Plant Methods

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Novel cultivation techniques for water lily (*Nymphaea micrantha* Guill. & Perr) production based on in vitro technology

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Abstract

Water lily (*Nymphaea micrantha* Guill. & Perr) is an aquatic plant that is well known for its nutritional value and medicinal uses. The lack of adequate information regarding propagation and farming techniques has led to the low utilization of this valuable plant. To address the knowledge gap in the use of water lily rhizomes as explants in tissue culture, in this study, the processes of sterilization, induction, proliferation, and rooting in water lily tissue culture are examined. Laboratory experiments were conducted to determine the best methods, materials, and concentrations to develop an ideal method for producing water lily plants. Disinfection with 75% C_2H_5OH for 2 min + 0.1% $HgCl_2$ for 15 min produced the best results, with a contamination rate of 30% and a browning rate of 25%, according to the data. The results indicated that indole-3-butyric acid (IBA) is the optimal plant growth regulator for the induction of water lily rhizomes. Medium containing 3 mg L^{-1} of 6-BA was most suitable for the induction of water lily adventitious shoots, with an induction rate of up to 80% and a yield of 2 to 8 shoots. The induction rate of water lily adventitious shoots approached 80% in medium supplemented with 3 mg L^{-1} 6-benzylaminopurine (6-BA). The best medium for inducing root development contained IBA at a concentration of 0.5 mg L^{-1}, which resulted in rapid root elongation. The best tissue culture techniques identified in the present study were successful in growing full water lily plants with good and vigorous growth from tuberous rhizomes to flowering plants. This early success in water lily tissue culture technology provides crucial technical assistance for ex vitro preservation and water lily seedling growth. This study offers a workable answer to one of the most significant obstacles preventing the spread of the use of water lily culture by outlining a good technique that results in robust and healthy seedlings, which helps lower the cost of cultivation.

Keywords Aquatic plants, Explant, Tissue culture, Disinfection, Induction, Seedlings

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Introduction

Water lily (*Nymphaea micrantha* Guill. & Perr) is a perennial floating-leaved aquatic herbaceous plant renowned as the"aqueous botanical sovereign", flourishing in tropical and subtropical temperate regions [1, 2]. Water lily, as a botanical entity, boasts a commendable economic and ecological value, presenting expansive avenues for development [3–5]. Water lily is a rooted, aquatic, tuberous herb that thrives in temperate and tropical climates worldwide, growing continuously from a submerged stem anchored in the mud, where its rootlets provide stability and asexual shoots emerge above the water's surface [6–8]. As of now, approximately 868 publications are indexed in the PubMed database, with the annual number rising rapidly from just eight in 2000 to 71 in 2021, highlighting the growing research interest in various types of water lilies [7]. The limited utilization of edible aquatic plants, e.g., water lilies, is primarily due to insufficient information on their nutritional value, cultivation, production, harvesting, processing, and food preparation techniques [4, 9]. The starch-rich rhizomes of water lily are used for food and fermentation, while its edible stalks, petioles, and flavonoid-rich petals with hypoglycemic and antioxidant properties make it suitable for tea, dried flowers, and health supplements [10–14].

Water lily plants mainly propagate vegetatively [10]. However, owing to limited proliferation, among other factors, the cost of water lily seedlings remains prohibitively high [4, 14]. This constrains the industrial and scalable development of water lily. The application of this plant in organized agriculture as an effective means of sustainable development depends mainly on quickly gathering a large stock of seedlings [3, 5]. Regrettably, comprehensive research on water lily production remains insufficient, and challenges such as significant pollution, low induction rates, and poor roots hinder the agricultural advancement of this valuable species [5, 6, 15]. In vitro methods swiftly and efficiently generate standardized, disease-free planting material, especially for the long-term breeding of many plant species and for commercial uses [16]. Different microarthropods (mites, ticks, and their vectors), microorganisms (filamentous fungi, yeasts, and bacteria), viruses, and viroids can all cause contamination in plant tissue cultures [17, 18]. Nevertheless, specific preparation processes for each crop and cultivar must be developed to render in vitro culture a financially viable production strategy; for instance, sterilization methods suitable for some plants may not be totally appropriate to the sterilization of water lily explants [17, 19, 20]. The complete life cycle of water lily plants transpires in an aquatic environment, with roots and stems penetrating the substrate, so promoting a proliferation of fungus, bacteria, and endophytes [7–9]. The scarcity of studies on water lily tissue culture, along with contamination and low rooting rates, hinders its agricultural development. [1, 6, 7, 13, 14].

To date, there are no reports of tissue culture using water lily rhizomes as explants. To address this gap, in this study, tissue culture techniques that utilize water lily rhizomes as explants are explored, with the goal of identifying the optimal sterilization method as well as the best induction, proliferation, and rooting culture media and micropropagation techniques. Through these endeavors, preliminary success in water lily tissue culture has been achieved, providing valuable technical support for the ex situ preservation and propagation of water lily seedlings.

Materials and methods

Plant materials, reagents, and equipment

The rhizomes of a petite floral water lily (*Nymphaea micrantha* Guill. & Perr) were selected as explants for the current study. The source of the rhizomes was Haikou Experimental Station, Chinese Academy of Tropical Agriculture Sciences, Haikou, Hainan, China. The pristine rhizomes of healthy, pest-free small water lilies were collected on a clear day. Rhizomes were cut into 2 cm pieces and used in all experiments. Agar, 6-benzylaminopurine (6-BA), and 1-naphthylacetic acid (NAA) were obtained from BIOFROXX (Hangzhou, China); indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) from Biosharp (Hefei, China); absolute ethanol, mercuric chloride ($HgCl_2$), and sodium hypochlorite (NaClO) from Xilong Scientific Co., Ltd. (Guangdong, China); and the fungicide S106 and sucrose from the Innovation Tissue Culture Technology Research Institute (Jiangxi, Wannian, China). The garden soil, nutrient-rich soil, seedling bags, perforation-free flower pots, etc., were provided by the Haikou Experimental Station. The autoclaves, tissue culture bottles, culture bags, parchment paper, dispensers, high-pressure sterilizers, aseptic workstations, dissecting knives, forceps, sealing devices, beakers, dissecting scissors, and balances used were provided by Haikou Experimental Station.

Treatments and experimental design

Sterilization procedures

Ethyl alcohol (C_2H_5OH) was added for 2 min, and different mixtures of mercury chloride ($HgCl_2$) and sodium hypochlorite (NaClO) were used as disinfectants. The efficiency of the tested disinfection procedures was evaluated by calculating the browning and contamination rates. The tested sterilization procedures are shown in Table 1. The rhizomes of the water lily were delicately placed in a receptacle filled with water and transported to the laboratory. Initially, the surface sediment on the rhizomes was rinsed away with tap water,

and the root hairs were carefully removed from their surfaces using fine forceps. The rhizomes were subsequently cleansed with laundry detergent (Tween 20), and once the surface impurities had been thoroughly removed, they were rinsed under running tap water for 30 min. Thereafter, they were carefully arranged on aseptic workbenches for sterilization experiments. Following each sterilization treatment, it was imperative to meticulously rinse away any residual sterilizing solutions with sterile water. The various sterilization methods were applied to the explants (Table 1), which were then subjected to 5 thorough rinses with sterile water. The explants were subsequently soaked in sterile water for 5 min to eliminate any remnants of the sterilizing solutions, thus preventing harm to the explants and minimizing the browning rate.

This process was repeated 5 times with sterile water rinses, followed by blotting dry with sterile filter paper. To ensure the removal of the outer layer of rhizome surfaces in contact with the sterilizing agent, a sterile scalpel was used. The treated explants were then cultured on Murashige and Skoog (MS) + S106 medium according to Murashige and Skoog [21], with each treatment having three replicates and each replicate comprising 60 bottles, with one explant per bottle. After a period of 10 days, the outcomes were meticulously recorded. The contamination rate (%) was determined as the ratio of contaminated explants to the total number of explants inoculated, multiplied by 100%. The browning rate (%) was calculated as the ratio of browning explants to the total number of explants inoculated, which was also multiplied by 100%.

Induction methods

The ability of various induction chemicals to induce water lily explants was tested by adding them to MS contained 30 g/L of sugar and 7 g/L of agar. Culture medium was added to each bottle at the dose of 50 mL per bottle. Implanting uncontaminated explants into different induction culture media (B1-B4) was performed (Table 2). Each treatment consisted of three replicates, with each replicate comprising 60 bottles, with one explant per bottle. The experiment was performed in a culture medium containing sterile water (just sufficient to cover the surface of the explant) to simulate the water lily growth environment. This was achieved by maintaining them at 28 °C in a cultivation chamber, initially in darkness for 5–7 days, followed by exposure to light at an intensity of 4000 lx, with a photoperiod of 10–14 h per day (based on primary experiments). The results were recorded after 30 days. The induction rate (%) was calculated as follows: induced adventitious shoots per explant/total inoculated explants × 100%.

Breeding methods for the regenerated water lily buds

The induced regenerated water lily buds were inoculated into different proliferation culture media (30 g/L of sugar and 7 g/L of agar), designated C1 through C5 (Table 3). Each treatment consisted of three replicates, with each replicate comprising 60 bottles, with one single regeneration bud per bottle and 50 mL of media per bottle. The culture medium contained sterile water (just enough to cover the surface of the rhizomes) to simulate the water lily growth environment. The inculcated bottles were placed in a cultivation chamber at 28 °C, initially in darkness for 5–7 days, followed by cultivation at 28 °C, with a light intensity of 4000 lx (280 small LED light bulbs) and a photoperiod of 10 to 14 h per day. After 20–25 days, adventitious shoot induction and proliferation were observed, and the results were recorded after 30 days.

Table 1 Different disinfection methods

Abbreviation	Disinfection methods
A1	Sterile water (control)
A2	75% C_2H_5OH 2 min + 20% NaClO 10 min
A3	75% C_2H_5OH 2 min + 20% NaClO 15 min
A4	75% C_2H_5OH 2 min + 20% NaClO 20 min
A5	75% C_2H_5OH 2 min + 0.1% $HgCl_2$ 10 min
A6	75% C_2H_5OH 2 min + 0.1% $HgCl_2$ 15 min
A7	75% C_2H_5OH 2 min + 0.1% $HgCl_2$ 20 min

Ethyl alcohol C_2H_5OH, *sodium hypochlorite* NaClO, and *mercury chloride* $HgCl_2$

Table 2 Different induction medium formulations

Abbreviation	Culture medium
B1	MS + S106 1 mL/L
B2	MS + 6-BA 2.0 mg/mL + IBA 0.5 mg/mL + S106 1 mL/L
B3	MS + 6-BA 2.0 mg/mL + NAA 0.5 mg/mL + S106 1 mL/L
B4	MS + 6-BA 2.0 mg/mL + IAA 0.5 mg/mL + S106 1 mL/L

Murashige and Skoog (MS) culture medium, fungicide (S106), 6-benzylaminopurine (6-BA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA)

Table 3 Different breeding medium formulations

Abbreviation	Culture medium
C1	MS + 6-BA 1.0 mg/mL + IBA 0.5 mg/mL + S106 0.5 mL/L
C2	MS + 6-BA 2.0 mg/mL + IBA 0.5 mg/mL + S106 0.5 mL/L
C3	MS + 6-BA 3.0 mg/mL + IBA 0.5 mg/mL + S106 0.5 mL/L
C4	MS + 6-BA 4.0 mg/mL + IBA 0.5 mg/mL + S106 0.5 mL/L
C5	MS + 6-BA 5.0 mg/mL + IBA 0.5 mg/mL + S106 0.5 mL/L

MS Murashige and Skoog culture medium, *S106* fungicide, *6-BA* 6-benzylaminopurine, *IBA* indole-3-butyric acid

Inducing root development in water lily seedlings

The induced seedlings were introduced into various rooting culture media (30 g/L of sugar and 7 g/L of agar), designated D1 through D7 (Table 4), for robust seedling root development. Each treatment consisted of three replicates, and each replicate 30 vessel each contained one explant. The rooting culture medium took the form of a semisolid substrate (50 mL per each one), with each vessel accommodating three explants. Ten such vessels were employed, and they were positioned within a cultivation chamber maintained at a temperature of 28 °C. An initial period of incubation in darkness for 2–3 days was followed by exposure to a temperature of 28 °C with a luminous intensity of 4000 lx (280 small LED light bulbs) and a light cycle lasting 10–14 h per day. The cultivation process was carried out under these specific conditions. The results were documented after 20–25 days. The rooting percentage was determined as the number of rooted seedlings divided by the number of explants initially introduced multiplied by 100%.

Cultivation of water lily seedlings

Plants in bottles

The water lily seedlings produced using the optimal methods of tissue culture in the current study were planted as follows. Once well-established seedlings with intact roots were obtained, the young plants were placed in an indoor environment with a light diffusion level ranging from 50 to 70%, and the temperature was maintained at approximately 25 ± 2 °C. The luminous intensity was gradually increased from 20 to 40 μmol/(m^2·s) to 75 to 100 μmol/(m^2·s). The bottle cap was slightly loosened after 7 to 10 days and then partially opened after an additional 1 to 2 days. When a cuticular layer formed on the surface of the plants, the seedlings were delicately extracted from the bottles using sterile techniques. The plants were submerged in a basin of clean water, and any residual culture medium adhering to the plant roots was carefully removed. The seedlings were subsequently immersed in a solution of 50% wettable powder containing a 500- to 800-fold dilution of fungicide for a duration of 20 to 30 min. The abovementioned processes must precede the transplantation of the seedlings to an external cultivation vessel.

Table 4 Different rooting medium formulations

Abbreviation	Rooting medium formulations
D1	MS
D2	MS + IBA 0.2 mg/L
D3	MS + IBA 0.5 mg/L
D4	MS + IBA 1.0 mg/L
D5	MS + NAA 0.2 mg/L
D6	MS + NAA 0.5 mg/L
D7	MS + NAA 1.0 mg/L

Murashige and Skoog (MS) culture medium, indole-3-butyric acid (IBA), and α-naphthaleneacetic acid (NAA)

Plants outside of bottles

First, the clay or mud substrate treated with a 0.1% to 0.4% potassium permanganate solution or a wettable mancozeb powder, ensuring even application through spraying. Next, we mixed 3% to 5% hydrated lime thoroughly, creating a conical soil heap. We covered and sealed it with polypropylene plastic film and then allowed it to ferment under high-temperature conditions for 7 to 10 days. Alternatively, the substrate was sterilized using a high-temperature dry heat method. Once the sterilized soil cooled to room temperature, it was transferred into seedling cups or nonwoven seedling bags with a diameter ranging from 9 to 12 cm and a height ranging from 7.5 to 14.5 cm. The well-cultivated water lily seedlings were placed into the prepared seedling cups and nurtured in the shade until new leaves sprouted. After approximately 20 to 30 days, they were moved into regular sunlight. The shade level was maintained at 70% to 90% for another 7 to 10 days before being moved to full daylight conditions.

Statistical analysis

Completely Randomized Design (CRD) with three replicates was used to setup all the examined treatments. SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis, i.e., Tukey's test. The data in all figures are mean ± standard deviation.

Results

Efficiency of the water lily explant disinfection procedures

Different mixtures of mercury chloride ($HgCl_2$), sodium hypochlorite (NaClO), and ethyl alcohol (C_2H_5OH) were utilized as disinfectants at various times. The efficiency of the tested disinfection procedures in reducing browning and contamination rates is shown in Figs. 1A and B. The browning rate ranged between 0 and 87%; the highest values were found in the A4 treatment (75% C_2H_5OH for 2 min + 20% NaClO for 20 min). The efficiency of A5 and A6 in reducing the browning rate was very similar, with browning rates of 20 and 25%, respectively. The contamination rate ranged between 27 and 100%; the highest values were found in the control, while the lowest values were found in A7 (75% C_2H_5OH for 2 min + 0.1% $HgCl_2$ for 20 min). The experimental results indicated that disinfection method A6 (75%

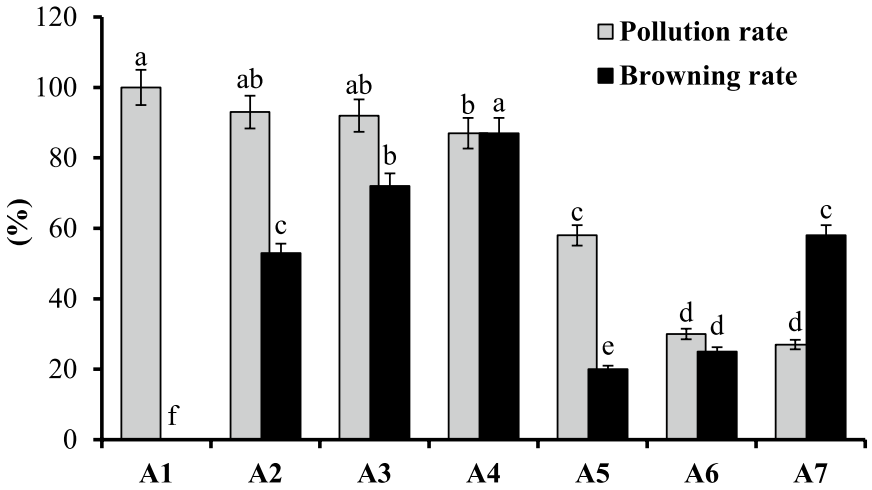

Fig. 1 Effects of different disinfection procedures on the browning and pollution rate (percentage) in water lily. Means ± standard deviations with various letters indicate significant differences at $p < 0.05$

C_2H_5OH for 2 min + 0.1% $HgCl_2$ for 15 min) was the most suitable approach for sterilizing water lily rhizomes, with a contamination rate of 30% and a browning rate of 25%. Although the contamination rate of A7 (75% C_2H_5OH for 2 min + 0.1% $HgCl_2$ for 20 min) was the lowest (27%), the browning rate was alarmingly high, reaching 58%. The use of sodium hypochlorite as a disinfectant in A2, A3, and A4 resulted in a high contamination rate and severe browning of the explants. Increasing the time for all disinfection treatments led to an increase in the browning rate. Therefore, mercuric chloride emerged as the more suitable disinfectant for water lily rhizomes for only 15 min. The findings of this research indicated that A6 is the optimal method for sterilizing water lily rhizomes.

Influence of various plant growth regulators on the induction of water lily explants

Different induction compounds were added to Murashige and Skoog (MS) culture medium to test their ability to induce water lily (Table 5). Compared with the control, the tested compounds, i.e., 6-benzylaminopurine (6-BA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA), had significant effects (Fig. 2 A and B). The induction rate ranged between 37 and 77%; the lowest values were found in the control, whereas the highest values were found in B2, which contained IBA. The results indicate that the plant growth regulator IBA is the most suitable hormone for inducing water lily rhizomes, with an induction rate reaching 77% and the presence of two or more adventitious shoots. In contrast, the control group, as well as the IAA and NAA treatments, yielded some shoots, mostly singular in number, and their growth vigor was not comparable to that of the shoots treated with IBA. Consequently, IBA emerged as the optimal plant growth regulator for the induction of water lily rhizomes.

Influence of 6-benzylaminopurine (6-BA) on the proliferation of water lily shoots

The growth regulator 6-benzylaminopurine (6-BA) was added to the culture medium at doses ranging from 1 to 5 mg L^{-1} (Fig. 3 A and B, Table 6).

Table 5 Influence of various plant growth regulators on the induction of water lily explants

Treatments	Bud growth
B1	After 15 days, a single, fragile shoot with a slow growth rate had appeared, lacking any clustered shoots
B2	After nine days, sprouts with two or more strong shoots that were growing quickly began to appear
B3	After a span of twelve days, solitary shoots began to emerge, devoid of clustered growth, exhibiting a more gradual development compared to the vigor of B2, with the seedlings manifesting a diminutive stature
B4	One branch appeared within 10 days, and some of them produced 1–2 buds. These seedlings had a slender and elongated shape

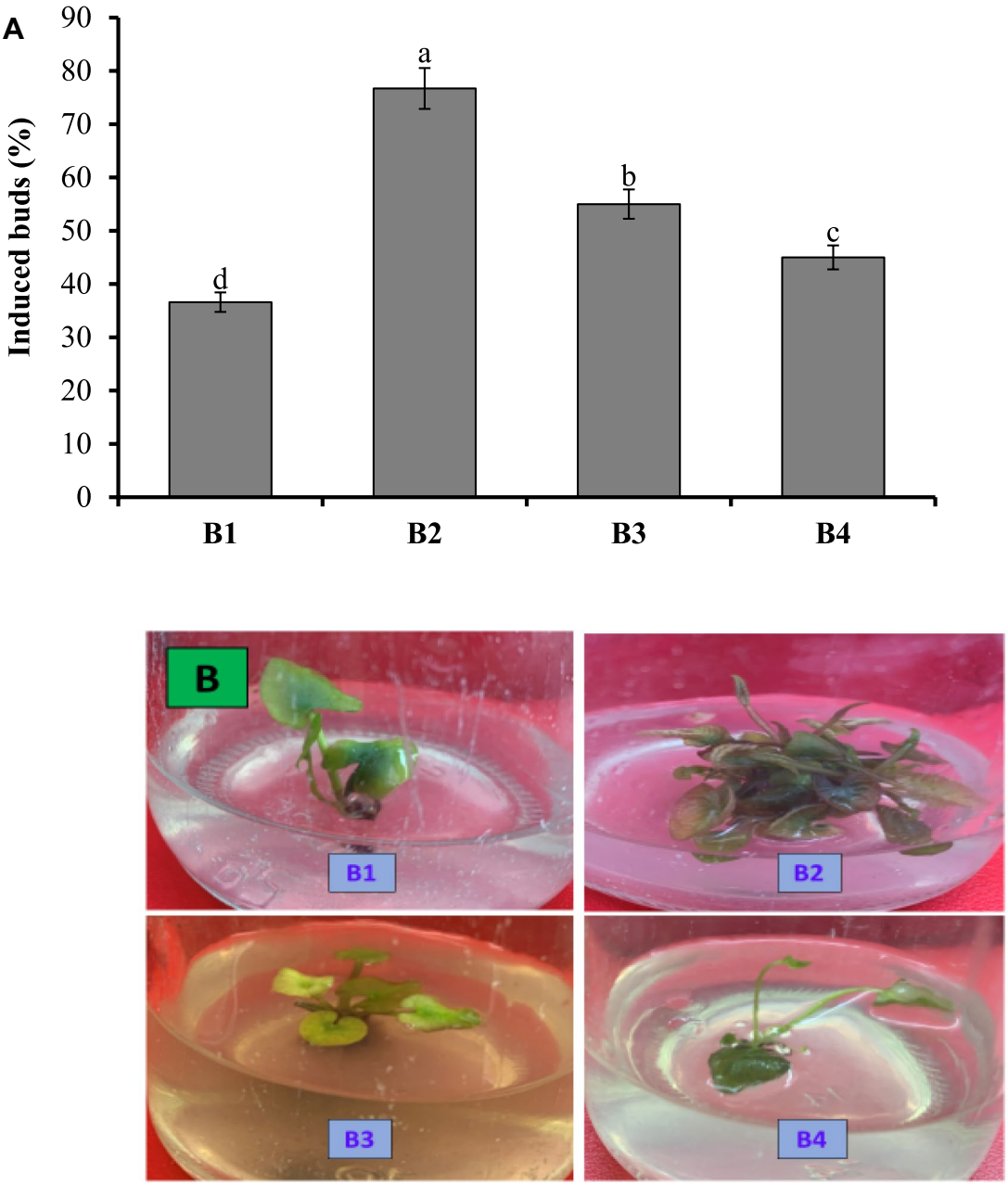

Fig. 2 The influence of various plant growth regulators on the induction of *Nymphaea tetragona* explants. **A**: Graphs indicating the induction (percentage) and **B**: photographs of the tested treatments. Means ± standard deviations with various letters indicate significant differences at $p < 0.05$

These results indicate that C2 and C3, which contain 2 and 3 mg/L of 6-BA, respectively, were more suitable for water lily proliferation, with induction rates reaching 77% and 80%, respectively. The medium formulation of C3, which contains 3 mg L^{-1} of 6-BA, was more suitable for the induction of adventitious shoots in water lily, with an induction rate reaching 80% and a yield of 2 to 8 shoots. As the concentration of 6-benzylaminopurine (6-BA) increased, the induction rate also increased.

However, once the 6-BA concentration reached a certain level, the induction rate decreased. Moreover, higher 6-BA concentrations induced tissue damage to the shoots, potentially resulting in their death. The number of regenerated shoots decreased, tissue damage increased,

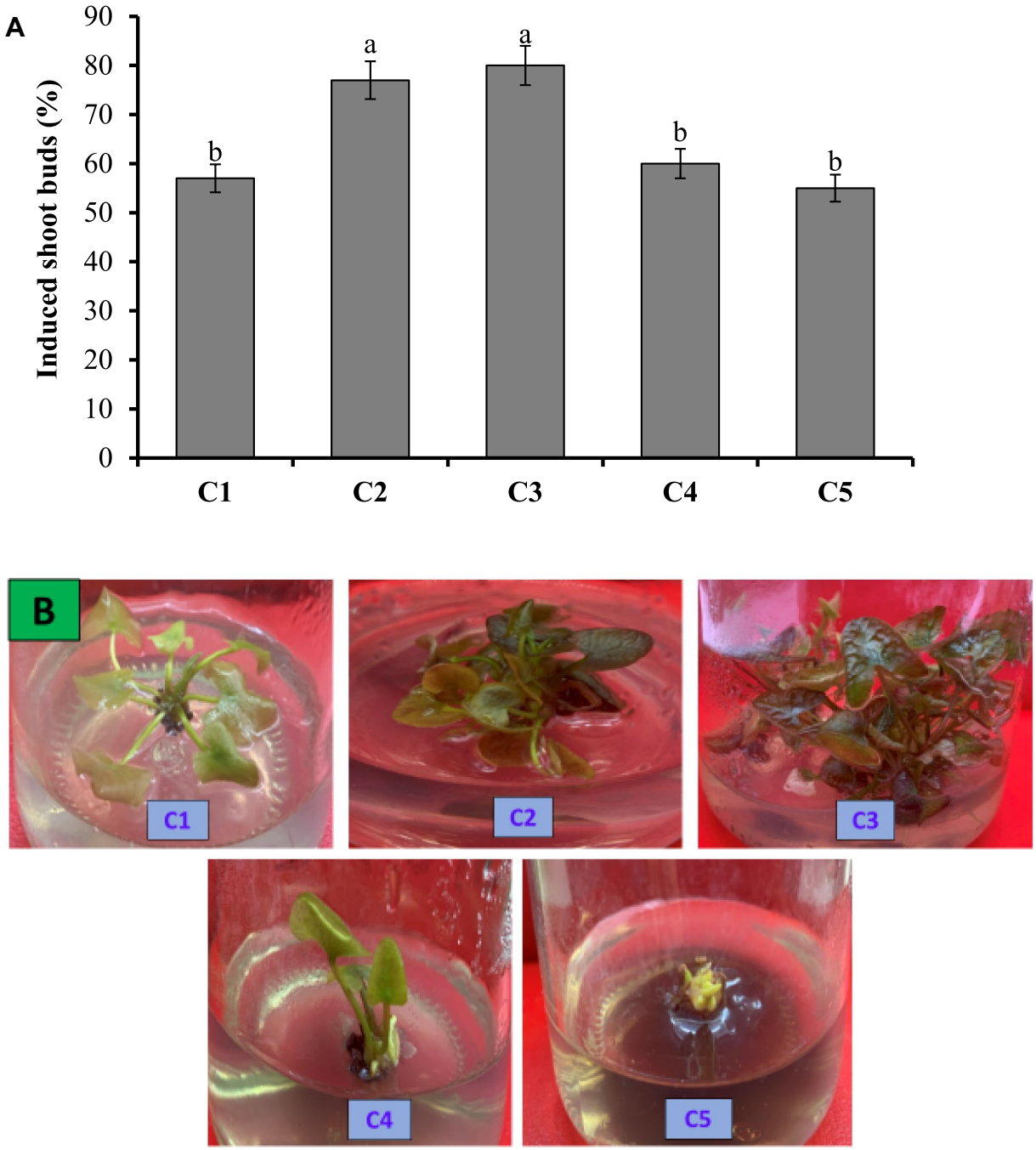

Fig. 3 The influence of 6-benzylaminopurine (6-BA) on the proliferation of *Nymphaea tetragona* shoots. **A**: Graphs indicating the induction of shoot buds (percentage) and **B**: photograph of the tested treatments. Means ± standard deviations with various letters indicate significant differences at $p < 0.05$

and growth vigor was weakened when the concentration of 6-BA was greater than 4.0 mg L^{-1}. Thus, the optimal proliferation culture medium formulation consists of MS + 6-BA (3.0 mg L^{-1}) + IBA (0.5 mg L^{-1}) + S106.

Influence of various plant growth regulators (PGR) and their concentrations on the rooting of water lily tissue-cultured seedlings

Water lily rooting was improved by the application of indole-3-butyric acid (IBA) and naphthaleneacetic

acid (NAA) at various doses (Fig. 4 and Table 7). These results indicate that IBA is the most effective stimulant for root development in water lily. The use of IBA as a root-inducing hormone resulted in the proliferation of robust and vigorously flourishing root systems in tissue culture-generated water lily seedlings. Specifically, tissue culture-generated water lily seedlings cultivated on medium D3, which contained IBA at a concentration of 0.5 mg L^{-1}, exhibited rapid root elongation. After just nine days, the roots started to grow, yielding 5–10 strong roots that stood out for their significant length and girth. These seedlings displayed vigor and flourished,

Table 6 Influence of varying concentrations of 6-benzylaminopurine (6-BA) on the proliferation of water lily shoots

Treatments	Bud growth
C1	The burgeoning shoots showed no signs of injury; there were 2–3 thriving shoots, exhibiting robust growth
C2	The burgeoning shoots showed no signs of injury; there were 2–5 thriving shoots, exhibiting robust growth
C3	The burgeoning shoots showed no signs of injury; there were 3–8 thriving shoots, exhibiting robust growth
C4	The cluster of shoots exhibited occasional callus formation, typically comprising 1 to 2 shoots, with a generally moderate growth trajectory
C5	The proliferation of shoots led to increased injury, with the emergence of one to two such shoots. These shoots had poor growth vigor, and injury-induced necrosis and browning lead to their death

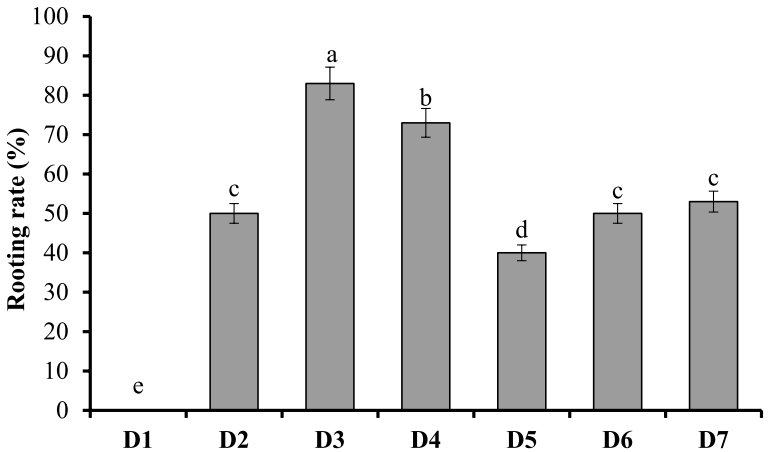

Fig. 4 The influence of various botanical growth regulators and their concentrations on the rooting of tissue culture-generated water lily seedlings. Means ± standard deviations with various letters indicate significant differences at $p < 0.05$

Table 7 The influence of various botanical growth regulators and their concentrations on the rooting of tissue culture-generated water lily seedlings

Treatments	Rooting formulation and growth
D1	Despite the seedlings growing nicely, none of them rooted
D2	Twelve days later, a major root and one to two subsidiary roots had developed. Although the seedlings'primary roots were very short, they had a strong potential for growth
D3	After nine days, a root system with five to ten thick, aggressively growing roots that were notable in both length and girth developed. All of these are signs of healthy energy and good progress
D4	After 9 days, a primary root emerged, typically with four to six subsidiary roots, exhibiting slight swellings. Seedling growth was generally moderate
D5	After a fortnight, the roots emerged with one to two delicate tendrils. These roots were slender and short in nature, but the sapling robustly flourished
D6	After 12 days, 1–3 slender roots formed, exhibiting fine root structures, and the seedlings displayed robust growth
D7	After 12 days, a primary root had emerged, typically with 1–3 secondary roots. These roots were slender, and the seedling growth rate was generally moderate

with exemplary growth vitality. Consequently, D3, which contains MS + IBA at a concentration of 0.5 mg L^{-1}, emerged as the superlative medium for fostering optimal root development. On the other hand, the use of NAA as the root-inducing hormone for tissue culture-generated water lily seedlings resulted in the production of a small number of roots that are relatively short and narrow and were characterized by sluggish root development. This observation substantiates the assertion that IBA is the most judicious choice as a root-inducing hormone for tissue culture-generated water lily seedlings. In summary, the ideal medium for inducing root development in tissue culture-generated water lily seedlings is unequivocally D3, which contains MS + IBA at a concentration of 0.5 mg L^{-1}.

Performance of water lily seedlings resulting from the best tissue culture techniques

In this investigation, the most effective techniques for rooting, bud induction, shoot multiplication, and sterilizing water lilies were tested. The regeneration percentage from rhizome as explant was 75%. The water lily seedlings resulting from the best tissue culture techniques in the current study were cultivated to produce completely healthy plants. The results of the cultivation process are shown in Fig. 5. The percentage of regenerated plants successfully acclimatized was 90%. The best methods identified in the present study succeeded in producing complete plants with good, strong growth.

Discussion

The best tissue culture methods used in the present study produced water lily seedlings that were then grown into full, healthy plants. The water lily is an aquatic plant that lives in humid conditions. Therefore, sterilization techniques and growth-stimulating materials that are appropriate for the nature of the plant must be chosen. In vitro culture-based plant biotechnology requires contamination-free culture [22]. The most effective sterilization method may vary depending on the type of plant because different plants have different bacteria dwelling in the tissue culture-relevant area [23–25]. The results of the present study confirmed the high efficiency of mercury chloride over sodium hypochlorite (Fig. 6). Sodium hypochlorite resulted in a high contamination rate and severe browning of the explants, whereas mercuric chloride emerged as a more suitable disinfectant for water lily rhizomes. Sodium hypochlorite and mercuric chloride are widely used disinfectants in plant tissue culture, with NaOCl acting through oxidation and membrane disruption, while $HgCl_2$ denatures proteins and inhibits enzymes; however, their efficiency varies depending on microbial load, plant tissue sensitivity, and environmental safety concerns [22–26].

Proteins and metabolites that affect plant tissues and change the content and/or pH of the culture medium can be released by contaminants [23–25]. Using disinfectants to reduce the activity of microbes and reduce their secretion into the environment provides an opportunity for the success of the tissue transplantation process [25]. Huasha et al. [13] conducted sterilization experiments using various parts of water lilies, including rhizomes, roots, leaves, leaf stalks, and seeds, as explants. The results of Huasha et al. [13] indicated that rhizomes and seeds yielded more favorable sterilization outcomes. Nonetheless, even when rhizomes were used as explants, contamination rates remained as high as 70%, with a browning rate of up to 45% [13]. However, the findings of the present study revealed that the rhizomes of water lily sterilized with 75% C_2H_5OH for 2 min + 0.1% $HgCl_2$ for 15 min presented the lowest browning and contamination rates. The choice of sterilization method is determined based on the type of plant [22–24].

The most prevalent auxins for many species are indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA) [27, 28]. Our research revealed that IBA is the best plant growth regulator for the induction of water lily rhizomes, with an induction rate of 77% (Fig. 6). IBA is the auxin that is most frequently employed because of its strong induction potential [28–30]. The superior efficiency of indole-3-butyric acid (IBA) in inducing water lily rhizomes can be attributed to its stability, efficient conversion to indole-3-acetic acid (IAA), targeted accumulation in rhizogenic zones, lower cytotoxicity, and its role in regulating auxin-cytokinin interactions for optimal morphogenesis [30, 31].

On the basis of the findings of the present study, the induction rate of water lily adventitious shoots can approach 80% when 3 mg L^{-1} of 6-BA is added to the medium, resulting in the production of 2 to 8 shoots. One of the cytokinin types that is most frequently used in shoot propagation is 6-benzylaminopurine (6-BA), which is strong, resistant to deterioration, and readily available [32, 33]. Cytokinin promotes cell proliferation and plant regeneration by promoting the sprouting of calluses into shoots; however, an excessive amount can be harmful to plant tissue [33, 34]. In general, 6-BA is favored over other cytokinin types [32, 33]. Although cytokinin in the medium is required for proliferation, doses that enhance a rapid increase in shoot number can hinder shoot elongation [33, 34]. The best doses to promote an increase in shoot length and number are 1 and 3 mg L^{-1}, while the ideal concentrations vary depending on the type of plant [32, 34, 36]. Our findings showed that 2.0 to 3.0 mg L^{-1}

Fig. 5 Water lily tissue culture technology

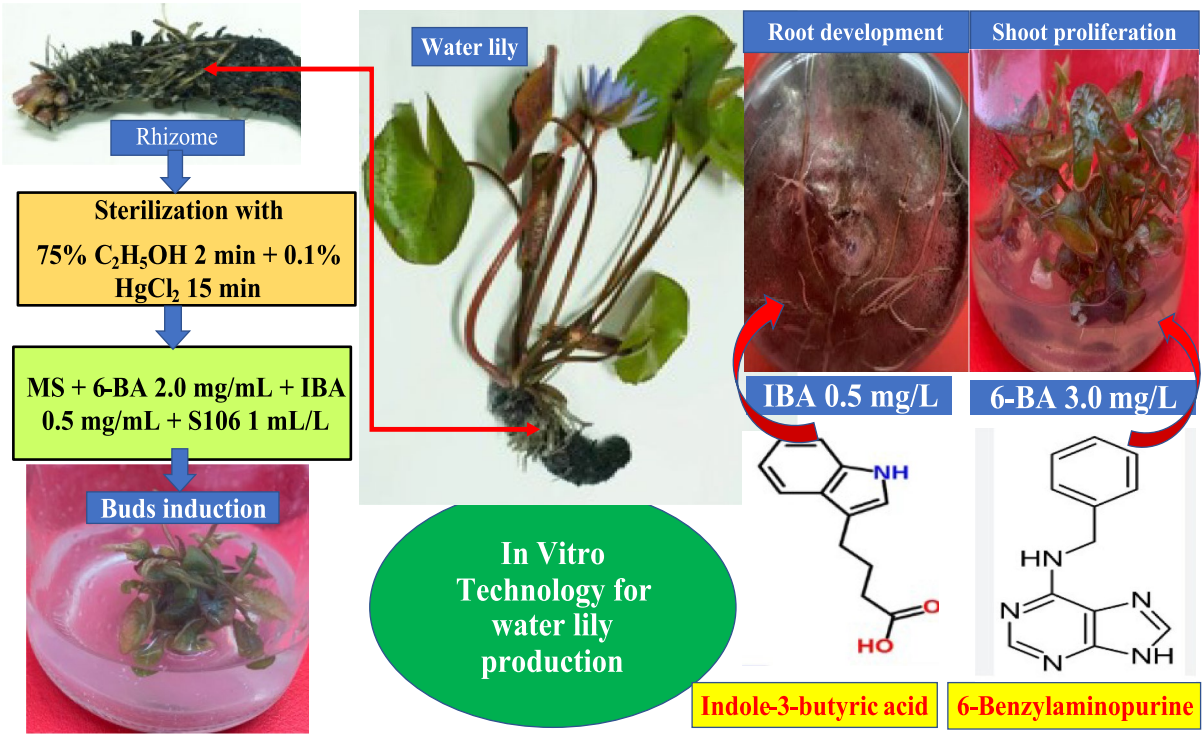

Fig. 6 Schematic diagram exploring water lily cultivation techniques

of 6-BA was consistently effective at promoting water lily proliferation in in vitro tests. A critical stage in micropropagation, adventitious root production influences how well a plant performs once it is planted in soil [28, 37, 38]. The evaluated plants, i.e., water lily, produced a high percentage of rooted shoots and a high-quality root system after successful rooting treatment using IBA and NAA. Different auxins, particularly IBA and NAA, have been employed to achieve rooting in previous studies, e.g., Justamante et al. [29], De Klerk et al. [37], and da Costa et al. [38]. According to the results of this investigation, IBA at a dose of 0.5 mg L^{-1} is clearly the best for promoting root development in tissue culture-generated water lily seedlings. IBA is the auxin that is most frequently utilized because of its strong induction capability [28, 29]. The results revealed that IBA is the best rooting hormone to stimulate rooting in water lily, and the results are consistent with those of other studies [28, 29, 39].

Conclusion

Water lily (*Nymphaea micrantha* Guill. & Perr) is an aquatic plant valued for its nutritional and medicinal uses. However, limited knowledge regarding its propagation and cultivation has hindered its widespread utilization. This study aimed to address this gap by evaluating tissue culture techniques for water lily rhizomes, focusing on sterilization, induction, proliferation, and rooting. Laboratory experiments were conducted to determine optimal methods and materials for efficient micropropagation. The results demonstrated that disinfection with 75% ethanol (C_2H_5OH) for 2 min followed by 0.1% $HgCl_2$ for 15 min yielded the lowest contamination (30%) and browning (25%) rates. Indole-3-butyric acid (IBA) was identified as the most effective plant growth regulator for rhizome induction, while a medium containing 3 mg L^{-1} 6-benzylaminopurine (6-BA) resulted in an adventitious shoot induction rate of approximately 80%, producing 2 to 8 shoots per explant. Rooting was best achieved with 0.5 mg L^{-1} IBA, leading to rapid root elongation. These optimized tissue culture techniques enabled the successful propagation of water lily plants from tuberous rhizomes to flowering plants. While this study presents an effective method for water lily micropropagation, certain limitations should be acknowledged. The observed contamination rate of 30% indicates that further refinement of sterilization protocols may be necessary to enhance explant survival. Additionally, the study was conducted under controlled laboratory conditions, and the field performance of tissue-cultured plants requires further evaluation. Future research should explore genetic stability, acclimatization efficiency, and large-scale

propagation feasibility. By establishing a reliable propagation technique, this study provides a foundation for the conservation and commercial cultivation of N. micrantha, potentially reducing cultivation costs and increasing its accessibility for various applications.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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Not applicable.

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The authors declare no competing interests.

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