ISOLATION AND CULTURE OF *Celosia cristata* L. CELL SUSPENSION PROTOPLASTS

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ABSTRACT

*Developmental competence of **Celosia cristata** L. cell suspension-derived protoplasts was investigated. The protoplasts were isolated from 3- to 9-d old cultures in enzyme solution containing 2% (w/v) Cellulase YC and 0.5% (w/v) Macerozyme R-10 which was dissolved in washing solution (0.4 M mannitol and 10 mM $CaCl_2$) at pH 5.6 for 3 hours. The highest number of viable protoplasts was released from 5-d old culture of a homogenous cell suspension. Subsequently, three kinds of protoplast culture media were simultaneously examined with four kinds of concentration of gelling agent. Culturing the protoplasts on KM8p medium solidified with 1.2% agarose significantly enhanced plating efficiency as well as microcolony formation. Afterwards, the microcalli actively proliferated into friable watery callus when they were subcultured on MS medium supplemented with 0.3 mg/l 2,4-D and 1.0 mg/l kinetin. Although the plant regeneration from the protoplasts-derived calli has not yet been obtained, the reproducible developmental step from protoplasts to callus in this study may facilitate the establishment of somatic hybridization using **C. cristata** as one parent.*

Key words: *cell suspension, **Celosia cristata**, agarose, protoplast culture, protoplast isolation*

INTRODUCTION

Higher plants are divided into two groups, C_3 and C_4 plants, depending on the mechanisms of their photosynthetic carbon assimilation. C_3 and C_4 plants differ on their leaf anatomy, carbon fixation mechanism, CO_2 compensation, photorespiration, response of photosynthesis to ambient O_2 concentration and optimum temperature for photosynthesis (Raghavendra and Das, 1977). Characteristics of C_4 plants associated with the capacity of photosynthesis provide C_4 plants to have higher efficiency than C_3 plants in hot dry weather. Researches on C_3 species indicated that reducing photorespiration have little possibility for increasing net photosynthesis (Nelson and Langdale, 1992). Therefore, the introduction of photosynthetic characters of C_4 plants into C_3 species then be considered as the most logical approach to reduce photorespiration and to improve photosynthetic potential (Brown and Bouton, 1993).

The principle of hybridization has been carried out for C_3 and C_4 plants. Characteristics of C_4 plants have been transferred into C_3 crop species by conventional crosses. However, to produce a new hybrid plant containing combination of the desirable traits from C_3 and C_4 species through sexual crosses is still difficult (Brown *et al*., 1983; Brown and Bouton, 1993). Somatic hybridization through protoplast fusion has also been attempted to produce C3/ C4 hybrid (Terada *et al*., 1987, Murty and Cocking, 1988, and Vasil *et al*., 1988).

Amaranthaceae is a family containing genus with either the C_3 types of photosynthesis or C_4 types. This family offers unique possibility for the investigation of somatic hybridization. Somatic hybridization using protoplast systems needs an accumulation of sufficient information on parental protoplasts including the competence of protoplasts to regenerate cell wall, to divide and ultimately differentiate into normal plants.

The genus *Celosia* is annual herbs which are distributed throughout the tropical and temperate regions of Asia, Africa and America. *Celosia* comprises both weedy and ornamental species. The weedy species produce seeds which have high protein content and vitamins such as vitamin B1, B2, C, E, and beta carotene (Weng *et al*., 1994; Prakash *et al*., 1992; Prakash *et al*., 1995). In India and China the seeds of *Celosia* are widely used for medicinal purposes (Hase *et al*., 1996; Imaoka *et al*., 1994; Shah *et al*., 1993). From 56 species of *Celosia*, two common flowering species are *C. cristata*, which has a wide comb-shaped inflorescence, and *C. plumosa* with feather-like flowers (Porat *et al*., 1995). *Celosia cristata*, is one of the C_3 species of Amaranthaceae.

Studies on karyology (Wakakuwa, 1931), cytology (Grant, 1954), speciation and basic chromosome number (Grant 1961), nuclear DNA content (Nath *et al*., 1992) and nutritional component as well (Weng *et al*., 1994) of *C. cristata* have been published. However, the investigation of *C. cristata* in tissue culture is very scarce. Development of fasciations achieved from the excised shoot apices of *C. cristata* was reported by Driss-Ecole (1981).

In order to produce C_3/C_4 somatic hybrid plant involving *C. cristata* as one parent, the aim of this study was to examine the regeneration capacity of its protoplasts.

MATERIALS AND METHODS

Establishment of Cell Suspension Culture

The small (1-1.5 mm in diameter), lenticular-shaped, tan-colored seeds of *Celosia cristata* L. cv. Pink charm (Takii, Kyoto) were surface sterilized with 70% alcohol (30 sec) and 5% sodium hypochlorite (15 min). Afterwards, the disinfectant was removed by three rinses in autoclaved distilled water. The sterilized seeds were aseptically sown on MS basal medium (Murashige and Skoog, 1962) supplemented with 3 mg/l 2.4-D and 0.1 mg/l kinetin. The produced callus was transferred into callus maintenance (CM) medium (MS medium supplemented with 0.3 mg/l 2.4-D, 1.0 mg/l kinetin and 3% sucrose at pH 5.7 and solidified with 0.8% agar). All cultures were incubated under cool-white fluorescent lamps (1000 lux) at 23-24º C.

Cell suspension cultures were initiated from about 2 g fresh weight of friable, fast growing white calli of *C. cristata* that inoculated into 20 ml CM liquid medium in 100 ml Erlenmeyer flask. Flasks were maintained on a rotary shaker (100 cycles/min) at 23-24º C under a dim light. To stabilize cell growth subculture was performed at weekly intervals by transferring about 5 ml of original cell suspension cultures into 20 ml fresh medium by filtration with stainless steel sieve. It took four or five subculturing to get a homogenous cell suspension culture (Figure 1a), which was suitable for the protoplast isolation.

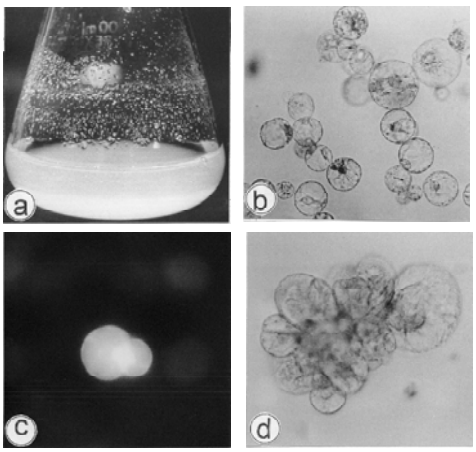

Figure 1. Development of protoplasts isolated from 5 day-old *C. cristata* cell suspension cultured in KM8p medium solidified with 1.2% agarose. a. Cell suspension cultures as protoplast sources, b. Freshly isolated protoplasts with translucent cytoplasm (×800), c. First division of protoplast (×800), d. Protoplast-derived microcolony detected after 2 weeks of culture (×820).

Protoplast Isolation, Purification and Culture

Approximately 1-1.5 g of centrifuged and packed fine cells was inoculated in 2.5 ml filter sterilized enzyme solution and poured into 50 mm falcon petri dish. The enzyme solution (pH 5.6) contained 2% (w/v) cellulase YC and 0.5% (w/v) Macerozyme R-10 which was dissolved in washing solution (0.4 M mannitol and 10 mM $CaCl_2$). Protoplast isolation was carried out on a reciprocal shaker at 23-24° C in the dark for 3 hours. The released protoplasts were separated from the undigested cell suspension by filtration through double layers of 350 μm nylon mesh and pelleted by centrifugation at 100 g for 3 min in 55 × 100 mm test tube. The protoplast pellet was resuspended in washing solution, centrifuged at 100 g for 2 min and purified by floating over 21% (w/v) sucrose. The sucrose solution was then centrifuged at 100 g for 3 min and band of purified protoplasts formed over the sucrose solution interface was gently removed with a Pasteur pipette into washing solution. Following centrifugation at 100 g for 2 min protoplasts were removed into culture medium and were centrifuged in the same method in washing solution. Protoplast yield was counted after purification and final wash (before plating in protoplast culture medium) by a Haemacytometer. To observe a suitable age for producing high protoplast number the isolation was undertaken from three to nine days after subculture of cell suspension.

The protoplasts culture density was adjusted before plating at 10^5/ml. Three different media, (Table 1) were tested for culturing protoplasts. Composition of KM8p1 medium was similar to KM8p medium (Kao and Michayluk, 1975) except 0.5 g/l benzyl adenin (BA), while MBK medium was a combination of MS, B5 (Gamborg *et al*., 1968) and KM8p basal medium.

Table 1. Media composition of *Celosia cristata* L. var. Pink Charm protoplast cultures

Constituent	Media of Protoplast Cultures		
	KM8p[a]	KM8p1	MBK
Mineral salt	KM8p	KM8p	MS[b]
Vitamins	KM8p	KM8p	B5[c]
Sucrose	KM8p	KM8p	KM8p
Glucose	KM8p	KM8p	KM8p
Other sugars and sugar alcohols	KM8p	KM8p	KM8p
Casein hydrolysate	KM8p	KM8p	KM8p
Coconut water	KM8p	KM8p	KM8p
Hormones (amount in mg/l):			
2,4-D	0.2	0.2	0.2
Zeatin	1.5	0.5	0.5
NAA	2.0	1.0	2.0
BA	-	0.5	-
pH	5.6	5.6	5.6

[a]Kao and Michayluk (1975); [b]Murashige and Skoog (1962); [c]Gamborg *et al.* (1968)

Culture medium containing protoplasts was mixed with an equal volume of agarose solution before embedding in Sea Plaque agarose. The mixture of protoplasts and agarose solution was dispensed as droplets on the bottom of petri dish (50 mm in diameter) and bathed in 2.5 ml liquid medium. Four agarose concentrations (0.6%, 0.8%, 1.0%, and 1.2%) were tested. All culture dishes were sealed with Parafilm and initially incubated in the dark at 23-24° C in the first week. Afterwards, they were exposed to dim (± 100 lux) and bright light (1000 lux) in the second and third week, respectively. During culture in KM8p medium, the osmolarity was progressively reduced according to Dhir *et al.* (1991). Removing the original liquid medium and replacing it with fresh KM8p:KM8 medium in the ratios of 2:1, 1:1, 0:1, 0:1 was performed at day 7, 14, 21, and 28, respectively. Reduction of osmolarity in KM8p1 and MBK medium was done at weekly interval by reducing the glucose concentration. Fresh medium containing glucose of 0.30 M, 0.25 M, 0.20 M and 0.15 M was added at day 7, 14, 21, and 28, respectively. In three media tested original liquid medium was removed and replace with CM liquid medium at day 35. When the microcalli grew to 1-2 mm in diameter, they were transferred onto various regeneration media. Plating efficiency was defined as the number of cells divided/total protoplasts plated. Microcolony formation was defined as the number of cell colonies of 4-10 cells/total protoplasts plated. Plating efficiency and microcolony formation were evaluated one week and two weeks after culture, respectively. Five random microscopic fields per droplet, for three droplets per plate and for three plates per treatment were examined. All experiments were performed three times with at least three plates for each treatment treatment.

Viability and Cell Wall Formation of Protoplasts

Viability of protoplasts was determined by fluorescein diacetate (FDA) staining technique (Widholm, 1972). The percentage of viability was calculated from the number of protoplasts giving yellow fluorescence out of the total number of protoplasts. Initiation of cell wall regeneration was visualized with fluorescence microscopy using Calcofluor white.

RESULTS

The purified protoplasts of cell suspension were spherical with many scattered vacuoles or rich in cytoplasm (Figure 1b) with average diameter (n = 48) was 53.4 ± 18.4 μm. The viable protoplasts showing yellow fluorescence of FDA were more than 80% (Figure 2). Under the enzyme solution used the highest yield (12.3 × 10^5 protoplasts/ml) was derived from 5 d old culture of cell suspension (Figure 2). Isolating from 6 d onward reduced the released protoplasts.

Either three different media or four different agarose concentrations tested were not different in initiation time of cell wall regeneration. Initiation of new cell wall regeneration was observed in some protoplasts 4 hours after isolation as indicated by weak fluorescence of Calcofluor white. Twenty-four hours after culture the protoplasts exhibited a ring of blue fluorescence around the plasma membrane. After 3-4 d of culture the first mitotic division was initially observed (Figure 1c).

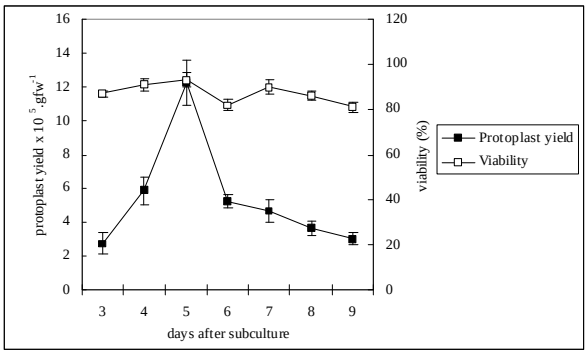

Figure 2. Yield and viability of cell suspension-derived protoplasts isolated on 3 to 9 days after subculture. Values are means of three independent experiments (n = 16)

One week after culture, KM8p medium showed a capability to give a higher plating efficiency than other two media (Figure 3). The plating efficiency and microcolony formation appeared to be strongly dependent on the concentration of agarose as gelling agent. In KM8p medium, plating efficiency increased with increasing agarose concentration. The highest level of plating efficiency was obtained in the medium solidified with 1.2% agarose.

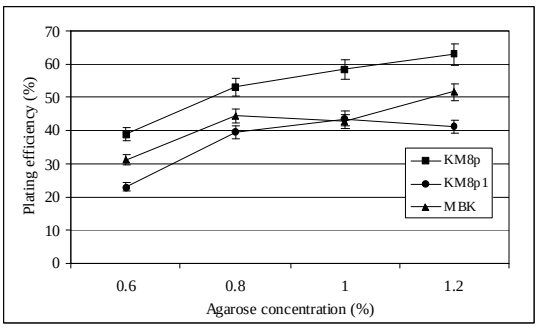

Figure 3. Effect of agarose concentrations on plating efficiency (%) of *C. cristata* cell suspension-derived protoplasts one week after culture in three kinds of protoplast culture media (KM8p, KM8p1, MBK). Values are means of three independent experiments (n = 45)

Microcolonies containing 4-10 cells could be detected after two weeks of culture (Figure 1d). At this stage the response of microcolony formation of *C. cristata* protoplasts to different concentrations of agarose in three kinds of culture media tested showed a different trend (Figure 4). KM8p medium solidified with 1.2% agarose still provided the highest number of microcolonies. Microcolony formation in MBK medium was not so different in four concentrations of agarose. While microcolony formation in KM8p1 medium exhibited a low number in 0.6% agarose, markedly increased in 0.8% and 1.0% agarose and finally decreased in 1.2% agarose. Although all three protoplast media tested were able to induce protoplast division, however, KM8p medium was the only one medium which stimulated a sustained division leading to microcallus formation.

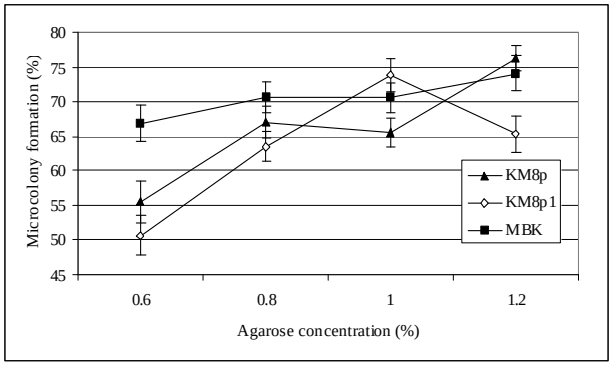

Figure 4. Effect of agarose concentration on microcolony formation (%) of *C. cristata* cell suspension-derived protoplasts two weeks after culture in three kinds of protoplast culture media (KM8p, KM8p1, MBK). Values are means of three independent experiments (n = 45)

After four weeks of culture the visible microcalli in KM8p1 and MBK medium were few (Figures 5a, b). In contrast, numerous microcalli were observed in each droplet of agarose-solidified KM8p medium (Figure 5c). Following subculture into CM medium the microcalli grew fast into yellowish, friable calli (Figure 5d). Unfortunately, attempts to induce organ formation from these calli have been unsuccessful.

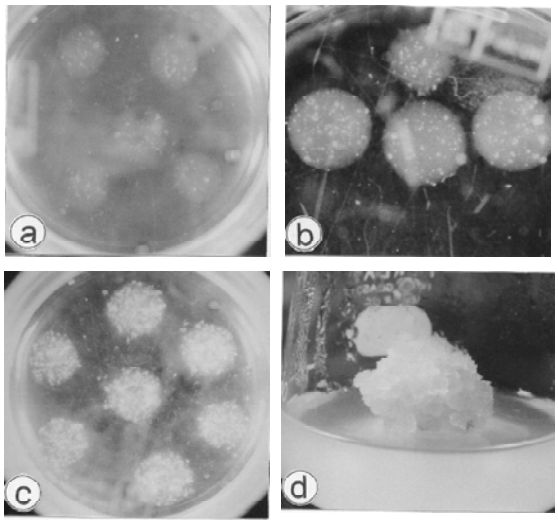

Figure 5. Development of microcalli in protoplast culture and callus maintenance (CM) medium. a. Visible microcalli in KM8p1 medium solidified with 1.2% agarose. b. Visible microcalli in MBK medium solidified with 1.2% agarose. c. Visible microcalli in KM8p medium solidified with 1.2% agarose. d. Friable callus four weeks after transfer into callus maintenance medium

DISCUSSION

Suspension cultures which are maintained in log phase by frequent subcultures are generally superior as a source of protoplasts than callus cultures because they have a higher growth rate and are more homogenous (Arcioni, 1994). The quality of protoplasts source is the most important factor for successful regeneration. In the first two weeks of inoculation in liquid medium callus produced fine cell suspension which consisted of fine and highly dispersed cell clusters. However, when subculture was done without filtration formation of large clumps occurred in time. These large clumps were considered as an undesirable component since they released protoplasts in lower number rather than fine cell clusters (data not shown). Therefore, a regular filtration had to be performed to maintain *C. cristata* cell suspension in homogenous condition. Correlation between heterogeneity of cells and protoplast yield was also showed on wheat cell suspension (Yang *et al*., 1994).

The growth conditions of protoplast source are very important to achieve success in protoplast experiment, because an optimal growth will increase the yield and viability of the released protoplasts. Many factors including age and physiological conditions of the material tissues as well as procedure of isolation and culture of protoplast were found to be critical for obtaining viable protoplasts capable of undergoing divisions and subsequent colony formation. Five-d old cultures of *C. cristata* cell suspension seems to be the most suitable source for isolating protoplasts in terms of ease of enzymatic digestion and subsequent viability of protoplasts. The results show that the optimum age released the highest number of protoplasts. This nature indicated that susceptibility of protoplast source to enzymatic digestion may decrease over time (Bhojwani *et al*., 1977; Imbrie-Mulligan and Hodges, 1986). In contrast, the stability of protoplast viability regardless of age of protoplast source indicated that the susceptibility of *C. cristata* cell suspension-derived protoplasts to damage by the isolation procedure remain fairly constant from 3 to 9 days. Elmer *et al*. (1989) showed that the viability of *Asparagus* callus-derived protoplasts remained constant up to 30 days after subculture of callus.

Under the culture condition used *C. cristata* cell suspension protoplasts showed the capacity to regenerate cell wall. The regeneration of a new cell wall at the surface of plasma membrane has been regarded as the first significant step in the development of isolated protoplasts because biosynthesis of the wall is an important process in cytodifferentiation, particularly regarding the capacity of protoplasts to divide and to regenerate plants (Meyer and Abel, 1975; Cocking, 1972).

The plating efficiency and microcolony formation appeared to be strongly dependent on the concentration of agarose as gelling agent. Similar results were also showed by Asano *et al*. (1994) who reported that plating efficiency of protoplast culture of bentgrass remarkably improved by increasing agarose concentration in the medium over the conventionally used level. Increasing the agarose concentration not only increases the plating efficiency but also increases the visible colony formation after a prolonged culture period.

Medium composition also played an essential role in successful protoplast culture. The composition of the protoplast culture medium was critical to obtain higher plating efficiency and microcolony formation. The variation in plating efficiency on three media tested showed that the growth of *C. cristata* protoplasts required medium containing more complete mineral nutrition (KM8p medium) in which *C. cristata* protoplasts easily divided, formed microcolony and proliferated into callus. KM8p medium seems to be the best medium for protoplast growth allowing obtaining a lot of microcalli after several weeks on culture. While reduced growth in KM8p1 medium containing the similar composition to the KM8p medium except addition of BA suggested that for callus proliferation there was a necessity of specific interaction of plant hormones.

The present study has proved the developmental competence of protoplasts from *C. cristata* cell suspension. The protoplasts were easily isolated and regenerated into calli. Culture medium containing complex nutrition was preferable to sustain an active proliferation of *C. cristata* protoplasts. Although plant regeneration from protoplast-derived calli of *C. cristata* has been unsuccessful, however, the results of this study could be expected to provide the basic condition for somatic hybridization in the further.

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