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Factors Affecting the Protoplast Isolation and Culture of *Anubias nana* Engler

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Abstract: The effects of various factors on the isolation and culture of *Anubias nana* Engler protoplast were investigated. Several factors, such as kind and concentration of enzymes, mannitol concentration in digestion solution, incubation time, plant material age and sucrose concentration in purifying solution were extremely important to obtain highest number and viability of protoplasts. The highest yield ($4.79\pm0.48\times10^6$ per gram fresh weight) and viability (82.90±4.31%) was obtained when protoplasts were digested from *in vitro* six-week-old leaves with 2% Cellulase Onozuka RS, 0.2% Pectolyase Y-23, 0.6 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM morpholinoethane sulfonic acid (MES), pH 5.6 for 4 h in the dark and purified with 18% sucrose gradient centrifugation. Purified protoplasts were cultured on KM8P medium supplemented with 0.2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg L^{-1} α-naphthalene acetic acid (NAA), 0.5 mg L^{-1} zeatin by agarose-bead with thin layer liquid culture. The protoplasts regenerated cell walls within 24 h. First cell division was observed after culturing for 7-9 days and micro-colonies were formed within 30 days. The culture medium, culture method and plant growth regulator were significant factors influencing cell division and survival of protoplast.

Key words: *Anubias nana* Engler, protoplast isolation, protoplast culture

INTRODUCTION

Aquatic plants are an important economic crop in Thailand. Many species of aquatic plant have been successfully commercialized around the world. Anubias is one of the major exported genera. The genus *Anubias* comprises eight species. Of these, *Anubias nana* Engler (Dwarf Anubias) is commercially cultivated for aquaria (Mühlberg, 1982). *A. nana* Engler is a monocotyledonous species belonging to Araceae (Cook, 1996). It is a small attractive plant with thick creeping roots, petiole of up to 5 cm long and glabrous leaf blade of up to 10 cm long and about 2.08 cm broad. It grows slowly and the leaves survive for several years. Traditionally, it is propagated vegetatively by rhizome division (Rataj and Horeman, 1977; Allgayer and Teton, 1986).

An isolated protoplast is a plant cell in which the outer wall has been mechanically or enzymatically removed (Eriksson, 1989). Protoplasts have diverse applications including the study of cell physiology and genetics, plasma membrane properties, cell-cell communication and particle uptake. In terms of its agricultural implications, the technology has played an important role in the study of host-parasite interactions and plant genetic transformations (Sinha *et al*., 2003). Protoplasts are also useful materials for production of novel genotypes of flower and ornamental plants through somatic hybridization (Nakano and Mii, 1993; Mizuhiro *et al*., 2001; Horita *et al*., 2003) and somaclonal variation (Frearson *et al*., 1973). Somatic hybridization through protoplast fusion allows production of hybrids between distantly related species offering chances for generating novel cultivars that cannot be obtained by conventional hybridization techniques (Nakano *et al*., 1996).

Therefore the development of techniques for protoplast culture and fusion of Anubias may lead to the production of new varieties. This would increase the market of *Anubias* species in ornamental plant industry. However, for the successful application of this technique the availability of an efficient procedure for protoplast isolation and culture is prerequisite, but, so far, the protoplast isolation and culture of this species has not been reported. In this study, we attempted to establish efficient protoplast isolation and culture procedure of *A. nana* Engler.

MATERIALS AND METHODS

Tissue culture of *A. nana* Engler: Shoots of *A. nana* Engler were obtained from plants growing under a

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hydroponics system in a greenhouse of Aquatic Plants and Ornamental Fish Research Institute, Bangkok, Thailand. Tissue culture of *A. nana* was carried out according to Pongchawee (2004). The shoots were rinsed in 70% (V/V) ethanol for 1 min and washed twice with sterile distilled water. They were surface sterilized in 2% NaOCL with 1 drop of tween 20 per 100 m L^{-1} solution for 10 min and then immersed in 1% NaOCL with 1 drop of tween 20 per 100 m L^{-1} solution, stirred for 10 min and washed with sterile distilled water three times. The shoot tips of explants were cultured on semi-solid MS medium (Murashige and Skoog, 1962) containing 8 μM of benzyladenine (BA), 30 g L^{-1} sucrose and 1.6 g L^{-1} gelrite (Sigma, USA). Cultures were incubated in a 16 h photoperiod at 25±1°C and subcultured onto the same fresh medium at 6 weeks intervals.

Factors affecting the protoplast isolation

Kind and concentration of enzymes: Protoplasts were isolated from one gram fresh weight of young leaves of *in vitro* 6-week-old plants using five different enzyme mixtures (Table 1). The leaves were transversely sliced into small pieces in a petri dish containing a washing solution (0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES, pH 5.6) and plasmolysised for 30 min. The washing solution was pipetted off and replaced with 5 m L^{-1} of the filter-sterilized (Satorius, pore size 0.20 μm) enzyme mixtures. The enzyme-tissue mixture was incubated in the dark on a gyratory shaker (40 rpm) at 25±1°C for 4 h. The incubated enzyme-tissue mixture was then gently filtrated through nylon meshes of 60 and 40 pore size to remove undigested cell clumps and debris. The filtrate was centrifuged for 5 min at 750 rpm. The supernatants were discarded and the pellets were washed twice by centrifugation at 750 rpm for 3 min in the washing solution. The protoplast pellets were pipetted into 20% sucrose solution and centrifuged at 1000 rpm for 10 min. The purified protoplasts were collected then washed with the washing solution through centrifugation at 750 rpm for 3 min and resuspended in the washing solution. Protoplast viability was determined using fluorescein diacetate (FDA) staining (Widholm, 1972). Protoplast yield was measured using a haemacytometer under an inverted microscope (Nikon ECLIPSE TE 300, Japan) (Gleddie, 1995).

Mannitol concentration: The enzyme solution composing of 2% Cellulase RS, 0.2% Pectolyase Y-23, 2.5 mM $CaCl_2.2H_2O$, 5 mM MES (pH 5.6) and various mannitol concentrations (0.4, 0.5, 0.6 and 0.7 M mannitol) were tested for their affects on the isolation of *A. nana* Engler protoplasts. The protoplasts were isolated and collected as previously described. Protoplast yield and viability were determined.

Incubation time: One gram of six-week-old *in vitro* leaves was incubated in 5 m L^{-1} of enzyme mixture containing 0.6 M mannitol as previous described. The digestion was performed on a gyratory shaker (40 rpm) for 3, 4, 5 or 6 h in the dark. Then the protoplasts were collected and purified as previously described. The viability and yield of protoplasts were recorded to determine the effect of degradation periods.

Plant material age: The affect of plant material age (four, six, eight and ten week old) on protoplast yield and quality was examined. The protoplasts were isolated using enzyme mixture as previously described. Protoplast yield and viability were determined.

Purification by various sucrose concentrations: The protoplasts were isolated by enzyme mixture as previously described. The protoplasts was purified with varying levels of sucrose solution (16, 18, 20 and 22%) and centrifuged at 1000 rpm for 10 min. Then the protoplasts were washed with the washing solution and centrifuged at 750 rpm for 3 min. Protoplast yield and viability were determined.

Protoplast culture

Media: The purified protoplast suspensions density of 5×10^5 protoplasts mL^{-1} was cultured in two different media: MS (Murashige and Skoog, 1962) and KM8P (Kao and Michayluk, 1975) containing 0.2 mg L^{-1} 2,4-D,

Table 1: Components of enzyme mixture used for protoplast isolation of *Anubias nana* Engler

	Enzyme concentration (% w/v)			
	Cellulase		Pectinase	
Enzyme mixture	Cellulase R10[1/]	Cellulase RS[1/]	Macerozyme R10[1/]	Pectolyase Y23[2/]
E1	2	-	2	-
E2	2	-	-	0.2
E3	-	2	2	-
E4	-	2	-	0.2
E5	-	2	2	0.1

Enzyme mixture: Enzymes, 0.5 M Mannitol, 2.5 mM $CaCl_2$, 5 mM MES (pH 5.6) [1/] Yakult Honsha, Japan; [2/] Kaowa Chemical, Japan

1 mg L^{-1} NAA and 0.5 mg L^{-1} zeatin using agraose bead method. The culture dishes (60×15 mm) were sealed with parafilm and incubated at 25±2°C in the dark. The protoplast survival rate and plating efficiency were determined on the 10th day of culture according to Balestri and Cinelli (2001).

$$\text{Plating efficiency (\%)} = \frac{\text{No. of dividing protoplasts} \times 100}{\text{Total No. of plated protoplasts}}$$

Type and concentration of plant growth regulator: Three plant growth regulator combinations: M1 (1.5 mg L^{-1} NAA and 0.4 mg L^{-1} BA), M2 (0.2 mg L^{-1} 2,4-D, 1 mg L^{-1} NAA and 0.5 mg L^{-1} zeatin) and M3 (0.2 mg L^{-1} 2, 4-D, 2 mg L^{-1} NAA and 0.5 mg L^{-1} zeatin) were tested in KM8P medium using agarose bead method. The cultures were incubated in the dark at 25±2°C. Protoplast survival and cell division were assessed after 10 days.

Culture method: The purified protoplasts at a density of 5×10^5 protoplasts mL^{-1} were cultured using the liquid thin layer and agarose bead methods. For the liquid thin layer method, 1.5 mL^{-1} of protoplast suspension in liquid KM8P medium was poured onto the bottom of a 6 cm petri dish (Kim *et al*., 2003). For agarose bead method, one volume of the protoplast suspension was gently mixed with one volume of KM8P medium containing 0.2 mg L^{-1} 2,4-D, 1 mg L^{-1} NAA and 0.5 mg L^{-1} zeatin with 1.2% (w/v) agarose (SeaPrep®, FMC BioProducts, USA). The protoplast suspension was pipetted and dropped into a 6 cm petri dish. After gelling of agarose, the droplets were covered with 3 m L^{-1} of modified liquid KM8P medium. The Petri dishes were sealed with parafilm and incubated at 25°C in the dark for 10 days, dim light for 10 days, and then in the light for 30 days. Cell wall regeneration was observed with 0.01% (w/v) calcofluor white staining under a fluorescence microscope (Phansiri *et al*., 1992). Colony formation, cell division and survival percentage were determined after 10, 30 and 50 days of culture.

Data analysis: The experiment had three replicates. All data were assessed by one-way analysis of variance (ANOVA) and the means were compared by the Tukey test at 95% confidence of interval ($p<0.05$). The significance of difference in plating efficiency and survival rate as influenced by the culture media and culture methods were assessed by independent sample t-test. All statistical analyses were carried out using SPSS 11.0 software (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Factors affecting protoplast isolation

Kind and concentration of enzyme mixtures: Five different enzyme mixtures were tested for isolated protoplasts from *A. nana* Engler leaves. The results indicated that the kind and concentration of enzymes significantly affected protoplast yield and quality ($p<0.05$). E4 (2% Cellulase RS and 0.2% Pectolyase Y-23) gave the highest yield of protoplasts ($10.96\pm1.63\times10^5$ protoplasts g^{-1} FW) (Fig. 1A). However, viability of protoplast obtained from E4 (71.27±3.49%) was not significantly different from that of E2 (55.51±5.13%), E3 (58.00±3.94%) and E5 (62.37±2.26%) at $p>0.05$ (Fig. 1A), but was significantly different from E1 (48.17±4.19%) ($p<0.05$). An enzyme mixture containing 2% Cellulase RS and 0.2% Pectolyase Y-23 was suitable for isolating mesophyll protoplast of *A. nana* Engler. This combination could produce higher yield of protoplasts

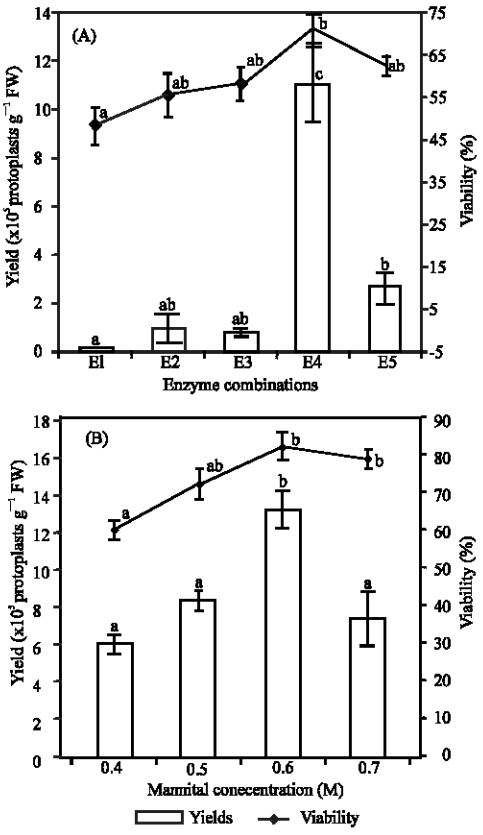

Fig. 1: Effect of different enzyme mixtures (A), and mannitol concentrations (B) on yield and viability of *Anubias nana* Engler. Data represent mean±standard error of three replicates

than 2% Cellulase R10 and 0.2% Pectolyase Y-23 (E2). Therefor Cellulase RS was more effective for protoplast isolation of *A. nana* Engler than Cellulase R10. The similar results were previously reported in rice (Toyama *et al.*, 1989). Pectolyase Y23 was more effective than Macerozyme R10 in isolating protoplasts from leaves of *A. nana* Engler. The Pectolyase Y-23 has been used successfully for isolating mesophyll protoplasts in many species such as *Brussonetia papyrifera* (Phansiri *et al.*, 2001) *Lotus corniculatus* (Vessabutr and Grant, 1995) *Dendrobium Yukidaruma* Queen (Yasugi, 1989) *Pistum sativum* (Durieu and Ochatt, 2000) and *Musa* spp. (Assani *et al.*, 2002). Nagata and Ishii (1979) indicated that Pectolyase Y-23 has about 50 times stronger endo-polygalacturonase activity than Macerozyme R-10.

Effect of mannitol concentration: The effect of various mannitol concentrations 0.4, 0.5, 0.6 and 0.7 M on protoplast isolation showed that the mannitol concentration in enzyme solution significantly influenced the yield and viability of protoplasts at $p<0.05$ (Fig. 1B). Mannitol at 0.6 M gave the highest yield ($13.12\pm1.04\times10^5$ protoplasts g^{-1} FW) and viability (82.26±3.59%). When using mannitol with at 0.4 or 0.7 M concentration, number of protoplasts were low. Generally, protoplasts burst in hypotonic solution and collapse in hypertonic solution (Maeda and Hagiwara, 1974; Ohshima and Toyama, 1989). It was concluded that 0.6 M has suitable osmotic pressure for protoplast isolation of *A. nana* Engler. This concentration was different from that used in aquatic plant *Cryptocoryne wendtii* De Wit (0.5 M mannital) (Pongchawee *et al.*, 2005).

Incubation time: The effect of time in protoplasts digestion (3, 4, 5 and 6 h) found that incubation time significantly ($p<0.05$) influenced yield and viability of protoplasts (Fig. 3). Optimal time of incubation was 4 h, which gave the highest number ($14.24\pm1.27\times10^5$ protoplats g^{-1} FW) and viability of (81.18±2.07%) protoplasts ($p<0.05$). While the incubation time was longer than 4 h, The yield and viability of protoplasts decreased for over digestion (Fig. 2A). The prolonged incubation of leaves in enzyme solution could potentially lead to protoplast mass breaking and disfunction. Damage could be decreased by modifying the enzyme solution (lower concentration of enzymes) or duration (shortening) of enzymatic treatment (Navrátilová *et al.*, 2000).

Plant material age: *In vitro* young leaves of four-, six-, eight and ten-week-old *A. nana* were used for protoplast isolation. It was found that the age of leaves

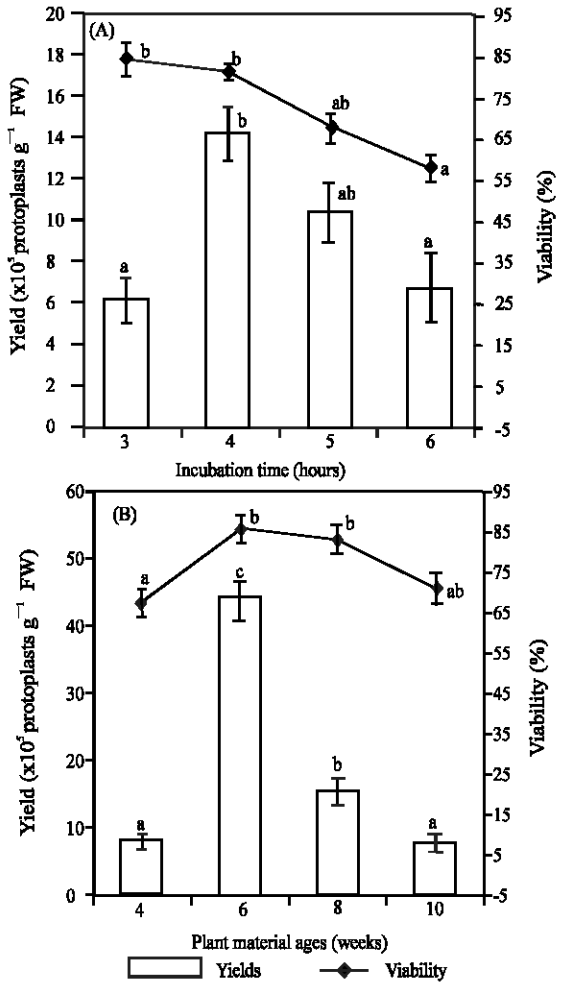

Fig. 2: Effect of incubation time (A) and plant material ages (B) on yield and viability of *Anubias nana* Engler. Data represent mean±standard error of three replicates

influenced the viability and yield of protoplasts. Six-month-old leaves provided the highest yields ($43.73\pm2.81\times10^5$ protoplasts g^{-1} FW) and viability (85.91±3.32%) of protoplasts ($p<0.05$) (Fig. 2B). While isolated protoplast from older leaves (eight- and ten-week-old) or younger leaves (four-week-old) gave the lower protoplast yield and viability. Similar experiments were showed that age of leaves could influence the number of isolated protoplasts such as *Allium ampeloprasum* (Buiteveld and Creemers-Molenaar, 1994), *Medicago sativa* (Levee *et al.*, 2005) and *Sesbania bispinosa* (Zhao *et al.*, 1995). It was difficult to isolate protoplasts from older leaves because more lignin substances accumulate in cell walls of old cells. Therefore, a six-week-old leaf was the appropriate age for protoplast isolation of *A. nana* Engler (Fig. 4A). The isolated protoplasts were

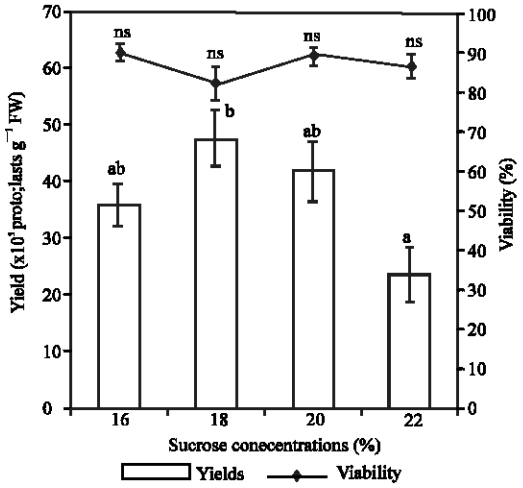

Fig. 3: Effect of sucrose concentrations on yield and viability of *Anubias nana* Engler. Data represent mean±standard error of three replicates

spherical and contained many chloroplasts (Fig. 4B). The viable protoplasts fluoresced a yellow-green color after staining with FDA (Fig. 4C).

Purification by various sucrose concentrations: There was a significant difference between the yield of protoplasts, which centrifuged in the four sucrose concentrations, but not significantly differences in the viability (Fig. 3). Purification with 18% sucrose solution gave the highest yield of $47.95\pm4.80\times10^5$ protoplasts g^{-1} FW with the viability of 82.90±4.31% ($p<0.05$). Protoplasts purified in 22% were greatly decreased in number of protoplasts. The general procedure for *A. nana* Engler protoplasts purification established here was different to that developed for *Cryptocoryne wendtii* De Wit which using 16% sucrose solution (Pongchawee *et al.*, 2005). This method could remove cell debris and needle-like crystals which have been found in protoplast isolation of *A. nana* Engler.

Factors affecting the protoplast culture

Culture media: The effects of MS and KM8P media on protoplast culture was compared. Plating efficiency and survival rate of protoplasts in KM8P medium were significantly different from protoplasts in that of MS medium ($p<0.05$) (Table 2). Cell wall synthesis occurred within 24 h and could be observed by calcofluor white staining (Fig. 4D). The first cell division of the protoplasts in KM8P medium was observed within 7-9 days after being cultured (Fig. 4E). Cell division occurred at an average of 15.28±1.30% after 10 days culture. The protoplast survival percentage (55.92±8.65%) was obtained at 10 days of culture in KM8P medium. No cell division was observed in MS medium. This suggests that KM8P medium was appropriate for culturing *A. nana* Engler protoplasts. According to KM8P was enriched with vitamins, organic acids, sugar, sugar alcohols, casamino acids and coconut water (Tamura *et al.*, 1995). The KM8P medium has been successfully used for protoplast culture in various plant species including *Broussonetia papyrifera* (Phansiri *et al.*, 2001) and *Pelargonium odoratissinum* (Miyazaki *et al.*, 1992). However, protoplasts of many plants such as *Primula malacoides* and *P. obconica* (Mizuhiro *et al.*, 2001), *Posidonia oceanica* and *Cymodocea nodosa* (Balestri and Cinelli, 2001) and *Rosa hybridia* (Kim *et al.*, 2003), could be divided and regenerated into plantlets when cultured in MS medium.

Type and concentration of plant growth regulator: The protoplasts were cultured in KM8P medium containing different types and concentrations of plant growth regulator: M1 (1.5 mg L^{-1} NAA + 0.4 mg L^{-1} BA), M2 (0.2 mg L^{-1} 2,4-D + 1 mg L^{-1} NAA + 0.5 mg L^{-1} Zeatin) and M3 (0.2 mg L^{-1} 2,4-D + 2 mg L^{-1} NAA + 0.5 mg L^{-1} Zeatin). Type and concentration of plant growth regulator significantly affected cell division and survival of protoplasts ($p<0.05$). The best results were observed in M2. The maximum survival of protoplasts and cell division were 18.33±1.32 and 56.25±1.91%, respectively (Table 3). Protoplasts cultured in 1.5 mg L^{-1} NAA + 0.4 mg L^{-1} BA did not divide and died. Growth of protoplasts is dependent on the interaction between naturally occurring endogenous growth substances and growth regulators added to the medium. The auxins (NAA and 2,4-D) and cytokinins (BA and Zeatin) are generally needed in the protoplast culture medium (Veilleux *et al.*, 2005). The kind of plant growth regulator and the balance between auxin and cytokinin are important factors controlling organ differentiation (Jomori *et al.*, 1995).

Culture method: Two different protoplast culture methods were tested. Significant differences in plating efficiencies and protoplast survival were found ($p<0.05$) (Table 4 and 5). No cell division was observed in liquid medium. Protoplasts did not divide and died.

Table 2: Effect of culture medium on plating efficiency and survival of *Anubias nana* Engler protoplasts after 10 days of culture

Culture medium	Plating efficiency (%)	Survival rate (%)
MS	0.00±0.00^a	0.00±0.00^a
KM8P	15.28±1.30^b	55.92±8.65^b

Data are means±standard error of triplicate. Means in the same column not sharing the common superscript letter are significantly different determined by Tukey's test ($p<0.05$)

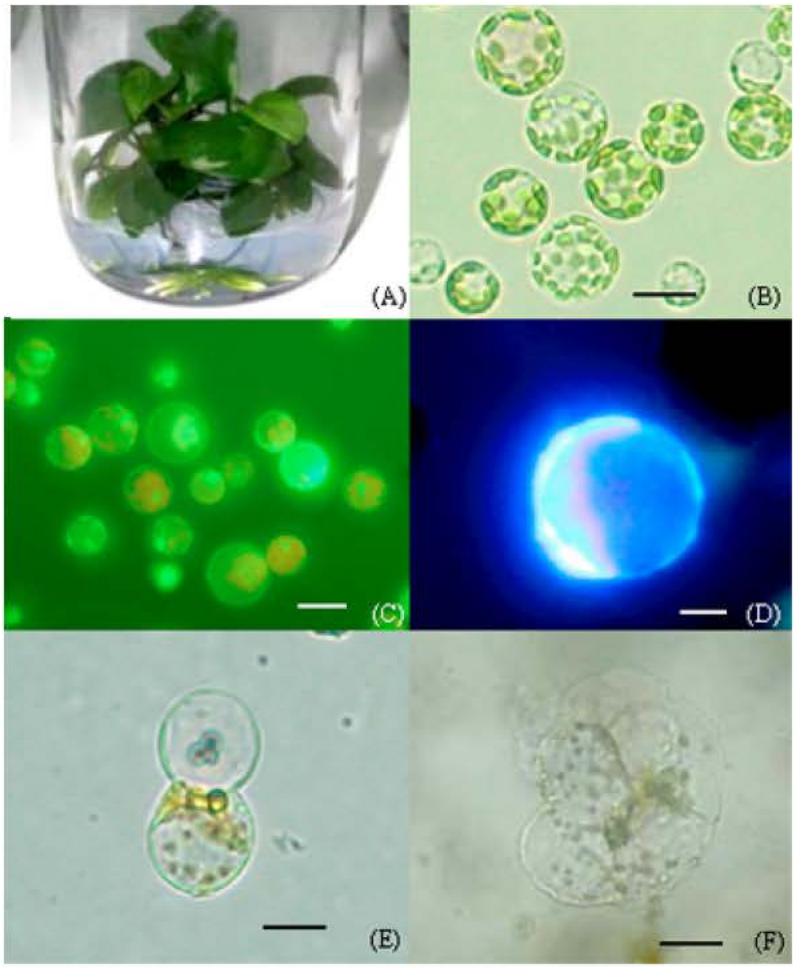

Fig. 4: Isolation, culture and cell division of *Anubias nana* Engler protoplasts. Six-week-old plantlets suitable for the isolation of leaf protoplasts (A), protoplasts after purification with 18% sucrose solution (B), vigorous protoplasts fluoresce a yellow-green color when stained with FDA (C), protoplast culture in agarose bead; cell wall regenerated within 24 h with detected by calcofluor white staining (D), first cell division after 7-9 days of culture (E), small cell colonies after culturing for 30 days (F). Bar = 20 μm

Table 3: Effect of plant growth regulators on plating efficiency and survival (10 days culture) of *Anubias nana* Engler protoplasts

PGRs combinations (mg L^{-1})	Plating efficiency (%)	Survival rate (%)
M1:1.5 mg L^{-1} NAA + 0.4 mg L^{-1} BA	0.00±0.00^{a}	0.00±0.00^{a}
M2:0.2 mg L^{-1} 2,4-D + 1 mg L^{-1} NAA + 0.5 mg L^{-1} zeatin	18.33±1.32^{c}	56.25±1.91^{c}
M3:0.2 mg L^{-1} 2,4-D + 2 mg L^{-1} NAA + 0.5 mg L^{-1} zeatin	10.51±2.57^{b}	40.82±1.28^{b}

Data are means±standard error of triplicate. Means in the same column not sharing the common superscript letter are significantly different determined by Tukey's test ($p<0.05$)

Table 4: Effect of culture methods on cell division (plating efficiency) of *Anubias nana* Engler protoplasts in KM8P medium

	Plating efficiency (%)		
Culture method	Day 10	Day 30	Day 50
Liquid medium	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Agarose bead	19.95±3.92^{b}	18.99±1.78^{b}	11.76±2.37^{b}

Data are means±standard error of triplicate. Means in the same column not sharing the common superscript letter are significantly different determined by Tukey's test ($p<0.05$)

Table 5: Effect of culture methods on survival rate of *Anubias nana* Engler protoplasts in KM8P medium

	Survival rate (%)		
Culture method	Day 10	Day 30	Day 50
Liquid medium	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Agarose bead	47.08±5.20^{b}	40.52±5.87^{b}	29.53±4.02^{b}

Data are means±standard error of triplicate. Means in the same column not sharing the common superscript letter are significantly different determined by Tukey's test ($p<0.05$)

Culturing protoplasts using agarose-bead with thin layer liquid culture method resulted in high cell division and plating efficiencies. The first cell division was observed after 2-3 days of culture. Plating efficiency was 19.95±3.92, 18.99±1.78 and 11.76±2.37% after 10, 30 and 50 days in culture, respectively. Survival rate of protoplasts cultured for 10, 30 and 50 days by this method were 47.08±5.20, 40.52±5.87 and 29.53±4.02%, respectively. Micro-colonies were formed within 30 days (Fig. 4F). However, the microcolonies stopped growing and died. The use of agarose has been proved to be useful for avoiding the agglutination of protoplasts and to be beneficial for cell division and colony formation in various plant species such as *Broussonetia papyrifera* (Phansiri *et al*., 2001) and *Curcumis melo* 'Green Delica' (Sutiojono *et al*., 1998).

CONCLUSIONS

We developed an efficient method for isolation and culture of protoplast of *A. nana* Engler. Protoplasts were successfully isolated from *in vitro* six-week-old leaves using an enzyme mixture comprising 2% Cellulase Onozuka RS, 0.2% Pectolyase Y-23, 0.6 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES, pH 5.6. Approximately $4.79\pm0.48\times10^6$ P g^{-1} Fw, with 82.90±4.31% viability were obtained after isolation for 4 h in the dark and purified with 18% sucrose gradient centrifugation, enzyme mixture, osmotic solution, incubation time, plant material age and sucrose concentration in purified solution influenced both yield and viability of protoplasts. The protoplasts regenerated cell walls within 24 h after culturing on modified KM8P medium supplemented with 0.2 mg L^{-1} 2,4-D, 1 mg L^{-1} NAA, 0.5 mg L^{-1} Zeatin by agarose-bead with thin layer liquid culture. First cell division was observed after culturing for 7-9 days and micro-colonies were formed within 30 days. Further research is in the process to find the optimal conditions for colony formation and plant regeneration in *A. nana* Engler.

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REFERENCES

Allgayer, R. and J. Teton, 1986. Aquarium Plants. Worlds Lock Ltd., London, pp: 157.

Assani, A., R. Haicour, G. Wenzel, B. Foroughi-Wehr, F. Bakry, F.X. Cote, G. Ducreux, A. Ambroise and A. Grapin, 2002. Influence of donor material and genotype on protoplast regeneration in banana and plantain culivars (*Musa* spp.). Plant Sci., 162: 355-362.

Balestri, E. and F. Cinelli, 2001. Isolation and cell wall regeneration of protoplasts from *Posidonia oceania* and *Cymdocea nodosa*. Aqua. Bot., 70: 237-242.

Buiteveld, J. and J. Creemers-Molenaar, 1994. Plant regeneration from protoplasts isolated from suspension culture of leek (*Allium ampeloprasum* L.). Plant Sci., 100: 203-210.

Cook, C.D.K., 1996. Aquatic Plant Book. SPB Academic Publ., Amsterdam, pp: 228 .

Durieu, P. and S.J. Ochatt, 2000. Efficient intergeneric fusion of pea (*Pistus sativum* L.) and grass pea (*Lathyrus sativus* L.) protoplasts. J. Exp. Bot., 51: 1237-1242.

Eriksson, T.R., 1989. Protoplast Isolation and Culture. In: Plant Protoplast. Fowke L.C. and F. Constabel. (Eds.). CRC Press., Florida, pp:1-20.

Frearson, E.M., J.B. Power and E.C. Cocking, 1973. The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol., 33: 130-137.

Gleddie, S.C., 1995. Protoplast Isolation and Culture. In: Plant Cell Tissue and Organ Culture: Fundamental Methods. Gamborg O.L. and G.C. Phillips (Eds.), Springer-Verlag, Berlin Heidelberg, pp: 167-180.

Horita, M., H. Moorhashi and F. Komai, 2003. Production of fertile somatic hybrid plants between oriental hybrid lily and *Lilium x formolongi*. Planta, 217: 597-601.

Jomori, H., Y. Tanaka and N. Kaizuma, 1995. Plant regenation from leaf-derived calli of gentians and their protoplasts culture. Acta Hortic., 392: 81-86.

Kao, K.N. and M.R. Michayluk, 1975. Nutritional requirement for growth of *Vicia hajaastana* cells and protoplasts at a very low population density in liquid media. Planta, 126: 105-110.

Kim, S.W., S.C. Oh, D.S. In and J.R. Liu, 2003. Plant regeneration of rose (*Rosa hybridia*) from embryogenic cell-derived protoplasts. Plant Cell, Tiss. Org. Cult., 73: 15-19.

Levee, V., M. Bartrand, M. Duval, P. Bilodeau, S. Aquin and L.P. Vezina, 2005. An efficient system for protoplast culture from alfafa (*Medicago sativa*) suitable for plant transformation and regeneration. http://www.medicago.com, July 16, 2005.

Maeda, E. and T. Hagiwara, 1974. Enzymatic isolation of protoplasts from the rice leaves and callus cultures. Proc. Crop Sci. Soc. Japan., 43: 68-76.

Miyazaki, H., N. Oka and M. Fukushima, 1992. Plant regeneration from protoplasts of scented-leaved geranium. Plant Cell, Tiss. Org. Cult., 9: 99-103.

Mizuhiro, M., Y. Kenichi, K. Ito, S. Kadowaki, H. Ohashi and M. Mii, 2001. Plant regeneration from cell suspension-derived protoplast of *Primula malacoides* and *Primula obconica*. Plant Sci., 160: 1221-1228.

Mühlberg, H. 1982. The Complete Guide to Water Plant . EP. Publ. Ltd., London, pp: 392.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

Nagata, T. and S. Ishii, 1979. A rapid method for isolation of mesophyll protoplast. Can. J. Bot., 57: 1820-1823.

Nakano, M. and M. Mii, 1993. Interspecific somatic hybridization in *Dianthus*: Selection of hybrids by the use of iodoacetamide inactivation and regeneration ability. Plant Sci., 88: 203-208.

Nakano, M., Y. Hoshino and M. Mii. 1996. Intergeneric somatic hybrid plantlets between *Dianthus barbatus* and *Gypsophila paniculata* obtain by electrofusion. Theor. Applied Genet., 92: 170-172.

Navrátilová, B., L. Rokytova and A. Lebeda, 2000. Isolation of mesophyll protoplasts of *Cucumis* spp. and *Cucurbita* spp. Acta Hortic., 510: 425-431.

Ohshima, M. and S. Toyama, 1989. Studies on culture of cells and tissue of crop plants, I. survey on isolation and culture of protoplast from rice leaf sheath. Jpn. J. Crop Sci., 58: 103-110.

Phansiri, S., H. Miyake, E. Maeda and T. Taniguchi, 1992. Studies on cell wall regeneration and cell division in soybean protoplasts using fluorescence and scanning electron microscopy. Jpn. J. Crop Sci., 61: 487-493.

Phansiri, S., R. Charoensub, P. Plasilmongkol and T. Taniguchi, 2001. Plant regeneration from protoplasts of paper mulberry, Thai variety. Proceeding of Paper Mulberry and Hand-Made Paper for Rural Development. March 19-24, 2001. Bangkok, Thailand, pp: 81-88.

Pongchawee, K., 2004. Micropropagation of *Anubias nana* Engler. 42th Kasetsart Annual Conference. February 3-6, 2004. Kasetsart University, Bangkok, Thailand, pp: 1-8.

Pongchawee, K., U. Na-Nakorn, S. Lamseejan, S. Poompuang and S. Phansiri, 2005. Isolation and culture of aquatic plant *Cryptocoryne wendtii* De Wit protoplast, The 5th National Symposium on Graduate Research. October 10-11, 2005, The Graduate School, Kasetsart University, Bangkok, Thailand, pp: 25.

Rataj, K. and T.J. Horeman. 1977. Aquarium Plant: Their identification, Cultivation and Ecology. T.F.H. Publ. Inc., West Sylvania, pp: 448.

Sinha, A., A.C. Wetten and P.D.S. Caligari, 2003. Optimisation of protoplast production in white lupin. Biol. Plant., 47: 21-25.

Sutiojono, E., H.M. Nonhebel and A.S. Kantharajah, 1998. Factors affecting protoplast culture of *Cucumis melo* Green Delica. Ann. Bot., 81: 775-777.

Tamura, M., R. Tao and A. Sugiura, 1995. Regeneration of Plants from Protoplasts of *Diospyros khaki* L. (Japanese Persimmon). In: Biotechnology in Agriculture and Foresty, Vol. 34: Plant Protoplasts and Genetics Engineering VI. Bajaj, Y.P.S. (Ed.). Springer-Verlag, Berlin, pp: 43-54.

Toyama, S., H. Katou and T. Imai, 1989. Studies on culture of cells and tissues of crop plants III. Isolation of rice mesophyll protolasts with a new cell wall lytic enzyme funcelase. Jpn. J. Crop Sci., 58: 635-640.

Veilleux, R.E., M.E. Compton and J.A. Saunders, 2005. Use of Protoplasts for Plant Improvement. In: Plant Development and Biotechnology. Trigiano, R.N. and D.J. Gray (Eds.). CRC Press, Florida, pp: 213-224.

Vessabutr, S. and W.F. Grant, 1995. Isolation, culture and regeneration of protoplasts from birdsfood trefoil (*Lotus corniculatus*). Plant Cell, Tiss. Org. Cult., 41: 9-15.

Widholm, J.M., 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol., 47: 189-194.

Yasugi, S., 1989. Isolation and Culture of Orchid Protoplasts. In: Biotechnology in Agriculture and Forestry, Vol. 8. Bajaj, Y.P.S. (Ed.). Springer-Verlag, Berlin Heidelberg, pp: 235-253.

Zhao, Y.X., P.J.C. Harris and D.Y. Yao, 1995. Plant regeneration from protoplasts isolated from cotyledons of *Sesbania bispinosa*. Plant Cell, Tiss. Org. Cult., 40: 119-123.