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Palacký University in Olomouc, Faculty of Science, Department of Botany, Olomouc, Czech Republic

Haploid and mixoploid cucumber (*Cucumis sativus* L.) protoplasts – isolation and fusion

Dagmar Skálová, Božena Navrátilová, Ivana Doležalová, Radim J. Vašut, Aleš Lebeda

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Summary

This paper reports on the isolation of haploid and mixoploid protoplasts in the genus *Cucumis*. The cucumber mixoploid plants (*C. sativus* L., 2x/4x; 2x = 14) were obtained after oryzalin treatments and the mesophyll protoplasts (2x/4x/8x) were isolated and cultivated by well known *in vitro* methods. The influence of oryzalin pretreatment on the average viability and density of protoplasts was tested. The average viability as well as the density is significantly influenced by the oryzalin concentration, whereas the time-span of the treatment doesn't have significant impact on the density and the viability. Callus formation was the highest level of regeneration in the experiments described in our study. Furthermore the isolation and cultivation of the cucumber and muskmelon (*C. melo* L.; 2x = 24) haploid protoplasts from young-stage pollen grains were improved. Subsequently, somatic hybridization between mixoploid cucumber protoplasts and muskmelon mesophyll and callus protoplasts, and kiwano (*C. metuliferus* E. Meyer ex Naudin; 2x = 24) mesophyll protoplasts, by chemical fusion with polyethylene glycol (PEG) 6000 was performed for the first time. Heterofusants were observed and developed into micro colonies. Additionally, the gametosomatic hybridization between mixoploid cucumber protoplasts and pollen muskmelon protoplasts was performed for the first time. Heterofusants and the first cell division were observed, however, the regeneration stopped in this stage. In conclusion, the different ploidy, especially the mixoploid character of isolated protoplasts, has positive influence on protoplasts isolation and the following fusion as represented by a higher regeneration capacity. In addition, both types of protoplasts, haploid and mixoploid, represent a unique systems for biochemical, molecular and genetic experiments. Especially, the haploid protoplasts could be used during *in vitro* fertilization.

Introduction

Many biotechnological methods are being used during cucumber breeding programmes (Gajdová et al., 2004; Skálová et al., 2004). Classical breeding methods of cucumber are intraspecific and interspecific hybridization (Chen and Adelberg, 2000; Skálová et al., 2008a). For example, somatic hybridization by isolation and fusion of protoplasts were reported (Gajdová et al., 2004). They provide the possibility of transferring suitable genes from muskmelon and wild *Cucumis* species to various diseases which have decreased the yield of cucurbit crops worldwide. These unique biotechnological methods and their products can be utilized also during selected biochemical (Navrátilová et al., 2008a) and molecular experiments (Ondřej et al., 2009). Also, the methods themselves and the factors affecting them were studied (Gajdová et al., 2007).

During polyploidization it is possible to isolate mixoploids (chimaeras consisting of diploid and tetraploid tissues). Mixoploidy is characterized by the presence and coexistence of the tissues with different chromosome number in a single cell. This trait is capable of significantly increase the adaptive potential of plants (hybrid production, prevalence in nature promotion – mixoploidy is characteristic of many highly productive commercial cultivars) (Kunakh et al., 2008). Hop (*Humulus lupulus* L.) mixoploid plants were isolated after colchicine treatment of diploid plants and plants displayed higher levels (%) of tetraploid nuclei in root tissue (Koutoulis et al., 2005). Oryzalin pretreatment induces mixoploid plants with lesser toxicity than colchicine (mitotic toxin) (Skálová et al., 2009; Skálová et al., 2010). However, colchicine is still used for chromosome doubling (Singh, 2003). Nilanthi et al. (2009) used high concentration of colchicine (30 - 240 mg/l) and duration of its treatment (7 - 30 days) of using petiole explants of *Echinacea purpurea* L. (2x = 22). The cucumber mixoploid plants (*C. sativus* L., 2x/4x; 2x = 14) and the mesophyll protoplasts isolated from them were used for the first time (Skálová et al., 2010). Embryo-rescue, *in vitro* pollination, and protoplasts isolation were tested. The utilization of *in vitro* mitotic polyploidization and following obtained mixoploid (or tetraploid) plants were recorded in other genera. Tetraploid regenerants were tested in intra- and inter-specific crosses for example in the genus *Solanum* (Greplová et al., 2009).

Haploid protoplasts were isolated from cucumber (*C. sativus*) and muskmelon (*C. melo*) pollen grains (young-stage pollen; mononucleate) for the first time by Skálová et al. (2008b). The mixture of pollen protoplasts and pollen grains were used for improving the isolation and cultivation procedures. Successful isolation of protoplasts was associated with the development of a microspore feeder cell system and the composition of the enzyme solution. By using a suitable cultivation medium and osmotic pressure, successful manipulation of pollen protoplasts was possible. For example, microspore protoplasts of *Brassica napus* L. were isolated and callus was induced by a feeder cell system with embryogenic microspores (Sun et al., 1999). In this case, microspores at late unicellular stage and pollen at early bicellular stage were used, and selected enzymes (cellulase, pectinase, macerozyme, pectolyase) in various combinations and concentrations were tested for protoplast isolation. The various types of co-cultivation of these protoplasts were attempted and modified KM8p medium was used for cultivation. Tanaka et al. (1987) isolated pollen protoplasts from pre-anthesis binucleate pollen (immature pollen grains) of *Lilium longiflorum* L. enzyme solutions containing macerozyme and cellulose were used. For protoplast cultivation, they tested modified White medium. The first production of hybrids between haploid protoplasts (pollen protoplasts and protoplasts from haploid plants) was reported for *Brassica* species (Liu et al., 2007). Trinucleate-stage pollen (mature pollen grains) of *Brassica oleracea* L. was suspended in enzyme solution (Sun et al., 1999). Purified pollen protoplasts were cultured in liquid medium (in this case ½ strength of the medium). Recently, the gametosomatic hybridization between isolated egg cell protoplasts and mesophyll protoplasts was recorded in *Petunia* × *hybrida* E. Vilm. (Sangthong et al., 2009a; Sangthong et al., 2009b). Enzymatic solution contained cellulase and macerozyme supplemented with 2-morpholinoethane-sulfonic acid (MES), mannitol and potassium dextran sulphate.

The main aim of this research was to optimize of the manipulation with mixoploid and pollen (haploid) protoplasts of selected *Cucumis*

spp. For the first time, the influence of polyploidization pretreatment by oryzalin on the cucumber mixoploid protoplasts viability, density and regeneration capacity was examined. Isolation and cultivation procedures for both types of protoplasts have been demonstrated. The composition of enzyme solutions and cultivation media for pollen protoplasts were developed. Previously tested procedure for diploid cucumber protoplast was retested using mixoploid cucumber protoplasts. Finally, somatic (mixoploid with diploid protoplasts) and gametosomatic (haploid (pollen) protoplasts with mixoploid protoplasts) hybridization experiments were done for the first time in cucurbits. The results were compared with the knowledge of the diploid cucurbits protoplasts and with the protoplast of other genera also. Especially, for the haploid (pollen) cucurbits protoplasts and for gametosomatic hybridization unique results were obtained, which have not been published before. Additionally, both types of protoplast culture established represent unique systems for biochemical, molecular and genetical experiments.

Materials and methods

Plant material

The list of accessions of *Cucumis* species used for protoplasts isolation and fusion are given in Tab. 1. The plant material originated from the vegetable germplasm collection of the Research Institute of Crop Production (Prague), Department of Gene Bank, Olomouc, Czech Republic and from the USDA-ARS North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa, USA. Plants were cultivated in a glasshouse (25°C/15°C day/night) of the Department of Botany, Palacký University in Olomouc, Olomouc, Czech Republic.

Tab. 1: Plant material.

Cucumis species	Abbreviation	Accession number
C. sativus (Marketer 430)	CS	CZ 09H390121
C. melo var. Charentais	CM	CZ 09H401116 (PI 385965)
C. melo PMR 45	CMx	CZ 09H400597
C. melo WMR 29	CMx	CZ 09H400598
C. melo PMR 5	CMx	CZ 09H400599
C. metuliferus	CMe	CZ 09H410587 (CGN 2315)

Polyploidization *in vitro*

Embryos of cucumber (cv. Marketer 430) were treated with oryzalin ($C_{12}H_{18}N_4O_6S$) in cultivation medium (1/2 MS medium; Murashige and Skoog, 1962) in Petri dishes. Two concentrations (25 and 45 μM oryzalin solutions) and three times of treatment (8, 24, and 48 h) were used. The seedlings developed from these treated embryos were cultivated on medium OK (Skálová et al., 2008a). The ploidy of regenerated plants (4-6 week-old) was determined by flow cytometry (Skálová et al., 2009; Skálová et al., 2010).

Isolation of mixoploid protoplasts

Mixoploid cucumber plants (2x/4x; obtained after *in vitro* polyploidization) were used for isolating mesophyll protoplasts (2x/4x/8x). Protoplasts were isolated and cultivated according to Navrátilová et al. (2008). Average density, viability and the level of regeneration in protoplasts isolated from mixoploid cucumber mesophyll after oryzalin treatments were observed. 1) CD (cell division; the first and other cell divisions were passed, but no micro calluses were observed); 2) MC (micro calluses; micro calluses were developed, the calluses smaller than 2 mm); 3) C (calluses; the calluses larger than 2mm) and 4) P (plant development; this level was not determined in our experiment). The isolated protoplasts were cultivated in modified LCM1 medium (Navrátilová et al., 2008a) approximately for 2 weeks in the dark and were transferred to the cultivation room (day/night temperatures 22 °C / 18°C and a 16 h day / 8 h night photoperiod, 32 - 36 $\mu molm^{-2}s^{-1}$). Experiments were repeated and the variability in the system was reflected using standard deviation. The effect of the oryzalin concentration and the time-span of the treatment on the average density and average viability were analyzed by factorial ANOVA tests in statistica 9.0 (StatSoft, Inc. 2009). For all statistical tests, results were considered significant at $p < 5.000 \times 10^{-2}$.

Isolation of haploid (pollen) protoplasts

The isolation of mononucleate cucumber and muskmelon pollen grains and pollen protoplasts was based on procedure described by Skálová et al. (2008b, 2009). We optimized suitable medium for long-term cultivation of pollen protoplasts. Media previously tested for protoplasts – i.e., LCM1, LCM2 medium (Gajdová et al., 2007; Navrátilová et al., 2008a); KM medium (Kao and Michayluk, 1975) were used. However, they were not suitable for long-term

Tab. 2: New types of media for muskmelon pollen protoplasts.

Basic medium	**Media for protoplasts cultivation / the composition of media**					
CPW *	**CPW1**	**CPW2**	**CPW3**	**CPW4**	**CPW5**	**CPW6**
Manitol (g/l)	70	0	45.5	45.5	0	70
Sorbitol (g/l)	0	70	45.5	0	45.5	70
CPW *	**CPWa**	**CPWb**	**CPWc**	**CPWd**	**CPWe**	
Manitol (g/l)	70	0	35	35	0	-
Glucose (g/l)	0	70	35	0	35	
CPW *	**CPWA**	**CPWB**	**CPWC**	**CPWD**		
Manitol (g/l)	70	35	35	17.5	-	-
Agarose (g/l)	0	0	30	0		
B5**	**B5-1**	**B5-2**	**B5-3**	**B5-4**		-
Glucose (g/l)	**(B5-1A***)**	**(B5-2A)**	**(B5-3A)**	**(B5-4A)**		
	160	240	400		600	

Explanatory notes: * - CPW medium was supplemented with 1 g/l glycine; **B5 medium was supplemented with 1g/l glycine; 10 g/l sucrose; 1 g 2,4-D; *** - 2% agarose was added to cultivation media B5-1 – B5-4.

cultivation of pollen protoplasts of cucumber and muskmelon. Thus, we modified the other media. The haploid protoplasts require high osmotic pressure, so the high concentration of osmotic reagents were combined and tested in CPW-medium (Divakaran et al., 2008) and B5 medium (Gamborg et al., 1968). We used mannitol, sorbitol, glucose and sucrose in various combinations. The specific composition of media was summarized in Tab. 2. Method of co-cultivation and fusion (by PEG 6000) with mesophyll cucumber protoplasts was examined. Agarose (2%) was tested for the more successful cultivation of haploid protoplasts.

Fusion of cucumber and muskmelon (or kiwano) protoplasts

The isolated cucumber mixoploid (2x/4x/8x) protoplasts were fused with protoplasts, derived from callus, mesophyll and pollen, of muskmelon (*C. melo*) and mesophyll of kiwano (*C. metuliferus*). The rate of fused protoplasts was 2:1 (mixoploid protoplasts : mesophyll, callus or pollen protoplasts). The chemical fusion by PEG 6000 was realised according to Navrátilová et al. (1997); Gajdová et al. (2004), and Greplová et al. (2004). The formation of fused protoplasts (especially heterofusants) was observed and the number of successful fusion was detected. In case of pollen and callus protoplasts, the heterofusants contained either colourless callus or pollen part with green mesophyll part. On the other hand, the detection of heterofusants developed only from mesophyll protoplasts, required the method how to differentiate it. The mesophyll protoplasts of fusion partners for mixoploid cucumber protoplasts were coloured by the neutral red, which was added to cultivation medium. This modified medium was used only for the part of fused protoplasts because of the negative influence of colorant on the viability of fused protoplasts. The level of regeneration of fused protoplast was determined and there was used the same regeneration level as mentioned above for isolated mixoploid protoplasts (CD, MC, C, P).

Results and discussion

The mixoploid (2x/4x) plants were obtained by *in vitro* oryzalin treatment of cucumber diploid (2x) plants. Concentration in cultivation media and the duration of oryzalin treatment did not have influence on the ploidy level of regenerants. The ratio of non-affected to mixoploid to tetraploid regenerants was 0:100:0 (100% plants were mixoploid). Thus, polyploidization pretreatment was successful. Greplová et al. (2009) compared efficiency of polyploidization by colchicine and oryzalin and reported the rate of non-affected to mixoploid to tetraploid regenerants of selected *Solanum* species. Oryzalin was more effective (14:2:1) than colchicine (22:2.5:1) ($P = 0.1$). In another study, colchicine was used for inducing tetraploids from petiole explants in *Echinacea purpurea* L. (Nilanthi et al., 2009). Long cultivation (28 days) in medium supplemented with 120 mg/l colchicine produced 23.5% tetraploids. We did not use colchicine in *in vitro* treatment ever, because of the less toxicity and higher efficiency of oryzalin in *in vivo* polyploidization pretreatment (Skálová et al., 2010).

Mesophyll protoplasts were isolated from mixoploid plants of cucumber (Fig. 1A-B). The ploidy of these protoplasts was checked using flow cytometry. Mixoploidy (2x/4x/8x) was confirmed and protoplasts were isolated and cultivated. The average viability, density and the level of regeneration of the isolated protoplasts were observed (Tab. 3) and shown in Fig. 2 - 5. Different concentration of the oryzalin pretreatment has significant impact on the viability as well as density ($p = 0.807 \times 10^{-2}$). In contrast, the time-span of the oryzalin pretreatment appeared to have not significant influence ($p = 7.126 \times 10^{-2}$). The highest average viability (87 ± 15%) was detected in the 25 µM concentration of oryzalin with 48 h treatment (Fig. 6). The most suitable oryzaline pretreatment for the highest average density was 25 µM concentration of oryzalin with 8 h time ($7.1 \pm 3.8 \times 10^{6}$). These values were higher than those reported by Navrátilová et al. (2011). Generally, the lower concentration of oryzalin (25 µM) showed better results with respect to viability and density. On the other hand, the highest level of regeneration and callus formation was observed for 45 µM oryzalin treatment for 48 h. The successful isolation, which means the isolation recorded cell division or production of micro calluses or calluses, was similar in majority of oryzaline pretreatments (50%). However, the lowest success in regeneration was observed in 45 µM concentration of oryzalin with 24 h time (23%) and the highest regeneration rate was obtained in 25 µM oryzalin for 8 h (60%). The average regeneration capacity after oryzalin pretreatment was 50.2%. Gajdová et al. (2007) observed 25% regeneration ability of diploid cucumber protoplasts. Navrátilová (2004) recorded 20% regeneration of hypocotyl protoplasts of *B. napus* measured as the percentage of protoplasts that was able to divide and create micro calluses and calluses. Generally, the ability of protoplasts to divide may range between 0 - 80% (Navrátilová, 2004). Kaur et al. (2006) observed high frequency of dividing cells in *Brassica* genotypes.

Fig. 1A: The mixoploid cucumber plants (45 µM oryzalin / 8 h)

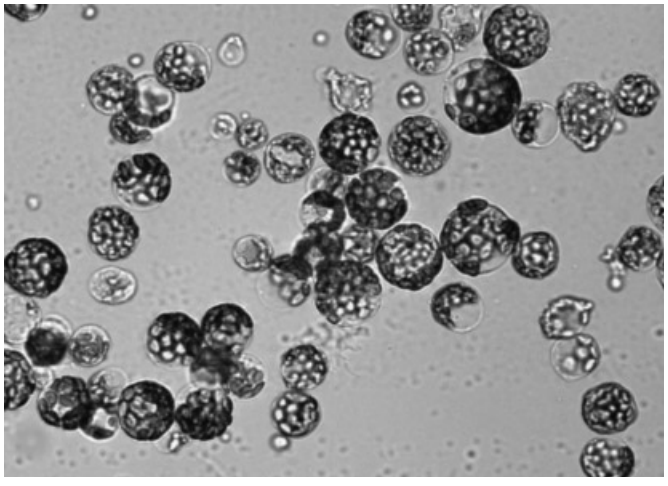

Fig. 1B: The mixoploid cucumber protoplasts (25 oryzalin µM / 48 h)

Tab. 3: The average viability and density, and the type of regeneration of isolated mixoploid protoplasts.

Time of influence/ oryzaline concentration	8h			24h			48h		
	D* (× 10^6)	V* (%)	R**	D* (× 10^6)	V* (%)	R**	D* (× 10^6)	V*	R** (%)
25 µM	7,1±3,8	83±20	0-3 CD-4 MC-5	4,7±2,8	76±24	0-4 CD-5 MC-2	5,1±2,6	87±15	0-6 CD-5 MC-6
45 µM	4,3±2,2	83±12	0-2 CD-4 MC-0	3,6±1,9	74±13	0-8 CD-3 MC-1	3,1±1,2	79±9	0-1 CD-1 MC-5 C-1

***Explanatory notes*:** D – average density of isolated protoplasts, V – average viability of isolated protoplasts; R – type of regeneration (the final level of regeneration) ; CD – cell division in suspension of isolated protoplasts; MC – micro calluses; C – calluses; * - the variability of the data is represented by standard deviation; ** - the number of isolations (the isolations with contamination were not summarized here).

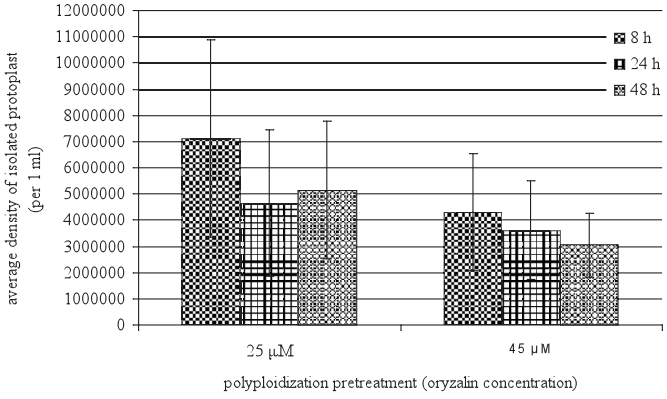

Fig. 2: Influence of polyploidization pretreatment on average density of isolated protoplasts.

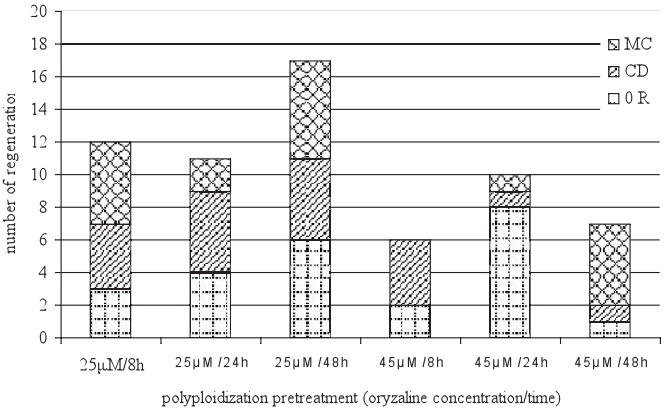

Fig. 4: The type of regeneration of isolated mixoploid protoplasts.

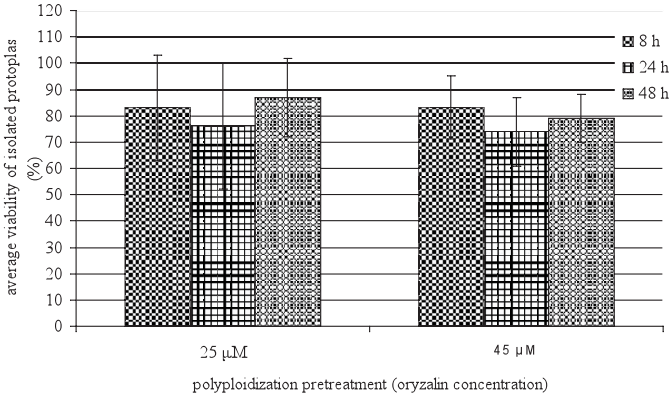

Fig. 3: Influence of polyploidization pretreatment on average viability of isolated protoplasts.

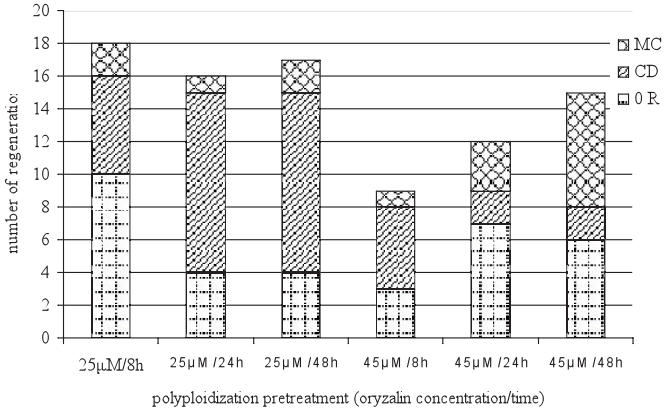

Fig. 5: The type of regeneration after the fusion of isolated mixoploid protoplasts.

The regeneration efficiency was about 80% for *B. oleracea* and 50% for *B. napus*. The highest level of regeneration we performed with mixoploid cucumber protoplasts and it was callus. However, the calluses obtained after cultivation of protoplasts were small (maximum 5 mm) and yellow (maximum light green) (Fig. 7). In cultivation of diploid cucumber protoplasts, NAVRÁTILOVÁ et al., (2007) observed green, soft and big (around 20 mm) calluses. In *Brassica* or *Solanum* species the intact plants were obtained after isolation and cultivation of protoplasts. In *Brassica* species, regeneration frequency of calluses developed from protoplasts was 69 - 75% (*B. oleracea*) and 2 - 3% (*B. napus*) (KAUR et al., 2006). For *Solanum* species, GREPLOVÁ et al. (2008) reported

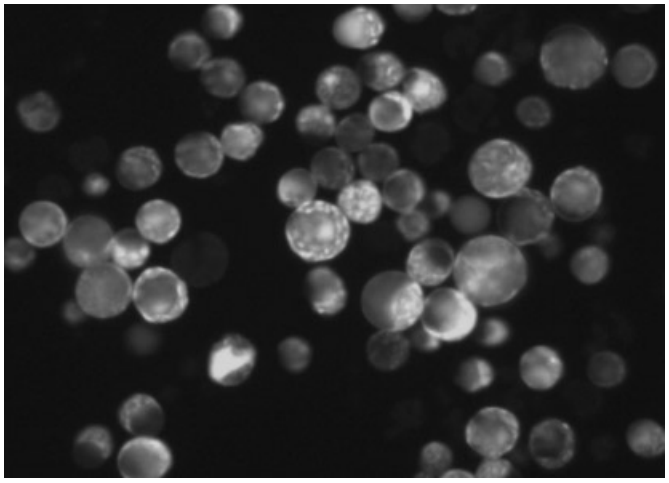

Fig. 6: The viability of mixoploid cucumber protoplasts (25 μM oryzalin / 48 h) (staining by FDA)

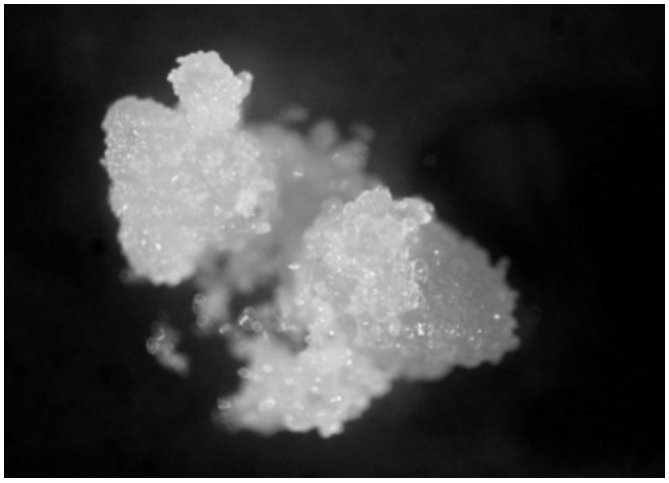

Fig. 7: Growing callus from mixoploid cucumber prototoplasts (45 μM oryzalin / 24 h)

plant regeneration from selected dihaploid *Solanum* genotypes (*S. tuberosum tbr* dh 243; *tbr* dh 299; *tbr* cv. Bintje).

The pollen protoplasts from cucumber and muskmelon were isolated in this study (Fig. 8A-B). Previously tested methods for the isolation procedures were used (SKÁLOVÁ et al., 2008b, 2009). These methods were related to the isolation of mononucleate pollen grains (SKÁLOVÁ et al., 2008b) and the selection of suitable enzyme solutions (SKÁLOVÁ et al., 2009). We used a combination of cellulase (Onozuka R-10), pectinase, and pectolyase Y-23 that was dissolved in CPW medium supplemented with D-mannitol and sorbitol. The average density of isolated cucumber protoplasts per one bud was $3.1 \pm 1.4 \times 10^3$ and for muskmelon protoplasts were $9.1 \pm 3.7 \times 10^3$ (for CM - marks for different muskmelon genotype – Tab. 1) and $8.3 \pm 3.8 \times 10^3$ (for CMx). The influence of muskmelon genotype on the average protoplasts density is summarized in Fig. 9. The balanced enzymatic solution was very important for isolation of sufficient protoplasts from pollen grains. LIU et al. (2007) used the same enzymatic combination for trinucleate *B. oleracea* var. *italica* Plenck. They observed numbers of protoplasts per bud ranging from 0.3×10^4 to 3.9×10^4. ZHAO et al. (2004) used a combination of cellulase, pectinase, and hemicellulase in MS basic salt medium with mannitol for lily (*Lilium longiflorum* L.). The culture density of lily pollen protoplasts was $1.5 - 3.0 \times 10^4$. To isolate lily pollen protoplasts, TANAKA et al. (1987) combined cellulase (Onozuka R-10) and macerozyme R-10 in White's modified medium with sucrose and potassium dextran sulphate. Hardly any protoplasts were isolated from mononucleate pollen grains (immature pollen grains). The same combination of enzymes was used for post-anthesis binucleate pollen grains (mature pollen grains) of *N. tabacum* (DESPREZ et al., 1995). The other important step for the manipulation of pollen protoplasts was to find a suitable medium for long-term cultivation. The isolated cucumber and muskmelon protoplasts cultivated together with pollens were very susceptible to the osmotic pressure of the medium. New types of media were tested in this study (Tab. 2) and Tab. 4 summarized the results. We observed that protoplast survived to the second day of cultivation in modified B5 medium only. The best variation of modified B5 medium was the variant B5 - 4. This medium had the highest level of osmotic potential (60% glucose in medium). The muskmelon pollen protoplasts survived on this medium for a long period; the last control was done three month after the isolation and they appeared without deformation and elongated (Fig. 10). Unfortunately, cell division was not observed. A similar result (elongation of protoplasts during the long-term cultivation of pollen protoplasts with one case of cell division) was observed in *Brassica* genotypes (NAVRÁTILOVÁ et al.,

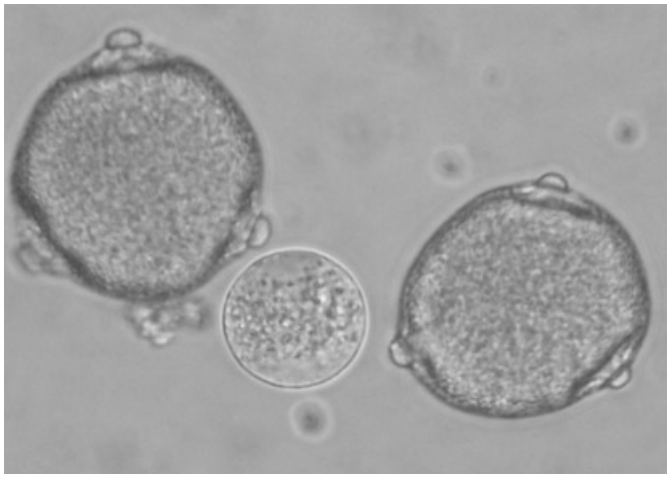

Fig. 8A: Cucumber pollen protoplasts

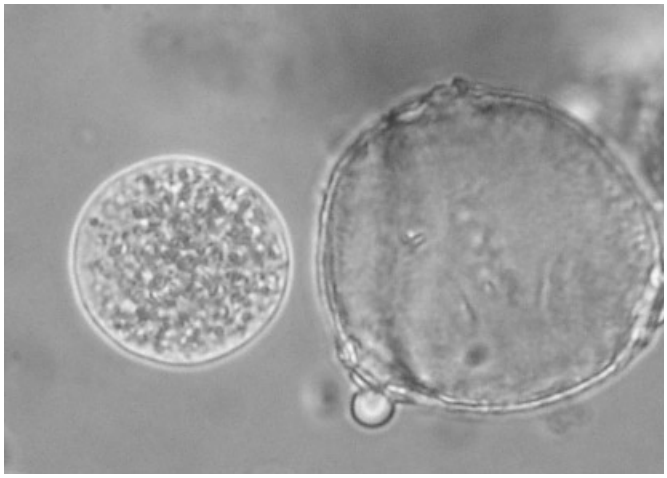

Fig. 8B: Muskmelon pollen protoplasts

2008b). ZHAO et al. (2004) used MS medium with sucrose, 2,4-D, and Km8p mixed organic acids and mixed sugars for cultivation of lily (*L. longiflorum*) protoplasts. Lily was also cultivated on Whites medium supplemented with yeast extract and sucrose (TANAKA et al., 1987).

The isolated pollen protoplast were usually cultivated (co-cultivated) or fused with other protoplasts. LIU et al. (2007) observed viable and green calluses after the fusion of haploid pollen protoplasts of *B. oleracea* with haploid mesophyll protoplasts of *B. rapa* in PEG. The successful regeneration of calluses, i.e. the presence of shoot primordia, ranged from 16.7 - 100.0%. DESPREZ et al. (1995) discussed the PEG fusion of tobacco (*N. tabacum*) pollen protoplasts with mesophyll protoplasts. The transfer of chloroplasts into the pollen protoplasts was evident after one day

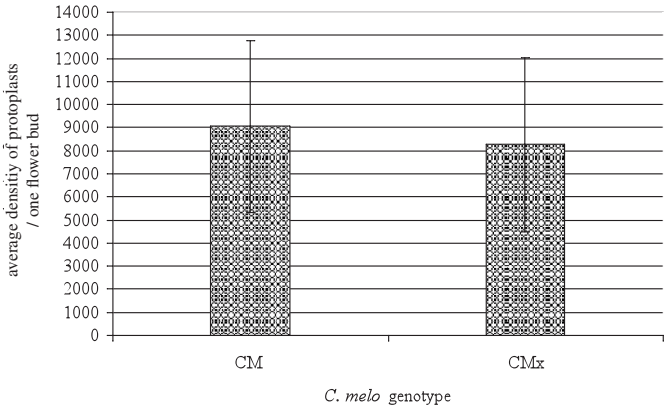

Fig. 9: Influence of muskmelon genotype on the average density of pollen protoplasts.

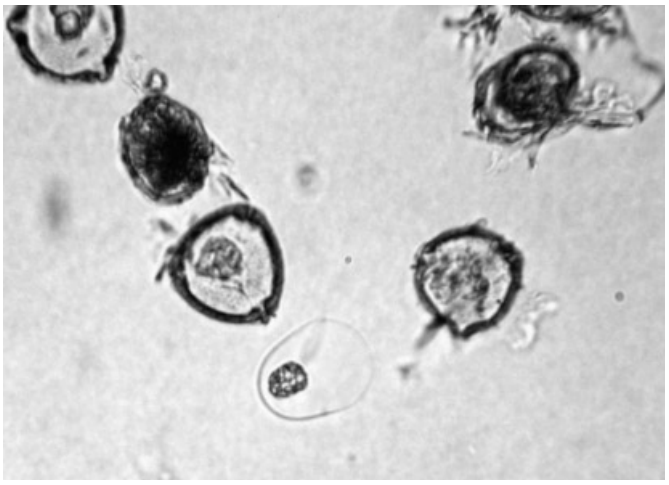

Fig. 10: The elongation of muskmelon pollen protoplasts

Tab. 4: The results of cultivation haploid (pollen) cucumber and muskmelon protoplasts on B5 modified medium.

Medium / No. of cultivation	1.	2.	3.	4.
B5 1	CM + CMx[1]	CM + CMx	CM + CMx	CM + CMx
	P (deformed)[2] *	0	P (deformed)	P (deformed)
	0[3]	0	0	P (deformed)
B5 1A	CS	-	-	-
	P (deformed)			
	0			
B5 2	CS	CM + CMx	CM + CMx	CM + CMx
	P (deformed)	P (deformed)	P (deformed)	P (deformed)
	0	0	0	P (differentiation)**
B5 2A	CS	CS	CM + CMx	CM + CMx
	0	P (deformed)	P (deformed)	P (deformed)
	0	0	0	P (deformed)
B5 3	CM + CMx	CM + CMx	CM + CMx	CM + CMx
	P (deformed)	P (deformed)	P (deformed)	P (deformed)
	0	P (differentiation)	P (differentiation)	P (deformed)
B5 3A	CM + CMx	CM + CMx	CM + CMx	-
	P (deformed)	P (deformed)	P (deformed)	
	0	0	P (differentiation)	
B5 4	CM + CMx	CM + CMx	-	-
	P	P		
	P (differentiation)	P (differentiation)		
B5 4A	CM + CMx	-	-	-
	P			
	P (differentiation)			

Explanatory notes: 1 – the genotype used for the isolation of pollen protoplasts; 2 – the checking of presence of protoplasts the second day after the cultivation on media (there were occurred three possibilities: 0 – no protoplasts; P (deformed) – deformed protoplasts; P – protoplasts without deformation; 3 – the checking the protoplasts after one week to three months on cultivation media (there were occurred three possibilities: 0 – no protoplasts; P (deformed) – deformed protoplasts without changes; P (differentiation) – normal protoplasts after some changes – starting the differentiation by the extended the protoplasts, but without cell division).

of culture. In our experiments with haploid protoplasts, we tried to co-cultivate cucumber and muskmelon pollen protoplasts with mesophyll cucumber protoplasts. In the second step, the fusion by PEG was tested. These experiments did not show positive results. We suggested, that it was due to the different osmotic pressure in cultivation media, which were used for pollen and for mesophyll protoplasts (the medium for pollen protoplasts required higher osmotic pressure, than the medium for mesophyll protoplasts). The mesophyll protoplasts did not function as the nurse-cells, because of the misbalance of the osmotic pressure in mixed cultivation medium. In next experiments, the pollen protoplasts (muskmelon) were tested also in the PEG fusion with cucumber mixoploid mesophyll protoplasts (Tab. 5). In this case, the successful fusions were observed and the first division was noted in the mixture of pollen and mesophyll protoplasts in 45% of the successfully fused cells. Micro colonies were observed only in one case (fusion with 45 µM / 8 h mixoploid cucumber plants). Nevertheless, the fusion of mixoploid protoplasts with other protoplasts (mesophyll or callus of mentioned *Cucumis* spp.) showed better results. Micro colonies or micro calluses were observed (19% of successfully fused cells) (Fig. 11). The best results were observed during the fusion with mesophyll protoplasts of *C. metuliferus* (especially the 25 µM / 24 h and 48 h). Regarding somatic fusion in other cases, they usually showed more successful results, especially a higher level of regeneration, than our mixoploid cucumber or haploid muskmelon protoplasts. NAVRÁTILOVÁ et al. (2006) reported that only the micro calluses and calluses were obtained after the electrofusion of protoplasts in *Brassica*, *Solanum* and *Cucumis* genera (for example, electrofusion between *C. sativus* + *C. melo*). The same results (gain of calluses) were obtained after chemical fusion between *C. sativus* + *C. melo* and *C. metuliferus* (GREPLOVÁ et al., 2004). On the contrary, the intact plants were usually observed after the somatic fusion of the genus *Brassica* (NAVRÁTILOVÁ, 2004; BERÁNEK et al., 2007) and *Solanum* (GREPLOVÁ et al., 2008).

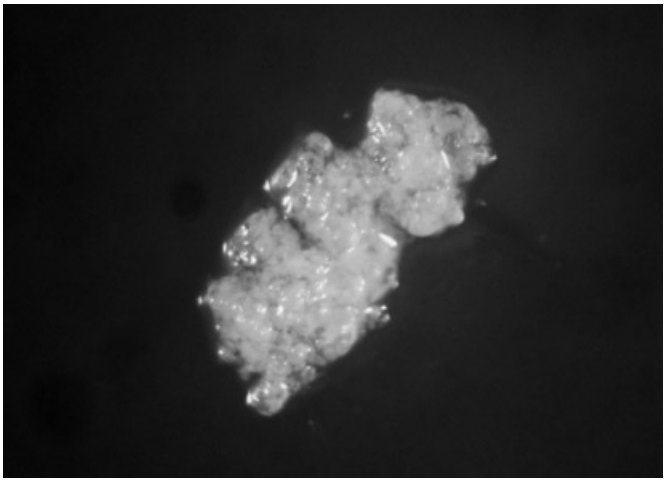

Fig. 11: The micro colonies (microcalluses) of muskmelon pollen protoplasts

Conclusion

Isolation, cultivation and fusion of haploid (pollen) and mixoploid protoplasts of *Cucumis* spp. were successful in this study. We have developed new isolation procedures and compositions of cultivation media. Micro calluses were obtained during cultivation and fusion experiments with mixoploid cucumber protoplasts. Only the elongation of isolated protoplasts was observed during the long-term cultivation of haploid muskmelon protoplasts. As mentioned above, these unique protoplasts systems could be utilize in other experiments (in another fusion experiments for obtain putative hybrid calluses or plants; in biochemical, genetic or molecular experiments).

Tab. 5: The summarization of proceeded fusion between mixoploid *C. sativus* protoplasts and the selected fusion partners.

Type of fusion partners / type of mixoploid cucumber plants		***C. melo*** **(CM)** (callus)	***C. melo*** **(CM)** (mesophyll)	***C. melo*** **(CMx)** (pollen)	***C. metuliferus*** **(CMe)** (mesophyll)
25 µM / 8h	F (SF)	-	-	5 (5)	14 (13)
	CD	-	-	2	6
	MC	-	-	0	2
25 µM / 24 h	F (SF)	-	-	5 (5)	12 (11)
	CD	-	-	2	9
	MC	-	-	0	1
25 µM / 48 h	F (SF)	-	-	4 (4)	14 (13)
	CD	-	-	2	9
	MC	-	-	0	2
45 µM / 8 h	F (SF)	-	-	4 (4)	5 (5)
	CD	-	-	2	3
	MC	-	-	1	0
45 µM / 24 h	F (SF)	6 (6)	4 (2)	-	7 (4)
	CD	1	0	-	1
	MC	2	0	-	1
45 µM / 48 h	F (SF)	9 (7)	4 (3)	-	6 (5)
	CD	1	0	-	1
	MC	4	0	-	3

Explanatory notes: F – the number of all proceeded fusion; SF – the number of successful proceeded fusion (the formation of fused protoplasts – heterofusants); CD – cell division; MC – microcalluses; - – not proceeded fusion.

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Address of the authors:
RNDr. Dagmar Skálová, Ph.D.*; RNDr. Božena Navrátilová, Ph.D.; RNDr. Ivana Doležalová, Ph.D.; RNDr. Radim J. Vašut, Ph.D.; Prof. Ing. Aleš Lebeda, DrSc.; Department of Botany, Faculty of Science, Palacký University in Olomouc, Šlechtitelů 11, 783 71 Olomouc – Holice, Czech Republic.
*corresponding author (E-mail: dagmar.skalova@upol.cz;
Tel.: +420585634811; Fax: +420585634824)