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Factors affecting protoplast isolation and cultivation of *Cucumis* spp.

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Summary

Protoplasts of *Cucumis anguria*, *Cucumis melo* (3 accessions), *Cucumis metuliferus* and *Cucumis sativus* were isolated from leaves, growing apices, hypocotyls and calluses. Plants were cultured on 2 concentrations of sucrose. The effect of plant culture medium, explant age and explant type on protoplast viability were investigated. The protoplasts were cultured in 3 types of culture medium and at two temperatures. Optimal age range for protoplast isolation was 1-5 weeks depending on explant type and genotype. Viabilities ranging between 50 % and 80 % were obtained from all explants and genotypes. Increased concentration of sucrose had negative impact on viability of protoplasts. The highest level of regeneration achieved was callus, regenerated from leaf protoplasts of *C. melo* cv. 'Charentais' and *C. melo* 'MR-1'. The lowest regeneration capability was observed in hypocotyls. Liquid LCM medium (B5 macro and microelements ($1\ g \cdot l^{-1}\ CaCl_2$), B5 vitamins with $1 g \cdot l^{-1}$ myo-inositol, $2\ mg \cdot l^{-1}$ ascorbic acid, $0.8\ mg \cdot l^{-1}$ glycine, $20\ mg \cdot l^{-1}$ glutamine, $100\ mg \cdot l^{-1}$ casein hydrolysate, $70\ g \cdot l^{-1}$ mannitol, $10\ g \cdot l^{-1}$ sucrose, $5\ g \cdot l^{-1}$ glucose, $585\ mg \cdot l^{-1}$ MES, 5.37 $\mu mol \cdot l^{-1}$ NAA, 2.26 $\mu mol \cdot l^{-1}$ 2,4-D, 2.22 $\mu mol \cdot l^{-1}$ BA) was optimal for protoplast regeneration. Agarose-solidified medium caused a decrease in the number of cell divisions (used in *C. melo* 'PI 124112'). Cultivation at 25ºC resulted in a higher frequency of cell divisions (tested in *C. metuliferus*).

Abbreviations

2,4-D – 2,4-dichlorphenoxyacetic acid; BA – benzylaminopurine; IBA – indole-3-butyric acid; IPA – indolylpropionic acid; MES – 2-N-morfolin-etansulfonic acid; NAA – α-naphthaleneacetic acid

Introduction

Cucurbit downy mildew (*Pseudoperonospora cubensis*) and cucurbit powdery mildews (*Podosphaera xanthii, Golovinomyces cichoracearum*) are responsible for significant loss of cucurbit crops worldwide annually (Jahn et al., 2002; Lebeda and Widrlechner, 2003). There are different methods used to control outbreaks of such diseases but these are all chemical based treatments which are not only sometimes inefficient but also have significant economic and environmental costs (Hollomon and Wheeler, 2002; Lebeda and Widrlechner, 2003; McGrath, 2001; Urban and Lebeda, 2006). Classical methods of cucumber breeding like intraspecific and interspecific hybridisation have not been reported to confer viable resistance (Chen and Adelberg, 2000; Lebeda et al., 2007). Recent studies have shown that by using protoplast fusion hybrid plants may be obtained, however these did not produce viable seeds (Gajdová et al., 2004). In order to address the problems with somatic hybridisation we continued on work with previously reported accessions of wild and cultivated species of *Cucumis* (Gajdová et al., 2007).

Various protocols were developed and some successfully used in protoplast cultures of Cucurbitaceae. Many of them led to regeneration of normal plants able to form roots in soil and produce fruits (Gajdová et al., 2004). Despite this progress, fusion experiments are still problematic, because even when regeneration of hybrid plants was achieved, the genes of one of the parents may gradually become silenced (Yamaguchi and Shiga, 1993).

Recently several studies have reported on factors influencing protoplast yield, viability and regeneration capability, such as genotype, explant type, explant pre-treatment, presence of glycine in the enzyme solution, and culture medium composition (e.g. growth regulator concentration) (Fellner and Lebeda, 1998; Sutiojono et al., 1998, 2002). McCarthy et al. (2001b) studied the effect of light on protoplast division, alongside temperature and agarose effects. They also demonstrated the influence protoplast density has on division rates and microcallus production (McCarthy et al., 2001b). Despite improving the culture technique and increasing of the culture efficiency of protoplasts, the regeneration of plants was not always achieved (Gajdová et al., 2004; McCarthy et al., 2001a, b; Sutiojono et al., 1998, 2002).

To improve the protocol for protoplast isolation and cultivation it is first necessary to increase the protoplast viability (the percentage of protoplasts surviving the isolation and purification procedure). Second is to increase the regeneration efficiency (the percentage of protoplasts regenerating into dividing cells and subsequently into calluses or plants).

The aim of this investigation was: a) to contribute towards a better understanding of regeneration from protoplasts which could then be used in subsequent somatic hybridisation; b) to find out optimal plant or callus age, plant culture media, donor plant organs, temperature and composition of culture media for cultivation and regeneration of plants.

Material and methods

Plant material and explants

Cucumis spp. genotypes that in previous experiments indicated good regenerative capabilities (calluses obtained) (Gajdová et al., 2007) were used for this study. The following four accessions were selected for good regenerative capabilities: *Cucumis melo*[a] '600' (MR-1, CZ 09-H40-0600), *Cucumis melo*[a] '16' (cv. Charentais, CZ 09-H40-1116), *Cucumis metuliferus*[a] (CZ 09-H41-0587), *Cucumis sativus*[a] line SM 6514 (CZ 09-H39-0768). Both *Cucumis anguria* var. *anguria*[b] (Ames 23536) and *Cucumis melo*[b] '12' (PI 124112) were selected because they have increased yield and viability of protoplasts. The plant material originated from the vegetable germplasm collection of the Research Institute of Crop Production (Prague) [a], Department of Gene Bank (Olomouc, Czech Republic), and Regional Plant Introduction Station[b] (Ames, Iowa, USA).

Seeds were surface sterilised with 8 % Chloramin B for 30 minutes and rinsed 3 times with sterile distilled water. The seat coat was excised and they were germinated on half strength MS (Murashige and Skoog, 1962) medium in Petri dishes (60 mm) in the dark at 25°C. After germination, hypocotyls were detached and seedlings planted on OK medium (MS medium supplemented with $20\ g \cdot l^{-1}$ sucrose,

20 mg·l^{-1} ascorbic acid, 0.8 % agar, 0.049 µmol·1^{-1} IBA and 0.044 µmol·1^{-1} BA) in plastic boxes (volume 800 ml) or Erlenmeyer's flasks (100 ml). Plants were cultivated in culture room with a 16 hour day (light intensity 32 - 36 µmol · m^{-2} · s^{-1}) and the temperature at 22 ± 2°C. They were subcultured every 3 weeks. Hypocotyls were used either for protoplast isolation or for callus derivation. Leaves and growing apices served as sources of protoplasts. Calluses were derived from hypocotyls of *in vitro* plants and in the case of *C. melo* '12' and *C. sativus* from leaves. Leaves for callus derivation were cut into quarters, hypocotyls were cut in to several pieces and then halved and placed on MSC medium (MS with 30 g·l^{-1} sucrose, 13.43 µmol·l^{-1} NAA, 4.4 µmol·l^{-1} BA and 0.8 % agar) to induce callus growth and growing calluses were subcultured every 2 weeks. Calluses were cultivated in the dark at 25°C.

Protoplasts isolation and culture

Protoplast isolation enzyme solutions for leaves, growing apices and hypocotyls contained 1% (w/v) Cellulase Onozuka R-10 (Duchefa) and 0.25 % (w/v) Macerozyme R-10 (Duchefa) dissolved in PGly washing solution (DEBEAUJON and BRANCHARD, 1992; composition: 27.2 mg·l $^{-1}$ KH_2PO_4, 101 mg·l^{-1} KNO_3, 1117.6 mg·l^{-1} $CaCl_2$, 246 mg·l^{-1} $MgSO_4 \cdot 7H_2O$, 0.16 mg·l^{-1} KI, 0.025 mg·l^{-1} $CuSO_4 \cdot 5\,H_2O$, 11.5 g·l^{-1} glycine, 18.016 g·l^{-1} glucose, 0.58572 g·l^{-1} MES and 65.58 g·l^{-1} mannitol). Enzyme solution containing 2% Cellulase Onozuka R-10, 1% Macerozyme R-10 and 0.3 % Driselase (Fluka) was used for callus protoplast isolation. Hypocotyls, growing apices and the youngest fully developed leaves were cut into fine strips or pieces and incubated in the enzyme solution for 16-17 hours in the dark at 25°C (approximately 2 ml of enzyme solution for 100-200 mg of tissue). Approximately 5 ml enzyme solution was used for 1 g of callus. Calluses were cut into pieces and placed on a shaker (80 rpm) for 30 minutes, then in an incubator (25°C, dark) for 17 hours and finally on the shaker for 30 minutes again. The protoplast suspension was filtered through nylon mesh (72 µm), mixed with PGly solution (approximately 5 ml) and centrifuged at 100 × *g* for 5 minutes. After pouring off the supernatant the pellet was resuspended in 20 % (w/v) sucrose (4 ml) and overlaid with the PGly washing solution (2 ml) ensuring they did not mix. They were centrifuged at 100 × *g* for 10 minutes, protoplasts were isolated from the layer between sucrose and PGly using Pasteur pipette. Protoplasts were mixed with approximately 3 ml of PGly solution and centrifuged at 100 × *g* for 5 minutes.

Viability of protoplasts was established after purification using an Olympus fluorescent microscope 'BX60' and fluorescein diacetate stain (LARKIN, 1976) and a BW filter. Results were calculated by using the percentage of the protoplasts which were living; ten readings were made for each sample. Protoplasts were cultured in modified liquid LCM medium (DEBEAUJON and BRANCHARD, 1992) containing: B5 macro and microelements (1 g·l^{-1} $CaCl_2$), B5 vitamins with 1g·l^{-1} myo-inositol, 2 mg·l^{-1} ascorbic acid, 0.8 mg·l^{-1} glycine, 20 mg·l^{-1} glutamine, 100 mg·l^{-1} casein hydrolysate, 70 g·l^{-1} (0.38 mol·l^{-1}) mannitol, 10 g·l^{-1} sucrose, 5 g·l^{-1} glucose, 585 mg·l^{-1} MES, 5.37 µmol·l^{-1} NAA, 2.26 µmol·l^{-1} 2,4-D, 2.22 µmol·l^{-1} BA, in Petri dishes (diameter 40mm), approximately 1.5 ml of medium per dish, at density 10^5 protoplasts·ml^{-1}. Protoplast cultures were placed in the dark at 25°C for 14 days and then transferred into a culture room with 16/8 hours day/night cycles with light intensity 32-36 µmol · m^{-2} · s^{-1} at 22±2°C. At this time LCM2 medium containing 3.3 µmol·l^{-1} BA and no mannitol (DEBEAUJON and BRANCHARD, 1992) was added to dishes (approximately 1 ml) and protoplasts from each dish were subdivided between two dishes (using a pipette). Growing microcalluses (after 4 weeks of culture) were transferred onto a solid F medium (MS macro and microelements, B5 vitamins, 10g·l^{-1} sucrose, 0.537 µmol·l^{-1} NAA, 2.2 µmol·l^{-1} BA, 0.8% agar) (PELLETIER et al., 1983) and the resultant calluses were subcultured after 2-3 weeks onto a fresh medium.

Effect of plant culture media on viability of isolated protoplasts

Plants were cultured on OK medium with 30 g·l^{-1} of sucrose. Leaves and growing apices were detached between 15-63 days. Protoplast isolation was carried out according to the protocol in the previous section. Protoplasts were cultured in liquid LCM medium. The viability of isolated protoplasts was compared to those derived from plants grown in standard OK medium.

Optimal age of plants, seedlings and calluses for protoplast isolation

Leaves were detached from plants between 8-107 days old, growing apices from 7-56 days old, hypocotyls from seedlings 4-17 days after sowing and calluses were used for protoplast isolation 14-96 days after starting their cultivation. The viabilities of isolated protoplasts were used as criteria to find the optimal age of *in vitro* materials for protoplast isolation. The yield of protoplasts was usually sufficient if the viability was high.

Regenerative capability of protoplasts isolated from different types of explants

The level of regeneration from protoplasts was investigated for all tissue types. Levels of regeneration were categorised as the following: cell division (<0.2 mm), microcallus (colonies of cells 0.2-2 mm which were visible by the eye) and callus (>2 mm in diameter). Results were recorded by the percentage of samples showing each level (category) of regeneration.

Protoplast culture technique

Cucumis melo '12' leaf protoplasts were cultivated both in filter sterilised liquid medium and in solidified (0.6% agarose) LCM medium. The number of divisions observed after 14 days represented the regeneration efficiency when expressed as a percentage of total sample number. In *Cucumis anguria* modified CML medium (without edamin) (COLIJN-HOOYMANS et al., 1988) was compared to LCM. CML medium contained MS macro and micro elements, 25 µmol·l^{-1} NAA and 15 µmol·l^{-1} IPA, 2 g·l^{-1} sucrose, 0.25 mol·l^{-1} mannitol. *Cucumis metuliferus* leaf protoplasts were used to compare effect of culture temperature during first 14 days of cultivation. Temperatures of 25°C and 27°C were compared. Due to constraints of time it was not possible to carry replicates of each variable with each species within this study, future work will focus on the most responsive candidates.

Results and discussion

Effect of plant culture media on viability of isolated protoplasts

It is evident that using of the culture medium with a higher concentration of sucrose (30 g·l^{-1}) resulted in decreased protoplast viability (Fig. 1). Protoplasts were obtained in normal yields, and being normal in size and shape. They were however, not able to survive digestion and purification procedure. In *C. melo* '16', *C. metuliferus* and *C. sativus* a big decrease in viability was recorded, particularly in leaf protoplasts. However, in *C. melo* '600' an increase of viability was seen in protoplasts from growing apices. This suggests that concentration of sugar, namely sucrose in culture medium can have strong effect on subsequent isolation of protoplasts from *Cucumis* tissue dependent on genotype.

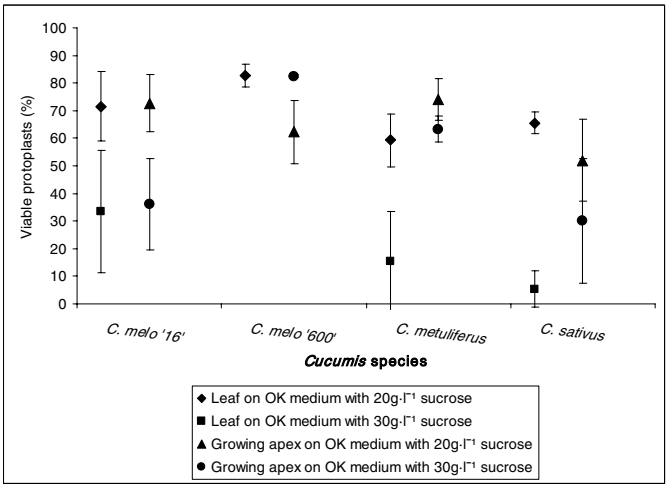

Fig. 1: Effect of sucrose concentration on protoplast viability. Data points comprised of a minimum of 16 individual explants. Y-error bars represent standard deviation.

There are reports about positive effect of a high concentration of sucrose (85.6 $g \cdot l^{-1}$) on somatic embryogenesis in cotyledon tissue (LOU and KAKO, 1995) but no studies on sucrose concentration in donor plant culture medium have been reported. MORENO et al. (1984) have reported increasing protoplast divisions when donor plants were cultivated on medium with yeast extract, but this medium also caused strong decrease of protoplast yield. This suggests that composition of culture medium can greatly influence protoplast physiological properties.

Optimal age of plants, seedlings and calluses for protoplast isolation

The optimal age for protoplast isolation varies both among genotypes and explants (Tab. 1). For leaves, the highest viabilities were obtained from 3-5 weeks old plants (2-3 weeks after subculture), but in some genotypes the suitable age was up to 10 weeks. Plants of *C. anguria* and *C. metuliferus* grew rapidly and exhibited increased vitality *in vitro* for prolonged intervals (up to several months) but the optimal age for *C. metuliferus* was only 3-5 weeks. Flowers of the *C. sativus* started budding after 4 weeks and plant growth decreased rapidly thereafter. Plants of *C. melo* grew slowly from the beginning with poor rooting and leaves were rather tough in texture. Despite this, these plants provided good results. COLIJN-HOOYMANS et al. (1988) and DEBEAUJON and BRANCHARD (1992) reported plant regeneration from leaf protoplasts isolated from 3 week old plants of *C. melo* and *C. sativus*. Growing apices generated good protoplast viability from 1-8 weeks, depending on genotype. Soft and fine grained calluses only were suitable for protoplast isolation, optimally up to 5 weeks for most genotypes, 7-14 days after last subculture. BURZA and MALEPSZY (1995) reported plant regeneration from callus protoplasts used 7-10 days after subculture, and after 4-8 subcultures. Hypocotyls need to be prepared within 8 days after sowing seeds. This is in line with published data from *C. melo* 'Green Delica' and 'Fastoso' (SUTIOJONO et al., 2002) where it was reported that hypocotyls older than 8 days had lignified cell walls which prevented successful isolation of protoplasts.

Effect of donor explant variation on protoplast viability

Fig. 2 shows viabilities of protoplasts which were isolated within their optimal explant age ranges for respective genotypes. Differences among species and varieties and especially among explant types within one genotype are visible in the graph. In *C. melo* '12' and *C. melo* '600' leaf protoplasts were the most viable. All explant types of *C. melo* '16' were suitable for protoplast isolation, in *C. metuliferus* growing apex and callus gave the best results using our isolation protocol. In *C. sativus* callus protoplasts were the most viable. Different species have different requirements for cultivation and isolation, for example in *C. melo* '600' better results were achieved for growing apices by growing plants on OK medium with higher concentration of sucrose (Fig. 1). To also increase the protoplasts viability in other explant types it is necessary to optimise the culture protocols and subsequent isolation techniques for each individual genotype. For example hypocotyls of *C. metuliferus* were much softer than in other species so they may need a lower concentration of digesting enzymes or cutting into bigger pieces then other studied species. The isolation technique has been developed and optimised for *C. melo* which is why its protoplast viabilities are increased over that of the other species. The yield also differed among explant types being approximately ten times higher in leaves and growing apices than in calluses. Hypocotyls gave lower yields than leaves and growing apices (unpublished data). Other authors have reported differences among *Cucumis* species and tissue types as well (GAJDOVÁ et al., 2007; MCCARTHY et al., 2001b; SUTIOJONO et al., 2002).

Tab. 1: Optimal age of plants, cultivated calluses and seedlings used for protoplast isolation

	Leaf (days after planting)		Growing apex (days after planting)		Callus (days of cultivation)		Hypocotyls (days after sowing)	
Genotype	Tested Range	Optimal	Tested Range	Optimal	Tested Range	Optimal	Tested Range	Optimal
C. anguria	15-74 (22)	21-74	—	—	—	—	—	—
C. melo '12'	12-40 (51)	20-27	—	—	17-96 (22)	33-34	—	—
C. melo '16'	20-35 (4)	20-35	7-35 (7)	7-22	14-94 (10)	14-28	—	—
C. melo '600'	8-107 (18)	30-64	8-34 (3)	8-34	18-42 (13)	35-42	4-15 (4)	4-8
C. metuliferus	22-91 (8)	22-35	—	—	18-42 (12)	32-35	—	—
C. sativus	11-75 (16)	22-34	11-39 (6)	11-39	—	—	6-17 (5)	6-8

The sample number is represented in brackets, each sample comprises of 2-4 explants for leaves, 8 explants for growing apices, 4 dishes for callus and 10 explants for hypocotyls.

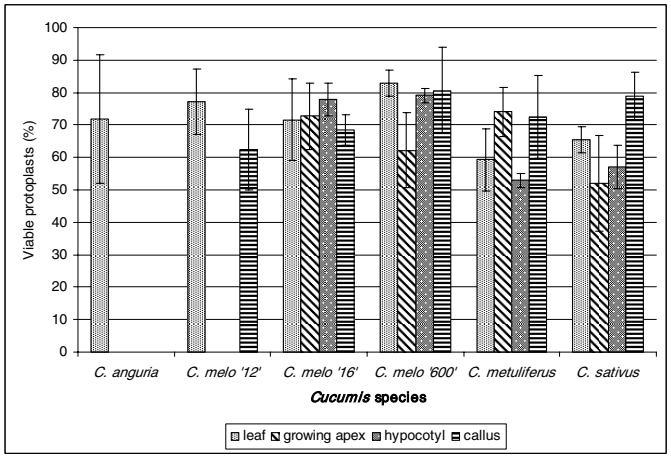

Fig. 2: Effect of donor explant variation on viability of protoplasts. Each value in the graph is based on at least 16 leaf explants, 24 growing apices, 20 hypocotyls and 12 dishes of callus. Y-error bars represent standard deviation.

Regenerative capability of protoplasts isolated from different types of explants

The highest level of regeneration was achieved using leaf protoplasts in all tested genotypes (Tab. 2) despite that leaf protoplasts were not as viable as other types of protoplasts in most genotypes. Calluses were regenerated from leaf protoplasts in *C. melo* '16' and *C. melo* '600'. Microcalluses were obtained in *C. metuliferus* and *C. sativus* leaf protoplasts. Using the same technique calluses are also possible using *C. metuliferus* and *C. sativus* (GAJDOVÁ et al., 2007). Protoplasts from growing apices did not achieve this level of regeneration but had higher regeneration efficiency than hypocotyl and callus protoplasts. Using growing apices as source of protoplasts has not been reported yet. Hypocotyl and callus derived protoplasts exhibited very low regenerative capability. It has been reported that callus protoplasts require a different medium for regeneration (with 0.045 $\mu mol \cdot l^{-1}$ 2,4-D and 0.913 $\mu mol \cdot l^{-1}$ zeatin) whereas leaf protoplasts of the same *C. sativus* variety had the highest regeneration rate using 25 $\mu mol \cdot l^{-1}$ NAA and 14.8 $\mu mol \cdot l^{-1}$ 2iP (BURZA and MALEPSZY, 1995). For this study the same regeneration protocol was used for all types of protoplasts. Hypocotyl protoplasts have not been

Tab. 2: Effect of plant tissue on regeneration of protoplasts

Genotype	Level of regeneration	Regeneration efficiency[1] (%)	Number of cultivated samples[2]
C. anguria			
Leaf	Cell division	58	12
C. melo '12'			
Leaf	Cell division	25	28
Callus	No regeneration	100	15
C. melo '16'			
Leaf	Callus	50	2
Growing apex	Microcallus *and* cell division	33 *and* 33	3
Callus	Microcallus *and* cell division	14 *and* 7	14
C. melo '600'			
Leaf	Callus *and* cell division	4 *and* 38	26
Growing apex	No regeneration	100	3
Hypocotyl	No regeneration	100	1
Callus	Cell division	14	14
C. metuliferus			
Leaf	Microcallus *and* cell division	5 *and* 53	19
Growing Apex	Cell division	100	3
Hypocotyl	Cell division	25	4
Callus	No regeneration	100	12
C. sativus			
Leaf	Microcallus *and* cell division	25 *and* 25	8
Growing apex	Microcallus	50	2
Hypocotyl	No regeneration	100	1
Callus	No regeneration	100	8

Only samples with viabilities of over 40% were used for regenerative capability assessment. [1]Regeneration efficiency is calculated as percentage of regenerating samples given for each level of regeneration. [2]One sample consists of at least 2-4 explants for leaves, 8 explants for growing apices, 4 dishes for callus and 10 explants for hypocotyls. In cases, where two levels of regeneration were observed, the regeneration efficiencies are given respectively.

regenerated into plants yet in *Cucumis* spp. (GAJDOVÁ et al., 2004). SUTIOJONO et al. (1998) have reported that leaf protoplasts had the highest regeneration potential, however cotyledon protoplasts divided slower and hypocotyl protoplasts did not divide at all. Plants were regenerated from protoplasts isolated from leaves (DEBEAUJON and BRANCHARD, 1992; ORCZYK and MALEPSZY, 1985), cotyledons (ROIG et al., 1986; COLIJN-HOOYMANS et al., 1988) and calluses (BURZA and MALEPSZY, 1995) in *C. melo* and *C. sativus*. Cotyledon protoplasts were mostly tetraploid and regenerated shoots were not normal however leaf protoplasts were diploid and produced normal plants (COLIJN-HOOYMANS et al., 1988). Regarding this, we did not use cotyledons in our study.

In *C. melo* '12' the highest regeneration efficiency was achieved with protoplasts from plants at 20-27 days old (which was also optimal age for protoplast isolation as shown in Tab. 1). In all other genotypes protoplasts from explants of various age were regenerating. Calluses from *C. melo* '16' and *C. melo* '600' were regenerated from 20-28 days old plant-derived protoplasts. Regenerated calluses grew several months but organogenesis did not occur on the media used in this study. Although no plant growth was observed in this study, further investigation into the callus regeneration media would likely yield plants (DEBEAUJON and BRANCHARD, 1992; ORCZYK and MALEPSZY, 1985).

Protoplast culture technique

The number of cell divisions decreased using agarose-solidified medium in our experiment (Tab. 3). Interestingly, several studies showed increased plating efficiency using this technique compared to liquid culture (COLIJN-HOOYMANS et al., 1988; MCCARTHY et al., 2001b; SUTIOJONO et al., 1998). The decrease in number of cell divisions in our study may have been caused by heat stress when embedding protoplasts in warm agarose medium (approximately 40°C). In our experiments the LCM medium was much better for protoplast regeneration of *C. anguria* leaf protoplasts than CML medium (Tab. 4). LCM medium has been successfully used for plant regeneration from cotyledon and leaf protoplasts in *C. melo* (DEBEAUJON and BRANCHARD, 1992). Similar concentrations of growth regulators (2.69 $\mu mol \cdot l^{-1}$ NAA, 4.56 $\mu mol \cdot l^{-1}$ 2,4-D and 2.22 $\mu mol \cdot l^{-1}$ BA) were used in *C. sativus* and leaf and cotyledon protoplast of *C. melo* (DABAUZA et al., 1991; ROIG et al., 1986). CML medium was successfully used for plant regeneration from leaf protoplasts of *C. sativus* (ORCZYK and MALEPSZY, 1985; COLIJN-HOOYMANS et al., 1988). Both temperatures used in this study had similar effects on cell division (Tab. 5). No increase was seen when using 27°C. MCCARTHY et al. (2001a) have obtained increased rates of cell division and microcalluses in cotyledon protoplasts of *C. metuliferus* cultured at 30°C over those cultured at 25°C.

Conclusions

From this study it is evident that the most important factor for obtaining the highest viability of *Cucumis* protoplasts was donor explant age (optimal age range was mostly 1-5 weeks, varying according to explant type and genotype). Using the plant culture medium with increased level of sucrose resulted in production of protoplasts with poor viability except with *C. melo* '600' growing apex protoplasts. Explant type was also important for viability, but there was no 'high viability explant' common to all genotypes. All explants were able to produce over 50 % (some over 80 %) viable protoplasts. *C. melo* varieties gave slightly higher protoplast viabilities than *C. metuliferus* and *C. sativus*. The regenerative capability of protoplasts was primarily dependent on source explant type, with the highest regeneration level (callus) observed in leaf protoplasts. Calluses were obtained only

Tab. 3: Effect of viscosity of the protoplast culture medium on cell division

Tested species	Cell divisions in liquid medium		Cell divisions in agarose solidified medium	
Cucumis melo '12'	Regeneration efficiency[1] (%)	Number of cultivated samples[2]	Regeneration efficiency[1] (%)	Number of cultivated samples[2]
	25	28	5	20

[1]Regeneration efficiency is the percentage of regenerating samples. [2]Each sample consisted of 2-4 leaves.

Tab. 4: Effect of the protoplast culture medium composition on cell division

Tested species	Cell divisions in LCM medium		Cell divisions in CML medium	
Cucumis anguria	Regeneration efficiency[1] (%)	Number of cultivated samples[2]	Regeneration efficiency[1] (%)	Number of cultivated samples[2]
	58	12	10	10

[1]Regeneration efficiency is the percentage of regenerating samples. [2]Each sample consisted of 4-8 leaves.

Tab. 5: Effect of culture temperature on cell division

Tested species	Cell divisions in 25°C		Cell divisions in 27°C	
Cucumis metuliferus	Regeneration efficiency[1] (%)	Number of cultivated samples[2]	Regeneration efficiency[1] (%)	Number of cultivated samples[2]
	75	8	63	8

[1]Regeneration efficiency is the percentage of regenerating samples. [2]Each sample consisted of 8-16 leaves.

in *C. melo* '16' and *C. melo* '600'. Temperature and culture medium composition affected number of cell division but there were no differences in level of regeneration obtained. This study has highlighted some of the important factors in promoting healthy regeneration of protoplasts. Future work will concentrate on further optimising the growth conditions of regenerating microcalluses specific to the donor species. Investigation on the effects of protoplast density in culture medium may help increase the plating efficiency. These may yield the answer in producing viable plants from protoplast fusions in the future.

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