



Callus Induction of *Piper betle* Var Nigra Using 2,4-Dichlorofenoxyacetic Acid and 6-Benzil Aminopurin

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Abstract

Piper betle L. var Nigra (black betel) is a member of Piperaceae family which has potential as medicinal plant due to its secondary metabolites. Callus culture is one of the alternative methods to elevate production of secondary metabolites. This study was aimed to determine the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzil aminopurine (BAP) towards callus induction and growth, also to determine the most optimal variation of 2,4-D and BAP concentration for callus induction of black betel leaf explant. This study was a laboratory experimental study with complete randomized design. Black betel leaf explant was planted in Murashige and Skoog (MS) medium supplemented with 2,4-D and BAP growth regulators at concentration of 0.0 mg/L, 0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L respectively. Parameter recorded for callus induction and growth including callus induction time (days), percentage of explant forming callus, fresh weight, dry weight, color and texture. After callus planted for 8 weeks, analysis was performed statistically. Result showed that 2,4-D and BAP supplementation to medium affected the growth of black betel leaf explants. Additional concentration of 0.5 mg/L 2,4-D and 1.0 mg/L BAP growth regulators showed the fastest response in callus formation, at 7.25 days. Growth regulators of 2,4-D 0.5 mg/L and BAP 2.0 mg/L concentration produced the highest fresh and dry weight, at 0.6802 g and 0.0670 g respectively. The best treatment was used as a basis to produce secondary metabolites.

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INTRODUCTION

Black betel (*Piper betle* L. var *Nigra*) which belong to Piperaceae family (Pradhan *et al.*, 2013; Rahminiwati *et al.*, 2014) is a multifunctional plant species due to its utilization as both decoration and medicinal plant (Rekha *et al.*, 2014). As medicinal plant, black betel possesses interesting prospect due to secondary metabolites it contained, such as alkaloids, flavonoids, saponins, tannins, steroids, triterpenoids, and polyphenolates (Rija'i, 2015; Junairiah *et al.*, 2017).

Essential oils contained in black betel leaf were found to be able to inhibit the growth of *Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus viridans*, *Actinomyces viscosus*, and *Staphylococcus aureus* bacteria (Hermawan, 2007). Phenols in black betel are also toxic for microbes as it can inhibit their enzymatic activity (Suliantari *et al.*, 2008). Saponins and tannins have antiseptic properties towards surface injuries, playing role as bacteriostatic which can be used against dermal and mucosal infection, while flavonoids can be applied as anti-inflammation (Mursito, 2002). Black betel also contains nitrogen, proteins, carbohydrates, fibers, vitamin A, B complex, C, D, and E, sodium, potassium, magnesium, phosphor, iron, copper, and zinc (Yanti, 2012).

Up to now, secondary metabolites is obtained by means of direct extraction from various organ of plants. However, this method requires large amount of fresh plants as ingredient, in addition to costly process of extraction, isolation, and purification (Kartika, *et al.*, 2013). Because of those reasons, special techniques are applied. One of the alternatives used to retain plant availability, increase secondary metabolites production, and requires shorter production time is plant tissue culture (Filova *et al.*, 2014; Rajkumar *et al.*, 2010; Karuppusamy, 2009). Tissue culture or *in-vitro* culture is a technique of growing cells, tissue, and organ in aseptic condition (Sharma *et al.*, 2011). One of the tissue culture method applied to produce secondary metabolites is callus culture (Chowdury *et al.*, 2011; Radfar *et al.*, 2012; Tambe *et al.*, 2013).

Growth regulators are consisted of cytokinin and auxin (Ngumuo *et al.*, 2013). Auxin is a type of compounds able to stimulate cell elongation. Auxin in medium plays role in stimulating callus growth, cell augmentation, root growth, and morphogenesis regulation. Meanwhile, cytokinin is a derivate of adenine, purine base that formed DNA and RNA (Guilfoyle and Hagen, 2002). The interaction of auxin and cytokinin is highly required for explants growth. Application

of both regulators at the right concentration will induce optimal callus growth from explant (Bajguz and Pitrowska, 2009). Cytokinin promotes cell division in tissue culture by increasing the transition of G₂ to mitosis, because it elevates the rate of protein synthesis (builder protein or enzyme requires for mitosis). Protein synthesis can be promoted by stimulating higher mRNA formation encoding the proteins (Riefler *et al.*, 2006; Lee *et al.*, 2010).

The application of 2,4-dichlorophenoxyacetic acid (2,4 D) and 6-benzil aminopurine (BAP) had previously performed by Radhakrishnan and Ranjithakumari (2007) who had found that growth regulators 2,4D and BAP at 4.5 µM and 4.4 µM respectively are able to induce callus of *Glycine max*. In the other hand, callus induction of *Rheum webbiaarum* explant was accomplished in Murashige and Skoog (MS) medium supplemented with combination of 0.5 mg/L 2,4D and 3 mg/L BAP (Rashid *et al.*, 2014).

Based on elaboration above, the addition of growth regulators is found to be able to stimulate callus formation. This study was designed in aim to determine the effect of various concentration of 2,4-D and BAP combination in inducing black betel leaf callus (*Piper betle* L. var *Nigra*). The importance of this study result was to provide information on plant growth regulator that could induce callus of black betel leaf explants. It could further be used to produce bioactive compounds contained in black betel using plant tissue culture technique.

METHODS

Plant material used in this study was young leaves of *Piper betle* L. (black betel), grown on the second to fourth nodes from the upper tip. Black betel plant used was obtained from Bratang Flower Market, Surabaya. Leaves were washed with detergent water and rinsed with running tap water to clean the dirt in the explant surface, then rinsed again with distilled water. Leaves were sterilized under laminar air flow by shaking it in 20% chlorox solution for 5 minutes before rinsed with sterile distilled water three times.

Leaves were cut at ± 1 cm² wide, disposed of its margin to be used as explants. Explants were planted in culture bottles filled with callus induction medium supplemented with growth regulators at various concentrations. Culture bottles were covered with aluminum foil. Bottles were maintained in incubation room at 25°C ± 2°C temperature under 40-watt TL 2600 lux storage lamp. All processes related to explant plant-

ing was performed aseptically, under laminar air flow with Bunsen burner and blower.

This study was a laboratory experimental study. In this research there were two factors, 2,4D (0, 0.5, 1, 1.5, and 2 mg/L) and BAP (0, 0.5, 1, 1.5, and 2 mg/L). Study was designed as complete randomized design consisted of 25 treatments with 12 replications of each. Explant used was black betel leaf which planted on MS medium supplemented with various 2,4-D and BAP growth regulators concentration of 0, 0.5, 1, 1.5, and 2 mg/L. Explants were planted for 8 weeks before being harvested and weighted.

Data recorded in this study were qualitative data; callus morphology which analyzed descriptively, and quantitative data; callus induction time, percentage of explant forming callus, fresh

and dry weight. The callus was ovened for five days at 50 degrees Celsius, which were analyzed statistically using SPSS for Windows 22.0. Callus induction time, fresh weight, and dry weight were analyzed using Kruskal Wallis t-independent test then continued with Mann-Whitney test to determine difference between treatments. All tests were performed at 5% margin of error.

RESULT AND DISCUSSION

Effect of additional 2,4-D and BAP at various concentration towards callus induction time and percentage of explant forming callus of black betel leaf (*Piper betle L.*)

Planting of black betel leaf explant in MS

Table 1. Mean callus induction time and percentage of explants forming callus from black betel leaf explants planted in MS medium supplemented with various combination of 2,4-D and BAP growth regulators concentration.

Growth regulators concentration (mg/L)	Mean of callus induction time (days)	Percentage of explants forming callus (%)
0.0 2,4-D + 0.0BAP	38.17 ± 0.389 ^o	100
0.0 2,4-D + 0.5 BAP	26.25 ± 0.452 ⁿ	100
0.0 2,4-D + 1.0 BAP	22.00 ± 0.000 ^m	100
0.0 2,4-D + 1.5 BAP	17.50 ± 0.522 ⁱ	100
0.0 2,4-D + 2.0 BAP	21.50 ± 0.522 ^l	100
0.5 2,4-D + 0.0BAP	16.25 ± 0.452 ^h	100
0.5 2,4-D + 0.5 BAP	13.00 ± 0.000 ^e	100
0.5 2,4-D + 1.0 BAP	7.25 ± 0.452 ^a	100
0.5 2,4-D + 1.5 BAP	13.33 ± 0.492 ^{ef}	100
0.5 2,4-D + 2.0 BAP	17.75 ± 0.522 ⁱ	100
1.0 2,4-D + 0.0BAP	12.50 ± 0.522 ^d	100
1.0 2,4-D + 0.5 BAP	18.33 ± 0.492 ^j	100
1.0 2,4-D + 1.0 BAP	12.25 ± 0.452 ^d	100
1.0 2,4-D + 1.5 BAP	15.50 ± 2.153 ^g	100
1.0 2,4-D + 2.0 BAP	11.00 ± 0.000 ^c	100
1.5 2,4-D + 0.0BAP	13.75 ± 0.452 ^f	100
1.5 2,4-D + 0.5 BAP	16.00 ± 0.000 ^{gh}	100
1.5 2,4-D + 1.0 BAP	16.25 ± 0.452 ^h	100
1.5 2,4-D + 1.5 BAP	15.50 ± 0.522 ^g	100
1.5 2,4-D + 2.0 BAP	13.75 ± 0.452 ^f	100
2.0 2,4-D + 0.0BAP	9.00 ± 0.000 ^b	100
2.0 2,4-D + 0.5 BAP	19.25 ± 0.452 ^k	100
2.0 2,4-D + 1.0 BAP	13.75 ± 0.452 ^f	100
2.0 2,4-D + 1.5 BAP	17.42 ± 0.515 ⁱ	100
2.0 2,4-D + 2.0 BAP	17.42 ± 0.515 ⁱ	100

Note: Different letters after number indicated significant difference based on t-independent test and Mann-Whitney test at 5% margin of error.

medium supplemented with growth regulators 2,4-D and BAP at various concentration resulted in a range of response. This response difference was possibly due to different combination of growth regulators applied to medium in addition to physiological condition of respective explants. Result showed that all leaf explants from all treatments formed callus (Table 1). The fastest callus induction occurred at average time of 7.25 days from explant given 0.5 mg/L 2,4-D and 1.0 mg/L BAP combination. Callus given no growth regulators was induced at the longest period of 38.17 days. Percentage of explants forming callus was determined by comparing explants able to form callus to total number of explants planted. Result showed that 100% explants were induced to grow callus. Leaf explants of *Vanda* sp was grown in MS medium with 2,4-D 2 ppm produced 83.3% callus (Budisantoso *et al.*, 2017). This results showed that the combination of 2,4-D and BAP is better compared to 2,4-D treatment only.

Callus induction was initiated with explant tissue thickening at the part where leaf was wounded. This marked the interaction between explant with medium, growth regulators, and environments, thus explant increased in size (Yelnitis and Komar, 2010). Previous study evidenced that induction of *Calophyllum inophyllum* Linn. leaf callus given combination of 0.5 mg/L 2,4-D and 2 mg/L BAP did not occurred until 13th days of planting (Indah and Ermavitalini, 2013). In current study, explant given 0.5 mg/L 2,4-D and 1.0 mg/L BAP had grown callus in the shortest period, at the 1st week of planting. This was due to nutrient contained in the MS medium could induce callus formation. In addition, combination of growth regulators at 0.5 mg/L 2,4-D and 1.0 mg/L BAP was able to stimulate cells division, thus this combination was the most balance to induce cells in the explant to divide continuously and differentiate, leading to faster callus formation (Hardiyanto *et al.*, 2004).

In the current study, all leaf explants planted given various growth regulators combination had resulted in callus formation. Compared to previous study, percentage of callus formation in *Citrullus colosynthis* leaf explants planted in the same MS medium supplemented with 2,4-D (1.5 mg/L) and BAP (1.0 mg/L) was 65.1% (Savitha *et al.*, 2010). Combination of 2,4-D (13.50 μ M) and BAP (4.50 μ M) was also found to be able to induce *Hymenocallis littoralis* callus at 100% percentage (Sundarasekar *et al.*, 2012), while 100% callus of *Byrsonima verbascifolia* was induced by combination of 4.52 μ M-18.10 μ M 2,4-D and

4.44 μ M or 17.75 μ M BAP (Castro *et al.*, 2016).

Fresh and Dry weight of black betel leaf (*Piper betle* L.) callus planted in MS medium given various combination of 2,4-D and BAP growth regulators concentration

Mean resulting fresh and dry weight of black betel leaf callus supplemented with various combination of 2,4-D and BAP concentration was presented in Table 2. Different combination of ...produced different fresh and dry weight. Callus given combination of 0.5 mg/L 2,4-D and 2.0 mg/L BAP resulted in the highest mean of fresh and dry weight, at 0.6802 g and 0.0670 g, respectively. Lowest mean of fresh and dry weight was obtained from callus given no growth regulators, at 0.0673 g and 0.0061 g respectively (Table 2).

Supplementation of 0.5 mg/L 2,4-D and 2.0 mg/L BAP combination resulted in the most optimal and significant fresh and dry weight of black betel leaf callus. This indicated that this combination was the most balanced concentration between auxin (2,4-D) and cytokinin (BAP), thus resulted in forming an optimal mass of callus of *Raufofia serpentina* (Aryani *et al.*, 2005). Explants given no growth regulators was not capable in optimizing fresh and dry weight of callus, resulting in lowest fresh weight of 0,0673 \pm 0.0141 g and dry weight of 0.0061 \pm 0.0031 g. Treatment of 2,4D 0.5 mg/L and BAP 2 mg/L on leaf explants of *Justicia gendarussa* produced wet weight and dry weight 1.230 g and 0.078 g respectively (Wahyuni *et al.*, 2017).

The difference of growth rate is affected by the ability of explant tissue in absorbing available nutrients. Addition of growth regulators at various concentration is purposed to reach balanced level of endogenous auxin and cytokinin in the explants. Balanced level of auxin and cytokinin in *in-vitro* culture will be able to stimulate formation of callus via interaction to achieve cells enlargement and division. Higher callus weight can also be indicated from the size of callus formed (Wulandari *et al.*, 2004).

Plant production usually more accurate if recorded in dry weight compared to fresh weight, because plant fresh weight is highly affected by environment via metabolism activity and humidity (Muryanti *et al.*, 2005), thus callus dry weight is more stable compared to fresh weight. The difference of callus fresh and dry weight is also affected by medium supplemented with auxin and cytokinin growth regulators at various concentration, thus producing different result from the

Table 2. Mean fresh and dry weight (g) of black betel leaf callus after 8 weeks of culture period.

Combination of growth regulators concentration (mg/L)	Mean fresh weight (g)	Mean dry weight (g)
0.0 2,4-D + 0.0BAP	0.0673 ± 0.0141 ^k	0.0061 ± 0.0031 ^g
0.0 2,4-D + 0.5 BAP	0.1216 ± 0.0242 ^j	0.0118 ± 0.0025 ^{fg}
0.0 2,4-D + 1.0 BAP	0.1159 ± 0.0178 ^j	0.0110 ± 0.0019 ^{fg}
0.0 2,4-D + 1.5 BAP	0.2116 ± 0.0684 ⁱ	0.0202 ± 0.0077 ^{efg}
0.0 2,4-D + 2.0 BAP	0.2357 ± 0.0497 ⁱ	0.0241 ± 0.0049 ^{defg}
0.5 2,4-D + 0.0BAP	0.3613 ± 0.0407 ^{fg}	0.0359 ± 0.0046 ^{bcdefg}
0.5 2,4-D + 0.5 BAP	0.3377 ± 0.0598 ^{fgh}	0.0343 ± 0.0061 ^{bcdefg}
0.5 2,4-D + 1.0 BAP	0.5215 ± 0.0421 ^{de}	0.0505 ± 0.0039 ^{bcde}
0.5 2,4-D + 1.5 BAP	0.4842 ± 0.0487 ^e	0.0492 ± 0.0057 ^{bcde}
0.5 2,4-D + 2.0 BAP	0.6802 ± 0.0949 ^a	0.1141 ± 0.1624 ^a
1.0 2,4-D + 0.0BAP	0.5505 ± 0.0712 ^{bcd}	0.0554 ± 0.0070 ^{bcd}
1.0 2,4-D + 0.5 BAP	0.2925 ± 0.0508 ^h	0.0302 ± 0.0095 ^{bcdefg}
1.0 2,4-D + 1.0 BAP	0.3718 ± 0,0521 ^f	0.0385 ± 0,0047 ^{bcdef}
1.0 2,4-D + 1.5 BAP	0.3073 ± 0,0307 ^{gh}	0.0303 ± 0,0020 ^{cdefg}
1.0 2,4-D + 2.0 BAP	0.6002 ± 0,0522 ^b	0.0601 ± 0,0056 ^{bc}
1.5 2,4-D + 0.0BAP	0.4995 ± 0,0230 ^{de}	0.0503 ± 0,0027 ^{bcde}
1.5 2,4-D + 0.5 BAP	0.6594 ± 0,0605 ^a	0.0656 ± 0,0069 ^b
1.5 2,4-D + 1.0 BAP	0.5358 ± 0,0571 ^{cde}	0.0538 ± 0,0058 ^{bcd}
1.5 2,4-D + 1.5 BAP	0.5509 ± 0,0778 ^{bcd}	0.0553 ± 0,0080 ^{bcd}
1.5 2,4-D + 2.0 BAP	0.5771 ± 0,1249 ^{bc}	0.0574 ± 0,0112 ^{bc}
2.0 2,4-D + 0.0BAP	0.5324 ± 0,0537 ^{cde}	0.0533 ± 0,0059 ^{bcd}
2.0 2,4-D + 0.5 BAP	0.3519 ± 0,0548 ^{fg}	0.0354 ± 0,0059 ^{bcdefg}
2.0 2,4-D + 1.0 BAP	0.4901 ± 0,0455 ^e	0.0499 ± 0,0047 ^{bcde}
2.0 2,4-D + 1.5 BAP	0.3256 ± 0,0531 ^{fgh}	0.0323 ± 0,0054 ^{cdefg}
2.0 2,4-D + 2.0 BAP	0.2388 ± 0,0519 ⁱ	0.0273 ± 0,0050 ^{defg}

Note: Different letters after mean number indicated significant difference based on t-independent test and Mann-Whitney test at 5% margin of error.

cultured explants.

Morphology of black betel (*Piper betle* L.) leaf callus supplemented with growth regulators 2,4-D and BAP at various concentration

Administration of growth regulators 2,4-D and BAP at various concentration to black betel leaf explant resulted in various response in the development of callus. Different responses were shown in explant by curving, rolling, widening, swelling, or callus growing at the surface or margin of the explant. The formed callus color was found to change every week during the culture period (Fig. 1). Indicators of explant growth in *in-vitro* culture are callus color and texture.

Results showed that all treatment of growth regulators formed compact callus. Com-

compact texture of callus was an effect from cytokinin that played role in nutrient transport. Cytokinin transport system ranged from basal to apex part of plants brings water and nutrients via transport vessel and affects osmotic potential in the cells. Additional sucrose in the medium flows through phloem vessel, resulting in turgor pressure. Turgor pressure emerges due to difference in fluid concentration, thus water and nutrients (sucrose) from medium will enters the cells through osmosis. This will cause cell walls to be increasingly rigid, resulting in a compact callus (Castro *et al.*, 2016; Mahadi *et al.*, 2016; Romanov, 2009).

Generally, callus formation occurs in the injured parts of midrib which touch the medium. Formation of callus in those parts are aimed to close the wound with cell proliferation from parent tissue or explant. The emergence of cal-

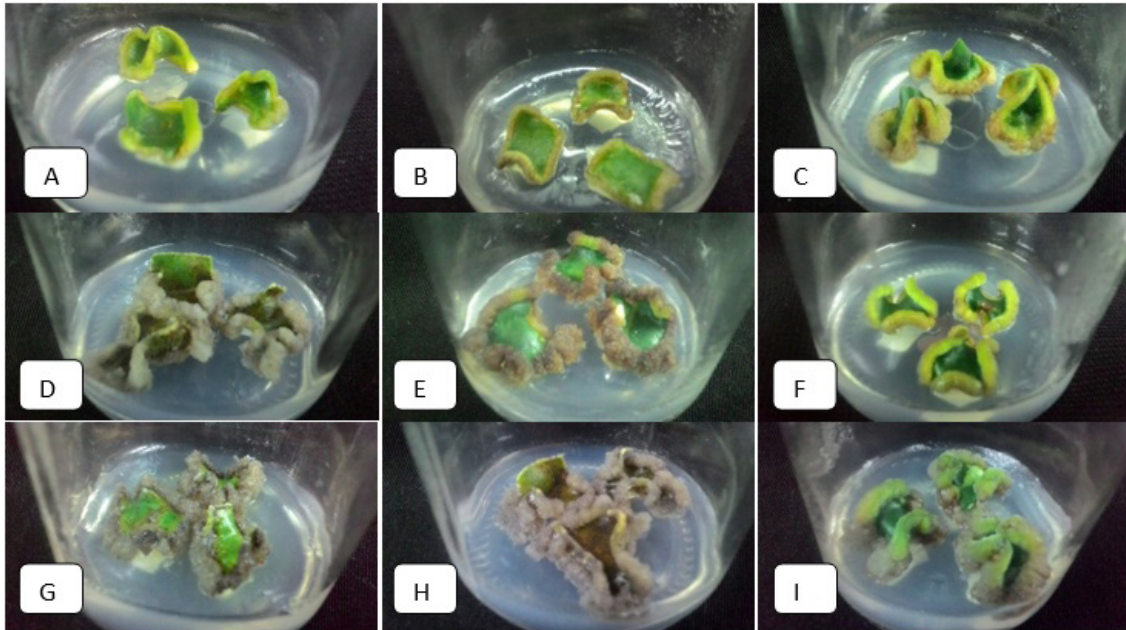


Figure 1. Various color of *Piper betle* L var Nigra callus (A) greenish yellow, (B) greenish white, (C) brownish yellow, (D) white, (E) brown, (F) yellowish green, (G) gray, (H) brownish white, (I) greenish brown.

lus at the margin part of explant (injured part) as observed from several groups in current study was marked with explants swelling, emergence of wave structure, then the growing of callus. In addition, callus could also emerge at the explant surface as observed in several callus given different treatments. This was possibly due to stimulation from explant tissue. Parts of explant that forms callus is caused by cells contacted the medium are promoted to transform into meristematic tissue, leading to cells become actively dividing. Cell division leading to callus formation occurs from response towards wound and both natural and artificial hormone supply in the explant (Ajithkumar *et al.*, 2013; Walirrahman, *et al.*, 2015; Sherma *et al.*, 2017).

Compact callus is a feature of organogenic callus, in which the growth seemed to be slow, because its growth is more inclined to differentiation process (Rashid *et al.*, 2009). Callus with compact texture is characterized with glossy flat or smooth serrated surface.

White color of callus possibly due to parenchymal tissue containing starch particles, which functions as polysaccharide storage of the plant (Desriatin, 2010). At the 7th week, color of several callus had turned into brownish white, while others brown and gray. This change of color was due to the aging of explant, showing signs of browning. Browning occurs in callus because of enzymatic reaction of phenolic compounds for-

mation (Chaudhary and Dantu, 2015), Browning of an explant indicates that it contains high level of phenols (Hendaryono and Wijayani, 1994). In the current study, phenolic compounds formed in callus as a response of explants towards injury. If the formed phenols are oxidized, callus color will turn into brown (Chaudhary and Dantu, 2015). Gray color in callus is caused by explants containing high level of stevioside compounds (Liu-dianto, 2003).

Treatment of various 2,4-D and BAP growth regulators concentration also affected shape of the leaf sheet. Curving leaf was an effect of auxin and turgor pressure (Uno *et al.*, 2001). Auxin induces cell walls to relax and stretch out. Relaxation of cell walls occurs due to acid secretion by activation of an enzyme at certain pH level. This enzyme will cut off the bound between cellulose molecules in the cell walls. With the stretching of cells, cells can perform elongation. Turgor pressure happens if cells absorb water molecules as a response of increasing concentration of soluble substances in the vacuoles, thus supporting expansion of cellsin size.

All explants from every treatment were found to be able to be induced to grow callus, with most of them colored brown and textured compact. From 25 combinations of growth regulator concentration, callus grown from each group were found to have only slight difference. Initial growth of callus was averagely the same,

which was firstly came from the margin of explants. Callus at its initial growth was colored transparent white or translucent like dews. At the next week, most callus changed color; several became white, greenish yellow, yellowish green, brownish white, dark brown, and gray.

CONCLUSION

Variation of 2,4-D and BAP growth regulators concentration affected induction period of black betel (*Piper betle* L.) leaf callus. Combination of 0.5 mg/L 2,4-D and 1.0 mg/L BAP resulted in shortest time of callus induction among other combination of concentration, at 7.25 ± 0.452 days. Variation of 2,4-D and BAP concentration affected percentage of explants forming callus. Variation of 2,4-D and BA concentration combination also affected callus fresh and dry weight. Optimal concentration of growth regulators resulting in highest fresh and dry weight of black betel leaf callus, at 0.6802 ± 0.0949 g and 0.0670 ± 0.1624 g respectively, was 0.5 mg/L 2,4-D and 2.0 mg/L BAP. Variation of growth regulators 2,4-D and BAP concentration affected callus morphology of black betel leaf explant. Morphology of formed callus varied in color and texture. Most of the callus formed were colored brown and had compact texture. Compact callus texture could accumulate more secondary metabolites.

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