

Isolation of Protoplasts from Suspension Culture and Subsequent Shoot Regeneration in Sugarcane

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Abstract

Cell suspension cultures with an ability to regenerate plants were initiated from 10 sugarcane clones tested. Cultures were initiated and maintained in modified N6 liquid medium containing 2 mg/l 2,4-D, 500 mg/l casein hydrolysate and 3% sucrose. Protoplasts isolated from these suspensions were embedded in 1.2% agarose (modified KM8P medium) and cultured in modified KM8P medium with the addition of nurse culture cells from suspension culture. After 5 to 8 weeks of culture, calluses were obtained in 5 clones. Calluses derived from protoplasts could not form any organs in the following culture on R2 regeneration medium. Only, protoplast-derived calluses of NiF4 (cultivar) cultured for 2 weeks on PR4 medium regenerated green shoots on R9 medium. These shoots could not develop to plant, and died in the following culture.

Discipline: Biotechnology

Additional key words: agarose beads, nurse culture

Introduction

In sugarcane (*Saccharum* spp.), plant regeneration from protoplasts has been reported by Srinivasan and Vasil⁹⁾ and Chen et al.²⁾. Thereafter, Sugimoto et al.¹⁰⁾ and Taylor et al.¹²⁾ reported on the regeneration of albino shoots and shoot-like structures from protoplasts, respectively. However, stable, fertile plant regeneration from protoplasts has not been reported, and protoplast culture is still difficult in sugarcane.

Establishment of a suspension culture system with regeneration ability is important for protoplast culture in cereals¹⁴⁾. Also in sugarcane, embryogenic suspension cultures were studied to develop a basic technique to obtain totipotent protoplasts^{4,11)}. In those studies, the difficulties in the development and

maintenance of a suspension culture with regeneration ability, and differences in cultivars have been reported.

The objective of the current study was to develop an effective suspension culture system with regeneration ability that could be applied to a wide range of *Saccharum* spp., and to induce plant regeneration from protoplasts isolated from suspension cultures.

Materials and methods

1) Plant materials

Calluses were initiated from the following 10 sugarcane clones: Yellow Caledonia, Chune, US 76-9, NiF3, NiF4, NiF8, F172, NCo310, Co290 and Trojan. Yellow Caledonia belongs to *Saccharum officinarum*, Chune to *S. barberi*, US76-9 is an F₁ clone of *Saccharum* spp. hybrid × *S. spontaneum*, and others are *Saccharum* spp. hybrids.

This study was supported in part by a grant for "A Comprehensive Research Program of Biotechnological Plant Breeding" from the Ministry of Agriculture, Forestry and Fisheries, Japan.

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Table 1. Composition of N6-based media¹⁾ used for cultures

Components	Medium (concentration, mg/l)					
	N6-1	N6-2	R1	R2	R9	PR4
2,4-D	2.0	2.0	0	0	0	0.4
IAA	0	0	0	0	0.5	0
BA	0	0	0	1.0	1.0	0
Kinetin	0	0	0	0	0	0.1
Abscisic acid	0	0	0	0	0	0.5
Aspartic acid	0	0	0	0	0	1,000
Glutamine	0	0	0	0	0	1,000
Casein hydrolysate	0	500	0	500	1,000	1,000
Sucrose (g/l)	30	30	30	30	15	15
Sorbitol (g/l)	0	0	0	0	15	15
Agar (% w/v)	0.9	0	1.0	1.0 ²⁾	1.0 ²⁾	1.0

1): N6 medium according to Chu et al. (1975), but with 0.025 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025 mg/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 mg/l $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ and 100 mg/l myo-inositol. All the media were adjusted to pH 5.8.

2): For the calluses derived from protoplasts, 1.2% agarose was used.

2) Initiation of callus and cell suspension culture

Explants for culture were obtained from young leaf base and were cultured on the modified N6 medium³⁾ containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose and 0.9% agar (N6-1, Table 1). Cultures were incubated at 26°C in the dark for 4 weeks. The calluses formed were subcultured every 4–5 weeks under the same conditions as those described above.

Suspension cultures were initiated by transferring 0.5 g (fresh weight) of calluses subcultured for 3 months to 30 ml of N6-2 medium (Table 1) in 100 ml flasks. Cultures were maintained by transferring 1 ml volume of cell aggregates to 30 ml of fresh N6-2 medium every 7 days, and cell aggregates were split into small pieces (1 mm in diameter) by passing through a stainless steel net every 14 days. Suspensions were incubated on an orbital shaker (90 rpm) at 26°C in a room using light with moderate intensity under 12 h photoperiod.

The cell aggregates grown in suspensions were tested for shoot formation on R2 medium (Table 1). Cultures were incubated in the dark for 2 weeks, and subsequently under fluorescent light (5,000 lux) and 12 h photoperiod. Shoots formed from the calluses were transferred to R1 medium (Table 1) for root induction.

3) Protoplast isolation and culture

Protoplasts were isolated from 3 to 6 months old suspension cultures, 4 to 5 days after subculture. Cell aggregates of 1 ml were collected and suspended

in 10 ml of enzyme solution containing 3% Cellulase Onozuka RS, 0.05% Pectolyase Y23, 3 mM CaCl_2 , 1% potassium dextran sulfate and 0.55 M mannitol (pH 5.5). The enzyme-cell aggregate mixture was incubated at 30°C for 10 to 12 h, then filtered through 38 μm nylon meshes. Protoplasts were pelleted by centrifugation at 140 \times g for 3 min, and were washed 3 times in a 0.55 M mannitol solution containing 0.3 mM CaCl_2 . Washed protoplasts were suspended in a culture medium (modified KM8P medium: KM8P medium⁶⁾ containing 0.46 M glucose, 0.09 M sucrose and 2 mg/l 2,4-D) and were counted with a haemocytometer. Then, the protoplasts were embedded in 1.2% molten agarose (modified KM8P medium) at a density of 0.5 to 1.0×10^6 protoplasts/ml and plated as a layer (0.5 ml each) in petri dishes. Protoplasts embedded in agarose were batched in 6 ml modified KM8P medium with the addition of nurse culture cells from suspension cultures of NiF4, and incubated at 26°C in the dark. During the culture, the osmolarity of the medium was gradually reduced by replacing the KM8P medium with N6-2 medium. Nurse cells were removed by washing 3 times with fresh N6-2 medium, after cell colonies regenerated from protoplasts. Subsequently, cell colonies in agarose were collected and cultured in the N6-2 medium by applying the same suspension culture method as that described above.

4) Protoplast-derived callus culture

Cell suspensions derived from protoplasts were cultured for 1 to 2 weeks in N6-2 medium until

cell aggregates developed to calluses 1–2 mm in diameter. Then, calluses were transferred to solid R2 medium (1.2% agarose). Following incubation in the dark for 2 weeks, cultures were incubated under fluorescent light (5,000 lux) and 12 h photoperiod.

Cell suspensions derived from protoplasts of NiF4a were transferred to PR4 medium (Table 1, 1% agar) and were incubated in the dark for 2 weeks. Then, calluses developed to 3–5 mm in diameter were transferred to R9 medium (Table 1, 1.2% agarose), and were incubated under fluorescent light (5,000 lux) and 12 h photoperiod.

Results and discussion

1) Culture initiation and maintenance

Compact, yellowish-white calluses were induced in all the 10 sugarcane clones tested. However, part of the calluses of NiF4, NiF3 and F172 was soft and friable. Only compact and yellowish-white calluses were selected and subcultured. These compact calluses displayed a regeneration ability, and the form of calluses was similar to that of the embryogenic calluses reported by Ho & Vasil⁵⁾, Chen et al.¹⁾, Taylor et al.¹¹⁾ and Fitch & Moore⁴⁾. Plant regeneration via somatic embryogenesis, however, could not be confirmed in the present experiment.

Cell suspension cultures were established from 10 sugarcane clones, by incubating compact callus in N6–2 liquid medium containing 2 mg/l 2,4-D and 500 mg/l casein hydrolysate. Suspension cultures of 10 sugarcane clones consisted of hard cell aggregates like a compact callus and fewer small aggregates with elongated cells. Suspensions of NiF4, however, showed the 2 types, one consisting of the same type of cell aggregates as described above (NiF4a), and the other consisting of homogeneous small cell aggregates with actively dividing cells (NiF4b), after selective subculture for 3 months. These 2 types of suspension cultures were similar to the heterogeneous and homogeneous suspension cultures, respectively, reported by Taylor et al.¹¹⁾. These suspension cultures could be maintained with each keeping its characteristics.

The cell aggregates which grew in heterogeneous cell suspension cultures and were 3 months of age were able to form shoots on R2 medium in all the sugarcane clones tested, although differences among the clones were observed in the frequency of shoot formation. These shoots produced roots on R1 medium and finally formed plantlets. The cell aggregates in homogeneous cell suspension of NiF4b had lost

their regeneration ability during the subculture for 3 months.

In our previous study⁸⁾, we reported that modified N6 medium was more suitable than MS medium for suspension culture of sugarcane, and growth of suspension was accelerated by the addition of 500 mg/l casein hydrolysate to the medium. In the present study, we established suspension cultures with a regeneration ability for all the 10 sugarcane clones tested. It is thus suggested that the culture method and N6–2 medium applied here were effective for the establishment of suspension cultures in a wide range of *Saccharum* spp.

2) Protoplast culture

Protoplast yields from suspension cultures of 10 sugarcane clones ranged from 0.8 to 5×10^6 protoplasts/ml. The lowest yield of protoplasts was obtained from suspensions of Trojan, while the highest yield was obtained from suspensions of US76–9 and NiF3 (Table 2, Plate 1a).

The first division of protoplasts isolated from suspension cultures of Y. Caledonia, US76–9, NiF3, NiF4a, NiF4b, NiF8 and F172 was observed after 2 to 3 days of culture in modified KM8P medium (Plate 1b). The highest frequency of first divisions observed was 21% for plated protoplasts derived from the suspensions of NiF4b. However, protoplasts isolated from US76–9, NiF3, NiF4a, NiF4b, NiF8 and F172 could develop only to visible cell colonies after 21 to 28 days of culture (Plate 1c). Nurse culture

Table 2. Isolation and culture of protoplasts in 10 sugarcane clones

Clones	Protoplast yield ($\times 10^6$ protoplasts/ml)	Protoplast culture ¹⁾
Yellow Caledonia	1.6	+
Chunee	2.6	–
US76–9	5	++
NiF3	5	++
NiF4a ²⁾	4	+++
NiF4b	2.6	+++
NiF8	2.6	++
F172	3.2	++
NCo310	1.6	–
Co290	1.9	–
Trojan	0.8	–

1): – ; No division, + ; First division, ++ ; Some cell colonies (1–30 cell colonies/agarose plate), +++ ; Many cell colonies (30–several hundreds of cell colonies/agarose plate).

2): a ; Homogeneous suspension culture, b ; Heterogeneous suspension culture.

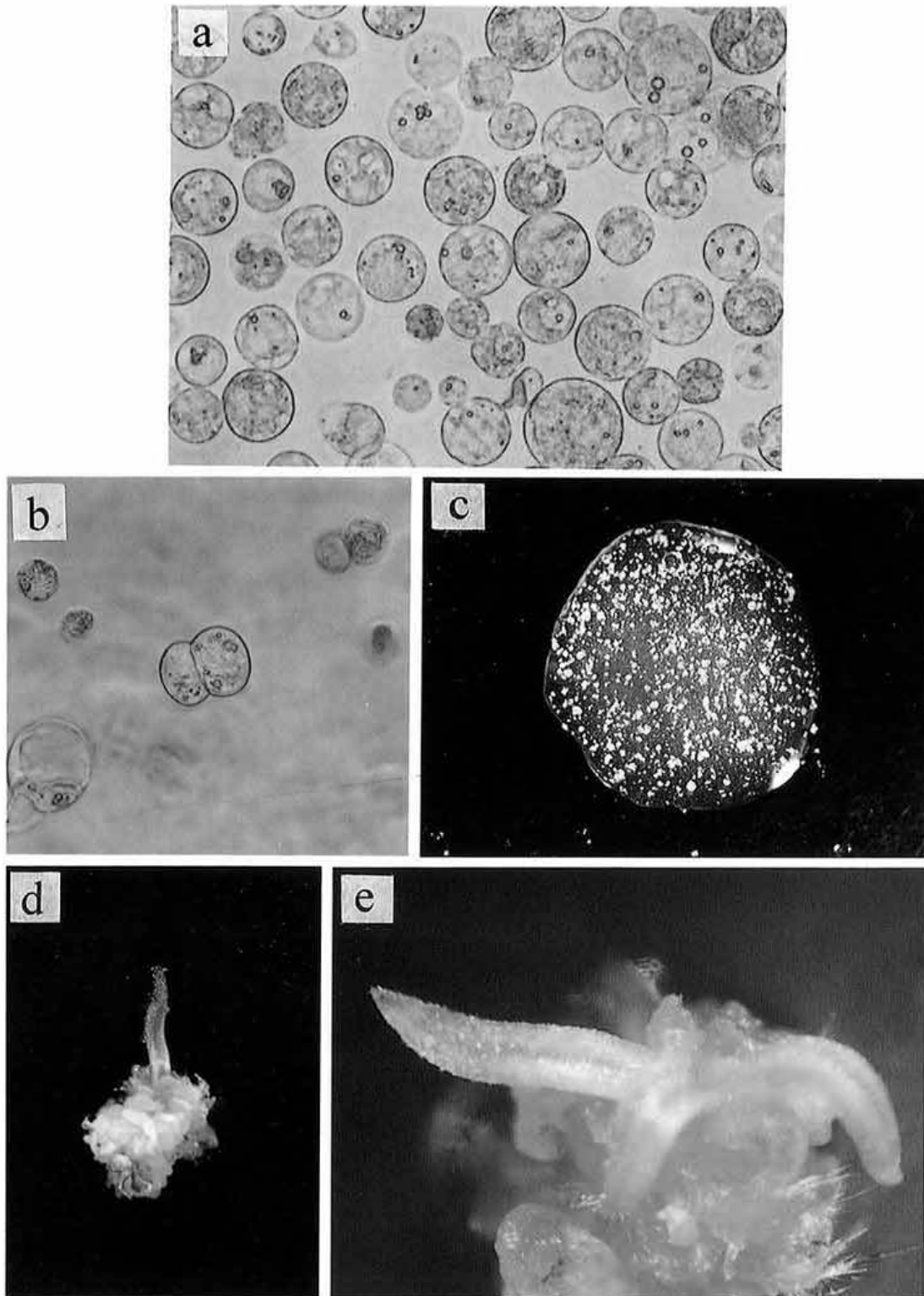


Plate 1. Shoot regeneration from protoplasts of cell line NiF4a
a: Freshly isolated protoplasts,
b: First division of protoplasts on 3rd day,
c: White visible colonies formed in agarose plate after 21 days,
d, e: Regeneration of green shoot from protoplast-derived calluses.

technique using nurse cells of NiF4b was effective to induce cell division of protoplasts. When protoplasts were cultured without nurse cells, only a few divisions of protoplasts were observed in NiF4a and NiF4b, but no divisions were observed in the other clones. After the formation of visible cell colonies, nurse cells were removed by washing and agarose plates were cultured continuously in N6-2 medium. After 7 to 14 days of culture, cell colonies were collected by breaking the agarose plate. Collected cell colonies were resuspended in the N6-2 medium and they re-established suspension cultures.

3) Shoot induction from protoplast-derived calluses

After 7 to 14 days of culture, the cell aggregates grown in suspension cultures derived from protoplasts of 6 cell lines were transferred to R2 medium and were incubated in the dark. During the 2-week period of culture in the dark, most of the cell aggregates formed white and friable calluses. These friable calluses could not develop to any organs, became brown and died during incubation under fluorescent light and 12 h photoperiod.

To induce compact calluses with a regeneration ability, and to improve the regeneration rate of callus, PR4 and R9 medium (Table 1) were tested. The R9 medium which contains 0.5 mg/l 3-indole acetic acid (IAA) and 1 mg/l 6-benzylaminopurine (BA)⁷⁾ was effective for shoot regeneration from callus derived from suspension cultures in several sugarcane clones. In the case of the calluses derived from suspensions of NiF4a, the frequency of shoot regeneration on R2 medium was 26%, and on R9 medium 61%⁸⁾. The PR4 medium is based on P10 medium used in preculture of callus for regeneration in rice anther culture¹³⁾, with the concentrations of sucrose and sorbitol were modified to 1.5% each. Culture for 2 weeks on PR4 medium in the dark increased the frequency of the formation of white and compact calluses from cell aggregates of suspension cultures. In addition, calluses subjected to preculture on PR4 medium were more effective in promoting the frequency of shoot regeneration, and less browning occurred than without preculture, after the transfer of calluses to R9 medium (data not shown). On the basis of these results, the cell aggregates derived from protoplasts of NiF4a were incubated on PR4 and R9 medium.

After 2 weeks of culture on PR4 medium, many white, compact calluses were formed from protoplast-derived cell aggregates of NiF4a. About 200 pieces of calluses were transferred to R9 medium and in-

cubated under fluorescent light and 12 h photoperiod. After 2 weeks, green shoot primordia were formed in some of the calluses. These shoot primordia developed into green shoots (5–10 mm in length) in the following 2 to 4 weeks of culture on R9 medium (Plate 1d,1e). The number of protoplast-derived calluses which developed green shoots totaled nearly 50 during the culture. Shoots separated from the calluses were transferred to R1 medium for rooting. However, these shoots gradually turned brown and died off on R1 medium within 4 weeks of culture. Taylor et al.¹²⁾ also reported that protoplast-derived shoots failed to develop roots for unknown reasons.

Although many green shoots were developed from protoplast-derived calluses, plants were not regenerated in the present study. Further studies should be carried out to establish stable and totipotent protoplast cultures in sugarcane.

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(Received for publication, Jan. 9, 1995)