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OPTIMIZATION OF CONDITIONS FOR ELECTROFUSION IN SUGARCANE PROTOPLASTS

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Abstract

Somatic hybridization studies were performed using leaf mesophyll as well as homogeneous cell suspension-derived protoplasts in sugarcane (*Saccharum* spp. *hybrid* cvs. CoL-54 and CP-43/33). The conditions standardized for electrofusion were 40 V of primary voltage for 30 seconds of AC for pearl chain formation and 2.0 KV cm^{-1} for 20 µS in DC, 4 times with repeat intervals of 5S for fusion. About 60% of the protoplasts underwent fusion event (pearl chain formation) and single pair fusion was observed in about 21% of the protoplasts. Microcalluses from such protoplasts were successfully achieved on KM8P-K8P medium after 20 days of culture.

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, Solanum* 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 & 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. 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 & 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 & Iqbal, 2001). In the present study, the experimental conditions for somatic hybridization in sugarcane (*Saccharum* spp. *hybrid* cvs. CoL-54 and CP-43/33) using electrofusion technique have been reported.

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Materials and Methods

Electrofusion was carried out on Shimadzu somatic hybridizer SSH-1 ver.1.4 (Shimadzu, Tokyo, Japan).

Protoplasts were isolated from the mesophyll and cell suspension cultures of the two cultivars of *Saccharum* spp., *hybrid* cvs. CoL-54 and CP-43/33 as reported earlier (Aftab *et al.*, 1996). Isolated protoplasts were mixed in appropriate (1:1) ratio and resuspended in electrofusion buffer at 2.5 x 10^5 protoplasts ml^{-1}. The electrofusion buffer consisted of mannitol (0.72M or 13%) and 0.1% MES, pH 5.8.

For pearl chain formation between the protoplasts from the two sources, protoplasts in electrofusion buffer were gently pipetted between the electrodes of any particular chamber in use. The chamber was placed under inverted microscope for visual observations during the whole fusion treatment. The sample was exposed to an AC pulse in a range of 10, 20, 30 and 40 volts in combination with frequency of alternating current (AC) ranging from 0.5, 1.0 to 2.0 MHz. To achieve pearl chain formation, different AC pulse and frequency combinations were manipulated for different time periods ranging from 1-90 seconds.

After pearl chain formation, the sample was subjected to direct current (DC) pulse/pulses (number = n = 1 –10). DC pulses were so manipulated that the field strength did not increase beyond 2.0 KV/cm in a particular fusion chamber. Pulse width (PW) was kept in accordance with the field strength. PW range of 100-500 μS (longer duration) was applied at or less than 1.0 KV/cm of field strength (lower range) whereas for 1-2.0 KV/cm (higher range) of field strength, the range of PW monitored was 10-100 μS (shorter duration). The interval between the two DC pulses was also optimized and a range of 0.1-10 seconds was monitored. The AC field was applied during the interval between the DC pulses (range 1-20 V). After the fusion experiment, the treated protoplasts were left for an hour at 26-28°C and observed for complete single pair fusion. The hybridized protoplasts were visually selected and counted under the bright field of inverted microscope (x40). Light green coloured mesophyll protoplasts were clearly distinguished from colourless protoplasts of cell suspension culture of the other source. The treated protoplasts were cultured as described earlier (Aftab *et al.*, 1996).

Results

Electrofusion

Standardized and reproducible conditions for pearl chain formation and single pair fusion between the protoplasts of two cultivars showed that the optimum density of protoplasts from the two sources *i.e.*, mesophyll protoplasts and ECSC-derived protoplasts for electrofusion was 2.5 x 10^5 ml^{-1} (Table 1). The chamber volume was 0.8 ml and the interelectrode distance (electrode spacing) of 2.0 mm. The other conditions standardized for electric fields were 1 MHz of frequency, 40V of primary voltage for 30S of AC for pearl chain formation and 2.0 KV cm^{-1} for 20 μS in DC, 4 times with a repeat interval of 5S for fusion. The electrofusion process repeatedly produced hybrid cells in a number of experiments. Using this protocol, about 60% of the protoplasts underwent fusion event (pearl chain formation) and single pair fusion was observed in about 21% of the protoplasts (Fig.1).

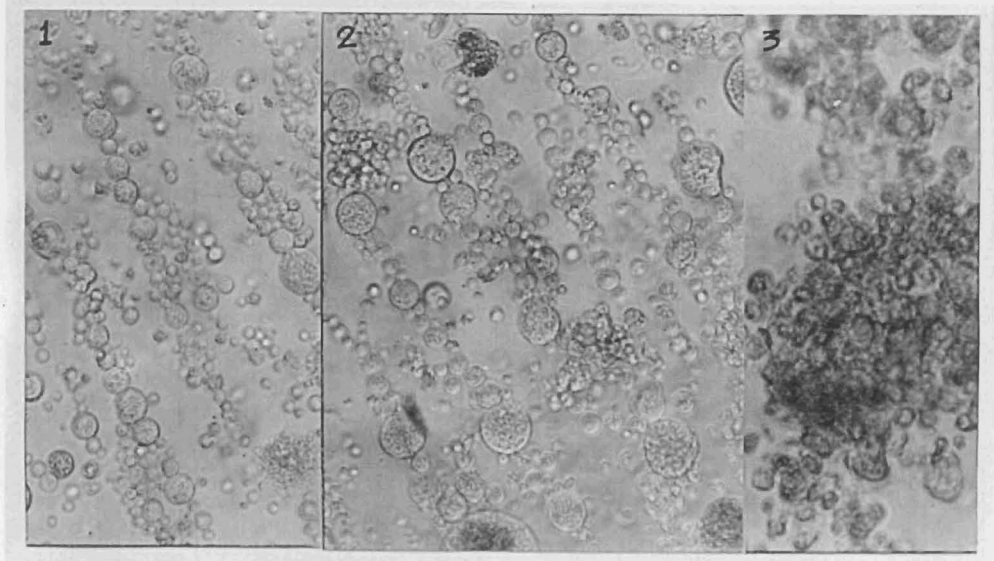

Fig. 1. Pearl chain formation between mesophyll protoplasts of cv. CP-43/33 and cell suspension protoplasts of cv. CoL-54 (x250). Fig. 2. Heterokaryons produced: *Note* the larger sized chloroplast-containing protoplasts (x250). Fig. 3. Microcolony developed from hybridized protoplasts in KM8P-K8P agarose-solidified medium after 20 days of culture (x250).

Table 1: Optimum conditions for electrofusion of protoplasts in *Saccharum* spp., *hybrid* cv. CP-43/33 (mesophyll protoplasts) and cv. CoL-54 (ECSC protoplasts)*.

	Parameters	Standard condition
1.	Optimum protoplast density (cv. CP-43/33 mesophyll protoplasts + cv. CoL-54 ECSC protoplasts)	2.5 x 10^5 $p.ml^{-1}$ (final density of protoplasts from both sources).
2.	Electrofusion buffer	13% mannitol and 0.1% MES; pH 5.8.
3.	Suitable fusion chamber	SSH-CO3
4.	Chamber capacity	0.8 ml
5.	Electrode spacing	2.0 mm
6.	Chamber constant	0.05
7.	Frequency	1 MHz
8.	Volts AC (primary)	40 volts
9.	Initial time	30 seconds
10.	Pulse width	20 μS
11.	Volts DC	400 volts
12.	Field strength	2.0 KV cm^{-1}
13.	Repeat interval	5 seconds
14.	n = number of DC pulses	4
15.	VDC decreasing rate	80%
16.	VAC decreasing rate	70%
17.	Temperature	27±1°C

* Electrofusion conditions as optimized on Shimadzu somatic hybridizer, Model SSH-1 Ver.1.4.

Microscopic Observations and Identification of Fusion Products

Bright field microscopy clearly differentiated hybridized protoplasts from the donor protoplasts. Hybrid protoplasts were comparatively larger in size and contained chloroplast (Figs. 1 and 2).

Culture and Regeneration Studies of Hybridized Protoplasts

Microcalluses from hybrid protoplasts were successfully achieved on KM8P-K8P medium after 20 days of culture. Despite numerous manipulations further growth of microcalluses could not be obtained (Fig. 3).

Discussion

Several factors viz., PEG molecular form and concentrations, $CaCl_2.2H_2O$ concentration, pH, protoplast density and somatic hybridization methods governed the PEG-mediated somatic hybridization in sugarcane (Aftab & Iqbal, 2001). The results of the present investigation depicted that electrofusion approach was a better option since the fusion products could undergo divisions and develop up to microcallus stage. Maximum of 17% heterokaryons were produced using PEG-6000. This percentage was also improved during the present study and single pair fusion was observed in about 21% of the protoplasts. The formation of microcalluses from hybridized protoplasts (via electrofusion) as achieved in this study is a new observation. Divisions in hybridized protoplasts between two sugarcane cultivars leading to any organized cellular stage eg., microcalluses as observed in the present study does not appear to have been reported so far. It is interesting to note that 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 earlier by Tabaeizadeh *et al.*, (1986) where plant regeneration could not be achieved. In the present study also, though microcalluses were achieved from hybridized protoplasts, the limiting factor was again the lack of regeneration from hybridized microcalluses.

Somatic hybridization for producing useful variation in sugarcane holds promise since it is a polyploid and vegetatively propagated crop. Further work on these lines is in progress for producing useful variation in sugarcane.

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