Notas Científicas

Somatic hybridization between *Citrus sinensis* (L.) Osbeck and *C. grandis* (L.) Osbeck

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Abstract – This work had as objective to produce citrus somatic hybrids between sweet oranges and pummelos. After chemical fusion of sweet orange embryogenic protoplasts with pummelo mesophyll-derived protoplasts, plants were regenerated by somatic embryogenesis and acclimatized in a greenhouse. The hybrids of 'Hamlin' sweet orange + 'Indian Red' pummelo and 'Hamlin' sweet orange + 'Singapura' pummelo were confirmed by leaf morphology, chromosome counting and molecular analysis. These hybrids have potential to be used directly as rootstocks aiming blight, citrus tristeza virus, and *Phytophthora*-induced disease tolerance, as well as for rootstocks improvement programs.

Index terms: genetic improvement, protoplast fusion, rootstock.

Hibridação somática entre *Citrus sinensis* (L.) Osbeck e *C. grandis* (L.) Osbeck

Resumo – O objetivo deste trabalho foi produzir híbridos somáticos entre laranjas doces e toranjas. Após fusão de protoplastos embriogênicos de laranja doce com protoplastos derivados de mesófilo foliar de toranjas, plantas foram regeneradas por embriogênese somática e aclimatizadas em casa de vegetação. Os híbridos laranja 'Hamlin' + toranja 'Indian Red' e laranja 'Hamlin' + toranja 'Singapura' foram confirmados pela morfologia foliar, contagem do número de cromossomos e análise molecular. Esses híbridos apresentam potencial para serem testados como porta-enxertos tolerantes a declínio, tristeza e doenças causadas por *Phytophthora*, bem como em programas de melhoramento de porta-enxertos.

Termos para indexação: melhoramento genético, fusão de protoplastos, porta-enxerto.

Somatic hybridization is an important tool for supplying new varieties to be used directly as cultivars or in genetic improvement programs of cultivated species of *Citrus*. In Brazil, citrus protoplast fusion approaches have been developed since 1995, leading to the production of somatic hybrids for rootstock improvement aiming at the control of important diseases, such as blight and citrus tristeza virus (CTV) (Mendes-da-Glória et al., 2000; Mendes et al., 2001; Latado et al., 2002; Costa et al., 2003).

Gummosis, the most important fungal disease in Brazil, is caused by *Phytophthora parasitica* and *P. citrophthora*. It generally affects the trunk resulting in foot rot and gum-producing lesions, being able to expand to the main root up to 20–30 cm under ground level and up the trunk. Thus, trunk infections usually girdle and kill the tree. In the seedbeds, these fungi cause seedlings damping-off with brown and depressed lesions that enlarge and result in seedling death (Prates & Pelegrinetti, 1995). The obtainment and use of resistant rootstocks can be considered as the most important handling strategy against *Phytophthora*-induced diseases.

Among the rootstocks, *Poncirus trifoliata*, 'Swingle' citrumelo, citranges and sour orange are highly resistant to those pathogens. The mandarins, tangelos and lemons are moderately susceptible or tolerant, while sweet oranges, including 'Caipira' sweet orange, are highly susceptible (Davies & Albrigo, 1994). However, *P. trifoliata* and citranges showed to be susceptible to blight and lowly tolerant to drought, besides being incompatible with 'Pera' sweet orange, the most important cultivar for the Brazilian citrus industry (Pompeu Junior, 2001). Several interspecific and intergeneric somatic hybrids have already been produced to control *Phytophthora*-induced diseases, and their performance in the field has been tested (Grosser et al., 2000).

Pummelos (*C. grandis* L. Osbeck) have no commercial importance in Brazil. However, hybrids involving this species show potential to blight tolerance, adaptation to salinity conditions and high soil pH (Grosser et al., 2000). The objective of this research was the production of interspecific somatic hybrids that combine complementary traits namely disease resistance, using protoplast chemical fusion and pummelos as one of the parentals.

Protoplast fusion experiments were performed between 'Hamlin' sweet orange and 'Indian Red' grafted pummelo or 'Singapura' selected pummelo seedling cultivated in a greenhouse. 'Hamlin' sweet orange protoplasts were isolated from ovule-derived embryogenic callus, whereas protoplasts from the two pummelo cultivars were obtained from mesophyll, after incubation into an enzymatic solution of cellulase, macerozyme and pectolyase Y-23 (Costa et al., 2003).

After isolation, protoplasts were purified and fused by polyethylene-glycol. Protoplasts were cultivated in a liquid solution, in the dark, for about 30 days, and then transferred to solid medium for spontaneous somatic embryogenesis. Somatic embryos were germinated, then transferred to a rooting medium, and finally acclimated in containers with a commercial substrate in a greenhouse (Costa et al., 2003).

Cytological, molecular and morphologic analyses were performed to confirm the hybrid nature of regenerated plants. Characteristics like petiole shape, leaf color and thickness were evaluated. For chromosome counting, young-root tips were collected from regenerated plants and treated with 0.002 M 8-hydroxyquinoline following Vieira et al. (1993) protocol, and the ploidy level was verified. Randomly amplified polymorphic DNA (RAPD) profiles also were used to analyze the regenerated plants, with primers OPAA1, OPAA2, OPAA4, OPAA7, OPAA10, OPAA15, and OPAA18.

The hybrids 'Hamlin' sweet orange with 'Indian Red' pummelo (Figure 1) and 'Hamlin' sweet orange with 'Singapura' pummelo seedling (Figure 2) were obtained. From these fusions, a great deal of abnormal embryos was formed, with a low conversion rate into plantlets, demonstrating the genotype influence on regeneration of fusion-derived cultures.

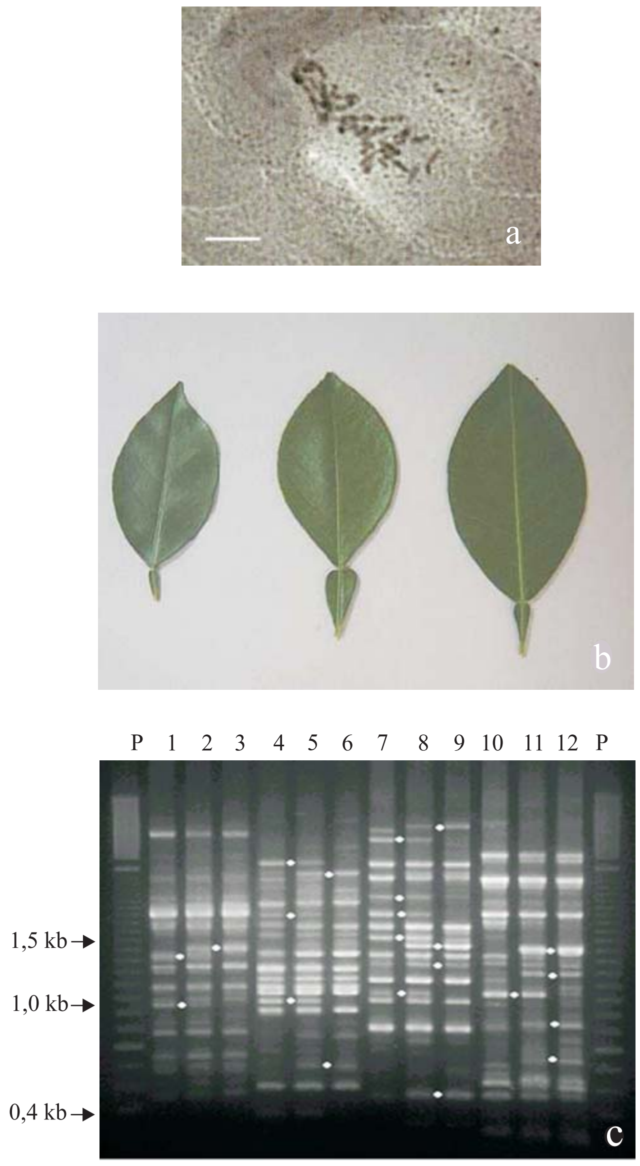

Figure 1. Characterization of somatic hybrid between 'Hamlin' sweet orange and 'Indian Red' pummelo. a) Mitotic metaphase of a somatic hybrid plant showing 2n=4x=36 chromosomes (bar = 1 μm); b) Leaf morphology of *Citrus sinensis* (left), somatic hybrid (center) and *C. grandis* (right); c) RAPD fragments of the parental lines 'Hamlin' (lanes 1, 4, 7 and 10) and 'Indian Red' grafted pummelo (lanes 3, 6, 9 and 12) and their somatic hybrid (lanes 2, 5, 8 and 11), amplified with primers OPAA4 (lanes 1–3), OPAA10 (lanes 4–6), OPAA15 (lanes 7–9) and OPAA18 (lanes 10–12). P = 1.0 kb ladder.

Mitotic analyses confirmed the polyploid nature of the cells (2n=4x=36) (Figures 1a and 2a). Leaf morphology of the hybrid plants was intermediate to the parents (Figures 1b and 2b) as demonstrated by previous research (Mourão Filho et al., 1996).

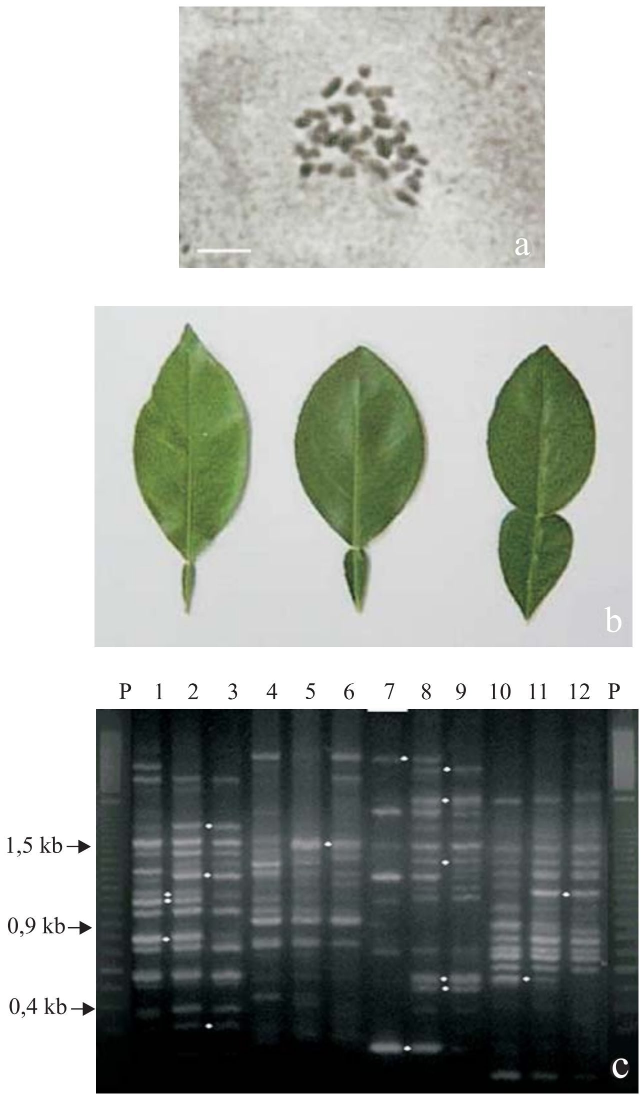

Figure 2. Characterization of somatic hybrid between ‘Hamlin’ sweet orange and ‘Singapura’ selected pummelo seedling. a) Mitotic metaphase of a somatic hybrid plant showing 2n=4x=36 chromosomes (bar = 1 μm); b) Leaf morphology of *Citrus sinensis* (left), somatic hybrid (center) and *C. grandis* (right); c) RAPD fragments of the parental lines ‘Hamlin’ (lanes 1, 4, 7 and 10) and ‘Singapura’ selected pummelo seedling (lanes 3, 6, 9 and 12) and their somatic hybrid (lanes 2, 5, 8 and 11), amplified with primers OPAA1 (lanes 1–3), OPAA2 (lanes 4–6), OPAA7 (lanes 7–9) and OPAA10 (lanes 10–12). P = 1.0 kb ladder.

Among the primers presenting polymorphism between ‘Hamlin’ sweet orange and ‘Indian Red’ grafted pummelo, OPAA4, OPAA10, OPAA15 and OPAA18 showed high-quality amplification patterns (Figure 1c). Considering the parents ‘Hamlin’ sweet orange and ‘Singapura’ pummelo seedling, the primers OPAA1, OPAA2, OPAA7 and OPAA10 amplified DNA sequences from both parents in the hybrid genotype (Figure 1c). The RAPD technique was able to prove the hybrid nature of the fusion products.

‘Hamlin’ is characterized for inducing good production of good quality oranges, but it is not used as rootstock due to its susceptibility to *Phytophthora*-induced diseases and blight (Louzada et al., 1992). Sweet oranges have been used as parents in several somatic hybridizations aiming for checking out traits of tolerance to blight. Somatic hybrids have been produced combining ‘Hamlin’ sweet orange with *P. trifoliata* cv. Flying Dragon (Grosser et al., 1988), ‘Rangpur’ lime (Louzada et al., 1992), *Severinia buxifolia*, ‘Rough’ lemon (Grosser et al., 1992) and ‘Femminello’ lemon (Tusa et al., 1990).

Pummelos, in turn, have been used as germplasm source, being able to contribute with tolerance to diseases such as *Phytophthora*-induced disease and CTV. A new gene providing resistance to CTV was identified in ‘Chandler’ pummelo, being denoted as *Ctv2* and controlled by only one dominant gene, quite similar to another gene identified in *P. trifoliata* (Fang & Roose, 1999). Pummelos show tolerance to saline stress (Moore et al., 2000) and high soil pH values. Pummelos were recently included in somatic hybridization programs in Florida.

Hybrids resulting from fusions between ‘Hamlin’ sweet orange and ‘Indian Red’ grafted pummelo and ‘Singapura’ pummelo seedling have potential to be used as rootstock, being expected to present tolerance to blight, CTV, and *Phytophthora*-induced diseases and its progeny to be highly nucelar, which is quite desirable in a rootstock variety.

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References

COSTA, M.A.P.; MENDES, B.M.J.; MOURÃO FILHO, F.A.A. Somatic hybridisation for improvement of citrus rootstock: production of five combinations with potential for improved disease resistance. **Australian Journal of Experimental Agriculture**, v.43, p.1151-1156, 2003.

DAVIES, F.; ALBRIGO, L. **Citrus**. Wallingford: CAB International, 1994. 254p.

FANG, D.Q.; ROOSE, M.L. A novel gene conferring citrus tristeza virus resistance in *Citrus maxima* (Burm.) Merrill. **HortScience**, v.34, p.334-335, 1999.

GROSSER, J.W.; GMITTER JUNIOR, F.G.; CHANDLER, J.L. Intergeneric somatic hybrids plants of *Citrus sinensis* cv. Hamlin sweet orange and *Poncirus trifoliata* cv. 'Frying Dragon'. **Plant Cell Reports**, v.7, p.5-8, 1988.

GROSSER, J.W.; GMITTER JUNIOR, F.G.; LOUZADA, E.S.; CHANDLER, J.L. Production of somatic hybrid and autotetraploid breeding parents for seedless citrus development. **HortScience**, v.27, p.1125-1127, 1992.

GROSSER, J.W.; OLLITRAUT, P.; OLIVARES-FUSTER, O. Invited review: somatic hybridization in *Citrus*: an effective tool to facilitate variety improvement. **In Vitro Celular & Developmental Biology - Plant**, v.36, p.434-449, 2000.

LATADO, R.R.; DERBYSHIRE, M.T.V. de; TSAI, S.M.; TULMANN NETO, A. Obtenção de híbridos somáticos de limão 'Cravo' e tangerina 'Cleópatra'. **Pesquisa Agropecuária Brasileira**, v.37, p.1735-1741, 2002.

LOUZADA, E.S.; GROSSER, J.W.; GMITTER JUNIOR, F.G.; NIELSEN, B.; CHANDLER, J.L. Eight new somatic hybrid citrus rootstock with potential for improved disease resistence. **HortScience**, v.27, p.1033-1036, 1992.

MENDES, B.M.J.; MOURÃO FILHO, F.A.A.; FARIAS, P.C.M.; BENEDITO, V.A. Citrus somatic hybridization with potencial for improved blight and CTV resistance. **In Vitro Cellular Developmental Biology - Plant**, v.37, p.256-261, 2001.

MENDES-DA-GLÓRIA, F.J.; MOURÃO FILHO, F.A.A.; CAMARGO, L.E.A.; MENDES, B.M.J. Caipira sweet orange + Rangpur lime: a somatic hybrid with potential for use as rootstock in the Brazilian citrus industry. **Genetics and Molecular Biology**, v.23, p.661-665, 2000.

MOORE, G.A.; GUY, C.L.; TOZLU, I.; WEBER, C.A. Mapping quantitative trait loci for salt tolerance and cold tolerance in *Citrus grandis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf. hybrid populations. **Acta Horticulturae**, v.535, p.37-45, 2000.

MOURÃO FILHO, F.A.A.; GMITTER JUNIOR, F.G.; GROSSER, J.W. New tetraploid breeding parents for triploid seedless citrus cultivar development. **Fruit Varieties Journal**, v.50, p.76-80, 1996.

POMPEU JUNIOR, J. Rootstocks and scions in the citriculture of the São Paulo state. In: INTERNATIONAL CONGRESS OF CITRUS NURSERYMEN, 6., Ribeirão Preto, 2001. **Proceedings**. Ribeirão Preto: International Society of Citrus Nurserymen, 2001. p.75-82.

PRATES, H.S.; PELEGRINETTI, J.R. **Controle sanitário e cultural**: legislação e relação de defensivos para citros. Campinas: Fundação Cargill, 1995. 40p.

TUSA, N.; GROSSER, J.W.; GMITTER JUNIOR, F.G. Plant regeneration of 'Valência' sweet orange, 'Femminelo' lemon and the interspecific somatic hybrid following protoplast fusion. **Journal of the American Society for Horticultural Science**, v.115, p.1043-1046, 1990.

VIEIRA, M.L.C.; AGUIAR-PERECIN, M.L.R.; MARTINS, P.S.M. A cytotaxonomic study in twelve Brazilian taxa of *Stylosanthes* Sw., Leguminosae. **Cytologia**, v.58, p.305-311, 1993.

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