

## Caipira sweet orange + Rangpur lime: a somatic hybrid with potential for use as rootstock in the Brazilian citrus industry

Fernanda Januzzi Mendes-da-Glória<sup>1</sup>, Francisco de Assis Alves Mourão Filho<sup>2</sup>, Luis Eduardo Aranha Camargo<sup>3</sup> and Beatriz Madalena Januzzi Mendes<sup>4</sup>

### Abstract

Protoplast culture following polyethylene glycol-induced fusion resulted in the regeneration of somatic hybrid plants between Caipira sweet orange (*Citrus sinensis* L. Osbeck) and Rangpur lime (*C. limonia* L. Osbeck). The plants were confirmed as somatic hybrids by leaf morphology, chromosome number and RAPD profile. All regenerated plants were tetraploid ( $2n = 4x = 36$ ), with intermediate leaf morphology and complementary RAPD banding profile of both parents. This combination may be useful as a rootstock for the citrus industry in Southeastern Brazil since this somatic hybrid could combine the drought tolerance and vigor of Rangpur lime with the blight tolerance of Caipira sweet orange.

### INTRODUCTION

The State of São Paulo, in southeastern Brazil, accounts for one third of the world's orange production (Neves and Boteon, 1998). Almost 90% of the oranges produced in the state (estimated at 16.5 million metric tons in 1997/98) are converted into frozen concentrated juice, so that for every 10 cups of orange juice consumed in the world, eight come from the citrus groves of São Paulo State (Neves and Neves, 1996; Amaro and Maia, 1997). However, in the last few years important problems have threatened this orange production. Pests, diseases and climatic problems have limited expansion of the industry and have affected the quality of the harvest. Among the diseases, bacterial canker, citrus variegated chlorosis and blight are the most important (Fundecitrus, 1999).

A series of limitations, such as nucellar polyembryony (apomixis), heterozygosity, self and cross incompatibility and long juvenility, has made citrus breeding through conventional methods a difficult task (Soost and Cameron, 1975). However, recently developed technologies have helped breeders to overcome these difficulties. Among these techniques, somatic hybridization through protoplast fusion has proven to be a useful tool for developing inter-specific, and intra- and inter-generic allotetraploid somatic hybrids ( $2n = 4x = 36$ ). Somatic hybridization has been used in citrus breeding programs to combine complementary, elite scion varieties that can be used as pollen donors in interploid sexual hybridization to produce new seedless triploid fruit varieties (Grosser *et al.*, 1992, 1998a; Mourão Filho *et al.*, 1996); to produce allotetraploid hybrids among existing culti-

vars with complementary characteristics for rootstock development (Grosser *et al.*, 1994; Louzada *et al.*, 1992; Grosser *et al.*, 1998b) and to combine *Citrus* species with related, sexually incompatible genera that possess important traits for germplasm enhancement (Grosser *et al.*, 1996).

The first example of somatic hybridization involving the genus *Citrus* was reported by Ohgawara *et al.* (1985) who obtained an intergeneric allotetraploid hybrid from the fusion of embryogenic callus-derived protoplasts of Trovita sweet orange (*C. sinensis*) and mesophyll protoplasts of *Poncirus trifoliata*, a sexually compatible *Citrus*-related species. Since then, several citrus breeding programs involving somatic hybridization have been started in countries such as the United States (Grosser and Gmitter Jr., 1990), France (Ollitrault and Luro, 1995), Japan (Kobayashi and Ohgawara, 1988; Miranda *et al.*, 1997) and Israel (Vardi *et al.*, 1987). More than 150 *Citrus* somatic hybrids have been reported during the last 15 years (Ohgawara *et al.*, 1994; Grosser *et al.*, 1995) with some hybrids displaying interesting agronomic traits such as ovule fertility (Kobayashi *et al.*, 1991) and reduced tree size when used as rootstocks (Grosser *et al.*, 1995).

A routine procedure for somatic hybridization of Brazilian citrus varieties has been described by Mendes-da-Glória *et al.* (1999). We report the production of somatic hybrids between Rangpur lime and Caipira sweet orange. This combination is expected to combine good horticultural traits from Rangpur lime, such as drought and citrus tristeza virus tolerance and good vigor, with blight tolerance from Caipira.

<sup>1</sup>Curso de Pós-Graduação em Fitotecnia, Departamentos de

<sup>1,2</sup>Produção Vegetal, and <sup>3</sup>Entomologia, Fitopatologia e Zoologia Agrícola, ESALQ/USP, Piracicaba, SP, Brasil.

<sup>4</sup>Laboratório de Biotecnologia Vegetal, CENA/USP, Caixa Postal 96, 13400-970 Piracicaba, SP, Brasil. Send correspondence to B.M.J.M.

## MATERIAL AND METHODS

### Plant material

An embryogenic cell suspension culture derived from Caipira sweet orange (*Citrus sinensis* L. Osbeck) nucellar callus was maintained in liquid EME medium (Grosser and Gmitter Jr., 1990) at 100 rpm, in the dark, at 27°C, on a 2-week culture cycle. Rangpur lime plants (*Citrus limonia* L. Osbeck) derived from nucellar seedlings were grown in RMAN medium (Grosser and Gmitter Jr., 1990) at 27°C under 16 h of light.

### Protoplast isolation, fusion and culture

Protoplasts were isolated from cell suspension cultures and leaves and were chemically fused as described by Mendes-da-Glória *et al.* (1999). Cells from suspension cultures were used for protoplast isolation seven days after subculture. After fusion, the protoplasts were cultured in 0.7 M BH3, 0.7 M EME or BH3:EME medium (1:1 v/v, Grosser and Gmitter Jr., 1990), in 58 x 15-mm plastic dishes sealed with parafilm and incubated in the dark at 27°C. After 3-5-week incubation, cultures were supplemented with medium for osmotic reduction (Mendes-da-Glória *et al.*, 1999). For the development of somatic embryos, calli were transferred to EME medium containing 25 g of sucrose/l. Well-formed embryos were germinated in EME medium with gibberellic acid (1 mg/l). Regenerated plants with 4-5 leaves were transferred to a greenhouse for acclimatization.

### Analyses of regenerated plants

Confirmation of the hybrid nature of the regenerated plants was based on morphology (leaf thickness and petiole shape), chromosome number and RAPD analyses.

For the determination of chromosome number, root tips from regenerated plants were excised 3-4 h after the onset of light and placed in 0.2 mM hydroxyquinoline for 3 h at room temperature. After this incubation, the root tips were rinsed in distilled water and fixed in a solution of 100% ethanol:acetic acid (3:1, v/v) for 18 h. Root tips were then rinsed in distilled water, hydrolyzed at 60°C in 1 N HCl for 8 min, rinsed again in distilled water for 5 min and stained in Schiff solution (leuco basic fuchsin) for 45 min in the dark (Vieira, 1988). After staining, the root tips were rinsed in distilled water and spread on a glass slide containing a drop of 45% acetic acid solution. Cell preparations were mounted in Entellan and scanned for metaphase cells at 200X magnification. Chromosomes were counted at 1250X magnification.

Leaves from the parental plants and from the putative somatic hybrids were collected for total DNA extraction, according to Hoisington *et al.* (1994). One or two leaves (about 200 mg) were ground in liquid nitrogen for

approximately 1 min and an aliquot (500 µl) of extraction buffer (100 mM Tris, pH 7.5, 700 mM NaCl, 50 mM EDTA, pH 8.0, 1% CTAB (hexadecyltrimethylammonium bromide), 140 mM β-mercaptoethanol) was added to the samples, which were then incubated at 65°C in a water bath for 60 min prior to extraction with an equal volume of a 24:1 chloroform:isoamyl alcohol (CIA; 24:1, v/v) followed by centrifugation for 10 min at 16000 g. The supernatant was transferred to another tube and extracted with 400 µl of Tris-equilibrated phenol. The tubes were centrifuged again for 10 min at 16,000 g and the supernatant transferred to a clean tube for DNA precipitation with 400 µl of 100% ethanol. The samples were placed in a freezer (-20°C) for 10 min and then centrifuged as described above. The pellets were rinsed in 75% ethanol for 10 min, air-dried, resuspended in 480 µl of Tris-EDTA (TE) buffer, pH 8.0, and re-precipitated with 130 µl of 5 M NaCl and 1 ml 100% ethanol as described above. After a double rinse, the DNA was resuspended in 50 µl of TE buffer, pH 8.0.

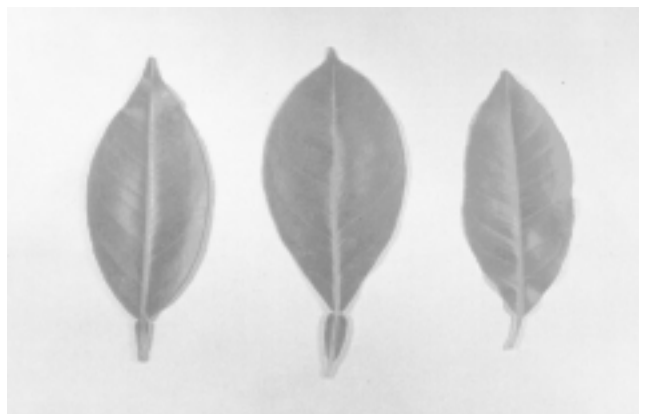
Total genomic DNA fragments from both parents were amplified with 21 10-mer primers (Operon Technologies) by the polymerase chain reaction (PCR). Reactions were conducted with a PTC-100 thermal controller (MJ Research) using an initial denaturation step of 93°C for 2 min followed by 43 cycles at 92°C for 1 min, 37°C for 1 min, and 72°C for 2 min. A final elongation step was carried out at 72°C for 5 min. Each 20 µl of reaction mixture contained 100 mM Tris-HCl, pH 9.0, 500 mM KCl, 2.0 µl 10X taq polymerase buffer, 2.5 mM MgCl<sub>2</sub>, 100 µM of each dNTP, 30 ng of primer, 1 U of taq DNA polymerase and 30 ng of total genomic DNA. The amplification products were separated by electrophoresis (45 V for 4.5 h at room temperature) in ethidium bromide-stained 1.5% agarose gels (0.5 µg EtBr/ml of gel) with 0.5X 45 mM Tris, 45 mM boric acid, 1 mM EDTA (TBE) buffer. The gels were photographed under UV light.

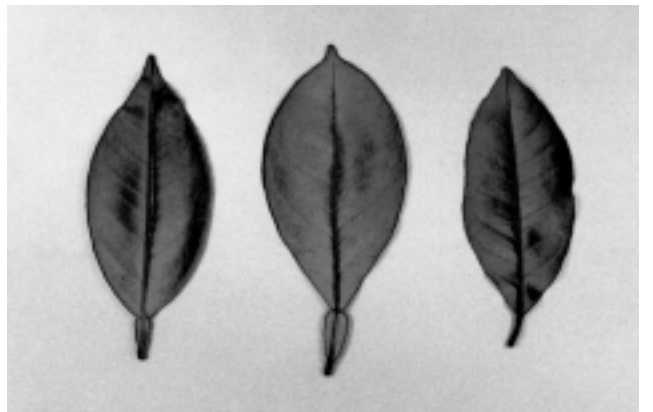
## RESULTS AND DISCUSSION

Protoplast fusion allows the production of interspecific and intergeneric allotetraploid somatic hybrids and generates new combinations unobtainable by conventional breeding methods (Ohgawara *et al.*, 1994; Grosser *et al.*, 1996). This should be useful for citrus breeding programs.

Initial callus culture on regular EME (Grosser and Gmitter Jr., 1990) does not induce somatic embryogenesis. The number of embryos induced on this medium is very low in spite of several reports showing that sucrose is the carbon source for citrus somatic embryogenesis (Kobayashi and Ohgawara, 1988; Grosser and Gmitter Jr., 1990; Ohgawara *et al.*, 1994; Grosser *et al.*, 1996; Mourão Filho *et al.*, 1996). We have observed, in fusion experiments involving other parental combinations, that many embryos developed in the same EME basal medium







when sucrose was substituted by maltose (25 g/l) (Perez *et al.*, 1998). Several studies have demonstrated the positive influence of other carbon sources, including lactose (Ling *et al.*, 1990; Kunitake *et al.*, 1991), galactose (Kochba *et al.*, 1978, 1982) and glycerol (Vu *et al.*, 1993), on the somatic embryogenesis of citrus plants.

Recovered somatic embryos have a shape different from that of typical somatic embryos. Secondary embryogenesis is also observed (Ohgawara *et al.*, 1985; Louzada *et al.*, 1992), with pluricotyledonary embryos, multiple shoot meristems, fused embryos and fasciation (Jumin and Nito, 1996), but this does not interfere with germination and plant recovery. Embryo development is better in EME medium with 25 g of sucrose/l than with 50 g of sucrose/l (Grosser and Gmitter Jr., 1990). Lower sucrose levels influenced embryo development and suppressed callus growth. More than 100 embryos were regenerated after callus culture. Most of these embryos were recovered from protoplast culture on 0.7 M BH3 or BH3:EME medium (1:1 v/v). These embryos continued to be subcultured on solid EME for development. Embryo culture on media with a higher concentration of malt extract (1500 mg/l) (Grosser and Gmitter Jr., 1990) was not beneficial for embryo development in this case. Some small embryos transferred to this media showed callus formation and no development. Embryo germination occurred in EME medium containing 1 mg of GA3/l. A large number of these embryos germinated (about 75% germination) and produced morphologically normal plants, which were transferred to a greenhouse (Figure 1). Twenty-five plants were recovered and well acclimated in the greenhouse. Protoplast fusion to plant regeneration lasted about 18 months.

The leaf morphology of all the recovered plants was intermediate to that of the parents. In addition, leaves from somatic hybrid plants had a bigger petiole wing and thicker leaf blade compared to both parental types (Figure 2), a finding typical of somatic hybrids and polyploids (Miranda *et al.*, 1997). Chromosome counts confirmed the tetraploid nature of the regenerated plants (Figure 3).

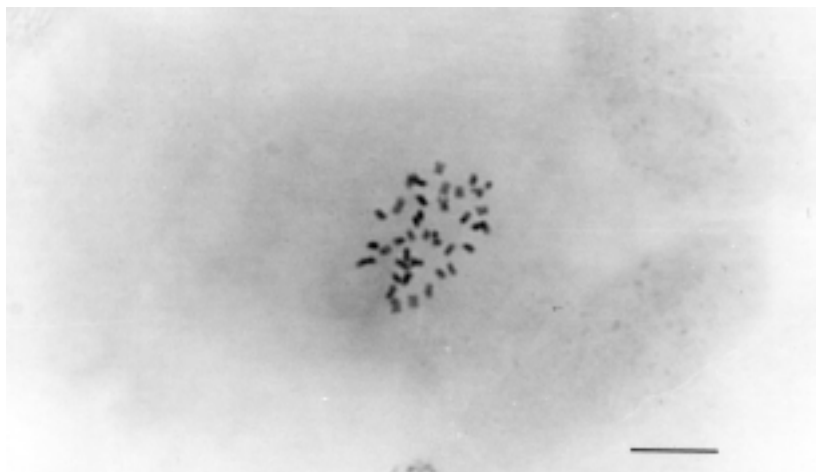
Somatic hybridization can be further confirmed by the analysis of molecular markers such as isozymes (Grosser *et al.*, 1988; Ling and Iwamassa, 1994), rDNA restriction endonuclease digestion patterns (Ohgawara *et al.*, 1985; Miranda *et al.*, 1997), and RAPD-PCR (Grosser *et al.*, 1996; Mourão Filho *et al.*, 1996). RAPD analysis confirmed the somatic hybridization and revealed complementary banding patterns in the somatic hybrid Caipira sweet orange + Rangpur lime, indicating the presence of DNA from each parent in the corresponding hybrid (Figure 4). Six out of 21 primers showed polymorphic bands. DNA fragments amplified with primers OPAA3, OPAB5 and OPAB7 indicated the presence of Caipira sweet orange DNA, whereas DNA amplified using the primers OPAA7 and OPAB1 confirmed the presence of Rangpur lime DNA in the somatic hybrids. DNA



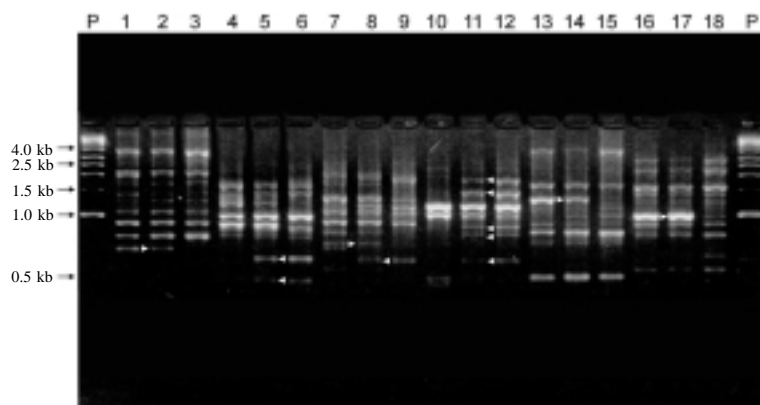
**Figure 1** - Regenerated somatic hybrid plant of Caipira sweet orange + Rangpur lime.



**Figure 2** - Leaf morphology of *Citrus sinensis* (left), somatic hybrid (center), and *C. limonia* (right).



**Figure 3** - Mitotic metaphase of a somatic hybrid plant showing  $2n = 4x = 36$  (Bar = 1  $\mu\text{m}$ ).



**Figure 4** - RAPD fragments of the parental lines Caipira (lanes 1, 4, 7, 10, 13 and 16) and Rangpur lime (lanes 3, 6, 9, 12, 15 and 18) and their somatic hybrid (lanes 2, 5, 8, 11, 14 and 17) amplified with primers OPAA3 (lanes 1-3), OPAA7 (lanes 4-6), OPAA18 (lanes 7-9), OPAB5 (lanes 10-12) and OPAB7 (lanes 16-18). P = 1.0 kb ladder.

amplified with the primer OPAA18 revealed the contribution of DNA from both parents.

Somatic hybrid regeneration confirmed the efficiency of selection medium with a high content of sucrose when sweet orange is a parent (Ohgawara *et al.*, 1985; Motomura *et al.*, 1995) as only fused protoplasts formed embryos. It also demonstrated the efficiency of the protocol adopted for Brazilian citrus cultivars (Mendes-da-Glória *et al.*, 1999). The regenerated somatic hybrids are currently maintained under greenhouse conditions and show adequate vigor. This hybrid may be useful as a rootstock since it should combine the drought tolerance and vigor of Rangpur lime with the blight tolerance of Caipira sweet orange, though it may have some susceptibility to *Phytophthora*. Another combination of sweet orange + Rangpur lime was previously described by Louzada *et al.* (1992) with the somatic hybridization between 'Hamlin' sweet orange and Rangpur lime.

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#### RESUMO

Híbridos somáticos de laranja doce (*Citrus sinensis* L. Osbeck) e limão Cravo (*C. limonia* L. Osbeck) foram regenerados após a fusão (polietileno glicol) e cultura de protoplastos. Os híbridos somáticos foram confirmados pela análise da morfologia das folhas, determinação do número de cromossomos e marcadores moleculares (RAPD). Todas as plantas analisadas revelaram-se tetraplóides ( $2n = 4x = 36$ ), possuíam folhas de morfologia intermediária e uma combinação do padrão de bandas

de RAPD de ambos os parentais. Esta combinação pode se tornar útil como porta-enxerto para a Região Sudeste da indústria cítrica brasileira. Este híbrido somático potencialmente combinará as características de tolerância à seca e o vigor do limão Cravo com a tolerância ao declínio da laranja Caipira.

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