EFFECT OF COPPER SULPHATE ON BIOMASS PRODUCTION AND BIOACTIVE COMPOUND IN CALLUS CULTURE OF PIPER BETLE L. VAR NIGRA

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Article History: Received: 12.12.2022 **Revised:** 29.01.2023 **Accepted:** 15.03.2023

Abstract

Callus culture is the method for secondary metabolite production. Using elicitors can stimulate the production of secondary metabolites. The objective is to determine the elicitor effect on biomass and bioactive compounds in Piper betle L. var Nigra callus. The elicitor used copper sulfate ($CuSO_4$) with concentrations of 0.5; 1.0; 2.5 mg/L, with six replications. The culture medium used Murashige and Skoog (MS) medium. The incubation time was eight weeks. Identification of bioactive compounds carried out using Gas Chromatography - Mass Spectrometry (GC-MS). The results showed that 0.5 mg/L $CuSO_4$ produced the highest fresh weight (0.6711 g) and contained the bioactive compound salicylic acid hydrazide (21.23%). In general, these compounds act as antimicrobials.

Keywords: bioactive compound; callus; copper sulphate

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DOI: 10.31838/ecb/2023.12.1.257

1. Introduction

Indonesia has the highest biodiversity third in the world. Herbal plants in Indonesia are abundant and interesting study object. Herbal plants have many benefits that are important to develop them, nearly 30,000 plant species and more than 900 plant species. Among them are efficacious as medicine, only about 22% have been cultivate and the rest is still taken directly from the forest (Suharno et al., 2016; Alamgir, 2017). About 80% of the world's population depends on traditional medicine that uses plants as raw materials (Parveen et al., 2015). Black betel (Piper betle L. var Nigra) is one of the medicinal herbals that can produce secondary metabolites.

P. betle L. var Nigra contain flavonoids, triterpenoids, alkaloids, tannins, saponins, steroids, and polyphenols as secondary metabolites (Rija'i, 2015). The highest types of compounds in a group of secondary metabolites are terpenoid (±25,000 compounds) whereas alkaloids (±12,000 compounds), phenols (±8,000 compounds). Terpenoids are secondary metabolites that have potential in pharmacology and industry (Radulovic et al., 2013). Terpenoids used as a fragrance, color, and insecticide in the agriculture and medicinal industry (Roberts, 2007). Several studies have shown natural products against various diseases such as cancer, malaria, Alzheimer, and HIV/AIDS (Guimarães et al., 2014; Choudhary et al., 2021). In addition, the ethanolic extract of P. betle L. var Nigra leaf inhibit growth of Candida albicans (Ummah, 2014).

Therefore, the increasing demand for plants as medicinal raw materials reaches more than 1,000 tons per year. The availability of raw materials for medicinal plants from cultivation is 18 species and direct exploitation of their natural habitat is 13 species. But the cultivation technique is carried out conventionally, thus considered less effective and has a dependence on season and climate which results in limited yields. (Gupta, 2017; Singh & Chaturvedi, 2010; Dobranszki & da Silva, 2010). The lack of information nutrient and mineral content in planting caused produce fluctuating and not optimal in secondary metabolites (Yang et al., 2018). The process of fulfilling the demand for medicinal plants obtained from their natural habitat also causes decrease in plant populations, fragmentation, degradation of natural habitats, and loss of species due to overexploitation of plants (Corlett, 2016).

Callus culture is an alternative method of intensive and efficient propagation of medicinal plants that is needed to overcome the problem of fulfilling plant materials which produce secondary metabolites (Chandana et al., 2018). Callus culture is the most effective biotechnological methods due to can reduce the exploitation of plants from the original habitat and is an appropriate method for producing large amounts of callus biomass and the secondary metabolite components accumulation (Bakhtiar et al., 2016; Khalil et al., 2015). The optimum production of secondary metabolites through callus culture increased by elicitation. The method that refers to natural phenomena in the defense mechanism of host plant cells against pathogens by producing secondary metabolites (Bhaskar et al., 2021). In plant cells, biotic and abiotic elicitors can produce secondary metabolites to protect plants from various environmental stresses (Ramakrishna & Ravishankar, 2011). Elicitors are signals that induce stress responses in plant cell defense systems (Narayani & Srivastava, 2017). Physiological, morphological, biochemical, and molecular in plants caused by the stress response and triggers from treatment (Ahmad et al., 2012).

The concentration of cobalt chloride ($CoCl_2$) affecting callus biomass and produce higher terpenoid profile of P. betle L. var Nigra in a concentration of 1.0 mg/L (Junairiah et al., 2020). The callus culture of Artemisia annua L. induced in 2 mg/L $CoCl_2$ and has highest of total phenolic content (8.40 mg/g dry weight) compared to control (6.66 mg/g dry weight) (Zarad et al., 2021). Another study reported that copper sulfate ($CuSO_4$) is an abiotic elicitor. It affected saponin content of adventitious roots of Talinum paniculatum in the concentration of 7 mg/L. The concentration of 45 mg/L CuSO4 elicitors in Bacopa monnieri shoot culture can increase bacoside content by 8.73 mg/g dry weight than in control cultures (6.14 mg/g dry weight) (Sharma et al., 2015). The objective to determine whether using CuSO4 as an abiotic elicitor could stimulate an increase in biomass production and terpenoid content profile of Piper betle L. var Nigra callus to obtain the optimum abiotic elicitor concentration.

2. Method

Plant material

The explants used were the meristematic leaves from shoots of P. betle L. var Nigra. The explants were collected from Bratang Flower Market, Surabaya, East Java, Indonesia.

Medium preparation

The compounding of MS medium with the capacity of 1000 mL requires a stock of macronutrients 10 mL of (macro I : KNO_3, $MgSO_4.7H_2O$, macro II : $CaCl_2.2H_2O$, and macro III : KH_2PO_4). Then added micronutrient nutrient stock (1 mL), 5 mL of iron,

4 mL of vitamins, 0.5 mg/L 2,4-D (2,4-Dichlorophenoxyacetic acid), and 2.0 mg/L BAP (Benzyl Amino Purine). It homogenized with 500 mL of distilled water. Furthermore, Myo-inositol and sucrose were added to the mixture. The volume of medium was added to 1000 mL by distilled water. After the homogeneity, the pH value of the solution was measured using a pH indicator (pH=5.8). Agar was added to make a solid medium, and labeled according to the treatment. The medium was sterilized using an autoclave (1.2 atm, 121°C, 15 minutes) (Manuhara, 2014).

Explants Sterilization

The black betel leaf was soaked using liquid detergent for 5 minutes and 70% alcohol for 6 minutes, then rinsed 3 times with sterile distilled water. Next, the explants were immersed in 20% sodium hypochlorite (NaOCl) for 10 minutes, then rinsed with sterile distilled water for 3 times. The next step, the leaf were cut into 1x1 cm and planted in MS medium. Explants were cultured for 5 weeks at 25±2°C, lighting 3000-3500 lux for 24 hours.

Preparation of Elicitor

Preparation of the $CuSO_4$ stock solution was carried out by dissolving 0.1 g of $CuSO_4$ in 50 mL of distilled water, after homogeneous and clear, then distilled water was added to the final volume of 100 mL. The stock solution was stored in the refrigerator. Variations of $CuSO_4$ added to solid MS medium were 0.5 mg/L, 1.0 mg/L, and 2.5 mg/L.

Preparation of MS Medium with $CuSO_4$ concentration

1 L of MS medium was added with $CuSO_4$ with treatment (0.5; 1.0; 2.5 mg/L). The bottle was closed with aluminum foil and labeled according to the treatment. The medium in bottles was sterilized and stored in a sterile room.

Elicitation of P. betle var Nigra callus

After five weeks, the P. betle L. var Nigra callus was transferred in MS medium which had added $CuSO_4$ concentration according to predetermined concentration and harvested after eight weeks of culture.

Harvesting P. betle var Nigra callus

Callus was weighed using an analytical balance, then dried using the oven at 60°C for 72 hours until it reach the stable weight. Dry callus was stored and labeled according to treatment for the extraction step.

Callus Extraction and GC-MS Analysis

Identification of bioactive compounds was done using GC-MS. The 0.5 g dry callus were mashed using mortar until a fine powder. The powder was then extracted by maceration using 5 mL of methanol. Extraction was carried out in a water bath at a temperature of 60°C for 5 minutes and then filtered and concentrated to a volume of 2 mL. The dried black betel leaves were extracted in the same way as callus extraction to compare them (Belhadi et al., 2020).

3. Results and Discussion

The callus that was elicited with $CuSO_4$ in various concentrations at the age of eight weeks were harvested. The results showed the variation of the mean fresh weight and dry weight of callus. The following is a table of mean fresh and dry weight of black betle callus elicited by $CuSO_4$ elicitor (Table 1).

Table 1. The mean fresh and dry weight of P. betle var Nigra callus under $CuSO_4$ treatment.

Treatment	Replication	Fresh Weight (g)	Mean of Fresh Weight (g)	Dry Weight (g)	Mean of Dry Weight (g)
Control	1	0.2873	0.2870	0.0330	0.0307
	2	0.3188		0.0337	
	3	0.2678		0.0278	
	4	0.3032		0.0262	
	5	0.2679		0.0218	
	6	0.2769		0.0414	
0.5 mg/L $CuSO_4$	1	0.6632	0.6711	0.0491	0.0513
	2	0.6608		0.0510	
	3	0.6319		0.0454	
	4	0.6790		0.0518	
	5	0.6931		0.0492	

	6	0.6984		0.0613	
1.0 mg/L $CuSO_4$	1	0.5856	0.5986	0.0488	0.0485
	2	0.5812		0.0607	
	3	0.6508		0.0450	
	4	0.5560		0.0445	
	5	0.5819		0.0443	
	6	0.6362		0.0474	
2.5 mg/L $CuSO_4$	1	0.5429	0.5727	0.0570	0.0554
	2	0.5521		0.0544	
	3	0.5308		0.0462	
	4	0.5729		0.0426	
	5	0.6425		0.0553	
	6	0.5950		0.0768	

Table 1 showed that the treatment of 0.5 mg/L $CuSO_4$ obtained the highest mean of fresh weight (0.6711 g). In the majority, the callus growth of black betel leaf started to induce callus for the first time in the second week, the edges of the explant began to wavy and the size of the explants began to lengthen and widen. It was also a swollen wound cover that was beginning callus induction (Figure 1). The callus color in this abiotic elicitor treatment was generally yellow-brown and green-brown with a compact callus texture (Figure 2).

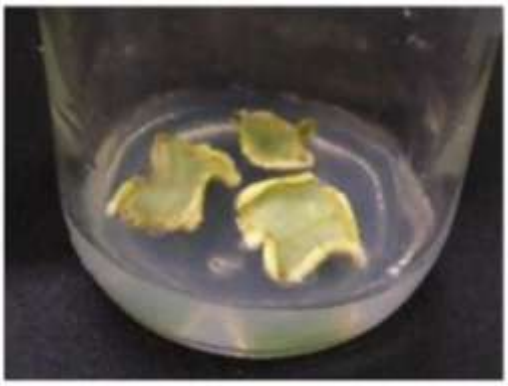

Figure 1. Callus induction at second weeks

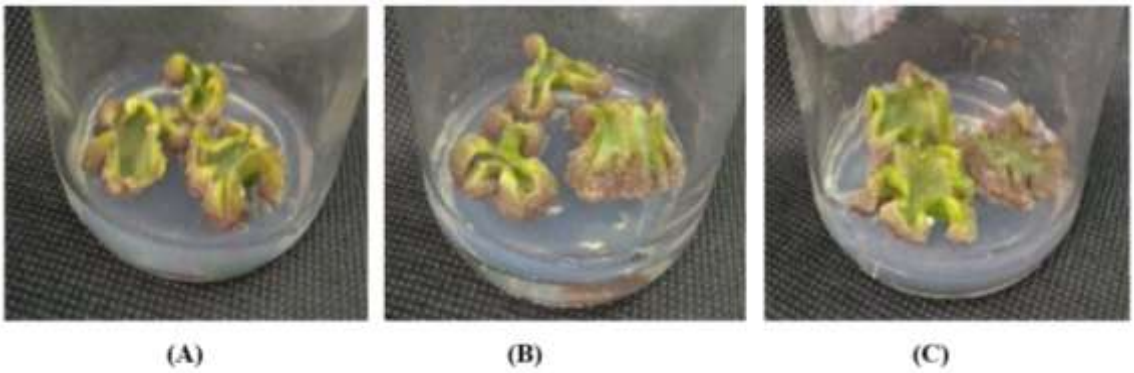

Figure 2. Callus morphology with $CuSO_4$ concentration at eight weeks. (A) 0.5 mg/L, (B) 1.0 mg/L, and (C) 2.5 mg/L.

The results of bioactive compound identification of black betel callus from $CuSO_4$ elicitor (0.5, 1.0 and 2.5 mg/L) as seen in Table 2-4.

Table 2. The results of bioactive compounds identification in black betel leaf callus extract on elicitation treatment of $CuSO_4$ 0.5 mg/L.

No.	Retention Time	Compounds	% Area Width
1.	5.292	2-Piperidinenethanamine	3.15
2.	10.358	Octanoic acid	10.12
3.	10.723	Cordianine	5.31
4.	11.660	Salycylic acid hydrazide	21.23
5.	14.806	Hexadecanoic acid	8.29
6.	14.991	1,2-Benzisothiazole	17.39

Table 3. The results of bioactive compounds identification in black betel leaf callus extract on elicitation treatment of $CuSO_4$ 1 mg/L.

No.	Retention Time	Compounds	% Area Width
1.	1.828	Oxaluric acid	3.00
2.	5.303	Hexadeuterobenzene	10.16
3.	9.703	Dodecanoic acid	2.33
4.	10.322	Carbamimic acid	19.88
5.	10.724	7-Methylindole	6.90
6.	14.801	Guanidine	3.10

Table 4. The results of bioactive compounds identification in black betel leaf callus extract on elicitation treatment of $CuSO_4$ 2.5 mg/L.

No.	Retention Time	Compounds	% Area Width
1.	1.400	Trideuteroacetone	2.87
2.	1.474	Acetic acid, methyl ester	10.63
3.	5.303	1-Penten-3-one	1.39

Elicitors play an important role in biosynthetic pathways. It can elevate the production of secondary metabolite on a larger scale (Patel & Khrisnamurthy, 2013). Elicitors have two types, namely biotic and abiotic elicitors. Biotic elicitors have come from pathogens or the plant itself. While abiotic elicitors consist of physical and chemical substances (Narayani & Srivastava., 2017). Abiotic elicitors, on the other hand, are derived from non-living sources and consist of physical and chemical substances. Physical elicitors include mechanical damage, such as insect feeding, and exposure to light, temperature, or radiation (Bostock et al., 2014). Copper has an important role in physiological and biochemical processes. Such as photosynthesis, conversion of nitrogen compounds, respiration, transport of carbohydrates and constituent of the protein components of several enzymes in plants, especially those that participate in electron flow, catalyzing redox reactions in mitochondria, chloroplasts, walls cells, and cytoplasm of plant cells (Al-Mayahi, 2014; Ahanger et al., 2016). Based on the results showed that the $CuSO_4$ concentration of 0.5 mg/L increased fresh weight callus. However, in another study, the addition of Copper sulfate in the medium inhibited callus formation. The elicitor $CuSO_4$ concentration of 50 µM inhibited callus formation from Origanum majorana L. shoot explants and Panax ginseng root explants growth (Korkor et al., 2017). In callus extraction with $CuSO_4$ treatment, quite a lot of bioactive compounds were identified. Other bioactive compounds such as 0.5 mg/L $CuSO_4$ contained salicylic acid hydrazide which appeared at the retention time of 11.660 as much as 21.23%. Compounds in general act as antimicrobial compounds (National Center for Biotechnology Information, 2019). In addition, there are other bioactive compounds such as 2-piperidinenethanamine (3.15%), carbamic acid/urea (19.88%) as an inhibitor for several types of bacteria, and 1-penten-3-one (1.39%). The addition of 100 µM concentration of Fe EDTA and 20 µM concentration of CuSO4 showed a good response as indicated by higher betaxanthin gain (Prativa et al., 2014). Callus extract elicited with CuSO4 contains 1,2-benzisothiazole. This bioactive compound has the potential as an antimicrobial against Staphylococcus aureus, Salmonella typhosa, and Aspergillus niger bacteria. Octanoic acid is a fatty acid that is an important ingredient and precursor that is widely used in the chemical industry (Wernig et al., 2021).

4. Conclusion

Based on the result, CuSO4 as an elicitor can increase the biomass production of P. betle var Nigra callus. The concentration of 0.5 mg/L CuSO4 was the highest fresh weight at 0.6711 g. Bioactive compounds were produced from various

concentrations. Salicylic acid hydrazide was the highest percentage of the bioactive compound with 21.23%. The resulting bioactive compounds have the potential as antimicrobial.

Competing Interests

The authors have declared that they have no competing interests.

Acknowledgment

Author thanks to Directorate General of Higher Education, Research and Technology, Ministry of Education, Culture, Research and Technology for providing supporting research funds through PDUPT in 2021.

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