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Plant regeneration from mesophyll protoplasts of lisianthus (*Eustoma grandiflorum*) by adding activated charcoal into protoplast culture medium

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Abstract

Plant regeneration from isolated protoplasts of 8 cultivars of lisianthus, *Eustoma grandiflorum* (Griseb.) Schinners, has been established by using activated charcoal. Protoplasts were isolated from lisianthus leaves grown *in vitro* and started to divide within 3-4 days of culture, but successful colony formation was only achieved by adding gellan gum blocks containing 1% (w/v) activated charcoal immediately after culture. Colonies consisting of as many as 50-100 cells formed after 30 days of culture and were transferred to fresh medium for callus proliferation and shoot regeneration, respectively. These shoots rooted on MS medium containing 0.5 mg l^{-1} indolebutyric acid(IBA) and the plantlets were finally transplanted to pots. Morphological characteristics, growth habit and pollen fertility of protoplast-derived plants of one cultivar were not different from those of seed-grown plants as control.

Abbreviations: BA – 6-benzylaminopurine; NAA – 1-naphthaleneacetic acid; MS – Murashige & Skoog (1962) medium; IBA – indolebutyric acid; MES – 2-N-morpholinoethane sulfonic acid

Introduction

Lisianthus (*Eustoma grandiflorum* (Griseb.) Schinners) belongs to the Gentianaceae and is native to the American prairies ranging from South Nebraska to Louisiana and Texas. The breeding of this plant has been mainly done in Japan since its introduction in 1950s and many varieties with various flower color such as purple, plum, white, pink and bicolors have been produced. Some dwarf forms and double petaled flower types have also been bred. Lisianthus is now very popular as a cut flower or pot plant in Japan and its popularity in the United States and Europe is increasing (Halevy & Kofranek 1984; Griesbach & Semeniuk 1987; Uematsu 1989).

Establishment of a reproducible plant regeneration system from cultured protoplasts would be useful for the application of techniques such as gene transfer and somatic hybridization. In the family Gentianaceae, plant regeneration from protoplasts was first reported in gentian (*Gentiana scabra* Bungei) (Takahata & Jomori 1989). In lisianthus, a protoplast culture system was recently reported by O'Brien & Lindsay (1993), who isolated protoplasts from either cotyledon or mature leaves of 5 cultivars and succeeded in regenerating whole plants by using an agarose bead method.

In this report, we describe an alternative and efficient protocol for regeneration of plants from protoplasts isolated from mature leaves of 8 cultivars of lisianthus and the morphological characteristics observed from protoplast-derived plants (referred to as 'protoclones' hereafter).

Materials and methods

Plant material

Nine cultivars of lisianthus (*Eustoma grandiflorum* Schinners) used throughout this study were 'Genjizakura', 'F_1 White Star', 'Early Bicolor Purple', 'Azumano Yosooi', 'Azumano Asa', 'Azumano Kiri', 'Holy Small Lady', 'Sky Friend' and 'Wakamurasaki'. Seeds of these 9 cultivars were sterilized by using 70% (v/v) ethanol for 30 sec and 1% (v/v) sodium hypochlorite for 7 min, successively, followed by 3 washings with distilled water. The seeds were sown into an 300 ml conical beaker (height 13 cm, Iwaki Glass JAPAN) containing 50 ml of MS (Murashige & Skoog 1962) medium with 3% (w/v) sucrose and 0.2% (w/v) gellan gum (Kelco, Division of Merck & Co. Inc., San Diego, California). Three to 4 seeds per conical beaker were cultured at 25 °C under continuous illumination of 38 μmol m^{-2} s^{-1}. The *in vitro* plantlets with 5–6 mature leaves obtained after 40 days of culture were used for protoplast isolation. The degree of hyperhydricity of mature leaves in these cultivers was visually observed at this stage.

Protoplast isolation and culture

The upper 4–5 mature leaves of *in vitro* grown 40 day-old seedlings (1 g fresh weight) were cut into small pieces and incubated with 10 ml of filter-sterilized (Millipore, 0.45 μM) enzyme solution containing 1% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.05% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 5 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, and 0.6 M sorbitol, at pH 5.7. The mixture was incubated on a rotary shaker (60 rpm) for 3–4 h, at 25 °C to liberate protoplasts. Protoplasts were collected by filtration through a nylon sieve (60 μm) and harvested by centrifugation at $100 \times g$ for 5 min in a 0.6 M mannitol solution. The pellet was resuspended in 0.65 M sucrose solution and centrifuged at $100 \times g$ for 5 min. The floating protoplasts were collected and washed twice with 0.65 M mannitol solution. Protoplast yield was estimated using a hemocytometer, and the culture density of protoplasts was adjusted before plating.

The viability of protoplasts was assessed using the fluorescein diacetate (FDA) staining method (Widholm 1972). Protoplasts were suspended in culture medium containing 0.01% (w/v) FDA for 2–5 min and observed under a fluorescent microscope (Olympus model FLM). The percentage of viability was calculated from the number of protoplasts giving green fluorescence out of the total number of protoplasts counted.

Protoplasts were cultured in a modified MS (-NH_4NO_3) liquid medium containing 2 mg l^{-1} NAA, 1 mg l^{-1} BA, 3% (w/v) sucrose and 0.5 M mannitol at a density of 1×10^5 ml^{-1} in 60×15 mm plastic petridishes containing 3 ml of culture medium which were sealed with Parafilm and maintained at 25 °C in the dark.

The effect of activated charcoal on colony formation from protoplasts was investigated. Gellan gum blocks containing MS medium supplemented with 0.6 M glucose and 1% (w/v) activated charcoal (referred to as 'charcoal blocks' hereafter) were added to the liquid protoplast culture medium. The timing of addition and quantity of the charcoal blocks was investigated in order to obtain a high frequency of colony formation. Using the best conditions obtained, genotypic differences in colony formation from protoplasts were also investigated among several cultivars. As a measure of colony formation, the number of colonies which attained the size of approximately 0.5 mm in diameter was counted after 30 days of culture.

Callus proliferation and plant regeneration

After 2 months of culture, protoplast-derived colonies (ca. 1 mm in diameter) were transferred to MS medium containing 2 mg l^{-1} NAA, 1 mg l^{-1} BA, 3% sucrose and 0.8% agar to induce callus proliferation. After proliferation, the calluses were transferred for shoot regeneration to test tubes containing 0.8% (w/v) agar-solidified 1/2 MS media supplemented with different concentrations of NAA and BA. For each growth regulator treatment, ten replicate test tubes were used and 200 mg of callus was inoculated onto 20 ml of medium in each tube. These were cultured under the same conditions as for callus proliferation. The induction frequency of adventitious shoots was defined as the percentage of protoplast-derived calluses which produced adventitious shoots after one month of culture. This experiment was repeated twice.

The shoots regenerated from the calluses were detached and were transferred for rooting to 1/2 MS medium containing 0.5 mg l^{-1} IBA, 3% sucrose and 0.8% agar. Rooted plants were potted in vermiculite and peatmoss (1:1 mixture), placed in an incubator for acclimatization for about 3 weeks at 25 °C under con-

Table 1. Differences in protoplast yield and colony formation among 9 cultivars of lisianthus.

Cultivars	A degree of hyperhydricity[a]	Protoplast yield[b] ($\times 10^5$ cells/gFW±SE)	Colony formation[c] (Av. number± SE/dish)
Genjizakura	+	14.9±4.4	17.3±14.6
F_1 White Star	+++	1.5±1.6	327.3±62.3
Wakamurasaki	++	13.6±3.8	453.8±96.7
Early Bicolor Purple	+	16.0±3.9	967.8±88.2
Azumano Yosooi	++	4.6±3.0	683.2±72.3
Azumano Asa	+	11.6±2.5	454.3±98.3
Azumano Kiri	++	11.9±3.3	0
Holy Small Lady	+	2.1±1.1	368.8±37.8
Sky Friend	++	10.1±1.5	642.3±31.2

[a]Degree of hyperhydricity of mature leaves was classified as follows: +++; high, ++ medium, +; low

[b]Number of protoplasts isolated from 1 g mature leaves after 4 h of maceration. The values represent the mean ± SE of at least 5 independent experiments.

[c]Number of colonies / dish after 2 months of culture. The values represent the mean ± SE of 2 experiments, each with 5 replicates.

tinuous illumination of 38 μmol m^{-2} s^{-1}, and were then transferred to a glasshouse. The morphological characteristics and growth habit of the protoclones 'Wakamurasaki' were compared with seedlings grown from seed. The pollen fertility of protoclones and seedlings was checked by staining with 1.0% aceto-carmine.

Results

Protoplasts were readily isolated from mature leaves of *in vitro* grown plants after 3–4 h of enzyme treatment. They were successfully purified by floating on 0.65 M sucrose solution and showed a very low level of contamination with undigested cells. The protoplasts were heterogeneous in size ranging from 20 to 60 μm in diameter and the yields ranged from 1.5 to 16.0×10^5 /gram (fresh weight) among 9 cultivars of lisianthus (Table 1). 'Early Bicolor Purple' showed the best isolation with 16.0×10^5/gram (fresh weight). About 85% of them were viable as determined by FDA staining method. Cultured plants of 'F_1 White Star' and 'Holy Small Lady' showed high level of hyperhydricity and gave a low yield.

Purified and washed mesophyll protoplasts were initially cultured in liquid MS medium containing 2 mg l^{-1} NAA, 1 mg l^{-1} BA, 3% sucrose and 0.5 M mannitol. In this medium, protoplasts could divide but never reached beyond a 4–6 cell colony stage. Removal of NH_4NO_3 from this medium slightly improved the situation and the protoplasts started to divide after 3–4 days of culture, and attained an approximately 10-cell stage in this modified MS medium. Subsequently, the colonies in this medium gradually turned brown and died. In an attempt to prevent necrosis, the effect of activated charcoal on colony formation was examined. When charcoal blocks were added to the liquid cultures, cell division continued, browning was inhibited, and individual colonies consisted of as many as 50–100 cells were observed after 30 days of culture.

The timing of addition of the charcoal block was important in obtaining successful growth of the colonies (Fig. 1). High colony formation was obtained (approximately 700 colonies/dish), when a charcoal block was added to the culture at an early stage of protoplast culture (0–7 days after culture). However, the addition of the charcoal block after 14 days of culture did not have any significant effect on the colony formation. The weight of the charcoal blocks also affected colony formation (Fig. 2). In the two cultivars examined, addition of a 200 mg charcoal block gave the highest colony formation, 630 in 'Azumano Yosooi' and 240 colonies per dish in 'F_1 White Star', respectively. More than 400 mg of the charcoal block resulted in a much lower colony formation. Using the optimum conditions with respect to timing and weight of charcoal blocks added differences in colony formation were examined among 9 cultivars (Table 1). Colony formation was observed in 8 cultivars with averages from

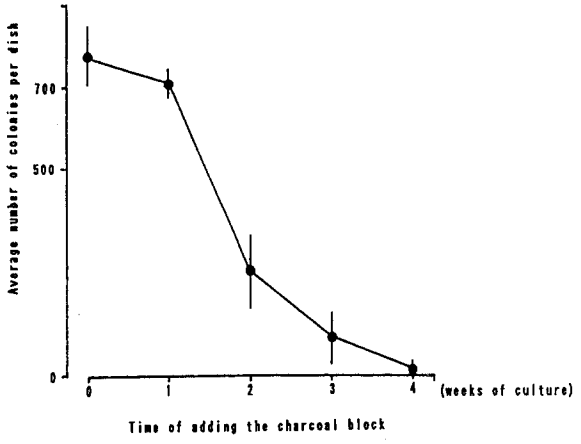

Fig. 1. Effect of time of adding the charcoal block to the culture medium on colony formation from protoplasts of lisianthus cv. 'Azumano Asa'. Two hundred mg of activated charcoal block were added to 3 ml of protoplast culture medium. Two independent experiments, each with 5 replicates were carried out. The data were recorded after 2 months of culture. Each value represents average number of colony per dish ± S.E..

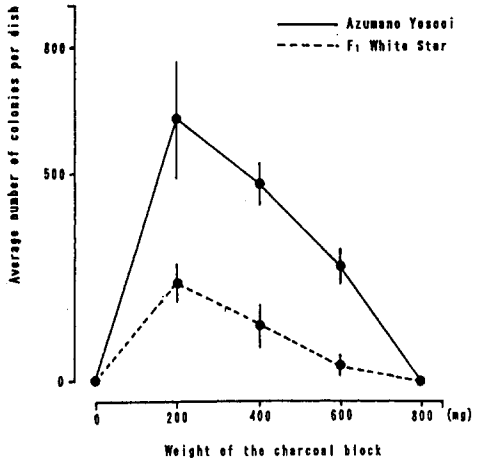

Fig. 2. Effect of the charcoal block on colony formation from protoplasts of lisianthus cv. 'Azumano Yosooi' and F_1 White Star. The charcoal block was added to 3 ml of protoplast culture medium after a week of culture. Two independent experiments, each with 5 replicates were carried out. The data were recorded after 2 months of culture. Each value represents average nurber of colony per dish ± S.E..

17 to 968/dish, and 'Early Bicolor Purple' showed the best colony formation, whereas 'Azumano Kiri' did not produce any colonies.

After 2 months of culture, colonies were transferred onto MS medium containing 2 mg l^{-1} NAA, 1 mg l^{-1} BA, 3% sucrose and 0.8% agar, where they grew rapidly into green compact calluses. The effect of growth regulators on adventitious shoot formation was investigated using protoplast derived-calluses of 'Azumano Asa' (Table 2). Three to 4 weeks after transfer, adventitious shoots began to develop on the surface of the calluses. The highest regeneration frequency of adventitious shoots (70%) was obtained on 1/2 MS medium containing 2 mg l^{-1} BA. However, the average number of adventitious shoots per callus was the highest at 1mg l^{-1} BA (12.6 shoots), whereas 4.3 shoots/callus were obtained at 2mg l^{-1} BA. Shoot regeneration from 8 cultivars of protoplast–derived calluses were observed on 1/2 MS medium containing 2 mg l^{-1} BA, and differencies in its shoot regeneration rate showed between these cultivars (data not shown).

When the shoots became 0.5–1.0 cm in height, they were transferred to 1/2 MS medium containing 0.5 mg l^{-1} IBA. Roots rapidly initiated at the base of the shoots 2 weeks after transfer.

Thirty five protoclones of 'Wakamurasaki' were transplanted first into pots, and acclimatized in an incubator and then transferred to a glasshouse. Leaf development of the protoclones was slower than that of seed-grown plants immediately after acclimatization, but no difference in growth was observed thereafter. Four months after acclimatization, all protoclones flowered normally (Fig. 3). No differences were observed in flower and leaf characters such as shape, thickness and color between protoclones and seed-grown plants. Aceto-carmine staining also showed no difference in pollen fertility (approximately 95%) between them.

Discussion

By adopting the method used in the present study, viable mesophyll protoplasts were successfully isolated from several cultivars of lisianthus, although they showed differences in their protoplast yields. One of the main reasons for the differences may have been the occurrence of hyperhydricity in the mature leaves of *in vitro* plants, which was probably genotype dependent. O'Brien & Lindsay (1993) previously showed that hyperhydricity of source-plant material prevented successful protoplast isolation and suggested that calcium ions played an important role in the occurrence the hyperhydricity. Furthermore, it is generally accepted that improvements in tissue culture conditions such as light intensity, gas exchange rates and concentration of CO_2 are key factors for preventing hyperhydricity of source-plant material (Binding 1974; Kao & Michayluk 1974; Shepard & Totten 1977; Kozai & Iwanami 1988). Therefore, using non-vitrified leaves

Table 2. Effect of growth regulators on the regeneration of adventitious shoots from protoplast-derived calluses of lisianthus cv. 'Azumano Asa'.

Growth regulators ($mg\ l^{-1}$)		Callus weight (g ± SE)	Adventitious shoot formation	
BAP	NAA		Regeneration frequency (%)[a]	Average number[b]
0	0	1.41±0.41	0	0
1	0	1.61±1.13	30.0	12.6
2	0	1.36±0.61	70.0	4.3
0	0.5	1.62±0.67	50.0	0.1
1	0.5	1.88±0.92	0	0
2	0.5	2.27±0.96	0	0

About 200 mg (fresh weight) of protoplast-derived calluses were transferred to each tube which contained 10 ml of culture medium. Two independent experiments, each with 10 replicates, were carried out, and the data were recorded 2 months after transfer.

[a] Percentage of calluses showing adventitious shoot formation

[b] Average number of adventitous shoots per callus

Fig. 3. Protoplast-derived plant of lisianthus cv. Wakamurasaki with normal flowers.

produced by improving these tissue culture conditions is important for isolating sufficient numbers of viable protoplasts.

The phenomenon of browning often imposes a serious obstacle to protoplast, tissue and organ cultures of higher plants (Hu & Wang 1983). So far, elimination of ammonium ions (Eriksson 1985), replenishing of the protoplast culture medium (Doughty & Power 1988) and addition of high concentration of organic components (Doughty & Power 1988; Kouider *et al.* 1984; Nizeki *et al.* 1983; Kunitake & Mii 1990a, 1990b) have been used as procedures for decreasing cell browning. In the present study, browning of cells could be inhibited and cell division continued by eliminating ammonium ions and by adding charcoal blocks to the protoplast culture medium. Several reports have shown that a reduction or elimination of ammonium ions from the culture medium has a beneficial effect on protoplast division and subsequent growth of cell colonies (Ochatt & Caso 1986; Ochatt & Power 1988; Oka & Ohyama 1985). However, there are only a few examples regarding the utilization of activated char-

coal in protoplast culture. According to Fridborg & Eriksson (1975) and Fridborg *et al.* (1978), the effect of activated charcoal on somatic embryo formation in carrot cell cultures was mainly due to adsorption of phenolic substances. Carlberg *et al.* (1983) reported that the pronounced effect of the activated charcoal was to decrease brown exudates accumulating in the potato protoplast medium. A similar effect of activated charcoal seemed to occur in the protoplast culture of lisianthus in the present study. The importance of the timing of addition and quantity of the activated charcoal blocks in obtaining high colony formation in our study suggests that these growth inhibitory substance were secreted from the beginning of protoplast culture. The inhibition of colony formation by additional amount of charcoal may be due to the adsorption of essential components of the culture medium such as NAA and BA.

Semeniuk & Griesbach (1987) first observed shoot regeneration in lisianthus from callus on the modified MS medium supplemented with NAA and BA. They reported that most of these regenerated plants showed altered phenotypic characteristics of a single stem with branching from the mid to the top portion of the plant, and a strong basal branched characteristic was exhibited in several regenerated plants (Griesbach & Semeniuk 1987). However, no such morphological characteritics have so far been observed in our protoplast-derived plants, in the limited number examined.

Successful regeneration of whole plants from protoplasts was achieved in most of the lisianthus cultivars used in the present study. This suggests that the use of the appropriate amount of activated charcoal block at early satge of culture would also be applied to the other cultivars of this species as well as the other plant species which have the problem of cell browning during protoplast culture.

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