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SOMATIC EMBRYOGENESIS IN PROTOPLAST CULTURES DERIVED FROM MESOPHYLL AND EMBRYOGENIC CALLUS OF SUGARCANE (*SACCHARUM* SPP. *HYBRID* CV.COL-54)

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Abstract

Protoplasts were isolated both from leaf mesophyll tissue as well as from compact globular white embryogenic callus of mesophyll origin in sugarcane (*Saccharum* spp. hybrid cv. COL 54). Totpotent protoplasts with an yield of 1×10^6 and 1×10^5 ml^{-1} with 72 and 68% viability were obtained respectively from mesophyll and embryogenic callus cultures. A heterogeneous population of protoplasts (30-60μm ϕ) was obtained from both sources. Protoplasts isolated from compact globular white embryogenic callus were less heterogeneous (30-40μm ϕ) and cytoplasmically dense. Of the several protoplast culture methods tried, the best results were achieved using protoplast embedding in agarose-solidified medium. Medium P9 proved good for somatic embryogenesis from mesophyll-derived protoplasts in which further differentiation of somatic embryos was not possible. Protocallus formation from protoplasts isolated from embryogenic callus cultures was achieved in dark using agarose-solidified KPR medium in 16 weeks. Shoot formation was achieved from such protocalluses by further transferring to MS basal medium containing 9μmol (2 mgl^{-1}) 2,4-D for 8 weeks under 16h photoperiod condition. Transfer of such proliferated callus masses to MS basal medium containing 9.29μmol (2 mgl^{-1}) Kinetin + 5.37 μmol (1.0 mgl^{-1}) NAA and 200 mgl^{-1} activated charcoal for 10 weeks resulted in shoot formation.

Introduction

There have been extensive efforts for sugarcane crop improvement using cell and tissue culture during the past few decades (Nickell, 1961; Heinz & Mee, 1969; Nadar & Heinz, 1977; Ho & Vasil, 1983a,b; Nagai *et al.*, 1991). Sugarcane protoplasts has become an established tool for the genetic improvement (Chen *et al.*, 1987; Franks & Birch, 1991; Bower & Birch, 1992; Chowdhury & Vasil, 1992; Rathus & Birch, 1992a,b; 1993).

For plantlet regeneration from protoplasts, an important prerequisite, has been the establishment and development of a homogeneous (preferably embryogenic) cell suspension culture (Srinivasan & Vasil, 1986; Chen *et al.*, 1988; Taylor *et al.*, 1992a; Liu, 1994; Aftab *et al.*, 1996). There is a general belief about the non-totipotent nature of mesophyll-derived protoplasts in the members of Poaceae (Vasil & Vasil, 1992). There does not appear to be any report on the isolation of totipotent protoplasts from callus cultures and their subsequent development into organized structures such as shoot formation. The present report describes the somatic embryogenesis from mesophyll-derived protoplasts and shoot regeneration from embryogenic callus culture-derived protoplasts in sugarcane (*Saccharum* spp. *hybrid* cv. CoL-54).

Materials and Methods

Establishment of Embryogenic Callus Cultures: Embryogenic calluses were initiated from inner two whorls of young leaves of sugarcane (*Saccharum* spp. hybrid cv. CoL-54) as described earlier (Aftab *et al.*, 1996).

Protoplast Isolation and Culture: Protoplasts were isolated both from leaf mesophyll as well as embryogenic callus. For leaf mesophyll protoplast, inner two to three whorls were used as donor material. The leaves were cut into $1mm^2$ pieces in 60-90mm ϕ Petri plates. For embryogenic callus protoplasts, one mm^2 fragments of 1g callus piece from compact globular white embryogenic callus cultures were taken. The enzyme solution for protoplast isolation consisted of 2% cellulase R-10, 0.5% macerozyme R-10 (both enzymes from Yakult Honsha Co. Ltd. Japan), 0.1% pectolyase Y-23 (Sigma), 2% PVP (polyvinyl pyrrolidone; Sigma) and antibiotics (ampicillin 40 mgl^{-1};tetracycline 10 mgl^{-1} and gentamycin 10 mgl^{-1}; all antibiotics of Sigma grade) dissolved in CPW medium (Frearson *et al.*, 1973) containing 13% mannitol and $CaCl_2.2H_2O$ (5mM) at pH 5.8. Rest of the procedure for embryogenic protoplast isolation from the two sources was the same as reported earlier for cell suspension cultures of sugarcane (Aftab *et al.*, 1996). Previously reported culture media and growth conditions were also followed for protoplast as well as protoplast-derived calluses and regeneration studies (Aftab *et al.*, 1996).

Results

Protoplast Isolation: For mesophyll protoplasts, the best results were achieved using inner two to three whorls of young leaves. Amongst several callus types produced in this study, only compact globular white embryogenic callus of mesophyll origin proved to be a good source material for protoplasts (Fig.1). Protoplasts obtained at 8-10 days of fresh subculturing of these calluses were best for regeneration; though protoplasts up to 4 months could be used for regeneration. After 4 months callus lost its potential for high yield of totipotent protoplasts. Pre-plasmolysis of leaf and embryogenic callus cultures (ECC) in 10-15% mannitol solution for 30-60 minutes failed to improve the yield of protoplasts (data not shown). Therefore, pre-plasmolysis incubation was omitted. Protoplast yield of $1x10^6$ and $1x10^5$ ml^{-1} with 72 and 68% viability was obtained for mesophyll and ECC, respectively. The use of PVP (2%) in protoplast isolation medium was critical for the isolation of totipotent protoplasts from the two sources. Although PVP did not improve protoplast yield or viability, lack of PVP in protoplast isolation medium resulted in excessive browning during protoplast isolation and hence a complete experimental failure. Such a protoplast culture system could not be developed further. Lower concentrations of PVP (less than 2%) did not control browning during protoplast isolation. Higher concentrations were not tried since 2% PVP was found quite effective for the control of excessive browning during protoplast isolation from the two sources. The role of PVP seems to be of a strong antioxidant in the present investigation. Apart from this, any other role PVP might have played during this study needs examination.

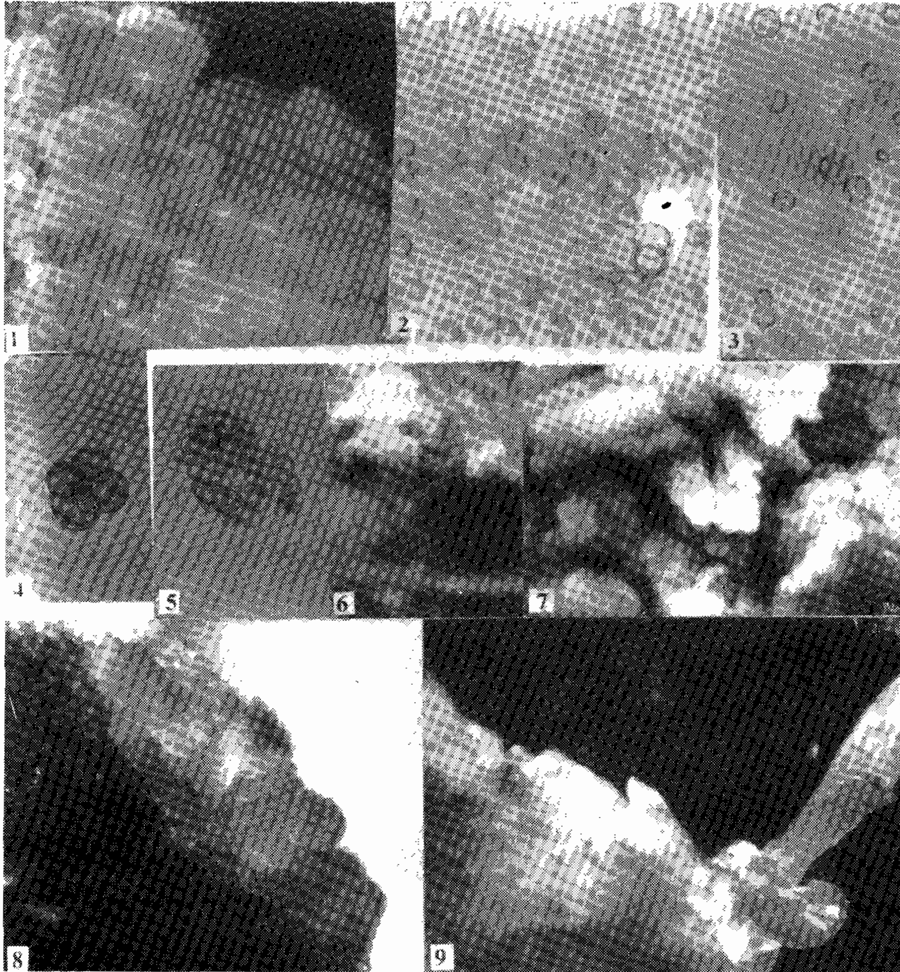

Fig.1. Compact globular white embryogenic callus on MS2 medium after 8 weeks of culture in dark (X40).

Fig.2. Crude mesophyll preparation in CPW13M (X250).

Fig.3. Purification of mesophyll protoplasts (from Fig.2; X250).

Fig.4. Cell division initiation in mesophyll protoplasts in P9 medium after 4 days of culture in dark (XZ50).

Fig.5. Mesophyll protoplast-derived microcolonies after 20 days of culture in P9 medium (X125).

Fig.6. Mesophyll protoplast-derived cell colonies plated in P9 medium under 16 h photoperiod showing sign of somatic embryo formation (X4).

Fig.7. Stereomicrograph representing multicellular aggregates having proembryonic structures formed on the surface of the mesophyll-derived protocallus after 16 weeks of culture in P9 medium under 16 h photoperiod (X40).

Fig.8. ECC-derived protocallus on KPR medium showing sign of somatic embryogenesis after 8 weeks of growth on MS medium supplemented with 2,4-D (2 mgl^{-1}) under 16 h photoperiod (X25).

Fig.9. Stereomicrograph showing germination of somatic embryo leading to shoot regeneration under 16 h photoperiod (X25).

Table 1. Effect of various levels of sucrose concentration (0.1-1.0M) at three different centrifuge speeds on band forming pure protoplasts of mesophyll and ECC.

Sucrose (M)	Band forming protoplasts (%)					
	500 rpm		700 rpm		1000 rpm	
	M*	ECC**	M	ECC	M	ECC
0.1-0.4	a	a	a	a	a	a
0.5	a/b	a/b	32.97	34.53	26.37	33.68
0.6***	a/b	a/b	65	68.09	52.18	54.47
0.7****	a	a/b	a	75	a	61.18
0.8-1.0	a	a	a	a	a	a

*:Mesophyll protoplasts

**:Embryogenic callus culture derived protoplasts.

***:Critical sucrose concentration for purification of mesophyll protoplasts.

****:Critical sucrose concentration for the purification of ECC** protoplasts.

a:No pure/sharp protoplast band achieved.

b:A diffused band contaminated with debris/intact cells

Table 2. Effect of different media (KM8P, KPR and P9) and 2,4-D levels on mesophyll protoplast culture and somatic embryogenesis in *Saccharum* spp., hybrid cv. CoL-54.

Medium	2,4-D (mgl^{-1})	Division efficiency (%)		Plating efficiency (%)		Protocallus formation		Embryo/ 100 proto-calluses[a]	
		M	ECC	M	ECC	M	ECC	M	ECC
KM8P[b]	0.2 2,4-D	19 (± 0.63)	21.1 (± 0.98)	0.20 (± 0.01)	0.25 (± 0.02)	Yes	Yes	0	0
KPR[c]	0.5	21 (± 0.95)	22.9 (± 0.74)	0.60 (± 0.03)	0.66 (± 0.04)	Yes	Yes	0	0
P9[d]	3.0	18 (± 0.63)	20.1 (± 0.66)	1.17 (± 0.01)	0.90 (± 0.03)	Yes	Yes	1.01 (± 0.95)	0

*:Division and plating efficiencies were measured after 20 and 30 days of culture respectively. Protocalluses were scored for somatic embryogenesis after 60 days of culture in agarose-solidified medium. Data comprises means (±S.E.) from five independent experiments.

a) Observations for somatic embryogenesis at early protocallus stage, 60 days after culture.

b) Modified protoplast culture medium 8P of Kao & Michayluk (1975).

c) Modified protoplast culture medium based on KPR medium (Kao, 1977).

d) P9 medium (Chen & Shih, 1983).

For protoplast purification, the effect of sucrose concentrations vis-a-vis protoplast band formation for mesophyll and ECC at three different rpm using Sorvall (R) RC-5B Refrigerated Superspeed Centrifuge (Sorvall Instruments, SS-34 Rotor) is given in Table 1, Figs.2 & 3. As evident from the table that for mesophyll, maximum protoplasts, i.e., 65% of the total yield, formed a sharp band using 0.6M sucrose concentration at 700 rpm, while ECC-derived protoplasts formed a sharp band at 700 rpm using 0.7M sucrose concentration.

A heterogeneous population was obtained from mesophyll tissue. The heterogeneity was due to differences in cell size and shape of cells comprising mesophyll tissue. The larger protoplasts were highly vacuolated. The smaller ones, were cytoplasmically rich, containing several starch grains and chloroplasts. Thus the colour of mesophyll protoplasts was green. The diameter of mesophyll protoplasts ranged from 30-60μm (Fig.3). As compared to mesophyll, the protoplast preparation from compact globular white embryogenic callus culture (ECC) was less heterogeneous with protoplast diameter ranging from 30-40μm. The protoplasts were cytoplasmically dense.

Protoplast Culture: The effect of 2,4-D supplementation to KM8P, KPR and P9 media on mesophyll and ECC protoplast growth are given in Table 2. In KM8P medium having 0.2 mgl^{-1} 2,4-D, 19% division efficiency was obtained. Of the cultured protoplasts, only 0.20% formed microcolonies capable of further growth. The protocallus thus achieved was of smooth and shiny type without any morphogenic potential. It is interesting to note that no other growth regulator except 2,4-D supplementation in KM8P, KPR and P9 media induced division in cultured protoplasts. Moreover, the protoplast growth response was achieved only at given concentrations of 2,4-D (KM8P, 0.2; KPR, 0.5 and P9, 3.0 mgl^{-1}). A minor change in above mentioned concentration in each case resulted in complete suppression of protoplast division. In KM8P medium, division efficiency was 21% and plating efficiency 0.60%. In P9 medium 18% division of cultured mesophyll protoplasts along with 1.17% of plating efficiency was observed. Somatic embryos were produced @ 1.01% of the protocalluses. For ECC-derived protoplasts, KM8P medium showed 21.1% division efficiency and 0.25% plating efficiency. Protocallus formation was also achieved. KPR medium supported protoplast division up to 22.9% accompanied by plating efficiency of 0.66%. Protocallus formation also took place in this medium. The use of P9 medium resulted in 20.1% division efficiency and 0.9% microcolonies. These microcolonies later on transformed into protocallus after 45 days.

Cultured mesophyll protoplasts in P9 medium showed vigorous growth of microcolonies within the same Petri plates (Figs.4 & 5). These rapidly proliferating colonies were picked after 8-10 weeks and transferred to fresh medium under 16h photoperiod. After about 8 weeks of further growth, distinct globular embryoids developed (Fig.6 & 7). Efforts to improve embryogenesis in protoplast cultures failed and only a low proportion (up to 1.01%) of microcolonies formed embryoids. Embryoids produced in P9 medium could not be regenerated to plantlets in any of the employed protoplast regeneration media.

The protoplasts isolated from leaf-derived ECC, 4 months old line formed protocallus in agarose solidified KPR medium in 16 weeks. Protocallus was further proliferated in MS medium having 2,4-D (2 mgl^{-1}) for 2 months at 16h photoperiod.

Transferring these embryogenic calluses on MS + Kinetin (2 mgl^{-1}) + NAA (1.0 mgl^{-1})+ activated charcoal (200 mgl^{-1}) for 10 weeks favoured shoot formation (Fig.8 & 9). A maximum of 45% regeneration frequency was obtained with an average number of 2 plants per protocallus on regeneration medium. By employing the reported protocols vigorous rooting was not achieved which is a pre-requisite to raise plantlets to maturity.

Discussion

Attempts to culture mesophyll protoplasts in sugarcane have largely been unsuccessful. In this regard, the success in obtaining embryogenesis from leaf mesophyll-derived protoplasts as observed in the present study is a step forward. A similar report in rice has been made by Gupta & Pattanayak (1993), which were also in contrast to the results of earlier studies by various groups utilizing mesophyll protoplasts. Though plant regeneration could not be achieved from sugarcane mesophyll protoplasts in the present study, but looking at the results from this investigation and those from Gupta & Pattanayak (1993), it may be concluded that mesophyll protoplasts are totipotent and are fully capable of undergoing sustainable divisions and hopefully plant regeneration.

It is difficult to manipulate protoplast cultures of the members of Poaceae, workers have emphasized the need for use of embryogenic cell suspension cultures as an appropriate material to yield totipotent protoplasts capable of plant regeneration (Taylor *et al.*, 1992a,b; Liu, 1994). Of the few earlier studies utilizing callus cultures as protoplast source, only protocallus could be achieved (Koblitz, 1976). Though a callus culture having embryogenic potential has routinely been used in the establishment of cell suspensions (Mordhorst & Lorz, 1992; Yin *et al.*, 1993; Golds *et al.*, 1994; Yang *et al.*, 1994), protoplast isolation directly from embryogenic callus cultures is not a common practice. It is inferred from the present investigation that plant regeneration from protoplasts may be obtained from any available source provided the source material is so manipulated that it does not lose its cellular totipotency as well as an optimum level of growth during the course of subculturing. In the present study, it was primarily due to this reason that shoot regeneration from compact white globular embryogenic callus-derived protoplasts was achieved.

The presence of 2,4-D @ 0.2, 0.5 and 3.0 mgl^{-1} in agarose solidified KM8P, KPR and P9 media respectively, was found to be very important for sustainable protoplast division. The presence of 2,4-D has also been reported by a number of workers to be of prime importance in sugarcane as well as other protoplast culture system for the members of Gramineae (Srinivasan & Vasil, 1986; Yang *et al.*, 1994; Wang & Lörz, 1994; Golds *et al.*, 1994). The effective role of other hormones/growth regulators only seems significant at later stages of protoplast-derived callus development (protocallus stage). For the early protoplast divisions, presence of 2,4-D as sole growth regulator seems quite effective (Vasil & Vasil, 1984).

Initial incubation of protoplasts in dark was observed to promote early wall formation and division leading to microcolony formation. It was only after the formation of microcalluses that these cultures were transferred to 16h photoperiod. It is interesting to note that KPR or KM8P media as used in this study have been reported to become phytotoxic to protoplasts under light conditions (Kao & Wetter, 1977).

Abbreviations: MS- salts of Murashige & Skoog (1962) basal medium, 2,4-D- 2,4-dichlorophenoxyacetic acid, CH- casein hydrolysate, KM8P- protoplast culture medium of Kao & Michayluk (1975), KPR-protoplast culture medium of Kao (1977), P9- protoplast culture medium (Chen & Shih, 1983), BA-Benzyladenine, Picloram-4-amino-3,5,6-trichloropicolinic acid, NAA- naphthalene acetic acid.

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