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PEG-MEDIATED SOMATIC HYBRIDIZATION STUDIES IN SUGARCANE (*SACCHARUM* SPP. *HYBRID* CVS. CoL-54 AND CP-43/33)

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Abstract

Somatic hybridization studies were performed using leaf mesophyll as well as homogeneous cell suspension-derived protoplasts. The optimum results were achieved when leaf mesophyll protoplasts from cv. CP-43/33 and homogeneous cell suspension-derived protoplasts from cv. CoL-54 (at $2.0x10^5$ ml^{-1} final density) were used. Polyethylene glycol (PEG) at 35% level was found optimum for maximum protoplast agglutination (32%). Maximum heterokaryon formation (17%) was obtained employing 50 mM l^{-1} $CaCl_2.2H_20$ at pH 10.5 as an eluting solution. The hybridized protoplasts were cultured to monitor the divisions and further growth.

Introduction

Sugarcane is one of the important cash crops in tropical and sub-tropical countries of the world where about 60% of the world's cane-sugar production is based on this crop. Sugarcane crop improvement using modern techniques fall under three broad categories i.e., a) electroporation-mediated transformation studies (Rathus & Birch, 1992a,b), b) microprojectile bombardment technique to produce transgenic plants (Bower & Birch, 1992; Chowdhury & Vasil, 1992), and c) somatic hybridization (Tabaeizadeh *et al.*, 1986). Sugarcane is a polyploid crop. Hence, apart from the other two techniques mentioned above, somatic hybridization in a polyploid vegetatively propagated crop like sugarcane becomes even more advantageous and holds a lot of promise for genetic improvement. The problem of plant regeneration from protoplasts in the past seems to limit the success as far as the potential of somatic hybridization for crop improvement in sugarcane is concerned. Consequently very little research work has been done in this field. Using this technique, only somatic hybrid embryos could be produced using sugarcane (*Saccharum officinarum* clone B43-62) and pearl millet [*Pennisetum americanum* (L.) K. Schum; Gahi 3, cytoplasmic male slerile] protoplasts (Tabaeizadeh *et al.*, 1986). Alongwith electroporation of protoplasts, somatic hybridization might have produced much more than expected. The present paper thus highlights the importance to undertake work on somatic hybridization in sugarcane cultivars (*Saccharum* spp. *hybrid* cvs. CoL-54 and CP-43/33) alongwith the technique of electroporation-mediated and biolistic transformation to achieve the benefits that such techniques offer for crop improvement.

Materials and Methods

The following protocols were utilized for intraspecific somatic hybridization in *Saccharum* spp., *hybrid* cvs. CoL-54 and CP-43/33. The protoplasts were isolated from leaf mesophyll and homogeneous cell suspension cultures of cvs. CoL-54 and CP-43/33 respectively employing previously reported protocols (Aftab *et al.*, 1996). Chemically

induced fusions using high pH and Ca^{2+} concentration were carried out after Keller & Melchers (1973) and Melchers & Labib (1974). As far as PEG (polyethylene glycol) MW 4000 and 6000 induced fusions are concerned, the method described by Kao (1976) was followed. Treated protoplasts were pelleted at 500 rpm for 10 minutes and cultured as reported earlier (Aftab *et al.*, 1996).

Results

Effects of PEG (polyethylene glycol) molecular forms and concentrations

In experiments using PEG-1500, the results were not satisfactory. Satisfactory fusion was accomplished using PEG-4000 and PEG-6000. For PEG-4000, a concentration range of 15-45 % was used. The effects of this range on protoplast agglutination (tight adhesions between two or more protoplasts) are shown in Table 1. Maximum number of agglutinated protoplasts were achieved at 40% level. Observations using haemocytometer revealed that approximately 24% protoplasts out of $2x10^5$ ml^{-1} originally treated, i.e., $4.8x10^9$ ml^{-1} underwent agglutination process leading to tight adhesions. In few cases even complete fusion was noted at this stage of PEG treatment.

Table 1. Effects of different concentrations of PEG-4000/PEG-6000 alongwith 10.5 m Ml^{-1} $CaCl_2.2H_2O$ and 0.7 m Ml^{-1} $KH_2PO_4.H_2O$ on protoplast fusion initiation and agglutination.*

Treatment PEG %	Number of protoplasts per treatment (ml^{-1})	Agglutinated protoplasts (ml^{-1})		% of agglutinated protoplasts	
		PEG-4000	PEG-6000	PEG-4000	PEG-600
15	$2.0x10^5$	1.0×10^4	1.8×10^4	06	09
20	$2.0x10^5$	1.6×10^4	3.0×10^4	08	15
25	$2.0x10^5$	2.4×10^4	3.8×10^4	12	19
30	$2.0x10^5$	3.0×10^4	5.2×10^4	15	26
35	$2.0x10^5$	3.6×10^4	6.4×10^4	18	32
40	$2.0x10^5$	4.8×10^4	5.6×10^4	24	28
45	$2.0x10^5$	4.2×10^4	4.0×10^4	21	20

*Protoplast source:

a. Mesophyll protoplasts of cv. CP-43/33.

b. Homogeneous cell suspension cultures of cv. CoL-54 developed in AA medium.

Where PEG-6000 at a concentration range of 15-45 % was used (Table 1). Maximum number of agglutinated protoplasts were obtained at 35% level (32% agglutinated protoplasts, i.e., $6.4x10^4$ ml^{-1} protoplasts). However, 30 and 40% PEG-6000 levels also favoured 26 and 28% adhesions, respectively which was a fairly good response.

Effects of $CaC1_2.2H_20$ concentration and pH on fusion with PEG-4000 and PEG-6000

A maximum of 24 % of protoplast adhesions were achieved using PEG-4000 + 10.5 m Ml^{-1} $CaC1_2.2H_20$ + 0.7 m Ml^{-1} $KH_2P0_4.H_20$ ($4.8x10^4$ ml^{-1} agglutinated protoplasts out of $2.0x10^5$ protoplasts originally treated). Out of 24% agglutinated protoplasts, 50.1% complete single pair fusions were achieved where $CaC1_2.2H_20$ was used at 50 m Ml^{-1} as an 'eluting solution' at pH 10.5. In final analysis only 12.0% heterokaryons were obtained by PEG-4000 and $CaC1_2.2H_20$ treatment. pH 9.0, 9.5, 10.0 and 11.0 at 50 m Ml^{-1} $CaC1_2.2H_20$ considerably reduced the fusions (Table 2).

Table 2. Effects of different concentrations of $CaCl_2.2H_2O$ at different pH levels on heterokaryon formation. The data depicts heterokaryon formation after the PEG-4000 and PEG-6000 treatment.

Serial Number	$CaCl_2.2H_2O$ m Ml^{-1}	pH	PEG-4000					PEG-6000				
			9.0	9.5	10.0	10.5	11.0	9.0	9.5	10.0	10.5	11.0
1	30	Complete heterokaryon formation	5856	9984	12048	14448	12048	5632	12672	17216	22400	19008
		% heterokaryon formation to agglutinated protoplasts	12.2	20.8	25.1	30.1	25.1	8.8	19.8	26.6	35.0	29.7
		% heterokaryon formation to total number of protoplasts treated	29.2	4.99	6.02	7.22	6.02	2.81	6.33	8.60	11.2	9.50
2	40	Complete heterokaryon formation	8736	17088	18816	21600	19296	10560	19136	22336	25664	23744
		% heterokaryon formation to agglutinated protoplasts	18.2	35.6	39.2	45.0	40.2	16.5	29.9	34.9	40.1	37.1
		% heterokaryon formation to total number of protoplasts treated	4.36	8.54	9.41	10.8	9.64	5.28	9.568	11.16	12.83	11.87
3	50	Complete heterokaryon formation	9648	19248	21600	24000	20160	16000	25920	30016	34000	27008
		% heterokaryon formation to agglutinated protoplasts	20.1	40.1	45.0	50.1	42.0	25.0	40.5	46.9	53.12	42.2
		% heterokaryon formation to total number of protoplasts treated	4.82	9.62	10.8	12.0	10.08	8.0	12.96	15.00	17.0	13.50
4	60	Complete heterokaryon formation	4848	7440	9648	18720	12000	8192	10560	13312	27520	16640
		% heterokaryon formation to agglutinated protoplasts	10.1	15.1	20.1	39.0	25.0	12.8	16.5	20.8	43.0	26.0
		% heterokaryon formation to total number of protoplasts treated	2.42	3.72	4.82	9.36	6.0	4.0	5.28	6.65	13.76	8.32

Maximum adhesions were achieved using 35 % of PEG-600 + 10.5 m Ml^{-1} of $CaC1_2.2H_20$ and 0.7 m Ml^{-1} $KH_2P0_4.H20$ (Table 1). Out of 32% of protoplasts that agglutinated during this treatment ($6.4x10^4$ ml^{-1}), 53.12% of protoplasts underwent complete fusion at 50 m Ml^{-1} $CaC1_2.2H_20$ and pH 10.5. Thus the percentage of complete single pair fusion to actual protoplasts treated was 17.0 (Table 2).

Effects of protoplast density

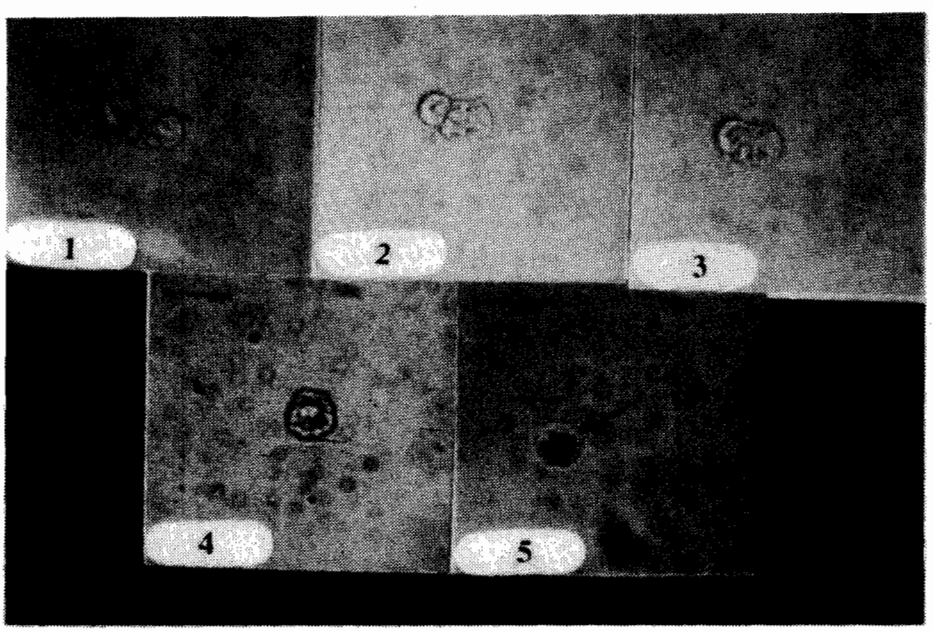
Lower than $2.0x10^5$ protoplasts ml^{-1} density resulted in a negligible number of protoplast fusions with better results at $2.0x10^5$ ml^{-1}. At this density 12% heterokaryon formation was achieved. Higher protoplast density than the optimum value ($2.0x10^5$ ml^{-1}) resulted in multiple agglutination thus resulting in multiple protoplast fusions (multiple pair fusions). Consequently, protoplast density of $2.0x10^5$ ml^{-1} was taken as standard in all of subsequent fusion treatments involving PEG-6000.

Effects of chemically induced somatic hybridization methods

Two centrifuge treatments reduced the number of agglutinated protoplasts to $3.0x10^4$ ml^{-1} (15%) and $4.0x10^4$ ml^{-1} (20%) in comparison with $4.8x10^4$ ml^{-1} (24%) and $6.4x10^4$ ml^{-1} (32%) in small scale method. The number of single pair fusion was further reduced to $1.6x10^4$ ml^{-1} (8.0%) and $2.2x10^4$ ml^{-1} (11.0%) by centrifuging twice in washing solution. Consequently, the small scale method for protoplast fusion was preferred in this study.

Microscopic observations, identification and culture of fusion products

The PEG-6000 mediated fusion treatment followed by $CaC1_2.2H_2O$ (50mM; pH 10.5) as an 'eluting solution' resulted in a gradual protoplast fusion extended over a period of 30-45 minutes (Figs. 1-5).



Figs.1-5. Sequential view of protoplast fusion by chemical means (PEG-6000 and 50 m Ml^{-1} $CaCl_2.2H_2O$ at pH 10.5). A protoplast of cell suspension origin undergoing process of agglutination and membrane fusion with two already fused mesophyll protoplasts resulting in the formation of a heterokaryon involving three protoplasts (x250).

As far as the identification of hybridized protoplasts was concerned, bright field microscopy clearly differentiated such protoplasts as comparatively larger chloroplast-containing protoplasts. The hybridized protoplasts achieved in chemically induced protoplast fusion methods could not undergo divisions.

Discussion

Since successful protoplast regeneration was achieved in sugarcane (Aftab *et al.*, 1996), attempts were focussed on somatic hybridization to achieve the benefits it offers in sugarcane. Attempts using PEG-1500 yielded poor results. Higher molecular weight polyethylene glycol (PEG-4000 and PEG-6000) yielded quite a number of heterokaryons in the present investigation. PEG-6000 has been found to be better than PEG-4000 as a maximum of 32% of protoplasts underwent agglutinations at its 35% level as compared to PEG-4000 (40%) where 24% protoplasts agglutinated. Effective role of higher molecular weight as PEG-6000 for somatic hybridization is also supported by Armstrong *et al.*, (1990) in PEG-mediated stable transformations of maize protoplasts. Another merit of PEG-4000 and PEG-6000 induced fusions has been the formation of the most binucleates rather than large protoplast fusion (Kao, 1977). PEG-induced fusions were non-specific. In addition to fusions between homogeneous cell suspension (ECSC) and mesophyll-derived protoplasts, fusion between protoplasts derived from the same source (either mesophyll to mesophyll or ECSC to ECSC protoplasts) have also been routinely observed as suggested earlier by Kao *et al.*, (1974).

In the present study, the best method for PEG-mediated protoplast fusion was the combination of (a) PEG-induced protoplast fusion as described by Kao (1976) and (b) the high pH, calcium ion method as developed by Keller & Melchers (1973).

Repeated centrifugation, after the fusion treatments, in large scale method adversely affected the yield and viability of hybridized protoplasts. Therefore, to standardize PEG-mediated protoplast fusion in this study, initially the technique of fusing the protoplasts on coverslip (small scale method) was preferred (Kao, 1976; Chen *et al.*, 1987). In the present study, however, the complete heterokaryon formation was achieved in 12% of total protoplasts treated in PEG-4000 and $CaC1_2.2H_20$ at pH 10.5 and 17% in PEG-6000.

In an earlier study, involving somatic hybridization between sugarcane and *Pennisetum americanum*, selection of somatic hybrid cell lines and formation of somatic hybrid embryos (using an amino acid-analog-resistant cell line and metabolic inhibitors) has been reported by Tabaeizadeh *et al.*, (1986). However, plant regeneration could not be achieved in their studies. In the present study, the limiting factor was the inability of hybridized protoplasts to divide. Somatic hybridization in a polyploid and vegetatively propagated crop such as sugarcane has a good potential for producing useful variation.

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