a superoptimal concentration of GA are all due to a unidirectional effect, i.e. the inhibition of GA's action by CFL.

1. Schneider, G., *Annu Rev. Plant Physiol.*, 1970, **21**, 499–536.
2 Mathew, T, *Curr Sci.*, 1987, **56**, 1186–1188.
3. Krelle, E. and Libbert, E., *Planta*, 1967, **76**, 179–181.
4. Jones, R. L. and Varner, J. E., *Planta*, 1967, **72**, 155–161.
5. Jackson, D. I, *J. Exp. Bot.*, 1971, **22**, 613–619.
6. Gibson, R. A. and Paleg, L. G., *Aust. J. Plant Physiol.*, 1982, **5**, 623–628.
7. Varner, J. E. and Mense, R. M., *Plant Physiol.*, 1972, **49**, 187–189.

ACKNOWLEDGEMENT I thank Dr S. D. Mishra for a critical evaluation of the manuscript.

Received 5 July 1993; revised accepted 22 November 1994

Plant regeneration from protoplasts of scented indica rice using heterologous feeder system

J. N. Gupta*, G. S. Cheema, Ravinder Kaur and H. S. Dhaliwal

Punjab Agricultural University, Ludhiana 141 004, India
*Present address: Department of Biotechnology, Directorate of Rice Research, Rajendranagar, Hyderabad 500 030, India

Protoplasts were isolated from 4–5-month-old embryogenic calli induced from mature seeds of the rice variety Pusa Basmati-1. The isolated protoplasts were cultured in the well formed by a ring of feeder cells of *Panicum maximum*, that were maintained as suspension cultures by periodic subculturing in R_2 medium. Six-month-old cell suspensions of *P. maximum* were found more suitable as a feeder for culturing of rice protoplasts than *Saccharum officinarum*. The microcalli obtained from protoplast culture were proliferated on modified N_6 medium and plantlets were obtained on MS regeneration medium supplemented with 1 mg l^{-1} IBA and 4% sucrose.

SEVERAL reports are available on regeneration of plants from protoplasts of rice[1–10]. These reports also suggested that feeder cells are a critical requirement for sustained division of protoplasts in the initial stage of culture. In most cases suspension cells of *japonica* rice varieties were used as feeder cells. When feeder cells and protoplasts of the same species are used together, often doubts are expressed with regard to the origin of calli and plantlets, whether, they are truly from protoplasts. In order to ensure that the plants regenerated are from protoplasts rather than from suspension cells, we used a heterologous system of feeder cell suspensions developed from a species distinctly different from cultured rice protoplasts.

Dehusked scented rice variety Pusa Basmati-1 (*Oryza sativa* ssp. indica) was surface-sterilized in 70% ethanol for 1 min and with 0.1% mercuric chloride for 5 min, followed by rinsing in sterile distilled water thrice. The sterilized seeds were cultured on modified MS medium[11] with 2 mg l^{-1} 2,4-D and variable concentration of either BAP or kinetin. After four weeks of incubation in the dark at 26 ± 1°C, the scutellar region of the seed embryos developed either nodular/globular whitish embryogenic calli (E-calli) or unorganized rough-surfaced light yellowish to creamy type of non-embryogenic calli (NE-calli). The embryogenic calli were separated under stereomicroscope and proliferated on the same callus induction medium. For isolation of protoplasts, the embryogenic calli were incubated in 15 ml of an enzyme mixture consisting of 2% cellulase 'Onozuka' RS (Yakult Honsha Co. Ltd, Tokyo, Japan) and 0.5% pectolyase Y-23 (Seishin Pharmaceutical Ltd. Co., Tokyo, Japan) in 5 mM MES buffer (2(*N*-morpholino)ethanesulfonic acid) and CPW salts[12] with 13% mannitol. The enzyme digestion was carried out in 90 mm Petri dishes for 4 h on a gyratory shaker at 50 rpm, followed by 1 h stationary period in the dark at 26 ± 1°C. The released protoplasts were sieved through a set of 64, 45 and 30 µm sterile nylon mesh and purified by sucrose density gradient[6]. The protoplasts were washed thrice in CPW salts[12] with 13% mannitol and pelleted by centrifugation at 1000 rpm. The viability and cell contamination of protoplasts were determined by staining with Trypan blue and Calcoflour white, respectively.

Calli were induced on MS medium[11] supplemented with 3.0 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP from young inflorescence of *Panicum maximum*, leaf spindle of *Saccharum officinarum*, mature seed of *Triticum aestivum* and *Oryza barthii*. The 2-month-old calli of *P. maximum* and *S. officinarum* were used to develop a cell suspension for preparation of feeder cells. Suspension cultures were initiated in liquid R_2 medium[13] for *P. maximum* and AA medium[14] for *S. officinarum* and maintained through

Table 1. Effect of growth regulators on callus induction and embryogenic callus formation from seed embryos of Pusa Basmati-1

Basal MS medium with			Frequency of callus formation (%)	Growth of E-callus (%)
2,4-D (mg l^{-1})	BAP (mg l^{-1})	Kinetin (mg l^{-1})		
2	0 2	–	87.5	88 9
2	0 5	–	73.3	43 8
2	–	0 5	70 0	44 0
2	–	1	66 6	24 4
2	–	–	75 0	62 6

*For correspondence

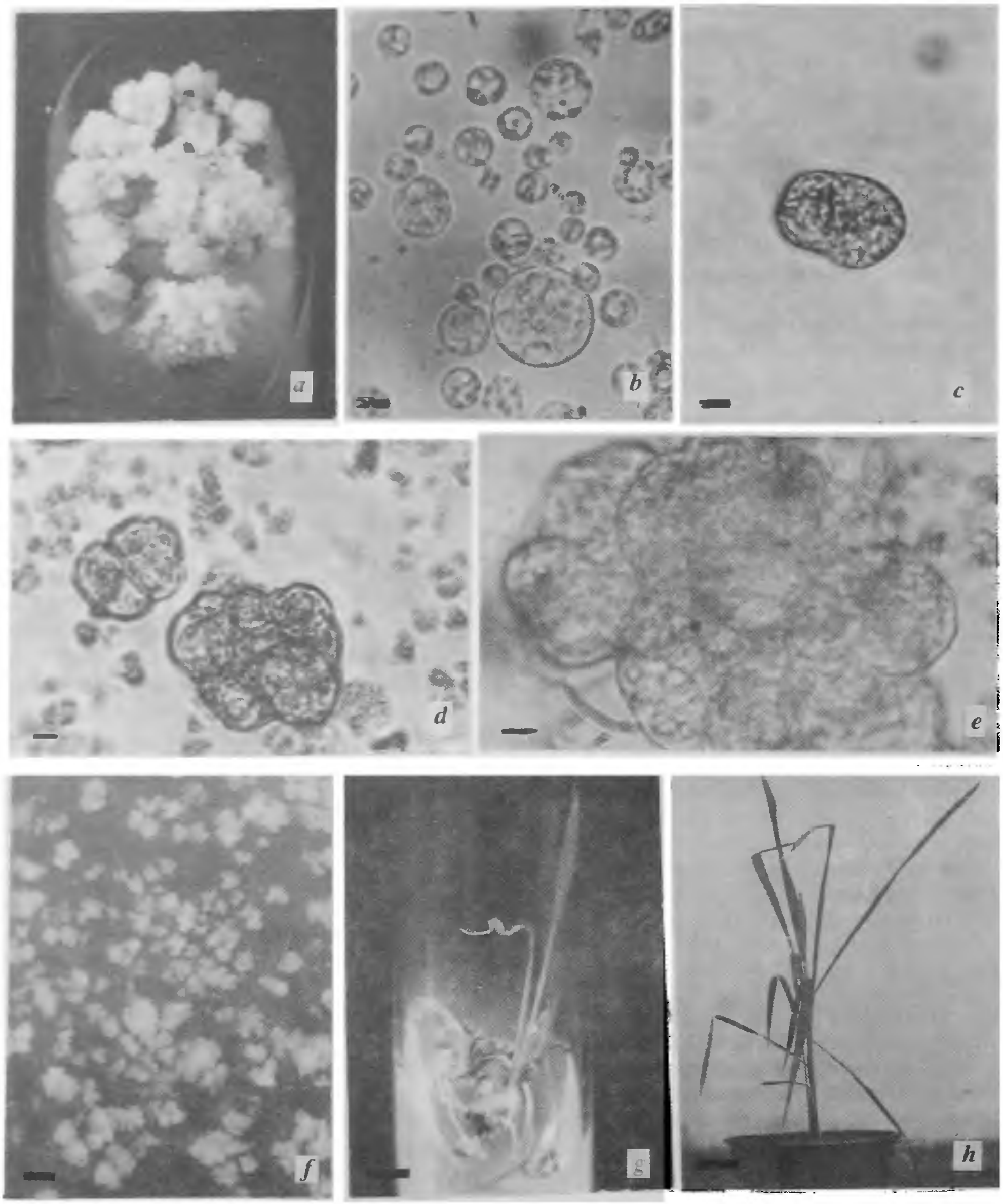

Figure 1. Plant regeneration from protoplasts of scented indica rice cv. Pusa Basmati-1. *a*, Embryogenic calli; *b*, isolated protoplasts; *c*, predividing protoplast after 4 days of culture; *d*, two-cell stage and further dividing protoplast; *e*, microcalli after 20 days of culture; *f*, protoplast-derived calli on callus proliferation medium; *g*, regenerated plantlet; *h*, plant in pot. Bar: *a*, 1 cm; *b*, 10 µm; *c*, 5 µm; *d*, 5 µm; *e*, 20 µm; *f*, 2 mm; *g*, 1 cm and *h*, 10 cm.

periodic subculturing in the same media.

For preparation of feeder cells, 1.5 ml of actively growing cell suspensions were mixed with 10 ml of N_6 medium[15] containing 2.0 mg l^{-1} 2,4-D, 0.2 mg l^{-1} zeatin, 500 mg l^{-1} casein acid hydrolysate, 0.5 M glucose and 0.6% agarose. 1.5 ml of this mixture was poured along the inner periphery of a 35 mm Petri dish in the form of a ring with a well at the centre.

One ml of purified protoplasts at a density of 1×10^6 ml^{-1} in N_6 medium[15] with 1.5 mg $^{-1}$ 2,4-D, 0.2 mg l^{-1} zeatin, 500 mgl^{-1} casein acid hydrolysate, 1.1 M glucose and 0.15% sea plaque agarose were plated in the well.

The cultured protoplasts were fed with fresh N_6 medium[15] supplemented with 1.5 mg l^{-1} 2,4-D, 0.2 mg l^{-1} zeatin, 500 mg l^{-1} casein acid hydrolysate at day 8 with reduced osmoticum (0.8 M glucose) and subsequently every five days with further reduction in osmoticum (0.4 M glucose). After 30 days, the microcalli formed from protoplasts were transferred to N_6 medium supplemented with 2.0 mg l^{-1} 2,4-D, 0.2 mg l^{-1} zeatin, 500 mg l^{-1} casein acid hydrolysate, 3% sucrose and 0.8% agarose for proliferation and embryoid formation. For differentiation, these calli were further transferred on MS salts with 1 mg l^{-1} IBA, 4% sucrose and 0.8% agarose and kept initially in the dark for one week and then transferred to a 16/8 h light/dark regime. The regenerated plantlets at 2–3-leaf stage were transferred to semisolid MS/2 medium for root development and hardened in liquid MS/2 medium (without sucrose) before transfer from tube to soil.

Of all the combinations of growth regulators tested for callus induction and embryogenic calli formation, MS medium supplemented with 2 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BAP was found to be the best, both in terms of frequency of callus induction (87.5%) and in terms of growth of embryogenic calli (88.9%) (Table 1). Substitution of 0.5 mg l^{-1} BAP with kinetin did not show any better response.

The protoplasts isolated from 1-month-old primary calli were found to be highly vacuolated. Further, a large number of protoplasts were seen budding, whereas 4–5-month-old embryogenic calli (Figure 1 *a*) yielded only small (8–15 µm), round and densely cytoplasmic protoplasts (Figure 1 *b*) in very large numbers (about 6.7×10^7 protoplasts per gram fresh wt of callus). The viability of protoplasts was also higher (83%) when isolated from 4–5-month-old embryogenic calli compared to younger calli. The size of protoplasts showed 18–30% increase after 4 days of culture (Figure 1 *c*). The first complete protoplast division was observed after 6 days of culture and further divisions led to colony formation in 10 days (Figure 1 *d*) and 20 days of culture (Figure 1 *e*). Reduction of osmoticum after 8 days was found beneficial for sustained divisions of protoplasts, confirming our earlier observations[6–8]. Morphologically distinguishable type of calli, i.e. with respect to colour and texture, were considered essential for use as feeder cells to ensure that regeneration is only from protocalli and not from feeder cells. Calli of *P. maximum* and *S. officinarum* were obtained and initiated for establishment of cell suspension culture. The calli of *P. maximum* were grey in colour, making them distinguishable from rice protocalli, but *S. officinarum*, *O. barthii* and *T. aestivum* did not show such distinction. Further, the cell sizes of *P. maximum* and *S. officinarum* in suspension culture were larger and clearly distinguishable from rice cells. Therefore, established cell suspensions of *P. maximum* and *S. officinarum* were selectively used as feeder cells. Collapsing of cultured protoplasts was observed within 36 h in feeders prepared from younger (2–3 months) cell suspension of *S. officinarum*. With feeder cells of *P. maximum*, collapsing was delayed and noticeable only after 4 days of culture. Six-month-old cell suspension of *P. maximum* supported the sustained divisions of protoplasts. The frequency of dividing protoplasts at day 15 was found to be 1.87%. The calli were transferred on N_6 medium supplemented with 2 mg l^{-1} 2,4-D, 0.2 mg l^{-1} zeatin, 500 mg l^{-1} casein acid hydrolysate, 3% sucrose and 0.8% agarose for proliferation and embryoid formation. The protocalli showing embryoids (Figure 1 *f*) were transferred to plant regeneration medium. About 66.3% of calli differentiated to form plantlets (Figure 1 *g*). The hardened plantlets were transferred to pots for further growth and development (Figure 1 *h*).

1. Kyozuka, J., Otoo, D. and Shimamoto, K., *Theor. Appl. Genet.*, 1988, **76**, 887–890.
2. Lee, L., Schroll, R. E., Grimes, H. D. and Hodges, T. K., *Planta*, 1989, **178**, 325–333.
3. Li, Z. J. and Murai, N., *Plant Cell Rep.*, 1990, **9**, 216–220.
4 Guderdoni, E. and Chair, M., *Plant Cell Rep* , 1992, **11**, 618–622.
5. Su, R. C., Rudert, M. L. and Hodges, T. K., *Plant Cell Rep.*, 1992, **12**, 45–49.
6. Gupta, J. N., Ph D Thesis, Gorakhpur University, Gorakhpur, 1992, pp. 211.
7. Gupta, J. N., *Rice Biotechnol. Quart.*, 1993, **16**, 2–3.
8. Gupta, J. N., Pattanayak, A. and Gupta, H. S., *Rice Genet. Newslett.*, 1993, **10**, 113–116.
9. Ghosh Biswas, G. C. and Zapata, F. J., *J. Plant Physiol.*, 1993, **139**, 523–527.
10. Gupta, H. S. and Pattanyak, A., *Bio/Technology*, 1993, **11**, 90–94.
11. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–497.
12. Frearson, E. M., Power, J. B. and Cocking, E. C., *Develop. Biol.*, 1972, **33**, 130–137.
13. Ohira, K., Ojima, K. and Fujiwara, A., *Plant Cell Physiol.*, 1973, **14**, 1113–1121.
14. Muller, A. J. and Grafe, R., *Mol. Gen Genet.*, 1970, **161**, 67–76
15. Chu, C. C., Wang, C. C., Sun, C. S , Hsu, C., Yin, K. C., Chu, C. Y. and Bi, F. X., *Sci. Sinica*, 1975, **18**, 659–668.

ACKNOWLEDGEMENTS. JNG is grateful to N. P. Sarma, Department of Biotechnology, Directorate of Rice Research, Hyderabad, for valuable suggestions during preparation of the manuscript and the Indian Council of Agricultural Research, India, for financial support.

Received 21 February 1994; revised accepted 25 November 1994