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Induction of indirect somatic embryogenesis in Borneo ironwood

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Induction of indirect somatic embryogenesis in *Eusideroxylon zwageri*, an endangered and hardest timber tree species in Borneo tropical rainforest, was initiated from immature leaves. Embryogenic callus cultures were induced on Murashige and Skoog medium (MS) containing 3% sucrose, 0.24% Gelrite, and various concentrations and combinations of BAP, NAA and GA3 after 4 weeks of culture in darkness. A higher response (76%) of embryogenic callus was induced on MS medium with 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} GA3. Higher numbers of globular- (31), heart- (30), torpedo-(28), and cotyledon-stage (25) embryos per explant were obtained by culturing embryogenic callus on MS with 3% sucrose, 0.24% Gelrite without plant growth regulators after 8 weeks culture in darkness. These results on the induction of indirect somatic embryo in *E. zwageri* could be used for mass propagation and to select useful traits of this tree species at the cellular level. However, further work needs to be done on the conversion of the regenerated embryos.

Highlighted Conclusion
Murashige-Skoog medium is effective for the induction of indirect somatic embryogenesis in *Eusideroxylon zwageri* by using leaf as the main source of the explant.

Borneo is estimated to comprise of about 15,000 plant species and may well have the highest plant diversity of any region on Earth (WWF 2016). Borneo's tropical rainforests and climate offer the ultimate conditions for an estimate of 3,000 species of trees, more than 1,700 species of orchids and more than 50 carnivorous pitcher plant species to thrive (WWF 2016). *Eusideroxylon zwageri* Teijsm. & Binn., commonly known as Borneo Ironwood, is one of that 3,000 tree species of the tropical rainforest which can be found in Borneo Island and economically very crucial for a source of hardwood timber. This species is also known as Belian in Malaysia, Ulin in Indonesia, Borneo Ironwood in European Union and Biliran in The Philippines. This hardwood timber tree species with specific gravity of around 0.88-1.19 g cm^{-3} (Irawan 2004) is classified into timber of Class Of Strength I and timber of Durabilty of Class I. Borneo Ironwood have very dense, termite resistance silica and contain heartwood extractives known as Eusiderin which makes this species durable (Irawan and Gruber 2003) and therefore can survive of 40 years of rotting process and in the dry condition it can survive up to a century (Martawijaya et al. 1989). The wood of *E. zwageri* can subsist under hazardous condition either in ground contact or submerged in the water without losing its strength due to its anatomical features and its contents of extractives (Wong and Singh 1995). Besides, the heartwood of Belian species is very resistant to preservative treatment and therefore used in furniture industries (Irawan 2004).

Eusideroxylon zwageri is traditionally propagated through sexual reproduction by seed (Martawijaya et al. 1989). The recalcitrant characteristic of the seed makes it difficult to break the seed dormancy thus becomes the main limitation for the conventional propagation of this species (Irawan and Gruber 2003). Apart from that, the germination takes around nine to twelve months in its natural habitat and it requires almost 200 years or more for this species to reach it mature size (Hidayat 2007). Another alternative of propagation through cutting can be also used for the regeneration of Borneo Ironwood but the rooting rate of cutting is still very low (Irawan 2004).

In Sabah, Ironwood is categorized as a species that almost extinct (UNEP-WCMC 2007) whereas according to World Conservation Union and the IUCN Red List of threatened species (IUCN 2010), Borneo Ironwood is classified as a "Vulnerable species" as per the criteria A1cd+2cd. According to IUCN (2015), any of a species

under “Vulnerable” category is not critically endangered or endangered but this species may face a high threat of extinction in the wild in the medium-term future. In Indonesia, mainly on the flat lowlands of southern Sumatra, for example, the vast stands of ironwood a species of great commercial importance producing an exceptionally durable timber, have been almost entirely destroyed (WWF 2016).

Due the reason above, it is obligatory to regenerate and preserve this species through tissue culture technique. A tissue culture technique such as using indirect somatic embryogenesis has been proved important for cloning superior trees with similar adeptness that can be applied to other organisms (Mark and Dean 2000). A previous preliminary study on the induction of somatic embryogenesis of *E. zwageri* by using half strength of MS medium was successfully developed but however the maturation of the somatic embryos were still very low (Gibson and Rebicca 2016). Both direct and indirect somatic embryogenesis protocol in Lauraceae species has been successfully developed such as in *Persea americana*, *Ocotea catharinensis*, *Cinnamomum camphora* and *Cinnamomum verum* (Chen and Chang 2009). In this study, the somatic embryos of *E. zwageri* were successfully induced by using young leaf as the main source of explants. These young leaves were cultured into Murashige and Skoog (MS) medium augmented with various concentrations and combinations of different 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and Gibberilic acid GA3 under an aseptic condition.

MATERIAL AND METHODS

Plant material. In this study, the young leaf explants were collected from two to three years old of *E. zwageri* seedlings originally obtained from the forest and maintained in the pot culture outside Plant Tissue Culture Laboratory of Universiti Malaysia Sarawak (UNIMAS), Malaysia.

Explant preparations and culture conditions. The leaves explants were placed under running tap water for about one hour before soaked with 0.1% Benomyl for 30 min. The young leaf explants were surface sterilized using 15% Sodium Hypochlorite solution with 3 drops of Tween 20 for 5 minutes. After sterilization, these young leaves were thoroughly washed three times with sterile distilled water. The leaf explants were cut into 0.5 to 1.0 cm and were used as the initial explants source to induce somatic embryos.

Induction of somatic embryogenesis. Explants were placed in Petri dishes (100 X 20 mm) containing 25 mL of modified Murashige and Skoog (Murashige and Skoog 1962) medium (MS; M499, Phyto Technology Laboratories, Shawnee Mission, KS) supplemented with 3% (w/v) sucrose, 0.24% (w/v) Gelrite™ (P-8169, Sigma-Aldrich, St. Louis, MO), BAP (1.0 mg L^{-1}), NAA (0.5 mg L^{-1}), GA3 (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) and the dishes sealed with Parafilm® M (Pechiney Plastic Packaging, Menasha, WI). These cultures were maintained in the darkness at 25±2 °C for 4 weeks, and additional control cultures were maintained under a 16-h light photoperiod (50 µmol m^{-2} s^{-1}, cool white fluorescent tubes) at 25±2 °C. All the cultures were transferred to fresh MS supplemented with 3% sucrose, 0.24% Gelrite, devoid of growth regulators, and maintained under the same two conditions. All media were autoclaved at 121 °C for 20 min. after adjusting the pH to 5.7 with 0.1 N NaOH. Twenty-five explants were cultured per treatment with five explants per Petri dish. Control cultures were also initiated on MS devoid of any growth regulators. Cultures were observed weekly and data recorded. The experiment was replicated three times.

Statistical analysis. All experimental data on percent response and embryo formation were subjected to analysis of variance and significant ($P<0.05$) means were determined with Tukey test to distinguish differences between treatment means at $\alpha=0.05$ level using SPSS Software Version 22.0.

RESULTS AND DISCUSSION

The initiation of embryogenic calli has been shown to be affected by several factors, including PGRs (Gill et al. 2003). The young leaves cultured on MS medium supplemented with 3% sucrose, 0.24% Gelrite™, and various concentrations and combinations of BAP, NAA and GA3 became swollen and produced compact white to yellowish embryogenic callus when cultured in the darkness. Callus initiation occurred from the cut ends of the explants within 3 weeks of culture in darkness. Embryogenic callus yielded globular, heart, torpedo, and cotyledon-stage somatic embryos when transferred to MS medium containing 3% sucrose, 0.24% Gelrite™ without plant growth regulators after 8 weeks of culture in darkness. Explants cultured on MS supplemented with 1.0 mg L^{-1} BAP,

0.5 mg L^{-1} NAA and 1.0 mg L^{-1} GA3 yielded a higher percentage (76±0.25) of embryogenic callus (Table 1) after 4 weeks of culture in darkness (Figure 1a).

Table 1. Effect of BAP, NAA in combination with GA_3 on somatic embryo induction from young leaves of *Eusideroxylon zwageri.*

BAP + NAA + GA_3 (**mg** L^{-1})	**Embryogenic callus** response (%)	Number of embryos per explants per developmental stage			
		Globular	Heart	Torpedo	Cotyledonary
1.0 + 0.5 + 0.5	11±0.35^{c}	10±0.45^{a}	10±0.45^{b}	7±0.55^{c}	3±0.55^{d}
1.0 + 0.5 + 1.0	76±0.25^{a}	31±0.55^{a}	30±0.71^{b}	28±0.00^{c}	25±0.55^{d}
1.0 + 0.5 + 1.5	45±0.15^{b}	21±0.45^{a}	20±0.00^{b}	17±0.55^{c}	16±0.55^{d}
1.0 + 0.5 + 2.0	29±0.55^{bc}	20±0.89^{a}	19±0.45^{b}	15±0.55^{c}	10±0.55^{d}

Column means followed by different letters differ significantly according to Tukey's test. α = 0.05. Cultures were initially maintained in darkness for 4 weeks and then transferred to growth regulator-free medium and maintained in darkness. Twenty-five explants were used for each treatment and the experiment was repeated three times. Mean values ± SD.

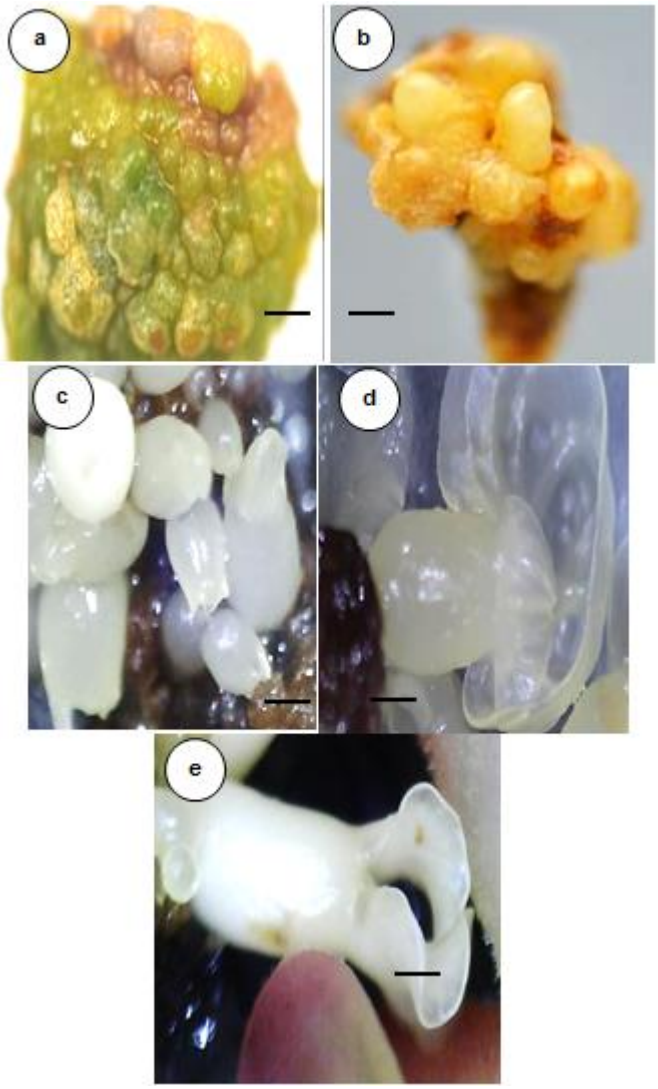

Figure. 1 Various developmental stages of *Eusideroxylon zwageri* somatic embryos induced from young leaves explants.

a. Embryogenic callus obtained from MS medium containing 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} GA3 4 weeks culture in darkness (bar = 1.5 mm).

b. Globular stage embryo (bar = 1.5 mm).

c. Heart-stage embryo (bar = 1.5 mm).

d. Torpedo-stage embryo (bar = 1.5 mm).

e. Cotyledon stage embryo (bar = 1.5 mm).

Embryogenic callus obtained on 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} GA3 when transferred to MS medium devoid of plant growth regulators yielded a higher number of globular embryos (31±0.55; Figure 1b). Globular embryos developed into heart-stage embryos (30±0.71; Figure 1c), and these embryos further developed into torpedo-stage embryos (28±0; Figure 1d), and finally into normal mature white to yellowish opaque cotyledon-stage embryos (25±0.55; Figure 1e). The percentage of explant response and the number of somatic embryos per explant was highest at 1.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} GA3 (Table 1) for the various concentrations of BAP, NAA in combination with GA3 tested. Increasing or decreasing BAP, NAA in combination with GA3 concentration resulted in a decline for both explant response and number of embryos per explant (Table 1).

In this present study, the maturation of somatic embryos from leaves explants was successfully obtained by using MS medium augmented with 1.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA in combination with either 1.0, 1.5 or 2.0 mg L^{-1} of GA3. The similar finding was also obtained in a preliminary study on the induction of somatic embryogenesis in *E. zwageri* (Gibson and Rebicca 2016) by using half strength MS medium. In this preliminary study, the maturation of somatic embryos up to cotyledonary stages was very low as compared to when MS medium was used as the somatic embryos induction medium as in this study. Similarly, on Lauraceae species such as in somatic embryogenesis of *C. kanehirae* (Chen and Chang 2009) claimed that the addition of 1.0 and 2.0 mg L^{-1} GA3 in combination with 1.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA into the MS culture medium have induced the highest percentage of somatic embryos maturation.

Research on Lauraceae species such as in *P. americana* also discovered the same result in which the addition of 1.0 mg L^{-1} of GA3 into MS culture medium for the maturation of somatic embryos was essential in order for the maturation of somatic embryos (Sanchez et al. 2005). The application of 0.5 to 2.0 mg L^{-1} of GA3 to induce somatic embryos of *E. zwageri* up to cotyledonary-shaped was also applied similarly for the formation and germination of somatic embryos of *Cocos nucifera* (Ashton 2011). At the concentration of 0.5 to 2.0 mg L^{-1}, the addition of GA3 into MS culture medium has increased 1.5 fold the number of calli induced somatic embryos and two folds the number of somatic embryos per callus in culture of *C. nucifera*. A research on somatic embryogenesis of Bermuda grass also proved that the addition of GA3 into MS medium triggers the maturation and the germination of the somatic embryos (Li and Qu 2002).

In a review of previous studies of somatic embryogenesis in Lauraceae species, primary explants for the somatic embryogenesis was usually from the zygotic embryos such as in *S. ramdaoemse* (Chen and Wang 1985), *C. camphora* (Cheng and Ma 1990, Du and Bao 2005), *P. americana* (Monney and Van 1987, Witjaksono and Litz 1999), *L. nobilis* (Canhoto et al. 1999), *O. odorifera* (Catarina et al. 2001) and *O. catharinensis* (Catarina et al. 2003). This research for *E. zwageri* was another research of the Lauraceae species that induced the indirect somatic embryogenesis by the use of the leaves explants after the first research of *C. kanehirae* (Chen and Chang 2009).

In conclusion, the manipulation of embryo induction medium was significantly affected the germination and maturation of somatic embryogenesis in *E. zwageri*. MS medium was also effective for the induction of somatic embryogenesis in *E. zwageri* by using leaf explant. The maturation of these globular somatic embryos were obtained in MS medium with BAP, NAA and GA3 in which the highest mean number of the induction of somatic embryos up to the cotyledon stage was observed on MS medium with 1.0 mg L^{-1} of BAP, 0.5 mg L^{-1} of NAA in combination with 1.0 mg L^{-1} of GA3. This study proved that MS medium was also effective for the induction of indirect somatic embryogenesis in *E. zwageri* by using leaf as the main source of the explant.

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