In vitro induction of haploid plants in unpollinated ovules, anther and microspore culture of *Cucumis sativus*

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Abstract
In order to increase the yield of haploid cucumber plants obtained from in vitro culture of unpollinated ovules, anther and microspore, we have studied several factors: the nature and concentration of plant growth regulators, the genotypes, the flower bud lengths, and the stages of microspore development. Among the different growth regulators tested, thidiazuron (TDZ) was the best for gynogenesis induction with an optimal concentration of 0.2 mg.L\(^{-1}\). Out of the ten evaluated genotypes, only cultivar ‘Gordion’ was able to develop the haploid plant through in vitro gynogenesis. Based on the histological studies we found that the best stages of the anther development for haploid induction are the late uninucleate microspore and the early bicellular pollen grains. Anther and microspore cultures differed in the embryogenic callus formation; callus was formed from anther somatic cells and from vegetative cells of pollen grain in anther and microspore cultures respectively.

INTRODUCTION
The androgenesis, gynogenesis and parthenogynesis are widely used for modern breeding programs of vegetables as a source of doubled haploid lines (DHLs). The parthenogynesis is mostly used for cucumber DHLs production, while the application of androgenesis and gynogenesis to *Cucumis sativus* is still limited (Gemes-Juhasz et al. 2002; Ashok Kumar and Murthy 2004; Claveria et al. 2005; Song et al. 2007). Such factors as the plant genotype, growth conditions of the donor plants, the microspore developmental stage, pre-treatment of flower buds, the media and the culture conditions can influence androgenesis and gynogenesis (Bajaj 1990). The object of the present investigation was to determine the effect of different growth regulators, plant genotype, and stage of microspore development on induction of gynogenesis and androgenesis in cucumber.

MATERIAL AND METHODS

Plant material and surface sterilization of flower buds
Ten cultivars of *C. sativus*, namely, ‘Hiziz’, ‘Gordion’, ‘Hana’, ‘Malen’, ‘Kadet’, ‘Asak’, ‘Reisa’, ‘Tristan’, ‘Tarantutka’, and ‘Rostovchanin’ were used in experiments with unpollinated ovules culture. Cultivars ‘Wodolei’ and ‘Rodnichok’ were used in experiments with anther and isolated microspores cultures. Buds and
Ovaries were excised and surface-sterilized in 0.1% HgCl\textsubscript{2} for 10 min, followed by three 5-min long rinses with sterile-distilled water.

**Ovules culture**

Unfertilized ovaries were harvested 6h before anthesis, during anthesis, and two days after anthesis. Ovules were isolated from ovaries and cultured on induction MSm medium (Masuda et al. 1981) supplemented with 5% sucrose and various plant growth regulators. Ovule culture was incubated for two weeks at 22°C in the dark. Proliferated ovules were transferred onto MSm medium with 3% sucrose, 0.05 mg.L\textsuperscript{-1} NAA (1-naphthaleneacetic acid) and 0.2 mg.L\textsuperscript{-1} BA (6-benzylaminopurine) and incubated under a 14-h photoperiod (cool-white fluorescent light, 2-2.500 lx,) at 22°C for two weeks. Differentiated callus was subcultured onto MSm medium with 3% sucrose, 0.02 mg.L\textsuperscript{-1} NAA, and 0.4 mg.L\textsuperscript{-1} BA. The developed shoots and plantlets were transferred to the same medium without growth regulators.

**Evaluation of genotypes and hormones**

Ovules of the ten cultivars were cultured on MSm medium supplemented with 5% sucrose and various plant growth regulators: MSm with 2.0 mg.L\textsuperscript{-1} GA (gibberellic acid); MSm with 0.1 mg.L\textsuperscript{-1} TDZ and 0.2 mg.L\textsuperscript{-1} BA; MSm with 2.0 mg.L\textsuperscript{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) and 4.0 mg.L\textsuperscript{-1} BA; MSm with 0.2 mg.L\textsuperscript{-1} 2,4-D.

**Evaluation of hormone concentrations**

Ovules of the cultivar ‘Gordion’ were cultured on MSm medium supplemented with 5% sucrose and various concentration of plant growth regulators: 0.2 mg.L\textsuperscript{-1} TDZ and 0.2 mg.L\textsuperscript{-1} BA; 0.1 mg.L\textsuperscript{-1} TDZ and 0.1 mg.L\textsuperscript{-1} BA; 0.05 mg.L\textsuperscript{-1} TDZ and 0.05 mg.L\textsuperscript{-1} BA.

**Anther culture**

The flower buds of different length containing microspores and pollen grains at the different developmental stages were harvested (Tab. 1). Anthers were placed on MSm medium containing 8% sucrose, 100 mg.L\textsuperscript{-1} serine, 800 mg.L\textsuperscript{-1} glutamine and various plant growth regulators (2.0 mg.L\textsuperscript{-1} 2,4-D and 1.0 mg.L\textsuperscript{-1} BA; 1.0 mg.L\textsuperscript{-1} 2,4-D and 0.5 mg.L\textsuperscript{-1} BA; 0.4 mg.L\textsuperscript{-1} 2,4-D and 0.2 mg.L\textsuperscript{-1} BA). The anther cultures were kept in the dark at 35°C for 72 h followed by four weeks at 22°C. The developed embryogenic callus was subcultured onto MSm medium supplemented with 2% sucrose, 0.2 mg.L\textsuperscript{-1} BA and 0.05 mg.L\textsuperscript{-1} NAA. The cultures were incubated in the dark at 22°C for one week after inoculation and then at 22°C and 14-h photoperiod at 2-2.500 lx.

In order to get microspore culture, anthers were macerated in NLN liquid medium (Lichter 1982) with 10% sucrose and without the growth regulators at pH 5.8. Microspores were obtained by filtration through a nylon screen, and were then washed three times with NLN medium by centrifugation at 100 x g for 3 min. Microspores were suspended and cultured in NLN medium containing 10% sucrose, 2.0 mg.L\textsuperscript{-1} 2,4-D. Microspore cultures were incubated at 22°C in the dark.

**Cytological studies**

Anthers were fixed in fixative (3:1: ethanol, acetic acid) for 24 h, washed with 70% ethanol and stained with 2% acetocarmine solution.
RESULTS AND DISCUSSION

Unpollinated ovules culture

Out of the ten cultivars tested, only embryo sac of cultivar ‘Gordion’ resulted in increase volume when the unpollinated ovules were cultured on induction MSm medium with TDZ. Although the cells of embryo sac became viable, they were unable to divide and yield callus and embryos. Ovules of other cultivars did not develop and cells of embryo sac degenerated. Maximum frequency of gynogenesis was 3.5%, when the ovules of cultivar ‘Gordion’ were cultured in MSm medium containing 0.2 mg L$^{-1}$ TDZ and 0.2 mg L$^{-1}$ BA. The maximal yield of viable plants (0.5%) was obtained on MSm medium supplemented with 3% sucrose, 0.02 mg L$^{-1}$ NAA, and 0.4 mg L$^{-1}$ BA. The regenerated plants differed from donor-plant in leaf size and color and in type of flowering. Thus, the supplementation with 0.2 mg L$^{-1}$ of TDZ was optimal for gynogenic induction of cucumber as opposed to the gynogenic induction of carrot, onion, and sugar beet (Campion and Alloni 1990; Shmikova and Tjukavin 1994; Znamenskaya et al. 1994).

Figure 1. Anther culture of C. sativus L. (cultivar ‘Rodnichok’) on MSm medium supplemented with 2 mg L$^{-1}$ 2,4-D and 1.0 mg L$^{-1}$ BA. (a) Section of anther after two weeks of culture (x160). Arrows indicate division of somatic cells. (b) Compact callus developed from somatic cells after four weeks of culture (x10).

Figure 2. Development of C. sativus L. (cultivar ‘Rodnichok’) callus in microspore culture (x720). (a) Freshly isolated microspore at the late uninucleate stage. Nucleus displaces to a peripheral position. (b) Freshly isolated vacuolated bicellular pollen grain. (c), (d), and (e) Initial stage of callus formation from vegetative cell of pollen grain after two weeks of culture in NLN medium supplemented with 2,4-D.

Anther and microspore culture

Anthers culture produced embryogenic callus from low differentiated cells of endothecium and connectiva (Fig. 1). Cytological analysis of microspore culture showed that induction of androgenesis was observed only in anthers harvested from the flower buds longer than 6mm (magnification = 720×) (Tab. 1, Fig. 2 a, b). The anthers contained the late uninucleate microspores and early bicellular pollen grains. It was shown that embryogenic callus was formed from vegetative cell of pollen grain.
when microspores were cultured in NLN medium supplemented with 2,4-D (Fig. 2 c, d, e).

The present results suggest that using microspore culture is more effective than anther culture for induction of haploid plants of cucumber. Studies of plant regeneration from callus obtained from microspore culture are currently being carried out.

Table 1. Microspore and pollen grain development in anther of cucumber

<table>
<thead>
<tr>
<th>Length (mm)</th>
<th>calyx</th>
<th>corolla</th>
<th>Stages of microspore and pollen grain development</th>
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<tr>
<td>4.0-5.0</td>
<td>2.0</td>
<td>Tetrads, microspores released from tetrads</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>2.5</td>
<td>Lightly vacuolated young microspores with centrally located nucleus</td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>3.0</td>
<td>Microspores with increased vacuole and peripherally located nucleus</td>
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</tr>
<tr>
<td>6.0</td>
<td>4.0</td>
<td>Microspores division and development of vegetative and generative cells</td>
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<tr>
<td>8.0</td>
<td>10.0</td>
<td>Bicellular vacuolated pollen grains</td>
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</tr>
<tr>
<td>8.0</td>
<td>11.0</td>
<td>Devacuolation of vegetative cell</td>
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<tr>
<td>10.0</td>
<td>15.0</td>
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<tr>
<td>12.0</td>
<td>18.0</td>
<td>Disappearance of vacuole</td>
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<td>20.0</td>
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<tr>
<td>15.0</td>
<td>25.0</td>
<td>Mature pollen grains</td>
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Literature cited


