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Production of Tetraploid Somatic Hybrid Breeding Parents for Use in Lemon Cultivar Improvement

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Abstract. **Allotetraploid somatic hybrid plants of 'Hamlin' sweet orange *(Citrus sinensis* L. Osbeck) + 'Femminello' lemon *(C. limon* L. Burm. f.), and Milam lemon (purported hybrid of *C. jambhiri* Lush) + 'Femminello' lemon were regenerated via somatic embryogenesis following protoplast fusion. 'Hamlin' and Milam protoplasts were isolated from undeveloped ovule-derived embryogenic callus cultures and fused using a polyethylene glycol method with seedling leaf-derived protoplasts of 'Femminello' lemon. Somatic hybrids were identified on the basis of leaf morphology, root-tip cell chromosome number, and electrophoretic analyses of phosphoglucose isomerase, phosphoglucose mutase, and 6-phosphogluconate dehydrogenase leaf isozymes. The somatic hybrids will be used in interploid crosses with lemon in an effort to generate seedless triploid lemon types with improved tolerance to mal secco disease.**

Somatic hybridization techniques for *Citrus* are well developed and are being employed to facilitate *Citrus* scion and rootstock improvement (Grosser and Gmitter, 1990a). *Citrus* somatic hybrids can be propagated and tested directly as *Citrus* rootstocks (Grosser and Gmitter, 1990a, 1990b, 1990c), or they may be used as tetraploid breeding parents in interploid crosses to generate seedless triploid zygotic progeny for selection of specific traits (Tusa et al., 1990). The production of a vigorous 'Valencia' sweet orange + 'Femminello' lemon somatic hybrid was reported by Tusa et al. (1990), which, if fertile, will be crossed with diploid lemons to combine the desirable quality and performance of 'Femminello' lemon with the cold hardiness and mal secco tolerance of 'Valencia' sweet orange (Tusa et al., 1990).

Improved tolerance of mal secco, a disease caused by the systemic fungus *Phoma tracheiphila* (Petri) Kantsch. & Gik. (Solel and Salerno, 1988), is an important lemon breeding objective in the Mediterranean and Black Sea areas (Tusa et al., 1990). Typical symptoms of this disease include veinal chlorosis, shedding of leaves, and subsequent dieback of twigs and branches (Reichert and Chorin, 1965).

The objective of the present research was to produce additional somatic hybrid combinations with potential for use as tetraploid breeding parents in lemon improvement. 'Hamlin' sweet orange is tolerant of ma1 secco, more cold-hardy than 'Femminello' lemon, and ripens much earlier than 'Valencia' sweet orange (≈5 months). Milam is a selection of rough lemon *(C. jambhiri)* that produces lemon-type fruit (Swingle and Reece, 1967). However, Milam leaf isozyme banding patterns indicate a hybrid origin and suggest that sweet orange could have been the pollen parent (Moore and Castle, 1988). Milam has not been tested for ma1 secco tolerance, but its genetic contribution to a somatic hybrid breeding parent, as compared to sweet orange, may lead to a greater frequency of lemon-type triploid zygotic progeny from subsequent interploid crosses.

Leaf-derived protoplasts of 'Femminello' lemon were isolated from nucellar seedlings maintained in a growth chamber (16-h photoperiod, 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, 26-30C) as previously described (Grosser and Chandler, 1987; Grosser and Gmitter, 1990a). 'Hamlin' sweet orange and Milam lemon protoplasts were isolated from undeveloped, presumably unfertilized ovule-derived embryogenic callus cultures initiated and maintained on MT (Murashige and Tucker, 1969) basal medium containing (in $g \cdot liter^{-1}$) 0.50 malt extract, 50 sucrose, and 8.0 agar, according to the method of Grosser and Gmitter (1990a). Protoplasts were purified by passage through a 45-µm stainless steel filter followed by centrifugation on a sucrose-mannitol gradient as previously described (Grosser and Gmitter, 1990a; Tusa et al., 1990). Protoplasts were mixed and fused using a polyethylene glycol (PEG) method de-

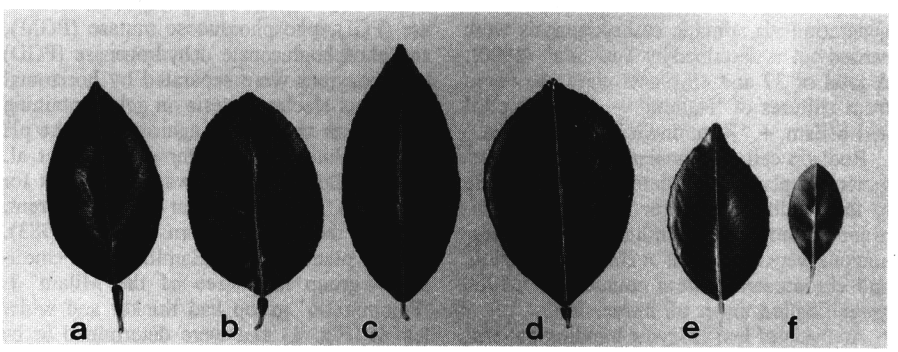

Fig. 1. Leaf morphology (left to right) of: (a) 'Hamlin' sweet orange, (b) 'Hamlin' + 'Femminello' somatic hybrid, (c) 'Femminello' lemon, (d) Milam + 'Femminello' somatic hybrid, (e) Milam lemon, and (f) Milam variant regenerated from unfused callus-derived protoplasts.

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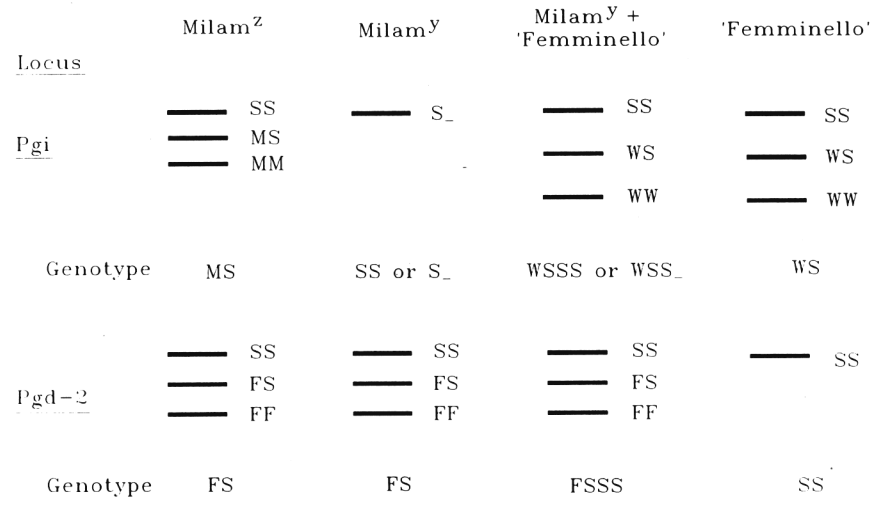

Fig. 2. Schematic diagram of PGI and PGD-2 isozyme banding patterns from Milam, 'Femminello', and their somatic hybrid. The origin is at the top of the diagram, and migration is toward the anode.
[z]Standard Milam.
[y]Milam callus line and protoplast regenerants.

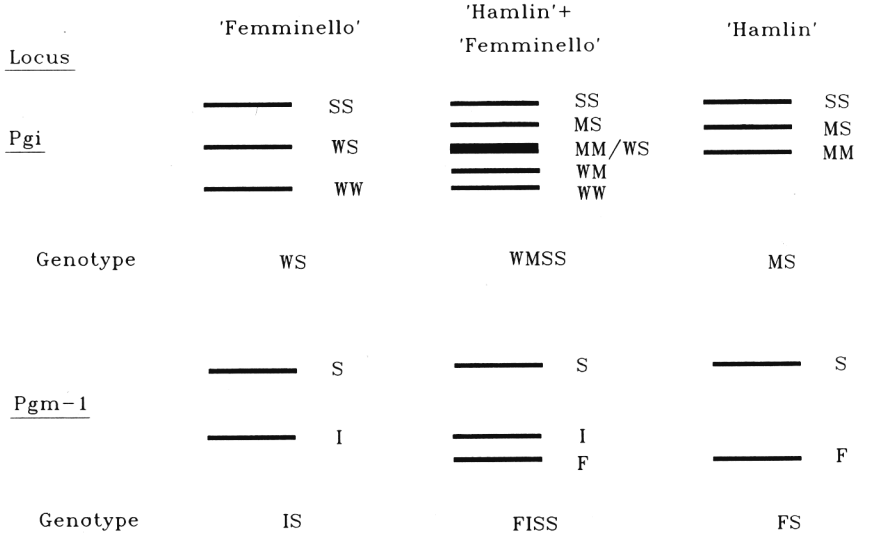

Fig. 3. Schematic diagram of PGI and PGM-1 leaf isozyme banding patterns from 'Femminello' lemon, 'Hamlin' orange, and their somatic hybrid. The origin is at the top of the diagram, and migration is toward the anode.

scribed by Grosser and Gmitter (1990a). Protoplast culture and subsequent plant regeneration via somatic embryogenesis were carried out as described by Tusa et al. (1990). A total of 37 and 48 plants were recovered from cultures of 'Hamlin' + 'Femminello' and Milam + 'Femminello', respectively.

Root-tip cell chromosome numbers of regenerated plants were determined according to the modified hematoxylin staining technique of Grosser and Gmitter (1990a). Preparations were scanned at × 200 magnification, and chromosomes were counted at × 1000 magnification under oil immersion.

Analysis of leaf isozyme banding patterns was performed using crude extracts of 'Femminello' lemon, 'Hamlin' sweet orange, and Milam lemon; regenerated plants; and the Milam callus itself. Phosphoglucose isomerase (PGI), phosphoglucose mutase (PGM), and phosphogluconate dehydrogenase (PGD) leaf isozymes were separated by horizontal starch gel electrophoresis on gels containing 10% starch and 0.15% agarose with the pH 5.7 histidine-citrate buffer of Cardy et al. (1981). Electrophoresis was carried out for 3 h at 4C and a constant 60 mA current. Staining recipes were from Vallejos (1983).

Two plants of the 'Hamlin' + 'Femminello' group and three of the Milam + 'Femminello' group had thicker and wider leaves (Fig. 1) and were determined to be tetraploids (2n = 4x = 36) by chromosome counts. These vigorous plants were later verified as somatic hybrids by leaf isozyme analysis (Figs. 2 and 3). Morphologically, the two groups of hybrid plants were distinguishable, but uniform, within each group. The Milam + 'Femminello' somatic hybrid was verified on the basis of leaf isozyme banding pattens of PGI and PGD. No single isozyme locus provided direct and complete evidence that these plants were hybrid because of the lack of unique parental alleles. The somatic hybrid pattern appeared the same as 'Femminello' for PGI (WSSS or WSS_). Milam is known to be MS at *Pgi* (Moore and Castle, 1988), but electrophoresis of callus tissue and leaf tissue of plants regenerated from callus-derived protoplasts revealed only the S band. It cannot be determined at this time whether the M allele has been lost or inactivated in this callus line and its regenerants, including the somatic hybrids, or if this callus line possibly originated from a self-derived zygotic embryo. PGD was assumed to be represented by two loci, with the faster migrating locus designated *Pgd-1,* as per Durham (1990). 'Femminello' is homozygous for a slow band at *Pgd-2* (SS), but Milam is heterozygous (FS) at *Pgd-2;* the somatic hybrid pattern contained the same bands as Milam. Therefore, the conclusion that these plants were, indeed, hybrid was based on the fact that they appeared identical to 'Femminello' at *Pgi* and to Milam at *Pgd-2.*

Complementary banding patterns were observed from the 'Hamlin' + 'Femminello' somatic hybrid for both PGI and PGM. 'Femminello' is WS and 'Hamlin' is MS at *Pgi;* the somatic hybrid genotype was found to be WMSS. The expected six bands corresponding to all possible dimeric combinations could not be distinguished because migration rates of several dimers were similar, and we were unable to separate them by the electrophoretic methods described. However, several repeated electrophoresis runs consistently revealed expression of all alleles (W, M, and S) from the donor parents in the somatic hybrid. Banding patterns of PGM (a monomeric enzyme) from the somatic hybrid clearly verified hybridity by expression of the F allele of 'Hamlin', the I allele of 'Femminello', and the common S allele.

The remaining diploid plants of both populations apparently were regenerated from unfused callus-derived protoplasts (35 'Hamlin' sweet orange and 45 Milam lemon). Unlike previous results (Tusa et al., 1990), no 'Femminello' lemon plants were recovered from unfused leaf protoplasts, perhaps because the seedlings used in our experiments were a few months older than those used for the earlier experiments. Although uniform, the regenerated Milam lemon plants were not typical of standard Milam (Fig. 1); they had shorter internodes, rounded leaf tips, and poor color and vigor, perhaps because of a mutation(s) or chromosomal rearrangement in the Milain callus line.

If fertile, these somatic hybrids can be used

in interploid crosses with diploid lemons to generate triploid seedless lemon types that can be selected for quality comparable to 'Femminello' and improved ma1 secco tolerance. Fertility of *Citrus* somatic hybrids has been adequate in the first three examined (Grosser, 1992; Kobayashi et al., 1991; Oghawara et al., 1991), and triploid hybrids have been recovered from a cross using a somatic hybrid as the pollen parent (Oiyama et al., 1991).

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