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Research Article

Protoplast Isolation in Lupin (*Lupinus mutabilis* Sweet): Determination of Optimum Explant Sources and Isolation Conditions

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Abstract: Effects of cultural factors on the yield, viability and division of protoplasts were investigated in *Lupinus mutabilis* Sweet containing a high protein content as well as a reasonable oil content which may make this species an alternative crop to soybean in Turkey. Explants from different in vitro seedling parts were evaluated on the suitability of protoplast isolation and viability. Leaf mesophyll was the most suitable tissue as a protoplast source. Pectinases as well as cellulases were essential for the isolation of protoplasts. Nine percent (w/v) mannitol was suitable to stabilise osmotic pressure together with low salt concentrations in washing and isolation solutions while 9% (w/v) glucose gave better results in culture medium. Shoot tip protoplasts exhibited a higher viability than other protoplast sources. Prolonged viability was observed when protoplasts were cultured in low density media. Techniques such as nurse cultures and electro-stimulation were ineffective. Sustained division of protoplasts in lupin (all sources) was not possible. However, techniques applied in this study may help other researchers, especially those studying protoplast culture of recalcitrant plant species, as well as further studies on this species.

Key Words: *Lupinus,* protoplast, enzyme, leaf mesophyll.

Lüpende (*Lupinus mutabilis* Sweet.) Protoplast İzolasyonu: Optimum Eksplant Kaynağı ve İzolasyon Şartlarının Tespiti

Özet: Türkiye'de soya fasulyesine alternatif olabilecek yüksek bir protein oranı ile orta seviyede yağ oranına sahip bir lüpen türü olan *Lupinus mutabilis* sweet'in farklı kaynaklardan elde edilen protoplastlarında verim, canlılık ve bölünme üzerine değişik kültür şartlarının etkisi araştırılmıştır. *In vitro* şartlarda yetiştirilen çeşitli bitki parçalarının protoplast izolasyonuna uygunluğu değerlendirilmiştir. Yaprak mezofil hücreleri en uygun protoplast kaynağını oluştururken, izolasyonda hem pektinaz hem de selülaz grubu enzimlerin kullanılması gerekli bulunmuştur. Düşük tuz konsantrasyonu ile birlikte, %9 manitol hem izolasyon hem de yıkama solüsyonunda, en uygun osmotik basınç dengeleyici olurken, kültür ortamında %9 glikoz daha iyi sonuçlar vermiştir. Sürgün uçları en canlı protoplast kaynağını oluştururken, düşük yoğunlukta kültür protoplastların daha uzun süre canlı kalmasını sağlamıştır. Nörs kültürleri ve elektrik uyarımı gibi diğer teknikler sürekli bölünmede etkili olmamıştır. Bu çalışma, uygulanan teknikler açısından benzer konuda ve özellikle, protoplast izolasyonun zor olduğu bitki türleri ile bu türde çalışacak araştırıcılara ışık tutabilecektir.

Anahtar Sözcükler: *Lupinus,* protoplast, izolasyon, yaprak mezofili.

Introduction

Lupins contain the highest protein content among grain legumes, including soybean, ranging between 28.0 and 47.6% depending on the species (1). Lupins should be considered as a model for low input plants, especially in marginal lands where no other crops can be grown profitably (2). *Lupinus albus* L., *L. luteus* L. and *L. angustifolius* L. are Old World species whereas *L. mutabilis* Sweet is a New World species (2n=52) originating from South America and exhibiting day-neutral photoperiodism, which may allow this species to adapt to temperate climates. Generally, high protein and oil contents in any one cultivar is the main goal of lupin breeding. *L. mutabilis* is the only large-seeded lupin with an oil content (13-19%) which makes it economically valuable for oil extraction, as well as high protein content (45%). *L. mutabilis* can compete with soybean (36% protein, 18% oil) if higher yielding varieties can be improved since this species is lower in yield in Europe and Turkey (3), compared with other cultivated lupin species, especially *Lupinus albus*, which is the only cultivated species, but lower in protein content with negligible oil content.

Interspecific sexual hybridisation was attempted between *L. mutabilis* and Old World species of lupins but always resulted in a failure of embryo set mainly due to

post-zygotic incompatibility problems (4). Similarly, attempts to rescue hybrid embryos failed to give normal embryos and plants. Vuillaume (5) suggested that best characteristics of *L. albus* and *L. mutabilis* should be united in one genotype in order to get a high yielding crop with high protein and oil content. For this, protoplast isolation and fusion (somatic hybridisation) in lupins may provide the opportunity for by-passing reproductive isolation barriers (6).

Compared with other crop species, grain legumes including lupins, are generally recalcitrant to tissue cultural applications or even less responsive to protoplast culture and manipulations. Leaf protoplasts were isolated in low yield from different *Lupinus* L. species except *L. mutabilis* but failed to divide (1,7). *Pisum sativum* L. (8), *Glycine argyrea* Tind. (9) and *Vicia faba* L. (10) are the only successful crop species regenerated into plants from protoplasts.

There are no reports to date describing the isolation of protoplasts from *L. mutabilis*. In this paper the effects of cultural factors on the yield, viability and division of protoplasts were investigated in order to develop a protoplast-to-plant system for use in somatic hybridisation and other cell manipulation studies.

Materials and Methods

Self-pollinated seeds of *L. mutabilis* cv. Potosi were kindly provided by Dr. P. Roemer (Südwestdeutsche Saatzucht, Im Rheinfeld 1-13, 7550, Rastatt, Germany). This species possesses 17.8% oil and 44.4% protein in seeds and does not contain alkaloids which make grain bitter. All aseptic works were carried out in a laminar air flow cabinet. Fully expanded leaves from in vitro grown seedlings (21 d old) in 175 ml screw-capped glass-jars containing 50 ml of semi-solid (agar, 0.8% w/v) MS (11) with 3% sucrose (w/v) (pH: 5.8), were used for the initial assessments to optimise enzymatic requirements for leaf protoplast isolation. Also, whole shoot tips (0.5-2.0 cm) excised from 8, 10, 12, 14 d, unexpanded or newly expanded leaves excised from 10, 12, 14, 21, 28 and 35 d seedlings were chopped, pre-plasmolysed in appropriate plasmolysation solutions and incubated in suitable enzyme mixtures (Tables 1, 2). Mature excised embryos, immature cotyledons, seedling cotyledons, hypocotyls, epicotyls, and seedling root tips as alternative protoplast sources, were also sliced and incubated in enzyme solutions. Alternatively, seedlings were grown for 7, 14, 21 d on MS agar (0.8%, w/v) medium with 2% (w/v) sucrose and 1.0 mg l^{-1} BAP or Zeatin. Leaves from these seedlings were collected and incubated in appropriate enzyme solutions.

Pre-treatments of tissues prior to enzyme incubation

Removal of the lower epidermis was not possible for young leaves (7-14 d). To minimise these problems, donor seedlings were incubated in the dark (48 h) prior to removal of explants. Alternatively, leaves were bruised with a soft brush or with the cutting edge of a scalpel. In other cases, leaves were finely chopped after removal of the mid-ribs. All other tissues were finely chopped.

Table 1. Protoplast washing and enzyme preparation solutions.

Components	Concentration (mg l^{-1})	
	PW9M	CPW 9M[a]
KH_2PO_4	-	27.2
$NaH_2PO_4.2H_2O$	100	-
$CaCl_2.2H_2O$	1480	1480
$CuSO_4.5H_2O$	-	0.025
KNO_3	101	101
KI	-	0.16
$MgSO_4.2H_2O$	-	246
MES buffer	1000	1000
Casamino acids	1000	-
Mannitol	90000	90000
pH	5.8	5.8

[a]To prepare CPW21S, 21% (w/v) sucrose was added to CPW salts solution.

Immediately after preparation, all tissues (1.0 g f. wt.) were either enzymatically digested directly or exposed (1 h) to an osmotic régime to induce plasmolysis [10 ml of CPW (12) salts solution with 9 or 13% (w/v) mannitol, CPW9M or 13M, Table 1].

Enzyme mixtures and incubation conditions

The pre-plasmolisation solution was replaced, initially by a single enzyme preparation [(1.0% (w/v) each of Cellulase-R10 and Macerozyme-R10] in CPW9M and 1.0 g l^{-1} MES, pH 5.6 (Table 1). New plasmolisation solution (PW9M), which contained low salts and casamino acids (1.0 g l^{-1}), and enzyme combinations were prepared (Table 2), based upon the initial response of tissues in terms of protoplast release. Several anti-oxidants [citric acid (100-200 mg l^{-1}), L-glycine (10-100 mg l^{-1}), PVP-10 (1.0-2.0%, w/v) and L-arginine (10, 100 mg l^{-1})] were individually incorporated into enzyme solutions. All solutions were filter sterilised (0.22 µm pore size, Sartorius filter), dispensed in universal bottles as 10 ml aliquots and frozen (-20 °C) until required.

All incubations were carried out at 26±1°C, either in the dark or under continuous illumination (6.25 µE cm^{-2} s^{-1}, cool white fluorescent tubes). Samples of the incubation mixtures were taken at 1 h intervals (up to 24 h) both from shaken cultures (40 or 80 cycles/min) and static cultures. Each experiment was replicated three times with three dishes per individual treatment.

Protoplast purification

Protoplasts were released by squeezing the digested tissues (using a wide-bore Pasteur pipette) and passage of the incubation mixture through two nylon sieves (100 µm and 64 µm pore sizes). Any undigested tissues were rinsed with 10 ml CPW9M or PW9M solutions. The filtrate was dispensed into 16.0 ml screw-capped glass tubes, centrifuged (100 x g, 10 min) and the supernatant discarded. Two strategies were followed for protoplast purification:

i) Protoplasts resuspended in PW9M and were passed through nylon sieves of decreasing pore size (80, 64, 45 and 30 µm respectively),

ii) A protoplast pellet was resuspended in 2.0 ml of washing solution and layered on top of 10.0 ml of CPW21S (Table 1), in 16.0 ml tubes, and centrifuged (5 min, 100 x g). Protoplasts were collected at the interface and removed.

Protoplasts were washed twice by resuspension and centrifugation (100 x g, 10 min) in the respective washing solutions and were finally resuspended in 10 ml of washing solution.

Protoplast viability

Protoplasts were mixed with an equal volume of the appropriate wash solution containing FDA (0.1 ml of a 5.0 mg ml^{-1} stock in acetone per 10 ml of medium) and viability was determined, under UV illumination, using a Nikon inverted microscope. Protoplasts exhibiting a green/yellow fluorescence were regarded as being viable. Results were expressed as the percentage of viable (fluorescing) protoplasts per field with each count including at least 500 randomly-chosen protoplasts.

Cell wall

For assessments of cell wall regeneration, an aliquot (ca. 100 µl) of protoplasts was mixed with an equal volume of 0.1 % (w/v) Calcofluor white in appropriate washing solutions. Preparations were examined under

Table 2. Enzyme mixtures tested for protoplast isolation[a].

Code	ENZYME CONCENTRATION[b] (%, w/v)						
	Cellulases				Pectinases		
	C-R10	C-RS	C-YC	Dri.	M-R10	Pecto.	Pecti.
PE1	1.0	-	-	-	1.0	-	-
PE2	-	1.0	-	-	1.0	-	-
PE3	-	1.1	-	-	1.3	0.25	-
PE4	-	-	0.5	-	-	0.5	-

[a]Each enzyme was prepared using each solution in Table 1. [b]C-R10, Cellulase-R10; C-RS, Cellulase-RS; C-YC, Cellulase-YC; Dri., Driselase; M-R10, Macerozyme-R10; Pecto., Pectolyase Y-23; Pecti.= Pectinase (Serva).

UV illumination using a Nikon inverted microscope Diaphot TMD (high pressure mercury vapour lamp HBO 100 w/z) fitted with a B1 filter IF 420-485, dichroic mirror DM510 and eyepiece absorption filter 570. Protoplasts with incomplete or undigested cell walls showed blue fluorescence. Results were expressed as a mean percentage of the original protoplast population.

Protoplast yield

Protoplasts in respective washing solutions were counted using a modified Fuchs Rosenthal haemocytometer (Northern Media). Results were expressed as yield per gram fresh weight (g. f. wt) of starting tissue. All yield assessments were repeated at least 5 times.

Protoplast size

The diameter was determined for at least 100 randomly selected protoplasts of each type/treatment using a calibrated eyepiece graticule fitted to a Nikon inverted microscope [x 40 objective].

Protoplast culture

During preliminary assessments, the viability of protoplasts exhibited a marked decline during purification and the early culture stages, irrespective of enzymatic treatment.

For all sources, protoplasts were initially cultured at a density of $2.5x10^5$ protoplasts ml^{-1}, in liquid MSP19M (13), KM8P, KM8, K8, K8P (14, 15; 9) and KPR medium (K8P plus 0.4 mg l^{-1} 2,4-D).

Six basal media [(MS with B5 vitamins, MS with casamino acids (0.2%/, w/v), MS with 1060 mg l^{-1} $CaCl_2$ $2H_2O$, NH_4NO_3-free MS (lacking ammonium ions) with KM vitamins, B5 with L-glutamine (730 mg l^{-1}) and KM with KM supplements] were also evaluated with 100 factorial combinations of growth regulators with at least two replications. These consisted of NAA x BAP, NAA x TDZ and 2,4-D x KIN, all at concentrations of 0.1, 0.5, 1.0, 2.0 or 4.0 mg l^{-1}. All media contained 9% (w/v) mannitol or glucose as osmoticum.

Plant growth regulators were added prior to filter sterilisation. All culture media were prepared at single (for liquid culture) or double strength (for semi-solid culture) and stored in the dark, at room temperature, until required. For all media MES buffer was added (1.0 g l^{-1}) since it was observed to be essential for protoplast stability.

Culture methods

Protoplasts in their appropriate culture medium were dispensed in 5.5 cm Petri dishes as either liquid layers (5.0 ml aliquots), semi-solid layers (5.0 ml aliquots), or 15 (100 µl each) semi-solid droplets submerged in 3.5 ml of liquid medium. Sea-plaque agarose (0.6%, w/v) was used for all semi-solid media by mixing (1:1, v/v) liquid double-strength medium with molten (60 °C) agarose (1.2%, w/v). Protoplasts were suspended in the liquid media or mixed with semi-solid media (ca. 40 °C) to give the final required plating density. Semi-solid droplets were submerged in the appropriate liquid media counterpart. For some experiments, the osmoticum was omitted in the liquid medium.

Cultural conditions and assessments

Protoplasts were plated at initial densities of 0.1, 0.5, 1.0, 2.5 and 5.0 $x10^5$ ml^{-1} of media. All cultures were maintained at 26±1°C, either in the dark or under continuous illumination (19.5 µE m^{-2} s^{-1}). Each medium was replicated at least five times and all experiments repeated twice.

Reduction in osmotic pressure of protoplast culture medium

Protoplasts were either left undisturbed or appropriate dilutions of the culture media were made following the first mitotic division. In experiments where protoplast division was not observed (by 2-3 w) the culture media were diluted osmotically.

Alternative protoplast culture techniques

Nurse cultures

Five ml settled cell volume of a fast growing cell suspension of *Lolium multiflorum* (5-year-old, non-embryogenic), maintained by weekly subcultures in N6 medium (16), was harvested after 3 d of subculture and suspended in 100 ml liquid KPR medium made semi-solid with 0.8% (w/v) Sea-plaque agarose. Five ml aliquots were dispensed into 5 cm diameter Petri dishes. Dishes were sealed and cultured (26±1 °C) for 1 d in the dark. The surface of the medium, containing nurse cells, was covered, 0.5 h before culture, with a sterile, 47 mm diameter Millipore polyvinyliedene difluoride membrane (type GV, Cat. No: GVW PO 4700, Millipore). Protoplast suspensions in a range of media and densities ($0.5x10^5$-$2.5x10^5$ protoplasts ml^{-1}) were spread out (200 µl) on the surface of the membranes. Ten membranes from each culture were weighed after spreading the protoplast

suspension for future measurements of division or colony formation. Alternative assessments were based on direct observations of the membrane under a light microscope. The membranes were sometimes washed to release protoplasts.

Embedding of protoplasts in sodium alginate

After purification, protoplasts were cultured using the method of Tegeder et al. with modified mannitol concentrations. Protoplasts were suspended in 10.0% (w/v) mannitol solutions, at a density of $5x10^5$ protoplasts ml^{-1} and subsequently embedded in sodium alginate by mixing equal volumes of the protoplast suspensions and mannitol (8.0%) containing 2.0% (w/v) sodium alginate to obtain a final density of $2.5x\ 10^5$ protoplasts ml^{-1}. Aliquots (0.5 ml) of this mixture were distributed in 5.5 cm Petri dishes containing 9% (w/v) mannitol respectively with 2.94 g l^{-1} $CaCl_2\ 2H_2O$ to polymerise the alginate layer. Four ml of liquid protoplast culture medium containing 0.5 mg l^{-1} each of 2,4-D, NAA and BAP was added. Protoplasts were cultured in the dark at 26 °C. Protoplasts were either left undisturbed or 0.5 volume of culture media was replaced (after 7 d) with fresh medium lacking mannitol.

Electro-enhancement

An evaluation was carried out to determine whether the repeated application of electric pulses would stimulate division of lupin protoplasts. Protoplasts were aligned in an electric field (AC) at a frequency of 1 MHz and a field strength of 200 V cm^{-1} for 30 sec, a DC pulse of 20 or 40 ms duration at 250, 500, 750 and 1000 V cm^{-1} was applied. The electrofusion apparatus used was that described by Jones et al. (17). Voltages were applied to the protoplasts using seven parallel nickel-silver plate electrodes with a 2.8 mm separation. Protoplasts were suspended at a density of 2×10^5 ml^{-1} in 0.5 mM MES buffer containing 0.5 mM $CaCl_2$ and 9% (w/v) mannitol (pH 5.8). Aliquots (0.5 ml) of resuspended protoplasts were placed in the individual wells of a 5x5 plastic dish. The electrode assembly was first sterilised in 80% (v/v) ethanol solution (1 h) and allowed to air dry, prior to electrofusion. Following electro-treatment, protoplasts were cultured using appropriate culture media. Cell wall regeneration and division of protoplasts were also monitored.

Results

Isolation of protoplasts

Attempts to isolate protoplasts from mature excised embryos, immature and mature cotyledons and seedling roots were unsuccessful and no further isolations were attempted.

Plasmolysis of tissues prior to enzyme incubation increased the protoplast viability (Table 1), in agreement with Ochatt and Power (18). The use of MES buffer (1.0 g l^{-1}) in all media (plasmolysis, enzyme solution and culture media) was a necessity for maximising the stability of protoplasts. Leaf tissues had a lower pectinase requirement. Cellulase RS was the most efficient cellulase and the use of more purified enzymes (such as Cellulase YC) did not increase viabilities. Mesophyll protoplast release was enhanced when leaves were incubated in the dark with shaking (80 cycles/min) at 26 °C. The addition of PVP-10, citric acid, L-glycine and L-arginine did not improve viability. The use of MES buffer (5 mM) in either enzyme or washing solutions was crucial. Low salt solution containing casamino acids (1.0 g l^{-1}) during pre-plasmolysis, in the enzyme solution and in the purification step (PW9M), enhanced protoplast viability and survival rates compared with CPW salts solution. Plasmolysis of tissues, prior to enzyme incubation, increased yield and viability (Table 3). Protoplasts were successfully purified using the series of nylon sieves of decreasing pore size. Floatation in CPW21S solution was unsuitable.

Comparable and optimum results were obtained, in terms of yield and viability, from either peeled or chopped leaves when pre-plasmolysed for 1 h in PW9M solution prior to incubation [6 h, shaken (80 rpm)] in enzyme solution PE3.

The results demonstrate that PE3 enzyme solution gave workable yields ($3.10\pm0.22 \times 10^6$ ml^{-1}) of viable (65±4%) protoplasts from chopped, fully-expanded leaves of 21 d old seedlings, pre-plasmolysed for 1 h in PW9M medium prior to incubation (Table 3). This was the basis for further isolation studies involving shoot tips and newly expanded leaves. The results are given in Table 4.

Good yields of highly viable protoplasts were obtained from whole shoot tips (8 d) which were chopped, plasmolysed and incubated in enzyme solution (6 h) followed by shoot tips from 10 and 12 d old seedlings and newly expanded leaves excised from 10 d old in vitro seedlings (Figs 1a, b). For all subsequent assessments newly expanded leaves from 10 d old seedlings were used for protoplast isolation. Among the enzyme mixtures tested, only PE3 gave acceptable yields and viabilities for other explant sources i.e., hypocotyl and epicotyl (8 d) tissues (Table 4).

Leaf protoplasts fell within the range of 20-30 µm diameter whereas protoplasts from shoot tips ranged

Table 3. Effect of pre-treatments and enzyme mixtures on the release and viability of mesophyll protoplasts from fully expanded leaves of 21 d old seedlings.

Enzyme Code[a]	Washing and enzyme solution[b]	Peeled leaves				Chopped leaves			
		Untreated		Plasmolysis		Untreated		Plasmolysis	
		Yield[c]	Viability[d]	Yield	Viability	Yield	Viability	Yield	Viability
PE1	CPW 9M	0.42±0.24	10±4	0.91±0.24	16±4	0.62±0.31	14±5	1.06±0.11	30±6
	PW9M	0.51±0.22	13±3	1.01±0.12	24±3	0.60±0.24	21±3	1.03±0.09	32±2
PE2	CPW9M	1.82±0.24	26±6	2.52±0.23	34±5	1.46±0.33	31±4	2.08±0.27	43±5
	PW9M	1.74±0.36	31±3	2.44±0.27	39±2	1.44±0.19	40±2	2.14±0.21	54±2
PE3	CPW9M	2.48±0.29	35±5	3.25±0.30	41±4	2.16±0.21	39±4	2.95±0.29	54±3
	PW9M	2.52±0.34	41±2	3.22±0.24	50±3	2.21±0.14	49±3	3.10±0.22	65±4
PE4	CPW9M	1.28±0.19	31±4	1.64±0.19	39±3	0.97±0.17	28±3	1.44±0.14	46±4
	PW9M	1.36±0.27	35±2	1.47±0.13	45±2	1.01±0.09	41±2	1.36±0.11	58±2

[a,b]see Table 1, 2 for plasmolysis, protoplast washing and enzyme solutions, [c]yield was expressed as 10^6 protoplasts/g. f. wt., [d]viability was expressed as a %. Data are the mean (± SD) of three replicates.

Table 4. Effect of age on the protoplast yield and viability of various tissues used for protoplast isolation[a].

Seedling age	Hypocotyl slices		Epicotyl slices		Chopped shoot tips		Chopped leaves	
(day)	Yield	Viability	Yield	Viability	Yield	Viability	Yield	Viability
8	0.62±0.14	51±4	0.26±0.08	53±5	4.81±0.38	76±4	N/Tb	N/T
10	0.45±0.09	50±3	0.22±0.09	52±5	4.64±0.32	74±5	3.80±0.22	71±4
12	0.43±0.08	37±3	0.18±0.09	47±6	4.21±0.40	64±5	3.47±0.24	65±3
14	N/T	N/T	N/T	N/T	3.62±0.21	58±4	3.22±0.36	64±3
21	N/T	N/T	N/T	N/T	N/T	N/T	3.10±0.22	65±4
28	N/T	N/T	N/T	N/T	N/T	N/T	2.87±0.44	57±6
35	N/T	N/T	N/T	N/T	N/T	N/T	2.54±0.57	50±7

[a]PE3 enzyme mixture was used for all tissues. All digests were sieved through nylon sieves of 80, 64, 45 µm pore size respectively, with an additional 30 µm sieve when shoot tips were digested. Pre-plasmolysis (1 h), enzyme preparation and all washings were done with PW9M. Hypocotyl and epicotyls were sliced, other tissues were chopped, pre-plasmolised for 1h. Yield and viabiliy was determined as in Table 3.[b]N/T= not tested due to unavailability/unsuitability. Data are the mean (± SD) of three replicates.

from 15 to 25 µm diameter. Larger protoplasts (>30 µm diam.) became vacuolated during culture with budding, with cell elongation but no division. Smaller protoplasts remained unchanged in shape and consequently senesced (Fig 1c).

Protoplast culture

Initial experiments revealed that KM8P, K8P and KPR (10% glucose as osmoticum) media supported a percentage survival of mesophyll protoplasts while other media resulted in protoplast lysis during the early hours of culture.

Following culture (all treatments) a decline in survival (>50% after 3 d) was observed for mesophyll protoplasts irrespective of media used. Higher plating densities (>$2x10^5$ protoplasts ml^{-1}) resulted in rapid browning and loss of viability (>80% in 7 d). The

optimal medium was KM-based, lacking coconut milk, with 9% (w/v) glucose and the plant growth regulators of KPR medium (0.5 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA and 0.2 mg l^{-1} Zeatin (coded as P-LP medium). Survival of protoplasts in culture did not vary significantly between PGR treatments. Protoplasts cultured in all media remained spherical for 4-7 d of culture. Budding (8%) was most frequently observed in media containing NAA with CPPU or BAP. Protoplast survival was prolonged in liquid media compared with agarose-solidified media, which resulted in the lower percentage of protoplast survival (20% after 3 d). No beneficial effects of including antioxidants in protoplast culture media were apparent.

Pre-treated leaf-derived protoplasts displayed a prolonged survival in P-LP medium (20-24 d) where protoplasts (10%) were elongated with limited cell wall formation (3%). Division was not initiated (Figure 1d).

Encapsulation of protoplasts in sodium alginate beads or electro-stimulation did not promote cell wall regeneration or division. In contrast, budding and survival (up to 28-32 d) of leaf protoplasts was promoted with nurse culture but in practical terms did not provide a protoplast-baseline for further study.

Discussion

Successful isolation of protoplasts from newly expanded leaves of in vitro-generated seedlings was partly due to the fact that the cell wall becomes thinner as the rate of cell division is high in young cultures. This can also be a result of less pectic substances accumulated in young cell walls than in older cells.

The requirement for a low plating density for prolonged survival in mesophyll protoplasts, may, in part, be associated with reduced phenolic accumulation since at higher plating densities, the diffusion of phenolic compounds from non-viable cells may adversely affect the growth of neighbouring cells.

The use of high concentrations of sucrose and sorbitol are other factors which can stimulate protoplast budding (18). Although glucose was used as the sole carbon source in the optimum culture medium, budding incidence

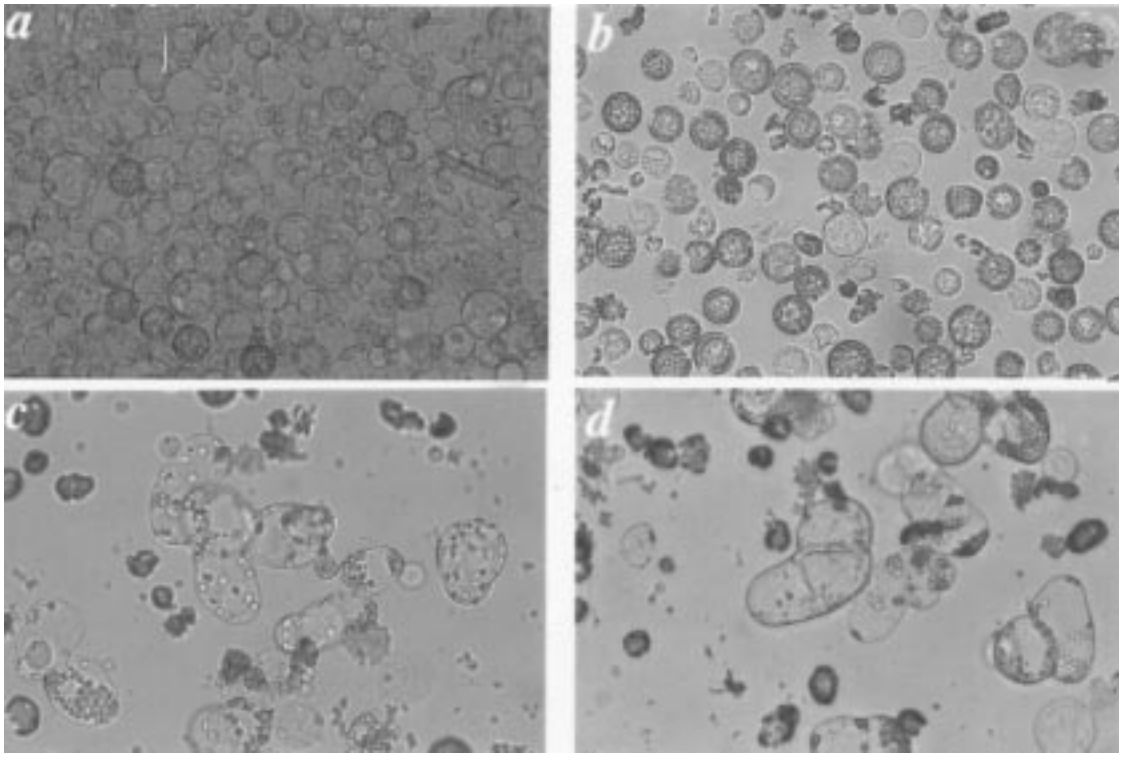

Figure 1. Protoplasts isolated from various tissues of *Lupinus mutabilis* cv. Potosi. a. Shoot tip protoplasts from 8 d old *in vitro* seedlings x40. b. Leaf mesophyll protoplasts from 10 d old *in vitro* seedlings x40. c. Elongation and budding of protoplasts cultured x40. d. Elongation and partial division of protoplasts x40.

occurred and lack of division is clearly a genetic-based texture.

Leaves taken from seedlings grown on cytokinin-containing media did not give dividing protoplasts, in contrast to Adzuki Bean protoplasts, which displayed sustained division (19).

Further studies should be concentrated on the stimulation of cell wall formation in cultured protoplasts It is also worthwhile to establish embryogenic cell suspension cultures in lupins for the exploitation of protoplasts as tools for genetic transformation and somatic hybridisation.

The lack of inducible mitotic division in protoplasts of the *Lupinus* species might also be a result of programmed cell death (apoptosis) which might be the result of i) a developmental event, or ii) as a cellular response to an unknown stimulus (20) or of iii) the enzymatic digestion process, which was reported to inhibit nuclear division in some protoplast systems (21). Hence, mechanical isolation of lupin protoplasts should be considered.

Use of nurse cultures did not make any remarkable contribution and non-transparent membranes used in this study did not allow practical microscopic examinations of the protoplasts during culture. Feeder cells from other leguminous species may provide more appropriate conditions for the culture of protoplasts coupled with the use of translucent or transparent membrane filters.

The possible stimulatory effects of non-ionic surfactants (e.g. Pluronic F-68) together with oxygenated perfluorocarbons (PFCs) (22) to be added either in feeder layers in studies involving nurse cultures or their direct supplementation into protoplast culture media should be considered in further studies. Such agents may be especially advantageous in static cultures of protoplasts, as in the case of lupins, which are extremely sensitive in culture with rapid decreases in viability.

The possible detrimental effect of ammonium ions (18) or the stimulatory potential of Ca^{2+} ions for cell wall formation (19, 23), seem not to apply to *Lupinus* protoplasts. The use of young shoot tips as a source of protoplasts, coupled with the embedding of isolated protoplasts in sodium alginate (10), was the key success for division, callus formation and shoot regeneration in peas. Shoot tips (8 d) gave the highest protoplast yields but subsequent culture in alginate disks did not result in protoplast division indicating that variations exist among the protoplasts in grain legumes in relation to their culture behaviour.

Isoflavones (e.g., genistein, luteone and wighteone) in *Lupinus* species are characteristic secondary plant metabolites and were considered to be one of the primary defence mechanisms in lupins against pathogens (24). Isoflavones in lupins were also considered to have an additional role in controlling some processes of plant cell development, possibly by interacting with cell wall peroxidases (CWP) in epigenetic control of CWP, activities which are involved in lignification and are higher in younger than in older lupin tissues (25). Such relationships should be investigated.

Growth of Adzuki bean (*Phaseolus angularis* W. Wigt) seedlings in MS solidified medium containing 1.0 mg l^{-1} Zeatin and 2% (w/v) sucrose was reported to be necessary for sustained division and plant regeneration from leaf protoplasts (19). Similarly, protoplast yield was markedly increased with significantly higher plating efficiencies when leaflets of alfalfa were pre-cultured in a medium enriched with sugars, antioxidants and growth regulators for 36-48 h (18). Also, cell wall regeneration and subsequent division of soybean hypocotyl-derived cell suspension protoplasts were both markedly enhanced by electrofusion treatments, which presumably stimulate pore formation of protoplast membranes, facilitating the uptake of ions and nucleic acids from the surrounding medium (26). Ammonium ions, in particular, have been found to be detrimental to protoplast survival. In such circumstances, a reduction in the ammonium ion concentration (or total elimination) can have dramatic effects on survival and division of protoplasts. The converse applies, in terms of Ca^{2+} ion concentration, which promotes membrane stability (18). High Ca^{2+} ion concentrations ($CaCl_2$, 900 mg l^{-1}), in protoplast culture medium [normal concentrations being 600 mg l^{-1} (KM); 440 mg l^{-1} (MS) media] was effective for increasing the plating efficiency of Adzuki bean leaf mesophyll protoplasts (19). However, attempts to mimic those systems did not work for *L. mutabilis*.

The limited progress achieved in this study in determination of optimum explants and enzyme solutions for use in isolation of protoplast from *Lupinus* species will however, provide a basis for future work on the further development of a protoplast-to-plant regeneration system. This study may also present some useful information and techniques to researchers willing to study protoplast isolation especially using grain legume species.

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