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Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (*Saccharum* spp. hybrids)

Paul W.J. Taylor[1], Hian-Lien Ko[1,2], Stephen W. Adkins[3], Carl Rathus[1,4] & Robert G. Birch[5]
[1]*Bureau of Sugar Experiment Stations, PO Box 86, Indooroopilly, Queensland, Australia 4068*; [2]*present address: Redlands Research Station Horticulture Centre, PO Box 327, Cleveland, Queensland, Australia 4163*; [3]*Department of Agriculture, The University of Queensland, Australia 4072*; [4]*present address: CSIRO, Division of Tropical Crops and Pastures, 306 Carmody Road, St Lucia, Queensland, Australia 4067*; [5]*Department of Botany, The University of Queensland, Australia 4072*

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Abstract

For 18 sugarcane cultivars, four distinct callus types developed on leaf explant tissue cultured on modified MS medium, but only Type 3 (embryogenic) and Type 4 (organogenic) were capable of plant regeneration. Cell suspension cultures were initiated from embryogenic callus incubated in a liquid medium. In stage one the callus adapted to the liquid medium. In stage two a heterogeneous cell suspension culture formed in 14 cultivars after five to eight weeks of culture. In stage three a homogeneous cell suspension culture was developed in six cultivars after 10 to 14 weeks by selective subculturing to increase the proportion of actively dividing cells from the heterogeneous cell suspension culture. Plants were regenerated from cell aggregates in heterogeneous cell suspension cultures for up to 148 days of culture but plants could not be regenerated from homogeneous cell suspension cultures. High yields of protoplasts were obtained from homogeneous cell suspension cultures (3.4 to 5.2×10^6 protoplasts per gram fresh weight of cells [$gfwt^{-1}$]) compared to heterogeneous cell suspension cultures (0.1×10^6 protoplasts $gfwt^{-1}$). Higher yields of protoplasts were obtained from homogeneous cell suspension cultures for cultivars Q63 and Q96 after regenerating callus from the cell suspension cultures, then recycling this callus to liquid medium (S-cell suspension cultures). This process increased protoplast yield to 9.4×10^6 protoplasts $gfwt^{-1}$. Protoplasts isolated from S-cell suspension cultures were regenerated to callus and recycled to produce SP-cell suspension cultures yielding 6.4 to 13.2×10^6 protoplasts $gfwt^{-1}$. This recycling of callus to produce S-cell suspension cultures allowed protoplasts to be isolated for the first time from cell lines of cultivars Q110 and Q138.

Introduction

Most direct gene transfer techniques for crop improvement depend on the regeneration of transformed plants from protoplasts. For the cereals there have been few successes; plants have been regenerated from protoplasts isolated from cell suspension cultures of pearl millet (Vasil & Vasil 1980), barley (Lührs & Lörz 1988), rice (Abdullah et al. 1986; Yamada et al. 1986; Ogura et al. 1987; Lee et al. 1989), maize (Rhodes et al. 1988) and wheat (Vasil et al. 1990; Wang et al. 1990). Embryogenic cell suspensions are an important source of totipotent protoplasts capable of cell division and plant regeneration in Gramineae (Vasil & Vasil 1984).

In sugarcane, Srinivasan & Vasil (1986) regenerated five green plants from protoplasts of the

cultivar B4362 derived from two- to six-month-old embryogenic cell suspension cultures and Chen et al. (1988b) regenerated plants from protoplasts derived from embryogenic cell suspension cultures of the cultivar F164. However, both groups have been unable to repeat their earlier successes (I. Vasil, pers. comm., W. Chen, pers. comm.).

Large numbers of protoplasts are required to regenerate genetically transformed plants from protoplasts because of the low plating efficiencies associated with the culture of protoplasts (Thompson et al. 1986) and reduced viability of protoplasts during gene transfer procedures (Fromm et al. 1985).

The aim of this research is to develop reliable techniques for gene transfer into sugarcane for cultivar improvement. Published techniques for embryogenic callus culture, cell suspension culture, protoplast culture and plant regeneration in sugarcane have been developed and tested using only a few cultivars. We therefore undertook to evaluate and adapt techniques for application to a range of commercial sugarcane cultivars. This paper describes the establishment of sugarcane embryogenic callus cultures, regenerable cell suspension cultures, and cell suspension cultures yielding high numbers of protoplasts.

Materials and methods

Callus cultures

Callus cultures were established for 18 sugarcane (*Saccharum* spp. hybrid) cultivars (Table 1) by culturing 3 mm segments of the innermost four to five young furled leaves, aseptically taken from 1 to 50 mm above the apical meristem. The segments were cultured on solid (0.8% agar; Agar Agar 750, Langdon Co., Sydney, Aust.) Murashige & Skoog's medium (MS) as modified by Ho & Vasil (1983a) with the addition of 50 mg l^{-1} arginine, 3 mg l^{-1} 2,4-D and 50 ml l^{-1} coconut water. Cultures were incubated in the dark at 27°C (Ho & Vasil 1983a) and subcultured at three-week intervals by selectively transferring embryogenic callus onto the same medium.

For scanning electron microscope studies, callus pieces were fixed for 18 h at 4°C in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.8, then fixed in 1% osmium tetroxide, dehydrated through a graded ethanol series, critical point dried and sputter coated with gold. Observations and photographs were made with a Philips 505 scanning electron microscope.

Leaf explants of cultivars B4362, Q63, Q96 and Q117 were cultured in 12-well plates (Lin-

Table 1. Establishment of embryogenic callus and cell suspension cultures for a range of sugarcane cultivars.

Cultivar	Callus cultures from leaf explant	Heterogeneous cell suspension cultures	Homogeneous cell suspension cultures
Q44	+[a]	+	−
Q63	+	+	+
Q87	+	−	−
Q90	+	+	+
Q95	+	−	−
Q96	+	+	+
Q110	+	+	+
Q117	+	+	+
Q137	+	+	−
Q138	+	+	+
B4362	+	+	−
B629	+	+	−
CP44-101	+	−	−
F164	+	+	−
MQ72-2011	+	+	−
MQ72-4143	+	+	−
MQ72-4157	+	+	−
NCo310	+	−	−

[a] + successful establishment −not successful

bro, USA) on MS callus medium as modified by Ho & Vasil (1983a) containing 0.5, 1, 1.5, 2, 3 and 5 mg l^{-1} 2,4-D to determine the optimum concentration of 2,4-D for embryogenic callus initiation. There were three replicates of each treatment and the experiment was repeated three times. The percentage of embryogenic callus formation was determined after 28 days incubation by rating the proportion of embryogenic callus to the total callus volume.

Cell suspension cultures

Cell suspension cultures were initiated for cultivars (Table 1) by incubating 0.5 g (fresh weight-fwt) of embryogenic callus in 50 ml flasks containing 10 ml of liquid MS medium as modified by Ho & Vasil (1983b) supplemented with 50 ml l^{-1} coconut water, 500 mg l^{-1} casein hydrolysate and 3 mg l^{-1} of the plant growth regulators 2,4-D, picloram or dicamba. Cultures were incubated on an orbital shaker at 120 rpm in darkness at 27°C (Ho & Vasil 1983b).

Cell suspension cultures were developed in three stages (Table 2). First, the medium was replaced every three to four days to minimise the accumulation of phenolic compounds produced by the callus. Second, the callus and cells were transferred to 100 ml flasks and made up to 20 ml volume with fresh medium; 10 ml of medium was replaced every three to four days. Five to eight weeks after transfer of callus to liquid medium, a heterogeneous cell suspension culture developed and was maintained by dividing the culture equally into two flasks and adding fresh medium to 35 ml volume every seven days.

Third stage suspension cultures were established by methods similar to those of Ho & Vasil (1983b). After the second stage cell suspension cultures were manually shaken for 5 sec, larger callus clumps settled to the bottom of the flask, vacuolated cells and cellular debris remained in the upper portion and the middle portion of the suspension culture consisted primarily of small callus aggregates and densely cytoplasmic and actively dividing cell groups. A 15 ml subculture was taken with a wide-bore pipette from the middle of the cell suspension and added to 20 ml of fresh culture medium. The cell cultures were enriched for actively dividing cells by changing the subculture protocol according to cell culture density. Initially, a 20 to 25 ml subculture was taken at seven-day intervals, but as the cell cultures became more dense and homogeneous, the subculture volume and frequency were reduced to 10 ml at three- to four-day intervals. Homogeneous cell suspension cultures were established 10 to 14 weeks after the transfer of callus to the liquid medium and maintained by transferring 10 ml of culture into 25 ml of fresh medium each three to four days.

Callus regenerated from homogeneous cell suspension cultures was transferred to liquid medium to initiate a secondary homogeneous cell suspension culture, designated an S-cell suspension culture.

Table 2. The three developmental stages of cell suspension cultures for sugarcane.

Stage	I	II	III
Period (weeks)	2–3	3–8	6–14
State of culture	Embryogenic callus starts to grow: phenolics produced	Callus dissociates into small cell aggregates: formation of heterogeneous cell suspension cultures	Enrichment for actively dividing cells: formation of homogeneous cell suspension cultures
Subculture interval (days)	Medium replaced at three- to four-day intervals	7–14	3–7

Regeneration of plants from cell suspension cultures

Heterogeneous cultures

Heterogeneous cell suspension cultures were filtered sequentially through 500, 200 and 100 μm nylon filters, and 0.5 to 1 g (fwt) of callus and cells from each fraction was transferred onto solid MS medium (Ho & Vasil 1983a – 0.8% agar) supplemented with 50 ml l^{-1} coconut water, 2 mg l^{-1} 2,4-D, 500 mg l^{1} casein hydrolysate with and without 10 g l^{-1} activated charcoal (Fisher FA 50–200 mesh, USA) and incubated in darkness at 27°C for two weeks.

Pieces (1 to 2 g fwt) of the resulting callus were transferred to solid MS media with successively lower 2,4-D concentrations (0.75, 0.25 mg l^{-1}) with and without activated charcoal, with a two-week incubation between transfers. Finally, callus was transferred to medium without plant growth regulators and incubated under diffuse lights with a 12-h photoperiod at 27°C.

Homogeneous cultures

The morphogenic capacity of homogeneous cell suspension cultures was assessed by transferring 1 ml of a two- to five-day-old suspension culture onto solid medium and incubating as described above.

Protoplast isolation and culture

Protoplast isolation was attempted within one week of the establishment of heterogeneous homogeneous suspension cultures, and repeated at two-week intervals until protoplasts were isolated. For suspension cultures that did not yield protoplasts after 20 to 25 weeks, protoplast isolation was attempted at four- to six-week intervals.

Protoplasts were isolated from homogeneous suspension cultures three to four days after subculture and seven days after subculture for heterogeneous suspension cultures. Homogeneous and heterogeneous suspension cultures were filtered sequentially through 500 and 200 μm nylon filters. One gram of cells from the heterogeneous cell suspension cultures collected on the 500 and 200 μm filters, and 0.5 to 1 g from homogeneous cell suspension cultures collected on the 200 μm filter, were incubated in Petri dishes (9 cm diameter) with 10 ml of enzyme solution (Srinivasan & Vasil 1986) on an orbital shaker at 60 rpm in darkness at 27°C. After four to five hours incubation the mixture was filtered sequentially through 100, 50 and 25 μm nylon filters. Protoplasts were pelleted by centrifugation at 100 *g* for 5 min and washed three times in Kao & Michayluk (1975, K & M) medium as modified by Vasil & Vasil (1980) and Srinivasan & Vasil (1986). Protoplasts were resuspended in a defined volume of modified K & M culture medium, counted with a haemocytometer and stained with fluorescein diacetate (FDA; Wildholm 1972) to assess viability.

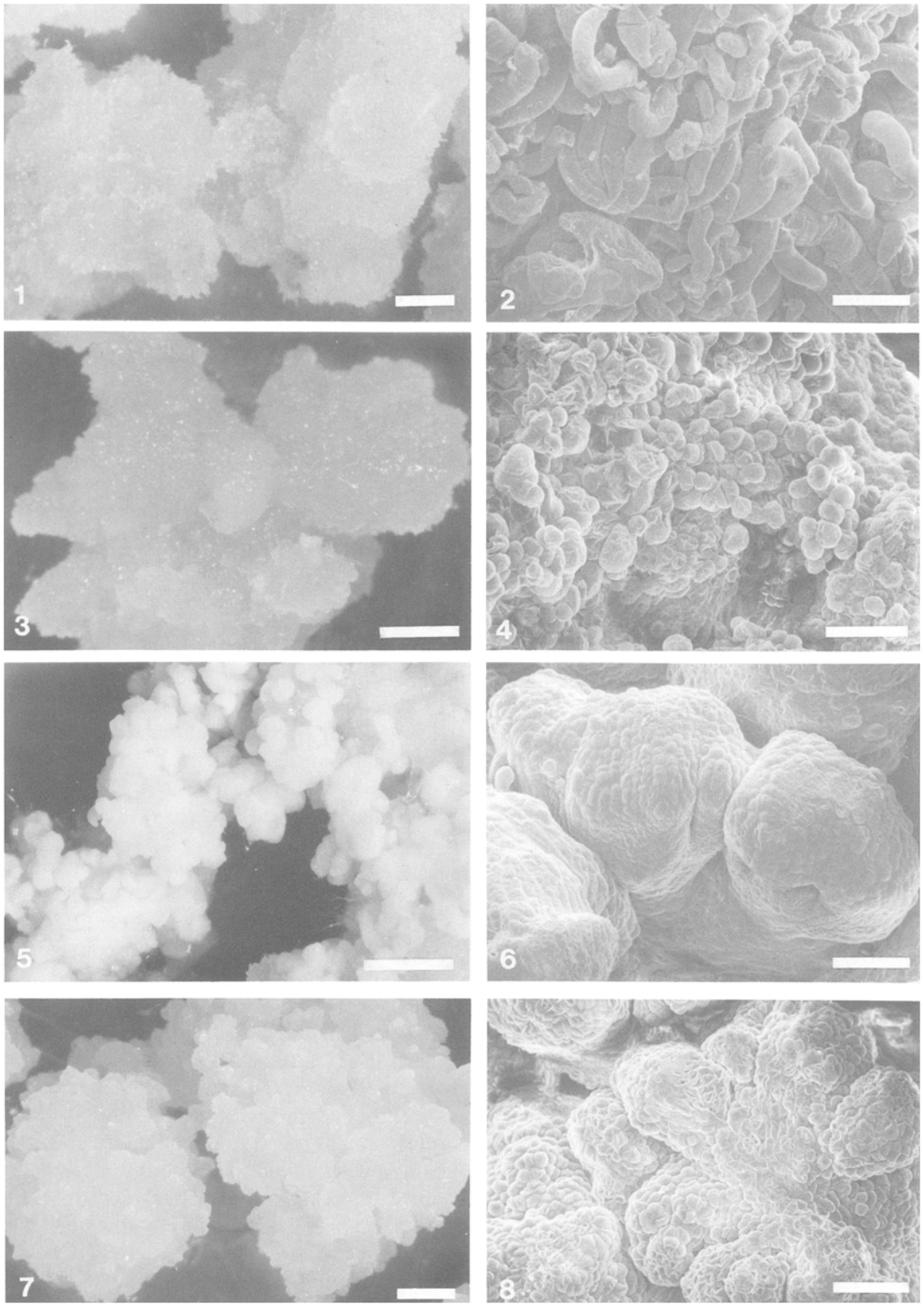
Protoplasts isolated from homogeneous and heterogeneous cell suspension cultures were cultured at a density of approximately 5×10^5 protoplasts ml^{-1} in agarose (0.6% Seaplaque FMC Co. USA) droplets, 0.05 to 0.1 ml volume, in 9 cm diameter Petri dishes bathed in 10 ml of modified K & M medium.

Callus regenerated from protoplasts isolated from homogeneous S-cell suspension cultures of Q63, Q96 and Q138 was transferred to liquid medium to initiate a third type of homogeneous cell suspension culture, designated as an SP-cell suspension culture.

Results and discussion

Establishment of embryogenic callus cultures

Four callus types developed on sugarcane leaf explant tissue. Type 1 callus consisted of semi-translucent callus containing loose, large, elongated cells and was first observed at the cut ends of the leaf explants after five days of culture (Figs 1, 2). Type 2 callus developed amongst the Type 1 callus after 13 days of culture, and was a soft, mucilaginous, grey-yellow callus consisting of round, vacuolated, highly dissociated cells (Figs 3, 4). Type 3 embryogenic callus developed on all 18 cultivars (Table 1) between 13 and 21 days and formed at the cut ends of the leaf explant around the vascular tissue. This callus was compact, hard, yellow with smooth surfaced globular structures that became white in later stages of development and consisted of small, round, densely cytoplasmic cells (Figs 5, 6). Type 4 callus also developed on all cultivars



Figs. 1–8. Sugarcane cultivar Q63 callus types. (*1 & 2*). Type 1 – semi-translucent callus containing large elongated cells. (*3 & 4*). Type 2 – mucilaginous callus consisting of round, dissociated cells. (*5 & 6*). Type 3 – embryogenic callus containing smooth-surfaced, globular embryo structures consisting of small, round, densely cytoplasmic cells. (*7 & 8*). Type 4 – friable callus consisting of semi-organised structures composed of small, round, densely cytoplasmic cells. (Scale bars: 2.0 mm for Figs 1, 3, 5 & 7 [reflected light micrographs]; and 0.1 mm for Figs 2, 4, 6 & 8 [SEM]).

between 13 and 21 days and was yellow, friable, with clusters of semi-organised structures consisting of small, round, densely cytoplasmic cells (Figs 7, 8). These clusters of semi-organised structures were unable to develop into the well-defined globular embryos which are characteristic of embryogenic callus. Types 1 and 2 callus were non-morphogenic, i.e. no plants were regenerated from these callus types after removal of 2,4-D from the solid medium. In contrast, after transfer onto 2,4-D free medium, plants regenerated from Type 3 embryogenic callus and to a lesser extent from Type 4 callus. Since Type 4 callus appeared not to form somatic embryos, plant regeneration from this callus must have occurred by organogenesis. Chen et al. (1988a) have previously observed plant regeneration from sugarcane callus by both somatic embryogenesis and organogenesis.

The degree of embryogenic callus formation varied depending on the cultivar and concentration of 2,4-D tested (Table 3). More embryogenic callus formed on explants of cultivar Q117 after 28 days at 2,4-D concentrations of 2 to 5 mg l^{-1} than for cultivars B4362, Q63 and Q96 at similar 2,4-D concentrations.

Callus cultures consisting primarily of embryogenic Type 3 callus were established after 70 to 84 days in culture by isolating and subculturing embryogenic callus. Embryogenic callus from all 18 cultivars was maintained on medium containing 3 mg l^{-1} 2,4-D for up to 26 weeks. The amount of embryogenic callus and the plant regeneration capability of this callus began to decrease after six months of maintenance for all cultivars. Similar results have been obtained by others; Chen et al. (1988a) were able to maintain

Table 3. Degree of embryogenic callus formation on leaf explant after 28 days incubation.

2,4-D mg l^{-1}	Cultivars			
	B4362	Q63	Q96	Q117
0.5	–[a]	–	–	–
1	+	+	–	+
1.5	++	+	–	++
2	++	+	+	+++
3	++	++	++	+++
5	++	++	++	+++

[a] – = 0, + = 5 to 15, ++ = 20 to 45, +++ = 50 to 75 embryogenic callus as a percentage of total callus volume.

embryogenic callus for 30 months after callus initiation by alternating subculture on a 2,4-D regime of 1 and 3 mg l^{-1} at monthly intervals. Similarly, Ho & Vasil (1983a) found the optimum concentration of 2,4-D for induction of embryogenic callus for sugarcane cultivar 68-1067 was 0.5 to 1.5 mg l^{-1}, and the optimum for maintenance was 2.5 to 3 mg l^{-1}. In contrast, Guiderdoni (1986) induced callus on leaf explants of Co6415 and Q75 on MS medium supplemented with 7 mg l^{-1} 2,4-D and after four weeks reduced the 2,4-D concentration to 1 mg l^{-1} for maintenance of callus. It appears from our results that for many sugarcane cultivars, culture on modified MS medium containing 3 mg l^{-1} of 2,4-D is simple and reliable for initiation and short-term maintenance of embryogenic callus.

Establishment of regenerable cell suspension cultures

Cell suspension cultures were established for a range of sugarcane cultivars by incubating embryogenic callus in liquid medium containing 3 mg l^{-1} 2,4-D. The plant growth regulators 2,4-D and picloram at 2 to 3 mg l^{-1} have been used to establish embryogenic suspension cultures of sugarcane (Fitch 1986; Ho & Vasil 1983b; Chen et al. 1988b). However, no differences were observed in the present study for the initial growth rates of heterogeneous cell suspension cultures established with the different plant growth regulators. Suspension cultures containing dicamba and picloram produced more organics, presumably phenolics, than cultures containing 2,4-D. The cultures grown with picloram and dicamba died after 9 and 15 weeks respectively despite regular changes of medium.

Three stages in the establishment of cell suspension cultures were identified (Table 2). These stages were similar to those reported by Lührs & Lörz (1988) for the establishment of barley cell suspension cultures. During the first stage the embryogenic callus adapted to a change from solid medium to liquid medium. The callus was from 10 to 23 weeks old when transferred to liquid medium, and had been through three to six subculture cycles. Callus from some cultivars

and some batches of callus from the same cultivar did not adapt to the liquid medium. Non-adapted callus turned brown, presumably producing phenolic compounds that accumulated in the medium and died within two weeks of transfer. Callus of B4362 and Q117 rapidly turned brown during adaptation. Regular replacement of the liquid medium minimised the accumulation of phenolics and enhanced the adaptation of this callus to liquid medium. Variation in adaptation of callus to liquid medium has been reported for barley suspension cultures (Lührs & Lörz 1988) but not previously for sugarcane. Ho & Vasil (1983b) and Chen et al. (1988b) reported replacement of the liquid medium, every two to three days for cultivar 68-1067 and three to five days for cultivar F164 respectively, to minimise the accumulation of phenolics. This frequent replacement of the medium would have assisted the adaptation of the callus to the liquid medium.

The second stage of development began after approximately two to three weeks of callus growth with the release of small cell aggregates and single cells into the liquid medium. Heterogeneous cell suspension cultures formed after five to eight weeks of culture. These suspensions consisted of single, elongated, vacuolated cells, larger aggregates of callus, and a few small, highly cytoplasmic and actively dividing cells. Heterogeneous cell suspension cultures were obtained from most cultivars tested (Table 1); however, only Q63, Q96, B629, B4362, F164, MQ72-2011, MQ72-4143 and MQ72-4157 could be maintained as heterogeneous cell suspension cultures. Other cultivars either developed into third stage cell suspensions or grew slowly and eventually died. Reasons for slow growth and death of these cultures are unknown, and tested modifications to the medium and culture conditions were not effective to maintain growth (data not shown).

Selective subculturing of heterogeneous cell suspension cultures resulted in the formation of homogeneous cell suspension cultures for cultivars Q63, Q90, Q96, Q110, Q117 and Q138 (Table 1). These cell suspension cultures consisted of small, compact, aggregates of densely cytoplasmic and actively dividing cells with prominent nuclei. The cells resembled the embryogenic cells described by Ho & Vasil (1983b) and Srinivasan & Vasil (1986) for cultivars 68-1067 and B4362 respectively.

Frequently, the establishment of homogeneous cell suspension cultures required several sequential, selective subcultures at weekly intervals from the one heterogeneous cell suspension culture. This inconsistency may have been due to a lack of critical density of actively dividing cells in the initial 15 ml subculture from the heterogeneous cell suspension culture. A critical density of cells may be required to sustain rapid cell growth and allow the formation of homogeneous cell suspension cultures.

S-cell suspension cultures were established by transferring non-morphogenic, friable callus (Type 4) regenerated from homogeneous cell suspension cultures to liquid medium. SP-cell suspension cultures were similarly established from callus regenerated from protoplasts isolated from homogeneous S-cell suspension cultures. This callus required little time to adapt to the liquid medium and a homogeneous cell suspension culture was established within two weeks.

Embryogenic callus of Q110 and Q138 did not completely adapt to liquid medium although homogeneous cell suspension cultures were established. The solution around cells of these suspensions gradually turned brown and cells died after 42 weeks of culture. During this time the cell suspension cultures grew slowly and required large (20 ml) subcultures. However, Q110-S and Q138-S homogeneous cell suspension cultures were established within two weeks of callus transfer to liquid medium. The cultures continued to grow rapidly and could be maintained indefinitely by subculture at three- to four-day intervals. This suggests that the death of the initial cell suspension cultures for these cultivars may have been due to effects of a subpopulation of cells that can be avoided by plating and recyling callus from the initial suspension cultures.

Regeneration of plants from cell suspension cultures

Callus and cell aggregates collected on the 500 μm filter from heterogeneus cell suspension cultures regenerated friable, yellow callus (Type

4; Figs 7, 8) and to a lesser extent white, globular, compact, embryogenic callus (Type 3; Figs 5, 6). Plants were regenerated from only Type 3 callus from cultures of MQ72-4157 up to 148 days after transfer of callus to liquid culture medium; MQ72-2011 up to 41 days old; and B4362 up to 100 days old. Few callus and cell aggregates from heterogeneous cell suspension cultures were collected on the 200 μm filter but small cell aggregates were collected on the 100 μm filter. These cells regenerated mucilaginous and friable callus (Types 2 and 4). Plants could not be regenerated from these callus types.

Homogeneous cell suspension cultures grown on solid MS regeneration medium formed friable callus (Type 4). On transfer of callus to a solid medium without plant growth regulators, no proembryoids or shoot meristems developed, although small thin roots often were produced. These results with sugarcane are similar to those obtained with rice where plant regeneration did not occur from finely-divided, fast-growing cell suspensions (Abdullah et al. 1986). In contrast, Ho & Vasil (1983b) regenerated plants from finely-divided, fast-growing embryogenic cell suspension cultures of sugarcane cultivar B4362.

The plant regeneration capability of embryogenic callus transferred to liquid medium was reduced as the callus dissociated into smaller cell groups and ultimately to a homogeneous cell suspension. For example, few plants were regenerated from cell aggregates of Q63 which had been in liquid medium for between 8 and 14 weeks. Plants could not be regenerated from S- and SP- cell suspension cultures.

Protoplast isolation and culture

The period required between the transfer of callus into liquid medium and the first isolation of protoplasts varied between cultivars and cell lines (Table 4). Homogeneous cell suspension cultures required 10 to 14 weeks for establishment but homogeneous S- and SP-cell suspension cultures required only two weeks to establish after the transfer of callus to liquid medium. We assume that the initial establishment of cell suspension cultures enrich for a cell-type that can more readily re-establish cell suspension cultures from regenerated callus.

The optimum interval for subculture of established homogeneous cell suspension cultures to maintain cells in a rapid growth phase to maximise protoplast yields was between three and four days.

Protoplast yield from heterogeneous cell suspension cultures was low (0.1 to 2.2×10^6 protoplasts per gram fresh weight of cells [$gfwt^{-1}$]) compared to non-recycled homogeneous cell suspension cultures (3.4 to 5.2×10^6 protoplasts $gfwt^{-1}$) (Table 4). Heterogeneous cell suspen-

Table 4. Age of cell suspension cultures when protoplasts were first isolated and average yields of protoplasts from subsequent isolations[a].

Cultivar/ Cell line	Time from initiation of cell suspension to first protoplast isolation (weeks)	Protoplast yield ($\times 10^6$ $gfwt^{-1}$)	
Heterogeneous suspension cultures			
Q63	8	0.1 ± 0.2	(27)[b]
Q96	5	0.2 ± 0.1	(3)
B629	6	<0.1	(1)
B4362	11	<0.1	(4)
F164	–[c]	0	
MQ72-2011	–	0	
MQ72-4143	28	2.2 ± 2.7	(5)
MQ72-4157	44	<0.1	(5)
Homogeneous suspension cultures			
Q63	15–20	4.4 ± 4.0	(32)
Q96	30	5.2 ± 1.8	(10)
Q110	–	0	
Q117	10	3.4 ± 2.6	(13)
Q138	–	0	
Q63-S[d]	5	9.4 ± 6.0	(19)
Q96-S	3–6	9.4 ± 4.0	(5)
Q110-S	5	2.6 ± 1.8	(3)
Q138-S	3–4	1.8 ± 1.4	(9)
Q63-SP[d]	3–5	6.4 ± 3.2	(63)
Q96-SP	3–5	13.2 ± 4.6	(6)
Q138-SP	3–5	1.8 ± 0.7	(5)

[a] Protoplast isolations were attempted at two-weekly intervals (refer to text).
[b] Number of experiments assessed.
[c] No protoplasts were isolated.
[d] S and SP suspensions (refer to text).

sion cultures may have given these relatively low yields of protoplasts because the enzymes were unable to digest cell wall components of the less actively dividing heterogeneous cells.

Higher yields of protoplasts were obtained from homogeneous cell suspension cultures of the cultivars Q63 and Q96 after recycling regenerated callus to liquid medium. The S-cell suspension cultures produced 9.4×10^6 protoplasts $gfwt^{-1}$ and the SP-cell suspension cultures produced 6.4 to 13.2×10^6 protoplasts $gfwt^{-1}$ (Table 4). The diameter of protoplasts of cultivars Q63 and MQ72-4143 isolated from heterogeneous cell suspension cultures was 19 μm and varied from 22 to 27 μm for protoplasts of cultivars Q63, Q63-S, Q138-S, Q63-SP and Q96-SP isolated from homogeneous cell suspension cultures. This is comparable to the range from 21 to 30 μm observed by I. Vasil (pers. comm.) for protoplasts from embryogenic cell suspension cultures of sugarcane cultivar B4362. Viability of protoplasts isolated from all homogeneous cell suspension cultures was 95 to 98%, but only 85 to 90% from heterogeneous cell suspension cultures. The high yield of viable protoplasts from Q63-S, Q63-SP, Q96-S and Q96-SP cell suspension cultures makes these cultures suitable for direct gene transfer studies that require large numbers of protoplasts.

The recycling of callus regenerated from homogeneous cell suspension cultures to establish S-cell suspension cultures also resulted in isolation of protoplasts from cell lines of Q110 and Q138. Attempts to isolate protoplast from slow-growing suspension cultures of these cultivars over 40 weeks were unsuccessful. However, satisfactory protoplast yields were obtained within three to five weeks of initiating S-cell suspension cultures of these cultivars.

Microcolonies formed from protoplasts isolated from most cell lines but further development to callus stage occurred only from protoplasts isolated from homogeneous cell suspension cultures.

Conclusions

The results from a range of sugarcane genotypes suggest the following generalisations for establishment of sugarcane embryogenic callus, cell suspension cultures and protoplast culture systems.

- Embryogenic callus can be initiated on leaf explant tissue taken from a range of genetically diverse sugarcane cultivars by culture on a modified MS medium containing $3\ mg\ l^{-1}$ 2,4-D. This callus is readily regenerated to plants, and appears to represent an ideal target for gene transfer by microprojectile bombardment.
- Four distinct callus types can be recognised developing on leaf explants, but only Type 3 (embryogenic) and Type 4 (organogenic) are capable of plant regeneration, and these must be selectively subcultured if this characteristic is required.
- Heterogeneous cell suspension cultures form within five to six weeks after transfer of embryogenic callus to liquid medium and are capable of plant regeneration. For some cultivars, homogeneous cell suspension cultures can be selected after 10 to 14 weeks, but these may not be capable of plant regeneration.
- Homogeneous cell suspension cultures yield higher numbers of protoplasts than heterogeneous cell suspension cultures. Homogeneous cell suspension cultures described in this report have several similarities to the embryogenic sugarcane cell suspension cultures described by Srinivasan & Vasil (1986). We are evaluating the capability for plant regeneration from protoplasts isolated from homogeneous and heterogeneous cell suspension cultures of sugarcane.
- Callus regenerated from homogeneous cell suspension cultures, or callus regenerated from protoplasts isolated from homogeneous cell suspension cultures, may establish S- and SP-homogeneous cell suspension cultures respectively within two weeks of transfer to liquid medium. This approach allows the establishment of stable homogeneous cell suspension cultures from some cultivars that otherwise tend to die in liquid culture.
- Establishment of S-cell suspension cultures provides a route to protoplast isolation for some, otherwise recalcitrant, sugarcane cultivars. The high yield of protoplasts from the S- and SP-cell suspension cultures is also advantageous for direct gene transfer studies.

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