Technique for Scanning Electron Microscopy of Fungal Structures within Plant Cells

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ABSTRACT

A procedure for the removal of host cytoplasm from infected cells and the preservation of fungal structures for scanning electron microscopy (SEM) is described. The technique permits the use of SEM to study the morphology and spatial relationships of intracellular structures produced by parasitic fungi. Haustoria of pathogenic fungi and arbuscules of a vesicular-arbuscular mycorrhizal fungus are illustrated.

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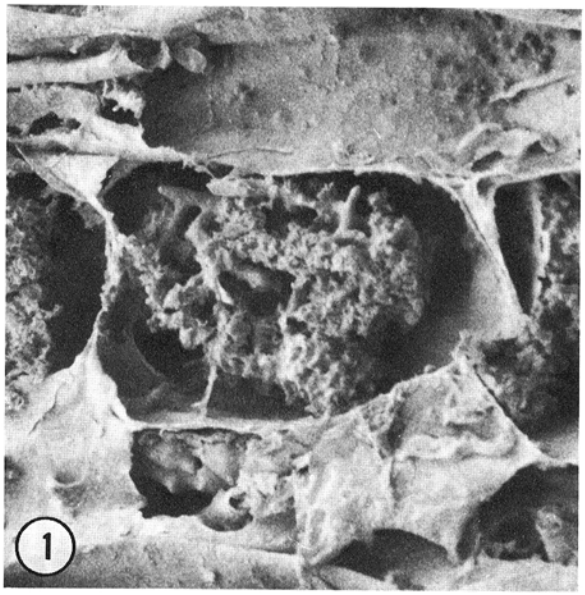
Recently, scanning electron microscopy (SEM) has been employed to elucidate the distribution and physical relationships of fungi within plant tissues (3, 4, 7, 10). Intracellular hyphae of *Cronartium ribicola* in pine bark (10) and of *Phytophthora infestans* in potato tubers (3) have been demonstrated, but the haustoria produced by these fungi were not observed or could not be identified in these studies. Scanning microscopy of haustoria and, in our experience, of arbuscules in vesicular-arbuscular mycorrhizal roots, is complicated by the presence of cytoplasm and organelles of the host cell. The following technique, devised to remove host cytoplasm and to reveal the morphological features of arbuscules of *Endogone* sp. in mycorrhizal roots of yellow poplar, was employed with natural infections of wheat leaves by *Erysiphe graminis* DC. f. sp. *tritici* Em Marchal and *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. and with barley leaves infected by *Puccinia hordei* Otth. to evaluate its effectiveness for SEM studies of haustorial morphology.

MATERIALS AND METHODS.—The specimens were fixed with 3% glutaraldehyde-3% acrolein in a 0.1 M K-K_2 phosphate buffer, pH 6.8 - 7.2, at room temperature for 6 hours or longer depending upon sample size. This buffer system was employed for all buffered solutions. After three or four rinses to remove excess fixative and an overnight wash in the buffer, the samples were postfixed in buffered 2% OsO_4 for 6 hours or longer at 4 C to harden the tissue. The specimens were rinsed thoroughly in buffer and cut with a sharp razor blade along the longitudinal axis of infected host cells to expose the maximum interior area. Tissue pieces which served as controls were randomly selected from each tissue type. These samples received no hydrolytic treatment and, consequently, cytoplasm and organelles were retained in the fractured host cells. Attempts to remove cytoplasm from fractured host cells by KOH hydrolysis (8) indicated that, with osmium-fixed tissues, it was not possible to achieve complete removal without introducing gross morphological artifacts at the SEM level. Consequently, in order to minimize treatment with KOH to hydrolyze host cytoplasm, oxidation of bound osmium to the soluble tetroxide form with periodic acid (5) was employed. Those samples from which host cytoplasm was to be removed were treated with 1.0% aqueous periodic acid for 2-3 min. They were rinsed five or six times in distilled water over a 10 minute period, then treated with 4.0% aqueous KOH for 30 minutes at 55 C. Following a 5-minute exposure in 1.0% acetic acid, the samples were rinsed with eight to ten changes of distilled water over a 1 hour period and were placed in buffered OsO_4 for a minimum of 6 hours to re-harden them for further processing. Treatment with OsO_4 for periods up to several days, either at this stage or previously, did not alter specimen morphology adversely. The second OsO_4 treatment was essential in reducing drying artifacts later in the preparation procedure. Excess OsO_4 was removed from both treated and control samples with five or six buffer rinses prior to dehydration in a graded ethanol series (20, 40, 60, 80, 95, and 100%). The ethanol was replaced with amyl acetate in an ethanol-amyl acetate series (30, 50, 70, 90, and 100%) and the specimens were critical-point dried (1). Dried specimens were mounted on holders with conductive cement, coated with gold in a vacuum evaporator and examined with a JEOL JSM-S1 scanning electron microscope operating at 10 Kev.

Fig. 1-7. Scanning electron micrographs of intracellular fungal structures demonstrating the effect of periodic acid-KOH hydrolysis in comparison to untreated control samples. **1, 2)** Cortical cells of yellow poplar root containing arbuscules of *Endogone*; control and treated, respectively (both × 1,500). **3,4)** Wheat leaf epidermal cells containing digitate haustoria of *Erysiphe graminis tritici;* control and treated, respectively. Haustoria of *Puccinia graminis tritici* (arrows) are present in Fig. 4 (both × 1,500). Fig. 5, 6) Barley leaf mesophyll containing *Puccinia hordei;* control and treated, respectively (both × 2,000). **5)** Host chloroplasts (arrows) and hypha-like structure of *P. hordei.* **6)** Hypha-like structure and bulbous haustorium of *P. hordei.* **7)** Haustorium of *P. hordei* with haustorial neck visible in treated sample (× 3,000).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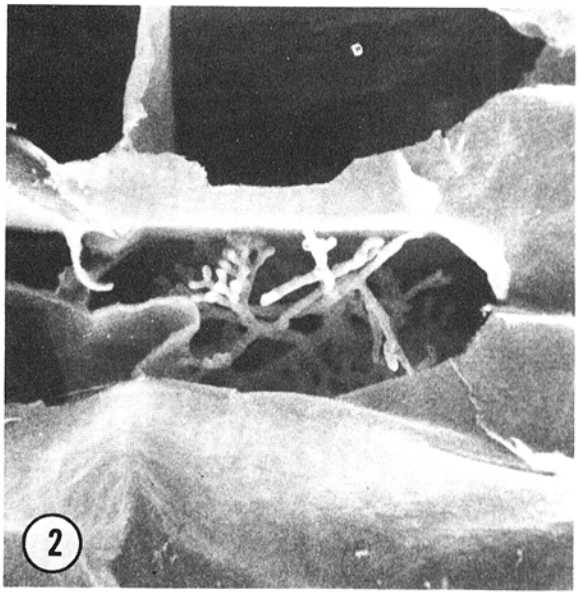
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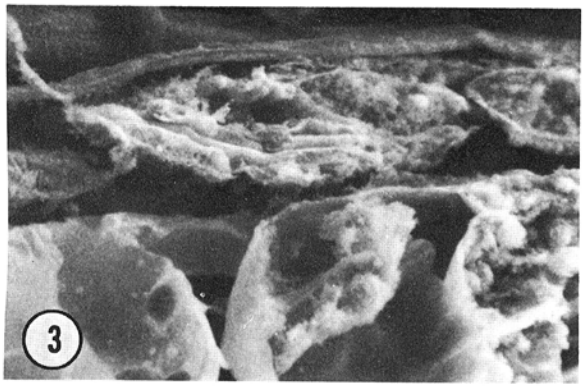
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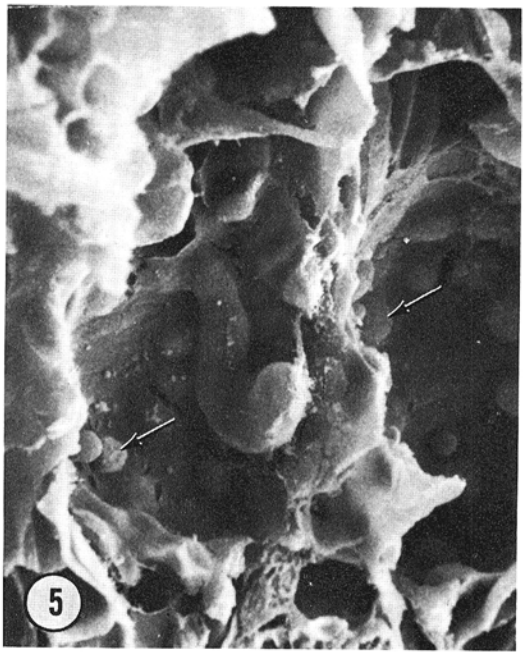
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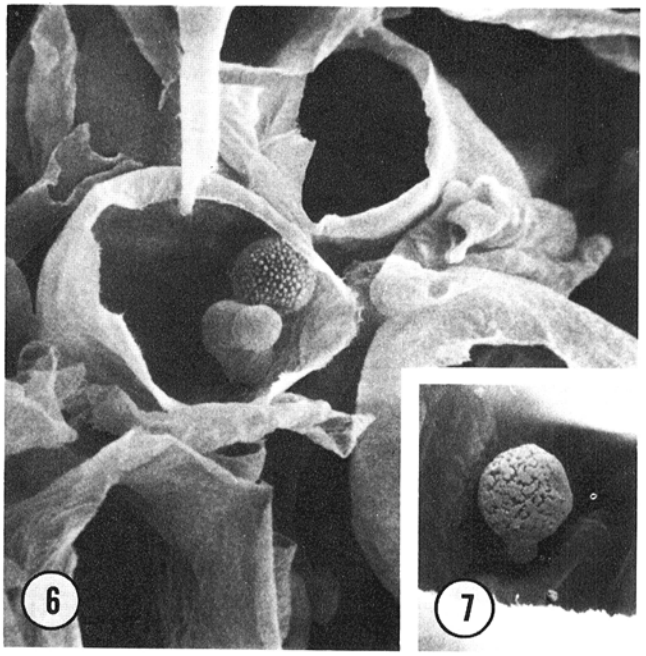
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RESULTS AND DISCUSSION.—While arbuscules of *Endogone* and haustoria of *E. graminis tritici* were easily located in control samples (Fig. 1, 3), little of their three-dimensional structure could be discerned. However, these structures were well preserved and clearly visible in samples subjected to periodic acid-KOH treatment (Figs. 2, 4) which completely removed cytoplasm and all membrane bound organelles from fractured host cells. Occasionally, individual starch grains were observed in treated samples. The sample shown in Fig. 4 supported a dual infection of *E. graminis tritici* and *P. graminis tritici*. Haustoria of both pathogens were easily differentiated in treated samples but those of *P. graminis tritici* were infrequently observed in control samples. The haustoria of *P. graminis tritici* approximate the size of chloroplasts and were difficult to differentiate from these and other host organelles displaced during specimen preparation. Bulbous haustoria and hypha-like structures of *Puccinia hordei* were observed in treated barley samples. Bulbous haustoria (Figs. 6, 7) were abundant in these samples but they could not be distinguished from host nuclei in control preparations. The surface texture of bulbous haustoria in treated samples varied from smooth to verrucose or foveate, with the latter predominating. Differences in surface texture may represent stages of haustorial development and/or differential degradation of materials comprising the encapsulation. Elongate, hypha-like structures (Figs. 5, 6) also were observed in both control and treated samples. It was not determined whether these structures represent a stage of haustorial development or hyphal ramification of host cells, such as illustrated by Bolley and Pritchard (2).

This technique has provided a reliable method for removal of host cytoplasm and organelles as well as excellent morphological preservation of intracellular fungal structures at the SEM level in various host-parasite associations. It is possible that more mild oxidants, such as peracetic acid (6) or oxone (9), also may be used to remove bound osmium in SEM preparations. Such treatments, in combination with enzymatic hydrolysis, may provide a means of defining the chemical composition of the encapsulation or sheath surrounding haustoria of pathogenic fungi.

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