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Plant regeneration from embryogenic cell suspension cultures of wild sorghum (*Sorghum dimidiatum* Stapf.)

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Abstract A simple and efficient protocol is described for regeneration of wild sorghum (*Sorghum dimidiatum*) from cell suspension cultures. Fast-growing cell suspensions were established from shoot-meristem-derived callus. Plating of the suspension on Murashige and Skoog agar medium supplemented with 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in the formation of embryogenic calli. High-frequency (80%) somatic embryogenesis from small cell clusters (300–400 μm) was observed when the cultures were initially maintained in liquid medium with reduced levels of 2,4-D (0.25 mg l^{-1}), followed by transfer to regeneration medium. Direct plating of these small clusters on regeneration medium or transfer to liquid regeneration medium containing kinetin and 6-benzylaminopurine resulted in the development of mature somatic embryos and plantlets. The regenerants developed to maturity and were all phenotypically and cytologically normal.

Key words *Sorghum* species · Cell suspension · Somatic embryogenesis · Plant regeneration

Abbreviations *BAP* 6-Benzylaminopurine · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *MS* Murashige and Skoog

Introduction

Totipotent embryogenic cell suspension cultures facilitate the generation of mutant or variant cell lines, isolation of protoplasts, genetic transformation, and physiological and biochemical studies. Somatic embryogenesis in suspension cultures was first encountered in cultivated carrot, *Daucus carota* (Steward et al. 1958). Since then, it has been observed in many dicotyledons and most gramineous species (Vasil 1987).

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most important cereal crop of arid regions in the world and is used for food, spirits, vinegar, pulp and feed. As in other cereal crops, wild species represent an important source of germplasm, as repositories of genes for conferring resistance to a wide range of pathogens and tolerance to environmental stress (Brown 1982). Since wild species of sorghum cannot be readily crossed with cultivated sorghum, modern biotechnological methods such as somatic hybridization must be developed for transferring useful genes.

A number of studies have been carried out on tissue culture of cultivated sorghum (Gamborg et al. 1977; Brettel et al. 1980; Wernicke and Brettell 1982; Bhaskaran et al. 1987; Wen et al. 1991; Mythili et al. 1997). Plant regeneration from wild species, viz., *S. arundinaceaum* (Boyes and Vasil 1984; Guo and Liang 1993), *S. almum* (George and Eapen 1988; Guo and Liang 1993), *S. versicolor* (Eapen and George 1990; Guo and Liang 1993), *S. nilolium*, *S. miliaceum*, *S. virgatum*, *S. plumosum*, *S. sudanensis* and *S. aethiopicum* (Guo and Liang 1993), has also been reported. However, there are very few reports on cell suspensions of cultivated and wild sorghums (Chourey and Sharpe 1985; Wei and Xu 1990). In this communication, we describe the establishment of cell suspension cultures and high-frequency somatic embryogenesis in a wild species of sorghum, *S. dimidiatum*, as an initial step in our efforts to develop efficient methods for somatic hybridization in this crop plant.

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Materials and methods

Establishment of cell suspensions

Three-month-old rapidly growing friable yellowish embryogenic calli were used to initiate cell suspension cultures. The calli were initiated from the shoot meristem (0.5–0.8 mm in length) using the pro-

tocol developed by N. Seetharama, R. V. Sairam, and T. Shyamala (unpublished data). For culture initiation, approximately 1 g of callus was teased into small pieces and transferred to 100-ml flasks containing 25 ml of the culture medium. The initial culture medium was composed of Murashigae and Skoog (1962) (MS) salts with 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Cultures were placed on a gyratory shaker set to 100 rpm in a dark room at 23±2 °C. Every 3–4 days, half of the supernatant was removed and replaced by an equal volume of fresh medium (Vasil and Vasil 1982). This procedure was repeated twice a week for 3 months. Established suspensions were maintained by adding 5 ml of the culture to 25 ml of fresh medium (MS + 2.5 mg l^{-1} 2,4-D) at 5-day intervals.

Growth measurements

A three-month-old embryogenic suspension was used for growth studies. Fresh weight, dry weight and packed cell volume (PCV) were measured at 2-day intervals from the day of subculture (day 0) to 10 days. The growth rate is represented as the percentage increase in growth rate. Cell fresh weight was obtained by removing the medium from 5 ml of suspension by passing it through a preweighed filter paper followed by reweighing. The dry weight was recorded after drying the sample overnight in an oven at 60 °C. PCV was determined by centrifuging 5 ml of suspension at 2000 rpm for 5 min. Five replicates were used for each experiment. For studying callus induction, 5 ml of suspension was plated directly onto solid medium supplemented with the same concentration of 2,4-D used for culture initiation.

Somatic embryogenesis and plant regeneration

The regeneration capacity of suspension cultures was studied after transfer to solid or liquid regeneration medium. Three methods were used to initiate somatic embryos in suspension cultures (1) Transfer of 4-week-old callus produced from suspension plated on callus induction medium to regeneration medium [MS + 0.5 mg l^{-1} kinetin + 0.5 mg l^{-1} 6-benzylaminopurine (BAP)]. (2) Direct dispensing of suspension on regeneration medium. (3) Regeneration completely in liquid medium. For the second and third methods, subculture of suspensions was continued with medium containing three different reduced concentrations of 2,4-D (1.0, 0.5, 0.25 mg l^{-1}) for 2–3 weeks. At the end of the 3rd week, suspension was directly plated on regeneration medium for method 2, and subculture was continued with liquid regeneration medium for method 3. At this stage, the liquid cultures were maintained on a gyratory shaker at 25 rpm in a culture room (23 °C day/night with 16-h photoperiod at 2000 lux). For regeneration studies using methods 2 and 3, MS medium supplemented with two different concentrations (0.5 and 0.25 mg l^{-1}) of kinetin and BAP separately, or both together at 0.25 mg l^{-1}, were used.

For mitotic analysis, root tips of regenerated plants were pretreated with cold water (0–4 °C) for 24 h, fixed in 3:1 (wt/vol) solution of methanol and glacial acetic acid and stored in 70% methanol until further use. Prior to cytological analysis, material was hydrolyzed in 1 N HCl at 60 °C for 10 min, washed thoroughly, blotted and stained with 1% Feulgen prepared following the method of Simpson (1982).

Results

A fine cell suspension was established after 3 months of repeated subculture. The cell suspensions at this stage were well dispersed and contained small groups of embryogenic cells (Fig. 1A) which were small, actively dividing, and richly cytoplasmic. Changes in the PCV, fresh weight, and dry weight are shown in Fig. 2. There was no marked lag phase after transfer of cells to fresh medium. Growth measurements showed a rapid increase in PCV after 6 days. The maximum PCV was achieved on the 8th day after subculture. By the end of the subculture period, the cell fresh weight had nearly tripled. Very little increase in cell dry weight was observed between day 6 and 8. The suspension showed no change in growth rate during weekly subcultures for 6 months. Suspensions plated on MS medium solidified with 0.8% agar and 2.5 mg l^{-1} 2,4-D formed friable, yellowish embryogenic calli within 4 weeks (Fig. 1B). The callusing ability of the small clusters was much lower than that of larger clusters measuring 0.5–1.0 mm in diameter (Table 1). Plants could be directly regenerated from these small clusters without plating them on callus induction medium.

Four-week-old calli from plated suspension, when transferred to solid regeneration medium, became nodular and formed somatic embryos in 3–4 weeks (Table 1, Fig. 1D). Plantlets with green shoots and roots were developed from the embryos after 2–3 weeks. The percent somatic embryogenesis in the small clusters was very low (Table 1). When the suspension containing small clusters was maintained in liquid medium containing reduced levels of 2,4-D, the percent embryogenesis increased. In the medium containing 0.25 mg l^{-1} 2,4-D, the frequency of embryogenesis was 60% (Table 2). Development of many organized structures (proembryos) with a distinct epidermis was observed in the liquid medium. They showed no further growth when maintained in the same medium. However, if they were directly plated on MS regeneration

Table 1 Effect of cell cluster size on embryogenic callus formation and regeneration in *Sorghum dimidiatum*

Cluster size	Number of calli developed	Embryogenesis (%)
0.5–1.0 mm	139.4±12.91	86.02±2.01
300–400 µm	67.0±8.36	44.50±1.58

Table 2 Effect of 2,4-D concentration on embryogenesis of small clusters in *S. dimidiatum*

2,4-D (mg l^{-1})	Number of clustered observed	Embryogenesis (%)
1.0	250	45.60±3.78
0.5	300	51.66±5.47
0.25	240	60.00±9.35

Table 3 Effect of kinetin and BAP on somatic embryogenesis in small clusters in *S. dimidiatum*

Hormone	Concentration (mg l^{-1})	Embryogenesis (%)	Number of plantlets/ aggregate
Kinetin	0.5	32.1±5.0	1.8
	0.25	55.0±7.90	3.2
BAP	0.5	40.0±3.53	2.0
	0.25	45.2±9.75	2.8
Kinetin + BAP	0.25 0.25	80.0±7.90	3.4

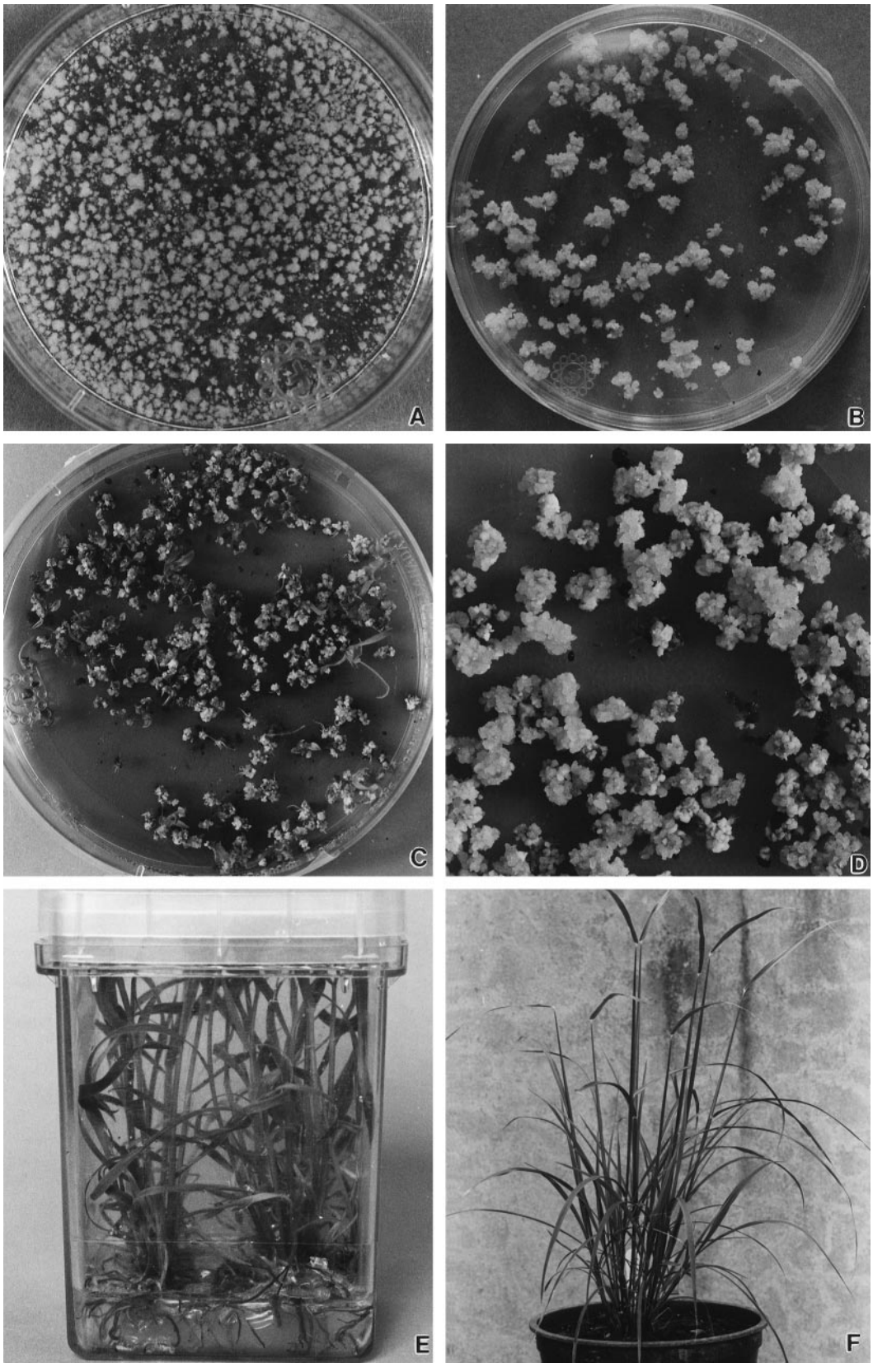

Fig. 1A–F Regeneration of fertile plants from suspension cultures of *Sorghum dimidiatum*. **A** Established cell suspension showing clusters of cells. **B** Cell suspension plated showing development of embryogenic calli. **C** Suspension directly plated on solid regeneration medium showing differentiation. **D** Suspension-derived calli showing embryo formation. **E** Rooted regenerated plantlets. **F** Regenerated plants in glasshouse

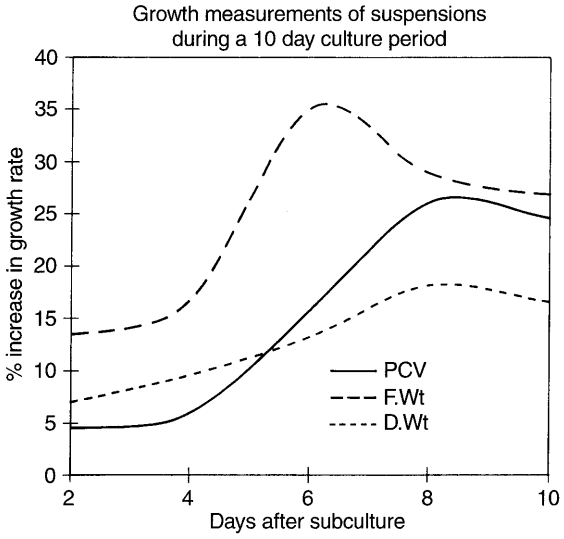

Fig. 2 Growth curve of cell suspension cultures of *S. dimidiatum* (*PCV* packed cell volume, *F.Wt* fresh weight, *D.Wt* dry weight)

medium containing kinetin and BAP, further differentiation took place in 2–3 weeks and plantlets with green shoots and roots were observed (Fig. 1C). Kinetin and BAP in combination (at 0.25 mg l^{-1}) promoted greater somatic embryogenesis (Table 3) than when each was added separately to the culture medium. Under these optimal conditions, each aggregate produced three to four plantlets. The plantlets were transferred to MS basal medium for further growth and establishment (Fig. 1E). The plants were finally transferred to soil, moved to a greenhouse (Fig. 1F), and grown to maturity.

The regeneration potential of the small clusters was also studied by retaining them in liquid regeneration medium. In the first step, when the suspensions were maintained in liquid medium with 0.25 mg l^{-1} 2,4-D, a number of embryos developed from the peripheral regions of the small clusters. Various embryo developmental stages were seen 6–10 days after resuspending the suspensions in liquid regeneration medium containing 0.25 mg l^{-1} kinetin and 0.25 mg l^{-1} BAP (Fig. 3A). The embryoids (50–60%) germinated while still in the liquid medium (Fig. 3B) and developed into plantlets, which appeared phenotypically normal. Budding from the developing embryoids and secondary proliferation of embryoids was commonly observed. However, further studies are needed on the conditions responsible for this type of proliferation and plantlet recovery from these embryoids. The regenerated plants flowered and were fertile. Karyotypes from a minimum of 50 root meristems of the regenerated plants showed a normal somatic chromosome number of 2n = 10.

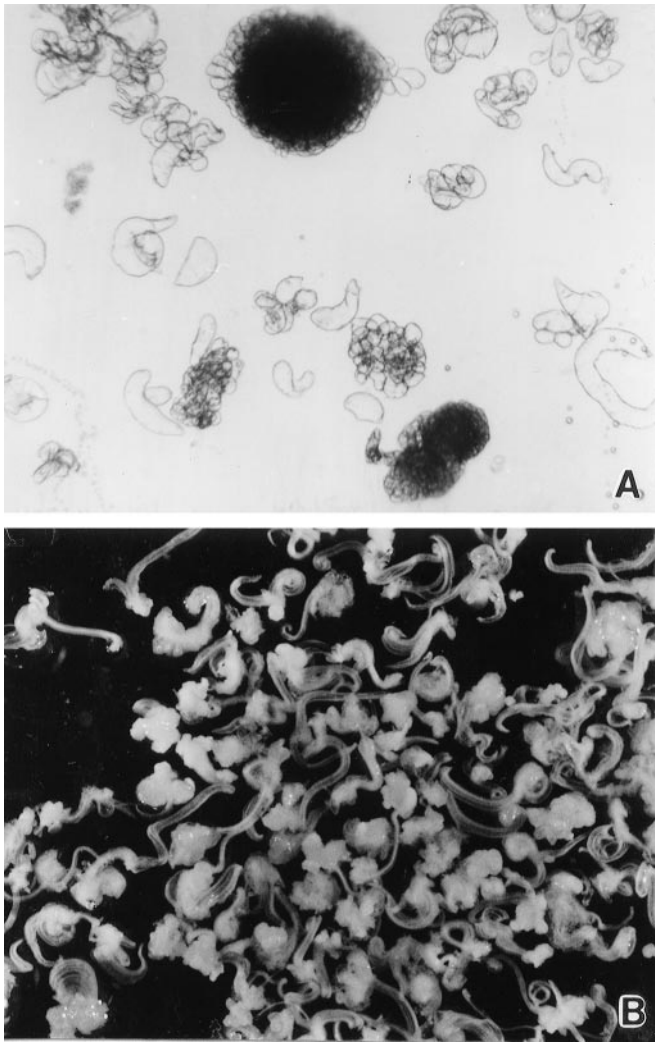

Fig. 3A, B Different developmental stages of somatic embryos and regeneration from small clusters of suspensions of *S. dimidiatum* **A** Different stages of somatic embryo development. **B** Germination of somatic embryos in liquid medium

Discussion

The availability of cell suspensions with high regeneration capacity is crucial for successful isolation of protoplasts, and for transformation experiments in monocotyledonous species (Vasil 1987). As with the successful reports of regeneration from embryogenic suspensions of other cereal crops (Lu and Vasil 1981; Vasil and Vasil 1981, 1982, 1986; Green et al. 1983; Ho and Vasil 1983; Wang and Nguyen 1990; Jahne et al. 1991), the protocol described here will facilitate the application of advanced technologies for sorghum. Embryogenic suspensions were friable and fast growing without any marked lag phase, which is essential for the maintenance of embryogenic capacity. Similar results have been reported for *Panicum* and *Pennisetum purpureum* (Vasil and Vasil 1984; Karlsson and Vasil 1986). In the present study, both the establishment of suspensions as well as embryogenesis was obtained in a single medium (MS) with varying concentrations of a single auxin (2,4-D). Thus, it seems that complex media combinations and supplements are not necessary for the system described here. 2,4-D is the most commonly used growth regulator

in cereal tissue culture, and embryogenic callus induction in the presence of 2,4-D alone has also been reported in sorghum (for review see Bhaskaran and Smith 1990). Somatic embryogenesis increased markedly when the suspension was cultured with reduced 2,4-D (0.25 mg l^{-1}), followed by kinetin and BAP (0.25 mg l^{-1}). The concentration of 2,4-D had a significant effect on cell differentiation in the suspensions, as has been reported earlier (Vasil and Vasil 1982). High-frequency embryogenesis (80%) of small clusters in regeneration method 2 is more efficient as it does not involve callusing. Regeneration in liquid medium is simple and if synchronized will be useful in biochemical studies. The ability of small cell clusters to regenerate both on solid and liquid medium is important as reported in rice (Ozawa and Komamine 1989; Ozawa et al. 1996). Finally, it can be concluded that establishment of suspension cultures needed a high concentration of 2,4-D (2.5 mg l^{-1}), while MS with 0.25 mg l^{-1} kinetin and 0.25 mg l^{-1} BAP greatly enhanced the regeneration capacity. The same combination was found suitable for regeneration in liquid medium. The uniformity of the regenerated plants in the present study can be attributed to the development of somatic embryos from the finer cell clusters (Swedlund and Vasil 1985).

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