Eight New Somatic Hybrid Citrus Rootstocks with Potential for Improved Disease Resistance

Eliezer S. Louzada1, Jude W. Grosseti, Frederick G. Gmitter, Jr.2, Beatriz Nielsen3, and J.L. Chandler4

Department of Horticultural Sciences, Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850

Xiu Xin Deng
Department of Horticulture, Huazhong Agricultural University, Wuhan Hubei 430070, People’s Republic of China

Nicasio Tusa
Centro di Studio per il Miglioramento Genetico degli Agrumi, Consiglio Nazionale delle Ricerche, Male delle Scienze, Palermo 90128, Sicily

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Abstract. Protoplast culture following polyethylene glycol-induced fusion resulted in the regeneration of vigorous tetraploid somatic hybrid plants from eight complementarily parental rootstock combinations: Citrus reticulata Blanco (Cleopatra mandarin) + C. aurantium L. (sour orange), C. reticulata (Cleopatra mandarin) + C. jambhiri Lush (rough lemon), C. reticulata (Cleopatra mandarin) + C. volkameriana Ten. & Pasq. (Volkamer lemon), C. reticulata (Volkamer mandarin) + C. limonia Osb. (Rangpur), C. sinensis (L.) Osb. (Hamlin sweet orange) + C. limonia (Rangpur), C. aurantium (sour orange) + C. volkameriana (Volkamer lemon) zygotic seedling, C. aurantium hybrid (Smooth Flat Seville) + C. jambhiri (rough lemon), and C. sinensis (Valencia sweet orange) + Carrizo citrange [C. paradisi Macf. × Poncirus trifoliata (L.) Raf.]. Diploid plants were regenerated from nonfused callus-derived protoplasts of Valencia sweet orange and Smooth Flat Seville and from nonfused leaf protoplasts of sour orange, Rangpur, rough lemon, and Volkamer lemon. Regenerated plants were classified according to leaf morphology, chromosome number, and leaf isozyme profiles. All somatic hybrid plants were tetraploid (2n = 4x = 36). One autotetraploid plant of the Volkamer lemon zygotic was recovered, apparently resulting from a homokaryotic fusion. These eight new citrus somatic hybrids have been propagated and entered into field trials.

Somatic hybridization via protoplast fusion has become a powerful tool in Citrus for combining superior genomes to produce viable allotetraploid plants. New genetic combinations produced by protoplast fusion include interspecific somatic hybrids (Grosser et al.; 1989, 1991; Kobayashi et al., 1988a, 1988b; Ohgawara et al., 1989, 1990), intergeneric somatic hybrids from sexually compatible parents (Deng et al., 1991; Grosser and Gmitter, 1990a; Grosser et al., 1988a, 1991; Ohgawara et al., 1985) and sexually incompatible parents (Grosser and Gmitter, 1990c; Grosser et al., 1988b, 1990, 1991), and cybrids (Vardi et al., 1987, 1989). Recent reviews of protoplast culture (Vardi and Galun, 1988) and somatic hybridization in Citrus (Grosser, 1992; Grosser and Gmitter, 1990a, 1990b) are available.

Primary pathological factors that dictate citrus rootstock choice in Florida are citrus blight, citrus tristeza virus, Phytophthora-induced disease, and nematodes (Castle, 1987; Castle et al., 1989; Whiteside et al., 1988). Horticultural performance and resistance to abiotic stresses must also be adequate. No commercial rootstock is completely satisfactory for all selection criteria. One strategy that applies somatic hybridization techniques to rootstock improvement is the production of tetraploid somatic hybrid rootstocks that combine the intact genomes of complementary rootstock parents (Grosser and Gmitter, 1990b). The objective of our research was to produce a population of somatic hybrids that combine all of the necessary disease and pest resistance into horticulturally desirable rootstocks adapted to the various environmental conditions that exist in Florida.

Rough lemon, Volkamer lemon, and Rangpur have been used as rootstocks, because trees on these stocks are vigorous, high-yielding, and tristeza and drought-tolerant (Castle, 1987; Castle et al., 1989). However, their use has declined because they are highly susceptible to citrus blight. Cleopatra mandarin has become an increasingly important rootstock that is less susceptible to citrus blight than the rootstocks named above, tolerant to tristeza, and cold-hardy. Trees grafted on Cleopatra mandarin are moderately vigorous but are slow to reach full bearing potential (Castle et al., 1989). Sweet orange is tolerant of citrus blight, but is not used as a rootstock due to Phytophthora susceptibility. Somatic hybridization of Cleopatra mandarin with rough lemon, Volkamer lemon, and Rangpur; ‘Hamlin’ sweet orange with Rangpur; and ‘Valencia’ sweet orange with Carrizo citrange was attempted to generate vigorous productive rootstocks with acceptable horticultural characteristics but improved blight tolerance.

Sour orange, formerly the most important rootstock in almost every citrus-growing region in the world, is still important in some areas because of its yield potential, favorable influence on fruit quality, wide soil adaptability, and tolerance to cold, Phytophthora-induced diseases, and citrus blight (Castle, 1987; Castle et al., 1989). However, its extreme susceptibility to tristeza-induced decline with sweet orange or grapefruit scions has drastically reduced or eliminated its use in most citrus-growing regions, including Florida and Brazil. Somatic hybridization of sour orange with tristeza-tolerant Cleopatra mandarin and Volkamer lemon, and Smooth Flat Seville with tristeza-tolerant rough lemon was attempted to generate sour orange-type rootstocks that are tolerant to tristeza virus and citrus blight.

Leaf protoplasts of sour orange, rough lemon, Volkamer lemon, and Carrizo citrange were isolated from young nucellar seedlings maintained in a growth chamber (16-h photoperiod, 300 μmol-m-1’s light intensity, 26 to 30°C; Grosser and Chandler, 1987). Protoplasts of Cleopatra mandarin, ‘Hamlin’ and ‘Valencia’ sweet oranges, sour orange, and Smooth Flat Seville were isolated from friable, nucellus-derived, embryogenic callus cultures maintained on either EME or H + H solid medium (Grosser and Gmitter, 1990a). All protoplasts were purified by passage through a 4.5-mm stainless steel filter followed by centrifugation on a 25% sucrose-13% mannitol gradient before mixing (Grosser and Gmitter, 1990a; Tusa et al., 1990). Approximately equal volumes of embryogenic callus-derived protoplasts of one parent and leaf protoplasts of the second parent were mixed and fused for each parental combination (Table 1) by use of the polyethylene glycol (PEG) method of Grosser and Gmitter (1990a). Following fusion, protoplasts were cultured directly in 60 x 15mm fusion petri
Fig. 1. Leaf morphology of: (a) Smooth Flat Seville; (b) Smooth Flat Seville + rough lemon somatic hybrid; (c) rough lemon; (d) Cleopatra mandarin + rough lemon somatic hybrid; (e, h, p) Cleopatra mandarin; (f) Cleopatra mandarin + sour orange somatic hybrid; (g, v) sour orange; (i) Cleopatra mandarin + Rangpur somatic hybrid; (j) Rangpur; (k) ‘Hamlin’ sweet orange + Rangpur somatic hybrid; (l) ‘Hamlin’ sweet orange; (m) ‘Valencia’ sweet orange; (n) ‘Valencia’ + Carrizo citrange somatic hybrid; (o) Carrizo citrange; rough lemon, Volkamer lemon, Rangpur, and regenerated plants. Peroxidase (PER), phosphoglucomutase (PGM), and phosphoglucose isomerase (PGI) isozymes were separated by horizontal starch gel electrophoresis on 10% Connaught starch gels with the pH 5.7 histidine citrate buffer of Cardy et al. (1981). Electrophoresis was carried out for 3 h at 4°C and constant 60 mA current. Gels were stained as described by Vallejos (1983).

Plants regenerated following protoplast fusion are summarized in Table 1. The shapes of recovered somatic embryos were often abnormal, but this did not interfere with germination and plant recovery. For the ‘Hamlin’ sweet orange + Rangpur combination, embryo germination appeared to be most efficient on BGN medium. Embryo germination for the Cleopatra mandarin + Rangpur and sour orange + Volkamer lemon combinations appeared to be most efficient on DBA3 medium, and shoots also developed via adventitious budding on the cut surfaces of dissected embryos of these hybrid genotypes on this medium. Embryo germination for the remaining combinations was adequate on B + embryo germination medium. The Cleopatra mandarin + Rangpur and ‘Hamlin’ sweet orange + Rangpur combinations showed vigor throughout the protoplast to plant cycle, as demonstrated by the large populations of hybrid plants recovered (Table 1). The Cleopatra mandarin and sour orange callus lines apparently lost their regeneration capability, because no plants were regenerated from nonfused protoplasts of these lines. Plant regeneration of ‘Hamlin’ sweet orange from nonfused callus-derived protoplasts was inhibited by the high-sucrose content of EMEP protoplast culture medium, as previously reported (Grosser et al., 1988a, 1988b; Ohgawara et al., 1985). Numerous diploid plants of sour orange, the zygotic Volkamer lemon, and a few plants of Rangpur were regenerated from nonfused leaf protoplasts. A tetraploid plant of the zygotic Volkamer lemon was also recovered, apparently resulting from a homokaryotic fusion. These results probably depended on co-culture with embry-

Table 1. Summary of *Citrus* plant regeneration following protoplast fusion.

<table>
<thead>
<tr>
<th>Embryogenic parent (embryogenic callus)</th>
<th>Leaf parent</th>
<th>Plants from</th>
<th>Somatic hybrid* (no.)</th>
<th>Embryogenic parent (no.)</th>
<th>Leaf parent (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleopatra mandarin <em>(C. reticulata)</em></td>
<td>Sour orange <em>(C. aurantium)</em></td>
<td>171*</td>
<td>0</td>
<td>223</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rough lemon <em>(C. jambhiri)</em></td>
<td>5*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rangpur <em>(C. limonia)</em></td>
<td>&gt;1000*</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Volkamer lemon <em>(C. volkameriana)</em></td>
<td>8*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hamlin sweet orange <em>(C. sinensis)</em></td>
<td>Rangpur</td>
<td>&gt;1000*</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Valencia sweet orange <em>(C. sinensis)</em></td>
<td>Carrizo citrange</td>
<td>7*</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Smooth Flat Seville <em>(C. aurantium)</em></td>
<td>Rough lemon</td>
<td>3*</td>
<td>0</td>
<td>77*</td>
<td></td>
</tr>
<tr>
<td>Sour orange <em>(C. aurantium)</em></td>
<td>Volkamer lemon</td>
<td>113*</td>
<td>0</td>
<td>77*</td>
<td></td>
</tr>
</tbody>
</table>

*Indicates the number of plants regenerated but not necessarily the number of plants recovered from individual fusion events.

1Phosphoglucomutase used to confirm somatic hybridity.

2Peroxidase used to confirm somatic hybridity.

3Phosphoglucose isomerase used to confirm somatic hybridity.

4Includes one autotetraploid plant.
Table 2. Designated leaf isozyme genotypes of the donor *Citrus* parents for peroxidase (PER), phosphoglucone isomerase (PGI), and phosphoglucone mutase (PGM).

<table>
<thead>
<tr>
<th>Donor</th>
<th>PER</th>
<th>PGI</th>
<th>PGM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleopatra mandarin</td>
<td>FF</td>
<td>MM</td>
<td>FF</td>
</tr>
<tr>
<td>Sour orange</td>
<td>FS</td>
<td>WS</td>
<td>FS</td>
</tr>
<tr>
<td>Rough lemon</td>
<td>SS</td>
<td>MS</td>
<td>FI</td>
</tr>
<tr>
<td>Volkamer lemon</td>
<td>SS</td>
<td>MS</td>
<td>FI</td>
</tr>
<tr>
<td>Volkamer lemon zygotic</td>
<td>SS</td>
<td>MS</td>
<td>FI</td>
</tr>
<tr>
<td>Rangpur</td>
<td>SS</td>
<td>MS</td>
<td>FI</td>
</tr>
<tr>
<td>Hamlin/Valencia orange</td>
<td>FF</td>
<td>MS</td>
<td>FS</td>
</tr>
<tr>
<td>Smooth Flat Seville</td>
<td>FS</td>
<td>MS</td>
<td>FS</td>
</tr>
<tr>
<td>Carrizo citrange</td>
<td>FP</td>
<td>SS</td>
<td>PS</td>
</tr>
</tbody>
</table>

do not show the S allele that we expected to be contributed by both parents. The S allele was either lost or its expression blocked in these plants for reasons we cannot explain. All sour orange + Volkamer lemon zygotic somatic hybrid plants were clearly hybrid at the PGI locus, showing the expected WMSS banding pattern. We have previously observed and explained missing isozyme bands in somatic hybrid plants on two occasions. An expected M band at the PGI locus was not present in Hamlin lemon (*Citrus jambhiri* Lush purported hybrid) + femminello lemon (C. limon L. Burm. F.) somatic hybrid plants, but the M band was also missing in the parental Hamlin lemon callus line and plants regenerated from this line (Tusa et al., 1992). An expected S band at the PGI locus was not present in ‘Hamlin’ sweet orange (C. sinensis) + Severinia buxifolia Pon. Teacher, but these plants were also missing chromosomes (2n = 27, whereas the expected chromosome number was 2n = 4x = 36).

Plants of the somatic hybrid rootstocks reported herein have been propagated and entered into field trials. Determining their characteristics and the nature of the genetic control of important traits should provide a valuable basis for selecting parents for future somatic hybridizations.

**Literature Cited**


