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Protoplast Isolation and Culture of Aquatic Plant *Cryptocoryne wendtii* De Wit

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ABSTRACT

The optimum conditions for protoplast isolation and culture of *Cryptocoryne wendtii* De Wit were investigated. Protoplasts were successfully isolated from *in vitro* four-week-old leaves using an enzyme mixture comprising 2% Cellulase Onozuka R-10, 0.2% Pectolyase Y-23, 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM 2 (*N*-morpholino)-ethanesulfonic acid (MES), pH 5.6. Approximately $1.04\pm0.06 \times 10^7$ protoplasts per gram fresh weight with 90.79±4.80% viability were obtained after incubating in enzyme solution for 4 hours in the dark and purified with 16 % sucrose gradient centrifugation. Protoplasts were cultured on modified MS medium supplemented with 0.2 mg/l 2,4-dicholorophenoxyacetic acid (2,4-D), 1 mg/l α-naphthalene acetic acid (NAA), 0.5 mg/l zeatin, 0.15 M sucrose and 0.3 M mannitol by agarose-bead with thin layer liquid culture. The protoplasts regenerated cell walls within 24 hours. First cell division was observed after culturing for 2-3 days, and micro-colonies were formed within 4 weeks. Enzyme mixture, osmotic solution, incubation time, age of leaves, and sucrose solution concentration were found to influence both yield and viability of protoplasts. Culture media, plant growth regulators and method of culture affected protoplast division.

Key words: aquatic plant, *Cryptocoryne wendtii* De Wit, protoplasts isolation, protoplasts culture

INTRODUCTION

The Cryptocoryne genus is a member of Araceae with more than 50 different species. They are distributed throughout Southeast Asian coastal zones. Some species are commercially cultivated as aquarium plants (Mühlberg, 1982). *Cryptocoryne wendtii* De Wit is an important species used in the aquarium plant trade (Rajaj and Horeman, 1977). It is a medium-sized species with thin rhizomes and runners, able to grow emerged or submersed and is propagated by runners (Mühlberg, 1982). The aerial leaves are oblong with round or heart shaped base, 8 to 10 cm long by 2 to 3 cm wide and below water. The blade are narrower (Allgayer and Teton, 1986).

In order to increase the value of exports and to cope with international market demand, the improvement of new aquatic plant varieties for desirable traits such as variable leaf color and form are the key to success. Related or relevant genera of cultivated crops contain a large reservoir of

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genes covering a variety of desirable traits (Liu *et al.*, 2005). However, reproductive incompatibility generally prevents simple hybridization between taxa. Somatic cell fusion enables nuclear and cytoplasmic genomes to be combined, fully or partially, at the interspecific and intergeneric levels to circumvent naturally occurring sexual incompatibility barriers (Davey *et al*., 2005). There have been many reports of the transfer of useful agronomic traits by protoplast fusion for production of triploid (Fu *et al*., 2003) and polyploid (Mizuhiro *et al.*, 2001) plants and increasing plant vigour (Cheng *et al*., 2003). This technique may be a possible alternative for the genetic improvement of *Cryptocoryne*. For successful protoplast fusion, a reliable procedure for protoplast isolation and culture is a prerequisite. Up to date, there are a few reports of protoplast isolation and culture of aquatic plants such as seagrass (Balestri and Cinelli, 2001).

In this study, the procedures for isolation and culture of *C. wendtii* protoplasts were established for the first time. The information obtained from this study will greatly benefit further genetic improvement of Cryptocoryne.

MATERIALS AND METHODS

Plant materials

Shoot tip explants of *C. wendtii* were surface-sterilized by immersion in 50% (V/V) ethanol for 1 min and 1.05 % NaOCl containing 1 drop of Tween-20 per 100 ml for 12 min, followed by rinsing three times with sterile distilled water (Kane *et al*., 1999). Explants were cultured on semi-solid MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/l 6-benzyladenine (BA), 0.25 mg/l NAA, 30 g/l sucrose and 1.6 g/l gelrite (Sigma, USA). The cultures were incubated under 16/8 h light/dark photoperiod at 25°C. Plantlets derived from shoot tips were subcultured into the same medium every four weeks. Leaves of plantlets were used as the explants for protoplast isolation.

Factors affecting the protoplast isolation

1. Enzyme mixtures

Five enzyme mixtures (Table 1) were examined for the suitable protoplast isolation. The tested enzyme mixtures were dissolved in 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM 2-N-morpholino-ethanesulfonic acid (MES) pH 5.6. One gram of *in vitro* four-week-old leaves were cut transversely into 1-2 mm wide strips in a washing solution (0.45 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES, pH 5.6). The sliced tissues were plasmolysed by immersion in washing solution for 30 minutes. The plasmolysis solution was pipetted off, replaced with 5 ml of the filter-sterilized (Satorius, pore size 0.20 μm) enzyme mixtures and incubated in the dark on a gyratory shaker (40 rpm) at 25°C for 4 hr. The protoplasts

Table 1 Components of enzyme mixtures used for protoplast isolation of *C. wendtii*

Enzyme mixtures	Enzyme concentration (% w/v)			
	Cellulase		Pectinase	
	Cellulase R-10[a]	Cellulase RS[a]	Macerozyme R-10[a]	Pectolyase Y-23[b]
E1	2	-	2	-
E2	2	-	-	0.2
E3	-	2	2	-
E4	-	2	-	0.2
E5		2	2	0.1

[a] Yakult, Tokyo.

[b] Seishin, Tokyo.

were then gently filtered through a 60 and 40 µm nylon mesh to remove undigested tissue and debris. The filtrate was centrifuged for 5 min at 750 rpm. The same process was repeated once more. The protoplast pellets were purified by floating on 20 % sucrose solution and centrifuged at 800 rpm for 10 min. The purified protoplasts were further washed twice with washing solution. Protoplast yield was estimated by a hemocytometer (Gleddie, 1995). Viability of protoplasts was assessed using 0.01% (w/v) fluorescein diacetate staining (FDA) (Sigma, USA) followed by observation with a UV fluorescence microscope (Widholm, 1972).

2. Concentration of osmoticum solution

The best result of experiment 1 was used in experiment 2. One gram of four-week-old *in vitro* leaves was incubated in 5 ml of filter-sterilized enzyme mixture, 2% (w/v) Cellulase Onozuka R-10 (Yacult Honsha, Japan), 0.2% (w/v) Pectolyase Y-23 (Kyowa Chemical, Japan) in washing solution of four varied mannitol concentrations; 0.4, 0.5, 0.6 or 0.7 M. The protoplasts were isolated and purified as previously described. Protoplast yield and viability were determined.

3. Incubation period

The best result of experiment 2 was used in experiment 3. One gram of four-week-old *in vitro* leaves was incubated in 5 ml of enzyme mixture, 2% Cellulase Onozuka R-10, 0.2% Pectolyase Y-23, 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES. The digestion was performed for 3, 4, 5 or 6 hr in the dark. The protoplasts were then harvested and purified as previously described. Protoplast yield and viability were determined.

4. Age of leaves

One gram of four-, six-, eight- and ten-week-old leaves was isolated using enzyme mixture, 2% Cellulase Onozuka R-10, 0.2% Pectolyase Y-23, 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES, and incubated in the dark on a gyratory shaker (40 rpm) at 25°C for 4 hr. The protoplasts were then harvested and purified as previously described. Protoplast yield and viability were determined.

5. Sucrose concentrations for purification

One gram of four-week-old *in vitro* leaves was incubated in 5 ml of enzyme mixture, 2% Cellulase Onozuka R-10, 0.2% Pectolyase Y-23, 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES. The protoplasts were harvested and purified with varying levels of sucrose solution; 16, 18, 20 and 22 % and centrifuged at 800 rpm for 10 min. Protoplast yield and viability were determined.

Factors affecting the protoplast culture

1. Culture medium

The purified protoplasts at the density of 5×10^5 protoplasts/ml were cultured in two kinds of liquid culture media; MS (Murashige and Skoog, 1962) and KM8P (Kao and Michayluk, 1975) containing 0.2 mg/l 2,4-D, 1 mg/l NAA, 0.5 mg/l zeatin, 0.15 M sucrose and 0.3 M mannitol incubated at 25°C in the dark. The cell division was observed periodically with an inverted microscope. The plating efficiency (% of plated protoplasts which were under cell division) and the survival rate of protoplasts were determined after 10 days of culture.

2. Plant growth regulators

The protoplasts were cultured in liquid MS medium containing various combinations of growth regulators. Three culture media tested for protoplast culture were M1 (1.5 mg/l NAA and 0.4 mg/l BA), M2 (0.2 mg/l 2,4-D, 1 mg/l NAA and 0.5 mg/l zeatin) and M3 (0.2 mg/l 2,4-D, 2

mg/l NAA and 0.5 mg/l zeatin) incubated at 25°C in the dark. The plating efficiency and percentage of survival were evaluated after 10 days of culture.

3. Culture method

Protoplasts were cultured using two methods, namely, the liquid thin layer and agarose bead methods. For the liquid thin layer method, protoplasts in liquid MS medium at the density of 5×10^5 protoplasts/ml were poured into a 6 cm Petri dish. For agarose bead method, one volume of the protoplast suspension was gently mixed with one volume of modified MS medium containing 0.2 mg/l 2, 4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l NAA and 0.5 mg/l Zeatin with 1.2 % (w/v) agarose (SeaPrep®, FMC BioProducts, U.S.A.). The protoplast suspension was dropped into a 6 cm Petri dish. The droplets were covered with 3 ml of modified liquid MS medium 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 using 0.01% (w/v) calcofluor white staining under a fluorescence microscope (Phansiri *et al.*, 1992). The plating efficiency and percentage of protoplast survival were examined after 10, 30 and 50 days of culture.

Statistical analysis

All data were assessed by one-way analysis of variance (ANOVA), and the means were compared by the Turkey test at 95% interval of confidence (*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 analysis were carried out using SPSS 11.0 software (SPSS, Chicago, IL, USA).

RESULTS

Factors affecting the protoplast isolation

1. Enzyme mixtures

Among five enzyme mixtures tested, E2 (2% Cellulase Onozuka R-10, 0.2% Pectolyase Y-23, 0.5 M mannitol, 2.5 mM $CaCl_2$ and 5 mM MES) was most appropriate for protoplast isolation, since it produced the highest yield of 81.87×10^5 protoplasts/g FW with the highest viability of 91.78 % (Figure 1). This was significantly different from other enzyme solutions (*P<0.05). The protoplasts isolated with E1 and E3 showed the lowest yield and viability.

2. Concentration of osmoticum solution

The concentration of mannitol in the enzyme solution significantly affected the yield and viability of the protoplasts (Figure 2). A 0.5 M mannitol solution was found to be most efficient for regulation of the osmotic pressure in protoplast isolation. It gave the highest yield of 80.56×10^5 protoplasts/g FW with the highest viability of 85.01 %, and was significantly different from other concentrations (*P<0.05). In addition, there was a significant decrease (*P<0.05) in protoplast viability as the mannitol concentration increased.

3. Incubation period

The incubation period during enzyme digestion significantly affected (*P<0.05) the yield and viability of the protoplasts. The highest yield of 84.36×10^5 protoplasts/g FW, with the highest viability of 85.10 % was obtained at the incubation period of 4 hr (Figure 3). The viability of protoplasts decreased with prolonged incubation period. The lowest viability of 59.27 % (*P<0.05) was recorded in protoplasts incubated in enzyme solution for 6 hr.

4. Age of leaves

The age of leaves also influenced the viability and yield of protoplasts. It was found that four- (Figure 5A) and six-week-old leaves were more suitable for protoplast isolation than eight- and ten-week-old leaves. The isolated protoplasts were spherical and contained many chloroplasts

(Figure 5B). Their viability was 87.14 % and 82.76 % for the four- and six-week-old leaves, respectively, as determined by FDA staining (Figure 5C). Protoplast viability decreased significantly with the increase in leaf age (Figure 4). It was also found a remarkable number of raphids when using the leaves as a source of protoplasts.

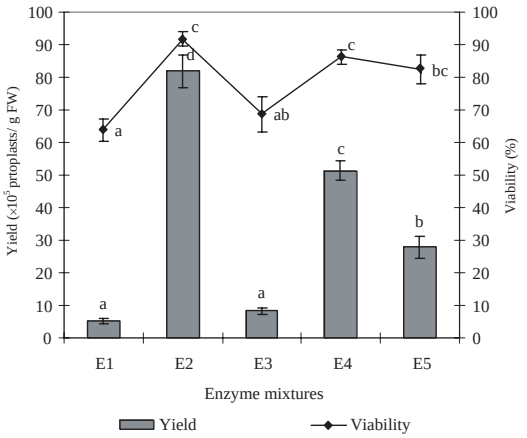

Figure 1 Effect of different enzyme mixtures on yield and viability of *C. wendtii* protoplasts. Data represent mean ± standard error of three replicates.

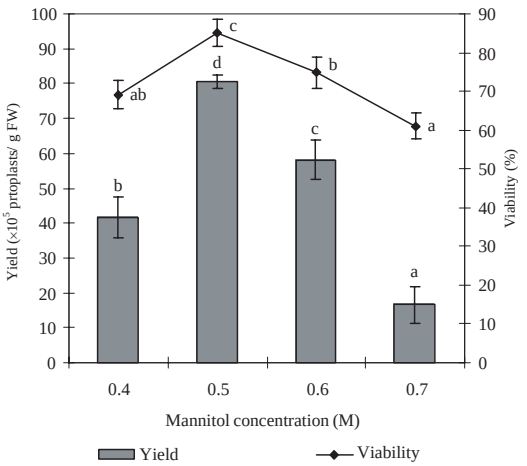

Figure 2 Effect of mannitol concentrations in the enzyme mixture on yield and viability of *C. wendtii* protoplasts. Data represent mean ± standard error of three replicates.

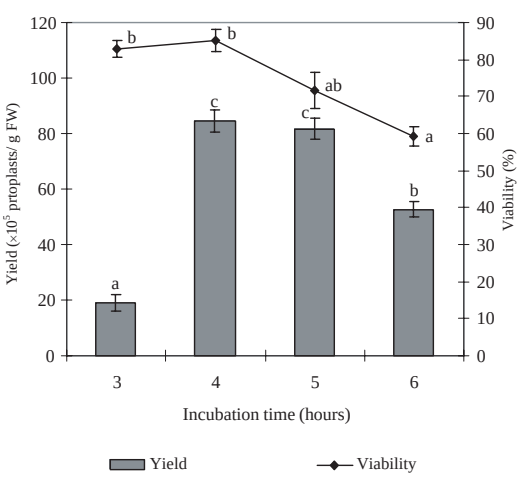

Figure 3 Effect of incubation time on yield and viability of *C. wendtii* protoplasts. Data represent mean ± standard error of three replicates.

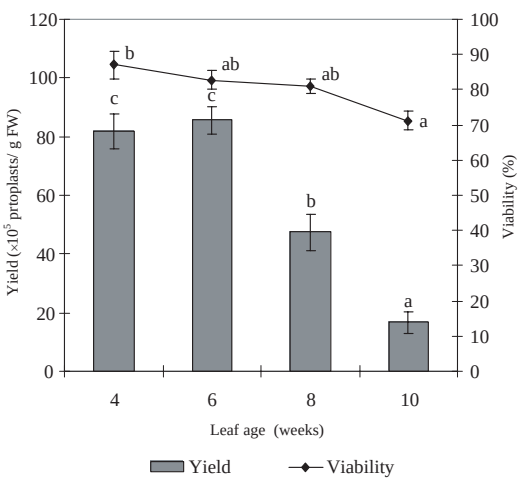

Figure 4 Effect of leaf age on yield and viability of *C. wendtii* protoplasts. Data represent mean ± standard error of three replicates.

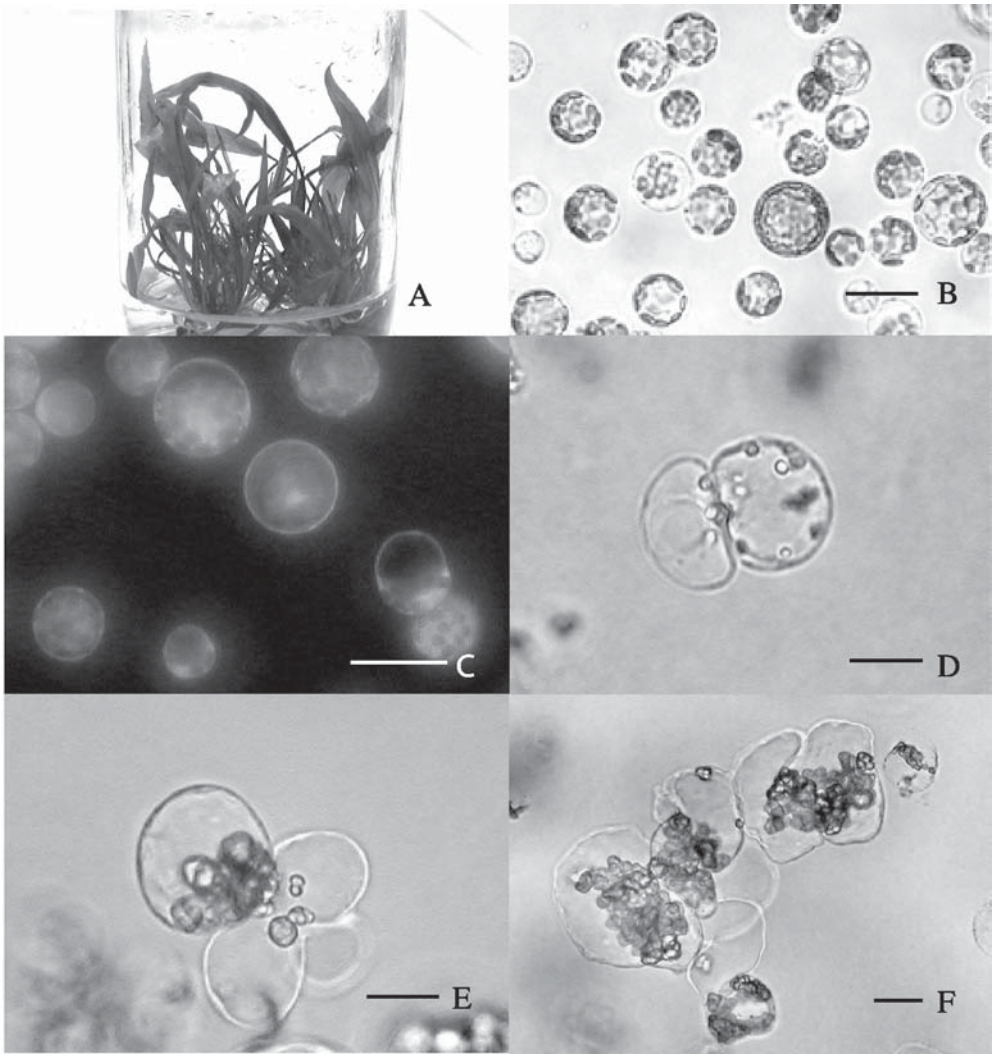

Figure 5 Isolation, culture and cell division of *Crytocoryne wendtii* protoplasts. Four-week-old plantlets suitable for the isolation of leaf protoplasts (A), protoplasts after purification with 16 % sucrose solution (B), vigorous protoplasts fluoresce a yellow-green color when stained with FDA (C), first cell division of protoplast culture in agarose bead after a few days of culture (D), second cell division after 10 days of culture (E), small cell colonies after culturing for 30 days (F). Bar = 20 μm.

5. Purification by various sucrose concentrations

There was a significant difference between the yield of protoplasts centrifuged in the four sucrose concentrations tested, but no significant difference in the viability (Table 2). Purification with 16 % sucrose solution gave the highest yield of 103.62×10^5 protoplasts/g FW with the viability of 90.79 %, and without cell debris.

Factors affecting the protoplast culture

1. Culture medium

MS medium was found to be more effective than KM8P medium. The first cell

division was found within 2-3 days in MS medium supplemented with 0.2 mg/l 2,4-D, 1 mg/l NAA and 0.5 mg/l Zeatin, 0.15 M sucrose and 0.3 M mannitol. The plating efficiency and survival rate at 10 days after culture were 21.27 % and 60.44 %, respectively (Table 3). In contrast, the protoplasts cultured in KM8P medium with the same growth regulator as MS medium did not divide but turned brown and died after 10 days of culture. This indicates that MS medium was suitable for culturing mesophyll protoplasts of *C. wendtii*.

2. Plant growth regulators

Protoplasts did not divide after being cultured in M1 (1.5 mg/l NAA, 0.4 mg/l BA) for 10 days. The highest plating efficiency (21.27 %) and cell survival (57.11 %) were observed in M2 (0.2 mg/l 2,4-D, 1 mg/l NAA and 0.5 mg/l Zeatin), which was statistically similar to that in M3 (0.2 mg/l 2,4-D, 2 mg/l NAA and 0.5 mg/l Zeatin) (Table 4).

3. Culture method

The freshly isolated protoplasts cultured in liquid and agarose bead culture regenerated cell walls within 24 hr. The first division of protoplasts was observed in 2-3 days (Figure 5D). After 10, 30 and 50 days there were no significant differences within the plating efficiency and survival rate of both culture methods (Table 5, 6). The plating efficiency and survival rate decreased

Table 2 Effect of sucrose concentration on yield and viability of *C. wendtii* protoplasts.

Sucrose (%)	Yield ($\times 10^5$ protoplasts/g FW)	Viability (%)
16	103.62±5.63 ^{c}	90.79±4.80^{ns}
18	80.38±1.78^{b}	84.74±3.23 ^{ns}
20	81.04±1.78 ^{b}	80.27±4.52 ^{ns}
22	58.79±2.96 ^{a}	76.96±1.50 ^{ns}

Data represent mean ± S.E. of three replicates. Means in the same column sharing the same superscript letter are not significantly different as determined by Turkey's test (*P>0.05).

Table 3 Effect of culture medium on cell division and survival of *C. wendtii* protoplasts after culturing for 10 days.

Culture media	Plating efficiency (%)	Survival rate (%)
MS	21.27 ± 1.32^{b}	60.44 ± 3.61^{b}
K8	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

Data represent mean ± S.E. of three replicates. Means in the same column not sharing the same superscript letter are significantly different as determined by Turkey's test (*P<0.05).

Table 4 Effect of plant growth regulator on plating efficiency and survival of *C. wendtii* protoplasts after culturing for 10 days.

PGRs combinations (mg/l)	Plating efficiency (%)	Survival rate (%)
M1:1.5 mg/l NAA + 0.4 mg/l BA	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
M2:0.2 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/l Zeatin	22.71 ± 3.02^{b}	57.11 ± 4.89^{b}
M3:0.2 mg/l 2,4-D + 2 mg/l NAA + 0.5 mg/l Zeatin	18.09 ± 2.82^{b}	48.62 ± 5.71^{b}

Data represent mean ± S.E. of three replicates. Means in the same column not sharing the same superscript letter are significantly different as determined by Turkey's test (*P<0.05).

as the culture period increased. Some protoplasts survived, divided and developed to small colonies only in agrarose bead (Figure 5F). However, callus was not formed, they turned brown and finally died.

DISCUSSION

The success in protoplast isolation of *C. wendtii* was influenced by the enzyme mixture, osmoticum solution, incubation period, age of leaves, and sucrose concentration. The combination of enzyme solution has been reported to be an important factor on yield and viability of protoplasts in many plant species such as *Artemisia judaica* L. and *Echinops spinosissimus* Turra (Pan *et al*., 2003) and *Echinacea augustifolia* (Zhu *et al*., 2005). Cellulase Onozuka R-10 was a preferred enzyme for leaf protoplast isolation of *C. wendtii* rather than Cellulase RS which had higher cellulase activity (Marchant *et al*., 1997). Cellulase Onozuka 10 combined with Pectolylase Y-23 was the most efficient for protoplast isolation of *C. wendtii*. Pectolyase Y-23 was efficient for digestion of mesophyll protoplast (Nagata and Ishii, 1979; Eriksson, 1985) due to Pectolyase Y-23 having endo-polygalacturonase activity about 50 times stronger than Macerozyme R-10 (Nagata and Ishii, 1979).

In isolating protoplasts, the wall pressure must be replaced by osmotic pressure in the isolation mixture. Mannitol is considered to be relatively inert metabolically and infuses slowly into the protoplast (Eriksson, 1985). The concentration of mannitol in the enzyme solution was another important factor affecting *C. wendtii* protoplast release. The yield and viability of protoplasts were shown to decrease with the increasing of mannitol concentration due to the protoplasts being plasmolyzed (Sinha, 2003). The prolonged incubation period decreased the yield and viability of protoplasts because of the over digestion (Zhu *et al*., 2005).

The ages of the leaves were also critical for the successful protoplast isolation of *C. wendtii*. The younger leaves gave the maximum of both viability and yield because less pectic substances accumulate in young cell walls than in the old cells (Babaoğlu, 2000), and the cell wall of a rapidly expanding leaf is thinner (Marchant *et al*., 1997). There were many calcium oxalate needles found when leaves were used as the source of protoplasts. These crystals are able to puncture and burst protoplasts during isolation (Price and Earle, 1984;

Table 5 Effect of culture methods on plating efficiency of *C. wendtii* protoplasts in MS medium.

Culture method	Plating efficiency (%)		
	Day 10	Day 30	Day 50
Liquid	28.61 ± 4.72^{ns}	18.77 ± 3.50^{ns}	13.60 ± 1.80^{ns}
Agarose bead	25.82 ± 2.46^{ns}	20.66 ± 4.67^{ns}	14.76 ± 2.14^{ns}

Data represent mean ± S.E. of three replicates. Means in the same column sharing the same superscript letter are not significantly different as determined by Turkey's test (P>0.05)

Table 6 Effect of culture methods on survival rate of *C. wendtii* protoplasts in MS medium.

Culture method	Survival rate (%)		
	Day 10	Day 30	Day 50
Liquid	67.25 ± 5.46^{ns}	57.47 ± 4.65^{ns}	33.67 ± 4.06^{ns}
Agarose bead	68.12 ± 5.37^{ns}	54.93 ± 5.65^{ns}	36.57 ± 3.08^{ns}

Data represent mean ± S.E. of three replicates. Means in the same column sharing the same superscript letter are not significantly different as determined by Turkey's test (P>0.05)

Kunasukdakul and Smitamana, 2003). However, all raphids and debris could be successfully removed by centrifugation of protoplasts with 16 % sucrose solution.

For the protoplasts culture of *C. wendtii*, the culture media, culture method and plant growth regulators were important factors affecting plating efficiency and survival rate. The protoplasts could divide in liquid as well as in agarose bead culture. However, microcolonies were formed only in agarose bead culture. The agarose bead culture methods have been found to be an efficient method for cell division and microcolony formation in many crop species including *Lavatera thuringiaca* (Vazquez-Tello *et al*., 1995); *Rosa hybrida* (Marchant *et al*., 1997) and *Cucumis melo* 'Green Delica' (Sutiojono *et al*., 1998). The enhanced protoplast division observed in bead culture was due to the dilution of substances having inhibitory effects on protoplast division which are secreted from the cell to the medium (Mizuhiro *et al*., 2001).

Colony formation was observed after culturing protoplasts in MS medium supplemented with 0.2 mg/l 2,4-D, 1 mg/l NAA, 0.5 mg/l Zeatin, 0.3 M mannitol, and 0.15 M sucrose for 30 days. However, it did not form a callus but turned brown and finally died. It has been reported that the protoplasts isolated directly from leaves of monocotyledons, except rice, was very difficult to culture (Kuehnle and Nan, 1990). It was suggested that leaf cells rapidly lose totipotency thus preventing cells from dedifferentiating and reentering the cell cycle (Krautwig and Lörz, 1995). Plant regeneration has been found possible when callus and cell suspension were used as the source of protoplast isolation and culture (Kobayashi *et al*., 1993; Pauk, *et al*., 1994).

CONCLUSION

The procedure for simple and reliable isolation and culture of *C. wendtii* protoplasts has been described for the first time. It might lead to the improvement of the *Cryptocoryne* through somatic hybridization, somaclonal variation and genetic engineering by using the protoplast technique. Even though the viable protoplasts of *C. wendtii* could form microcolonies, further research is needed to develop the efficient procedure for the protoplast regeneration.

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