# Optimizing culture medium for commercial production of tropical day-blooming waterlily (*Nymphaea colorata* Peter)

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## ABSTRACT

**Background and Objective:** Tropical day-blooming waterlily, the queen of aquatic ornamental plants, has steadily increased commercial demand. However, the traditional propagation methods result in a low number of plants and lack consistency, which is inefficient in meeting the demands of the global export industry. Therefore, this study aims to develop a protocol for rapid mass propagation of tropical waterlily using tissue culture techniques.

**Methodology:** Individual aseptic shoots of *Nymphaea colorata* Peter were cultured on different culture media, including Murashige and Skoog (MS) and Oil Palm Culture Medium (OPCM), with various types and concentrations of plant growth regulators. The experiment was designed as a completely randomized design (CRD) with 20 replications, each replication consisting of 5 shoot tips. After 4 weeks of culture, shoot multiplication rate and number of shoots per explant were recorded. Shoots were transferred to a rooting medium, transplanted in aquatic plant substrates, and transferred directly to a freshwater tank with a 20 cm water depth under greenhouse conditions. The survival rate was recorded.

**Main Results:** OPCM medium supplemented with 3.0 mg/L BAP and 0.5 mg/L NAA resulted in significantly ($P < 0.05$) highest growth and shoot proliferation, producing 19.2 shoots per explant. For mass shoot proliferation, culturing shoot explants on OPCM medium supplemented with 0.5 mg/L TDZ in combination with 1.0 mg/L BAP, and 0.5 mg/L NAA showed a significant ($P < 0.05$) highest response rate of 86.66% and produced the highest number of shoots at 31.15 shoots/explant. Plantlets exhibited robust growth and achieved a high survival rate of 90%, achieving 100%, as evidenced by the production of new floating leaves and shoots under greenhouse conditions.

**Conclusions:** OPCM medium supplemented with 0.5 mg/L TDZ in combination with 1.0 mg/L BAP, and 0.5 mg/L NAA was suitable for mass propagation of tropical day-blooming waterlily.

**Keywords:** Tissue culture, ornamental waterlily, tropical waterlily, shoot growth, mass shoot production

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## INTRODUCTION

Thailand is a leading producer and exporter of ornamental waterlilies, renowned for its year-round production of outstanding, elegant, and impressive tropical waterlily hybrids. The demand for new, exotic hybrids has surged rapidly, contrasting sharply with the low production rates achieved through conventional methods, leading to missed commercial opportunities. Plant tissue culture has emerged as a high-performance platform for mass clonal propagation of plants, providing a controlled environment for the rapid and efficient production of disease-free clones (Thakur *et al*., 2024). However,

for the past decade, there has been a lack of review on efficient protocols for waterlily micropropagation.

Therefore, this study aims to evaluate the fundamental factors that influence the production of plantlets to achieve the appropriate quality and quantity for commercial purposes. We focused on various cultural media and PGRs that facilitated mass shoot production. *Nymphaea colorata* Peter, an important waterlily species widely used for ornamental purposes and hybridization programs, was used to replicant high-performance ornamental tropical waterlily. A significant challenge in waterlily *in vitro* is not only high rates of microbial contamination during sterile shoot establishment but also low shoot multiplication rates (Jenks *et al*., 1990; Kane *et al*., 1991; Lakshmanan, 1994; Nammai *et al*., 2024).

Previous studies had reported a low shoot number (1.0–4.1 shoots/explant) and poor quality of plantlets under aseptic conditions (Lakshmanan, 1994; Donjanthong *et al*., 2017; Yu *et al*., 2018). Furthermore, shoot establishment is hindered by poor responsiveness to the culture medium, stunted shoot growth, yellowing leaves, and undeveloped roots, resulting in long-term commercial production challenges. The type of culture media plays a crucial role in plant growth and development. Research indicates that the choice of media, such as Murashige and Skoog (MS), significantly impacts plant propagation and quality (Sugiarto *et al*., 2023). Variations in media components, including mineral content, agar concentration, and the addition of plant growth regulators, can influence the success of micropropagation (Bashi *et al*., 2023; Dat *et al*., 2024). In aseptic culture, cytokinins such as 6-Benzylaminopurine (BAP) and Thidiazuron (TDZ) have been demonstrated efficacy to be effective in promoting shoot proliferation in aquatic plants. The studies on *N. pubescens* demonstrated that liquid MS medium with BAP (2.0 mg/L) significantly induces direct shoot organogenesis (4.0 shoots/explant) from tubers (Ubonprasirt *et al*., 2011). Additionally, research on *N.* 'Chalong Kwan' indicates that bulbil explants respond positively to semi-solid MS medium supplemented with 10 µM BAP and overlaid with sterilized water (Noimai, 2012).

## MATERIALS AND METHODS

### Plant Material Preparation

Turions were collected from mature rhizomes of *Nymphaea colorata* Peter between March to June 2021. The sixteen months old of mother plants were grown in floating paddy fields under full sun and fertile loamy clay soil. All plant materials were supplied by a specialized waterlily collector (Baufah Garden 401 Watcharapol Road, Tha Raeng, Bang Khen district, Bangkok, Thailand). The axenic shoot was established from sterile turions cultured in plant growth regulator-free (PGRs-free) liquid Murashige and Skoog (MS) medium for 8 weeks. The vigorous shoot clumps derived from this culture were excised into individual shoots, each comprising 4–5 leaves and measuring 0.5–1.0 cm in length, which served as the initial explants. The experiment was conducted at Crop Biotechnological Laboratory, Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University.

### Different Culture Media on Shoot Growth

To evaluate the efficacy of different culture media, two types of media (MS and Solidified Oil Palm Culture Medium, OPCM) were tested with and without activated charcoal (AC) supplementation. All media were fortified with 3.0 mg/L BAP and 0.5 mg/L NAA. Semi-solid media were dispensed a 10 mL into 5.0 × 6.8 cm glass bottles. Each bottle contained three shoots, with twenty replicates/treatment. Growth efficacy was assessed by measuring the number of shoots and the number of leaves. The leaf width and petiole length were determined by measuring the largest leaf and longest petiole in each shoot clump after 4 weeks of culture.

### PGRs on Shoot Multiplication

For mass shoot proliferation, semi-solid OPCM medium was fortified with various combinations and concentrations of Thidiazuron (TDZ; 0.0–0.5 mg/L), BAP (0.0–3.0 mg/L), NAA (0.5 mg/L) and compared with PGRs-free MS medium. Each bottle contained five individual shoots,

with ten replicates/treatment. The efficacy of PGRs was determined by evaluating the number of shoots, the number of leaves, and visible morphological characteristics after 8 weeks of culture.

### Root Induction and Acclimatization

Excised shoot clumps containing 2–3 shoots, derived from the medium with the highest shoot multiplication efficiency (OPCM supplemented with 0.5 mg/L TDZ, 1.0 mg/L BAP, and 0.5 mg/L NAA), were transferred to a rooting medium (Rodboot *et al*., 2024). Root induction was achieved after two weeks of culture. The rooted shoot clumps were then transplanted into sterilized aquatic plant substrates overlaid with half-strength liquid OPCM medium supplemented with 0.5 mg/L NAA for an additional two weeks. Once a well-developed root system formed, the plantlets were transferred directly to a freshwater tank with a 20 cm water depth under greenhouse conditions. After producing floating leaves, the plantlets were subsequently transplanted into loamy clay soil under field conditions.

### Culture Conditions

All culture media contained 30 g/L sucrose and solidified with 4.5 g/L agar to form a semi-solid medium. The pH of the culture medium was adjusted to 5.8 with 1 N NaOH or HCl, and 10 mL was dispensed into each 5 × 6.8 cm glass bottle before autoclaved at 121°C for 15 minutes. The cultures were incubated under 26 ± 2°C with a 10/14 -h day-night cycle photoperiod under 12 μmol $m^{-2}$ $s^{-1}$ provided by fluorescent lamps (LED BEC SET-SPIRIT T8 18W/6500K daylight).

### Statistical Analysis

All experiments above were arranged in a completely randomized design (CRD). Data from all parameters were expressed as mean values ± standard deviation (SD) and statistically analyzed using analysis of variance (ANOVA). The mean among treatments was separated by Duncan's multiple range test (DMRT) using the Statistical Package for the Social Sciences (SPSS) 17.0 program for Windows (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

### Effect of Different Culture Media on Shoot Growth and Multiplication

The first challenge we encountered in the growth of waterlily shoots under *in vitro* conditions was the explant's poor response to the culture medium. This was evidenced by stunted shoot growth, yellowing leaves, and undeveloped roots, which hindered initial shoot establishment. A semi-solid MS and OPCM basal medium was tested to optimize a culture medium that promotes efficient shoot growth after multiplication. The experiment was conducted on 5 different culture media (Table 1) with all containing 3.0 mg/L BAP and 0.5 mg/L NAA accepted control. After 4 weeks of culture, the result showed the number of shoots varied significantly ($P < 0.05$) across different culture media (Table 1).

The control (PGRs-free MS medium) produced the lowest number of shoots at 1.5 shoots/explant (Figure 1A), while the OPCM medium supplemented with 3.0 mg/L BAP and 0.5 mg/L NAA produced ($P < 0.05$) highest number of shoots significantly at 19.2 shoots/explant (Figure 1D). The derived shoots were robust and showed a new clump of shoot bud formation. This indicates a strong positive effect of OPCM combined with PGRs on shoot multiplication. The addition of 0.1% AC to OPCM (Table 1; Figure 1E) resulted in a lower number of shoots compared to OPCM without AC but still significantly ($P < 0.05$) higher than the control. The number of leaves was significantly ($P < 0.05$) higher in all media supplemented with PGRs compared to the control. The highest number of leaves with normal expansion was observed in the MS medium with PGRs at 41 leaves/clump (Table 1; Figure 1B). The addition of AC to the MS medium reduced the number of leaves and also provided abnormal leaves compared to the same medium without AC. Petiole length was significant ($P < 0.05$), highest in the MS medium with PGRs at 2.01 cm (Figure 1B).

**Table 1** Effects of different culture media on shoot growth and shoot multiplication after culture for 4 weeks

| Culture media | Number of shoots (shoot) | Number of leaves/clump (leaf) | Leaf width (cm) | Petiole length (cm) | Root formation (%) |
|---|---|---|---|---|---|
| PGRs-free MS medium (control) | 1.50 ± 0.67$^{e}$ | 9.70 ± 1.55$^{e}$ | 0.35 ± 0.06$^{d}$ | 1.20 ± 0.20$^{c}$ | 10 |
| MS + 3.0 mg/L BAP + 0.5 mg/L NAA | 11.10 ± 3.72$^{b}$ | 41.00 ± 7.52$^{a}$ | 1.01 ± 0.04$^{a}$ | 2.01 ± 0.46$^{a}$ | 10 |
| MS + 3.0 mg/L BAP + 0.5 mg/L NAA + 0.1% AC | 9.90 ± 1.04$^{c}$ | 39.20 ± 3.18$^{c}$ | 0.65 ± 0.05$^{c}$ | 1.54 ± 0.14$^{b}$ | 90 |
| OPCM + 3.0 mg/L BAP + 0.5 mg/L NAA | 19.20 ± 2.27$^{a}$ | 38.70 ± 4.36$^{d}$ | 0.81 ± 0.12$^{b}$ | 1.95 ± 0.37$^{ab}$ | 30 |
| OPCM + 3.0 mg/L BAP + 0.5 mg/L NAA + 0.1% AC | 8.40 ± 1.85$^{d}$ | 40.20 ± 3.78$^{b}$ | 0.62 ± 0.12$^{c}$ | 1.51 ± 0.20$^{b}$ | 100 |
| F-test | ** | ** | ** | ** | |
| CV (%) | 23.06 | 34.13 | 23.88 | 29.45 | |

**Note:** Data are presented as mean ± standard deviation. Different superscript letters ($^{a, b}$) within the same column indicate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test (DMRT).

The addition of AC to both MS and OPCM media generally resulted in intermediate leaf and petiole lengths. Root formation varied widely among the different culture media and the addition of 0.1% AC to MS medium significantly increased rooting formation to 90%. The OPCM medium with 3.0 mg/L BAP and 0.5 mg/L NAA had a moderate rooting formation rate of 30%, which further increased to 100% with the addition of 0.1% AC. The MS basal medium is widely used in plant tissue culture and significantly affects plant growth and development (Phillips and Garda, 2019).

The nutrient-rich MS medium with a high concentration of macronutrients and a specific concentration of BAP and NAA is efficient for the rapid multiplication of shoots (Mohamed *et al*., 2019; Dogan, 2022). Compared to MS medium, Woody Plant Medium (WPM) has a lower concentration of salts, which can reduce the risk of osmotic stress and toxicity, particularly for sensitive plants (Cooper and Dumbroff, 1973; Santiago *et al*., 2019). The lower levels of macronutrients but higher levels of certain micronutrients and vitamins may support the unique metabolic needs of plants. OPCM medium contained half of MS with half of WPM nutrients (Kerdsuwan and Te-chato, 2016), which would provide a balanced nutrient environment that is potentially beneficial for a waterlily species. This combination might offer the advantages of both media are sufficient nutrients for waterlily shoot growth and development.

OPCM medium with 3.0 mg/L BAP and 0.5 mg/L NAA promotes robust shoot multiplication obviously in single shoot culture of *N. colorata*. The AC can benefit culture media by adsorbing inhibitory substances, reducing browning, and promoting root formation (Van Winkle and Pullman, 2003). However, its non-selective adsorption can lead to removing essential nutrients and growth regulators, complicating medium preparation. Based on the findings, the OPCM medium without AC supplementation provided a high quantity of shoot. While OPCM medium with AC supplementation provided low response of shoot and obviously distorted the leaf characteristics but is beneficial for root formation and high performance on quality of root subsequent success in plantlet acclimatization. Therefore, we suggest that an assessment of the concentration of AC is needed.

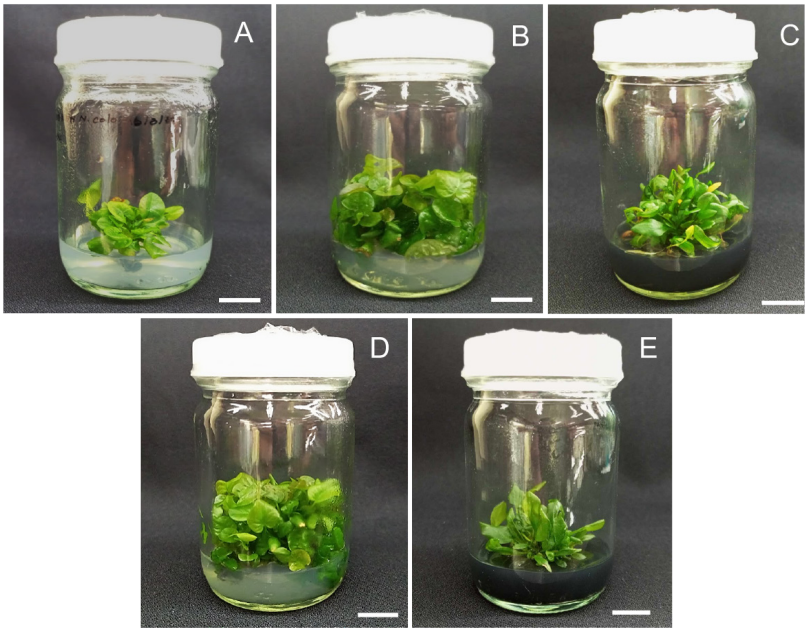


**Figure 1** Characteristics of shoot responses on different culture media using shoot clump after culture for 4 weeks (bar = 1 cm): (A) PGRs-free MS medium (control) showed low shoot multiplication rate and slow shoot growth with normal expanded leaf, (B) MS + 3.0 mg/L BAP + 0.5 mg/L NAA showed medium-high shoot multiplication rate and vigorous shoot and leaf with normal expanded leaf, (C) MS + 3.0 mg/L BAP + 0.5 mg/L NAA + 0.1% AC showed medium-low shoot multiplication rate with abnormal leaf but high rate of root formation, (D) OPCM + 3.0 mg/L BAP + 0.5 mg/L NAA showed high shoot multiplication rate and densely vigorous shoot and leaf with normal expanded leaf, (E) OPCM + 3.0 mg/L BAP + 0.5 mg/L NAA + 0.1% AC showed medium shoot multiplication rate with abnormal leaf but high rate of root formation.

**Effect of PGRs on Shoot Multiplication**

A major problem in the *in vitro* propagation of waterlily is not only the high rate of microbial contamination during sterile shoot establishment but also the low shoot multiplication rate (Jenks *et al*., 1990; Swindells, 1990; Lakshmanan, 1994). Previous research has indicated that the number of shoots under aseptic conditions is relatively low (Noimai, 2012). Consequently, *in vitro* commercial production has been unsuccessful for a decade.

To optimize the efficacy of PGR combinations for mass shoot production, the sterile individual excised shoots were inoculated with various types and concentrations of PGRs (TDZ, BA, and NAA). The response rate showed shoot proliferation varied significantly ($P < 0.01$) across different concentrations of PGRs. The highest response rate at 100% was observed in the medium supplemented with the combination of TDZ, BAP, and NAA (Table 2). In contrast, the lowest response rate at 13.33% was seen in the control (Figure 2A). These results indicated that high concentrations of cytokinins (0.5 mg/L TDZ and 1.5–3.0 mg/L BAP) significantly improve the shoot multiplication rate from single shoot explants of *N. colorata*.

**Table 2** Effect of different concentrations and combinations of TDZ, BAP, and NAA on *in vitro* shoot multiplication after culture for 8 weeks

| PGR concentration (mg/L) | | Shoot multiplication rate (%) | Clump size (cm) | Number of shoots/clump (shoot) | Number of leaves/shoot (leaf) | Leaf width (cm) |
|---|---|---|---|---|---|---|
| TDZ | BAP | | | | | |
| 0 | 0 | 13.33 | 2.78 ± 0.44$^{e}$ | 1.13 ± 0.35$^{t}$ | 5.76 ± 1.59$^{d}$ | 0.98 ± 0.13$^{b}$ |
| 0.05 | | 50.00 | 2.95 ± 0.18$^{d}$ | 5.40 ± 1.40$^{q}$ | 4.53 ± 0.81$^{o}$ | 0.87 ± 0.08$^{c}$ |
| 0.1 | | 60.66 | 2.58 ± 0.26$^{g}$ | 6.42 ± 1.59$^{p}$ | 4.56 ± 0.78$^{o}$ | 0.69 ± 0.04$^{e}$ |
| 0.5 | | 63.66 | 2.49 ± 0.22$^{h}$ | 6.57 ± 1.78$^{o}$ | 4.80 ± 0.99$^{m}$ | 0.62 ± 0.07$^{f}$ |
| | 0.5 | 60.00 | 2.51 ± 0.14$^{h}$ | 1.78 ± 0.76$^{s}$ | 5.36 ± 0.80$^{g}$ | 0.56 ± 0.04$^{fg}$ |
| | 1.0 | 66.66 | 2.69 ± 0.24$^{f}$ | 3.25 ± 0.76$^{r}$ | 5.13 ± 0.73$^{j}$ | 0.51 ± 0.07$^{gh}$ |
| | 1.5 | 100.00 | 3.30 ± 0.17$^{a}$ | 17.50 ± 2.87$^{g}$ | 5.23 ± 0.77$^{i}$ | 0.44 ± 0.07$^{hi}$ |
| | 3.0 | 100.00 | 3.29 ± 0.14$^{a}$ | 21.30 ± 3.50$^{e}$ | 4.86 ± 0.86$^{l}$ | 0.47 ± 0.06$^{hi}$ |
| 0.05 | 0.5 | 53.33 | 2.91 ± 0.49$^{d}$ | 7.44 ± 1.85$^{n}$ | 6.43 ± 1.73$^{c}$ | 0.62 ± 0.07$^{f}$ |
| 0.05 | 1.0 | 76.66 | 2.94 ± 0.38$^{d}$ | 11.21 ± 2.24$^{k}$ | 5.66 ± 1.32$^{e}$ | 0.72 ± 0.06$^{de}$ |
| 0.05 | 1.5 | 86.66 | 2.95 ± 0.31$^{d}$ | 10.65 ± 3.67$^{l}$ | 5.73 ± 1.25$^{d}$ | 0.76 ± 0.06$^{d}$ |
| 0.05 | 3.0 | 83.33 | 3.04 ± 0.21$^{c}$ | 9.04 ± 1.75$^{m}$ | 4.73 ± 1.17$^{n}$ | 1.03 ± 0.11$^{b}$ |
| 0.1 | 0.5 | 66.66 | 2.63 ± 0.53$^{fg}$ | 18.55 ± 2.01$^{f}$ | 5.33 ± 1.06$^{gh}$ | 1.10 ± 0.17$^{a}$ |
| 0.1 | 1.0 | 80.00 | 2.82 ± 0.25$^{e}$ | 15.58 ± 2.15$^{h}$ | 5.30 ± 1.29$^{h}$ | 1.03 ± 0.15$^{b}$ |
| 0.1 | 1.5 | 96.66 | 2.90 ± 0.27$^{d}$ | 14.35 ± 2.17$^{j}$ | 5.50 ± 1.07$^{f}$ | 0.62 ± 0.11$^{f}$ |
| 0.1 | 3.0 | 96.66 | 2.96 ± 0.26$^{d}$ | 14.44 ± 2.17$^{i}$ | 6.40 ± 1.54$^{c}$ | 0.45 ± 0.05$^{i}$ |
| 0.5 | 0.5 | 80.00 | 2.92 ± 0.22$^{d}$ | 30.08 ± 2.74$^{b}$ | 6.83 ± 1.31$^{a}$ | 0.48 ± 0.05$^{hi}$ |
| 0.5 | 1.0 | 86.66 | 3.08 ± 0.22$^{c}$ | 31.15 ± 2.92$^{a}$ | 6.63 ± 1.27$^{b}$ | 0.73 ± 0.07$^{de}$ |
| 0.5 | 1.5 | 96.66 | 3.19 ± 0.25$^{b}$ | 27.86 ± 2.29$^{c}$ | 6.43 ± 1.56$^{c}$ | 1.00 ± 0.18$^{b}$ |
| 0.5 | 3.0 | 100.00 | 3.21 ± 0.18$^{b}$ | 26.43 ± 1.74$^{d}$ | 5.06 ± 1.20$^{k}$ | 1.01 ± 0.10$^{b}$ |
| F-test | | | *** | *** | *** | *** |
| CV (%) | | | 10.04 | 15.02 | 21.71 | 13.61 |

**Note:** Data are presented as mean ± standard deviation. Different superscript letters ($^{a, b}$) within the same column indicate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test (DMRT).

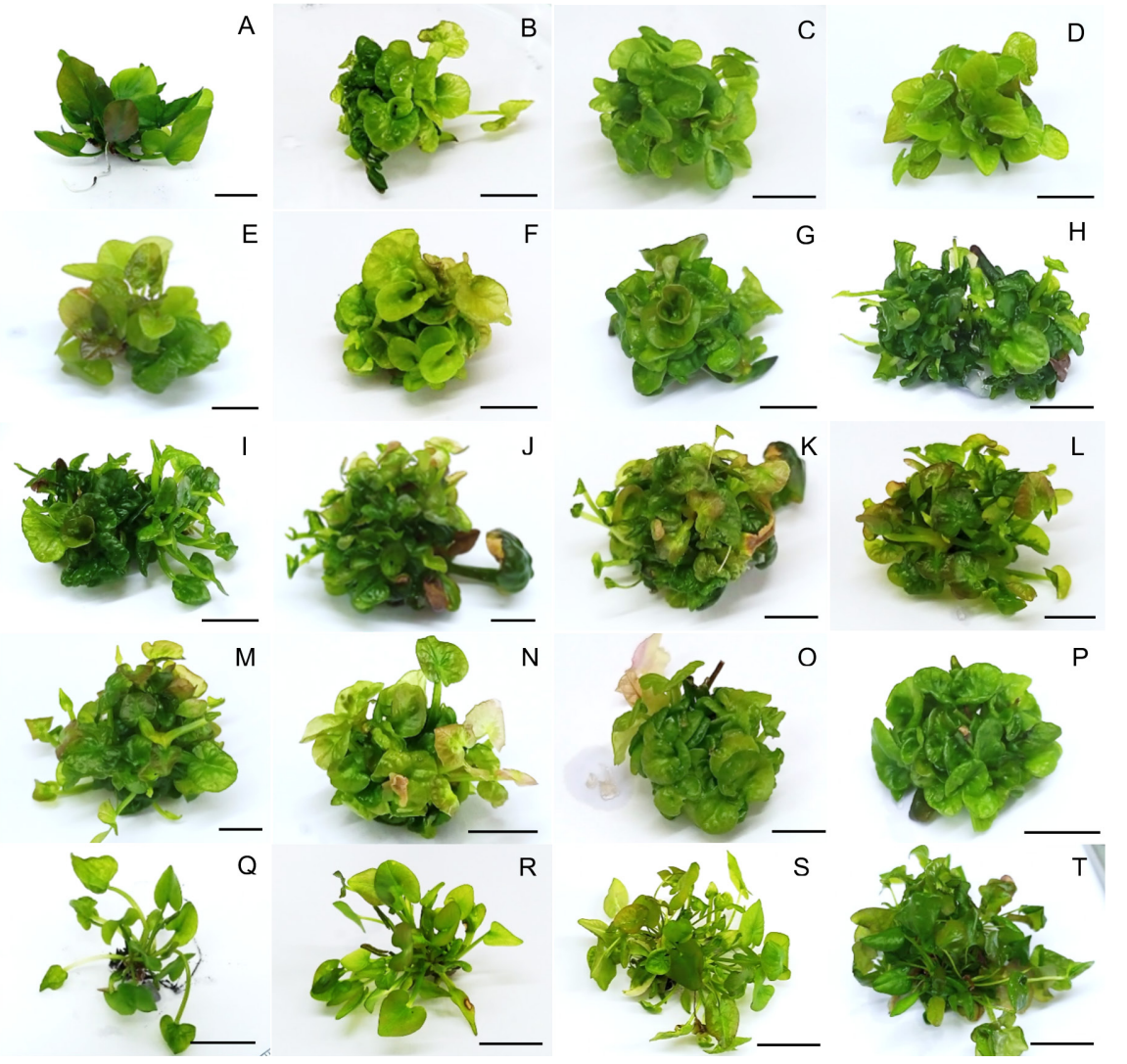


**Figure 2** Various characteristics of multiplied shoot clump responses across different combinations of types and concentrations of PGRs (T = TDZ, B = BAP, N = NAA) after culture for 8 weeks (bar = 1 cm): (A) PGR-free MS medium (control), (B) 0.05T + 0.5B + 0.5N, (C) 0.05T + 1.0B + 0.5N, (D) 0.05T + 1.5B + 0.5N, (E) 0.05T + 3.0B + 0.5N, (F) 0.1T + 0.5B + 0.5N, (G) 0.1T + 1.0B + 0.5N, (H) 0.1T + 1.5B + 0.5N, (I) 0.1T + 3.0B + 0.5N, (J) 0.5T + 0.5B + 0.5N, (K) 0.5T + 1.0B + 0.5N, (L) 0.5T + 1.5B + 0.5N, (M) 0.5T + 3.0B + 0.5N, (N) 0.05T + 0.5N, (O) 0.1T + 0.5N, (P) 0.5T + 0.5N, (Q) 0.5B + 0.5N, (R) 1.0B + 0.5N, (S) 1.5B + 0.5N, (T) 3.0B + 0.5N.

For clump size, the result showed significantly ($P < 0.01$) varied depending on the PGR combinations (Table 2). The largest clump size at 3.29–3.30 cm was obtained in the medium with 1.5–3.0 mg/L BAP and 0.5 mg/L NAA (Figures 2S-2T), while the smallest clump size at 2.49 cm was observed in the medium with 0.5 mg/L TDZ and 0.5 mg/L NAA (Figure 2Q). The results indicated that BAP more effectively enlarges the clump of shoot than TDZ. These findings are compared with the visualized quite plumped shoot and short petiole length characteristics of TDZ-derived shoot clump.

The number of shoots per clump was significantly ($P < 0.01$) affected by the different concentrations of TDZ and BAP. The maximum number of shoots/clumps at 30.08–31.15 shoots/clump were obtained with 0.5 mg/L TDZ, 0.5–1.0 mg/L BAP and 0.5 mg/L NAA, respectively (Figures 2J-2K). The shoots were robust and densely compacted. After excising the shoot clump into individual shoots, each shoot showed a high performance in rooting, with some readily multiplying (Figure 3). The minimum number of shoots at 1.13 shoots/clump was found in the control. These findings indicate that combining two different types of cytokinins with an auxin is more effective in increasing shoot numbers than using either TDZ or BAP with NAA. The number of leaves per shoot varied significantly ($P < 0.01$) among the treatments. The highest number of leaves at 6.83 leaves/shoot was found in the medium with 0.5 mg/L TDZ, 0.5 mg/L BAP, and 0.5 mg/L NAA (Figure 2J) and the lowest at 4.53 leaves/shoot in the medium with 0.05 mg/L TDZ and 0.5 mg/L NAA. Leaf width also showed significant ($P < 0.01$) differences across the different PGR combinations. The widest leaves at 1.10 cm were observed in the medium with 0.1 mg/L TDZ, 0.5 mg/L BAP, and 0.5 mg/L NAA (Figure 2F), while the shortest leaves at 0.44 cm were found in the medium with 1.5 mg/L BAP and 5.0 mg/L NAA.

Based on the research findings, BAP (1.5–3.0 mg/L) effectively promoted shoot multiplication with normal shoot growth. However, TDZ outperformed BAP regarding shoot multiplication, producing more shoots per explant. This suggests that while BAP benefits normal shoot growth and overall shoot quality, TDZ is more effective in achieving a greater number of shoots. TDZ is recognized for its cytokinin-like activity and ability to induce shoot regeneration in a wide range of plant species (Govindaraj, 2018). At optimal concentrations, TDZ enhances efficiency by overcoming monopodial growth habits and stimulating axillary shoot development (Novikova and Zaytseva, 2018).

The combination of TDZ, BAP, and NAA significantly influences shoot multiplication, leaf number, and clump size. Kaviani *et al*. (2019) reported that the treatment of 0.50 mg/L TDZ, 4.00 mg/L BA, and 0.10 mg/L NAA on *Aglaonema widuri* produced maximum nodes (13.25 per explant). Generally, TDZ and BAP promote shoot proliferation, while NAA supports overall growth and root formation (Hutchinson *et al*., 2014; Cárdenas-Aquino *et al*., 2023). When comparing the effects of TDZ and BAP on shoot characteristics, it is observed that TDZ can induce morphological abnormalities in plants. TDZ-treated shoots exhibited plumped shoot clumps with short and somewhat vitrified leaves (Vinoth and Ravindhran, 2018). These findings align with those of Novikova and Zaytseva (2018), who reported that low concentrations of TDZ can lead to vitrification *in vitro*, resulting in stunted or abnormal shoots. Based on these results, a combination of TDZ and BAP is recommended for mass-shoot production of *N. colorata*. However, we would suggest that subculturing the shoots to a PGR-free OPCM medium could mitigate the adverse effects of TDZ.

**Figure 3** Excised shoot clump into individual shoot derived from the most suitable treatment on mass shoot production of *N. colorata* (semi-solid OPCM medium with 0.5 mg/L TDZ, 1.0 mg/L BAP, and 0.5 mg/L NAA) (bar = 1 cm)

**Plantlet Acclimatization**

Published protocols for micropropagation often challenge the acclimatization of waterlily plantlets. However, this study presents a simplified and effective acclimatization process designed for commercial application, building upon the method developed by Rodboot *et al*. (2024). The process involves strengthening plantlets in aquatic soil and enhancing root development through the addition of NAA before transplantation (Figure 4).

After two weeks, plantlets exhibited robust growth and achieved a high survival rate of 90% under greenhouse conditions (Figure 4D). Plantlets grown in aquatic soil were directly transplanted into water tanks without requiring cleaning or removal of the soil. By using uncapped bottles as protective covers, plantlets developed well-established root systems and produced floating leaves within the first week under greenhouse conditions (Figure 4E).

Once acclimatized, these plantlets were transplanted into loamy clay soil under field conditions. They demonstrated excellent adaptation to the ambient environment, achieving a 100% survival rate, as evidenced by the production of new floating leaves and shoots (Figure 4F). This efficient and reliable acclimatization protocol represents a significant advancement for the commercial propagation of ornamental waterlilies, offering a practical solution to support large-scale production efforts.

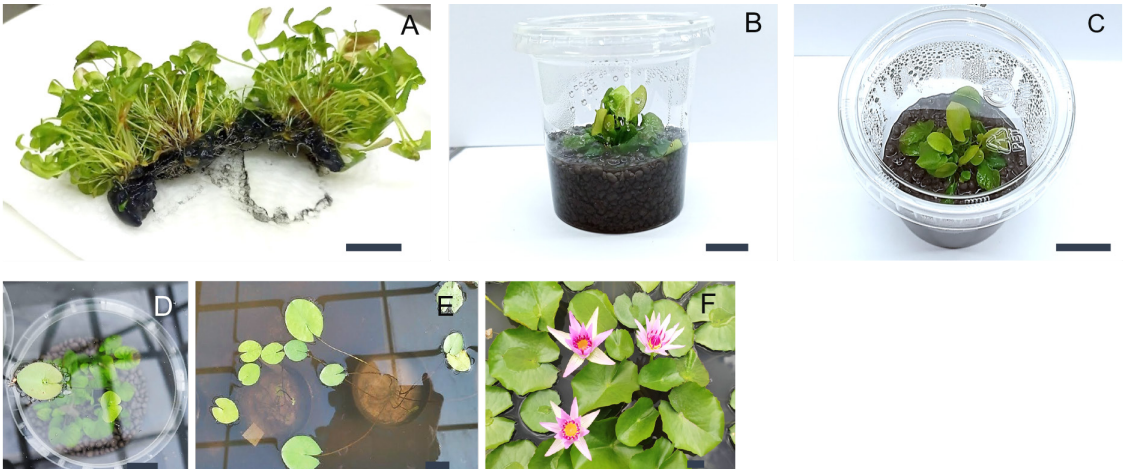


**Figure 4** Plantlet acclimatization process (bar = 1 cm): (A) rooted shoot clump, (B-C) transplanted plantlets into sterilized aquatic soil with half liquid OPCM medium added 0.5 mg/L NAA, (D) plantlet produced floating leaves after being submerged in a water tank under greenhouse conditions, (E) soil transplanted plantlet under greenhouse conditions, (F) matured plant (2 months) under field-grown conditions.

## CONCLUSIONS

The semi-solid OPCM medium was optimal for individual shoot growth of *N. colorata* Peter under aseptic conditions. For effective mass shoot production, the OPCM medium supplemented with 0.5 mg/L TDZ, 1.0 mg/L BAP, and 0.5 mg/L NAA was highly efficient. Based on our results, we recommend further optimization of activated charcoal (AC) concentration to enhance the medium's efficiency in promoting high-quality shoot and root formation for future waterlily production.

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