

## 論文 (Original article)

# Polyethylene glycol improves somatic embryo maturation in big-leaf mahogany (*Swietenia macrophylla* King, Meliaceae)

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### Abstract

The effect of polyethylene glycol (PEG) treatment on somatic embryo maturation in *Swietenia macrophylla* was investigated. The addition of PEG to the medium stimulated maturation of somatic embryos and subsequently enhanced germination and conversion efficiency. The best results for cotyledonary somatic embryo production, frequency of germination and plantlet conversion were achieved using medium supplemented with 7.5% PEG. Compared with the control (PEG-free treatment), significant enhancement was recorded in the number of cotyledonary embryos harvested (7 to 56), germinated embryos (25 to 59%) and embryos converted into plantlets (5 to 32%). Regenerated plantlets were successfully acclimatized and transferred to a glass-house.

**Key words** : embryogenesis, mahogany, polyethylene glycol, *Swietenia macrophylla*, tissue culture, tropical tree

### Introduction

Big-leaf mahogany (*Swietenia macrophylla* King) is the most commercially important timber tree in the Neotropics, being the source of the renowned “mahogany wood”, one of the most beautiful woods in the world. Almost all mahogany wood in the commercial market is provided by *S. macrophylla* harvested from natural forests in southern Mexico through Central and South America to Brazil, Peru and Bolivia, with most exports destined for North America and Europe. At present, however, available natural stands of *S. macrophylla* have become increasingly scarce as a result of intense extraction pressure during recent decades. In addition, the establishment of artificial stands has been severely limited due to repeated attacks by the mahogany shoot-borer (*Hypsipyla grandella*, Lepidoptera: Pyralidae), the most harmful insect pest of the Meliaceae family (Maruyama et al., 1989; Maruyama, 2006). The difficulty of ensuring its regeneration in logged-over forests led to its inclusion in Appendix II of the Convention on International Trade in Endangered Species of Wild fauna and Flora (CITES) in 2003.

For the purpose of insect-resistant-tree breeding, the selection and propagation of resistant clones is essential. Somatic embryogenesis technology has great potential for rapid-large-scale micropropagation and for genetic

manipulation. Somatic embryogenesis in *S. macrophylla* was previously reported (Maruyama and Ishii, 1999). However, the reported conversion of somatic embryos into normal plants was very low. In this study, attempts were made to improve plant conversion efficiency from somatic embryos using polyethylene glycol (PEG) treatment during the maturation stage.

### Materials and Methods

#### Embryogenic cultures

Embryogenic cultures of *S. macrophylla* were initiated and maintained using the method described by Maruyama and Ishii (1999) (Fig. 1A). Embryogenic cultures were subcultured into fresh liquid or solid media at intervals of 4-6 weeks. Media was adjusted to pH 5.8 before autoclaving for 15 min at 121°C (1.1 kg cm<sup>-2</sup>). Cultures were kept in a culture room at 25°C under white fluorescent lighting of about 25-35 μmol m<sup>-2</sup> s<sup>-1</sup> photon flux density (400-700 nm) with a regime of 16 h light and 8 h dark.

#### Somatic embryo maturation

Embryogenic cultures about 2 years old were used as plant material in the experiments for somatic embryo maturation. Embryogenic cultures (about 50 mg fresh

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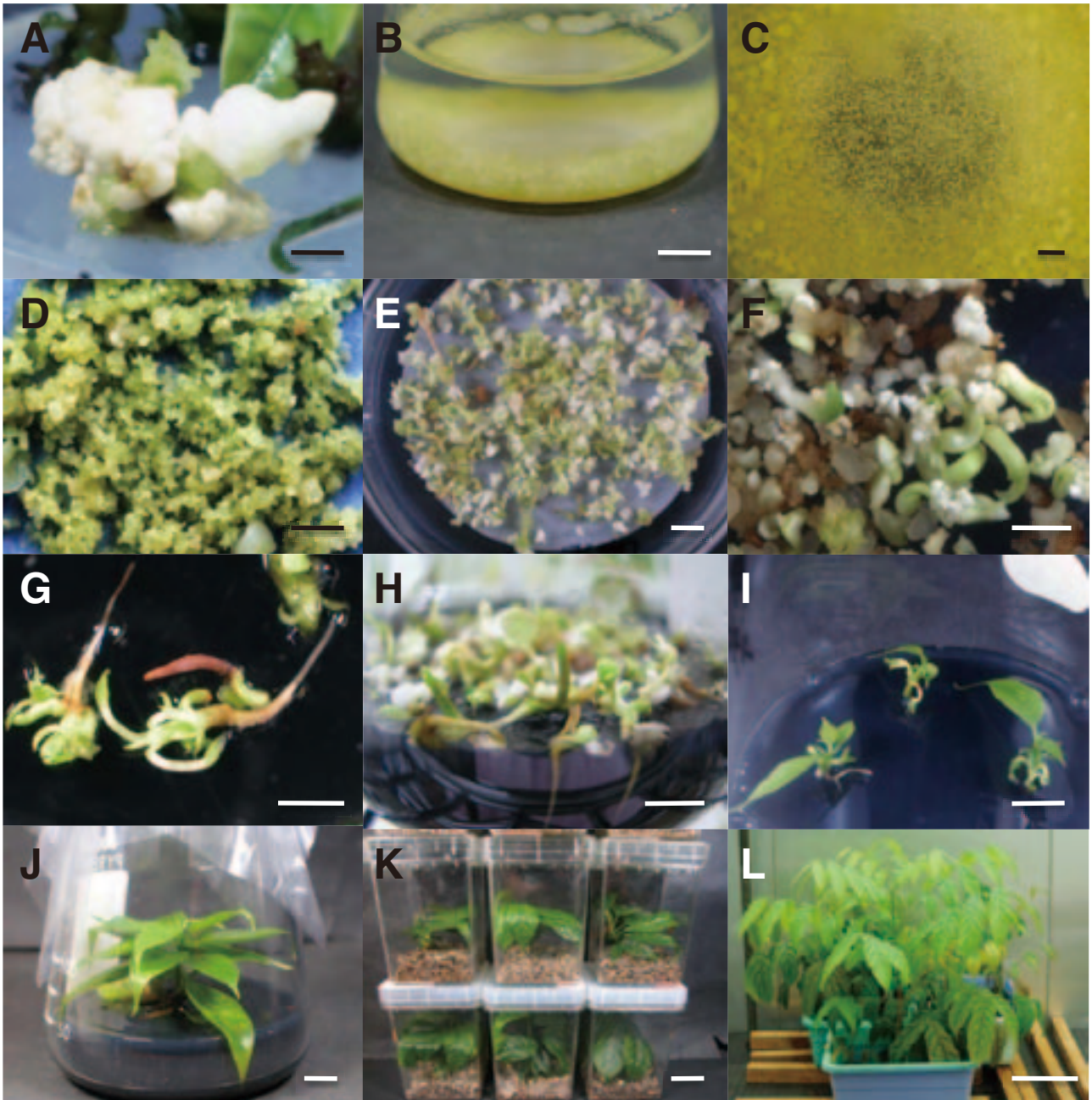


Fig.1. Somatic embryogenesis in *Swietenia macrophylla*: **A** primary embryogenic tissues; **B-C** proliferation of secondary embryogenic tissues in liquid medium; **D-F** maturation of somatic embryos; **G** germination of somatic embryos; **H-K** growth of emblings; **L** acclimatized somatic plants. Bars 1 cm (**A-J**); 3 cm (**K**); 20 cm (**L**).

weight) were subcultured into 100-ml flasks containing 30-40 ml of WP (Woody Plant) (Lloyd and McCown, 1980) liquid media supplemented with different plant growth regulators (PGRs) (Table 1), and cultured at 25°C for 4 weeks in a bio-shaker at 70 rpm under photon flux density of 25-35  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with 16 h photoperiod before transferring to maturation media (Fig. 1B-C). About 150 mg fresh weight of embryogenic cultures was poured onto 70 mm diameter filter paper disks (Advantec No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) over 90 x 20 mm Petri

dishes containing 30-40 ml of WP medium containing glutamine (0.5 g/l), asparagine (0.3 g/l), arginine (0.1 g/l), proline (0.04 g/l), lysine (0.04 g/l), and activated charcoal (AC) (2 g/l), and supplemented with 0-15% polyethylene glycol 4000 (PEG) (Wako Pure Chemical Industries, Osaka, Japan). The media was solidified with 3 g/l gellan gum (Gelrite®; Wako Pure Chemical Industries, Osaka, Japan). Cultures were kept at the same conditions as described above.

Table 1. Effect of plant growth regulator on proliferation of *S. macrophylla* embryogenic cultures

| Plant growth regulator<br>(concentration in $\mu\text{M}$ ) | Fresh weight (mg) after 1 month of culture |           |
|---|--|-----------|
|   | Range                                      | Mean (SE) |
| Growth regulator free                                       | 500-650                                    | 580 (44)a |
| BA (1)  | 350-580                                    | 460 (67)a |
| BA (10)   | 380-690                                    | 520 (90)a |
| 2,4-D (1)   | 550-650                                    | 610 (31)a |
| 2,4-D (10)  | 420-690                                    | 560 (78)a |
| BA (1) + 2,4-D (1)  | 500-610                                    | 550 (32)a |
| BA (1) + 2,4-D (10)   | 440-480                                    | 460 (12)a |
| BA (10) + 2,4-D (1)   | 360-600                                    | 480 (69)a |
| BA (10) + 2,4-D (10)  | 320-480                                    | 410 (46)a |
| KIN (1)   | 350-550                                    | 460 (58)a |
| ZEA (1)   | 300-550                                    | 440 (74)a |
| TDZ (1)   | 310-490                                    | 400 (52)a |
| 4-PU (1)  | 290-540                                    | 410 (72)a |
| CPPU (1)  | 250-500                                    | 380 (72)a |
| 2iP (1)   | 330-510                                    | 420 (52)a |

Embryogenic cultures (about 50 mg fresh weight) were subcultured into 100-ml flasks

SE: standard errors of means from three replications of each treatment

BA: 6-benzylaminopurine

2,4-D: 2,4-dichlorophenoxyacetic acid

KIN: kinetin

ZEA: trans-zeatin

TDZ: thidiazuron

4-PU: N-(4-pyridyl)-N'-phenylurea

CPPU: N-(2-chloro-4-pyridyl)-N'-phenylurea

2iP: 6-( $\gamma,\gamma$ -Dimethylallylamino)purine

Means followed by same letter were no significantly different at a level  $P < 0.05$  according to Tukey's multiple comparison test

### Germination and plantlet conversion

Cotyledonary somatic embryos were collected from maturation media after about 15 weeks of culture (Fig. 1D-F) and transferred to 90 x 20 mm Petri dishes containing 30-40 ml of germination medium. The germination medium containing the basal salts and vitamins of the maturation medium was supplemented with 20 g/l sucrose and 2 g/l AC, and solidified with 10 g/l agar. Germinated somatic embryos (Fig. 1G) were transferred into 300-ml flasks containing 100 ml of the same fresh medium (Fig. 1H-J) or into Magenta® vessels (Sigma, St. Louis, USA) containing Florialite® (Nisshinbo Industries, Inc., Tokyo, Japan) irrigated with 0.1% (v/v) Hyponex 5-10-5 plant-food solution (Hyponex, Osaka, Japan) (Fig. 1K) and were kept under photon flux density of  $65 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Regenerated plantlets were transplanted into pots filled with vermiculite and were acclimatized in plastic boxes with transparent covers (Fig. 1L) as described by Maruyama et al. (1997).

### Statistical analysis

Standard errors of means were calculated from three

replications of each treatment. Data presented in the Tables 1 and 2 were analyzed using ANOVA and the differences between means were determined using Tukey's multiple comparison test at the 5% significance level.

## Results

### Effect of PGRs on proliferation of embryogenic cultures

The best results on proliferation of embryogenic cultures in terms of fresh weight harvested after 1 month of culture were achieved in medium supplemented with  $1 \mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D). However, as shown in Table 1, no significant differences were observed among the PGR treatments.

### Effect of PEG on somatic embryo maturation

The range of cotyledonary somatic embryos harvested per Petri dish varied from 0-141 depending on the maturation media and type of PGR used in the previous proliferation culture (Table 2). When embryogenic cells were cultured on PEG-free medium, an average of only

Table 2. Effect of PEG treatment on maturation of *S. macrophylla* somatic embryos

| Plant growth regulator<br>in proliferation culture<br>(concentration in $\mu\text{M}$ ) | Number of cotyledonary somatic embryos per Petri dish after<br>PEG treatment (SE) |                          |                          |
|---|---|--------------------------|--------------------------|
|   | PEG (0%)  | PEG (7.5%)               | PEG (15%)                |
| Growth regulator free   | 5 (1) <sup>ab</sup>   | 125 (47) <sup>ef</sup>   | 15 (4) <sup>abcd</sup>   |
| BA (1)  | 33 (6) <sup>abcd</sup>  | 141 (56) <sup>f</sup>    | 99 (25) <sup>odef</sup>  |
| BA (10)   | 23 (6) <sup>abcd</sup>  | 126 (45) <sup>ef</sup>   | 69 (23) <sup>abcde</sup> |
| 2,4-D (1)   | 4 (2) <sup>ab</sup>   | 101 (39) <sup>def</sup>  | 2 (1) <sup>a</sup>       |
| 2,4-D (10)  | 0 (0) <sup>a</sup>  | 2 (1) <sup>a</sup>       | 0 (0) <sup>a</sup>       |
| BA (1) + 2,4-D (1)  | 3 (2) <sup>ab</sup>   | 24 (7) <sup>abcd</sup>   | 20 (5) <sup>abcd</sup>   |
| BA (1) + 2,4-D (10)   | 1 (1) <sup>a</sup>  | 7 (3) <sup>ab</sup>      | 3 (1) <sup>ab</sup>      |
| BA (10) + 2,4-D (1)   | 2 (1) <sup>a</sup>  | 15 (4) <sup>abcd</sup>   | 38 (7) <sup>abcde</sup>  |
| BA (10) + 2,4-D (10)  | 1 (1) <sup>a</sup>  | 6 (3) <sup>ab</sup>      | 0 (0) <sup>a</sup>       |
| KIN (1)   | 5 (2) <sup>ab</sup>   | 47 (10) <sup>abcde</sup> | 21 (5) <sup>abcd</sup>   |
| ZEA (1)   | 8 (1) <sup>abc</sup>  | 35 (6) <sup>abcde</sup>  | 22 (8) <sup>abcd</sup>   |
| TDZ (1)   | 0 (0) <sup>a</sup>  | 25 (9) <sup>abcd</sup>   | 5 (2) <sup>ab</sup>      |
| 4-PU (1)  | 6 (3) <sup>ab</sup>   | 62 (14) <sup>bcd</sup>   | 40 (9) <sup>abcde</sup>  |
| CPPU (1)  | 2 (1) <sup>a</sup>  | 27 (6) <sup>abcd</sup>   | 25 (10) <sup>abcd</sup>  |
| 2iP (1)   | 7 (3) <sup>ab</sup>   | 94 (14) <sup>bcd</sup>   | 25 (5) <sup>abcd</sup>   |
| Mean  | 7 (1) <sup>x</sup>  | 56 (9) <sup>y</sup>      | 26 (5) <sup>x</sup>      |

PEG: polyethylene glycol (4000)

SE: standard errors of means from three replications of each treatment

BA: 6-benzylaminopurine

2,4-D: 2,4-dichlorophenoxyacetic acid

KIN: kinetin

ZEA: trans-zeatin

TDZ: thidiazuron

4-PU: N-(4-pyridyl)-N'-phenylurea

CPPU: N-(2-chloro-4-pyridyl)-N'-phenylurea

2iP: 6-(*γ,γ*-Dimethylallylamino)purine

Means followed by same letter were no significantly different at a level  $P < 0.05$  according to Tukey's multiple comparison test

7 cotyledonary somatic embryos per dish was observed. The addition of PEG to the maturation medium resulted in enhanced production of cotyledonary-stage embryos. An average of 56 and 26 cotyledonary somatic embryos per dish was obtained after 7.5% and 15% PEG treatment, respectively. The highest number of cotyledonary embryos (141) per dish was achieved with maturation medium containing 7.5% PEG using embryogenic cells previously cultured in proliferation medium containing 1  $\mu\text{M}$  BA (6-benzylaminopurine).

#### Germination and plantlet conversion

Plantlet conversion of *S. macrophylla* somatic embryos was characterized by first emergence of root and subsequent shoot elongation. The start of germination was observed 1-2 weeks after transfer to germination medium. As shown in Table 3, maturation of somatic embryos on PEG-supplemented media subsequently increased both

the frequency of germination and the number of embryos converted into plantlets. Compared with the control (0% PEG), when PEG (7.5-15%) was included in the maturation media, a significant increase range of 1.6-2.4 and 4.2-6.4 fold was recorded for frequency of germination and plantlet conversion, respectively. Regenerated plantlets were successfully acclimatized and transferred to a glass-house.

#### Discussion

For the large-scale propagation of trees using selected clones or genetically engineered genotypes, one of the most desirable propagation systems targets somatic embryogenesis. Plant regeneration of *S. macrophylla* via somatic embryogenesis was first reported about 10 years ago (Maruyama and Ishii, 1999). In that report, a major limitation of the system used was the low conversion rate of somatic embryos into plants. As in other species, the



Table 3. Effect of PEG treatment on germination and plantlet conversion of *S. macrophylla* somatic embryos

| PEG (%) | Number of embryos tested | Germinated embryos | Frequency of germination (%) | Converted embryos | Frequency of conversion (%) |
|---------|--------------------------|--------------------|------------------------------|-------------------|-----------------------------|
| 0       | 100                      | 25                 | 25                           | 5                 | 5                           |
| 7.5     | 200                      | 118                | 59                           | 64                | 32                          |
| 15      | 150                      | 60                 | 40                           | 31                | 21                          |

PEG: polyethylene glycol (4000)

low plant conversion rate in *S. macrophylla* is due to poor quality of somatic embryos. Although there was frequent germination of somatic embryos derived from both direct and indirect secondary (repetitive) embryogenesis, many of the germinated embryos did not have a viable apical meristem or well-developed epicotyl, and generally developed into leaf-like, bud-like or cotyledonary structures. Consequently, the conversion of somatic embryos into normal plants was very rare. The aim of this study was to improve the plant conversion efficiency by investigating the effect of PGRs and PEG on the maturation of somatic embryos.

According to the results presented in Table 1, no notable differences were observed among the PGRs in terms of effect on cell proliferation after 1 month of culture (harvested fresh weight). However, significant differences were recorded among the PGRs in terms of effect on cotyledonary somatic embryo production after transfer to the maturation media (Table 2). Although the addition of both 2,4-D and BA to the media is a widespread protocol for maintenance and proliferation of embryogenic cells in a number of species (Jain et al., 1995a; 1999; Rout, 2005), BA alone at a concentration of 1-10  $\mu$ M proved to be the best PGR. In contrast, 2,4-D (either alone or in combination with BA) at a concentration of 10  $\mu$ M recorded the worst results among the PGRs tested. Independent of the effect of PGRs, the addition of PEG stimulated cotyledonary embryo production. The number of cotyledonary-stage embryos produced was significantly enhanced after treatment with 7.5% PEG (8-fold more than that produced on PEG-free medium) but the stimulus was halved after treatment with 15% PEG (4-fold). In the absence of PEG, repetitive somatic embryogenesis was evident and the production of normal cotyledonary embryos was very poor. The addition of PEG to the media is a common procedure for the maturation of somatic embryos of conifers (Jain et al., 1995b; Maruyama et al., 2002; 2005a; 2005b; 2005c; 2007; Maruyama and Hosoi, 2007). The beneficial effect of PEG on embryo maturation may be related to its giving

rise to water stress and inducing storage reserve synthesis (Roberts et al., 1990; Attree and Fowke, 1993; Yeung, 1995). In angiosperms, the addition of sucrose, maltose, sorbitol or mannitol as osmoticum in the culture media has been frequently reported to effectively promote somatic embryo maturation in a number of species (Jain et al., 1995a; 1999; Corredoira et al., 2003). In contrast, in a number of other species, no addition of osmotic agents to the media has been reported in the plant regeneration via somatic embryogenesis. Rout (2005) reported somatic embryogenesis and plant regeneration in *Azadirachta indica* (Meliaceae) without the addition of PEG in the maturation media. Viana and Mantell (1999) reported that levels of sorbitol exceeding 0.125 M inhibited growth and stimulated browning of the cotyledonary-stage embryos in *Ocotea catharinensis*. In addition, Okamura et al. (2001) demonstrated that the addition of 7.5% PEG in the culture media was not found effective for improving the maturation of somatic embryos of *Quercus acutissima*.

In this study, even though PEG at both 7.5 and 15% effectively improved the maturation efficiency, PEG at 7.5% in the maturation media proved to be the best concentration for somatic embryo production and subsequent plant conversion. Germination frequency of about 60% and conversion frequency of more than 30% was achieved, which represents a notable improvement when compared to previous reports on *S. macrophylla*. Actually, studies on somatic embryogenesis are currently in progress, including on the response of different plant materials, embryo maturation using several osmotic agents, and post-maturation treatments to improve the efficiency of plant regeneration system for practical purposes.

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## オオバマホガニー不定胚の成熟化における ポリエチレングリコールの影響

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### 要旨

オオバマホガニー (*Swietenia macrophylla*, センダン科) 不定胚の成熟化におけるポリエチレングリコール (PEG) の効果を検討した。PEG を不定胚成熟化培地に添加したことにより、発芽及び個体再生率が向上した。PEG の最適添加濃度は 7.5% であった。成熟化培地に PEG を 7.5% 添加した場合には無添加の場合と比較して 8 倍以上の成熟不定胚が得られた。また、得られた成熟不定胚は発芽伸長用の培地上で発芽成長し、PEG 無添加培地由来の不定胚個体再生率は 5% であったのに対し、7.5% 添加培地由来の場合には 30% 以上の不定胚が幼植物体を形成した。再生植物体は枯死することなく順化され、温室内で正常に生育している。

キーワード：不定胚形成、マホガニー、ポリエチレングリコール、*Swietenia macrophylla*、組織培養、熱帯樹

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