

Application of Scanning Electron Microscopy to Paraffin-Embedded Plant Tissues to Study Invasive Processes of Plant-Pathogenic Fungi

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ABSTRACT

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Scanning electron microscopy (SEM) applied to paraffin-embedded tissue sections is compared with the traditional techniques of light microscopy and surface SEM for the study of invasion by a plant-pathogenic fungus. SEM of paraffin-embedded sections of wheat leaves infected by *Coprinus psychromorbidus* consistently yielded high-quality micrographs showing three-dimensional views of both internal and external

disease development processes. When the orientation of the specimen in the SEM is manipulated, the specimen can be viewed from different perspectives. The technique is simple and inexpensive and combines the advantages of great depth of focus and high resolution of the SEM with the simple preparatory techniques employed for light microscopy.

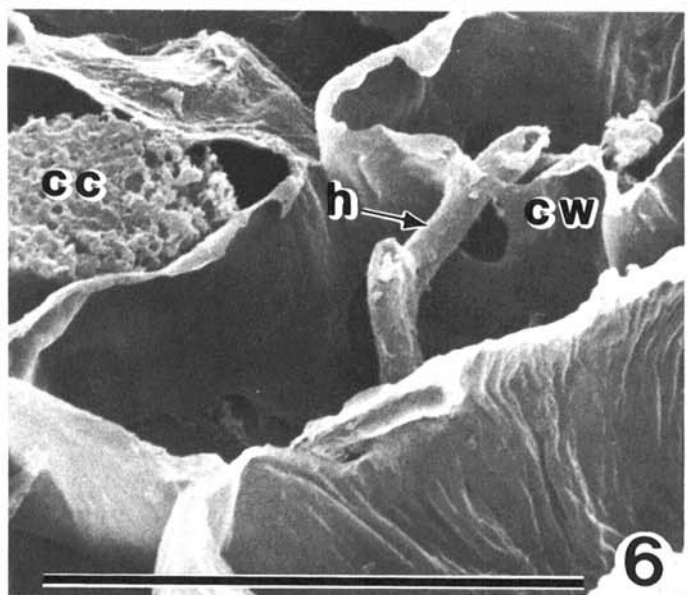
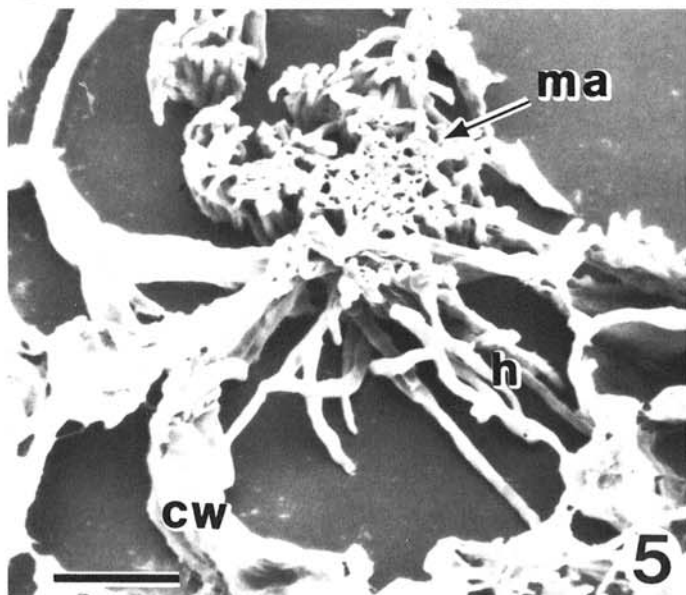
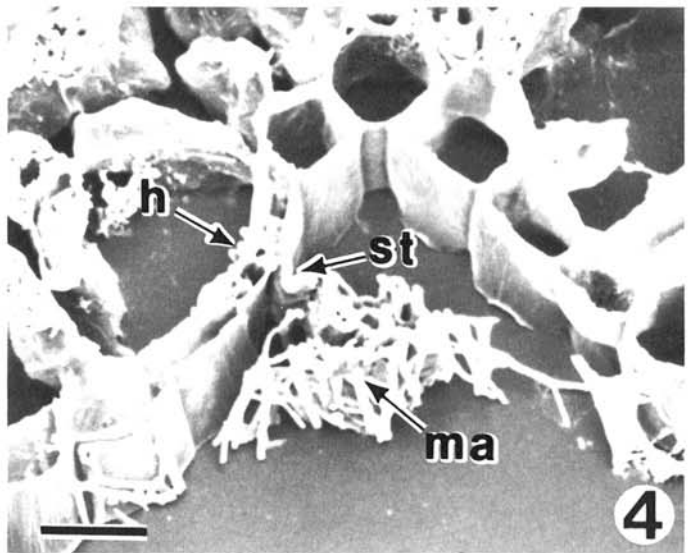
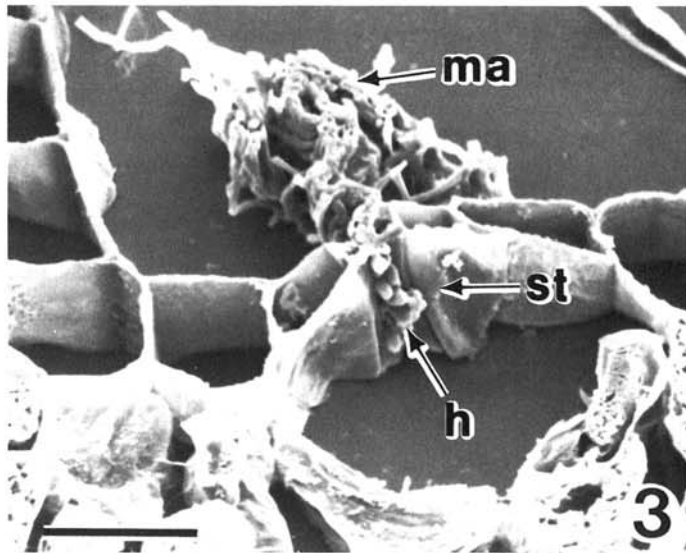
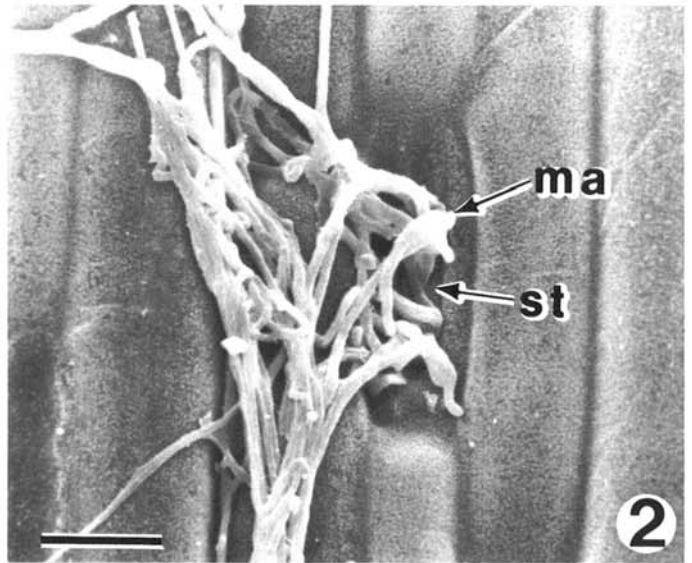
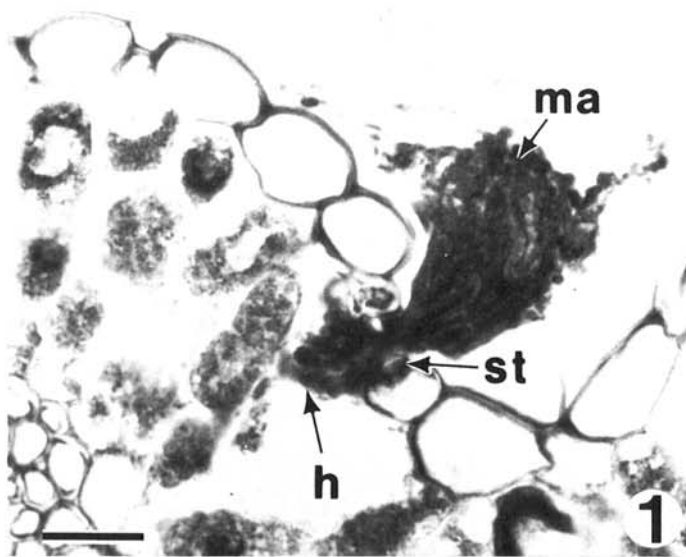
Additional key words: correlative microscopy, low-temperature basidiomycete, transmission electron microscopy, *Triticum aestivum*.

Scanning electron microscopy (SEM) of tissues and cells is normally limited to studies of surface phenomena. Methods involving freeze fracture can be useful for exposing internal structures of tissues, but these techniques are often complex and insufficiently precise for observing and quantifying highly localized histological events (4,6). Using SEM on sectioned, paraffin-embedded tissue combines the advantages of serial sectioning and differential staining used for standard light microscopy (LM), with

the high magnification, increased depth of focus, and higher resolution attainable by SEM. Visualization of three-dimensional orientations of both internal and external structures is made possible. Furthermore, this technique allows for direct correlation of LM images with those obtained by SEM, thereby improving the interpretation of scanning electron micrographs (1,3,4). The application of SEM to anatomical studies of paraffin-embedded sections has been demonstrated with human (1,2) and plant tissues (8).

This paper demonstrates the advantageous use of SEM with deparaffinized sectioned tissue in studying the penetration of winter wheat leaf tissue by the low-temperature pathogen *Coprinus psychromorbidus* (7). It exemplifies the numerous potential applications for this technique for studying the development of plant pathogenic fungi.

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Figs. 1-6. Micrographs of wheat leaves infected with *Coprinus psychromorbidus*. **1 and 2**, light microscopy (LM) and surface scanning electron microscopy (SEM), respectively, of mycelial aggregates (ma) and hyphae (h) penetrating a stoma (st). **3-6**, SEM of paraffin-embedded leaf sections. **3 and 4**, Hyphae (h) penetrating a stoma (st) in the same section, viewed from nearly opposite angles. **5**, Cell wall (cw) distortion and loss of cell contents associated with the spread of hyphae (h) in the leaf tissue. **6**, Hyphae (h) penetrating a cell wall (cw) adjacent to a healthy cell with normal-appearing cell contents (cc). Scale bars = 20 μ m.

MATERIALS AND METHODS

Cultivar Winalta winter wheat was grown in pasteurized soil in 15-cm-diameter pots in a growth cabinet at 20 C for 3 wk (18-hr day length), hardened at 2 C for 3 wk (12-hr day length), and inoculated with a pathogenic strain of *C. psychromorbidus* previously grown on sterilized rye grain. The inoculum was spread on the soil surface, and plants were covered with moistened absorbent cotton and incubated in snow-mold chambers (7) at -3 C. Infected leaf sections were collected at 2-wk intervals for 8 wk.

Paraffin-embedded tissues. Leaf sections were fixed in formalin-acetic acid-alcohol (FAA), dehydrated in Johansen's *t*-butyl alcohol series (5), and embedded in paraffin. Serial cross-sections, 10- μ m thick, were cut on a rotary microtome and affixed to an 18 \times 18-mm glass coverslip (for SEM) or to a glass slide (for LM) with Haupt's adhesive (5) and dried at 37 C for 24 hr.

For LM, sections were deparaffinized and stained according to Johansen's quadruple staining method (5). For SEM, sections were deparaffinized in xylene, rinsed in absolute ethanol, and air-dried. The coverslips were attached to standard aluminum specimen stubs with silver paint, sputter-coated with gold, and examined with a Hitachi S500 scanning electron microscope.

Conventional SEM. Excised leaf sections were fixed for 16 hr at 4 C with 4% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.0), buffer-rinsed, and postfixed in 2% osmium tetroxide in cacodylate buffer for an additional 4 hr. Material was then buffer-rinsed, dehydrated in an ethyl alcohol series, critical-point dried in CO₂, mounted in colloidal silver paste onto aluminum stubs, sputter-coated with gold, and examined with a Hitachi S500 scanning electron microscope.

RESULTS

Figs. 1 and 2 are "typical" illustrations of LM and SEM as used in a histological investigation. Figs. 3-6 illustrate results obtained by using the SEM technique with deparaffinized tissues.

In Fig. 1, a mycelial aggregate has formed above a stoma through which the individual hyphae have penetrated. Fig. 2 reveals surface detail of the initial formation of the mycelial aggregate and penetration of hyphae through a stoma. Figs. 3 and 4 show penetration through a stoma on the same leaf section, viewed from nearly opposite angles. Note that both surface and internal hyphae are visible. In Fig. 5, loss of cell contents and subsequent distortion and collapse of the cell wall associated with the spread of the pathogen through the leaf tissue is clearly depicted. Fig. 6 reveals details of the penetration of a plant cell wall by a hypha of *C. psychromorbidus*. Note the smooth surface of the borders of the penetration hole and the cell contents of an adjacent, apparently normal, healthy cell. Details of both inter- and intracellular surface topography are readily discernible.

DISCUSSION

SEM of deparaffinized tissue sections of winter wheat infected by *C. psychromorbidus* consistently yielded high-quality micrographs of external and internal disease development processes. In addition to increased magnification and depth of focus, SEM of deparaffinized sections offers several advantages over standard techniques presently used for LM, SEM, and

transmission electron microscopy (TEM). This technique is simple and inexpensive, especially when routine paraffin embedding techniques are employed. Appropriate sections may be verified by LM before the effort is expended in preparing the specimens for SEM. Information available from LM may be directly correlated with the higher resolution available with SEM. Manipulating the orientation of the section in the SEM provides great flexibility in viewing the same subject from different perspectives. This flexibility, combined with the high resolution attainable by SEM, permitted detailed examination of fungal penetration of the cell wall (Fig. 6). Lack of any observable tearing along the edges of the penetration hole suggests that enzymatic dissolution of the cell wall has occurred.

Techniques have been described for removing epoxy resins normally used in preparing sections for TEM so that the specimen may be viewed by LM as well as SEM (9). However, the procedures for dissolving the polymerized plastic are complex and often cause severe distortion of the specimen (6). Because ultrathin sections (50-100 nm thick) are required for TEM, the advantage of the increased depth of focus obtainable with SEM is lost. Ultrathin sections are not appropriate for the localization of relatively rare events such as penetration and disease progression. Due to the extreme thinness of the sections, literally thousands are needed to cover a small subject area. SEM of cryofractured tissues permits examination of internal structures, but the technique can be complex, requiring specialized low-temperature sectioning and critical-drying equipment (5). Furthermore, because the line of fracture cannot be controlled, a single, almost random line of cleavage will occur instead of the continuous, precisely oriented serial sections of known thickness that are obtained by sectioning paraffin-embedded tissue.

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