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ISOLATION OF PROTOPLASTS FROM LEAF MESOPHYLL CELLS OF *HEVEA BRASILIENSIS*

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A method has been standardised for the isolation of large number of protoplasts from young leaves of *Hevea brasiliensis*, which could be another source for culturing protoplasts for somatic hybridisation. It was observed that selection of suitable enzymes, their combinations and concentrations, pH of the incubation medium, duration and temperature of incubation are very important in the protoplast isolation procedure. Standardisation of these parameters in the preparation of leaf tissues for enzyme digestion, purification and protoplast viability is discussed. In this study, 95 per cent healthy and viable protoplasts were obtained from young leaves of one-year-old plants digested with 1.0 per cent Macerozyme R10 and 1.5 per cent Cellulase Onozuka R10 along with 0.7 M mannitol as osmoticum at pH 5.5 at 35 ^{0}C. Isolated protoplasts were finally purified using sucrose gradient (0.7 M and 0.5 M sucrose solutions) method. Purified protoplasts were found as light green layer between the 0.7 M and 0.5 M sucrose layers. Viability of protoplasts was ascertained using Fluoscein-di-acetate (FDA) staining. The study also indicated the potential of utilising leaf mesophyll cells of young *H. brasiliensis* plants for protoplast isolation and its use for somatic hybridisation studies in crop improvement programmes.

Keywords: Enzymatic digestion, *Hevea brasiliensis*, Mesophyll cells, Protoplasts isolation, Viability.

INTRODUCTION

Protoplasts isolated from higher plants provide a fairly uniform population of genetically similar single units. Plant protoplasts open up a new avenue to utilise them in many molecular biology techniques including the genetic modification of plants. The successful isolation of protoplasts from plant tissues is a prerequisite for their use in physiological, biochemical and virological studies. In addition, protoplast technology can offer a better tool for achieving crop improvement, if plant regeneration from protoplasts can be achieved. *In vitro* fusion of plant protoplast with subsequent regeneration of hybrid plants has been suggested as a technique for introducing greater genetic diversity in plants for breeding purposes. In recent years, a number of methods have been described for accomplishing protoplast fusion (Power *et al.*, 1970; Compton *et al.*, 1999). Somatic hybridisation bypasses biological barriers and creates new evolutionary opportunities that would be difficult to accomplish through natural or conventional breeding techniques.

Isolation of plant protoplasts using enzymatic degradation of cell walls, developed by Cocking (1960) opens up a new

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area of fundamental and applied research in genetic engineering. Protoplasts from various organs of a wide range of crop plants have been isolated and cultured (Smith, 1974). Protoplasts can also be induced to fuse and form somatic hybrid cells (Power *et al.*, 1970) that can be regenerated *in vitro* to develop a new genetically modified (GM) plant. Though attempts made in the past for isolation of protoplasts from various tissues or cultured cells of *Hevea* were promising (Othman and Paranjothy, 1980; Cazaux and d'Auzac, 1994; Sushamakumari *et al.*, 1999), no reports are available regarding the successful protoplast isolation from leaf mesophyll cells.

The present investigation involves the protocol using leaf mesophyll cells of *H. brasiliensis* for the isolation of protoplasts that could be used further in various crop improvement programmes.

MATERIALS AND METHODS

Immature leaves at light green stage from the upper-most whorl of one-year-old polybag plants of RRIM 600 *Hevea* clones were collected and washed thoroughly in a detergent (Teepol). Leaves were surface sterilised with 0.2 per cent $HgCl_2$ for 10 min and rinsed thoroughly in sterile water. Leaves were then cut into fine strips and suspended in 0.6 M mannitol solutions for 10 min. to increase the turgidity of the cells before enzyme treatment (Shepard and Totten, 1975). Different pH levels from 4 to 6 were tested in the treatment medium. Among the sugars like sucrose, sorbitol and mannitol tested as osmoticum, mannitol was selected as a better one in this study.

Enzymes manufactured by Sigma Chemical Co. USA (pectinase and cellulase) and Yakult Pharmaceuticals Co., Japan (Macerozyme R10 and Cellulase Onozuka R10) were used for cell wall digestion. Of these enzymes, Macerozyme R10 and Cellulase Onozuka R10 were found more effective to digest the cell wall. Enzyme solutions were prepared using 0.7 M mannitol solution with 0.1 per cent $CaCl_2.2H_2O$ as supplement. The combinations and concentrations of enzymes used are given in Table 1.

Table 1. **Combinations and concentrations of enzymes used for protoplast isolation**

Enzyme	Concentration (%)				
	I	II	III	IV	V
Macerozyme R10	0.5	0.5	1.0	1.0	1.5
Cellulase Onozuka R10	0.5	1.0	1.0	1.5	1.5

The leaf tissues, suspended in different enzyme mixtures, were incubated at different temperatures ranging from 20–40 ^{0}C. Approximately 2 g of leaf tissues were incubated in 100 ml of the enzyme solution (Quazi, 1975; Shepard and Totten, 1975). After various incubation periods ranging from 2-5 h, the cells/protoplasts suspensions were filtered through a nylon mesh (40-50 μm) and centrifuged at 100-200 rpm for 3-4 min. The pellet was washed twice with 0.7 M mannitol solution.

Isolated protoplasts were finally purified using sucrose gradient method (Huges *et al.*, 1978; Piwowarczyk, 1979; Bhojwani and Razdan, 1983) by which intact protoplasts, free of enzymes and debris, could be obtained by a single spinning. Sucrose density gradient was used in a centrifuge tube using a layer of 0.7 M sucrose solution at the bottom followed by a layer of 0.5 M sucrose solution and finally by pouring

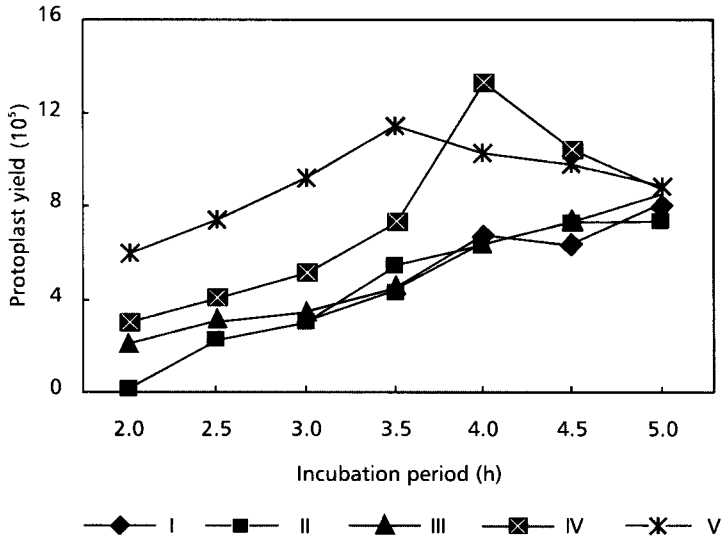

Fig. 1. Effect of different enzyme mixtures on protoplast isolation from leaf mesophyll cells

the protoplast suspension in 0.7 M mannitol solution with 0.1% $CaCl_2$, very slowly along the side of the centrifuge tube. The tubes were centrifuged 300 rpm for 2 min. The pure protoplasts layer was drawn out using a Pasteur pipette. Protoplast counts were made using haemocytometer.

Viability of protoplasts was tested (Wildholm, 1972; Evans and Bravo, 1983) using fluorescein diacetate (FDA). FDA – acetone solution (5 mg/ml) was prepared and diluted to a final concentration of 0.01 per cent. Equal volume of FDA solution and protoplast suspension was taken. Protoplasts were observed under UV microscope after 5 min.

RESULTS AND DISCUSSION

Shredding of the leaves into fine segments was found to be more effective than using the whole leaves or leaf discs.

Among the five different enzyme concentrations and combinations tried, enzyme mixture containing 1 per cent Macerozyme R10 and 1.5 per cent Cellulase Onozuka R10 (No. IV) and 1.5 per cent Macerozyme R10 and 1.5 per cent Cellulase Onozuka R10 (No.V) were found to be the better combinations (Fig. 1). Between these two, combination IV was found better for

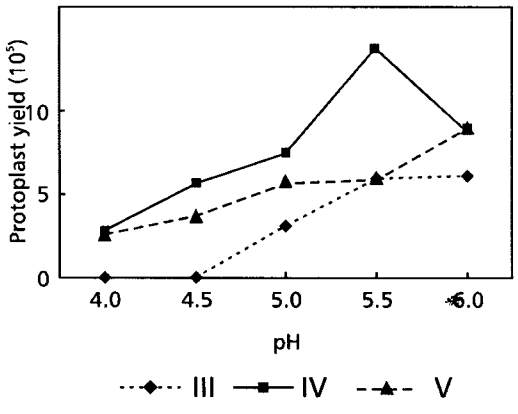

Fig. 2. Effect of pH of enzyme solutions on protoplast isolation

isolating more number of protoplasts without affecting their viability. With the combination V, though the initial protoplast yield was higher that of combination IV, it declined with prolonged incubation that of time (beyond 3.5 h). In this study, it was observed that pH and incubation temperature played critical roles in releasing protoplast cells as both activate enzyme digestion. The effects of pH and temperature on isolation of protoplasts from young leaves are presented in Fig. 2 and Fig. 3 (A-D). The optimum pH and temperature were found to be 5.5 and 35 °C respectively for an incubation period of 4 h. The pH below 5.5 and temperature below 35 °C had resulted in either incomplete digestion of cell walls

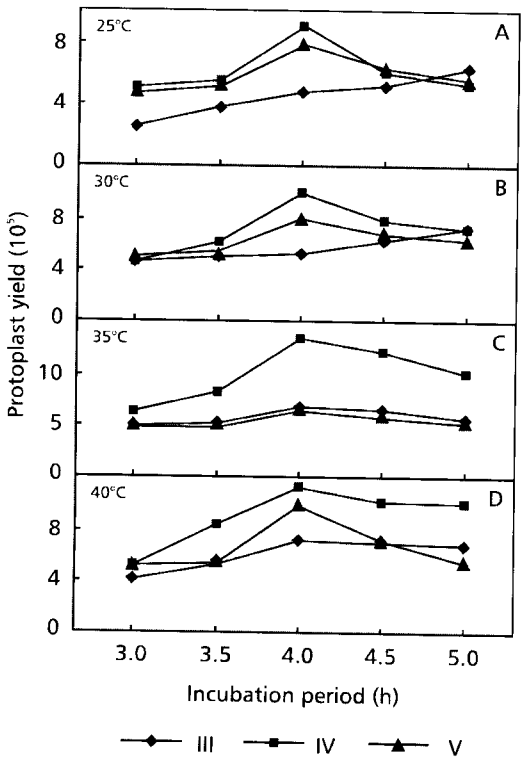

Fig. 3. Effect of incubation temperature (A) 25°C, (B) 30°C, (C) 35°C and (D) 40°C on protoplast isolation

or poor release of protoplasts. These results support the observations of Othman and Paranjothy (1980) where the protoplasts were isolated from pith tissues of *H. brasiliensis* for cell culture studies.

Selection of proper osmoticum in the digestion mixture also played an important role in isolation of protoplasts from *Hevea* leaves. Hypotonic solutions, generally, result in the bursting or budding of the protoplasts whereas slightly hypertonic solutions yield more stable protoplasts. The different concentrations of mannitol solutions tested showed that 0.7 M mannitol concentration was found to be the optimum (Fig. 4) for producing maximum number of stable protoplasts from leaf mesophyll cells. This finding supported the observations of Cazaux and d'Auzac (1994) and Sushamakumari *et al.* (1999) in the production of culture cell protoplasts of *Hevea*. Higher concentrations of mannitol resulted in the shrinkage of protoplasts, which could be due to exosmosis from the protoplasm of the cells.

Incubation period was also found to be very critical even if the pH and temperature were optimum. An incubation period of four hours was found to be the optimum time for isolation of protoplasts from *Hevea* leaf mesophyll cells when pH and temperature were maintained at 5.5 and 35 °C respectively. However, extended incubation period beyond five hours caused the bursting of protoplasts.

During the process of cell wall digestion, the enzyme mixtures contain debris like vascular elements, undigested cells and also some sub-cellular elements along with broken protoplasts. The debris was removed by straining through nylon mesh and then by centrifuging at low speed

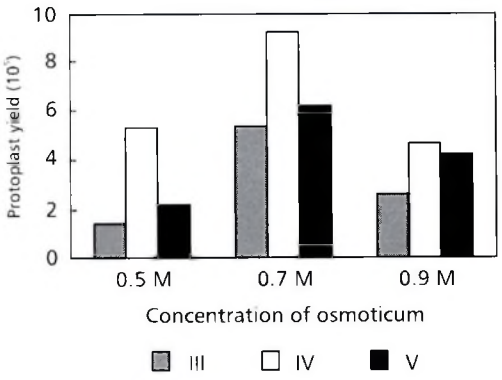

Fig. 4. Effect of different concentration of mannitol (osmotic) solution

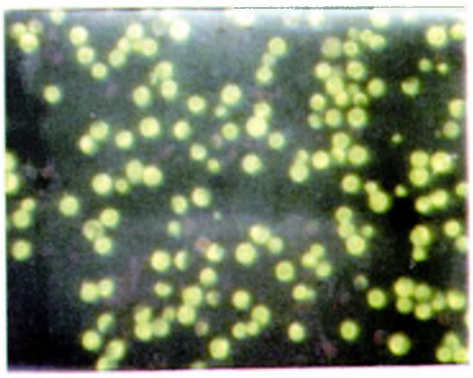

Fig. 5. Intact active protoplast

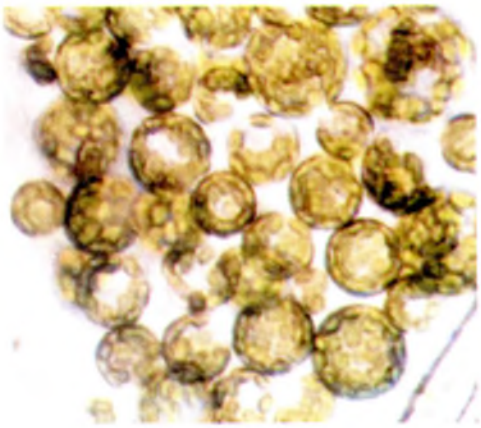

Fig. 6. Isolated protoplast

(100-200 rpm). It was frequently observed that the protoplasts were damaged if higher spinning speed was applied. The pellet was collected and resuspended in osmoticum and the procedure followed was repeated for purification again.

After final purification protoplasts (free from all debris) were visible as a pale yellow ring just above the layer of 0.7 M sucrose gradient and the debris was deposited at the bottom. Intact active protoplasts treated with FDA acetone solution absorbed the stain and exhibited yellow green fluorescence under UV microscope (Fig. 5), whereas dead protoplasts did not show the yellow-green fluorescence. However, the dead mesophylls may be seen as fluorescent red because of the autofluorescence of the chlorophyll. This observation was also reported by Compton *et al.* (1999). In this study, 95 per cent protoplasts were found viable (Fig. 6) that may be used for cell culture explant studies.

It was concluded from the present investigation that young leaves could be used successfully as a potential source for isolating protoplasts which may further be utilised in the advanced crop improvement programmes.

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