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RESEARCH ARTICLE

Study on micropropagation and acclimatization of hippeastrum 'Double king' flowers in An Giang province of Vietnam

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Abstract

Hippeastrum 'Double King' is a beautiful flower with high economic value and has recently been introduced to Vietnam; therefore, the process of producing this flower has not been systematically established. The study was conducted to find out the suitable media for the micropropagation process of the hippeastrum 'Double King' flowers and the multiplication of tissue-cultured plants in greenhouse conditions. In this study, four experiments were carried out, including shoot regeneration from bulb scales, *in-vitro* shoot multiplication, complete plantlet generation and evaluation of the adaptability and development of tissue-cultured seedlings in greenhouses. The result showed that MS medium supplemented with 2 mg/L BAP was the best medium for shoot regeneration and the two-scale explants produced the most shoots in this medium. For the shoot multiplication experiment, the quarter-bulb explants cultured in the MS medium supplemented with 0.5 mg/L NAA and 2 mg/L BAP produced the greatest number of shoots, the best plant height and the highest number of leaves per bulb explant. The *in-vitro* complete plantlet generation experiment showed that MS medium supplemented with 2 mg/L NAA, 0.5 g/L activated carbon and 40 g/L sucrose was the most suitable for rooting seedlings. The substrate of 75 % sand + 25 % rice husk ash was suitable for the growth and development of seedlings in the nursery. The results of this study initially help to optimize the culture and production of 'Double King' hippeastrum flowers in An Giang province, Vietnam.

Keywords: hippeastrum 'Double King'; *in-vitro*; medium; micropropagation; tissue culture

Introduction

The Hippeastrum Herb. plant, commonly known in Vietnam as the red trumpet lily, hippeastrum, Mac Chu Lan or Tu Dien, belongs to the *Hippeastrum* genus within the Amaryllidaceae family (1). The *Hippeastrum* genus comprises over 60 species (2).

The hippeastrum, widely recognized for its beauty, is cultivated both in pots and gardens and prized as a cut flower (3). It is commercially grown on a large scale in countries such as the Netherlands, South Africa, Japan, Brazil and the United States (4). Today, hippeastrum flowers are available in a remarkable array of colors, shapes, sizes and varieties (5). However, the high cost of bulbs, especially for 'Double King' varieties, poses a challenge, as most bulbs are imported from the Netherlands, Taiwan and Australia. In An Giang, the hippeastrum 'Double King' is highly sought after for its vibrant red color, large, elegant blooms with multiple petal layers and delightful fragrance. Yet, the price remains steep due to limited bulb availability. Thus, research into the mass propagation of these bulbs is crucial to reduce costs and meet the growing demand among flower enthusiasts.

Compared to conventional propagation methods such as bulb division, tissue culture propagation of hippeastrum bulbs exerts significant advantages, including a higher multiplication rate and the production of robust, healthy bulbs with superior growth potential, thanks to aseptic planting conditions. This technique has been extensively employed commercially to meet the rising demand for this popular flower (6-8). In various studies on hippeastrum, bulbs have consistently been used as the initial explant material for shoot regeneration (9-11).

In Vietnam, tissue culture propagation of hippeastrum has only recently been studied, driven by the rising popularity of imported varieties known for their vibrant and diverse colors. Typically, this method involves culturing bulb scales. For example, researchers used double or multiple bulb scales as explants, with bulbs cut into 8 or 16 sections, achieving a multiplication rate of 2.79-3.75 shoots per explant (12).

An experiment was conducted using seven different methods of bulb sectioning. After four months, it was found that cutting the bulb into 48 parts yielded the highest number of shoots, with 34 shoots per explant (13). In other study of propagating hippeastrum bulbs by tissue culture method, where small in vitro-grown bulbs, derived from double bulb scales of *Hippeastrum hybridum* "Apple Blossom," were cut into two or four parts and cultured for 10-12 weeks, resulting in a multiplication rate of up to 100 shoots per initial explant (14). Micropropagation of *Crinum macowanii* flowers, a species belonging to the Amaryllidaceae family, was successfully recorded in producing 700-1000 plants from a single bulb over 12 months (15).

Although many hippeastrum varieties have been successfully propagated *in vitro*, an optimal protocol for the 'Double King' variety has yet to be established. Therefore, this research was conducted to develop and optimize an *in vitro* propagation and bulb acclimatization process for the hippeastrum 'Double King' in An Giang province. This will enable the production of a large quantity of high-quality plantlets to meet the needs of flower growers.

Materials and Methods

Place and time

This study was conducted at the Plant Tissue Laboratory, the Laboratory Complex at An Giang University and the My Quy Farm, Long Xuyen city, An Giang province, Vietnam. The experiments were carried out from October 2020 to August 2022.

Materials

Plant materials

Hippeastrum 'Double King' bulbs were imported from the Dutch-bulbs company at Carolus Clusiuslaan 3d, 2215 RV, Voorhout, Netherlands and grown in a greenhouse until their bulbs reached 40-50 mm in diameter.

Chemicals

The study used Murashige and Skoog (MS) medium as the nutrient medium in tissue culture and plant growth regulators (hormones) such as α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and thidiazuron (TDZ). These chemicals were purchased from Duchefa Biochemie B.V., Netherlands.

Explant preparation

After harvesting, the stems, leaves and roots were removed. The mature bulbs were thereafter rinsed with tap water and sterilized with 0.1 % mercuric chloride ($HgCl_2$) for ten min. Following sterilization, the bulbs were divided into smaller sections and cultured in MS nutrient medium and adjusted to a pH of 5.6 (16).

Experiment 1 - Shoot regeneration from bulb scales

This experiment applied four kinds of explants, single (1), twin or double (2), four (4) and eight (8) scales, to evaluate *in-vitro* shoot regeneration ability (Fig. 1). These explants were cultured on two different media for 28 days (4 weeks) (7, 12, 13). The MSB medium was prepared from MS medium supplemented with 2 mg/L BAP, while the MSBN was from MS medium supplemented with 2 mg/L BAP and 0.5 mg/L NAA. Observation criteria in this experiment included the number of regenerated shoots per explant, shoot height and number of leaves per explant.

Experiment 2 - *In-vitro* shoot multiplication

The 1 cm diameter bulblets obtained from experiment 1 were cut into halves and quarters longitudinally. The bulb explants, including whole bulbs, half bulbs and quarter bulbs, were incubated for ten weeks in MS medium with 0.5 mg/L NAA, (0 or 2 mg/L) BAP and (0 or 2 mg/L) TDZ (Fig. 2). Treatments of this experiment were described in Table 1. Observation criteria in

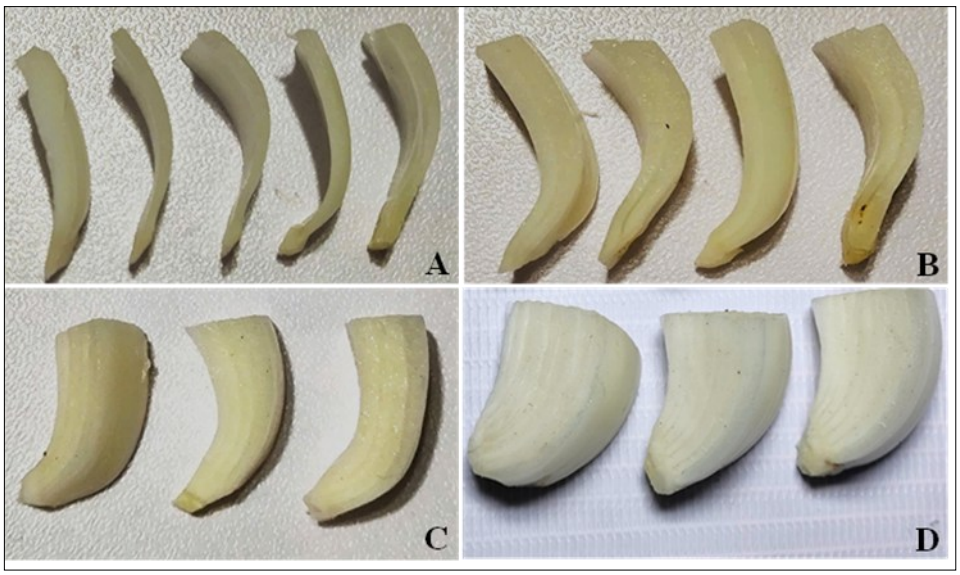

Fig. 1. Single (A), twin or double (B), four (C) and eight scales (D) used in the regeneration experiment.

Fig. 2. Whole bulb (A), half bulb (B) and quarter bulb (C) used in *in-vitro* shoot multiplication experiment.

this experiment included the number of regenerated shoots per bulb explant, plant height and number of leaves per bulb explant.

Experiment 3 - Complete plantlet generation

The strong bulbs from shoot multiplication were collected for the rooting experiment. *In-vitro* bulblets were placed in different rooting media, MS medium supplementing NAA (0, 1, or 2 mg/L), sucrose (30 or 40 g/L) and active charcoal (0 or 0.5 mg/L) for ten weeks. Treatments of this experiment were described in Table 2. Observation criteria in this experiment included the number of roots per plant, root length and plant height.

Experiment 4 - Evaluation of the adaptability and development of tissue-cultured seedlings in greenhouses

After complete plantlet generation, the 'Double King' plantlets were transferred to greenhouse conditions. They were grown in the C5 plastic pot (10.5 × 11.0 × 7.5 cm) containing sand, soil, rice husk and rice husk ash. The follow-up period was 12 weeks at the greenhouse. Treatments of this experiment were described in Table 3. The experiment was designed with three replications, each replication had two pots and one bulb was grown in each pot. Observation criteria in this experiment included survival rate, plant height, the number of leaves per plant and bulb diameter.

Data analysis

Data processing was performed using Microsoft Excel. Variance analysis and comparisons of treatment means (where applicable) were then conducted using SAS 9.1 software.

Table 1. Treatments in the experiment of *in-vitro* shoot multiplication

Treatment	**MS medium + 0.5 mg/L NAA**	
Types of explant	**+ 2 mg/L BAP**	**+ 2 mg/L TDZ**
Whole bulb	B1	B4
Half bulb	B2	B5
Quarter bulb	B3	B6

Note: BAP: 6-benzylaminopurine and TDZ: thidiazuron

Table 2. Treatments in the experiment of complete plantlet generation

Treatment	**MS medium**		
	+ NAA (mg/L)	**+ Sucrose** (g/L)	**+ Active charcoal**
C1 (control)	0	30	0
C2	1	30	0
C3	2	30	0
C4	1	30	0.5
C5	2	30	0.5
C6	1	40	0
C7	2	40	0
C8	1	40	0.5
C9	2	40	0.5

Notes: C1-C9: treatments of culture media.

Table 3. Treatments in the evaluation of the adaptability and development of tissue-cultured seedlings in greenhouses

Treatment	**Sand**	**Rice husk ash**	**Rice husk**	**Soil**
D1	100 %	0 %	0 %	0 %
D2	75 %	25 %	0 %	0 %
D3	50 %	50 %	0 %	0 %
D4	75 %	20 %	5 %	0 %
D5	50 %	20 %	5 %	25 %
D6	0 %	20 %	5 %	75 %

Notes: D1-6: treatments of substrate for growing hippeastrum 'Double King'.

Results and Discussion

Shoot regeneration from bulb scales

Different parts of explants were used for *in-vitro* bulb regeneration of amaryllis (hippeastrum), such as bulb scales, leaf base, floral stems and callus from immature flower buds (7). However, bulb scales, especially twin scales, were widely used as explants (17-19). In addition, the combination of BAP and NAA was common in the micropropagation of hippeastrum (9, 20-22). In this study, after four weeks, shoots from scale explants were regenerated with different quantities and morphologies (Fig. 3).

The highest number of regenerated shoots (4.3 ± 0.24) was observed on twin-scale explants cultured on MSB medium and the lowest ratio (2.5 ± 0.20) was recorded on single-scale explants on MSB medium. On the MSBN, single-scale and quadruple-scale explants had the highest regenerated shoot quantity (3.2 ± 0.17) while twin-scale explants obtained the lowest quantity (2.7 ± 0.4). The number of regenerated shoots of single-scale and quadruple-scale explants on MSBN was higher than those on MSB, however, the number of regenerated shoots of twin-scale explants on MSBN was lower than those on MSB (Fig. 4A). The height of these regenerated shoots in the MSBN medium was better than in the MSB medium (Fig. 4B). The average number of leaves per shoot in the two media was similar (Fig. 4C). As a result, through the experiment of shoot regeneration from 'Double King' bulb scales in the *in-vitro* environment, the type of twin-scale explant cultured in MSB medium (MS medium supplemented with 2 mg/L BAP) gave the most regenerated shoots.

In-vitro shoot multiplication

The *in vitro* formed bulblets were used as explants to improve and speed up the micropropagation of hippeastrum in the previous study (17). After ten weeks, shoots from bulb explants were regenerated with different quantities and morphologies in the media (MS + 0.5 mg/L NAA) plus 2 mg/L BAP or 2 mg/L TDZ (Fig. 5). The number of regenerated shoots per bulb explant in B1, B2 and B3 (MS medium + 0.5 mg/L NAA + 2 mg/L BAP) media was similar to or lower than that in B4, B5 and B6 (MS medium + 0.5 mg/L NAA + 2 mg/L TDZ) media (Fig. 6A). However, this difference was not significant. The significant differences between the two kinds of media (+BAP and +TDZ) were recorded in the plant height of shoots and the number of leaves per bulb explant. The plant height of shoots regenerated from different kinds of bulbs on media of (MS + 0.5 mg/L NAA) plus 2 mg/L BAP was 1.7 to 5.2 times higher than that plus 2 mg/L TDZ (Fig. 6B). Of which, the explant of quarter bulb on MS medium plus 0.5 mg/L NAA and 2 mg/L BAP had the highest value in plant height with 12.0 cm. Similarly, the number of leaves per bulb explant on media of (MS + 0.5 mg/L NAA) plus 2 mg/L BAP was 2.2 to 3.3 times higher than that plus 2 mg/L TDZ (Fig. 6C). The explant of quarter bulb on MS medium plus 0.5 mg/L NAA and 2 mg/L BAP was also recorded with the greatest number of leaves (4.3 leaves per bulb explant). Therefore, quartered bulbs and medium of (MS + 0.5 mg/L NAA + 2 mg/L BAP) were proposed as optimum materials for *in-vitro* shoot multiplication.

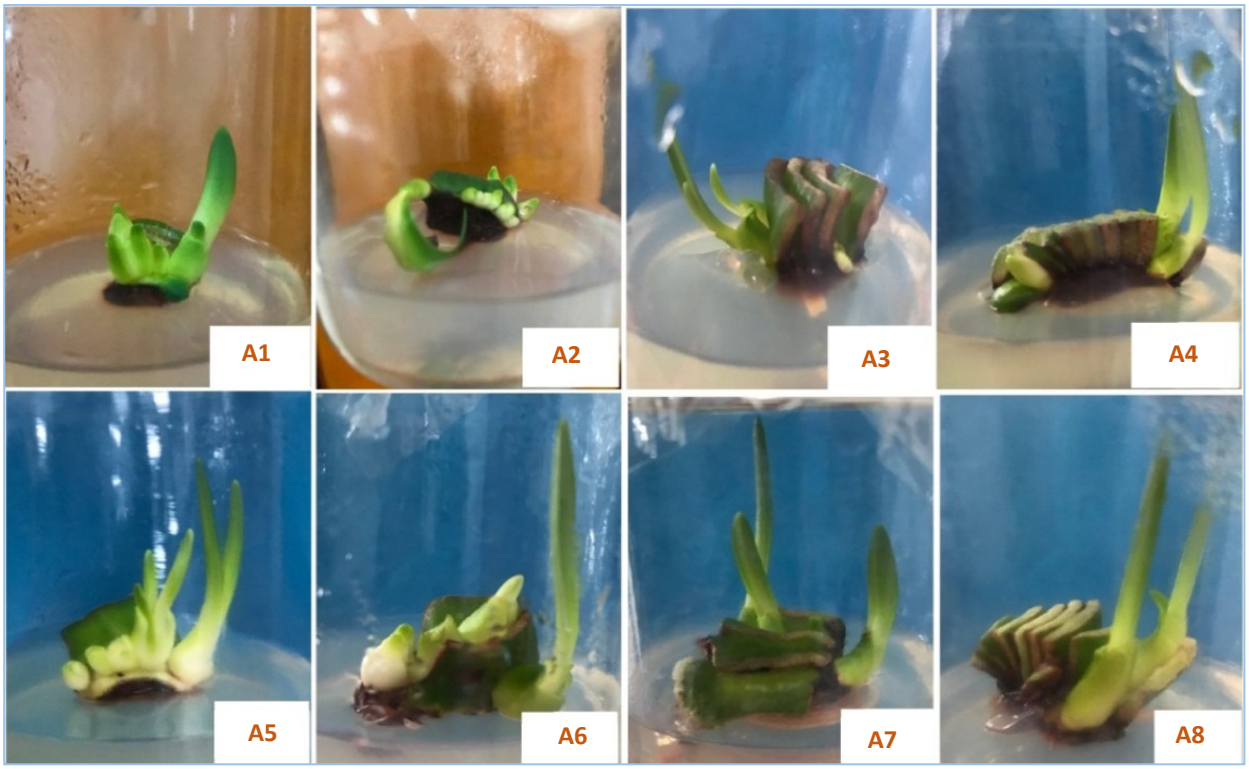

Fig. 3. Morphology of regenerated shoots from bulb scales. Notes: A1, A2, A3 and A4: Single, twin, four and eight scales in MSB medium, respectively; A5, A6, A7 and A8: Single, twin, four and eight in MSBN medium, respectively.

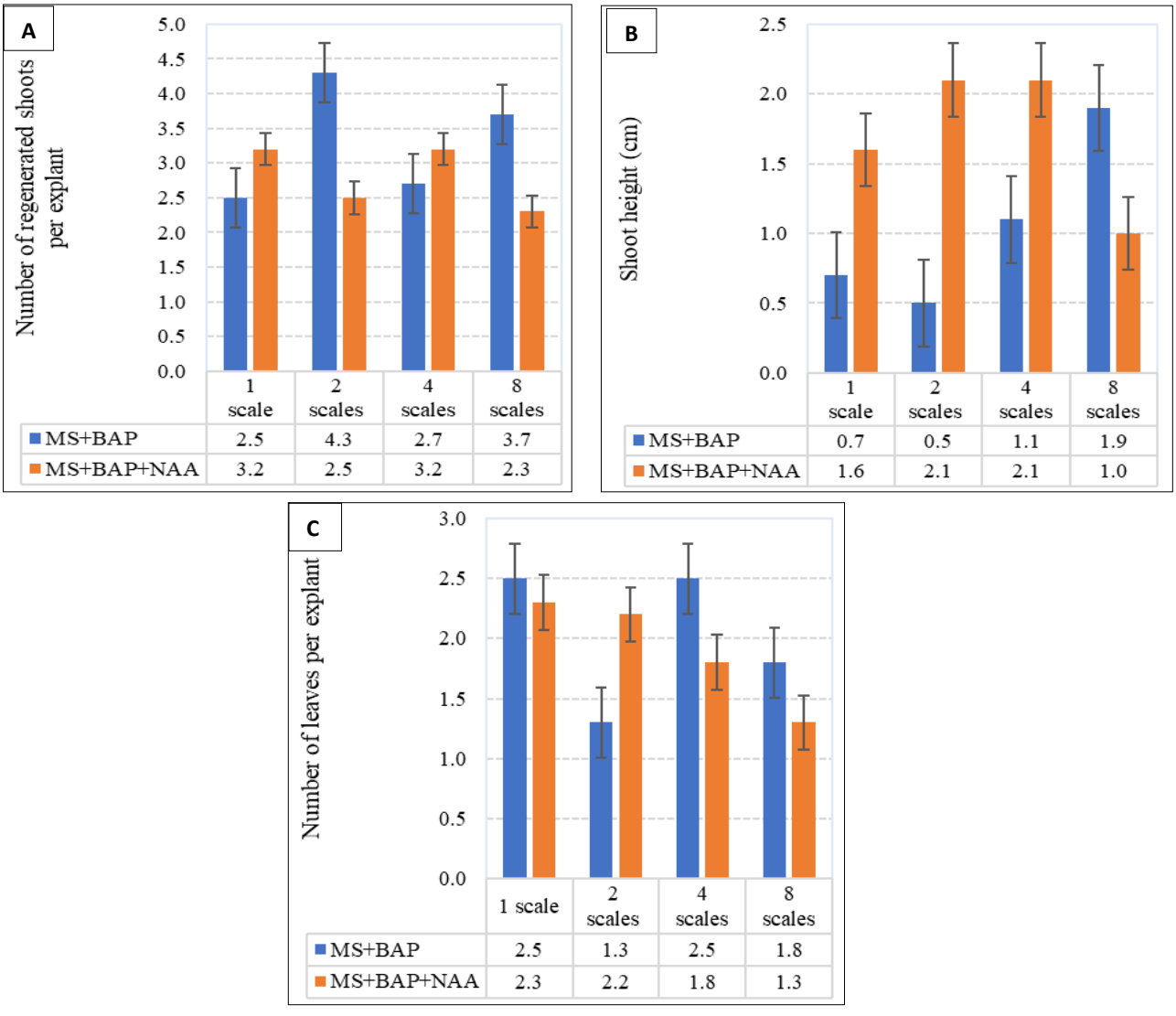

Fig. 4. Effect of plant hormones and type of bulb scales on shoot regeneration for four weeks. (A) number of regenerated shoots per explant; (B) plant height and (C) number of leaves per explant.

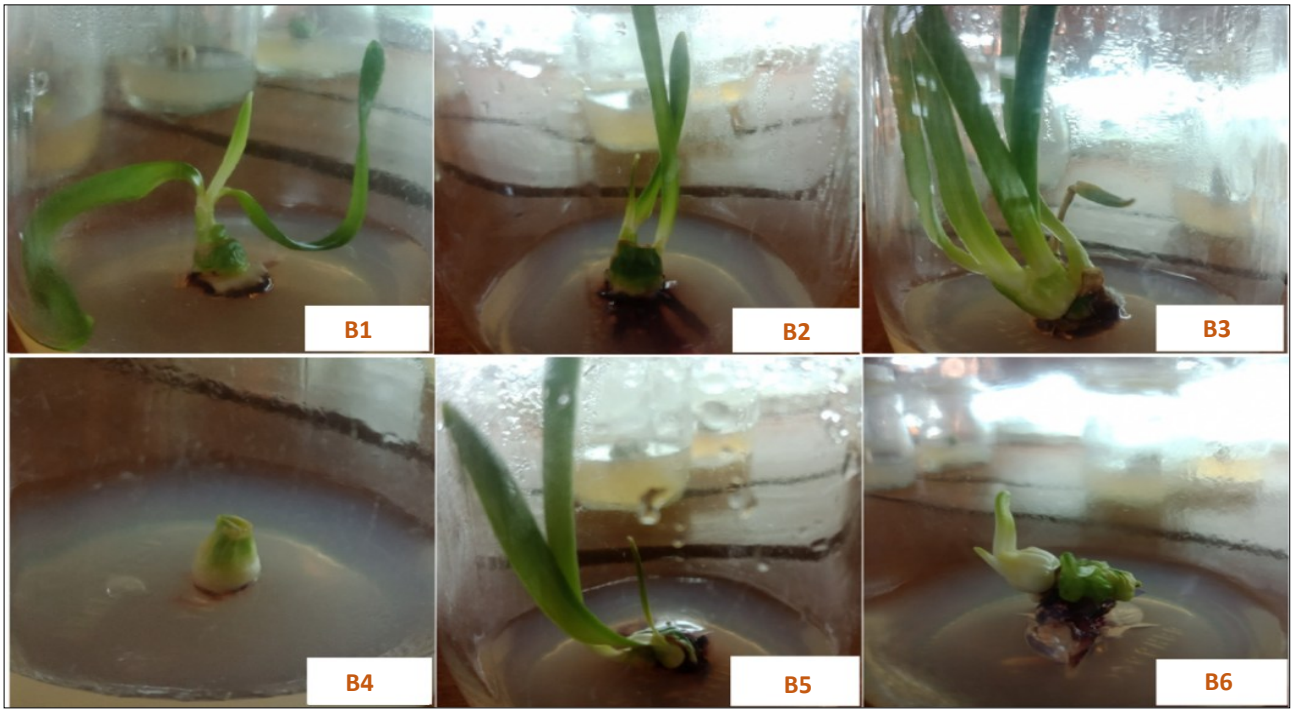

Fig. 5. Morphology of regenerated shoots from bulb explants. Notes: B1, B2, B3: whole, half and quarter bulbs in MS medium + 0.5 mg/L NAA + 2 mg/L BAP, respectively; B4, B5, B6: Whole, half and quarter bulbs in MS medium + 0.5 mg/L NAA + 2 mg/L TDZ, respectively.

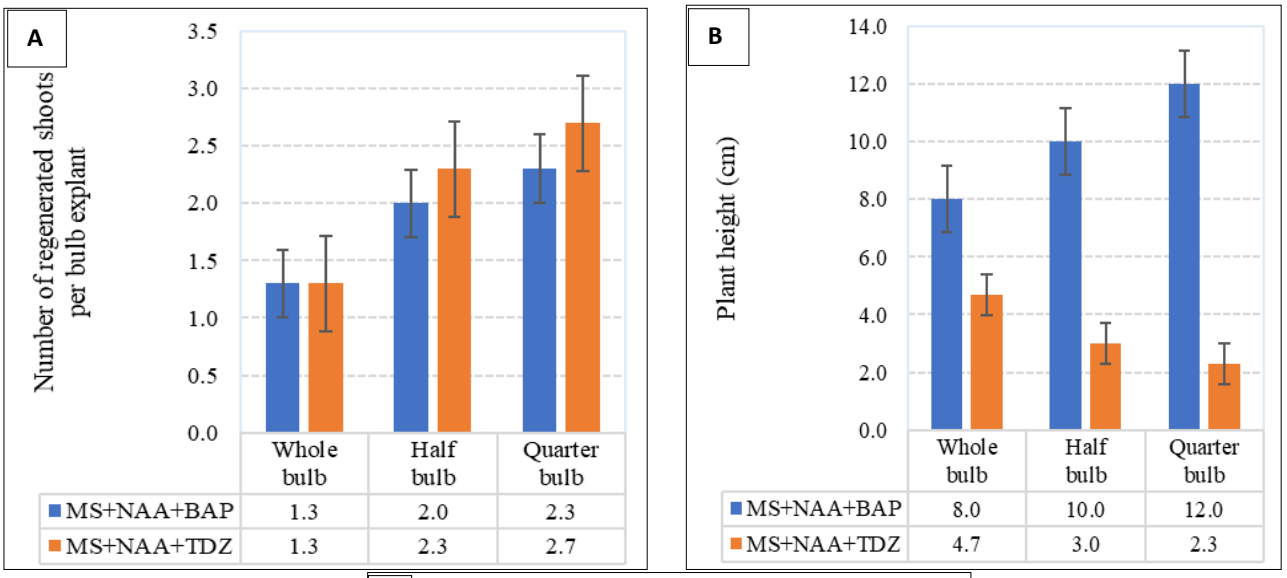

Fig. 6. Effect of plant hormones and type of bulbs on *in-vitro* shoot multiplication for 10 weeks. (A) number of regenerated shoots per bulb explant; (B) plant height and (C) number of leaves per bulb explant.

Complete plantlet generation

The bulbs formed after ten weeks of shoot multiplication will be collected and transferred to the rooting media. The NAA was widely used to promote the rooting of many plants, including hippeastrum (23-25). To generate complete plantlets, *in vitro*-formed bulbs were transferred to rooting media consisting of MS medium supplemented with NAA, sucrose and active charcoal with different concentrations (Table 2). The result showed that the number of roots per plant generated from bulbs on MS medium containing 1 or 2 mg/L NAA (from 9.6 to 16.0 roots) was 4.4 - 7.3 times more than that on MS medium without NAA (2.2 roots). Roots induced by rooting media with NAA (treatments C2-C9) were thicker and stronger, but shorter than roots induced by the control medium (treatment C1) (Table 4, Fig. 7). Besides, MS medium containing 2 mg/L NAA (treatments C3, C5, C7 and C9) helped bulbs generate roots more than MS medium containing 1 mg/L NAA (treatments C2, C4, C6 and C8). This implies that NAA plays a role in root development. A previous study also confirmed that a well-developed root system was achieved in the MS media supplemented by 0.2 mg/L NAA (26). Among the treatments of complete plantlet generation, the C9 treatment helped bulbs generate roots best. For the sucrose supplement, bulbs cultured on the rooting media with 40 g/L sucrose generated more roots than those with 30 g/L sucrose. Meanwhile, active charcoal assisted with root generation; however, the result has been recorded that there was no significant difference between treatments with and without active charcoal.

Treatments of culture media affected plant height and the number of leaves per plant. The result showed that NAA helped plant growth. Plant height in the medium with 2 mg/L NAA was better than that in the medium with 1 mg/L NAA. The number of leaves per plant in the treatments ranged from 1.2 to 2.2 leaves and was significantly higher than that in the control medium (Table 4, Fig. 7).

Therefore, the MS medium supplemented with 2 mg/L NAA, 0.5 g/L activated carbon and 40 g/L sucrose assists in the formation and development of roots in hippeastrum 'Double King' bulbs.

Evaluation of the adaptability and development of tissue-cultured seedlings in greenhouses

The tissue-cultured seedlings had a good survival rate, ranging from 75.0 % to 100 %, wherein the seedlings in D1, D2 and D3 treatments survived completely with a survival rate of 100 % (Table 5). In different environmental treatments, hippeastrum 'Double King' seedlings grew and developed differently (Table 5, Fig. 8). Plant height of seedlings fluctuated from 28.3 to 35.3 cm, of which the seedlings under the D2 treatment exhibited the tallest plants. The treatments from D2 to D6, which included the use of rice husk ash, showed an increase in height that was not significantly different from each other but was distinct from the D1 treatment, which did not use husk ash. The number of leaves per plant among seedlings under treatments was not small different and ranged from 3.5 to 4.8. The bulb diameter of seedlings ranged from 1.3 to 1.6 cm. In bulb diameter, the treatments D2, D4, D5 and D6 showed no statistically significant differences, but they were different from the treatments D1 and D3. Thus, after 12 weeks of cultivating hippeastrum 'Double King' seedlings in the greenhouse, it was shown that the substrate of 75 % sand + 25 % rice husk ash (D2) was suitable for acclimatizing seedlings in the nursery. A similar result in a previous study also showed that the optimal substrate for acclimatizing hippeastrum flowers to outdoor conditions was a sand and rice husk ash mixture at a 3:1 ratio (12).

Table 4. Generation of roots and development of plants in the different culture media

Treatment	Number of leaves/plant	Plant height (mm)	Number of roots/plant	Root length (mm)
C1	1.0^{c}	124.8^{cd}	2.2^{f}	79.2^{a}
C2	2.2^{a}	132.0^{c}	9.6^{e}	54.8^{c}
C3	1.2^{bc}	200.2^{b}	11.6^{cd}	70.2^{ab}
C4	2.0^{ab}	199.2^{b}	9.6^{e}	63.6^{bc}
C5	1.6^{abc}	228.0^{a}	9.8^{de}	62.2^{bc}
C6	1.2^{bc}	100.2^{de}	12.8^{bc}	57.6^{bc}
C7	1.2^{bc}	90.8^{e}	14.2^{ab}	56.4^{bc}
C8	1.2^{bc}	117.6^{cd}	11.0^{cde}	63.8^{bc}
C9	2.0^{ab}	141.8^{c}	16.0^{a}	32.2^{d}
P value	**	**	**	**
CV %	27.5	8.5	9.5	11.4

Notes: C1-C9: treatments of culture media.

Fig. 7. The hippeastrum 'Double King' seedlings after 12 weeks of cultivation. Notes: C1-C9: Treatments of culture media.

Table 5. Adaptation and development of tissue-cultured 'Double King' seedlings in the greenhouse after 12 weeks of cultivation

Treatment	Survival rate (%)	Plant height (cm)	Number of leaves per plant	Bulb diameter (cm)
D1	100	28.3^{b}	3.9^{cd}	1.4^{b}
D2	100	35.2^{a}	4.1^{bcd}	1.5^{a}
D3	100	33.1^{a}	3.5^{d}	1.3^{b}
D4	87.5	35.3^{a}	4.3^{abc}	1.6^{a}
D5	87.5	33.7^{a}	4.6^{ab}	1.5^{a}
D6	75.0	33.5^{a}	4.8^{a}	1.5^{a}
P value	-	*	*	*
CV (%)	-	5.7	10.1	6.3

Notes: Within the same group of average values, means followed by the same letter were not significantly different from each other; *: Significance at the 5 % level; D1-6: Treatments of substrate for growing hippeastrum 'Double King'.

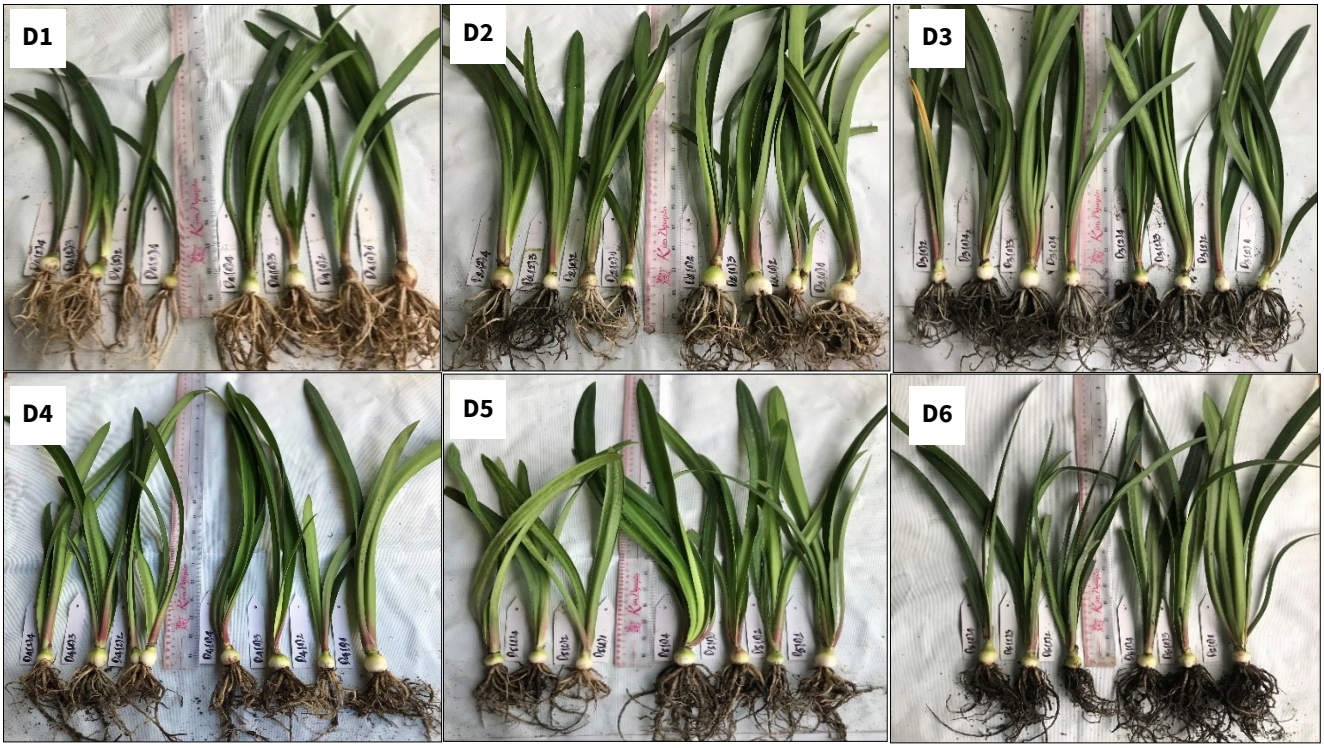

Fig. 8. The hippeastrum 'Double King' seedlings under different substrates in a greenhouse after 12 weeks. Notes: D1-6: Treatments of substrate for growing hippeastrum 'Double King'.

Conclusion

The study found the optimal media for micropropagation and acclimatization of hippeastrum 'Double King' flowers and a good substrate for their cultivation under greenhouse conditions in An Giang province, Vietnam. The conclusions from this study could be described as follows: (1) The type of twin-scale explant cultured in the MS medium supplemented with 2 mg/L BAP results in the best shoot regeneration; (2) Quartered bulbs under the medium of MS with 0.5 mg/L NAA and 2 mg/L BAP are proposed in *in-vitro* shoot multiplication; (3) The MS medium enriched with 40 g/L sucrose, 0.5 g/L activated carbon and 2 mg/L NAA promotes root formation and growth in hippeastrum 'Double King' bulbs; (4) The substrate of 75 % sand + 25 % rice husk ash is suitable for acclimatizing seedlings in the nursery. The results of this research will help optimize the production process of hippeastrum 'Double King' and promote large-scale production of bulbs at lower costs to promptly supply local flower growers.

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Authors' contributions

NTMD conceived the study, designed and coordinated experiments and drafted the manuscript. TTTH and DNTH carried out the experimental work and participated in the sequence alignment. BPT edited the content, images and format of the manuscript. The authors have read and agreed to the published version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process: The authors used Grammarly to review the writing of the manuscript at the final stage. After using this tool, the authors carefully reviewed and edited the content as needed and take full responsibility for the final version of the publication.

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