ANTHER-DERIVED CALLUS OF *DOLICHOS BIFLORUS* L, ITS PROTOPLAST CULTURE AND THEIR MORPHOGENIC POTENTIAL

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ABSTRACT

Anthers of *Dolichos biflorus* cv BR 10 produced callus on SS-A-8 medium irrespective of different stages of microspore development. Maximum response (91.5%) was obtained with anthers containing pollen mother cells (PMC). Callus derived from anthers at this stage differentiated globular somatic embryos on medium containing 88 μM BA and 88 μM GA_3. These developed further into heart and torpedo-shaped embryos on medium containing an auxin or an auxin with a cytokinin. Plantlets were recovered on the SS-A-8 medium supplemented with 50% coconut milk.

Callus derived from anthers containing PMC was used to produce suspensions of single cells and aggregates in liquid medium. Protoplasts isolated from frequently subcultured (every 2nd day) suspensions showed a high frequency of division. These calli differentiated globular structures and then somatic embryos but failed to produce plantlets or even roots.

INTRODUCTION

GRAIN legumes provide an important source of vegetable protein and contribute substantially to the nitrogen enrichment of soils through symbiosis with *Rhizobium*. The application of tissue culture and genetic engineering techniques to legumes is dependent on our ability to regenerate plants from cultured tissues, cells and protoplasts. Only limited success has been reported for *in vitro* organogenesis or regeneration from callus or single cells of seed legumes[1]. Regeneration of plants from protoplasts derived calli of legumes has been reported recently in *Medicago sativa*[2–5], *M. coerulea* and *M. glutinosa*[6], *Onobrychis vicifolia*[7], *Crotalaria juncea*[8], *Glycine max* and *G. tabacina*[9] and *Trifolium repens*[10,11] though isolated protoplasts from a wide variety of legumes have been reported to divide and form callus[12].

The objective of this study has been to establish methods and procedures for the regeneration of the whole plants from cultured tissues and protoplasts of some of the important tropical grain legumes. *Dolichos biflorus*, a grain legume which is a rich source of vegetable protein and the enzyme Urease, is grown in drought-prone areas of India, Bangladesh and Pakistan. The present communication describes the regeneration of plants from anther derived callus of *D. biflorus* and induction of embryoids from protoplasts derived from this callus.

Materials and Methods

Anther culture: Seeds of *Dolichos biflorus* cultivar BR10 were sown in pots and maintained in net house. Buds developing during the first one and half months from the day of the first flower emergence were used for anther culture. Buds were surface-sterilized in 0.2% mercuric chloride solution for 4 min and then rinsed thoroughly in sterile water. Ten anthers were carefully removed from each bud. One of the anthers was used for the identification of the pollen stage, the remaining were cultured in 25 × 150 mm culture tubes containing 15 ml agar solidified modified SS-K-8 medium[13]. The new medium, designated SS-A-8, consisted of (in mg/l): KNO_3, 2500; NH_4NO_3, 500; KH_2PO_4, 300; $MgSO_4$, $7H_2O$, 500; NaH_2PO_4, H_2O, 100; $CaCl_2$, $2H_2O$, 500; Biotin 0.5; Folic acid, 0.5; Sucrose, 60000; BA, 1–5; NAA, 1–5. The pH of the medium was adjusted to 5.8. Other components were identical with SS-K-8 medium. Anther cultures were incubated at 25 ± 1°C and illuminated continuously with white fluorescent tubes (Phillips TLF Reflectalite) at 6 μE m^{-2} sec^{-2}.

Morphogenesis: To induce differentiation, anther-derived callus after two 30 day passages were transferred to medium containing mineral salts and vitamins of SS-A-8 medium, *m*-inositol, 100 mg/l; sucrose, 1%; coconut milk, 10%; agar, 0.8% and pH, 5.8. Two cytokinins BA, (6-benzyladenine) and kinetin (6-

furfurylaminopurine) and four auxins IAA (indole-3-acetic acid); NAA (α-naphthaleneacetic-acid); 2,4-D (2,4-dichlorophenoxyacetic acid) and picloram (4-amino-3,5,6-trichloropicolinic acid) and GA_3 (gibberellic acid) were tested in different combinations. Five replicates were kept for treatment except GA_3-BA combination which was tested with 10 replicates under 8 hr dark/16 hr light (60 μE m^{-2} sec^{-2}) period at $25 \pm 1°C/28 \pm 1°C$.

Suspension culture: Suspension cultures were initiated by transferring actively growing anther callus to 50 ml of SS-K-8 medium. The flasks were incubated on a gyratory shaker at 120 rpm in dark. After establishment, suspension cultures were subcultured every 2nd day by mixing equal volumes of medium and suspension cultures.

Isolation and culture of protoplasts: Protoplasts were isolated from rapidly growing cells in liquid medium. Cells were suspended in enzyme solution in the ratio of 1 part cells to 10 parts enzyme solution in 100 ml Erlenmeyer flasks and incubated on a waterbath shaker (20 rpm) at 30°C. The enzyme mixture contained 2% cellulase 'Onozuka' R10, 0.5% macerozyme R10 in a solution of mannitol (osmotic pressure 615 mOs/kg of H_2O). The pH of the mixture was maintained at 6.0. The enzyme cell mixture was passed through a 100 μm stainless steel sieve after 3 hr of incubation. Protoplasts were collected by low speed centrifugation (80 g) and washed 3–4 times with culture medium and purified by repeated flotation on 25% sucrose solutions. Protoplasts were cultured in 40 μl droplets of SS-K-8 medium in 60 mm falcon petridishes. The medium contained mineral salts and vitamins of SS-K-8 medium plus sucrose 15 g; coconut milk, 15%; biotin, 0.5 mg; folic acid, 0.5 mg; BA, 1 mg; picloram, 0.6 mg and pH 5.8. The medium was modified with mannitol to give a final osmotic pressure of 615 mOs/kg H_2O. After 15 days of culture, an equal volume of culture medium without mannitol but supplemented with xylose, mannose, fructose, rhamnose (100 ml/l each) was added to each droplet. This medium was modified with glucose to give an osmotic pressure of 430 mOs/kg H_2O. After 25–30 days of culture, droplets were collected and spread over agar solidified (0.4% Difco Bacto-Agar) SS-K-8 medium containing other carbohydrates as mentioned above. All cultures were incubated at $27 \pm 1°C$ under continuous white fluorescent light (13 μE m^{-2} sec^{-2}). The plates are kept in transparent plastic boxes containing moist filter paper to maintain high humidity and provide light. Calli obtained from protoplasts were transferred on SS-K-8 medium containing BA or zeatin with GA_3 to induce organogenesis.

RESULTS

Anther culture and plant regeneration: The time required for the initiation of callus from anthers varied from 3–12 days. Anthers produced callus on SS-A-8 medium at varying frequency irrespective of the stages of microspore development (table 1). Only 4.4% of the anthers containing microsporocytes or tetrads responded to culture treatment. Maximum response (91.5%) was obtained with anthers containing pollen mother cells. Amongst different auxins tested, only NAA in combination with BA or Kn produced callus which survived the first subculture. Various concentrations of NAA (1–5 mg/l) were found to be equally suitable.

Proliferation of anther wall or connective tissue and filament was observed in anthers containing microspore cells. The calli were light to dark brown and compact in texture. Cytological examination of anthers, fixed after 10 days in culture, showed that none of the microspore cells participated in callus formation. Anthers containing pollen mother cells showed swelling within 5 days of inoculation and callus emerged through the ruptured anther wall after 10–12 days which were distinctly different from other calli observed. These calli were yellowish white and turned green when exposed to light. Ploidy of these tissues cannot be determined, though all the available techniques for staining of *in vitro* cultured cells were followed. However, these observations revealed that

Table 1 *Effect of pollen stage on callus formation in anther culture of Dolichos biflorus**

Pollen Stage	No. of anthers cultured	No. of anthers producing callus	Percentage of anthers producing callus
Pollen mother cells	246	209	91.5
Meiotic and tetrad stage/ cells	273	12	4.4
Uninucleate stage	1285	654	50.9
First mitosis	357	139	38.9
Binucleate cells	286	116	40.6

* The SS-A-8 medium contained 4 mg/l NAA and 2 mg/l BA

10–12 days old culture anthers contained highly cytoplasmic dividing cells. The calli gradually enlarged in size and were so friable as to crumble readily and separated into individual cells and small group of cells when agitated at low speed in liquid medium.

To study the morphogenic potential of calli obtained from anthers containing pollen mother cells as well as anthers containing microspore cells, these were transferred to media containing various combinations of phytohormones. Studies indicated that none of the available auxins independently and in combination with a cytokinin induced organogenesis (table 2). Globular structures appeared within 12 days on the surface of calli derived from anthers containing pollen mother cells, when tissues were cultured on medium containing 88 μM BA and 88 μM GA_3. Within 20–25 days, these structures dedifferentiated and the chlorophyllous callus turned brown. Thus, it became necessary to transfer these calli to some other media for further differentiation (table 3). These globular struc-

Table 2 *Response of calli derived from anthers containing pollen mother cell to various combinations of GA_3 and BA in SS-A-8 medium*

Growth regulators (GA_3 + BA) (in μM)	Morphogenic response	No. of explants cultured	No. of explants showing globular structures or roots	Percentage of anthers showing globular structures or roots
28 + 28	Roots	10	3	30.0
28 + 48	—	9	—	—
28 + 68	—	10	—	—
48 + 68	Roots	10	6	60.0
68 + 68	Roots	9	1	11.1
88 + 68	Roots	8	1	12.5
88 + 88	Globular structures	10	8	80.0
88 + 108	—	8	—	—

Table 3 *Morphogenic response of calli with globular structures to different auxins independently and in combination with cytokinins in SS-A-8 medium*

Growth regulators	Morphogenic response	No. of explants cultured	No. of explants showing organogenic response	No. of organs/explant	No. of* shoots/explant	No. of days taken for organ appearance	No. of* plants regenerated
40 μM NAA	Roots	7	3	5–7	–	15	–
20 μM NAA	Embryos	6	3	8–13	3–4	21	–
10 μM NAA + 5 μM BA	Embryos	7	4	15–19	5–6	18	–
40 μM IBA	Roots	6	2	12–18	–	12	–
10 μM IBA	Roots	5	1	6	–	13	
8 μM 2,4-D	Roots	6	2	2–4	–	9	–
8 μM 2,4-D + 6 μM BA	Embryos	6	3	3–8	5–8	17	5
4 μM Picloram	Embryos	7	6	6–13	2–5	19	–
6 μM Picloram + 4 μM BA	Embryos	6	6	18–25	10–12	9	15
20 μM IAA	Roots	6	3	5–8	–	12	–
12 μM IAA + 8 μM Kn	Roots	6	2	12–18	–	16	
10 μM GA_3	Roots	6	4	5–11	–	16	–

* Results of experiment conducted on hormoneless medium (see text).

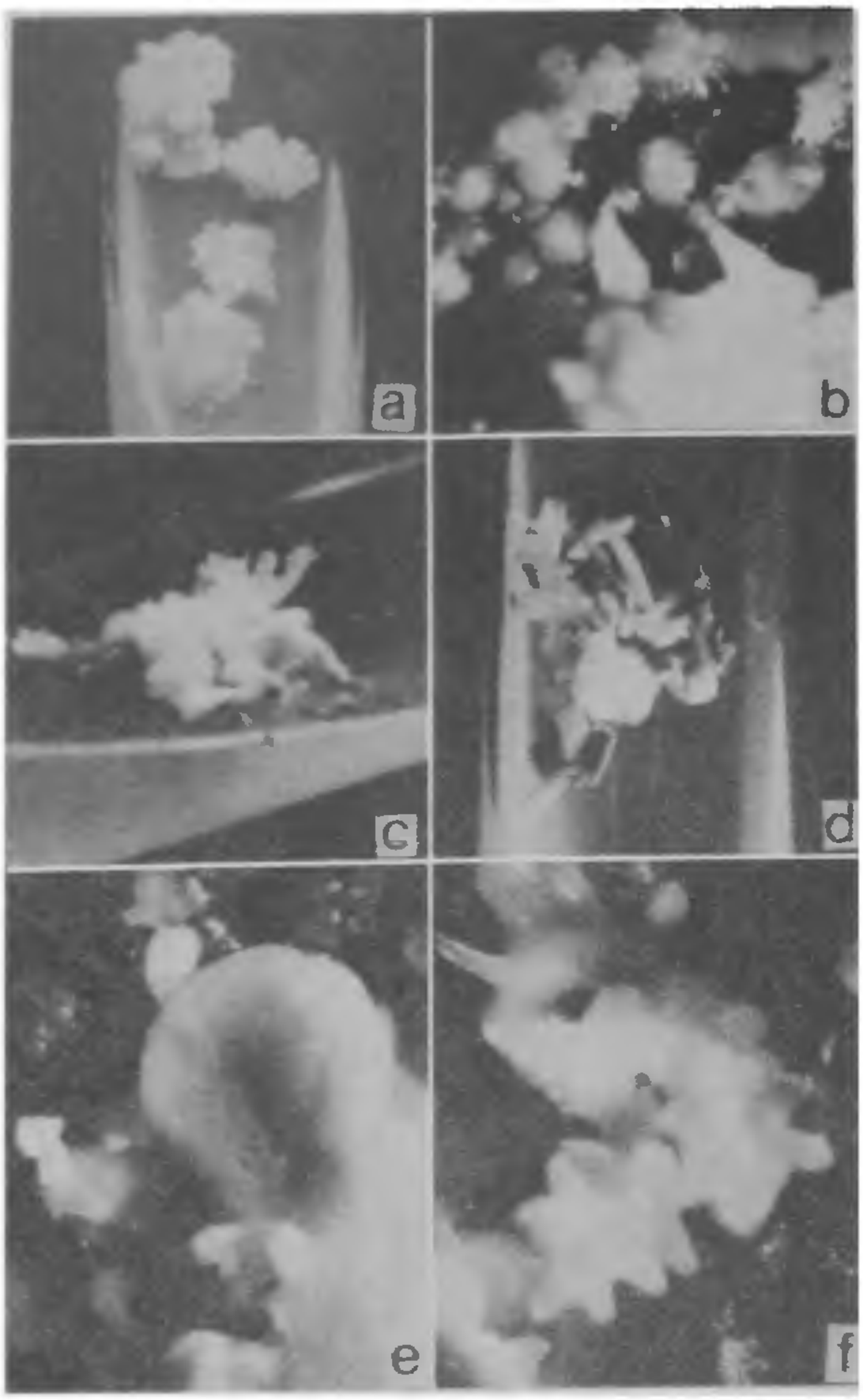

Figure 1A. 40-day-old callus derived from PMC containing anthers on medium containing 2 mg/l NAA and 1 mg/l BA: **B.** Proliferating callus with embryoidal structures of different stages; **C, D.** Small plantlets arising from somatic embryos; **E.** Close-up view showing cotyledonary leaf and embryoids (arrow); **F.** A morphogenic callus with somatic embryos and roots.

tures differentiated further and formed heart and torpedo-shaped embryos (figure 1B) in five media where auxins have been supplemented independently or with a cytokinin. The maximum response was recorded in cases where the medium contained 6 μM picloram and 4 μM BA. On this medium 100% explants responded to embryogenesis and 18–25 embryos/explant were observed. Media supplemented with auxins or GA_3 alone favoured rhizogenesis in most of the cases. The calli containing somatic embryos were subcultured on to hormoneless medium supplemented with 50% coconut milk (figure 1C). Two subsequent subcultures on the same medium at an interval of 15 days stimulated shoot growth giving rise to two cotyledonary leaves (figure 1D, E). These plantlets were separated and cultured on coconut milk depleted medium for 20–25 days. After a thorough washing of the agar, the plants were transferred to soil

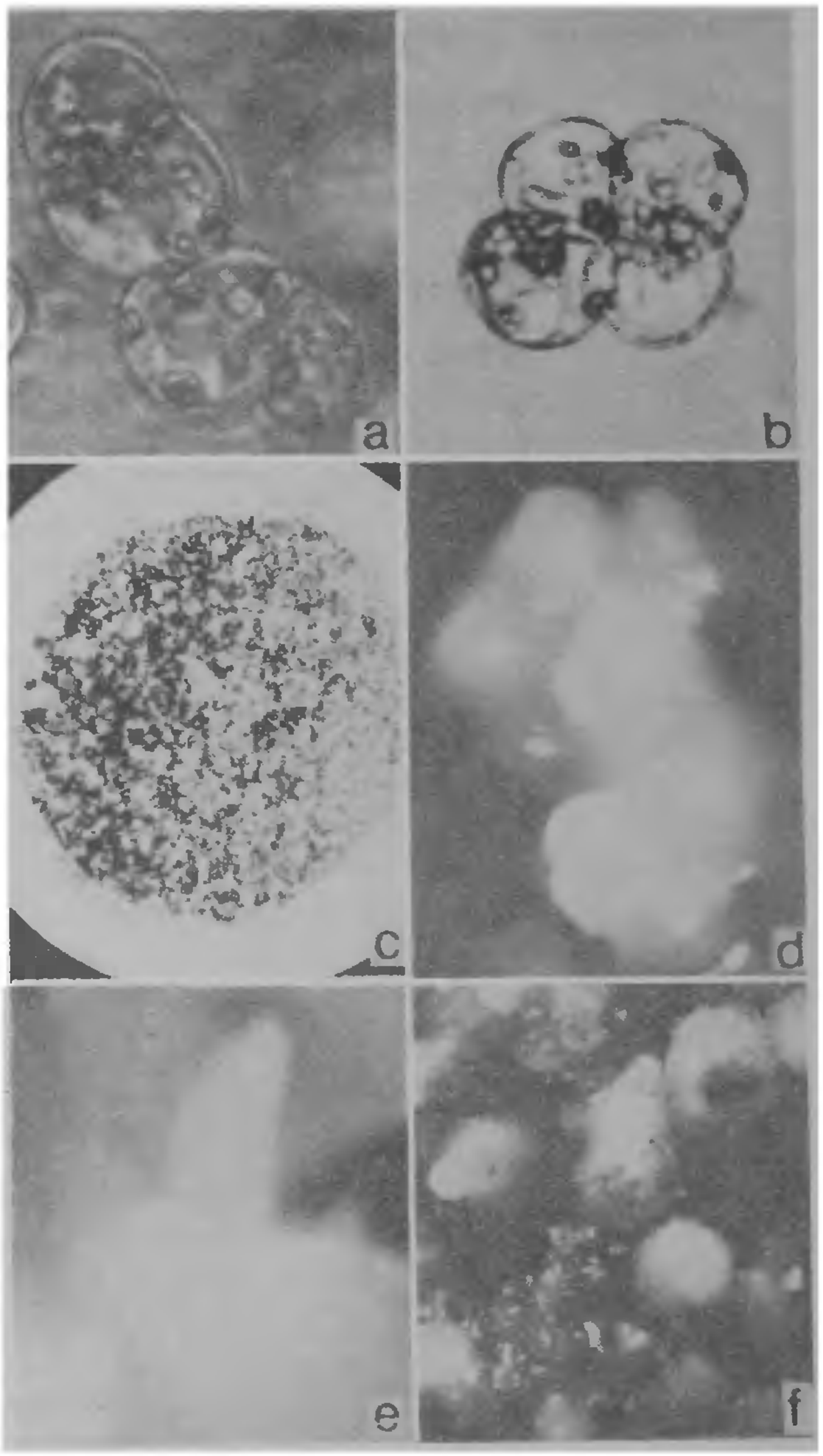

Figure 2A. Cell division in regenerated cells from cell culture derived protoplasts, 4 days after isolation; **B.** Second division after 6 days; **C.** Protoplast derived cell colonies 30 day after transfer to semi-solid medium; **D.** Globular structures appearing after 20 days of growth on 88 μM GA_3 and 70 μM BA containing medium; **E.** Somatic embryos after 40 days of growth; **F.** Dedifferentiating embryos on 2,4-D and BA containing medium.

under high humidity conditions. About 13% plants survived after transfer to the field.

Protoplast culture and embryogenesis: Suspension cultures initiated from callus derived from anthers containing pollen mother cells grew mainly as single cells or aggregates of 8–32 cells and had a cell doubling time of 24.5 hr during the exponential phase of growth. About $84.6 \pm 9.2\%$ cells were converted into protoplasts after 3 hr of enzymatic digestion of the cell wall and $89.8 \pm 3.2\%$ of these protoplasts were found to be viable.

Swelling of protoplasts was usually observed during the first 24 hr in SS-K-8 medium containing 1 mg/l BA and 0.6 mg/l picloram, some protoplast budding and shape distortion were apparent within 36 hr, indicating cell wall synthesis. Initial division occasionally occurred within 60 hr. About $23.6 \pm 8.4\%$ regenerated cells underwent one or two divisions (figure 2A, B) within 6 days and within 10 days small cell colonies of 4–8 cells were observed. In one of the experiments some droplets showed 60–70% division. The cell colonies after 25–30 days of culture were transferred on agar solidified SS-K-8 medium supplemented with xylose, mannose etc (see materials and methods) and 0.1 mg/l BA and 0.6 mg/l picloram. When these calli attained the size of 2–5 mm (figure 2C) they were transferred to media containing various combination of GA_3 and BA or zeatin. Globular structures (figure 2D) and somatic embryos (figure 2E) differentiated on the surface of callus mass within 20–25 days of culture on medium containing 88 μM GA_3 and 70 μM BA. The same sequence of treatments as in the case of anther callus tissue as well as media formulations previously used for morphogenesis of other plant species were offered, but no growth was observed; however, these structures dedifferentiated (figure 2F). All efforts to induce these proembryoidal structures to grow further have failed so far.

DISCUSSION

The morphogenic studies with anther derived calli revealed that only callus derived from anthers containing pollen mother cell and their protoplast derived calli produced somatic embryos. Niizeki and Grant[14] recovered diploid plants from anther callus of *Lotus* species. They explained their results on the basis of Konar and Nataraja's[15] observations on *Ranunculus sceleratus* anther cultures that plants have been regenerated from somatic tissue rather than microspore cells. Efforts to induce morphogenesis in calli derived from other plant parts of *D. biflorus* have been futile[16].

The results also demonstrate that by manipulating the GA_3-BA balance of the medium, development of somatic embryos can effectively be controlled. Only GA_3 in combination with BA at only one particular combination induced embryogenesis. BA and GA_3 appeared to have triggered embryo initiation which was not observed with auxin and cytokinin. However, the embryoids remained dormant when maintained on the same medium. Substitution of GA_3 with other auxins allowed development of embryoids into heart and torpedo shape stages. It has been reported earlier that GA_3 stimulates shoot development in callus[17–19]. In *D. biflorus* GA_3 appears to have a synergistic effect with BA in stimulating embryogenesis.

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ANNOUNCEMENTS

SECOND INTERNATIONAL SYMPOSIUM ON BENEFICIATION AND AGGLOMERATION ISBA '86

The Regional Research Laboratory (CSIR) Bhubaneswar in association with the Bhubaneswar Chapter, Indian Institute of Metals have organised the Second Symposium on beneficiation and agglomeration of minerals during *December 17–19, 1986.*

Original papers relating to any of the following topics are invited: 1. Mineral characterisation of very fine particles, 2. Recent developments in techniques and methods for mineral processing for the recovery of fine particles and their agglomeration. 3. Pilot plant and plant experiences in processing of low grade ores, ore fines and concentrates. 4. Techniques and processes towards energy conservation in mineral processing and agglomeration. 5. Developments in the area of treatment of mining and metallurgical wastes. 6. Characterisation of agglomerates.

Details can be had from D. N. Dey, Convener, ISBA-86, Regional Research Laboratory, Bhubaneswar 751 013, India.

INFLUENZA VACCINES FOR 1986–1987—MODIFIED COMPOSITION RECOMMENDED

A change in the composition of the influenza vaccine is now recommended by representatives of the World Health Organization (WHO) Collaborating Centres for Reference and Research on Influenza. The current composition has been used since 1984 and during the consultation, held in mid-February in Geneva, experts recommended that the vaccine for use in the 1986–1987 season should be trivalent and contain the following antigens:

- an A/Christchurch/4/85(H3N2)-A/Mississippi/1/85(H3N2)-like antigen
- an A/Chile/1/83(H1N1)-like antigen, and
- a B/Ann Arbor/1/86-like antigen

Details of the influenza epidemiology, antigenic and vaccine studies leading to the recommendations for the influenza vaccine appear in the WHO *Weekly Epidemiological Record*, No. 9, (WHO Press Release, No. 7, 28 February 1986; World Health Organization, Media Service, 1211, Geneva, 27; Switzerland)