Callus induction and flavonoid production on the immature seed of *Stelechocarpus burahol*

IOP Conf. Series: Journal of Physics: Conf. Series **983** (2018) 012186 doi:10.1088/1742-6596/983/1/012186

Callus induction and flavonoid production on the immature seed of *Stelechocarpus burahol*

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Abstract. *Stelechocarpus burahol* [(Bl.) Hook. f. & Th.] is one of the medicinal plants. *In vitro* callus induction studies on *S. burahol* were carried out to determine phytohormone requirement for optimum callus induction. Immature seed explants were cultured on MS medium by adding different kinds and different concentrations of plant growth regulators (picloram and 2,4-D) under light and dark conditions. The results showed that callus formation was initiated on the 18,50th to the 55th days. The best condition for optimum callus induction was found on MS medium, which was supplemented with 7.5 mg/L picloram and was maintained in the dark condition. The callus induction varied from 60% to 100%. The callus that produced the highest flavonoid was grown on the medium with the addition of 10 mg/L of 2,4-D. In conclusion, the results represented a suitable medium for *S.burahol* callus induction.
Keywords: Seed; callus; *Stelechocarpus burahol;* flavonoid

1. Introduction

Stelechocarpus burahol (Blume) Hook. F. & Thomson is one of the plants having potential medicinal properties. The active substances in this plant in the form of secondary metabolites have been utilized as an antioxidant [1], gout medication [2], oral contraceptives [3] and natural deodorant [4] Flavonoid content in this plant is known for its anti hyperuricemic ability, and it is developed for gout medication [2]. However, commercial production of *S. burahol* secondary metabolites is challenging due to the limited availability of this plant in its natural habitat nowadays. Therefore, tissue culture technique is a promising way to obtain *S. burahol* callus for secondary metabolites production.

However, callus formation is strongly influenced by the growth regulator. For instance, Yücesan *et al*. [5] reported that callus of Ivanina was formed on the medium by enrichment of BA and NAA. Callus induction on seed pieces of *Gardenia resinifera* was also optimum on the MS medium enriched

with 2,4-D and BAP, whereas callus induction from internodes was successfully obtained from the medium with NAA and BAP [6]. Moreover, utilization of 2,4-D and picloram for callus induction was also reported in *Rollinia mucosa* [7]. Another finding indicated that the optimal callus induction on *Cynodon dactylon* seed explants was achieved on medium enriched with 40 μM 2,4-D, resulting in 92% callus induction [8], whereas the percentage of callus induction from seed explants of *Festuca arundinaceae* reached only 19% [9]. Also, the frequency of callus induction (FCI), the time of callus initiation (TCI), the rate of callus growth (RCG) and the appearance of callus depended on the types of explants and illumination conditions [10]. The light appeared to affect growth of *Eustoma grandiflorum* callus. The fresh weight of *Eustoma grandiflorum* callus at the end of incubation period in the light environment was higher than the fresh weight of the callus incubated in dark condition [11]. However, the earliest and maximum callus induction responses were observed on hypocotyl explants *of Catharanthus roseus* under dark conditions [12].

Synthesis of bioactive compounds in cell culture varies greatly in each species. Most cell cultures can produce higher secondary metabolites compared to intact plants. Production of secondary metabolites *in vitro* culture is influenced by several factors, such as, concentration of growth regulators [13],[14],15], and the stages of differentiation [16],[17]. Plant growth regulators (PGRs) play an important role not only in the growth of tissue cell lines but also in the production of secondary metabolites compounds [18]. Recently, the information on callus induction and flavonoids production by *in vitro* culture of *S. burahol* have not been reported. Therefore, the objective of this study is to obtain the best conditions for callus induction and flavonoid production on *S. burahol.* In this study, the culture medium optimization is adapted from the *Rollinia mucosa* callus induction method. We assume that this technique would be optimum since *S. burahol* and *R. mucosa* belong to the same Annonaceae family. Based on our best knowledge, this study is the first report on the *S. burahol* callus induction from immature seed.

2. Methods

2.1 Stelechocarpus burahol explant preparation

This preliminary investigation was conducted in the Laboratory of Plant Tissue Culture, Faculty of Biology, Gadjah Mada University, Indonesia. The immature seed explants (1-2 cm) were taken from unripe fruits of *S. burahol* trees at Gadjah Mada University area. Then, the fruits were washed in running water and soaked in a liquid detergent solution (1 ml in 50 ml distilled water) for 15 minutes. Next, they have immersed in 5.25% NaClO solution for 30 minutes. These fruits were sterilized in the laminar air flow cabinet using 5.25% NaClO solution for 10 minutes. Finally, seeds were taken from the fruits after three items of washing in sterilized distilled water.

2.2 Tissue culture media preparation

Murashige and Skoog medium (1962) was used as a medium for explants growing. 2,4-D plant growth regulators at 10, 15 and 20 ppm concentrations and picloram at 5, 7.5 and 10 ppm concentrations were used for callus induction. During 2,4-D treatment, 0.09 ppm BAP was added to the media as well. The investigation was carried out using 12 combination treatments, i.e. Dark condition: DP5 (Pic 5 ppm), DP7.5 (Pic 7.5 ppm), DP10 (Pic 10 ppm), DD10 (2,4-D 10 ppm), DD15 (2,4-D 15 ppm), DD20 (2,4-D 20 ppm). Light condition: LP5 (Pic 5 ppm), LP7.5 (Pic 7.5 ppm), LP10 (Pic 10 ppm), LD10 (2,4-D 10 ppm), LD15 (2,4-D 15 ppm), LD20 (2,4-D 20 ppm). The experiment unit was two seeds in 1 culture flask, and the experiment was repeated through triplicate trial on each unit.

2.3 Callus harvesting method

The sterile explants were cultured in each treatment medium; then, it was maintained at 24-25 ^{o}C under two different light environments, i.e., 24 h light condition (1000 lux) and 24 h darkness (without any light provided). An incubation room condition with 60 ± 5% relative humidity and light intensity of 985-1000 lux was provided using two cool white fluorescent lamps (18 W). The distance between lamps and culture of explants was ±25 cm. Luxmeter LX-1010B was used to measure light intensity.

The dark environment was prepared by covering the culture rack using thick black paper. The culture was carefully maintained for approximately five months. The culture was regularly transferred to fresh media every month. Next, callus harvesting was performed, followed by callus weighing to determine its fresh weight. Subsequently, the callus was dried in an oven at 60 °C for 48 hours to determine its dry weight and flavonoids content. Further observations were performed on the callus growth and morphology characteristics. The percentage of explants with successful callus formation, rapidity of callus formation, callus fresh weight, callus of dry weight and callus morphology were recorded.

2.4 Flavonoid content determination

Dried callus (0.2 g) was ground into fine powder using mortar and pestle. Then, the powder was extracted with methanol containing 1% of (v/v) HCl, followed by addition of 2 N HCl (equal volume) and was incubated at 90 ºC for one h. After the incubation, acid-hydrolyzed extracts were dried and were resuspended in methanol[19]. Total flavonoid content was determined following the procedure specified by Zou *et al* [20]. A 0.5-mL aliquot of the appropriately diluted sample solution was mixed with two 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 two 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. The 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. Measurements performed on three samples.

3. Results and discussion

As shown in Table 1, the period of callus induction and growth of callus varied. They depend on the type and the concentration of growth regulators and lighting conditions. Callus induction ranged from 60% to 100%, i.e., the explants maintained in dark condition showed a greater callus formation (90-100%) than the one maintained in the light environment (60-100%). The induction of callus in explants occurred within 18.50 to 55 days after inoculation (Table 1). The fastest callus formation occurred in the explants grown on a medium enriched with 10 mg/l of picloram in dark room. The results reveal that the dark environment affects callus growth. It grew faster and greater than callus grown in light condition. According to Lakshmi and Reddy [6], it is believed that the release of phenolic compounds can be reduced in the dark environment.

Moreover, based on the result, the formation of callus from the immature seed of *S. burahol* requires a longer period than formation in other plants. For example, the best callus formation (92% growth) from *Cynodon dactylon* seed explants was obtained in a medium with 40 μM 2,4-D within five days [8]. However, not all callus formation from seed explants were successful; for instance, the percentage of callus formation from seed explants of *Festuca arundinaceae* was very low and reached only 19% growth [9]. Fresh and dry weights callus-that were formed in each treatment- represent the growth of callus. The result of this study shows that the growth of callus varied in each treatment (Table 2).

The explant that was inoculated in a medium supplemented with 7.5 mg/l of picloram under dark condition showed the best growth with 3.42 ±0.34 wet weight and 0.272 ±0.0015 dry weight. The lower the concentration of picloram, the less growth of plant callus was obtained. Picloram has been reported to be an inductor for faster growth and higher biomass in the culture suspension of *Rollinia* that is incubated in a dark condition[7]. Also, the presence of picloram in culture medium enhances callus proliferation from durian young leaf explants. The fastest callus proliferation was found on a medium supplemented with 4.0 mg of L-1 picloram[21]. The segments of immature inflorescences taken from barley *(Hordeum vulgare L.)* cultured on the LS medium containing 7.5 mg/l of picloram gave the highest values of callus induction rate[22]. The results showed that effects of picloram concentrations on the callus induction from explants were significantly different.

Table 1. The day of callus formed and the percentage of immature seed explants that formed callus in many treatments medium and light conditions

Treatment		Average of callus formation (day)	Callus induction (%)
Lighting condition	PGR		
Dark	P 5	20.33±1.97[a]	100
	P7.5	19.00±1.41[a]	100
	P10	18.50±1.64[a]	100
	D10	33.33±2.66[cd]	100
	D15	32.17±1.70[c]	100
	D20	40.83±1.72[e]	90
Light	P 5	34.83±2.79[d]	90
	P7.5	29.17±1.47[b]	70
	P10	34.17±1.60[cd]	60
	D10	55.00±1.26[f]	60
	D15	28.67±2.66[b]	80
	D20	41.50±2.43[e]	100

Means within a column followed by the same letters are not significantly different by Duncan's multiple range test ($P > 0.05$).

Table 2. Response of callus growth on immature seed explants at various medium and light condition

Treatment		Average fresh weight (g)	Average dry weight (g)
Lighting	PGR		
Dark	P 5	2.64±0.11[b]	0.22±0.018[b]
	P7.5	3.42±0.34[a]	0.27±0.015[a]
	P10	1.12±0.11[f]	0.12±0.013[e]
	D10	0.46±0.04[i]	0.06±0.006[h]
	D15	2.01±0.13[c]	0.17±0.01[c]
	D20	0.89±0.07[g]	0.09±0.008[g]
Light	P 5	0.32±0.02[i]	0.12±0.004[e]
	P7.5	0.76±0.05[gh]	0.09±0.004[g]
	P10	0.69±0.06[h]	0.11±0.013[f]
	D10	0.41±0.04[i]	0.13±0.014[e]
	D15	1.61±0.09[d]	0.16±0.009[c]
	D20	1.30±0.10[e]	0.14±0.009[d]

Means within a column followed by the same letters are not significantly different by Duncan's multiple range test ($P > 0.05$).

Similar to the other auxin, picloram and 2,4-D induces callus. Auxin signaling is transduced via Auxin Response Factor (ARF) transcription factors, especially ARF7 and ARF19, to activate the expression of Lateral Organ Boundaries Domain (LBD) family transcription factors, LBD16, LBD17, LBD18, and LBD29. These LBDs, in turn, induce E2 Promoter Binding Factor an (E2Fa), a transcription factor that plays a central role in cell cycle reentry[23]. Friable callus was produced on most of the treatment medium as shown in Figure 1 and Figure 2.

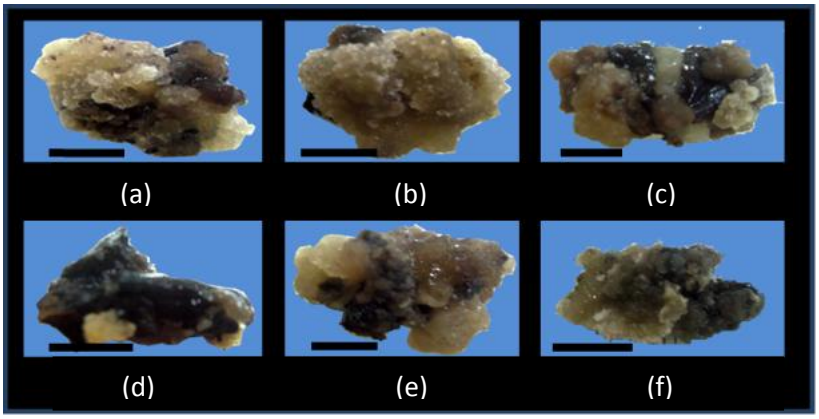


Figure 1. Friable callus results of explant immature seed planting in dark condition in the medium with the addition of 5 mg/l Picloram (a), 7.5 mg/l Picloram (b), 10 mg/l Picloram(c), 10 mg/l 2,4-D (d), 15 mg/l 2,4-D (e), 20 mg/l 2,4-D (f). Barr = 1 cm

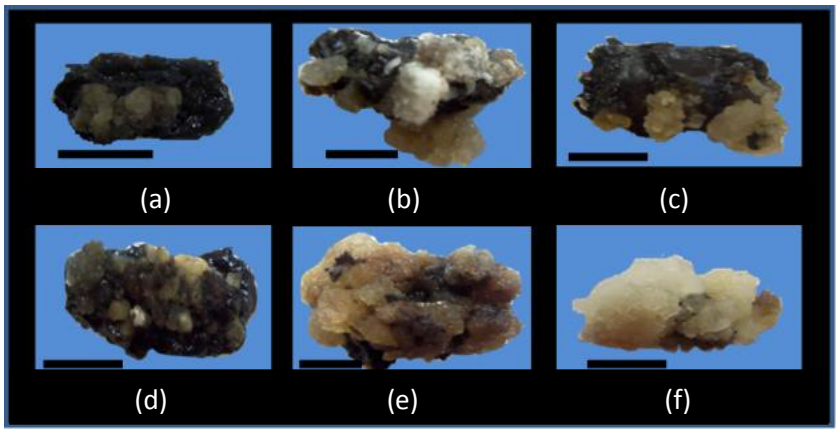
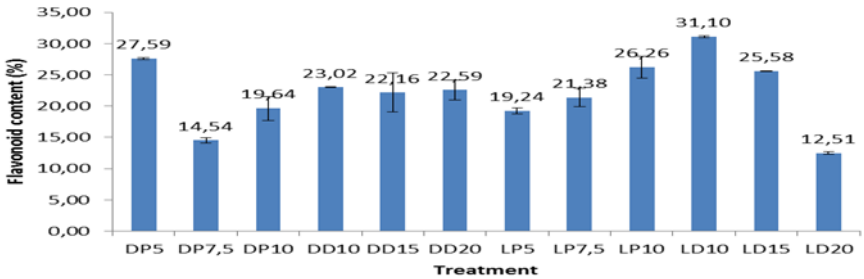


Figure 2. Friable callus results of explant of immature seed planting in light condition in the medium with the addition of 5 mg/l Picloram (a), 7.5 mg/l Picloram (b), 10 mg/l Picloram(c), 10 mg/l 2,4-D (d), 15 mg/l 2,4-D (e), 20 mg/l 2,4-D (f). Barr = 1 cm

Callus growth in all treatments produced a various amount of flavonoids as presented in Figure 3. According to the result of the study by Purwantiningsih et al.[2], it was proved that flavonoid compounds in *S. burahol* had a potential *anti-hyperuricemic* activity. One of the flavonoid compounds in *S. burahol* is 3, 7, 3', 4'-tetrahidroxy-5- methyl-flavone; it shows the highest antioxidant activity in comparison with the other flavonoids [24].



Note: D:Dark; L:Light; 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

Figure 3. Responses of flavonoid production of immature seed explants at various medium

The results also reveal that callus with the highest flavonoid production was the growing callus from the explants in a medium with an addition of 10 mg/L of 2,4-D. It is in line with the fact that the production of secondary metabolites in callus is strongly influenced by hormones. The signal of hormone regulates the physiological processes in plant culture[25]. The exogenous growth-regulating substances can alter the accumulation of secondary metabolites by regulating the expression of genes involved in the synthesis of secondary metabolites. The regulation of gene expression is performed on the transcription stages by regulating gene transcription factor. The transcription factor plays an important role in plant development process, including synthesis of secondary metabolites [26],[27]. The plant-specific transcription factor, i.e., *NAM/ATAF/CUC (NAC)*, plays a critical role in a variety of developmental processes including defense responses. NAC transcription factor domain is allegedly organized at the transcription level by environmental factors, such as hormone concentrations. The induction at the transcriptional level *PmNAC1* is associated with the increasing concentrations of auxin in culture medium [27]. In this study, the addition of picloram from 5 mg/L to 10 mg/L to the growth medium showed a significant increase of flavonoid content in both dark and light conditions. It suggests that the supplementation of growth regulator substances significantly affected the secondary metabolites production in the callus.

4. Conclusion

The explant, which was maintained in MS medium by supplementation of 7.5 mg/L picloram under dark condition, was proved to be the best condition for optimum callus induction (fresh and dry weight). Picloram was shown to be the most effective for induction and production of friable callus. The callus induction varied, ranging from 60% to 100%. Moreover, callus in all treatments demonstrated flavonoid compound production. Also, the highest production of flavonoid compounds occurred in the callus that was maintained on medium with the addition of 10 mg/L of 2.4-D. Further investigation on flavonoid production using *S. burahol* mesocarp explants in the form of cell culture suspension is urgently needed. Therefore, the results of this study suggest a noble start for the future experiment.

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