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Callus Induction of Porang Plants (*Amorphophallus muelleri* Blume) with 2,4- D on Various Explant Sources In Vitro

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ABSTRACT

This study aims to determine the effect of a concentration of 2,4-D added to the Murhasige and Skoog (MS) basic media on porang callus induction and the effect on various explant sources. The study was conducted in the form of experiments in the Laboratory of Bioscience and Plant Reproduction Biotechnology, Department of Agriculture, Faculty of Agriculture, Hasanuddin University from August to December 2021. The experiments were based on a Completely Randomized Design (CRD) pattern arranged in a factorial way with two treatment factors. The first factor was the concentration of 2,4-D consisting of five levels of treatment: without 2,4-D (0.0 mg L^{-1}), 0.5 mg L^{-1}, 1.0 mg L^{-1}, 1.5 mg L^{-1}, and 2.0 mg L^{-1}. The second factor was the source of explants consisting of three levels: the petiole, leaf midrib, and leaf blade. The results after five months showed that the interaction between the concentration of 2.0 mg L^{-1} 2,4-D with the petiole explants had the best effect on callus induction time (12.25 days) and callus weight (2.97 g). The concentration of 2.0 mg L^{-1} 2,4-D had the best influence on the callus induction percentage (83.33%), and petiole-derived explants had the best influence on the callus induction percentage (91.67%). Administration of 2,4-D at a concentration of 2.0 mg L^{-1} and the petiole-derived explant gave the best results in this study; therefore, it is expected to be applied to the development of porang plant seeds in vitro.

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Keywords:
2,4-D; Callus; Explant Source; In Vitro; Porang

1. Introduction

The porang plant (*Amorphophallus muelleri* Blume) is a tuberous plant with high economic value and has potential for development in Indonesia. Porang is a member of the Araceae family native to Indonesia where it is abundant in the forests of Java Island and is known in Japan as Jawa Mukago Konnyaku.

Data from the Ministry of Agriculture, Directorate General of Food Crops (2020) show that the export of porang increased from 11,720 tons in January to July 2019 to 14,568

tons within the same period in 2020. The export destinations of porang are China, Vietnam, Thailand, Japan, and Hong Kong. The government has allocated an area of 17,886 ha in the Provinces of Java, Banten, East Nusa Tenggara, and South Sulawesi to develop porang in 2020. The porang cultivation area expansion brings about the necessity of porang seed propagation.

Porang can be propagated and bred vegetatifly using stem tubers and leaf tubers (bulbils) or generatively using seeds. The use of stem tubers is not effective as it could reduce the porang production, while porang propagation through bulbil and seeds is also limited and is time-consuming because, in one growth period, it produces only one bulbil, in two periods 4-7 bulbils, and in three periods 10-20 bulbils. The availability of seeds for multiplication is also limited as the porang starts flowering after the tubers are three years of age, and from flower to mature seeds needs approximately one year (Nisak, 2020). An alternative propagation technique to produce many porang plants in a short time is the in vitro culture technique.

The in vitro culture is a technique for growing plant parts, either cells, tissues, or organs, under aseptic culture conditions in vitro. In vitro propagation has several advantages, including a high multiplication capacity in a short time, producing disease-free plants, and producing offspring with the same genotype and phenotype as the parent plant. In vitro culture of porang plants generally uses a variety of explant sources, such as young shoots emerging from tubers, leaves, cultured seeds, and shoots from seeds (Kumar & Reddy, 2011; Aziz, 2014). In addition, the propagation of porang plants, using several explant sources including: bud eyes from stem tubers, stem tubers, petioles (petioles), seeds and leaves has been widely practiced (Ibrahim, 2019).

In vitro propagation or regeneration can be conducted by organogenesis and somatic embryogenesis pathways, either directly or indirectly through the callus phase. Somatic embryogenesis is growing an embryo from a somatic cell without fertilization. It could also be defined as a process of explant regeneration through the formation of an embryo-like texture (embryoid) from somatic cells that already have a radicle and shoots (Sintha, 2017).

Induction of porang calluses using the in vitro culture technique requires a precise formulation of the plant growth regulator substances. The plant growth regulators widely used in in vitro culture in plants are auxin and cytokinin. Generally, auxin and cytokinin initiate a callus and organogenesis and can increase secondary metabolite production. The balance and interaction between cytokinin and auxins determine the growth and morphology of plants in vitro (Glory et al., 2017).

Growth regulators often used to support callus growth are 2,4-dichlorophenoxyacetic acid and Indole Acetic Acid (IAA). Compared with IAA, 2,4-D is more stable, as it is not easily broken down by enzymes secreted by plant cells or by heat during the sterilization process (Bustami, 2011). According to Imelda et al. (2008), adding 2,4-D to solid MS media can induce callus formation in taro (*Colocasia esculenta* var. esculenta) explants. Aziz et al. (2014) reported that the concentration of 2,4-D on MS medium used to induce calluses are 0.5 mg L^{-1}, 1.0 mg L^{-1}, and 1.5 mg L^{-1}.

The initial step for obtaining porang calluses that can thrive during cultivation is to conduct the study about In Vitro Callus Induction of Porang Plants (*Amorphophallus muelleri* Blume) using 2,4-D on Various Explant Sources. The purpose of this reported study was to determine the effect of the addition of different concentrations of 2,4-D to

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an MS basic media on porang callus induction and its effects on various porang explant sources.

2. Materials and Methods

The study was conducted in the laboratory Bioscience and Plant Reproduction Biotechnology, Department of Agronomy, Faculty of Agriculture, Hasanuddin University from August to December 2021.

The equipment used in the study was a laminar water flow (LAF) cabinet also called a transfer box, digital scales (±0.009), an oven, a hot plate, a stirrer, an autoclave, graduated cylinders, culture vessels (jam jars), a refrigerator, beakers, micropipettes, lighters, Petri dishes, Bunsen burners, spatulas, and dissection tools which consist of a scalpel, tweezers, and blades, a ruler, and a camera. The materials used in this study were porang explants, Murashige & Skoog (MS) base media, 2,4-D growth regulator, agar, distilled water, sugar, dish soap, 70% and 96% alcohol, household bleach, sterile tissues, and labels.

The study was based on a Completely Randomized Design (CRD) arranged factorially with two treatment factors. The first factor is the 2,4- D concentration consisting of five levels: 0.0 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, 1.5 mg L^{-1}, and 2.0 mg L^{-1}. The second factor is the of explants consisting of three treatment levels: petiole, leaf bone, and leaf blade. Out of these two factors, 15 treatment combinations were obtained. Each treatment combination was repeated four times. Three plantlets units were used for each treatment combination, with a total of 180 explants

2.1 Tool Sterilization

The tools (petri dishes, beakers, culture vessels), and dissection tools (scalpels, tweezers, and blades) were sterilized using these steps: they were washed using detergent and rinsed with clean water. Then they were sterilized using an autoclave at a temperature of 121 °C for 30 minutes, then heated in an oven for 3 hours at 150 °C.

2.2 Explant Sterilization

The plant materials used as the explant source is the porang plant leaf. Before the porang leaf was isolated, the leaf was washed using dishwashing liquid and rinsed with running water. After that, the leaf was immersed in fungicide and bactericide for 10 minutes each and rinsed using distilled water. Isolation was conducted in the laminar airflow cabinet. The leaf was sterilized with 70% alcohol, rinsed three times using distilled water then immersed in 20% bleach for 1-2 minutes, and then rinsed with distilled water three times. Then the leaf was placed on sterile filter paper to absorb any sap released by the explant. The leaf tissue was then cut into 1×1 cm square and inoculated. After that, the jars were sealed using the lid, wrapped, and stored in an air-conditioned room at ±24 °C. Explant was sub culturing every two weeks.

The parameters observed were callus induction time, callus weight, fresh callus weight, success level percentage, callus texture (classified into three groups: (1) compact calluses, (2) mixed/intermittent calluses, (3) crumb calluses), and color.

2.3 Data Analysis

Data obtained from the observation results were collected and tabulated. The tabulated data were then analyzed using analysis of variance (ANOVA). The analysis of variance showed that results were significant and were tested further using Tukey's honest significance difference (HSD) of 5% or 0.05.

3. Results and Discussion

3.1. Research Implementation

3.1.1. Callus Induction Time (DAP)

The analysis of variance shows that 2,4-D concentration treatment (p) explant source (e) and the interaction had a very significant effect on the callus induction time.

Table 1. Average callus induction time (DAP) with several concentrations of 2,4-D on various explant sources

2,4D	Explant Source			HSD NP
	Petiole (e1)	Midrib (e2)	Blade (e3)	0,05
0.0 mg L^{-1} (p0)	30.75 (1.50)^{a} p	23.25 (1.35)^{c} p	0.00 (0.00)^{b} q	
0.5 mg L^{-1} (p1)	24.50 (1.39)^{ab} q	55.75(1.75)^{a} p	0.00 (0.00)^{b} r	
1.0 mg L^{-1} (p2)	26.25 (1.41)^{ab} q	40.00 (1.60)^{b} q	57.75 (1.42)^{a} p	15.41
1.5 mg L^{-1} (p3)	14.00 (1.18)^{ab} r	50.00 (1.51)^{ab} q	75.50 (1.87)^{a} p	
2.0 mg L^{-1} (p4)	**12.25 (1.12)**^{ab} q	19.50 (1.31)^{c} q	68.25 (1.84)^{a} p	
HSD NP 0.05%		15.41		

Remark: Numbers followed by the same letter in rows (a,b,c) and columns (p,q,r) were not significantly different in the HSDα=0.05 follow-up test.

The $HSD_{\alpha=0.05}$ test in Table 1 shows that the treatment with a concentration of 2 mg L^{-1} (p4) had the callus induction time average time at 12.25 days post-planting; however, it was not significantly different with the 2,4-D concentrations of 0.5 mg L^{-1} (p1), 1.0 mg L^{-1} (p2), 1.5 mg L^{-1} (p3), but was significantly different with the control (p0).

The $HSD_{\alpha=0.05}$ in Table 1 shows the treatment of petiole explants (e1) in the administration of 2.0 mg L^{-1} of 2,4-D (p4) had the lowest in average callus induction time 12.25 days but it was not significantly different from midrib-sources explant (e2) and was significantly different from the blade-source explant (e3).

3.1.2. Callus Weight (g)

The analysis of variance showed that the concentration of the 2,4-D treatment and explant source had a very significant effect on the callus weight.

The HSDα=0.05 test in Table 2 shows that the 2,4-D treatment concentration of 2.0 mg L^{-1} (p4) on petiole-source explants (e1) produced the best average callus weight in average 2.965 g and was significantly different from the other treatments.

Table 2. Average callus weight (g) using several concentrations of 2,4-D on various explant sources.

2,4D	Explant Source			HSD NP 0,05
	Petiole (e1)	Midrib (e2)	Blade (e3)	
0.0 mg L^{-1} (p0)	0.547^{d} p	0.362^{c} p	0.000^{c} q	
0.5 mg L^{-1} (p1)	1.490^{c} p	0.659^{bc} q	0.000^{c} r	
1.0 mg L^{-1} (p2)	2.160^{b} p	1.327^{a} q	0.759^{b} r	0.347
1.5 mg L^{-1} (p3)	1.882^{b} p	0.539^{c} r	1.274^{a} q	
2.0 mg L^{-1} (p4)	**$2,965^{a}$** p	0.923^{b} r	1.424^{a} q	
HSD NP 0.05%		0.347		

Remark: The numbers followed by the same letter in rows (a,b,c) and in columns (p,q,r) were not significantly different in the HSDα=0.05 test follow-up test.

3.1.3. *Callus Induction Percentage (%)*

The results of the average callus percentage and variance are presented in Appendix Tables 3a, 3b, and 3c. The analysis of variance showed that the 2,4-D treatment concentration and explant source had a significant effect on the callus induction percentage.

The $HSD_{\alpha=0.05}$ test in Table 3 shows that treatment using 2,4-D at 0.5 mg L^{-1} (p1), 1.0 mg L^{-1} (p2), 1.5 mg L^{-1} (p3), and 2.0 mg L^{-1} (p4) on petiole-source explants (e1) resulted in the highest average percentage of callus at 100.00% it was not significantly different from the control treatment (p0).

Table 3. The average percentage of callus (%) with several concentrations of 2,4-D in various explant sources.

2,4D	Explant Sources			HSD NP 0,05
	Petiole (e1)	Midrib (e2)	Blade (e3)	
0.0 mg L^{-1} (p0)	6.67^{b} p	41.50^{b} q	0.00^{b} r	
0.5 mg L^{-1} (p1)	91.67^{a} p	58.34^{b} q	0.00^{b} r	
1.0 mg L^{-1} (p2)	100.00^{a} p	75.00^{ab} q	66.67^{a} q	19.92
1.5 mg L^{-1} (p3)	100.00^{a} p	83.34^{a} q	58.34^{a} q	
2.0 mg L^{-1} (p4)	100.00^{a} p	83.34^{a} pq	66.67^{a} q	
HSD NP 0.05%		17.00		

Remark: The numbers followed by the same letter in rows (a,b,c) and in columns (p,q,r) were not significantly different in the HSDα=0.05 test follow-up test.

The $HSD_{\alpha=0.05}$ in Table 3 shows that the 2,4-D treatments of 0.5 mg L^{-1} (p1), 1.0 mg L^{-1} (p2), 1.5 mg L^{-1} (p3), and 2.0 mg L^{-1} (p4) resulted in the best average callus percentage in the petiole-explant (e1) at 100.00% but it was not significantly different from the treatment of midrib-source bone explant (e2) and was significantly different from blade-derived explants (e3).

3.1.4. Callus Color and Texture

The qualitative data observed in this study were the callus color and texture. The data were obtained through visual observation from the beginning of planting until the end. The color of the callus is an indicator in describing the appearance of a callus whether the callus still has actively dividing cells or is dead. The texture of the callus is an indicator for evaluating the quality of the callus.

Table 4. The callus color and are texture produced under several concentrations of 2,4-D on various explant sources

Treatment	Color	Texture
p0e1	Gray-brown group N199 (Strong yellowish-brown D)	Compact
p0e2	Green-white group 157 (Pale yellow-green C	Compact
p0e3	Black	Compact
p1e1	White group 155 (Greenish white C)	Compact
p1e2	Brown Group 200 (Grayish reddish-brown B)	Compact
p1e3	Black	Compact
p2e1	Yellow-white group 158 (Yellowish white D)	Compact
p2e2	Green-white group 157 (Pale yellow-green C)	Compact
p2e3	White group 155 (Greenish white C)	Compact
p3e1	Green-white group 157 (Pale yellow-green C)	Compact
p3e2	White-group NN155 (White C)	Compact
p3e3	Green-white group 157 (Pale yellow-green C)	Compact
p4e1	White group 155 (Yellowish white B)	Compact
p4e2	Orange-white group 159 (Pale orange yellow B)	Compact
p4e3	Grayed-orange group 164 (Moderate orange yellow c)	Compact

Remark: The color reference color was the 2015 edition RHS Color chart.

The resulting callus color is uneven so that the callus color visualization is taken from the dominant color in the callus and followed by additional colors that appear. Callus texture resulting from all treatments are compact type could be viewed in Table 4.

Figure 1. Callus obtained from porang explants after 8 weeks of incubation: (a) callus from petiole explants, (b) callus from leaf blade explants with and (c) callus from leaf blade explants.

The color of the calluses was uneven, so the callus color was observed from the dominant color followed by additional colors. The textures of the resulting calluses from all treatments were the compact type and can be seen in Table 4 and Figure 1.

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3.2. *Discussion*

3.2.1. Influence of the Interaction between the 2,4-D Treatment and Explant Source

The analysis of variance shows that there was a very significant interaction between 2,4-D treatment and explant sources concerning the callus induction time parameter and callus weight The use of 2,4-D concentrations with various types of explants affects the induction of porang callus where the addition of 2,4-D 2.0 mg L^{-1} and 1.5 mg L^{-1} using petiole gives a faster callus emergence time of 12.25 h and 14.00 h compared to control and other treatments. This is because the use of explant sources and the right concentration of growth regulators can encourage callus induction. Imelda et al. (2008) Stated that to obtain optimal results in callus induction, an appropriate source of explants and concentration plant growth regulators is required. In line with the research of Mahood (2022) who used stalk explants treated with 1.5 mg L^{-1} 2,4-D followed by leaves with 1.0 mg L^{-1} 2,4-D are two treatments resulting in higher callus induction, 90% and 80%.

The best treatment to encourage porang callus induction is the addition of 2.0 mg L^{-1} 2,4-D using a petiole-source explant. Petioles are an easily obtained source of explants that are also sterile because using meristem tissues can reduce contamination. The use of an appropriate explant source and the supplementation of plant growth regulators will encourage callus growth. This is supported by Hendriyani et al. (2020) who stated that there is an abscission zone on the petiole. The abscission zone is a zone that has a thinner cell wall than the cells in the leaf blade. Therefore, the cells in the petiole are more responsive in absorbing nutrients and plant growth regulators from the callus induction media. This is why it is easier for calluses to form in the petiole than in other parts of the plant.

The concentration of 2,4-D combined with the petiole explant on the callus induction time parameter had a significant effect. The induction of a callus shows a natural response to a wound on the explant, that is to seal the wounded tissue. Indah and Ermavitalini (2013) stated that the response of plant tissue when wounded is cell division which will lead to callus induction, and if it is supported by a supply of plant growth hormones, it will accelerate the callus induction time.

3.2.2. Influence 2,4-D Treatment

The results of the experiment showed that the 2,4-D treatment had a very significant effect on the callus induction time and callus weight analysis of variance table parameters. Supplementation with 2,4-D acting as a plant growth regulator that promotes callus induction showed different callus responses in each treatment. In the treatment without 2,4-D on the petiole, midrib, and blade explant sources, the growth level was low compared to those with the administration of 2,4-D. This was because of the absence of supplemented regulator growth hormones. According to Aziz et al. (2014), when the media is not supplemented with hormones, the protoplasm will die.

The addition of 2.0 mg L^{-1} of 2,4-D hormone for callus growth had a significant effect compared to the treatment without the 2,4-D hormone. This shows that a higher concentration of 2,4-D in the media will accelerate callus growth. Asmono and Sari (2016) stated that an increase in the concentration of 2,4-D will increase the cell division and cell size growth processes. Cell division and size growth will affect the callus weight and texture. Carsono et al (2021) The use of higher concentrations of 2,4-D (> 4.02 mg L^{-1})

can decrease the mitotic index, which inhibits cell division. In addition, it can reduce the rate of callus development because in vitro totipotency is influenced by genes that control hormone levels in cells and the threshold of sensitivity to hormones.

Cell division and size growth affect wet callus weight because of the high-water content of the callus. The wet weight of a callus is strongly dependent on the ability of cells to divide, multiply, and enlarge the callus. Indah and Ermavitalini (2013) stated that 2,4-D in media will encourage the callus morphogenesis and callus induction process. 2,4-D plays a role as a plant growth regulator that could cause physiological and biochemical changes in the plant through enzyme regulation. 2,4-D is used in the binding of protein membranes that have the potential for enzymatic activity. The binding results are used in activating the enzyme and altering the substrate into several new products. The newly formed product will cause a series of secondary reactions, one of which is the production of secondary metabolites.

3.2.3. *Explant Source Treatment Effect*

The test results show that the explant source treatment had a very significant effect on the analysis of variance table callus induction time and callus weight parameters. The blade-derived explant without the supplementation of 0.5 mg L^{-1} 2,4-D and 2,4- D did not experience growth. According to Aziz et al. (2014), this occurred because the explants did not have the complete physiological information or equipment and, therefore, could not enter the cell division cycle.

Petiole-derived explants resulted in better callus growth compared to midrib and blade-derived explants. This was because petioles contain meristematic tissue. According to Prayana et al. (2017), young petioles have more advantageous meristematic tissue because younger tissues from calluses are better compared to old explants. After all, in old explants, the meristematic tissue is no longer actively growing. Sitinjak (2010) also added meristem tissues are generally used for reproduction purposes. The use of explants that contain meristem tissues can also reduce contamination.

3.2.4. *Callus Texture and Color*

The study resulted in a compact callus texture (Table 4 and Figure 1), where a compact callus texture has a cell arrangement that is dense, tight, and difficult to separate single clones during the subculture process. This is due to the influence of PGR used and the plant's endogenous auxin. According to Fauzy et al. (2016), endogenous auxin in the callus will loosen the fibers in the cell wall, which causes the diffusion process, the entry of nutrients from the media to the cell wall. This will continue until the water potential and osmotic potential are balanced then the cell swells due to the entry of fluid from outside to the cell. When the cell swells, the supplementation of cytokinin can affect cell division and cause cell wall formation to accelerate and the callus to become compact.

Callus texture can be classified into two groups, crumb textured calluses and compact textured calluses, depending on the type of plant used and media composition. According to Idris & Pasang (2019), the texture of calluses could vary from compact to crumb, depending on the type of media used, composition of media nutrients, plant growth regulator, and environmental conditions.

In addition to texture, callus color is also a visual marker to judge the callus growth and success. Differences in callus color indicate the developmental stage of cell growth. In the beginning, the callus appears yellowish-white. As its ages, the callus changes from

yellow, to greenish-yellow, brownish yellow, brown, dark brown, and finally black. The color of the callus could indicate the existence of chlorophyll as well as any starch globules within. The observation of callus color can be seen in Table 4. The average petiole explants are greenish white, while the leaf bone explants have varying colors depending on the concentration level ranging from greenish yellow, brown, white and brownish yellow. While the leaf blade is black, greenish yellow and yellow. It can be concluded that petiole explants produce callus that is quite good compared to other explants when viewed from the average color produced, namely greenish white. Mahadi et al. (2016) stated that the white and yellow pigments indicate good callus growth, and the callus becomes greenish white because the callus contains chlorophyll due to the addition of a plant growth promotor which plays a role in the production of chlorophyll as a reaction to light, allowing chloroplasts to conduct photosynthesis. Lestari et al. (2009) added that a change of callus color from brownish or yellow to yellowish white then greenish indicates morphogenesis or that the regenerated callus may form a shoot.

The callus color is an indicator of callus quality. A green callus indicates good callus quality, while a lighter color or white indicates a fairly good quality callus, as the white in the callus is embryonic tissue that does not yet contain chloroplasts but contains many starch granules (Purba et al., 2017). Some calluses aged 7-8 WAP experience a color change to darker brown or even black. Fauzy et al. (2016) stated that browning, where a brownish or black color is observed on the callus, could inhibit the callus growth. This is a natural occurrence if the callus experiences a physiological setback causing the callus to change color to brown (browning), ultimately leading to callus death. Browning is believed to be due to a delay in the subculture process, or it could also be due to prolonged contact between the callus and external air during the sub culturing process.

4. Conclusion

Interaction between the 2,4-D concentration of 2.0 mg L^{-1} with petiole explants produced the best effect on callus induction time (12.25 days) and callus weight (2.97 g). The 2.0 mg L^{-1} concentration of 2,4-D produced the best influence on the callus induction percentage (83.33%) while petiole explants produced the best effect on the callus induction percentage (91.67%).

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