Secondary somatic embryogenesis and plant regeneration of Troyer citrange from hypocotyl segments of nucellar embryos

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ABSTRACT
A protocol for secondary somatic embryogenesis and plant regeneration of Troyer citrange has been established. Embryogenic callus was induced on hypocotyls of 20-30-mm plantlets derived from nucellar embryos cultured on half-strength Murashige and Tucker medium (MT) supplemented with sucrose and gibberellic acid. Callus was then transferred on MT media differing by the carbon source: somatic embryos were obtained on lactose or galactose containing medium. Their formation was retained over successive subcultures on the lactose medium. Another way for embryogenesis was the multiplication of a callus stock on solid or liquid MT containing sucrose and malt extract: secondary somatic embryos differentiated after transfer of callus on MT medium containing lactose. Vitroplantlets were produced on solid half-strength MT medium supplemented with sucrose and gibberellic acid.

KEYWORDS
Citrus, citranges, plant propagation, *in vitro* culture, culture techniques, somatic embryos, callus, embryonic development.

Embryogenèse somatique secondaire et régénération de plants de citrange Troyer à partir de segments d'hypocotyles d'embryons nucellaires.

RÉSUMÉ
Un protocole d'embryogenèse somatique secondaire et de régénération de plants de citrange Troyer a été mis au point. Des cals embryogènes ont été obtenus sur des hypocotyles de plantules, issues d'embryons nucellaires cultivés sur milieu de Murashige et Tucker (MT) dilué de moitié et enrichi en saccharose et en acide gibbérellique. Ils ont été transférés, ensuite, sur un milieu MT présentant une autre source de carbone (lactose ou galactose) ; des embryons somatiques ont été obtenus et leur formation s'est maintenue sur plusieurs cycles de culture. Une autre méthode d'embryogenèse a consisté à multiplier un stock de cals sur milieu MT, solide ou liquide, contenant du saccharose et de l'extrait de malt : des embryons somatiques secondaires ont été différenciés après transfert du cal sur un milieu MT contenant du lactose. Des vitroplants ont été produits sur milieu solide MT dilué de moitié, additionné de saccharose et d'acide gibbérellique.

MOTS CLÉS
Citrus, citrange, multiplication des plantes, culture *in vitro*, technique de culture, embryon somatique, cal, développement embryonnaire.

Embriogénesis somática secundaria y regeneración de plantas de citranjo Troyer a partir de segmentos de hipocotilos de embriones nucelares.

RESUMEN
Se elaboró un protocolo de embriogénesis somática y de regeneración de plantas de citranjo Troyer. Se obtuvieron callos embriogenos a partir de hipocotilos de plantulas, procedentes de embriones nucelares cultivados en medio de Murashige y Tucker (MT) enriquecido en sucrosa y en ácido giberélico. Luego fueron transferidos en un medio MT presentando otra fuente de carbono (lactosa o galactosa); se obtuvieron unos embriones somáticos y su formación se mantuvo durante varios ciclos de cultivo. Otro método de embriogénesis consistió en multiplicar una reserva de callos en medio MT, sólido o líquido, conteniendo sucrosa y extracto de malta : unos embriones somáticos secundarios fueron diferenciados después de haber transferido el callo en medio MT conteniendo lactosa. Se produjeron vitroplantas en medio sólido MT, adicionado de sucrosa y de ácido giberélico.

PALABRAS CLAVES
Citrus, citranjo, propagación de plantas, cultivo *in vitro*, técnicas de cultivo, embrión somático, desarrollo embrionario.

introduction

The health and productivity of the citrus industry is often dependent on the rootstock used. *Citrus aurantium,* formerly the most important rootstock in every citrus-growing region of the world, is still important in some areas because of its yield potential, favorable influence on fruit quality and tolerance to cold, *Phytophthora*-induced diseases and citrus blight. However, its great susceptibility to tristeza-induced decline with sweet orange or grapefruit scions has drastically reduced or eliminated its use in most citrus-growing regions (LOUZADA *et al*, 1992; BROADBENT and GOLLNOW, 1993). *Poncirus trifoliata* is a major germplasm source for citrus tristeza virus (CTV) resistance, and for instance, Troyer citrange (*Citrus sinensis* (L) Osb x *Poncirus trifoliata* (L) Raf), a vigorous productive rootstock with good horticultural characteristics, is also immune to CTV (BROADBENT and GOLLNOW, 1993).

Somatic embryogenesis from ovule-derived nucellar callus cultures of *Citrus* has been extensively studied (KOCHBA *et al*, 1972; JUAREZ *et al*, 1976; KUNITAKE *et al*, 1991; VU *et al*, 1993) and numerous publications emphasize the genetical stability of the produced plants from polyembryonic cultivars. However, reproductive protocols for a continuous production of somatic embryos (SE) are limited and aberrant *Citrus* plants were sometimes obtained by somatic embryogenesis of nucellus from monoembryonic cultivars cultivated *in vitro* (NAVARRO *et al*, 1985). The aim of this work was to expand the applicability of somatic embryogenesis techniques from *Citrus* to Troyer citrange and to develop a method for promoting somatic embryogenesis from Troyer citrange nucellar embryos (NE) and the subsequent plant regeneration.

materials and methods

plant material and culture conditions

Nucelli of immature seeds of Troyer citrange were removed from fruit 3 months after fertilization and cultured onto a MURASHIGE and TUCKER (1969) medium (MT) containing sucrose (50 g/l) and different malt extract (ME) concentrations (500, 1000 and 1500 mg/l). The media were adjusted to pH 5.6, gelled with 0.6% Difco-Bacto agar, autoclaved at 110°C for 20 min and distributed in 90 × 15 petri dishes. When developed NE had reached a sufficient size (0.5-1 mm), they were removed from nucellar tissue and transferred on a solid half-strength MT supplemented with 20 g/l sucrose and 1 mg/l filtered gibberellic acid (GA3). Nucellar plantlets were formed and some showed a callus at the hypocotyl region.

Secondary somatic embryogenesis induction was initiated on the hypocotylar callus excised from these plantlets and subcultured on 20 ml solid MT medium containing several carbon sources. Experiments were realized in MT medium supplemented with sucrose, galactose or lactose at 50 g/l as carbon source, ME (1500 mg/l), abscisic acid (ABA: 0.5, 1 or 2 mg/l) and GA3 (1 mg/l).

Acclimatable vitroplantlets were obtained after culture of mature NE and SE for 60-90 days on a solid half-strength MT medium supplemented with sucrose (20 g/l) and GA3 (0.5 or 1 mg/l), in cultures tubes of 20 × 160 mm.

All the cultures were incubated in a growth chamber under a 16 h light/8 h dark cycle with a photon fluency rate of 50 $\mu mol/m^2/s$ (General Electric deluxe cool white fluorescent lamps). The temperature was 25 ± 2°C in the light and 20 ± 2°C in the dark.

histological study

Some fragments of embryogenic callus and individual SE were fixed in FAA (40% formalin: 6.5 ml; 50% ethanol: 100 ml; acetic acid: 3.5 ml), dehydrated and embedded in a glycol metacrylate hydrophilic resin (JB.4®, Polysciences Ltd, Eppelheim), cut to 3 µm thickness with an ultramicrotome Sorvall MT1 and collected in a drop of water containing NH_4OH. They were stained for polysaccharides by periodic acid Schiff (PAS) and for proteins by naphthol blue black (FISHER, 1968).

results and discussion

Troyer citrange nucellar embryos were formed from nucelli cultured both onto a growth regulator-free solidified MT medium without malt extract (ME), or with ME added at tested concen-

trations (table I). The mean comparison by Student's-Fisher test at 0.1% indicated that the embryo production was most effective in medium containing 1500 mg/l ME. The embryos developed from cultured nucelli of *Citrus* were assumed to have arisen from embryogenic cells that occurred in undeveloped ovules: they were produced *in vitro* by 15 of the 17 representative polyembryonic cultivars examined by MOORE (1985), while the ovules of five monoembryonic cultivars did not become embryonic. Anatomical studies in unfertilized undeveloped seeds of facultative apomictic *Citrus* showed that adventive embryos developed from initial cells at all positions in the nucellus (WAKANA and UEMOTO, 1987). In Troyer citrange floral buds, 8-10 embryonic cells were present in the nucellus. Callose deposits in the walls of the initial cells made their identification easier (BELKOURA *et al*, 1995) and could be an early cytological marker of the embryogenic differentiation process (DUBOIS *et al*, 1991).

When gibberellic acid (GA3) was added to the half-strength MT medium, Troyer citrange NE conversion to plantlets was observed. About 45% of 2-month-old plantlets (20-30 mm long) presented a small callus on the hypocotyl (photo 1). The white friable aspect of this callus resembled the embryogenic calli of Trovita orange (KOBAYASHI *et al*, 1983) and Satsuma mandarin (KUNITAKE *et al*, 1991). KOCHBA *et al* (1972) and GMITTER and MOORE (1986) had already indicated that GA3 supplemented medium was sufficient to stimulate germination of *Citrus* somatic embryos (SE). Exogenous GA3 at low concentrations (0.5 or 1 mg/l) ensured a balanced germination and successful conversion of SE initiated in Troyer citrange callus; higher concentrations (2 or 5 mg/l) gave rise to abnormal embryos and necrosis of shoot apex.

Secondary Troyer citrange somatic embryogenesis was induced upon the transfer of nucellar embryo hypocotyl-derived callus to a growth regulator-free solidified MT medium supplemented with lactose or galactose. Embryogenic callus was never obtained with sucrose. Green globular proembryos were formed after 4-5 weeks of culture. When these structures remained on lactose or galactose containing media, further stages of embryo development appeared. Photo 2 shows a typical 2-month-old embryogenic culture, with a fugacious callus, numerous proembryos and some globular, heart-shaped and cotyledonary green embryos. The number of somatic embryos (SE) and the weight of embryogenic callus (callus and proembryos) were determined on 2-month-old cultures. Lactose was a better source of carbon than galactose for induction and development of SE (table II). Our results agree with those of KOCHBA *et al* (1982) who demonstrated the stimulatory effect of galactose and lactose on embryogenesis with nucellar callus in several *Citrus* cultivars (only lactose stimulated embryogenesis in *Citrus aurantium*), while fructose, glucose and sucrose were less effective in stimulation of embryogenesis. In 1983, BEN-HAYYIM and NEUMANN reported the ability of several cell cultures of *Citrus* to grow with glycerol as the sole carbon source and a stimulatory effect of this polyalcohol in the embryo formation. More recently, somatic embryogenesis was also obtained in *Citrus deliciosa nucellus by replacing sucrose by galactose at the same concentration* (CABASSON *et al*, 1995).

Table I
Effect of malt extract (ME) on nucellar embryo (NE) production from Troyer citrange nucelli after 6 weeks of culture. Basal medium was Murashige and Tucker with 50 g/l sucrose. Results were mean of NE ± SD.

ME concentration (in mg/l)	*Number of cultured nucelli*	*No of NE produced per treatment*	*Mean number of NE per nucellus*
0	150	691	4.61 ± 1.35
500	150	831	5.54 ± 1.80
1000	150	1002	6.68 ± 1.77
1500	150	1226	8.17 ± 2.06

Table II
Effects of lactose and galactose on somatic embryogenesis of Troyer citrange hypocotyl-derived callus. Experiments were conducted with 100 embryogenic calli (2 mg fresh weight/callus) on Murashige and Tucker medium. Results (mean ± SD) were collected after 2 months of culture. GGE, globular green embryos. DSE, developed somatic embryos (heart-shaped and cotyledonary stages). FW, fresh weight.

Sugars (50 g/l)	*Embryogenic callus (mg FW)*	*GCE No/callus*	*DSE No/callus*
Galactose	82.0 ± 7.1	86.8 ± 16.6	7.1 ± 2.1
Lactose	302.2 ± 9.3	221.3 ± 55.0	45.0 ± 5.1

Embryogenic callus potentialities were conserved over several subcultures on solidified media containing lactose or galactose, but once established, the embryogenic potentialities could also be maintained on sucrose (50 g/l) containing MT medium. Histological sections made on embryogenic callus showed a mass constituted by small groups of isodiametric, starch-containing cells with high nucleoplasmic ratio, a dense cytoplasm and a quite large nucleolus (photo 3). Such cells were characteristic of proembryogenic masses, as indicated by MICHAUX-FERRIÈRE and SCHWENDIMAN (1992) on coffee, oil palm and other species and by BARCIELA and VIEITEZ (1993) on *Camellia japonica*.

Germination and conversion into plantlets of heart-shaped and cotyledonary secondary SE were obtained onto half-strength MT medium added with 20 g/l sucrose and 0.5 or 1 mg/l GA3. Under these conditions, some of them showed a hypocotyl callus which might be used to initiate another embryogenic cycle. So, citrange SE which developed without the need of a maturation step were comparable with 'self-controlled somatic embryos' of *Coffea arabica* (NEUENSCHWANDER and BAUMANN, 1992). White or green globular embryos did not differentiate under these conditions. Addition of abscisic acid (ABA) to make the maturation step easier (KIM and JANICK, 1989) did not increase the formation of germinating embryos and plantlets. On the contrary, in medium supplemented with ABA, some globular embryos proliferated to form a compact and brown nodule or a white callus. Others produced adventitious embryos directly on their surface (photo 4). The behavior of these structures differed with regard to media used: adventitious embryogenesis was maintained through subcultures of globular SE and nodules on MT supplemented with sucrose (50 g/l) and a low concentration of ABA; best results were obtained with 0.5 mg/l ABA. Adventitious embryogenesis was also noted at the root region of developed SE. The white callus behaved like embryogenic hypocotylar callus when cultured in presence of lactose or galactose, without ABA.

Histological sections of nodules cultured in ABA-containing medium showed the presence of two external layers of cells with embryogenic characteristics. The presence of such cells might explain potentialities of nodules to form directly adventitious embryos on their surface. Reculture of nodules onto MT supplemented with lactose or galactose (50 g/l) and without ABA allowed the extension of embryogenic cell characteristics to most of the inside cells (photo 5). One month after the transfer, proembryogenic masses were observed at the surface of the nodules and, 2 months later, the external cell layers burst and embryogenic calli were formed, in which globular embryos appeared (photo 6).

Transfer of nodules and embryogenic callus onto MT with ME was attempted to induce proliferation of a callus without formation of embryos. Such a callus was obtained over successive subcultures on solid MT medium supplemented with ME (1500 mg/l) and sucrose (50 g/l). Subcultures of this callus in liquid-agitated MT medium did not enhance the biomass production (photo 7). SE were formed on these long-term calli following transfer to growth regulator-free MT added with lactose or galactose. Germinating embryos converted into normal plantlets on solid half-strength MT medium with 20 g/l sucrose and 1 mg/l GA3 (photo 8).

conclusion

This study allowed to propose a six-step micropropagation procedure (fig 1):

1) development of embryos from nucelli of immature seeds;

2) conversion of nucellar embryos into plantlets with an hypocotylar callus;

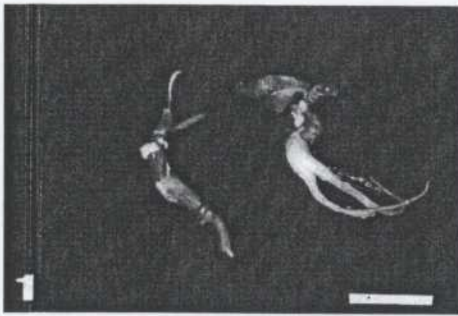
3) obtention of embryogenic callus and formation of secondary somatic embryos;

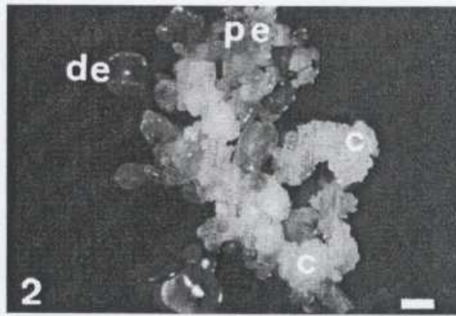
4) germination and conversion of somatic embryos and acclimatization of plantlets;

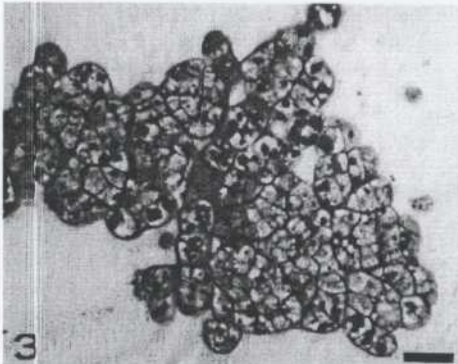
5) subculture of callus on solid or in liquid agitated medium, with retention of embryogenic potentialities (callus stock); and

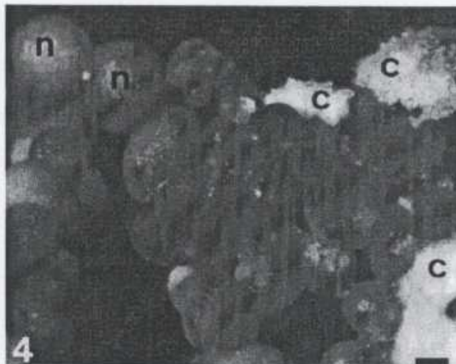
6) induction and development of secondary somatic embryos, after transfer of the callus to a growth regulator-free MT medium containing lactose.

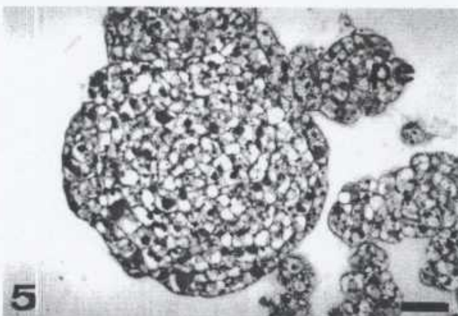
Secondary somatic embryo number produced through the first 5 months of culture was also evaluated. Under the best conditions 180 developed somatic embryos per nucellus were obtained and the conversion rate into plantlets was

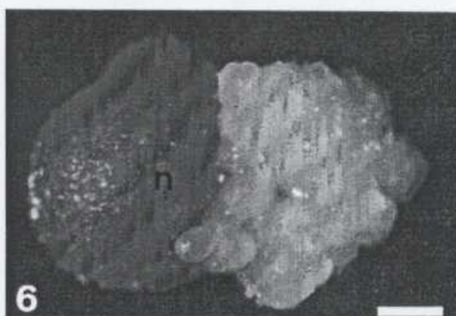


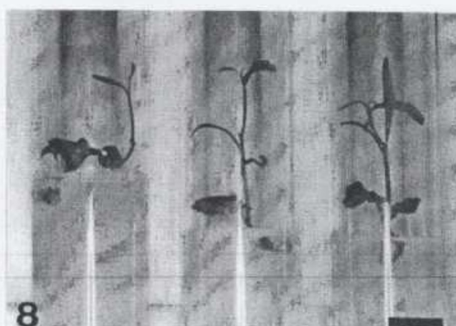












Photos 1-8.
***1**. Two-month-old plantlets with hypocotylar callus derived from nucellar embryos. Bar = 10 mm.*
***2**. Two-month-old embryogenic culture with callus (c), proembryos (pe) and developed embryos (de). Bar = 1 mm.*
***3**. Histological section of embryogenic callus. Double staining: PAS (periodic acid Schiff)/naphtol-blue-black. Bar = 50 µm*
***4**. White callus (c), compact nodule (n) and mass of adventitious embryos from proliferation of globular somatic embryos on Murashige and Tucker (MT) + abscisic acid (ABA) medium. Bar = 1 mm.*
***5**. Histological section of a young nodule. Note numerous embryogenic cells with starch and formation of adventitious proembryos (pe) on the surface. Double staining: PAS (periodic acid Schiff)/naphtol-blue-black. Bar = 50 µm.*
***6**.Embryogenic callus protruding from a nodule (n) cultured for 2 months on Murashige and Tucker (MT) without abscisic acid (ABA) medium. Bar = 1 mm.*
***7**. Subculture of long-term nodular callus onto Murashige and Tucker (MT) + sucrose (50 g/l) + malt extract (ME) (1500 mg/l) medium. Bar = 10 µm.*
***8**. Development of plantlets on solid 1/2 Murashige and Tucker (MT) + sucrose (20 g/l) + gibberellic acid (GA3) medium. Bar = 10 mm.*

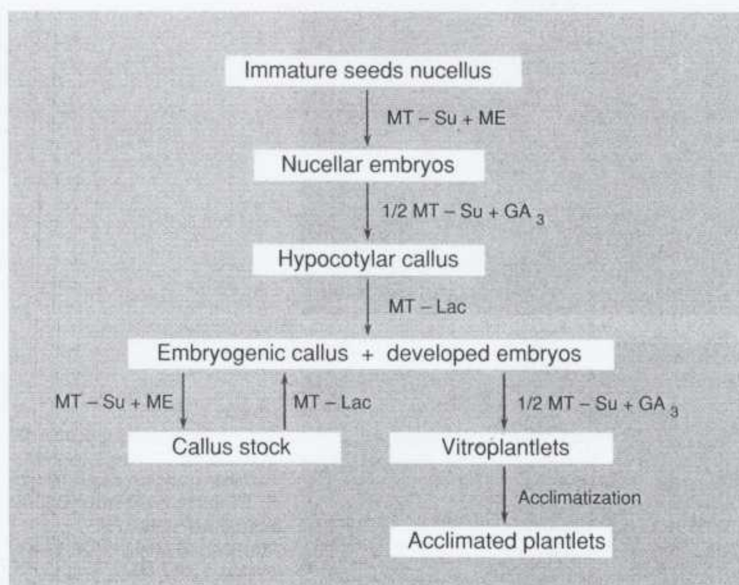


Figure 1
Diagrammatic scheme of somatic induction and plantlet development. MT, Murashige and Tucker medium; ME, malt extract; Lac, lactose; Su, sucrose; GA_3, gibberellic acid.

about 25%. The acclimated plantlets were phenotypically conform to developed plantlets obtained from nucellar embryos. Two other ways could contribute to increase plantlet production: nodules formed from globular embryos produced embryogenic calli and secondary, somatic embryos were formed from adventitious embryogenesis at different stages of the culture.

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