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Somatic embryogenesis of Sandalwood (*Santalum album* L.)

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Abstract

Sandalwood (*Santalum album* L.) is native species of Indonesia, especially in East Nusa Tenggara, is one of the twenty two species of the genus Santalum in the world. Sandalwood is an important tree because it has high economic value can produce sandal oil these can be used for perfumes, cosmetics, pharmaceuticals, and are often used in religious ceremonies. *In vitro* particularly somatic embryogenesis has been widely applied in the propagation of sandalwood. The Objective of this research is to obtain regeneration of sandalwood through somatic embryogenesis using leaves explant from various clones. Medium for embryo induction is MS (Murashige and Skoog, 1962) solid medium containing treatment of 2,4-D (2,4-Dichlorophenoxyacetic acid) at various concentrations. To the media 0,15 mg /l kinetin, 40 g/l sucrose, and 2,5 g/l gelrite were added. Culture were incubated in the dark. Medium for Embryo development (maturation) is MS solid medium containing treatment of BAP (Benzyl-amino-purine) at various concentrations. To the media 0,01 mg /l NAA (Napthalene-acetic-acid), 40 g/l sucrose, and 2,5 g/l gelrite were added. Culture were incubated in the light. To study the specific structure of sandalwood somatic embryo early detection was conducted using histological analysis. Results of anova showed that the clones, media, and interaction between clones with media did not significantly affect the development of sandalwood callus percentage. Results of anova showed that the clones and BAP concentration significantly effect to the embryo development of sandalwood.

Keywords : Somatic embryogenesis, Santalum album, Embryogenic callus, maturation.

Introduction

Sandalwood (*Santalum album* L.) as a native species of Indonesia (Asian Regional Workshop, 1996), is one of the best Santalum species in the world because of the timber terrace and sandal oil content is high (Rao and Bapat, 1995). Sandalwood is a potential commodity for the economy of Indonesia, especially in East Nusa Tenggara. The oil is widely exported to Europe, USA, China, Hong Kong, Korea, Taiwan, and Japan. While the woods were used for handy crafts such as sculpture, handy fan, beads, and rosary.

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The problem is in the supply of raw material of sandal oil still comes from natural forests, which supply over time tends to decrease. Gap between the ability and providing the raw material damage ecosystems and therefore needs conservation of forest resources (Forest Service of East Nusa Tenggara, 1998). According to IUCN (International Union for Conservation Nature and Natural Resources), sandalwood is one of tree in vulnerable category / VU A^1D^1 and endangered category/EN A^1A^2. It is serious matter and requires conservation and mass propagation of the plants (Pusat Penelitian dan Pengembangan Hutan Tanaman, 2006).

Sandalwood seedlings can be reproduced through germinated seeds, but the current problem is the difficulty to obtain good quality of seed. Conventional vegetative

propagation through shoot cuttings and root cuttings has been done, but its success is still low (Surata, 2003). *In vitro* regeneration technique can be used to encounter difficulties of conventional propagation methods. Somatic embryogenesis using vegetative materials using young leaves explants derived from epicormic shoot from branch soaked in tap water.

The Objective of this research is to obtain regeneration of sandalwood through somatic embryogenesis.

Materials and Methods

Exploration activity of sandalwood vegetative material was done in Genetic Conservation area in Watusipat, Playen, Gunung Kidul, Yogyakarta, Indonesia. Somatic embryogenesis culture was done in Tissue Culture Laboratory of Center of Forest Biotechnology and Tree Improvement (CFBTI) in Kaliurang, Yogyakarta, Indonesia. The explants (young leaves) from various clones were immersed in 0,1 per cent (w/v) fungiside for fiveteen minutes, surface sterilized and were washed thoroughly in running tap water and then washed with 70 percent ethanol. The explants were then immersed in 2 per cent sodium hypochlorite (NaOCl) solution for ten minutes, and rinsed 3 times in sterile distilled water. All the culture were maintain on MS (Murashige and Skoog, 1962) basal media.

Somatic embryogenesis of Sandalwood were initiated from young leaves explants that obtained from the epicormic shoot multiplication of branchs its were soaked in tap water in green house from selected sandalwood tree of 10 years old. *In vitro* plant materials were cultivated in bottles in a growth chamber at 24 ^{o}C, 70% humidity, and a photoperiod of 16-h with a white light.

Culture media

Medium for embryo induction is MS solid medium containing CND1 = 2,4-D (2.4 Dichlorophenoxyacetic acid) 1 mg/l; CND3 = 2,4-D 3 mg/l; and CND5 = 2,4-D 5 mg/l , 0,15 mg /l kinetin, 40 g/l sucrose, and 2,5 g/l gelrite. Culture were incubated in the dark.

Embryo development medium (maturation) is MS solid medium containing B0 = BAP (Benzyl-amino-purine) 0 mg/l; B1 = BAP 1 mg/l; B2 = BAP 2 mg/l; and B3 = BAP 3 mg/l, 0,01 mg /l NAA (Napthalene-acetic-acid), 40 g/l sucrose, and 2,5 g/l gelrite. Culture were incubated in the light.

Histological studies

Histological studies were carried out on embryogenic tissue which were cultured for (30 – 60) days. The explant were fixed in FAA(formalin 5 % (v/v); acetic-acid 5 % (v/v); ethanol 90 % (v/v), dehydrated in an ethanol series and embedded in para Yn wax. Section, 10 – 16 µm thick were cut and stained with toluidine blue – O (Feder and O'Brien, 1968).

Results and Discussion

Embryo induction

Somatic embryogenesis is a process inwhich somatic cells (both haploid and diploid) developed into new plants through specific stages of embryonic development without gamet fusion.

Initiation of embryo development, at the globular stage was easily detected by a light yellow to cream color in the callus, which is characteristic of the sandalwood somatic embryo as early as the globular stage.

During first 7 days, leaflets come in rolls and first appeared callus to day 21. The process of embryogenesis starts with formation of crumb and yellow beige color. Up to day 75 callus induction increases 1-2 times. 69 % of the explants react positively. In this first step of the protocol induction of somatic embryos are promoted by 3 mg/l 2,4-D in combination with the cytokinin 0,15 mg/l Kinetin. Induction and development of the structures on MS medium take around 60 days. This first step of the protocol resemble of the first two steps of the protocol described by Opabode *et al.* (2011) but the period for induction and development is

Table 1. The respond genotype of each clone and concentration 2,4-D on growthrate sandalwood callus induction after incubation for 8 weeks

Clones	Percentage callus induction (%)			Average callus induction (%)
	CND1	CND3	CND5	
A.II.4.5				
Average Embryo induction	60 %	40 %	20 %	40 %
A.II.2.3				
Average Embryo induction	20 %	0	0	6,7 %
A.IV.Ii.12				
Average Embryo induction	20 %	40 %	0	20 %
B.VI.7.14				
Average Embryo induction	0	0	0	0
T.A.12				
Average Embryo induction	20 %	20 %	20 %	20 %
A.II.4.16				
Average Embryo induction	60 %	60 %	0	40 %
B.VI.Ii.12				
Average Embryo induction	20 %	0	40 %	30 %
TA.1				
Average Embryo induction	60 %	40 %	60 %	54 %
K				
Average Embryo induction	80 %	80 %	20 %	60 %
Average Embryo induction				**30** %

Table 2. Anova sandalwood callus induction after incubation in the dark for 8 weeks

Source	Sum of Square	df	Mean Square	F	Sig
Clone	13924,275	8	1740,534	1,967^{ns}	0,059
Media	930,254	2	465,127	0,526^{ns}	0,593
Clone x Media	8695,671	16	543,479	0,614^{ns}	0,865
Galat	84935,450	96	884,744		
Total	176059,000	123			

Description : ns = non significant

shorter with 21 days. Sub-culture explants were conducted every 30 days on fresh MS medium, when embryos appeared at the surface of the explants it the time to transfer them carefully.

The results of study on the effect of various clones and concentration of 2,4-D as plant growth regulator (PGR) used to induce the sandalwood callus after incubation for 8 weeks (Table 1).

The results of induction of somatic embryogenesis using MS media for eight weeks, of the nine clones obtained average embryo induction 30 %. Friable embriogenic tissue produced from leaf explants only reached 30%, this is according to the research conducted by Rugkla and Jones, 1998 which stated that friable embriogenic tissue produced from the explants derived from nodal segments was low. However embryo induction using MS medium clone K gave the best response on development of embryogenic callus, with an average embryo induction 60%.

To verify the response of clones and the media to sandalwood callus induction followed by anova as shown in Table 2 below.

Results of anova showed that the clones, media, and interaction between clones with media did not significantly affect the development of sandalwood callus percentage. Development of callus induction (Fig. 1 and Fig. 2).

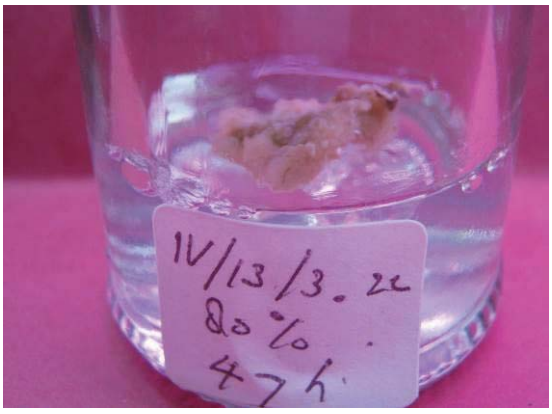

Figure 1. Early development of friable callus after 47 days incubation in the dark condition

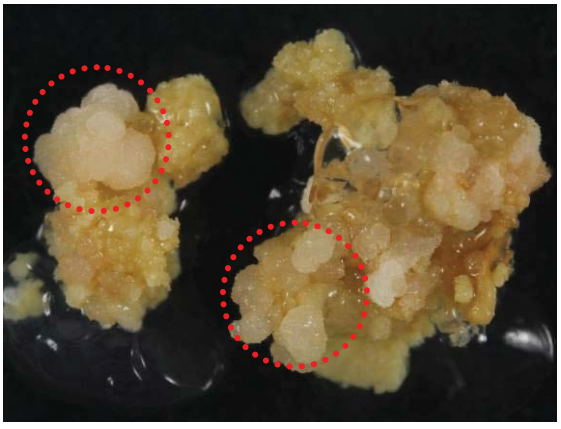

Figure 2. Growth of embryogenic callus induction Globular stage is marked with a red circle

The results of these studies suggested that growth and development of somatic embryogenesis is influenced by many factors, including plant species, genotype, age, explant type, season, carbon source, type and concentration of exogenous growth regulators. In the present study, we investigated the production of somatic embryos in excised young leaves Sandalwood from epicormic shoot at various concentration of 2,4-D. Our study showed that young leaves gave responsive explants. Successful induction and maturation of somatic embryos in woody plants using leaf explants have been documented (Rugkhla and Jones, 1998; Jain *et al*., 2000).

Embryogenic calluses were induced with 2,4-D and the growth regulator affected the number of embryogenic calluses obtained from young leaves explants. 2,4-D alone and in combination with other plant growth regulators, has been widely used to induce somatic embryos in leguminous woody plants (Das, 2010). Induce stress responses on explants is another role of 2,4-D by which the somatic embryo is inducted (Zavattieri *et al*., 2010). It has been suggested that the embryogenic effect of 2,4-D probably derives from its methylation on the nuclear DNA (George *et al*., 2008).

In somatic embryo proliferation phase showed that the use of MS medium containing PGR 2,4-D 1- 5 mg/l 2,4-D + 0,15 mg/l kinetin provide good response, this is accordant with research conducted by Revathy and Arumugam, 2011 described that high number direct somatic embryo proliferation from leaves explant was observed in MS medium containing 3 mg/l 2,4-D.

Embryogenic callus formation occurs because there is the process of proliferation, occurs because there are physiological constraints on growth. The process of callus formation that used of 2,4-D PGR occurred in dark conditions on ± 24^{o} C temperature and 70% humidity.

Embryo development

When white globular embryos of Sandalwood were transfered on to new media containing BAP and Kinetin, it will stimulate the formation and further development of embryo structure, they produced a number of secondary somatic embryo (Fig. 3). A mixture of somatic embryo and friable embryogenic tissue was produced in 2 mg/l BAP.

Maturation of sandalwood embryos showed that treatment clone I / 1.B5.A and media MS + 2 mg/l BAP gave the best response to the development of sandalwood

Table 3. The respond genotype of each clone and concentration of BAP on growthrate sandalwood embryo maturation after incubation for 8 weeks

Clone	Replication	BAP Concentration			
		CND0	CND1	CND2	CND3
I/1.B.5A	1	0.3	0.2	0.4	0.4
	2	0.5	0.1	0.2	0.2
	3	0.2	0.1	0.5	0.3
B. PROV.01	1	0.1	0.3	0.4	0.2
	2	0.1	0.1	0.2	0.1
	3	0.1	0.3	0.2	0.1
K	1	0.2	0.1	0.4	0.1
	2	0.1	0.1	0.3	0.2
	3	0.2	0.1	0.1	0.2
I/2.B.08	1	0.2	0.3	0.1	0.1
	2	0.4	0.2	0.3	0.1
	3	0.5	0.2	0.4	0.2

Description : CND0 = 0 mg/l BAP ; CND1 = 1 mg/l BAP 1; CND2 = 2 mg/l BAP; CND3 = 3 mg/l BAP

Table 5. Anova embryo maturation of sandalwood after incubation in the dark for 8 weeks

Source	Sum of Square	df	Mean Square	F	Sig.
Clone	0,099	3	0,033	3,045*	0,043
Media	0,107	3	0,036	3,301*	0,033
Clone x Media	0,172	9	0,019	1,736^{ns}	0,115
Galat	0,347	32	0,011		
Total	3,110	48			

Description : * = significant, ^{ns} = non significant

embryogenic callus maturation. There was a significant (P<0.05) influence of the clones and concentration of BAP on the embryo development of sandalwood. Similarly, the clones had significant (P<0.05) effect on the embryo development (Table 6). Clone I/1.B.5A had the highest callus weight (0,2833 g**).** The concentrations of BAP had significant (P<0.05) effect on the embryo development (Table 7). Medium supplemented with 2 mg/l BAP had the highest callus weight concentrations had the highest callus weight (0,2917 g**).**

To determine the level of difference followed by DMRT test, the results of the analysis are presented in Table 6 and 7.

Means within the same column followed by same letters are not significantly by Duncan's Multiple Range Test at 5 % probability level. The values are means of twelve replications.

Table 6. The effect genotype and BAP concentration to the Sandalwood maturation

Treatment	Clone
K	0,1750a
B.Prov.01	0,1833a
½.B.08	0,2500ab
1/1.B.5A	0,2833b

Table 7. The effect of PGR BAP concentration to the Sandalwood Maturation

Treatment	PGR BAP concentration
CND1	0,1750a
CND3	0,1833a
CND0	0,2417ab
CND2	0,2917b

Means within the same column followed by same letters are not significantly by Duncan's Multiple Range Test at 5% probability level. The values are means of twelve replications.

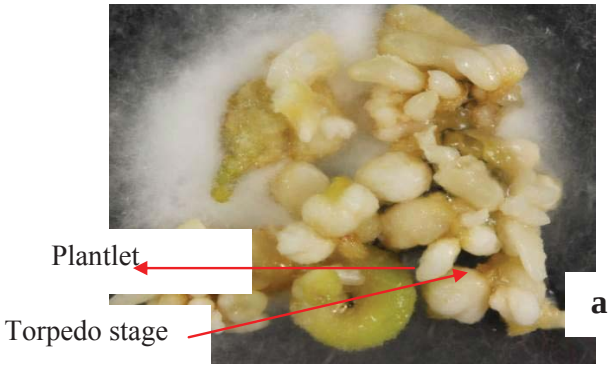

Figure 3a. Embryo development (maturation) of Sandalwood

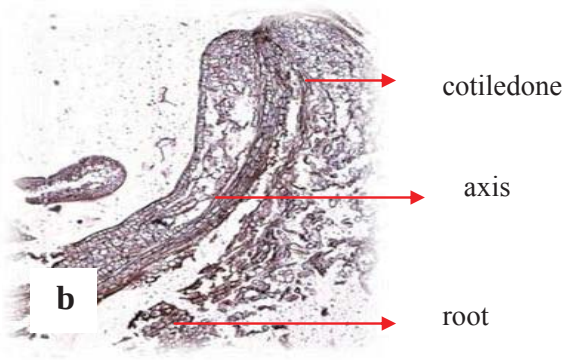

Figure 3b. Histologycal torpedo stage of Sandalwood

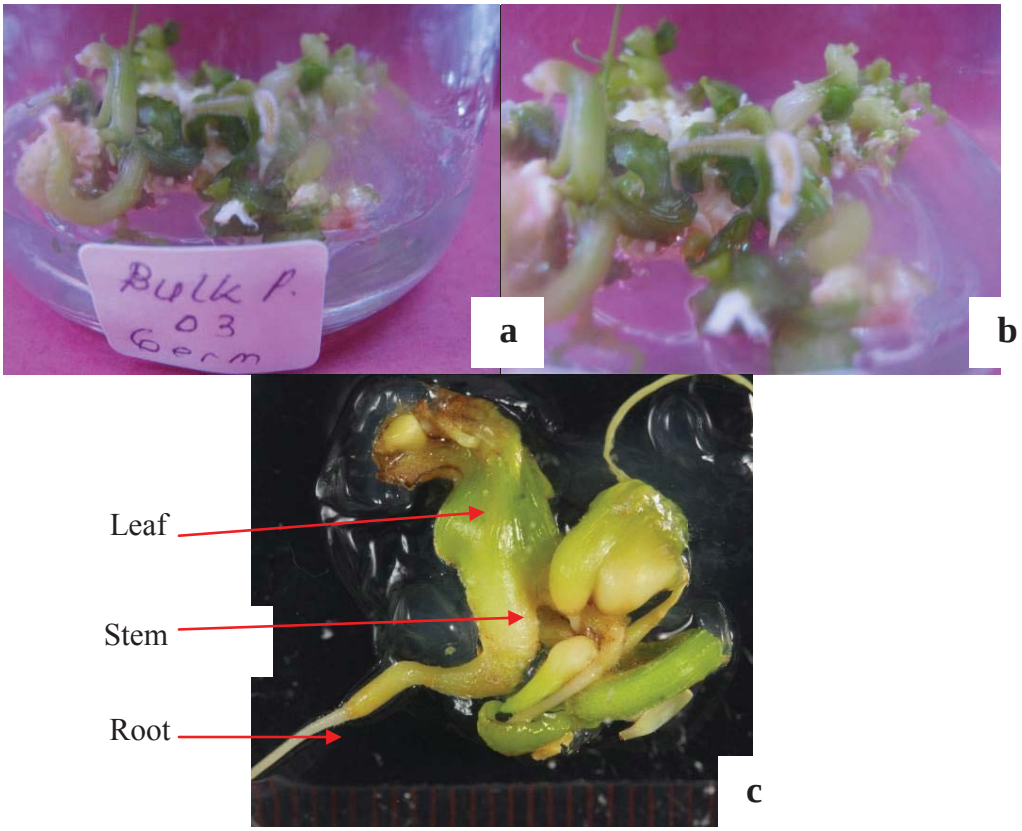

Figure 4. (a, b, and c) Sandalwood plantlet 8 weeks after somatic embryogenesis

Effect of various genotype on sandalwood clones and PGR BAP concentrations to maturation of Sandalwood can be seen in Fig. 3 and 4.

Media culture is one of the mportant factor for plant micro propagation in this study. Medium were used Murashige and Skoog, 1962 because MS is a privilege of the media content of nitrate, ammonium and potassium high. It contains a decent amount of inorganic nutrients to meet the needs of different types of plant cells in culture (Wetter dan Constabel, 1982).

When explants (calluses with embryogenic structures) were transferred to medium supplemented with different

concentrations of BAP, they all survived. However, the concentrations of BAP had a significant ($P<0.05$) effect on the percentage of matured somatic embryos (Table 7).

The second step of the protocol is related to embryo convertion to plantlets and roots formation. This step is performed on CND2 medium. Embryo structures with well-developed cotyledonary leaves are detached very carefully from the explants and planted on petri dishes containing CND2 medium (Fig. 4). For a period of 60 days, embryo develop into plantlet and roots formation.

Conclusion

The result showed that the young leaves explants gave good responce to induce friable callus of sandalwood. MS medium suitable to be used in somatic embryogenesis of sandalwood. Callus induction using MS medium showed that clone K gave the best response on development of embryogenic callus. High number direct somatic embryo proliferation from leaves explant was observed in MS medium containing 3 mg/l 2,4-D. The solid MS medium + 2 mg/l BAP gave the best response to develop embryogenic callus of sandalwood. The result suggested that somatic embryogenesis response of sandalwood was affected by several factors including plant species, genotype, age, explant type, season, carbon source, type and concentration of exogenous growth regulators.

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