J Wood Sci (2012) 58:64–68
DOI 10.1007/s10086-011-1212-z

NOTE

Plant regeneration by somatic embryogenesis from mature seeds of *Magnolia obovata*

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Received: 24 May 2011 / Accepted: 29 July 2011 / Published online: 21 December 2011

Abstract We attempted to develop a method for the regeneration of plantlets from mature seeds of medically important *Magnolia obovata* via the induction of somatic embryogenesis in vitro. We initially cultured halves of mature seeds on either Murashige and Skoog (MS) medium or B_5 medium that contained 0, 1, 5 or 10 μM gibberellic acid (GA_3) for 1 month and then transferred the half-seeds to half-strength MS basal medium or B_5 basal medium for further culture in the absence of GA_3. Proembryogenic masses (PEMs) were observed 1 month after the transfer of the halved mature seeds to the medium without GA_3. The frequency of formation of PEMs was higher (28%) after initial culture in MS basal medium plus 1 μM GA_3 than in other tested media (0 or 4%). Somatic embryos that had been developed from PEMs were cultured on half-strength MS basal medium or B_5 basal medium for completion of maturation and then transferred to fresh aliquots of the same medium for initiation of germination. The frequency of germination, with the formation of normal primary leaves and roots, was above 80%. We transferred the somatic embryo-derived plantlets to soil for acclimatization and the plantlets continued to thrive.

Keywords Gibberellic acid · *Magnolia obovata* · Mature seed · Proembryogenic masses · Somatic embryo

Introduction

Magnolia obovata is a broad-leaved tree that belongs to the family Magnoliaceae and is native to Japan. The trees themselves are valued by landscape gardeners and the wood is used for furniture and various industrial arts. In addition, the bark is a source of valuable medicinal compounds, such as magnolol and honokiol [1, 2]. Magnolia species can be propagated from seeds and rooted cuttings and by grafting. However, the rate of germination of *M. obovata* seeds is relatively low (approximately 35%) [3]. Therefore, we postulated that a tissue culture technique might be useful for the propagation of *M. obovata*.

There have been several reports of culture in vitro of a few species in the family Magnoliaceae. Merkle and Sommer [4] reported the regeneration of *Liriodendron tulipifera* by somatic embryogenesis from immature embryos, and somatic embryogenesis from immature seeds, with subsequent germination, has been reported for other members of Magnoliaceae, such as *M. virginiana*, *M. fraseri*, *M. acuminata, M. macrophylla* and *M. pyramidata* [5–7]. More recently, Martin et al. [8] established a plant-regeneration system that included somatic embryogenesis for *M. dealbata*.

In contrast, efforts at the regeneration of *M. obovata* using tissue culture techniques have met with limited success. Since explants of *M. obovata* contain many phenolic compounds and the rate of contamination is very

Part of this study was presented at the 59th Annual Meeting of the Japan Wood Research Society, Matsumoto, Japan, March 2009.

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high, their callus formation is frequently inhibited [2]. Nakamura et al. [9] reported that multiple shoots were induced during culture of shoot apices of *M. obovata*, but no roots were produced. Kim et al. [10] reported successful somatic embryogenesis and the regeneration of plants from immature seeds of *M. obovata*. However, to our knowledge, there are no published reports of somatic embryogenesis and plant regeneration from mature seeds of *M. obovata*.

The main aim of the present study was to induce the formation of proembryogenic masses (PEMs) and the regeneration of complete plantlets from mature seeds of *M. obovata*. Mature seeds can be stored for a longer time than immature seeds and, thus, establishment of a method for embryogenesis from mature seeds should have economical benefits. This is the first report, to our knowledge, of the regeneration of *M. obovata* by somatic embryogenesis from mature seeds.

Materials and methods

Plant materials

Mature seeds were collected from an approximately 60-year-old specimen of *M. obovata* on the campus of the Tokyo University of Agriculture and Technology in Fuchu, Tokyo, Japan, on 16 August 2007 (Fig. 1a, b). The surface of seeds was sterilized by washing seeds as follows: immersion in 70% ethanol for 2 min; immersion in a solution of 6% sodium hypochlorite that contained a few drops of TWEEN 20® per 300 mL for 30 min; immersion in sterile distilled water for 10 min (repeated a total of five times); immersion in 0.01 M HCl for 3 min; and immersion in sterile distilled water for 5 min (repeated a total of three times). Hard mature seeds were bisected longitudinally with nippers, and the halves were placed, cut surface downward, on the indicated medium in a 90-mm plastic Petri dish.

Induction of PEMs

The halves of mature seeds were initially cultured on Murashige and Skoog (MS) basal medium [11] or on B_5 basal medium [12] that contained 0, 1, 5 or 10 μM GA_3 (gibberellic acid; Wako, Japan) for 1 month because MS basal medium plus GA_3 was used to induce somatic embryos from root explants of *Carica papaya* [13] and immature seeds of *Quercus robur* [14]. Then, we transferred the mature half-seeds to half-strength MS basal or B_5 basal medium for further culture in the absence of GA_3 to induce PEMs.

All media were supplemented with 30 g/L sucrose (Wako) and solidified with 3 g/L gellan gum (Wako). In addition, MS but not B_5 basal medium was supplemented with *myo*-inositol (100 mg/L). All media were adjusted to pH 5.7 with 1 N KOH and then autoclaved at 121°C for 20 min. GA_3 was added to the media as a filter-sterilized solution. All cultures were incubated at 25°C in darkness.

Induction and germination of somatic embryos

For the induction of somatic embryos that had been developed from PEMs, they were cultured on half-strength MS basal or B_5 basal medium plus 30 g/L sucrose and 3 g/L gellan gum with replacement by fresh medium at monthly intervals at 25°C in darkness.

For germination of somatic embryos, a piece of filter paper was placed on top of the same gellan gum-solidified medium. All cultures were maintained at 16-h photoperiod at 25°C. Plantlets were cultured for 4 months in vitro and then transferred to potting soil (peat moss, vermiculite, perlite; v/v, 1:1:1).

Results and discussion

Induction and maturation of PEMs

We observed PEMs 1 month after the mature half-seeds had been placed on half-strength MS basal medium or B_5 basal medium without GA_3 (Fig. 1c). PEMs were yellowish and compact with evidence of considerable proliferation of cells. The frequencies of induction of PEMs were different among the eight initial media tested, although we did not observe repeated times (Table 1). The frequencies of induction of PEMs were 28, 4 and 4% on initial basal MS medium supplemented with 1, 5 and 10 μM GA_3, respectively. In the absence of GA_3, no PEMs were induced on MS basal medium. On initial B_5 basal medium without GA_3 and on initial B_5 basal medium supplemented with 5 μM GA_3, the frequencies of induction of PEMs were 4 and 8%, respectively. No PEMs were induced on B_5 basal medium supplemented with 1 and 10 μM GA_3. Thus, initial MS basal medium with 1 μM GA_3 appeared to be the most frequent formation of PEMs from mature half-seeds of *M. obovata*.

One month after PEMs had been transferred to half-strength MS basal medium or B_5 basal medium without GA_3, we observed somatic embryos at the globular, heart, and torpedo stages (Fig. 1d–f). One month later, we observed approximately 90 somatic embryos at the torpedo and cotyledon stages (Fig. 1g).

In contrast to our study with mature seeds, Merkle and Watson-Pauley [6] cultured immature seeds of *M. macrophylla* in the yellow-poplar induction medium that had been developed by Merkle and Wiecko [5] and contained

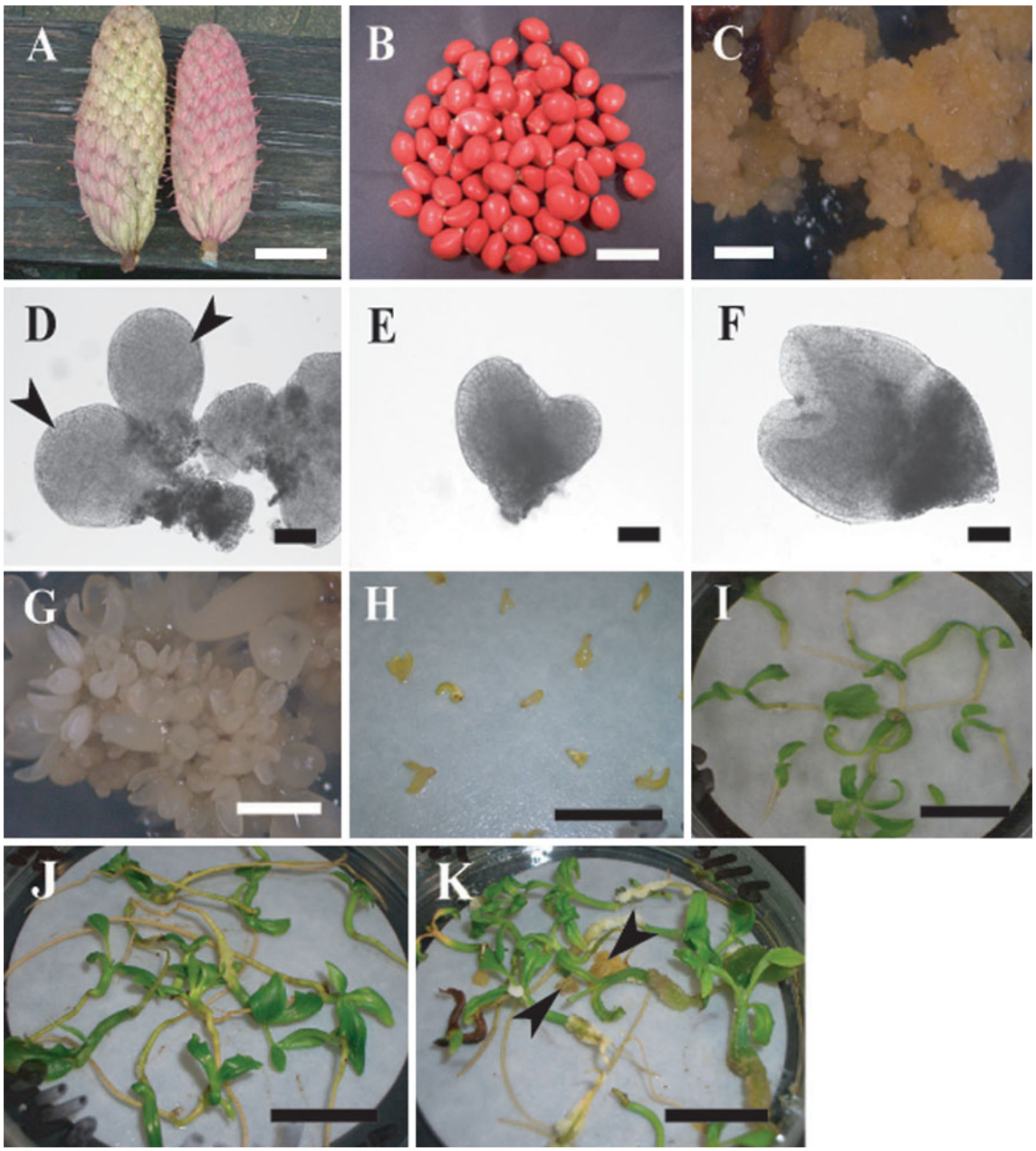

Fig. 1 Regeneration of plants by somatic embryogenesis from mature seeds of *Magnolia obovata*. **a** Fruits collected on 16 August 2007. *Scale bar* 5 cm. **b** Mature seeds from a fruit. *Scale bar* 2 cm. **c** PEMs induced after initial culture on MS basal medium that contained 1 μM GA_3 for 1 month and transfer to half-strength MS basal medium without GA_3 for 1 month. *Scale bar* 2 mm. **d** Globular-stage somatic embryos (*arrowheads*). *Scale bar* 100 μm. **e** Heart-stage somatic embryo. *Scale bar* 100 μm. **f** Torpedo-stage somatic embryo. *Scale bar* 100 μm. **g** Torpedo- and cotyledon-stage somatic embryos generated from PEMs. *Scale bar* 2 mm. **h** Mature somatic embryos derived from the torpedo- and cotyledon-stage embryos shown in **g** after 2 further weeks in culture. *Scale bar* 2 cm. **i** Germination from cotyledon-stage somatic embryos shown in **h** after a further 2 weeks in culture, with cotyledons and roots. *Scale bar* 2 cm. **j** Generation of plantlets from somatic embryos. *Scale bar* 2 cm. **k** Plantlets with secondary embryos at the base of the stem and the end of the root (*arrowheads*). *Scale bar* 2 cm

9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.1 μM benzyl adenine (BA). They observed the induction of both PEMs and somatic embryos. Kim et al. [10] reported that addition of 1.0 mg/L (approximately 4.5 μM) 2,4-D alone or in combination with 0.01 mg/L (approximately 0.5 μM) thidiazuron to MS basal medium induced the formation of embryogenic calli from immature seeds of *M. obovata*. However, we failed to observe the formation of PEMs and of somatic embryos from mature seeds of *M. obovata* when we added the combination of 9.0 μM 2,4-D and 1.1 μM BA to half-strength MS basal medium or B_5 basal medium (data not shown). Therefore, neither

Table 1 Numbers of proembryogenic masses (PEMs) induced under various conditions and the frequencies of germination from mature seeds of *Magnolia obovata*

Initial culture medium[a]	Culture medium[b]	No. of half-seeds cultured	No. of explants producing PEMs[c]	Germination (%)[d]
MS				
No GA_3	Half-strength MS basal medium	25	0 (0)	–
1 μM GA_3		25	7 (28)	82 ± 3
5 μM GA_3		25	1 (4)	90 ± 3
10 μM GA_3		25	1 (4)	85 ± 4
B_5				
No GA_3	Full-strength B_5 basal medium	25	1 (4)	83 ± 1
1 μM GA_3		25	0 (0)	–
5 μM GA_3		25	2 (8)	81 ± 2
10 μM GA_3		25	0 (0)	–

[a] All mature half-seeds were initially cultured on MS basal medium or B_5 basal medium that contained GA_3 (1, 5 and 10 μM) or without GA_3 for 1 month

[b] Culture medium for maturation and germination of somatic embryos that had developed from PEMs

[c] Frequency (%) of induction of PEMs in parenthesis

[d] Values are averages ± standard errors ($n = 5$). Frequencies of germination, with production of normal primary leaves and roots, were determined after 1 month of culture in basal medium

2,4-D nor BA appeared to be necessary for the formation of PEMs and somatic embryos from mature seeds of *M. obovata*.

Germination

We transferred the somatic embryos individually to half-strength MS basal medium or B_5 basal medium without GA_3 and incubated them with 16-h photoperiod for germination. After 2 weeks, the formation to cotyledons was apparent (Fig. 1h). Within 1 month, the embryos formed roots and hypocotyls (Fig. 1i). Cotyledons elongated, reaching close to 2 cm in length. The somatic embryos developed more rapidly and turned green after they were exposed to 16-h photoperiod.

After 1 month in culture, almost all the somatic embryos had germinated, and we counted the numbers of germinated somatic embryos with normal primary leaves and roots (Table 1). The frequencies of normal germination were 82, 90 and 85% for the somatic embryos that had been cultured initially on MS basal medium with 1, 5 and 10 μM GA_3, respectively. The frequencies of germination were 83 and 81% for somatic embryos that had been cultured initially on B_5 basal medium without GA_3 and with 5 μM GA_3, respectively. The remaining plantlets with abnormal cotyledons formed well-developed roots. After 1 month of elongation of cotyledons and hypocotyls, most plantlets were approximately 5 cm long and had developed several pairs of leaves (Fig. 1j). We sometimes observed the formation of secondary embryos at the base of the stem and ends of roots during the germination stage (Fig. 1k). After 3 months, most plantlets had reached approximately 15 cm in height and had developed sizeable leaves (Fig. 2a). We transferred the plantlets to pots of soil for acclimatization and the plants continued to grow (Fig. 2b).

Fig. 2 Plants obtained by somatic embryogenesis from mature seeds of *Magnolia obovata*. **a** Plantlets derived from somatic embryos. *Scale bar* 5 cm. **b** Acclimatized plants 4 months after the transfer of plantlets to potting soil

Abscisic acid (ABA) was used to induce the germination and normal maturation of embryos of *Liriodendron tulipifera* [15], and Kim et al. [10] observed the germination of somatic embryos derived from immature seeds of *M. obovata* on the half-strength MS basal medium plus 1.0 mg/L (approximately 3 μM) GA_3. By contrast, the germination from somatic embryos was observed on yellow-poplar induction medium without growth regulators in the case of several species of *Magnolia* [5–7], such as *M. virginiana*, *M. fraseri*, *M. acuminata*, *M. macrophylla*, and *M. pyramidata*. Martin et al. [8] also observed that the germination rate of somatic embryos of *M. dealbata* in woody plant (WP) basal medium without growth regulators was close to 90%. Similarly, we found that more than 80% of somatic embryos germinated on half-strength MS basal medium or on B_5 basal medium without growth regulators. We found that neither ABA nor GA_3 was needed for the maturation and germination of somatic embryos from mature seeds of *M. obovata*.

Conclusion

We produced plantlets from mature seeds of *M. obovata* by somatic embryogenesis. Our results indicate that the addition of 1 μM GA_3 to initial MS basal medium might promote the induction of PEMs from mature half-seeds of *M. obovata*. More than 80% of somatic embryos germinated normally. Therefore, we obtained approximately 70–80 plantlets with normal primary leaves and roots from one half-seed that had induced PEMs. Approximately 10 months were required for the regeneration, from mature seeds, of plantlets that could be transferred to soil. The culture system for somatic embryogenesis developed in the present study should be useful for the propagation of medically important *M. obovata.*

Acknowledgments This work was supported, in part, by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (nos. 17580137, 19580183, 21380107 and 22.00104).

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