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ENHANCING DEVELOPMENT OF IMPROVED ROOTSTOCKS BY TISSUE CULTURE PROPAGATION AND FIELD PERFORMANCE OF SELECTED ROOTSTOCKS

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Abstract. **A recently established Cooperative Research and Development Agreement between the USDA and Twyford International to develop micropropagation procedures for new hybrid rootstocks promises to significantly expand the range of germplasm that can be effectively used as rootstocks for citrus, and accelerate testing and release of promising selections. Micropropagation of rootstock cultivars makes it possible to rapidly obtain thousands of uniform plants from a few buds of source material through tissue culture, regardless of whether the original source has fruit, seed, or comes true-to-type from seed. Many citrus relatives and hybrids that previously could not be used as rootstocks because they did not grow uniformly from seed can now be rapidly and uniformly propagated. Micropropagation has other potential advantages, including promoting the rapid distribution of new cultivars and encouraging the production of healthy and high quality plants. Outstanding performance in two field trials is reported for several new hybrid rootstocks for which efficient micropropagation procedures are being developed.**

Introduction

Profitability of citrus production in Florida is limited by the rootstock. Efforts to develop improved citrus rootstocks are limited by genetic traits that are available in the usable germplasm. One important trait for citrus rootstocks that has generally been critical in determining acceptability of rootstocks for commercial use is nucellar embryony, a type of apomixis. Common commercial propagation of citrus utilizes seed propagated rootstock liners that are genetically identical to the source tree because of nucellar embryony. Species or hybrids that do not produce at least 80-90% apomictic seeds have usually been excluded from testing as citrus rootstocks, and avoided as parents in making new hybrid rootstocks because of this reliance on seed propagation. Even when selections that are not highly apomictic have been tested as rootstocks, variation in tree performance due to genetic variability in the seedling liners has been a serious negative trait.

Advances in methods for vegetative propagation of citrus rootstocks through tissue culture (micropropagation) have now made it possible to economically produce large numbers of genetically identical plants from rootstock selections regardless of the natural mode of reproduction. After a short acclimation period in soil, these plants from tissue culture can be budded and grown as though they were seedling rootstocks. Although some types of tissue culture manipulations can produce genetic changes, selection of proper conditions for micropropagation can maintain genetic mutations at levels that are comparable to those observed in rootstock seedlings and scion budwood of common commercial cultivars.

Several new rootstocks that are currently under advanced testing by the USDA appear very promising for commercial use. Most of these new rootstocks can be effectively seed propagated. However, micropropagation may be useful in propagating these rootstocks because plants in culture can easily be maintained free of disease, multiplication is unaffected by seasonal variations, and the system facilitates rapid increase and distribution of new cultivars even when sufficient seed is not available. Also, there are other new rootstocks under development that are difficult or impossible to propagate by seed and are even more suitable for micropropagation.

Diversity of Rootstock Germplasm

The rootstocks currently used in Florida citrus production are not genetically diverse. Nearly all rootstocks in commercial use are composed of genetic material from six species (Table 1). About 90% of Florida citrus trees are growing on rootstocks that are mandarin (*Citrus reticulata* Blanco) or hybrids between trifoliate orange (*Poncirus trifoliata* [L.] Raf.) and sweet orange (*C. sinensis* [L.] Osbeck) or grapefruit (*C. paradisi* Macf.). The number of sex-

Table 1. Genetic diversity represented in Florida citrus rootstocks.

Species	Common name	Proportion of germplasm[1]	Nucellar Embryony
Citrus aurantium	sour orange	6	yes
C. limon	lemon	4	yes
C. paradisi	grapefruit	26	yes
C. reticulata	mandarin	14	some
C. sinensis	sweet orange	12	yes
Poncirus trifoliata	trifoliate orange	38	yes
Citrus aurantifolia	lime	0	yes
C. grandis	pummelo	0	no
C. indica	Indian wild orange	0	no
C. medica	citron	0	no
C. tachibana	tachibana orange	0	yes
C. ichangensis		0	no
C. hystrix		0	no
C. macroptera		0	no
C. micrantha		0	no
C. celebica		0	no
C. latipes		0	no
Microcitrus (6 species)		0	no
Eremocitrus glauca		0	no
Fortunella (4 species)		0	no
Clymenia (1 species)		0	yes?

[1]Proportion of this germplasm in rootstocks of new citrus trees (%), calculated from trees propagated in last 7 years according to FL, DPI Annual Report; Hybrid rootstocks are counted as H each parental type.

ually compatible species within *Citrus* can be debated, with numbers of officially named species exceeding 150 (Swingle and Reece, 1967). There are five genera (*Poncirus, Eremocitrus, Microcitrus, Fortunella*, and *Clymenia*) that can be sexually hybridized with *Citrus* and yield healthy plants. With the exception of hybrids with *Poncirus*, these types of hybrids have largely been untested as rootstocks. There is tremendous genetic variation and many potentially useful traits within the citrus group that have not yet been adequately evaluated or used in producing new rootstock hybrids. Many of these sources of useful traits have not been used because of the difficulty in propagating uniform trees for testing and the probability that they would never be used commercially because they do not come true to type from seed. The integration of commercial citrus micropropagation with rootstock cultivar development will allow the expansion of rootstock breeding into previously untapped resources and facilitate testing and release of superior new rootstocks.

Cooperative Research and Development Agreement

A recently established Cooperative Research and Development Agreement (CRADA) between the USDA-ARS Rootstock Breeding Program and Twyford International will facilitate the joint development of cultivar specific methods for micropropagation of superior new rootstocks. These propagation methods will enable the commercial use of new citrus rootstock selections that are not efficiently reproduced true-to-type from seed, thus expanding the germplasm base and potential for developing improved rootstocks. During the initial phase of the CRADA, the project will primarily focus on shortening the time needed to prepare a promising new seed-propagated rootstock (such as those listed in Tables 2 and 3) for release to the industry, promoting the rapid propagation and distribution of newly released rootstock cultivars, and encouraging the production of healthy disease-free plants. During the second phase, the focus of the cooperative project will shift to rootstocks with superior traits that cannot be seed propagated and are dependent on micropropagation for commercial use.

Table 2. St. Cloud Rootstock Trial for 'Hamlin.'

Rootstock	Parentage	Totals (7 years) Boxes/ tree	Lbs. solids/ box	Lbs. solids/ tree	Tree height (% Swingle)
HRS-802	Siamese × Trif. Or.	43.9	4.0	176	130
HRS-852	Changsha × Trif. Or.	40.3	4.3	173	90
Swingle	Duncan × Trif. Or.	32.8	4.3	141	100
HRS-801	Changsha × Trif. Or.	32.1	3.9	125	90
HRS-896	Cleopatra × Trif. Or.	28.6	4.5	129	80
HRS-897	Cleopatra × Trif. Or.	24.2	4.2	102	70

Commercial Micropropagation

Citrus can be propagated by tissue culture (Kobayashi, 1987; Murashige and Tucker, 1969; Singh et al., 1994), but there has been little commercial use of micropropagation for rootstocks because nearly all cultivars that have been shown to possess good rootstock characteristics can be easily and inexpensively propagated by nucellar apomictic seed. In the past, micropropagation has been somewhat more expensive than seed propagation for cultivars that are uniformly propagated by seed. However, recent advances in micropropagation methods for citrus have made prices for micropropagated liners more competitive with seedling liners.

Twyford International has been propagating citrus rootstocks for commercial use for several years. To date, several thousand micropropagated liners have been produced by Twyford, budded with commercial scions, and planted into the field, mostly from Sun Chu Sha mandarin, Benton citrange, and citrumelos F80-5, F80-7, and F80-18. Procedures for micropropagation of other rootstocks, including new superior USDA rootstock hybrids, are being developed. The micropropagation system used by Twyford can be summarized as follows:

Stage 1—Initiation of cultures. Plant material is moved into tissue culture by treating seeds or softwood node cuttings with sterilant chemicals such as sodium hypochlorite. Node cuttings from ex situ or seedlings produced in culture are then placed on a medium consisting of Murashige and Skoog basal salts (Murashige and Skoog, 1962) with 1.0 mg/l benzyladenine (BA), 0.5 mg/l kinetin,

Table 3. Lynchburg rootstock trial for 'Valencia.'

Rootstock	Parentage	Lbs. solids per tree 1995	1996	1997	Total	Tree height (% Swingle)
HRS-812	Sunki × Trif. Or.	4.7	6.9	12.6	24.2	115
HRS-942	Sunki × Trif. Or.	4.9	7.0	11.9	23.8	100
Vangasay	Lemon	5.4	4.6	13.7	23.7	130
HRS-849	SFS × Trif. Or.	4.6	5.2	9.9	19.7	95
HRS-827	Rangpur × Trif. Or.	3.7	4.2	11.2	19.1	115
HRS-952	Pearl × Trif. Or.	4.6	6.3	8.1	19.0	100
Swingle	Duncan × Trif. Or.	3.7	4.4	10.2	18.3	100
Carrizo	Navel × Trif. Or.	3.0	2.9	9.1	15.0	105
Sour #2	Sour orange	0.9	0.4	2.5	3.8	60

and 0.5 mg/l 1-naphthaleneacetic acid (NAA), with agar as a solidifying agent (Singh et al., 1994). Plant material is transferred periodically to fresh media with a cycle time of about 5 weeks. It takes about 6 to 8 months to develop uniform multiplying cultures from new plant material.

Stage 2—Multiplication of cultures. Multiple shoot clusters are produced by alternating between media containing: 1) Murashige and Skoog basal salts with 1.0 mg/l BA, 0.5 mg/l kinetin, and

0.5 mg/l NAA, and 2) Half strength Murashige and Skoog (H macro elements) basal salts with 0.8 mg/l BA and 2.0 mg/l indole acetic acid. Multiple shoot clusters are divided and placed in new media on a cycle of about 5 weeks.

Stage 3—Rooting of shoots. Single shoots are harvested from the multiple shoot clusters at the time of cluster division and sections with two to three nodes are placed onto media containing Murashige and Skoog basal salts with 2.0 mg/l NAA and GelRite as the solidifying agent. The rooting cycle lasts about 6 weeks.

Stage 4—Transfer plants to soil. Rooted shoots are removed from media and placed into soilless potting mix (containing peat moss, perlite, and vermiculite) under high humidity conditions. Humidity is gradually reduced until plants are adapted to ambient greenhouse conditions. Handling of micropropagated plants is similar to that of seedling liners after this point.

As mentioned previously, micropropagation has several advantages in comparison to conventional seedling propagation of citrus rootstocks. Micropropagation of citrus rootstocks also has some disadvantages in comparison with seed propagation. First, despite improvements in the system, micropropagation will probably remain at least slightly more expensive. Second, off-type plants can be obtained from culture. The frequency of off-type plants produced from micropropagated citrus is greatly influenced by the source of plant material (genetic uniformity of the source material) and the particular methods employed to multiply the plants in culture. Careful selection of uniform source material and the use of methods for multiplication that minimize mutations can produce plants from culture that appear no more variable in traits than seedling liners of common commercial rootstocks. Third, micropropagation is better suited to uniform year-round production than to seasonal peak production. The fourth difference to be noted in the micropropagation system may, in fact, be an advantage or disadvantage depending on the situation. The root system of micropropagated plants generally does not develop a taproot like seedlings during the early stages of growth, but instead has a more spreading root system. It is not yet known how the root structure of mature trees on micropropagated rootstocks will compare with that of trees on seedling rootstocks of the same cultivar. The effect of a more spreading root system on tree performance in the field will require further study (Castle, 1977). Generally it is thought that a deep taproot is advantageous on a deep well-drained soil, while a more spreading root system is preferred for a shallower flatwood type soil.

New rootstocks being developed

Advanced rootstock selections with superior field performance are currently under development by USDA. Many of these rootstocks can be economically and uniformly propagated by seed, and for these selections, micropropagation would probably only be advantageous during the first few years after release when seed may be in short supply or not available. Other rootstocks under development cannot be propagated uniformly by seed and would require micropropagation for commercial use. The integration of micropropagation with the rootstock breeding program is resulting in a broader germplasm base being entered into field testing. Eventually, the expansion of the germplasm base will yield more good rootstock choices and increase the potential for profits to Florida citrus growers. Partial information from two field trials is described here to give an indication of the potential performance from new hybrid rootstocks. Micropropagated plants for experimental use are now being produced from most of the new rootstocks listed in these trials. However, all of the rootstocks listed, except HRS-852, can be effectively seed propagated and micropropagation would probably only be advantageous when sufficient seed was not available.

The first rootstock trial is a cooperative planting with Mr. Orie Lee in Osceola County. The trees in this block were planted in December 1986 with Hamlin 8-1-5 XE scion at a spacing of 13.5 ft × 22 ft. Fifteen different rootstocks were originally included in the trial, although several were replaced during the course of the testing because of poor performance. Trees were arranged in a randomized complete block design with six replicates of three trees for each rootstock. More details of the experiment and results for the first 4 years from this trial were described previously (Wutscher and Hill, 1995). A summary of production from trees on Swingle citrumelo, and new hybrids in this block through 10 years of age shows that at least five new hybrids have exciting potential (Table 2). In total production over the 7 year period, trees on the hybrid rootstocks HRS-802 and HRS-852 have yielded about 25% more pounds-solids per tree than those on Swingle citrumelo. This superior production has been consistent over the 7 years that yield was measured (data not shown). HRS-802 is vigorous and trees on this rootstock are considerably larger than trees on Swingle citrumelo. In contrast, trees on HRS-852 are lower in height than trees on Swingle. Trees on three other hybrids, HRS-801, HRS-896, and HRS-897, were less productive than those on Swingle, on a per tree basis, but may be useful in high density plantings because the trees are small and much of the fruit can be harvested from the ground. All five of these new hybrid rootstocks have been observed to be relatively cold tolerant, resistant to phytophthora, and tolerant of tristeza. None of the trees on these five new rootstocks have developed blight through 11 years of age, unlike trees on other rootstocks in the block.

The second trial is on the property of Mr. Patrick T. Bentley in Polk County. This block was planted in May 1991 with a Valencia scion at a spacing of 8 ft. × 18 ft. Twenty-one different rootstocks were used in this trial, including several standard cultivars and thirteen new hybrids. Trees were arranged in a randomized complete block design with six replicates of four trees for each rootstock. Preliminary results from this trial indicate that two new hybrids are yielding better than any commercially available rootstock and three other new hybrids are yielding better than any commercial rootstock except lemon (Table 3). Trees on the new hybrids HRS-812 and HRS-942 have yielded at least 30% greater pounds solids per tree than on Swingle or Carrizo over the three year period 1995-97. Tree performance will need to be monitored for several more years to fully assess productivity, tree size, and tolerance of cold and disease. The poor performance of trees on sour orange probably indicates the presence of a severe tristeza isolate in the block.

Germplasm used in citrus rootstocks for Florida is too limited. Incorporation of greater diversity in good rootstocks has potential to provide new superior rootstocks with good adaption to problem situations and enhance the opportunities for profit to Florida citrus growers. Cooperative research in micropropagation of new rootstocks promises to expand the range of rootstock genetic diversity that can be tested and commercially used by the Florida citrus industry. A commercial-scale micropropagation system is being developed for several superior new rootstocks under test by USDA.

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DEVELOPMENT OF IMPROVED SWEET ORANGE CULTIVARS USING TISSUE CULTURE METHODS

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Abstract. **Sweet orange improvement by conventional breeding techniques has been inhibited by the complex biology of sweet orange, e.g. large plant size, extended juvenility, and the presence of nucellar embryos in the seed. Common sweet orange cultivars widely grown today probably originated as selections from chance seedlings or from naturally occurring mutations. Therefore, we are pursuing alternative methods to improve sweet orange, and one such method is to take advantage of a phenomenon called somaclonal variation, which is defined as genetic variation that is either uncovered or induced by a tissue culture process. This approach to cultivar improvement is attractive for sweet orange because of potential to identify superior clones with positive genetic changes while maintaining sweet orange integrity. Targeted traits for improvement include altered maturity dates, increased soluble solids, and improved color. We are currently evaluating approximately 2000 trees of 'Hamlin' and 'Valencia' sweet orange clones in the field, including the following four populations of each variety: organogenic (regenerated via adventitious shoot buds); embryogenic (regenerated from secondary embryogenic callus via somatic embryogenesis); protoplast-derived (regenerated via somatic embryogenesis); and nucellar seedlings as a control. Significant stable variation has been observed for the following general tree characteristics: canopy size/shape; leaf size/shape; ploidy level; and juvenility/thorniness/vigor. Fruit characteristics showing significant variation include brix, acid, ratio, color (fruit/juice), maturity date, size, rind thickness, and juice content. Of particular interest are clones of 'Hamlin' showing improved color, and clones of 'Valencia' showing significantly earlier maturity.**

Sweet orange (*Citrus sinensis* L. Osbeck) is the most horticulturally important and widely grown *Citrus* species, and it is highly polyembryonic. With the exception of the controversial 'Ambersweet' (Hearn, 1989), no sweet orange cultivars have been produced through conventional breeding techniques (Hearn, 1973). The use of conventional breeding techniques in sweet orange improvement has been inhibited by large plant size, extended juvenility, and primarily to nucellar embryony (sweet oranges generally contain from one to many adventive nucellar embryos). Zygotic sweet orange hybrids are difficult to obtain, and are often weak and do not produce fruit that resembles sweet orange. It is generally accepted that commonly grown sweet orange cultivars probably originated from the selection of a chance seedling well-adapted to a particular area or from a mutation in a particular cultivar or seedling (Nishiura, 1965; Hodgson, 1968). Spontaneous mutations visible as bud or limb sports or sectors on chimeric fruits occur frequently in citrus (Soost and Cameron, 1975). Recently, there has been great interest in using tissue culture methods to induce or uncover beneficial genetic variation in regenerated plants, and the

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