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IN VITRO POLLINATION OF ISOLATED OVULES OF SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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A model system for in vitro self-pollination of isolated sunflower (*Helianthus annuus* L.) ovules was developed. The pollinated ovules were cultured on modified MS medium supplemented with 3% sucrose, glycine, casein hydrolysate, and growth regulators including indole–3-acetic acid (IAA), kinetin (KIN) and gibberellic acid (GA_3). The frequency of developed seedlings and dissected embryos after in vitro pollination was rather low, 2.2% and 2.3%, respectively. Gynogenic seedlings were not produced in culture of unpollinated ovules. Embryological study revealed abnormalities in embryo and endosperm development after in vitro pollination. In vitro culture did not stimulate egg cell induction or autonomous endosperm development in unpollinated ovules cultured on the same media.

Key words: *Helianthus annuus*, in vitro pollination, ovules, embryos, endosperm, germination, pollen.

INTRODUCTION

In vitro pollination and fertilization are terms referring to pollen tube penetration of the embryo sac by means other than the normal in situ process (Stewart, 1981). The techniques of in vitro pollination and fertilization were pioneered by scientists at the University of Delhi, India. The formation of viable seeds in vitro after the application of pollen to the ovule surface of excised placentae was first reported in the early 1960s for the poppy *Papaver somniferum* (Kanta et al., 1962). Further work on in vitro fertilization focused mainly on application of the original method used for *Papaver* to other plant material. The best results were obtained in species with large ovaries containing many ovules, from Brassicaceae, Caryophyllaceae, Papaveraceae, Liliaceae, Primulaceae and Solanaceae (for review see: Rangaswamy, 1977; Zenkteler, 1999).

Direct in vitro pollination of ovules may be useful in overcoming some stigma/style incompatibility barriers. For example, Shivanna and Rangaswamy (1969) used this method to eliminate gametophytic self-incompatibility in *Petunia axillaris*. Another use of placental or ovular pollination is production of interspecific and intergeneric hybrids (Zenkteler, 1980). Recently, a report of wide crosses between angiosperms and gymnosperms demonstrated the possibility of germination and pollen tube growth on placentas and ovules of very distant genera (Zenkteler and Relska-Roszak, 2003). In vitro pollination has also been successfully applied for induction of haploids by distant pollination or by pollination with inactive pollen (Hess and Wagner, 1974; Musiał and Przywara, 1998). Finally, in vitro pollination is useful for studying pollen physiology and fertilization (Dupuis and Dumas, 1989, 1990).

In vitro pollination of single ovules is a technique much more complicated than placental pollination, that is, pollination of ovaries with large placentas containing many ovules. Up to now, only three successful experiments with in vitro pollinated isolated ovules have been reported. Seedlings were obtained in self-pollinated excised ovules of *Brassica oleracea* (Kameya et al., 1966) and *Cichorium intybus* (Castaño and de Proft, 2000), and in interspecific cross-pollination of *Gossypium* (Stewart, 1981). Experiments with fertilization of isolated ovules of several species of Fabaceae did not result in seedling formation. Embryo development was arrested at the globular stage (Tilton and Russel, 1984; Zenkteler, 1980, 1990). Globular embryos were formed after in vitro self-pollination of isolated ovules of *Trifolium rubens*, in experiments of inter-genera (*Trifolium rubens* × *Cytisus albus*) in vitro pollination (Zenkteler, 1980, 1990), and as an effect of inter-cultivar in vitro pollination of *Prunus* (Dziedzic et al., 1999).

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The aim of the presented work was to develop a model system for in vitro self-pollination of isolated ovules of sunflower, and to achieve embryo and seedling development using this technique. The method could be useful in future experiments with in vitro fertilization of isolated embryo sacs of sunflower.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *Helianthus annuus* L. cv. Frankasol obtained from Systeme O.C.D.E. (Semences CARGILL, 40305 Peyrehorade, France) were sown in pots. Seedlings were cultivated in the greenhouse of the Department of Plant Physiology, Polish Academy of Sciences in Cracow, under a 16 h photoperiod at 28˚C/22˚C (day/night). At blooming, mature inflorescences were hand-pollinated. Fertilized flowers were used as control material.

POLLEN GERMINATION AND VIABILITY

Pollen germination was tested on media solidified with 2% agar and gelatine, supplemented with 3, 10, 20, 30 or 40% sucrose. Pollen grains were put on a slide with a layer of medium and kept in a humid chamber under a 16 h photoperiod (cool white fluorescent tubes, avg. 70–100 mol $m^{-2}s^{-1}$) at 25 ± 3˚C.

Pollen grain viability was determined using fluorescein diacetate (FDA, Sigma). Stock solution (5 mg/ml in acetone) was diluted with 0.6 M sucrose to make a 10 µg/ml solution (Yang, 1986; Wędzony, 1996). One drop of staining solution was added to 1 ml medium with fresh pollen. After 5 min incubation the slides were observed with a fluorescence microscope (Nikon HB–10101 AF with a Nikon FX–35 DX camera) at 485 nm. Viable pollen grains and pollen tubes stained yellowish green. In total, 600 pollen grains originating from three flower heads (200 per inflorescence) were analyzed.

IN VITRO POLLINATION AND OVULE CULTURE TECHNIQUE

For in vitro pollination, 1,762 ovules were isolated from tubular florets with fully extended, opened anthers and without visible stigma. In tubular florets (stage I) the ovules contained mature, unfertilized embryo sacs with an egg apparatus at the micropylar pole and a central cell with a secondary nucleus resulting from the fusion of two polar nuclei (Popielarska and Przywara, 2003).

To find optimal culture conditions for development of fertilized ovules, 120 ovules isolated from in situ pollinated flowers were cultured: 70 ovules represented stage II with a zygote or few-celled proembryo, and 50 stage III with a globular embryo (Popielarska and Przywara, 2003).

TABLE 1. Media used for in vitro pollination and culture of isolated sunflower ovules

Ingredient	Medium	
	A	B
Macroelements	MS[a]	MS
Microelements	MS	MS
Vitamins	MS	MS
Sucrose (%)	3	3
Casein hydrolysate (mg l^{-1})	500	500
IAA (mg l^{-1})	4	0.5
Kinetin (mg l^{-1})	0.5	0.5
GA_3 (mg l^{-1})	5	4
Glycine (mg l^{-1})	9.5	7.5
Agar (%)	0.8	0.8

[a]Murashige and Skoog (1962).

For gynogenesis induction, 262 unpollinated ovules excised from florets at stage I were cultured on the same media.

Before sterilization, the sepals and the corolla tube with style and anther filaments were removed. Intact ovaries were sterilized in a diluted commercial bleach solution for 10 min and washed three times with sterile distilled water. The ovules were dissected from the ovaries and inoculated (10–15 ovules per Petri dish). A total of 2,144 ovules were cultured.

The pollen used for in vitro pollination originated from opened, non-disinfected anthers of the same floral head as the isolated ovules. The ovules were self-pollinated with pollen placed directly on their micropylar part and on medium near the micropylar part of the ovule.

The ovules were placed horizontally on solid medium containing MS (Murashige and Skoog, 1962) macronutrients, micronutrients, vitamins, 3% sucrose, glycine, protein and two combinations (medium A and B) of growth regulators (Tab. 1). The pH was adjusted to 5.8 and the medium was autoclaved. Solutions with hormones, glycine and proteins were filter-sterilized (Millipore) and added to the autoclave-sterilized parts of the medium.

Petri dishes with inoculated ovules were placed in a culture room under a 16 h photoperiod (cool white fluorescent tubes, avg. 70–100 µmol $m^{-2}s^{-1}$) at 25 ± 3˚C and 60% relative humidity.

EMBRYOLOGICAL STUDY

Unpollinated and in vitro pollinated ovules after 10 days of culture, and ovules 10 days after in situ pollination were fixed in glutaraldehyde. Then the material was washed four times in phosphate buffer (PBS), dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 96%), 15 min in each concentration, and kept overnight in absolute ethanol at room temperature. Samples were infiltrated in 3:1, 1:1 and 1:3 solutions of absolute ethanol and Technovit (Heraeus Kulzer

GmbH, Germany) for 1 h in each solution and kept for 12 h in pure Technovit. The resin was polymerized with the addition of hardener. The material was sectioned at 3–4 μm using a rotary microtome (Microm, Adamas Instrumenten, Walldorf, Germany), placed on slides, stained with toluidine blue, mounted in Entellan (Merck) and covered with a cover slip.

The microscope images were processed using a digital JVC TK-C 1380 color video camera and analyzed with Image-Pro Plus, ver. 3.0 for Windows.

KARYOLOGICAL STUDY

Root tips of seedlings produced after in vitro pollination of isolated ovules were pretreated with a saturated solution of α-bromonaphtalene and stored for 24 h at 4˚C. After three washes with distilled water, the material was fixed in acetic alcohol (1:3 glacial acetic acid/96% ethanol) for 2 h. Part of the material was macerated in 1N HCl solution (10 min at 60˚C) and squashed in a drop of 45% acetic acid, and the cover glasses were removed with dry ice. Then the slides were air-dried, stained with toluidine blue and mounted in Entellan (Merck). The rest of the material was stained in toto with acetic orcein for 3–7 days and squashed in a drop of 45% acetic acid

RESULTS

VIABILITY AND GERMINATION OF POLLEN GRAINS

Pollen grains germinated poorly in vitro on all media used. The frequency of germinating grains was rather low 20–30 min after inoculation (Tab. 2). The pollen grains started to germinate but later their growth was stopped. The pollen tubes were swollen, twisted and/or burst. High sucrose concentrations (30 and 40%) arrested pollen germination completely.

The frequency of viable pollen grains in the FDA test was very high, reaching 97.5%. To determine the duration of pollen tube viability, germinated pollen grains were incubated in FDA solution. After 1–3 h incubation, 87–95% of the germinated pollen tubes showed fluorescence (Tab. 3). After 7 h incubation, pollen grain viability was dramatically reduced to 0.7%.

IN VITRO POLLINATION AND OVULE CULTURE

In vitro pollination of isolated ovules

A total of 1,762 isolated ovules were self-pollinated in vitro. After germination, pollen tubes grew both toward and away from isolated ovules. Some pollen tubes were twisted.

During culture the isolated ovules enlarged slightly, with no change in their yellowish white color. Developing green embryos were clearly visible through the ovule wall. No ovule-derived calli were observed.

TABLE 2. Efficiency of pollen germination; average (%) of 3 replicates

Medium with sucrose concentration (%)	No. of germinated pollen grains
3	32.5
10	21.1
20	4.3
30	-
40	-

TABLE 3. Viability test of pollen grains and pollen tubes, tested by fluorescein diacetate; average (%) of 3 replicates

Time of inoculation (h)	Viability of pollen grains and pollen tubes
0	97.5
1	95.2
3	86.7
4	59.3
5	23.1
6	7.8
7	0.7

Seedlings developed two to four weeks after in vitro pollination; 50% of the seedlings germinated 12–21 days after inoculation. No pericarp was observed because the ovary wall was removed before culture. The seedlings showed great variability in their structure. Some seedlings had leaves with a shoot apex and no radicula (Fig. 3), and others formed leaves and radicula. Endosperm attached to developing seedlings was frequently seen (Figs. 1, 2). Seedling-derived calli were observed occasionally (Fig. 1b). Outgrowing endosperm ruptured the ovule wall in a few cases (Fig. 2); no embryos were detected in such ovules.

Seedlings were transferred to hormone-free MS medium 4–6 weeks after germination; 4–6 weeks after that transfer, the plants developed flower buds (Fig. 4), and 28.2% of the plants produced abnormal leaves (Fig. 5).

Six weeks after inoculation, embryos at different stages of development (globular, heart, torpedo-shaped) were dissected (Tab. 4), with globular-stage embryos most frequent. There were no significant differences between the media used (t-test for independent samples; $p < 0.05$).

In vivo fertilized ovules isolated and cultured on the same media as in vitro pollinated ovules developed with high efficiency. The frequency of obtained seedlings from ovules isolated at the zygote, few-celled proembryo and globular embryo stage was relatively high, reaching 56% and 98%, respectively.

No seedlings or embryos were noted from the 262 unpollinated ovules cultured on the same media.

TABLE 4. Results of culture of isolated, in vitro pollinated sunflower ovules; % in parenthesis

	Medium A					Medium B				
	No. of inoculated ovules	No. of obtained seedlings	No. of dissected embryos*			No. of inoculated ovules	No. of obtained seedlings	No. of dissected embryos*		
			glob	heart	torp			glob	heart	torp
	121	-	-	-	1 (0.8)	86	1 (1.1)	1 (1.1)	-	-
	150	5 (3.3)	2 (1.3)	-	1 (0.6)	125	3 (2.4)	1 (0.8)	-	-
	210	-	2 (0.9)	-	2 (0.9)	195	2 (1.0)	2 (1.0)	1 (0.5)	2 (1.0)
	130	8 (6.1)	1 (0.7)	-	-	112	3 (2.6)	2 (1.7)	1 (0.8)	1 (0.8)
	105	5 (4.7)	3 (2.8)	1 (0.9)	2 (1.9)	87	-	1 (1.1)	-	-
	94	4 (4.2)	4 (4.2)	-	3 (3.1)	135	4 (2.9)	-	-	2 (1.4)
	97	2 (2.0)	3 (3.0)	-	-	115	2 (1.7)	2 (1.7)	-	-
Total	907	24 (2.6)	14 (1.5)	1 (0.1)	9 (0.9)	855	15 (1.7)	11 (1.2)	2 (0.2)	5 (0.5)
				25 (2.8)					16 (1.9)	
		2.9±2.3**		3.0±2.5**			1.6±1.0**		1.7±0.9**	

*stage of embryo development: glob – globular; torp – torpedo-shaped.
**mean ± standard deviation

EMBRYOLOGICAL STUDY OF ISOLATED OVULES CULTURED IN VITRO

Embryological analysis of ovules isolated 10 days after in situ pollination showed a heart-shaped embryo and cellular endosperm. In ovules 10 days after in vitro pollination, a globular embryo and cellular endosperm were observed, indicating delayed embryo development. Ovules with degenerated embryo sacs and pollen tube remnants in the micropylar part accompanied normally developed ovules (Fig. 6). Globular embryos formed after in vitro pollination differed from the control embryos in shape, structure, size, localization (toward the chalazal pole) and lack of a suspensor (Figs. 7–9). In most analyzed sections, the endosperm was callus-like tissue with large, vacuolated cells. No sporophytic tissue proliferation was observed.

Unpollinated ovules fixed 10 days after inoculation contained degenerated embryo sacs. No signs of egg cell parthenogenesis and/or autonomous endosperm induction were noted.

KARYOLOGICAL STUDIES

The somatic chromosome number of *Helianthus annuus* is 2n = 34, as established previously (Bolkhovskikh et al., 1969). Karyological variability, both intra- and interplant, was found in the root meristematic tissue of four seedlings produced by in vitro pollination (Tab. 5). Descending aneuploidy (2n = 32 – 22) but not polyploidy was involved in karyological differentiation. Analysis of 17 metaphase plates suggests that the obtained seedlings were zygote-derived, as the haploid number (n = 17) was not counted.

DISCUSSION

Although pollen germination was observed on isolated ovules, it is evident that not all ovules were fertilized. Pollen tube viability and localization were checked simultaneously with FDA staining. Although Yang (1986) reported that a single pollen tube could keep fluorescence 24 h or even longer after fluorochrome incubation, in the current experiment no viable pollen tubes were observed after 6 h of staining. That finding, along with the low rate of pollen tube growth, might indicate that the culture conditions were not optimal for pollen germination. Sugar concentrations and addition of borate and calcium are the most important factors for pollen tube germination (Higashiyama et al., 1998; Vervaeke et al., 2004). Modification of the medium and semi in vivo techniques could improve pollen germination and tube growth in sunflower.

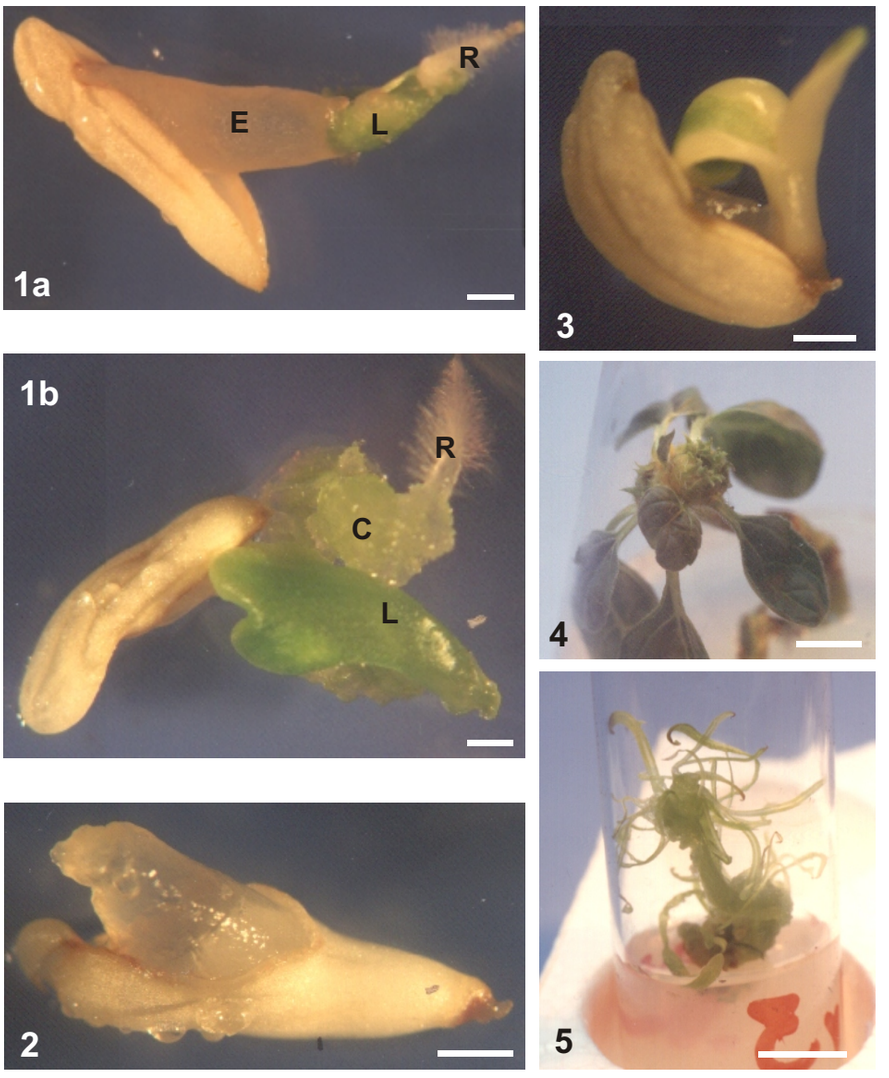
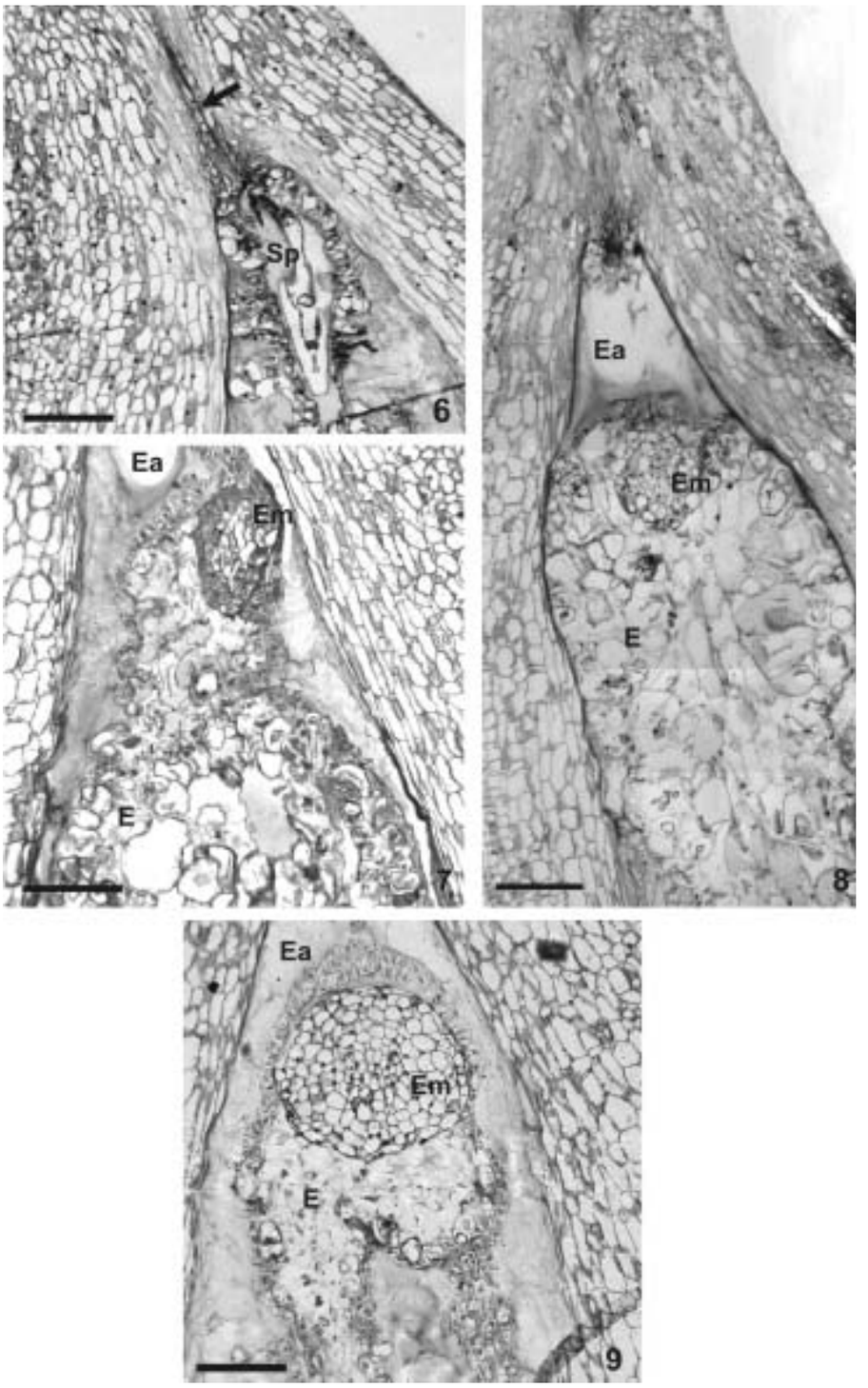


Fig. 1. Germination of ovules after 22 (**a**) and 26 (**b**) days of culture; endosperm (E) has been resorbed during plantlet development. **Fig. 2.** Endosperm outgrowing the ovule after 21 days of culture. **Fig. 3.** Plantlet germination without the formation of callus after 18 days of culture. **Fig. 4.** Plant producing flower buds after 6 weeks of culture. **Fig. 5.** Plants with abnormal leaves after 5 weeks of culture. C – callus; E – endosperm; L – leaf; R – radicula. Bars = 1 mm in Figs. 1, 2, 3; 1 cm in Figs. 4, 5.

The abnormalities accompanying embryo development after in vitro self-pollination of isolated ovules were similar to those found in embryo sac culture (Popielarska and Przywara, 2003). In the current study, globular embryos resulting from in vitro pollination differed in size and shape (pear-like) from that described by Newcomb (1973a,b). Additionally, the embryos had atypical localization in the embryo sac: they were deep in the embryo sac, far from the micropylar region. The empty area observed between the embryo and the micropylar part of the embryo sac probably was due to the lack of a suspensor. Abnormal positioning of embryos developed in culture is a known phenomena, observed in gynogenic embryos of *Allium cepa* (Musiał et al., 2001).

The absence of endosperm or its rapid digestion at the zygote or young embryo stage in ovule culture has been reported in different plant species (Kapoor, 1959; Töpfer and Steinbiss, 1985; Zenkteler and Nitsche, 1985). There are some examples of endosperm development in ovules pollinated in vitro, for example in *Prunus* (Dziedzic et al. 1999). This was also observed in the present experiments. Endosperm accompanied the developing embryos. In some ovules, endosperm was so



Figs. 6–9. Isolated in vitro pollinated ovules after 10 days of culture; longitudinal sections of the micropylar part of the ovules, stained with toluidine blue. Bars = 300 µm. **Fig. 6.** Degenerating shrinkage protoplast (Sp) of embryo sac; arrow indicates remnants of pollen tube. **Figs. 7–9.** Abnormal globular-stage embryo (Em) with cellular endosperm (E); embryo is located close to chalazal part of embryo sac; near micropylar part an "empty" area (Ea) is visible.

TABLE 5. Results of karyological study of seedlings obtained via in vitro pollination

No. of plant	No. of metaphase plates	Chromosome number
1.	4	22; 26; 29; 30
2.	4	29; 31; 31; 31
3.	8	25; 27; 28; 29; 30; 30; 32; 34
4.	1	30

abundant that it disrupted the ovule tissues. Moreover, outgrowing endosperm quite frequently persisted during the early stage of seedling germination. In a few cases, embryo formation did not accompany the endosperm, which developed into a mass disrupting the ovule wall. The absence of an embryo can result from (1) inhibition of embryo development at an early stage by proliferating endosperm, resulting finally in embryo degeneration, (2) the lack of egg cell fertilization, with only the secondary nucleus of the central cell fertilized, or (3) lack of fertilization of both the egg cell and the secondary nucleus, with endosperm developed autonomously. Autonomous endosperm induction but not egg cell parthenogenesis was found in culture of unpollinated ovules of *Viola odorata* (Wijowska et al., 1999a,b), *Allium cepa* (Musiał et al., 2001) and *Brassica napus* (Rojek et al., 2002).

The efficiency of in vitro pollination of isolated sunflower ovules was quite low, with 4.5% developed embryos. Only 2.2% of the ovules developed into seedlings; 2.3% stopped development at the globular, heart or torpedo-like torpedo-shaped stage. Examination of a number of dissected globular embryos has confirmed that this stage of embryo development is a critical one (e.g., Góralski and Przywara, 1998). Higher efficiency of embryo development was achieved after in vitro cross-pollination of plum ovules; 21% to 40% of the pollinated ovules contained embryos at different stages of their development (Dziedzic et al., 1999). In contrast, in self-incompatible *Cichorium intybus* only 0.7% of in vitro self-pollinated isolated ovules produced seedlings (Castaño and de Proft, 2000). The low frequency of seedling development resulted from embryo degeneration at the globular or heart-shaped stages. The high percentage of developed embryos in *Prunus* could be the result of culture conditions. In vitro pollination was done on medium with 15% sucrose. For regeneration, ovules were transferred to medium with 3.4% sucrose and supplemented with BAP (Dziedzic et al., 1999). The positive influence of this growth regulator was also noted in culture of isolated embryo sacs of *Helianthus annuus* (Popielarska and Przywara, 2003). It should be emphasized, however, that no seedlings were obtained after in vitro pollination of isolated plum ovules. Moreover, in the present study, in vivo pollinated ovules isolated at zygote or globular embryo stage developed very well on the same media.

Karyological study indicated the diploid level of sunflower plantlets obtained from in vitro pollinated ovules, suggesting their zygote origin. However, karyological variability was found in the analyzed material, with chromosome numbers ranging from 2n=22 to 2n=34. Intra- and interplant chromosome number differentiation observed in roots could be an effect of in vitro culture conditions. Structural chromosome aberrations, genome mutations (reduction or multiplication) and disturbances in mitotic divisions resulting in aneuploidy are phenomena well known in material cultured in vitro (for review see: Bayliss, 1975; D'Amato, 1995).

We succeeded in obtaining seedlings after in vitro pollination of isolated sunflower ovules. The low percentage of developed embryos and plantlets may be the result of disturbances in embryo and endosperm development under in vitro conditions and/or the lack of ovule fertilization, as pollen germination was rather low despite high pollen viability. Nevertheless, successful application of the model system for in vitro pollination of single isolated ovules is possible in sunflower. The present data should prove useful in future studies of in vitro pollination and fertilization.

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