Explant surface sterilization protocol for micropropagation *of Amorphophallus muelleri* Blume

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ABSTRACT
The success of tissue culture is greatly influenced by the explant surface sterilization technique. The presence of bacterial and fungi contamination, and the occurrence of browning on the explants can interfere with the process of culture propagation. High concentration of sterilant agents will inhibit cell division and the growth of explant tissues. This study aims to determine the optimum type and concentration of sterilant agents and how to use them to sterilize explants without causing much damage to explant tissue. The surface sterilization treatment of bulbil explant by soaking in a 250 mg.L-1 cefotaxime antibiotic solution for 30 minutes, and followed by immersion in 0.2% HgCl2 for 7 minutes, followed by soaking in NaOCl 1:2 for 10 minutes showed the best results obtained 60% sterile explants and faster growth of callus cells from explants. Callus grows to form adventitious buds within a period of 7 weeks.

Keywords*: aseptic technique, bulbil, sterilant agent, sterile explant*

1. INTRODUCTION

Porang plant (*Amorphophallus muelleri*) is a member of Araceae family and easy to find in forest, slopes, along the river in tropical regions [1]. Porang plant is potential to be further developed in Indonesia to fulfill the market demand [2]. The useful part of porang is bulbil for its glucomant content, high fiber content, and no cholesterol [3]. Glucomant is widely used as food materials, i.e., flour and manosse, and it is also used for cosmetics, emulsifier, pharmacy industry, and others [4]. Unfortunately, the cultivation of porang plant has been facing some problems, such as slow propagation of porang, long life cycles that lead to low commercial production of porang (3 years after planted), and low yield production which causes some diseases to attack, i.e., soft rot (*Pectobacterium carotovora*) and blight seedlings (*Sclerotinium rolfsii* Sacc.) [5]. Plant tissue culture technique can be chosen to solve the problems.

The success of plant tissue culture technique depends on their explant surface sterilization [6]. Microbe contamination, i.e., fungi, bacteria, and others can decrease the growth of plant [7], hence the explant surface sterilization should be carried out. However, sterilant agents are toxic and can also kill plant tissue, so the proper duration of exposing, concentration sterilant, and type of sterilant must be specified. The requirement of duration, concentration, and type of sterilant can vary from one plant to another depending on their morphological tissue characters [8]. The sterilization method should decrease the contamination, but the plant tissues should also survive.

Various sterilant agent widely used, i.e., sodium hypochlorite (NaOCl), ethanol, $HgCl_2$, and antibiotics, i.e. cefotaxime and carbenicillin. NaOCl, ethanol, and $HgCl_2$ are used to minimize explants surface contamination rate due to chemical toxicity. Ethanol is a powerful sterilizing agent, but phytotoxic, hence the duration of exposure should only be a short time [9]. Cefotaxime antibiotics can also be used to decrease explants endophytic contamination.

According to literature, the optimal explant surface sterilization method for porang's bulbils is indeterminate. So many surface sterilizations of porang's bulbil achieve success for in vitro propagation, but the percentage of sterile explants and regeneration tissues are low [10]. Thus, this research aims to determine the proper explant surface sterilization of porang's bulbils with its regeneration after sterilization.

2. MATERIALS AND METHODS

2.1 Materials

Plant materials in this research were bulbils of porang (*A. muelleri*) obtained from local farmer of porang, Klangoon, Madiun, Indonesia.

2.2 Surface Sterilization

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Bulbils of *A. muelleri* were harvested, then washed under running tap water using commercial detergent solution 5 mL, which contain active compound, such as antibacterial agent 0.3%, biodegradable surfactant, and emillient. Next, the bulbils were soaked in 2 $g.L^{-1}$ fungiside dithane M-45 according to table 1. After that, they were rinsed thoroughly and soaked in 2 $g.L^{-1}$ bacteriside Agrept (table 1). Then, the bulbils were rinsed thoroughly and sterilized in Laminar Air Flow (LAF) Cabinet by soaked in NaOCl:Aquadest (1:1 or 1:2 or 2:1) or 70% ethanol or $HgCl_2$ (0.1% or 0.2%) or 250 $mg.L^{-1}$ cefotaxime antibiotic according to each treatment of explant surface sterilization (table 1). Bulbils were rinsed thoroughly with sterile Aquadest after soaking them in sterilant agent. The bulbils were cultured in Murashige and Skoog Medium (MS) with 5 $mg.L^{-1}$ 6-Benzylaminopurine (BAP) and 0.2 $mg.L^{-1}$ Naphthalene Acetic Acid (NAA). Then, they were incubated at 26°C under a blue light with photoperiod of 16 h light and 8 h dark.

Treatment of surface sterilization shown at table 1 below:

Table 1. Treatment of *Amorphophallus muelleri*'s Bulbils Explant Surface Sterilization

Sterilization Method Code	Sterilant Agent		Protocol	Duration (Minutes)
T1	Outside LAF	Detergent	washed under running tap water	clean
		Fungicide	soaked	60
		Bacteriside	soaked	60
	Inside LAF	NaOCl 1:1	soaked	10
		NaOCl 2:1	soaked	15
		Sterile aquadest	rinsed	clean
T2	Outside LAF	Detergent	washed under running tap water	clean
		Fungicide	soaked	120
		Bacteriside	soaked	120
	Inside LAF	70% Ethanol	soaked	3
		NaOCl 1:1	soaked	10
		Sterile aquadest	rinsed	clean
T3	Outside LAF	Detergent	washed under running tap water	clean
		Fungicide	soaked	120
		Bacteriside	soaked	120
	Inside LAF	0.1% $HgCl_2$	soaked	5
		NaOCl 1:1	soaked	10
		Sterile aquadest	rinsed	clean
T4	Outside LAF	Detergent	washed under running tap water	clean
		Fungicide	soaked	180
		Bacteriside	soaked	120
	Inside LAF	0.2% $HgCl_2$	soaked	7
		NaOCl 1:1	soaked	10
		Sterile aquadest	rinsed	clean
T5	Outside LAF	Detergent	washed under running tap water	clean
		Fungicide	soaked	180
		Bacteriside	soaked	120
	Inside LAF	0.2% $HgCl_2$	soaked	7
		NaOCl 1:2	soaked	15
		Sterile aquadest	rinsed	clean
T6	Outside LAF	Detergent	washed under running tap water	clean
		Fungicide	soaked	180
		Bacteriside	soaked	120
	Inside LAF	250 $mg.L^{-1}$ Cefotaxime	soaked	30
		0.2% $HgCl_2$	soaked	7
		NaOCl 1:2	rinsed	10
		Sterile aquadest	soaked	clean

2.3 Data Analysis

Bulbils were sterilized using various methods (table 1) and the percentage of explant condition (sterile, total emerged fungi, total emerged bacteria, and deceased plant) were observed until 4 weeks after treatment (table 2). After that, steril bulbils will be further observed regarding their regeneration in culture media. Regeneration of bulbil's explant observed by the diameter bulbil's explant (table 3). The diameter of bulbil's explant were statistically analyzed using one way ANOVA (Analysis of Variance). Duncan's Multiple Range Test (DMRT) at 5% error level (α=0.05) was used in the case of significant difference was observed (table 3).

3. RESULTS AND DISCUSSION

Contamination is one of the most problems on plant tissue culture, which can inhibit the growth of cultures. Contamination is commonly caused by endophytic contaminants, environment, culture medium, and others [11]. To solve the problems, proper surface sterilization is required. Surface sterilization is a method to decontaminate microbe from explant, but sterilant agent is also toxic for plant tissue, hence the proper concentration, duration, and type of sterilant agent must be determined [9]. The percentage of sterile explant and contaminant during culture initiation stage for 4 weeks can be seen at table 2 below:

Table 2. Percentage of sterile explant and contaminant during culture initiation stage for 4 weeks

Sterilization Method Code	Week-1	Week-2	Week-3	Week-4	Explant Condition	Percentage (%)
T1	10	5	2	0	Sterile	0
	1	4	4	6	Fungi	30
	9	10	12	12	Bacteria	60
	0	1	2	2	Deceased explant	10
T2	6	4	1	0	Sterile	0
	7	7	8	8	Fungi	40
	5	7	7	8	Bacteria	40
	2	2	4	4	Deceased explant	20
T3	2	0	0	0	Sterile	0
	8	9	9	9	Fungi	45
	5	6	6	6	Bacteria	30
	5	5	5	5	Deceased explant	25
T4	13	12	10	8	Sterile	40
	2	3	4	4	Fungi	20
	1	1	2	4	Bacteria	20
	4	4	4	4	Deceased explant	20
T5	15	10	8	7	Sterile	35
	1	4	6	7	Fungi	35
	2	3	3	3	Bacteria	15
	2	3	3	3	Deceased explant	15
T6	18	15	12	12	Sterile	60
	1	2	5	5	Fungi	25
	0	0	0	0	Bacteria	0
	1	3	3	3	Deceased explant	15

Note: 20 total explants for each treatment

For the first step, explant was washed with detergent to remove any foreign contaminants and rinsed thoroughly under running tap water. According to table 1, the increasing duration of explant exposure to fungicide can decrease the percentage of fungi contamination (table 2), which can be seen at method T1, T2, and T3. Fungicide is usually used to control of certain fungus disease of ex vitro vegetables, fruits, crops, and in vitro propagation. Moreover, NaOCl is commonly used as sterilant agent, when NaOCl is diluted into water, the hypochlorite salts lead to formation of HClO with bactericidal activity. It may be due to lethal DNA damage [12]. However, using only NaOCl for sterilization of bulbils was not effective since the percentage of sterile explant was 0% (table 2).

$HgCl_2$ and 70% ethanol are usually chosen to combine with others sterilant agents, but they are classified into toxic sterilant agents, hence, the duration of explant exposure to its sterilant agents should only be a short time to prevent toxicity for themselves tissues. To improve effectiveness of ethanol, it is commonly used earlier for treatment with other compounds, but 70% ethanol is not effective in this research because there is no sterile

explants obtained (table 2). Exposing explants to $HgCl_2$ may lead to the browning and death of explants [13]. However, according to table 1, 0.2% $HgCl_2$ was more effective to remove the contaminants than 0.1% $HgCl_2$ (table 2), where the contamination of fungi decreased from 45% (T3) to 20% (T4). 0.2% $HgCl_2$ was also effective to remove the bacteria contamination (table 2).

According to table 2, the proper surface sterilization method was T6 with longer duration exposure of fungicide than T1, T2, and T3, which was more effective to remove fungi on explant culture. Moreover, combination sterilant agents of cefotaxime antibiotic, 0.2% $HgCl_2$, and NaOCl 1:2 were effective to reduce contamination by producing 60% sterile explants. Cefotaxime antibiotic was used to reduce endophytic contaminants. According to [14], using 500 $mg.L^{-1}$ cefotaxime was effective to reduce contamination on Elite Enset (*Ensete ventricosum* Welw). Although $HgCl_2$ is toxic, in the proper duration and concentration it would be an effective sterilant agent. The appropriateness of T6 method can be seen not only by the lowest contamination, but also by the deceased explants. T6 method also resulted the lowest deceased explants, which is expected to facilitate the regeneration of explants after damaged of sterilant agents.

Regeneration of bulbils's explant must be observed after the surface sterilization because it does not only sterilize the explant, but it also may regenerate into plantlet. Regeneration of bulbil's explant observed by the diameter bulbil's explant can be seen on table 3 below:

Table 3. Regeneration of bulbils's explant during 4 weeks after surface sterilization

Sterilization Method Code	Diameter bulbils's explant (cm)			
	Week-1	Week-2	Week-3	Week-4
T1	0^a	0^a	0^a	0^a
T2	0^a	0^a	0^a	0^a
T3	0^a	0^a	0^a	0^a
T4	0.02 ± 0.003^b	0.09 ± 0.006^b	0.18 ± 0.005^c	0.35 ± 0.008^b
T5	0.02 ± 0.004^b	0.05 ± 0.007^a	0.12 ± 0.008^b	0.29 ± 0.006^b
T6	0.08 ± 0.013^c	0.17 ± 0.015^c	0.32 ± 0.021^d	0.85 ± 0.002^c

Note: Values followed by the different letter in the same column were significantly different ($p < 0.05$ by DMRT).

The optimal surface sterilization was soaked into 250 $mg.L^{-1}$ cefotaxime during 30 minutes and followed by 0.2% $HgCl_2$ during 7 minutes. After that, it was soaked at NaOCl 1:2 for 10 minutes, which resulted 60% sterile explants with low percentage of deceased explant (15%), but reached high regeneration (table 2 and table 3). According to table 3, T6 method can result in the highest diameter bulbil's explant, i.e. (0.85±0.002) cm. In addition, there was a formation of white nodular callus visually within 4 weeks as seen in figure 1.

Regeneration of *A. muelleri* bulbils's explant to callus and adventitious shoot during 7 weeks after explant surface sterilization can be seen in figure 1 below:

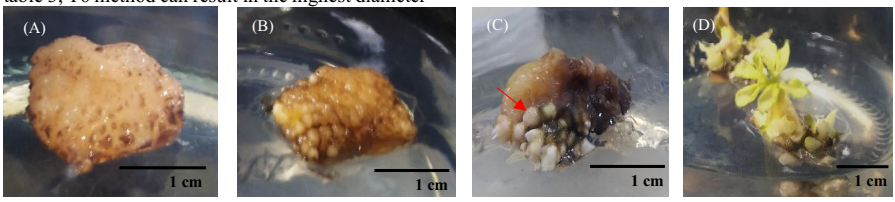

Figure 1 Regeneration of *A. muelleri* bulbils's explant to callus and adventitious shoot
(A) 1 week after explant surface sterilization
(B) 2 weeks after explant surface sterilization
(C) 4 weeks after explant surface sterilization
(D) 7 weeks after explant surface sterilization
Note: white nodular callus (red arrow 1C)

4. CONCLUSION

The proper explant surface sterilization for bulbils of *A. muelleri* was soaking it at fungicide and bacteriside on the outside of LAF, then soaked with 250 mg.L^{-1} cefotaxime antibiotic during 30 minutes, 0.2% $HgCl_2$ during 7 minutes, and 50% NaOCl (NaOCl 1:2) during 10 minutes, with resulted 60% sterile explants of bulbils's *Amorphophallus muelleri*.

AUTHORS' CONTRIBUTIONS

F.I drafted and corrected the manuscript**; A.N.W** drafted the manuscript**; W.D.S** drafted the manuscript; analyzed the data; **A.M** performed the experiments and collect data; **M.A.T** performed the experiments and collect data; **P.H.H** drafted and corrected the manuscript, supervised, funding, and resources, and also designed the experiment. All the authors have read and agreed to this manuscript.

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