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In vitro plant regeneration from immature cotyledon explants of macadamia (*Macadamia tetraphylla* L. Johnson)

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Abstract The macadamia tree, an Australian native, is highly valued for its nuts. Macadamia improvement programs so far have relied on conventional breeding and selection. The production of improved cultivars required to meet future demands could be accelerated by the application of modern biotechnological techniques, but this requires an efficient and reproducible regeneration system that has not yet been established for macadamia. We report here shoot regeneration from immature cotyledon explants of macadamia. Adventitious buds were induced on the cotyledon explants from fruits collected at 140 and 190 days after full bloom (DAFB) on MS medium supplemented with either 10 or 15 μM TDZ. The addition of 2% coconut milk (CM) to 10 μM TDZ containing media resulted in enhanced adventitious bud induction from 190 DAFB explants. Further shoot development from the induced buds was depressed in media containing TDZ + CM; the addition of 0.001 μM IAA to this combination doubled shoot development, from 1.9–3.9 shoots per explant. The transfer of bud clumps to media supplemented with 8.8 μM BA alone or in combination with either 0.14 μM GA_3 or 0.001 μM IAA significantly increased shoot production from the previously induced explants by 1.5–2 times of that observed in TDZ + CM medium. Histological examinations revealed that shoot regeneration was primarily by organogenesis originating from cells on or just below the cut surfaces of explants.

Keywords Macadamia · Somatic embryogenesis · Organogenesis · Histology

Abbreviations BA: 6-Benzylaminopurine · GA: Gibberellic acid · IAA: Indoleacetic acid · TDZ: Thidiazuron · IBA: Indolebutyric acid

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Introduction

Macadamia trees (*Macadamia tetraphylla* L. Johnson and *M. integrifolia* Maiden & Betche) are native to the tropical rain forests of coastal northeastern New South Wales and southern Queensland regions of Australia (between latitudes 25 and 32°S). Macadamia nuts have been produced commercially for more than 70 years and are the only commercialized food crop native to Australia. The macadamia nut is one of the world's finest gourmet nuts because of its unique delicate flavour, fine crunchy texture and rich creamy colour. The nut is growing in popularity due to being one of the functional foods that can reduce cholesterol and the incidence of heart disease in humans (Colquhuon et al. 1993, 1996). Macadamia nuts have a high oil content (>72%), but this is offset by the absence of *trans* fatty acids (Stephenson 2005) and the oil being the most monounsaturated one available (Mavis 1997); they are also high in fibre, selenium and phytic acid, all of which are associated with reducing the risk of cancer in humans (Mavis 1997). Macadamia nuts can be eaten raw, roasted, coated in chocolate, in cakes, as butter or as oil.

Despite the growing importance of macadamia, crop improvement has continued to rely upon conventional hybridization and clonal selections of elite types that are then multiplied for distribution to growers. However, this method of crop improvement is expensive and very slow due to the long generation times required to produce a single improved selection (given that it takes 8–12 years

for a macadamia tree to bear fruit). Meeting the predicted future demand requires new and improved high-yield cultivars. The production of the improved elite cultivars for macadamia can be accelerated by the application of in vitro and molecular manipulation techniques. These techniques can be aimed at targeted gene integration to achieve specific crop improvement goals such as insect–pest resistance, fungal- or bacterial-disease resistance and improved nut quality.

Successful molecular manipulation requires a well-established, effective and reproducible in vitro plant regeneration protocol (Korban et al. 1992; Obeidy and Smith 1993; Pooler and Scorza 1995). Several reports of the regeneration and genetic transformation of woody crop plants have appeared in recent years (Jain and Gupta 2005; Gray et al. 2005), and many of these crops are now at various stages of development and field testing (TRANSGEN 2005). However, there are no reports on attempts to develop in vitro culture technology for macadamia. A successful plant regeneration system in macadamia would be an effective tool for inducing variants and recovering genetically transformed plants following the transformation of plant cells with genes of agronomic interest.

In our work with macadamia, we have observed the plant to be recalcitrant to regeneration from leaf, petiole or young stem tissues and the only reported propagation of macadamia is via bud proliferation (Mulwa and Bhalla 2000; Bhalla and Mulwa 2003). This report describes a method for the reliable in vitro induction of organogenesis and recovery of viable plantlets from immature cotyledon explants of macadamia.

Materials and methods

Plant materials

Developing fruits from tagged racemes of an open pollinated *M. tetraphylla* tree were collected at 40, 90, 140 and 190 days after full bloom (DAFB). After each harvest, the fruits were disinfested by thorough washing in detergent solution for 20 min and rinsing under running tap water for 30 min. Surface disinfestation was performed by placing fruits in 1-l flasks containing 300-ml solutions of 5% v/v Clorox bleach (2.5% NaOCl) with 0.01% (v/v) Tween 20® for 20 min, and then rinsing four times with sterile distilled water.

Fruits picked at 40 DAFB were carefully dissected to remove the husks, and the developing cotyledons were cut longitudinally into four sections of approximately 3 mm × 4 mm. Fruits harvested at 90 and 140 DAFB were halved longitudinally using a sterile stainless steel hacksaw. The cotyledons were carefully extracted and dissected into rectangular pieces approximately 3 mm × 4 mm × 2 mm (length × width × thickness). Fruits collected at 190 DAFB (which had shells that were fully developed and turning brown) were dehusked and, following disinfestation as described above, held in a small sterilized bench vice and cut into halves, and then their cotyledons were extracted and sectioned as described above. Explant pieces from all the fruit growth stages were placed on 25 ml of variously constituted agar-solidified culture media contained in 100-mm plastic Petri dishes.

In vitro shoot induction experiments

To induce shoot regeneration, explants from the various fruit development stages were placed in Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 0, 5, 10 and 15 μM thidiazuron (TDZ) alone or in combination with 2% v/v coconut milk (CM). All media also contained 30 g l^{-1} sucrose and 6 g l^{-1} bacto agar (Sigma Aldrich, Australia), and were adjusted to pH 5.8 before autoclaving for 20 min (growth regulators were added before pH adjustment and autoclaving). Explants were incubated for 2 weeks in the dark (25 ± 1°C) and then transferred to a culture room maintained at 25 ± 1°C with a photoperiod of 16 h of light and 8 h of darkness. Cultures were maintained for a total of 12 weeks with one transfer to fresh media after the first 4 weeks. The percentage of responding explants (explants turning green or forming calluses) was determined at the end of 8 weeks, and the total number of buds forming on each replicate plate was computed after 12 weeks. The number of buds per responding explant was determined, and the means analysed to select the most appropriate adventitious bud-induction medium.

Shoot development experiments

Shoot development from bud clumps induced in media containing 10 or 15 μM TDZ + 2% CM was evaluated by placing 3 mm × 3 mm bud clump pieces on various MS basal media supplemented with growth regulators as follows: control (no growth regulators), 15 μM TDZ + 2% CM, 15 μM TDZ + 0.001 μM IAA + 2% CM, 8.8 μM BA, 8.8 μM BA + 0.14 μM GA_3 and 8.8 μM BA + 0.001 μM IAA. Shoot development was assessed every 2 weeks, in which developing shoots were excised and placed in rooting medium while the original explants were placed back in the media to develop more shoots. The experiment was carried out over a period of 12 weeks, and explants were transferred to fresh media after 6 weeks. The mean number of shoots collected per explant and the length of shoots in each treatment were determined to select the best shoot development medium. Excised shoots were rooted in 1.5 MS medium containing 2 μM IBA and 1% w/v activated charcoal. The regenerated plants were transferred under glasshouse conditions as described by Mulwa and Bhalla (2000).

Experimental design and statistical analyses

Shoot induction experiments were conducted in a randomized design with four replicate plates per treatment, each containing 10 explants, while shoot development experiments were set up with three replicates per treatment, each

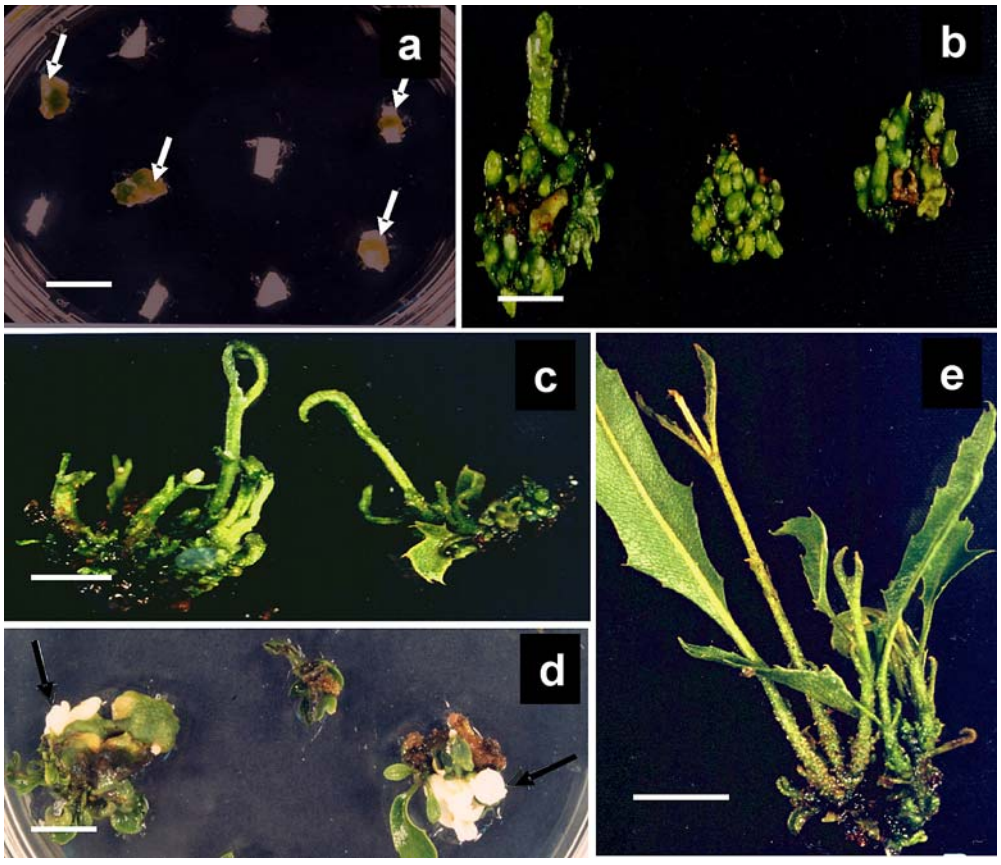

Fig. 1 In vitro plant regeneration from cotyledon explants of macadamia. **a** Explants showing morphogenic responses on the MS medium containing 15 μM TDZ + 2% CM (bar = 4 mm). **b** Formation of adventitious buds (bar = 2 mm). **c** Shoot development from induced buds (bar = 1 cm). **d** Formation of embryonic structures (arrows, bar = 3 mm). **e** Elongated adventitious shoots ready for excision and root induction (bar = 1 cm)

containing 10 explants. All experiments were repeated at least three times, and data were pooled to compute representative treatment means. Data from all experiments were subjected to one-way analysis of variance, and means were separated by Tukey's Studentized range test at $P \leq 0.05$ using the SAS computer program (SAS Institute, 1990).

Histology of shoot regeneration

To study the mode of shoot regeneration, explant samples from the 190-DAFB harvest were collected from the medium containing 15 μM TDZ + 2% CM after culturing for 1, 3 and 8 weeks. The tissues were fixed overnight in 4% paraformaldehyde in 50 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES buffer, pH 8.0) followed by two 15-min washes in 50 mM PIPES buffer and serial washes (20 min each) in graded ethanol series followed by xylene series (Bancroft and Stevens 1990). Tissue samples were embedded in paraffin wax blocks and sectioned at 5 μm with a rotary microtome (Reichert Histostat, Germany). Sections were dewaxed, stained with toluidine blue and examined under a fluorescence microscope (Leica, Germany). Images were taken to document the various stages of shoot regeneration.

Results

Regeneration

The explants almost doubled in size within the first 2 weeks of dark incubation. This was accompanied by a colour change from white to green and a concomitant development of nodular greenish-yellow calluses on the surfaces of explants (Fig. 1a). The highest explant responses for callus induction were observed in media supplemented with 10 or 15 μM TDZ (Table 1) from 140 and 190 DAFB explants.

Green adventitious buds developed on most of the responding explants by the end of 12 weeks (Fig. 1b), with callus and/or bud development being greatest at the higher concentrations of TDZ (10 and 15 μM, Table 2). Explant responses to TDZ treatments also increased with the age of the explants with those collected at 140 and 190 DAFB producing significantly higher frequencies of calli and adventitious buds than those collected at 40 and 90 DAFB. The number of buds formed per responding explant varied significantly with the treatments (Table 2). The number of buds produced per explant generally increased with the TDZ levels (Table 2). Explants collected at 40 and 90 DAFB produced the highest numbers of buds per

Table 1 Effect of the plant growth regulator, TDZ, on tissue culture response of macadamia cotyledon explants after 8 weeks in culture

Growth regulators		Callus formation from cotyledon explants (% response)[a]			
		Explant age (DAFB)			
TDZ (μM)	Coconut milk (2%)	40	90	140	190
0	−	13.3 bc	11.7 c	16.7 cd	9.2 d
5	−	17.5 b	36.7 b	30.0 c	25.3 cd
10	−	64.2 a	57.5 a	71.7 b	62.5 b
15	−	57.5 a	59.2 a	84.2 ab	72.5 ab
0	+	8.3 c	19.2 bc	11.7 d	10.8 d
5	+	22.5 b	20.0 bc	26.7 cd	35.0 c
10	+	61.7 a	60.0 a	82.5 ab	82.5 a
15	+	54.2 a	58.3 a	90.8 a	85.8 a

DAFB: days after full bloom

[a]Means within columns followed by the same letter series are not significantly different by Tukey's students' test at $P \leq 0.05$

Table 2 Effect of the plant growth regulator, TDZ levels on bud formation from macadamia cotyledon explants

Growth regulators		Mean number of buds per responding explant[a]			
		Explant age (DAFB)			
TDZ (μM)	Coconut milk (2%)	40	90	140	190
0	−	0.0 c	0.0 c	0.0 c	0.0 c
5	−	0.7 c	0.7 c	5.4 a	2.4 bc
10	−	3.7 b	6.6 ab	4.4 a	4.3 b
15	−	6.6 a	7.5a	4.9 a	7.9 a
0	+	0.5 c	0.4 c	1.3 a	0.5 c
5	+	0.7 c	0.9 c	3.0 ab	3.3 b
10	+	0.5 c	4.4 b	5.0 a	8.1 a
15	+	0.9 c	4.6 b	5.1 a	9.0 a

DAFB: days after full bloom

[a]Means within columns followed by the same letter series are not significantly different by Tukey's students' test at $P \leq 0.05$

responding explant in media containing 15 μM TDZ, with means of 6.6 and 7.5, respectively (Table 2). The addition of CM to the media had a negative effect on bud initiation at 10 and 15 μM TDZ in explants collected at 40 DAFB and in 15 μM TDZ at 90 DAFB explants. In contrast, explants collected at 190 DAFB exhibited an increase in the number of buds per explants when they were cultured in medium supplemented with 10 μM TDZ + 2% CM (Table 2).

Shoot development

Shoot development was observed when bud clumps were transferred to shoot development media. Shoot regeneration was characterized by the formation of clusters of primordial leaves followed by extension of the shoots (Fig. 1c). Even though medium supplemented with 15 μM TDZ + 2% CM induced significantly more buds at the induction stage, it was not suitable for further shoot development since it resulted in very low shoot numbers; however, this was improved when 0.001 μM IAA was included in this medium (Table 3). Shoot development in media containing TDZ (especially 15 μM TDZ + 2% CM + 0.001 μM IAA) was also accompanied by the formation of white embryonic structures in most of the explants (Fig. 1d), but these did not develop into recognizable somatic embryos. Overall, shoot formation was observed in all the media tested, but with significant variations in the numbers produced per explant and in the shoot lengths. Explants placed in medium devoid of growth regulators produced the lowest number of shoots per explant and the shortest shoots followed by those in medium containing 15 μM + 2% CM. There were no statistical differences in the number of shoots produced in media containing 8.8 μM BA alone or in combination with 0.14 μM GA_3 or 0.001 μM IAA. New adventitious shoots continued to develop and elongate as older shoots were excised from cultures. The highest number of shoots produced per explant was 7.0 in medium supplemented with 8.8 μM BA. All of the tested shoot development media produced shoots that were long enough to be easily excised and placed in rooting medium, with the longest shoots recorded in media containing GA_3 (Table 3, Fig. 1e).

Table 3 Effect of the plant growth regulators on shoot formation from adventitious buds induced on macadamia cotyledon explants

Growth regulator combinations	Mean numbero of shoots per explant[a]	Mean shoot length (mm)[a]
Control (no growth regulators)	1.1 d	19.2 d
15 μM TDZ + 2% CM	1.9 d	22.8 cd
15 μM TDZ + 2% CM + 0.001 μM IAA	3.9 c	37.4 c
8.8 μM BA	7.0 ab	60.7 b
8.8 μM BA + 0.14 μM GA_3	5.3 a	86.2 a
8.8 μM BA + 0.001 μM IAA	5.2 a	57.8 b

[a]Means within columns followed by the same letter series are not significantly different by Tukey's students' test at $P \leq 0.05$

Histological examinations of the regeneration process revealed areas of active cell division in the parenchyma tissue below the cut surfaces of the explants after 1 week of culturing (Fig. 2a). These developed into meristematic regions that appeared on the surfaces of explants and formed recognizable shoot meristems by the third week of culture (Fig. 2b, c). Further examination of the late bud and shoot development stages revealed the initiation of early stages of somatic embryogenesis. Observations at this stage revealed two phases of somatic embryogenesis: the globular stage and a vascularized bipolar embryonic body (Fig. 2d, e). However, we did not obtain viable mature somatic embryos during the culture processes.

Discussion

This study investigated the potential of plant regeneration from developing seed explants of macadamia. The results obtained suggested the presence of regeneration through

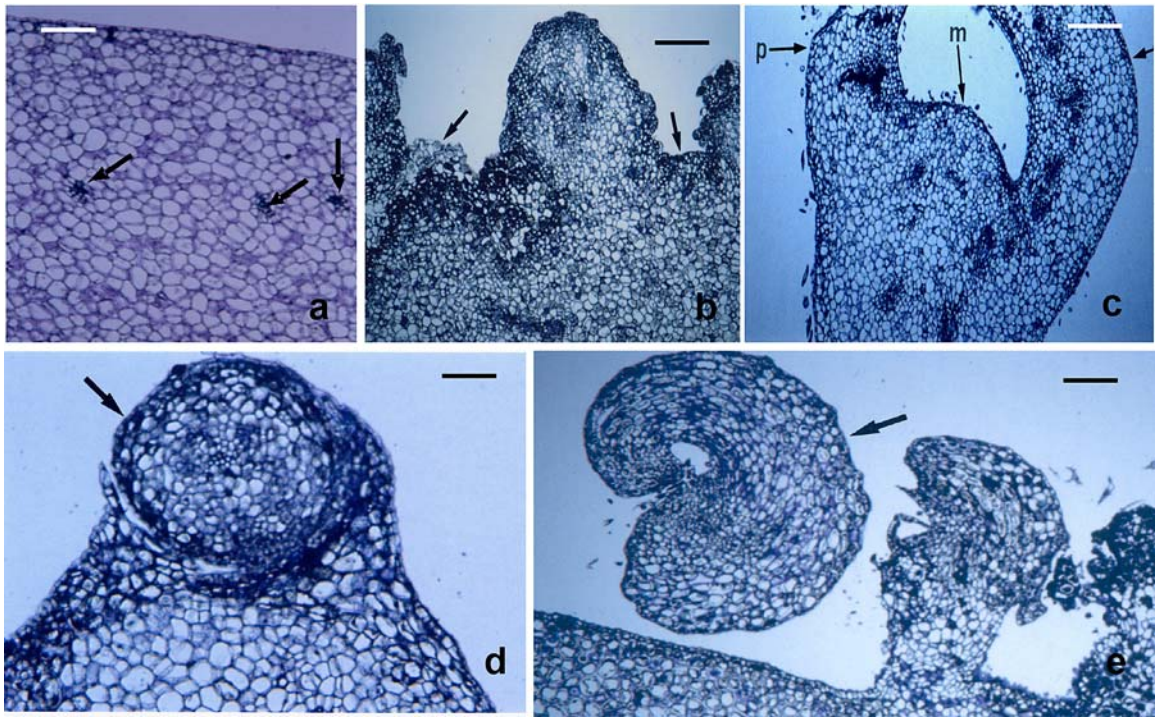

Fig. 2 Histology of cotyledon explants regeneration. **a** Active cell division as indicated by arrow 1 week after culture (bar = 20 μm). **b** Organization of meristematic tissues 3 weeks after culture (bar = 20 μm). **c** Developed meristem showing the dome (m) and primordial leaves (p) (bar = 50 μm). **d** Globular embryo (bar = 40 μm). **e** Bipolar embryo (bar = 40 μm)

organogenesis and, to some extent, somatic embryogenesis. Our histological examinations indicated that the bulk of shoots produced on explants were from organogenesis on cells located on or near the outer surfaces of the explants. This study also indicates that the ability to regenerate from tissues varies with the maturity of the fruits. Young fruit tissues produced callus and buds only in the presence of very high levels of growth regulators, in contrast to their more mature counterparts. Correlations between developmental stages and regenerative capacities have also been reported for seed tissue cultures of other fruit and nut crops. Organogenesis and somatic embryogenesis in pecan were reportedly possible only when immature nuts were used as explant sources (Yates and Wood 1989; Yates and Reilly 1990), although Obeidy and Smith (1993) managed to regenerate pecan plants by direct organogenesis from mature nut tissues. In peach, Pooler and Scorza (1995) reported efficient shoot regeneration from cotyledons of mature stored seeds of four cultivars, in contrast to the previous reports of regeneration only be attainable in immature seed cotyledons (Mante et al. 1989). Our study revealed a higher regeneration capacity in seed tissues from immature nuts collected just before the pericarp started turning brown.

Our results also demonstrate the requirement for growth regulators to initiate organogenic responses in macadamia cotyledon explants. Positive morphogenic responses were only observed in explants cultured in media supplemented with TDZ. TDZ is a potent growth regulator for woody plant tissue cultures, and its effectiveness in inducing organogenesis in various types of plant tissue has been reported in several species (Korban et al. 1992; Obeidy and Smith 1993; Pooler and Scorza 1995). Ainsley et al. (2001) reported the optimal induction of shoot regeneration from immature seed cotyledons of almond in a medium containing 10 μM TDZ.

In the present study, the addition of CM to media improved morphogenic responses from the explants except those collected at 40 DAFB. Several studies have alluded to the effectiveness of CM in various tissue culture procedures. Sinha and Mallick (1991) reported that the addition of CM to media containing BA or kinetin accelerated bud organogenesis from cotyledon calli of *Sesbania bispinosa*. However, apart from the claimed morphogenic effects of CM in tissue cultures (Halperin 1966), its exact role remains unknown.

While the combination of 15 μM TDZ + 2% CM was optimal for inducing bud formation in the most responsive explants, it depressed shoot development from the induced adventitious buds. This prompted us to evaluate other growth regulator combinations. A combination of 15 μM TDZ + 2% CM + 0.001 μM IAA doubled shoot development from induced bud clumps, but only to 3.9 shoots per explant. Culturing bud clumps in media containing 8.8 μM BA alone or in combination with 0.001 μM IAA (an auxin) or 0.14 μM GA_3 significantly increased the development of shoots in the explants. Korban et al. (1992) also reported that the induction of organogenesis and subsequent shoot regeneration from leaf explants of different apple genotypes required a combination of TDZ and an auxin (NAA). Additionally, our work with

macadamia shoot regeneration revealed that the combination of 8.8 μM BA + 0.14 μM GA_3 was optimal for the in vitro elongation of macadamia shoots from nodal explants (Mulwa and Bhalla 2000; Bhalla and Mulwa 2003).

The occurrence of somatic embryogenesis during the culture of cotyledon sections, as demonstrated in the present study, indicates the potential usefulness of pursuing investigations on somatic embryogenesis in macadamia. Although most reports on the somatic embryogenesis of tree species have indicated the necessity for complex multistep culture procedures, this method of in vitro plant regeneration is proving to be a powerful tool for the rapid clonal propagation of forest species (Merckle and Wiecko 1989; Bueno et al. 1992; Merckle 1995) and may also serve as an alternative to organogenesis in trees. Thus the successful development of a somatic embryogenesis system for macadamia would add to the methods of in vitro propagation that will be available for this crop.

In conclusion, we have demonstrated plant regeneration from cotyledon explants of macadamia via organogenesis and, to some extent, somatic embryogenesis. To our knowledge, this is the first report on regeneration of macadamia nuts in vitro. The regeneration techniques described here can now be used for genetic transformation studies.

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