Isolation of Protoplasts from Stem and Hypocotyl of the Legume Vigna sinensis and Some Factors Affecting Their Regeneration

SHASHI BHARAL* and A. RASHID

Department of Botany, University of Delhi

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Summary

Cut segments of stem and hypocotyl of in vitro grown seedlings were incubated in 1% cellulase, 0.6 M mannitol and a salt solution at pH 5.5 for 10 hours. When these segments were left undisturbed the protoplasts formed did not escape into the enzyme solution which was removed by decantation. Also the tissue could be washed and liquid removed by decantation. Finally the protoplasts were released by shredding the tissue followed by gentle shaking. Undigested material was removed by sieving. The preparation thus obtained hardly had any cell requiring purification. In this way protoplasts could be obtained without centrifugation—a necessary evil in current techniques of harvesting of protoplast. These protoplasts readily regenerated if isolated from plants grown at 3,500 lux. The growth, however, did not proceed beyond a few-celled stage unless the pH was adjusted to 6.0. For sustained divisions osmotic concentration of the nutrient medium also had to be lowered to 0.5 M. This method, however, could not be applied to mesophyll protoplasts which required centrifugation for washing and purification resulting in heavy losses in yield and destabilization of protoplasts which failed to divide.

Keywords: Cell differentiation; Physiological factors; Protoplast culture; Vigna sinensis.

1. Introduction

Hybridization of somatic cells is reckoned to be one of the novel methods of broadening the pool of genetic variability for plant improvement. The potential of protoplasts in the production of improved varieties can become limiting in the absence of regeneration potential and unlike other plants the regeneration of protoplasts of legumes and cereals—our most important crop plants—have proved to be difficult. Among the legumes regeneration has been reported from mesophyll protoplasts of Pisum sativum (CONSTABEL, KIRKPATRICK, and GAMBORG 1973), Phaseolus vulgaris (PELCHER, GAMBORG, and

* Correspondence and Reprints: Department of Botany, University of Delhi, Delhi 110007, India.
Kao 1974), Vicia narbonensis (Donn 1978), Vicia faba (Binding and Nehls 1978) and Vigna sinensis (Davey, Bush, and Power 1974). However, our inability to regenerate mesophyll protoplasts of Vigna sinensis led up to look for tissues other than leaves as source of protoplasts. A comparative study of protoplasts isolated from hypocotyl, stem, stem callus, and leaves of Vigna seedlings (Bharal and Rashid 1979 a) indicated that the hypocotyl and stem are suitable systems for obtaining high yield of viable protoplasts—here as many as 80% of the cells could be transformed into viable protoplasts whereas in case of callus and leaf the isolation efficiency did not exceed 20%. The present communication is the result of an inquiry into the regenerative capacity of protoplasts obtained from hypocotyl, stem, and leaves of V. sinensis.

2. Material and Methods

Seeds of Vigna sinensis var. Pusa Phalguni were surface-sterilized with chlorine water for 5 minutes, rinsed with sterile water and sown on mineral medium B5 (Gamborg and Evelope 1968) containing 5 × 10^{-5} M FeSO4 and 5 × 10^{-5} M Na2 EDTA as iron source and 2% sucrose. Stem, hypocotyl and leaves excised from 8-day-old seedlings at 2-leaf-stage were cut longitudinally and subdivided into 2 cm long pieces. About 1 g of the material was incubated in 10 ml of the enzyme mixture comprising the enzymes cellulase and pectinase, alone or in combination, mannitol and the following salts (mg/l): KH2PO4 272, KNO3 101, CaCl2 1480, MgSO4 7H2O 246, KI 0.16, CuSO4 5 H2O 0.025. The pH of the incubation mixture was adjusted with HCl or NaOH. During incubation stem and hypocotyl segments were left undisturbed and leaf tissue was gently shaken every 2 hours. At the end of incubation period the enzyme solution from flasks having stem and hypocotyl segments was decanted. It was followed by four washings and the liquid was removed by decantation. Also, during washing care was taken not to disturb the tissue. The tissue washed free of enzymes was shredded in liquid medium with forceps followed by gentle shaking to liberate protoplasts. The protoplast suspension was filtered through a 45 micron stainless steel mesh to remove the debris. As for leaf tissue, the debris and enzymes were removed by sieving and centrifugation, at 500 rpm for 5 minutes, and purified by floatation on 25% sucrose which was later removed by centrifugation.

For washing and culture of protoplasts B5 medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), benzylaminopurine (BAP), all at level of 1 mg/l, and mannitol was employed. For plating an equal volume of protoplast suspension and culture medium, with 1.2% agar kept molten at 40 °C, was mixed and about 0.2 ml of this was dispensed over pre-jelled, 0.6% agar, culture medium. The density was kept at 5 × 10⁶ protoplasts/ml. The petridishes were sealed with cellotape and incubated at 25 ± 2 °C in diffuse light (250 lux). For callusing and organogenesis the colonies were transferred to B5 medium containing 2,4-D, indoleacetic acid (IAA), NAA, BAP or zeatin.

3. Results

A time-course study of protoplast liberation from stem and hypocotyl, at different enzyme levels, indicated that the process begins after 2 hours and continues up to 10 hours. With 1% cellulase, the optimal period of incubation was 10 hours and at higher concentrations (2–4%) it was 8 hours. For further