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ARTICLE

From Callus to Plantlet: unveiling the optimal hormonal synergy and sucrose supplementation for *Zamioculcas zamiifolia* micropropagation

Do calo à muda: revelando a sinergia hormonal ideal e a suplementação de sacarose para a micropropagação de *Zamioculcas zamiifolia*

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Abstract: Zamiifolia (*Zamioculcas zamiifolia*) is an important plant in ornamental markets, characterized by a slow growth rate and low propagation efficiency. To establish an efficient propagation protocol for this species, two experiments were conducted. The first experiment aimed to optimize the micropropagation of Zamiifolia through callus induction. The second experiment evaluated the effects of the callogenesis medium on the growth and performance of regenerated plants in greenhouse. Leaf explants were incubated on the half-strength Murashige and Skoog medium (½MS) containing different combinations of benzyladenine (0, 4.4, and 8.8 µM BA) and naphthalene acetic acid (0, 2.7, 5.4, and 10.8 µM) and sucrose (2.0% and 3.0%). The highest callus induction rate and weight were obtained using 0.0 µM BA+2.7 or 5.4 µM NAA+3.0% sucrose. The obtained calluses were incubated on ½MS medium supplemented with different combinations of BA (0.0, 2.2, and 8.8 µM) and NAA (0.0, 1.1, and 2.7 µM) for shoot regeneration , with the highest proliferation efficiency on medium containing 2.2 µM BA. Rooting of shoots evaluated in ½MS containing indole-3-butyric acid (0.0, 2.5 and 5.0, µM IBA) and NAA (0.0, 37.2, and 2.7 µM) and 2.5 µM IBA medium was suggested, respectively. In the second experiment, plantlets were regenerated from calli induced on the four most effective media, and their growth was evaluated under greenhouse conditions. The best growth and health of micropropagated plants were obtained from callogenesis medium containing 2.7 µM NAA+3.0% sucrose. Excessive cytokinin accumulation following BA treatment impaired root and overall growth of micropropagated plants.
Keywords: callus, chlorophylls, micropropagation protocol, rooting, soluble carbohydrates.

Resumo: A zamioculca (*Zamioculcas zamiifolia*) é uma planta importante nos mercados ornamentais, com taxa de crescimento lenta e baixa eficiência de propagação. Para estabelecer um protocolo de propagação eficiente para esta espécie, foram conduzidos dois experimentos. O primeiro experimento visou otimizar a micropropagação da zamioculca por meio da indução de calos. O segundo experimento avaliou os efeitos do meio de calogênese no crescimento e desempenho de plantas regeneradas em casa de vegetação. Explantes foliares foram incubados em meio Murashige e Skoog de meia força (½MS) contendo diferentes combinações de benziladenina (0,0; 4,4 e 8,8 µM BA) e ácido naftaleno acético (0,0; 2,7; 5,4 e 10,8 µM) e sacarose (2,0% e 3,0%). A maior taxa e peso de indução de calos foram obtidos usando 0,0 µM BA + 2,7 ou 5,4 µM ANA + 3,0% de sacarose. Os calos obtidos foram incubados em meio ½MS suplementado com diferentes combinações de BA (0,0; 2,2 e 8,8 µM) e ANA (0,0; 1,1 e 2,7 µM) para regeneração de brotos, com a maior eficiência de proliferação em meio contendo 2,2 µM BA. O enraizamento dos brotos foi avaliado em meio ½MS contendo ácido indol-3-butírico (0,0; 2,5 e 5,0 µM AIB) e ANA (0,0; 37,2 e 2,7 µM), e o meio com 2,5 µM AIB foi sugerido, respectivamente. No segundo experimento, plântulas foram regeneradas a partir de calos induzidos nos quatro meios mais eficazes, e seu crescimento foi avaliado em condições de casa de vegetação. O melhor crescimento e qualidade das plantas micropropagadas foram obtidos a partir do meio de calogênese contendo 2,7 µM ANA + 3,0% de sacarose. O acúmulo excessivo de citocinina após o tratamento com BA prejudicou o enraizamento e o crescimento geral das plantas micropropagadas.
Palavras-chave: calos, clorofilas, carboidratos solúveis, enraizamento, protocolo de micropropagação.

Introduction

Shiny and attractive dark green leaves, resistance to pests and diseases, adaptability to low-light environments, and minimal maintenance requirements have made Zamiifolia (*Zamioculcas zamiifolia*) a popular indoor ornament. Traditionally, Zamiifolia is propagated through rhizome division or leaf cuttings. The efficiency of these methods is low, as regeneration occurs at a slow pace, taking approximately seven months to form new tubers and roots (Nirmala, et al., 2019). Micropropagation offers an effective alternative technique for plants that cannot be easily reproduced using conventional methods (Kharrazi et al., 2023). However, optimizing micropropagation protocols is time-consuming and costly, as it requires species-specific adjustments. To date, only a limited number of studies have focused on optimizing callus induction and organogenesis media for Zamiifolia. Leaves are suitable explants for callus proliferation in this species (Sayadi Nejad and Sadeghi, 2019). In most studies, a combination of auxins and cytokinins has been recommended for callogenesis. However, the effects of growth regulator types and concentrations vary significantly across different experiments. Pan *et al.* (2007) evaluated various NAA and BA combinations in MS medium and obseerved the highest callus proliferation rate and growth with 0.5 mg L^{-1} NAA+0.5 mg L^{-1} BA. Sayadi Nejad and Sadeghi (2019) achieved the fastest and most extensive callus formation using 8.8 µM BA+5.4 µM NAA in ½MS. Vanize-canton and Leonhardt (2009) identified ½MS supplemented with 0.88 µM BA+18.1 µM 2,4-D as optimal conditions for callogenesis. Shi and Liang (2003) suggested MS medium supplemented with 8.8 µM BA+4.5 µM 2,4-D for cell proliferation. Ni (2015) reported the highest callus growth on MS medium with 8.8 µM BA+0.55 µM NAA. Kharrazi et al. (2023) achieved maximum callogenesis and growth using MS medium containing 0.88 µM BA+21.6 µM NAA. Given the variety of growth regulators used for callogenesis in Zamiifolia, an important question arises: Can these compounds influence the performance of micropropagated plants? One of the aims of this study is to address this gap by evaluating the effects of callus-inducing media on the growth and health of regenerated plantlets.

Sucrose is another detrimental factor in callogenesis. Sucrose serves as both an osmotic agent and a cost-effective carbon source in plant tissue culture media (Thaneshwari, 2018). While the effects of sucrose levels on callus formation in Zamiifolia have not been thoroughly investigated, its impact on callogenesis has been well-documented in other species. Wahyuni et al. (2020) evaluated sucrose concentrations (0.0 to 5.0%) in

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the culture of *Justicia gendarussa* leaf explants and found the optimal callus growth with 3.0% sucrose. Thaneshwari (2018) studied the effects of sucrose concentrations (0.0% - 12.0%) on callus induction in *Tagetes* spp. leaves, reporting the highest proliferation at 4.0% sucrose. Sari et al. (2018) demonstrated that 3.0% sucrose yielded the highest callus formation in *Myrmecodia tuberosa* cotyledon explants. They noted that higher sucrose concentrations could inhibit callus formation due to osmotic stress.

In addition to callus induction, shoot regeneration and rooting of new shoots require optimization. Ni (2015) recommended MS medium supplemented with 8.8 µM BA+2.7 µM NAA for shoot proliferation and ½MS medium containing 0.1 µM NAA for rooting. Shi and Liang (2003) introduced MS medium with 1.1 µM NAA+13.2 µM BA for shoot regeneration from callus. They reported the best root growth on ½MS medium supplemented with 2.7 µM NAA+8.8 µM BA. Sayadi Nejad and Sadeghi (2019) achieved the fastest shoot growth (stem length and leaf number) using 10.8 µM NAA. They suggested 4.4 µM BA+2.5 µM NAA as the optimal combination for root development.

This study aimed to develop an efficient protocol for Zamiifolia propagation through callus proliferation and subsequent organogenesis. Developing such methods is essential to reduce the need for importing Zamiifolia and to enhance its propagation speed in temperate regions. Additionally, the effects of the callus-inducing medium on the growth and performance of regenerated plants were evaluated under greenhouse conditions. The findings from this section revealed significant insights that could inform the management of growth regulators in micropropagation protocols, ultimately improving the efficiency and quality of propagated plants.

Material and methods

Experiment I: optimizing Zamiifolia micropropagation via callus regeneration

Step 1: Callus induction

In vitro propagation experiments were conducted using fully expanded leaflet explants from mature plants of *Z. zamiifolia.* The leaflets were collected from the middle portion of healthy, mature leaves, ensuring that neither overly young nor senescent leaflets were used. The leaflets were surface-sterilized using a multi-step protocol. Initially, the leaves washed with distilled water containing a few drops of Tween-20 for 20 min to remove surface contaminants. Next, they were submerged in 70% ethanol for 30 sec, followed by three rinses with sterilized distilled water at intervals of 2, 5, and 10 min. Finally, the samples were disinfected with 2.0% sodium hypochlorite for 5 min and rinsed again with sterilized distilled water. The sterilized leaves were aseptically cut into segments of approximately 32 mm in length and 11 mm in width, with each piece weighing about 250 mg. The cuts were made to achieve uniform explants. The leaf explants were placed horizontally on half-strength Murashige and Skoog medium (½MS) with the abaxial (lower) surface in direct contact with the agar surface. This orientation ensured maximum and uniform contact with the culture medium, facilitating optimal uptake of nutrients and hormones. This configuration was consistently applied across all treatments to ensure reproducibility and experimental uniformity.

The culture medium was supplemented with various concentrations of benzyladenine (BA; 0.0, 4.4, and 8.8 µM), naphthalene acetic acid (NAA; 0.0, 2.7, 5.4, and 10.8 µM), and sucrose (2.0% and 3.0%) (Fig. 1A). After adding vitamins and adjusting the pH to 5.8, 5.0 g L^{-1} agar was incorporated into the medium. The prepared media were autoclaved at 121 °C and 121 kPa for 20 min and distributed into sterilized jars (8.0 × 10.5 cm). Each culture jar was filled with 30 mL of medium. This volume was standardized across all treatments to ensure consistent nutrient availability and optimal space for explant growth and gas exchange. The experiment was conducted as a factorial arrangement in a completely randomized design, consisting of 24 treatments, each with three replicates and three explants per replicate. The list of treatments can be seen in Table 1. The cultures were maintained at 25 - 27 °C under a 16-h photoperiod provided by white fluorescent lamps at an intensity of 50 µmol m^{-2} s^{-1} measured at the culture vessel level. After three weeks, the induced calli were subcultured onto the same hormone-supplemented medium for the first culture. For subsequent subcultures (second and third), calli were transferred to a hormone-free ½MS medium to prevent over-accumulation of growth regulators and potential adverse effects on the explants. After 90 days, the percentage of callus initiation, the fresh weight of each callus mass, total fresh weight of callus in jar, and the number of days to callus formation were recorded.

Step 2: Shoot regeneration

The callus obtained from the previous step was transferred to ½MS medium (1 g per jar; Fig. B and C) supplemented with various combinations of benzyladenine (BA; 0.0, 2.2, and 8.8 µM) and naphthalene acetic acid (NAA; 0.0, 1.1 and 2.7 µM). After adding vitamins, adjusting the pH to 5.8, and incorporating 5 g L^{-1} agar, the media were autoclaved at 121 °C and 121 kPa for 20 min and distributed into sterile jars (8 × 10.5 cm). Each culture jar was filled with 30 mL of medium.

This experiment was conducted as a factorial experiment using a completely randomized design. The factors included three concentrations of BA and three concentrations of NAA, resulting in a total of nine treatments. Each treatment was replicated three times, leading to a total of 27 jars. The explants were incubated under the same environmental conditions as Step 1. Shoot regeneration was evaluated after 50 days of culture. Due to the acaulescent nature of Zamiifolia, which lacks a distinct aerial stem, the number of newly formed leaves per culture vessel was used as a reliable indicator of shoot proliferation and regenerative capacity. Each emerging leaf arises from an axillary bud and represents a potential shoot development, making leaf number a suitable and biologically relevant proxy for proliferation efficiency in this species. The number of leaves and time to first leaf emergence were recorded (Fig. 1C).

Step 3: Root induction

Uniform shoots from the previous step were cultured on ½MS medium supplemented with indole-3-butyric acid (IBA; 0.0, 2.5 and 5.0 µM IBA) and naphthalene acetic acid (NAA; 0.0, 2.7 and 5.4 µM) to induce rooting. Each culture jar was filled with 30 mL of medium. The explants were maintained at 25 - 27 °C under a 16-hour photoperiod. This experiment followed a factorial design based on a completely randomized structure. Nine treatments were investigated, each replicated three times, resulting in a total of 27 jars (8 × 10.5 cm). After 30 days, the time until the first root was observed, the number and length of roots and the percentage of rooted explants were recorded (Fig. 1D).

Experiment II: investigating the performance of regenerated plants derived from different callus induction media

Regenerated plantlets derived from the most effective callus-inducing media (2.7 µM NAA+3.0% sucrose, 2.7 µM NAA+4.4 µM BA+2.0% sucrose, 5.4 µM NAA+3.0% sucrose, and 5.4 µM NAA+4.4 µM BA+2.0% sucrose) (Table 1) were carefully removed from culture vessels, and the agar and residual medium were gently rinsed off with sterile distilled water. Plantlets were then transplanted into transparent plastic containers filled with a moist mixture of cocopeat and perlite (1:1, v v^{-1}). Immediately after transplantation, the plantlets were misted with sterile distilled water and the containers were sealed with transparent lids to maintain high internal humidity (Fig. 1E). Acclimatization of regenerated plantlets was carried out under controlled environmental conditions (25 ± 2°C, 35% relative humidity). The containers were kept under a 16-h photoperiod provided by white fluorescent lamps at an intensity of 75 µmol m^{-2} s^{-1}. To prevent desiccation, plantlets were misted every three days during the first week. From the second week onward, the misting interval was extended to every four days. Weekly fertilization with a compound fertilizer (Eurosolids WS NPK 12-12-36+2MgO+TE) solution (500 mg L^{-1}) was initiated one week after transplantation. Concurrently, the containers were gradually opened starting from the second week to allow a stepwise reduction in internal humidity, and were completely uncovered by the end of the fourth week. Subsequently, the adapted plants were transferred to plastic pots (12 × 10 cm) containing the same cocopeat and perlite mixture and grown under shade in a greenhouse (Fig. 1F). The greenhouse environment was maintained at an average temperature of 25 °C and a relative humidity of 80%. After 30 days, various physiological and biochemical traits were measured. This experiment was conducted using a completely randomized design with three replicates and for plants per replicate.

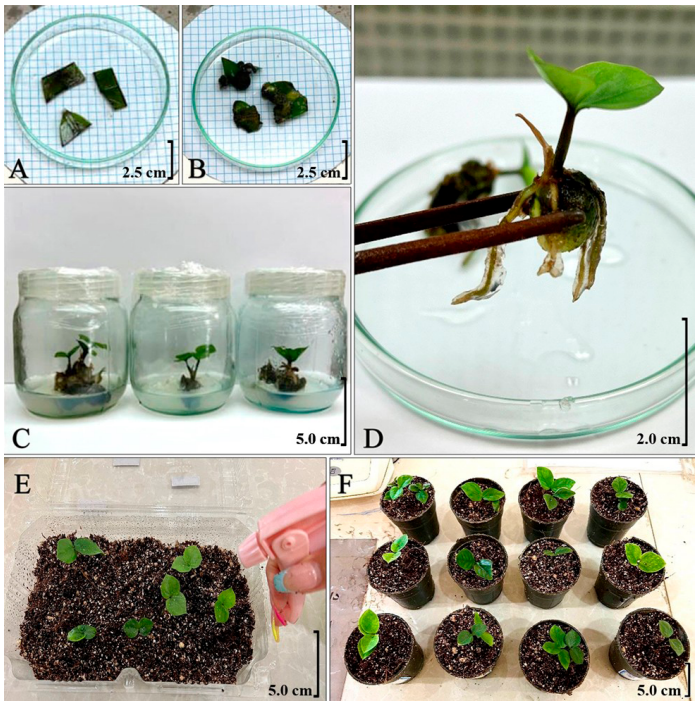

Fig. 1. Steps of Zamiifolia micropropagation via callus induction: A) Leaf explant preparation; B) Callus tissue for shoot regeneration; C) Regenerated shoot from callus; D) Rooted plantlets; E) Acclamatization of plantlets in the controlled conditions; F) Established plants in the greenhouse.

Biochemical Analyses

Fresh leaf samples (0.5 g) were extracted using 80% acetone and centrifuged. The absorbance of the supernatant was measured at wavelengths of 663 nm, 646 nm, and 480 nm. The concentrations of chlorophylls and carotenoids were calculated using the method described by Martins et al. (2020). To determine soluble carbohydrates, 0.10 g of leaf tissue was extracted with 80% ethanol and reacted with anthrone reagent in sulfuric acid. The absorbance of samples was measured at 625 nm using the method described by Sahraie et al. (2025).

Statistical Analysis

Each step of experiments 1 and 2 was arranged as a factorial experiment following a completely randomized design. Data were subjected to analysis of variance (ANOVA), and means were compared using Duncan's Multiple Range Test (DMRT) at a significance level of $p \leq 0.01$. All statistical analyses were performed using SPSS v. 22 software.

Results

Experiment I: optimizing Zamiifolia micropropagation via callus regeneration

Step 1: The effect of growth regulators and sucrose on callus proliferation

Callogenesis was affected by the BA and NAA treatment and their interaction. The effects of BA, NAA, sucrose and their triple interaction were significant ($p \leq 0.01$) on the number of days until callus formation, the average weight of callus and the total weight of callus in jars.

The highest callogenesis percentage (100%) was observed in media containing 2.7 or 5.4 µM of NAA without BA or 10.8 µM NAA+4.4 µM BA. Callus initiation in these treatments was also similar to the control (94.4%), the media with 10.8 µM NAA (94.4%) or 4.4 µM BA+2.7 µM NAA (88.9%). Increasing BA concentration reduced callus initiation and the 8.8 µM BA+10.8 µM NAA treatment completely prevented callus formation (Fig. 2).

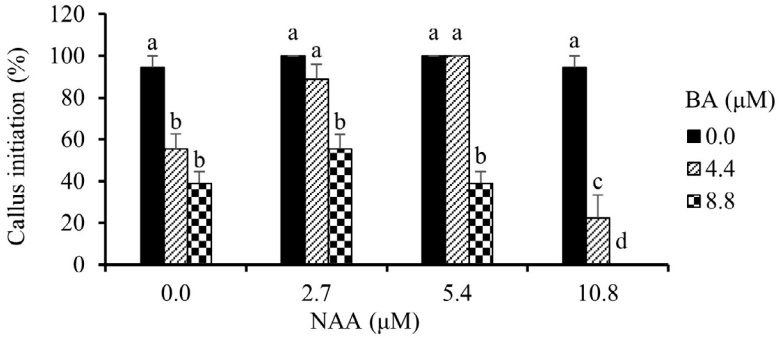

Fig. 2. The effect of using different concentrations of naphthalene acetic acid (NAA) and benzyladenine (BA) in ½MS medium on the percentage of callogenesis in Zamifolia leaf explants. Means (±SE) with the same letters are not statistically different (DMRT at $p \leq 0.01$).

The highest total fresh weight of callus and mean fresh weight per callus mass were observed in media containing 2.7 µM NAA+3.0% Suc and 5.4 µM NAA+3.0% Suc, while the medium 5.4 µM NAA+8.8 µM BA+3.0% Suc produced the lowest total and mean callus mass weight. No callus formation was observed in media containing 10.8 µM NAA+4.4 µM BA+2.0% Suc, 10.8 µM NAA+8.8 µM BA+3.0% Suc or 10.8 µM NAA+8.8 µM BA+2.0% Suc (Table 1). The fastest callus initiation occurred 24 days after culture in media supplemented with 2.7 µM NAA+3.0% Suc, 2.7 µM NAA+2.0% Suc, and 5.4 µM NAA+3.0% Suc (Table 1). The longest time to callus formation (73.3 Days) was observed with the medium containing 5.4 µM NAA+8.8 µM BA+2.0% Suc.

Table 1. The interaction effect of different concentrations of naphthalene acetic acid (NAA), benzyladenine (BA) and sucrose (Suc) in ½MS medium on formation and growth of callus from Zamiifolia leaf explants 90 days after culture.

NAA (µM)	BA (µM)	Suc (%)	Total callus weight (g)	Callus mass weight (g)	Days to callus formation
0.0	0.0	3.0	4.04^{bc}	1.35^{bc}	45.0^{g}
0.0	0.0	2.0	3.89^{bc}	1.30^{c}	50.7^{f}
0.0	4.4	3.0	0.93^{gh}	0.31^{h}	53.0^{ef}
0.0	4.4	2.0	1.44^{g}	0.48^{gh}	61.3^{c}
0.0	8.8	3.0	2.24^{f}	0.74^{gf}	55.7^{ed}
0.0	8.8	2.0	0.77^{ghi}	0.26^{hi}	62.7^{c}
2.7	0.0	3.0	7.97^{a}	2.66^{a}	24.0^{k}
2.7	0.0	2.0	3.34^{cde}	1.11^{de}	25.3^{jk}
2.7	4.4	3.0	2.70^{ef}	0.91^{def}	35.0^{h}
2.7	4.4	2.0	3.89^{bc}	1.30^{bc}	37.3^{h}
2.7	8.8	3.0	2.81^{def}	0.37^{h}	34.3^{hi}
2.7	8.8	2.0	1.13^{gh}	0.26^{hi}	36.7^{h}
5.4	0.0	3.0	7.59^{a}	2.53^{a}	26.0^{jk}
5.4	0.0	2.0	4.45^{b}	1.48^{b}	29.7^{ji}
5.4	4.4	3.0	3.52^{cd}	1.17^{cd}	32.6^{hi}
5.4	4.4	2.0	2.46^{f}	0.82^{f}	34.0^{hi}
5.4	8.8	3.0	0.43^{hi}	0.20^{hi}	63.0^{c}
5.4	8.8	2.0	0.67^{ghi}	0.74^{fg}	73.3^{b}
10.8	0.0	3.0	2.58^{ef}	0.86^{ef}	34.4^{hi}
10.8	0.0	2.0	0.61^{hi}	0.94^{def}	37.7^{h}
10.8	4.4	3.0	1.44^{g}	0.48^{gh}	59.3^{cd}
10.8	4.4	2.0	0.0^{i}	0.0^{i}	>120.0^{a}
10.8	8.8	3.0	0.0^{i}	0.0^{i}	>120.0^{a}
10.8	8.8	2.0	0.0^{i}	0.0^{i}	>120.0^{a}

Means with the same letters are not statistically different according to DMRT at $p \leq 0.01$.

Step 2: The effect of growth regulator treatments on branching from callus tissue

The presence of NAA in the culture medium delayed leaf emergence from callus explants (Fig. 3A). The fastest leaf emergence occurred in NAA-free media, whereas the slowest was observed in the medium containing 2.7 µM NAA (Fig. 3A). A treatment promoted leaf induction. The longest delay in leaf appearance was recorded in the absence of BA, while the fastest emergence was achieved with 8.8 µM BA. This treatment did not differ significantly from 2.2 µM BA (Fig. 3B). The highest number of leaves was observed in media lacking NAA, and increasing NAA concentration reduced leaf formation. The lowest number of leaves was obtained in the medium with 2.7 µM NAA (Fig. 3C). The number of leaves increased with increasing BA concentration in the culture medium. Leaf number increased progressively with higher BA concentrations. The fewest leaves were observed in the BA-free medium, which did not differ significantly from the 2.2 µM BA treatment. The highest leaf count was achieved in the medium supplemented with 8.8 µM BA (Fig. 3D).

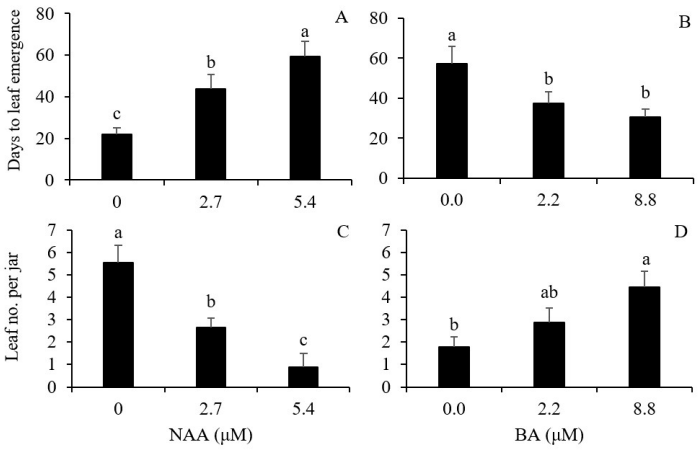

Fig. 3. The number of days until leaf inducton on Zamiifolia callus in response to using A) naphthalene acetic acid (NAA) and B) benzyladenine (BA); and the number of leaves per jar in response to adding C) NAA and D) BA to ½MS medium. Means (±SE) with the same letters are not statistically different (DMRT at $p \leq 0.01$).

Step 3: The effect of growth regulator treatments on rooting from shoots

The effects of NAA treatment and the interactive effect of NAA and IBA were significant on the number of days until root emergence and the number of roots at $p \leq 0.05$. In the control treatment (lacking plant growth regulators), roots emerged from regenerated shoots after 24 days, with an average of 4.3 roots per shoot. In the absence of IBA, increasing NAA concentration up to 2.7 µM accelerated root emergence compared to the control; however, treatment with 5.4 µM NAA did not differ significantly from the control (Fig. 4A). When NAA was omitted, supplementation with 2.5 or 5.0 µM IBA accelerated root formation, with no significant difference between the two concentrations (Fig. 4A). Using 2.5 and 5.0 µM IBA in the absence of NAA accelerated root formation and there was no difference between these treatments. Combination of IBA and NAA delayed root emergence, although these effects were not significantly different from the control treatment (Fig. 4A). Overall, IBA proved to be more effective than NAA in promoting shoot rooting. In the control treatment, an average of 4.3 roots were formed per branch. In the absence of IBA, supplementation with NAA did not affect the number of roots per shoot. In the absence of NAA, the highest number of roots (8.3) was produced in the culture medium with a concentration of 5.0 µM IBA without a significant difference from 2.5 µM IBA. The combination of IBA and NAA significantly reduced the number of roots compared to the control (Fig. 4B).

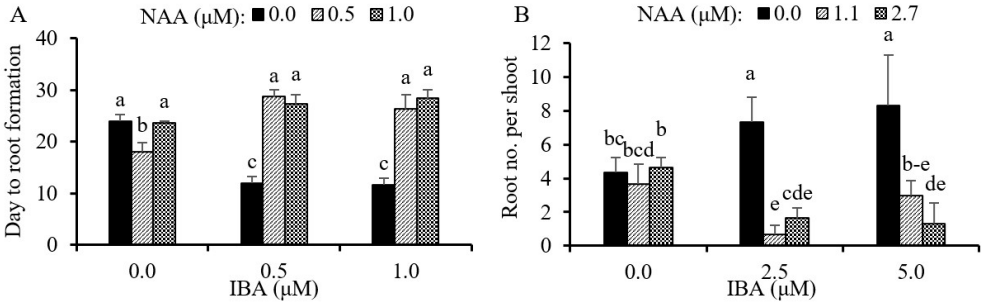

Fig. 4. Effects of using naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) in ½MS medium on A) number of days until root formation and B) number of roots per shoot of Zamiifolia. Means (±SE) with the same letters are not statistically different (DMRT at $p \leq 0.01$).

Experiment II: Effects of callus induction medium on micropropagated plants

The callogenesis step had no significant effect on leaf weight, tuber weight, tuber diameter, and leaf area. The average weight of the leaves and tubers of the plants was 0.205 and 2.81 g, respectively. The tuber diameter was 3.07 cm and the plant leaf area was 0.274 m^2. However, its effect was significant on the total plant weight, root volume, root length, plant height at $p \leq 0.05$ and root weight at $p \leq 0.01$ (Table 2). The highest total plant weight (28.06 g) was observed in medium containing 2.7 µM NAA+3.0% Suc and plants in media supplemented with 2.7 µM NAA+4.4 µM BA+2.0% Suc and 5.4 µM NAA+4.4 µM BA+3.0% Suc had the lowest value (21.64 g). The highest stem weight (0.37 g) and height (3.20) were observed in plants from 5.4 µM NAA+3.0% Suc medium and the lowest values were found in medium containing 5.4 µM NAA+4.4 µM BA+3.0% Suc. Plants from 2.7 µM NAA+ 3.0% Suc medium had the highest (1.00 g) and plants from 5.4 µM NAA+4.4 µM BA+ 3.0% Suc. medium had the lowest (0.18 g) root weight. The highest root volume (2.33 cm^3) and length (3.26 cm) were observed in 2.7 µM NAA+3.0% Suc medium and the lowest was in 5.4 µM NAA+4.4 µM BA+3.0% Suc medium.

Table 2. The effect of callus induction medium conditions on the morphological characteristics of micropropagated Zamiifolia plantlets after acclimatization and transferring to the greenhouse.

Medium	Plant FW (g)	Shoot FW (g)	Plant height (cm)	Root FW (g)	Root vol. (cm^3)	Root length (cm)
NAA2.7 + 3.0% Suc	28.0^{a}	0.15^{bc}	2.68^{ab}	1.00^{a}	2.33^{a}	3.26^{a}
NAA2.7+BA4.4 + 2.0% Suc	21.6^{b}	0.31^{ab}	2.97^{a}	0.37^{bc}	1.33^{b}	2.27^{ab}
NAA5.4 + 3.0% Suc	25.3^{ab}	0.37^{a}	3.20^{a}	0.81^{ab}	1.33^{b}	2.69^{b}
NAA5.4+BA4.4 + 3.0% Suc	24.6^{ab}	0.08^{c}	2.03^{b}	0.18^{c}	1.00^{b}	1.93^{b}

Means with the same letters are not statistically different according to DMRT at $p \leq 0.01$.

Callogenesis significantly affected concentrations of photosynthetic pigments and soluble carbohydrates in leaves of micropropagated plants. The plants obtained from medium containing 2.7 µM NAA+3.0% Suc had the highest concentrations of chlorophylls (0.68 mg g FW^{-1}) and carotenoids (0.61 mg g FW^{-1}) compared to other media (Fig. 5 A and 5B). The concentration of chlorophylls in the plants obtained from media supplemented with 2.7 µM NAA+4.4 µM BA+2.0% Suc or 5.4 µM NAA+4.4 µM BA+3.0% Suc was the lowest, but they had no significant difference in concentration of carotenoids (Fig. 5 A and 5B).

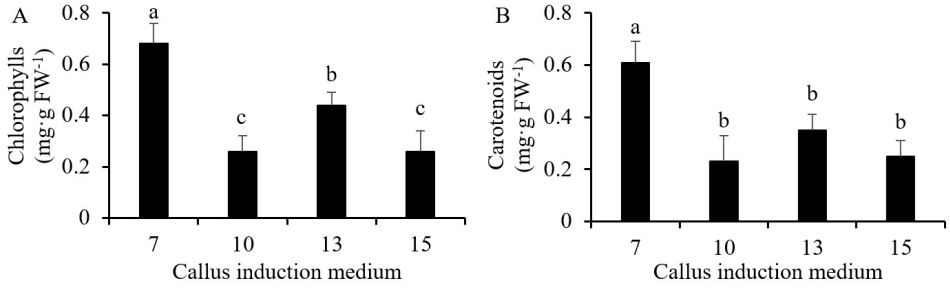

Fig. 5. Effect of callus formation medium on concentration of A) chlorophylls and B) carotenoids in leaves of micro-propagated Zamiifolia plants in greenhouse. Means (±SE) with the same letters are not statistically different (DMRT at $p \leq 0.01$).

The plants obtained from 2.2 µM NAA+3.0% Suc had the highest soluble carbohydrate concentration (56.6 mg g FW^{-1}) compared to other media. The lowest soluble carbohydrate concentration was observed in plants from medium containing 2.7 µM NAA+4.4 µM BA+2.0% Suc (Fig. 6).

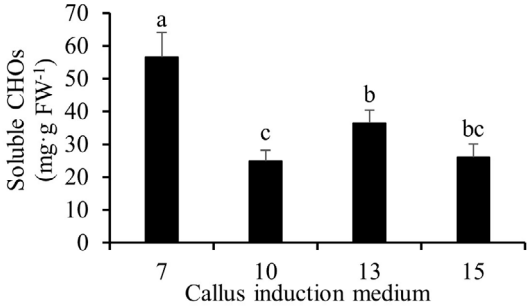

Fig. 6. Effect of callus formation medium on concentration of soluble carbohydrates (CHOs) in leaves of micropropagated Zamiifolia plants in greenhouse. Means (±SE) with the same letters are not statistically different (DMRT at $p \leq 0.01$).

Discussion

The present study demonstrates that the success of *Zamioculcas zamiifolia* micropropagation is highly dependent on the precise balance of exogenous growth regulators and carbon supply during callus induction, with lasting effects on subsequent organogenesis and greenhouse performance. A key finding is the suppression of callogenesis by benzyladenine (BA), particularly at higher concentrations and in combination with elevated NAA levels. This contrasts with many plant species where cytokinin-auxin synergy promotes cell division and callus formation (Sayadi Nejad and Sadeghi, 2019; Fathy et al., 2022; Pourhassan et al., 2023), suggesting a species-specific sensitivity in *Z. zamiifolia*. The optimal callus induction occurred in the absence of BA and with moderate NAA (2.7 - 5.4 µM) supplemented with 3.0% sucrose. This pattern implies that endogenous cytokinin levels in leaf explants are sufficient to drive cell proliferation, and exogenous BA may disrupt hormonal homeostasis, potentially triggering feedback inhibition or ethylene-mediated stress responses (An et al., 2020). Such overstimulation can lead to cellular dedifferentiation without organized growth, explaining the reduced callus biomass in high-BA treatments. Specific combinations of growth regulators significantly accelerated both callus initiation and subsequent growth.

The critical role of sucrose as both a carbon source and an osmotic regulator is evident in the superior callus growth on 3.0% sucrose media. Sucrose not only provides energy for cell division but also influences the uptake and activity of nutrients and growth regulators (Thaneshwari, 2018). Higher sucrose levels may stabilize membrane integrity and enhance metabolic activity, thereby supporting greater biomass accumulation. This aligns with findings in *Justicia gendarussa* (Wahyuni et al. , 2020), reinforcing the importance of carbon availability in callogenesis. Based on the results, the optimum conditions for Zamiifolia callogenesis were achieved in media containing 2.7 µM NAA+3.0% sucrose and 5.4 µM NAA+3.0% sucrose.

During shoot regeneration, BA significantly promoted leaf emergence and branching, while NAA exerted inhibitory effects. This reinforces the classical auxin-cytokinin antagonism, where cytokinins promote shoot formation and auxins suppress it. The most efficient shoot induction occurred with 2.2 - 8.8 µM BA in the absence of NAA, indicating that exogenous auxin is unnecessary and detrimental at this stage. This may be due to sufficient endogenous auxin production in developing meristems, which, when combined with high exogenous NAA, leads to auxin overdose and ethylene synthesis, ultimately inhibiting growth (An et al., 2020). This phenomenon has been observed in other Araceae members, which often exhibit heightened sensitivity to auxin concentrations (Pourhassan et al., 2023).

In rooting, IBA outperformed NAA in both root initiation and development, with 2.5 µM IBA yielding optimal results. The poor performance of NAA, especially in combination with IBA, suggests interference in auxin metabolism or transport. Unlike NAA, which is highly stable and can accumulate to inhibitory levels (Nanda et al., 2004), IBA is metabolized more gradually into active IAA (Van der Krieken et al., 1993), providing a sustained and physiological auxin signal conducive to root primordia formation (Roychoudhry and Kepinski, 2022). Moreover, IBA conjugates may act as auxin reservoirs during later stages of root development (Van der Krieken et al., 1993), explaining its superior efficacy. These results support the recommendation of IBA over NAA for Zamiifolia micropropagation, consistent with findings in Paulownia (Zayova et al., 2014).

Perhaps the most significant insight from this study is the carry-over effect of the callus induction medium on greenhouse performance. Plants regenerated from calli induced on 2.7 µM NAA+3.0% sucrose exhibited superior growth, root development, and photosynthetic pigment content. This suggests that early hormonal and nutritional programming during callogenesis can have long-term epigenetic or physiological impacts on plant vigor. High chlorophyll and carotenoid levels in these plants indicate enhanced photosynthetic capacity and better stress resilience (Li and Kim, 2022; Karimi et al., 2020). Similarly, elevated soluble carbohydrates reflect improved carbon fixation and energy storage, crucial for acclimatization to *ex vitro* conditions (Salam et al., 2021). Moreover, soluble carbohydrates act as osmotic regulators, enhancing water uptake and retention under abiotic stress, which is critical for the successful

acclimatization of micropropagated plants to greenhouse conditions (Karimi et al., 2018).

Conversely, plants from media containing 4.4 µM BA (especially the medium supplemented with 5.4 µM NAA+2.2 µM BA+3.0% Suc) showed stunted growth and poor root development. This supports the hypothesis that excessive cytokinin exposure during the callus phase can impair root meristem establishment or alter auxin transport, leading to long-term growth limitations. Similar negative carry-over effects have been reported in *Alstroemeria* (Kristiansen et al., 1999), *Codiaeum variegatum* (El-Gedawey et al., 2020), and *Valeriana jatamansi* (Nazir et al., 2022), emphasizing the need for cautious cytokinin use in micropropagation protocols.

Conclusions

In conclusion, optimal callus induction in *Zamioculcas zamiifolia* was achieved using 2.7 - 5.4 µM NAA and 3.0% sucrose in ½MS medium, without the need for BA. Shoot regeneration was most effective with 2.2 µM BA in the absence of NAA, while 2.5 µM IBA alone induced the highest rooting efficiency. The results highlight that minimalist hormonal regimes - relying on endogenous hormone pools and optimal carbon supply - can yield superior results in *Z. zamiifolia* micropropagation. Crucially, the composition of the callus induction medium had a lasting impact on plant performance: Using BA during callogenesis suppressed root system development, which in turn limited the overall growth and vigor of regenerated plants in the greenhouse. These findings underscore the importance of considering long-term plant performance when designing *in vitro* systems. Future studies could explore hormonal balance and alternative growth regulators for minimizing adverse effects on root development while maintaining high propagation efficiency.

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Author Contributions

NA: Investigation, Formal Analysis, Writing – Original Draft. **SDD:** Conceptualization, Methodology, Supervision. **SK:** Validation, Writing – Review & Editing.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

Data will be made available upon request to the authors.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that the use of AI and AI-assisted technologies was not applied in the writing process.

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