Pak. J. Bot., 33(2): 125-132, 2001.

PROTOPLAST ISOLATION IN SUGARCANE

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Abstract

A protocol was established for isolation of sugarcane protoplasts, cell wall regeneration and cell division. Protoplast were isolated from cell suspension initiated from fresh leaf calli and produced on agar medium by enzymatic treatment, 4% cellulase, 2% macerozyme, 1% pectinase and 0.7M mannitol at pH 5.4 under a defined conditions.

Introduction

Sugarcane is a vegetatively propagated crop. Somatic hybridization and genetic manipulation are two of the most important components of plant biotechnology which assist in the improvement of sugarcane. Both require regeneration of plants from protoplast and/or single cell. It is now possible to regenerate plants from protoplast of many species (Dale, 1983; Vasil, 1983). The culture of graminaceous protoplast have been refined though it is extremely difficult (Chen & Shih, 1983). An enzymatic isolation of sugarcane protoplast was first reported by Ferenczy & Maretzki (1970). Subsequent induction of cell division has been obtained from the protoplast derived from cell culture (Maretzki & Nickell, 1973), rolled young leaves (Chen & Liu, 1976) and shoot apices with immature leaves (Krishnamurthi, 1976; Evans *et al.*, 1980). Protoplast isolated from embryogenic cell suspension cultures of sugarcane have also been reported to form calli which undergo limited organogenesis. Protoplast isolation, suspension culture, organogenesis from coleoptiles or roots has also been reported (Yan *et al.*, 1985). Tabaeizadeh *et al.*, (1986) have obtained somatic hybrid cell after fusion of sugarcane and pearl millet protoplast with a limited morphogenetic ability. Srinivasan & Vasil (1986) successfully regenerated sugarcane protoclones from embryogenetic cell suspension.

The procedure of previous protoplast isolation methodologies were tedious and did not give a good yield of protoplast under our conditions and the tested genotypes did not respond good enough to these methods. Efforts were therefore made to establish practically reformed conditions for isolation of protoplast where a comparative study of protoplast isolation from cell suspension and callus obtained from solid medium were made.

Materials and Methods

Young immature leaves of sugarcane clones viz., BL4, AEC81-0819 and AEC81-8415 were explanted on basic MS medium (Murashige & Skoog, 1962) containing 4mg/l 2,4-D for callus induction. After one month of incubation actively growing callus was separated and sub-cultured on solid medium and liquid medium. One gram of callus was transferred in 40 ml of liquid medium containing 4mg/l 2,4-D, 50 ml coconut water and

500 mg/l casein hydrolysate and placed on a shaker having 120 rpm for obtaining cell suspension. The suspension were sub-cultured after every 4-5 days by inoculating 10 ml of suspension in 40 ml of fresh medium. In case of solid medium one gram of callus was sub-cultured on 20 ml of solid medium containing 4 ml/l 2,4-D for proliferation.

Procedure A: Five days before the isolation of protoplasts, the liquid medium was sub-cultured on every alternate day to keep the cells in active division phase because the active cells are more prone to enzymatic treatment and liberate robust protoplasts. Actively growing cells were collected from cell suspension by filteration through 50μM sieve. The collected cells were resuspended in 20 ml of enzyme mixture filter sterilized, containing 4% cellulase Onozuka RS, 2% macerozyme R10 and 1% pectinase R10 with 0.35 mM KH_2PO_4 and 3 mM $CaCl_2$ while pH was adjusted at 5.4 and osmotic pressure was maintained with 0.7M mannitol. The mixture was placed on a shaker at 60 rpm for 4-11 hours at room temperature.

Procedure B: For protoplast isolation, calli were sub-cultured on agar medium. These calli were attained in actively growing phase within 10-15 days after sub-culturing. These cells were harvested, smashed and suspended in the enzyme mixture. Rest of the procedure was the same as mentioned in procedure A.

Protoplast purification: After enzyme treatment, in both the procedures the enzyme protoplast mixture was passed through nylon sieves of 38μM pore size to remove the undigested cells. The filterate was transferred to 25 ml screw-capped centrifuge tube and the protoplasts were pelleted by centrifugation at 900 rpm for 3-4 minutes. Protoplast were washed twice in 0.7 M mannitol solution containing 0.35 mM KH_2PO_4 and 3mM $CaCl_2$ by centrifugation at 900 rpm for two minutes. Protoplast were counted by haemocytometer. Washed protoplasts were resuspended in a defined liquid culture medium for microcolony formation.

Protoplast culture media: MS media comprising of the following adjusted to pH 5.6 were used for microcolony formation.

Ammonium nitrate	1650.00 mg/l	Potassium iodide	0.83 mg/l
Potassium nitrate	1900.00 mg/l	Sodium molybdate	0.25 mg/l
Calcium chloride	440.00 mg/l	Copper sulphate	0.025 mg/l
Magnesium sulphate	370.00 mg/l	Cobalt	0.025 mg/l
Potassium diphosphate	170.00 mg/l	Thiamine HCl	1.00 mg/l
Boric acid	6.20 mg/l	Myo-inositol	100.00 mg/l
Manganese sulphate	22.30 mg/l	Mannitol	0.70 mg/l
Zinc sulphate	8.60 mg/l	Sucrose	30.00 mg/l
Ferrous sulphate	27.80 mg/l	2,4-D	2.00 mg/l
Sodium EDTA	37.30 mg/l	NAA	2.00 mg/l

Results and Discussion

A large number of protoplasts with high capability of cell colony formation were produced from the calli on agar media as well as from the cell suspensions subcultured only once after the last subculture on agar media (Table 1 & 2; Figs. 1 & 2). The most uniform preparations, free of debris and undigested cells were obtained from the suspensions. Microcolony formation was successfully accomplished (Fig.3) but no green plantlets were obtained. Clone BL4 showed very high capability of microcolony formation as well as high yield of protoplasts. High protoplasts yield was also obtained from AEC81-8415 and initial cell division was also observed but no microcolony formation was achieved, whereas AEC81-0819 did not produce even very low quantity of protoplast as compared to BL4 and AEC81-8415.

Table 1. Isolation and culture of protoplasts through procedure "A" (Suspension culture).

Variety	Age of callus (weeks)	Yield of protoplasts and duration of enzymatic treatment	
		6 hours	8 hours
BL 4	4-6 weeks	+	+++
	6-8 weeks	++	+++
	8-10 weeks	-	+
AEC81-8415	4-6 weeks	+	++
	6-8 weeks.	+	++
	8-10 weeks	-	-
AEC81-0819	4-6 weeks	-	+
	6-8 weeks	+	++
	8-10 weeks	-	-

+ = Fair; ++ = Good; +++ = Excellent; - = No isolation

Table 2. Isolation and culture of protoplasts through procedure "B" (solid medium).

Variety	Age of callus (weeks)	Yield of protoplasts and duration of enzymatic treatment	
		6 hours	8 hours
BL 4	4-6 weeks	+	+++
	6-8 weeks	+	++
	8-10 weeks	-	+
AEC81-8415	4-6 weeks	+	++
	6-8 weeks	+	+
	8-10 weeks	-	-
AEC81-0819	4-6 weeks	-	+
	6-8 weeks	-	+
	8-10 weeks	-	-

+ = Fair; ++ = Good; +++ = Excellent; - = No isolation

Fig. 1. Protoplasts isolation from callus.

Fig 2. Protoplasts isolation from cell suspension culture.

It was observed that the growth phase of the cells also plays an important role in protoplast isolation. Under microscopic examination the cells with abundant cytoplasm when treated with enzyme mixture yielded high quantity of protoplasts as compared to cells with low cytoplasmic contents. Moreover low debris (due to cell bursting) were obtained in the purification of protoplasts of the former cells. In case of solid medium if freshly subcultured callus was treated with enzyme mixture, high quantity of protoplasts as compared to aged calli were produced.

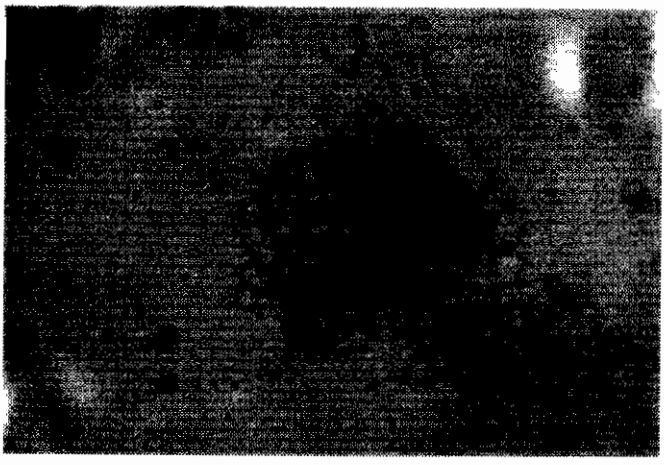

Fig. 3. Accomplishment of microcolony formation in the isolated protoplast.

Where the enzyme mixture with cells were reciprocally shaken at 120 rpm, most of the protoplasts were injured and turned incapable of division (Table 3). When enzyme mixture with cells were shaken at 60 rpm, huge amount of protoplasts (5×10^{-5} cell/ml) with high mitotic cell division potential were produced. At 30 rpm the protoplast yield was low (0.3×10^{-5} cell/ml) but cells were less injured. Duration of enzymatic treatment also exhibited its effect on protoplast yield. Best yield was obtained at 6-8 hours of treatment at 60 rpm whereas enzymatic treatment of more than 8 hours caused over digestion of cells and less duration of 4 hours showed less protoplasts due to inadequate digestion.

Table 3. Effect of reciprocal shaking of enzyme and cell mixture on protoplast yield.

Variety	Duration of enzyme treatment	Shaking (rpm) of enzyme and cell mixture	Protoplast yield
BL 4	8 hours	30	++
	8 hours	60	+++
	8 hours	120	+
AEC81-8415	8 hours	30	++
	8 hours	60	+++
	8 hours	120	+
AEC81-0819	8 hours	30	++
	8 hours	60	+++
	8 hours	120	+

+ = Fair; ++ = Good; +++ = Excellent;

Fig 4. High mortality rate of protoplasts due to lack of nutrients during growth phase.

Fig. 5a. Cell division in the isolated protoplasts.

When protoplasts were cultured at low (0.5×10^{-5} cell/ml) density, fewer cell division were observed. On the contrary, when protoplast density was high (5×10^{-5} cell/ml), protoplasts culture turned brown very rapidly and it needs quick replenishment of the medium. Moreover high mortality rate of protoplasts was observed (Fig. 4) and it may be due to lack of nutrients during growth phase (Larkin, 1982) and polyphenols which act as suppressors of cell division (Krishnamurthi, 1986). The optimum protoplast density for culture was $1.5\text{-}2 \times 10^{-5}$ cell/ml. It was also observed that protoplast were very sensitive to light if given from direct radiation source (Krishnamurthi, 1976) and high temperature of 32°C. Therefore it is important to keep protoplasts in diffused light or darkness at low temperature during culture conditions.

Fig. 5b. Colony formation in the isolated protoplasts.

Response of sugarcane protoplast culture to the osmotic pressure is different from that of many other crops. To initiate cell colony formation, it was not necessary to lower the osmotic pressure except for rare instances in which plasmolysis was observed. Cell division and colony formation were observed (Fig.5 a & b) in both the media. Half strength MS medium was more suitable for cell division and colony formation as compared to MS medium.

References

Chen, W.H. and M.C. Liu. 1976. Nuclear behaviour and cell wall regeneration in protoplast from sugarcane young leaves. *Rept. Taiwan Sugar Res. Inst.*, 71: 1-9.

Chen, W.H. and S.C. Shih. 1983. Isolation of protoplast from cell culture and subsequent callus regeneration in sugarcane. *SSCT Proc, X VIII Congress*.vol 2: 632-639.

Dale, P.J. 1983. Protoplast culture and plant regeneration of cereals and other recalcitrant crops. *Exper. Supp (Basel)*, 46: 31-41.

Evans, D.A., O.J Crocomo and De M.T.V. Carvalho. 1980. Protoplasts isolation and subsequent callus regeneration in sugarcane. *Z. Pflanzenphysio*, 98: 355-358.

Ferenczy, L. and A. Maretzki. 1970. Success in preparing sugarcane protoplasts. *Ann. Rept. Exp. Sta.* HSPA, pp. 64-65.

Krishnamurthi, M. 1976. Isolation, fusion and multiplication of sugarcane protoplasts and comparison of sexual and parasexual hybridization. *Euphytica*, 25: 145-150.

Krishnamurthi, M. 1986. Sugarcane protoplasts *ISSCT Proc. XIX pp.* 1208-1214.

Larkin, P.J. 1982. Sugarcane tissue and protoplasts culture. *Plant Cell Tissue and Organ Culture*, 1: 149-164.

Maretzki, A. and L.G. Nickell. 1973. Formation of protoplasts from sugarcane cell suspension and the regeneration of cell culture from protoplasts. *Collog. Intern. Centre Natle. Rech. Sci.* Paris, 212: 51-63.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*, 15: 473-497.

Srinivasan, C. and I.K. Vasil. 1986. Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L) *J. Plant Physiol.*, 126: 41-48.

Tabaeizadeh, Z., R.J. Feri and I.K. Vasil. 1986. Somatic hybridization in the Gramineae. *Saccharum officinarum* L + *Pennisetum americanum* (L) K. Schum. (Pearl millet). *Proc. Nat. Acad. Sci.*, USA, 83: 5616-5619.

Vasil, I.K. 1983. Regeneration of plants from single cells of cereals and grasses. In: Genetic Engineering in Eukaryotes Lurquin, P and Kleinhofs, A. (Eds): Plenum Press, New York. pp. 233-252.

Yan, Q., X. Zhang and Z. Chen. 1985. Organogenesis from sugarcane protoplast. *Kexue Tonbbao*, 30: 1392-1395.

(Received for publication 14 February 2000)