Massive *In Vitro* Propagation of Sandalwood Through Friable Embryogenic Callus

Supatmi[1*], Nurdiya Ardiyanti[1], Nurhamidar Rahman[1] and Enny Sudarmonowati[1]

[1]Research Center for Biotechnology, Indonesian Institutes of Sciences (LIPI)
Cibinong Science Center, Jl. Raya Bogor KM 46, Cibinong 16911

Abstract

Sandalwood (*Santalum album*), which belongs to Santalaceae family, is a commercially important tree in Indonesia due to its essential oil contents used for important essence of perfume in the perfumery industry. However, its population has significantly depleted since the planting materials of this tree using conventional methods are difficult to be provided. This study was conducted to mass propagate sandalwood using *in vitro* methods through friable embryogenic callus (FEC). The somatic embryos were formed using leaves in length of 1-3 and 4-7 mm cultured in MS medium containing 0.5 mg/l and 1 mg/l indole acetic acid (IAA), MS medium supplemented with 1 mg/l IAA and 0.2 mg/l kinetin and half concentration of MS medium supplemented with 1 mg/l Gibberellic acid (GA3). Primary somatic embryos (PSE) and secondary somatic embryos (SSE) then formed friable embryogenic callus when it repetitively transferred into MS medium supplemented with 1.7 mg/l BAP, 1 mg/l proline or 1.5 mg/l BAP and 1.2 mg/l kinetin every 3 weeks. The FEC shows its optimum maturation and regeneration in the MS medium supplemented with 1.5 mg/l BAP and 1.2 mg/l kinetin for 4-8 weeks. The acclimatization of sandalwood plantlets were perfectly conducted in the medium containing soil, sand and compos in ratio of 1:1:1 with the companion plant namely *Murraya paniculata*, (L) Jack which gave the best percentage of survival rate and the lowest percentage of fallen leaves. These findings may improve the massive propagation of sandalwood through FEC as well as a useful material for further genetic improvement of sandalwood by using FEC as material for genetic transformation.

Keywords: *Santalum album*, somatic embryos, friable embryogenic callus, plantlets

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[*] Corresponding author:
Research center for Biotechnology
Indonesian Institute of Sciences (LIPI)
Jl. Raya Bogor Km.46, Cibinong 16911, Indonesia
Tel. +62-21-8754587, Fax.+62-21-87754588 Email: patmi_bio@yahoo.com

Introduction

Sandalwood (*Santalum album*), which belongs to Santalaceae family, is a commercially important tree due to its many applications. The essential oil contents in the heartwood of this tree are used widely as essence of perfume in the perfumery industry (Rugkhla & Jones, 1998; Pothala *et al*., 2010; Teixeira da Silva *et al*., 2016). In Indonesia, sandalwood has been traded as a premium commodity since a thousand years ago. It can be found abundantly in East Nusa Tenggara (NTT) and East Timor (Badan Penelitian dan Pengembangan Kehutanan, 1992). Naturally, the population of these trees plummets since its regeneration rate is not in an equal number to the harvesting rate. During the harvesting, the entire tree of sandalwood is usually removed which may cause the loss of genetic diversity and agronomic characters (Mujib, 2005; Muthan *et al*., 2006). In addition, the increase of illegal logging activity, forest burning and livestock pasture have worsened the scarcity of sandalwood population, which may place the sandalwood as endangered tree species (Rai and McComb, 2002; Muthan *et al*., 2006).

Conventional breeding of sandalwood has been hampered due to the sexual incapability, long generation time and heterozygous nature while the vegetative propagation of sandalwood are not yet available (Rugkhla & Jones, 1998; Mujib, 2005; Mo *et al*., 2008; Sukmadjaja, 2005). *In vitro* approach prior to sandalwood regeneration has been still limited due to the difficulty in the root development and acclimatization (Muthan *et al*., 2006). Most propagation technique of sandalwood was applied through micrografting (Muthan *et al*.,

2006), organogenesis (Du & Pijut, 2008; Sukmadjaja, 2005; Rahman *et al*., 2012) and somatic embryogenesis (Rai and McComb, 2002; Sukmadjaja, 2005, Bele *et al*., 2012), which has been in progress. The direct use of somatic embryos in both primary and secondary somatic embryos in genetic transformation has not been recommended since their histological structure seems to have multicellular origin. This may cause the production of chimeras leading to undesirable somaclonal variation (Raemaker *et al*., 1997; Quiroz- Figueroa *et al*., 2006). In addition, results obtained in these techniques, in correlation to published methods, were unreliable and depended on many factors such as explants, growth regulators and environment (Shekhawat *et al*., 2008; Sudarmonowati *et al*., 2009). Therefore, a new approach to produce sandalwood plantlets in a large scale with an existing root axis through friable embryogenic callus (FEC) would be a promising way to solve the problem.

The FEC, which initially produced from embryogenic callus, is highly disorganized and regenerate through a single cell origin (Taylor *et al*., 2004; Sudarmonowati *et al*., 2009). Moreover, FEC can be maintained for a long time without losing its capability to regenerate embryos (Bespalhok & Hattori, 1998). Because of these reasons, FEC become preferred materials for propagation and efficient materials for transgene integration (Bull *et al*., 2009; Taylor *et al*., 2012). The FEC has been successfully applied as an efficient material for genetic transformation in African marigold (*Tagetes erecta* L.) (Bespalhok & Hattori, 1998) and cassava model cultivar 60444 (Hankoua *et al*., 2006). Established propagation through FEC production would open further development of sandalwood to address the genetic conservation and supply of this material for industries demand in Indonesia. Since the FEC induction is mostly the same as embryogenic callus which are affected by many factors (Sudarmonowati *et al*., 2009), therefore; the objectives of this research were to induce friable embryogenic callus of sandalwood *in vitro* utilizing different explant source and media composition as well as improved its regeneration and acclimatization.

Materials and Methods

Plant Materials. Different size of leaves in length of 1-3 mm (A) and 4-7 mm excised from sandalwood shoot *in vitro* originally provided by Centre for Forest Biotechnology and Tree Improvement (CFBTI), Yogyakarta, were used as plant materials.

Induction Of Embryogenic Callus. Two different sizes of leaf-lobes 1-3 mm (A) and 4-7 mm (B) in length were excised from *in vitro* mother plants using an aseptic scalpel and placed on to petri dish contain different media supplemented with different concentration of CuSO4, amino acids and growth regulators as shown in Table 1. All cultures were incubated in the dark for 4 weeks during stage-1 culture prior to inducement of the embryogenic callus. Each treatment consists of 5 explants in three technical replicates. After 30 days of performances, explants were observed and recorded in terms of survival rate and callus form.

Table 1. The media composition for embryogenic callus induction of sandalwood.

No	Media composition	References
1.	MS+5 mg/L CuSO4+5 mg/L picloram	Atehnkeng, *et al* (2006) with modification
2.	MS+ 10 mg/L CuSO4+ 10mg/L picloram	Atehnkeng, *et al* (2006) with modification
3.	½ MS+ ½ GD + 100 mg/L tyrosin	Koehorst-van Putten, *et al* (2012) with modification
4.	½ MS+ ½ GD	Koehorst-van Putten, *et al* (2012) with modification
5.	MS+ 0.3mg/L CuSO4+ 0.15 mg/L1 mM glutamine+ 18 mg/l picloram	Koehorst-van Putten, *et al* (2012) with modification

Note: MS: Murashige-skoog; GD: Gresshoff and Doy; CuSO4: Cupric sulphate

Induction and Multiplication Of Primary Somatic Embryos (PSE) And Secondary Somatic Embryos (SSE) Callus. Organized embryogenic structures obtained from embryogenic callus medium were excised from non-embryogenic callus in small clumps and then subcultured in various PSE and SSE mediums, which contain different plant growth regulators as shown in Table 2 for 4 weeks. Each treated medium consisted of 10 embryogenic callus clumps in 4 technical replicates. All cultures were subsequently incubated under the light at 16 h photoperiod at 25±1ºC from 4 weeks to 8 weeks, depends on the response of embryogenic callus to produce PSE/SSE in the various media. Callus performances were observed and recorded in terms of survival rate and the response of explants for inducing callus.

Table 2. The media composition for primary and secondary embryogenic callus induction of sandalwood

No	Media composition	References
1.	MS+0,5 mg/l IAA	Sukmadjaja, 2005
2.	MS+1 mg/l IAA	Sukmadjaja, 2005
3.	MS+1 mg/l IAA+0,2 mg/l Kinetin	Rugkhla & Jones, 1998 with modification*
4.	MS+0,5 mg/l GA3	Sukmadjaja, 2005
5.	MS+1mg/l GA3	Sukmadjaja, 2005
6.	0,5 MS+1 mg/l GA3	Sukmadjaja, 2005

Note: MS: Murashige-skoog , IAA: Indole acetic acid; GA3: Gibberellin acid. *The modification of media composition,used in this experiment, in terms of the concentration of IAA and Kinetin, were different with those used in the references.

Production and Maturation Of FEC. Induction of FEC was initiated from PSE and SSE obtained from previous various media depending on the purpose, i.e for proliferation, regeneration or maturation. Prior to inducement of FEC, PSE/ SSE which proliferated in the nodular stage in the previous medium were then transferred on to MS medium supplemented with amino acid and cytokinin regulator growths, i.e MS + 1.7 mg/l BAP + 1 mg/l proline and MS + 1.5 mg/l BAP + 1.2 mg/l kinetin. All cultures were subcultures every 4 weeks until 3 cycles time. The maintenance of FEC has been conducted by spreading the super layer of FEC on to MS medium containing 1.7 mg/l BAP + 1 mg/l proline every 3-4 weeks for 3 cycle times. The regeneration and maturation of FEC were conducted by transferring FEC into medium 1.5 mg/l BAP + 1.2 mg/l kinetin for 4-8 weeks until the cotyledon stage has been formed. The regenerated FEC were then separated in single plantlet and transferred to MS medium prior to development of the high shoot for acclimatization purposes.

Acclimatization Of Regenerated Plantlets. Acclimatization of plantlets from regenerated FEC was conducted by growing plantlets on to three different mediums i.e; A (sand, soil and compos in ratio of 1:1:1); B (cocopit and compos in ratio of 1:1); C (old fern dipped in atonik solution overnight). Plantlets were then carefully removed from agar medium. Subsequently, roots of plantlets were washed with aquadest. The height of plantlets, number and length of roots and number of leaves were measured. Plantlets were then planted in polybags together with the companion plant namely *M. paniculata*, (L) Jack and subsequently covered with transparent plastic bags. Watering had been conducted every three days. The performances of plantlets were observed in terms of number of survival plants, the leaves appearances and the rate of fallen leaves every month.

Statistical Analysis: Data obtained from observation were then analyzed with an analysis of variance (ANOVA) using SPSS 16.0 version followed with a Duncan's comparison test to distinguish differences between treatments at the 95% level of significant confidence.

Results

Response of Diverse Size of Leaves and Different Media for Embryogenic Callus Induction. Response of different size of leaves and media supplemented with amino acids and auxin to the induction of embryogenic callus of

sandalwood have been shown in Table 3. Results show that either leaves in length of 1-3 mm (A) or 4-7 mm (B) gave the same response to varied induction medium. Specifically, the performances of explants were changed in terms of rolling leaves, callus and browning leaves (Table 3). Unfortunately, the callus formation obtained from these diverse media have been categorized as non-embryogenic callus which failed to develop further into plantlets. Interestingly, the addition of CuSO4 in three different induction media (Table 1) could maintain the colour of explants in green colour. Of that reason, the explants cultured in those media (MS supplemented with 5 mg/l CuSO4 and 5 mg/l picloram; MS supplemented with 10 mg/l CuSO4 and 10 mg/l picloram) had been excised and cultured in a new induction medium supplemented with diverse plant growth regulators.

Table 3. The effect of different size of leaves and induction mediums on sandalwood *in vitro*.

No	Media	Rolling leaf (%)		Callus formation (%)		Browning leaves (%)	
		A	B	A	B	A	B
1.	MS+5 mg/l CuSO4+5 mg/l picloram	93	60	0	0	0	27
2	MS+ 10 mg/l CuSO4+ 10mg/l picloram	100	100	0	0	0	0
3	½ MS+ ½ GD + 100 mg/l tyrosin	73	73	33	53	100	93
4	½ MS+ ½ GD	100	100	73	100	93	47
5	MS+ 0.3 mg/l /l CuSO4+0.15 mg/l glutamine+ 18 mg/l picloram	87	100	100	67	40	47

Note: The size of leaves in length of 1-3 mm (A) and 4-7 mm (B).

Somatic Embryo Induction. Prior to inducing somatic embryos of sandalwood, explants cultured in previous embryogenic callus medium (Table 1) had been transferred to MS medium supplemented with diverse plant growth regulators such as IAA, Kinetin and GA3 as shown in Table 2. Our results show that the survival rate of explants cultured in different culture media was in variety. Of these five-induction media, MS medium supplemented with IAA (0,5 and 1 mg/l) or both 1 mg/l IAA and 0,2 mg/l Kinetin gave the best response ($P \leq 0.05$) for the survival rate of explants compared to that obtained in other media after 2 weeks observation time (Fig.1). Interestingly, half concentration of MS medium supplemented with 1 mg/l GA3 also gave a high survival rate in sandalwood explants (Fig.1). Meanwhile, explants cultured in normal concentration of MS medium supplemented with 1 mg/l GA3 had the lowest survival rate of those entire survival rate obtained in other media.

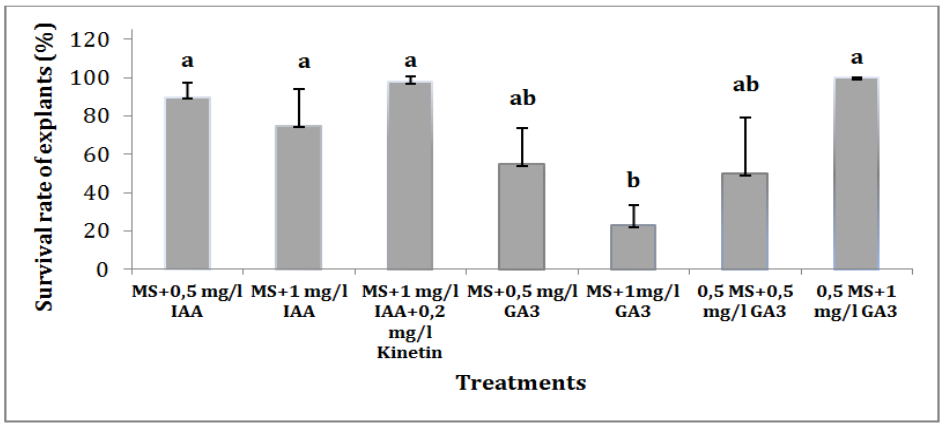

Figure 1. The survival rate of *in vitro* sandalwood explants grown in various MS medium containing different plant growth regulators. Means in each diagrams followed by the different letters are significantly different at $P \leq 0.05$ by ANOVA.

The performances of explants had been observed further for 4 weeks. Observation of explants performances show that somatic embryos and direct organogenesis of sandalwood *in vitro* has been developed after 4 weeks cultured in diverse media supplemented with plant growth regulators (Table 4; Figs. 2A&B). Almost 98 to 100 % somatic embryos have been developed in three treated media i.e MS medium supplemented with 1 mg/l IAA, MS medium supplemented with 1 mg/l IAA and 0.2 mg/l Kinetin, and MS medium supplemented with 0.5 mg/l MS supplemented with 1 mg/l GA3 ($P \leq 0.05$).. Meanwhile, organogenesis performances of sandalwood *in vitro* have been formed in MS medium supplemented with GA3 in ranged of 0,5 and 1 mg/l GA3 and also 0.5 mg/l MS medium supplemented with 0,5 mg/l GA3 ($P \leq 0.05$). Somatic embryos appearance obtained in these media was mostly in proembryogenic stages such as nodular and tubular (Fig. 2a) while direct organogenesis has been formed from the appearance of small shoot or root after 4 weeks observation time using stereo microscope (Fig. 2b).

Table 4. The effect of the various medium containing different plant growth regulators on the induction of somatic embryos of sandalwood *in vitro*.

Medium	Response of explants	
	Somatic embryos (%)	Organogenesis (%)
MS+0,5 mg/l IAA	52 ± 11.7 [b]	50 ± 11 [b]
MS+1 mg/l IAA	100 ± 0.0 [a]	0 ± 0.0 [c]
MS+1 mg/l IAA+0,2 mg/l Kinetin	98 ± 5.0 [a]	5 ± 5.0 [c]
MS+0,5 mg/l GA3	20 ± 10.8 [c]	97 ± 2.8 [a]
MS+1mg/l GA3	17 ± 23.0 [c]	100 ± 0.0 [a]
0,5 MS+0,5 mg/l GA3	0 ± 0.0 [c]	100 ± 0.0 [a]
0,5 MS+1 mg/l GA3	100 ± 0.0 [a]	0 ± 0.0 [c]

Note: Means ± standard error within a column followed by the different letters are significantly different at $P \leq 0.05$ by ANOVA

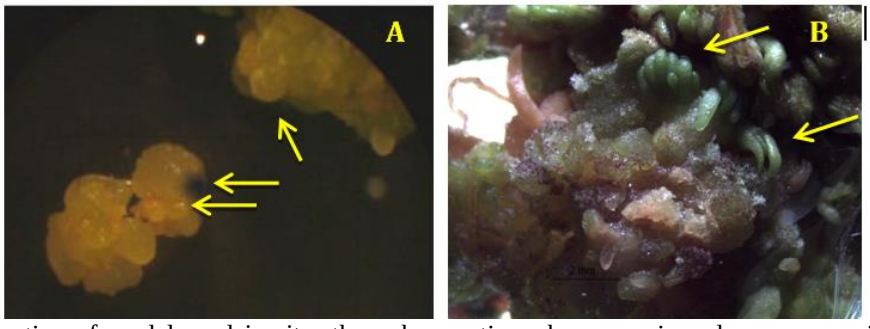

Figure 2. Regeneration of sandalwood *in vitro* through somatic embryogenesis and organogenesis in different MS medium containing different plant growth regulators. A. Somatic embryos induced in MS medium supplemented with1 mg/l IAA and 0,2 mg/l Kinetin. The arrows show the nodular stages in the somatic embryos development; B. Organogenesis induced in MS medium supplemented with 1mg/l GA3. The arrows show the initial shoot formation during direct organogenesis development.

Production And Maturation Of FEC. The monolayer/super layer of somatic embryos had been transferred in the same medium every 4 weeks to multiply the white globular embryos of sandalwood. The multiplication of these repetitive somatic embryos in the same medium produced a number of secondary somatic embryos or mixture of somatic embryos and friable embryogenic tissue. To produce FEC, the early nodular stage of PSE or SSE were transferred to two different media: MS + 1.7 mg/l BAP + 1 mg/l proline and MS + 1.5 mg/l BAP + 1.2 mg/l kinetin for 4 weeks. These cultures had been regularly transferred into new medium until three cycle times. The results show that these cultures growth either in MS medium supplemented with two different cytokinin or amino acid and cytokinin produced FEC masses enormously (Fig.3a & 3b). Remarkably, sandalwood FEC, which were transferred continuously, every 3-4 weeks in MS medium supplemented with 1.7 mg/l BAP and 1 mg/l

proline, could maintain FEC in the same condition without losing their embryogenic potential for at least 2-3 years. Meanwhile, FEC which were cultured in MS medium supplemented with 1.5 mg/l BAP and 1.2 mg/l kinetin for 4 weeks or more without retransferring them in new medium could develop FEC in to the next step of maturation (Fig.4), as can be seen in the development of proembryogenic mass from nodular and globular to cotyledonary like-tissues, which were then transformed into plantlet (Fig. 4). Unfortunately, not all of the plantlet obtained from these maturation was regenerated with both shoot and root formation. Some FEC had been regenerated in the higher abnormal plantlets, which lack of root or shoot part or fused cotyledons (Fig.4d).

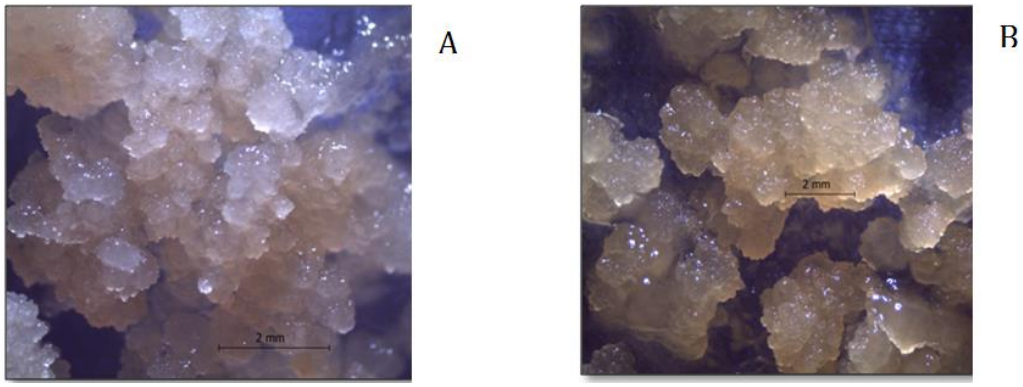

Figure 3.The development of FEC sandalwood which was cultured in FEC induction medium for 4 weeks. A. FEC development in MS medium supplemented with 1.7 mg/l BAP and 1 mg/l proline; B. FEC development in MS medium supplemented with 1.5 mg/l BAP and 1.2 mg/l kinetin.

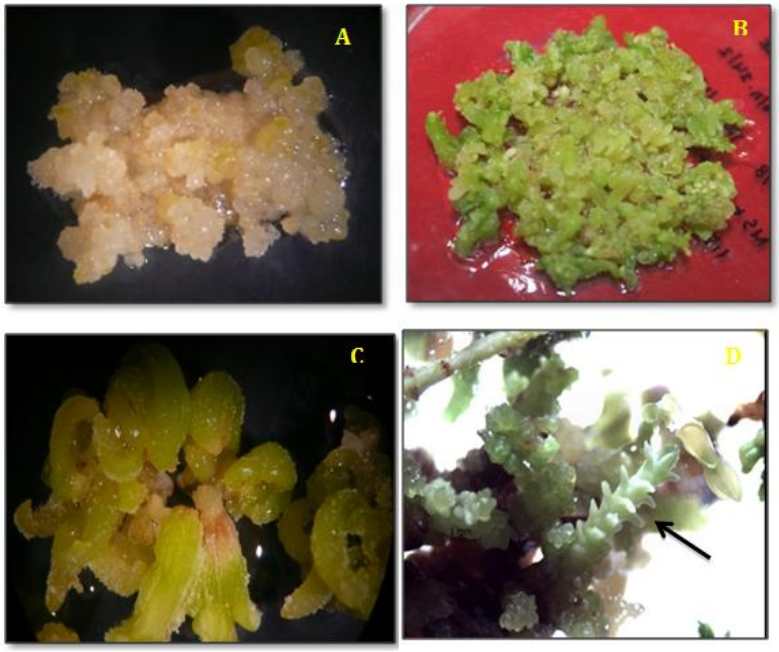

Figure 4. The production and regeneration of FEC sandalwood. A. FEC proliferation; B. FEC maturation; C. Stage cotyledonary phase; D. Plantlet development. Arrow shows the shoot formation of FEC sandalwood.

Since not all plantlets collected from FEC regeneration produced a root formation, the shoots were transferred into different rooting media supplemented with different concentration of IAA for 12 weeks. The results show that shoots transferred in WPM (woody plant media) produced 100% roots compared with other media (Table 5). Root performances of plantlets were mostly a tap root with short and single root formation (Fig.6B).

Table 5. The various media supplemented with different IAA concentration prior to root induction of sandalwood shoots *in vitro*.

Media	Root formation (%)
WPM	100%
0,5 MS+ 1,6 mg/l IAA	0%
0,5 MS + 0,5 mg/l IAA	25%
0,5 MS	25%

Note: WPM: Woody plant media. The number of shoots used in this experiment consisted of 5 explants per-bottle with 6 bottles as replication.

Acclimatization Of Sandalwood Plantlets. Three different media had been used in the acclimatization of sandalwood *in vitro* such as A (Sand, soil and compos in ratio of 1:1:1); B (Cocopit and compos in ratio of 1:1); C (Old fern dipped in atonik solution overnight) (Fig.6). Plantlets used in this acclimatization were selected by considering the same age of plantlets such as 1 months in the regenerated medium and height of plantlets which in the range of 5 cm. Moreover, these selected plantlets have already produced the short root (Fig. 6A). The companion plants namely *M. paniculata*, (L) Jack was used in the acclimatization because sandalwood lives naturally near to some companion plants. The results show that the survival rate of plantlets planted in medium A were higher and increase from the first three months to second three months (Table 6). The same result was confirmed from the percentage of fallen leaves during the acclimatization observation. The colour of leaves has turned from green to yellowish green during eight months of observation (Table 6). This acclimatization process using these three different media had been repeated in two independent experiments with the same results that medium A (sand, soil and compos in ratio of 1:1:1) gave the higher survival rate compared to that obtained in other medium. However, re-transferring plants from green house to the field is limited by the decrease of their survival rates due to uncontrolled environmental factors such as temperature and humidity.

Table 6. The plantlet performances treated with three different media prior to acclimatization.

Ages (Months)	**Treatment**	**Number of Survival Plants (%)**	**Leaves Characteristics**	
			The colour of leaves	**Falling leaves (%)**
3	A	83	Fresh green	12
	B	67	Fresh green	4
	C	75	Fresh green	4
4	A	96	Yellowish green	25
	B	58	Yellowish green	38
	C	92	Yellowish green	54
5	A	100	Yellowish green	25
	B	63	Yellowish green	25
	C	71	Yellowish green	38
6	A	100	Yellowish green	42
	B	58	Yellowish green	29
	C	63	Yellowish green	42
7	A	100	Yellowish green	33
	B	54	Yellowish green	38
	C	63	Yellowish green	54
8	A	87	Yellowish green	21
	B	54	Yellowish green	29
	C	67	Yellowish green	50

Note: A (Sand, soil and compos in ratio of 1:1:1); B (Cocopit and compos in ratio of 1:1); C (Old fern dipped in atonik solution overnight)

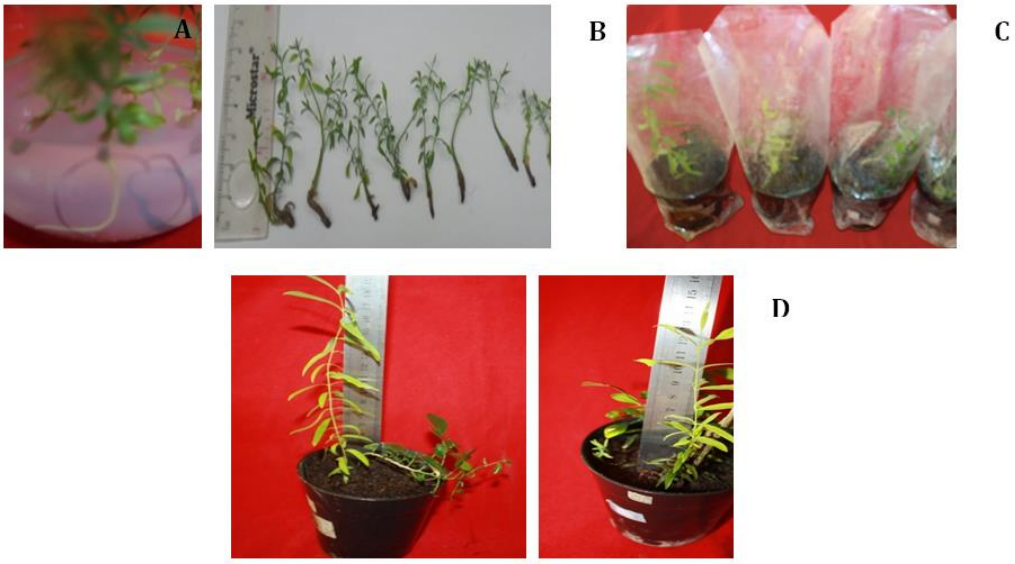

Figure 6. The acclimatization process of sandalwood treated with three different medium. A. Plantlet of sandalwood with root formation in MS medium; B. Plantlets which will be acclimatized; C. Plantlets covered with transparent bag during acclimatization; D. The survival plants of sandalwood after 8 months in the pots; left : plant treated in medium A (Sand, soil and compos in ratio of 1:1:1); Right: (Cocopit and compos in ratio of 1:1).

Discussions

This study has firstly shown that sandalwood can produce friable embryogenic callus (FEC) from repetitive somatic embryos (PSE and SSE), which were initially induced from leaves explants. Since FEC is firstly produced from embryogenic callus (Taylor *et al.,* 2004), two different sizes of leaves and diverse media composition were used to induce embryogenic callus of sandalwood. The induction media of embryogenic callus in this study comprised MS, GD, amino acids (tyrosine and glutamine) and also picloram. The varied concentration of these media was modified from success methods of FEC cassava induction (Atehnkeng, *et al.,* 2006; Koehorst-van Putten, *et al.,* 2012). In this present study, the response of explants to different combination and concentration of induction media (Table 1) was in variety. There were changes in performances of explants in terms of rolling leaves, callus and browning leaves. Unfortunately, callus obtained from these induction media was categoryzed as non-embryogenic callus. Although, these callus have been subcultured in the same medium, they can not turn into embryogenic callus. The composition of embryogenic callus media used in this study (Table 1) apparently did not work well in sandalwood although it was succesfully applied in cassava. These results were in line with Rukghla and Jones (1998) who reported that the development of embryogenic tissue callus in sandalwood is genotype dependent. They stated that the addition of amino acids and high concentration of auxin have not successfully induced embryogenic tissue callus in *S. spicatum* but it gave a highly positive response in *S. album* (Rukghla and Jones,1998). Interestingly, the appearance of explants cultured in media containing $CuSO_4$ was apparently maintained in green condition. This might be correlated with $CuSO_4$ function as activator of many enzyme involved in electron transport and also the inhibitor of ethylene precursor (maturation precursor) which enabled explants to maintain their performances in the fresh condition (Purnhauser and Gyulai, 1993, Nirwan & Kothari, 2003). Since the embryogenic callus is initially crucial step for FEC induction, green explants obtained from those media were transferred in to new induction medium consisting of different plant growth regulators.

It has been reported that the combination of auxin (IAA, 2,4D, picloram) and cytokinin (BAP, Kinetin, TDZ) induce production of embryogenic callus around 50 to 100% in a high reproducibility (Rukghla and Jones, 1998; Sukmadjaja, 2005; Atehnkeng, *et al.*, 2006; Koehorst-van Putten, *et al.*, 2012; Bele *et al.*, 2012). In this study, almost 98 to 100 % explants cultured in MS media supplemented with 1 mg/l IAA or both 1 mg/l IAA and 0.2 mg/l Kinetin and also 0.5 mg/l MS supplemented with 1 mg/l GA3 enormously produced somatic embryos by

using sandalwood leaves in the length of 1-3 mm and 4-7 mm. Interestingly, half concentration of MS medium containing 0,5 mg/l GA3 could also induce embryogenic callus but a normal concentration of MS medium supplemented with 1 mg/ l GA3 or half concentration of MS medium supplemented with 0,5 mg/l GA3 resulted in direct organogenesis of sandalwood. The results of sandalwood organogenesis were consistent with those obtained from published paper (Sukmadjaja, 2005). However, the results of somatic embryos obtained from this study were in contrast to Sukmadjaja (2005), who reported that somatic embryos of sandalwood have been developed directly in MS medium without containing IAA. These different results might occur due to the used of different explant source in the somatic embryos induction of sandalwood. The zygotic embryos used in previous published paper (Sukmadjaja, 2005) may already contain a high level of endogenous auxin compared to that in leaves explants, thus; somatic embryos could be induced in the absence of exogenous auxin. On the other hand, the induction of somatic embryos using leaves explants should be added with an exogenous auxin prior to inducing somatic embryos.

In this study, successful production of sandalwood FEC were obtained after PSE or SSE had been transferred into MS medium supplemented with 1.7 mg/l BAP and 1 mg/l proline or MS medium supplemented with 1.5 mg/l BAP and 1.2 mg/l kinetin every 3-4 weeks. The FEC was produced from the super layer of PSE and SSE, which was cultured in to FEC media until 3 cycle times. Maintenance and proliferation of FEC sandalwood were also best performed using repetitive culture of FEC for every 3 weeks into MS medium supplemented with 1.7 mg/l BAP and 1 mg/l Proline. Meanwhile, efficient maturation and regeneration of FEC sandalwood can be conducted by prolonging FEC cultures on to MS medium supplemented with 1.5 mg/l BAP and 1.2 mg/l kinetin for 4-8 months. The regenerated FEC sandalwood can be subsequently transferred in to MS medium prior to acclimatization. However, not all of the plantlets formed from regenerated FEC were successfully forming a root. This might happen due to the addition of BAP or another plant growth regulators leading to the abnormality performances of regenerated FEC transform to plantlets (Rukghla and Jones, 1993). Of these reasons, the plantlets, which were still in shoot formation, were transferred into several rooting media. Apparently, plantlets could produce 100% root after being transferred in WPM medium for 12 weeks. It seems that WPM medium is an appropriate medium to trigger the root formation since this medium composition might be specifically formulated for plant forestry *in vitro (*Sudarmonowati *et al*., 2009).

The acclimatization of sandalwood plantlets were done by using varied media [(A (Sand, soil and compos in ratio of 1:1:1); B (Cocopit and compos in ratio of 1:1); C (Old fern dipped in atonik solution overnight)] to develop entire plantlets. The results show that effective acclimatization of sandalwood plantlets can be conducted in the medium containing soil, sand and compos in ratio of 1:1:1 with the companion plant namely *M. paniculata*, (L) Jack. The effectiveness of this media could be seen in the highest percentage of survival rate and the lowest percentage of fallen leaves compared to those obtained from another media treatments. Sandalwood commonly lives near companion plants because of its character as a partial-parasitic plant; it clearly depends on another plant specifically in nutrient supply during their early plant development (Musselman & William, 1978). Despite sandalwood plantlets could survive until 8 months observation time, the percentage of fallen leaves increased and the performances of leaves colour apparently turned to yellow. This might happen due to the competition between plantlets and the companion plant in terms of macro nutrition elements supply such as NPK, which resulted in yellow colour. Therefore, the application of fertilizer and re-potting in new medium may increase the survival rate and decrease the percentage of fallen leaves of sandalwood *in vitro*. Besides, improvement on the technical methods to transfer plantlets from greenhouse to the field is needed since the environment factors such as temperature and humidity are uncontrolled.

This study highlighted the initial success of FEC sandalwood production *in vitro* by utilizing leaves with different size in length and various induction media supplemented with diverse concentration and plant growth regulators, as well as acclimatization media of sandalwood plantlets. Although the somatic embryo production as an important material for initial FEC induction was obtained from media which were based on published paper (Sukmadjaja, 2005), this study comes into conclusion that MS

media containing 1.7 mg/l BAP and 1 mg/l proline as well as 1.5 mg/l BAP and 1.2 mg/l kinetin are the best media for induction and maturation in the purpose of production FEC sandalwood. The regeneration of FEC sandalwood and its acclimatization resulted in this study definitely improve the high possibility of sandalwood *in vitro* for being transferred in the field. These findings may shed light for improving the massive propagation of sandalwood through FEC and a useful material for further genetic improvement of sandalwood by using FEC as material for genetic transformation.

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