Production of artemisinin in shoot cultures of *Artemisia cina* irradiated callus

Produksi artemisinin pada kultur tunas hasil iradiasi kalus *Artemisia cina*

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Abstract

Artemisinin is an antimalaria agent that is only found in *Artemisia sp*. medicinal plant. This compound is occurred at very low level in that plant. Irradiated callus of *Artemisia cina* have been conducted by the dose of 40 Gy to improve artemisinin content in shoot cultures. Regeneration of irradiated callus to shoot cultures formation has been successful in Murashige & Skoog (MS) medium combined with Naphtalene Acetic Acid (NAA) 1 mg/g, Benzyl Amino Purin (BAP) 2 mg/L and 15 % coconut water. Ten mutant lines of shoot cultures have been analyzed for the artemisinin content. The artemisinin content of mutant lines were higher than the original plant. The highest artemisinin content is 5 mg/g found in mutant line number 404, this line also has wider leaf area than original plant.
Key word : artemisinin, *Artemisia cina*, shoot cultures, irradiated callus

Abstrak

Artemisinin adalah senyawa antimalaria yang hanya diproduksi oleh tanaman obat *Artemisia sp*. Senyawa ini sangat sedikit terdapat dalam tanaman tersebut. Iradiasi kalus terhadap *Artemisa cina* dengan dosis 40 Gy telah meningkatkan kadar artemisinin dalam kultur tunas. Regenerasi dari iradiasi kalus untuk membentuk tunas telah berhasil dengan menggunakan media Murashige & Skoog (MS) yang dikombinasi dengan Naftalen Asam Asetat (NAA) 1 mg/L dan Benzyl Amino Purin (BAP) 2 mg/L dan air kelapa15 %. Sepuluh galur mutan dari kultur tunas telah dianalisis kadar artemisininnya. Kadar artemisinin galur mutan lebih tinggi dari tanaman induknya. Kadar tertinggi diperoleh 5 mg/g pada galur mutan nomor 404, galur ini juga memiliki daun lebih lebar daripada tanaman induknya.
Kata kunci : artemisinin, *Artemisia cina*, kultur tunas, iradiasi kalus

Introduction

Malaria is one of infectious disease caused by *Plasmodium falciparum*, this disease is serious threat to people in the tropical country included Indonesia. The medication using chloroquine due to plasmodium resistance against this drug, therefore, new drugs over come this resistant parasites are urgently needed.

Artemisinin is a sesquiterpene lactone which is very active substance as antimalarial and anti tumor agents. Artemisinin based-compound it called Artemisinin Combined Theraphy is recommended by World Health Organization (WHO) for malaria treatment in Africa. Artemisinin has been found in leaves and top of flower of *Artemisia annua*. Artemisinin content in the port of the plant is influenced by environmental factors such as light intensity, altitude and cultivation condition (Ferreira and Janicks, 1996).

Several studies have focused on the production of artemisinin such as fertilizing by nitrogen and potassium, in vitro cultures and mutation induction by gamma rays. Gamma ray is electromagnetic wave which has high energy

produce some free radicals from radiolysis of water. Free radicals will attack the DNA when the plant is treated by gamma rays. The effect of gamma rays on plants can be shown by phenotypic and genomic changes of plants. Callus is a mass of undifferentiated cells of *A.cina*, it very sensitive on gamma rays, and it will produce the mutant line with wider leaves, many branches and increasing of artemisinin content.

Artemisia cina (Compositae) is one of medicinal plant which has been used traditionally for tumor and malaria diseases (Aryanti *et.al*, 2001 and 2006) because they contain of artemisinin bioactive compound. However, the content of this compound is very low level. Artemisinin content in this plant is lower than in *A.annua*, so that the improving of this compound by mutation induction is needed. *A.cina* is found in Papua Province compared to *A.annua* is imported from abroad.

The aim of this study was to improve the artemisinin content by mutation induction of callus of *Artemisia cina*.

Methodology

Materials. The species of *Artemisia cina* of Compositae medicila plants is used in this experiment. All chemicals used are produced by Merck Germany with pro analytic quality.

Callus cultures. To obtain the friable callus, the petioles of *A.cina* was cultured in Murashige & Skoog (MS) medium was combined by Benzyl Amino Purin (BAP), Naphtalene Acetic Acid (NAA) growth regulators, by the concentrations 0.05 to 2 mg/L. The callus formation was observed up to 6 weeks. The best callus was selected for irradiation samples.

Irradiation. Callus was prepared in Petri-dish and irradiated by the dos of 40 Gy. The irradiated callus was then transferred to fresh MS medium combined with BAP, NAA and 10 and 15 % coconut water for regeneration of irradiated callus.

Extraction, and determination of artemisinin content. The growing of plantlets was observed during shoot cultures such as height of plantlet, leaves and umber of branches. To obtain the artemisinin content, the dried leaves from 5 sub cultures was extracted by n-hexane and then separated by small chromatography column with gradients of organic solvent. The ethyl acetate fraction from above separation then injected to High Performance Liquid Chromatography using Bondapak C-18 column, UV detector and acetonitril/water (7/3) as mobile phase. The artemisinin content was calculated by comparing to artemisinin pure standard.

Results and Discussion

The callus cultured on MS medium supplemented with BAP and NAA with combination from 0.05 mg/L to 2 mg/L concentrations (Table 1). The combination auxin and cytokinin at the same concentration, the callus formation was better than medium with higher auxin than cytokinin.

The irradiated callus was then regenerated in several kinds of mediums. The regeneration based on totipotency concepts. Totipotency is the ability of all living cells potentially to regenerate whole new plant if the cells are grown in suitable medium and environment. Table 2 is shown the ability of irradiated callus to regenerate into whole plants at several kinds of mediums.

The best medium for regeneration of irradiated callus was combination 1 mg/L NAA, 2 mg/L BAP and 15 % of coconut water after 6 weeks callus culture. The induction callus by this medium was better than the others because of the differentiation of callus to shoot cultures formation need cytokinine more than auxin (Woerdenbag *et.al*, 1993 and Geldre *et.al* 1997). Coconut water also influence on regeneration of callus cultures.

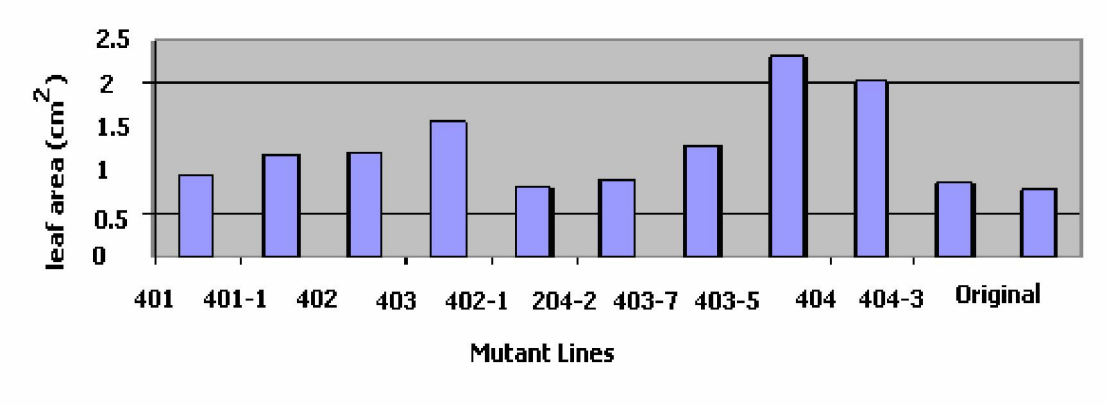

Figure 1. Leaf area (cm^2) of mutant lines and original plant.

Table I. Effect of growth regulators on *A.cina* callus induction

Culture	Growth regulator combination	Concentration (mg/L)	Callus formation	Appearance
1	BAP/NAA	0.05/0.05	-	No callus
2	BAP/NAA	0.5/0.5	+++	Friable, green
3	BAP/NAA	1/0.5	+	compact,yellowish
4	BAP/NAA	1/1	++++	Friable, green
5	BAP/NAA	2/2	++	Friable, green

Note :
- : not detectable, + : bad, ++ : moderate, +++ : good, ++++ : best
For the initial callus was trying several petioles. Young leaves were the best source of explants. The best medium for the callus induction was MS combination with BAP/NAA by 1/1 mg/L concentration.

Table II. Effect of growth regulators on regeneration of irradiated callus

Irradiated callus	NAA/BAP(mg/l)/CW (%)	Number of shoots/callus
1	1/0.5/10	3.68c
2	1.5/0.5/10	5.00d
3	2/0/10	2.83b
4	1/2/15	8.84e
5	1.5/1/15	4.48c
6	1/1/15	2.90b
7	1/0/15	1.81a

The letter in the same column is significantly different each other at 5 % level

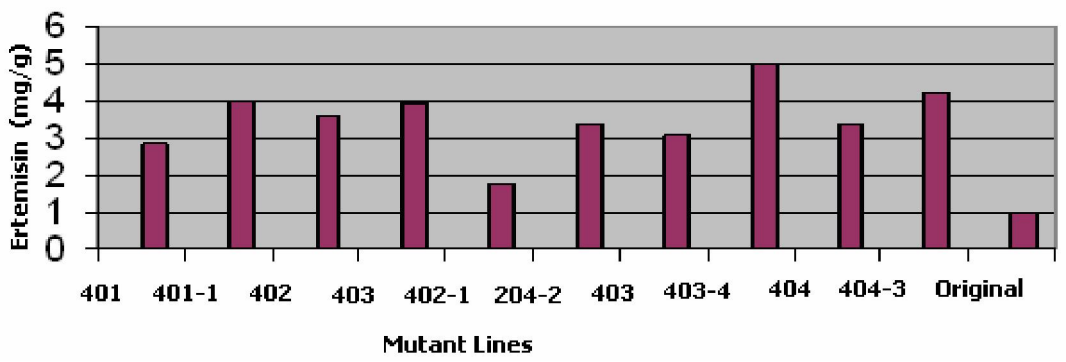

Figure 2. Artemisinin content of *A.cina* mutant lines

Coconut water is contain some minerals, amino acid (serin and sichimic acid), organic acid, and growth hormone i.e., zeatin, Indole Acetic Acid (IAA) and gibereline-like.

Serin and sichimic acid amino acid are precursor for triphtophan formation. Zeatin a kind of cytokinine growth hormone was inducer for cell division and shoot formation (Aryanti, 2001; Nin *et.al*, 1996). Shoot formation from the best medium (no. 4) was then propagated to MS medium without growth hormone, and then leaf area of mutant lines and original plant were observed during culturing. Furthermore the propagated shoot from this medium is called mutant line number 40. After selection at second sub culture based on the performance and artemisinin content, the selected mutant lines were then sub cultures until 5 time. Figure 1 is shown the leaf area of mutant lines and original plant, only selected lines are shown in this figure.

Artemisinin is accumulated in leaves of *A.cina*, the widest leaf of mutant line is found in 404 mutant lines. The leaf area of mutant lines were significant different with original plant (F=5 %). Kelsey and Shafizadeh (1980) reported that 35 % of the mature leaf surface is covered with capitate glands which contain most of the monoterpenes and virtually all of sesquiterpene lactone. The widest leaf area is correlated with artemisinin content (Figure 2).

Figure 2 shown that, the highest artemisinin from mutant line of *A.cina* was 40-4 line with the concentration was 5.00 mg/g. All the mutant lines have higher artemisinin content than original plant and significant different between artemisinin content of mutant line and original plant (F=5 %). The concentration of artemisinin in between mutant lines no significant different. According to Croteau and McCaskill (1994), the formation of sesquiterpene-lactone is through of mevalonic-acetic pathway with 3–hidroxy-3 methyl-coenzyme A reductase (HMGR) as regulator. The most part for the expression of HMGR 1L at leaves, and flowers of *Artemisia sp.* The enhancing of artemisinin content by gamma rays is assumed that the free-radicals enhance the pathway of sesquiterpene-lactone formation.

Conclusions

The shoot cultures from regenerated irradiated callus of *A.cina* were contain higher artemisinin than original plant. The highest artemisinin is found 5 mg/g at mutant line number 404, this line also have widest leaf area compared to original plant.

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