Biosaintifika_Flavonoid Production in Callus Cultures from Mesocarp of Stelechocarpus burahol.pdf

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Submission date: 01-Sep-2021 04:20PM (UTC+0700)

Submission ID: 1639376794

File name: Biosaintifika_Flavonoid Production in Callus Cultures from Mesocarp of Stelechocarpus burahol.pdf (409.36K)

Word count: 4776

Character count: 24910

Biosaintifika 8 (2) (2016) 214-221

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Flavonoid Production in Callus Cultures from Mesocarp of *Stelechocarpus burahol*

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DOI: 10.15294/biosaintifika.v8i2.6632

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History Article

Received 20 February 2016
Approved 10 June 2016
Published 18 September 2016

Keywords:
mesocarp; callus; *Stelechocarpus burahol*; flavonoid

Abstract

Stelechocarpus burahol is one of the medicinal plants that contains flavonoids. The study was carried out to know flavonoid production of cultures in vitro *S. burahol* from mesocarp explants. Mesocarp explants were cultured on MS medium containing different combination and concentration of plant growth regulators i.e. picloram (5, 7.5 and 10 mg/L) and 2, 4-D (10, 15 and 20 mg/L) under dark condition. Induction of callus formation started on the 20.29th to the 29.86th days. Medium supplemented with Picloram and dark state proved to be the best condition for optimum callus induction from mesocarp explants of *S. burahol*. Callus grown on medium with the addition of 7.5 mg/l Picloram produces the highest flavonoid. The maximum production of the secondary metabolite was obtained from 8 weeks old callus. However, by the time of callus ageing, its output has declined. It could be concluded that callus cultures from mesocarp *S. burahol* can be used for flavonoid production.

How to Cite

Habibah, N. A., Moeljopawiro, S. Dewi, K. & Indrianto, A. (2016). Flavonoid Production in Callus Cultures from Mesocarp of *Stelechocarpus burahol*. *Biosaintifika: Journal of Biology & Biology Education*, 8(2), 214-221.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Stelechocarpus burahol (Blume) Hook. F. & Thomson) is one of the potential medicinal plants from Annonaceae family which has the synonym Uvaria *burahol*, Blume. It naturally spread in Southeast Asia and the islands of Salomon (Siregar, 2005). In Indonesia, it is called by its local name Kepel. Kepel has the anti-hyperuricemic flavonoid compounds which can be potentially developed as gout medication. A study on the ability of Kepel as gout treatment was performed by Purwatiningsih et al. (2010). The study results showed that both ethanol and hexane extracts of Kepel have the effect on lowering blood uric acid levels. The ethanol extract of Kepel was effective at 200 mg/kg b.w., whereas the hexane extract was effective at 100 mg/kg b.w One of the most active antioxidants of flavonoid compounds in Kepel is 3, 7, 3 ‘, 4’-methyl-5- tetrahydroxy-flavone (Sunarni et al., 2007). Kepel has traditionally been used as a traditional perfume, particularly among the palaces in Java Island. Kepel is usually consumed by the queen and princesses since Kepel acts as a natural body, breath, and urine deodorant (Darusman et al., 2012). The previous study on Kepel as a natural deodorant showed that Kepel can eliminate the smell of sweat, breath, and faeces by absorbing the ammonia (NH_3) and methyl mercaptan (CH3SH). Also, Kepel leads to increase the population and the activity of probiotic bacteria *Bifidobacter* sp. with growing population. The growing population of probiotic bacteria will combat the people of odor-producing bacteria (Darusman et al., 2012).).

Flavonoids are secondary metabolites produced by various parts of the plant with low molecular weights. It has varied functions of bioactive activities among others. For example: as an antioxidant (Sunarni et al., 2007), an anti-cancer (Lin et al., 2008), an anti-gout (Purwatiningsih et al., 2010), an antihypertensive (Cassidy et al., 2011) and an anti-bacterial (Khan et al., 2013).

The production of secondary metabolites by *in vitro* culture is influenced by several factors, i.e. the concentration of growth regulators (Amoo & Staden, 2012, Amoo et al., 2012, Gurel et al., 2013) and the stages of tissue development (Aslam et al., 2010 , Keul et al., 2012, Balasubramanya et al., 2012, Misra & Dey, 2013). The exogenous growth regulating substances can alter the accumulation of secondary metabolites by regulating the gene expression involved in the metabolites synthesis. The regulation of gene expression performs during the transcription process (it occurs in the transcription factor of the genes associated with the metabolites synthesis) (Zhao et al., 2011, Rosa et al., 2013). Lewis et al., (2011) found that the addition of exogenous auxin is reported to induce the gene encoding enzymes involved in flavonoid biosynthesis pathway. In fact, all plants have the same flavonoid biosynthesis pathway; however, the results of flavonoid types and concentrations produced are different in each species.

This phenomenon occurs since there is varied gene expression patterns associated with the primary enzymes and transcription factors involved in the biosynthesis of flavonoids depending on the stage of organ development, response to the hormonal treatment, and response to the stimulation of the wound (Zhao et al., 2013). Chalcone synthase (CHS) is a key enzyme in the biosynthesis of all classes of flavonoids in the plant. This enzyme catalyses the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin-chalcone (Kreuzaler & Hahlbrock (1975) in Moriguchi et al., 1999).

There are some researches related to *kepel* potencies that have been investigated (Tisnadjaja et al., 2006, Purwantiningsih et al., 2010, Sunardi, 2010, Darusman et al., 2012). Research on the *in vitro* culture of the family Annonaceae have been reported in *Annona muricata* L., *Rollinia mucosa, Annona cherimola* and *Annona gabra* (Lemos and Lleger, 1998, Figueiredo et al., 1999, Figueiredo et al., 2000, Figueiredo et al., 2001, Padilla and Encina, 2004, Oliveira et al., 2008).

Recently, the information on callus induction and flavonoids production by *in vitro* culture of *kepel* that used mesocarp as explant has not been reported. Therefore, the study was carried out to know flavonoid production of cultures in vitro *S. burahol* from mesocarp explants.

METHODS

Establishment of Callus Culture

Mesocarp explants were taken from young fruit of kepel trees at University of Gadjah Mada area. The fruit was washed by running water and soaked in a solution of liquid detergent (1 ml in 50 ml aquadest) for 15 minutes, then immersed in 100% bleach solution (containing 5.25% NaClO) for 30 minutes. In the laminar air flow cabinet, fruits were sterilised using 100% bleach solution (containing 5.25% NaClO) for 10 minutes. Finally, mesocarp was taken from the fruit after washing with sterile distilled water for three times. the mesocarp was taken from the fruit.

Murashige and Skoog (1962) (MS) medium was used as a medium for the growth of

explants. Plant growth regulators 2,4-D at concentrations of 10, 15 and 20 ppm and Picloram at concentrations of 5, 7.5 and 10 ppm used for callus induction. In the treatment of 2,4-D, the medium is was added 0.09 ppm BAP as well. The investigation was carried out using six combination treatments (Table 1).

Table 1. Treatment of Plant Growth Regulator

PGRs		Treatment
Picloram	2,4-D	
5	-	P5
7.5	-	P7.5
10	-	P10
-	10	D10
-	15	D15
-	20	D20

After the medium preparation, subsequently, the sterile explants were cultured in the treatment medium. The cultures were maintained under total darkness at 25±2 °C. The culture was maintained for three months. Every month the culture was transferred to fresh media. Calli were harvested and weighed to determine the wet weight. Then callus dried in an oven for 48 hours at 60°C to determine the dry weight and analysed the content of flavonoids. Observations were made on growth and morphology of the callus. Percentage of explants that formed the callus speed callus formation, callus fresh weight, dry weight and morphology of the callus were recorded.

Callus Growth Determination

The callus growth was observed by inoculating 1 g callus in the optimum medium obtained from the previous experiment. The experiment was set up in a completely randomised design with 12 treatments (number of weeks in culture) and three replicates. Evaluations were made at 1-week intervals up to the 12th week. Each treatment consisted of one flask, from which cell fresh and dry weights, and flavonoid content was determined.

Total Flavonoid Determination

Dried callus (0.2 g) was ground to a powder with a mortar and pestle. The powder was extracted with methanol containing 1% (v/v) HCl, followed by addition of 2 N HCl (equal volume) and 1 h incubation at 90°C. Acid-hydrolyzed extracts were dried and resuspended in methanol (Hao et al., 2009). The total flavonoid content was determined using the procedure reported by Zou et. al., (2004). A 0.5-mL aliquot of appropriately diluted sample solution was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and allowed to stand for 6 min, then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 mL, then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus a prepared water blank. Quercetin was the standard of choice for the expression of results at 510 nm.

Data analysis and Statistical Testing

Analysis of variance calculated for the callus induction, callus growth, cell growth, and flavonoid content. All the results expressed as means ± standard errors from three replications. Data were analysed using the SPSS statistical system. Where the F test showed significant differences among means, multiple range test were performed at the 0.05 level of probability.

RESULTS AND DISCUSSION

Establishment of Callus Culture

The endophytic species live in the Kepel' mesocarp is causing a laborious and tedious sterilisation process. However, the sterilisation process on mesocarp is easier than the leaves. The sterilisation process on leaves requires gradual processes with the low success rate (Habibah et al., 2013).

The results of callus induction frequency, days to callus formation, and fresh weight from mesocarp explants in S. burahol in response to Picloram and 2, 4-D in MS media is presented in Table 1. The calluses were developed in all treatments. However, the growth of calluses was significantly differed (Table 2). Growth medium supplemented with 7.5 mg/L Picloram proved to be the best condition for the optimum fresh weight of callus. The maximum callus induction frequency for mesocarp explants (100%) was observed in the presence of 5 mg L-1 Picloram. Explants on callus induction occurred within 20.29 to 29.86 days after inoculation. The fastest growth of callus from mesocarp explant was observed at medium by addition of 10 mg/ L Picloram. The explant growth at medium with 10 mg/L 2,4-D, it showed the slowest growth rate (29.86 days). The control treatment (MS medium without any plant hormone supplementation) was not capable

of inducing callus growth. The optimum callus induction using immature seeds explant was also obtained on MS medium supplemented by Picloram 7.5 mg/L (Habibah et al., unpublished data).

However, when compared with the culture of mesocarp of other plants, the callus induction on Kepel requires longer time. Mesocarp of *Psidium guajava* produced callus within three days with a percentage of 92% on MS medium supplemented with 2.4-D 2 mg/L. Mesocarp of immature fruit is more responsive than the mature one (Chandra et al., 2004). The growth of callus on Vitis mesocarp was induced 15 days on a medium supplemented with 1.0 mM kinetin and 0.5 mM a-naphthaleneacetic acid (Calderon et al., 1995). Peach mesocarp was induced within two weeks on White medium with kinetin and coconut water. Kepel mesocarp was failed to produce callus when it has grown in a medium with 2.4-D concentrations lower than 10 mg/L (data not shown). According to the results, it could be assumed that the induction of callus formation on Kepel requires higher auxin concentration than in other plants. Induction of callus formation requires auxin and cytokinin in balance concentration that derived from endogenous or exogenous hormones.

Callus growth is shown by the wet weight and dry weight of the callus generated at each treatment. These results indicated that the growth of callus varies in each treatment. The explants grown in the medium supplemented with Picloram 7.5 mg/L produced the optimum callus growth. It showed the wet weight of 3.76 ± 0.7. Inversely, the explants were grown in the medium supplemented with Picloram 10 mg/l produced the lowest callus growth with the wet weight of 3.13±0.22. Picloram reportedly also can induce the most rapid growth and produce the highest biomass in suspension cultures of Rollinia (Figueiredo et al., 2000). Fruit explants (epicarp and hypoderm) of *Aronia melanocarpa* grown in medium supplemented with Kinetin combination, IAA and 2.4-D with various concentrations produced callus but the intensity, the growth index and morphology differs depending on the type and dose of PGR. The explants of *Aronia melanocarpa* were grown in medium supplemented with 2.4-D showed the highest callus intensity than medium supplemented with NAA. Mesocarp with the addition of Kin, BAP and 2.4-D does not produce callus (Calalb et al., 2014). The results of this studies reinforce that types of plant growth regulators (auxin and cytokinin), and the ratio between the two are important not only for increasing the percentage of callus formation but also for callus growth (Zenk 1978).

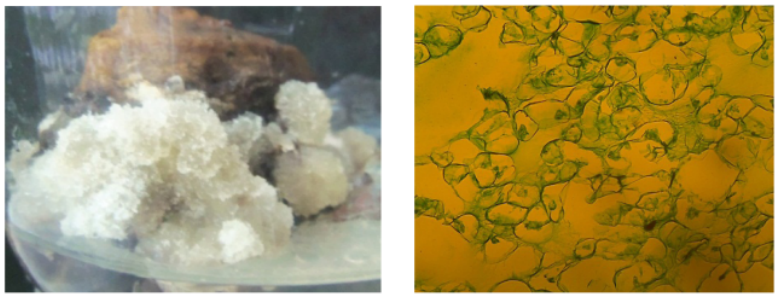
Auxin showed different effects on cell division, proliferation and subsequent regeneration of auxin in plants. Auxin acts by inducing non-dividing cells that are sensitive to auxin, which is at the G1 phase, to enter S phase and mitosis. The time duration of this process depends on the type and concentration of auxin (Barro et al., 1998). Callus produced on most of the treatment medium was friable callus (Figure 1). Callus formed are the yellowish white colour at the beginning of its formation, and it turns into brownish associated with an increased content of phenolic compounds on the callus.

The resulting callus on all treatments produced flavonoids with varying amounts (Figure 2). Flavonoids in Kepel has an antioxidant activity and one of the flavonoids contained in Kepel is 3, 7, 3 ‘, 4’-methyl-5- tetrahydroxy-flavone which has the most active antioxidant activity than other flavonoids (Sunarni et al., 2007). Flavonoids in *S. burahol* also have an anti-hyperuricemic activity. The results of in vivo tests of both ethanol and hexane extracts of Kepel has a potential in lowering blood uric acid levels. Flavonoids in S. burahol have the ability as a xanthine oxidase in-

Tabel 2. Effects of Picloram and 2,4-D in MS medium on callus induction frequency, days to callus formation, and fresh weight from mesocarp explants in *S. burahol*

PGRs Concentration (ppm)	Callus induction frequency (%)	Days to callus formation (days)	Fresh weight (g)
Picloram 5	100	24.14±0.90	3.51±0.22^{b}
7.5	80	21.57±1.27	3.76±0.16^{a}
10	80	20.29±0.49	3.13±0.22^{c}
2,4-D 10	28	29.86±2.41	3.23±0.18^{c}
15	68	25.29±1.38	3.32±0.13^{bc}
20	36	29.71±1.80	3.38±0.15^{bc}

Note : different letters in the same column indicate significant differences between treatments.



(A) (B)

Figure 1. (A) Callus from mesocarp of *S. burahol* on MS medium supplemented with 7.5 mg/l of Picloram after a culture period of two months. (B) The anatomy of callus (3 months old) as the results of mesocarp inoculation on MS medium with the addition of 7.5 mg/l Picloram

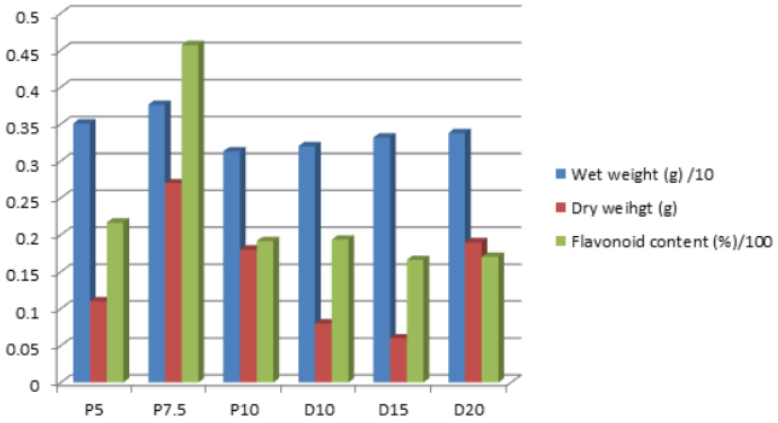


Figure 2. Growth and Flavonoid content in *S. burahol* callus in different medium
Note: P5:MS+Picloram 5 mg/l; P7,5:MS+Picloram 7,5 mg/l; P10:MS+Picloram 10 mg/l; D10:MS+2,4-D 10 mg/l; D15:MS+2,4-D 15 mg/l; D20:MS+2,4-D 20 mg/l

hibitor which inhibits the formation of uric acid as well as gout medication using Allopurinol (Purwantiningsih et al., 2010).

A callus that produces the highest flavonoid was the callus that grew in the medium containing 7.5 mg / l Picloram. Conversely, the lowest level of callus growth and flavonoid compound were found in a medium supplemented with 15 mg/l 2.4-D. Picloram and 2.4-D significantly increased the accumulation of biomass and production of withanolides in *Withania somnifera*. High concentration of auxin often inhibits cell growth and production of withanolides in culture *Withania somnifera* (Sivananthan et al., 2013). The use of 2,4-D with higher concentrations of BAP can improve the quality but not the quantity of bioactive compound in Hawthorn callus culture (Chaabani et al., 2015)

Hormones strongly influence the production of secondary metabolites in callus. The signal hormone can regulate the physiological processes in plant culture (Molchan et al., 2012). Exogenous growth regulating substances can alter the accumulation of secondary metabolites by regulating the expression of genes involved in the synthesis of secondary metabolites. Regulation of gene expression performed on the stages of transcription by regulating gene transcription factor that plays a major role in the process of plant development including synthesis of secondary metabolites (Zhao et al., 2011, Rosa et al., 2013). Plant-specific transcription factor NAM / Ataf / CUC (NAC) plays a critical role in a variety of developmental processes including defence responses. NAC transcription factor domain was allegedly organised at the level of transcription by

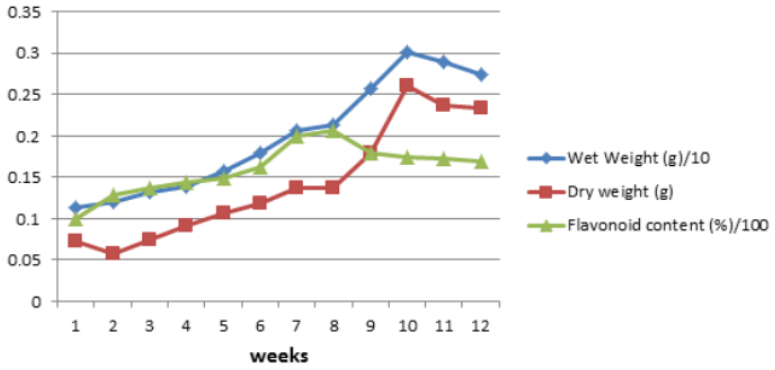


Figure 3. Growth and Flavonoid content of *S. burahol* callus in P7.5 medium at variable time duration

environmental factors, such as hormone concentrations. Induction at the transcriptional level PmNAC1 associated with increased levels of auxin in the culture medium (Rosa et al., 2013). Auxin is also reported to induce gene encoding enzymes involved in flavonoid biosynthesis pathway. The addition of 1 µm IAA induces the accumulation of mRNA chalcone synthase (CHS) and flavonol synthase (FLS) 3 times greater than the control (Lewis et al., 2011).

The production of flavonoids in the callus culture of *S. burahol* ran from the first week and peaked at the 8th week (Figure 3). The growth of callus in the first week until the eighth week showed the lag phase. Subsequently, started from the 8th to the 10th week, it represents the log phase and then entered the stationary phase. Both steps showed that the production of flavonoids is not in line with the growth of callus. At the time of early log phase of callus growth, the highest flavonoid concentration was obtained. The optimum callus growth was reached in the 10th week. On the date of log phase, it requires an abundant amount of primary metabolites. Therefore, the production of secondary metabolites is decreased. When a stationary phase on the callus is reached, the secondary metabolite production will increase.

However, the supply of nutrients is limiting the process of secondary metabolites production to not to rise in its concentration. Verbascoside and linear in production were associated with growth reaching their highest metabolite production when cell growth was in the stationary stage. Verbascoside was the primary phenylpropanoid produced in vitro cultures (root, white and green callus, while linear in and hydroxycinnamic acid production were low. Verbascoside and linear in production were improved in cell suspension culture (Estrada-Zu´n˜iga et al., 2009).

CONCLUSIONS

The optimum callus induction from mesocarp explants was obtained in the growth medium supplemented with 7.5 mg/L Picloram in dark conditions. Picloram induces the formation of friable callus. The callus induction was varied from 28% to 100%. Callus in all treatments expressed the flavonoid compound production. The callus that produced the highest flavonoid content was the callus maintained in the medium with the addition of 7.5 mg/1 Picloram. The maximum flavonoid production was obtained at eight weeks old callus.

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