Journal of the American Pomological Society 58(1):21-28 2004

Production of Twelve New Allotetraploid Somatic Hybrid Citrus Breeding Parents with Emphasis on Late Maturity and Cold-Hardiness

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Abstract

The rapid development of adapted, high-quality, easy-to-peel, seedless mandarin/mandarin hybrid cultivars is paramount for the future success of the Florida tangerine industry. The primary approach of the CREC cultivar improvement team to achieve this objective is to develop superior triploid cultivars, which should be seedless regardless of cross pollination. At present, the most efficient method for producing large populations of genetically diverse triploids for selection is via interploid crosses of monoembryonic diploid females with tetraploid pollen parents. A broad germplasm base will facilitate the identification of superior interploid parental combinations. We have been using somatic hybridization to expand our base of superior tetraploid breeding parents, and in this report we introduce 12 new hybrids produced from parents selected for fruit quality, cold-hardiness, and late maturity as follows: 'Murcott' tangor + 'Sunburst' tangerine; 'Murcott' + ['Clementine' x 'Satsuma' hybrid]; 'Murcott' + 'Washington' navel orange; 'Murcott' + 'Osceola' mandarin hybrid; 'Murcott' + 'Ortanique' tangor; 'Itaborai' sweet orange + G96 trifoliate hybrid; 'Nova' mandarin hybrid + 'Osceola'; 'Nova' + 'Ortanique'; 'Meiwa' kumquat + 'Changsha' mandarin; 'Meiwa' + 'Dancy' mandarin; 'Succari' sweet orange + LB8-9 tangelo; and 'Succari' + 'Changsha'. Coldy-hardy late-maturing seedless triploid cultivars should provide excellent future opportunities for fresh-citrus growers in Florida and worldwide.

Introduction

Seedlessness has become the primary objective for fresh market mandarin improvement programs worldwide. Consumer demand for convenience has placed a high premium on easy-peel seedless tangerines. In Florida and the USA, popular Florida cultivars 'Sunburst' and 'Murcott' are being displaced in the marketplace by 'Clementine' imported from Spain, Morocco, and more recently California. To date, 'Clementine' cultivars do not perform adequately in Florida and usually produce seedy fruit due to cross-pollination. No seedless alternative tangerine cultivars are currently available to Florida growers. Because sweet orange and grapefruit are widely planted in Florida (for processing and fresh market), mandarin cultivars that must be protected

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Acknowledgements

This research was financially supported by the Florida Agricultural Experiment Station, and a grant from FCPRAC, approved for publication as Journal Series No. R-09580. The authors thank the Florida Citrus Production Research Advisory Council for a continuing block-grant supporting research on citrus variety improvement. The authors also thank Wayne Sherman for providing the G96 hybrid, Fred Gmitter for providing the LB8-9 hybrid, and George Riegler for providing the ('Clementine' x 'Satsuma') hybrid.

from cross-pollination for seedlessness are not practical. Therefore, the CREC cultivar improvement program is focusing on the development of seedless cultivars adapted to the humid subtropical conditions of Florida via triploidy. Parthenocarpic triploids are generally seedless regardless of cross-pollination. The most efficient method for producing triploid citrus is via interploid crosses of monoembryonic diploid females with tetraploid pollen parents. Crosses are generally done in this direction because monoembryonic tetraploids are rare and generally unavailable (14). In such crosses, embryo rescue is required to efficiently generate triploids, due to endosperm failure (8). Our program has had great success using somatic hybridization techniques to build a broad-based collection of quality tetraploid breeding parents for use in mandarin interploid crosses (5,6,8,11). Herein, we report on 12 new allotetraploid mandarin hybrids that were produced with potential to transfer cold-hardiness and/or late-maturity to subsequent triploid progeny.

Historically, the best area to produce mandarin/mandarin hybrids in Florida has been the northern part of the central sandridge. Colder nights in this region contribute to better external color and fruit quality. However, citrus production in this region has greatly diminished due to several severe freezes during the 1980s. Freezes during the past century have also interrupted Satsuma production in northern Florida. Therefore, cold-hardiness is also an important breeding objective for mandarin improvement. Successful manipulation of maturity date could also provide fresh mandarin growers with new opportunities. Considering the international market, Satsumas capture the early market, and the early-mid to mid season is dominated by 'Clementine' selections. In Florida, the latest maturing peelable tangerine cultivar is the seedy 'Murcott', which has a mid-late season maturity date. No seedless easy-peelers are presently available that ripen after 'Murcott'. Huge marketing opportunities for fresh citrus growers are available for seedless cultivars that mature mid-late to late season, thus the emphasis on late maturity in the present report.

Description of Fusion Parents:

Embryogenic parents: 'Murcott', also known as the Honey tangerine, is a purported tangor (*C. reticulata* Blanco x *C. sinensis* L. Osbeck) of unknown parentage, possibly created by W.T. Swingle (12). It is thin-skinned and peelable, and has exceptional internal color and quality. Previously produced somatic hybrids of 'Murcott' with 'Valencia' and 'Succari' sweet oranges have inherited its internal quality and color (J.W. Grosser, unpublished data). As mentioned, 'Murcott' generally ripens during mid-late season, and should be a good source of genes for later maturity. 'Nova' mandarin hybrid was produced from a cross of the 'Fina' Clementine (*C. reticulata* Blanco) with Orlando tangelo ['Duncan' grapefruit (*C. paradisi* Macf.) x 'Dancy' tangerine (*C. reticulata* Blanco)] made by F.C. Gardner and J. Bellows in Florida in 1942 (12). 'Nova' trees are vigorous and produce fruit with exceptional internal quality, which are seedless when protected from cross-pollination. Fruit quality diminishes quickly if harvest is delayed.

A previous somatic hybrid of 'Nova' with 'Succari' sweet orange is exceptionally vigorous, and has been used as a pollen parent to generate hundreds of triploid progeny (under the direction of F.G. Gmitter, Jr.) (8). 'Itaborai' sweet orange (*C. sinensis* L. Osbeck) is an early maturing selection with high quality fruit and exceptional early season juice color (2). 'Meiwa' kumquat (*Fortunella crassifolia* Swingle) is considered to be the best eating variety of kumquat. Kumquats exhibit outstanding cold-hardiness due to earlier and longer dormancy than citrus (9). 'Meiwa' also has a highly colored and edible rind. Kumquats are also a source of resistance to citrus canker caused by *Xanthomonas axonopodis* pv. *Citri.* 'Succari' sweet orange (*C. sinensis* L. Osbeck) is a low-acid selection that transmits low-acidity to its progeny (H.C. Barrett, personal communication). Since high acidity has been a problem in mandarin hybrids produced by conventional breeding (C.J. Hearn, personal communication), use of this selection should facilitate production of higher frequencies of progeny with acceptable acid levels. Previously produced somatic hybrids of 'Succari' + 'Page' tangelo and 'Succari' + 'Minneola' tangelo indeed have low acidity (J.W. Grosser, unpublished data).

***Leaf Parents*:** 'Sunburst' mandarin hybrid was produced from a cross of sister cultivars 'Robinson' and 'Osceola' ['Clementine' (*C. reticulata* Blanco) x 'Orlando' tangelo (*C. reticulata* Blanco x *C. paradisi* Macf.)] made by P.C. Reece in 1961 (12). 'Sunburst' has been the primary fresh mandarin in Florida due to its attractive deep reddish-orange external color and superior shelf life (12). However, recent sales have plummeted due to competition from superior imported cultivars. 'Sunburst' matures early-mid season. The 'Clementine' x 'Satsuma' hybrid (*C. reticulata* Blanco x *C. unshiu* Hort. ex Tan.) was provided by Florida hobbyist George Riegler. This selection is an easy-peeler with mild but pleasant flavored fruit that mature early-mid season. It is not yet known if this selection inherited the cold-hardiness of 'Satsuma'. 'Washington' navel orange (*C. sinensis* L. Osbeck) is a standard navel cultivar grown in Florida and elsewhere. 'Osceola' mandarin hybrid was produced from a cross of 'Clementine' x 'Orlando' tangelo made in 1942 by F.C. Gardner and J. Bellows (12). The rind has a beautiful deep reddish-orange color at maturity, and the fruit is easy to peel. However, the rind oil has an unusual aroma that is unacceptable to some consumers. It matures in November in Florida (early mid-season). 'Ortanique' tangor (*C. reticulata* Blanco x *C. sinensis* L. Osbeck) was discovered in Jamaica by C.P. Jackson in 1920 (12). Trees are vigorous and productive, and the fruit matures at the same time as the late orange 'Valencia' (late season). Fruit has excellent flavor and juice color, and can be stored on the tree with minimal loss of quality. This selection should be an excellent source of genes for late maturity. 'Changsha' mandarin (*C. reticulata* Blanco) is considered to be the most cold-hardy of the sweet mandarins. It produces highly colored, seedy, but delicious fruit. It should be an excellent source of genes for fruit quality and cold-hardiness. 'Dancy' mandarin (*C. reticulata* Blanco or *C. tangerina* Tanaka) is an easy-peel mandarin with excellent flavor, and was formerly the most popular tangerine grown in Florida. It was subsequently replaced by 'Sunburst' due to its lack of shelf-life. It is considered to be moderately cold-hardy (9). LB8-9 tangelo was produced from a cross of 'Clementine' x 'Minneola' tangelo made by A. Pierenger (F. G. Gmitter, personal communication). Trees are vigorous and produce fruit that matures early-mid season with excellent external color and outstanding aromatic flavor. The fruit is similar in shape and flavor to 'Minneola', but matures slightly earlier and peels more easily. This selection will soon be named and released by F. G. Gmitter, University of Florida, CREC. This selection has excellent cold-hardiness, as the original tree survived three devastating freezes occurring during the 1980s in central Florida. The complex G96 hybrid ([*C. grandis* x (*C. paradisi* x *P. trifoliata*)] x [(*C. paradisi* x *P. trifoliata* x *C. reticulata*)]) was produced by W.B. Sherman (University of Florida, Gainesville, FL) in efforts to move genes for cold-hardiness and CTV (citrus tristeza virus) resistance from the inedible trifoliate orange (*Poncirus trifoliata* (L.) Raf.) into hybrids producing edible fruit with dooryard cultivar potential. This hybrid produces a fruit similar to a sweet orange, but with lesser quality. Trees have shown excellent cold-hardiness in northern Florida.

Materials and Methods

Protoplast Isolation, Fusion, and Culture

Protoplasts of embryogenic parents 'Murcott', 'Itaborai', 'Nova', and 'Succari' were isolated from suspension cultures maintained on H+H agar-free medium with a two-week subculture cycle according to Grosser and Gmitter (4). Protoplasts of embryogenic parent 'Meiwa' were isolated directly from callus cultures maintained on solid H+H medium (0.8 g/l agar) with a six-week subculture cycle. Protoplasts of leaf parents 'Sunburst', ('Clementine' x 'Satsuma') hybrid, 'Washington' navel, 'Osceola', 'Ortanique', 'Changsha', and 'Dancy' were isolated from fully expanded young leaves taken from nucellar seedlings maintained in a heavily shaded greenhouse (double shadecloth). Protoplasts of leaf parents G-96 and LB8-9 were isolated from newly budded plants maintained in the same greenhouse. Protoplasts from all sources were purified by passage through a 45 μm stainless steel mesh to remove large debris and undigested material, followed by centrifugation on a sucrose-mannitol gradient to remove smaller debris from broken protoplasts according to Grosser and Gmitter (4). Protoplasts from parental combinations were mixed in equal volumes and fused using the highly successful standard PEG (polyethylene glycol) protocol of Grosser and Gmitter (4). This protocol has been used at the CREC to generate somatic hybrid plants from more than 150 parental combinations (8). Following fusion, protoplasts were cultured in a 1:1 v:v mixture of 0.6M BH3 and 0.6M EME protoplast culture media in 60x15 Falcon petri dishes sealed with Nescofilm according to Grosser and Gmitter (4). Osmoticum reduction was conducted as described by Grosser and Gmitter (4).

Plant Regeneration and Somatic Hybrid Verification

Recovered embryoids were enlarged on 1500 medium, and well-formed embryos were transferred to B+ medium for germination (4). Embryos not germinating on B+ medium and all abnormal embryos were sliced in half and cultured on DBA3 shoot induction medium (3). One-three transfers (3-4 week intervals) on DBA3 medium were required to recover somatic hybrid plants from most combinations. Recovered shoots were rooted on RMAN medium in Magenta boxes according to Grosser and Gmitter (4). Tetraploid regenerants were identified by flow cytometry using a Partec table-top ploidy analyzer. The allotetraploid nature of recovered somatic hybrids was verified by RAPD (Random amplified polymorphic DNA) analysis using customized 12-mer primers A-19 (sequence AAGGCGCGAACG) and C-11 (sequence AGGTACGCCCGA) (sequences provided by K. Kepenek, personal communication, and synthesized by Operon Technologies, Alameda, CA, USA). The following PCR reaction conditions were used: one cycle of 2 min at 94°C; followed by 34 cycles for 1 min of denaturing at 94°C, 1 min of annealing at 42°C and a 2 min extension at 72°C; a final extension for 10 min at 72°C. Amplification products were separated on a 1.8% agarose gel containing 1X TAE buffer and 0.5 μg/ml ethidium bromide for 1.5 h at 2.3 V/cm, and visualized under UV light. Complementary banding patterns revealed a genetic contribution from both parents in each new somatic hybrid.

Results and Discussion

Allotetraploid somatic hybrid plants were recovered from all parental combinations provided in Table 1. RAPD analysis showing complementary banding patterns for each parental combination are provided in Figure 1. Successful fusion experiments of the 'Succari' sweet orange + LB8-9 tangelo combination were more difficult, because yields of LB8-9 leaf

Table 1. Embryogenic culture and leaf parents of twelve new allotetraploid somatic hybrid plant combinations produced by protoplast fusion.

Embryogenic parent	Leaf parent	Vigor of hybrid
'Murcott' tangor	'Sunburst' mandarin hybrid	very poor
'Murcott' tangor	('Clementine' x 'Satsuma') hybrid	low
'Murcott' tangor	'Washington' navel orange	low-medium
'Murcott' tangor	'Osceola' mandarin hybrid	low-medium
'Murcott' tangor	Ortanique' tangor	medium
'Itaborai' sweet orange	G-96 hybrid	medium
'Nova' mandarin hybrid	'Osceola' mandarin hybrid	high
'Nova' mandarin hybrid	'Ortanique' tangor	high
'Meiwa' kumquat	'Changsha' mandarin	medium
'Meiwa' kumquat	'Dancy' mandarin	medium
'Succari' sweet orange	LB8-9 tangelo	medium
'Succari' sweet orange	'Changsha' mandarin	medium

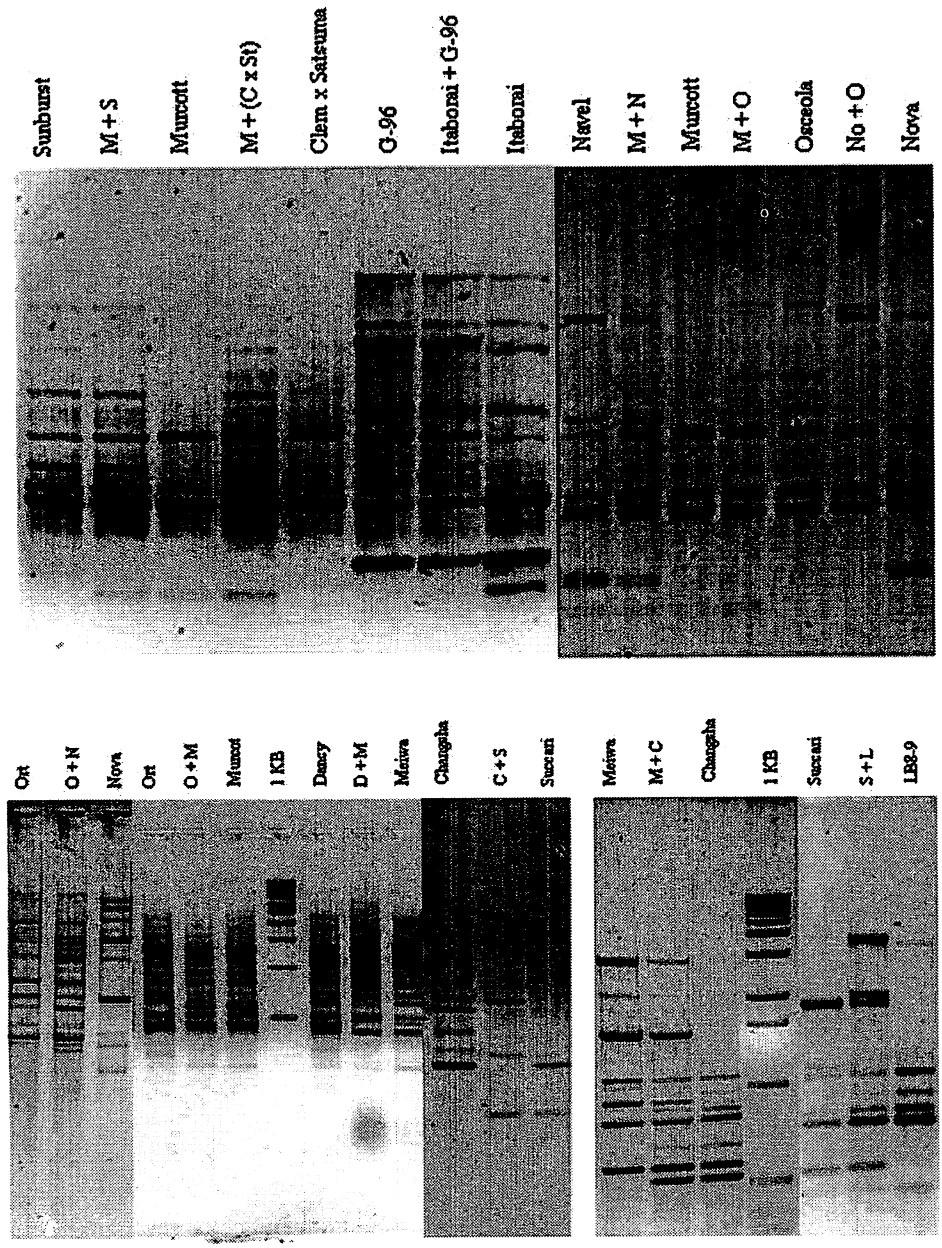

Figure 1. RAPD analysis verifying twelve new somatic hybrid combinations. The upper and lower right-hand gels were produced using primer C-11, and the lower left-hand gel with primer A-19.

protoplasts were usually inadequate. Diploid plants morphologically resembling the leaf parent were recovered from fusions of 'Murcott' + 'Ortanique', 'Itaborai' + G96 hybrid, and 'Succari' + LB8-9 tangelo. These plants are putative cybrids, as citrus leaf protoplasts are not totipotent unless cybridization occurs (1,7,10). It has been proposed that cybridization quantitatively increases mitochondria content of cells which can provide adequate energy for cell-wall resynthesis and regeneration (10). Attempts to confirm the cybrid nature of these plants by molecular analysis has not been conducted. These putative cybrid plants are being evaluated for potentially useful somaclonal variation.

An estimate of the vigor of the somatic hybrid plants is also described in Table 1. Only two hybrid plants of the 'Murcott' + 'Sunburst' combination were recovered, and they were both very weak and eventually died. This could be due to some genetic incompatibility between these two parents. All other somatic hybrids were successfully budded to rootstocks (i.e. Carrizo citrange) and planted in the field for subsequent use as pollen parents in interploid crosses. The 'Nova' + 'Osceola' and 'Nova' + 'Ortanique' combinations produced highly vigorous plants (Figure 2) that have already flowered this spring (2003), and their pollen was used in interploid crosses with selected females conducted under the direction of F.G. Gmitter, Jr. Because LB8-9 is monoembryonic, the somatic hybrid combining it with 'Succari' sweet orange may also be monoembryonic, in which case it could be used as a female in interploid crosses. Crosses utilizing monoembryonic tetraploids as females do not require embryo rescue for triploid recovery (13).

Overall, the research reported herein has greatly expanded the CREC germplasm base for interploid crosses by providing eleven new allotetraploid breeding parents. These new hybrids will greatly expand

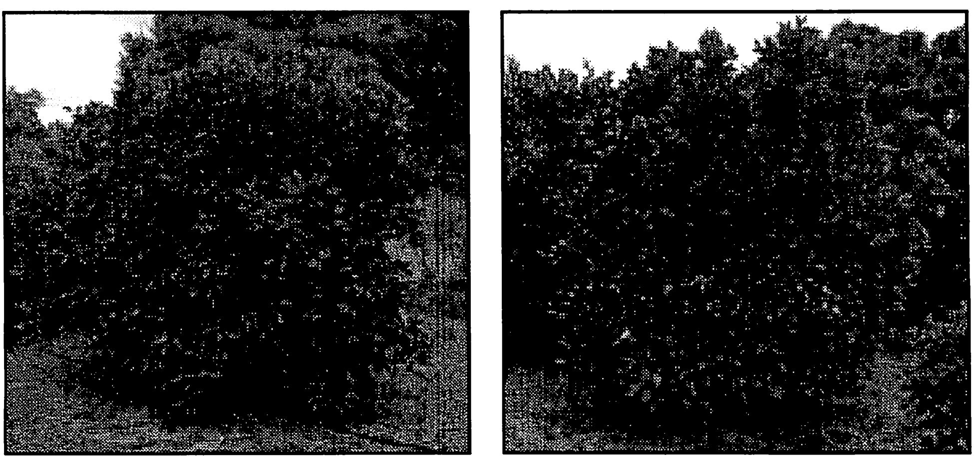

Figure 2. Left: Field tree of 'Nova' + 'Osceola' somatic hybrid and right: field tree of 'Nova' + 'Ortanique' somatic hybrid, budded to somatic hybrid rootstock 'Hamlin' + rough lemon.

possible interploid crosses, which will eventually lead to the identification of superior interploid parental combinations. Under the direction of F. G. Gmitter, the CREC is presently producing several hundred to several thousand triploid hybrids annually, and many produced during the past 6 years have been fathered by somatic hybrids. The new allotetraploid somatic hybrids reported herein should be excellent sources of genes for fruit quality, disease resistance, cold-hardiness and late maturity in subsequent triploid progeny. The overall CREC cultivar improvement program is expected to provide fresh-citrus growers with new alternative seedless, easy-to-peel cultivars of various maturity dates in the not too distant future.

Literature Cited

1. Cabasson, C. M., F. Luro, P. Ollitrault, and J. W. Grosser. 2001. Non-random inheritance of mitochondrial genomes in Citrus hybrids produced by protoplast fusion. Plant Cell Rep. 20: 604-609.
2. Castle, B. 1999. Promising new selections of sweet orange cultivars. Citrus Industry Magazine 80: 24-28.
3. Deng, X. X., J. W. Grosser, and F. G. Gmitter, Jr. 1992. Intergeneric somatic hybrid plants from protoplast fusion of *Fortunella crassifolia* cultivar 'Meiwa' with *Citrus sinensis* cultivar 'Valencia'. Scientia Hort. 49: 55-62.
4. Grosser, J. W. and F. G. Gmitter, Jr. 1990. Protoplast fusion and citrus improvement. Plant Breeding Rev. 8: 339-374.
5. Grosser, J. W., F. G. Gmitter, Jr., E. S. Louzada, and J. L. Chandler. 1992. Production of somatic hybrid and allotetraploid breeding parents for seedless citrus development. HortScience 27: 1125-1127.
6. Grosser, J. W., J. Jiang, F. A. A. Mourao-Fo, E. S. Louzada, K. Baergen, J. L. Chandler, and F. G. Gmitter, Jr. 1998. Somatic hybridization, an integral component of citrus cultivar improvement: I. Scion improvement. HortScience 33: 1057-1059.
7. Grosser, J. W., F. G. Gmitter, Jr, N. Tusa, G. R. Recupero, and P. Cucinotta. 1996. Further evidence of a cybridization requirement for plant regeneration from citrus leaf protoplasts. Plant Cell Rep. 15: 672-676.
8. Grosser, J. W., P. Ollitrault, and O. Olivares-Fuster. 2000. Somatic hybridization in Citrus: An effective tool to facilitate variety improvement. In Vitro Cell. Dev. Biol. – Plant 36: 434-449.
9. Hodgson, R. W. 1967. Horticultural Varieties of Citrus. Chapter 4. In: W. Reuther, H. J. Webber, and L. D. Batchelor (Eds.). The Citrus Industry. 1: 431-591.
10. Moreira, C. D., C. D. Chase, F. G. Gmitter, Jr., and J. W. Grosser. 2000. Inheritance of organelle genomes in citrus somatic cybrids. Molec. Breed. 6: 401-405.
11. Mourao, F. A. A.-Fo., F. G. Gmitter, Jr., and J. W. Grosser. 1996. New tetraploid breeding parents for triploid seedless citrus cultivar development. Fruit Var. J. 50: 76-80.
12. Saunt, J. 1990. Citrus varieties of the world. Sinclair International Limited, Norwich, England.
13. Soost, R. K. and J. W. Cameron. 1980. 'Oroblonco', a triploid pummelo-grapefruit hybrid. HortScience 20: 1134-1135.
14. Starrantino, A. and G. Reforgiato-Recupero. 1981. Citrus hybrids obtained from 2x females and 4x males. Proc. Intl. Soc. Citricult. p.31-32.