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The effects of 2,4-Dichlorophenoxyacetic acid and leaf surface orientation on callus induction of black betel (*Piper betle* L. var. *nigra*)

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ABSTRACT. Black betel (*Piper betle* L. var. *nigra*), which has pharmacological properties against a number of infectious disorders, contains secondary metabolites like alkaloids, terpenoids/steroids, flavonoids, and tannins. While preserving plant availability, callus culture can quickly enhance the production of these secondary metabolites. This study aims to determine the combination of the concentration of growth regulator 2,4-Dichlorophenoxyacetic acid (2,4-D) and the optimal position of leaf explants for the induction of black betel callus. This study is an experimental study using a complete randomized design with combinations of treatments between concentrations of 2.4-D growth regulators (0.0 mg/l; 1.5 mg/l; 2.5 mg/l; 3.5 mg/l) and leaf explant position (abaxial contact with media and adaxial contact with media). Quantitative data obtained were analyzed using the Kruskal-Wallis test and Mann-Whitney test on the SPSS program. Meanwhile, qualitative data in callus morphology were analyzed descriptively. The treatment of 1.5 mg/l 2,4-D using abaxial leaf position can be chosen as optimal combination of treatment. The results showed no significant difference (P>0.05) for many of the observed variables between this treatment compared to treatment with higher concentration of 2,4-D. MS media with 1.5 mg/l 2,4-D and abaxial leaf position can induce callus at 2.4 weeks after planting, caused 100 % percentage of explants forming callus, browning score of 2.1, 10% explant contamination, callus growth score of 2.6 and callus morphology in the form of yellowish-white callus color and compact callus texture.

Keywords: Abaxial and adaxial contact; callus induction; leaf surface orientation; sirih hitam; 2,4-Dichlorophenoxyacetic acid

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INTRODUCTION

One in eight deaths in 2019 were due to bacterial infections, which are the second biggest cause of death globally, including Indonesia (Global Burden of Diseases, 2020), but usually cured using antibiotics. However, as a result of the possibility of pathogenic resistance to widely used antibiotics, excessive usage of antibiotics will have a negative effect (Friedman *et al*., 2016; Mancuso *et al*., 2021). An alternate strategy to limit the harmful use of antibiotics is to use natural antibacterial capabilities of plants, such as black betel (*Piper betle* L. var. *nigra*), a native of Indonesia.

Black betel is a multifunctional plant that can be used as an ornamental plant or medicinal plant (Rekha *et al*., 2014), there has yet to be plenty more scientific study on this plant. Black betel contains alkaloids, flavonoids, saponins, tannins, steroids, triterpenoids, and polyphenols (Rija'i, 2015; Junairiah *et al*., 2018a; Junairiah *et al*., 2019), and its leaves are a source of natural antioxidant (Biswas *et al*., 2022), and traditionally used for the treatment of several diseases such as bad breath/halitosis (Oktanauli *et al*., 2020), wounds, inflammations (Rahayu *et al*., 2019), chronic cough coughs, and indigestion (Vijayalakshmi *et al*., 2019; Widowati *et al*., 2020). Until recently, direct extraction was the means used to obtain secondary metabolites (Junairiah *et al*., 2018a). However, this is problematic because direct extraction requires large quantities of fresh plants and requires expensive costs for extraction, isolation, and purification processes which requires a long time to carry out. One approach to resolving this issue is tissue culture, specifically callus culture, which can boost the production of secondary metabolites while preserving plant availability.

The success of tissue culture is influenced by several factors such as explants source, medium (organic and inorganic components), growth regulators, light, and temperature (Hazrati *et al*., 2022; Mehbub *et al*., 2022). Plant growth regulators (PGR) are compounds that can affect plant physiological processes and are added to the medium of a certain type and concentration depending on the desired growth response. Auxin growth regulators such as 2,4-Dichlorophenoxyacetic acid (2,4-D) is often used for callus induction (Sugiyarto & Kuswandi, 2014; Junairiah 2018b; Suhartanto *et al*., 2021). Auxin is effective in differentiated tissue, such as the leaves, where it promotes cell elongation, cell enlargement, callus formation, and root growth (Majda & Robert, 2018; Pacheco-Villalobos *et al*., 2016). Leaf position in plant tissue culture is known to affect the percentage of explants forming calluses (Mitić *et al*., 2012) and the number of shoots produced due to the side of the leaf that is placed on the media and the bending of the leaf abaxial side (Schuchovski *et al*., 2020).

Given the impact of explant location on the outcome, we must locate the optimum media and determine the ideal explant position to produce callus from medicinal plants. The aim of this study was to ascertain which 2,4-D concentration and leaf position combination induced callus more quickly. The callus produced by this study has the potential to be employed for the extraction of medicinally effective substances.

MATERIALS AND METHODS

This study is an experimental study using a complete randomized design (CRD) with two factors of a concentration of growth regulators of 2.4-D (0.0 mg/l; 1.5 mg/l; 2.5 mg/l; 3.5 mg/l) and two leaf explant positions (abaxial and adaxial) in contact with culture media (Fig. 1).

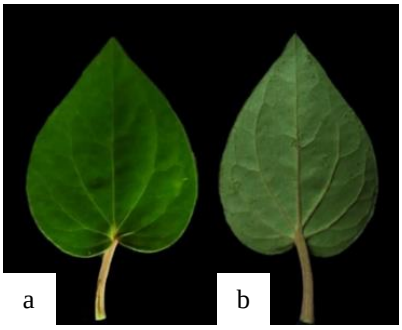

Fig. 1. The two leaves orientation used in this research: a. Abaxial position, where the lower surface is facing down; b. Adaxial position (right) where upper surface is facing down

The concentration of 2,4-D was adapted from Junairiah *et al.* (2019). The media used was Murashige and Skoog (MS) with vitamins from CV. AgriBiotech (Yogyakarta). There were in total eight treatment combinations (Table 1) and for each treatment explants were planted in five replicates with each petri dish containing two pieces of explants.

Table 1. Treatment codes with each combination of 2,4-D concentration and leaf position

No	Treatment code	Combination of 2,4-D concentration (mg/l) and leaf position
1	D_0P_1	0 + abaxial
2	D_0P_2	0 + adaxial
3	$D_{1.5}P_1$	1.5 + abaxial
4	$D_{1.5}P_2$	1.5 +adaxial
5	$D_{2.5}P_1$	2.5 +abaxial
6	$D_{2.5}P_2$	2.5 +adaxial
7	$D_{3.5}P_1$	3.5 +abaxial
8	$D_{3.5}P_2$	3.5 +adaxial

The explants used were the second to the fourth leaves from the tip of the plant. For sterilization, the leaves were washed using liquid detergent (Sunlight) for 5 min then rinsed using running water.

In the Laminar air flow (LAF) cabinet, the leaves were soaked in 70% alcohol for 6 min, and rinsed using sterile aquadest 3 times. Sterilization was then carried out using a 20% sodium hypochlorite (Bayclin®) solution for 10 min, and rinsed using sterile aquadest 3 times. The leaves were cut to a size of ±1 cm^2 then planted on each type of media (Table 1). The parameters observed were callus induction time, level of callus growth, level of browning, and percentage of contamination. Scores for callus growth and browning is described in Table 2 as adapted from Dewi *et al.* (2012) and Admojo & Indrianto (2016). Callus morphology (type and colour) was analyzed descriptively using description adapted from Khaniyah *et al.* (2012) and Kristianto & Setyorini (2021).

Table 2. Score for callus growth and browning on explants

Score	Callus description	Browning description
0	Callus does not form callus	Explants do not undergo browning
1	Explants show bending or swelling	> 0 – 25% browning of the explant
2	> 0 – 20% callus covers explants	> 25 – 50% browning of the explant
3	>20 – 40% callus covers explants	> 50 – 75% browning of the explant
4	> 40 – 60% callus covers explants	> 75 – 100% browning of the explant
5	> 60 – 80% callus covers explants	
6	>80 – 100% callus cover explants	

Data analysis. The quantitative data from scoring (time of callus induction and callus growth at week 8) were analyzed using the SPSS ver. 26. Test of normality and homogeneity was undertaken. The data was found to be non-parametric thus the Kruskal-Wallis test and post-hoc Mann-Whitney test were used.

RESULTS AND DISCUSSION

Callus induction time. The results of this study showed that the position of the adaxial leaves on the media with and without 2,4-D resulted in callus induction time approximately one week faster than planting with the abaxial position on the media (Table 3). Leaf explant position on a tissue culture media can affect the effectivity of absorption. In black betel, trichomes are present on the abaxial surface of the leaves as also seen in other *Piper* species (Raman *et al*., 2012). Trichomes on the lower leaf surface (abaxial) can interfere with the absorption process of the medium so can slow down the time of callus induction. When placed on the abaxial side, the black betel leaf for a curved/curled up shape that also reduce the surface area in contact with the medium. However, when the leaf position is on adaxial side, a larger leaf surface area can touch the media thus can help increase the absorption of nutrients from the media causing callus to grow faster.

The influence of leaf position in inducing explants in tissue culture was also found in other studies but with different results. Such as the *Talinum paniculatum*, where the abaxial planting position produces a greater number of main roots than the adaxial position (Solim *et al*., 2017) and in *Fragaria vesca*, where callus and shoot induction showed significant difference between abaxial and adaxial side up (Sarker *et al*., 2020).

Table 3. The average time of callus induction and the percentage of black betel leaf explants form callus

Combination treatment		Treatment code	Mean callus induction time	Percentage of explants
2.4-D (mg/l)	Leaf position		(weeks after planting) ± SD	forming callus
0	Abaxial	D_0P_1	3.9 ± 0.2236^{d}	100 %
0	Adaxial	D_0P_2	2.9 ± 0.5477^{ab}	100 %
1.5	Abaxial	$D_{1.5}P_1$	2.4 ± 0.6519^{abc}	100 %
1.5	Adaxial	$D_{1.5}P_2$	2.2 ± 0.2739^{c}	100 %
2.5	Abaxial	$D_{2.5}P_1$	2.3 ± 0.6708^{abc}	100 %
2.5	Adaxial	$D_{2.5}P_2$	2.3 ± 0.4472^{ac}	100 %
3.5	Abaxial	$D_{3.5}P_1$	3.2 ± 0.5701^{b}	100 %
3.5	Adaxial	$D_{3.5}P_2$	2.1 ± 0.2236^{c}	100 %

Notes: Mean accompanied by different notations show a significance difference ($P<0.05$).

Media MS with 1.5 mg/l 2.4-D was able to induce callus in 2.2 weeks which did not differ significantly from the treatment using 3.5 mg/l 2.4-D. This is in accordance with research conducted by Junairiah *et al.* (2019) which showed that addition of 1.5 mg/l 2,4-D in culture media was able to induce callus from black betel leaves faster than at a lower or higher concentrations. Endogenous hormone in the black betel leaves plays an important factor in its response to external hormone/plant growth regulators added in the culture media because plant development is regulated by hormonal signals that depend on the specific balance of hormones such as auxin and cytokinin (Saeedpour *et al*., 2021).

Callus growth. The utilization of medicinal plants can be developed through extracts of their active ingredients directly from *in vitro*-induced callus. In this study, callus growth was observed so that not only the speed at which the callus appeared was important but the development of callus could also determine the level of active ingredient that could be produced.

Table 4. Average growth score of black betel leaf explant callus at week 8

Combination treatment Concentration 2.4-D (mg/l)	Leaf position	Treatment code	Mean callus growth score± SD
0	Abaxial	D_0P_1	2 ± 0.0000^{a}
0	Adaxial	D_0P_2	2 ± 0.2236^{a}
1.5	Abaxial	$D_{1.5}P_1$	2.6 ± 0.2236^{b}
1.5	Adaxial	$D_{1.5}P_2$	2.3 ± 0.4183^{ab}
2.5	Abaxial	$D_{2.5}P_1$	2.4 ± 0.0000^{b}
2.5	Adaxial	$D_{2.5}P_2$	2.5 ± 0.2739^{b}
3.5	Abaxial	$D_{3.5}P_1$	2.4 ± 0.3536^{ab}
3.5	Adaxial	$D_{3.5}P_2$	2.7 ± 0.4472^{b}

Notes: Mean accompanied by different notations show a significance difference ($P<0.05$)

The average callus growth score was analyzed in the eighth week after planting. Scoring was based on description in Table 2. The average callus score for all combination of treatments were less than 4 (less than 50% explant coverage) at 8 weeks after planting. Treatment with 3.5 ppm 2.4-D + adaxial showed the highest callus score while the lowest was in the control (without 2,4-D in the media) (Table 4) but was not significantly different to treatment with 1.5 mg/l 2.4-D + abaxial. Thus, it could confirm that adding 1.5 mg/l of 2,4-D is more effective and can reduce cost to produce the same amount of callus. The development of callus in the 1.5 mg/l 2,4-D media compared to the control media is presented in Fig. 2, showing white callus forming on the sides of the explants compared to the control (D_0P_1) which shows no callus and only browning occurred.

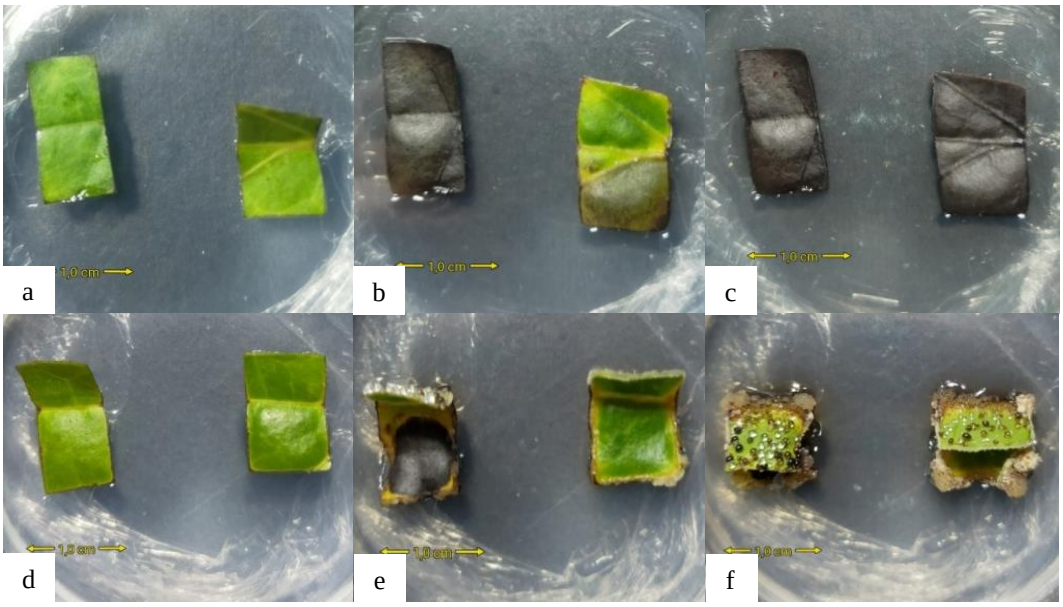

Fig. 2. Callus in treatments in week 8 after planting. a-c: D_0P_1; d-f: $D_{1.5}P_1$. Scale bar = 1cm

Due to its involvement in boosting osmotic pressure, cell permeability, protein synthesis, and cell plasticity, which results in an ease of the cell wall, the growth plant regulator 2,4-D is able to generate callus in leaf explants, such as *Piper* sp. (Santos *et al*., 2016; Wasti & Pant, 2019; Kristianto & Setyorini, 2021). Those processes will then increase the plasticity and solubility of cellulose in the cell wall so oxygen, water and minerals can pass easily through cell membranes.

Levels of callus browning. Browning on explants is the appearance of a brown colour that can inhibit the growth of the explants. This happens because of polyphenol oxidation causing an accumulation of phenolic compounds that cause the brown colour (Mustafa *et al*., 2013; Zhao *et al*., 2021). All treatments showed browning in the explants but had different scores. Observation was undertaken for eight weeks and results show an increase the number of explants that showed browning both in the callus and original leaf tissue (Fig. 3). In the 8th week, the lowest browning score was 2.1 in the treatment with 1.5 mg/l 2.4-D + abaxial and 2.5 mg/l 2.4-D + abaxial (Fig. 3). The highest browning score was 3.7 in the control D_0P_2 (0 mg/l 2.4-D + adaxial). However, statistical analysis of the browning score at the eighth week was carried out using the Kruskal-Wallis test and the result showed that there is no significant difference (P>0.05) between treatments.

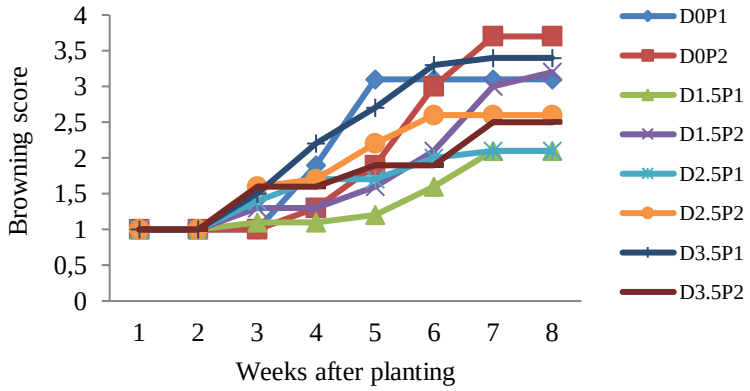

Fig. 3. Browning scores for the different treatments on black betel leaf explants for eight weeks after planting

Phenolic compounds are known to be found in *Piper* species, including in black *Piper betle* (Prasetya *et al*., 2021). Browning, which results from wounding of plant tissue during tissue culture can cause ineffective growth of explants as it can reduce growth, causing lower regeneration percentage and can lead to tissue death (Amente & Chimdessa, 2021). The different media contains 2,4-D variation or leaf position did not significantly affect browning so there is potential to find an alternative method to reduce browning in the explants and can support explant to induce better callus.

Fig. 4. Browning in explants: a. 0mg/l 2,4-D+adaxial (D_0P_2); b. 2.5mg/l 2,4-D+abaxial ($D_{2.5}P_1$)

Contamination of callus. Contamination in plant tissue culture is generally caused by fungi and bacteria that can originate externally (the working environment) and internally (the surface and inside the explant tissue). Contamination by fungi is characterized by the appearance of fungal colonies that are white, gray, green, pink and black, while contamination by bacteria is characterized by the

appearance of a yellowish-white liquid (Nalavade *et al*., 2017; Rahmadi *et al*., 2020; Andriani & Heriansyah, 2021). No contamination was observed in treatment 3.5 mg/l 2.4-D + adaxial ($D_{3.5}P_2$) and the highest in 0 mg/l 2,4-D+ abaxial (D_0P_1) and in 1.5 mg/l 2,4-D+adaxial ($D_{1.5}P_2$).

Analysis on the number of contaminated explants in the eighth week was carried out using the Kruskal-Wallis test with a confidence level of 95% and the results showed that there was no significant difference between treatments (P>0.05). Therefore, the variation in 2,4-D concentrations and leaf position did not affect the percentage of contamination in a black betel leaf explants which shows that the sterilization technique is suitable for black betle leaf explant for in vitro culture.

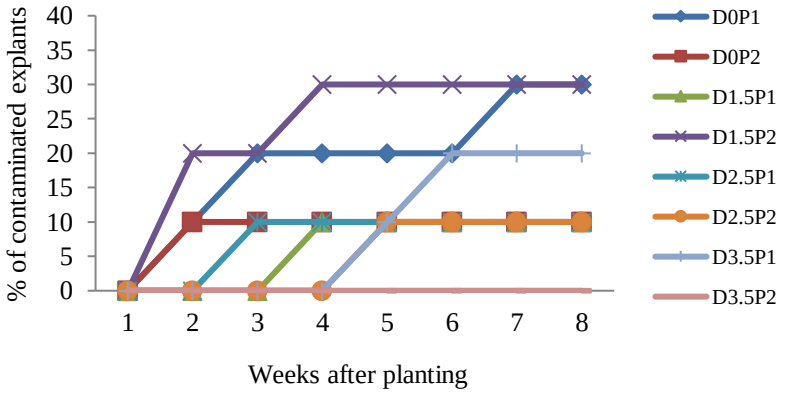

Fig. 5. The percentage of contaminated black betel leaf explants in each treatment for eight weeks after planting

Callus morphology. The stages of development and the efficacy of the treatment can be determined by the morphology of an *in vitro*-induced callus. The cells in calluses actively proliferate to generate the colours white, yellow, or green, which contain chlorophyll (Winson *et al*., 2020; Chen *et al*., 2021). The callus expected for the production of secondary metabolites as medicinal materials should have a compact texture with cells that actively divide. Higher antioxidant activity was found from compact callus of *Hovenia dulcis* compared to samples of *in vitro* propagated plants and field grown plants (Ribeiro *et al*., 2015). This showed that in vitro production of medicinal compounds by callus culture can be used as an alternative method of production. However, friable callus can also produce certain compounds such as terpenoids which was found in black *Piper betle* callus induced using 0.5-2.5 mg/l 2,4-D (Junairiah *et al*., 2019).

Table 5. Morphology of black betel callus from leaf explant at week 8 after planting

Combination treatment		Treatment code	Callus colour	Callus texture
Concentration 2.4-D (mg/l)	Leaf position			
0	Abaxial	D_0P_1	Brown	Friable
0	Adaxial	D_0P_2	Brown	Friable
1.5	Abaxial	$D_{1.5}P_1$	Yellowish white	Compact
1.5	Adaxial	$D_{1.5}P_2$	White-brown	Compact
2.5	Abaxial	$D_{2.5}P_1$	Yellowish white	Compact
2.5	Adaxial	$D_{2.5}P_2$	Yellowish white	Compact
3.5	Abaxial	$D_{3.5}P_1$	Yellowish white	Compact
3.5	Adaxial	$D_{3.5}P_2$	Yellowish white	Compact

Based on the type and colour of the callus, treatments that can potentially produce callus with more optimal secondary metabolite content are the treatments: 1.5 mg/l 2,4-D + abaxial, 2.5 mg/l 2,4-D + abaxial, 2.5 mb/l 2,4-D + adaxial, 3.5mg/l 2,4-D + abaxial and 3.5 mg/l 2.4-D + adaxial (Table 3). Those five treatments produced yellowish-white callus and a compact callus structure while other combination of treatments produced brown or friable callus.

CONCLUSION

The optimal treatment for callus induction and black betel callus growth was 1.5 ppm 2,4-D using abaxial leaf position. That combination of treatment was able to induce callus at 2.4 weeks after planting, caused 100 % percentage of explants forming callus, browning score of 2.1, 10% explant contamination, callus growth score of 2.6 and callus morphology in the form of yellowish-white callus color and compact callus texture. Using a higher concentration of 2,4-D (2.5 and 3.5 mg/l) did not result in significant difference than 1.5 mg/l 2,4-D.

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