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Explant-dependent receptivity to isolation and a cell-wall resynthesis in protoplast culture of recalcitrant yellow lupin

Wpływ eksplantatu na izolację i odbudowę ściany komórkowej w kulturze protoplastów łubinu żółtego

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Abstract
Cell-wall resynthesis was studied in protoplast culture of yellow lupin (*Lupinus luteus* L.). We optimized protoplast isolation and found that explants excised from young seedling were more suitable sources of protoplasts, in contrast to callus tissue. Incubation in 2% cellulase R-10, 1% pectinase and 0.5% macerozyme solution for 3h effectively released protoplasts from majority of tested explants. Furthermore, we determined the optimal developmental age of explants which was 4, 21, 25 and 35 days for hypocotyls, cotyledons, *in-vitro* leaf mesophyll and *ex-vitro* leaf mesophyll, respectively. Explant type, culture medium and genotype influenced both a rate and a pattern of the cell wall regeneration. After 10 days of culture, the number of regenerated cells reached 44%-59% in hypocotyl, 84%-91% in cotyledonary, and 31%-42% in mesophyll protoplasts. Our results show that the earlier wall regeneration begins, the wall surface will be more incomplete. We suggest that unbalanced and inefficient cell-wall resynthesis likely contributes to recalcitrance of yellow lupin to manipulations in protoplast technology.

Keywords: cell wall, Fabaceae, *Lupinus luteus* L., protoplast culture, viability

Abstrakt
W przedstawionej pracy badano resyntezę ściany komórkowej w kulturze protoplastów łubinu żółtego (*Lupinus luteus* L.). Zoptymalizowano proces izolacji protoplastów i wykazano, że fragmenty siewek są lepszym źródłem protoplastów niż tkanka kalusowa. Inkubacja tkanek w mieszaninie 2% celulazy R-10, 1% pektynazy i 0.5% macerozymu przez 3 h umożliwiła uzyskanie protoplastów z większości badanych eksplantatów. Ustalono optymalny wiek eksplantatów, pozwalający na uwolnienie dużej liczby żywotnych protoplastów: 4 dni dla hypokotyli, 21 dni dla liścieni, 25 dni dla liści z warunków *in vitro* oraz 35 dla liści roślin rosnących *ex vitro*. Rodzaj eksplantatu, pożywka oraz genotyp miały wpływ zarówno na tempo, jak i wzór odtwarzania ściany komórkowej przez protoplasty. Po 10 dniach kultury, liczba zregenerowanych komórek wyniosła 44%-59% w kulturze protoplastów hypokotylowych, 84%-91% w kulturze protoplastów liścieniowych i 31%-42% w kulturze protoplastów mezofilowych. Wyniki pokazały, że im szybciej odtwarzana jest ściana komórkowa w kulturze, tym bardziej niekompletna jest jej struktura na

powierzchni komórki. Sugeruje się, że niezbalansowana i nieefektywna odbudowa ściany komórkowej może być przyczyną wysokiej wrażliwości łubinu żółtego na manipulacje w kulturze protoplastów.

Słowa kluczowe: Fabaceae, *Lupinus luteus* L., protoplasty, ściana komórkowa, żywotność

Introduction

Significance of lupins in modern agriculture as well as their potential importance in other industries are the main reasons for wide interest in genetic enhancement of the genus (Kuchuk et al. 2000). In addition to their applications in agriculture, lupins are exploited in horticultural practice, for example as ornamental plants in rustical gardens. There are also attempts to introduce lupins into human food chain as potential vegetable crops. The agricultural and nutritional value of yellow lupin (*Lupinus luteus* L.) significantly increased due to the conventional breeding programmes. The plant that has traditionally been cultivated as an ornamental plant only, became one of the most important species in animal feed and crop rotation (Święcicki et al. 2000). On the other hand, as a result of intensive breeding and selection, natural variability within genus dramatically decreased (Kuchuk et al. 2000). Limited gene pool of lupin inhibits construction of new cultivars and lines that would be adapted to rapidly changing requirements of global agriculture. Protoplast isolation and fusion (somatic hybridization) may facilitate the improvement of yellow lupin, provided that existing recalcitrance is bypassed (Davey et al. 2005).

Therefore, it is crucial to develop comprehensive optimization of the isolation of lupin protoplasts. Studies on protoplast isolation conditions have identified various parameters, including enzyme combination, the choice of the explant and its physiological state within a range of plant species (Davey et al. 2005). Here we report the results of investigation into factors that influence protoplast release from yellow lupin tissues. To date, there are no studies on the elaboration of reproducible isolation protocol of yellow lupin. Similar studies undertaken on other lupin species, such as white lupin (Sinha et al. 2003) and pearl lupin (Babaoglu 2000) proved that optimization of isolation procedure is essential to further utilization of protoplast culture technique in the improvement of those recalcitrant crops.

The ability of protoplasts to undergo divisions and thus regenerate plants is dependent on numerous processes involved in cytodifferentiation. A crucial step in gaining totipotency in protoplast culture is biosynthesis of the cell wall, as shown in pioneer studies (Meyer and Abel 1975; Nagata and Takebe 1970). We investigated cell-wall regeneration by protoplasts of different origin. The purpose of the study was to insight into protoplast behavior during early days of culture. We hypothesize that the recalcitrance of yellow lupin to protoplast culture conditions may result from disturbances at primary stages of morphogenetic activity acquirement.

Material and Methods

Plant material

Experiments were conducted on three Polish yellow lupin cultivars: „Parys", „Taper" and „Mister". Hypocotyls, cotyledons, young leaves of both *in vitro* and *ex vitro* grown seedlings as well as callus tissue were tested as explant donors of protoplasts for isolation. Protoplast isolation efficiency was determined using explants either at the

control age: 8 days for hypocotyls, 18 for cotyledons, 21 for *in vitro* leaves, 35 for *ex vitro* leaves, or of the control origin: 21-day old callus induced on medium L1 (see below).

Additionally, we examined the influence of explants age on protoplast production. Material donors were etiolated hypocotyls at the age of 4, 10 and 12 days, cotyledons at the age of 10, 14, 21 and 25 days, and *in vitro* leaves 14, 18, and 25 days old.

Ex vitro seedlings were grown in greenhouse conditions. Fully expanded leaves at the age of 35, 42 and 49 days were used for protoplast isolation after surface sterilization in 70% (v/v) ethanol for 60 s and in mercuric chloride for 180 s.

Protoplasts were also isolated from callus obtained on 2 mm hypocotyl slices cultured for three weeks on four induction media, based on B_5 mineral salts and vitamins (Gamborg et al. 1968), supplemented with 0.1 g/l ascorbic acid, 0.15 g/l citric acid and various treatments of growth regulators: medium L1 contained 1.0 mg/l 1-naphthaleneacetic acid (NAA) with 2.0 mg/l zeatin, medium L2 - 1.0 mg/l NAA with 2.0 mg/l meta-topolin, medium L3 – 0.5 mg/l NAA with 0.5 mg/l kinetin, whereas medium L4 – 2.0 mg/l NAA, 0.5 mg/l kinetin and 0.5 mg/l zeatin. An indirect effect of media composition (callus origin) on protoplast yield and viability was further tested.

Protoplast isolation

We evaluated the suitability of ten enzyme mixtures for protoplast release (compositions are given in Table 1). Chopped or sliced explants were incubated in 10 ml of filter sterilized enzyme solution, supplemented with sorbitol in concentration suitable for each explant (Table 2) and 0.1% MES buffer [2-(N-morpholino)ethanesulfonic acid] in CPW salts (Frearson et al. 1973). Lower epidermis was removed from leaves prior to incubation. To test the effect of incubation regime on protoplast yield, explants were incubated either statically for 18 h or for 3 h on a gyratory shaker at 80 rpm, depending on applied enzyme mixture. Obtained protoplasts were purified by filtration through 70 μm nylon mesh filter and afterwards were rinsed three times with sorbitol in CPW salts.

Table 1. Composition of enzyme mixtures used for protoplast isolation from various explants of yellow lupin
Tabela 1. Skład mieszanin enzymów użytych do izolacji protoplastów z różnych eksplantatów łubinu żółtego

	Enzyme concentrations (%, w/v)									
Enzyme type	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Cellulase R-10 (Kinki Yakult)	0.5	0.5	1.0	0.5	2.0	2.0	2.0	2.5	3.0	-
Hemicellulase (Sigma)	-	-	0.1	0.2	-	-	-	1.0	1.0	-
Pectinase (Sigma)	0.05	-	-	-	-	1.0	1.0	1.0	1.0	-
Macerozyme R-10 (Kinki Yakult)	0.05	-	0.1	-	1.0	-	0.5	0.5	0.5	0.3
Pectolyase Y-23 (Duchefa)	-	0.12	-	0.05	-	-	-	-	-	0.04
Driselase (Sigma)	-	-	-	-	-	-	-	1.0	-	-
Meicelase (Meiji Seika Kaisha)	-	-	-	-	-	-	-	-	1.0	4.0
Rhozyme (Rohm & Haas Co.)	-	-	-	-	-	-	-	-	-	2.0

Table 2. Enzymatic mixtures used for protoplast isolation from tested explants

Tabela 2. Mieszaniny enzymatyczne do izolacji protoplastów z testowanych eksplantatów

Explant type and sorbitol concentration		Enzyme mixture E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Hypocotyl	12%	+	+	+	+	+	-	+	-	-	-
Cotyledon	14%	+	+	+	+	+	-	+	-	-	-
In vitro leaf	9%	+	+	+	+	+	+	-	-	-	-
Ex vitro leaf	13%	-	-	-	-	+	+	+	-	-	-
Callus	8%	-	-	-	-	-	-	-	+	+	+

Protoplast culture

Culture density was adjusted to 2 × 10^5 protoplasts/ml using a haemocytometer. Protoplasts were suspended in 4 ml of nutrient medium in small Petri dishes (55 mm diameter). Cultures were established using three liquid media: 1) modified B_5 medium according to Gamborg et al. (1968), containing 0.5 mg/l NAA, 0.5 mg/l 6-benzylaminopurine (BAP), 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l 2-isopentenyladenine (2iP) and 0.5 mg/l gibberelic acid (GA_3), 2) K8P medium according to Kao (1977), containing 1.5 mg/l NAA and 0.5 mg/l BAP, 3) V-47 medium according to Binding (1974), containing 1.0 mg/l NAA, 1.0 mg/l 2,4-D and 0.5 mg/l zeatin (Table 3). Dishes were sealed with parafilm and incubated in the dark at 27°C. Cell wall regeneration was investigated every two days during 10 days of culture, starting at the second day.

Table 3. Composition of liquid media used for protoplast culture
Tabela 3. Skład pożywek płynnych do kultury protoplastów

Macro- and microelements	Vitamins and other organic compounds (mg/l)	Growth regulators (mg/l)
B_5 (Gamborg et al. 1968)	200 glycine, 100 mioinositol, 1.0 pyridoxine, 10 thiamine, 0.1 biotin; 40 L-asparagine, 60 L-glutamine, 100 L-tyrosine, 10000 sucrose	0.5 NAA, 0.5 BA, 0.5 2,4-D, 0.5 2iP, 0.5 GA_3
K8P (Kao 1977)	1.0 niacin, 100 mioinositol, 1.0 pyridoxine, 10 thiamine, 1.0 choline chloride, 125 fructose, 125 xylose, 125 mannose, 125 ribose, 125 casein hydrolysate, 10 citric acid, 0.2 folic acid, 10 malic acid, 0.2 p-aminobutyric acid, 0.5 calcium panthotenate, 0.1 riboflavin, 0.005 vitamin A, 0.01 cobalamin, 6000 glucose, 250 sucrose	1.5 NAA, 0.4 BAP
V-47 (Binding 1974)	2.0 glycine, 0.5 niacin, 100 mioinositol, 0.5 pyridoxine, 0.1 thiamine	1.0 NAA, 1.0 2,4-D, 0.5 zeatin

Data collection and statistical analyses

Data regarding protoplast yield and viability were collected on day of isolation. A haemocytometer was used to estimate isolation efficiency, which was expressed in yield per gram fresh weight of starting tissue (f. w. t.). Viability was determined using

the Evans Blue staining procedure, where protoplasts exhibiting a blue absorption were regarded as being non-viable. Cultural behaviour of protoplasts was evaluated every two days, starting at the second day, using Delta Optical inverted microscope. Cell wall regeneration was studied by visualisation of regenerated cellulose fibres with Fluorescent Brightener (Sigma) followed by observation in fluorescence microscope Zeiss Imager M2 Axio at λ=365 nm. The treatments were independently replicated three times, with three isolations constituting a replicate. For studies of cultural behaviour, three Petri dishes constituted a replicate. For statistical analyses data concerning protoplast viability and cell wall regeneration (the percentages) were transformed to arcsine values. All results were subjected to STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA) ANOVA analysis; post-hoc Tukey"s test was used afterwards to study differences between respective variants at $P < 0.05$.

Results

Effect of explant type and enzyme mixture on protoplast isolation

We observed protoplast release from tissues of lupin independently on the enzyme mixture used. However, protoplast yield differed significantly between mixtures. Results of protoplast yield are given in Table 4. The highest isolation efficiency was achieved from leaf tissue, originating from both *in vitro* and *ex vitro* conditions. Incubation of leaves in mixtures containing 2% cellulase, 1% pectinase, with or without 0.5% macerozyme (E5 and E6) gave very high protoplast yield, considerably exceeding 10^8 protoplasts/1 g of starting tissue (Table 4). The same mixtures were appropriate for protoplast isolation from cotyledonary explants and hypocotyls. Satisfactory tissue digestion was achieved also in the mixture of 0.5% cellulase and 0.12% pectolyase (E2) (Table 4). Enzyme solutions E1 and E3, with reduced amount of cellulase (0.5-1%), although containing various enzymes of macerating activity (macerozyme, pectinase, hemicellulase), proved to be unsuitable for protoplast production from yellow lupin tissues (Table 4).
Donor tissues excised from young seedlings were suitable for protoplast isolation in applied enzyme treatments, and, as a consequence, the isolation procedure was easy and highly efficient. In contrast, the results of protoplast isolation from callus tissue were not satisfactory. Among three tested mixtures, reasonable yield of protoplasts was obtained only after incubation in 1% solution of driselase (mixture E8) (Table 4). Although protoplast yield exceeding 10^4 may be considered low, it is still sufficient for cytological studies and even culture initiation. Notwithstanding the differences between mixtures in the effectiveness of protoplast isolation, for each of tested explants it was possible to choose at least one enzyme combination that ensured rather satisfactory protoplast yield. Among mixtures designated for overnight incubation, only mixture E2 effectively released protoplasts during gentle treatment. The most appropriate mixtures for specific explants (bolded in Table 4) were consequently used in further experiments.

Table 4. Efficiency of protoplast isolation from various explants of three yellow lupin genotypes using ten enzyme mixtures
Tabela 4. Wydajność izolacji protoplastów z różnych eksplantatów pochodzących z trzech genotypów łubinu żółtego przy użyciu dziesięciu mieszanin enzymów

	Protoplast isolation efficiency (protoplast number $\times 10^5$/ g f.w.t.)									
	18 h incubation				3 h incubation					
Explant type	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10

					„Parys"					
Hypocotyl	0.02c	2.07b	1.83b	2.30b	**14.5**[1]a	-	13.4a	-	-	-
Cotyledon	2.10d	21.7c	0.68d	2.20d	43.7b	-	**127**a	-	-	-
In vitro leaf	0.06e	1700ab	24.0d	777c	1010b	**2000**a	-	-	-	-
Ex vitro leaf	-	-	-	-	277c	1270b	**2030**a	-	-	-
Callus	-	-	-	-	-	-	-	**0.27**a	0.01b	0.01b
					„Taper"					
Hypocotyl	0.09c	0.81b	0.29c	0.16c	0.87b	-	**4.07**a	-	-	-
Cotyledon	1.20bc	10.0b	0.44c	1.57bc	7.30b	-	**14.0**a	-	-	-
In vitro leaf	0.23bc	1130ab	78.3b	220b	1230a	**1390**a	-	-	-	-
Ex vitro leaf	-	-	-	-	292b	**1500**a	1040a	-	-	-
Callus	-	-	-	-	-	-	-	**1.07**a	0.01b	0.08b
					„Mister"					
Hypocotyl	0.05c	0.63c	0.08c	0.19c	1.13bc	-	**2.53**ab	-	-	-
Cotyledon	1.70c	14.3bc	0.53c	2.13c	18.0b	-	**113**a	-	-	-
In vitro leaf	0.13c	383a	50.0b	84.6b	380a	**597**a	-	-	-	-
Ex vitro leaf	-	-	-	-	263c	1060b	**1670**a	-	-	-
Callus								**0.34**a	0.05b	0.03b

The means followed by the same letter in each line do not significantly differ at $P = 0.05$.
[1] Bolded values were obtained by using the most appropriate enzyme mixture for specific explant.

The age of primary explants

Our results show that both protoplast yield and viability significantly differed depending on the explant age (Table 5). The tendencies observed within the genotypes and specific explants were also distinct. Considering the yield of hypocotyl protoplasts, tested cultivars responded differently. The highest yield was obtained from the youngest explants in „Parys", whereas in „Taper" the best protoplast source was 10-days old hypocotyls. Interestingly, protoplasts of „Mister" yielded equally in most of tested terms. However, protoplast viability was the highest in the youngest hypocotyls. In the case of cotyledonary protoplasts, genotype did not influenced on a relation between protoplast yield and explant age. At the beginning isolation efficiency increased substantially with the increase of the explant age. When explants were older than 21 days, the yield decreased. Similarly to hypocotyl tissue, the fall in protoplast viability was associated with the increase of the age of cotyledons. The highest isolation efficiency of mesophyll protoplasts was obtained using 21-days-old *in vitro* leaves and 35-days-old *ex vitro* leaves, regardless of the genotype. The overall viability of protoplasts obtained from both types of mesophyll was significantly lower than those of other examined explants and did not exceed 70%. To sum up, the best terms for harvesting donor material for protoplast production were 4th day in hypocotyls, 21st day in cotyledons, and 25th day *in vitro* leaves and 35th day in *ex vitro* leaves.

Table 5. The influence of primary explant age and secondary explant origin on the yield and viability of protoplasts isolated from three yellow lupin genotypes
Tabela 5. Wpływ wieku eksplantatu pierwotnego i pochodzenia eksplantatu wtórnego na plon i żywotność protoplastów izolowanych z trzech odmian łubinu żółtego

Explant age/origin[1]	„Parys"		„Taper"		„Mister"	
	yield (×10^5/g f.w.t)	viability (%)	yield (×10^5/g f.w.t)	viability (%)	yield (×10^5/g f.w.t)	viability (%)
			Hypocotyl			
4	18.30 ±0.6 a[2]	93±1 a	1.13 ±0.6 b	91±3 a	2.37 ±1.1 a	94±2 a
10	0.61 ±0.02 b	74±3 bc	3.97 ±0.3 a	78±3 b	3.27 ±0.3 a	76±4 bc
12	0.59 ±0.08 b	68±2 c	2.43 ±0.7 b	73±4 bc	3.00 ±0.5 a	70±3 c
			Cotyledon			

10	2.07 ±0.03 d	88±1 a	4.47 ±2.0 b	91±1 a	1.13 ±0.1 d	90±2 a
14	13.7 ±1.8 c	89±2 a	13.3 ±1.9 a	92±1 a	17.3 ±4 c	93±3 a
21	143.0 ±10 a	61±1 b	16.7 ±2.8 a	71±2 b	303.0 ±80 a	69±3 b
25	62.0 ±12.0 b	63±1 b	2.77 ±1.2 b	71±3 b	127.0 ±20 b	68±4 b
			In vitro leaf			
14	1350.0 ±100 c	67±3 a	1070.0 ±230 b	61±3 b	1200.0 ±600 b	72±2 a
18	1630.0 ±70 b	69±1 a	1130.0 ±410 b	73±3 a	1030.0 ±300 b	73±3 a
25	2740.0 ±600 a	69±1 a	2730.0 ±100 a	61±2 b	3130.0 ±200 a	64±3 b
			Ex vitro leaf			
35	2030.0 ±500 a	63±3 b	1040.0 ± 60 a	72±4 a	1670.0 ±120 a	68±1 a
42	1200.0 ±100 b	60±4 b	947.0 ±110 a	63±2 b	1330.0 ±70 ab	63±2 b
49	1230.0 ±280 b	56±3 b	922.0 ±50 a	61±3 b	1270.0 ±100 b	56±5 b
			Callus			
L1	0.24 ±0.01 a	76±2 a	1.07 ±0.20 a	73±3 a	0.34 ±0.01 a	71±3 a
L2	0.25 ±0.03 a	64±2 bc	1.13 ±0.40 a	61±5 b	0.26 ±0.02 ab	65±2 b
L3	0.19 ±0.04 a	58±4 c	0.86 ±0.24 a	57±2 b	0.19 ±0.02 b	50±1 d
L4	0.22 ±0.02 a	61±2 bc	1.02 ±0.10 a	60±3 b	0.23 ±0.01 b	56±4 c

[1] Days of culture for hypocotyl, cotyledon and mesophyll-derived protoplasts; callus induction medium for callus-derived protoplasts

[2] Letters within columns indicate the effect of explant age on the yield and viability of isolated protoplasts

The origin of secondary explant

Composition of initiation medium affected mainly the viability of callus-derived protoplasts, in a genotype-dependent manner. The vitality of „Taper" protoplasts obtained from tissue cultured on medium L1 amounted to 73%, whereas on other media was about 60% (Table 5). Isolation efficiency in cultivar „Taper" was the highest among tested cultivars and did not depend on medium composition. „Parys" and „Mister" yielded moderately, with 20-30 000 protoplasts being released from 1 g of tissue. In all cases better results were obtained using media with elevated concentration of cytokinins, especially medium L1, which was clearly superior to other tested formulations.

Cell wall regeneration

Hypocotyl protoplasts slowly regenerated a new cell wall. On the second day of culture 2%-5% protoplasts presented visible fragments of cellulosic fibers (Table 6), regardless of applied medium. Two days later resynthesis accelerated and ca. 50% protoplasts rebuilt new cell wall, at least partially. In subsequent days the number only slightly increased and after 10 days of cultivation regenerated cells constituted 44%-59% of culture population, depending on the genotype (Table 6).

Table 6. The influence of genotype, explant type and culture medium on cell wall regeneration in protoplast culture of yellow lupin
Tabela 6. Wpływ genotypu, rodzaju eksplantatu i pożywki na regenerację ściany komórkowej w kulturze protoplastów łubinu żółtego

Culture medium	Days of culture	Cell wall regeneration (%)								
		Cotyledon			Hypocotyl			Leaf		
		„Mister"	„Parys"	„Taper"	„Mister"	„Parys"	„Taper"	„Mister"	„Parys"	„Taper"
B_5	2.	72	81	73	1	2	2	9	7	7
	4.	73	82	77	32	34	28	13	11	10
	6.	79	84	81	31	37	37	17	16	20
	8.	84	87	89	39	43	37	26	34	32
	10.	89	91	91	45	47	44	37	42	40
			a[1]			b			a	

K8P	2.	44	45	49	3	4	3	5	4	5
	4.	50	53	59	39	48	33	10	15	18
	6.	66	67	72	42	51	35	19	22	26
	8.	77	74	81	43	59	40	26	27	30
	10.	87	88	88	53	59	52	31	36	32
			a			a			a	
V-47	2.	44	41	43	3	5	5	6	4	3
	4.	59	55	51	33	46	30	9	14	7
	6.	72	67	59	32	46	36	24	27	19
	8.	80	75	71	37	51	42	30	29	25
	10.	88	88	84	49	53	48	33	31	34
			a			ab			a	

[1] Letters within columns indicate the effect of applied medium on cell wall regeneration in cultures originating from specific explants, analyzed at the 10th day of culture

The highest efficiency of new wall formation was observed in „Parys" (59%) but considering the overall rate of regeneration, no differences occurred between tested cultivars. Optimal medium for cell wall regeneration in hypocotyl protoplast culture was modified K8P. Cellulose deposits were usually arranged evenly and accrued gradually over the cell surface (Fig. 1A,B). In non-spherical cells, thicker cellulose layers were visible in those parts of a cell that were changed in shape (Fig. 1C,D). In contrast, cotyledonary protoplasts rebuilt their walls relatively quickly. On the second day of culture cellulosic layers were detected in 70%-80% of protoplasts in B_5 medium, and 40%-50% in other media. Afterwards, resynthesis rate increased, however after ten days complete walls were observed in about 80%-90% cells. The highest percentage of regenerated cells was recorded in B_5 medium, while the lowest in V-47 medium (Table 6). The rate of the cell wall reconstruction was comparable between tested genotypes. Interestingly, applied culture medium influenced a pattern of cell wall arrangement. Regular distribution of cell wall components on cotyledonary protoplasts, similar to that found in hypocotyls-derived culture, was observed in media K8P and V-47 (Fig. 1E, G). Thicker layers of wall material were visible in non-spherical areas, especially in the budding fragments. At the initial stage of cell wall reconstruction cellulose fibers were arranged as rings embracing the entire protoplast (Fig. 1F,G). In B_5 medium, we observed deviation in wall regeneration pattern. During early stages of resynthesis, cellulose fibers were regularly deposited on cell surface. Later on, starting on the sixth day, regenerated cell walls had thinner fragments and clearly noticeable „holes" (Fig. 1H-J). In such places cell wall was thin or even absent. In mesophyll protoplast culture cell wall regeneration proceeded slowly. On the second day of culture cell walls had been reconstructed in only a few percent of protoplasts. Nevertheless resynthesis rate varied between tested genotypes, the number of cells was gradually increasing, reaching finally 30%-40% on the 10. day (Table 6). In the case of leaf explant, applied culture media did not influence the rate of cell-wall resynthesis. Cell wall structure was irregular, with both thinner and thicker cellulose deposits (Fig. 1M-P). However, cellulose layer was visible on the whole cell surface. Further thickening of a cell wall in changing areas was not revealed during early morphogenetic processes.

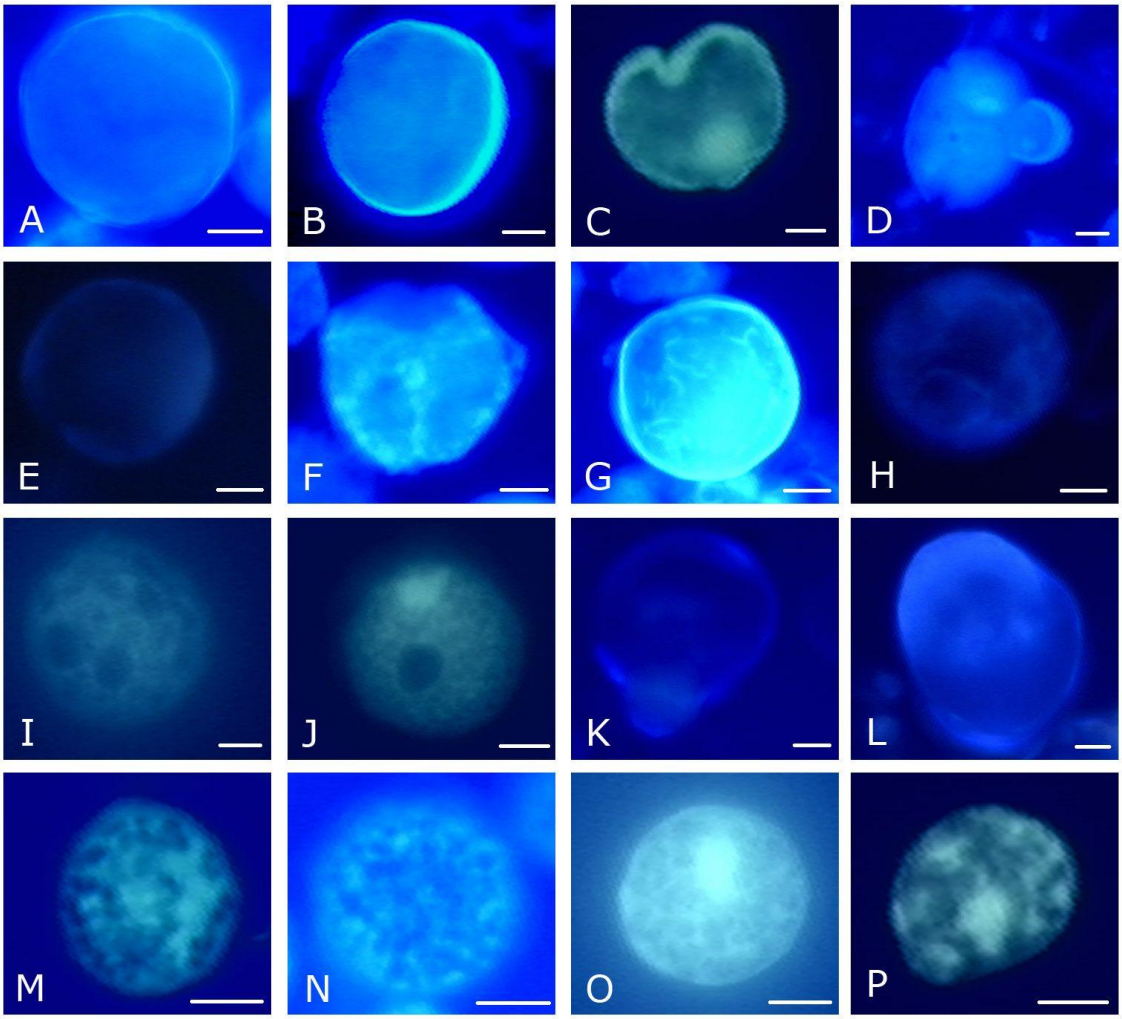

Figure 1. Cell wall regeneration in protoplast cultures of *Lupinus luteus* L. A-D, hypocotyl-derived protoplasts and budding cells of „Parys" cultured in medium K8P. E-G, cotyledonary protoplasts of „Mister" in media K8P (E-F) and V-47 (G). H-J, cotyledonary protoplasts of „Mister" in medium B_5. K-L, early morphogenetic response in „Parys" cotyledon-derived culture in medium K8P. M-P, mesophyll-derived protoplasts and cells of „Taper" in medium K8P. Bar 10 µm.
Rysunek 1. Regeneracja ściany komórkowej w kulturach protoplastów *Lupinus luteus* L. A-D, protoplasty hypokotylowe i pączkujace komórki odmiany „Parys" w pożywce K8P. E-G, protoplasty liścieniowe odmiany „Mister" w pożywkach K8P (E-F) i V-47 (G). H-J, protoplasty liścieniowe odmiany „Mister" w pożywce B_5. K-L, wczesna odpowiedź morfogenetyczna w kulturze protoplastów liścieniowych odmiany „Parys" w pożywce K8P. M-P, protoplasty i komórki odmiany „Taper" w kulturze protoplastów uzyskanych z mezofilu liści w pożywce K8P. Podziałka 10 µm.

Discussion

Enzymatic isolation applied to yellow lupin tissues proved to be an effective procedure for obtaining large populations of viable protoplasts. Because the proper choice of enzyme mixture compounds is a crucial step in optimising of protoplast isolation, we have tested numerous mixtures, dissimilar in relation to both enzyme type and concentration. For cell wall removal in primary explants the cellulase R-10

was chosen. This enzyme is highly efficacious in degrading cellulose fibres and is successfully exploited in protoplast isolation in a range of Fabaceae species, i.e. lucerne (Mizukami et al. 2006; Zafar et al. 1995), clover (Radionenko et al. 1994), pea (Xiao and Koster 2001) and soybean (Dhir et al. 1991). Enzyme mixtures applied to callus tissues were more complex and contained driselase, meicelase and rhozyme. The reason for the supplementation of mixture with cellulases exhibiting diverse activities is usually a difference in cell wall composition in callus cells in comparison with those of primary explant cells. In yellow lupin the most effective was the mixture containing driselase. This is in agreement with the results of studies on protoplast production from callus of other leguminous plants (Rybczyński 1997; Schaefer-Menuhr 1988). In our study pectinolytic enzymes such as macerozyme, pectinase and pectolyase Y-23 were used for the separation of cells. Apart from the similar activity, these enzymes demonstrate a certain selectivity in interactions with various cells of the tissue. Different pectinolytic enzymes act like “biochemical sorters” of cells, releasing them preferentially (Sinha and Caligari 2004). For that reason the choice of the maceration enzymes somehow influences further morphogenetic responses of protoplasts in culture. Taking into account that applied enzyme can indirectly stimulate or inhibit prolific mitoses, special attention should be given to the optimisation of tissue maceration step.

The results of our study demonstrate the suitability of tested primary explants for protoplast isolation in yellow lupin. In studies on other *Lupinus* species, the protoplast isolation from leaves was usually similarly effective as described here (Babaoglu 2000; Schaefer-Menuhr 1987; Sinha et al. 2003a; Sonntag et al. 2009). However, it has to be emphasized that various explants of particular lupin species have differential susceptibility to the tissue digestion. For example, cotyledonary tissue was found to be unsuitable source of *L. mutabilis* protoplasts (Babaoglu, 2000), while in *L. albus* this was considerably well reacting material (Sinha et al. 2003a,b). In yellow lupin a reasonable yield of protoplasts was obtained from hypocotyls, whereas in other lupins this explant was decidedly unresponsive to isolation protocols (Babaoglu 2000; Sinha et al. 2003a).

We also show significant efficiency of protoplast isolation from callus tissue and, to our knowledge, the isolation of protoplasts from lupin callus is reported here for the first time. In numerous Fabaceae species callus is considered valuable source of highly totipotent protoplasts. Such protoplasts can be used not only to regenerate entire plantlets but also can be exploited in somatic hybridisation and in the improvement of selection methods in protoplast cultures (Hou and Jia 2004; Luo et al. 2005; Mizukami et al. 2006). On the other hand, the primary callus is not suitable tissue for biotechnological experiments, due to its genetic instability. Therefore, prior to further studies, callus culture stabilization in several subcultures is required to obtain the tissue as much homogeneous as possible.

On the basis of obtained results concerning the effect of the primary explant age on protoplast yield and viability, we established reproducible protocols for protoplast isolation from each tested organ. The dependence between isolation parameters and the age of donor explant has been also described in other legumes. For example, high yield and purity of *Vigna mungo* protoplasts was achieved only when protoplasts were isolated from young, 4-5 days old hypocotyls, whereas in *V. sublobata* the best were protoplasts obtained from fresh, one week old callus (Gill et al. 1987). Babaoglu (2000) showed that young organs of Andean lupin: 8 days old shoot tips, epicotyls and 10 days old leaves of the seedlings, are better source of protoplasts than older organs. Similarly to yellow lupin, in *L. mutabilis* the viability of protoplasts decreased

with the increase of explant age. In white lupin higher number of protoplasts was isolated from older explants, however the percentage of viable forms systematically dropped (Sinha and Caligari 2003a).

Yellow lupin protoplasts regenerated a cell wall in a different mode, depending on the donor explant and nutrient medium. This was particularly pronounced in the culture of cotyledonary protoplasts. We observed a relationship between the rate of cell wall resynthesis and accumulation of abnormalities in cell wall structure. In a rather unsuitable culture medium B_5, newly regenerated walls were incomplete and unevenly distributed around the cell. On the other hand, resynthesis began there relatively quickly. The phenomenon can be though partially explained by lower NAA concentration in medium B5 (0.5 versus 1.5 and 1.0 mg/l in medium K8P and V-47, respectively). Recently this synthetic auxin has been proven to delay cell wall synthesis in ovule culture of cotton (Singh et al. 2009). It is probable that higher doses of NAA applied to yellow lupin protoplasts in media K8P and V-47 caused a similar effect, allowing the cell wall to be rebuilt slowly and therefore more precisely. According to Ochatt and Power (1992), the process of cell wall formation correlates with cell division. In particular, cytokinesis is strongly affected by cell wall structure and is often arrested when cellulose layers are not properly bounded (Suzuki et al. 1998). The disturbances in cell wall regeneration may be therefore a reason for strongly limited mitotic activity observed in the course of our experiment.

Hypocotyl and mesophyll-derived protoplasts of yellow lupin regenerated walls relatively slowly, in comparison with plant species regarded as non-recalcitrant. For example, in tobacco and sunflower protoplast cultures cell wall resynthesis began a few hours after isolation and on 3. day of culture all protoplasts had cellulose deposits on their surface (Caumont et al. 1997; Nagata and Takebe 1970). We therefore suggest that in yellow lupin both the rate and the pattern of cell wall regeneration contribute to limited morphogenetic response of cultured protoplasts. However, the responsible mechanisms remain unknown. Manipulations during protoplast preparation may affect the process of cell wall resynthesis. Such factor as osmoticum treatment and fast plasmolysis may influence the stability of plasmalemma and subsequent cellulosic microfibril alignment on the surface of plasma membrane. Cell wall regeneration may be also affected by the presence or absence of outside skeleton made from gelling agents. It was previously reported that embedding of white lupin protoplast in agarose increases division frequency (Sinha and Caligari 2005), however, studies on cell wall resynthesis were not there conducted. Therefore, an important task to be held in future is to compare between patterns of cellulose deposition in protoplasts suspended in liquid medium and embedded in outer matrix.

Conclusions

Yellow lupin is potentially a higher yielding protoplast source than other *Lupinus* species examined to date. Furthermore, freshly obtained protoplasts are viable and morphologically regular. Here, isolation as a crucial step of protoplast utilization was comprehensively optimized, taking into account various factors affecting the efficiency of the procedure. Efficiently produced protoplasts are a suitable material for fundamental investigations, such cell wall regeneration or cytoskeleton stability and organization. Better understanding of factors influencing protoplast isolation, as well as their interactions in applied experimental system, provide a necessary basis for successful studies in the field of protoplast totipotency and recalcitrance in yellow

lupin. Moreover, by defining the essential parameters for the isolation of yellow lupin protoplasts, this work lays foundations for the efficient protoplast production from the other recalcitrant *Lupinus* species.

It is also possible that an important reason for yellow lupin recalcitrance to protoplast culture may be incorrect cell wall regeneration in experimental conditions. We provided evidence that in unsuitable culture environment newly synthesized cell wall exhibit anomalous architecture. Further studies are now in progress to evaluate the composition of walls during early stages of various culture systems of yellow lupin protoplasts (i.e. liquid and solid medium), as well as the involvement of cytoskeleton in cell wall reconstruction and cytodifferentiation.

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