Isolation and culture of protoplasts from the medicinal plant *Centella asiatica*

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ABSTRACT: Isolation and culture of protoplasts from the medicinal plant *Centella asiatica*. In the present investigation, protoplasts were isolated from cell suspensions initiated from leaf laminae and petioles using an enzyme mixture consisting of 1.5% (w/v) Cellulase R10, 1.0% (w/v) Macerozyme R10 and 0.5% (w/v) Driselase in CPW salts solution with 13% (w/v) mannitol as osmotic stabilizer. Yields and viabilities of isolated protoplasts were $1.2 \times 10^5 \pm 0.1$ g^{-1} fresh weight and $20.8 \pm 4.4\%$ for protoplasts from lamina-derived cell suspensions and $7.9 \times 10^5 \pm 1.5$ g^{-1} fresh weight and $79.3 \pm 13.4\%$ for protoplasts from petiole-derived cell suspensions. Protoplasts from lamina explant-derived cell suspensions were cultured at plating densities of $0.25 \times 10^5 – 2.0 \times 10^5$ protoplasts ml^{-1} in half-strength B5 based medium containing 0.1 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.3 mgl^{-1} zeatin, dispensed as semi-solid agarose droplets (each approx. 70 µl in volume) in 5.5 cm diameter Petri dishes (10 droplets per dish). First mitotic divisions of protoplast-derived cells were observed after 4 d of culture at an optimum plating density of 0.5×10^5 protoplasts ml^{-1}, giving an initial plating efficiency at this time of $12.7 \pm 0.6\%$. After 42 d of culture, protoplast-derived cell colonies were creamy-white in colour and each approx. 1 mm in diameter, with a final plating efficiency of $0.6 \pm 0.2\%$. Cell colonies transferred to semi-solid proliferation medium containing 2,4-D (4.0 mgl^{-1}) and zeatin (0.2 mgl^{-1}) were creamy-yellow in appearance, whereas colonies cultured on medium devoid of these growth regulators became light green and compact. In the case of protoplasts from petiole-derived cell suspensions, culture in Murashige and Skoog (1962)-based medium supplemented with 2.0 mgl^{-1} alpha-naphthaleneacetic acid and 0.5 mgl^{-1} 6-benzylaminopurine resulted in an initial plating efficiency of $19.3 \pm 4.2\%$ at an optimum plating density of 1.0×10^5 protoplasts ml^{-1}. However, mitotic division was not sustained after this stage. Plant regeneration studies are on-going from protoplasts isolated from lamina-derived cell suspensions.

Key words: *Centella asiatica*, cell suspensions, medicinal plant, protoplasts

INTRODUCTION

Centella asiatica (L.) is a classic ethnomedicinal species used by diverse ancient civilisations and tribal groups. The plant extracts seem to have been used for medicinal purposes by the people of Java and Malaysia for many years, as both topical and internal agents for the healing of wounds, diarrhoea, eye infections and partial relief from leprosy. It is also used as an anti-diarrheic, anti-dysentheric, circulatory stimulant, hypotensive, tonic (Goh et al., 1995).

A protoplast is a wall-less plant cell where the cell wall has been degraded by a range of hydrolytic enzymes (cellulases, pectinases). The potential use of protoplast technology for the genetic improvement of plants is immense, however, the application of the technology to medicinal plant species is relatively poorly exploited. Their usefulness for medicinal plants has at least been realised. They may be employed for genetic modification of chemical composition or other characters such as disease resistance (Xia et al., 1996). Somatic hybridisation provides the possibility of producing novel medicinal products in hybrids. Furthermore, protoplast culture is one of the most effective ways to isolate truly single cell origin lines selected for perhaps high secondary metabolite productivity (Lou et al., 1999). To date, there is no report on the isolation, culture and plant regeneration from protoplasts of *Centella asiatica*. This paper describes the development of protocols for isolations and cultures of *C. asiatica* protoplasts derived from cell suspension cultures.

MATERIAL AND METHOD

Actively growing *C. asiatica* cell suspensions induced from young leaves and petioles growing in MS based medium supplemented with 0.1 mg/l BAP and 0.5 mg/l 2,4-D and MS based medium containing 0.23 mg/l BAP and 0.22 mg/l 2,4-D (Solet et al., 1993) respectively, were used as source materials for protoplast isolations. Cell suspension cultures were 6 - 27 months post-initiation and routinely subcultured every 14 days.

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Cells from *Centella* suspension cultures (lamina- and petiole-derived) were harvested (3 d post-subculture) and incubated with an appropriate volume of enzyme solution (1.0 g f. wt. cell suspension in 10 ml of enzyme solution) in a 9.0 cm diam. Petri dish (Bibby Sterilin Ltd., Stone, UK). Enzyme solution contained 1.5% (w/v) Cellulase R10 (Yakult Honsha Co., Tokyo, Japan), 1.0% Macerozyme R10 (Yakult Honsha Co., Tokyo, Japan) and 0.5% (w/v) Driselase (Kyowa-Hakko Kogyo Co., Tokyo, Japan) were prepared by dissolving enzymes in CPW salt solution containing 13% (w/v) mannitol. The pH of the enzyme solutions was adjusted to 5.8. Enzyme solutions were filter-sterilised through 0.2 μm membrane filters (Milipore High-Flow, Sartorius, Germany). The dishes containing cells and enzymes were sealed with Nescoflim (Nippon Shoji Kaisha Ltd., Osaka, Japan) and incubated for 16 h on a rotary shaker (20 - 30 r.p.m.) in the dark at 25 ± 2°C.

For purification, digested cells callus and enzyme solution were filtered through a sterile 80 μm mesh nylon sieve (Wilson Sieves, Nottingham, UK) to remove coarse and undigested materials. The collected enzyme with protoplasts was transferred to 16 ml capacity screw-capped centrifuge tubes (Corning Ltd., New York, USA) and centrifuged twice (80 x g) for 7 min. The supernatant was removed and the protoplast pellet was washed twice by resuspension in 10 ml of CPW salts solution (*Power et al.*, 1989) supplemented with 13% (w:v) mannitol (designated CPW 13M wash solution).

Yields of protoplasts were determined using a double-chamber haemocytometer (Modified-Fuchs Rosenthal rulings, model B.S. 74B; Weber Scientific Internaional Ltd., Teddington, UK). The density of the protoplast population was adjusted according to requirement.

Viability of protoplasts was assessed by uptake and cleavage of fluorescein diacetate (FDA, Sigma) (Widholm, 1972). Counts of viable protoplasts were made from at least 4 fields of view from each slide and the percentage viability was determined.

Protoplasts from lamina-derived cell suspensions were cultured on half strength B5 based medium (Gamborg et al., 1968) containing 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l zeatin (ZN) at plating densities of 0.25 - 2.0 x 10^5 protoplasts ml^{-1}. Protoplasts were cultured in 70 μl semi-solid [0.8% (w/v); SeaPlaque agarose, FMC, Rockland, USA] beads (10/dish) surrounded by 3 ml aliquots. Protoplasts from petiole-derived cell suspensions were cultured in Murashige and Skoog (1962)-based medium supplemented 2.0 mg/l alpha-naphthaleneaceatic acid (NAA) and 0.5 mg/l 6-benzyleaminopurine (BAP). Petiole-derived protoplasts were cultured in 5.5 cm Petri dish containing 3ml liquid medium at 5 plating densities (0.5 - 5.0 x 10^5 protoplasts ml^{-1}). A minimum of 200 protoplasts per Petri dish per medium per density was scored; evaluation at each plating density was repeated 3 times.

Protoplast plating efficiency was determined at the Initial Plating Efficiency (IPE) and the Final Plating Efficiency (FPE). The Initial plating efficiency (IPE) was estimated by counting the originally plated protoplasts that had undergone at least one mitotic. The Final Plating Efficiency (FPE) was defined as the percentage of the initially plated protoplasts that developed colonies (> 1.0 mm in diam.) by 45 d.

Protoplast-derived callus (1 - 2 mm diam.approx.) were transferred to one of several semi-solid [0.8% (w:v) agar (Sigma, St. Louis, USA)], osmoticum-free, regeneration media. A total of 34 media based on MS salts were evaluated for plant regeneration from such protoplast-derived colonies. Six growth hormones at various concentrations and combinations were evaluated. MS medium with 0, 0.5, 1.0, 2.0, 3.0, 4.0 mg/l BAP and 0.05 mg/l NAA; 0, 0.05, 0.1, 0.15, 0.2, 0.25 mg/l IBA plus 0.25 mg/l KN; 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5 mg/l NAA and 3 .0 mg/l BAP; 0, 0.1, 1.0, 2.0, 4.0 mg/l 2,4-D; 0, 0.1, 1.0, 2.0, 4.0 mg/l 2,4-D with 0.1 mg/l KN; 0, 0.05, 0.1, 0.15, 0.2 mg/l ZN; 0.25 mg/l NAA, 4 mg/l BAP plus 2 mg/l KN (Patra *et al.,* 1998). All protoplast-derived colonies were maintained under a 16 h illumination (25 mmol m^{-2} s^{-1}; provided by type TLD 58 W Daylight fluorescent tubes; Philips Lighting Ltd., Croydon, UK) at 25 ± 2°C.

RESULT

Protoplasts were successfully isolated from both sources using the enzyme mixture. Yields of protoplasts of lamina-derived cell suspension were 1.2 x 10^5 ± 0.1 protoplasts g $f.wt^{-1}$ with 20.8 ± 4.4% viability while that of petiole-derived cell suspension were 7.9 x 10^5 ± 1.5 protoplasts g $f.wt^{-1}$ with 79.3 ± 13.4% viability from Results are summarised in Table 1.

TABLE 1. Protoplast yields and viabilities from lamina- and petiole-derived cell suspension.

Source of protoplasts (3 d post-subculture)			
Lamina-derived		Petiole-derived	
Protoplast yield (protoplast g^{-1} f. wt. ± s.d.)	Viability (%± s.d.)	Protoplast yield (protoplast g^{-1} f. wt. ± s.d.)	Viability (% ± s.d.)
1.2 x 10^5 ± 0.1	20.8 ± 4.4	7.9 x 10^5 ± 1.5	79.3 ± 13.4

First mitotic division of protoplasts from lamina-derived cell suspension was observed after 4 d of culture (Figure 1B) and second cell divisions took placed within 7 d (Figure 1C). After 14 d, 10-celled aggregates were formed and, importantly by this time, browning of regenerated protoplasts was not observed. After 6 - 7 weeks culture, colonies were creamy white in colour and approximately 1 mm in diameter (Figure 1E). The optimum plating density for protoplasts from lamina-derived cell suspension was 0.5 x 10^5 protoplasts ml^{-1}, this density gave an initial plating efficiency of 12.7 ± 0.6% and a final plating efficiency of 0.6 ± 0.2% after 42 days culture. Results on plating efficiency revealed that as plating density was increased, there was a concomitant reduction in the final plating efficiency; at the lowest plating density (0.25 x 10^5 protoplast ml^{-1}), final plating efficiency was negligible (Table 2).

First cell divisions of petiole-derived cell suspension protoplasts was observed after 7 d of culture. The initial plating efficiency of 19.3 ± 4.2% was observed at plating density of 1.0 x 10^5 protoplasts ml^{-1}. However, mitotic division was not sustained after this stage and protoplasts became necrotic after 10 - 14 d. Results are shown in Table 2.

TABLE 2. Plating efficiencies of protoplasts from lamina- and petiole-derived cell suspension.

Plating density (protoplast ml^{-1})	Protoplasts from lamina-derived cell suspension		Protoplasts from petiole-derived cell suspension	
	[1]InitialPlating Efficiency (% ± s.d.) (after 4 days)	Final Plating Efficiency (% ± s.d.) (after 42 days)	[1]Initial Plating Efficiency (% ± s.d.) (after 7 days)	Final Plating Efficiency (% ± s.d.) (after 42 days)
0.25 x 10^5	< 10	0.0	NT	NT
0.5 x 10^5	12.7 ± 0.6	0.6 ± 0.2	5.9 ± 4.7	NC
1.0 x 10^5	17.2 ± 0.5	0.3 ± 0.1	19.3 ± 4.2	NC
2.0 x 10^5	25.4 ± 2.3	0.1 ± 0.0	3.4 ± 1.5	NC
2.5 x 10^5	NT	NT	1.8 ± 1.5	NC
5.0 x 10^5	NT	NT	1.5 ± 1.5	NC

Values are mean ± s.d. of at least 200 initially plated protoplasts counted in 3 replicates.
[1]Protoplasts had undergone at least one mitotic division. NT = not tested; NC = no colonies formed.

Microcolonies transferred to semi-solid regeneration media survived with variable frequencies (0 - 100%) with those transferred onto MS medium containing BAP (2 mg/l) and NAA (0.5 mg/l) (Figure 1F) exhibiting the highest survival frequency (30 - 100%). Growth of protoplast-derived callus was highly variable between protoclones; their morphological appearance reflecting the media used. Microcolonies cultured on media devoid of 2,4-D and ZN became light green and eventually developed into compact green calli. In some media, compact green calli became nodular (Figure 1G). In contrast, protoplast-derived microcolonies cultured on media containing 2,4-D and ZN had a creamy yellow appearance throughout culture. Those on media with 2,4-D were more friable compared to those cultured on media containing ZN. Shoot regeneration and/or rhizogenesis were not observed from such protoplast-derived callus.

DISCUSSION

In this study, both lamina- and petiole-derived suspensions gave protoplast yield lower than counterpart studies involving other Umbelliferae species, such as *Anthriscus sylvestris* (Xia et al., 1996) and *Bupleurum scorzonerifolium* (Xia et al., 1992). The enzyme mixture contained 2 types of cellulase, Cellulase R10 and Driselase, the later had resulted in increased yields of protoplasts for both cell suspensions. Preliminary results using enzyme mixture without Driselase had produced low protoplasts yields (data not shown).

The optimum plating efficiency for protoplasts from lamina-derived suspensions of *C. asiatica* was influenced by IPE. For *Centella*, a plating density of 0.5 x 10^5 protoplast ml^{-1} gave the highest plating efficiency, this is similar to that required by *Bupleurum falcatum* (Bang et al., 1999). Optimum plating densities of other species in the Umbelliferae (*e.g A. sylvestris*) lied in the 2 - 5 x 10^5 protoplast ml^{-1} (Xia et al., 1996). The requirement for a relatively lower plating density for *Centella asiatica* may be associated with inherent phenolic production; at higher densities, the diffusion (cross-feeding) of phenolic compounds from non-viable cells to viable, dividing cells will adversely

affect growth overall (Yu et al., 2000). A lower plating density also reduces the nutrient deficiency effect which can limit growth, since plating at higher densities elicits a nutritional-limitation to growth, as suggested for protoplasts of rose (Marchant et al., 1997).

This present study also suggested that divisional responses of *C. asiatica* protoplasts medium was influenced by the original source of cell suspensions for protoplasts, whereby only protoplasts from lamina-explant derived suspensions divided to form microcolonies. Protoplast culture media are often modified from original formulations to suit the requirements of protoplasts from specific species (Davey et al., 2000). The choice of growth regulators is always critical for successful protoplast culture. In the present study, although the hormonal content of media from other species of Umbelliferae were modified and evaluated for *C. asiatica* protoplasts, sustainable division of protoplasts from petiole suspensions was surprisingly never achieved. Sustained division of protoplasts from lamina suspensions of *C. asiatica* was though maximised using ½ B5 medium and agarose droplet culture. The composition of the culture medium has been shown to profoundly affect protoplast division capacity and colony formation, especially the presence of ammonium ions (Ochatt & Power, 1992). B5 medium contains a lowered ammonium ion concentration (Gamborg et al., 1968); by reducing the strength of B5 medium as in this medium, further reduced the ammonium ion content. Protoplasts of other species, like *Centella* require either an ammonium ion-free culture medium or a low ammonium ion containing medium, such as those of *Ginkgo biloba* (Laurain et al., 1993) and *Daucus carota* (Grambow et al., 1972).

Despite the detailed screening of several media with a wide range of growth regulator combinations previously used to induce protoplast-calli embryogenesis in species from the family Umbelliferae, the production of shoots (or roots) from protoplast-derived calli was not achieved for *Centella*. The failure to induce plant regeneration from protoplast-derived callus of *C. asiatica* may be due to the fact that cell suspensions had lost morphogenesis potential since cultures were 6 - 27 months old. With time, suspensions lose their morphogenic (totipotency) potential (Folling & Olesen, 1999). The reasons for decline in competence of morphogenesis of cell suspensions are not fully understood but cytological studies of cell suspensions have revealed that extensive chromosome losses and rearrangements occur during prolonged culture (Wang et al., 1992). It is a fair assumption that an increase of aneuploid cells during prolonged culture time is correlated with a decrease in morphogenesis (Krautwig & Lorz, 1995). Future study will require the use of protoplasts isolated from younger cell suspensions.

This study is the first on protoplast culture of *C. asiatica*. Although plant regeneration was not achieved, results provide a realistic basis for future work on the development of a protoplast-to-plant regeneration system, possibly leading to isolation of *C. asiatica* plants (protoclones) with high asiaticoside productivity.

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