www.czasopisma.pan.pl PAN www.journals.pan.pl

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 52/1: 111–115, 2010
DOI: 10.2478/v10182-010-0014-8

OPTIMIZING CULTURE FOR IN VITRO POLLINATION AND FERTILIZATION IN *CUCUMIS SATIVUS* AND *C. MELO*

DAGMAR SKÁLOVÁ*, BOŽENA NAVRÁTILOVÁ, VLADAN ONDŘEJ, AND ALEŠ LEBEDA

Palacký University in Olomouc, Faculty of Science, Department of Botany, Šlechtitelů 11, 783 71 Olomouc-Holice, Czech Republic

Received July 3, 2009; revision accepted March 20, 2010

In vitro pollination can be used to overcome crossing barriers in interspecific hybridization within the genus *Cucumis*. This technique offers a way to produce viable interspecific hybrids. We tested two types of media, designated CP and YS, for in vitro pollination in *Cucumis sativus* and *C. melo*. Pollen grains were isolated by centrifugation or directly from mature male flowers and were cultured with mature ovules. We assessed pollen grain viability, fertilization ability, and fertilized ovule development. The developing ovules (becoming enlarged and green) were transferred to media supporting embryogenesis (with ascorbic acid, caseinhydrolysate, coconut water and gibberellic acid). The highest level of regeneration after in vitro pollination was callus formation from ovules. We found caseinhydrolysate to be the most beneficial component during in vitro pollination (CP medium) and during development of fertilized ovules (ON medium). The hybrid character of fertilized ovules arisen from crosses between cucumber and muskmelon was checked but not confirmed by RAPD analysis, for reasons we suggest. The in vitro protocol needs to be optimized further to obtain a high yield of potential hybrid embryos.

Key words: *Cucumis* spp., cucumber, muskmelon, intraspecific hybridization, interspecific hybridization, in vitro pollination, in vitro fertilization, RAPD analysis.

Cucumber (*Cucumis sativus* L.) ranks among the ten most important vegetables in world production (Lebeda et al., 2007), but it is susceptible to many pathogens and pests. Valuable sources of disease resistance have been found in wild African species of *Cucumis*. For introgression of resistance genes in cucumber, one very useful method entails interspecific hybridization technique, including in vitro culture (Lebeda et al., 2007). In some cases the interspecific hybridization barrier is attributed to differences in chromosome number between parents. *Cucumis sativus* belongs to the Asian group and contains n=7 chromosomes. Wild *Cucumis* spp. and *C. melo* belong to the African group and contains n=12 chromosomes (Jeffrey, 2001; Křístková et al., 2003; Lebeda et al., 2007).

Optimization of tissue culture protocols will become increasingly important for successful interspecific hybridization within the genus *Cucumis* and for production of viable *C. sativus* and *C. melo* hybrids (Lebeda et al., 1999, 2007; Skálová et al., 2004). Various methods have been used to rescue embryos in interspecific hybridization, particularly between *C. sativus* and *C. melo* L. (Lebeda et al., 1996, 1997, 1999; Skálová et al., 2006). In vitro pollination and fertilization have been used to overcome pre-zygotic (factors hindering effective fertilization) and post-zygotic (barriers occurring during or after syngamy) cross-incompatibility in the genus *Cucumis* (Ondřej et al., 2002a). For successful in vitro pollination the pollen grains must be selected at the right stage, since in vitro manipulation of pollen is limited. Studies have focused on establishing optimal culture conditions for isolation, sterilization and viability of cucumber pollen grains (Vižintin and Bohanec, 2004). In vitro pollination has been reported by many authors (e.g., Tilton and Russell, 1984; Castano and De Proft, 2000; Zenkteler et al., 2005; Popielarska, 2005).

Here we report our work on isolation and culture of pollen grains, pollination, fertilization and ovule development of cucumber (*C. sativus*) and muskmelon (*C. melo*) in vitro. The developed media facilitated pollination and fertilization, and helped in rescuing potential interspecific hybrids.

For the experiments we used these selected genotypes of *Cucumis* species: *C. sativus* Stela F1, 09H390744 (CS); *C. melo* PMR 45, 09H400597 and

*e-mail: dagmar.skalova@upol.cz

PL ISSN 0001-5296

TABLE 1. Media composition

Medium	Composition of media	Reference
YS	600 mg/l Ca (NO_3), 100 mg/l H_3BO_3, 8% sucrose	Ondřej et al., 2002a
CP	MS + 9.5 mg/l glycine, 500 mg/l caseinhydrolysate, 4 mg/l IAA, 0.5 mg/l KIN, 4mg/l GA_3, 4% sucrose	Castano and De Proft, 2000
OK	MS + 20mg/l ascorbic acid, 0.01mg/l IBA, 0.01mg/l BA, 2% sucrose	Skálová et al., 2007; 2008
ON	MS + 1g/l caseinhydrolysate, 0.01mg/l IBA, 0.01mg/l BA, 2% sucrose	Skálová et al., 2007; 2008
CW	MS + 5% coconut water, 200mg/l α- glutamine, 0.01mg/l IBA, 0.01mg/l BA, 6% sucrose	Skálová et al., 2007; 2008
GA	MS + 0.3mg/l GA_3, 0.01mg/l IBA, 0.01mg/l BA, 2% sucrose	Skálová et al., 2007; 2008

MS – Murashige and Skoog (1962); IBA – indole-3-butyric acid; BA – benzyladenin; KIN – kinetin; IAA – indole-3-acetic acid; GA_3 – gibberellic acid; 8 g/l agar was added in all types of media.

PMR 5, 09H400599 (CM). Materials were obtained from the vegetable germplasm collection of the Research Institute of Crop Production (Prague), Olomouc Research Station, Czech Republic (www.vurv.cz/, part databases, EVIGEZ). Plants were grown in a glasshouse (25°C/15°C day/night) of the Department of Botany, Palacký University of Olomouc (Czech Republic). They were watered daily and fertilized weekly with Kristalon Start fertilizer (NU3 B.V., Vlaardingen, Netherlands) (10 ml fertilizer per 10 l water) without chemical treatment.

Immature female and male cucumber and muskmelon flowers were sterilized for 1 min in 70% (v/v) ethanol and 10 min in 2.5% chloramine, rinsed three times in sterile water in aseptic conditions and then cultured on MS medium (Murashige and Skoog, 1962) at 25°C for 3 days in darkness. Mature female cucumber flowers were excised in aseptic conditions and ovules were transferred on solid media (CP medium, Castano and De Proft, 2000; YS medium, Ondřej et al., 2002a); media composition is specified in Table 1. Pollen grains were isolated from anthers of mature male flowers of cucumber and muskmelon by centrifugation (10-5-5-min., 900 rpm) in washing solution (10 ml modified NLN 13 medium; Lichter, 1981). They were transferred on and around ovules of *C. sativus* in the culture medium. Petri dishes with ovules (10 per dish) and pollen grains were cultured for 2–3 days at 25°C in darkness. Pollen tube growth and in vitro pollination and fertilization were observed with a microscope (OLYMPUS CK40). The origin of the zygote and the subsequent development to globular-stage embryo were described in previous work (Ondřej et al., 2001; Ondřej et al., 2002a). Fertilized ovules were transferred to previously tested media supporting embryogenesis (OK, ON, CW, GA medium; Skálová et al., 2007; 2008; Table 1 specifies the medium composition. The pollination ability of pollen grains isolated directly from the mature male flowers of CS and CM was tested. CP and YS media were again used for in vitro pollination, and CW and GA media for embryogenesis.

Our experiments also compared the viability of pollen isolated by centrifugation in washing solution (modified NLN-13 medium) and pollen isolated directly from anthers and cultured on CP and YS media. Pollen grains were stained with fluorescein diacetate (FDA) and viability checked with an Olympus BX60 fluorescence microscope (Larkin, 1976) with a BW filter.

All experiments were repeated (ten ovules per dish; final numbers summarized in Tables 2 and 3). The results are expressed as means ±SD.

We used DNA extraction and RAPD analysis to verify the hybrid character of the cucumber ovules fertilized by muskmelon pollen grains. Genomic DNA was isolated from young leaves of the parental plants by the CTAB method. DNA from fertilized ovules was extracted using the REDExtract-N-Amp Seed PCR Kit (Sigma) according to the manufacturer's protocol. DNA from parents and from ovules was amplified with REDExtract-N-Amp PCR reaction mix (Sigma) with RAPD primer 5´-GTGT-GCGATCAGTTGCTGGG-3´ (Koo et al., 2005). PCR products were analyzed by electrophoresis in 1% agarose gel and detected by ethidium bromide staining.

Germination of isolated pollen grains in culture is a prerequisite for several biotechnological manipulations in cucumber (Vižintin and Bohanec, 2004). Pollen grains germinate in vitro, enabling the process of sexual reproduction to proceed (Ondřej et al., 2002a). Indeed, we achieved successful in vitro pollination after selfing and interspecific crossing within the genus *Cucumis*. For *in vitro* pollination we isolated pollen grains of *C. sativus* and *C. melo* by centrifugation (Tabs. 2, 3; Fig. 1). YS medium gave better pollen tube growth following fertilization (42 ± 12%) than CP medium (32 ± 9%), but ovules cultured on CP medium showed better subsequent development (59 ± 12%) than those cultured in YS medium (27 ± 15%). Tilton and Russell (1985) observed that medium composition plays a significant role in pollen tube growth and ovule viability. Subsequent

TABLE 2. Number of isolated cucumber ovules (♀) with cucumber pollen grains (♂) isolated by centrifugation and directly; number of successful intraspecific in vitro pollinations, fertilizations and progressed ovules in CP and YS- media

♀	♂	No. of isolated ovules (♀) [cultivated together with pollen grains (♂)]		No. of successful fertilizations (growth and penetration of pollen tubes)		No. of progressed ovules (ovules becoming enlarged and green; callus formation)	
		CP	YS	CP	YS	CP	YS
CS	CS (pollen grains isolated by centrifugation)	660	660	150	190	15 (OK) 45 (ON) 35 (CW) 95	8 (OK) 25 (ON) 24 (CW) 57
	(pollen grains isolated directly)	90	90	50	50	12 (CW) 10 (GA) 22	23 (CW) 20 (GA) 43

CP medium (Castano and De Proft, 2000); YS medium (Ondřej et al., 2002a) (media for in vitro pollination); OK, ON, CW, GA – media used for culture of ovules after in vitro pollination (basic MS medium supplemented with specific additives supporting embryogenesis (Skálová et al., 2007, 2008).

TABLE 3. Number of isolated cucumber ovules (♀) with muskmelon pollen grains (♂) isolated by centrifugation and directly; number of successful interspecific in vitro pollinations, fertilizations and progressed ovules in CP and YS- media

♀	♂	No. of isolated ovules (♀) [cultivated together with pollen grains (♂)]		No. of successful fertilizations (growth and penetration of pollen tubes)		No. of progressed ovules (ovules becoming enlarged and green; callus formation)	
		CP	YS	CP	YS	CP	YS
CS	CM (pollen grains isolated by centrifugation)	160	160	100	100	25 (ON) 9 (CW) 7 (GA) 41	10 (ON) - 7 (GA) 17
	(pollen grains isolated directly)	90	90	60	60	4 (CW) 4 (GA) 8	10 (CW) 8 (GA) 18

CP medium (Castano and De Proft, 2000); YS medium (Ondřej et al., 2002a) (media for in vitro pollination); OK, ON, CW, GA – media used for culture of ovules after in vitro pollination (basic MS medium supplemented with specific additives supporting embryogenesis (Skálová et al., 2007, 2008).

development of fertilized ovules on OK, ON, CW, and GA media was profuse. The best results were for ON medium containing caseinhydrolysate (CP also contained it). Custers (1981) and Ondřej et al. (2002b) used caseinhydrolysate to good effect in cucumber embryo culture. We evaluated the effect of caseinhydrolysate in early stages of embryo development after in vitro pollination. Of all the types of in vitro pollination (Tabs. 2, 3), the (♀) *C. sativus* × (♂)*C. sativus* cross was more successful (yielding 29 ± 12% developed ovules) than the (♀)*C. sativus* ×(♂)*C. melo* cross (18 ± 11%).

In vitro pollination with pollen grains isolated directly from mature anthers was successful. The key results from this experiment are given in Tables 2 and 3. CP and YS media did not differ significantly in the success of in vitro pollination, but more ovules were regenerated in YS (34 ± 14%) than in CP (17 ± 11%).

Of the media for embryogenesis, the results were better in CW medium containing coconut water than in GA medium with gibberellic acid. The intraspecific cross within *C. sativus* gave a higher percentage of developed ovules (36 ± 12%). The development percentages were higher for ovules fertilized with pollen taken directly from anthers than for ovules fertilized with pollen isolated by centrifugation. Developing ovules fertilized by pollen obtained using both types of isolation reached maximum (2 mm length), and only callus formation was observed. Similar results were reported by Ondřej et al. (2002a).

The pollen obtained by direct isolation had higher average viability (88 ± 12% for *C. sativus*;

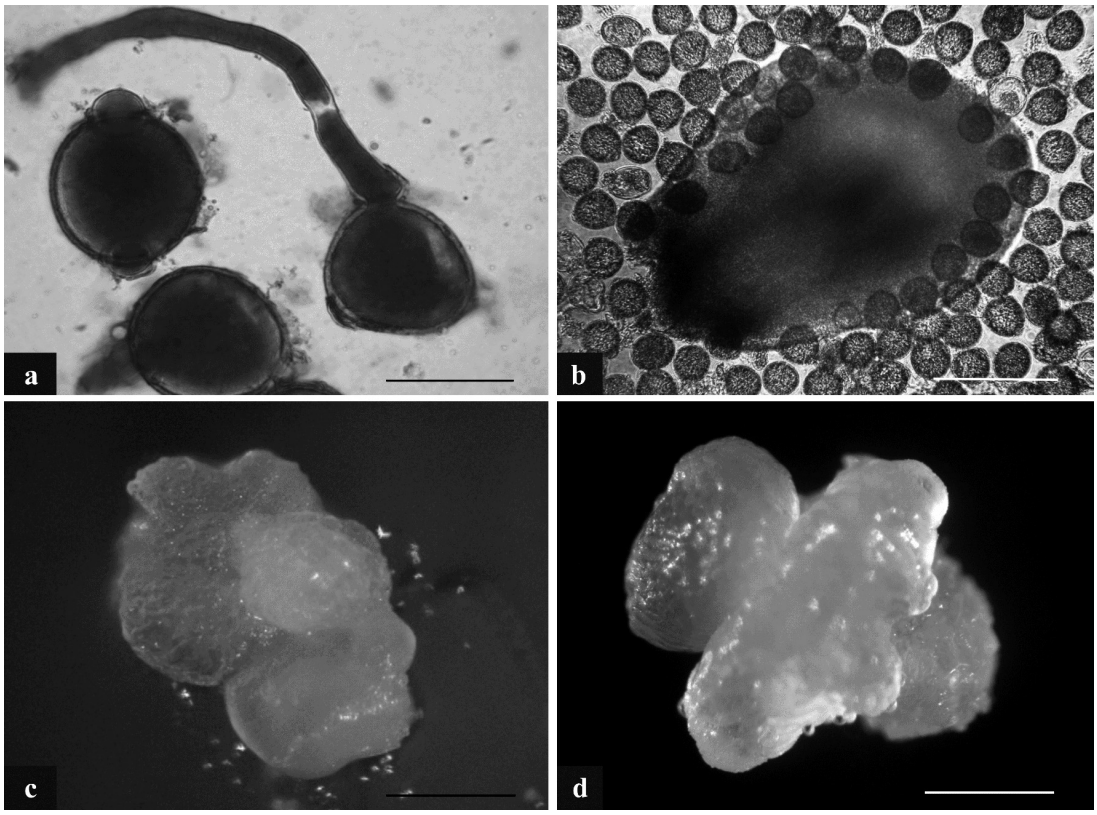

Fig. 1. Mature pollen grains with pollen tubes **(a)**, pollen grains isolated by centrifugation, around ovule **(b)**, developed ovules (CS × CM) fertilized on CP medium, transferred on CW medium, 6 weeks old **(c)**, developed ovules (CS × CS), fertilized on YS medium, transferred on GA medium, 6 weeks old **(d)**. Bar in (a) = 50 μm; (b) = 220 μm; (c) = 340 μm; (d) = 650 μm.

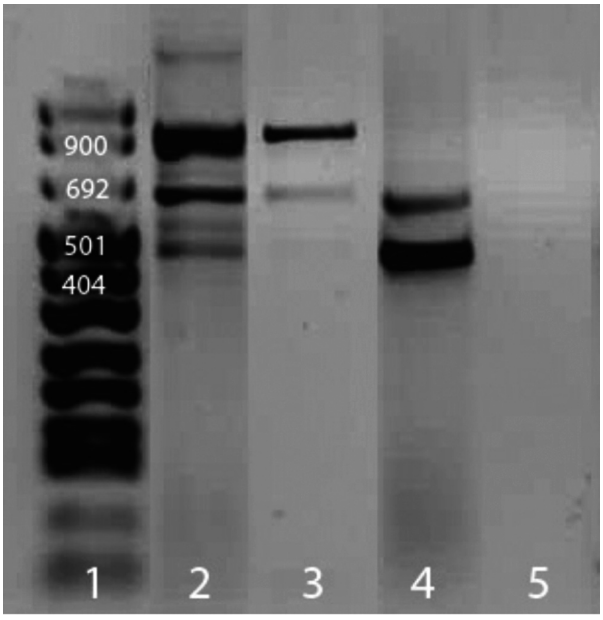

Fig. 2. Comparison of RAPD profiles of parental plants and fertilized ovules. Lane 1 – ladder with marked fragments (bp), lane 2 – profile of *C. sativus*, lane 3 – fertilized ovules showing *C. sativus* patterns, lane 4 – profile of *C. melo*, lane 5 – control sample.

76 ± 13% for *C. melo*) than pollen isolated using centrifugation (68 ± 19% for *C. sativus*; 64 ± 12% for *C. melo*). CP medium gave the best viability of pollen immediately after isolation; for *C. sativus* pollen it gave the highest viability of all the treatments (95 ± 8%).

The success of in vitro pollination, fertilization and embryogenesis depends on a number of exogenous and endogenous factors. The main exogenous factor is the composition of culture media. The most important endogenous factor seems to be ovule and pollen grain maturity. Adding caseinhydrolysate to CP medium increased pollen viability and callus formation. On the other hand, YS basal medium for in vitro pollination increased the number of growing pollen tubes.

RAPD analysis did not confirm the hybrid character of fertilized ovules obtained from in vitro interspecific pollination between cucumber and muskmelon. Only cucumber RAPD patterns were detected (Fig. 2). This result may be attributable to the low amount of muskmelon genome DNA versus the DNA present in the huge mass of cucumber ovule tissue surrounding the possibly hybrid proembryo.

ACKNOWLEDGEMENTS

We thank Dr. Goruin Rogers (University of Sydney, Sydney, Australia) for reading and commenting on the first draft of the manuscript. This research was supported by the Ministry of Agriculture of the Czech Republic (NAZV No. QF 4108) and the Ministry of Education of the Czech Republic (No. MSM 6198959215).

REFERENCES

CASTANO CI, and DE PROFT MP. 2000. *In vitro* pollination of isolated ovules of *Cichorium intybus* L. *Plant Cell Reports* 19: 616–621.

CUSTERS JBM. 1981. Heart shape stage embryos of *Cucumis* species more successful in embryo culture than advanced stage embryos. *Cucurbit Genetics Cooperative Reports* 4: 48–49.

JEFFREY C. 2001. Cucurbitaceae. In: Hanelt P [ed.], *Mansfeld´s Encyclopedia of Agricultural and Horticultural Crops*, 1510–1557. Vol. 3. Springer-Verlag, Berlin, Heidelberg, New York.

KŘÍSTKOVÁ E, LEBEDA A, VINTER V, and BLAHOUŠEK O. 2003. Genetic resources of the genus *Cucumis* and their morphological description (English – Czech version). *Horticultural Science (Prague)* 30: 14–42.

KOO DH, CHOI HW, CHO J, HUR Y, and BANG JW. 2005. A high-resolution karyotype of cucumber (*Cucumis sativus* L. Winter Long) revealed by C-banding, pachytene analysis, and RAPD-aided fluorescence in situ hybridization. *Genome* 48: 534–540.

LARKIN PJ. 1976. Purification and viability determinations of plant protoplasts. *Planta* 128: 213–216.

LEBEDA A, KŘÍSTKOVÁ E, and KUBALÁKOVÁ M. 1996. Interspecific hybridization of *Cucumis sativus* × *Cucumis melo* as a potential way to transfer resistance to *Pseudoperonospora cubensis*. In: Gómez-Guillamón ML, Soria C, Cuartero J, Torés JA, and Fernández-Muòoz R [eds.], Cucurbits Towards 2000. Proceedings of the VIth Eucarpia Meeting on Cucurbit Genetics and Breeding, 28–30 May 1996, 31–37. Malaga, Spain.

LEBEDA A, KUBALÁKOVÁ M, KŘÍSTKOVÁ E, NAVRÁTILOVÁ B, DOLEŽAL K, DOLEŽEL J, and LYSÁK M. 1997. Interspecific hybridization of *Cucumis sativus* L. × *C. melo* L. In: Kobza F, Pidra M, and Pokluda R [eds.], Biological and Technical Development in Horticulture, 9–12 September, 1997, 49–52. Lednice na Moravì, Czech Republic.

LEBEDA A, KUBALÁKOVÁ M, KŘÍSTKOVÁ E, NAVRÁTILOVÁ B, DOLEŽAL K, DOLEŽEL J, and LYSÁK M. 1999. Morphological and physiological characteristics of plants issued from an interspecific hybridization of *Cucumis sativus* × *Cucumis melo*. *Acta Horticulturae* 492: 149–155.

LEBEDA A, WIDRLECHNER MP, STAUB J, EZURA H, ZALAPA J, and KŘÍSTKOVÁ E. 2007. Cucurbits (Cucurbitaceae; *Cucumis* spp., *Cucurbita* spp., *Citrullus* spp.), Chapter 8. In: Singh R [ed.], *Genetic Resources, Chromosome Engineering, and Crop Improvement Series*, vol. 3 – *Vegetable Crops*, 271–376. CRC Press, Boca Raton, FL, U.S.A.

LICHTER R. 1981. Anther culture of *Brassica napus* in a liquid culture medium. *Zeitschrift für Pflanzenphysiologie* 103: 229–237.

MURASHIGE T, and SKOOG F. 1962. A revised medium for rapid growth and bio–assays with tobacco tissue cultures. *Physiologia Plantarum* 115: 473–497.

ONDŘEJ V, NAVRÁTILOVÁ B, and LEBEDA A. 2001. Determination of crossing barriers in hybridization of *Cucumis sativus* and *Cucumis melo*. *Cucurbit Genetics Cooperative Reports* 24: 1–5.

ONDŘEJ V, NAVRÁTILOVÁ B, TARKOWSKI P, DOLEŽAL K, and LEBEDA A. 2002a. *In vitro* pollination as a tool of overcoming crossing barriers between *Cucumis sativus* L. and *Cucumis melo* L. *Acta Facultatis Rerum Naturalium Universitatis Comenianea Botanica* 41: 81–88.

ONDŘEJ V, NAVRÁTILOVÁ B, and LEBEDA A. 2002b. *In vitro* cultivation of *Cucumis sativus* ovules after fertilization. *Acta Horticulturae* 588: 339–341.

POPIELARSKA M. 2005. In vitro pollination of isolated ovules of sunflower (*Helianthus annuus* L.). *Acta Biologica Cracoviensia Series Botanica* 47/1: 85–92.

SKÁLOVÁ D, LEBEDA A, and NAVRÁTILOVÁ B. 2004. Embryo and ovule cultures in *Cucumis* species and their utilization in interspecific hybridization. In: Lebeda A, and Paris HS [eds.], Progress in Cucurbit Genetics and Breeding Research. Proceedings of Cucurbitaceae 2004, the 8th EUCARPIA Meeting on Cucurbit Genetics and Breeding. Palacký University in Olomouc, 415–430. Olomouc, Czech Republic.

SKÁLOVÁ D, NAVRÁTILOVÁ B, and LEBEDA A. 2006. Embryo culture as a tool of interspecific hybridization of *Cucumis sativus* and wild *Cucumis* spp. In: Holmes G [ed.], *Cucurbitaceae*, 51–59. North Carolina State University, Raleigh, NC, U.S.A.

SKÁLOVÁ D, DZIECHCIARKOVÁ M, LEBEDA A, NAVRÁTILOVÁ D, and KŘÍSTKOVÁ E. 2007. Interspecific hybridization *C. anguria* × *C. zeyheri*, *C. sativus* × *C. melo* and *C. sativus* × *C. metuliferus* through embryo cultures. *Acta Horticulturae* 731: 77–82.

SKÁLOVÁ D, NAVRÁTILOVÁ B, and LEBEDA A. 2008. Embryo-rescue of cucumber (*Cucumis sativus*), muskmelon (*C. melo*) and some wild *Cucumis* species (*C. anguria*, *C. zeyheri*, and *C. metuliferus*). *Journal of Applied Botany and Food Quality* 82: 83 – 89.

TILTON VR, and RUSSELL SH. 1984. Applications of in vitro pollination/fertilization technology. *BioScience* 34: 239–242.

VIŽINTIN L, and BOHANEC B. 2004. *In vitro* manipulation of cucumber (*Cucumis sativus* L.) pollen and microspores: Isolation procedures, viability tests, germination, maturation. *Acta Biologica Cracoviensia Series Botanica* 46: 177–183.

ZENKTELER M, BAGNIEWSKA-ZADWORNA A, and ZENKTELER E. 2005. Embryological studies on ovules of *Melandrium album* pollinated *in vitro* with *Lychnis coronaria* pollen grains. *Acta Biologica Cracoviensia Series Botanica* 47/1: 135–138.