Influence of Rootstock Propagation Method on Traits of Grafted Sweet Orange Trees

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Abstract. The rootstock plays a large role in modern citrus production because of its influence on tolerance to adverse abiotic and biotic soil-borne stresses, and on the general horticultural characteristics of the grafted scion. In recent years, rootstock has received increased attention as a management strategy to alleviate the devastating effects of the bacterial disease huanglongbing (HLB), also known as “citrus greening.” In commercial citrus nursery production, rootstocks are typically propagated by seed. Because of the increased demand for HLB-tolerant rootstocks, seed supply is often inadequate for the most popular cultivars. Cuttings and tissue culture (TC) propagation are alternative methods to supply adequate quantities of genetically identical rootstocks to be used as liners for grafting. However, there are concerns among nursery owners and citrus growers regarding the possible inferiority of rootstocks that are not propagated by seed. This study investigates the influence of rootstock propagation method on traits of sweet orange trees grafted on four commercially important rootstock cultivars during the nursery stage and during the first year of growth in a commercial citrus orchard. Several of the measured traits during the nursery stage, including rootstock sprouting, grafted tree growth, and root mass distribution were significantly influenced by the rootstock propagation method, but traits were also influenced by the rootstock cultivar. Our results also suggest that for tissue culture-propagated plants, differences in the starting material and the culturing method can affect the grafted tree behavior. Except for canopy spread and scion to rootstock trunk diameter ratio, tree growth during the orchard stage was determined by the combination of propagation method and rootstock, rather than by propagation method alone.

Rootstock selection has played an important role in the history of citrus production (Castle, 2010). The rootstock has a significant impact on tolerance to biotic and abiotic stress factors as well as on fruit quality, yield,

and other horticultural parameters of a grafted citrus tree (Bowman and Joubert, 2020; Bowman et al., 2016a; Castle, 1995; Wutscher and Hill, 1995; Wutscher and Bowman, 1999). Since the arrival of the bacterial disease huanglongbing (HLB, a.k.a. citrus greening) in Florida, citrus production has declined steadily from 242 million boxes of citrus before 2005 to 74 million boxes in the 2018–19 production year (www.nass.usda.gov/fl). Most commercial citrus cultivars are susceptible to HLB, and disease management in Florida is focused on eliminating the disease vector and improving tree health and productivity using different horticultural strategies (Morgan et al., 2016; Stansly et al., 2014).

Rootstocks have received increased attention as a management strategy to alleviate the devastating effects of HLB. In contrast to most scion cultivars, several rootstock cultivars are tolerant to HLB and enable a grafted tree to remain productive for a longer time (Albrecht and Bowman, 2011, 2012; Bowman et al., 2016a). The physiological mechanism for rootstock effects on the scion under HLB endemic conditions have not yet been elucidated, but it may be associated with

their ability to influence the scion metabolically (Albrecht et al., 2019; Killiny et al., 2018) or indirectly by affecting absorption of water and nutrients from the soil. The ability to influence uptake of water and nutrients is related to the root system architecture, which can vary considerably among rootstocks (Castle and Youtsey, 1977).

The phenomenon of nucellar embryony found in citrus and other fruit tree crops allows the clonal production of most rootstock cultivars from seed (Koltunow et al., 1995; Kumar and Rani, 2013), and seed propagation remains the preferred method of rootstock production in commercial citrus nurseries. In recent years, the increased demand for HLB-tolerant rootstocks has led to an inadequate seed supply for the most popular cultivars. Consequently, alternative propagation methods, namely cuttings and tissue culture propagation, are required to produce the desired quantities of trees.

Propagation by cuttings and tissue culture will result in genetically identical rootstocks that can be used as liners for grafting (Albrecht et al., 2017a; Bowman and Albrecht, 2017). For new cultivars, both propagation methods are indispensable to produce trees for field testing until seed source trees have reached sexual maturity (Bowman and Albrecht, 2017). In addition, for those rootstocks that produce few or no nucellar embryos but have other desirable traits (Barcelos Bisi et al., 2020), vegetative propagation is the only option to produce genetically identical plants.

Seed-grown rootstocks usually develop a single taproot during initial growth in the nursery. In contrast, rootstocks propagated by cuttings and tissue culture produce multiple adventitious roots (Albrecht et al., 2017b). Based on traditional views, there is concern among citrus growers and citrus nurseries that the different root architectures arising from vegetative propagation will result in trees of inferior quality. The use of rooting hormones and the maturity of the source plant (Ferguson et al., 1985) are additional sources of concern as they may affect growth behavior of rootstock liners prior and during the grafting stage.

In citrus, early studies regarding growth and root architectures of trees produced other than by seed were mostly conducted with the intent to compare scion cuttings with scion seedlings or grafted trees rather than rootstock propagation methods. These studies reported shallower and sparser root systems of cuttings compared with seedlings (Halma, 1931; Savage et al., 1945). However, Palma et al. (1997) suggested that the multiple adventitious roots produced by rooted micro-cuttings of *Citrus macrophylla* would be more efficient for nutrient uptake than the single perpendicular root of the seedlings. Castle (1977) found that despite differences in some root traits, the root distribution and root density of sweet orange trees on cuttings compared favorably with that of trees on seedlings. In addition to differences among propagation methods, considerable

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differences among rootstock genotypes were reported (Castle and Youtsey, 1977).

Studies on temperate fruit tree crops showed varying results depending on the propagation method, culturing conditions, and cultivar used (Webster, 1997 and references therein). Results also vary in other tree crops. For example, African plum trees propagated by vegetative methods had a lower root density than seed-grown trees and were suggested to be less competitive in intercropping systems (Asaah et al., 2012). A study on different *Eucalyptus* species found that micropropagation yielded an inferior root system compared with macropropagation during the first 16 months of field growth (Mokotedi et al., 2010). In contrast, a comparison of tissue culture cultivars and seedlings of *Eucalyptus camaldulensis* discovered no above- or belowground architectural differences that were associated with the propagation method (Bell et al., 1993).

A recent study conducted in our laboratory (Albrecht et al., 2017b) compared root architectures of rootstock liners produced by seed, cuttings, and tissue culture during the early weeks of growth and before grafting. In the present study, we are expanding our research to investigate root architectures, tree growth, and other traits of grafted sweet orange (*Citrus sinensis*) trees in the nursery and in a commercial citrus orchard. Our objectives were to investigate if the propagation method affects 1) growth traits of trees during the early weeks after grafting in the nursery, 2) root architectures and other traits of grafted field-ready nursery trees, and 3) grafted tree growth during the first year of establishment in a commercial citrus orchard. Four commercial citrus rootstock cultivars were included in the study to investigate the influence of rootstock and the combination rootstock and propagation methods.

Materials and Methods

Rootstock production

Rootstock cultivars. Four commercially important citrus rootstocks were used: 'US-802' ('Siamese' pummelo [*Citrus maxima*] × 'Gotha Road' trifoliate orange [*Poncirus trifoliata*]), 'US-812' ('Sunki' mandarin [*C. reticulata*] × 'Benecke' trifoliate orange), 'US-897' ('Cleopatra' mandarin × 'Flying Dragon' trifoliate orange), and 'US-942' ('Sunki' mandarin × 'Flying Dragon' trifoliate orange), four hybrid rootstocks that have gained major commercial importance in Florida since their release by the U.S. Department of Agriculture (USDA) (Bowman and Joubert, 2020; Bowman et al., 2016b).

Seed propagation. Seeds of the rootstock cultivars were extracted from fruits as described in Albrecht et al. (2017b). Plants were grown in the USDA Horticultural Research Laboratory (USHRL) greenhouses in Fort Pierce, FL beginning with sowing seeds into premoistened, soilless potting mix (Pro Mix BX; Premier Horticulture, Inc., Quakertown, PA) using racks of 3.8-cm × 21-cm cone cells (Cone-tainers; Stuewe and Sons, Tangent,

OR). After germination, plants were irrigated as needed and fertilized biweekly using a water-soluble fertilizer with micronutrients (20N–10P–20K; Peters Professional, The Scotts Company, Marysville, OH) at a rate of 400 mg N/L. Insecticides were applied as needed. Any off-types arising from zygotic embryos were identified based on leaf morphological traits and discarded.

Cuttings propagation. Plants were grown in the USHRL greenhouses in Fort Pierce, FL. Single node cuttings of ≈2.5 cm in length were excised from woody sections of 1- to 2-year-old greenhouse-grown nucellar rootstock plants. Leaves remained attached to each node but were trimmed to reduce the leaf size to 20% to 30%. The basal end of each cutting was dipped in a commercial rooting powder (Hormodin 2, E.C. Geiger, Inc., Harleysville, PA) containing 0.3% indole-3-butyric acid (IBA), and cuttings were inserted into 3.8-cm × 21-cm cone cells (Cone-tainers; Stuewe and Sons) containing the same potting medium described above. Cones were placed on a mist bench, and misting was applied for a duration of 6 weeks as described in Bowman and Albrecht (2017); during this time period, the shade-cloth on the greenhouse was closed from 9:00 AM to 6:00 PM daily. After 4 weeks, plants received a liquid fertilizer application as described above. Two weeks later, plants received another liquid fertilizer application at the same rate but including chelated iron (Sequestrene 138 Fe; Ciba-Geigy Corp., Greensboro, NC). Starting in week 7, the shade-cloth remained open, and plants were maintained in the same manner described for seedlings.

Tissue culture propagation. Plants were grown in two different commercial citrus nurseries and produced using two different methodologies described in Albrecht et al. (2017b). The source of explants for tissue culture method 1 (TC1) were nucellar embryos from seeds obtained from fruit collected from foundation trees at the Bureau of Citrus Budwood Registration, Florida Department of Agriculture and Consumer Services, Chiefland, FL. Disinfected embryos were placed into clear polypropylene 473-ml deli containers containing Murashige and Skoog (MS) agar-nutrient medium (Murashige and Skoog, 1962) without added growth regulators. Embryos were dissected after pregermination of seeds and identified as nucellar based on leaf morphology and uniformity of growth of the regenerated plant. Multiple shoot clusters were produced by alternating between media containing MS medium with 1.0 mg/L benzyladenine (BA), 0.5 mg/L kinetin, and 0.5 mg/L naphthalene acetic acid (NAA) (Bowman et al., 1997), and MS medium or EXS-III basal medium (PhytoTechnology Laboratories, Lenexa, KS) with no added growth regulators. Clusters were divided and placed in new media on a cycle of about 5 weeks. Elongated shoots suitable for rooting were produced by serial transfers on hormone-free medium. Single shoots were obtained by removing sections

with at least four nodes and placing them on a MS basal medium containing 2.0 mg/L NAA and 1 g/L active charcoal. After rooting for a period of about 6 weeks, plantlets were removed from the medium, roots were trimmed to a 3–6 cm length, and placed into 3.8-cm × 21-cm cone cells containing the same potting medium described for seedlings. Plants were kept in high humidity in a plant growth chamber (EGC Model M36, Environmental Growth Chambers, Chagrin Falls, OH) with a 16-h light (200 μmol·m⁻²·s⁻¹)/8-h darkness photoperiod. Humidity was gradually reduced over 3–4 weeks to allow plants to acclimate to ambient greenhouse conditions. Plants were then transferred to the greenhouse and maintained as described for seedlings.

Explants for tissue culture method 2 (TC2) were buds from young apical shoots from certified disease-free budwood from the Bureau of Citrus Budwood Registration citrus germplasm collection in Chiefland, FL. Disinfected buds were cultured in test tubes containing a MS-based agar nutrient medium (Agromillora, composition proprietary). Cultures were maintained in a growth room under controlled conditions at a temperature of 24 to 26 °C and a 16-h light/8-h darkness photoperiod. Buds were transferred to jars and subcultured every 2 weeks to fresh nutrient medium. After several cycles, depending on the number of plants to be produced, explants were transferred to new cultures in which the shoot elongation was promoted. In these cultures, plantlets were maintained in the growth room under the same conditions as described above for 8–10 d until they reached a height of 6–8 cm. Elongated plants were individualized by cutting off the stem at the base, planted in 3.8- × 4.4-cm paper pots (Ellepots) containing a mix of peat (Pelemix, Las Salinas, Spain) and coconut fiber (Klasmann-Deilmann, Geeste, Germany), and transferred to misting tunnels inside the greenhouse for acclimatization. After 2–3 weeks, rooted plants were moved to growth benches, grown until 18–20 cm in height (8–10 weeks), and then moved to the USHRL greenhouses in Fort Pierce, FL, where they were transplanted into cone cells as described above.

Grafted nursery plants

Rootstock liners were divided into two sets. One set remained in the USHRL greenhouse and the other set was transported to a commercial citrus nursery. Liners that remained at the USHRL greenhouse were transplanted into 15.2-cm × 15.2-cm × 30.5-cm plastic tree pots (Stuewe & Sons) containing Pro Mix BX potting medium. Plants were arranged in a randomized design with rootstock and propagation method as fixed effects and each replication consisting of 9 plants. When stem diameters were of a suitable size (5–6 mm), liners were budded with certified disease-free 'Valencia' (*C. sinensis* L.) budwood using the inverted T method (Albrecht et al., 2017b). Plants were irrigated as needed using a handheld sprayer and

fertilized biweekly using a water-soluble fertilizer (20N–10P–20K; Peters Professional, The Scotts Company) at a rate of 400 mg N/L, and insecticides were applied as needed.

In the commercial citrus nursery, rootstock liners were transplanted into 10.2-cm × 10.2-cm × 34.3-cm plastic tree pots (Stuewe & Sons) containing a coir-based potting medium (Pelemix, Kiryat Gat, Israel). When liners reached a suitable size (5–6 mm stem diameter) they were budded with certified disease-free ‘Valencia’ budwood using the inverted T method. Plants were arranged in the nursery in a design favored by the nursery owner that is optimized for rapid nursery production. Arrangement was by rootstock cultivar, with each block of rootstock containing all propagation methods. Plants were maintained in the greenhouse under conditions typical for the commercial nursery using an automated drip irrigation system (Netafim, Tel Aviv, Israel). Plants were fertigated daily using 20N–10P–20K with micronutrients (Totalgro, SDT Industries, Winnsboro, LA) at a rate of 150 ppm N. Plants were grown until ready for field planting, which was ≈6 months after budding.

Field-grown trees

Field-ready grafted trees were planted in a commercial citrus orchard near Felda, Hendry County, FL (lat. 26°36′33.3″ N, long. 81°26′42.1″ W). The soil type at this location is a sandy spodosol with little organic matter, low cation exchange capacity, poor drainage, and low water holding capacity (Mylavarapu et al., 2016). Trees were planted in six single rows on raised 6.5-m wide beds, separated by 6.5-m wide furrows, with a spacing of 2.4 m between trees and 1–2 border trees at the end of each row. Trees were planted in a split plot design with rootstock as the main plot and propagation method as the subplot and six replications (rows). Each subplot consisted of three trees. Tree management followed grower standards and included regular root drenches with neonicotinoids and foliar sprays of other insecticides as needed. Irrigation was by under-tree microjets. Trees were assessed 1 year after planting.

Plant assessments

Young grafted nursery trees. Plants maintained in the USHRL greenhouses were evaluated for sprout formation and bud survival during the first 12 weeks after budding (WAB). Sprout formation was expressed as the number of rootstock sprouts per plant and bud survival was expressed in percent. In addition, scion height and stem diameters were measured at 12 WAB and at 22 WAB, respectively.

Field-ready nursery trees. A subset of field-ready grafted trees from the commercial nursery was dissected to assess the plant biomass distribution and root architectures. Six trees of each scion/rootstock combination were used. Plants were separated into leaves, stems, and roots. Leaves were counted, scanned with a Cannon MG3620 scanner,

and leaf area was determined using Assess 2.0 (Lakhdar Lamari, American Phytopathological Society) image analysis software. Leaves were dried in an oven at 53 °C to a constant weight to determine leaf dry weight and calculate the specific leaf area (SLA). SLA was calculated as the ratio of leaf area (cm²) to leaf dry weight (g). Stem diameters were measured 2.5 cm above and below the graft union, and trees were separated at the graft union. Roots were removed from the potting medium and washed with tap water. The number of primary roots, defined as roots directly arising from the root crown (including tap root and adventitious roots), were counted, and root diameters were measured at their point of origin on the root trunk. Roots were separated into large-diameter roots (>0.5 cm in diameter), medium-size diameter roots (0.2–0.5 cm), and fibrous roots (<0.2 cm in diameter). A subset of fibrous roots was scanned, and root length was determined using Assess 2.0 software. Roots and stems were dried as described for leaves to determine stem and root dry weights and calculate the specific root length (SRL). Leaf, stem, and root biomasses were expressed as percentage of the total plant biomass. Shoot-to-root ratio was determined by dividing combined the dry weights of stems and leaves by the dry weight of roots. Biomasses of the different root classes (large, medium, and fibrous) were expressed as percentage of the total root biomass. The SRL was calculated as the ratio of length (m) to dry weight of fibrous roots (g).

Field-grown trees. Trees were assessed 1 year after field planting. Trunk diameters were measured at 5 cm above and below the graft union using a digital caliper. Tree height was determined from the soil level to the top of the canopy using a digital measuring pole (Sokkia, Kanagawa, Japan), excluding any erratic shoots. Canopy spread was determined as the average of canopy width across and parallel to the row. HLB foliar disease symptoms were assessed by visual ratings on a scale of 1 to 5, with 1 = no foliar disease symptoms, 2 = foliar symptoms on less than 25% of leaves, 3 = 25% to 50% of leaves with symptoms, 4 = 50% to 75% of leaves with symptoms, 5 = more than 75% of leaves with symptoms. Canopy thickness and canopy color were also rated on a scale of 1 to 5, with a rating of 1 representing the thinnest and most unhealthy (yellow) canopy and a rating of 5 representing a very thick and healthy/green canopy. Two ratings per tree were conducted on two opposite sides parallel to the row, and ratings were expressed as the average.

Statistical analysis

Analysis of variance (ANOVA) was conducted using TIBCO Statistica v13.3 (TIBCO Software Inc., Palo Alto, CA), and main effect means were separated using Tukey’s honestly significant difference post hoc test. For young grafted nursery-grown trees, factorial ANOVA was employed with rootstock and propagation method as fixed effects. For

field-ready nursery plants, factorial ANOVA was employed for all variables expressed as relative values (biomass ratios, specific leaf area, specific root length, etc.). For absolute measures (total biomass, leaf area, scion and rootstock trunk diameters, and primary root diameter) analysis was by one-way ANOVA across all rootstock cultivars. For field trials, factorial ANOVA was employed with rootstock and propagation method as fixed effects, and block included as a random factor. Means were separated by Tukey’s honestly significant difference test. Differences were defined as statistically significant when the *P* value was <0.05.

Results

Young grafted nursery trees. The percentage of live buds measured 12 weeks after budding was similar among rootstocks propagated by seed, cutting, and tissue culture (85.4% to 94.4%), and there was a significant interaction among propagation methods and rootstock (Table 1). ‘US-942’ propagated by TC1 had the lowest percentage (52.8%) of live buds compared with most of the other rootstocks.

The number of rootstock sprouts measured during the first 12 weeks after budding varied significantly among propagation methods and rootstocks. Rootstocks propagated by TC2 had the largest number of sprouts (4.53) compared with the other propagation methods. Among the different rootstock cultivars, the largest number of sprouts were produced on ‘US-812’ and ‘US-942’ (3.69 and 3.82) and the lowest on ‘US-802’ (2.65).

A significant interaction was found among propagation methods and rootstock cultivar for the scion height. Scions grafted on ‘US-942’ propagated by seed were largest (19.5 cm), whereas scions grafted on ‘US-802’ produced by TC2 were smallest (6.4 cm). A significant interaction among propagation methods and rootstock cultivar was also found for scion diameters. Scion diameters were largest in scions grafted on ‘US-942’ and ‘US-812’ (5.36 mm and 4.95 mm) produced by seed, and on ‘US-942’ produced by TC1 (5.07 mm), and smallest in scions on ‘US-802’ produced by TC2 (2.48 mm).

Field-ready nursery trees. Because of the commercial nursery requirements, plants were arranged by rootstock cultivar on the greenhouse benches; absolute measures (total plant biomass, leaf area, scion and rootstock trunk diameters, and primary root diameter) were therefore only compared among propagation methods, and not among rootstock cultivars. The total biomass of plants was 52.3 g on average and did not vary significantly (*P* = 0.1950) among propagation methods (data not shown). The same was observed for the leaf area, which was 2179 cm² on average, with no significant differences (*P* = 0.1234) among propagation methods (data not shown). No significant differences among propagation methods were observed for scion trunk diameters

Table 1. Bud survival, rootstock sprouts, and other traits of young nursery-grown ‘Valencia’ trees grafted on different rootstocks propagated by seed (SD), cuttings (CT), and tissue culture (TC).

Factor	% Buds alive 12 WAB	No. of RS sprouts 0–12 WAB	SC ht 12 WAB (cm)	SC stem diam 22 WAB (mm)
Propagation method				
SD	92.4	2.94 b	17.1 a	4.83 a
CT	87.4	3.12 b	13.5 b	3.88 bc
TC1	85.4	2.60 b	13.1 b	4.25 b
TC2	94.4	4.53 a	13.0 b	3.79 c
	$P = 0.2002$	$P < 0.0001$	$P = 0.0021$	$P < 0.0001$
Rootstock				
US-802	97.9 a	2.65 b	10.8 b	3.26 c
US-812	89.6 ab	3.82 a	13.0 b	4.15 b
US-897	93.7 a	3.08 ab	16.3 a	4.48 b
US-942	77.8 b	3.69 a	16.8 a	4.93 a
	$P = 0.0012$	$P = 0.0013$	$P < 0.0001$	$P < 0.0001$
Propagation method × rootstock				
SD × US-802	100.0 a	1.5	18.0 ab	4.40 abc
SD × US-812	80.6 ab	3.28	15.7 abc	4.95 a
SD × US-897	100.0 a	3.03	15.3 abc	4.58 abc
SD × US-942	88.9 a	3.94	19.5 a	5.36 a
CT × US-802	100.0 a	2.53	9.8 bcd	3.17 def
CT × US-812	86.1 ab	3.64	14.0 abcd	3.70 bcde
CT × US-897	80.6 ab	3.03	16.1 abc	4.44 abc
CT × US-942	81.5 ab	3.33	14.3 abcd	4.34 abcd
TC1 × US-802	97.2 a	2.47	9.1 cd	2.99 ef
TC1 × US-812	94.4 a	3.28	12.0 abcd	4.38 abc
TC1 × US-897	97.2 a	2.36	15.6 abc	4.58 abc
TC1 × US-942	52.8 b	2.31	15.6 abc	5.07 a
TC2 × US-802	94.4 a	4.08	6.4 d	2.48 f
TC2 × US-812	97.2 a	5.08	10.4 bcd	3.57 cdef
TC2 × US-897	97.2 a	3.89	18.1 ab	4.31 abcd
TC2 × US-942	88.9 a	5.08	17.0 abc	4.82 ab
	$P = 0.0133$	$P = 0.2744$	$P = 0.0168$	$P = 0.0096$

Different letters within columns indicate significant differences according to Tukey’s honestly significant difference test. WAB = weeks after budding; RS = rootstock; SC = scion.

($P = 0.9152$), and rootstock trunk diameters ($P = 0.8166$), which were 8.6 mm and 12.0 mm, respectively, on average (data not shown).

The scion-to-rootstock trunk diameter ratio of the field-ready grafted trees was not influenced by the propagation method, but by the combination of rootstock cultivar and propagation method (Table 2). Among the plants with the highest scion-to-rootstock trunk diameter ratio (0.76–0.78) were plants grafted on ‘US-942’—regardless of the propagation method. The lowest ratios (0.63–0.65) were measured for plants grafted on ‘US-802’.

The scion-to-rootstock biomass ratio varied significantly among propagation methods. Plants on rootstocks propagated by cuttings and TC2 had the highest ratio (3.23 and 3.03), and plants on seed-propagated rootstocks had the lowest ratio (2.60). Significant differences were also measured among rootstock cultivars, with plants on ‘US-942’ having the highest (3.24) and plants on ‘US-802’ having the lowest (2.65) ratio.

The specific leaf area of the grafted plants ranged from 150.7 to 169.8 cm²/g, but no significant differences were measured among rootstock propagation methods and rootstock cultivars. The percentage of leaf biomass did not vary among rootstock propagation methods. However, significant differences were found among rootstock cultivars. The highest percentage of leaves was produced by trees on ‘US-942’ and ‘US-897’ (27.2% and 26.5%), and the lowest on ‘US-802’ (23.1%).

The percentage of stem biomass and root biomass varied significantly among rootstock propagation methods. Trees on rootstocks propagated by cuttings and TC2 had a higher proportion (51.8% and 50.6%) of stem tissue than trees on seed- and TC1-propagated rootstocks (46.3 and 48% respectively). Trees on seed-propagated rootstocks had a larger proportion of roots (27.7%) than trees on cuttings- and TC2-propagated rootstocks (23.5% and 24.6%). The root mass fraction also differed among rootstock cultivars, with the largest fraction found for ‘US-802’ (27.2%) and the lowest for ‘US-942’ and ‘US-897’ (23.5% and 24.5%).

Rootstock propagation method significantly influenced the number of primary roots and ranged from 1.4 for seed-rootstock to 4.9 for cuttings-propagated rootstocks (Table 3). No significant differences were observed among rootstock cultivars, although there was a trend ($P = 0.0651$) for ‘US-942’ producing the largest number (3.6) of roots. Rootstock propagation method also significantly ($P = 0.0001$) affected the diameter of the primary roots. Primary roots of seed- and TC1-propagated rootstock had the largest diameter (12.3 mm and 10.9 mm), and cuttings-propagated rootstocks had the lowest diameter (6.3 mm) (data not shown). Typical root systems of field-ready trees produced by seed, cuttings, and tissue culture are presented in Fig. 1.

Root size distribution was significantly affected by the propagation method but not by the rootstock cultivar. Seed and

TC1-propagated rootstocks had a larger proportion (55.8% and 54.4%) of large roots than cuttings- and TC2-propagated rootstocks (32.4% and 42.8%). The reverse was observed for the proportion of medium-size roots, which was largest in cuttings- and TC2-propagated rootstocks (40.3% and 34.2%) and lowest in seed- and TC2-propagated rootstocks (20.6% and 21.9%). A significant interaction between propagation method and rootstock cultivar was found for the proportion of fibrous roots. Most of the cuttings-propagated rootstocks were among those with the largest proportion (26.6% to 29.1%) of fibrous roots. The lowest proportion (14.2%) was found for ‘US-802’ when propagated by TC2.

The specific root length (SRL) of fibrous roots was not significantly influenced by the rootstock propagation method but by the rootstock cultivar. SRL was higher for ‘US-897’ and ‘US-942’ (26.1 and 24.0 m/g) than for ‘US-802’ and ‘US-812’ (20.1 and 20.9 m/g).

Field-grown trees. Tree survival was 100% for trees grafted on seed-, cuttings-, and TC1-propagated rootstocks and 94.4% for trees grafted on TC2-propagated rootstocks. The average tree height of trees after 1 year of growth in a commercial citrus orchard was significantly influenced by the combination of propagation method and rootstock cultivar (Table 4). ‘US-802’ propagated by TC2 induced the largest tree size (113.8 cm); and ‘US-897’ propagated by SD, TC1 and TC2, and ‘US-942’ propagated by cuttings induced the smallest tree size.

Table 2. Biomass distribution of leaves, stem, roots, and other plant traits of field-ready, nursery-grown ‘Valencia’ trees grafted on different rootstocks propagated by seed (SD), cuttings (CT), and tissue culture (TC).

Factor	SC/RS trunk diam ratio	SC/RS biomass ratio	SLA (cm ² /g)	Leaf mass (%)	Stem mass (%)	Root mass (%)
Propagation method						
SD	0.71	2.60 b	163.4	26.0	46.3 b	27.7 a
CT	0.72	3.23 a	157.8	24.7	51.8 a	23.5 b
TC1	0.71	2.84 ab	165.2	26.2	48.0 b	25.8 ab
TC2	0.71	3.03 a	160.9	24.9	50.6 a	24.6 b
	<i>P</i> = 0.4860	<i>P</i> = 0.0009	<i>P</i> = 0.1866	<i>P</i> = 0.3850	<i>P</i> < 0.0001	<i>P</i> = 0.0006
Rootstock						
US-802	0.64 c	2.65 c	159.1	23.1 b	49.6	27.2 a
US-812	0.75 ab	2.78 bc	161.3	25.0 ab	48.7	26.3 ab
US-897	0.72 b	3.04 ab	161.6	26.5 a	49.0	24.5 bc
US-942	0.76 a	3.24 a	165.3	27.2 a	49.4	23.5 c
	<i>P</i> < 0.0001	<i>P</i> = 0.0012	<i>P</i> = 0.3862	<i>P</i> = 0.0015	<i>P</i> = 0.7760	<i>P</i> = 0.0011
Propagation method × rootstock						
SD × US-802	0.64 c	2.56	165.7	23.6	48.4	28.0
SD × US-812	0.74 a	2.42	159.5	26.9	43.9	29.2
SD × US-897	0.71 a	2.55	160.0	25.8	46.1	28.1
SD × US-942	0.78 a	2.94	168.5	27.8	46.9	25.3
CT × US-802	0.63 c	2.93	150.7	22.3	52.3	25.4
CT × US-812	0.75 a	2.67	159.7	21.0	51.8	27.2
CT × US-897	0.76 a	3.79	156.4	27.6	51.6	20.9
CT × US-942	0.76 a	3.85	164.4	28.1	51.4	20.6
TC1 × US-802	0.65 bc	2.51	164.3	25.9	46.1	28.0
TC1 × US-812	0.71 ab	2.84	162.6	26.6	47.5	25.9
TC1 × US-897	0.71 ab	2.99	169.8	25.5	49.6	24.9
TC1 × US-942	0.78 a	3.08	163.9	26.8	48.8	24.4
TC2 × US-802	0.63 c	2.62	155.8	20.7	51.7	27.6
TC2 × US-812	0.78 a	3.31	163.3	25.4	51.6	23.0
TC2 × US-897	0.71 ab	3.09	160.2	27.2	48.7	24.0
TC2 × US-942	0.74 a	3.22	164.2	26.1	50.3	23.6
	<i>P</i> = 0.0104	<i>P</i> = 0.2161	<i>P</i> = 0.7219	<i>P</i> = 0.0991	<i>P</i> = 0.2515	<i>P</i> = 0.1942

Different letters within columns indicate significant differences according to Tukey’s honestly significant difference test. SC = scion; RS = rootstock; SLA = specific leaf area.

Table 3. Biomass distribution of large, medium, and fibrous roots, and other root traits of field-ready, nursery-grown ‘Valencia’ trees grafted on different rootstocks propagated by seed (SD), cuttings (CT), and tissue culture (TC).

Factor	Number of primary roots	Large size root mass (%)	Medium size root mass (%)	Fibrous root mass (%)	SRL (m/g)
Propagation method					
SD	1.4 d	55.8 a	20.6 b	23.6	24.1
CT	4.9 a	32.4 b	40.3 a	27.3	22.3
TC1	2.3 c	54.4 a	21.9 b	23.7	22.3
TC2	3.6 b	42.8 b	34.2 a	23.1	22.4
	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> = 0.0616	<i>P</i> = 0.0903
Rootstock					
US-802	3.0	0.493	0.282	0.225	20.1 b
US-812	2.8	0.435	0.323	0.242	20.9 b
US-897	2.7	0.487	0.272	0.242	26.1 a
US-942	3.6	0.439	0.293	0.268	24.0 a
	<i>P</i> = 0.0651	<i>P</i> = 0.3502	<i>P</i> = 0.6398	<i>P</i> = 0.1055	<i>P</i> < 0.0001
Propagation method × rootstock					
SD × US-802	1.5	49.2	27.1	23.7 ab	20.5
SD × US-812	1.8	54.4	22.0	23.6 ab	23.4
SD × US-897	1.0	60.9	18.8	20.3 ab	27.1
SD × US-942	1.2	58.9	14.5	26.6 a	25.3
CT × US-802	4.8	34.8	36.3	28.9 a	19.9
CT × US-812	4.3	31.5	43.8	24.7 ab	19.3
CT × US-897	4.3	34.6	38.8	26.6 a	26.4
CT × US-942	6.0	28.7	42.2	29.1 a	23.5
TC1 × US-802	1.8	60.4	16.6	23.0 ab	21.1
TC1 × US-812	2.0	48.8	26.8	24.5 ab	20.2
TC1 × US-897	2.0	63.2	16.4	20.3 ab	23.9
TC1 × US-942	3.3	45.2	27.8	27.0 a	23.9
TC2 × US-802	3.8	52.9	32.9	14.2 b	18.8
TC2 × US-812	3.2	39.4	36.6	24.1 ab	20.5
TC2 × US-897	3.5	35.9	34.7	29.4 a	26.9
TC2 × US-942	3.8	42.8	32.6	24.6 ab	23.3
	<i>P</i> = 0.4167	<i>P</i> = 0.2714	<i>P</i> = 0.7925	<i>P</i> = 0.0130	<i>P</i> = 0.5045

Different letters within columns indicate significant differences according to Tukey’s honestly significant difference test. SRL = specific root length.

Canopy spread was significantly influenced by propagation method and rootstock cultivar. Trees on seed- and cuttings-propagated root-

stocks were wider (92.1 cm and 89.9 cm) than trees on tissue culture-propagated rootstocks (83.4–83.7 cm). Among the rootstock culti-

vars, ‘US-812’ produced had the widest canopy (90.4 cm) and ‘US-897’ the narrowest (84.2 cm).

Fig. 1. Root systems of nursery-grown, field-ready ‘Valencia’ trees grafted on ‘US-897’ rootstock propagated by seed (SD), cutting (CT), and tissue culture (TC1 and TC2). The yellow bar represents a scale of 5 cm.

Table 4. Tree height, canopy spread, and other traits of field-grown ‘Valencia’ trees grafted on different rootstocks propagated by seed (SD), cuttings (CT), and tissue culture (TC).

Factor	Tree ht (cm)	Canopy spread (cm)	RS trunk diam (mm)	SC trunk diam (mm)	SC/RS trunk diam ratio
Propagation method					
SD	104.9	92.1 a	35.1 a	24.2 ab	0.69 b
CT	105.8	89.9 a	34.3 ab	24.9 a	0.72 a
TC1	107.3	83.4 b	33.8 ab	23.3 b	0.69 b
TC2	107.1	83.7 b	33.0 b	23.7 ab	0.72 a
	$P = 0.4185$	$P < 0.0001$	$P = 0.0274$	$P = 0.0067$	$P < 0.0001$
Rootstock					
US-802	108.2 a	87.3 ab	38.0 a	23.7 bc	0.62 c
US-812	107.7 a	90.4 a	35.1 b	26.0 a	0.74 a
US-897	103.4 b	84.2 b	31.5 c	22.5 c	0.71 b
US-942	105.7 ab	87.3 ab	31.7 c	24.0 b	0.76 a
	$P = 0.0111$	$P = 0.0357$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Propagation method × rootstock					
SD × US-802	106.2 ab	93.1	39.1 a	24.2 bcde	0.62
SD × US-812	106.2 ab	95.7	36.8 ab	26.6 ab	0.72
SD × US-897	102.2 b	86.6	31.4 cd	21.3 e	0.68
SD × US-942	105.2 ab	92.8	33.2 bcd	24.9 abcd	0.75
CT × US-802	109.4 ab	89.4	39.1 a	24.5 abcde	0.63
CT × US-812	110.1 ab	95.2	35.8 abc	27.5 a	0.77
CT × US-897	100.9 b	87.6	32.4 bcd	24.3 abcde	0.75
CT × US-942	102.7 b	87.6	30.0 d	23.3 cde	0.78
TC1 × US-802	103.3 ab	80.5	35.7 abc	21.9 de	0.61
TC1 × US-812	110.8 ab	85.7	36.2 abc	25.5 abc	0.70
TC1 × US-897	108.9 ab	83.9	31.7 cd	22.2 de	0.70
TC1 × US-942	106.1 ab	83.3	31.7 cd	23.6 bcde	0.75
TC2 × US-802	113.8 a	85.9	38.3 a	24.3 abcde	0.63
TC2 × US-812	103.7 ab	84.7	31.3 cd	24.2 bcde	0.78
TC2 × US-897	101.4 b	78.6	30.5 d	22.2 de	0.73
TC2 × US-942	109.0 ab	85.4	31.7 cd	24.2 bcde	0.76
	$P = 0.0012$	$P = 0.6934$	$P = 0.0072$	$P = 0.0043$	$P = 0.0959$
Block					
	$P < 0.0001$	$P < 0.0001$	$P = 0.0007$	$P < 0.0001$	$P = 0.0003$
Block × rootstock					
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.0823$

Different letters within columns indicate significant differences according to Tukey’s honestly significant difference test. RS = rootstock; SC = scion.

Rootstock and scion trunk diameters were significantly influenced by the combination of propagation methods and rootstock. ‘US-802’ propagated by seed, cuttings, and TC2 had the largest rootstock trunk diameter (38.3–39.1 mm), and ‘US-897’ propagated by TC2 and ‘US-942’ propagated by cuttings had the smallest (30.5 and 30.0 mm). Largest scion diameters were induced by ‘US-812’ propagated by cuttings and the smallest by ‘US-897’ propagated by seed.

Both propagation method and rootstock cultivar significantly influenced the scion-to-rootstock trunk-diameter ratio. Trees with rootstocks propagated by cuttings and by TC2 had a larger ratio (0.72) than trees on rootstocks propagated by seed and by TC1 (0.69). Among rootstock cultivars, US-812 and US-942 had the largest scion-to-rootstock trunk-diameter ratio (0.74 and 0.76), and US-802 had the smallest (0.62).

The trees in this experiment were not much affected by HLB after 1 year of growth

in the orchard. HLB disease indices ranged from 1.10 to 1.24 and were neither influenced by rootstock propagation method nor by rootstock cultivar (Table 5). Canopy thickness and canopy color indices ranged from 3.13 to 3.41 and from 4.06 to 4.34, respectively, with no significant effect of propagation method or rootstock cultivar.

For all of the measured variables, block was a significant factor; and significant interactions with rootstock cultivar—but not propagation method—were observed.

Table 5. Huanglongbing (HLB) foliar disease symptoms, canopy thickness, and canopy color indices of field-grown ‘Valencia’ trees grafted on different rootstocks propagated by seed (SD), cuttings (CT), and tissue culture (TC).

Factor	Foliar HLB symptoms	Canopy thickness	Canopy color
Propagation method			
SD	1.18	3.36	4.32
CT	1.22	3.38	4.31
TC1	1.14	3.19	4.16
TC2	1.13	3.19	4.10
	$P = 0.3248$	$P = 0.3542$	$P = 0.1892$
Rootstock			
US-802	1.21	3.41	4.28
US-812	1.10	3.32	4.34
US-897	1.13	3.13	4.21
US-942	1.24	3.25	4.06
	$P = 0.4674$	$P = 0.6402$	$P = 0.4725$
Propagation method $\times$ rootstock			
	$P = 0.7170$	$P = 0.1525$	$P = 0.1368$
Block			
	$P = 0.0775$	$P = 0.3914$	$P = 0.6020$
Block $\times$ rootstock			
	$P = 0.0003$	$P = 0.0008$	$P = 0.0072$

Indices were determined by ratings on a scale of 1 to 5, with 1 being the best and 5 being the worst for foliar HLB symptoms and 1 being the worst and 5 being the best for canopy color and canopy density.

Discussion

To cope with the devastating effects of HLB in Florida, the demand for superior rootstocks has increased dramatically, exceeding the available supply of seeds to be used for propagation of some rootstocks. One of the rootstocks dramatically affected by this is ‘US-942’, for which the commercial demand for trees far exceeds the seed supply. During 2018, of the 846,608 trees propagated on ‘US-942’ in Florida, more than 82% of nursery trees made use of liners propagated by tissue culture or cuttings (FDACS records). This study presents information on the suitability and effects of alternative methods of rootstock propagation on the grafted tree growth in the nursery and during the first year of establishment in the field.

Many of the tree traits during the early weeks after grafting were influenced by the propagation method; these include the number of rootstock sprouts, the scion height, and the scion stem diameter. Although trees with seed-propagated rootstocks appeared to induce more vigorous scion growth compared with the other propagation methods, effects depended on the rootstock cultivar. This is not unexpected, as the four rootstocks used in this study are known for their differences in vigor (Bowman and Joubert, 2020; Bowman et al., 2016a, 2016b). Among the four rootstocks studied, ‘US-802’ is the highest vigor-inducing rootstock and ‘US-897’ the lowest.

Some nursery owners reported observing excessive sprouting of rootstock liners propagated by tissue culture (Nate Jameson, personal communication). More sprouting was also observed in our study, but only when liners were propagated by TC2, not by TC1. One of the differences between these two methods is the source of the plant tissue used as explant. Whereas in TC1, cultures were initiated from embryos, in TC2, they were initiated from buds of mature trees. The type or the maturity of the explant used for regeneration may have been a determining factor

for the differences in sprout formation. Although in tissue culture and in mature trees, shoot regeneration or sprouting usually diminishes with age (Bond and Migley, 2003; Yildiz, 2012), Webster (1995) reported that in temperate fruit trees, micropropagated rootstocks frequently sucker profusely because of rejuvenation. Other dissimilarities between the two tissue culture methods in our study, such as the use of plant growth hormones and time in culture, may be responsible for the observed differences in sprouting. Grant and Hammat (1999) reported that total time in culture is the most important factor causing physiological changes in micropropagated apple and cherry. Additional research is necessary to determine the differences in rootstock sprouting of micropropagated citrus and ways of effectively limiting or managing this. In addition to the propagation method, sprouting was influenced by the rootstock cultivar. A cultivar-specific influence was also observed in micropropagated and conventional-propagated apple rootstocks (Jones and Hadlow, 1989).

The biomass distribution of the field-ready nursery plants was influenced both by rootstock propagation method and by rootstock cultivar. In general, plants on seed- and TC1-propagated rootstocks were similar for most traits and different from plants on cuttings- and TC2-propagated plants. Relative root masses were larger in the former, and the reverse was found for the relative stem masses. A similar trend was observed in our previous study on young rootstock liners (Albrecht et al., 2017b), suggesting the persistence of morphological traits throughout the grafted-tree stage in the nursery. A larger root system is generally associated with a greater ability to withstand wind. This is particularly important in Florida where citrus production is threatened by tropical storms and hurricanes. Studies on apple trees found that trees on seedling-propagated rootstocks were more upright after 5 years of exposure

to westerly winds than trees on micropropagated or dwarfing rootstocks (Larsen and Higgins, 1993). Similarly, uprooting resistance of Eucalyptus trees was lower for micropropagated plants than for macropropagated plants (rooted cuttings) and seedlings after 16 months of field growth (Mokotedi et al., 2010). It must be noted that in the study on Eucalyptus, micropropagated trees had the lowest number of roots, which is different to our findings in citrus. Whether the propagation method influences uprooting resistance in field-grown citrus trees is currently being examined in our laboratory. The relative root mass was also influenced by the rootstock cultivars. In addition, rootstock influenced the relative leaf mass of the scion, with ‘US-942’ and ‘US-897’ having the largest. Both rootstocks are known for inducing production of high-quality fruit (Bowman and Joubert, 2020; McCollum and Bowman, 2017).

As previously reported for rootstock liners (Albrecht et al., 2017b), the number of primary roots of the grafted field-ready trees increased from seed- to vegetative-propagated rootstocks with the most (adventitious) roots observed for cuttings. Consequently, in seed-propagated rootstocks, the biomass fraction was larger for large-size roots and smaller for medium-size roots compared with the other propagation methods, except TC1. According to Mokotedi et al. (2010), the number of roots is a strong predictor of uprooting resistance. This would suggest a possible advantage of citrus trees on vegetative-propagated over seed-propagated rootstocks, should differences in the root number persist under field conditions.

It is surprising that TC1-propagated rootstocks were more similar in their root morphological traits to seed-propagated rootstocks than to TC2-propagated rootstocks. One possible reason for this finding is that rootstocks produced by seed and by TC1 originated from juvenile tissue (embryos), whereas rootstocks

from cuttings and TC2 originated from stems and buds, respectively. As discussed above, this suggests an influence of the type or maturity of the explant on the morphological traits of the regenerated plant. The maturity of the explants was attributed to differences in morphological traits of field-grown plants in other tree species (Gupta et al., 1991). For example, Chinese fir plants regenerated from explants of juvenile trees were vigorous, whereas plants regenerated from explants of mature trees were slow and plagiotrophic in growth (Bigot and Engelmann, 1987). It is probable that other factors involved in the propagation process, such as composition of the culture medium, transition to the potting medium, and early growth conditions, were at least partially responsible for the different traits of plants in our study. In a study on Japanese persimmon, the field performance of micro-propagated own-rooted trees was affected by the initial growing environment, supporting this notion (Tetsumura et al., 1998). This may also explain the varying reports on the effects of propagation methods in other tree crops.

Contrary to our previous study (Albrecht et al., 2017b), which focused on young rootstock liners, in this study the specific root length was not influenced by propagation method. This is explained by the fact that in the present study, the fibrous roots used to determine SRL only comprised a small portion of the root system; whereas in the previous study, roots of the young liners were mostly fibrous. The fibrous root mass fraction was not influenced by the propagation method, but by the combination of propagation method and rootstock cultivar. Because of their role in water and nutrient uptake, fibrous roots are the most important part of the root system (Anderson and Ingram, 1993). Eissenstat (1991) found that citrus rootstocks of high SRL had a faster growth rate of root proliferation and a greater rate of water extraction compared with rootstocks of lower SRL. Our findings suggest that the ability to uptake water and nutrients in a grafted citrus tree seems to be more influenced by the rootstock cultivar than by the method by which they are propagated. As previously reported (Albrecht et al., 2017b; Bowman and Albrecht, 2017), 'US-942' and 'US-897' had the largest SRL, which may contribute to their positive influence on the juice total soluble solids content.

Tree survival and establishment during the first year of field growth is important for the future success of the orchard. In our field study, only two trees did not survive the first year in a commercial citrus orchard; both had rootstocks propagated by TC2. This is a small percentage of trees and does not necessarily suggest an inferiority of this propagation method, as a small percentage of tree death is expected in any new citrus planting. Tree height after 1 year of field growth was determined by the combination of propagation method and rootstock but not by propagation method alone. Interactions were also found

for rootstock and scion trunk diameters. In contrast, rootstock propagation method influenced canopy spread and scion-to-rootstock trunk-diameter ratios, but these traits were also influenced by the rootstock cultivar. A larger influence of the genotype than the propagation method on tree growth and root morphology were also observed in Eucalyptus trees (Bell et al., 1993). Marín et al. (2003) compared peach and nectarine trees grafted on cuttings- and tissue culture-propagated rootstock and found no influence of propagation method on the field performance during the first year of growth. Seed-propagated rootstocks were not included in these studies.

The similarity of root traits between seed- and TC1-propagated rootstocks observed in the field-ready nursery trees did not transfer to aboveground traits during 1 year of growth in the field. This suggests that in the absence of seeds, vegetative propagation may provide a viable alternative for the rapid production of true-to-type rootstocks.

In conclusion, our results suggest that despite differences in the root architectures associated with the propagation method, growth of young grafted citrus trees appears to be affected more by the rootstock cultivar than by the propagation method. Whether this trend continues throughout the productive years is the subject of continuing investigations. The differences in some of the morphological traits of plants regenerated from different tissue culture methods provide an opportunity for further research on the role of the source and/or maturity of the explant in the short- and long-term growth traits of citrus trees.

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