Rootstock breeding strategies for the mediterranean citrus industry; the somatic hybridization potential

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Received 19 February 1998
Accepted 1 May 1998

Fruits, 1998, vol. 53, p. 335–344

Resumen Español, p. 344

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Abstract — General trends of the mediterranean citrus industry. Sour orange was the dominant rootstock used in the mediterranean basin, however, because of the spread of citrus tristeza virus (CTV), there is an urgency to develop alternative rootstocks resistant to tristeza and well adapted to the constraints of the area. **Constraints for rootstocks prevailing in the mediterranean context**. The major problems affecting this citrus area are salinity, alkaline pH due to calcareous soils, tristeza, nematodes and *Phytophthora*. A new generation of rootstocks exhibiting good grafting compatibilities with local cultivars of citrus, and cumulating tolerance to these constraints must be worked out. **Possible strategies for new rootstock development**. Interesting traits of tolerance for biotic and abiotic stresses are present in the *Citrus* and *Citrus*-relative gene pools. To use this genetic diversity, the sexual recombination presents some limits because of the citrus reproductive biology and the high degree of heterozygosity of most citrus cultivars and rootstocks; on the other hand, the somatic hybridization is more promising for obtaining good agronomical traits and tolerances to pathogens such as tristeza and *Phytophthora*. **Somatic hybridization program for citrus rootstock breeding**. A total of 17 callus lines with potential interest for rootstock breeding was obtained by the Cirad-flhor team from ovule and seed culture. They were used to isolate protoplasts after electrofusion and somatic hybrids were selected after plantlet regeneration, of which a vigorous and resistant to tristeza promising intergeneric somatic hybrid between *P. trifoliata* and *C. deliciosa*, presently propagated by soft-wood cutting for further multilocal evaluations. However, the use of the embryogenic callus obtained after fusion during the regeneration process would be an interesting alternative for mass propagations. (© Elsevier, Paris)

mediterranean zone / *Citrus* / plant biotechnology / selection criteria / rootstocks / somatic hybridization

Sélection de porte-greffes d'agrumes pour la production méditerranéenne ; potentialités de l'hybridation somatique.

Résumé — Les tendances de la production d'agrumes en région méditerranéenne. Le bigaradier est le principal porte-greffe utilisé jusqu'à présent en région méditerranéenne, cependant l'extension du virus de la tristeza des agrumes (CTV) impose de devoir développer rapidement un nouveau porte-greffe résistant à cette maladie et bien adapté aux contraintes de cette zone de culture. **Facteurs limitant le développement des porte-greffes en région méditerranéenne**. Les principaux problèmes affectant cette zone sont la salinité, l'alcalinité des sols calcaires, la tristeza, les nématodes et le *Phytophthora*. De nouveaux porte-greffes offrant une bonne compatibilité avec les cultivars d'agrumes locaux et tolérants à ces diverses contraintes doivent être trouvés. **Stratégies possibles permettant le développement de nouveaux porte-greffes**. D'intéressants caractères de tolérance vis-à-vis de stress biotiques et abiotiques existent dans les espèces du genre *Citrus* et des genres apparentés. La recombinaison génétique, utilisable pour exploiter cette diversité génétique, est limitée par la biologie de la reproduction des agrumes et la forte hétérozygotie de la plupart des cultivars et porte-greffes ; en revanche, l'hybridation somatique apparaît comme étant une voie plus favorable au cumul des caractères d'intérêt agronomique et de tolérances à des pathogènes tels que la tristeza et le *Phytophthora*. **Programme d'hybridation somatique pour l'amélioration des agrumes**. À partir de cultures d'ovules ou de graines, l'équipe du Cirad-Flhor a constitué 17 lignées de cals, présentant un intérêt potentiel pour la sélection de porte-greffes. Elles ont permis d'isoler des protoplastes par électrofusion ; puis, après régénération des plantules, des hybrides somatiques ont été sélectionnés, dont un hybride intergénérique entre *P. trifoliata* and *C. deliciosa*, vigoureux et résistant à la tristeza, actuellement multiplié par bouturage pour de prochaines évaluation multilocales. Cependant, l'utilisation du cal embryogène formé après fusion au cours du processus de régénération serait une voie intéressante pour la propagation de masse. (© Elsevier, Paris)

zone méditerranéenne / *Citrus* / biotechnologie végétale / critère de sélection / porte-greffe / hybridation somatique

1. general trends of the mediterranean citrus industry

With citrus introduction tracing back some 20 centuries ago, the mediterranean basin is recognized as the first historical non-native region. As such, it is an important secondary center of genetic diversification, namely for sweet oranges, lemons, and mandarins. The wider mediterranean area has a global annual citrus output amounting to 17 Mt of which 87% are channelled to the fresh market and only 13% to juice processing. This is in sharp contrast with the North and South American production that is dedicated to juice processing for 80% of its global output [1].

Mediterranean citrus growers have thus focused increasingly their effort on the fresh fruit market. Sweet orange is still the main production of the area with, respectively, 2.6 Mt and 1.4 Mt for fresh fruit export and processing. This area has a pre-eminent position for easy peelers, especially for clementines, that are strongly attracting European consumers, with about 95% of the fresh world export (1.4 Mt) and 50% of fruit processing (0.3 Mt). The mediterranean area is also of significant importance for lemon production.

Sour orange was the dominant rootstock used in the mediterranean basin and 60% of the orchard area are still grafted onto this rootstock. However, the use of sour orange has declined and will soon be hampered with the spread of citrus tristeza virus (CTV). There is an urgency to identify or to develop alternative rootstocks resistant to tristeza and well adapted to the other biotic and abiotic constraints of the area listed below. Moreover, the predominance of the fresh fruit market requires the selection of rootstock inducing higher fruit quality and good fruit size [2].

2. constraints for rootstocks prevailing in the mediterranean context

Salinity is a major problem affecting the mediterranean citrus area; saline stress affects the agricultural productivity and the survival of many crops, including citrus [3]. Some citrus rootstocks such as Rangpur lime, Cleopatra mandarin, *Citrus macrophylla* or *C. amblycarpa* have an interesting tolerance to salinity while *Poncirus trifoliata* and its hybrids are very sensitive [4, 5].

Alkaline pH due to calcareous soils is an another serious concern for the mediterranean basin citrus industry. It is associated with debilitating iron chlorosis, that is particularly prevalent in semi-arid regions where irrigation is high in bicarbonates. Crop tolerance to iron deficiency has been proved to be genetically controlled [6]. For citrus, good tolerances have been identified in Mexican lime, Eureka lemon, Rough lemon, Sour orange, *C. macrophylla C. volkameriana* and *C. taiwanica* [7–10].

Tristeza, caused by the CTV is one of the most destructive diseases of citrus. Since 1957, the strains that are prevalent in the mediterranean area have already caused the death of more than 13 million trees grafted on sour orange, and CTV tolerant rootstocks have been recommended although they do not stop necessarily virus multiplication and evolution. The spread of severe strains and the potential risk of *Toxoptera citricidus* support the objective of selecting resistant, and not only tolerant, genotypes [11]. Garnsey, et al. [12] found immunity or a high level of resistance to CTV in seven lines of *Poncirus trifoliata*, in *Swinglea glutinosa* (Blanco) Merr., and in *Severinia buxifolia* (Poir.) Tenore. *P. trifoliata* immunity to CTV seems to be controlled by a single dominant gene [13]. In addition to *P. trifoliata*, the Carrizo and Troyer citranges are used as CTV-resistant rootstocks. Although CTV resistant, these rootstocks will not stand the adverse effect of exocortis or those of calcareous soil and salinity.

Nematodes (such as *Tylenchulus semipenetrans)* have some economic incidence on the citrus industry. Nematicides are expensive and environmentally unfriendly. Therefore, citrus rootstocks tolerant to nematodes are needed. Resistances have been found in *P. trifoliata*, various hybrids of *P. trifoliata* and *Severinia buxifolia* [14–16]. However some biotypes of citrus burrowing nematodes are able to circumvent the resistance of *P. trifoliata* [17].

Phytophthora foot and root rots are the most important fungal citrus diseases worldwide. Various species of *Phytophthora* sp. can attack citrus [18], however, *P. parasitica* and *P. citrophthora* are the most frequent in the mediterranean basin. Even if chemical treatments prove effective for containing *Phytophthora* attacks [19], tolerance to *Phytophthora* sp. is a fundamental requirement for citrus rootstocks. Such tolerance is found in *C. aurantium*, *P. trifoliata* and some mandarins, however, these rootstocks have other drawbacks such as tristeza susceptibility for *C. aurantium*, or calcareous, salinity and exocortis sensitivities for *P. trifoliata*.

As a whole, there are some limitations to the actual choice of conventional rootstocks. For instance, *P. trifoliata* and some of its hybrids (citranges and citrumelos) conferring interesting resistances to biotic stresses (tristeza, nematodes, *Phytophthora* sp., etc.) are susceptible to calcareous soils and salinity. On the other hand, most of the rootstocks of the 'lemon group' tolerant to tristeza confer a poor fruit quality and some degrees of intolerance to *Phytophthora* sp. and nematodes, especially in replant situation. Cleopatra mandarin, which displays good tolerances to salinity, tristeza and *Phytophthora* sp., has a rather low agronomical potential and is sensitive to cachexia, a viroid commonly found in the mediterranean area.

Even if practical solutions are temporarily found by combining the screening of conventional existing rootstocks and better policies of sanitation, it is clear that a new generation of rootstocks exhibiting good grafting compatibilities with local cultivars of sweet orange, mandarin, grapefruit and lemon, and cumulating tolerance to *Phytophthora* sp., nematodes, tristeza, alkalinity or salinity, must be worked out for the mediterranean citrus areas.

3. possible strategies for new rootstock development

Interesting traits of tolerance for biotic and abiotic stresses are present in the citrus and citrus relative gene pools (*table I*). Complementary genitors can be identified and two major breeding methods are offered for taping this genetic diversity: sexual recombination or somatic hybridization.

3.1. limits of the conventional sexual breeding

Progress with citrus genetic improvement and subsequent cultivar development by conventional breeding is a difficult task, mainly for the following two reasons.

– Reproductive biology: partial apomixis due to nucellar embryogeny, widespread in citrus, is seriously hampering the successful recovery of zygotic recombinants, even if morphological markers as trifoliate leaves or isozyme analysis [20] are useful for identifying zygotic progenies. The effective harnessing of genetic diversity of citrus and related genera remains also a difficult task [21]. Indeed, the low fertility of the intergeneric hybrids obtained between *Citrus* sp., *Fortunella* sp., *Poncirus* sp., *Microcitrus* sp. and *Eremocitrus* sp. [22] hinders the production of a second generation generally necessary for further breeding [23].

– Heterozygosity: molecular markers studies [24–26] have shown the high degree of heterozygosity of most citrus cultivars and rootstocks. This heterozygosity leads to important sexual recombination of genes in progenies and then is associated with low probability to obtain recombinant hybrids cumulating the desirable characters of the two parents (*table II*).

As a result, if several good rootstocks such as citranges and citrumelos have been selected in the United States by intergeneric sexual hybridization, they are generally not suitable for areas with greater constraint of salinity and alkalinity. New programs focusing on these constraints might be successful but require

Table I.
Distribution of some tolerance traits for abiotic (alkalinity, chlorides, boron, drough) and biotic (tristeza, nematodes, *Phytophthora*) constraints in the citrus gene pool.

Citrus varieties or species	Alkalinity	Chlorides	Boron	Drough	Tristeza	Nematodes	*Phytopthora*
Cleopatra mandarin	pT	TT	pT	pT	T	S	pT
Sour orange	T	pT	T	T	S	S	T
Sweet orange	pT	S	pT	T	T	S	S
Citrus amblycarpa	T	T	?	S	T	S	T
C. taiwanica	T	T	pT	pT	S	S	T
Lemon group							
Rangpur lime	pT	TT	T	TT	T	S	S
Rough lemon	TT	pT	PT	T	T	S	S
C. macrophylla	T	T	TT	T	S	S	T
C. volkameriana	T	pT	?	T	T	S	T
Mexican lime	T	pT	pT	T	S	S	S
Citrus relatives							
Eremocitrus glauca	T	TT	TT	TT	T	S	T
Severinia buxifolia	pT	TT	TT	pT	T	T	T
Poncirus trifoliata	S	S	S	pT	TT	TT	TT

TT: very tolerant; T: tolerant; pT: poorly tolerant; S: susceptible.

efficient early screening methodology at the greenhouse and even at the molecular level [27] to analyze very large progenies in order to identify good multilocus recombinants.

3.2. potential of somatic hybridization

In 1985, Ohgawara et al. [28] obtained the first somatic hybrid between *C. sinensis* and *P. trifoliata*. Since then, numerous intergeneric combinations were produced by various research teams [29, 30, etc.]. The addition of nuclear genomes from interspecific and intergeneric parents is relatively easy. This technique seems very promising for rootstock breeding as it cumulates all dominant genes for tolerance to biotic and abiotic stresses, irrespective of the heterozygosity of both parents (*table II.*). There is also a wider possibility of harnessing natural biodiversity as 1) somatic hybrids have been successfully developed between sexually incompatible species and genera [23, 31], 2) somatic hybridization enables to overcome sterility and to obtain fertile tetraploid hybrids from sterile diploids [32] and 3) it is easy to combine two polyembryonic parents.

Somatic hybridization has already been widely exploited for rootstock breeding in Florida by the Institut of Food and Agricultural Sciences. Promising results have been obtained at the agronomical level and for some tolerances to pathogens such as tristeza and *Phytophthora* [29]. This research team has also proved that tetraploidy itself confers some level of tolerance to nematodes [33]. So far, however, less information is available regarding the heritability of tolerances to abiotic factors such as alkalinity and salinity.

4. somatic hybridization program for citrus rootstock breeding

4.1. establishment of a callus line library

The establishment of a callus library of the potential genitors is a prerequisite for

Table II.
Example of allele transmission with sexual and somatic hybridization for nine independant loci (with only one homozygous locus for the tolerant allele among one of the parents).

Plant type	Allelic structure									Frequency
Parental genotypes										
Parent A	T	T	S	T	S	T	T	S	T	1
	S	S	S	S	S	S	S	S	T	
Parent B	S	T	T	S	T	S	S	T	S	1
	S	S	S	S	S	S	S	S	S	
Sexual progeny*										
parent A × parent B	S	T	S	T	S	T	S	S	T	0.5^8
	S	T	T	S	S	S	S	T	S	
	T	S	S	S	S	T	T	S	T	0.5^8
	S	T	S	S	T	T	S	T	S	
	S	S	S	T	S	S	S	S	T	0.5^8
	S	T	S	S	S	S	S	S	S	
	T	T	S	T	S	T	T	S	T	0.5^8
	S	T	T	S	T	S	S	T	S	
	T	T	S	T	S	T	T	S	T	0.5^8
	S	S	T	S	T	S	S	T	S	
	T	S	S	T	S	T	T	S	T	0.5^8
	S	T	T	S	T	S	S	T	S	
Somatic hybrid somatic	T	T	S	T	S	T	T	S	T	The tetraploid
progeny:	S	S	S	S	S	S	S	S	T	hybrid possesses
parent A + parent B	S	T	T	S	T	S	S	T	S	tolerant alleles for
	S	S	S	S	S	S	S	S	S	all the loci

S: susceptible allele; T: tolerant allele.
*: only 3×0.5^8 of the progenies possess all the tolerant alleles.

an efficient somatic hybridization program. Indeed, successful regeneration from heterofusion protoplasts is dependent on the callus embryogenic potential. Moreover, it is easier to isolate protoplasts from callus lines than from leaves of adult trees. True-to-type genetic origin is also easier to assess for callus lines than for in vitro seedlings that are classical explants for mesophyll protoplast isolation.

It has been proved that somatic hybrid cells are generally more competitive at the cellular level than the parental embryogenic callus one [34]. Consequently, it is not necessary to rely on a selection strategy by differential metabolic requirements of protoplasts arising from different explant sources (embryogenic callus protoplast and leaf protoplasts) as the one proposed by Grosser et al. [35]. Finally, somatic hybrids can also be obtained easily with embryogenic callus protoplasts from both parents.

Our callus lines were induced by ovule culture on Murashige and Tucker medium added with 1 $mg \cdot L^{-1}$ kinetin [25], or by seed culture on the medium described by Jumin and Nito [36]. A total of 17 callus lines with potential interest for rootstock breeding are presently being run (*table III*). The established embryogenic callus lines from ovule culture can be amplified in hormone free medium over several years or eventually cryopreserved in liquid nitrogen [37]. More difficulties are arising from the callus obtained at the hypocotyl

Table III.
Callus lines with potential interest for rootstock breeding obtained by Cirad-Flhor.

Embryogenic callus line	Callus line from mature seeds
Cleopatra mandarin	*Citrus macrophylla*
Bintangor mandarin	*C. amblycarpa*
Sunki mandarin	Rangpur lime
Sour orange	*Poncirus trifoliata* cv. Pomeroy
Willow leaf mandarin	*P. trifoliata* cv. Flying dragon
Shamouti sweet orange	Carrizo citrange
Star ruby grapefruit	*Muraya paniculata*
Mexican lime	*Clausena excavata*
Lakeland limequat	

of seedlings in the Jumin and Nito medium. In fact, the stabilization of embryogenic callus lines was successful only for limequat cv. Lakeland and Bintangor mandarin. The other lines require subculture on media supplemented with auxin and cytokinin as they frequently become compact calli.

4.2. electrofusion and hybrid selection methodology

Embryogenic callus lines of one parent versus leaves of the other or callus lines of the two parents are used to isolate protoplasts according to the method of Grosser and Gmitter [38]. Protoplast suspensions are then submitted to an AC electric field to produce the alignment of protoplasts; some pulses of 225 V (DC) are then emitted to induce protoplast fusions.

After electrofusion, the suspension of protoplasts in BH3 0.6 M medium [39] modified by Grosser and Gmitter [38] is poured into an MT 0.6 M sucrose solid medium [40] in 90 × 15 mm petri dishes. Protoplasts are then cultivated under dark conditions for 3 weeks at 28 °C. Subsequently, they are transferred to a culture room at the same temperature with 12 h of light daily. One month after electrofusion, the pro-embryos and cell lines are subcultured in MT 0.15 M sucrose or MT 0.15 M galactose solid medium for further development. When cotyledonary embryos are obtained, they are subcultured on MT medium added with 1 $mg \cdot L^{-1}$ GA3 and 0.1 M sucrose for root and shoot elongation.

Somatic hybrids are selected after plantlet regeneration by isozyme analysis and ploidy evaluation by flow cytometry [34].

4.3. obtention of a first promising hybrid

Cirad-Flhor has recently undertaken a rootstock breeding program, by somatic hybridization, with a view of addressing the mediterranean citrus rootstock challenge. Several interspecific and intergeneric combinations have been produced. A promising intergeneric somatic hybrid between *P. trifoliata* and *C. deliciosa* has already been developed for merging the tolerance traits towards biotic constraint of *P. trifoliata* with tolerance traits towards abiotic factors, such as alkalinity of *C. deliciosa*. This intergeneric hybrid has a vigorous growth (*figure 1*) and proved resistant to tristeza (Grisoni, personal communication). It is presently being propagated by soft-wood cutting (*figure 2*) for further multilocal evaluations concerning tolerance and agronomical traits.

4.4. methodologies for the propagation of new somatic hybrid rootstocks

Expedient propagation techniques of new somatic hybrids are essential for assessing their agronomical potential and for commercial applications. Considering the long juvenile phase, it is necessary to develop vegetative amplification methods from juvenile material.

The development of an amplification process by soft-wood cuttings is already experimented for the *C. deliciosa* × *P. trifoliata* hybrid [41] (*figure 1*). This technique relies on the induction of rooting of soft-wood microcuttings comprised of two or three internode segments in artificial controlled conditions of light, temperature and humidity. The principle is that, after flushing, axillary buds of each microcutting develop in shoots bearing new nodes. A period of 45 d after excision was necessary to enable the microcuttings to develop simultaneously an aerial and root system. An average of 90% of rooted cutting recovery was achieved, the roots having originated from the basal section of the explants. After acclimatization, 95% of the rooted micro-cuttings were transferred successfully on soil and rock wool blocks in greenhouse. Without delay, these nodes will be harvested for a next cycle of multiplication. This technique is convenient for low range production rates enabling evaluation trials. However, higher rates of multiplication and safer methodologies concerning disease-free materiel production will be required for the establishment of commercial plantations. In vitro micro-cutting techniques similar to the ones already developed for *Poncirus* Flying Dragon [42] would also be applied for somatic hybrids.

Somatic embryogenesis run from embryogenic callus obtained during somatic hybrid rooting (*figure 3*) should be an interesting alternative way for mass propagation. This method would allow for very high amplification rates and offers the possibility of storing the hybrid lines by cryopreservation [37] while the rootstock evaluation phase is carried out. Preliminary studies on somatic embryogenesis induction by carbohydrate content [43] or osmotic regulation [44] and embryo development [45] have already been performed. Additional studies are necessary, however, for the assessment of somatic embryo conformity and the optimization of embryo germination and plantlet recovery.

Figure 1.
Allotetraploid somatic hybrid between *Citrus deliciosa* and *Poncirus trifoliata*.

Figure 2.
Soft-wood cutting of the *Citrus deliciosa* × *Poncirus trifoliata* somatic hybrid.

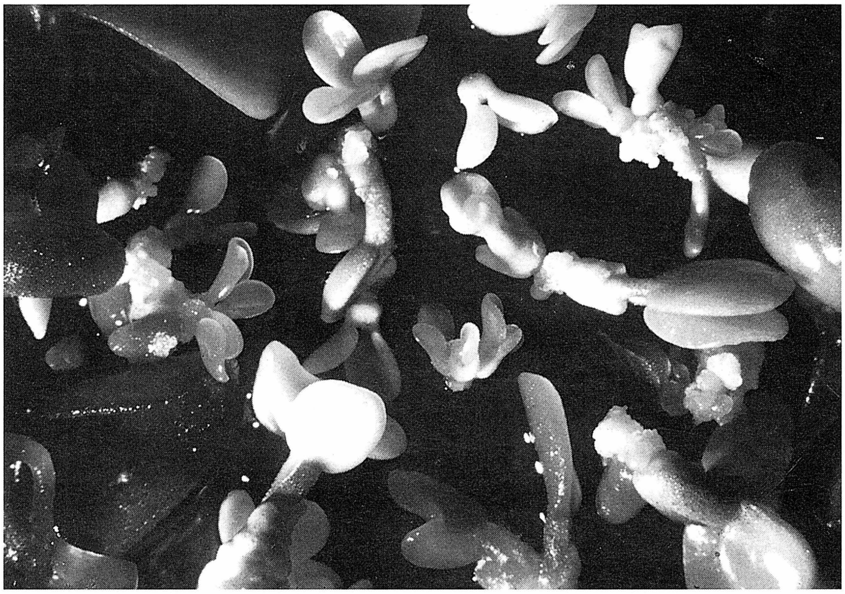

Figure 3.
Citrus amplification by somatic embryogenesis.

acknowledgement

The authors thank Michel Grisoni (Cirad-Flhor, Reunion) for the evaluation of tristeza tolerance.

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Selección de porta injertos de agrios para la producción mediterránea; potencialidades de la hibridación somática.

Resumen — Las tendencias de la producción de agrios en región mediterránea. El naranjo amargo es el principal porta injerto utilizado hasta el momento en región mediterránea, no obstante la extensión del virus de la tristeza de los agrios (CTV) hace imprescidible desarrollar rápidamente un nuevo porta injerto resistente a esta enfermedad y bien adaptado a las limitaciones de esta zona de cultivo. **Factores que limitan el desarrollo de los porta injertos en región mediterránea**. Los principales problemas que afectan esta zona son la salinidad, la alcalinidad de los suelos calcáreos, la tristeza, los nematodos y el *Phytophthora*. Se tienen que encontrar nuevos porta injertos que ofrecen una buena compatibilidad con los cultivares de agrios locales y tolerantes a estas varias limitaciones. **Posibles estrategias que permiten desarrollar nuevos porta injertos**. Interesantes caracteres de tolerancia frente a los estreses bióticos y abióticos existen en las especies del género *Citrus* y en los géneros aparentados. La recombinación genética, utilizable para explotar esta diversidad genética, se halla limitada por la biología de la reproducción de los agrios y la fuerte heterozigotia de la mayoría de los cultivares y porta injertos; en cambio, la hibridación somática resulta ser una vía más favorable a la acumulación de los caracteres de interés agronómico y de tolerancias a patogènos tales como la tristeza y la *Phytophthora*. **Programa de hibridación somática para el mejoramiento de los agrios**. A partir de cultivos de óvulos o de semillas, el equipo del Cirad-flhor ha constituido 17 descendencias de callos, que presentan un interés potencial para la selección de porta injertos. Permitieron aislar protoplastas por electro fusión; y luego, después de regeneración de las plántulas, se seleccionaron híbridos somáticos, entre ellos un híbrido inter genérico entre *P. trifoliata* and *C. deliciosa*, vigoroso y resistente a la tristeza, actualmente multiplicado por esqueje para próximas evaluaciones en distintos sitios. Sin embargo, la utilización del callo embriogeno formado después de fusión durante el proceso de regeneración sería una vía interesante para la propagación de masa. (© Elsevier, Paris)

zona mediterrànea / *Citrus* / biotecnología vegetal / criterios de selección / portainjertos / hibridación somática