Electron Optics: Principles, Techniques, and Applications in Horticulture[1]

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Excellent, though limited, use has been made of electron optical equipment in the field of horticulture. The increasing availability and the refinements in such instruments as the electron microprobe X-ray analyzer, the scanning electron microscope and the transmission electron microscope have made them invaluable tools for the horticultural scientist. In an effort to extend the use of such equipment this paper discusses the operation, present and potential applications, sample preparation techniques and problems, and a glimpse of the future of electron optical equipment in horticulture.

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The inquisitive mind of man has historically given him the drive to get "a closer look" climbing the mountain to see what was on top, entering the forest to see what grows on the floor, and holding an object ever closer to the eye to resolve more detail. The limitations of the unaided eye were frustrating and stimulated the search for a way to see more.

Light microscope (LM)

In the 17th century Leeuwenhoek and Hooke first used the simple and compound light microscopes to describe bacteria and cells of higher organisms. Many of the early microscopes were limited because of defects in the lenses.

In the early days of microscope manufacture, instruments were built for their external appearance as well as their internal capability. Tubes were inlayed with gold, silver, brass and leather, yet it wasn't until the mid 1850's that a microscope manufacturer engaged the services of a mathematician to help overcome the severe limitations in the construction and design of lenses. The combined efforts of Carl Zeiss and Ernst Abbé resulted in lenses which reached the theoretical limits of resolution with the light microscope.

Transmission electron microscope (TEM)

To extend the magnification range of microscopes, scientists considered the use of X-rays, which have shorter wave lengths than visible light. This approach was abandoned because such rays could not be focused sharply. The challenge was to find some system which could focus short wavelengths of energy in the same manner glass lenses focused visible light.

In 1924, de Broglie proposed that a beam of electrons could be considered as radiation with certain wave characteristics. This concept may not have stimulated the construction of the first transmission electron microscope (51), but it did contribute greatly to future development of the TEM. The first working model of a TEM was conceived and built by Ruska and Knoll (80) in 1931. It employed the first electromagnetic and electrostatic lenses to control the diameter (focus) of a beam of electrons. Several years of experimentation and redesign were necessary to reduce the magnitude of lens abberations which were similar to those encountered by light microscopists.

Scanning electron microscope (SEM)

The limited depth of field of the LM and TEM prevented the study of the morphological and anatomical characteristics of biological specimens and the surface characteristics of both biological and non-biological samples. The limitations of this depth of field for each instrument will be discussed later.

The concept of an SEM is credited to Knoll (60). In 1935 he suggested that characteristics of a sample surface could be observed by focusing a scanning electron beam on the surface and recording the emitted current as a function of beam position. Unlike the TEM, which uses ultra-thin sections, the SEM samples would not be sectioned at all. The first functional SEM was constructed in 1938 by von Ardenne (7) based on Knoll's concept.

A basic difference between the SEM and other microscopes is the use of scanning coils to drive the beam in the Y direction while being deflected in the X direction and the detection and display of low energy secondary electrons. After several modifications in the original SEM, von Ardenne was able to demonstrate resolutions of about 50 nm. The first commercial instrument became available in 1965. Instruments available today have resolution limits of 6-10 nm.

Many different detection modes and displays are available in modern SEM's such as: backscattered electrons, sample current, cathodoluminescence, Y modulation, transmitted electrons, voltage contrast, mirror microscopy and X-ray analysis.

Although commercial SEM's have resolution limits of 10 nm, some experimental instruments (23) are capable of 1.0 nm resolution with modified electron sources, lenses and detection systems. The SEM is rapidly becoming necessary to plant studies along with the LM and TEM.

Electron microprobe X-ray analyzer (MP)

The history of the MP as a working instrument dates to 1949 when a French graduate student, Castaing, developed the instrument as *part* of his PhD thesis (21). Prior to that time, Hillier (55) applied for a patent on the principle of microprobe analysis in 1943. Independent of the work of Hillier and Castaing, Borovskii (1953) developed the microprobe concept and published a description of the technique in Russia (16). The first commercial instrument was made available in 1961 and was initially employed in metallurgy and geology. Early MP's were destructive to biological tissue due to excessive heat from the electron beam.

Application of the microprobe to biological tissue was made in the early 1960's. Hard tissues such as bone and teeth were relatively easy to examine and could be cut and polished similar to geological samples (10, 18). Examination of plant tissue with the MP was reported in 1966 (62) when Laüchle and Schwander demonstrated the distribution of calcium, potassium, strontium, iron, silicon, phosphorus and sulfur in the leaves of corn (*Zea mays* L.).

MP's are capable of detecting all elements, except hydrogen, helium, lithium and beryllium, at varying concentrations. The instrument identifies the element, its location and its quantity.

Definition of terms

In the following discussion of electron optical equipment it will be useful to use abbreviations or other short terminology. A list of terms is provided below.

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Optical contrast:
Ratio of defracted rays to direct rays. Ability to differentiate organelles.

Depth of field:
Distance between two distinct planes of a specimen with both planes exhibiting acceptable detail.

Back scattered electrons:
Electrons from the primary beam which elastically bounce from the sample surface with energies approaching the incident beam.

Sample current:
Electrons conducted through the specimen.

Cathodoluminescence:
Light generated in the visible region upon interaction of the specimen with the electron beam.

Secondary electrons:
Low energy electrons (0-100 ev) emitted from the first 10 nm of the surface of the sample.

X-ray:
Electromagnetic radiation used in microprobe analysis, wavelengths of 0.1-10 nm.

Energy dispersive X-ray Analysis:
Separation of X-rays by energy level in multi-channel analyzers. Principally a qualitative technique.

Wavelength dispersive X-ray Analysis:
Separation of X-rays by wavelength using crystals. Principle of microprobe analysis, qualitative and quantitative.

Resolution:
The ability to distinguish two particles as being separate and distinct.

Limitations of equipment in optical labs

It is useful to know the limits of the equipment discussed above in order to more fully utilize their capabilities. The parameters most often examined are: magnification range, possible resolution, and depth of field. In addition, the ease of use, reliability and expense (both initial and recurring) of the various instruments are important.

In its simplest form, magnification is a function of the distance between an object and the eye. As things are brought closer to our eyes, they appear larger until they reach the point (about 10 cm from the eye) at which they can no longer be focused. The primary purpose of magnification is to allow resolution of individual details of the objects examined. At the closest possible working distance our eyes can resolve points approximately 0.2 mm apart. When light or electron microscopes are used to increase magnification, resolution of fine details is correspondingly improved. Resolution of very fine details does require magnification as indicated above. More important, however, is the distinct nature of features in the magnified image. The resolving capability of the LM and TEM is a function of wavelength of the illuminating radiation and the quality of the lenses – that is their freedom from distorting aberrations. The SEM resolving power is more directly related to the size and shape of the focused scanning beam.

Because visible light is the illuminating radiation in light microscopes, wavelength is dependent upon the color of light used. Blue light with 400 nm wavelength results in resolution of ca. 200 nm in a light microscope. Electrons accelerated by a potential of 100 kV (100,000 V) have a calculated wavelength difference of about 1/100,000 of that of the LM and should result in a similar improvement in resolution. In practice, however, TEM's can achieve about 0.2 nm resolution which is only a 1000 fold increase over the LM. The limiting factors are in electronmagnetic lens design, construction and operation.

Characteristically a great deal of what we see with the unaided eye appears to be in focus simultaneously. That is, both near and far objects are clearly defined. This depth of field combined with the mixing of images from both eyes gives us a 3-dimensional image of our surroundings. In the light microscope the nature of the lens systems employed greatly reduces the depth of field that is in focus at one time. Indeed as the magnification is increased on the LM the depth of field decreases. In practice only a very small portion of the specimen will be in focus. The depth of field can be calculated by dividing the illuminating radiation wavelength by the square of the numerical aperture of the lens. Thus in a TEM with .004 nm wavelength and a NA of 10^{-3} in the objective lens, 4 μm may be in focus at one time. This is a considerable increase over the LM, but is very small compared to that of the SEM where depth of field can exceed a millimeter and this greater depth gives a 3-D appearance to the micrographs in addition to the clarity of details provided (Fig. 1).

Working distance varies among instruments. To achieve high magnification in the LM it is necessary to place the objective lens very close to the specimen. It becomes almost impossible to do any manipulation of the specimen while viewing it under these conditions. The TEM is similarly limited. In the SEM a distance of about 12 mm between the last lens and the specimen allows manipulation of the specimen during observation.

The LM, TEM and SEM can be contrasted in a number of additional ways (Table 1). Each instrument has particular strengths and many investigations could benefit from a combined instrument approach.

Although the MP has added another dimension to the electron optics field, it

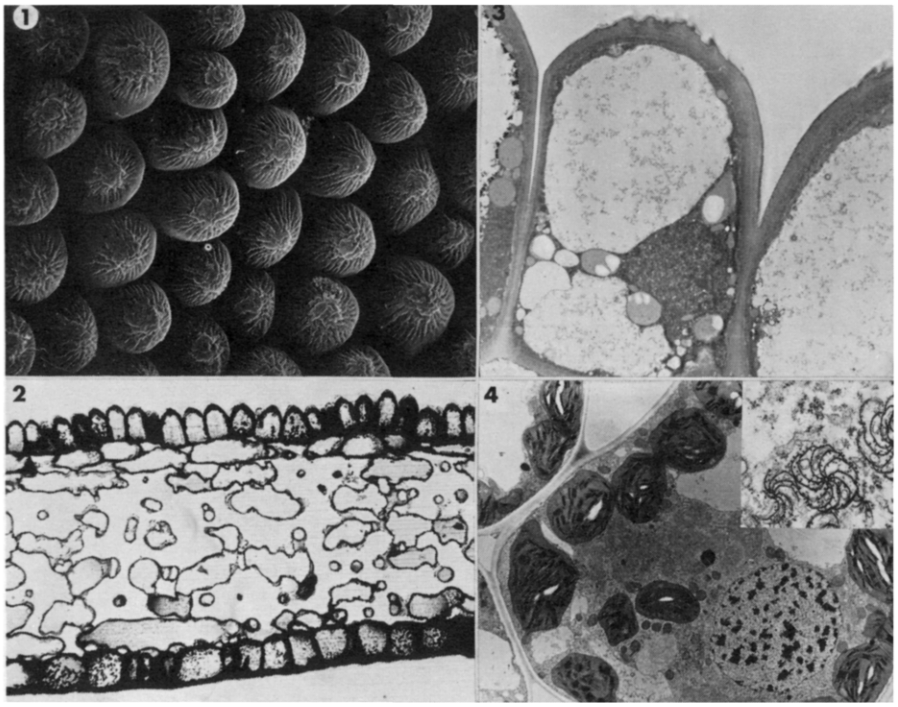

Fig. 1-4. Photographs of light and electron optical instrument images: Fig. 1 - Scanning electron microscope image of the upper surface of a rose peta. 600×. Fig. 2 - Light microscope image of a rose petal cross section. 350×. Fig. 3 - Transmission electron micrograph of a rose petal cross section. 3500×. Fig. 4 - Ultrastructure of a mesophyll cell from wheat infected with spindle streak mosaic virus. 2400×. Insert shows virus at a later stage, pinwheel inclusions evident. 15,000×.

too has its limitations. The beam diameter of the MP is approximately 50 nm (newer MP's approach 10 nm), and the current necessary to excite sufficient X-rays is 10^{-8} amps compared to 10^{-12} amps for the SEM. Therefore, more sample damage is possible in the MP. With the excited volume of the specimen several times larger than the electron beam, resolution is reduced. The detection of most elements except H, He, Li, Be is possible. As a rule of thumb 10^{-15} grams of actual element or 0.01% of the matrix is the lowest detectable concentration (77).

Instrument type and information obtainable

Many types of light microscopy are in use today, basically to increase optical contrast because the resolution limits have been achieved. Several excellent books (87) are available on the use of the light microscope; therefore, a brief listing of available techniques will suffice:

1) Bright field (use of stain necessary), 2) dark field (special condensor required), 3) phase contrast (accentuates the difference between light diffraction of the organelle and its surroundings without staining), 4) polarized light (presence or absence of molecular orientation or the presence of crystals), 5) reflected light (limited analysis of specimen surface), 6) ultra violet (limited use in horticulture), 7) infrared (limited use in horticulture), 8) interference (quantitative data obtainable i.e., dry weight and optical path difference for cell structures), 9) stereo (good depth of field at low magnifications, photography difficult). A typical light micrograph is shown in Fig. 2.

As is evident from Fig. 3 and 4, the TEM not only increases magnification but also resolution. Once the sample has been fixed, sectioned and stained (47), it is placed under the electron beam where electrons are scattered, absorbed or transmitted resulting in the creation of an image on either a phosphorescent screen or a photographic film. Since the specimen is extremely thin, the depth of field is not exceeded and everything is in focus. This factor may lead to misinterpretation of the photomicrographs. For example, if the sample is tilted during sectioning, resulting in a bias cut, the cell wall width will appear to be much greater than the true thickness.

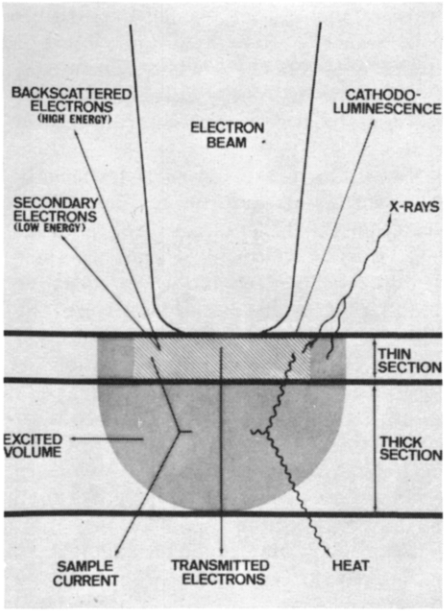

Fig. 5. A diagramatic representation of the interaction of the electron beam with the sample and the signals generated.

At this point in our discussion it seems appropriate to amplify on the interaction of the sample with the electron beam. Fig. 5 diagrammatically illustrates the signals generated during electron bombardment. Irrespective of the electron instrument in use, all this information is generated, and with the proper detector, the information can be collected and displayed.

The TEM, SEM and MP have been designed to give maximum information from a given signal; the remaining signals, if collected, will be of inferior quality as compared to the instrument built for that purpose. For example, the generation of X-rays characteristic for a given element is dependent on the concentration of that element in the excited volume of the sample; if a thin section is used the concentration may not be high enough to be detected above the background level. With a MP the sample is usually thicker, the element in higher concentration and therefore more X-rays are generated. Where elements occur in very low amounts the MP would be the instrument of choice.

In addition to secondary electron detection (Fig. 1) and readout on a cathode ray tube (CRT), which is intensity modulated, the SEM, with proper detectors, can be used for wavelength dispersive and energy dispersive X-ray analysis. In the energy dispersive X-ray mode, the SEM is usually more effective because of its small beam, rastering characteristic and the use of infinitely thick specimens.

The CRT is intensity modulated (more electrons – brighter image) and there may be times when certain important surface features are not clearly elucidated. An operational mode call *Y-modulation* (Fig. 6) produces a deflection modulated display which may accentuate the surface characteristics (51). Similarly if one is interested in the relative height of surface features stereo pairs may be produced which under a stereo-viewer allow a 3-dimensional analysis. Plane depth in stereo pairs can be quantified using the procedure of Boyd (17). Stereo pair studies are also possible on the TEM but the instrument must be equipped with an optional goniometer (tilting) stage. A tilting stage is standard on the SEM and newer MP's. Stereo procedures are straight-forward in that two photographs are taken at different tilt angles around the same axis and later viewed as paired micrographs in a

Table 1. A comparison of selected characteristics of light and electron microscopes.

Feature	Light microscope	Transmission electron microscope	Scanning electron microscope
Illuminating radiation	Visible light	High speed electrons	High speed electrons
Wavelength of above	400 nm-700 nm	.006 nm (40 kV) – .004 nm (100 kV)	.04 nm (1 kV) – .007 nm (30 kV)
Best resolution	200 nm	.2nm	10 nm
Magnification range	10X - 1000X	500X - 500,000X	20X - 50,000X
Depth of field	.002 – .05 mm	.004 to .006 mm	.003 to 1 mm
High magnification working distance	2 mm	–	12 mm
Lens type	glass	electromagnetic	electromagnetic
Image formation	on eye by lenses	on phosphorescent plate by lenses	Built on cathode ray tube by scanning spot
General use	Surface morphology and sections	Sections (40-150 nm) or small particles on thin membranes	Surface morphology

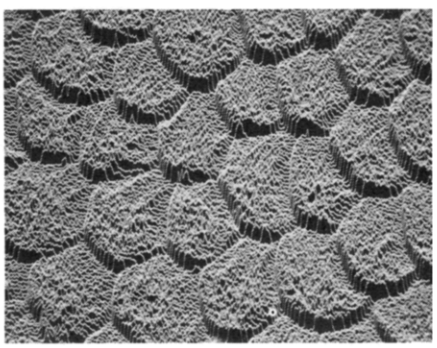

Fig. 6. Scanning electron microscope Y-modulation image of the upper surface of a rose petal. 520x.

stereo viewer.

Phase contrast effects are possible in the TEM and SEM and are used to a very limited extent by horticulturists. They are particularly useful where small particles such as isolated organelles or viruses are being studied. Dark field techniques are discussed thoroughly by Nathan (69) and the theory and application of phase contrast is covered by Johansen (58).

Preparation of materials for electron optical analysis

TEM. Because electrons accelerated at 40-100 kV will not pass through very thick objects it is necessary to use a minimum size specimen in the TEM. To study internal structures, sectioned material should not be more than about 150 nm thick. As previously noted, blue light has a wavelength of 400 nm, which gives some indication of how thin the TEM sections must be. Particulates (viruses, bacteria, isolated cell constituents) can be placed on thin plastic films and examined directly whereas surface features of plants must be examined by replica techniques. A brief account of these techniques as used in horticultural and other botanical studies follows.

Sectioning procedures for the TEM are much like those used for light microscopy. Tissues are killed and fixed, dehydrated, embedded and hardened and finally sectioned and stained. Killing and fixing for TEM requires careful techniques since high magnification reveals even small artifacts due to faulty preservation. While each tissue may require specific combinations of buffers and fixatives a general procedure is to fix in 3-6% buffered aldehyde (usually glutaraldehyde) in the cold followed by a buffer rinse and post fixation in 1-3% buffered osmium tetroxide. Fixation may be carried out for 1-12 hr depending upon tissue size and other characteristics. The material is dehydrated in a graded ethanol or acetone series and then gradually infiltrated with embedding material such as plastics or epoxy resins. Epoxy resins, which are most commonly used, are very hard after curing and ultra thin sections can be cut from the block with glass or diamond knives on an ultramicrotome. The sections are picked up on fine mesh metal grids and stained by floating them on solutions containing compounds such as lead or uranyl salts.

More recently methods have been developed whereby fresh tissues can be sectioned directly utilizing a freezing attachment on the ultramicrotome.

In plant physiological or pathological studies, small particles must often be examined. These particles can be separated from other cellular constituents and the resulting centrifuge pellets can be embedded and sectioned as above. It is faster and easier, however, to allow some of the suspended pellet material to dry on a plastic film-coated grid.

Since the untreated particles usually lack sufficient density to be clearly visible in the TEM a variety of methods have been developed to enhance their visibility. The technique of negative staining is often employed. Here the film is flooded with a solution of electron opaque compounds such as phosphotungstic acid or uranyl salt solutions. When the solution dries, it coats the film surface but not the particles, which then appear in sharp relief as electron transparent areas in an opaque background.

Another common technique is to evaporate metals under vacuum onto the particles as they rest on film coated grids. The metal is applied at a 30-45° angle which results in a buildup of metal on the source side of each particle and a metal free "shadow" on the other side. Particle height and shape can therefore be calculated by shadows.

In some cases it is not possible to view plant materials directly in the TEM. For example, the surface of a leaf may have topographical features of interest but thin sections would not allow examination of sufficient surface area. Or it may be desirable to examine surface features of frozen fractured tissues using plastic and/or metal films that will preserve faithfully very fine surface detail. In the former example the replicating materials can be applied directly at room temperature, dried and then used as a mold to make another cast of the features to be examined. In the latter example tissues are fractured under vacuum while frozen and etched slightly by sublimation of surface ice. While still frozen and in the vacuum they are coated with carbon and platinum and the replica removed from the thawed sample for examination. A complete discussion of freeze etching and other foregoing techniques is available in recent texts (19).

Techniques such as histochemistry and autoradiography are mentioned in many current studies involving SEM, TEM, and MP. Histochemistry as used in these studies may involve the formation *in situ* of specific reaction products that are detectable by microscopic examination, e.g., localization of acid hydrolase in corn root tips (41) or general staining of whole classes of compounds, e.g., ruthenium red staining of polysaccharides. Specific digestion of fixed-sectioned materials may also be employed.

Autoradiographic electron microscopy differs from that used in light microscopy primarily in sophistication of emulsion composition and application (74). Silver grains developed after exposure to radiation can be viewed as electron dense spots over thin sections in the TEM or detected via X-ray analysis in TEM, SEM or MP (12).

SEM. The preparation of plant material for TEM is often a long, difficult process. Many projects have been abandoned because of inability to match the time and effort required to do TEM work. Sample preparation for the SEM is not as difficult or time consuming. The preparation technique used depends on both the plant material and experimental requirements. Methods must: 1) preserve morphological detail; and 2) reduce or eliminate charging under the electron beam.

To preserve morphology of living tissue, it is necessary to dry the specimen. The adverse effects of drying on plant material must be minimized. Samples may be prepared as follows: air drying; drying via organic solvents; freeze drying (48); and critical point drying (6). Some plant materials, e.g. woody tissues, are not altered appreciably during drying and preparation may be by air drying at room temperature or slightly higher. Air drying is equally effective where the specimen is not affected by drying artifacts. For example, the nature of surface waxes on leaves may not require preservation of sub-surface detail and any treatment other than air drying may alter the wax structure. While certain materials can be directly dried from the fresh state, fixation prior to drying may improve results. Fixatives used in TEM, e.g., aldehydes and osmium tetroxide, may be used for SEM preparation. Stabilization and partial solidification of cytoplasmic contents by fixation adds structural support to prevent distortion during drying. A simple fixation procedure for tissues such as petals or glandular leaf surfaces consists of exposure of fresh tissue to vapors from osmium tetroxide solutions or crystals.

Dehydration can be faciliated by using a graded alcohol or acetone series. After reaching 100% solvent the tissues are either air dried at room temperature, dried in a heated air blast, or critical-point dried.

Freeze-drying and critical point drying are the most common methods for preserving plant materials for SEM. In the former technique fresh material is plunged into liquid nitrogen and placed, frozen, into a vacuum apparatus to remove water by sublimatiom. This procedure is time consuming and is most effective with small specimens (1 mm^3).

Both fresh and chemically fixed specimens can be critical point dried. The material is usually dehydrated in an organic solvent, then transferred into a

chamber where liquified gas under pressure is exchanged for the solvent. The liquid gas (CO_2, freons and N_2O are commonly used) is heated under pressure to the point at which the gas vapor and liquid phase have the same density. At this point there is no distinction between gas and liquid (no surface tension) and the heated chamber is exhausted. This eliminates the great pressure exerted by receding water surfaces during drying (estimated by Anderson (6) at 42,000 kg/cm^2) (Fig. 7).

Fresh-frozen tissue can be examined in the SEM with a cold stage or some modification to maintain the frozen state of the material (70). Formation of frost from condensing vapors, crushing of tissues as they dehydrate and specimen charging or other problems may be encountered with frozen specimens.

Internal structures of plants can be examined by fracturing tissues frozen in water or ethyl alcohol and critical point drying them. Similarly, resin embedded TEM samples can be observed by partially dissolving away the epoxy until the specimen is revealed.

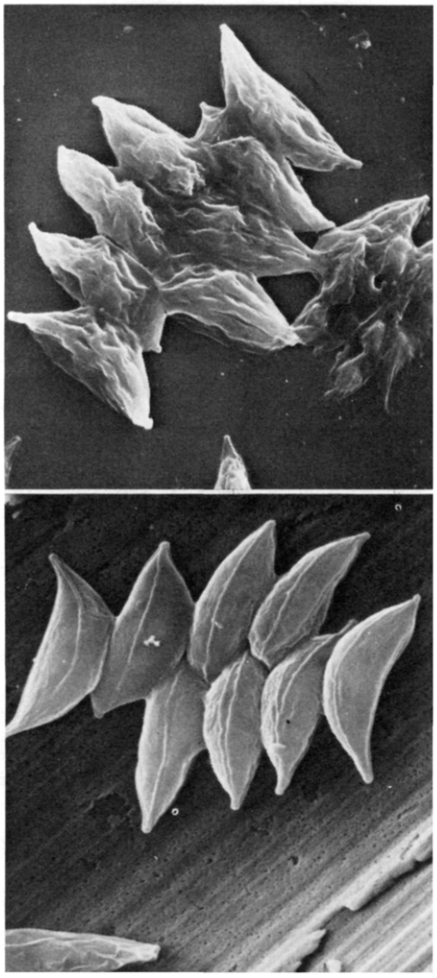

Fig. 7. Photomicrographs of a spineless mutant of the algae Scenedesmus. 2200. (Top) Cells fixed and air dried. (Bottom) Cells fixed and critical point dried.

A negative charge may build up on a non-conductive specimen under examination resulting in beam distortion (charging). Frozen or fresh samples may contain enough electrolytes in the water to allow the charge to reach earth potential through the metal stub to which it is affixed. Dried materials, however, must be rendered conductive. A thin (5-20 nm) layer of metal (gold, silver, gold/palladium) and/or carbon evaporated or sputtered onto the mounted specimen eliminate charging and increase secondary electron emission. Sputter coating (cold cathode) is recommended because of its speed, ease of application, low temperature and low expense. Photography in the SEM is accomplished by placing a camera over a separate, short retention, phosphorescent recording CRT.

MP. Specimen preparation for the MP is governed by some of the same needs as in the SEM. It is desirable to preserve morphological relationships and to avoid charging of the specimen. Since the MP is designed to detect X-rays from elements an additional problem is encountered. The specimen must be so prepared and mounted that X-rays are generated only from the desired material. For example, aluminum mounting stubs would completely negate efforts to find aluminum in plant tissue sections. Most samples are mounted on pure polished carbon disks or quartz slides. If surface features are to be analyzed, preparation may be air drying or freeze drying. Specimens are affixed to the carbon disks with adhesives such as Tube-Koat. Usually the specimen is vacuum coated for conductivity with carbon alone. Critical point drying is useful in MP studies only where the elements desired are not subject to washing out by the solvents and/or gases employed.

Sectioned material is commonly analyzed in the MP (11, 12, 35, 42, 73, 78). While sections of paraffin embedded tissues can be mounted and the paraffin removed by solvents, it is much more satisfactory to use a cryostat or freezing microtome to cut fresh frozen tissues. Sections can then be mounted and air or freeze dried. Material embedded in epoxy resins can be sectioned for MP analysis, but frequently traces of a number of elements remain in the resin and confound the analysis.

Heat from the electron beam may damage the tissue or volatilize desired elements. A requirement in preparation and examination in the MP is selection or proper tissue size and employment of the most efficient beam parameters. It is also useful to use standards in the form of known amounts of the desired elements. For example, the element can be suspended in agar and the agar sectioned in the cryostat and further analyzed as if it were a tissue section.

The primary goal in tissue preparation for the MP is to retain the elements in the same spatial arrangements as the fresh tissue.

Application of microscopy in modern horticultural studies

The area of use of electron optics in horticultural studies is vast. The very basic problems of flower bud formation, the search for mode of entry and action of herbicides, mechanisms of fruit ripening or fruit thinning, and indeed almost any area where physiology or morphology are important are now being actively examined via electron microscopes.

TEM. Microscopy has traditionally been an extension of the eye. With the advent of X-ray equipment on electron microscopes we have extended the laboratory bench. In plant studies the use of electron microscopes has been extensive, particularly in the traditional areas of anatomy and morphology. A recent bibliography of the use of SEM's and MP's in plant studies lists over 1100 titles (30). A similar listing of TEM studies in plant sciences would be much more extensive due to the length of time TEM's have been available and their commonplace occurrence at most research and teaching facilities. The study of plant anatomy and cytology has been greatly furthered by the use of modern electron microscopes (64) and most botany or horticulture texts contain abundant electron micrographs. Through the use of histochemistry, particulary with the TEM, a wedding of plant physiology and plant morphology-cytology is occurring. The examples cited below illustrate only a portion of the studies that have been carried out.

Practically all horticultural crops suffer from pests and pathogens. The latter group includes bacteria, fungi, viruses and mycoplasma as disease agents. While bacteria and fungi can be observed in the light microscope, the extent and nature of the diseases they cause is much more apparent in the SEM and TEM. Mycoplasma and viruses are either at or below the resolution limits of LM's and near those of most SEM's; hence they are in the domain of the TEM. Indeed mycoplasma-like organisms were not even known in plants until 1967 when Japanese workers discovered the organisms in phloem of plants thought to be virus infected (28). Now the "yellows" diseases are known to be primarily mycoplasma infections (67).

Plant viruses can be very elusive

problems. They cause diseases that range from subtle losses in productivity to world-wide disasters such as those caused by citrus tristeza in oranges (1). The TEM is used routinely to check for viruses in foundation stock or budwood of a number of fruit and vegetable crops and has added immeasurably to both applied and fundamental studies of diseases caused by very small pathogens (34, 81). More recently SEM's have proven capable of visualizing virus particles and some of the mechanics of virus transmission may now be easier to understand (29).

While fungi can be observed in the LM, much of the detail of the interface between pathogen and host was unknown until the TEM became available. In particular, studies of obligate parasites such as rusts and powdery mildews have proven fruitful (31). Actual exchange of certain materials between fungus and plant has been detected by employing X-ray analysis in the MP (22). Many workers in plant pathology have recognized the value of using both LM and TEM techniques to study pathogenesis. An excellent example involved the study of the club root disease of cabbage (2). Here a new look at one of the first recognized plant diseases was presented.

Microbe and plant relationships, such as mycorrhizal and bacterial nodule as mycorrhizal and bacterial nodule associations in plants, are not necessarily physiological investigations of the bacteria in legume nodules have been done in several crops. The TEM has been invaluable in exploring the physical cell-bacterium association (13, 14, 27).

Mineral imbalance is common in many horticultural crops and symptoms often resemble those caused by disease. Low temperatures and certain chemical treatments may have similar effects. Electron microscopy of affected plants is an aid to proper diagnosis and a means of pinpointing action sites of the physical or chemical agents. The SEM, for example, has been used to view damage involving both frost and fungal parasites on grapes (15) and the mechanism of cell killing of a chemical pruning agent, methyl decanoate, has been clarified with the TEM (71).

The effect of mineral nutrient deficiency is often apparent in alteration of leaf cell structures such as chloroplasts when they are viewed in the TEM (86). By employing energy dispersive X-ray analysis on the TEM both ion transport and ion compartmentalization studies become possible (84).

The relationships between structures and physiological processes in plants have not often been understood. In some cases extensive physiological investigations and morphological studies in the same plants have paralleled each other for years before finally coming together. The electron microscope has often led to the merger. A good example is the study of C_4 vs C_3 photosynthesis. Certain plants, e.g., sugar cane and *Atriplex* spp., were known to produce C_4 dicarboxylic acids as primary products of photosynthesis. Light and electron microscopic examination revealed a certain organization of mesophyll and leaf veins as well as size and structural differences in chloroplasts and other organelles unique to C_4 plants (61).

SEM. In the late 1960's and early 1970's the SEM began to provide for the horticultural scientist a new means to observe plants. Prior to this time, text books contained photographs and detailed drawings of wood sections, cells, and leaf surfaces. LM photographs lacked the depth of field necessary for true three dimensional images.

The intricate structure of pit fields (82), vessel elements (83), and bordered pits including the torus was demonstrated in 1968 (79).

The particulate matter content of phloem, xylem sap and cellular cytoplasm is readily observed with the SEM. In *Euphorbia* spp. Mahlburg (66) found that the morphological character and size of starch grains varied not only between species but also between locations within the same plant.

Wolf (88) used the SEM to determine the effect of isolating techniques and treatments on the protein bodies in soybeans. Many of the subcellular particles in seeds could be observed and their reaction to treatment characterized.

Taxonomically (85) and physiologically (89), pollen characteristics, stage of development and dehydration can be observed and characterized with the SEM (see cover). Studies of foreign pollen content, percentage of aborted pollen, and pollen tube development and penetration into the stigma and through the style would be of importance in all aspects of horticulture.

The LM enabled us to view changes in the conversion of a meristem from the vegetative to the reproductive stage but great difficulty was encountered in reconstructing a three dimensional image from 2 dimensional observation in the LM. The organogenesis of meristems by SEM analysis gives a total picture of meristimatic activity and morphology (36). The lily (32) and carnation (33) developmental stages have been clearly identified and characterized. Mutational alteration of the reproductive apex in carnation (33) has led to a better understanding of the sequence of events in carnation flower production.

Leaf development, in particular the development of wax forms on the leaf surface, has received increased attention with the SEM. Plants which are normally aquatic and produce no wax were found to develop wax when exposed to air (46). Leaves which were normally aerial produced no wax when surrounded by water.

Regeneration of wax after mechanical abrasion was found to be slow in mature leaves but rapid in expanding leaves (3). Many studies have described the wax characteristics of both deciduous (8, 9, 26) and evergreen (43, 44) species.

The effect of toxic levels of aluminum on root morphology was seen in the SEM as an arresting of root elongation by inhibition of the root tissue external to the endodermis (45). Elongation inside the endodermis resulted in a shearing and separation in the cortical tissue (Fig. 8).

The application of spray materials for chemical pruning (39, 40) was found to vary directly in its effectiveness with the number of trichomes on the stem surface of chrysanthemum. Similarly the distribution of herbicides varied depending on the wax type and trichome distribution on leaf surfaces (52). The herbicides were identified using cathodoluminescence.

As earlier stated, the diseases of horticultural crops lend themselves readily to SEM analysis. The mode of entry of many pathogens into the leaf is easily observable (Fig. 9), as are their

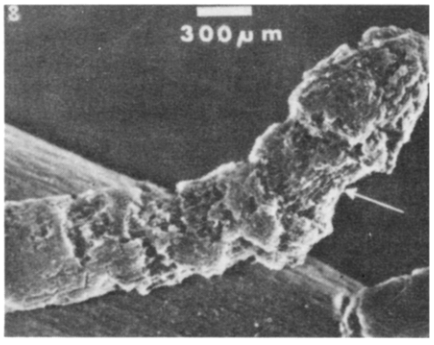

Fig. 8. Photomicrograph of a secondary root of corn (*Zea mays*) grown at toxic Al levels. Cell shearing evident at arrow.

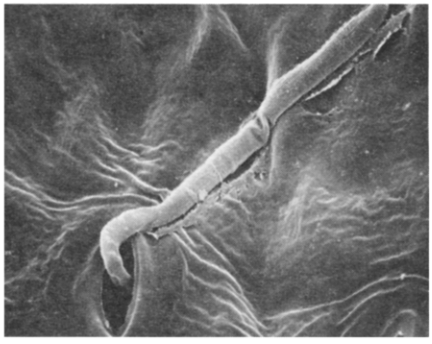

Fig. 9. Cherry leaf spot fungus (*Coccomyces hiemalis*). Hyphae penetrating a leaf stomate. 1000x.

associations with other superficial tissues (65) (Fig. 10).

Albrigo (3, 4) and Albrigo and Brown (5) have studied the surface characteristics of both leaf and fruit of orange under normal developmental conditions, of fruit with stem end rind breakdown and of fruit treated with a plastic spray. They studied wax form and found it to be different at different locations on the fruit surface. Wax development of leaves and fruit of orange differed in its consistency, chemistry and structure.

Our laboratory has extensively studied the characteristics of flower petal surfaces (Fig. 11), leaf and stem surfaces and meristems.

Examples of SEM applications to horticulture are numerous. Carr (20) and Hollenberg and Erickson (56) review applications and potential of the SEM in biology.

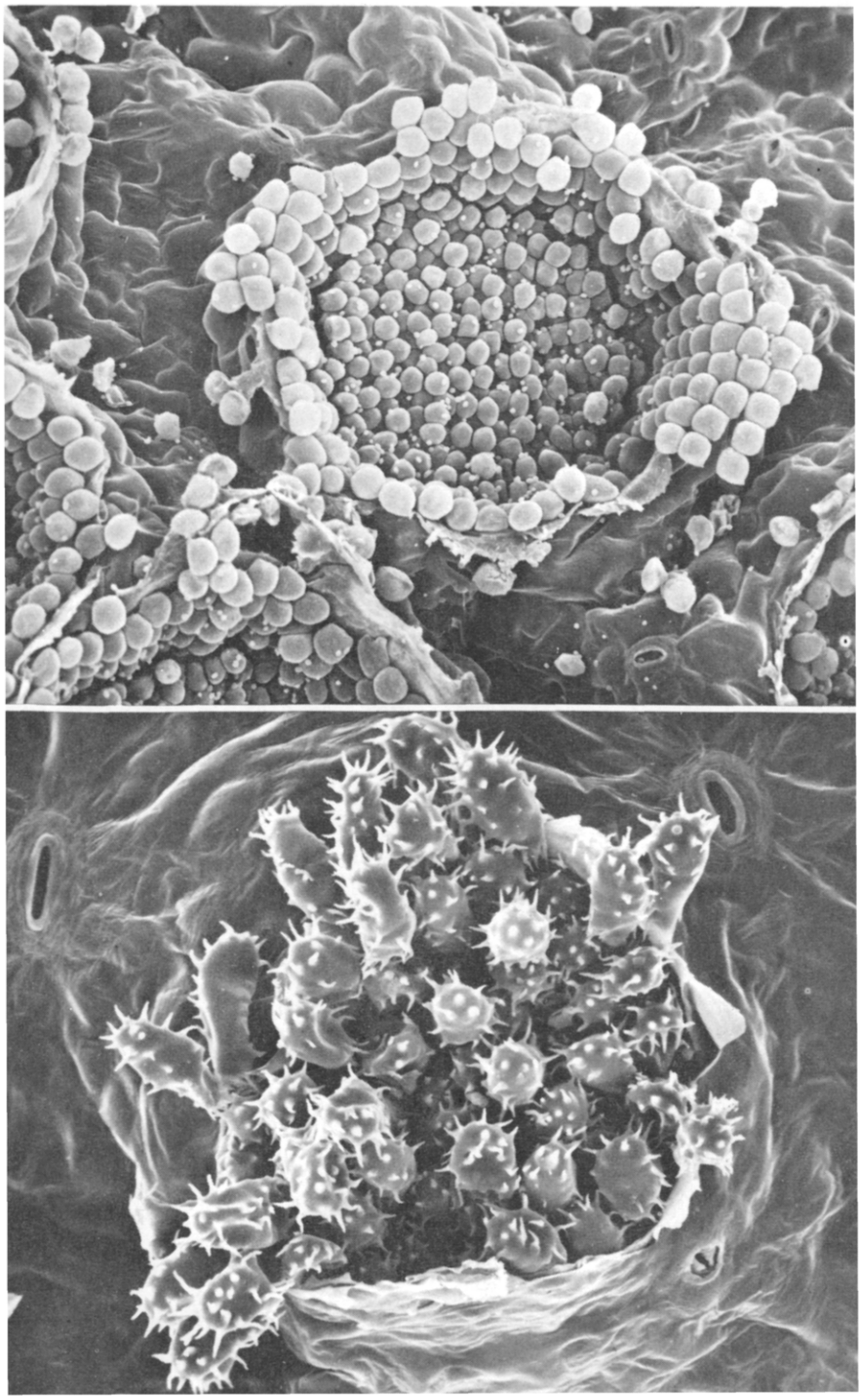

Fig. 10. Photomicrographs of leaf rust fungus (*Puccinia podophylia*) on the lower surface of mayapple. (Top) Aecial stage. 250×. (Bottom) Telial stage. 500×. (Courtesy K. L. O'Donnell).

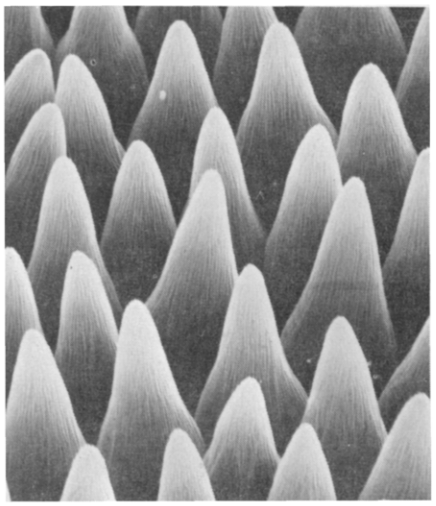

Fig. 11. Photomicrograph of upper surface of snapdragon (*Antirrhinum majus*) petal. 350×.

MP X-ray analysis

Many studies of the distribution and concentration of elements in plant tissues have been made. Many techniques have been developed such as X-ray diffraction, spectrographs, autoradiography, histochemistry and etc. Most methods developed do not allow an *in situ* analysis but require either a destruction of the tissue or the application of exogenously applied tracers.

There are limitations and problems associated with the MP but the "state of the art" is overcoming many of them. Some of the most recent applications of the MP and corresponding techniques were recently discussed in a symposium on the use of the MP in cells and tissues (42).

As with the SEM the MP has been used for analysis of most plant tissues. When aluminum was present at toxic levels in corn roots (45, 76, 78) most Al was present at the root surfaces with the primary mode of entry along an emerging secondary root.

Similar studies were carried out to determine the effect of toxic levels of copper on iron distribution in bean plants (25). High levels of copper caused the precipitation of iron as iron phosphate resulting in chlorosis of the plant. Minor elements concentrations may be below detection limits. However, information is readily available for major elements and for toxic levels of most elements.

Movement of elements in soils has been studied by MP analysis (77). Hill and Sawhney (53) have reviewed the use of MP and problems associated with soil analysis.

Most elements which enter through the roots are distributed throughout the

shoot and become a critical factor in the growth of plants. The elements must remain in their original sites during MP analysis if distribution factors or patterns are to be meaningful. Several techniques have been employed such as freeze drying, freeze substitution, cryostat sectioning and fresh frozen tissue. Using freeze substituted corn leaf sections, Pallaghy (73) found K and Cl concentration differences between bundle shealth and mesophyll cells and between nuclei, chloroplasts, cytoplasm and vacuoles.

In many monocots silicon has been shown to be present in high concentrations. Utilizing the MP, Hayward and Parry (50) have demonstrated non-uniform distribution throughout the shoot. At the nodes radial walls contained more Si than tangential walls, the flag leaf contained the highest concentration and Si was present in the stomata, sclerenchyma and vascular bundle regions.

Other plant tissues such as pollen (89), necrotic apple tree bark (24), abscission zones (75), and graft unions (35) have yielded new information with MP analysis.

A procedure which combines MP and microautoradiography has proven valuable in determining the distribution of CO_2 fixation in C_3 and C_4 plants (12) and in determining the site of entry of chemical pinching compounds in chrysanthemum (39). The procedure is to expose and develop the autoradiogram and then subject it to analysis for silver distribution on the MP. As can be seen from Fig. 12, semiquantiative data for iostope distribution can be made.

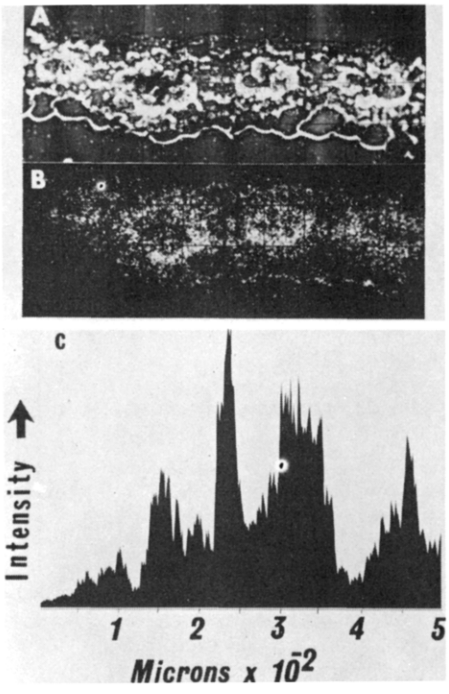

Fig. 12. Electron microprobe analysis of a microautoradiogram used to localize $^{14}CO_2$ in corn leaves *Zea mays*. A-Sample current image. B-X-ray distribution for silver. C-Line scan for the distribution of silver across the leaf cross section.

The future

The application of tried and proven techniques in electron microscopy will be the major thrust in horticulture. Studies combining the LM, SEM and TEM on the same specimen (39) will find wider application.

Techniques now being developed such as observation of fresh frozen tissue with the SEM (70) will have application.

Sophistication of micrograph interpretation (54) with computers and the combination of multichannel analysis and computers for MP analysis will be frequently used. Energy dispersive (38) X-ray systems for total elements and improved MP and ion microprobe systems will give us a closer look at elemental distribution.

New sample preparation techniques will continue to be developed (39).

Instrumentation i.e., high voltage TEM's and SEM's with lens improvements for existing equipment will increase resolution. The development of scanning X-ray microscopes (57) and scanning ion microscopes are possibilities. In general, the improvement in instrumentation will be greater than our utilization in the field of horticulture. The opportunities are ours to greatly expand our knowledge of plants by the use of electron optics.

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