HAYATI Journal of Biosciences 24 (2017) 105–108

HOSTED BY

Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Original Research Article

Induction of Somatic Embryogenesis in Sengon (*Falcataria moluccana*) With Thidiazuron and Light Treatments

Ari Sunandar,[1,4] Dorly,[2] Ence Darmo Jaya Supena[2,3*]

[1] *Plant Biology Graduate Program, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16680, West Java, Indonesia.*
[2] *Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor 16680, West Java, Indonesia.*
[3] *Research Center for Bioresources and Biotechnology, Bogor Agricultural University, Bogor 16680, West Java, Indonesia.*
[4] *Biology Education Program, Faculty of Teaching Training and Education, Universitas Muhammadiyah Pontianak, Pontianak 78124, West Kalimantan, Indonesia.*

ARTICLE INFO

Article history:
Received 25 January 2017
Received in revised form
4 July 2017
Accepted 22 August 2017
Available online 12 September 2017

KEYWORDS:
fast growing tree,
secondary somatic embryogenesis,
somatic embryo,
woody legume

ABSTRACT

Falcataria moluccana is important for reforestation and afforestation in Indonesia. However, epidemic of gall rust disease in *F. moluccana* plantations decreases its productivity. Genetic engineering is an alternative solution to against gall rust disease. Somatic embryogenesis is an efficient *in vitro* plant regeneration for successful plant improvement through genetic engineering. The objective of this study was to investigate the effect of thidiazuron and light treatments on the induction of somatic embryogenesis of *F. moluccana*. The effects of thidiazuron concentration (5, 10 or 15 μM) and light (continuous light, 7 days of dark followed by light, or continuous dark) on the induction of somatic embryogenesis in leaf explants were assessed. The highest production of somatic embryos was obtained in 5 μM thidiazuron and dark treatments for 7 days followed by light in Murashige and Skoog medium supplemented with 1.2 g/L proline. Histological analysis in globular and cotyledon stages confirmed that cells had progressed to secondary somatic embryogenesis. This research needs more improvements to become a successful and efficient somatic embryogenesis method and as a potential method for successful plant improvement through genetic engineering in *F. moluccana*.

1. Introduction

Sengon [*Falcataria moluccana* (Miquel) Barneby & Grimes] is a fast-growing tree, which is planted for reforestation and afforestation in Indonesia. The wood of *F. moluccana* is useful for fuelwood and light construction, and is a source of material for making musical instruments and paper pulp (Soerianegara and Lemmens, 1994). However, epidemic of gall rust disease in *F. moluccana* plantations, especially in Java Island makes the productivity of *F. moluccana* decrease (Lestari *et al.* 2013). Genetic engineering is alternative solution to against gall rust disease in *F. moluccana*. An efficient *in vitro* plant regeneration system is needed for successful plant improvement through genetic engineering. Somatic embryos are an alternative target tissue for Agrobacterium-mediated transformation in *Hevea brasiliensis* (Huang *et al.* 2015).

To date, there are only a few studies on somatic embryogenesis of *F. moluccana* compared with other legume plants. Induction of somatic embryogenesis of *F. moluccana* using various types of explants and plant growth regulators obtained embryogenic calli only (Damanik, 1999; Sumiasri *et al.* 2006; Hartati, 2011). In contrast, regeneration of plants through somatic embryogenesis was successful for some other legume plants including the use of thidiazuron alone for *Lens culinaris* (Chhabra *et al.* 2008) and *Cajanus cajan* (Aboshama, 2011), or thidiazuron combined with 6-benzylaminopurine for the regeneration of *Vigna umbellata* (Saini and Chopra, 2012). In addition, the regeneration of *Acacia arabica*, *Acacia catechu*, *Hardwickia binata*, and *Dalbergia sissoo* through somatic embryogenesis was conducted successfully using a combination of proline and several plant growth regulators (Das, 2011). The success of plant regeneration through somatic embryogenesis was reported to be influenced by light. For example, dark conditions for 9 days were required in the early stage of induction of somatic embryogenesis in *Tetrapleura tetraptera* leaves (Opabode *et al.* 2011).

The objective of this study was to investigate the effects of thidiazuron and light treatments on the induction of somatic

* Corresponding author.
E-mail address: encedarmo@ipb.ac.id (E.D.J. Supena).
Peer review under responsibility of Institut Pertanian Bogor.

http://dx.doi.org/10.1016/j.hjb.2017.08.002

embryogenesis of *F. moluccana*. Successful induction of somatic embryogenesis would represent a useful starting point for plant breeding of *F. moluccana* via genetic transformation.

2. Materials and Methods

2.1. Seed germination

Mature dried seeds of *F. moluccana* were washed with a detergent under running tap water for 15 min and then soaked in hot water (100°C) for 3 min. The seeds were soaked in distilled water overnight at room temperature. The seeds were sterilized using 20% (v/v) commercial sodium hypochlorite (NaOCL) and two drops tween-20 for 15 min, and washed three times with sterilized distilled water. The sterilized seeds were incubated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% sucrose and 0.3% agar (gellan gum) and maintained at 25°C ± 2°C with 16-h photoperiod for 4 weeks.

2.2. Induction of somatic embryogenesis

Induction of somatic embryogenesis began with the induction of compact calli. Mature leaflet explants were collected from the 2nd and 3rd leaflets from 2 weeks *in vitro* seedlings and inoculated aseptically on the MS medium with different concentrations of thidiazuron (5, 10, or 15 μM) (Chhabra *et al.* 2008), and different light treatments (continuous light, 7 days of dark followed by light, or continuous dark) at 25°C ± 2°C and 16-h photoperiod for 4 weeks.

Compact calli obtained from thidiazuron and light treatments were transferred to MS medium supplemented with 1.2 g/L proline (Murch *et al.* 1999), using the same thidiazuron concentrations and light treatment conditions as those used for the induction of compact calli. Morphological changes of calli and the number of somatic embryos were noted every 2 weeks for all treatments.

2.3. Histological assessment of *F. moluccana* somatic embryos

Histological assessment of somatic embryos was conducted by fixing the somatic embryos in formaldehyde:acetic acid glacial:ethanol 70% (5:5:90) solution for 24 h. The somatic embryos were dehydrated and cleared using serial solutions of Johansen I–VII (Johansen 1940), then embedded in paraffin wax and sliced using a rotary microtome at 10 μm thickness. Sliced somatic embryos were stained using 2% safranin and 1% aniline blue, and then observed under a light microscope.

2.4. Experimental designs and data analysis

Complete randomized design in factorial arrangement (thidiazuron × light) was used in this study. Each treatment consisted of five bottles, each bottle containing five compact calli. The experiment was repeated four times. All experimental data were subjected to analysis of variance, and significant $p < 0.05$ means were determined with the Duncan multiple range test, to distinguish differences between treatment means at the $\alpha = 0.05$ level using SPSS software, version 16 (SPSS Inc, Chicago, IL).

3. Results

3.1. Effect of thidiazuron and light treatments on somatic embryogenesis

In this study, compact calli were the starting point for the induction of somatic embryogenesis in *F. moluccana*. Compact calli were obtained in all thidiazuron and light treatments (Figure 1A). The treatments caused different color responses in the compact calli. Compact calli turned green under continuous light and 7 days of dark followed by light treatment, but yellowish brown under continuous dark conditions. Embryogenic calli were obtained on MS medium supplemented with 1.2 g/L proline in the presence of 5 μM thidiazuron and 7 days of dark followed by light treatments. Embryogenic cells developed into globular, heart, torpedo, and cotyledon stages (Figure 1).

Thidiazuron and light treatments had significant impact on the induction somatic embryogenesis. However, there was no evidence suggesting an interaction between thidiazuron and light treatments (Table 1). The highest induction frequency for somatic embryogenesis was obtained with 5 μM thidiazuron on MS medium supplemented with 1.2 g/L proline over 7 days of dark followed by light treatment (Table 1; Figure 1). No significant effect on the induction of somatic embryogenesis was obtained with the other thidiazuron concentrations (10 and 15 μM) under either continuous light or continuous dark treatments.

3.2. Influence of light treatment on the timeline development of somatic embryogenesis

Thidiazuron and light treatments not only had an effect on the number of somatic embryos, but also influenced the timeline development of somatic embryogenesis induction. In 7 days of dark followed by light treatment with 5 μM thidiazuron and 1.2 g/L proline, the globular, heart, and cotyledon stages were formed at 2, 4, and 7 weeks post-treatment, respectively. In continuous light treatment with 5 μM thidiazuron and 1.2 g/L proline, the globular and heart stages were formed at 2 and 13 weeks post-treatments, respectively, but failed to reach further stages of development. In continuous dark treatment with 5 μM thidiazuron and 1.2 g/L proline, the globular and heart stages were formed at 2 and 17 weeks post-treatment, respectively, but failed to reach further stages of development.

3.3. Histology of somatic embryo

Somatic embryos of *F. moluccana* were failed to germinate. The longitudinal section at the globular and cotyledon stages showed the development of secondary embryogenesis. Asymmetric division was found in the epidermal tissue at the globular stages, whereas embryogenic globular structures were found at the cotyledon stage (Figure 2). Both globular and cotyledon stages that progressed to secondary embryogenesis showed no further developmental growth.

4. Discussion

In this study, somatic embryogenesis of *F. moluccana* was induced. Thidiazuron and light treatments had effect on the induction somatic embryogenesis, but there was no evidence to suggest an interaction between thidiazuron and light treatments, implying each has different roles in the induction of somatic embryogenesis. Thidiazuron at 5 μM is the optimal concentration to induce somatic embryogenesis of *F. moluccana* than 10 and 15 μM. In other study, thidiazuron at 5 μM is the optimal concentration to induce somatic embryogenesis of *L. culinaris* (Chhabra *et al.* 2008). It was reported that thidiazuron induced the endogenous plant growth regulators, in particular auxin and cytokinin (Murthy *et al.* 1995). Cytokinin increases cell division and differentiation (Aboshama 2011), whereas auxin contributes to cell specification, dedifferentiation, and differentiation (Prasad and Dhonukshe 2013). A previous study showed that light with 2,4-Dichlorophenoxyacetic acid was a signal for G protein, nucleoside-diphosphate kinase, and arrestin protein for the induction of cell proliferation and differentiation in somatic embryogenesis in *Triticum aestivum* (Nato *et al.* 2000). In this study, thidiazuron was effective at relatively high concentrations as reported previously in *Cinnamomum pauciflorum*, which need 2.5 μM thidiazuron (Kong *et al.* 2009).

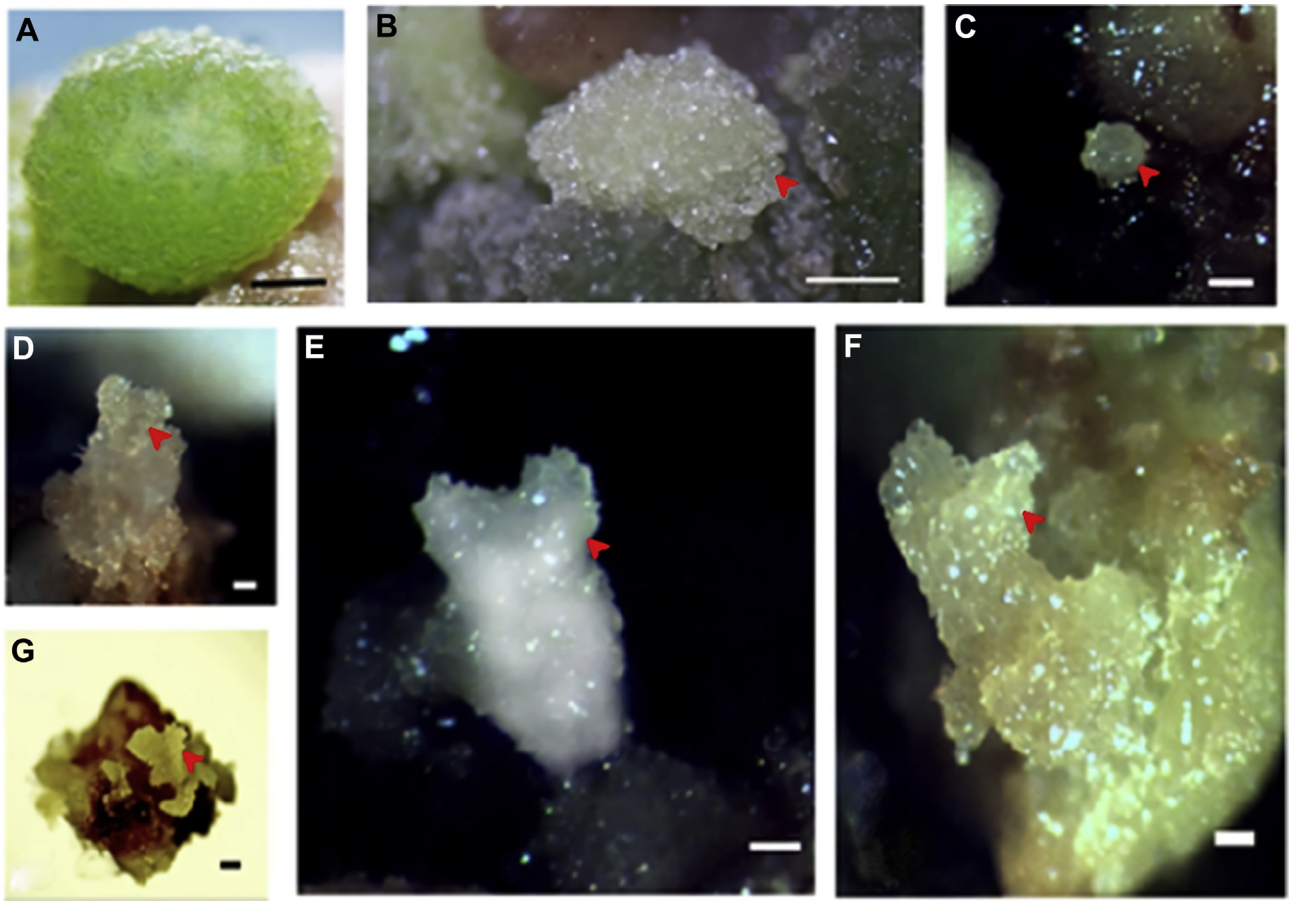

Figure 1. Induction of somatic embryogenesis of *F. moluccana*. (A) Compact callus on MS medium supplemented with 5 μM thidiazuron in 7 days of dark conditions followed by continuous light treatment at 4 weeks post-treatment, (B) embryogenic callus (red arrow head), (C) globular stage (red arrow head), (D) heart stage (red arrow head), (E) torpedo stage (red arrow head), (F) early cotyledon stage (red arrow head), and (G) late cotyledon stage (red arrow head). (B–G) On MS medium supplemented with 1.2 g/L proline and 5 μM thidiazuron in 7 days of dark conditions followed by continuous light treatment at 1, 2, 4, 6, 7, and 8 weeks post-treatment, respectively. (A–G) Bar 1 mm. MS = Murashige and Skoog.

Table 1. Effects of thidiazuron and light treatments on the mean of somatic embryos in 17 weeks post-treatment

Light treatments				
Thidiazuron, μM	Continuous light	7 d of dark conditions followed by continuous light treatment	Continuous dark	Means*
5	1.2	2.5	0.8	1.5[a]
10	0.5	1.2	0	0.6[b]
15	0.3	0.8	0	0.3[b]
Means*	0.6[b]	1.5[a]	0.3[b]	–

* Each value represents mean in globular stages (minimum). Means followed by different letter are significant according to Duncan multiple range test ($p = 0.05$).

Furthermore, in our study, light treatments had significant influence on the induction of *F. moluccana* somatic embryogenesis, and we found that 7 days of dark treatment followed by light to be the optimal treatment condition for the induction of somatic embryogenesis. Dark treatment early in the induction of somatic embryogenesis provides a signaling cue for cells to decide on growth direction, whether to increase the endogenous hormone level, or increase the sensitivity to plant growth hormones (Zobayet and Saxena 2003). Dark treatment was reported previously to decrease tissue browning (George 1993) that was caused by inactivation of an oxidation enzyme in plant tissue (Zobayet and Saxena 2003). In the induction of somatic embryogenesis of geranium, it was found that continuous light treatment decreased the level of endogenous plant growth regulators and then inhibited somatic embryogenesis (Hutchinson *et al.* 2000). In addition, in cauliflower, continuous dark treatment could increase ethylene synthesis (Sasaki 2002). A previous study showed that increased ethylene synthesis inhibited somatic embryogenesis in *D. sissoo* (Sahu *et al.* 2014).

In this study, somatic embryos were failed to germinate, and longitudinal section showed asymmetric division at globular stage and embryogenic globular structures at cotyledon stage. High concentrations of endogenous auxin induced asymmetric division at the epidermal globular stage as well as an embryogenic globular structure, which is in agreement with a previous report of asymmetric division and embryogenic globular structure formation in *Alyssum borzaeanum* (Păunescu 2008). High auxin concentration sustained over a long period could also inhibit auxin polar transport (Goldsmith 1982), thus inducing abnormal somatic embryos in Scots pine (Abrahamsson *et al.* 2012). Auxin transport is required for the developmental transition from the globular to heart stage in *Picea abies* (Ramarosandratana and van Staden 2004), but the presence of auxin in the late globular stage inhibits differentiation, causing the tissue return to a less differentiated state (Terzi and Lochiavo 1990).

In addition, it should be noted that in this study, induction of somatic embryogenesis of *F. moluccana* requires a higher proline concentration (1.2 g/L) than *D. sissoo*, *A. catechu*, *Acacia arabica*, and *H. binata*, which need 0.6 g/L proline (Das 2011). The roles of proline in somatic embryogenesis include that as a nitrogen storage pool,

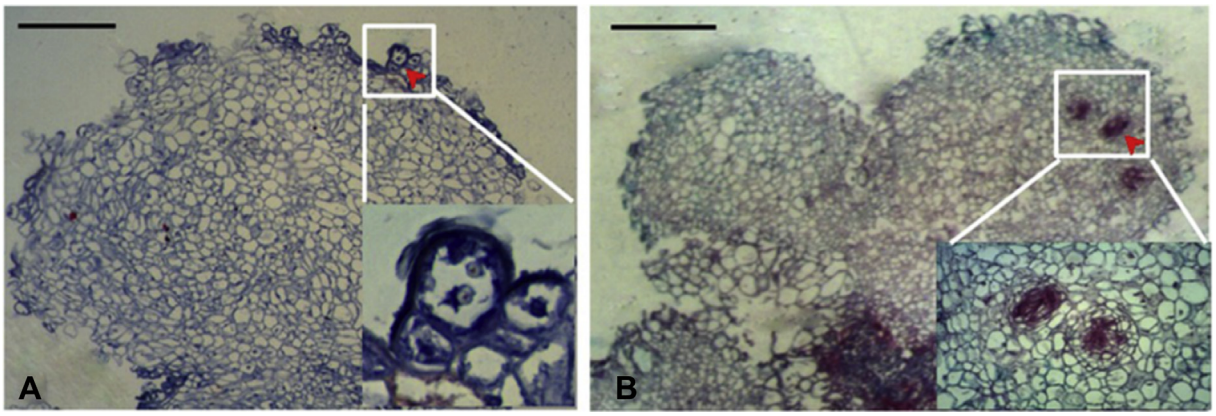

Figure 2. Longitudinal section of somatic embryo in 5 μM thidiazuron treatment in combination with 7 days of dark conditions followed by continuous light treatment on MS medium supplemented with 1.2 g/L proline. (A) Asymmetric division at 2 weeks post-treatment globular stage (red arrow head), (B) embryogenic globular structures at 6 weeks post-treatment cotyledon stage (red arrow head). Bar 100 μm.

an osmoticum, and a source of $NADP^+$ for embryonic development (Ghanti *et al.* 2009).

This study shows the benefit of thidiazuron and light treatments in the induction of somatic embryogenesis of *F. moluccana*. Thidiazuron at 5 μM concentration and 7 days of dark followed by light treatment were the optimal treatment conditions for the induction of somatic embryogenesis of *F. moluccana* on MS medium supplemented with 1.2 g/L proline. This research needs more improvements to become a successful and efficient somatic embryogenesis method and as a potential method for successful plant improvement through genetic engineering in *F. moluccana*.

Conflict of interest

There is no conflict of interest.

Acknowledgements

The authors gratefully appreciate the postgraduate scholarship program from the Directorate of Higher Education (DIKTI) of Ministry of Education and Cultures and they also grateful to Nia Dahniar for assisting them during the research.

References

Aboshama HMS. 2011. Somatic embryogenesis proliferation, maturation and germination in *Cajanus cajan*. *World J Agric Sci* 7:86–95.

Abrahamsson M, Valladares S, Larsson E, Clapham D, Von Arnold S. 2012. Patterning during somatic embryogenesis in Scots pine in relation to polar auxin transport and programmed cell death. *Plant Cell Tissue Organ Cult* 109:391–400.

Chhabra G, Chaudhary D, Varma M, Sainger M, Jaiwal PK. 2008. TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). *Physiol Mol Biol Plants* 14:347–53.

Damanik RI. 1999. Development of Regeneration Method in Somatic Embryogenesis and In Vitro Shoot Proliferation in Sengon (*Paraserianthes falcataria* (L) Nielsen) [Dissertation]. Bogor: Bogor Agricultural University, Bogor.

Das P. 2011. Somatic embryogenesis in four tree legumes. *Biotechnol Res Int* 2011: 1–8.

George EF. 1993. Plant Propagation by Tissue Culture Part 1. England: Exegetics Ltd, England.

Ghanti SK, Sujata KG, Rao S, Udayakumar M, Kishor PBK. 2009. Role of enzymes and identification of stage specific proteins in developing somatic embryos of chickpea (*Cicer arietinum* L.). *In Vitro Cell Dev Biol Plant* 45:667–72.

Goldsmith MEM. 1982. A saturable site responsible for polar transport of indole-3-acetic acid in sections of maize coleoptiles. *Planta* 155:68–75.

Hartati NS. 2011. Modification of Lignin Content of Wood of Sengon (*Paraserianthes falcataria* (L.) Nielsen) by Engineered Gene of 4-Coumarate CoA Ligase (4CL) [Ph.D thesis]. Bogor: Bogor Agricultural University, Bogor.

Huang TD, Li J, Li YT, Huang HS, Hua YW. 2015. Somatic embryo, an alternative target tissue for Agrobacterium-mediated transformation in *Hevea brasiliensis*. *J Rubb Res* 18:171–88.

Hutchinson MJ, Senaratna T, Sahi SV, Saxena PK. 2000. Light mediates endogenous plant growth substances in thidiazuron-induced somatic embryogenesis in geranium hypocotyl cultures. *J Plant Biochem Biotech* 9:1–6.

Johansen DA. 1940. Plant Microtecniques. London: Mc-Graw Hill Book Co. Inc, London.

Kong L, Dai D, Shang M, Li K, Zhang CX. 2009. Thidiazuron-induced somatic embryos, their multiplication, maturation, and conversion in *Cinnamomum pauciflorum* Nees (Lauraceae). *New Forest* 38:131–42.

Lestari P, Rahayu S, Widiyatno. 2013. Dynamic of gall rust disease on Sengon (*Falcataria moluccana*) in various agroforestry patterns. In: *Procedia Environmental Sciences of the 3rd International Conference on Sustainable Future for Human Security, Japan*, Vol 17. Amsterdam: Elsevier, Amsterdam. pp. 1016.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–97.

Murch SJ, Victor JMR, Krishnaraj S, Saxena PK. 1999. The role of proline in thidiazuron-induced somatic embryogenesis of peanut. *In Vitro Cell Dev Biol Plant* 35:102–5.

Murthy BNS, Murch SJ, Saxena PK. 1995. Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): endogenous growth regulator levels and significance of cotyledons. *Physiol Plant* 94:268–76.

Nato A, Fresneau C, Moursalimova N, De Buyser J, Lavergne A, Henry Y. 2000. Expression of auxin and light-regulated arrestin-like proteins, G proteins and nucleoside diphosphate kinase during induction and development of wheat somatic embryos. *Plant Physiol Biochem* 38:483–90.

Opabode JT, Akinyemiju OA, Ayeni OO. 2011. Plant regeneration via somatic embryogenesis from immature leaves in *Tetrapleura tetraptera* (schum. & thonn.) Taub. *Arch Biol Sci* 63:1135–45.

Păunescu A. 2008. Histological investigation of the secondary somatic embryogenesis of *Alyssum borzaeanum (Brassicaceae)*. *Phytol Balcan* 14:111–7.

Prasad K, Dhonukshe P. 2013. Polar auxin transport: cell polarity to patterning. In: Chen R, Baluška F (Eds.). *Polar Auxin Transport, Signaling and Communication in Plants*. Berlin: Springer, Berlin. pp. 25–44.

Ramarosandratana AV, van Staden J. 2004. Effects of auxins and 2,3,5–triiodobenzoic acid on somatic embryo initiation from Norway spruce zygotic embryos (*Picea abies*). *Plant Cell Tissue Organ Cult* 79:105–7.

Sahu J, Khan S, Sahu RK, Roy A. 2014. Micropropagation of *Dalbergia sissoo* Roxb. through tissue culture technique. *Pak J Biol Sci* 17:597–600.

Saini R, Chopra AR. 2012. *In vitro* plant regeneration via somatic embryogenesis in rice-bean *Vigna umbellata* (Thunb.) Ohwi and Ohashi: an underutilized and recalcitrant grain legume. *J Environ Res Develop* 6:452–7.

Sasaki H. 2002. Brassinolide promotes an adventitious shoot regeneration from cauliflower hypocotyl segment. *Plant Cell Tissue Organ Cult* 71:111–6.

Soerianegara I, Lemmens RHMJ. 1994. Timber Trees: Major Commercial Timbers. Bogor (ID): Plant Resources of South-East Asia, Bogor (ID).

Sumiasri N, Priadi D, Yokota S, Yoshizawa N. 2006. Tissue culture of fast growing tropical trees in Indonesia: Mangium (*Acacia mangium* Wild) and Sengon (*Paraserianthes falcataria* (L) Nielsen). In: Imamura Y, Umezawa T, Hata T (Eds.). *Sustainable Development and Utilization of Tropical Forest Resources*. Kyoto: Research Institute for Sustainable Humanosphere Kyoto University, Kyoto. pp. 123–30.

Terzi M, Loschiavo F. 1990. Somatic embryogenesis. In: Bhojwani SS (Ed.). *Plant Tissue Culture: Applications and Limitations*. Amsterdam: Elsevier, Amsterdam. pp. 54–66.

Zobayet SMA, Saxena PK. 2003. *In vitro* regeneration of *Echinacea purpurea* L.: enhancement of somatic embryogenesis by indolebutyric acid and dark preincubation. *In Vitro Cell Dev Biol Plant* 39:605–12.