Studies on Cell Wall Regeneration in Protoplast Culture of Legumes – the Effect of Organic Medium Additives on Cell Wall Components

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Abstract

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The cell wall regeneration in mesophyll protoplasts of yellow lupin and grass pea was studied. The occurrence of cell wall components: cellulose, callose and arabinogalactan proteins was analysed during 15 days of culture. Protoplasts were cultured in different media to test the effect of culture environment on the cell wall regeneration. Medium supplementation with 2 mg/l chitosan resulted in prolonged viability, more balanced cellulose resynthesis, increased callose formation and induction of mitotic divisions in protoplast-derived cells of both examined legumes. In chitosan-enriched medium arabinogalactan proteins were detected in cell plates of divided cells. Medium rich in additional organic compounds, i.e. free amino acids, organic acids and monosaccharides, was inferior to media of simpler composition. In both species the relatively quick cellulose resynthesis negatively affected the viability of protoplast-derived cells. In grass pea cellulose appeared during 24 h of culture. In yellow lupin the process started significantly later and after 10 days the frequency of walled cells did not exceed 50%. Callose was detected in cultures of both species and its pattern suggested that the synthesis was unlikely to be a result of protoplast wounding. Arabinogalactan proteins were localized in cell walls of different types of cells: dividing, elongating, but predominantly in degenerating ones. Occurrence and organization of the cell wall components studied were discussed in relation to recalcitrance of grass pea and yellow lupin protoplasts.

Keywords: arabinogalactan proteins; cell wall; cellulose; chitosan; *Fabaceae*; protoplast culture

Protoplasts of numerous legume crops are considered recalcitrant to *in vitro* culture, mainly due to their limited ability to divide. Examples of such crops are yellow lupin (*Lupinus luteus* L.) and grass pea (*Lathyrus sativus* L.) that have not been regenerated into plants using protoplast culture. Attempts are being made to enhance the regeneration potential of protoplasts through the selection of responsive genotypes, donor explants and culture optimisation (Sinha *et al*. 2003; Wiszniewska & Pindel 2009). Improved protocols are desired as a source of novel gene pools, a method for overcoming cross-incompatibility barriers and for the somatic hybrid production. The insight into cellular and molecular mechanisms controlling recalcitrance may contribute to the broader exploitation of legume protoplast culture in modern breeding. Therefore it is essential to distinguish cellular events related to the protoplast inability to maintain morphogenetic competences (Wiszniewska *et al*. 2012).

The cell wall structure and its dynamic changes may be precise markers of developmental competences of the cell (Malinowski & Filipecki 2002). Cell wall reconstruction and further alterations in the rate of resynthesis, as well as in the composition and physical-chemical properties of regenerated wall affect the mitotic activity of cells (Wang *et al.* 1991; Suzuki *et al.* 1998).

During cell wall regeneration two polysaccharides – cellulose and callose, are particularly important. Cellulose (β-1,4-glucan) is the most widespread poly-

mer in plant walls, constituting 30–90% of structural polysaccharides (Lee *et al*. 2011). In easily regenerating protoplasts its synthesis may start as early as a few hours after culture establishment (Tylicki *et al.* 2001). A unique cell wall polysaccharide is callose, a 1,3-glucan, whose occurrence is specific and restricted to cell plates, pollen tubes, plasmodesmata and wounded cells (Lee *et al*. 2011).

The response of cultured protoplasts may also be attributed to the presence of arabinogalactan proteins (AGPs) in the cell wall. These highly glycosylated proteins are regarded as identity markers of a plant cell and information carriers in intercellular communication (Malinowski & Filipecki 2002; Rumyantseva 2005). It has been proven that AGPs play a role in the proper binding of components in the new wall (Rumyantseva 2005). Gao and Showalter (1999) suggested that some types of AGPs could be markers of cells entering the apoptosis pathway. In this respect, apoptosis relates to the dedifferentiation process during morphogenesis.

Studies on cultured protoplasts allow an analysis of the pattern of cell wall regeneration (arrangement of cellulose fibres, a sequence of polysaccharide synthesis), as well examination of changes in the composition of cell wall during the acquisition of mitotic potential. Differential cell wall resynthesis in unresponsive and totipotent protoplast cultures has been reported for several plant species (Wang *et al*. 1991; Katsirdakis & Roubelakis-Angelakis 1992; Tylicki *et al.* 2001). However, there is only scarce information on the cell wall renewal in recalcitrant legumes, such as grass pea and yellow lupin (Wiszniewska *et al*. 2012). In these species the main obstacles in protoplast culture are limited mitotic potential and low viability. Therefore the studies have to be restricted to events occurring during early stages of culture. Nevertheless, these results may give an insight into the wall regeneration capacity of recalcitrant legume protoplasts that would be of considerable importance, from both the fundamental and practical point of view.

We hypothesized that the recalcitrance may be correlated with disturbed/improper resynthesis of the cell wall. Therefore, the process of its regeneration in mesophyll protoplasts of yellow lupin Parys and grass pea Krab was examined. The occurrence of cell wall components: cellulose, callose and arabinogalactan proteins was analysed during early days of culture. Protoplasts were cultured in different media to test the influence of the culture environment on the cell wall regeneration.

MATERIAL AND METHODS

Plant material and protoplast preparation. Polish cultivars of grain legumes: grass pea (*Lathyrus sativus* L.) Krab and yellow lupin (*Lupinus luteus* L.) Parys were used as plant material. Protoplasts were isolated from leaves of three-weeks-old *in vitro* seedlings. Seedlings were obtained as described previously (Wiszniewska *et al.* 2012). Leaves with the lower epidermis removed were incubated overnight in an enzyme mixture containing 1% Cellulase Onozuka R-10 (Kinki Yakult Co., Tokyo, Japan), 0.5% Macerozyme Onozuka R-10 (Kinki Yakult Co.), sorbitol as osmotic stabilizer (11% for grass pea, 9% for yellow lupin) in CPW salts (Frearson *et al.* 1973). Protoplasts were filtered through 100 µm nylon mesh filter and purified afterwards.

Protoplast culture. Protoplasts were suspended in a liquid medium at a density of 1×10^5 protoplasts/ml and cultured in plastic Petri dishes (4 cm in diameter). Three culture media were used, all based on Gamborg *et al*. (1968) formulation: B5A medium, B5A medium supplemented with 2 mg/l chitosan (Sigma, St. Louis, USA), and modified AS medium (according to Schäfer-Menuhr & Stürmer 1987). All media contained the same growth regulators: NAA, BAP, 2,4-D, 2iP, GA_3, each in a concentration of 0.5, and 20 mg/l ascorbic acid. Cultures were maintained in the dark at 27°C.

Viability was determined after 1, 2, 5, 7, 10 and 15 days of culture using Evans Blue staining (Gaff & Okang'o-Ogola 1971). The results were expressed as a percentage of viable protoplasts/cells per microscope field (magnification 160×) in each count including at least 100 randomly chosen protoplasts/cells. To visualise nuclei, cells were stained with DAPI (4',6-diamidino-2-phenylindole) for 10 min and observed at 420 nm in an Axio Imager M2 multifunctional microscope (Zeiss, Jena, Germany).

Cell wall staining. Protoplast-derived cells were analysed for the presence of cell wall polysaccharides after 1, 2, 5, 7, 10 and 15 days of culture. Cellulose was stained with 0.01% Calcofluor White ST (Sigma, St. Louis, USA) for 5 min. Callose was detected using 0.1% aniline blue (Sigma) staining for 5 min. Cellulose fluoresced blue, while callose yellow-light blue, in UV light and were observed at 400 nm in an Axio Imager M2 multifunctional microscope (Zeiss). Arabinogalactan proteins (AGPs) were localised in cell walls after 5, 7 and 10 days of culture, using 0.01% β-D-glucosyl Yariv reagent (Biosupplies, Australia Pty. Ltd., Bundoora, Australia). Cells were stained

for 30 min and examined in a Nikon Eclipse E400 microscope (Nikon Corp., Tokyo, Japan). Cell walls containing AGPs stained red-brown.

The results were expressed as a percentage of cells containing the examined component per microscope field (magnification 160×) in each count including at least 100 randomly chosen cells. Counting was repeated thrice and materials were sampled from randomly selected Petri dishes.

Statistics. The treatments were independently replicated three times (3 replicates), five Petri dishes (4 cm in diameter) per treatment within each replicate. All results were subjected to STATISTICA 9.0 one-way ANOVA analysis; post-hoc Tukey's test was used afterwards to study differences between respective variants at $P < 0.05$.

RESULTS

Viability. For both species examined, stable viability of cultured protoplasts and protoplast-derived cells was observed in B5A medium containing 2 mg/l chitosan. In this medium survival rate of yellow lupin protoplasts after 10 days of cultivation was the highest (8.8%). Viability of grass pea protoplasts was higher than that of lupin, and on the 10th day of culture it amounted to 47.7% (Table 1). Unsupplemented B5A medium was less effective in maintaining viability. After 10 days of culture, in unsupplemented medium the mean viability reached 5.4% for grass pea and 2.9% for yellow lupin. In AS medium the viability exceeded 80% after 24 h. However, on subsequent days the number of viable protoplasts decreased, to 11.2% in yellow lupin (Table 1). After 15 days of culture, viable grass pea protoplasts were present in both variants of B5A medium (18.5 and 1.6%, respectively), while those of lupin stayed viable only in the medium with chitosan (3.1%) (Table 1).

Cellulose. In grass pea cellulose resynthesis occurred relatively quickly. After 24 h cellulose fibres were detected in 31.6–56.1%, depending on the medium (Table 1). Interestingly, in both B5A media

Table 1. The effect of culture medium on the viability, cellulose and callose synthesis in protoplast cultures of grass pea and yellow lupin (in %)

Days	Grasspea Krab			Yellow lupin Parys		
	B5A[1]	B5A+ch	AS	B5A	B5A+ch	AS
Viability of protoplasts and protoplast-derived cells						
1	61.0^{b}	82.5^{a}	89.4^{a}	35.7^{b}	40.0^{b}	81.9^{a}
2	38.8^{c}	81.0^{a}	59.9^{b}	30.8^{a}	36.2^{a}	11.2^{b}
5	12.8^{c}	68.5^{a}	24.3^{b}	21.4^{b}	37.1^{a}	2.1^{c}
7	11.4^{b}	59.1^{a}	8.6^{b}	17.1^{a}	14.6^{a}	2.4^{b}
10	5.4^{b}	47.7^{a}	3.7^{b}	2.9^{b}	8.8^{a}	0.7^{b}
15	1.6^{b}	18.5^{a}	0.0^{c}	0.0	3.1^{a}	0.0
Cells with cellulose						
1	44.8^{b}	31.6^{c}	56.1^{a}	0.0	0.0	0.0
2	62.8^{a}	44.9^{b}	58.1^{a}	4.6^{b}	3.1^{b}	25.4^{a}
5	90.9^{a}	60.0^{b}	47.4^{b}	26.1^{a}	11.8^{b}	29.5^{a}
7	88.7^{a}	76.2^{ab}	55.8^{b}	27.7^{b}	26.4^{b}	42.0^{a}
10	96.4^{a}	90.5^{a}	58.0^{b}	33.4^{b}	42.1^{a}	37.1^{ab}
15	100.0^{a}	93.7^{a}	57.1^{b}	–[2]	–	–
Cells with callose deposits						
1	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0
5	4.6^{a}	5.7^{a}	1.4^{b}	3.3^{a}	2.9^{a}	0.0^{b}
7	8.8^{b}	15.9^{a}	3.2^{c}	3.0^{b}	11.5^{a}	0.0^{c}
10	8.4^{b}	32.4^{a}	7.4^{b}	3.8^{b}	9.8^{a}	0.0^{c}
15	6.1^{b}	30.8^{a}	7.9^{b}	–	–	–

[1]medium code; [2]not conducted; means with the same letter are not significantly different at $P < 0.05$ according to one-way ANOVA and post-hoc Tukey's test (the effect of the medium was tested on each day of culture, separately for both examined species)

the number of cells with the reconstructed cell wall increased gradually (up to 100% on the 15th day), while in AS medium the number was constant (from 47.4 to 58.1%) throughout the culture period. Cellulose was evenly distributed over the cell surface (Figure 1A). No abnormalities in fibre alignment were observed. Despite the presence of complete cellulosic cell walls, protoplasts budded intensively (Figure 1B). The first and second protoplast divisions occurred only in the medium supplemented with chitosan, with the highest frequency of 3.2% after 7 days of culture. A thick cell plate was visible in divided cells (Figure 1C).

In yellow lupin, cellulose appeared on the cell surface after 48 h of culture and was seldom distributed regularly. At this time, the highest number of complete cells was present in AS medium (Table 1). Interestingly, the slowest increase in the number of walled cells was observed in the medium with chitosan. After 10 days, the number of regenerated cells was comparable in all tested media – the mean percentage did not exceed 43%. In comparison with AS medium, in both B5A media protoplasts rebuilt new walls more slowly and remained viable for longer. Cellulose fibres were distributed irregularly on the cell surface, forming thicker and thinner deposits in different areas of the cell (Figure 1D). Fibres not attached tightly to the cell wall were also visible, as well as cellulose bundles loosely connected to the surface of the cell (Figure 1E). At the beginning of cell wall regeneration, cellulose appeared on those parts of the plasmalemma where chloroplasts were gathered (Figure 1F). After 10 days, cell walls became thicker, however still irregular. The majority of the cells had regenerated cell walls consisting of a net of unequally thick cellulosic strands (Figure 1G). In non-spherical and budding cells, thicker cellulose deposits were formed around malformed parts of the cell. In such cells karyokineses were observed using DAPI staining, but no cytokineses occurred (Figure 1I–J).

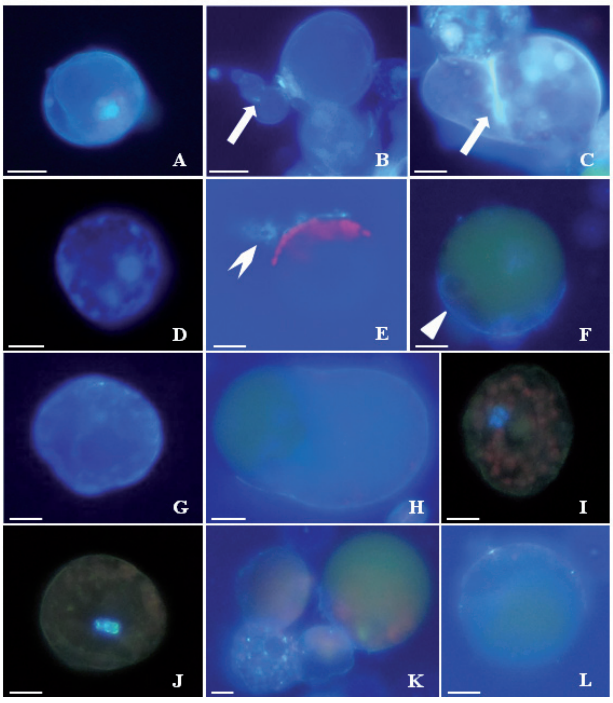

Figure 1. Regeneration of cellulose in protoplast culture: (A)–(C) *Lathyrus sativus*; (A) – protoplast with regular cell wall; (B) – budding cell (arrow); (C) – cell plate (arrow) in dividing cell in the medium with chitosan; (D)–(L) *Lupinus luteus*; (D),(G) – irregular alignment of cellulose on the 5th day (D) and 10th day (G); (E) – cellulosic bundles (arrowhead) loosely connected to the cell surface; (F) – cellulose nearby chloroplast clusters (triangle); (H) – budding protoplast; (I),(J) – karyokinesis; (K)–(L) – cellulosic wall on the 30th day; bar – 10 µm

Additional observations were conducted on 30-day lupin cultures in the medium containing chitosan. Thin, regular cellulose layers covering around 50–75% of the cell were then observed (Figure 1H, K–L).

Callose. In grass pea cultures, callose was absent until the fifth day of culture, irrespective of the applied medium (Table 1). Small callose deposits appeared above chloroplast clusters (Figure 2A). On subsequent days a callose layer covered the majority of the cell

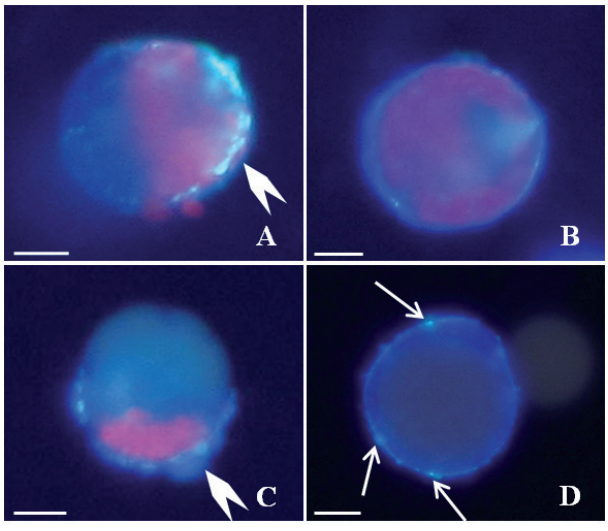

Figure 2. Detection of callose in protoplast culture: (A), (B) – *Lathyrus sativus* protoplasts; (C), (D) – *Lupinus luteus* protoplasts; (A), (C) – callose (arrowheads) above chloroplast clusters; (B), (D) – callose layer (B) and spots (arrows) (D); bar – 10 µm

Table 2. The influence of culture medium on the presence of arabinogalactan proteins (AGPs) in cell walls of grass pea and yellow lupin protoplast-derived cells

Days	Cells containing AGPs (%)								
	dividing			non-dividing[1]			degenerating		
	B5A[2]	B5A+ch	AS	B5A	B5A+ch	AS	B5A	B5A+ch	AS
Grasspea Krab									
5	0	0.6[a]	0	12.3[a]	14.3[a]	18.5[a]	26.2[b]	14.6[c]	42.1[a]
7	0	3.2[a]	0	15.1[b]	19.1[a]	22.4[a]	34.6[b]	26.2[b]	61.6[a]
10	0	2.9[a]	0	4.3[c]	13.9[b]	16.3[a]	41.2[b]	32.1[c]	58.5[a]
Yellow lupin Parys									
5	0	0	0	5.4[b]	8.6[a]	0.8[c]	39.8[b]	31.2[c]	49.7[a]
7	0	0	0	5.9[b]	9.1[a]	5.1[b]	65.2[a]	52.3[b]	66.8[a]
10	0	0	0	4.5[b]	12.3[a]	6.4[b]	61.4[b]	48.7[c]	82.4[a]

[1]non-dividing spherical, elongated and budding cells; [2]medium code; means with the same letter are not significantly different at $P < 0.05$ according to one-way ANOVA and post-hoc Tukey's test (the effect of the medium was tested on each day of culture, separately for both examined species)

surface (Figure 2B). Up to 30% of cells cultured in chitosan-enriched medium contained this glucan (Table 1). In the remaining media, the number of cells with callose did not exceed 9%. Although cells divided in the medium with chitosan, the presence of callose in cell plates was not confirmed.

In yellow lupin, callose was absent during the whole experimentation period in AS medium, while in both B5A media, small callose deposits were visible from the 5th day (Table 1). Similarly to grass pea cultures, callose was initially present nearby chloroplast clusters (Figure 2C). Subsequently, callose appeared as small dots distributed on the plasmalemma (Figure 2D). Again, the occurrence of callose in cell plates was not observed.

Arabinogalactan proteins. Arabinogalactan proteins were detected in dividing, non-dividing (elongated, budding) and degenerating cells of grass pea (Table 2). In the medium supplemented with chitosan both symmetric and asymmetric divisions were observed (around 3% after 7 days). In mitotic cells, AGPs were localized in cell plates, and were absent in the remaining parts of the cell wall (Figure 3A–D). In non-dividing, but non-spherical cells, AGPs were distributed in altered parts of the cell wall (Figure 3E). In contrast, AGP(+) degenerating cells had stained both the cell wall and the cytoplasm (Figure 3F). The number of such cells was significantly higher in AS medium than in both B5A media.

In yellow lupin cultures, AGPs were found after 5 days in some elongating and most of degenerating cells (Table 2). Degenerating AGP(+) cells exhibited serious irregularities of the cell wall (irregular surface, cytoplasm emerging from perforated cell wall, "vesicles" and "bundles" on the cell surface) (Figure 3G,H). The majority of elongating cells, as

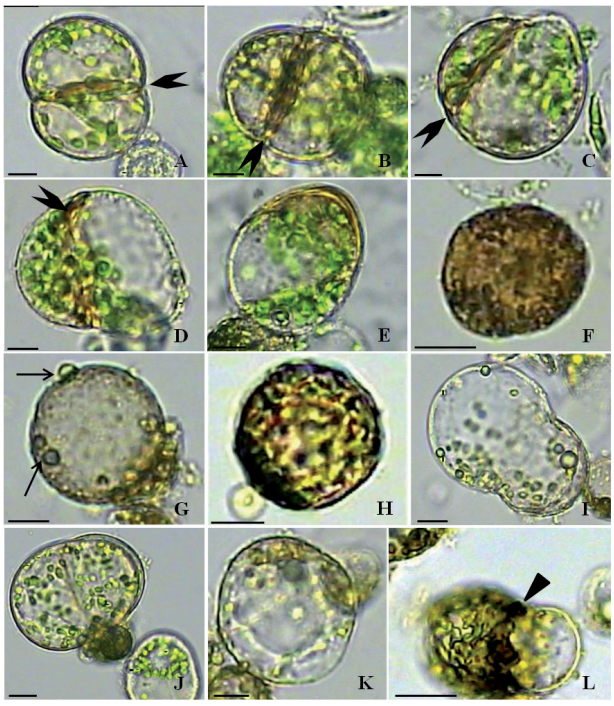

Figure 3. Detection of arabinogalactan proteins (AGPs) in protoplast culture: (A)–(F) – *Lathyrus sativus*; (G)–(L) – *Lupinus luteus*; (A)–(E) – AGPs in cell plates of symmetrically (A), (B) and asymmetrically (C), (D) dividing cells and in non-spherical cell (E); (F)–(G) – AGP(+) degenerating protoplast with "bundles" on the cell wall (arrows) (G) and irregular surface (F); (I)–(K) – AGPs(-) elongated cells; (L) – thick and irregular cell plate (triangle) after mitosis; bar – 10 μm

well as those in pre-mitotic stage, were AGP(-) (Figure 3I–K). Incipient mitotic cells contained AGPs in the cell plate area, but cell plates were abnormally thick and irregular (Figure 3L). The number of degenerating cells was the lowest in the medium supplemented with chitosan. In this medium incipient mitoses (frequency lower than 0.1%) were noted, as well as the highest percentage of elongating cells. In the remaining media no divisions were observed.

DISCUSSION

The cell wall renewal is often regarded as a key step in reprogramming and dedifferentiation in protoplast culture. The majority of studies on cell wall regeneration report on the process occurring in protoplast cultures of responsive, easily dividing genotypes and cell lines (van Amstel & Kengen 1996; Tylicki *et al*. 2001). Observations made on such materials allow one to elaborate models of the cell wall resynthesis that can then be compared with processes occurring in protoplasts of recalcitrant genotypes.

The results of this study indicate that the main factor affecting cell wall regeneration and protoplast behaviour in the species examined was the culture medium. Media differed solely in their content of organic compounds. B5A media contained basal vitamins, while AS medium was enriched with free amino acids, monosaccharides, organic acids and additional vitamins (Schäfer-Menuhr & Stürmer 1987). Surprisingly, the richer medium was decidedly the less optimal for maintaining viability in grass pea and lupin cultures. On the other hand, in this medium we observed the highest percentage of walled protoplast-derived cells during the first day of culture. The composition of AS medium seemed to accelerate cellulose resynthesis on the protoplast surface, but inhibit its deposition on subsequent days. In contrast, in media without additional organic ingredients, cellulose synthesis was slightly slower, but the number of wall-regenerated cells increased constantly during culture. Our results are unexpected, since in prevailing protocols for the protoplast culture of legume species, including lupins and grass pea, media rich in organic compounds are considered the most suitable for culture development (Schäfer-Menuhr & Stürmer 1987; Durieu & Ochatt 2000; Sinha & Caligari 2009). We reported previously that in cotyledonary protoplasts of yellow lupin the relatively fast cellulose resynthesis negatively affected protoplast viability (Wiszniewska *et al.* 2012). Here we observed the same phenomenon in mesophyll cultures. Moreover, a similar relationship seems to occur in grass pea cultures, although to a lesser extent. Although the reason for such negative correlation remains unclear; it may be directly related to protoplast recalcitrance of the examined species. Such a reaction may be a result of the unbalanced osmotic status leading to osmotic stress, and for that reason the reduction of medium osmolarity is usually essential for proper regeneration processes (Davey *et al*. 2005). However, our previous studies on yellow lupin protoplasts revealed that gradual osmolarity reduction had no effect on the number of viable protoplasts and the frequency of mitoses (unpublished). Such a reaction is quite rare, but sometimes reported for protoplast systems of other species (Yu *et al.* 2000). In grass pea cultures the osmolarity of the medium was not reduced before the 10th day of culture, so early events of cell wall resynthesis occurred also in a medium of relatively high osmotic potential. Further studies are necessary to clarify the relation between the osmolarity of culture medium and cell wall regeneration in the examined species.

In suboptimal media, yellow lupin protoplasts regenerated a cellulosic wall only at a restricted surface area. According to van Amstel and Kengen (1996), the occurrence of that pattern may be interpreted as indicative of diminished cell integrity. Such tobacco cells regenerated from protoplasts degenerated easily, in agreement with our observations.

The addition of chitosan to the culture medium caused a significant increase in protoplast viability, as well as an improvement in their response during cultivation. In grass pea cultures, the first and subsequent divisions of protoplast-derived cells were observed. Even in decidedly less responsive yellow lupin incipient mitoses also occurred. This indicates that the cell wall was properly reconstructed, at least in some cells. Chitosan is quite often used in plant tissue culture as a cell-stimulating additive (Kowalski *et al*. 2006; Nge *et al*. 2006). This compound induces cellular defence responses against various stresses, including callose formation (Köhle *et al*. 1985). This is in agreement with our results, since for both species callose deposits were intensively synthesized in the chitosan-containing medium. Köhle *et al*. (1985) speculated that an elicitation of callose is mainly due to controlled electrolyte leakage, which seems to be chitosan-concentration dependent. Elevated concentrations of chitosan resulted in a full destruction of soybean cells. This also corroborates our preliminary observations that

higher concentrations of chitosan negatively affect the viability of cultured protoplasts (unpublished data). It is possible that in grass pea and yellow lupin protoplasts chitosan induced controlled destabilization of the plasma membrane and stimulated callose formation to prevent irreversible damage of the cells.

The pattern of callose deposits may provide further information on the protoplast condition in culture. Van Amstel and Kengen (1996) reported the presence of dispersed granular callose deposits in undamaged tobacco protoplasts, since in the wounded ones intense radiant spots surrounding the unstained area were observed. Punctate deposits detected in our experiment suggest that callose formation in grass pea and yellow lupin cultures was unlikely to be a result of protoplast wounding. Callose was not detected in cell plates of grass pea and yellow lupin protoplasts. This was surprising, since callose is known to be their component (Lee *et al*. 2011). However, in a protoplast culture of *Marchantia polymorpha,* callose was not detected between divided cells either (Shibaya & Sugawara 2009). The authors speculated that the reduced level of β-1,3-glucan (or even its absence in cell plates) may be a result of some perturbations of arabinogalactan proteins. In legume protoplast cultures an interesting relationship between AGPs and callose during the cell plate formation may be related to protoplast recalcitrance and therefore requires further studies.

The beneficial effect of chitosan was also seen in the increased frequency of mitoses, especially in grass pea. For the first time, it was possible to detect arabinogalactan proteins in cell plates of divided protoplast-derived cells of legume species. Previously, the involvement of AGPs in the cell plate formation was reported only in protoplast cultures of the bryophyte *Marchantia polymorpha* (Shibaya & Sugawara 2009). Recently, Yu and Zhao (2012) proposed the contribution of AGPs not only to the cell plate construction, but also to a decision on its position, resulting in either symmetric or asymmetric division of tobacco zygote and proembryo.

In yellow lupin, AGPs were also detected in cell plates, however daughter cells quickly degenerated. This may suggest that although the cell wall regeneration was successful in the chitosan-enriched medium, further processes, including mitosis itself, were still defective. Cell death and abnormally thick cell plates are visible results of aberrations that are still unclear. Nevertheless, in recalcitrant material such as protoplasts of grass pea or yellow lupin, the detection of AGPs in cell plates may be a considerable contribution to the existing knowledge of protoplast response to culture conditions.

A stimulatory action of chitosan on cell wall regeneration and cell divisions may also be attributed to the structure of AGPs. N-acetylglucosamine (monomer of the chitosan molecule) is present in AGP carbohydrate moiety and seems to be also present in the side polysaccharides of AGPs (Rumyantseva 2005). Chitooligosaccharides in AGP carbohydrate chains are supposed to be biologically active (van Hengel *et al.* 2001).

In the cultures of grass pea and lupin, cells containing AGPs were predominantly degenerating. AGPs seem to play a certain role in triggering apoptosis (Rumyantseva 2005). Since the separation of the plasma membrane from the cell wall is a sign of apoptosis in cultured plant cells (Pennell & Lamb 1997), it is probable that the majority of protoplasts were unable to recover from isolation stress and their cell status was therefore switched to programmed cell death. To verify this hypothesis, further studies would be necessary on totipotent cultures, which are however difficult to establish.

To conclude, the course of cell wall regeneration in the legume crops examined depended on culture environment and had an impact on the developmental pathway of cultured cells. Such detailed studies on the process may contribute to a better understanding of legume recalcitrance to protoplast culture. Other potential reasons for recalcitrance that should be considered in the future include a strong stress reaction and inefficient defence mechanism, a lack of reprogramming and dedifferentiating signals, and defective reorganisation of remaining cellular components, i.e. cytoskeleton.

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