Effect of Pb and Cd Elicitors on Growth and Content of Vetiver Oil Adventitious Root *In Vitro* of Vetiver (*Vetiveria zizanioides* L. Nash)

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Abstract

This research aimed to evaluate the effect of Pb and Cd elicitors on root growth and essential oil content of vetiver oil on the root culture of vetiver (*Vetiveria zizanioides* L. Nash). Roots were induced by culturing of the shoot on MS media supplemented with 0.1 mg.L^{-1} kinetin and 7 mg.L^{-1} NAA. Elicitation was done by culturing roots on MS media with 0.1 mg.L^{-1} kinetin and 7 mg.L^{-1} NAA + 0.1 mM Pb or Cd. Roots then observed for fresh weight, dry weight, number, and root length, and vetiver oil contents were analyzed using GC-MS. The addition of Pb and Cd heavy metal elicitor affected the formation and the content of vetiver oil compounds of root culture. Pb elicitor increased the number of roots, while Cd elicitor increased root length. However, the addition of Pb and Cd elicitor on culture media decreased the fresh weight and root dry weight. Fifty eight vetiver oil compounds in adventitious roots of vetiver plants were identified. The main compounds of vetiver oil in roots culture were Khusimone (6.94%), Khusimol (6.05%), Khusimene (4.85%), α Vetivone (3.70%), β Vetivone (3, 53%), Vetiverol (3.22%), Prezizaene (2.35%), and Zizaene (1.91%). Elicitation with Pb and Cd increased the composition of the main compounds of vetiver oil. Cd elicitor increased the composition of the main compound vetiver oil higher than the Pb elicitor.

Keywords: adventives root, elicitor, in vitro, Pb and Cd, vetiver oil

INTRODUCTION

Vetiver plant (*Vetiveria zizanoides*) is an essential oil-producing plant and widely used for industrial needs as a basic ingredient of the perfume, cosmetics, and soap [1]. The main compounds composing vetiver essential oil are sesquiterpenic hydrocarbons such as cadenene, clovene, aromadendrine, and carbon compounds such as vetivone and khusimone which are the main compounds producing odor, as well as characteristic compounds of vetiver oil [2].

Essential oil production has reached 250 tons per year on a global scale. The needs of essential oils have not been fulfilled due to production that were dominantly done using conventional technology. The use of conventional technology produces oils with quality that does not meet consumer needs. One way to increase the production and quality of essential oils is by using the culture method. Tissue culture techniques can be carried out through callus culture, cell suspension, and root culture (adventitious and root tip) [3]. Large-scale production of ginsenoside successfully carried out through ginseng adventitious plant root culture [4].

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Culture techniques to increase secondary metabolite production can be done using elicitor [4]. Elicitors are compounds which stimulate physiological disorders in plants that can be used to increase the synthesis of secondary metabolites. Previous study have stated that the addition of Pb metal elicitor in vetiver plant increased the activity of enzymes that play a role in the synthesis of vetiverol [5]. Cd metal elicitor in *Datura stramonium* root culture increased the production of sesquiterpenoid compounds [6]. Therefore, this study aims to determine the effect of Pb and Cd elicitor on growth and components of vetiver oil on the vetiver plant by in vitro.

MATERIALS AND METHOD

Induction of Adventitious Root *In vitro* of Vetiver

Adventitious roots induced by culturing shoots (±2 cm) on MS media with the addition of 0.1 mg.L^{-1} Kinetin + 7 mg.L^{-1} NAA. Root cultures incubated with 600 lux light at temperature of 25-26°C for four weeks. Formed roots multiplied on liquid MS media with the same type of growth regulator.

Elicitation of Root *In vitro* with Pb and Cd

Roots (0.1 g) were cultured in liquid MS media + 0.1 mg.L^{-1} Kinetin + 7 mg.L^{-1} NAA + 0.1 mM Pb or Cd elicitor. The medium without any addition of an elicitor was used as a control. Cultures incubated with 600 lux light at temperature of

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25-26°C for four weeks. Each treatment was repeated five times. The culture was evaluated for the growth of roots (fresh and dry weight, number and length, of the root). Vetiver oil components analyzed by the GC-MS method.

Analysis of *Vetiver oil* compound content of Root using *Gas Chromatography-Mass Spectrometry* (GC-MS)

Roots were pulverized and weighed at 0.1 g. The samples were extracted three times with hexane in a ratio of 1:5 (sample: hexane) and stirred until homogeneous. Samples were incubated overnight at cold temperatures. The solution filtered with an Erlenmeyer vacuum filter, so that filtrate and residue were obtained. The filtrate were combined and evaporated using a rotary evaporator to separate hexane solvents so that an extract obtained. The extracted solution was centrifuged at 5000 rpm for 20 minutes to obtain the supernatant. The supernatant was then diluted using hexane. The supernatant was vortexed and filtered with 0.45 µm membrane cellulose acetate. Vetiver oil compound analysis was carried out by the Gas Chromatography-Mass Spectrometry (GC-MS) Shimazu QP 2010 SE method. The GC-MS apparatuses consist of 15 Kpa helium as the carrier gas, ZB-AAA column type (10 m x 0.25 mm), column temperature of 110-320°C, and injector temperature of 200°C.

RESULT AND DISCUSSION

Effect of Pb and Cd Elicitors on The Growth of Root *In vitro*

Initial growth responses of root explants were indicated by shoot formation after one week of culture. Formation and growth of roots occur after two weeks of culture. The roots of the control media were yellowish-white (Fig. 1A), whereas on the culture media with the addition of Pb elicitor were yellowish-white, and green (Fig. 1B). The root of the culture medium with the addition of Cd elicitor had a color variation were yellowish-white, green, and several others were green with an orange-colored end (Fig. 1C).

Figure 1. *In vitro* roots of elicitation treatment. **Description:** (A) Control, (B) Pb elicitor, (C) Cd Elicitor.

Elicitation of heavy metals in root culture affected fresh weight and roots dry weight. The addition of Pb and Cd elicitor on culture media significantly reduced fresh weight and dry weight compared to the control. The fresh and dry weight of roots in the control media were 144 mg and 12 mg, while the fresh and dry weight of the media with the addition of elicitor were 118 mg and 10 mg with Pb elicitor and 134 mg and 11 mg with Cd elicitor. The decreasing of roots fresh and dry weight in media with the addition of Pb elicitor was higher than that of the Cd elicitor (Fig. 2).

The addition of Pb and Cd elicitor on culture media affected the number and length of the roots. The addition of the Pb elicitor inhibited the growth of root length, but did not significantly affect the number of roots, although there was an increase in the number of roots. The number of roots on the addition of Pb and Cd elicitor was not significantly different compared to the control. However, in the Pb elicitation media, the number of roots produced was significantly higher than the number of roots on the Cd elicitation medium. The addition of Pb on the elicitation media inhibited the growth of root length significantly compared to the control. The roots on the Cd elicitation medium were significantly longer compared to the roots on control and Pb elicitation treatment (Fig. 3).

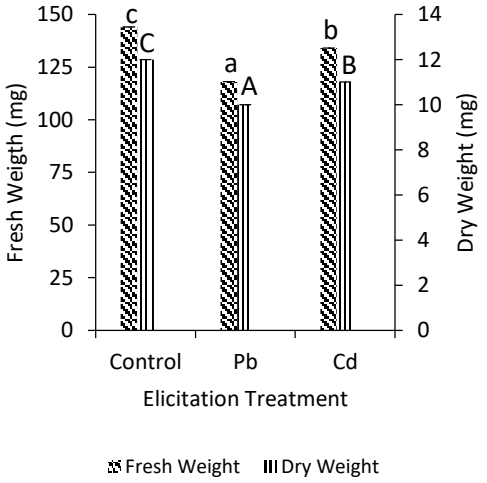

Figure 2. Effect of Pb and Cd licitors on the fresh weight and dry weight of roots *in vitro* of vetiver. **Note:** the same letter on each bar showed not significant difference according to Duncan test (α=0.05).

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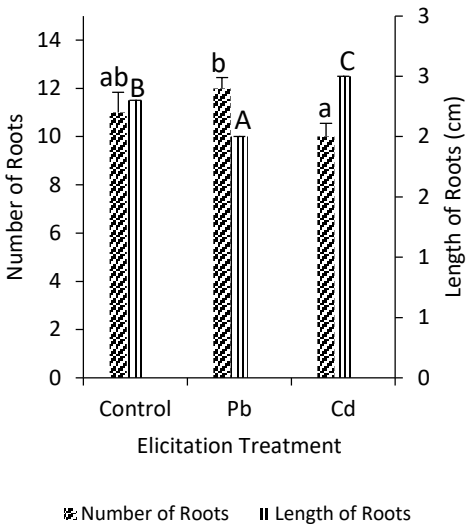

Figure 3. Effect of Pb and Cd elicitors on the number and length of roots *in vitro* of vetiver. **Note:** the same letter on each bar showed not significant difference according to Duncan test (α=0.05).

Elicitor divided into two types, namely biotic and abiotic. Heavy metal was one of the effective abiotic elicitors and had often been used to stimulate the production of secondary metabolites in tissue culture [7]. The use of $CdCl_2$ elicitor increased roots growth and formation in *Brugmansua candida* roots culture [8]. The low concentration of Cd elicitor (5.0 µM) gave the best response to increase the number of roots, and an increase in secondary metabolite compounds of andrographolide in *Andrographis paniculata* plants [9].

Effect of Pb and CD Metal Elicitors on the Component of *Vetiver Oil* compound of *Roots*

The results chromatogram in roots culture on control medium showed 58 compounds (Fig. 4A), but on medium with Pb elicitor, 52 compounds were identified (Fig. 4B), and on medium with Cd elicitor, 50 compounds were identified (Fig. 4C). The retention time for vetiver oil compounds in each treatment was the same, but the percentage composition of each compound was different for each treatment.

The main compounds of vetiver oil were identified both in control roots and treated roots namely khusimone, khusimol, khusimene, α vetivone, β vetivone, vetiverol, prezizaene, and zizaene (Fig. 4). The identified components of the main vetiver oil appear at different times. Zizaene was the first major compound of vetiver oil identified at 24.7 minutes, followed by prezizaene at 25 minutes, khusimene in minute 25.1, khusimone in minutes 29.9, vetiverol at minute 32, β vetivone in minutes to 35.8 and α Vetivone in minutes 36.5 (Table 1).

The composition of main compounds of vetiver oil in the control treatment were zizaene 1.81%, prezizaene 2.23%, khusimene 4.59%, khusimone 6.58%, vetiverol 3.05%, khusimol 5.73%, β vetivone 3.35% and α vetivone 3.50%. Khusimone was the compound that has been identified as having the highest composition, while zizaene has the lowest composition compared to the other main compounds. The treatment of Pb and Cd elicitation caused an increase in the composition of the main compounds of vetiver oil in roots culture. Cd elicitor was able to increase the composition of the main compound in vetiver oil in root culture higher than Pb heavy metal elicitor (Table 1 & Fig. 5).

In the elicitation treatment, fewer vetiver oil compounds were identified, 52 of which were elicited with Pb, and 50 in the results of elicitation with Cd. This amount is less than in controls (58 compounds). Among these compounds, 6 compounds were not detected in the results of Pb elicitation compared to controls, namely Isoeugenol, δ Selinene, Elemol, β Atlantol, 10 Epi γ eudesmol, and Anhydro β rotunol.

The elicitation treatment with Cd caused some compounds not to be detected compared to controls, namely Myrcene, Isoeugenol, δ Cellinene, Agarospirol, 10 Epi γ eudesmol, Cubenol, and Anhydro β rotunol. Some of the compounds were not identified in the elicitation treatment with Pb and Cd. It possibly caused by the elicitation properties used, namely heavy metals, which cause toxicity resulting in inhibition of the synthesis process.

The main components of vetiver oil consist of sesquiterpenes, sesquiterpenol, and sesquiterpenon such as benzoate acid, vetiverol, furfurol, α and β vetivone, vetivene, and vetivenyl vetivenate [10]. The type of elicitor influenced the content of secondary metabolites produced. Also, the concentration of elicitor added to culture media also affected the content of secondary metabolites in a sample. Each elicitor had the ability to increase secondary metabolites, but the concentration of each component was not the same. The optimum concentration of an elicitor was able to increase the content of secondary metabolites in a sample [11].

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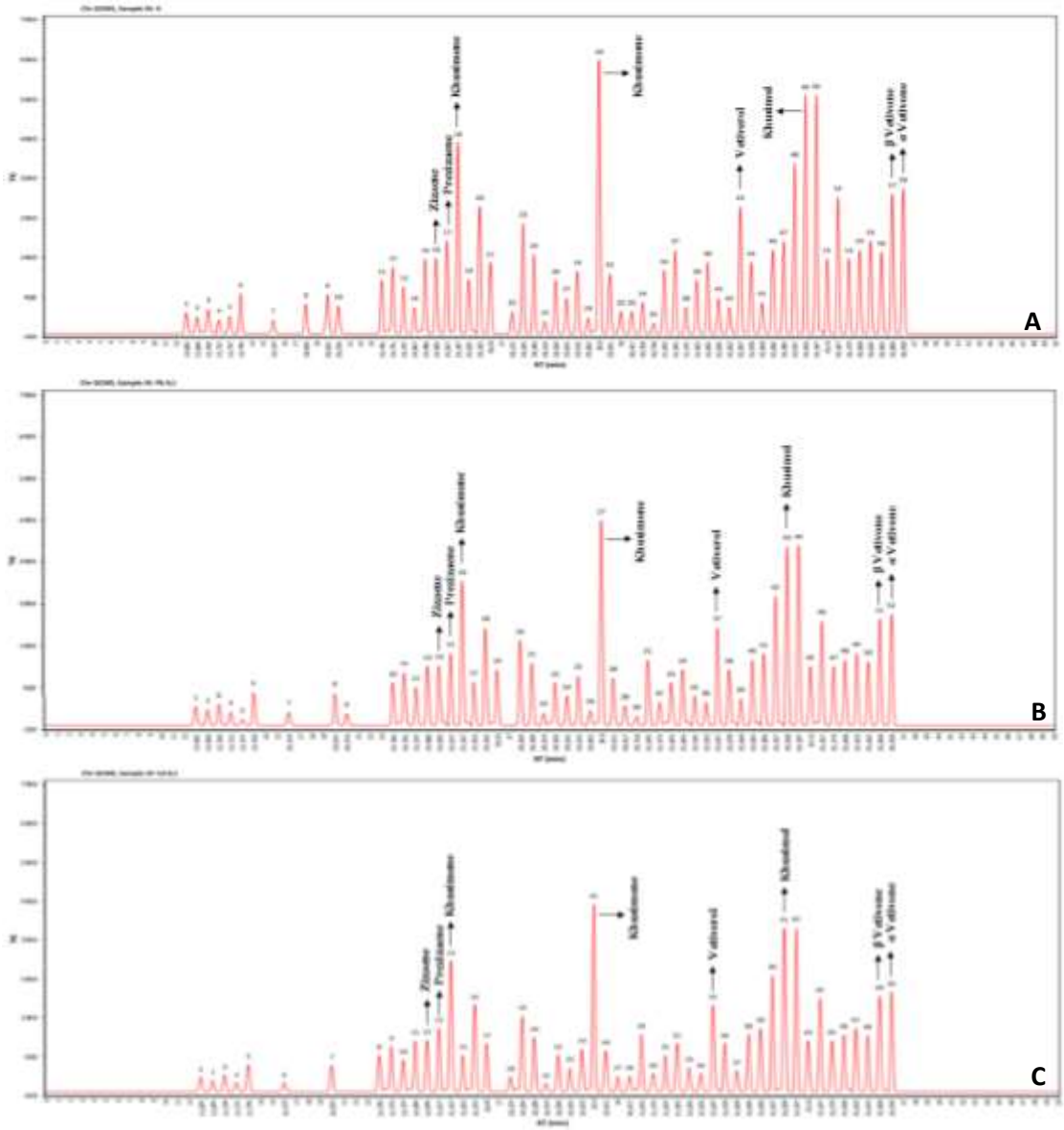

Figure 4. Chromatogram of GC-MS *vetiver oil* of roots *in vitro* of vetiver with elicitation treatment (A) Control, (B) Pb, (C) Cd.

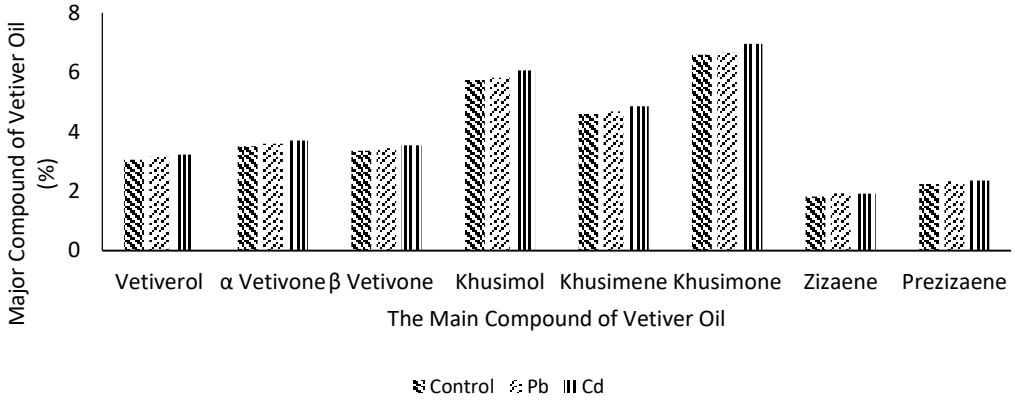

Figure 5. Effect of Pb and Cd Elicitors on *Vetiver Oil* Main Compound of Adventitious Root of Vetiver

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Table 1. Percentage of chemical compound of *vetiver oil* from adventives roots of *in vitro* vetiver plants result of elicitation with metal

No	Compounds Name	Formula Structure	Retention time (minutes)	Percentage/Content of *Vetiver Oil* Compounds on Elicitation Treatment		
				Control	Pb	Cd
1	4 Vinylphenol	C_8H_8O	13.682	0.49679	0.61116	0.52442
2	α Thujene	$C_{10}H_{16}$	13.694	0.39244	0.50764	0.41427
3	α Pinene	$C_{10}H_{16}$	13.704	0.57125	0.68504	0.60303
4	Camphene	$C_{10}H_{16}$	13.722	0.31486	0.43068	0.33238
5	Myrcene	$C_{10}H_{16}$	13.737	0.41916	0.19530	-
6	Limonene	$C_{10}H_{16}$	13.746	0.94590	1.05673	0.99851
7	Linalool	$C_{10}H_{18}O$	16.119	0.31380	0.42962	0.33126
8	Isoeugenol	$C_{10}H_{12}O_2$	18.636	0.69722	-	-
9	Khusitene	$C_{14}H_{22}$	20.015	0.91834	1.02939	0.96943
10	Cadalene	$C_{15}H_{18}$	20.376	0.65955	0.38959	-
11	α Funebrene	$C_{15}H_{24}$	24.785	1.27265	1.38090	1.34344
12	Zizanene	$C_{15}H_{24}$	24.791	1.58895	1.69470	1.67734
13	Acora 4,9 diene	$C_{15}H_{24}$	24.797	1.11669	1.22617	1.17880
14	δ Selinene	$C_{15}H_{24}$	24.807	0.63073	-	-
15	Valencene	$C_{15}H_{24}$	24.986	1.79981	1.90390	1.89993
16	**Zizaene**	$C_{15}H_{24}$	24.995	1.81533	**1.91930**	**1.91631**
17	**Prezizaene**	$C_{15}H_{24}$	25.017	2.23138	**2.33206**	**2.35550**
18	**Khusimene**	$C_{15}H_{24}$	25.167	4.59593	**4.67794**	**4.85158**
19	γ Gurjunene	$C_{15}H_{24}$	25.925	1.28152	1.38970	1.35281
20	β Vetispirene	$C_{15}H_{22}$	26.242	3.05781	3.15197	3.22791
21	γ Amorphene	$C_{15}H_{24}$	26.35	1.69342	1.79835	1.78761
22	Elemol	$C_{15}H_{26}O$	28.125	0.51115	-	0.53958
23	β Vetivenene	$C_{15}H_{22}$	28.283	2.65284	2.75020	2.80041
24	α Vetispirene	$C_{15}H_{22}$	28.289	1.90469	2.00795	2.01064
25	β Bisabolol	$C_{15}H_{26}O$	28.314	0.228624	0.40228	0.302216
26	Maaliol	$C_{15}H_{26}O$	28.583	1.27365	1.38190	1.34450
27	Juniper camphor	$C_{15}H_{26}O$	29.015	0.83244	0.94417	0.87875
28	Viridiflorol	$C_{15}H_{26}O$	29.525	1.48340	1.58999	1.56591
29	Agarospirol	$C_{15}H_{26}O$	29.662	0.37137	0.48673	-
30	**Khusimone**	$C_{14}H_{20}O$	29.9	6.58222	**6,64856**	**6.94837**
31	Cadinane	$C_{15}H_{26}$	29.911	1.42490	1.53195	1.50416
32	β Atlantol	$C_{15}H_{24}O$	30.000	0.52563	-	0.55486
33	Junenol	$C_{15}H_{26}O$	30.417	0.52562	0.63976	0.55485
34	10 Epi γ eudesmol	$C_{15}H_{26}O$	30.708	0.73726	-	-
35	Cubenol	$C_{15}H_{26}O$	30.758	0.24404	0.29603	-
36	Anhydro β rotunol	$C_{15}H_{20}O$	31.005	1.51022	-	-
37	1,7 Di epi α cedrenal	$C_{15}H_{22}O$	31.042	2.00915	2.11159	2.12092
38	α Cadionol	$C_{15}H_{26}O$	31.375	0.63197	0.74528	0.66713
39	Mustakone	$C_{15}H_{22}O$	31.825	1.27257	1.38082	1.34336
40	Zizanal	$C_{15}H_{22}O$	31.842	1.69447	1.79939	1.78873
41	Caryophyllene oxide	$C_{15}H_{24}O$	32.035	0.83304	0.94476	0.87938
42	Epikhusinol	$C_{15}H_{24}O$	32.053	0.63102	0.74434	0.66613
43	**Vetiverol**	$C_{15}H_{24}O$	32.067	3.05302	**3.14722**	**3.22285**
44	Zizanol	$C_{15}H_{24}O$	32.078	1.69510	1.80001	1.78939
45	Junicedranol	$C_{15}H_{26}O$	32.658	0.73727	0.84975	0.77828
46	Nootkatol	$C_{15}H_{24}O$	33.083	2.00900	2.11143	2.12075
47	Vetiselinenol	$C_{15}H_{24}O$	33.383	2.22073	2.32150	2.34427
48	Z β curcumin 12 ol	$C_{15}H_{24}O$	33.567	4.10643	4.19232	4.33486
49	**Khusimol**	$C_{15}H_{24}O$	34.058	5.73647	**5.80949**	**6.05558**
50	Khusol	$C_{15}H_{24}O$	34.187	5.75563	5.82849	6.07580
51	Isovalencenol	$C_{15}H_{24}O$	35.15	1.80051	1.90459	1.90066
52	Spirovetiva 3,7(11) dien 12 ol	$C_{14}H_{22}O_2$	35.267	3.28391	3.37628	3.46658
53	Allo khusiol	$C_{15}H_{26}O$	35.279	1.79958	1.90367	1.89968
54	Nootkatone	$C_{15}H_{22}O$	35.608	2.00808	2.11053	2.11978
55	Isokhusenic acid	$C_{15}H_{22}O_2$	35.633	2.22467	2.32541	2.34842
56	Zizanoic acid	$C_{15}H_{22}O_2$	35.642	1.96217	2.06498	2.07132
57	**β Vetivone**	$C_{15}H_{22}O$	35.858	3.35089	**3.44273**	**3.53729**
58	**α Vetivone**	$C_{15}H_{22}O$	36.558	3.50513	**3.59576**	**3.70011**

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Increased secondary metabolites with elicitor occur through activation of secondary pathways in response to stress, which results in a metabolic process that increases the activity of enzymes involved in the biosynthesis of secondary metabolites [12]. The addition of the Pb elicitor increases the component of Vetiverol compounds, higher than the control. Whereas, the addition of Cd activates the enzyme activity that plays a role in the synthesis of alpha sinensal and alpha amorphene in vetiver callus culture [5].

The addition of the Cd^{2+} elicitor gave a positive response to produce the production of alkaloids in the *Beta vulgaris* plant. The addition of Cd elicitor with a concentration of 1.0 mM gave the best response in increasing the production of secondary metabolites A and C inophyllums, as well as calophyllolide, whereas in callus suspension culture, increased the production of B and P inophyllums [13]. The addition of $CdCl_2$ with a concentration of 1 mM on culture media was able to effectively increase the production of andrographolide in *Andrographis paniculata* cell suppression culture by 4.14 times higher than the control [14]. Addition of 0.005 $mg.L^{-1}$ CdCl2 in *Rubia tinctorum* L. callus culture increased the flavonoid content of 57-64% compared to controls [15].

CONCLUSION

The addition of Pb and Cd heavy metal elicitor affected the formation and the content of vetiver oil compounds of root culture. Pb elicitor increased the number of roots, while Cd elicitor increased root length. However, the addition of Pb and Cd elicitor on culture media decreased the fresh weight and root dry weight. Fifty-eight vetiver oil compounds in the adventitious roots of vetiver plants were identified. The main compounds of vetiver oil in roots culture were Khusimone (6.94%), Khusimol (6.05%), Khusimene (4.85%), α Vetivone (3.70%), β Vetivone (3, 53%), Vetiverol (3.22%), Prezizaene (2.35%), and Zizaene (1.91%). The Cd elicitor increased the composition of the main compound in vetiver oil, that higher than the Pb elicitor.

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