

Somatic embryogenesis in tamarillo (*Cyphomandra betacea*): approaches to increase efficiency of embryo formation and plant development

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Abstract Somatic embryogenesis induction and somatic embryo development of the solanaceous tamarillo tree were previously established and successfully used for plant regeneration from different explants and varieties. Somatic embryogenesis was induced in Murashige and Skoog medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram and high sucrose concentrations (0.25 M). The embryogenic tissues were transferred to an auxin-free medium, with reduced sucrose levels, to permit embryo development and conversion into plantlets. This two-step protocol is often impaired by an ineffective transition from the proembryogenic masses to embryo development. In this work, attempts to optimize the somatic embryogenesis system of tamarillo by improving the quality of somatic embryo and embryo conversion were carried out. The results showed that the presence of a high number of abnormal somatic embryos did not significantly inhibit plant conversion, hence indicating that shoot apical meristem development was not affected in abnormal somatic embryos. It was also shown that the manipulation of sucrose concentration in the development medium (0.11 M) and dark conditions before conversion increased the number of morphologically normal somatic embryos. The comparison between mature cotyledonary zygotic and somatic embryos showed an

inefficient accumulation of storage compounds, mainly lipids, in somatic embryos. These reduced levels of lipid storage could be responsible for the abnormal patterns of embryo development found in tamarillo somatic embryos.

Keywords Abscisic acid · Dark conditions · Embryo maturation · Histochemical analysis · Storage compounds · Sucrose

Introduction

Cyphomandra betacea (Cav.) Sendt. is a solanaceous tree native to South America and commonly known as tamarillo (Barghchi 1998). Used mainly because of its high nutritional edible fruits, this species has spread to several world regions, such as Central America, Southern Europe and New Zealand, which is nowadays the main producer and exporter of this fruit crop (Meadows 2002). Tamarillo is also cultivated in temperate regions of the Northern hemisphere, where it blossoms mostly during the transition from summer to fall, although flowering may also occur during other times of the year depending on climate.

Somatic embryogenesis induction and somatic embryo development of tamarillo have been successfully obtained from different explants such as mature zygotic embryos, cotyledons, hypocotyls, roots, and from leaves of in vitro proliferating shoots (Canhoto et al. 2005; Lopes et al. 2000). More recently, an induction system to clone adult trees was also developed (Correia et al. 2011). The assays performed with these different explants have given important insights about the conditions for somatic embryogenesis induction in this species.

For tamarillo, as for many other woody species, the development of efficient somatic embryogenesis protocols

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of plant regeneration may represent a useful method for clonal mass propagation of selected material, genetic transformation and germplasm cryopreservation (Park 2002; von Arnold 2008). Nevertheless, in species in which plant regeneration through somatic embryogenesis occurs through a two-step system (Corredoira et al. 2003; Hussain et al. 2009), as tamarillo, one of the major problems is an ineffective transition from the proembryogenic masses to embryo formation and development, which is often impaired by the occurrence of abnormal embryos and precocious germination. This situation may be caused by an inadequate maturation of the embryos, an important phase of somatic and zygotic embryo development following the classic morphogenic phases from globular to cotyledonary embryos.

During maturation, embryo cells undergo various physiological changes, which become evident by the deposit of storage materials, repression of germination and acquisition of desiccation tolerance (Jiménez 2005; Vahdati et al. 2008). In some species, particularly among gymnosperms, such as Norway spruce (Bozhkov et al. 2002; von Arnold 2008), effective protocols for somatic embryo development and maturation have been implemented and, as a consequence, the yield of plant conversion could be increased. Also, in some angiosperms (Mauri and Manzanera 2003; Pinto et al. 2008; Prakash and Gurumurthi 2010), the manipulation of the culture conditions gave a major contribute to boost the rate of embryo conversion.

To increase the number of somatic embryos formed from tamarillo embryogenic tissues, following transfer to an auxin-free medium, modifications in the composition of culture media and in culture conditions were tested and evaluated in terms of their effects on somatic embryo development and conversion. To better understand somatic embryo development, a comparison of storage compounds was also performed between cotyledonary somatic embryos and zygotic embryos through histochemical and biochemical techniques.

Materials and methods

Establishment and maintenance of embryogenic tissues

Embryogenic tissues were obtained and maintained following the methodology described by Lopes et al. (2000) and Correia et al. (2011). The most apical expanding leaves were excised from in vitro cloned shoots of tamarillo (red cultivar) and cultured in the induction medium containing the nutrients of the MS formulation (Murashige and Skoog 1962), 0.25 M sucrose, 20 μ M picloram, 0.25% phytigel and pH adjusted (with KOH) to 5.7. Leaf explants were randomly punctured and placed (abaxial side down) in test

tubes (15 \times 2.2 cm) containing 15 ml of the induction medium. Cultures were incubated at $24 \pm 1^\circ\text{C}$ in the dark for 12 weeks. After this period, embryogenic areas were isolated and subcultured at 4-week intervals in the same medium. All the embryogenic tissues used for proliferation and conversion studies were taken from a single embryogenic line (Fig. 1a) that was multiplied and maintained for 1 year before the subsequent assays.

Yield of the embryogenic process

To determine the yield of the embryogenic system in tamarillo the number of somatic embryos produced per embryogenic tissue was registered. Somatic embryos were divided into normal and abnormal. Normal somatic embryos were considered those that reached the cotyledonary stage of development and displayed a normal phenotype: two well formed cotyledons and an embryo axis. Embryos displaying some kind of abnormality, such as an altered number of cotyledons, enlarged size, fused embryos or any other bizarre aspect were considered abnormal. The assay was performed transferring an initial mass (an average of 0.043 ± 0.004 g) of embryogenic tissue to test tubes containing the development medium (MS plus 0.07 M sucrose, 0.6% agar, pH 5.7). The cultures were placed under a 16 h daily illumination regime of $15\text{--}20 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation provided by cool-white fluorescent lamps at $24 \pm 1^\circ\text{C}$. One month later, the fresh weight of callus and somatic embryos was registered, as well as the number of normal and abnormal embryos (Table 1). These embryogenic tissues were then transferred to new test tubes containing the same culture medium for a 2-month period under the same conditions. At the end of the assay, after a total of 3 months of culture, the total fresh weight and the number of plantlets per explant were registered.

Effect of the development media and culture conditions on somatic embryo development

To increase the number of somatic embryos reaching the cotyledonary stage, the embryogenic tissue was transferred to different development media and placed under various culture conditions. White opaque clusters, consisting, in average, of 100 mg of embryogenic tissue each, were removed from the induction medium and transferred to 100 ml flasks containing 25 ml of solidified medium for a 4-week period under different conditions. Five replicates per treatment were used, and the experiments were repeated twice.

The influence of medium composition on somatic embryo development was tested using full strength MS basal medium supplemented with different sucrose concentrations:

Table 1 Mass proliferation (measured as fresh weight—FW) and total number of somatic embryos (SE) and emblings obtained after 1 and 3 months on the embryo development medium (MS medium, 0.07 M sucrose) and under photoperiod conditions

Explant	Initial embryogenic tissue FW (g)	Callus + SE FW after 1 month (g)	Number of SE per explant (after 1 month of culture)		Number of emblings per explant (after 3 months)
			Normal/cotyledonary SE	Abnormal SE	
1	0.049	1.06	8	8	2
2	0.052	1.32	2	13	7
3	0.026	1.16	3	33	23
4	0.041	0.96	10	28	41
5	0.040	1.57	8	15	23
6	0.050	1.06	6	28	32
Mean ± SE*	0.043 ± 0.004	1.19 ± 0.1	6.2 ± 1.3	20.8 ± 4.1	21.3 ± 6.0
Number of SE/emblings per g of initial embryogenic tissue FW*			143.9 ± 30.2	482.6 ± 95.3	494.2 ± 139.5

*Mean number ± standard error

Table 2 Tamarillo embryogenic masses proliferation under different developmental conditions

	Treatment conditions*			Embryogenic tissue fresh weight (g)**	
	Sucrose concentration	ABA 8 μM (yes/no)	Light (L)/dark (D)	Initial	After maturation
S1L	0.07 M	No	L	0.107	0.812 ± 0.120 ^a
S1D		No	D	0.113	0.813 ± 0.155 ^a
S1AL		Yes	L	0.106	0.406 ± 0.630 ^c
S1AD		Yes	D	0.118	0.546 ± 0.221 ^b
S2L	0.11 M	No	L	0.119	0.275 ± 0.302 ^c
S2D		No	D	0.105	0.553 ± 0.188 ^b
S2AL		Yes	L	0.111	0.496 ± 0.883 ^b
S2AD		Yes	D	0.104	0.423 ± 0.280 ^c
S3D	0.16 M	No	D	0.116	0.395 ± 0.408 ^c

*MS medium was used as basal medium, solidified with 0.6% (w/v) agar, pH 5.7

**Values are means ± standard error of 5 replicates from two experiments. Values followed by the same superscript letter are not significantly different at $P < 0.05$, according to the Tukey test

0.07 M (S1), 0.11 M (S2) and 0.16 M (S3), and either free of growth regulators or supplemented with 8 μM abscisic acid (ABA, S1A and S2A). S1 medium (0.07 M sucrose without ABA) was used as control.

To evaluate the influence of light, the above treatments (S1, S1A, S2, S2A) were performed under two light conditions (Table 2): one group was maintained in the dark, at $24 \pm 1^\circ\text{C}$ (S1D, S1AD, S2D, S2AD), and the other group was incubated under a 16 h daily illumination regime of $15\text{--}20 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation provided by cool-white fluorescent lamps at $24 \pm 1^\circ\text{C}$ (S1L, S1AL, S2L, S2AL).

The efficiency of the development conditions was assessed by counting the number of mature cotyledonary and abnormal somatic embryos after 3 weeks of culture. At the end of this period, the fresh weight was registered and a statistical analysis (SPSS Statistics 17.0) was performed through an analysis of variance (one-way ANOVA) with

the significantly different means determined by the Tukey test ($P = 0.05$).

For somatic embryo conversion into plantlets, mature cotyledonary somatic embryos were transferred to test tubes containing MS basal medium, 0.07 M sucrose, 0.6% (w/v) agar, pH 5.7 and cultured for 1 month under a 16 h daily illumination regime, at $24 \pm 1^\circ\text{C}$.

Extraction and quantitative analyses of storage compounds

In order to determine the levels of storage compounds present in cotyledonary zygotic and somatic embryos, intact zygotic embryos were carefully removed from sterilized seeds (7% w/v calcium hypochlorite, for 20 min) while somatic embryos were isolated from the embryogenic cultures after a 3-week period in the development medium. Both zygotic and somatic embryos were first

rinsed with sterilized water, dried on filter paper, weighted and fast-frozen in liquid nitrogen before being kept at -80°C . Three replicates of each sample were analysed per assay. Statistical analysis (SPSS Statistics 17.0) was performed using the Levene test for homogeneity of variance and the T test for independent samples ($P = 0.001$, $\alpha = 0.05$). The applied procedures for each type of compound were as follows.

Total lipid extraction and quantification: previously frozen samples (2.5 g) were lyophilised and all the subsequent treatments were made in terms of dry weight. The extraction was made using a modified Folch procedure (Folch et al. 1957) with chloroform:methanol (2:1) as the extraction solvent. The lyophilised samples were homogenized in 7 ml of extraction solvent, followed by centrifugation (10 min) at 3,000 rpm. The supernatant was removed and the pellet was washed twice with 1 ml aliquots of methanol:chloroform:water (MCW, 48:3:47), which was then evaporated under vacuum conditions, on a rotary evaporator, at 45°C . The residuum was collected at the bottom of the flask, transferred to a glass vial, dissolved in 2 ml of chloroform and evaporated again. This final residuum was redissolved in 1 ml of chloroform and kept at -20°C until quantitative analyses were performed.

Total lipid quantification was performed based on the reaction of lipid degradation products with aromatic aldehydes, which resulted in a red coloration quantified at 528 nm (Zöllner and Kirsh 1962).

Total protein extraction and quantification: frozen samples (100 mg) were grounded into a fine powder in a pre-cooled mortar using liquid nitrogen. Tissue powder was transferred to sterile tubes and re-suspended with 200 μl of an ice-cold extraction buffer [50 mM sodium citrate pH 5.5, 5% w/v sodium dodecyl sulphate (SDS), 0.01% w/v bovine serum albumin (BSA), 150 mM calcium chloride, 2% β -mercaptoethanol; protease inhibitors as indicated by the manufacturer (SIGMA FAST™ Protease Inhibitor)] and centrifuged (30 min, 13,200 rpm). After centrifugation, the supernatant was transferred to a new tube and kept on ice until quantification.

The protein levels were determined by the colorimetric method of Bradford (1976) using coomassie brilliant blue G-250 (Sigma). The absorbance was read at 595 nm and BSA used as standard.

Starch extraction and quantification: the procedures described by McCready et al. (1950) were used. For total soluble sugars extraction, samples were macerated using 2 ml of MCW (12:5:3), and centrifuged for 10 min at 2,000 rpm. The supernatant was recovered and the pellet was re-extracted using 2 ml of MCW. One part chloroform and 1.5 part water were added for each four parts of the supernatant, followed by a new centrifugation for 10 min at 2,000 rpm, from which two phases were obtained. The upper

aqueous phase (soluble sugars) was removed and the pellets were grounded with 1 ml of 30% (v/v) perchloric acid and centrifuged for 15 min at 10,000 rpm. The supernatant containing starch was transferred to a new tube and the pellets were re-extracted twice. The supernatants were then combined and the pellets discarded (McCready et al. 1950). The sugar and starch concentrations were calculated using anthrone (0.2%) and glucose as standard, according to Umbreit et al. (1964). The absorbance was read at 620 nm.

Histochemical analysis of cotyledonary somatic and zygotic embryos

Storage compounds were also histochemically analysed. Cotyledons of zygotic and somatic embryos were both fixed for 2 h in a 2.5% (w/w) glutaraldehyde and 0.2 M sucrose solution prepared with 0.1 M sodium cacodylate buffer, pH 7.2, and post-fixed, at room temperature, for 1 h, in 1% (w/v) osmium tetroxide solution prepared with the same buffer. Samples were thoroughly dehydrated in an ethanol series (20, 40, 60, 80, 95 and 100% v/v) and embedded in Spurr's resin (Spurr 1967). Following polymerisation at 65°C overnight, semi-thin (0.5–1 μm) cross sections of the cotyledons were obtained with a LKB ultra-microtome, using glass knives, and stained with Sudan Black B (Bronner 1975) for lipids, mercury bromophenol blue (Mazia et al. 1953) for proteins and periodic acid-Schiff (PAS) reaction (McManus 1948) for starch detection.

Results

Evaluation of somatic embryo and embling formation

Embryogenic masses of tamarillo (Fig. 1a) were formed by clumps of isodiametric meristematic cells that continued to grow without somatic embryo formation while maintained in an auxin containing medium. When transferred to an auxin-free medium, with reduced sucrose levels, the embryogenic masses started to organize into somatic embryos (Fig. 1b) which went through the typical phases of somatic embryo development (Fig. 1c). After 1 month in this medium, fresh weight increased as much as 30 times, mainly due to the developing embryos (Table 1; Fig. 1d). The average number of cotyledonary somatic embryos formed was 143.9 per g of initial tissue, whereas the total number of abnormal somatic embryos was about three times higher, reaching a value of 482.6 embryos per g of initial embryogenic tissue (Table 1). The fresh weight at the end of the experiment was much higher than at the end of the first subculture (1 month) and the number of developing plantlets (494.2 per g of initial embryogenic tissue) clearly outnumbered the normal somatic embryos initially

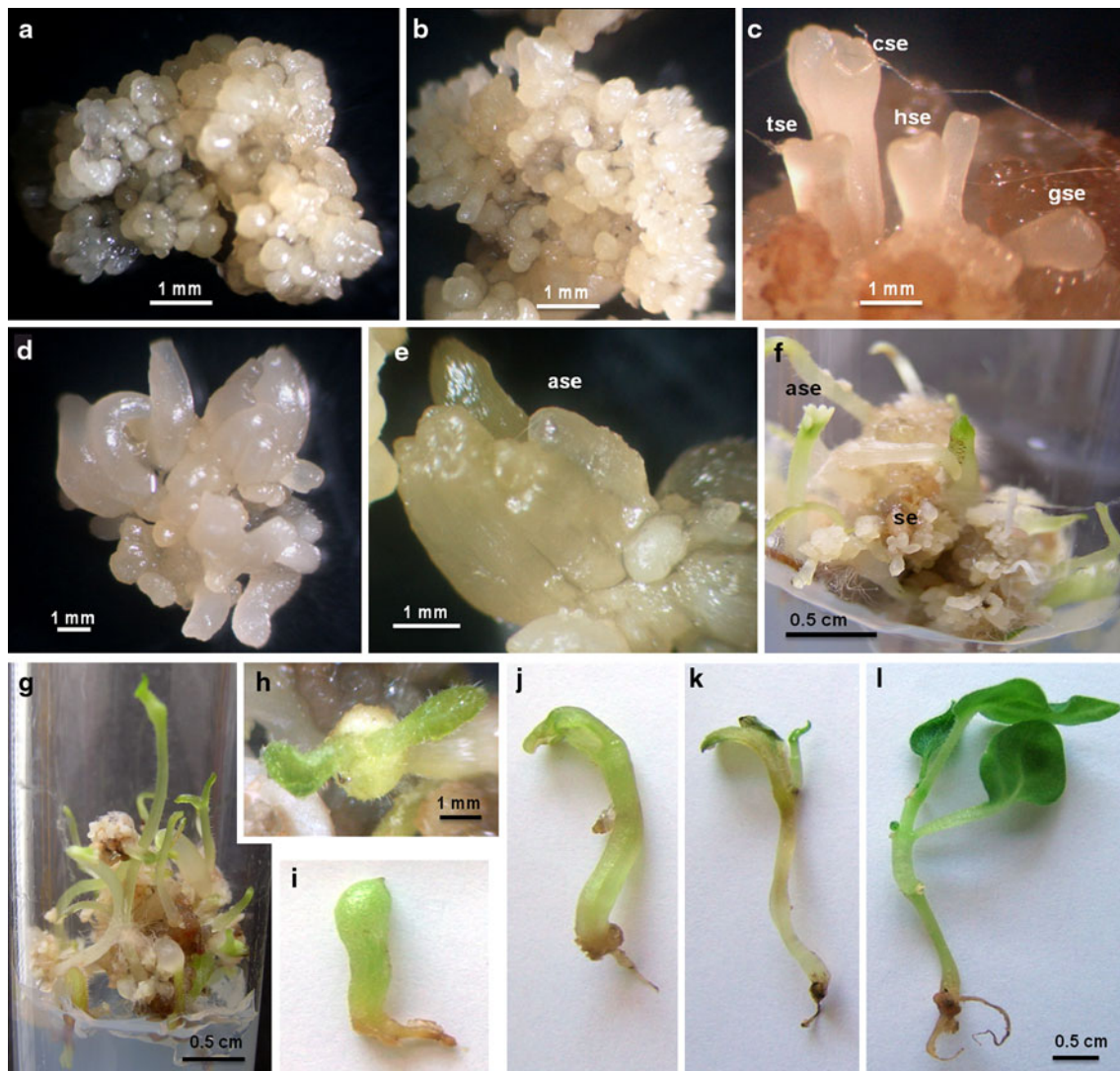


Fig. 1 Tamarillo somatic embryo development and conversion in MS medium supplemented with 0.07 M sucrose and photoperiod conditions **a** Embryogenic tissue induced from leaves **b** Somatic embryos start to develop after 1 week, through an asynchronous process, as observed in **c**, where, after 2 weeks, several stages of somatic embryo development can be seen (*gse* globular, *hse* heart-shaped, *tse* torpedo and *cse* cotyledonary somatic embryos) **d–e** Somatic embryos, after a 4-week development period. Note the

presence of large-sized abnormal somatic embryos (*ase*) **f** Somatic embryo conversion into plants after 8 weeks in culture - masses of embryogenic tissue and developing somatic embryos (*se*), and abnormal somatic embryos (*ase*) are still present **g–l** Plantlet conversion after 10 weeks in culture showing shoot and root development. Note the characteristic development of the shoot apical region from inside a sheath-like structure and the adventitious root formation

developed, reaching a value equivalent to the amount of both normal and abnormal somatic embryos (Table 1). Abnormal somatic embryos displayed different phenotypes such as fused, “cup-like” or absent/non-developed cotyledons, and fused and/or large-sized embryos (Fig. 1e). At the end of the experiment, many somatic embryos at different morphological stages were still present in the cultures (Fig. 1f, g), indicating that many more plants could have been obtained if more subcultures were carried out.

A prominent and regular feature observed was the presence of a sheath-like structure involving the shoot apex of the developing plantlets (Fig. 1g, h). In spite of the

evident morphological abnormalities, most of these embryos were able to germinate and be converted into viable plantlets (Fig. 1i–l). It was also observed that, in some of the embryos formed, a root apical meristem seems to be absent since the roots were adventitious, formed after the conversion in the development medium (Fig. 1j–l).

Effect of the media composition and culture conditions on somatic embryo development

In all media and conditions tested, embryogenic masses developed into somatic embryos similar to those obtained in

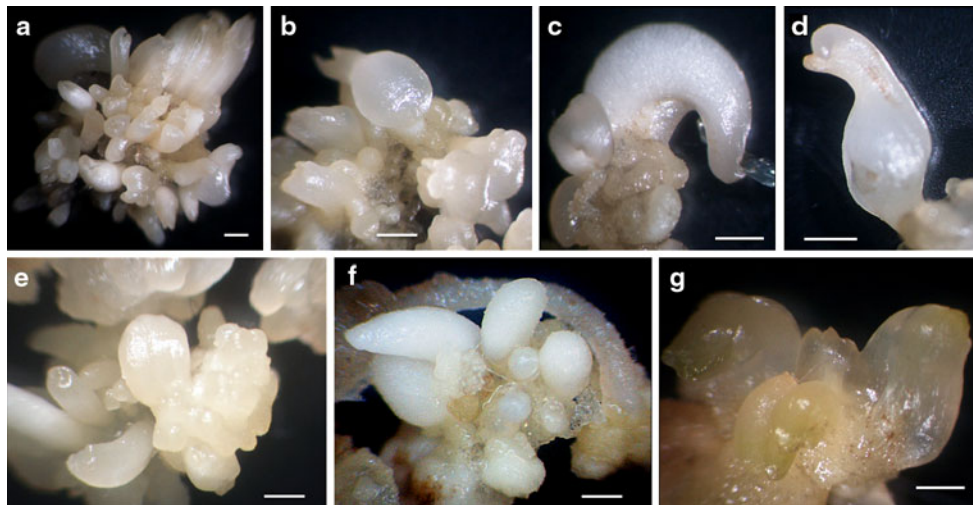


Fig. 2 Tamarillo somatic embryo development, after 4 weeks, under different culture conditions **a–d** Somatic embryos obtained in MS medium supplemented with 0.11 M sucrose and dark conditions; note the somatic embryo asynchronous development and the presence of large-sized abnormal embryos, with fused or “cup-shaped” cotyledons **e** Somatic embryos developed in MS medium supplemented

with 0.16 M sucrose in the dark **f–g** Comparison between embryo development in MS medium supplemented with 0.11 M sucrose and 8 μ M ABA, in the dark (**f**) and MS medium supplemented with 0.07 M sucrose, in light conditions (**g**). Note the white compact color of the somatic embryos in **f** when compared to the translucent somatic embryos in **g**. Bars 1 mm

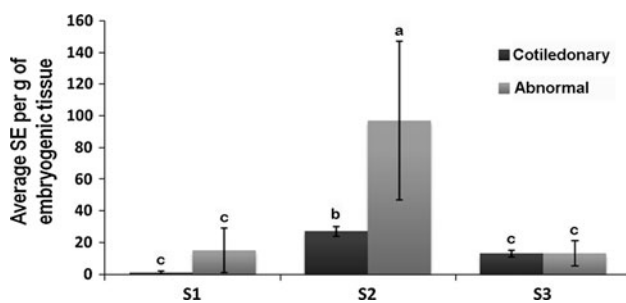


Fig. 3 Effect of sucrose concentration in the development medium on somatic embryo development under dark conditions. The values are means \pm standard error of 5 replicates from two experiments. Different superscript letters indicate significant differences at $P < 0.05$, according to the Tukey test

the initial experiment, including phenotypically normal somatic embryos and different types of abnormalities (Fig. 2a, b). Somatic embryo development was not synchronized and different stages were found in the same embryogenic tissue (Fig. 2a). The presence of a large number of abnormal somatic embryos (Fig. 2c, d) was a constant in spite of the treatments. The results clearly indicated that sucrose has a crucial role in the development of somatic embryos, with the best results being obtained when 0.11 M sucrose (S2) was used (Fig. 3). Besides, the number of abnormal embryos continued to exceed the number of normal somatic embryos formed. When higher sucrose concentrations (0.16 M) were tested, the number of somatic embryos sharply dropped and was not significantly different from the number obtained with the lowest sucrose concentration (0.07 M, S1, Fig. 3). These somatic embryos were

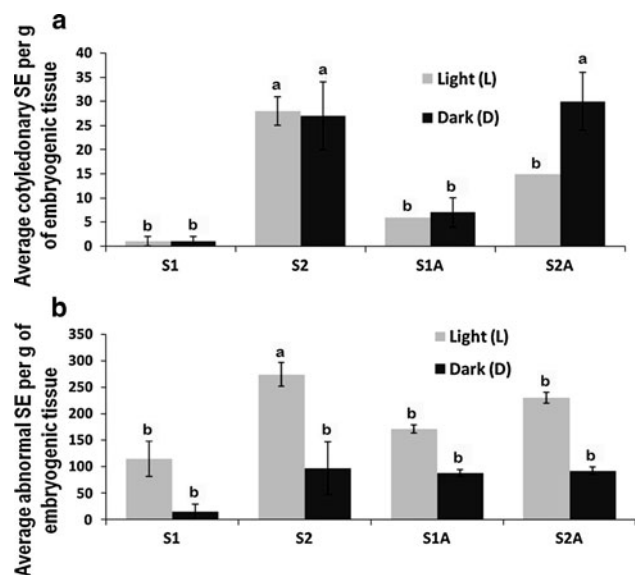


Fig. 4 Effect of medium composition (sucrose concentration and presence/absence of 8 μ M ABA) and light on somatic embryo development. The values are means \pm standard error of 5 replicates from two experiments. Different superscript letters indicate significant differences at $P < 0.05$, according to the Tukey test

also morphologically very similar to those obtained with the lowest sucrose concentration (Fig. 2e). The level of sucrose in the medium also affected tissue proliferation. When this parameter was analysed, the results showed that lower sucrose levels (0.07 M) presented the best results both in light and dark conditions (Table 2).

The results also indicated that ABA is an important factor for somatic embryo development. In the presence of

ABA, the number of normal cotyledonary embryos was significantly higher, but only when 0.11 M of sucrose was used and the explants were kept under dark conditions (Fig. 4a). Nevertheless, no significant differences were found between the number of normal embryos obtained in the dark, with MS 0.11 M sucrose supplemented with ABA (S2A), and the number of embryos obtained with MS 0.11 M sucrose not supplemented with ABA (S2), either in light or dark conditions (Fig. 4a). The formation of abnormal somatic embryos was significantly promoted in S2 under photoperiod conditions and, although not at a significant level, it was consistently higher in all the other situations under light conditions (Fig. 4b).

The morphological observation of the somatic embryos showed that embryos formed under dark conditions, in MS medium with 0.11 M sucrose and ABA (Fig. 2f), were more whitish and opaque than the more translucent ones formed under light conditions (Fig. 2g).

Biochemical and histochemical analysis of storage compounds

A general staining with toluidine blue indicated that the cells of the cotyledonary zygotic embryos were large, more or less isodiametric and completely packed with reserves (Fig. 5a). Histochemical analysis showed that the reserves were mainly lipids (Fig. 5c) and proteins (Fig. 5e). In somatic embryos, reserves were scarcer and cells were more vacuolated (Fig. 5d, f). Another important difference was found in starch content. Starch grains could not be found in cells of zygotic embryos, but their presence was a general feature in somatic embryos (Fig. 5b). The results obtained by the biochemical assays were not completely consistent with the histochemical data. Thus, although the total lipids were higher in zygotic embryos than in somatic ones, as observed in the histological studies, the levels of total proteins were not significantly different in both types

Fig. 5 Histochemical analysis of the cotyledons of mature somatic and zygotic embryos. **a** General staining, with toluidine blue, of a mature zygotic embryo's cotyledon (*n* nucleous, *ob* oil bodies, *pb*, protein bodies) **b** Total starch detection, in somatic embryos' cotyledons, with the PAS procedure **c–d** Sudan Black B staining of total lipids from the cotyledons of a mature zygotic embryo (**c**) and of a cotyledonary somatic embryo (**d**) **e–f** Total protein analysis, through bromophenol blue staining, of a mature zygotic embryo (**e**) and of a cotyledonary somatic embryo (**f**). Arrows indicate the presence of the identified compound. Bars 100 μ m

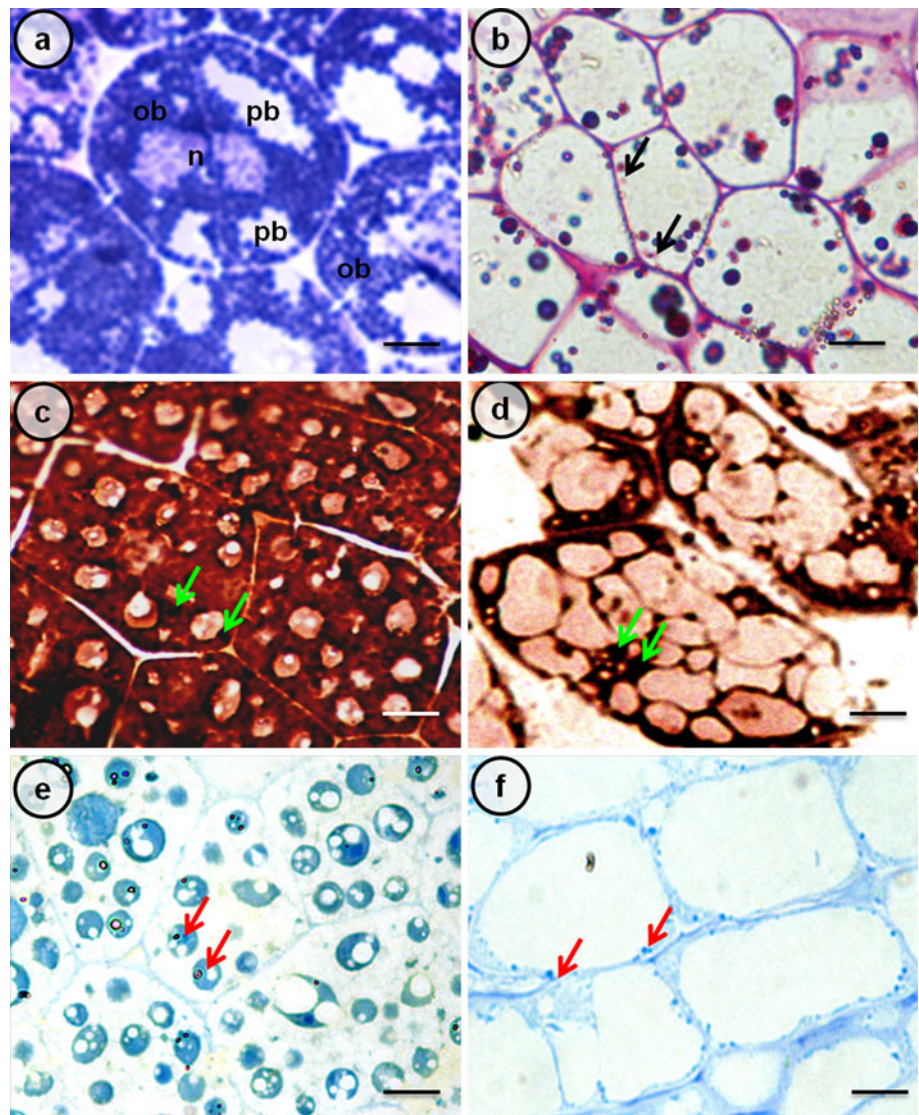


Table 3 Levels of lipids, proteins, soluble sugars and starch (mg/g fresh weight—FW) of tamarillo cotyledonary-staged somatic and zygotic embryos

Cotyledonary embryos (100 mg FW)	Total lipids (mg/g FW)	Total proteins (mg/g FW)	Soluble sugars (mg/g FW)	Starch (mg/g FW)
Somatic	2.3 ± 0.04 ^b	2.4 ± 0.76 ^a	7.70 ± 0.03 ^a	6.93 ± 0.01 ^a
Zygotic	46.6 ± 0.72 ^a	2.9 ± 0.06 ^a	6.58 ± 0.05 ^a	5.92 ± 0.05 ^a

The values presented are the mean ± SE of three replicated treatments

Values followed by different letters inside parameters indicate significant differences according to the *T* test for independent samples ($P = 0.001$, $\alpha = 0.05$)

of embryos, although slightly higher in the zygotic ones (Table 3). Also, the starch content was similar in both types of embryos (Table 3), but this could not be established with the histochemical studies in which, as above mentioned, starch could not be detected in zygotic embryos. Like starch, soluble sugars were present in similar amounts in both types of embryos.

Discussion

Previous work with tamarillo has contributed to a well established somatic embryogenesis induction protocol. However, it has been shown that the embryo quality is often poor, leading to the occurrence of morphological abnormalities, germination difficulties and precociously germinated embryos (Canhoto et al. 2005). In the literature, those abnormalities have been frequently related to malformations of the apical meristems or to constraints occurring during somatic embryo development (Corredoira et al. 2003; Hussain et al. 2009; Stasolla and Yeung 2003). Preliminary results with tamarillo (Lopes et al. 2000), and also with other species (Canhoto et al. 1999; Correia and Canhoto 2010), have demonstrated that somatic embryo cells are often vacuolated, hence suggesting a weak ability to accumulate storage compounds during maturation with negative impacts on embling production.

The results of the present work showed that plantlet formation was not conditioned by the high number of abnormal somatic embryos formed, since the number of plantlets was 3 times higher than the number of normal somatic embryos obtained after the maturation period. This seems to indicate that the meristematic zones were well formed and not negatively affected by the culture conditions, at least in what concerns the SAM. In fact, during conversion it was found that primary root development often occurred from adventitious root formation. Further studies concerning the characterization of apical meristems in somatic and zygotic embryos must bring some light in these aspects. An interesting feature observed during tamarillo somatic embryo conversion was the formation of a sheath-like structure completely surrounding the shoot

apex and roughly resembling the coleoptiles of gramineous plants. Attempts to find similar structures described in the literature were unsuccessful. The structural/functional role of this sheath must be analysed in detail in further studies.

Since the high number of abnormal somatic embryos did not prevent the conversion of most of them, the transition from embryogenic tissue to plant development seems to be more critical than conversion. Thus, assays to improve plant conversion through the establishment of more appropriate culture conditions for somatic embryo development and maturation were performed. The obtained results indicated that sucrose concentrations of 0.11 M are better for embryo development than the lower concentrations (0.07 M) previously used. The type and concentration of carbohydrates in the culture media have been pointed out as factors that strongly affect somatic embryo maturation and conversion (Chen et al. 2010; Corredoira et al. 2003; Troch et al. 2009). Sucrose has shown to improve maturation when present in high concentrations (Mauri and Manzanera 2003), but higher sucrose levels can also be deleterious (Shi et al. 2009). These results are in accordance to what was found for tamarillo, suggesting that intermediate sucrose levels (0.11 M) could reduce the drastic change from the induction medium (0.25 M sucrose) to the development medium conditions. Besides, higher sucrose availability in the culture medium could be mobilized to compensate the low levels of storage compounds detected in the developing tamarillo somatic embryos.

Although in some woody plants, maturation and germination of somatic embryos have been obtained in media lacking plant growth regulators (Canhoto et al. 2005; Shi et al. 2009), in most cases, the application of specific treatments is necessary. Abscisic acid and osmotic stresses are known to be important factors for seed maturation in many angiosperms (Rai et al. 2011; Vahdati et al. 2008). The inclusion of ABA into the culture medium during the final phases of somatic embryo development, simulating the natural increase occurring in zygotic embryos (Jiménez 2005), has been recognized as a factor which promotes normal development and maturation of somatic embryos in several species, such as cyclamen (You et al. 2011), Japanese larch (Zhang et al. 2010), Norway spruce (Bozhkov

et al. 2002), Persian walnut (Vahdati et al. 2008) or Spanish red cedar (Peña-Ramírez et al. 2011). For tamarillo, the presence of ABA did not improve the development process. Instead, 0.11 M sucrose and dark conditions were more effective for the improvement of somatic embryo development/maturation than culture in a medium supplemented with ABA.

Regarding light conditions, most authors have used photoperiod or darkness during somatic embryogenesis. Nevertheless, systematic studies on the effects of light in somatic embryogenesis are limited. Assays with *Eucalyptus globulus* showed that light may influence the quality of the maturation process, suggesting that darkness should be maintained until the cotyledonary stage (Pinto et al. 2008). Also for *Campanula punctata*, culture periods of 2 weeks in the dark followed by 3 weeks under light resulted in higher frequencies of embryo formation (Sivanesan et al. 2011). These observations correlate to what was observed for tamarillo somatic embryos.

Since the maturation and development of somatic embryos should mimic equivalent processes in zygotic embryos (Ikeda and Kamada 2005), the biochemical and histochemical differences between cotyledonary somatic embryos and mature zygotic embryos were also analysed. The results showed that somatic embryos have significant reduced levels (about 20 times) of total lipids when compared to zygotic embryos. Histological analysis revealed that cells of zygotic embryos were packed with lipid bodies, indicating that the quantified lipids are in fact storage lipids. Lipid storage in embryo cells, during the maturation stage of zygotic embryo development, is a common feature for many species (Bewley and Black 1994). Those lipids are mobilized during the early stages of germination. The correlation of lipid accumulation following ABA treatments (Kharenko et al. 2011) suggests that the culture conditions used for tamarillo somatic embryo maturation can still be improved, namely with the use of better suited ABA concentrations.

The histological analysis of storage proteins indicated that protein accumulation is more reduced in somatic embryos, but the biochemical quantification showed equivalent values for both types of embryos. One explanation could be related to the fact that storage proteins are highly glycosylated, giving origin to large protein bodies with a lower protein content than expected. Supporting this observation is the fact that the PAS technique, presumably specific for carbohydrates, also stain protein bodies. Other possibility is that, in somatic embryos, the protein cannot accumulate so intensively in protein bodies and may have a more diffuse distribution in the cytoplasm. Ultrastructural studies comparing the subcellular organization of cells in both types of embryo cotyledons may help clarifying these contradictory observations.

Histological data showed that zygotic embryos do not accumulate starch in their cells whereas somatic embryos usually possess starch grains. The accumulation of starch in somatic embryos is probably the result of a modification in cellular metabolism that might be a consequence of the high sucrose levels used in the culture medium (Pinto et al. 2011). Some studies (Merkle et al. 1995; Thorpe and Stasolla 2001) have focused on the association between synthesis and accumulation of starch and the development of somatic embryos. The results showed an increase in starch levels in the late development stages, indicating that starch accumulated at higher amounts in somatic embryos. These studies also suggested metabolic differences between zygotic and somatic embryos, the later being less efficient in converting carbohydrates into lipids and storage proteins.

The results so far obtained seem to indicate that an inefficient accumulation of storage compounds in developing tamarillo somatic embryos is related to the low rates of morphologically normal embryos obtained but does not interfere with plantlet formation. Further studies should clarify if the improved development conditions promote the accumulation of particular storage compounds in somatic embryos. A subject also deserving further analysis is the structural and functional characterization of the sheath-like structure found in the germinating somatic embryos which is absent in their zygotic counterparts.

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