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Influence of copper and zinc sulfates on *in vitro* propagation efficiency of orchids (*Dendrobium phalaenopsis*): A step towards optimizing clonal propagation protocols

K.P.G. Dilshika Madushani Polwaththa and A.A. Yasarathna Amarasinghe *

Department of Export Agriculture, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, Belihuloya, Sri Lanka.

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Abstract

The Orchidaceae family, particularly *Dendrobium* orchids, plays a significant role in the global floriculture industry, yet developing efficient clonal propagation protocols is challenging. This study investigates the effects of copper sulfate ($CuSO_4$) and zinc sulfate ($ZnSO_4$) supplementation on the *in vitro* propagation of *Dendrobium phalaenopsis* orchids. Various concentrations of $CuSO_4$ (2.0, 4.0, 6.0, and 8.0 mg/L) and $ZnSO_4$ (4.0, 8.0, and 12.0 mg/L) were tested individually and in combination using Murashige and Skoog's (MS) media, supplemented with different auxins and a constant cytokinin level. *Dendrobium* seeds (0.01 g per culture bottle) served as the starting material, with germination recorded by the first appearance of protocorms. Media with 6.0 mg/L $CuSO_4$ resulted in the fastest protocorm emergence (31.56 days), while the combination of 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ proved most effective overall, yielding a shorter germination time of 39 days, the highest shoot production (14 shoots), and the tallest shoots (12.56 cm) one-month post-germination. This combination also achieved the highest shoot multiplication rate (27.33) and enhanced chlorophyll content (2.41 mg/g) three months after transfer to multiplication media. Notably, during root formation, this treatment accelerated root initiation (31.11 days) and improved root vigor. The carryover effects of $CuSO_4$ and $ZnSO_4$ from *in vitro* to *in vivo* conditions were evident, with a 92.22% survival rate and no morphological differences from seed-grown plants. These findings highlight the synergistic benefits of $CuSO_4$ and $ZnSO_4$ in establishing an efficient method for orchid propagation with improved success rates in both *in vitro* and *in vivo* environments.

Keywords: *Dendrobium*; Copper sulfate; Zinc sulfate; Synergistic effect; Carry over effect

1. Introduction

The Orchidaceae family, with approximately 750 genera and 28,000+ species [1], is the largest and one of the most complex plant families. Members of this diverse group are found on every continent except Antarctica, with the greatest species richness in tropical regions such as Southeast Asia, South America, and Central America. Orchids exhibit an extraordinary range of growth habits; approximately 70% of all orchid species are epiphytes, meaning they grow on other plants, but terrestrial, aquatic, and lithophytic species are also present [2]. Among the Orchidaceae, the *Dendrobium* genus is particularly notable due to its vast diversity in vegetative and floral traits, and it is a key component of the global floriculture industry. The high ornamental value of *Dendrobium* hybrids has made them one of the most sought-after genera in horticulture, particularly for their use in flower arrangements and as potted plants [3].

One of the defining features of orchids is their seeds, which are uniquely adapted for wind dispersal. Orchid seeds are extremely small and contain an undifferentiated embryo that lacks the enzymes required to metabolize polysaccharides, which are necessary for germination [4-5]. The seed coat, is thin yet hard, and although orchid embryos contain sugars such as sucrose, fructose, and glucose, these sugars are present in insufficient quantities to support germination and plantlet development [4]. In nature, orchids often rely on a symbiotic relationship with mycorrhizal fungi to facilitate

* Corresponding author: A.A.Y. Amarasinghe

germination, but *in vitro* culture techniques allow researchers to bypass this requirement by providing artificial nutrients and conditions necessary for seed germination and growth.

Despite the advances in tissue culture techniques, the *in vitro* propagation of *Dendrobium* species has not yet reached optimal efficiency. Several propagation methods have been attempted using various explants, such as shoot tips [6], protocorms and protocorm-like bodies [7], nodal segments [8], seeds [9], and callus tissues [10], but these methods are often hindered by long culture periods, low rates of survival, and limited success in obtaining healthy, fully developed plantlets. In particular, propagation via seed culture in *Dendrobium* has yet to consistently yield high survival rates, and improving *in vitro* protocols remains a key challenge in orchid cultivation.

Micronutrients such as copper (Cu) and zinc (Zn) play a crucial role in plant tissue culture, particularly in enhancing plant growth and development. Copper has been identified as an important element in tissue culture protocols for various crops, such as rice [11] and anthurium [12]. Zinc, on the other hand, is essential for a variety of biosynthetic processes, including enzyme activation, protein synthesis, and DNA replication [13]. Both Cu and Zn have been shown to positively influence plant development when applied at optimal concentrations *in vitro*. Therefore, it is reasonable to hypothesize that incorporating copper sulfate and zinc sulfate into the culture medium could enhance the efficiency of *in vitro* propagation in *Dendrobium* orchids. This could lead to improved shoot proliferation, root formation, and overall plantlet survival, while also accelerating the germination process, ensuring quicker and more robust seedling development.

Objectives of the study

This study aims to investigate the effects of copper sulfate and zinc sulfate on the *in vitro* propagation of *Dendrobium phalaenopsis* species. Specifically, it will focus on the modification of the Murashige and Skoog (MS) basal medium by adding these micronutrients to determine their impact on the growth and development of *Dendrobium phalaenopsis* plantlets. By optimizing the concentrations of copper and zinc in the culture media, this research seeks to establish an improved clonal propagation protocol that could significantly enhance the survival rates of *Dendrobium phalaenopsis* plants when transferred to *in vivo* conditions.

2. Materials and methods

2.1. Source of plant materials and surface sterilization

Green, healthy capsules of *Dendrobium phalaenopsis*, seven months after hand-pollination, were collected from reliable private growers who regularly supply capsules for plant production. The capsules were obtained from greenhouses of these growers. They were immediately wrapped in aluminum foil and stored at 7 °C until further use.

Prior to surface sterilization, capsules were submerged in water containing a few drops of Teepol solution, followed by thorough rinsing under running tap water for 30–40 minutes to remove external debris and dirt. Surface sterilization was performed under aseptic conditions in a laminar air flow cabinet. The capsules were first immersed in 70% ethanol for 1 minute, then transferred to a 1% sodium hypochlorite solution containing two drops of Tween 20 for 5 minutes. This was followed by an additional 5-minute treatment in fresh 1% sodium hypochlorite solution without Tween 20. Capsules were subsequently rinsed 3–4 times with sterile double-distilled water. As a final sterilization step, the capsules were briefly flamed for two to three seconds after being dipped in 70% ethyl alcohol for 8 to 10 minutes. Using a sterile scalpel, the capsules were longitudinally split open and clusters of immature orchid seeds were extracted.

The shoots from the optimal germination media, identified at the end of the establishment stage, were selected for transfer to the multiplication media. Similarly, shoots from the most effective multiplication media, screened at the end of the multiplication stage, were chosen for transfer to the rooting media. To ensure an adequate supply of explants for the initiation of each *in vitro* stage, additional stock cultures were maintained for each treatment throughout the experimental period under the same conditions provided to the replicates.

2.2. Media Preparation

All media were prepared using the Murashige and Skoog's (MS) basal medium [14], supplemented with vitamins, 3% sucrose, and 7.0 g/L agar (Vetec®). To investigate the individual effects of copper sulfate ($CuSO_4$) and zinc sulfate ($ZnSO_4$), the basal media were supplemented with four concentrations of $CuSO_4$ (2.0 mg/L, 4.0 mg/L, 6.0 mg/L, and 8.0 mg/L) and three concentrations of $ZnSO_4$ (4.0 mg/L, 8.0 mg/L, and 12.0 mg/L), separately. Additionally, the same concentrations of $CuSO_4$ and $ZnSO_4$ were combined in all possible combinations to explore their interaction effects.

The media with $CuSO_4$ or $ZnSO_4$ or their combinations were supplemented with a fixed concentration of 0.1 mg/L BA throughout the culture period, along with varying types and concentrations of auxins: 0.2 mg/L 2,4-D for germination media, 0.2 mg/L NAA for multiplication media, and 0.1 mg/L IBA for rooting media, respectively. The pH of the media was adjusted to 5.8. All media were then sterilized by autoclaving at 121 ^{0}C and 1.1 kg/cm^2 for 20 minutes.

2.3. Culture Conditions

All culture bottles, during the germination, multiplication, and rooting stages, were carefully sealed and maintained at a controlled temperature of 25 ± 1 ^{0}C. The cultures were subjected to a consistent 16/8-hour light/dark cycle, with a light intensity of 36 µmol/m^2/s, ensuring optimal growth conditions throughout each stage of development until they were ready to be transferred to the next phase of the process.

2.4. Establishment stage (Seed germination)

Under sterile conditions in a laminar airflow cabinet, 0.01 g of seeds were cultured in each bottle containing 40 mL of the various germination media. Throughout the culture period, careful observations were made to monitor changes in the explants and media. Germination was marked by the development of a protocorm, a tuberiform structure typical of orchid seed germination [15]. Daily observations were conducted to record the number of days taken for the first protocorm to emerge, indicating seed germination.

Furthermore, the number of shoots produced per 0.01g of cultured seeds, along with their height, was recorded one month after germination, just prior to transferring them to the multiplication media.

2.5. Multiplication stage (Shoot multiplication)

One-month-old shoots grown in high-performance germination media were transferred to culture bottles containing 40 ml of multiplication media, with three shoots per bottle. Data collection took place one month after the transfer to the multiplication media, where the number of new shoots produced was recorded, and the multiplication rate was calculated as given below.

$$Multiplication\ Rate\ = \frac{Total\ number\ of\ new\ plants\ or\ shoots}{Initial\ number\ of\ plants\ or\ shoots}$$

Shoot height and leaf chlorophyll content were measured three months after the shoots were transferred to multiplication media, just before they were transferred to the rooting media. To assess chlorophyll content, 0.1 g of leaf material was extracted using 30 mL of 80% acetone. The extracts were filtered, diluted to 50 mL, and analyzed using a spectrophotometer at wavelengths of 645 nm and 663 nm. The total chlorophyll content was then calculated using Arnon's Method [16], based on absorbance measurements and applying the formula as given below.

Total Chlorophyll (mg/L) = 20.2 (A_{645}) + 8.02 (A_{663})

Where: A_{645} and A_{663} are the absorbances measured at 645 nm and 663 nm, respectively.

$$Chlorophyll\ content\ (mg/g) = \frac{Chlorophyll\ concentration\ (mg/L)\ X\ Volume\ of\ solvent\ (L)}{Mass\ of\ tissue\ (g)}$$

2.6. Rooting stage (*In vitro* rooting)

Shoots from high-performing multiplication media were selected 3 months after growing on these media and transferred into culture bottles, each containing 40 ml of rooting medium, with five shoots per bottle. In addition to the compositions of the rooting media described above, 3.0 g/L of activated charcoal was added at a lower concentration than recommended to promote better rooting and allow easier observation of root initiation and development. Careful observations were made from the day the shoots were transferred to the rooting media, specifically monitoring the first root initiation to record the days taken for root emergence in each treatment containing $CuSO_4$, $ZnSO_4$, and their combinations. The number and length of roots were also recorded two months after the shoots were transferred to the rooting media, just before weaning the plants for acclimatization.

2.7. Acclimatization stage (*In vivo* growth of plants)

The plantlets two months after initiation of roots grown *in vitro* rooting media were carefully removed from the culture bottles and placed in lukewarm water containing Captan, a fungicide, to remove any agar clots attached to the roots and

to protect them from fungal attack during the initial stages. Thirty plants from each treatment were then planted in pots, treatment-wise, containing a sterilized potting mixture of 50% Sphagnum moss, 30% fine vermiculite, and 20% coir fiber. The plants were placed inside a humidity dome to maintain a high humidity level of 90-100% initially, with gradual airflow introduced over the course of a week to acclimatize them to outdoor conditions. All necessary aftercare measures were applied, and the survival rates (%) for each treatment were calculated one month after transferring the rooted plants to *in vivo* conditions.

2.8. Data Analysis

All experiments were conducted following a Complete Randomized Design (CRD) with three replicates per treatment, each consisting of three 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 = 0.05.

3. Results and discussion

3.1. Seed Germination

The results of germinating 0.01g of orchid seeds cultured per bottle across 20 different treatments, with germination media supplemented with $CuSO_4$, $ZnSO_4$, or their combinations, are summarized in Table 1. The shortest time for the first protocorm to emerge, indicating seed germination, was 31.56 days in the medium containing 6.0 mg/L $CuSO_4$. Seeds cultured in a medium containing both 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ exhibited the second shortest germination time of 39 days. Both results are significantly different from the other treatments at P = 0.05. These findings suggest that $CuSO_4$ has a positive effect on orchid seed germination when optimized in the medium, whereas $ZnSO_4$ alone does not exhibit a similar effect. However, when both $CuSO_4$ and $ZnSO_4$ are used together in the germination medium, the amount of $CuSO_4$ required can be reduced if $ZnSO_4$ is optimized, while still achieving similar effects as using $CuSO_4$ alone. This is because copper plays a vital role in seed germination by regulating physiological and biochemical processes, although excessive levels can reduce or suppress germination.

This was also demonstrated by studies on copper nanoparticles in the *in vitro* seed germination of wheat (*Triticum aestivum* L.), where it was reported that copper nanoparticles can enhance seed germination rates and seedling growth by improving water uptake and promoting enzymatic activities necessary for seed metabolism [17].

Table 1 Effects of $CuSO_4$ and $ZnSO_4$ on *in vitro* seed germination and seedling growth of *Dendrobium phalaenopsis*

Treatment Number	Treatment	Days to Seed Germination[1]		Number of Shoots [2]		Height of Shoots [3] (mm)	
1	MS+1mg/L BA+0.2mg/L 2,4-D (C) - Control	61.67 ± 2.00	a[4]	4.89 ± 1.17	def	9.44 ± 0.69	cb
2	C+2mg/L $CuSO_4$	55.56 ± 2.22	bc	4.11 ± 1.07	f	9.56 ± 0.84	cb
3	C+4mg/L $CuSO_4$	54.89 ± 0.51	bcd	4.44 ± 1.02	ef	9.56 ± 1.02	cb
4	C+6mg/L $CuSO_4$	31.56 ± 1.35	g	7.89 ± 0.69	c	9.44 ± 0.51	cb
5	C+8mg/L $CuSO_4$	49.33 ± 0.58	e	4.11 ± 1.54	f	9.67 ± 1.20	cb
6	C+4mg/L $ZnSO_4$	58.78 ± 0.19	ab	4.78 ± 0.51	ef	7.89 ± 2.22	c
7	C+8mg/L $ZnSO_4$	54.78 ± 1.71	cd	6.56 ± 0.77	cd	8.89 ± 0.69	cb
8	C+12mg/L $ZnSO_4$	55.11 ± 0.38	bc	4.56 ± 0.19	ef	8.89 ± 0.84	cb
9	C+2mg/L $CuSO_4$+4mg/L $ZnSO_4$	55.22 ± 0.19	bc	4.67 ± 0.33	ef	8.78 ± 0.51	cb
10	C+2mg/L $CuSO_4$+8mg/L $ZnSO_4$	51.00 ± 1.73	de	4.33 ± 0.67	f	9.67 ± 0.88	cb
11	C+2mg/L $CuSO_4$+12mg/L $ZnSO_4$	52.89 ± 1.17	cde	4.67 ± 0.88	ef	9.22 ± 0.77	cb
12	C+4mg/L $CuSO_4$+4mg/L $ZnSO_4$	53.56 ± 0.38	cd	4.22 ± 0.51	f	9.22 ± 1.07	cb
13	C+4mg/L $CuSO_4$+8mg/L $ZnSO_4$	52.89 ± 0.51	cde	10.11 ± 1.02	b	9.89 ± 0.38	cb
14	C+4mg/L $CuSO_4$+12mg/L $ZnSO_4$	39.00 ± 6.96	f	14.00 ± 0.67	a	12.56 ± 0.51	a

15	C+6mg/L $CuSO_4$+4mg/L $ZnSO_4$	52.44 ± 3.02	cde	6.22 ± 1.50	de	10.78 ± 1.02	b
16	C+6mg/L $CuSO_4$+8mg/L $ZnSO_4$	52.67 ± 0.33	cde	5.11 ± 0.38	def	9.11 ± 0.51	cb
17	C+6mg/L $CuSO_4$+12mg/L $ZnSO_4$	55.33 ± 0.88	bc	5.11 ± 1.02	def	9.11 ± 2.17	cb
18	C+8mg/L $CuSO_4$+4mg/L $ZnSO_4$	52.67 ± 0.58	cde	4.56 ± 0.77	ef	9.44 ± 0.96	cb
19	C+8mg/L $CuSO_4$+8mg/L $ZnSO_4$	59.33 ± 2.19	a	3.56 ± 0.51	f	9.11 ± 1.07	cb
20	C+8mg/L $CuSO_4$+12mg/L $ZnSO_4$	61.33 ± 2.19	a	4.22 ± 1.71	f	9.00 ± 0.33	cb

1. Average number of days to emerge the first protocorm; 2. Average number of shoots produced per 0.01g seeds cultured in the media one (01) month after germination (at the time of transferring them to the multiplication media); 3. Average height of shoots one (01) month after germination (at the time of transferring them to the multiplication media); 4. 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's basal medium

Copper also stimulates enzymatic activities by enhancing nutrient availability to provide energy for germination. This was supported by previous experiments on cotyledonary carbohydrate status during bean seed germination [18], which noted that copper regulates cotyledonary carbohydrate status during germination, aiding in the mobilization of nutrients necessary for seedling development. Importance of $CuSO_4$ was also reported by the studies of the impact of $CuSO_4$ on the *in vitro* propagation of anthurium [12]. Conversely, the negative effects of copper at higher concentrations were reported in research on rice seed germination, where the interaction of copper with hormones such as abscisic acid (ABA) suppressed seed germination under copper stress conditions [19]. Also, it was reported that $ZnSO_4$ enhances improving water absorption, enzymatic activities, and mobilization of nutrients [20-21] which is important for seed germination.

One month after germination, the average number of shoots (14) produced per 0.01 mg seeds was significantly higher (P=0.05) in the medium supplemented with 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$. The second highest average number of shoots (10.11) was recorded in the medium containing 4.0 mg/L $CuSO_4$ and 8.0 mg/L $ZnSO_4$, which was also significantly different from other treatments at P=0.05. Similarly, the tallest shoot height, 12.56 cm, one month after germination, was observed in the medium with 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$, which was significantly different as well. These results suggest that the combined effects of $CuSO_4$ and $ZnSO_4$ at their optimal concentrations are more potent than their individual effects during the seed germination and growth of shoots. In contrast, during seed germination, $CuSO_4$ alone also had a positive impact. The combined effects of $CuSO_4$ and $ZnSO_4$ exhibit a synergistic influence on seed germination and seedling growth by enhancing nutrient uptake, enzyme activity, and root development. This finding aligns with previous studies on rapeseed plants, which demonstrated that the combined application of $CuSO_4$ and $ZnSO_4$ enhances seed germination and root growth by regulating oxidative stress and promoting nutrient mobilization [22]. Similarly, research on *Vigna mungo* reported that $CuSO_4$ and $ZnSO_4$ improve enzyme activity and physiological processes, thereby enhancing root development and overall seedling growth [23].

3.2. Shoot Multiplication

Although the germination medium containing 6.0 mg/L $CuSO_4$ alone resulted in the shortest time to seed germination, it was not significantly effective in promoting further seed germination to produce more shoots on the germination media or increasing shoot height during the first month of shoot growth. In contrast, the medium containing 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$, which showed the second shortest time to germination and the highest number of germinated seeds with the tallest shoots, was screened for obtaining shoots to transfer to the multiplication media containing the same concentration of $CuSO_4$, $ZnSO_4$ and their combinations as used in germination media, one month after germination. Table 2 presents the results of the number of shoots produced one month after transfer to the multiplication media, including the calculated multiplication rate.

Additionally, it provides data on shoot height (mm) and total chlorophyll content of the leaves (mg/g) three months after transfer to the multiplication media. The supplementation of 4.0 mg/L $CuSO_4$ in combination with either 8.0 or 12.0 mg/L $ZnSO_4$ significantly improved new shoot production and multiplication rates (25.44 and 8.48, and 27.33 and 9.11, respectively) compared to other media supplemented with varying concentrations of $CuSO_4$ and $ZnSO_4$, as previously described. However, the differences between these two media were not statistically significant at P = 0.05.

Table 2 Effects of $CuSO_4$ and $ZnSO_4$ on *in vitro* seedling multiplication of *Dendrobium phalaenopsis*

Treatment Number	Treatment	Number of New Shoots[1]		Multiplication Rate[2]		Height of Shoots[3] (mm)		Chlorophyll Content in leaves[4] (mg/g)	
1	MS+1mg/L BA+0.2mg/L NAA (C) - Control	12.00 ± 1.33	b[5]	4.00 ± 0.44	b	28.67 ± 0.67	cde	1.19 ± 0.10	e
2	C+2mg/L $CuSO_4$	11.11 ± 0.51	b	3.70 ± 0.17	b	29.00 ± 0.67	cde	1.38 ± 0.08	d
3	C+4mg/L $CuSO_4$	11.11 ± 1.17	b	3.70 ± 0.39	b	28.33 ± 1.15	de	1.36 ± 0.10	d
4	C+6mg/L $CuSO_4$	12.44 ± 1.92	b	4.15 ± 0.64	b	28.11 ± 0.84	de	1.62 ± 0.03	b
5	C+8mg/L $CuSO_4$	10.67 ± 2.52	b	3.56 ± 0.83	b	28.78 ± 0.19	cde	1.42 ± 0.05	cd
6	C+4mg/L $ZnSO_4$	11.00 ± 1.76	b	3.67 ± 0.59	b	29.22 ± 1.17	cde	1.43 ± 0.09	cd
7	C+8mg/L $ZnSO_4$	11.11 ± 1.58	b	3.70 ± 0.53	b	27.89 ± 0.77	e	1.44 ± 0.06	cd
8	C+12mg/L $ZnSO_4$	12.44 ± 3.34	b	4.15 ± 1.11	b	29.00 ± 0.58	cde	1.56 ± 0.06	bc
9	C+2mg/L $CuSO_4$+4mg/L $ZnSO_4$	12.00 ± 0.33	b	4.00 ± 0.11	b	29.11 ± 0.51	cde	1.41 ± 0.01	cd
10	C+2mg/L $CuSO_4$+8mg/L $ZnSO_4$	12.67 ± 0.00	b	4.22 ± 0.00	b	30.44 ± 1.50	bc	1.44 ± 0.03	cd
11	C+2mg/L $CuSO_4$+12mg/L $ZnSO_4$	12.67 ± 2.40	b	4.22 ± 0.80	b	29.44 ± 1.50	cde	1.46 ± 0.02	cd
12	C+4mg/L $CuSO_4$+4mg/L $ZnSO_4$	12.22 ± 1.95	b	4.07 ± 0.65	b	29.78 ± 1.17	cde	1.49 ± 0.02	bcd
13	C+4mg/L $CuSO_4$+8mg/L $ZnSO_4$	25.44 ± 1.02	a	8.48 ± 0.34	a	31.78 ± 1.54	b	1.51 ± 0.14	bcd
14	C+4mg/L $CuSO_4$+12mg/L $ZnSO_4$	27.33 ± 5.17	a	9.11 ± 1.72	a	41.67 ± 1.00	a	2.41 ± 0.24	a
15	C+6mg/L $CuSO_4$+4mg/L $ZnSO_4$	14.89 ± 2.69	b	4.96 ± 0.89	b	30.11 ± 1.50	bcd	1.40 ± 0.01	cd
16	C+6mg/L $CuSO_4$+8mg/L $ZnSO_4$	13.33 ± 0.58	b	4.44 ± 0.19	b	28.22 ± 0.77	de	1.45 ± 0.03	cd
17	C+6mg/L $CuSO_4$+12mg/L $ZnSO_4$	12.67 ± 0.88	b	4.22 ± 0.29	b	28.56 ± 0.84	cde	1.46 ± 0.03	bcd
18	C+8mg/L $CuSO_4$+4mg/L $ZnSO_4$	12.44 ± 1.68	b	4.15 ± 0.56	b	28.44 ± 1.58	cde	1.44 ± 0.08	cd
19	C+8mg/L $CuSO_4$+8mg/L $ZnSO_4$	11.89 ± 2.78	b	3.96 ± 0.93	b	29.33 ± 0.88	cde	1.41 ± 0.06	cd
20	C+8mg/L $CuSO_4$+12mg/L $ZnSO_4$	11.89 ± 2.12	b	3.96 ± 0.71	b	28.67 ± 0.58	cde	1.39 ± 0.07	d

1. Average number of new shoots produced one (01) month after transferring them to multiplication media; 2. Multiplication rate one (01) month after transferring shoots to the multiplication media. It was calculated by; $Multiplication\ Rate = \frac{Total\ number\ of\ new\ plants\ or\ shoots}{Initial\ number\ of\ shoots}$; where initial number of shoots per culture bottle containing multiplication medium is three (03). 3. Average shoot height three (03) months after transferring shoots to multiplication media (at the time of transferring them to rooting media); 4. Chlorophyll content of leaves three (03) months after transferring shoots to multiplication media (at the time of transferring them to rooting media); 5. 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's basal medium

However, the shoot height and total chlorophyll content of the leaves (measuring 41.67 mm and 2.41 mg/g, respectively, at the end of the 3-months multiplication period) were significantly enhanced by media supplements containing 4.0

mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ during the multiplication phase. Therefore, the combination of 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ exhibited a synergistic effect, significantly improving shoot production, shoot height, multiplication rates, and total chlorophyll content, making it more effective than other media formulations in promoting overall plant growth and multiplication. $CuSO_4$, as a cofactor for enzymes like polyphenol oxidase and superoxide dismutase, enhances stress response and cell elongation, while $ZnSO_4$ supports protein synthesis, gene regulation, and auxin production. Copper is essential for the formation of plastocyanin, a protein involved in electron transfer in the photosynthetic electron transport chain, while zinc influences the structure and function of proteins involved in chlorophyll biosynthesis. Together, these micronutrients increase chlorophyll production and photosynthetic efficiency, promoting faster shoot multiplication and overall plant health. Previous studies on *Stevia rebaudiana*, *Withania somnifera* and *Dendrocalamus strictus* have also shown significant increases in shoot induction and growth when these elements are combined [24-26]. Similar type of synergistic effect of $CuSO_4$ has also been explained by the previous studies carried out on effects of $CuSO_4$ and $CoCl_2$ on *in vitro* performances of traditional indica rice (*Oryza sativa* L.) varieties in Sri Lanka [27].

3.3. *In vitro* Root Formation

Table 3 Effects of $CuSO_4$ and $ZnSO_4$ on *in vitro* rooting and their carryover impact on the *in vivo* survival of *Dendrobium phalaenopsis*

Treatment Number	Treatment	*In vitro*						*In vivo*	
		Days to Root Initiation[1]		Number of Roots[2]		Length of Roots[3] (mm)		Survival rate[4] (%)	
1	MS+1mg/L BA+0.1mg/L IBA (C) - Control	44.89 ± 2.78	a[5]	2.78 ± 0.19	d	28.00 ± 2.96	e	50.00 ± 8.82	c
2	C+2mg/L $CuSO_4$	44.89 ± 0.77	a	3.00 ± 0.67	cd	30.56 ± 2.27	de	55.56 ± 5.09	c
3	C+4mg/L $CuSO_4$	42.33 ± 2.00	a	3.67 ± 1.53	abcd	32.22 ± 1.07	cd	58.89 ± 1.92	bc
4	C+6mg/L $CuSO_4$	32.78 ± 1.90	b	3.78 ± 0.77	abcd	38.33 ± 1.53	b	70.00 ± 8.82	b
5	C+8mg/L $CuSO_4$	43.00 ± 3.38	a	4.11 ± 1.02	abcd	33.22 ± 1.07	cd	56.67 ± 5.78	c
6	C+4mg/L $ZnSO_4$	42.33 ± 1.76	a	3.78 ± 0.69	abcd	32.44 ± 3.15	cd	53.33 ± 8.82	c
7	C+8mg/L $ZnSO_4$	44.89 ± 2.36	a	3.67 ± 0.67	abcd	32.67 ± 0.88	cd	60.00 ± 8.82	bc
8	C+12mg/L $ZnSO_4$	43.33 ± 1.53	a	4.22 ± 0.69	abcd	37.33 ± 2.91	b	55.56 ± 6.94	c
9	C+2mg/L $CuSO_4$+4mg/L $ZnSO_4$	43.78 ± 1.92	a	3.67 ± 0.88	abcd	32.11 ± 1.07	cd	60.00 ± 3.33	bc
10	C+2mg/L $CuSO_4$+8mg/L $ZnSO_4$	42.78 ± 0.51	a	3.11 ± 0.19	bcd	33.22 ± 2.87	cd	56.67 ± 8.82	c
11	C+2mg/L $CuSO_4$+12mg/L $ZnSO_4$	44.33 ± 0.00	a	4.33 ± 0.88	abc	34.00 ± 0.88	cd	51.11 ± 6.94	c
12	C+4mg/L $CuSO_4$+4mg/L $ZnSO_4$	43.67 ± 1.53	a	4.11 ± 0.19	abcd	34.33 ± 2.33	c	56.67 ± 8.82	c
13	C+4mg/L $CuSO_4$+8mg/L $ZnSO_4$	43.22 ± 0.84	a	3.78 ± 0.77	abcd	33.00 ± 1.00	cd	63.33 ± 3.33	bc
14	C+4mg/L $CuSO_4$+12mg/L $ZnSO_4$	31.11 ± 1.58	b	4.33 ± 1.00	abc	47.67 ± 1.76	a	92.22 ± 5.09	a
15	C+6mg/L $CuSO_4$+4mg/L $ZnSO_4$	43.33 ± 3.06	a	4.78 ± 0.38	a	33.22 ± 0.84	cd	56.67 ± 3.33	c
16	C+6mg/L $CuSO_4$+8mg/L $ZnSO_4$	44.78 ± 3.34	a	4.56 ± 0.51	ab	33.33 ± 1.15	cd	61.11 ± 5.09	c

17	C+6mg/L $CuSO_4$+12mg/L $ZnSO_4$	44.56 ± 3.60	a	4.11 ± 0.69	abcd	33.78 ± 1.71	cd	62.22 ± 3.85	bc
18	C+8mg/L $CuSO_4$+4mg/L $ZnSO_4$	45.78 ± 3.15	a	4.33 ± 1.20	abc	32.56 ± 1.07	cd	53.33 ± 10.00	c
19	C+8mg/L $CuSO_4$+8mg/L $ZnSO_4$	44.78 ± 1.26	a	3.89 ± 0.51	abcd	32.56 ± 0.38	cd	60.00 ± 5.78	bc
20	C+8mg/L $CuSO_4$+12mg/L $ZnSO_4$	44.00 ± 1.00	a	3.33 ± 0.58	abcd	33.00 ± 0.88	cd	57.78 ± 6.94	bc

1. Average number of days to initiate roots; 2. Average number of roots two (02) month after root initiation (at the time of weaning them for acclimatization); 3. Average length of roots two (02) month after root initiation (at the time of weaning them for acclimatization); 4. survival rate one (01) month after transferring rooted plants to *in vivo* conditions; 5. 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's basal medium

According to the results presented in Table 3, the addition of 6.0 mg/L $CuSO_4$ to the rooting media significantly enhanced root initiation at P=0.05, leading to root initiation within 32.78 days. This effect was further improved by combining $CuSO_4$ with $ZnSO_4$, where the requirement for $CuSO_4$ was reduced from 6.0 mg/L to 4.0 mg/L when supplemented with an optimized concentration of 12.0 mg/L $ZnSO_4$, resulting in root initiation within 31.11 days. Although the individual effect of 6.0 mg/L $CuSO_4$ and the combined effect of 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ on the days taken for root initiation were not significantly different, both treatments produced significantly faster root initiation compared to media prepared with other concentrations of $CuSO_4$, $ZnSO_4$, or their combinations at P=0.05. Although the efficiency of increasing root number was not significantly affected by varying concentrations of $CuSO_4$ and $ZnSO_4$, a comparison with the control, where no $CuSO_4$ and $ZnSO_4$ were added, revealed a clear trend toward improved root formation when these supplements were included in the media. However, the subsequent root growth was significantly enhanced when 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ were added together in the rooting media, resulting in a shoot length of 47.67 mm two months after root initiation, demonstrating a synergistic effect on root development. Furthermore, the root systems of these plants were more vigorous and developed more root hairs compared to those grown in other media. This is because $CuSO_4$ and $ZnSO_4$ work together to enhance root formation by supporting critical biological processes.

Copper aids in activating enzymes involved in lignin biosynthesis, a key process for root cell wall strengthening, while zinc boosts auxin production, a key hormone responsible for root elongation and initiation. Additionally, both elements play roles in DNA replication and protein synthesis, driving cell division in the root system. This combined action helps plants develop stronger, healthier, more extensive roots, provided the concentrations of $CuSO_4$ and $ZnSO_4$ remain within optimal levels. Studies have shown that these micronutrients can enhance adventitious root development by regulating the enzymatic and hormonal activities involved in root induction.

In particular, $CuSO_4$ has been found to improve the rooting efficiency by aiding cellular differentiation and elongation, while $ZnSO_4$ promotes the development of root primordia in date palm (28-29]. Elevated levels of both $CuSO_4$ and $ZnSO_4$ have been shown to enhance root formation in *Mucuna pruriens* [30]. However, the concentrations must be carefully controlled, as excess amounts can have inhibitory effects on root growth [31].

3.4. Survival Rate

The addition of 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ to the media throughout the entire *in vitro* culture period, starting from the germination stage, significantly improved the survival rate of the plants under *in vivo* conditions (P=0.05). This resulted in a 92.22% survival rate, the highest recorded for *in vitro*-grown *Dendrobium phalaenopsis* orchids, one month after being transferred to *in vivo* conditions, demonstrating the carryover effects of $CuSO_4$ and $ZnSO_4$. There are some previous studies reported on the same where the carry-over effects of $CuSO_4$ and $ZnSO_4$ in *in vitro* cultures have been shown to influence plant growth, tissue regeneration, and metabolic activities. The use of $CuSO_4$ and $ZnSO_4$ can enhance seed germination, shoot regeneration and rooting in plant species like *Rauvolfia tetraphylla* and date palm, but excessive concentrations may lead to toxic effects, altering DNA integrity and inducing oxidative stress (29, 32]. Furthermore, there were no observable morphological differences between the *in vitro* propagated plants and the seed-grown plants of the same developmental stage.

4. Conclusions

This study demonstrates that the supplementation of copper sulfate ($CuSO_4$) and zinc sulfate ($ZnSO_4$) significantly enhances the *in vitro* propagation efficiency of *Dendrobium phalaenopsis* orchids by optimizing them in various developmental stages. Supplementation of the media with $CuSO_4$ alone too is effective in promoting shoot and root

initiation. However, the combination of $CuSO_4$ and $ZnSO_4$ is more effective in enhancing overall shoot and root growth, as well as further development. The combination of 4.0 mg/L $CuSO_4$ and 12.0 mg/L $ZnSO_4$ was particularly effective, resulting in shorter germination times, increased shoot production and height, and faster root initiation compared to other treatments. This specific combination showed a synergistic effect, leading to the improvement of seed germination, the highest shoot multiplication rates, more vigorous root development, and improved chlorophyll content. Furthermore, the optimized media resulted in a 92.22% survival rate of plants when transferred to *in vivo* conditions, with no morphological differences from seed-grown plants, underscoring the carryover benefits of these micronutrient supplements. These findings highlight the potential for $CuSO_4$ and $ZnSO_4$ to significantly improve clonal propagation protocols, offering a reliable and scalable method for cultivating *Dendrobium phalaenopsis* orchids with higher success rates.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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Authors short Biography

K.P.G.D.M. Polwaththa is a graduate of Sabaragamuwa University of Sri Lanka, where she earned a B.Sc. in Agricultural Sciences and Management, graduating with Second-Class Upper Division Honors. Her research focuses on plant biotechnology, particularly on enhancing *in vitro* regeneration efficiencies of improved plant varieties. As an agricultural graduate, she is eager to leverage her strong commitment to the field of agriculture and to enhance her experience through continuous learning and collaboration. Currently, she is a researcher and one of the research fellows in the Plant Cell and Tissue Engineering Laboratory at the Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka.

A.A.Y. Amarasinghe, a Senior Professor at Sabaragamuwa University of Sri Lanka, is an expert in plant biotechnology with advanced knowledge of modern biotechnological tools. He holds a B.Sc. in Agriculture with Upper Second-Class Honors from the University of Peradeniya, an M.Phil. from the same university, and a Ph.D. from South China Agricultural University. As the in-charge of the Plant Cell and Tissue Engineering Laboratory, he leads a research team focused on enhancing *in vitro* regeneration efficiencies, developing improved plant varieties, and advancing tissue culture techniques. His extensive academic and administrative experience enables him to actively contribute to solving global agricultural challenges through innovative research.