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# Culture and regeneration of mesophyll-derived protoplasts of sorghum [*Sorghum bicolor* (L.) Moench]

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**Abstract** A protocol for plant regeneration from mesophyll/protoplasts of sorghum [*Sorghum bicolor* (L.) Moench] was developed. The yield of intact protoplasts, their subsequent divisions and regeneration were genotype-dependent. The genotype 296B was always more responsive than IS 32266. For 296B, the sixth leaf from 18-day-old plants kept in dark for 2 days before harvesting was found to be the most suitable source of viable protoplasts. The first division was observed 10–12 days after plating, and the second division after 12–14 days. The maximum plating efficiency was 4.8% in 296 B, followed by 2.48% in IS 32266. Microcolonies were visible after 25–30 days, and microcalli after 60–75 days. Whole plants were obtained after 6–8 weeks of culture of microcalli on MS medium containing 0.2 mg $l^{-1}$ kinetin and 2 mg $l^{-1}$ BAP. The frequency of regeneration in 296B and IS 32266 was 12.80% and 10.58%, respectively. Ten plants transferred to pots in the glasshouse established well. The seeds collected from glasshouse-grown plants were sown in the field where plants were grown to maturity.

**Key words** Sorghum · (Mesophyll) protoplasts · Cereals · Tissue culture · Regeneration

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**Abbreviations** *BAP* 6-Benzylaminopurine · *MS* Murashige and Skoog's medium · *KM8* Kao and Michyaluk's medium · *NAA* α-Naphthaleneacetic acid · *2,4-D* Dichlorophenoxyacetic acid · *IAA* Indole aceatic acid

## Introduction

The development of efficient and reproducible techniques for the regeneration of fertile plants from protoplasts opens up opportunities for genetic transformation through the direct uptake of DNA or through electroporation (Potrykus 1990). Further, it facilitates the fusion of protoplasts to produce somatic hybrids between incompatible species.

In contrast to several reports on protoplast culture in cereals such as rice and wheat, only five has been published on sorghum. Karunaratne and Scott (1981) were the first to isolate protoplasts derived from 1-week-old sorghum seedlings. Brar et al. (1980) and Chourey and Sharpe (1985) studied the growth of protoplasts derived from suspension cells, but they could not regenerate whole plants. In a preliminary report, Murthy and Cocking (1988 a,b) discussed a few factors affecting mesophyll protoplast yield and observed three to four divisions in 12-day-old cultures. Wei and Xu (1990) reported success in regenerating plants from the protoplasts isolated from inflorescence-derived calli of two sorghum cultivers. In all of these investigations, except for the one by Murthy and Cocking (1988 a,b), cell suspension cultures were used. However, the leaf tissue is the most convenient source of plant protoplasts because it allows the isolation of a large number of relatively uniform protoplasts without destroying the plant or removing the panicle.

We have developed a procedure for regenerating sorghum plants from mesophyll protoplasts. To maximize protoplast yield and the regeneration of plants, we studied the factors that affect protoplast yield and their subsequent division under culture. We

have investigated the effects of media composition and genotype differences and other conditions of the donor (mother) plants such as temperature, light, age and positions of the leaf on protoplast yield and regeneration.

## Materials and methods

Eight sorghum genotypes, 296 B (seed parent), IS 32265, IS 32266, IS 33911, IS 4807 (landrace), M35–1 (selection from landrace), CSH 9 and CSH 12 R (hybrids) were used in an initial experiment designed to choose appropriate genotypes for the subsequent investigation. Detailed studies were conducted only with the two genotypes 296B (most popular seed parent in India) and IS 32266 (a salt tolerant forage variety in northern India). Seeds were sown in a soil-sand (1:1) mix in pots kept in a glasshouse equipped with evaporative coolers to control temperatures (day/night: 30°/20 °C). Unless otherwise mentioned, six recently expanded leaves (as soon as the ligule had just become visible) were harvested and carried to the laboratory in sealed polybags kept on ice.

### Protoplast isolation, culture and regeneration

The leaves were first washed with a solution of detergent (Teepol), then surface-sterilized with 0.1% (w/v) mercuric chloride for 3 min and finally rinsed five times with sterile double-distilled water. The leaves were placed in a sterile chamber, and the midribs were removed before cutting them into 3- to 4-mm-long strips. They were then treated with the enzyme solutions to digest the cell wall.

### Two-step procedure of protoplast isolation

In the first step, the leaf segments were vacuum-infiltrated for 5 min with an enzyme mixture (Table 1) and incubated in dark for 6 h on a rotatory shaker set to 30 rpm. The freed cells were filtered through a nylon mesh and collected after centrifugation (100 *g*, 10 min). In the second step, cells isolated in step 1 were treated with 2% cellulase "Onozuka" RS (Yakulto Co, Japan) for 90 min to dissolve the cell walls. The liberated protoplasts were gathered and washed by centrifugation at 100 *g*.

### Single-step procedure

The leaf strips were plasmolyzed in CPW 13 M (Power et al. 1987) for 1 h before floating on a suitable enzyme mixture. Normally 5.0 ml of enzyme mixture RS10 Y-23 (Table 1) consisting Cellulase, Pectolyase Y 23 (Seishin Co, Japan) was added to 0.2 g of fresh leaf tissue. After an incubation period of 5–6 h the enzyme mixture together with the leaf tissue was filtered through 50 μm mesh. Single protoplasts and cell fragments passed through the mesh, and larger multicellular debris was retained. The filtrate was centrifuged (100 *g*, 10 min) to pellet the protoplasts. The small subcellular debris remained suspended.

The pelleted protoplasts obtained in both procedures were further purified by flotation on 18% sucrose in a 50 ml centrifuge tube and centrifuged at 100 *g* for 5 min using a horizontal rotor (RC5 C, Sorvall instruments, DuPoint, Newtown, Cann.).

### Culture of protoplasts

The isolated protoplasts were cultured in the dark at a concentration of $5 \times 10^5$ ml$^{-1}$ and embedded in KM8 (Kao and Michyaluk 1975) medium solidified with 1.20% agarose (Sea-Plaque agarose, FMC Co, Rockland, Me.) supplemented with 2 mg l$^{-1}$ 2,4-D and 0.5 mg l$^{-1}$ NAA in sterile plastic disposable petri plates (95 × 15 mm, TC-quality, Greiner). These cultures were left undisturbed, and microcolonies were isolated after 20–25 days and cultured on fresh KM8 agarose medium.

### Viability and protoplast density

Staining with fluorescein diacetate (FDA) tested the viability of the protoplasts. With FDA, protoplasts were observed under a fluorescence microscope using a blue filter (380 nm) combination. The concentration of the protoplasts was estimated using a haemocytometer.

### Plating efficiency

This was calculated as the percentage of colonies greater than 0.1 mm in diameter that developed in a plate after 20–25 days of culture.

### Factors affecting the yield and regeneration of protoplasts

*Effect of light and temperature during growth of mother plants*

An experiment was conducted with three replications to determine the effect of light and temperature on protoplast yield and divisions. The main treatments included plants kept: (1) under normal glasshouse conditions (1400–1500 lux, 23 °C), (2) in a partly lighted area inside a service anteroom in a glasshouse with reduced light (650–700 lux, 23–24 °C), and (3) outside the glas-

**Table 1** Compositions of enzyme solutions used for protoplast isolation

| Enzyme 155 13M for two-step procedure | | RS 10 Y23 for single-step procedure | |
|---|---|---|---|
| Macerozyme | 0.5 g l$^{-1}$ | Cellulase | 10 g l$^{-1}$ |
| CPW salts[a] | For 1 l | Pectolyase Y23 | 1 g l$^{-1}$ |
| Mannitol | 130 g | MES buffer | 5 m*M* |
| Ampicillin | 400 mg | CPW salts[a] for 1 l | |
| Gentamycin | 10 mg | | |
| Tetracycline | 10 mg | | |
| Potassium dextran Sulphate | 3 g l$^{-1}$ | | |
| Step 2 | | | |
| Cellulase | 20 g l$^{-1}$ | | |
| CPW salts[a] | For 1 l | | |
| Distilled water to make up to 1 l and filter sterilized | | | |

[a] CPW salts, Power et al. (1987)

shouse in open air (max. light 3000 lux; minimum temperature of 25 °C, and maximum temperature of 32 °C). The three sub-treatments included: mother plants kept in the dark for 1 day, the same for 2 days, and a control (under light, on both days).

### Plant regeneration

Proliferating colonies derived from protoplasts were plated on Murashige and Skoog's (MS, 1962) medium supplemented with 0.2 mg $l^{-1}$ kinetin, and 2 mg $l^{-1}$ BAP and solidified with 1% agarose and cultured at 23 °C under a 16 h photoperiod. Regenerated plants were transplanted to charcoal (0.3%) medium containing half strength MS salts with full-strength vitamins with 0.5 mg $l^{-1}$ IAA. After 15 days, they were transplanted to pots containing sterile vermiculite and soil (1:1) in the glasshouse. All the experiments were repeated several times. Data were analyzed statistically by analysis of variance, and the means were evaluated by standard error.

## Results and discussion

### Isolation of protoplasts

The yield of protoplasts from leaves of different genotypes varied at least twofold. For example, leaves of IS 32265 yielded only $4.5 \times 10^3$ protoplasts per gram of fresh tissue, while those of 296 B produced $6.45 \times 10^5$ protoplasts. Genotypes IS 33911 and IS 4807 failed to respond to culture. Two of the genotypes, 296B and IS 32266, gave consistently better results than the others in all the initial experiments, the plating efficiency was also much better in these two genotypes (Table 2). Therefore, all subsequent experiments were confined to these two genotypes only.

### Single-versus double-step procedure

The isolation of protoplasts with the double-step procedure was quicker than that with the single step. However, the protoplast yields were lower and the subsequent growth was poorer in the double-step procedure than with the single-step procedure. Therefore, we used the single-step procedure in all subsequent experiments.

### Protoplast culture

The optimum period of incubation with enzyme solution RS10 Y23 was found to be 6–8 h at 23 °C. After plasmolysis, to prolong the period of incubation so that protoplasts could be isolated the next morning, we incubated the tissue in a dilute enzyme mixture (1 part of enzyme mixture added to 3 parts of CPW 13 M). Isolation of protoplasts was carried out in hypertonic solutions to avoid protoplast bursting due to internal turgor. Vasil and Vasil (1980) supposed that preplasmolysis reduces the amount of uptake by endocytosis during contraction.

### Viability of protoplasts

The protoplasts isolated after enzymatic dissolution of their walls appeared spherical in shape. A thin membrane-like structure (Fig. 1A) enclosed the densely packed plastids. All freshly isolated protoplasts, when stained with FDA and observed under UV light, fluoresce yellow-green, indicating their viability. Most of the protoplasts remained viable even after 48 h of culture. This was also confirmed by staining with methylene blue. Less than 5% of the protoplasts cultured for 48 h took up stain, indicating the retention of viability in more than 95% of the protoplasts.

### Callus formation and regeneration of plants

#### *Induction of callus*

The protoplasts started regenerating the cell wall within 10–12 days after being embedded in agarose medium (Fig. 2B). This was revealed through staining with calcoflour. Further divisions could be seen after 12–14 days (Fig. 1C,D). Microcolonies in 20- to 25-day-old cultures were visible to the naked eye (Fig. 1E). These colonies continued dividing, leading to small nodular structures. These nodules, when cultured later on KM8 medium, led to the formation of microcalli (Fig. 1F) which were measuring 0.5–1.0 mm in size. These were sub-cultured frequently (every 10–15 days) for further proliferation to form larger calli.

**Table 2** Effect of genotype on plating efficiency of cultured protoplasts

| S/no. | Genotype | Description | Plating efficiency (%) (mean of three replications) |
|---|---|---|---|
| 1 | 296 B | Most popular seed parent of sorghum hybrids | 4.80 |
| 2 | IS 32266 | Salinity-tolerant forage line | 2.48 |
| 3 | IS 32265 | Salinity-tolerant forage line | 0.34 |
| 4 | IS 33911 | Salinity-tolerant forage line | 0.00 |
| 5 | IS 4807 | Salinity-tolerant forage line | 0.00 |
| 6 | M 35-1 | Drought-resistant sorghum | 1.20 |
| 7 | CSH 9 | Hybrid | 1.57 |
| 8 | CSH 12 | Hybrid | 1.19 |

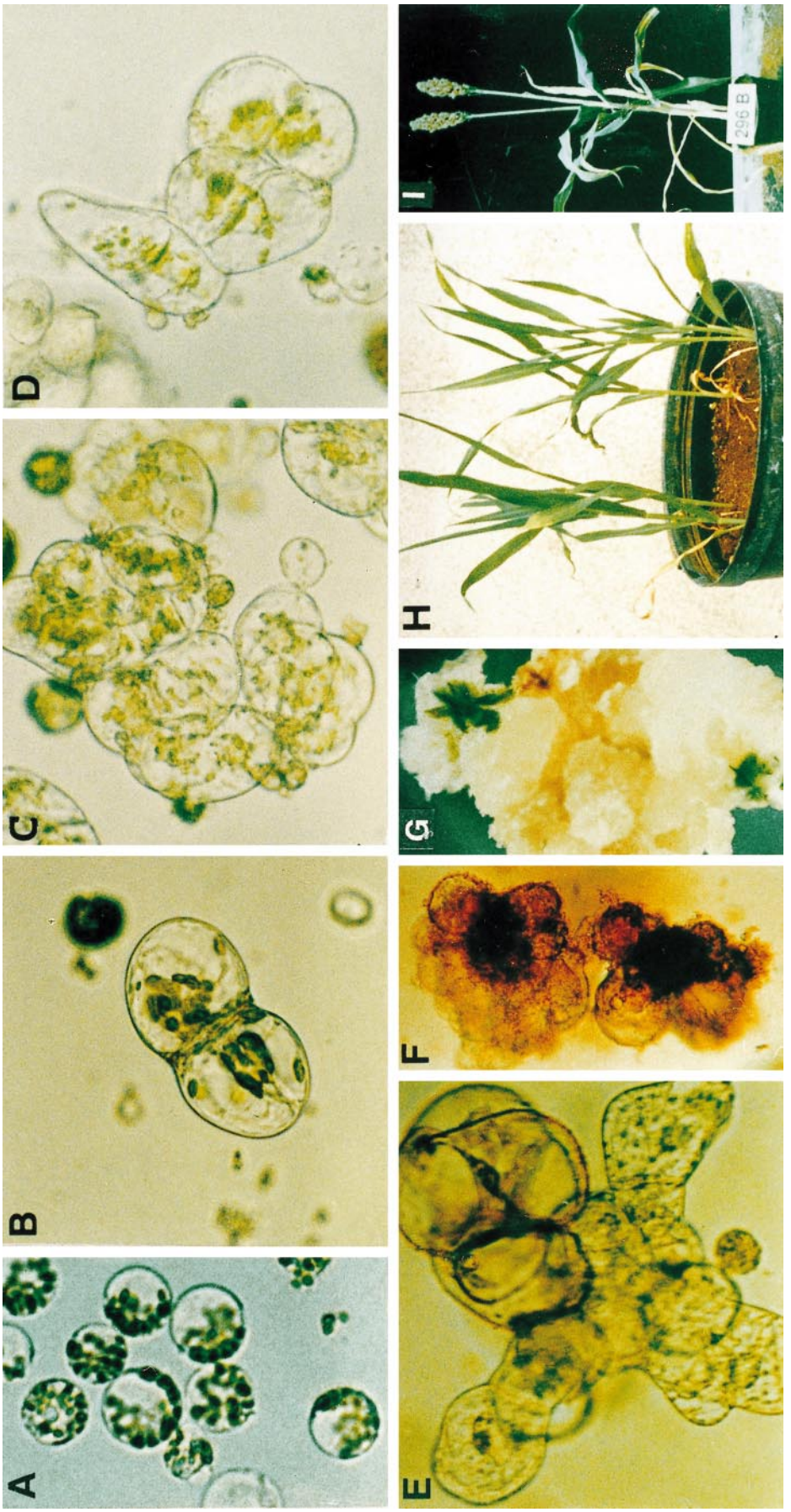


**Fig. 1A–I** Plant regeneration from mesophyll protoplasts of sorghum. **A** Freshly isolated mesophyll protoplasts of genotype 296B, **B** two cell division in protoplasts, **C** 15-day-old dividing protoplasts, **D** 20-day-old dividing protoplasts, **E** microcolonies from 4-week-old cultures, **F** microcalli after 45 days of culture on KM8 medium, **G** calli induced from protoplasts showing green shoots, **H** shoots transferred to soil in glasshouse, **I** fertile plants of 296 B in glasshouse

**Table 3** Effect of genotype on plant regeneration from protoplast-derived calli

| S/no. | Genotype | Micro-colonies | Microcalli (mean)[a] | Number of shoots (mean)[a] | Plant regeneration (%) |
|---|---|---|---|---|---|
| 1 | 296 B | 150 | 60–65 | 8 | 12.80 |
| 2 | IS 32266 | 60 | 18–20 | 2 | 10.58 |

[a] Mean of three replications

*Regeneration of plants from protoplast induced callus*

For shoot induction, calli of 3–4 mm in size were transferred to MS medium fortified with 0.2 mg $l^{-1}$ kinetin and 2 mg $l^{-1}$ BAP. Green spots appeared on the callus 2 weeks after the transfer (Fig. 1G). After the first subculture of callus, up to three shoots appeared from the callus mass. In about 4–5 weeks the shoots rooted and gave rise to plantlets (Fig. 1H). The plantlets established well after transfer to pots (Fig. 1I). The regeneration frequency of 296 B and IS 32266 was 12.80% and 10.58%, respectively (Table 3). The support of feeder cells has been found to be essential for sustained divisions of rice (Gupta and Pattanayak 1993; Ghosh-Biswas et al. 1994) and barley protoplasts (Stöldt et al. 1996). We found it impossible to control the growth of the feeder layer and, therefore, we could not be sure of the origin of the microcolonies. However, in the present investigation the protoplasts of 296B and IS 32266 showed divisions even in the absence of a feeder layer. Plant regeneration from protoplast-derived callus has been achieved with *Pennisetum americanum* (Vasil and Vasil 1980) without the use of nurse cells.

Factors affecting protoplast yield and regeneration

*Position of leaf*

The seventh leaf of 296 B yielded the highest number of intact protoplasts ($6.45 \times 10^5$ $ml^{-1}$) (Table 4). Exactly, the opposite response was seen in IS 32266, in which the third leaf yielded the highest number of protoplasts. The proportion of intact protoplasts was also higher in 296B than in IS 32266. The frequency of division was 4.80% and 2.48% in the sixth and third leaves of 296B and IS 32266, respectively. These results highlight the importance of testing the suitability of starting material for each genotype and are consistent with the earlier studies of Murthy and Cocking (1988a,b).

*Age of the leaf*

The effect of leaf age on protoplasts was statistically significant. The maximum yield of intact protoplasts and their division frequency was observed in protoplasts isolated from a fully emerged leaf (ligule just visible). The ratio of intact to broken protoplasts was nearly the same for leaves of all ages except for the leaf with the just-emerged ligule, in which case it was three times higher.

*Effect of growth conditions of mother plants (light and temperature)*

The plants grown in the glasshouse and retained in the dark for 2 days yielded nearly two-three times more intact protoplasts than plants grown in dark for 1 day and also the control plants (Table 5). Shepard and Totten (1977) reported that the exposure of donor plants or explants of *Solanum* and *Lycopersicum* to low-light intensities, shortened photoperiods or even

**Table 5** Effect of growth conditions (light and temperature) of mother plants on protoplast yield

| Treatment/ environment | Intact protoplasts ($\times 10^5$ $g^{-1}$) when kept under | | |
|---|---|---|---|
| | 2 days in dark | 1 day in dark | Control |
| Glasshouse | 8.25 | 3.00 | 2.25 |
| Corridor | 7.50 | 1.87 | 3.75 |
| Sun | 6.88 | 1.88 | 3.38 |

**Table 4** Effect of leaf position (at nodes 3, 5, 6 and 7) on yield and division of protoplasts in genotypes 296 B and IS 32266

| Leaf position | Intact protoplasts of 296 B ($\times 10^5$ $ml^{-1}$) | Division (%) | Intact protoplasts of IS 32266 ($\times 10^5$ $ml^{-1}$) | Division (%) |
|---|---|---|---|---|
| 3 | 2.95 | 0.41 | 4.05 | 2.48 |
| 5 | 3.25 | 2.14 | 3.50 | 0.63 |
| 6 | 5.22 | 4.80 | 1.78 | 1.60 |
| 7 | 6.45 | 2.12 | 1.10 | 0.54 |

the dark for a defined period of time resulted in a consistently higher yield of viable protoplasts. One explanation for these results could be the synthesis of thinner cell walls with lower concentrations of pectate at low light intensities (Cassels and Barlass 1976). The other reason could be the mobilization of starch deposited in starch grains which otherwise would disrupt fragile protoplasts during plasmolysis or centrifugation (Chang and Loescher 1991).

Thus, our results confirm that mesophyll tissue of sorghum is as good a source material for protoplast isolation and culture as in other cereals. These results should encourage the use of sorghum protoplasts for gene transfer and protoplast fusion to generate novel genotypes of sorghum.

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