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Optimation of Callus Induction from *Piper betle* L. var. Nigra Explants with Various Concentrations of Coconut Water and Addition of 2,4-D and BAP

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ABSTRACT

Black betle [*Piper betle* (L.) var. Nigra] is one of the endemic species from Indonesia that has potential as medical plant because this plant can produce secondary metabolites such as alkaloids, flavonoids, tannins, steroids, terpenoids and saponins. Secondary metabolites are isolated from callus culture with proper medium formulation to get optimal result. This study aimed at knowing the influence of variations in the concentration of coconut water and combination of 0.5 mg/l 2,4-D and 2.0 mg/l BAP towards induction of callus from *P. betle* L. var. Nigra's leaf explants. The experiment consisted of five treatments which were repeated for six times. Observation was performed for eight weeks in parameters including percentage of callus formed from explants, callus induction time, callus fresh weight, dry weight and morphological features (texture and colour). Results showed that various concentrations of coconut water combined with addition of 0.5 mg/l 2,4 D and 2.0 mg/l BAP had effects on callus from *Piper betle* L. var. Nigra leaf explants. The best combination for callus induction from *P. betle* L. var. Nigra's leaf explants was addition of 0.5 mg/L 2,4 D and 2.0 mg/l BAP combined with 5% coconut water, which resulted in the highest dry weight at 0.09 g and fastest induction time at 13.17 days. Callus of *P. betle* L. var. Nigra had compact texture in all treatments and dominant colour was brownish yellow but callus in eighth week mostly turned to black colour.

Key words : Callus, *Piper betle* L. var. Nigra, coconut

INTRODUCTION

One of the plants known to have medicinal advantages is black betel (*Piper betle* L. var. Nigra). Betel plant has long been used by local community to do menginang (chewing betel), which is done not only by Indonesian, but also by people in India, Pakistan and South Africa (Jaiswal *et al.*, 2014). Black betel is reported to contain secondary metabolites such as alkaloids, flavonoids, saponins, terpenoids and steroids which have potential as antibacteria, antifungi, anti-diabetes, anti-ulcer, anti-platelet, anti-fertility, anti-tumor, anti-mutagen and anti-helminth (Jaiswal *et al.*, 2014; Junairiah *et al.*, 2018). Black betel leaves extracted using n-hexane, ethyl acetate and methanol solvent also reported having biological activity as antifungal and antibacterial agent against *Candida albicans* ATCC 10232, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922 (Junairiah *et al.*, 2017).

In order to fulfil demand of highly potential medicinal plants, such as black betel which has high potential, conventional method is generally not enough due to the long time and large area it needs in addition to weather-dependent. Up until now, secondary metabolites are obtained from direct extraction from plant organs. This method needs fresh ingredients in large scale and costly extraction, isolation and purification process (Jones and Kinghorn, 2012). Thus, alternative method to obtain secondary metabolites more efficiently in large scale with good quality is needed. This is the reason why tissue culture is chosen to perform propagation of medicinal plants.

Growth regulators are one of the important factors in determining the success of plant tissue culture. There are two types of growth regulators commonly used in tissue culture; auxin and cytokinin (Rademacher, 2015). 2,4-D is one of the synthetic auxins able to promote callus growth from explants. BAP has the same basic structure as kinetin, but it is more

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effective due to benzyl groups. In addition, most plants have better response towards BAP compared to other types of cytokinin, because BAP has stronger and more stable activity, thus, it is more effective for *in vitro* shoot production (Rademacher, 2015).

Based on Baque *et al.* (2011), coconut water is a complex organic material containing sugar, vitamins, minerals, amino acids and phytohormones. Hormone with most significant level in coconut water is cytokinin (Kokilavani *et al.*, 2017). Lima *et al.* (2015) identified cytokinin types contained in coconut water as trans-zeatin, zeatin glucoside and zeatin riboside. Kokilavani *et al.* (2017) reported that coconut water also contained diphenil urea which had activity like cytokinin. The levels of coconut water recommended to be added into medium were 15% (Saraswat and Kumar, 2019). Lima *et al.* (2015) mentioned that 10-15% coconut water supplementation resulted in the best callus of spinach plant. The addition of coconut water in tissue culture at overly high concentration caused lowered growth and induced abnormal culture morphology of orchid callus (Baque *et al.*, 2011).

Study on Piperaceae had been extensively performed, mainly in black betel. Previous study was conducted to induce black betel callus with various combinations of growth regulators; 2,4-D and BAP, IAA and kinetin, IAA and BAP, IBA and BAP, and IBA and kinetin. From the five combinations, the best callus induction was found from 0.5 mg/l 2,4-D and 2.0 mg/l BAP, indicated by highest fresh and dry weight. This study continued the previous study to optimize callus formation with combination of growth regulators 0.5 mg/l 2,4-D and 2.0 mg/l BAP and coconut water, which expected to be able to induce callus at higher quality and quantity.

MATERIALS AND METHODS

Murashige and Skoog (MS) medium for culture was prepared at 1000 ml, by dissolving macronutrient chemicals (1650 mg NH_4NO_3; 1900 mg KNO_3; 440 mg $CaCl_2.2H_2O$; 370 mg $MgSO_4.7H_2O$; 170 mg KH_2PO_4) one by one into 500 ml distilled water. After all macronutrients dissolved, 5 ml iron, 1 ml micronutrient and 4 ml vitamin of stock solutions were added. Then, 100 mg myoinositol and 30 g sucrose were mixed into medium before coconut water and growth regulators (2,4-D and BAP) were added in concentration previously determined. Acidity was measured using pH paper and medium pH was adjusted to range of 5.6-5.8 using either KOH 1 N or HCl 1 N. Lastly, distilled water was added until medium volume was 1000 ml.

After all chemicals were mixed in, 8 g agar was added into medium and dissolved in heated medium. Medium was filled into culture bottle at±10 ml/bottle. Culture bottle was covered with alumunium foil and labelled accordingly. Bottle with solidified medium was then sterilized using autoclave at 121°C for 15 min, 1.2 atm before being stored in incubation room.

Black betel leaf was washed using detergent, then rinsed with flowing water for three instances before sterilized in LAF. Leaf surface was sterilized by soaking leaves in 10% chlorox while shaken for 7 min. Leaves were rinsed with sterile distilled water three times. Explants were put in petri dish layered with sterile filter paper. Leaves were cut in 1 cm^2 pieces to be planted on MS medium in culture bottles. Every bottle was filled with three explants, labelled, covered tightly, and stored in incubation room at 25 °C under 20-watt continuous neon lighting.

RESULTS AND DISCUSSION

This study was performed to determine the effect of various combinations of coconut water and growth regulators 2,4-D and BAP in callus induction time, callus wet weight, dry weight and morphology of black betel leaf callus. In various combination, response of black betel callus was found to be different. Observation in the current study was performed for eight weeks of explant culture period.

Treatment of black betel leaf explants in MS medium with addition of 5% coconut water combined with 0.5 mg/l 2,4-D and 2.0 mg/l BAP was able to induce fastest callus growth compared to other treatment at average time of 13.16 days (Table 1), while on previous study (Junairiah *et al.*, 2018) black betel leaf explants on MS medium with combination of 0.5 mg/l 2,4-D and 2.0 mg/l BAP was able to induce callus in 17.75 days. There was difference of black betel leaf callus induction time on MS medium with combination of 0.5 mg/l 2,4-D and 2.0 mg/l BAP with and without coconut water addition.

Culture of black betel explants in MS medium with addition of coconut water and growth

Table 1. Callus induction time and percentage of black betel leaf explant growing callus on MS medium with additional various concentrations of coconut water (CW) and growth regulators 2,4-D and BAP ($D_{0.5}B_2$)

Combination of coconut water and 2,4-D and BAP (mg/l)	Callus induction time (days)	Percentage of explant growing callus
$CW_0+D_{0.5}B_2$	14.5±0.836^{ab}	100
$CW_5+D_{0.5}B_2$	13.17±0.4068^{a}	100
$CW_{10}+D_{0.5}B_2$	14±0^{ab}	100
$CW_{15}+D_{0.5}B_2$	14.17±0.408^{ab}	100
$CW_{20}+D_{0.5}B_2$	15.67±0.516^{b}	100

Different superscripts indicate significant difference based on Mann Whitney test (α=0.05).

regulators 0.5 mg/l 2,4-D and 2.0 mg/l BAP were able to grow callus faster compared to without additional coconut water. This was probably due to diphenyl urea content in coconut water, which had cytokinin-like activity to induce cell division in tissue culture. In addition, coconut water contained various vitamins, such as thiamins and pyridoxins, which were used as synthetic vitamin substances in MS medium beside macronutrients, such as N, P and K. Micronutrients in coconut water possibly developed further as micro- and macronutrient substances and carbon sources, specifically sucrose.

In addition to cytokinin source, the addition of coconut water also increased nutrient availability in medium, in line with Buah and Agu-Asare (2014) that additional coconut water to medium meant to raise medium nutrient, consisted of complex organic materials and growth regulators. In the current study, all combinations were able to induce 100% of callus growth from explants planted. The shortest callus induction time was in line with Khan *et al.* (2015), who explained that 1.5 mg/l 2,4-D and 10% coconut water combination was able to induce callus of green grape (*Vitis vinifera* L.).

Fresh and dry weight callus was recorded at the end of observation period (eight weeks). Only explants growing callus was weighted, thus explants were cleaned and weighed. Combination of 10% coconut water combined with 0.5 mg/l 2,4-D and 2.0 mg/l BAP produced the highest fresh weight at 1.048±0.098 g (Table 2).

Based on Shekhawat and Manokari (2016), recommended concentration of coconut water to be added to culture medium was at 10-15% per litre. In the current study, shortest induction time was found from additional 5% coconut water combined with 0.5 mg/l 2,4-D and 2.0 mg/l BAP, while the highest fresh weight was produced from addition of 10% coconut water combined with the same levels of growth regulators. In contrast, higher concentration of coconut water lowered fresh and dry weight and increased callus induction time. This showed that the most optimal levels of coconut water addition were at 5-10%. Other study reported that 1.5 mg/l 2,4-D and 10% coconut water combination was able to produce green grape (*Vitis vinifera* L.) callus with highest fresh and dry weight (Khan *et al.*, 2015). Combination of 3 mg/l 2,4-D and 10% coconut water was also found to be able to induce spinach callus with highest fresh and dry weight.

Results showed that all combination grew compact callus (Table 3). Compact texture of callus was the effect of cytokinin and auxin addition, which affected water potential. This promoted water absorption from medium to cells, resulting in rigid texture. Callus induced with cytokinin addition had compact texture

Table 2. Fresh and dry weight of black betel leaf callus after eight weeks culture period

Combination of coconut water and growth regulators (mg/l)	Callus fresh weight (g)	Callus dry weight (g)
$CW_0+D_{0.5}B_2$	0.8335±0.0563^{a}	0.0647±0.0099^{a}
$CW_5+D_{0.5}B_2$	0.9297±0.0483^{b}	0.0924±0.0055^{c}
$CW_{10}+D_{0.5}B_2$	1.0488±0.0989^{c}	0.083±0.0167^{bc}
$CW_{15}+D_{0.5}B_2$	0.7601±0.0303^{a}	0.0687±0.0093^{ab}
$CW_{20}+D_{0.5}B_2$	0.8652±0.0339^{bc}	0.0740±0.0046^{c}

Different superscripts indicate statistical difference based on Duncan test (α=0.05).

Table 3. Morphology of black betel (*Piper betle* L. var. Nigra) leaf callus with various combinations of coconut water and growth regulators 2,4-D and BAP

Combination of coconut water and 2,4-D and BAP ($D_{0.5}B_2$) (mg/l)	Figure	Callus morphology
$CW_0+D_{0.5}B_2$		Callus colour blackened, compact-textured. Callus covered all explant surface.
$CW_5+D_{0.5}B_2$		Callus colour blackened, compact-texture. Callus covered all explant surface.
$CW_{10}+D_{0.5}B_2$		Callus colour blackened, partially brownish green, compact- textured. Callus covered all explant surface.
$CW_{15}+D_{0.5}B_2$		Callus colour blackened, partially brownish green, compact-textured. Callus covered all explant surface.
$CW_{20}+D_{0.5}B_2$		Callus colour blackened, partially brownish white, compact-textured. Callus covered all explant surface.

compared to without cytokinin (Khan *et al.*, 2015), thus supplementation of coconut water, which contained growth regulators zeatin and ribozeatin and diphenyl urea that had cytokinin-like activity, supported formation of callus texture. Callus with compact texture was preferable in producing secondary metabolites (Joanne *et al.*, 2014).

Callus texture was one of the parameters used in evaluating callus quality. Difference of callus morphology was hypothesized due to different tissue ability in absorbing nutrients and growth regulators from culture medium. This was in line with Saraswat *et al.* (2019), who elaborated that callus type varied from compact to crumbly, depending on plant species, nutrient composition in medium, growth regulators and culture environment.

Results showed that changes occurred to colour of black betel leaf explants. Initial colour of white turned to yellowish white, to greenish white up to yellowish green. Main colour produced during callus formation was white and green, young callus was coloured white, then colour turned to green, yellow and brown along with callus aging (Kumar *et al.*, 2015). In addition, different callus morphology of color was thought to be caused by varying ability of tissue in absorbing nutrients and growth regulators contained in medium.

Change of callus colour into brownish in black betel explants occurred in seventh to eighth week. Explants were browned, then further blackened. Different from current study, *Virginia pine* callus was browned starting from third until fourth week of culture period (Tang and Newton, 2013). Browning occurred as natural progression and adaptive change of plant organs due to physical aspects, such as peeling or cutting. Browning indicated physiological senescence of explants (Jones and Saxena, 2013).

In the other study, 1.5 mg/l 2,4-D and 10% coconut water produced compact-textured callus coloured brownish green of green grape (*Vitis vinifera* L.). Other study suggested that alfalfa (*Medicago sativa* L.) cultured with supplementation of 3 mg/l 2,4-D and 10% coconut water grew compact callus with brownish yellow colour. The application of auxin induced chlorophyll synthesis in callus. Brown callus indicated senescence of cells, as

found in the current study, in which addition of coconut water produced callus with yellowish or brownish green colour.

CONCLUSION

Based on the study conducted, 5% coconut water combined with 0.5 mg/l 2,4-D and 2.0 mg/l BAP could shorten callus induction time and produced highest fresh and dry weight, in addition to form compacted callus. Further study can explore secondary metabolites contained in black betel callus grown in coconut water-supplemented medium.

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