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Induction Of Somatic Embryogenesis In Dendrobium Sp. Orchids Through In Vitro Application Of Combined 2,4-D And Sucrose Concentrations

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Abstract:
Background*: Dendrobium orchids are among the most commercially valuable ornamental plants due to their vibrant floral diversity and horticultural appeal. However, conventional propagation methods, including seed germination and shoot division, remain inefficient for mass production. Somatic embryogenesis offers a promising alternative by enabling rapid in vitro propagation of genetically uniform and disease-free plantlets. This study aimed to identify the optimal combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and sucrose concentrations to induce somatic embryogenesis in Dendrobium sp. explants.*
Materials and Methods*: The experiment was conducted using a solid MS medium supplemented with 16 treatment combinations of 2,4-D (1, 2, 3, and 4 ppm) and sucrose (20, 30, and 40 g L^{-1}), arranged in a completely randomised design with three replicates. Data were analysed using DMRT at a 5% significance level. Key parameters observed included the survival rate of embryogenic calli, callus formation percentage, days to callus initiation, fresh callus weight, and morphological characteristics.*
Results*: The best performance was recorded in the treatment containing 3 ppm 2,4-D and 4% sucrose, yielding 88% callus survival, 77.67% embryogenic callus formation, the earliest initiation time (28.33 days after culture), and a fresh weight of 6.30 g. The calli produced were green to light green and had a friable to compact texture, indicating high embryogenic potential.*
Conclusion: *These findings provide a foundational step in developing an efficient and reproducible somatic embryogenesis protocol for Dendrobium orchids.*
Key Word*: Somatic Embryogenesis, Dendrobium sp., 2,4-D, Sucrose, Plant Tissue Culture, Orchid Micropropagation.*

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I. Introduction

Dendrobium orchids are among the most economically significant ornamental plants in Asia, known for their aesthetic diversity, long blooming periods, and adaptability to tropical climates. In Indonesia, Dendrobium constitutes the dominant group of cultivated orchids, accounting for approximately 60–70% of national production (Direktorat Jenderal Hortikultura, 2023). The annual output of orchids in the country surpasses 22 million pots, reflecting their high demand in both domestic and export markets. These orchids are widely distributed to countries such as Japan, Singapore, the Netherlands, and Taiwan, positioning Indonesia as an active participant in the global floriculture industry. With such demand, the development of reliable and scalable propagation systems becomes essential for ensuring the continuity of supply. However, existing propagation methods are often constrained by inefficiencies in multiplication and a lack of standardised protocols.

Conventional propagation methods, including seed germination and vegetative division, remain widely used but are not without limitations. Seed propagation in orchids is time-consuming and produces genetically variable offspring due to their cross-pollinated nature, while vegetative propagation is labour-intensive and limited in scale. These methods often fall short in fulfilling commercial requirements, particularly for large-scale production of uniform and disease-free plantlets. Consequently, in vitro culture techniques such as somatic embryogenesis have become a promising alternative. Somatic embryogenesis enables the regeneration of complete plants from somatic cells, producing clonal populations with high genetic fidelity under controlled environmental conditions. This technique is particularly advantageous for orchids, which often exhibit slow natural propagation rates and are sensitive to environmental fluctuations.

The success of somatic embryogenesis depends on several critical factors, including the physiological status of explants, medium composition, and hormonal balance. Auxins, especially synthetic types such as 2,4-

dichlorophenoxyacetic acid (2,4-D), are widely recognised for their ability to induce dedifferentiation and stimulate the formation of embryogenic callus (Purnamaningsih, 2002). The application of 2,4-D triggers cellular reprogramming by influencing endogenous hormone levels and activating genes associated with embryogenic competence. At the same time, carbohydrates, particularly sucrose, serve a dual role as a primary carbon source and osmotic stabiliser in plant tissue culture (Heriansyah, 2019). Sucrose influences cell elongation, energy metabolism, and the regulation of morphogenic responses during somatic embryo induction. Therefore, the interaction between growth regulators and sucrose concentration plays a crucial role in the successful induction and development of somatic embryos.

Several studies have demonstrated the effectiveness of 2,4-D and sucrose in promoting embryogenic callus formation in orchids. Dewanti et al. (2023) reported that 3.5 ppm 2,4-D induced up to 92% embryogenic callus formation in Dendrobium sp., with a notably fast response within 14 days. In contrast, Rohmiati and Semiarti (2023) found that a lower concentration of 1 ppm 2,4-D yielded better results for Dendrobium lineale, indicating species-specific responses to auxin levels. The effectiveness of sucrose concentration has also been documented, with Heriansyah et al. (2023) reporting that 50 g L^{-1} sucrose combined with 1 mg L^{-1} kinetin significantly enhanced root and shoot formation in somatic embryos of Dendrobium from Riau Province. These findings highlight the need for optimising both hormone and carbohydrate components to achieve desirable in vitro responses. However, excessive concentrations of either 2,4-D or sucrose may induce callus browning, inhibit growth, or lead to somaclonal variation (Puspita Sari et al., 2018). Hence, a balanced formulation of these components is critical for ensuring both efficacy and reproducibility.

Despite the increasing interest in orchid tissue culture, research focusing on the interactive effects of 2,4-D and sucrose concentrations remains limited, particularly for local Dendrobium varieties in Indonesia. Many previous studies have treated these factors in isolation, neglecting their potential synergistic roles in embryogenic callus development. Moreover, protocols optimised for foreign cultivars may not be directly applicable to native Indonesian germplasm, which may exhibit different physiological responses to in vitro treatments. This gap in the literature hinders the development of locally adapted, standardised propagation systems that are vital for supporting commercial orchid cultivation and biodiversity conservation. Addressing this issue is essential for enhancing Indonesia's capacity to sustainably cultivate and commercialise its rich orchid resources. A context-specific, evidence-based approach is therefore needed to improve micropropagation outcomes for local Dendrobium lines.

This study aimed to investigate the interactive effects of different concentrations of 2,4-D and sucrose on the induction of somatic embryogenesis in Dendrobium sp. explants. Specifically, the study sought to determine the optimal combination that maximises callus viability, embryogenic potential, and biomass production under in vitro conditions. Additionally, the research examined morphological characteristics of the Callus, such as colour and texture, as indirect indicators of embryogenic potential. By systematically exploring the interplay between auxin and carbohydrate levels, the findings are expected to contribute to the development of an effective somatic embryogenesis protocol tailored to local orchid genotypes. This work not only offers a practical solution for mass propagation but also supports long-term strategies for the conservation and commercial utilisation of Indonesian Dendrobium. Ultimately, the study provides a scientific basis for enhancing orchid biotechnology in tropical horticulture.

II. Material And Methods

Study Location and Duration

The experiment was conducted at the Plant Tissue Culture Laboratory, Department of Agrotechnology, Faculty of Agriculture, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia. This laboratory is equipped with standard facilities for in vitro culture, including laminar airflow cabinets, autoclaves, growth chambers, and analytical tools. The study was carried out over a three-month period, from July to September 2022, during which culture preparation, inoculation, incubation, and observation were completed. Temperature and photoperiod were consistently monitored to maintain optimal environmental conditions for orchid tissue growth. The laboratory also ensured aseptic techniques throughout the entire process to avoid microbial contamination. All procedures were carried out following institutional biosafety guidelines for plant tissue culture research.

Plant Material

The explants used in this study were derived from three-month-old in vitro *Dendrobium sp.* plantlets, which were previously subcultured to induce callus formation. Callus were taken from the basal parts of the leaf segments that exhibited healthy, uniform morphology and were free from necrosis or contamination. The plant material was obtained from the laboratory's local orchid germplasm collection and maintained under controlled growth conditions. Prior to experimentation, callus pieces were standardised in size (approximately 0.5–1.0 cm^3) to minimise variation in initial biomass. The choice of explant source was based on previous findings indicating

high embryogenic responsiveness in juvenile tissues. All explants were selected aseptically and handled using sterile instruments under a laminar airflow cabinet.

Media Preparation

Murashige and Skoog (MS) basal medium was used as the culture medium, with the addition of 100 mg L^{-1} myo-inositol, 7 g L^{-1} agar, and varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and sucrose. A factorial combination of 2,4-D (1, 2, 3, and 4 ppm) and sucrose (20%, 30%, and 40%) was applied, resulting in 16 distinct treatment combinations. The medium pH was adjusted to 5.8 before autoclaving at 121°C for 15 minutes under 1.1 kg cm^{-2} pressure. All media were poured into sterile culture jars (approximately 20 mL per jar) and allowed to solidify at room temperature. The sterilisation process ensured both media and glassware were free from contaminants before use. All chemicals and reagents used were of analytical grade and obtained from certified suppliers.

Explant Inoculation and Culture Conditions

Explant sterilisation was performed using a standard protocol, including rinsing in sterile distilled water, immersion in 0.1% detergent solution, followed by treatment with commercial sodium hypochlorite (Bayclin®) for surface disinfection. Explants were then washed three times with sterile distilled water to remove any residues and transferred to the culture medium under aseptic conditions. The inoculated culture jars were sealed and incubated in a growth chamber at 25 ± 2°C with a 16-hour photoperiod and light intensity of approximately 40 µmol m^{-2} s^{-1}. Relative humidity was maintained between 60–70% to support optimum callus growth and prevent desiccation. Cultures were observed weekly to monitor contamination and initial morphological responses. No subculturing was performed during the experimental period to maintain treatment integrity.

Experimental Design and Data Collection

The experiment was arranged in a Completely Randomised Design (CRD) with 16 treatments and three replicates per treatment, resulting in a total of 48 experimental units. Each replicate consisted of one culture jar containing a single explant, enabling independent observation and measurement. The observed parameters included: (1) percentage of callus survival, (2) percentage of embryogenic callus formation, (3) days to callus initiation, (4) fresh weight of callus at the end of the culture period, and (5) morphological characteristics (colour and texture). Callus colour was recorded using Munsell colour notation, while texture was categorised as friable or compact based on visual and tactile assessment. Data were collected at the end of the 12-week culture period. Observations were documented with photographs to support quantitative and qualitative analysis.

Statistical Analysis

All quantitative data were subjected to Analysis of Variance (ANOVA) using standard statistical procedures for factorial experiments. When significant effects were detected, Duncan's Multiple Range Test (DMRT) was applied at a 5% significance level to separate means among treatment combinations. The statistical software used for analysis was SPSS version 16.0, which allowed for accurate comparison of treatment effects. Normality and homogeneity of variance were tested prior to performing ANOVA to ensure data suitability. Graphs and tables were constructed to clearly present the results and support interpretation. Statistical analysis was conducted in consultation with a research methodologist to ensure validity and reliability of the findings.

III. Result

The survival percentage of embryogenic Callus reflects the capacity of explants to maintain viability and physiological activity under in vitro conditions. In this study, it was found that the combination of 2,4-D and sucrose concentrations significantly affected the survival rate of Dendrobium sp. Callus after 12 weeks of culture. The highest survival percentage (88.00%) was observed in the treatment containing 3 ppm 2,4-D and 4% sucrose. This combination likely created a favourable microenvironment that supported metabolic activity and reduced physiological stress. In contrast, the lowest survival rate (75.17%) was recorded in the treatment with 1 ppm 2,4-D and 5% sucrose. (Tabel 1).

Table 1. Survival Percentage and Embryogenic Callus Formation of Dendrobium Orchids in Response to MS Medium Supplemented with 2,4-D and Sucrose at 12 Weeks After Culture

No	Treatment	Embryogenic Callus Survival Rate (%)	Percentage of Embryogenic Callus Formation in Dendrobium sp. Orchids (%)
1	MS + 2.4-D 1 ppm + Sukrosa 2 %	78.50 ^{cde}	43.67 ^{e}
2	MS + 2.4-D 1 ppm + Sukrosa 3 %	76. 00 ^{de}	60.33 ^{c}

3	MS + 2.4-D 1 ppm + Sukrosa 4 %	79.17 bcd	60.33 c
4	MS + 2.4-D 1 ppm + Sukrosa 5%	75.17 e	53.33 d
5	MS + 2.4-D 2 ppm + Sukrosa 2 %	79.50 bcd	46.00 e
6	MS + 2.4-D 2 ppm + Sukrosa 3 %	80.17 bc	60.00 c
7	MS + 2.4-D 2 ppm + Sukrosa 4 %	79.00 bcd	61.00 c
8	MS + 2.4-D 2 ppm + Sukrosa 5%	80.17 bc	63.33 bc
9	MS + 2.4-D 3 ppm + Sukrosa 2 %	80.17 bc	44.33 e
10	MS + 2.4-D 3 ppm + Sukrosa 3 %	82.87 b	66.67 b
11	MS + 2.4-D 3 ppm + Sukrosa 4 %	88.00 a	77.67 a
12	MS + 2.4-D 3 ppm + Sukrosa 5%	80.50 bc	61.33 c
13	MS + 2.4-D 4 ppm + Sukrosa 2 %	79.83 bc	52.00 d
14	MS + 2.4-D 4 ppm + Sukrosa 3 %	79.83 bc	61.00 c
15	MS + 2.4-D 4 ppm + Sukrosa 4 %	79.17 bcd	59.00 c
16	MS + 2.4-D 4 ppm + Sukrosa 5%	79.00 bcd	43.67 e
Average		**79.81**	**53.33**

Note: Values followed by different letters within the same column are significantly different according to Duncan's Multiple Range Test at the 5% level.

Based on the experimental results, explants of *Dendrobium sp.* callus demonstrated that the combination of 2,4-D and sucrose concentrations had a significant effect on the percentage of embryogenic callus formation under in vitro conditions (Table 1). Table 1 clearly shows that the interaction between 2,4-D and sucrose concentrations in MS medium significantly influenced the embryogenic callus formation percentage in *Dendrobium sp.* orchids. The highest percentage (77.67%) was obtained with the combination of 3 ppm 2,4-D and 4% sucrose.

In general, the time of embryogenic callus emergence refers to the period when callus tissue begins to develop and exhibits embryogenic characteristics during plant tissue culture. According to Table 2, the combination of 2,4-D and sucrose concentrations in MS medium had a significant effect on the timing of embryogenic callus emergence in Dendrobium sp. callus explants. The most favourable result was observed in the treatment with 3 ppm 2,4-D and 4% sucrose, which exhibited the earliest initiation time of 28.33 days after culture. In contrast, the longest induction time (36.33 days) was recorded in treatments with 1 ppm 2,4-D combined with 2% and 5% sucrose, respectively.

Table 2. Mean Time of Embryogenic Callus Emergence (Days After Culture) and Fresh Weight of Embryogenic Callus (g) in Dendrobium sp. Explants Under Various Treatments

No	Treatment	Time of Embryogenic Callus Emergence (Days After Culture)	Fresh Weight of Embryogenic Callus at 12 Weeks After Culture (gram)
1	MS + 2.4-D 1 ppm + Sukrosa 2 %	36.33 a	3.87 h
2	MS + 2.4-D 1 ppm + Sukrosa 3 %	36.00 a	4.30 g
3	MS + 2.4-D 1 ppm + Sukrosa 4 %	35.00 ab	4.23 g
4	MS + 2.4-D 1 ppm + Sukrosa 5%	36.33 a	3.77 h
5	MS + 2.4-D 2 ppm + Sukrosa 2 %	32.33 d	4.90 ef
6	MS + 2.4-D 2 ppm + Sukrosa 3 %	32.33 d	5.63 bc
7	MS + 2.4-D 2 ppm + Sukrosa 4 %	31.67 de	5.07 df
8	MS + 2.4-D 2 ppm + Sukrosa 5%	33.67 bcd	4.73 f
9	MS + 2.4-D 3 ppm + Sukrosa 2 %	31.67 de	5.60 bc
10	MS + 2.4-D 3 ppm + Sukrosa 3 %	30.33 e	5.83 b
11	MS + 2.4-D 3 ppm + Sukrosa 4 %	28.33 f	6.30 a
12	MS + 2.4-D 3 ppm + Sukrosa 5%	33.00 cd	4.90 ef
13	MS + 2.4-D 4 ppm + Sukrosa 2 %	35.00 ab	5.50 c
14	MS + 2.4-D 4 ppm + Sukrosa 3 %	32.33 d	5.50 c
15	MS + 2.4-D 4 ppm + Sukrosa 4 %	33.67 bcd	5.37 cd
16	MS + 2.4-D 4 ppm + Sukrosa 5%	34.67 abc	5.33 cd

Note: *Values followed by different letters within the same column are significantly different according to Duncan's Multiple Range Test at the 5% significance level.*

Table 3. Effects of 2,4-D and Sucrose Combinations on Colour and Texture of Dendrobium sp. Embryogenic Callus at 12 Weeks After Culture

No	Perlakuan	Embryogenic Callus Colour	Munsell Notation	Callus Texture
1	MS + 2,4-D 1 ppm + Sukrosa 2%	Cream and Light Green	10YR 8/4 dan 2.5GY 8/4	Friable
2	MS + 2,4-D 1 ppm + Sukrosa 3%	Cream and Light Green	10YR 8/4 dan 2.5GY 8/4	Friable
3	MS + 2,4-D 1 ppm + Sukrosa 4%	Light Green	2.5GY 8/4	Friable
4	MS + 2,4-D 1 ppm + Sukrosa 5%	Light Green	2.5GY 8/4	Friable
5	MS + 2,4-D 2 ppm + Sukrosa 2%	Light Green	2.5GY 8/4	Remah
6	MS + 2,4-D 2 ppm + Sukrosa 3%	Light Green	2.5GY 8/4	Friable-Compact
7	MS + 2,4-D 2 ppm + Sukrosa 4%	Green	2.5GY 6/4	Friable-Compact
8	MS + 2,4-D 2 ppm + Sukrosa 5%	Green	2.5GY 6/4	Friable-Compact
9	MS + 2,4-D 3 ppm + Sukrosa 2%	Light Green	2.5GY 8/4	Friable
10	MS + 2,4-D 3 ppm + Sukrosa 3%	Light Green	2.5GY 8/4	Friable-Compact
11	MS + 2,4-D 3 ppm + Sukrosa 4%	Light Green	2.5GY 8/4	Friable-Compact
12	MS + 2,4-D 3 ppm + Sukrosa 5%	Light Green	2.5GY 8/4	Friable-Compact
13	MS + 2,4-D 4 ppm + Sukrosa 2%	Light Green	2.5GY 8/4	Friable-Compact
14	MS + 2,4-D 4 ppm + Sukrosa 3%	Light Green	2.5GY 8/4	Friable-Compact
15	MS + 2,4-D 4 ppm + Sukrosa 4%	Light Green	2.5GY 8/4	Friable-Compact
16	MS + 2,4-D 4 ppm + Sukrosa 5%	Green	2.5GY 6/4	Friable-Compact

Note : Cream = 10YR 8/4. Green =2.5GY 8/4. Green= 2.5GY 6/4 (Munsell Notation)

Table 3 presents the influence of different combinations of 2,4-D and sucrose concentrations on the colour and texture of Dendrobium sp. embryogenic Callus at 12 weeks after culture. Colour and texture are critical visual indicators used to identify the embryogenic potential of in vitro-grown Callus. Cream-coloured Callus typically reflect low metabolic activity and are considered non-embryogenic, whereas light green to bright green hues are associated with chloroplast formation and increased metabolic activity—both of which are linked to the early stages of somatic embryogenesis (Bhojwani & Razdan, 1996; Muliati et al., 2017).

IV. Discussion

Embryogenic Callus Survival Rate

The findings in Table 1 that show low value suggests a potential imbalance in hormonal signalling or the presence of osmotic stress, which may have disrupted cell division and nutrient uptake were in line with those of Dewanti et al. (2023), who reported that concentrations of 3–3.5 ppm 2,4-D effectively stimulated the formation and survival of embryogenic callus in Dendrobium sp.. Auxins such as 2,4-D are known to activate cell dedifferentiation and division when applied at moderate concentrations. However, deviations from the optimal range may lead to inhibitory or toxic effects, which negatively affect cell viability. In the context of this study, the 3 ppm 2,4-D treatment appears to strike a balance between promoting growth and avoiding hormonal overstimulation. Moreover, the 4% sucrose concentration provided sufficient carbon for metabolic processes without inducing osmotic stress. This balance between hormone and carbohydrate levels is essential for sustaining embryogenic tissue under artificial conditions.

The results are also supported by Wibowo (2019), who found that 4% sucrose optimally supports cellular metabolism in tissue culture systems. Lower sucrose levels, such as 2%, may lead to limited energy availability, while higher levels, like 5%, can result in osmotic inhibition. In orchid cultures, proper osmotic pressure is necessary for water absorption and nutrient uptake, both of which are vital for cell survival. The observed interaction between 2,4-D and sucrose in this study underscores the importance of precise media formulation. Growth regulation by auxins and osmotic control by sucrose must be harmonised to optimise explant responses. Therefore, the combination of 3 ppm 2,4-D and 4% sucrose may serve as an optimal baseline for enhancing embryogenic callus survival in Dendrobium culture systems.

Percentage of Embryogenic Callus Formation in *Dendrobium sp.* Orchids

The result in Table 1 indicates that 3 ppm 2,4-D is the most effective auxin concentration for inducing embryogenic callus formation, especially when combined with 4% sucrose as a carbon source. The effectiveness of this treatment is supported by the role of 2,4-D in activating key developmental genes such as LEC1, BBM, and SERK, which are essential for embryogenesis (Singh et al., 2023). However, when 3 ppm 2,4-D was combined with 2% or 5% sucrose, its effectiveness declined significantly (44.33% and 61.33%, respectively), highlighting the importance of maintaining a hormonal and osmotic balance.

The application of 4% sucrose has been shown to support optimal cell division and metabolic activity, whereas lower concentrations (2%) may fail to provide sufficient energy, and higher concentrations (5%) are suspected to cause physiological stress that inhibits callus morphogenesis. The lowest embryogenic callus formation (43.67%) was recorded in the treatment with 1 ppm 2,4-D and 2% sucrose. This finding suggests that 2,4-D concentrations below 2 ppm tend to induce non-embryogenic or necrotic callus tissue. Similarly, 4 ppm 2,4-D did not produce better results and, in some cases—such as in combination with 5% sucrose—resulted in reduced embryogenic callus formation (43.67%). These results are consistent with the reports of Ahmad et al. (2021) and Radhakrishnan et al. (2021), who stated that excessive 2,4-D concentrations may be toxic and inhibitory to embryogenesis. Zakaria et al. (2024) also emphasised that the precise sucrose concentration is critical for efficient callus formation in Clitoria ternatea, while Puspita Sari (2018) reported that excessive osmotic pressure due to high sucrose levels may interfere with cell division and inhibit embryogenic callus formation.

Time of Embryogenic Callus Emergence

Based on Table 2 shows findings that highlight the importance of an optimal hormonal and nutritional balance in accelerating the onset of embryogenic responses. A well-formulated medium enables explants to respond more rapidly to in vitro induction cues. The effectiveness of 3 ppm 2,4-D in accelerating callus formation is attributed to its function as a synthetic auxin that stimulates both cell division and dedifferentiation—two essential processes in early callus development (Narmani et al., 2021). Increasing 2,4-D concentration to an optimal level enhances the physiological responsiveness of the explant to hormonal stimuli. This enables a more rapid transition from the latent phase to the active mitotic phase, thus reducing the induction period. The timely reactivation of the cell cycle is crucial for embryogenic callus initiation, particularly in species with slow or variable morphogenic responses. Without adequate hormonal stimulation, cellular reprogramming is delayed, resulting in longer induction times. Therefore, 3 ppm 2,4-D appears to offer the most balanced stimulus for early morphogenic activation in Dendrobium sp.

In addition, the supplementation of 4% sucrose played an important role in providing sufficient energy to support early metabolic activities. Wang et al. (2022) reported that optimal sucrose concentrations can enhance cellular activity and promote in vitro callus formation. On the other hand, a low concentration of 2,4-D (1 ppm) likely results in delayed emergence due to insufficient hormonal signals to trigger the onset of cell division. Excessive 2,4-D levels (4 ppm) also appeared to inhibit callus formation, potentially due to mild phytotoxicity that disrupts early physiological processes before the cells can adapt and initiate dedifferentiation (Putri et al., 2020). These findings confirm that embryogenic callus emergence is highly dependent on achieving the correct hormonal and osmotic conditions. Hence, the interplay between auxin and sucrose must be carefully regulated to ensure timely induction and optimal callus performance.

Fresh Weight of Embryogenic Callus in Dendrobium sp.

Fresh weight of embryogenic callus serves as a quantitative measure of tissue mass that possesses regenerative potential to develop into whole plants. Based on the data presented in Table 2, the application of various combinations of 2,4-D and sucrose concentrations in MS medium significantly affected the average fresh weight of Dendrobium sp. embryogenic Callus at 12 weeks after culture. The most optimal treatment was observed in MS medium supplemented with 3 ppm 2,4-D and 4% sucrose, which produced the highest fresh weight of 6.30 grams. This treatment differed significantly from all others and demonstrated that the combination effectively supports embryogenic callus development and proliferation. In contrast, the combinations of 1 ppm 2,4-D with 2% and 5% sucrose resulted in the lowest fresh weights, recorded at 3.87 g and 3.77 g, respectively. These findings suggest that both hormonal stimulation and energy availability must be finely balanced to maximise callus biomass.

An increase in 2,4-D concentration from 1 ppm to 3 ppm tended to enhance fresh weight accumulation, particularly when combined with sucrose concentrations of 3% to 4%. This result is consistent with the findings of Situmorang et al. (2023), who reported that 2–3 ppm 2,4-D was effective in inducing callus formation in Phalaenopsis orchids. The increase in callus biomass is likely due to enhanced cell division and tissue expansion stimulated by sufficient auxin signalling. In parallel, the results align with the findings of Putri and Handayani (2021), who stated that moderate sucrose concentrations (3%–4%) can provide optimal energy for biomass synthesis and callus cell proliferation in Dendrobium tissue cultures. These concentrations also ensure the maintenance of osmotic balance, which is crucial for water uptake and metabolic function. Therefore, the synergistic effect of moderate auxin and sucrose levels creates a conducive environment for embryogenic callus development.

Additionally, the results are supported by Inayah (2017), who noted that sucrose concentrations between 3% and 4% represent optimal conditions for enhancing embryogenic callus production in orchids through increased synthesis of structural polysaccharides and secondary metabolites. Conversely, treatments

involving either 2% or 5% sucrose across all 2,4-D levels generally resulted in lower fresh weights. A 2% sucrose level may not sufficiently support energy-demanding processes such as cell proliferation and anabolic metabolism. Meanwhile, higher concentrations (e.g., 5%) may lead to osmotic stress that inhibits water absorption and interferes with mitotic activity. These outcomes reinforce the importance of finely tuning both hormonal and nutritional components of the culture medium. Establishing this balance is essential for promoting efficient biomass accumulation and enhancing the regenerative capacity of orchid Callus.

Colour and Texture of Embryogenic Callus

The application of various concentrations of 2,4-D and sucrose produced Callus with a spectrum of colours ranging from cream to bright green and with textures varying from friable to friable-compact (Table 3). These morphological characteristics reflect differences in physiological activity and developmental phase depending on the treatment applied. Such features provide useful criteria for preliminary screening of embryogenic competence prior to microscopic or molecular confirmation. Callus treated with 1 ppm 2,4-D and 2%–3% sucrose primarily displayed cream to light green pigmentation (Munsell notations 10YR 8/4 and 5GY 8/4), along with friable textures. These features indicate an early stage of callus growth with low cellular differentiation. Increasing the concentration of 2,4-D to 2–3 ppm and sucrose to 3%–5% resulted in Callus with brighter green colouration (5GY 7/4 to 5G 6/6) and more compact friable textures, which are characteristic of actively embryogenic tissues. The green pigmentation is indicative of chloroplast accumulation and the transition of callus tissue into the embryogenic phase (Muliati et al., 2017, in Sasmita et al., 2022). Treatments involving 4 ppm 2,4-D combined with 4%–5% sucrose produced Callus with deeper green pigmentation and firmer friable-compact textures. These features suggest a more advanced embryogenic state, possibly poised for somatic embryo differentiation (George & Debergh, 2008).

The morphological characteristics of Callus were clearly influenced by the interaction between 2,4-D and sucrose concentrations. Treatments involving 2–4 ppm 2,4-D and 3%–5% sucrose consistently generated Callus with green pigmentation and friable-compact textures—morphological indicators typically associated with embryogenic competence. This combination appears to be the most effective in supporting somatic embryo formation. However, in certain cases, the development of dark green Callus was observed, which may not be ideal for regeneration. Such pigmentation could reflect hormonal stress or osmotic imbalance, potentially limiting embryogenic progression. Therefore, colour and texture not only serve as diagnostic features but also provide insight into the physiological conditions and potential developmental trajectory of the callus tissue.

V. Conclusion

This study demonstrated that the combination of 3 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) and 4% sucrose significantly enhanced somatic embryogenesis in Dendrobium sp. under in vitro conditions. This treatment yielded the highest survival rate of embryogenic Callus (88%), the greatest callus formation (77.67%), the earliest initiation time (28.33 days after culture), and the highest fresh biomass (6.30 g). Morphologically, the Callus exhibited a light green to green hue with a friable-to-compact texture, which are characteristic indicators of high embryogenic competence. These results suggest that the synergistic regulation of auxin concentration and carbohydrate availability plays a critical role in optimising somatic embryogenesis in Dendrobium orchids.

Based on the findings, the 3 ppm 2,4-D + 4% sucrose formulation is recommended as a baseline protocol for the induction of embryogenic Callus in Dendrobium sp. via somatic embryogenesis. For future research, it is suggested to advance the protocol toward complete plantlet regeneration and to investigate the expression of embryogenesis-related genes (e.g., LEC1, BBM, SERK) as molecular markers of totipotency. Additionally, applying this protocol to other local Dendrobium cultivars could support mass propagation and conservation efforts, contributing to the development of a sustainable orchid biotechnology platform in Indonesia and Southeast Asia.

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