negatively charged fluorescent vital stain (Ex/Em: 495/519).

Microscopy. Microscopic observations were made using a Carl Zeiss Axion Scope A-1equipped with a Cannon EOS Rebel T3I camera and a Carl Zeiss fluorescent stereoscope.

Results

Lasered area. For our study, we selected a dot matrix pattern that would maximize the amount leaf area exposed to applied substances (Fig. 1). Each pin-hole depression across the cuticle had an average diameter of 190 μm. With a pattern consisting of 313 pin-holes per rectangle, the exposed area totaled 17.78 mm^2 per leaf.

Experimental solutions. Table 1 presents all the solutions tested, their physical characteristics and concentrations applied. 2-NBDG rapidly penetrated mesophyll cells as depicted by the fluorescence emanating from the pin-hole depressions and surrounding area in Fig. 2A. By comparison, lasered leaves with no solutions added displayed cuticular fluorescence, but the central area remained opaque and there was not fluorescence in the inter-lasered spaces (Fig. 2B). Control un-lasered leaves supplied with 2-NBDG on the adaxial cuticle and rinsed before observation showed no fluorescence (dark field micrographs, not shown).

When treated leaves were viewed under fluorescence microscopy at low magnification 2 h after application, a strong fluorescence signal delineated minor and main veins (Fig. 3A). These results demonstrated that 2-NBDG was mobilized down from the treated area likely by phloem cells. A cross section of the petiole 4 h after treatment showed strong fluorescent signal within the vascular tissue (Fig. 3B).

In a separate experiment, a total of 5 treated leaves were treated and left on the tree for 24 h before observation. In these trees, portions of the bark over 30 cm below treated leaves showed fluorescence (Fig. 4A) compared to control trees (Fig. 4B) confirming the down flow of 2-NBDG through the phloem tissue in the direction of the bulk flow.

Carboxyfluorescin-diacetate, a cell permeant fluorescent compound (Aeschbacher et al., 1986), gave similar results to 2-NBDG. In contrast, artificial fluorescent "Q-dots" (15 nm CdSe/ZnS crystals), Dextran-TR (mw 3,000) and Alexa-488 (membrane impermeable fluorescent probe) did not move beyond the area of application.

Discussion

At the moment, there are no effective weapons in the fight against HLB. Because of the bacterial nature of the causing agent,

Fig. 1. Dot matrix pattern that would maximize the amount leaf area exposed to applied substances.

Table 1. List of fluorescent substances used to determine penetration and movement into lasered citrus leaves. Substances were individually applied onto laserd and control un-lasered citrus for 4 and 24 h. Fluorescence was followed using a Carl Zeiss Axion Scope A-1.

Name	MW	Concentration	Ex/Em (nm)
2-NBDG	342.26	30 mM	465/540
5(6) Carboxyfluorescein diacetate	460.39	4.85 mM	492/517
Dextran-Texas Red	3000	3.5 mM	595/615
Alexa-488	884.91	4.5 mM	495/519
Q-Dots 565, 15 nm	≈ 110,000	2 μM	405-525/565

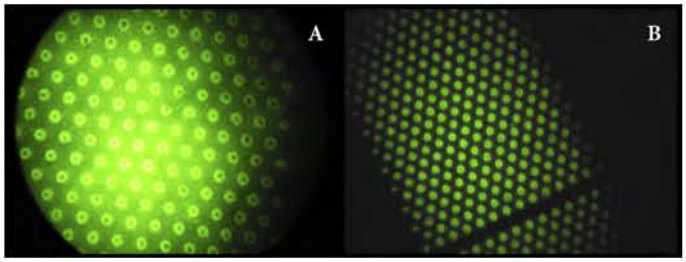

Fig. 2. Citrus leaves after being lasered using a rectangular dot pattern and observed under fluorescent microscopy. A. Lased leaf 20 min after addition of 5 μL fluorescent deoxy-glucose (NGDG). Observe the spread of fluorescence under the lazed dots.

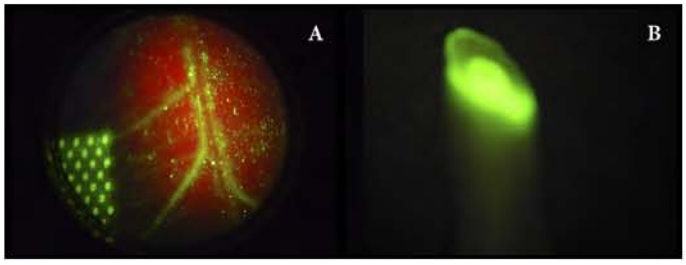

Fig. 3. Fluorescent micrograph of a portion of a lased citrus leaf showing: A. the site of 2-NBDG application, and fluorescent secondary and the main veins. B. Excised petiole showing 2-NBDG fluorescence after 4 h.

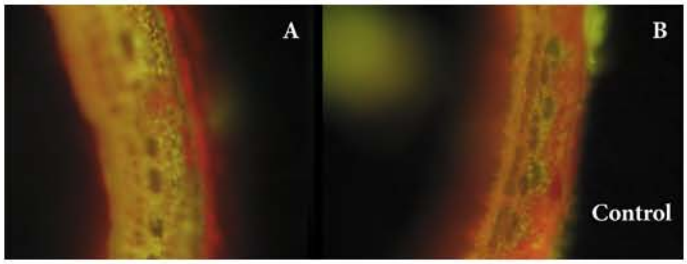

Fig. 4. Tree bark taken 30 cm below treated leaves with 2-NBDG 24 h after application (A), and control untreated leaves (B).

antimicrobials are being considered as potential tools in efforts to extend the productive life of citrus trees until a more permanent solution is found. However, to be effective, antimicrobials not only have to find their way to the phloem cells, but they also have to be taken up and transported to the rest of the plant.

Our investigation demonstrated the effectiveness of laser light technology in enhancing penetration of foliarly applied substances. When compared to unlasered control leaves, phloem mobile substances were able to penetrate and to be transported down the phloem stream. Among test substances, the phloem mobile compounds 2-NBDG, a fluorescent analog of glucose (a natural sugar found in plants) and carboxyfluorescin (a membrane permeable fluorescent compound) showed high degree of mobility. Uptake of these substances into the leaves was confirmed by the spread of fluorescence within the lased area (Fig. 2A, 3A) and down the stem after 24 h (Fig. 4A).

Q-dots and Dextran-TR were not capable of moving beyond the point of application. These substances have Stokes radii larger than the cellulose fiber inter spaces (calculated at approximately 5 nm; Carpita et al., 1979), which made them too large to move through the cell wall matrix. Alexa-488, although a small negatively charge molecule, was unable to move through the also negatively charged cell wall (Dainty, 1990).

Without a system to help penetrate the plant body, the foliar use of antimicrobials alone can be futile. The data presented in this communication demonstrated the usefulness of laser light in enhancing penetration of test substances into the leaf and movement through the tree.

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