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Seed set after pollination with in-vitro-matured, isolated pollen of *Triticum aestivum*

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Summary. Immature pollen of two varieties of *Triticum aestivum*, at the stage right after the first pollen mitosis, was isolated from individual anthers and cultured in microcultures of microliter droplets. In a specifically designed medium, some of the pollen grains developed to maturity. These were applied to excised stigmas on agar, where they produced pollen tubes. Application to flowers in vivo led to seed set. Pollen was matured in vitro from a variety that produced a different protein banding pattern on SDS-PAGE as compared to the variety that was pollinated. The protein banding in the produced seeds showed the hybrid pattern, demonstrating that the seeds were not produced by self-pollination in this in-breeding species but by pollination with the in-vitro-matured pollen.

Key words: Pollen culture – Pollen maturation – Pollination – *Triticum aestivum* – Gliadins

Introduction

Control of pollen development has a number of applications in plant breeding. Whole plant chemical treatments and expression of nuclear as well as cytoplasmic genes can lead to pollen abortion (male sterility). Whole plant or flower treatments (temperature, CO_2) have been used to overcome self-incompatibility. Isolation of anthers or pollen and their development in vitro can lead to haploid plant formation, and the diploidized plants can be used for dihaploid breeding.

We recently reported that isolated tobacco pollen, when cultured in vitro, can develop to maturity under particular culture conditions (Benito Moreno et al. 1988). After pollination with the in-vitro-matured pollen, seeds were formed. It was suggested that pollination with in-vitro-matured pollen can have interesting applications in plant breeding. One possibility would be to rescue sterile pollen and self-fertilize otherwise self-sterile plants. Similarly, it should be possible to overcome self-incompatibility. Selection on the haploid male gametophytic level that is usually performed during pollen tube growth could be extended to the period of pollen development. Finally, we suggested that genes be transferred into immature pollen grains, and that the in-vitro-matured pollen be used as a super vector to transfer these genes into the embryo sac (Alwen et al. 1990).

For these potential applications, it was necessary to demonstrate that this technique not only works in the model plant tobacco but also in a crop plant. We report here that in-vitro-matured pollen of wheat (*Triticum aestivum*) can also be used for pollination in vivo.

Materials and methods

Plants and growth conditions

$HgCl_2$ (0.1% plus 0.1% saponine) treated seeds of the winter varieties H77022 and Diplomat of *Triticum aestivum* were allowed to imbibe in water overnight and were then germinated under vernalization conditions (4 °C for 4–6 weeks). The seedlings were then transferred to soil and raised on the terrace of the institute. Fertilizers and insecticides were applied when necessary.

Seeds of the summer variety Orofen (courtesy of Dr. B. Barnabás, Martonvásár, Hungary) were vernalized for 2 weeks only and were then grown in the same way as the winter varieties.

Microculture of pollen

Tillers containing immature spikes were cut and the spikes were taken out in a sterile hood. A spike was dipped into ethanol (96%) for 30–40 s and was then washed with sterile, distilled water. The three anthers of a floret were removed with fine tweezers and were transferred to 50-µl droplets of medium in a petri dish. Depending on the desired pollen density, one to three anthers (of the same floret) were placed in a droplet. The petri

dish was positioned under an inverted microscope in the sterile hood. An anther was held with the tweezers and small cuts were performed with a scalpel over the entire surface of the anther. Pollen was released spontaneously during dissection. The petri dish was viewed under the inverted microscope to determine the precise developmental stage of the pollen. Staging was performed without staining.

After staging, 100 µl of medium was added to the 50 µl droplet. After 5–10 min, the pollen had gathered in the center of the droplet and, after removal of the anther pieces, the medium was carefully sucked off from the periphery of the droplet. This washing step was repeated twice. Finally, the droplet of ca. 50 µl was transferred to a new petri dish. In this way, droplets could be grouped according to the stage of the pollen.

The culture medium was derived from the MR26-medium that has been used for in vitro pollen maturation in tobacco (Benito Moreno et al. 1988). However, sucrose (Su) was used at half the concentration (0.25 *M*), and 0.28 *M* mannitol (Ma) was used to provide optimal osmotic conditions for the immature wheat pollen (MR31 medium).

During in vitro pollen development, the MR31-medium was replaced by media with lower osmolarity (MR31A: 0.2 *M* Su and 0.2 *M* Ma; MR31B: 0.15 *M* Su and 0.15 *M* Ma). From a 50-µl droplet, 25 µl of medium was removed and 25 µl of new medium was added.

Semi-in vitro pollination

Approximately 7 days before pollen maturity, the anthers were removed from 16 main florets in spikelets from the center of a spike. The spikelets from the top and bottom part of the spike and the third florets of the central spikelets were removed. The spike was bagged. After 7 days, the stigmas of the emasculated florets were cut off of the pistil and were placed in an agar medium containing Murashige and Skoog (1962) minerals, 1 mg/l thiamin-HCl, 0.8% Difco Agar Purified, and 10% sucrose at pH 7 (before autoclaving).

On each stigma, 5 µl of pollen suspension (in MR31B medium) with about 1,000 pollen grains were applied. From these grains, usually 8–10 were considered to be mature.

After 30 min, the pollinated stigmas were fixed in ethanol (96%) and in lactic acid for 20 min. After washing in a 0.5 *M* mannitol solution, the stigmas were stained with cotton blue (0.2% cotton blue in lactophenol) for 10 min. After one washing step in the mannitol solution, a second staining was performed with propiono-carmine for 15 min. After a final washing step in the mannitol-solution, the stigmas could be viewed under the microscope (Chandra and Bhatnagar 1979; B. Barnabàs, personal communication).

In vivo pollination

Emasculation was performed as described for the semi-in vitro pollination. The emasculated florets were pollinated with 5 µl droplets of pollen suspension and the spike was bagged. The bags were shaken with a granulous fungicide (Arbosan).

SDS-PAGE for seed protein separation

Extracts were obtained from half seeds without embryo by grinding them in a mortar with extraction buffer (10 m*M* HEPES, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, and 1 m*M* 1,4-dithio-DL-threitol), containing a mixture of protease inhibitors (1 m*M* benzamidine, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 100 µg/ml bacitracin, 1 µg/ml antipain, 0.1 m*M* phenyl-methyl-sulfonyl-fluoride). The extract was centrifuged at 10,000 rpm in a Sigma table-top centrifuge for 10 min. Proteins were precipitated by adding 5 vol. of cold acetone to the supernatant and incubated at −20 °C for 2 h. After centrifugation at 10,000 rpm for 10 min, the pellet was dried in a vacuum exsiccator. The pellet was taken up in sample buffer (Laemmli 1970).

Proteins were separated in one-dimensional SDS-PAGE (Laemmli 1970) in a HOEFER Mighty Small II Slab Gel electrophoresis unit. A 8% polyacrylamide gel of 0.75 mm thickness was used. Silver staining was according to Bürk et al. (1983).

Results

After isolation, 80–90% of the early binucleate pollen in a typical microculture was viable (Fig. 1 A) and, after 3 days, 40% still remained viable. In these grains, the two nuclei were still visible and starch grains became visible in the cytoplasm (Fig. 1 B).

Shortly before and during the first pollen mitosis, microspores did not incorporate starch under the conditions applied and media used. Stages later than the early binucleate stage also did not show any apparent development.

Lowering the osmotic value during pollen maturation promoted pollen quality. More pollen grains were densely packed with starch grains than without medium change. The optimal scheme for medium change was after 4 days into MR31A and after a further 2 days into MR31B medium.

A further reduction of the osmotic value was not beneficial. It is assumed that the beneficial effect of lowering the osmotic value is an adaptation to the lower turgor pressure inside the pollen grains caused by the conversion of sugar into starch or lipids.

From day 4 to 7, the generative cell detached from the pollen wall (not shown) and the number and size of starch grains in the vegetative cell increased strongly, mainly around the vegetative nucleus. After resorption of the vacuole, the starch grains filled the entire lumen of the vegetative cells. Maturity was judged by the filling with starch grains and the structure of the germ pore. Usually, the pollen reached maturity after 8 days. Mature pollen had the operculum still closed (Fig. 1 C), while at a too advanced stage, the operculum had opened and the contents of the vegetative cell (including starch grains) were ejected into the medium. The number of mature pollen grains in the microcultures was quite variable. In good cultures, up to 1% of the cultured pollen grains were considered mature.

Unlike pollen from many other species, in vitro germination of wheat pollen in liquid or agar media is not possible. In order to test whether the in-vitro-matured pollen grains were able to produce pollen tubes, an in vitro germination test had to be developed. Microdroplets of in-vitro-matured pollen in MR31B medium were transferred onto excised stigmas in agar, and the formation of pollen tubes was observed under the microscope

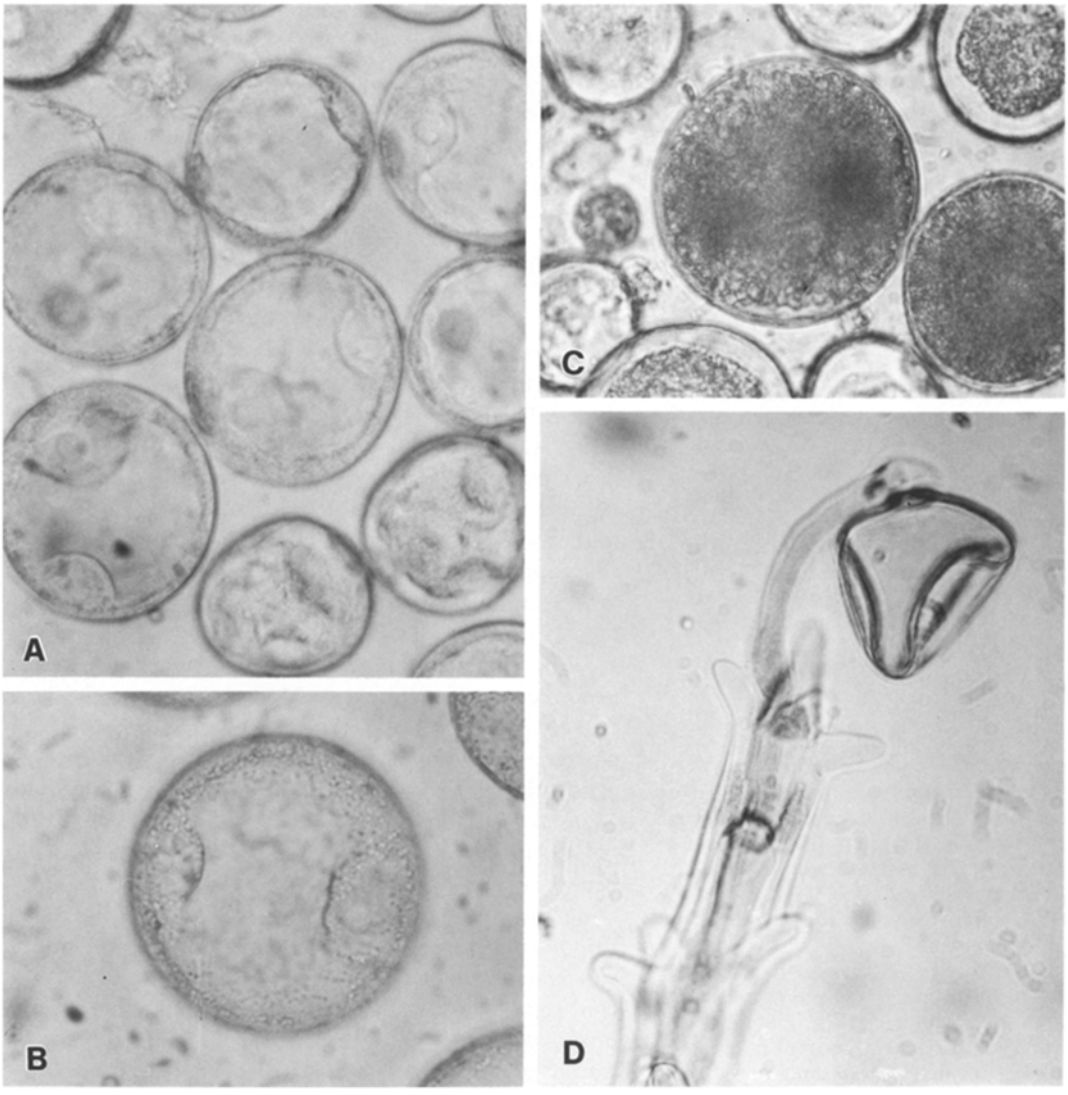

Fig. 1. **A** Wheat pollen at the early binucleate stage in microculture shortly after isolation. The pollen grains have a diameter of 50–60 μm, contain a vacuole, and the generative cell is attached to the intine. Magnification × 700. **B** Initial, early binucleate pollen grain after 3 days of culture. Starch grains form in the cytoplasm, preferentially around the vegetative nucleus. Magnification × 900. **C** Two mature pollen grains formed from initial, early binucleate pollen after 8 days of culture. They are completely filled with starch grains. Dead pollen grains surround the mature ones. Magnification × 900. **D** Germinating pollen grain that had matured in vitro. The pollen tube has left the now empty pollen grain and has penetrated the stigma. Magnification × 900

after staining with cotton blue and propiono-carmine. Pollen tubes were observed in or on the stigmas after pollination (Fig. 1 D).

For in vivo pollinations with the in-vitro-matured pollen, small droplets of pollen suspension were applied to stigmas in emasculated florets and the spikes were bagged. After application, the suspension quickly dried on the stigma surface. The presence of a fungicide in the bag effectively prevented infections. Such in vivo pollinations led to the formation of 12 seeds (Table 1).

Most of the seeds were of normal size. Some seeds, however, were small but contained an embryo. Of the 12 seeds, the 9 normal ones were surface-sterilized with $HgCl_2$ and were cut transversely into an embryo-containing and an embryo-free half.

The embryo-containing halves were placed on the medium used for the semi-in vitro pollination. All of them germinated and the plants grew normally.

The embryo-free halves were used to prepare crude protein extracts that were separated by SDS-PAGE. In the gels of the two seeds produced by pollination of Diplomat plants with in-vitro-matured pollen of H77022 (Fig. 2, lane 1), it was possible to detect a band that was present in the pollen donor (H77022, lane 3) but not in the pistil donor (Diplomat, lane 2). Control pollinations of Diplomat with normal, in-vivo-matured pollen of

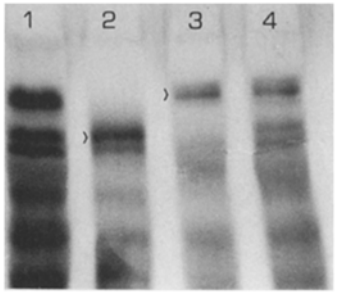

Fig. 2. Separation on SDS-PAGE of seed proteins present in crude extracts of embryo-free seed halves of the wheat varieties Diplomat (*lane 2*) and H77022 (*lane 3*), as well as of hybrids produced by pollination of Diplomat plants with either in-vivo- (*lane 4*) or in-vitro-matured pollen of H77022 (*lane 1*). The *arrows* in *lanes 2* and *3* indicate bands used as variety-specific markers

Table 1. Number of seeds produced after pollinations with in-vitro-matured pollen

Polli-nation	Genotype of pistil donor	Genotype of pollen donor	No. of seeds	Electro-phoresis
1	Diplomat	Diplomat	3	yes
2	Diplomat	H77022	2	yes
3	H77022	Diplomat	4	yes
4	Diplomat	Diplomat	1	no
5	Diplomat	H77022	1	no
6	Orofen	H77022	1	no

H77022 showed the same band (lane 4). In the four seeds produced by pollinations of H77022-plants with in-vitro-matured pollen of Diplomat, a lower band (indicated in lane 2) could be used as a marker to identify the hybrids (data not shown). In "self" pollinations of Diplomat plants with in-vitro-matured Diplomat pollen, only the Diplomat marker band was detected (data not shown).

Discussion

In this work, we show that pollination with in-vitro-matured pollen of wheat can lead to seed set. This demonstrates that the technique not only works in the model plant tobacco, but also in a crop plant. The possibility now exists to use this technique as a breeding tool for overcoming crossing barriers or for the selection of new traits on the haploid, gametophytic level (Heberle-Bors 1989).

It became evident, however, that only one particular stage (the early binucleate one) could be successfully cultured to maturity and that the yield of in-vitro-matured pollen was very low in wheat. In tobacco, in contrast, the stages from the late microspore stage upwards can be used successfully and the frequency of matured pollen is much higher (Benito Moreno et al. 1988).

Wheat belongs to the plant species with trinucleate pollen (two sperm cells within the vegetative cell in the mature pollen). We were able to see three nuclei in a few in-vitro-matured pollen grains only, while the majority was binucleate (one vegetative, one generative). We do not know whether only the few trinucleate ones were fertile. It is possible that second pollen mitosis in the in-vitro-matured pollen took place during pollen tube growth, as in the species with binucleate pollen.

Since wheat is a self-pollinating plant, it was necessary to prove that seed set had occurred through pollination with the foreign, in-vitro-matured pollen, and not by contaminating self-pollen left behind or introduced by inadequate emasculation. This proof was obtained by the detection of marker proteins present only in the pollen parent.

It is not completely clear which seed proteins were isolated with the aqueous, salt-containing extraction buffer. Extraction with 70% ethanol that dissolves gliadins showed similar bands in the same molecular weight range in both the parent and the hybrid lines. The differences were not so clear, however, as with the aqueous extraction procedure followed by acetone precipitation. We assume, therefore, that these bands correspond to gliadins. The major bands in the top part of the gel should represent individual gliadins of the omega group that are inherited in a codominant manner (Metakovsky et al. 1984).

In any case, the parallel occurrence of the bands in seeds obtained after pollination with in-vitro- and in-vivo-matured pollen indicates that these bands are valid markers for identifying the two varieties and the hybrids.

Of the six hybrid seeds tested in SDS-PAGE, all exhibited the expected hybrid character. The production of a false-positive result by natural cross-pollination was unlikely since (a) wheat, as a self-pollinating plant, does not release much pollen; (b) the two varieties used for crossing were kept separate; (c) the pollinated spikes were bagged; (d) the seeds obtained by "self-pollinations" of diplomat with in-vitro-matured pollen showed only the Diplomat marker band.

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