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Callus Induction of Leaf Explant *Piper betle* L. Var Nigra with Combination of Plant Growth Regulators Indole-3-Acetic Acid (IAA), Benzyl Amino Purin (BAP) and Kinetin

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Abstract. The purpose of this research was to determine the combination of plant growth regulators IAA, BAP and kinetin towards callus induction and growth of leaf explants *Piper betle* L. VarNigra. Explants from leaf of *Piper betle* L. VarNigra was cultured on MS medium with 24 treatment combinations of plant growth regulators IAA and BAP and 24 treatment combinations of plant growth regulators IAA and kinetin with 0.0;0.5;1.0;1.5;2.0 mg/L concentration respectively, the observed variable were the length of time the formation of callus, callus morphology, fresh and dry weight of callus. The results of this research showed that the combination of growth regulators IAA with BAP and kinetin had effects on leaf growth of *Piper betle* L. VarNigra. During 8 weeks observation, it indicated that the combination of concentration IAA 0.5 mg/L and BAP 2.0 mg/L showed fastest callus formation at 8.5 days. Combination of concentration IAA 1.0 mg/L and BAP 1.5 mg/L showed the highest of fresh weight at 0.6596 grams, and the highest dry weight was obtained from the combination of concentration IAA 0.5 mg/L and BAP 0.5 mg/L at 0.0727 grams. Combination of concentration IAA 1.0 mg/L and kinetin 1.5 mg/L had the highest of fresh weight at 0.2972 grams and the highest dry weight at 0.1660 grams. Callus of *Piper betle* L. VarNigra had two textures, that were compact and friable, and also showed various kind of colors, like white, greenish white, yellowish white, tanned white, brown and black. Based on this research, that concentration IAA 1.0 mg/L and 1.5 mg/L kinetin was the best combination for induction of callus from leaf of *Piper betle* L. Var Nigra.

INTRODUCTION

Piper betle L. Var Nigra (Piperaceae), commonly known as black betel is distributed in the eastern part of the coast of Africa, Zanzibar island, Bonin island, Fuji islands, and the Indonesian islands [1]. It is rapidly growing perennial herb reaching 5 meters in height or up to 15 meters. The colour of stems are blackish red and the colour of leaves are blackish green. Falling leaf will leave a ring-shaped marks on the stem, the leaves have a distinctive aromatic odor [2].

Piper betle L. Var Nigra is known to have high medicinal value such as alkaloid, flavonoid, saponin, tannin, steroid, triterpenoid, and polifenolat [3]. It is used in bronchitis, anti-asthma, epistaxis, and anti-fungal [1,4]. As the plant *Piper betle* L. Var Nigra found to contain several important secondary metabolites, until now these secondary metabolites is obtained through extraction of organs in plants directly, but this method requires a supply of fresh herbs on a large scale furthermore the process of extraction, isolation, and purification costs are relatively expensive. One of the alternatives that can be done to keep the availability and increasing the production of secondary metabolites in shorter time is through callus culture.The purpose of this research was to determine the combination of plant growth regulators Indole-3-Acetic Acid (IAA) concentration of Benzyl Amino Purine (BAP) and kinetin

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towards on callus induction and growth of leaf explants *Piper betle* L. VarNigra. This is the first tissue cultural report in *Piper betle* L. VarNigra.

MATERIALS AND METHODS

The whole plants of *Piper betle* L. Var Nigra were collected from the bratang flowers market, Surabaya, East Java, Indonesia. Healthy and young leaves were selected as explants source. The leaves were initially washed with liquid detergent followed by rinsing with running tap water for 5 minutes. Then the leaves were treated with 70% alcohol for 6 minutes and washed running tap water for 3 times. Later, the leaves were treated with 20% clorox for 10 minutes and washed running tap water for 3 times. The last, the leaves are cut to a size of ± 1 cm^2, and the explants were inoculated into culture bottle inside the Laminar Air Flow.

Culture media

The basal MS medium (Murashige and Skoog, 1962) with 3% sucrose solidified with 0.8% agar was used. Several concentration and combination of growth regulators were tested including IAA, BAP, and kinetin. The pH of the medium was adjusted to 5.6-5.8 before autoclaving at 121^{o}C for 15 minutes. The cultures were maintained at 20-25^{o}C.

Statistical analysis

Experiments were carried out with 49 replicates, and were repeated 6 times. One control treatment, 24 combination concentration of IAA and BAP treatments (Table 1), and 24 combination concentration of IAA and kinetin treatments (Table 2). After 8 weeks, the cultures were visually observed for formation of callus texture, coloration, fastest callus formation, fresh weight, and dry weight.

RESULTS AND DISCUSSION

This research was conducted to know the effect of plant growth regulators IAA, BAP, and kinetin on callus induction time, callus morphology, fresh weight, and dry weight callus black betel leaf. In a various of treatment with a combination of growth regulators IAA, BAP, and kinetin showed different responses.

Table 1 shows the most rapid callus induction occurs in the combination of concentration IAA 0.5 mg/L and BAP 2.0 mg/L with a mean length of time of induction of 8.5 days, while the longest callus induction occurred in the MS basal medium with a mean length of time of induction of 30.5 days (control). The mean fresh weight and dry weight of callus which has been given to the treatment of the various combinations of concentrations IAA and BAP can be seen in Table 1. The combination of concentration IAA and BAP on fresh weight and dry weight of callus showed mean values were different. In the treatment with a combination of growth regulators IAA 1.0 mg/L and BAP 1.5 mg/L indicated the highest average value of the fresh weight with 0.6596 grams, while the highest average value of dry weight treated with combination of growth regulators IAA 0.5 mg/L and BAP 0.5 mg/L was 0.0727 grams.

TABLE 1. *The fastest average callus formation, fresh weight, and dry weight of callus induction from explants black betel formed on MS medium concentrations of various combinations of growth regulators IAA and BAP.*

The combination of concentration (mg/L)		The fastest average callus formation (days)	The average fresh weight (grams)	The average dry weight (grams)
IAA	**BAP**			
0.0	0.5	15.5	0.1155	0.0155
0.0	1.0	13.5	0.1441	0.0061
0.0	1.5	10.5	0.2604	0.0128
0.0	2.0	11.3	0.2689	0.0152
0.5	0.0	16.5	0.0460	0.0054
0.5	0.5	9.5	0.5021	**0.0727**
0.5	1.0	10.5	0.2652	0.0143
0.5	1.5	9	0.2195	0.0193
0.5	2.0	**8.5**	0.6016	0.0614
1.0	0.0	12	0.0119	0.0021
1.0	0.5	10.5	0.2186	0.0323
1.0	1.0	9	0.5447	0.0491
1.0	1.5	11.5	**0.6596**	0.0450
1.0	2.0	12.5	0.4673	0.0296
1.5	0.0	12.5	0.0384	0.0047
1.5	0.5	11.5	0.0380	0.0056
1.5	1.0	9.5	0.3075	0.0288
1.5	1.5	12	0.6356	0.0367
1.5	2.0	11	0.5362	0.0269
2.0	0.0	16	0.0221	0.0044
2.0	0.5	11.3	0.2652	0.0282
2.0	1.0	9.5	0.4415	0.0419
2.0	1.5	9.5	0.4701	0.0318
2.0	2.0	10	0.2852	0.0261
MS basal medium (control)		30.5	0.0031	0.0012

Table 2 shows the most rapid callus induction occurs in the combination of concentration IAA 0.5 mg/L and kinetin 0.5 mg/L (10 days) (Figure 1i). The combination of concentration IAA 1.0 mg/L and kinetin 1.5 mg/L showed the highest fresh weight with 0.2972 grams and the highest dry weight with 0.1660 grams (Figure 1j).

TABEL 2.*The fastest average callus formation, fresh weight, and dry weight of callus induction from explants black betel formed on MS medium concentrations of various combinations of growth regulators IAA and kinetin.*

The combination of concentration (mg/L)		The fastest average callus formation (days)	The average fresh weight (grams)	The average dry weight (grams)
IAA	Kinetin			
0.0	0.5	14	0.0345	0.0049
0.0	1.0	11.5	0.0210	0.0040
0.0	1.5	11	0.0473	0.0054
0.0	2.0	12	0.0723	0.0112
0.5	0.0	26	0.0038	0.0012
0.5	0.5	**10**	0.1528	0.0702
0.5	1.0	12	0.2280	0.0874
0.5	1.5	12	0.1504	0.0658
0.5	2.0	11.5	0.0581	0.0116
1.0	0.0	13.5	0.0099	0.0023
1.0	0.5	12	0.1802	0.0181
1.0	1.0	11	0.1865	0.1005
1.0	1.5	10.5	**0.2972**	**0.1660**
1.0	2.0	11	0.0760	0.0121
1.5	0.0	13	0.0810	0.0253
1.5	0.5	13	0.1097	0.0482
1.5	1.0	12	0.1636	0.0741
1.5	1.5	11.5	0.0971	0.0109
1.5	2.0	11	0.0593	0.0074
2.0	0.0	27	0.0013	0.0007
2.0	0.5	13	0.0884	0.0290
2.0	1.0	12	0.2005	0.0891
2.0	1.5	12	0.1305	0.0473
2.0	2.0	10.5	0.1099	0.0616

The callus of *Piper betle*L. VarNigra had two textures, that were compact (Figure 1a) and friable (Figure 1b), and also showed various kind of color, like white (Figure 1c), greenish white (Figure 1h), yellowish white (Figure 1d), tanned white (Figure 1f), brown (Figure 1e) and black (Figure 1g).

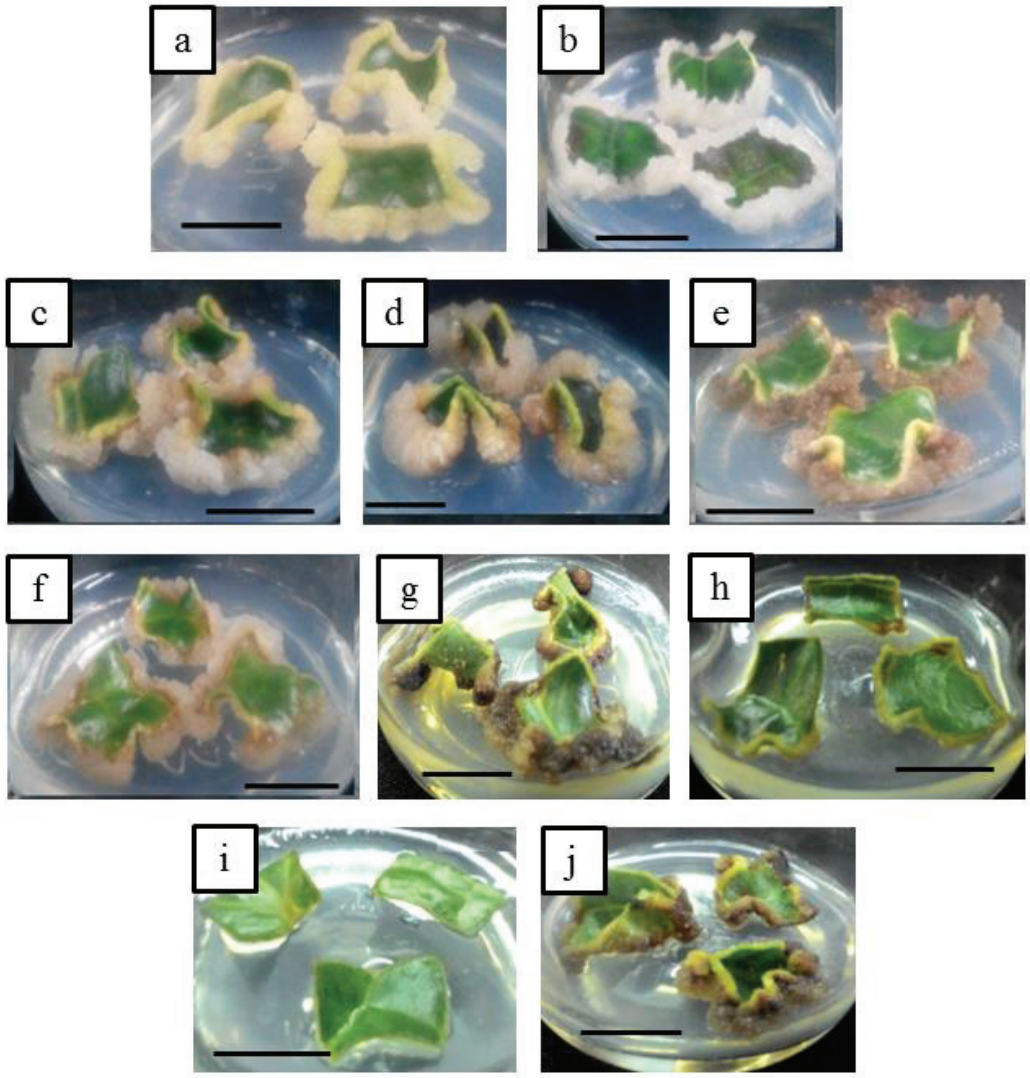

FIGURE 1. Callus morphology of *Piper betle*L. VarNigra in some treatments. **(a)** IAA 1.0 mg/L and BAP 1.0 mg/L, 6 weeks of culture period, **(b)** IAA 1.5 mg/L and BAP 1.5 mg/L, 8 weeks of culture period, **(c)** IAA 0.5 mg/L and BAP 2.0 mg/L, 8 weeks of culture period, **(d)** IAA 0.0 mg/L and BAP 2.0 mg/L, 8 weeks of culture period, **(e)** IAA 0.5 mg/L and BAP 0.5 mg/L, 8 weeks of culture period, **(f)** IAA 0.5 mg/L and BAP 1.0 mg/L, 8 weeks of culture period, **(g)** IAA 0.5 mg/L and kinetin 1.0 mg/L, 8 weeks of culture period, **(h)** IAA 2.0 mg/L and kinetin 0.5 mg/L, 7 weeks of culture period, **(i)** IAA 0.5 mg/L and kinetin 0.5 mg/L, 2 weeks of culture period, **(j)** IAA 1.0 mg/L and kinetin 1.5 mg/L, 8 weeks of culture period. Scale bar = 1 cm.

Callus formation was observed from leaf segment after 8 weeks of inoculation in MS medium supplemented with different combinations of IAA, BAP and kinetin (Table 1 and 2). The fastest explant for callus formation was observed in the MS medium supplemented with combination of IAA 0.5 mg/L and BAP 2.0 mg/L (± 8.5 days). Combination of IAA and kinetin, the fastest explant for callus formation was observed in the MS medium supplemented with combination of IAA 0.5 mg/L and kinetin 0.5 mg/L (10 days). Higher concentration of cytokinin than auxin on MS medium can induce calus faster because the number of cells undergoing division will increase [5]. That BAP are active in the growth and proliferation of callus [6]. Auxin can stimulate plant growth by increasing cell elongation process [7]. Callus formation depend on the several factors including the culture, environment, nature of explants and hormonal and non-hormonal regulators which may act synergistically in determining the proper induction, proliferation and regeneration of callus into planlets [8].

While the slowest explant for callus formation was observed in the basal MS medium/control (± 30.5 days). Differences in growth rate is influenced by absorbing nutrient substances ability of tissue. It is caused by administration of combination concentration of growth regulator in media which equalize auxin concentrations of exogenous and endogenous cytokinin contained hearts explants. Equilibrium concentration of auxin and cytokinin in culture can be known to stimulate callus formation through interaction enlargement and cell division [9]

The growth of the leaf callus cultures was measured in terms of fresh weight and dry weight. The highest explant for fresh weight of callus was observed in the MS medium supplemented with combination of IAA 1.0 mg/L and BAP 1.5 mg/L (0.6596 grams) and the highest for dry weight explant of callus was observed in the MS medium supplemented with combination of IAA 1.0 mg/L and kinetin 1.5 mg/L (0.1660 grams). The high value of the dry weight due to increased activity of the callus. The balance of auxin and cytokinin concentrations in vitro are known to stimulate callus formation through interaction with the enlargement and cell division [9]

Difference in fresh weight and dry weight of callus was also influenced by media which contained combination treatment concentration of growth regulator auxin and cytokinin that gave different results on explants culture. The most influencing factor in plant culture in vitro is the interaction and balance between the supply of plant growth regulators in the media and the production of endogenous growth regulators by cultured cells. Auxin and cytokinin may experience some kind of interaction such as antagonistic, or synergistic [10] However, the interaction between auxin and cytokinin are synergistic. Auxin plays role in regulating cell growth and elongation, while cytokines plays role in cell division. It is easy to understand because cellular auxin plays role in cell elongation, while cytokines trigger cell division [11].

The treatment of combination concentration of growth regulators IAA, BAP, and kinetin on black betel leaf explants gave different responses. Current study found that constituents of the solid medium or the requirement of the plant growth hormones and the nitrogenous source (nitrate/ammonium salts and amino acids) have produced morphological effects of the grown tissues that from the initial explants. For example, high concentration of auxin is responsible for the high multiplication of roots whereas an excess cytokinin may yield shoots. Balance concentration of auxin and cytokinin will often generate an unorganised and undifferentiated growth of cells, called callus [12]. The different responses can be observed visually indicated on a weekly basis during the culture period. The changes that occurred in the first week and the second week of explants black betel are curved or curled (explants wavy edges). After that in the second week and third week of the explant thickened edges / swell followed by the growth of callus on the edge of the explant.

Indicator explant growth in vitro culture also can be seen on color and texture of the callus. Based on the observations suggest that the majority of treatment produce friable callus (Figure 1b). Compact texture of callus (Figure 1a) is the effect of cytokinin that play role in the transport of nutrients. Cytokinin transport system from the basal to apical will bring water and nutrients through the carrier vessels and affects the osmotic potential inside the cell. The addition of sucrose in the medium will flow through the phloem vessels and raises turgor pressure. These pressures arise because of difference in concentration of the solution, thus water and nutrients (sucrose) from medium entered into cells via osmosis. This will make the cell walls more rigid, so that the cells will be compact callus [13]. The increasing concentration of IAA that added to the medium resulted in callus color which tends to yellow. This is proved by treatment combination of IAA 0.0 mg / L and BAP 2.0 mg / L color: white callus resulting yellowish (Figure 1d). In addition, this study also found other colors shown by black betel leaf explants callus, they were white, greenish white, brown and white, brown and black. This change was presumed due to change of pigmentation in the callus which reduced green pigment (chlorophyll).

Morphological observation of black betel leaf explants callus was done for eight weeks of explant culture. Callus of *Piper betle* L. Var Nigra had two textures, that were compact and friable, and also showed various kind of colors, like white, greenish white, yellowish white, tanned white, brown and black (Figure 1). Previous research has stated that the concentration of IAA 0.1 mg / L combined with BAP (1-2 mg / L) on the callus *Sempervivum tectorum* L. showed a wide variety of colors, such as white, yellowish white, amber, and green light [14].

In this research, the color of black betle's callus on a combination IAA 0.5 mg/L + kinetin 1.0 mg/L and IAA 0.5 mg/L + BAP 0.5 mg/L were black and tanned. Black betel is a tropical plant that has high content of secondary metabolites, one of them is kavikol which is a derivative of phenol [3]. Phenol compound is oxidized when the cell is injured (George and Sherrington, 1984). As a result, the tissue change to brown or blackish and failure to thrive. Tissue browning occurs because the oxidase enzyme activity is released or synthesized and available on oxidative conditions when the tissue is wounded [15,16].

CONCLUSION

Based on current study, 1.0 mg/L IAA and 1.5 mg/L kinetin was the best combination of plant growth regulators for induction of callus from leaf of *Piper betle* L. Var Nigra, as it resulted in callus with highest fresh weight of 0.2972 g and dry weight of 0.1660 g.

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