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Plant regeneration from protoplasts of embryogenic suspension cultures of orchardgrass (*Dactylis glomerata* L.)

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ABSTRACT

An embryogenic suspension culture of orchardgrass (*Dactylis glomerata* L.) consisting of small, embryogenic cell clusters was obtained from callus formed on basal sections of young leaves through a process of selective enrichment. These suspensions were used as a source of protoplasts. The isolated protoplasts divided at a frequency of 0.5-10% when plated in an agarose solidified culture medium. Conditioned medium, in which embryogenic *Dactylis* suspension cultures had been grown, was found to increase the rate of cell colony formation. Protoplast-derived colonies grew rapidly in a bead-type culture system of floating agarose slabs in liquid medium. New suspension cultures formed as the colonies grew out of the agarose. These cultures were embryogenic and formed green plantlets when plated on a solid medium lacking auxin. The plantlets were established in soil and grown to mature plants.

Abbreviations: B5, medium according to Gamborg *et al.*(1968); SH-x, medium according to Schenk and Hildebrandt (1972) supplemented with x μM dicamba; dicamba, 3,6-dichloro-o-anisic acid; KM-8p, medium 8p of Kao and Michayluk (1975).

INTRODUCTION

The monocotyledenous plant family of the *Gramineae* contains a large number of economically important species. It includes maize, sorghum, sugarcane, rice, wheat and barley, as well as important forage crops such as *Festuca*, *Lolium* and *Dactylis*. The *Gramineae* have also been among the most difficult to manipulate *in vitro* (Vasil 1988). In particular, the regeneration of whole soil-grown plants from protoplast-derived cells and tissues has been accomplished only recently in sugarcane (Srinivasan and Vasil 1986), in rice (Abdullah *et al.* 1986; Thompson *et al.* 1986; Yamada *et al.* 1986; Kyozuka *et al.* 1987), in maize (Rhodes *et al.* 1988) and two forage grasses, *Lolium multiflorum* and *Festuca arundinacea* (Dalton 1988).
Orchardgrass (*Dactylis glomerata* L.) is an important hay crop in the central United States and Europe. Conger and colleagues have described the regeneration of orchardgrass plants from somatic embryos formed on callus or in suspension cultures (Hanning and Conger, 1982; Gray *et al.* 1984). In this species, somatic embryos together with embryogenic callus were shown to form directly from basal sections of young leaves. Embryogenic callus has subsequently been used to initiate heterogenous submersed callus cultures which were capable of differentiating somatic embryos (Gray *et al.* 1984).

Here we report the successful regeneration of orchardgrass plants from cultured protoplasts. The protoplasts were isolated from an improved suspension culture consisting predominantly of small embryogenic cell clusters. Callus produced from the protoplasts was able to regenerate plants. The regenerated plants were then established in soil.

MATERIALS AND METHODS

Induction and culture of embryogenic callus. Embryogenic callus was initiated from basal sections of the youngest, innermost leaves of greenhouse-grown orchardgrass plants (supplied by B.V. Conger) as previously described by Hanning and Conger (1982). Sterilized leaf sections were placed on Schenk and Hildebrandt (1972) medium (SH medium) containing 30 μm dicamba and solidified with 0.8% (w/v) SeaPlaqueR agarose (FMC Corp.) in the dark at 25°C. Callus appearing within 2-6 weeks after plating was maintained by subculturing onto the same medium every 2-4 weeks.

Preparation of an embryogenic suspension culture. Embryogenic suspension cultures were initiated by putting 0.5 g of embryogenic callus into 50 ml of SH medium containing 45 μM dicamba and 4 g/l casein hydrolysate as described by Gray and Conger (1985). The suspension cultures were grown at 27°C under a photoperiod of 16 h light (40 $\mu Em^{-2}s^{-1}$) and 8 h dark on a gyratory shaker at 130 rpm in 125 ml Delong flasks sealed with a metal cap and parafilm. The light source used was Sylvania 'Cool White' fluorescent tubes, unless otherwise specified. After approximately four weeks the larger cell clumps in the suspension were allowed to settle and 10 ml aliquots of the supernatant containing small cell clusters were removed and transferred to 50 ml of fresh medium. This process was repeated every 3-4 weeks.

Plant regeneration from embryogenic suspension cultures. Embryogenic cell clusters were plated onto solid SH medium lacking an auxin-type growth regulator for embryo development. Mature embryos germinated to produce plantlets which were transplanted to soil at the 6-12 leaf stage, hardened off and grown to maturity in the greenhouse as described below.

Isolation and purification of protoplasts. Protoplasts were isolated from the small embryogenic cell clusters of the suspension cultures. The cells were collected onto a 0.2 μm filter membrane (Nalgene, Inc.). About 0.5 g fresh weight cells were then added to each 12.5 ml of filter-sterilized protoplast enzyme mixture in a petri dish. The enzyme mixture which gave the highest protoplast yield and subsequent plating efficiency consisted of 2% (w/v) Cellulase 'Onozuka' RS (Yakult Honsha Co. Ltd., 1.1.19 Higashi-Shinbashi, Minato-ku, Tokyo 105, Japan), 7 mM $CaCl_2$, 0.7 mM NaH_2PO_4, 3 mM morpholinoethane sulfonic acid (MES; pH 5.6) and glucose (approx. 0.45 M to give an osmolality of 550 mOs/kg H_2O). The cells in enzyme mixture were placed on an orbital shaker at 50 rpm in dim light (< 5 $\mu Em^{-2}s^{-1}$) for about 4 hours at room temperature. Protoplast release was monitored under the inverted microscope. When sufficiently digested, the mixture was filtered through a stainless steel sieve (94 μm mesh), distributed into 12 ml sterile plastic centrifuge tubes and centrifuged at about 100 x*g* for 10 min. The sedimented protoplasts were washed three

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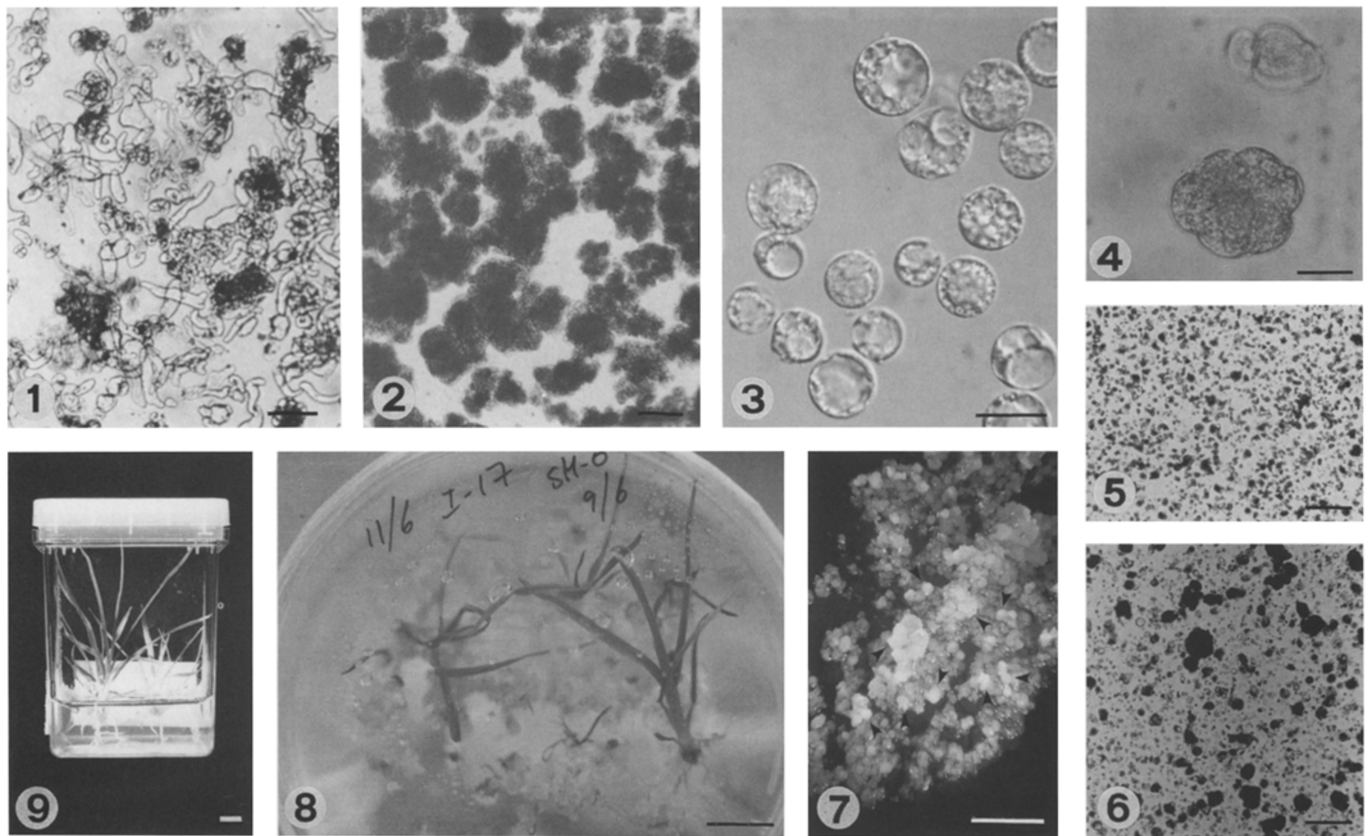

Figures:

1. Coarse suspension culture of *Dactylis glomerata* after initiation from embryogenic leaf base derived callus. Note heterogeneity and relative abundance of different cell types. Bar = 100 μm.
2. Improved embryogenic suspension culture after enrichment for small clusters of embryogenic cells. Bar = 100 μm.
3. Freshly isolated protoplasts from embryogenic suspension cultures. Bar = 20 μm.
4. Cell division and cell colony formation from protoplasts plated in agarose, 15 days after plating in KM-8p medium. Bar = 100 μm.
5. Cell division and cell colony formation from protoplasts plated in agarose, 15 days after plating in KM-8p medium. Bar = 1 mm.
6. Effect of conditioned medium on colony formation from agarose plated protoplasts, 15 days after plating in a 1:1 mixture of KM-8p and conditioned suspension culture medium. Bar = 1 mm.
7. Protoplast-derived cell colonies plated on SH-30 medium showing signs of somatic embryo formation and differentiation (arrows). Bar = 5 mm.
8. Germination of somatic embryos and development of *D.glomerata* plantlets from embryogenic callus derived from cultured protoplasts. Bar = 1 cm.
9. *D.glomerata* plantlets derived from cultured protoplasts, growing in a GA7 container. Bar = 1 cm.

times with protoplast culture medium (KM-8p with glucose (approx. 0.4 M for a final osmolality of 550 mOs/kg H_2O). In some experiments the washed protoplasts were layered atop 10 ml of KM-8p culture medium made 700 mOs/kg H_2O with sucrose. After centrifugation for 10 min at about 100 xg, protoplasts banding at the interface were collected.
Finally, the protoplasts were resuspended in KM-8p culture medium and filtered through a 20 μm mesh size stainless steel mesh screen.

Protoplast culture. The purified protoplasts were plated at a density of $5x10^5$ protoplasts per ml in KM-8p culture medium containing 1.2% (w/v) SeaPlaqueR agarose (3 ml per 6 cm diameter petri dish). In some experiments, conditioned medium was added to the KM-8p medium in a proportion of 10 to 50%. The conditioned medium was prepared from embryogenic *Dactylis* suspension cultures 3 to 4 weeks after subculturing to fresh medium. The medium was filtered free of cells, its osmolality was adjusted to 550 mOs/kg H_2O with glucose and the pH readjusted to 5.8. The medium was then filter-sterilized and mixed in the desired proportion with KM-8p medium. Protoplasts were plated as above.
The plates were incubated in the dark at 28°C. After 14 days the agarose was cut into slices which were placed into 'bead culture' Shillito *et al.* 1983) using 20 ml of liquid SH-45 medium supplemented with 4 g/l casein hydrolysate per each 3 ml of original agarose embedded culture. The plates were placed on a platform shaker and agitated at 50 rpm in the light (8 $\mu Em^{-2}s^{-1}$). Suspension cultures were formed as the colonies grew out of the agarose and released cells into the liquid medium. The resultant suspensions were plated onto SH-30 medium solidified with 0.8% agar and placed in the dark at 25°C.
Alternatively, individual cell colonies growing out of the agarose medium were picked and transferred to callus maintenance medium. The callus was subcultured every 3-4 weeks on the same medium.

Regeneration of plants. For regeneration, the callus was plated on SH-0 medium solidified with 0.24% (w/v) Gelrite, and placed in the light (30-60 $\mu Em^{-2}s^{-1}$). The embryo-forming callus and the maturing somatic embryos were subcultured every 2-3 weeks. Embryos germinated to form small plantlets. The plantlets were transferred onto the same medium or onto Murashige and Skoog (1962) medium with the vitamins of B5 medium (Gamborg *et al.* 1968), and solidified with 0.8% purified agar (Difco DF 0560-01) for rooting. Lighting was provided from equal numbers of Grolux and 'Daylight' fluorescent tubes at 60-100 $\mu Em^{-2}s^{-1}$. Plantlets were moved to the greenhouse when roots were adequately developed. Plantlets were potted in Metromix 220 (Grace Products, Cambridge, Mass., USA) in 7.5 cm peat pots, and covered with

clear plastic cups and cheesecloth to reduce water loss and light intensity. The plants were fertilized twice in the first week with Peters Plant Starter (9/45/15; Grace Products). The cups were removed after 5 days, and the cloth 2 days later. The plants were transferred to 15 cm pots when the roots reached the sides of the peat pots. Plants were fertilized weekly with Peters 20/20/20 fertilizer. Plants were grown in natural light with supplementary lighting to 300 $\mu Em^{-2}s^{-1}$ from high pressure sodium lamps to give a 16 hour day, with 22°C day and 18°C night temperature.

RESULTS AND DISCUSSION

Suspension cultures. By subculturing the supernatant after allowing the larger cell clumps to settle it was possible to gradually enrich the proportion of small embryogenic cell clusters relative to the non-embryogenic cells. After 5-8 transfers the remaining suspensions displayed properties quite different from their initial appearance: These suspensions were finely dispersed and uniform and essentially free of non- embryogenic cells. The majority of the embryogenic cell clusters were about 150-200 μm in diameter. Figures 1 and 2 show the different appearance of freshly initiated coarse suspensions (Fig. 1) as compared to an improved culture after several rounds of selective subculturing (Fig. 2). Selective subculturing may be used to maintain the special phenotype of these suspensions. Sieving was not usually required.
When selective subculturing was practiced for several subcultures, it was noted that the propensity of somatic embryos to mature in the suspension was gradually reduced. As a result the suspensions became more and more homogenous, consisting predominantly of small clusters of densely cytoplasmic cells and representing very early developmental stages of somatic embryos. When plated on solidified SH-30 medium, the cultures proliferated as a callus containing abundant embedded embryos and embryogenic cell clusters. When plated on solid SH medium lacking dicamba (SH-0), the embryogenic cell clusters readily developed into the later stages of somatic embryos. Within the first eight months of suspension culture close to 100% of plated mature embryos were able to germinate and form whole, apparently normal plants. Plants were regenerated from the embryogenic suspensions for more than two years.

Protoplast culture. The enzyme mixture described above is a modification of one by Lu *et al.* (1981) and was found to be superior to others tested. Yields of 5 to 30x10^6 protoplasts per gram fresh weight of suspension culture cells were obtained using this mixture. With regard to yield and subsequent plating efficiency, glucose was clearly superior to sucrose as the osmoticum used during the isolation of protoplasts. Protoplasts obtained after sieving through the 20 μm mesh screen averaged 12-15 μm in diameter and were densely cytoplasmic (Fig. 3). Contamination of the protoplast preparation not floated on sucrose with whole undigested cells was generally less than 0.1% and was even less when flotation on sucrose was used. However, plating efficiencies were lower when the sucrose flotation step was included. Protoplast yields and subsequent plating efficiencies were optimal if the source suspension cultures were subcultured 7 to 10 days prior to protoplast isolation.
The first cell divisions were seen two days after plating the protoplasts. Subsequent divisions occurred in a non-synchronous fashion as first divisions, together with small cell colonies (Fig. 4), could still be seen after 7 days.
Of several culture media tested, KM-8p gave the best plating efficiency. Others tested were RY-2 (Yamada *et al.* 1986), CC (Potrykus *et al.* 1979), and SH-30 (Hanning and Conger 1982), all appropriately adjusted to 550 mOs/kg H_2O with glucose. Using KM-8p culture medium the formation of macroscopic cell colonies capable of forming callus varied from 0.5 to 10% in different experiments. Glucose was superior to sucrose in supporting growth of freshly plated protoplasts.
The addition of 30-40% (v/v) conditioned suspension culture medium to the protoplast culture medium was found to accelerate cell division in the protoplast-derived colonies formed within the first weeks of culture. Figures 5 and 6 show the difference in colony size after 15 days using supplementation with conditioned medium (Fig. 6) as compared to unsupplemented medium (Fig.5). Good colony growth was observed when KM-8p medium with glucose or sucrose was used as the liquid component of the bead

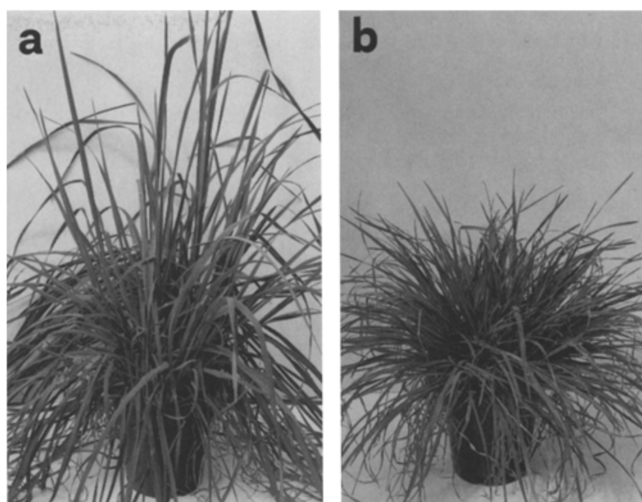

Figure 10:
a) *Dactylis glomerata* (orchardgrass) plant derived from seed.
b) *Dactylis glomerata* plant derived from cultured protoplasts about 3 months after establishment in soil.

culture system. However, colonies grew faster in SH-45 medium supplemented with 4 g/l casein hydrolysate (the medium used to maintain the source suspension cultures). Within two to three weeks after initiating the bead cultures, new suspension cultures could be observed in the plates. Microscopic examination of the agarose slabs revealed that some of the colonies closest to the surface had grown out into the liquid and were apparently releasing small clumps of cells while still anchored in the agarose. The new suspensions grew rapidly, and after another two weeks were transferred as suspension cultures in the usual manner or plated onto SH-30 for callus proliferation and plant regeneration.

Regeneration of Plants. Maturation of somatic embryos occurred (Fig. 7) when the suspension cultures obtained from protoplasts were plated onto SH-30 medium for two to five weeks and subsequently onto SH-0 medium. Plantlets formed on the callus (Fig. 8) over a period of more than one year. All of the plantlets formed were green. Plantlets were removed from the callus and transferred to fresh SH-0 medium for further growth (Fig. 9). Initially, some plantlets had somewhat narrow or twisted leaves, and tillers were produced in profusion. When tillers were separated and subcultured to fresh medium, plantlets with a normal morphology were obtained. The production of one to five tillers on each plant during each subculture afforded an easy way to propagate the plantlets in culture, while also allowing clonal plants of the same origin to be transferred to the greenhouse. Roots formed on SH-0 medium were thick, and had few laterals. However, plantlets grew better on this medium than on MS medium when first removed from the callus. Roots formed in MS medium were thinner and formed abundant laterals. Consequently, plantlets were moved to the MS medium once roots had formed.
At the six to twelve leaf stage, plants were transferred to the greenhouse (Fig. 10), hardened off gradually as described, and grown to maturity. The transplanting efficiency generally was above 80%. Once transplanted to soil most of the plants regenerated were normal. However, some had variations in leaf width, and occasionally twisted leaves were observed. This variation was not unexpected and may result from physiological disturbances related to the *in vitro* culture conditions.

Our results have demonstrated the regeneration of whole plants from cultured protoplasts of the grass species, *Dactylis glomerata* L. The procedure described uses protoplasts from embryogenic suspension cultures as the starting material. In using actively growing tissue with proven plant regeneration potential we hoped to obtain protoplasts which would divide and form plants. In at least some species of the *Gramineae*, embryogenic suspension cultures have been shown to yield protoplasts which could be induced to divide and regenerate to whole plants (Vasil 1988).

We believe the early, heterogenous, unselected suspensions of *Dactylis* cells to be a poor starting material for the isolation of protoplasts which are capable of dividing, forming embryogenic callus and regenerating plants. Protoplasts were difficult to isolate and did not divide (Conger, unpublished results). However, when the suspension cultures were enriched for small embryogenic cell clusters, protoplast cultures capable of dividing and forming plants have been obtained. Hence, the results of the present work suggest that the inherent quality of the source suspension, i.e. its composition of small embryogenic cell clusters, has a controlling effect on the subsequent protoplast culture. The procedure we describe has been successful in producing embryogenic suspension cultures from which large numbers of plants can be regenerated. These suspension cultures also exhibit a greatly improved capacity to yield protoplasts capable of dividing and forming plants. With a working protoplast culture system now developed, experiments aimed at direct gene transfer, protoplast fusion and organelle transfer are feasible.

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REFERENCES

Abdullah R, Cocking EC, Thompson JA (1986) Bio/Technology 4:1087-1090

Dalton SJ (1988) J Plant Physiol 132:170-175

Gamborg OL, Miller RA, Ohyama K (1968) Exptl Cell Res 50:151-158

Gray DJ, Conger BV, Hanning GE (1984) Protoplasma 122:196-202

Gray DJ, Conger BV (1985) Plant Cell Tissue Organ Cult 4:123-133

Hanning GE, Conger BV (1982) Theor Appl Genet 63:155-159

Kao KN, Michayluk MR (1975) Planta 126:105-110

Kyozuka J, Hayashi Y, Shimamoto K (1987) Mol Gen Genet 206:408-413

Lu C-Y, Vasil V, Vasil IK (1981) Z Pflanzenphysiol 104: 311-318

Murashige T, Skoog F (1962) Physiol Plant 15:473-497

Potrykus I, Harms CT, Lörz H (1979) Theor Appl Genet 54:209-214

Rhodes CA, Lowe K, Ruby K (1988) Bio/Technology 6:56-60

Schenk RV, Hildebrandt AC (1972) Can J Bot 50:199-204

Shillito RD, Paszkowski J, Potrykus I (1983) Plant Cell Rep 2:244-247

Srinivasan C, Vasil IK (1986) J Plant Physiol 126:41-48

Thompson JA, Abdullah R, Cocking EC (1986) Plant Sci 47:123-133

Vasil IK (1988) Bio/Technology 6:397-402

Yamada Y, Zhi-Qi Y, Ding-Tai T (1986) Plant Cell Rep 5:85-88.