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LEAF-DISC GRAFTING FOR THE TRANSMISSION OF *CANDIDATUS* *LIBERIBACTER ASIATICUS* IN CITRUS (*CITRUS SINENSIS*; *RUTACEAE*) SEEDLINGS¹

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- *Premise of the study:* The search for resistance/tolerance to the devastating citrus huanglongbing disease (syn. HLB or citrus greening) is generating an increasing number of new plants of diverse genetic makeup. As the increasing number of new plants require more space, resources, and time, the need for faster and more efficient HLB screening tests becomes crucial.
- *Methods and Results:* The leaf-disc grafting system described here consists in replacing a disc of leaf tissue with a similar disc from an infected plant. This can be performed in young seedlings not yet big enough to endure other types of grafting. Graft success and infection rates average approximately 80%.
- *Conclusions:* We describe the successful adaptation of leaf-disc grafting as a powerful screening tool for HLB. The system requires minimal plant material and can be performed in seedlings at a very young age with increased efficiency in terms of time, space, and resources.

Key words: citrus greening; disease transmission; grafting efficiency.

Grafting, the process of fusing parts of two separate (but compatible) plants, has long been used in horticulture to generate plants with shared and improved horticultural properties (Hartmann and Kester, 1975; Goldschmidt, 2014). The technique is based on taxonomic compatibility between rootstock and scion, both carrying desired agronomical traits, forming a composite grafted plant. Compatibility consists of a successful formation of a graft union and connection of xylem and phloem vessels (Asahina and Satoh, 2015). Desired agronomical traits for rootstocks include improved nutrient uptake, reduced juvenility period, tree size, and adaptation to soil abiotic and biotic factors. For scions, sought-after traits include flower and fruit loading capacity and fruit quality (Rogers and Beakbane, 1957; Jones, 1984).

Grafting is also used in epidemiological studies to facilitate the transmission of communicable diseases from one plant to another (Lee and Bar-Joseph, 2003). In breeding programs, grafting regularly functions as a screening tool to test for the resistance/tolerance to a given disease by newly developed germplasm material (White et al., 1991). In this regard, the search for resistance/tolerance to huanglongbing disease (syn. HLB or citrus greening), which is caused by the phloem-limited bacterium *Candidatus Liberibacter asiaticus* (CLAs), is currently a major focus of citrus breeding programs worldwide, as the disease continues to spread and devastate existing groves (Gottwald, 2010).

Conventional grafting methods for disease transmission involve the placement of a portion of infected wood into a

receptor stem through diverse forms of bark cuts (Hilf and Lewis, 2016). Transmission can also be achieved by inserting shards of infected leaves or bark into the receptor tree trunk (Blue et al., 1976). More recently, a method consisting of inserting the petiole of an infected leaf into the bark of a decapitated plant has been reported (Hilf and Lewis, 2016). In all these instances, however, the receptor tree has to be of sufficient age and girth to have developed adequate bark to allow for tissue insertion and manipulation. To reach a manageable size, citrus trees require prolonged growth times and resources, and they take up additional greenhouse space due to the inherently larger size (Lopes et al., 2009; Hartung et al., 2010). Given the increased frequency and large numbers of new germplasm produced via sexual and asexual methods, earliest detection of HLB resistance/tolerance offers the benefits of space and time and, consequently, a significant increase in efficiency.

Leaf-disc grafting—the replacement of a leaf tissue disc from a healthy plant with that of a diseased one (Blue et al., 1976)—offers the advantage that it can be performed on a single receptor leaf. This method, using a paper hole puncher, has been used exclusively in citrus to examine the transmission of citrus tristeza virus, citrus exocortis virus, vein enation virus, and psorosis virus (Blue et al., 1976). Conceptually, leaf-disc grafting voids the need for mature bark, making possible its use in young seedlings. Earlier versions of leaf grafting using free-hand triangle cuts or a cork borer had been used (also in citrus) for the transmission of viruses and viroids (Cohen, 1972; Schwartz, 1972; Vogel, 1973), but these methods are physically less precise. Given the benefits provided by leaf-disc grafting, we proceeded to study the possible adaptation of this technique to very young and immature trees as a means to accelerate studies in HLB.

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MATERIALS AND METHODS

Plant material—Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) fruits were hand-picked from a Valencia grove at the Citrus Research and Educational Center, University of Florida, Lake Alfred, Florida, USA. Seeds were immediately extracted by longitudinally cutting the fruit in half, without damaging the integrity of the seeds. After removal of mucilage with sodium hydroxide, seeds were dried at ambient temperature and the coat removed to facilitate germination. Seeds were sown in yellow potting tubes (3 cm diameter) with potting mix (Fafard Custom Citrus Mix; BWI Companies, Apopka, Florida, USA), watered as necessary, and kept at an approved secured U.S. Department of Agriculture–Animal and Plant Health Inspection Service (USDA–APHIS) greenhouse (28–32°C). Seedlings were transferred to small pots (10 cm diameter) at two months old, as the first true leaves matured. Seedlings were watered twice a week and fertilized with water-soluble fertilizer 20-10-20 (Winfield Solutions, Apopka, Florida, USA) once a week.

Leaf-disc grafting—Forty plantlets were selected for the leaf-disc grafting technique seven days after transferring to new pots (Fig. 1A). Previously confirmed HLB–PCR-positive Valencia orange plants, kept in a screenhouse, were used as infection source material. A paper hole puncher was used to create leaf discs and leaf holes of the same size. A leaf disc was excised from the second true leaf of the healthy plants (taking care that the midrib was at the center of the excised tissue) and replaced with a similar disc from an infected tree. The midrib of the infected leaf disc was aligned with that of the healthy leaf respecting both the abaxial/adaxial position and the basipetal/acropetal direction (Fig. 1B). One single HLB-affected leaf was source to several grafts, while only one disc was replaced per leaf on three separate leaves for each of the 40 healthy plantlets. To keep the infected tissue disc in contact with the healthy leaf, clear Scotch tape (3M, St. Paul, Minnesota, USA) was used to support the graft in place on both the adaxial and abaxial leaf surfaces (Fig. 1C, Appendix 1). Grafted seedlings (Fig. 1D) were then kept in the same greenhouse, isolated from healthy material inside a mesh cage, under the same irrigation and fertilization schedules described above. To minimize the potential of rotting along the grafting edges, overhead irrigation and fertilization were avoided. Twelve plantlets were not grafted and kept under the same conditions to be used as negative control.

Tissue sampling and microscopy—At determined times after grafting, mature symptomatic leaves from the new flush were collected, individually tagged,

bagged, and stored at –20°C until PCR analysis to confirm presence of the bacteria. Graft unions were corroborated by light microscopy using a Wild Heerbrugg stereoscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Canon EOS T3i camera (Canon, Tokyo, Japan).

DNA extraction and real-time PCR—DNA from citrus leaf was extracted using potassium acetate buffer and TissueLyser II (QIAGEN, Valencia, California, USA). Briefly, 300 mg was placed in a 2-mL Safe-Lock microcentrifuge tube with a 5-mm stainless steel bead (QIAGEN). The tubes were immersed in liquid nitrogen for up to 5 min and then were placed into the TissueLyser adapter (kept in the freezer) and fixed into the TissueLyser clamp. Immediately, samples were processed for 30 s at 30 Hz. The vibration was repeated three times with rotation of the adapter. Eleven hundred microliters of extraction buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris [pH 9.0], 10 mM DTT) was added to each tube and brief centrifugation at 5000 rpm for 1 min was performed to discard the debris. One milliliter of supernatant was recovered into a new 2-mL tube and 20 µL of SDS 20% was added. The mix was incubated at 65°C in a water bath for 45 min, then 500 µL of 5 M potassium acetate was added. After vortexing, the tubes were incubated in ice for 20 min and then were subject to centrifugation under cooling at 12,500 rpm for 10 min. One milliliter of supernatant was recovered and mixed with 1 mL of ice-cold isopropanol. The tubes were centrifuged at 12,500 rpm for 20 min at 4°C. After discarding the supernatant, 1 mL of ice-cold 70% ethanol was added to the pellet, and tubes were well vortexed and then centrifuged at 12,500 rpm for 10 min at 4°C. Supernatant was pipetted carefully and discarded. Pellets were dried under an N₂ stream. DNA pellets were resuspended in 20 µL of RNase-free water, and concentration was measured using a NanoDrop ND1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Extracted DNA was used for quantitative real-time PCR (qPCR) using primers and probe as described by Li et al. (2006).

RESULTS

Real-time PCR—qPCR was performed as described by Li et al. (2006) for all 40 plantlets. The HLB-causing agent was confirmed in 32 of 40 plantlets or an 80% success rate. We considered plantlets positive if the cycle threshold was less than 28. The majority had a cycle threshold of between 18 and 22. The 12 control plantlets (not grafted with infected leaf discs) remained PCR negative.

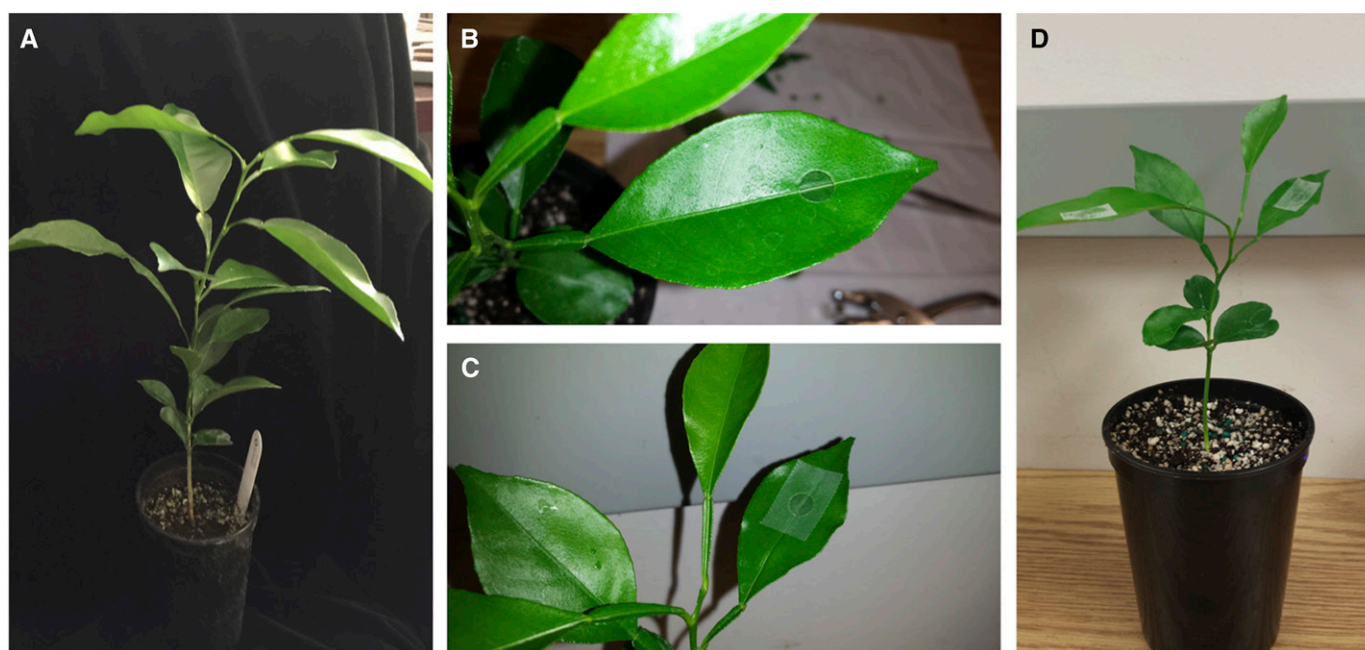


Fig. 1. Citrus leaf-disc grafting. (A) Two-month-old Valencia seedling used for the leaf-disc graft method for HLB transmission. (B) Leaf from a healthy seedling grafted with a CLas-infected leaf disc. (C) Grafted leaf disc kept in place with tape on both sides of the leaf. (D) Valencia seedling showing two leaf-disc grafts.

Grafting success rates and symptom development—In approximately 85% of grafted young trees, all CLAs-infected leaf-disc grafts remained green throughout the experimental period. In the remaining trees, where one or more discs became yellowish or necrotic, leaf-disc grafts were replaced after one month. In these cases, younger but mature leaves from the original grafted trees were used. For observation of successful grafts, three grafted leaves were excised and viewed from both the abaxial and adaxial surfaces. The grafted discs were observed tightly joined to the leaf around the circumference of the disc (Fig. 2A). Both the leaf and grafted disc were joined by a lignified ring of tissue (Fig. 2B).

HLB symptoms began appearing three months after grafting. Widespread HLB symptoms could be observed in the majority of plantlets after six months (Fig. 2C). Symptoms were the classic blotchy mottle and corky texture.

DISCUSSION

The rapid spread of HLB across continents and the imminent threat it poses to the survival of the citrus industry worldwide has demanded accelerated efforts to develop resistant/tolerant germplasm (Stover et al., 2016). As the number of new plants of diverse genetic makeup are produced both sexually and asexually at increasing rates, the need for faster and more efficient HLB screening tests becomes crucial (Hilf and Lewis, 2016). Breeding programs can make new germplasm testing more efficient by decreasing the time and space required as well as the materials needed.

For most grafting techniques, either the budwood, the test plant, or both require a physiological and structural maturity that needs a considerable amount of time and significant space to develop. In this report, we describe the successful adaptation of leaf-disc grafting (Blue et al., 1976) as a screening tool for

HLB, a bacterial disease. The main advantages of this procedure are several, and can be described in terms of:

1. Time: Shorter times are required before challenging the test plants with CLAs, given that grafting can be carried out in young plants still in their primary growth phase. This system bypasses the need to have cortex/bark of workable girth for other types of grafting (which can take considerable time) as only one leaf is required.

2. Space: The space needed to keep experimental plant material is reduced due to the smaller size of trees ready for leaf-disc grafting, and the inherent faster turnover opens up space for new germplasm.

3. HLB tissue source: A reduction in HLB-affected plants to be used as sources of leaf discs is possible because a much larger number of HLB-affected leaf discs can be obtained from an HLB-donor plant. It should be noted that, although three discs per plant were used in this experiment, we were successful in transmitting HLB with only one disc (data not shown).

4. Infection rate: Rates of HLB transmission in the 80% range are comparable to or higher than other grafting methods, e.g., the bud-stick method (yielding between 67% and 88% success rate; Lopes et al., 2009) or the individual leaf method (67% average success rate; Hilf and Lewis, 2016).

It is noteworthy that the method presented here was intended for testing young seedlings for tolerance or resistance to infection or development of disease symptoms and not to compare its efficiency to other grafting methods. However, the simplicity of leaf-disc grafting provides additional advantages not only to the study of HLB in citrus, but also to the study of other vascular pathogens in citrus and elsewhere. For example, the grafted disc marks the precise initial infection point, which allows the researcher to follow the progression of the disease and/or movement of the pathogen. In addition, a grafted leaf disc does not alter the natural

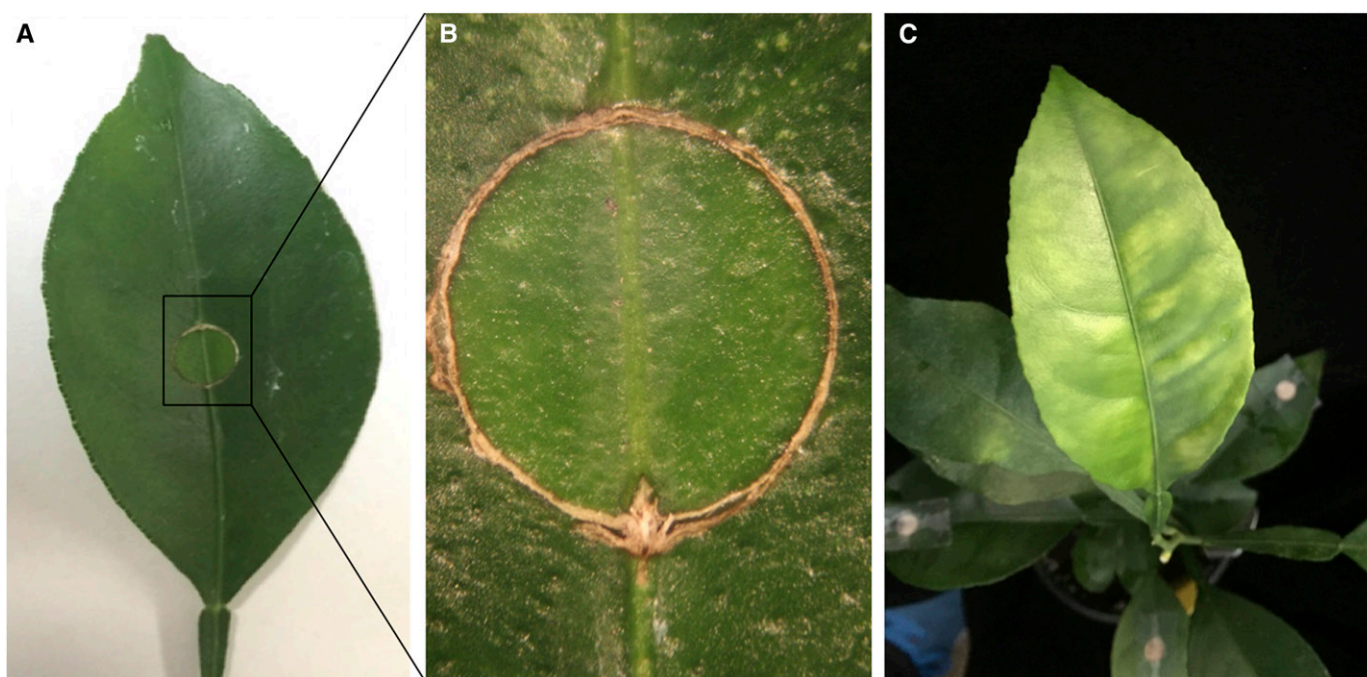


Fig. 2. (A) Leaf disc three months after grafting. (B) Close-up of a successful graft union formation, which took approximately one month. (C) HLB symptoms six months after grafting.

vasculature of a plant, which is beneficial for epidemiological studies in which parastichies and other properties of the vasculature system need to remain intact. In conclusion, the leaf-disc grafting method for HLB transmission reported here offers a new tool to enable earlier screening of new germplasm against HLB.

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APPENDIX 1. Supply list and protocol sheet.

A. Materials

1. Citrus seedlings
2. Infected leaf material (HLB-affected material or any other infectious citrus disease to be tested)
3. Paper hole puncher
4. Scotch tape (3M, St. Paul, Minnesota, USA)

B. Leaf-disc grafting

1. Excise a leaf disc from the second true leaf of a healthy young plant, making sure that the midrib is at the center of the excised tissue.
2. Place a piece of Scotch tape on the abaxial side of the leaf, covering the hole.
3. Replace the excised leaf disc with a similar disc from an infected leaf. The midrib of the infected leaf disc should be aligned with that of the healthy leaf in respect to both the abaxial/adaxial position and the basipetal/acropetal direction.
4. Fasten the disc on the adaxial side of the leaf using another piece of Scotch tape.