

Factors affecting maturation of avocado somatic embryos

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Abstract

The effect of mineral salts, sucrose, gellan gum, abscisic acid and coconut water on maturation of avocado (*Persea americana* Mill.) somatic embryos was studied. Use of B5 major salts was essential to obtain white-opaque embryos. Sucrose at 175 mM, gellan gum (6.8 g l⁻¹) or coconut water (10–20%) also enhanced the recovery of white-opaque embryos. Abscisic acid slightly enhanced the appearance of white-opaque embryos. White-opaque embryos were further grown on MS, modified B5 medium supplemented with 10% coconut water or Jensen–MS media and they subsequently germinated on M1 medium. Higher germination rates (11.11%) were obtained with embryos that matured on modified B5 medium supplemented with 10% coconut water, although larger embryos developed on MS medium. Thus, embryo size during maturation does not seem to be correlated with germination capacity. Shoots obtained from germinated embryos could be multiplied in the presence of 4.44 μM benzylaminopurine and successfully rooted (80% rate) after a 3 days treatment with 4.92 μM indole-3-butyric acid. Over 90% of the rooted shoots survived acclimatization.

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Keywords: Avocado; *Persea americana* Mill.; Maturation phase; Somatic embryogenesis

Abbreviations: ABA, abscisic acid; BA, benzylaminopurine; B5, Gamborg major salts; B5m, Gamborg major salts and Murashige and Skoog minor salts and vitamins; cw, coconut water; IBA, indole-3-butyric acid; M1, germination medium of Skene and Barlass; MS, Murashige and Skoog; w-o, white-opaque

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1. Introduction

Application of biotechnology in breeding programs with tree species requires efficient *in vitro* regeneration methods. In this sense, somatic embryogenesis is preferred to adventitious regeneration, e.g. single-cell origin with less risk of genetic chimeras (Shoemaker et al., 1986) and simultaneous production of organized root and shoot axes (Mathews and Wetzstein, 1993). The development of an efficient regeneration system in avocado, via somatic embryogenesis, would be of key importance to obtain transformed plants with genes conferring tolerance to root rot caused by *Phytophthora cinnamomi*, a disease having tremendous negative impacts on commercial orchards of avocado throughout the world (Zilberstaine and Ben-Ya'acov, 1999).

Embryogenic cultures derived from immature avocado zygotic embryos have been obtained by different authors (Pliego-Alfaro, 1981; Mooney and van Staden, 1987; Witjaksono and Litz, 1999a). Nevertheless, the final conversion into plants is difficult (Pliego-Alfaro and Murashige, 1988; Witjaksono and Litz, 1999b). Low rates of conversion are common for somatic embryos (Janick, 1993), limiting the exploitation of the technique. Poor quality and incomplete maturation of the somatic embryos produced are considered to be the main factors limiting the conversion of embryos into plants (Ammirato, 1987).

The aim of this work was to improve development and maturation of avocado somatic embryos. The effects of different media components on the production of white-opaque (w-o) somatic embryos have been studied. White-opaque somatic embryos have been considered as good quality embryos because their aspect may reflect that deposition of storage substances (starch and proteins) has been initiated (Cailloux et al., 1996). In addition, the influence of different media on further maturation and subsequent embryo germination was tested.

2. Materials and methods

2.1. Induction of embryogenic cultures

Embryogenic avocado (*Persea americana* Mill.) cultures were established from immature zygotic embryos, cultivar Anaheim, according to Pliego-Alfaro and Murashige (1988) on MS medium (Murashige and Skoog, 1962) supplemented with 0.41 μM picloram and 6 g l⁻¹ agar (Sigma A-1296). Cultures were incubated in darkness at 25 \pm 1 °C. The embryogenic cultures were maintained on the same medium with the same culture conditions, with subculturing at monthly intervals.

2.2. Development of white-opaque somatic embryos

Embryogenic suspensions were initiated following the protocol of Witjaksono and Litz (1999a) by inoculating 0.5 g of friable callus into 15 ml of the same medium used for culture initiation in 50 ml Erlenmeyer flasks. Suspensions were allowed to grow on a rotary shaker (50 rpm) in semi-darkness during two recultures of 15 days. Embryogenic suspensions were

filtered successively using 2 and 1 mm screens and the fraction retained between the two screens was selected to initiate development of w-o somatic embryos. Inoculum consisted of 40 mg of this fraction per test tube (25 mm × 150 mm) containing 25 ml of semisolid medium.

2.2.1. *Effect of basal medium*

Two different basal media were tested: MS and B5m medium (major salts of the B5 formulation (Gamborg et al., 1968) with MS minor salts and vitamins). Both contained 88 mM sucrose and 555 μM *m*-inositol and were gelled with 1.7 g l^{-1} gellan gum (Gelrite, Kelco).

Cultures were maintained in the dark at 25 °C and subcultured onto fresh medium at 4-week intervals.

2.2.2. *Effects of sucrose, gellan gum, abscisic acid and coconut water*

The effects of sucrose (175, 263 mM), gellan gum (3.4, 6.8 g l^{-1}), abscisic acid (ABA) (1, 10, 100, 1000 μM) and coconut water (cw) (10 and 20% (v/v)) on formation of w-o somatic embryos were studied. ABA and cw (Sigma C-5915) were filter sterilized and added after autoclaving. Basal medium consisted of B5m medium with 88 mM sucrose, 555 μM *m*-inositol and it was solidified with 1.7 g l^{-1} gellan gum.

2.3. *Maturation of somatic embryos*

For maturation, w-o somatic embryos >5 mm in length that developed in B5m medium gelled with 6.8 g l^{-1} gellan gum were transferred individually onto the following media: B5m medium supplemented with 10% (v/v) cw (B5m + cw) during 10 weeks; MS medium during 10 weeks; Jensen (Jensen, 1977) medium 5 weeks and MS medium 5 weeks.

Maturation treatments were carried out in darkness at 25 ± 1 °C with reculturing at 5 week intervals.

2.4. *Germination of somatic embryos*

Mature somatic embryos with cotyledons partially excised were transferred to M1 medium (Skene and Barlass, 1983) to induce germination. Germination occurred at 25 ± 1 °C under a 16 h light photoperiod provided by GroLux lamps (Sylvania, Germany) (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The embryos were kept in this medium during three recultures of 5 weeks each.

2.5. *Data and statistical analysis*

The number of cultures per treatment ranged between 18 and 20 in experiments to induce formation of w-o embryos. Experiments were observed during three subcultures.

In the first series of experiments, percentage and average percentage of cultures giving rise to w-o somatic embryos as well as number and average number of w-o somatic embryos per culture were recorded. At the end of each subculture, w-o embryos were removed and only selected embryogenic cultures were transferred onto fresh medium.

In the maturation and germination experiment, the number of somatic embryos per treatment ranged between 25 and 32. Weight increase and percentage of necrotic cultures were evaluated after 5 and 10 weeks on maturation medium. Percentage of germinating as well as necrotic cultures and type of germination (shoot, root, or shoot and root) were recorded at the end of each reculture (5, 10 and 15 weeks) on germination medium.

The Kruskal–Wallis nonparametric test was applied. Experiments with percentage data were subjected to a frequency analysis with the $R \times C$ test of independence. The significance level was 0.05 in all cases.

3. Results

3.1. Recovery of white-opaque somatic embryos

3.1.1. Effect of basal medium

Noticeable differences were found between cultures on MS and B5m basal media. Although a higher weight increase was achieved on MS medium (data not shown), cultures on MS medium were quite homogeneous with a lot of proembryonic masses (PEMs) and translucent somatic embryos in the earlier developmental stages. By contrast, cultures that developed on B5 major salts mainly consisted of somatic embryos that reached the cotyledonary stage appearing w-o in color. In B5m basal medium, w-o somatic embryos were obtained beginning with the first subculture (Fig. 1) although somatic embryos >5 mm were obtained only from the second subculture onwards.

3.1.2. Effect of sucrose

Sucrose concentrations higher than the standard (88 mM) significantly enhanced the development of w-o structures with optimum results obtained in the presence of 175 mM sucrose (Fig. 2). In this treatment, an important increase in the production of high quality (w-o and >5 mm) embryos was observed in the second subculture. This result was mainly due to the increase in the percentage of cultures forming w-o embryos. In the following subculture, the production of w-o somatic embryos decreased, especially those >5 mm.

Roots appeared in 47.37% of the cultures grown for 10 weeks in the presence of 175 mM sucrose with an average of 1.53 roots per culture. The emergence of roots decreased with time and disappeared after the fifth subculture (data not shown).

3.1.3. Effect of gellan gum

The production of w-o somatic embryos increased significantly with gelling agent concentration attaining the highest values with respect to frequency and number per culture with 6.8 g l⁻¹ Gelrite (Fig. 3). Moreover, in the second subculture a high proportion of the w-o somatic embryos obtained were >5 mm (86% for 3.4 g l⁻¹ and 68% for 6.8 g l⁻¹). The maximum w-o somatic embryo production was anticipated as the Gelrite concentration increased.

3.1.4. Effect of ABA

Different ABA concentrations did not have a clear effect on the development of w-o somatic embryos (data not shown). Presence of ABA did not significantly affect the production

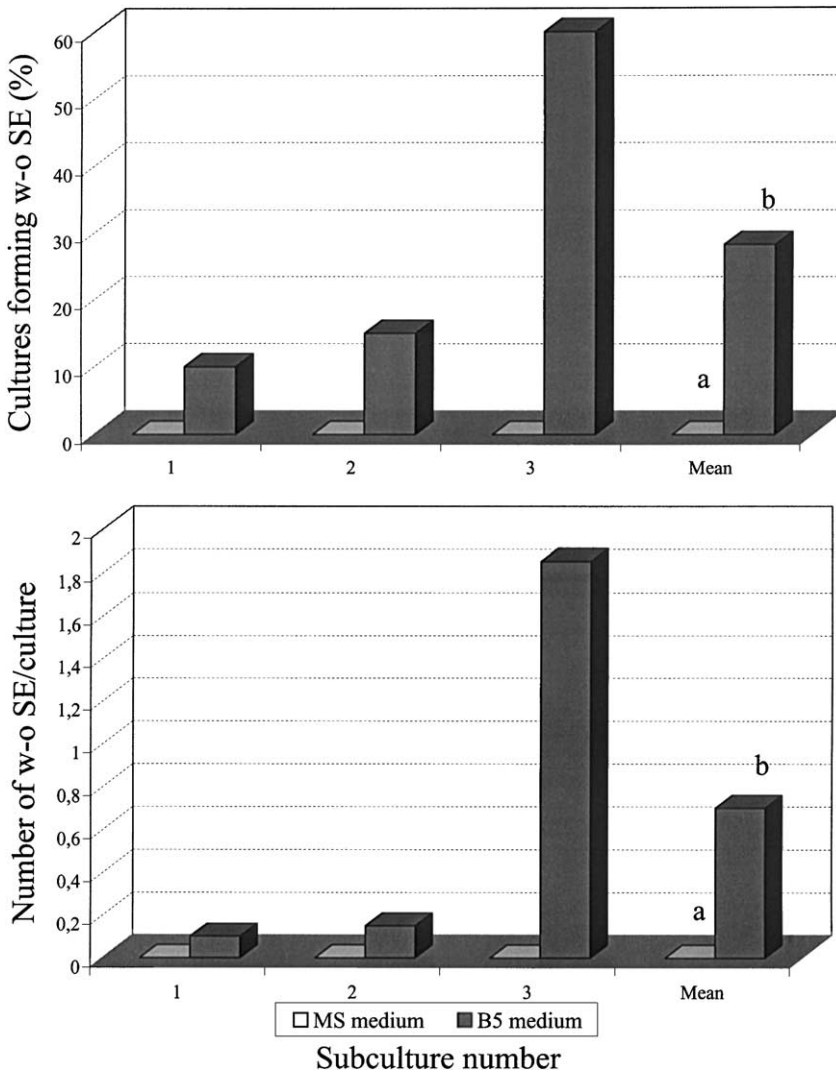


Fig. 1. Effect of MS and B5m media on formation of w-o avocado embryos, cv. Anaheim. Data were taken during three subcultures (4 weeks each). Different letters indicate significant differences obtained by frequency analysis and the Kruskal–Wallis test at 5%.

frequency of w-o somatic embryos. However, the total number of w-o somatic embryos per culture was always higher in the presence of ABA with fluctuations in the range between 1.12 (10 μ M) and 2.40 (100 μ M) versus 0.94 w-o somatic embryos per culture in the control. Interestingly, formation of hyperhydric globular somatic embryos was significantly increased in the presence of ABA (data not shown).

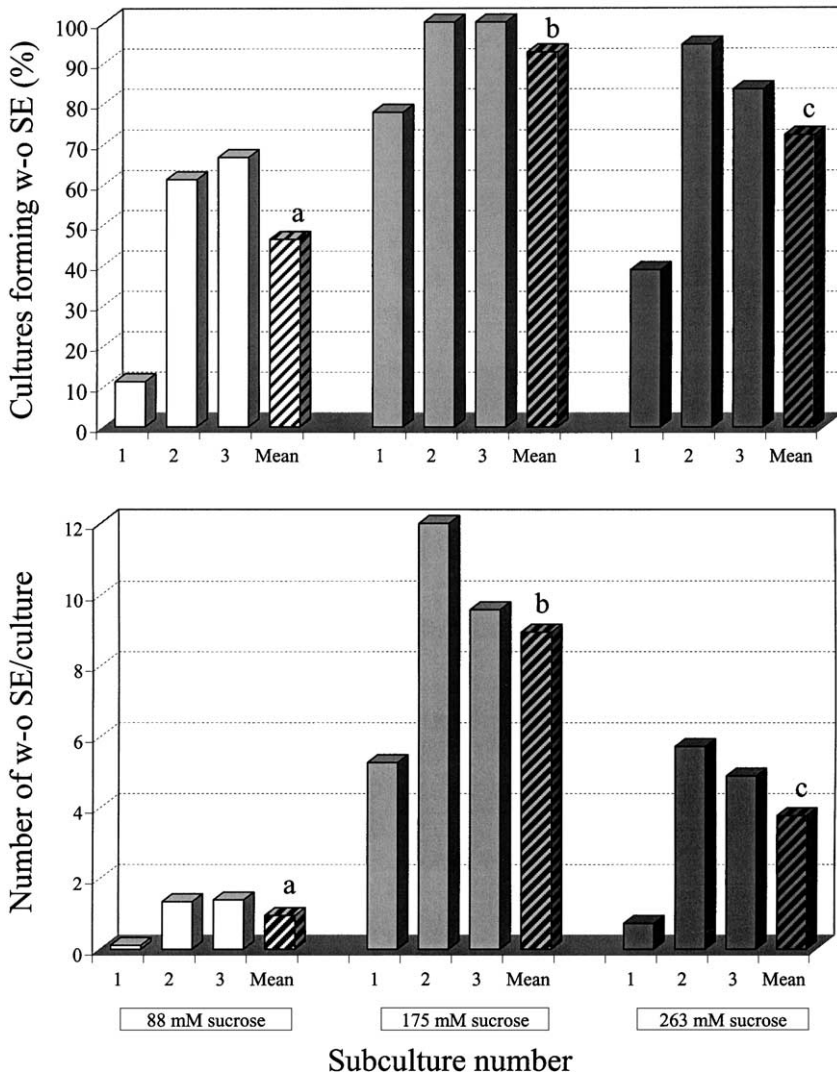


Fig. 2. Effect of sucrose concentration on formation of w-ovocado embryos, cv. Anaheim. Data were taken during three subcultures (4 weeks each). Different letters indicate significant differences obtained by frequency analysis and the Kruskal–Wallis test at 5%.

3.1.5. Effect of cw

The incorporation of cw in B5m basal medium significantly improved the production of w-o somatic embryos (Fig. 4). However, the effect of cw was limited to the production of w-o somatic embryos of small size (<5 mm). Differences between treatments were detected only when the average number of w-o somatic embryos produced per culture was considered, e.g. 2.45 in the presence of 20% cw versus 1.40 with 10% cw. Production of w-o somatic embryos drastically increased during the third subculture.

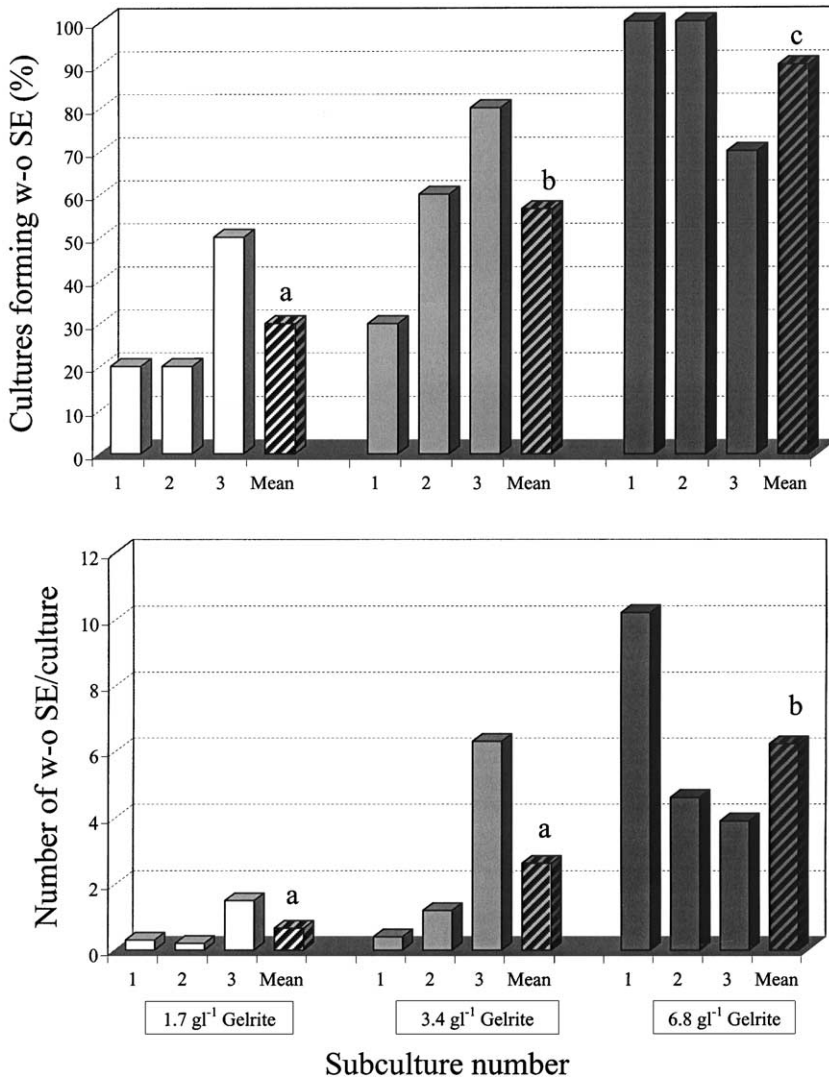


Fig. 3. Effect of gellan gum concentration on formation of w-o avocado embryos, cv. Anaheim. Data were taken during three subcultures (4 weeks each). Different letters indicate significant differences obtained by frequency analysis and the Kruskal–Wallis test at 5%.

3.2. Effect of different maturation media on germination

White-opaque somatic embryos >5 mm developed on B5m medium with 6.8 g l⁻¹ Gelrite (Fig. 5(1)) and matured (Fig. 5(2)), showing different weight increases depending on the maturation medium. The average weight increase of w-o embryos on MS medium (0.88 g) was significantly greater than that of embryos on B5m + cw (0.63 g) or Jensen–MS (0.20 g) media. Weight increases were significant since the initial fresh weight of the embryos used

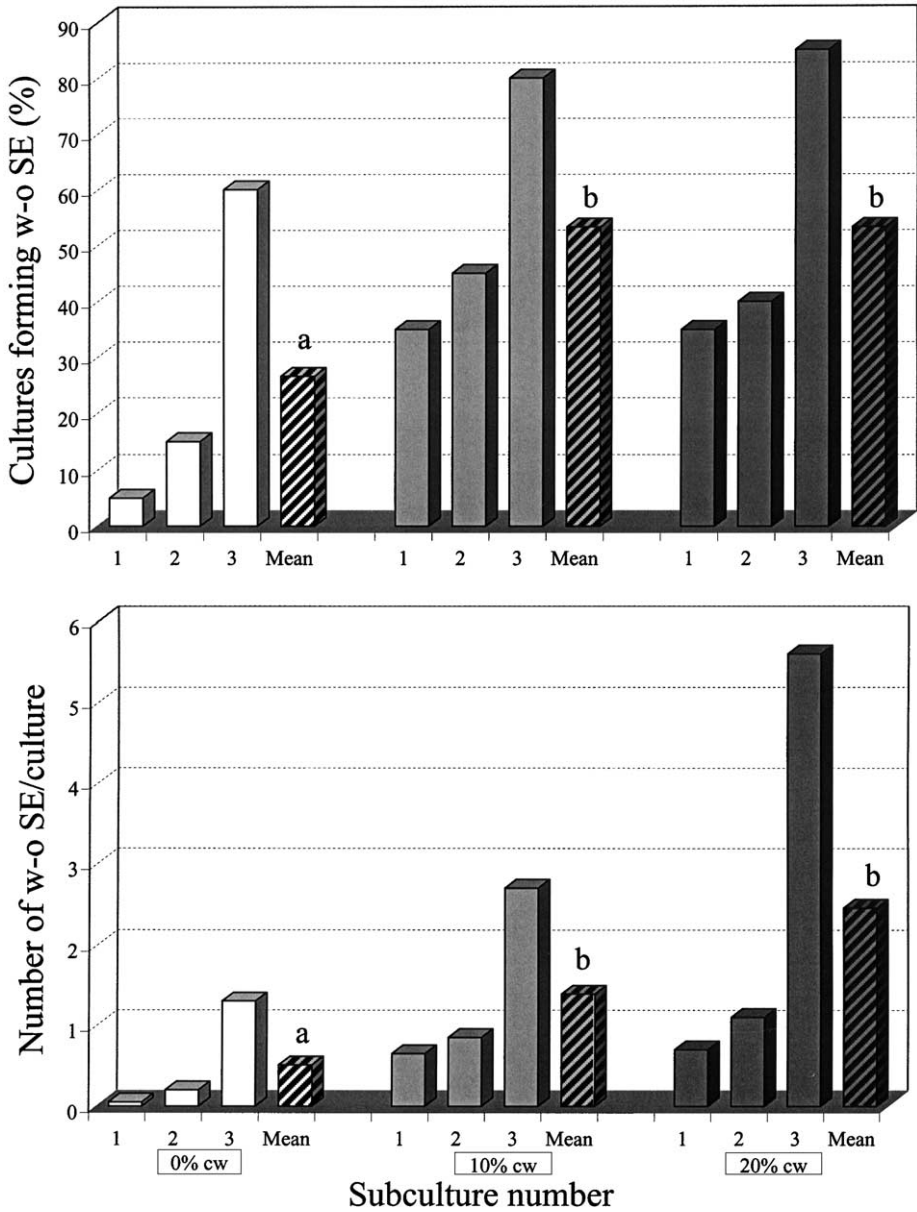


Fig. 4. Effect of cw concentration on formation of w-o avocado embryos, cv. Anaheim. Data were taken during three subcultures (4 weeks each). Different letters indicate significant differences obtained by frequency analysis and the Kruskal–Wallis test at 5%.

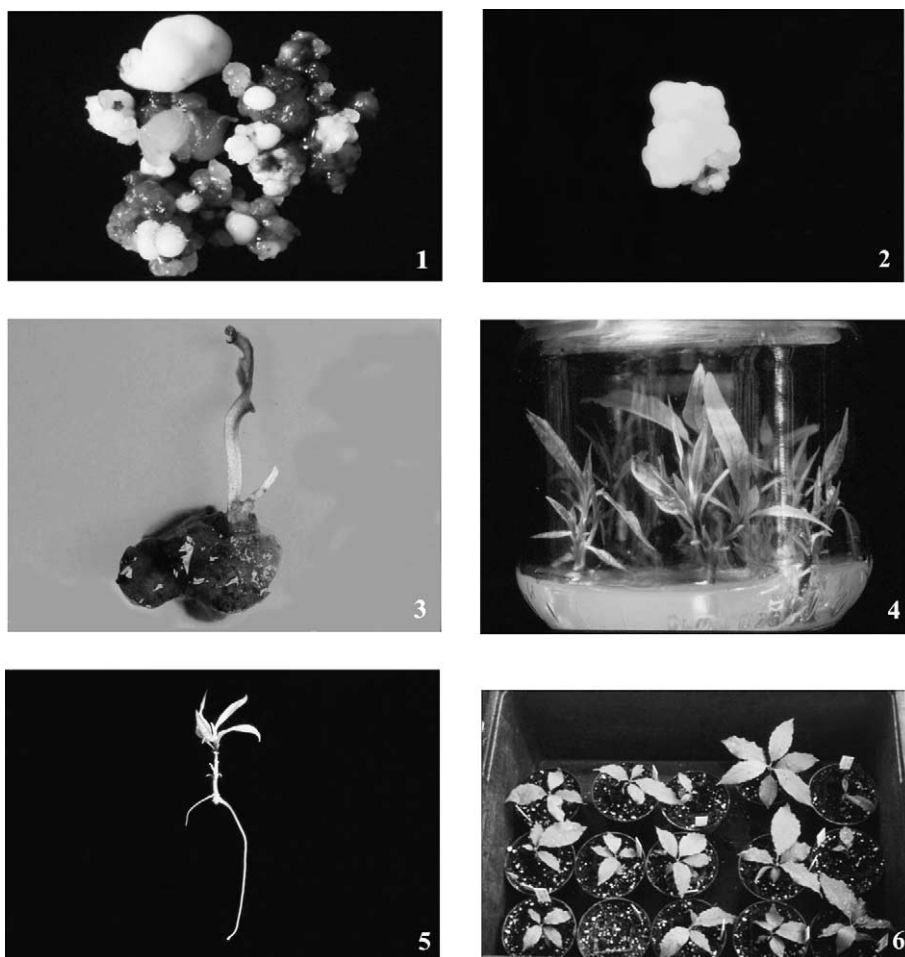


Fig. 5. Regeneration of avocado, cv. Anaheim, via somatic embryogenesis: (1) white-opaque somatic embryos developed on B5m medium solidified with 6.8 g l^{-1} Gelrite; (2) avocado somatic embryo after the maturation phase; (3) avocado somatic embryo germinated on M1 medium; (4) shoots derived from somatic embryos in proliferation phase; (5) rooted shoot; (6) acclimatization of avocado plantlets derived from somatic embryos.

averaged 0.06 g . Necrotic rates for w-o somatic embryos cultured on B5m + cw, MS and Jensen-MS were 11.11, 36 and 31.25%, respectively. The highest germination percentage (11.11%) was obtained for embryos that developed on B5m + cw (Table 1). Very low germination percentages (3.13–4%) were obtained with the other maturation treatments. In relation to the type of germination, there were differences too, e.g. with B5m + cw medium the embryos produced shoots ($6.33 \pm 4.16 \text{ mm}$ long) in all cases, whereas in other maturation media only root formation was observed. Shoots (Fig. 5(3)) could be micropropagated on the medium developed by Barceló-Muñoz et al. (1990) for juvenile avocado (Fig. 5(4)),

Table 1
Germination percentages on avocado somatic embryos cv. Anaheim

Treatment	Shoot germination (%)	Radicle germination (%)	Shoot and radicle germination (%)
B5m + cw	11.11 (3/27) a	0 (0/27) a	0 (0/27) a
MS	0 (0/25) b	4 (1/25) a	0 (0/25) a
Jensen–MS	0 (0/32) b	3.13 (1/32) a	0 (0/32) a

White-opaque embryos larger than 5 mm were matured over 10 weeks in different media: B5m + cw, MS or Jensen–MS in darkness and were germinated in M1 medium, under light conditions. Different letters within each column indicate significant differences obtained by frequency analysis.

e.g. shoots proliferated in the presence of 4.44 μM benzylaminopurine (BA) and could be rooted following a 3 days exposure to liquid MS medium with macroelements at 0.3 \times and a 4.92 μM indole-3-butyric acid (IBA) supplement. Using this procedure, 80% of micro-propagated shoots rooted (Fig. 5(5)) and survival rate after 8 weeks under in vivo conditions (Fig. 5(6)) was 92%.

4. Discussion

According to Ammirato (1983) culture conditions must be changed in a sequential manner during somatic embryo development and germination. Quatrano (1987) indicated that maturation is a key phase between embryo development and germination. In our experiments the parameter chosen as indicator of maturation was the appearance of w-o structures (Cailloux et al., 1996). When comparing the effect of MS or B5m salts on formation of w-o embryos, noticeable differences were found, e.g. 60% of cultures formed w-o embryos at the end of the third subculture in presence of B5m salts versus 0% in presence of MS salts. Our results are in accordance with observations of DeWald et al. (1989a) with mango and contrast with observations of Witjaksono and Litz (1999b) in other avocado cultivars (Booth 8, Isham, M25864, T362) since these authors recommended the use of MS medium supplemented with 8 g l⁻¹ agar.

During maturation, the accumulation of storage substances needed for embryo germination takes place. The beneficial effects of low osmotic potential on somatic embryo quality is well known (McKersie and Brown, 1996). Dodeman et al. (1997) indicated that ABA and water stress induced gene expression responsible for the synthesis and accumulation of storage and late embryogenesis abundant (LEA) proteins. According to Morris et al. (1988) water stress does not necessarily cause an increase in endogenous ABA; rather, both factors may operate in a complementary manner (Benech-Arnold et al., 1991). ABA has also been shown to regulate the amount of storage lipids in zygotic (Finkelstein and Somerville, 1989) as well as somatic (Kim and Janick, 1991) embryos. In cacao zygotic embryos, the effects of ABA and high sucrose are qualitatively similar, both increasing the amount of storage lipids (Pence, 1992). In *Hevea brasiliensis*, presence of ABA and high sucrose in the culture medium favored accumulation of starch and protein reserves (Etienne et al., 1993). In avocado cv. Anaheim culturing in presence of 175 mM sucrose increases the percentage of cultures forming w-o embryos as well as the number of w-o embryos per

culture. Under these conditions many embryos formed roots, as observed in *Arachis hypogaea* L. by Mhaske et al. (1998). According to these authors, this precocious germination could be avoided by increasing sucrose concentration or by continuous culture on medium with relatively high sucrose levels. Our results confirmed these observations since no roots appeared after several subcultures in presence of 175 mM sucrose or in cultures growing in 263 mM sucrose; however, the latter induced toxic effects in avocado cultures, probably due to the high permeability of sucrose as suggested by Mhaske et al. (1998).

Water relations between the embryo and its environment, in vivo or in vitro, play an important role in embryo development and particularly during maturation phase (Adams and Rinne, 1980). To decrease water availability of the medium without the interference of osmotically active substances as sucrose, the concentration of gellan gum was increased and a noticeable increase in number of w-o somatic embryos was observed confirming results of Witjaksono and Litz (1999b) in other avocado cultivars. Increased concentration of gelling agent probably caused the conversion of translucent to w-o embryos, as suggested by Monsalud et al. (1995) in mango. Moreover, in our case, w-o embryos obtained in medium with high concentrations of gelling agent showed a higher germination capacity than those obtained in presence of high sucrose (data not shown). In *Pinus strobus*, increasing gellan gum concentration also increased the number of w-o embryos as well as their conversion into plants (Klimaszewska and Smith, 1997).

Abscisic acid concentrations in the range 10–50 μM have been used to stimulate somatic embryo maturation. Generally, 1 month treatment is considered to be adequate, although prolonged exposures increase the number of mature embryos formed (von Arnold et al., 2002). In avocado, 4–12 weeks exposures to concentrations in the range 1–1000 μM had no clear effect on the formation rate of w-o embryos, although an increase in average number of these structures was obtained in ABA-treated cultures as compared to the control. Unexpectedly, the number of immature translucent embryos was much higher in the presence of ABA (data not shown). Perhaps, ABA might not have a direct effect on avocado embryo maturation, and the increased number of w-o embryos observed could be a consequence of the higher number of embryos which are formed in the presence of ABA.

The beneficial effects of cw in the development of very young embryos of *Datura stramonium* was first suggested by van Overbeek et al. (1942). Since then, different authors have reported its positive effects on somatic embryo development of cereals (Vasil and Vasil, 1981), carrot (Halperin and Wetherell, 1964) and mango (DeWald et al., 1989b). In somatic embryos of *Borago officinalis*, Quinn et al. (1989) related its enhancing effect on embryo maturation to the 40% increase in total fatty acids content observed in embryos cultured in the presence of 10% cw. In avocado, we observed a significant effect of this organic supplement in the formation of w-o embryos.

Nyman et al. (1987) point out the importance of equilibrated embryo growth in order to obtain adequate germination. Our results confirm these observations since culture conditions during maturation inducing higher weight increase (MS medium) were not optimum for germination. The best results were obtained when maturation medium was B5m + cw.

For embryo germination, M1 medium (Skene and Barlass, 1983) containing MS medium at half strength and 2.22 μM BA was used. In our case, cotyledons were removed since they were found to inhibit germination of avocado zygotic embryos (Perán-Quesada, 2001). In *Aesculus hippocastanum* L. Radojevic (1988) also recommends removal of cotyledons for

germination. In avocado, virtually all germinated somatic embryos formed shoots probably due to the presence of BA. The positive effects of this cytokinin on somatic embryo germination has previously been reported in *Carya illinoensis* (Mathews and Wetzstein, 1993) and *Laurus nobilis* (Canhoto et al., 1999).

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