PROTOCOL OF SOMATIC EMBRYOGENESIS: TAMARILLO (*CYPHOMANDRA BETACEA* (CAV.) SENDTN.)

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1. INTRODUCTION

Cyphomandra betacea, commonly known as tamarillo or tree tomato, is a soft wooded, fast growing, evergreen shrub or small tree seldom reaching 5 m. The species, a member of the *Solanaceae* family, is native of median altitude Andean regions, in South America where it has been under cultivation for a long time and has become one of the most popular local fruits (Dawes and Pringle 1983; http://www.crfg.org/pubs/ff/tamarillo.html). From South America it has spread to Central America and West Indies and, later on, to the Portuguese islands of Azores and Madeira and to Southern Europe (Atkinson and Gardner 1993; Hooker 1899). By the end of the XIX century it had reached Australia and New Zealand (Slack 1976; Symon 1981). This last country is nowadays the main producer and exporter of tamarillo and also is the main place where most progress in crop improvement has occurred (Dawes and Pringle 1983). Depending on the cultivar, tamarillo fruits are red, orange or yellow egg-shaped berries with 2-3 inches long and 2 inches in diameter (Fouque 1973; Hooker 1899). The fruits of the red cultivar are most popular due to their more striking appearance and better flavour (Carloto et al. 1999; Slack 1976). Tamarillo crop has high nutritional value and contains relatively high contents of protein and fibres, rich in vitamins B6, C and E, provitamin A, citric acid and mineral elements (iron and potassium); and low in carbohydrates and calories (Cacciopo 1984; McCane and Widdowson 1992).

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Although tamarillo can be grown in a variety of soils and climates, there are some limitations to its cultivation. In temperate regions this plant is usually grown as an outdoor ornamental although some attempts have been made to explore this species commercially due to the high prices attained by its fruits in market places (10 – 15 euros/Kg). The plant requires a period of low temperatures along the year but very low temperatures, occurring in winter and spring, and autumn frosts are important limitations to a large-scale cultivation of tamarillos (Lopes et al. 2000).

Propagation of tamarillo can be achieved from seeds or from cuttings. Seeds germinate easily and have been used for the development of new cultivars (Slack 1976). However, if the objective is to maintain the genetic characteristics of the cultivars they could not be used. Since young cuttings are soft and difficult to manipulate mature cuttings (30-40 cm in length and 1 –2.5 cm diameter) should be used for rooting. Micropropagation techniques can also be used with advantage over conventional methods. Among these techniques somatic embryogenesis has several potential advantages such as large-scale production (Thorpe and Stasolla 2001), regeneration of transgenic plants from transformed cells (Dandekar 1995) and possibility of artificial seed production (Deverno 1995). It also provides a model system for the study of plant development (Leyser and Day, 2003). In the case of tamarillo, somatic embryogenesis associated with genetic transformation may play a crucial role in developing new cultivars more tolerant against biotic and abiotic factors.

2. EXPLANT SOURCE, STERILIZATION AND CULTURE

In tamarillo, somatic embryogenesis or embryogenic calli can be induced from different source of explants (Guimarães et al. 1988, Guimarães et al. 1996; Lopes et al. 2000) such as: mature zygotic embryos, hypocotyl segments, internodes and young leaves (from seedlings or from shoots of *in vitro* propagated adult plants) using different approaches. Recently we found (data not published) that explants from yellow cultivars produce embryogenic calli more readily than from red cultivars and calli are also more stable in culture. Mature embryos or young leaves isolated from seedlings of this cultivar are routinely being used in embryogenic studies. Following desiccation, seeds can be stored in plastic recipients at room temperature for extended periods of time before use.

Cultures are initiated with seed sterilization for 20 min in 7.5% calcium hypochlorite solution followed by 3 rinses with double distilled water. Leave seeds for over night in sterile water. Induce embryogenic callus and somatic embryos by using whole isolated zygotic embryos or leaf sections taken from 6-week-old seedlings. Zygotic

embryos are cultured on MS (Murashige and Skoog, 1962) medium containing one of the auxins: naphthaleneacetic acid (NAA), picloram or 2,4-dichlorophenoxyacetic acid (2,4-D). Seedlings for leaf sections are obtained by culturing aseptically isolated zygotic embryos on MS medium containing 1% sucrose, under a 16h light daily regime. Leaf sections are cut into 4-6 pieces after removing the main vein, which are immediately platted, adaxial surface down, on MS induction medium added with 2,4-D or Picloram. Leaf sections and internode segments from shoots obtained through micropropagation of adult plants, are also used for the induction of embryogenic calli. In this case, nodal segments from adult plants are first established in MS medium containing 0.5mg/l benzylaminopurine (BA). Axillary shoot proliferation is achieved in the same medium. Leaves and internodal segments (5 mm long) from the resulting axillary shoots are taken after one month growth and cultured in media containing combinations of 2,4-D or Picloram with kinetin (Kn). For somatic embryogenesis induction or embryogenic calli production cultures are kept in the dark at 25ºC. The pH of all media is adjusted to 5.7 before autoclaving at 121ºC. Difco Bacto-agar (0.8% w/v) is added to all media. Normally, two embryos or one leaf section or internode segment are inoculated per test tube containing 15 ml culture media. Embryogenic callus, 80-100 mg (fresh weight), is sub-cultured (4-6 weeks) to the fresh culture media.

3. SOMATIC EMBRYOGENESIS INDUCTION

Mature zygotic embryos of tamarillo are cultured in the induction media containing different NAA concentrations (0.1 to 10 mg/l) and 3% sucrose. After 3-4 weeks, callus is produced and 2-3 weeks later, somatic embryos develop from calli (Fig. 1a) (Guimarães et al. 1996). Calli develop mainly from the hypocotyledonary region, the best results are obtained when 2.0 mg/l NAA is used. Under these conditions, about 80% explants produce calli, out of which 42% show somatic embryo formation. The inclusion of higher sucrose levels (9%) in the induction media strongly increases somatic embryo formation raising the efficiency of somatic embryogenesis induction to 85% (Guimarães et al. 1996). The number of somatic embryos per induced explant is generally low and, in most cases, less than 10 somatic embryos are produced. During development most somatic embryos pass through different morphological phases similar to those occurring during zygotic embryogenesis (globular, heart-shaped, torpedo and cotyledonary). However, morphological abnormalities such as fused cotyledons, altered number of cotyledons and precocious germination are commonly seen, especially when low levels of sucrose are used. Calli induced in the presence of NAA are unable to maintain their embryogenic potential and rapidly lose ability to further differentiate somatic embryos. Under these conditions, induced somatic embryos are able to proceed with their development up to the cotyledonary

stage in the same culture medium. This process of somatic embryogenesis induction has been called “one step somatic embryogenesis” (Sharp et al. 1980) and has also been observed in other species studied in our lab such as *Feijoa sellowiana* (Canhoto and Cruz 1996) and *Myrtus communis* (Canhoto et al. 1999a).

4. INDUCTION AND MAINTENACE OF EMBRYOGENIC CALLI

When zygotic embryos, leaf sections from six-week-old seedlings or from one-month-old shoots were cultured in the presence of the auxins 2,4-D or Picloram a slow growing callus was induced after 4 to 6 weeks of culture (Lopes et al. 2000 ; Maia 2002). By the 8th to 10th week of culture whitish clusters of embryogenic cells (Fig. 1b and c) were formed in some areas of the callus, which kept on proliferating. These cultures have been called “indirect somatic embryogenesis” (Sharp et al. 1980) and this type of embryogenesis, occurring in a large number of angiosperms and gymnosperms, has been recently reviewed (Raemakers et al. 1999). In the following sections we describe the particular conditions used for the induction and maintenance of embryogenic calli for each of the different explants tested.

Zygotic embryos: A wide range of 2,4-D concentration (1 to 25 mg/l) is able to induce embryogenic calli. Best results (33%) are obtained with 10 mg/l of the auxin. Since we have found that 2,4-D concentrations of 5 mg/l or higher have some deleterious effect on further callus growth and embryo development, 2,0 mg/l of 2,4-D is currently used in our experiments. Calli maintenance is done with the transfer of small portions (80–100 mg) of embryogenic calli to the same culture medium by monthly transfer. Embryogenic calli can also be transferred to liquid medium (Fig. 1d) of the same composition with subcultures made at 3 weeks interval. Similar results are observed when Picloram is used instead of 2,4-D.

Leaf sections from seedlings: Explants are cultured on a medium containing 5.0 mg/l Picloram and 3% sucrose. Small whitish embryogenic areas can be seen at the leaf surface, after 6 weeks of culture, together with fast growing non-embryogenic calli. In these conditions about 40% of the explants produced embryogenic calli (Lopes et al. 2000). Increasing the level of sucrose in the culture medium to 9% allowed that induction rates over 70% were attained. Lower Picloram concentrations (1.0 or 2.0 mg/l) also can induce embryogenic calli but at lower frequencies (15 and 27%, respectively). A medium containing 5.0 mg/l Picloram and 9% sucrose is currently used to maintain embryogenic. Subcultures are made at 6-week intervals.

Leaf sections and internodes from shoots: *in vitro* shoots (Fig. 1e) from field-growing adult plants are established and multiplied according with a protocol developed by

Barghchi (1998). Among them, the best results are obtained with internodes (35% of the explants showing embryogenic calli) cultured on a medium containing 5.0 mg/l 2,4-D, 0.5 mg/l (Kn) and 9% sucrose (Maia 2002). Comparatively, leaf sections produce embryogenic calli at lower frequencies, with best results (25% explants producing embryogenic calli) being achieved when the Kn concentration is raised from 0,5 to 1,0 mg/l. Assays of embryogenic calli of internodal origin show that the medium containing 2.0 mg/l 2,4-D produce the best proliferation rate, with a 5x increase of the original fresh weight after 8 weeks of culture (Maia 2002). Accordingly this medium is being used to maintain calli of internodal origin.

Embryogenic callus formation and maintenance offers a great potential for large-scale production and for genetic transformation (Merkle et al. 1995). Embryogenic calli of tamarillo can be maintained in the conditions described for several years without loss of the embryogenic potential. However, our experiments have also shown that embryogenic calli become very unstable in culture, especially those maintained for periods longer than one year. RAPD analysis showed polymorphisms among embryogenic calli of the same origin (Lopes et al. 2000). Chromosome abnormalities, including the occurrence of tetraploid plantlets, are observed by chromosome counting (data not published). Studies at our lab have also shown that plantlets obtained from 5-year-old embryogenic calli display more abnormalities than those regenerated from younger embryogenic calli (one year or younger). Differences are also observed in the multiplication rate among several callus lines and among cultivars

Independently of the conditions and explant source, embryogenic calli do not produce embryos on a medium containing auxin. Subsequent embryo development only occurs when the embryogenic calli are transferred to a medium without auxin as described in the next section.

5. SOMATIC EMBRYO DEVELOPMENT

Embryogenic calli are formed from densely stained isodiametric meristematic cells (Fig. 1c). When pieces of embryogenic calli (80 to 100 mg), originate from leaves, zygotic embryos or internodes, are transferred to a medium (liquid or solidified) containing 2% sucrose (reduced level) and without growth regulators, the embryogenic masses develop into somatic embryos morphologically identical to those obtained by “one step somatic embryogenesis”. Under these conditions, morphologically abnormal somatic embryos are also found. Somatic embryo development is not synchronized and different phases can be found in the same callus.

However, after three to four weeks on this medium most of the embryos are at the cotyledonary stage and ready to conversion. The effect of several growth regulators during somatic embryo development shows that abscisic acid (ABA) and Kn do not improve somatic embryo quality (Guimarães et al. 1996). However, reduced levels (0.1mg/l) of gibberellic acid (GA_3) strongly improved somatic embryo development (Guimarães et al. 1996).

The inclusion of GA_3 in the development medium also reduces time for somatic embryo conversion. The number of somatic embryos per embryogenic callus is dependent of several factors such as the initial explant, genotype, callus lines, maintenance medium and the period of callus maintenance in culture. However, values as high as 140 embryos per 100 mg (fresh weight) of embryogenic calli can be obtained after 4 weeks in the development medium. Particularly relevant is the fact that embryogenic calli obtained from zygotic embryos produce a higher number of somatic embryos than calli of internode origin (Maia 2002).

Although an auxin-free medium is necessary for somatic embryo development from embryogenic calli, use of auxin polar transport inhibitors has shown that endogenous auxin is required for normal somatic embryo development. In fact, the presence of auxin-polar transport inhibitors such as triiodobenzoic acid (TIBA) or chlorophenoxy methyl propionic acid (CFA) in the development medium sharply decreases the number of somatic embryos attaining the torpedo stage (Lopes et al. 2000). Furthermore, some of the embryos show fused cotyledons indicating a disturbance during transition from radial to bilateral symmetry (Hadfi et al. 1998; Liu et al. 1993).

6. SOMATIC EMBRYO CONVERSION

For somatic embryo conversion cotyledonary embryos are isolated and transferred to the conversion medium. Five somatic embryos are cultured on test tubes containing the same medium as for development: MS plus 2% sucrose. Cultures are kept at 25ºC under 16h photoperiod of 96 $\mu mol.m^{-2}\ s^{-1}$ photosynthetically active radiation provided by cool white fluorescent lamps. The results show low frequency rate (37% inoculated embryos) are of conversion into plantlets (Fig. 1f). The inclusion of GA_3 (0.1 mg/l) or charcoal (15g/l) don't improve somatic embryo conversion rate. The way in which somatic embryos are formed in the presence of NAA, 2,4-D or Picloram, also don't influences somatic embryo conversion. Although the somatic embryos cultured on the germination medium are morphologically normal, they must have some kind of abnormalities that impair somatic embryo conversion such as poor meristem differentiation, defficiencies in

the process of embryo maturation or abnormalities in chromosome number and strucuture. Preliminary histochemical and ultrastructural studies have shown that cotyledonary cells of the somatic embryos show lower levels of proteins and lipids as compared to zygotic embryos. In some cases, conversion of the embryos occur by shoot development with out the concomitant root development. However, adventitious roots often arise at the base of these shoots that makes plant regeneration possible.

7. ACCLIMATISATION

Following root formation, the plantlets are transferred to the greenhouse (Fig. 1g). Agar is removed gently from roots with tap water. The plantlets are immersed for 1min in 0.6g/l fungicide (Benlate) solution. Transfer plantlets in "Melfert" bags (220 ml) containing vermiculite and "Osmocote" slow release fertilizer (0.6 g/plantlet). Place plantlets in containers (60 x 40 cm) and cover them with plastic sheet to maintain high humidity environment. Containers are placed on an irrigation sheet in the greenhouse. Gradually reduce humidity by raising the plastic sheets.

Allow plants to grow for a period of 3 months in the greenhouse. The survival rate of the plants (Fig. 1h) is 55%. Transfer plantlets to the field and observe growth and fruit production. Our experience shows plants perform well in terms of growth and fruit production.

During the initial phases of plant development some plantlets show morphological abnormalities, further growth recovered the normal phenotype. High levels of plant mortality during this phase are probably related with abnormal plant development rather than with the growing conditions. In fact, similar experiments carried out with plantlets obtained by axillary shoot proliferation showed a survival rate of about 100%.

Figure 2 resumes the protocols used in our labotatory for somatic embryogenesis induction and plant regeneration in tamarillo.

8. CONCLUDING REMARKS

Somatic embryos of tamarillo can be induced from various explant types under different culture conditions. With this protocol, a large number of plantlets can be obtained. However, some problems still persist that reduce the success of the technique. One of the major problems is the occurrence of genetic alterations in embryogenic callus cultures, especially older than one year, that could be prevented

with cryopreservation or encapsulation of young embryogenic calli (Sakai 1995; Tremblay et al. in this book). Alternatively, modify the maintenance medium by reducing auxin that may prevent or minimise genetic alterations in among regenerated plants derived from callus, maintained on the modified medium. Factors leading to abnormal somatic embryo development should be determined, e.g. events leading to genetic modifications. Anomalous somatic embryo formation is a common feature in numerous woody species (Canhoto et al. 1999b) and the factors controlling embryo development must be better understood. The process of somatic embryo maturation in tamarillo is being monitored by histochemical and ultrastrucutural analysis. Our results demonstrate that several somatic embryo cells of the cotyledons and hypocotyl are often vacuolated and have reduced number of lipid and protein bodies. In contrast, cells of the cotyledonary zygotic embryos are completely filled with those type of reserve organelles. Poor somatic embryo maturation can also impair further somatic embryo conversion into normal plantlets (Thorpe and Stasolla, 2001).

In some of the media tested, embryogenic and non-embryogenic calli can be obtained from the same explant, and their cultures are maintained as embryogenic and non-embryogenic lines. Important insights about the process of somatic embryo induction and development can be obtained from biochemical and molecular studies that are being carried out with this material. Previous work have helped us to isolate and characterize a cDNA specific of non-embryogenic calli of tamarillo (Faro et al. 2003).

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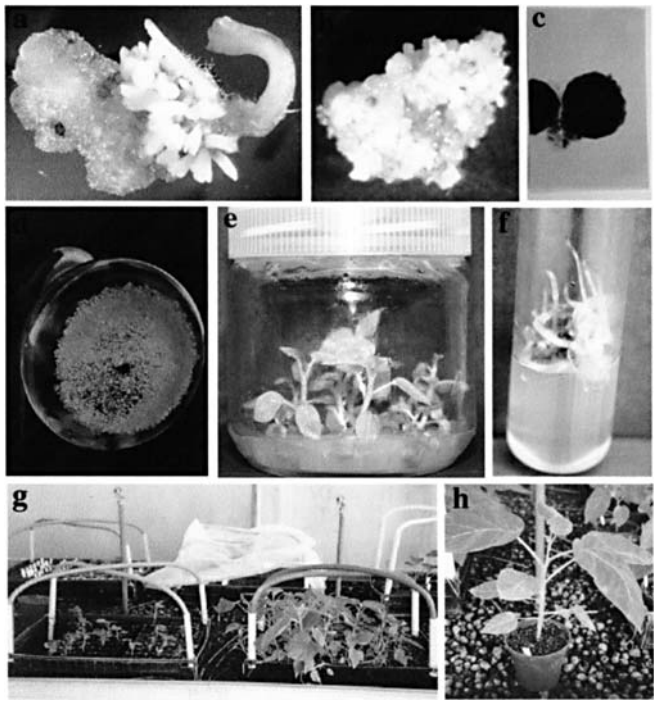

Figure 1. Somatic embryogenesis in tamarillo : (a) Somatic embryos produced in a medium containing NAA. Embrygenic calli (b) and embrygenic masses (c) obtained through the culture of leaf sections in a 2,4-D containing medium. (d) Embryogenic calli maintained in liquid medium. (e) Axillary shoot proliferation. (f) Somatic embryo conversion. (g) Plantlet acclimatation. (h) Plant of tamarillo regenerated by somatic embryogenesis and ready to go to field conditions.

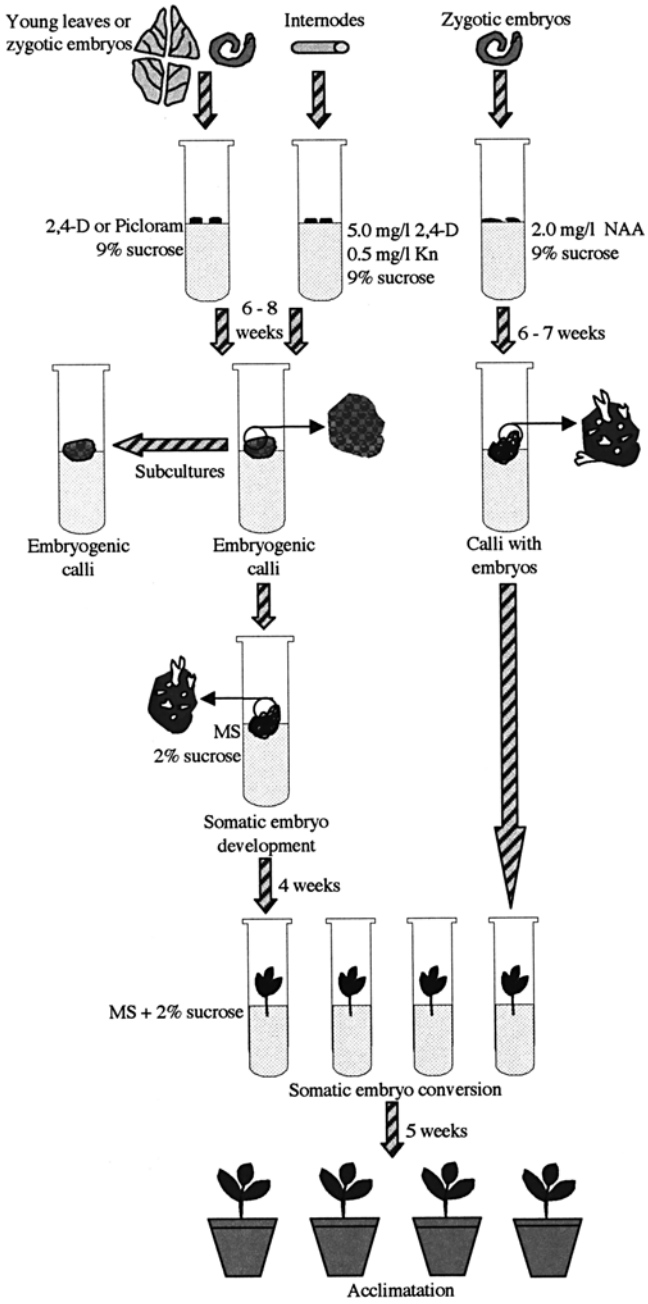

Figure 2. Schematic representation of the protocols for somatic embryogenesis induction in tamarillo