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EFFECT OF ABIOTIC AND BIOTIC ELICITORS ON CALLUS AND SUSPENSION FROM PIPER BETLE L. VAR. NIGRA

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Abstrak

Piper betel L. var. Nigra (black betel) contains secondary metabolites and has biological activity as antibacterial, antifungal, antioxidant, etc. To increase the production of secondary metabolites, an alternative method is needed, namely cell suspension culture. This study aims to determine the effect of abiotic and biotic elicitors on callus biomass produced from cell suspension culture. Leaf explants were grown on Murasige and Skoog (MS) medium with the addition of growth regulator 2.4-D 0.5 mg/L and BAP 2.0 mg/L with abiotic elicitors CuSO4, ZnSO4, HgCl2, and CoCl2 with a concentration of 0.5; 1.0 and 2.5 mg/L. The biotic elicitor used was Aspergillus niger with a concentration of 0.025%; 0.050% and 0.1%. The cultures were incubated for 8 weeks. 0.5 g callus was subcultured on a 25 mL cell suspension medium. The suspension culture was shaken at 110 rpm. In this suspension culture, the callus was incubated for 3 weeks. After 3 weeks of age callus on suspension medium was harvested and weighed the fresh and dry weight. The results showed that the highest average dry weight was found in the treatment with abiotic elicitor CuSO4 0.5 mg/L at 0.088 g. Conclusion, the results showed that the highest average dry weight was found in abiotic elicitor treatment of CuSO4 0.5 mg/L, which was 0.088 g. Cell biomass is a very important factor in measuring growth.

Keywords: Callus, Cell Suspension, Elicitors, Piper betle L. Var. Nigra.

INTRODUCTION

Piper plant consists of more than 1000 species scattered in various tropical regions, several species of this genus are used as food, medicine, stimulant, analgesic, anti-inflammatory, antiseptic, antidepressant, and antioxidant (Meena et al., 2010);(Santos et al., 2016). Various extracts of Piper have high antioxidant activity (Barua et al., 2014);(Akbar et al., 2014);(Hafizah et al., 2010);(Anggreni et al., 2019);(Dasgupta & De, 2004). One of the species with high potential is Piper betle L. var. Nigra (black betel). Several secondary metabolites were identified from black betels such as alkaloid, terpenoid, steroid, flavonoid, polyphenol, saponin, and tannin (J. Junairiah et al., 2019);(Rija'i, 2015). Moreover, black betel leaf extracts have antibacterial and antifungal activity against Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Candida albicans ATCC 10231, and

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Streptococcus pneumonia (N. I. Z. Junairiah et al., 2020);(Ummah, 2014);(Rajeshbabu et al., 2011).

The high potential of the plant affects demanding for medicinal raw materials, but there are obstacles, one of them being limited availability. The exploitation of plants directly from nature cause population degradation and conventional black betel propagation, hence the production of secondary metabolites from harvest fluctuates and is not optimum (Singh & Chaturvedi, 2010);(Manorma et al., 2011);(Dal Toso & Melandri, 2011). Therefore, other appropriate optimization methods are needed for the fulfillment of secondary metabolites, one of which is the cell suspension culture method.

The results of the cell suspension culture of Piper permucronatum and Piper solanum showed an increase in secondary metabolites, also in the callus of other plants (Santos et al., 2016);(Balbuena et al., 2009);(Jamil et al., 2018);(Khanpour-Ardestani et al., 2015). Callus extracts from cell suspension cultures of Nigella sativa L. were reported to have optimum antimicrobial and antioxidant activity (Chaudhry et al., 2015). This study is a first report because previously there was no information about the activities of antimicrobials and antioxidants from black betel callus resulting from cell suspension culture.

RESEARCH METHODS

Plant Materials and Explant Sterilization

Black betel was obtained from a flower market in Bratang, Surabaya, East Java. Explants originated from young or meristematic leaves. The leave was washed with running water to remove dirt on the surface of the explants, soapy water, and rinsed with running water. In the laminar air flow cabinet (LAF), leaves were immersed in 20% Clorox for five minutes, and rinsed with distilled water three times.

Callus Induction

Planting of black betel leaf explants for callus induction was carried out by cutting leaves with the size of ±1 cm2 and the edge of leaves was removed, then placed in culture bottles that already contained callus induction media with growth regulator 2,4-D 0.5 mg/L. + BAP 2.0 mg/L with abiotic elicitor CuSO4, ZnSO4, HgCl2 and CoCl2 with concentration of 0.5; 1.0 and 2.5 mg/L. In addition, with biotic elicitor Aspergillus niger in the concentration of 0.025%; 0.050%, and 0.1%. Each bottle was filled with three explants, then the mouth of the culture bottle was closed with aluminum foil and incubated for eight weeks. After eight weeks, the parameter observed was callus wet weight. The incubation chamber temperature is 25± 2°C.

Cell Suspension Culture

Callus weighing 0.5 g was subcultured on a cell suspension medium. The volume of the medium was 25 mL. The suspension culture was shaken at speed of 110 rpm. In this suspension culture, the callus was incubated for three weeks. After three weeks of age, the callus on the suspension medium was harvested and weighed wet and dry weight. The subculture medium was MS medium with growth regulator 2,4-D 0.5 mg/L + BAP 2.0 mg/L and abiotic elicitor CuSO4, ZnSO4, HgCl2, and CoCl2 with

concentrations of 0.5; 1.0 and 2.5 mg/L. In addition, the biotic elicitor Aspergillus niger with concentrations of 0.025%; 0.050%, and 0.1%.

RESULTS AND DISCUSSION

The results of this study were presented in tables and figures. The data obtained were callus induction time, percentage of explants forming callus, wet weight of callus on solid medium, and also wet weight and dry weight on cell suspension culture medium.

A. Callus Induction Time and Percentage of Explants Forming Callus

The first stage in this study was to induce callus explants from black betel leaf (Piper betel L. var Nigra) on solid Murashige and Skoog medium with the addition of growth regulators 2.4 D 0.5 mg L, BAP 2.0 mg L and elicitor abiotic CuSO4, ZnSO4, HgCl2, CoCl2, and biotic elicitor Aspergillus. The cultures were incubated for eight weeks. At this stage, parameters were observed in callus induction time (days) and percentage of explants forming callus (%). The callus induction time data and percentage of explants forming the callus can be seen in Table 1.

Table 1. Induction time and percentage of black betel callus formation on solid MS medium + 2,4-D 0.5 mg/L + BAP 2.0 mg/L with the addition of various types of abiotic and biotic elicitor concentrations (Aspergillus niger) during eight weeks.

No	Elicitor Type	Concentration (mg/L)	Induction Time (Day)	Percentage of Callus Formation (%)
		0,5	14	100%
1	$CuSO_4$	1,0	14	100%
		2,5	15	100%
		0,5	14	100%
2	$ZnSO_4$	1,0	14	100%
		2,5	13	100%
		0,5	15	100%
3	$HgCl_2$	1,0	14	100%
		2,5	13	100%
		0,5	13	100%
4	$CoCl_2$	1,0	13	100%
		2,5	12	100%
		0,025%	14	100%
5	*Asp*	0,050%	14	100%
		0,100%	15	100%
6	KOZ	-	17	100%

As shown in Table 1, it showed that the fastest callus induction time was 12 days in CoCl2 elicitor treatment with a concentration of 2.5 mg/L. However, the longest callus induction time was in the control treatment, which was 17 days. All treatments were able to induce callus, hence the percentage of explants forming callus was 100%.

Following the study of callus induction in Trachyspermum ammi plants. In this study, growth regulators 2,4D and BAP were used. The results showed that usage of BAP 0.25 mg/L and 2 mg/L optimized callus production, hence affecting callus weight, callus volume, and callus color. The best explanation for callus

induction was shoot (Fazeli-Nasab, 2018). In another study, namely Hymenocallis littoralis, growth regulators 2,4D and BAP were used. The use of 13.50 M 2, 4-D, and 4.50 M BAP was able to induce callus with a percentage of 93.75%. However, a concentration of 22.50 M 2, 4-D, and 9.50 M BAP gave a percentage of callus induction of 12.50%. Callus induction time was 15 days, and longer when compared to treatment with a CoCl2 elicitor (Sundarasekar et al., 2012).

B. Wet Weight of Callus on Murashige and Skoog Medium

After eight weeks callus culture will be harvested and weighed with wet weight. The wet weight data could be seen in Table 2. Callus morphology at the age of eight weeks is displayed in Figure 1. Table 2. Average fresh weight of black betel callus on solid MS medium + 2,4-D 0.5 mg/L + BAP 2.0 mg/L with the addition of various types of abiotic and biotic elicitor concentrations (Aspergillus niger) for eight weeks.

No	Elicitor Type	Concentration (mg/L)	Fresh Weight (g)					Average
			1	2	3	4	5	
		0,5	0,763	0,768	0,777	0,927	0,763	0,800
1	**$CuSO_4$**	**1,0**	0,907	0,898	0,810	0,885	0,722	0,844
		2,5	0,601	0,676	0,641	0,611	0,758	0,657
		0,5	0,731	0,990	0,708	0,724	1,090	0,849
2	**$ZnSO_4$**	**1,0**	1,413	0,739	0,945	0,937	0,811	0,969
		2,5	0,827	1,044	0,876	0,869	0,787	0,881
		0,5	0,969	1,388	1,373	0,786	0,755	1,054
3	**$HgCl_2$**	**1,0**	0,824	1,054	0,867	1,075	0,914	0,947
		2,5	0,953	1,136	0,862	0,859	0,877	0,937
		0,5	0,871	0,738	1,035	0,911	0,725	0,856
4	**$CoCl_2$**	**1,0**	1,256	1,196	1,003	1,017	0,950	1,084
		2,5	1,080	0,788	0,871	1,038	0,959	0,947
		0,025%	0,898	0,954	0,869	0,791	0,813	0,865
5	***Asp***	**0,050%**	1,087	0,905	1,165	0,776	0,943	0,975
		0,100%	0,762	0,904	0,578	1,049	0,521	0,763
6	**KOZ**	**-**	1,072	1,010	0,976	0,837	0,740	0,927

Figure 1. Morphology of black betel callus on eight weeks of MS medium

Based on the results of weighing wet weight, the highest callus weight was found in two treatments, namely abiotic elicitor CoCl2 concentration of 1.0 mg/L which was 1.084 g and elicitor HgCl2 concentration of 0.5 mg/L was 1.054 g. In the induction of callus, Cordia myxa also used elicitor CoCl2 and ZnSO4 0.5; 1.0, and 2.5. The results showed that abiotic elicitor treatment of CoCl2 with a concentration of 0.5 mg/L resulted in a wet weight of 559 mg and a dry weight of 56 mg. In the treatment with abiotic elicitor ZnSO4 0.5 mg/L, the wet weight of the callus produced was 659 mg and the dry weight of the callus was 65 mg (Alaa, 2016). Elicitation is one of the most effective techniques used to increase the production of secondary metabolites.

Elicitor is a compound that play role in plant defense, increases secondary metabolites, and protects cells in the whole plant. There are two kinds of elicitors, namely abiotic elicitors and biotic elicitors. Abiotic elicitor is non-biological substances usually in the form of inorganic salt compounds such as AgNO3, AlCl3, CaCl2, CdCl2, CoCl2, CuCl2, HgCl2, KCl, MgSO4, NiSO4. The majority of biotic elicitor is recognized by specific receptor bound to the cell membrane. These stimuli are then transferred to the cell by a signal transduction system, resulting in changes that ultimately lead to the formation of phytoalexins (Ramirez-Estrada et al., 2016).

C. Wet Weight and Dry Weight of Callus from Cell Suspension Culture

Furthermore, a callus weighing 0.5 g was subcultured on a cell suspension medium. The volume of the medium was 25 mL. The suspension culture was shaken at speed of 110 rpm. In this suspension culture, the callus was incubated for three weeks. After three weeks of age callus on suspension medium was harvested and weighed wet and dry weight. Wet weight and dry weight data could be seen in Table 3. Table 3. Average fresh weight and dry weight of black betel callus in the subculture of liquid MS medium + 2,4-D 0.5 mg/L + BAP 2.0 mg/L with the addition of various concentrations of abiotic and biotic elicitor (Aspergillus niger) during three weeks.

Elicitor Type	Concentration (mg/L)	Fresh Weight (g)			Average	Dry Weight (g)			Average
		1	2	3		1	2	3	
	0,5	1,579	0,542	0,473	0,865	0,189	0,032	0,044	0,088
$CuSO_4$	**1,0**	0,982	0,596	0,737	0,772	0,088	0,048	0,057	0,064
	2,5	1,132	0,836	0,796	0,921	0,100	0,058	0,058	0,072
	0,5	1,707	0,958	0,986	1,217	0,096	0,031	0,032	0,053
$ZnSO_4$	**1,0**	1,481	0,787	1,157	1,142	0,084	0,034	0,051	0,056
	2,5	1,501	0,701	0,625	0,942	0,068	0,034	0,037	0,046
	0,5	1,149	0,820	0,798	0,922	0,074	0,033	0,033	0,047
$HgCl_2$	**1,0**	0,808	1,495	0,685	0,996	0,026	0,093	0,022	0,047
	2,5	1,595	0,991	0,636	1,074	0,100	0,030	0,025	0,052
	0,5	0,483	0,587	0,473	0,514	0,039	0,060	0,038	0,046
$CoCl_2$	**1,0**	0,514	0,460	0,458	0,477	0,025	0,044	0,029	0,033
	2,5	0,481	0,545	0,524	0,517	0,031	0,035	0,046	0,037
Asp	**0,025%**	0,679	0,702	0,732	0,704	0,036	0,042	0,034	0,037

	0,050%	0,652	0,681	0,821	0,718	0,033	0,035	0,042	0,037
	0,100%	0,791	0,777	0,838	0,802	0,036	0,045	0,052	0,044
KOZ	-	0,483	0,483	0,641	0,536	0,021	0,021	0,023	0,022

Based on the observation table, it showed that in suspension culture, the highest average wet weight was found in ZnSO4 treatment with a concentration of 0.5 mg/L with a wet weight of 1.217 g and ZnSO4 treatment with a concentration of 1.0 mg/L was 1.142 g. The average dry weight of CuSO4 treatment with the concentration of 0.5 mg/L was 0.088 g and CuSO4 treatment at 2.5 mg/L was 0.072 g. A study of Andrographis paniculata suspension culture has been carried out using a CuSO4 elicitor. The explants used were hypocotyl and cotyledon. The medium used was Skoog and Hilderbrandt (SH) with a growth regulator of 2,4D and BAP with a concentration of 2.0 g/mL and 0.1 g/mL, which could increase andrographolide production by 29.42±0.31 mg/g (Dawande & Sahay, 2021). Callus elicited in cell suspension culture in liquid MS medium with the addition of abiotic elicitor are shown in Figures 2, Figure 3, and Figure 4.

Figure 2. Callus morphology from cell suspension culture with $CuSO_4$ as an abiotic elicitor

Figure 3. Callus morphology from cell suspension culture with $HgCl_2$ as an abiotic elicitor

Figure 4. Callus morphology from cell suspension culture with $ZnSO_4$ as an abiotic elicitor

Figure 5. Callus morphology from cell suspension culture with *Aspergillus* as a biotic elicitor

CONCLUSION

The results showed that the highest average dry weight was found in abiotic elicitor treatment of CuSO4 0.5 mg/L, which was 0.088 g. Cell biomass is a very important factor in measuring growth. The type and concentration of the elicitor will determine biomass. Elicitors with high concentrations will give a hypersensitive response and sometimes cause cell death. Therefore, we need the right type of elicitor and optimal concentration to get high cell biomass.

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