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Authors: Etxeberria, Ed, Gonzalez, Pedro, Borges, Ana Fanton, and Brodersen, Craig

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Application Article

The use of laser light to enhance the uptake of foliar-applied substances into citrus (*Citrus sinensis*) leaves[1]

Ed Etxeberria[2,4], Pedro Gonzalez[2], Ana Fanton Borges[2,3], and Craig Brodersen[2,3]

[2]Citrus Research and Education Center (CREC), University of Florida, 700 Experiment Station Road, Lake Alfred, Florida 33850 USA; and [3]Yale School of Forestry and Environmental Studies, Yale University, New Haven, Connecticut 06511 USA

- *Premise of the study:* Uptake of foliar-applied substances across the leaf cuticle is central to world food production as well as for physiological investigations into phloem structure and function. Yet, despite the presence of stomata, foliar application as a delivery system can be extremely inefficient due to the low permeability of leaf surfaces to polar compounds.
- *Methods:* Using laser light to generate microscopic perforations in the leaf cuticle, we tested the penetration of several substances into the leaf, their uptake into the phloem, and their subsequent movement through the phloem tissue. Substances varied in their size, charge, and Stokes radius.
- *Results:* The phloem-mobile compounds 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG), lysine, Biocillin, adenosine triphosphate (ATP), trehalose, carboxyfluorescein-SE, and poly(amidomine) (PAMAM) dendrimer G-4 nanoparticles (4.5 nm in size) showed a high degree of mobility and were able to penetrate and be transported in the phloem.
- *Discussion:* Our investigation demonstrated the effectiveness of laser light technology in enhancing the penetration of foliar-applied substances into citrus leaves. The technology is also applicable to the study of phloem mobility of substances by providing a less invasive, highly repeatable, and more quantifiable delivery method. The implied superficial lesions to the leaf can be mitigated by applying a waxy coating.

Key words: *Citrus*; foliar sprays; foliar uptake; laser light; phloem diseases; phloem movement.

In agriculture, aerial sprays are preferred as a means to deliver agrichemicals over large areas, given that leaves are the most readily accessible tissues and represent a significant proportion of the total plant body (Fernández and Eichert, 2009). However, penetration of externally applied substances into trees is generally prevented by the presence of protective layers such as the lipidized area of the epidermal cell wall (Buschhaus and Jetter, 2011) on leaves and young branches, although variations in roughness and chemical composition are also determinants of the liquid-solid interaction (Fernández and Khayet, 2015). Although the primary functions of these protective layers are to prevent water loss and to act as a defense from pathogens and pests (Kerstiens, 1996), these surface properties also make foliar applications of agrichemicals (e.g., foliar nutrients, pesticides) difficult. The movement of substances across the cuticle and into leaves is most feasible through the stomatal openings (Burkhardt et al., 2012) found almost exclusively on the abaxial leaf surface in *Citrus* L. leaves (Reed and Hirano, 1931), through the polysaccharide matrix (Guzmán et al., 2014), and through occasional cracks in the cuticle itself. Stomata significantly contribute to the overall exchange of hydrophilic substances across leaf surfaces (Eichert et al., 2008); nevertheless, the collective area of stomatal openings that would allow for penetration of externally supplied solutions into the leaf is still reduced, even under optimum circumstances, because: (1) stomata often close under a variety of biotic and abiotic conditions (Daszkowska-Golec and Szarejko, 2013), (2) only a small percentage of the opened stomata participate in the uptake process (Eichert and Burkhardt, 2001), and (3) the entire stomata opening is not available to the movement of aqueous solutions because stomates are protected against infiltration by their geometry and pore walls (Schönherr and Bukovac, 1972).

An emerging approach to extend the productive life of tree crop species, like citrus, against fastidious bacterial infections is the use of antimicrobial substances. Aside from regulatory considerations, antimicrobial efficacy is necessarily dependent on the degree of penetration into the plant and on the systemic distribution by translocating tissues. For example, the citrus industry worldwide is currently struggling with "Huanglongbing" (HLB; syn. citrus greening), a disease caused by a phloem-limited bacterium (*Candidatus* Liberibacter asiaticus, *C*Las). Effective treatment of this disease has largely been limited by the inability to deliver antibacterial substances to the phloem. Given the presumed phloem-limited nature of *C*Las (Islam et al., 2012) and the basipetal direction of phloem flow, foliar application of antimicrobial substances should come into contact with existing populations of *C*Las residing in the phloem.

To increase penetration of externally supplied substances across the lipidized leaf surface and subsequent transport into the phloem, we examined the use of laser (light amplification by stimulated emissions of radiation) light as a tool to increase the permeability of the cuticle without extensive damage to the underlying leaf tissue. A laser beam, where all of the photons

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[4]Author for correspondence: eetxeber@ufl.edu

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are in a coherent state, uses light to stimulate electrons in a "gain medium" into an excited state (called optical pumping). When the electrons collapse into the lower-energy unexcited state (called population inversion), they emit photons. When applied to plant surfaces, the energy released by the beam disperses the cuticle and, if desired, epidermal cells, creating microscopic and superficial indentations of approximately 250 µm in diameter (Etxeberria et al., 2009) that would allow penetration of applied substances. This technology (Sood et al., 2008, 2009) has been approved by the Food and Drug Administration (FDA) for labeling fruits and vegetables (Patent no. 5,660,747 and 5,897,797; FDA Docket no. FDA-2007-F-0390) and can be adapted for agronomical purposes as well as for physiological investigations (e.g., introducing dyes into the phloem sap). To test for the possible use of laser light in enhancing antimicrobial and foliar nutrition treatments, we used several phloem-mobile fluorescent substances and demonstrated that laser light greatly enhances penetration, uptake, and transport of substances into the phloem and throughout the tree.

MATERIALS AND METHODS

***Plant material*—**Two-year-old 'Valencia' orange (*Citrus sinensis* (L.) Osbeck) trees on Swingle (*Citrus paradisi* × *Poncirus trifoliata*) rootstock grown in 4-L pots were kept in a greenhouse with natural lighting. When needed for experimental treatment, trees were brought to the laboratory and treated accordingly.

***Laser treatment*—**Leaf surfaces were perforated with a laboratory model, low-energy, CO_2 laser-etching machine (model XY Mark-10; GPD Technologies, Peachtree City, Georgia, USA) located at the University of Florida's Citrus Research and Education Center in Lake Alfred, Florida. Laser specifications used were those already reported for use in citrus fruits (Sood et al., 2009). We used the dot matrix pattern where the surface area of one dot was approximately 3.14×10^{-4} cm^2 and the energy per surface area of one dot = 7.85 mW dot^{-1} 10^{-6} s.

Depending on the objective of the experiment, leaves were lasered once on each side of the midvein; while for others, etching was made across the midvein. Immediately after lasering, 10 µL of test solutions were manually applied to each lasered area. After the solution dried off, a layer of natural oil (Johnson's baby oil [Johnson & Johnson, New Brunswick, New Jersey, USA]) was applied to prevent desiccation. All leaves were rinsed before observation in the microscope to eliminate background fluorescence produced by unabsorbed solutions.

***Penetration and mobility of experimental solutions*—**To evaluate the mobility of solutions through perforations in the cuticle and epidermis generated by the laser treatment, we applied a series of fluorescent dyes and nanoparticles. In this way, we were able to determine how readily solutions moved into the leaf, through the cell wall, and their loading and transport into the phloem. These solutions were of a variety of sizes and physical and chemical properties, some of which are listed in Table 1. Each solution was tested at least three times on separate leaves from different trees. Some trees were used to test more than one solution.

***Microscopy*—**Microscopic observations were made using a Carl Zeiss Axio Scope.A1 (Carl Zeiss Microscopy GmbH, Göttingen, Germany) equipped with a Canon EOS Rebel T3i camera (Canon, Tokyo, Japan). Low-magnification images were taken with a Zeiss Stemi SV11 fluorescent stereoscope (Carl Zeiss Microscopy GmbH).

***Fluorometry*—**To measure the presence and velocity of phloem-mobile dye as it moved from the leaf blades to the petioles, we developed a system based on the methods of Savage et al. (2013). We positioned the terminus of a bifurcated optical fiber probe (Ocean Optics QR600-7-UV-125F; Ocean Optics, Dunedin, Florida, USA) at the petiole midvein approximately 2 cm from the dye application position to monitor fluorescence signals emitted from the dye (n = 3). One fiber bundle within the cable was coupled to a light-emitting diode (LED) light source with a peak wavelength of 490 nm (Ocean Optics LLS-470) operating in continuous illumination mode that delivered an excitation wavelength for the majority of the dyes used in this study (Table 1). The second fiber bundle carried the fluorescence signal to a spectrometer (Ocean Optics USB 4000) tuned to the specific emission wavelength (520 nm) of the dye. After the leaf surface was perforated and carboxyfluorescein-succinimidyl ester (CF-SE) dye applied, we waited 20 min to allow the dye to penetrate into the leaf. At this point, the emission signal receptor was initiated and began recording continuously for ca. 2–3 h, depending on the movement of the dye in the tissue. By recording the dye application time and then determining when the fluorescence signal slope significantly deviated from the background signal, we were able to determine a relative velocity for the solution over a known distance. Using this method, our estimate of phloem sap velocity included the phloem-loading step.

TABLE 1. List of compounds, molecular weight or Stokes radius, and concentrations used to determine the effectiveness of laser light in their penetration of citrus leaves.

Compound[a]	Molecular weight (g·mol^{-1})[b]	Conc. (mM)[c]	λ Ex/Em (nm)	Leaf penetration
Fluorescent deoxyglucose, 2-NBDG	342	30	465/540	+
Carboxyfluorescein	376	5	492/517	+
Ethidium bromide	394	1	285/605	—
Carboxyfluorescein-SE	460	4.9	492/517	+
Lucifer Yellow	522	1	428/536	—
5-TAMRA lysine	559	9	545/575	+
Bocillin-FL penicillin	661	3	504/511	+
FITC-Trehalose	744	3	492/517	+
Alexa Fluor 488	885	4.5	495/519	—
BODIPY-FL-ATP	933	5	504/513	+
CellTrace Calcein Green, AM	995	1	488/515	—
BODIPY-FL vancomycin	1723	100	504/510	—
Dextran, Texas Red	3000	3.5	595/615	—
PAMAM dendrimers (generation-4-Alexa 488)[d]	14 kD	0.2	495/519	+
BODIPY-Lysine-tRNA	27 kD	1	502/510	—
Quantum dots (Qtracker 565)[d]	110 kD	2 µM	405–525/565	—

Note: + = compounds that penetrated and moved down the phloem; — = compounds that remained in the application site; λ Ex/Em = wavelength excitation/emission.

[a] 2-NBDG = 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose; PAMAM = poly(amidomine).

[b] Molecular weight is in g·mol^{-1} unless other units are specified.

[c] Concentration is in mM unless other units are specified.

[d] PAMAM dendrimers: ø = 4.5 nm; quantum dots: ø = 10 nm.

***Determination of solution infiltration*—**To quantify the uptake of applied solutions, fluorescent vancomycin (100 µM) was used in a parallel experiment. This dye was applied over laser-perforated and control non–laser-perforated leaves and allowed to infiltrate for 4 h. At this time, leaf surfaces were rinsed with distilled water to eliminate unabsorbed residual solution. From each laser-perforated rectangle, a leaf disc was excised using a paper hole-puncher and placed in a 2.5-mL homogenizing tube containing 0.5 mL of water and four metal beads. Tissue was homogenized in 0.5 mL of water using a Precellys homogenizer (Bertin Technologies, Ampère Montigny-le-Bretonneux, France). The tubes were centrifuged and supernatant measured at excitation/emission (Ex/Em) 490/520 nm in a Bio-Rad VersaFluor fluorometer (Bio-Rad, Hercules, California, USA). Data are expressed in relative fluorescence units (RFU). A relative fluorescence unit is described as the increase in the fluorescence scale of one numeric unit at λ 520. The experiment was carried out three times.

RESULTS

***Characterization of citrus leaf surface*—**Citrus leaves are protected by a thick lipidized cuticle on both the adaxial and abaxial sides (Fig. 1A). The subtending epidermis consists of a single layer of cells with cuticular material extending into the tangential cell walls (Fig. 1B). On the adaxial side, the cuticle is fairly uniform and the epidermis is devoid of stomata (Fig. 1C), whereas the abaxial surface contains numerous stomata (Fig. 1D). Our observations agree with earlier measurements of mature 'Valencia' orange leaf stomata estimated at an average of 23 µm in length and 17 µm in width, with an average density of 450 stomata/mm^2 (Reed and Hirano, 1931).

***Properties of laser-generated perforations*—**Perforation size through the cuticle increased both in width and depth with increasing exposure time to the laser light (Fig. 2). With a standard energy output of 1.9×10^6 J/cm^2 and at 30 µs exposure, the cuticle was superficially affected (Fig. 2A). Cuticle and epidermis perforation increased substantially between 30 and 60 µs (Fig. 2B–C) and began leveling off up to 80 µs (Fig. 2D). For the current study, we selected 35 µs exposure and a dot matrix laser pattern that would maximize the amount of leaf area exposed to applied substances (Fig. 3A, B). The laser-perforated area consisted of five successive rectangles (15 rows of 10 perforations each) for a total of 150 perforations per rectangle.

Each perforation across the cuticle had an average diameter of 200 µm (Fig. 3C). With a pattern consisting of 150 perforations per rectangle, the exposed area totaled 4.85 mm^2 per rectangle or 48.5 mm^2 per leaf. Under the set conditions of energy and focal aperture, each laser pore penetrated into the epidermis and circumscribed approximately seven to 10 epidermal cells (Fig. 3D). The depth of the perforations can reach the palisade tissue at longer exposure times (Fig. 2D). Preliminary experiments

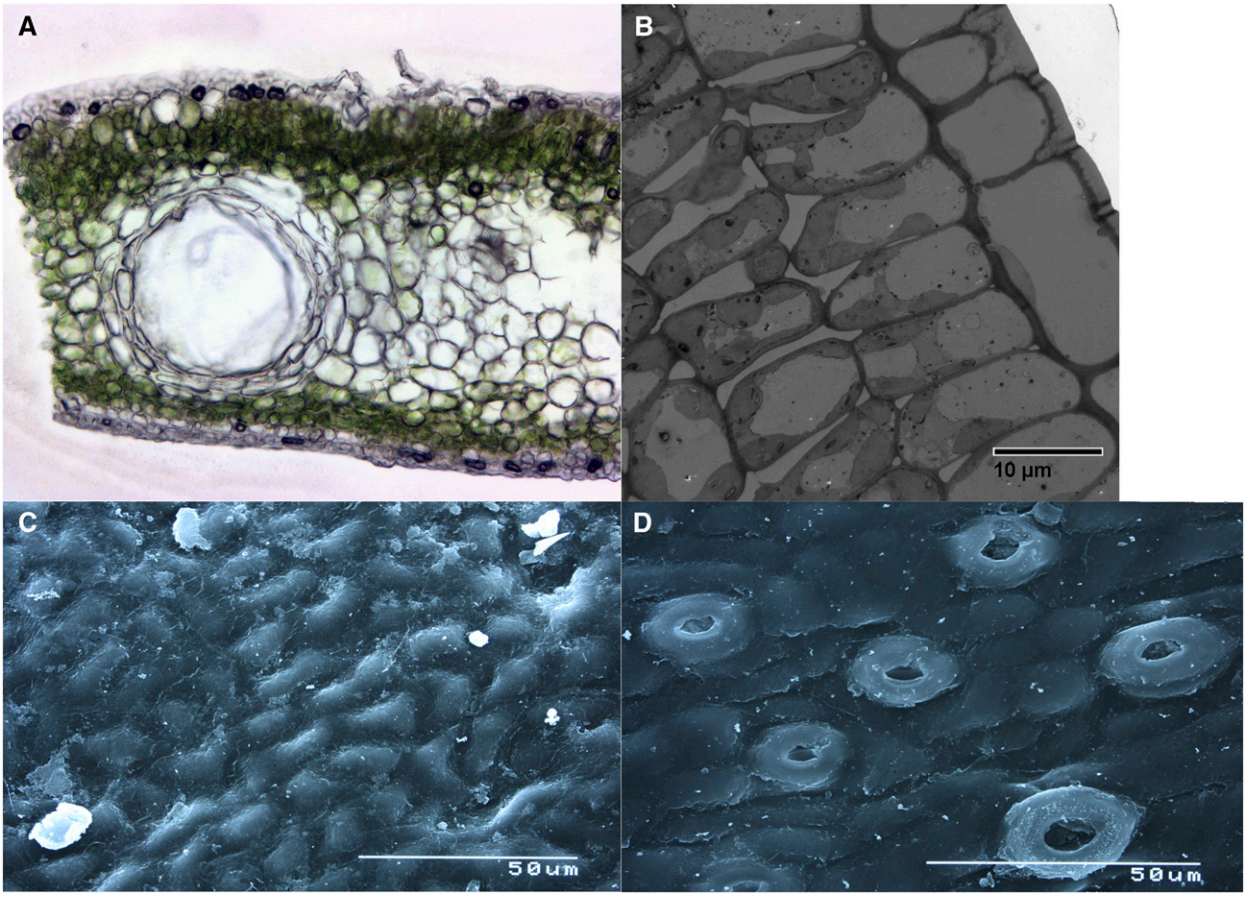

Fig. 1. Anatomical features of citrus leaves. (A) Light micrograph of a leaf cross section showing the thick cuticles on both the abaxial and adaxial surfaces. (B) Transmission electron micrograph showing a single epidermal layer and the cuticular material extending between the epidermal cells. (C) Scanning electron micrograph of the adaxial leaf surface. (D) Scanning electron micrograph of the abaxial leaf surface.

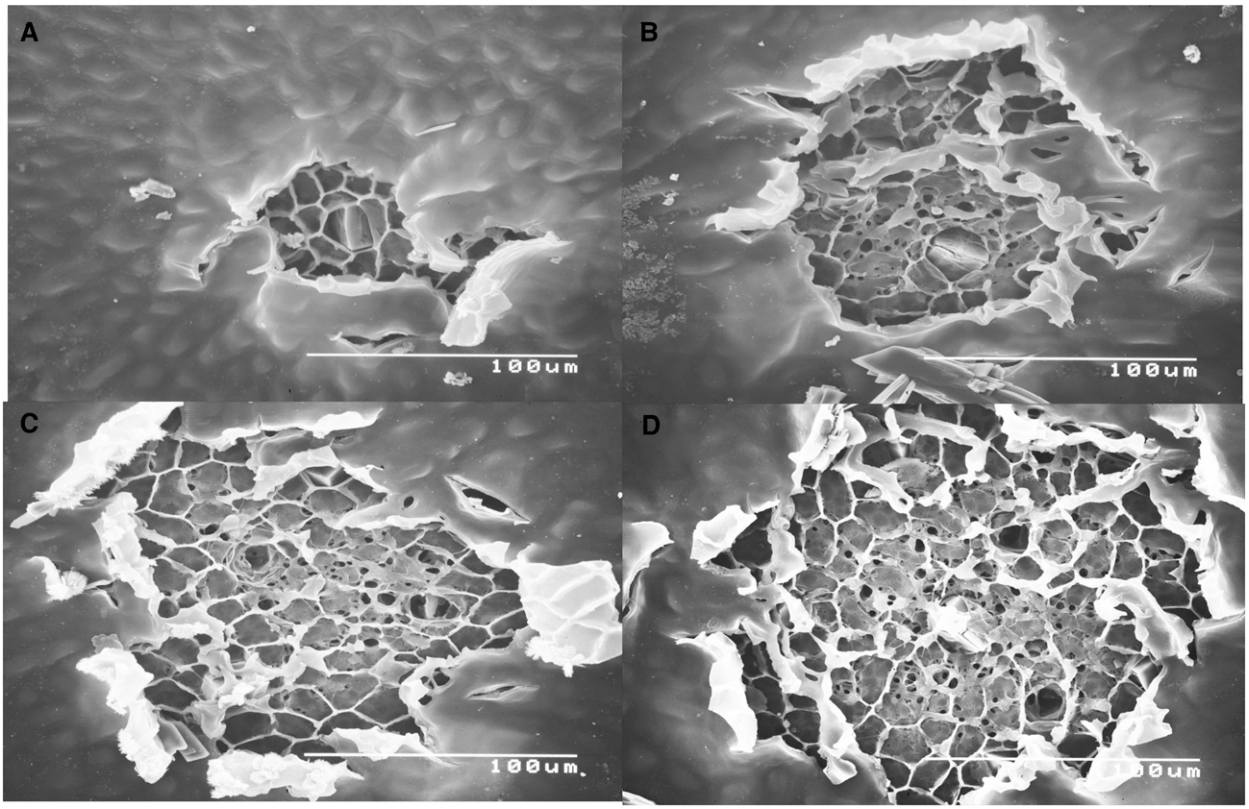

Fig. 2. Scanning electron micrographs of laser perforations at different times of exposure to a standard energy output of 1.9×10^6 J/cm^2. (A) Perforations at 30 µs exposure. (B) Perforations at 40 µs exposure. (C) Perforations at 60 µs exposure. (D) Perforations at 80 µs exposure.

have demonstrated that the use of oils prevented desiccation with no apparent side effect, which was corroborated in these experiments.

Characteristics, leaf penetration, and mobility of experimental solutions—Table 1 presents all solutions tested, their molecular weights, physical characteristics, and concentrations applied. Our initial testing was carried out with 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG), a fluorescent glucose analog already demonstrated to be taken up by plant cells (Etxeberria et al., 2005). 2-NBDG rapidly penetrated into the apoplastic space and mesophyll cells as depicted by the fluorescence emanating from the pinhole perforations and surrounding leaf area (Fig. 4A, B). By comparison, leaves that were equally laser perforated but that did not have solutions added displayed cuticular autofluorescence, but the central area remained opaque and there was no fluorescence in the interlasered spaces (Fig. 4C, D). The contrast between the green background color in Fig. 4B and the red of Fig. 4D demonstrates that 2-NBDG entered the leaf, because its green fluorescence signal is visible after entering living cells, whereas the green points of light in control leaves are due to cuticle autofluorescence (Fig. 4D). Control unlasered leaves supplied with 2-NBDG on the adaxial cuticle and rinsed before observation showed no green fluorescence signal and only background autofluorescence (not shown).

When treated leaves were viewed under fluorescence microscopy at low magnification 2 h after application, a strong fluorescence signal delineated minor and central veins within the leaf tissue (Fig. 5A). A cross section of the petiole 4 h after treatment showed a strong fluorescent signal within the vascular tissue, specifically the phloem (Fig. 5B). These results demonstrated that 2-NBDG entered the laser perforation pore, moved through the apoplast, was taken up by the phloem cells, and mobilized downstream from the treated area, indicating a basipetal movement of the applied substance.

In a separate experiment, we applied the laser perforation treatment to a total of five successive leaves on one single branch and then treated those target areas with 2-NBDG. The experimental leaves were left on the tree for 24 h before observation. In these trees, portions of the bark 30 cm below the treated leaves were excised with a razor blade such that the bark and phloem separated from the rest of the stem at the cambial interface. When viewed under the microscope, the bark tissue showed fluorescence (Fig. 5C) compared to controls (Fig. 5D), confirming the basipetal movement of applied 2-NBDG through phloem tissue.

Other fluorescent analogs of naturally occurring substances (adenosine triphosphate [BODIPY-FL-ATP; Thermo Fisher Scientific, Waltham, Massachusetts, USA]; Fig. 6A, B) and a fluorescent derivative of the antibiotic penicillin (Bocillin-FL penicillin [Thermo Fisher Scientific]; Fig. 6C, D) gave similar results to 2-NBDG, as fluorescence was observed in cross sections of leaf blades (Fig. 6A, C) and down in the petiole (Fig. 6B, D). In all of the substances noted above, fluorescence was not limited to the phloem cells and often appeared emanating from the xylem and parenchymatous tissue. It is likely that some of the phloem sap escaped the phloem when the petiole

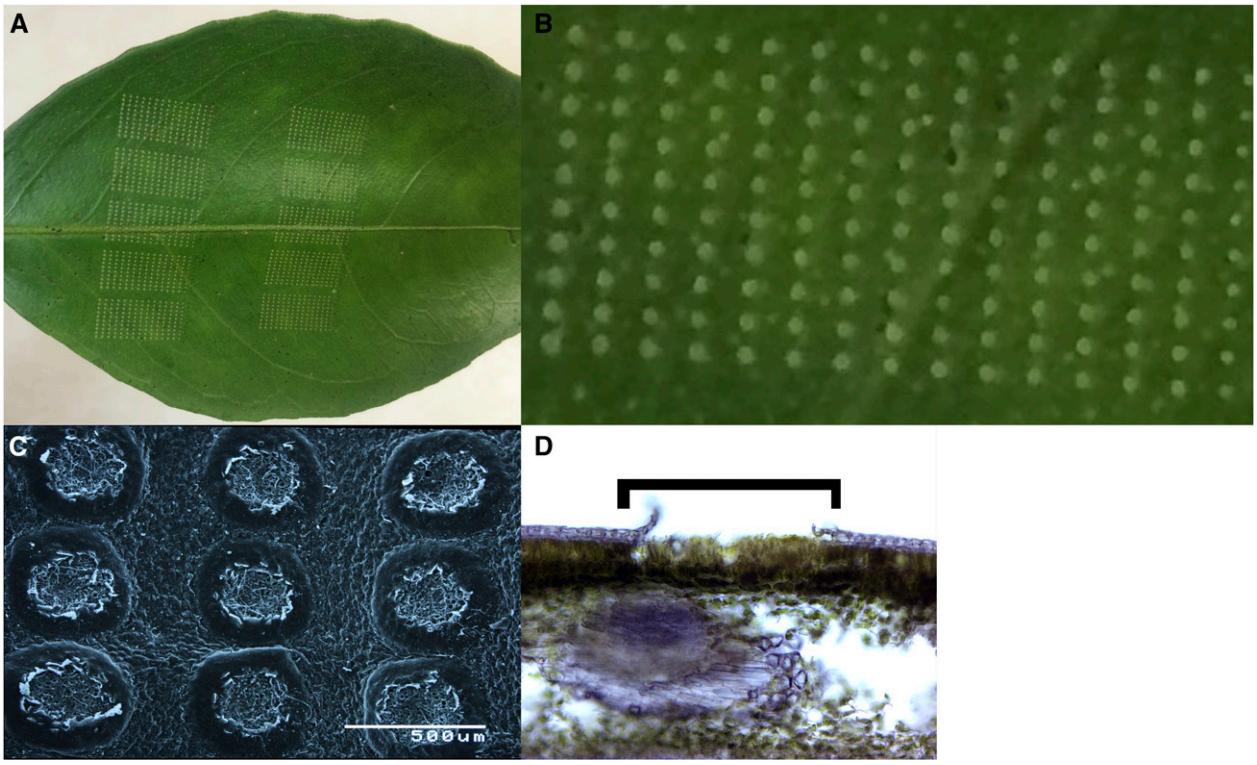

Fig. 3. Spatial pattern of laser perforations and structural changes resulting from the laser treatment on citrus leaves. (A) Citrus leaf lasered with the selected dot matrix perforation pattern. (B) Close-up of a single rectangle laser perforation pattern in A showing the cuticle perforations. (C) High-magnification scanning electron micrograph of a laser-perforated leaf surface. (D) Transverse light micrograph of a citrus leaf showing the site of a laser perforation hole (note the removal of the cuticle on the adaxial surface, bracket).

was sectioned prior to observation. Furthermore, it is also probable that these substances are being released or unloaded by the phloem to heterotrophic cells of the vascular tissue as they would with naturally occurring photoassimilates traveling in the phloem sap.

The use of CF-SE allowed us to corroborate the above observations without the complications imposed by the distribution of natural substances (or their analogs) to heterotrophic cells. CF-SE is a cell-permeant nonfluorescent probe that yields a fluorescein-labeled cell-impermeable product upon esterase removal of the SE group in live cells. The resulting ester (SE) reacts with intracellular amines and becomes trapped within the cell (Aeschbacher et al., 1986). Therefore, if CF-SE were to penetrate the phloem elements around the application site, it would be trapped in the phloem upon hydrolysis and transported downstream with the bulk phloem sap flow. In our experiments, fluorescence was observed exclusively in the living cells of the leaf palisade (Fig. 7A) and in the phloem tissue of the petiole (Fig. 7B). It is worth noting the contrast between the opaque laser perforation in Fig. 7A and those treated with fluorescent probes (i.e., Fig. 6A, C), as CF-SE only fluoresces inside living cells.

Other tested substances (Table 1) of a variety of sizes and chemical properties (e.g., quantum dots, lysine-tRNA) did not move beyond the application site (Fig. 8). These substances range from small ionic fluorescent vital stains (Lucifer Yellow, Alexa Fluor 488) to larger nanoparticles (quantum dots, ø = 15 nm) with Stokes radii larger than the estimated wall spaces (Carpita et al., 1979).

Movement of CF-SE as determined by a noninvasive phloem sap velocity measurement—Our assessment of CF-SE movement within the phloem using the noninvasive phloem sap velocity method confirmed the microscopic investigation. These data revealed that CF-SE moved approximately 3.94 $\mu m \cdot s^{-1} \pm 2.71$ SD from the dye application time to when the fluorescence signal slope significantly deviated from the background signal (Fig. 9).

Laser-enhanced solution penetration—To estimate the increased penetration of applied substances resulting from the application of laser light to the upper cuticle, we applied fluorescent vancomycin (BODIPY-FL vancomycin) to laser-perforated leaf surfaces. Similar to Alexa 488 and others, vancomycin penetrates the laser aperture and is immobilized by the cell wall, facilitating sample collection and estimation. Compared to untreated control leaves, laser treatment alone created a negligible fluorescent background signal (Table 2). Application of BODIPY-FL vancomycin on untreated leaves resulted in minimal uptake as determined by the marginal increase in fluorescence. However, when BODIPY-FL vancomycin was applied to laser-perforated leaves, uptake increased 2511% over non–laser-perforated leaves.

DISCUSSION

Treatment of vascular diseases in woody plants is a challenging undertaking due to the difficulty in delivering therapeutic

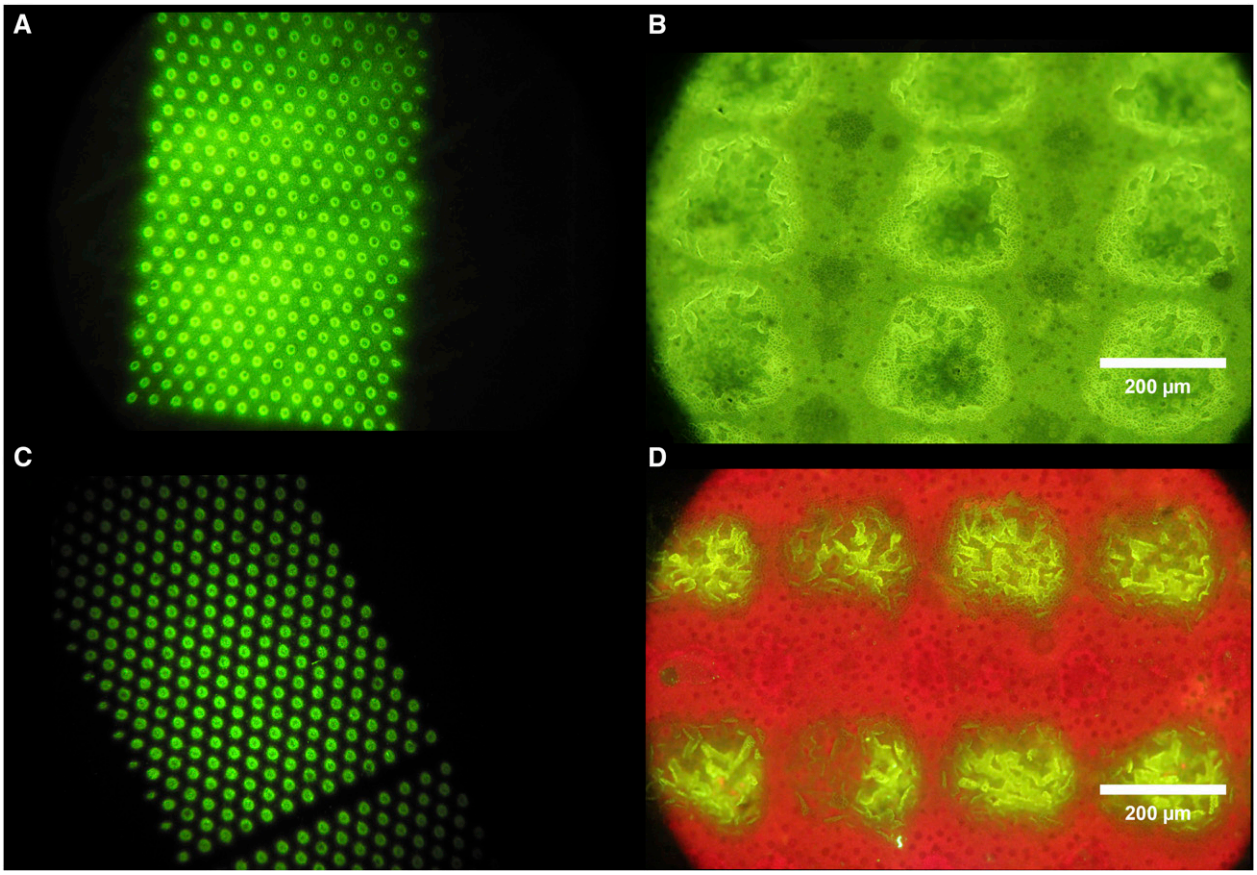

Fig. 4. Fluorescence micrographs of laser-perforated areas on citrus leaves after dye application. Exposure time for both treatments was 35 µs. (A) Low-magnification image of a laser-perforated area 2 h after application of 2-NBDG. (B) Close-up of A. (C) Low-magnification image of a control leaf 2 h after laser treatment. (D) Close-up of C.

materials to the affected tissue. In the case of phloem diseases, the problem is exacerbated given that phloem elements are one of the smallest cell types in plants and are surrounded by several other cell types (Knoblauch and Oparka, 2012). At the moment, there are no effective treatments in the fight against HLB in Florida and worldwide. Because of the bacterial nature of the causative agent, antimicrobial compounds are being considered as a potential tool in efforts to extend the productive life of citrus trees until a more permanent solution is found. However, the use of antibiotics to treat phloem-limited plant diseases like HLB is challenged by the inability to effectively introduce such substances into the phloem.

Foliar applications to treat a variety of biotic (Williams et al., 2003) and abiotic conditions (e.g., nutrient deficiencies; Knoche, 1994) have become an essential part of many horticultural crop production systems (Zhang and Brown, 1999). However, unless aimed to treat an external condition, foliar sprays can be inefficient due to their difficulty in penetrating the leaf surface. For example, given the low penetration rates of foliar nutrients and other substances such as growth regulators (Greene and Bukovac, 1974; Knoche et al., 1998), the concentrations needed for effective uptake may lead to considerable waste and often results in leaf injury and ground pollution. Furthermore, substances are generally only absorbed while the spray remains dissolved in a liquid media on the leaf surface or is rewetted by dew or high humidity (Allen, 1970).

Although influenced by many factors such as drop/surface interaction (Fernández and Khayet, 2015), the rate-limiting step in foliar spray efficiency is the diffusive process (Bondada et al., 2006). To overcome the complex array of factors and conditions affecting penetration and uptake of foliar application of nutrients and other substances, a more effective delivery system that circumvents the cuticular barrier is essential to guarantee higher penetration efficiency, and at the same time, lower the environmental impact. Without a system to help penetrate the plant body, foliar application of antimicrobials alone can be inefficient, especially in citrus leaves where stomates are almost exclusively located in the abaxial side of the leaf (Fig. 1). The data presented here demonstrate the utility of laser light perforation in enhancing penetration of test substances into the leaf and movement through the tree. By creating openings in the leaf surface, laser light establishes a field of high-density direct-entry points into the apoplast, where mobile substances can find passage to the phloem where other factors may play a separate role in the uptake (Fernández and Brown, 2013) and mobilization by the sieve cells.

In our investigation, the use of laser light technology significantly enhanced the penetration of foliar-applied substances across the cuticle of citrus leaves (Table 2). The creation of multiple pores through the cuticle allowed for the applied substances to reach the apoplast where, depending on their chemical composition, they either moved apoplastically to the phloem

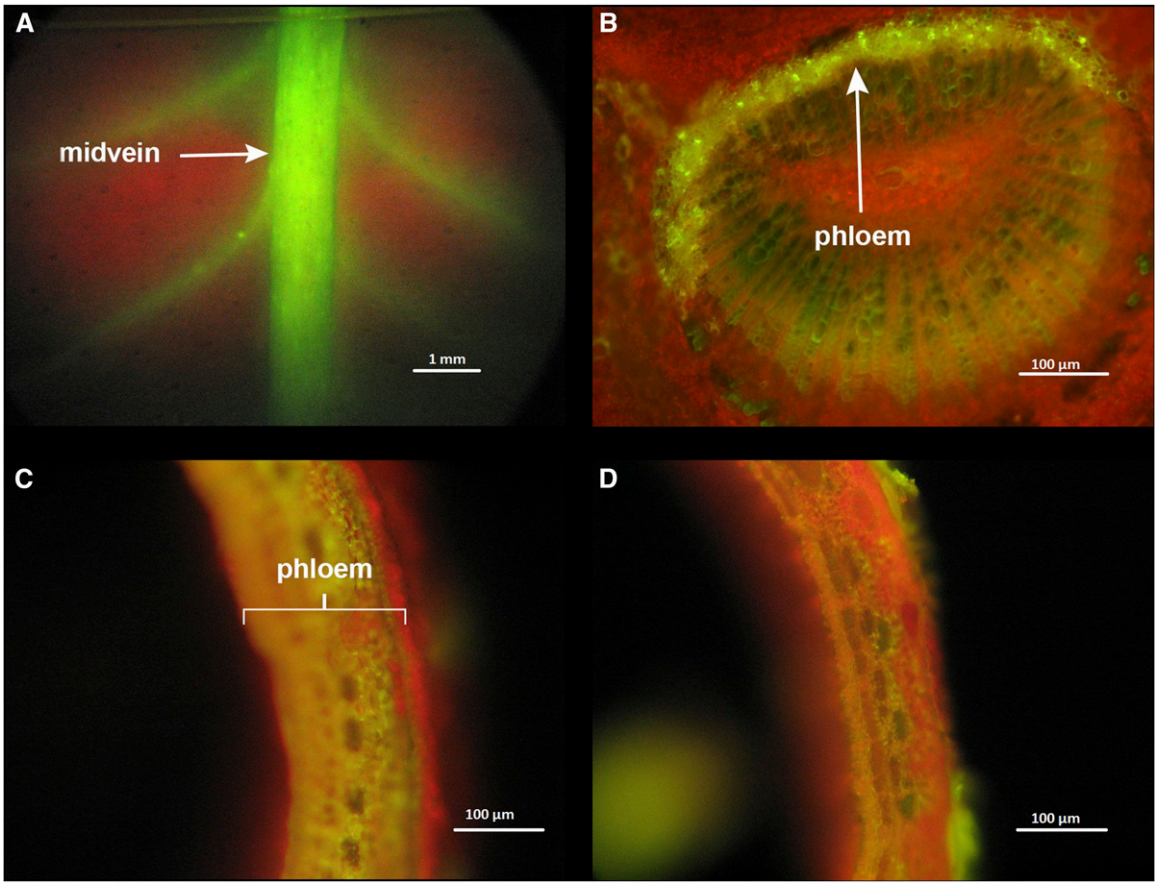

Fig. 5. Fluorescent micrographs. (A) Abaxial side of a citrus leaf 2 h after laser treatment and 2-NBDG application. (B) Cross section of a citrus leaf petiole 4 h after laser treatment and 2-NBDG application on the upper (adaxial) leaf surface. (C) Cross section of citrus bark 30 cm below the laser perforation site treated with 2-NBDG. (D) Control bark of an untreated tree 30 cm from a leaf.

(Figs. 6 and 7) and were carried down the leaf, or remained in the application point (Fig. 8). Phloem-mobile substances were also able to penetrate and be transported down the phloem stream (Figs. 5–8). Among test substances, the phloem-mobile compounds 2-NBDG, lysine, Biocillin, ATP, trehalose, CF-SE, and poly(amidomine) (PAMAM) dendrimer G-4 nanoparticles (4.5 nm in size) showed a high degree of mobility. Uptake of these substances into the leaves was confirmed by the spread of fluorescence within the lasered area (Figs. 4A, B and 6A, C), down the petiole after 4 h (Figs. 5A, B; 6B, D; and 7B) and stem (Fig. 5C, D), and by the increase in vancomycin uptake compared to the control (Table 2).

Q-dots (10 nm diameter) and Dextran, Texas Red (molecular weight 3000 $g \cdot mol^{-1}$) were not capable of moving beyond the point of application. These substances either have Stokes radii larger than the cellulose fiber interspaces (calculated at approximately 5 nm; Carpita et al., 1979) or their linear size made them too large to move through the cell wall matrix. Alexa 488 and Lucifer Yellow, although small charged molecules, were unable to move through the negatively charged cell wall (Richter and Dainty, 1990).

Other factors not considered in this communication need further scrutiny for large-scale agricultural applications. For example, the increase in exposed surface area created by the laser perforations could lead to excess water loss and serve as a pathway for invading organisms. However, these same issues have been resolved for packinghouse operations by the application of a waxy coating immediately after laser perforation. This procedure eliminated water loss and prevalence of decay organisms (Sood et al., 2009; Danyluk et al., 2010) and could theoretically be used in conjunction with the laser perforation method. In our studies, and under the specified conditions, there was no permanent damage to the leaves when oil was applied as determined by the fact that leaves remained intact.

Aside from practical field applications, this method can be very useful in the study of phloem physiology. Recently, a number of phloem-mobile dyes have been evaluated for their use in studying the velocity of phloem sap flow (Savage et al., 2013; Knoblauch et al., 2015). Yet, methods for delivering phloem-mobile dyes into the sieve elements can be imprecise (e.g., physical abrasion of the leaf cuticle and epidermis; Savage et al., 2013) or require additional compounds applied to the leaf surface to increase cuticle permeability, which appear to currently be the best options (Knoblauch et al., 2015). Our results in phloem transport velocity were significantly slower compared to a previous report (Savage et al., 2013); although we used a similar methodology, this variation may correspond with differences in plant species, age, organs measured, and conduit diameters. Our results show phloem transport velocity from a mature leaf to its petiole in a fully developed citrus plant, which differs considerably from the herbaceous species used in the Savage

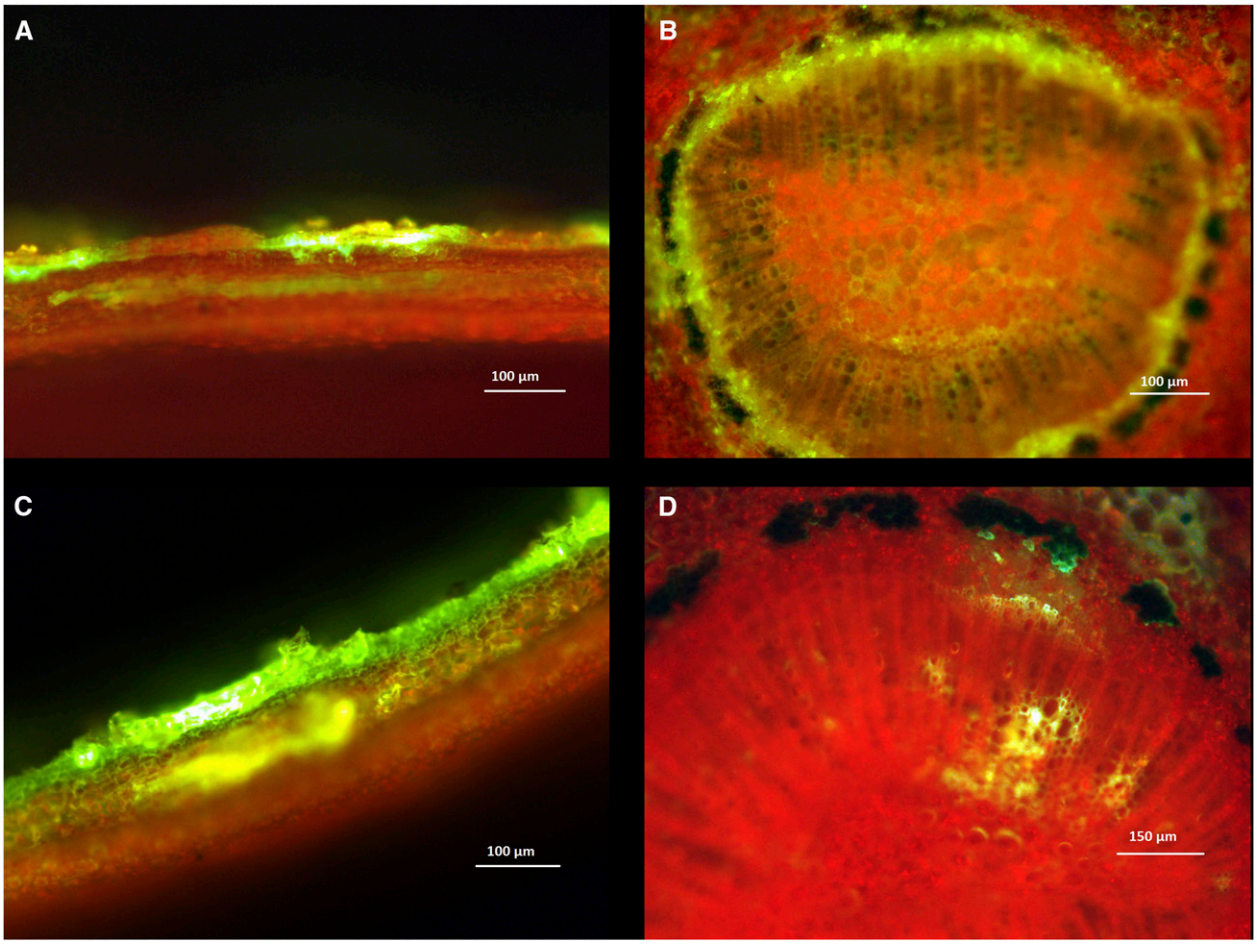

Fig. 6. Fluorescence micrographs. (A) Cross section of a lasered citrus leaf blade 2 h after BODIPY-FL-ATP application. (B) Cross section of a lasered citrus leaf petiole 4 h after laser treatment and BODIPY-FL-ATP application. (C) Cross section of a lasered citrus leaf blade 2 h after Bocillin-FL penicillin application. (D) Cross section of a lasered citrus leaf petiole 4 h after laser treatment and Bocillin-FL penicillin application.

et al. (2013) study. In addition, phloem loading in citrus depends on an apoplastic loading system (Nolte and Koch, 1993), which could have led to slower uptake and translocation into the sieve tubes. Nonetheless, the laser perforation method described here is highly repeatable, and the precise surface area and depth of the perforations can be customized, quantified,

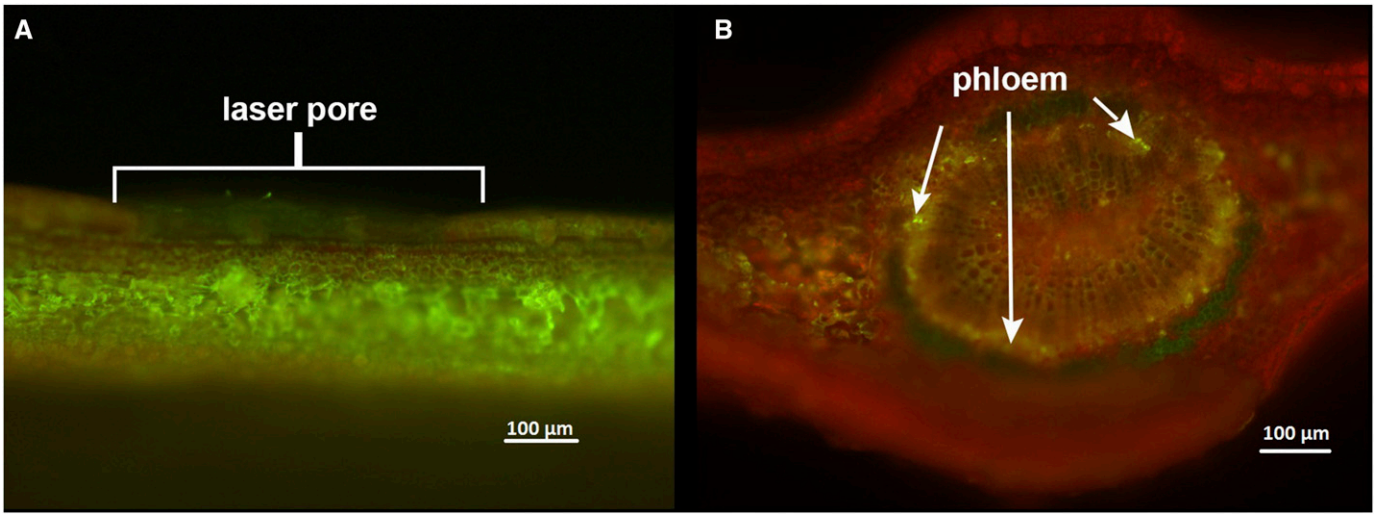

Fig. 7. Fluorescence micrographs. (A) Cross section of a lasered citrus leaf 2 h after CF-SE application. (B) Cross section of a lasered citrus leaf petiole 4 h after laser treatment and CF-SE application.

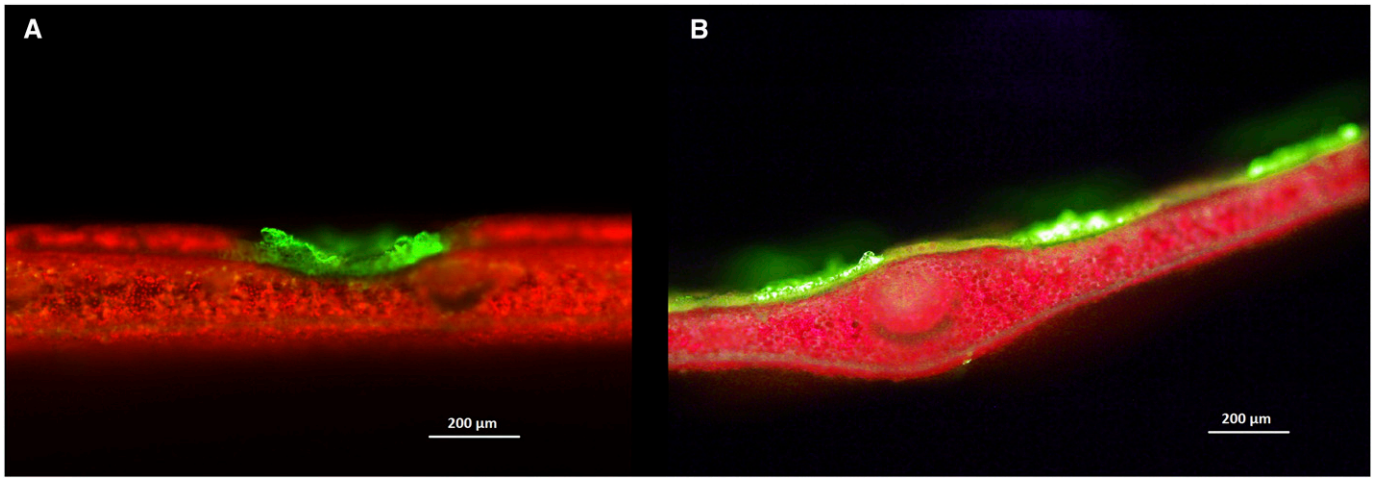

Fig. 8. Cross section of a lasered citrus leaf blade 24 h after application of (A) CdSe/ZnS quantum dots (10 nm diameter) and (B) Lysine-tRNA (molecular weight 26.5 kD). In both instances, the applied fluorescent substances remained in the application site.

and standardized between experiments. Because the laser system can be tuned to deliver different levels of penetration, it could also be used to specifically target the application of dyes to different cell types at known depths within the tissue.

At the present time, this technology is being developed as part of a continuous effort to curb HLB in citrus. For large-scale field applications, a scaled-up and more flexible model of the instrument containing multiple nozzles for the laser light, antimicrobial spray, and wax application is being developed. In principle, the system is also useful to other agronomical crops with somewhat similar plant architecture and leaf form, although individual application parameters need to be altered to account for differences in leaf thickness, surface properties, and overall leaf size. The overall system also offers the added advantage of lower application frequencies and hence reduction in chemical use, a condition that lessens environmental impact.

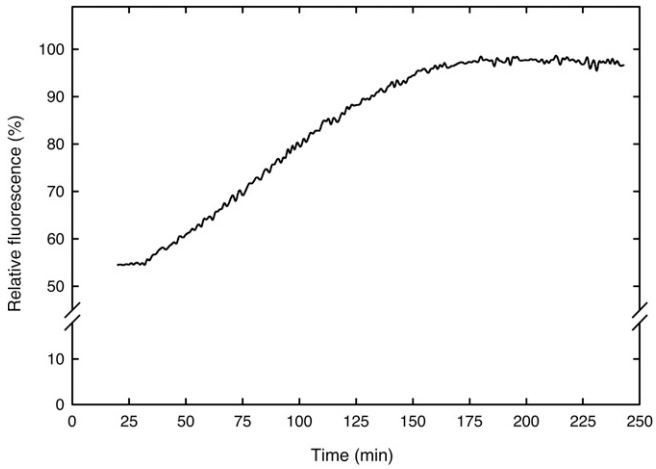

Fig. 9. Velocity of CF-SE through the phloem of a citrus leaf petiole. CF-SE dye was applied at T = 0 min; dye-tracing method started at T = 20 min.

Table 2. Uptake of fluorescent vancomycin by control and laser-treated citrus leaves.

Treatment	RFU[a]	Uptake (RFU)	% increase + laser
Untreated leaf	104 ± 20	—	—
Leaf + vancomycin	144 ± 46	44[b]	—
Lasered leaf	127 ± 65	—	—
Lasered leaf + vancomycin	1232 ± 139	1105	2511

Note: RFU = relative fluorescence units.

[a]A relative fluorescence unit is the increase in the fluorescence scale of one numeric unit at λ 520.

[b]Differences are significant at $P < 0.05$.

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