Micropropagation of Sumatra Benzoin (*Styrax benzoin* Dryander) to Obtain Plant Seedling

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Abstract: The strategy to produce high quality seedling of Sumatra Benzoin (*Styrax benzoin* Dryander) is very urgent as a technique to provide seedling for industry and forestry purposes in Indonesia. The limitation number of good quality seedling becomes the main problem because generative propagation is the only strategy that has been conducted to produce *Styrax benzoin* seedling, which are obtained naturally grown from the seed around the mature styrax trees. Micropropagation is an alternative to produce good quality seedling of Sumatra Benzoin. The study is aimed to obtain optimum conditions for micropropagation of Sumatra Benzoin as a step in the production of styrax seedling for forestry purposes. The study is performed in a completely randomized design with using the concentrations of growth stimulators NAA (0 - 3 mg/L) and BAP (0 - 3 mg/L). The results showed that growth stimulator influenced the growth of the calli. The NAA and BAP was effective to induce calli of the benzoin in culture medium. The concentration combination of 3 mg/L NAA and 3 mg/L BAP was the best condition to obtain the heaviest callus. The micropropagation step obtained in this study is promising for mass production of *Styrax benzoin* seedling.

1 INTRODUCTION

The conservation of forest by using woody plants that containing bioactive compounds is very strategic in Indonesia to preserve the trees as well as avoid the logging since the tree can be used as a source of income to the people around the forest. One of the potential trees is Sumatra Benzoin that are grown well in Indonesia. The skin saps of Styrax produce incense containing bioactives that can be used in medical and cosmetics purposes (Singh, *et al.* 2017). Sumatra Benzoin is a Divisio of Spermatophyta, sub Divisio of Angiospermae, where the Class is Dicotyledonae, Ordo is Ebenales, the Family is Styraceae, Genus is Styrax, and the Spesies *Styrax benzoin* Dryander (Nurwahyuni and Elimasni, 2006). Sumatra Benzoin grown well in North Sumatera Indonesia and become forest commodity, and it becomes income generate to the people around the forest in many regencies in Indonesia (BPS, 2017). The insence of benzoin was commonly use as traditional and modern medicine (Fan, *et al.* 2017; van Wyk, 2009; Sianipar and Simanjuntak, 2000). The Sumatra Benzoin has been known to be valuable tree and have high prospect, however, the cultivation of Sumatra Benzoin has not been done properly in Indonesia. Production of high quality styrax seedling become a problem since the peasants in North Sumatera are still depend on a generative propagation for Benzoin seedling. Therefore, the existence of Sumatra Benzoin was merely obtained from naturally grown in the forest due to the difficulty to obtain good quality of styrax seedling. It has been predicted that the existence of Sumatra Benzoin will be lower down in the future if there is no action being carried out to plant the Sumatra Benzoin in North Sumatera Indonesia.

Micropropagation technique is a good alternative to produce high quality seedling of wood trees. Mass production of the seedlings similar to its parent plant can be provided with this technique (Mirani, *et al.* 2017; Pniewski, *et al.* 2017; Martínez-Estrada, *et al.* 2017; Gashi, *et al.* 2015). The source of explant for micropropagation can be come from seed, leaf blade, petiole, stem segment, axilar shoot, and meristemic axilar from young plant with active cell (Panigrahi, *et al.* 2017; Pedro, *et al.* 2017; Ahmed and Anis, 2014; Chen, *et al.* 2014). The strategy was made with plated the explant in MS medium containing mineral salts, amino acid, vitamin, glucose and growth stimulator at certain composition (Zhang, *et al.* 2016; Murashige and Skoog, 1962; Murashige and Tucker, 1969). Various types of growth stimulators have been introduced such as auxin (NAA, 2,4-D, IBA, etc.) and

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cytokinin (BA, kinetin, dan zeatin) for plants propagation (Trettel, *et al.* 2017; Quiroz, *et al.* 2017; Yakhin, *et al.,* 2017). It has been obtained that the growth stimulators influenced the development of the plants (Quiroz, *et al.* 2017; Shinde, *et al.* 2016). It has been known that low concentration cytokinine is found to be effective to generate the callus and the shoot from stem segment (Nurwahyuni, *et al.* 2017; Nurwahyuni, *et al.* 2015), and the auksin to regenerate the roots (Shukla, *et al.* 2017; Alatar, *et al.* 2017). Therefrore, the strategy has to be made to optimise the concentration of growth stimulators in the regeneration of new target plants (Thiem, *et al.* 2017); Nurwahyuni and Sinaga, 2014; Soni and Kaur, 2014). Based on literature studies, the paper publish on propagation of benzoin is very limited, only to study the activity of cytotoxisity (de Oliveira, *et al.* 2016) and *in vitro* propagation (Nurwahyuni and Elimasni, 2006; Nurwahyuni, 2005). Therefore, Benzoin micropropagation techniques applied in this study was carried out followed the success for propagation of medicinal woody plants (Baskaran, *et al.* 2017; Cardoso, *et al.* 2017; Wu, *et al.* 2014). This research was aimed to implement micropropagation technique to propagate Sumatra Benzoin (*Styrax benzoin* Dryander) as a strategy to obtain good quality of styrax seedling.

2 RESEARCH METHODS

2.1 Materials and Method

The study was conducted at Universitas Sumatera Utara, in the Department of Biology. Mother plant of Sumatra Benzoin (*Styrax benzoin* Dryander) was obtained from the forest at Kecamatan Pergetteng-Getteng Sengkut, Kabupaten Pakpak Bharat, North Sumatera, Indonesia. The procedures carried out in this study are consisted of sterilization of equipments, preparation of medium solution, micropropagation, and data collections followed the micropropagation procedures explained previously (Nurwahyuni and Sinaga 2018; Nurwahyuni, 2016).

2.2 Sterilization of Equipment

The equipments were sterilized by soaking them in hot detergent, followed by rinsing with sterile water flow and keep to dry. They are then wrapped in aluminium foil and sterilized in oven at 180^{o} C for 2 hours. Glass wares are sterilized by using autoclave at 121 ^{o}C, 15 psi for 15 minutes. The laminar air flow cabinet (LAFC) was sterilized using UV light and alcohol (70%).

2.3 Preparation of Medium Culture

Medium culture used in this study was MS medium containing sugar, nutrients (macro, micro, and trace), with supplementation of α-napthaleneacetic acid (NAA) and benzyl amino purin (BAP) in various combination concentrations (Nurwahyuni and Sinaga 2018; Nurwahyuni, 2016). The solution was prepared in sterile water and transferred in to an erlenmeyer and the stock solution was stored in the fridge when not in use. The buffer solutions (pH 5.8 - 6.8) were prepared and they were sterilized (121 ^{o}C, 15 lb) for 20 minutes. The variation compositions of growth stimulator (0 - 3 mg/L) BAP and (0 - 3 mg/L) NAA were prepared with five replicates (Zar, 1996) as summarized in Table 1. The medium consisted of sucrose that are enriched with growth stimulator of NAA and BAP. Callus initiation was performed in MS culture medium enriched with growth stimulator (Murashige and Skoog, 1962).

Tabel 1: Experimental design for micropropagation of Sumatra Benzoin (*Styrax benzoin* Dryander) in MS medium containing of α-napthaleneacetic acid (P) and benzyl amino purin (Q). The experiments are carried out with 5 replicates.

		Citokinine BAP (Q)			
		0	1	2	3
Auxine NAA (P)	0	P_0Q_0	P_0Q_1	P_0Q_2	P_0Q_3
	1	P_1Q_0	P_1Q_1	P_1Q_2	P_1Q_3
	2	P_2Q_0	P_2Q_1	P_2Q_2	P_2Q_3
	3	P_3Q_0	P_3Q_1	P_3Q_2	P_3Q_3

P_0 = Naphthalene acetic acid 0.0 mg/L; P_1 = Naphthalene acetic acid 0.5 mg/L; P_2 = Naphthalene acetic acid 1.0 mg/L; P_3 = Naphthalene acetic acid 3.0 mg/L; Q_0 = Benzyl Amino Purin and BAP 0.0 mg/L; Q_1 = Benzyl Amino Purin and BAP 0.5 mg/L; Q_2 = Benzyl Amino Purin and BAP 1.0 mg/L; Q_3 = Benzyl Amino Purin and BAP 3.0 mg/L.

2.4 Micropropagation Procedures

The explants used in the study are obtained from mature seeds of Sumatra Benzoin (*Styrax benzoin* Dryander) that was taken from good quality benzoin (Fig. 1a). The seed is then opened and the embryo is taken (Fig. 1b). The explants for micropropagation is prepared from healthy benzoin seeds (Fig. 1c) which are then sterilized, and were then cut (0.5 - 1.0 cm long), successively washed in water and detergent, followed by immersion in 70% (v/v) ethanol for 5

min, in a solution of 20% (v/v) sodium hypoclorate (0.8 %w/v NaClO) for 20 min, followed by rinsed with sterile water three times. After sterilization process have been conducted, the explant was cut and inoculated in culture medium with variation in the concentration of 2,4-D and BAP in the culture medium (Fig. 1d). The incubation of cultures were then kept at 25±2 °C. The culture was daily illumination with UV light for 16-h and spray with alcohol 70% regularly.

Figure 1. Preparation of healthy benzoin seeds as sources of explants for micropropagation: (a) A mature seed obtained from good quality Sumatra Benzoin (*Styrax benzoin* Dryander), (b) Typical inner side of Sumatra Benzoin seed, (c) The closed view of Styrax seeds to be used as a source of explant, (d) The explant is planted in cultur media for incubation.

3 RESULTS AND DISCUSSION

3.1 Callus Induction of Sumatra Benzoin

Induction of callus is very important in the propagation of Sumatra Benzoin as it was known that the ability of cell multiplication and differentiation are influenced various factors such as culture medium components, the concentration of growth stimulators, and the intensity of UV light (Nurwahyuni and Sinaga 2018; Nurwahyuni, 2016). Incubation of Sumatra Benzoin (*Styrax benzoin* Dryander) culture has been carried out at various treatment conditions (Figure 2a). It has been observed that the callus induction was starting after 20 days, that was begin with the cell multiplication to become bigger explant until the callus has grown at week three. The culture regenerated to become callus. Growth intensity of the callus are vary depends on the variation of the concentration of growth stimulator in culture medium. Typical growth of the culture is presented in Figure 2b where the callus started to grow from the edge of the explant (Figure 2c-d).

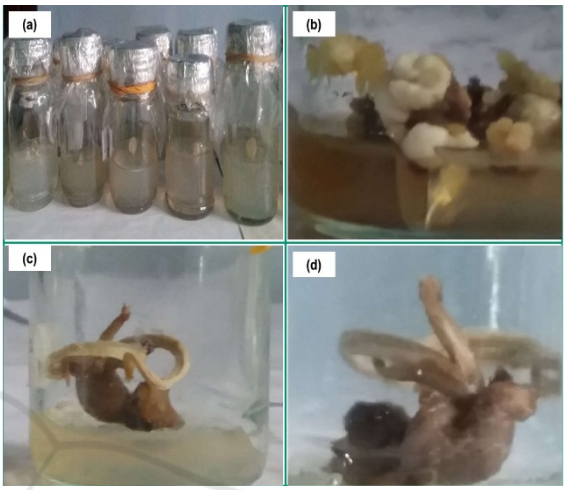

Figure 2. Typical growth of callus in micropropagation of Sumatra Benzoin (*Styrax benzoin* Dryander): (a) Callus initiation of the benzoin in culture medium at various treatment conditions at room temperature; (d) The development of explant to become callus in cultur media, (c) Typical growth of the callus after 21 days, (d) The closed view of the callus,

Micropropagation condition has been optimised to obtain the best condition in the callus induction. The growth of the culture of Sumatra Benzoin with the variation of BAP and NAA in MS culture medium is observed after seven weeks as presented in Table 1. It was observed that in the first and second weeks, the explants are all transformed to bigger explant condition, and some callus are grown after three weeks. The growth intensity of the callus are assigned to be low to high intensity, depends on the variation of growth stimulator supplemented in the culture media. The formation of callus have covered the explants in some treatment conditions, however, some of the explants are having callus in low and medium growth intensity after seven weeks while many of the callus growth are in high intensity (see the results in Table 2). Some cultures are browning without callus due to contamination.

Table 2: Typical growth development of the callus culture on micropropagation of Sumatra Benzoin (*Styrax benzoin* Dryander) in medium culture after incubation for seven weeks.

Experimental Treatment	Growth of the callus (week)						
	1	2	3	4	5	6	7
P_0Q_0	*	*	*	*	*	*	*
P_0Q_1	*	*	+	+	+	+	+
P_0Q_2	*	*	+	+	++	++	++
P_0Q_3	*	*	++	++	+++	+++	+++
P_1Q_0	*	*	+	+	+	+	+
P_1Q_1	*	*	+	+	++	++	++
P_1Q_2	*	*	++	++	+++	+++	+++
P_1Q_3	*	*	+	+	+	++	++
P_2Q_0	*	*	*	*	+	+	+
P_2Q_1	*	*	+	+	++	++	++
P_2Q_2	*	*	+	+	+	+	+
P_2Q_3	*	*	+	+	++	++	++
P_3Q_0	*	*	++	++	+++	+++	+++
P_3Q_1	*	*	+	+	+	+	+
P_3Q_2	*	*	+	+	++	++	++
P_3Q_3	*	*	++	++	+++	+++	+++

P_0 = Naphthalene acetic acid 0.0 mg/L; P_1 = Naphthalene acetic acid 0.5 mg/L; P_2 = Naphthalene acetic acid 1.0 mg/L; P_3 = Naphthalene acetic acid 3.0 mg/L; Q_0 = Benzyl Amino Purin and BAP 0.0 mg/L; Q_1 = Benzyl Amino Purin and BAP 0.5 mg/L; Q_2 = Benzyl Amino Purin and BAP 1.0 mg/L; Q_3 = Benzyl Amino Purin and BAP 3.0 mg/L. (*) The explant grow bigger, (+) low callus growth intensity, (++) medium callus growth intensity, (+++) high callus growth intensity,

3.2 Optimization of Growth Stimulator

The weight of the callus in the culture media have been observed with results from the variation of growth stimulator supplemented in the study. The growth development of the culture was categorised very slow. The weight of the callus obtained at different experimental treatments is presented in Table 3. The results showed that variation of the experimental conditions influenced the weight of callus. The percentage of styrax culture survive until week seven are lies between 50-83%, where treatment condition on P_3Q_1 are only survive 50%, while P_1Q_0 and P_2Q_0 are 83%. It has been observed that the weight of callus increased as increasing the growth stimulator in the culture medium. The heaviest callus is obtained at P_3Q_3 (1.275 gram), that is in the experiment condition with combination of 3 mg/L NAA and 3 mg/L BAP. The growth trend has showed in the data that the concentration of BAP is significantly improved the growth of the callus. Data analysis has revealed that high concentration of NAA and BAP influenced growth intensity of Sumatra Benzoin callus (F_{count} 27.39 > F_{table} 2.44), with significant level at 0.01. The effect of each of the growth stimulator onto the growth intensity of the callus of Sumatra Benzoin has been observed. It was found that the concentration of the NAA in MS media highly influenced the growth intensity of the callus (F_{count} 113.45 > F_{tablet} 4.23). The results also revealed that the concentration of BAP influenced the growth intensity of the Benzoin callus (F_{count} 11.70 > F_{crit} 4.23). The interaction effects for both the NAA and BAP are also found influenced the growth intensity of styrax callus (F_{count} 3.94 > F_{table} 2.44). The concentration of growth stimulators that are supplemented in the culture media become crucial factor in the micropropagation of the Sumatra Benzoin.

Table 3: The influences of the concentration variation of the growth stimulators (NAA and BAP) onto the growth intensity of the callus of Sumatra Benzoin (*Styrax benzoin* Dryander). The results are obtained after incubation seven weeks.

Experimental Treatment	Weight of Callus (Gram) / Replication					Total	Average*
	I	II	III	IV	V		
P_0Q_0	0.016	0.018	0.017	0.015	-	0.066	0.017^{f}
P_0Q_1	0.028	0.030	-	0.028	0.027	0.113	0.028^{ef}
P_0Q_2	0.030	-	0.030	0.030	0.030	0.120	0.030^{ef}
P_0Q_3	0.031	0.031	0.031	0.031	-	0.124	0.031^{ef}
P_1Q_0	0.040	0.039	0.040	0.060	0.031	0.210	0.042^{ef}
P_1Q_1	0.042	0.042	0.041	0.042	-	0.167	0.042^{ef}
P_1Q_2	0.050	0.049	0.051	0.050	-	0.200	0.050^{ef}
P_1Q_3	0.053	0.054	0.053	0.052	-	0.212	0.053^{ef}
P_2Q_0	0.065	0.070	0.060	0.065	0.065	0.325	0.065^{de}
P_2Q_1	0.067	0.068	0.065	0.070	-	0.270	0.068^{de}
P_2Q_2	0.073	0.070	0.075	0.073	-	0.291	0.073^{de}
P_2Q_3	0.088	0.093	0.090	0.102	-	0.373	0.093^{cd}
P_3Q_0	0.147	0.141	0.150	0.147	-	0.585	0.146^{b}
P_3Q_1	0.160	0.165	0.158	-	-	0.483	0.161^{b}
P_3Q_2	0.180	-	0.202	0.191	0.185	0.758	0.190^{b}
P_3Q_3	0.186	-	0.275	0.302	0.512	1.275	0.319^{a}
Total	1.256	0.870	1.338	1.258	0.850	5.572	1.114
Average	0.079	0.067	0.089	0.084	0.142	0.343	0.086

P_0 = Naphthalene acetic acid 0.0 mg/L; P_1 = Naphthalene acetic acid 0.5 mg/L; P_2 = Naphthalene acetic acid 1.0 mg/L; P_3 = Naphthalene acetic acid 3.0 mg/L; Q_0 = Benzyl Amino Purin and BAP 0.0 mg/L; Q_1 = Benzyl Amino Purin and BAP 0.5 mg/L; Q_2 = Benzyl Amino Purin and BAP 1.0 mg/L; Q_3 = Benzyl Amino Purin and BAP 3.0 mg/L. (-) The culture was not grown.

*Data shown are mean of five experiments followed by notation letter are significant according to Duncan's multiple range analysis (P = 0.05)

4 CONCLUSIONS

Micropropagation of Sumatra Benzoin (*Styrax benzoin* Dryander) has successfully conducted to produce callus as a primary step in the propagation of local benzoin. The effect of NAA and BAP onto the growth of the styrax callus has been formulated to obtain optimum condition in the propagation procedures, and become a good step in the production of benzoin seedling. The results have shown that the concentration variation of growth stimulators alone (NAA or BAP), and the combination of both the NAA with BAP, are all acted to induce the callus of Sumatra Benzoin in MS medium, and showed significantly effect to the weight intensity of the Benzoin callus. The best condition for micropropagation of the Sumatra Benzoin was obtained when using combination concentration of 3 mg/L NAA and 3 mg/L BAP (P_3Q_3) that was produced 1.275 gram callus. Supplementation of high concentration of NAA and BAP is effective to improve the growth of callus of Sumatra Benzoin

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