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ORIGINAL PAPER

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A Comparative Study on Isolation, Culture and Regeneration of Cell Suspension Protoplasts of *Solanum Tuberosum* L.

Abstract: Protoplasts were isolated from stem-derived cell suspensions of *S. tuberosum* cvs. Desiree and Maris Piper using three enzyme mixtures. The enzyme mixture containing 1% cellulase ("Onozuka" R-10) and 0.1% Pectolyase Y-23 in 0.5 M Mannitol with an incubation period of 3h resulted in the highest protoplast yield. Isolated protoplasts were cultured in different media. In MS-KM liquid medium, they divided and formed small cell colonies. Resulting calluses were transferred either to MS medium containing 0.05 µM NAA, 2.85 µM zeatin riboside and 0.03 µM GA_8 or to modified MS medium containing 0.54 µM NAA, 0.29 µM IAA, 2.22 µM BAP, 2.28 µM zeatin, 0.93 µM kinetin and 0.58 µM GA_3 for shoot regeneration. Shoot regeneration was 2 weeks earlier on the former medium.

Keywords: Plant regeneration, potato, protoplasts, *Solanum tuberosum*, suspension culture.

الأنعزال والغرس وتجدد خلق النباتات من بروتوبلاستات الخليات المعلقه لبطاطة

م أ أنجم

المستخلص: عزلت البروتوبلاستات من خلايا ساق البطاطا المعلقة بأستخدام ثلاث مخاليط من عدة أنزيمات. وتحصلنا على إنتاج اكثر من البروتوبلاستات بحضانة الخلايا المعلقة ، الناتج من تفاعل 1% Cellulase Onozuka, %0.1 Pectolyase Y-23 المخلوطة في 5% المولر Mannitol لفترة ثلاث ساعات . تم إستنبات البروتوبلاستات المعزولة في ثلاث مستنبتات . الأول MS-KM (وسط سائل)، انقسمت فية البروتوبلاستات المعزولة الى مستعمرات خلايا صغيرة. الثاني MS والذي أضفنا فية 0.05, 0.03, 2.85 ميكروفولر NAA ، Zeatenriboside ،GA3 على التوالي . أما في المستنبت الأخير MS المعدل كانت إضافة 4 0.5, 0.29, 2.22, 2.28 GA_3, Kinetin, Zeatin,BAP, IAA, NAA ميكرومولز 0.58, 0.93, على التوالي لتجديد خلق براعم النباتات والتي لاحظنا سبق استنباتها في المستنبت الأول MS الوسط السائل بأسبوعين منها في المستنبت الأخير MS المعدل.

كلمات مدخلية: نبات–بطاطس– تجديد البراعم . بروتوبلاستات –مستنبت. خلايا معلقة

List of abbreviations: BAP: 6-benzylaminopurine, GA_3: Gibberellic acid, IAA: Indole-3-acetic acid, MES: 2(N-morpholino) ethanesulphonic acid, NAA: α-Naphthaleneacetic acid, RNA: Ribonucleic acid, KM: Kao and Michayluk (1975), MS: Murashige and Skoog (1962).

Introduction

Plant protoplasts are considered to be useful tools for plant improvement. Their capability to regenerate into plants makes them potentially interesting material for somatic hybridization, plant transformation and selection studies. However, before starting plant improvement work, isolation and culture procedures should be developed including the selection of starting material, enzyme mixture and culture medium.

Leaf mesophyll cells and suspension-cultured cells are the most common sources for protoplast isolation. In potato, leaf mesophyll cells have been used more frequently than any other source (Bokelmann and Roest, 1983; Feher, *et al.* 1989; Cardi, *et al.* 1990 Kochevenko, *et al.* 1996; Anjum, 1998). Cell suspension cultures are an excellent source for the isolation of non-green protoplasts, which are advantageous in somatic hybridization experiments with mesophyll protoplasts because heterokaryons can be easily identified. Furthermore, they are already aseptic, respond better to *in vitro* culture conditions and usually require a lower quantity of cell-separating enzyme. Therefore in this study, an attempt was made to compare different media and develop an efficient method for the large-scale isolation of protoplasts from cell suspension cultures of two potato cultivars, Desiree and Maris Piper, and to regenerate them into plants.

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Materials and Methods

Protoplast isolation

Cell suspension cultures were established from stem callus of *S. tuberosum* cvs. Desiree and Maris Piper as described earlier (Anjum and Mahmud, 1998). Cells were obtained by centrifuging 5 - 7 day-old cell suspensions at 100g for 5 minutes (Chung and Sim, 1986). 2ml packed cell volume (PCV) was mixed with 10 ml of enzyme solution for digestion. Three different enzyme combinations were examined (Table 1). The mixtures were incubated at 27°C in the dark on a rotary shaker at 40 rpm. The incubation period varied with the enzyme composition used (Table 1). Protoplasts were separated from cell debris by filtration through a nylon mesh (70 µm). The filtrate was transferred to sterile plastic centrifuge tubes (15ml) and centrifuged at 70g for 5 minutes. Protoplasts were washed twice by suspending the pellet containing protoplasts in washing medium (0.5 M mannitol) and centrifuging at 70g for 3 minutes (De Vries and Bokelmann, 1986). Protoplast yield was determined by using a modified Fuchs-Rosenthal haemocytometer (chamber depth, 0.2 mm). Viability of freshly isolated protoplasts was estimated using the 0.01% fluorescein diacetate (Widholm, 1972) or 0.4% Evans' blue (Gaff and Okong'O-ogola, 1971). Viability was expressed as the percentage of viewed protoplasts.

Protoplast culture

Freshly isolated protoplasts were cultured in three media i.e. modified MS medium (De Vries and Bokelmann, 1986), V-KM medium (Bokelmann and Roest, 1983) and MS-KM medium (containing inorganic salts of modified MS medium and the organic salts of V-KM medium). Protoplasts were suspended in the culture medium at a final density of 1 x 10^5 protoplasts ml^{-1}, and 2 ml of protoplast suspension was plated into plastic petri dishes (Ø 55 mm). The petri dishes were sealed with Parafilm and incubated at 25 ± 1°C in continuous light at an irradiance of 70 µmol $m^{-2}s^{-1}$ from cool-white fluorescent lamps. After one week, the protoplast cultures were diluted to twice their volume with fresh liquid culture media. The following week, an equal volume of culture medium plus 0.4% agar was added, and the cultures incubated for 2 weeks. Subsequently, the small calluses, about 0.5 mm in diameter, were plated in Ø 90 mm plastic petri dishes on solidified MS medium (Murashige and Skoog, 1962) containing 0.8% agar, 3.0% sucrose plus 4.44 µM BAP and 1.07 µM NAA, and incubated at 25 ± 1°C in continuous light at 200-250 µmol $m^{-2}s^{-1}$.

Plantlet regeneration

Protoplast-derived calluses were transferred either to MS medium containing 0.05 µM NAA, 2.85 µM zeatin riboside and 0.03 mM GA_3 (Bokelmann and Roest, 1983) or to modified MS medium containing 0.54 µM NAA, 0.29 µM IAA, 2.22 µM BAP, 2.28 µM zeatin, 0.93 µM kinetin and 0.58 µM GA_3 (Lam, 1977) for shoot regeneration. Cultures were incubated at an irradiance of 200-250 mmol $m^{-2}s^{-1}$ in a 16h photoperiod at 20 ± 1°C. Regenerated adventitious shoots about 1 - 2 cm long were periodically excised and transferred to MS medium containing 3.0% sucrose, 0.8% agar plus 8.39 µM Ca-pantothenic acid and 0.72 µM GA_3 for root formation.

Table 1. Composition of enzyme mixtures; I (De Vries and Bokelmann, 1986), II (Chung and Sim, 1986) and III (Tavazza, *et al.* 1988), and incubation periods used for protoplast isolation.

	Enzyme mixture		
Constituents	I	II	III
Enzymes (w/v)			
Cellulase (Onozuka R-10)	1%	2%	3%
Macerozyme (R-10)	—	1%	2%
Pectolyase Y-23	0.1%	—	—
Mineral salts			
Salt mixture	—	MS (exc. NH_4NO_3)	—
$CaCl_2.H_2O$	—	—	408 mM
Osmoticum			
Mannitol	0.5 M	0.5 M	0.3 M
Buffer			
MES	—	—	5 mM
pH	5.5	5.7	5.5
Incubation time	3 hours	5 hours	12 hours

Results and Discussion

Protoplast isolation

The most important factors for the liberation of protoplasts from cultured cells are the physiological state of the donor culture, the type and concentration of enzymes, and the incubation period. In the present work, 5 to 7 day-old cell suspensions were used and three enzyme compositions were tested. The number of protoplasts isolated from one ml of PCV varied depending upon the enzyme composition and incubation period (Table 2)

Table 2. Yields (number of protoplasts) per ml of PCV and viabilities of *S. tuberosum* cvs. Desiree and Maris Piper protoplasts isolated from stem-derived cell suspensions by using different enzyme mixtures.

	Enzyme mixture (see Table 1)		
	I	II	III
Yield (x 10^5)			
Desiree	3.2	0.85	2.7
Maris Piper	2.8	1.0	2.5
Viability (%)			
Desiree	85	82	75
Maris Piper	80	84	72

Data represent means of 3 replicates

For both Desiree and Maris Piper, yields of protoplasts were higher when cells were incubated in enzyme mixture I, containing 1% cellulase Onozuka and 0.1% Pectolyase Y-23 in 0.5 M mannitol, for 3h. The lowest protoplast yields were obtained with 2% cellulase Onozuka and 1% Macerozyme R-10 (enzyme mixture II) and an incubation period of 5 hours. However, when concentrations of cellulase Onozuka and Macerozyme R-10 were increased to 3 and 2% respectively (enzyme mixture III) and incubation period extended to 12h, the yields of protoplasts increased 2.5 to 3-fold. This was still lower than the yield with enzyme mixture I (Table 2) and also a lowered protoplast viability resulted. Similar variations in the influence of enzyme composition on protoplast yields have been reported by several workers (Handley and Sink, 1985, Chung and Sim, 1986, Tavazza, *et al.* 1988). In the present work, Pectolyase Y-23 was proved to be an effective macerating agent, and in combination with cellulase Onozuka released a large number of protoplasts. Similar results were reported for protoplast isolation from mesophyll cells of pea (Nagata and Ishii 1979).

The viabilities of freshly isolated protoplasts ranged from 72 to 85% in both Desiree and Maris Piper. In enzyme mixtures I and II, with incubation periods of 3 and 5 hours respectively, protoplast viabilities were 80 - 85% but for protoplasts isolated using enzyme mixture III, with an incubation period of 12 hours, the viabilities of protoplasts decreased to 72 - 75% (Table 2) indicating the harmful effects of enzymes during the prolonged incubation, as the excessive enzymes would be toxic for plant cells.

Protoplast culture

For protoplast culture, modified MS medium (lacking NH_4NO_3) has been used by several workers (Handley and Sink, 1985; De Vries and Bokelmann, 1986; Tavazza, *et al.* 1988), while some workers used V-KM medium (Bokelmann and Roest, 1983; Feher, *et al.* 1989; Cardi, *et al.* 1990). Both of these media have been shown to generate calluses from potato protoplasts. In the present study, modified MS medium almost failed to support the growth of protoplasts while in V-KM medium protoplast plating efficiency (the percentage of plated protoplasts which underwent cell division), determined after 14 days of culture, was only 3 - 5% (Table 3). Therefore, a new MS-KM medium, a modification of V-KM medium (Bokelmann and Roest, 1983), was developed in which the inorganic salts of V-47 medium (Binding 1974) were replaced with MS inorganic salts. Hence this medium was composed of inorganic salts of modified MS medium and the organic salts of V-KM medium.

In MS-KM medium, protoplasts began dividing after 3 days of culture (Fig. 1, B). In the second week, the cultures were diluted by adding an equal volume of fresh culture medium. Protoplast plating efficiency, determined after 14 days of culture, was 21% in Desiree and 15.5% in Maris Piper (Table 3). Cell colonies (0.5 - 1 mm in diameter) were obtained two weeks after adding soft medium (MS-KM plus 0.4% agar), and were subsequently plated over solidified MS medium for further growth. Due to the higher irradiance (200-250 $\mu mol\ m^{-2}s^{-1}$), small calluses became pale green and grew to about 2 - 3 mm in diameter during 2 weeks (Fig. 1, C). These calluses were then transferred to regeneration medium for shoot development. Higher protoplast plating efficiencies were recorded in Desiree as compared to Maris Piper. Considerable differences among potato genotypes have already been noticed in protoplast plating efficiency and subsequent growth (Carputo, *et al.* 1995). This could be because

different genotypes may have specific nutritional and hormonal requirements to initiate and/or sustain division of their protoplasts, which is prerequisite for colony formation (Anjum, 1998).

Plantlet regeneration

Shoot initiation from calluses started on medium of Bokelmann and Roest (1983) after 8 weeks of transfer in both Desiree and Maris Piper but it took 10 weeks on medium of Lam (1977). This earliness of 2 weeks in shoot regeneration from calluses subcultured onto the former medium than those subcultured onto the latter medium was probably because these media contained different growth regulators in different concentrations. Cytokinins could possibly act through transfer RNA, and the former medium contained zeatin riboside which was probably readily available to the plant cells (Anjum, 1998).

According to Carputo, *et al.* (1995) shoot regeneration is strongly influenced by the medium utilised. In the present study, shoot regeneration frequencies (the percentage of calluses which produced shoots) were higher on the medium of Bokelmann and Roest (1983) than on medium of Lam (1977), in both cultivars (Table 3). This indicates that the former medium had better composition of nutrients and balanced auxin/cytokinin ratio. The regenerated shoots, 1 - 2 cm long (Fig. 1, D), were removed and transferred to the rooting medium where 90% shoots developed roots within 3 weeks. Then they were transferred to small plastic pots containing sterilised compost. These pots were covered with transparent plastic containers to maintain high humidity around the plantlets. After 2 weeks the plantlets were transferred to pots containing soil mix (John Innes No. 2) and moved to a greenhouse under non-sterile conditions (Fig. 1, E) where they continued their growth and also formed tubers.

TABLE 3. Protoplast plating efficiencies and shoot regeneration frequencies from the protoplasts-derived calluses of *S. tuberosum* cvs. Desiree and Maris Piper on different media.

Media used	Desiree	Maris Piper
Plating efficiency (%)		
Modified MS	1.0 ± 0.3	0.5 ± 0.2
V-KM5.0 ± 1.0	3.3 ± 0.6	
MS-KM	21.0 ± 3.2	15.5 ± 2.8
Regeneration frequency (%)		
Bokelmann & Roest (1983)	52.0 ± 6.1	54.0 ± 5.8
Lam (1977)	44.0 ± 5.5	38.0 ± 5.6

Data (± SD) represent means of 3 replicates

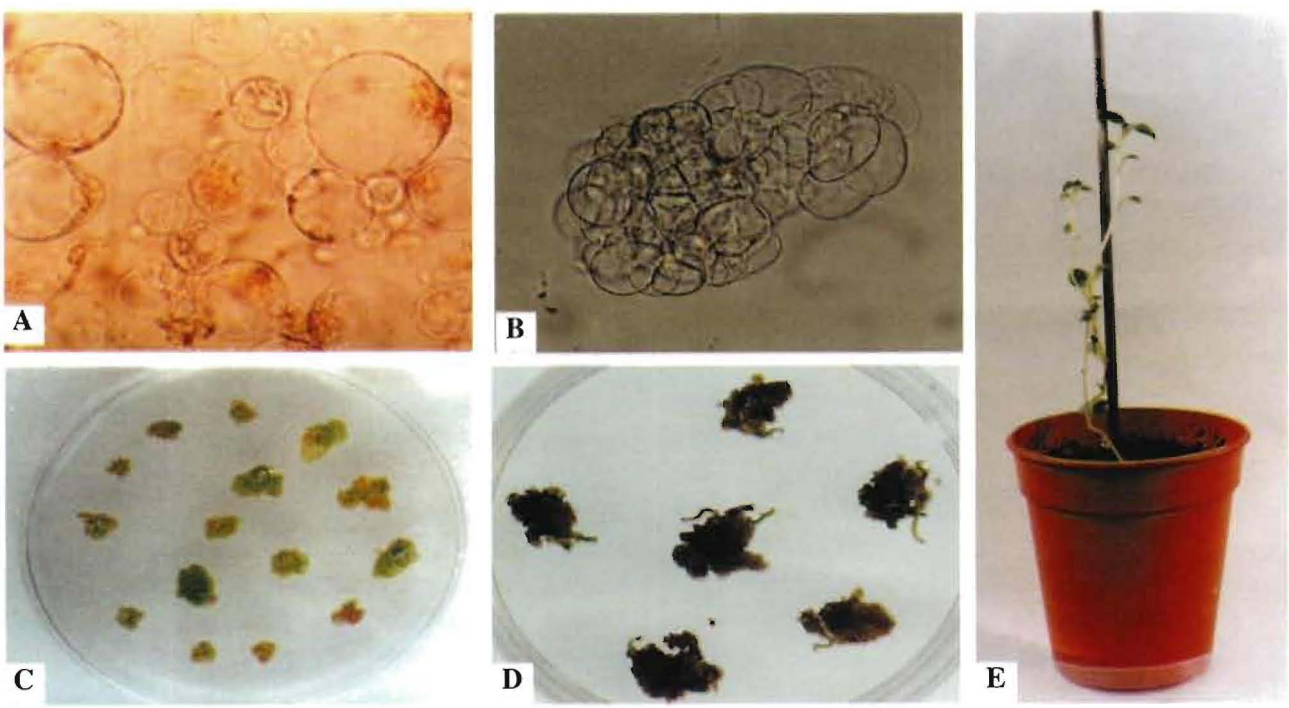

Fig. 1. A) Protoplasts of cv. Desiree isolated from cell suspension cultures; B) A cell colony developed from a protoplast, after 10 days of culture in MS-KM medium; C) Protoplast-derived calluses transferred to solidified MS medium, photographed after 2 weeks; D) Shoot regeneration from protoplast-derived calluses, photographed after 13 weeks of culture; E) A plantlet regenerated from protoplast-derived callus and transferred to a pot containing soil mix.

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