Ultrastructural studies on pollen embryogenesis in maize (Zea mays L.)

B. Barnabas 1, P. F. Fransz 2, and J. H. N. Schel 2

1 Agricultural Research Institute, Hungarian Academy of Sciences, Martonvásár, P.O. Box 19, H-2462 Hungary
2 Department of Plant Cytology and Morphology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

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ABSTRACT
Maize anthers have been induced on modified N6 medium to produce embryoids. Different stages from the cultures were sampled and prepared for microscopical examination. The microspores at the onset of culture were in an early developmental stage, with the nucleus and numerous organelles centred in the middle, surrounded by many small vacuoles with a lipid content. The binuclear pollen grains contained small vesicles and much starch. The partially condensed vegetative nucleus indicated participation of the vegetative component in the formation of multicellular pollen grains (MPGs). Several MPGs have been observed which differed in morphology. We suggest, on the basis of these ultrastructural observations, that in maize mainly the vegetative cell contributes to the MPG which further develops directly into embryoids.

INTRODUCTION
Since the last decade considerable efforts have been made to get haploid plants from anther cultures (for reviews, see e.g. Clapham 1977, Maheswari et al. 1982, Heberle-Bors 1985, Hu and Yang 1986, Raghaven 1986). For maize, results were initially obtained by Chinese groups (401 Research Group 1975, Xu et al. 1978, Kuo 1978, Miao et al., 1978, Ting et al. 1981) but more recently also other laboratories have reported on maize anther culture (Brettell et al. 1981, Genovesi and Collins 1982, Nitsch et al. 1982, Janos 1985, Petolino and Jones 1986, Tsay et al. 1986a). Because of the strong genotype dependency of the anther response during the in vitro culture of maize it is unfortunately time consuming to find a good embryogenic genotype after testing numerous ones. This might be the reason why much less research was carried out on the ultrastructural aspects of maize pollen embryogenesis, while several publications can be found about the cytochemical and ultrastructural features of the in vitro androgenesis in other species of the Gramineae (Sunderland et al. 1979, Genovesi and Magill 1982, Tsay et al. 1986; for a survey, see Huang 1986). There is obviously a lack of information about maize in this field.

The aim of the present work was to use a relatively highly embryogenic maize genotype (Barnabas et al., in prep.) for ultrastructural studies of pollen embryogenesis and to observe different features of multicellular pollen grain formation and production of embryoids.

MATERIALS AND METHODS
In vitro anther culture
A field grown maize hybrid (Mv Exp. 2804), supplied by the breeders of the Martonvásár Institute, was used as the experimental material. The plants were raised in the greenhouse during springtime at 20/17 °C for 3 weeks and then at 22/18 °C for the further development of the plants. The anthers containing microspores in the early developmental stage were sampled and cultured at 29 °C in Petri dishes on modified N6 medium with 15% sucrose (Chu 1978) for 30 days in the dark. The medium was supplemented with 25 ppm Na-MoO4·2H2O, 25 ppm CuSO4·5H2O and 25 ppm CoC12·7H2O (Dr. Jia Xu, personal communication). Light microscopy and cytochemistry
In general, 10 anthers were randomly chosen from the selected tassels as a control and from the Petri dishes after 6, 8 and 29 days in culture. The anthers were then fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h and postfixed in 1% OsO 4 solution in the same buffer for another 2 h at room temperature. After dehydration they were embedded in Epona. Semithin and ultrathin sections were then prepared of two anthers selected from each stage, using a LKB Ultramicrotome V. The semithin sections were stained with 0.5% toluidine blue O in 0.1% sodium carbonate. Lipid material was detected with a saturated solution of Sudan Black B in 70% ethanol. Polysaccharides were stained with periodic acid-Schiff's reagent (PAS). At various time intervals 2-3 anthers were stained with aceticarmine and squashed. For all methods, see O'Brien and McCully (1981).

Electron microscopy
Ultrathin sections were post-stained with uranyl acetate and lead citrate using a LKB Ultrastainer. They were examined with a Philips EM 301 electron microscope at 60 kV.

RESULTS
Squash preparations of about 1 week cultures from early microspores showed pollen grains in varying stages of androgenic development. In Fig. 1A a grain is shown in which most likely the vegetative nucleus has divided (arrows) while in Fig. 1B many nuclei are present (arrows) which seem to have originated from repeated divisions of the vegetative nucleus. The generative nucleus, as deduced from its position, also divided (Fig. 1B, arrowheads). Although, in our opinion, squash
preparations are no conclusive evidence because of possible shifts in nuclear positions induced by the mechanical stress, we have used this information to focus the further ultrastructural studies on this stage of culture, i.e. 6-8 days after onset. After one month in culture the anthers gave rise to small clumps of callus and embryoids (Fig. 1C). After prolonged culture small plantlets developed (Barnabas et al., in prep.).

**Early microspores**
The peak response of pollen embryogenesis in cereals is achieved with microspores before the first mitosis. In general, the so-called mid-uninucleate or early unicellular stage is optimal. This was confirmed for maize by Miao et al. (1978) who found an induction frequency of 7.0% at the mid-uninucleate stage against 4.35% at the late uninucleate stage. We prefer, however, to speak about early microspores because they are, by definition, uninucleate.

An electron micrograph, showing this early stage of a maize microspore is given in Fig. 2A. The nucleus (n) is centred in the middle of the grain. The chromatin is diffuse and often a prominent nucleolus is visible (not shown in Fig. 2A, but see Fig. 2B). Also located in the center, adjacent to the nucleus, is a region with many organelles, a.o. proplastids, mitochondria and dictyosomes (Fig. 2A, arrows). The microspore center is surrounded by many small vacuoles (v) containing electron-dense material. After staining with toluidine blue the vacuoles remained colorless (Fig. 2B) but with Sudan Black they were darkened (Fig. 2C) indicating the presence of lipid-like components. The exine layer was thick, with channels, while the intine layer was hardly developed.

![Fig. 1A. Light micrograph of a squash preparation after 6 days in culture, stained with acetocarmine. The generative nucleus is visible (gn); the vegetative nucleus has divided (arrows). Fig. 1B. The same preparation, but now showing many nuclei (arrows) obviously originated from the vegetative nucleus. The generative nucleus has also divided (arrowheads). Fig. 1C. Anther (a) cultures after one month showing some callus (c) formation and embryoids (em). Fig. 2A. Electron micrograph of an early microspore at the onset of culture. Note the central position of the nucleus (n) surrounded by many small vacuoles (v). A region with many organelles is visible (arrows). Fig. 2B. Light micrograph of an early microspore stained with toluidine blue. Vacuoles are not stained. A distinct nucleolus (arrow) is visible. Fig. 2C. Same preparation, stained with Sudan Black. Many lipid-like components (li) are visible.](image-url)