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Micropropagation of *Anubias barteri* var. *Nana* from Shoot Tip Culture and the Analysis of Ploidy Stability

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Abstract

Plant regeneration of *Anubias barteri* var. *Nana* was achieved through organogenesis in shoot tip cultures. Multiple shoots were induced from cultured shoot tips on a modified MS (Murashige and Skoog, 1962) medium supplemented with BA and kinetin. The maximum green shoot numbers were best obtained on MS medium containing 3 mg/L BA with 5 shoots. Rooting in all regenerated shoots was promoted on MS medium devoid of plant growth regulators or kinetin singly. Acclimatization and survival when transferred to field conditions were shown to be 100% in the regenerated plants. Cytological and flow cytometric analyses of the mother plants and *in vitro* grown plants derived from 5 years old cultures showed no differences in ploidy level, they were all diploid (2n = 2x = 48) with a 2C peak indicating that ploidy alteration did not occur.

Keywords: aquatic plant, *Araceae*, flow cytometry, nuclear DNA content

Introduction

The genus *Anubias* of the family Araceae is divided into many varieties such as *Anubias barteri* var. *Barteri*, *A. barteri* var. *Angustifolia*, *A. barteri* var. *Caladiifolia*, *A. barteri* var. *glabra*, and *A. barteri* var. *Nana (*Kasselmann*, 2003)*. The most cultivated and commercially important species is *A. barteri* var. *Nana* which is commonly grown in aquaria. *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 large scale multiplication of elite plants (Carter *et al*., 2011; Myung *et al*., 2010). However, information concerning details of media and growth regulator amendments is still a fundamental requirement of the intense commercial production of *A. barteri*.

The chromosome number of *A. barteri* var. *Nana* is very difficult to assess since they are small and numerous. Flow cytometry is being used to analyze DNA content in a number of plant species; which require only a small amount of tissue and is therefore non-destructive. In this context, this study investigated an efficient protocol for *A. barteri* var. *Nana* multiplication. The study also investigated the effects of plant growth regulators on number of chromosomes using flow cytometry as rapid methods for detecting ploidy levels in regenerated plants.

Materials and methods

Plant materials

Young plantlets of *A. barteri* var. *Nana* were obtained from the Aquatic Plant Center Co., Ltd., Thailand. They were surface sterilized using 0.5% (w/v) mercuric chloride solution containing 2 drops of Tween-20 emulsifier per 100 ml solution for 3 min. The treated plantlets were washed three times with sterile distilled water to remove traces of disinfectant. The explants were then surface sterilized again using a dilution of 10% (v/v) commercial Clorox™ which yields 5.25% NaOCl and 2 drops of Tween 20 per 100 mL solution for 15 min, followed by 5% (v/v) Clorox™ for 5 min. After the surface decontamination was completed the explants were rinsed 3 times with sterile distilled water. Following disinfection 3-5 mm shoot tip explants were excised prior to culture on MS (Murashige and Skoog, 1962) basal medium containing 3% sucrose to grow the explants.

Medium preparation and culture conditions

After 6 weeks of culture, well developed shoots were obtained. The small shoots with a pair of leaves were transferred to MS medium supplemented either with 0, 1, 3, or 5 mg/L BA or 0, 1, 3, or 5 mg/L kinetin. All culture media consisted of MS salts and vitamins supplemented with 3% sucrose and 0.82% Mermaid™ agar. The pH of media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121°C for 20 min. Cultures were

maintained at 25±1°C in a culture room with a 16-h light photoperiod. All explants were subcultured at 8-week intervals. Cultures were maintained at 25±1°C in a culture room with a 16/8 h light/dark photoperiod under an illumination of 20 μmolm^{-2} s^{-1} photosynthetic photon flux intensity provided by cool white fluorescent light. Plant materials were stored in glass-capped culture jars (115 ml capacity) each containing 20 ml of medium.

Chromosome counting

To determine an accurate ploidy level, chromosome counting was carried out on young root tips of *in vitro* grown plants. Actively growing root tips ca. 5-10 mm in length were excised and pretreated with saturation solution of Para dichlorobenzene for 24 h at 4°C. They were fixed in fresh solution of Carnoy's fluid (3 parts 95% ethanol and 1 part glacial acetic acid) for 24 hours and stored in 70% ethanol at 4°C. This treatment was followed by hydrolysis in 1N HCl at 60°C for 5-6 min. Finally, they were rinsed with tap water and stained in carbol fuchsin. The stained regions of root tips (0.5-1 mm long) were cut and squashed on a slide and cover with a cover slip. The chromosomes counts were carried out at 1000x magnification under light microscope (Olympus model CH 30, Japan) and the chromosomes of 7-8 cells were counted in three replications.

Flow cytometry analysis

Approximately 20-30 mg of fully expanded young leaves of the mother plants and *in vitro* grown plants were harvested and transferred to glass Petri dish containing nuclei extraction buffer. The glass Petri dish was placed on top of ice in a bucket and nuclei were mechanically isolated by chopping leaf materials using a sharp razor blade. After chopping, the suspension was filtered through a 42 μm nylon mesh and CyStain UV Ploidy (DAPI staining solution) was added. The fluorescence of a minimum of 5000 DAPI-stained nuclei per sample was estimated using a PA-II flow cytometer (Partec, Germany). The reference standard plant (*Zea mays* cv. CE-777; 2C = 5.43 pg) was kindly provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic. The reference standard peak was adjusted to show at channel 100 of relative fluorescence intensity for instrument calibration. The 2C DNA content was calculated according to the formula

$$2\,\text{C DNA} = \frac{\text{Sample G1 peak mean} \times \text{Standard 2 C DNA content}}{\text{Standard G1 peak mean}}$$

Statistical analysis

All experiments were carried out at least 3 times with 5-10 replicates per treatment. The fluorescence histograms were resolved into G0/G1 (2C), S and G2/M (4C) cell-cycle compartments with a peak-reflect algorithm using two Gaussian curves (WinMDI version 2.8). Data were analyzed by ANOVA and the differences among the means were compared using Scheffe's test at $p \leq 0.05$.

Results and discussion

The results of *in vitro* organogenesis in *A. barteri* var *Nana* are shown in Tab. 1. Both BA and kinetin supplements resulted in different morphogenetic responses in

Tab. 1 Effect of different concentrations of BA and kinetin combinations on shoots leaves and root regeneration of *Anubias barteri* var. *nana*

BA (mg/L)	Kinetin (mg/L)	Number of shoots per explant (Mean±SD)	Number of leaves (Mean±SD)	Number of leaves per shoot	Number of roots per shoots (Mean±SD)	Root formation (%)
0	0	1.4±0.89^{c}	3.8±2.16^{c}	3.0±1.87^{abcd}	2.0±2.00^{abc}	60
	1	1.2±0.45^{c}	4.4±1.34^{c}	4.0±1.41^{ab}	2.4±1.95^{ab}	80
	3	1.2±0.45^{c}	4.2±2.38^{c}	3.7±2.44^{abcd}	3.8±4.27^{a}	60
	5	1.4±0.89^{c}	3.8±3.19^{c}	2.1±1.52^{cd}	2.2±3.03^{abc}	40
1	0	1.6±0.89^{c}	5.4±2.79^{c}	3.47±0.51^{abcd}	0.0±0.00^{c}	0
	1	1.2±0.45^{c}	4.4±1.14^{c}	4.4±1.14^{a}	0.0±0.00^{c}	0
	3	1.4±0.55^{c}	5.8±1.78^{bc}	4.4±1.08^{a}	0.0±0.00^{c}	0
	5	1.0±1.58^{bc}	7.8±4.43^{bc}	2.9±0.96^{abcd}	0.0±0.00^{bc}	0
3	0	5.0±2.12^{a}	13.4±5.52^{a}	2.9±0.42^{abcd}	0.0±0.00^{c}	0
	1	2.4±1.14^{bc}	5.0±2.12^{c}	2.0±0.71^{d}	0.0±0.00^{c}	0
	3	1.8±1.06^{bc}	5.2±2.80^{c}	3.87±1.04^{abc}	0.0±0.00^{c}	0
	5	1.8±1.10^{bc}	7.2±4.65^{bc}	4.1±1.02^{ab}	0.0±0.00^{c}	0
5	0	3.4±1.34^{b}	10.4±3.04^{ab}	3.3±0.97^{abcd}	0.0±0.00^{c}	0
	1	3.0±2.00^{bc}	8.20±3.76^{bc}	3.1±0.82^{abcd}	0.0±0.00^{c}	0
	3	2.0±1.41^{bc}	6.6±4.27^{bc}	3.5±0.50^{abcd}	0.0±0.00^{c}	0
	5	2.4±0.89^{bc}	6.0±3.46^{bc}	2.4±0.55^{bcd}	0.0±0.00^{c}	0

The different letters within column show significant difference (Mean ± SE.) analyzed by Scheffe's test at $p<0.05$.

terms of shoot, leaf and root formation, number of leaves per shoot, and percentage of root formation. At low concentration of BA (0, 1 mg/L) regenerated single shoot while an increase in BA concentration from 3 to 5 mg/L resulted in increased number of shoots per explant. The data in Tab. 1 revealed that the maximum number of 5±2.12 shoots per explant was obtained on MS medium supplemented with 3 mg/L BA

The results show that BA used singly was important for induction of axillary bud outgrowth in *A. barteri* var. *Nana*. By successive subculture on MS medium containing 3 mg/L BA, masses of proliferating shoot cultures were established (Fig. 1a).

A significant difference in the number of leaves was detected among the treatments containing BA and kinetin. Regenerated shoots had higher number of leaves on MS medium supplemented with kinetin alone. Leaves were formed at a high frequency of 13.4 ($p \leq 0.05$, Tab. 1) on MS medium supplemented with 3 mg/L BA. No leaf was observed on the control explants. The feature of leaves developed on MS medium containing 1-5 mg/L BA and 1-5 mg/L kinetin showed broad and dark green leaves (Fig. 1a). BA seemed to inhibit root formation since the root formation was recorded only on culture media containing MS medium (control) or kinetin singly (Tab. 1). The roots arising from the basal end of shoots were large and vigorous (Fig. 1a). The explants were subcultured for 5 years successfully and did not show any morphological abnormality when compared with the non tissue cultured plants.

The cytological study of root tips at metaphase of long term cultures revealed complete accurate counts of 48 chromosomes (Fig. 1b). An occurrence of chromosome changes has been often observed during application of tissue culture, especially in plants, callus or cells that were maintained for long-term cultures *in vitro* (Hao and Deng, 2002). The distribution of the nuclei extracted from both the mother plants and *in vitro* grown plants displayed a prominent peak at 2C indicating that they consisted of cells with G0/G1 phase of cell cycle thus no ploidy variation occurred (Fig. 2). The mean 2C DNA content of the mother plants and *in vitro* grown plants are 5.43 and 5.45 pg $2C^{-1}$, respectively. This finding confirmed that the alteration of DNA content was not observed among *in vitro* grown plants compared to the mother plants probably due to the reason that plants regenerated from well-developed meristematic tissues that had minimum tendency of genetic variation (Rout *et al*., 1998).

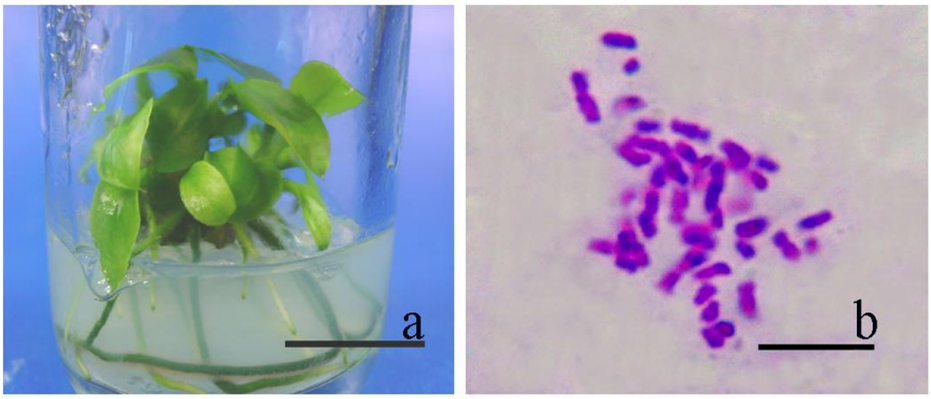

Fig. 1. *In vitro propagation* of *Anubias barteri* var. *Nana* (a) Multiple shoots formation from a single shoot explant after eight weeks cultured on MS medium supplemented with 3 mg/L BA (Scale bar =10 mm) (b) Mitotic metaphase of root tips showing diploid 2n = 2x =48 chromosomes (Scale bar = 50 μm)

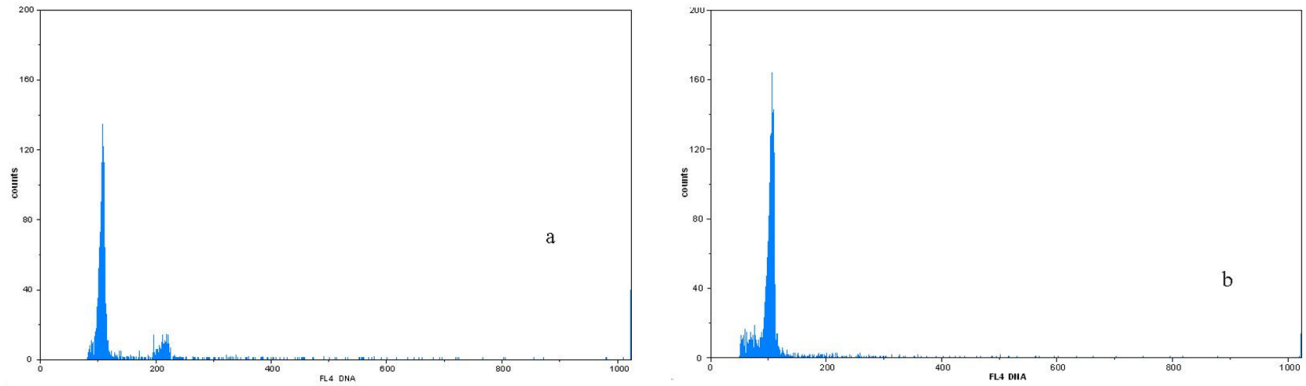

Fig. 2. Flow cytometric histograms of the relative nuclear DNA content (in channel numbers) of *A. barteri* var. *Nana* with (a) diploid profiles of standard plant (b)diploid profile of 5 years old *in vitro* grown plant

In conclusion, we demonstrated that the establishment of rapid *in vitro* plant propagation of *A. barteri* var. *Nana* can be achieved. The nuclear DNA content value for *A. barteri* var. *Nana* was provided. Ploidy variations were not observed during subculture in 5 years as detected by cytological study and flow cytometry. A combination of cytological study and flow cytometry achieved better results in terms of both accuracy and rapidity.

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References

Carter J, Gunawardena AHLAN (2011). Regeneration of the aquatic monocot *Aponogeton madagascariensis* (lace plant) through callus induction. Aquat Bot 94:143-149.

Dolezel J, Greilhuber J, Lucretti S, Meister A, Lysak MA, Nardi L, Obermayer R (1998). Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot 82:17-26.

Hao YJ, Deng XX (2002). Occurrence of chromosomal variations and plant regeneration from long-term cultured citrus callus. *In Vitro* Cell Dev Biol Plant 38:472-480.

Huang L, Chang Y, Chang Y (1994). Rapid *in vitro* multiplication of the aquatic angiosperm, *Anubias barteri* var. *undulata*. Aquat Bot 47:77-83.

Kane ME, Gilman EF, Jenks MA, Sheehan TJ (1990). Micropropagation of the aquatic plant *Cryptocoryne lucens*. HortSci 25:687-689.

Kane ME, Greg L, Davis1 GL, McConnell DB, Gargiulo JA (1999). *In vitro* propagation of Cryptocoryne wendtii Aquat Bot 63:197-202.

Kasselmann C (2002). Aquarium Plants. Malabar, FL: Krieger Publishing Co., 104 p.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Planta 15:473-479.

Myung JO, Hye RN, Hong-Keun C, Jang RL, Suk WK (2010). High frequency plant regeneration system for *Nymphoides coreana* via somatic embryogenesis from zygotic embryo-derived embryogenic cell suspension cultures. Plant Biotechnol Rep 4:125-128.

Predieri S (2001). Mutation induction and tissue culture in improving fruits. Plant Cell Tiss Org Cult 64:185-210.

Rout GR, Das P, Goel S, Raina SN (1998). Determination of genetic stability of micropropagated plants of ginger using random amplified polymorphic DNA (RAPD) markers. Bot Bull Acad Sin 39:23-37.

Trewavas AJ, Cleland RE (1983). Is plant development regulated by changes in concentration of growth substances or by changes in the sensitivity? Trends in Biochem Sci 8:354-357.

Zhou C, An S, Jiang J, Yin D, Wang Z, Fang C, Sun Z, Chen Qian C (2006). An *in vitro* propagation protocol of two submerged macrophytes for lake revegetation in east China. Aquat Bot 85:44-52.