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Research

Effect of Auxins on Growth Enhancement of Cell Suspension Culture of Tongkat Ali Hitam (*Polyalthia bullata*)

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ABSTRACT

Polyalthia bullata, a Southeast Asian plant, is valued for its bioactive compounds with pharmaceutical potential. To prevent overharvesting and extinction, cell suspension culture offers a sustainable method for the mass production of these compounds. Despite its effectiveness, no studies on *Polyalthia bullata* cell suspension culture have been established. Therefore, this study aimed to establish the culture by evaluating growth and biomass production. To achieve the objective, leaf derived callus of *Polyalthia bullata* was multiplied on Murashige and Skoog (MS) + 30 µM dicamba medium. Subsequently, cell suspension initiation and multiplication were carried out using half-strength MS basal medium (½ MSO) supplemented with 5, 15, 25, and 30 µM of 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA), respectively. In this study, suspension cells in the ½ MSO recorded the highest increment in fresh (4.455 ± 1.170 g FW) and dry weight (0.220 ± 0.033 g DW**)** but produced dark brown cells. Meanwhile, cells grown on ½ MS medium supplemented with 30 µM NAA recorded the highest increase in fresh weight (3.472 ± 0.694 g FW) and dry weight (0.190 ± 0.012 g DW), displaying a light yellowish-brown cell. Although the ½ MSO yielded the highest biomass, the cell suspension cultures supplemented with 30 µM NAA showed promising results, achieving higher biomass compared to other auxin treatments and exhibiting a light yellowish-brown cell. This suggests that 30 µM NAA is a more efficient auxin utilization in reducing the occurrence of dark brown cells. In conclusion, optimizing auxin concentrations is crucial for high-quality *Polyalthia bullata* cell suspension culture. This study can provide insight into sustainable cultivation practices for the plant, serving as a potential bio-factory for mass-producing bioactive compounds.

Key words: Auxins, cell growth, cell suspension culture, dry weight, fresh weight, *Polyalthia bullata*

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INTRODUCTION

Plant cell and tissue culture have become important tools in the biotechnology field, facilitating the production of active compounds and enabling research into plant-based medicines (Farjaminezhad *et al*., 2013). Various factors, such as plant growth regulators (PGRs), explant age, explant type, and culture media, contribute to the outcomes of plant tissue culture (Zakaria *et al*., 2011).

Cell suspension culture is one method that has been widely used in plant tissue culture. It is a technique in which plant cells grow or aggregate in a liquid culture medium under constant agitation (Motolinía-Alcántara *et al*., 2021). This technique is preferred for its cost-effectiveness and its capacity to produce compounds that are identical to those present in their parent cells (Gonçalves *et al*., 2018; Isah *et al*., 2018). Furthermore, previous studies from Özyiğit *et al*. (2023) have revealed that cell suspension cultures are suitable for large-scale production of secondary metabolites in plants due to their availability of carbon and nutrient sources supporting culture growth, resulting in consistent secondary metabolites production (Moscatiello *et al*., 2013).

Auxins are known to promote cell growth in plant tissue culture. Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), and α-Naphthaleneacetic acid (NAA) are among the auxins

that have been utilized in cell suspension studies. The IAA is the naturally occurring auxin while NAA is the auxin analog closest to natural IAA that displays a similar function but is more stable (Pasternak & Steinmacher, 2024). Meanwhile, IBA is the natural auxin that showed an identical structure as IAA in which their contribution to overall auxin level varies among the plant species (Damodaran & Strader, 2019). It has been reported that auxin plays a vital role in controlling plant growth and development via the promotion of cell division (proliferation), growth (expansion, elongation), and differentiation (Majda & Robert, 2018). The study on the effect of the IAA, IBA, and NAA on the growth of *Polyalthia bullata* callus has been reported by Kamarul Zaman *et al*. (2020), which shows different responses of these auxins on callus growth in solid media. However, the effect of these auxins on the cell suspension culture of *Polyalthia bullata* is yet to be studied.

Several studies have been conducted to determine the effect of different auxins on cell suspension culture. Pinto (2023) reported that the use of a half-strength MS liquid medium supplemented with 30 µM dicamba in *Polyalthia bullata* cell suspension culture was the most effective in promoting cell growth, producing the highest weight of cells (3.898 ± 0.092 g FW and 0.091 ± 0.005 g DW). Meanwhile, 15 µM picloram produced the highest alkaloid content, suggesting its potential for secondary metabolite production in *Polyalthia bullata* cells. In addition, optimum biomass accumulation was found in the cell suspension culture of *Withania somnifera* (L.) Dunal grown in MS supplemented with 9.05 µM 2,4-D (Nagella & Murthy, 2010). Tan *et al*. (2010) reported that the use of 2 µM 2,4-D produced the maximum flavonoid yield in the suspension culture of *Centella asiatica* L. Urban. Moreover, studies by Krishnan *et al*. (2019) also showed that the use of a half-strength MS medium supplemented with 10.74 µM NAA resulted in higher production of camptothecin (CPT) in its suspension culture. These studies emphasize the importance of auxins in enhancing cell growth and biomass accumulation in cell suspension cultures, making the technique promising for enhancing the growth and biomass production of plant cells, including *Polyalthia bullata.*

Polyalthia bullata is a tropical plant native to Southeast Asia, specifically found in many countries such as Malaysia, Indonesia, and Thailand. It belongs to the *Annonaceae* family, known for its diverse plant species with medicinal properties (Harahap *et al*., 2022). Within this family, the genus *Polyalthia*, to which *Polyalthia bullata* belongs, has attracted attention due to its bioactive compounds, including alkaloids, flavonoids, and triterpenoids (Paarakh & Khosa, 2009; Jothy *et al*., 2013; Yao *et al*., 2019). These studies reveal the diverse medicinal activities of these compounds, ranging from anticancer and anti-inflammatory to antibacterial properties, offering various health benefits (Dias *et al*., 2012; Perviz *et al*., 2016; Wang *et al*., 2017; Wang *et al*., 2019). In addition, the therapeutic potential of secondary metabolites from *Polyalthia* species is further highlighted by Chen *et al*. (2021), especially in the context of chemoprevention. These findings emphasize the special medicinal qualities of *Polyalthia bullata*, making it an attractive target for additional investigation using advanced plant cell culture techniques. Therefore, we aimed to determine the growth and biomass production of *Polyalthia bullata* cells in cell suspension culture containing different types of auxins.

MATERIALS AND METHODS

Plant material

The experiment was conducted in the Plant Biochemistry and Biotechnology Laboratory at the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor. The callus was derived from the leaf of *Polyalthia bullata* with identification number PID 170820-13, provided by the Forest Research Institute Malaysia (FRIM).

Multiplication of *Polyalthia bullata* callus

For the multiplication process, a solidified MS medium (Murashige & Skoog, 1962) supplemented with 30 µM dicamba was used due to its optimal growth, which was observed as early as week 3, resulting in higher callus biomass with friable morphology (Kamarul Zaman *et al*., 2020). The callus of *Polyalthia bullata* was multiplied using the MS media containing 30 µM dicamba (Kamarul Zaman *et al*., 2020). Five clumps of calli, each weighing 100 mg, were cultured on fresh media of MS supplemented with 30 µM dicamba. The cultures were then incubated under dark conditions at 24 ± 2°C for 3 weeks.

Multiplication of *Polyalthia bullata* cell suspension culture

The multiplication of cell suspension culture was carried out by culturing 1g of 3-week-old chopped friable calli in a 100 mL conical flask containing 25 mL of ½ MSO liquid medium (control) and ½ MSO containing different types of auxins (NAA, IAA & IBA) at concentrations of 5, 15, 25, and 30 µM,

respectively. The conical flasks were wrapped with aluminum foil and placed on an orbital shaker at 120 rpm at 24 ± 2°C in the dark for 18 days. The friable callus was obtained from the multiplication of callus in MS medium supplemented with 30 µM dicamba, as mentioned in the previous section. The 18-day observation period was selected based on a previous study by Pinto (2023), which showed that cell growth had already reached the stationary phase after 18 days of incubation.

Preparation of cell suspension culture growth curve

The growth curves of cell suspension cultures were determined by measuring the fresh and dry weight of cells (Pinto, 2023). For fresh weight, the suspension culture was harvested by filtering the cells with filter paper (Whatman No. 1). A clean and dry container was weighed, and the initial weight was recorded. The filtered cells were then transferred to the pre-weighed container by scrapping the cells using a spatula, and the final weight was measured. The fresh weight was determined by weighing the callus. For the determination of dry weight, the cells were dried in an oven at 50°C for at least 48 hr or until a constant weight was reached. The dry weight of the cells was calculated using Equation 1. Recorded data for both fresh and dry weight were utilized to construct the growth curve. The analysis was run in triplicate, and data were collected at 3-day intervals for 18 days.

Dry weight (g) = (Final weight of the container with cells) - (Initial weight of the container) - Equation 1

Statistical analysis

The data for fresh and dry weight were subjected to statistical analysis using a two-way analysis of variance (ANOVA) and a completely randomized design (CRD) using R Statistical Software (RStudio). The significant differences (p<0.05) between group means were further evaluated using Tukey's Honest Significant Difference (Tukey's HSD) test. This post-hoc test performed pairwise comparisons to identify specific treatment groups that exhibited statistically significant differences. Then, the bar graph was plotted using Microsoft Excel (Microsoft 365).

RESULTS AND DISCUSSION

Establishment of cell suspension culture

Based on Figures 1, 2, and 3, ½ MSO (control) exhibited the highest increase in the weight of cell suspension culture on day 18, with a fresh weight of 4.455 ± 1.170 g FW (Figure 1a, 2a, 3a) and dry weight of 0.220 ± 0.033 g DW (Figure 1b, 2b, 3b). The highest biomass production in the ½ MSO might be due to an optimal balance of nutrients that support the growth of *Polyalthia bullata* cells as described by Murthy *et al*. (2014). A previous study by Pinto (2023) aligns with the present study, indicating that the ½ MSO basal medium was the most effective for the multiplication of *Polyalthia bullata* cells compared to full-strength MS and woody plant medium WPM basal media. However, current findings and those of Pinto (2023) differ from a study conducted by Goyal *et al*. (2023), where they observed liquid MSO was unable to support cell division in *Cenchrus ciliaris* L. cell suspension cultures due to browning contamination.

Among the NAA treatment, as shown in Figure 1(a) and Figure 1(b), ½ MS with 30 µM NAA induced the growth in cell suspension for both fresh and dry weight (3.472 ± 0.694 g FW & 0.190 ± 0.012 g DW) on day 18. Meanwhile, ½ MS with 15 µM NAA showed the least increase in weight (2.009 ± 0.094 g FW & 0.129 ± 0.007 g DW) on day 18. Based on the results obtained, there were no significant differences in the growth of the cell suspension culture in the ½ MSO media. This observation may be due to the minimal nutrient composition of ½ MSO, which serves as a control medium to evaluate the effects of various growth regulators. The lack of significant growth differences suggests that any variations observed in other treatments are primarily attributable to the influence of the added auxins rather than the basal medium itself.

The results observed in the cell suspension culture treated with ½ MS supplemented with 30 µM NAA showed a positive effect on the cell growth and biomass production of *Polyalthia bullata* cells. The optimal concentration of 30 µM NAA might have promoted higher rates of cell division, resulting in the highest cell growth and biomass within the suspension culture. This led to a biomass increase of 30.16% (5 µM NAA), 42.14% (15 µM NAA), and 18.98% (25 µM NAA) on day 18 compared to other NAA concentrations. In the callus culture of *Polyalthia bullata*, the study by Kamarul Zaman *et al*. (2020) found that a concentration of 30 µM NAA showed optimal growth, which aligns with the findings in its suspension culture present in this study. The results obtained by Mamdouh and Smetanska (2022) showed that the suspension culture of *Lycium schweinfurthii* supplemented with 10.74 µM NAA was the

best in terms of higher biomass, phenolics, and flavonoids accumulation. These findings contradicted the study by Farjaminezhad and Garoosi (2021), where the fresh cell weight of the suspension culture of *Azadirachta indica* decreased with an increase in NAA concentration. A high concentration of NAA could disrupt auxin signaling pathways, leading to reduced cell division or prioritized cell wall strengthening over biomass accumulation (Wei *et al*., 2022). This suggests that the effect of NAA may vary depending on plant variety, possibly due to differences in auxin sensitivity, metabolic rates, or hormonal balance across species. In comparison, the 15 µM NAA showed the lowest increase in both fresh and dry weight. *Polyalthia bullata* cells may not be as responsive to NAA at this concentration compared to other concentrations. This variability in response could be attributed to the inherent differences in sensitivity and reactions to changes in auxin levels among different species and cell types (Leyser, 2017). Moreover, the observed growth pattern may be specific to the stage of the cell culture. Optimal cell growth is typically observed during the exponential phase of the growth curve, where maximum cellular division and the highest biomass accumulation occur. This is followed by the stationary phase, during which no further increase in culture weight is observed, and finally, the decline phase, where cellular death occurs (Kamarul Zaman *et al*., 2020). As the cultures were observed for 18 days, the control group might be at a stage exhibiting higher growth rates compared to the NAA treatments. Apart from that, the effects of NAA on cell growth could be time-dependent. Hence, the selected time points for cell growth observations might capture stages where the control group exceeds the NAA treatment groups.

In the IAA treatment shown in Figure 2(a), ½ MS with 25 µM IAA showed the highest increase in fresh weight of cell suspension cultures (2.468 ± 0.071 g FW) on day 18. Meanwhile, the least increment in weight was recorded in ½ MS with 5 µM IAA (1.855 ± 0.455 g FW) on day 18. The data obtained were significantly different from the growth of the cell suspension culture in the ½ MSO media. For dry weight, as shown in Figure 2(b), the highest increase in biomass was recorded in ½ MS with 15 µM IAA (0.218 ± 0.020 g DW) on day 18. On the other hand, the least increase in weight was observed in ½ MS with 5 µM IAA (0.173 ± 0.034 g DW) on day 18. Based on the statistical analysis, the recorded data were not found to be statistically significant when compared to the ½ MSO group.

The results of the IAA treatment further highlight the relationship between PGR concentration and the growth of *Polyalthia bullata* cells. Based on the findings, 25 µM IAA promoted a steady increase in both fresh and dry weight of the *Polyalthia bullata* in cell suspension culture, indicating efficient resource utilization and optimal auxin signaling. However, previous studies have shown that higher IAA concentrations can trigger the production of reactive oxygen species (ROS) (Nguyen *et al*., 2016). This ROS can potentially damage cells and tissues, leading to growth inhibition and a reduction in biomass production (Mansoor *et al*., 2022).

In contrast to higher concentrations, lower concentrations of IAA are more effective in promoting cell growth (Khalid & Aftab, 2020). A study by Kamarul Zaman *et al*. (2020) reported that a lower concentration of IAA (7 µM) enhanced growth in *Polyalthia bullata* callus, while higher concentrations of IAA (14 µM) inhibited growth. These findings differ from the results obtained in this study, as 5 µM IAA showed lower cell growth compared to other concentrations.

Notably, 15 µM IAA recorded the highest increase in dry weight (0.128 ± 0.02 g DW) on day 18, which differed from its fresh weight (Figure 2b). The steady increase in dry weight might reflect the plant's response to strengthen its structure or accumulate secondary metabolites in response to the stress induced by high IAA (Jing *et al*., 2023). This could lead to an increase in dry weight due to the accumulation of secondary metabolites (Özyiğit *et al*., 2023). Findings from Shmarova *et al*. (2019) also found that high concentrations of IAA (7.38 µM) increased the dry weight as well as the phenolics and flavonoids content of the cell suspension cultures of *Lactuca sativa* but resulted in a decrease in the fresh weight and their growth index.

For IBA treatment, as shown in Figure 3(a) and Figure 3(b), ½ MS with 15 µM IBA recorded the highest increase in both fresh (2.862 ± 0.375 g FW) and dry weight (0.220 ± 0.018 g DW) on day 18. Furthermore, the cell suspension in a higher concentration of IBA, at 30 µM, exhibited the least increase in fresh and dry weight on day 18 (2.313 ± 0.713 g FW and 0.175 ± 0.045 g DW). Regarding the fresh weight, the data obtained were statistically significant when compared to the ½ MSO group. However, there were no significant differences in the dry weight of the *Polyalthia bullata* cells when compared to the ½ MSO group.

Notably, 15 µM IBA promoted the most consistent growth, suggesting efficient utilization of this auxin at this concentration, leading to enhanced cell division, and increasing biomass production. This is supported by the steady increase in fresh and dry weight, aligning with ½ MSO.

In contrast, the 30 µM IBA treatment exhibited fluctuations in fresh and dry weight, potentially

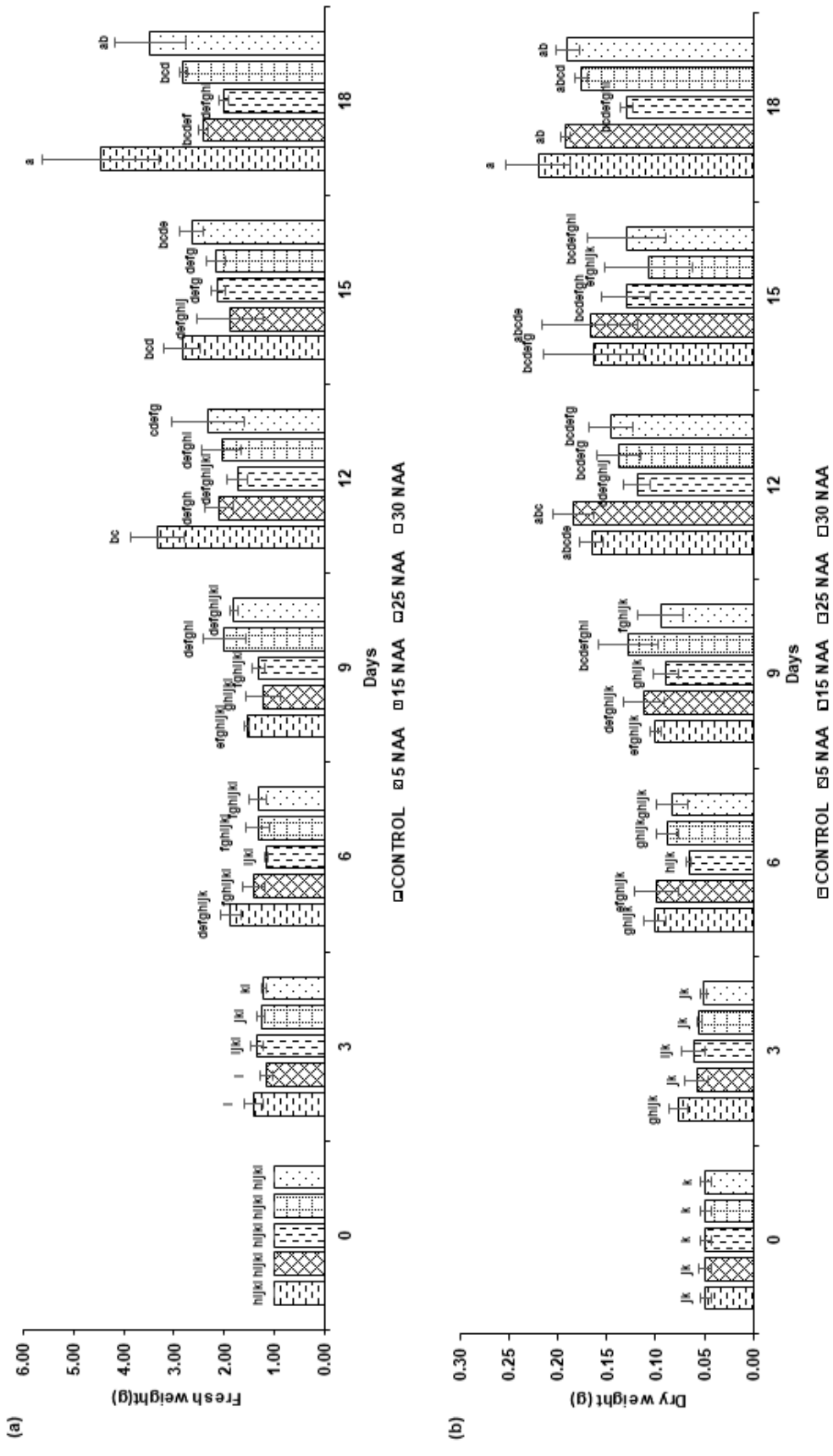


Fig. 1. The growth of *Polyalthia bullata* cell suspensions in ½ MS media with different concentrations of NAA for 18 days. (a) The graph of the fresh weight of *Polyalthia bullata* cells in ½ MSO (Control) and ½ MS media with 5 µM, 15 µM, 25 µM, and 30 µM NAA. (b) The graph of the dry weight of *Polyalthia bullata* cells in ½ MSO (Control) and ½ MS media with 5 µM, 15 µM, 25 µM, and 30 µM NAA. Different letters indicate statistically significant differences (p<0.05) between treatments tested using Tukey's HSD test.

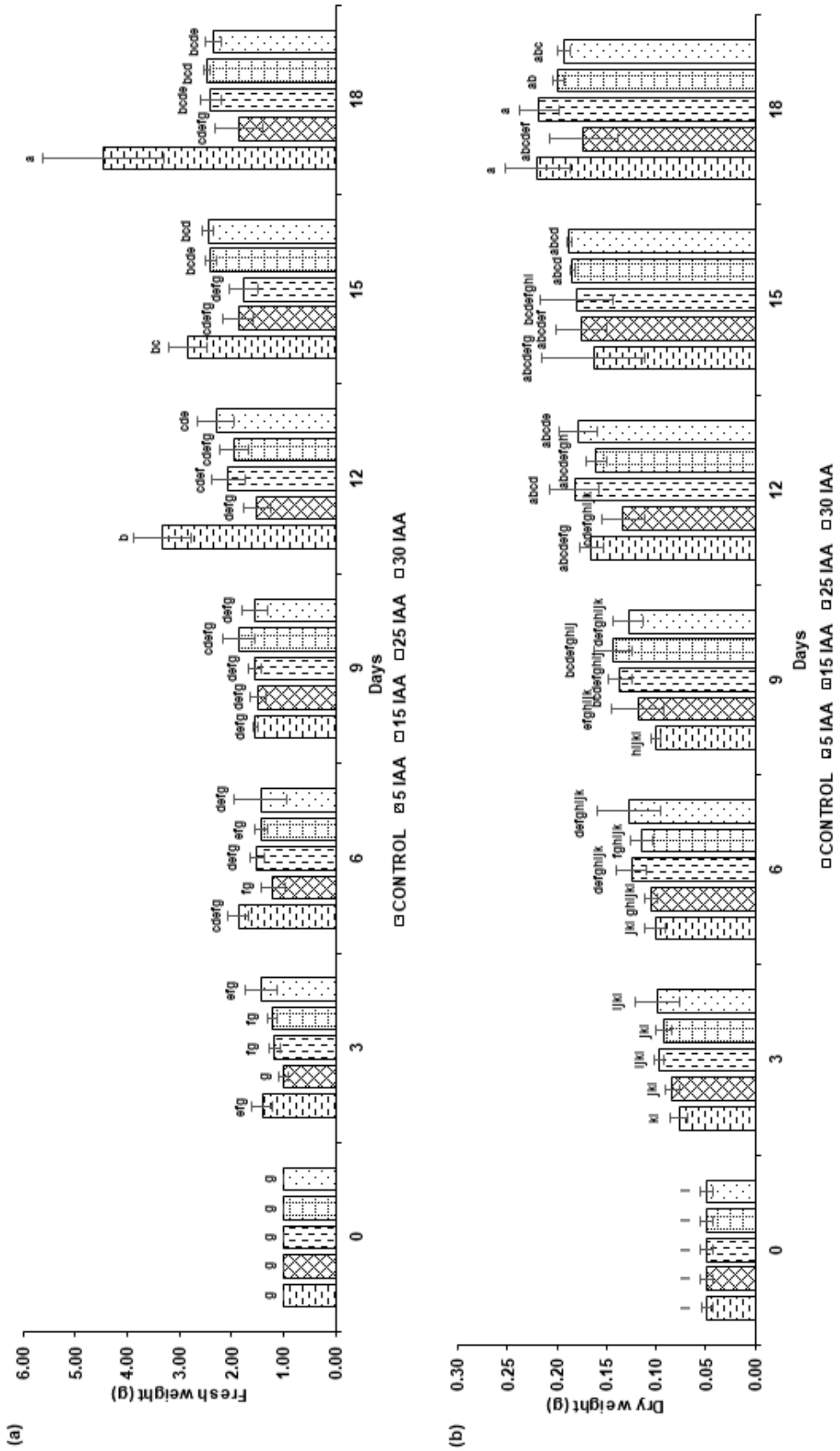


Fig. 2. The growth of *Polyalthia bullata* cell suspensions in ½ MS media with different concentrations of IAA for 18 days. (a) The graph of the fresh weight of *Polyalthia bullata* cells in ½ MSO (Control) and ½ MS media with 5 µM, 15 µM, 25 µM, and 30 µM IAA. (b) The graph of the dry weight of *Polyalthia bullata* cells in ½ MSO (Control) and ½ MS media with 5 µM, 15 µM, 25 µM, and 30 µM IAA. Different letters indicate statistically significant differences ($p<0.05$) between treatments tested using Tukey's HSD test.

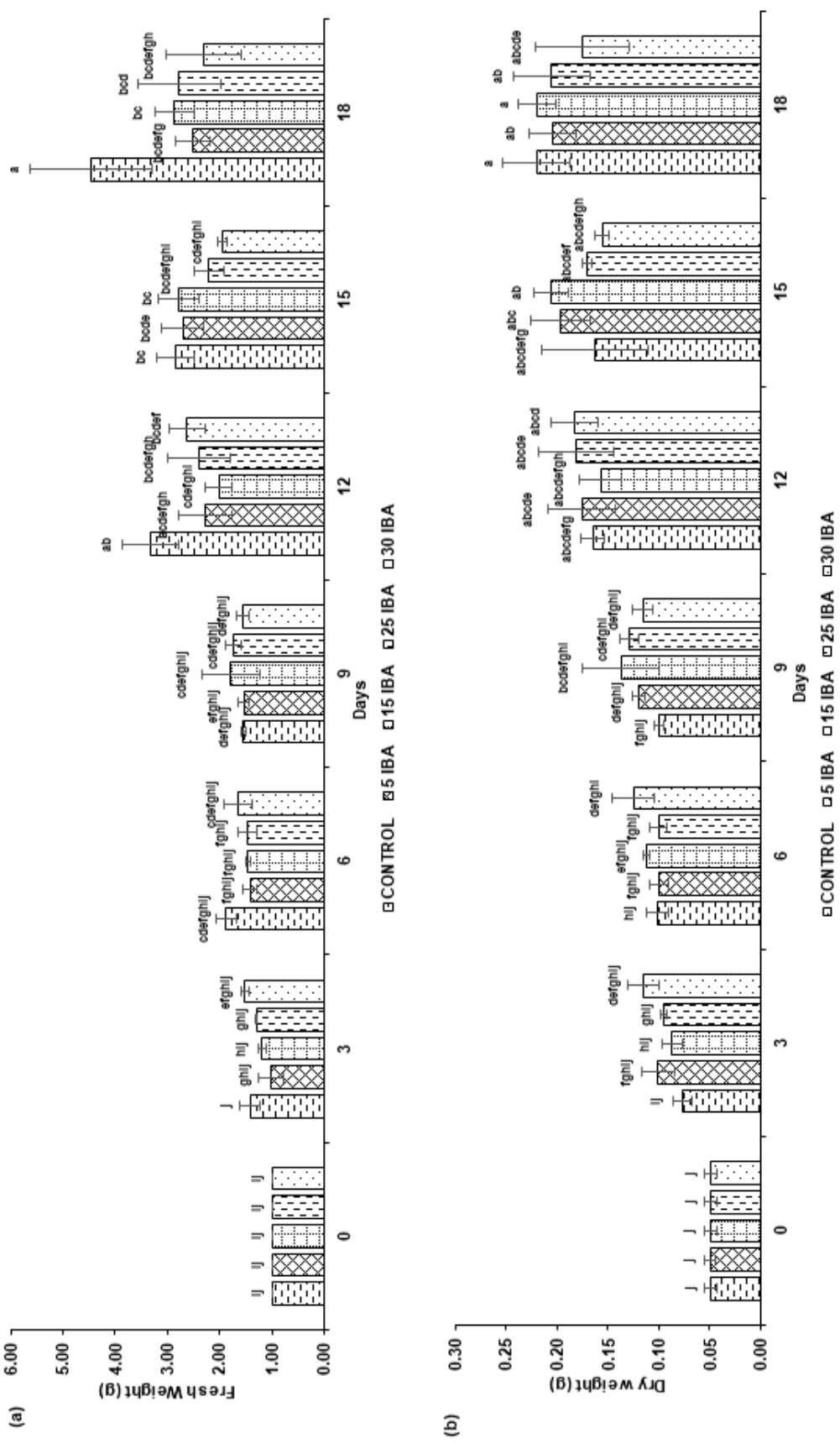


Fig. 3. The growth of *Polyalthia bullata* cell suspensions in ½ MS media with different concentrations of IBA for 18 days. (a) The graph of the fresh weight of *Polyalthia bullata* cells in ½ MSO (Control) and ½ MS media with 5 µM, 15 µM, 25 µM, and 30 µM IBA. (b) The graph of the dry weight of *Polyalthia bullata* cells in ½ MSO (Control) and ½ MS media with 5 µM, 15 µM, 25 µM, and 30 µM IBA. Different letters indicate statistically significant differences ($p<0.05$) between treatments tested using Tukey's HSD test.

indicating a stress response. A high concentration of IBA may lead to excessive production of IAA, which is the primary active form of auxin in plants (Frick & Strader, 2017). IBA may overstimulate the auxin signaling pathway and cause negative feedback regulation of auxin transporters, receptors, and transcription factors (Leyser, 2017). Consequently, this disturbance in auxin homeostasis influences the expression of auxin-responsive genes, which play pivotal roles in cell growth, development, and stress tolerance (Zhang *et al*., 2022). These stress responses can divert resources from growth towards antioxidant defense mechanisms, leading to fluctuations in fresh and dry weight as growth rates change.

The present study highlights the role of PGR in optimizing *Polyalthia bullata* cell growth in suspension culture. Notably, the calli grown in ½ MSO recorded the highest fresh and dry weight when compared to all treatment groups. These findings suggest that a balanced nutrient composition without exogenous auxins might provide the most favorable conditions for growth under the studied conditions. However, among the auxin treatments, 30 µM NAA produced the highest callus biomass (Figure 4).

Morphology of cell suspension culture

Based on cell morphology as shown in Table 1 and Table 2, the control group displayed dark brown color. For the NAA treatment, all cells were yellowish to brown with different intensities. Meanwhile, 5 and 15 µM NAA exhibited a dark yellowish-brown color, while 25 and 30 µM NAA showed a light yellowish-brown. For IAA, at 5 µM the cells exhibited light yellowish-brown. However, at 15, 25 and 30 µM, the cells exhibited dark yellowish-brown in color. In the case of IBA, the treatment with 30 µM IBA displayed a light yellowish-brown color. Dark yellowish-brown was observed in the cells treated with 5, 15, and 25 µM IBA. The yellowish-brown color may reflect a transition phase as the cells adjust to hormonal stimulation.

Among the color changes, the yellowish brown displayed better quality of cells, as observed in cells grown in 30 µM NAA. This result is further supported by parallel observations of cell growth recorded for 30 µM NAA, suggesting efficient utilization of this concentration without significant stress, potentially reflected in the light yellowish-brown cells.

The brown coloration in the control group could indicate the accumulation of phenolic compounds, which act as antioxidants and defense molecules (Pratyusha, 2022). However, since the phenolics are typically associated with reducing the cell division rate in plant culture (Lattanzio, 2019), the results obtained from this study showed an increase in cell weight despite browning observed in the cell suspension culture. This suggests the presence of growth enhancers that stimulate the callus proliferation of *Polyalthia bullata*.

Table 1. Morphology of the cell suspensions and biomass produced in treatments with NAA, IAA, and IBA after 18 days of growth

Concentration of auxins(µM)			Morphology of cell suspensions
NAA	IAA	IBA	
0	-	-	Dark brown
5	-	-	Dark yellowish-brown
15	-	-	Light yellowish-brown
25	-	-	Light yellowish-brown
30	-	-	Light yellowish-brown
-	0	-	Dark brown
-	5	-	Light yellowish-brown
-	15	-	Dark yellowish-brown
-	25	-	Dark yellowish-brown
-	30	-	Dark yellowish-brown
-	-	0	Dark brown
-	-	5	Dark yellowish-brown
-	-	15	Dark yellowish-brown
-	-	25	Light yellowish-brown
-	-	30	Dark yellowish-brown

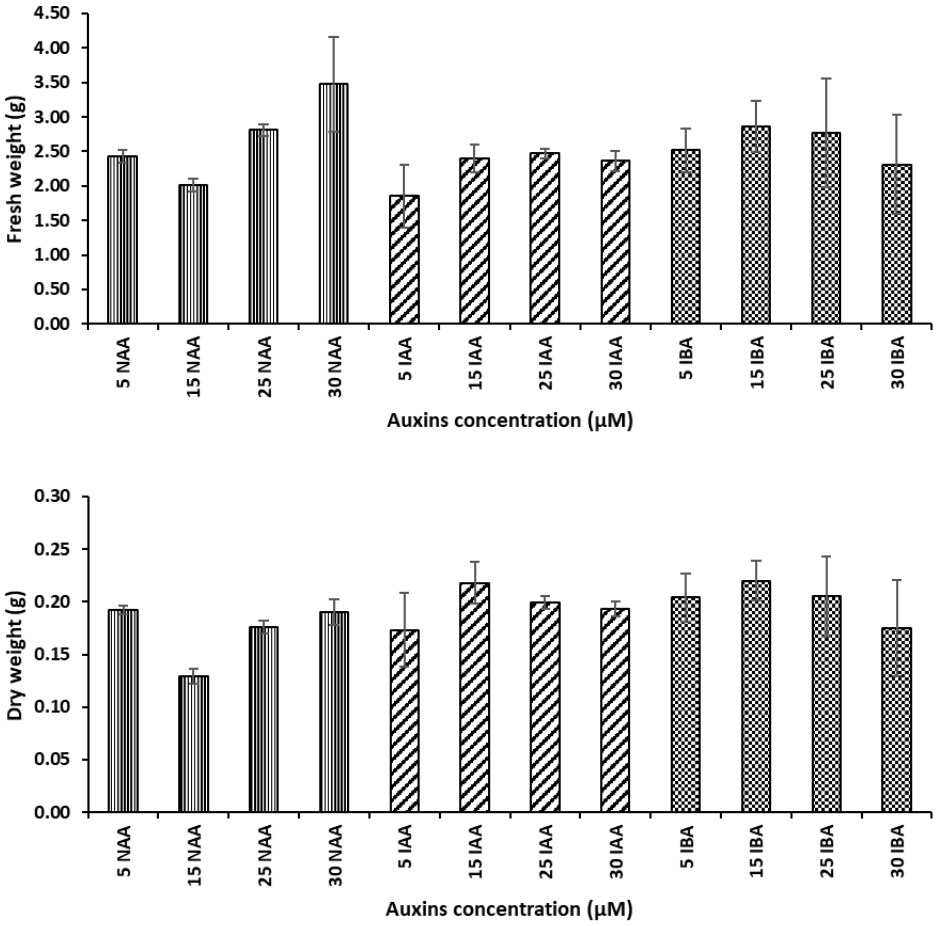
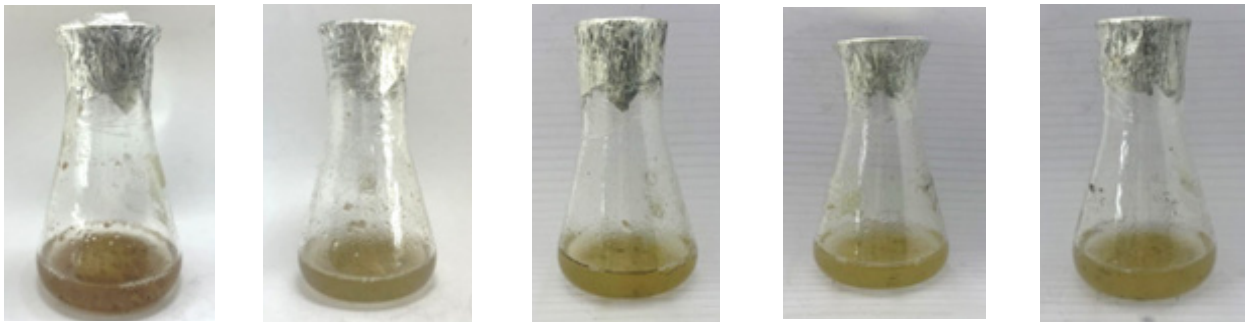
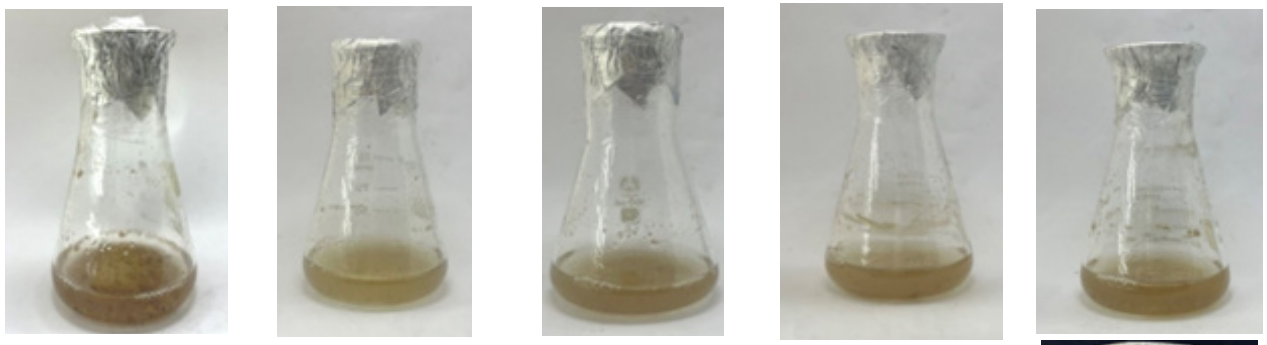
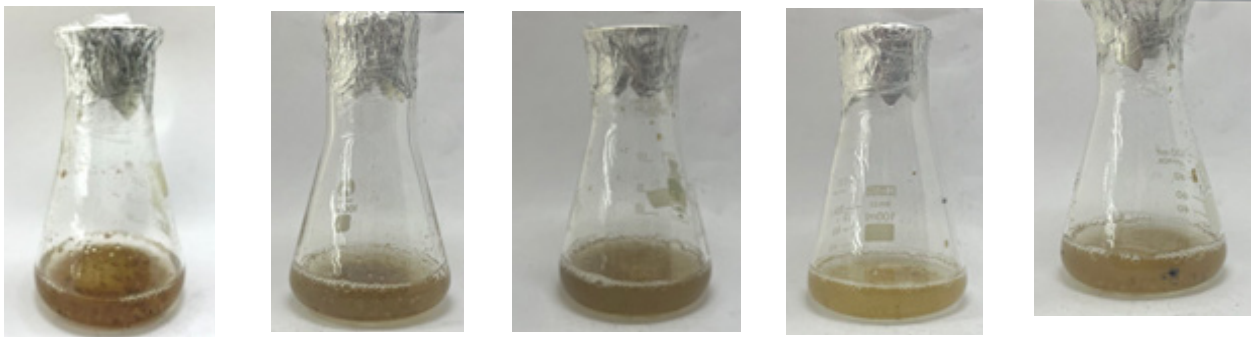


Fig. 4. The fresh and dry weights of *Polyalthia bullata* cell suspensions in ½ MS media supplemented with different concentrations of NAA, IAA, and IBA on day 18.

Table 2. Morphology of cell suspensions in treatment media after 18 days

Media	Samples				
	½ MS + 0 µM	½ MS + 5 µM	½ MS + 15 µM	½ MS + 25 µM	½ MS + 30 µM
NAA					
IAA					
IBA					

CONCLUSION

In conclusion, different types and concentrations of auxins affected cell suspension growth as well as morphology. The control group recorded the highest fresh and dry weight on day 18 (4.455 ± 1.170 g FW & 0.220 ± 0.033 g DW), but the cells produced a dark brown color. Meanwhile, among the auxin treatments, 30 µM NAA recorded the highest fresh and dry weight on day 18 (3.472 ± 0.694 g FW & 0.190 ± 0.012 g DW) with a light yellowish-brown color. Despite the lower biomass production recorded in the treatment groups of NAA, IAA, and IBA, it is important to note that there were significant differences in growth and biomass between days and concentrations of PGRs used in this study.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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