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The Effect of Different Sterilization Methods on Obtaining Sterile Leaf Explants of Porang (*Amorphophallus muelleri* B.)

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ABSTRACT

Porang (*Amorphophallus muelleri B.*) is a type of tuber that contains glucomannan, which is used as a raw materials for the food, chemical, pharmaceutical and cosmetic industries. In vitro culture as porang propagation method using plant parts (leaf explants) was developed under aseptic environmental conditions. Sterilization is an important aspect in the success of micropropagation in obtaining sterile explants. This research method used a Completely Randomized Design (CRD) with 2 factors, namely clothes bleach sterilant containing NaOCl 5.25% (Bayclin®) at 30% concentration for 15 minutes (Treatment A), and using clothes bleach containing NaOCl 5.25% (Bayclin®) at 30% concenteration for 15 minutes + 70% alcohol for 15 seconds (Treatment B). Each treatment was filled with 3 explants, and every treatment was repeated 27 times, so that experimental units were obtained with a total of 162 explants. Treatment B is the best treatment in obtaining sterile explants, because it is more effective in removing contamination and inhibiting contamination and browning with survival the highest rate of 51.85% and the lowest mortality rate of 48.15%, it can also suppress and inhibit the appearance of contamination and browning compared to treatment A.

INTRODUCTION

Porang *(Amorphophallus muelleri B.)* is a type of tuber that contains glucomannan. Glucomannan is a hydrophilic polysaccharide (starch) derived from plant tubers, and is produced through processing, extraction and purification which is utilized because it has biological properties such as immunomodulatory, anti-tumor, and anti-diabetic (Shi, 2020), Effectively lowers blood sugar and cholesterol levels (Alamsyah, 2019), as an additive in the manufacture of artificial rice based on *modified cassava flour* (mocaf) (Yuwono, 2013), coating material on microcapsules, because it has the potential as a natural biopolymer in the food microencapsulation process (Anwar, 2017), paper adhesives, drug tablet fillers, and cosmetics

(Sari, 2015), and make porang as a plant producing raw materials in the food, chemical, pharmaceutical and cosmetic industries.

Glucomannan in porang tubers, which has many benefits, increases the need and interest for porang plants in fresh and processed forms, making porang plants an export commodity for the food crop sub-sector in several destination countries. According to statistical data on the export of porang plants in 2017-2020, the total exports of porang plants have increased every year. In 2017 as many as 5,505.38 tons (BPS, 2017), and in 2020 as many as 16,002.2 tons (BPS, 2020), with a total increase in export volume reaching 191% and total export volume in 2011 reaching 41,549.49 tons.

The strategy to increase the production of porang plants, by mass procurement and improvement of planting material, can be carried out using conventional in vitro culture techniques or biotechnology. In the conventional method, constraints such as planting material in the form of stem tubers and leaf tubers/tubers that are dormant in the dry season, the growth cycle is quite long, which is around 38-43 months (Sumarwoto, 2005), generative reproduction takes 3-4 years to produce flowers, fruit and seeds (Hidayat, 2013), and planting material in the form of tubers with natural flower buds, it takes about 4 weeks to reach the anthesis phase with optimum seeds can be harvested after 8-9 months of the anthesis phase (Sari, 2019), cause difficulties and delays in production activities.

In vitro culture technique is applied as a method of porang plant propagation, because it has advantages such as relatively fast clone propagation, genetic uniformity, provision of micro plant stocks, year-round crop production, and can reproduce plants that are difficult to propagate conventionally (Zulkarnain, 2018). In vitro culture as a development of plant biotechnology techniques, is used to reproduce plants that are difficult to reproduce generatively, by vegetative way using plant parts containing somatic cells, which are bred in aseptic environmental conditions, and optimally based on three principles like totipotency cell, redifferentiation, and competence (Widyastuti, 2018).

Contamination is one of the factors that affect the success of plant tissue culture activities. Contamination occurs in consequence of growth and development of microorganisms such as fungi and bacteria. Contamination can also be supported by several factors such as planting media and environmental factors such as temperature and humidity that encourage the growth of contaminants. Contamination can also cause death in explants if not immediately handled or rescued. Explants contaminated with bacterial or fungal contaminants will quickly spread to the entire surface of the explants, so that they can infect other healthy explants contained in one bottle of culture media. This can occur because of the competition for media nutrient absorption between explants and contaminants (Handayani et al, 2021)

Sterilization is one of the important aspects in the success of micropropagation on tissue culture. Sterilization is the initial stage on tissue culture technique method in obtaining sterile explants, which determines the success or failure of a micropropagation in vitro culture technique, because if the sterilization stage is not successful then the culture technique is impossible to do. Failure of the sterilization method is usually caused by contamination of

microorganisms such as fungi and bacteria which can damage cells or tissues in the cultured explants and cause stunted growth or even death of the explants (Adawiyah *et al*, 2021). Sterile materials such as detergent, tween 80, bactericide, fungicide, clorox/bleach (NaOCl), 70% alcohol, PPM solution, vitamin C/*ascorbic acid*, and *povidone-iodine* or antiseptic are sterilants commonly used in sterilization activities, and can affect the quality of the explants produced based on the color of the post-sterilization explants (Surya, 2020). The purpose of this study was to determine the effect of different sterilization techniques on obtaining of sterile explants of porang (*Amorphophallus muelleri* B.).

METHOD

Location and Time

The research was carried out at the Plant Ecophysiollogy and Tissue Culture Laboratory of the Agronomy Study Program, Faculty of Agriculture, University of Jember.. This research was carried out from September 2021 to February 2022

Tools and Materials

Laminar Air Flow Cabinet (LAFC), culture bottles, tweezers, Bunsen lamps were used in this research as tools were used in this research as a tools. Porang leaves (ex vitro) explant were obtained from culture collection in Echophisiology and Plant Tissue Culture Laboratory, Faculty of Agriculture Jember University, clothes bleach (Bayclin®) with the active ingredient NaOCl 5.25%, alcohol 70%, aquadest and Murashige and Skoog Basal media (MS Basal) with additives such as sucrose (30 $g.L^{-1}$), agar (8 $g.L^{-1}$) used in this study as an materilas.

Method of Collecting Data

This research method used a Completely Randomized Design (CRD) with 2 factor, that are the sterilization method uses a sterilizing agent in the form of clothes bleach (Bayclin®) solution concentration of 30% for 15 minutes (Treatment A), and the sterilization method uses a sterilizing agent in the form of clothes bleach (Bayclin®) for 15 minutes + 70% alcohol for 15 seconds (Treatment B). Each treatment was filled with 3 explants, and every treatment was repeated 27 times, so that experimental units were obtained with a total of 162 explants.

Observation Variable and Data Analysis

The observed variables on the effect of the sterilization method include:

1. Explant Mortality and Survival Rate

Mortality rate was observed visually every day, and by counting the number of dead explants which were differentiated based on the cause of death (bacterial contamination, fungal contamination, browning) by the number of explants planted in each replication, and survival rate was observed visually every day, and by counting the number of living explants with the number of explants planted in each replication, with the following equation:

Bacterial contamination percentage : $\frac{\Sigma \text{ Explant Contaminated with Bacteria}}{\Sigma \text{ Explants Planted}} x100\%$

Fungal contamination percentage : $\frac{\Sigma \text{ Explant Contaminated with Fungus}}{\Sigma \text{ Explants Planted}} x100\%$

Browning percentage : $\frac{\Sigma \text{ Explant Browning}}{\Sigma \text{ Explants Planted}} x100\%$

Percentage of live explants : $\frac{\Sigma \text{ Explant Survived}}{\Sigma \text{ Explant Planted}} x100\%$

2. The Early Day of Explant Contamination and Browning

The early day of explant contamination was carried out by observing the explants every day, which was expressed in days after planting notation by looking at the signs of explants that showed visual contamination, and observing morphological changes. browning usually indicates a change in color to brown.

The data were analyzed qualitatively and quantitatively. Quantitative data tested statistically by using descriptive statistic, and qualitative data were observed visually will analyzed descriptively.

Research Procedures

1. Leaf explants from the field were washed using soap or detergent and running water in the sink. Washing by gently rubbing the top and bottom surfaces of the leaf explants from dirt, until they are completely clean. Sterilization of explants at LAFC by inserting leaf explants in a bottle containing a sterile solution.
2. The first method uses *Sodium Hypochlorite* (NaOCl) derived from clothes bleach (Bayclin®) which has an active ingredient of 5.25% NaOCl with a concentration of 30% which has been diluted using aquadest, which is used as a disinfectant solution and shaken gently for ± 15 minutes.
3. The second method is a combination of 30% NaOCl (Bayclin®) solution which has been diluted with aquadest, gently shaken for ± 15 minutes and then followed by 70% alcohol solution and shaken for ± 15 seconds.
4. Sterilization by soaking all parts of the leaf explants and shaking them slowly, so that the explants are thoroughly exposed to the disinfectant solution. The explants that have gone through the sterilization stage are then washed using aquadest and gently shaken until clean for ± 3-6 minutes for 3 replications before being planted in MS media culture bottles.

RESULT AND DISCUSSION

Mortality and Survival Rate

The sterilization method used in this study was sterilization using clothes bleach (Bayclin®) with the active ingredient NaOCl (*Sodium hypochlorite*) 5.25% at a solution concentration of 30% for 15 minutes (Treatment A), and a combination of clothes bleach sterilant (Bayclin®).) 30% concentration for 15 minutes and 70% alcohol concentration for 15 seconds (Treatment B). The treatment of different sterilization methods aims to determine the effect and level of effectiveness of the sterilant methods and materials in cleaning explants from contamination and preventing browning of explants, which gives different results and tendencies towards explant mortality rates and explant survival rates in each sterilization treatment (Table 1).

Table 1. The effect of sterilization method on mortality rate and survival rate explants

Sterilization Treatment	Explants Planted	Explants Death (%)	Mortality Rate			Survival Rate (%)
			Bacterial Contamination (%)	Fungal Contamination (%)	Browning (%)	
A	81	61,73	44,44	3,70	13,58	31,27
B	81	48,15	29,63	3,70	14,82	51,85

According the results of the data (Table 1), that the highest percentage of explant deaths occurred in treatment A by 61.73% (50 explants died), which was caused by contamination with the highest type of contaminant originating from bacterial contaminants of 44.44% (36 explants were contaminated with bacteria), followed by browning 13.58% (11 explants browning), and fungal contaminants 3.70% (3 explants contaminated with fungi), with a survival rate of 31.27% (31 explants survived). In treatment B the highest percentage of explant death was also caused by contamination of 48.15% (39 explants died) with the highest type of contaminant coming from bacteria at 29.63% (24 explants contaminated with bacteria), followed by browning 14.82% (13 browning explants), and 3.70% fungal contaminants (3 explants contaminated with fungi), with a survival rate of 51.85% (42 explants survived).

The phenomenon of high types of contamination caused by bacterial contaminants in each treatment is thought to be influenced by several factors such as planting material. Planting material from the field (not from tissue culture) has a higher risk of contamination and is susceptible to infection, compared to explants from tissue culture which are relatively more sterile. Explants from the field, especially leaf explants are more susceptible to fungal or bacterial contamination, that presumably because the leaves are in direct contact with the air, which is a source for microorganism, bacteria and fungal spores, so that potentially increasing contaminants attached to the leaf surface (Shofiyani, 2010), as well as the morphological structure of flat leaves, causing reduced effectiveness during the sterilization process because the sticking of leaf surfaces to each other (Juarna, 2016).

The high percentage of explant deaths in treatment A was due to contamination compared to treatment B, presumably because the sterilant material, concentration, and soaking time of explants were less effective and in cleaning contaminants on the surface of the explants, so that contaminants were still attached to the explants and were not lost during the sterilization process. , causing explants to be contaminated when planted and become one of the causes of explant death. The high bacterial contamination was thought to have come from explants from the field and contact with microorganisms attached to the leaf surface, while fungal contamination was suspected that fungal spores still attached to the explant surface. During the soaking process, the explants became less effective, resulting in the formation of spores fungi can still grow and develop along with the development of explants in culture bottles.

Browning occurs consequence to incision wounds in explant cutting, being one of the main causes of browning, because it can stimulate stress and cause increased activity of PAL (*phenylalanine ammonia lyase*), which is an enzyme in *phenylpropanoid* that causes browning followed by production followed by phenol metabolism (Lestari, 2018). The sterilization method is thought to increase browning due to the high concentration of the sterilant and the time of explants were immersed during sterilization process, so that they could damage the surface of the explants, especially leaf explants. Sterilization materials can also cause browning of explants, especially explants that are relatively young, causing a slowdown in the growth and development of explants as well as decreasing physiological functions, and even death of the explants (Wulandari, 2014).

The high percentage of explant deaths due to browning in treatment B compared to treatment A, presumably due to the sterilization method using a combination of Bayclin® 30% solution for 15 minutes and 70% alcohol for 15 seconds, having a high concentration, so it has the potential to damage the surface of leaf explants and cause browning or blackening, and can cause the explant to die. The relatively soaking time of explants increases the risk of explants browning, because the explants will be damaged and the risk of pigment damage to the tissue, because sterilant such as alcohol and NaOCl (*sodium hypochlorite*) have a fairly high toxicity, so that the increase in concentration and the soaking time of explants during sterilization does not can only reduce contamination, but also increase the mortality of culture material (explants) (Pratiwi, 2021).

The Early Day of Explants Contamination and Browning

Bacterial contamination was characterized by the appearance of clear mucus to yellowish white from the surface of the explant, which then spread to the entire surface of the culture medium over time. As time goes by, the color of the bacterial mucus changes to brownish white and the expansion of contaminated area until explant potentially death. So the longer the bacterial contamination is ignored (there is no handling or rescue), the faster the bacteria spreads. Bacterial contamination that extends to the entire surface of the culture medium, can also infect other explants grown in the same culture bottle (Figure 1).

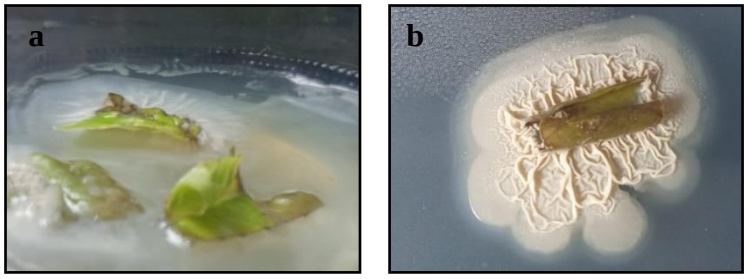

Figure 1. Bacterial contamination (a) clear mucus (b) brownish white mucus

According the results of visual observations, that bacterial contamination comes from the surface of the explant attached to the surface of the medium. Bacterial contamination is characterized by the emergence of bacterial colonies that cause the media around the explants to be brown and yellow, then the explants will be wet or slimy, because the bacteria directly attack the tissues of the plant body (Wati, 2020).

Fungal contamination occurs when the surface of the explant has been infected with fungal spores attaches to the surface of the culture medium. ccording the results of visual observations, that fungal contamination in explants was characterized by the appearance of a colonies of white threads or called hyphae colonies with white and blackish brown color around the explants infected with fungal spores, then expanded and spread to all parts of the surface of the culture media. (Figure 2). Explants contaminated with fungi showed symptoms of the appearance of white to blackish-brown hyphae, and thrived on culture media and explants, because they were good substrates for fungal growth. (Oratmangun, 2017).

Figure 2. Fungal spores form colonies of hyphae attached to the explant surface and media

Browning is a phenomenon caused by the physiological activity of explants after being injured. Browning is generally caused by phenolic compounds that usually appear and accumulate in injured explants (Guntur, 2019). Secondary metabolites that are synthesized by plants under normal growth or can be caused by stressful plant conditions such as injury, infection or exposure to UV radiation produce

phenolic compounds (Kuncoro, 2018). The phenomenon of browning in the sterilization process is suspected by the high concentration sterilization method which causes stress conditions on the explant surface and the injured part of the explant, increasing the occurrence of browning. Browning was observed visually characterized by a change in color on the surface of the explant to brown to blackish brown (Figure 3).

Figure 3. Browning of the explant surface and the injured part

The formation of phenolic compounds is influenced by several factors, that is internal factors, such as chemical structure, plant species, plant biological processes, and developmental stages, as well as external factors, such as injury to explant tissue that triggers stress and causes increased browning, which can inhibit growth and development of explants, which can generally be avoided by transferring explants to a new medium in vitro culture (Hutami, 2008).

The early day of explants contamination and browning was observed visually every day by calculating the average day of contaminant or browning emergence in each replication in each treatment, and the range time required for contaminants to infect explants indicated by symptoms of bacterial or fungal contamination or the range time required for explants to browning, which proved to give different results. (Figure 3).

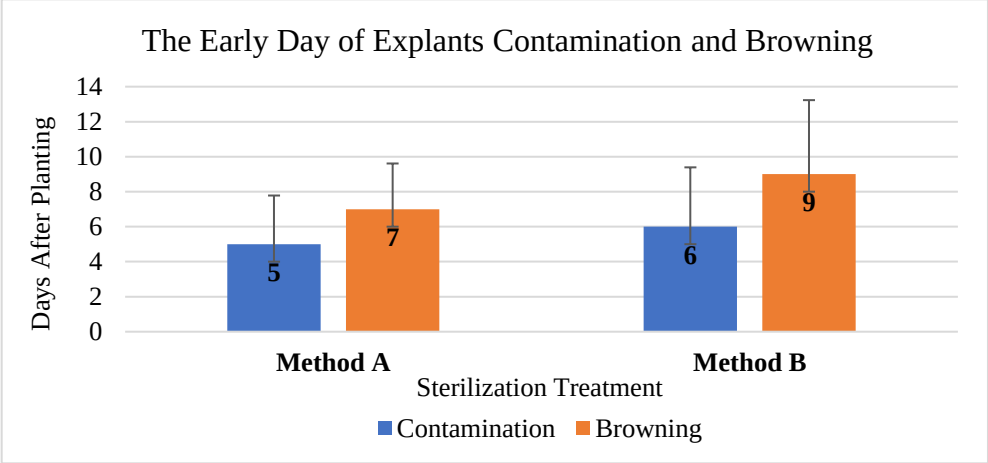

Figure 4. The effect of sterilization method on the early day of explants contamination and browning

According data on the graph (Figure 4), the average day of contaminant emergence in each replication in each treatment occurred 5 days after planting in method A and 6 days after planting in method B, so it took less than a week for the explants to be infected by bacteria or fungi. The early day of contamination is related to the source of contamination in the explants. It is suspected that contaminants or microorganisms that cause contamination (bacteria and fungi) are still attached to the surface of the explants, especially leaf explants from the field that are susceptible to exposure to contaminant agents. Microorganisms that cause contamination (contaminants) such as bacteria are not

only on the surface, but also sometimes on the inside of the planting material (explants), and if the contaminants are still on the surface, the response of the infected explants is faster and can be seen within two days (Fitriani, 2019). Sources of contamination are classified into two types, namely external contamination from contaminants on the surface of the explants and will appear 2-3 days after planting, and internal contamination from contaminants from plant tissues (endophytes) and will appear 4 days after planting or more (Andriani, 2021).

Basically, explant planting material from the field is more susceptible to contamination because the growing environment is in a septic environment, so that the possibility of exposure to plants with contaminants is greater, and makes it easier for contaminants to grow on the surface or plant tissue. Therefore, the selection of planting material becomes important before being used as an explant for in vitro culture, because field explants have more external and internal sources of contaminants, compared to sterile explants (plantlets), especially internal contaminants because higher isolation of endophytic contaminants was found in field explants.

Based on the graphic data (Figure 4), each treatment showed a different tendency at the early day of browning in explants. The average day of browning emergence in each replication in each treatment appeared 7 days after planting in method A and 9 days after planting in method B, so that in less than one week the explants indicated browning.

The early day of explants browning is the average day required for explants to browning, which is indicated by a morphological color change on the explant surface to brown which is related to the sterilization method, type and age of the explant used. Inappropriate sterilization methods such as sterilization with too high a concentration are thought to damage the tissue in explants, and explants that are too old or mature can increase the activity of phenolic compounds, and cause faster browning.

The use of strong sterilants with high concentrations, especially sterilants containing NaOCl (Bayclin®) and 70% alcohol, will cause tissue or cell death in explants (Asmono, 2021). The type of explant used can also affect the rate of browning, such as leaf explants which are thin explants that are more sensitive to exposure to high concentrations of sterilants and have lower resistance (Rodinah, 2016), the age and size of explants with an older and longer explant size (> 1.5 cm) used can increase the rate and cases of browning (Fauzan, 2017) which is the maturity level of explants that can affect the rate of browning (Handayani, 2021).

CONCLUSIONS

The sterilization method with combination clothes bleach (Bayclin®) 30% concentration for 15 minutes + 70% alcohol concentration for 15 seconds (Treatment B) is the best treatment in obtaining sterile explants, because it is more effective in removing contamination and inhibiting contamination and browning with survival the highest rate of 51.85% and the lowest mortality rate of 48.15%. Treatment B can also suppress and inhibit the appearance of contamination and browning as evidenced by the slower rate of contamination and browning compared to treatment A.

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