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RESEARCH ARTICLE

OPTIMIZATION OF CONDITIONS FORFUSION IN SUGARCANE PROTOPLASTS (*SACCHARUM* COMPLEX.)

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ABSTRACT

The experimental was carried out on two sugarcane varieties, namely CoN -05071 and CoC-671 at the Sugarcane Tissue Culture Laboratory of Main Sugarcane Research Station, Navsari Agricultural University, Navsari during 2016-017. The investigation deals with protoplast fusion such as agglutinated protoplasts, and heterkaryon formation And Molecular character. In this experiment, PEG 6000(19%) has been found to be better than PEG 4000, CaCl2.2H2O(35µm) and 10.5pH was found to be better for total agglutination and heterokaryon formation in sugarcane cultivars. Somatic hybridization in a polyploid and vegetatively propagated crop such as sugarcane has a good potential for producing useful variation. Maximum polymorphism is observed among the genotypes and has accumulated maximum genetic changes in comparison.

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INTRODUCTION

Somatic hybridization between various sexually compatible and non-compatible plant species has been reported (Harms, 1986; Finch *et al.,* 1990). Most of the reported work on somatic hybridization deals with the members of the family Brassicaceae and Solanaceae. This is because of the fact that regeneration of plants from protoplasts isolated from almost all *Brassica* species and members of family Solanaceae such as *Nicotiana, Datura, Petunia, Solatium* and *Lycopersicon* has been reported (Sihachakr *et al.* 1988; Kirti *et al.,* 1991). Consequently, work on somatic hybridization in *Brassica* and other members of Brassicaceae as well as Solanaceae has yielded meaningful results (Narasimhulu *et al.,* 1994; Stattmann *et al.,* 1994). Apart from above mentioned plants where protoplast manipulation has been relatively easier, experimentation involving protoplasts have generally proved difficult in many other plant groups. As far as the family Gramineae is concerned, almost all of its members pose extreme recalcitrance in *in vitro* manipulation of cells and protoplasts (Vasil and Vasil, 1992). Sugarcane, a member of the family Gramineae, is an important crop in many parts of the world. Unlike other members of Gramineae, sugarcane is a polyploid crop.

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Hence, somatic hybridization seems to offer an opportunity for sugarcane crop improvement. Unfortunately, the work on somatic hybridization in sugarcane is extremely scanty (Tabaeizadeh *et al.,* 1986). A major reason for this until recently has been the lack of 'protoplast to plant' system. However, now a number of reports exist describing successful plant regeneration from sugarcane protoplasts (Taylor *et al.,* 1992; Liu, 1994; Aftab *et al.,* 1996; Aftab and Iqbal, 1999). Keeping in view the above mentioned aspects, work on somatic hybridization in sugarcane was initiated. Somatic hybridization via chemical means have been reported earlier (Aftab and Iqbal, 2001). In the present study, the experimental conditions for somatic hybridization in sugarcane (*Saccharum* spp. CoN -05071 and CoC-671) using fusion technique have been reported

MATERIALS AND METHODS

Salient feature of sugarcane varieties

CoN-05071 –High yielding, Early mature, Medium resistant to red rot and wilt, CoC-671-Early mature, high sugar content, but susceptible to red rot and wilt. Per cent agglutinated protoplasts. The quantified protoplast through haemocytometer were considered and the protoplast density is checked and standardized. Standardized protoplast density at different levels of PEG (4000, 6000) applied to one ml cell mix and at different incubation levels agglutinated protoplasts were

counted from the no. of protoplast per treatment. Per cent heterokaryon formation to total number of protoplast treated. Per cent agglutinated protoplast includeboth homokaryon and heterokaryon fusions. Fusion levels induced by application of different levels of $CaCl_2.2H_2O$ and pH.

Molecular characterization

Molecular characterization, inner two leaf whorls of two to three months old shoot tops of each variety CoN -05071 and CoC-671 were grown in field used for genomic DNA isolation for Randomly Amplified polymorphic DNA (RAPD).

Electrophoretic Quality

To check quality of DNA (supercoiled, linear or sheared) and RNA contamination of isolated genomic DNA was run electrophoretically on 0.8 % agarose gel and quality was judged by viewing the image of separated DNA fragments.

Table 1. The components of PCR mix

1	Taq buf fer (10 x) wi th 15 mM MgCl 2l	2.5 µl
2	Primer (10 pmoles/ µl)	1.0 µl
3	dNTPs mix (10 mM each	0.5 µl
4	Taq DNA polymerase (5U/ µl)	0.3 µl
5	Template DNA (50ng/ µl)	2.5 µl
6	Sterile distilled water	18.2 µl
Total		25.0 µl

All the PCR reactions were carried out in 200 µl thin walled PCR tubes. PCR tubes containing reaction mixture were tapped gently with short spinning (5000 rpm for 30 seconds). The tubes were then placed in the Thermal Cycler (Biometra T. Gradient, Germany) for cyclic amplification

Table 2. List of RAPD primers

Sr. No.	RAPD Primer	Sequence (5 '- 3')
1	OPK-03	CCAGCTTAGG
2	OPK-04	CCGCCCAAAC
3	OPK-08	GAACACTGGG
4	OPK-09	CCCTACCGAC
5	OPK-10	GTGCAACGTG
6	OPK-11	AATGCCCCAG
7	OPK-15	CTCCTGCCAA
8	OPK-17	CCCAGCTGTG
9	OPK-18	CCTAGTCGAG
10	OPK-20	GTGTCGCGAG
11	OPL-02	TGGGCGTCAA
12	OPL-03	CCAGCAGCTT
13	OPL-04	GACTGCACAC

RESULTS

As far as PEG (poly ethylene glycol) MW 4000 and 6000 induced fusions are concerned, the method described by Kao (1976) was fallowed. Treated protoplasts were pelleted at 600 rpm for 2 minutes and culture procedures were followed as earlier reported by (Aftab *et al*., 1996). In the fusion culture, two gradients of PEG 4000, 6000 with different concentrations of $CaCl_2.2H_2O$ and pH level were utlized. Satisfactory fusion was accomplished at different levels of implication of treatment combinations. Concentration range of 30-40 per cent PEG, 30-40 µm/L $CaCl_2.2H_2O$ and 10, 10.5 and 11 of pH levels were checked. The effects of these different ranges in combination on protoplast agglutination (tight adhesion between 2 or more protoplast) are shown in table 4.5and figure4.10 and Plate 9 Maximum number of agglutinated protoplasts were achieved at 35 per cent level of PEG 6000, 35 µm/L $CaCl_2.2H_2O$ and 10.5 pH. Observations using haemocytometer revealed that approximately 19 per cent protoplast out of 2×105 ml originally treated is 3.8×104 ml underwent agglutination process leading to tight adhesion, where PEG 4000, 30µm/L $CaCl_2.2H_2O$ and 10 pH resulted into (30 %)very low number of agglutinated protoplast (5%) out of 1.0×104 ml originally treated, underwent agglutination process. However, 30 and 40 per cent of PEG 6000 levels also favoured 13 and 11 per cent adhesion, respectively.

Table 2. Effect of different concentrations and combinations of PEG 4000/6000, $CaCl_2.2H_2O$ and pH Levels on agglutinated protoplast

Sr. no	Treatment	Total agglutinated protoplast
1	$P_1C_1Ca_1H_1$	1.0* (1.2)
2	$P_1C_2Ca_2H_2$	1.8 (1.52)
3	$P_1C_3Ca_3H_3$	1.2 (1.30)
4	$P_2C_1Ca_1H_1$	2.6 (1.76)
5	$P_2C_2Ca_2H_2$	3.8 (2.07)
6	$P_2C_3Ca_3H_3$	2.2 (1.64)
S.Em±		0.04
CD 0.05%		
C.V %		3.47

Figures in parenthesis are Square Root + 0.5 transformed values
*Figures are original value.
Where,
P_1=4000 MW, P_2=6000 MW, C_1= 30%, C_2=35%, C_3=40%, Ca_1=$CaCl_2$ @30 ml, Ca_2= $CaCl_2$ @35 ml, Ca_3= $CaCl_2$ @40 ml
H_1=pH @10, H_2= pH @10.5, H_3= pH @11

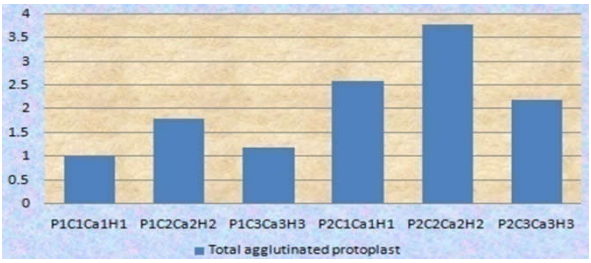


Fig. 1. Effect of different concentration and combination of PEG 4000/6000, $Cacl_2 \cdot 2H_2o$ and pH levels on agglutinated protoplast

Table 3. Effect of different concentrations and combinations of PEG 4000/6000,$CaCl_2.2H_2O$ and PH Levels on agglutinated protoplast

Sr. no.	Treatment	No. of protoplast/ treatment	Agglutinated protoplast	Agglutinated % Protoplasts.
1	T1	2.0×10^5	1.0×10^4	5
2	T2	2.0×10^5	1.8×10^4	9
3	T3	2.0×10^5	1.2×10^4	6
4	T4	2.0×10^5	2.6×10^4	13
5	T5	2.0×10^5	3.8×10^4	19
6	T6	2.0×10^5	2.2×10^4	11

Table 4. Per cent heterokaryon formation to total agguluminated protoplast

Sr. no.	Treatment	% agglutinated protoplast	% heterokaryon
1	T1	5	12
2	T2	9	8.8
3	T3	6	14.1
4	T4	13	11.5
5	T5	19	17.10
6	T6	11	13.7

Maximum of 19 per cent protoplast adhesions were achieved using PEG 6000 + 35% µm CaCl2.2H2O (3.8×104 ml agglutinated protoplast), out of 2.0×105 protoplast originally treated. Out of 19 percent agglutinated protoplast, 50 per cent complete single pair fusions were achieved.

Table 5. Effect of different concentrations $CaCl_2$ at different pH levels on heterokaryon formation

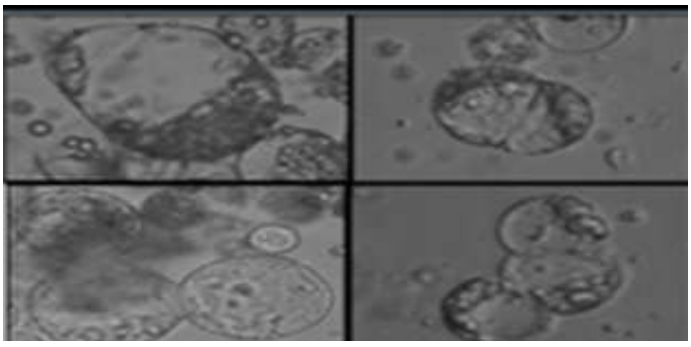
Sr. no.	Conc. of $CaCl_2$	PEG 4000			PEG 6000		
		pH 10	pH 10.5	pH 11	pH 10	pH 10.5	pH 11
1	30	1200* (12%)	1350 (13.50%)	900 (9%)	2000 (11.5%)	900 (3.45%)	1600 (6.1%)
2	35	1100 (6.1%)	1600 (8.8%)	2500 (13.8%)	1200 (3.1%)	6500 (17.10%)	2900 (7.6%)
3	40	800 (6.6%)	1300 (10.8%)	1700 (14%)	900 (4.00%)	3600 (16.3%)	2800 (12.7%)

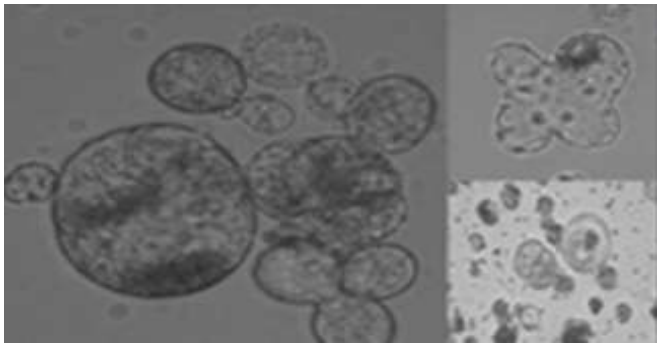
Figure indicated * are Complete heterokaryon formation
Figures are in parenthesis % heterokaryon formation

Table 6. Qualitative and quantitative analysis of genomic DNA using nanodrop spectrophotometer of different sugarcane lines

CoN-05071			CoC-671		
Line No.	Genomic DNA Conc. (ng/ µl)	Optical Density 260:280 Ratio	Line No.	Genomic DNA Conc. (ng/ µl)	Genomic DNA Conc. (ng/ µl)
1	569.2	1.87	1	524.3	1.99
2	455.1	1.91	2	601.8	1.86
3	432.0	1.95	3	527.0	1.95
4	472.4	1.95	4	648.0	1.80
5	526.9	1.87	5	425.4	1.99
6	518.4	1.88	6	346.3	1.98
7	507.7	1.91	7	525.2	1.95
8	515.7	1.90	8	599.0	1.85
9	447.2	1.98	9	425.2	1.94
10	535.8	1.93	10	548.9	1.87
11	617.7	1.88	11	1038.9	1.85
Average	508.9	1.91	Average	564.5	1.90

P = Parent 1-10 = Mutant lines





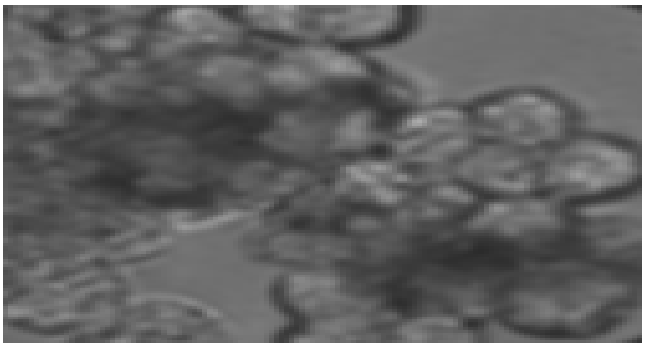


Fig. 2. Maximum number of agglutinated protoplast at 35 percent level of PEG 6000 MW, 35 µm/L and 10.5 pH

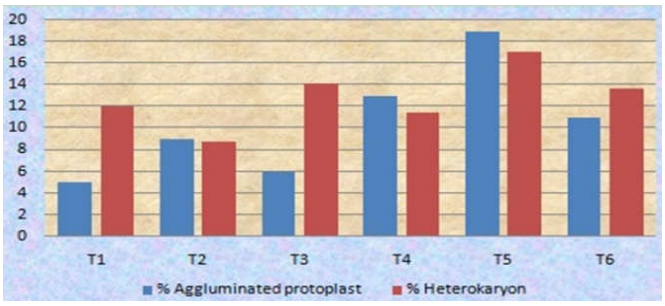


Fig. 3. Percent heterokaryon formation to total agglutinated protoplast

In final analysis, only 17.1 per cent heterokaryon were obtained by PEG 6000, 35 µm $CaCl_2.2H_2O$ and 10.5 pH level. High pH levels and high $CaCl_2.2H_2O$ concentrations considerably reduced the fusion [(Table 3), (Table 4) and (Table 5)]. Fig. 3 and Fig. 4. Qualitative and Quantitative analysis of genomic DNA.

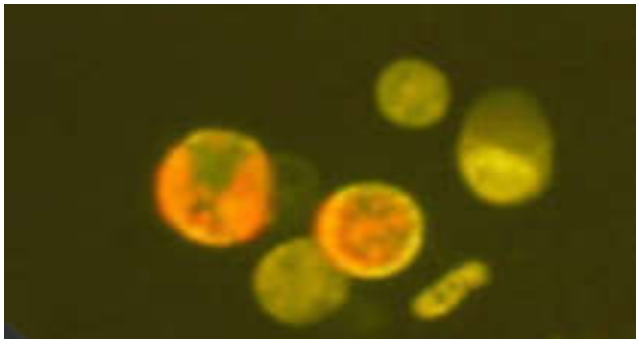


Fig. 4. Heterokaryon formation to total agglutinated protoplast

In present investigation (Table 7) the average concentration of DNA was 508.9 ng/µl and 564.5 ng/µl in lines of CoN-5071 and CoC-671 respectively, measured by nanodrop spectrophotometer.

The line 11 and line 1 showed highest concentration as 617.7 ng/µl and 569.2 ng/µl respectively. However, line 3 showed lowest concentration of 432.0 ng/µl in cv. CoN-5071. The line 11 and line 4 showed highest concentration as 1038.9 ng/µl and 648.0 ng/µl, respectively however, line 6 showed lowest concentration of 346.3 ng/µl in cv. CoN-05071. Quantitative analysis of DNA was carried out from uniform working stock dilution of all lines for further PCR analysis. The quality of DNA was decided by optical density 260:280 ratio. All the lines have optical density 260:280 ratio between1.8 to 2.0. The integrity of DNA was also confirmed on agarose gel electrophoresis. It showed compact band without RNA contamination.

Ten decamers oligonucleotide primers were used for a RAPD analysis. On an average each primer gave nine bands. The amplification products range from 0.1 Kb to 1 Kb. In genotype CoC-671, primer OPK-4 produced maximum 12 bands out of which one was polymorphic. The primer OPK-20 produced 3 monomorphic bands out of total 9 bands in cv. CoN-05071. Primer OPK-10 produced maximum polymorphism (100%) followed by primer OPK-04 (91.67%), OPK-15 (88.89%) and OPL-03 (88.89%) in genotype CoC-671. In genotype CoN-05071, primer OPK-8 produce 12 band out of which 2 were monomorphic. Primers OPK-17 and OPL-04 produced maximum polymorphism (100%) followed by primer OPK-04 (88.89%), OPK-15 (85.71%) and OPK-08 (83.33%) in genotype CoN 05071. The RAPD profiles revealed genetic polymorphism among selected plant in both the genotypes. The NTSYSpc programme was used to calculate Jaccard's simila rity coef ficient. The genetic similarity between mother and regenerated plants was an average. In genotype CoC-671, line 8 had showed maximum genetic variation to the control plant (0.543) followed by line 10 (0.556) and line 2 (0.565) .

Conclusion

Higher molecular weight PEG 4000 and 6000 yielded quite a number of heterokaryon in the present investigation. PEG 6000 has been found to be better than PEG 4000 (19%). 35µm CaCl2.2H2O and 10.5 pH was found to be better for total agglutination and heterokaryon formation in sugarcane cultivars. Somatic hybridization in a polyploid and vegetatively propagated crop such as sugarcane has a good potential for producing useful variation. Maximum polymorphism is observed among the genotypes and has accumulated maximum genetic changes in comparison.

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