Merton F. Brown and Jerry A. White
University of Missouri, Columbia

Applications of Scanning Electron Microscop

Since the time of Leeuwenhoek and Hooke, microscopists have sought to push back the barriers imposed by the limitations of the human eye. In the quest to see ever smaller objects, the light microscope has been developed to an unparalleled state of perfection. Although several sophisticated types of light microscopy have been developed in the past 75 years, the limit of resolving power for the light microscope was reached around the turn of the century. Since the ultimate resolving power of any microscope is a function of the wavelength of the illumination used, the sole means of significantly increasing resolution and, consequently, useful magnification is to utilize illumination of shorter wavelengths. Below the spectrum of visible light, the most suitable radiation for this purpose is the electron beam. With a wavelength of less than 0.1 Å, the electron beam possesses a resolving power a thousand times greater than can be attained with light in the visible spectrum.

Transmission electron microscopes (TEM), which project a broad electron beam through the sample, were the first to be developed and sold commercially. Although transmission electron microscopes are capable of very high magnification and high resolution, the biologist interested in the morphology of cells, tissues, and organs is limited primarily to the two-dimensional images yielded by ultrathin sections. External morphology can be studied by transmission electron microscopy only through the use of surface replicas of bulk specimens or, in the case of certain samples such as bacteria and viruses, by direct shadow-casting and/or negative staining techniques. While the resolution achieved with such preparations is much better than can be achieved with scanning microscopy, the attainable depth of focus is extremely shallow. Consequently, the application of transmission electron microscopy to most morphological studies is quite restricted.

The theoretical concepts that form the basis for scanning microscopy date back to the 1930s, and functional prototypes were developed in several laboratories during the 1940s and 1950s. However, the first commercial scanning electron microscopes (SEM) did not enter the marketplace until 1965. These early instruments yielded a depth of focus some 300 times greater than the light microscope, had a resolving power of about 250 Å (10 times better than the best light microscopes), and had a magnification range from as low as 15× to more than 50,000×.

Clearly, the SEM was ideally suited to bridge the gap between the light microscope and the TEM. In the past 15 years, significant improvements in imaging modes, signal processing, and analytical capabilities have further expanded the usefulness of scanning microscopy. Resolution performance has also been improved so that most modern scanning microscopes, such as that shown in Figure 1, are capable of resolving structures in the 50–70 Å range. While the primary applications of the SEM in the biological sciences have been in morphological studies, X-ray analysis capabilities have extended its use to studies in which data relating to elemental composition and distribution are desirable.

Image Formation in the SEM

A comprehensive consideration of the electron optics involved in the scanning electron microscope is clearly beyond the scope of this article. However, a brief description of processes involved in image formation may be of interest since they differ significantly from those employed for light or transmission electron microscopy. In most scanning microscopes currently available, the electron beam is generated from a small-diameter tungsten wire within a high-voltage electron gun situated at the top of a column which is maintained under a vacuum of better than 10^{-5} torr. The electron beam is projected through a series of electromagnetic lenses that progressively reduce the diameter of the electron probe to 50 Å or less. This small-diameter electron probe is scanned over the surface of the sample in a raster pattern of closely spaced parallel lines causing atomic excitation of the sample. Several different types of radiation are emitted that can be collected by appropriate detector systems to define different characteristics of a specimen. Those most useful in biological research are shown in Figure 2 along with their applications.

Of the modes of operation shown in Figure 2, the one used most extensively by biologists is the secondary electron mode, which yields three-dimensional morphological images. In this mode of operation, low-energy (secondary) electrons emitted from any given point on the sample are attracted to an electron detector and are then converted to an electrical impulse that appears as a single visible light spot on a cathode ray tube (CRT). The location of this spot on the CRT corresponds to the position of the electron probe on the specimen. The intensity of the spot reflects the relative quantity of secondary electrons emitted and collected from the particular location on the surface of the specimen. The number of secondary electrons emitted from each such data point varies proportionately with the surface area intersected by the electron probe, ie, secondary electron emission increases as obliqueness of the sample surface to the impinging beam increases. Also, a greater proportion of secondary electrons emitted are collected from those data points facing the detector. Consequently, as the electron probe traverses the sample, a topographic image created by varying quantities of secondary electrons detected along each scan line is formed point-by-point on the CRT in the same manner that a television image is produced. The image can be altered during examination by moving the sample along the X and Y axes, or rotating or tilting it to achieve the desired orientation. Ultimately, the image is photographed on a high-resolution CRT.

Because of the way in which the secondary electron image is formed, the scanning micrograph photographically records the specimen as though it were illuminated by a diffuse but directional light source. Since most objects we observe are lighted in a similar manner, scanning electron micrographs look realistic and require virtually no conscious effort for interpretation of three-dimensional relationships. This capability to portray the specimen in an easily interpreted fashion and at high magnification has made the SEM a valuable tool in both teaching and research applications. An artistically composed scanning micrograph of a well-preserved organism or structure stresses the validity of an accompanying descriptive text because it *looks* lifelike.

Despite the complexity of the scanning microscope, operation of the instrument is undoubtedly the least demanding aspect of studies employing biological materials. Since most biological scanning microscopy is performed at magnifications below 20,000×, the necessary operator

Missouri Agricultural Experiment Station Journal Series No. 8784.

0191-2917/82/040 28209/$03.00/0
©1982 American Phytopathological Society

skills can usually be achieved with only a few hours' instruction. This is not to imply that expertise with the instrument is not required; it is. However, manufacturers of scanning microscopes have incorporated such a wide range of "convenience" accessories in recent instruments that much of the guesswork formerly involved in SEM work has been eliminated. Once one learns basic operational sequences and has defined those operational parameters best suited to the material being examined, the balance of the SEM study essentially rests with the ability to produce suitable preparations for SEM examination. Unfortunately, the scanning microscope does not discriminate between fact and artifact—both are revealed in three-dimensional perspective. Consequently, the major challenge to the biological scanning microscopist is to reduce the frequency of artifacts to an acceptable level. While one would hope to produce artifact-free preparations, this is probably never achieved using even the best techniques because these methods incorporate treatments that produce dimensional changes in the specimen. Nevertheless, careful application and manipulation of existing techniques will generally yield preparations in which alterations are so subtle as to be indistinguishable at the SEM level.

Because scanning microscopy can be employed with such a broad spectrum of biological materials, a multitude of preparative techniques have been developed that yield excellent preparations of many organisms and tissues (2,7). As might be expected, preparative requirements for SEM are highly specimen-dependent, not only with respect to the type of organ and or organism involved, but also with regard to the particular component(s) of the sample to be preserved and to whether external or internal morphological features are to be examined. Clearly, there is no universal solution to the preparation of specimens for scanning microscopy any more than there is a single protocol suitable to all biological materials for light microscopy.

Sample Preparation

A minimal criterion for acceptable preservation of any biological specimen for SEM examination would be that the structure(s) examined accurately reflect three-dimensional morphological characteristics as well as they can be defined at the light microscopic level. Without this low-magnification confirmation of the faithful preservation of known characteristics, morphological characteristics of a much finer order seen in the SEM must be considered suspect. A biologist is confronted with two significant problems that must be resolved if high-quality micrographs of surface structure are to be obtained. First, since the sample is subjected to a vacuum of 10^{-5} to 10^{-6} torr in the microscope, all free and bound water in the specimen must be removed in such a manner as to minimize morphological alterations and desiccation artifacts. The second difficulty arises because dry biological specimens are very poor electrical conductors and, consequently, must be made conductive to obtain acceptable secondary electron images of them. An optimal preparation retains the morphological features of the living specimen and is uniformly conductive. The scanning microscopist must select or devise preparative techniques that satisfy these requirements and simultaneously fulfill the objectives of a particular study.

While nonbiological materials usually require very little preparation before they are examined in the SEM, the means employed to remove water from biological specimens profoundly influence the preservation of morphological features of the specimen. Air-drying is obviously the most straightforward way of removing most of the water from biological specimens but only a few inherently rigid types of specimens can withstand the enormous surface tension stresses that accompany the evaporation process. When samples are air-dried, surface tension stresses, which may be equivalent to more than 40,000 kg cm^{-2}, are exerted on the specimen as the liquid-gas phase boundary moves through it. Forces of this magnitude are sufficient to cause gross distortions or collapse of nearly all types of biological structures. To illustrate this point, we selected a smaller European elm bark beetle that had been collected in 1968 and stored in an entomological museum as a dry mount. The beetle was sonicated briefly in an aqueous detergent solution, rinsed in distilled water, and then allowed to air-dry. Conidia from a fresh culture of *Ceratocystis ulmi*, the Dutch elm disease fungus, were deposited on the beetle in an aqueous suspension and were also allowed to air-dry. Morphological characteristics of the beetle that would be of interest to an insect taxonomist are well preserved, despite the rather rough treatments imposed upon the specimen (Fig. 3A). Many of the fungal spores, however, are collapsed or depressed as a consequence of surface tension stresses associated with air-drying (Fig. 3B). More complex specimens are typically so

Fig. 1. A high-resolution scanning electron microscope equipped with an energy dispersive X-ray analyzer.

distorted following air-drying that they bear little resemblance to their true morphology.

Water loss through air-drying of a freshly collected specimen, a common problem encountered with field-collected specimens, usually causes irreversible desiccation artifacts that are subsequently preserved by SEM preparative sequences. Even fungal cultures within petri dishes may show significant artifacts in the SEM if they have been studiously examined under intense illumination before preparation. Prerequisites to good preparations are to start with fully turgid specimens bearing the desired structures and to initiate the preparative sequence immediately before desiccation can occur. This requires that one know quite precisely what one seeks to resolve with the SEM *before* the specimen is collected, as well as how the specimen is to be processed. An awareness of the importance of preventing desiccation is a significant step toward good SEM preparations in the laboratory. Field collections require that the specimens be maintained in a cool moist chamber from the time they are collected until the SEM preparation sequence can be initiated.

With representative fresh specimens in hand, the initial step in the preparation of biological samples for SEM is stabilization of morphological structure. One of the first means by which this was accomplished, and one that is still required for the preservation of such structures as mucilaginous spore droplets (Fig. 3C), is physical stabilization by means of quick freezing. The water (ice) contained in the specimen is subsequently removed by sublimation under vacuum. Freeze-drying has been employed successfully with a broad variety of biological materials and, as one might suspect, the conditions that must be fulfilled to employ this technique effectively have become increasingly sophisticated (1). Samples must be quick-frozen at temperatures approaching that of liquid nitrogen within microseconds and they must be maintained at temperatures of no less than −60 C throughout the sublimation period. These conditions are achieved by quenching small samples in Freon 12, or a similar fluid, cooled with liquid nitrogen. The frozen samples are placed in a vacuum evaporator or freeze-drying unit equipped to maintain the sample at a temperature of −60 C, under a vacuum of approximately 5×10^{-3} torr, throughout the sublimation period. When the drying process is judged to be complete, which may require several days for large specimens, the sample is warmed to room temperature over a period of several hours before returning it to atmospheric pressure. Although freeze-drying techniques can be used with chemically fixed specimens, or samples frozen in dehydrating solvents such as ethanol or acetone, these procedures have been essentially replaced by other methods more suitable to specimens that have been immersed in liquid fixatives or an aqueous medium.

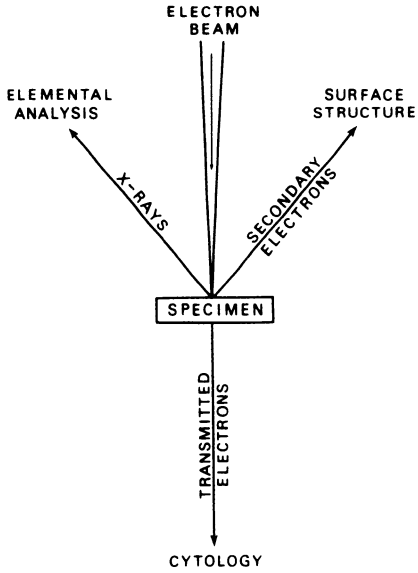


Fig. 2. Diagram of biological applications of radiation generated by the electron beam in a scanning electron microscope.

Major disadvantages of freeze-drying techniques are that the rate of freezing must be both extremely fast and uniform throughout the sample to avoid ice crystal formation, the quenching phase of the procedure may cause severe mechanical damage to aerial structures, and ice recrystallization may occur during the sublimation period causing rather dramatic artifacts. Despite these detrimental aspects, freeze-drying is currently the only means by which instantaneous stabilization of all components of any given specimen can be achieved. Highly mobile organisms or components of samples that are water-soluble or are solubilized by the organic solvents employed in other preparative routines may be preserved successfully by freeze-drying of unfixed or vapor-fixed specimens.

The most widely used drying procedure for SEM of biological specimens is critical-point-drying. This technique requires chemical fixation of the sample in an appropriate fixative solution such as glutaraldehyde and/or osmium tetroxide, gradual dehydration in an organic solvent, and, finally, drying by the critical-point method (3). Fixation accomplished by immersion in aldehyde or osmium solutions is not instantaneous and does not stabilize all components of all specimens. These fixatives stabilize primarily the proteinaceous components of biological specimens, and the degree of structural stabilization achieved depends not only on the chemical composition of specific components of the sample but also on the solubility of these components in a dilute aqueous environment.. Consequently, external mucopolysaccharides are often partially or totally solubilized in liquid fixatives and are incompletely preserved, whereas insoluble structural components of the sample are usually well preserved by exposure to these fixative solutions. In many instances, the loss of external soluble substances is actually advantageous in SEM studies, since underlying structural components of the specimen are more clearly exposed to view. In other cases, specimens may require deliberate cleaning procedures to solubilize superficial substances before chemical fixation is attempted in order to obtain an unobstructed view of the desired structures. Specimens produced in nutrient solutions, for example, are washed with a suitable buffer before fixation to prevent deposition of components of the medium on the surface of the sample. Buffers employed in fixatives are also removed by rinsing in distilled water before dehydration with acetone or ethanol. Once the sample has been thoroughly dehydrated, it is placed in a critical-point apparatus and the dehydrating solvent is replaced by liquid CO_2 or Freon. When the temperature of the apparatus is elevated above the critical point of the liquid used (ie, CO_2), the liquid CO_2 instantaneously converts to a supersaturated gas without forming a liquid-gas interface. Since no phase boundary forms, surface tension stresses are absent and their effects on the specimen are circumvented. The dry sample is returned to atmospheric pressure by gradual release of the gas.

While the protocol required for critical-point-drying is relatively simple, the numerous solution changes which must be performed introduce a high probability that the specimen will be damaged mechanically or that the sample will be exposed to a liquid-air interface at some stage in the sequence. Both conditions cause artifacts that are later preserved by the drying operation. Careful manipulation of samples is therefore required throughout the processing sequence.

The dry specimen is subsequently mounted directly on a metal specimen holder that is compatible with the SEM to be used or, alternatively, is attached to a metal, mica, or glass disk which in turn is fastened to the specimen holder. Electrically conductive adhesives, such as silver or graphite cements, are generally used for large sample pieces, while particulates may be deposited on double-stick tape fastened to the holder or other substrate. Before SEM examination, a 100–300 Å layer of carbon, gold, or gold alloy is deposited on the sample either by sputter coating or vacuum evaporation in order to render the specimen electrically conductive (4). Specimens may also be made conductive by employing solution-coating techniques that increase the quantity of osmium tetroxide bound to

the sample surface (6). These procedures involve treatment of osmium-fixed samples with thiocarbohydrazide, tannic acid, or other agents that attach to osmium molecules bound to the surface of the specimen. A second treatment with osmium tetroxide results in an additional layer of conductive osmium being deposited on the sample surface. Solution-coating techniques may reduce the likelihood of obscuring very fine surface details, which can sometimes occur with conventional gold-coating techniques. The probability of introducing mechanical damage to delicate aerial structures is increased, however, since the samples must be thoroughly rinsed following each step in the coating sequence. The pine wilt nematode (*Bursaphelenchus xylophilus*) shown in Figure 3D was fixed in aqueous osmium tetroxide, osmium-coated using thiocarbohydrazide, and then dehydrated and critical-point–dried.

Morphological Studies

While the majority of SEM studies relating to plants, insects, and pathogens have been of a basically morphological nature, the diversity of specific types of materials examined is as broad as the disciplines involved in plant protection. Most have involved the direct examination of biological material ranging from host tissue surfaces to submicroscopic structures of pathogens. The illustrations presented here comprise a representative

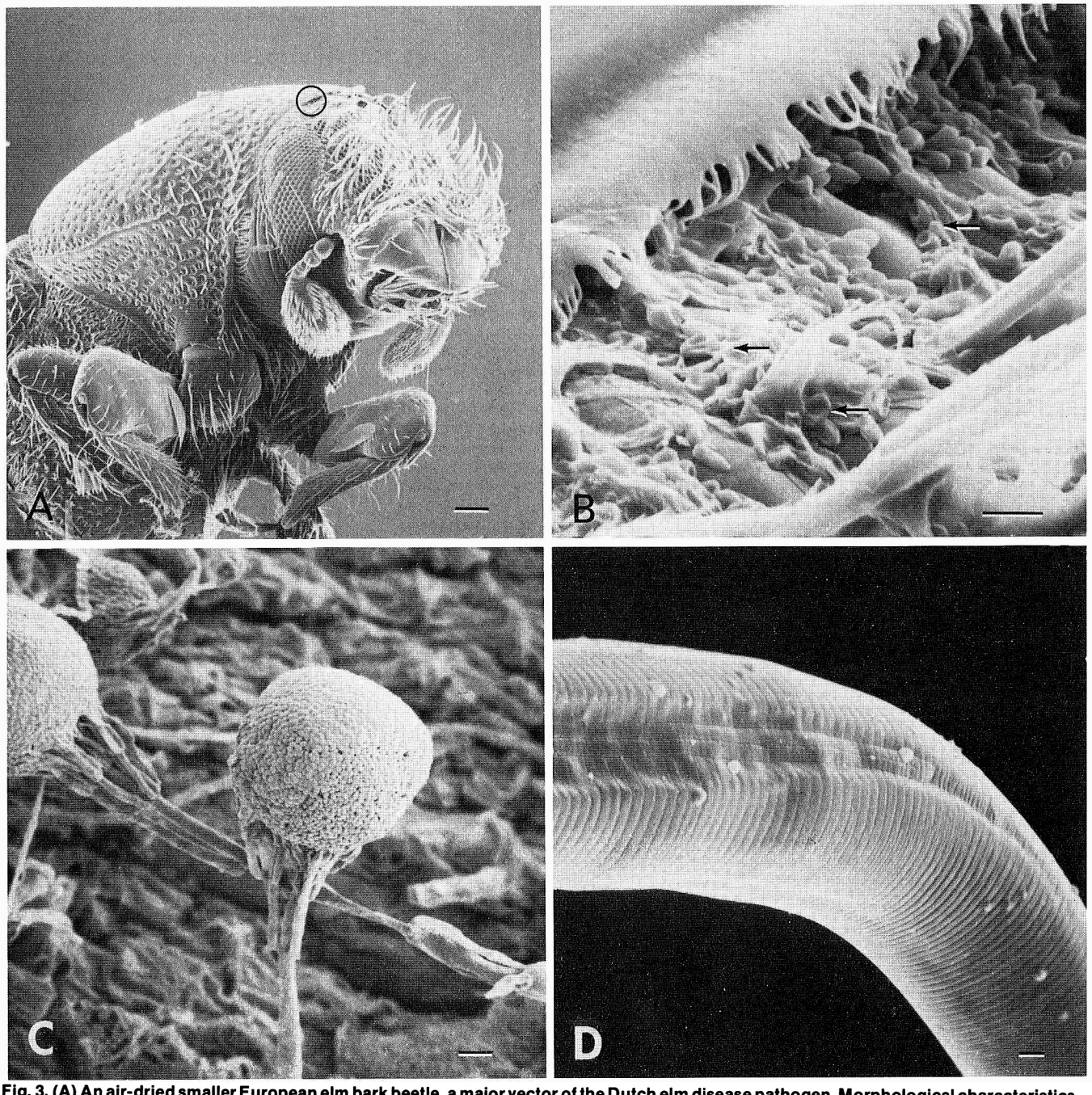
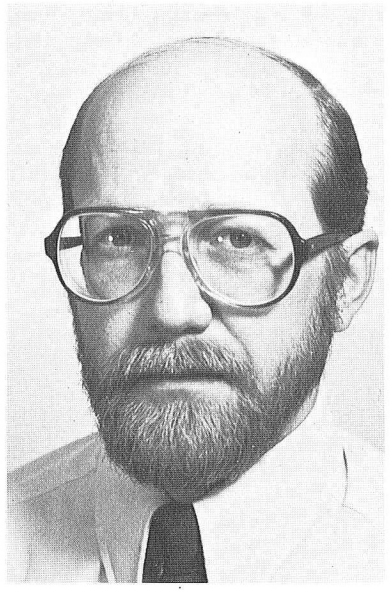


Fig. 3. (A) An air-dried smaller European elm bark beetle, a major vector of the Dutch elm disease pathogen. Morphological characteristics of taxonomic interest are well preserved. Sexual dimorphism is most readily defined by the relative abundance and length of hairs on the forward portion of the head; the male, shown here, has numerous long hairs. ×55. Bar = 100 μm. (B) An enlargement of the area circled in A. Air-dried conidia of the Dutch elm disease fungus, *Ceratocystis ulmi*, are shown in the depression between the thorax and head of the vector. While some conidia of the fungus have been protected by mucilage in the spore droplet, many are collapsed because of surface tension stresses associated with air-drying (arrows). ×2,000. Bar = 5 μm. (C) A conidiophore of *Gliocladium deliquescens* bearing a characteristic mucilaginous droplet of conidia. Members of this genus of the Fungi Imperfecti produce a *Penicillium*-like conidiophore, but the conidia are produced in a slime droplet that can be preserved only by freeze-drying. ×500. Bar = 10 μm. (D) A critical-point–dried third-stage larva of *Bursaphelenchus xylophilus*, the pine wilt nematode, showing the differentiation of components comprising the forward portion of a lateral incisure. The head of the nematode is to the lower right. ×4,000. Bar = 1 μm.

Merton F. Brown

Dr. Brown is professor of plant pathology and manager of the Agricultural Experiment Station Electron Microscope Facility at the University of Missouri, Columbia. He obtained his B.S. and M.S. degrees at the University of Maine–Orono and received his Ph.D. from the University of Iowa in 1966. Since 1968 he has been responsible for the development and administration of an interdisciplinary training and research laboratory in electron microscopy at the University of Missouri. His research has included scanning electron microscopy of plant-pathogenic fungi and ultrastructural studies of host-endophyte interactions in vesicular-arbuscular mycorrhizae. He has authored a text with H. G. Brotzman, "Phytopathogenic Fungi: A Scanning Electron Stereoscopic Survey," published in 1979.



Jerry A. White

Dr. White is a member of the Department of Plant Pathology and assistant manager of the Agricultural Experiment Station Electron Microscope Facility at the University of Missouri, Columbia. He holds a B.S. in biology and an M.S. in botany. He received his Ph.D. in horticulture from the University of Missouri in 1971. He has been involved with graduate-level instruction in electron microscopic techniques for the past 9 years and has served as a primary consultant and collaborator for scanning and transmission electron microscopic applications in many disciplines at UMC. He has also authored several publications involving ultrastructural studies in plant pathology. His present area of interest is energy dispersive X-ray analysis of biological materials.

range of organisms and host materials that have been studied by SEM in our laboratory. Although they by no means encompass the entire scope of applications to plant protection, these examples serve to illustrate several different ways in which scanning microscopy can be employed in morphological studies of plants, insect pests, and plant pathogens.

Much of the scanning microscopy performed in our laboratory is conducted on a service basis for faculty researchers who clearly recognize the value of SEM studies in their research programs but who have no personal experience with the preparation of specimens for scanning microscopy. Our approach to satisfying their requirements is to determine as precisely as possible what the investigator wishes to resolve by SEM, the nature of the specific specimens to be examined, the chemical composition and solubility of components of interest in aqueous media, and, of primary importance, in what condition the specimens would be received. With this information, it is generally possible to determine those preparative techniques best suited both to the specimens and the objectives of the study.

One recent study involved the feeding activities of a leafhopper, *Forcipata loca*, on tall fescue. Two entirely different types of specimens were involved: the mouthparts of the insect and the feeding wounds it produces on the host tissues. The leafhoppers were fixed cryogenically by placing a liquid nitrogen-cooled metal probe close to their feeding sites. The frozen insects were then deposited directly in absolute ethanol, rinsed briefly to remove extraneous debris, and air-dried. Air-drying from ethanol reduced surface tension stresses imposed on the samples by a factor of 3 in comparison to air-drying from water and was considered acceptable in view of the inherent rigidity of the structures to be examined (Fig. 4A). Feeding wounds on the host, however, required preservation of both the surface morphology of fescue epidermal cells and the proteinaceous salivary droplets deposited by the insect at the feeding site. Consequently, samples of fescue leaves were preserved by chemical fixation, sequential dehydration, and critical-point–drying (Fig. 4B). The latter specimens may also have been preserved by freeze-drying techniques with the possibility of preserving feeding leafhoppers in situ, although that was not a point of interest in this particular study.

Of the different groups of plant pathogens, more fungi have undoubtedly been examined with the SEM than any other. While the growth of certain fungal pathogens on plant surfaces and penetration phenomena have been investigated, the majority of the studies performed have been concerned with sporulative structures and sporogenesis. In virtually all instances, these studies have been performed with either freeze-dried preparations (Fig. 3C) or critical-point–dried specimens such as the conidiophores of *Aspergillus parasiticus* shown in Figure 4C, depending on whether external mucilage is present and whether preservation of it was desired. Although these conventional procedures are suitable for most mycological studies, there are frequent instances when scanning microscopy can be applied to the examination of structures that may be obscured in the usual SEM preparations or may be produced within rather than on host tissues. One means by which structures not visible in conventional SEM preparations can be exposed for SEM study involves preparation of the specimen following the usual embedment procedure for transmission electron microscopy. The embedded samples can be microtomed and the embedding medium extracted by suitable solvents, leaving the structures of interest exposed in sectional view. These sections may subsequently be critical-point–dried, coated with gold or gold alloy, and examined in the SEM. The sectional view of the apex of a conidiophore of *A. flavus* (Fig. 4D) demonstrates the capability to obtain quite precisely oriented sectional views of small structures while maintaining the structural integrity of components of the sample. In this instance, the specimen was embedded in an epoxy resin and sections were cut with a glass knife on an ultramicrotome.

An alternative method of obtaining sectional views of botanical or pathological specimens, employed extensively in our facility for examination of fungal fructifications in host tissues (2), is to section the material without embedment following fixation and to process the sections through dehydration and critical-point–drying in the normal manner (Fig. 5A). While a sectional view of a mature uredium is shown to illustrate one application of the use of sections obtained without embedment, the

procedure could as well be applied to earlier stages of the development of fungal or other pathogens in host tissues provided the sectioning procedure or subsequent processing would not displace the structures of interest from their position within the host. An example of one situation in which neither of the sectioning routines indicated would be entirely suitable, because the probability of loss of the pathogen would be so high as to preclude confidence in the SEM observations, involves the occurrence and/or distribution of bacteria within host tissues. In studies of this sort, despite the frequently conspicuous mechanical damage to some host tissues, it is desirable to prepare the infected samples or inoculated tissues in large pieces to minimize loss or internal redistribution of the pathogen during processing, then expose the pathogen in situ by manually slicing the sample along the most advantageous plane. Figure 5B shows the distribution of cells of *Erwinia amylovora* in the primary xylem vessels of an apple petiole prepared in this manner.

To this point, a number of techniques and illustrations have been presented that permit the use of the SEM to view surface morphology and/or distribution of various pathogens in different types of host tissues. This approach has been extended further to include the examination of microorganisms such as rust fungi, host wall alterations associated with nematode invasion, and structures of nonpathogenic symbiotic organisms such as nitrogen-fixing bacteria and mycorrhizal fungi. With specimens of this sort, it is usually necessary not only to "open up" the particular host involved but also to employ additional treatments to remove cytoplasmic components of the host cells that may enclose the particular

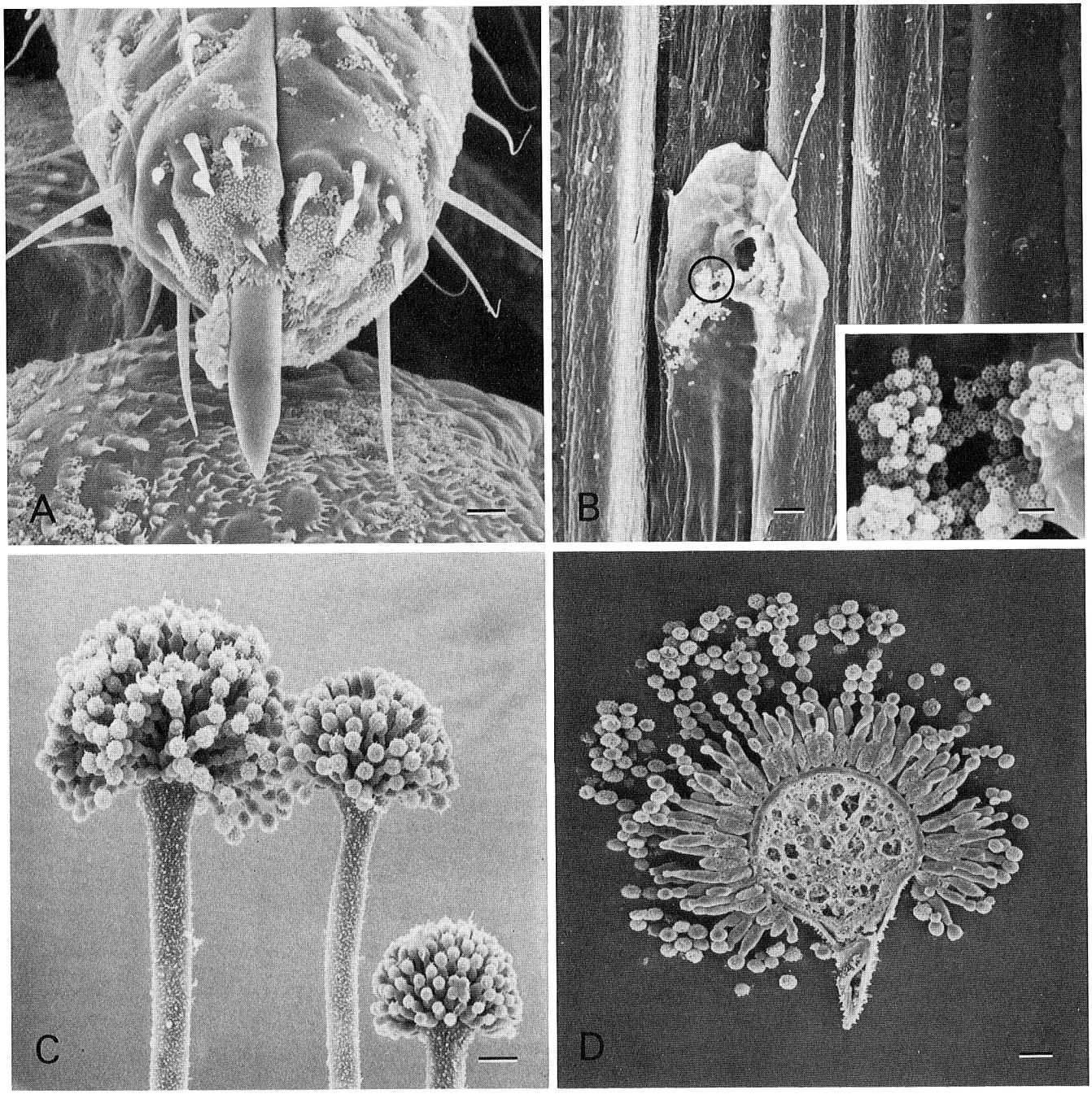


Fig. 4. (A) Mouthparts and tip of the retracted stylet of the leafhopper *Forcipata loca*. The insect was air-dried from absolute ethanol without chemical fixation. ×1,200. Bar = 5 μm. (B) A feeding site of *F. loca* on the upper epidermis of a tall fescue leaf. ×1,000. Bar = 5 μm. The salivary droplet includes perforated spherical particles (insert). ×6,000. Bar = 1 μm. The sample was chemically fixed and critical-point-dried. (C). Conidiophores of *Aspergillus parasiticus* showing a progression of conidial development from right to left. The sample was prepared by chemical fixation and critical-point-drying. ×650. Bar = 10 μm. (D) Sectional view of the apex of a conidiophore of *A. flavus* obtained by extracting the epoxy embedding medium from a 5-μm-thick section. ×540. Bar = 10 μm.

structures one wishes to examine. Figure 5C illustrate one such intracellular structure, an arbuscule of a vesicular-arbuscular mycorrhizal fungus, which was exposed for SEM examination by alkaline hydrolysis of host cytoplasm in hand-sectioned yellow poplar roots before dehydration and critical-point-drying. This basic technique of sectioning fixed, but unembedded, material and then selectively hydrolyzing certain components can also be applied to sporulation processes within such fungal fructifications as acervuli and pycnidia (2).

Analytical Scanning Microscopy

In addition to obtaining information pertaining to morphology, it is frequently advantageous to be able to define the chemical composition of certain structures that are observed. Chemical analysis in the SEM is made possible by the addition of an energy dispersive X-ray spectrometer to the microscope. Energy dispersive X-ray analysis is a technique that permits nondestructive qualitative and quantitative elemental analysis (5). X-ray analysis is based on the fact that atoms, when excited by a high-energy electron beam, emit X-rays of a wavelength and energy that are characteristic for each element. The energy dispersive system analyzes the various energies of all X-rays collected within a predetermined energy range and is capable of detecting all elements between sodium (Z = 11) and

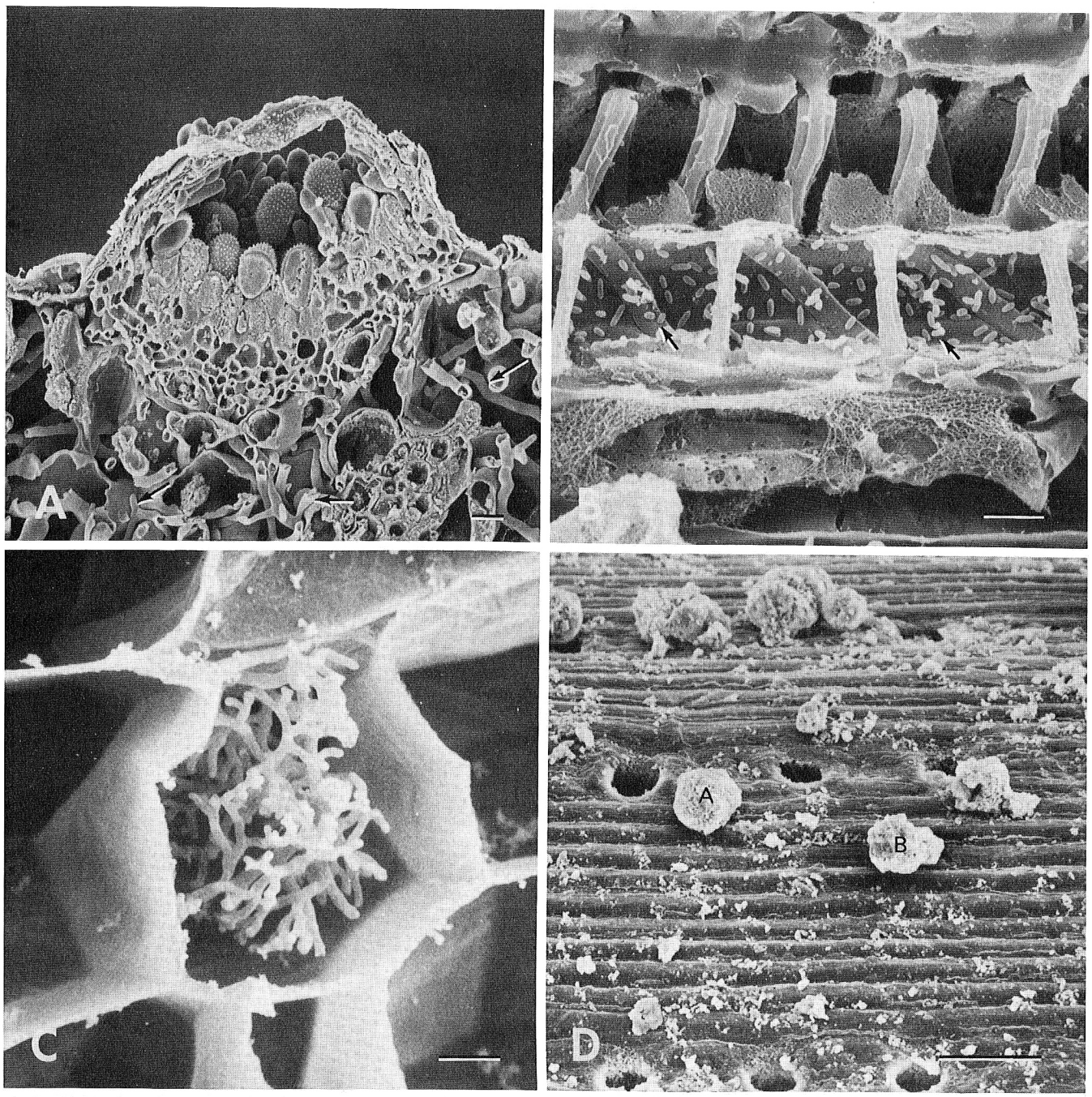


Fig. 5. (A) Section of a soybean leaf bearing a uredium of the soybean rust fungus, *Phakopsora pachyrhizi*. The leaf was sectioned without embedment after chemical fixation and was subsequently critical-point-dried. Hyphae of the pathogen (arrows) can be seen in the intercellular spaces of the host mesophyll tissue. ×500. Bar = 10 μm. (B) Cells of *Erwinia amylovora*, the fire blight pathogen, within primary xylem vessels of an apple petiole. Distribution of the bacterial cells (arrows) in relation to an inoculation point was defined by hand-sectioning the host tissue longitudinally after drying to minimize both displacement and loss of bacterial cells. ×2,000. Bar = 5 μm. (C) An arbuscule of *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus, within a cortical cell of yellow poplar root. This haustorium-like structure, normally enclosed by host cytoplasm, was exposed by alkaline hydrolysis of the sectioned root before critical-point-drying. ×2,000. Bar = 5 μm. (D) Particles of Bordeaux mix and Dithane on a pine needle. X-ray spectra obtained from particles A and B are shown in Figure 6. ×300. Bar = 25 μm.

uranium (Z = 92). There are three basic ways in which the X-ray signals from a sample can be related to morphological structure. Their application depends largely on the concentration of elements present and the objectives of the analysis. The first, and the most commonly employed mode of operation, is essentially a spot or static probe analysis of all elements present at a given location on the specimen. In this type of analysis, the electron beam is held stationary on the desired structure and the X-rays emitted from that location are analyzed. Distribution "maps" of a selected element can also be made to determine where, in a selected field of view, a particular element is located. This map can then be compared with the conventional secondary electron micrograph to equate areas of concentration of the selected element with morphological structures. A third means of analysis also relates localized concentrations of a selected element to a particular morphological structure. In this mode of operation, the electron beam is scanned in a single line across the structure of interest and the X-ray signal of the selected element is used to modulate the vertical deflection of the line on the CRT. X-ray analysis has been employed successfully with many biological materials and, although it has been used only to a limited extent in areas directly related to plant protection, there are many potential applications.

For purposes of demonstrating one application of X-ray analysis, that of identification of fungicide granules on host tissue, a combination of Bordeaux mixture and Dithane M-45, a coordination product of zinc ion and manganese ethylene bisdithiocarbamate, was deposited on pine needles and examined with the SEM to compare the morphology of the fungicide granules (Fig. 5D). A spot analysis X-ray spectrum for two particles of similar morphology shown in Figure 5D is illustrated in Figure 6. Both X-ray spectra include peaks of gold (Au) and palladium (Pd) due to the use of a gold-palladium alloy coating to confer conductivity to the sample. Manganese, but not zinc, was detected in the Dithane particle. The particle of Bordeaux mix analyzed is shown in Figure 7A at a higher magnification in combination with a line profile analysis for copper. A distribution map for copper, correlating to Figure 7A, is shown in Figure 7B. Areas of high copper concentration are indicated by

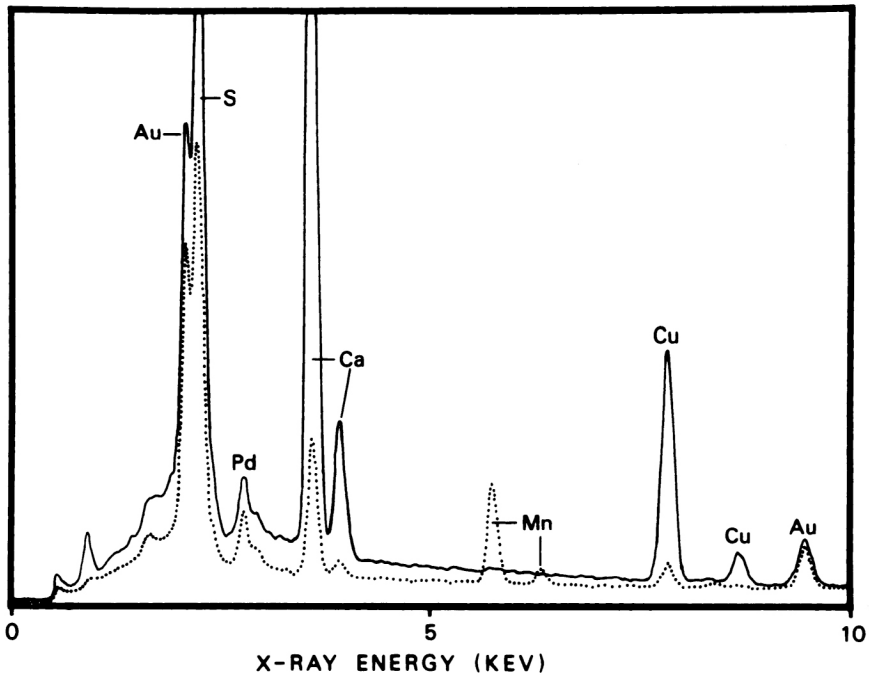


Fig. 6. Energy dispersive X-ray spectra of particles shown in Figure 5 D. Particle A (.....) is identified as Dithane because of the presence of S and Mn. Particle B (———) is Bordeaux mix because it contains Cu, Ca, and S. Gold (Au) and palladium (Pd) are present in both spectra because the sample was coated with Au:Pd alloy to obtain the secondary electron image.

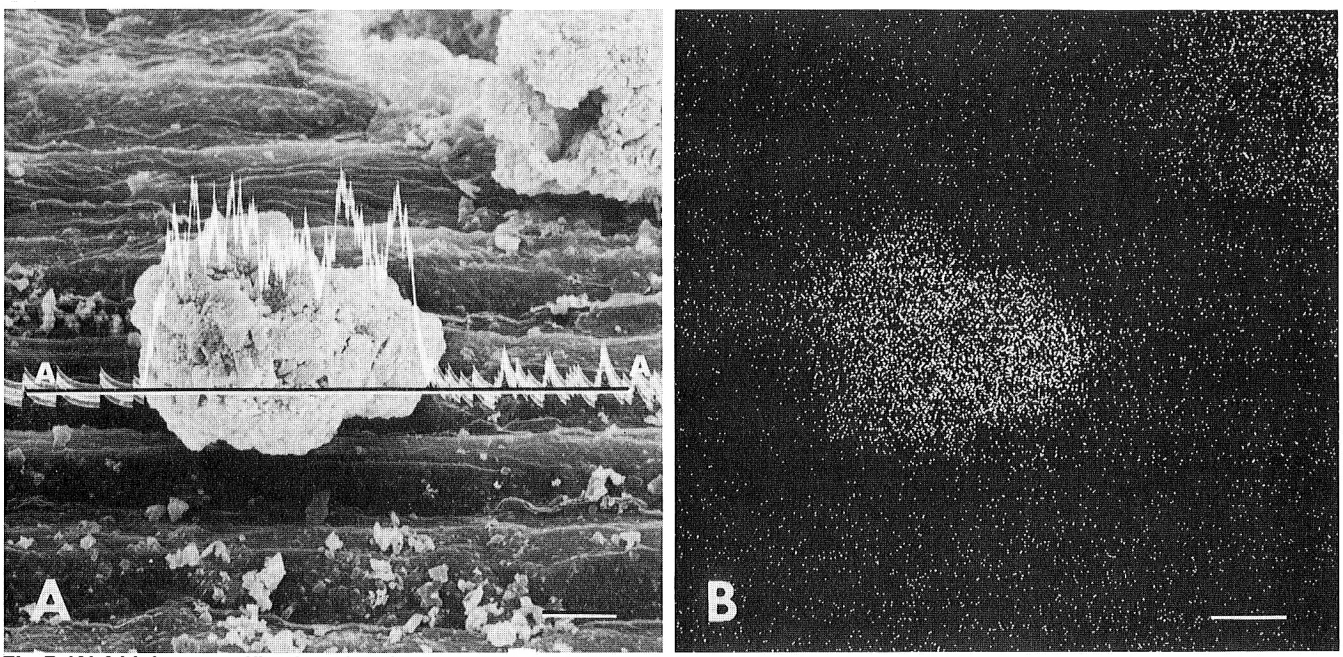


Fig. 7. (A) A higher magnification of the Bordeaux mix particle shown in Figure 5D. A line scan profile of copper concentrations along the line A—A is superimposed on the secondary electron image. ×1,000. Bar = 10 μm. (B) Area distribution map indicating the areas of copper concentration in A. High-density areas of white dots indicate sources of X-rays emitted from copper atoms. Although the particle in the upper right of A was not subjected to a spot analysis, it can be identified as a Bordeaux particle because of its copper content, an element not present in Dithane. ×1,000. Bar = 10 μm.

increasing density of white dots in the map represent ng sources of copper X-ray emission. Although the particle in the upper right of Figure 7A was not analyzed by the spot method, its high concentration of copper in the map indicates that i is a Bordeaux particle.

The third mode of operation described in Figure 2, the transmitted electron mode, extends the analytical capabilities of the SEM to include thin sections. This technique requires the addition of a transmitted electron detector situated below the specimen. A scanning microscope equipped with both an X-ray spectrometer and a transmitted electron detector is capable of performing elemental analysis on thin-sectioned material as well as bulk specimens. While the quality of the transmitted image cannot equal that produced by a TEM, this mode of operation may enjoy greater popularity as specimen preparation techniques and instrumentation improve. Most X-ray studies involving thin sections are better performed with instruments specifically designed for that purpose, ie, scanning transmission electron microscopes equipped with X-ray spectrometers.

Summary

In the 15 years since its introduction, scanning microscopy has been employed to explore a previously inaccessible world of microstructure ranging from structural analysis of moon rocks to ontogenetic studies of plant pathogens. It has not only become a valuable and often essential tool in research laboratories in all disciplines concerned with surface morphology but it has also provided a new sense of the realism of microscopic structures relating to highly technical disciplines which is readily appreciated by both students and an interested public. It is as useful in demonstrating features of relevance to the content of introductory courses as it is to the determination of new morphogenetic processes. The application of the SEM in both instances is essentially the same—only the objectives differ. Scanning microscopy is fundamentally an endeavor directed toward the demonstration, photographically, of morphological attributes with a resolution and three-dimensional perspective attainable by no other means. Recent developments in X-ray analysis also provide capabilities for relating chemical composition to morphological structures seen in the SEM. The current and potential applications of scanning microscopy in plant protection are restricted only by the resolution capabilities of the instrument, the nature of the specimen to be studied and one's ability to suitably prepare it for microscopy, and the imagination or ingenuity of the investigator.

Literature Cited

1. Boyde, A. 1974. Histological and cytological methods for the SEM in biology and medicine. Pages 308-336 in: Scanning Electron Microscopy. O. C. Wells, ed. McGraw-Hill, New York.
2. Brown, M. F., and Brotzman, H. G. 1979. Phytopathogenic Fungi: A Scanning Electron Stereoscopic Survey. Extension Division, University of Missouri, Columbia.
3. Cohen, A. L. 1974. Critical point drying. Pages 44-112 in: Principles and Techniques of Scanning Electron Microscopy. Vol. 1. M. A. Hayat, ed. Van Nostrand Reinhold, New York.
4. Echlin, P. 1975. Sputter coating techniques for scanning electron microscopy. Pages 218-224 in: Scanning Electron Microscopy. O. Johari and I. Corvin, eds. IIT Research Institute, Chicago.
5. Marshall, A. T. 1975. Electron probe X-ray microanalysis. Pages 103-173 in: Principles and Techniques of Scanning Electron Microscopy. Vol. 4. M. A. Hayat, ed. Van Nostrand Reinhold, New York.
6. Murphy, J. A. 1978. Non-coating techniques to render biological specimens conductive. Pages 175-193 in: SEM/1978/II. SEM Inc., AMF O'Hare, IL.
7. Postek, M. T., Howard, K. S., Johnson, A. H., and McMichael, K. L. 1980. Scanning Electron Microscopy. A Student's Handbook. Ladd Research Industries, Burlington, VT.