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Callus induction and protoplast isolation from tissues of *Cucumis sativus* L. and *C. melo* L. seedlings

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Abstract

Hypocotyls, cotyledons and true leaves of *in vitro* seedlings of 10 cucumber and melon genotypes resistant to downy and powdery mildew were cultured on several combinations of initiation and multiplication media to produce callus and subsequently cell suspensions as suitable sources for isolation and culture of protoplasts. Cotyledons of both species were shown to be the most responsive to variation in culture media. However, calli and cell suspensions derived from hypocotyls generally provided higher number of protoplasts by treatment with several enzymatic solutions. The protoplasts formed new cell walls after 12 h of culture in liquid culture medium and first cell division was observed 2 d later with more frequent divisions after one week of culture.

Additional key words: cell suspension cultures, protoplast fusion, interspecific somatic hybrids, resistance to *Pseudoperonospora cubensis, Erysiphe cichoracearum, Sphaerotheca fuliginea.*

Introduction

Despite great progress in cucumber breeding (Tatlioglu 1993) the genetic variation in *C. sativus* is relatively very limited (Staub *et al.* 1987, Knerr *et al.* 1989). Resistance to several important diseases and pests have been located in wild *Cucumis*

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Abbreviations: BAP - 6-benzylaminopurine; CPW - salts of Banks and Evans (1976), FDA - fluorescein diacetate; FM - fresh mass; MES - 2-[N-morpholino] ethanesulfonic acid; MS - Murashige and Skoog (1962) medium; MS 0 - Murashige and Skoog (1962) medium without growth regulators; NAA - naphthalenacetic acid; WM - washing medium; 2,4-D - dichlorophenoxyacetic acid.

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species (Leppik 1966, Lebeda 1984, 1992a, Láska and Lebeda 1989, Lhotský *et al.* 1991) which belong to subgenera *Melo* - African group (x = 12). Interspecific hybridization is very important for broadening of the genetic base of cucumber (Nijs and Custers 1990), especially for resistance breeding [*e.g.* in *C. sativus* sources of resistance against downy mildew [*Pseudoperonospora cubensis* (Berk. and Curt.) Rostow.] are not available (Lebeda 1992b, Lebeda and Prášil, 1994)]. Several important sources of resistance have been located in *C. melo* (Thomas 1986, Cohen and Eyal 1987, Lebeda 1991) which could be valuable if transferred to *C. sativus*.

The subgenus *Melo* is cross-sterile with the subgenus *Cucumis* (x = 7) (Raamsdonk *et al.* 1989) and classical methods have not been successful in obtaining fertile hybrid plants (Nijs and Custers 1990). The transfer of genes responsible for resistance to downy mildew may be achieved by embryo rescue culture (Lebeda *et al.* 1996) and/or by somatic hybridization via protoplast fusion.

Attempts to isolate and regenerate protoplasts of *C. sativus* and *C. melo* were firstly described by Coutts and Wood (1977) and Moreno *et al.* (1984). Most investigators have used mesophyll, cotyledons or true leaves as source tissues for protoplast isolation (Orczyk and Malepszy 1985, Roig *et al.* 1986, Colijn-Hooymans *et al.* 1988a,b, Debeaujon and Branchard 1992). Cucumber embryogenic calli were used for protoplast isolation and regeneration for the first time by Burza *et al.* (1992). Recent information relating to the somatic embryogenesis in *Cucurbitaceae* has been summarized by Debeaujon and Branchard (1993).

The aim of this work was to choose the source material necessary to derive callus and cell suspension of *C. sativus* and *C. melo* for protoplast isolation, culture and fusion. The genotype effect being studied with special reference to the interspecific transfer of resistance genes to downy and powdery mildew of cucurbits.

Materials and methods

Plants: Seeds of *Cucumis sativus* L. (cvs. Bílské, Admira F1, Pálava) originated from the Vegetable Breeding Station, Smržice (Czech Republic). Seeds of *Cucumis melo* L. accessions (line MR-1, PI 124111, PI 124112, PI 200819, PI 234607, PI 321005, CGN 2365) were kindly provided by Dr. M. Pitrat (Institut National de la Recherche Agronomique, Montfavet, France), Dr. M.P. Widrlechner (Plant Introduction Station, Iowa State University, Ames, USA) and Mr. S. Zijlstra (Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands). Accessions of *C. melo* under study are characterized by resistance to downy mildew (*Pseudoperonospora cubensis*) and powdery mildew (*Erysiphe cichoracearum, Sphaerotheca fuliginea*) (Thomas 1986, Cohen and Eyal 1987, Pitrat 1990, Widrlechner - personal communication, Lebeda - unpublished).

Seeds of *C. sativus* and *C. melo* were surface sterilized [wetted in water for 15 min, rinsed with 70 % ethanol, transfered for 30 min in 8 % *Chloramine B* (sodium-benzensulfochloramidium) and then rinsed three times in sterile bidistilled water].

Callus and cell suspension derivation: Sterilized seeds were placed on a medium containing BDS mineral salts and vitamins (Dunstan and Short 1977) supplemented with 20 g dm^{-3} saccharose and 8 g dm^{-3} agar, but without hormones. Seeds were germinated in the dark at 25 °C. After germination, they were grown under 16-h photoperiod (irradiance of 32 - 36 μmol(PAR) m^{-2} s^{-1}) and temperature 25 °C and grown to small green seedlings. Hypocotyls, cotyledons and true leaves from 4-d to 6-d-old seedlings of *C. sativus* cv. Bílské and five genotypes of *C. melo* (PI 124112, PI 200819, PI 234607, line MR-1, CGN 2365) were then cut into segments (3 × 3 mm) and placed (10 to 15 pieces per 100 cm^3 Erlenmayer flask) on the solid initiation media containing various levels of growth regulators (MS 10/2 = 10 μM 2,4-D, 2 μM kinetin; MS 100/5 = 100 μM 2,4-D, 5 μM kinetin; MS-DNB = 20 μM 2,4-D, 10 μM NAA, 0.5 μM BAP). Explants were cultured in the dark at 25 °C.

After one or two weeks, the explants were transferred to multiplication medium either MS 0 (without growth regulators) or MS 10/05 (with 10 μM NAA and 0.5 μM BAP) and cultured under the same conditions. Derived calli were evaluated after three weeks and then transferred every month to the same fresh multiplication media and cultured in the dark at 25 °C. A piece of callus was transferred after evaluation onto the MS 10/0.5 and maintained under irradiance 32 - 36 μmol m^{-2} s^{-1} for a 16-h photoperiod and temperature 25 °C. All the above mentioned media contained 30 g dm^{-3} saccharose and pH was adjusted to 5.7 before autoclaving. Callus size was evaluated by measuring the diameter; root formation and callus colour were also evaluated.

Cell suspensions were initiated from selected friable calli of *C. sativus* and *C. melo*, genotype PI 234607, derived on the initiation MS 10/2 medium and cultured on MS 10/0.5 medium in the dark for both species. 5 g of callus were transferred into 250 cm^3 Erlenmayer flasks containing 50 cm^3 of liquid medium MS 10/0.5. For both plant species, the flasks were shaken either in the dark or in light. One-week old cell suspensions were filtered through a 200 to 700 μm plastic mesh filter to remove large clumps of callus. They were then further cultured under the same conditions. The suspensions were diluted in fresh medium to suitable density (10^5 - 10^6 cells cm^{-3}) every 7 to 10 d, dependent on growth.

Protoplast isolation: Selected calli and cell suspensions of *C. sativus* cv. Bílské, cv. Admira F1 (SM 5048 F1) and cv. Pálava, and *C. melo* genotypes PI 124111, PI 124112, PI 234607, PI 321005 and PI 200819 were used for protoplast isolation. Some of these materials were obtained in previous experiments (Lebeda *et al.* 1993) (see Tables 4 and 5). This material was cultured for 4 weeks on MS medium, supplemented with 5.4 μM 2,4-D and 2.3 μM kinetin, and then maintained either on MS medium supplemented with 2 μM 2,4-D and 2 μM kinetin (MS 2/2) or on MS 10/0.5 medium.

One week after transfer to fresh medium, calli were cut into small pieces (for cell suspensions, centrifuged cells were used) and placed into 60 or 100 mm Petri dishes containing the enzyme solution (5 cm^3 per 1.5 g fresh mass). All enzyme solutions (Table 1) were sterilized by filtration through a 0.2 μm filter. Digestions were

performed in the dark at 25 °C without shaking for 8 to 17 h depending on the plant material and the enzyme solution.

Table 1. Enzymatic mixtures used for isolation of protoplasts from calli and cell suspensions of *Cucumis sativus* and *Cucumis melo*.

Enzyme mixture	Composition	Osmolality [mOs kg^{-1}]	pH
BC*	1.2 % cellulase Onozuka R-10, 1.2 % Macerozyme R-10, 0.3 % driselase, 3 mM MES, 0.1 M glycine, CPW salts, mannitol	478	5.7
BC-d	same as BC but free of driselase	464	5.7
KFK**	2 % cellulase Onozuka R-10, 0.2 % Macerozyme R-10, 1 % pectinase, 0.9 % $CaCl_2$, mannitol	688	5.6
KFK	2 % cellulase Onozuka 0.1/0.1 R-10, 0.2 % Macerozyme R-10, 1 % pectinase, 0.1 M glycine, 0.1 % $CaCl_2$, 3 mM MES, mannitol	759	5.7
KFK-I-CS	2 % cellulase P-25, 0.4 % Macerozyme R-10, 2 % pectinase, 0.9 % $CaCl_2$, mannitol	643	5.6
OMA	2 % cellulase Onozuka R-10, 1.5 % Macerozyme R-10, 1 % pectinase, 0.5 % dextran sulphate, 3 mM MES, 0.1M glycine, mannitol	624	5.7
OMA2	same as OMA but supplemented with 0.1 % $CaCl_2$	490	5.6
EPS	2 % cellulase Onozuka R-10, 0.5 % Macerozyme R-10, 0.5 % driselase, 1 % pectinase, 0.1% $CaCl_2 . 2H_2O$, 100 cm^3 dm^{-3} MES stock solution (stock solution of MES: 0.585 mg cm^{-3} MES + 0.1 mg cm^{-3} NaH_2PO_4), mannitol	504	5.7
DBMC	0.8 % cellulase Onozuka R-10, 0.2 % Macerozyme R-10, 0.9 0.9 % $CaCl_2$, mannitol	560	5.6

* - from Burza *et al.* 1992; ** - from Fellner and Havránek 1994

Culture of protoplasts: Protoplasts were isolated with BC enzyme solution (Table 1), strained through a 57 or 72 μm mesh nylon filter and centrifuged at 40 *g* for 8 min. The pellet was resuspended in a washing medium (WM) containing 1 mM $CaCl_2 . H_2O$ and glucose as osmoticum (osmolality was adjusted to 540 mOs $kg^{-1}(H_2O)$; pH 5.7) and recentrifuged at 50 *g* for 8 min. A further purification by density gradient was performed. Protoplasts (4 cm^3) were partitioned by a centrifugation at 75 *g* for 20 min between a two phase mixture containing 20 % (m/v) saccharose (3 cm^3) and WM (3 cm^3). Most of debris pelleted and purified protoplasts were layered at the glucose-saccharose interphase. Protoplasts were collected and washed once more in WM by a centrifugation at 44 *g* for 8 min. The final pellet was resuspended in a culture medium at the density of 2×10^5(protoplasts) cm^{-3}.

The protoplasts were cultured in the dark at 25 °C in 60 mm plastic Petri dishes. The MS 2/2 medium used was the same as for cell suspensions. osmolality was adjusted by mannitol to 490 mOs kg^{-1} (pH 5.7). The viability of protoplasts was determined by FDA staining (Widholm 1972) and cell wall regeneration by the UV-excited optical brightener *Rylux BSU* staining (Hejtmánek *et al.* 1990)

Results

Growth of calli and cells in suspension: Callus of *C. sativus* was produced from hypocotyls and cotyledons cultured on all combinations of the initiation and multiplication media used (Table 2). The calli derived from hypocotyls reached a larger size when grown in the presence of 2,4-D for 7 d. The largest calli were produced on the combination MS 10/2 - MS 10/0.5 and DNB - MS 10/0.5 media without any root formation. With the multiplication MS 0 medium, a sporadic production of roots was observed, independent of the time on initiation medium. Calli from cotyledons were larger with vigorous roots on all combinations of media. Root formation was not dependent on the type of initiation medium. The best media for callus production were MS 10/2 or MS 10/0.5, as for hypocotyls. The calli were generally yellow with exception of those induced from cotyledons placed 7 d on initiation media, where calli were yellow-green or yellow-brown.

Hypocotyls as well as cotyledons of *C. melo*, genotype PI 234607, formed calli on all medium combinations (Table 3). The size of induced calli were comparable with calli induced from hypocotyls and cotyledons of *C. sativus*, however, the calli from *C. melo* were very heterogeneous in colour as well as in their structure. The medium combinations MS 10/2 - MS 10/0.5 were found to be the best for callus growth of *C. melo* PI 234607, from both hypocotyls and cotyledons. For this genotype, we did not observe any difference in callus production from hypocotyls placed for 7 or 14 d on medium containing 2,4-D. However, those calli almost never formed roots. In contrast, cotyledons of the same genotype cultured for 7 d on medium containing 2,4-D formed roots. The callus of *C. melo* PI 234607 was usually more friable than callus of *C. sativus*.

On the basis of our preliminary results, only hypocotyls of the genotypes PI 124112, PI 200819 and line MR 1, and media combinations MS 10/2 - MS 10/0.5 and DNB - MS 10/0.5 were used for callus induction. Callus from those three genotypes was friable and yellow to yellow-brown. The callus of line MR-1 and PI 200819 often became necrotic. Genotype PI 200819 formed smaller calli than did genotypes MR-1 and PI 124112 which produced the largest calli of all the genotypes of *C. melo*. The calli of PI 200819 may have been internally contaminated by bacteria. This contamination ceased to be evident during long-term culture of calli on MS 10/0.5 medium.

For callus induction of *C. melo* genotype CGN 2365, only true leaves were used. Explants were cultured on medium combinations MS 10/2 - MS 10/0.5 and DNB - MS 10/0.5 with a 7 d initiation. However, the explants did not form any calli under our conditions and usually became necrotic.

Part of calli of *C. melo* transferred to light after evaluation and grown on medium MS 10/0.5 were mostly yellow-green to green; they were friable and grew vigorously during subculture. The calli of *C. sativus* grew weakly, under the same light culture conditions, on medium MS 10/0.5 when compared with *C. melo* (data not shown).

Cell suspensions initiated from calli of *C. sativus* and *C. melo* genotype PI 234607, cultured on medium MS 10/0.5 under light, reached a stationary phase from the 7th to

Table 2. Callus production in *Cucumis sativus* cv. Bílské in relation to donor initial explant and culture media. Size of induced calli after 3 weeks on the multiplication media: 0 - no calli, 1 - calli 2 to 4 mm, 2 - 4 to 8 mm, 3 - ≥ 8 mm. Average number of non-contaminated explants was 23.

Donor explant	Initiation medium	Length of culture [d]	Multiplication medium	Number of calli	Size 0 [%]	1 [%]	2 [%]	3 [%]	Rooting [%]	Callus colour
Hypocotyl	MS 1/2	7	MS 0	16	25	50	25	0	50	yellow
	MS 1/2		MS 1/0.5	6	0	0	100	0	0	yellow
	MS 1/5		MS 0	5	20	20	60	0	0	yellow
	MS 1/5		MS 1/0.5	10	0	100	0	0	0	yellow
	DNB		MS 0	7	0	100	0	0	85.7	yellow
	DNB		MS 1/0.5	18	11.1	77.8	11.1	0	0	yellow
	MS 1/2	14	MS 0	6	100	0	0	0	33.3	yellow
	MS 1/2		MS 1/0.5	24	0	91.7	8.3	0	0	yellow
	MS 1/5		MS 0	4	0	100	0	0	0	yellow
	MS 1/5		MS 1/0.5	13	46.2	53.8	0	0	0	yellow
	DNB		MS 0	7	0	100	0	0	0	yellow
	DNB		MS 1/0.5	26	19.2	80.8	0	0	0	yellow
Cotyledon	MS 1/2	7	MS 0	68	39.7	50	10.3	0	73.5	yellow-green
	MS 1/2		MS 1/0.5	39	0	17.9	43.6	38.5	69.2	yellow
	MS 1/5		MS 0	38	10.5	13.2	76.3	0	42.1	yellow
	MS 1/5		MS 1/0.5	20	0	65	35	0	10	yellow-brown
	DNB		MS 0	27	0	37	29.6	33.3	92.6	yellow-green
	DNB		MS 1/0.5	47	0	29.8	31.9	38.3	36.2	yellow-brown
	MS 1/2	14	MS 0	46	2.2	58.7	39.1	0	47.8	yellow
	MS 1/2		MS 1/0.5	9	0	22.2	77.8	0	66.7	yellow
	MS 1/5		MS 0	30	3.3	76.7	20	0	53.3	yellow
	MS 1/5		MS 1/0.5	16	6.2	75	18.8	0	0	yellow
	DNB		MS 0	57	5.3	38.6	56.1	0	47.4	yellow
	DNB		MS 1/0.5	16	0	62.5	37.5	0	50	yellow

Table 3. Callus production in *Cucumis melo* genotypes in relation to donor initial explant and culture media. Size of induced calli after 3 weeks on the multiplication media: 0 - no calli, 1 - calli 2 to 4 mm, 2 - 4 to 8 mm, 3 - ≥ 8 mm. Average number of non-contaminated explants was 36.

Genotype	Donor explant	Initiation medium	Length of culture[d]	Multiplication medium	Number of calli	Size 0 [%]	1 [%]	2 [%]	3 [%]	Rooting [%]	Callus colour
PI 234607	hypocotyl	MS 10/2	7	MS 10/0.5	4	0	0	100	0	0	yellow
		MS 100/5		MS 10/0.5	6	16.7	66.6	16.7	0	0	light yellow
		DNB		MS 0	5	0	100	0	0	2.8	yellow-brown
		DNB		MS 10/0.5	11	9	45.5	45.5	0	0	light yellow
		MS 10/2	14	MS 0	7	14.3	57.1	28.6	0	0	yellow
		MS 10/2		MS 10/0.5	3	0	66.7	33.3	0	0	yellow
		MS 100/5		MS 0	6	16.7	83.3	0	0	0	yellow
		DNB		MS 0	8	0	100	0	0	0	yellow-brown
		DNB		MS 10/0.5	6	0	100	0	0	0	yellow-green
	cotyledon	MS 10/2	7	MS 0	41	22	31.7	46.3	0	65.9	light yellow
		MS 100/5		MS 0	39	20.5	43.6	35.9	0	35.9	light yellow
		DNB		MS 0	13	15.4	46.1	38.5	0	69.2	light yellow
		DNB		MS 10/0.5	29	27.6	69	3.4	0	0	light yellow
		MS 10/2	14	MS 0	7	28.6	71.4	0	0	0	yellow-green
		MS 10/2		MS 10/0.5	13	7.7	92.3	0	0	0	yellow
		MS 100/5		MS 0	21	76.2	23.8	0	0	4.8	yellow-brown
		MS 100/5		MS 10/0.5	13	100	0	0	0	0	yellow-brown
		DNB		MS 0	14	28.6	71.4	0	0	7.1	yellow-brown
		DNB		MS 10/0.5	28	50	50	0	0	0	yellow-brown
MR-1	hypocotyl	MS 10/2	7	MS 10/0.5	102	0	3.9	66.7	29.4	0	yellow-brown
		DNB		MS 10/0.5	99	1	4	91	4	0	yellow-brown
PI 124112	hypocotyl	MS 10/2	7	MS 10/0.5	168	0	9.5	62.5	28	0	yellow
		DNB		MS 10/0.5	152	0	87.5	11.8	0.7	0	yellow
PI 200819	hypocotyl	MS 10/2	7	MS 10/0.5	54	0	98.1	1.9	0	0	dark yellow
		DNB		MS 10/0.5	48	0	100	0	0	0	dark yellow
CGN 2365	leaf	MS 10/2	7	MS 10/0.5	17	100	0	0	0	0	-
		DNB		MS 10/0.5	21	100	0	0	0	0	-

10th day of culture after the last dilution and they also formed cell clusters every time. In suspensions of *C. sativus*, some embryogenic configurations were also formed.

Isolation and protoplast culture. Protoplast isolation was evaluated on the basis of protoplast yield per gram of FM. The maximal yield of protoplasts [more than 10^6 protoplasts g^{-1}(FM)] was obtained from the cell suspension culture of *C. sativus* cv. Bílské which was cultured in MS 2/2 medium in light using the BC enzymatic solution, or from calli of the same genotype cultured under the same conditions but using a KFK-I-CS enzymatic solution (Table 4). The optimal digestion time was 17 h in both cases. Other enzymatic solutions tested proved unfit with an insufficient yield of protoplasts to wash and recover useful numbers of protoplasts for culture.

Highest protoplast yields of *C. melo* [more than 10^6 protoplasts g^{-1}(FM)] were obtained from the cell suspension of genotype PI 124112 cultured in MS 2/2 medium in light, and from calli of genotype PI 234607 cultured in either darkness or light, by using the BC enzymatic solution (8 h digestion) (Table 5). The enzymatic solution OMA was also useful for protoplast production from calli of genotype PI 234607. KFK enzymatic solution was used to isolate the most protoplasts from cell suspension of genotype PI 124112, cultured with light on the medium MS 10/0.5. Calli of genotype PI 124112 cultured in the dark on the medium MS 10/0.5 produced many protoplasts with enzyme solution KFK-I-CS. Other genotypes gave poor yields of protoplasts for culture experiments.

The size of freshly isolated protoplasts was dependent on the plant material and also on the osmolality of enzymatic solutions. In the case of BC solution (479 mOs kg^{-1}), the size of protoplasts of *C. melo* varied between 32 and 92 μm, and for protoplasts of *C. sativus* cv. Bílské between 41 and 140 μm. Viability of fresh protoplasts varied between 69.1 and 76.7 %.

The experiments on protoplast culture were carried out with protoplasts of *C. melo*, genotype PI 124112, which were maintained with light on MS 2/2 medium at 25 °C. The viability of freshly isolated protoplasts was 72.4 % on average. During the course of culture, the mean viability of protoplast-derived cells decreased to 15.4 % after 2 weeks. Cell wall formation by fresh protoplasts was observed after 12 h of culture, and after 24 h 19.6 % of protoplasts showed the regeneration of new cell wall. Additional evidence for the presence of new cell walls included the loss of spherical shape by protoplasts and initiation of weak budding. Two days after protoplast isolation, the formation of small cell clusters was evident. The first cell divisions were observed on the 2nd day of culture. On the 4th day of culture, the initial medium osmolality 490 mOs kg^{-1} was adjusted to 370 mOs kg^{-1} by adding of fresh culture medium free of mannitol. More frequently, the first cell division was observed on the 7th or 8th day of culture, when the mean viability of protoplasts was 42.6 %. However, the frequency of cell division was very low (no more than 1 %). Ten days later, the osmolality of medium was decreased to 280 mOs kg^{-1} (by fresh medium). Second cell divisions and subsequent cellular development were not observed.

Table 4. Protoplast isolation from *Cucumis sativus* genotypes. Hours of enzymatic treatment in parentheses. 0 - no protoplasts, + - 10^3 to 5×10^5 protoplasts, ++ - 5×10^5 to 9×10^5 protoplasts, +++ - $\geq 10^6$ protoplasts g^{-1}(FM).

Genotype/ cultivar	Initial explant	Multiplicative medium	*In vitro* culture	Conditions	Enzymatic mixtures BC	BC-d	KFK-I-CS	OMA	OMA2	DBMC
Bílské	cotyledon*	MS 10/0.5	callus	dark	+ (17)			+ (16)		
nakládačky	hypocotyl	MS 10/0.5	callus	dark	-+ (8)	0 (16)			+ (16)	+ (17)
	hypocotyl	MS 10/0.5	callus	light	-+ (14)	++ (16)		+ (16)	+ (16)	
	cotyledon*	MS 2/2	callus	light			+++ (17)			
	cotyledon	MS 10/0.5	suspension	dark		0 (16)				
	cotyledon*	MS 2/2	suspension	light	+++ (17)	++ (16)		+ (16)	+ (16)	
Admira F1	cotyledon*	MS 2/2	suspension	light					+ (16)	
Fálava	cotyledon*	MS 2/2	suspension	light					+ (16)	

* - material described in Lebeda *et al.* 1993

Table 5. Protoplast isolation from *Cucumis melo* genotypes. Hours of enzymatic treatment in parentheses. 0 - no protoplasts, + - 10^3 to 5×10^5 protoplasts, ++ - 5×10^5 to 9×10^5 protoplasts, +++ - $\geq 10^6$ protoplasts g^{-1}(FM).

Genotype/ accession	Initial explant	Multiplication medium	*In vitro* culture	Conditions	Enzymatic mixtures BC	BC-d	KFK	KFK 0.1/0.1	KFK-I-CS	OMA	OMA2	EPS	DBMC
PI 124111	cotyledon*	MS 2/2	suspension	light		0 (16)					0 (16)		
PI 234607	hypocotyl	MS 10/0.5	callus	dark	+++ (17)					+++ (16)	0 (16)		
	hypocotyl	MS 10/0.5	callus	light	++ (17)	+ (16)				++ (15)	++ (16)		
	cotyledon	MS 10/0.5	callus	dark	0 (8)	0 (16)							
	cotyledon	MS 10/0.5	callus	light	+++ (8)							0 (8)	
	hypocotyl	MS 10/0.5	suspension	dark							0 (8)		
	hypocotyl	MS 10/0.5	suspension	light			+ (17)	+ (17)					
	hypocotyl*	MS 2/2	suspension	light	0 (17)	0 (16)				0 (16)	0 (16)		
PI 321005	hypocotyl*	MS 2/2	suspension	light	0 (17)		0 (17)	0 (17)					
PI 124112	hypocotyl	MS 10/0.5	callus	light			++ (17)						
	cotyledon*	MS 10/0.5	callus	dark					+++ (17)				+ (17)
	cotyledon*	MS 2/2	callus	light					+ (17)				0 (17)
	cotyledon*	MS 10/0.5	suspension	light	+ (17)	+ (16)	+++ (8)			+ (16)	++ (16)	+ (14)	
	cotyledon*	MS 2/2	suspension	light	+++ (8)					0 (16)			
	hypocotyl	MS 10/0.5	callus	dark					0 (17)				0 (17)

* - material described in Lebeda *et al.* 199

Discussion

Calli of *C. sativus* were formed from cotyledons and hypocotyls on every combinations of initiation and multiplication media tested. However, the optimal conditions for callus formation from cotyledons and hypocotyls were: initiation on MS medium supplemented with 10 μM 2,4-D + 2 μM kinetin, and subsequent multiplication on MS medium with 10 μM NAA + 0.5 μM BAP, or 20 μM 2,4-D + 10 μM NAA + 0.5 μM BAP. Callus morphology was generally not dependent on the amount of auxin and cytokinin supplemented in our experiments.

Under almost the same culture conditions, Chee (1990) induced calli from cotyledons of *C. sativus* after 1 month on MS medium with 9 μM 2,4-D + 2.3 μM kinetin. Comparable, combinations of 2,4-D and BAP were used for induction of calli from *in vitro* cotyledon and leaf explants of *C. sativus* by Kim *et al.* (1988) and Custers *et al.* (1990). Recently Colijn-Hooymans *et al.* (1994) showed that the decrease in regeneration capacity of *C. sativus* cotyledons from 7-d-old and older seedlings was lowered dramatically. The decrease in regeneration competence correspond with the change in DNA content of the cotyledonary cells. Also our results confirmed a close link between the polyploidization and the loss of totipotency *in vitro* in *C. sativus* (Kubaláková *et al.* 1996). The same relationship is known in *C. melo* (Ezura and Oosawa 1994).

As with *C. sativus*, also for *C. melo* the cotyledons seemed to be more responsive to the culture medium than were hypocotyls. There were larger calli and more frequent root formation when explants were cultured 7 d on initiation medium than for a 14 d exposure.

Oridate and Oosawa (1986) induced callus production from cotyledons and hypocotyls of *C. melo* after 3 weeks on MS medium with 4.5 μM 2,4-D + 0.4 μM BAP and 0.5 μM 2,4-D + 0.4 μM BAP. They reported some delay in callus derivation from hypocotyls compared with cotyledons and later, the inability of hypocotyls to form embryos. This is in accord with our results showing less responsiveness of hypocotyls to culture conditions compared with cotyledons. Also Tabei *et al.* (1991) observed the MS medium supplemented with 0.5 μM 2,4-D + 0.4 μM BAP as optimal for callus induction from cotyledons, leaf explants, petioles and hypocotyls of *C. melo*. Recently similar results were published by Molina and Nuez (1995a,b).

Thus, a combination of growth regulators such as 2,4-D and BAP or kinetin seems to be necessary for the formation and differentiation of calli from *C. sativus* and *C. melo* explants. In addition, hypocotyl-derived calli and cell suspensions were noted, to be better sources for protoplast production than cotyledon-derived cultures.

Many other researchers recently successful in the culture and regeneration of protoplasts of *C. melo* and *C. sativus* have used cotyledon or mesophyll cells of *in vitro* plants as sources for the isolation of protoplasts (Kantharajah and Dodd 1990, Punja *et al.* 1990, Bokelmann *et al.* 1991, Dirks and Van Buggenum 1991, Debeaujon and Branchard 1992, Tabei *et al.* 1992, Jarl *et al.* 1995). A new system for *C. sativus* protoplast regeneration has also been described (Burza *et al.* 1992).

We studied the possibility of isolating the protoplasts from calli and cell suspensions derived from both species, *C. melo* and *C. sativus*. For isolation, we used

the procedure described by Burza *et al.* (1992) for callus from *C. sativus* with modification, that the enzymes were dissolved in CPW salts and the solution supplemented with 3 mM MES and 0.1 M glycine, as Orczyk and Malepszy (1985) used for isolation of leaf protoplasts of *C. sativus*. Our yield of protoplasts from a suspension of *C. sativus* cv. Bílské was higher than 10^6 protoplast g^{-1}(FM). From the calli of *C. sativus* we obtained the same protoplast yield as reported by Burza *et al.* (1992). However, unlike these authors, we used 25 °C as an incubation temperature.

To our knowledge, this is the first attempt to use calli of *C. melo* for protoplast isolation and culture. The isolation procedure developed was optimal for protoplast isolation from cell suspensions and calli of two genotypes of *C. melo* (PI 124112, PI 234607) with a yield of more than 10^6 protoplasts g^{-1}(FM). The yield was dependent on the choice of the starting material and on the conditions in which the material was cultured before isolation (*e.g.* culture medium, light or darkness). However, for the induction of normal cell division and for successful culture and regeneration of callus-derived protoplasts of *C. melo*, it is still necessary to find the most suitable melon genotypes and to optimize culture conditions.

From previous and recent results it can be concluded that there are available reasonable techniques for protoplasts isolation of both *Cucumis* species and also genotypes carrying the resistance to important diseases. Somatic fusion of protoplasts may provide a means of genetic interchange with aim of creation of interspecific hybrids with resistance to downy and powdery mildews. Until now there is the failure to obtain plants from the assymetric protoplast fusion products. This is due to the irregular ploidal situation or some somatic incompatibility reaction between *C. sativus* and *C. melo* (Jarl *et al.* 1995, Fellner *et al.* 1996)

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