agronomy

MDPI

Article

Development and Optimization of a Rapid In Vitro Micropropagation System for the Perennial Vegetable Night Lily, *Hemerocallis citrina* Baroni

Gaoya Zuo [1], Ke Li [1], Yining Guo [1], Xiaorun Niu [1], Lijin Yin [1], Zhiqiang Wu [2], Xiaomin Zhang [3], Xiaojing Cheng [1], Jie Yu [1], Shaowen Zheng [1], Yanfang Wang [1], Guoming Xing [1,4], Sen Li [1,4] and Feifan Hou [1,*]

[1] College of Horticulture, Shanxi Agricultural University, Jinzhong 030801, China; zuogaoya2020@163.com (G.Z.); saulisen@163.com (S.L.)
[2] Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518120, China
[3] Guangling County Rural Reform and Economic Affairs Center, Yangzhou 037500, China
[4] Datong Daylily Industrial Development Research Institute, Datong 037000, China
* Correspondence: zc1393543@163.com

Abstract: The perennial herbaceous night lily, *Hemerocallis citrina* Baroni, is an important vegetable crop with an increasing production and consumption in China. The long lifecycle and slow growth of the night lily are becoming bottlenecks for the large-scale production of elite lines and various genetic and breeding studies. There is a lack of a protocol for rapid and efficient micropropagation for this crop. Here, we reported the systematic investigation and optimization of in vitro plant regeneration through tissue-culture-based organogenesis in the night lily variety 'Datong Huanghua'. We evaluated various factors affecting the efficiency of callus induction and subculture, shoot regeneration, rooting and plantlet establishment, including explant type and age, inoculation methods, basal culture media and the type and concentration of plant growth regulator (phytohormones) in various growth media. We developed an optimized protocol, as follows. The highest efficiency of callus induction was observed on Murashige and Skoog (MS) medium supplied with 22.7 μM TDZ (thidiazuron) using the young scape (flower stem or stalk) as the explant, which was cut longitudinally in half to produce a segment approximately 0.5 cm in length. Callus subculture and proliferation were more efficient on MS medium containing 9.0 μM 2,4-D (2,4-dichlorophenoxyacetic acid) under light culture conditions. Shoot regeneration showed the highest efficiency on MS medium supplemented with 8.9 μM 6-BA (6-benzylaminopurine) + 5.4 μM NAA (α-naphthaleneacetic acid), while the best rooting medium was MS medium containing 2.7 μM NAA. After transplanting, the transplanted regenerated seedlings showed the highest survival rate (96%) on a substrate mixture with a 2:1:1 ratio of peat/perlite/vermiculite. A protocol and flowchart for the rapid in vitro micropropagation of night lily plants is proposed that will facilitate various genetic, genomic and breeding studies on this crop.

Keywords: *Hemerocallis*; night lily; tissue culture; micropropagation; organogenesis

Citation: Zuo, G.; Li, K.; Guo, Y.; Niu, X.; Yin, L.; Wu, Z.; Zhang, X.; Cheng, X.; Yu, J.; Zheng, S.; et al. Development and Optimization of a Rapid In Vitro Micropropagation System for the Perennial Vegetable Night Lily, *Hemerocallis citrina* Baroni. *Agronomy* **2024**, *14*, 244. https://doi.org/10.3390/agronomy14020244

Academic Editor: Valeria Cavallaro

Received: 8 December 2023
Revised: 8 January 2024
Accepted: 17 January 2024
Published: 24 January 2024

1. Introduction

The night lily (or long yellow daylily), *H. citrina* Baroni, is one of the most economically important plant species in the genus *Hemerocallis* (family Asphodelaceae, previously Hemerocallidaceae or Liliaceae) [1]. In East Asian countries, the night lily is a popular specialty vegetable crop, and its dried immature flower buds serve as a primary food source [1,2]. In recent years, a marked increase in the production and consumption of night lilies has occurred in China. For instance, in 2018, more than 60,370 hectares of night lilies were cultivated, yielding 561,300 tons of immature flowers, translating to an

economic value of nearly CNY 4 billion [1–3]. Research on night lilies has made significant progress, including the collection and molecular characterization of germplasm resources and the development of various genetic and genomic resources, such as genome sequences and molecular markers [1,2,4–8]. This research lays a solid foundation for expediting the breeding of this crop through molecular mapping.

The night lily is an herbaceous perennial and it is commercially propagated asexually by dividing the crown or by making cuttings, while seed propagation is used for breeding [3,9,10]. In sexual propagation, the night lily undergoes a prolonged juvenile (vegetative) growth phase, typically requiring two to three years to reach the flowering stage. After several years of growth, the night lily plants are composed of several interconnected crowns, which can be separated into individual plants. There are two primary challenges in the propagation of night lilies. In seed production, the extended growth period necessary for new plant development is particularly cumbersome in genetics and breeding research, especially when developing segregating populations for molecular-marker-related studies [5,11]. In commercial production, the annual rate of new crown development is limited, averaging an 8:1 ratio depending on the genotype [10,12]. Thus, for a newly released variety, it may take more than 10 years to produce enough plants to meet market demand [9].

One solution for addressing these bottlenecks in plant production is plant tissue culture and micropropagation, which rely on the totipotency of plant cells for regenerating plants [13]. During in vitro plant regeneration, an explant undergoes cell division and differentiation, during which organs and tissues form throughout its growth. This process can be realized through somatic embryogenesis or organogenesis [14–16]. In vitro micropropagation can accelerate vegetative propagation and has been widely used for commercial production of many horticultural crops [17–21]. Krikorian and Kann were the first to demonstrate the totipotency of *H. citrina* plants [22]. Since then, extensive studies have been conducted on tissue culture and micropropagation in night lilies. Factors affecting callus induction, shoot and plant regeneration in the night lily have been investigated to optimize various culture conditions, including plant genotypes, explants, basal growth media and supplemental plant growth regulators (PGRs) [10,23–26]. For explants, micropropagation has been performed with young inflorescences, flower stems (scapes) or petals [26–30], ovaries [22], flower stems (scapes), leaves [11], suspension culture cells [31,32], isolated protoplasts [23,33], anther filaments and immature seed embryos [10,34]. More recently, Matand et al. [30] reported high-frequency in vitro regeneration of adventitious shoots in daylily stem tissue (scapes) using Murashige and Skoog (MS) medium [35] supplemented with thidiazuron (TDZ), which enhances culture efficiency in various tissues and genotypes [36,37]. The authors suggested this protocol for the mass multiple shoot formation and rapid propagation of this crop to meet market demands and expedite exogenous gene transfer and breeding selection processes.

Currently, there is no established protocol for the large-scale production of night lily plants through in vitro micropropagation. Additionally, the perennial growth habit of these plants indicates the seasonal availability of explants for night lilies. Hence, this study aimed to develop an efficient and rapid protocol for tissue culture of night lilies. We evaluated various factors that affect the propagation of the night lily, including basal growth media, plant growth regulators (PGRs), explants for callus induction, shoot regeneration, subculture, rooting and transplanting. All our experiments were performed with the night lily cultivar 'Datong Huang Hua' ('Datong Yellow Flower'), which is a popular variety that has been widely grown in Shanxi Province for many years and has a high yield [1,2]. Its genome is also being assembled in the senior author's laboratory (unpublished data). In this work, we proposed a protocol for the rapid and large-scale production of night lily plants through micropropagation.

2. Materials and Methods

2.1. Plant Materials and Explant Culture Conditions

Young leaf or flower stem (scape) segments from the night lily variety 'Datong Huanghua' were used as explants in tissue culture in this study. This variety was grown in the *Hemerocallis* germplasm nursery at Shanxi Agricultural University (Taigu, Shanxi Province, China) for 10 years. The leaf was collected in late March when the leaf started to grow. The scape was collected in June before the flower bud started to expand.

The explants were initially rinsed with tap water and placed in sterile jars. Subsequently, the explants were rinsed once with sterile deionized water (dH_2O) on an ultraclean bench, the surface disinfected with 75% ethanol for 30 s, and rinsed twice with dH_2O, followed by sterilization in 0.1% $HgCl_2$ for 10 min with continuous shaking. Afterwards, the explants were rinsed five times with dH_2O. The entire procedure was performed under sterile conditions in a laminar flow hood. Detailed information (names and vendors) on all the chemicals and components of the growth media used in this study is provided in Supplemental Files 1a and 1b.

The explants were cultured in 200 mL glass jars containing approximately 25 mL of culture medium containing MS basal salts, vitamins, sucrose (30 g/L) and phytagel (2 g/L), along with different concentrations of PGRs depending on the specific treatment (see below). The pH of the MS medium was adjusted to 5.8~6.2 with 1 M NaOH, followed by autoclaving at 121 °C for 20 min. The tissue culture room condition was 25 ± 2 °C incubation temperature. There were two types of photoperiod conditions: dark culture conditions (24 h of total darkness) and light culture conditions (16 h photoperiod (6:00~22:00)). The explants were subcultured onto fresh media every four weeks throughout the entire experimental period.

2.2. Comparison of Callus Indcution Efficieny Using Leaf and Scape Exaplants

Young leaf segments of Datong Huanghua plants (1.0 cm × 0.5 cm) were inoculated on MS medium supplemented with 2.2 μM 6-BA and 9.0 μM 2,4-D for callus induction [38]. The effect of TDZ on callus induction was tested at five different concentrations (0.5, 4.5, 11.4, 22.7 and 45.4 μM) in a completely randomized design (CRD); each treatment had 6 jars and each jar had 8 explants. The explants were incubated in the dark and then transferred to light following callus emergence. The rate of callus formation was calculated after 60 days of light culture, along with observations of callus division and differentiation.

When young scapes were used as the explants, scape segments with 0.5 cm in length obtained using Method 3 described below were inoculated in MS medium supplemented with different concentrations of TDZ (0.5, 4.5, 11.4, 22.7, 45.4, 68.1 and 90.8 μM), 6-BA (0, 2.2, 6.7 and 11.1 μM) and 2,4-D (0, 0.5, 2.3 and 4.5 μM) for a total of 23 treatments; each treatment had 9 jars and each jar had 5 explants. The callus formation rate was recorded after 30 days of dark culture and the shoot regeneration rate was recorded after 30 days of light culture. Observations of the callus growth status were also made.

From this experiment, the scape was shown to be a better explant than the leaf for callus induction and shoot regeneration. Thus, the scape was used as the explant in all subsequent investigations.

2.3. Evaluation of Callus Indcution Efficiency among Different Cutting Methods for Scape Explant Preparation

Three cutting/inoculation methods for scape tissue were evaluated for explant culture (detailed in the Results section). Method 1: A 1.0 cm long scape segment was cut into halves longitudinally [30]. Each half was further cut at the lower end (root end) aiming to maximize the wounding. The cut surface was placed in contact with the medium for callus formation. Method 2: The scape was cut into 0.1 cm slices along their cross-sections, which were inoculated with the lower end facing upwards on the medium. Method 3: A 0.5 cm segment of the scape was cut longitudinally at its lower end and inoculated on the medium with the lower end facing upwards. Scape explants from different methods were

cultivated in MS medium supplemented with 22.7 μM TDZ. For each treatment (cutting method), 12 jars were used with 5 explants within each jar. The explants were cultured in the dark and observed daily. The callus formation and degeneration rates were recorded after 30 days of culture.

2.4. Evaluation of Basic Culture Media on Callus Indcution and Shoot Regeneration from Scape Explant

The efficiency of the four basic culture media for callus induction and shoot regeneration from scape explants was assessed. These media included MS, ½MS, Gamborg B-5 (B5) [39] and Chu N6 (N6) [40] (refer to Supplemental File 1a for details). In each case, TDZ (22.7 μM TDZ) was added to the medium. Each treatment had 6 jars, with 5 scape explants inoculated per jar. The explants were cultured in the dark for culture condition and observed daily. Callus formation and browning rates were recorded after 30 days of dark culture. Subsequently, cultures were transferred to light conditions, with shoot regeneration rates recorded after an additional 30 days of light culture.

2.5. Callus Subculture, Shoot Regeneration and Rooting

The embryogenic-like calli (light yellow in color and compact) generated from scape explants in the primary culture were selected for subculture/proliferation. These calli were segmented into pieces (~0.5 cm^3), weighed and subsequently inoculated on MS proliferation medium containing different concentrations of NAA and 2,4-D (13 treatments in total; see below for details). Each treatment included 3 jars with three calli in each jar. Light and dark cultures were generated separately in the culture chamber, and the weight of the proliferated calli was recorded after 30 days. Additionally, the average growth rate of the calli was calculated.

The embryogenic-like calli of scape explants from the primary culture were selected and cut into small pieces (~0.5 cm^3) and inoculated on MS shoot regeneration medium containing different concentrations of 6-BA (4.4, 8.9 and 13.3 μM) and NAA (2.7 and 5.4 μM) (for a total of 6 treatments). Each treatment had 9 jars; there were five calli in each jar. The shoot regeneration rate was recorded after 30 days of light cultivation.

Shoots with a height of ~3 cm in height were transplanted into rooting media including full-strength, ½ and ¼ MS, each of which was supplemented with different concentrations of NAA and 2,4-D (see below). For each treatment, there were 6 jars and 5 shoots in each jar. The rooting rate, the average number of roots and the rooting conditions of rooted seedlings were obtained after culture for 30 days.

2.6. Transplanting

Seedlings with well-developed root systems and robust growth were transplanted into plastic trays containing a mixture of peat, perlite and vermiculite as the growth substrate. Four treatments were evaluated for their effect on seedling establishment and growth: the ratios of peat, perlite and vermiculite were 3:1:1, 2:1:1 and 1:1:1, respectively, and the proportions of peat and perlite were 1:1. For each treatment, 50 tissue-cultured seedlings were evaluated. The seedling survival rate was recorded 40 days after transplanting.

2.7. Data Analysis

In different experiments, various parameters were calculated to evaluate the effects of treatments, described as follows:

Callus production rate = (# of calli formed/# explants inoculated) × 100%
Callus mortality rate = (# dead calli/total # of calli) × 100%
Callus browning rate = (# calli with browning/total number of calli) × 100%
Shoot regeneration rate = (# of explants with shoots/# calli inoculated) × 100%
Callus growth rate = Mean weight of calli at harvest − initial weight of the calli
Rooting rate = (# rooted seedlings/total # seedlings inoculated) × 100%
Average number of roots = total number of roots/total number of rooted seedlings

Seedling survival rate = (# seedlings survived/# seedlings transplanted) × 100%

All the experiments were analyzed with a completely randomized design (CRD). Except for the rooting substrate optimization experiment, the data from all the other experiments were analyzed with one-way ANOVA using SPSS Statistic 23.0. All plots and graphs were generated with Graph Pad Prism 9.0.0. The data are presented as the mean ± standard deviation (SD). Duncan's multiple range test was used to assess significant differences between means at $p < 0.05$.

3. Results

3.1. Callus Induction Efficiency from Leaf and Scape Explants

Post-inoculation, leaf explants retained their freshness during prolonged dark culture. Callus initiation, marked by the enlargement and curling of the cut site, occurred around 120 days. Once transferred to light culture, the explants underwent differentiation, with calli becoming visible after 60 days (Figure 1a). Over time, leaf explants without calli progressively browned and lost their differentiation potential, while the calli continued to proliferate, appearing green and loose (Figure 1b).

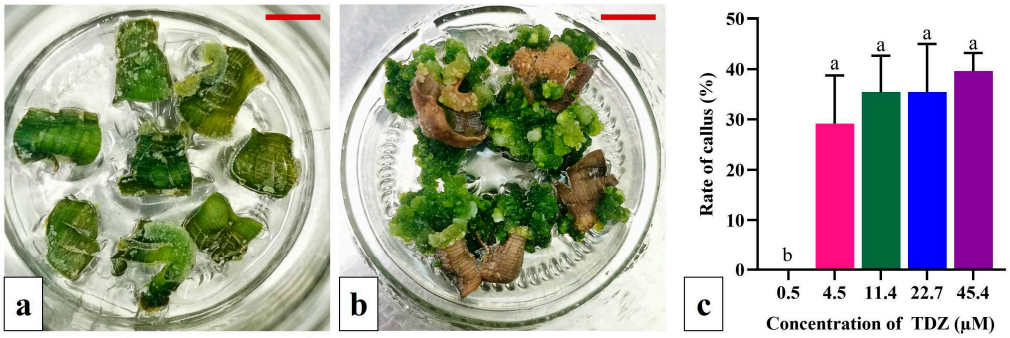

Figure 1. Callus induction from leaf explants. (**a**) Callus formation at 180 days. (**b**) Loose and enlarged callus at 240 days with browning leaf tissues. (**c**) Effects of different TDZ concentrations on the rate of callus formation. Different letters indicate significant differences of means among treatments ($p < 0.05$). Bars = 1.0 cm.

Callus induction rate under different PGR treatments was recorded after 60 days of light culture (Figure 1c). When the concentrations of 6-BA and 2,4-D were fixed, the calli induction rate was positively correlated with a certain range of TDZ concentration. No callus was induced from the explants when the TDZ concentration was only 0.5 μM. When the TDZ concentration was 4.5 μM, the induction rate was increased substantially (28%), but a further increase in TDZ concentration up to 45.4 μM did not seem to increase the rate significantly (Figure 1c). The calli from leaf explants divided continuously but failed to generate shoots, even with prolonged incubation in the same medium.

3.2. Optimization of TDZ Concentration for Callus Induction and Shoot Regeneration from Scape Explant

The scape explants produced abundant white calli under dark culture conditions for up to 30 days on MS medium supplemented with different concentrations of TDZ (Figure 2a). The frequencies of callus induction and shoot regeneration at seven TDZ concentrations are detailed in Table 1. Echoing the leaf explant results, a positive correlation existed between callus induction rate and TDZ concentration up to 22.7 μM. Under light culture, the white calli gradually turned green and differentiated into adventitious buds and shoots (Figure 2b,c). However, increasing TDZ beyond 22.7 μM did not significantly improve shoot generation efficiency (Table 1). Thus, we propose 22.7 μM TDZ as the optimal concentration for callus induction/shoot regeneration when the scapes were used as the explants. On the other hand, after extended incubation, the adventitious buds showed vitrification with

dwarf curled morphology (Figure 2d), suggesting that dedicated shoot regeneration growth medium was needed.

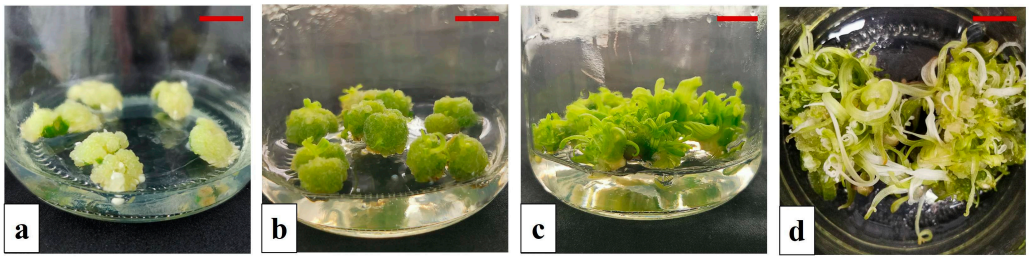

Figure 2. Differentiation of scape explants in medium supplemented with 22.7 μM TDZ. (**a**) White callus formed after 30 days of dark culture condition. (**b**) Callus greening under light culture condition. (**c**) Differentiated adventitious buds. (**d**) Bud vitrification. Bars = 1.0 cm.

Table 1. Effects of TDZ concentrations on callus production and shoot regeneration from scape explants.

Treatment	TDZ/(μM)	Callus Production Rate (%)	Shoot Regeneration Rate (%)
1	0.5	15.56 ± 0.58 d	27.78 ± 0.26 d
2	4.5	42.22 ± 2.52 c	51.85 ± 0.03 c
3	11.4	84.44 ± 1.53 b	73.51 ± 0.05 b
4	22.7	97.78 ± 0.58 ab	97.78 ± 0.04 a
5	45.4	100.00 ± 0.00 a	100.00 ± 0.00 a
6	68.1	97.78 ± 0.58 ab	100.00 ± 0.00 a
7	90.8	100.00 ± 0.00 a	100.00 ± 0.00 a

Different letters indicate significant differences among means at $p < 0.05$.

3.3. Optimization of Phytohormone Concentrations on Callus Induction and Shoot Regeneration from Scape Explants

Sixteen treatments involving various concentrations of 6-BA and 2,4-D concentration (Table 2) were evaluated. Scape explants were cultured under dark conditions for 30 days for callus induction and then transferred to light culture for shoot regeneration. The absence of 6-BA and 2,4-D levels below 4.5 μM resulted in no calli or shoot formation (Table 2). Generally, 6-BA concentrations of 2.2 μM or higher increased callus induction rates. However, as Table 2 illustrates, the interactions between 6-BA and 2,4-D were complex. At a constant 6-BA concentration, the responses to different concentrations of 2,4-D were complex. MS medium supplemented with 11.1 μM 6-BA without 2,4-D had the highest callus production rate (97.78%) and shoot generation rate (85.67%). Under these conditions, the calli appeared healthy and embryogenic; there was also no vitrification during the shoot growth (Figure 3a,b) and normal rootless seedlings were formed directly in the same medium (Figure 3c).

Table 2. Effects of 6-BA and 2,4-D on callus induction and shoot regeneration from scape explants.

Treatments	6-BA/(μM)	2,4-D/(μM)	Callus Production Rate (%)	Shoot Regeneration Rate (%)
1	0	0	0 ± 0.00 f	0 ± 0.00 e
2	0	0.5	0 ± 0.00 f	0 ± 0.00 e
3	0	2.3	0 ± 0.00 f	0 ± 0.00 e
4	0	4.5	0 ± 0.00 f	0 ± 0.00 e
5	2.2	0	37.78 ± 1.53 e	42.06 ± 0.08 d
6	2.2	0.5	44.44 ± 2.08 e	69.26 ± 0.22 abc
7	2.2	2.3	42.22 ± 2.89 e	66.67 ± 0.29 abc
8	2.2	4.5	48.89 ± 1.53 de	63.49 ± 0.05 abcd
9	6.7	0	91.11 ± 1.53 ab	83.57 ± 0.09 a
10	6.7	0.5	68.89 ± 2.31 bcd	76.35 ± 0.09 ab
11	6.7	2.3	57.78 ± 3.06 cde	56.56 ± 0.10 bcd

Table 2. *Cont.*

Treatments	6-BA/(μM)	2,4-D/(μM)	Callus Production Rate (%)	Shoot Regeneration Rate (%)
12	6.7	4.5	57.78 ± 0.58 cde	65.28 ± 0.11 abcd
13	11.1	0	97.78 ± 0.58 a	85.67 ± 0.01 a
14	11.1	0.5	91.11 ± 1.15 ab	85.64 ± 0.13 a
15	11.1	2.3	82.22 ± 1.53 ab	48.88 ± 0.08 cd
16	11.1	4.5	75.56 ± 2.31 abc	54.76 ± 0.13 bcd

Different letters indicate significant differences of means among treatments at $p < 0.05$.

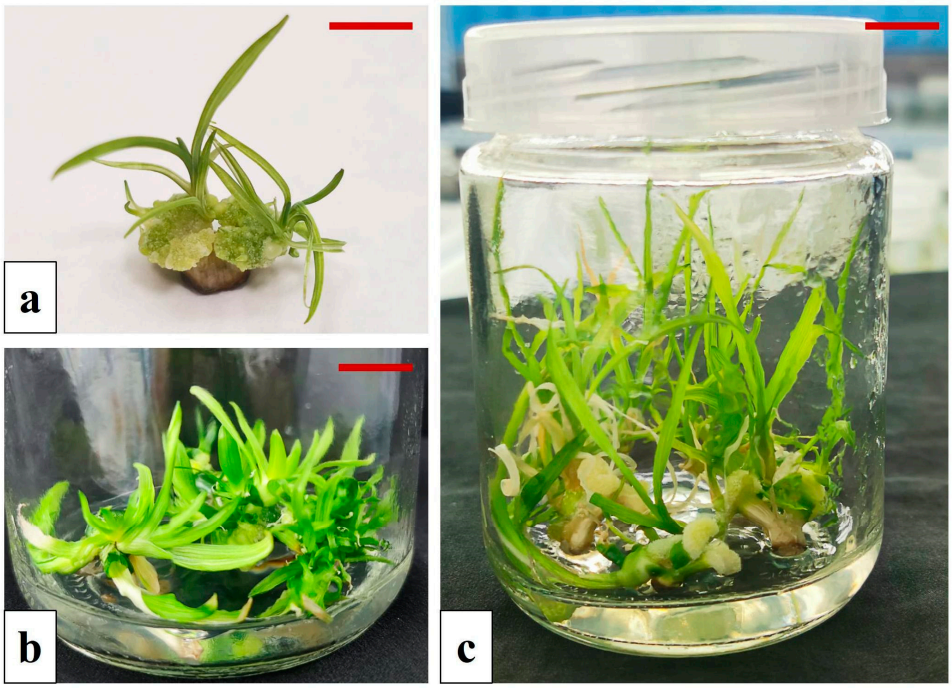

Figure 3. MS medium supplemented with 11.1 μM 6-BA results in high callus induction rate and high shoot regeneration. (**a**) Adventitious buds differentiated from the green calli under light culture conditions. (**b**) Normal growth of adventitious buds. (**c**) Direct seedling establishment under the same medium condition. Bars = 1.0 cm.

3.4. Callus Induction Efficiency of Scape Explants with Different Cutting Methods

We prepared scape explants using three different cutting methods (Figure 4a) and found that different cutting and inoculation methods for scape explants exhibited different rates of callus induction. We found that the scapes inoculated after cutting into segments were most likely to produce calli from the cut at the lower parts of the explants. When the cut surface at the lower part of the scape cutting was inoculated in contact with the medium, the calli became trapped inside the medium, causing poor aeration and thus affecting their differentiation (Figure 4b). A 0.5 cm scape segment produced the most calli, which were associated with vigorous division (Figure 4d) and the lowest mortality. The lowest callus production rate was obtained from scape explants with 0.1 cm slices, which was probably due to the excessive water loss from the slices and death of the explants (Figure 4c). The 1.0 cm long scape segments had a relatively low callus production rate of 55.7%. As the incubation time increased, the explants exhibited severe browning due to the larger wound area (Figure 4b), resulting in greater mortality. This result was not significantly different from the results obtained from inoculating the 0.1 cm cuttings. Therefore, cutting the scape into 0.5 cm sections along the cross-section, followed by a longitudinal cut at the lower end to increase the wound area for callus formation, and inoculating with the cut side up proved most effective.

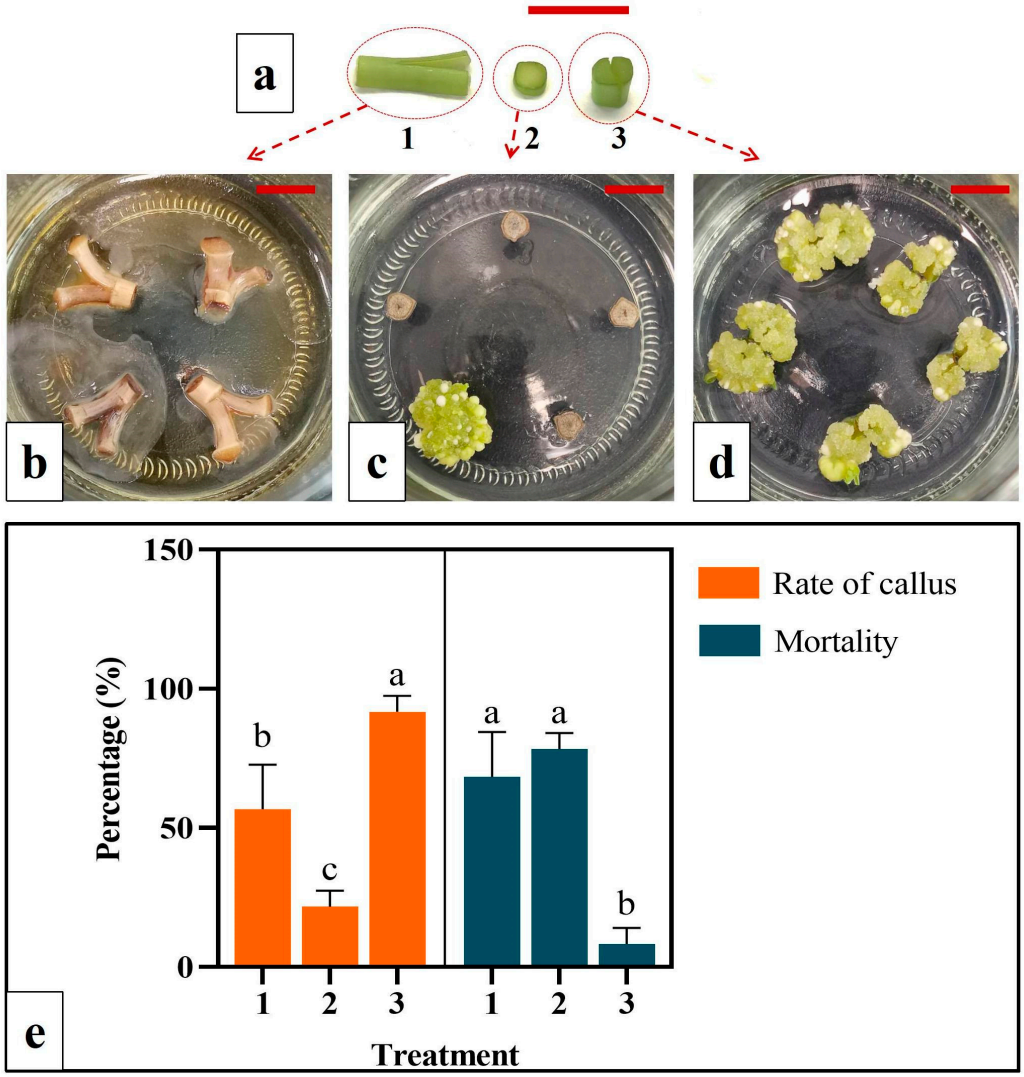

Figure 4. Callus induction with different cutting and inoculation methods of scape explants. (**a**) Three cutting methods (1, 2 and 3). (**b**) Browned explants of cutting type 1. (**c**) Dead and a single surviving explants of cutting type 2. (**d**) Differentiated normal calli from explants of cutting type 3. (**e**) Effects of the different cutting and inoculation methods on the callus production and mortality rate of scape explants. Different letters indicate significant differences ($p < 0.05$). Bars = 1.0 cm.

3.5. Effects of Basic Culture Media on Callus Induction of Scape Explants

The effects of four growth media (MS, ½MS, B5 and N6) on callus formation and shoot generation from scape explants. Representative images and statistics are presented in Figures 5a–d and 6a–c, respectively. These media, varying in salts and vitamins (see Supplemental File 1a), significantly influence callus and adventitious bud induction. Similar callus induction rates were observed on full-strength MS and ½MS media, but MS yielded significantly more shoots than ½MS (Figure 6a,b). The least effective for callus production was the B5 medium (Figure 6a), and calli on B5 and N6 exhibited limited shoot regeneration (Figure 5c,d). Callus browning was more prevalent in ½MS, B5 and N6 than in MS medium (Figure 6c), indicating that full-strength MS is the optimal growth medium for scape explants.

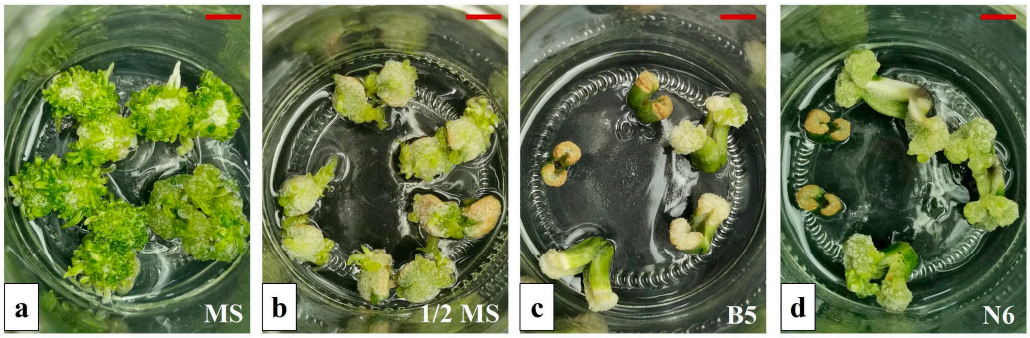

Figure 5. Effects of growth media on callus formation and shoot generation from scape explants. (**a**) Large number of healthy green calli and shoots from scape explants on MS medium. (**b**) White calli with browning edges in ½MS medium containing a few differentiated buds. (**c**) In B5, the calli were mostly white and failed to differentiate into buds. (**d**) There were green calli in the N6 medium but with a low number of differentiated buds. Bars = 1.0 cm.

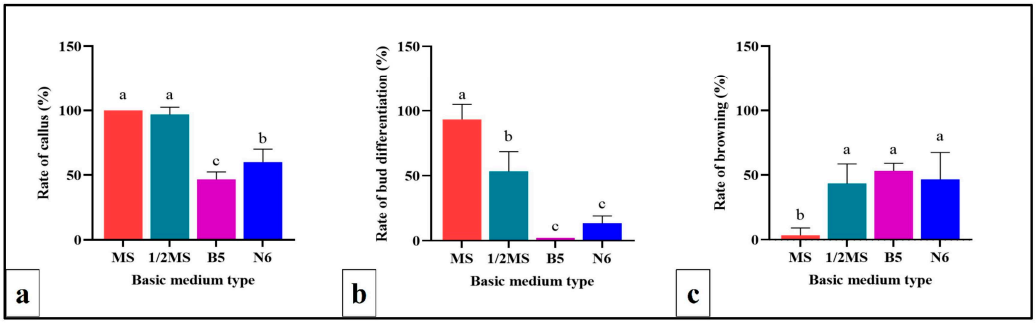

Figure 6. Callus induction rate (**a**), shoot generation efficiency (**b**) and frequency of callus browning (**c**) in culture using scape explants on four different growth media. Different letters indicate significant differences among means from one-way ANOVA at $p < 0.05$.

3.6. Effects of Lights and PGRs on Callus Subculture/Proliferation

After initiation and growth, the calli were transferred to different growth media for subculture and proliferation. The impact of light conditions and PGRs on callus proliferation was investigated. Calli proliferated under light culture were dark green (Figure 7a), whereas those in dark culture appeared lighter in color (Figure 7b). The proliferation calli had a loose texture and did not differentiate into adventitious buds, preventing browning or vitrification.

Table 3. Callus proliferation media with different concentrations of PGRs.

Treatment	NAA/(μM)	2,4-D/(μM)
CK	0	0
1	2.7	4.5
2	2.7	9.0
3	2.7	13.6
4	0	4.5
5	0	9.0
6	0	13.6
7	5.4	2.3
8	10.7	2.3
9	16.1	2.3
10	5.4	0
11	10.7	0
12	16.1	0

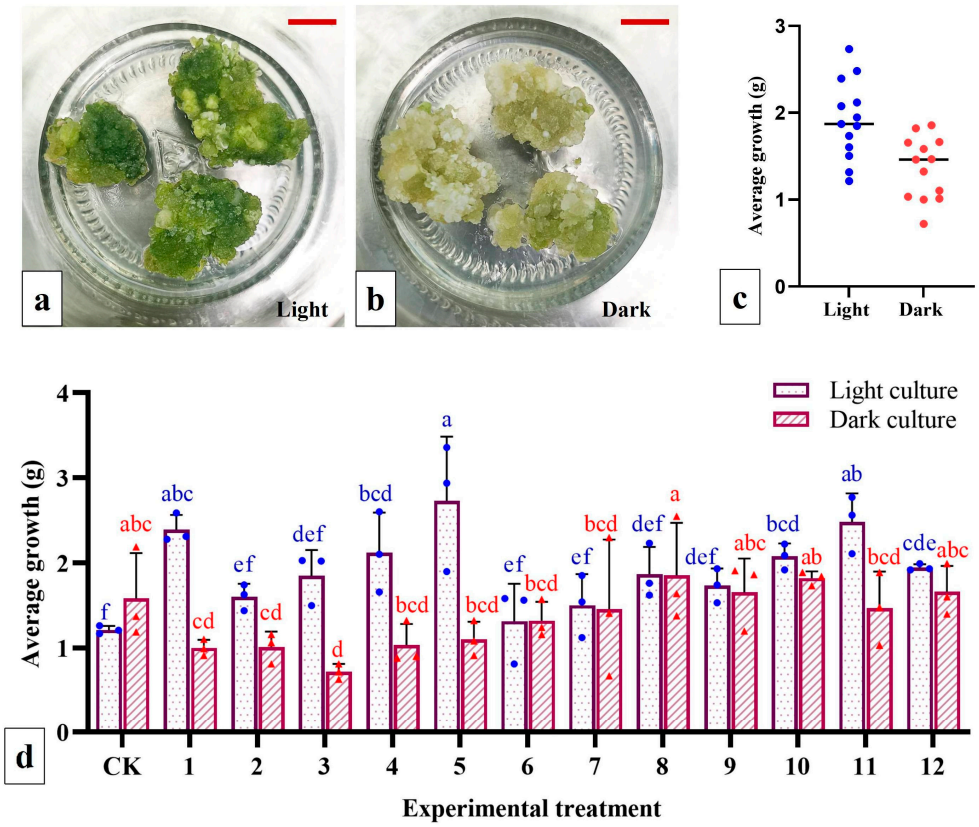

Figure 7. Effects of light and PGRs on callus subculture/proliferation. (**a**,**b**). Appearances of calli from callus proliferation under light (**a**) and dark (**b**) culture. Bars = 1.0 cm. (**c**) Light significantly promotes callus growth during proliferation. Data were collected 30 days after culture. (**d**) Effects of different PGR concentrations on callus proliferation. The X-axis indicates 13 treatments (Table 3). Different letters indicate significant differences among means ($p < 0.05$).

We assessed callus proliferation on MS medium supplemented with various PGR concentrations (NAA and 2,4-D) under two light conditions. Thirteen treatments were included, as detailed in Table 3. Contrary to the CK treatment (media without PGRs), the average growth of calli in PGR-supplemented media under dark culture conditions was less than or equal to that under light culture conditions **(**Figure 7c,d). In light culture, treatment 5 (9.0 µM 2,4-D) had the highest callus growth in 13 treatments (Figure 7d), reaching 2.73 g. In dark culture, treatment 8 (10.7 µM NAA + 2.3 µM 2,4-D) had the highest callus growth but with no significant difference from the control (Treatment 1). Consequently, for optimal callus proliferation under light conditions, MS medium supplemented with 9.0 µM 2,4-D is recommended.

3.7. Optimization of Culture Conditions for Shoot Regeneration

PGRs play critical roles in shoot regeneration from calli. We compared shoot generation frequency using MS medium infused with various concentrations of 6-BA and 2,4-D across the six treatments (Table 4). With a constant NAA level, the shoot regeneration frequency was positively correlated with the 6-BA concentration (Table 4). Generally, higher concentrations of both PGRs promoted shoot regeneration. The most effective shoot generation occurred with combinations of 13.3 µM 6-BA + 2.7 µM NAA, 13.3 µM 6-BA + 5.4 µM NAA and 8.9 µM 6-BA + 5.4 µM NAA, with no significant differences among these treatments (Table 4). Although higher shoot production was observed with 13.3 µM 6-BA (Treatments 3 and 6), this led to overcrowding and brittle shoots, hampering growth. Therefore, for effective shoot regeneration, we recommend MS medium supplemented with 8.9 µM 6-BA and 5.4 µM to optimize both shoot quantity and quality.

Table 4. Effects of PGR concentration on shoot regeneration from night lily calli.

Treatment	6-BA/(μM)	NAA/(μM)	Shoot Regeneration Rate (%)
1	4.4	2.7	22.22 ± 0.58 c
2	8.9	2.7	55.56 ± 1.53 b
3	13.3	2.7	86.67 ± 1.00 a
4	4.4	5.4	8.89 ± 1.53 c
5	8.9	5.4	82.22 ± 0.58 a
6	13.3	5.4	84.44 ± 2.31 a

Different letters indicate significant differences among means ($p < 0.05$).

3.8. Induction of Adventitious Roots

We evaluated the strengths of MS medium and various concentrations of NAA and 2,4-D on rooting efficiency (Table 5). After the shoots were transferred to the rooting media, white roots were gradually induced around 15 days of incubation under light conditions (Figure 8a). As the incubation time increased, the roots elongated and coiled at the bottom of the medium and gradually changed to yellow or light green (Figure 8b).

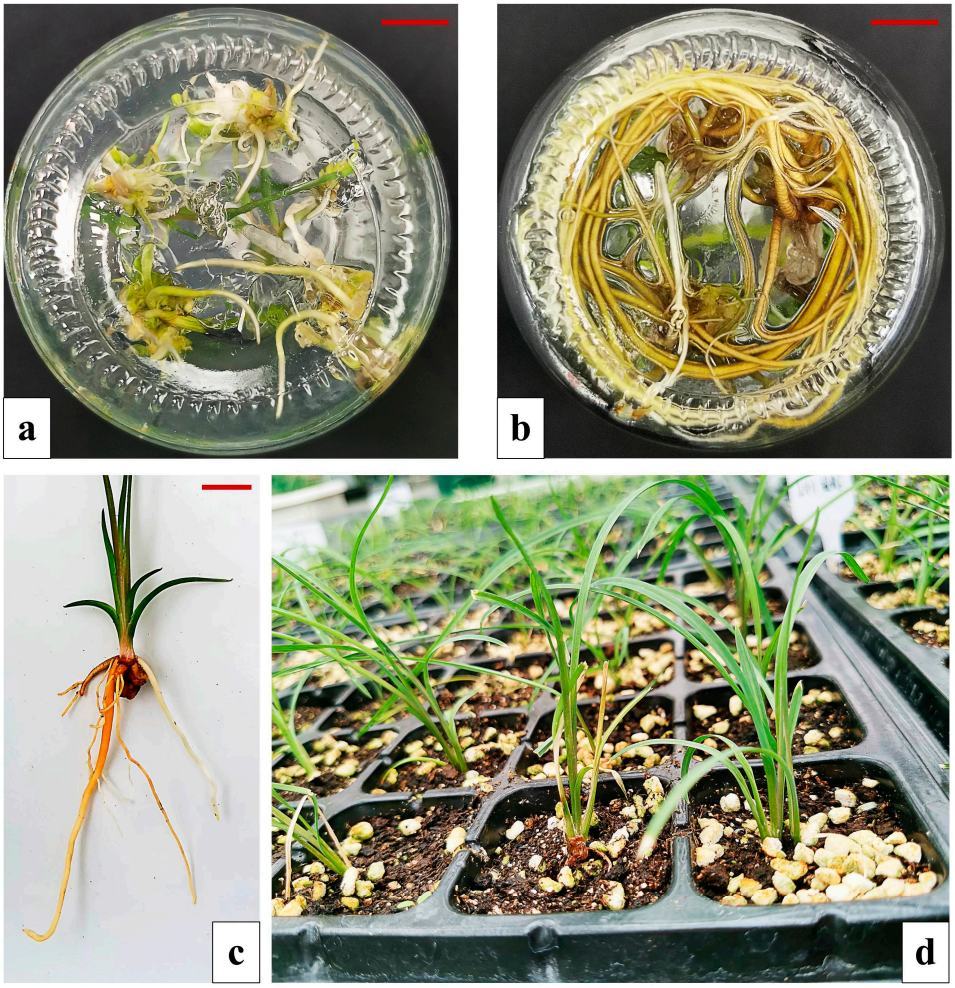

Figure 8. Rooting and seedling transplanting. (**a**) White roots induced in MS medium supplemented with 2.7 μM NAA. (**b**) Seedlings with yellow coiled roots under extended maintenance in the media. (**c**) Seedlings with fibrous roots 40 days after transplanting. (**d**) Seedlings grown in a 2:1:1 substrate mixture of peat, perlite and vermiculite. Bars = 1.0 cm.

Table 5. Effects of MS strength and PGRs on rooting efficiency.

Basic Media	Treatments	NAA/(μM)	2,4-D/(μM)	Rooting Rate (%)	Rooting Number per Seedling
MS	1	2.7	0	90.00 ± 1.00 a	2.19 ± 0.20 ab
	2	0	2.3	73.33 ± 1.53 b	2.36 ± 0.14 ab
	3	1.3	1.1	43.33 ± 0.58 c	1.23 ± 0.25 bc
½MS	4	2.7	0	83.33 ± 1.53 a	2.00 ± 0.48 abc
	5	0	2.3	46.67 ± 2.31 bc	2.71 ± 0.87 a
	6	1.3	1.1	33.33 ± 0.58 cd	1.50 ± 0.41 bc
¼MS	7	2.7	0	30.00 ± 1.00 cd	1.89 ± 0.46 abc
	8	0	2.3	23.33 ± 1.53 cd	2.29 ± 1.15 ab
	9	1.3	1.1	10.00 ± 1.00 d	1.33 ± 0.76 d

Different letters indicate significant differences among means ($p < 0.05$).

The highest rooting rate (90%) was observed in MS or ½MS medium enriched with 2.7 μM NAA (Table 5). The greatest average number of roots per seedling (2.71) was recorded in ½MS medium containing 2.3 μM 2,4-D, although this did not significantly differ from the other treatments (Table 5). Seedlings grown in MS medium generally exhibited robust growth, characterized by green leaves and strong roots (Figure 8c). Conversely, seedlings in ½MS medium developed numerous but weaker roots, necessitating careful post-transplant management for survival. The nutrient-deficient ¼ MS medium resulted in stunted seedling growth and leaf yellowing after extended cultivation. Based on these findings, MS medium supplemented with 2.7 μM NAA is recommended for efficient rooting induction.

3.9. Effects of Substrates on Establishment of Transplanted Seedlings

In micropropagation, transplanting is a critical step for the survival of tissue-cultured seedlings. We observed that the transplanting substrate exerts a certain influence on the growth conditions of tissue-cultured seedlings under the same management practices. Four different substrates were evaluated for use in seedling transplantation (Table 6). The survival rate in substrates consisting of peat, perlite and vermiculite exceeded 90%, compared to approximately 86% in the peat and perlite mixture. The highest survival rate (96%) was recorded for the substrate with a peat:perlite:vermiculite ratio of 2:1:1 (Table 6). This specific mixture facilitated robust growth (Figure 8d) and a well-developed root system (Figure 8c), making it the most effective substrate for transplanting tissue-cultured night lily seedlings.

Table 6. Effects of different substrates on the transplanting survival rate.

Treatments	Substrate Formulae	# Transplanted Seedlings	# Survived Seedlings	Survival Rate (%)
1	peat:perlite:vermiculite = 3:1:1	50	47	94.00
2	peat:perlite:vermiculite = 2:1:1	50	48	96.00
3	peat:perlite:vermiculite = 1:1:1	50	45	90.00
4	peat:perlite = 1:1	50	43	86.00

3.10. Development of an Optimized Micropropagation System for Night Lily

Based on our studies, we have established a rapid micropropagation protocol for night lily using young scapes as explants. The essential steps are outlined in Figure 9, with representative images provided in Supplemental file 2. Briefly, sterilized scape explants are sectioned into 0.5 cm segments and placed on callus induction medium containing 22.7 μM TDZ, with their lower ends facing upwards (Supplemental File 2 Figure S1a–c). The explants are incubated in callus induction medium in the dark for ~30 days until calli are produced (Supplemental File 2 Figure S1d). The calli are transferred to shoot generation medium (MS + 8.9 μM 6-BA + 5.4 μM NAA or 9.0 μM 2,4-D) (Supplemental File 2 Figure S1e). Once

shoots reach ~3 cm in length, they are moved to rooting medium (MS + 2.7 µM NAA). It takes ~15 days for roots to be visible (Supplemental File 2 Figure S1f,g). After washing off the growth medium, rooted seedlings are transplanted into the recommended substrate (Supplemental File 2 Figure S1h,i). The entire cycle from explant preparation to rooted seedling establishment takes approximately 3 months.

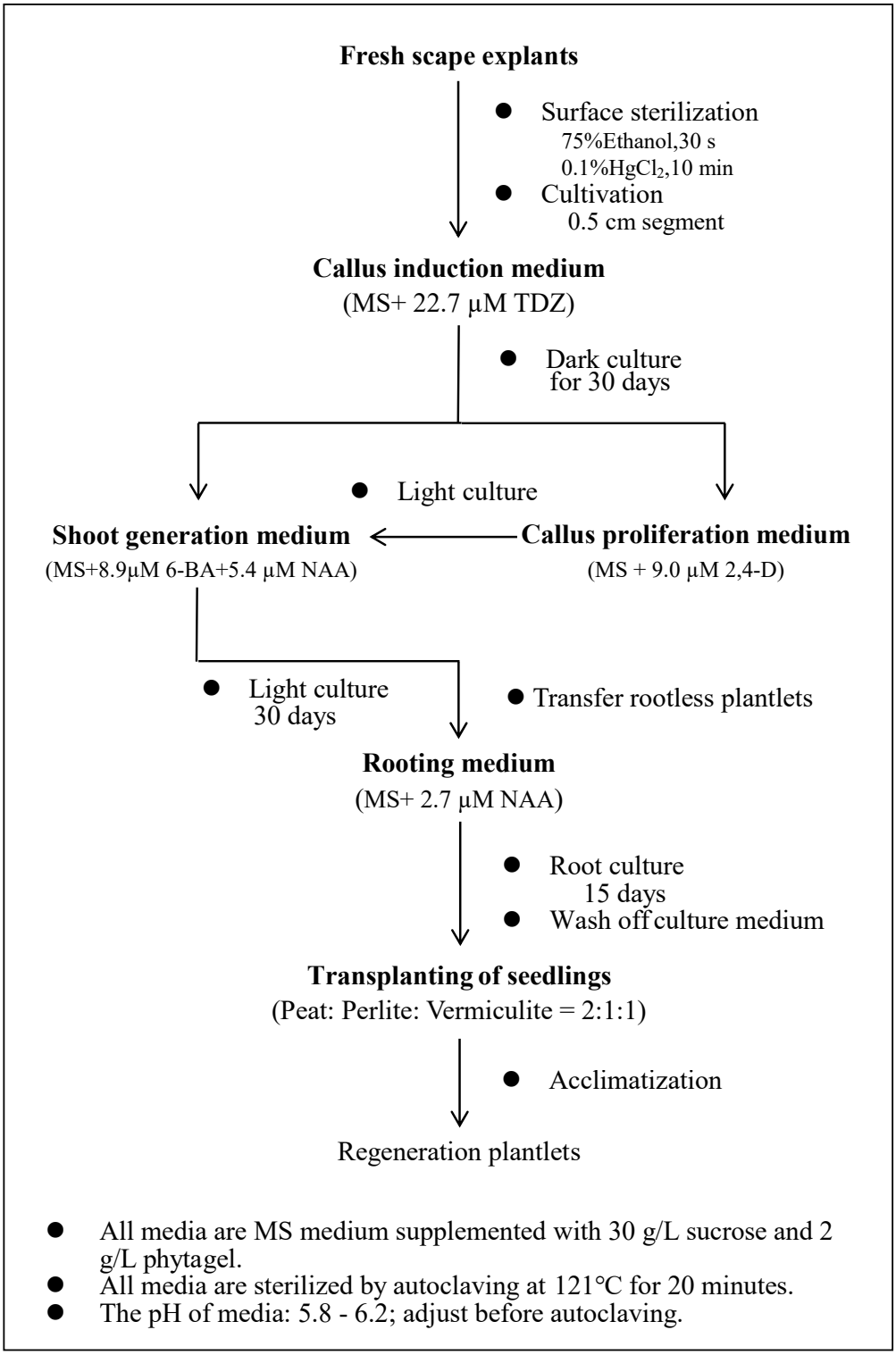

Figure 9. Flowchart of the rapid in vitro micropropagation of night lily plants using young flower stem (scape) as the explant. One cycle typically takes approximately 3 months.

4. Discussion

4.1. Selection of Explants in Micropropagation in Night Lily

Cell totipotency, the ability of various plant tissues and organs to form a complete regenerative plant, is fundamental in plant tissue culture [41]. The selection of explants capable of differentiation is a primary consideration in this process [42]. In preliminary experiments, we attempted to use the rhizome of the night lily 'Datong Huanghua' as an explant. Although shoots were generated within 10 days, thorough sterilization of the rhizome was challenging due to its underground location and association with symbiotic endophytic bacteria. Consequently, rhizomes were excluded from consideration as explants in the present study. Considering that the differentiation ability of juvenile explants is high, this study selected young scapes sampled from the field for in vitro culture and established a complete and reliable system for *H. citrina* mass propagation for the first time. The regenerative abilities of different explants vary significantly [28], and leaf explants are commonly used in daylily tissue culture. However, despite their abundance, leaves may not be ideal for rapid and mass propagation of night lilies due to their low callus induction rate and extended differentiation period [11,43]. Our work reported herein was consistent with this early notion. In daylily, Matand et al. [30] developed an in vitro tissue culture system using the scapes as the explants, and high callus induction efficiency and shoot regeneration efficiency were facilitated with the use of TDZ. We took a similar approach in this study for night lily, but we conducted more comprehensive investigations to optimize various key factors, which should be more informative for *H. citrina* propagation.

4.2. The Role of PGRs in Tissue Culture

PGRs are pivotal in plant morphogenesis within tissue culture [44]. Auxins are mainly used to induce cell division and promote root differentiation. Cytokinins are mainly used to promote cell division and shoot regeneration from calli or organs [45]. Selecting PGR concentrations and the proper ratios of different PGRs is critical for tissue culture research [46]. TDZ is a highly effective cytokinin used in the tissue culture of various plants, while 6-BA, NAA and 2,4-D are often used in combination to induce adventitious buds or roots [47,48]. Although Matand et al. [30] reported that TDZ induced numerous buds from scapes, they did not analyze the subsequent growth of the buds. In our study, we found that TDZ was effective for callus induction from scape explants; however, prolonged culturing time in the same medium resulted in vitrification of the shoots if not transferred to fresh medium in time. A high concentration of TDZ seems to affect shoot growth or negatively affect the differentiation of buds. Chen et al. [49] reported that, in night lily, the addition of either NAA or 2,4-D promoted callus proliferation. Furthermore, the addition of PGRs enhanced night lily shoot differentiation: calli redifferentiated into buds when cultured on media supplemented with 6-BA and NAA. We achieved normal bud formation in MS medium supplemented with 11.1 μM 6-BA and direct rooting in the same medium formulation, resulting in a 'one-step seedling formation' process, a novel finding not reported in previous studies.

4.3. Other Factors Affecting the Success of Night Lily Tissue Culture

Pretreatment of explants before inoculation is important for successful tissue culture since it prevents contamination, which is the primary limiting factor [50]. The surfaces of plant explants taken from the field often contain numerous bacteria; therefore, sterilization with a suitable disinfectant before inoculation is an indispensable step to ensure the sterility of the explant surface. If the sterilization time is too short, the sterilization will be ineffective; thus, the explants can be sterilized twice if necessary [51]. However, prolonged sterilization might negatively impact the explants, such as cell rupture or browning, thus inhibiting their normal growth [52,53]. Common disinfectants used in *H. citrina* tissue culture include alcohol, sodium hypochlorite, mercury, hydrogen peroxide and formalin [54]. In our experiment, a combination of 75% alcohol and 0.1% mercury solution effectively sterilized both the leaf and scape explants.

Different basic media contain varying amounts of macronutrients and micronutrients [55]. It is known that ammonium nitrogen and potassium ions in the medium are conducive to embryo formation, whereas an increased inorganic phosphorus content promotes organogenesis and the absence of reduced nitrogen is beneficial for root formation [56]. Almeida and Shepherd [57] compared the ammonium and nitrate ratios in MS and B5 media, finding that the B5 medium had significantly reduced ammonium ion levels and lower total nitrogen. Explants cultured on B5 medium exhibited superior bud and particularly root growth compared to those on MS medium. However, our study showed that MS medium plays a greater role in the callus induction and budding stages of night lily callus from scape explants. The callus did not differentiate in the B5 and N6 media. We used MS, ½MS and ¼MS media for rooting. Previous studies suggest that different strengths of MS medium (¼, ½ or full strength) do not significantly affect rooting [58]. Yet, we found that ½MS induced more roots during the rooting process of the night lily tissue-cultured seedlings, although the overall growth was not as robust as in full-strength MS medium. Organic additives, such as coconut water, tomato juice, mashed potatoes and mashed bananas, also significantly influence plant cultures ex vivo [59,60]. Their effects on *H. citrina* tissue culture remain unreported, presenting an opportunity for future research.

5. Conclusions

The following major conclusions were reached from this study. (1) The young scape seems to be an ideal explant. (2) The MS medium is suitable for night lily micropropagation. The optimal concentrations of supplemental PGRs at different stages of tissue culture were as follows: callus induction: 22.7 μM TDZ; callus subculture/proliferation: 9.0 μM 2,4-D); shoot generation: 8.9 μM 6-BA + 5.4 μM NAA; and rooting: 2.7 μM NAA. Substrate for transplanting: peat:perlite:vermiculite = 2:1:1. Using our established rapid propagation system, the entire cycle from explant preparation to rooted seedling generation was approximately 3 months (callus induction: ~30 days; shoot generation: ~30 days; rooting: ~15 days). With the protocol developed herein, we were able to produce to obtain more than 2000 plantlets in less than a year. With abundant resources and labor, we believe that higher production efficiency and larger scales can be achieved. Hence, the application of such tissue culture systems in the mass propagation of *Hemerocallis citrina* Baroni is significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14020244/s1, File 1a: Culture media for night lily micropropagation; File 1b: Chemicals and stock solution preparation for night lily micropropagation; File 2: Figure S1: Key steps for rapid in vitro micropropagation of night lily through tissue culture.

Author Contributions: G.Z., F.H. and S.L. conceived and designed the research. G.Z., X.C. and J.Y. performed the experiments. K.L., Y.G., X.N. and L.Y. analyzed the data. G.Z. and X.Z. wrote the manuscript. S.Z., Z.W., Y.W., G.X. and F.H. advised on the project and participated in the revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Key Research and Development Program Project in Shanxi Province (202102140601009), the National Key Research and Development Program Project (2021YFD1600301), the 2022 National College Students Innovation and Entrepreneurship Training Program (202210113149) and the Science and Technology Innovation Project of Colleges and Universities in Shanxi Province (2019L0375).

Data Availability Statement: Data are contained within the article.

Acknowledgments: We are grateful to researcher Jin Xu (Shanxi Agricultural University) for providing serious help in this paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

MS	Murashige and Skoog growth medium
B5	Gamborg B-5 growth medium
N6	Chu N6 growth medium
PGR	plant growth regulator
TDZ	thidiazuron
6-BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
NAA	α-naphthaleneacetic acid

References

1. Li, S.; Liu, J.; Gong, F.F.; Wang, N.; Du, W.; Zhang, L.L.; Kang, X.P.; Xing, G.M. Studies on flowering characteristics and breeding system of *Hemerocallis citrina* 'Datong Huanghua'. *Hortic. Plant J.* **2021**, *48*, 589–601.
2. Li, S.; Ji, F.F.; Hou, F.F.; Shi, Q.Q.; Xing, G.M.; Chen, H.; Weng, Y.Q.; Kang, X.P. Morphological, palynological and molecular assessment of *Hemerocallis* core collection. *Sci. Hortic.* **2021**, *285*, 110181. [CrossRef]
3. Xie, Y.; Chen, T.; Ren, H. Rhizome weight and number of sectioning per rhizome determine plantlet growth and propagation rate of *Hemerocallis citrina* Baroni in cutting propagation. *Agronomy* **2022**, *12*, 2777. [CrossRef]
4. Li, S.; Ji, F.F.; Hou, F.F.; Cui, H.L.; Shi, Q.Q.; Xing, G.M.; Weng, Y.Q.; Kang, X.P. Characterization of *Hemerocallis citrina* transcriptome and development of EST-SSR markers for evaluation of genetic diversity and population structure of *Hemerocallis* collection. *Front. Plant Sci.* **2020**, *11*, 686. [CrossRef]
5. Tomkins, J.P.; Wood, T.C.; Barnes, L.S.; Westman, A.; Wing, R.A. Evaluation of genetic variation in the daylily (*Hemerocallis* spp.) using AFLP markers. *Theor. Appl. Genet.* **2001**, *102*, 489–496.
6. Ou, X.B.; Liu, G.; Wu, L.H. The complete chloroplast genome of *Hemerocallis citrina* (Asphodelaceae), an ornamental and medicinal plant. *Mitochondrial DNA Part B* **2020**, *5*, 1109–1110. [CrossRef]
7. Qing, Z.X.; Liu, J.H.; Yi, X.X.; Liu, X.B.; Hu, G.A.; Lao, J.; He, W.; Yang, Z.H.; Zou, X.Y.; Sun, M.S.; et al. The chromosome-level *Hemerocallis citrina* borani genome provides new insights into the rutin biosynthesis and the lack of colchicine. *Hortic. Res.* **2021**, *8*, 89–98. [CrossRef]
8. Zhang, K.; Wang, Y.H.; Zhang, X.; Han, Z.P.; Shan, X.F. Deciphering the mitochondrial genome of *Hemerocallis citrina* (Asphodelaceae) using a combined assembly and comparative genomic strategy. *Front. Plant Sci.* **2022**, *13*, 1051221. [CrossRef]
9. Dunwell, W.C. *Hcznemcallis* (clavlily) propagation. *Perenn. Plants* **1998**, *6*, 9–13.
10. Gulia, S.K.; Singh, B.P.; Carter, J.; Giiesbach, R.J. Daylily: Botany, Propagation, Breeding. *Hortic. Rev.* **2009**, *35*, 193–220.
11. Li, Z.; Mize, K.; Campbell, F. Regeneration of daylily (*Hemerocallis*) from young leaf segments. *Plant Cell Tissue Organ Cult.* **2010**, *102*, 199–204. [CrossRef]
12. Dunwell, W.C. Ilemerocallis (davlily) propagation. *Proc. Int. Plant Prop. Soc.* **1996**, *46*, 590–594.
13. Feher, A. Callus, dedifferentiation, totipotency, somatic embryogenesis: What these terms mean in the era of molecular plant biology? *Front. Plant Sci.* **2019**, *10*, 536. [CrossRef] [PubMed]
14. Birnbaum, K.D.; Alvarado, A.S. Slicing across kingdoms: Regeneration in plants and animals. *Cell* **2008**, *132*, 697–710. [CrossRef] [PubMed]
15. Dinneny, J.R.; Benfey, P.N. Plant stem cell niches: Standing the test of time. *Cell* **2008**, *132*, 553–557. [CrossRef] [PubMed]
16. Ikeuchi, M.; Ogawa, Y.; Iwase, A.; Sugimoto, K. Plant regeneration: Cellular origins and molecular mechanisms. *Development* **2016**, *143*, 1442–1451. [CrossRef] [PubMed]
17. Oseni, O.M.; Pande, V.; Nailwal, T.K. A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. *Int. J. Curr. Microbio. App. Sci.* **2018**, *7*, 3778–3786. [CrossRef]
18. Arnholdt-Schmitt, B.; Lörz, H. Plant tissue culture—100 years since Gottlieb Haberlandt. *Plant Sci.* **2003**, *166*, 257. [CrossRef]
19. Vir, R.; Kumar, V. Role of plant tissue culture in improvement of horticultural crops. *Ann. Hortic.* **2021**, *14*, 1–6. [CrossRef]
20. Bidabadi, S.S.; Jain, S.M. Cellular, Molecular, and physiological aspects of in vitro plant regeneration. *Plants* **2020**, *9*, 702. [CrossRef]
21. Mehbub, H.; Akter, A.; Akter, M.A.; Mandal, M.S.H.; Hoque, M.A.; Tuleja, M.; Mehraj, H. Tissue Culture in Ornamentals: Cultivation Factors, Propagation Techniques, and Its Application. *Plants.* **2022**, *11*, 3208. [CrossRef]
22. Krikorian, A.D.; Kann, R.P. Micropropagatiun of daylilies through aseptic culture techniques: Its basis, status, problems and prospectus. *Hemerocallis J* **1979**, *33*, 44–61.
23. Ling, J.T.; Sauve, R.J. Isolation and culture of daylilv mesuphvll protoplasts. *Plant Cell Rep.* **1995**, *15*, 293–296. [CrossRef] [PubMed]
24. Chen, J.X.; Hall, D.E.; Luca, V.D. Effects of the growth retardant Paclobutrazol on large-scale micropropagation of daylily (*Hemerocallis* spp.). *Vitr. Cell Dev Biol.* **2005**, *41*, 58–62. [CrossRef]
25. Adelberg, J.; Delgado, M.; Tomkins, J. In vitro sugar and water use in diploid and tetraploid genotypes of daylily (*Hemerocallis* spp.) in liquid medium as affected by density and plant growth regulators. *HortScience* **2007**, *42*, 325–328. [CrossRef]
26. Matand, K.; McGowan, Z.; Li, C. Shoot organogenesis and pluripotency profile in daylily whole flower bud. *J. Biotech Res.* **2021**, *12*, 42–51.

27. Heuser, C.W.; Apps, D.A. In vitro plantlet formation from flower petal explants of *Hemerocallis* cv. Chipper Cherry. *Can. J. Bot.* **1976**, *54*, 616–618. [CrossRef]
28. Chen, L.; Gosukonda, R.; Carter, J. Evaluation of different types of daylily explants for somatic embryogenesis. *HortScience* **2004**, *39*, 847D–847. [CrossRef]
29. Matand, K.; Wu, N.; Conley, S.; Acquaah, G. A more improved protocol for in vitro shoot organogenesis in daylily (*Hemerocallis* sp.). *Afr. J. Biotechnol.* **2013**, *12*, 820–825.
30. Matand, K.; Shoemake, M.; Li, C. High frequency in vitro regeneration of adventitious shoots in daylilies (*Hemerocallis* sp) stem tissue using thidiazuron. *BMC Plant Biol.* **2020**, *20*, 31. [CrossRef]
31. Krikorian, A.D.; Kann, R.P. Plantlet production from morphogenetically competent cell suspensions of daylily. *Ann. Bot.* **1981**, *47*, 679–686. [CrossRef]
32. Smith, D.L.; Krikorian, A.D. Growth and maintenance of an embryogenic cell culture of daylily (*Hemerocallis*) on hormone-free medium. *Ann. Bot.* **1991**, *67*, 443–449. [CrossRef] [PubMed]
33. Fitter, M.S.; Krikorian, A.D. Recovery of totipotent cells and plantlet production from daylily protoplasts. *Ann. Bot.* **1981**, *48*, 591–597. [CrossRef]
34. Krikorian, A.D. Regeneration of Plants from Protoplasts of *Hemerocallis* (Daylily). In *Part of the Biotechnology in Agriculture and Forestry*; Book Series: Plant Protoplasts and Genetic Engineering VI; Springer: Berlin/Heidelberg, Germany, 1995; Volume 34, pp. 70–78.
35. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **1962**, *15*, 473–497. [CrossRef]
36. Saxena, P. Multiplication of Apple 'Gala' (*Malus domestica* L.) by Thidiazuron (Germination, In Vitro Root-Based Regeneration, and Micrografting). Ph.D. Dissertation, University of Guelph, Guelph, ON, Canada, 2018.
37. Govindaraj, S. Thidiazuron: A potent Phytohormone for In vitro regeneration. In *Thidiazuron: From Urea Derivative to Plant Growth Regulator*; Ahmad, N., Faisal, M., Eds.; Springer: Singapore, 2018; pp. 393–418.
38. Chu, H. Construction of Rapid Propagation System and Rescue of Hybrid Embryo on *Hemerocallis* spp. Master's Dissertation, Shanxi Agricultural University, Jinzhong, China, 2017.
39. Gamborg, O.L.; Miller, R.A.; Ojima, K. Nutrient requirements of suspension culture of soybean root cells. *Exp. Cell Res.* **1968**, *50*, 15–158. [CrossRef] [PubMed]
40. Chu, C.; Wang, C.C.; Sun, C.; Hsu, C.; Yin, K.; Chu, C.Y.; Bi, F. Establishment of an efficient medium for anther culture of rice through comparative experiments of the nitrogen sources. *Sci. Sin.* **1975**, *18*, 659–668.
41. Su, Y.H.; Tang, L.P.; Zhao, X.Y.; Zhang, X.S. Plant cell totipotency: Insights into cellular reprogramming. *J. Integr. Plant Biol.* **2021**, *63*, 228–243. [CrossRef] [PubMed]
42. Javed, S.B.; Alatar, A.A.; Anis, M.; El-Sheikh, M.A. In vitro regeneration of coral tree from three different explants using thidiazuron. *HortTechnology* **2019**, *29*, 946–951. [CrossRef]
43. Churikova, O.A. Microclonal propagation and some regularities of morphogenesis of daylily and hosta in vitro. *Mosc. Univ. Biol. Sci. Bull.* **2008**, *63*, 89–94. [CrossRef]
44. Zahid, G.; Iftikhar, S.; Shimira, F.; Ahmad, H.M.; Kaçar, Y.A. An overview and recent progress of plant growth regulators (PGRs) in the mitigation of abiotic stresses in fruits: A review. *Sci. Hortic.* **2022**, *309*, 111621. [CrossRef]
45. Erland, L.A.; Shukla, M.R.; Glover, W.; Saxena, P.K. A simple and efficient method for analysis of plant growth regulators: A new tool in the chest to combat recalcitrance in plant tissue culture. *Plant Cell Tissue Organ Cult.* **2017**, *131*, 459–470. [CrossRef]
46. Zareian, B.A.Z.; Kamali, A.A.K.; Tabandeh, S.A. Comparison of different culture media and hormonal concentrations for *in-vitro* propagation of pomegranate. *Int. J. Fruit Sci.* **2020**, *20*, S1721–S1728. [CrossRef]
47. Gaspar, T.; Kevers, C.; Penel, C.; Greppin, H.; Reid, D.M.; Thorpe, T.A. Plant hormones and plant growth regulators in plant tissue culture. *Vitr. Cell. Dev. Biol. Plant* **1996**, *32*, 272–289. [CrossRef]
48. Baday, S.J. Plant growth regulators importance and effects: A review. *Asian J. Microbiol. Biotechnol.* **2019**, *4*, 74–83.
49. Chen, X. Optimum callus production and complete plant regeneration. *Agrotechnology.* **2021**, *10*, 1.
50. Herman, E.B. Plant tissue culture contamination: Challenges and opportunities. *Acta Hortic.* **2017**, *1155*, 231–237. [CrossRef]
51. Cassells, A.C. Pathogen and biological contamination management in plant tissue culture: Phytopathogens, vitro pathogens, and vitro pests. *Plant Cell Cult Prot.* **2012**, *877*, 57–80.
52. Orlikowska, T.; Nowak, K.; Reed, B. Bacteria in the plant tissue culture environment. *Plant Cell Tissue Organ Cult.* **2017**, *128*, 487–508. [CrossRef]
53. Jones, A.M.P.; Saxena, P.K. Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: A novel approach to reduce oxidative browning in plant tissue culture. *PLoS ONE* **2013**, *8*, e76802. [CrossRef]
54. Singh, C.R. Review on problems and its remedy in plant tissue culture. *Asian J. Biol. Sci.* **2018**, *11*, 165–172. [CrossRef]
55. Long, Y.; Yang, Y.; Pan, G.; Shen, Y. New insights into tissue culture plant-regeneration mechanisms. *Front Plant Sci.* **2022**, *13*, 2112. [CrossRef]
56. Dezhsetan, S.; Farzaneh, G.R.; Jahanbakhsh, S. Evaluation the effects of media and plant growth regulators on *Stevia rebaudiana* micropropagation. *J. Int. Sci. Publ. Agric. Food* **2017**, *5*, 405–413.
57. Almeida, V.P.D.; Shepherd, S.L.K. *Sinningia allagophylla* (Gesneriaceae): In vitro cultivation of a native plant of the Brazilian cerrado. *Braz. J. Bot.* **1999**, *22*, 381–384. [CrossRef]

58. Rani, G.; Arora, S.; Nagpal, A. Direct rhizogenesis from in vitro leaves of *Withania somnifera* (L.) Dunal. *J. Herbs Spices Med. Plants* **2003**, *10*, 47–54. [CrossRef]
59. Dewi, P.Y.A.; Astarini, I.A.; Kriswiyanti, E. PENYELAMATAN EMBRIO *Dendrobium anosmum* Lindl. MELALUI KULTUR IN VITRO. *METAMORFOSA J. Biol. Sci.* **2016**, *3*, 129–139.
60. Seeni, S.; Latha, P.G. Foliar regeneration of the endangered red vanda, *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell Tissue Organ Cult.* **1992**, *29*, 167–172. [CrossRef]