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Regeneration of *Acacia mangium* through somatic embryogenesis

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Abstract Somatic embryogenesis and whole plant regeneration were achieved in callus cultures derived from immature zygotic embryos of *Acacia mangium*. Embryogenic callus was induced on MS medium containing combinations of TDZ (1–2 mg/l), IAA (0.25–2 mg/l) and a mixture of amino acids. Globular embryos developed on embryogenic callus cultured on the induction medium. Nearly 42% of embryogenic cultures with globular embryos produced torpedo- and cotyledonary-stage embryos by a two-step maturation phase. The first stage occurred on 1/2-strength MS basal medium containing 30 g/l sucrose and 5 mg/l GA_3 followed by the second stage on 1/2-strength MS basal medium containing 50 g/l sucrose. Of the cotyledonary-stage somatic embryos, 11% germinated into seedlings that could be successfully transferred to pots. Light- and scanning electron microscopy showed that the somatic embryos originated from single cells of the embryogenic callus. Further, a single cell layer could be detected beneath the developing somatic embryos that appeared to be a demarcation layer isolating the somatic proembryonic structure from the rest of the maternal callus. A suspensor-like structure connected the globular embryos to the demarcation layer. This is the first successful report of plant regeneration through somatic embryogenesis for this economically important tropical forest species.

Keywords *Acacia mangium* · Somatic embryogenesis · Regeneration · Thidiazuron

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Abbreviations *Asn* L-Asparagine monohydrate · *6-BA* 6-Benzylaminopurine · *CH* Casein enzymatic hydrolysate · GA_3 Gibberellic acid · *Gln* L-Glutamine · *IAA* Indole-3-acetic acid · *KT* Kinetin · *MS* Murashige and Skoog (1962) · *NAA* α-Naphthaleneacetic acid · *Pro* L-Proline · *TDZ* 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron) · *Vc* Vitamin C (L-ascorbic acid)

Introduction

Acacia, a leguminous genus in the family *Mimosaceae,* comprises more than 1,200 species that grow mainly in tropical and subtropical regions (Simmons 1987). *Acacia mangium* Willd. is a fast-growing tropical tree that is being increasingly used for plantation, reforestation and soil rehabilitation in degraded soil. Many *A. mangium* plantations have been established in acidic soil or abandoned land. *A. mangium* is used as raw material for the pulp industry due to its high yield and high-quality fibre (Tsai 1988). Many paper and pulp mills in Indonesia are increasingly relying on plantations as a source of wood, and *A. mangium* is the preferred choice. It has been estimated that by 2004 the Asia Paper and Pulp group will obtain all of its wood from plantations consisting mainly of *A. mangium* (Bayliss 1998a, b). This group planted 123,000 ha of land with *A. mangium* in 1996, indicating the tremendous economic value of this species.

The flowers of *A. mangium* possess the ability for self- and cross-pollination. Interspecific pollination *with Acacia auriculiformis* has also been reported for *A. mangium* (Sedgley et al. 1992; Sornsathapornkul and Owens 1999). These characteristics of reproduction are disadvantageous to commercial propagation and plantation through seeds. Therefore, the clonal propagation of superior trees will be of great importance in the management of *A. mangium* plantations.

Plant regeneration has been reported in a few *Acacia* species, such as *A. catechu* (Rout et al. 1995)

and *A. auriculiformis* (Rao and Prasad 1991). In *A. mangium*, protoplast isolation (Toshihiro and Sonoko 1999) and plant regeneration through nodal bud culture (Ahmad 1991; Bhaskar and Subhash 1996; Galiana et al. 1991a, b) have been reported. However, there is no published information on somatic embryogenesis and plant regeneration in this species. In our laboratory, we were able to induce embryogenic callus and regenerate complete plantlets from embryo axes of immature seeds. This is the first report of the regeneration of *A. mangium* through somatic embryogenesis.

Materials and methods

Plant materials

Immature green pods were collected from 20-m-high adult *Acacia mangium* trees growing naturally at Science Park Drive, Singapore. Green immature seeds were gently isolated from the pods and washed four to five times with sterile double-deionised water (ddH_2O), then surface sterilised with 30% market Clorox® bleach (5.25% sodium hypochlorite) for 5 min and washed again with sterile ddH_2O five times. The seeds were further sterilised with 0.1% $HgCl_2$ for 6 min and washed with sterile ddH_2O five times. Embryos were isolated aseptically, and the embryo axes (Fig. 1A) were used as explants for embryogenic callus induction.

Media preparation and culture conditions

All media were readjusted to pH 5.8 with 1 *N* KOH after autoclaving at 121 °C for 25 min. Plant growth regulators were added into media after autoclaving. All cultures were maintained at 28 °C under warm-white fluorescent light at an irradiance between 23 and 26 $\mu mol\ s^{-1}\ m^{-2}$ and a 16 h (light)/8 h (dark) photoperiod.

Embryogenic callus induction

Embryo axes were cultured for 2 months on MS basal medium containing different combinations of 2,4-D (0.5, 1.0, 2.0, 5.0, 40.0 mg/l) and KT (0.5,1.0, 3.0 mg/l), 2,4-D (0.5, 1.0, 2.0 mg/l) and 6-BA (0.5, 1.0, 3.0 mg/l), NAA (0.5, 1.0, 2.0 mg/l) and 6-BA (0.5, 1.0, 3.0, 20.0 mg/l), NAA (0.5, 1.0, 2.0 mg/l) and KT (0.5, 3.0 mg/l) or IAA (0.25–2.0 mg/l) and TDZ (0.022–5.0 mg/l). Each treatment was conducted with 30 embryo axes and repeated twice. All media were supplemented with 100 mg/l Vc, 150 mg/l Asn, 100 mg/l CH, 150 mg/l Gln, 150 mg/l Pro, and 30 g/l sucrose solidified with 0.3% (w/v) phytagel.

Somatic embryo maturation and germination

Embryogenic calli with globular embryos (Fig. 1B, C) induced on induction medium containing 0.25 mg/l IAA and 2.0 mg/l TDZ (Table 1) were cultured for 1 month on 1/2-strength MS basal medium supplemented with 0–5.0 mg/l GA_3, 30–50 g/l sucrose, 100 mg/l Vc, 150 mg/l Asn, 100 mg/l CH, 150 mg/l Gln, 150 mg/l Pro and 1 g/l activated charcoal solidified with 0.35% phytagel (Table 2). Somatic embryos were then transferred onto 1/2-strength MS basal medium containing 50 g/l sucrose and 0.35% phytagel (AM-425) for 40 days (Table 2). Each treatment was conducted with 80 embryogenic cultures and repeated twice.

Cotyledon somatic embryos at the cotyledonary stage (mature somatic embryos) were germinated on 1/2-strength MS basal medium enriched with 5 mg/l GA_3 and 30 g/l sucrose and solidified with 0.35% phytagel (AM-424) for 40 days (Table 2). Seedlings were transferred to 1/2-strength MS basal medium containing 20 g/l sucrose and 0.35% phytagel and cultured for 2 months before being transferred to pot soil (3 parts peat to 1 part sand, v/v).

Histology

Embryogenic calli were fixed in 2.5% glutaraldehyde in 50 m*M* sodium phosphate buffer (pH 7.2) overnight at room temperature, then dehydrated through a graded ethanol series (20%, 30%, 50%, 70% and 90%) sequentially for 20 min at each step, each step being repeated three times, and finally in 100% ethanol for three times 30 min. All tissues were embedded in plastic-embedding medium (Leica Plastic Embed Kit) and sectioned at a thickness of 5–10 μm. All sections were stained in 0.025% toluidine blue O for 1 min, dried at 42 °C, mounted with DPX (BDH) and examined under a light microscope (Leica).

Scanning electron microscopy

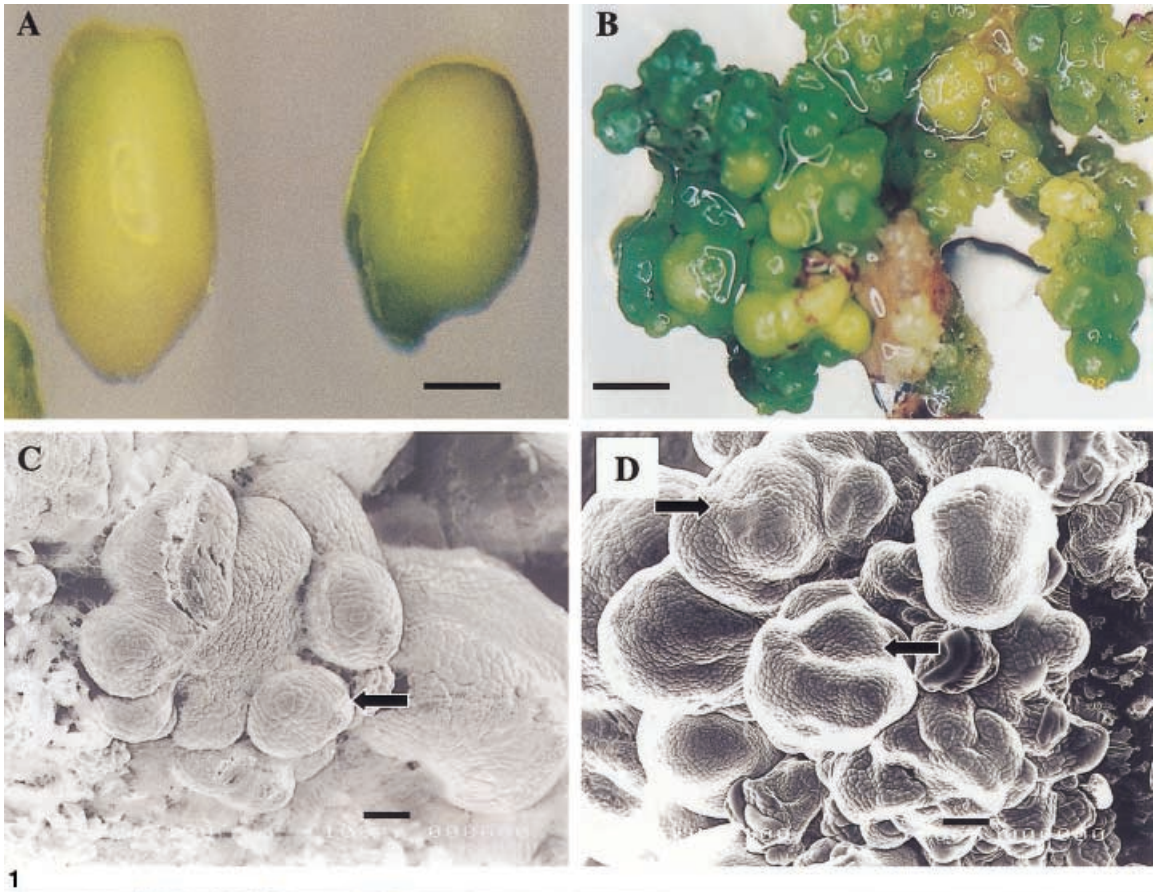
Embryogenic calli were fixed with liquid nitrogen and examined by low-vacuum scanning electron microscopy (JSM-5310LV, JEOL Co, Japan).

Results and discussion

Somatic embryogenesis

Of all the plant growth regulators used, embryo axes of *A. mangium* cultured on the media containing only TDZ (1.0–2.0 mg/l) and IAA (0.25–2.0 mg/l) produced green-yellowish and friable embryogenic calli (Table 1, Fig. 1B). The medium containing 2.0 mg/l TDZ and 0.25 mg/l IAA was the most efficient (16.41%) for inducing embryogenic calli (Table 1). Embryogenic calli produced globular- and heart-shaped embryos (Fig. 1B–D) on the induction medium itself. Embryogenic calli with globular- and heart-shaped embryos were used for further maturation experiments.

Rout et al. (1995) reported that the combination of 13.9 μ*M* (2.99 mg/l) KT and 2.7 μ*M* (0.5 mg/l) NAA promoted *A. catechu* somatic embryogenesis from immature cotyledon. However, a similar combination of KT (3.0 mg/l) and NAA (0.5 mg/l) (medium AM-23) did not induce embryogenic callus from *A. mangium* immature embryonic axes (data not shown). Also, the other tested combinations of NAA (0.5–2.0 mg/l) and 6-BA (0.5–20 mg/l) or NAA (0.5–2.0 mg/l) and KT (0.5–3.0 mg/l) induced adventitious roots or loose white-yellowish callus instead of embryogenic callus (data not shown). 2,4-D or NAA has been reported to induce efficient soybean somatic embryogenesis (Shoemaker et al. 1991; Trick et al. 1997). The combination of 2,4-D and 6-BA induced endosperm cultures of *A. noltina* to form somatic embryos (Garg et al. 1996). In our experiments, the tested combinations of 2,4-D (0.5–40.0 mg/l) and KT (0.5–3.0 mg/l) or 2,4-D (0.5–2.0 mg/l) and 6-BA (0.5–3.0 mg/l) did not induce somatic embryogenic calli in *A. mangium* (data not shown). The calli induced on the media containing 2,4-



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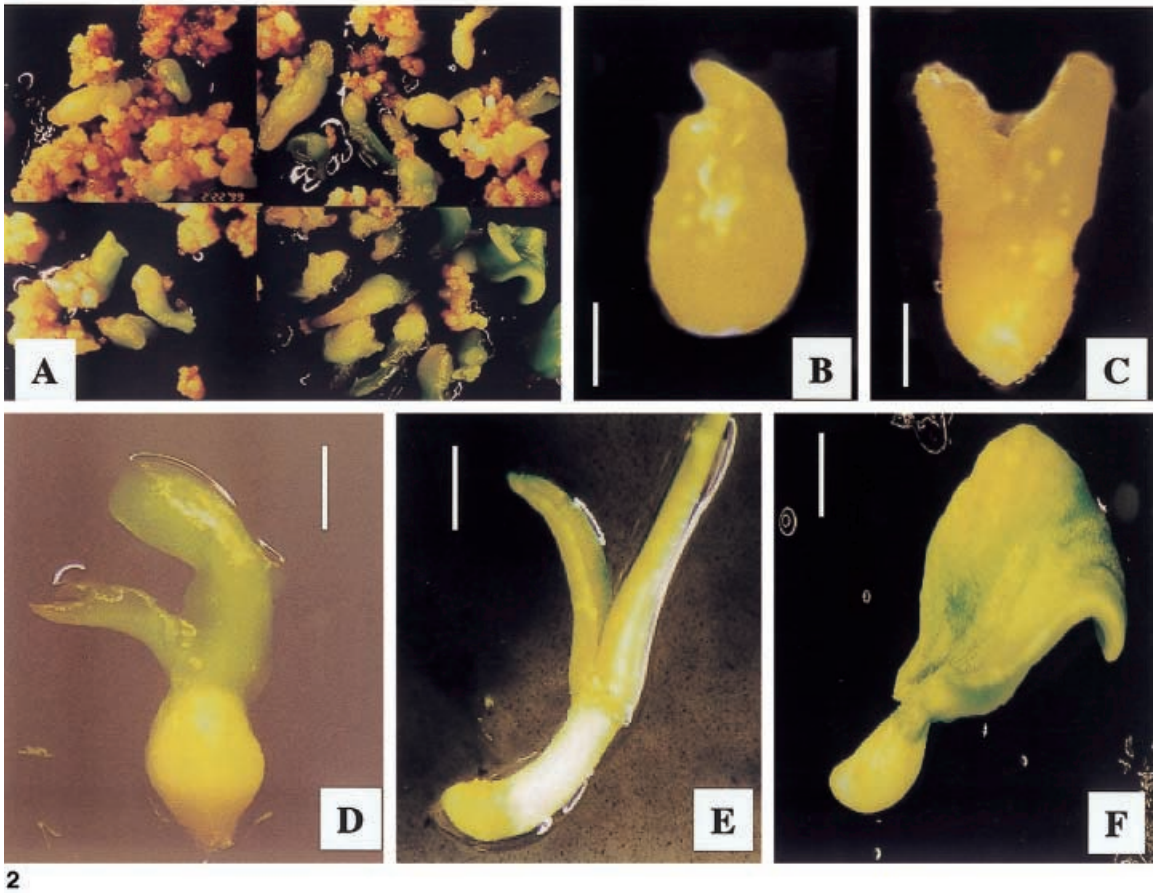

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Fig. 1A–D Somatic embryo induction. **A** Embryo axis used as the explant to induce embryogenic callus (*bar*: 0.5 mm), **B** embryogenic callus induced on the medium containing 2.0 mg/l TDZ and 0.25 mg/l IAA (*bar*: 0.5 mm), **C, D** scanning electron microscopy of *Acacia mangium* somatic embryos: **C** of globular stage (*arrow*) (*bar*: 100 μm), **D** of heart-shaped stage (*arrow*) (*bar*: 100 μm)

Fig. 2A–F Somatic embryo maturation. **A** Maturation of somatic embryos on 1/2-strength MS basal medium containing 50 g/l sucrose and 0.35% phytagel (AM-425), **B** torpedo stage (*bar*: 0.4 mm), **C** early cotyledonary stage (*bar*: 0.6 mm), **D, E** cotyledonary stage (*bar*: 0.8 mm), **F** somatic embryo with single cotyledon (*bar*: 0.8 mm)

Table 1 The effects of different combinations of IAA and TDZ on embryogenic callus induction. Data correspond to average results of three independent repeated experiments, each with 30 embryo axes

IAA (mg/l)	TDZ (mg/l)	Frequency of explants forming embryogenic callus (%)
0.25	0.022	0
0.25	0.1	0
0.25	0.25	0
0.25	1.0	11.06 ± 0.94[a]
0.25	2.0	16.41 ± 4.75
0.25	5.0	0
2.0	0.022	0
2.0	0.1	0
2.0	0.25	0
2.0	1.0	3.51 ± 3.35
2.0	2.0	1.67 ± 0.58
2.0	5.0	0

[a] Mean ± standard deviation

Table 2 The effects of different media on somatic embryo maturation. Embryogenic calli with globular embryos were treated for 1 month and then transferred onto medium AM-425 for 40 days. Data correspond to average results of three independent repeated experiments, each with 80 embryogenic cultures

Maturation media			Frequency of embryogenic calli producing torpedo and cotyledon somatic embryos (%)
Media no.	GA_3 (mg/l)	Sucrose (g/l)	
AM-423	0	30	0
AM-425	0	50	0
AM-467	2.5	30	18.67 ± 6.11[a]
AM-424	5.0	30	42.33 ± 13.65
AM-426	5.0	50	26.67 ± 8.33

[a] Mean ± standard deviation

D and 6-BA were a mixture of white-yellowish structures that were empty inside and distinct from embryogenic calli. *Acacia* genus comprises more than 1,200 species, and significant morphological variations among species have been documented (Simmons 1987). There have been reports of significant differences among genotypes of peanut and soybean with respect to somatic embryo formation (summarised by Lakshmanan and Taji 2000). This variation of responses to plant growth regulators may be due to differences in genetic make-up among different species.

TDZ has been used for the in vitro regeneration of legumes and woody plants, especially recalcitrant species (reviewed by Lakshmanan and Taji 2000; Lu 1993). Replacing 6-BA and KT with TDZ increased peanut plant recovery through somatic embryogenesis (Chengalrayan et al. 1997). TDZ effectively induced direct somatic embryogenesis in five genotypes of annual medics (*Medicago spp*.) (Iantcheva et al. 1999). Similarly, our results showed that TDZ effectively induced somatic embryogenesis in *A. mangium*.

Somatic embryo development stopped at the heart-shaped stage on the media containing TDZ (2.0 mg/l) and IAA (0.25 mg/l) (Figs. 1D, 3H). As secondary somatic embryos formed if heart-shaped embryos were kept longer on the same induction medium, a maturation treatment was necessary for the further development of *A. mangium* somatic embryos. This was in agreement with the results of somatic embryogenesis in soybean (Ranch et al. 1986) and *Picea abies* (Jalonen and Arnold 1991). Maturation methods vary in different species. Ranch et al. (1986) reported that the addition of 6% maltose to the medium promoted soybean somatic embryo histodifferentiation and maturation. ABA treatment was found to be necessary for *Picea abies* somatic embryo maturation (Jalonen and Arnold 1991). In our experiments, neither 6% maltose nor 0.5–1.0 mg/l ABA could induce somatic embryo maturation in *A. mangium* (data not shown). Instead, a two-step procedure involving GA_3 and high concentrations of sucrose was found to be effective in inducing embryo maturation. Firstly, embryogenic calli with globular somatic embryos (Fig. 1B) were cultured on medium containing 2.5–5.0 mg/l GA_3 solidified with 0.35% phytagel (maturation medium AM-424, AM-426 and AM-467) for 1 month (Table 2). These somatic embryos were then transferred onto medium containing 50 g/l sucrose and 0.35% phytagel for 40 days (maturation medium AM-425) for desiccation. The second step was also used to prevent the formation of callus from somatic embryos after culturing on media with GA_3. Nearly 42% of the embryogenic calli with globular embryos (Fig. 1B, C) cultured initially on medium AM-424 and then on AM-425 produced torpedo- and cotyledonary-stage embryos (Fig. 2, Table 2).

The development from globular-stage somatic embryos to torpedo- and cotyledonary-stage ones did not occur during the first step on maturation media without GA_3 (AM-423 and AM-425) (Table 2), indicating the importance of GA_3 in promoting somatic embryo maturation in this species. This is in contrast to the situation reported in geranium (*Pelargonium* × *hortorum* Bailey) where the presence of GA_3 was detrimental to somatic embryo formation during both the

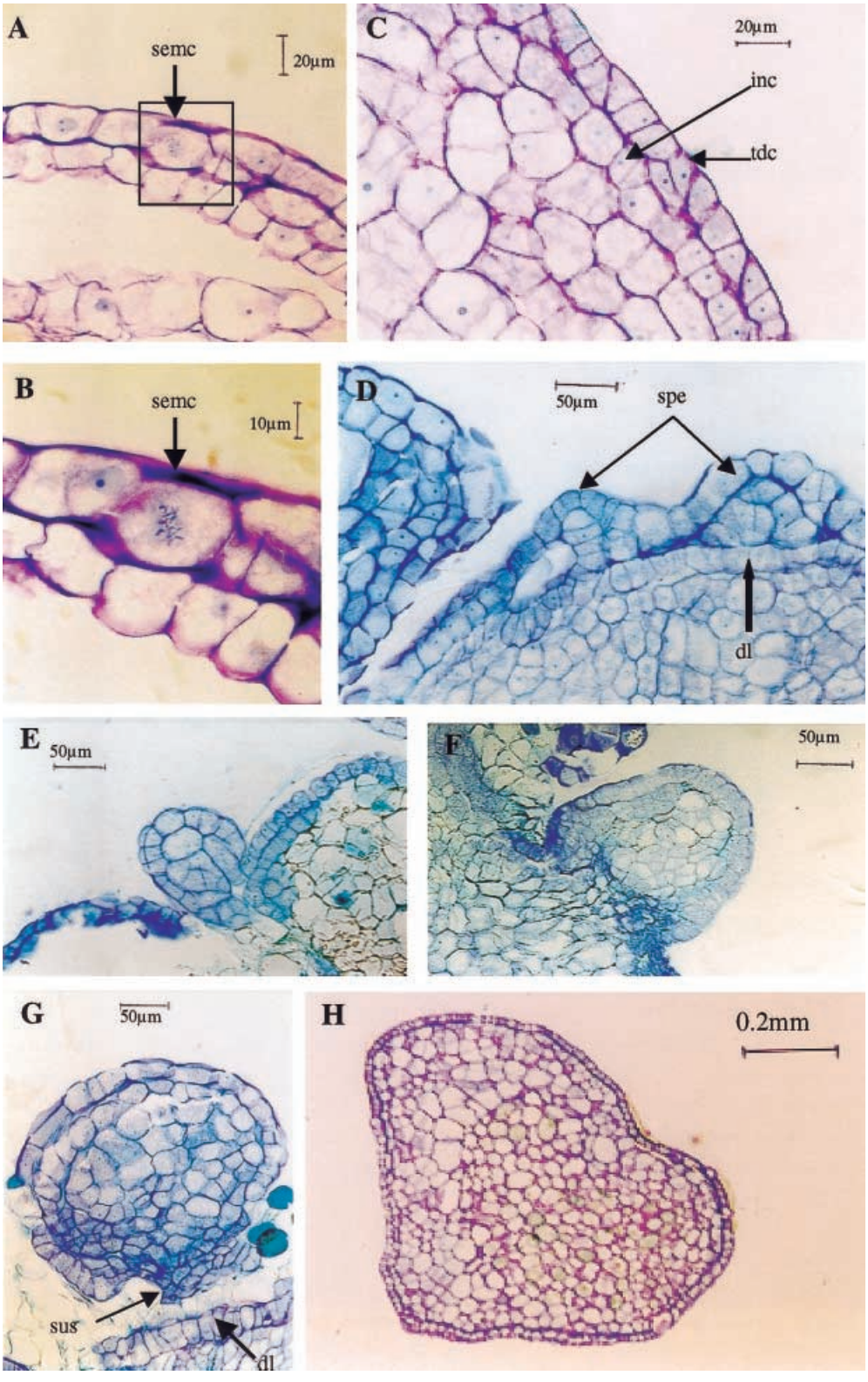

Fig. 3A–H Histology of *Acacia mangium* somatic embryogenesis. **A–D** Somatic embryo initiation from a single cell at metaphase stage of division (**A, B**, *arrow*), two daughter cells (**C**, *arrow*) and somatic proembryonic structure (**D**), **E–G** globular embryo formation, **H** heart-shaped embryo. *dl* Demarcation layer, *inc* inward neighbour cell division, *semc* somatic embryo mother cell, *spe* somatic proembryonic structure, *sus* suspensor-like structure, *tdc* two daughter cells

induction and expression phases of somatic embryogenesis (Hutchinson et al. 1997).

In our experiment, somatic embryo maturation was completed in the presence of a relatively high sucrose concentration during the second maturation step. Similar results have been reported in *Populus* somatic embryogenesis where a sucrose concentration of 50 g/l stimulated somatic embryo differentiation (Michler and Bauer 1991). Biahoua and Bonnean (1999) also reported that a high sucrose concentration (350 m*M*,

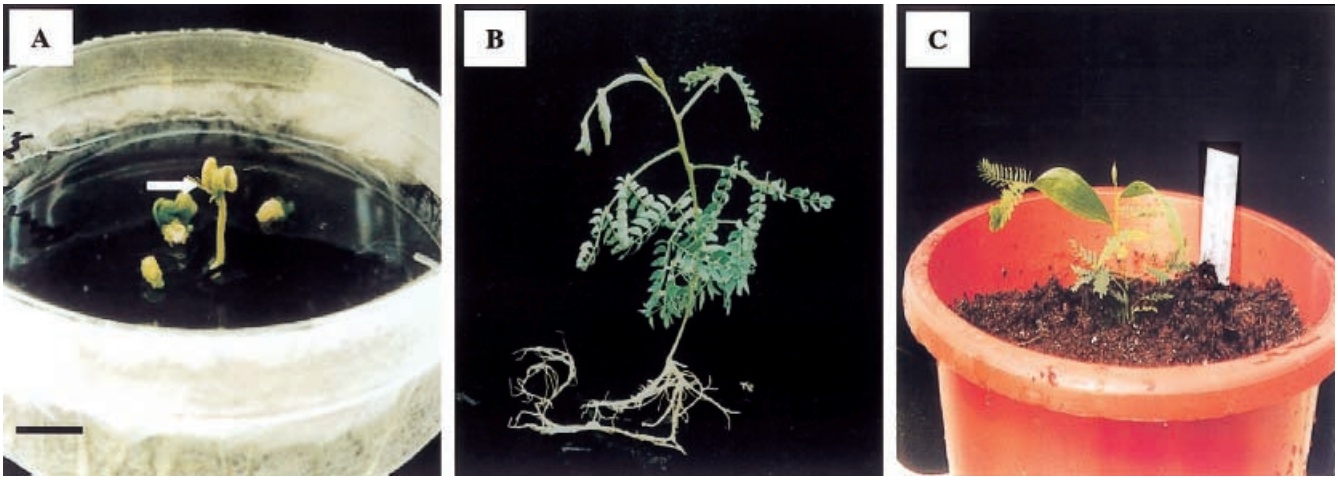

Fig. 4A–C Somatic embryo germination. **A** Germinating mature somatic embryo with the pinnate leaf (*arrow*) (*bar*: 1.5 cm), elongated hypocotyl and radicle in medium, **B** fully developed plantlet before transfer, **C** plant growing in peat:sand (3:1, v/v)

120 g/l) gave the highest efficiency of *Euonymus europaeus* somatic embryogenesis. The precise role of high levels of sucrose in enhancing somatic embryogenesis is not evident. However, many previous studies suggest that sucrose has both a nutritive and an osmotic effect on embryogenesis in several species (Parrott et al. 1992). In our experiment we observed abnormal somatic embryos, for example, with only one cotyledon (Fig. 2F) or with three cotyledons, which could not germinate into seedlings. Similar observations have also been reported in soybean (Ranch et al. 1986) and peanut (Chengalrayan et al. 1997).

Commercial mass propagation through embryogenic culture requires efficient protocols for repetitive or recurrent somatic embryogenesis, and these have only been reported for a few legume crop species, such as alfalfa (Ianthchana et al. 1999; Ninkovic et al. 1995), soybean (Finer and Nagasawa 1988) and peanut (Durham and Parrot 1992). Successful repetitive embryogenesis in legume tree species has not been reported. When we subcultured embryogenic callus or heart-shaped embryos on the solid induction medium containing 2.0 mg/l TDZ and 0.25 mg/l IAA (Table 1), secondary embryogenesis induction was observed. The capacity of repetitive somatic embryogenesis was maintained for over 6 months by subculturing once a month (data not shown). Our protocol with the option of recurrent embryogenesis provides the possibility for large-scale commercial propagation, and this is the first such report for any leguminous tree species.

Histology of somatic embryogenesis

Somatic embryogenesis has been considered to be a model system for studying plant embryo development (Warren 1993). Two distinct types of somatic embryogenesis, direct and indirect, have been recognised. In direct somatic embryogenesis, a single cell (or group of cells) of the primary explant commences meristematic growth and develops into an embryo (Warren 1993). In indirect somatic embryogenesis, an embryo develops from one or more cells from an embryogenic callus. The somatic embryogenesis of *A. mangium* reported here is of the indirect type of embryogenesis. Histological sections showed that somatic embryogenesis developed from one cell (termed as somatic embryo mother cell in this paper) in the outmost layer of embryogenic callus (Fig. 3A, B). Periclinal division of the mother cell produced two daughter cells (Fig. 3A–C), one outer cell and one inner cell (Fig. 3C).

The continued cell division of these two daughter cells resulted in the formation of a somatic proembryonic structure (Fig. 3D). The somatic proembryonic structures developed further into globular- and heart-shaped embryo (Fig. 3D–H). In the light of histological analysis, we suggest that the anticlinal division of the outer daughter cell develops into epidermal cells of the somatic embryo, while the periclinal and anticlinal division of the inner daughter cell develops into somatic embryo body (Fig. 3D). It is also suggested that the inward neighbour cells next to the mother cell do not form somatic embryo but their anticlinal division forms a layer of cells to isolate the developing somatic embryo from maternal callus (Fig. 3C–D, G). This layer of cells is termed the demarcation layer in this paper (Fig. 3D, G). During the transition from somatic proembryonic structure to globular embryo, a suspensor-like structure developed and connected the globular embryos to the demarcation layer (Fig. 3G).

On the induction medium, embryo development stopped at the heart-shaped embryo stage (Figs. 1D, 3H). Through maturation, globular embryos developed into torpedo- and cotyledonary-stage embryos (Fig. 2B–D). In conclusion, somatic embryogenesis of *A. mangium* originated from one cell (somatic embryo mother cell) of the embryogenic callus and underwent distinct stages of development as in zygotic embryogenesis (Figs. 2, 3).

Somatic embryo germination

Normal somatic embryo germination of *A. mangium* included the elongation of the radicle and hypocotyl and the expansion of the first pinnate leaf. On the 1/2-strength MS basal medium containing 5.0 mg/l GA_3 (AM-424) (Table 2), 11% of the cotyledonary-stage somatic embryos germinated into seedlings with root, elongated hypocotyl and the first pinnate leaf (Fig. 4A, arrow). Only those mature somatic embryos consisting of radicle, hypocotyl, cotyledon and the first pinnate leaf structure could germinate into seedlings (Fig. 4B, C).

A combination of embryogenesis and *Agrobacterium*-mediated transformation has been successful in many woody species, such as English walnut, pecan and cherry, and will be very important in tree breeding (Kozlowski and Pallardy 1997). *A. mangium* is a commercially important tropical leguminous tree for the paper and pulp industry and a leading tree for plantation. A combination of an efficient regeneration system through somatic embryogenesis with biolistic or *Agrobacterium*-mediated transformation will provide a valuable alternative breeding method for introducing desirable commercial traits to *A. mangium*.

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