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Vetiver Oil Production from Root Culture of *Vetiveria zizanioides*

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***Abstract*—**Vetiver oil is secondary metabolite that accumulates in *Vetiveria zizanioides* roots. The aim of this study was to obtain best type of root culture which produce high amount of vetiver oil, and was similar to metabolites produce from its mother plant. Protein analysis was also conducted to detect protein, related to putative enzymes, which have a role in terpenoids synthesis in the root culture. The results showed that root culture derived from crown explant produced the best root growth. The root culture produced primary and lateral roots, ca. 40 branches. The vetiver oil produced from root culture was analyzed by using GC-MS., and the highest content of terpenoids from roots of crown explant attained 19.024%. The result of SDS PAGE showed proteins which were ±61 kD and ± 68 kD, each might be related to putative monoterpene synthase and sesquiterpenes complex, respectively.

***Keywords*—**Protein, Root culture, Terpenoids, Vetiver oil.

I. Introduction

VETIVER oil is essential oils produced usually by refining roots of *Vetiveria zizanioides*. It has a complex composition, which consists of over 300 components with the main cyclic and bicyclic sesquiterpenoid structures like compound, e.g. vetivone α-, β-vetivone, khumisol, cadanene, cedrene and β-humulene [1]. It has an earthy fragrant aroma and a high fixative property, so it is widely used as raw material for industries such as in the manufacture of perfumes, cosmetics, deodorant, soaps, medicines, as well as insect repellent. Hence the economic value of vetiver oil is very high. However, vetiver plants also serve as barriers to erosion and soil rehabilitation, resulting in increasingly limited land for the planting of vetiver devoted to the production of vetiver oil. Therefore, alternative technologies are required to solve this issue.

Tissue culture methods have been shown as beneficial method in the production of some secondary metabolites in plants [2]. Secondary metabolites are easily purified, and can be produced without depending on climate and soil conditions [3]. Through tissue culture method, a physiological process that occurs in invivo bioprocess can be engineered in a controlled environment. Therefore, cells can be propagated to produce a particular metabolite. In addition, this method can also overcome the contradictions of land use, in this case between land for the production of vetiver oil with land used as barriers to erosion and soil conservation

Culture studies on the roots of vetiver plants had been conducted by [4]. However, these studies indicated that root growth was very small, amounting to 2cm for the early growth and only 1mm after subculture. This suggested that the research produced sub optimal root growth in culture, which might be due to a combination of plant growth regulators (PGR) used was not optimum, while the development of plant culture was strongly influenced by the combination of auxin and cytokinin, both exogenously and endogenously

Research conducted by [5] showed that callus culture which differentiated into root produced more various compounds and higher terpenoids concentrations than the callus culture, but it was still lower than that of roots of plants in nature (*in vivo*). This might be caused by the character of secondary metabolites synthesis in plants, which sometime was affected by the differentiation process since it was associated with the availability of the enzyme as well as functional precursor compounds [6]. Enzyme plays a very important role in determining the synthesis of metabolites. Therefore, the objectives of this research were to produce the best root culture type of vetiver containing components of vetiver oil, and to analyze protein patterns (related to putative enzymes) detected in the root culture.

II. Materials and Methods

A. Culture Preparation

Tissue culture need an aseptic condition, therefore all materials and medium were sterilized using an autoclave for 15 minutes at a temperature of 121°C and a pressure of 15 psi.

Explant used was the root tip and stems near the basal part of the roots of the plant *Vetiveria zizanioides* (crown). All explants were surface sterilized with 70% ethanol and then rinsed with sterile distilled water, followed by soaking in 0.8% NaClO for 10 minutes. After washing with distilled water, explants were then transferred into a sterile petri dish.

B. Root Culture Initiation

A Sterile explants, i.e. 5cm root tip and crown/stem consisting of small roots (± 2cm) were cut and cultured into MS liquid medium, which contained 1.0ppm naphthalene acetic acid (NAA), and 0.5ppm indole acetic acid (IAA). Cultures were then incubated in a shaker with agitation speed of 90 rotations per minute (rpm) in dark conditions.

C. Terpenoid Analysis Using GC - MS

Sample of roots (1.0g) were dried, and extracted with 10 mL of n-hexane at room temperature. The sample was then agitated for 24 hours. Extract were analyzed qualitatively and quantitatively using gas chromatography-mass spectrometry

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(GC-MS), linked to hot ionization detector (flame ionization detector), using a column of 5% phenyl methyl silox (30 X 0.25mm X 0.25lm). Vetiver oil content was analyzed, both compounds and percent of availability

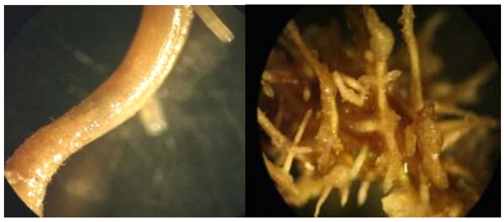
D.Protein Analysis

One gram of root samples were frozen and ground with liquid nitrogen. Samples were then precipitated with 15mL of tricloro acetic acid (TCA)-acetone (10% w / v) for 16 hours at -20˚C. Subsequently, samples were centrifuged for 30 minutes at 5000x g at 4˚C and the supernatant discarded. The sample was then added by 10mL of acetone (ice cold) and centrifuged for 10 minutes at 5000x g at 4˚C and the supernatant was discarded (this step was repeated 3 times). Furthermore pellet sample was added to a solution of 250mL lytic buffer.

Protein separations were performed using 10% SDS-PAGE and 5% stacking gel. Gel was placed in the electrophoresis buffer in the chamber. Samples: 0.5mg protein was added to the loading buffer (1: 1) and heated at 100˚C for 5 minutes. Samples and marker were loaded into wells in the gel and run at a voltage of 100 V for approximately 2 hours. The Gel was colored with dye solution containing 0, 05% coomasine brilliant blue-R 250 for 2 hours. Excess dye was removed by washing the gel in a solution of de staining solution for 12 hours.

III. RESULT

Result of root cultures showed that roots of explants derived from root tips and crown responded differently to MS medium with the addition of 1.0 ppm IAA and 0.5 ppm NAA. Explants derived from the root tip of vetiver plants produced the primary branch with an average of 3 branches of roots and lateral branch with average length of 0.108cm (Fig. 1 (a)). Explants derived from vetiver crown produced massive first branch and second branch formation (Fig. 1 (b)). Average first branch formation was as much as 3.3 branch roots with an average length of 5,667cm per branch, while the average number of lateral branch was 40.3 and the average root length of lateral branches was 0.487cm.



(a) (b)

Fig. 1 Root culture derived from root tip (a) and crown explants (b) responded to MS medium containing 1 ppm NAA and 0.5 ppm NAA

The use of different explants on induction of *Vetiveria zizanioides* root tissue culture was conducted to find the best explants that could form roots. It was based on concept that every part of plants (explants) used contained endogenous auxin and cytokinin differently, so they had different ability of morphogenesis although cultured in medium containing the same growth regulator [7]. Research of [8], also indicated that the basal part of the shoots on *Ulmus gabra* produced better results than the apical portion of the root buds of this plant, although they were given the same growth regulator. The apical and basal parts of the shoots on *Ulmus gabra* have different hormone content, hence produced different response on morphogenesis.

Results of the GC-MS chromatogram analysis showed that in roots culture (of explants: the root tip and crown/stem) some vetiver oil constituent compounds were detected (Table I). The highest content of vetiver oil produced in this study was in root cultures derived from crown/stem explants, which amounted to 19.02%. This value was higher than vetiver oil content resulting from root tip or rooted callus extract (10.35%), including also in the previous [5] study. According to [9], the ability of culture to produce secondary metabolites was often associated with the ability to form a specific organ culture. It can be due to the differentiation processes in plants [6], which also related to the availability of the enzyme, the type of synthesis, biosynthetic pathways, and the formation of space allocation precursor synthesis and accumulation [10].

TABLE I
THE CONTENT OF VETIVER OIL

Root culture type	% Vetiver oils compounds	%Terpenoid (sesquiterpen & monoterpen)	Sesquiterpen type	Monoterpen type
Root tip explant	1,68± 0.097	0,65±0,167 (0,39±0,084 & 0,26±0,083)	Phenol,2,4,6-tris(1-methylethyl)	4-ethyl-m-xylene, Ψ -cumene, dan durene
Crown explant	19,02 ±1,672	9,62±0,071 (5,03±0,396 & 4,59±0.325)	Phenol,2,4,6-tris(1-methylethyl)	4-ethyl-m-xylene, Ψ -cumene, 2-etil-1,4-dimetil-benzen, 1-3 dimetil -2-etil benzene dan durene
Root cultures of crown explants undergo dedifferentiation into callus.	10,28±1,2374	4,89±0,275 (4,64±0,985 & 0,23±0,017)	Phenol,2,4,6-tris(1-methylethyl)	4-ethyl-m-xylene, Ψ -cumene, 2-etil-1,4-dimetil-benzen, dan durene
Rooted callus	10,35±2,82	4,95±1,089 (3,07±1,34 & 1,88±0,2545)	Phenol,2,4,6-tris (1-methylethyl)	4-ethyl- m-xylene, Ψ -cumene, 2-etil-1,4-dimetil-benzen, dan durene

Vetiver oil content in root culture was lower when compared with vetiver oil content of plant roots *V. zizanioides* derived from nature. This might be resulted from different ages, in this study the roots of the culture was 2 weeks old, while the root extract of *V. zizanioides* derived from nature were generally aged 9-12 months. According [11], plant age affect the amount of secondary metabolites produced by plants. In addition, plant roots of *V. zizanioides* in nature usually were in symbiosis with bacteria and endomikoriza, which plays a role in the biotransformation of vetiver oil and increase the availability of nutrients, especially phosphorus [12]. This caused the amount of the metabolites of plant roots

V. zizanioides in nature was higher than in vitro culture [13].

SDS-PAGE results in this study indicated that there were two major protein bands between molecular weight of 70 kD and 55 kD. In addition, there was also a protein band between the molecular weight of 25 kD and 15 kD in protein extracts derived from the root of the culture of root culture (root tip explants and crown/stem) and roots that undergo dedifferentiation into callus and adventitious roots (roots callus) as available in Fig. 2. 12. Results of analysis showed that proteins between 70kD and 55kD were at ± 61kD and 68 kD, whereas the protein that resides between the molecular weight of 25kD and 15kD was at ± 23kD (Fig. 2).

SDS-PAGE analysis performed in this study was aimed to separate the total plant based protein molecular weight, so that can know the type of protein (enzyme) that was at the root of the culture of *V. zizainioides*. Research by [14] and [15] also showed that the results of SDS-PAGE analysis in the form of bands with molecular weight of a particular protein could be used as an indicator to determine the type of specific proteins, such as enzymes in plants. In this study the focus was monoterpene synthase, sesquiterpene synthase and FPP synthase enzyme.

Protein band with a molecular weight of 68kD was estimated as enzyme that plays a role in the formation of sesquiterpenes, i.e. the enzyme FPP synthase, an enzyme complex sesquiterpene synthase or terpene synthase enzyme. The putative enzyme in the range of molecular weights approximately 67kD was FPP synthase [16], sesquiterpene synthase which has a molecular weight of approximately 68.8 kD [17] and terpene synthase enzyme complex was approximately 67.4kD molecular weight, as an enzyme that acts to catalyze change in compound GPP and FPP into cyclic terpenoid compounds [18]. Molecular weight protein bands estimated to ± 61kD was estimated as an enzyme that plays a role in the formation of monoterpenoid, namely monoterpene synthase. This was because the monoterpene synthase enzymes in Quercus ilex have the molecular weight ± 59kD [19]. Protein that has a similar molecular weight usually when analyzed by SDS-PAGE will be on the same bands, but differences in voltage, temperature, and the gel buffer may also affect results in the migration of a particular protein. Tholl [20] indicated that the enzymes in *Arabidobsis thaliana*, e.g. sesquiterpene synthase were detected in a molecular weight of 63, 38 and 64kD. Furthermore, according to [21] some of the enzymes which have different molecular weights but has the same activity called isoenzymes. Therefore, the results of this study can be used as the basic information necessary for engineering gene to increase synthesis of vetiver oil, among others, by over-expression of genes that play a role in the synthesis and activity of sesquiterpene synthase enzyme.

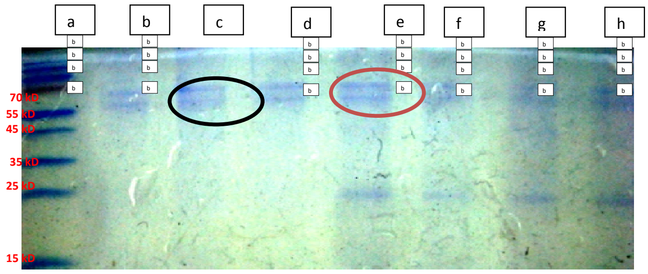


Fig. 2 SDS-PAGE of marker (a), 4 months in vivo roots(b), 9 month in vivo roots(c), 12 months in vivo roots (d) , root cultures derived crown explant (e), rooted callus (f), root cultures derived root tip explants (g), and dedifferentiation root (h)

IV. Conclusion

1. The results of quantitative and qualitative analysis of root cultures showed that cultures contained components of vetiver oil.
2. Growth and vetiver oil were obtained best from *Vetiveria zizanioides* root cultures derived from crown/stem explants.
3. Protein analysis (identification of the enzyme) in cultured roots of *Vetiveria zizanioides* showed protein bands which were thought to be a putative enzymes that plays a role in the formation of monoterpenes and sesquiterpenes (FPP synthase, monoterpene synthase and sesquiterpene synthase). These enzymes were allegedly very influential in the formation of vetiver oil in the root culture of *V. zizanioides.*

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