



Research Article

## In vitro growth of *Coelogyne* hybrid (*Coelogyne pandurata* × *Coelogyne rumphii*) on 2,4-D and BAP media

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### ABSTRACT

*Coelogyne* is a genus of ornamental orchids renowned for its distinctive floral characteristics and high conservation and economic value, particularly *Coelogyne pandurata*, which is commonly referred to as a black orchid. Efforts in cultivating black orchids in vitro require media modification with the addition of growth regulators to optimize plant development. Growth regulators that can be used include auxins and cytokinins. This study evaluated the effects of 2,4-D (2,4-Dichlorophenoxyacetic acid) and BAP on the in vitro growth and shoot proliferation of *Coelogyne pandurata* × *Coelogyne rumphii* hybrid seedlings. The experiment used a completely randomized design with two factors: 2,4-D and BAP, each with four concentration levels (0, 0.5, 1.0, and 1.5 ppm), resulting in 16 treatment combinations. The variables observed were the percentage of callus formation, callus color, shoot number, root number, leaf number, and plant length. The results showed that the optimal callus growth was achieved with a combination of 2,4-D at 0.5 ppm and BAP at 0.5 ppm, resulting in 100% callus formation. The 2,4-D treatment yields the optimum results in terms of leaf numbers (23.56) and plant height (2.15 cm), while the 1.5 ppm BAP treatment achieves the optimum results in terms of leaf numbers (31) and shoot numbers (22.39).

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**Keywords:** auxin; black orchid; callus; cytokinin; plant growth regulator

### INTRODUCTION

The production of ornamental orchids has developed quite rapidly, and countries with advanced technology dominate the global orchid export market (Restanto, et al., 2016). According to Volza (2024), between 2023 and 2024, orchids were exported by 117 countries and imported by 200 countries, indicating the strong and growing demand for this commodity. This global trend presents both challenges and opportunities for Indonesia, particularly in strengthening its capacity for in vitro orchid cultivation as a means to reduce dependence on imported varieties.

Orchids have long captivated people with their exotic beauty and remarkable diversity. They are cultivated commercially worldwide, stimulated by increasing demand and improved propagation techniques (Tiwari et al., 2024). Indonesia is among the countries with the greatest number of diverse orchid species, having more than 25,000 or even 30,000 wild orchid species that come from 750 genera and about 5,000 orchid species that have been discovered, classified, and identified. According to Lalla and Sudiarta (2022), Indonesia has around 4000-5000 species of orchids. The black orchid is a species of orchid native to Borneo. This orchid has a characteristic that there are black

markings on the lips that extend to the inside of the flower, while the crown and petals of black orchids are bright green (Kartiman et al., 2018).

In vitro culture is an effective technique for black orchid conservation and extinction prevention. Habitat loss due to land conversion, over-logging, and the challenge of conventional cultivation are the reasons for black orchids. Another factor that led to extinction is the relatively short flowering period and the difficulty of crossing. Orchid seeds do not have an endosperm and therefore have very little chance of growing unless cultivated in vitro (Jainol & Gansau, 2017). This is because in vitro culture provides a controlled environment that supplies external nutrients, enabling active cells to proliferate even in the absence of endosperm, which is essential for germination under natural conditions. In vitro techniques enable the production of new plants within a short period and ensure genetic uniformity.

Black orchid tissue culture requires precise modification of the culture medium using plant growth regulators to allow them to grow. Growth regulators that can be used include 2,4-D, which possesses the ability to stimulate callus growth (Mostafiz & Wagiran, 2018). BAP promotes cell growth, apical dominance, and shoot differentiation, especially when combined with auxins (Gethami & El Sayed, 2020). The experiment evaluated the effects of 2,4-D (2,4-Dichlorophenoxyacetic acid) and BAP on the in vitro growth and shoot proliferation of *Coelogyne pandurata* × *Coelogyne rumphii* hybrid seedlings.

## MATERIALS AND METHODS

This study was conducted at the Laboratory of Plant Physiology and Biotechnology, Faculty of Agriculture at Sebelas Maret University, Surakarta, Indonesia. The plant materials used were orchid plantlets derived from a hybrid of *C. pandurata* × *C. rumphii*. Each culture bottle had four plantlets, each with 4 to 5 leaves.

The culture medium used was Murashige & Skoog (1962) medium containing macro- and micronutrients, vitamins, and sucrose, modified by adding plant growth regulators. The experiment consisted of two factors: 2,4-D and BAP, each with four concentration levels (0, 0.5, 1.0, and 1.5 ppm), resulting in 16 treatment combinations.

The experimental design was a completely randomized design with four replications. The observed variables included the percentage of callus formation, callus color, shoot number, root number, leaf number, and plant height.

Data were analyzed using ANOVA at a 5% significance level. Significant differences among treatments were further analyzed using DMRT at the 5% level.

## RESULTS AND DISCUSSION

### *Percentage of callus formation*

Indicators of callus formation were observed weekly from the initiation stage until 9 weeks after treatment (WAT). Callus formed from orchid buds from a hybrid *Coelogyne pandurata* × *Coelogyne rumphii* began to form at week 5<sup>th</sup> after planting on the give treatment. According to Novak et al. (2017), the hormone 2,4-D can induce embryogenic callus formation in orchids. The percentage of callus formation is presented in Table 1.

Table 1. Callus formation of orchids from the hybrid *C. pandurata* × *C. rumphii* at 9 weeks after planting

Growth regulators		Percentage of callus formation (%)	Callus color
2,4 D (ppm)	BAP (ppm)		
0	0	0	-
	0.5	0	-
	1.0	0	-
	1.5	0	-
0.5	0	50	Yellow
	0.5	100	Yellow
	1.0	0	-

Growth regulators		Percentage of callus formation (%)	Callus color
2,4 D (ppm)	BAP (ppm)		
1.0	1.5	50	Green
	0	50	Brown
	0.5	0	-
	1.0	50	Yellow
1.5	1.5	50	Yellow
	0	25	Greenish yellow
	0.5	0	-
	1.0	25	Yellow
	1.5	25	Yellow

Based on observations, the callus started to grow around five weeks after planting. Research by Haque and Ghosh (2017) explained that orchid callus growth is initially yellowish-white. Callus formation begins with swelling at the base of the plant part used, and this area contains meristematic tissue that interacts directly with growth hormones.

In the present experiment, the highest callus formation (100%) was observed when using a combination of 0.5 ppm 2,4-D and 0.5 ppm BAP (Table 1). However, Romeida et al. (2016) found that 1 ppm 2,4-D performed best for callus formation. This is likely because they worked with different orchid species. Budisantoso et al. (2017) also noted that the effects of plant hormones can vary depending on the plant species.

The application of 2,4-D at concentrations of 0.5–1.5 ppm resulted in good callus growth, with callus formation percentages ranging from 50% to 100%, as presented in Table 1. This result aligns with the findings of Khalida et al. (2019) in wax orchids (*Aerides odorata*), which showed good callus growth with 2,4-D. Treatments without 2,4-D did not produce callus but instead stimulated shoot development, indicating that 2,4-D plays a crucial role in callus induction.



Figure 1. Callus formation at 9 weeks after treatment. (A) 2,4-D 1.5 ppm + BAP 1 ppm, (B) 2,4-D 0.5 ppm + BAP 0.5 ppm, (C) 2,4-D 0.5 ppm + BAP 1.5 ppm

Callus quality was assessed based on callus color and texture. In the present study, yellow callus was considered of good quality because it was actively dividing and exhibited healthy growth, indicating high viability. Initially, the callus appeared yellowish-white, then progressed to yellow as it developed, which corresponds to vigorous meristematic activity. Some calli turned green or brown, indicating aging or phenolic compound accumulation, which can inhibit further growth and reduce viability (Rasud & Bustman, 2020; Mayerni et al., 2020). Browning usually occurs due to oxidation of phenolic compounds induced by light exposure, which negatively affects callus development (dos Santos & Paz, 2016; Dreger et al., 2019). Therefore, actively dividing yellow callus represents the best quality in terms of potential for further proliferation and subculturing.

#### Root number

Observations showed that both 2,4-D and BAP had significant effects on root formation. The best results were achieved by applying 2,4-D at 1 ppm, resulting in an average of 13.25 roots per plantlet (Table 2). These results contrast with those of Widiastoety (2017), who reported that 1 ppm 2,4-D had the least effect on root and leaf formation. She noted that growth regulators do not solely influence root growth, but are

also influenced by environmental factors such as light intensity. Endogenous plant growth regulators become more active in stimulating root formation under lower light conditions.

Table 2. Effect of 2,4-D on the average root number, leaf number, and plant height of hybrid orchid plants *C. pandurata* × *C. rumphii* at 20 weeks after treatment

Concentration of 2,4-D (ppm)	Root number	Leaf number	Plant length (cm)	Shoot number
0	6.00b	0.43b	0.80b	3.24a
0.5	10.25a	2.81b	0.90b	2.90a
1.0	13.25a	3.06b	1.32b	4.86a
1.5	8.25b	23.56a	2.15a	3.26a

Note: Values followed by the equal alphabet in each column indicate that there is no significant difference based on the DMRT at  $\alpha=5\%$ .

As shown in Table 2, increasing the concentration of 2,4-D to 1.5 ppm led to decreased root formation, indicating that the optimal level had been exceeded. Goswami et al. (2016) explained that high concentrations of auxins can inhibit root elongation and adventitious root formation, although auxins do promote root branching. Root formation was less pronounced in media supplemented with BAP alone compared to those with 2,4-D. Mose et al. (2017) found that root growth in *Dendrobium* orchids is slower when BAP is used as the sole regulator.

#### Leaf number

The results of ANOVA at a 5% significance level showed that 2,4-D and BAP significantly affected leaf number. Among the 2,4-D treatments, the highest average leaf number was observed at 1.5 ppm, reaching 23.56 leaves per plantlet (Table 2). In BAP treatments, increasing cytokinin concentration also enhanced leaf development, with 1.5 ppm BAP producing the highest leaf number (31 leaves per plantlet), although this difference was not statistically significant based on DMRT analysis (Table 3).

Table 3. Effects of BAP on the average shoot number, roots, and leaves of hybrid orchid plants *C. pandurata* × *C. rumphii* at 20 weeks after treatment

Concentration of BAP (ppm)	Root number	Leaf number	Plant length (cm)	Shoot number
0	0.12b	8.75b	2.84a	1.25c
0.5	1.31b	13.75b	3.56a	1.50c
1.0	1.75b	24.25a	3.57a	10.18b
1.5	9.43a	31.00a	4.56a	22.39a

Note: Values followed by the equal alphabet in each column indicate that there is no significant difference based on the DMRT at  $\alpha=5\%$ .

Higher leaf numbers were observed under BAP treatments alone, with 1.5 ppm BAP producing an average of 31.00 leaves per plantlet (Table 3), indicating that BAP had a stronger effect on leaf development than 2,4-D. Widiastoety (2017) reported that 2,4-D had minimal influence on leaf development. These differences may be due to the synergistic interaction between exogenous hormones and the plant's endogenous hormones. 2,4-D, a synthetic auxin, is commonly used to promote cell division in plant tissue culture media. Leaf development requires optimal energy input, which is supported by the presence of both auxins and cytokinins. Cytokinins promote cell division and the growth of various plant organs, including leaves. Additionally, they can delay senescence by regulating the degradation of chlorophyll and proteins, thereby maintaining leaf vitality (Ashokhan et al., 2020).

#### Shoot numbers

MS medium is a classic plant tissue culture medium used for the culture of new shoots and consists of important nutrients such as nitrogen, potassium, sulphur, and zinc. In this study, the ANOVA test at a 5% significance level showed that both BAP alone and the combination of 2,4-D with BAP significantly affected shoot formation in the orchid

hybrid *C. pandurata* × *C. rumphii*. The first shoots were visible in the 6 Weeks After Planting (WAP). This contrasts with Pradhan et al. (2018), who mentioned that the first primordia of the shootlets of their species were observed in week 8 after subculturing. Chin et al. (2019) also noted that repeated subculturing may influence shoot multiplication rates, leading to experimental variability. Such variation might be caused by environmental factors, including temperature, air purity, humidity, and the physiological state of the material responsible for the odor.

Table 4. Effect of the combination of 2,4-D and BAP on the average shoot number of orchids from the hybrid *C. pandurata* × *C. rumphii* at 20 weeks after treatment

Concentration growth regulators		Average shoot number
2,4 D (ppm)	BAP (ppm)	
0	0	18.75b
	0.5	33.75a
	1.0	24.25ab
	1.5	31.00a
0.5	0	0.25c
	0.5	2.00c
	1.0	3.75c
	1.5	2.25c
1.0	0	3.00c
	0.5	2.50c
	1.0	1.00c
	1.5	4.25c
1.5	0	0.75c
	0.5	1.75c
	1.0	4.25c
	1.5	4.00c

Note: Values followed by the equal alphabet in each column indicate that there is no significant difference based on the DMRT at  $\alpha=5\%$ .

Among the treatments, the combination of 0 ppm 2,4-D and 0.5 ppm BAP produced the highest shoot number, averaging 33.75 shoots per plantlet (Table 4). Similarly, analysis of BAP treatments alone (Table 3) showed that increasing BAP concentration also promoted shoot formation, with the highest shoot number observed at 1.5 ppm BAP (22.39 shoots), indicating that BAP is the main factor stimulating shoot proliferation in the orchid hybrid. In contrast, the application of 2,4-D at all concentrations did not significantly affect shoot number (Table 2), suggesting that auxin primarily influenced other growth parameters rather than shoot induction. According to Tunjung et al. (2021), BAP is a kind of cytokinin hormone that plays a vital role in cell division and the development of shoots and leaves. Cytokinins stimulate cell growth and differentiation and are major growth promoters in most plant development aspects (Feng et al., 2017). Nutrient media enriched with appropriate growth regulators can stimulate in vitro plant development, including in orchids (Sherif et al., 2016). Adding plant growth regulators at appropriate concentrations enhanced shoot proliferation in plantlets cultured in MS medium.

#### Plant length

Plant elongation and height are primarily driven by cell division and cell elongation processes occurring in meristematic tissues, particularly at the shoot apex. In this study, plant length was mainly influenced by the application of 2,4-D, while BAP did not significantly affect plant length at any concentration (Table 3). The use of MS medium supplemented with 2,4-D had a significant effect on plant height, with the tallest average plant height (2.15 cm) being recorded under the 1.5 ppm 2,4-D treatment (Table 2). According to Sipayung et al. (2018), plant height is closely associated with root development. Roots play a crucial role in nutrient absorption from the culture medium,

which supports the assimilation process and the synthesis of food reserves (e.g., starch and cellulose) that contribute to stem elongation.

## CONCLUSIONS

The in vitro growth and shoot proliferation of *Coelogyne pandurata* × *Coelogyne rumphii* hybrid seedlings were significantly affected by the application of 2,4-D and BAP in the culture medium. The combination of 0.5 ppm 2,4-D and 0.5 ppm BAP was the most effective treatment for callus induction, achieving 100% callus formation. For vegetative growth, 2,4-D at 1.5 ppm enhanced plant height and leaf number compared to the control. However, in terms of in vitro propagation efficiency, BAP at 1.5 ppm was identified as the optimal treatment, as it produced the highest shoot proliferation (22.39 shoots per plantlet) and leaf development (31.00 leaves per plantlet). These findings demonstrate that BAP at an appropriate concentration plays a dominant role in shoot multiplication, which is the key parameter in the mass propagation of this hybrid orchid.

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