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LIQUID SUBSTITUTION: AN ALTERNATIVE PROCEDURE FOR LEAF SURFACE STUDIES WITH SCANNING ELECTRON MICROSCOPY

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Abstract

A scanning electron microscopy (SEM) preparation method that maintains the leaf surface in terms of ultrastructural morphology and contaminants as closely as possible to the *in vivo* state is described. Organic solvents used in critical point drying may cause severe alterations of the leaf surface. Cryo-methods are an expensive alternative and do not exclude the problem of artifacts. In this study, the water content of tobacco leaves was substituted with triethylene glycol without the leaf surface being contaminated in any way. This method excluded dehydration with organic solvents. Liquid substitution enabled the examination of uncoated and very thinly coated (5 nm) material with optimal results. This method was found to be superior for studies on leaf surfaces by SEM.

Key Words: Scanning and transmission electron microscopic preparation, triethylene glycol, epicuticular wax, leaf surface, uncoated specimens.

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Introduction

The interpretation of physiological processes related to surface phenomena in higher plants requires a comprehensive understanding of the physical and chemical properties of the cuticle, epicuticular wax, surface contaminants and trichomes. The leaf surfaces of plants have become of increasing taxonomic, agronomic and commercial significance. Knowledge of leaf surface properties including the wax deposits of *Nicotiana tabacum* L. have become increasingly important in our studies with regard to the determination of the characteristics of the leaf boundary layer in relation to water stress resistance properties. The antimalarial drug artemisinin, a sesquiterpenoid lactone, is produced by *Artemisia annua*, yet little is known about the development and structure of the gland in which it is assumed to accumulate (Duke and Paul, 1993). Some of the secondary products found in glandular trichomes of higher plants are of commercial importance. They provide the flavours of foliar-derived spices, i.e., oregano and essential oils used in flavouring, such as mint (Duke, 1994). From the literature, it is evident that the young stems and flowers of many species have considerable agronomic importance (Lazzaro and Thomson, 1989) because their viscous resinous exudate clogs machinery, hindering seed harvest. Trichome characteristics provide a wealth of taxonomically (Amarasinghe *et al*., 1991) useful information in all 13 taxa of *Eriodictyon* Bentam (Hydrophyllaceae), and may prove useful in the study of related genera of the Hydrophyllaceae (Hannan, 1988). A factor that influences the successful application of a herbicide to the foliage is the chemical and physical nature of epicuticular wax (Bitterlich and Upadhyaya, 1990). Literature cited show that the epicuticular wax may be influenced by temperature, light, humidity and rain. Important cuticular properties such as regulation of gas and water exchange and leaching, protection from environmental contaminants and absorption of foliar applied compounds (Baker, 1974; Reed and Turkey, 1982; Baker and Hunt, 1986; Osborn and Taylor, 1990) could therefore be affected.

To understand cuticular structure - function relations, it is important to characterize the cuticle physically and chemically at the ultrastructural level by using isolation

and preparation procedures that maintain the cuticle as closely as possible to the *in vivo* state. Waxes are a major cuticular component responsible for many cuticular properties (Martin and Juniper, 1970). In recent years, concern has been expressed over the widespread use of dehydration with organic solvents followed by critical point drying (CPD). Such concerns have revolved around the extraction of cellular components, shrinkage, the loss of extracellular components, and the possible safety hazards associated with operating these devices, which develop high pressures (Crang, 1988). CPD gives excellent results with many tissues, but the solvents used may destroy the epicuticular wax layer. Better results are often obtained if freshly mounted untreated or briefly coated (with gold in a sputtering device) tissues are examined with a low accelerating voltage that minimizes charging (Juniper and Jeffree, 1983). In order to prepare biological specimens that often contain 70-80% water for electron microscopy, it is necessary to dehydrate the material. Dehydration agents like ethanol and acetone are strong organic solvents, and they inevitably cause shrinkage and extraction of cellular constituents (Nei, 1974; Gabriel, 1982; Reed, 1982; Hayat, 1989). The low density of biological tissue results in a relatively large interaction volume of the beam with the tissue. As a result, electrons can be generated far from the point of impact, which decreases resolution. Coating the sample with a layer of heavy metal only partially alleviates this problem. However, coating the sample may change its surface characteristics and render the results of the scanning electron microscopy (SEM) examination inadmissible.

In the study of leaf surfaces, alternative methods that replace conventional preparation methods for SEM may limit artifacts imposed by dehydration and coating. In our study of the leaf surface of *N. tabacum*, we used the method described by Ensikat and Barthlott (1993). The underlying principle of this method is that certain liquids with a very low vapour pressure, such as glycerol or triethylene glycol, can be used to infiltrate biological specimens in order for it to be observed in the scanning electron microscope without drying. The added conductive properties of the substitution fluids allow specimens to be examined either uncoated or with very thin coatings (Ensikat and Barthlott, 1993).

The dual purpose of this paper is to evaluate/refine the triethylene glycol (TEG) method and to stress the need to strive to keep tissue samples as close to the *in vivo* state as possible when studying leaf surfaces. In this regard, it is important to determine the objective of the study and then select the best method. Examination of a specimen by light microscopy in its native state may be of use prior to SEM sample preparation so that preparation-induced artifacts can be recognized and avoided. In our study, the criteria of Bowers and Maser (1988) were used in assessing and judging the quality of fixation. This entailed comparing the morphology of living and fixed specimens and comparing the morphology of specimens fixed by different means. The surfaces of fresh tobacco leaves were studied with a Wild MZ 8 stereomicroscope and compared with the fixed leaf surfaces studied with a SEM. This monitoring procedure was conducted in order to allow identification of artifacts that could originate during SEM investigations of leaf surfaces.

Figure 1. The short glandular trichomes collapsed with primary fixation in 2% glutaraldehyde for 1 hour and post-fixation in 2% OsO_4 for 2 hours. Bar = 10 µm.

Figure 2. The short glandular trichomes were well preserved with primary fixation in 2% OsO_4 for 2 hours and when simultaneously fixed in 2% OsO_4 damp and 2% glutaraldehyde for 2 hours as post-fixation (Falk *et al*., 1971). Bar = 10 µm.

Figure 3. Epidermal cells and short glandular trichomes appeared collapsed with 2% OsO_4 for 1 hour as primary fixation when the scarred side of the leaf faced away from the fixative, whilst allowing the trichomes to float in the fixative. Bar = 10 µm.

Figure 4. The branched glandular trichomes were well preserved with 2% OsO_4 for 1 hour as primary fixation with the scarred side of the leaf either facing to or away from the fixative. Bar = 100 µm.

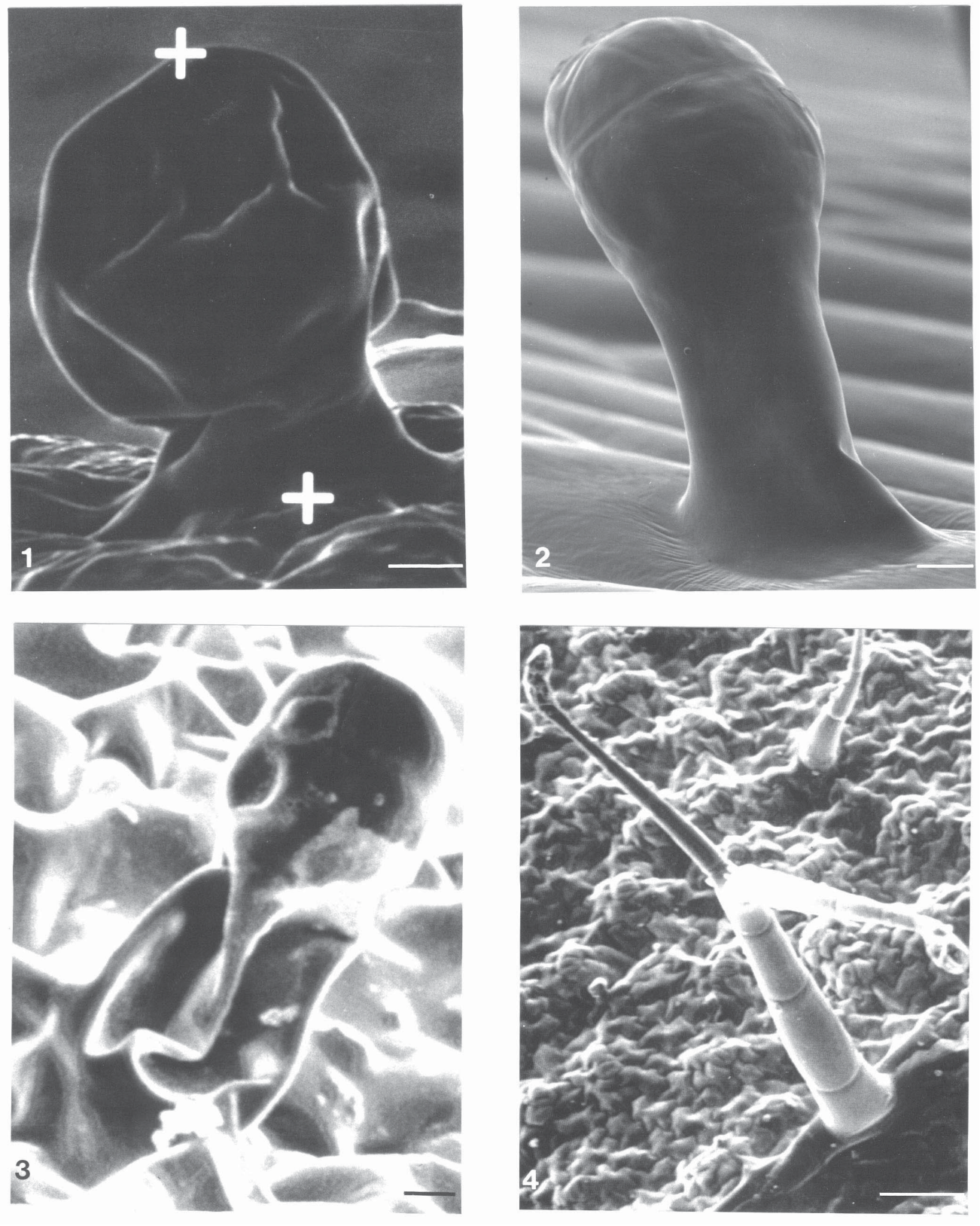
Materials and Methods

Plant material

Four cultivars of *Nicotiana tabacum* L., TL33, CDL28, GS46, and ELSOMA (in sequence of increasing drought tolerance) were used in this study. Seed was germinated in soil in pots. The young seedlings developed under glasshouse conditions with optimal water application. Before the onset of experimentation, the plants were moved to a growth room and allowed to acclimatize for a period of 98 hours. The growth chambers were set for 13 hours of daylight at 25°C followed by an 11 hour dark period at 16°C. Experimentation started when the plants were approximately 90 days old (for more details, see Van Rensburg *et al*., 1993).

Stereomicroscopy

The sixth youngest leaf was used as it represented a mature, non-senescent nearly fully expanded leaf (Van Rensburg *et al*., 1993). Samples of 0.25 cm^2 were cut from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the center of the lamina, lateral to the midrib. A Wild MZ 8 Leica stereomicroscope was used to study the morphology of the trichomes of tobacco. This investigation enabled recognition of artifacts caused by SEM preparation procedures.

Transmission electron microscopy

Samples of 1 mm^2 were cut from the sixth leaf in the same position as described above. The samples were fixed in 2% OsO_4 vapour for 3 hours (Falk *et al*., 1971) and post-fixed in 2% aqueous OsO_4 for 2 hours. After two rinse sessions of 3 minutes each with distilled water, the samples were dehydrated in two changes of 100% acetone for 15 minutes each, at room temperature. Infiltration was in 50% Spurr resin (Spurr, 1969) for 12 hours and twice in 100% Spurr for 8 hours each. Polymerization took place at 70°C for 12 hours. Sectioning was done on a Reichert-Jung (Vienna, Austria) Ultracut E microtome using glass knives. Thin sections were contrasted with 5% uranyl acetate for 20 minutes (Watson, 1958) and lead citrate for 20 minutes (Reynolds, 1963). The sections were observed and photographed in a Philips CM10 transmission electron microscope operated at an accelerating voltage of 100 kV (Philips Electronic Instruments, Mahwah, NJ).

Scanning electron microscopy

Critical point drying Two millimeter squares of leaf tissue were immersed in 1% OsO_4 (aqueous) fixative for 1 hour. After three rinses of 5 minutes each in 0.1 M cacodylate buffer at pH 7.2, the materials were post-fixed in 2.5% glutaraldehyde with 0.1 M cacodylate buffer. Dehydration was achieved by passage through an ethanol series of 30%, 50%, 70%, 90% and 100%, three times in each concentration for 5 minutes in each. The ethanol in the tissue was substituted with CO_2 over a period of 2 hours in the CPD-bomb before CPD. The tissue were mounted with carbon adhesive on stubs, coated with gold (~3 nm) and examined in the scanning electron microscope (JSM 840, JEOL, Tokyo, Japan).

Triethylene glycol (TEG) substitution Samples of 2 mm^2 were cut from the sixth leaf at the center of the lamina, lateral to the midrib. Either the adaxial or abaxial side of the leaf was scarified by scraping the epidermis and mesophyll with a razor blade. Stereomicroscopically it was revealed that the trichomes were not damaged during the process of removing the epidermis and mesophyll. Adaxial as well as the abaxial sides of the tobacco leaf samples were studied. Five fixation schedules were performed: (1) primary fixation in 2% glutaraldehyde for 1 hour and post-fixation in 2% OsO_4 for 2 hours, (2) primary fixation in 2% OsO_4 vapour for 2 hours with post-fixation in 2% glutaraldehyde for 2 hours (Falk *et al*., 1971), (3) primary fixation in 2% OsO_4 for 1 hour with (A) the scared side of the leaf floating on the fixative and with (B) the scarified side of the leaf facing away from the fixative, whilst allowing the trichomes to float in the fixative, (4) primary fixative in 4% glutaraldehyde for 1 hour and (5) primary fixation in 2% OsO_4 and 4% glutaraldehyde at 4°C for 1 hour and post-fixation in 2% OsO_4 for 1 hour (Franke *et al*., 1969). All fixatives were buffered with 0.1 M sodium cacodylate, pH 7.2. The scarified side of the leaf faced downward unless otherwise stated. After every fixation schedule, the samples were washed for 3 times (10 minutes each) in the buffer. No dehydration with either acetone or ethanol was used during this procedure. The water in the tissue was substituted with triethylene glycol ($C_5H_{14}O_4$) {Merck art. 808245, Darmstadt, Germany}, the properties of which are described in detail in the paper of Ensikat and Barthlott (1993). The scarified tissue was floated on an ascending series (increments of 10%) of aqueous solutions of TEG starting with a 10% concentration and ending with a concentration of 100%. The samples remained on each drop of a specific concentration in the series for 4 hours. The specimens were then removed from 100% TEG and placed on Whatmann filter paper to permit the drainage of excess TEG. The unscarified surface was not submerged in liquid at any time. Great care was taken to keep it dry at all times. Curved microforceps was used to transfer the sample from one drop to another without immersing it in any liquid. The scarified side of the leaf was glued to the stub with a carbon adhesive. Samples were viewed either uncoated or coated very thinly with gold. The specimens were investigated with a Jeol JSM 840.

Results

In a comparison of results for the five fixation schedules, the short glandular trichomes appeared collapsed (Fig. 1), while the long glandular trichomes and non-glandular trichomes were well preserved with fixation schedule 1. In contrast, all the branched glandular trichomes collapsed completely. With fixation schedule 2, the short glandular trichomes were well preserved (Fig. 2) with the four head cells discernible, and the curved stalk did not appear collapsed as was the case with schedule 1. An explanation for this improvement could be that OsO_4 stabilizes lipids by crosslinking or otherwise rendering them insoluble in organic solvents (Glauert, 1978; Bowers and Maser, 1988). For, although the exudate of the trichomes of tobacco is rich in lipids (Akers *et al*., 1978), and glutaraldehyde does not fix lipids (Bowers and Maser, 1988), all the trichomes including branched glandular trichomes, were well preserved. Branched glandular trichomes and long glandular trichomes were well preserved with fixation schedule 3A and B but the epidermal cells and short glandular trichomes appeared collapsed with schedule 3B opposed to schedule 3A (Figs. 3 and 4). Fixation with 4% glutaraldehyde for 1 hour (schedule 4) did not yield good results. Schedule 5

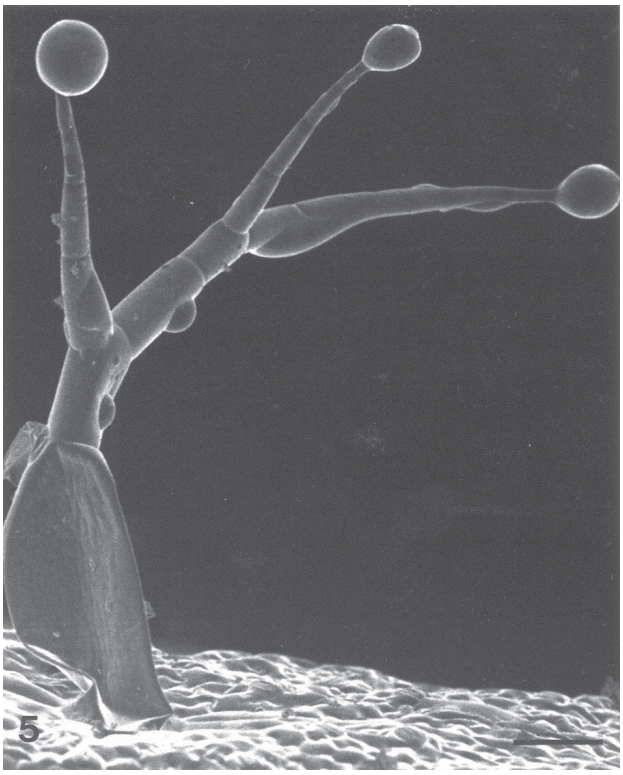

Figure 5. Long diffusion pathways might cause the collapse of base cells of long and branched glandular trichomes. Bar = 100 µm.

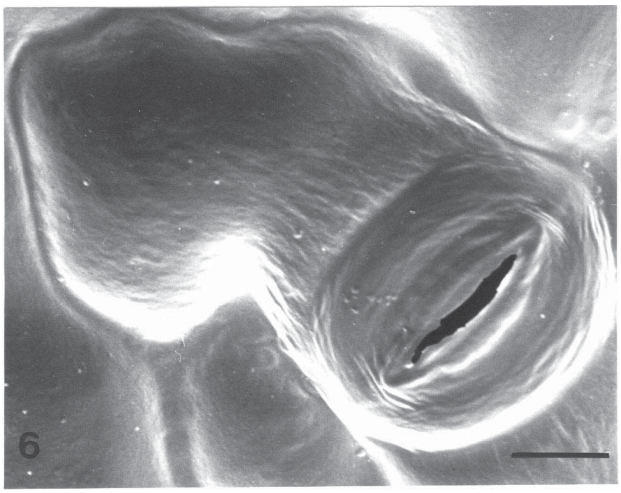

Figure 6. Stomata and epidermal cells were very well preserved with the TEG method. Bar = 10 µm.

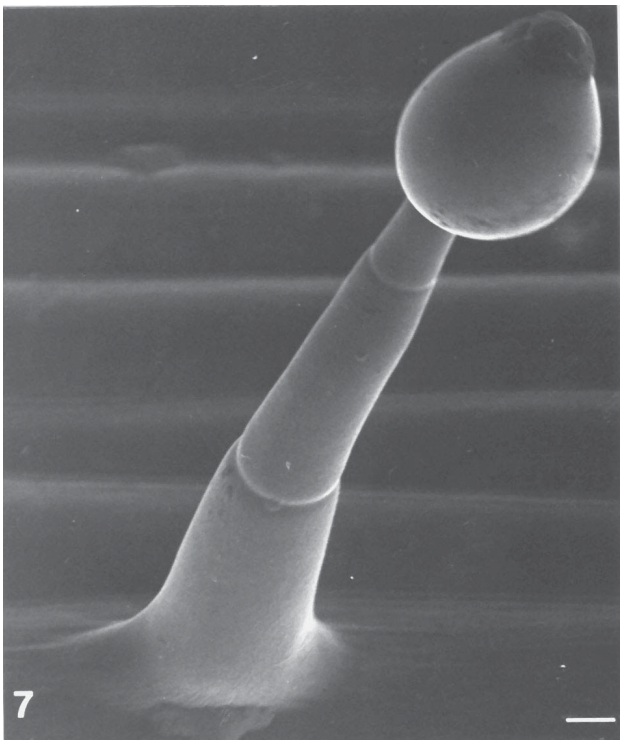

Figure 7. Long glandular trichomes were very well preserved with the TEG method. Bar = 10 µm.

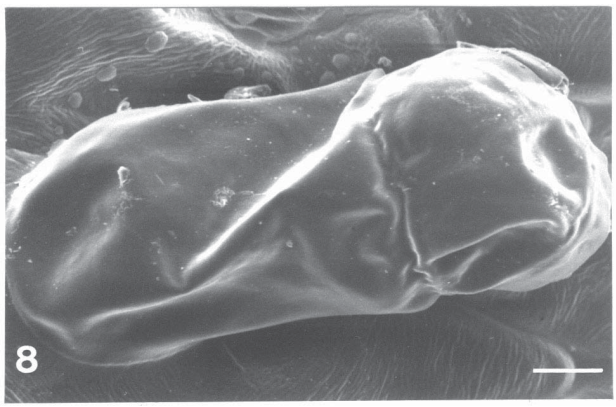

Figure 8. Dehydration with ethanol for CPD caused severe shrinkage of the short glandular trichomes. Bar = 10 µm.

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preserved the epidermal cells as well as all the trichome types very well.

The viscosity of triethylene glycol is 20.9 mPas at 293 K, which is considerably lower than that of glycerol (1499 mPas) (Ensikat and Barthlott, 1993). As the high viscosity of glycerol may result in shrinkage during dehydration, TEG was used in this study to improve infiltration. In our preparation of the leaf specimens, the adaxial or abaxial surface was scarified to improve infiltration, as

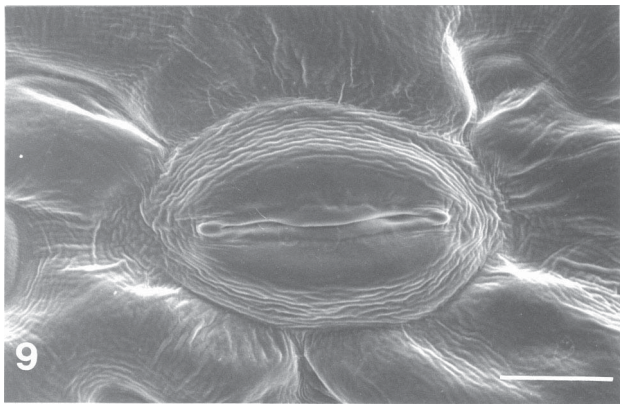

Figure 9. Dehydration with ethanol for CPD caused severe shrinkage of stomata and epidermal cells. Bar = 10 μm.

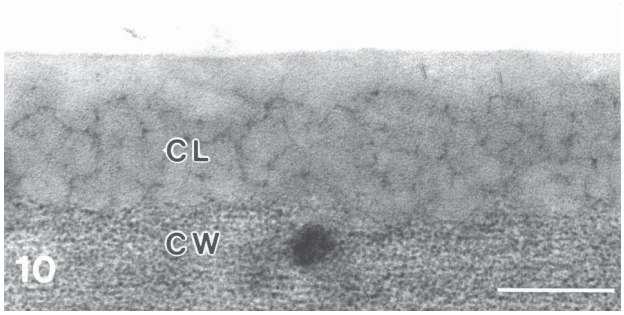

Figure 10. Reticulate cuticular layer (CL) and part of cellin wall (CW) of adaxial epidermis of *Nicotiana tabacum* L. with no epicuticular wax discernible. Bar = 0.1 μm.

suggested by Ensikat and Barthlott (1993), which gave optimal results. The branched and long glandular trichomes again posed special problems because of their length, (100 to 350 μm). This is in accordance with the comments of Ensikat and Barthlott (1993) that shrinkage and collapses are problems caused by long diffusion pathways. Some of the long and branched glandular trichomes collapsed at the base cell of the trichome (Fig. 5). With the TEG method, the amounts of dehydration steps were increased and concentrations decreased to 10% to avoid osmotic shock. Pease (1966) found the most critical period of dehydration to be during the intermediate stages of the alcohol series when the rate and kind of molecular exchange are of great importance. Success is thought to depend upon stabilization and immobilization the macromolecular systems before cytomembranes are damaged. Pease (1966) found the most effective agents to be ethylene glycol, glycerol and glucose syrup, the first of these being preferred.

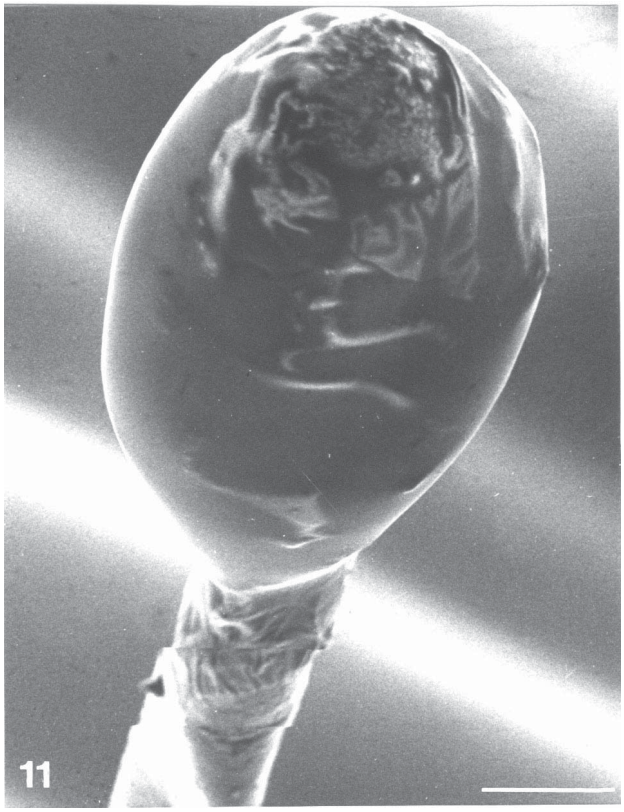

Figure 11. The multicellular head of a long glandular trichome was not discernible as exudate covered the head of the trichome, prepared with the TEG method. Bar = 10 μm.

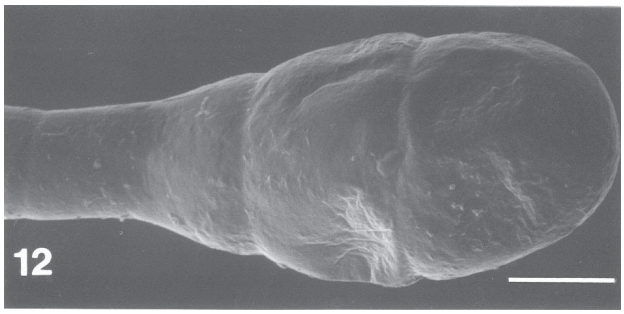

Figure 12. With the CPD method, the multicellular head of a long glandular trichome was discernible as the exudate was washed off and dissolved by ethanol. Bar = 10 μm.

Glycols, glycerol and glucose can all be fitted into the lattice structure of water, and need not seriously disturb the hydrophobic bonding of macromolecules (Pease, 1966). The TEG method therefore gives optimal results, as displayed

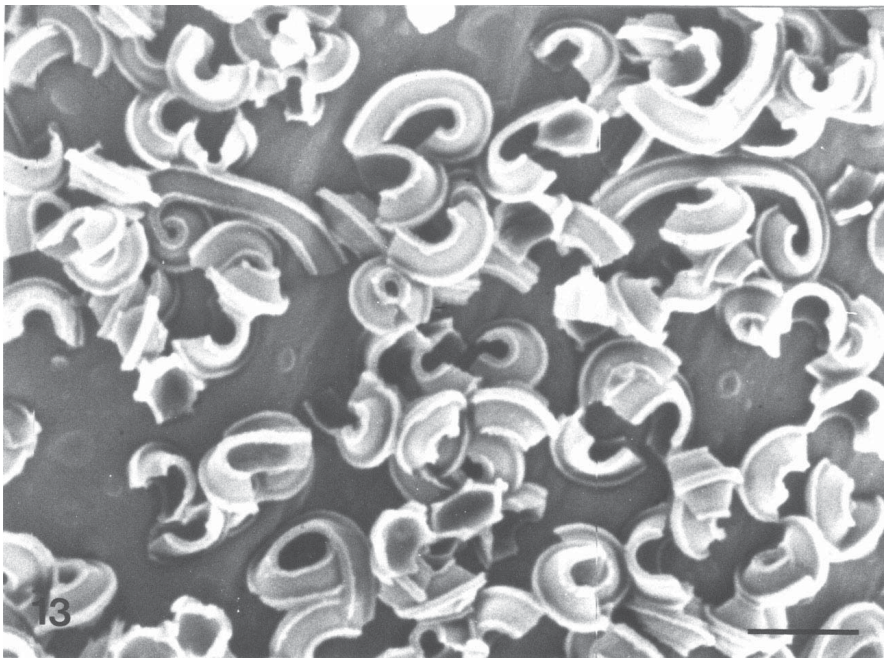

Figure 13. Wax particles of the *Bemesia tabai* whitefly found intact on the leaf surface after using the TEG method. Bar = 5 μm.

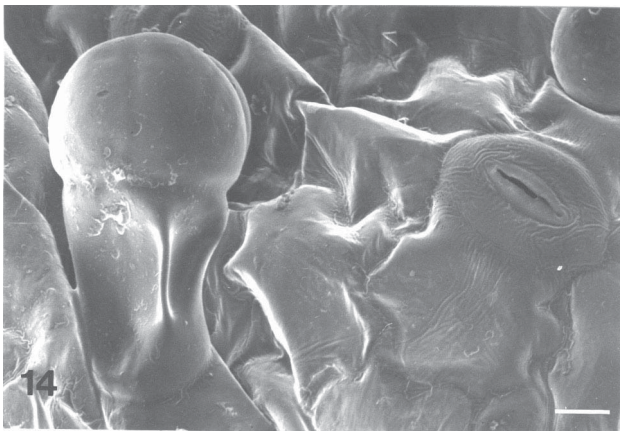

Figure 14. The disadvantage of the TEG method is the short time available to observe the specimens, as the tissue collapse under the high vacuum. Bar = 10 μm.

by the well-preserved epidermal cells, stomata, and trichomes of *N. tabacum* (Figs. 6 and 7). With the TEG method, no acetone or ethanol is used during the drying process of the specimen, which makes this method optimal for leaf surface studies.

Dehydration with organic solvents like acetone and ethanol before CPD cause shrinkage (Figs. 8 and 9) and extraction of surface and cuticular waxes. Reed (1982) showed that both 50% and 70% ethanol, methanol and acetone caused slight alternations of the epicuticular waxes at 0-4°C and 22°C, whereas 95% and 100% caused moderate changes at 0-4°C and severe alternations at 22°C. Approximately 18% of the cuticular wax was extracted with all dehydrants at both temperatures. Dykstra (1992) warns that if a refrigerated dehydration series is used, it should be recognized that samples will tend to develop increasing water contents after each exposure to room temperature, probably due to condensation of moisture from the air as it comes in contact with the cold solvent. Testimony of the fact that acetone and ethanol dissolve epicuticular wax can be seen in Figure 10, in which no epicuticular wax was discernible (also see Krüger *et al*., 1996). It should be noted that the Spurr medium could also have dissolved the wax, as specimens embedded in "Spurr" are heated up to 70°C, which is around the melting point of many of the waxes. Furthermore, even if the wax crystals remain intact, they will be hardly visible because they have no contrast against the embedding medium. This cross section of the cuticle of tobacco where no wax was discernible was dehydrated for 30 minutes in 100% acetone. This confirms that plant cuticles and epicuticular wax can easily be altered during the CPD procedure (Eveling and McCall, 1983; Sargent, 1983).

In Figure 11, a multicellular head of a glandular trichome covered with exudate, prepared with the TEG method, is shown. The multicellular head is not discernible, in fact, it was never seen with the TEG method. This can probably be ascribed to the presence of diterpenes, ∝ and β 4,8,13 - duvatriene - 1,3 - diol, as their biosyntheses have been observed, in intact glandular heads from trichomes of *N. tabacum* (Michie, 1968; Keene and Wagner, 1985). These

products, having some of the general properties of lipids, consist of a complex mixture of non-volatile terpenes with the result that dehydration with acetone or ethanol will extract a large percentage of these lipids and in doing so, alter the leaf surface of tobacco. However, in Figure 12, the multicellular head is clearly discernible, as all the exudate was dissolved by the ethanol during dehydration and CPD. Many homopteran insects are characterized by copious amounts of extracuticular wax that cover their bodies. In whiteflies, this wax is extruded from wax plates as a continuous ribbon, but is broken off as curly particles when the animals' hind tibiae pass over the plates. This wax forms tight curls of approximately 1 μm in diameter that are found not only on the insect but also on surrounding surfaces and these may be mistaken as leaf surface structures (Byrne and Bellows, 1991; Nelson *et al*., 1994). Sargent (1988) found the wax of the whitefly *Aleyrodes brassicae* on the leaf surface of cabbage. These wax particles were readily dislodged during specimen preparation and dissolved in the solvents used during CPD, but cryo-preservation retains them intact. *Bemesia tabai* is a whitefly found on tobacco. In our study, we found wax particles of *B. tabai* on the leaf surface of *N. tabacum* (Fig. 13). In accordance with the results of Sargent (1988), CPD dislodged and dissolved the wax. However, the TEG method preserved the wax just as well (Fig. 13) as the cryo-preservation method did.

The vast majority of dried biological specimens do not possess sufficient electrical and thermal conductivity for viewing with secondary electron imaging in the SEM. Metal and carbon deposits on the surface of the specimen are normally in the range of 10-25 nm thick (Crang, 1988). The inherent high electrical conductivity of TEG samples allows for the application of very thin metal coatings, enabling high-resolution imaging. In contrast, dried specimens require a thicker coating to prevent charging, particularly on rough surfaces, that may obliterate fine detail (Ensikat and Barthlott, 1993). In our study, the leaf surface was either not coated (Fig. 7) or very thinly coated (5 nm) with gold (Fig. 5). Leaf surface studies are normally done at low magnification, and thus the low accelerating voltage of 5 kV is in itself not a problem. We found a thinly coated leaf surface to give optimal results with an accelerating voltage of 3-5 kV. Metal coatings not only aid in thermal and electrical conductivity, but lend some mechanical stability to fragile biological specimens. A stronger secondary electron yield will be obtained from specimens coated with gold or other heavy metals, as it will result in an improved signal-to-noise-ratio in the SEM operation. In numerous cases, we found that working with an accelerating voltage between 12-20 kV enhanced charging, especially if the glandular trichomes were covered with lipids. The TEG sublime during examination and bleed-off excess charge, thus reducing specimen charging. The use of different metals like platinum/iridium/carbon in the correct ratio may produce coatings more finely grained than gold and may thus enhance the resolving power (Wepf *et al*., 1991). It is also important to note that the TEG method lends itself to uncoated material in the SEM, thus revealing the true structures.

Discussion

For specimens with a dry, unwetable surface, such as plant surfaces covered with epicuticular wax and trichomes, we observed, as also noted by Ensikat and Barthlott (1993), that the TEG substitution method displayed optimal results because the surface remains unaffected. This makes liquid substitution a simple and versatile procedure for SEM that offers an alternative to other preparation techniques such as critical point drying and cryo-methods (Ensikat and Barthlott, 1993). The advantages of the TEG method include the retention of lipids, waxes, loose particles and surface contaminants. The concept of liquid substitution by infiltration with glycerol or other fluids is by no means novel. Pease (1965, 1966, 1967) dehydrated rat tissue with glycol, glycerol and dextrose for TEM work. Mozingo *et al*. (1970) prepared the leaf surface of *Dionaea muscipula* Ellis for SEM using glycerol. Falk *et al*. (1971) used glycerol to infiltrate specimens in their comparative study of fixation methods. Panessa and Gennaro (1974) described a method of liquid substitution with 50% aqueous glycerol for intracellular observation of plant specimens for SEM. What makes the TEG method unique is that specimens are not dried, but the water is instead substituted for a liquid, triethylene glycol that evaporates very slowly under high vacuum.

When we compare the results obtained with this technique with others techniques we find that though critical point drying is a method favoured by many researchers and often gives superior results, this method may not always be suitable for leaf surface studies, in spite of its wide use (Akers *et al*., 1978; Werker and Kislev, 1978; Lyshede, 1980; Bosabalidis, 1990; Duke and Paul, 1993; Figueriedo and Pais, 1994). Although cryo-preservation preserved the leaf surface of broccoli very well (Eveling and McCall, 1983; Sargent, 1988), and the leaf surface and trichomes of *Nepeta racemosa* L. were very well preserved with cryo-preservation (Bourett *et al*., 1994), for many materials, the artifacts induced by CPD unfortunately outweigh those CPD was intended to avoid (Sargent, 1988). As reported in the study of Sargent (1983), CPD resulted in distortion of leaf epidermal cells and signs of abrasion were evident. As previously stated, cuticle preservation without damage to the surface is of paramount importance in leaf surface studies. Thus, a method that includes dehydration with ethanol or acetone is not suitable for leaf surface studies.

The literature is often inconclusive or contradictory

in regards to the relative merits of using either fresh, critical point dried or freeze-dried material when examining cuticular surfaces. Juniper and Jeffree (1983) examined fresh material briefly sputter coated with gold using a low accelerating voltage that minimizes charging. The major advantage of this method is that suitable specimens, such as leaves with a very impermeable cuticle, can be examined in their natural state, with or without metal coating. Furthermore, this preparation technique is very fast; the water content avoids charging; the contrasts of uncoated fresh material are similar to that of TEG- or glycerol-infiltrated specimens and differ from that of gold-coated material. But, the lower stability (against beam damage) makes high resolution work difficult or impossible. Bitterlich and Upadhyaya (1989) used fresh leaf material for examining epicuticular wax and trichomes, but the leaf surface appeared shrunken and showed high contrast; additionally, the long unbranched trichomes seemed to have collapsed as a result of the SEM vacuum. The dehydration in the vacuum of the SEM probably causes cells to collapse, but it is difficult to predict the susceptibility of a particular specimen to vacuum chamber-induced collapse. Another disadvantage of this method is that shrinkage cause continual movement of the specimens as they lose water to the vacuum, making it difficult to take micrographs. Freeze-drying preserved the form of the wax structures of Broccoli, but their orderly orientation was completely destroyed, making it impossible to recognize the outlines of underlying cells. This resulted from the shrinkage and distortion of the underlying epidermal cells that support the wax structures (Eveling and McCall, 1983; Sargent, 1983, 1988). However, it should be noted that Reed and Tukey (1982) reported no wax or tissue damage with freeze-drying for Brussels Sprouts and "White Sim" carnations. Freeze-substitution is effectively similar to dehydration in that shrinkage induced by extraction occurs (Gabriel, 1982). Most cryo-substitution is done using polar solvents such as acetone (which is the most widely used agent) for substitution (Harris, 1991). Freeze-substitution is therefore limited by the same problems, as dehydration followed by CPD is not optimal for leaf surface studies (Nei, 1974; Roos, 1991).

Freeze-fracture replication can give false impressions of plant surface microtopography (Holloway and Baker, 1974), and the actual process of fracturing can lead to production of furrows and steps that do not represent true morphological features (Stolinski and Breathnach, 1975). Specimens with a high degree of fine three-dimensional relief may not produce very satisfactory replicas, especially if the surface is normally covered with delicate structures such as hairs, bristles, cilia, etc. (Crang, 1988). Thus, freeze-fracture replication would not be suitable for tobacco where long trichomes cover the leaf surface. The objective of cryo-techniques is to maintain the three-dimensional structure of the specimen, solidify the soft tissue by freezing, and lessen the release of chemical substances (Nei, 1974). The most serious artifact of cryo-techniques is ice crystal formation. It is when these hexagonal ice crystals are formed in frozen biological specimens that severe structural damages take place. However, when cooling is rapid, small cubic ice crystals that cause little damage can be formed (Dubochet *et al.*, 1991).

High pressure freezing (HPF) preserves cells and tissues in a vitrified state and prevents ice crystal formation up to a depth of 500 μm (Stelzer and Lehmann, 1993). In literature cited by Ding *et al*. (1992), excellent freezing was achieved for all cell types of *N. tabacum*, including epidermal cells, vascular cells and mesophyll cells prepared with high pressure freezing and propane-jet freezing. However, when material frozen with a propane-jet freezer were compared with those frozen with high-pressure, microfilament bundles of the material often had a frayed or loosened appearance. Ding *et al*. (1992) suggest that such an appearance of the microfilament bundles of the high-pressure frozen material could be an artifact, presumably caused by the high pressure developed prior to freezing.

With high-pressure freezing, plunge-freezing and propane-jet freezing the specimen is mounted on one of two metal planchets or sheets. In high-pressure freezing, it helps to reduce the risk of the sample being crushed under the high pressure applied, and in the propane-jet freezing, it protects the surface of samples from the high velocity propane jet. In all three techniques, the sandwich eliminates the self-defeating transfer of thermal energy within a relatively massive heat reservoir caused by the bulky, solid supports to the cooling specimen. Some of this energy must exit through the specimen and must impair its cooling. Specimens cool faster when isolated from the relatively large thermal mass of solid support (Ryan, 1992). The long trichomes and delicate cuticle of tobacco may impose problems when sandwiched between two sheets - this has yet to be addressed. With slam-freezing (Lemke *et al*., 1994), the specimen is slammed on a pre-cooled metal block; this in itself may damage surface structures like trichomes (Harris, 1991). Consequently, this technique is at present also sub-optimal for leaf surface studies.

The major disadvantage of the TEG method is the short time available to observe some specimens as tissue collapses because of the high vacuum (Fig. 14). It is advisable, therefore, not to put all the material in the SEM at once, as the observation time allowed depending on the vacuum varies between 15 minutes to 5 hours. Therefore, as for fresh material and cryo-preserved specimens, TEG specimens unfortunately cannot be retained for future observations.

Conclusions

Liquid substitution offers a viable alternative that could be further developed to constitute an important method for the preparation of leaf surface material for SEM, as the surface stays unaltered. We are of the opinion that it is a simple, inexpensive and versatile method with great potential. Cryo-preservation is an alternative for optimal studies on the leaf surface but is expensive with the possible artifact problem not entirely ruled out. CPD has the disadvantage of dehydration that alters the leaf surface and is thus not recommended for leaf surface studies. However, it should be stated that CPD preparation may be of help with the interpretation of the features not revealed by the TEG method. With the CPD method, exudate was removed which made the multicellular head of the long glandular trichomes discernible. In our opinion, cryo-preservation technique and the TEG method fall within the same class of superiority in terms of the retention of surface features.

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Discussion with Reviewers

G.M. Roomans: It is surprising that the environmental scanning electron microscope is not mentioned in this paper. How does this compare to the liquid substitution technique?
Authors: We are conducting a comprehensive study in this regard, and it will be the subject of another paper.

G.M. Roomans: Did you try to use an accelerating voltage

lower than 3 kV?
Authors: We were not successful at accelerating voltages lower than 3 kV.