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Enhancing somatic embryogenesis in avocado (*Persea americana* Mill.) using a two-step culture system and including glutamine in the culture medium

Carlos López Encina[a], Ann Parisi[b], Chris O'Brien[b], Neena Mitter[c,*]

[a] Institute of Horticulture Subtropical and Mediterranean 'La Mayora' (IHSM) CSIC-UMA, Experimental Station La Mayora, 29750 Algarrobo-Costa, Málaga, Spain
[b] Agri-Science Queensland, Department of Agriculture, Fisheries and Forestry, QLD 4067, Australia
[c] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD 4067, Australia

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ABSTRACT

The development of a more efficient *in vitro* regeneration system for somatic embryos (SEs) of avocado (*Persea americana*) would facilitate the development of new superior cultivars for this valuable horticultural crop. In this study, we report a new and efficient method for maintenance and regeneration of avocado SEs. Avocado SEs of four cultivars remained healthy and viable *in vitro* for 11 months on a medium used for mango somatic embryogenesis, compared with 3–4 months on Murashige and Skoog medium. Various supplements and media modifications were investigated to improve the low conversion rate of regenerated plants from avocado SEs reported previously. The one-step system for regeneration of white-opaque somatic embryos (WOSEs) used solid medium only over a period of 12–14 weeks (subculturing every 6 weeks). Addition of proline and glutamine improved the total regeneration from 0 to 17.5% and 10.5%, and plant/shoot recovery from 0 to 12.5% and 5%, respectively. A two-step culture system involving the transfer of WOSEs of cultivar 'Reed' after 6 weeks on solid to liquid medium for 12–15 days as an intermediate step, followed by subculturing again onto solid medium for 6 weeks improved total regeneration to 29% and plant/shoot recovery to 18.3 from 0% when regenerated by subculturing on solid medium only. Supplementation with proline in the solid as well as liquid medium in the two-step culture system at 0.4 g/L increased total regeneration to 35% and plant/shoot recovery to 20%. We were able to achieve highest regeneration using glutamine at 1 g/L in the two-step culture system in terms of both total regeneration (58.3%, including 43.3% bipolar regeneration) and plant/shoot recovery (36.7%) rates, which were significantly higher than in any other treatment investigated.

1. Introduction

Avocado (*Persea americana* Mill.), a subtropical evergreen tree of the Lauraceae family, is an economically important fruit species cultivated in tropical and subtropical areas worldwide. It originated from central Mexico and West Indies, and was introduced to other places such as Australia, South Africa, Israel and California from the early 1800s (Ben-Ya'acov and Michelson, 1995). In 2011, total production of avocado in the world was 4.4 million metric tonnes (MT), with 1.2 million MT of total production contributed solely by Mexico, making it the largest avocado producing country (FAO, 2011). Avocado production in Australia has increased by 65% since 2003 and is expected to increase from 28,196 tonnes to 64,000 tonnes by 2013/14. The most economically important avocado cultivar is 'Hass' because of its rich, pale yellow-green flesh and taste (Newett et al., 2002). Avocado is considered a good source of protein, carbohydrates, minerals and vitamins (Knight, 2002). The beta-sitosterols found in avocado help maintain healthy cholesterol levels and prevent cardiovascular diseases (Duester, 2001).

The factors that limit the production of avocado include diseases such as Phytophthora root rot, anthracnose and avocado sunblotch viroid (Marais, 2004; Palukaitis et al., 1979). The losses caused by these diseases run into millions of dollars and severely affect the growth, production and international trade of avocado. The search for resistance to these and other diseases within the species is extremely difficult (Litz et al., 2005). Breeding programs to incorporate disease resistance and other desirable traits in avocado are delayed by its long juvenile period and heterozygosity, the time required for evaluation of progeny of controlled pollination,

* Corresponding author Tel.: +617 3346 6513; fax: +617 3346 6501.
E-mail address: n.mitter@uq.edu.au (N. Mitter).

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and the absence of molecular markers for important horticultural traits (Raharjo et al., 2008). Implementation of biotechnological approaches including genetic transformation, *in vitro* mutagenesis and protoplast-based technologies is hindered by the lack of an efficient regeneration protocol for single cells of elite selections.

Regeneration from embryogenic cultures is problematic in most woody species (Márquez-Martín et al., 2012). Different explants such as immature zygotic embryos and nucellus have been used to study the embryogenic regeneration pathway of avocado (Mooney and van Staden, 1987; Pliego-Alfaro, 1981; Pliego-Alfaro and Murashige, 1988; Raviv et al., 1998; Suarez et al., 2006; Witjaksono and Litz, 1999a,b). Witjaksono and Litz (1999a) reported that avocado SEs can lose their morphogenic competence in as little as 3–4 months after induction, depending on the genotype. In addition to the loss of viability over time, the main factor limiting the conversion of SEs into plantlets is incomplete maturation (Ammirato, 1987). Perán-Quesada et al. (2004) worked with the cultivar 'Anaheim' and showed that there were several factors which effected the maturation of avocado SEs, i.e. mineral salts, sucrose concentration, gellan gum, coconut water and abscisic acid. In particular the formation of white-opaque somatic embryos (WOSEs) was significantly increased with the use of B5 nutrients (60% WOSEs) verses Murashige and Skoog (MS) nutrients (0% WOSEs) and higher germination rates were obtained when B5 media was supplemented with 10% coconut water.

Although the production of avocado SEs occurs with high efficiency, the percentage of high quality, bipolar embryos with shoot as well as root regeneration from avocado SEs is extremely low and is genotype-dependent (Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999b; Raharjo and Litz, 2003). This low rate of SE conversion is currently the main bottleneck in avocado regeneration via somatic embryogenesis (Litz et al., 2005). Avenido et al. (2009) reported success in 3 different trials of plant/shoot regeneration at 16.3, 23 and 20.7% with the cultivar 'Semil'. Witjaksono and Litz (2004) recorded shoot development of 2–10% in cultivar 'T362' and 16–37% in cultivar 'Fuerte' while root emergence varied from 5–23% for 'T362' and 0–3% for 'Fuerte'. Avenido et al. (2009) recorded a shoot regeneration of 17.8–26.9% after gamma irradiation, however, they still found the procedure to obtain plants ready to transplant remained slow and time consuming and required long-term shoot culture and micro-propagation through micro-grafting techniques. Efendi (2003) and Raharjo et al. (2008) were able to obtain GM avocado plantlets from SE cultures after transformation procedures but at extremely low percentages. Recently, Palomo-Ríos et al. (2012) reported a significant improvement in the germination rate of genetically transformed avocado SEs by introducing a step of culturing in liquid medium, however, the conversion rate to mature plantlets remained low at 0.5–2%.

Here, we report a new and efficient method for avocado SE regeneration that improves on existing protocols for maintenance, regeneration and plant recovery. This two-step culture method will pave the way for the application of genetic transformation for desired traits, as well as cryopreservation of SEs for germplasm conservation.

2. Materials and methods

2.1. Induction of SEs

Zygotic embryos were removed from 1–2 mm fruits of avocado (*Persea americana* Mill.) cultivars 'Reed', 'Hass', 'Duke 7' and 'A10' following the protocol of Pliego-Alfaro and Murashige (1988). Embryogenic cultures were then established from immature zygotic embryos that were placed on an induction medium (Witjaksono and Litz, 1999a) used to initiate embryogenesis. This induction medium was based on a modified B5 medium containing B5 major salts without NH_4NO_3 (Gamborg et al., 1968), MS minor salts (Murashige and Skoog, 1962), 0.1 mg/L picloram, 4 mg/L thiamine-HCl, 100 mg/L inositol, 30 g/L sucrose and 8 g/L agar (Sigma A-1296) pH 5.7 (Witjaksono and Litz, 1999a). The embryogenic cultures were incubated in the dark at 25 ± 1 °C in Petri dishes (60 × 15 mm) containing 15 mL of medium and sealed with Parafilm® for a maximum of 3 months.

2.2. Maintenance of SEs

After induction, the avocado embryogenic cultures consisting of SEs were subcultured at monthly intervals. Each petri dish (100 × 15 mm) containing either 30 mL of Murashige and Skoog (1962) basal salts supplemented with 0.1 mg/L picloram, 4 mg/L thiamine, 100 mg/L myo-inositol, 30 g/L sucrose and 8 g/L agar (Sigma A-1296) pH 5.7 (Witjaksono and Litz, 1999b) or mango medium for somatic embryo induction (MMSE) which consisted of Gamborg's B5 major salts (Gamborg et al., 1968), MS minor salts (Murashige and Skoog, 1962), MS vitamins (Murashige and Skoog, 1962), MS Fe-EDTA (Murashige and Skoog, 1962), 0.4 g/L glutamine, 0.5 mg/L 2, 4-dichlorophenoxyacetic acid, 100 mL/L coconut water, 60 g/L sucrose and 2.5 g/L gellan gum (Gelrite® Merck) pH 5.7 (Patenã et al., 2002) was seeded with approximately 50 mg of SEs, sealed with Parafilm® and grown at 25 ± 1 °C in the dark. The transfer of SEs was carried out monthly for a maximum of 11 months on each medium starting with a specified number of plates for each cultivar. Only the viable SEs without any necrosis were harvested from all plates and re-seeded onto new plates at the same seeding rate of approximately 50 mg per plate. The non-viable/dead SEs which showed necrosis were discarded.

2.3. Germination of WOSEs on solid media

The SE germination experiments were carried out only with the commercial cultivar 'Reed', the most commonly used rootstock in Australia. For germination experiments, WOSEs with a diameter of approximately 5–8 mm were selected from actively growing embryogenic cultures on MMSE medium. These embryos were induced to germinate on solid somatic embryo development media (S-SED) containing MS major and minor salts (Murashige and Skoog, 1962), 4 mg/L thiamine-HCl, 100 mg/L inositol, 0.5 mg/L 6-benzyladenine (BA), 1 mg/L gibberellic acid (GA_3), 45 g/L sucrose, 100 mL/L coconut water and 6 g/L gellan gum (Gelrite®, Merck). The S-SED medium was supplemented with various concentrations of glutamine (0.5, 1, 2 or 4 g/L) or proline (0.1, 0.2 or 0.4 g/L). A minimum of 50 WOSEs were used per treatment and each experiment was conducted in duplicate. The WOSEs were placed on S-SED medium in Petri dishes (100 × 20 mm) containing 40 mL of medium with or without any supplements and sealed with Parafilm® and grown at 25 ± 1 °C in a culture room under standard light conditions (42 μmol m/s) provided by cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania) for 6 weeks. The WOSEs were subcultured once to fresh S-SED medium with or without the proline or glutamine concentrations for a period of 6–8 weeks. Results were recorded in terms of SE survival (number of SEs without necrosis), number of green embryos, unipolar (shoot only regeneration, no roots) or bipolar (regeneration of shoot along with a root), and plant/shoot recovery (growth of bipolar shoot to a stage suitable for transfer to soil or unipolar shoot to a stage suitable for micro-grafting or root induction).

2.4. Germination of WOSEs using a two-step regeneration system

The two-step regeneration system was trialled with and without supplementation of proline (0.4 g/L) or glutamine (1 g/L) in the

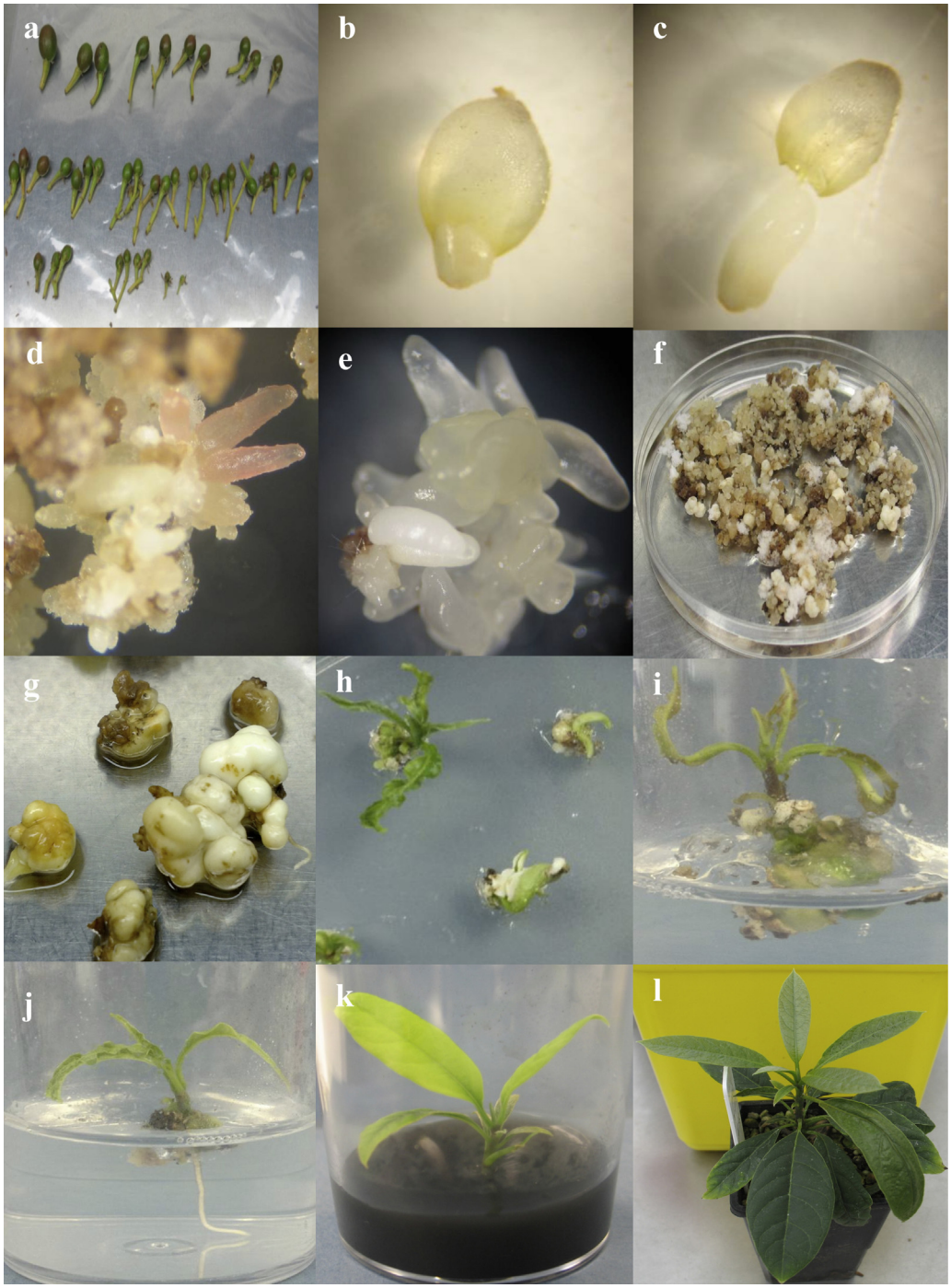

Fig. 1. Induction, multiplication, maintenance and regeneration of 'Reed' avocado SEs: (a) immature avocado fruitlets 5–10 mm in size, (b) and (c) immature avocado embryo, (d) primary SEs, (e) secondary SEs, (f) SEs on MMSE media, (g) WOSEs on S-SED, (h) germinating embryos, (i) unipolar shoot, (j) bipolar shoot, (k) complete plantlet on medium supplemented with activated charcoal and (l) Acclimatised plant.

S-SED as well as in the liquid somatic embryo development (L-SED) medium. WOSEs 5–8 mm in size were selected from MMSE plates and placed on S-SED medium for 6 weeks (Fig. 1g). After 6 weeks WOSEs were transferred from S-SED media to L-SED as an intermediate step. Ten WOSEs were placed in a 250 mL Erlenmeyer flask containing 25 mL of L-SED medium. A minimum of 50 WOSEs were used per treatment and each experiment was conducted in duplicate. Flasks were maintained on a rotary shaker at 80–85 rpm for 12–15 days. The WOSEs were transferred to S-SED medium for a further period of 6 weeks. The results for regeneration of WOSEs were recorded as described above in Section 2.3.

2.5. Plantlet growth

Bipolar plants recovered from the germination treatments were subcultured on S-SED medium plus 1 g/L activated charcoal for 15 days (minus growth hormones) in 120 mL containers for shoot/plant development. Unipolar shoots which did not have a root and were 1.5–2.0 cm in length were induced to root by subculturing them on MS medium minus ammonium nitrate (NH_4NO_3) supplemented with 25 mg/L indole-3-butyric acid (IBA) for 3 days (Barceló-Muñoz et al., 1999; Pliego-Alfaro, 1988). After 3 days, the shoots were transferred onto MS medium (without NH_4NO_3) minus

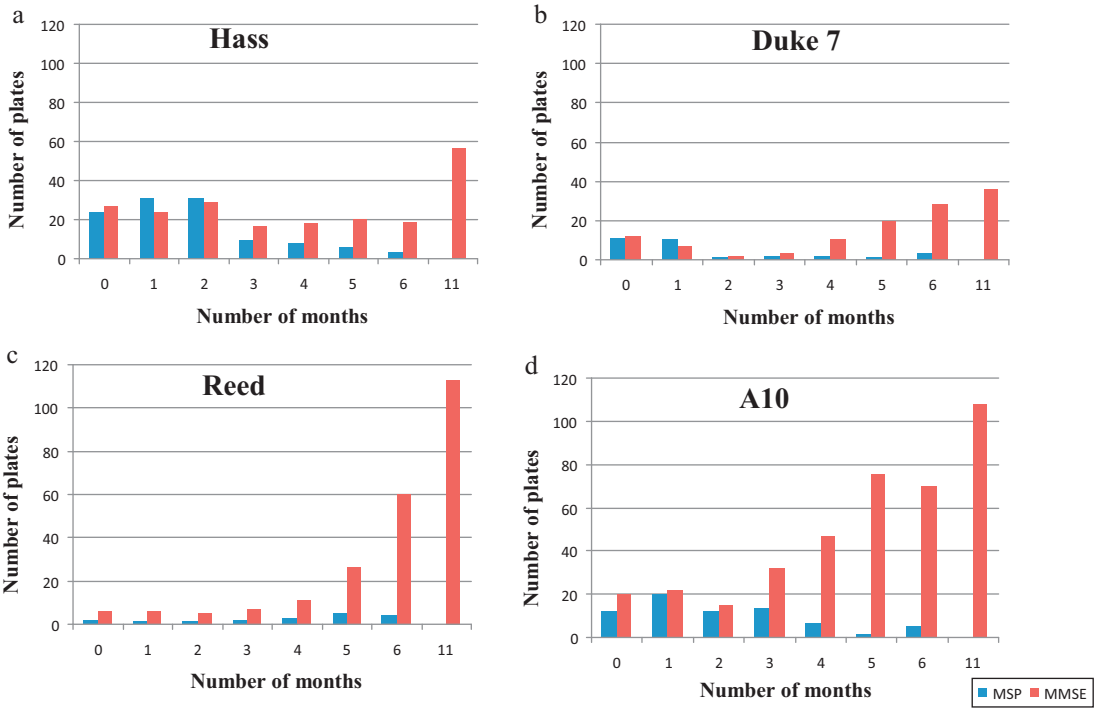

Fig. 2. Multiplication of SEs of (a) 'Hass', (b) 'Duke7', (c) 'Reed' and (d) 'A10' grown on MSP or MMSE media subcultured monthly for 11 months. The seeding rate was maintained at 50 mg/plate at each subculture.

IBA but supplemented with 1 g/L activated charcoal and placed in a plant growth room as described above.

2.6. Statistical analysis

Statistical analysis was carried out using the SAS (Littell et al., 2002) and GenStat (Payne et al., 2011) software packages. The variables (percentage survival, regeneration and plant/shoot recovery) were analysed by maximum likelihood analysis of variance. Treatment means were compared with a contrast test.

3. Results

3.1. Induction, multiplication and maintenance of SEs

Avocado embryogenic cultures consisting of SEs were induced in Petri dishes from immature zygotic embryo explants (Fig. 1a–c). Secondary embryos were generated successfully from primary SEs and maintained on either MSP or MMSE media (Fig. 1d–f). MMSE medium sustained a higher rate of multiplication after 11 months compared with MSP for all the cultivars tested. SEs of 'Reed' showed lower multiplication rate on MSP at all time points, whereas the number of MMSE plates increased significantly over the time period of 5–11 months (Fig. 2c). SEs of 'A10' showed a higher proliferation rate on MMSE from 3 to 11 months (Fig. 2d) while their proliferation rate on MSP gradually declined over this time period. Statistical analysis of SE multiplication of the four cultivars showed quite clearly that the maintenance on the two media tested was different with MSP having a negative slope over time and MMSE having a positive one (Fig. 3). Prolonged subculturing on MSP resulted in a decrease in multiplication of SEs and no viable SEs could be recovered after 11 months. SEs on MMSE media on the other hand continued to multiply with each subculture for a period of up to 11 months (Fig. 3).

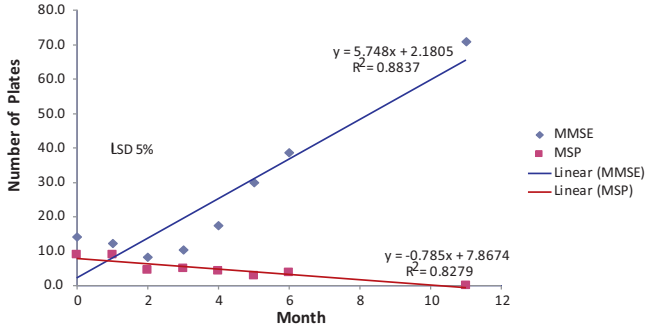

Fig. 3. Linear regression trend lines for the effect of media on the multiplication rates of avocado SEs of the four cultivars over a time period of 11 months. The plot shows that the difference between the two media was significant after 3 months with negative slope on MSP and positive slope on MMSE.

3.2. Maturation and germination of SEs

Although, a 95% survival rate for WOSEs was recorded (data not shown) on S-SED medium, none of the embryos showed unipolar or bipolar regeneration (Table 1). Also, WOSEs did not show any signs of greening on S-SED medium (data not shown). The S-SED medium was then supplemented with various concentrations of the amino acids proline (0.1–0.4 g/L) and glutamine (0.5– 4 g/L) to increase the level of available nutritional reserves for the SEs (Table 1) and make it easier for them to fully mature and germinate. All treatments showed excellent survival rates of over 85% and the percentage of WOSEs that became green during the incubation time varied from 65 to 93% (data not shown).

The overall percentages of unipolar shoot regeneration remained quite low, with a maximum of 12.5% in the 0.4 g/L proline treatment, which also showed 5% bipolar regeneration. The remaining proline treatments produced less than 10% unipolar regeneration rates and no bipolar regeneration (Table 1).

Table 1
Percentage regeneration of 'Reed' WOSEs on S-SED medium supplemented with proline and glutamine after 12–14 weeks.

Treatment	Shoot regeneration (unipolar) (A)	Shoot + root regeneration (bipolar) (B)	Total regeneration (A + B)	Plant/shoot recovery
S-SED/S-SED	0 c	0 b	0 d	0 c
S-SED/S-SED with proline 0.1 g/L	7.5 b	0 b	7.5b c	7.5 b
S-SED/S-SED with proline 0.2 g/L	5 b	0 b	5 c	5 b c
S-SED/S-SED with proline 0.4 g/L	12.5 a	5 a	17.5 a	12.5 a
S-SED/S-SED with glutamine 0.5 g/L	0 c	0 b	0 d	0 c
S-SED/S-SED with glutamine 1 g/L	10.5 a	0 b	10.5 b	5b c
S-SED/S-SED with glutamine 2 g/L	10 a	0 b	10 b	3.3b c
S-SED/S-SED with glutamine 4 g/L	0 c	0 b	0 d	0 c

Means with the same letter within a same column are not significantly different ($p<0.05$) according to the contrast test.

For glutamine, the percentage of unipolar shoot regeneration was statistically similar at 1 and 2 g/L. It was also statistically similar to proline at 0.4 g/L. An increase in glutamine concentration to 4 g/L resulted in no regeneration. None of the glutamine treatments on S-SED medium induced any bipolar regeneration. Plant/shoot recovery after regeneration was highest on proline at 0.4 g/L followed by glutamine at 1 and 2 g/L. The developed shoots usually showed hyperhydricity that disappeared with the rapid growth of the regenerated plants. The regenerated shoots also showed other abnormalities like scythe leaves, apical necrosis and callus overgrowth during the initial steps of the regeneration process, probably because of the high concentration of GA_3 (1 mg/L) in the S-SED medium.

3.3. Germination of SEs using a two-step regeneration system

A survival rate of 90–95% (data not shown) showed that SE survival was not affected by introducing an intermediate L-SED step. A total plant regeneration rate of 29% was recorded after introducing an intermediate liquid step (Table 2) compared with 0% when SEs were regenerated on S-SED medium only (Table 1). Both L-SED and S-SED media were then supplemented with either glutamine (1 g/L) or proline (0.4 g/L) to improve regeneration. The addition of proline to the media gave a total plant regeneration rate of 35%, which was statistically similar to the treatment without supplementation. The addition of glutamine to the L-SED and S-SED media produced the most promising results, with a significantly higher percentage of total plant regeneration (58.3%) and 43.3% of the regenerates showing bipolar regeneration (Table 2).

Plantlets were obtained from both unipolar (Fig. 1i) and bipolar regenerates (Fig. 1j) by subculture on S-SED medium plus 1 g/L activated charcoal for 15 days (Fig. 1k) followed by transfer to larger containers containing S-SED medium. Rooting of unipolar shoots was induced by subculturing 1.5–2.0 cm shoot explants in MS medium without NH_4NO_3 supplemented with 25 mg/L IBA for 3 days. The fully grown *in vitro* plantlets (Fig. 1l) were acclimatized in potting mix before being moved to a glass house for further development. The plant/shoot recovery percentage obtained on 1 g/L glutamine medium (36.7%) was significantly higher than on media without supplements (18.3%) or supplemented with 0.4 g/L proline (20%) (Table 2).

4. Discussion

Somatic embryogenesis in avocado was first reported by Pliego-Alfaro (1981) using immature zygotic embryos of cultivar 'Hass' and has since been accomplished with zygotic embryos of various cultivars (Mooney and van Staden, 1987; Perán-Quesada, 2001; Raviv et al., 1998; Witjaksono and Litz, 1999b). More recently, induction of embryogenic cultures from the nucellus of immature fruits has also been reported (Márquez-Martín et al., 2012; Suarez et al., 2006; Vidales-Fernández et al., 2003). Witjaksono and Litz (1999a) optimised the growth of embryogenic cultures from immature zygotic embryos as the initial explants and reported that loss of embryogenic potential under maintenance conditions is cultivar-dependent and can vary from 3 months to more than a year. In our investigation initiation of SEs on the media described by Witjaksono and Litz (1999a) followed by multiplication on MMSE gave the best outcomes for the cultivars tested. For the four cultivars used in the current study, MMSE medium was found to support improved proliferation of the SEs over a period of 11 months. The improved proliferation and formation of WOSEs seen on MMSE could be due to the medium using gellan gum as the gelling agent versus MSP which contains agar. Adams and Rinne (1980) showed that water relations between the SE and its environment *in vitro* were particularly important during the maturation phase. Witjaksono and Litz (1999b) and Perán-Quesada et al. (2004) also found that WOSEs were significantly increased by decreasing water availability through higher concentrations of gellan gum and no interference in sucrose availability. In our study we also noted the increase of WOSEs on MMSE medium which uses gellan gum. Another major difference between the MMSE and MSP medium was the use of B5 macro nutrients in MMSE medium. Perán-Quesada et al. (2004) and Avenido et al. (2009) showed that the use of B5 nutrients was essential in the formation of WOSEs that were able to germinate. It should also be noted the MMSE medium contains coconut water which has been shown to increase fatty acid content in SEs (Quinn et al., 1989) and has a positive effect on germination in many other species. MMSE medium has previously been reported to be the best medium for mango somatic embryogenesis and regeneration of plantlets for 14 mango varieties (Patenã et al., 2002).

After many years of research on avocado somatic embryogenesis by various authors, it is clear that the initial steps of SE induction take place without any problems (Witjaksono and Litz, 1999a,b). However, conversion of SEs into plantlets with bipolar germination occurs at very low frequencies (Pliego-Alfaro and Murashige, 1988; Raharjo and Litz, 2003; Witjaksono and Litz, 1999b). Pliego-Alfaro and Murashige (1988) reported that the meristems fail to organize in the majority of avocado SEs, which is a major barrier for germination. During their maturation, SEs accumulate or store products and change colour from translucent to white opaque (Cailloux et al., 1996). This morphological feature has been used as an indicator for the efficiency of avocado SE maturation and regeneration (Witjaksono and Litz, 1999b). Márquez-Martín et al. (2012) reported that SE-and PEM-type embryogenic cultures have cells that are physiologically different from each other and therefore go through separate maturation processes that in turn have unique regeneration capabilities. In general, small SEs in the late torpedo stage usually fail to germinate, probably because of inadequate development of the embryo; hence, it is important to use embryos over 5 mm in germination experiments as reported by Perán-Quesada et al. (2005). WOSEs with a diameter of approximately 5–8 mm (Fig. 1g) were also used as starting materials in

Table 2
Percentage regeneration of 'Reed' WOSEs using an intermediate liquid step supplemented with proline (0.4 g/L) or glutamine (1 g/L) after 14 weeks.

Treatment	Shoot regeneration (unipolar) (A)	Shoot + root regeneration (unipolar) (B)	Total regeneration (A + B)	Plant/shoot recovery
S SED/L-SED/S-SED	9 b	20 b	29 b	18.3 b
S-SED/L-SED/S-SED with 0.4 g/L proline	25 a	10 c	35 b	20 b
S-SED/L-SED/S-SED with 1 g/L glutamine	15 b	43.3 a	58.3 a	36.7 a

Means with the same letter within a same column are not significantly different ($p<0.05$) according to the contrast test.

the current regeneration study. The greening of embryos observed on regeneration media can indicate a change in the physiological status of the SE because of the development of chloroplasts in the cotyledonary tissues and embryo axis, but until now it has been impossible to correlate greening with real development and germination of the SE. In our experiments, the high percentages of SEs showing a change in colour did not correlate with the germination percentages obtained. However, the green-opaque SEs obtained in various treatments showed much reduced (1–2%) callus production and a longer survival time without degeneration or necrosis (data not shown).

Amino acids are known to play a role in cellular growth and differentiation and are often components of plant cell culture media (Márquez-Martín et al., 2012). Though the regeneration of 'Reed' SEs on S-SED supplemented with 0.5 mg/L BA, 1 mg/L GA_3 and various concentrations of proline and glutamine was improved over media without these supplements, the plant/shoot recovery percentage was very low, varying from 5 to 7.5% (Table 1). In accordance with Mooney and van Staden (1987), Pliego-Alfaro and Murashige (1988), and Witjaksono et al. (1999) we also detected frequent failure of bipolarity in SEs on S-SED/S-SED medium with or without supplements and the occurrence of various types of abnormalities.

We investigated a two-step culture system involving a liquid intermediate step with or without amino acid supplementation to improve bipolar regeneration as well as plant shoot recovery. Von Arnold (2008) reported better synchronisation of embryogenic cultures in suspension. Cerezo et al. (2011) showed that culturing olive calli in liquid medium for 4 weeks followed by sieving through a mesh did not affect the recovery of mature SEs, and even increased the size of mature embryos. Palomo-Ríos et al. (2012) also reported a significant improvement in the germination rate of genetically transformed avocado SEs when they were cultured in liquid medium with 0.44 μM BA and 2.89 μM GA_3 for 3 days in a roller drum and later transferred to the same medium gelled with agar. However, the conversion rate they recorded was still low at 0.5 to 2%. The current culture system which included growing SEs on S-SED/L-SED/S-SED with proline or glutamine supplementation, resulted in total regeneration of 29–58.3% and plant/shoot recovery rates of 18.3–36.7% for 'Reed' SEs in the various treatments investigated (Table 2). During the 12–15 days incubation on L-SED as an intermediate step, the WOSEs germinate and develop shoots and roots which grow actively. Subsequent subculturing on S-SED is required to finish plant development and to overcome the hyperhydric anomalies occurring during the incubation in L-SED.

Due to the low percentage of bipolar regeneration, the procedure to obtain plants ready to transplant remains inefficient, slow and time consuming, requiring long-term shoot culture and micropropagation through micro-grafting techniques. Witjaksono and Litz (2004) reported a higher shoot regeneration rate of 16–37% with 'Fuerte', but the bipolar regeneration rate obtained was only 3%. A high bipolar regeneration rate of 43.3% was obtained for 'Reed' SEs when glutamine was added as a supplement in the two-step culture system. In this work, we did not record the regeneration of roots alone as a significant event as these could never be developed into full plants. However, micro-grafting of regenerated shoots can be used to obtain rooted plants from unipolar shoot regeneration as reported by Raharjo and Litz (2003). The unipolar shoots obtained in the present investigation were subcultured on MS medium without NH_4NO_3 following the procedure of Barceló-Muñoz et al. (1999).

The process to obtain plants ready for acclimatization from WOSEs using our two-step method took approximately 14 weeks with a highest plant/shoot recovery rate of 36.7% on medium supplemented with 1 g/L glutamine. The total time required for SED/SED method or S-SED/L-SED/S-SED method is similar, and may depend on the behaviour of the individual regenerates, but the two-step method is more efficient in terms percentage of germination and quality of the shoots. The long-term maintenance of avocado SEs and increased efficiency of regeneration processes for avocado plants via somatic embryogenesis can add considerable value to developmental and genetic transformation studies for the generation of new and improved avocado varieties as well as cryopreservation of SEs for germplasm conservation.

5. Conclusion

Long term maintenance and regeneration of plantlets from avocado SEs has been a major barrier in the development of new avocado varieties. Multiplication on MMSE was found to give significantly better proliferation rates of SEs over a time period of 11 months. A two-step regeneration system involving the transfer of WOSEs to liquid medium as an intermediate step for inducting germination between subcultures in solid medium, significantly enhanced shoot/plant development from SE tissue. The total time required for SED/SED method or S-SED/L-SED/S-SED methods is similar, and maybe dependent on the behaviour of the individual regenerates, but the two-step method is always more efficient in terms percentage of germination and quality of the shoots.

Liquid incubation in a media supplemented with 1 g/L glutamine for 12–15 days followed by the subculture of the regenerants in the same solidified media increased the shoot regeneration and regenerants survival from 10 and 5% in solid medium to 58.3 and 36.7%, respectively. The work paves way for further studies to increase the efficiency of regeneration process of avocado plants via somatic embryogenesis as well as to increase the quality of the regenerants using different genotypes of avocado. This can add considerable value to developmental and genetic transformation studies on the generation of new and improved avocado varieties as well as cryopreservation of SEs for germplasm conservation.

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