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Establishment of the *In Vitro* Culture and Plant Regeneration of *Anubias barteri* var. *nana* 'Mini'

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Anubias barteri **is an ornamental aquatic plant of economic importance worldwide. Effective removal of contaminants has been a major problem for the *in vitro* propagation of *A. barteri*. Hydrogen peroxide (H_2O_2) was applied as a pre-disinfectant to treat rhizome bud explants of *A. barteri* var. *nana* 'Mini' followed by disinfection with mercuric chloride ($HgCl_2$) to eliminate *in vitro* contamination. However, application of 0.1% $HgCl_2$ for 5 min without pre-disinfecting with H_2O_2 significantly reduced the contamination rate to 44.4% compared to other combinations. Moreover, *in vitro* shoots of *A. barteri* var. *nana* 'Mini' were used as plant materials for determining the basal requirement of inorganic minerals. Optimal plant growth was achieved on 1/2 Murashige and Skoog (MS) basal medium. Rhizome bud explants were cultured on 1/2 MS basal medium supplemented with 6-benzyaminopurine (BAP) or thidiazuron (TDZ) in combination with α-napthaleneacetic acid (NAA) to screen for the optimal combinations for shoot proliferation. BAP or TDZ alone at 1 mg L^{-1}, and the combination of 0.5 mg L^{-1} TDZ and 1 mg L^{-1} NAA showed significantly higher shoot proliferation rate ranged from 2.4 to 2.7-fold after being cultured for 5 weeks. *Ex vitro* plantlets after acclimatization adapted well in aquarium. Successful plant regeneration of *A. barteri* var. *nana* 'Mini' was established through direct shoot organogenesis from rhizome buds and could be used for mass propagation.**

Keywords: *Anubias barteri*, aquatic plant, *in vitro* contamination, mass propagation, plant growth regulator

Abbreviations: BAP - benzyaminopurine, MS - Murashige and Skoog, NAA - napthaleneacetic acid, TDZ - thidiazuron

INTRODUCTION

The genus *Anubias*, Araceae family, consists of many varieties that are commercially cultivated for aquariums, and possess the potential for export market development (Kanchanapoom et al. 2012). *Anubias barteri* var. *nana* 'Mini', a miniature variety, can grow over a wide range of light intensities without supplemental carbon dioxide and is as undemanding and robust as the wild type (Walstad 2003). However, export quarantine and stable production of aquascaping plants have limited the development of the aquatic plant industry (Barpete et al. 2019). *Anubias* can be propagated vegetatively using rhizome division; however, the proliferation rate is low and inefficient for commercial production. Micropropagation is currently applied to aquatic plants as a tool for large-scale multiplication of elite plants (Carter and Gunawardena 2011; Sereda et al. 2017). Nevertheless, information concerning details of media and growth regulator amendments is still a fundamental requirement for the intense commercial production of *A. barteri*.

Effective removal of contaminants has been a major problem for the *in vitro* propagation of aquatic plants. The most frequently used disinfectants for killing microbes on explants are hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), and sodium hypochlorite (NaOCl). The disinfection process was applied on shoot tip explants of *A. barteri* var. *nana*, including disinfection with 0.5% $HgCl_2$ for 3 min, followed by 5.25% NaOCl for 15 min and lastly 2.6% NaOCl for 5 min (Kanchanapoom et al. 2012). However, less information is available on the efficiency of these disinfectants for the surface disinfection of microorganisms naturally harbored on *A. barteri* and its varieties (Barpete et al. 2019). Thus, we evaluated the supplementary effects of H_2O_2 as a pre-

disinfectant followed by $HgCl_2$ on rhizome bud explants of *A. barteri* var. *nana* 'Mini' to decrease *in vitro* contamination and maintain the regenerative capacity. The hypothesis was that H_2O_2 could work synergistically with $HgCl_2$ to optimize the disinfection efficiency and explant viability. In addition, *in vitro* shoots of *A. barteri* var. *nana* 'Mini' were also used as explants and cultured in different strengths of Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) to assess mineral requirements. Half-strength MS basal medium was supplemented with 6-benzyaminopurine (BAP) or thidiazuron (TDZ) in combination with α-naphthaleneacetic acid (NAA) to screen for the optimal combination of PGRs for shoot proliferation. Substrates used for *ex vitro* acclimatization significantly affect the transition of *in vitro* aquarium plant from photomixotrophic to photoautotrophic growth (Pinker et al. 2007). Therefore, the effects of three different supporting substrates on *ex vitro* acclimatization of *A. barteri* var. *nana* 'Mini' were also evaluated in the study.

The aim of this study was to develop an efficient disinfection method of rhizome explants for *in vitro* culture of *A. barteri* var. *nana* 'Mini' and optimize the basal requirements of inorganic minerals and plant growth regulators (PGRs) for plantlet regeneration and mulitplication. In addition, we also investigated appropriate aquarium substrates for the adaptation of plantlets during acclimatization after *ex vitro* transfer. Optimization of *in vitro* and *ex vitro* culture conditions may facilitate the development of a protocol for the efficient mass propagation of *A. barteri* var. *nana* 'Mini'.

MATERIALS AND METHODS

Plant Materials

In the study, young plantlets of *A. barteri* var. *nana* 'Mini', grown in plastic pots (5.5 cm in diameter × 5.0 cm in height) containing rockwool, were purchased from local aquarium gardens in Taipei, Taiwan.

Disinfection Conditions and Establishment of *In Vitro* Cultures

The whole plants (Fig. 1A) were cleaned with running tap water and thoroughly soaked in 2.5 mg L^{-1} of glutaraldehyde (Alfa Aesar, Haverhill, MA, USA) for 1 week at 50 rpm in an orbital shaker, followed by surface sterilization with 70% ethanol (v/v) for 30 s. Any damaged rhizome tissues were excised with a scalpel and the processed rhizome materials were blotted on paper towels.

We designed a two-stage disinfection experiment for the sterilization of rhizome explants. In the first stage, the cut rhizomes were disinfected with 0% or 5% H_2O_2 (Sigma -Aldrich, St. Louis, MO, USA) for 5 min by hand agitation, rinsed three times with ddH_2O. Continuously, in the second stage, pre-disinfected rhizomes were disinfected with 0%, 0.05%, or 0.1% $HgCl_2$ containing 0.01% Tween 20 for 5 min, and rinsed three times again with ddH_2O. The condition of 0% H_2O_2 and 0% $HgCl_2$ was used as control. Each disinfection treatment consisted of seven to ten replicated Petri dishes, with six explants per replicate.

Rhizome bud segments with a length of 3~5 mm were dissected from the disinfected rhizome as explants. Explants were cultured on shoot induction medium which consisted of ½ MS basal medium with 2 mg L^{-1} of BAP and 0.5 mg L^{-1} NAA, which was modified from Huang et al. (1994) and Kanchanapoom et al. (2012). Half-strength MS basal medium contained 1/2-strength MS salts and full-strength vitamins (Murashige and Skoog 1962), 100 mg L^{-1} myo-inoisitol, 2 mg L^{-1} glycine, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 0.1 mg L^{-1} thiamine HCl, 30 g L^{-1} sucrose, and 2.5 g L^{-1} gelrite. The *pH* of the medium was adjusted to 5.7, and the medium was dispensed as 20 mL aliquots into Petri dishes after autoclave for the disinfection experiments.

Explants were cultured in a growth chamber (CH-202-A, Chin-Hsin, Taipei, Taiwan) at 25 ± 1°C with a light intensity of 64 μmol m^{-2} s^{-1} provided by cool white fluorescent lights, a 12-h photoperiod, and 70% relative humidity. Visible contamination of the explants and shoot numbers were recorded after *in vitro* culture for 3 weeks. The percentage of contaminated explants was calculated as the number of contaminated explants divided by the total number of explants.

Effects of MS Mineral Nutrition on Shoot Development and Plant Growth

To evaluate the optimal MS salt strength of the basal medium for shoot development, full, 1/2-, 1/4-, and 1/8-strength MS salt solutions were examined, and the other components of the medium were as previously described. The medium was dispensed as 15 mL aliquots into test tubes, and 15 explants were used in each treatment. Shoot materials were induced from rhizome bud explants on 1/2 -MS basal medium containing 2 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, and these were used as explants. Numbers of shoots, roots, and leaves were recorded after culture of 6 weeks under various concentrations of MS salt. Plant width was defined as the maximum width of the entire plantlet.

Effects of Plant Growth Regulators on Shoot Proliferation and Root Formation

After 6 weeks of the culture period, well-developed shoots were obtained, and all explants were subcultured at 4 week intervals. Rhizome bud explants with a length of 2~3 mm were cut and cultured on 1/2 strength MS medium supplemented with 0, 0.5, or 1.0 mg L^{-1} NAA combined with either TDZ (0, 0.5, or 1.0 mg L^{-1}) or BAP (0, 0.5, 1.0, or 2 mg L^{-1}). One explant was placed in each test tube with 15 mL aliquots of medium, and 10 or 20 explants were cultured for each treatment. Culture conditions in the growth chambers were as described above. The rate of shoot proliferation and root formation was calculated as the number of explants with shoot proliferation and root formation divided by the total number of explants, respectively.

Acclimatization

In vitro plantlets of at least 1.5 cm in height and 5 leaves and 3 roots were randomly cultivated in three different substrates, including premium soil (ISTA, Tzong Yang Aquarium, Taipei, Taiwan), sphagnum moss (Hsien-Chinis, Chile) and hydroponic cotton, and water was used as control. Each treatment consisted of 15 replicates and each replicate contained one plantlet. Prior to *ex vitro* acclimatization, plantlets were carefully cleaned and the residual media were removed to prevent pathogenic contamination. The cleaned plantlets were then planted in water saturated substrates and covered with a transparent plastic cover to maintain a high relative humidity. After six weeks of *ex vitro* acclimatization, the cover was removed, and the plants were kept in the greenhouse and watered once a day. Plant growth parameters, including plant height and width and leaf length and width, were recorded.

Statistical Analysis

All experiments were arranged in completely randomized designs. Data were subjected to analysis of variance (ANOVA), with a significance level of $p < 0.05$ using CoStat statistical software (Cohort Berkeley, Monterey, CA, USA). However, data (expressed as percentages) were transformed using arcsine square root transformation prior to ANOVA and converted back to original scale (Compton, 1994). Synergistic effects of H_2O_2 and $HgCl_2$ on disinfection of the rhizome bud explants were analyzed by a two-factor completely randomized ANOVA. Significant differences were determined using the least significant difference (LSD) test at $p < 0.05$, 0.01, or 0.001.

RESULTS

Effect of Pre-disinfection with H_2O_2 on the Sterilization Efficiency of $HgCl_2$

ANOVA results of the main effects of H_2O_2, $HgCl_2$, and their interaction effect on rhizome bud explants are summarized in Table 1. Contamination rates significantly differed at the 0.05 and 0.01 significance levels for $HgCl_2$ treatment alone and the interaction effect, respectively. However, the main effect of treatment with H_2O_2 was not significant at eliminating contaminants *in vitro* (Table 1). When disinfected with 0.05%, 0.1%, or 0.5% $HgCl_2$ either pre-disinfection with H_2O_2 or not, *in vitro* contamination of rhizome bud explants significantly decreased to lower levels, ranging from 44.4% ~ 95.2% (Table 2). In particularly, sterilization of 0.1% and 0.5% $HgCl_2$ without pre-disinfection with H_2O_2 significantly decreased *in vitro* contamination rates to 44.4% and 52.1%, respectively. Furthermore, all explant tissues were contaminated when disinfected without $HgCl_2$. Apparently 5% H_2O_2 and 0.05% $HgCl_2$ had slight synergistic effects on eliminating contaminants for *in vitro* culture.

Effect of MS Mineral Nutrients on Shoot Development of *A. barteri* var. *nana* 'Mini'

For shoot development of *A. barteri* var. *nana* 'Mini', four different ionic strengths of MS medium were used, and results are shown in Table 3. The highest and lowest

Table 1. Disinfection efficiency of *Anubias barteri* var. *nana* 'Mini' affected by H_2O_2, $HgCl_2$, and their interaction based on

Source of Variance	Degree of Freedom	MS	*F value*	*P value*
H_2O_2	1	689.2	0.98	0.33 ^{NS}
$HgCl_2$	3	7736.2	10.9	<0.0001***
H_2O_2 x $HgCl_2$	3	2145.8	3.04	0.04*

NS, non-significant

Table 2. Disinfection effects of H_2O_2 and $HgCl_2$ on *Anubias barteri* var. *nana* 'Mini'.

H_2O_2 (%)	$HgCl_2$ (%)	Contamination Rate (%) [1]
0	0.00	100.0±0.0^{a}
0	0.05	95.2±2.7^{b}
0	0.10	44.4±13.3^{c}
0	0.50	52.1±12.8^{c}
5	0.00	100.0±0.0^{a}
5	0.05	81.7±10.5^{b}
5	0.10	83.3±7.0^{b}
5	0.50	52.4±13.3^{c}

[1]Percent contamination = [Number of contaminated explants/ Total number of explants] x 100

Each treatment contained seven to ten replicates in Petri dishes, with six explants each. Percentage data were arc sine transformed prior to ANOVA analysis; means ± standard errors within a column followed by different letters indicate significant differences according to Fisher's protected LSD test at $p < 0.05$.

Table 3. Effects of levels of Murashige and Skoog (MS) mineral salts on plant growth of *Anubias barteri* var. *nana*

MS Strength	Shoot Number	Leaf Number	Root Number	Plant Width (cm)
Full MS	1.4 ± 0.2[b1]	6.4 ± 0.4[a]	4.3 ± 0.4[b]	1.8 ± 0.1[a]
1/2 MS	1.9 ± 0.2[a]	6.8 ± 0.4[a]	6.8 ± 0.6[a]	1.8 ± 0.1[a]
1/4 MS	1.6 ± 0.2[ab]	6.3 ± 0.5[a]	6.6 ± 0.5[a]	1.4 ± 0.1[b]
1/8 MS	1.8 ± 0.2[a]	6.7 ± 0.3[a]	5.0 ± 0.4[b]	1.2 ± 0.1[c]

[1] Different letters within each column of the same culture time indicate significantly different means at $p < 0.05$ by Fisher's protected LSD test. n = 15.

numbers of shoots (1.9 ± 0.2 and 1.4 ± 0.2) and roots (6.8 ± 0.6 and 4.3 ± 0.4) were observed in 1/2 MS medium and full strength MS media, respectively. Leaf numbers were unaffected by the concentration of MS, however, the plant width significantly decreased with lowering the concentration of MS minerals. Half strength of MS medium showed optimal shoot, leaf, and root development, and whole plant width compared to other levels of inorganic MS salts. Subsequently, 1/2 MS basal medium containing half-strength inorganic MS minerals was used as the basal medium for the shoot proliferation study.

Effects of Plant Growth Regulators on Shoot Proliferation and Root Formation of Rhizome Bud Explants of *A. barteri* var. *nana* 'Mini'

Combination of TDZ or BAP with NAA significantly affected shoot and root formation, and leaf numbers of rhizome bud explants of *A. barteri* var. *nana* 'Mini'. Table 4 revealed that shoots were formed at a high frequency of 100% on 1/2 MS medium supplemented with 0.5 and 1 mg L^{-1} BAP or 0.5 mg L^{-1} TDZ alone or treatments with 1 mg L^{-1} NAA combined with 0.5 or 1 mg L^{-1} TDZ. The composition of 1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP showed the lowest shoot proliferation rate (40%) and shoot number (0.5). The maximum number of 2.4 ~ 2.7 shoots per explant was obtained on treatments with 1 mg L^{-1} BAP (Fig. 1B), 0.5 or 1 mg L^{-1} TDZ alone, and the combination of 1 mg L^{-1} NAA and 0.5 mg L^{-1} TDZ. Roots formed at frequencies of 10% ~ 60% on all treatments, except that no roots were observed on 1/2 MS medium supplemented with 1 or 2 mg L^{-1} BAP alone. The combination of 0.5 mg $^{-1}$ NAA and 0.5 mg L^{-1} BAP and the control treatment resulted in the highest number of roots formation. Number of roots decreased when 1/2 MS medium with NAA concentration increased up to 1.0 mg L^{-1} without BAP or TDZ. Nevertheless, root numbers increased when 1/2 MS medium with NAA concentration increased up to 1.0 mg L^{-1} combined with 1.0 or 2.0 mg L^{-1} BAP. A significant difference in the number of fully expanded leaves was detected in the control (1.8 ± 0.4) and treatment with 0.5 mg L^{-1} of BAP (1.7 ± 0.4) compared to other treatments. No fully expanded leaves were observed with treatments of 1.0 mg L^{-1} NAA combined with 0.5 and 1 mg L^{-1} BAP or 0.5 mg L^{-1} TDZ or the combination of 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} TDZ. In general, an increase in the NAA concentration from 0 to 1 mg L^{-1} resulted in a decrease in the number of fully expanded leaves per explant. Subsequently, multiple shoots were divided into individual shoots and subcultured on 1/2 MS basal medium, and the expanding

Table 4. Effects of plant growth regulators on shoot proliferation and root formation of rhizome bud explants of *Anubias barteri* var. *nana* 'Mini'.

NAA (mg L^{-1})	BAP (mg L^{-1})	TDZ (mg L^{-1})	Shoot Proliferation Rate (%)	Shoot Number	Root Formation Rate (%)	Number of Roots	Number of Fully Expanded Leaves
0.0	0.0	0.0	85.0±8.2[ab]	1.5±0.2[ab]	50.0±11.5[a]	1.4±0.5[a]	1.8±0.4[a]
0.5	0.0	0.0	65.0±10.9[abc]	1.1±0.2[b]	35.0±10.9[ab]	0.8±0.3[b]	0.3±0.1[cd]
1.0	0.0	0.0	70.0±10.5[abc]	1.5±0.3[ab]	30.0±10.5[ab]	0.3±0.1[c]	0.3±0.1[cd]
0.0	0.5	0.0	100.0±0.0[a]	2.0±0.3[ab]	30.0±15.3[ab]	0.3±0.1[c]	1.7±0.4[a]
0.5	0.5	0.0	60.0±16.3[abc]	0.8±0.1[c]	60.0±16.3[a]	1.4±0.4[a]	0.2±0.1[d]
1.0	0.5	0.0	40.0±16.3[c]	0.5±0.1[d]	30.0±15.3[ab]	0.3±0.1[b]	0.0±0.0[e]
0.0	1.0	0.0	100.0±0.0[a]	2.4±0.3[a]	0.0±0.0[b]	0.0±0.0[d]	0.8±0.3[b]
0.5	1.0	0.0	60.0±16.3[abc]	0.7±0.2[c]	30.0±15.3[ab]	0.3±0.1[c]	0.3±0.1[cd]
1.0	1.0	0.0	70.0±15.3[abc]	0.8±0.2[c]	60.0±16.3[a]	1.2±0.4[ab]	0.0±0.0[e]
0.0	2.0	0.0	50.0±16.7[bc]	1.2±0.2[b]	0.0±0.0[b]	0.0±0.0[d]	0.5±0.2[c]
0.5	2.0	0.0	70.0±15.3[abc]	1.0±0.1[bc]	10.0±10.0[ab]	0.3±0.1[c]	0.2±0.1[d]
1.0	2.0	0.0	90.0±10.0[ab]	1.9±0.2[ab]	30.0±15.3[ab]	0.4±0.2[c]	0.2±0.1[d]
0.0	0.0	0.5	100.0±0.0[a]	2.5±0.4[a]	10.0±10.0[ab]	0.2±0.1[c]	0.3±0.1[cd]
0.5	0.0	0.5	90.0±10.0[ab]	1.3±0.3[b]	20.0±13.3[ab]	0.2±0.1[c]	0.2±0.1[d]
1.0	0.0	0.5	100.0±0.0[a]	2.7±0.5[a]	20.0±13.3[ab]	0.2±0.1[c]	0.0±0.0[e]
0.0	0.0	1.0	70.0±15.3[abc]	2.6±0.4[a]	30.0±15.3[ab]	0.3±0.2[c]	0.4±0.2[c]
0.5	0.0	1.0	70.0±15.3[abc]	1.5±0.4[ab]	10.0±10.0[ab]	0.2±0.1[c]	0.0±0.0[e]
1.0	0.0	1.0	100.0±0.0[a]	1.7±0.3[ab]	30.0±15.3ab	0.3±0.2[c]	0.2±0.1[d]

[1] Percentage data were arc sine transformed prior to ANOVA analysis; means ± standard errors within a column followed by different letters indicate significant differences according to Fisher's protected LSD test at $p < 0.05$. n = 10-20.

leaves of shoot became visible (Fig. 1C). During 4 weeks after subculture, *in vitro* shoots showed oval, erect, and dark green leaves, and then developed into healthy plantlets with vigorous root growth (Fig. 1D). The *in vitro* plantlets of *A. barteri* were successfully subcultured every 4 weeks for half a year and showed no morphological abnormalities compared to their parental plants (Fig. 1E). In addition, the plantlets developed an extensive root system.

Effects of Culture Substrates on *Ex Vitro* Acclimatization of *A. barteri* var. *nana* 'Mini'

All *in vitro* plantlets adapted and grew well after *ex vitro* acclimatization for 6 weeks, and a hundred percent survival was obtained irrespective of the culture substrates or water. The growth parameters of *ex vitro* plantlets were measured, and the progressive growth was observed after the 6 weeks of acclimatization (Fig. 2). The height and width of *ex vitro* plantlets cultured in water only were superior to other treatments (Table 5). In contrast, the average leaf number of plantlets cultured on the substrates (10.3 - 10.9) was significantly higher than those in water (7.9). Moreover, there were no significant differences in other tested growth parameters among those three substrates (premium soil, sphagnum, and hydroponic cotton), suggesting that *ex vitro* plantlets of *A. barteri* can be transplanted to aquarium directly after acclimatization.

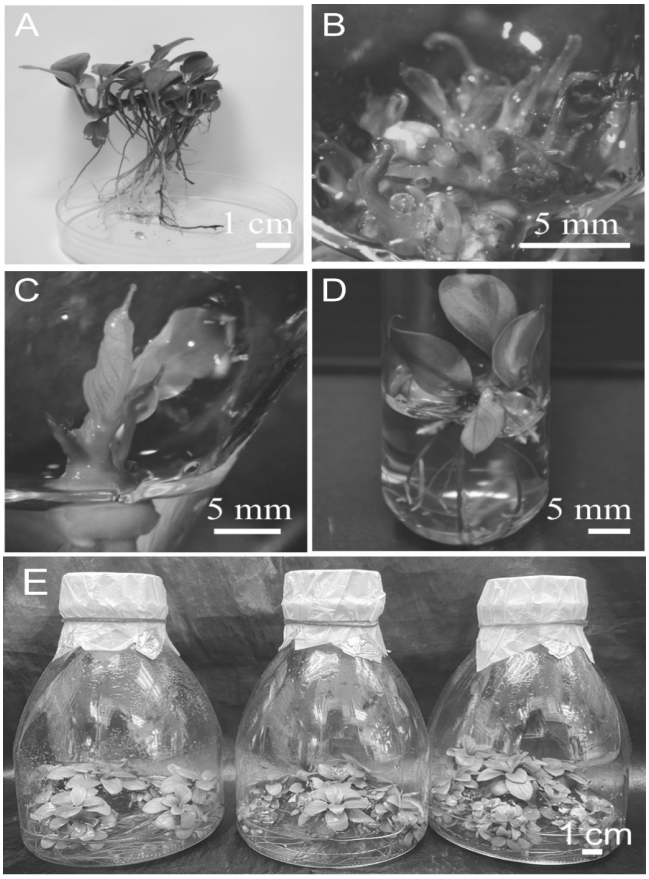

Fig. 1. Establishment of *in vitro* plant regeneration of *Anubias barteri* var. *nana* 'Mini'. (A) Plant material, (B) Multiple shoot induction from rhizome bud explants on 1/2 Murashige and Skoog (MS) basal medium containing 1 mg L^{-1} 6-benzyaminopurine (BAP) after four weeks of culture in ½ MS basal medium, (C) Expanding leaves from shoots were visible after subculturing on 1/2 MS basal medium for 1 week, (D) Root formation of shoots after subculturing on 1/2 MS basal medium for 4 weeks, (E) *In vitro* plantlets subcultured on 1/2 MS basal medium every 4 weeks for six months.

DISCUSSION

Explant disinfection is a prerequisite for *in vitro* culture of aquatic plants and different sterilizing agents or procedures have been used for different plants. Repeated disinfection procedure, including 0.5% $HgCl_2$, 5.25% NaOCl and then 2.63% NaOCl, was applied for *A. barteri* var. *nana* (Kanchanapoom et al. 2012) or higher concentration of disinfectant with 16% H_2O_2 for *Limnophilla aromatica* (Karatas and Aasim 2015). Lower concentration of disinfectant was also ultilized, including application of 0.1% $HgCl_2$ on node bud explants of *Staurogyne repens* for 5 min (Sereda et al. 2017) and 0.01% NaOCl plus 0.01% benzalkonium chloride on rhizome explants of *Marsile quadrifolia* L. for 15 min (Rolli et al. 2015). However, the sterilization efficiency of the above mentioned methods on aquatic plants has not been studied. H_2O_2 can rapidly penetrate membranes of microorganisms to trigger oxidative stress (Linley et al. 2012). H_2O_2 was used as pre-disinfectant and worked synergistically with ClO_2 to optimize the sterilization efficiency and viability of rhizome bud explants in *Zantedeschia aethiopica* L. (Chen et al. 2017). Mercuric chloride is efficient for reducing contamination and increasing the survival rate of recalcitrant explants (Moradpour et al. 2016). We combined the advantages of H_2O_2 and $HgCl_2$ to disinfect rhizome bud explants of *A. barteri* var. *nana* 'Mini', while simultaneously preventing damage to plant cells. Although pre-disinfection with 5% H_2O_2 for 5 min effectively enhanced the sterilization efficiency (52.4%) of 0.5% $HgCl_2$ for propagating *A. barteri in vitro*, those explants disinfected with 0.1% $HgCl_2$ for 5 min without pre-disinfection with H_2O_2 exhibited a low incidence of contamination (44.4%). Pre-disinfection with 5% H_2O_2 for 5 min might not supplement the sterilization efficiency with 0.1% or 0.5% $HgCl_2$, but still retained a higher viability of explants for shoot development. Disinfection with 0.1% or 0.5% $HgCl_2$ without H_2O_2 offers advantages of a high sterilization efficiency and low phytotoxicity, which can overcome disadvantages of conventional disinfection methods with hypochlorite, fungicides, or antibiotics.

Table 5. The effect of medium on *ex vitro* acclimatization of *Anubias barteri* var. *nana* 'Mini'.

Medium	Number of Leaves	Leaf Length (cm)	Leaf Width (cm)	Plant Height (cm)	Plant Width (cm)
Water	7.9 ± 0.7^{b1}	2.1 ± 0.1^{a}	1.1 ± 0.0^{a}	2.6 ± 0.2^{a}	4.2 ± 0.2^{a}
Premium soil	10.9 ± 0.7$_{a}$	1.9 ± 0.1^{ab}	1.0 ± 0.0^{a}	1.8 ± 0.1^{b}	3.9 ± 0.1^{ab}
Sphagnum	10.7 ± 1.0^{a}	1.9 ± 0.1^{b}	1.0 ± 0.0^{a}	1.8 ± 0.1^{b}	3.8 ± 0.1^{ab}
Hydroponic cotton	10.3 ± 0.9^{a}	1.9 ± 0.1^{b}	1.1 ± 0.1^{a}	1.7 ± 0.1^{b}	3.7 ± 0.2^{b}

[1] Means within each column followed by the same letter are not significantly different at $p < 0.05$ by Fisher's protected LSD test. n =20.

Full-strength MS mineral salts are used as basal medium for shoot development and proliferation in most aquatic plants, including *A. barteri* var. *nana* (Kanchanapoom et al. 2012; Sheeja et al. 2015), *A. barteri* var. *undulata* (Huang et al. 1994), *Limnophilla aromatic* (Karatas and Aasim 2015), *Lindernia antipoda* (Jabir et al. 2016), and *Staurogyne repens* (Seredaet al. 2017). However, half-strength MS minerals were used as the basal medium for *Aponogeton madagascariensis* (Carter and Gunawardena 2011) and *Marsilea quadrifolia* (Rolli et al. 2015). In our study, *in vitro* shoots of *A. barteri* var. *nana* 'Mini' showed optimal development with half-strength MS minerals compared to 1/4- and 1/8-strengths. Aquarium plants differed in their requirements of mineral elements for optimum plant growth, and full- or half-strength MS mineral salts was favored in most aquatic plants (Barpete et al. 2019).

There were differences in the effects of PGR on shoot proliferation of *A. barteri* and other aquatic plants. A high concentration of 3 mg L^{-1} BAP was used to induce direct shoot organogenesis from shoot tip explants of *A. barteri* var. *nana* with 5-fold proliferation (Kanchanapoom et al. 2012). However, for *in vitro* shoot tip culture of *A. barterivar*. *nana* 'Petite', 0.2 mg L^{-1} BAP was suggested (Sheeja et al. 2015), but for that of *A. barteri* var. *undulata*, the combination of 0.3 mg L^{-1} BAP, 0.01 mg L^{-1} TDZ, and 0.1 mg L^{-1} NAA was optimal (Huang et al. 1994). In this experiment of *A. barteri* var. *nana* 'Mini', 1 mg L^{-1} BAP or 0.5 or 1 mg L^{-1} TDZ alone, and the combination of 0.5 mg L^{-1} TDZ and 1 mg L^{-1} NAA were recommended for shoot proliferation and TDZ was more effective than BAP in inducing shoot formation.

Fig. 2. Plant morphology of *Anubias barteri* var. *nana* 'Mini' after 6 weeks of *ex vitro* acclimatization. (A) water, (B) premium soil, (C) sphagnum, (D) hydroponic cotton.

In other aquatic plants, the combination of 2 mg L^{-1} BAP and 0.5 mg L^{-1} IAA was applied on axillary nodes of *Staurogyne repens* for direct shoot organogenesis (Sereda et al. 2017). Shoot organogenesis of *Nymphoides indica* was established from petiole explants in MS basal medium supplemented with 2.3 mg L^{-1} BAP and 3.5 mg L^{-1} IAA (Jenks et al. 2000). For the shoot tip culture of *Cryptocoryne wendtii* and *C. becketti*, MS media supplemented with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IBA (Stanly et al. 2011) or 0.1 mg L^{-1} IAA (Kane et al. 1999) showed a high efficiency of shoot proliferation, different from *C. lucens*, Linsmaier and Skoog medium (Linsmaier and Skoog 1965) containing 4.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA (Kane et al. 1990). Application of 2 mg L^{-1} BAP and 2 mg L^{-1} NAA was used on immature inflorescences of *Aponogeton madagascariensis* for indirect shoot organogenesis (Carter and Gunawardena 2011). These studies indicate that lower concentration of IAA or IBA is indispensable for the optimal medium of shoot induction. However, for *A. barteri* var. *nana* 'Mini', NAA is not necessary for shoot formation, and higher concentrations of NAA seemed to inhibit leaf expansion. NAA seemed to inhibit leaf growth since higher leaf numbers were mostly recorded on culture media containing 1/2 MS medium (control) or only BAP. Interestingly, the control medium also produced the highest root numbers, indicating that the use of a PGR was ineffective, and its influence led to arrest of root formation. However, Sheeja et al. (2015) reported that maximum proliferation of the *in vitro* shoots (derived from basal buds of *A. barteri* var. *nana* 'Mini') was obtained on full-strength MS medium supplemented with 0.2 mg L^{-1} BAP. Moreover, maximum number of *in vitro* roots was found on full-strength MS medium only or supplemented with a combination of 0.1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. In the *in vitro* culture of *Limnophilla aromatic*, NAA at 0.25 ~ 1.0 mg L^{-1} promoted root formation (Karatas and Asim 2015). The reason for the adverse effect of NAA with TDZ and BAP might be due to the endogenous level of hormones within the explants, which altered the growth response, as it is well known that balanced auxin and cytokinin concentrations are required for the onset of regeneration (Skoog and Miller 1957).

Under heterotrophic conditions, the relative humidity is usually greater than 95% and *in vitro* plantlets may not

develop a waxy cuticle and functional stomata to adapt the autotropic conditions (Pospíšilová et al. 1999). Plant survival and growth of aquarium plant *Echinodorus aschersonianus* were influenced by the substrates used for *ex vitro* acclimatization (Pinker et al. 2007). However, in this study, supporting substrates had only slight effects on *ex vitro* acclimatization of *A. barteri* var. *nana* 'Mini'. The acclimatized plantlets showed a high survival rate of 100% and adapted well to the aquariums, suggesting that the vigorous growth of *A. barteri* var. *nana* 'Mini' resulted from its nature of robustness in development (Walstad 2003).

CONCLUSION

In vitro regeneration of *A. barteri* var. *nana* 'Mini' from rhizome bud explants was successfully established. The technique of *in vitro* culture and plant regeneration would be useful for the commercial mass propagation of *A. barteri* and can also be applied in conservation and restoration of other endangered species as well.

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