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Short communication

Isolation and culture of *Lens culinaris* Medik. cv. Eston epicotyl protoplasts to calli

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Abstract

Protoplasts of *Lens culinaris* Medik. cv. Eston were isolated from epicotyl tissues of seedlings grown on Murashige & Skoog basal medium. For isolating the protoplasts, epicotyl tissues were digested for 12–14 h at 25°C in an isolation mixture (pH 5.7) containing 1% Cellulase RS, 0.5% Driselase, 0.25% Pectolyase Y23, 0.2 M calcium chloride, 10 mM mannitol and 10 mM MES. Protoplasts were purified by flotation over 20% sucrose and washed with 0.2 M calcium chloride solution supplemented with 10 mM mannitol. Purified protoplasts were cultured at a density of 10^5 ml^{-1} in agarose (Seaplaque, 0.6%) blocks which were suspended in an identical but liquid KM8P culture medium lacking amino acids, ammonium nitrate, and coconut water but containing 0.35 M glucose and a growth regulator complement of either 2.2 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.7 μM naphthaleneacetic acid (NAA), 2.3 μM N-(2-furanylmethyl)-1H-purine-6-amine (kinetin), 2.2 μM benzylamino purine (BAP), 2.3 μM 2-methyl-4-(1H-purine-6-ylamino)-2-buten-1-ol (zeatin), and 1.4 μM gibberellic acid (GA_3), or 5.4 μM NAA and 2.2 μM each of 2,4-D and BAP. The osmotic potential of the liquid culture medium was gradually reduced over a period of 3 weeks by replacing the spent medium with a fresh medium containing 0.25, 0.1 and 0 M glucose at weekly intervals. About 6% of the dividing protoplasts developed into cell colonies after 3 weeks of culture at 25°C in diffuse light (10 μE m^{-2} s^{-1}). In 35–42 days the microcolonies were about 1 mm in diameter and developed into calli on transfer to agar-solidified B5 medium supplemented with growth regulators used in the protoplast culture medium and 5 mM glutamine. Attempts to regenerate plants from protoplast-derived calli have so far been unsuccessful.

Introduction

Lens culinaris (lentil) is an important grain legume cultivated world-wide as a human food source [2] because of its nutritious seed. In recent years, it has gained considerable popularity as a speciality cash crop [3]. In addition to conventional breeding, in vitro cell culture techniques are also expected to contribute to improvement in seed quality, productivity, and disease resistance of lentil. Currently, plant regeneration from callus cultures of *Lens culinaris* has been successful both via organogenesis [13] and somatic embryogenesis [10]. The rescue of embryos from interspecific crosses in *Lens* species has also been achieved [1]. However, the introduction of foreign genetic material using protoplast fusion and direct gene transfer requires production of viable protoplasts and a protocol for regeneration of plants. To date, there has been no such report for lentil. Stiff et al. [12] described the culture of leaf protoplasts from the cv. Laird. However, cell divisions were accompanied by the accumulation of a brown exudate which inhibited the development of viable cell colonies; only sporadic

microcalli were obtained. The present communication describes the isolation of viable protoplasts from lentil epicotyl tissue and their further development to form calli.

Materials and methods

Seeds of *Lens culinaris* Medik. cv. Eston were surface-sterilized by immersion in 95% (v/v) ethanol for 30 s and then in a 25% (v/v) solution of Javex (Bristol Meyers, Toronto, Ontario, Canada) for 10 min. The sterilization procedure was repeated with fresh Javex solution and the seeds were washed 4–5 times with sterile distilled water. Ten to twenty seeds were placed on MS basal medium [8] solidified with 0.8% (w/v) Difco agar in 100 × 10 mm Petri dishes. The dishes were incubated at 25°C in the dark to promote elongation of the epicotyls. Seeds were incubated for at least 4 days to provide sufficient epicotyl tissues. Epitotyls were excised and cut into 1–2 cm segments which were then sliced longitudinally several times with a scalpel. About 100 mg of the sliced tissues in 5 ml of an enzyme solution in a 60 × 15 mm Petri dish were incubated for 12–14 h at 25°C in the dark on a horizontal gyrotory shaker at 25 rpm. The enzyme mixture (pH 5.7) consisted of (w/v) 1% Cellulase RS, 0.25% Pectolyase Y-23, 0.5% Driselase and 10 mM MES (2[N-morpholino]ethanesulfonic acid) in combination with five different test osmotica: (1) 0.2 M $CaCl_2 \cdot 2H_2O$ and 10 mM mannitol; (2) 5 mM $CaCl_2 \cdot 2H_2O$ and 0.5 M mannitol; (3) 12% sucrose; (4) 0.125 M KCl, 0.125 M NaCl, and 0.5 mM $CaCl_2 \cdot 2H_2O$; (5) 0.125 M KCl and 0.125 M $CaCl_2 \cdot 2H_2O$.

Released protoplasts were separated from coarse debris by passage through a 85 μm filter and collected in a disposable centrifuge tube (15 ml). After centrifuging (70 *g*; 10 min), the enzyme solution was pipetted off and the protoplasts were resuspended in 5 ml of a solution containing 20% (w/v) sucrose, 0.5% (w/v) $CaCl_2 \cdot 2H_2O$, and 10 mM MES solution (pH 5.7). Two ml of osmotic solution (containing 10 mM MES) of the same composition used in the five enzyme solutions was gently layered on top of the sucrose solution. The tube was recentrifuged (50 *g*; 10 min) whereby the protoplasts floated to the interface between the two solutions. The band of protoplasts was removed with a Pasteur pipette and transferred to a centrifuge tube and 5 ml of osmotic solution as used in the enzyme mixture were added to the tube. This suspension was centrifuged (70 *g*; 5 min) to pellet the protoplasts and wash any remaining enzymes. The wash procedure was repeated and the pellet was suspended in 1 ml of culture medium. The protoplasts were counted on a haemocytometer (Hausser Scientific, Blue Bell, Pa., USA).

Protoplasts were cultured at a density of 10^5 ml^{-1} in liquid or solid medium or in a combination of solid and liquid medium. Three replicates were prepared for each treatment. For liquid culture, 2.5 ml volumes of the protoplast suspension were transferred to 35 × 10 mm Petri dishes and the dishes were gently rotated to distribute the suspension uniformly. To culture the protoplasts in solid medium, 1.25 ml of the suspension adjusted to a density of 2 × 10^5 protoplasts ml^{-1} was mixed with 1.25 ml of culture medium containing 1.2% (w/v) agarose (Seaplaque) held in molten state at 45°C in a water bath and the mixture was pipetted into a 35 × 10 mm Petri dish. In experiments where protoplasts were cultured in agarose blocks, the protoplast suspension (6.0 ml) was plated in a 60 × 15 mm Petri dish as described above and the gel was cut into 14–16 blocks of ca. 1.0 cm^2. Half of the agarose blocks (7–8) were transferred to another dish and to both the dishes liquid protoplast culture medium (2.5 ml) was added. Agarose block cultures were kept on a horizontal gyrotory shaker set at 50 rpm. The media used were based on the KM8P [6] and contained 0.35 M glucose, 1 g l^{-1} sucrose, 600 mg l^{-1} or 1 g l^{-1} $CaCl_2 \cdot 2H_2O$, and a growth regulator complement of either 2.2 μM 2,4-D, 2.7 μM NAA, 2.2 μM BAP, 2.3 μM kinetin, 1.4 μM GA_3 (KM8P-5H), or 5.4 μM NAA and 2.2 μM each of 2,4-D and BAP (KM8P-3H). Coconut water, casamino acids and ammonium nitrate were deleted from all media. The pH of the media was adjusted to 5.7 with KOH prior to filter sterilization (0.22 μm, Nalgene). All cultures were incubated at 26°C in diffuse light (10 μE m^{-2} s^{-1}) provided by cool white fluorescent tubes (Westinghouse, Pittsburgh, USA) with a photoperiod of 16 h.

After 14 days of culture, the osmotic potential of the culture media was lowered in all three types of cultures. In liquid cultures, the old medium along with protoplast-derived cells was transferred into a

centrifuge tube and allowed to stand for 15 min. The medium was pipetted out carefully without disturbing the resulting pellet and 2.5 ml of a fresh culture medium of reduced osmolarity were added. The suspension was transferred to a fresh Petri dish. In solid cultures, the agarose gel containing protoplast-derived cells was cut into two sectors which were then transferred into 100 × 10 mm Petri dish containing a fresh agarose (0.6%)-solidified medium of reduced osmolarity. In agarose block cultures, the surrounding liquid medium was replaced with a fresh medium of reduced osmolarity. The osmolarity of the protoplast culture medium was reduced every week for a total of 3 weeks by reducing the concentration of the osmoticum (glucose) to 0.25, 0.1, and then 0 M. Microcolonies, about 1 mm^2, developed in 35–42 days after protoplast isolation and were gently teased out of the agarose gel and transferred to 100 × 10 mm Petri dishes containing solidified (0.8% Difco agar) B5 medium [4] with a growth regulator complement of either 2.2 μM 2,4-D, 2.7 μM NAA, 2.3 μM kinetin, 2.2 μM BAP, 1.4 μM GA_3 (B5-5H) or 5.4 μM NAA and 2.2 μM each of 2,4-D and BAP (B5-3H) and 5 mM glutamine. Plating efficiency (%) was determined after a week of culture and referred to the number of protoplasts which divided at least twice to develop 2–4-celled clusters. Percentage cell colony formation was calculated on the basis of the total numbers of dividing protoplasts and the cell colonies recovered after 5 weeks of culture. All experiments were repeated twice.

Results and discussion

Protoplast release started within 2 h of enzymatic digestion but 12–14 h incubation was necessary to complete an isolation. All 5 osmotic solutions maintained intact protoplasts, however, some did not result in suitable yields. With 0.5 M mannitol + 5 mM $CaCl_2 \cdot 2H_2O$ and 12% sucrose, significant numbers of protoplasts remained suspended in the supernatant during initial centrifugation to pellet the protoplasts. An increase in the speed of centrifugation up to 200 *g* was ineffective in enhancing protoplast collection with these solutions. The use of isotonic salt solutions of 0.125 M KCl + 0.125 M $CaCl_2 \cdot 2H_2O$, or 0.125 M KCl + 0.125 M NaCl + 0.5 mM $CaCl_2 \cdot 2H_2O$ allowed efficient pelleting but resulted in poor viability since the protoplasts died after 2 days of culturing. Only the 0.2 M $CaCl_2 \cdot 2H_2O$ + 10 mM mannitol solution resulted in efficient pelleting (i.e. few protoplasts remaining in the supernatant) of protoplasts that were subsequently washed, counted and found capable of division and growth. The enzyme mixture and wash solution with $CaCl_2 \cdot 2H_2O$ and mannitol as osmoticum produced protoplast yields averaging 4.2 × 10^6 per gram of epicotyl tissues. Salts are not commonly used as osmotica for plant protoplast isolation. Only in certain cases, for example in lentil (present study) and an auxotrophic variant of *Datura innoxia* [9], has the use of salts proved necessary for protoplast isolation.

The protoplasts (Fig. 1A) obtained through the protocol described were 20–30 μm in diameter and light-green in colour. Subsequent culturing of the protoplasts was only successful when they were suspended in agarose (0.6%)-supplemented KM8P medium. In all test liquid culture media, the protoplasts exhibited severe clumping within 24 h. Although the protoplasts developed cell walls in liquid cultures and some occasional divisions occurred, no microcolonies developed (Table 1). By contrast, agarose-supplemented media prevented protoplast clumping; thus, promoted development of protoplasts. Agarose has been found to support protoplast division in several other systems [5, 7, 11] and this effect has been attributed to the neutral and less toxic nature of agarose [7]. The observation of the suitability of agarose in culturing *Lens* protoplasts is in contrast to that of Stiff et al. [12] who found that embedding protoplasts in agarose caused lysis of protoplast-derived cells within a few days of culture. Cell wall formation, as evidenced by a change in the shape of protoplasts from spherical to oval, occurred within 3 days and first divisions were observed on day 4 (Fig. 1B). In the agarose-supplemented media, cell division was sustained and led to microcolonies (Fig. 1C). Following 14 days of culture, the osmotic potential of the protoplast culture medium was lowered at weekly intervals.

When cultured in agarose-supplemented KM8P-5H medium, upto 54% protoplasts underwent first division but only 0.6% of the dividing population formed colonies visible to an unaided eye (Table 1).

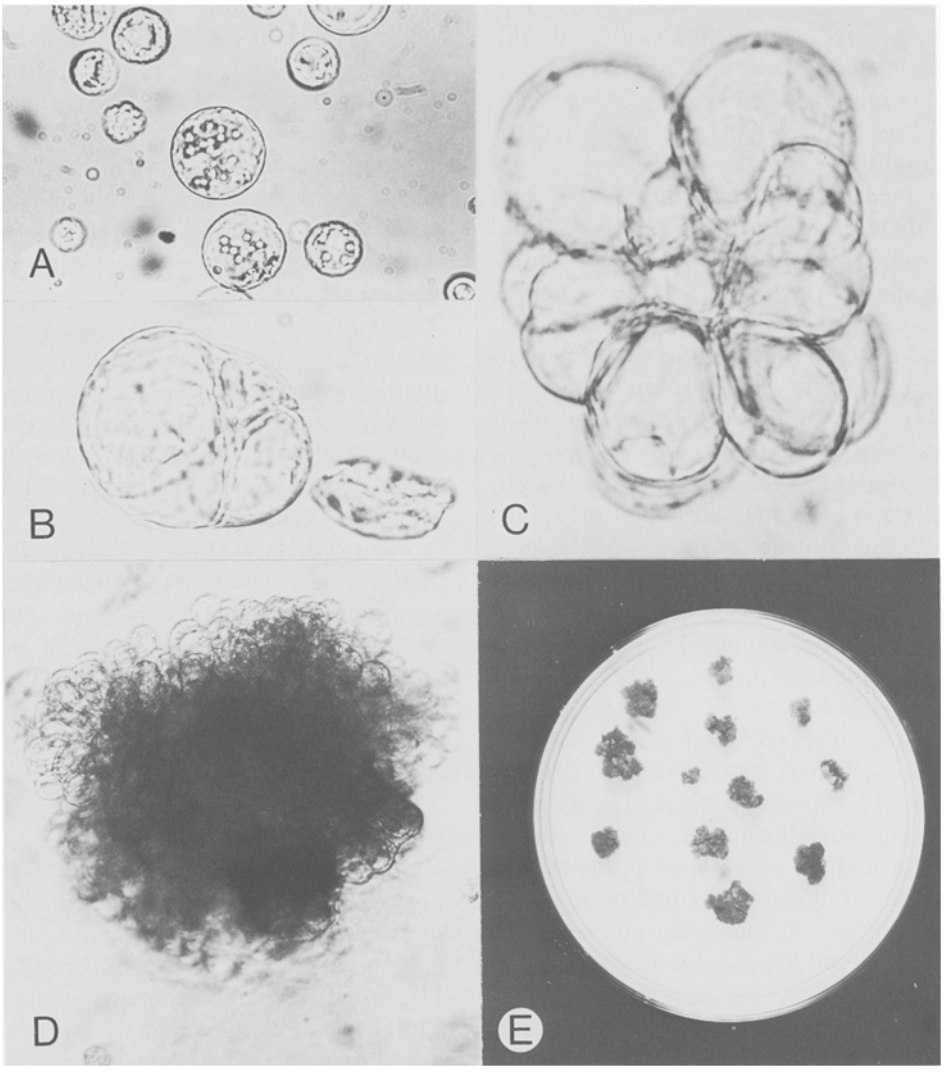

Fig. 1. Isolation and culture of epicotyl-derived protoplasts of *Lens culinaris* Medik cv. Eston. (*A*) Freshly isolated protoplasts. (*B*) Initiation of cell division. (*C, D*) Protoplast-derived cell colonies after 2 and 5 weeks of culture, respectively. (*E*) Protoplast-derived calli obtained after 8 weeks of culture.

Table 1. The effect of culture media and methods on cell colony formation from epicotyl protoplasts of *Lens culinaris* cv. Eston.

Method of culture	Media	Plating efficiency (% ± SE)	Cell colony formation (% ± SE)
Liquid culture	KM8P-5H	5.0 ± 2.0	n.d.*
	KM8P-3H	4.6 ± 0.6	n.d.
Agarose culture	KM8P-5H	54 ± 6.2	0.65 ± 0.2
	KM8P-3H	46 ± 4.1	0.94 ± 0.1
Agarose blocks/ Liquid medium	KM8P-5H/KM8P-5H	52 ± 3.0	3.2 ± 0.42
	KM8P-5H/KM8P-3H	56 ± 5.3	3.6 ± 0.33
	KM8P-3H/KM8P-3H	60.4 ± 3.4	6.6 ± 0.63
	KM8P-3H/KM8P-5H	61 ± 2.0	4.8 ± 0.66

* n.d. = not detected as protoplasts did not progress beyond 4–8 cell stage.

However, maximum frequency of cell colony formation was achieved with KM8P-3H used both in preparing the agarose blocks and for culture of the blocks, indicating that the initially used complement of five hormones is not necessary for protoplast development. Instead, the suspension of agarose blocks in identical liquid medium supported optimum growth of protoplast-derived cell colonies as evident from the data given in Table 1. In agarose-solidified KM8P-3H medium, 46% of the protoplasts divided initially but only less than 1% of these dividing cells formed cell colonies, whereas 6.6% cell colony formation was achieved in cultures showing 60% plating efficiency using a combination of agarose blocks and liquid medium (Table 1). The combination of agarose blocks and liquid medium was introduced by Shilito et al. [11] and has been reported to be beneficial for a number of protoplast culture systems [7, 11]. Up to 20% of the lentil protoplasts showed budding. When the $CaCl_2 \cdot 2H_2O$ was increased to $1\,g\,l^{-1}$, budding increased up to 60% and division and growth of protoplasts was reduced.

After approximately 6 weeks in culture, many of the larger microcolonies (ca. 1 mm in diameter) (Fig. 1D) started to release a brown exudate. Such cell colonies were then transferred to fresh B5-5H or B5-3H media containing 5 mM glutamine to circumvent this problem. This prevention of the brown exudate in our experiments is in contrast to the observations made with protoplast cultures of *L. culinaris* cv. Laird, where browning could not be eliminated by several treatments including the use of activated charcoal, culture at low cell density and incubation in dark or at high temperature [12].

Over 95% microcalli from agarose-solidified KM8P-5H and KM8P-3H media grew on B5-5H or B5-3H media supplemented with glutamine, and after 6–8 weeks of growth the green calli reached a size of $6\,mm^2$ (Fig. 1E).

Presently, conditions are being investigated to improve the frequency of cell colony formation and induce organogenesis and/or somatic embryogenesis from the protoplast-derived calli.

References

1. Cohen D, Ladizinsky G, Ziv M, Muehlbauer FJ (1984) Rescue of interspecific *Lens* hybrids by means of embryo culture. Plant Cell Tissue Organ Culture 3: 343–347
2. Duke J A (1981) Handbook of Legumes of World Economic Importance (pp 110–113), Plenum Press, New York
3. Erskine W (1984) Evolution and utilization of lentil germ plasm in the international breeding program. In: Genetic Resources and their Exploitation — Chickpeas, Fababeans and Lentils (pp 225–241), Martinus Nijhoff/Dr W Junk Publishers, The Hague
4. Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res 50: 151–158
5. Holbrook L A, Reich T J, Iyer VN, Hoffner M, Miki BL (1985) Induction of efficient cell division in alfalfa protoplasts. Plant Cell Rep 4: 229–232
6. Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells at a very low population density in liquid media. Planta 126: 105–110
7. Lorz H, Larkin PJ, Thomson J, Scowcraft WR (1983) Improved protoplast culture and agarose media. Plant Cell Tissue Organ Culture 2: 217–226
8. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
9. Saxena PK, King J (1987) Optimal conditions for isolation and culture of protoplasts from an isoleucine-valine-requiring auxotroph of *Datura innoxia* P. Mill. Can J Bot 65: 1376–1740
10. Saxena PK, King J (1987) Morphogenesis in lentil: plant regeneration from callus cultures of *Lens culinaris* Medik. via somatic embryogenesis. Plant Sci 52: 223–227
11. Shillito RD, Paszkowski J, Potrykus I (1983) Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. Plant Cell Rep 2: 244–247
12. Stiff CM, Kleinhofs A, Lurquin PF, Letourneau D (1986) Isolation and culture of lentil (*Lens culinaris* var. Laird) protoplasts. Abstracts 6th Int Congr Plant Tissue Cell Culture, Minnesota (p 232)
13. Williams DJ, McHughen A (1986) Plant regeneration of the legume *Lens culinaris* Medik. (lentil) in vitro. Plant Cell Tissue Organ Culture 7: 149–153