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In Vitro Micropropagation of the Vulnerable Chilean Endemic *Alstroemeria pelegrina* L.

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Abstract: The Chilean rhizomatous geophyte *Alstroemeria pelegrina* (*A. pelegrina*), a species endemic to the Coquimbo (31°45′ S) and Valparaíso (33°12′ S) regions, is currently classified as vulnerable and experiencing population decline due to loss of habitat. This heightened threat underscores the need to develop effective mass propagation techniques to support its conservation efforts. Consequently, the present study aimed to establish an optimized in vitro propagation protocol tailored specifically for *A. pelegrina* to facilitate its large-scale propagation and promote the preservation of this vulnerable geophyte. In our experiment, explants obtained from in vitro germination were used. These explants were then cultured on a Murashige and Skoog (MS) medium solidified with agar (6 g L^{-1}) and supplemented with 2.22 μM 6-Benzylaminopurine (BAP) and 30 g L^{-1} sucrose. The results indicated an average of 4.6 new shoots produced per initial explant. The implementation of light-emitting diode (LED) illumination with a red:blue ratio of 3:1 and 2.22 μM BAP resulted in an average shoot length of 6.2 cm. For rhizome induction, the addition of either 5.37 μM 1-naphthaleneacetic acid (NAA) or 5.37 μM indole-3-butyric acid (IBA) resulted in average rhizome lengths of 1.9 cm and 1.7 cm, respectively, with fresh weights of the vitroplants ranging from 2.9 g to 3.1 g, and rooting percentages between 100% and 96%, respectively. Notably, the survival rate of rooted plants obtained through in vitro propagation was 90% after a 4-week acclimatization period in a cold greenhouse, indicating the efficacy of the developed protocol for mass propagation and conservation efforts of this vulnerable geophyte species.

Keywords: Mariposa de Los Molles; in vitro culture; geophyte; flowers; *Alstroemeria*; *Alstroemeriaceae*

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1. Introduction

The genus *Alstroemeria*, belonging to the *Alstroemeriaceae* family, is native to South America, comprising approximately 90 species distributed across Chile, Brazil, Argentina, Peru, Bolivia, Paraguay, and Venezuela [1–3]. This genus has gained substantial commercial importance as an ornamental crop, extensively cultivated for its captivating floral forms and diverse color palette, which has driven its global production and trade both as cut flowers and potted plants [4–7].

Propagation of this genus primarily relies on vegetative techniques such as rhizome division. However, these methods are associated with several drawbacks, including low efficiency, time-consuming procedures, risks of disease dissemination (e.g., fungal and viral infections), reduced productivity, and seasonal limitations [8,9]. In contrast, seed-based propagation is not widely adopted due to challenges related to embryonic and

combined dormancy, genotypic and physical constraints, and consequently diminished germination capacity in most *Alstroemeria* species [10,11].

However, the wild and endemic Chilean species *A. pelegrina* seeds undergo physical dormancy. Scarification techniques were employed to enhance its germination capacity, leading to the development of a successful in vitro germination protocol [12]. Currently, in vitro propagation techniques, particularly those associated with interspecific hybridization, mutagenesis, polyploidization, and in vitro embryogenesis, are used in this genus to address the need for commercial variety development and the difficulties associated with seed germination [13–18].

Micropropagation of *Alstroemeria* can be achieved through embryo rescue. Following manual pollination, seeds are collected 10–23 days later. Excised ovules are then cultured on Murashige and Skoog (1962) (MS) medium in darkness to promote embryo development. The culture medium is optimized to enhance this process [13–18]. Regeneration rates reported in vitro vary between 2 and 30%, with a culture period of 6–11 months [19]. Various explants for in vitro multiplication, including stems, leaves, flower buds, and fragmented rhizomes/meristems, were tested, and meristematic explants were the most successful, achieving multiplication rates of 2–3 shoots [20–24].

Apical bud dominance was identified as a major challenge in micropropagation. The highest shoot induction rate was achieved by incubating of Alstroemeria sp. explants in darkness for 15 days at 8 °C, followed by 15 days at 25 °C, under a 16/8 h light/dark cycle [20–24]. In contrast, the use of greenhouse-grown rhizome-derived plants cultured in vivo on a substrate present significant contaminations that were difficult to overcome [25]. In addition, in vitro propagation studies in this genus focused on optimizing the effect of gelling agent concentration, using between 3 and 7 g L^{-1}. Conversely, the use of liquid medium resulted in hyperhydricity or vitrification, reducing growth, causing explant translucency, and potentially leading to necrosis. Cytokinins are the most commonly used growth regulators, with 6-benzyladenine and 6-benzylaminopurine at concentrations of 2.22 to 8.88 μM being particularly effective in promoting shoot growth and bud multiplication [26–30].

Chile harbors a high diversity of Alstroemeria plant genus, with 58 taxa documented (including 37 species, 11 subspecies, and 10 varieties). Notably, 82% of these taxa are endemic to the Chilean Mediterranean climate zone [31]. However, these endemic wild plants face threats due to human activities (urbanization) and climate change, which lead to a decline in their reproductive capacity [32,33]. ⊚ ne such threatened species is *Alstroemeria pelegrina*. It is found along the Chilean coast, distributed from the Coquimbo Region (31°45′ S) south to Quintay in the Valparaíso Region (33°12′ S). It typically occurs at heights up to 120 m a.s.l. (above sea level), inhabiting coastal cliffs, rocky areas, and mountain slopes. *A. pelegrina* is currently listed as vulnerable by the Chilean Ministry of the Environment due to overcollection and desertification, factors that diminish its natural habitat [32–34].

In vitro seed germination offers significant advantages for the conservation of vulnerable plant species. Compared to traditional seed germination, in vitro seed germination reduces germination time and allows more uniform seedling emergence [35,36]. In addition, micropropagation increases overall germination percentages and provides a rapid obtaining of plantlets. This contributes to maintaining genetic diversity in ex situ conservation programs by incorporating a broader range of genotypes, ultimately enhancing the viability of existing vulnerable plant populations. Micropropagation of shoots obtained from seeds germinated in vitro ensures this by preserving genetic variability within the propagated material, effectively reducing the risk of genetic bottlenecks that can occur with traditional seed-based propagation [35,36]. This leads to populations with greater adaptability, which is crucial for their long-term survival in the face of environmental challenges, and reduces the risk of extinction.

Given the critical need to preserve Chile's unique biological heritage, developing methods for large-scale propagation of this *Alstroemeria* genus is essential. In vitro culture

provides a viable option for mass propagation. Therefore, this study aimed to develop a protocol for the in vitro propagation of the vulnerable species, *A. pelegrina*.

2. Materials and Methods

Plant material

Alstroemeria pelegrina fruits were collected in January and February 2021 in the city of Quillota (32°54′ S, 71°16′ W), Chile, from a collection of 15 plants maintained at the Propagation Laboratory of the Pontificia Universidad Católica de Valparaíso. Thirty fruits of *A. pelegrina* at physiological maturity were randomly collected [12]. The seeds were manually taken out for germination.

Explant acquisition

Explants for in vitro propagation were obtained from seedlings of *A. pelegrina* following an established protocol for seed germination and seedling establishment [12]. This protocol involved breaking the physical dormancy of the seeds by transversally cutting the apical zone to a depth of 0.1 to 0.5 mm and then establishing them on a medium composed of half-strength MS [37] mineral salts. The pH was adjusted to 5.7 ± 0.1 and the medium was solidified with 6 g L^{-1} agar (Algas Marinas S.A., Santiago, Chile).

2.1. In vitro Multiplication Stage

To investigate the influence of light and BAP concentration on shoot multiplication of *A. pelegrina*, shoots obtained from in vitro germination, characterized by the presence of their first true leaf were used (Figure 1). Three artificial light sources were evaluated: L1: cool white fluorescent lamps (Philips TL-D 36W/54) emitting light with a wavelength range of 400–700 nm and a photosynthetic photon flux density (PPFD) of 5.27 μmol m^{-2} s^{-1}. L2: red and blue LED light in a 3:1 ratio (PARALED PARALED_FRRB 55W system, Ciencia Pura SpA. Santiago, Chile). L3: red and blue LED light in a 1:1 ratio (PARALED PARALED_FRRB 55W system, Ciencia Pura SpA. Santiago, Chile). To avoid light contamination, each treatment was separated by a white screen. The effects of these light sources were assessed in combination with three different concentrations of 6-Benzylaminopurine (BAP): BAP1 (0 μM), BAP2 (2.22 μM), and BAP3 (4.44 μM). The PPFD and red:blue ratios for each light source were measured using a spectrophotometer (Lighting Passport Standard Pro model, Allied Scientific ProTM, Gatineau, QC, Canada). In addition, the PPFD photon irradiation provided by each light source was measured in the blue (400–499 nm) and red (600–699 nm) spectral regions. Detailed information on the PPFD measurements is provided in Table 1.

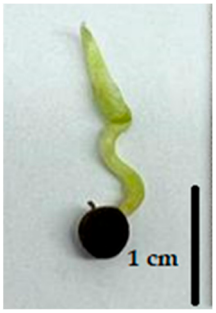

Figure 1. *A. pelegrina* seedling with first true leaf obtained by in vitro germination. Scale bar 1 cm.

Table 1. Photosynthetic photon flux density (PPFD) and red:blue ratios of light sources used for the in vitro multiplication of *A. pelegrina*.

Light Type	Red	Blue	Red:Blue Ratio
	PPFD, μmol m^{-2} s^{-1}		
LIGHT1	1	1	1:1
LIGHT2	11	4	≈3:1
LIGHT3	6	6	1:1

The plant material was cultured on MS medium supplemented with vitamins and sucrose (30 g L^{-1}) for 30 days. The pH of the medium was adjusted to 5.7 ± 0.1 and gelled with 6 g L^{-1} agar. Four plantlets were cultured per 250 mL flask and maintained in a growth chamber under controlled conditions of 25 ± 1 °C temperature and a 16:8 h photoperiod (light/dark cycle).

A completely randomized factorial design with a 3 × 3 arrangement was employed. Each treatment consisted of 20 shoots with three replicates. Shoot length and number of shoots were evaluated. Data were analyzed using two-way analysis of variance (AN◎ VA) and variance component analysis. Differences between treatments were determined using Tukey's test with a significance level of $p \leq 0.05$. Minitab 19 statistical software (Minitab Inc., State College, PA, USA) was used for the analyses.

2.2. Rhizome and Root Induction Stage

Uniform 3 cm long shoots obtained from the multiplication stage were used. The effect of MS basal medium supplemented with different concentrations of 1-Naphthaleneacetic acid (NAA) and Indole-3-Butyric Acid (IBA) was evaluated. Five treatments were employed: T1: control (no growth regulator); T2: 2.69 μM NAA; T3: 5.37 μM NAA; T4: 2.69 μM IBA; and T5: 5.37 μM IBA. Ten shoots per treatment with three replicates were cultured in a growth chamber under controlled conditions: 25 ± 1 °C temperature, cool white fluorescent lamps (Philips TL-D 36W/54) providing light with a wavelength range of 400–700 nm and a PPFD of 5.27 μmol m^{-2} s^{-1}, and a 16:8 h photoperiod (light/dark cycle).

Rhizome length, fresh weight (g) per plant, and rooting percentage were evaluated. Rooting percentage was calculated as the ratio of the number of rooted plants (with a root length of 0.5 cm) to the total number of shoots used for each treatment. Percentage values were transformed by natural logarithm for analysis.

The experiment was designed completely at random. ◎ne-way analysis of variance (AN◎ VA) and variance component analysis were performed. Differences between treatments were determined using Tukey's test with a significance level of $p \leq 0.05$. Minitab 19 statistical software (Minitab Inc., State College, PA, USA) was used for the analyses.

2.3. Ex Vitro Acclimatization

After rooting, all plants were transplanted into 200 cm^3 containers filled with a 1:1 (*v*/*v*) mixture of peat moss and perlite. Each plant was covered with a transparent 200 cm^3 container and transferred to a cold greenhouse for acclimatization. Temperatures inside the cold greenhouse fluctuated between 18 and 30 °C. The transparent containers were gradually removed in four discrete steps over a four-week period, allowing for a stepwise decrease in relative humidity. After four weeks, the containers were completely removed.

During this acclimatization stage, the survival rate (%) of *A. pelegrina* plants was evaluated. Survival rate was calculated as the percentage of living plants relative to the total number of acclimated plants.

3. Results

3.1. In Vitro Multiplication Stage

Analysis of variance (AN◎ VA) revealed a significant interaction ($p \leq 0.05$) between light source and BAP application on shoot length (SL) (Table 2). The interaction of L2BAP2 (red:blue light 3:1 with 2.22 μM BAP) and L1BAP2 (white light with 2.22 μM BAP) resulted in the greatest shoot length, reaching 6.2 cm and 5.4 cm, respectively ($p \leq 0.05$). Interactions L3BAP2 (red:blue light 1:1 with 2.22 μM BAP), L2BAP3 (red:blue light 3:1 with 4.44 μM BAP), L1BAP3 (white light with 4.44 μM BAP), L3BAP3 (red:blue light 1:1 with 4.44 μM BAP), L2BAP1 (red:blue light 3:1 with 0 μM BAP), and L3BAP1 (red:blue light 1:1 with 0 μM BAP) promoted an average shoot length of 4.5 cm, 4.1 cm, 3.7 cm, 3.5 cm, 3.3 cm, and 2.7 cm, respectively. Notably, L1BAP1 (white light with 0 μM BAP) combination produced the shortest shoots, averaging 2.4 cm. These findings indicate that shoot height in *A. pelegrina* is positively influenced by the synergistic effects of different light spectra and BAP concentrations.

Table 2. Effect of light spectra and BAP concentration on *A. pelegrina* shoot length and shoot number.

Led Lights	BAP Concentration	SL (cm)	SN
L1 (white light)	BAP1 (0 μM)	2.4 e	2.9 bc
L1 (white light)	BAP2 (2.22 μM)	5.4 a	4.8 a
L1 (white light)	BAP3 (4.44 μM)	3.7 bc	3.5 abc
L2 (3:1 red:blue)	BAP1 (0 μM)	3.3 cd	3.1 abc
L2 (3:1 red:blue)	BAP2 (2.22 μM)	6.2 a *	4.6 ab
L2 (3:1 red:blue)	BAP3 (4.44 μM)	4.1 bc	4.1 abc
L3 (1:1 red:blue)	BAP1 (0 μM)	2.7 de	2.6 c
L3 (1:1 red:blue)	BAP2 (2.22 μM)	4.5 b	4.3 abc
L3 (1:1 red:blue)	BAP3 (4.44 μM)	3.5 cd	3.6 abc
F lights × BAP		$p = 0.017$	$p = 0.86$

* Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$). SL: shoot length. SN: Shoot number.

In contrast to shoot length, shoot number (SN) was not significantly influenced by the different light spectra ($p \geq 0.05$) (Table 2) and was solely determined by BAP concentration ($p \leq 0.05$) (Table 3). The highest SN value (4.6 shoots; $p \leq 0.05$) was achieved when 2.22 μM BAP was added to the culture medium, while BAP concentrations of 4.44 μM resulted in average SN values of 3.7. ◎n the other hand, the lowest shoot number was obtained without BAP (Table 3 and Figure 2).

Table 3. Effect of 6-Benzylaminopurine (BAP) concentration on *A. pelegrina* shoot number.

Treatment	BAP Concentration (μM)	SN
BAP1	0	2.9 c
BAP2	2.22	4.6 a *
BAP3	4.44	3.7 b
	F_{BAP}	$p = 0.00$
	F_{LED}	$p = 0.32$
	$F_{BAP \times LED}$	$p = 0.86$

* Different letters indicate significant differences according to Tukey's test ($p \leq 0.05$). SN: Shoot number.

Figure 2. Effect of 6-Benzylaminopurine (BAP) concentration on *A. pelegrina*. BAP1: 0 µM; BAP2: 2.22 µM; BAP3: 4.44 µM. Scale bar 1 cm.

3.2. Rhizome and Root Formation Stage

After four weeks of culture on rhizome and root formation medium (Table 4), *A. pelegrina* seedlings significantly differed in rooting percentage, plant fresh weight, and rhizome length based on the auxin treatment applied. Treatments T3 (5.37 µM NAA) (Figure 3) and T5 (5.37 µM IBA) resulted in the highest rooting percentages, reaching 100% and 96%, respectively ($p \leq 0.05$). Furthermore, these concentrations promoted significant increases in plant fresh weight (2.9 and 3.1 g) and average rhizome length (1.9 and 2.1 cm) compared to the control and other treatments ($p \leq 0.05$). Treatments T2 (2.69 µM NAA) and T4 (2.69 µM IBA) allowed us to achieve moderate rooting percentages (53% and 51%), plant fresh weights (1.9 and 1.7 g), and rhizome lengths (1.4 and 1.6 cm), respectively ($p \leq 0.05$). As expected, the control treatment (T1) lacking auxins caused the lowest rooting percentage (11%), with a significantly lower average fresh weight (1.3 g) and rhizome length (0.8 cm) compared to all auxin-supplemented treatments ($p \leq 0.05$).

Table 4. Effect of different NAA and IBA concentrations on rooting, fresh weight, and rhizome length of *A. pelegrina.*

Treatments	Auxin Type	Auxin Concentration (µM)	Rooting (%)	Plant Fresh Weight (g)	Rhizome Length (cm)
T1	Control	0	11 ± 1.0 c	1.3 ± 0.2 c	0.8 ± 0.2 c
T2	NAA	2.69	53 ± 1.5 b	1.9 ± 0.1 b	1.4 ± 0.1 b
T3	NAA	5.37	100 ± 2.0 a *	2.9 ± 0.1 a *	1.9 ± 0.1 a *
T4	IBA	2.69	51 ± 1.3 b	1.7 ± 0.3 b	1.6 ± 0.2 b
T5	IBA	5.37	96 ± 1.7 a	3.1 ± 0.2 a	2.1 ± 0.3 a

* Letters indicate significant differences according to Tukey's test ($p \leq 0.05$). Mean ± standard deviation.

Figure 3. Rooting of an *A. pelegrina* shoot. Treatment 3 (ANA 5.37 µM). Scale bar 1 cm.

3.3. Ex Vitro Acclimatization

Ex vitro acclimatization of rooted *A. pelegrina* shoots (Figure 4a) resulted in a high survival rate of 90% after four weeks of transfer to a 1:1 (*v*/*v*) peat moss/perlite substrate. Following six weeks of acclimatization (Figure 4b), continued growth and development of *A. pelegrina* plants were observed, including the emergence of new shoots and the expansion of the root system, with the formation of storage roots (Figure 4c).

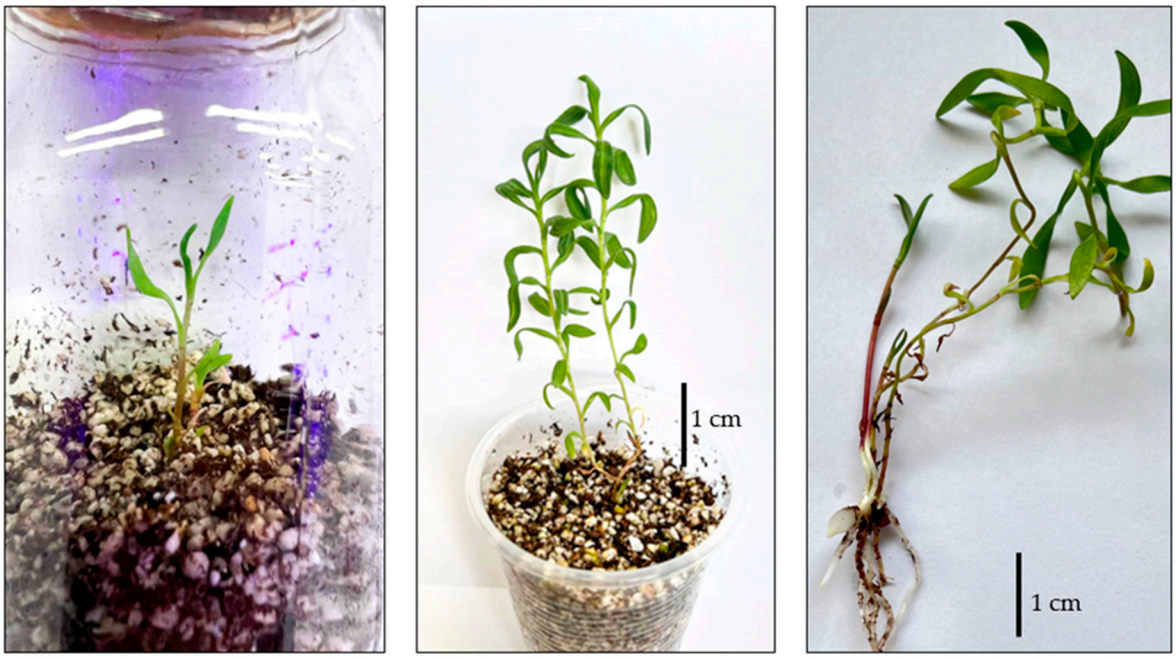

Figure 4. *A. pelegrina* plant during ex vitro acclimatization. (**a**) Plant after 2 weeks of acclimatization. (**b**) Acclimatized plant after 6 weeks. (**c**) Acclimatized plant after 6 weeks with storage roots. Scale bar 1 cm.

4. Discussion

This study presents an efficient micropropagation protocol for *A. pelegrina* regeneration using MS medium with its corresponding vitamins, supplemented with 30 g L^{-1}

sucrose, 6 g L^{-1} agar, and 2.22 μM BAP for shootmultiplication. Subsequently, rooting was achieved with the application of 5.37 μM NAA or 5.37 μM IBA during the rooting stage.

Previous studies reported micropropagation outcomes in various *Alstroemeria* species. In *Alstroemeria* var. Fiesta de 15 INTA, a regeneration rate of 76%, shoot length of 2.4 cm, and 3.4 leaves per explant were achieved using MS medium supplemented with 1.07 μM NAA [38]. In addition, the combination of 1.07 μM NAA and 4.44 μM BA resulted in 5.2 shoots [27]. For *Alstroemeria pallida*, an endemic Chilean species, the highest proliferation rate was observed in MS medium supplemented with 8.88 μM BAP and solidified with 3.5 g L^{-1} agar, producing 3.5 shoots [19]. These findings are comparable to the results of this study, where an average of 4.6 shoots was obtained using 2.22 μM BAP. However, in other *Alstroemeria* genotypes, the application of 2.22 μM BAP for 3 weeks allowed us to obtain a 3.0-fold multiplication of shoots and a 2.3-fold increase in rhizomes [19].

In the *Alstroemeria* genus, the application of the cytokinin BAP was reported to promote shoot and rhizome multiplication [4]. In the case of the hybrid *Alstroemeria ligtu*, the application of 2.22 μM BAP and 2.69 μM NAA resulted in the development of 2.25 rhizomes and 2.75 roots per shoot [24]. In addition, BAP was demonstrated to reduce apical dominance in shoots [39], which could explain the observed increase in shoot number in *A. pelegrina*.

Light intensity and quality are crucial factors in micropropagation, influencing plant growth and development. In this study, the spectral energy distribution of blue light corresponded to an emission peak of 420–440 nm, while red light was 660 nm. Notably, these wavelengths coincide with the absorption spectra of chlorophyll A (430 and 663 nm) and chlorophyll B (453 and 642 nm), optimizing photosynthetic efficiency [40]. The combination of 3:1 red:blue LED lights and 2.22 μM BAP resulted in the highest shoot height (6.2 cm) among the tested treatments.

In contrast, studies in *Cymbidium* revealed that red light promoted leaf growth but decreased chlorophyll content [41]. However, *Curcuma longa* micropropagated under 2:1 red:blue LED lights (25 μmol m^{-2} s^{-1} red and 11 μmol m^{-2} s^{-1} blue) characterized with a proliferation rate of 5.12 plants per explant with an increased number of shoots [42]. Conversely, in *Vaccinium corymbosum*, blue LED lights promoted chlorophyll accumulation, while red LED lights reversed this effect [43].

The combination of blue and red light is essential for chlorophyll synthesis, enhancing plant growth and development in vitro [44]. *Lycium barbarum* micropropagated under RB LED lights exhibited a 68% increase in shoot number, a 41% increase in leaf number, and a 64% increase in shoot length compared to white fluorescent lights [45]. Similar findings were observed in *Carica papaya* and *Bacopa monnieri*, where the red:blue ratio strongly stimulated shoot number [46,47].

Red and blue light spectra serve as the primary energy sources for plant photosynthesis, enabling the assimilation of C_2. LEDs offer precise control over wavelengths, allowing them to match the absorption spectra of plant photoreceptors. Consequently, LEDs can influence plant morphology and metabolism, making them a valuable tool in in vitro culture technology [48–50]. By manipulating light spectra, precise control over plant growth, development, and nutritional quality can be achieved. The introduction of LEDs into in vitro culture represents a significant innovation in the field of plant propagation.

This study also evaluated the average fresh weight of micropropagated plants. After four weeks of culture on rooting medium supplemented with 5.37 μM NAA and 5.37 μM IBA, fresh weights of 2.9 and 3.1 g and rhizome lengths of 1.9 and 1.7 cm were achieved, respectively. These results differ from those obtained in *A. pallida*, where BAP concentrations of 4.44 and 8.88 μM were used, resulting in a fresh weight of 4.6 g after eight weeks [30].

Furthermore, the use of MS medium supplemented with 5.37 μM NAA and 5.37 μM IBA promoted rooting in *A. pelegrina*, giving 100% and 96% of rooted plants, respectively.

5. Conclusions

This study reports the development of an efficient in vitro micropropagation protocol for the vulnerable species *A. pelegrina*. Shoots derived from in vitro seed germination were used as explants. MS medium supplemented with 2.22 μM BAP facilitated a multiplication rate of 4.6 plantlets per initial shoot. LED illumination further enhanced plant production potential. Following 4–5 months in culture, rooting success rates of 96–100% were achieved, accompanied by increased fresh weight and improved rhizogenic capacity. The protocol incorporates a critical acclimatization phase conducted in a cool greenhouse using a substrate. This final step resulted in a 90% survival rate after 6 weeks.

The successful establishment of this in vitro micropropagation protocol for *A. pelegrina* opens up exciting possibilities for the mass propagation of this vulnerable species. In vitro culture serves as a high-impact strategy for addressing conservation challenges associated with vulnerable or endangered plant species. The protocol's efficiency, with a multiplication rate of 4.6 plantlets per initial shoot and high rooting success rates, makes it an attractive tool for large-scale production.

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Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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