TARIM BİLİMLERİ DERGİSİ 1996, 2 (2) 73-79

DOI: 10.1501/Tarimbil_0000000674

Protoplast Isolation in Lupin (*Lupinus spp*) *

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Geliş Tarihi : 25.03.1996

Abstract: The aim of this work was to find out a reliable method for isolation of+ protoplasts from various lupin (*Lupinus spp*) species. A1 10 ml l^{-1}, $CaCl_2$. $2H_2O$ 0.103 g l^{-1}, Cellulase Onozuka R-10 1.5 %, Macerozyme R-10 0.1%, mannitol 8.96 % and BSA 0.1% have been found to be a suitable enzyme mixture for isolating protoplasts from the leaves of glasshouse-grown lupin species. Digestion in the dark in the refrigerator (4 ^{0}C) for 14-15 hours followed by incubation at room temperature for 30- 60 minutes and then shaking at 70 rpm at 25 ^{0} C for 30 minutes released a high quality and number of protoplasts in all lupin species examined.

Key words: *Lupinus*, lupin, protoplast, isolation

Lupin'den (*Lupinus spp*) Protoplast İzolasyonu

Özet: Bu araştırmanın amacı değişik lüpin türlerinden (*Lupinus spp*) protoplast izole etmek için uygun yöntem geliştirmektir. A1 10 ml l^{-1}, $CaCl_2$. $2H_2O$ 0.103 g l^{-1}, Cellulase Onozuka R-10 1.5 %, Macerozyme R-10 0.1%, mannitol 8.96 % ve BSA 0.1% karışımı serada yetiştirilen lüpin türleri yapraklarından protoplast elde etmeye uygun bir enzim karışımı olarak belirlendi. Karanlıkta, buzdolabında (+4 ^{0} C) 14-15 saat hücre duvarını enzimle uzaklaştırma bunu takiben de oda sıcaklığında 30-60 dakika inkube etme ve daha sonra 25 ^{0} C de 70 devir/ dk. 30 dakika sallama ile araştırmada kullanılan Bütün lüpin türlerinden yüksek kalite ve miktarda protoplast elde edildi.

Anahtar kelimeler: Lupin, *Lupinus*, protoplast, izolasyon

Introduction

There are two *in vitro* culture techniques with the potential to overcome the crossing barriers that exist between lupin species; embryo rescue of abortive embryos and protoplast fusion. Although there are some reports about tissue -culture studies in lupin, there is a limited number of reports on isolation of protoplasts and the obtaning of plants from protoplasts. Schafer-Menuhr (1987, 1988, 1989,1990, 1991) studied different enzyme mixtures, plasmolysis solutions to obtain protoplasts from lupin. Protoplasts of the hybrid *L. mutabilis X L. hartwegii* were fused with protoplasts of suspension cultures of *L. polyohyllus* and plants were obtained from these fused homokaryon, heterokaryon protoplast mixtures (Schafer-Menuhr, 1990).

The aim of this work was to find out a reliable method for isolation of protoplasts from various lupin species. A reliable method giving a high yield of viable protoplasts is the first step required for effective protoplast fusion and culture.

Material and Methods

There are more then 300 species in genus *lupinus*. In this experiment 11 lupin species were used to obtain protoplast including *L. albus*, *L. luteus*, *L. angustifolius* and *L. mutabilis* which have achieved an agricultural importance.

L .albus, *L. luteus*, and *L. angustifolius* originated in mediterranean area and *L. mutabilis* originated in South America. There is a crossing incompatibility between lupin species except for a partial compatibility between *L. luteus* with *L. hispanicus* and *L. pilosus* with *L. palaestinus*.

Leaves taken from the glasshouse grown plants were weighed and 0.3g samples were used for every enzyme application. Surface sterilization was done by putting leaves into 0.8% sodiumhypochlorite with 0.01% Tween 20 for 10 minutes then rinsing them three times in sterilized distilled water. After sterilization leaves were pretreated overnight (23-24) hours by incubating at 4 ^{0}C in the dark in conditioning medium (Appendix.I).

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* Part of M.Sc. Thesis

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After conditioning, leaves were sliced into strips (1-2 mm wide) with a sterile scalpel.Then these strips were incubated at 25 ^{0}C for 30 minutes in plasmolysis medium (Appendix IV.). The plasmolysis medium was replaced with 6 mls of enzyme medium.Total cells and protoplasts seen were counted on the inverted light microscope (x200 magnification) using a cell counting chamber from three fields of view of protoplasts settled on the bottom of culture dish. After counting, protoplasts were left on the shaker (70rpm) at 25 ^{0}C in the dark for 30 minutes, and then counts were repeated. The protoplasts were then seperated from the undigested tissues by passage through a 50 micron nylon filter into 5 cm petri dishes. Filtered medium and protoplasts were pipetted into 10 cm^{3} sterile plastic centrifuge tubes (Sterilin) and centrifuged at 80g for 5 minutes. The enzyme solution was then poured off and the pellet resuspended in a known volume of protoplast wash medium (Appendix. II)

Enzyme mixtures were sterilized by passage through a 0.22 micro m filter and stored in a deep freezer (-20 ^{0}C) until they were needed. Protoplast yield was estimated using a 0.2 mm deep haemocytometer. A sample was taken from the known volume of protoplast suspension and 5 counts were used to estimate the total yield. Protoplast viability was determined using fluorescein diacetate (FDA) solution (Larkin, 1976)

A series of experiments was carried out as follows:

Determination of optimum enzyme mixture for protoplast isolation

5 different enzyme solutions (Appendix III.) were used and digestion was carried out overnight (14-15h) in the dark at 25 ^{0}C. All observations were taken as described above.

Effect of epidermis peeling on protoplast yield

The enzyme mixture D was used for protoplast isolation and peeling was done after conditioning and before plasmolysis.

Digestion Duration and Temperature

Lupinus angustifolius and *Lupinus luteus* line 1154 were digested at 25 ^{0}C for 4 hours. On the other hand, for overnight isolation, *Lupinus polyphyllus*, *Lupinus mutabilis* line J82 and *Lupinus luteus* line 1154 were left in the growth room (25 ^{0}C) and refrigerator (4-6 ^{0}C) for overnight (14-15) dark for digestion.

Protoplast yield from overnight digestion of a range of lupin species

Using enzyme mixture A (Appendix III) protoplasts were isolated at 4 ^{0}C and 25 ^{0}C overnight (14-15h) from the other lupin species available in glasshouse.

Results and Discussion

Determination of optimum enzyme mixture for protoplast isolation

It was shown that enzyme mixture A (Appendix III) released a superior quantity and quality of protoplasts than the other enzyme mixtures. (Table 1) pectolyase Y23 was added in enzyme mixture A to increase the protoplast yield, but this had the effect of decreasing the protoplast to total cell ratio for *L. albus* line 1050 and *L. mutabilis* line J 82 , although it increased slightly the total released cell number from 492 to 575, for *L. albus* line 1050 and from 523 to 860 for *L. mutabilis* line J82. When the concentration of cellulase onozuka R-10 was increased to 2% in enzyme mixture A (Enzyme mixture C) , the number of released protoplasts did not increase, and protoplast to total cell ratio decreased. Enzyme mixture D (meicelase 1.5 %, macerozyme R-10 0.05 % in CPW medium) also released a lower quantity and quality of protoplasts than that of enzyme mixture A. Enzyme mixture E (driselase 2% in CPW medium) released the highest number of protoplasts for *L. albus* line 1050, but for the other species it reduced the protoplast to total cell ratio in digestion medium. Meicelase has cellulase activity but it is not so highly purified as cellulase and is less specific, while pectolyase Y23 is used for difficult species as a pectinase. The use of meicelase and pectinase enzymes in the current study has shown that they do not increase yield and quality of lupin protoplasts. Driselase has both cellulase and pectinase activity (Davay & Kumar, 1983), and in the current study it was shown to release lower quality protoplasts than enzyme mixture A except in *L. albus* line 1050. Schafer-Menuhr (1987) has reported that onozuka 1 % + pektinase 0.5% and driselase 1% + pektinase 0.5% enzyme combinations released the highest number of protoplasts from the leaf of *L. angustifolius* c.v Kubesa. However protoplasts obtained by using enzyme combination onozuka 1 % +pektinase 0.5 % were more stable than the protoplasts obtained using enzyme combination driselase 1% + pektinase 0.5%. Schafer-Menuhr (1989, 1991) has also obtained protoplasts from the leaves of hybrid 33 from the cross *L. mutabilis x L. hartwegii* using 1 % cellulase onozuka R-10 + 0.5 % macerozyme enzyme mixture. Protoplast isolation studies from cell suspensions of *L. polyphyllus* have shown that driselase 1 % and onozuka R-10 2% + macerozyme 1% enzyme solutions resulted in higher protoplast yield than the other enzyme solutions used, but, onozuka R-10 2% + macerozyme 1 % enzyme combination released higher yield and quality protoplasts than driselase 2 % enzyme solution (Schafer-Menuhr, 1988). In the current study, it has also been observed that protoplasts obtained by using driselase 2% are not stable. Gilbert (1991) has reported that the use of meicelase and pectolyase as isolating enzymes in potato have a detrimental effect on protoplast regeneration and after 5 days in culture the percentage of dead protoplasts was higher and elliptical cells lower than in cultures isolated with cellulase and macerozyme. The current study has also shown that the cellulase onozuka R-10 + macerozyme enzyme combination is better than other enzyme

combinations used for releasing high yield and quality protoplasts . Lee et al (1989) have reported that a higher concentration of cellulase and macerozyme decreased protoplast viability, and in the current study it has also been observed that cellulase onozuka R-10 1.5% + macerozyme 0.1% enzyme combination is better than cellulase onozuka R-10 2 % + macerozyme 0.1% enzyme combination. Protoplast isolation appeared to be species-dependent and there are differences between species in terms of protoplast yield and protoplast to total cell ratio for the same enzyme mixtures. Shaking at the end of digestion for 30 minutes at 70rpm is necessary for high protoplast yield isolation and is adequate and it that it does not burst protoplasts, although most workers use lower than 70 rpm for other species. As a result of these observations enzyme mixture A was used for the other protoplast isolation works (except peeling experiment) and protoplast quality was increased with modification of isolation conditions.

Effect of peeling epidermis on protoplast yield.

In an effort to increase protoplast yield, peeling the lower epidermis of leaves was examined. It was found that peeling slightly increased protoplast yield in *L. mutabilis* line J82 but decreased the yield in *L. albus* var Boetec (Table 2). Peeling of lower epidermis is difficult in lupin and it does not increase protoplast yield. To facilitate the penetration of enzyme solution into the intercellular spaces of leaf for effective digestion, various methods are employed, such as peeling lower epidermis and cutting the leaves into small pieces. Cutting the leaf into small pieces has been found useful where peeling of epidermis is not possible or inconvenient (Bhojwani & Razdan, 1983). In the current study, it has been found that cutting the leaf in to 2mm wide slices released a high quantity and quality of protoplasts.

Digestion duration and temperature

The protoplast isolation study from *L. angustifolius* and *L. luteus* line 1154 has shown that 4 hours incubation with enzyme mixture A does not release a high protoplast yield for *L. luteus*, but 4 hours incubation releases high quality and of quantity protoplasts for *L. angustifolius*. Schafer-Menuhr (1978, 1989,1991) has reported that protoplast yield reached the optimal level after 4-6 hours incubation for *L. angustifolius* c.v Kubesa in different enzyme combinations while 3-4 hours incubation for isolating protoplasts from cell suspensions of *L. polyphyllus* and 2-3 hours incubation for the isolating protoplasts from leaves of *L. mutabilis x L. hartwegii* has also been reported by the same researcher. Optimum protoplast isolation duration varies from species to species and with tissue used as a source material. While several minutes is sufficient for isolation of protoplasts from the pollen of some *Allium* species (Fellner & Havranek, 1992), 16 hours released the highest yield of protoplasts in sunflower (Dupuis et al, 1990). However, there are some reports indicating that longer incubation in enzyme solution decreases the viability of protoplasts (Dupuis et al,1990; Lee et al., 1989). Since short incubation duration was not enough for *L. luteus* line 1154, and in addition, because it was important to have sufficient time to purify, wash, count and carry out viability counts, the overnight digestion (14-15 h) was used for later studies at the two different digestion temperatures. Although optimal temperature for the activity of enzymes is 40-50 ^{0}C, 25-30 ^{0}C is reported to be adequate for short as well as overnight isolation of protoplasts (Bhojwani & Razdan, 1983). But digestion at 14 ^{0}C has been suggested by Vasil& Vasil (1980) for isolation of protoplasts from corn. In the current study, it has been found that although there is not significant difference between the yield of protoplasts obtained at the 25 ^{0}C and 4 ^{0}C, the incubation at 4^{0}C released a significantly higher protoplast to total cell ratio and there was lower debris concentration in the 4^{0}C digested solution (Table 3). In addition, protoplasts obtained at the 25 ^{0}C incubation were more vulnerable to collapse during subsequent filtering, washing and purification studies.

Protoplast yield for overnight digestion from a range of lupin species

As can be seen in Table 4 protoplast isolation appeared to be species-dependent. This may be due to differences between cell wall structure in different lupin species. *L. palaestinus, L. subcarnasus* and *L. hispanicies* sierre de france released lower quantity and quality of protoplasts. The other species released very high quality and quantity of protoplasts. The reason why *L. subcarnasus* and *L. hispanicies* sierre de france released lower quantity and quality of protoplasts, maybe, because they were not digested at 4 ^{0}C. They should be also digested at 4 ^{0}C to compare with other species. Protoplasts obtained from *L. angustifolius, L. luteus* line 1154, *L. mutabilis* and *L. albus* line 1050 were cultured and all of them showed regeneration after culturing in initial culture medium between 26 days and 15 days.

Conclusion

The first step required for effective protoplast fusion and culture is obtaining clean, viable high quantity of protoplasts. The method described above can be used for this purpose. But, there is a great diversity in genus *Lupinus*, and there are significant differences between species interms of protoplast isolation, thus, this protoplast isolation procedure should be applied and improved for other species. Next step is to fuse protoplasts and obtain plants from fused protoplasts. However, there is also a limited number of work in this area and there is need to improve *in vitro* techniques in lupin.

Table 1. Total released cell, protoplast and protoplast to cell ratio means (mean of 3 fields of view of protoplasts settled on the bottom of the culture dish containing digested tissue in 6 ml enzyme medium, digested overnight at 25 ^{0} C) for different enzyme mixtures. (See Appendix. III for enzyme mixture)

Lupin Species	Enzyme A	Enzyme B	Enzyme C	Enzyme D	Enzyme E
angustifolius Total cell protoplast Pro/To.cell	610 410 62 %				
luteus 1154 Total cell protoplast Pro/To.cell	311 202 64.9%				
albus 1050 Total cell protoplast Pro/To.cell	492 92 18.7%	575 21 3.6 %	389 25 6.4%		643 164 25.5%
mutabilis Total cell protoplast Pro/To.cell	523 169 32.3%	860 101 11.7%	660 83 12.6%	504 63 12.5%	810 155 19.1%
luteus Boec (1121) Total cell protoplast Pro/To.cell		119 28 23.5%	230 122 53%		386 166 43%
albus boetec Total cell protoplast Pro/To.cell				333 94 28.2%	
angustifolius c.v Blusa 3 (1154) Total cell protoplast Pro/To.cell					521 303 58.1%
polyphyllus Total cell protoplast Pro/To.cell	523 161 30.7%				
hispanicies sierra defrance Total cell protoplast Pro/To.cell	303 121 39.9%				
perennis Total cell protoplast Pro/To.cell	479 184 38.4%				
subcarnasus Total cell protoplast Pro/To.cell	454 273 60.1%				

Table 2. Mean total cell, protoplast and protoplast to total cell percentages (mean of 3 fields of view of protoplasts settled on the bottom of the culture dish containing digested tissue in 6 ml enzyme medium)

	L. albus var. Boetec	*L. mutabilis* J82
No peeling		
Total cell	333	504
Protoplast	94	63
Protoplast/total cell Ratio (%)	28.2	12.5
Peeling		
Total cell	264	750
Protoplast	67	46
Protoplast/total cell Ratio (%)	25.4	6.1

Table 3. Mean protoplast, total cell numbers (mean of 3 fields of view of protoplasts settled on the bottom of the culture dish containing digested tissue in 6 ml enzyme medium, two replication) and protoplast and total cell yield (number of protoplast obtained from 1 g of leaf)before purification at two digestion temperature.

	L. mutabilis J82		*L.luteus* (1154)		*L. polyphyllus*	
	4°C	25°C	4°C	25°C	4°C	25°C
Before shaking						
Total cell	176	288	4	21	86	195
Protoplast	84	155	2	11	23	125
Prot/total cell (%)	47.72	53.82	50	52.38	26.74	64.10
After shaking						
Total cell	897	700	266	278	638	394
Protoplast	774	399	149	122	499	157
prot/total cell (%)	86.28	57.00	56.01	43.88	78.21	39.85
Yield (x10^6)/g leaf fresh weight						
Total cell						
Protoplast	15.36	11.91	3.43	5.25	10.71	8.60
Prot/Tot. cell(%)	14.25	6.11	3.10	2.79	6.54	1.75
	92.77	51 30	90.38	53.14	61.06	20.35

Table 4. Mean protoplast yields (g^{-1} fresh weight of leaf) from different Lupin species

Species	Total cell x 10^6	protoplast x 10^6
mutabilis J82 (at 4°C)	12.58	9.18
polyphyllus (at 4°C)	11.91	7.75
albus 1050 (at 4°C)	10.68	6.39
perennis (at 4°C)	11.81	6.86
angustifolius (at 4°C)	8.93	7.12
luteus 1154 (at 4°C)	3.94	2.3
palaestinus (at 4°C)	4.85	1.31
pilosus (at 4°C)	14.11	13.47
hartwigii (at 4°C)	14.92	10.32
subcarnasus (at 25°C)	5.62	1.644
hispanicies sierre de france (at 25°C)	4.62	1.35

APPENDICES

Appendix I: Conditioning medium for whole leaf treatment.

	g l^{-1}	Stock	Amount (ml l^{-1})
A1			
KNO_3	0.190		
$CaCl_2.2H_20$	0.044	x100	10
$MgSO_4.7H_2O$	0.037		
KH_2PO_4	0.017		
A2			
Na_2EDTA	3.7	x100	10
$FeSO_4.7H_2O$	2.8		
A3			
H_3BO_3	0.60		
$MnCl.4H_2O$	2.00	x1000	1
$ZnSO_4.7H_2O$	0.90		
KI	0.08		
A4			
$Na_2MoO_4.2H_2O$	0.03		
$CuSO_4.5H_2O$	0.003	x1000	1
$CoSO_4.5H_2O$	0.003		
A5			
Thiamine HCl	0.05		
Glycine	0.20		
Nicotinic acid	0.50		
Pyridoxine HCl	0.05	x1000	1
Folic acid	0.05		
Biotin	0.005		

P^H 5.6 , Sterilization: autoclave at 121 ^{0}C for 15 minutes.

Apendix II.Protoplast wash medium

A1	10 ml l^{-1}
mannitol	93.5 g l^{-1}

P^H 5.6, sterilise-autoclave at 121 ^{0}C for 15 minutes

Appendix III. Enzyme media for isolation of protoplasts

Enzyme medium A	A1	10 ml l^{-1}
	$CaCl_2.2H_2O$	0.103 g l^{-1}
	Cellulase onozuka R-10	1.5% (w/v)
	Macerozyme R-10	0.1%(w/v)
	Mannitol	8.96% (w/v)
	Bovine Serum Albumin (BSA)	0.1% (w/v)
Enzyme medium B	A1	10 ml l^{-1}
	$CaCl_2.2H_2O$	0.103 g l^{-1}
	Cellulase onozuka R-10	1.5% (w/v)
	Macerozyme R-10	0.1%(w/v)
	Pectolyase Y23	0.1% (w/v)
	Mannitol	8.96% (w/v)
	Bovine Serum Albumin (BSA)	0.1% (w/v)
Enzyme medium C	A1	10 ml l^{-1}
	$CaCl_2.2H_2O$	0.103 g l^{-1}
	Cellulase onozuka R-10	2 % (w/v)
	Macerozyme R-10	0.1%(w/v)
	Mannitol	8.96% (w/v)
	Bovine Serum Albumin (BSA)	0.1% (w/v)
Enzyme medium D	Meicelase	1.5% (w/v)
	Macerozyme R-10	0.05% (w/v)
	Dissolved in CPW 13 medium	
Enzyme medium E	Driselase	2% (w/v)
	Dissolved in CPW 13 medium	

P^H 5.6, Filter sterilise through a 0.22 micro m filter.

Appendix IV: Plasmolysis medium for cell plasmolysis

A1	10 ml l^{-1}
Mannitol	81.97 (0.45 M)

P^H 5.6, Sterilise-autoclave at 121 ^{0}C for 15 minutes.

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