Full Length Research Paper

Plant regeneration of *Michelia champaca* L., through somatic embryogenesis

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Michelia champaca L. is a woody ornamental tree species which has high commercial value to be used as a basic material for perfume, cosmetic, and medicine. The development of an efficient plant regeneration system for *M. champaca* is essential for the production of Champaca planting material and precondition for genetic manipulation. Plant regeneration systems of *M. champaca* through somatic embryogenesis derived from immature seed of *M. champaca* L. which was successfully developed in this study. MS medium supplemented with 2 mgL^{-1}, NAA produced highest percentage of embryogenic callus formation of (43%). The embryogenic cells proliferated and formed somatic embryos (30%) after four to six months of culture in the same medium. Meanwhile, on germination of somatic embryos, hormone free of MS medium resulted in highest percentage of normal plantlets produced (45%) compared with other germination medium which is containing different GA$_3$ concentrations with ranges from 1 - 8% somatic embryos germinated.

Key words: *Michelia champaca*, immature seed, α-naphthaleneacetic acid, 2,4 dichlorophenoxyacetic acid, gibberellic acid, somatic embryogenesis.

INTRODUCTION

Michelia champaca L. known as champaca is belonging to the family of *Magnoliaceae.* It consists of 12 genera and 220 species of evergreen trees and shrubs, native to tropical and subtropical South and Southeast Asia (Indomalaya), including southern China. There are three species of *Michelia* available in Malaysia. They are *Michelia alba* (white chempaka), *M. champaca* (orange chempaka) and *Michelia figo* (dwarf chempaka) with *M. champaca* and *M. alba* being the most popular species within the family (Ibrahim et al., 2005). There is, however, no established variety or cultivar from *M. champaca* has been reported recently. *M. champaca* is a small tree with glossy leaves and yellow or orange flowers. It has

commercial value from almost every parts of the plant especially the flower. The flower has a number of other cosmetic, medicinal and economic uses. Fresh flowers can be taken as natural fragrant and also can be extracted into perfumes and medicinal products such as cure for coughs and rheumatism. Some cosmetic products such as Joy, J'adore and Dior contain *M. champaca* fragrant extracts in their ingredient composition (Warren, 1998). Commonly, *M. champaca* is propagated by means of seed and vegetative pathways via layering. However, seed propagation is time consuming (5 weeks to 4 months to germinate) and generally low percentage in germination and quick lost of viability (Zabala, 1990). On the other hand, vegetative propagation through layering does not produce large number materials for large scale planting within short period of time.

The difficulties of *M. champaca* propagation through conventional technique cause researchers to try to look for the alternative to solve the problems. Meanwhile, the expansion in Champaca industry has led to an increasing

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demand for plant. It is therefore, the *in vitro* system or tissue culture technique which is the suitable approach that can be employed in plant propagation of *M. champaca* (Noraishah et al., 2009; Armiyanti, 2009).

In-vitro propagation either through organogenesis or somatic embryogenesis has become an important and popular method to reproduce crops (Kozai et al., 1997) which are difficult to propagate conventionally (Rugini and Guitierrez-Pesce, 2003). Somatic embryogenesis is the *de novo* production of structures resembling zygotic embryos from somatic cells and to be classified as a somatic embryo. The somatic embryo is a bipolar structure possessing both root and shoot poles and it has a closed vascular system (Merkle, 1999). Somatic embryogenesis has a number of advantages over other micropropagation techniques, which include very high multiplication rates and do not need root induction study (Jain et al., 2000). Through this technique the uniformity and the availability of planting materials could also be assured. This research work focuses on plant regeneration of *M. champaca* L. through somatic embryogenesis from immature seed.

MATERIALS AND METHODS

Explant materials and surface sterilization procedure

Explant materials for this research was immature seed of *M. champaca*. The immature seeds were obtained from the golf field of Agricultural University Park, Universiti Putra Malaysia Serdang, Selangor, Malaysia. The immature seeds were separated from the fruit aggregates and sterilized using 0.2% (w/v) benlate solution for 15 min followed by 70% (v/v) of ethanol for 2 min and finally 20% (v/v) clorox solution for 15 min. The explants were then rinsed with sterile distilled water for three times.

Explants preparation and treatments

After seed surface sterilization process, the immature seeds were isolated from the fruit by removing fruit pericarp. The seeds taken from seed carpel were then cut and end at the corner side of seed by using scalpel blade. The seeds explants were cultured on a basal MS medium (Murashige and Skoog, 1962) that contained 30 gL^{-1} of sucrose and solidified with 3.9 gL^{-1} gelrite agar, and which was supplemented with 2,4-D (2,4-dichlorophenoxy acetic acid) and NAA (α-naphthaleneacetic acid) with their varying concentrations (0, 2, 4, 6, 8 and 10 mgL^{-1}) for somatic embryo induction.

Furthermore, the somatic embryos obtained from immature seed of *M. champaca* explants at the sixth month old of cultured were transferred on solid MS hormone free medium for maturation and to remove the residual effect of plant growth regulators (PGRs) from earlier experiments. Within two weeks on hormone free MS medium, the somatic embryos were then transferred on germination medium containing different concentrations of gibberellic acid (GA_3) 0, 0.5, 1 and 1.5 mgL^{-1} for germination of somatic embryos. The medium was adjusted to pH 5.8 and the cultures were kept at 25 ± 2°C in growth room (incubation room) with a daily fluorescence lighting of 16 h with the light intensity of 13.8 $\mu molm^{-2}s^{-1}$.

Parameters measured

Parameters measured in the somatic embryogenesis stages, were percentage of explants form callus (%), percentage of explants form embryogenic callus (%) and percentage of explants form somatic embryo (%). Data were measured every month until the sixth months of culture. Meanwhile, parameters on somatic embryo germination that are recorded were percentage of somatic embryo germination (%), percentage of normal plantlets (%) and percentage of abnormal plantlets (%). Data were recorded until the 12th week of culture, while growth characteristics were observed daily.

Experimental design and statistical analysis

The experiments were arranged in a completely randomized block design (CRBD). Data were analyzed using the analysis of variance (ANOVA) and Duncan New Multiple Range Test (DNMRT) p ≤ 0.05 for comparison between treatment means. Statistical parameters such as percentage, mean, and standard deviation were estimated by using MS excel program and Duncan's new multiple range test was estimated using the SAS program version 6.12 (SAS institute, Cary NC, USA).

RESULTS AND DISCUSSION

Induction of somatic embryo from immature seed of *M. champaca*

Somatic embryo was successfully induced from immature seeds of *M. champaca* by using different concentrations and auxin types (2,4-D and NAA). All auxin types (2,4-D and NAA) and their concentrations tested successfully induced callus formation (Figure 1). Within period of culture, callus formed faster when the immature seed explants cultured on MS medium containing different 2,4-D concentrations compared to the immature seed explants which were cultured on MS medium containing different NAA concentrations. Based on the observation, the explants started to form callus on the 4th week of culture on all MS medium containing different concentrations of 2,4-D. Meanwhile, on MS medium containing different concentrations of NAA, the explants formed callus on the 6th week of culture. The calli grew and enlarged within two to three months of culture on MS medium containing different concentrations and auxin types (Figure 4b).

Highest percentage of callus formation was observed on 2,4-D treatments rather than NAA treatments. MS medium containing 6 and 8 mgL^{-1} of 2,4-D supported the highest percentage of callus formation (90%) followed by other 2,4-D treatments and 8 and 10 mgL^{-1} NAA treatments. This result is in line with those of Rodriguez and Wetzstein (1994) who reported that 2,4-D treatment produced more callus compared with NAA treatment in their experiment on induction of somatic embryos of pecan *(Carya illinoinensis)*. However, based on the result in this study, although all auxin types tested (2,4-D and NAA) produced callus (Figure 1), yet not all callus produced were embryogenic callus.

Figure 2, depicts the effect of different auxin types (2,4-D and NAA) and their concentrations on the percentage of embryogenic callus formation after six months of
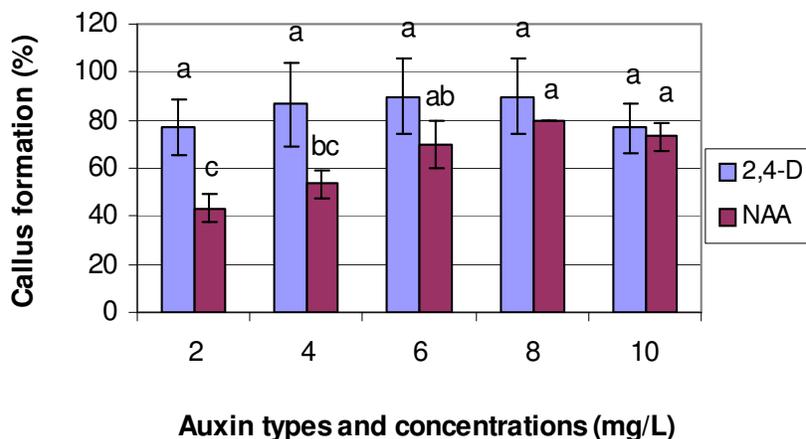
Figure 1. Effect of different auxin types (2,4-D and NAA) and their concentrations on the percentage of callus formation from immature seed of *M. champaca* after six months of culture. Vertical bars represent ± standard deviation (SD). Bars followed by the same letter(s) are not significantly different using DNMRT at p ≤ 0.05.
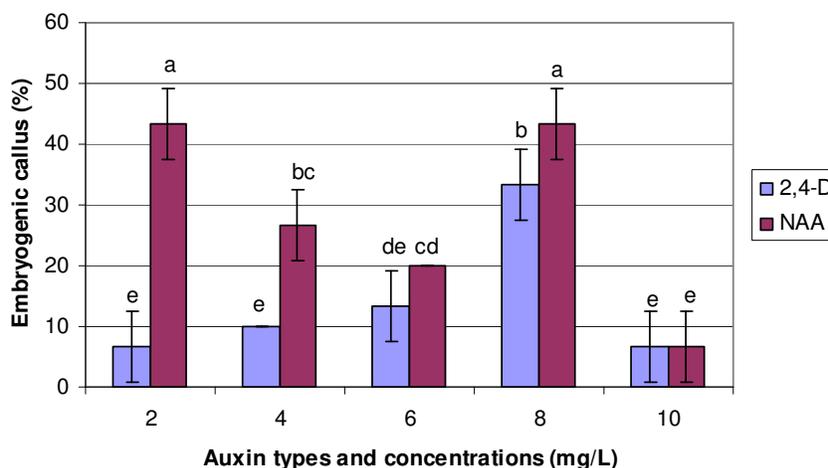

Figure 2. Effect of different auxin types (2,4-D and NAA) and their concentrations on the percentage of embryogenic callus formation from immature seed of *M champaca* after six months of culture. Vertical bars represent ± standard deviation (SD). Bars followed by the same letter(s) are not significantly different using DNMRT at p ≤ 0.05.

culture. The result showed that, the NAA treatments produced more embryogenic callus compared with the 2,4-D treatments. Based on the observation, the calli which are initiated from 2,4-D treatments resulted in translucent form (Figure 4c). Meanwhile, calli which are produced from NAA treatments were yellowish and nodular structures grew at the calli surface, that is, indicating embryogenic callus (Figure 4d). The embryogenic callus with the yellowish characteristics and friable texture was observed on treatment containing 2 and 8 mgL[-1] NAA after the fourth month in culture. These treatments (2 and 8 mgL[-1] NAA) produced the highest

embryogenic callus formation (43%) which is significantly different compared with other treatments. This finding is similar to that of Zhang et al. (2007) who revealed that the induction of somatic embryo on *Pinus bungeana*, and proliferation of embryogenic tissue were poor when it is cultured on 2,4-D only. Nevertheless, the addition of NAA in the media improved both tissue growth and quality. Techato and Rungnoi (2000) reported also that MS medium containing 1 μM NAA combined with 10 μM Kinetin was the best treatment for induction of friable embryogenic callus from leaves of sadao chang (*Azadirachta excelsa*).

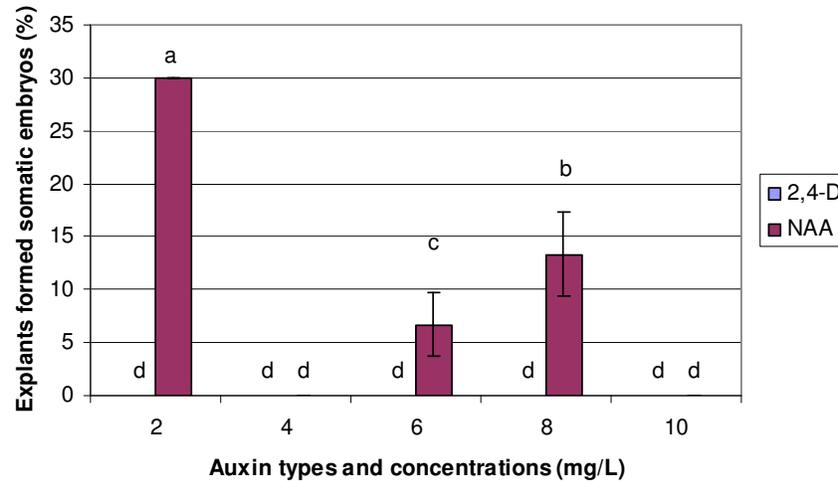The work done by Gray (1996) revealed that auxins

Figure 3. Effect of different auxin types (2,4-D and NAA) and their concentrations on the percentage of explants forming somatic embryos after six months of culture. Vertical bars represent ± standard deviation (SD). Bars followed by the same letter(s) are not significantly different using DNMRT at p ≤ 0.05.

induce cells to become embryogenic and subsequently to promote repetitive cell division of embryogenic cell population and that high concentration of auxin prevented cell differentiation and embryo growth. However, Rodriguez and Wetzstein (1998) stated that callus which was induced with NAA had embryogenic regions composed of homogeneous, isodiametric, and meristematic cell. Furthermore, Stasolla and Yeung (2003) stated that embryogenic tissue can be characterized as a transculent mass of immature somatic embryos often referred to as filamentous embryos or embryonal suspensor masses.

Based on Figure 2, a fluctuate percentage of explants forming embryogenic callus was observed within NAA treatments. A decrement in the percentage of explants forming embryogenic callus occurred when the NAA concentration increased from 2 to 4 and 6 mgL^{-1}. However, the percentage of explants that formed embryogenic callus increased again on treatment containing 8mgL^{-1} and decreased at 10mgL^{-1}. According to Jimenez (2005), fluctuation of embryogenic callus formation from auxins treatment was caused by many reasons. Every explant tissues consist of cells and have distinct capacity to respond on induction treatment to become embryogenic cells. The majority of cells sometimes do not acquire embryogenic capacity. Furthermore, Trewavas (1981) reported that, the sensitivity of the plant cells, tissues and organ to plant hormones is different among them. The sensitivity of explants to hormone is important for the hormone to modulate several processes in plants.

Bell et al. (1993) and Somleva et al. (1995) stated that, involvement of sensitivity during induction of somatic embryo could be evidenced by the fact that only responsive tissues react to the Plant Growth Regulator (PGR) contents in culture media. However, the experimental report of Dudits et al. (1995) showed that, explant

sensitivity to auxin differs between plant species, genotypes or cells in the same explants or in explants with different origin, in their capability to become embryogenic. Further work by Jimenez (2005) also showed that, the developmental process in plant expression of somatic embryogenesis is triggered by different factors, depending on species, cultivar, and physiological conditions of the donor plant. While the use of only 2,4-D (1 mgL^{-1}) led to the induction of embryogenic callus formation from immature seeds of *Magnolia obovata* (Kim et al., 2007), the induction of embryogenic callus had also been effectively achieved through treatment combination of auxin and cytokine (Bhansali et al., 1990). Hence, individual or combined treatment of auxin produced embryogenic callus and somatic embryos (Chalupa, 1990; Wachira and Ogada, 1995; Kim et al., 2003).

Based on the observation, somatic embryogenesis from immature seed of *M. champaca* started to form somatic embryos after four to six months of culture. Somatic embryos at the heart stage were formed almost at the fifth month of culture and followed by torpedo somatic embryo stage at the sixth month of culture (Figures 4e and 4f). Figure 3 depicts the effect of different auxin types (2,4-D and NAA) on the percentage of immature seeds of *M. champaca* formed somatic embryo after six months of culture. Significant differences were observed on the percentage of immature seeds of *M. champaca* forming somatic embryo after six months of culture. MS medium containing 2 mgL^{-1} NAA produced highest percentage of explants formed embyogenic callus formation (30%) and differed significantly from other treatments tested. Meanwhile, all 2,4-D treatments did not produce somatic embryos. The calli produced turned brown and finally died. The browning of culture in this study was caused by phenolic compounds. According to Lai and Lee (1994),
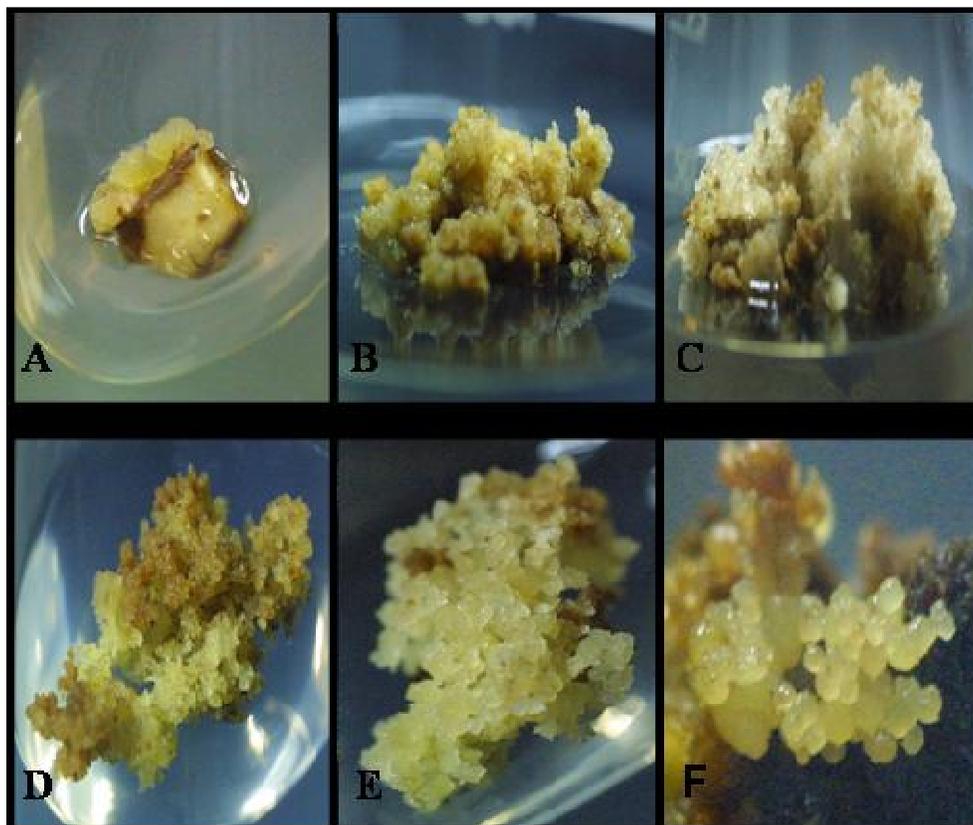
Figure 4. (A) The immature seed started to form callus on the 6th week of cultured on NAA treatment; (B) callus formation from immature seed of champaca on the 3rd month of culture; (C) callus formation produced from 2,4-D treatment; (D) embryogenic callus showing friable texture and yellowish in colour; (E) somatic embryos at the heart stage; and (F) somatic embryos at the torpedo stage.

the 2,4-D concentrations (1 µM) affected faster callus growth and faster browning. Meanwhile, Kim et al. (2007) reported that callus induction from immature seeds of *M. obovata* was very poor and most explants became brown after three days of culture because of the phenolic compounds. However, the developmental process in plant expression of somatic embryo was triggered by different factors, depending on species, cultivar, and physiological conditions of the donor plant (Jimenez, 2005). According to Wetzstein et al. (1990), the types and concentrations of auxin influenced somatic embryo formation and percentage of somatic embryogenesis. Work done by George (1993) also showed that media containing high level of auxin is often used for induction of somatic embryogenesis. However, somatic embryos do not develop further unless the auxin concentration is reduced.

Germination of somatic embryos

Based on the observation, the somatic embryos germinated into plantlets by ten weeks of culture and developed into plantlets completely by the 12th week of culture. Figure (5) shows the developmental stages of somatic embryos germination into plantlet. A significantly high percentage of germination (56%) of somatic embryos was observed on hormone free MS medium (G1). In this treatment, the percentage of somatic embryos germination into normal plantlets was also the highest (45%) and showed significantly the highest percentages for both parameters measured compared with other treatments (G2, G3 and G4). On the percentage of abnormal plantlets produced from the somatic embryos after twelve week of culture, no significant differences were observed among the GA_3 treatments used. Hormone free MS medium was found producing better results compared with MS treatments containing the different concentrations of GA_3 (0.5, 1 and 1.5 mgL^{-1}).

In contrast to Kim et al. (2007), the addition of 1 mgL^{-1} of GA_3 in half strength MS medium solidified with 0.8% agar for germination of somatic embryos of *M. obovata* resulted in 25% production of normal plantlets. Meanwhile, Sanchez et al. (2003) reported that, the addition of 3 mgL^{-1} of GA_3 in germination medium of somatic embryos of oak (*Quercus robur* L.) did not improve the germination
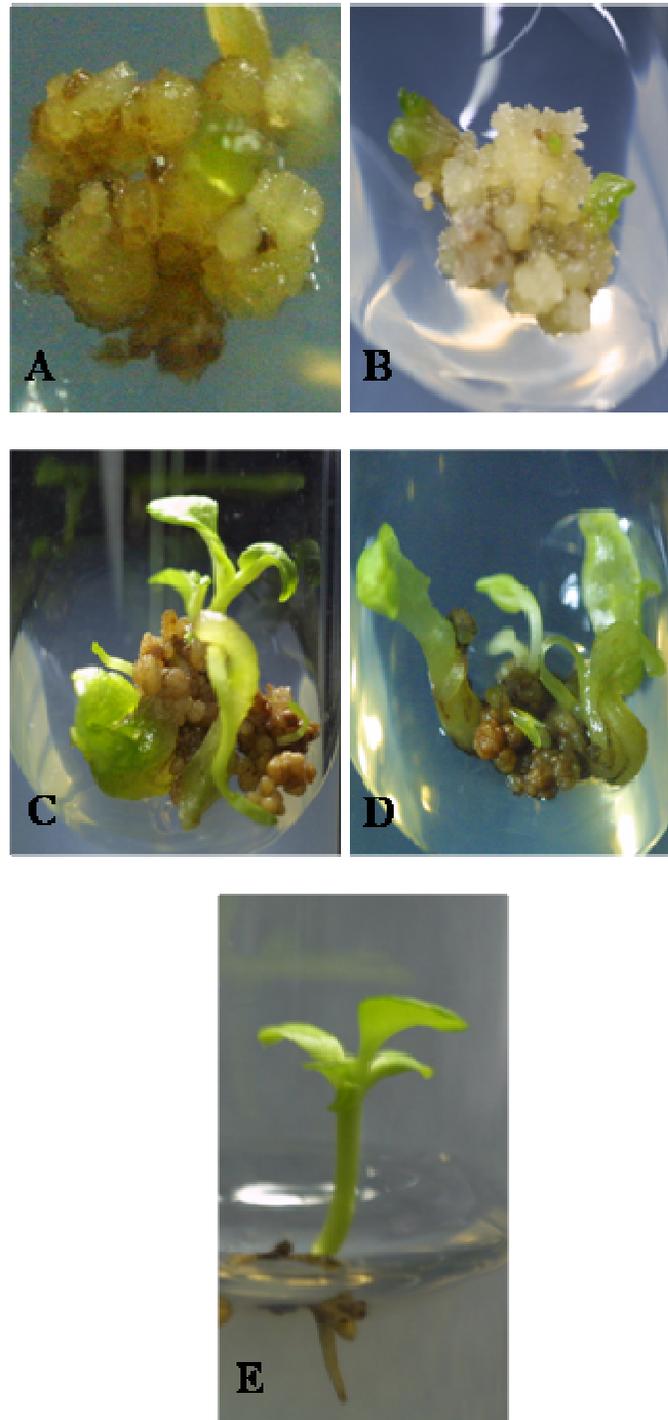
Figure 5. (a) A clump of somatic embryos consisting of 10 to 15 of somatic embryos; (b) somatic embryos started to germinate; (c) somatic embryos developed into plantlets; (d) abnormal plantlets; and (e) normal plantlet.

rates. Whereas, Canhoto et al. (1999) reported that the highest germination rate of somatic embryos of feijoa was obtained on germination medium containing GA$_3$ combined with kinetin.

A study carried out by Stasolla and Yeung (2003) showed that mature somatic embryos of conifer germinated into plantlets in a growth regulator free MS medium with a low sucrose concentration. This finding is

Table 1. Effect of different concentrations of GA3 on the percentage of germination of somatic embryos produced through solid culture medium, the percentage of normal plantlet produced and percentage of abnormal plantlets produced after 12 weeks of culture.

Treatments (mgL^{-1})	Code	Somatic embryos germination (%)	Normal plantlets produced (%)	Abnormal plantlets produced (%)
MSO (control)	G1	56[a]	45[a]	11[a]
0.5 GA$_3$	G2	12[b]	8[b]	4[a]
1 GA$_3$	G3	8[b]	4[b]	4[a]
1.5 GA$_3$	G4	6[b]	1[b]	5[a]

Means followed by the same letter(s) are not significantly different using Duncan New Multiple Range Test (DNMRT) at $p \leq 0.05$.

medium with a low sucrose concentration. This finding is similar to that of Garcia-Martin et al. (2001) who reported that, high sucrose concentration do not improve the germination of somatic embryos of *Quercus suber* L. However, Sanchez et al. (2003) reported that the germination of somatic embryos of oak (*Q. robur* L.) was influenced by the carbon source and concentration in the germination medium. It therefore appears to be, according to Arnold et al. (2002) that, only mature embryos with a normal morphology and which have accumulated enough storage materials and acquired desiccation tolerance at the end of the maturation stage develop into normal plants. Based on the findings of this study, hormone free MS medium could be chosen as the ideal germination medium for somatic embryo of *M. champaca* L.

Conclusion

Plant regeneration systems of *Michelia champaca* through somatic embryogenesis was successfully developed from immature seed. The study found that 43% of embryogenic callus and 30% of somatic embryos were formed when *M. champaca* immature seeds were continuously cultured on the MS medium supplemented with 2mgL^{-1} NAA. Meanwhile, hormone free MS medium resulted in germination of 40% somatic embryos into normal plantlet. The protocol of somatic embryogenesis from immature seeds of *M. champaca* developed in this study could serve as a potential tool with a suitable application in genetic transformation and production of large number and quality of planting materials.

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