Kasetsart J. (Nat. Sci.) 31 : 55 - 59 (1997)

Isolation and Culture of Protoplasts from Different Explants of Cotton (*Gossypium hirsutum* L.)

Prapon Boonrumpun[1], Catherine Pannetier[2], and Yves Chupeau[2]

ABSTRACT

Protoplasts were isolated from callus, cotyledons, hypocotyls and leaves of cotton (*Gossypium hirsutum* L. var. Coker 310) using different digestion media. Protoplast yield per gram of fresh mass of leaves, callus cotyledons and hypocotyls was 22.2, 5.5, 1.7 and 1.1 million respectively. Protoplasts were most viable in basic medium in presence of thidiazuro 0.663 gm/l and 2, 4, 5-trichlorophenoxy acetic acid 7 mg/l. Hypocotyl and leaf-derived protoplasts formed microcolonies. Addition of $CaCl_2$ and glutamine to the basic medium improved protoplast survival, and division of hypocotyl and leaf-derived protoplasts. The growth conditions, such as the light and carbon source of donor plant were the important factors for protoplast isolation and culture.

Key words : *Gossypium hirsutum*, protoplast culture, protoplast isolation, hypocotyl-derived protoplast, and leaf-derived protoplast.

INTRODUCTION

Reports on protoplast culture of cotton are now meager, not numerous though it is common to obtain isolated protoplasts from tissues, callus, cotyledons, hypocotyl, formation and regeneration of normal plantlets reveal limited.

For protoplast cultures, 3 basic media are used; MS media (Murashige and Shoog, 1962), B5 media (Gamborg *et al.*, 1987) and K3 media (Kao *et al.*, 1974). For the first time, callus formation was observed from the isolated protoplasts of main stem callus of *G. hirsutum* (Saka *et al.*, 1987). Peeters (1994) reported that plantlets were obtained from generated protoplast, which derived from embryogenic suspension of cotton (*G. hirsutum)* variety Coker 312 and Coker 210. The protoplasts were isolated from embryogenic suspension, which initiated from hypocotyl callus. At last it was found that usage of feeder layer was necessary to get embryogeneic callus from protoplasts.

The objective of this research work was to determine the source of effective cotton tissue that produces high viable protoplast.

MATERIAL AND METHODS

Cotton protoplast used in this experiment were from two types of staring material. The first source of protoplasts was coming from cotton (*G. hirsutum)* variety Coker 310 plantlets which germinated in vitro. At the outset, cotton seeds were surface sterilized for 30 minutes then germinated on hormone-free (MS medium

[1] DORAS Center, Kasetsart University, Bangkok 10900, Thailand.
2 CIRAD-INRA, Versailles, France.

(Murashige and Skoog, 1962) and incubated at 25°C in 16 h. day light of 60 mol $m^{-2}s^{-1}$ from fluorescent lamp , 40 W Philips. After plantlet developed, cotyledons, hypocotyls and young leaves were used to produce protoplasts. The second source of protoplasts was embryogenic callus, which initiated from hypocotyl-derived callus on MS medium without growth substance.

Protoplast Isolation

Protoplasts were isolated and selected from plantlet cotyledons and hypocotyls of 10-14 day-old seedling, young leaves of approximately 3 week-old plants and embryogenic callus of 2 week-old. Plant material were incubated on GSG medium (Chuppeau *et al.*, 1989) with digestion enzyme (macerozyme R 10, 0.02%) at 22°C in dark for 16 h. To infiltrate the tissue with enzyme mixture, staring material were chopped with scalpel in to strip of 0.5-1.0 mm.

Protoplast purification

Protoplasts were separated from undigested tissues by filtration through stainless steel sieves of 40 μm for young leaves, 80 μm for cotyledon 140 μm for hypocotyls and embryogenic callus. Then centrifugation at 70 g was achieved for 6 minutes. Pellet of protoplasts from centrifugation were resuspened in 25 ml of washing solution of Chupeau *et al.*, (1989) and recentrifuged at 70 g for 6 minutes. The procedure was repeated twice for two more times before plating. Protoplast number was estimated by a Malassez glass.

Protoplast Culture

Protoplasts of 5.0×10^4/ml density were cultured in a modified MS medium containing KNO_3 20 $mg.l^{-1}$ NH_4NO_3 100 $mg.l^{-1}$ $CaCl_2H_2O$ 420 $mg.l^{-1}$ $MgSO_4.7H_2O$ 185 $mg.l^{-1}$ KH_2PO_4 20 $mg.l^{-1}$ microelement of Heller (1953) vitamin of Morel and Wetmore (1951) sucrose 2% 2,4,5-Trichlorophenocy acetic acid 3 $mg.l^{-1}$ thidiazuron 0.663 $mg.l^{-1}$ pH 5.5. The protoplast were distributed in 5 cm Petridishes (3 ml/plate), and incubated at 25°C in the dark.

RESULTS AND DISCUSSION

Protoplast Isolation

Efficiency of isolation depends on the age of callus (Bhojhwani *et al.*, 1977:Finer and Smith, 1982). In this experiment, callus at the age of 14 – 18 days produced the best yield of protoplasts. For the explants of plantlets germinated in vitro, cotyledons and hypocotyls from 10 – 14 day-old seedlings produced less protoplast yield than young leaves of approximately 3 – 4 week-old.

The explants of cotton were important factor affecting protoplast yield. Generally, young leaves produced highest number of protoplasts (Table 1).

Table 1 Results of protoplast isolation from different explants.

Explant	Age (days)	Yield 10^6 protoplasts/g. fresh weight.
Callus	14	5.5
Cotyledons	10	1.7
hypocotyls	14	1.1
leaves	21	22.2

The size of hypocotyl protoplasts were relatively large, 40 – 100 μm in diameter. Young leaves protoplasts were smaller in size, 10 - 30 μm but more uniform.

Protoplasts Culture

Protoplasts were most viable in basic medium with thidiazuron at 0.633 $mg.l^{-1}$ and 2,4,5 – trichlorophenocy acetic acid at 7 $mg.l^{-1}$. Culture density was an important factor influencing protoplast development (E. Firoozabady and de Boer, 1986). The optimum culture density discovered in vitro were $5 - 6 \times 10^6$ $protoplast.ml^{-1}$. Generally, first division of protoplasts of hypocotyls and young leaves occurred in 4-5 days and microcolonies (of 2-8 cells for hypocotyls and 20 – 30 cells for young leaves) appeared within 2 weeks.

The protoplast culture efficiency of different explants were showed in Table 2. Only hypocotyls and young leaves derived protoplasts formed microcolonies respectively of 2-8 cells (Figure 1) and 20-30 cells (Figure 2). Callus and cotyledon derived protoplasts entered only first division but

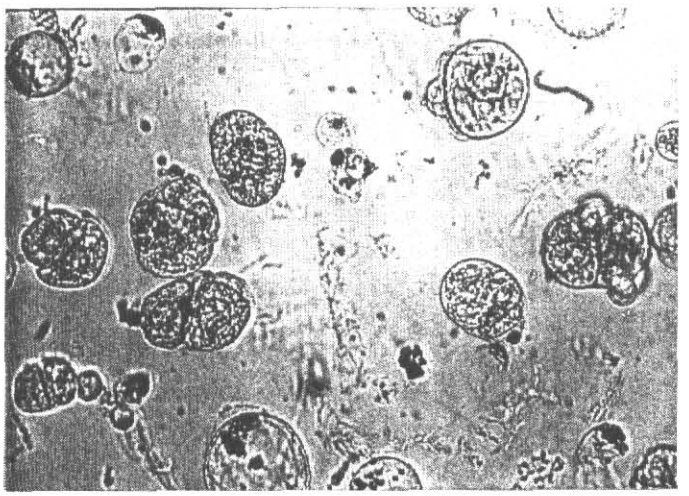

Figure 1 Microcolonies of hypocotyl-derived protoplast at 8 days after culture.

Table 2 Protoplasts culture efficiency of different explants of *G. hirsutrm.*

Explant	Protoplasts survival*	Division	Microcolonies
Callus	+++	+	-
Cotyledons	+	+	-
hypocotyls in dark	++	++	+
hypocotyls in light	++	+++	+++
leaves	+	++	+

* 3 days after culture

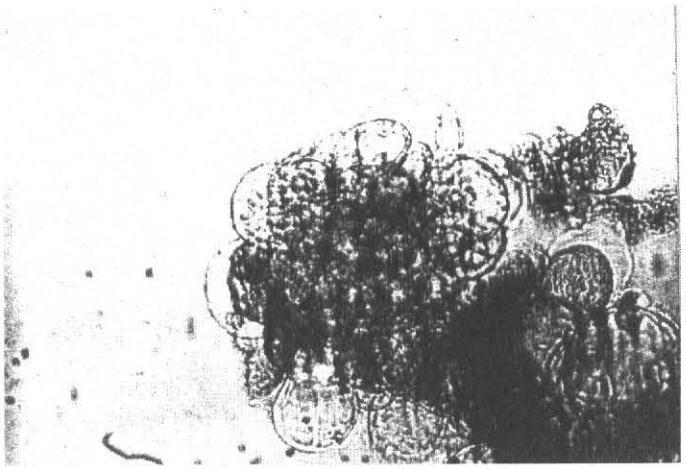

Figure 2 Microcolonies of leaf-derived protoplast at 45 days after culture.

survival of callus-derived protoplasts was superior. Attempt to produce microcalli from this procedure was not successful, but we obtained microcolonies from young leaves successfully for the first tine.

Two organic substances were used to improve protoplast survival: adenosine and glutamine. Adenosine at the rate 100 $mg.l^{-1}$ was most effective but the glutamine at rate of 146 $mg.l^{-1}$ improved protoplast survival.

Results suggested that adding $CaCl_2$ in the basic medium could improve the protoplast survival for all explants. The optimal dose was mot determined but results on hypocotyl and leave protoplast showed that the rate up to 700 $mg.l^{-1}$ improved protoplast survival.

Protoplast derived from hypocotyl exposed in light gave more efficiency than cell division and microcolony formation than in the dark.

A few numbers of large size cellular microcolonies issued from culture of leaf-derived protoplast is promising for improving the culture of mesophyll protoplast by further experiment.

LITERATURE CITED

Bhojwani S. S., J. B. Power, and E. C. Cocking. 1977. Isolation, culture and division of cotton callus protoplast. Plant Sci. Lett. 8:85-89.

Chupeau M. C., C. Bellini, P. Guerche ,B. Maisonneuve, G. Vastra, and Y. Chupeau. 1989. Transgenic plants of lettuce *(Lactuca sativa)* obtained throug electroporation of protoplasts. Biotechnology 7:503-508.

Finer J. J. and R. H. Smith. 1982. Isolation and culture of protoplasts from cotton *(Gossypium klotzchianum* Anderss*)* callus cultures. Plant Sci. Lett. 26:147-151.

Firoozabady E. and D. L. De Boer. 1986. Isolation, culture and cell division in cotyledon protoplasts of cotton *(Gossypium hirsutum* and *G. barbadense).* Plant Cell Reports 5:127-131.

Gamborg O. L., R. A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exptl. Cell Res. 50:151-158.

Heller R. 1953. Recherches sur la nutrition minérale des tissus végétaux cultivés in vitro Ann. Sci.

Nat. Bot. Biol. Veg. 14:1-223.

Kao K. N., F. Constabel , M. R. Michayluk and O. L. Gamborg. 1974. Plant protoplast fusion and growth of intergeneric hybrid cells. Planta 120:215-227.

Morel G. and R.H. Wetmore. 1951. Fern callus tissue culture. Am. J. Bot. 38:141-143.

Murashige T. and F. Skoog. 1962. A revised medium of rapid growth and bio-assay with tobacco. Physiol. Plant. 15:473-479.

Peeters M. C., K. Willems, and R. Swennen. 1994. Protoplasts-to-plant regeneration in cotton *(Gossypium hirsutum* L. cv. Coker 312) using feeder layers. Plant Cell Reports 13:208-211.

Saka K., F. R. Katerman, and J. X. Thonas. 1987. Cell regeneration and sustained division of protoplasts from cotton *(Gossypium hirsutum* L.). Plant Cell Report 6:470-472.