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Research article

Influence of MS Medium Strengths and Types on *In Vitro* Shoot Multiplication and Development of *Nymphaea colorata* Peter

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Abstract: Efficient *in vitro* propagation systems are essential for the large-scale commercial production and conservation of the important tropical waterlilies (*Nymphaea* spp.), particularly elite ornamental varieties and species. This study aimed to optimize culture medium conditions by evaluating the effects of Murashige and Skoog (MS) medium strengths and types on the *in vitro* growth of *Nymphaea colorata*, using it as a plant model. The derived shoots obtained from sterilized turions were cultured on different MS medium concentrations and types. The results demonstrated that full-strength MS medium significantly enhanced the survival percentage (100%), the number of shoots/explant (1.53 shoots), the number of leaves/explant (18.86 leaves), leaf width (2.66 cm), and petiole length (11.50 cm) compared to diluted formulations. Among different medium types, semi-solid MS medium effectively supported shoot growth while reducing hyperhydration, a common issue observed in liquid cultures. Shoots cultured on semi-solid MS medium exhibited well-developed leaves and elongated petioles, making them more suitable for subsequent acclimatization. These findings underscore the importance of optimizing both the nutrient composition and physical state of the culture medium to enhance micropropagation efficiency in *Nymphaea colorata*. The use of full-strength MS medium in combination with semi-solid culture conditions offers a promising approach for high-quality plantlet production, subsequent large-scale propagation, and conservation of elite waterlily genotypes.

Keywords: Waterlily micropropagation; Semi-solid medium; *Nymphaea colorata*

1. Introduction

Waterlilies (Nymphaeaceae) are emergent aquatic herbs comprising over 100 species distributed worldwide across tropical and subtropical regions [1, 2, 3]. Among these, ornamental waterlilies are widely cultivated due to their aesthetic appeal, playing a significant role in aquatic landscaping and the global horticultural market [4, 5]. Their striking floral colors, pleasant fragrance, and diverse foliage characteristics contribute to their increasing commercial demand. Among wild waterlily species, *Nymphaea colorata* (subgenus Brachyceras), native to East Africa, is particularly notable [6, 7]. This species is distinguished by its vibrant violet flowers, a rare trait among waterlilies, and is extensively utilized in breeding programs due to its ability to transmit unique floral colors and patterns to its progeny [8, 9, 10]. Beyond its ornamental significance, *N. colorata* serves as a valuable model species for studies on floral development, pigmentation, and waterlily genetics [1, 6, 11]. Notably, it is one of the few waterlily species with a sequenced genome, offering key insights into angiosperm evolution and the genetic mechanisms underlying aquatic adaptation [12-14].

Despite the growing demand for ornamental waterlilies in landscaping and horticulture, conventional propagation methods are limited by low multiplication rates and restrict large-scale commercial production [15]. Micropropagation presents an efficient alternative, enabling the rapid clonal propagation of elite genotypes, particularly for rare or hybrid varieties that are challenging to propagate through traditional means [16-17]. This technique ensures the production of genetically uniform plants while preserving desirable horticultural traits such as flower color, size, and disease resistance [18]. Additionally, *in vitro* propagation can overcome limitations associated with slow growth rates and seasonal constraints in natural propagation [19- 21]. By integrating micropropagation techniques, the ornamental waterlily industry can enhance production efficiency, support commercial expansion, and contribute to the conservation of genetically valuable or endangered species [15].

Murashige and Skoog (MS) medium is one of the most widely used plant culture media for micropropagation due to its well-balanced nutrient composition, effectively supporting shoot and root development in various plant species, such as *Dioscorea* sp., grapes, and *Aponogeton ulvaceus* [22-26]. The concentration, composition, and type of culture media play a critical role in regulating in vitro growth and development across different water lily species. Variations in nutrient levels and types of medium can significantly influence shoot regeneration, root induction, nutrient uptake, and overall plant health [27]. Several studies have demonstrated that optimizing these parameters can lead to substantial differences in developmental outcomes, emphasizing the necessity of precise media formulation to achieve optimal *in vitro* propagation efficiency [28-30]. Explant hyperhydration is a physiological disorder typically found in plant tissue culture, particularly in liquid medium systems. It is characterized by excessive water accumulation in tissues, leading to translucent, swollen, and brittle plantlets [31]. This condition can negatively affect growth, morphogenesis, and survival during acclimatization. For waterlily micropropagation, hyperhydration is a significant concern as it severely impacts plantlet establishment during ex vitro transition [14]. This phenomenon is strongly influenced by the composition of the culture medium, including the type and concentration of gelling agents, plant growth regulators (PGRs), and osmotic agents [31]. Given the importance of culture media optimization, this study aims to evaluate the key factors —medium concentration and type —that influence the production of healthy waterlily plantlets. The findings will contribute to the establishment of an efficient waterlily micropropagation protocol, ensuring high-quality and high-yield plant production for future commercial applications.

2. Materials and Methods

2.1 Plant material collection and preparation

The mature, bare-root rhizomes of *N. colorata* Peter, measuring 3.0–5.0 cm in diameter and 10.0–15.0 cm in length, along with their leaves, flowers, and turions, were collected (Figure 1a). The healthy attached turions, free from scars and diseases, were selected and used as the initial explant. The mother plants were cultivated in floating paddy fields under full sun and fertile loamy clay conditions. All plant materials were sourced from Baufah Garden (401 Watcharapol Road, Tha Raeng, Bang Khen District, Bangkok) between March and June. These turions served as the initial explants for *in vitro* propagation experiments. Turions of *N. colorata* with robust shoots were collected (**Figure** 1b). To remove residual dirt and dust, the explants were submerged in running tap water for 3 hours and gently brushed. The attached leaves, roots, and scale leaves were carefully removed. The outer layer of the turion was cleaned using pointed tweezers (Figure 1c). Finally, trichomes on the shoot crown were carefully removed under running tap water using forceps. The cleaned explants were sterilized according to a protocol by Rodboot et al. [14]. The procedure involved immersion in a 0.1% (w/v) Carbendazim solution (a systemic fungicide; Anhui Guangxin Agrochemical Co., Ltd., PRC) for 2 hours, followed by two rinses with sterilized distilled water. The explants were then shaken in 50% ethyl alcohol for 1 minute, treated with 20% (v/v) Clorox® containing five drops of Tween 20 for 20 minutes, and shaken in 0.1% (w/v) $HgCl_2$ for 15 minutes. Finally, any residual disinfectants were removed by five successive washes with sterilized distilled water. The disinfected explants were blotted dry on autoclaved tissue paper

and then cultured in liquid MS medium, free from plant growth regulators, for 8 weeks. The cultures were maintained on an orbital shaker optimized at 90 rpm.

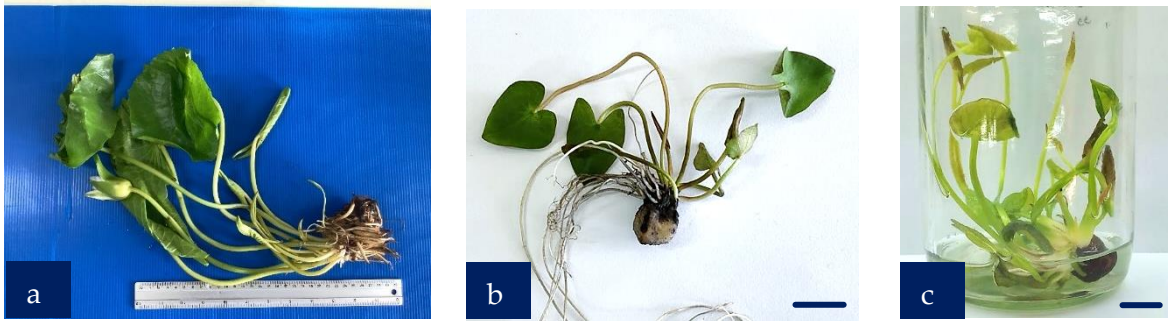

Figure 1. Initial explant establishment (bar = 1 cm)
(a) Mother plant
(b) Raw turion separated from the mother tuber, remaining leaves, and roots
(c) Sterilized turion in plant growth regulator-free liquid MS medium after culture for 8 weeks, exhibiting slightly hyperhydrated shoots

2.2 Effect of different strengths of MS basal medium on in vitro growth

Individual shoots removed from the turions at 8 weeks old in PGR-free liquid MS media, which had 2-3 mature, expanded leaves and measured 3-5 centimeters in length, were used to encourage healthy shoot growth. (Figure 1c). Different strengths of liquid MS medium (full, 1/2, 1/4, and 1/8 MS) were dispensed into 20 ml in 6.3 x 10.8 cm glass bottles. Each culture bottle contained a single explant. The experiment was repeated twice, with twenty replicates for each treatment. After culturing for 4 weeks, the survival rate, number of shoots, leaf length, petiole length, and visible characteristics were assessed.

2.3 Effect of different types of MS basal medium on in vitro growth

To evaluate the optimal type of culture medium for shoot growth, various types of MS basal medium (liquid, semi-solid, and bilayer) were tested. Individual shoots excised from the turions at 8 weeks old, grown in PGRs-free liquid MS medium consisting of 2-3 leaves and 3-5 cm in length, were used. Different types of MS medium were dispensed into 20 mL in 6.3 x 10.8 cm glass bottles. Each culture bottle contained a single explant. The experiment was repeated twice, with twenty replicates for each treatment. After culturing for 4 weeks, the survival rate, number of shoots, leaf length, petiole length, and visible characteristics were recorded.

2.4 Culture conditions

All experiments used the MS basal medium containing 30 g L^{-1} sucrose. The pH of the culture medium was adjusted to 5.7 with 1 N NaOH or HCl before being autoclaved at 121°C for 15 min. The cultures were incubated at 26 ± 2 °C with a 10/14 day-night cycle photoperiod under 12 μmol m^{-2} s^{-1} provided by fluorescent lamps.

2.5 Statistical analysis

All experiments above were arranged in a completely randomized design (CRD). Each treatment consisted of 20 replicates. Data from all parameters were expressed as mean values ± standard error (S.E.) and statistically analyzed using analysis of variance (ANOVA). The means among treatments are separated by Duncan's multiple range test (DMRT) using the SPSS 17.0 program for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1 Effect of different strengths of MS basal medium on in vitro growth

The excised shoots were cultivated in liquid MS media of several strengths (full, 1/2, 1/4, and 1/8 MS), supplemented with 30 g/L sucrose, until the optimal medium strength for shoot growth was established. After four weeks of culture, all treatments exhibited a 100% survival rate; however, the explants displayed differential growth responses depending on the medium strength (Table 1, Figure 2). A gradual reduction in MS medium

concentration was associated with a decreased growth rate and a lower number of shoots. Full-strength MS medium proved to be the most effective in promoting shoot growth in *N. colorata*, yielding the highest average values across all measured parameters ($p \leq 0.05$) (Table 1). Specifically, the full-strength MS medium resulted in an average of 1.53 shoots/explant, which was not significantly different from the 1/2-strength MS medium (1.40 shoots/explant).

The number of leaves was also highest in full-strength MS medium, with an average of 18.86 leaves per explant. These leaves exhibited the greatest width (2.66 cm) and the longest petioles (11.50 cm). Explants cultured in full-strength MS medium demonstrated the most vigorous growth, characterized by dark green, fully expanded leaves and elongated petioles. In contrast, explants grown in diluted media exhibited slow growth, smaller leaves, and shorter petioles (Table 1, Figures 2b-d). In addition to superior growth, explants cultured in full-strength MS medium exhibited rapid shoot development. Within three weeks, the petioles extended beyond the bottleneck of the culture vessel, and the leaves were fully expanded with dark green coloration, indicative of healthy growth. However, hyperhydration, brittle leaf, and petiole structures were occasionally observed. These findings align with previous studies on some varieties of *Nymphaea* micropropagation, which reported that full-strength MS medium supports optimal shoot and leaf development from various explant sources, including tubers, turions, and rhizomes [32-35]. Full-strength MS medium is widely recognized as an optimal formulation for micropropagation due to its well-balanced composition of macro- and micronutrients [36-37]. The balanced presence of nitrate (NO_3^-) and ammonium (NH_4^+) ensures efficient nitrogen uptake, promoting protein synthesis and enzymatic activity essential for organogenesis [26, 38]. Furthermore, the inclusion of sucrose provides an external energy source, which is crucial for the development of non-photosynthetic explants [39-41]. When supplemented with appropriate PGRs, full-strength MS medium enhances direct shoot formation, shoot multiplication, and root formation. Its comprehensive nutrient profile makes it suitable for a wide range of aquatic plant species; however, adjustments may be necessary to prevent salt accumulation and pH stress in more sensitive species [42].

Reducing MS medium strength significantly enhanced root formation and overall plant quality under *in vitro* conditions. In the current experiment, we found that roots developed at the basal portion of shoots within 3–4 weeks in both 1/2- and 1/4-strength MS media, with approximately 35% of explants forming roots. This finding is consistent with previous studies, where 1/2-strength MS medium improved root formation in *Ceratonia siliqu* [43] and, when supplemented with 0.2 mg/L IBA, promoted maximum root and shoot production in *Tylophora indica* [44]. Additionally, nitrogen deficiency has been shown to increase salicylic acid levels, thereby modulating root growth [45]. Lowering macronutrient concentrations, particularly nitrogen, alleviates the inhibitory effects of high salt concentrations, thereby enhancing root induction [46-47]. An optimal nutrient balance in diluted MS medium not only minimizes osmotic stress but also enhances root elongation, nutrient uptake, and subsequent ex vitro acclimatization. However, excessive dilution may lead to nutrient deficiencies, resulting in weaker plantlets with reduced shoot vigor and lower survival rates.

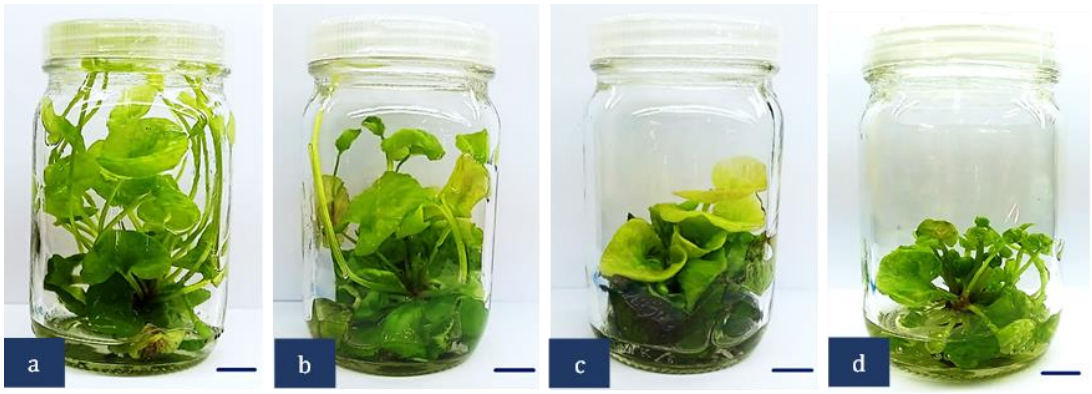

Figure 2. The different characteristics of shoot growth in various strengths of liquid MS medium without PGRs after culture for 4 weeks (bar = 1 cm)

(a) Full (b) 1/2 (c) 1/4 (d) 1/8

Table 1. Effects of different strengths of PGRs-free liquid MS medium on *in vitro* growth after culture for 4 weeks

Strengths of the medium	Survival rate (%)	No. of shoots (shoot)	No. of leaves (leaf)	Petiole length (cm)	Leaf width (cm)
Full	100	1.53[a]	18.86[a]	11.50[a]	2.66[a]
1/2	100	1.40[ab]	15.40[b]	6.72[b]	2.40[b]
1/4	100	1.33[b]	10.60[c]	4.67[b]	2.06[c]
1/8	100	1.33[b]	8.80[d]	3.95[d]	1.05[d]
F-test		*	**	**	**
C.V. (%)		26.77	38.39	30.3	34.66

* significantly different ($p \leq 0.05$), ** significantly different ($p \leq 0.01$)
Means ±SD having different letters in the same column are statistically significant differences by DMRT

3.2 Effect of different types of MS basal medium on in vitro growth

To determine the optimal culture medium for shoot growth, three types of MS basal medium were evaluated: liquid, semi-solid, and bilayer media. The bilayer medium consisted of two variants: a thin layer of sterile distilled water (SDW) or liquid MS medium overlaid on a semi-solid medium. After four weeks of culture, all explants exhibited a 100% survival rate, with varying growth responses depending on the type of medium. Among the treatments, the bilayer medium consisting of semi-solid MS overlaid with liquid MS medium produced the highest number of shoots (1.77 shoots/explant) and leaves (12.45 leaves/explant), with minimal occurrence of hyperhydration (Table 2). However, these differences were not statistically significant ($p \leq 0.05$). Liquid MS medium was identified as the most effective in promoting waterlily shoot growth, as explants cultured in this medium exhibited the highest number of leaves (13.66 leaves/explant), longest petioles (4.45 cm), and widest leaves (2.55 cm) (Table 2, Figure 3a). However, the number of shoots per explant (1.55 shoots) was not significantly different from that observed in other treatments ($p \leq 0.05$). The explants cultured in liquid MS medium exhibited rapid growth, characterized by dark green, fully expanded leaves and elongated petioles. Despite these positive growth attributes, plump, hyperhydrated, and brittle shoots were frequently observed, consistent with findings from Experiment 1.

The choice of culture medium has a significant influence on the growth and development of *N. colorata* during *in vitro* propagation. Each medium type has distinct advantages and limitations that affect shoot proliferation and overall plantlet quality. Several studies have reported that a liquid medium enhances shoot growth and development in water lilies [32, 34, 48]. Moreover, liquid MS medium plays a crucial role in plant tissue culture by improving nutrient uptake, accelerating shoot proliferation, and facilitating large-scale propagation, particularly in suspension cultures [49-50]. The absence of gelling agents in the liquid medium reduces production costs. However, challenges such as hyperhydration, inadequate mechanical support, and increased risk of contamination remain significant drawbacks. Excessive water uptake may lead to physiological abnormalities, while limited aeration can inhibit root development [31].

In contrast, semi-solid MS medium exhibited lower growth performance across all measured parameters (Table 2, Figure 3b). Explants cultured in this medium developed significantly shorter petioles (2.59 cm), leaf width (2.13 cm), and produced narrower leaves compared to those in liquid culture (Figure 3). Nevertheless, semi-solid MS medium offers distinct advantages, including structural support, reduced hyperhydration, and lower contamination risks, making it a suitable option for stable *in vitro* plant growth and structurally robust shoot formation [51]. Additionally, semi-solid medium regulates nutrient absorption, preventing excessive water uptake and thereby supporting balanced shoot and root development [36, 49-50]. These findings align with previous reports. For instance, a single-node explant of *Stevia rebaudiana* exhibited superior shoot elongation and a higher number of nodes when cultured in semi-solid medium, with minimal hyperhydration. Similarly, in *Phoenix dactylifera*, a semi-solid medium type was found to be more conducive

to normal shoot development, whereas a liquid medium resulted in hyperhydration and morphological abnormalities [52]. Despite the higher cost associated with gelling agents, semi-solid MS medium remains a practical choice for maintaining plant structure and reducing handling difficulties in micropropagation systems.

In the present study, explants cultured on semi-solid MS medium exhibited the lowest incidence of hyperhydration compared to those in liquid or bilayer media, underscoring their potential for *in vitro* micropropagation protocols where hyperhydration is a concern. Therefore, we propose that the semi-solid medium type is the most suitable solution for facilitating the stable growth and development of *N. colorata* under *in vitro* conditions.

Figure 3. The characteristics of shoots cultured in different types of medium after culture for 4 weeks (bar = 1 cm)
(a) Liquid MS medium
(b) Semi-solid MS medium
(c) Solid MS medium overlaid with sterilized distilled water
(d) Solid MS medium overlaid with liquid MS medium

Table 2. Effects of different MS medium types on in vitro growth after culture for 4 weeks

Types of medium	Survival rate (%)	No. of shoots (shoot)	No. of leaves (leaf)	Petiole length (cm)	Leaf width (cm)
Liquid (control)	100	1.55 ± 0.41	13.66 ± 2.44^{a}	4.45 ± 1.46^{a}	2.55 ± 0.49^{a}
Semi-solid	100	1.00 ± 0.0	10.04 ± 0.37^{b}	2.59 ± 0.43^{ab}	2.13 ± 0.3^{a}
Semi-solid/ SDW	100	1.11 ± 0.15	12.92 ± 1.42^{ab}	2.42 ± 0.71^{ab}	1.75 ± 0.58^{b}
Semi-solid/ liquid MS medium	100	1.77 ± 0.56	12.45 ± 0.62^{ab}	2.22 ± 0.22^{b}	2.49 ± 0.38^{c}
F-test		ns	*	*	*
C.V. (%)		32.35	46.09	35.55	22.94

ns; not significantly different, * significantly different ($p \leq 0.05$)
Means ±SD having different letters in the same column are statistically significant differences by DMRT

4. Conclusions

This study highlights the significant influence of MS medium strength and type on the *in vitro* growth and development of *Nymphaea colorata*. Full-strength MS medium was identified as the most effective for promoting explant survival percentage and overall plant growth, supporting optimal growth performance compared to diluted formulations. In addition to medium strength, the physical state of the culture medium played a crucial role in shoot development. Based on these findings, semi-solid medium is recommended for long-term subculture and subsequent plantlet acclimatization, as it effectively reduces hyperhydration while maintaining normal growth performance. Therefore, for the commercial production of waterlilies, the use of full-strength semi-solid MS medium is recommended to ensure robust and healthy plant development.

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Author Contributions: N.R. carried out the experiments and writing - original draft; S.Y. writing - review and editing: S.T. visualization. All authors read and approved the final manuscript.

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