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Callus induction and proliferation of *Centella asiatica* L. generated from leaves and petioles in the presence of Dicamba and BAP

Abstract. Centella's need for industrial raw materials is high as a medicinal plant. These needs can be met through rapid multiplication using tissue culture techniques. In this study, induction and proliferation for a callus of centella cv. Castina 3 was conducted in the MS basal medium plus 4 mgL^{-1} Dicamba withand enriched with 7 concentrations of BAP (0, 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 mgL^{-1}). Two kinds of explant were used, i.e., leaf and petiole. The results revealed that the addition of BAP in MS plus Dicamba medim stimulated better and produced a higher callus growth rate, both from leaf and petiole explants, than that media with Dicamba alone. Furthermore, 4 mgL^{-1} Dicamba + 1.1 mgL^{-1} BAP had a friable callus in the induction phase and a friable-compact callus in the proliferation phase. From this finding, it can be considered to use a combination of 4 mgL^{-1} Dicamba with 1.1 mgL^{-1} BAP in callus induction and proliferation for Centella rapid multiplication.

Keywords: BAP · Callus induction · Centella · Dicamba · Proliferation

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Introduction

Centella (*Centella asiatica* L.) is commonly used for medicinal purposes, as this plant is rich in bioactive compounds, which are included in the triterpenoid, such as asiacotide, madecassoside, asiatic acid, and madecassic acid (Prasad et al., 2019; Anam et al., 2023; Diniz et al., 2023). Centella is one of the most important raw materials in the industry of jamu or herbal tonic (Elfahmi et al., 2014; Prasad et al., 2014), medicine (Thuraisingam et al., 2023; Vijayakumar et al., 2023), health-care supplement (Sardrood et al., 2019; Ogunka-Nnoka et al., 2020), and cosmetisc (Subha & Ranjit, 2014; Karlina et al., 2022; Liu et al., 2022; Sari et al., 2022). This leads to the increasing demand for centella from time to time. Unfortunately, few efforts are carried out to cultivate this plant, and to meet industrial needs, as people tend to have this product directly from nature. Continuous harvesting, tends to overexploitation without restoration, that leads to decline this plant in the near future (Kor et al., 2021).

Centella could be propagated *in-vitro* using an indirect regeneration strategy via the callus phase (Rahayu et al., 2016; Gururajan et al., 2021; Luthra et al., 2022). Callus is frequently used in basic research and industrial applications (Kruglova et al., 2023). Apart from producing a large number of seedlings using a piece of original plant (Mohapatra et al., 2021), this technique can be employed as well in culture of calluas and cell suspension to produce secondary metabolites, particularly asiacotide, the principal pharma-cological active component in centella (Ncube et al., 2017; Luthra et al., 2022; Kruglova et al., 2023).

Adding Dicamba to growth media helps the formaction of centella callus, with 4 mgL^{-1} being the optimum dose (Rahayu et al., 2016), even though both the amount and the quality of calli generated remained inadequate. It is assumed that adding cytokinin, such as BAP, will be more effective in creating more calli because this hormone is an essential regulator for cell architecture and proliferation (Schaller et al., 2015). The utilization of BAP is predicted to boost the number and quality of calluses. This study evaluated the effect of combining Dicamba with BAP on centella callus production.

Materials and Methods

This activity was carried out in the Center for Standard Testing of Biotechnology Instruments and Agricultural Genetic Resources, Bogor's tissue culture laboratory from August to October 2021. Prior to tissue culture works, stolons were collected from centella plants cv. Castina 3 in the greenhouse and utilized as explants for *in vitro* culture. Stolon surface sterilization was performed with 0.5 gL^{-1} benomyl and 20 gL^{-1} streptomycin sulfate, each for one hour. Continuing sterilization with 70% alcohol, 15.75% sodium hypochlorite, and 10.5% sodium hypochlorite for 5, 7, and 9 minutes, respectively. Afterward, rinse using sterilized distilled water three times. The stolon apical shoots were then isolated and cultured for 5 weeks on MS medium (Murashige & Skoog, 1962) without growth regulators to develop plantlets.

Once the plantlets were fully developed, leaves and petioles were isolated in 0.5 x 0.5 cm and 1 x 0.15 cm, respectively. In the induction of callus, explants in the form of leaves and petioles were then placed on MS media with 3% sugar and pH 5.8, treated with Dicamba (3,6-dichloro-2-methoxybenzoic acid) and BAP then solidified with 0.3% agar. The culture was then incubated at 22°C, 300 lux light intensity, and a photoperiod of 16 hours. The present tratment was a combination of Dicamba and BAP, as follow A : Dicamba 4 mgL^{-1}; B : Dicamba 4 mgL^{-1} + BAP 0.1 mgL^{-1}; C : Dicamba 4 mgL^{-1} + BAP 0.3 mgL^{-1}; D : Dicamba 4 mgL^{-1} + BAP 0.5 mgL^{-1}; E : Dicamba 4 mgL^{-1} + BAP 0.7 mgL^{-1}; F : Dicamba 4 mgL^{-1} + BAP 0.9 mgL^{-1}; G : Dicamba 4 mgL^{-1} + BAP 1.1 mgL^{-1}. A randomized complete design with ten replications was utilized. Observations were made on the percentage of callus induction, fresh weight of callus, rate of callus growth, and color and texture of callus. The rate of callus growth is measured using the following scoring:

0 = no growth of callus
1 = growth of callus on 1 - 25% of explant
2 = growth of callus on 26 – 50% of explant
3 = growth of callus on 51 – 75% of explant
4 = growth of callus on 76 – 100% of explant

The callus induction was observed four weeks after transplanting, followed by observation in the proliferation phase for the next 4 weeks. Analysis of Variance (ANOVA) was used to examine the acquired data, followed by Least Significant Difference (LSD) at 5% to distinguish between treatments.

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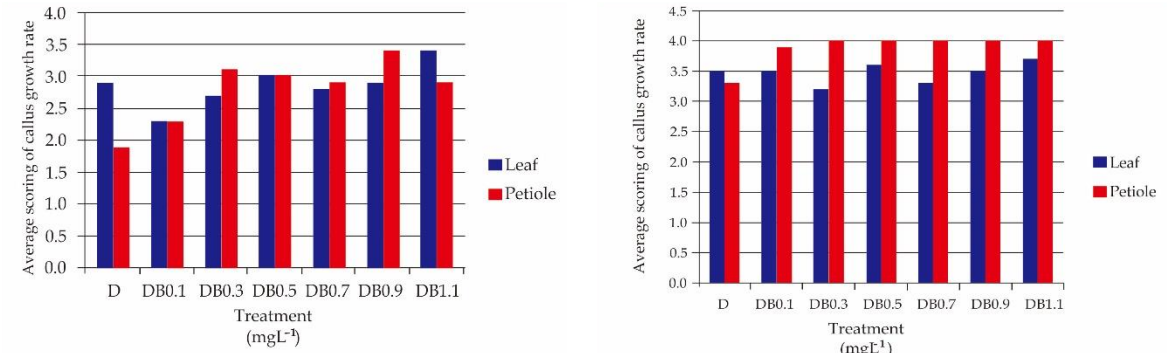
Results and Discussion

In the present study, callus occurred in all treatments after 4 weeks of culture incubation. All treatments affected each parameter observed in both leaf and petioles explants. The addition of 4 mgL^{-1} Dicamba together with BAP to the medium enhanced callus formation and callus fresh weight during the induction and proliferation phases. The callus growth rate, determined by the percentage of callus formation, varied between treatments (Figure 1). In the induction and proliferation phases, callus from petiole explants grown on a medium containing a combination of Dicamba and BAP at all concentrations grew faster than control media (treatment A or only Dicamba). Furthermore, callus originating from petioles grew faster than from leaves during the induction phase in several treatments.

The finding of this study, which indicated that callus originating from petioles grew faster than callus originating from leaves, contradicted the finding of previous research. Usha et al. (2015) discovered that leaf explants were the best for callus induction compared to other plant parts (Usha et al., 2015). The petioles used as explants in this study were obtained from *in vitro* plants, so the cell tissue may still be in a juvenile state. Combining Dicamba, a type of auxin, with BAP, a type of cytokinin, promoted callus growth more effectively than Dicamba alone. This is consistent with previous findings that auxin and cytokinin in the form of BAP encourage callus formation (Anwar & Isda, 2020; Gururajan et al., 2021).



A B

Figure. 1. The rate of callus growth at induction (A) and proliferation phase (B)

(Treatment: D= Dicamba 4 mgL^{-1}; DB0.1= Dicamba 4 mgL^{-1} + BAP 0.1 mgL^{-1}; DB0.3= Dicamba 4 mgL^{-1} + BAP 0.3 mgL^{-1}; DB0.5= Dicamba 4 mgL^{-1} + BAP 0.5 mgL^{-1}; DB0.7= Dicamba 4 mgL^{-1} + BAP 0.7 mgL^{-1}; DB0.9= Dicamba 4 mgL^{-1} + BAP 0.9 mgL^{-1}; DB1.1= Dicamba 4 mgL^{-1} + BAP 1.1 mgL^{-1})

Table 1. Effect of combination of Dicamba dan BAP treatment on the fresh weight of callus generated from leaf and petiole explant in the induction and proliferation phase

Treatment	Induction phase		Proliferation phase	
	Leaf explant	Petiole Explant	Leaf Explant	Petiole Explant
A	0.252 e	0.130 c	0.578 c	0.380 c
B	0.358 cde	0.208 c	0.791 bc	0.620 c
C	0.294 de	0.301 abc	0.609 c	0.946 b
D	0.521 bc	0.291 bc	0.995 b	0.974 b
E	0.465 cd	0.386 ab	0.963 b	1.045 b
F	0.545 b	0.483 a	1.215 a	1.585 a
G	0.752 a	0.370 ab	1.379 a	1.230 b

Means in the same column followed by the same letter are not significantly different at a 5% level of probability. A combination of Dicamba and BAP, as follow A : Dicamba 4 mgL^{-1}; B : Dicamba 4 mgL^{-1} + BAP 0.1 mgL^{-1}; C : Dicamba 4 mgL^{-1} + BAP 0.3 mgL^{-1}; D : Dicamba 4 mgL^{-1} + BAP 0.5 mgL^{-1}; E : Dicamba 4 mgL^{-1} + BAP 0.7 mgL^{-1}; F : Dicamba 4 mgL^{-1} + BAP 0.9 mgL^{-1}; G : Dicamba 4 mgL^{-1} + BAP 1.1 mgL^{-1}.

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Similarly, there were variations in the fresh weight of the callus between treatments, and the treatment combining Dicamba and BAP (treatments B - G) was significantly different from the control (treatment A) (Table 1). There was even a tendency for increasing the concentration of BAP to increase the fresh weight of the callus. This happened throughout the induction and proliferation phases with all types of explants. Treatment G (Dicamba 4 mgL^{-1} + BAP 1.1 mgL^{-1}) provided callus with the most significant fresh weight for callus formed from leaf explants at both the induction and proliferation stages. Meanwhile, treatment F (Dicamba 4 mgL^{-1} + BAP 0.9 mgL^{-1}) produced the highest fresh weight of callus developed from petioles.

These findings suggest that cytokinin, in this case BAP, significantly affects the induction and proliferation of centella callus. Callus initiation requires the addition of cytokinin and auxin to the growth media, as with other dicotyledonous plants (Schaller et al., 2015; Fehér, 2019). Cytokinin, along with auxin, is known to regulate cell division and morphogenesis (Fehér, 2019). The fresh weight of the callus is particularly essential when it comes to centella as a medicinal plant. Secondary metabolites are abundant in calluses with considerable fresh weight. Apart from that, it facilitates regeneration into plantlets for additional propagation material. However, when large amounts of secondary metabolites are desired, it is necessary to pay attention to the density level of the callus culture, where it is often necessary to subculture to maintain the freshness of the culture as well as to increase the number of cultures (Varshney & Anis, 2016).

Apart from callus growth, observations were also made on callus morphology, in this case, the texture and color of the callus. The observations' findings revealed variations in texture and callus color among the treatments applied, both throughout the induction and proliferation phases. During the induction phase, the texture of the callus originating from the leaves was friable and friable - compact. However the callus originating from the petiole was dominated by friable texture (Figure 2). Raising the BAP concentration in calluses originating from leaves tended to increase the number of calluses with a compact texture. Meanwhile, during the proliferation phase, the most friable texture was discovered in callus that got only Dicamba treatment (Figure 2).

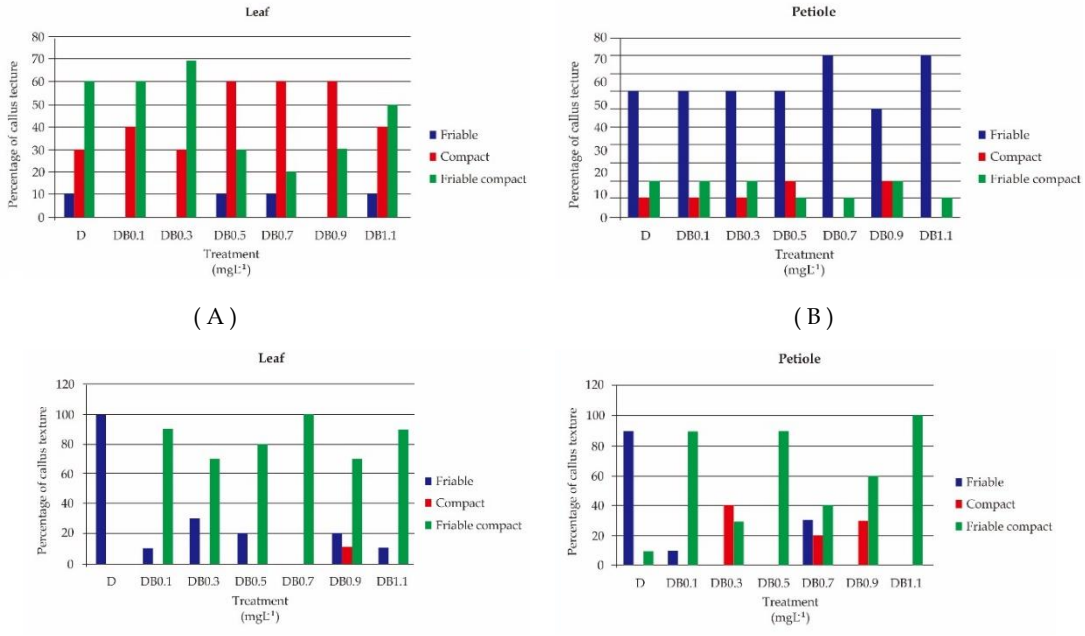


Figure. 2. The texture of callus generated from leaf and petiole explant in the induction phase (A and B) and proliferation phase (C and D)

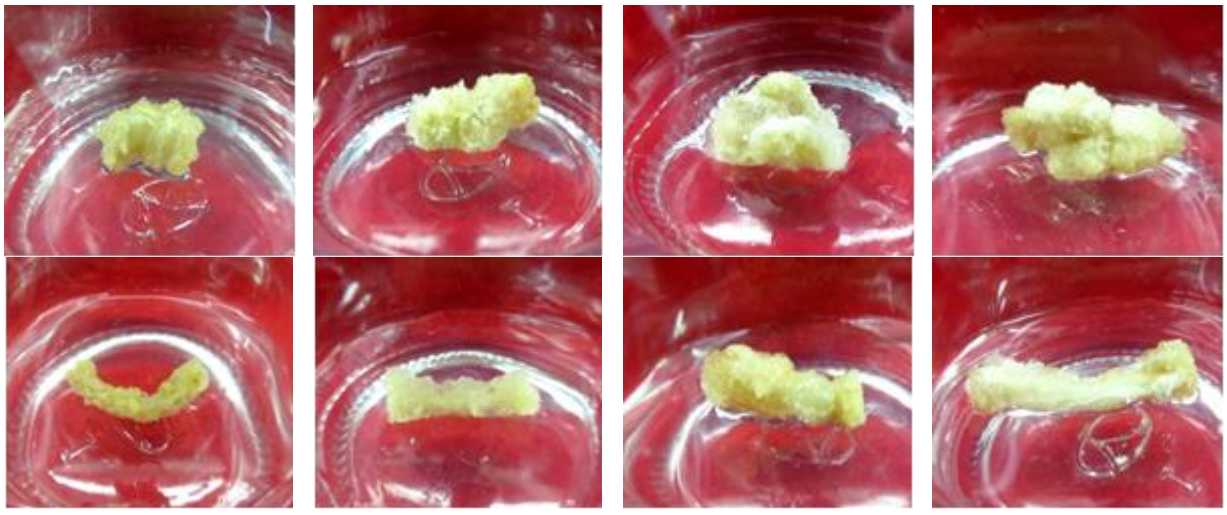
(Treatment: D= Dicamba 4 mgL^{-1}; DB0.1= Dicamba 4 mgL^{-1} + BAP 0.1 mgL^{-1}; DB0.3= Dicamba 4 mgL^{-1} + BAP 0.3 mgL^{-1}; DB0.5= Dicamba 4 mgL^{-1} + BAP 0.5 mgL^{-1}; DB0.7= Dicamba 4 mgL^{-1} + BAP 0.7 mgL^{-1}; DB0.9= Dicamba 4 mgL^{-1} + BAP 0.9 mgL^{-1}; DB1.1= Dicamba 4 mgL^{-1} + BAP 1.1 mgL^{-1})

The combination of Dicamba and BAP treatment produced calluses with a friable - compact texture. This applies equally to calluses resulting from both leaves and petioles. From this study, it was indicated that BAP added to the media increased callus compactness. The advantage of the compact callus texture is that it is good for regenerating into plantlets. However, this compact texture is unsuitable for cell suspensions to produce secondary metabolites. For cell suspensions, a friable callus texture is preferred.

Table 2 shows variations in callus color due to treatment and changes in callus color from the induction phase to the proliferation phase. The callus color variations observed varied from cream to brownish cream. Different callus color changes due to hormone treatment occur between explant types (leaves and petioles) during the induction phase, where the callus appears, as shown in Figure 3. Extending the culture period over the proliferation phase caused alterations in callus color. In previous studies, using auxin stimulates callus browning over long periods of culture (Usha et al., 2015; Rahayu et al., 2016). To prevent browning of the callus, it is necessary to sub-culture regularly after the culture reaches 4 weeks of age. Apart from that, adding cytokinin in the form of BAP is also expected to reduce the browning or darkening of the callus color. In this study, the callus in the proliferation stage changed color to brownish cream and brownish yellow cream compared to the preceding one in the induction phase, which was still brighter (cream, white cream, and greenish cream). The synthesis and accumulation of harmful compounds, such as phenol, that cause browning, as well as the reduction in nutrient content in the media due to prolonged use in closed jars, are thought to contribute to the change in callus color in the proliferation phase to become darker (Schaller et al., 2015; Anwar & Isda, 2020). Particularly in Centella, the concentration of methyl jasmonate, which induces browning, is known to increase as Centella grows (Ganie et al., 2022).

Table 2. The color of the callus generated from leaf and petiole explant in the induction phase and proliferation phase

Treatment	Induction Phase		Proliferation Phase	
	Leaf explant	Petiole explant	Leaf explant	Petiole explant
A	Cream	Cream	Brownish-cream	Brownish-cream
B	Brownish-cream	Whitish-cream	Brownish-cream	Brownish-cream
C	Brownish-cream	Whitish-cream	Brownish-cream	Brownish yellow-cream
D	Greenish-cream	Brownish-cream	Brownish-cream	Brownish yellow-cream
E	Greenish-cream	Brownish-cream	Brownish yellow-cream	Brownish yellow-cream
F	Whitish-cream	Brownish-cream	Brownish yellow-cream	Brownish yellow-cream
G	Whitish-cream	Whitish-cream	Brownish yellow-cream	Brownish yellow-cream



4 mgL^{-1} Dicamba | 4 mgL^{-1} Dicamba + 0.3 mgL^{-1} BAP | 4 mgL^{-1} Dicamba + 0.7 mgL^{-1} BAP | 4 mgL^{-1} Dicamba + 1.1 mgL^{-1} BAP

Figure 3. The appearance of callus generated from leaf explants (top) and petioles (bottom) in the induction phase

The color of the callus can indicate the quality of the callus. Callus, which is embryonic and quickly regenerates to form shoots, is generally green, while yellow callus tends to turn brown and stop growing because it is non-embryonic (Ganie et al., 2022). Several centella investigations found that media enriched with 1 mgL^{-1} BAP and 0.3 mgL^{-1} NAA, as well as 2 mgL^{-1} BAP and 2 mg/L Kn, produced green callus (Anwar and Isda, 2020; Gururajan et al., 2021). Callus cultured on media with NAA and BA is also bright green (Mikhovich & Teteryuk, 2020; Wante and Leung, 2022).

Conclusion

According to the results of the studies, the media containing a combination of 4 mgL^{-1} Dicamba and 1.1 mgL^{-1} BAP developed the most remarkable Centella callus growth, both in the induction and proliferation phases, as demonstrated by the high callus growth rate and callus fresh weight. In addition, the treatment of the Dicamba and BAP combination generated variation in the callus texture and color of the callus, with the addition of BAP tending to enhance the compactness of the callus texture.

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