

11. Morales, M. A., Jabbagy, A. J. and Terenzi, H. P., *Neurospora Newslett.*, 1973, **20**, 24.
12. Folch, J., Lees, H. and Sloane, G. H., *J. Biol. Chem.*, 1957, **226**, 497.
13. Shanmugasundaram, E. R. B., Jeyadass, T. and Shanmugasundaram, K. R., *J. Indian Inst. Sci.*, 1985 (under publication).
14. Shanmugasundaram, E. R. B., Parameswari, C. S. and Radha Shanmugasundaram, K., *Curr. Sci.*, 1984, **53**, 1290.
15. Rathinavelu, A., Doctoral thesis submitted to and approved for the Ph.D. degree from the University of Madras, 1984.

PLANT REGENERATION FROM HYPOCOTYL PROTOPLASTS OF MOTHBEAN (*VIGNA ACONITIFOLIA*)

RAVINDER GILL and SUSAN EAPEN

*Bio-Organic Division,
Bhabha Atomic Research Centre,
Trombay, Bombay 400 085, India.*

EVER SINCE the successful demonstration of regeneration of isolated protoplasts in plants, much was speculated about its potential use for genetic manipulation of important crop plants namely legumes and cereals. It is essential to obtain reproducible differentiation of plants from protoplasts at will, before attempting any genetic manipulation. Unfortunately, unlike other plants, the regeneration of protoplasts from grain legumes and cereals have proved recalcitrant, though sporadic reports are available^{1,2}.

In our laboratory, we have initiated a programme to develop protoplast, cell and tissue culture technique in mothbean, a widely cultivated drought resistant grain legume so as to use the system for somatic cell hybridization and plant cell transformation. In previous studies on moth bean, regeneration of protoplasts has been reported from mesophyll³ involving a complicated procedure of enzyme purification, and shoot-apex derived callus⁴, by use of non-commercial enzymes. The aim of the present communication was to obtain reproducible plant differentiation from protoplasts of yet another source—hypocotyl-derived callus under simpler and well-defined conditions.

Seeds of mothbean (*Vigna aconitifolia* Jacq Marechal) cv No. 88 were surface-sterilized with 70% alcohol for 30 sec followed by treatment with mercuric

chloride (0.1%) for 5 min. The seeds were rinsed five times with sterile distilled water and aseptically implanted on MS medium⁵ for germination. Hypocotyl of 4–5 day old seedlings were cut into very fine pieces and cultured on MS medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid at 2 mg/l) and coconut water (15%).

Protoplasts were isolated from 4–5 day old freshly raised callus. About 10 ml of filter-sterilized enzyme was used for a gram of callus tissue. Enzyme mixture comprised of 2.5% cellulase, 0.5% macerozyme (both from Kinki Yakult Biochemicals, Japan), 0.5% driselase (Kyowa Hakko Kogyo, Japan) and 0.5% hemicellulase (Sigma Chemicals, USA) in 0.5 M sorbitol. Incubation was done overnight at $25 \pm 1^\circ\text{C}$ by keeping tissue stationary in enzyme mixture. At the end of the incubation, undigested material was separated by filtration through a 43μ steel mesh. The resulting suspension was centrifuged at 300 g for 5 min and the pellet washed thrice by 0.5 M sorbitol. The final pellet was cleaned by floating on 25% sucrose solution⁶ followed by centrifugation at 100 g for 5 min. In the process, the purified preparation formed supernatant and the pellet was discarded. Protoplasts were recovered by a Pasteur pipette and cultured in liquid media in glass petri dishes at a final concentration of 5×10^4 to 10^5 protoplasts/ml. Various media namely Kao and Michayluk⁷, V-47⁸, MS⁵ and modified MS were used for culturing protoplasts. Hereafter the modified MS refers to a medium which contains mineral elements and iron of Murashige and Skoog⁵, vitamins after Lin and Staba⁹, along with 2,4-D (2 mg/l), kinetin (Kn 0.1 mg/l), caesin hydrolysate (100 mg/l), glutamine (800 mg/l), DL-tryptophan (Sigma Chemical Co. 25 mg/l), sucrose (3%) and sorbitol (9%). The petri dishes were sealed with parafilm and incubated in diffuse light at $25 \pm 2^\circ\text{C}$. Protoplast-derived colonies were transferred to agar-jelled modified MS with 3% sucrose alone either by plating¹⁰ or 'liquid over solid medium' method¹¹. For organogenesis such calli were transferred to modified agar-jelled MS medium supplemented with cytokinins like BA (benzyladenine) Z(zeatin) Kn or 2,i-P (6- γ , γ -dimethylallylaminopurine) at 2 mg/l + 2,4-D (0.1 mg/l). After one month, protoclonal were transferred to basal medium devoid of phytohormones.

With the above enzyme combination, protoplast yield varied between 3×10^6 /g and 5×10^6 /g of tissue used (figure 1). It was not found essential to add Ca^{++} in incubation period for their stabilization. However, Shekhawat and Galston³ had to use purified Driselase and also add Ca^{++} in the incubation medium. Protoplast yield was found to be dependent upon age