FOLIA OECOLOGICA – vol. 35, no. 2 (2008). ISSN 1336-5266

Cell wall regeneration of protoplasts isolated from Norway spruce tissue cultures in a liquid nutrient medium

Helena Ivanová

Branch of Woody Plants Biology, Institute of Forest Ecology of the Slovak Academy of Sciences, Akademická 2, 949 01 Nitra, Slovak Republic, E-mail: helena.ivanova@savzv.sk

Abstract

Ivanová, H. 2008. Cell wall regeneration of protoplasts isolated from Norway spruce tissue cultures in a liquid nutrient medium. *Folia oecol.*, 35: 18–24.

Protoplasts isolated from spruce tissue culture (*Picea abies* L.) Karst. were incubated in a medium supplemented with D-/^{14}C/ glucose, with the aim to provide releasing radioactive oligosaccharides and polysaccharides. The amount of both in the medium was increasing over the entire 90-hour incubation period. We have confirmed that the secerned oligosaccharides are not formed in processes of hydrolysis running at the cytoplasmic membrane. The first are cell wall components accumulated through synthesis and then released from the being-created cell wall. A fluorescence microscope and a light one were our tools for study of protoplasts´ regeneration. The fluorescence reagent primulin was fixed on the protoplasts´ surface. Since 24 hours after the incubation, polysaccharidic cell wall components were deposed on the surface. All the protoplasts were covered with polysaccharidic material in three days after the incubation.

Keywords

Picea abies, protoplasts, secondary cell wall, oligosaccharides, polysaccharides

Introduction

Protoplasts of plant cells are a topical experimental material for study of cell wall regeneration. Protoplasts were isolated from plants for the first time by Cocking in 1960 (Cocking, 1960). The author used enzymes. Since then, protoplasts have been getting of increasing importance in a variety of branches in experimental botany. Protoplasts provide the most up-to-date and focussed tool in experimental breeding, because they offer completely new approaches for a more insightful look into genetic variability of cultural plants, either through transformation of their inborn genes or through somatic hybridisation. Protoplasts provide a powerful tool for evaluating marker gene expression in plants. Protoplasts can be used to regenerate whole trees; consequently, implementation of protoplast techniques for conifers is of crucial importance to forest biotechnology.

In conifers, protoplasts have also been isolated from seedlings (embryos, cotyledons, shoots) preconditioned in vitro by culture on a medium-containing auxin and cytokine before the protoplast isolation (David et al., 1982; Lang and Kohlenbach, 1989; Gómez-Maldonado, 2001; Malinowski and Filipecki, 2002).

Although protoplasts have been isolated from haploid cells of conifers, including pollen of *Cupressus arizonica* (Duhoux, 1980) and female gamethophytes of *Picea abies* (Hakman et al., 1986), most of the efficient preparation methods use callus or suspension cultures as starting materials (Kákoniová and Labudová, 1987).

Material and methods

The tissue culture of Norway spruce was obtained from hypocotyls of germinated spruce seeds and cultivated on a basic MS medium, modified according to Brown-Lawrence (1968).

We started our experiments with tissue cultures obtained from the just discussed seedling hypocotyls. The mixture of enzymes (5% cellulase, Onozuka R-10“ and 2% macerozyme R-10 in 0.4 M manitol) was

supplemented with approx. 6 g spruce tissue culture. The incubation was running in a water bath, at 30 °C, for 5 hours, under continual stirring. Then the mixture was filtered, washed with 0.4 M manitol and processed in a centrifuge at 1500/min The sediment was separated through another centrifuging at 1500/min and a centrifuging at a dextrane gradient (15%, 10% and 5% dextrane, 6 ml each) at 3500/min. In 15 minutes, we succeeded to separate the protoplasts from cells or cell clusters. The fraction of pure protoplasts was obtained after removing dextrane through three times repeated washing with 0.4 M manitol and subsequent 10 min centrifuging at 1000/min.

We used the method of paper descending chromatography on Whatman 1 in the system S_1 – ethyl-acetate, n-propanol, water (1 : 7 : 2) and in the system S_2 – ethyl-acetate, pyridine, water (8 : 2 : 1).

Chemical detection of saccharides was carried out in solution of 20 µl saturated $AgNO_3$ dissolved in 40 ml of acetone and in solution of 0.5% NaOH dissolved in 75% ethyl alcohol.

Radioactivity of the samples was measured with a liquid scintillation equipment "Packard 3300" with scintillation solution consisting of: 5 g PBD, 100 mg POPOP, 1 l toluene.

Cleared protoplasts suspended in 0.4 M manitol were sealed into a liquid agar medium. The suspension of cleared protoplasts (100 µl) was supplemented with 50 µl of fluorescence reagent – primulin for staining polysaccharides. Photographing of microscopic specimens was performed with a fluorescence microscope (ZEISS, Jena, projector 4 : 1, objective G249, filter B-224 g).

Results and discussion

Protoplasts isolated from spruce tissue cultures at exponential phase of their growth were maintained in an environment consisting of 0.4 M manitol with stabilised osmotic pressure. The dimensions of cells embedded into this environment were the same as the original ones. Such treated protoplasts were transferred onto the liquid agar medium. Four or five days later, stable, non-lysing protoplasts began to appear. This means that these protoplasts had already synthetised cell wall components in sufficient amounts.

The cell wall regeneration of protoplasts was studied in experiments in which the cultivation medium without saccharose was added with D-/^{14}C/ glucose. Over the first four days, we explored radioactive carbohydrate metabolites released into the liquid nutrient medium. The incubation mixture consisted of 1 ml nutrient medium, 4 µci /^{14}C/ glucose and 2.10^4 protoplasts – suggesting that the protoplasts released oligosaccharides and polysaccharides into the cultivation medium. The amount of the released compounds was increasing with incubation time. This means that immediately after the embedding of the isolated protoplasts in the cultivation medium, the protoplasts started with synthesis of their cell wall components. The amount of radioactive glucose added to the medium at the beginning decreased rapidly with cultivation time, on the other hand, the amounts of released oligosaccharides and polysaccharides were progressively increasing.

The viability of protoplasts was evaluated on the amount and rate of utilization of D-/^{14}C/ glucose supplemented to the medium. We have found that the protoplasts (2.10^4) utilize D-/^{14}C/ glucose for the first seven days (Table 1). Later, there occurred changes in percent presence of the individual oligosaccharides (Table 2).

The released oligosaccharides can be separated in approx. three groups: A, B, C based on the chromatographic mobility of the first. Re-chromatography of the fractions in the system S_2 (48 h) did not resulted in any further distinction; therefore, we subjected the fractions to hydrolysis with 1 N HCl. The subsequent chromatography of the hydrolysate in the system S_2 (48 h) resulted in indication of presence of radioactive glucose and galactose and non-radioactive manose, xylose and arabinose.

The cultivation medium was supplemented with released oligosaccharides and polysaccharides. In a liquid cultivation medium, these compounds are evidently flushed away from the building cell wall, and their activity is dependent on biochemical composition of the cell wall (Fry et al., 1993; Malinowski and Filipecki, 2002). We needed to confirm that the released oligosaccharides did not originate in processes of hydrolysis taking place at the cytoplasmic membrane – either as the result of absorption of enzymes used in the protoplasts treating or as the result of presence of hydrolysing enzymes at the membrane. For this purpose, we transferred protoplasts incubated in the medium supplemented with D-/^{14}C/ glucose (4 µCi/ml) into the identical medium, but without D-/^{14}C/ glucose. At specified time intervals (30 min, 16, 22, 48, 54 and 120 h) we took aliquot samples of the medium, and investigated, through chromatography (Whatman 1, system S_2), the presence of radioactive oligosaccharides and polysaccharides expected to release into the liquid medium by effect of enzymes present in the cytoplasmic membrane. However, not even after a 90-hour incubation of protoplasts embedded into the nutrient medium, there were no radioactive oligosaccharides and polysaccharides released into the medium. In such a way, we have confirmed that the last are not originated in the cell wall hydrolysis at the cytoplasmic membrane, but that they are transported from the cytoplasm to the cytoplasmic membrane and to the cell wall being formed and through this cell wall, finally, into the liquid medium.

Table 1. Viability of protoplasts (2.$^{10-}$4) according to quantity and rate of use of D-/^{14}C/ glucose on medium MS with saccharose

Fractions / incubation time (hours)	½	24	48	168	192	240
1	0.54	3.59	5.69	7.46	7.11	8.61
2	0.42	1.42	2.05	3.56	2.33	2.40
3	1.31	2.23	3.68	8.67	6.56	7.55
4	0.56	2.33	4.15	7.09	4.59	2.37
5	97.16	90.41	84.40	73.28	79.40	79.05

Table 2. Radioactivity of D-/^{14}C/ glucose (imp/min.) used in separate fractions on MS medium without saccharose expressed in per cents

Incubation time (hours) / fraction	½	24	48	168	192	240
	Amount (%)					
1	5.04	35.80	35.81	42.09	38.30	40.47
2	0.72	7.52	7.82	4.75	8.41	6.40
3	0.88	11.29	17.02	23.93	22.49	24.18
4	1.48	19.28	16.87	12.32	15.31	13.70
5	91.86	26.10	22.46	16.89	15.47	16.23

It has been well recognised that some growth substances, eg IAA and 2.4-D affect the building of polysaccharides in cell walls. For this reason, we first examined the stability of protoplasts in the liquid nutrient medium added with growth substances IAA and 2.4-D (0.1; 1; 5; 20 and 50 mg l^{-1}) in varying concentrations. The protoplasts were not lysed, not even after the 18-hour incubation in the medium supplemented with the growth substances in the just mentioned concentrations. After increasing the concentration values to 10^{-2} M (1,750 mg l^{-1}) and 10^{-3} M (175 mg l^{-1}) IAA, the protoplasts turned brown and started getting lysed.

The influence of growth substances on secretion of radioactive metabolites was studied on protoplasts incubated with 1 and 5 mg l^{-1} IAA, 1 and 5 mg l^{-1} 2.4-D and 1 mg l^{-1} kinetin (20,000 protoplasts per 1 ml of medium containing radioactive D-/^{14}C/ glucose and growth substance in appropriate concentration). At the given time intervals (30 min, 16, 22, 48, 54 and 120 h) we took aliquot samples of the medium. The last were processed through chromatography and detected for saccharides presence with an alkaline $AgNO_3$. The measurement of the material radioactivity revealed that, already 30 minutes after the incubation, the medium contained radioactive oligosaccharides the amount of which was increasing with the incubation time. No important differences, however, were identified in secretion between the studied hydro-carbonic metabolites depending on different concentrations of the used growth substances (Table 3). This effect of growth substances IAA and 2.4-D was suggested by several authors (Steveninck, 1965; Power and Cocking, 1970; Ray, 1973; Tanimoto and Igari, 1976) to explain by changes in protoplasts membrane permeability.

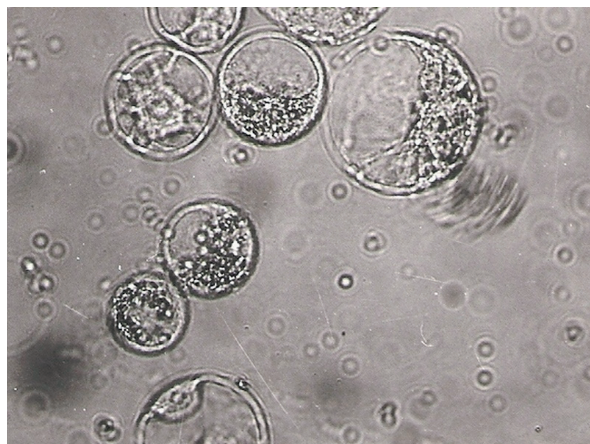
According to Capek et al. (2000), which works ourselves with research of polysaccharid galactoglucomannan from spruce, this polymer is not only a structural constituent of the secondary cell-wall, but lower fragments of this polymer (oligosaccharides) showed biological activity in elongation growth induced by auxin, in some morphogenic processes and in regenerating protoplasts.

Cell wall regeneration of protoplasts proving the viability of the isolated protoplasts was studied through fluorescence phenomenon – the fluorescence stain primulin was added to the medium containing protoplasts. The fresh isolated protoplasts only contained tiny residua of the cell walls (Fig 1a, b). After 24 hours of incubation, the protoplasts surface began to cover with cell wall components on which the stain is fixed (Fig 2a, b). After three days of incubation, we could observe deposition of polysaccharidic material on almost all the protoplasts (Fig 3a, b). Our results are in good accordance with the results obtained by Gómez-Maldonado et al. (2001), who prepared protoplasts by isolation from cotyledons of seedlings of *Pinus pinaster* by staining with fluorescein diacetate.

Table 3. Influence of growth substances on secretion of radioactive metabolites

Fraction / hours		0	15	22	48	54	120
	K	0.21	30.63	46.37	52.72	51.27	42.55
	IAA	0.22	26.24	46.73	56.79	54.36	45.27
1	IAA	0.25	34.27	45.18	45.30	54.26	53.90
	2.4-D	0.18	36.31	46.31	50.90	48.10	42.78
	2.4-D	0.23	17.37	35.78	54.77	57.21	60.30
	K	0.63	10.77	16.84	25.02	27.70	31.08
	IAA	0.58	14.31	21.75	31.64	28.01	34.06
2	IAA	0.61	13.70	21.20	30.46	31.89	32.24
	2.4-D	0.63	16.70	25.53	30.39	32.29	36.83
	2.4-D	0.57	6.79	12.96	24.08	25.28	20.66
	K	–	6.59	9.30	11.55	10.86	10.29
	IAA	–	7.57	10.02	10.43	9.03	9.53
3	IAA	–	7.01	9.40	13.74	15.11	6.92
	2.4-D	–	7.02	9.55	8.89	5.85	9.99
	2.4-D	–	4.59	7.71	9.92	8.28	5.19
	K	99.15	52.02	27.39	10.75	10.16	10.06
	IAA	99.18	44.34	21.47	11.24	8.58	10.23
4	IAA	99.13	45.01	24.23	10.47	8.72	6.86
	2.4-D	99.18	39.96	18.50	10.09	9.74	10.37
	2.4-D	99.18	71.23	43.52	11.22	9.21	5.75

IAA (3-indoleacetic acid) 1 and 5 mg l^{-1}
2.4-D (2,4-dichlorfenoxy acetic acid) 1 and 5 mg l^{-1}
K (kinetin) 1 mg l^{-1}



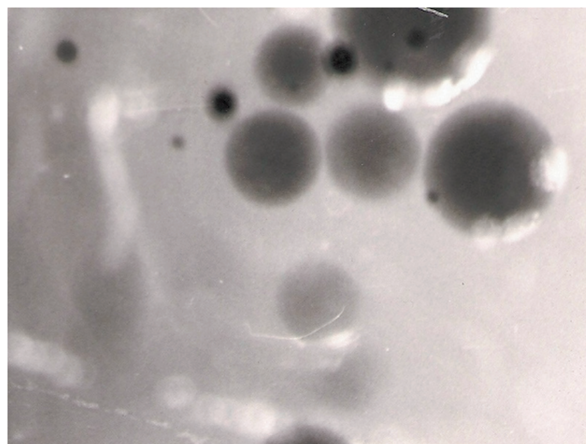
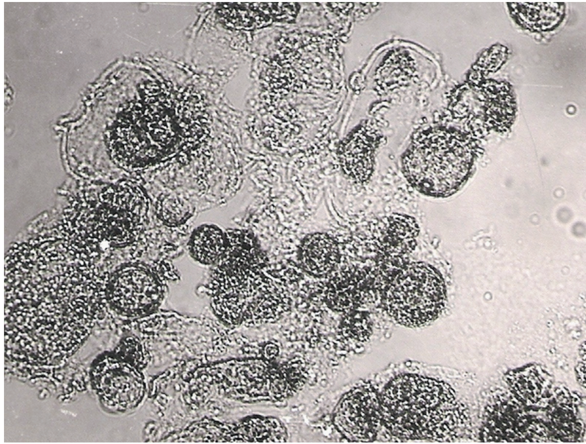


Fig 1a, b . Fresh isolated protoplasts only contained tiny residua of the cell walls
1a (light microscopy), 1b (fluorescence microscopy)



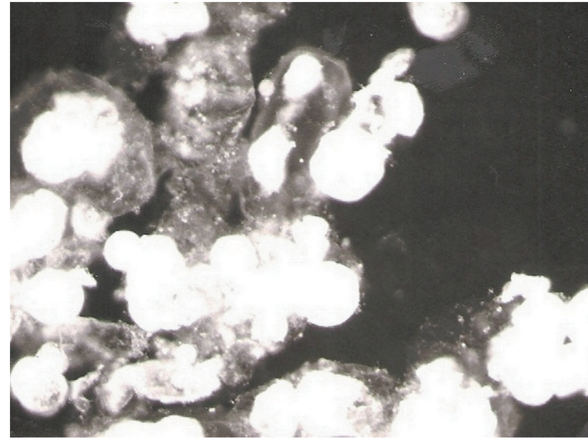
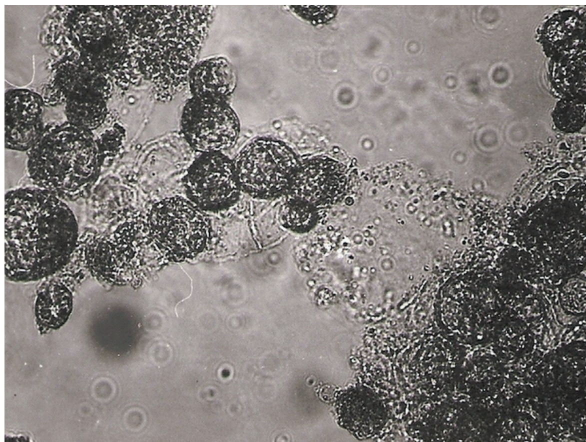


Fig 2a, b. After 24 hours of incubation, the protoplasts surface began to cover with cell wall components on which the stain is fixed, 2a (light microscopy), 2b (fluorescence microscopy)



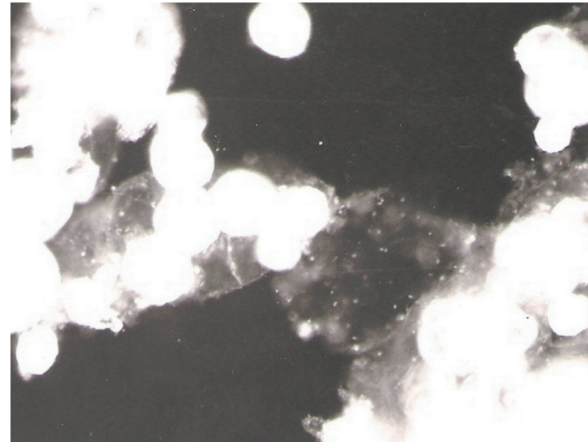


Fig 3a, b. Deposition of polysaccharidic material on almost all the protoplasts after three days of incubation 3a (light microscopy), 3b (fluorescence microscopy)

Conclusions

The method of protoplasts isolation from tissue culture of Norway spruce easily allows replications. Studying stability of protoplasts in medium with admixture of growth substances IAA and 2.4-D (0.1; 1; 5; 20; 50 mg l^{-1}) in varying concentrations, we have found that the protoplasts were not lysed, even after 128 hours of incubation. The concerned concentrations of growth substances had not significant influence on secretion of oligosaccharides and polysaccharides that are considered to be identifying the cell wall regeneration.

The incubation of protoplasts in the medium with admixture of D-/^{14}C/ glucose induced releasing radioactive oligosaccharides and polysaccharides into the medium. The amount of the released material increased over the whole 90-hour incubation period. It has been confirmed that the released saccharides were not a product of hydrolysing processes occurring at the cytoplasmic membrane, but that the first were synthesized cell wall components flushed away from the created cell wall.

The study of protoplasts viability confirmed that the protoplasts used D-/^{14}C/ glucose over the first seven days. In the following days, there occurred changes in percentages of the individual oligosaccharides caused by presence of hydrolytic enzymes in the cultivation medium.

The study of protoplasts regeneration pursued through fluorescence microscopy coupled with light microscopy revealed that there were no cell wall residues on surface of freshly isolated protoplasts. However, already after 24 hours of incubation, the protoplasts surface began to cover with polysaccharidic components of cell walls, fixing fluorescence stain primulin. After three days of incubation, we could observe polysaccharidic material deposition on practically all protoplasts.

Abbreviations

IAA 3-indoleacetic acid
2.4-D 2.4-dichlorfenoxy acetic acid
kinetin 6 furfurylaminopurine
primulin mono and bi-dehydro-p-toluidine sulphonate
MS Murashige and Skoog (1962)
BL Brown and Lawrence (1968)

References

BROWN C.L., LAWRENCE R.H. 1968. Culture of pine callus on a defined medium. *Forest Sci.*, 14: 62–64.

CAPEK P., KUBAČKOVÁ M., ALFÖLDI J., BILISICS L., LIŠKOVÁ D., KÁKONIOVÁ D. 2000. Galactoglucomannan from the secondary cell wall of Picea abies L. Karst. *Carbohydr. Res.,* 329: 635–645.

COCKING E.G. 1960. A method for the isolation of plant protoplasts and vacuoles. *Nature*, 187: 962–963.

DAVID H., DAVID A., MATEILLE T. 1982. Evaluation of parameters affecting the yield, viability and division of Pinus pinaster protoplasts. *Physiol. Plant.*, 56: 108–113.

DUHOUX E. 1980. Protoplast isolation of gymnosperm pollen. *Z. Pfl.-Physiol.*, 99: 207–214.

FRY S.C., ADLINGTON S., HETHERINGTON P.R., AITKEN J. 1993. Oligosaccharides as signals and substrates in the plant cell wall. *Pl. Physiol.*, 103: 1–5.

GÓMEZ-MALDONADO J., CRESPILLO R., ÁVILA C., CÁNOVAS F.M. 2001. Efficient preparation of maritime Pine (Pinus pinaster) protoplasts suitable for transgenic expression analysis. *Pl. molec. Biol. Reporter*, 19: 361–366.

HAKMAN I.C., HARNOLD S., FELLNER-FELDEGG H. 1986. Isolation and DNA analysis of protoplasts from developing female gametophytes of Picea abies (Norway spruce). *Pl. Sci.*, 38: 53–59.

KÁKONIOVÁ D., LABUDOVÁ I. 1987. Callus culture as substrate for the production of specific cell wall degrading enzymes for derived protoplast isolation. *Biotechnol. Lett.,* 9, 10: 721–724.

LANG H., KOHLENBACH H.W. 1989. Cell differentiation in protoplast cultures from embryogenic callus of Abies alba L. *Pl. Cell Rep.*, 8, 2: 120–123.

MALINOWSKI R., FILIPECKI M. 2002. The role of cell wall in plant regeneration. *Cell. molec. Biol. Lett.*, 7: 1137–1151.

MURASHIGE T., SKOOG F. 1962. A revised medium for rapid growth an bioassays with tobacco tissue cultures. *Physiol. Plant.*, 154: 473–497.

POWER J.B., COCKING E.C. 1970. Isolation of leaf protoplasts: Macromolecule uptake and growth substance response. *J. exp. Bot.*, 21: 64–70.

RAY P.M. 1973. Regulation of β-Glucan Synthetase Activity by auxin in Pea stem tissue. I. Kinetic aspects. *Pl. Physiol*., 51:609–614.

STEVENINCK J. VAN 1965. Effect of indolyl-3-acid on the permeability of membranes storage tissue. *Nature (Lond.)*, 205: 13–84.

TANIMOTO E., IGARI M. 1976. Correlation between β-galactooxidase and auxin-induced elongation growth in etiolated pea stems. *Pl. Cell Physiol.*, 17: 673–682.

Regenerácia bunkových stien protoplastov z pletivových kultúr smreka obyčajného (*Picea abies* L.) Karst. v tekutom živnom médiu

Súhrn

Regeneráciu protoplastov pripravených z pletivovej kultúry smreka obyčajného sme sledovali fluorescenčnou mikroskopiou v kombinácii so svetelnou mikroskopiou. Na čerstvo izolovaných protoplastoch nie sú zvyšky bunkových stien. Po 24 hodinovej inkubácii sa na povrch protoplastov ukladali polysacharidické komponenty bunkovej steny, na ktoré sa viaže fluorescenčné farbivo primulín. Po trojdňovej inkubácii sme prakticky na všetkých protoplastoch pozorovali ukladanie polysacharidického materiálu.

Pri práci s izolovanými protoplastami je dôležitá ich stabilita a životnosť. Stabilitu protoplastov sme sledovali v médiu s prídavkom rôznych koncentrácií rastových látok IAA a 2,4-D (0,1; 1; 5; 20; 50 mg l^{-1}). Protoplasty ani po 18-hodinovej inkubácii v tomto prostredí nelyzovali. Použité koncentrácie rastových látok nemali výrazný vplyv na sekréciu oligosacharidov a polysacharidov, ktoré sa považujú za znak tvorby bunkovej steny.

Zistili sme, že protoplasty inkubované v médiu s prídavkom D-/^{14}C/ glukózy secernujú do média rádioaktívne oligosacharidy a polysacharidy, ktorých množstvo sa počas 90-hodinovej inkubácie zvyšovalo. Potvrdili sme, že secernované oligosacharidy nie sú produktom hydrolyzačných dejov prebiehajúcich na cytoplazmatickej membráne, ale že sú to nasyntetizované komponenty bunkovej steny, ktoré sú odplavované od tvoriacej sa bunkovej steny.

Pri sledovaní životnosti protoplastov sme zistili, že protoplasty utilizujú D-/^{14}C/ glukózu počas prvých sedem dní. V nasledujúcich dňoch dochádza k percentuálnym zmenám v zastúpení jednotlivých oligosacharidov spôsobených hydrolytickými enzýmami, ktoré sú prítomné v kultivačnom médiu.

Received June 4, 2008
Accepted August 5, 2008