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Analysis of Secondary Metabolite Production in Somatic Embryo of Pasak Bumi (*Eurycoma longifolia*Jack.)

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Abstract

Pasak bumi (*Eurycoma longifolia* Jack.) has been known as a plants that can produce secondary metabolites for medicinal purposes such as: aphrosidiac, antimalaria, dysentri, antitumor, etc. Poor seed germination of pasak bumi will affect the avaibility of plant material for drug extraction. Over exploitation of this plant will also reduce plant population in its natural habitat. In vitro culture, i.e. through somatic embryogenesis, therefore, can be used as one of an alternative method for plant regeneration as well as for in vitro metabolite production. Based on this reason, the research has been done with an objective to analyze the presence of secondary metabolite in somatic embryo of pasak bumi. Seed-derived callus was used as an explant. This callus was maintained to proliferate in MS (Murashige&Skoog, 1962) medium supplemented with 2.25 mg/L 2,4-D and 2.0 mg/L kinetin. A half gram of callus from proliferation medium was transferred into the MS liquid medium containing 1.0 or 2.25 mg/L 2,4-D, and 2.0 mg/L BAP or 2.0 mg/L kinetin. Histochemical examination using Jeffrey's reagen and neutral red showed that alkaloid and terpenoid substances were presence in somatic embryo of pasakbumi. In accordance with histochemical test, GC-MS analyses showed that secondary metabolites was also synthesized by non embryogenic callus and the mixture ofembryogenic callus and somatic embryo, although the concentration in the mixture of embryogenic callus and somatic embryo was lower than those in non-embryogenic callus. Secondary metabolites, including 3-[(cyclohexyl-methyl-amino)-methyl]-3H-benzooxazole-2-one (0.06 %) and 2-furancarboxaldehyde, 5-(hydroxymethyl) (43.024 %) were found in embryogenic callus and somatic embryo. In addition, the mixture of embryogenic callus and somatic embryo also synthesized fatty acid and lipids (52.751 %) which was higher than non-embryogenic callus (24.789 %). Based on the result, the mixture of embryogenic callus and somatic embryo could produce secondary metabolites, such as alkaloid, terpenoid subtances, and phenol. The concentration of metabolites in the mixture of embryogenic callus and somatic embryo, however, was lower compare to non-embryogenic callus.

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1. Introduction

Pasak bumi (*Eurycoma longifolia* Jack.) is a plant species belong to the family of Simaroubaceae. It is popular,in some country of South East Asia, such as Indonesia and Malaysia, for its alleged sexual-enhancing properties[1]. Recent studies havereported that pasakbumican also be used for some medicinal purposes. Root of pasakbumi produce compounds such as quassinoids, canthin-6-onealkaloids, b-carboline alkaloids, tirucallane-type triterpenes, squalene derivatives and biphenylneolignans[2]. Some of these compounds has antiproliferative,anti-ulcer, antiplasmodial, anti-malaria and cytotoxicactivity[3, 4].

Poor seed germination of pasak bumi[5]will affect the avaibility of plant material for drug extraction. Moreover, over exploitation of this plant will also reduce plant populationin its natural habitat. Plant tissue culture, i.e. somatic embryogenesis, can be applied as an alternative method for plant regeneration as well as for *in vitro* metabolite production. Somatic embryogenesis has been successfully used for regeneration of some rare and endangered medicinal plant and also for production of secondary metabolite[6, 7]. *In vitro* production of secondary metabolite through somatic embryo has also been achieved in *Rauwolfia serpentine*[8],and *Salvia officinalis*[9].

Through *in vitro* culture, quality and quantities of secondary metabolite scan consistently be maintained, low cost, and do not need a large cultivation area[10]. Based on this reason, the research has been done with an objective to analyze the presence of secondary metabolite in the somatic embryo of pasak bumi.

2. Experiments

2.1. Callus Proliferation

Seed-derived callus was used as an explant for somatic embryo induction. This callus was maintained to proliferate in the MS basal medium[11] supplemented with 2.25 mg/L 2,4-D (2,4-Dichlorophenoxyacetic acid), 2.0 mg/L kinetin, 0,8 % agar, and 3% sucrose. Culture was then incubated at 25^{o}± 2^{o}C with 12 h photoperiod.

2.2. Somatic embryo induction

A half gram of callus from proliferation medium was transferred into the MS liquid medium containing 0.1 g/L activated charcoal, 3 % sucrose, 1.0 or 2.25 mg/L 2,4-D, and 2.0 mg/L BAP or 2.0 mg/L kinetin. These calluscultures were put into a shaker, agitated at 120 rpm, at room temperature and 16 h photoperiod for four weeks.

2.3. Histochemical Analysis

The present of alkaloid in callus, embriogenic and non embryogenic, was analyzed by using Jeffrey's reagent. The presence of alkaloid in cell was marked as yellow-brown color[12]. The terpenoid was detected using 0.03 % neutral red. The presence terpenoid in cell was marked as red color[12]. The observation was conducted under Nikon inverted microscope.

2.4. Gas-Chromatography Mass-Spectophotometric (GC-MS) Analysis

The quantitative analysis of secondary metabolites on callus in proliferation medium, the mixture of embryogenic callus and embryo somatic (including proembryo cell, globular embryo, heart-shaped embryo), and non embryogenic was conducted using GC-MS. Each of cell types was dried using freeze dryer. One gram of callus was diluted with 10 mL 96% ethanol and incubated on a shaker for two days. The callus and ethanol extract were filtered by using Whatman No.1 filter paper. This ethanol extract was then analyzed by using GC-MS.

Percent of total compounds was calculated according to equation below:

$$\%\ of\ Total = \frac{Corr\ area\ of\ compound}{Total\ corr\ area\ of\ desired\ compound}\ x\ 100\%$$

3. Results and Discussion

Pasak bumi contains secondary metabolites, such as alkaloids and quassinoids that can be used for medicinal purposes. Based on histochemical analysis, terpenoid could be detected in all type of callus, indicated by a red color change in the cells following reaction with neutral red (Fig. 1.). Meanwhile, only few of cell and callus could produce alkaloids following reaction using Jeffrey's reagent (Fig. 1.)

Some of alkaloid compounds could be found in all three types of callus (Table 1). The highest alkaloid content was detected in non-embryogenic callus (1.598 %), meanwhile the alkaloid content in embryogenic callus only about 0,06 %. The composition of alkaloid in all callus type was a bit different with those in root and stem[12]. The 2-furancarboxaldehide, 5-hydroximethyl produced in all of the cell type, around 49. 742 % of this compound could be obtained from callus in proliferation medium, 61.349 % from non embryogenic callus, and 43.024 % from embryogenic callus. The (2-furancarboxaldehide, 5-hydroximethyl) from non-embryogenic and embryogenic callus was also detected in the root and stem of pasak bumi[12]. Based on previous research, this compound is terpenoid derivative[12]. Instead of alkaloids and terpenoids, some phenolic compounds was also recognized in the three types of cell. The non embryogenic callus produced the highest phenolic compound concentration (9.404 %), while embryogenic callus only produced 0.583 %.

These result showed that the *in vitro* cells/tissues could synthesize alkaloids, terpenoids, and phenols. Nevertheless, the secondary metabolites in embryogenic callus were lesser than non embryogenic callus and callus in proliferation medium. This results was almost similar to the previous research conducted by some researcher. Only a fewsecondary metabolites was produced in somatic embryo of *Eleutherococcus senticosus*[7] and *Rauwolfia serpentina*[8].

The low content of secondary metabolites, either on somatic embryo or embryogenic callus, might be due to somatic embryo was still in growth stages. Growing somatic embryo generally require more primary metabolites to support cell growth and development. This result was supported by the result of GC-MS analysis which showed that relatively high concentration (52.791 %) of lipid was produced on embryogenic callus, including somatic embryos (Table 2). In the non embryogenic callus, only about 24.789 % lipid was detected and callus from proliferation medium produced about 37. 456% lipid . The high fatty acid was also obtained from somatic embryo of *Jatropha curcas, Daucus carota,* and *Brassica napus*[13-15].

Callus in proliferation medium, non-embryogenic callus, and embryogenic callus produced different composition of fatty acid and lipid. The highest composition of fatty acid on embryogenic callus and callus in proliferation medium was 9-octadecenoic acid, while on non embryogenic callus was *cis*-vaccenic. According to Avjioglu and Knox[7], somatic embryos show similar pattern like zygotic embryo when accumulate fatty acids and lipids, especially during maturation stage. However, the composition and amount of fatty acids and lipids produced by somatic embryo depend on the composition of the medium used for culturing[16, 15, 13].

Result of this study demonstrated that embryogenic and non-embryogenic callus, as well as somatic embryo, could synthesize alkaloid and terpenoid compound . The concentration of secondary metabolites on embryogenic callus and somatic embryos, however, was lesser than non embryogenic callus. Embryogenic callus and somatics embryo could produce higher fatty acids and lipids than non embryogenic callus due to they were still in growth stages.

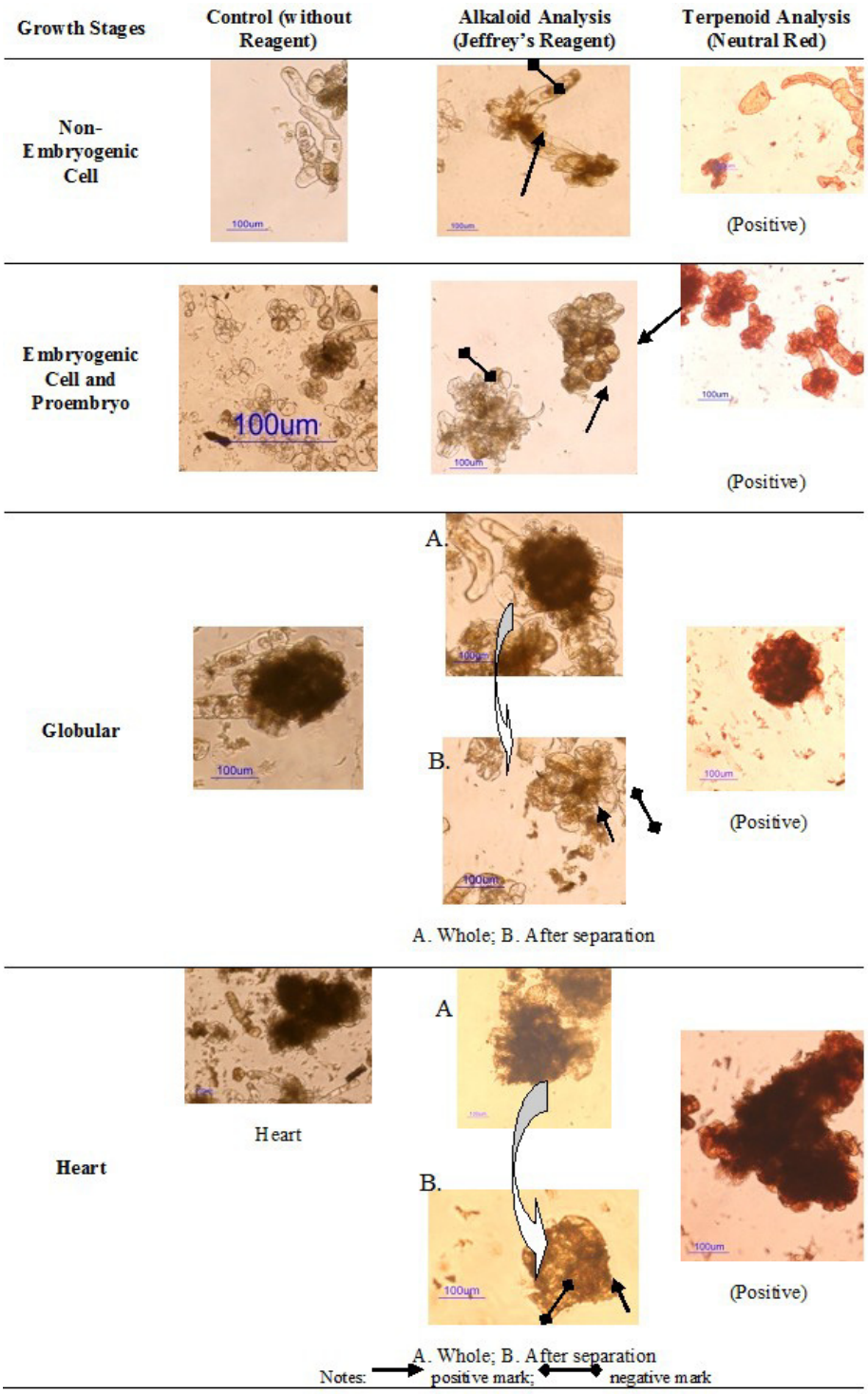

Fig. 1.Results of histochemical analysis on embryogenic, somatic embryo and non embryogenic of callus aftaer staining with the reagents to analyze alkaloid and terpenoid content

Table 1.The secondary metabolites using GC-MS analysis

No.	Compound Name	Callus in Proliferation Medium (%)	Non-embriogenic Callus (%)	The Mixture of Embriogenic Callus and Somatic Embryo (%)
Alkaloid				
1.	(1*E*)-Butanaldimethylhydrazone	-	0.335	-
2.	2,4,5- Trimethyl-3-oxazoline	-	0.193	-
3.	Propenoic acid, 2-trifluoroacetylamino	0.178	0.076	-
4.	8-Ethyl-2-methylthioindolizine	-	0.07	-
5.	5- (2-Oxohexahydro-1H-thieno [3,4-d] imidazol-4-yl) pentamide	-	0.081	-
6.	6,9-Eteno-5H-pyrrolo[1,2-A] azepin-5-one, 6,9-dihydro-7-phenyl	-	0.519	-
7.	1-(4-Methoxy-phenyl)-5,5-dioxo-hexahydro-5.λ.(6)-thieno[3,4-b]pyrrol-2-one	-	0.324	-
8.	Butanoic acid , 4-amino-4-oxo	0.162	-	-
9.	Benzenesulfonanilide	0.624	-	-
10.	2,5-Ethano-2H-azocino [4,3-b] indole, 4-ethylidene-1,3,4,5,6,7,hexahydro	0.146	-	-
11.	3-[(Cyclohexyl-methyl-amino)-methyl]-3H-benzooxazol-2-one	-	-	0.06
	Total	1.11	1.598	0.06
Aldehide				
1	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	49.742	61.349	43.024
Furfuran compound				
1	4,6-Dihydrothieno [3,4-b] Furan	0.085	-	
Phenol				
1.	Phenol, 2,6-bis (1,1-dimethylethyl)-4-methyl	-	0.496	0.583
2.	Phenol, 2-methyl-5-(1-methylethyl)	-	0.254	-
3.	Phenol, 2-(1,1- dimethylethyl)-4-(1,1,3,3-tetramethylbuthyl)	0.066	0.766	-
4.	Phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl	-	0.82	-
5.	2,3-dihydro-3,5-dihydroxi-6-methyl-4H-piran-4	7.668	7.068	-
	Total	7.734	9.404	0.583

Table 2. The fatty acid and lipid using GC-MS analysis

No.	Compound Name	Callus in Proliferation Medium (%)	Non-embriogenic Callus (%)	The Mixture of Embriogenic Callus and Somatic Embryo (%)
1.	Dodecanoic acid(10:0)	-	0.508	-
2.	Tetradecanoic acid (14:0)	0.243	1.448	0.213
3.	Tetradecyltrifluoroacetate (14:0)	-	0.217	-

4.	Hexadecanoic acid, methyl ester (16:0)	1.643	1.456	2.969
5.	Hexadecenoic acid (19-16:1)	-	0.201	-
6.	Palmitic acid (16:0)	-	3.399	-
7.	cis-Vaccenic acid	-	6.703	-
8.	9-Octadecenoic acid, methyl ester (9-18:1)	-	3.369	6.929
9.	Octadecanoic acid, methyl ester (18:0)	2.545	1.903	4.232
10.	Octadecanoic acid (18:0)	9.433	5.315	12.588
11.	Hydroxymethyl ethyl palmitate	-	0.27	-
12.	Tricosanoic acid	0.085	-	-
13.	Oleic acid	0.183	-	1.502
14.	Hexadecanoic acid(16:0)	4.585	-	5.076
15.	11-Octadecenoic acid, methyl ester (18:0)	4.538	-	-
16.	9-Octadecenoic acid (9-18:1)	11.372	-	18.821
17.	Methyl dihydromalvalate	0.186	-	-
18.	Cis-10-nonadecenoic acid	0,319	-	-
19.	2,3-Dihydroxypropyl elaidate	0.771	-	-
20.	2,3- Dihydroxypropyl (9Z)-9-Octadecenoate	1.553	-	-
21.	Nonanoic acid	-	-	0.14
22.	Stearic acid (18:0)	-	-	0.281
Total		37.456	24.789	52.751

4. Conclusion

Based on this experiment, the embryogenic callus could produced secondary metabolites, such as alkaloid, terpenoid, and phenolic. The concentration of metabolites in embryogenic callus, however, was lower compare to non-embryogenic callus.

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