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A Simple Method for Morphogenic Calli Formation from Protoplasts in Sugarcane

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Abstract
An attempt was made to produce protoplasts with a high potential of cell colony formation from sugarcane cell suspensions through an enzyme treatment. Those suspensions were intiated from fresh leaf calli as well as leaf and young panicle calli each on agar media. The key procedures presented in this study are summarized as follows: (1) To use calli with a high potentiality of plant regeneration in rapidly growing phase, i.e. 10-20 days after transfer; (2) To treat with enzyme the cell suspensions subcultured only once after the last subculture on agar media, or the calli o agar media directly; (3) To incubate the cell-enzyme mixture under shaking at 30-60 rpm at 32°C for 4-10 hr; (4) For effective formation of cell colonies, an AA liquid medium is most suitable, where reduction of osmotic pressure is not usually necessary; and (5) For morphogenic calli formation, formed calli must be transferred to another medium, such as 1/2 MS, MS, B5 or N6 medium supplemented with 5×10^{-6}M 2,4-D and 10^{-6}M NAA. In about a month after the treatment, a large number of cell colonies consisting of small, tightly packed cells were produced. They eventually developed into morphogenic calli differentiating roots.

Discipline: Biotechnology
Additional key words: biotechnology, calli on agar media, differentiating roots

Introduction

Regeneration of plants from protoplasts of sugarcane has been regarded rather difficult. Only a few successful achievements have been reported in differentiating protoplasts from sugarcane cell suspensions. The suspensions were initiated from calli in a liquid medium and subcultured intensely with short intervals[1–3].

Due to the requirement for short interval subculture involved, their methods are too complicated for practical use in a sugarcane breeding program. A simpler way is therefore needed in applying a biotechnology such as cell fusion in the breeding practices.

The authors attempted to produce morphogenic calli in a simple way from protoplasts of some domestic as well as exotic sugarcane varieties.

The proposed method in this paper is simpler than the others in the sense that it does not require a series of subculture with short intervals and that it can be applied even to the calli harvested directly from the solid media.

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Materials and methods

(1) Sugarcane cultivars used

Commercial sugarcane cultivars NCo310, NiF3 and NiF4 were used in Experiment A, while NiF4 and F172 were used in Experiments B and C.

(2) Initiation of calli and cell suspensions

Calli were initiated from leaf base explants on MS agar medium with 5×10^{-6} M 2,4-D, 10^{-6} M NAA and 30 g/l sucrose with adjusted pH 5.8.

The cultures were maintained at 26°C for 12 hr under a mild room-light illumination. The calli formed were subcultured 6–10 times every 3–4 weeks on fresh agar media. In the course of subculture, the calli that regenerated plants vigorously were selected.

The calli and cell suspensions for protoplasts initiation were prepared in the following three procedures:

Experiment A: Cell suspensions were initiated by inoculating 2.0 g f. wt. of calli which had been subcultured for 6–10 months and harvested 10–40 days after the last subculture in 40 ml of MS or AA liquid medium containing 10^{-5} M 2,4-D or 5×10^{-6} M 2,4-D + 10^{-6} M NAA + 30 g/l sucrose. The pH was adjusted to 5.8.

Experiment B: Cell suspensions were initiated from calli harvested 10–20 days after the last transfer of 2–3-month subculture.

Experiment C: Calli subcultured for 2–3 months on agar media and harvested 10–20 days after the last transfer were directly suspended in the enzyme solution.

(3) Protoplast isolation

Experiments A and B: Exponentially growing cell suspensions, which were cultured for 4–7 days in liquid media, were centrifuged at 900 rpm for 4–5 min. The collected cells were resuspended in 20 ml of enzyme solution containing 4% Cellulase Onozuka RS + 2% Macerozyme R10 with 0.35 mM KH_2PO_4 + 3 mM $CaCl_2$. The solution pH was adjusted at 5.5. Osmotic pressure was maintained by adding 0.7 M Mannitol. The mixture was reciprocally shaken at 30 or 60 rpm for 4–11 hr at 32°C.

Experiment C: One gram of rapidly growing calli were suspended in 10 ml of enzyme solution and shaken at 30 or 60 rpm at 32°C.

After the treatment in each experiment, the enzyme-protoplast mixture was passed through nylon sieves of 38 μm pore size to remove undigested cells. The filtrate was transferred to 25 ml screw-capped centrifuge tubes and the protoplasts were pelleted by centrifugation at 900 rpm for 3–4 min. Protoplasts were washed twice in 0.7 M Mannitol solution containing 0.35 mM KH_2PO_4 + 3 mM $CaCl_2$ (pH 5.8) through two cycles of resuspension and centrifugation (900 rpm for 2 min). Washed protoplasts were resuspended in a defined liquid culture media. Prior to the resuspension, the number of protoplasts was counted with a hemocytometer.

(4) Protoplast culture media

The following five basic media were examined: MS, 1/2 MS, AA (nitrogen of MS salts were replaced by amino acids), B5 and N6 of Chu, each of which was modified by two levels of growth regulating substances, 10^{-5} M 2,4-D or 5×10^{-6} M 2,4-D + 10^{-6} M NAA. All of them contained 30 g/l sucrose and the pH was adjusted to 5.8. Osmotic pressure was maintained by adding glucose.

(5) Protoplast culture in liquid media

Protoplast suspensions with a density of $0.1–1.0 \times 10^5$/ml in a liquid medium in 35 mm plastic petri dishes were kept at 26°C under a mild room-light illumination with 12-hr photoperiod. After cell divisions took place, a certain amount of fresh medium was added.

Cell suspensions were transferred 2–3 times to a fresh media. Osmotic pressure was lowered gradually by reducing glucose, though this procedure was not always necessary as in the case of other plant species, on the occasions where the re-differentiation process was extremely slow.

(6) Plating on an agar solidified medium

After one month of suspension culture, the cell colonies were transferred onto the surface of an MS agar medium with 5×10^{-6} M 2,4-D, 10^{-6} M NAA, 30 g/l sucrose and 10 g/l agar.

Calli formed on an agar solidified medium were subcultured several times on the same kind of medium for further growth and plant regeneration.

Results and discussion

The results of Experiments A, B and C are shown in Tables 1, 2 and 3, respectively. In all the three experiments, a large number of protoplasts with high capability of cell colony formation were produced

Table 1. Isolation and culture of protoplasts in Experiment A (1988)

Variety	Protoplast isolation				Protoplast culture	
	Age of callus[a]	Medium[b]	Time[c]	Yield[d]	Density[e]	Activity[f]
NCo310	10–20	MS-1	6	+	Low	−(MS-1, MS-2)
			8	++	Low	−(MS-1, MS-2)
		AA-2	8	++	Medium	±(MS-1, MS-2)
	20–30	MS-1	6	±	Low	−(MS-1, MS-2)
			8	±	Low	−(MS-1, MS-2)
NiF3	20–30	AA-1	4	+	Low	−
			6	++	Medium	−
NiF4 (Young panicle callus)	10–20	MS-1	4	+++	Low	−
					Medium	+++(AA-1,2), ++(MS-1) +(B5-1,2, 1/2MS-1,2)
					High	±
			6	++++	Medium	+++(AA-1,2), ++(B5-1,2) +++(MS-1,2, 1/2MS-1,2)
	20–30	MS-1	4	±	Low	−
			6	±	Low	−
NiF4 (Leaf callus)	10–20	MS-1	4	++	Low	++(AA-1), −(the others)
		AA-1	4	++++	Low	+(MS-2, 1/2MS-2), ±(B5-1,2)
					Medium	+++(AA-1,2), ++(the others)
			6	+++	Medium	+++(AA-1,2), ++(1/2MS-1,2) ++(MS-1,2, B5-1,2)
		AA-2	4	+++	Medium	++(MS-1,2, 1/2MS-1,2, B5-1,2)
			6	+++	Medium	++(MS-1, 1/2MS-1), ±(B5-2)
	30	MS-1	4	+	Medium	+(MS-2, 1/2MS-2)

a): Days of last subculture on an agar medium for increasing calli for suspension culture.
b): Medium of suspension culture for initiation of cell suspensions; 1: 10^{-5}M/l 2,4-D, 2: 5×10^{-6}M/l 2,4-D + 10^{-6}M/l NAA.
c): Hours for enzyme treatment to isolate protoplasts from calli or cell suspensions.
d): Yield of protoplasts under the enzyme treatments; ±: $0–0.5\times10^5$, +: $(0.5–1.0)\times10^5$, ++: $(1–3)\times10^5$, +++: $(3–10)\times10^5$, ++++: 10^6.
e): Protoplast density; Low: 0.5×10^5/ml, Medium: $(0.5–1.0)\times10^5$/ml, High: 1.0×10^5/ml<.
f): Activity of culture for cell division, colony formation, callus formation, root differentiation; −: no division, ±: first division, +: a few cell colonies, ++: some cell colonies, +++: many cell colonies.

Table 2. Isolation and culture of protoplasts in Experiment B (1988)

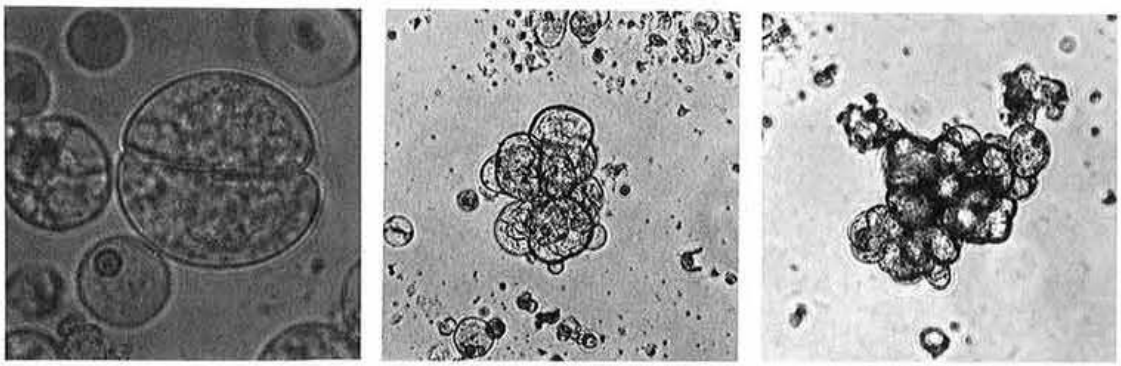
Variety	Protoplast isolation				Protoplast culture
	Medium[b]	Time[c]	Yield[d]	Frequency[g]	Activity[f]
NiF4	MS-1	9	++	60	+++(AA-2, 1/2MS-2)
		16	+	30	−(AA-2, 1/2MS-2)
	MS-2	9	++	60	++(AA-2, 1/2MS-2)
	AA-1	9	++	60	+++(AA-2, 1/2MS-2)
		16	+	30	−(AA-2, 1/2MS-2)
F172	MS-1	9	++	60	++(AA-2, 1/2MS-2)
	MS-2	9	+++	60	+++(AA-2, 1/2MS-2)
	AA-1	9	+	60	+++(AA-2), ++(1/2MS-2)
		11	+	60	−(AA-2, 1/2MS-2)

b), c), d), f): Refer to notes in Table 1.
g): Frequency of shaking for enzyme treatment (unit: rpm).

Table 3. Isolation and culture of protoplasts in Experiment C (1988)

Variety	Protoplast isolation			Protoplast culture
	Time[c)]	Yield[d)]	Frequency[g)]	Activity[f)]
NiF4	9	+	60	+(AA-2), −(1/2MS-2)
	11	+	60	+(AA-2), −(1/2MS-2)
	9	±	30	−(AA-2), −(1/2MS-2)
	11	+	30	+(AA-2), −(1/2MS-2)
F172	9	++	60	+++(AA-2), ++(1/2MS-2)
	11	+++	60	+++(AA-2), ++(1/2MS-2)
	11	++	30	+++(AA-2), ++(1/2MS-2)

c), d), f): Refer to notes in Table 1.
g): Refer to note in Table 2.



First division　　Following division　　Small cell colony

Plate 1. Early stage of cell colony formation from protoplasts

from the calli on agar solidified media as well as the cell suspensions subcultured only once after the last subculture on agar media. The main factors determining the efficiency of cell colony formation are described hereafter.

Genotypes played a crucial role. Whether they are derived from calli on solid media or cell suspensions, F172 and NiF4, particularly the former, showed a very high capability of cell colony formation as well as high yield of protoplasts. Protoplasts were also produced from NCo310 and NiF3. However, cell colony formation was limited in NiF3. No cell colony formation was observed in NCo310.

Growth phase of cells to be treated by enzyme was also very important. In Experiment C, it was essential to use calli of 10–20 days after the last transfer for the enzyme treatment. In Experiments A and B, the similar conditions of calli were also required to initiate suspension culture. Under these conditions, calli were in the status of "rapidly growing phase", which was composed of small cells with abundant cytoplasm. If aged cells were used, very few protoplasts were produced and they were hardly purified.

When the enzyme solution with cells was reciprocally shaken at 120 rpm, most of protoplasts produced could not divide. When the solution was reciprocally shaken at 60 rpm, a large number of protoplasts with high mitotic potential were produced (Plate 1). At 30 rpm, the protoplast yield was usually low, but it was occasionally as high as the case of 60 rpm and the protoplasts produced were less injured.

When the protoplasts produced were cultured at a density lower than 0.5×10^5/ml, fewer cells divided. Limited capability of cell division due to low density of protoplasts has been observed not only in sugarcane but in many other crops. In some

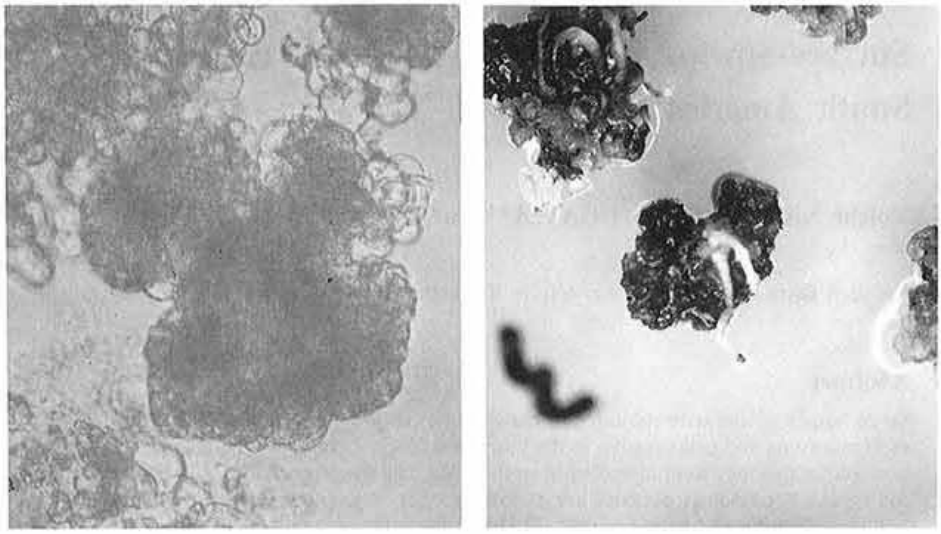


Plate 2. Cell colony in liquid medium and root on an agar solidified medium

instances of other crops, this limitation has been relaxed by employing nurse culture.

On the contrary, when the protoplast density was too high, many cells divided but did not develop further. Cultures turned brown and all the cells died. In such a case, when the medium was refilled before the color of the culture turned brown, the cells continued growing.

Response of the protoplast culture of sugarcane to osmotic pressure was different from that of many other crops. To initiate cell colony formation, it was not necessary to lower the osmotic pressure except for rare instances, where plasmolysis was observed.

Cell division and colony formation took place in all the media used. AA medium was most suitable for cell division and colony formation, being followed by 1/2 MS and MS medium. In regard to the growth regulating substances to accelerate cell division and cell colony formation, 10^{-5} M 2,4-D was better suited than 5×10^{-6} M 2,4-D + 10^{-6} M NAA.

Morphogenic calli, occasionally differentiating roots (Plate 2), were developed from cell colonies consisting of many small, tightly packed cells. These cell colonies were originally initiated from the calli selected under the subculture for high capability of plant regeneration.

The above observations are consistent with the well-accepted understanding that the cell lines with higher embryonic potential are suitable for regenerating protoplasts.

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