

Freeze-Fracture and Thaw-Fixation of *Vicia* Root Cells for Scanning Electron Microscopy (SEM)

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ABSTRACT Conditions of freezing and thawing of *Vicia* root cells for scanning electron microscopy (SEM) were investigated. The best result was obtained when the roots were frozen directly in liquid Freon without pretreatment with a cryoprotectant DMSO (dimethyl sulfoxide). The damage caused by the absence of DMSO, if present, was less than that caused by DMSO pretreatment. The composition of the thawing solution was important. A high concentration (40%) of DMSO in the thawing solution probably served to lessen the freeze-thawing damage.

KEY WORDS freeze-fracture/root meristem/*Vicia faba*/scanning electron microscopy

Introduction

With the advent of scanning electron microscopy, it became possible to observe structures three-dimensionally. To observe intracellular structures, a freeze-fracture method was devised. When a fixed cell was fractured, however, the fractured surface of the cell was usually featureless, because intracellular structures were hidden in the cell matrix. To solve the problem, Tanaka and coworkers (1981, 1984) developed a method for etching the freeze-fractured surface of fixed cells with a dilute osmic solution. Haggis and his associates (1977, 1979, 1981, 1983), on the other hand, devised a technique for freeze-fracture of unfixed cells, followed by thawing into a fixative. In their method, soluble materials were washed away from the fractured surface of the unfixed cells at the thawing stage. In order to minimize the freeze-thaw damage, they used DMSO (dimethyl sulfoxide) or glycerol as a cryoprotectant. Infiltrating unfixed cells with the cryoprotectant is, however, harmful for the preservation of ultrastructure. Haggis and his coworkers first used 25% DMSO (Haggis and Bond, 1979; Haggis, 1981), and later reduced the concentration of DMSO to 10% (Haggis *et al.*, 1983). We tried to freeze root tips of *Vicia* directly without using any cryoprotectant and succeeded to observe nucleoli (Maruyama and Okuda, 1982) and chromosomes (Maruyama, 1983). In the present study, we looked for a better condition for freezing and thawing the material and ascertained that nuclei and chromosomes in *Vicia* roots were preserved well by direct freezing without a cryoprotectant, if the freeze-fractured materials were thawed in an aldehyde fixative containing a high concentration of DMSO.

Materials and Methods

Roots of *Vicia faba* (broad beans) grown in vermiculite were treated with 0.02% colchicine for 5-7 hours. The root tips, 0.5-1 cm long, were incubated 10%, 20% and 40% DMSO (dimethyl sulfoxide) or glycerol in 0.05 M sodium phosphate buffer,

pH 7.2, for 1 hour each at 4°C. They were then frozen by plunging into liquid Freon 22 (cooled with liquid nitrogen) and transferred to liquid nitrogen. The frozen roots were fractured longitudinally in two with a razor blade and a hammer. The fractured roots were then thawed by dipping them into a solution of 2% paraformaldehyde, 2% glutaraldehyde and 40% DMSO in 0.05 M sodium phosphate buffer, pH 7.2, at 4°C. After 10–20 min, they were transferred to a solution of 2% paraformaldehyde and 2% glutaraldehyde in the same buffer, and further fixed overnight at 4°C. After the fixation, the materials were washed in the buffer solution, and post-fixed in 1% osmium tetroxide in the same buffer for 2 hours. They were then subjected to a conductive staining of tannic acid-osmium ligation to render tissues electrically conductive (Murakami, 1976). For this staining, the specimens were treated with a solution of 2% tannic acid and 4% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.2, for 5–6 hours at 4°C, washed with the buffer and then treated with 1% osmium tetroxide in the same buffer for 2–3 hours. This staining process was repeated two or three times. After dehydration in a graded series of acetone-water mixtures and pure acetone, the specimens were critical-point dried with liquid carbon dioxide. The fractured surface of the dried specimens was then sputter-coated lightly with platinum-palladium with an ion-coater (Eiko IB-5, Eiko Engineering Co., Japan). Specimens were observed with a scanning electron microscope (JEOL JSM-T200). Images were recorded on Polaroid 665 p/n films.

Results

Pretreatment with Cryoprotectant before Freezing.

When the roots were pretreated successively with 10%, 20%, and 40% DMSO each for 1 hour before freezing, they shrank considerably. When the cells were examined with the SEM, the ultrastructure of the cell was damaged. It was not possible to find metaphase chromosomes. In the interphase nucleus, as shown in Fig. 1, chromatin was coagulated. The surface of the nucleolus was granular. In Fig. 2, the fracture had passed through a nucleolus. In the center of the organelle, there was one large vacuole. The surface of the vacuole was also granular. The main part of the nucleolus (the periphery of the nucleolar vacuole) in the fractured organelle was flat and featureless. When the roots were treated with 10% DMSO for 3 hours before freezing, metaphase chromosomes could be found. However, they were condensed considerably and the fine fibers of chromosomes were not apparent. The nucleolus in the interphase nucleus showed pores on the surface (Fig. 3). However, chromatin was still coagulated. When the roots were pretreated with 5% DMSO for 3 hours before freezing, metaphase chromosomes showed fine fibers on the surface (Fig. 4). The nucleoli in the interphase nuclei showed pores on the surface and canals in the fractured surface of the organelle (Fig. 5). However, chromatin in these nuclei was still somewhat coagulated.

The preservation of the ultrastructure was much worse by the pretreatment with glycerol even at low concentrations.

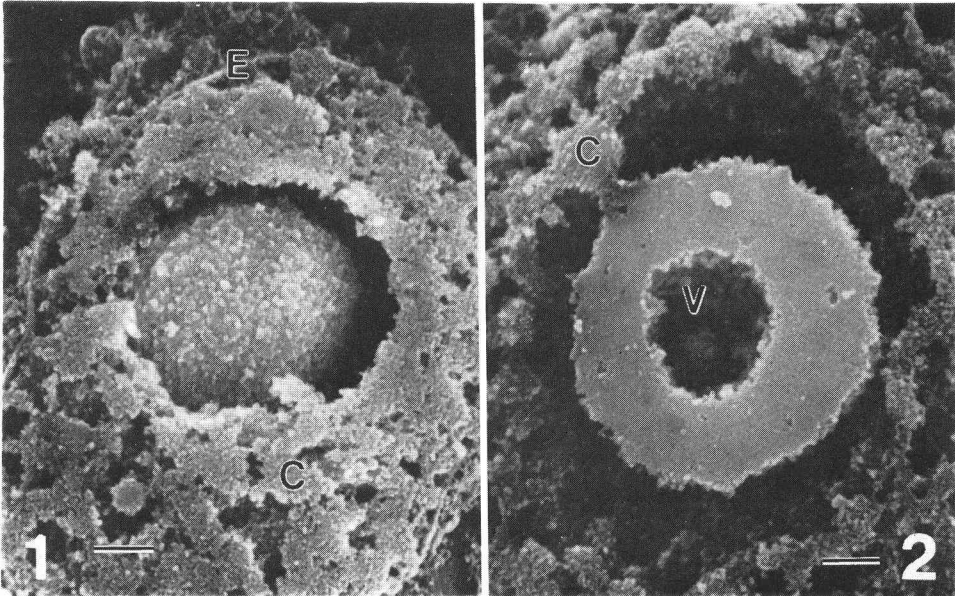


Fig. 1. The root was pretreated with 40% DMSO. The fracture passed above a nucleolus. E: nuclear envelope, C: chromatin. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

Fig. 2. Preparation as for Fig. 1. The fracture passed through a nucleolus. There is one large vacuole in the organelle. C: chromatin, V: nucleolar vacuole. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

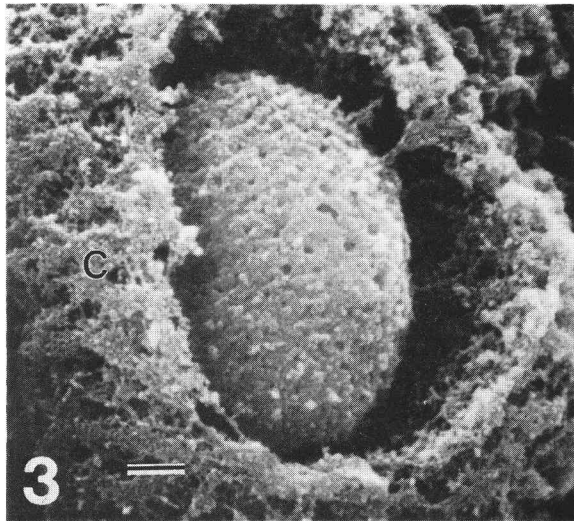


Fig. 3. The root was pretreated with 10% DMSO. The fracture passed above a nucleolus. C: chromatin. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

Direct Freezing

When the roots were frozen directly without any cryoprotectant present, and the freeze-fractured materials were thawed in the aldehyde fixative containing 40% DMSO,

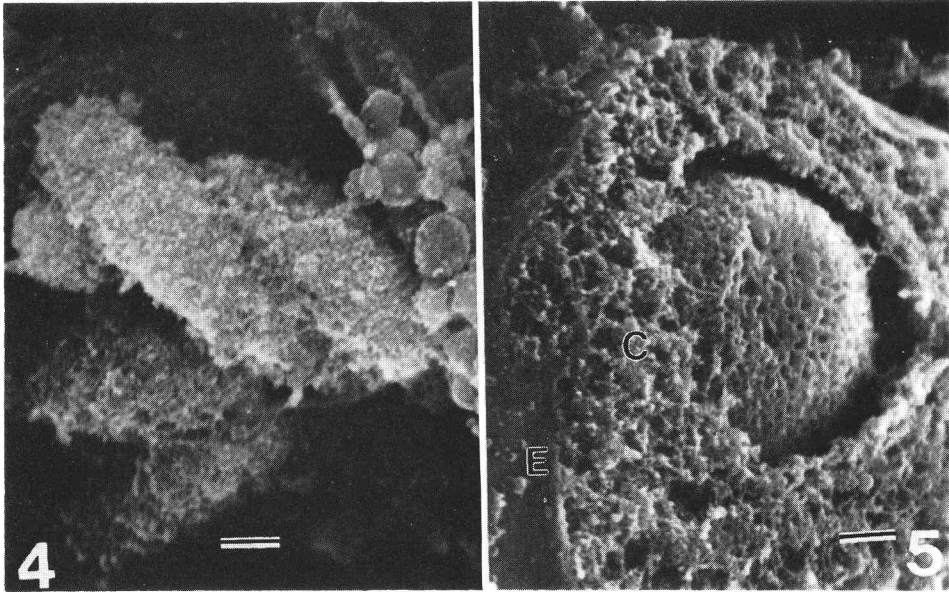


Fig. 4. The root was pretreated with 5% DMSO. Metaphase chromosomes. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

Fig. 5. Preparation as for Fig. 4. The fracture passed through a tip of a nucleolus. C: chromatin, E: nuclear envelope. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

metaphase chromosomes showed fine structural details clearly (Maruyama, 1983). In Fig. 6, prometaphase chromosomes were still wrapped up partially in sheets of the broken nuclear envelope. They were composed of knobby fibers about 500 Å in diameter. The fibers were randomly distributed in the chromosomes. The gross appearance of the chromosomes was similar to that described for water-spread chromosomes (Golomb and Bahr, 1971, 1974; Daskal *et al.*, 1976; Utsumi, 1981) and that for isolated chromosomes (Daskal *et al.*, 1976; Marsden and Laemmli, 1979; Adolph and Kreisman, 1983). As reported previously (Maruyama and Okuda, 1982), the nucleolus in the interphase nucleus showed pores and canals. In Fig. 7, the fracture had passed above a nucleolus. The organelle was embedded in tangles of fine chromatin threads 300–500 Å in diameter. There were many pores about 0.1–0.3 μm in diameter on the surface of the nucleolus. In Fig. 8, the fracture had passed through a nucleolus. There was one large vacuole, the surface of which was perforated. In the nucleolar main part, there were pores and canals, most of which were 0.1–0.3 μm in width. They may correspond to the *lacunae* observed by the transmission electron microscope (Lafontaine and Lord, 1973, 1974; Chouinard, 1974, 1975; Luck and Lafontaine, 1980). As a whole, the nucleolus appeared to be a hollow, sponge-like reticulum.

The composition of the thawing solution seemed to be important for the preservation of ultrastructure. When sucrose (0.2–0.7 M) was added to the thawing solution, small precipitates often stuck to structures. When the fractured roots were thawed in 40% glycerol, cells appeared similar to those thawed in DMSO, but the deposition of fine particles often obscured the fine details of structures. When the roots were thawed

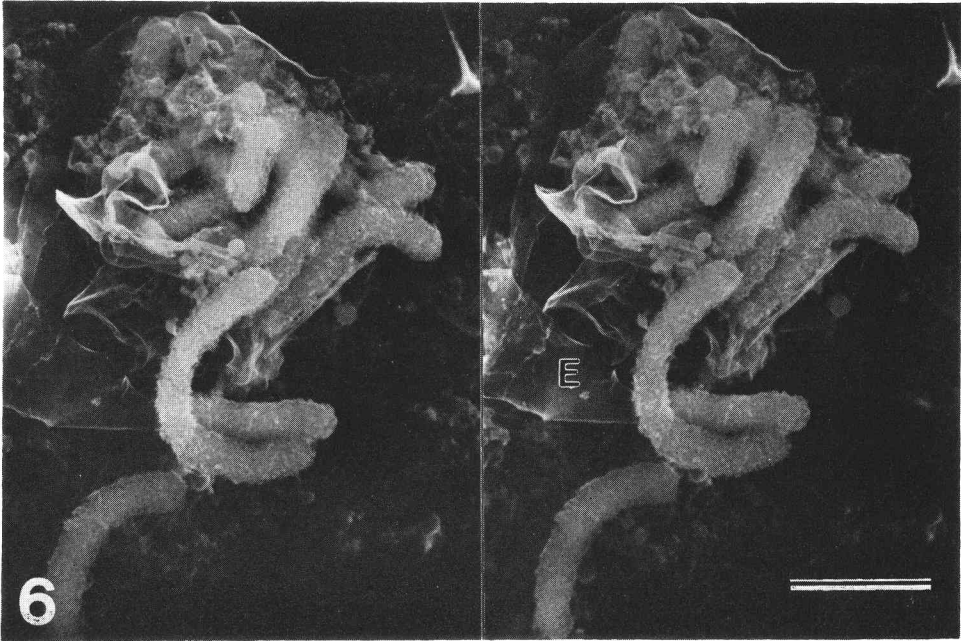


Fig. 6. The material was frozen directly without DMSO pretreatment. A prometaphase cell. E: remnant of the nuclear envelope. Magnification $\times 3,700$, bar = $5 \mu\text{m}$. Stereopair, tilt angles ± 2 .

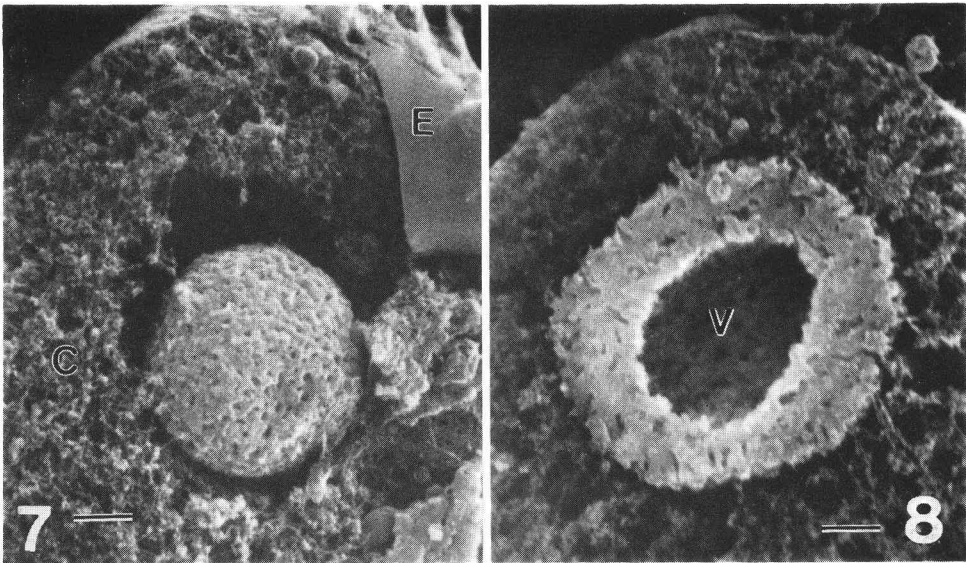


Fig. 7. Preparation as for Fig. 7. The fracture passed above a nucleolus. E: nuclear envelope, C: chromatin. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

Fig. 8. Preparation as for Fig. 7. The fracture went through a nucleolus. V: nucleolar vacuole. Magnification $\times 7,500$, bar = $1 \mu\text{m}$.

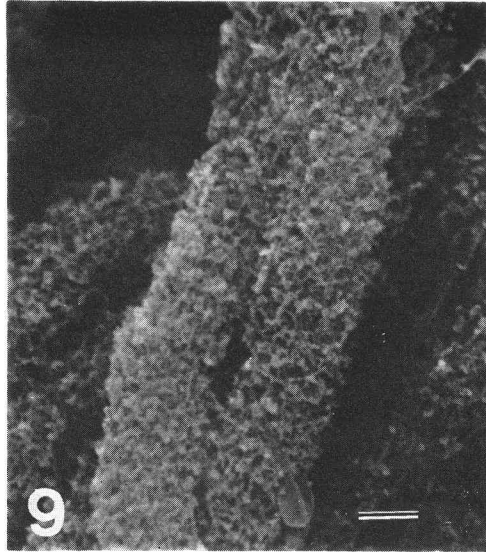


Fig. 9. The material was thawed in 40% DMSO without the fixative. Metaphase chromosomes consist of knobby 500 A-fibers. Magnification $\times 7,500$, bar = 1 μm .

in the aldehyde fixative containing lower concentrations of DMSO (20% or 10%), the nucleolus was difficult to identify in the interphase nuclei. When the roots were thawed in the fixative without DMSO, the interphase nuclei with the fine chromatin fibers could not be observed, although metaphase chromosomes were evident. When the freeze-fractured roots were immersed in 40% DMSO without the fixative for 1–2 min and were then fixed in the aldehyde fixative, it was also difficult to identify the nucleus and nucleolus, but metaphase chromosomes were easily found, and the knobby 500 A-fibers could be seen on the surface (Fig. 9).

Discussion

To permit clear observation with the scanning electron microscope of intracellular structures, it is necessary to devise a method by which the soluble cell-matrix can be removed. Haggis and his associates (1977, 1979, 1981, 1983) devised a technique of delaying fixation until after freeze-fracture and thawing. To avoid ice-crystal formation, they infiltrated cells with a cryoprotectant, DMSO or glycerol. However, unfixed cells did not tolerate the pretreatment with DMSO or glycerol. Initially, they made samples 100–150 μm thick and pretreated with the cryoprotectant at 25% (Haggis and Bond, 1979; Haggis, 1981). Later, they made samples only 15–20 μm thick from monolayers of lymphocytes attached to thin support films, and reduced the concentration of the cryoprotectant to 10% (Haggis et al., 1983). This method is, however, difficult to apply to the study of tissue cells. In root meristematic cells of *Vicia*, some toxic effects were apparent even when the concentration of DMSO was reduced to 5%. However, as shown in Figs. 6–8, nuclei and chromosomes were evident by direct fre-

eizing of whole roots without using any cryoprotectant. The damage by ice-crystal formation was not apparent. At least, the damage, if present, was less than that by the DMSO pretreatment.

The direct freezing method has the advantage of making specimens with a large plane for observation. It gives a good view of nuclei and chromosomes. A high concentration (40%) of DMSO in the thawing solution may serve to lessen the freeze-thawing damage. Lower concentrations (20%, 10%, or 0%) of DMSO failed to preserve nuclear and nucleolar fine morphology. Chromosomes were, however, evident even when the roots were thawed in the fixative without DMSO.

Thawing in 40% DMSO without the fixative also failed to preserve the nucleus. But metaphase chromosomes were evident. This provides further prospects to study the substructure of chromosomes by treating the freeze-fractured, unfixed cells with salts, detergents, or enzymes before fixation. This line of the study is now under way.

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