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The Effect of Metal Ion Elicitor on Growth of *Vetiveriazizanioides* (L.) Nash. Callus and Its Vetiver Oil Compounds.

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ABSTRACT

The aims of this research were to evaluate the effect of metal ion elicitor on vetiver callus growth and its vetiver oil compounds. For the research materials, callus was induced from tiller explant and cultured on Murashige and Skoog's (MS) medium supplemented with combination of 2.4-D 0.75 mg/L and kinetin 0.5 mg/L for 8 weeks. Elicitation was performed by culturing 0.2 gram callus on MS medium supplemented with 0.1 mM metal ion elicitor (Pb, Al, Cd) for 8 weeks. The vetiver oil compounds of metal ion-elicited callus were analyzed using TLC and GC-MS. The results of this research showed that metal ion elicitor inhibited vetiver callus growth and increased its vetiver oil compounds. Analysis with TLC showed that vetiver callus contained vetiver oil compounds. Based on GC-MS chromatogram, vetiver oil compounds contained in vetiver callus were vetiverol and vanillin. Vetiverol was detected in non-treatment callus and Pb and Al-elicited callus, while vanillin was found in non-treatment callus and Al and Cd-elicited callus. In addition, Cd elicitor was also able to induce the synthesis of other vetiver oil compound such as alpha sinensal and alpha amorphene. The highest vetiverol content was found in Pb-elicited callus, it was 4.37%, while vetiverol produced in non-treatment callus was only 3.99%.

Keywords: elicitation, metal ion, *Vetiveriazizanioides*, vetiver oil

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INTRODUCTION

Essential oil is a mixture of various compounds used in various industries, such as perfumes, cosmetics, foods, and medicines [1]. One of the plants producing essential oil is vetiver (*Vetiveriazizanioides* (L.) Nash.) and the essential oil produced is vetiver oil [2]. Vetiver oil contains a mixture of compounds consisting of sesquiterpenes (30-40%), sesquiterpenol (18-25%) and sesquiterpenone such as benzoic acid, vetiverol, furfurol, α and β vetivone, vetivene and vetivenilvetivenat [3].

Nowadays, the need of vetiver oil reaches 300 tons per year, but the average of national vetiver oil production is only 25-30 tons per year [4]. Therefore, it needs attempts to increase the vetiver oil production, in which one of them is through cell culture techniques. To increase the secondary metabolites production by cell culture techniques can be conducted through callus culture and cell suspension culture. The use of callus cultures in secondary metabolite enhancement has been performed in *Bacopamonnieri* [5] and *Plectranthusornatus*Codd [6].

The attempt of increasing the production of secondary metabolites through cell culture may be accomplished by addition of elicitors [7]. Addition of 8 µM $CdCl_2$ in callus culture increased the biosynthesis of isoflavone phytoestrogens daidzein and genistein 21 times and 18 times respectively higher than control [8], while culturing *Brugmansia candida* on medium supplemented with 250 µm$AlCl_3$for 48 hours was able to induce synthesis of tropane alkaloids (scopolamine) as much as 150% higher than control [9].

Compounds mixture separation can be carried out by using chromatography. Chromatography provides information about the number and type of compounds and the content of each compounds. Chromatographic separation methods include Thin Layer Chromatography (TLC) and Gas Chromatography-Mass Spectrophotometer (GC-MS). The GC-MS method is often used for the analysis of essential oil compounds because essential oils are volatile [10]. This research was performed in order to obtain information about the type and content of each compounds of vetiver oil produced from metal ion-elicited callus in vetiver.

MATERIALS AND METHODS

Plant material

Plants of *Vetiveriazizanioides* (L.) Nash. were collected from Sengklek, Pamalayan Village, Bayongbong District, Garut, West Java. These plants were washed by using running tap water, then trimmed about 4 cm from the base of shoot and separated from the root. The trimmed plants were disinfected using 96% alcohol for a minute, sterilized using 80% commercial whitening agent (containing 5.25% NaClO) for 25 minutes, and finally rinsed by using sterile aquadest twice each for five minutes. Sterile trimmed of plant was cleft, then the tiller was used as explant.

Callus Induction

About 0.2 cm of tiller explant was cultured on MS basal medium containing 2.4-D 0.75 mg/L and kinetin 0.5 mg/L. The culture was incubated at room temperature 25-26^{0}C with 600 lux light intensity for eight weeks. Callus was subcultured every two months. Two weeks callus after subculture was used as elicitation material.

Elicitation

Vetiver callus was cultured on MS basal medium containing 0.75 mg/L 2.4-D + 0.5 mg/L kinetin and supplemented with 0.1 mM metal ion elicitor (Pb, Al, and Cd).Callus cultured on MS basal medium + 0.75 mg/L 2.4-D + 0.5 mg/L kinetin without elicitor was used as control. The cultures were incubated at room temperature 25-26^{0}C with 600 lux light intensity for eight weeks. Each treatment was repeated ten times (ten bottles) and each bottle contained 0.2 g callus. The observation parameters were growth of callus including the morphology, then callus fresh weight and callus dry weight.

Analysis of vetiver oil compounds

Extraction of vetiver oil

The callus of elicitation treatments was dried at 65^0C for five minutes. Ten grams of dried callus was macerated in 50 ml n-hexane, then sonicated for 25 minutes, and homogenized by shaker at 120 rpm for about 24 hours. These steps were repeated three times. The n-hexane extract was filtered and the supernatant was evaporated using nitrogen gas until the concentrated extract was obtained. The vetiver oil extract was used as material of TLC and pure vetiver oil with 10^2dilution was used as the standard.

Thin Layer Chromatography (TLC) Analysis

Vetiver oil extract was qualitatively analysed by using TLC spotted on plate silica gel 60 F_{254}. The elusion was performed in benzene and ethyl acetate (19:1) as mobile phase. The spot visualization was executed under 254 and 366 nm UV. The Rf value were determined by dividing the compound distance with the eluent distance.

Gas Chromatography - Mass Spectrophotometer (GC-MS) analysis

Quantitative analysis of vetiver oil was conducted using Gas Chromatography - Mass Spectrometry (GC-MS) QP-2010 Ultra. The followings were the conditions of GC-MS for sample analysis: ionization modes EI (Electron Impact), Helium 68.3 KPa as carrier gas, column type HP-5MS, column length of 30 m, column diameter of 0.25 mm, column temperature of 50-300^{0}C, injector temperature of 250^{0}C, temperature detector of 310^{0}C, and gradient temperature rate 10^{0}C/min.

RESULTS

Effect of metal ion elicitor on vetiver callus growth

The addition of metal ion elicitor (Pb, Al, and Cd) in the culture medium influenced the vetiver callus growth. Callus cultured on medium without elicitors proliferated evenly on the entire surface of the explants, whereas callus cultured on the medium containing Pb and Al could only proliferate on some surface parts of the explants. Furthermore, on medium containing Cd, new callus cell was formed only on small parts of explant (Figure 1). Callus growth in the culture medium with the addition of metal ion elicitor was lower than that of callus growth on medium without elicitor. The average fresh weight of callus on medium without elicitor was 1.31 g, while fresh weight of metal ion-elicited callus was 0.62-1.10 g. On medium without elicitors, the average dry weight of callus reached 1.21 g, while dry weight of metal ion-elicited callus only 0.60 to 1.06 g. At the same concentration, each of ion elicitor provided different levels of callus growth inhibition. The highest to lowest growth inhibition level given by metal ion was Cd, Al, and Pb respectively (Figure 2).

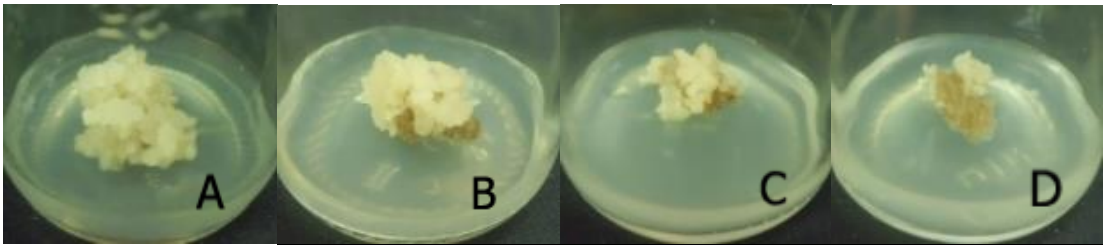

Figure 1: Callus growth on MS medium supplemented with 0.1 mM metal ion elicitor at 8 weeks after culture. (A) Control (B) Pb (C) Al (D) Cd.

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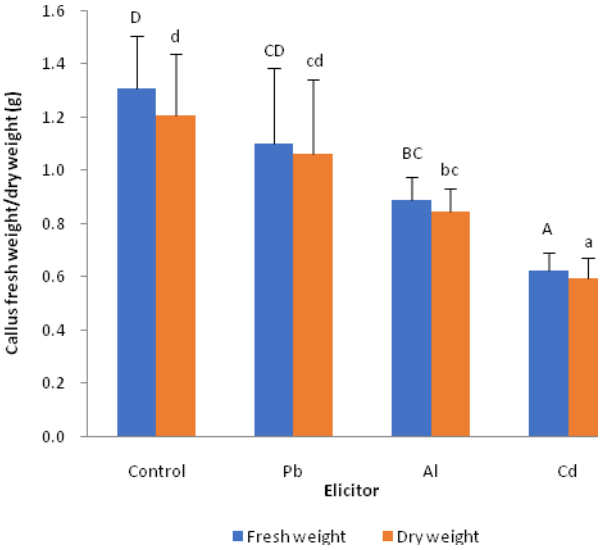

Figure 2: Fresh weight and dry weight of vetiver callus at 8 weeks after elicited with 0.1 mM metal ion. Note: the same letter of each bar showed no significant difference according to DNCT test (α = 0.05)

The difference level of growth inhibition in metal ion-elicited callus was probably caused by the different degree of toxicity and acidity of each elicitor. Based on their toxicity level, the toxicity of Cd metal ion was the highest among other metal ions. While based on their acidity, Pb had alkaline properties.

Effect of metal ion elicitor on vetiver oil compounds

The presence of vetiver oil contained in the callus vetiver was indicated by the spots in TLC plate under UV light after elution. Among these spots, there were three spots of callus samples corresponding to the standard vetiver oil, each spot was indicated by Rf value 0.14; 0.39; and 0.72. The similarity of Rf values between the sample and the standard vetiver oil provided preliminary information about the presence of vetiver oil chemical components in the oil extract from metal ion-elicited callus (Figure 3).

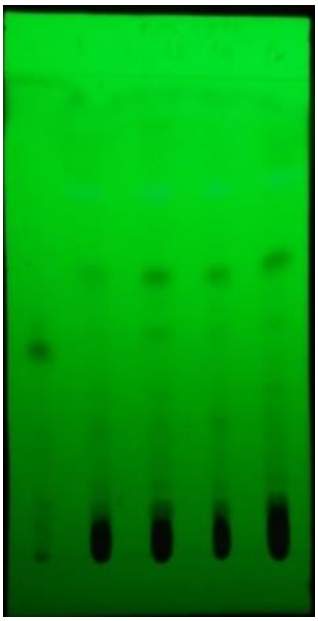

Figure 3: TLC of vetiver oil extract on metal ion-elicited vetiver callus at 8 weeks after culture under UV light at wavelength 254 nm. Note = 1: Standard, 2: Control, 3: Al, 4: Cd, 5 :Pb.

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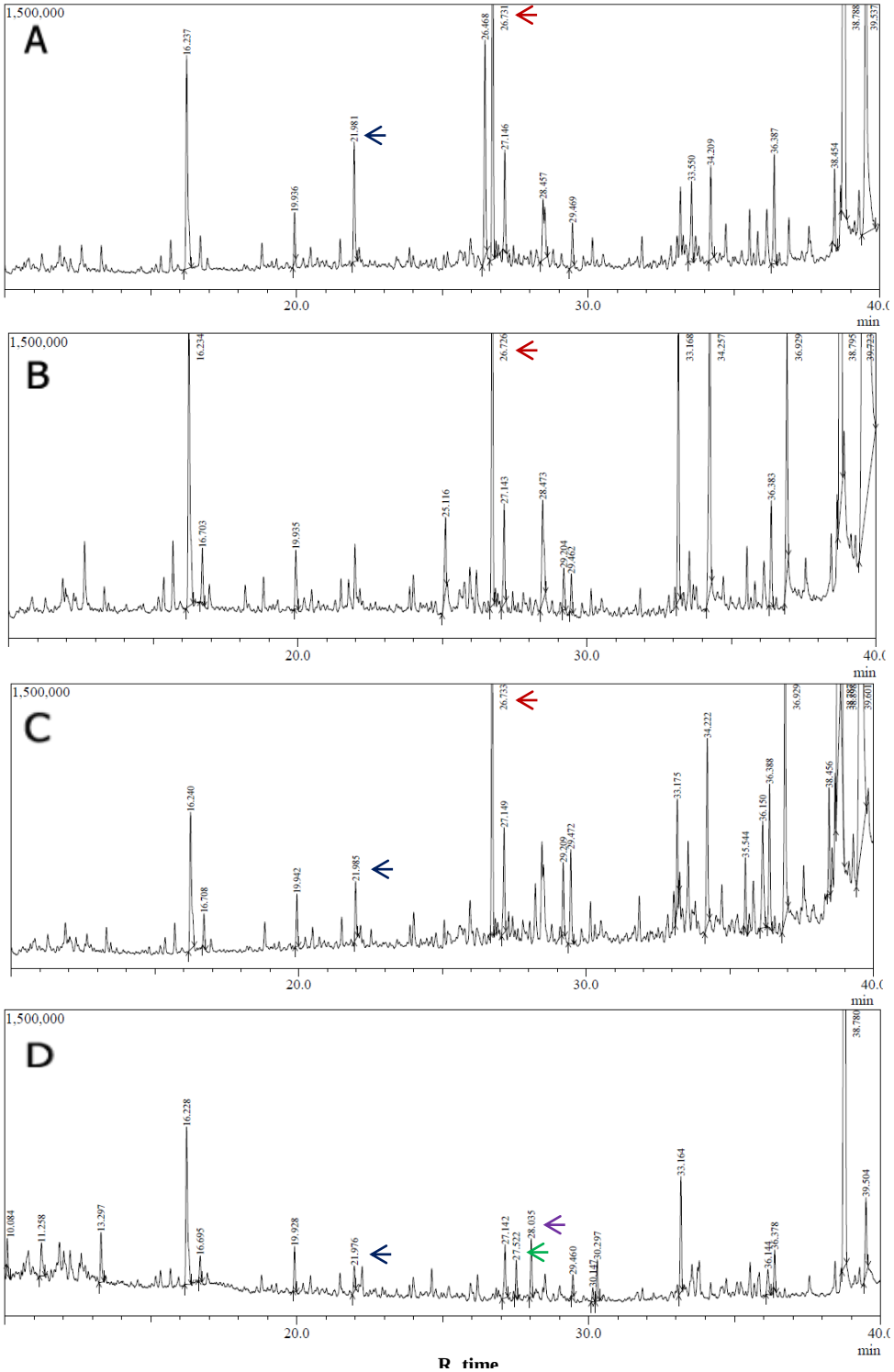
The results of GC-MS chromatogram in vetiver callus indicated the presence of 14 chemical components. The addition of Pb, Al, and Cd elicitors in the culture medium was effective to increase the number of chemical components of vetiver callus. The number of chemical components in Pb-elicited callus was 15 components, while in Al and Cd-elicited callus were 18 components (Figure 4). Several chemical components detected from chromatogram of callus were bioactive compounds belonging to vetiver oil (Table 1).



Note:

: vetiverol : alpha sinensal

: vanillin : alpha amorphene

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Figure 4: Chromatogram of vetiver callus at 8 weeks after elicited with 0.1 mM metal ion. (A) Control (B) Pb (C) Al (D) Cd

Table 1: Chemical component of GC-MS analysis of vetiver callus extract

No	Component Name	Chemical Formula	Molecular Weight	Real Time
1	Naphthalene	$C_{10}H_{18}$	138	11.2
2	Tridecane	$C_{14}H_{30}$	198	13.2
3	Benzothiazole	C_7H_5NS	135	16.2
4	(1-Methyl-Penta-2,4-Dienyl)-Benzene	$C_{12}H_{14}$	158	16.6; 16.7
5	Hexadecane	$C_{16}H_{34}$	226	19.9;27.1;33.5;38.4
6	Heptadecane	$C_{21}H_{44}$	296	19.9
7	Vanilin*	$C_8H_8O_3$	152	21.9
8	Oleic Acid	$C_{18}H_{34}O_2$	282	25.1
9	2,5Dimethoxy/Thermophylline-1,4-Benzoguinone	$C_8H_8O_4$	168	26.4
10	Vetiverol*	$C_{15}H_{24}O$	220	26.7
11	AphaSinensal*	$C_{15}H_{22}O$	218	27.5
12	Alpha Amorphene*	$C_{15}H_{24}$	204	28
13	Dodecanamide, N,N-Bis(2-Hydroxyethyl)	$C_{16}H_{33}NO_3$	287	28.4
14	Benzene, 1,1'-(Ethoxymethylene)	$C_{15}H_{16}O$	212	29.1
15	Propanoic Acid	$C_{16}H_{30}O_4$	286	29.4
16	Phenyl-Methane-1,1-Diol Di-N-Butanoate	$C_{15}H_{20}O_4$	264	29.4
17	3-(Hydroxy-Phenyl-Methyl)-3,4-Dimethyl-1-Phenyl-Pentan-2-One	$C_{20}H_{24}O_2$	296	29.4
18	Farnesane	$C_{15}H_{32}$	212	30.1
19	4-Tert-Butyl-2-(1-Methyl-2-Nitro-Ethyl)-Cyclohexanone	$C_{13}H_{23}NO_3$	241	33.1
20	Myristic Acid	$C_{14}H_{28}O_2$	228	34.2
21	3-Eicosene	$C_{20}H_{40}$	280	35.5
22	Stearic Acid	$C_{18}H_{36}O_2$	284	36.1
23	D-Galactit	$C_{14}H_{30}O_6$	294	36.1
24	1,2-Benzenedicarboxylic Acid	$C_{16}H_{22}O$	78	36.3; 38.7
25	Eicosane	$C_{15}H_{30}O_2$	242	38.4
26	Pentadecanoic Acid	$C_{20}H_{42}$	282	36.9; 39.5; 39.7
27	Palmitic Acid	$C_{16}H_{32}O_2$	256	39.6

Note: * = bioactive compounds belonging to vetiver oil

Some vetiver oil components resulted by each elicitation treatment was different. In control callus and Pb and Al -elicited callus, there was only one main component of vetiver oil, which was vetiverol. The Pb elicited-callus had the highest vetiverol content compared with control callus and other metal ion-elicited callus, which was 4.37%. Callus elicited with Cd did not contain vetiverol, but there were 2 vetiver oil

components which were not detected in control callus and other metal ion-elicited callus, i. e alpha sinensal and alpha amorphene, 0.17 and 0.51% respectively (Table 2) .

Table 2: The content of main component of vetiver oil from metal ion-elicited callus extract at 8 weeks after culture

No	Component Name	Content in each treatment (%)			
		Control	Pb	Al	Cd
1	Vetiverol	3.99	4.37	3.59	-
2	AphaSinensal	-	-	-	0.17
3	Alpha Amorphene	-	-	-	0.51

DISCUSSION

The lower fresh weight and dry weight of metal ion-elicited callus compared with control might be caused by the inhibition of cell division. According to [11], elicitor activity was also able to inhibit cell division and divert cell metabolism. The mechanism of inhibition of cell division by elicitor occurred by the absence of microtubules and inhibition of histone H1 kinase activity during DNA synthesis. While the diversion of cell metabolism took place through the process change, i.e. cells initially synthesized primary metabolite cells for growth and then cells switched to synthesize secondary metabolites as a defence response.

Callus cultures derived from vetiver were able to produce several vetiver oil compounds, among them there were three main components of vetiver oil, i.e. vetiverol, alpha sinensal, and alpha amorphene. As one of the main components of vetiver oil, vetiverol is a finger print in the determination of the quality of vetiver oil. The higher the vetiverol contained, the better the quality of the oil [12].

The addition of elicitor in culture medium was able to initiate and enhance the biosynthesis of secondary metabolites. The formation of secondary metabolites through the using of elicitor was possibly due to the activation of secondary pathways in stress response [13]. One of the effects caused by elicitor was the depolarization of plant cells, namely the occurrence of endogenous ion channel activation by the elicitor. Elicitor could also form pores to allow the ion to penetrate the membrane without being bounded to receptors and ion channel activation [14].

The elicitor added to the culture medium might be either belongs to biotic or abiotic. Abiotic elicitors are substance produced from non-biological origin, such as inorganic salts, metal ions, pH, and so on [15]. Some of ions used for elicitation include calcium, copper, manganese, cobalt, aluminium, lead, chromium, and so on. The addition of 5 mM CdCl2 in culture medium could increase andrographolide content of cell suspension culture in *Andrographispaniculata* 4.14 folds higher than control [16].

The success of elicitation to enhance the synthesis of secondary metabolite compounds are influenced by several factors, such as elicitor type, elicitor concentration, duration of elicitor contact, time of elicitor addition and cell growth phase, and nutrients contained in culture medium [17]. Each type of elicitor provides specific response to promote synthesis of secondary metabolites. Moreover, it is also influenced by the interaction between cell cultures and elicitors[18].

CONCLUSION

Vetiver callus could produce vetiver oil compounds. The addition of metal ion elicitors in culture medium inhibited callus growth and enhanced its vetiver oil content. Vetiver oil compounds produced in vetiver callus were vetiverol and vanillin. Vetiverol was contained in the control callus and Pb and Al-elicited callus, while vanillin contained in the control callus and Al and Cd-elicited callus. In addition, Cd metal ions were also able to induce the synthesis of other vetiver oil compounds; which were alpha sinensal and alpha amorphene. The highest vetiverol was contained in Pb-elicited callus.

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