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Isolation, culture and regeneration of avocado (*Persea americana* Mill.) protoplasts

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Abstract Protoplasts were isolated from embryogenic suspension cultures derived from avocado (*Persea americana* Mill.) zygotic embryos and nucellus in an enzyme digestion solution consisting of 1% cellulase Onozuka RS, 1% Macerase R10, 0.2% Pectolyase Y-23, 0.7 *M* mannitol. 24.5 m*M* $CaCl_2$, 0.92 m*M* NaH_2PO_4 and 6.25 2-[N-morpholino]ethanesulfonic acid (1.5 ml) mixed with 0.7 *M* MS⁻8P (2.5 ml). MS⁻8P medium consisted of Murashige and Skoog salts without NH_4NO_3, 1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 3.1 g l^{-1} glutamine and 8P organic addenda. Medium osmolarity was adjusted with 0.15 *M* sucrose and 0–0.55 *M* mannitol. Protoplast yields of 3.5×10^6 protoplasts g^{-1} were obtained. Growth and development of the protoplasts were significantly affected by osmolarity, nitrogen source, plating density and culture medium dilution. Under optimum conditions, proembryos developed directly from embryogenic protoplasts and subsequently into somatic embryos. Optimum conditions for somatic embryo development included the culture of protoplasts at a density of $0.8–1.6\times10^5$ ml^{-1} in 0.4 *M* MS⁻8P for 2–3 weeks, followed by subculture in 0.15 *M* MS⁻8P at a diluted density of 20–40× for 1 month in darkness to obtain somatic embryos. Mature somatic embryos were recovered on semisolid medium; however, a low frequency of plantlet recovery (≤1%) from protoplast-derived somatic embryos was observed.

Key words Avocado · Protoplast · Somatic embryo · *Persea americana*

Abbreviations *B5* Gamborg et al. (1968) · *BA* benzyladenine · GA_3 gibberellic acid · *IBA* indolebutyric acid · *MES* 2-[*N*-morpholino]ethanesulfonic acid · *MS* Murashige and Skoog (1962) · *PEM* proembryonic mass

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Introduction

Protoplast-based biotechnology approaches have been used to complement conventional breeding of a few fruit species (Ochatt et al. 1992). Protoplast regeneration protocols have been described for several *Citrus* species (Vardi et al. 1975; Sim et al. 1988; Vardi and Galun 1988). Protocols are also available for other species in the Rutaceae (Vardi et al. 1986; Jumin and Nito 1996a, b). Regeneration from protoplasts has been reported for species in the Rosaceae, including apple (*Malus* × *domestica* Borkh.) (Ding et al. 1995), sour cherry (*Prunus cerasus* L.) (Ochatt 1990), colt cherry (*Prunus avium* × *pseudocerasus*) (Ochatt et al. 1988) and pear (*Pyrus communis* L.) (Ochatt and Power 1988). Protoplast culture and regeneration protocols are also available for *Vitis* sp. (Vitaceae) (Reustle et al. 1995), *Actinidia deliciosa* var '*deliciosa*' 'Hayward' (Actinidiaceae) (Oliveira and Pais 1991), *Passiflora edulis* (Passifloraceae) (d'Utra Vaz et al. 1993) and *Diospyros kaki* (Ebonaceae) (Tao et al. 1991).

Protoplasts have been isolated from nonmorphogenic avocado (*Persea americana* Mill.) callus for studying sunblotch viroid replication (Blickle et al. 1986) and from fruit mesocarp tissue for studying fruit ripening (Percival et al. 1991). The purpose of the study presented here was (1) to develop protocols for protoplast isolation from embryogenic suspension cultures of avocado; and (2) to define optimum conditions for culture and somatic embryo regeneration from avocado protoplasts.

Materials and methods

Plant material

Avocado fruitlets, 0.3–2.0 cm in length without the calyx, were collected from 'T362' growing in the germplasm collection of the Uni-

versity of California, Riverside, Calif. After removal of the sepals and peduncles, fruitlets were surface-disinfested in a 10–20% solution of commercial bleach containing 10–20 drops of Tween 20® per liter for 10–20 min. Fruitlets were rinsed with two changes of sterile, deionized water and then bisected under axenic conditions. The zygotic embryo and nucellus were removed from each immature seed and transferred onto induction medium, which consisted of B5 (Gamborg et al. 1968) major salts and MS (Murashige and Skoog 1962) minor salts supplemented with the following: 0.41 μ *M* picloram, 0.4 mg/l thiamine HCl, 100 mg/l myo-inositol, 30,000 mg/l sucrose and 8 g/l TC agar (Carolina Biological Supply) (Witjaksono 1997). Plant growth medium was dispensed in 10-ml aliquots into sterile disposable petri dishes (60×15 mm). Induction medium was sterilized by autoclaving at 120 °C and 1.1 kg cm^{-2} for 15 min.

Embryogenic cultures were subcultured in filter-sterilized liquid MS medium supplemented as above (80 ml per 250-ml Erlenmeyer flasks maintained at 120 rpm and 25 °C in semidarkness) with a 2-week subculture period (Witjaksono 1997). Cultures consisted of proembryonic masses (PEMs) that proliferated repetitively.

Protoplast isolation

Approximately 0.8–1.2 g of 8- to 14-day-old embryogenic suspension cultures were incubated in a mixture consisting of 2.5 ml of 0.7 *M* $MS^{-}8P$ protoplast culture medium (see section *Protoplast culture medium*) and 1.5 ml enzyme digestion solution (1% cellulase Onozuka RS, 1% Macerase R 10, 0.2% Pectolyase Y-23, 0.7 *M* mannitol, 24.5 m*M* $CaCl_2$, 0.92 m*M* NaH_2PO_4, 6.25 m*M* MES, filter-sterilized) in 60×15-mm sterile plastic petri dishes (Grosser and Gmitter 1990). Mixtures were incubated in darkness at 25 °C on a rotary shaker at 50 rpm for approximately 16 h and then passed through a sterile stainless steel sieve (45 μm), and centrifuged at 100 g for 5 min. After the supernatants were removed, protoplast pellets were purified by gradient centrifugation using CPW25 S and CPW13 M [Frearson et al. (1973) as modified by Grosser and Gmitter (1990)]. Protoplasts at the interphase were collected with Pasteur pipettes and transferred to 15 ml sterile centrifuge tubes and washed once with 0.7 *M* $MS^{-}8P$ culture medium. Protoplasts were then repelleted and resuspended in 0.7 *M* protoplast medium to a volume of 20× or to a certain density. Protoplast yields were determined using a Fuchs Rosenthal haemacytometer, and viability was measured by fluorescence diacetate (FDA) staining according to the procedure of Huang et al. (1986).

Protoplast culture medium

Avocado protoplast culture medium contained MS basal salts, 1 g/l thiamine HCl, 100 mg/l myoinositol and 8P (Kao and Michayluk 1975) organic addenda as modified by Grosser and Gmitter (1990). Two medium formulations were utilized: $MS8P^{-}$ (standard MS salts) and $MS^{-}8P$ (no NH_4NO_3 and supplemented with 3.1 g/l glutamine). The 8P organic addenda contained (in mg/l) calcium panthothenate 1; ascorbic acid 2; choline chloride 1; *p*-aminobenzoic acid 0.002; folic acid 0.4; riboflavin 0.2; biotin 0.01; retinol 0.01; cholecalciferol 0.01; cyanocobalamine 0.02; sodium pyruvate 20; citric acid 40; malic acid 40; fumaric acid 40; fructose 250; ribose 250; xylose 250; mannose 250; rhamnose 250; cellobiose 250; galactose 250; mannitol 250; malt extract 1000; casein hydrolysate 250; and 2% liquid coconut endosperm. Sucrose was used at a concentration of 0.15 *M*, while mannitol varied from 0 to 0.55 *M*, depending on medium osmolarity. The protoplast culture medium was filter-sterilized.

Effect of nitrogen source, medium osmolarity and protoplast density

There were two nitrogen sources: $MS^{-}8P$ and $MS8P^{-}$ (See section *Protoplast culture medium*). Total nitrogen content of $MS^{-}8P$ was 61.2 m*M*, with 18.8 m*M* from NO_3^- and 42.4 m*M* from organic NH_4^+ (glutamine), whereas $MS8P^{-}$ had 60.0 m*M* nitrogen, with 39.4 m*M* from NO_3^- and 20.6 m*M* from inorganic NH_4^+ . Medium osmolarity was either 0.4 or 0.6 *M* (0.15 *M* sucrose + 0.25 or 0.45 *M* mannitol, respectively). Protoplasts were derived from embryogenic cultures that were induced from a 'T362' zygotic embryo. Protoplast plating density was either 0.8×10^5 or 1.6×10^5 protoplasts ml^{-1}. The number of microcalli or PEMs were counted in two adjacent squares (4 mm^2) at the center of a petri dish after it had been swirled to obtain an even distribution of microcalli and PEMs. The number of microcalli and PEMs were counted from three different areas of a petri dish, three petri dishes per treatment, 1 month after culture. Microcalli and PEMs were presented as percentages that were transformed with arc sine for ANOVA computation (SAS Institute 1992) and their non-transformed values were presented graphically. The relative number of microcalli and PEMs was also analyzed for ANOVA, and their means and standard errors were presented graphically.

Plating efficiency

Protoplasts derived from the nucellus of 'T362' were cultured in 2 ml 0.4 *M* $MS^{-}8P$ at a density of 1×10^5 ml^{-1} in 60×15-mm plastic petri dishes. Observations were recorded after 1, 5, 8 and 14 days. Three cultures were sampled at each observation, transferred to 15-ml centrifuge tubes and pelleted by centrifugation at 100 *g* for 3-5 min. Protoplasts and microcalli were rated according to aggregation, necrosis and division and counted with a haemacytometer. Data were presented as percentages (means and standard errors) of cells or microcallus within each category, and plating efficiency was calculated as the sum of the percentage from categories of cells and clusters of cells that divided.

Effect of subculture period and dilution on development

Protoplasts that were derived from embryogenic 'T362' nucellar cultures were cultured in 2 ml 0.4 *M* $MS^{-}8P$ with a density of 1×10^5 ml^{-1}. After 14, 21 and 28 days, protoplast-derived cultures were diluted with 2 ml liquid 0.15 *M* $MS^{-}8P$ to make dilutions of 3-, 6-, 12-, 24-, 48- and 96-fold. After 14 days, cultures consisted of PEMs (approx. 75 μm in diameter); at day 21, PEMs were 200–300 μm in diameter and in clumps (0.5–1.0 mm); at day 28, PEMs were dedifferentiating and were in clusters of approximately 1.3–1.5 mm. After 1 month of culture, three replicates (three petri dishes) from each dilution treatment and subculture period were randomly sampled and observed. Somatic embryos 2 mm diameter were counted from each replicate under a dissecting microscope and the culture was weighed. Analyses of variance were computed using PROC GLM (SAS Institute 1992). Means and standard errors were determined.

Somatic embryo maturation and germination on semisolid medium

Globular somatic embryos and PEMs (≥1.5 mm in diameter) developed after 1–2 months from protoplasts derived from 'T362' nucellar cultures on 0.4 *M* $MS^{-}8P$ medium diluted 20-fold and subcultured in 2 ml 0.15 *M* $MS^{-}8P$. These were used as inocula (0.01 g) for somatic embryo development on MS medium without picloram and supplemented with 6.0 g l^{-1} gellan gum (SED medium) (Witjaksono 1997). There were nine inocula per petri dish (150×20 mm). After 1–2 months, opaque somatic embryos (≥0.8 cm in diameter) were transferred individually onto semisolid MS medium supplemented with 4.44 μ*M* BA and 2.89 μ*M* GA_3 (maturation medium) and subcultured at 2-month intervals thereafter (Witjaksono 1997). Maturation medium was dispensed in 25-ml aliquots into 25×50-ml test tubes that were sealed with Kaputs and sterilized by autoclaving for 15 min at 1.1 kg cm^{-2} at 120 °C. Cultures were maintained in darkness at 25 °C until germination.

The pH of plant growth media was adjusted to 5.7 with either 0.1 *N* HCl or 0.1 *N* KOH prior to sterilization. Tissue cultures in sterile petri dishes were sealed with Nescofilm. After somatic embryos began to germinate, they were transferred to a 16-h photoperiod provided by cool white fluorescent bulbs (40 μ mol m^{-2} s^{-1}) at 25 °C.

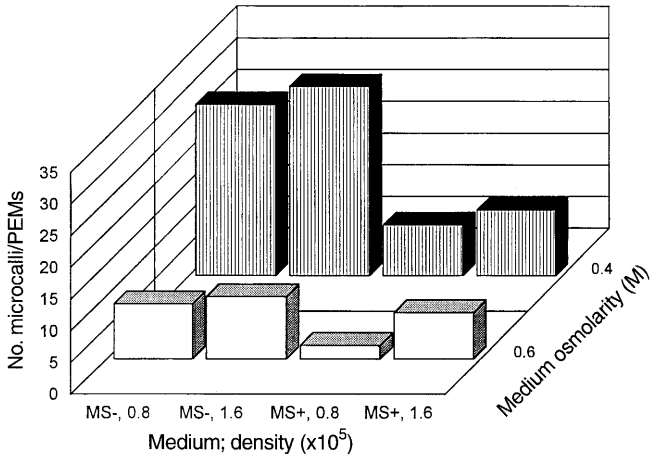


Fig. 1 Effect of nitrogen source, medium osmolarity and plating density on combined number of microcalli and PEMs that developed from 'T362' avocado zygotic embryo-derived protoplasts after 1 month in culture. *MS–* $MS^{-}8P$, *MS+* $MS8P^{-}$, *0.8* 0.8×10^5 protoplasts ml^{-1}, *1.6* 1.6×10^5 protoplasts ml^{-1}

ANOVA

Source	*df*	Mean square
Osmolarity (O)	1	834.26**
Nitrogen (N)	1	846.09**
Density (D)	1	49.59*
O · N	1	326.34**
O · D	1	0.51*
N · D	1	4.59
O · N · D	1	7.59

*,** Significant at $P\leq0.05$ and $P\leq0.01$, respectively

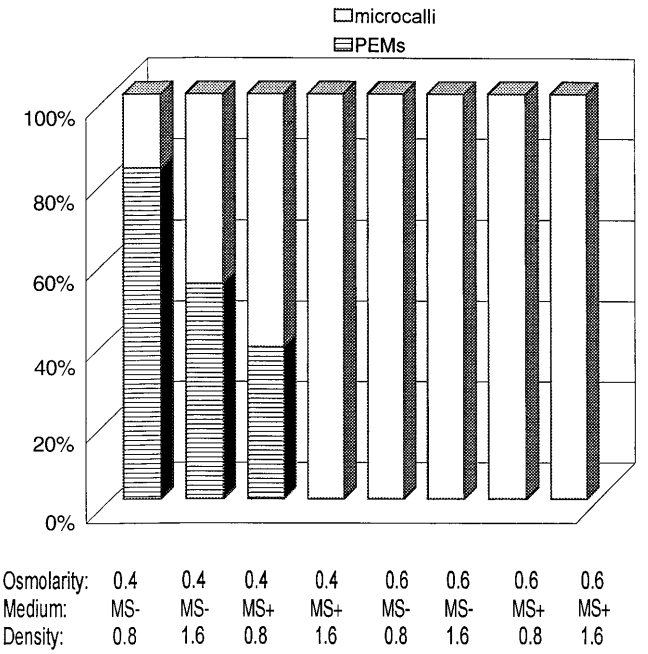


Fig. 2 Effect of nitrogen source, medium osmolarity and plating density on percentage of microcalli and PEMs that developed from 'T362' avocado zygotic embryo-derived protoplasts after 1 month in culture. *MS–* $MS^{-}8P$, *MS+* $MS8P^{-}$, *0.8* 0.8×10^5 protoplasts ml^{-1}, *1.6* 1.6×10^5 protoplasts ml^{-1}. Data represent percentage of microcalli and PEMs. Percentage data were transformed with arc sine transformation for analysis

ANOVA

Source	*df*	Mean square
Percentage microcalli and PEMs		
Osmolarity (O)	1	1.886**
Nitrogen (N)	1	1.037**
Density	1	0.135*
O · N	1	1.037**
O · D	1	0.135*
N · D	1	0.000
O · N · D	1	0.000
Error	16	0.017
Corrected total	23	

*,** Significant at $P\leq0.05$ and $P\leq0.01$, respectively

Results and discussion

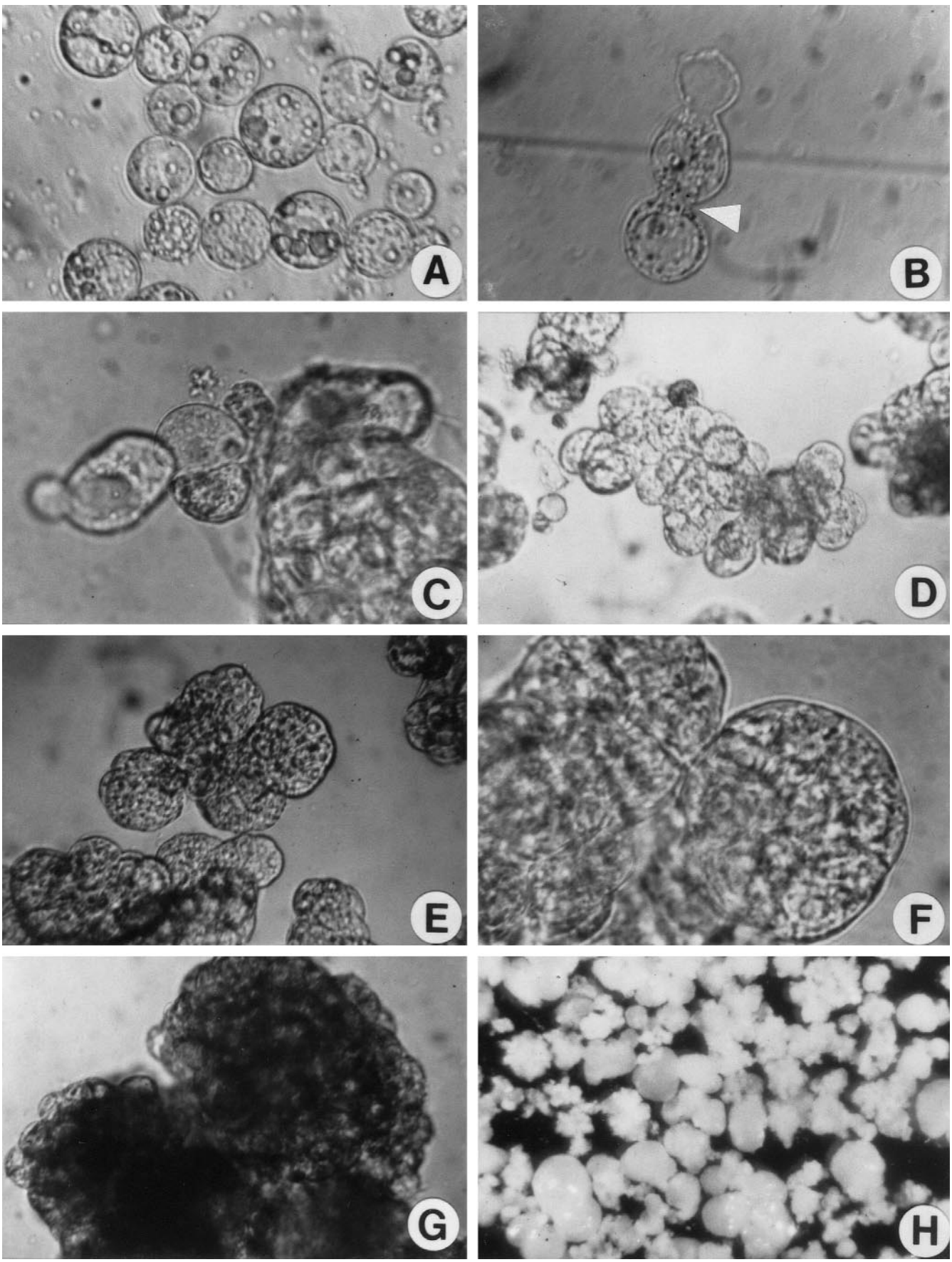
Yield

Protoplast yields from embryogenic suspension cultures derived from embryogenic 'T362' nucellar cultures were 3.5×10^6 g^{-1}, which is comparable to yields obtained with other woody species (Vardi et al. 1986; Ochatt and Power 1988; Ochatt 1990; Matsuta et al. 1986). PEM-derived avocado protoplasts were approximately 15–27 μm diameter Protoplast viability 24 h after isolation and purification was approximately 70–80%.

Effect of nitrogen source, medium osmolarity and protoplast density

After 1 month of culture, protoplasts derived from embryogenic zygotic embryo cultures had developed as either microcalli or PEMs (50–200 μm in diameter). Medium osmolarity, nitrogen source, plating density and the interaction of osmolarity and nitrogen source significantly affected the relative number of microcalli and PEMs (Fig. 1). With 0.6 *M* medium osmolarity, regardless of the nitrogen source of the medium and plating density, only microcalli developed (Fig. 2). The combined number of microcalli and PEMs was greatest with a low NO^{-}:NH_4^{+} ratio, lower (0.4 *M*) medium osmolarity and higher (1.6×10^5) plating efficiency. When microcalli and PEMs were considered separately, however, PEM development was dependent upon the source of nitrogen and protoplast density. Irrespective of the plating density, medium with glutamine ($MS^{-}8P$) and 0.4 *M* osmolarity allowed the formation of more PEMs than medium with NH_4NO_3 ($MS8P^{-}$). With a treatment combination of 0.4 *M* and $MS^{-}8P$, a plating density of 0.8×10^5 protoplast ml^{-1} allowed the development of more PEMs than the higher plating density (1.6×10^5 protoplast ml^{-1}).

Deletion of NH_4NO_3 from the basal medium has also been reported to stimulate microcallus development from


A
B
C
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E
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H

Table 1 Percentage of shriveled, healthy (nondividing) and dividing nucellus-derived avocado 'T362' protoplasts at 1, 5, 8 and 12 days after culture [a]

	(Protoplasts ±SE) %			
	Day 1	Day 5	Day 8	Day 12
Individual protoplasts				
Shriveled/brown	0.0±0.0	19.9±2.0	24.0±2.0	31.5±3.4
Healthy/transparent	48.0±7.0	19.2±1.6	17.6±1.3	3.4±1.6
1st division (2 cells)	0.0±0.0	1.5±0.8	3.7±2.2	0.7±0.7
2nd division (3–4 cells)	0.0±0.0	0.0±0.0	0.9±0.1	0.4±0.4
3rd division (5–8 cells)	0.0±0.0	0.0±0.0	0.4±0.3	0.4±0.4
Proembryos (≥ 9 cells)	0.0±0.0	0.0±0.0	0.2±0.2	0.4±0.4
Budding or chains of protoplasts				
Shriveled/brown	0.0±0.0	0.0±0.0	15.1±0.4	26.1±2.7
Healthy/transparent	51.2±7.0	56.1±3.6	33.6±2.6	15.1±4.5
1st division (2 cells)	0.0±0.0	3.6±0.5	3.8±1.0	11.2±0.2
2nd division (3–4 cells)	0.0±0.0	0.0±0.0	1.6±0.3	7.8±1.4
3rd division (5–8 cells)	0.0±0.0	0.0±0.0	0.5±0.3	1.7±0.4
Proembryos (≥ 9 cells)	0.0±0.0	0.0±0.0	0.2±0.2	1.7±1.1
Plating efficiency	0.0±0.0	5.1±0.6	9.2±1.5	25.0±1.2

[a] Protoplasts were cultured in 2 ml liquid medium of 0.4 *M* MS$^-$8P at a plating density of 1×10^5 protoplasts ml^{-1}

Pyrus spp. mesophyll protoplasts (Ochatt et al. 1992), and Grosser (1994) suggested that high NH_4NO_3 concentrations could be toxic to citrus protoplasts. The increased frequency of somatic embryo formation from avocado protoplasts following replacement of NH_4NO_3 with glutamine may be due either to the altered ratio of reduced nitrogen or the presence of the organic form of ammonium. Higashi et al. (1996) demonstrated that embryogenic carrot cell clusters required reduced nitrogen for somatic embryo development and that glutamine strongly affected the development of mature somatic embryos.

Plating efficiency of protoplasts and effect of medium dilution on somatic embryo development

After 1 day in culture, protoplasts (Fig. 3A) derived from embryogenic nucellar cultures began to aggregate, forming loose clusters of 3–16 protoplasts (Fig. 3B). Protoplast budding was observed in approximately 51% of the samples (Fig. 3B). After 5 days in culture, the protoplasts/cells were individual, clustered, budded and chained. Approximately equal numbers (20%) of individual protoplasts were either necrotic or healthy; however, only 5.1% of the protoplasts had undergone the first division (Fig. 3C; Table 1). The first cell division was observed in one or more cells within each cluster, indicating the probable single-cell origin of the microcalli and PEMs that develop from protoplasts. Direct somatic embryo regeneration from protoplasts derived from embryogenic cultures has also been reported for *Citrus* spp. (Kobayashi et al. 1985; Sim et al. 1988; Ling et al. 1990) and grapevine (Reustle et al. 1995). The second cell division was apparent at day 8 (Fig. 3C, D). Plating efficiencies at days 5, 8 and 12 after culture were 5%, 9% and 25%, respectively (Table 1). Protoplast-derived cells developed as PEMs (Fig. 3D–F), and produced secondary globular somatic embryos (Fig.3E, F) that were morphologically distinct from microcalli. Globular somatic embryos started to dedifferentiate after 21 days of culture (Fig.3G).

One month after subculture into diluted medium, cultures were composed of different sizes of globular somatic embryos (0.15–3.0 mm in diameter) and microcalli (0.5–1.5 mm in diameter) (Fig. 3H). The timing of subculture was critical for development of globular somatic embryos ≥2 mm diameter. Subculture age, dilution rate and their interaction significantly affected the number of somatic embryos per petri dish (Fig. 4). When protoplast-derived cultures were subcultured at day 15 with dilution rates of 6- to 48-fold, there were 6–12 globular (≥2 mm diameter) somatic embryos per petri dish. After subculture at days 21 and 28 with dilution rates of 49-to 96-fold, somatic embryos (≥2 mm diam) occurred at lower frequencies (max. five somatic embryos per petri dish) (Fig. 4).

Fresh weight of the cultures was significantly affect by dilution rate, subculture age and their interaction. It decreased with increasing dilution rate when the cultures were subcultured at days 15 and 21, except that the fresh weight of the older cultures was greater with dilution rates of 6-fold and higher (Fig.5). With subculture at day 28, the dilution rate had no effect on culture fresh weight, although dilution rates of 12- to 96-fold resulted in higher fresh

Fig. 3A–H Somatic embryogenesis from 'T362' avocado nucellus-derived protoplasts in 2 ml liquid 0.4 *M* MS$^-$8P in 60×15-ml petri dishes after 1–21 days. **A** Protoplasts after 1 day, **B** budding protoplast after 5 days, **C** dividing protoplast after 5 days, **D** chain of protoplasts after 5 days, **E** cluster of cells, some of which have formed PEMs, **E** PEMs in clusters after 14 days, **F** higher magnification of E, **G** disorganizing PEMs after 21 days, **H** PEMs (1–3 mm) after subculture to medium of lower osmolarity (0.15 *M*) and low density

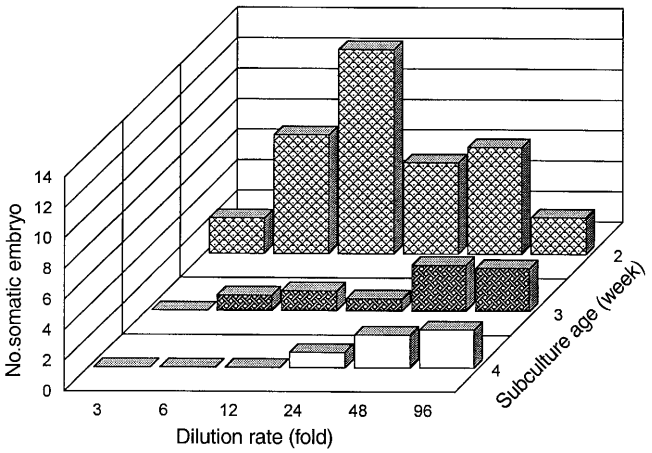

Fig. 4 Effect of subculture age and dilution rate in medium of low osmolarity (0.15 *M* MS^{-}8P) on the formation of ≥ 2-mm-diameter nodular PEMs from 'T362' avocado nucellus-derived protoplasts

ANOVA

Source	*df*	Mean square
Number of somatic embryos ≥ 2 mm		
Age	5	259.685**
Dilution rate	2	24.714**
Age · Dilution rate	10	32.608**

** Significant at $P \leq 0.01$

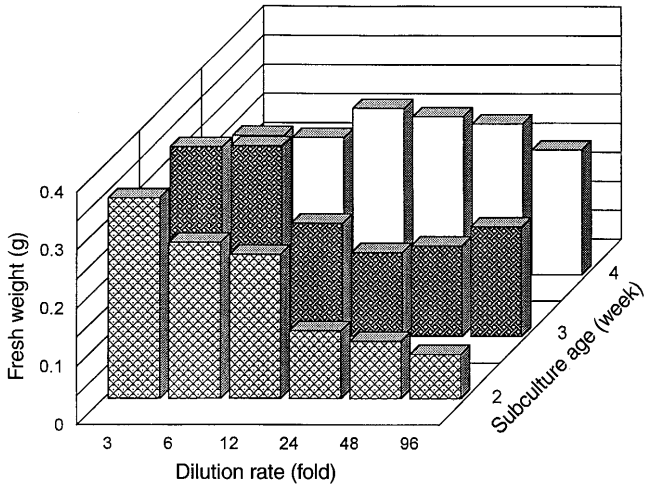

Fig. 5 Effect of subculture age and dilution rate in medium of low osmolarity (0.15 *M* MS^{-}8P) on fresh weight accumulation of PEMs from 'T362' avocado nucellus-derived protoplasts after 1 month

ANOVA

Source	*df*	Mean square
Fresh weight		
Age	5	0.022**
Dilution rate	2	0.049**
Age · Dilution rate	10	0.018**

** Significant at $P \leq 0.01$

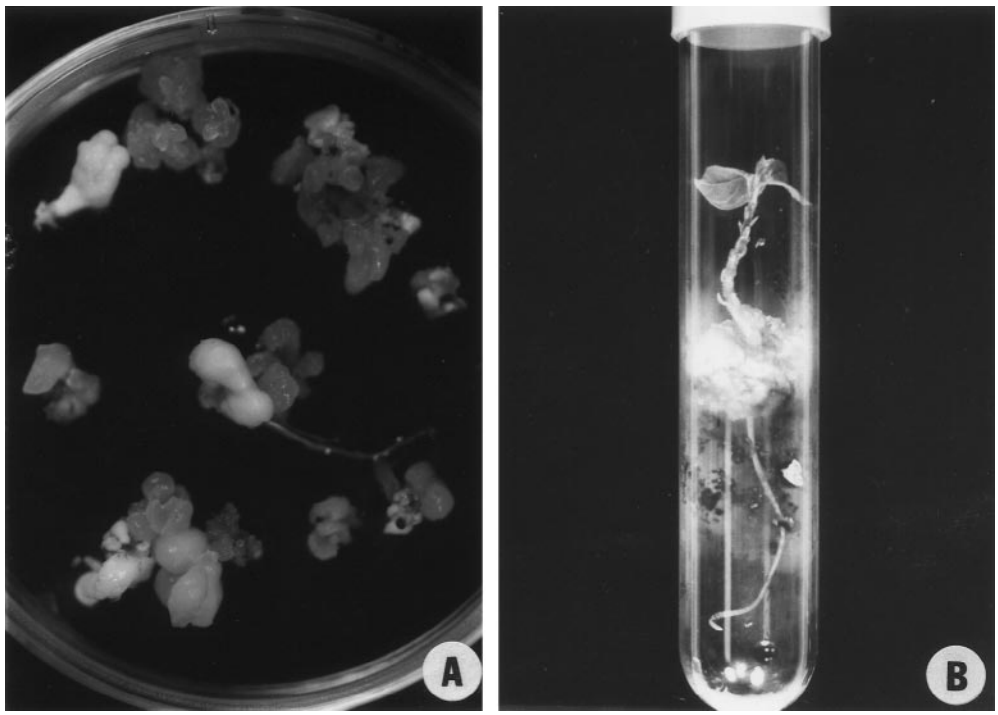

Fig. 6 **A** Mature somatic embryos from 'T362' nucellus-derived protoplasts. **B** Plantlet recovery from 'T362' nucellus-derived protoplasts

weight than treatments subcultured at days 15 and 21. Fresh weights of cultures from day 28 were as much as day-15 and -21 cultures at lower dilution rates, i.e., approximately 2.5–3.3 g per petri dish. Kobayashi et al. (1985) observed that reducing cell density derived from citrus protoplasts resulted in enhanced somatic embryo development and suggested that embryogenic potential might be repressed when cells are cultured at a high cell density.

Somatic embryo development, maturation and germination

When large (1–2 mm) protoplast-derived nodular PEMs and dedifferentiating PEMs derived from embryogenic nucellar cultures were used as inocula on SED medium, PEMs as well as somatic embryos of different sizes and stages of development and of varying hyperhydricity developed from the inoculum after 1–2 months (Fig. 6A). Only opaque somatic embryos ≥0.8 cm in diameter were transferred individually onto maturation medium on which they enlarged to approximately 1.0–1.5 cm in diameter. Approximately 60% of the somatic embryos produced secondary somatic embryos and/or disorganized PEMs on maturation medium, whereas the remainder developed normally. The conversion rate of somatic embryos was ≤ 1% (Fig. 6B). The low frequency of germination of protoplast-derived somatic embryos is not unexpected because of previous reports of low plant regeneration from avocado somatic embryos derived from zygotic embryo (Mooney and Van Staden 1987; Pliego-Alfaro and Murashige 1988; Witjaksono 1997) and nucellar (Witjaksono 1997) explants.

The most significant production problem of avocado is phytophthora root rot (PRR) caused by the soil-borne pathogen *Phytophthora cinnamomi* Rands. Avocado has no resistance to PRR, although other *Persea* spp. in the subgenus *Eriodaphne* are highly resistant to PRR; however, the species in subgenus *Eriodaphne* are sexually and graft-incompatible with avocado (Bergh and Ellstrand 1986). Interspecific somatic hybridization might provide a solution for developing a new generation of PRR-resistant avocado rootstocks (Pliego-Alfaro and Bergh 1992).

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