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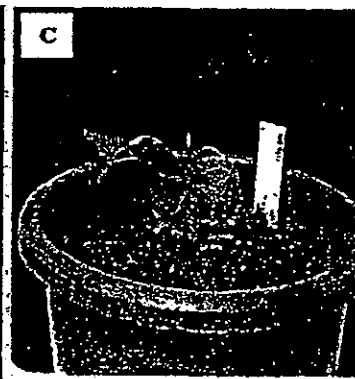
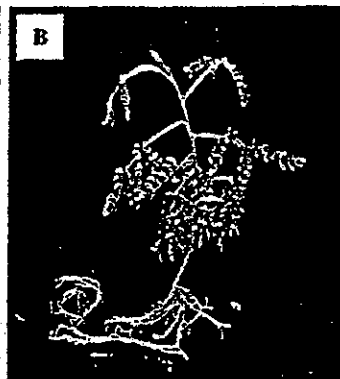
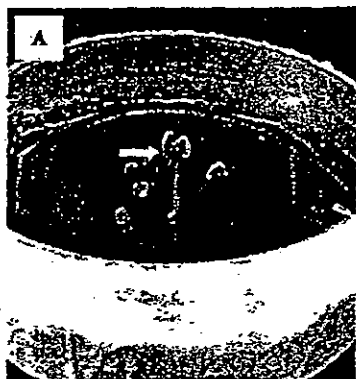
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(54) Title: SOMATIC EMBRYOGENIC REGENERATION OF ACACIA MANGIUM



(57) Abstract: The present invention relates to the field of somatic embryo production, particularly to methods for the regeneration of *Acacia mangium* through somatic embryogenesis. More specifically, the present invention relates to a method for regeneration of plants of *Acacia mangium* by culturing explants of immature zygotic embryos on callus induction medium to grow embryogenic tissue. Culturing of the embryogenic tissue is continued on somatic embryo maturation medium and germination medium. The germinated embryos are further converted to acclimatized plants for field planting. The method is well suited for producing clonal planting stock useful for reforestation.

TITLE OF THE INVENTIONSOMATIC EMBRYOGENIC REGENERATION OF *ACACIA MANGIUM*BACKGROUND OF THE INVENTION

The present invention relates to the field of somatic embryo production, particularly to methods for the regeneration of *Acacia mangium* through somatic embryogenesis. More specifically, the present invention relates to a method for regeneration of plants of *Acacia mangium* by culturing explants of immature zygotic embryos on callus induction medium to grow embryogenic tissue. Culturing of the embryogenic tissue is continued on somatic embryo maturation medium and germination medium. The germinated embryos are further converted to acclimatized plants for field planting. The method is well suited for producing clonal planting stock useful for reforestation.

The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the appended List of References.

Forests are very important to the world economy and for maintaining and preserving our ecosystem. Forest trees have a wide range of commercial uses (timber for construction, raw material for paper and pulp production, and as an energy source). The global demand for wood products (mostly for paper and pulp and firewood in developing countries) has been increasing year by year when the natural forests are in short supply. Re-forestation is the solution to meeting such increasing demand. Usually, the fast-growing, widely adapted tree species are chosen for re-forestation. Most tree-improvement programs are based on the management of genetic resources, including the selection of superior clones from existing forests, the conservation of genetic variability, partially controlled propagation and classical breeding for desired traits. In spite of the fact that it usually takes several generations to breed, this traditional breeding has been successful in getting elite trees with fast and uniform growth. However, many other traits such as disease and insect resistance, different lignin composition and content are difficult to acquire mainly due to high heterozygosity in tree species and big segregation population. Moreover, the gene(s) conferring certain phenotypes like disease resistance may not be in the gene pool at all. On the other hand, molecular breeding based on genetic transformation of tree species offers the possibility to introduce a particular phenotype without affecting the genetic background of a cultivar. Genetic transformation in *Populus* species and *Eucalyptus* species enabled some success in modification of lignin content (Tzfira et al., 1998; Robinson, 1999). The precondition of molecular breeding of forest tree species is the

availability of a reliable and reproducible genetic transformation method, which in turn relies on a system of regeneration of one whole plant from a single cell.

Genus *Acacia* comprises about 1200 tropical and subtropical tree species. It belongs to the family *Mimosaceae*. *Acacia mangium* is a multipurpose, fast growing and nitrogen fixing elite tropical legume tree. *A. mangium* has been increasingly used for plantation, reforestation and soil rehabilitation in degraded soil. Many *A. mangium* plantations have been established in acidic soil or abandoned land. Yielding high quality fibre, *A. mangium* has been increasingly planted in many regions of South East Asia like Malaysia (Tsai, 1988) and Indonesia as raw material for pulp industry. Many paper and pulp mills in Indonesia have been increasingly relying on plantation as the source of wood and *A. mangium* is the preferred choice. Asia Paper and Pulp group has two affiliate companies with total concession of 540,000 hectares. By 1996, one company had planted 123,000 hectares of *A. mangium*, about 90% of its plantation, which represented 180 million seedlings. It was estimated that by 2004, Asia Paper and Pulp group will virtually source all its wood from plantation, mainly *A. mangium* plantation (Bayliss, 1998a, 1998b). However, the flowers of *A. mangium* show weak protogyny and variable levels of andromonoecy. It possesses the characteristics of self-pollination, cross-intraspecific pollination or interspecific pollination with *A. auriculiformis* in nature (Sedgley et al., 1992; Sornsathapornkul and Owens, 1999). These characteristics of reproduction are disadvantageous to commercial propagation and plantation through seeds.

Regeneration is often used in woody tree propagation (Kozlowski and Pallardy, 1997). In *Acacia*, a few species were reported the success of regeneration, such as *Acacia catechu* regeneration via somatic embryogenesis (Rout et al., 1995) and *A. auriculiformis* regeneration through organogenesis (Rao and Prasad, 1991). *A. mangium* shoot propagation was reported (Bhaskan and Subbash, 1996; Ahmad, 1991; Galiana et al., 1991a, 1991b;) and Toshihiro (1999) also reported the isolation of protoplast from sterile *A. mangium* seedlings. Our PCT patent application No. PCT/SG00/00010 describes *A. mangium* regeneration through organogenesis. However, regeneration of *A. mangium* through somatic embryogenesis has not been described.

Somatic embryos are clonal in origin and thus multiplication using somatic embryos can have the potential for exceedingly high rates of vegetative increase and is therefore of considerable commercial interest. Regeneration via somatic embryogenesis is an attractive option for plant tissue culture. Somatic embryos reportedly provide more stable regenerants than shoots. Another advantage of regeneration systems using somatic embryos is their apparent single cell origin. This

means that it is unlikely that regenerants are of chimerical origin, since, if a regenerant originates from a cluster of cells rather than a single cell, the plant tissues may be chimerical or unstable and produce off-types. The availability of somatic embryogenesis protocols for potato, or other crop species recalcitrant to somatic embryogenesis, will permit these crops to take advantage of any new artificial seed technology advances.

Somatic embryos are suitable for transformation via *Agrobacterium tumefaciens* (Mathews et al., 1992), microinjection (Neuhaus et al., 1987) and particle bombardment (Wilde et al., 1992). In addition, somatic embryos can be cryopreserved using liquid nitrogen without loss of viability. Cryopreservation is an efficient means of maintaining germplasm and enables plant material to be transported over large distances. Furthermore, somatic embryos are suitable for the development of artificial seed technology (U.S. Patent No. 5,572,827; Bajaj, 1995a).

Thus, it is an object of the present invention to provide a method for regeneration of *A. mangium* through somatic embryogenesis.

SUMMARY OF THE INVENTION

The present invention relates to the field of somatic embryo production, particularly to methods for the regeneration of *Acacia mangium* through somatic embryogenesis. More specifically, the somatic embryogenesis process for *A. mangium* of the present invention can be divided into four general steps: (1) embryogenic callus induction, (2) embryo maturation, (3) embryo germination, and (4) conversion into acclimatized plants, i.e., regenerated plants or regenerants. The acclimatized plants are then transferred to the field. It is preferred to use a two step embryo maturation.

The present invention also relates to a process for preparing somatic embryos for *A. mangium*. This process generally comprises the first two previously described steps. The somatic embryos can be used as a source material for preparing transgenic plants, can be cryopreserved and can be developed into artificial seed.

Accordingly, one aspect of the present invention provides a method for preparing somatic embryos of *Acacia mangium* comprising:

- a) culturing an explant on callus induction medium to induce embryogenic callus and globular embryo formation; and
- b) culturing globular embryos on a maturation medium to form somatic embryos.

Another aspect of the present invention provides a method for regenerating *Acacia mangium* comprising:

3a

- a) culturing an explant on callus induction medium to induce embryogenic callus and globular embryo formation;
- b) culturing the globular embryos on a maturation medium to form somatic embryos; and
- c) culturing the somatic embryos on a germination medium to produce regenerated seedlings.

According to one embodiment of the present invention, somatic embryogenesis of *Acacia mangium* is achieved from callus induced from immature embryo axes on MS basal medium supplemented with the combinations of thidiazuron (TDZ) and indole-3-acetic acid (IAA) together with ascorbic acid, asparagine, casein enzymatic hydrolysate, glutamine, proline, sucrose and phytagel. Through two steps of maturation procedure by culturing somatic embryos first on ½ MS basal medium containing sucrose, phytagel and gibberellic acid (GA₃) and then on ½ basal medium

containing sucrose and phytigel, 42.33% of globular embryos developed into torpedo and cotyledon embryos. 11% of mature somatic embryos germinated into seedlings that were transferred to pot soil consisting of a mixture of peat soil and sand.

BRIEF DESCRIPTION OF THE FIGURES

5 Figures 1A-D show somatic embryo induction. Fig. 1A shows the use of the embryo axis as the explant to induce embryogenic callus. Fig. 1B shows embryogenic callus induced on the induction medium AM-304. Figs. 1 C-D show scanning electron microscopy of *Acacia mangium* somatic embryos with Fig. 1C showing globular stages (indicated by arrow; bar = 100 μ m) and Fig. 1D showing heart stage (indicated by arrow; bar = 100 μ m).

10 Figures 2A-F show somatic embryo maturation and germination. Fig. 2A shows maturation of somatic embryos on AM-425. Figs. 2B-F show various stages of somatic embryo maturation and germination with Fig. 2B showing torpedo stage, Fig. 2C showing early cotyledon stage, Fig. 2D showing cotyledon stage, Fig. 2E showing somatic embryo germination, and Fig. 2F showing a somatic embryo with single cotyledon.

15 Figures 3A-H show the histology of *A. mangium* somatic embryogenesis. Figs. 3A-D show somatic embryo initiation from epidermis cell as follows: metaphase (A and B, indicated by arrow), two-daughter cells (C, indicated by arrow), and proembryonic structure (D); Figs. 3E-G show globular embryo formation; and, Fig. 3H shows a heart embryo. Abbreviations used are: dc = demarcation cell layer; pe = proembryonic structure; sec = subepidermal cell; sus, suspensor; and, 20 tdc = two-daughter cell.

 Figures 4A-C show somatic embryo germination. Fig. 4A shows germinating mature somatic embryo with the pinnate leaf (indicated by arrow), elongated hypocotyl and radicle in medium. Fig. 4B shows seedling before planting into soil. Fig. 4C shows plant growing in soil (3:1).

DETAILED DESCRIPTION OF THE INVENTION

25 The present invention relates to the field of somatic embryo production, particularly to methods for the regeneration of *Acacia mangium* through somatic embryogenesis. More specifically, the present invention relates to a method for regeneration of plants of *Acacia mangium* by culturing explants of immature zygotic embryos on callus induction medium to grow embryogenic tissue. 30 Culturing of the embryogenic tissue is continued on somatic embryo maturation medium and

germination medium. The germinated embryos are further converted to acclimatized plants for field planting. The method is well suited for producing clonal planting stock useful for reforestation.

Propagation by somatic embryogenesis refers to methods whereby embryos are produced in vitro from small pieces of plant tissue or individual cells. The embryos are referred to as somatic because they are derived from the somatic (vegetative) tissue, rather than from the sexual process. Vegetative propagation via somatic embryogenesis has the capability to capture all genetic gain of highly desirable genotypes. Furthermore, these methods are readily amenable to automation and mechanization. These qualities endow somatic embryogenesis processes with the potential to produce large numbers of individual clones for reforestation purposes.

In one embodiment of the present invention, somatic embryos are produced by a process which can be divided into two general steps: (1) embryogenic callus induction and (2) embryo maturation. It is preferred to use a two step embryo maturation. In one aspect, somatic embryos are prepared by (a) inducing embryogenic callus on a callus induction medium comprising a basal medium supplemented with TDZ and IAA, (b) maturing the embryos on a first maturation medium comprising a basal medium supplemented with GA₃ and (c) maturing the embryos on a second maturation medium comprising a basal medium.

In a second embodiment of the present invention, regenerant plants of *A. mangium* are produced by a process which can be divided into four general steps: (1) embryogenic callus induction, (2) embryo maturation, (3) embryo germination, and (4) conversion into acclimatized plants. It is preferred to use a two step embryo maturation. The acclimatized plants can then be planted in the field. In one aspect, *A. mangium* regenerants are prepared by (a) inducing embryogenic callus on a callus induction medium comprising a basal medium supplemented with TDZ and IAA, (b) maturing the embryos on a first maturation medium comprising a basal medium supplemented with GA₃, (c) maturing the embryos on a second maturation medium comprising a basal medium, (d) germinating the mature embryos on germination medium comprising a basal medium and (e) acclimatizing the germinated plants in potting soil.

More specifically with respect to this latter embodiment, somatic embryogenesis of *Acacia mangium Willd.* was achieved from callus induced from immature embryo axes on MS basal medium supplemented with the combinations of TDZ (1-2 mg/l) and IAA (0.25-2 mg/l) together with ascorbic acid (Vc) 100 mg/l, asparagine (Asn) 150 mg/l, casein enzymatic hydrolysate (CH) 100 mg/l, glutamine (Gln) 150 mg/l, proline (Pro) 150 mg/l, sucrose 30 g/l and phytagel 0.3%. Through two steps of maturation procedure by culturing somatic embryos on ½ basal MS medium

containing 30-50 g/l sucrose, 0.1% activated charcoal, 0.35% of phytigel and 2.5 mg/l - 5.0 mg/l, preferably 5.0 mg/l, of GA₃ together with Vc 100 mg/l, Asn 150 mg/l, CH 100 mg/l, Gln 150 mg/l and Pro 150 mg/l, then on ½ basal medium containing 50 g/l of sucrose and 0.35% of phytigel together with Vc 100 mg/l, Asn 150 mg/l, CH 100 mg/l, Gln 150 mg/l, Pro 150 mg/l and 0.1% activated charcoal, 42.33% of globular embryos developed into torpedo and cotyledon embryos. With culturing the mature somatic embryos on ½ basal medium containing 30 g/l of sucrose, 0.35% of phytigel and 2.5 mg/l - 5.0 mg/l, preferably 5.0 mg/l, GA₃ together with Vc 100 mg/l, Asn 150 mg/l, CH 100 mg/l, Gln 150 mg/l, Pro 150 mg/l and 0.1% activated charcoal, 11% of mature somatic embryos germinated into seedlings. The seedlings were cultured on ½ basal medium containing 20 g/l of sucrose and 0.35% of phytigel and then transferred to pot soil consisting of peat soil: sand (3:1). The transplanted seedlings are then ready to be planted in the field.

Histological analysis showed that somatic embryogenesis was of unicellular origin from single epidermal cell of embryogenic callus. The somatic embryogenesis underwent 2-cell stage, proembryo stage, globular embryo stage, torpedo stage and cotyledon stage. Proembryonic structure was divided from maternal callus by one demarcation cell layer and globular embryo connected to callus by a suspensor-like structure.

Somatic embryogenesis has been used as the method of large-scale propagation of oil palm (Huong, 1999; Aberlenc-Bertossi et al., 1999) and soybean (Trick, 1997), and has been also used in other woody plants such as *Dalbergia sissoo* Roxb (Das et al., 1997), *Camellia sinensis* (L.) O. Kuntze (Ponsamuel et al., 1996), and *Picea abies* (L.) Karst (Westcott, 1994). One of important applications of somatic embryogenesis is for genetic modification. For example, soybean somatic embryogenesis provided the main approach to breed high quality transgenic soybean plants (Trick, 1997). Combination of embryogenesis and *Agrobacterium*-mediated transformation had also succeeded in English walnut, pecan, and cherry, and will be very important in breeding (Kozlowski and Pallardy, 1997). *A. mangium* is a commercially important tropical legume tree for paper and pulp industry and a leading tree for plantation. Combination of an efficient regeneration system through somatic embryogenesis, as described herein, with biolistic or *Agrobacterium*-mediated transformation provides a valuable alternative for breeding pest or disease resistance.

EXAMPLES

The present invention is further detailed in the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

EXAMPLE 1

Reagents and Culture Media

A. MS basic medium: MS medium (Murashige and Skoog, 1962)

	Name	Molecular formula	Concentration (mg/L)	Sigma Cat. No.
	<u>Macronutrients</u>			
10	Ammonium Nitrate	NH_4NO_3	1,650	A-3795
	Potassium Nitrate	KNO_3	1,900	P-8291
	Calcium Chloride dihydrate	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	C-2536
	Magnesium Sulfate Heptahydrate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	M-7774
15	Potassium phosphate monobasic, anhydrous	KH_2PO_4	170	P-8416
	Ferrous Sulfate Heptahydrate	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8	F-8263
20	Ethylenediamine-tetra-acetic acid (EDTA)	$\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (Na_2EDTA)	37.3	E-6635
	<u>Micronutrients</u>			
	Potassium Iodide	KI	0.83	P-8166
	Boric Acid	H_3BO_3	6.2	B-9645
25	Manganese Sulfate monohydrate	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	6.9	M-7899
	Zinc Sulfate	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	Z-1001
	Molybdic Acid (Sodium salt:dihydrate)	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	M-1651
30	Cupric Sulfate (Pentahydrate)	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	C-3036
	Cobalt Chloride	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	C-2911
	<u>Organic Reagents</u>			
	myo-Inositol	$\text{C}_6\text{H}_{12}\text{O}_6$	100	I-3011
	Nicotinic acid	$\text{C}_6\text{H}_5\text{NO}_2$	0.5	N-0765
35	Glycine	$\text{C}_2\text{H}_5\text{NO}_2$	2.0	G-6143
	Thiamine (Vitamin B1)	$\text{C}_{12}\text{H}_{17}\text{ClN}_4\text{OS} \cdot \text{HCl}$	0.1	T-3902
	Pyridoxine (Vitamin B6) Hydrochloride	$\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$	0.5	P-9755

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B. Other Organic Reagents

	Name	Molecular formula	Sigma Cat. No.
	L-Ascorbic acid (Vitamin C)	$C_6H_8O_6$	A-2174
	Casein Enzymatic Hydrolysate (CH)		C-7290
5	L-Glutamine (Gln)	$C_5H_{10}N_2O_3$	G-9273
	L-Asparagine monohydrate (Asn)	$C_4H_8N_2O_3 \cdot H_2O$	A-4284
	L-Proline (Pro)	$C_5H_9NO_2$	P-4655
	Sucrose		S-5390

C. Plant Growth Regulators

	Name	Sigma Cat. No.
10	2,4-Dichlorophenoxyacetic Acid (2,4-D)	D-8407
	Indole-3-acetic Acid (IAA)	I-2886
	α -Naphthaleneacetic Acid(NAA)	N-0640
	1-Phenyl-3-(1,2,3-thiadiazol-5-yl)Urea (Thidiazuron, TDZ)	P-6186
15	6-Benzylaminopurine (6-BA)	B-3408
	Kinetin (KT)	K-0753
	Gibberellic Acid (GA_3)	G-7645

D. Agar: Phytagel (Sigma catalog No.: P-8169)

E. Media for callus induction¹

20	AM-5	MS + 2,4-D 2.0 mg/L + KT 3.0 mg/L
	AM-6	MS + 2,4-D 2.0 mg/L + KT 0.5 mg/L
	AM-7	MS + NAA 2.0 mg/L + KT 3.0 mg/L
	AM-8	MS + NAA 2.0 mg/L + KT 0.5 mg/L
	AM-14	MS + 2,4-D 0.5 mg/L + 6-BA 3.0 mg/L
25	AM-22	MS + 2,4-D 2.0 mg/L + 6-BA 1.0 mg/L
	AM-27	MS + NAA 0.5 mg/L + 6-BA 3.0 mg/L
	AM-31	MS + NAA 0.5 mg/L + 6-BA 1.0 mg/L
	AM-230	MS + 2,4-D 5.0 mg/L + KT 3.0 mg/L
	AM-231	MS + 2,4-D 5.0 mg/L + KT 0.5 mg/L
30	AM-234	MS + 2,4-D 1.0 mg/L + KT 1.0 mg/L
	AM-265	MS + IAA 0.25 mg/L + TDZ 1.0 mg/L
	AM-304	MS + IAA 0.25 mg/L + TDZ 2.0 mg/L
	AM-308	MS + IAA 2.0 mg/L + TDZ 1.0 mg/L

¹ All media also contained CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, phytagel 0.30%, sucrose 30 g/L, pH 5.8 after autoclaving.

F. Media for Embryo Maturation and Germination¹

AM-423	$\frac{1}{2}$ MS + sucrose 30.0 mg/L
AM-424	$\frac{1}{2}$ MS + GA ₃ 5.0mg/L + sucrose 30.0 mg/L
AM-425	$\frac{1}{2}$ MS + sucrose 50.0 mg/L
AM-426	$\frac{1}{2}$ MS + GA ₃ 5.0 mg/L + sucrose 50.0 mg/L
AM-467	$\frac{1}{2}$ MS + GA ₃ 2.5 mg/L + sucrose 30.0 mg/L

¹ All media also contained CH 100 mg/L, Vc 100 mg/L, Gln 150 mg/L, Asn 150 mg/L and Pro 150 mg/L, phytigel 0.30%, activated charcoal 0.1%, pH 5.8 after autoclaving

G. Seedling Growth (Acclimatization)

$\frac{1}{2}$ MS, sucrose 20.0 mg/L, 0.35% phytigel

H. Transplantation

Peat soil:white sand 3:1

EXAMPLE 2

Plant materials

One and half months old immature green pods were collected from twenty-metre high adult *A. mangium Willd.* trees at Science Park Drive of Singapore. Green seeds were gently isolated from pods and washed with sterile double deionized water (ddH₂O) 4-5 times. After green seeds were surface sterilised with 30% market clorox bleach (NaClO) for 5 minutes and washed with sterile ddH₂O 5 times, the seeds were sterilised again with 0.1% HgCl₂ for 6 minutes and washed with sterile ddH₂O 5 times. Seed coats were removed with sterilised forceps and embryos were isolated. Embryo axes (EA) (Fig.1A) were gently separated with sterile forceps and used as explants for embryogenic callus induction.

EXAMPLE 3

Culture Conditions

All media were adjusted to pH 5.8 after autoclaving at 121 °C for 25min. Plant growth regulators tested in experiments were added into media after autoclaving but other additional substances were added into media before autoclaving. All cultures were maintained under the warm white fluorescent lights at a range of irradiance of 23.41-26.01 $\mu\text{mol.s}^{-1}\text{m}^{-2}$, with 16 h photoperiod and at 28° C.

EXAMPLE 4

Histology

Embryogenic calli were fixed in 2.5% glutaraldehyde in 50mM pH7.2 sodium phosphate buffer at room temperature overnight, then dehydrated through a graded ethanol series 20%, 30%, 50%, 70%, and 90% sequentially for 20 minutes thrice, at last in 100% ethanol for 30 minutes thrice. All tissues were embedded in plastics (Leica plastic embed Kit) and were sectioned at a thickness of 5-10µm. All sections were stained in 0.025% toluidine blue for 1 minute, dried at 42°C, mounted with DPX (BDH), and examined under a light microscope (Leica).

EXAMPLE 5

Scanning Electron Microscopy

Embryogenic calli were treated with liquid nitrogen, and examined by scanning electron microscopy (JSM-5310LV, JEOL Company, Japan).

EXAMPLE 6

Embryogenic Callus Induction

EAs were cultured for two months on MS basal medium (Murashige and Skoog 1962) supplemented with different combinations of plant growth regulators: 2,4-D (1.0-5.0 mg/l) and KT (0.5-3.0 mg/l), 2,4-D (0.5-2.0 mg/l) and 6-BA (1.0-3.0 mg/l), NAA (0.5 mg/l) and 6-BA (1.0-3.0 mg/l), NAA (0.5-2.0 mg/l) and KT (0.5-3.0 mg/l) or IAA (0.25-2.0 mg/l) and TDZ (1.0-2.0 mg/l). Each experiment was repeated twice. A total of 90 embryo axes were tested for embryogenic callus induction on each plant growth regulator combination. All media contained ascorbic acid (Vc) 100 mg/l, asparagine (Asn) 150 mg/l, casein enzymatic hydrolysate (CH) 100 mg/l, glutamine (Gln) 150 mg/l, and proline (Pro) 150 mg/l, sucrose 30 g/l and phytigel 0.3% (g/l) in this experiment.

It was reported that the initiation of somatic embryo production from callus occurs in the media containing high concentrations of auxin or a high ratio of auxin to cytokinin (Kozłowski and Pallardy, 1997). However, in *A. mangium* somatic embryogenesis, green-yellowish and friable embryogenic calli were obtained only on the media containing the combinations of 1.0-2.0 mg/l TDZ and 0.25-2.0 mg/l IAA, (Table 1; Fig.1B). The combination of TDZ 2.0 mg/l and IAA 0.25 mg/l was the most efficient for induction of embryogenic calli (16.41%) (Table 1), which then produced globular embryos and heart embryos (Figs.1B-D). This combination was used to induce more embryogenic calli. However, on the media containing 2,4-D (1.0-5.0 mg/l) and KT (0.5-3.0

mg/l), 2,4-D (0.5-2.0 mg/l) and 6-BA (1.0-3.0 mg/l), NAA (0.5 mg/l) and 6-BA (1.0-3.0 mg/l) or NAA (0.5-2.0 mg/l) and KT (0.5-3.0 mg/l), no embryogenic callus was obtained (Table 1). Rout et al. (1995) reported that the combination of kinetin 13.9 μ M (2.99 mg/l) and NAA 2.7 μ M (0.55 mg/l) promoted *A. catechu* somatic embryogenesis from immature cotyledon. However, the similar combination of NAA and KT did not induce any embryogenic callus from *A. mangium* immature embryonic axis (Table 1). The callus induced from embryonic axis was white and loose on the medium containing NAA and KT. The *Acacia* genus comprises more than 1,200 species and big variations in morphology (Simmons, 1987) were documented. Among other differences, an adult *A. mangium* is about 30 metres tall while one adult *A. catechu* is only 9-12 metres. The variation of responses to plant growth regulators may result from the differences of genetic background among species. TDZ was recommended in woody species tissue culture especially in recalcitrant species (Lu, 1993). Our results showed that TDZ was effective to induce embryogenic callus in *A. mangium*. Chengalrayan et al. (1997) reported that the substitution of 6-BA and KT with TDZ increased peanut plant recovery through somatic embryogenesis.

Table 1

The Effects of the Different Combinations of Plant Growth Regulators on Embryogenic Callus Induction

Media							Frequency of explants forming embryogenic callus ¹
Medium No.	2,4-D (mg/l)	NAA (mg/l)	IAA (mg/l)	TDZ (mg/l)	6-BA (mg/l)	KT (mg/l)	
20 AM-234	1.0					1.0	0
AM-6	2.0					0.5	0
AM-5	2.0					3.0	0
AM-231	5.0					0.5	0
AM-230	5.0					3.0	0
25 AM-14	0.5				3.0		0
AM-22	2.0				1.0		0
AM-31		0.5			1.0		0
AM-27		0.5			3.0		0
AM-23		0.5				3.0	0
30 AM-7		2.0				3.0	0
AM-8		2.0				0.5	0
AM-265			0.25	1.0			11.06 $\pm$ 0.94
AM-304			0.25	2.0			16.41 $\pm$ 4.75
AM-308			2.0	1.0			3.51 $\pm$ 3.35

¹ % (mean $\pm$ SD); data correspond to average results of three replicates.

The combination of TDZ (2.0 mg/l) and IAA (0.25 mg/l) promoted somatic embryogenesis of *A. mangium*. However, somatic embryo development stopped at the heart stage (Fig. 1D; Fig. 3H) and secondary somatic embryos formed if heart embryos were kept longer on the same induction medium. Somatic embryos could not develop forward to later stages. Thus, it was necessary to mature the somatic embryos.

EXAMPLE 7

Somatic Embryo Maturation and Germination

Globular embryos (Figs. 1B-C) induced from induction medium containing IAA 0.25 mg/l and TDZ 2.0 mg/l (AM-304) were cultured on $\frac{1}{2}$ MS basal medium supplemented with 0-5.0 mg/l of GA₃, 30-50 g/l of sucrose, Vc 100 mg/l, Asn 150 mg/l, CH 100 mg/l, Gln 150 mg/l, Pro 150 mg/l, phytigel 0.35% (g/l), and activated charcoal 0.1% for thirty days. Somatic embryos were then transferred onto maturation medium AM-425 and cultured for 40 days. Cotyledon somatic embryos (mature somatic embryos) were then transferred onto the medium AM-424 for germination. Each experiment was repeated twice with a total of 250 globular somatic embryos for each medium combination. Seedlings were transferred to $\frac{1}{2}$ MS basal medium containing sucrose 20 g/l as well as phytigel 0.35% and cultured for two months before being transferred to pot soil consisting of peat soil: sand (3:1).

The maturation of the somatic embryos was in agreement with the results from others that maturation was very important in plant regeneration via somatic embryogenesis (Kozłowski and Pallardy, 1997; Huong 1999). Two steps of maturation procedure were used for somatic embryo maturation. Firstly, somatic embryos were treated in medium containing 0.35% of phytigel and 2.5-5.0 mg/l of GA₃ (maturation medium AM-424, AM-426, and AM-467) (Table 2). Secondly, somatic embryos were transferred onto media containing 50 g/l of sucrose and 0.35% of phytigel (maturation medium AM-425) for desiccation. The treatment on medium AM-424 followed by AM-425 promoted 42.33% globular somatic embryos to develop into torpedo and cotyledon embryos (Fig. 2A; Table 2).

Table 2

the Effects of Different Media on Somatic Embryo Maturation

Maturation media				Frequency of globular embryos producing torpedo and cotyledon somatic embryos ¹
Media No.	Basal medium	GA3 (mg/l)	Sucrose (g/l)	
AM-424	½ MS	5.0	30	42.33±13.65
AM-467	½ MS	2.5	30	18.67±6.11
AM-426	½ MS	5.0	50	26.67±8.33
AM-423	½ MS	0	30	0
AM-425	½ MS	0	50	0

¹ % (mean ± SD); data correspond to average results of three replicates.

Further development of globular somatic embryos to torpedo stage and cotyledon stage was not observed on the maturation media without GA₃ (AM-423 and AM-425) (Table 2). These results indicated that GA₃ promoted somatic embryo maturation. Our results were opposite to the reports that the presence of GAs during both the induction and expression phases of geranium (*Pelargonium × hortorum* Bailey) somatic embryogenesis was detrimental to somatic embryo formation on the hypocotyl explants (Hutchinson et al., 1997). In our experiment, somatic embryo maturation was completed on a relatively high sucrose concentration. The similar results were reported in *Populus* somatic embryo embryogenesis that sucrose concentrations between 20-50 g/l stimulated somatic embryo differentiation (Michler and Bauer 1991). Biahoua and Bonneau (1999) reported that a high sucrose concentration of 350 mM (120 g/l) gave the most efficiency of *Euonymus europaeus* somatic embryogenesis and suggested two roles of the high sucrose concentration in somatic embryogenesis including one nutritious factor and the other osmotic potential in culture medium. Maturation of somatic embryogenesis was critical for many plants such as soybean somatic embryogenesis (Ranch et al. 1986) and *Picea abies* (Jalonen and Arnold 1991). Adding 6% of maltose in medium promoted soybean somatic embryo histodifferentiation and maturation (Ranch et al. 1986). For *Picea abies* somatic embryo maturation, ABA treatment was necessary in medium (Jalonen and Arnold 1991). In our experiment, abnormal cotyledon embryos, such as somatic embryos with only one cotyledon (Fig. 2F) or with three cotyledon structures, which could not germinate into seedling, were also observed.

EXAMPLE 8

Histology of the Somatic Embryogenesis

Somatic embryogenesis has been considered a model for the understanding of plant embryo development (Warren, 1993). Two distinct types of somatic embryogenesis have been recognized. One is direct embryogenesis, in which one single cell (or cell group) commences meristematic growth and all the descendants of this cell form part of embryo (Warren, 1993). The other is indirect embryogenesis, in which an embryo develops from one cell in a previously formed meristematic cluster (Warren, 1993). In *A. mangium*, the embryogenesis belongs to indirect somatic embryogenesis. Histological sections showed that somatic embryogenesis developed from one epidermal cell of embryogenic callus (Fig. 3A-D). Periclinal division of the cell produced 2-daughter cells (Figs. 3A-C), one outer daughter cell and one inner daughter cell (Fig. 3C). The continuous cell division of two daughter cells formed proembryonic structure (Fig. 3D) which developed further into globular embryo and heart embryo (Figs. 3E-H). In the light of histological analysis, it is believed that the outer daughter cell developed into epidermal cells of somatic embryo but the inner daughter cell developed into somatic embryo body. Histological sections also showed that sub-epidermal cells next to epidermal cell did not form somatic embryo but their anticlinal division formed a demarcation layer cells to divide somatic embryo and maternal callus (Figs. 3C-G). This structure of the layer cells between proembryonic structure and maternal callus to demarcate somatic embryo and maternal callus was called demarcation cell layer in this paper (Fig. 3D). From proembryonic structure to globular embryo, one suspensor-like structure formed at the base of globular embryo (Fig. 3G). On the induction medium, embryo development stopped at heart embryo stage (Fig. 1D; Fig. 3H). Through maturation, globular embryos developed forward to torpedo embryos (Fig. 2B) and cotyledon embryos (Figs. 2C-D). In conclusion, somatic embryogenesis underwent two-cell stage (Fig. 3C), proembryo stage (Fig. 3D), globular stage (Figs. 3E-G), heart stage (Fig. 1D; Fig. 3H), torpedo stage (Fig. 2B), and cotyledon stage (Figs. 2C-D).

EXAMPLE 9

Somatic Embryo Germination

Normal somatic embryo germination included radicle elongation, hypocotyl elongation and the first pinnate leaf expansion. Only those mature somatic embryos consisting of radicle, hypocotyl, cotyledon and the first pinnate leaf structure could germinate into free-living seedlings (Figs. 4B- C). On the medium AM-424 containing GA₃ 5.0 mg/l, 11% of somatic embryos

germinated to seedlings with root, the first pinnate leaf (Fig. 4A, arrow), and elongated hypocotyl. Somatic embryos with radicle and hypocotyl elongation but without the first pinnate leaf formation could not germinate to seedlings. Somatic embryos with the first pinnate leaf formation but without radicle elongation could be rescued by rooting or micropropagation. Abnormal embryos like those
 5 somatic embryos with big single cotyledon (Fig. 2F) or three cotyledon structures could not germinate into seedlings.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily
 10 occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A method for preparing somatic embryos of *Acacia mangium* comprising:
 - a) culturing an explant on callus induction medium to induce embryogenic callus and globular embryo formation; and
 - b) culturing globular embryos on a maturation medium to form somatic embryos.
2. The method of claim 1, wherein said step (b) comprises:
 - b1) culturing globular embryos on a first maturation medium and
 - b2) culturing the embryos on a second maturation medium.
3. A method for regenerating *Acacia mangium* comprising:
 - a) culturing an explant on callus induction medium to induce embryogenic callus and globular embryo formation;
 - b) culturing the globular embryos on a maturation medium to form somatic embryos;and
 - c) culturing the somatic embryos on a germination medium to produce regenerated seedlings.
4. The method of claim 3, wherein said step (b) comprises:
 - b1) culturing globular embryos on a first maturation medium and
 - b2) culturing the embryos on a second maturation medium.
5. The method of claim 3, wherein the regenerated seedlings are cultured on an acclimatizing medium.
6. The method of claim 4, wherein the regenerated seedlings are cultured on an acclimatizing medium.
7. The method of any one of claims 1 to 6, wherein the explant is an immature embryo.

8. The method of claim 7, wherein the callus induction medium comprises MS basal medium supplemented with a) thidiazuron, b) indole-3-acetic acid, c) casein enzymatic hydrolysate, d) L-ascorbic acid, e) L-glutamine, f) L-asparagine, g) L-proline, h) sucrose and i) phytagel.
9. The method of claim 8, wherein the callus induction medium comprises 1.0 mg/l to 2.0 mg/l thidiazuron and 0.25 mg/l to 2.0 mg/l indole-3-acetic acid.
10. The method of claim 8, wherein the first maturation medium comprises $\frac{1}{2}$ MS basal medium supplemented with a) gibberellic acid, b) casein enzymatic hydrolysate, c) L-ascorbic acid, d) L-glutamine, e) L-asparagine, f) L-proline, g) sucrose, h) activated charcoal and i) phytagel.
11. The method of claim 10, wherein the first maturation medium comprises 2.5 mg/l to 5.0 mg/l gibberellic acid and 30 g/l to 50 g/l sucrose.
12. The method of claim 8, wherein the second maturation medium comprises $\frac{1}{2}$ MS basal medium supplemented with a) casein enzymatic hydrolysate, b) L-ascorbic acid, c) L-glutamine, d) L-asparagine, e) L-proline, f) sucrose, g) activated charcoal and h) phytagel.
13. The method of claim 10, wherein the second maturation medium comprises $\frac{1}{2}$ MS basal medium supplemented with a) casein enzymatic hydrolysate, b) L-ascorbic acid, c) L-glutamine, d) L-asparagine, e) L-proline, f) sucrose, g) activated charcoal and h) phytagel.
14. The method of claim 8, wherein the germination medium comprises $\frac{1}{2}$ MS basal medium supplemented with a) gibberellic acid, b) casein enzymatic hydrolysate, c) L-ascorbic acid, d) L-glutamine, e) L-asparagine, f) L-proline, g) sucrose, h) activated charcoal and i) phytagel.
15. The method of claim 14, wherein the germination medium comprises 2.5 mg/l to 5.0 mg/l gibberellic acid.

16. The method of claim 10, wherein the germination medium comprises $\frac{1}{2}$ MS basal medium supplemented with a) gibberellic acid, b) casein enzymatic hydrolysate, c) L-ascorbic acid, d) L-glutamine, e) L-asparagine, f) L-proline, g) sucrose, h) activated charcoal and i) phytigel.
17. The method of claim 16, wherein the germination medium comprises 2.5 mg/l to 5.0 mg/l gibberellic acid.
18. The method of claim 13, wherein the germination medium comprises $\frac{1}{2}$ MS basal medium supplemented with a) gibberellic acid, b) casein enzymatic hydrolysate, c) L-ascorbic acid, d) L-glutamine, e) L-asparagine, f) L-proline, g) sucrose, h) activated charcoal and i) phytigel.
19. The method of claim 18, wherein the germination medium comprises 2.5 mg/l to 5.0 mg/l gibberellic acid.
20. The method of claim 8, wherein the acclimatizing medium comprises $\frac{1}{2}$ MS basal medium supplemented a) sucrose and b) phytigel.
21. The method of claim 18, wherein the acclimatizing medium comprises $\frac{1}{2}$ MS basal medium supplemented a) sucrose and b) phytigel.