

Light Microscopic Techniques for Detection of Plant Virus Inclusions

Plant virus inclusions are objective intracellular evidence of virus infection. Inclusions may consist of altered host constituents, aggregated viruses, aggregated coat-protein shells, and virus-coded proteins other than coat-protein, as well as mixtures of some of these with each other and with normal host constituents. Inclusions differ from the surrounding cytoplasm and organelles in structure and in staining reactions. Inclusions induced by a specific virus maintain a characteristic appearance over a host range. For example, inclusions similar to the banded inclusions (virus aggregates) of clover yellow mosaic virus (a potexvirus) shown in Figure 11 have been detected over a host range covering three plant families and three genera of one of these families, yet inclusion structure remains essentially the same.

Cytological studies with the electron microscope have resolved the distinctive structure and composition of many inclusions. Once these inclusion features were described at the ultrastructural level, stains capable of detecting and differentiating many of the same features in the light microscope were designed. The ability to identify a particular inclusion type with both the light and the electron microscope has enabled inclusions to be described in terms common to both levels of microscopy. For instance, an inclusion found by the electron microscope to consist of virus particles and sub-

sequently identified in the light microscope, or vice versa, can be described as a virus aggregate or crystal in the light microscope, even though individual particles cannot be resolved. Although in this article we deal exclusively with how inclusions appear in the light microscope, the descriptions have their basis in electron microscopy as well.

The Stains Used

When properly stained, most inclusions can be readily detected with a light microscope. Light microscopic recognition of inclusion types offers a reliable, practical method for identifying virus diseases at the virus group level and can often lead to a specific diagnosis when the virus host range is considered.

Unstained inclusions are hyaline, and many types are difficult to distinguish from the surrounding cytoplasm and organelles. Stains greatly enhance inclusion detection, and we have developed procedures (1,2) that provide rapid penetration and uniform distribution of stains, even in thick tissue pieces. The stains produce vivid contrast and make many structural details of inclusions visible and distinguishable from host constituents. The stains can also be used to differentiate inclusions.

Two stains that have proved valuable for detecting many plant virus inclusions are O-G, a combination of calcolmine orange 2RS and Luxol (E. I. du Pont de Nemours & Co., Inc., Wilmington, DE 19898) brilliant green BL dyes, and azure A. Azure A stain and the dyes for O-G stain are available from the Aldrich Chemical Company, P.O. Box 355, Milwaukee, WI 53201.

Separate stock solutions of calcolmine orange 2RS and Luxol brilliant green BL are prepared by suspending each dye in 2-methoxyethanol (1 g/100 ml), mixing thoroughly, and filtering through coarse filter paper. Both stocks remain stable

indefinitely at room temperature. A standard O-G staining solution is prepared by mixing the green dye, the orange dye, and water in a ratio of 8:1:1. The ratio can be varied according to the type of tissue being studied; this is best done by keeping the orange dye and water content at 1:1 and increasing or decreasing the green dye content. The stock solution of azure A in 2-methoxyethanol (0.1 g/100 ml) also remains stable at room temperature. The staining solution is prepared just before use by mixing the stock solution with 0.2 M dibasic sodium phosphate (Na_2HPO_4) in a ratio of 9:1. The stain should be prepared fresh in small quantities for each batch of tissue and *should not be reused*.

The cost of the stains and materials used in the light microscope techniques we describe is negligible. The only major piece of equipment needed is a compound light microscope with optics capable of about $\times 1,000$ magnification, although higher magnifications are useful for resolution of certain inclusion structures. Only a rudimentary knowledge of microscopy is needed, although some skill must be developed in preparing and staining fresh tissues and in recognizing the various inclusion types.

The Tissues Used

Epidermal tissues. Often, epidermal tissues are the easiest to obtain and contain numerous inclusions. These thin tissues stain readily. In plant species with turgid cells, the epidermis may be easier to remove if the tissue is allowed to wilt slightly. Epidermal strips can often be obtained from a dry, brittle sample by rehydrating the tissue for a few minutes.

The procedure for staining epidermal strips is the same for both O-G and azure A stains:

1. Strips are obtained by inserting the tips of sharp-pointed tweezers (e.g., Dumont No. 5) under the epidermis of a

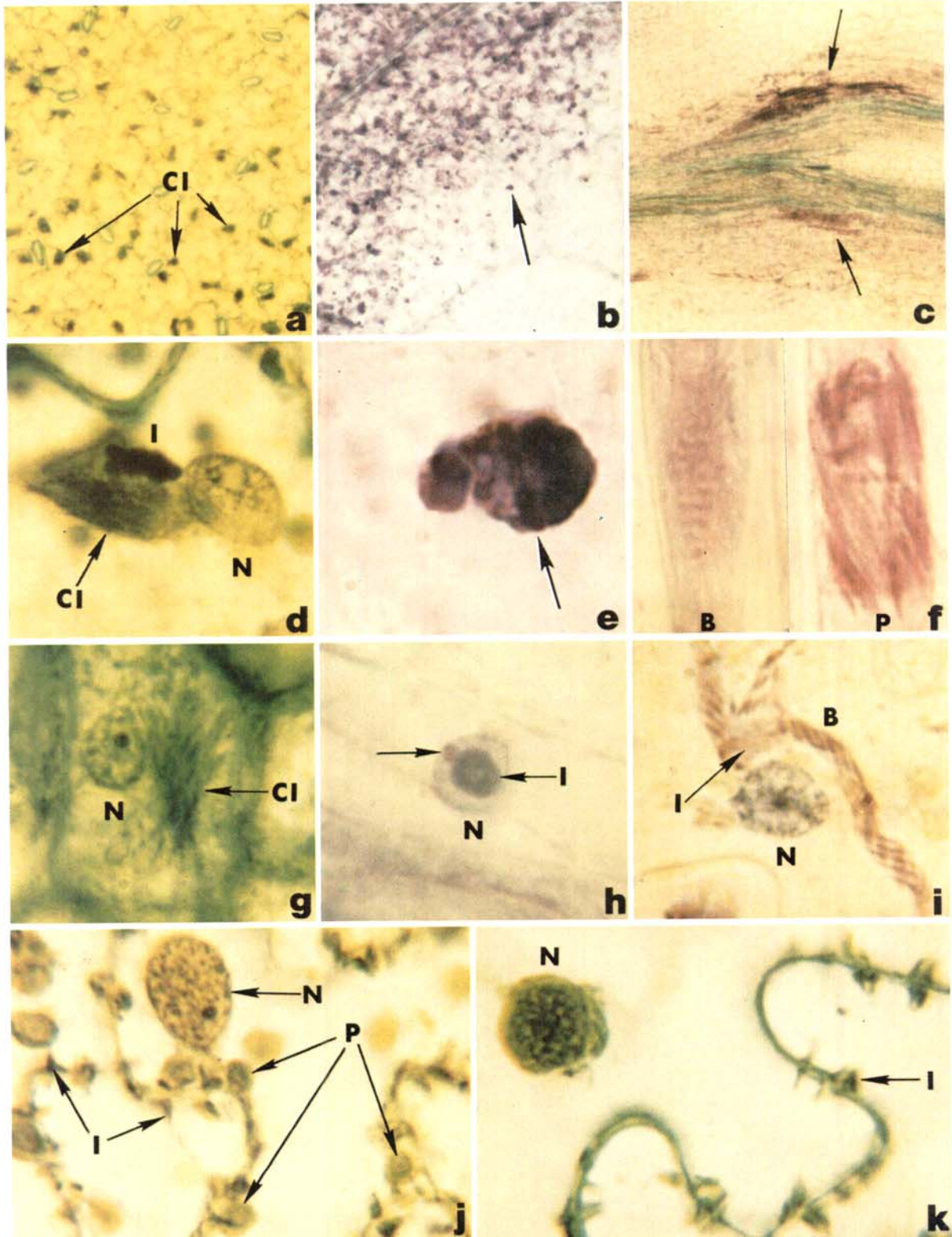
vein (preferably a vein junction) on the lower surface of a leaf and stripping the epidermis at an acute angle from the underlying tissues (Fig. 2A). In some species, a strip from the upper epidermis is easier to obtain. To prevent folding, the epidermal strip is brought into contact with the surface of the staining solution in the watch glass before being separated from the rest of the tissue (Fig. 2B). The torn surface should be in contact with the

solution, since the stain will not penetrate the cuticle. The tissue should float in the staining solution and not touch the glass surface.

2. After 5–10 minutes, the stain is removed. Excess stain is eliminated by several changes of 95% ethanol, 5–10 seconds per change for a total of about 30 seconds. (This is a convenient stopping point if the procedure must be interrupted; the ethanol can be removed and a small

amount of 2-methoxyethyl acetate added, since the stains are insoluble in this solution.)

3. After the excess stain has been eliminated, the epidermal strips are lifted out with a wooden applicator stick (to keep the tissue from folding) and mounted in a drop of Euparal (Carolina Biological Supply, Burlington, NC 27215) on a glass slide; regular Euparal is used with azure A and Euparal 'Vert' with



the O-G combination. Mounting media containing xylene should be avoided, since this solvent adversely affects the stains. A coverslip is placed over the tissue, and excess medium is removed and the tissue flattened by gentle blotting.

Low magnifications can be used to scan tissue for the presence of inclusions (Figs. 1A-C), but magnifications of $\times 1,000$ or higher are needed to resolve small inclusions and to distinguish many features that are useful for diagnosis (Figs. 1D-F).

The slide preparations are considered semipermanent but may last for several months to several years when properly stained and mounted. Refrigeration helps prolong their useful life. If a permanent record is desired, the slides should be photographed while the preparations are reasonably fresh.

When the O-G combination is used, stained plastids often obscure small inclusions. The plastids can be dissolved by floating the epidermal strip on a 5% solution of Triton X-100 (Rohm & Haas Co., Philadelphia, PA 19105) for 5 minutes before staining. The Triton X-100 solution is removed and the O-G stain added, but water is omitted from the staining solution because residual Triton X-100 replaces it. The staining time after Triton X-100 treatment is about one-half that for untreated tissue. After staining, the tissue is dehydrated and mounted as previously described.

The Triton X-100 treatment is especially useful for detecting the characteristic cylindrical inclusions of the potyvirus group. During early stages of infection, these inclusions develop at the cell periphery and often are so small that detecting them among the plastids and other cytoplasmic constituents in the epidermal strip is difficult. Figure 1J depicts tissues infected with soybean mosaic virus (a potyvirus) showing inclusions at an early stage of development. Figure 1K is of an adjacent piece of epidermis treated with Triton X-100 and illustrates how removing the plastids enhances detection of the inclusions. Although potyvirus inclusions are stable

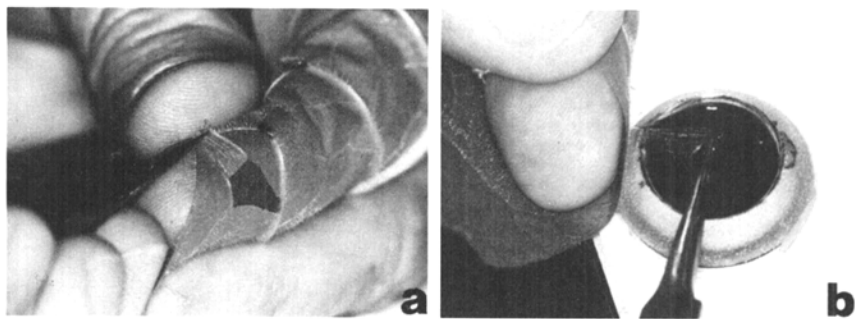


Fig. 2. Preparation of an epidermal strip: (a) The epidermis is kept parallel to the leaf surface while being pulled away from the underlying tissue. (b) To prevent folding, the torn surface of the epidermis is brought into contact with the staining solution before being separated from the leaf.

in Triton X-100, others, such as the crystalline inclusions of the tobamovirus group (Figs. 3G-I) and the banded inclusions of the potexvirus group (Fig. 1I) are dissociated by this treatment. Therefore, untreated controls should be included when the Triton X-100 procedure is used.

Triton X-100 does not remove starch grains, inorganic crystals, and proteinaceous microcrystals. Proteinaceous microcrystals are present in healthy as well as infected tissue, although they may be more numerous in diseased tissue. Study of healthy controls treated with Triton X-100 should eliminate these structures from consideration as being viral in nature.

The Triton X-100 treatment is normally not used with the azure A stain, which does not stain plastids and thus does not interfere with detection of inclusions.

Tissues other than the epidermis. Many virus inclusions are found predominantly in tissues other than the epidermis. Those induced by certain viruses are often more abundant in mesophyll tissues, whereas those induced by viruses of the closterovirus, geminivirus, and luteovirus groups appear in most cases to be associated predominantly with the veinal tissues. One of the most valuable techniques for detecting and studying these inclusions utilizes pieces from which the upper or the lower epidermis or both have been removed.

Such preparations open for inspection the palisade, spongy parenchyma, and veins of the leaf.

Thick tissue pieces. A thick leaf piece can be obtained by removing the epidermis as previously described. The piece of tissue from which the epidermis has been removed is cut out (Fig. 4A) and soaked in 2-methoxyethanol to remove chlorophyll and other pigments (Fig. 4B). This step usually takes about 30 minutes. The cleared tissue is stained for 10-15 minutes with azure A; the O-G combination stains the plastids and therefore is used only in special cases. After staining in azure A, the tissue is cleared of excess dye in 95% ethanol for about 15 minutes and mounted directly in Euparal. Tissues that are too blue for inspection because of residual dye can be returned to the alcohol until excess dye is removed and the nuclei and inclusions contrast sharply with the surrounding cytoplasm. An alternative method is to place the tissue into 2-methoxyethyl acetate after the first alcohol rinse. The acetate slowly removes the excess dissolved stain and leaves the cell nuclei and virus inclusions stained. While in the acetate solution, the tissue should be examined periodically, and when the nuclei and virus inclusions stand out sharply, the tissue can be mounted in Euparal. Figures 1B and 1E show cucumber mosaic virus inclusions in mesophyll cells of a thick piece of tobacco leaf tissue.

Fig. 1. Micrographs photographed at $\times 1,940$ unless otherwise stated. N = nucleus. (a) Portion of epidermal strip from *Chenopodium amaranticolor* infected with papaya ringspot virus (potyvirus) and stained with O-G combination. Cytoplasmic inclusions (CI) can be seen at relatively low magnification ($\times 200$). (b) Thick tissue preparation showing mesophyll cells of tobacco leaf infected with cucumber mosaic virus and stained with azure A ($\times 100$). Arrow points to virus crystal at periphery of infected area. (c) Freehand (Styrofoam mount) section of sweet orange leaf petiole infected with citrus tristeza virus (closterovirus) stained with azure A. Arrows point to inclusion-containing phloem cells ($\times 100$). (d) High-resolution micrograph of papaya ringspot virus inclusion taken from same area shown in (a). Group of proteinaceous cylindrical inclusions (CI) are stained green; these do not contain nucleic acid and therefore will not stain with azure A. Cytoplasmic irregular inclusion (I), induced by same virus, is stained deep green. (e) High-resolution micrograph of same cucumber mosaic virus crystal (arrow) shown in (b), demonstrating intensity of azure A stain for virus inclusions containing nucleic acid. (f) Composite micrograph showing banded (B) and paracrystalline (P) virus aggregates of citrus tristeza virus found in areas similar to those designated by arrows in (c). (g) Papaya epidermal cell infected with papaya ringspot virus. Leaf tissue was prepared by abrasion method, treated with Triton X-100, and stained with O-G combination. Arrow points to group of proteinaceous cylindrical inclusions (CI). (h) Phloem parenchyma cell nucleus of bean tissue containing inclusion (I) induced by bean golden mosaic virus (geminivirus) stained with azure A. These virus aggregates consist of DNA-containing particles and stain a distinct blue compared with RNA-containing nucleolus (arrow). (i) Epidermal cell of *Nicotiana benthamiana* infected with clover yellow mosaic virus (potexvirus) stained with azure A. Banded inclusions (B) are characteristic of this group and are similar over a host range. Their delicate structure is easily disrupted, and special techniques are used to ensure their preservation. Arrow points to vacuolate-vesiculate inclusion (I). (j) Epidermal cells of *N. benthamiana* infected with soybean mosaic virus (potyvirus) stained with O-G combination. Small, conical inclusions (I) typical of this virus group appear first at plasmalemma and are often obscured by plastids (P). (k) Epidermal cells infected with soybean mosaic virus taken from same leaf area shown in (j). Tissue was treated with Triton X-100 before staining with O-G combination to dissociate plastids and facilitate detection of inclusions (I).

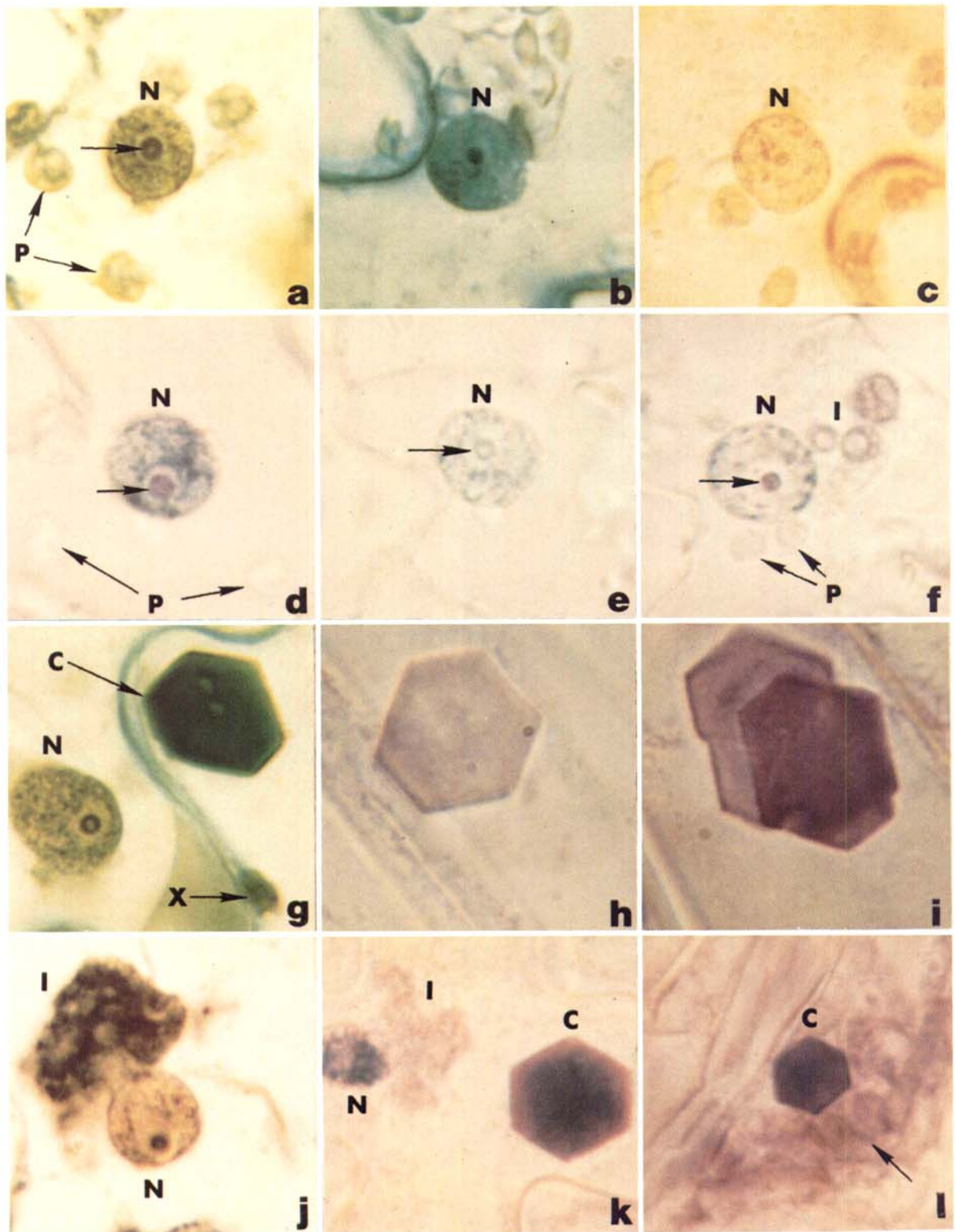


Fig. 3. Micrographs photographed at $\times 1,940$. N = nucleus. (a) Healthy tobacco epidermal tissue stained correctly with O-G combination. Nucleus has orange matrix with green chromatin and green nucleolus (arrow). Plastids (P) are also stained. (b) Healthy tobacco epidermal tissue understained with O-G combination. Tissue appears uniformly green. (c) Healthy tobacco epidermal tissue not differentiated with green and appearing uniformly orange. (d) Healthy tobacco epidermal tissue stained correctly with azure A. Chromatin is blue and nucleolus (arrow) is red-violet. Plastids (P) are not stained. (e) Healthy tobacco epidermal tissue not stained correctly with azure A. Chromatin is pale blue and nucleolus (arrow) is unstained. (f) Tomato mesophyll cell infected with tomato aspermy virus (cucumovirus) stained with azure A. Inclusions (I) are easily distinguishable from unstained plastids (P). (g) Tobacco epidermal cells infected with common tobacco mosaic virus (tobamovirus) stained with O-G combination. C = virus crystal, X = X-body. (h) Common tobacco mosaic virus crystal that failed to stain in azure A at room temperature. (i) Double crystal of common tobacco mosaic virus heated in azure A for 1 minute at 60 C. (j) Vacuolate inclusion (I) induced by cowpea mosaic virus (comovirus) stained with O-G combination. These complex inclusions pick up both orange and green dyes and appear brownish green. (k) Pea tissue infected with broad bean stain virus (comovirus) and stained with azure A at room temperature. Large vacuolate inclusion (I) is diffuse and stains light red. Denser inclusions similar to the one shown in (j) stain violet. Virus crystal (C) stains without heat. (l) Tobacco mesophyll cell infected with cucumber mosaic virus (cucumovirus) stained with azure A at room temperature. Cytoplasm and plastids (arrow) partially surrounding virus crystal (C) are lightly stained.

In many plant species, the epidermis is difficult to remove. A simple method (3) that overcomes this obstacle consists of gently abrading the tissue with a piece of 600-mesh emery cloth or sandpaper (Fig. 5A) until a portion of the tissue is thin enough to expose the opposite epidermis (Fig. 5B). In some cases, laying the tissue flat and abrading it with a small piece of sandpaper is easier. Dampening the tissue surface slightly with water may be helpful. The tissue from which the epidermis has been removed is cut out and cleared in 2-methoxyethanol, then processed the same as thick tissue obtained by epidermal stripping.

Areas that have been abraded to the extent that only the opposing epidermis (supported by the cuticle) is left can be handled in the same manner as an epidermal strip and be stained with either azure A or the O-G combination. When the O-G stain is used, the Triton X-100 treatment can be applied to remove any plastids interfering with detection of inclusions. This method is convenient for studying epidermal cells of plant species where the epidermis is virtually impossible to obtain by the simple strip method. Figure 1G is an epidermal cell containing cylindrical inclusions induced by papaya ringspot virus (a potyvirus) in papaya leaf tissue prepared by this method.

Thick tissue pieces can be mounted with either the torn surface or the uninjured epidermis next to the coverslip. When the epidermis is mounted next to the coverslip, the tissue can be studied in an uninjured state. This can be useful when fragile inclusions, such as the banded inclusions of certain members of the potyvirus group (Fig. 1I), are altered or destroyed during the tearing process. The palisade layer of tissue is also easily studied in this position by focusing down through the epidermis.

When the torn surface is mounted next to the coverslip, the spongy parenchyma and vascular tissues are easier to view. Observation of vascular tissues is important for detection of inclusions induced by phloem-associated viruses (e.g., the geminiviruses). Vascular tissues may also contain cytological evidence of other pathogens, such as phloem-oriented mycoplasmas (e.g., aster yellows) and xylem-located bacteria (e.g., that causing Pierce's disease).

After being viewed in one position, a thick tissue sample can be removed and remounted in the inverted position. In either position, inclusions lying beneath several cell layers can be detected.

Freehand and Cryostat Sections

Freehand sections. These can be very helpful in the study of inclusions located in tough leaf tissues, midribs, petioles, stems, bark, and fruit. Probably the simplest type of freehand section is obtained by a paradermal cut (Fig. 6). The periphery of such a section can be

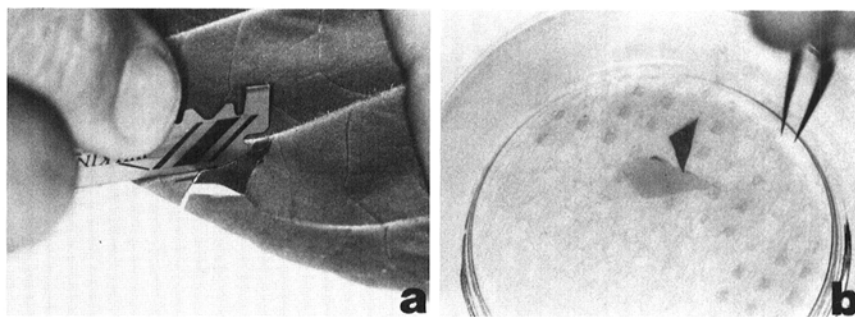


Fig. 4. Preparation of a thick tissue piece: (a) A razor blade is used to cut out a piece of tissue from which the epidermis has been removed. (b) The tissue is soaked in 2-methoxyethanol to remove pigments (the lower piece shown is clear of pigments and the other has just been placed in the solution).

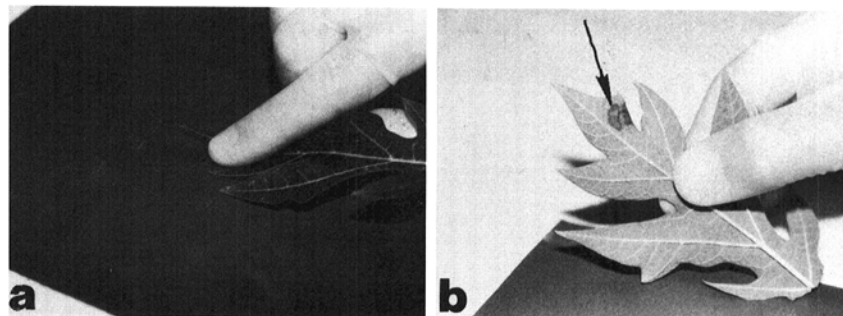


Fig. 5. Preparation of tissue by the abrasion technique: (a) The leaf surface is dampened with water and gently rubbed against 600-mesh sandpaper. (b) The opposite epidermis is evident (arrow) when abrasion is complete.

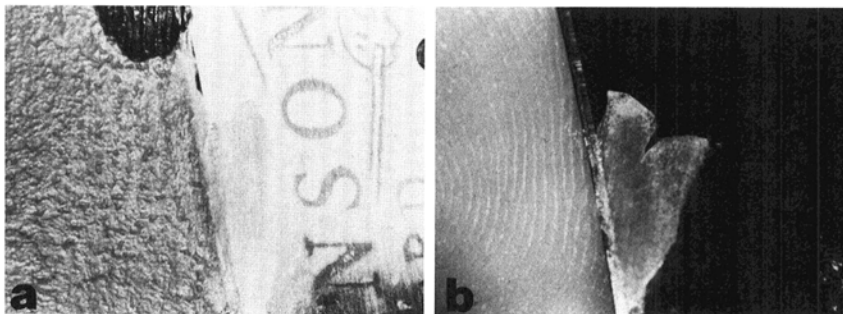


Fig. 6. Preparation of a paradermal section of an orchid leaf: (a) The cut is made with a razor blade parallel to the leaf surface. (b) The resultant section contains epidermal cells at the periphery and mesophyll tissues at the center.

treated as an epidermal strip, and the thicker portion, as a thick tissue piece. The thick pieces should be dehydrated and cleared in 2-methoxyethanol before staining.

Freehand sections can also be made by placing tissue into a slit made in a piece of Styrofoam packing material. Sections are cut with a slicing action, with the cut at an approximately 45° angle to the long axis of the tissue (Fig. 7). The Styrofoam should be as thin as possible. Both the tissue and the Styrofoam are placed into 2-methoxyethanol for a few minutes until the tissue is cleared. The sections are treated and stained in the same manner as epidermal strips. The Styrofoam technique was used to obtain the sections in Figures 1C and 1F showing inclusions induced by citrus tristeza virus in leaf petioles of sweet orange.

Dicing the tissue with a sharp razor

blade (Fig. 8) is a useful technique with young tissues that are difficult to handle with other methods. In chronic cucumber mosaic virus infections, for example, inclusions (virus aggregates) are often found only in leaves that are less than 2 cm long. These young leaves are difficult to handle by any of the previously described methods, but satisfactory sections can be obtained by dicing. The diced tissue is placed directly into 2-methoxyethanol for a few minutes to dehydrate and to remove chlorophyll. The sections are then handled as with the Styrofoam technique. Figure 3L shows an inclusion of cucumber mosaic virus in a 17-mm leaf taken from a tobacco plant with prominent symptoms on all mature leaves. This leaf was the largest in which inclusions were detected, illustrating the need for studying immature as well as mature tissues, especially in instances of

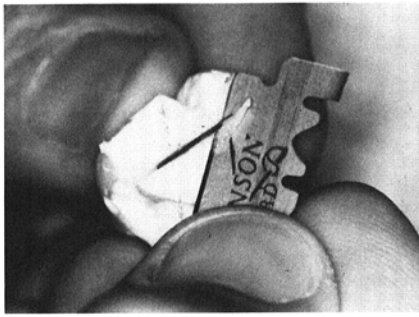


Fig. 7. Tough leaves, midribs, stems, and roots can be sectioned by supporting the tissue in a slit, made at an angle to the length of the tissue, in a piece of Styrofoam packing material.

chronic infection. Inclusions of many viruses often are abundant in young tissues but scarce or absent in older ones.

Cryostat sections. Cryostat sections are especially suitable for studying growing tips, anthers, ovules, stems, roots, and succulent leaf pieces. Since the staining solutions are principally non-aqueous, the sections adhere to the surface of the slide and do not wash off during the staining process. Adhesives can be used, but the O-G combination may stain the background slightly. A glass slide containing the sections is flooded with one of the staining solutions for an appropriate period, then is rinsed in 95% ethanol for 15–30 seconds,

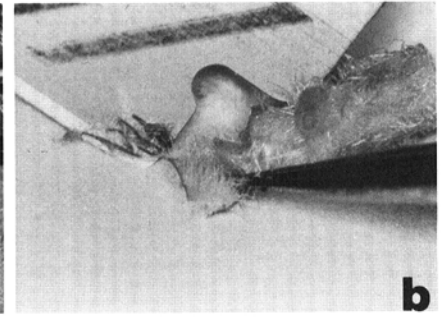


Fig. 8. (a) Delicate tissues such as this young tobacco leaf are best handled by dicing with a razor blade. (b) Diced leaf pieces on the edge of the blade.

followed by 2-methoxyethyl acetate for 1–2 minutes. The acetate solution is removed by touching the edge of the slide to a piece of blotting paper. The mounting medium and coverslip are applied before the acetate solution has dried.

Fixation with a 1–3% buffered glutaraldehyde solution for a few hours can render tissues easier to section after freezing and is especially helpful for sectioning growing points and immature tissues. Glutaraldehyde can be detrimental to certain types of inclusions, however, and should be used with appropriate controls.

Choice of Tissues and Methods

The choice of infected plant material can be critical to detection of inclusions,

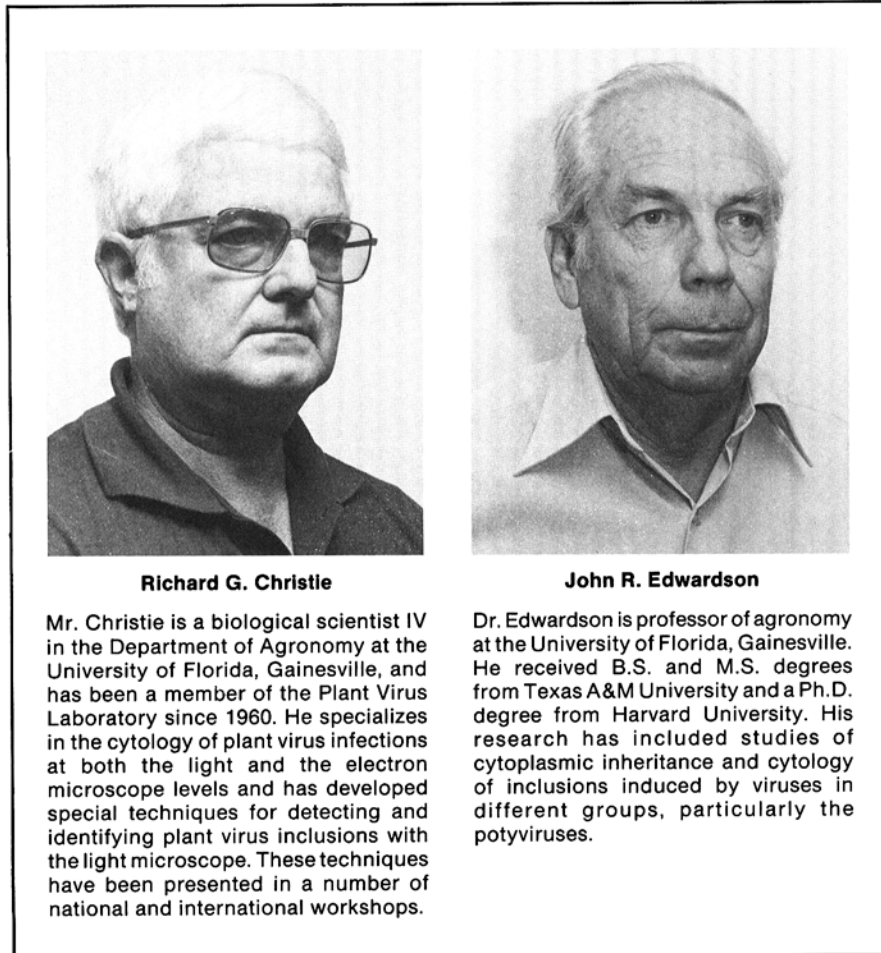
since symptom expression may not always be correlated with presence of inclusions. Inclusions may be difficult to detect when chlorosis or necrosis is severe because they may not have reached the size or stage necessary for recognition or they may have begun to disintegrate in dying cells. Often, inclusions are prominent just before symptom expression or in tissues with mild or undetectable symptoms. Therefore, samples should be taken not only from areas with symptoms but also from tissues of varying ages, regardless of symptom expression.

Epidermal cells are a rich source of inclusions of many virus groups and should be the starting point in the search for inclusions. Samples should be stained with both azure A and the O-G combination. When suitable tissue is limited, both stains can be used on the same piece, azure A preceding the O-G combination. Excess mounting medium is removed by soaking the tissue for a few minutes in 95% ethanol before restaining. If the epidermis is hard to remove from a sample, the abrasion method can be used to expose the epidermis opposite the abraded surface. Some of the epidermal tissue should be treated with Triton X-100, followed by the O-G stain. The rest of the epidermal samples can be stained with azure A.

If few or no inclusions are encountered in the epidermis, a thick tissue piece obtained by stripping the epidermis or abrading the tissue should be used. If inclusions are not detected in either the epidermis or the mesophyll of the leaves, freehand sections of stems, roots, immature tissues, and growing points should be tried. Either the O-G or the azure A procedure can be used on these tissues.

Some Comments on the Stains

Both the O-G combination and azure A stain certain host cell constituents present in healthy and virus-diseased tissues, and results are similar for all hosts (Table 1). Dead or dying tissues stain green with the O-G combination and blue with azure A. The organelles are not color-differentiated under these circumstances. Both stains work best when the water content is maintained at 10–20% by



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Table 1. Staining reactions of host cell constituents present in both healthy and virus-diseased tissues

Constituent	Stain	
	Azure A	O-G
Chromatin	Blue	Green
Nucleoplasm	Clear	Orange
Nucleolus	Red-violet	Green
Cell wall	Colorless	Yellow-green
Cytoplasm	Colorless ^a	Yellow-green
Plastids	Colorless ^a	Yellow-green
Microcrystals	Colorless	Green
P-protein (phloem)	Colorless	Green

^aMay stain reddish in diseased cells.

volume; solutions containing more than 20% water can drastically alter staining reactions. Tissues containing exceptionally high amounts of vacuolar sap should be dehydrated with 2-methoxyethanol before staining.

The O-G stain. The O-G combination differentially stains plant organelles and inclusions containing protein. The color reactions shown in Figure 3A of healthy tobacco epidermal tissue stained with the O-G combination are typical for most plant species. If plant organelles do not stain as shown in Figure 3A, the virus inclusions will *not* be correctly differentiated. Most inclusions containing protein stain green (Fig. 3G), but some stain both orange and green, resulting in a brownish green (Fig. 3J).

Tissue that appears too green is understained (Fig. 3B). This results when the orange dye penetrates slower than the green and can be corrected by removing the excess mounting medium from the tissue with alcohol and returning the tissue to the O-G stain for a longer period of time.

When tissue appears too orange (Fig. 3C), it has remained in the stain too long (the orange eventually replaces the green), it has remained in the alcohol too long (the green is more soluble in alcohol than the orange), or the mounting medium has leached out the green. All these conditions can be corrected by returning the tissue to an alcohol wash followed by restaining with green only, i.e., no water and no orange. The tissue is stained for about 30 seconds, dehydrated in alcohol, and mounted as previously described.

The azure A stain. The azure A stain is

designed for detecting inclusions containing nucleic acids. When used under the conditions described, azure A is metachromatic, rendering the chromatin blue and the nucleolus red-violet. In the healthy mature epidermal cell of tobacco stained with azure A shown in Figure 3D, only the nucleus is stained (blue chromatin and red-violet nucleolus); the other cellular organelles, including the plastids, are colorless. Virus inclusions containing ribonucleic acid (RNA) stain red-violet. The smaller, less dense inclusions tend to stain pink-red (Figs. 3F and 3K), whereas dense inclusions, particularly the virus aggregates, are more violet (Figs. 3I, 3K, and 3L). Inclusions containing deoxyribonucleic acid (DNA) stain blue (Fig. 1H). Virus inclusions contrast sharply with the surrounding cytoplasm. Because plastids remain almost colorless or stain only a light red (Fig. 3L), very small accumulations of virus particles or virus-related RNA that may mimic plastids can be detected (Fig. 3F).

Azure A stains most inclusions at room temperature but does *not* stain the crystalline (Fig. 3H), paracrystalline, or angled-layer aggregate inclusions induced by tobamoviruses unless heat is applied during staining. This is accomplished by placing the tissue in the staining solution and heating at 60 C for 1–2 minutes until the crystals stain (Fig. 3I). Because the crystals will rupture if overheated, a visual check should be made with the microscope at 30-second intervals while the tissue is in the stain. After staining, the tissue is rinsed in alcohol and mounted as previously described. This method distinguishes inclusions induced by tobamoviruses from those induced by viruses of other groups with similar outlines, e.g., the hexagonal crystals of broad bean stain virus, a comovirus (Fig. 3K), and cucumber mosaic virus, a cucumovirus (Fig. 3L).

Proper staining with azure A has been achieved when the chromatin of the nucleus is a distinct blue and the nucleolus is red-violet (Fig. 3D). If the chromatin is pale blue and the nucleolus unstained (Fig. 3E), the stain is not functioning properly. Faulty staining with azure A results in failure to differentiate inclusions. This problem can result from using water or monobasic phosphate in place of dibasic phosphate. If this does not appear to be the problem, the stain itself may be faulty and another batch or source should be tried.

If azure A is not available, azure B,

azure C, or toluidine blue may be substituted. These dyes, however, do not produce as intense a metachromatic reaction as azure A does.

Storing and Preserving Samples

The described procedures work best on fresh tissues. Samples that need to be transported should be sealed tightly in plastic bags and placed in a cooler. Processing should be done as soon as possible. Most inclusions are stable enough to last several days, however, if the samples are kept refrigerated.

If samples cannot be checked immediately or must be sent considerable distances for inspection, the tissues should be preserved. The epidermal strips, thick tissue pieces, or hand sections are prepared in advance and stored in a solution of 100% 2-methoxyethanol, which will preserve the tissues and maintain the integrity of the inclusions. Inclusions in samples that have remained in this solution for many years appear no different from those in fresh tissue.

In Summary

The detection of characteristic inclusions by light microscopy is useful in identifying many plant virus diseases. The light microscopic techniques described are specifically designed for this purpose. The procedures are simple, rapid, and inexpensive and require only a minimal knowledge of microscopy. The major difficulty lies in learning to recognize the different types of inclusions, but this skill is readily acquired with a little practice and experience.

Acknowledgments

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