Optimized Management of *Citrus* Embryogenic Calli for Breeding Programmes

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Fruits, vol. 49, n°5-6
p. 394-397 (English)
p. 477-478 (French)

Embryogenic calli were obtained for 15 Citrus *cultivars. Cryoconservation is now routinely used for long-term callus conservation. Isoenzyme and flow citometry techniques are applied for callus caracterization.*

introduction

Somatic embryogenesis is now widely used for protoplast fusion and gene transfer in *Citrus* breeding programmes. These latter techniques provide means to overcome some obstacles concerning genetic structures of cultivars and considerably expand the genetic base available to breeders. However, they require controlled management of embryogenic calli, the essential component of this system (Fig. 1). The main methodological advances jointly developed by CIRAD-FLHOR, INRA and ORSTOM are presented.

embryogenic callus induction

Friable embryogenic calli from polyembryonic varieties are conventionally induced by ovule culture. Depending on the genotype, the ovule can produce friable calli and embryos, compact chlorophyll calli, or just embryos. The results of isoenzyme studies (Ollitrault *et al.*, 1992b) and histological analyses (Cabasson, 1993) revealed that friable embryogenic calli are of nucellar somatic origin. Six months to 1 year after induction, such calli can be propagated on media without hormones. Callus strains have thus been obtained for 15 cultivars representing a wide-range of diversity within the *Citrus* genus. Embryogenic calli have also been formed around the hypocotyl in micropropagated somatic hybrids (Photo 1).

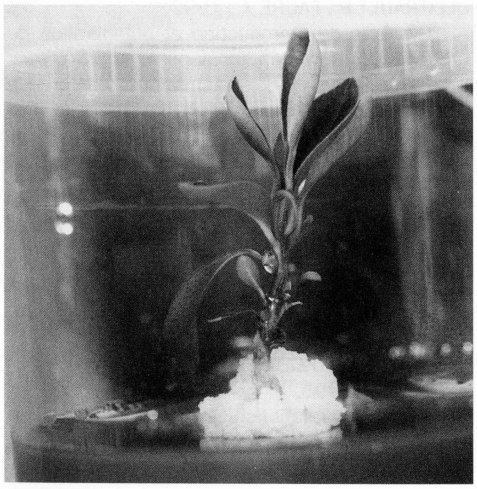

Photo 1
Formation of an embryogenic callus around the hypocotyl of a micropropagated somatic hybrid of C. deliciosa *and* F. japonica.

cryoconservation of embryogenic calli

A simplified technique for callus cryoconservation in liquid nitrogen has been developed (Engelmann *et al.*, 1994) to overcome callus induction problems with some genotypes and reduce somaclonal variations that could occur during successive subculturing. Calli are pretreated in a solution containing 0.15 M sucrose and 10% DMSO, placed in a small freezing module (Nalge Company) and then into a freezer set at −80°C. When the callus reaches −40°C, the cryotubes are plunged in liquid nitrogen. This technique is now routinely used for long-term callus conservation and can also be used to conserve duplicate fusion and transformation products during regeneration and evaluation processes.

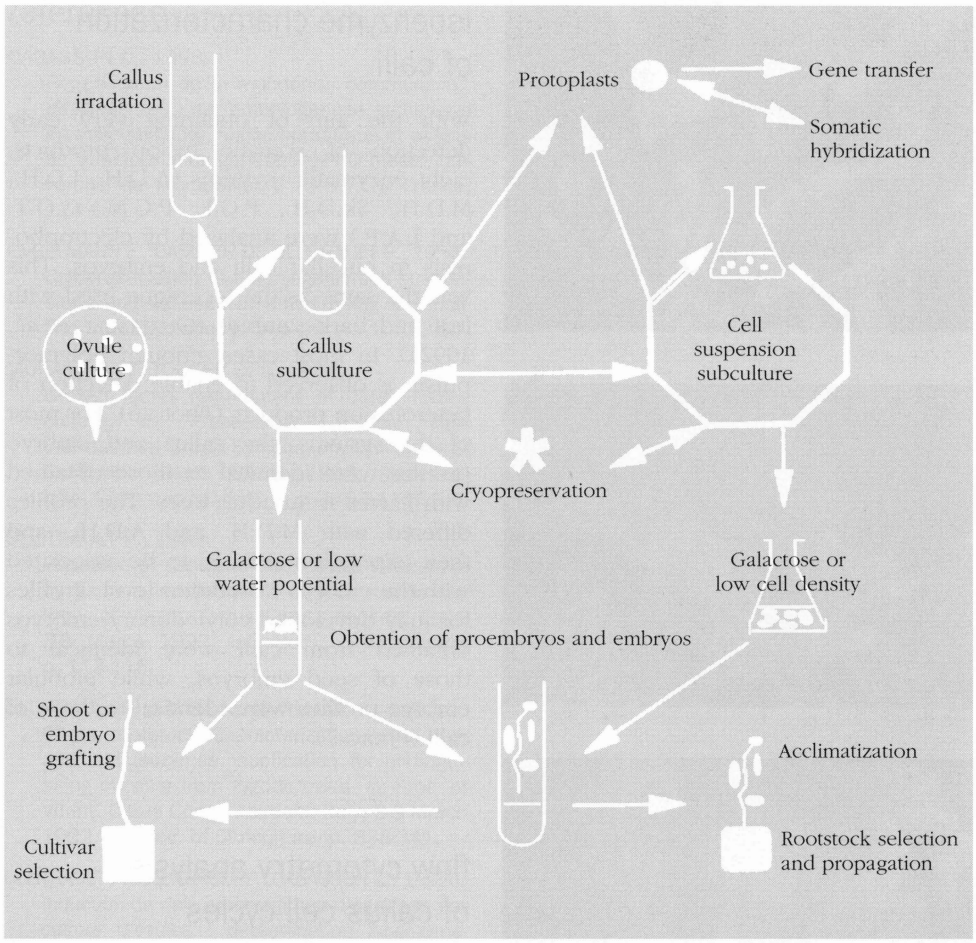

Figure 1
Management of embryogenic calli.

controlling embryo regeneration

Nucellar callus embryogenesis can be obtained on MURASHIGE and TUCKER medium (1969) without exogeneous hormones by modifying the form of the sugars contained in the medium (OLLITRAULT, 1992), or reducing the relative water content in the callus by increasing the gelling agent concentration (Fig. 2). With this original latter technique, embryos can be obtained for callus strains that do not respond to medium sugar modifications. Embryo development varies according to the callus strain and culture medium: cotyledonary embryos in cv Chios mandarin ("agar effect", Photo 2) and cv Sunki mandarin (galactose, Photo 3), and globular embryos in lemon (galactose, Photo 4). The observed suspensor-type structures (Photo 5) suggest that the embryos are of unicellular origin.

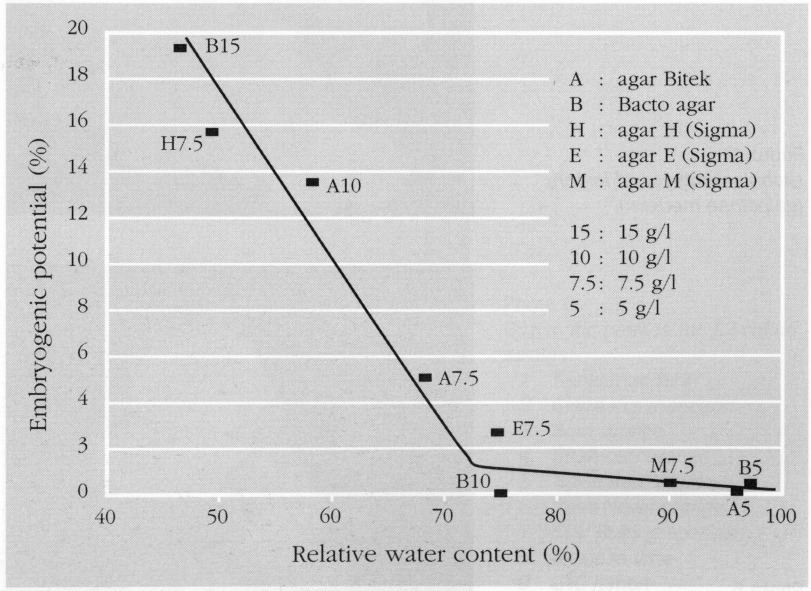

Figure 2
Control of embryogenesis in willow-leaf mandarin calli by modifying the type of agar and its concentration, in relation with the relative water content in the callus.

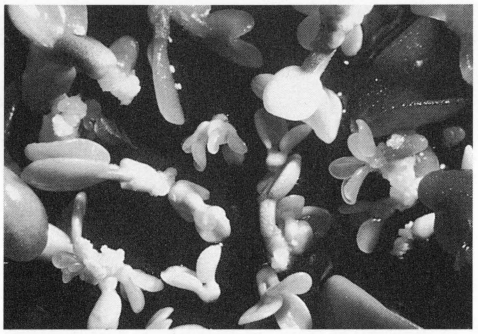

Photo 2
Cotyledonary embryos of cv Chios mandarin. Agar effect: MT medium (MURASHIGE & TUCKER, 1969) + 50 g/l sucrose + 4 g/l phytagel.

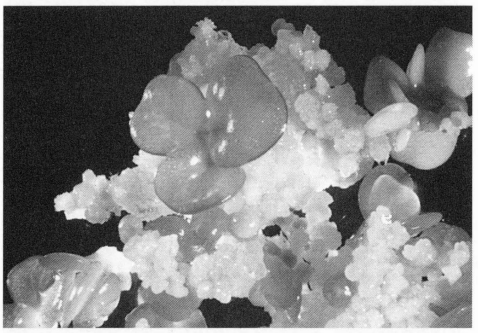

Photo 3
Cotyledonary embryos of cv Sunki mandarin. Galactose medium: MT medium + 30 g/l galactose + 2 g/l phytagel.

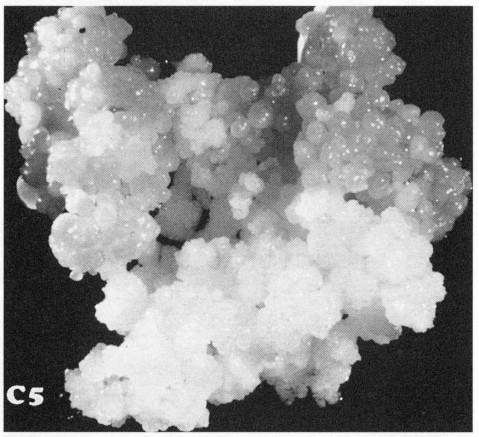

Photo 4
Globular embryos of lemon (galactose medium).

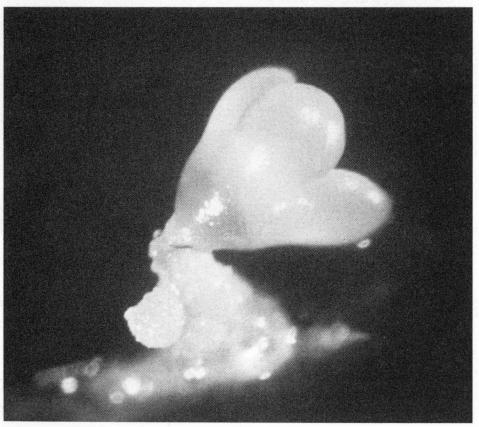

Photo 5
Embryo of C. deliciosa *attached to an embryogenic callus by a suspensor-type structure.*

isoenzyme characterization of calli

With the aim of obtaining very early detection of somatic fusion products, eight enzymatic systems (A.D.H., I.D.H., M.D.H., Sk.D.H., P.G.I., P.G.M. G.O.T. and L.A.P.) were analysed by electrophoresis in nucellar calli and embryos. This was the same as the technique used with leaf and bark samples (OLLITRAULT *et al.*, 1992a). In most cases, enough polymorphism is observed to enable detection of heterofusion products (Photo 6). For most of the systems, the callus and embryo profiles were identical to those obtained with leaves from adult trees. The profiles differed with M.D.H. and A.D.H., and their expression seemed to be associated with the embryo maturation level. Profiles for fully developed cotyledonary embryos obtained from calli were identical to those of seed embryos, while globular embryo profiles were identical to those of calli (Photo 7).

flow cytometry analysis of callus cell cycles and ploidy levels

Flow cytometry can be used to assess ploidy levels and relative proportions of cells in the G1 and G2 phases in organs and calli in the growth phase (OLLITRAULT and MICHAUX-FERRIERE, 1992). This latter information is important for isolating protoplasts to be used in somatic fusion and genetic transformation. Cytometry was used successfully to analyse protoplast nuclei obtained from embryogenic calli and stained with propidium iodide. In a preliminary study (Fig. 3), the highest levels of cells in the G2 phase (20%) were obtained 18 days after subculture. This technique is also useful for controlling ploidy stability of embryogenic calli formed around the hypocotyl of somatic tetraploid hybrids (Figure 1). This stability is necessary for mass propagation, involving somatic embryogenesis, of new rootstocks obtained in somatic hybridization programmes.

references

CABASSON C., 1993.
Régénération de la mandarine commune (*C. deliciosa* Ten.) par embryogenèse somatique en milieu liquide. Fusion somatique et essais de transformation génétique. PhD thesis, université de Montpellier II (U.S.T.L.), France, 124 p.

ENGELMANN F., DAMBIER D., OLLITRAULT P., 1994.
Cryopreservation of embryogenic cell suspensions and calluses of Citrus using a simplified freezing process. Cryo-Letters 15: 53-58.

MURASHIGE T. TUCKER DPH, 1969.
Growth factor requirement of citrus tissue culture. *In:* Proceedings of the First International Citrus Symposium, University of California, March 16-26, 1968. Riverside, USA, Chapman HD, vol. 3, p. 1155-1161.

OLLITRAULT P., 1992.
Research of seedless "willow leaf" mandarin by gamma irradiation of nucellar callus. *In*: Proc. of VII Int. Citrus Cong., Acireale, Italy, 3-8 March 1992. Int. Soc. of Citriculture, p. 113-116.

OLLITRAULT P., FAURE X., NORMAND F., 1992a.
Citrus rootstock characterization with bark and leaf isozymes; application for distinguishing nucellar from zygotic trees. *In*: Proc. of VII Int. Citrus Cong., Acireale, Italy, 3-8 March 1992. Int. Soc. of Citriculture, p. 338-341.

OLLITRAULT P., OLLITRAULT F., CABASSON C., 1992b.
Induction de cals embryogènes d'agrumes par culture d'ovules : détermination isoenzymatique de l'origine tissulaire des embryons. *Fruits* numéro spécial agrumes (47): 204-212.

OLLITRAULT P., MICHAUX-FERRIERE N., 1992.
Application of flow cytometry for Citrus genetics and breeding. *In*: Proc. of VII Int. Citrus Cong., Acireale, Italy, 3-8 March 1992. Int. Soc. of Citriculture, p. 193-198.

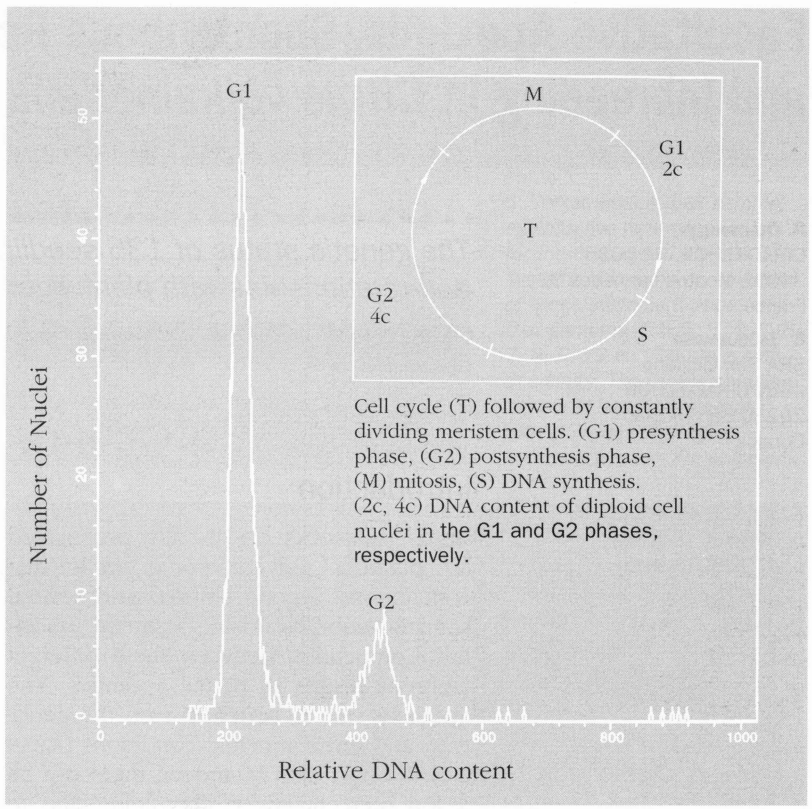

Figure 3
Analysis of the cell cycle of C. deliciosa *embryogenic calli (10 days after subculture).*

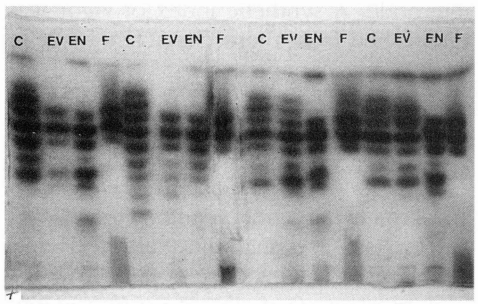

Photo 7
Comparison of MDH expression in calli (C), in vitro embryos (EV), nucellar seed embryos (EN), leaves (F), for four genotypes: cv Chios mandarin, cv Sunki mandarin, cv Shamouti orange and cv Star Ruby grapefruit.

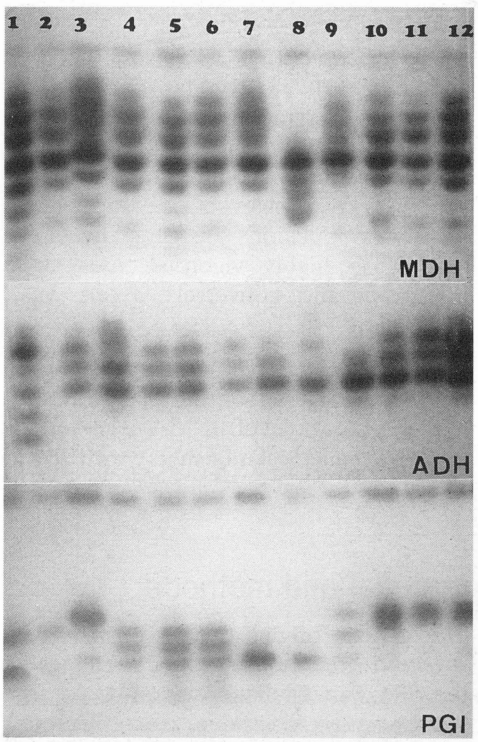

Photo 6
Enzymatic profiles for 12 callus strains:
1 *Sunki mandarin*
2 *Cleopatra mandarin*
3 *Sour orange*
4 *Shamouti orange*
5 *Navelate orange*
6 *Russ-Navel orange*
7 *Star Ruby grapefruit*
8 *Mexican lime*
9 *LAC lemon*
10 *Willow-leaf mandarin (G+)*
11 *Willow-leaf mandarin (G-)*
12 *Chios mandarin.*