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Efficient Plant Regeneration from Protoplasts of Sugarcane

Makoto MATSUOKA*,†, Hiroyuki FUKUOKA*, Taiichi OGAWA*,††, Hiroshi YANO*, Kunihiro UJIHARA** and Akira SUGIMOTO**

*Chugoku National Agricultural Experiment Station, Fukuyama, Hiroshima 721-8514, Japan
**Kyushu National Agricultural Experiment Station, Nishinoomote, Kagoshima 891-3102, Japan

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Establishment of a reliable totipotent protoplast culture method would greatly contribute to the advance in research of somatic cell genetics in sugarcane. Many studies in sugarcane protoplast culture have been undertaken and plant regeneration from protoplasts has been reported by several research groups [1-4]. However, the difficulties and differences in genotypes to maintain regenerable suspension cultures and the low rates of plant recovery from protoplasts still remain. We have reported plant regeneration from protoplasts of sugarcane clone US76-9 in a previous study [5]. However, the number of recovered plants was limited and efficient plant regeneration has not been established despite hundreds of shoots being regenerated from protoplast-derived calli. The aim of the present study was to establish a stable and reliable method of protoplast culture to regenerate plants based on the findings in our previous study. In this report we describe the modification of the protoplast culture method and efficient plant regeneration from protoplast-derived calli in two sugarcane clones.

Cell suspension cultures of sugarcane clones F177 and US76-9 were initiated and maintained in N6-2 medium as described elsewhere [5, 6]. F177 is a cultivar (*Saccharum* spp. hybrid) and US76-9 is a breeding material (F_1 clone of *Saccharum* spp. hybrid × *S. spontaneum*). Suspensions were incubated on an orbital shaker (110 rpm) at 26°C under fluorescent light (200 lux) with 16-h photoperiod.

Protoplasts were isolated from suspension cultures of F177 aged 38 and 62 weeks, and that of US76-9 aged 7 weeks. Three days after subculture, one ml of cell aggregates was collected and suspended in 10 ml of enzyme solution [5]. The cells were incubated at 30°C for 6 h, and filtered through a 30 μm nylon mesh. The filtrate was washed 3 times by centrifugation at 140×g for 3 min. with 0.55 M mannitol solution containing CPW salts [7]. Then, the isolated protoplasts were resuspended in the modified KM8P (mKM8P) medium [5, 8] and the number was counted with a haemocytometer. The protoplasts were finally embedded in 1.2% molten Sea Plaque agarose with the mKM8P medium at a density of 5×10^5 protoplasts/ml and plated as a layer (1 ml each) in 6 cm petri dishes. The agarose plate was batched in 6 ml of the conditioned mKM8P medium (1: 1 mixture of fresh and filter-sterilized mKM8P medium used for the culture of nurse cells for 2 days). Then 0.1 ml in packed cell volume of nurse cells [5] were added and incubated at 26°C in the dark. During the culture, the osmolarity of the medium was gradually reduced by replacing the conditioned mKM8P medium with the N6-2 medium. When cell colonies derived from the protoplasts appeared, nurse cells were removed by washing with fresh N6-2 medium, and cell colonies in agarose were collected and cultured in the N6-2 medium.

Cell suspensions derived from protoplasts were cultured for 1 to 2 weeks in N6-2 medium. Then, the cell aggregates were transferred to PR4 medium [5] and were incubated in the dark for 10 days. The calli reaching a diameter of 3-5 mm were transferred to the R9 medium [5] for shoot regeneration. The cultures were incubated in the dark for 7 days, then incubated under fluorescent light (3 klux) with 16-h photoperiod. Shoots regenerated from the calli of 5 to 10 mm in length were transferred to MS-R1 medium (hormon-free MS medium [9] containing 1 mg/l thiamine-HCl and 1% agar, pH 5.8) for root induction. To improve the rate of root induction from shoots, two different lighting conditions were tested. After being transplanted to MS-R1 medium, shoots were cultured under fluorescent light (3 klux) with 16-h photoperiod (Method 1). Alternatively, shoots were cultured under dark condition for the first 3 days, followed by 100 lux illumination with 16-h photoperiod for the next 3 days, then transferred to the same condition as Method 1 (Method 2). Sixty shoots regenerated were used in each treatment of F177 (aged 38 weeks) and US76-9. Other shoots

† Present address: *Japan International Research Center for Agricultural Sciences, Ishigaki, Okinawa 907-0002, Japan*
†† Present address: *National Institute of Agrobiological Resources, Tsukuba, Ibaraki, 305-8602 Japan*

Table 1. Isolation and culture of protoplasts in 2 sugarcane clones.

Clones	Age of suspension (weeks)	Protoplast yield (/1 m*l* cells)	No. of calli cultured on R9 medium	Frequency of green shoot formation* (%±S.D.)	No. of regenerated plants
F 177	38	2.0×10^6	218	81(±12.6)	151
F 177	62	2.8×10^6	190	60(± 8.2)	138
US 76-9	7	2.8×10^6	228	64(± 8.6)	32

* Frequency was calculated after 4 weeks of culture on the R9 Medium.
Values are the mean ±S.D. from 5 replicates each with 12 calli.

were cultured following the Method 2. The plantlets developed were transplanted to autoclaved soil in a growth room under the condition of Method 1. Plants which grew to about 10 cm in height were transferred to a greenhouse.

Cell suspension cultures were established both in F177 and US76-9 by incubating compact calli in N6-2 medium. These suspensions consisted of hard cell aggregates (1 to 3 mm in diameter) and fewer small aggregates of elongated cells like those reported in our previous study [6]. They retained the ability to regenerate plants.

Protoplast yield from suspension culture of F177 was 2.0 to 2.8×10^6 more protoplasts per one m*l* of cell aggregates (**Table 1**). The first division of protoplasts of F177 was observed after 2 to 3 days of culture in conditioned mKM8P medium with nurse cells. The use of conditioned mKM8P medium seemed to be more efficient on colony formation from protoplasts of F177 compared with fresh mKM8P in a preliminary experiment. Only a few colonies were formed without using conditioned mKM8P medium (Data not shown). Colonies derived from protoplasts of F177 in an agarose plate are shown in **Fig. 1-a**. After 4 weeks of culture, approximately a hundred visible, white cell colonies per plate were formed.

The cell aggregates reached a diameter of 1-2 mm were transferred to the PR4 medium. Most of these cell aggregates developed yellowish white, compact calli after 10 days of culture. These compact calli formed green shoots in the following 4 weeks of culture on R9 medium. The frequency of shoot formation from protoplast-derived calli of F177 at age 38 and 62 weeks were 81% and 60%, respectively (**Table 1, Fig. 1-b, c**). In a previous study [5] using PR4 medium for the culture of protoplast-derived cell aggregates of US76-9, compact, morphogenic calli were induced, and shoots were regenerated from these calli at a high frequency in the successive culture on the R9 medium. In this study, by using the same medium, we similarly succeeded in shoot regeneration from protoplast-derived calli of F177. These results indicate that the method used in this experiment for shoot induction from protoplast-derived calli was reproducible and effective.

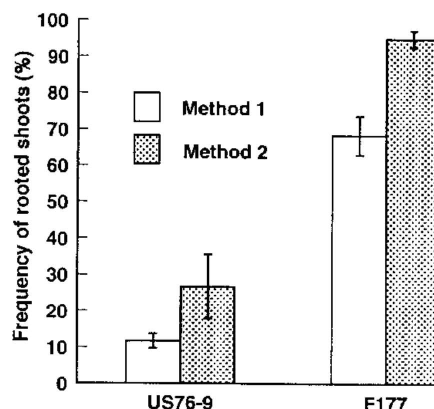

Fig. 2 Effect of pre-culture in the dark condition on rooting from regenerated shoots.
Frequency was calculated after 4 weeks of culture. Values are the mean ±S.D. from 5 replicates each with 12 shoots (F177) and 3 replicates each with 20 shoots (US76-9). See text for Method 1 and 2.

Lighting condition of the culture for root induction affected on rooting from regenerated shoots. The frequencies of rooted shoots in F177 by the Method 2 were apparently higher than that of Method 1, and a similar effect was observed in the experiment with US76-9 (**Fig. 2**). The importance of lighting condition for rooting is a new finding in tissue culture of sugarcane as far as the authors are aware.

The rooted plantlets established in soil easily, and few plantlets were lost during the acclimatization (**Fig. 1-d**). As a result, the total number of regenerated plants from protoplast-derived calli of F177 was 289, and that of US76-9 was 32 (**Table 1**). The rate of rooted shoots of US76-9 in this study increased in comparison with that in the previous study with the same method [5]. It is possible that the use of conditioned mKM8P medium had a favorable effect on the protoplast culture of sugarcane. Plants regenerated from protoplast-derived calli of F177 with a higher frequency than US76-9, even when protoplasts were isolated from suspension culture aged 62 weeks (**Table 1**). Generally, the shapes of shoots in F177 were almost normal, while irregular-shaped shoots

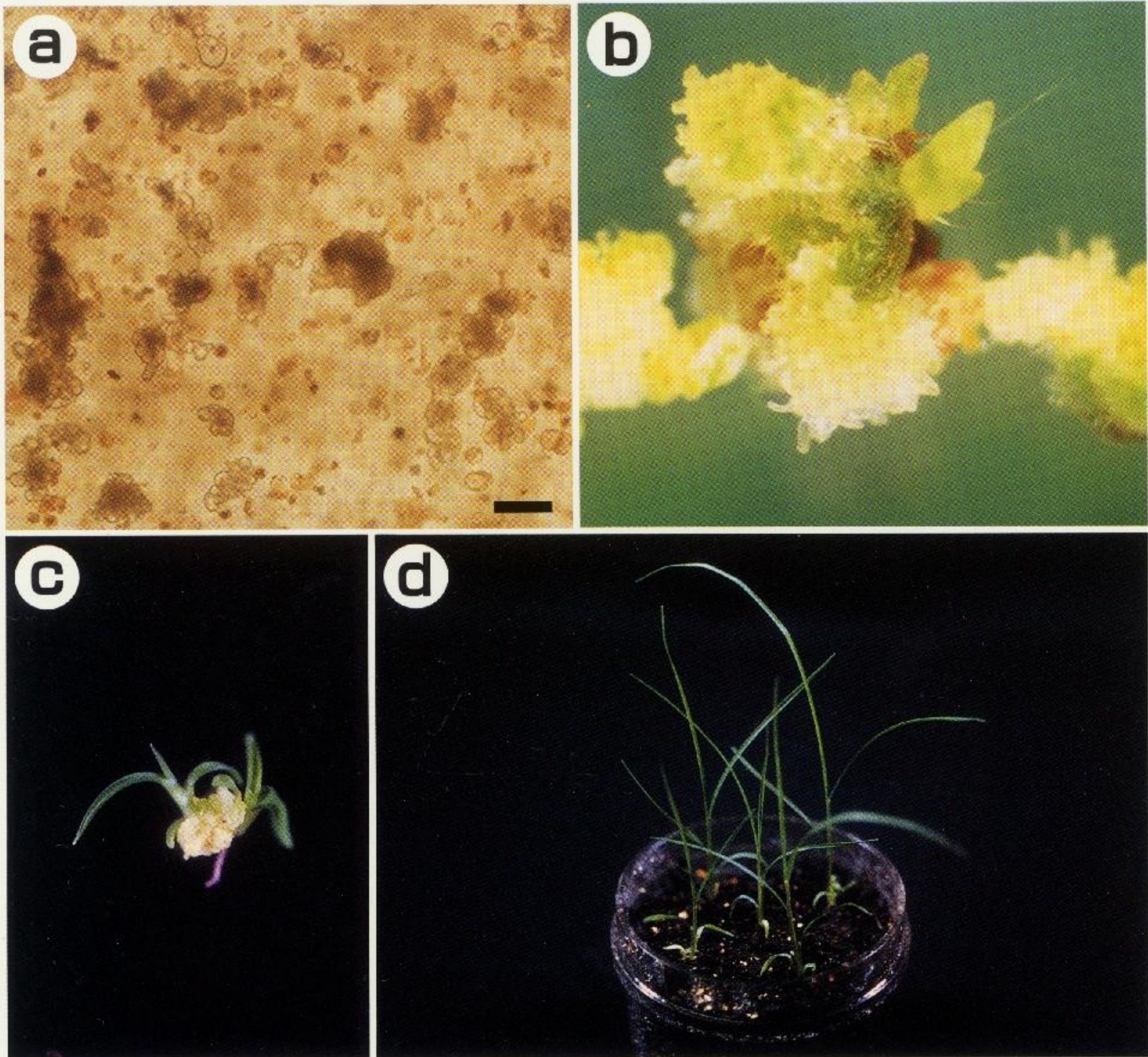

Fig. 1 Plant regeneration from protoplasts of F177.

a : Protoplast-derived colonies formed in agarose plate after 14 days of culture. Scale bar indicates 100 μm.

b, c : Regeneration of shoot primordia (b) and shoots (c) from protoplast-derived calli.

d : Protoplast-derived plants grown in pot.

were often observed in US76-9.

Plant regeneration from protoplasts of F177 has been reported by Liu [3]. Our results showed a more efficient culture method for plant regeneration from protoplast-derived calli in sugarcane. To date, no information concerning the recovery of plants from sugarcane protoplasts shows as many as presented in this report. Our results described in this report should facilitate further protoplast genetic manipulation studies in sugarcane.

The regenerated plants were transplanted in a field. The studies are under way to determine the presence of somaclonal variation in those plants.

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