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The Development of Scalable Micropropagation Protocols for *Alocasia zebrina* G.W. Johnson & R.Hogg

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ABSTRACT

Alocasia zebrina is a tropical ornamental species native to Southeast Asia, valued for its striking foliage and zebra-striped stems. This study aimed to develop an efficient *in vitro* propagation protocol to produce healthy, disease-free seedlings for commercial use. The protocol included explant sterilization, shoot proliferation, rooting, and acclimatization. The most effective sterilization method involved pre-treatment with a bactericide and a fungicide followed by 0.05% $HgCl_2$, resulting in 93% contamination-free explants. Shoot multiplication was optimized using MS medium supplemented with 0.05 mg/L TDZ and 0.75-1 mg/L BAP, yielding an average of over 18.9 shoots per explant. Rooting was most successful on MS medium containing 0.5 g/L activated charcoal and 1 mg/L NAA, producing robust plantlets with an average height of 6.2 cm, 6.2 roots, 4.2 leaves, and a stem diameter of 0.8 cm. During acclimatization, plantlets grown in a 2:1:1:1 mixture of husk charcoal, soil, organic manure, and volcanic sand achieved a 98% survival rate.

1. Introduction

Alocasia zebrina G.W. Johnson & R.Hogg, commonly known as the zebra plant, is a visually striking tropical ornamental species native to the Philippines. It belongs to the genus *Alocasia* Schott within the Araceae family (Hay 1999; Medecilo and Madulid 2013). The plant is highly valued for its unique zebra-striped petioles and bold, arrow-shaped green leaves, making it a popular choice in the ornamental plant market. In addition, *A. zebrina* holds potential as a medicinal plant besides being used as an ornamental plant (Siahaan *et al*. 2024). The Manobo tribe of Agusan del Sur, Philippines, uses its leaves and stems to treat allergies, cuts and wounds, snake and insect bites, and poisoning (Dapar *et al*. 2020). Currently, it is widely traded via social media and e-commerce platforms, resulting in significant market demand. Hence, further conservation efforts are essential to preserve it as a valuable source of germplasm.

Traditionally, propagation of *A. zebrina* relies on corms or seeds (Jo *et al*. 2008, 2009; Bhatt *et al*. 2013; Abdelbaset *et al*. 2020; Raju *et al*. 2022). However, these conventional methods are not only slow and inconsistent but also carry the risk of transmitting systemic plant diseases, particularly bacterial soft rot, which is ubiquitous in field-propagated Araceae. Propagation via corm division and suckers is further constrained by a low multiplication rate per parent plant, bulky logistics associated with transport, and strong seasonal dependency, as rainfall patterns dictate growth cycles and pathogen pressure, with corm rot being common in soil establishment. Seed-based propagation, while offering genetic diversity, suffers from rapid viability

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loss, long juvenile phases, and the inability to produce true-to-type clonal material, making it unsuitable for commercial-scale production. As a result, conventional methods are inadequate for meeting the growing demand for consistent, large-scale propagation, particularly in Indonesia's expanding ornamental plant industry.

Given the limitations of conventional propagation, micropropagation provides a reliable alternative that ensures uniformity, rapid multiplication, and disease-free planting material. Unlike corm- or seed-based methods, tissue culture offers controlled conditions that reduce pathogen transmission, overcome seasonal constraints, and enable large-scale production of genetically stable, true-to-type plants (Bhatt *et al*. 2013; Guerra *et al*. 2018; Abdelbaset *et al*. 2020). For *Alocasia* species, micropropagation not only accelerates growth but also preserves ornamental traits such as leaf morphology and coloration, ensuring consistency in commercial production (Rachmawati *et al*. 2025; Jianfen *et al*. 2015). Consequently, it has become a cornerstone technique for meeting the rising demand in Indonesia's ornamental plant industry, where efficiency and reliability are essential for scaling production. Micropropagation is generally divided into five stages (Iliev *et al*. 2010; Kumar & Reddy 2011), each critical for efficiency and reliability. Stage 0 involves preparing healthy, pathogen-free donor plants, as explant quality determines culture success (Monthony *et al*. 2021). Stage I establishes sterile explants, where effective sterilization prevents contamination (Ebrahimi *et al.* 2024). Stage II, multiplication, defines scalability, with optimized cytokinin combinations yielding dozens of shoots per explant (Hussain *et al*. 2018). Stage III ensures shoot elongation and rooting, producing robust plantlets enhanced by rooting hormones and activated charcoal (Thomas 2008). Stage IV, acclimatization, transitions plantlets to ex vitro conditions, where suitable potting media secure high survival and minimize transplant shock (Jo *et al*. 2009; Yunida *et al*. 2023). Collectively, these stages provide a systematic framework for disease-free, efficient, and commercially viable propagation of *Alocasia*. Micropropagation is recognized as a reliable and efficient technique for producing high-quality, pest- and disease-free planting materials of *Alocasia* species. Numerous *in vitro* propagation methods have been successfully developed (Jianfen *et al*. 2015; Abdulhafiz *et al*. 2020), with promising outcomes reported using cytokinins such as 6-benzylaminopurine (BAP) and thidiazuron (TDZ) (Hussain *et al*. 2018; Rachmawati *et al*. 2023). The combined use of TDZ and BAP has shown greater effectiveness in promoting shoot proliferation compared to individual applications, though careful management is required to avoid undesirable morphological effects.The present study aims to establish an optimized micropropagation protocol for *Alocasia zebrina*. This includes optimizing sterilization methods, evaluating the synergistic effects of BAP and TDZ on shoot multiplication, refining rooting conditions using activated charcoal and NAA, and identifying the most effective acclimatization media to enhance plantlet survival and growth.

2. Materials and Methods

The study was carried out from August 2024 to October 2025. The donor plant for explants was collected from Regar Orchids Nursery in Ciwidey, Bandung, Indonesia. Mature *A. zebrina* specimens, aged 2-3 years, were selected and maintained in a shaded greenhouse with 60% filtered sunlight. Prior to explant collection, plants received routine care, including weekly applications of 16:16:16 NPK fertilizer at 1 g/L, along with fungicide difenoconazole and insecticide deltamethrin applications according to the recommended dosages.

2.1. Sterilization Protocol Optimization

Three sterilization protocols were tested to establish aseptic shoot cultures (Table 1) using a completely randomized design (CRD) with nine replications. Each treatment included five bottles, each containing four explants. After sterilization, shoot tips were cultured on full-strength MS medium without plant growth regulators, supplemented with 2.5% (w/v) sucrose. The medium was adjusted to pH 5.8 ± 0.1, solidified with 11 g/L agar (Dolphin), boiled, and dispensed into 25 mL portions in jam bottles (6 cm diameter), then sealed with transparent plastic. Media were autoclaved at 121°C and 15 psi for 20 minutes. Contamination and explant growth were assessed after 8 weeks.

2.2. Shoot Initiation and Multiplications

Aseptic shoot tips (explants) successfully derived from the optimized sterilization stage (T2 method) were selected as the source material for subsequent stages. These explants were cultured on MS initiation medium supplemented with varying concentrations of TDZ (0.05 and 0.1 mg/L) and BAP (0.5 and 1 mg/L) (Duchefa Biochemie, Haarlem, The Netherlands). Cultures were incubated for 8 weeks, after which regenerated shoots were divided into four sections and subcultured on the same medium for another 8 weeks. The experiment

Table 1. Sterilization methods evaluated in the study

Treatment	Description
T-0	Washed corms (0.5-1 cm) were sterilized by soaking in 70% alcohol for 5 minutes, then in 2.5% sodium hypochlorite with Tween 20 for another 5 minutes, followed by five 2-minute rinses with sterile water under manual agitation. Under a stereomicroscope, shoot tips were trimmed to 1–1.5 mm and transferred to initiation medium.
T-1	Washed corms (0.5-1 cm) were surface sterilized in 70% alcohol for 5 minutes, followed by 2.5% sodium hypochlorite with Tween 20 for 5 minutes, then rinsed five times (2 minutes each) with sterile water under manual shaking. Under a stereomicroscope, shoot tips were trimmed to 1–1.5 mm, soaked in 0.1% $HgCl_2$ for 4 minutes, and rinsed five times again. The prepared shoot tips were then cultured on initiation medium.
T-2	Washed corms (0.5-1 cm) were treated with 1 g/L streptomycin sulfate and 1 g/L benomyl fungicide on a shaker at 100 rpm for 1 hour. They were then surface sterilized in 70% alcohol for 5 minutes, followed by 2.5% sodium hypochlorite with Tween 20 for 5 minutes, and rinsed five times (2 minutes each) with sterile water. Under a stereomicroscope, shoot tips were trimmed to 1–1.5 mm, soaked in 0.1% $HgCl_2$ for 4 minutes, rinsed five times again, and finally cultured on MS initiation medium.

followed a factorial Completely Randomized Design (CRD) with four replications, each consisting of five bottles containing four shoot clusters. Data collected included shoot height (cm) and the number of axillary shoots per shoot.

2.3. Root Induction

Axillary shoots were carefully separated and transferred to MS rooting media containing different combinations of activated charcoal (0.0 and 0.5 g/L) and NAA (0.5 and 1.0 mg/L). The experiment followed a Completely Randomized Design (CRD) with three replications, each consisting of five bottles containing three shoots. After 8 weeks, plantlet height, diameter, number of leaves, and number of roots were recorded.

2.4. Culture Incubation

All cultures were incubated in an incubation room under a 16-hour photoperiod with cool fluorescent lighting at an intensity of 13 µmol m^{-2} s^{-1}, maintained at 24±1°C and 70% relative humidity.

2.5. Plantlet Acclimatization

Vigorous, well-rooted shoots generated from the root induction phase were used for acclimatization. Plantlets (≈5 cm tall with 3-4 leaves) were gently removed using blunt tweezers, rinsed under tap water to remove agar, and soaked in 1 g/L benomyl (70%) fungicide for 1 hour. Plantlets were transplanted into seedling trays (50 cells) filled with one of three acclimatization media: (1) husk charcoal, (2) husk charcoal and cocopeat (2:1, v/v), or (3) a mix of husk charcoal, soil, organic manure, and volcanic sand (2:1:1:1, v/v/v/v). The experiment followed a CRD with nine replications, each treatment containing 50 plantlets. Trays were covered with transparent plastic and kept in a shaded glasshouse for 8 weeks. Afterward, plantlet survival and growth were assessed, and surviving plants were re-potted in the same media. Observed variables included survival rate (%), plant height (cm), number of leaves, and plant diameter (cm).

2.6. Data analysis

All experimental data were subjected to statistical analysis using analysis of variance (ANOVA) with SPSS version 27. Duncan's Multiple Range Test (DMRT) at a 95% confidence level ($p = 0.05$) was employed for further comparisons.

3. Results

3.1. Explant Sterilization Protocol

The study achieved aseptic rates ranging from 55% to 93%, with explant growth potential between 82% and 88% (Figure 1). The T-1 sterilization method, which combined sodium hypochlorite with $HgCl_2$, was more effective than the T-0 method using sodium hypochlorite alone, resulting in 75% contamination-free explants and 85% growth potential. The most effective treatment, T-2, involved pre-treatment with a bactericide and a fungicide prior to processing in the laminar airflow cabinet. This approach significantly reduced microbial contamination, yielding the highest aseptic rate of 93%.

3.2. Shoot Initiation and Proliferation

During the early multiplication stage, axillary shoots began to emerge clearly within 4-6 weeks after culture initiation. The shoots' height and number steadily increased. With elevated concentrations of BAP in the first and second cycles of subculture, both variables tended to increase. In combination with TDZ, both cytokinin hormones showed interactive effects, where reduced concentration of TDZ did not necessarily enhance shoot formation and growth. *In vitro* propagation of *A. zebrina* from explant to plant is presented in Figure 2.

Shoot explants cultured on MS medium with 0.05 mg/L TDZ and 1.0 mg/L BAP showed the most effective

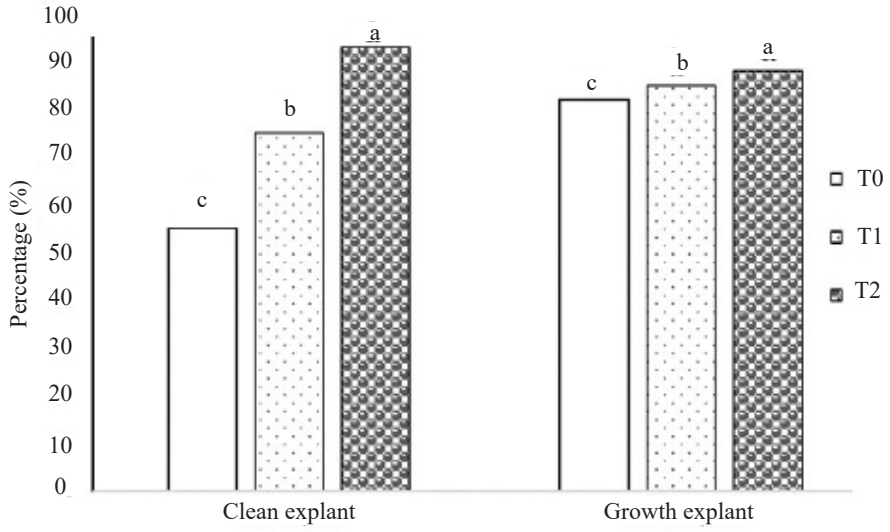


Figure 1. The effect of three different sterilization methods on the percentage of clean explants and explant growth potential in the initial stage of micropropagation of *Alocasia zebrina*. Bars represent mean values, and different letters indicate statistically significant differences based on DMRT at p = 0.05

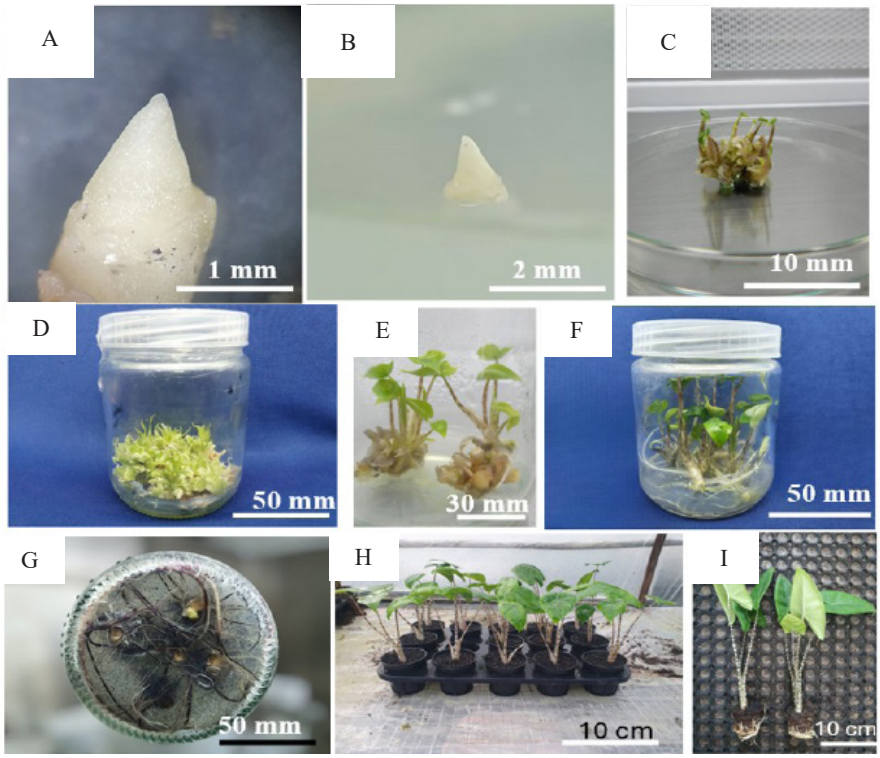


Figure 2. Developmental stages of *Alocasia zebrina* during micropropagation; (A) Micro shoot-tip explant prior to transfer onto MS medium, (B) sterile shoot-tip explant on MS initiation medium, 5 weeks after culturing on MS initiation medium, (C) early shoot proliferation during the first subculture on MS medium with 0.75 mg/L BAP and 0.05 mg/L TDZ, (D) adventitious shoot formation at the end of the second subculture on MS medium with 1 mg/L BAP and 0.05 mg/L TDZ, (E) initial shoot regeneration during the rooting and hardening phase on MS medium with 0.5 g/L activated charcoal (AC) and 1 mg/L NAA, (F) shoot elongation on MS medium supplemented with 0.5 g/L AC and 1 mg/L NAA, (G) root development in multiple plantlets cultured in vessels on MS medium with 0.5 g/L AC and 1 mg/L NAA, (H) newly emerged leaves on acclimatized plantlets grown in a 2:1:1:1 mix of husk charcoal, soil, organic manure, and volcanic sand, 8 weeks after transplant, (I)—growth performance of *Alocasia zebrina* plants after being removed from the potting medium, 6 months post-acclimatization

proliferation, producing an average of 2.8 axillary shoots in the first subculture and 25.7 in the second (Table 2). Among the treatments, the combination of 0.1 mg/L TDZ and 1.0 mg/L BAP also resulted in the second-best number of axillary shoots. In contrast, the lowest response was observed with 0.05 mg/L TDZ and 0.75 mg/L BAP.

3.3. Root Induction

The regenerated axillary shoots after the shoot multiplication stage were divided and subsequently subcultured onto rooting media. Root development was typically observed 4-6 weeks after the initiation of the culture. The application of different doses of AC in conjunction with NAA exhibited a significant interactive effect on root development and growth. The combination treatment of 0.5 g/l AC and 1 mg/l NAA had a substantial effect on root formation and growth in *A. zebrina*. The combination resulted in viable plantlets with shoots reaching 6.2 cm in height, 0.8 cm in diameter, an average of 6.2 roots per shoot, and 4.2 leaves per plantlet. The combination of 0.5 g/l AC and 0.5 mg/l NAA was shown to be the second most effective, while MS medium free of both AC and NAA produced the least favorable results (Table 3).

3.4. Plantlet Acclimatization

The selection of the acclimatization medium significantly influenced the survival and growth of *A. zebrina* plantlets. The most effective medium was a combination of husk charcoal, soil, organic manure, and volcanic sand in a 2:1:1:1 ratio (v/v/v/v), which resulted in a 98% survival rate. Plantlets grown in this medium also exhibited superior growth, with an average height of 6.4 cm, a stem diameter of 1.16 cm, and an average of 4.9 leaves per plant (Table 4). The second-best performance was observed in the medium combining husk charcoal and cocopeat, although it was not significantly better than husk charcoal alone in all variables observed.

4. Discussion

This study presents the successful establishment of a micropropagation protocol for *A. zebrina*, based on standardized plant tissue culture methodologies. The study initially aimed to maximize contamination-free explants by improving surface sterilization. Relying solely on sodium hypochlorite has shown low success rates (below 60%) in *Alocasia* species (Bhatt *et al*. 2013; Rachmawati *et al*. 2023). However, combining sodium hypochlorite with $HgCl_2$, as seen in *Echinodorus grisebachii* and *Phalaenopsis hybrid*, significantly increased sterilization success (Baby *et al*. 2024; Madhuri *et al*. 2021). Similarly, the T2 method in this study, using bactericide, fungicide, $HgCl_2$, and sodium hypochlorite, resulted in over 90% clean explants and 80% viable growth.

Shoot multiplication is a critical phase in micropropagation, typically requiring cytokinin hormones such as BAP or TDZ. BAP is widely used for its effectiveness, stability, and low cost, while TDZ is a

Table 2. Shoot proliferation on first and second subculture cycles of *Alocasia zebrina* on various combinations of TDZ and BAP

Plant growth regulator		Subculture cycle			
		First		Second	
BAP (mg/L)	TDZ (mg/L)	Shoot height	Shoot number	Shoot height	Shoot number
0.75	0.05	1.9^{c}	4.8^{c}	2.9^{c}	18.9^{c}
0.75	0.1	2.2^{bc}	5.4^{b}	3.5^{b}	16.8^{d}
1	0.05	2.8^{a}	6.2^{a}	4.0^{a}	25.7^{a}
1	0.1	2.5^{a}b	5.5^{b}	3.6^{b}	20.8^{b}

Means within a column followed by different letters are significantly different according to DMRT at p≤0.05

Table 3. The effect of various combinations of Activated Charcoal (AC) and Naphthalene Acetic Acid (NAA) on in vitro rooting of *Alocasia zebrina* 8 weeks after planting

AC (g/L)	NAA (mg/L)	Plant height (cm)	Diameter (cm)	Leaf number	Root number
0	0	5.1^{c}	0.58^{f}	3.4e	4.2^{c}
0.5	0	5.6^{b}	0.46^{d}	3.8^{cd}	5.1^{b}
0	0.5	4.6^{d}	0.54^{e}	4.0^{bc}	5.2^{b}
0.5	0.5	5.7^{b}	0.74^{b}	4.5^{a}	5.3^{b}
0	1	5.6^{b}	0.68^{c}	3.6^{de}	5.6^{b}
0.5	1	6.2^{a}	0.80^{a}	4.2^{b}	6.2^{a}

Means within a column followed by different letters are significantly different according to DMRT at p≤0.05

Table 4. Effects of three potting mixtures on the growth of *Alocasia zebrina* planlets eight weeks after planting

Potting mixture	Plant height (cm)	Diameter of bulb (cm)	No. of leaves	Plant survival %
Husk charcoal	5.4[b]	0.83[b]	3.6[b]	86[b]
Husk charcoal: cocopeat	5.3[b]	0,87[b]	4.2[ab]	88[b]
Husk charcoal: soil: organic manure: volcanic sand	6.4[a]	1.16[a]	4.9[a]	98[a]

Means within a column followed by different letters are significantly different according to DMRT at $p \leq 0.05$

potent cytokinin capable of inducing shoot regeneration at low concentrations (Ahmad and Faisal 2018; Desai *et al*. 2018; Guo *et al*. 2011). Previous studies have shown varied outcomes: in *Alocasia cuprea*, 1.0 mg/L TDZ with 0.5 mg/L BAP produced up to four shoots per explant (Rachmawati *et al*. 2025); similar effects were observed in Aglaonema and Musa (Nazihah *et al*. 2023; Zahara and Win 2020). In *Alocasia* cv. Verde Picante and *A. amazonica*, 3 mg/L BAP alone induced over six shoots per explant (Galvez Guerra *et al*. 2018; Abdel-Baset *et al*. 2020). In the present study, the combination of 1.0 mg/L BAP and 0.05 mg/L TDZ yielded over 20 shoots per explant in the second subculture—an eightfold increase from the first. These findings highlight the strong synergistic effect of low TDZ and high BAP concentrations in enhancing axillary shoot proliferation in *A. zebrina*.

Optimizing *in vitro* culture conditions significantly enhances plantlet survival during acclimatization. In this study, plantlets cultured on media supplemented with activated charcoal (AC) showed superior performance across all measured parameters compared to those grown on AC-free media. Activated charcoal (AC) enhances morphogenesis by adsorbing inhibitory compounds, reducing phenolic oxidation, and preventing browning (Thomas 2008). It also promotes growth by releasing beneficial substances, absorbing excess hormones like abscisic acid and ethylene, and darkening the medium to support root development (Amente and Chimdessa 2021). In this study, 0.5 g/L AC combined with 1 mg/L NAA significantly improved plantlet quality, yielding the highest root, leaf, bulb, and height values. Using the same treatment, optimum results were also obtained in *Alocasia azlanii*, where the rate of survival reached 93% at the acclimatization stage (Siregar *et al*. 2025). While NAA is widely used for *in vitro* root induction, IBA has shown better results in some species, such as *Alocasia amazonica* (Priyanka 2020; Raju *et al*. 2022; Yunida *et al*. 2023).

Potting media significantly influenced plantlet performance. The medium enriched with organic manure produced the highest values for plant height, bulb diameter, and leaf number. While the survival rate was slightly lower than the one reported in *Alocasia* using soil and sand mixtures (Jo *et al*. 2008), the incorporation of organic matter was proven effective in supporting robust growth and high survivability. These findings highlight the importance of media composition in optimizing acclimatization outcomes.

In Conclusion, An effective and scalable micropropagation protocol for *Alocasia zebrina* was successfully established, encompassing sterilization, shoot multiplication, rooting, and acclimatization. The optimized sterilization method (T2) achieved over 90% clean explants, ensuring reliable culture initiation. The combination of 1.0 mg/L BAP and 0.05 mg/L TDZ proved most efficient for shoot proliferation, producing an average of 25.7 shoots per explant in the second subculture—demonstrating a high multiplication rate. Subsequent rooting on MS medium supplemented with 0.5 g/L activated charcoal and 1 mg/L NAA yielded robust plantlets with superior morphological traits. During acclimatization, plantlets grown in organic manure-enriched media exhibited strong growth and a 98% survival rate. Overall, this protocol enables the production of more than 25 healthy plantlets from a single explant within one trial cycle, providing a reliable and efficient approach for large-scale propagation and conservation of *A. zebrina*.

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