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A Complete Micropropagation Protocol for Black-Leaved *Zamioculcas zamiifolia* (Lodd.) Engl. 'Dowon'

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Abstract: *Zamioculcas zamiifolia*, a drought-resistant plant in the family Araceae, is a popular ornamental potted foliage plant originating from tropical east and subtropical southeast Africa. The growth and propagation rate of this species is low in conventional propagation methods. Therefore, the current study aimed at developing a complete in vitro propagation protocol of black-leaved Raven® ZZ plant (*Z. zamiifolia* 'Dowon')—a novelty on the floricultural market. In order to initiate an axenic culture, the disinfection of leaf explants was performed with sodium hypochlorite and mercury chloride. Next, leaf segments were cultured on the Murashige and Skoog (MS) medium with the addition of 6-benzyladenine (BA) and/or α-naphthalene acetic acid (NAA) at various concentrations. The highest number of shoots (11) and leaves (22) per explant was obtained in a medium enriched with 2 mg·L^{-1} BA together with 0.5 mg·L^{-1} NAA. The maximum number of roots (3.33) was produced in microshoots cultured on the medium supplemented with 2 mg·L^{-1} NAA. On the other hand, the longest roots (2.66 cm) were produced on a medium containing 2 mg·L^{-1} NAA and 0.5 mg·L^{-1} BA. The combination of 0.5 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA was most effective in stimulating callus formation (78.33%). Rooted plantlets were transferred to plastic pots filled with coco peat and acclimatized to ambient greenhouse conditions with an average 68.19% survival rate. This is the first report on a complete micropropagation protocol of black-leaved zamioculcas.

Keywords: Araceae; in vitro propagation; ornamental plants; plant growth regulators

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1. Introduction

Zamioculcas is a monotypic genus in the family Araceae (aroids), comprising the single species *Zamioculcas zamiifolia* (Lodd.) Engl. It is a stem-less tropical herbaceous monocotyledonous originating from eastern Africa, from southern Kenya to northeastern South Africa, growing on the stony ground or tropical moist forest floor [1]. Zamioculcas (commonly named as 'ZZ plant') differs from most other aroids in possessing pinnate leaves [2]. It is grown as a medicinal-ornamental plant, mostly for its attractive glossy foliage and some pharmaceutical metabolites. Moreover, ZZ plant has the potential to reduce the concentration of pollutant gases such as benzene, ethylbenzene, xylene, and toluene from contaminated indoor air [1]. It is drought-resistant, can grow under low light conditions, and generates short sprouts from a thick underground tuber-like rhizome that stores water [3,4]. Consequently, it is described as a "unique" indoor foliage plant. The species is conventionally propagated through leaf cuttings (it rarely flowers), but this method is inefficient and time-consuming as ZZ plant is slow-growing, even under optimal production conditions [3]. For example, in a study by Prathibha et al. [5] an average of 2.2 shoots had formed per propagule at 180 days under in vivo conditions. Consequently, stock plants are expensive [2].

The development of an efficient micropropagation protocol for ZZ plant could increase the plant yield and decrease the production costs. Tissue culture techniques provide an opportunity for the large-scale production of an elite material and plants of commercial interest. The micropropagation of some Araceae genera such as *Aglaonema*, *Alocasia*, *Anthurium*, *Dieffenbachia*, *Homalomena*, *Philodendron*, *Spathiphyllum*, and *Syngonium* has been carried out [6,7]. In these studies, seedling fragments, leaves, shoots, inflorescences, and corms were used as explant sources and inoculated usually on the MS [8] medium. As for the plant growth regulators (PGRs), thidiazuron (TDZ) and 6-benzyladenine (BA) were extensively used as cytokinins, while α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), and 2,4-D (2,4-dichlorophenoxyacetic acid) were the most common auxins [7]. Among these PGRs, BA is the most effective in stimulating organogenesis in aroids. For example, Chen et al. [9] found that a 5 mg·L^{-1} BA treatment could induce the formation of adventitious shoot in *Aglaonema* 'White Tip'. In *Philodendron*, more shoots were regenerated on the BA- than kinetin-supplemented media [10]. The best conditions for in vitro multiplication of *Homalomena pineodora* were found in the MS medium with 0.5 mg·L^{-1} BA [11]. Nonetheless, there may be considerable cultivar differences in response to the type and concentration of cytokinins used.

Despite the popularity of the species, information on in vitro culture systems in *Z. zamiifolia* is limited to green-leaved cultivars and published in non-English articles [5,12]. Papafotiou and Martini [13] studied the effect of the inoculation position of leaf explants concerning PGRs during the micropropagation of ZZ plant. They found that medium supplementation with 2,4-D resulted in the formation of somatic embryos, NAA combined with BA induced mainly roots, and NAA alone produced tubers and roots, while BA alone resulted mainly in leaf development [13]. According to Sayadi Nejad and Sadeghi [14], the combination of BA and NAA was optimal for callus induction, while NAA alone stimulated shoot elongation if rhizome or petiole explants were inoculated in a half-strength (1/2) MS medium. Vanzie-Canton and Leonhardt [15] developed a protocol for the oryzalin treatment of *Z. zamiifolia* callus in a study aimed at producing a tetraploid plant in vitro.

Recently, the black-leaved attractive cultivar 'Dowon' has been created and patented by the South Korean breeder Hyuk Jin Lee [16] and offered on sale under the tradename Raven®. This cultivar has lime green new growth that turns a purple-black color as it ages, which brings an outstanding ornamental effect and gives the plant high popularity. As with other ZZ plants, it stores water in its thick petioles and a tuber-like rhizome, making it an exceptionally easy-to-grow houseplant that withstands even dark conditions. In some areas, this cultivar can be grown outdoors if the temperature does not fall below 15 °C [17].

Currently, there is no published information on the in vitro propagation of the black-leaved *Zamioculcas zamiifolia*. Considering the increasing demand for ZZ plants in the market, the present study aimed to evaluate the effect of BA and NAA on the mass proliferation of 'Dowon' cultivar through tissue culture technique.

2. Materials and Methods

2.1. Plant Material and Initiation of In Vitro Culture

Plant material (*Zamioculcas zamiifolia* (Lodd.) Engl. 'Dowon') was obtained from a greenhouse in Amol city, Mazandaran province, the northern part of Iran. The geographical coordinates of Amol are as follows: latitude: 36°28′10″ N, longitude: 52°21′02″ E, and elevation above sea level: 96 m. Leaf segments (15 mm × 15 mm) were used as primary explants. *Z. zamiifolia* 'Dowon' is a stem-less species and has underground rhizome. Therefore, the most appropriate organ for explant preparation is the leaf. To eliminate surface contamination, the leaves were washed with dishwashing liquid for 20 min. Next, the explants were placed under running tap water for half an hour and treated with 10% (*v/v*) sodium hypochlorite ($NaOCl/H_2O$ ± 3% active chloride, Chem-Lab, Zedelgem, Belgium) for 10 min. Then, the samples were disinfected with 0.1 mg·L^{-1} (*w/v*) mercury chloride ($HgCl_2$, Merck, Darmstadt, Germany) for 10 min followed by ethanol 70% for one min. Finally, the explants were washed thrice with sterile distilled water (5 min each). The mar-

gins of the explants were cut off to remove the parts damaged in the disinfection treatment. Each leaf was cut into four segments of 15 mm × 15 mm.

2.2. Organogenesis Induction

Disinfected explants were placed inside sterile glass-jars. Three explants were cultured in each jar filled with 50 mL of basal MS culture medium with 3% (*w/v*) sucrose (Merck, Darmstadt, Germany) and 0.7% (*w/v*) agar (SIGMA Aldrich, St. Louis, MO, USA). The media contained different concentrations of BA (0, 0.5, 1, and 2 mg·L^{-1}; i.e., 0, 2.22, 4.44, 8.88 μM) and NAA (0, 0.5, 1, and 2 mg·L^{-1}; i.e., 0, 2.69, 5.37, 10.75 μM) for the induction of organogenesis. Our aim was to evaluate the effect of 16 treatments on the performance of explants in vitro (direct or indirect organogenesis for both shoot multiplication and rooting). The PGRs combinations (treatments) stimulating simultaneous caulogensis and rhizogenesis were used in the further acclimatization step. The pH of the medium was adjusted to 5.6–5.8 after adding all components and before autoclaving at 121 °C and 105 kPa for 20 min. Cultures were kept in a growth room at 24 ± 2 °C with a 16 h photoperiod and light intensity of 50–60 μmol·m^{-2}·s^{-1} provided by cool-white fluorescent tubes and adjusted by a spectroradiometer. Plantlet number and length, leaf number, callus induction percentage, mean root length, and longest root length, all per explant, were measured after 12 weeks. To measure the traits and take pictures, the samples were removed from the glass containers and placed on Petri dishes.

2.3. Experimental Design and Data Analysis

The experiment was conducted in a completely randomized block design with three replications. Each experimental unit consisted of five glass-jars and three explants were cultured in each jar. Means were subjected to the analysis of variance and compared by Duncan's test at $p < 0.05$ using the SAS ver. 9.1 software (SAS Institute, 2003).

Considering that the interaction effects became significant, instead of comparing the averages of the main treatments, the average comparison between the levels of treatment compounds has been done. Therefore, the interaction effect of each treatment at different levels of the other treatment is well represented.

2.4. Acclimatization Process

All surviving and rooted microshoots (120-day-old) were removed from the culture jars and placed in the coco peat in 18 cm plastic pots (with a capacity of 4 kg) and watered with sterile water. In each pot, 3 plantlets obtained from the rooting step were planted (more than 100 pots were used in total). The pots were placed in a greenhouse with high humidity (70–80%), controlled photoperiod (16/8 light/dark with a light intensity not exceeding 100 μmol·m^{-2}·s^{-1}), and temperature (24 ± 2 °C) for acclimatization for 1.5–2 months. Irrigation was conducted by the rainy method and from the roof of the greenhouse. Plantlets were watered in this way every two weeks to prevent them from drying out. Light intensity was reduced by using two layers of canopy or nets. After 45 days of placing the plantlets in the adaptation pots, fully developed plants were transferred to a substrate (plot) containing cocopeat, peat moss, perlite, sand, and leaf soil in equal proportion for acclimatization and supply to the sales market. The survival (%) of the plants was evaluated after 8 weeks.

3. Results

3.1. Microshoot Proliferation

After disinfection (the efficiency of which reached over 85%), the explants were transferred to the proliferation culture medium. About 90% of explants regenerated shoots (Figure 1). A mean comparison of the data (Table 1) showed that the longest plantlets (2.06 cm) were obtained in MS medium augmented with 1 mg·L^{-1} NAA and 2 mg·L^{-1} BA. Their length was two- to four-fold higher than that of the other treatments. The shortest

plantlets (0.4 cm), on the other hand, were produced in a medium containing 0.5 mg·L^{-1} BA. Plantlet length was also low in the control treatment, i.e., medium without PGRs.

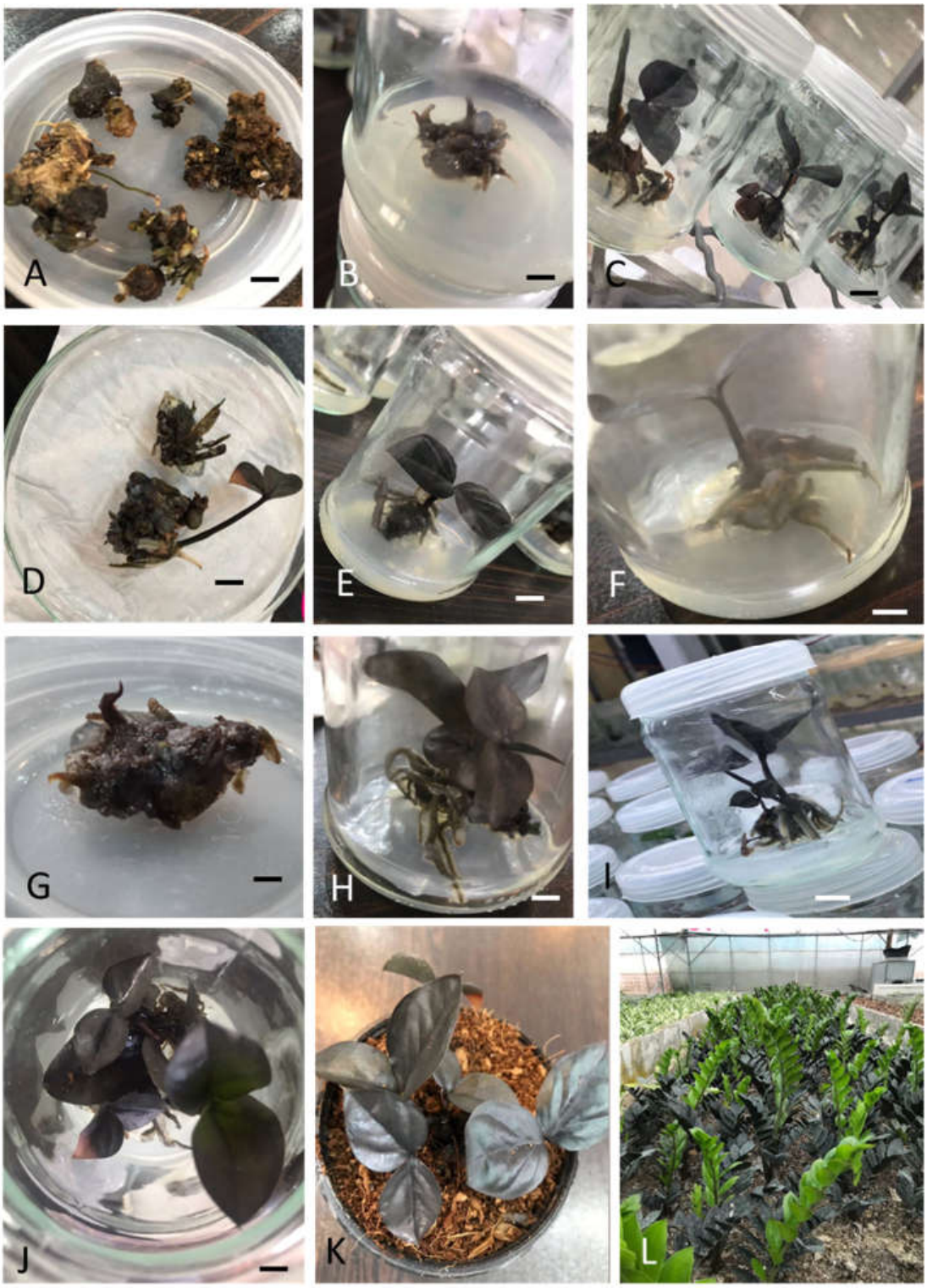

Figure 1. In vitro propagation of *Z. zamiifolia* 'Dowon'. Callus production in a medium augmented with 2 mg·L^{-1} NAA + 1 mg·L^{-1} BA (**A**) and the PGRs-free control (**B**); development and multiplication of shoots in a medium containing 0.5 mg·L^{-1} NAA ((**C**), left), 1 mg·L^{-1} NAA + 2 mg·L^{-1} BA

((**C**), medial), and 0.5 mg·L^{-1} NAA + 2 mg·L^{-1} BA ((**C**), right); comparison of plantlets produced in a medium containing 1 mg·L^{-1} NAA + 2 mg·L^{-1} BA ((**D**), down) and the control medium ((**D**), up); leaf development in a medium containing 2 mg·L^{-1} NAA + 2 mg·L^{-1} BA (**E**); root development in a medium containing 2 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA (**F**) and the control treatment (**G**); leaves developed in a medium supplemented with 0.5 mg·L^{-1} NAA + 2 mg·L^{-1} BA (**H**); plantlets produced in a medium enriched with 1 mg·L^{-1} NAA + 2 mg·L^{-1} BA (**I**); plantlets developed in a medium supplemented with 0.5 mg·L^{-1} NAA + 2 mg·L^{-1} BA, ready for acclimatization (**J**); plantlets cultivated ex vitro in a plastic pot filled with cocopeat (**K**); fully developed plants after one year in a plot containing cocopeat, peat moss, perlite, sand, and leaf soil in equal proportions (**L**). Age of cultures: (**A**–**J**): 60 days, (**K**): 105 days, (**L**): one year. Scale bars: (**A**,**B**,**D**,**I**) = 10 mm, (**C**,**F**) = 12 mm, (**E**,**H**) = 8 mm, (**G**) = 2 mm, and (**J**) = 5 mm.

Table 1. Effect of different concentrations of NAA and BA on the measured characteristics of *Zamioculcas zamiifolia* (Lodd.) Engl. 'Dowon' grown in vitro in the multiplication step.

NAA (mg·L^{-1})	BA (mg·L^{-1})	Plantlet Length (cm)	Plantlet Number per Explant	Leaf Number per Explant	Callus Induction (%)
0	0	0.54 ± 0.44 ^{c}	2.88 ± 0.19 ^{f}	5.66 ± 0.72 ^{f}	32.33 ± 5.80 ^{f}
0	0.5	0.40 ± 0.31 ^{d}	4.66 ± 0.28 ^{d,e}	9.33 ± 0.30 ^{d}	70.00 ± 20.00 ^{a,b}
0	1	0.56 ± 046 ^{c}	3.00 ± 0.36 ^{f}	7.33 ± 0.38 ^{e,f}	46.66 ± 15.30 $^{c–e}$
0	2	1.20 ± 0.30 ^{b}	4.33 ± 0.25 ^{d,e}	6.00 ± 0.27 ^{f}	33.33 ± 5.80 ^{f}
0.5	0	0.60 ± 0.11 ^{b,c}	3.66 ± 0.72 ^{e,f}	8.66 ± 0.482 ^{d}	45.00 ± 13.20 $^{c–e}$
0.5	0.5	0.53 ± 0.22 ^{c,d}	4.00 ± 0.15 ^{e}	7.33 ± 0.74 ^{e,f}	78.33 ± 7.60 ^{a}
0.5	1	0.60 ± 0.16 ^{b,c}	5.66 ± 0.17 ^{b,c}	8.00 ± 0.22 ^{e,f}	51.66 ± 7.60 ^{c,d}
0.5	2	1.16 ± 0.42 ^{b}	11.00 ± 0.42 ^{a}	22.00 ± 0.63 ^{a}	41.66 ± 7.60 ^{d,e}
1	0	0.66 ± 0.18 ^{c}	5.00 ± 0.53 $^{b–d}$	10.00 ± 0.62 ^{d}	40.00 ± 5.00 ^{d,e}
1	0.5	0.50 ± 0.37 ^{c,d}	3.66 ± 0.55 ^{e,f}	7.33 ± 0.29 ^{e,f}	56.66 ± 2.90 ^{b,c}
1	1	0.73 ± 0.44 ^{b,c}	4.66 ± 0.18 ^{d,e}	9.33 ± 0.28 ^{d}	46.66 ± 5.80 $^{c–e}$
1	2	2.06 ± 0.38 ^{a}	9.00 ± 0.34 ^{a,b}	18.00 ± 0.15 ^{b}	45.00 ± 5.00 $^{c–e}$
2	0	0.66 ± 0.11 ^{c}	5.00 ± 0.12 $^{b–d}$	10.00 ± 0.42 ^{d}	36.66 ± 2.90 ^{e,f}
2	0.5	0.63 ± 0.22 ^{c}	5.66 ± 0.15 ^{b,c}	11.33 ± 0.22 ^{c}	56.66 ± 5.80 ^{b,c}
2	1	0.66 ± 0.32 ^{c}	5.00 ± 0.44 $^{b–d}$	10.00 ± 0.15 ^{d}	45.00 ± 13.20 $^{c–e}$
2	2	0.93 ± 0.23 ^{b,c}	9.00 ± 0.23 ^{a,b}	18.00 ± 0.42 ^{b}	38.30 ± 2.90 ^{d,e}

Means with different letters in the same column are significantly different ($p < 0.05$) based on Duncan's test.

The highest number of plantlets per explant (11) was produced in the MS medium supplemented with 0.5 mg·L^{-1} NAA and 2 mg·L^{-1} BA and it was approximately four-fold higher than in the control object. Media with 1 mg·L^{-1} NAA together with 2 mg·L^{-1} BA and 2 mg·L^{-1} NAA with 2 mg·L^{-1} BA also induced high (9.0) plantlet number (Table 1). The smallest number of plantlets (2.88–3) was obtained in the control medium and medium with 1.0 mg·L^{-1} BA only.

A maximum number of leaves (22 per explant) was established in the MS medium with 0.5 mg·L^{-1} NAA and 2 mg·L^{-1} BA. Many leaves (18 per explant) were also produced in the media containing 1 mg·L^{-1} NAA together with 2 mg·L^{-1} BA and 2 mg·L^{-1} NAA with 2 mg·L^{-1} BA. A minimum number of leaves (5.66 per explant) was obtained in the medium without PGRs (Table 1).

The analysis of variance (Table 2) revealed that in terms of all studied characteristics related to plantlet proliferation, there was a significant effect of the tested PGRs concentrations.

3.2. Callus Induction Percentage

The concentration of BA and interaction effect between BA and NAA greatly affected the in vitro induction of callus in *Z. zamiifolia* 'Dowon' (Table 2, Figure 1A,B). The highest callus production (78.33% per explant) was obtained on the medium augmented with 0.5 mg·L^{-1} BA together with 0.5 mg·L^{-1} NAA (Table 1). The share of explants forming callus cultured on the medium containing 0.5 mg·L^{-1} BA was also high (70%). The lowest

rate of callus formation (32.33–33.33% of explants) was induced on the control object and medium with 2.0 mg·L^{-1} BA only (Table 1). Most of the calli were compact and firm.

Table 2. Analysis of variance of the effect of different concentrations of NAA and BA on the measured characteristics of *Zamioculcas zamiifolia* (Lodd.) Engl. 'Dowon' grown in vitro in the multiplication, rooting, and acclimatization steps.

Source of Variances	df	Plantlet Length	Plantlet Number	Leaf Number	Callus Percentage	Mean Root Length	Longest Root Length	Root Number	Acclimatization Efficiency
Block	2	119.73 *	0.11 **	0.13 **	119.00 ^{ns}	15.37 **	1.02 **	0.09 ^{ns}	74.70 ^{ns}
NAA	3	82.05 *	0.16 **	0.12 **	232.00 ^{ns}	24.94 **	2.49 **	2.72 **	735.00 **
BA	3	232.92 *	0.02 **	0.14 **	1839.00 **	2.72 **	0.53 **	0.19 ^{ns}	152.00 *
NAA × BA	9	44.11 *	0.12 **	0.11 **	994.00 *	10.43 **	0.70 **	1.10 **	298.00 **
Error	30	86.28	0.35	0.17	85.41	2.55	0.09	0.17	50.30
CV (%)	-	3.40	4.58	3.61	19.32	2.80	16.82	18.86	10.50

*, **: significant at the 0.05 and 0.01 probability level, respectively; ns: not significant; CV: coefficient of variation; df: degrees of freedom.

3.3. Microshoot Rooting

About 85% of the microshoots regenerated roots. The statistical analysis showed that there was a significant difference between various concentrations of NAA and BA (alone and in combination) regarding rooting effectiveness, i.e., mean root length and length of the longest root. The concentration of NAA alone and the interaction of NAA and BA had a significant effect on the root number (Table 2). The longest (2.66 cm) and shortest (0.26 cm) mean root lengths were found in the media enriched with 2 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA (Figure 1F) and 0.5 mg·L^{-1} BA alone, respectively. The length of roots produced in the medium without PGRs (control) was low (0.6 cm) (Table 3).

Table 3. Effect of different concentrations of NAA and BA on the measured characteristics of *Zamioculcas zamiifolia* (Lodd.) Engl. 'Dowon' grown in vitro in the rooting and acclimatization steps.

NAA (mg·L^{-1})	BA (mg·L^{-1})	Mean Root Length (cm)	Longest Root Length (cm)	Root Number	Acclimatization (%)
0	0	0.60 ± 0.29 ^{e}	1.73 ± 0.61 ^{c,d}	1.97 ± 0.00 $^{b–d}$	53.30 ± 5.80 ^{e,f}
0	0.5	0.26 ± 0.27 ^{f}	1.47 ± 0.25 ^{d,e}	2.00 ± 0.12 $^{b–d}$	60.00 ± 10.00 $^{c–f}$
0	1	1.16 ± 0.33 ^{c}	1.32 ± 0.12 ^{f,g}	1.33 ± 0.57 ^{d}	60.00 ± 10.00 $^{c–f}$
0	2	1.13 ± 0.45 ^{c}	1.73 ± 0.06 ^{c,d}	1.33 ± 0.27 ^{d}	70.00 ± 0.00 ^{b,c}
0.5	0	1.46 ± 0.12 ^{b}	1.77 ± 0.31 ^{c,d}	2.01 ± 0.00 $^{b–d}$	64.90 ± 5.80 $^{c–e}$
0.5	0.5	1.20 ± 0.55 ^{b,c}	1.40 ± 0.36 ^{e,f}	2.00 ± 1.00 $^{b–d}$	70.00 ± 10.00 ^{b,c}
0.5	1	1.30 ± 0.35 ^{b,c}	1.63 ± 0.12 $^{c–e}$	2.00 ± 0.00 $^{b–d}$	80.00 ± 0.00 ^{b}
0.5	2	1.20 ± 0.31 ^{b,c}	1.63 ± 0.25 $^{c–e}$	1.67 ± 0.42 ^{c,d}	70.00 ± 0.00 ^{b,c}
1	0	1.13 ± 0.46 ^{c}	1.57 ± 0.32 $^{c–e}$	2.33 ± 0.74 ^{b,c}	93.30 ± 5.80 ^{a}
1	0.5	1.36 ± 0.23 ^{b,c}	1.27 ± 0.15 ^{f,g}	2.00 ± 0.00 $^{b–d}$	70.00 ± 10.00 ^{b,c}
1	1	1.20 ± 0.36 ^{b,c}	1.57 ± 0.15 $^{c–e}$	2.33 ± 0.66 ^{b,c}	63.30 ± 5.80 $^{c–e}$
1	2	0.80 ± 0.20 ^{d}	1.14 ± 0.26 ^{g}	2.66 ± 0.58 ^{a,b}	80.00 ± 0.00 ^{b}
2	0	1.76 ± 0.22 ^{a,b}	2.60 ± 0.36 ^{b}	3.33 ± 1.15 ^{a}	63.00 ± 10.00 $^{c–e}$
2	0.5	2.66 ± 0.35 ^{a}	3.60 ± 0.46 ^{a}	2.67 ± 0.57 ^{a,b}	66.60 ± 5.80 ^{c,d}
2	1	1.60 ± 0.12 ^{a,b}	1.60 ± 0.10 $^{c–e}$	2.34 ± 0.35 ^{b,c}	56.60 ± 5.80 $^{d–f}$
2	2	1.76 ± 0.53 ^{a,b}	1.90 ± 0.26 ^{c}	2.66 ± 0.19 ^{a,b}	70.00 ± 10.00 ^{b,c}

Means with different letters in the same column are significantly different ($p < 0.05$) based on Duncan's test.

The highest root number (3.33 per microshoot) was regenerated in a medium containing only 2 mg·L^{-1} NAA. All other experimental objects regenerated less than 3 roots per microshoot. The lowest number of roots (1.33 per microshoot) was produced in the media with 1 and 2 mg·L^{-1} BA, both without NAA (Table 3).

3.4. Acclimatization

There was a significant impact of NAA concentration, BA, and interaction effect of both PGRs on the acclimatization efficiency of ZZ plants (Table 2). An average of 68.19% of the plantlets survived acclimation to the greenhouse environmental conditions (Figure 1J–L). Table 3 shows that a maximum survival rate (93.33%) in the acclimatization process was observed in plantlets generated on a medium supplemented with 2 mg·L^{-1} NAA. Plantlets produced in the control medium showed the lowest survival rate (53.3%).

4. Discussion

In vitro propagation on several aroids plants (Araceae family) has previously been carried out [7], but there is no published information on the micropropagation of the black-leaved ZZ plant. In some species, in vitro cultured plant tissues begin to grow upon encountering a cytokinin or auxin alone, but the presence of both of them is required to continue growth or increases the number of shoots regenerated per explant [18,19]. Exogenous application of these PGRs can increase the endogenous concentration of phytohormones. A study by Chen and Yeh [20] on the micropropagation of *Aglaonema* sp. reported that the shoot number increased linearly with elevating concentration of BA. Our work confirms this finding, but the addition of NAA was essential for caulogenesis; the highest number of plantlets (11) and leaves (22) per explant (after 12 weeks of culture) were produced in the medium supplemented with 0.5 mg·L^{-1} NAA and 2.0 mg·L^{-1} BA. These results are very promising, as Ghoochani Khorasani et al. [21] obtained a very low (2.62) multiplication ratio of green-leaved ZZ in vitro.

Several species need a low level of auxin in combination with a high concentration of cytokinins to increase shoot multiplication [22]. For example, Ali et al. [23] showed that excellent multiple shoots were formed in *Caladium bicolor* when 0.25 mg·L^{-1} NAA was added to 1 mg·L^{-1} of BA. Islam et al. [24] reported that the addition of NAA at variable concentrations in a medium containing 1 mg·L^{-1} BA induced shoot induction in *Anthurium andreanum* cv. Nitta. Treatment using 3 mg·L^{-1} BA and 0.2 mg·L^{-1} NAA recorded the highest shoot proliferation rate (6.0) and shoot length (7.75 cm) in *Aglaonema widuri*. Likewise, 4 mg·L^{-1} BA together with 0.1 mg·L^{-1} NAA and 0.5 mg·L^{-1} TDZ produced the maximum number of nodes (13.25 per explant) in this species. The highest number of leaves (4.25 per explant), on the other hand, was produced in the medium containing 3.5 mg·L^{-1} BA and 0.2 mg·L^{-1} NAA [6]. The present results are in agreement with these findings. Cytokinins are involved in many aspects of plant development, including the regulation of cell proliferation. Studies imply that these PGRs may elevate cell division rates by the induction of CycD3 expression, which encodes a D-type cyclin involved in the G1→M transition of the cell cycle [25]. Auxins, on the other hand, act as a signaling molecule to regulate many developmental processes throughout all stages of plant ontogenesis, e.g., cell elongation [26]. Interestingly, in the present study, callus, shoot, and root production were also observed in the PGRs-free culture medium, which suggested that the explants have enough endogenous phytohormones to stimulate organogenesis in vitro, although at a low rate. A similar phenomenon was reported by some researchers on a few aroid members [27–30]. The main advantage of this phenomenon is that there is no need to use exogenous PGRs and, as a result, reduction in propagation costs and somaclonal variation occurrence during micropropagation.

A study by Fang et al. [19] on *Aglaonema* 'Lady Valentine' reported that the longest shoots were produced on a medium with 5 mg·L^{-1} BA. Likewise, the use of BA was effective for axillary bud outgrowth in *Dieffenbachia compacta* [31], also a member of Araceae. In our study, the longest shoots were produced in the presence of 2 mg·L^{-1} BA together with 1 mg·L^{-1} NAA, probably due to the impact of auxin on the elongation of cells [26]. The superiority of BA over other cytokinins such as kinetin, N6-(Δ^2-isopentenyl) adenine (2-iP), and TDZ in promoting shoot elongation has been found in several other ornamental Araceae [19,23,32].

The current study revealed that leaf explants cultured on the MS medium containing 0.5 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA produced the greatest number of calli (response rate). Similar findings were reported in *Caladium bicolor* (Aiton) Vent. with 4 mg·L^{-1} BA along with 0.5 mg·L^{-1} NAA [33]. The auxin commonly used for callus induction is 2,4-D, but IAA and NAA are also used, alone or in combination with BA [34]. Yu et al. [35] achieved a compact callus mass from leaf and petiole explants in *Anthurium andreanum* Linden ex André by a combination of 2,4-D and BA, from which plant formation was obtained. Leaflet and petiole explants of *Z. zamiifolia* were cultured onto a callus induction medium composed of $\frac{1}{2}$ MS salts, 4 mg·L^{-1} 2,4-D, and 0.2 mg·L^{-1} BA by [15]. The cultures were transferred to a fresh medium biweekly and stored in the dark at a temperature of 25–27 °C. Callus was visible on the explants after about 4.5 weeks. Next, calli were transferred to a shoot induction medium containing $\frac{1}{2}$ MS enriched with 1 mg·L^{-1} BA. The development of adventitious buds was observed after 11 weeks of culture. All rooted plantlets transferred to the greenhouse developed normally [15]. Callus obtained from leaf laminas is a suitable source for the production of plantlets through indirect organogenesis and somatic embryogenesis. However, the genetic stability of such plants is uncertain, thus, the comparison of the PGRs-treated and control plantlets is necessary. In the present study, no phenotypical differences were observed among plants from various experimental objects, although further molecular analyses are recommended.

Our findings revealed the positive effect of NAA on root production in ZZ 'Dowon'. Microshoots cultured on a medium augmented with 2 mg·L^{-1} NAA produced the highest number of roots, whereas microshoots from the experimental objects containing 1 and 2 mg·L^{-1} BA without NAA regenerated the lowest number of roots. In a study on *Caladium* micropropagation, Ali et al. [23] reported that the best rooting was obtained on a medium augmented with 1.0 mg·L^{-1} NAA. Auxin type and concentration considerably affected rooting percentage and root length. In *C. bicolor*, the maximum root number was produced in a medium with 3 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA [20]. In *A. widuri*, the best root initiation and development (14.25 per explant) was found on a medium containing 3 mg·L^{-1} BA and 0.2 mg·L^{-1} NAA. On the other hand, the combination of 3.5 mg·L^{-1} BA and 0.2 mg·L^{-1} NAA was most suitable for root elongation (8.25 cm per explant) [6]. In contrast, microcuttings produced from tissue culture of *Aglaonema* rooted when treated with IBA and NAA [20]. Auxins trigger complex growth and developmental processes. They facilitate fast switching between gene activation and transcriptional repression via the auxin-dependent degradation of transcriptional repressors. The nuclear auxin signaling pathway consists of a small number of core components, but each component is represented by a large gene family [36]. Exogenous application of synthetic auxins, such as NAA, is crucial for root initiation and development [37].

It was found that the in vitro obtained plantlets of *Z. zamiifolia* 'Dowon' acclimatized suitably through cultivation in pots filled with coco peat. Different substrates such as sand, vermicompost, vermiculite, perlite, and organic humus have been used for the acclimatization of in vitro-regenerated Araceae plantlets, with high survival ratios ranging from 60% to 98% [30,38]. The high survival level of the plantlets obtained in the present study (reaching even 93.33%) confirms the suitability of the present protocol in the commercial reproduction of ZZ plant 'Dowon'.

5. Conclusions

This is the first complete report on the in vitro propagation of black-leaved *Z. zamiifolia* 'Dowon', which is a starting point for mass-propagation programs of this valuable cultivar. Our procedure led to an increased production of healthy and disease-free propagules compared to the traditional in vivo propagation systems. A combination of BA and NAA in suitable proportion induced most shoots and roots. The maximum number of shoots (11 per explant) and leaves (22 per explant) was obtained in a medium enriched with 2 mg·L^{-1} BA and 0.5 mg·L^{-1} NAA. On the other hand, the highest number of roots (3.33 per plant) was achieved in a medium with 2 mg·L^{-1} NAA. The survival of the plantlets during

the acclimatization process was high (even 93.33%) and they were phenotypically similar to the mother plant. The application of easily accessible PGRs allows for the effortless implementation of our protocol in horticultural practice, but also opens the possibility to further study the combined effect of BA and NAA with less conventional PGRs.

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References

1. Sriprapat, W.; Thiravetyan, P. Phytoremediation of BTEX from indoor air by *Zamioculcas zamiifolia*. *Water Air Soil Pollut.* **2013**, *224*, 1482. [CrossRef]
2. Chen, J.; Henny, R.J.; Henny, P.S. ZZ: A unique tropical ornamental foliage plant. *HortTechnol.* **2003**, *13*, 458–462. [CrossRef]
3. Lopez, R.G.; Blanchard, M.G.; Runkle, E.S. Propagation and production of *Zamioculcas zamiifolia*. *Acta Hortic.* **2009**, *813*, 559–564. [CrossRef]
4. Le Moullec, A.; Juvik, O.J.; Fossen, T. First identification of natural products from the African medicinal plant *Zamioculcas zamiifolia*—A drought resistant survivor through millions of years. *Fitoterapia* **2015**, *106*, 280–285. [CrossRef]
5. Prathibha, B.R.; Nirmala, K.S.; Satyanarayana, B.N.; Anithe, P.; Chinnaswamy, K.P. Induction of multiple shoots in *Zamioculcas zamiifolia* Engl. under in vivo conditions. *Int. J. Chem. Studies* **2018**, *6*, 667–671.
6. Kaviani, B.; Sedaghathoor, S.; Motlagh, M.R.S.; Rouhi, S. Influence of plant growth regulators (BA, TDZ, 2-iP and NAA) on micropropagation of *Aglaonema widuri*. *Iran. J. Plant Physiol.* **2019**, *9*, 2703–2718.
7. Chen, J.; Wei, X. Thidiazuron in micropropagation of aroid plants. In *Thidiazuron: From Urea Derivative to Plant Growth Regulator*; Faisal, M., Ahmad, N., Eds.; Springer: Berlin/Heidelberg, Germany, 2018; pp. 95–113.
8. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture'. *Plant Physiol.* **1962**, *15*, 473–497. [CrossRef]
9. Chen, J.; Henny, R.J.; Devanand, P.S.; Chao, C.T. AFLP analysis of nephthytis (*Syngonium podophyllum* Schott) selected from somaclonal variants. *Plant Cell Rep.* **2006**, *24*, 743–749. [CrossRef]
10. Chen, F.; Wang, C.; Fang, J. Micropropagation of self-heading philodendron via direct shoot regeneration. *Sci. Hortic.* **2012**, *141*, 23–29. [CrossRef]
11. Stanly, C.; Bhatt, A.; Sulaiman, B.; Keng, C.L. Micropropagation of *Homalomena pineodora* Sulaiman & Boyce (Araceae): A new species from Malaysia. *Hortic. Bras.* **2012**, *30*, 39–43.
12. Heping, S.; Peng, L. Plantlet regeneration from leaf explants of *Zamioculcas zamiifolia*. *Acta Hort. Sin.* **2003**, *5*, 621–622.
13. Papafotiou, M.; Martini, A. Effect of position and orientation of leaflet explants with respect to plant growth regulators on micropropagation of *Zamioculcas zamiifolia* Engl. (ZZ). *Sci. Hortic.* **2009**, *120*, 115–120. [CrossRef]
14. Sayadi Nejad, M.; Sadeghi, S. Optimization of callus production and regeneration of zamiifolia (*Zamioculcas zamiifolia*). *J. Hortic. Sci.* **2019**, *33*, 405–415. [CrossRef]
15. Vanize-Canton, S.D.; Leonhardt, K.W. In vitro callus induction and plantlet regeneration protocol developed for the oryzalin treatment of *Zamioculcas zamiifolia* (Lodd.) Engl. (Araceae). *Acta Hortic.* **2009**, *813*, 201–208. [CrossRef]
16. Lee, H.J. *Zamioculcas* Plant Named 'Dowon'. US Patent USPP30035P3, 25 December 2018.
17. Zamioculcas zamiifolia 'Dowon' Common Name(s): Jet-Black Raven, Raven™ ZZ. Available online: https://plants.ces.ncsu.edu/plants/zamioculcas-zamiifolia-dowon/ (accessed on 5 January 2023).
18. Dewir, Y.D.; Chakrabarty, E.; Hahn, A.; Paek, K. A simple method for mass propagation of *Spathiphyllum cannifolium* using an airlift bioreactor'. *Vitro Cell. Dev. Biol. Plant* **2006**, *42*, 291–297. [CrossRef]
19. Fang, J.Y.; Hsul, Y.R.; Chen, F.C. Development of an efficient micropropagation procedure for *Aglaonema* 'Lady Valentine' through adventitious shoot induction and proliferation. *Plant Biotechnol.* **2013**, *30*, 423–431. [CrossRef]
20. Chen, W.L.; Yeh, D.M. Elimination of in vitro contamination, shoot multiplication, and *ex vitro* rooting of *Aglaonema*. *HortSci* **2007**, *42*, 629–632. [CrossRef]
21. Ghoochani Khorasani, A.; Roohollahi, I.; Golkar, P. Effect of different concentrations of nitrogen and 2,4-D on callus and plantlet production of *Zamioculcas zamiifolia* Engl. under in vitro condition. *J. Hortic. Sci.* **2021**, *35*, 55–62.
22. Kaviani, B.; Hashemabadi, D.; Khodabakhsh, H.; Onsinejad, R.; Ansari, M.H.; Haghighat, N. Micropropagation of *Begonia rex* Putz. by 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA). *Int. J. Bioscie.* **2015**, *6*, 8–15.
23. Ali, A.; Munawar, A.; Naz, S. An in vitro study on micropropagation of *Caladium bicolor*. *Int. J. Agric. Biol.* **2007**, *9*, 731–735.
24. Islam, S.A.; Dewan, M.H.R.; Mukul, M.A.; Hossain, M.A.; Khatun, F. In vitro regeneration of *Anthurium andreanum* cv. Nitta. *Bangladesh J. Agril. Res.* **2010**, *35*, 217–226. [CrossRef]

25. Fathy, M.; Saad Eldin, S.M.; Naseem, M.; Dandekar, T.; Othman, E.M. Cytokinins: Wide-spread signaling hormones from plants to humans with high medical potential. *Nutrients* **2022**, *14*, 1495. [CrossRef]
26. Zhang, Y.; Yu, J.; Xu, X.; Wang, R.; Liu, Y.; Huang, S.; Wei, H.; Wei, Z. Molecular mechanisms of diverse auxin responses during plant growth and development. *Int. J. Mol. Sci.* **2022**, *23*, 12495. [CrossRef]
27. Viegas, J.; Rocha, M.T.R.; Ferreira-Moura, I.; Rosa, J.D.L.; Souza, A.; Correa, M.G.S.; Silva, J.A.T. *Anthurium andraeanum* (Linden ex Andre) culture: In vitro and ex vitro. *Floric. Ornam. Biotech.* **2007**, *1*, 61–65.
28. Liendo, M.; Mogollon, N. Multiplicacion clonal in vitro del anturio (*Anthurium andreanum* Lind. cv. Nicoya). *Bioagro* **2009**, *21*, 179–182.
29. Farsi, M.M.E.; Taghavizadeh, Y.; Qasemiomran, V. Micropropagation of *Anthurium andreanum* cv. Terra. *Afr. J. Biotechnol.* **2012**, *11*, 13162–13166.
30. Murillo-Gómez, P.A.; Naranjo, E.; Callejas, R.; Atehortúa, L.; Urrea, A. Micropropagation of the native species *Anthurium antioquiense* Engl. for conservation purposes. *Agronomia Colombiana* **2014**, *32*, 334–340. [CrossRef]
31. Azza, M.; El-Sheikh, K.; Mutasim, M.K. In vitro shoot micropropagation and plant establishment of an ornamental plant dumb cane (*Dieffenbachia compacta*). *Int. J. Curr. Res.* **2010**, *6*, 27–32.
32. Kozak, D.; Stelmaszczuk, M. The effect of benzyladenine on shoot regeneration in vitro of *Zantedeschia aethiopica* 'Green Goddess'. *Ann. UMCS Hortic.* **2009**, *19*, 14–18. [CrossRef]
33. Seydi, S.; Negahdar, N.; Andevari, R.T.; Ansari, M.H.; Kaviani, B. Effect of BAP and NAA on Micropropagation of *Caladium bicolor* (Aiton) Vent., an Ornamental Plant. *J. Ornament. Plants* **2016**, *6*, 59–66.
34. Kaviani, B. Some useful information about micropropagation. *J. Ornam. Plants* **2015**, *5*, 29–40.
35. Yu, Y.X.; Liu, L.; Liu, J.X.; Wang, J. Plant regeneration by callus-mediated protocorm-like body induction of *Anthurium andraeanum*. *Hort. Agric. Sci. China* **2009**, *8*, 572–577. [CrossRef]
36. Lavy, M.; Estelle, M. Mechanisms of auxin signaling. *Development* **2016**, *143*, 3226–3229. [CrossRef]
37. Matsuo, S.; Kikuchi, K.; Nagasuga, K.; Ueno, H.; Imanishi, S. Transcriptional regulation of auxin metabolic-enzyme genes during tomato fruit development. *Sci. Hortic.* **2018**, *241*, 329–338. [CrossRef]
38. Atak, C.; Celik, O. Micropropagation of *Anthurium andraeanum* from leaf explants. *Pakistan J. Bot.* **2009**, *41*, 1155–1161.