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In Vitro Rapid Multiplication of a Highly Valuable Ornamental Aquatic Plant *Anubias Heterophylla*

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Abstract

The present study focuses on the development of a micropropagation protocol for true to type plants of *Anubias heterophylla*, a valuable ornamental aquatic plant. Natural propagation of aquatic species is limited due to the production of a small number of plants with a long cultivation period, disease and requirement of a large space for propagation. Surface-disinfected shoot tip explants were cultured on MS medium containing 0.1 % (w/v) activated charcoal (AC) and supplemented with 6-benzylaminopurine (BAP) at different concentrations (0, 1.0, 3.0 and 5.0 mg/L) either singly or in combination with α-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) (0.5 and 1.0 mg/L). Among different auxins and cytokinin used, the number of shoots initiated per explant varied significantly among the different auxin and cytokinin combinations. The highest number of shoots per explants (3.60 ± 0.24) was formed in the culture medium containing 1.0 mg/L NAA in combination with 1.0 mg/L BAP, followed by in the culture medium with 3.0 mg/L BAP (2.40 ± 0.24). In the culture medium with 1.0 mg/L NAA and 1.0 mg/L BAP, 100 % of the cultured explants regenerated new shoots within 60 days. The maximum number of roots (19.80 ± 0.20 roots) per explant was observed after 60 days of culture on MS medium contained 0.1 % (w/v) AC and supplemented with 1.0 mg/L NAA and 1.0 mg/L BAP. With a 100% survival rate, the regenerated plantlets were effectively acclimatized to submerged underwater environments. Plantlets exhibited phenotypic traits that were comparable to those of their donor plant. This protocol is suited for commercial *Anubias heterophylla* propagation in the aquarium industry.

Keywords: Aquatic plant, Auxins, Cytokinins, Micropropagation, Plant growth regulators

Introduction

Anubias heterophylla is a popular aquaria plant. *A. heterophylla* is a typical *Anubias* species that may be found in planted aquarium tanks at medium to big proportions. *Anubias* can be found growing on rocks at stream margins, partially emerged and submerged in its natural environment, making it an excellent choice for aquarium tanks and terrarium-like, emerged settings. *A. heterophylla*, like most *Anubias*, is quite forgiving in nature, and is easily loved by hobbyists of all skill levels due to its hardy nature and ability to adapt well to a wide range of aquarium tank parameters. *A. heterophylla* is a bold green in hue and its leaves are fairly large with a slight wave. It has the potential to grow much larger when kept in its emerged state and will stay on the smaller side when kept in an aquarium fully submerged [1]. Like other *Anubias*, *Bucephalandra* and Java Ferns, *A. heterophylla* does best when attached securely to aquarium hardscapes such as aquascaping stones or aquarium driftwood. Alternatively, it can also be placed between cracks in an aquascape layout, and it will attach itself over time. Care should be taken to ensure its roots are not glued when attaching as this can promote the rotting of the plant. *Anubias* benefits from consistent fertilization, but light requirements are minimal as its leaves are susceptible to algae growth when placed under high lighting. CO_2 is optional but can promote faster growth and robust leaves when used [1].

Anubias can be propagated vegetatively using stolon division; however, stolon division is an inefficient propagation method for commercial purposes since the planting material has a very low

multiplication rate. Micropropagation is currently applied to aquatic plants as a tool for the large scale multiplication of elite plants [2,3]. Micropropagation is a common method for the rapid and large-scale production of many ornamental aquatic plant species, including some *Anubias* species, such as *Anubias barteri* var. *nana* [4], *Cryptocoryne wendtii* [5], *Aponogeton madagascariensis* [2] and *Nymphoides coreana* [3]. However, to the best of our knowledge, *in vitro* propagation of *A. heterophylla* has not been reported before. The present research aimed to develop an effective protocol for the micropropagation of this species.

In vitro propagation is an important approach for mass multiplication and genetic resource conservation of many plant species, especially those under the threat of extinction for destruction or other natural reasons [6-9]. Propagation via division has been the most commonly used method for bulbous plants; however, the use of division risks the permanent loss of mother bulbs, and divisions are often limited by their intrinsic low regeneration rate and long juvenility [10].

In this paper, we present a plantlet regeneration protocol based on *A. heterophylla* shoot tips culture. For clonal progenies that are phenotypically homogeneous and grow without chromosomal alterations or meiotic abnormalities, *in vitro* shoot tip culture is commonly employed. The protocol comprises only 1 part: the induction of *in vitro*-derived plantlets from a shoot tip explant. The current investigation deals with the initiation of aseptic cultures of *A. heterophylla* from shoot tip explants, optimization of culture medium for shoot proliferation and rooting micro shoots and hardening of rooted micro shoots. Therefore, the objective of this investigation was to study the effects of auxins with BAP on a shoot and root development of *A. heterophylla*.

Materials and methods

Plant materials, explant preparation and sterilization

Young plantlets of *A. heterophylla* were obtained from the Aquatic Plant Center Co., Ltd., Thailand. Shoots were excised from 90-day-old mother plants and were washed thoroughly under running tap water. The shoots were surface-sterilized using 15 % Clorox® (5.25 % sodium hypochlorite, NaOCl) and 2 - 3 drops of Tween-20 per 100 mL solution for 15 min, followed by rinsing 3 times with sterile distilled water. They were again surface sterilized for another 10 % Clorox® (5.25 % NaOCl) and 2 - 3 drops of Tween-20 per 100 mL solution for 10 min and 5 % Clorox® (5.25 % NaOCl) and 2 - 3 drops of Tween-20 per 100 mL solution for 5 min. The treated plantlets were washed 3 times with sterile distilled water to remove traces of disinfectant. The surface-sterilized shoot tip segments of 0.5 cm in length were excised aseptically and inoculated into bottles containing Murashige and Skoog (MS) medium [11] without plant growth regulators for 7 days. The culture bottles were sealed with Parafilm and incubated under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Culture media and conditions

Murashige and Skoog (MS) media [11] supplemented with 0.1 % (w/v) activated charcoal (AC) and 3 % (w/v) sucrose as a carbon source and gelled with 0.76 % (w/v) agar (commercial grade) were used as basal media throughout the experiment. Various concentrations and combinations of plant growth regulators (PGRs) (BAP; 0, 1.0, 3.0 and 5.0 mg/L, NAA; 0.5 and 1.0 mg/L, 2,4-D; 0.5 and 1.0 mg/L) were added to different cultures. The pH of the media was adjusted to 5.7 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. All cultures were aseptically maintained at 25 ± 2 °C air temperatures under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Plant materials were stored in glass-capped culture jars (115 mL capacity) each containing 25 mL of medium.

Effects of auxins with BAP on a shoot and root development of *Anubias heterophylla* after 60 days of culture

Shoot tip explants (0.5 cm in length) of 7 days old *in vitro* were placed on MS medium containing 0.1 % (w/v) activated charcoal (AC) and supplemented with 6-benzylaminopurine (BAP) at different concentrations (0, 1.0, 3.0 and 5.0 mg/L) either singly or in combination with α-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) (0.5 and 1.0 mg/L). The maximum percentage of shoot proliferation, number of shoots per explant, shoot length (mm), number of leaves per explant, leaf length (mm), leaf width (mm), the percentage of root formation, number of roots per shoot, and root length (mm) were recorded and compared statistically after 60 days of culture (starting from the initial day of inoculation). A set of cultures without plant growth regulators (PGRs) served as the control.

Hardening and establishment of tissue culture plantlets

Plantlets of *A. heterophylla* with well-developed shoots and roots were removed from the culture medium, washed gently with a soft brush in tap water to remove the adhering agar-agar with plant tissue. The plantlets were then transplanted into small clay pots containing tap water under greenhouse conditions. The plantlets were grown in the greenhouse with 80 - 90 % relative humidity and about 12 h photoperiod, 300 - 400 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (shaded sunlight) and 28 ± 2 to 24 ± 2 °C day/night temperature. After 30 days in the greenhouse, the survival rate of acclimatized plantlets was recorded.

Experimental design and statistical analysis

The data were collected after 60 days from a shoot and root development. All the experiments were conducted in a completely randomized design (CRD) with 5 replicates per treatment and the experiments were repeated 3 times. The results are expressed as mean ± SE of 1 experiment. The data were analyzed by ANOVA using SPSS version 20 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5 % probability level.

Results and discussion

Effects of auxins with BAP on a shoot and root development of *Anubias heterophylla* after 60 days of culture

An effective micropropagation method was done for the *in vitro* plant regeneration of *A. heterophylla.* For establishing a plant regeneration protocol, the current study investigated the effects of auxins with BAP on shoot and root development of *A. heterophylla* after 60 days of culture.

Regeneration potential of shoot tip explants of *A. heterophylla* was explored on MS medium containing 0.1 % (w/v) activated charcoal (AC) and supplemented with 6-benzylaminopurine (BAP) at different concentrations (0, 1.0, 3.0 and 5.0 mg/L) either singly or in combination with α-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) (0.5 and 1.0 mg/L) and the results were summarized in **Tables 1** and **2**. The synergistic influence of auxins with cytokinin was evident when the combination of optimal concentrations of cytokinin with different concentrations of NAA and 2,4-D (0.5 and 1.0 mg/L) were tested (**Table 1**). The results showed that there was a significant difference in the effect of type and concentration of PGRs on vegetative traits of *A. heterophylla* in the proliferation stage ($p \leq 0.05$). The lowest shoot proliferation percentage (30.00 ± 0.12) and the average number of shoots multiplied per single shoot tip explant (1.00 ± 0.00 shoot) developed in the absence of plant growth regulators (**Table 1** and **Figure 1A**). The number of shoots initiated per explant varied significantly among the different auxin and cytokinin combinations (**Table 1** and **Figure 1**). The highest number of shoots per explants (3.60 ± 0.24) was formed in the culture medium containing 1.0 mg/L NAA in combination with 1.0 mg/L BAP (**Table 1** and **Figure 1C**), followed by in the culture medium with 3.0 mg/L BAP (2.40 ± 0.24) (**Table 1** and **Figure 1B**). In the culture medium with 1.0 mg/L NAA and 1.0 mg/L BAP, 100 % of the cultured explants regenerated new shoots within 60 days. NAA also enhanced the number of shoots per explant (3.60 ± 0.24 - 2.00 ± 0.00 shoots per explant) but the presence of 2,4-D in the medium drastically reduced the number of shoots per explant. In the case of 2,4-D, the highest number of shoots per explant (1.40 ± 0.24 shoots) was achieved from shoot tip explants on MS medium containing 1.0 mg/L 2,4-D and 3.0 mg/L BAP (**Table 1** and **Figure 1E**). No callus formation was achieved when shoot tip segments were used as explants. The lowest shoot multiplication was observed on MS medium containing 0.1 % (w/v) AC (control, 1.00 ± 0.00 shoots) or MS medium contained 0.1 % (w/v) AC and supplemented with 0.5 mg/L 2,4-D and 1.0, 3.0, 5.0 mg/L BAP (1.00 ± 0.00 shoots) or 1.0 mg/L 2,4-D and 0.0, 1.0, 5.0 mg/L BAP (1.00 ± 0.00 shoots) (**Table 1**).

Figure 1 *In vitro* propagation of *Anubias heterophylla*; multiple shoots formation from shoot tip explants after 60 days cultured on agar-gelled MS medium supplemented with 0.1 % (w/v) activated charcoal (AC) and plant growth regulators. (A) Control, (B) 3.0 mg/L BAP, (C) 1.0 mg/L NAA+1.0 mg/L BAP, (D) 0.5 mg/L 2,4-D and (E) 1.0 mg/L 2,4-D+3.0 mg/L BAP (Scale bar = 1 cm).

In the case of BAP alone, the highest number of shoots per explant (2.40 ± 0.24 shoots), shoot length (72.40 ± 0.24) number of leaves (10.80 ± 0.73 leaves), leaf length (39.35 ± 0.12 mm), and leaf width (25.44 ± 0.82 mm) was achieved from shoot tip explants on MS medium contained 0.1 % (w/v) AC and supplemented with 3.0 mg/L BAP (**Table 1** and **Figure 1B**). The elongation of shoots was significantly better on medium containing 1.0 mg/L NAA and 1.0 mg/L BAP (76.40 ± 0.97 mm) (**Table 1** and **Figure 1C**) than in the presence of 1.0 mg/L 2,4-D supplemented with 1.0 mg/L BAP (54.60 ± 0.52 mm) (**Table 1**). The lowest elongation of shoots was observed on MS medium containing 0.1 % (w/v) AC (15.00 ± 0.00) (**Table 1** and **Figure 1A**). A significant difference in the number of leaves was detected among the treatments containing NAA, 2,4-D and BAP. Regenerated shoots had a higher number of leaves on MS medium supplemented with 0.1 % (w/v) AC and contained auxins with cytokinins. The maximum number of leaves per shoot (18.20 ± 0.73 leaves per explant), leaf length (42.44 ± 0.81 mm) and leaf width (26.20 ± 0.90 mm) were observed in MS medium containing 0.1 % (w/v) AC and supplemented with 1.0 mg/L NAA and 1.0 mg/L BAP. The leaves of the regenerated shoots were healthy with dark green color and did not show any sign of vitrification.

Table 1 Effects of auxins with BAP on shoot development of *Anubias heterophylla* after 60 days of culture. All culture mediums were added with 0.1% (w/v) AC.

Growth regulators (mg/L)			Shoot proliferation (%)	Number of shoots per explant	Shoot length (mm)	Number of leaves per explant	Leaf length (mm)	Leaf width (mm)
NAA	2,4-D	BAP	(mean ± SE)[a]	(mean ± SE)[a]	(mean ± SE)[a]	(mean ± SE)[a]	(mean ± SE)[a]	(mean ± SE)[a]
-	-	0.0	30 ± 0.12^{b}	1.00 ± 0.00^{e}	15.00 ± 0.00^{i}	15.00 ± 0.00^{b}	13.57 ± 0.27^{k}	10.30 ± 0.23^{k}
-	-	1.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	25.71 ± 0.85^{ghi}	9.20 ± 0.48^{hi}	25.80 ± 0.66^{ghi}	18.21 ± 0.57^{f}
-	-	3.0	100 ± 0.00^{a}	2.40 ± 0.24^{b}	72.40 ± 0.24^{a}	10.80 ± 0.73^{efgh}	39.35 ± 0.12^{b}	25.44 ± 0.82^{a}
-	-	5.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	27.00 ± 0.12^{fghi}	12.20 ± 0.48^{cde}	20.45 ± 0.47^{j}	12.31 ± 0.36^{j}
0.5	-	0.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	49.60 ± 0.97^{bcd}	12.60 ± 0.24^{cde}	31.88 ± 0.45^{de}	20.65 ± 0.55^{de}
0.5	-	1.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	48.40 ± 0.50^{bcd}	12.00 ± 0.00^{cdef}	32.80 ± 0.81^{d}	21.86 ± 0.62^{cde}
0.5	-	3.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	60.40 ± 0.63^{b}	11.60 ± 0.24^{def}	36.58 ± 0.76^{bc}	21.93 ± 0.40^{cde}
0.5	-	5.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	43.60 ± 0.39^{cd}	10.60 ± 0.24^{efgh}	27.62 ± 0.42^{fg}	17.62 ± 0.22^{fg}
1.0	-	0.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	72.40 ± 0.97^{a}	11.20 ± 0.73^{defg}	34.64 ± 0.83^{cd}	24.50 ± 0.60^{ab}
1.0	-	1.0	100 ± 0.00^{a}	3.60 ± 0.24^{a}	76.40 ± 0.97^{a}	18.20 ± 0.73^{a}	42.44 ± 0.81^{a}	26.20 ± 0.90^{a}
1.0	-	3.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	38.40 ± 0.97^{def}	13.00±0.00^{cd}	27.00 ± 0.54^{fgh}	17.26 ± 0.35^{fg}
1.0	-	5.0	100 ± 0.00^{a}	2.00 ± 0.00^{c}	55.60 ± 0.34^{bc}	10.60 ± 0.24^{efgh}	37.39 ± 0.14^{bc}	22.60 ± 0.61^{cd}
-	0.5	0.0	100 ± 0.00^{a}	1.20 ± 0.20^{de}	44.66 ± 0.60^{cd}	7.60 ± 0.24^{ij}	38.47 ± 0.15^{b}	23.36 ± 0.91^{bc}
-	0.5	1.0	100 ± 0.00^{a}	1.00 ± 0.00^{e}	41.80 ± 0.38^{de}	9.60 ± 0.10^{gh}	29.37 ± 0.17^{ef}	20.22 ± 0.11^{e}
-	0.5	3.0	100 ± 0.00^{a}	1.00 ± 0.00^{e}	30.80 ± 0.11^{efghi}	10.20 ± 0.37^{fgh}	26.49 ± 0.11^{gh}	18.15 ± 0.83^{f}
-	0.5	5.0	100 ± 0.00^{a}	1.00 ± 0.00^{e}	18.60 ± 0.81^{hi}	13.60 ± 0.14^{bc}	20.52 ± 0.55^{j}	13.60 ± 0.36^{ij}
-	1.0	0.0	100 ± 0.00^{a}	1.00 ± 0.00^{e}	40.40 ± 0.74^{de}	9.20 ± 0.91^{hi}	24.26 ± 0.70^{hi}	12.10 ± 0.48^{j}
-	1.0	1.0	100 ± 0.00^{a}	1.00 ± 0.00^{e}	54.60 ± 0.52^{bc}	6.20 ± 0.48^{j}	36.45 ± 0.82^{bc}	22.38 ± 0.86^{cd}
-	1.0	3.0	100 ± 0.00^{a}	1.40 ± 0.24^{d}	31.14 ± 0.82^{efg}	12.60 ± 0.40^{cde}	26.31 ± 0.12^{ghi}	14.92 ± 0.54^{hi}
-	1.0	5.0	100 ± 0.00^{a}	1.00 ± 0.00^{e}	23.40 ± 0.27^{ghi}	7.60 ± 0.60^{ij}	23.47 ± 0.10^{i}	16.05 ± 0.56^{gh}

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.
[a]Values represent means ± standard error.

In the present study, it was found that the development of root formation was significantly promoted by MS medium contained 0.1 % (w/v) AC and supplemented with BAP at different concentrations (0, 1.0, 3.0 and 5.0 mg/L) either singly or in combination with NAA and 2,4-D (0.5 and 1.0 mg/L) (**Figure 2**). The presences of both NAA and BAP on the medium significantly promote root development. The addition of auxin, NAA or 2,4-D led to an increase in the number of roots. The higher number of roots per explant was produced on a culture medium supplemented with both 1.0 mg/L NAA and BAP (**Table 2** and **Figure 2C**). Minimum root number (9.80 ± 0.20 roots) was obtained in the control medium (**Table 2** and **Figure 2A**). However, the length of roots was inversely proportional to the 1.0 mg/L NAA in combination with BAP concentration. The roots arising from the basal end of shoots were large and vigorous. The medium containing 3.0 mg/L BAP resulted in the maximum root length (62.40 ± 0.28 roots) (**Table 2** and **Figure 2B**).

Table 2 Effects of auxins with BAP on root development of *Anubias heterophylla* after 60 days of culture. All culture mediums were added with 0.1 % (w/v) AC.

Growth regulators (mg/L)			Root Formation (%)	Number of roots per explant	Root length (mm)
NAA	2,4-D	BAP	(mean ± SE)[a]	(mean ± SE)[a]	(mean ± SE)[a]
-	-	0.0	100 ± 0.00^{a}	9.80 ± 0.20^{ef}	31.64 ± 0.24^{h}
-	-	1.0	100 ± 0.00^{a}	18.60 ± 0.46^{ab}	39.52 ± 0.10^{efg}
-	-	3.0	100 ± 0.00^{a}	12.20 ± 0.20^{cdef}	62.40 ± 0.28^{a}
-	-	5.0	100 ± 0.00^{a}	16.00 ± 0.24^{abcd}	50.25 ± 0.21^{b}
0.5	-	0.0	100 ± 0.00^{a}	16.40 ± 0.97^{abcd}	33.75 ± 0.71^{gh}
0.5	-	1.0	100 ± 0.00^{a}	10.80 ± 0.48^{def}	46.85 ± 0.25^{bcd}
0.5	-	3.0	100 ± 0.00^{a}	13.20 ± 0.48^{bcdef}	50.90 ± 0.26^{b}
0.5	-	5.0	100 ± 0.00^{a}	10.00 ± 0.00^{ef}	35.90 ± 0.26^{fgh}
1.0	-	0.0	100 ± 0.00^{a}	13.20 ± 0.73^{bcdef}	46.84 ± 0.22^{bcd}
1.0	-	1.0	100 ± 0.00^{a}	19.80 ± 0.20^{a}	50.17 ± 0.16^{b}
1.0	-	3.0	100 ± 0.00^{a}	13.60 ± 0.14^{bcdef}	48.23 ± 0.16^{bc}
1.0	-	5.0	100 ± 0.00^{a}	8.80 ± 0.48^{f}	44.59 ± 0.27^{bcde}
-	0.5	0.0	100 ± 0.00^{a}	18.40 ± 0.11^{ab}	22.90 ± 0.10^{i}
-	0.5	1.0	100 ± 0.00^{a}	17.20 ± 0.91^{abc}	30.43 ± 0.11^{h}
-	0.5	3.0	100 ± 0.00^{a}	15.20 ± 0.23^{abcde}	43.15 ± 0.18^{cde}
-	0.5	5.0	100 ± 0.00^{a}	16.00 ± 0.16^{abcd}	40.33 ± 0.21^{def}
-	1.0	0.0	100 ± 0.00^{a}	13.60 ± 0.12^{bcdef}	39.52 ± 0.16^{efg}
-	1.0	1.0	100 ± 0.00^{a}	18.40 ± 0.97^{ab}	41.87 ± 0.23^{cdef}
-	1.0	3.0	100 ± 0.00^{a}	16.00 ± 0.54^{abcd}	39.21 ± 0.25^{efg}
-	1.0	5.0	100 ± 0.00^{a}	15.20 ± 0.33^{abcde}	42.63 ± 0.21^{cde}

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.
[a]Values represent means ± standard error.

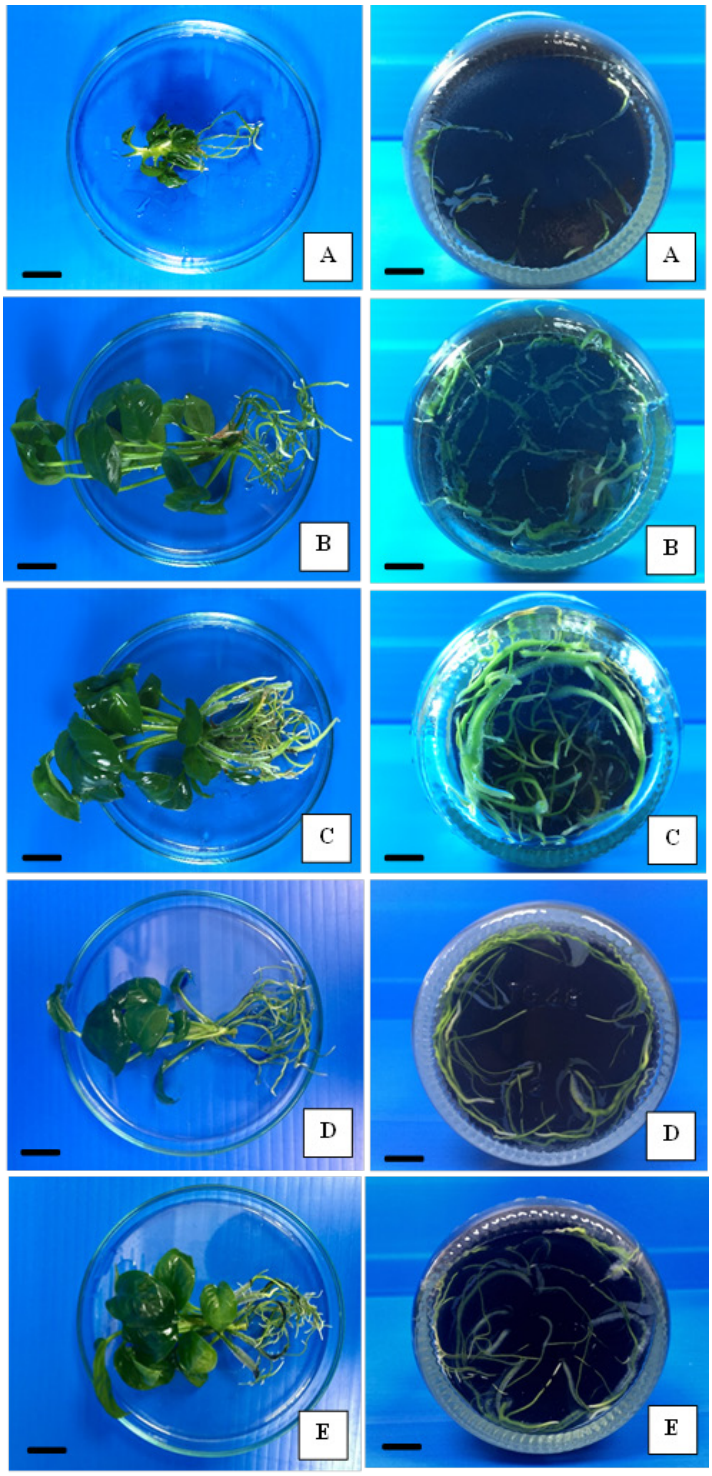

Figure 2 Effects of auxins with BAP on root development of *Anubias heterophylla* after 60 days of culture. (A; right-left) Control, (B; right-left) 3.0 mg/L BAP, (C; right-left) 1.0 mg/L NAA+1.0 mg/L BAP, (D; right-left) 0.5 mg/L 2,4-D and (E; right-left) 1.0 mg/L 2,4-D+3.0 mg/L BAP (Scale bar = 1 cm).

Hardening and establishment of tissue culture plantlets

Root formation and shoot elongation of *A. heterophylla* occurred at 60 days of culture. When the complete plantlets reach a length of 5 - 7 cm, agar was carefully washed from these plantlets. They were transferred to small clay pots containing tap water under greenhouse conditions. The percentage of

survival plantlets was 100 % under greenhouse conditions (**Figures 3A - 3F**). During the transferring process, the regenerated *A. heteropylla* plantlets showed normal phenotypic characteristics similar with their donor plant.

Figure 3 Acclimatization of *Anubias heterophylla* through shoot tip explants: (A - B) 60-day-old regenerated shoots on MS medium supplemented with plant growth regulators, (C - D) hardened plantlet of *Anubias heterophylla* into small clay pots containing with tap water under greenhouse conditions after 30 days and (E - F) the regenerated plantlets were successfully acclimatized in the greenhouse after 60 days (Scale bar = 1 cm).

Micropropagation offers a viable alternative for conventional methods because it can also be used as a complementary strategy for the conservation and utilization of genetic resources. Further, *in vitro* plant regeneration is an easy and economical way for obtaining a large number of consistently uniform and true to type plants within a short period of time [12]. The present study focuses on the development of a micropropagation protocol for true to type plants of *A. heterophylla*, a valuable ornamental aquatic plant. In the present study, the highest number of shoots per explants (3.60 ± 0.24) was formed in the culture medium containing 1.0 mg/L NAA in combination with 1.0 mg/L BAP, followed by in the culture medium with 3.0 mg/L BAP (2.40 ± 0.24). In the culture medium with 1.0 mg/L NAA and 1.0 mg/L BAP, 100 % of the cultured explants regenerated new shoots within 60 days.

Kanchanapoom *et al.* [4] has reported culture initiation and shoot multiplication from the lateral shoot tip explants of *A. barteri* var. *Nana* on MS medium containing 3.0 mg/L BAP. But in the present study, in *A. heterophylla*, a combination of NAA and BAP was found to be suitable for plant regeneration. The beneficial effect of BAP on shoot regeneration and proliferation and induction of multiple shoots was reported in other species [5,13-15]. Some species may require a low concentration of auxins in combination with high levels of cytokinins to increase shoot proliferation [16]. A study by Abadi and Kaviani [17] on micropropagation of *Aloe vera* L. using BA, NAA and IBA showed that the best proliferation of shoot per explants was shown on medium supplemented with 0.5 mg/L NAA containing 0.5 mg/L BA. In our work, the combination of NAA and BAP, both at 1.00 mg/L promoted shoot proliferation.

The presence of auxin (NAA or 2,4-D) along with BAP increased the shoot proliferation of organogenesis. The frequency and response of *in vitro* shoot regeneration are affected by the type of explants and the concentration of PGRs [18-20]. The promoting effect of auxin and cytokinin combinations on organogenic has been well documented [21,22]. *In vitro* shoot multiplication relies largely on medium formulations containing BAP as the major PGRs in combination with a low concentration of NAA [23]. Kim *et al.* [24] indicated that *in vitro* shoot proliferation and multiplication are largely based on media formulations containing cytokinins as major plant growth regulators, although low concentrations of auxins or GA_3 are also essential. The results of the present study demonstrated that the inclusion of 1.0 mg/L NAA and 1.0 mg/L BAP to the culture media increased the shoot multiplication and root formation. This result is similar to the findings on optimal NAA and BAP concentration [25]. This observation agreed with the earlier reports of Ahmad and Anis [26]. They suggested that the cytokinin: Auxin ratio is an important factor for the differentiation of adventitious shoots as elucidated by Skoog and Miller [27].

The effect of auxins on plant development has led many researchers to characterize this hormone as a plant morphogen [28-32]. Polar auxin transport via PIN proteins directs auxin to a specific location in the plant body during development and plays a central role in tropic growth, apical dominance, lateral root initiation, vascular development, and embryo patterning [28]. PGRs are the initial factors inducing morphogenic responses in plants *in vitro* [33]. Evidence from previous reports indicates that combinations of auxin and cytokinin are more effective for shoot organogenesis at equal ratios (1:1) or even lower [34-36]. Therefore, 2 combinations of PGRs at equal ratios (1 NAA:1 BAP and 1 2,4-D:1 BAP) were tested in this study at 2 different absolute concentrations; the medium concentration (1.0 mg/L NAA+1.0 mg/L BAP) stimulated shoot formation at a significantly faster and more efficient rate than did the other one concentration (**Table 1**). A reduced response was observed when the media were supplemented with a 2,4-D absolute concentration (**Table 1**), which may be mainly caused by auxins. As auxins are responsible for apical dominance [37], inhibiting effects on shoot organogenesis have also been reported *in vitro* [34]. Sensitivity to changes in hormone concentration (both endogenous and exogenous) is a key requirement for somatic cells to reach "embryogenic status". SE induction is favored by auxins [38], mostly 2,4-D [39, 40], with or without a low level of cytokinin [41]. In this study, a mixture of NAA and BAP in the media successfully induced shoots (**Table 1**). Auxin signaling affects somatic embryogenesis by altering the expression patterns of genes involved in auxin-induced growth and development [42]. Auxin signals can induce the synthesis of embryonic proteins during somatic embryogenesis but can act as negative factors by inhibiting the synthesis of protein needed for SE development [43].

In the present study, MS medium had a significantly high ($p \leq 0.05$) effect on both shoot multiplication and root formation. MS medium containing 0.1 % (w/v) AC and supplemented with 1.0 mg/L NAA and 1.0 mg/L BAP was found to perform better in terms of root formation. The addition of auxin, α-Naphthalene acetic acid (NAA) or 2,4-Dichlorophenoxy acetic acid (2,4-D) led to an increase in the number of roots. The maximum number of roots (19.80 ± 0.20 roots) per explant was observed after 60 days of culture on MS medium containing 0.1 % (w/v) AC and supplemented with 1.0 mg/L NAA and 1.0 mg/L BAP. Irrespective of the auxins used, it showed 100 % rooting. Our findings demonstrated that the addition of BAP and NAA in culture media was effective for increasing the number of root and root lengths. The current study showed the positive effect of NAA on root induction and root length. Some studies showed the positive effect of cytokinins on rooting [44]. Auxins are routinely used for induction of rooting for *in vitro* shoots [45-48]. The application of auxins promotes the enzyme activities that regulate various pathways of protein, carbohydrates, nitrogen, and polyphenolic metabolism which could influence root formation [46]. In the present experiment, the maximum number of roots (19.80 ± 0.20 roots) per explant was observed after 60 days of culture on MS medium containing 0.1 % (w/v) AC and supplemented with 1.0 mg/L NAA and 1.0 mg/L BAP. Previous studies have shown that in herbaceous

plant a very low concentration of strong auxins is effective in induction of rooting [49-51]. Apart from root number, their length is also critical in the survival of plants during hardening [52].

Regarding the *in vitro* acclimatization of *A. heterophylla* plantlets, our findings are consistent with the 100 % survival rate reported for this genus by several authors [4,53]. These results contrasted with those observed for some plant species; where a high mortality has occurred during the acclimatization process due to stomatal dysfunction, weak root systems, and/or poor cuticle development [54]. Therefore, the protocol established in this study also ensures rapid and inexpensive rooting, as well as an adequate survival rate of this commercially important species.

Conclusions

We have demonstrated the establishment of a rapid *in vitro* propagation of *A. heterophylla* through shoot tip culture. MS medium containing 0.1 % (w/v) AC in combination with 1.0 mg/L of both NAA and BAP was found to be the better medium for the regeneration of *A. heterophylla*. The plantlets formed from the study were well developed with healthy root and shoot before transferring into a nursery. All new progenies found survive following the transfer.

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