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Axenic leaf-derived callus induction of *Gynura pseudochina* (L.) DC. using combined 2,4-dichlorophenoxyacetic acid and benzylaminopurine

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Abstract

Background: *Gynura pseudochina* is a promising herbal plant belonging to the Asteraceae family. It contains many secondary metabolite compounds, including saponins, flavonoids, essential oils, and alkaloids. *In vitro* culture offers an efficient method for mass and rapid propagation of *G. pseudochina*. Callus induction is alternatively used to generate *in vitro* shoot proliferation and production of bioactive compounds. Previous study has reported that combined 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzyl Amino Purine (BAP) can effectively induce callus. However, the effect of this combination on *G. pseudochina in vitro* culture is still limited. Therefore, this study investigates the impact of auxin and cytokinin combination (2,4-D and BAP) on *G. pseudochina* callus formation *in vitro*.

Methods: Axenic nodal segments from *G. pseudochina* were used as explants and placed in Murashige and Skoog (MS) medium enriched with different 2,4-D and BAP combination for 60 days of culture. Subsequently, callogenic responses, callus emergence time, callus fresh weight and morphology were observed.

Results: We observed that 2,4-D was vital to induce callus formation. All axenic leaves were able to undergo callogenesis in all concentrations of 2,4-D. The combination of 1.5 mg/L 2,4-D and 1 mg/L BAP (A3B2) was the most effective treatment, producing 1.12 g fresh weight callus and showing rapid induction with callus emergence 12 days after culture. Meanwhile, calli obtained in this study were compact and friable with green, greenish-yellow and brown colors.

Conclusion: Our research demonstrates the effectiveness of combining 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) in inducing callus formation in *Gynura pseudochina* through *in vitro* culture. This study underscores the critical role of 2,4-D in promoting callogenesis, with the optimal callus formation achieved using a combination of 1.5 mg/L 2,4-D and 1 mg/L BAP.

Introduction

Gynura pseudochina is a widespread herbal plant in Southeast Asia, including Indonesia, and belongs to the Asteraceae family [1]. It contains numerous secondary metabolites, including saponins, flavonoids, essential oils, contributing to its diverse biological properties [2]. Like other herbal plants [3], this plant has demonstrated high antioxidant activity, which can scavenge massive production of oxidative radicals in cancer cells [2]. The leaves-derived flavonoid compounds exhibit an anti-inflammatory effect [4]. Moreover, vitamin K contained in the leaf extract of this herbal plant also aids in blood clotting process and regenerates blood cells [5]. Potential use of *G. pseudochina* in medicinal industries consequently increases their demand in the market. Therefore, efficient plant propagation and secondary metabolite production are required.

Stem cutting is a common conventional method of *G. pseudochina* propagation. However, this technique faces diverse problems, including low root formation, high susceptibility to pathogens and genetic non-uniformity [6]. *In vitro* micropropagation of plants could offer a solution to these limitations by producing rapid and large quantities of newly regenerated plants [7]. Furthermore, *in vitro* elicitor applications have been utilized to enhance plant bioactive compounds [8]. However, the type of elicitors and plant genotypes should be taken into consideration. Elicitors, which can be biotic (such as microbial extracts) or abiotic (such as methyl jasmonate, salicylic acid, heavy metals or UV light), are key agents in this process [1]. They mimic stress conditions, triggering the plant's defense mechanisms and stimulating the biosynthetic pathways responsible for secondary metabolite production. Previous work showed that methyl jasmonate enhanced flavonoid content in *G. pseudochina* via direct organogenesis using nodal explants [9].

Callus-derived production of secondary metabolites offers enormous advantages, especially in pharmaceutical industries. Callus consists of undifferentiated parenchymal cells that retain meristematic activity. Suspension cell culture derived from callus has been carried out in many medicinal plants to produce a substantial number of bioactive compounds. Some studies described that calluses might be potential cell factories, accumulating bioactive compounds. It includes a suspension cell in *Saxifraga stolonifera* Meerb. [10], *Celastrus paniculatus* [11], *Salvia miltiorrhiza* [12], *Lepidium sativum* [13]. Therefore, callus induction step in plant tissue culture, especially for metabolite production is substantial.

Callus developed through leaf, stem, and tuber explants [14]. However, appropriate culture media and plant growth regulators should be taken into account. Recently, developing calli from axenic leaf tissue is attracting many researchers due to its efficiency. Axenic leaves ensure a contamination-free environment, preventing microbial interference that could hinder callus induction and transformation processes. Additionally, callus derived from sterile explants tends to be more uniform and actively dividing [15]. The development of callus in plant cell culture involves auxins and cytokinins, in a particular dose, to promote cell division and differentiation [15]. The 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) are commonly used for initiating cell proliferation and callus formation. They might synergistically enhance callus induction by balancing cellular differentiation and proliferation. Some reports showed that 2.4-D and BAP combination effectively promotes callus formation in some medicinal plants, including *Amaranthus gangeticus* [16], *Gynura procumbens* [17], *Pogostemon cablin* [18], *Ficus religiosa* [19] *in vitro* culture.

The ratio of auxins to cytokinins is pivotal. The higher auxin-to-cytokinin ratio typically favors callus formation and root induction, while a lower ratio promotes shoot development. This balance is critical as it influences the type of callus formed—friable, compact, or embryogenic—which impacts subsequent plant regeneration processes [2]. However, the previous studies demonstrated that callus induction is also species and dose-dependent [20]. Nevertheless, nothing is known about the study that has been done on *G. pseudochina* cultured *in vitro*. Therefore, this study is conducted to ascertain the optimal dose of 2,4-D and BAP in callus induction of *G. pseudochina in vitro*.

Methods

Explant preparation, callus induction and culture conditions

G. pseudochina used in this study were obtained from the *in vitro* collection of Plant Bioscience and Technology Laboratory, Department of Biology, ITS Surabaya-Indonesia. Axenic immature leaves of approximately 1 cm^2 were excised from sterile plantlets and prepared to be subsequently planted in the MS medium with 2,4-D and BAP. The combination of 2,4-D and BAP was represented as 2,4-D (A) : BAP (B) (mg/L); A0B0 (0:0); A0B1 (0:0.5); A0B2 (0:1); A0B3 (0:1.5); A1B0 (0.5:0); A1B1 (0.5:0.5); A1B2 (0.5:1); A1B3 (0.5:1.5); A2B0 (1:0); A2B1 (1:0.5); A2B2 (1:1); A2B3 (1:1.5); A3B0 (1.5:0); A3B1 (1.5:0.5); A3B2 (1.5:1); A3B3 (1.5:1.5).

The MS medium was made using the procedure outlined by [15]. The leaf explants (abaxial part) were placed in contact with the media. We employed sixteen treatments, and each was triplicate. The explants were

cultured for 60 days at 23 ± 1 °C in a growing chamber. The photoperiod of the chamber was set at 24 h dark condition for callus culture development [21].

Growth parameters and data analysis

In this study, we measured the callus emergence time. Meanwhile, callogenic frequency was also measured according to the following formula:

$$Callogenic\ frequency = \frac{number\ of\ axenic\ explants\ forming\ callus}{total\ number\ of\ axenic\ explants}\ x\ 100\%$$

Visual observation considered in this study consisted of color and texture. After 60 days of cultivation, the callus's fresh weight was also measured. The average of fresh weight callus was statistically analyzed using two-way ANOVA and followed by Tukey post-hoc test.

Results

The formation of callus and its emergence time in *G. pseudochina* in vitro culture

In this study, axenic leaves of the *G. pseudochina* were inoculated in the MS medium containing several combinations of 2,4-D and BAP. The presence of auxin-type PGR used in this study was vital for callus induction. Callogenesis occurred only in treatments containing 2,4-D, whereas all media lacking 2,4-D produced no callus. Among effective treatments, callus emergence times varied, but callogenic frequency in all responsive treatments was 100%. (Figure 1).

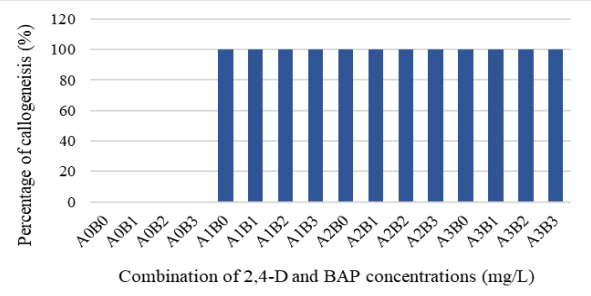

Figure 1: Callogenic frequency of *G. pseudochina* at combined concentrations of 2,4-D and BAP after 60 days of culture.

The observation of this study revealed that the callus emergence time was different in each treatment (Figure 2). The time required for callus formation varied from 12 to 15 days, with certain concentration combinations showing no response in callus formation (Figure 2). The shortest time (12 days after culture) for callus appearance occurred in A1B1 and A3B2 treatments (Figure 2). Meanwhile, callus formation at 14 days after culture was recorded in several treatments, particularly A1B3, A2B0, and A3B1. Conversely, the longest time for callus emergence was observed in A1B0; A1B2; and A2B1, averaging at 15 days after culture.

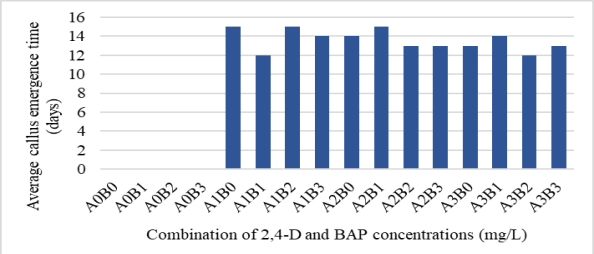

Figure 2: Callus emergence time of *G. pseudochina* at combined level of 2,4-D (A) and BAP (B).

Combined auxin and cytokinin (2,4-D and BAP) affect fresh weight of *G. pseudochina* callus.

Our findings indicated that combinations of the PGRs used affected the fresh weight of leaf-derived callus in *G. pseudochina in vitro* culture ($P \leqslant 0.05$). Varying concentrations of these PGRs led to varied fresh weights of the callus. We noted that the callus resulted from A1B1, A3B2 and A3B3 treatments demonstrated significantly different callus fresh weight compared to other treatments (Figure 3). A significant callus fresh weight came from A3B2 (1.12 g). On the other hand, the callus obtained from A1B0 treatment demonstrated the lowest fresh weight at 0.49 g. Our observation also showed that a higher amount of 2,4-D compared to BAP might result in higher callus fresh weight. However, low level of 2,4-D concentration with zero BAP resulted in minimum callus fresh weight. This indicates that 2,4-D is important for callus formation in *G. pseudochina in vitro* culture.

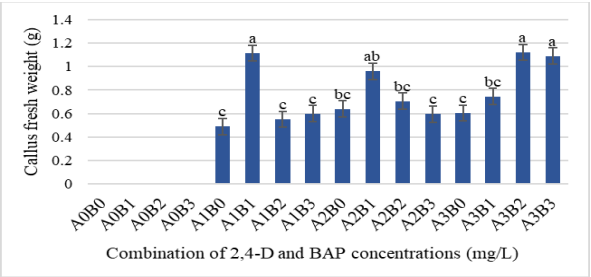

Figure 3: Impact of 2,4-D and BAP treatment on fresh weight of *G. pseudochina in vitro* culture after 60 days of culture. Notes: Numbers followed by the same letter in the same column are not significantly different based on the ANOVA test followed by the Tukey test (α = 0.05).

Variation of callus morphology *in G. pseudochina* micropropagation resulted from 2,4-D and BAP combination.

Leaf explants inoculated in the culture medium demonstrated distinct callus morphology and characteristics (Table 1). Two different callus textures were observed in this study: friable and compact. Friable callus was characterized by its delicate texture and watery consistency, is easily disintegrated. Meanwhile, compact callus appears as a dense mass, typically bright and white in color, with a hard texture

and low water content, making it resistant to disintegration [39]. Out of twelve treatments resulting in callus formation, 58.3% formed friable callus and the rest showed compact callus (Table 1).

PGRs concentrations (mg/L)		Callus texture	Callus color
2,4-D	BAP		
0.5	0	Compact	Greenish yellow
0.5	0.5	Friable	Green
0.5	1	Friable	Greenish yellow
0.5	1.5	Friable	Brownish yellow
1	0	Compact	Brown
1	0.5	Friable	Greenish yellow
1	1	Compact	Greenish yellow
1	1.5	Compact	Greenish yellow
1.5	0	Compact	Brown
1.5	0.5	Friable	Greenish yellow
1.5	1	Friable	Greenish yellow
1.5	1.5	Friable	Green

Table 1: Morphological characteristics of *G. pseudochina* callus under various combinations of 2,4-D and BAP concentrations.

Four different colors of calli were produced in this study. It includes greenish yellow, green, brownish yellow, and brown (Figure 4). About 58.3% of calli showed greenish yellow color, 25% of the callus demonstrated brown to brownish-yellow and a few portions (16.7%) of the calli possessed green color.

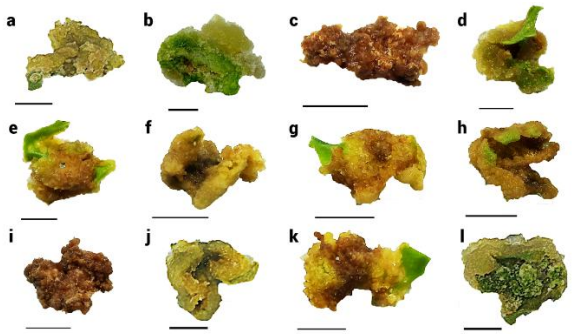

Figure 4: The morphology of *G. pseudochina* callus, resulted from combined 2,4-D and BAP after 60 days of culture. Scale bar: 1 cm.

Our reports suggest that the 2,4-D and BAP affect callus color in a dose-dependent and species-dependent. In addition, this study shows that combining specific amounts of 2,4-D and BAP enhances the formation of *G. pseudochina* leaf-derived callus *in vitro*. The optimal pairing of 2,4-D and BAP for inducing callus *in vitro* was found to be 2,4-D at a concentration of 1.5 mg/L combined with BAP at 1 mg/L. This combination resulted in calluses appearing as early as 12 days, achieving a 100% rate of callogenesis, with an average wet weight of 1.12 g. The callus displayed a green color and had a friable texture.

Discussion

Callus induction in plant tissue culture involves the dedifferentiation of plant cells from explants, typically leaves or stems, into an unorganized mass of cells under the influence of plant growth regulators (PGRs) [22]. In this study, we have successfully induced callus formation from axenic leaves of *Gynura pseudochina* using 2,4-D and BAP. Callus, a cluster of cells with irregular shapes, was initially formed from the wound side of the explants following the PGRs application [23]. Callus induction using the same PGRs have been also reported in previous studies such as *Astragalus membranaceus in vitro* culture [24], *Saxifraga stolonifera* [10], *Pogostemon cablin* [23], and *Axonopus compressus* [26]. Rashmi's research [27] also showed that *Nerium odorum* leaf explants treated with varying combined concentrations of 2,4-D and BAP (0.5 to 10 mg/L) led to a successful callus formation, whereas treatments without either auxin or cytokinin did not form callus. This highlights the profound effect of auxin and cytokinin on callus development. Consistent with these findings, Karuppiah and Hing [26] reported that using either auxin or cytokinin alone for callus initiation in *Talinum paniculatum* plants resulted in 0% callus growth. The 2,4-D regulates callus establishment due to its robust stimulation of cell differentiation. It encourages undifferentiated cells to start developing into specialized cell types, a critical step in the formation of callus tissue. In addition, this auxin-type PGR also suppress plant organogenesis. The 2,4-D directs the cells toward callus formation instead of organ development. Once callus tissue begins to form, 2,4-D also supports its continued growth and proliferation. Finally, 2,4-D can easily penetrate plant tissues, especially through cut surfaces or wounds. This ease of diffusion allows it to reach target cells efficiently, where it can exert its effects on cell differentiation and callus initiation [27].

The appropriate level of PGRs plays a pivotal role in eliciting an optimal response in cell division for callus formation. Balanced concentrations of auxin and cytokinin during callus induction encourage cell enlargement, leading to callus development. Elevating 2,4-D level might accelerate callus induction since 2,4-D easily permeates plant tissues through incisions, aiding endogenous auxin in stimulating cell division [28]. The provision of suitable growth regulators, properly initiate plant cells, resulting in callus formation. The successful induction of callus also hinges on the type of explants utilized. Combining 2,4-D and BAP proves to be an effective combination of growth regulators for generating callus from leaf explants, as they play roles in cell division. Leaf explants are meristematic and actively dividing, making them responsive to exogenous hormones like the auxin (2,4-D) and cytokinin (BAP) groups, which promote cell proliferation and organize cell structures poised for differentiation, as discused in previous study [29].

Our study also revealed that the emergence time of the callus varied according to the combination of 2,4-D and BAP concentrations. Interestingly, omitting 2,4-D from the medium completely prevented callus formation. This finding also aligns with [29], who demonstrated that higher level of 2,4-D compared to BAP was responsible for callus development on *Achyranthes aspera in vitro* culture within 9-11 days after culture. High 2,4-D level compared to BAP was reported to successfully induced callus formation in 11 days after culture in patchouli (*Pogostemon cablin*) tissue culture [1]. Moreover, it has been reported that equivalently low level of 2,4-D and BAP yielded a rapid callus formation, taking only 9 days after culture. Another study on patchouli *in vitro* culture also demonstrated that 1 mg/L 2,4-D alone was able to generate callus in 15 days after culture, whereas a combination of 2,4-D and BAP at 1.5 mg/L and 1 mg/L, respectively resulted in callus formation within 12 days. Callus fresh weight is a key indicator of cell proliferation and biomass accumulation during tissue culture. Higher callus fresh weight indicates significant plant cell growth and cell division [31]. Endogenous and exogenous applications of auxin and cytokinin have been reported to trigger callus initiation [32]. Numerous research investigations have indicated that the combination of cytokinins and auxins produces better results compared to using either cytokinins or auxins alone [33].Other reports also showed that 2,4-D possess a major role in callus formation [34]. However, the level of auxin and cytokinin are crucial in initiating callus formation. An increase of 2,4-D level resulted in enhance callus responses [35]. Nevertheless, some plant species might respond differentially [36]. For instance, in *Artemisia annua* tissue culture, higher levels of 2,4-D reduced the callogenesis response of the explant [37]. The combination of auxin and cytokinin also exert significant influence on callus, such as texture, and color. Higher concentrations of auxins often result in the formation of compact and hard callus structures, whereas lower concentrations tend to produce friable and soft callus. On the other hand, cytokinins foster cellular division and proliferation, leading to the development of larger callus establishment, with higher concentrations generally yielding compact callus [38]. Lee et al. [40] demonstrated that 2,4-D is highly potent PGR belonging to the auxin group, renowned for its efficacy in stimulating the formation of friable callus cells. This type of callus is preferentially used in cell suspension culture for multipurposes, including secondary metabolite productions [41, 42] and indirect organogenesis [43]. In addition, friable-textured callus has rapid growth with a high frequency of division [44]. Induction of friable callus by combined 2,4-D and BAP also reported in *Cyamopsis tetragonoloba* [45]. In this study, a relatively balanced concentration of 2,4-D and BAP at 1 mg/L produced compact callus. Similar results were also found in *Calotropis gigantea* [46]. High percentage of brownish to yellow-colored callus might indicate high level of phenol compound in the callus [47]. Other reports also suggest that 2,4-D administered in the culture medium interferes with chlorophyll biosynthesis, which then resulted in yellowish-colored callus in *Ficus religiosa* [17, 48]. Meanwhile, green callus was produced in *Jatropha curcas* after being treated with a slightly higher concentration of 2,4-D than BAP [49,50]. However, different results showed in *Ananas comosus in vitro* culture. The callus produced in this horticultural plant showed green and brown callus, in higher level of 2,4-D than BAP [51]. Auxins like 2,4-dichlorophenoxyacetic acid (2,4-D) play a crucial role in initiating callus formation by promoting cell division, while cytokinins such as benzylaminopurine (BAP) support proliferation and maintenance of callus growth. The balance between auxins and cytokinins is essential to determine callus characteristics, including texture, color, and regeneration potential. Moreover, combining PGRs with certain abiotic stresses can also be applied to enhance plant growth and metabolite production, even though different plant genotypes might potentially result in distinct responses [52,53]. Finally, optimizing the culture medium composition and growth conditions ensures successful callus induction, which serves as a foundation for further *in vitro* applications, such as plant regeneration and secondary metabolite production.

In conclusion, our study confirms the efficacy of combining 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) in callus induction of the *Gynura pseudochina* in vitro culture. This present study highlights the essential function of 2,4-D for callogenesis, with a combination of 1.5 mg/L 2,4-D and 1 mg/L BAP resulting in optimal callus formation. The calluses exhibited favorable characteristics, including early emergence and substantial fresh weight, with a variety of colors indicating different stages of development. Our study opens phytochemical perspectives for utilizing *G. pseudochina* callus as metabolic factory to produce the economically important metabolites. A detailed biochemical pathways elucidation of the secondary metabolites in *G. pseudochina* calluses should be performed to provide a comprehensive understanding of the phytochemical study. Additionally, investigation of the long-term stability and genetic fidelity of the calluses to ensure consistent quality in mass propagation also important to be conducted in the future. Finally, integrating molecular techniques could provide deeper

insights into the genetic factors influencing callus formation and secondary metabolite production, paving the way for advanced biotechnological applications.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Nurul Jadid (N.J) designed the study, supervised the research process, and contributed to the writing, reviewing, and editing of the manuscript. Prameswari Ayunda Safitri (P.A.S) conducted the investigation, performed the formal analysis, and contributed to the original draft preparation. Anisa Esti Rahayu (A.E.R) supported the formal analysis and prepared the visualizations. Ira Puspitaningtyas (I.P) participated in the investigation and formal analysis. Muhammad Rifqi Nur Ramadani (M.R.N.R) contributed to visualization and formal analysis.

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