IN VITRO PROPAGATION OF *Aloe* 'Pink Blush' (Kelly Griffin) – AN ATTRACTIVE ORNAMENTAL PLANT

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SUMMARY

Aloe 'Pink Blush' is a famous aloe cultivar with high ornamental values due to its attractive leaf patterns and color. However, traditional seedling production methods have not met market demand due to various propagation challenges. To overcome these issues, tissue culture technology offers a promising solution to mass-produce aloe seedlings with high quality. Therefore, this study was conducted to develop an efficient protocol for micropropagation of this special cultivar from self-pollinated seeds by optimizing the process of seed germination, shoot multiplication, and rooting under *in vitro* conditions. For initial stage, the highest germination rate was 80% for the seeds maintained on medium containing 0.1 µM GA_3 under continuous dark condition. Nodal segments of *in vitro* germinated seedlings were subsequently used for multiplication stage, in which half-strength MS medium supplemented with 1 µM BA showed the best responses with the highest shoot multiplication rate (7.07 shoots per explant), the longest shoots (4.31 cm), and the least hyperhydricity frequency (6.67%). Next, all shoots were effectively rooted in culture medium containing 10 µM IAA with an average of 7.20 roots per plant, and an average root length of 12.43 cm. Finally, over 60% of micropropagated plantlets survived after acclimatization and were successfully transferred to greenhouse condition. This study had successfully established a protocol for rapid shoot multiplication of *Aloe* 'Pink Blush', which not only promotes rapid propagation of this attractive aloe cultivar but also establishes a fundamental protocol for conserving other threatened succulent plants.

Keywords: Aloe 'Pink Blush', light condition, MS medium, plant growth regulators, seed explant, shoot multiplication.

INTRODUCTION

Aloe is a group of succulent plants belonging to the *Aloe* genus (Asphodelaceae family), widely distributed in the arid tropical regions around the world. These plant species are highly favorable for therapeutic and medicinal applications due to the high amount of important bioactive compounds that express antioxidative, anti-inflammatory, and anticancer effects. Apart from that, multiple aloes are also used as ornamental plants due to their distinctive leaf shapes and patterns that are suitable for decoration and landscape design (Grace, 2011). Amongst, *Aloe* 'Pink Blush' was originated as a particular aloe hybrid created by Kelly J. Griffin, a famous American plant breeder, since the 1990s and quickly gathered notable attraction from a huge number of plant collectors and breeders (Sterman, 2013). This miniature aloe is a dwarf clumping aloe which can be easily recognized by its signature neon-like white-and-green mottling patterns on the leaf blade and notable coral pink spines along the leaf margin. At maturity, an individual can grow up to 20 cm tall and wide and produce pink inflorescences with the blooming season ranging from September to December. *Aloe* 'Pink Blush' is an easy-to-grow plant that can be grown in most conditions without much care required, making it an excellent ornamental plant for any collectors. Until now, this cultivar has exhibited great preference around the world for ornamental purposes and quickly become one of the most economically valuable aloes in terms of selling price (Baldwin, 2021).

Unfortunately, conventional methods are not yet sufficient for aloe propagation to fulfill the market demand. Seedling multiplication via cuttings usually causes deformation or severe damage to the mother plant. Meanwhile, seed propagation poses various disadvantages, including long-time requirement, low seed production, and high risk of cross-pollination. Under this situation, tissue culture is a promising solution for rapid propagation of high quality true-to-type aloe seedlings. Up to date, tissue culture has been widely conducted for *Aloe vera* by culturing leaf or stem explants on MS medium (Murashige and Skoog, 1962) containing exogenous plant growth regulators (mainly auxin and cytokinin), showing notable multiplication rate that could reach up to 15.4 shoots per explant (Cristiano *et al.*, 2016). However, no specific attempt on micropropagation of the attractive *Aloe* 'Pink Blush' has been reported. Therefore, this study was conducted in order to investigate the *in vitro* multiplication from self-pollinated seeds of *Aloe* 'Pink Blush' towards an efficient protocol to rapidly propagate true-to-type seedlings of this attractive aloe.

MATERIALS AND METHODS

Plant materials and surface sterilization

Controlled self-pollinated seeds derived from an adult individual of *Aloe* 'Pink Blush', which was originated from Thailand and cultivated in Nha Trang (Vietnam) for five years, were used as initial materials in this study (Figure 1A). To obtain the self-pollinated seeds, pollens from ripe anthers were introduced to the receptable stigmas within the same blooming inflorescence using a small paintbrush, then the whole inflorescence was fully covered with transparent plastic bags to avoid any possible cross-pollination for a few days until the young fruits appeared. Three weeks later, mature fruits containing seeds were harvested (Figure 1B–C). These seeds were subsequently delivered to the laboratory of the Applied Biotechnology for Crop Development Research Unit (International University, VNU HCMC) within 24 hours.

Inside a laminar air flow cabinet, seed samples were surfaced sterilized with 10% commercial bleach (Myhao Ltd., containing 0.5% NaClO) for 10 minutes, followed by three rinses of sterile distilled water to remove all trace of chemicals. Then, sterilized seeds were used as explants for tissue culture initiation.

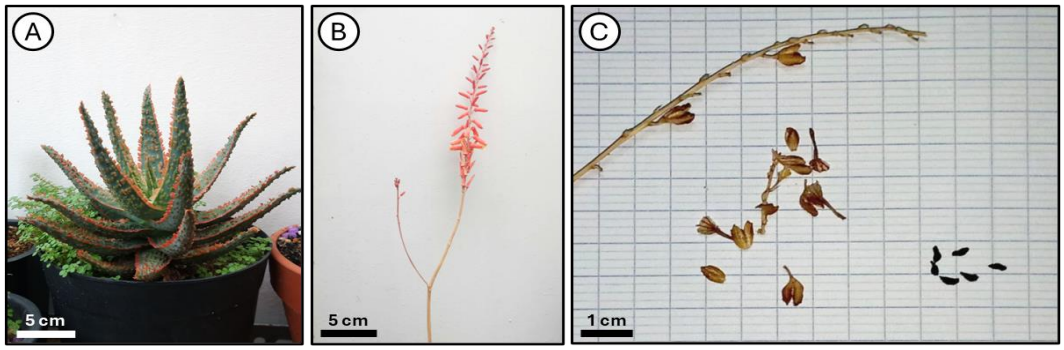

Figure 1. Plant materials of *Aloe* 'Pink Blush' used for *in vitro* initiation
(A) An adult individual used as mother plant. (B) A blooming inflorescence of the mother plant used for seed production. (C) Mature fruits and seeds of the mother plant obtained 3 weeks post pollination.

Medium preparation and culture conditions

This study was conducted with MS medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose 0.5 g/L activated charcoal, and 7.5 g/L agar. Depending on the experiments, the medium was supplemented with plant growth regulators including gibberellic acid (GA_3), 6-benzylaminopurine (BA), kinetin, or indole-3-acetic acid (IAA) at different concentrations. All reagents were tissue culture-graded (Duchefa Biochemie, Netherlands). Culture media were adjusted to pH 5.8 using 1M KOH/1M HCl prior to autoclaving for 20 minutes at 121^{o}C and 15 psi.

All cultures were maintained in a controlled growth room at 28±2^{o}C under 16-h photoperiod (100 µmol/m^2/s) provided by Cool Daylight Florescence light tubes (Philips, Vietnam). For dark treatment, cultures were kept inside a dark chamber fully enclosed with thick black cover.

In vitro seed germination

Seed explants were cultured on half-strength MS medium supplemented with GA_3 at different concentrations (0, 0.1, 0.5, 1.0 µM) and maintained under either dark or light conditions to induce *in vitro* germination. The effect of GA_3 and light conditions on seed germination was evaluated according to the final germination rate of the treated seeds after 8 weeks of culture. Then, explants were subcultured every 4 weeks for further growth.

Rapid shoot multiplication

Nodal segments, each containing one node, derived from 8-week-old germinated plantlets with all leaves and shoot tip eliminated were transferred to multiplication medium consisting of either half-strength or full-strength MS medium and either BA or kinetin at different concentrations (0, 1, 5, 10 µM). The efficiency of rapid shoot multiplication was evaluated according to the average number of shoots per clump, average number of leaves per shoot, average shoot length, and the rate of hyperhydricity after 4 weeks of culture.

Rooting and acclimatization

Individual shoots were separated from multi-shoot clusters and transferred to half-strength MS medium containing IAA at different concentrations (0, 5, 10, 15, 20 µM). Then, the efficiency of *in vitro* rooting was evaluated according to the rooting rate of cultured shoots, average number of roots per plantlet, and average root length after 4 weeks of culture.

For *ex vitro* acclimatization, plantlets with well-developed root system were rinsed thoroughly with tap water, transferred to plastic pots with a mixture of perlite:cocopeat:rice charcoal (1:1:1), and covered with transparent bags. After 8 weeks, the covers were removed, and surviving seedlings were allowed to continue growing under greenhouse conditions with natural sunlight and daily mist irrigation.

Statistical analysis

All experiments were conducted using a completely randomized design with three replicates. Each treatment included at least 10 individuals per replication. All data were presented as mean ± standard error between three replicates. Data were statistically analyzed with ANOVA and Duncan's test using SPSS software version 26.0 with p-value < 0.05 was considered as significantly different.

RESULTS AND DISCUSSION

In vitro seed germination

To optimize the conditions for *in vitro* germination, self-pollinated seeds of *Aloe* 'Pink Blush' was cultured with different GA_3 concentrations and light conditions. After 2 weeks of incubation, several seeds showed germination responses with the emergence of radicles and cotyledonary leaves in all treatments (Figure 2A). The results showed that both GA_3 and the light conditions significantly affected the seed germination rates, however, no interaction effect between the two factors had been recorded (Table 1). Among the tested treatments, seeds cultured in medium containing 0.1 µM GA_3 and maintained in the dark exhibited the highest germination rate (80.00%) compared to other treatments. However, as the amount of GA_3 went up and reached 1.0 µM, the germination rate was gradually reduced and even lower than the GA_3-free treatment. This observation was possibly explained by the need of gibberellin at proper amount to overcome seed dormancy by regulating the hormonal balance inside the seed and thus inducing germination while excessive or insufficient GA_3 concentrations could not exhibit this effect (Yang *et al.*, 2020).

Considering the effect of light condition, the germinating efficiency of all cultures maintained under light condition was beneath those in dark condition. This result suggested a potential inhibitory effect of light on seed germination, which potentially caused by the stimulating effect of light on abscisic acid activities, thus causing the maintenance of seed dormancy and preventing germination (Yang et al., 2020). However, opposite results were reported by Kulkarni *et al.* (2014) and Amoo *et al.* (2022), showing that *Aloe arborescens* preferred illumination for *in vitro* germination while no significant differences between light and dark conditions in the germination of *A. modesta, A. peglerae* and *A. reitzii* seeds. These observations suggest that the effect of light on seed germination is species-dependent in the *Aloe* genus.

Hence, medium with 0.1 µM GA_3 supplement in dark condition was chosen to be the most suitable treatment for seed germination of *Aloe* 'Pink Blush'. By subculturing into fresh medium, all germinated seedlings continued to grow and develop up to 7 true leaves, and ready for further experiment in shoot multiplication (Figure 2B–C).

Figure 2. *In vitro* germination of *Aloe* 'Pink Blush'
(A) A newly germinated seed with one emerging cotyledon after 2 weeks of culture.
(B) Germinated plantlets with functional leaves after 4 weeks of culture. (C) A grown-up plantlet after 8 weeks of culture.

Table 1. The effect of gibberellic acid and light conditions on the germination of *Aloe* 'Pink Blush' seeds

Treatment	Light condition	GA_3 (µM)	Germination rate (%)
T1	Dark	0	68.33 ± 4.41^{b}
T2		**0.1**	**80.00 ± 2.83^{a}**
T3		0.5	51.67 ± 4.41^{bc}
T4		1.0	58.33 ± 3.33^{cd}

T5	Light	0	46.67 ± 1.67^{de}
T6		0.1	45.00 ± 5.00^{de}
T7		0.5	38.33 ± 4.41^{e}
T8		1.0	36.67 ± 1.67^{e}
Light condition:			*p-value < 0.001*
GA_3:			*p-value = 0.001*
Light condition × GA_3:			*p-value = 0.063*

GA_3: Gibberellic acid. Data were presented as mean ± standard error. Data followed by the same letter (a, b, c…) within the same column were not significantly different from each other p-value < 0.05 according to two-way ANOVA and Duncan's test.

Shoot multiplication

Exogenous cytokinin has been proven to be essential for the proliferation of *in vitro* shoots of *Aloe* species (Bairu *et al.*, 2009). For *Aloe* 'Pink Blush', this study recorded that all explants exhibited new shoots formation in all treatments (Figure 3A–C), including the cytokinin-free media. Considering the shoot proliferation and development, shoot multiplication rate was rather determined by the application of exogenous cytokinin than the basal medium while both factors significantly affected the growth of shoots in terms of shoot length and average number of leaves (Table 2). Among all tested treatments, the half-strength MS medium supplemented with 1 µM BA gave the highest multiplication rate of 7.07 shoots per explants. This treatment also expressed similar or higher responses in terms of shoot elongation (4.31 cm in shoot length) and leaf formation (3.67 leaves per shoot) compared to other treatments, suggesting that this treatment was the suitable exogenous dose for shoot formation and development. In comparison to other *Aloe* species, optimal *in vitro* multiplication rate of *Aloe* 'Pink Blush' obtained in this study (7.07) was yet lower than one achieved in other species such as *A. ferox* (Bairu *et al.*, 2009), *A. vera* (Cristiano *et al.*, 2016), and *A. reitzii* (Amoo *et al.*, 2022) with 13.00, 13.39, and 15.40 shoots per explants respectively, indicating the notable difficulty in propagation of this ornamental aloe.

Moreover, during shoot multiplication, hyperhydricity was also observed among the developing shoots, appearing as glass-like shoot structures with excessive water accumulation and stunned growth (Figure 3D–F). The results showed that full-strength MS medium significantly increased the frequency of hyperhydricity (up to 53.33%) compared to half-strength medium while cytokinin did not show any notable effects. Similar results were reported and explained by *Jan et al.* (2021) as high mineral content was one of the major causes of this type of abnormality. Luckily, the treatment with optimal shoot multiplication (half-strength MS with 1 µM BA) only had 6.67% of hyperhydricity, which was considered to be acceptable.

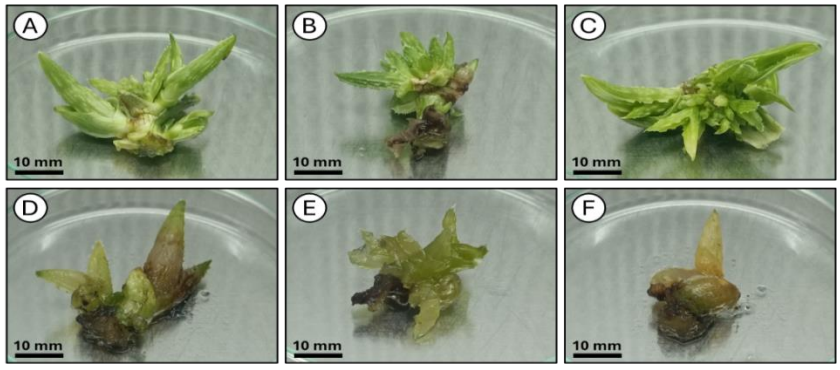

Figure 3. Shoot multiplication of *Aloe* 'Pink Blush' after 4 weeks of culture.
(A–C) Development of new shoots on the explant forming multi-shoot clumps. (D–F) Shoot clusters with hyperhydricity phenomenon.

Table 2. The effect of basal medium, BA, and kinetin on *in vitro* multiplication of *Aloe* 'Pink Blush'

Treatment	Basal medium	Cytokinin (µM)		Number of shoots per explant	Number of leaves per shoot	Shoot length (cm)	Hyperhydricity (%)
		BA	Kinetin				
T1	½ MS	0	0	5.07 ± 0.47^{def}	3.27 ± 0.48^{abcd}	3.46 ± 0.29^{cde}	6.67 ± 6.67^{cd}
T2		**1**	**0**	**7.07 ± 0.37^{a}**	**3.67 ± 0.27^{ab}**	**4.31 ± 0.17^{a}**	**6.67 ± 6.67^{cd}**
T3		5	0	6.13 ± 0.18^{b}	2.80 ± 0.12^{bcd}	2.40 ± 0.06^{f}	26.67 ± 13.33^{abcd}
T4		10	0	6.07 ± 0.33^{bc}	2.53 ± 0.24^{cd}	2.27 ± 0.12^{f}	26.67 ± 6.67^{abcd}

T5		0	1	5.13 ± 0.27^{cdef}	3.93 ± 0.29^{a}	3.97 ± 0.15^{abc}	0.00 ± 0.00^{d}
T6		0	5	5.53 ± 0.35^{bcdef}	3.67 ± 0.48^{abc}	4.13 ± 0.28^{ab}	13.33 ± 6.67^{bcd}
T7		0	10	6.27 ± 0.18^{ab}	4.00 ± 0.23^{a}	4.28 ± 0.13^{a}	6.67 ± 6.67^{cd}
T8		0	0	4.73 ± 0.29^{f}	2.93 ± 0.37^{abcd}	2.97 ± 0.19^{e}	26.67 ± 6.67^{abcd}
T9		1	0	6.00 ± 0.23^{bcd}	2.93 ± 0.18^{abcd}	3.55 ± 0.09^{cd}	26.67 ± 13.33^{abcd}
T10		5	0	5.80 ± 0.12^{bcde}	2.60 ± 0.42^{bcd}	2.04 ± 0.17^{f}	40.00 ± 11.55^{ab}
T11	MS	10	0	5.60 ± 0.35^{bcdef}	2.33 ± 0.27^{d}	1.93 ± 0.11^{f}	53.33 ± 6.67^{a}
T12		0	1	4.93 ± 0.29^{ef}	3.27 ± 0.37^{abcd}	3.31 ± 0.15^{de}	33.33 ± 13.33^{abc}
T13		0	5	5.60 ± 0.23^{bcdef}	3.47 ± 0.29^{abc}	3.56 ± 0.13^{cd}	33.33 ± 17.64^{abc}
T14		0	10	5.93 ± 0.24^{bcd}	3.47 ± 0.13^{abc}	3.66 ± 0.06^{bcd}	33.33 ± 6.67^{abc}
Basal medium:				*p-value = 0.078*	*p-value = 0.027*	*p-value < 0.001*	*p-value < 0.001*
Cytokinin:				*p-value < 0.001*	*p-value = 0.002*	*p-value < 0.001*	*p-value = 0.161*
Basal medium × Cytokinin:				*p-value = 0.425*	*p-value = 0.938*	*p-value = 0.839*	*p-value = 0.937*

BA: 6-benzylaminopurine, ½ MS: half-strength MS medium (Murashige and Skoog, 1962), MS: full-strength MS medium.

Data were presented as mean ± standard error. Data followed by the same letter (a, b, c…) within the same column were not significantly different from each other with p-value < 0.05 according to two-way ANOVA and Duncan's test.

Rooting and acclimatization

After 4 weeks of culture, generated shoots continued to grow and started to develop 3–10 roots at the shoot bases (Figure 4A–B). In general, shoots growing on culture media supplemented with IAA showed higher values of root formation rate and average number of roots compared to the control; however, no significant differences were recorded between the four tested concentrations of IAA (5–20 µM) as shown in Table 3. However, the use of IAA at high concentrations (15–20 µM) led to a remarkable decrease in average root length while lower treatments posed no significant impacts on this criterium of the testing plantlets. This phenomenon could be explained by the inhibitory effect of high-level auxin on root elongation because excessive auxin could result in hormonal imbalance in plant tissue as well as the rapid accumulation of growth retardants such as ethylene (Edelmann, 2022). Overall, the application of 10 µM IAA was considered as the optimal treatment that exhibited significantly better responses in rooting rate (100%) and number of roots (7.20 roots per plantlet) along with similar root length (12.43 cm) compared to the control. These results were similar to a study by Ahmad *et al.* (2022) which reported the optimal rooting efficiency of *in vitro A. vera* plantlet was 8.57–11.42 µM IAA, suggesting a similar influence of IAA on root formation process in different species and varieties within the *Aloe* genus.

After *in vitro* rooting, healthy and well-rooted plantlets were subjected to *ex vitro* acclimatization with more than 60% of plantlets survived and developed normally upon transferring to greenhouse condition (Figure 4C). This result was similar to the one reported in various *Aloe* species (including *A. vera*, *A. modesta*, *A. peglerae*, and *A. reitzii*) after 4–6 weeks of acclimatization, indicating the feasibility of transferring *Aloe* species to greenhouse conditions after *in vitro* rooting with exogenous auxin (Cristiano *et al.*, 2016; Amoo *et al.*, 2022). However, more studies should be established to optimize the survival rate of *ex vitro* seedlings to enhance the efficiency of micropropagation protocol for *Aloe* 'Pink Blush'.

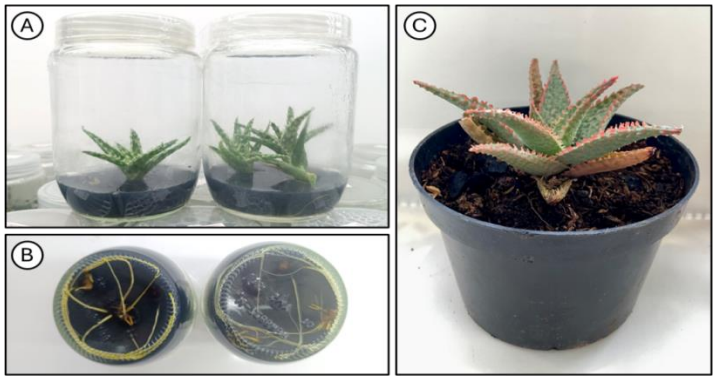

Figure 4. Rooting and *ex vitro* acclimatization of *Aloe* 'Pink Blush'
(A–B) Plantlets with healthy shoots (A) and well-developed root systems (B) ready for soil acclimatization. (C) A successfully acclimatized seedling after 8 weeks growing in greenhouse condition.

Table 3. The effect of IAA on *in vitro* rooting *Aloe* 'Pink Blush' plantlets

Treatment	IAA (µM)	Rooting rate (%)	Number of roots per plantlet	Root length (cm)
T1	0	73.33 ± 6.67^{b}	4.53 ± 0.35^{b}	10.10 ± 0.45^{a}
T2	5	93.33 ± 6.67^{ab}	6.40 ± 0.31^{a}	12.32 ± 1.08^{a}
T3	**10**	**100.00 ± 0.00^{a}**	**7.20 ± 0.50^{a}**	**12.43 ± 0.61^{a}**
T4	15	100.00 ± 0.00^{a}	6.33 ± 0.41^{a}	7.26 ± 1.02^{b}
T5	20	80.00 ± 11.55^{ab}	6.80 ± 0.46^{a}	4.79 ± 0.55^{b}
		p-value = 0.057	*p-value = 0.009*	*p-value < 0.001*

IAA: Indole-3-acetic acid.

Data were presented as mean ± standard error. Data followed by the same letter (a, b) within the same column were not significantly different from each other p-value < 0.05 according to one-way ANOVA and Duncan's test.

CONCLUSIONS

This study reported the optimization of light conditions, basal media, and various plant growth regulators for *in vitro* propagation of *Aloe* 'Pink Blush' (Kelly Griffin) from self-pollinated seeds to intact plantlets. Culture medium supplemented with 0.1 µM GA_3 achieved the highest seed germination rate of 80% under dark condition. For shoot multiplication, half-strength MS medium containing 1 µM BA was the most effective treatment, which was able to produce 7.07 shoots per explant with only 6.67% rate of hyperhydricity. For *in* vitro rooting, exogenous auxin IAA at 10 µM was proved to be optimal for the root induction, resulting in significantly higher rooting rate and average number of roots compared to the control. Finally, over 60% of micropropagated plantlets were successfully acclimatized to *ex vitro* condition.

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NGHIÊN CỨU NHÂN GIỐNG *IN VITRO* ĐỐI VỚI GIỐNG NHA ĐAM CẢNH *Aloe* 'Pink Blush' (Kelly Griffin)

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TÓM TẮT

Aloe 'Pink Blush' là giống nha đam cảnh có giá trị cao, nổi tiếng với bộ lá mang hoa văn và màu sắc độc đáo. Tuy nhiên, phương pháp sản xuất giống truyền thống chưa thể đáp ứng nhu cầu thị trường vì gặp nhiều hạn chế trong quá trình nhân giống. Nhằm khắc phục những nhược điểm đó, kỹ thuật nuôi cấy mô là một giải pháp tiềm năng nhằm sản xuất hàng loạt cây giống với chất lượng cao. Vì vậy, nghiên cứu này được thực hiện nhằm xây dựng quy trình vi nhân giống hiệu quả đối với giống nha đam đặc biệt này từ nguồn hạt tự thụ thông qua tối ưu hóa quá trình nảy mầm hạt, nhân nhanh chồi, và kích thích tạo rễ trong điều kiện *in vitro*. Với giai đoạn đầu nuôi cấy, tỷ lệ nảy mầm cao nhất đạt 80% đối với các mẫu hạt nuôi trên môi trường chứa 0,1 µM GA_3 và duy trì trong điều kiện tối. Sau đó, các mẫu đốt thân được dùng trong giai đoạn nhân nhanh chồi; trong đó, môi trường MS với nồng độ khoáng giảm một nửa và có bổ sung 1 µM BA cho thấy hệ số nhân chồi cao nhất (7,07 chồi/mẫu), chiều dài chồi lớn nhất (4,31 cm), và tỷ lệ thủy tinh thể thấp (6,67%). Tiếp đó, tất cả chồi non được kích rễ thành công trên môi trường chứa 10 µM IAA với số lượng rễ trung bình đạt 7,20 rễ/chồi, và chiều dài rễ trung bình là 12,43 cm. Cuối cùng, hơn 60% cây con nuôi cấy mô đã sống sót qua thuần dưỡng và được chuyển thành công sang giai đoạn vườn ươm. Nghiên cứu này đã triển khai thành công quy trình nhân nhanh chồi đối với giống nha đam *Aloe* 'Pink Blush'; kết quả này không chỉ góp phần thúc đẩy quá trình nhân giống loại nha đam cảnh này mà còn có thể được ứng dụng làm nền tảng xây dựng quy trình bảo tồn nhiều loại thực vật mọng nước khác.

Từ khóa: Aloe 'Pink Blush', chất điều hòa sinh trưởng thực vật, điều kiện chiếu sáng, môi trường MS, nhân nhanh chồi, mẫu hạt.

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