Somatic embryo grafting : a promising technique for citrus breeding and propagation.

P. OLLITRAULT*

SOMATIC EMBRYO GRAFTING :
A PROMISING TECHNIQUE FOR CITRUS BREEDING AND PROPAGATION.

P. OLLITRAULT.

Fruits, Numéro spécial Agrumes 1992, p. 213-218.

ABSTRACT - Increasing attention is being devoted to somatic embryogenesis in the citrus improvement programs. An other advantage of this technology is to offer the possibility of high rate multiplication of healthy plants. There are however some drawbacks concerning the growth and acclimatization of small vitro plants. Furthermore, the juvenile characters are a serious hindrance to the mass propagation of scion cultivars. Embryo micro-grafting was experimented in our laboratory with a view to bypassing some of the above problems. Somatic embryogenesis was induced by *in vitro* culture of ovules from four citrus species. The technique consisted in the excision of the radicular poles of *in vitro* young embryos (4 to 6 mm size), and their grafting on to one-year-old rootstocks. The percentage of recovery was fairly high, and the growth of the young embryos was fast. This is indeed a very useful technique to save embryos and speed up the development of weak haploids, mutants, or somatic hybrids. Interestingly, the plant reaches the flowering stage early. The combination of *in vitro* somatic embryogenesis and *in vivo* embryo grafting can also be promising for the propagation of disease-free varieties, if, as we expect, the juvenility phase is drastically reduced by a very early grafting.

LE GREFFAGE D'EMBRYONS :
UNE TECHNIQUE PROMETTEUSE POUR LES PROGRAMMES D'AMELIORATION DES AGRUMES.

P. OLLITRAULT.

Fruits, Numéro spécial Agrumes 1992, p. 213-218.

RESUME - L'embryogenèse somatique prend une importance croissante dans les programmes d'amélioration des agrumes, et permet par ailleurs, des taux de multiplication élevés de matériel sain. Cependant, les phases d'élongation et d'acclimatation des vitroplants sont souvent longues et délicates. De plus, les caractères de juvénilité sont un obstacle majeur au recours à l'embryogenèse somatique pour la propagation des cultivars. La technique de greffage d'embryons somatiques a été expérimentée dans notre laboratoire pour tenter d'éviter certains de ces problèmes. L'embryogenèse somatique est induite par culture *in vitro* d'ovules de quatre espèces d'agrumes. Après avoir incisé le pôle radiculaire, les petits embryons somatiques (entre 4 et 6 mm) sont greffés directement *in vivo*, sur des porte-greffe d'un an. Le taux de réussite est élevé et le développement de la pousse est rapide. Cette technique pourrait être très utile dans le cadre des programmes de création variétale pour sauver des embryons peu vigoureux (haploïdes, mutants, hybrides somatiques, ...) et obtenir plus rapidement les premières floraisons. La combinaison de l'embryogenèse somatique *in vitro* et du greffage d'embryon *in vivo* pourrait également déboucher sur la propagation de masse de plants sains à condition que la phase juvénile soit considérablement réduite par un greffage très précoce.

INTRODUCTION

Somatic embryogenesis has been achieved since the seventies for Citrus polyembryonic species (BUTTON and BORNMAN, 1971 ; KOCHBA and SPIEGEL ROY, 1973, ...). The embryogenic capacity of nucellar callus was successfully developed to isolate protoplasts and regenerate entire plantlets (VARDI *et al.*, 1975 ; VARDI and SPIEGEL ROY, 1982). In fact somatic embryogenesis and protoplasts technology have great potential for citrus breeding (mutagenesis, somatic hybridization, gene transfer, ...) and also for germplasm preservation or cultivar propagation. However, *in vitro* elongation of embryos and subsequent acclimatization is a long and sometime a difficult process (BUTTON and KOCHBA, 1977). Furthermore, juvenility characters delay significantly the selection procedure, and constitute important limitation for propagating citrus by somatic embryogenesis.

Therefore we attempted to develop the embryo grafting technique for avoiding some of the inconvenients of somatic embryogenesis. This report deal with our first results and develop the potential application of this technique.

* -CIRAD-IRFA-INRA - SRA San Giuliano - 20230 SAN NICOLAO Haute Corse

MATERIAL AND METHODS

Embryo obtaining.

Fertilised and unfertilised ovules of four Citrus species [*C. sinensis* (L.) Osbeck cv. 'Valencia late', *C. aurantium* L., *C. deliciosa* Tenore and *C. limon* (L.) Burn cv 'Eureka'] were axenically excised during the first month after anthesis, and placed on solidified medium in 10 cm Pétri dishes. According to VARDI and SPIEGEL ROY (1982) the medium consisted of Murashige and Tucker (1969) nutrient medium (MT), lacking phytohormones, containing 5 g/l sucrose and 500 mg/l malt extract. Before autoclaving, the pH was adjusted to 5.7 and supplemented with 10 g/l Difco Bacto agar. The dishes were maintained at 26°C and exposed to 16 h of dim light daily. Subcultures were made in the same medium every 4 or 5 weeks.

Control of the somatic origin of embryos using isozyme electrophoresis.

Isozymes can be used for the determination of the sort of tissue (somatic, gametic or zygotic) which generated the *in vitro* embryogenesis (OLLITRAULT *et al.*, 1992).

Isocitrate dehydrogenase (IDH), Malate dehydrogenase (MHD) and Phosphoglucose isomerase (PGI) were studied in starch gel according to TORRES *et al.* (1978 and 1982).

The embryo grafting technique.

In vitro cotiledonary embryos (between 4 and 6 mm) were chosen (Fig. 1b). The radicular poles of embryos were cut (Fig. 1c). Then the embryos were grafted (T grafting) *in vivo* on one year old *C. volkameriana*, which is a very vigorous rootstock (Fig. 1d). The grafted rootstocks were enclosed in transparent plastic bags in order to secure high hygrometry. The young grafts were maintained at 26°C with 16 h of dim light daily. When the new shoot was 6 to 8 cm long, the grafted plants were placed in a greenhouse.

In vitro plantlet regeneration and *in vivo* acclimatisation of whole plants.

In order to compare the embryo grafting technique and the traditional technique of vitroplants elongation and acclimatization, cotyledonary embryos were isolated and transfered to MT medium containing 5 g/l sucrose and 10 g/l Difco agar in cultures tubes. The culture was maintained at 26°C with 16 h photoperiods. When *in vitro* plantlets were about 3 to 4 cm long, they were transplanted *in vivo* in «Jiffy pots» and cultured in saturated humidity under the same temperature and light conditions as precedently.

RESULTS

Embryo obtaining.

Embryos were recovered from all tested cultivars (Fig. 1a). The frequency of callus induction and the nature of callus were variable according to the cultivars. Green and compact calli were obtained directly from «Eureka» lemon ; such calli displayed very low embryogenic capacity. For the other cultivars («willow leaf» mandarin, «Valencia late» sweet orange and sour orange), white and granular callus were recovered directly from ovules or secondary from globular embryos. Those calli (especially for the «willow leaf» mandarin) exhibited high embryogenic capacity and they produced many embryos at the begining of subculture.

Study of the origin of embryos.

All the embryos stemming from unfertilized ovules displayed the same isozyme patterns as the parental cultivars. For each cultivar, one or more loci are heterozygous (PGI for «commune» mandarine and sour orange, ICD and PGI for «Valencia late» sweet orange, ICD, MDH1 and PGI for «Eureka» lemon) ; therefore it was clear that, all the embryos obtained from unfertilized ovules were somatic embryos. On the contrary, zygotic embryogenesis can be obtained by *in vitro* culture of fertilized ovules. Such zygotic embryos were clearly identified for mandarin and sour orange.

Plant obtaining by the embryo grafting technique and the traditional method.

In this preliminary experiment, about 30 embryos of the different cultivars were grafted in *C. volkameriana*. Ten day after grafting, 19 of them had started to develop small shoots (Fig. 1 e). After one month, the shoots were 6 to 8 cm long, and the grafted plants were transfered to a greenhouse. Ten weeks after grafting the shoot were 15 to 25 cm long with morphological conformity (Fig. 1 f).

During the same time, 24 embryos of each cultivars were put *in vitro* for germination. The rate of germination was more than 90%, but the growth was very slow. After 10 weeks the shoots were only 3 to 4 cm long (Fig. 1 f) but were nevertheless transfered in «jiffy pots» for acclimatization. This last step took 4 to 6 weeks for obtaining 6 to 8 cm plantlet with a good root system. We yielded good results (> 90%) for acclimatization, however this step required a constant attention and a lot of traitments against fungal infection.

DISCUSSION

Somatic embryos were obtained from pollinated and unpollinated ovules of the four cultivars under study. Isozyme analysis indicated that zygotic embryogenesis could also be obtained by pollinated ovule culture. This result open the possibility of obtaining hybrids between polyembryonic species. Two kinds of callus lines were obtained. Green and compact «Eureka» lemon calli originated from integument as demonstrated by KOBAYASHI (1987 a) whereas white embryogenic calli were proved to be nucellar calli (BUTTON and BORNMAN, 1971 ; KOBAYASHI *et al.*, 1981). Such white calli are the basic step of breeding program using somatic embryogenesis (Fig. 2).

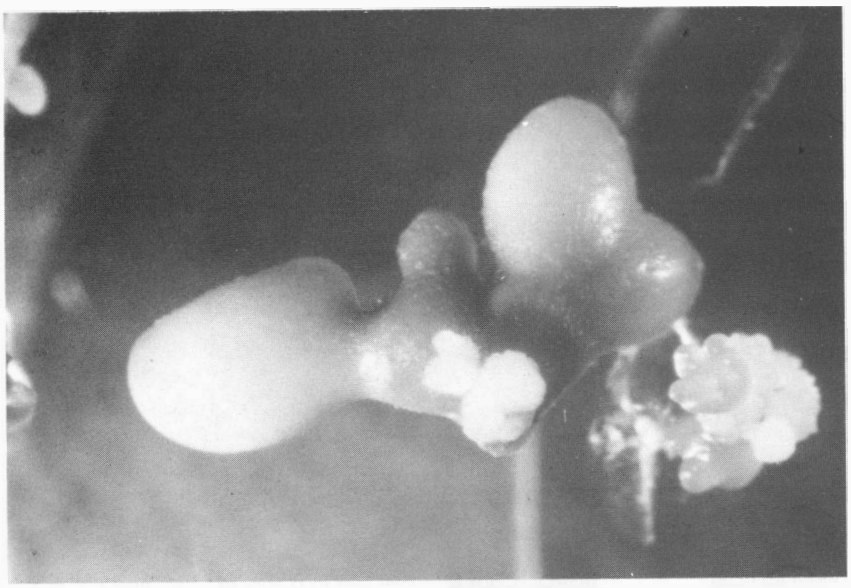
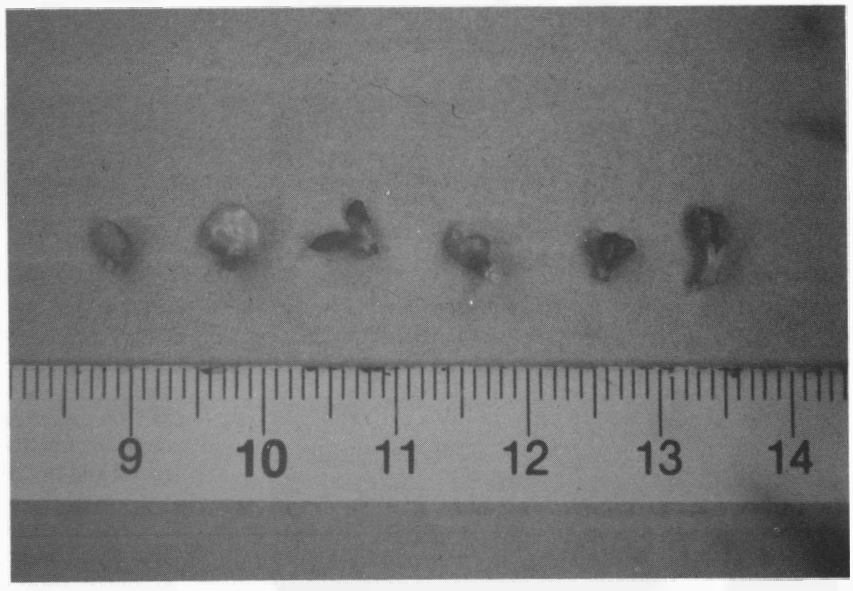
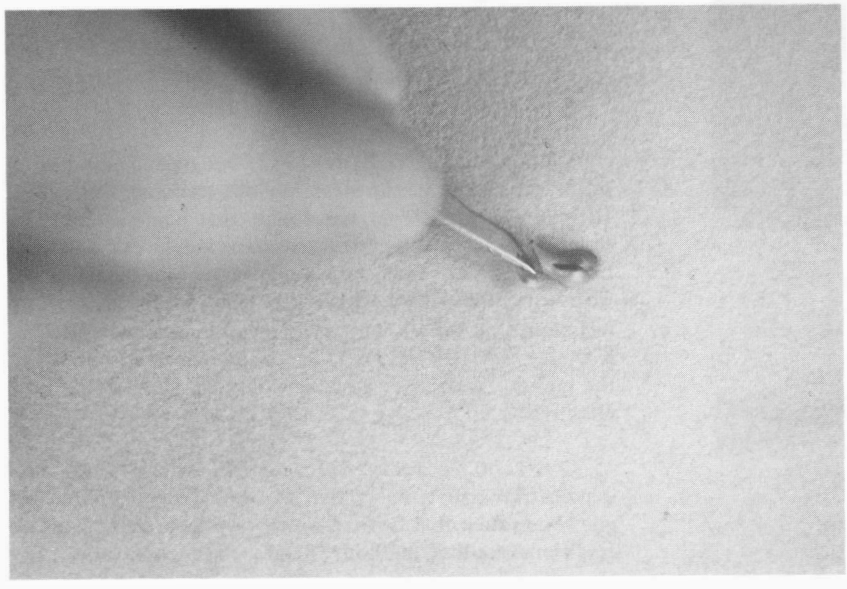


FIGURE 1 - Somatic embryos. grafting technique.

a : callus and somatic embryos from «willow leaf» mandarin.



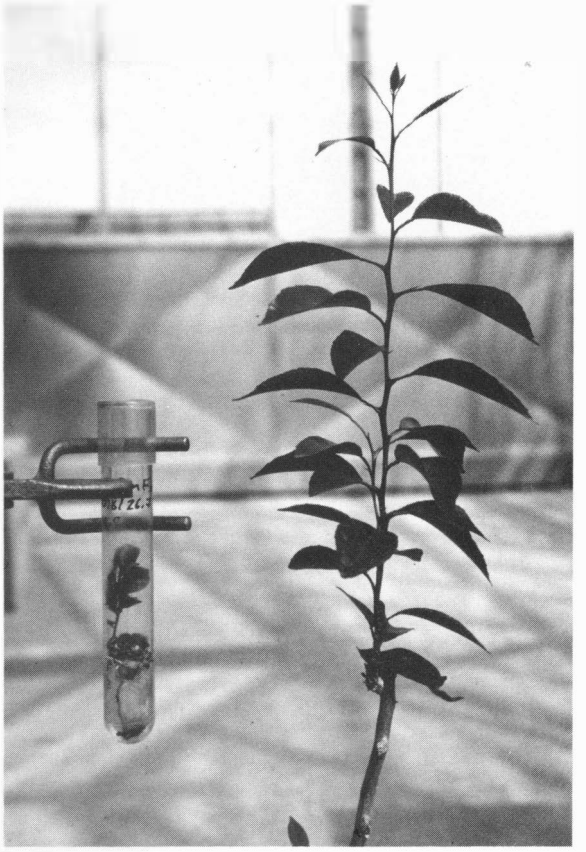
b : somatic embryos before grafting.



c : cutting of radicular pole.

d : embryo grafting.

e : development of the shoot 10 days after grafting.



f : comparison at ten weeks between embryo grafting and classical *in vitro* technique.

In this first experiment, the rate of success of embryo grafting (60%) was lower than that of traditional technique of elongation and acclimatization (80%). However it is clear that better results will be obtained with more experience and perhaps by using another technique of grafting.

The embryo grafting technique is much more simple and gives faster results than the traditional techniques of acclimatization

- the steps of *in vitro* elongation and acclimatization are suppressed,
- there is no more problem of *Phytophthora* or fungal infection of roots,
- the shoot development is much faster.

On coffea, COUTURON (1982) has grafted embryos of seeds in order to rescue weak haploid embryos. For citrus, HIDAKA *et al.* (1979) has found that haploid embryos, originating from anther culture, were very weak and that haploid plantlets were very sensitive to fungal infections. Therefore they have developed the approach grafting technique for the ultimate step of acclimatization (HIDAKA and KAJIURA, 1989) as for coffea, we think that the embryo grafting technique would be more simple and effective.

COUTURON (personnal communication) has also demonstrated that, on coffea, the embryo grafting technique allows their first flowering one or two years before the traditional seedling method. This is a considerable advantage

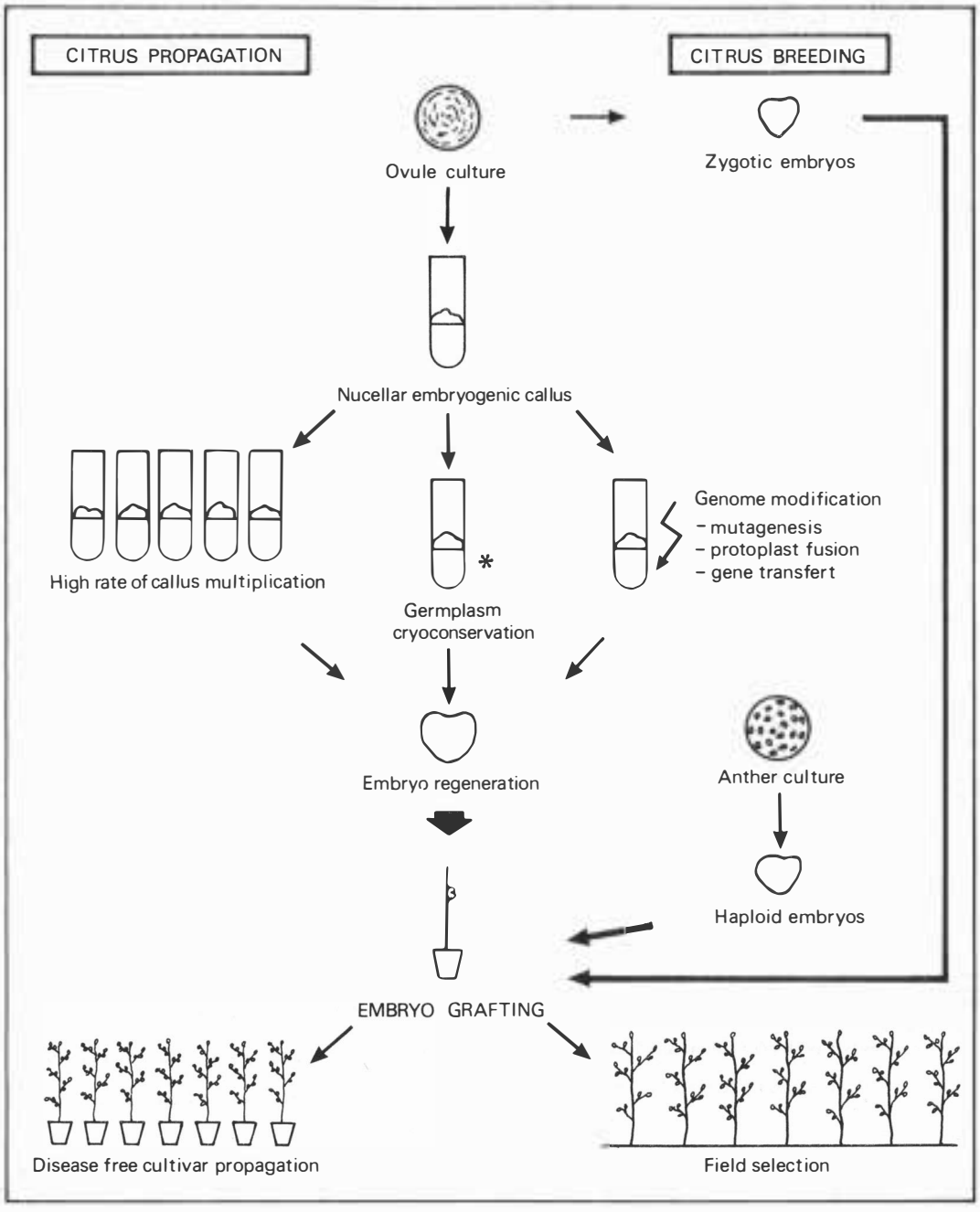


FIGURE 2 - Potential applications of *in vitro* embryogenesis and embryo grafting.

for breeders. So, if such a result is obtained for citrus, the embryo grafting technique would be a useful tool for all the variety breeding and conservation using the embryo pathway (Fig. 2) :

- Germplasm conservation by callus bank (HIDAKA and OMURA, 1989) or cryopreservation (SAKAI *et al.*, in press).

- Hybridization between polyembryonic species in combination with ovules cultures,

- Mutagenesis in callus or cell suspension (OLLITRAULT, 1992),

- Protoplast technology (KOBAYASHI *et al.*, 1988 a et b ; VARDI *et al.*, 1987 ...),

- Gene transfers (KOBAYASHI and UCHIMIYA, 1989 ; HIDAKA *et al.*, 1990).

A very high rate of multiplication can be obtained through somatic embryogenesis. Furthermore nucellar plants previously produced *in vitro* have responded negatively in tests for all identified citrus virus and virus like pathogens (BITTER *et al.*, 1972) and their genetic stability was demonstrated for polyembryonic species (STARRANTINO and RUSSO, 1983 ; KOBAYASHI, 1987 b). the ultimate application of somatic embryo grafting would be

mass propagation of disease free grafted trees. The effect of an early grafting on juvenility characters (such as thorns) is under study. A drastical reduction of these characters would be necessary for using embryo grafting as nursery tool.

CONCLUSION

This is the first of somatic embryo grafting for citrus. The results of this preliminary experiment will be confirmed and complemented by additional studies, but they are already very promising. This technique will be useful for breeding, not only for citrus but for all perenial crops. Its application for mass propagation will depend upon the reduction of juvenility characters.

REFERENCES

BITTER (W.P.), MURASHIGE (T.), RANGAN (T.S.) and NAUER (E.). 1972.
Investigation on establishing virus-free citrus plants through tissue culture.
In : *Proceeding of the 5th Conference of the International Organization of Citrus Virologist*, 267-271.

BUTTON (J.) and BORNMAN (C.H.). 1971.
Development of nucellar plants from unpollinated and unfertilized ovules of the Washington navel orange *in vitro*.
J.S. Afric. Bot., 37, 127-134.

BUTTON (J.) and KOCHBA (J.). 1977.
Tissue culture in the citrus industry.
In : *J. Reinert and Y.P.S. Bajaj (Eds). Applied and fundamental aspects of plant cell, tissue and organ culture. Springer-Verlag, Berlin, Heidelberg, New York*, p. 70-92.

COUTURON (E.). 1982.
Obtention d'haploïdes spontanés de *Coffea -Canephora* **PIERRE par l'utilisation du greffage d'embryons.**
Café, Cacao, Thé, 26 (3), 155-160.

HIDAKA (T.), YAMADA (Y.) and SHICHIJO (T.). 1979.
In vitro **differentiation of haploid plants by anther culture in** *Poncirus trifoliata* **(L.) Raf.**
Japan J. Breed, 29 (3), 248-254.

HIDAKA (T.) and KAJIURA (I.). 1989.
A simple method for acclimatization of *in vitro* **plantlets of Citrus.**
Bull. Fruit Tree Res. Stn., B. 16, 19-28.

HIDAKA (T.) and OMURA (M.). 1989.
Control of embryogenesis in Citrus cell culture.
Bull. Fruit Tree Res. Stn. B., 16, 1-17.

HIDAKA (T.), OMURA (M.), UGARI (M.), TOMIYAMA (M.), KATO (A.), OHSHIMA (M.) and MOTOYOSHI (F.). 1990.
Agrobacterium-mediated transformation and regeneration of *Citrus* **spp. from suspension cells.**
Japan J. Breed., 40, 199-207.

KOBAYASHI (S.). 1987 a.
Citrus protoplast technology : protoplast culture and somatic cell fusion.
In the breeding of horticultural crops, FFTC Book Serie n° 35, 123-135.

KOBAYASHI (S.). 1987 b.
Uniformity of plants regenerated from orange (*Citrus sinensis* **Obs.) protoplasts.**
Theor. Appl. Genet., 74, 10-14.

KOBAYASHI (S.), IKEDA (I.) and NAKATANI (M.). 1981.
Role of the primordium cell in nucellar embryogenesis in citrus.
in Proc. Int. Soc. Citriculture 1981, I, 44-48.

KOBAYASHI (S.), OHGAWARA (T.), OIYAMA (I.) and ISHII (S.). 1988 a.
Somatic hybridization between navel orange and murcott tangor.
Proceedings of the Sixth International Citrus Congress, Eds. R. Goren and K. Mendel. Margraf Scientific books, 135-140.

KOBAYASHI (S.), OHGAWARA (T.), OHGAWARA (E.), OIYAMA (I.) and ISHII (S.). 1988 b.
A somatic hybrid plant obtained by protoplast fusion between navel orange (*Citrus sinensis***) and satsuma mandarin (***C. unshiu***).**
Plant Cell, Tissue and Organ Culture, 14, 63-69.

KOBAYASHI (S.) and UCHIMIYA (H.). 1989.
Expression and integration of a foreign gene in orange (*Citrus sinensis* **Osb.) protoplasts by direct DNA transfer.**
Jpn. J. Genet., 64, 91-97.

KOCHBA (J.) and SPIEGEL-ROY (P.). 1973.
Effect of culture media on embryoid formation from ovular callus of «Shamouti» orange (*Citrus sinensis***).**
Z. Pflanzenzuchtg., 69, 156-162.

MURASHIGE (T.) and TUCKER (D.P.H.). 1969.
Growth factor requirements of Citrus tissue culture.
In : *Proc. First Int. Citrus Symp.*, vol. 3 (Ed. Chapman H.D.) 1155-1161. Riverside : Univers. California.

OLLITRAULT (P.). 1992.
Research of seedless «willowleaf» mandarin by gamma irradiation of nucellar callus.
VII Int. Citrus Cong. Acireale (Italy), Marsh 8-13.

OLLITRAULT (P.), OLLITRAULT (Frédérique) et CABASSON (Cécile). 1992.
Induction de cals embryogènes d'agrumes par culture d'ovules. Détermination isoenzymatique de l'origine tissulaire des embryons.
Fruits, Numéro spécial Agrumes 1992, p.204-212.

SAKAI (A.), KOBAYASHI (S.) and OIYAMA (I.).
Freeze preservation of nucellar callus of navel orange (*Citrus sinensis* **Osb. var.** *brasiliensis* **Tanaka) by a simple and novel method.**
Plant Cell Reports.

STARRANTINO (A.) and RUSSO (F.). 1983.
Reproduction of seedless orange cultivars from undeveloped ovules raised *in vitro*.
Acta horticulturae, 131, 253-258.

TORRES (A.M.), SOOST (R.K.) and DIEDENHOFEN (U.). 1978.
Leaf isozymes as genetic markers in Citrus.
Amer. J. Bot., 65, 869-881.

TORRES (A.M.), SOOST (R.K.) and MAU-LASTOVICKA (T.). 1982.
Citrus isozymes genetics and distinguishing nucellar from zygotic seedling.
J. Hered., 73, 335-339.

VARDI (A.), SPIEGEL-ROY (P.) and GALUN (E.). 1975.
Citrus cell culture isolation of protoplasts, planting densities, effect of mutagens and regeneration of embryos.
Plant Sci. Lett., 4, 231-236.

VARDI (A.) and SPIEGEL-ROY (P.). 1982.
Plant regeneration from Citrus protoplasts : Variability in methodological requirements among cultivars and species.
Theor. Appl. Genet., 62, 171-176.

VARDI (A.), BREIMAN (A.) and GALUN (E.). 1987.
Citrus hybrids : production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles.
Theor. Appl. Genet., 75, 51-58.

EL INJERTO DE EMBRIONES : UNATECNICA PROMETEDORA PARA LOS PROGRAMAS DE MEJORAMIENTO DE LOS CITRICOS.

P. OLLITRAULT.

Fruits, Numéro spécial Agrumes 1992, p. 213-218.

RESUMEN - La embriogenía somática va tomando una importancia creciente en los programas de mejoramiento de los cítricos, y permite, en particular niveles de multiplicación elevados del material sano. Sin embargo, las fases de alargamiento y de aclimatación de las vitroplantas son muchas veces largas y delicadas. Además, los carácteres de juvenilidad son un obstáculo mayor en el recurso a la embriogenía somática para la propagación de los cultivares. La técnica de injerto de embriones somáticos ha sido experimentada en nuestro laboratorio para tratar de evitar algunos de estos problemas. La embriogenía somática es inducida por cultivo *in vitro* de ovulos de cuatro especies de cítricos. Después de haber entallado el extremo radicular, los pequeños embriones somaticos (entre 4 y 6 mm) son interjados directamente *in vivo*, sobre porta-injertos de un año de edad. El porcentaje de éxito es elevado y el desarrollo del retoño es rápido. Esta técnica podría ser muy útil en el marco de los programas de creación varietal para salvar unos embriones poco vigorosos (haploïdes, mutantes, híbridos somáticos, ...) y obtener más rapidamente las primeras florescencias. La combinación de la embriogenía somática *in vitro* y del injerto de embrion *in vivo* podría igualmente resultar en la propagación masiva de plantas sanas con la condición de que la fase juvenil sea reducida considerablemente por medio de un injerto muy precoz.