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Three-dimensional observations on freeze-fractured frozen hydrated *Papaver dubium* pollen with cryo-scanning electron microscopy

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SUMMARY

The ultrastructure of generative cells and vegetative cells of *Papaver dubium* L. pollen was studied with low-temperature scanning electron microscopy (LTSEM). Liquid propane freezing resulted in a very fast fixation. The on-line fracturing and observation of the pollen grains was performed at −130°C. The specimens were fully hydrated and did not undergo morphological changes during the procedure. Unfractured and cross fractured organelles, generative cells, vegetative nuclei and membranes are visible in three-dimensional images. The LTSEM micrographs show a lobed vegetative nucleus with many nuclear pores and a wrinkled generative cell in longitudinal direction. The vegetative nucleus and the generative cell are situated close to each other but structural connections are not observed.

Key-words: cryo-scanning electron microscopy, generative cell, *Papaver dubium*, pollen, vegetative nucleus.

INTRODUCTION

Pollen of many plant species is an object for structural research. The size and behaviour of the generative cell and sperm cells in the vegetative cell and their association with the vegetative nucleus are current research objects (Keijzer 1988). In order to investigate the ultrastructural organization in pollen, different methods are in use, among which is, computerized three-dimensional reconstruction of serial sectioning of embedded material; a time consuming method. (Theunis *et al.* 1985; Wilms *et al.* 1986; McConchie *et al.* 1987).

Direct three-dimensional observation at the ultrastructural level is possible with scanning electron microscopy (SEM). To visualize cell organelles and membrane structures, techniques including chemical fixation, osmification, freeze-fracturing and maceration of the cytoplasmic matrix, followed by critical point drying, have been developed for high resolution SEM (Barnes & Blackmore 1984, 1986; Tanaka & Mitsushima 1984). Freeze-fracturing and osmium tetroxide maceration have been used to visualize generative cells and sperm cells in association with the vegetative nucleus (Keijzer *et al.* 1986; Barnes & Blackmore 1987). However, chemical fixation, dehydration and critical point drying

cause considerable changes in structure and size of the object (Robards 1984; Beckett & Read 1986). Hydrous fixatives for mature pollen introduce structural changes (Van Aelst & Wilms 1986).

The use of liquid propane for cryo-fixation enables rapid freezing (Robards & Sleytr 1985). It has already been applied for freeze-fracture studies of pollen (Kroh & Knusman 1985, Platt-Aloia *et al.* 1986; Emons *et al.* 1988).

This paper presents the recent developments in freeze-fracturing and low temperature SEM of fully hydrated plant material and the application of this technique to data collecting of the internal morphology of mature pollen of *Papaver dubium* L.

MATERIALS AND METHODS

Fresh pollen of *Papaver dubium* L. was dried for 24 h over silica gel at 20°C and subsequently stored for 4 months at −20°C. The pollen was rehydrated at 20°C in saturated moisture (RH = 100%) for 30 min. The vitality of rehydrated *Papaver* pollen grains was controlled by determining the germination on a solid medium (pH 5·9) containing: 0·6% Agar (Difco), 7% Saccharose, 0·1 mg/ml H_3PO_3, 0·3 mg/ml $Ca_2(NO_3)_2.4H_2O$, 0·2 mg/ml $MgSO_4.7H_2O$, 0·1 mg/ml KNO_3, 0·2 ml/mg citric acid (Hoekstra & Van Roekel 1988). To prevent displacement of the pollen by knife pressure during freeze-fracturing, the rehydrated pollen grains were mixed with Tissue Tek II OCT (Miles Laboratories, Naperville Illinois). Little droplets of the mixture were mounted on specimen carriers (Balzers Union, Liechtenstein) and quickly frozen by plunging into liquid propane. The specimens were stored for a short time in liquid nitrogen and subsequently fitted on the cryostage of a Balzers SCU 020 (Balzers Union, Liechtenstein). They were fractured with a cryotome in the preparation chamber at −130°C and subsequently placed on the second cryostage inside a Cambridge MK IIA SEM. The uncoated fractured droplets were briefly observed at −130°C until the desired fracture plane was present in the pollen grain. The specimens were replaced into the preparation chamber and spattered with a 10-nm layer of gold at −130°C. Ten fractured pollen grains were observed and recorded, at −130°C according to Mueller *et al.* (1986).

RESULTS AND DISCUSSION

The viability of the rehydrated pollen grains of *Papaver dubium* L. was 85%. The on-line freeze-fracturing and observation in SEM provided three-dimensional images within 20 min (Figs 1 and 2). The advantage of the cryomicrotome inside the preparation chamber is that the fracture procedure is well-controlled. Therefore, thin layers of about 7 μm can be sectioned from the specimens, resulting in cross fractured cells at a defined plane. The micrographs of cross-fractured pollen grains (Figs 1 and 2) show similarity with freeze-fractured pollen of *Zea, Phoenix* and *Collomia* (Platt-Aloia *et al.* 1986), due to the same method of cleavage of the frozen object. Notably the contours of the organelles in the fracture plane are similar. The difference between the technique we used and the freeze-fracture technique used in *Zea, Phoenix* and *Collomia* (Platt-Alioa *et al.* 1986), is the formation of the image. In SEM, data collection occurs from the frozen object by secondary electrons, in TEM the image is obtained by dispersion of the primary electrons in a shadowed replica of the fractured surface. Nevertheless, the distribution of contrast in the micrographs was very similar.

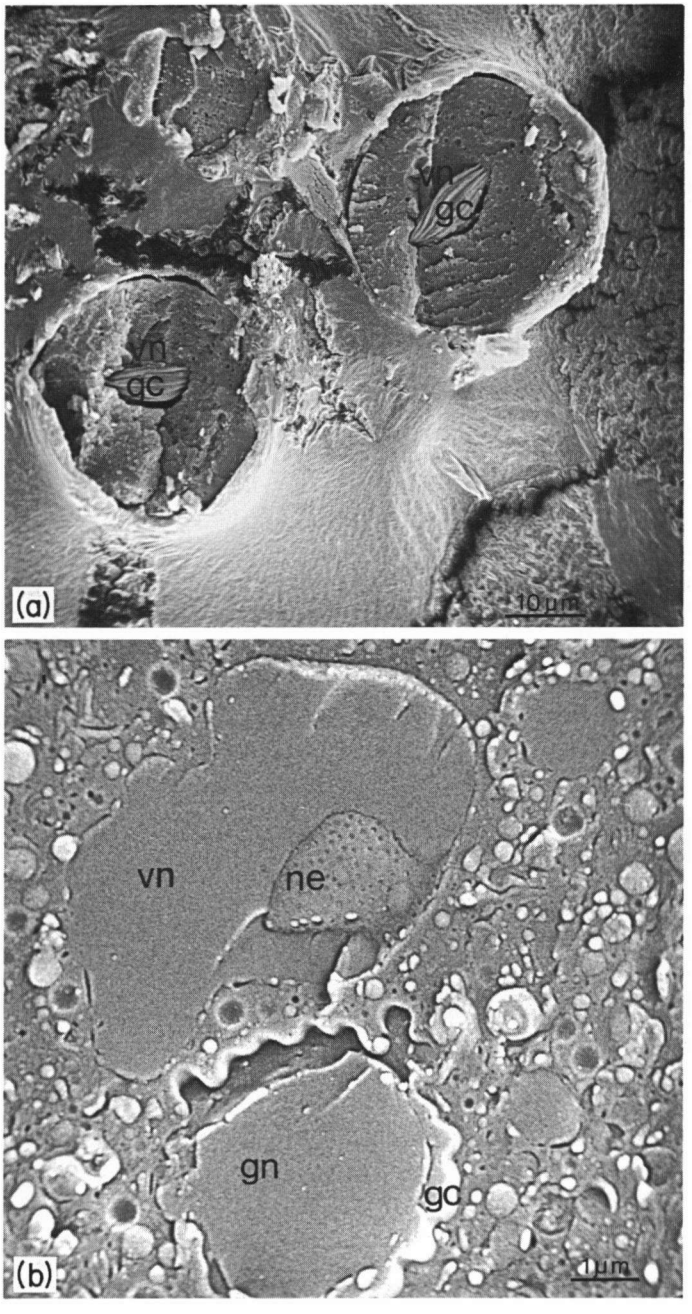

Fig. 1. Low temperature electron micrographs of freeze-fractured frozen hydrated bicellular *Papaver dubium* L. pollen. (a) Two fractured pollen grains, showing generative cells and vegetative nuclei. × 1170. (b) Detail of the vegetative cell surrounded by a thin layer of cytoplasm with nucleus and the generative cell with nucleus in cross fracture. The nuclear membrane of the vegetative cell shows many nuclear pores. × 9000. vn = vegetative nucleus, gn = generative nucleus, ne = nuclear envelope, gc = generative cell.

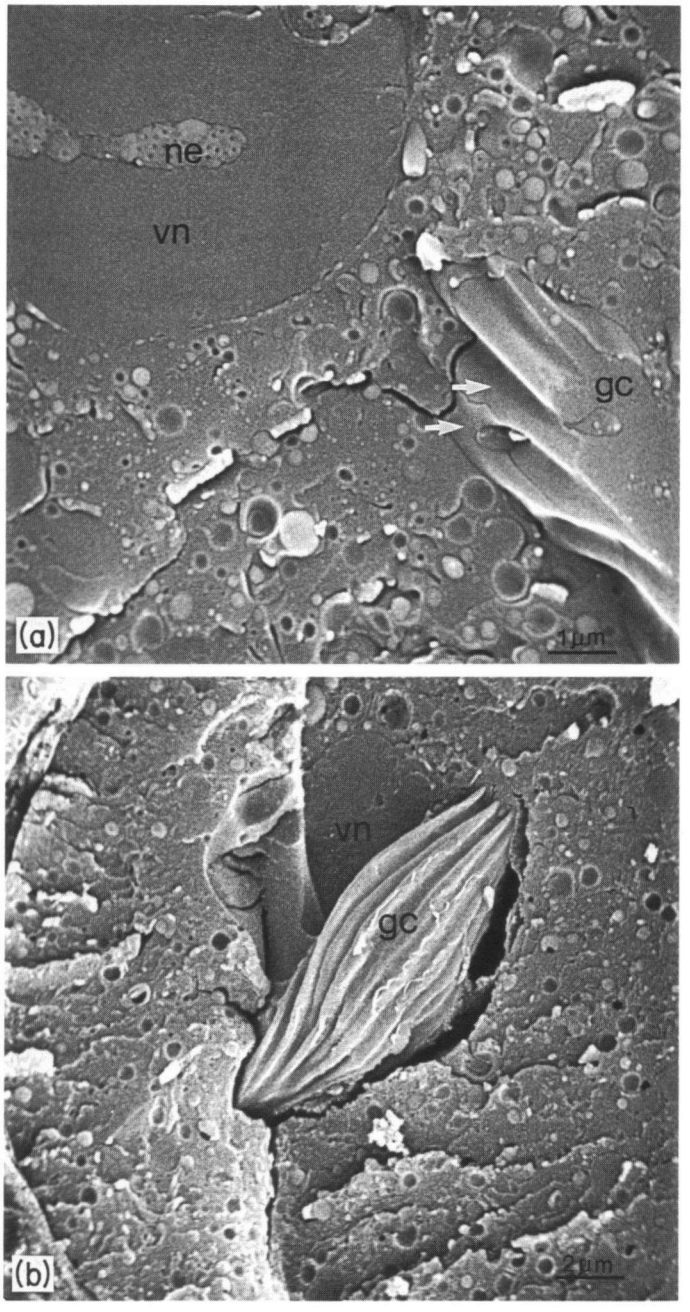

Fig. 2. Low temperature electron micrographs of freeze-fractured frozen hydrated bicellular *Papaver dubium* L. pollen. (a) Detail of the vegetative nucleus and the generative cell. Two membranes (arrows) are visible around the generative cell cytoplasm. No cell wall material is visible between the membranes ×9000. (b) Detail of the vegetative nucleus in cross fracture and a non-fractured generative cell showing wrinkles along its longitudinal axis ×4500. Abbreviations as for Fig. 1.

In the fractured cytoplasm of *Papaver* pollen no damage by formation of ice crystals was observed. In freeze fixation techniques, in which plunging is used for quick freezing, liquid propane has the highest cooling rate of the liquid cryogens available (Costello & Corless 1978; Robards & Sleytr 1985). A high sucrose concentration in water diminishes ice crystal growth dramatically at subzero temperatures (Franks 1986). The high sucrose (17% of the dry weight, Hoekstra & Van Roekel 1988) content inside the *Papaver* pollen grain is a natural cryoprotectant. The combination of liquid propane for quick freezing and the presence of sucrose inside the pollen grain explains the absence of visible ice crystals.

The vegetative nucleus and the generative cell including the generative nucleus are visible (Figs 1(b) and 2). The vegetative nucleus is visible in cross fracture and has a homogeneous content. The nuclear envelope is visible in cross fracture because of the varying space between the double membranes (Figs 1(b) and 2(a)). Nuclear pores are visible in places where the plane of fracture follows the nuclear envelope. The vegetative nucleus is undulated, patches of nuclear membrane can be observed in the fracture plane of the nucleus (Figs 1(b) and 2). By using three-dimensional reconstruction techniques large invaginations of the nuclear membrane were also seen in *Spinacea* (Wilms *et al.* 1986) and *Brassica* (McConchie *et al.* 1987).

The generative cell can be observed unfractured (Figs 1(a) and 2(b)), partly fractured (Fig. 2(a)) or completely cross fractured (Fig. 1(b)). The generative cell is situated very close to the vegetative nucleus and shows many wrinkles along its longitudinal axis. The shape of the generative cell has been described in a number of species (Cresti *et al.* 1984). In the micrographs no structrual evidence is present to indicate a morphological association between the generative cell and the vegetative nucleus (Figs 1(b) and 2). The plasma membranes of the generative and the vegetative cell appear to be closely connected (Fig. 2(a)). The vegetative cell membrane has partly been fractured from the generative cell membrane (Fig. 2(a) arrows). No cell wall is visible between the two plasma membranes. Also in the Lily no cell wall is present between the generative cell and the vegetative cell (Emons *et al.* 1988). The generative nucleus is present in cross fracture and no substructure is visible. The generative nucleus is surrounded by a thin layer of cytoplasm (Fig. 1(b)).

Ultrastructural studies of pollen development (Blackmore & Barnes, 1985, Barnes & Blackmore 1986), male germ units (Dumas *et al.* 1984; Barnes & Blackmore 1987), vegetative nucleus generative cell association and organelle polarity during microsporogenesis, pollen dehydration, rehydration and germination have been done with water-containing fixatives, and differ from the real *in vivo* configuration. Water-containing fixatives affect size, shape and polarity of cell organelles. This is partly due to the water sensitivity of pollen as being relatively dry cells (Van Aelst & Wilms 1986) and the rapidly occurring structural changes caused by chemical treatment with fixatives (Robards 1984). Using the low temperature SEM these hydration and fixation artifacts can be overcome and additional ultrastructural data can be obtained of the complex and quickly occurring morphological changes in pollen grains and tubes.

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REFERENCES

Barnes, S. & Blackmore, S. (1984): Freeze-fracture and cytoplasmic maceration in botanical scanning electron microscopy. *J. Microscopy* **136:** RP3–RP4.

— & — (1986): Plant ultrastructure in the scanning electron microscope. *Scan. Electron Microsc.* **1:** 281–289.

— & — (1987): Preliminary observations on the formation of the male germ unit in *Catanache caerulea* L. (Compositae: Lactuceae) *Protoplasma* **138:** 187–189.

Beckett, A. & Read, N.D. (1986): Low temperature scanning electron microscopy. In: Aldrich, H.C. & Todd, W.J. (eds): *Ultrastructure Techniques for Microorganisms.* 45–86. Plenum Press, New York, London.

Blackmore, S. & Barnes, S. (1985): Cosmos pollen ontogeny: a scanning electron microscopical study. *Protoplasma* **126:** 91–99.

Costello, M.J. & Corless, J.M. (1978): The direct measurement of temperature changes within freeze-fracture speciment during rapid quenching in liquid coolants. *J. Microscopy* **112:** 17–37.

Cresti, M., Ciampolini, F. & Kapil, R.N. (1984): Generative cells of some angiosperms with particular emphasis on their microtubules. *J. Submicrosc. Cytol.* **16:** 317–326.

Dumas, C., Knox, R.B., McConchie, C.A. & Russell, S.D. (1984): Emerging physiological concept in fertilization. *What's New in Plant Physiology* **15/5:** 17–20.

Emons, A.M.C., Kroh, M., Knuiman, B. & Platel, T. (1988): Intramembrane particle pattern in vegetative and generative plasma membranes of Lily pollen grain and pollen tube. In: Wilms, H.J. & Keijzer, C.J. (eds): *Plant Sperm Cells as a Tool for Biotechnology.* 41–48: PUDOC, Wageningen.

Franks, F. (1986): Metastable water at subzero temperatures. *J. Microscopy* **141:** 243–249.

Hoekstra, F.A. & Van Roekel, T. (1988): Desiccation tolerance of *Papaver dubium* L. pollen during development in the anther: possible role of phospholipid composition and sucrose content. *Plant Physiol.* **88:** 626–632.

Keijzer, C.J., Wilms, H.J. & Mogensen, H.L. (1988): Sperm cell research: current status and applications for plant breeding. In: H.J. Wilms & Keijzer, C.J. (eds): *Plant Sperm Cells as Tools for Biotechnology.* 3–8: PUDOC, Wageningen.

—, & —, A.C. Van Aelst & Leferink-Ten Klooster, H.B. (1986): Towards the isolation of sperm cells for androgenic purposes. In: Horn, W., Jensen, C.J., Odenbach & Schieder, O. (eds): *Genetic Manipulation in Plant Breeding.* 311–313. Walter de Gruyter, Berlin, New York.

Kroh, M. & Knusman, B. (1985): Exocytosis in non-plasmolyzed and plasmolyzed tobacco pollen tubes. *Planta* **166:** 287–299.

McConchie, C.A., Hough, T. & Knox, R.B. (1987): Ultrastructural analysis of sperm cells of mature pollen of *Zea mays. Protoplasma* **139:** 9–19.

Mueller, T., Guggenheim, R., Lueoend, G. & Dueggelin, M. (1986): On-line cryopreparation and cryomicroscopy in SEM with SCU 020. In: Imura, T., Maruse, S. & Suzuki, T. (eds): *Proceedings of the XIth International Congress on Electron Microscopy, Kyoto,* 2233–2234.

Platt-Aloia, K.A., Lord, E.M., Demason D.A. & Thomson W.W. (1986): Freeze-fracture observations on membranes of dry and hydrated pollen from *Collomia, Phoenix* and *Zea. Planta* **168:** 290–298.

Robards, A.W. (1984): A cool look at biological electron microscopy. *Proc. R. Microsc. Soc.* **19:** 195–208.

— & Sleytr, U.B. (1985): Low temperature methods in biological electron microscopy. In: Glauert, A.M. (ed.): *Practical Methods in Electron Microscopy.* Elsevier, Amsterdam.

Tanaka, K. & Mitsushima, A. (1984): A preparation method for observing intracellular structures by scanning electron microscopy. *J. Microscopy* **133:** 213–222.

Theunis, C.H., McConchie, C.A. & Knox, R.B. (1985): Three dimensional reconstruction of the generative cell and its wall connection in mature bicellular pollen of Rhododendron. *Micr. Microsc.* **16:** 225–231.

Van Aelst, A.C. & Wilms, H.J. (1986): The ultrastructure of mature pollen after wet and dry fixation. In: Williams, E.G., Knox, R.B. & Irvine, D. (eds): *Pollination '86.* 233–235. School of Botany, University of Melbourne.

Wilms, H.J., Leferink-Ten Klooster, H.B. & Van Aelst, A.C. (1986): Isolation of spinach sperm cells 1: Ultrastructure and three dimensional construction in the mature pollen grain. In: Mulcahy D.L., Bergamini-Mulcahy, G. & Ottaviano, E. (eds): *Biotechnology and Ecology of Pollen.* 307–312. Springer-Verlag, Berlin.