Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 9, Issue, 10, pp.58420-58424, October, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PROTOPLAST ISOLATION IN SUGARCANE (*SACCHARUM COMPLEX*.)

***Chaudhari, V. B., Parmar, V. L., Chaudhari, M. H. and Udutha, J.**

Department of Genetics and Plant Breeding, College of Agriculture, Navsari Agricultural University, Gujarat

ARTICLE INFO

Article History:
Received 18th July, 2017
Received in revised form
23rd August, 2017
Accepted 13th September, 2017
Published online 17th October, 2017

Key words:

Protoplast isolation such as protoplast Purification,
Protoplast density,
Protoplast viability.

ABSTRACT

The investigation 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 current study deals with the studies on protoplast isolation such as protoplast purification, protoplast density, protoplast viability in Sugarcane (*Saccharum* complex). In this experiment, successful isolation of protoplast from mesophyll cells of inner two leaf whorls and callus suspension cultures of sugarcane cultivars CoC-671 and CoN-05071 was obtained. Cultivars were standardized at 1 per cent cellulase, 0.5 per cent macerozyme, 1.0 per cent pectolyase and 0.5 M manitol. Maximum protoplast yield was observed at 600 rpm in callus suspension cells (68%) at 2 hrs of incubation period. Maximum density of protoplast was observed in callus suspension derived protoplast culture ((2.0×104, 2.2×104) in both the varieties at 2 hrs of incubation periods. Highest protoplast viability observed at 2.2x104 density level of callus suspension origin protoplast. In this study 2 hrs of incubation is optimal time for protoplast isolation from mesophyll cells and callus suspension cells of sugarcane cultivars.

Citation: Chaudhari, V. B., Parmar, V. L., Chaudhari, M. H. and Udutha, J. 2017. "Protoplast isolation in sugarcane (*Saccharum* complex.)", *International Journal of Current Research*, 9, (10), 58420-58424.

INTRODUCTION

Sugarcane 2n= 40 to 128 (*Saccharum* spp.) belongs to the family Poaceae (Graminae). It is an important cash crop of tropical and subtropical regions because of its high trade value. It is the main sugar producing crop that contributes more than 75% to the total sugar pool at the global level. Sugarcane is a vegetatively propagated crop. Somatic hybridization is the most important components of plant biotechnology which assist in the improvement of sugarcane. Both require regeneration of plants from protoplast and single cell. It is now possible to regenerate plants from protoplast of many species (Dale, 1983). The culture of graminaceous protoplast have been refined though it is extremely difficult (Chen and Shin, 1983). An enzymatic isolation of sugarcane protoplast was first reported by Ferenczy and Maretzki (1970). Subsequent induction of cell division has been obtained from the protoplast derived from cell culture (Maretzki and Nickell, 1973), rolled young leaves (Chen and 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 and chen, 1985). Tabaeizadeh *et al*., (1986) have obtained somatic hybrid cell after fusion of sugarcane and pearl millet protoplast with a limited morphogenetic ability. Plant regeneration from sugarcane protoplasts has only been achieved by Srinivasan and Vasil (1986) and this was at a low frequency (Vasil, pers. corn.). Practical application of established direct gene transfer systems to sugarcane depends on the regeneration of plants from protoplasts. It is, therefore, desirable to increase the frequency of regeneration from protoplasts and extend this technique to a range of cultivars.

******Corresponding author:* **Chaudhari, V. B.**
Department of Genetics and Plant Breeding, College of Agriculture, Navsari Agricultural University, Gujarat

MATERIALS AND METHODS

Enzymatic isolation

Protoplasts were isolated both from leaf Mesophyll as well as from callus tissue. For leaf Mesophyll protoplast 2-3 leaf whorls were used as donor material for both the varieties ofsugarcane (Coc-671, CoN-05071). The leaves were cut in to 1 mm2 pieces in 60-90 mm Petri plates. For callus protoplast 1 mm2 fragments of 1 g callus pieces from compact globular white callus cultures were taken. The enzyme solution for protoplast isolation consisted of different concentrations and combinations of cellulase R-10, macerozyme R-10 (YAKULT HONSHEA CO LIMITED, JAPAN), pectolyase Y-23 (sigma grade), PVP (Poly vinyl pyrolidone) and antibiotics ampicilin 40 mg/L, tetracycline 10 mg/L and gentamycin 10 mg/L were dissolved in CPW medium containing 13 per cent mannitol

$CaCl_2.2H_2O$ at pH 5.8. Leaves were surface sterilized in 70 per cent ethanol for 30 seconds and were rinsed with distilled water 3 times to remove trace elements. Leaves epidermis is scraped with sterilized blades and were sliced thinny in Petri dishes. The enzymes were added to the buffer solution and placed in water bath at 55 0C for 10 minutes to enhance enzyme solubility (Yoo *et al.*, 2007). The enzyme buffer solution was sterilized with a 0.22 µm syringe filter unit. In each Petri dish, 10 ml of the filter sterilized buffer enzymes solution was added to the thinny cut sections of the leaves and callus pieces separately. The Petri dishes were covered with parafilm and incubated for 2 hr. isolation was carried out in sterile environment provided by a laminar air flow unit. All the equipments and materials were sterilized by autoclaving to avoid contamination. The experiment was carried out twice with the four replicates each of with five Petri dishes. Similar procedure was carried out for callus protoplast isolation by selecting 1 mm2 fragments of 1 g callus pieces.

Table 1. Component of enzyme mixture used for enzymatic isolation

Enzyme mixture	Cellulase Onzuka R-10 % w/v	Macerozyme % w/v	Pectolyase
1	1.0	0.5	0.5
2	2.0	0.5	0.5
3	4.0	0.5	0.0
4	1.0	1.0	1.0
5	2.0	1.0	1.0
6	4.0	1.0	1.0

Protoplast Purification

After incubation, the Petri dishes/ culture tubes were agitated gently to release isolated protoplasts. The protoplast containing enzyme-buffer solution was diluted with equal volume of washing solution (125 µm $CaCl_2$, $NaCl_2$, KCl and pH 5.8) after which the released protoplasts were collected by sieving through 3 layers of cheese cloth, the protoplast containing solution was centrifuged at 600 rpm for 2 minutes and the pellet was resuspended in the washing solution. The procedure was continued twice. The resuspended pellet was overlayered on different sucrose concentrations (10%, 15%, 20% and 30% to make a gradient (Table 2). Protoplasts were collected at the interface of different sucrose concentrations to locate and retrieve protoplasts (Aftab and Iqbal, 2001). A sample of the collected protoplasts was viewed and photographed under the microscope.

Table 2. Solvents used for the dissolution of hormonal supplements

Hormones	Solvents
NAA	0.1N NaOH
2,4-D	70% Ethanol
BAP	0.1N NaOH

Protoplast quantification and viability test

Protoplasts were quantified by the use of haemocytometer. Viability of the protoplasts was examined by Trypan blue staining assay (Chamani *et al.*, 2012). Equal volume of 0.04% trypan blue in buffer solution was mixed with 100µl protoplast suspension, about 10µl of trypan blue/cell mix was pipetted on to a Haemocytometer (Reichert, USA) with cover slips. The protoplast suspension was allowed to fill the entire chamber. The filled heamocytometer slide was viewed under the light microscope at 100x magnification. Trypan blue was excluded by living protoplasts, whereas dead protoplasts and cell debris were stained a deep blue color. The number of unstained cells (viable protoplasts) was counted.

Table 3. Different supplementations in MS culture media

Media	NAA mg/l	BAP mg/l	2,4-D mg/l	Caseine hydrolysate mg/L
1	1.5	1.5	0.3	25
2	1.5	1.5	0.3	25
3	1.5	1.5	0.3	25

Table 4. Preparation of stock solutions for DNA extraction

S. No.	Solution
1.	1M TrisHCl(pH 8.0)
2.	0.5M EDTA (pH 8.0)
3.	5M NaCl
4.	70% Ethanol,500 ml
5.	Chloroform: Isoamyl alcohol (24:1),500 ml
6.	Ethidium Bromide (10mg/ml)

Table 5. Preparation of buffers for DNA extraction

S.No.	Buffer
1.	CTAB Extract ion buffer (3%) 10 ml
2.	TE buffer (0.1mM)100 ml 10mM Tr is HCl (pH 8.0) 0.1mM EDTA(pH 8.0)

RESULTS AND DISCUSSION

It was possible to isolate protoplasts from both of the cultivars of sugar cane tested (Coc-671 and CoN-05071). However cultivars responded differently to the isolation protocol, even the explant portion selected i.e. mesophyll cells and callus suspension tissue responded differently to the isolation protocol, and individual adjustment had to be performed for maximization of good quality protoplast. Quality of protoplast was defined by visual appearance under a microscope i.e. round cells, completely free of cell wall or easily detachable from cell wall debris, did not burst during enzymatic digestion and manipulation. Freshly isolated protoplast from sugar cane varieties CoC-671 and CoN-05071 of meshophyll cells and callus suspension tissue. Freshly isolated protoplasts were bright, spherical in shape and well separated. For meshophyll protoplast the best results were achieved using inner 2-3 whorls of young leaves. Protoplasts produced through callus culture were found to be good source. Compact globular white callus of inner leaf whorls of 8-12 weeks old callus found to be good source material for protoplast. In this experiment, up to four months age callus was used because of its ability and potentiality to regenerate. Trypson blue, a staining die, was used to monitor viability of the protoplast. Viable and non viable protoplast isolated from mesophyll cells and callus tissue. Pure protoplasts, which are lightly stained and clear in appearance were used for protoplast yield, density and viability studies. Diffused and debris materials were darkly stained.

Protoplast purification and isolation

Protoplast purification the effect of incubation of protoplast in the enzymatic solution viz., protoplast band formation for mesophyll and callus tissue of both the varieties at four different rpm levels using sigma 55-34 rotor is given in table 6 and 7.

Table 6. Effect of various incubation periods at four different centrifuge speeds on band forming pure protoplast of mesophyll and callus cells (Coc-671)

Band forming protoplast (%)								
Incubation (hr)	500rpm Mesophyll	Callus	600rpm Mesophyll	Callus	700rpm Mesophyll	Callus	1000rpm Mesophyll	Callus
2	a	a	51	68	30	42	4	10
4	a	a/b	18	31	12	18	a/b	a/b
6	a/b	a/b	b	a/b	a/b	b	b	b
8	a/b	a/b	b	a/b	b	a/b	a/b	a/b
10	a/b	a/b	a	b	a	a	a/b	a/b

*a - no pure protoplast band achieved
b - diffused band contaminated with debris /intact cells
a/b – mixture of pure and contaminated protoplast

Table 7. Effect of various incubation periods at for different centrifuge speeds on band forming pure protoplast of mesophyll and callus cells (CoN-05071)

Band forming protoplast								
Incubation (hr)	500rpm Mesophyll	Callus	600rpm Mesophyll	Callus	700rpm Mesophyll	Callus	1000rpm Mesophyll	Callus
2	a/b	ab	32	47	21	28	2	6
4	a	a/b	18	31	12	18	a/b	a/b
6	ab	a/b	b	a/b	a/b	b	B	b
8	a/b	a/b	b	a/b	B	a/b	a/b	a/b
10	a/b	a/b	a	b	a	a	a/b	a/b

*a - no pure protoplast band achieved
b - diffused band contaminated with debris /intact cells
a/b – mixture of pure and contaminated protoplas

Maximum protoplast yield was observed at 600 rpm with 2 hr incubation period followed by 700 rpm. No pure protoplast band formation was observed at 500 rpm and less protoplast band formation was observed at 1000 rpm. As incubation period of enzymatic solution increased from 2-10 hr, diffused and contaminated band formation was observed.

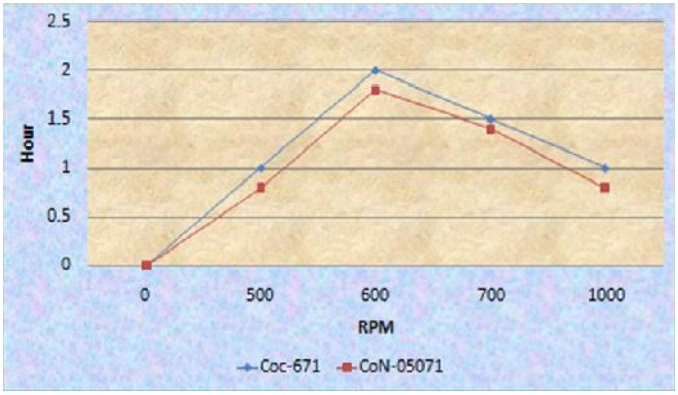

Fig. 1. Correlation of a incubation hours with different RPM levels of both cultivar CoC 671 and CoN 05071

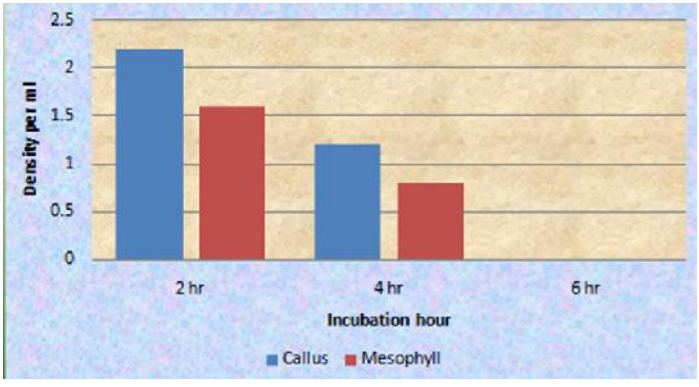

Fig. 2. Effect of incubation period on density in cultivar CoN 05071

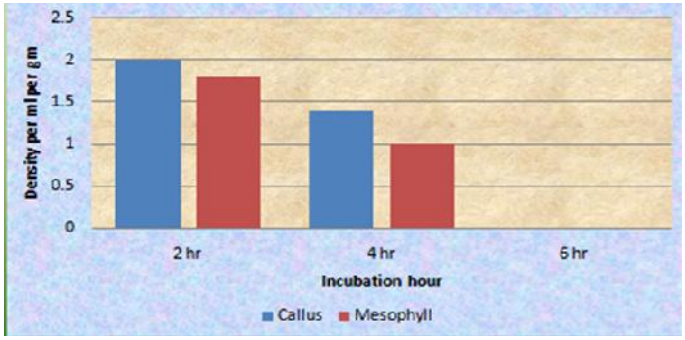

Fig. 3. Effect of incubation period on density in cultivar CoC 671

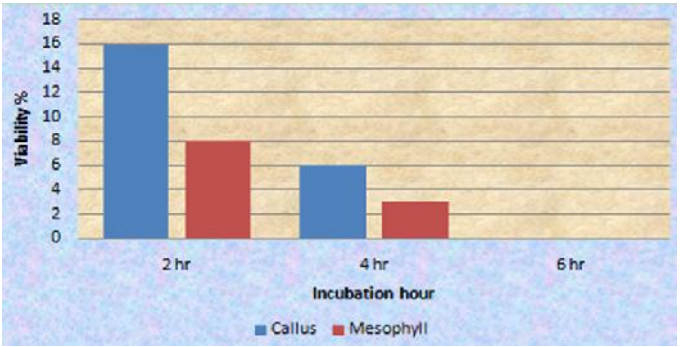

Fig. 4. Effect of incubation period on viability in cultivar Con 05071

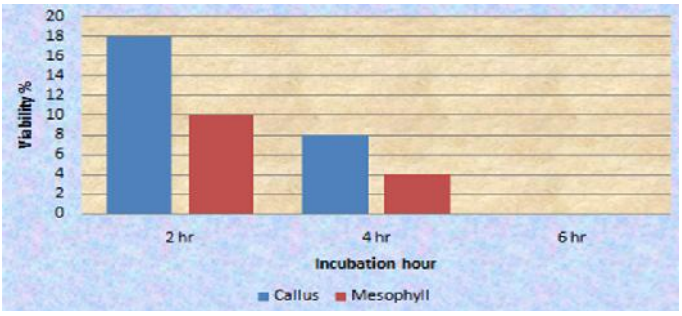

Fig. 5. Effect of incubation period on viability in cultivar CoC 671

Highest protoplast yield was observed at 600 rpm in callus tissue (68%) and mesophyll tissue (51%) at 2 hr incubation period. Lowest protoplast yield was observed at 1000 rpm in both mesophyll (4%) and callus tissue (10%) in cultivar CoC-671. In cultivar CoN-05071 maximum protoplast yield was observed at 600 rpm with 2 hr incubation period followed by 700 rpm. Diffused protoplast band formation was observed at 500 rpm and less protoplast band formation was observed at 1000 rpm. Cultivar CoN-05071 also responded in similar fashion of CoC-671. As incubation period increases to different levels diffused and contaminated band formation was observed in the (Table 6, Table 7 and Figure 1). From the figure 1, it is clearly seen that highest protoplast yield was observed at 600 rpm at 2 hr incubation period [callus tissue (47%), mesophyll (32%)]. Lowest protoplast yield was observed at 1000 rpm in both mesophyll and callus tissue.

Effect of explant material on density and viability of isolated protoplast

The effect of explant material on yield and density and viability of protoplast with 2% cellulase and 0.5% macerozyme in 0.5 µmmanitol buffer solutions are shown in (Table 8 and Table 9) and fig. 6,Fig. 7, Fig. 8 and Fig. 9 of both the varieties. The results showed that the protoplast digested from callus tissue exhausted higher density 2.2×104, 2.0×104 as compared to mesophyll cells of inner two leaf whorls (1.8×104, 1.6×104) in both the varieties at 2 hrincubation period.

Table 8. Effect of explant yield/density of isolated protoplast both cultivar CoC-671and CoN-05071

S. No.	Treatment	Density 104
1	G1E1	1.8
2	G1E2	2.0
3	G2E1	1.6
4	G2E2	2.2
S.Em±		0.0535
CD 0.05%		0.164
C.V. %		2.82

Table 9. Effect of explant viability of isolated protoplast both cultivar CoC-671and CoN-05071

S.No.	Treatment	Viability (%)
1	G1E1	10
2	G1E2	18
3	G2E1	8
4	G2E2	16
S.Em±		0.5
CD 0.05%		1.54
C.V. %		3.84

Where,
G1= CoC-671
G2=CoN-05071
E1=mesophyll cell
E2=Callus tissue

The protoplast viability was significantly higher (P<0.05) in both the varieties when compared with plant material used (callus and mesophyll). Protoplast density is in association with protoplast viability. There was positive correlation between protoplast density and viability as protoplast density increases there is an increase in viability per cent. (Standard no. of protoplast per treatment per ml to be considered 2.0×105) Fig. 6, Fig. 7 shows protoplast from callus tissue origin had higher protoplast density (2.0×104) in variety Coc-671.

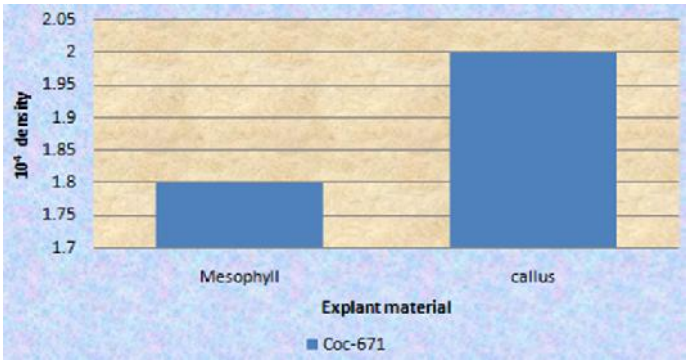

Fig. 6. Effect of explant on density of isolated protoplast variety CoC 671

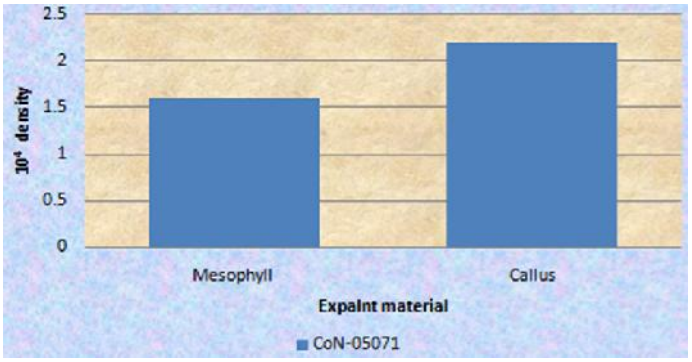

Fig. 7. Effect of explant on density of isolated protoplast CoN 05071

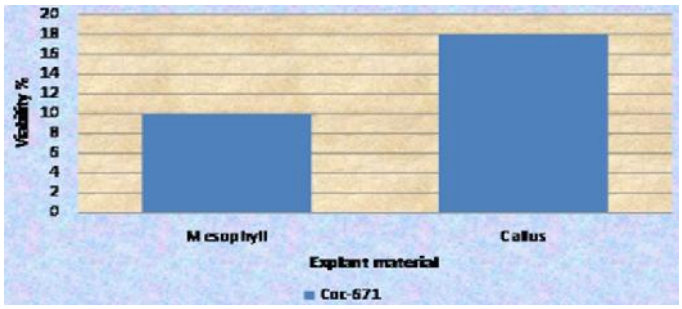

Fig. 8. Effect of explant on viability isolated protoplast variety CoC 671

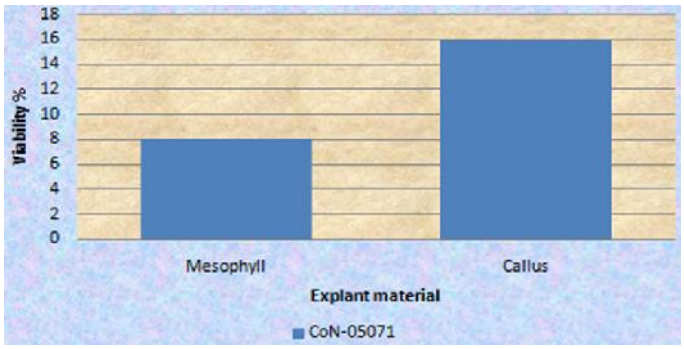

Fig. 9. Effect of explant on viability isolated protoplast variety on 05071

In cultivar CoN-05071, higher protoplast density was observed on callus tissue origin (2.2×104). Effect of density on viability shown in fig. 8 and Fig. 9 i.e. 18 per cent from callus tissue at 2.0×104 density of protoplast in both cultivar CoC-671,CoN-5071 Fig. 6 and Fig. 7 shows that higher protoplast viability was observed at density level 2.2 of callus tissue origin inboth cultivar CoC-671, CoN-05071. (Table 8 and Table 9)

Conclusion

In this experiment, successful isolation of protoplast from mesophyll cells of inner two leaf whorls and callus suspension cultures of sugarcane cultivars CoC-671 and CoN-05071 was obtained. Isolation as a crucial step of protoplast utilization was extensively optimized, taking into consideration various factors that could affect protoplast isolation. The optimal enzyme buffer solution for releasing good number of viable protoplasts from the leaves of sugarcane. Cultivars were standardized at 1 per cent cellulase, 0.5 per cent macerozyme, 1.0 per cent pectolyase and 0.5 M manitol. Maximum protoplast yield was observed at 600 rpm in callus suspension cells (68%) at 2 hrs of incubation period. Maximum density of protoplast was observed in callus suspension derived protoplast culture (2.0×104, 2.2×104) in both the varieties at 2 hrs of incubation periods. Highest protoplast viability observed at 2.2 density level of callus suspension origin protoplast. Highest protoplast density and viability was observed at 2 hrs of incubation of enzyme solution in both the varieties. In this study 2 hrs of incubation is optimal time for protoplast isolation from mesophyll cells and callus suspension cells of sugarcane cultivars.

REFERENCES

Aftab, F. and Iqbal, J. 2001. PEG-Mediated somatic hybridization studies in sugarcane (*Saccharum*spp.hybrid CVS.COL-54 and CP-43/33). *Pak.j.Bot*., 3: 233-238.

Chamani, E., Tahami, S. K., Zare, N., Asghari-Zakaria, R., Mohebodini, M. and Joyce, D. 2012. Effect of different cellulase and pectinase enzyme treatments on protoplast isolation and viability in LiliumledebeouriiBioss. NotulaeBotanicaeHortiAgrobotanici Cluj-Napoca.,40: 123-128.

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.1: 632-639.

Dale, P.J. 1983. Protoplast culture and plant regeneration of cereals and other recalcitrant cropsExper. 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. Stu. HSPA, pp. 64-65.

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

Maretzki, A, and L.G. Nickel 1973. Formation of protoplasts from sugarcane cell suspension and the regeneration of cell culture from protoplasts. Co/log. Intern. Centre Natte. Rech. Sci. Paris, 212: 51-63.

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

Tabaeizadeh, Z., R.J. Feri and UC Vasil. 1986. Somatic hybridization in the Gramineae. *Saccharumqfficinarum* L + *Permisetumamericamm* (L) K_ Schum (Pearl millet). Proc. Nat. Acad. Sci., USA, 83: 5616-5619

Yen, Q., X. Zhang and Z. Chen. 1985. Organogenesis from sugarcane protoplast Kexue Tonbbao, 30; 1392-1395.

Yoo, S. D., Cho, Y. H. and Sheen, J. 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocol*., 2: 1565-1572.

****