Methods of isolation of *Cucumis sativus* and *C. melo* pollen grains and their utilization in *in vitro* pollination

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**Abstract**

Optimizing manipulation with pollen grains is necessary for successful *in vitro* pollination. This pollination can be used for overcoming crossing barriers in interspecific hybridization in the genus *Cucumis*. Pollen grains from *Cucumis sativus* and *C. melo* were isolated by various methods. Different techniques of centrifugation were compared with direct isolation of pollen grains from anthers. The viability of pollen grains was evaluated and their ability to form pollen tubes was tested. Three isolation media (NLN, YST and VB) and two cultivation media (YS and CP) were used. Then pollen grains were cultivated with ovules of *C. sativus* and the level of regeneration was observed. The growing and green ovules were considered as the lowest level and the highest level was the formation of callus.

**INTRODUCTION**

The technique of *in vitro* placental or ovular pollination can be used for production of interspecific and intergeneric hybrids (Zenkteler 1980). It can help overcome crossing barriers to interspecific hybridization (Ondřej et al. 2002). Interspecific hybridization is an important research area in the genus *Cucumis* because of the possibility of transferring the genes for resistance to various diseases from wild *Cucumis* to the cucumber (*Cucumis sativus* L.) genome. For successful transfer of these genes, the use of some *in vitro* techniques has been suggested (Lebeda et al. 2007).

The first experiment of direct pollination of ovules was made by Kanta and Maheswari (1963) with some species of Papaveraceae. The whole process of sexual reproduction was observed. Well-developed seedlings were obtained after *in vitro* intraspecific pollination of *Cichorium intybus* L. by Castano and De Proft (2000), too. Zenkteler et al. (2005) used intergeneric *in vitro* pollination of *Melandrium album* and *Lychnis coronaria* and hybrid plants were observed.

The successful use of *in vitro* pollination has not yet been reported in the genus *Cucumis*. Manipulation with pollen and microspores of *C. sativus* has been tested (isolation procedures, viability tests, germination, maturation) (Vižintin and Bohanec 2004). Some attempts at *in vitro* pollination between *C. sativus* and *C. melo* were made, but no hybrid plants were observed and only calluses were obtained (Ondřej et al. 2002).

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The main aim of this study was to optimize the isolation of *C. sativus* and *Cucumis melo* pollen grains and use them for *in vitro* pollination of *C. sativus* ovules.

**MATERIALS AND METHODS**

**Plant material**
Two accessions of *Cucumis* species were used for testing the viability of pollen grains and *in vitro* pollination (*C. sativus* – CS; *C. melo* – CM) (*C. sativus*– ‘Stela F1’, CZ 09H3900744; *C. melo* – PMR, CZ 09H4000596, CZ 09H4000597, CZ 09H4000599; donor RICP). The plant material originated from the vegetable germplasm collection of the Research Institute of Crop Production (RICP, Prague), Department of Gene Bank, at Olomouc, Czech Republic (Web site: <www.vurv.cz/>, part databases, EVIGEZ). The plants were cultivated in a glasshouse (25°C/15°C day/night) at the Department of Botany, Palacký University, Olomouc, Czech Republic.

**Methods of isolation pollen grains**
Immature male flowers of CS and CM were sterilized (1 min in 70 % ethanol, 10 min in 2.5 % chloramine, three times rinsed in sterile water) and cultivated on ½ MS medium (Murashige and Skoog 1962) at 25°C in the dark for 3 days. Pollen grains were isolated from anthers directly or by one of three centrifugation techniques (Tab. 1). Three liquid isolation media used for centrifugation were compared: NLN (modified NLN 13 solution; Lichter 1981), YST [modified YST solution; Ondřej et al. (2002)], and VB [modified VB solution; Vižintin and Bohanec (2004)]. The viability of the isolated pollen grains was evaluated immediately after culture on YS medium and established using an Olympus BX60 fluorescent microscope, with BW filter and fluorescein diacetate stain.

**Table 1. Methods for isolation of pollen grains.**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description of isolation methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>“a”</td>
<td>pollen grains were isolated by squeezing anthers with a glass rod in isolation media; filtration; three times centrifugation (900 rpm, 10-5-5 min)</td>
</tr>
<tr>
<td>“b”</td>
<td>pollen grains were isolated by squeezing anthers with a glass rod in isolation media; centrifugation (500 rpm, 5 min); filtration; centrifugation (1000 rpm, 3 min)</td>
</tr>
<tr>
<td>“c”</td>
<td>pollen grains were isolated from anthers chopped with a razor blade in isolation media; centrifugation (500 rpm, 5 min); filtration; centrifugation (1000 rpm, 3 min)</td>
</tr>
<tr>
<td>“d”</td>
<td>pollen grains were isolated directly from anthers (transferred from anthers directly on to media )</td>
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**In vitro culture and in vitro pollination**
Immature female flowers of *C. sativus* (CS) were sterilized and cultured in the same way the male flowers were. The mature female flowers were then excised in aseptic conditions and ovules were transferred onto solid media [CP medium: Castano and De Proft (2000), and YS medium: Ondřej et al. (2002)]. Isolated pollen grains
were transferred on and around ovules. In the case of directly isolated pollen grains, they were cultivated in liquid isolation media in addition (NLN, YST, VB). The Petri dishes with ovules and pollen grains were cultured for 2 days at 25°C in the dark, and then fertilized ovules were transferred onto two types of media supporting embryogenesis (CW medium, GA medium; Skálová et al. 2007). The success of in vitro pollination was evaluated. Two levels of regeneration were described: green ovules (the ovules became green and grown) and calluses (max. 2 mm length; Fig. 1).

**RESULTS AND DISCUSSION**

The results are summarized in Table 2 and in Figures 2 and 3. No large differences in pollen grain viability were observed among centrifugation techniques. The highest viability was observed using *C. sativus* pollen grains in combination with isolation medium VB and method “c” (79%). Direct isolation showed better results (92% using *C. sativus* pollen grains on CP medium). Pollen grains of *C. melo* had lower viability (for centrifugation, 68% in isolation medium NLN and method “a”; for direct isolation 79% on CP medium). As ovules of *C. sativus* became grown and green after in vitro pollination, this was considered as the first level of regeneration. The highest level of regeneration was formation of callus. The same results were obtained by Ondřej et al. (2002). The higher regeneration was observed with in vitro pollination between *C. sativus* and *C. melo* (33% green ovules and 11% calluses). The values for regeneration of *C. sativus × C. sativus* were lower (20% green ovules and 9% calluses). Direct isolation of pollen grains offered better results again (*C. sativus × C. sativus* 36% green ovules and 3% calluses; *C. sativus × C. melo* 39% green ovules and 6% calluses). The positive results with directly extracted pollen grains were also observed by Castano and De Proft (2000). They obtained seedlings after in vitro pollination in *C. intybus*.

**Table 2.** Summary of number, viability and regeneration success of pollen grains.

<table>
<thead>
<tr>
<th>Pollen grains of Cucumis spp./ Type of isolation</th>
<th><em>C. sativus</em> / centrifugation</th>
<th><em>C. sativus</em> / directly</th>
<th><em>C. melo</em> / centrifugation</th>
<th><em>C. melo</em> / directly</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of isolated pollen grains</td>
<td>540</td>
<td>450</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>Average viability (%)</td>
<td>72</td>
<td>86</td>
<td>61</td>
<td>73</td>
</tr>
<tr>
<td>Average regeneration (%)</td>
<td>29</td>
<td>39</td>
<td>44</td>
<td>45</td>
</tr>
</tbody>
</table>
The major influence on viability of pollen grains was the condition of donor plants. No strong influence of different centrifugation procedures was observed on the viability of pollen grains. On the other hand, direct isolation was more favorable for pollen grains than using centrifugation. The most positive influence on developing ovules and embryos was medium CP. The results of Vižintin and Bohanec (2004) showed that the pollen germination rate is greatly influenced by different factors (genotypes of accessions, media composition, temperature, etc.). Further manipulation of pollen grains could bring positive results in in vitro pollination procedures.

![Diagram](image-url)

**Figure 2.** Viability of pollen grains isolated by centrifugation (a) and directly (b).
Figure 3. Regeneration (%) after *in vitro* pollination in *Cucumis* spp., pollen grains isolated by centrifugation (a) and pollen grains isolated directly (b).

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