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J. Amer. Soc. Hort. Sci. 101(3):224–228. 1976.

Structure of a Single Tissue Prepared for Analysis by Light, Scanning and Transmission Electron Microscopy[1]

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Additional index words. rose, *Rosa hybrida*

Abstract. **A technique was developed which permits observations of a single rose petal segment through the sterolight microscope (SLM), scanning electron microscope (SEM), transmission electron microscope (TEM), and light microscope (LM). The procedure consisted of viewing the fresh tissue with SLM, fixing and post-fixing in glutaraldehyde and osmium, respectively, and dehydrating in ethanol. The alcohol in the tissue was subsequently replaced with increasing concentrations of iso-amyl acetate, the tissue critical point dried, coated with C, and viewed in the SEM. The tissue was removed from the SEM mounting stub, pressure embedded in epoxy resin at 28 kg/sq cm (400 psi), polymerized, sectioned, stained, and viewed with both the TEM and LM. The technique of pressure embedding samples in epoxy resin eliminated the problem of rehydration and subsequent dehydration of tissue following SEM observation. Furthermore, this new technique reduced the time required for observation with multiple microscopic optical systems, while still offering latitude in the time between the various steps which has been a drawback in previous techniques.**

There are numerous techniques for studying biological tissues with electron or light microscopes (6, 7, 9, 11, 12, 15, 19). Recently, however, there has been interest in combining techniques so that correlative studies could be made on a single tissue block (2, 5, 14). Geissinger (8) developed a procedure whereby paraffin embedded sections could be viewed first on the light microscope (LM) and then the scanning electron microscope (SEM). Similar techniques (1, 16, 17) proved inadequate due to tissue shrinkage and distortion as a result of embedding. Also, the technique of Ayres et al. (1) was lengthy and involved rehydration of the tissue between SEM viewing and LM observation.

In a technique for viewing animal tissue in the SEM and transmission electron microscope (TEM) by Barber and Boyde (3), the tissue was partially embedded in Epon after which the surface of the tissue was washed with a jet of hot ethanol or propylene oxide. After SEM observation, polymerization was resumed and standard TEM preparations made. This procedure had several inherent problems when used with plant tissue. First, it was difficult to determine the point at which the excess embeddant was removed. Also, the hot solvent could remove or damage surface waxes often present on plant tissues, or dissolve cellular materials such as chlorophyll.

Panessa and Gennaro (18) obtained satisfactory SEM micrographs of plant tissues by a lengthy procedure involving prolonged fixation followed by glycerine substitution of water. Subsequent acetone dehydration and epoxy resin embedding of the same tissues was possible, but TEM images obtained were not of the highest quality. Also, tissues prepared in this manner deteriorated during prolonged storage.

Barber's (2) technique of rehydrating air or freeze dried SEM specimens and embedding them in Araldite for TEM appeared satisfactory for certain tissues, particularly those where air-drying did not cause cellular collapse visible in the SEM.

The most useful technique appeared to be that of Erlandsen et al. (7). Similar to the work of Barber and Boyd (3), the tissues were resin infiltrated as for TEM but, instead of washing partially polymerized resin prior to final encapsulation, fully polymerized blocks were washed with epoxy solvent until the surfaces were clean, prior to coating and examination in the SEM. This technique enabled the authors to examine the same tissue in the TEM and SEM. The tissues could also be reencapsulated with resin after SEM examination and used for TEM or LM studies. A recently presented technique by Brum-

[1]Received for publication April 14, 1975. Michigan Agricultural Experiment Station Journal Article Number 6628.

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mer et al. (5) also appears to be promising.

In many types of horticultural studies it is advantageous to sequentially examine the same tissues with stero-light microscopes (SLM) and SEM, then section the material for TEM and LM. Rehydrating and embedding tissues after SEM appeared to be the most satisfactory procedure to follow, but tissues were often hard to infiltrate with resin after freeze drying or critical point drying. We found (10) that resin could be forced into tissues under pressure. The objective of the current study was to examine the feasibility of using pressure infiltration of critical point dried plant specimens to enable multiple microscopic viewing.

Materials and Methods

Ten petals of a *Rosa hybrida* cv. Forever Yours flower were stripped prior to removal of a single petal for study. Subsequently a segment of about 5 mm^2 was removed and the upper surface was immediately photographed through an Olympus JM-TR SLM (Fig. 1).

The tissue was then fixed in a 3.2% solution of glutaraldehyde in 0.1 M phosphate buffer at pH 7.0 for 12 hr at 25°C. AFter 3 changes of buffer during a 30-min period, the tissue was post-fixed with 1.0% osmium solution in the same buffer for 1 hr at 25°. The tissue was dehydrated in an ethyl alcohol series (15%, 30%, 50%, 70% for 10 min each; 85% and 95% for 15 min each; and 100% for 1 hr). The alcohol was exchanged with iso-amyl acetate in a 2-step series (50% solution of iso-amyl acetate in absolute alcohol for 30 min and 100% iso-amyl acetate for 1 hr). The tissue was then critical point dried in a Denton DCP-1. The dried tissue was mounted on a SEM stub with a drop of Carbon TubeKoat (G. C. Electronics Co., Rockford, Illinois), coated with approximately 20 to 40 nm of C, and viewed in the SEM (Advanced Metal Research Model 900) at 21 kv accelerating potential (Fig. 1). Photomicrographs were also taken of a critical point dried petal without glutaraldehyde fixation or osmium post-fixation to serve as a control.

For TEM and LM viewing, the tissue was carefully removed from the SEM stub with a razor blade and placed in a small plastic cap in a 7:3 (v:v) epoxy resin mixture (13). The mixture containing the rose petal was placed in the critical point pressure chamber and slowly raised to 28 kg/cm (400 psi) with

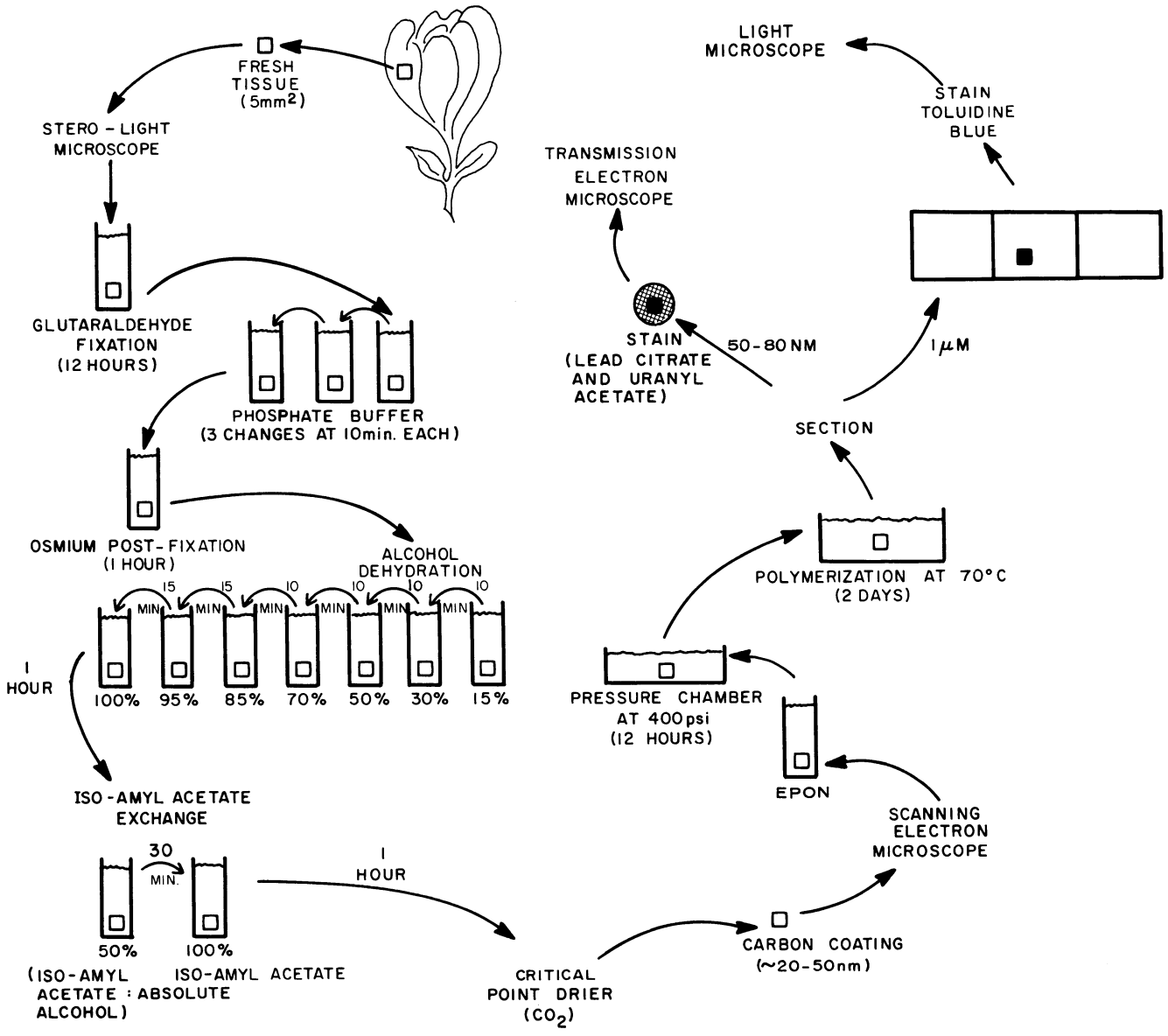

Fig. 1. Schematic diagram of the pressure embedding technique.

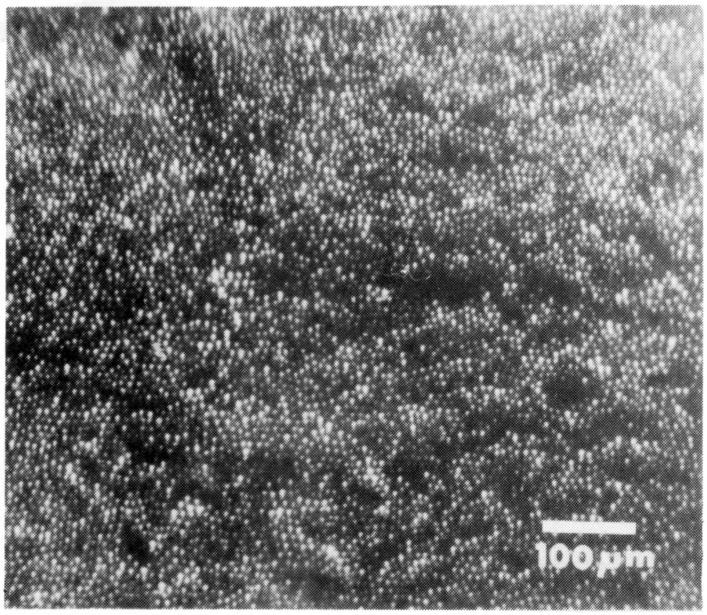

Fig. 2. Photomicrograph of the upper surface of a fresh rose petal through a stereo-light microscope (SLM).

CO_2 gas and held for 12 hr. The pressure was slowly lowered over a 30 to 60 min period to avoid damage to the tissue by bubbling the epoxy resin. Next, the Epon was polymerized at 70°C for 2 days and the tissue sectioned with a diamond knife to a thickness of 50 to 80 nm with a Porter-Blum MT-2 ultramicrotome. Sections were stained for 30 min in uranyl acetate, rinsed with water, stained for 5 min in alkaline lead citrate, rinsed in 0.2 N NaOH, and washed in water. TEM examination was made with either a Philips 100 or 300 TEM (Fig. 1). A control rose petal was prepared with standard glutaraldehyde and osmium fixation procedures for TEM viewing (9). After dehydration the control tissue was passed through a graded series of propylene oxide (PO) (1:1 mixture of 100% alcohol and PO for 15 min; 2 changes of 100% PO for 15 min followed by a 1:1 mixture of epoxy resin and PO for 24 hr). The tissue was transferred to a 100% epoxy resin and polymerized at 70°C for 2 days.

For LM observation, the tissue embedded with epoxy resin was sectioned to 1 um, mounted on a glass slide, stained with toluidine blue (4) and viewed through a Wild M-20 LM (Fig. 1).

Results and Discussion

The SLM offers a surface view of a fresh tissue which is believed to be free of artifact. Often, researchers have eliminated this step in analyzing tissues because of instrument limitations. Perhaps the most serious is resolution, but at the low magnification for which the instrument was designed this presents no real problem. Another concern is the loss of the "stereo-effect" in photomicrographs, but this could be eliminated by utilizing "stereo-pairs." With fragile tissue, the greatest potential problem with SLM use is the induction of artifacts from tissue drying and shrinkage while viewing. These concerns can be minimized, however, by using flourescent lighting to reduce temperatures and by proper instrument alignment to insure rapid tissue viewing and subsequent processing. These problems do not warrant the lack of use the SLM has received. Information about the true nature of the rose petal surface was obtained with the SLM (Fig. 2). For example, we observed that the upper surface is covered with small domes of approximately 10 to 15 um in diameter. Other advantageous features of the SLM include: 1) living tissue can be viewed, 2) the tissue has not been in contact with any solvent systems, nor is it subjected to high vacuums associated with the SEM, and 3) representative tissue color can be obtained. SLM therefore serves to record the structure of untreated tissue and thus to serve as a control for tissues treated for viewing with other instruments.

A SEM comparison of the control petal (Fig. 3A) with the fixed petal (Fig. 3B), which was prepared via the technique in Fig. 1, indicated that glutaraldehyde fixation and osmium post-fixation, necessary for later TEM viewing, did not alter the surface configurations of the rose petal. After double fixation, it was quite difficult to identify the upper side of the tissue due to the dark staining. This could present a problem with certain tissues being prepared for SEM observation.

After SEM analysis, the rose petal was prepared for observation with the TEM and LM. Rose tissue was prepared via the technique in Fig. 1 and control tissue prepared by standard TEM procedures (Fig. 4 and 5). Both techniques proved similar

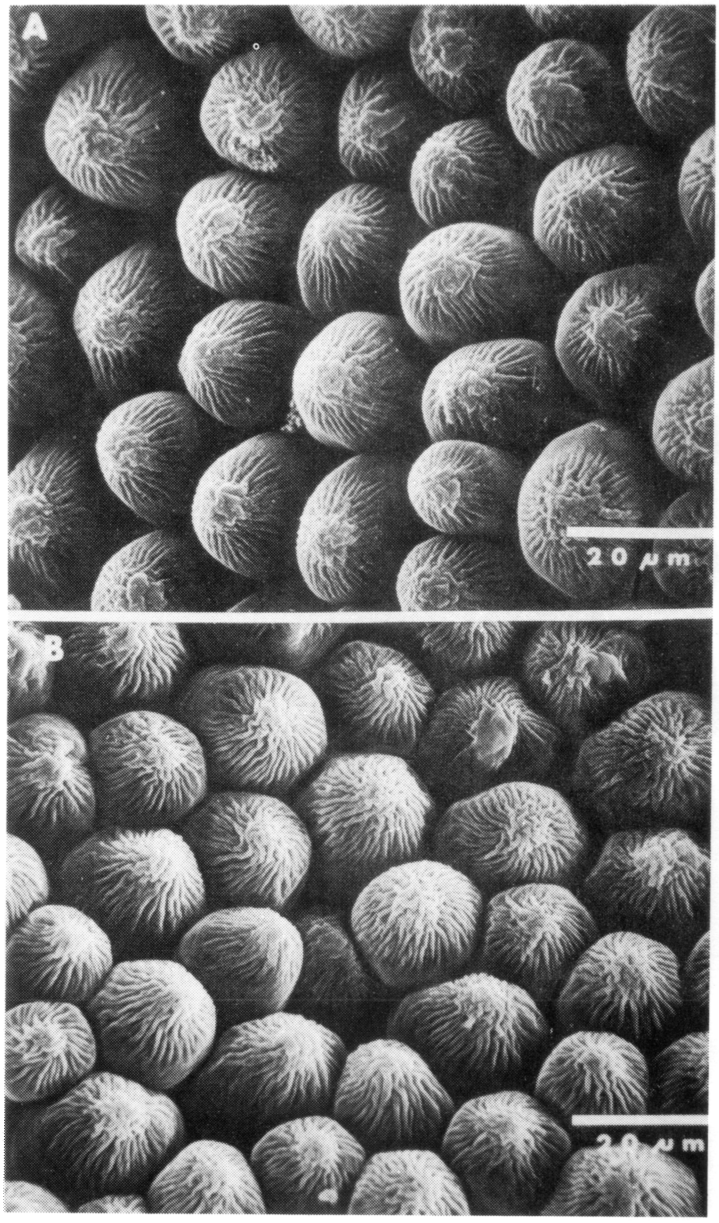

Fig. 3. Photomicrograph of the upper surface of a rose petal with the scanning electron microscope (SEM). A, Control petal was critical point dried only (CPD). B, Petal was fixed, post-fixed, and CPD following viewing on the SLM.

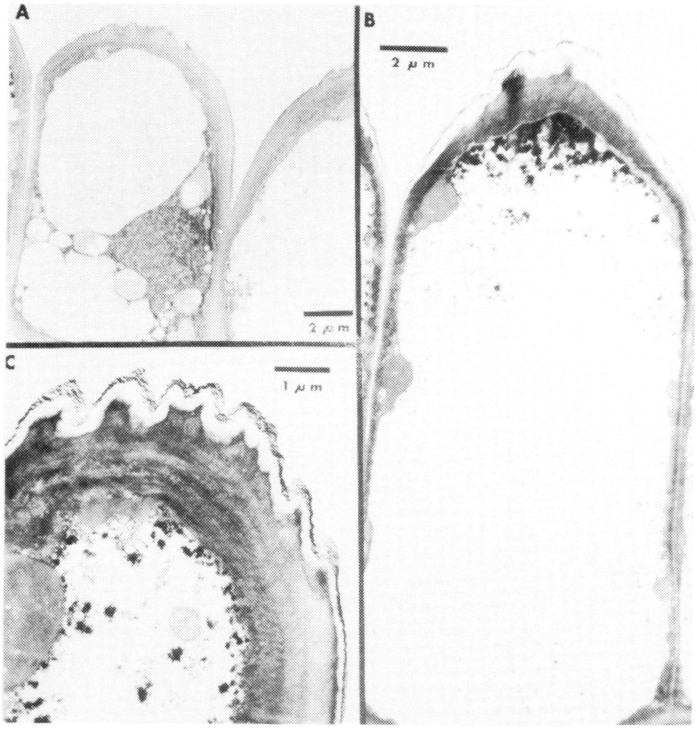

Fig. 4. Transmission electron photomicrographs of a longitudinal view of rose petal epidermal cells. A, Cells prepared by standard TEM techniques. B and C, Pressure embedded tissue that had been previously viewed in the SLM and SEM, respectively.

quality photomicrographs. The dome shaped epidermal cells of the upper surface of the control tissue are shown in Fig. 4A, while Fig. 4B and C show a similar view of the pressure embedded petal. Waxes on the tip of control epidermal cells are presented in Fig. 5A and B. Fig. 5C shows a similar view of a pressure embedded cell that had been C coated for SEM viewing.

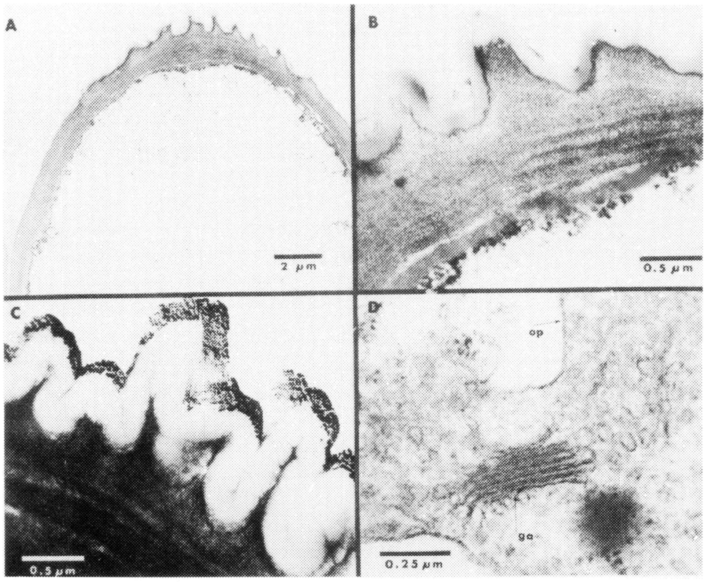

Fig. 5. Transmission electron photomicrographs of the internal anatomy of a rose petal. A and B, Cells that were prepared by standard TEM techniques. Note wax covering epidermal cell wall. C, Epidermal cell that had been pressure embedded. Note wax structure and C coating. D, Golgi apparatus (ga) and intact membrane (op) of a pressure embedded cell.

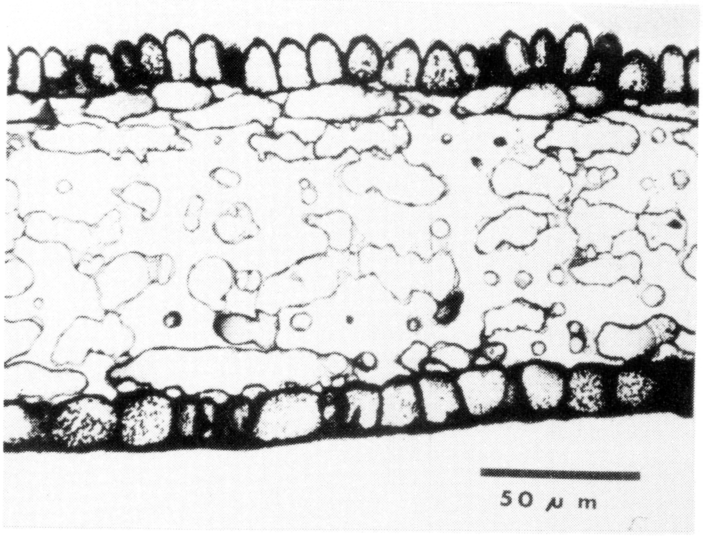

Fig. 6. Photomicrograph through the LM of the rose petal previously viewed through the SLM, SEM, and TEM.

The wax layer appeared more clearly on the C-coated cell than on the control, but the C layer may have more clearly delineated the outer wax surface. The amount of wax removed while processing the tissue through various solvent systems is unknown. However, it can be estimated from other work (unpublished data of E. A. Baker, Long Ashton Research Station, University of Bristol) that 5 to 10% of the total would be lost, while if the wax was composed mainly of aldehydes, fatty acids, and triterpenoid acids, practically none would be removed. Fig. 5D shows the golgi apparatus from one of the pressure embedded epidermal cells. The osmiophilic and osmiophobic layers of a membrane (op) were readily visible. Both the intact golgi and the bilayer of the membrane (Fig. 5D) provided evidence of good fixation. Uniform embedding of critical point dried tissue of this size was difficult when placed directly in the final embedding mixture. However, pressure embedding resulted in uniform embedding, easy sectioning, and no apparent tissue damage.

An LM photomicrograph of the pressure embedded petal (Fig. 6) shows the gross internal anatomy of the rose petal. This aided in interpretation of TEM photomicrographs. Also, histochemical analysis could be conducted with the LM prepared tissue. The lower surface of the petal was attached to the SEM stub with TubeKoat and as a result was destroyed for detailed analysis (Fig. 6). This was the main disadvantage of the technique, but this problem could be reduced by mounting with double sticky tape.

By following the pressure embedding technique, 4 different types of photomicrographs were prepared within 4 days, which is about the same time required for routine paraffin embedding for LM (12). With the technique of Panessa and Gennaro (18) or Erlandsen et al. (7) at least twice as much time was required. Furthermore, considerable latitude with respect to time in the various steps is possible; for example, the tissue could be held indefinitely after dehydration, after critical point drying, and after Epon polymerization. With pressure embedding, the rehydration step used by Barber (2) was eliminated. A much better understanding of the spatial relationships of features, unsuspected detail, and previously undescribed characters was obtained by using the four instruments in combination on a single tissue block. A single specimen must be viewed from several vantage points to make critical interpretations.

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J. Amer. Soc. Hort. Sci. 101(3):228–233. 1976.

Insecticide Effects on *Encarsia formosa* Gahan, Parasite of the Greenhouse Whitefly, *Trialeurodes vaporariorum* (Westwood)[1]

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Additional index words. endosulfan, malathion, nicotine sulfate, resmethrin, integrated control

Abstract. **Nicotine sulfate and resmethrin, applied at recommended rates, were less toxic to adults and larvae of *Encarsia formosa* than were endosulfan, malathion, or naled. Adult parasites were killed by contact with any of the 5 chemicals. Endosulfan and malathion left residues toxic to adult *E. formosa* for 2 to 3 weeks; malathion and naled killed many 10- to 15-day-old parasite larvae. The potential uses of nicotine sulfate and resmethrin were shown in theoretical models for integrated control of greenhouse whitefly.**

Encarsia formosa Gahan; (Hymenoptera: Aphelinidae), a parasitic wasp of the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood); (Homoptera: Aleyrodidae), was first observed in Idaho and Ohio (10). In England and Canada, *E. formosa* was reared and distributed to greenhouse growers in the late 1920's (1, 2, 17, 24, 27). Although *E. formosa* has been widely used (3, 19, 26), only recently have commercial procedures for exact release methods been proposed by McClanahan (16) and others (14, 20, 22, 23). Greenhouse growers have found it difficult to establish an effective balance between *E. formosa* and whitefly populations.

Whitefly resistance to insecticides, dangers associated with use of toxic insecticides, and increasing cost of insecticides have brought about a need for control systems which are more effective, safer, and cheaper (14). The potential for integration of chemicals to aid *E. formosa* was first reported by Speyer in 1929 (25) and later advocated by Balevski (3), who reported that *E. formosa* will emerge from parasitized nymphs fumigated with hydrogen cyanide. McClanahan (15) found that quinomethionate (Morestan^R) kills adult whiteflies and eggs without harming *E. formosa*. Quinomethionate does not have label clearance for use on greenhouse vegetable crops in the U.S., but McClanahan (personal communication) suggests maneb as a substitute. A few chemicals are relatively nontoxic to Hymenopterous insects (4), and pirimicarb (an aphicide) has been reported harmless to *E. formosa* when applied as a soil drench (13, 19).

This study evaluated naled, malathion, nicotine sulfate, resmethrin, and endosulfan as potential insecticides to use with *E. formosa* in an integrated program to control whitefly on greenhouse tomatoes. These chemicals have been cleared for most greenhouse plants, though resmethrin does not have label clearance for vegetable crops in the U.S.

Materials and Methods

Effect of 5 insecticides on adults and larvae of E. formosa. Recommended rates of 10 ml/4.4 liters (2 tsp/gal) 55% malathion, 10 ml/4.4 liters (2 tsp/gal) nicotine sulfate (40% alkaloid), 5 ml/4.4 liters (1 tsp/gal) Dibrom^R 8 emulsive (58% naled), 5 ml/4.4 liters (1 tsp/gal) 24% resmethrin, and 7.5 ml/4.4

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