Original Research Article

Investigating the Impact of Copper Sulfate on the *In Vitro* Propagation of Anthurium (*Anthurium andraeanum* Lind.)

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ABSTRACT

Aims: Anthurium (*Anthurium andraeanum* Lind.) is a valued ornamental plant, but efficient mass propagation methods are lacking. This study investigated the impact of copper sulfate ($CuSO_4$) on its *in vitro* propagation efficiency.
Study design: Complete Randomized Design (CRD)
Place and Duration of Study: Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, between November 2023 and June 2024.
Methodology: Callus induction and shoot regeneration were assessed using various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), Benzyladenine (BA), and $CuSO_4$. Callus induction from sterilized leaf explants and callus proliferation were conducted using Murashige and Skoog (MS) basal medium supplemented with concentrations of 2,4-D, BA, and $CuSO_4$ in the dark. Shoot regeneration from the calli was performed on a modified MS medium with different concentrations of growth regulators and $CuSO_4$ under light. Regenerated shoots were then transferred to a medium enriched with $CuSO_4$ for rooting, and the survival rate of the acclimatized plants was assessed.
Results: The results show $CuSO_4$ significantly enhanced callus induction and shoot regeneration rates. The optimal medium for callus induction consisted of MS medium with 0.3 mg/L 2,4-D, 1.0 mg/L BA, and 5.0 mg/L $CuSO_4$, leading to rapid induction (46 days) and a high induction percentage (86.67%) of calli. For shoot regeneration, the most effective medium contained 0.1 mg/L 2,4-D, 0.75 mg/L BA, and 5.0 mg/L $CuSO_4$, showing superior regeneration efficiency in total shoot number and average shoot length. The success of root initiation and acclimatization improved notably in plants derived from $CuSO_4$ treated media, with a survival rate of 92%.
Conclusion: This study highlights the significant role of $CuSO_4$ in enhancing the *in vitro* propagation of *Anthurium andraeanum* Lind., with optimal callus induction and shoot regeneration observed at specific concentrations. The inclusion of $CuSO_4$ led to rapid callus formation, superior shoot regeneration, and a high survival rate during acclimatization.

Keywords: Anthurium andraeanum, in vitro propagation, copper sulfate, callus induction, shoot regeneration, medium optimization

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1. INTRODUCTION

Anthurium (*Anthurium andraeanum* Lind.) is a popular ornamental plant valued for its vibrant and long-lasting nature and high postharvest durability of flowers [1]. The demand for Anthurium in the horticultural industry has been steadily increasing due to its aesthetic appeal and adaptability to indoor environments. The global sales volume of Anthurium ranks

second worldwide, trailing only behind orchids [2]. However, traditional methods of propagating Anthurium, such as seed and vegetative propagation, are limited in their efficiency and the time needed for propagation [3]. Anthurium was first propagated *in vitro* by Pierik in 1974 [4]. Even though *in vitro* propagation techniques offer a promising alternative for mass production, the efficiency of these methods for Anthurium has not yet been optimized.

The current state of *in vitro* propagation techniques for Anthurium is faced with challenges that hinder its optimal efficiency. These challenges include poor shoot multiplication rates, low rooting success, and high phenotypic variation. To address these limitations, there is a need to explore novel strategies that enhance the efficiency of *in vitro* propagation of Anthurium. Modifications in the composition of *in vitro* media are crucial for achieving maximum *in vitro* efficiency in Anthurium propagation. Optimizing and modifying nutrient concentrations, including macro and micronutrients, vitamins, and growth regulators, is essential to promote shoot multiplication and improve rooting success. Supplementing the media with additives like $CuSO_4$ may enhance shoot multiplication rates and reduce phenotypic variations [5].

The primary research problem revolves around the fact that the current *in vitro* propagation techniques for Anthurium have not reached an optimized level of efficiency. The challenges mentioned earlier contribute to this problem, impeding the production of Anthurium plants in large quantities with consistent quality and characteristics.

1.1 Objectives

The objective of this research is to improve the *in vitro* growth efficiency of Anthurium through the addition of copper sulfate to the culture media and to establish a highly efficient *in vitro* protocol for Anthurium propagation. This objective aims to evaluate specially the effects of copper sulfate on callus induction and shoot multiplication in Anthurium *in vitro* cultures. A comprehensive protocol encompassing shoot multiplication, rooting, and acclimatization stages will be established. The research aims to optimize *in vitro* propagation techniques by achieving this objective, leading to the mass production of high-quality Anthurium plants with uniform characteristics.

2. MATERIAL AND METHODS

2.1 Explant Materials and Aseptic Culture

Newly emerging immature very young leaves from vigorous and healthy mother plant stock maintained in the protected house were selected as explant materials for aseptic culture. The leaves were carefully excised and prepared for surface sterilization. Initially, any dirt or debris on the leaves was removed by cleaning them with detergent and rinsing them with running tap water for 30 minutes.

For surface sterilization, the leaves were subjected to a sequential treatment using 70% alcohol for 30 seconds. They were then transferred into 1% (v/v) sodium hypochlorite with 2-3 drops of Tween 20 for 10 minutes followed by 1% (v/v) sodium hypochlorite without Tween 20 for another 10 minutes with gentle agitation. Finally, the leaves were rinsed three times with sterile distilled water to remove residual sterilizing agents. The surface-sterilized leaves were then sectioned into approximately 1.0 cm^2 pieces.

2.2 Experiment 1 - Callus Induction and Proliferation

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Callus induction and development in *Anthurium andraeanum* Lind. were conducted using a Murashige and Skoog (MS) basal medium [6] supplemented with vitamins, 3% sucrose, and 3 g/L Gelrite. The media were further enriched with varying concentrations of 0 mg/L, 0.3 mg/L, 0.6 mg/L, 0.9 mg/L, and 1.2 mg/L 2,4-D in combination with a fixed concentration of 1.0 mg/L BA. Two different concentrations of $CuSO_4$, 0 mg/L, and 5.0 mg/L, were also incorporated into the media. This systematic approach aimed to determine the most effective combinations of 2,4-D, BA, and $CuSO_4$ in MS medium for promoting successful callus formation and development. The pH of the media was adjusted to 5.8.

For the initiation of callus, two pieces of surface-sterilized leaves (1.0 cm^2 each) were placed in culture bottles containing 40 ml of the prepared media, with the abaxial side down on the medium [7]. The bottles were sealed and the cultures were incubated under a dark condition with an average temperature of 27°C. [8-10]. During the culture period, careful observations were made to identify the changes in explants and the media. After one month from the initiation of culture, data collection was carried out on the days to callus induction and the callus morphology in each treatment. After 2 weeks from the callus initiation, callus induction frequency (%) was calculated and then, the calli were sub-cultured using the new medium containing the same composition for callus proliferation.

2.3 Experiment 2 - Shoot Regeneration

Following the data collection of callus development, the greenish white, compact, and granular calli formed from the most responsive callus induction media were identified. The selected callus pieces, approximately 0.5 - 0.6 cm^2 in size, were then inoculated into autoclaved culture bottles containing 40 ml of shoot regeneration medium. This medium consisted of modified half-strength MS salts, with ammonium nitrate (NH_4NO_3) reduced to 250 mg/L, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L thiamine, 0.5 mg/L folic acid, 0.05 mg/L biotin, 30 mg/L ferric sodium EDTA (Fe-NaEDTA), and 20 g/L sucrose. The media were prepared with different combinations of 2,4-D, BA, and $CuSO_4$ concentrations to determine their influence on shoot regeneration. Specifically, the media were supplemented with 5 different concentrations of BA: 0.5 mg/L, 0.75 mg/L, 1.0 mg/L, 1.25 mg/L, and 1.5 mg/L, in combination with a fixed concentration of 0.1 mg/L 2,4-D. Additionally, two different concentrations of $CuSO_4$ were used 0 mg/L and 5.0 mg/L.

Each culture bottle contained two callus pieces from the selected calli, allowing for simultaneous evaluation of shoot regeneration. The culture bottles were then placed in a controlled environment at a temperature of 27°C, under a 16/8-hour light and dark photoperiod, for a duration of 4 weeks. At the end of the 4-weeks, data were collected on two parameters including a total number of shoots per treatment and average shoot length. Regeneration percentage was then calculated.

2.4 Rooting and acclimatization

Regenerated shoots longer than 2 cm with a pair of true leaves obtained from the best-regenerated medium were transferred to rooting media consisting of the same basal medium used during shoot regeneration. The medium was supplemented with 1.0 mg/L Indole-3-butyric acid (IBA) and 0.04% active charcoal to initiate and development of roots from regenerated shoots [8]. Root regeneration was carried out at 27°C under a 16/8-hour light and dark photoperiod for 4 weeks. After root development, the rooted Anthurium plants were acclimatized by transferring them to pots with a sterilized potting medium containing coconut husk. The plants were gradually exposed to ambient conditions, receiving proper care. After a period of 4 weeks of transferring to *in-vivo* conditions, the survival percentage of the acclimatized plants was calculated.

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2.5 Data Analysis

All experiments were conducted following a Complete Randomized Design (CRD) with three replicates per treatment, each consisting of two bottles. Data analysis was carried out using Analysis of Variance (ANOVA) with SAS 9.0 software, and mean separation was conducted using Duncan's multiple range test at a significance level of P = .05.

3. RESULTS AND DISCUSSION

3.1 Experiment 1 - Callus induction of *Anthurium andraeanum*

The results of the experiments conducted on *Anthurium andraeanum* using newly emerging immature leaf explants cultured on MS basal medium supplemented with various combinations of 2,4-D, BA, and $CuSO_4$ for callus induction and development are presented in Table 1.

Results pertaining in Table 1 show that explants exposed to MS medium with 0.3 mg/L 2,4-D, 1.0 mg/L BA and 5.0 mg/L $CuSO_4$ (treatment 7) and MS medium with 0.6 mg/L 2,4-D, 1.0 mg/L BA and 5.0 mg/L $CuSO_4$ (treatment 8) take significantly the least number of days to induce callus. However, treatment 7 and 8 are not significantly different in terms of days to initiate calli. Accordingly, it is obvious that comparatively less concentration of 2,4-D initiates callus quickly along with 1.0 mg/L BA, and 5.0 mg/L $CuSO_4$. In terms of callus induction, treatment 7 showed a significantly higher callus induction percentage (86.67%) and resulted greenish white compact callus where $CuSO_4$ has shown positive impacts on both accelerating callus induction and increasing callus induction percentage. Nevertheless, these positive impacts were diminished with the higher concentration of 2,4-D in the medium. This is attributable to the suppression of the callus initiation due to the residual effect of 2,4-D on de-differentiation in the mitotic stage at a high concentration of 2,4-D [11]. A similar reduction in callus induction with increasing concentrations of 2,4-D was reported in *japonica* and *indica* rice, attributed to the browning or necrosis of cells for the same reason [12]. Hence, MS basal medium with 0.3 mg/L 2,4-D, 1.0 mg/L BA, and 5.0 mg/L $CuSO_4$ is the best medium composition for callus induction showing 46 days to callus induction and 86.67% callus induction rate of *Anthurium andraeanum* Lind.

Table 1. Influence of different BA, 2,4-D and CuSO4 on callus induction of *Anthurium andraeanum*

Treatment No	Treatment	Days to callus induction		Callus induction (%)		Appearance of the callus
1	MS+1.0 mg/L BA	100.00 ± 1.00	a[1]	6.67 ± 3.33	e	Yellowish white friable
2	MS+0.3 mg/L 2,4-D+1.0 mg/L BA	99.33 ± 0.67	a	20.00 ± 5.77	de	Greenish White friable
3	MS+0.6 mg/L 2,4-D+1.0 mg/L BA	88.67 ± 2.33	b	60.00 ± 10.00	b	Greenish White Compact
4	MS+0.9 mg/L 2,4-D+1.0 mg/L BA	74.00 ± 2.65	c	50.00 ± 5.77	bc	Yellowish Brown Compact
5	MS+1.2 mg/L 2,4-D+1.0 mg/L BA	79.00 ± 1.00	c	16.67 ± 3.33	de	Brownish friable

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6	MS+1.0 mg/L BA+5.0 mg/L $CuSO_4$	61.50 ± 3.50	d	6.67 ± 3.33	e	Greenish White Compact
7	MS+0.3 mg/L 2,4-D, 1.0 mg/L BA+ 5.0 mg/L $CuSO_4$	46.00 ± 2.08	e	86.67 ± 8.82	a	Greenish White Compact
8	MS+0.6 mg/L 2,4-D, 1.0 mg/L BA+ 5.0 mg/L $CuSO_4$	45.67 ± 2.19	e	50.00 ± 5.77	bc	Greenish White Compact
9	MS+0.9 mg/L 2,4-D, 1.0 mg/L BA+ 5.0 mg/L $CuSO_4$	75.00 ± 3.00	c	33.33 ± 8.82	cd	Yellowish Brown nodular
10	MS+1.2 mg/L 2,4-D, 1.0 mg/L BA+ 5.0 mg/L $CuSO_4$	97.67± 2.03	a	16.67 ± 3.33	de	Yellowish compact

[1]Means followed by the same small letters in the same column are not significantly different at 5% level in Duncan's Multiple Range Test.

MS – Murashige and Skoog basal medium [6]

3.2 Experiment 2 - Shoot regeneration of *Anthurium andraeanum*

The results of the calli, obtained from the screened callus induction medium (Treatment 7), subsequently transferred to a modified half-strength MS basal medium with different combinations of 2,4-D, BA, and $CuSO_4$ for *in vitro* shoot regeneration are given in Table 2.

Results from Table 2 showed the variations of shoot regeneration efficacy from Anthurium calli under different compositions of 2,4-D, BA, and $CuSO_4$ in the medium. Based on the findings, the regeneration percentage, total number of shoots per treatment, and average shoot length were increased with the addition of $CuSO_4$ to the medium. However, these positive impacts observed with $CuSO_4$ diminished with the increase of the concentration of BA in the medium. This was confirmed even without $CuSO_4$ by previous findings. The highest plant regeneration in *Anthurium andraeanum* was observed in a modified MS medium containing 1.0 mg/L BA and it declined when the BA concentration was increased [13]. Accordingly, calli culture on the modified MS medium containing 0.1 mg/L 2,4-D, and 0.75 mg/L BA supplemented with 5.0 mg/L $CuSO_4$ show significantly higher shoot regeneration efficiency in terms of a total number of shoots and average shoot length which is much higher than the previously reported values. Hence, the addition of $CuSO_4$ has significantly enhanced the i*n vitro* performance by improving the efficiency and effectiveness of the processes involved. This improvement is evidenced by increased reaction rates and higher yields of desired outcomes, demonstrating the beneficial impact of $CuSO_4$ on *in vitro* propagation of Anthurium.

Table 2. Influence of different BA, 2,4-D and CuSO4 on shoot regeneration of *Anthurium andraeanum*

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Treatment No	Treatment	Regeneration percentage (%)	Total number of shoots per treatment	Average Shoot length (cm)
1	MMS+0.1mg/L 2,4-D+0.5 mg/L BA	41.67 ± 8.33 de[1]	8.67 ± 2.40 f	1.20 ± 0.12 cd
2	MMS+0.1 mg/L 2,4-D+0.75 mg/L BA	50.00 ± 0.00 cde	32.67 ± 3.93 de	1.97 ± 0.07 bc
3	MMS+0.1 mg/L 2,4-D+1.0 mg/L BA	66.67 ± 8.33 bc	64.00 ± 6.11 b	2.87 ± 0.39 ab
4	MMS+0.1 mg/L 2,4-D+1.25 mg/L BA	58.33 ± 8.33 cd	40 .00± 8.96 cde	1.97 ± 0.18 bc
5	MMS+0.1 mg/L 2,4-D+1.5 mg/L BA	33.33 ± 8.33 e	10.33 ± 5.21 f	0.97 ± 0.29 d
6	MMS+0.1 mg/L 2,4-D+ 0.5 mg/L BA+5.0 mg/L $CuSO_4$	50.00 ± 0.00 cde	59.00 ± 7.37 bc	1.57 ± 0.28 cd
7	MMS+0.1 mg/L 2,4-D+ 0.75 mg/L BA+5.0 mg/L $CuSO_4$	91.67 ± 8.33 a	103.33 ± 7.88 a	3.40 ± 0.21 a
8	MMS+0.1 mg/L 2,4-D+ 1.0 mg/L BA+5.0 mg/L $CuSO_4$	83.33 ± 8.33 ab	96.33 ± 3.76 a	1.97 ± 0.38 bc
9	MMS+0.1 mg/L 2,4-D+ 1.25 mg/L BA+ 5.0 mg/L $CuSO_4$	58.33 ± 8.33 cd	53.33 ± 9.74 bcd	1.80 ± 0.53 cd
10	MS+0.1 mg/L 2,4-D+ 1.5 mg/L BA+5.0 mg/L $CuSO_4$	33.33 ± 8.33 e	23.67 ± 8.01 ef	1.73 ± 0.22 cd

[1]*Means followed by the same small letters in the same column are not significantly different at 5% level in Duncan's Multiple Range Test.*

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MMS – Modified Murashige and Skoog basal medium [6]

The results of the present study clearly showed that the incorporation of $CuSO_4$ into the MS medium positively affected both callus induction and regeneration of shoots. These findings revealed that the copper level in the MS basal medium is not optimum for the callus induction and regeneration of shoots in *Anthurium andreanum*. This result is supported by the numerous studies reported previously. The highest efficiency in callus proliferation and *in vitro* regeneration in *indica* rice were observed in the medium supplemented with $CuSO_4$ [5]. Increasing the $CuSO_4$ concentration in the medium dramatically increased the amount of callus and the number of plantlets regenerated in *Sorghum bicolor* (L) [14]. Both callus dry weight, fresh weight, and total phenolic content of *Artemisia annua* were increased by enhancing the medium with $CuSO_4$ [15]. $CuSO_4$ increased the mean number of regenerated plants per explant constantly in *Oryza sativa* L [16]. The optimization of copper levels resulted in increased plant regeneration from callus cultures of Barley as well [17].

3.3 *In vitro* Rooting

The new shoots generated from the highly effective regeneration medium were subsequently transferred to a rooting medium enriched with $CuSO_4$. The medium shows higher efficiency in root initiation during the 4-week period. After 4 weeks from acclimatization, the survival percentage of the plants was 92%. Copper is a component of plastocyanin, a protein that operates as an electron carrier between the two photosystems in the photosynthetic electron transport chain and superoxide dismutase, Cu-containing chloroplast protein which also influences the electron transport chain by redirecting harmful free radicals away from the thylakoid membrane [18]. Hence, copper ions directly involved in the increase of photosynthetic efficiency in plants. Thus, it might be a reason for the higher survival percentage obtained from the acclimatized plantlets which are obtained from the $CuSO_4$ treated media. Additionally, the carryover effect of $CuSO_4$ and its synergistic effect on plant growth regulators used in the medium may improve the survival rate, morphological characteristics, and overall performance of plants, resulting in more uniform traits.

Copper is recognized as a vital micronutrient due to its regulatory effect on plant growth and development and it is a component or activator of many enzymes involved in electron transport, protein and carbohydrate biosynthesis, and polyphenol metabolisms and these enzymes might play an important role in regeneration (14, 16). Hence, it has specific role in the plant cells in terms of growth and development. This study also proves these findings by enhancing the *in vitro* growth efficiency of Anthurium, specifically in terms of callus induction, shoot regeneration, and survival rate through the incorporation of $CuSO_4$. Even though, $CuSO_4$ has positive impacts on plant tissue culture in various ways, an excessive level of copper can be toxic to plant cells [15].

4. CONCLUSION

The MS basal medium supplemented with 5.0 mg/L $CuSO_4$ significantly accelerated callus induction and shoot regeneration in *Anthurium andraeanum* Lind. However, these positive impacts on callus induction and shoot regeneration were diminished with the higher concentration of 2,4-D and BA respectively in the media. Accordingly, the optimal medium composition for callus induction consists of MS basal medium with 0.3 mg/L 2,4-D, 1.0 mg/L BA, and 5.0 mg/L $CuSO_4$, while the most effective composition for shoot regeneration is a modified MS medium containing 0.1 mg/L 2,4-D, and 0.75 mg/L BA, supplemented with 5.0 mg/L $CuSO_4$. In addition, the regeneration of roots and the survival rate of the acclimatized

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plants obtained from the $CuSO_4$ treated *in vitro* media were much higher in *Anthurium andraeanum* Lind compared with previously published reports.

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