11 Tisserat, B and Van der Cook, C E, *Plant Cell Tissue Organ Culture*, 1985, **5**, 107–117
12 De Bry, L, *IAPTC Newslett*, 1986, **49**, 2–22
13 Weathers and Giles, K L, *In Vitro Cell Dev Biol.*, 1987, **23**, 69 (Abstract)
14 Levin, R, Gaba, V, Tal, V, Hirsch, S, De-Nola, D and Vasil, I K, *Biotechnology*, 1988, **6**, 1035–1040
15 Alvard D, Cote, F and Teisson, C, *Plant Cell Tissue Organ Culture*, 1993, **32**, 55–60
16 Pachauri, R K and Dhawan, V, in *Applications of Biotechnology in Forestry and Horticulture* (ed Dhawan, V), Plenum Press, New Delhi, 1988, pp 275–282.

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Plants regenerated from protoplasts of sugarcane (*Saccharum officinarum*)

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Protoplasts were isolated from calli derived from leaf spindles of sugarcane cultivar CoJ-76, released for commercial plantation. The purified protoplasts cultured in Kao and Michayluk (KM) medium were provided gradually reduced osmoticum by addition of amino acids-containing KM medium at weekly intervals. Regeneration of plants required the developing microcalli to be initially cultured on callus proliferation medium, followed by Murashige and Skoog (MS) basal plant regeneration medium. The regenerated plants were transferred to modified MS liquid medium for shoot proliferation and root development. Plants with well-developed roots were successfully transferred to soil for further development.

THE regeneration of complete plants from protoplasts is one of the most reliable ways to introduce foreign gene(s) for the production of transgenic plants in monocots. Regeneration of plants from protoplasts has been reported in several cereal crops like rice[1–6], wheat[7,8], barley[9] and oat[10].

In sugarcane, however, this system is limited due to poor release of good-quality, round-shaped protoplasts[11], the release of polyphenolic compound(s) from cultures[12] and difficulties in regeneration of plants from protoplasts of sugarcane[13–15] In this study, we have overcome the problems of release of (i) low numbers of good-quality protoplasts by identification of the callus type and (ii) release of polyphenolic compound(s) by use of amino acids in Kao and Michayluk (KM) medium[16] during osmoticum reduction of cultured protoplasts.

The materials for this study comprised sugarcane cv. CoJ-76, released for commercial plantation in the state of Punjab. The calli were obtained from the innermost young unfurled leaf spindle, cultured on MS medium[17] supplemented with 3.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l 6-benzylaminopurine (BAP). These cultures were maintained in the dark at 26 ± 1°C. The calli were separated according to morphological appearance and maintained by regular subculture on MS medium supplemented with 2.0 mg/l, 2,4-D and 0.2 mg/l BAP at every 15 days intervals.

Four–five-month old calli were used for isolation of protoplasts. Two grams of eight-day-old subcultured calli were incubated in a 90 mm Petri dish containing 15 ml of an enzyme mixture of 3% Cellulase 'Onozuka' *RS (Yakult Honsha Co. Ltd., Japan)*, 1% Macerozyme R-10 (Yakult Honsha Co. Ltd., Japan), 0.5% Pectolyase Y-23 (Seihin Pharmaceutical Ltd., Japan), 5 mM MES buffer and CPW salts[18] with 13% mannitol. The mixture was kept on a gyratory shaker and maintained at a speed of 50 rpm for 10 h, followed by 2 h stationary incubation at 26 ± 1°C in the dark. After incubation, the mixture was passed through a set of 64, 45 and 30 µm pore size sterile nylon mesh. The relatively uniform isodiametric protoplasts were obtained by sucrose density gradient technique[3]. The purified protoplasts were washed thrice in CPW salts with 13% mannitol and collected by pelleting at 1000 rpm. The viability of purified protoplasts was 76% when it tested by trypan blue. The protoplasts were cultured on agarose bed at a density of 1×10^6 protoplasts/ml in KM medium[16] supplemented with 0.8 M glucose and 0.15% agarose (Seaplaque, LGT, FMC, Rock Land, USA). The culture dishes were sealed with parafilm and incubated in the dark at 26 ± 1°C.

The agarose bed was prepared in KM medium supplemented with 0.4 M glucose and 0.8% agarose. The osmoticum of protoplast cultures was reduced at 8 days by feeding 0.5 ml of KMA medium (prepared by addition of amino acids of AA medium[19] and 0.5 M glucose in KM medium[16] instead of NH_4NO_3 and KNO_3). This was followed by KMA medium with 0.2 M glucose at weekly intervals. After 40 days of culture, the protoplast-derived calli were transferred on to callus proliferation medium (MS medium supplemented with 3.0 mg/l 2,4-D, 0.2 mg/l BAP, 30 g/l sucrose and 0.8% agarose) for 15 days, followed by transfer on to modified MS medium (MS salts with various combinations and concentrations of auxins with kinetin (e.g. 0.5 mg/l NAA and 3.0 mg/l kinetin, 1.0 mg/l NAA and 3.0 mg/l kinetin, 0.5 mg/l IAA and 3.0 mg/l kinetin, 1.0 mg/l IAA and 3.0 mg/l kinetin, 30 g/l sucrose and 0.8% agarose) for differentiation of calli. The cultures initially maintained in the dark for a week were transferred to a 16/8 h light/dark regime using an assortment of fluorescent light (2500 lux) at 26 ± 1°C. 8–10 cm plantlets dif-

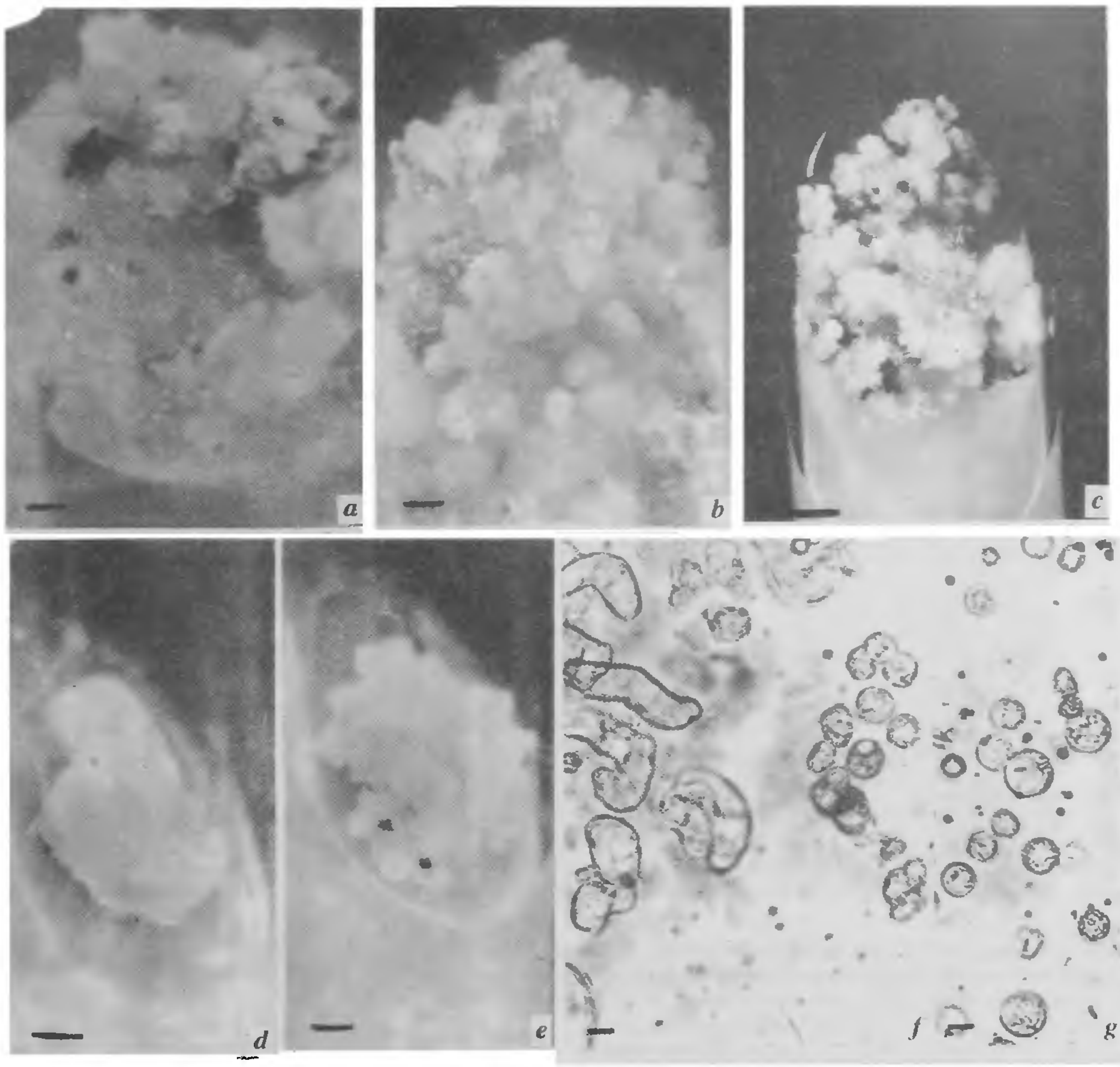

Figure 1. *a*, Primary callus, *b*, callus type I, *c*, callus type II, *d*, callus type III, *e*, callus type IV, *f*, freshly isolated protoplasts along with elongated cells; *g*, purified protoplasts. Bar scale, *a*–*e*, 1 cm; *f* and *g*, 15 µm

ferentiated from calli were transferred to modified liquid MS medium (MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l kinetin and 30 g/l sucrose) for shoot proliferation. Then these shoots were transferred to modified liquid MS medium (MS medium supplemented with 5 mg/l NAA and 7% sucrose) for rooting. The plantlets with well-developed roots were transferred to pots containing sterilized compost and soil (1:1 v/v) for further growth and development.

MS medium supplemented with 3.0 mg/l 2,4-D and 0.2 mg/l BAP was found to be suitable for callus induction from the innermost leaf spindle of sugarcane. The explants formed calli at the cut ends of the leaf spindle after 2–3 weeks and primary callus after 4–5 weeks of culture. Primary calli (Figure 1 *a*) consisted of four distinguishable types of calli. After two passages of subculture, these calli were more prominent and easily identified according to their morphological appearance: callus type I (organized, yellowish, nodular/globular embryoid containing compact callus, Figure 1 *b*); callus type II (smooth, semi-organized, light yellowish, differentiated callus, Figure 1 *c*); callus type III (spongy-type, soft, whitish, rough-surface callus, Figure 1 *d*); Callus type IV (unorganized, light yellowish, soft, rough-surface callus, Figure 1 *e*). Callus type III and callus type IV were unable to regenerate the plantlets. However, callus type I and callus type II showed high potential for morphogenesis of sugarcane. Eight-day-old subcultured calli were found suitable for isolation of a large number of protoplasts. The protoplasts were iso-

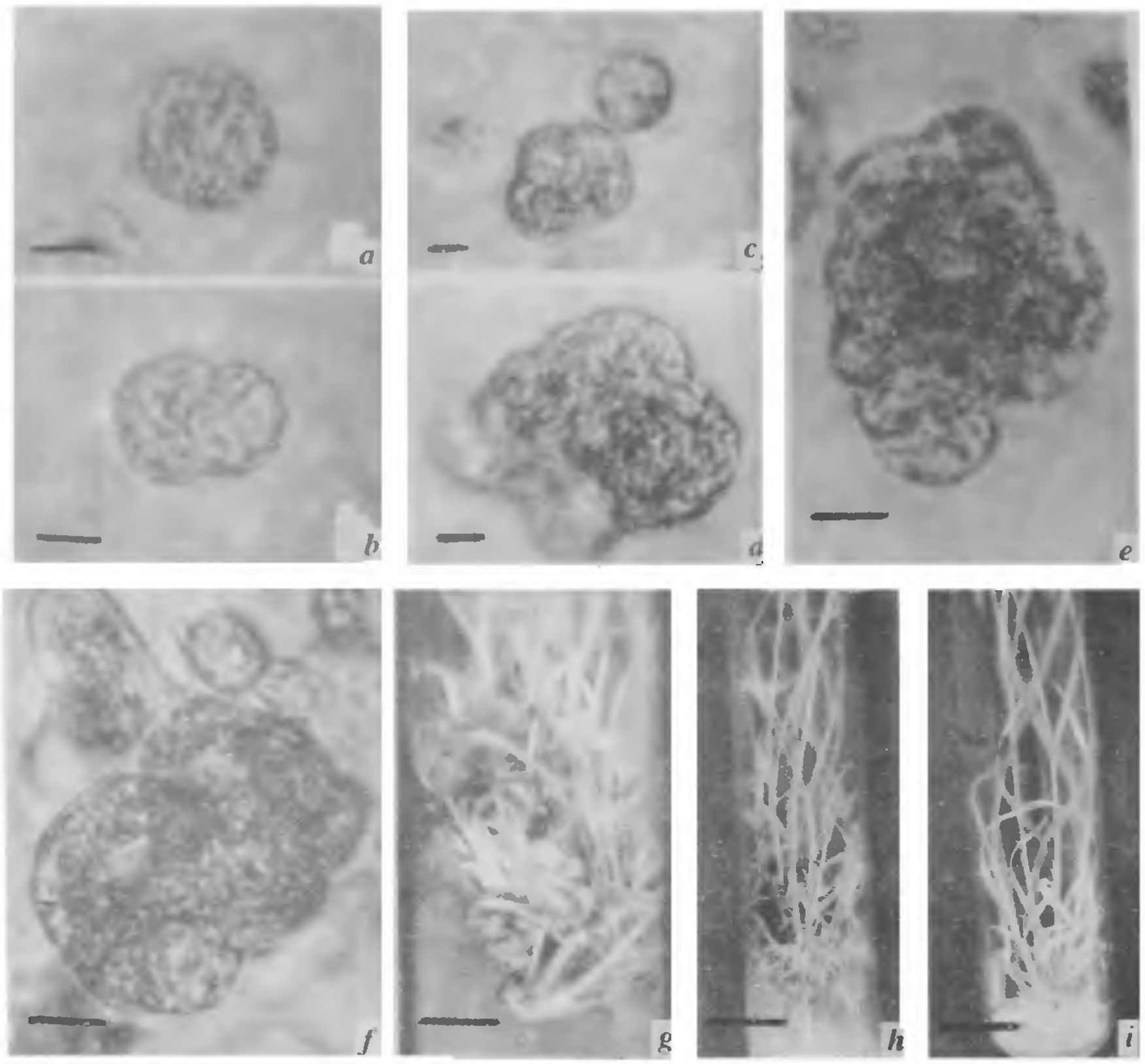

Figure 2. *a*, Single cell derived from protoplast. *b*, dividing protoplast after 7 days of culture, *c*, four-cell stage; *d*, microcalli derived from protoplast; *e* and *f*, macrocalli derived from protoplast, *g*, differentiated plantlets, *h*, plantlet with roots and shoots, *i*, plants in liquid medium for hardening Bar scale. *a–c*, 15 μm, *d*, 20 μm; *e* and *f*, 0 1 mm, *g*, 1 cm, *h* and *i*, 2 cm.

lated from all these types of calli. The release of round-shaped protoplasts from primary callus was very low (4.2×10^4 protoplasts per gram of inoculum). However, callus type III and callus type IV could not release round protoplasts. The release of round protoplasts from callus type I and callus type II was 4.6×10^6 and 8.0×10^5 protoplasts per gram fresh weight of callus, respectively (average of five independent experiments). The freshly isolated protoplasts were small, nearly isodiametric (16–25 μm), densely cytoplasmic, along with elongated cells (Figure 1 *f*). The application of sucrose density gradient technique was more effective for separation of round-shaped protoplasts (Figure 1 g).

The cell wall formation was completed after 4 days (Figure 2 *a*) and first cell division was observed after 7 days (Figure 2 *b*) of culture. Further division, i.e. four-cell stage, (Figure 2 *c*) led to the formation of colonies (Figures 2 *d–f*) after 15–25 days of culture. The frequency of dividing cells at 15 days was about 1.8% (average of 25 random fields per culture plate). The protoplast-derived cell colonies were of two types, viz. small, densely cytoplasmic and tightly packed or larger vacuoles and loosely packed. Both proliferated rapidly and formed compact cell clumps on callus proliferation medium.

The release of polyphenolic compound(s) is cited as a major problem for maintaining the sustained divisions of protoplast in sugarcane[12]. A similar problem was also reported in oat[20] and cotton[21]. This problem was effectively sorted by the use of high concentrations of BAP during callus induction[21], and 2,4-D[22], Ca^{2+} (ref. 12) and Mg^{2+} with arginine[21] during isolation and culture of protoplasts. The above modifications, i.e. high concentrations of growth regulators created the problem of callusing and regeneration of plantlets and high concentrations of Ca^{2+} in protoplast culture medium inhibited the sustained divisions in our experiment. The protoplasts cultured in Seaplaque agarose were found more

amenable for enhancement of divisions[3, 12, 23] and minimized the release of polyphenolic compound(s) from cultures. Gradual reduction of osmolarity (about one-third with glucose in amino acids-containing KM medium) at weekly intervals was effective in resuming the divisions and also prevented the synthesis of polyphenolic compound(s), by culture. This medium was also found suitable for minimizing the formation of loosely packed cell colonies. The protoplast-derived calli (protocalli) were transferred on to callus proliferation medium for proliferation and embryoid formation. These embryoids were selected and transferred on to MS salts with various combinations and concentrations of auxins with kinetin for differentiation and plant regeneration. MS salts with 0.5 mg/l NAA, 3.0 mg/l kinetin, 30 g/l sucrose and 0.8% agarose produced 30–50 plantlets per tube (Figure 2 *g*). Such a number of plantlets could not be produced from any other combination (data not shown), probably, due to inappropriate concentrations and combinations of growth regulators. These regenerants (Figure 2 *h*) were separated after 15 days, then transferred to modified liquid MS medium for proliferation of shoots and roots development (Figure 2 *i*). Although liquid MS/2 medium was also tried for root development, the high concentrations of NAA and sucrose showed drastic effect on root development, the observed survival of plants in *ex vitro* conditions being 80–85%.

1 Kyozuka, J , Otoo, D and Shimamoto, K , *Theor Appl. Genet.*, 1988, **76**, 887–890.
2. Datta, K , Potrykus, I. and Datta, S. K , *Plant Cell Rep.*, 1992, **11**, 229–233
3 Gupta, J N , Ph D Thesis, Gorakhpur University, 1992, pp 211
4 Gupta, J N , *Rice Biotechnol Quart* , 1993, **16**, 2–3
5. Gupta, J N , Pattanayak, A. and Gupta, H S , *Rice Genet Newslett* , 1993, **10**, 113–117.
6 Gupta, J N , Cheema, G. S , Kaur, Ravinder and Dhaliwal, H S , *Curr Sci* , 1995, in press
7. Harris, R , Wright, M , Byrne, M , Varnum, J., Brightwell, B and Schubert, K , *Plant Cell Rep* , 1988, 7, 337–340
8 Wang, H B , Li, X H , Sun, Y. R., Chen, J , Zhu, Z , Fang, R , Wang, P and Wei, J K , *Sci China (Series B)*, 1990, **33**, 294–305
9 Luhrs, R and Lorz, H , *Planta*, 1988, **175**, 71–81
10 Jahne, A , Lazzeri, P A and Lorz, H , *Plant Cell Rep.*, 1991, **10**, 1–6
11 Rathus, C and Birch, R G , *Plant Sci* , 1992, **21**, 65–74
12 Chen, W H , Davey, M R , Power, J B and Cocking, E. C , *Plant Cell Rep* , 1988, 7, 344–347
13 Srinivasan, C. and Vasil, I K , *J Plant Physiol* , 1986, **126**, 141–148
14 Taylor, P. W J , Ko, H L., Adkins, S W , Rathus, C. and Birch, R G , *Plant Cell Tissue Organ Cult* , 1992, **28**, 69–78
15 Gambley, R L., Ford, R and Smith, G R , *Plant Cell Rep* , 1993, **12**, 343–346
16 Kao, K N and Michayluk, M R , *Planta*, 1975, **126**, 105–110
17 Murashige, T and Skoog, F , *Physiol Plant*, 1962, **15**, 473–497
18 Frearson, E. M , Power, J. B and Cocking, E C , *Dev Biol* , 1973, **33**, 130–137
19 Muller, A J and Grafe, R , *Mol Gen Genet* , 1970, **161**, 67–76
20 Kaur-Sawhney, R , Rancilloc, M , Staskawiiz, M and Adams, W R , *Plant Cell Physiol* , 1977, **18**, 1309–1317
21 Thomas, J C. and Katterman, C., *Plant Sci Lett.*, 1984, **36**, 149–154
22 Davies, M E., *Planta*, 1972, **104**, 66–77
23 Ghosh-Biswas, G C. and Zapata, F. J., *Plant Breed* , 1990, **105**, 95–100

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'Punarnavine' profile in the regenerated roots of *Boerhaavia diffusa* L. from leaf segments

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Roots were regenerated from the leaf segments of *Boerhaavia diffusa* L. when cultured *in vitro* on MS (Murashige and Skoog) medium containing sucrose and indole-3-acetic acid (IAA). At the end of four weeks, about 17 roots, 10 cm long and containing 0.15% of the alkaloid punarnavine, developed from leaf segments of the third leaf (in serial order of development from the apex) when the IAA level of the medium was 0.5 µM/l. Increase in IAA levels of the culture media not only reduced the number of roots regenerated from the leaf segment but also reduced their length and alkaloid content. Replacing IAA by α-naphthaleneacetic acid caused no morphogenic response. However, treatment with 2,4-dichlorophenoxyacetic acid induced the development, from leaf segments, of callus which differentiated into roots having no capacity for alkaloid accumulation. Histological observations revealed that the roots were initiated from the phloem parenchyma of the cultured leaf segments.

BOERHAAVIA diffusa L. (family Nyctaginaceae) is a perennial herb commonly known as *punarnava*. Root extracts of this plant find applications as antihepatotoxic[1] and antiviral agents[2]. It cures corneal ulcers and night blindness[3]. The active principle lies in the alkaloid fraction – known as *punarnavine*[4] – of the root extract. Experimental work done on screening of the roots from garden-grown *in vivo* plants of *Boerhaavia diffusa* of different ages has shown that maximum alkaloid content (2%) was accumulated in the roots of three-year-old mature plants[5]. Due to extensive industrialization in and around Baroda, the high alkaloid yielding roots of *Boerhaavia diffusa* are scanty. An alternative possibility which is gaining importance nowadays, is producing