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Protoplast studies in cotton (*Gossypium* spp.)

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Abstract

Different explants like tender leaves, cotyledonary leaves, hypocotyl and hypocotyl derived callus of *G. hirsutum* cv. Abadhita and *G. herbaceum* cv. Jayadhar were used for protoplast isolation. Protoplasts were isolated in the enzyme mixture consisting of 5 per cent cellulase, 5 per cent pectinase, 7.5 per cent mannitol and inorganic salts (pH-5.8). Cotton protoplasts originating from different explants varied in size and shape. The variation in size was more evident in callus derived protoplasts. Callus derived protoplast yield was highest followed by tender leaves and least in hypocotyl segments. Protoplast viability test based on Evan's blue staining indicated that per cent of nonviable protoplasts was more from hypocotyl explants followed by cotyledonary leaf and least in callus. Protoplasts derived from different explants did not show any cell division but callus derived protoplasts cultured on MS liquid medium +2.5 g/l glucose+0.l mg/l NAA developed cell wall and microcolonies after 12-15 days of incubation.

Key words : Cotton, protoplast, explant, hypocotyl, cotyledon, callus and viability

Introduction

In recent years much attention has been paid to the development of protoplast technology, because protoplast culture is a feasible system for direct gene transfer, mutant selection and somatic hybridization through protoplast fusion. The first report on regeneration of plants from protoplast of tobacco [1, 2] appeared 11 years after the report on enzymatic release of protoplast [3]. Subsequently, regeneration through protoplast culture was reported in 320 higher plant species representing 146 genera and 49 plant families [4, 5]. Investigations on optimization of isolation and culture conditions were carried for cotton protoplasts isolated from callus cultures [6-10] and cotyledonary segments [11]. Successful regeneration of plants from cotton protoplasts was reported only in Cocker 312 genotypes [10]. The response of *in vitro* technique in other adapted varieties is necessary to add desirable characters through the tools of biotechnology. The present study was carried to find out regeneration possibility via protoplast in plant system where regeneration via callus cultures was not possible. The purpose of such study was to identify and exploit regeneration possibility of cells by exposing them to proper nutrient nourishment because nourishment at protoplast level is more optimum than cells in clumps.

Materials and methods

Materials : 1) *Genotypes* : Two genotypes, Abadhita (4x) belonging to *G. hirsutum* and Jayadhar (2x) belonging to *G. herbaceum* were studied. Abadhita and Jayadhar are popularly grown as bollworm tolerant and drought resistant varieties respectively in South Zone of Indian cotton growing area.

2) *Enzymes and Osmoprotectants* : Cellulase, pectinase (1.5 units/mg, E-Merk Company, Germany) and mannitol (s.d. fine Chemical, India) as osmoprotectants were used for isolation of protoplasts. The different concentrations of chemicals used in the study are presented in Table 1.

Table 1. Different treatment combinations of cellulase, pectinase and mannitol

Sl. No.	Cellulase (%)	Pectinase (%)	Mannitol (%)
1	2.5	2.5	7.50
2	2.5	2.5	10.0
3	2.5	5.0	7.50
4	2.5	5.0	10.0
5	2.5	7.5	7.50
6	2.5	7.5	10.0
7	5.0	2.5	7.50
8	5.0	2.5	10.0
9	5.0	5.0	7.50
10	5.0	5.0	10.0
11	5.0	7.5	7.50
12	5.0	7.5	10.0
13	7.5	2.5	7.50
14	7.5	2.5	10.0
15	7.5	5.0	7.50
16	7.5	5.0	10.0
17	7.5	7.5	7.50
18	7.5	7.5	10.0

3) *Stain for protoplast viability study* : Evan's blue, 0.3 per cent solution was prepared in a culture medium. Viable protoplasts do not take stain while nonviable protoplasts take stain and appear blue.

4) *Media used for callus development from protoplast* : Macro and micronutrients of Murashige and Skoog [12] medium supplemented with B5 organics [(Thiamine HCl (5 mgl^{-1}), Nicotinic acid (1 mgl^{-1}), Pyridoxine HCl (1 mgl^{-1}), Biotin (0.5 mgl-1) and glycine (0.5 mgl^{-1})], 100 mg/l inositol, 7.2g/l glucose, 125 mg/l each of sucrose, fructose, ribose, mannose, cellobiose and mannitol was used. The growth regulators like 2, 4 Dichlorophenoxy acetic acid (2,4-D @ 0.1 mg/1) and 1-Phenyl-3 (1-2-3 Thiadiazole-5yl) urea (TDZ @ 0.5 mg/l) were used. The macro and micronutrients + organics and 2, 4-D were analytical grade from S.D. Fine Chemicals, Mumbai, while TDZ was from Sigma.

Methodology : 1) Preparation of explant source for protoplast isolation

a) *Hypocotyl, cotyledon segments and tender leaves* : Seeds of *G. hirsutum* cv. Abadhita and *G. herbaceum* cv. Jayadhar were aciddelinted and surface sterilized with 90 per cent ethyl alcohol followed by 15 min in 0.05 per cent $HgCl_2$ (w/v). Then, seeds were washed three times with sterile water and soaked for 24 hours. The germinated seeds were cultured on hormone free 1/2 strength MS medium [12] and incubated at 28°C with 12 hours light. One mm hypocotyl and 1 sq. mm cotyledon segments were obtained from three to five days old seedlings and 1 sq. mm. segments of tender leaves were obtained from 13-15 days old plants.

b) *Callus induction* : The hypocotyls obtained from aseptically grown 3-5 days old seedlings were sectioned into 2-3 mm segments and cultured in MS medium supplemented with 0.1 mg/l 2,4-D and 0.5 mg/l TDZ to induce callus.

2) *Isolation of Protoplast* : Hypocotyl segments (1.00 mm long), cotyledon segments (1.00 sq. mm) and tender leaf segments (1.00 sq.mm) and fresh callus mass ($\simeq$ lg) were mixed with various protoplasts isolation combinations mentioned in Table 1. The pH of the solution was maintained at 5.8. The cultures were incubated for 5 hours at 26±20°C under gentle shaking (85 rpm). The digested homogenous cell material was passed through sterilized muslin cloth to remove the debris and the filtrate was centrifuged (3 x 100g) for 5 minutes. The supernatant was discarded and the pellet was washed thrice by repeated suspension and centrifugation in the wash solution consisting of NaCl (8g/l), $CaCl_2$ (18.4g/l), KCl (0.4 g/l) glucose (lg/l) and mannitol (30 g/l) with pH 5.8.

3) *Protoplast variability and viability* : Variability in protoplast size originating from different cultivars and explant sources was recorded using stage and ocular micrometer. Number of protoplasts was calculated based on ten microscopic (15X x l0X) field counts. This value indicates total yield of protoplasts which was taken as the measure to compare relative yield of protoplasts originating from different explants. The total yield of the protoplast consisting of large, medium and small sized protoplasts was calculated. Each category (size) of protoplasts was expressed as percentage of the total protoplast yield. Viability of protoplasts was tested by staining with 0.3 per cent Evan's blue solution. Small sample of protoplasts were transferred to glass microscopic slide and one drop of Evan's blue stain was added. Slides were observed after 5-10 minutes.

4) *Protoplast culture establishment* : Two culture methods, the droplet method and culturing on filter paper placed on liquid media having glass bead, have been followed. The second method facilitates continuous supply of fresh medium which may stimulate gaseous exchange and absorb residual enzymes. Suspension culture of protoplasts was incubated in petriplates drop wise in one method and in second method protoplasts were placed over filter paper put on liquid medium. Physical factors like temperature, light and humidity for incubation of protoplast culture were 26±2°C, 12 hours white fluorescent light (1000 lux) and 80-85 per cent, respectively.

Results and discussion

Before putting the explants in the enzyme mixture, they were pretreated with plasmolysis solution (10 per cent sucrose) for easy accessibility of cell wall for the action of enzymes (Fig. 1). The yield of protoplasts primarily depends on optimum concentration and combination of mascerating enzymes and osmotic agents. Among the 18 treatments (Table 1) consisting of combination of different concentrations of cellulase, pectinase and matinitol, one treatment combination with five per cent cellulase, 5 per cent pectinase and 7.5 per cent mannitol resulted in higher protoplast yield (Fig. 2).

Cotton protoplasts originating from different explant sources varied in size and shape [6]. Though they are characteristically spherical in shape, different shapes like round, oval and oblong were observed. The variability in cell shape in callus derived protoplasts may primarily be attributed to the heterogenity of the cultured cell populations [13]. Variation in size (Table 2) and shape might be due to variability of cell types in tissues of different explants. The variation in size of the protoplast obtained from two cultivars, Abadhita (4x) and Jayadhar (2x) was almost similar irrespective of explant source. However, range of variation in size was more in protoplasts derived from tender leaves (25-60µ and 22-60µ in Abadhita and Jayadhar respectively) and callus (20-60µ and 25-60µ in Abadhita and Jayadhar respectively) (Table 3).

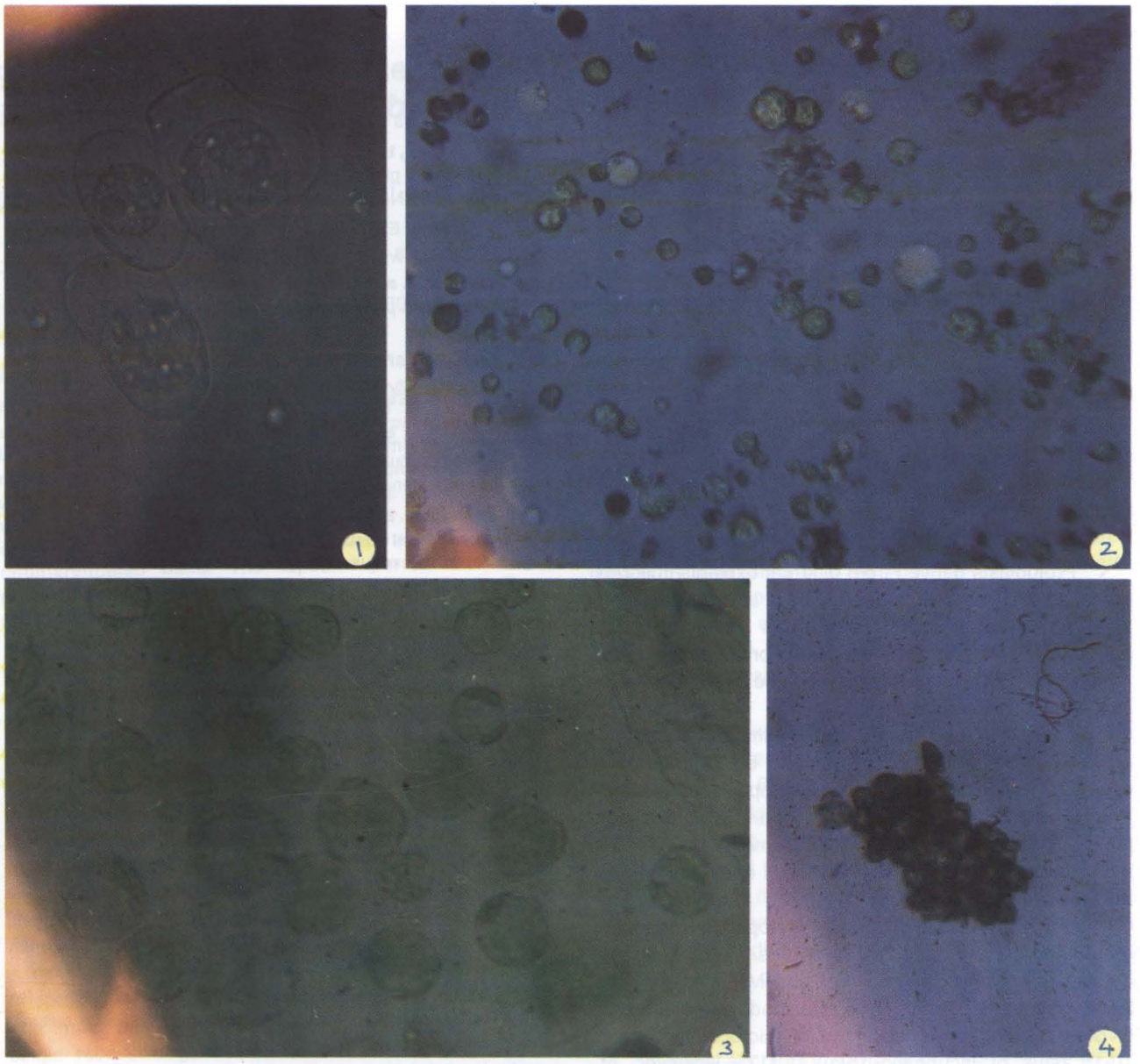

Fig. 1. Callus cells treated with plasmolysis solution; 2. Protoplast viability test with Evan's blue stain; 3. Isolated free protoplasts; 4. Protoplast derived microcolony

Table 2. Frequency of different sized protoplasts obtained from different cultivars and explant sources

Explant	Cultivars									
	Abadhjta					Jayadhar				
	Big	Medium	Small	Total	Nonviable	Big	Medium	Small	Total	Nonviable
Tender leaf	40.0 (57.73)	17.85 (25.76)	11.42 (16.48)	69.28	8.80 (12.70)	30.85 (54.44)	7.50 (13.23)	18.33 (32.35)	56.66	9.33 (16.46)
Cotyledonary leaf	16.36 (24.11)	28.22 (41.59)	23.26 (34.28)	67.84	13.26 (19.54)	8.70 (15.60)	29.00 (52.00)	18.06 (32.38)	55.76	12.78 (22.91)
Hypocotyl Segment	12.42 (20.55)	35.62 (58.95)	12.38 (20.48)	60.42	19.65 (32.52)	7.62 (14.29)	33.39 (62.65)	12.28 (23.04)	53.29	17.17 (32.21)
Hypocotyl derived callus	22.34 (31.83)	34.28 (48.85)	13.55 (19.31)	70.17	6.00 (8.55)	14.44 (22.16)	35.62 (54.66)	15.1 (23.17)	65.16	5.28 (2.10)

Table 3. Variation in size (m) of protoplasts originating from different genotypes and explant sources

Genotypes	Tender leaves	Cotyledonary leaves	Hypocotyl Segment	Hypocotyl derived callus
Abadhita	25-60	21-55	20-56	22-60
Jayadhar	20-60	20-50	19-50	25-60

Callus derived protoplast yield was highest (70.17 in Abadhita and 65.16 in Jayadhar), followed by tender leaf (69.28 in Abadhita and 56.66 in Jayadhar) and least in hypocotyl segments (Table 2). The major proportion of callus, hypocotyl and cotyledons derived protoplasts were medium in size whereas majority of protoplasts derived from tender leaf were bigger (57.73%

in Abadhita and 54.44% in Jayadhar). This indicates that protoplast yield and their size depends on type of explants [8, 9].

Protoplasts obtained from calli were light green in color with prominent central vacuole and peripherally aligned nucleus. Tender leaf and hypocotyl derived protoplasts were also light green in color with sparsely distributed green granule like structures on them. But protoplasts from cotyledonary leaf were dark green with rich cytoplasm.

Protoplast viability based on Evan's blue staining test indicated that the per cent of nonviable protoplasts were more in hypocotyl derived protoplasts (32.52% in Abadhita and 32.21% in Jayadhar) followed by cotyledonary leaf (19.54% in Abadhita and 22.91 in Jayadhar) and least in callus (8.55% in Abadhita and 8.10% in Jayadhar) (Table 2 and Fig. 3).

Protoplasts derived from different explants cultured on liquid MS medium did not show any cell division and degenerated after 8-10 days of incubation. Favorable conditions for rapid and continued cell wall regeneration in protoplasts may be the prerequisite for sustained division of protoplasts [6]. In few reports, the presence of Ca^{+2} ions, ammonium ions and poly ethylene glycol (PEG) were found to be important for cell wall formation and further protoplast division in *Phaseolus vulgaris* [14], Tobacco [15] and *Datura carota* [16]. On liquid MS medium supplemented with 100 mg/l inositol + 7.2g/l glucose + 125mg/l each of sucrose, fructose, ribose, mannose, cellobiose and mannitol + 0.1mg/l 2,4-D + 0.5mg/l TDZ, protoplasts remained green and free without any cell division. Only callus derived protoplasts cultured on liquid MS medium with 2.5g/l glucose + 0.1mg/l NAA remained green and cell division resulted in development of microcolonies after 10-15 days of incubation (Fig. 4) [17]. However, at very low frequency, protoplasts isolated from other explants exhibited cell division but did not divide beyond 2 cell stage, on any of the culture media. Nutrient and growth regulator management helped only for regeneration of cell wall and division of cells to form callus. But absence of regeneration in these callus clearly indicates need for the gene cassette for differentiation from de-differentiated cells in cotton cultivars which would further pave a way for the exploitation of protoplast cultures in genetic engineering of cotton.

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