

**ROLE OF BOUNDARY LAYER RESISTANCE AND WALL ULTRASTRUCTURE IN
DETERMINING DIFFERENTIAL DROUGHT TOLERANCE IN TOBACCO.**

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adnatus valere

**Dedicated to my sister Lorraine
and
my friend Sheila**

ABSTRACT

ROLE OF BOUNDARY LAYER RESISTANCE AND WALL ULTRASTRUCTURE IN DETERMINING DIFFERENTIAL DROUGHT TOLERANCE IN TOBACCO.

A holistic approach was followed to propose a new model for the leaf surface of tobacco to explain epidermal wall ultrastructure and permeability with the emphasis on drought stress. *Nicotiana tabacum* L. leaves of two drought sensitive and two drought tolerant cultivars were used.

A triethylene glycol liquid substitution method together with a Flexible Image Process System was found excellent for studying the external micro-morphology of the leaf surface. Differences were found to exist between the leaf micro-morphology parameters quantified for the drought sensitive and drought tolerant cultivars. From these results, it is concluded that plant fitness for tolerating drought stress may be maximised by the carbon gaining capacity of the leaf.

Contrary to most permeability studies, freshly harvested epidermal wall strips, with stomata and trichomes, were used. Results are discussed in terms of the limitations when using composite membranes with stomata and trichomes. The differential permeability coefficient values calculated for the transport of water in tobacco epidermal wall strips agreed with those reported for isolated cuticular membranes of various other species. The epidermal wall permeability coefficient seems to have merit as selection criterion for identifying drought tolerant genotypes.

The cuticular membrane is not an amorphous layer but consists of small globules (referred to as nitis globules) which group together in the larger globules (referred to as nichrista globules). The tobacco epidermal cell wall has five layers consisting of a matrix and microfibrils with diameters of 4 – 10 nm. The fibrils of the cuticular membrane are possibly a pathway for water, wax and cutin transport. The possibility of resistance factors, other than the soluble cuticular lipids, contributing to water permeability during drought stress, was investigated.

OPSOMMING

DIE ROL VAN GRENSLAAGWEERSTAND EN SELWAND-ULTRASTRUKTUUR BY DIE BEPALING VAN DIFFERENSIËLE DROOGTE VERDRAAGSAAMHEID IN TABAK

'n Holistiese benadering is gevolg om 'n nuwe model voor te stel om die ultrastruktuur en permeabiliteit van die epidermisselwand van die blaaroppervlak van tabak te verklaar met die klem op vogstremming. Twee droogtesensitiewe en twee droogteverdraagsame *Nicotiana tabacum* L. kultivars se blare is gebruik.

'n Tri-etileenglikol vloeistofuitruilmethode is saam met die 'Flexible Image Process System' gebruik en het uitstekende resultate gelewer vir die bestudering van die mikromorfologie vir die buite oppervlak van die blaar. Verskille is gevind in die blaar mikro-morfologie parameters wat gekwantifiseer is vir die droogtesensitiewe en droogteverdraagsame kultivars. Uit dié resultate word tot die gevolgtrekking gekom dat koolstofopname kapasiteit van die blaar, die plant se vermoë om droogte te verdra, optimaliseer.

In teenstelling met meeste ander permeabiliteitsstudies, is strokies vars epidermiswand met huidmondjies en trigome gebruik. Die beperkings wat deur die gebruik van saamgestelde membrane met huidmondjies en trigome op resultate geplaas word, word bespreek. Die differensiële permeabiliteitskoeffisiënt wat op hierdie wyse bereken is vir die vervoer van water deur die tabak epidermiswand strokies, vergelyk goed met die resultate verkry met gebruik van geïsoleerde kutikulamembrane van verskeie ander spesies. Die epidermiswand permeabiliteitskoeffisiënt kan moontlik as kriterium gebruik word om droogteverdraagsame genotipes te identifiseer.

Die kutikulamembraan is nie 'n amorfiese laag nie, maar bestaan uit klein globule (hier nitis globule genoem) wat saam gegroepeer is binne die groter globules (hier nichrista globule genoem). Die tabak epidermisselwand het vyf lae wat uit 'n matrys en mikrofibrille, wat 'n deursnee van 4 – 10 nm het, bestaan. Die fibrille van die kutikulêre membraan is moontlik die weë waardeur water, was en kutien vervoer word. Die moontlikheid dat ander faktore, behalwe oplosbare kutikulêre lipiede bydra tot permeabiliteitsweerstand gedurende vogstremming, is ondersoek.

Keywords: boundary layer, cell wall, cuticle, cuticle ontogeny, FE-SEM, permeability, wax

Sleutelwoorden: grenslaag, FE-SEM, kutikula, kutikula ontogenie, permeabiliteit, selwand, was

LIST OF ABBREVIATIONS

α	Stomatal aspect ratio
a	Semi-major axis
A_m	Exposed membrane area
A_w	Total area of water-filled pores
$A_w/\Delta\chi$	Pore area per unit diffusion path
A_w/A_m	Fractional pore epidermal membranes
β	Stomatal spacing ratio
b	Semi-minor axis
$\Delta\chi$	Thickness of the membrane
C_{don}	Donor concentration
CL	Cuticular layer
CM	Cuticular membrane
CP	Cuticle proper
CPD	Critical point dried
C_{rec}	Receiver concentration
CW	Cell wall
D	Diffusion coefficient
DMSO	Dimethylsulfoxid
ECW	Epicuticular wax
F	Flow rate
FE-SEM	Field emission scanning electron microscope
FIPS	Flexible Image Processing System
HPF	High pressure freezing
l_c	Boundary layer thickness
l_d	Increased boundary layer thickness
L_{eaf}	Mean length of the leaf in downwind direction
LM	Light microscope
LN_2	Liquid nitrogen
L_p	Nondimensional pore depth
LTP	Lipid transfer protein
MIP	Major intrinsic protein

N	Number of stomata per leaf area
P	Permeability coefficient
P_d	Differential permeability coefficient
PL	Pectin layer
PVP	Polyvinylpyrrolidone
R_a	Unstirred boundary layer resistance
r_c	Hypothetical radius
R_{cav}	Cavity resistance
R_e	End effect correction
R_{ent}	Entry diffusion resistance
R_{st}	Stomatal pore resistance
SCL	Soluble intracuticular lipids
SEM	Scanning electron microscope
T	Nondimensional boundary layer thickness
t_e	Hold-up time
TEG	Triethylene glycol
TEM	Transmission electron microscope
THO	Titrated water
V_{air}	Ambient air speed

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CHAPTER ONE
INTRODUCTION

INTRODUCTION

Drought stress is one of the most important limiting factors in agriculture in South Africa and elsewhere in the world. Sustainable agriculture is the only solution to feed an ever-increasing population in an environment that is constantly being degraded by human interference. The dwindling water resources as well as the poor quality of the available water in those parts of the world with the highest population growth, are becoming critical.

Although genetic engineering may supply solutions to the problem of establishing sustainable food production by producing stress tolerant crop plants, the morphological, physiological and biochemical concepts underlying stress tolerance in plants are still not clearly understood. This information is vital if stress tolerant varieties are to be selected for establishment in agriculture.

The literal meaning of the word “stress” (derived from the Latin *stringere*) is a constraining or impelling force (Larcher 1995). But what is stress? Larcher (1995) defined stress as a significant deviation from the optimal conditions for life, and that elicits changes and responses at all functional levels of the organism, which, although at first reversible, may become permanent. Even if the stressful event is temporary, the vitality of the plant is progressively reduced the longer the stress is maintained. Stress due to drought, in contrast to many other stressful events, does not occur abruptly, but develops slowly and increases in intensity over time (Larcher 1995). The influence of drought stress on different plant processes is discussed by Hsiao (1973).

Drought stress is manifested in almost every aspect of a plant's growth, including anatomical, morphological, physiological and biochemical properties (Tomati & Galli 1979). In the past, cuticular resistance to water loss was assumed to be constant (Parkhurst 1986) or negligible (Cowan 1982), but was found to increase reversibly during the day (Meidner 1986) and under soil water deficit conditions (Svenningsson & Liljenberg 1986). The cuticular permeability of most plant leaves is low in comparison to stomatal conductance and hence most transpiration occurs *via* the stomatal pathway. There are occasions, however, when cuticular transpiration takes place under drought conditions when stomata are supposedly closed. Thus, Larsson and Svenningsson (1986) concluded that cuticular transpiration *per se* could be useful as a selection criterion when breeding for drought tolerance.

Consequently, in this study a holistic approach was followed to propose a new model for the leaf surface of tobacco with the aid of epidermal wall ultrastructure and permeability data. In the past, research on epidermal wall permeability focused on the cuticular membrane permeability, while cell wall permeability was largely considered negligible (Larsson & Svenningsson 1986, Meidner 1986, Geyer & Schönherr 1990, Chamel *et al.* 1991). Permeability studies are not often correlated with the ultrastructure of the cuticular membrane and the cell wall. It is therefore important to determine exactly what the resistance factors are in the water permeability of the leaf surface, from the leaf to the environment, as well as to obtain some clarity with regard to the role of the cuticular wax in the resistance pathway. The results will not necessarily be directly extrapolatable to typifying other plant species, as the epidermal wall is a complex heterogeneous system. This system may, however, be related to those plant species that Holloway (1982) classified as Type 1 for cuticular membrane ultrastructure, as there may be some similarities. Freshly harvested stomatous epidermal wall strips with trichomes were used in this study. Kerstiens (1996) stated that no reliable method exists for the determination of cuticular water permeability of stomata-bearing leaf surfaces. The primary reason for this is that the presence of stomata makes the system of leaf conductance more complex. This causes considerable uncertainty as to the fractional contribution of cuticular water loss to minimum leaf conductance. The latter may include a very substantial contribution from imperfectly closed stomata, particularly in intact leaves in the field (Kerstiens 1996). It is important to distinguish between cuticular conductance and minimum leaf conductance, especially in leaves that do have stomata. The necessity to treat the leaf surface as a holistic system that interacts with the environment becomes apparent when the statement made by Kerstiens (1996) is taken into account, namely that: ‘cuticular transpiration may interact with stomatal response to water vapour pressure difference by affecting water relations within the epidermis, but the relationship between local gradients of water potential and stomatal aperture becomes fundamentally different when transpiration rates approach the maximum a leaf can sustain’.

The bilayered model for the cuticle as described in 1847 by Von Mohl (Jeffree 1996) has been the accepted model to the present. The general model for the mature tobacco leaf, classified as having a Type 1 cuticular membrane is shown in Fig. 1. This general epidermal wall model includes an epicuticular wax layer that forms a thin layer on the cuticle (Fig. 1). The cuticular membrane consists of a lamellate cuticle proper and a reticulate cuticular layer (Fig. 1). The cuticle proper consists of electron-dense and electron-lucent lamellae while the cuticular layer has fibrils that extend from the cell wall into the cuticle (Fig. 1). It is believed that the electron-

lucent lamellae contain soluble cuticular lipids and that these lipids are the ultimate barrier to water permeability within the cuticle. The cell wall model taken from the TEM work does not give much information on the ultrastructure as it only reveals an amorphous layer with granules (Fig. 1). Some models include a pectin layer between the cuticular membrane and the cell wall and some do not. Models that focus on the cuticle seldom, if ever, include detail of the cell wall such as protein and hemicellulose. The cell wall models are usually devoid of detail regarding cuticle ultrastructure. The plasma membrane is included in some models.

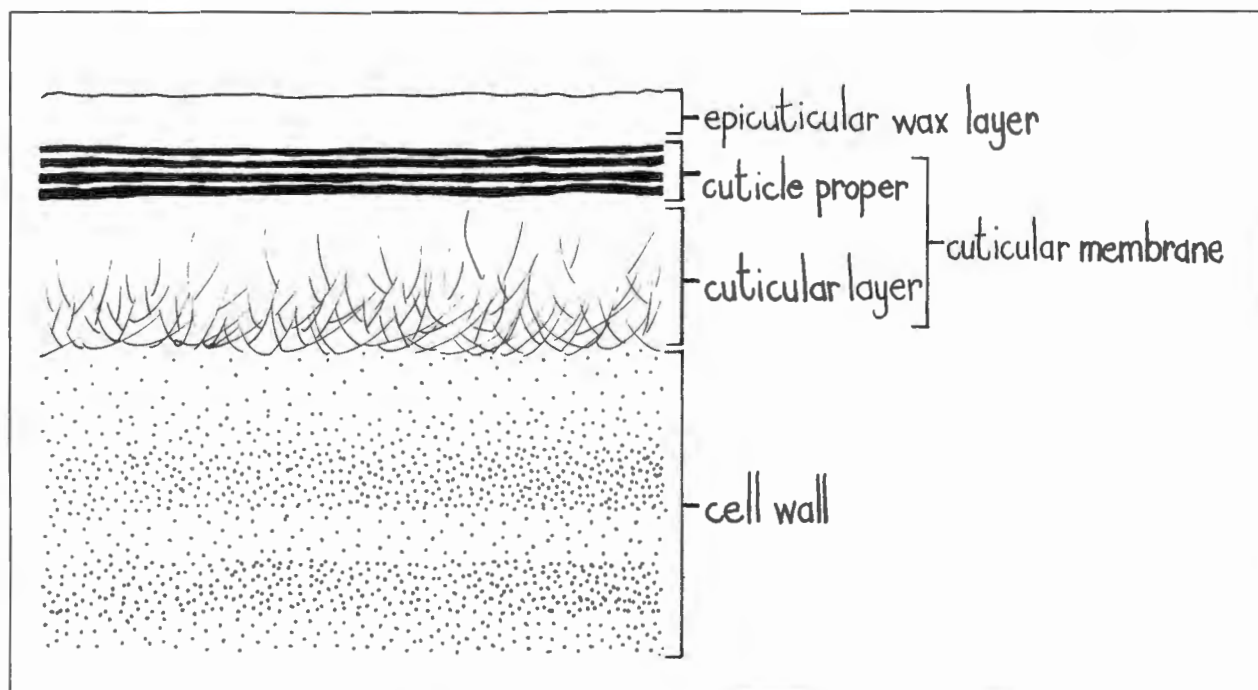


Fig. 1 A generalised model of a mature leaf epidermal wall. Epi-cuticular wax forms a layer on the bilayered cuticular membrane, including the cuticle proper and the cuticular layer. The cuticle proper is lamellate and the cuticular layer reticulate. The cuticular membrane and the primary cell wall constitute the epidermal wall (compiled from Holloway (1982), Jeffree (1996), Krüger *et al.* (1996) and Lyshede (1982)).

The main aim of this study was to elucidate the role of leaf surface permeability and ultrastructure as it relates to drought stress. A second aim was to identify whether leaf surface permeability has merit as selection criterion for identifying drought tolerant genotypes. Two drought sensitive and two drought tolerant cultivars of *Nicotiana tabacum* L. leaves was used in this study. In sequence of increasing drought tolerance, the four cultivars under investigation were TL33, CDL28, GS46 and Elsoma. These cultivars were selected on the bases of their differing performances in the field during drought-stress periods (Van Rensburg & Krüger 1993). Physiological and anatomical changes was investigated in tobacco, focusing on these

four cultivars and emphasising their differences in drought tolerance (Van Rensburg & Krüger 1993, Van Rensburg *et al.* 1993, Van Rensburg & Krüger 1994).

Three aspects of the leaf surface of tobacco namely the external micro-morphology, epidermal wall permeability and ultrastructure were studied. Firstly, the external micro-morphology of the tobacco leaf surface was quantified. The role of the boundary layer, along with the stomatal geometry and spacing, in determining the diffusional resistance to CO₂ and H₂O in the four cultivars were investigated. The trichome type, density and dimensions were determined as they determine the dimensions of the boundary layer, possibly the final resistance barrier to water loss. Secondly, the permeability of the epidermal wall was quantified, determining whether the permeability rate differed between the four cultivars. Freshly harvested stomatous epidermal wall strips, which included trichomes, were used. Thirdly, the ultrastructure of the epidermal wall and the cuticle ontogeny were investigated with the aid of electron microscopy. These results were evaluated to determine the possible pathway and resistance barriers of water through the epidermal wall. The epidermal wall permeability was also evaluated as a drought tolerance selection criterion. From this information a new leaf surface model for *Nicotiana tabacum* L. was constructed.

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CHAPTER TWO

LIQUID SUBSTITUTION: AN ALTERNATIVE PROCEDURE FOR LEAF SURFACE STUDIES WITH SEM

Scanning Microscopy 2000 (in press)

ABSTRACT

A SEM preparation method, which 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 CPD may cause severe alterations of the leaf surface. Cryo-methods are expensive alternatives 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 tobacco leaf surfaces for SEM.

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 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* L., yet little is known about the development and structure of the gland in which it is assumed to accumulate (Duke & Paul 1993). Some of the secondary products found in glandular trichomes of higher plants are of commercial importance and provide the flavours of foliar-derived spices, i.e. oregano and essential oils used in flavouring, for example various mints (Duke 1994). From the literature it is evident that the young stems and flowers of many species have considerable agronomic importance (Lazzaro & 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 & Upadhyaya 1990). Literature cited show that temperature, light, humidity and rain may influence the epicuticular wax. Important cuticular properties such as regulation of gas and water exchange and leaching, protection from environmental contaminants and absorption of foliar applied compounds (Reed & Tukey 1982, Baker & Hunt 1986, Baker 1974, Osborn & Taylor 1990) could therefore be affected.

To understand cuticular structure-function relations, it is important to characterise 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 & 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 minimises charging (Juniper & 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 (Hayat 1989, Reed 1982, Gabriel 1982, Nei 1974). 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 SEM examination inadmissible.

For the study of leaf surfaces alternative methods that replace conventional preparation methods for scanning electron microscopy (SEM) may prove to limit artefacts imposed by dehydration and coating. In our study of the leaf surface of *Nicotiana tabacum* L. we used the method as 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 & Barthlott 1993).

The dual purpose of this paper is to evaluate the triethylene glycol (TEG) method and to stress the necessity 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 artefacts can be recognised 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 as to be able to identify artefacts that could originate during SEM investigations of leaf surfaces.

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 acclimatise for a period of 98 h. The growth chambers were set for 13 hours daylength at 25°C followed by 11 hours dark period at 16°C. Experimentation started when the plants were approximately 90 days old (for more detail 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 cm² were cut from the centre 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 was conducted to be able to recognise artefacts caused by SEM preparation procedures.

Transmission electron microscopy

Samples of 1 mm² were cut from the sixth leaf in the same position as described above. The samples were fixed in 2% OsO₄ vapour for 3 h (Falk *et al.* 1971) and post-fixed in 2% aqueous OsO₄ for 2 h. After two rinse sessions of 3 min each with distilled water, the samples were dehydrated in two changes of 100% acetone for 15 min each, at room temperature. Infiltration was in 50% resin (Spurr 1969) for 12 h and twice in 100% resin (Spurr 1969) for 8 h each. Polymerisation took place at 70°C for 12 h. Sectioning was done on a Reichert-Jung Ultracut E microtome using glass knives. Thin sections were contrasted with 5% uranyl acetate for 20 min (Watson 1958) and lead citrate for 20 min (Reynolds 1963). The sections were observed and photographed in a Philips CM10 transmission electron microscope operated at 100 kV.

Scanning Electron Microscopy

Critical point drying

Two millimeter squares of leaf tissue were immersed in 1% aqueous OsO₄ fixative for 1 h. After three rinses of 5 min 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% 100%, three times in each concentration for 5 min in each. The ethanol in the tissue was substituted with CO₂ over a period of 2 h in the CPD-bomb before CPD. The tissue was mounted with carbon adhesive on stubs, coated with gold (ca. 3 nm) and examined in the SEM (JEOL JSM 840).

Triethylene glycol (TEG) substitution

Samples of 2 mm² were cut from the sixth leaf at the centre 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. Stereo microscopically 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 h and post-fixation in 2% OsO₄ for 2 h, (2) primary fixation in 2% OsO₄ vapour for 2 h with post-fixation in 2% glutaraldehyde for 2 h (Falk *et al.* 1971), (3) primary fixation in 2% OsO₄ for 1 h with (a) the scarred side of the leaf floating on the fixative (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 h and (5) primary fixation in 2% OsO₄ and 4% glutaraldehyde at 4°C for 1 h and post-fixation in 2% OsO₄ for 1 h (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 three times (10 min 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 (C₅H₁₄O₄) [Merck art. 808245], 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 final concentration of 100%. The samples remained on each drop of a specific concentration in the series for 4 h. The specimens were then removed from

100% TEG and placed on Whatman® 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 were 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

When comparing the results of 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 stabilises lipids by crosslinking or otherwise rendering them insoluble in organic solvents (Glauert 1978, Bowers & Maser 1988), as, although the exudate of the trichomes of tobacco is rich in lipids (Akers *et al.* 1978), and glutaraldehyde does not fix lipids (Bowers & 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 (Fig. 3 and Fig. 4). Fixation with 4% glutaraldehyde for 1 h (schedule 4) did not yield good results. Schedule 5 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 & 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 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 collapsing of tissue are problems caused by long diffusion pathways. Some of the long and branched glandular trichomes collapsed at the basal cell of the trichome (Fig. 5). With

the TEG method the number 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 stabilisation and immobilisation the macromolecular systems before cytomembranes are damaged. Pease (1966) found the most effective agents to be ethylene glycol, glycerol and glucose syrup, and the first of these being preferred. Glycol, 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 by the well-preserved epidermal cells, stomata, and trichomes of *N. tabacum* (Fig. 6 and Fig. 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 such as acetone and ethanol before CPD cause shrinkage (Fig. 8 and Fig. 9) and extraction of surface and cuticular waxes. Cross section of the cuticle of tobacco where no wax was discernible was dehydrated for 30 min in 100% acetone. Reed (1982) showed that both 50 and 70% ethanol, methanol and acetone caused slight alterations of the epicuticular waxes at 0 - 4°C and 22°C, whereas 95 and 100% caused moderate changes at 0 - 4°C and severe alterations at 22°C. Approximately 18% of the cuticular wax was extracted with all dehydrants at both temperatures. Dykstra (1992) warned that if a refrigerated dehydration series is used, it should be recognised 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 Fig. 10 in which no epicuticular wax was discernible (also see Krüger *et al.* 1996). It should be noted that the resin medium (Spurr 1969) could also have dissolved the wax, as specimens embedded in resin are heated up to 70°C which is about 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 confirms that plant cuticles and epicuticular wax can easily be altered during the CPD procedure (refer also to Eveling & McCall 1983, Sargent 1983).

In Fig. 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, α and β 4,8,13 -

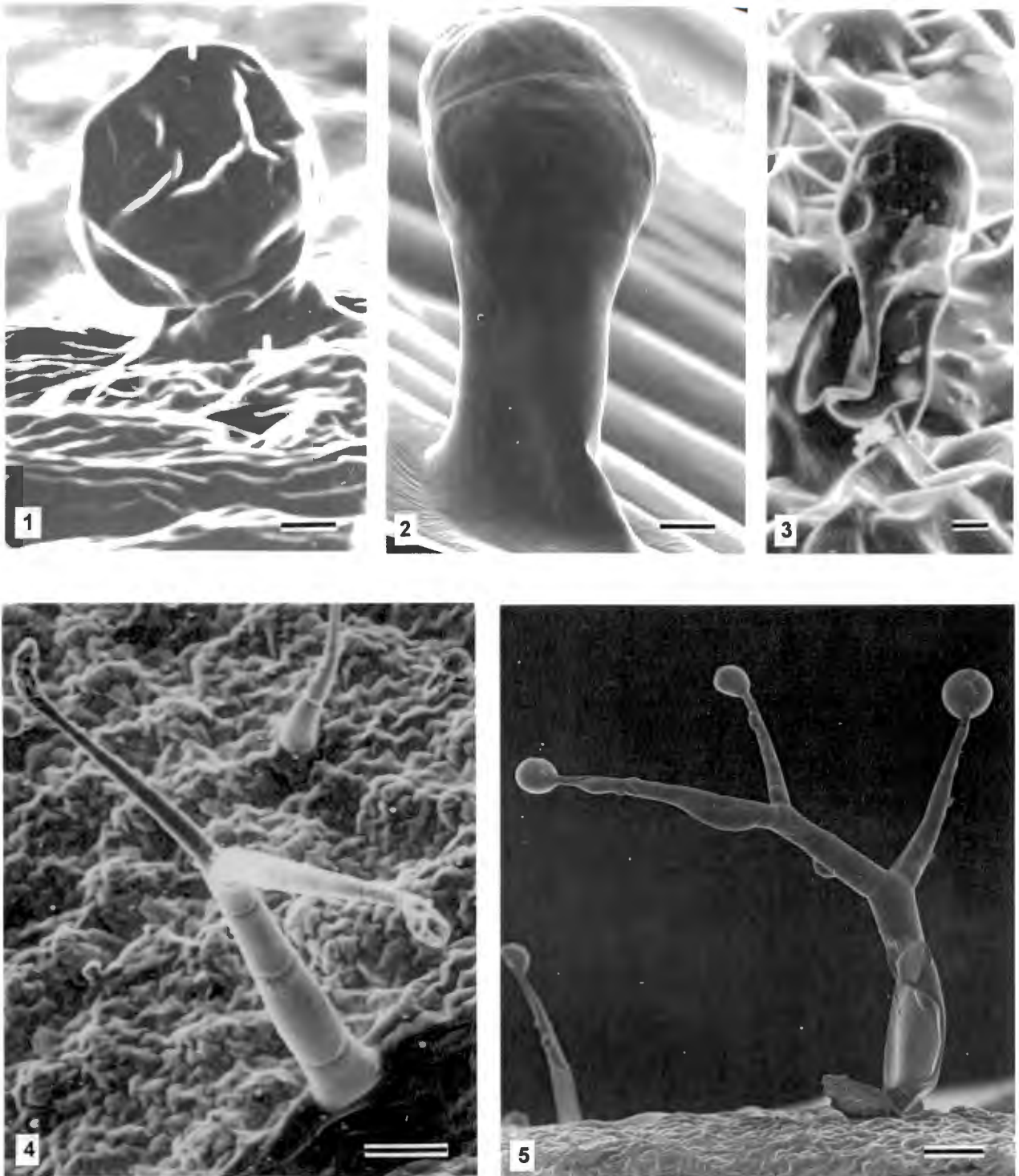


Fig. 1. The short glandular trichomes collapsed with primary fixation in 2% glutaraldehyde for 1 h and post-fixation in 2% OsO_4 for 2 h. Bar = 10 μm

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

Fig. 3. Epidermal cells and short glandular trichomes appeared collapsed with 2% OsO_4 for 1 h 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

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

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

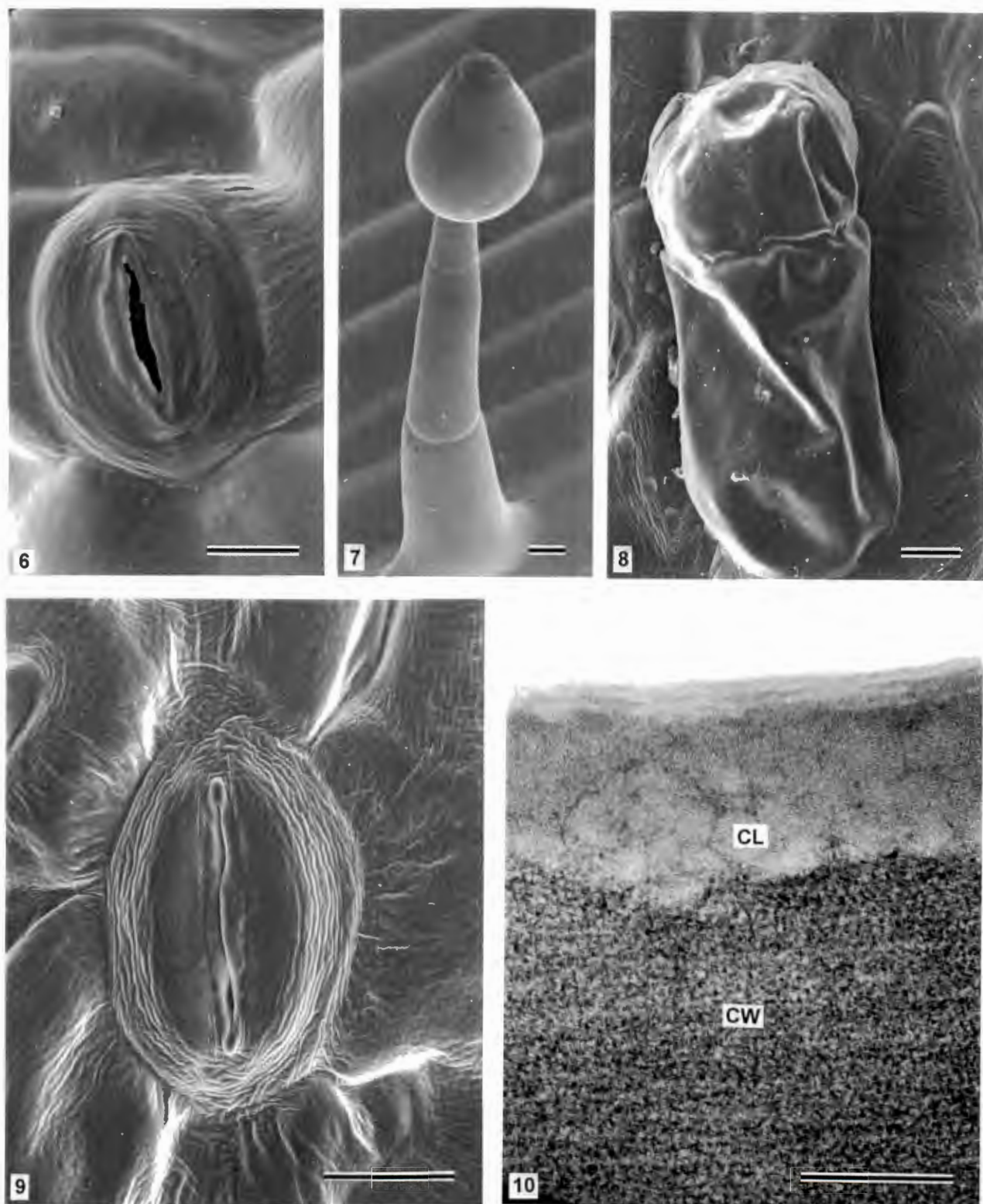


Fig. 6. Stomata and epidermal cells were very well preserved with the TEG method. Bar = 10 μ m

Fig. 7. Long glandular trichomes were very well preserved with the TEG method. Bar = 10 μ m

Fig. 8. Dehydration with ethanol followed by CPD caused severe shrinkage of the short glandular trichomes. Bar = 10 μ m

Fig. 9. Dehydration with ethanol followed by CPD caused severe shrinkage of stomata and epidermal cells. Bar = 10 μ m

Fig. 10. Reticulate cuticular layer (CL) and part of cell wall (CW) of adaxial epidermis of *Nicotiana tabacum* L. with no epicuticular wax discernible. Bar = 0.1 μ m

duvatriene - 1,3 - diol, as their biosynthesis have been observed, in intact glandular heads from trichomes of *N. tabacum* (Michie 1968, Keene & 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 so-doing alter the leaf surface of tobacco. However, in Fig. 12 the multicellular head is clearly discernible, as all the exudate was dissolved by the ethanol during dehydration and CPD. Many homopteran insects are characterised 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 animal's hind tibiae pass over the plates. This wax forms tight curls of approximately 1µm 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 & 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 & 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 3 – 5 kV. Metal coatings not only aid in thermal and electrical conductivity, but also 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

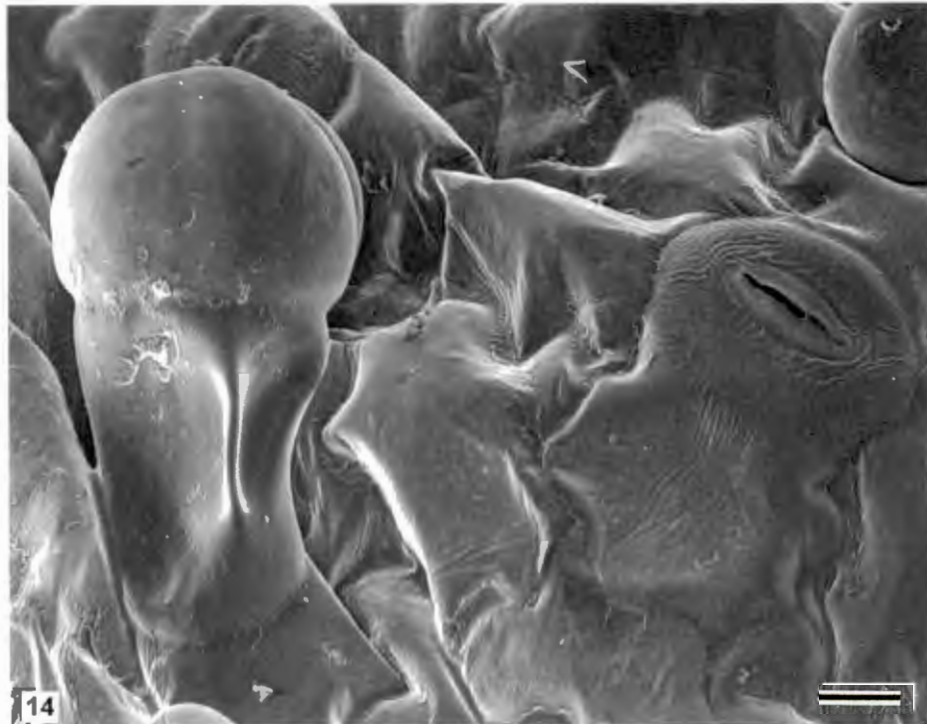
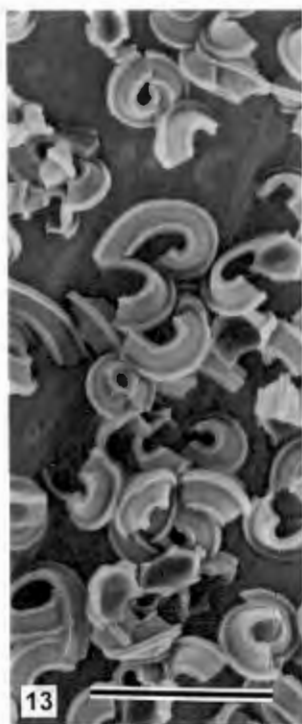
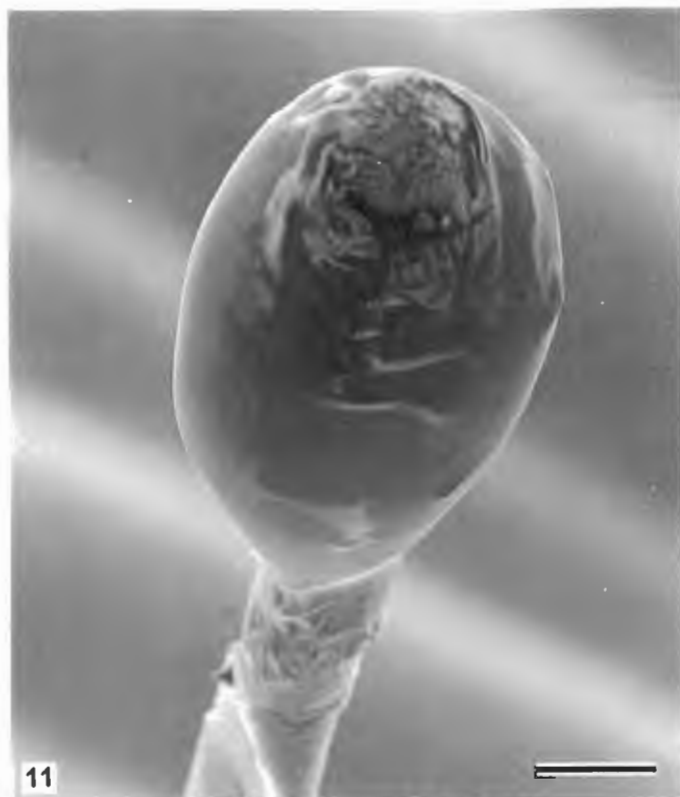


Fig. 11. The multicellular head of a long glandular trichome was not discernible as exudate covered the head of the trichome, prepared with the TEG method. Bar = 10 μ m

Fig. 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 μ m

Fig. 13. Wax particles of the *Bemisia tabai* whitefly found intact on the leaf surface after using the TEG method. Bar = 5 μ m

Fig. 14. The disadvantage of the TEG method is the short time available to observe the specimens, as the tissue collapsed under the high vacuum. Bar = 10 μ m

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, unwettable surface, such as plant surfaces covered with epicuticular wax and trichomes, we observed as was noted by Ensikat and Barthlott (1993) that the TEG substitution method displays 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 & 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 other 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 & Kislev 1978, Lyshede 1980, Bosabalidis 1990, Duke & Paul 1993, Figueiredo & Pais 1994). For although cryo-preservation preserved the leaf surface of broccoli very well (Eveling & 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 artefacts induced by CPD unfortunately outweigh those it 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 its 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 as to the relative merits of using either fresh, CPD 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 minimises charging. The major advantage of this method is those 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 and 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 (1990) used fresh leaf material for examining epicuticular wax and trichomes, but the leaf surface appeared shrunk, showed high contrast and the long unbranched trichomes appeared 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 causes 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 (Reed & Tukey 1982), 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 & McCall 1983, Sargent 1983, Sargent 1988). However, it should be noted that Reed and Tukey (1982) reported no wax or tissue damage with freeze-drying for brussel sprouts and "White Sim" carnations. Freeze-substitution is effectively similar to dehydration, in that shrinkage induced by extraction does occur (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 & Baker 1974) and the actual process of fracturing can lead to production of furrows

and steps that do not represent true morphological features (Stolinski & 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 a suitable method 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 to lessen the release of chemical substances (Nei 1974). The most serious artefact of cryo-techniques is ice crystal formation. It is when these hexagonal ice crystals are formed in frozen biological specimens that severe structural damage takes 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 & 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 was compared with that 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 artefact, 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 onto a pre-cooled metal block and this in itself may damage surface structures like trichomes (Harris

1991). Consequently, this technique is at present considered to be sub-optimal for leaf surface studies.

The major disadvantage of the TEG method is the short time available to observe some specimens as the tissue collapses due to 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 min to 5 h. As for fresh material and cryo-preserved specimens, TEG specimens can unfortunately not 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 remains unaltered. We are of the opinion that it is a simple, inexpensive and versatile method with great potential. Cryo-preservation is the other alternative for optimal studies of the leaf surface but is expensive with the possible artefact 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 assist 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 the 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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CHAPTER THREE

BOUNDARY LAYER, STOMATAL GEOMETRY AND -SPACING, IN RELATION TO DROUGHT TOLERANCE IN FOUR *Nicotiana tabacum* L. CULTIVARS

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ABSTRACT

Although numerous studies, both phenomenological and mathematical, have related gaseous interchanges at the plant leaf surface and atmospheric interface specifically with regard to drought tolerance, the important role of trichomes and the boundary layer has not been recognised. In this investigation the focus was restricted to the role of the boundary layer along with stomatal geometry and –spacing in determining the diffusion resistance to CO₂ and H₂O in four *Nicotiana tabacum* L. cultivars of different, but known, drought tolerance. Resistances were expressed both in terms of resistance components on a unit leaf area basis and in terms of dimensionless quantities. The abaxial leaf surface had the highest trichome frequency, more so in the drought tolerant than in the drought sensitive cultivars, being 9.7% and 6.9% higher than on the adaxial surface for TL33 and CDL28, as opposed to being 21.2% and 26.8% higher for GS46 and Elsoma, respectively. The frequency of the long glandular trichomes of epidermal cells, and consequently also the calculated stomatal index, was significantly ($p < 0.01$) higher on the adaxial surface of the drought tolerant cultivars. Both the fractional cavity and pore contributions to the overall entry resistance were higher for the drought tolerant cultivars, being 24.1% (H₂O) and 20.6% (CO₂) for the stomatal cavity, and 9.5% (H₂O) and 7.4% (CO₂) for the stomatal pore contributions, respectively. The fractional contribution of the boundary layer was, however, on average 3.4% (H₂O) and 6.5% (CO₂) higher for the drought sensitive cultivars. From the results presented it is concluded that plant fitness for tolerating drought in this mesophytic crop plant may be maximised not by minimising water loss or by maximising the photosynthetic to transpiration ratio, but by maximising the carbon gaining capacity of the leaf.

INTRODUCTION

From the voluminous amount of literature, based largely on observations of increasing pubescence with aridity along geographical lines and increased pubescence within a single habitat as drought progresses in the growing season (Ehleringer 1980), it would seem obvious that leaf pubescence is a means of increasing plant fitness in arid habitats, and may therefore be of adaptive value. Though several possibilities have been mentioned as probable function(s) for leaf pubescence (Brewer *et al.* 1991), the means by which leaf pubescence is adaptive is not so clear (Ehleringer 1980). One of the possibilities mentioned is that trichomes could modify the leaf boundary layer conditions (Ehleringer & Mooney 1978). But what is the boundary layer? For the purpose of this investigation we will describe the boundary layer as that laminar sublayer in contact with the leaf surface which can be subdivided (after Campbell 1977) into: (i) a top layer that exerts most of the drag on the air movement above the trichomes where the air speed in this layer decreases exponentially with distance downward from the top of the trichomes and has the same direction as the main air movement above the trichomes, (ii) a second layer which occupies mainly the stalk lengths of the trichomes (here the air movement may be quite unrelated, in both speed and direction, to the air movement above the trichomes), and (iii) a third layer where the air profile is similar to the above trichome logarithmic profile (the profile of this layer is primarily influenced by the architecture of the epidermal surface). As stated by Aphalo and Jarvis (1993), the role of the boundary layer both in terms of its involvement in (i) the mechanism of stomatal response to the condition of the bulk air, and (ii) the operational stomatal responses to the mole fraction of water vapour at the leaf surface and in the air outside the boundary layer, as well as with wind speed, has remained largely unexplored, even more so when it has to be related to drought tolerance.

As early as 1953 Bange (as cited by Rand & Cooke 1996) used an approximate analysis in order to consider a realistic geometry for the leaf interior as well as a still layer outside the leaf, and found that as the air speed increased, i.e. the thickness of the still air boundary layer decreased, the stomata played an increasingly important role in controlling gaseous fluxes. Cooke (1969) and Holcomb and Cooke (1977) considered the interaction effects between neighbouring stomatal pores, and by using separation variables showed that the flux depends on both the spacing between stomata on the leaf surface and the boundary

layer thickness. Mathematical models have been presented to describe the mechanism of stomatal functioning (Cooke *et al.* 1976, Upadhyaya *et al.* 1980) and stomatal patterning (Korn 1993). But, according to Holcomb and Cooke (1977), there is no optimal pore spacing to maximize the diffusion rate per unit leaf area, nor according to Sachs (1994) are most of the observations regarding stomatal patterning in accordance with any simple, intuitive model.

In a recent publication on the application of fluid dynamics in plant biology, Rand and Cooke (1996) gave an excellent historical overview on how the knowledge of gas exchange in leaves has progressed since this problem was first investigated from a mathematical point of view (Brown & Escombe 1900). The pathway for gaseous loss from the leaf to the ambient environment is generally thought to occur from the cell walls of mesophyll cells or the inner side of leaf epidermal cells, through the intercellular air spaces to the stomata and then into the outside air. According to Nobel (1991), of the four components involved in this process, two are strictly anatomical (intercellular air spaces and cuticle), one depends on anatomy and yet responds to metabolic as well as environmental factors (stomata) and one depends on the leaf morphology (boundary layer). These resistances can be conveniently expressed both in terms of dimensionless quantities (Cooke & Rand 1980) and resistance components (scm^{-1}) on a unit leaf area basis (Holcomb & Cooke 1977). In this paper we restrict our focus to the role of the boundary layer along with the stomatal geometry and –spacing in determining the diffusion resistance to CO_2 and H_2O in four *Nicotiana tabacum* L. cultivars of different, but known, drought tolerance.

MATERIAL AND METHODS

Plant material and growth conditions

In sequence of increasing drought tolerance, the four *Nicotiana tabacum* L. cultivars under investigation were TL33, CDL28, GS46 and Elsoma (Van Rensburg & Krüger 1993, 1994). Seeds were germinated in soil in 24 litre pots (from which excess water could freely drain) under glasshouse conditions, with optimal water application. Before the onset of sampling, plants were moved to computerised growth rooms (photon flux density, $600 \mu\text{molm}^{-2}\text{s}^{-1}$; relative humidity, 35%) and allowed to acclimatise for a period

of three weeks. During the acclimatisation period the plants were watered twice daily to field capacity to avoid their experiencing any drought stress and possibly hardening (an ability which may differ among the different cultivars) because of the slightly higher growth room temperature. The growth rooms were lit for 13 hours at 25°C, followed by 11 hours dark period at 16°C. Experimentation started when the plants were 90 days old.

Sampling and quantification procedure

Determinations were conducted on leaf samples obtained from a leaf on the sixth youngest node, as this can be regarded as a mature leaf before the onset of senescence. Stomatal geometry and frequency were quantified by making use of leaf samples that were fixed with 2% glutaraldehyde for 1 h and post-fixed with 2% OsO₄ for 2 h. Both fixatives were buffered with 0.1 M sodium cacodylate, pH 7.2. No dehydration with acetone or ethanol was used in this method. The water in the tissue was substituted by triethylene glycol (C₆H₁₄O₄). Further details on this procedure are given in Peacock *et al.* (2000). The leaf surface of tobacco was studied with a JEOL JSM 840 SEM. Ten micrographs were used for each quantification of the adaxial and abaxial side of the leaf for each cultivar. All the micrographs were taken at the same magnification and 5 kV. The epidermis cell and stomatal frequency were quantified by counting cells and stomata on the leaf surface with the aid of micrographs. Stomatal geometry was quantified with the aid of a Flexible Image Processing System (FIPS). Samples of 2 mm² were cut from the centre of the lamina, lateral to the midrib. The samples were fixed as previously described. However, these leaf samples were dehydrated in an acetone series (30-100%) and were infiltrated with resin (Spurr 1969). For light microscopy semi-thin sections were made and stained with toluidine blue O (Trump *et al.* 1961).

The stomatal index was calculated [(the number of stomata per unit area)/(number of stomata + number of epidermal cells per unit area) x 100] and expressed as a percentage. The advantage of the stomatal index is that if any variable during the growth conditions affected the epidermal cell expansion, its effects would largely have disappeared when the stomatal index was calculated, because to have an impact on the stomatal index the variable should have affected the proportion of guard cell initials (Beerling & Chaloner 1993). Dimensions of the stomatal apertures were calculated as described by Gorton *et al.* (1989).

Average trichome frequency was calculated by counting the trichomes on the leaf surface with the aid of a Wild MZ 8 Leica stereomicroscope. Six samples of 0.25 cm² were cut from the centre of the lamina, lateral to the midrib. The 0.25 cm² sample blocks were cut into smaller strips to make counting easier and more precise. Trichome lengths and widths were determined with the aid of FIPS using fresh leaf strips as already described. Because of the reasons cited by Brewer *et al.* (1991), both trichome heights and width (canopy) are reported.

Calculation of gaseous exchange predictions

The equations presented by Cooke and Rand (1980) were largely used for the expression of the following dimensionless quantities:

The stomatal aspect ratio (α) for the elliptical pore, i.e. the ratio of the semiminor axis, b, to the semimajor axis, a ($0.05 \leq \alpha \leq 0.5$):

$$\alpha = (b/a) \quad (1)$$

The stomatal spacing ratio (β), i.e. the ratio of the hypothetical radius of influence, r_c , to the length of the semimajor axis of the elliptical pore a ($\beta \geq 2$):

$$\beta = r_c/a = (\pi N)^{-1/2}/a \quad (2)$$

where N is the number of stomata per leaf area.

The nondimensional boundary layer thickness (T), i.e. the ratio of the dimensional boundary layer thickness l_c , [modified by us to include the possible effect of the long glandular trichomes acting as bluff bodies (see Nobel 1991)], to the length of the semimajor axis of the elliptical pore ($T \geq \beta \geq 2$):

$$T = [l_c + l_d f]/a \quad (3)$$

where l_c is the boundary layer thickness in mm, that is equal to the square root of L_{eaf} , (being the mean length of the leaf in the downwind direction in cm), divided by V_{air} (the ambient air speed in cms⁻¹), which is then multiplied by the factor of 0.4 for reasons cited

by Nobel (1991). l_d is the increased boundary layer thickness in mm due to the presence of trichomes, and is equal to the square root of d (the average stalk diameter of a long glandular trichome in centimeter divided by V_{air} (the ambient air speed in cm s^{-1}), which is then multiplied by the factor of 0.58 (see Nobel 1991). This value obtained for l_d is then multiplied by the factor f (i.e. the number of long glandular trichomes per surface area).

The nondimensional pore depth (L_p), i.e. the ratio of the dimensional pore depth, d , [modified by us to be 4% of the stomatal cavity depth for H_2O and 87% of the stomatal cavity depth for CO_2 (see Rand and Cooke 1996)] to the semimajor axis of the elliptical pore ($L_p \geq 0$):

$$L_p = (D_{\text{H}_2\text{O}} \text{ or } D_{\text{CO}_2} / a) \quad (4)$$

The resistance components in scm^{-1} on a unit leaf basis were used as given by Holcomb and Cooke (1977), where for the diffusion coefficient, D , a value of $0.25 \text{ cm}^2 \text{ s}^{-1}$ was used for H_2O and a value of $0.16 \text{ cm}^2 \text{ s}^{-1}$ for CO_2 as given by Cooke and Rand (1980). Furthermore, R_{cav} is the cavity resistance between mesophyll cell walls and the inner end of the pore and R_{st} is the stomatal pore resistance from the inner and to the outer end, whilst R_a represents the unstirred air or boundary layer resistance and R_e the end effect corrections. The entry diffusion resistance, R_{ent} , is the sum of the resistance of the substomatal cavity, stomatal pore and air boundary layer, provided that $\beta \geq 2$, $0.05 \leq \alpha \leq 0.5$, and $T \geq \beta$ (Cooke & Rand 1980).

Data were statistically analysed with the Student's t-test (to sample equal and unequal variance) function in Excel® (Version 5.0) in order to determine whether two samples were likely to have come from the same two underlying populations which had the same mean.

RESULTS

Average trichome frequency (Table 1) for the combined adaxial and abaxial leaf surfaces of both drought sensitive cultivars (TL33, CDL28) was 32% higher, when compared to that of the drought tolerant (GS46, Elsoma) cultivars. The abaxial leaf surfaces had the highest trichome frequency (Table 1), more so in the drought tolerant than in the drought

sensitive cultivars, being 9.7% and 6.9% higher than on the adaxial surface for TL33 and CDL28, as opposed to being 21.2% and 26.8% higher for GS46 and Elsoma, respectively. The long glandular trichomes on the adaxial leaf surface (Table 1), were the only trichome type that differed statistically significantly ($p < 0.01$) in frequency between the sensitive and tolerant tobacco cultivars, being less in the latter cultivars. Irrespective of cultivar, side of the leaf or drought tolerance, none of the trichome dimensions monitored (Table 2), differed statistically significantly.

Table 1. Trichome frequency per 0.25 cm² leaf area of four *N. tabacum* cultivars which differ with regard to their drought tolerance

Trichome type	Cultivars							
	Drought sensitive				Drought tolerant			
	TL 33		CDL 28		GS 46		ELSOMA	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Branched	20 ± 20	1.0 ± 1.0	4.0 ± 2.0	1.0 ± 1.0	20 ± 20	1.0 ± 1.0	3.0 ± 2.0	1.0 ± 1.0
Glandular (long)	75.2 ± 13.4 _a	93.3 ± 25.8	74.5 ± 15.9 _a	107.7 ± 20.5	39.2 ± 2.9 _b	75.0 ± 19.9	28.0 ± 10.4 _b	55.7 ± 10.9
Glandular (short)	19.2 ± 10.6	8.8 ± 2.8 _a	33.8 ± 15.9	23.3 ± 3.0 _b	25.7 ± 10.4	13.3 ± 7.3	18.7 ± 8.3	13.8 ± 5.3 _b
Non-glandular	11.5 ± 5.6 _a	16.3 ± 6.1 _c	73.0 ± 9.4 _b	67.0 ± 13.7 _d	48.5 ± 16.1 _b	57.0 ± 21.2 _a	13.7 ± 6.5 _c	15.5 ± 6.0

Means (n = 20) ± SD across column followed by no, or the same letter, were not significantly different at P = 0.05

On comparison the stomatal frequency (Table 3) for the combined abaxial and adaxial leaf surfaces were on average 10.7% higher for the drought sensitive cultivars, whilst the combined adaxial and abaxial epidermal cell frequency was on average 38.1% higher for the drought sensitive cultivars. The frequency of epidermal cells on the adaxial surface did, however, differ statistically significantly ($p < 0.01$) between the respective cultivars, being less for the drought tolerant cultivars. Consequently, the calculated stomatal index was also significantly ($p < 0.01$) higher for the adaxial surface of the drought tolerant cultivars. Of the substomatal cavity dimensions monitored (Table 4), only the surface area of the substomatal cavity was less ($p < 0.05$) in the drought tolerant cultivars.

Table 2. Trichome dimensions (μm) of four *N. tabacum* cultivars which differ with regard to their drought tolerance

Trichome type	Cultivars			
	Drought sensitive		Drought tolerant	
	TL33	CDL28	GS46	ELSOMA
Branched				
Total height	114.8 $\pm$ 28.1	115.0 $\pm$ 20.2	106.29 $\pm$ 31.1	122.3 $\pm$ 35.5
Stalk length	54.2 $\pm$ 20.5	62.2 $\pm$ 20.7	48.3 $\pm$ 25.1	55.2 $\pm$ 26.2
Width	36.6 $\pm$ 7.5	50.6 $\pm$ 24.9	45.8 $\pm$ 19.9	45.8 $\pm$ 9.8
Glandular (long)				
Multi-cellular head	302.4 $\pm$ 56.1	348.9 $\pm$ 75.0	314.1 $\pm$ 74.9	360.9 $\pm$ 71.9
One cellular head	189.4 $\pm$ 31.1	220.2 $\pm$ 34.3	204.4 $\pm$ 19.9	204.9 $\pm$ 36.8
Glandular (short)				
Height	53.3 $\pm$ 7.4	59.7 $\pm$ 7.1	57.59 $\pm$ 5.6	52.9 $\pm$ 8.1
Width	83.3 $\pm$ 2.7	91.7 $\pm$ 3.4	89.2 $\pm$ 3.3	99.8 $\pm$ 0.6
Non-glandular	148.6 $\pm$ 42.8	198.8 $\pm$ 33.1	175.7 $\pm$ 24.2	206.7 $\pm$ 33.3

Means ($n = 20$) $\pm$ SD across column followed by no, or the same letter, were not significantly different at the $P = 0.05$

Table 3. Number of stomata and epidermis cells per surface area (0.25 cm^2) in four *N. tabacum* cultivars of known, but different drought tolerance

Trichome type	Cultivars							
	Drought sensitive				Drought tolerant			
	TL 33		CDL 28		GS 46		ELSOMA	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Epidermal cells	132.0 $\pm$ 19.9 _a	144.6 $\pm$ 8.1 _c	143.0 $\pm$ 6.1 _a	179.3 $\pm$ 19.6 _c	83.0 $\pm$ 2.7 _b	114.6 $\pm$ 13.4 _c	83.0 $\pm$ 5.6 _b	90.3 $\pm$ 5.5 _b
Stomata	10.3 $\pm$ 6.8	36.6 $\pm$ 4.0	13.0 $\pm$ 2.3 _a	49.3 $\pm$ 2.3	15.6 $\pm$ 5.8	33.6 $\pm$ 2.1	19.7 $\pm$ 1.5 _b	28.6 $\pm$ 12.5
Stomatal index	7.24 $\pm$ 0.31 _a	20.19 $\pm$ 0.37 _c	8.33 $\pm$ 0.29 _b	21.56 $\pm$ 0.31 _c	15.32 $\pm$ 0.17 _b	22.67 $\pm$ 0.49 _c	19.15 $\pm$ 0.27 _b	24.05 $\pm$ 1.02 _d

Means ($n = 20$) $\pm$ SD across column followed by no, or the same letter, were not significantly different at $P = 0.05$

Table 4. Sub-stomatal cavity dimensions in four *N. tabacum* cultivars of known, but different drought tolerance

Sub-stomatal cavity dimensions	Cultivars			
	Drought sensitive		Drought tolerant	
	TL 33	CDL 28	GS 46	ELSOMA
Length of cavity (μm)	57.14 $\pm$ 13.51	77.48 $\pm$ 13.39	54.64 $\pm$ 17.07	57.38 $\pm$ 15.20
Width of cavity (μm)	57.48 $\pm$ 9.94	54.71 $\pm$ 9.82	43.26 $\pm$ 5.43	47.25 $\pm$ 22.34
Area of cavity (μm)	2626 $\pm$ 737.81 _a	2389 $\pm$ 661.38 _a	1473 $\pm$ 245.52 _b	1280.91 $\pm$ 398.55 _b

Means ($n = 20$) $\pm$ SD across column followed by no, or the same letter, were not significantly different at $P = 0.05$

Table 5. Dimensionless resistance in the boundary layer, stomatal pore and sub-stomatal cavity for the adaxial surfaces of four *N. tabacum* cultivars that differ in terms of drought tolerance

Parameter	Cultivars			
	Drought sensitive		Drought tolerant	
	TL 33	CDL 28	GS 46	ELSOMA
α	0.333	0.333	0.333	0.333
β	4.882	4.346	3.967	3.530
T	440.854	437.027	244.074	182.853
L_pH_2O	0.127	0.172	0.121	0.128
L_pCO_2	2.762	3.745	2.640	2.773
$R_{cav}H_2O$	2.132	1.689	1.408	1.115
$R_{st}H_2O$	0.654	0.702	0.413	0.343
R_aH_2O	31.741	31.466	17.573	13.165
R_eH_2O	1.781	1.377	1.122	0.861
$R_{ent}H_2O$	36.308	35.234	20.516	15.484
$R_{cav}CO_2$	3.332	2.640	2.200	1.742
$R_{st}CO_2$	22.217	23.869	14.022	11.665
R_aCO_2	49.596	49.166	27.458	20.571
R_eCO_2	2.782	2.151	1.753	1.345
$R_{ent}CO_2$	77.982	77.825	45.434	35.323

Of the dimensionless quantities calculated (Table 5), both the stomatal spacing ratio, β , and the nondimensionless boundary layer thickness, T, were on average respectively 18,8% and 51.4% higher for the drought sensitive cultivars. The average fractional contribution to the overall entry resistance, R_{ent} , was significantly less for the stomatal cavity (R_{cav}/R_{ent}) and stomatal pore (R_{st}/R_{ent}) than for the boundary layer (R_a/R_{ent}), both for H₂O and CO₂. Average cavity resistance was 6.2% for water and 4.4% for CO₂, and for pore resistance it was 2.0% for H₂O and 30.8% for CO₂. In contrast the average fractional contribution of (R_a/R_{ent}) was 86.9% for H₂O and 61.4% for CO₂. Both the fractional cavity and pore contributions to the overall entry resistance were higher for the drought tolerant cultivars, being 24,1% (H₂O) and 20.6% (CO₂) for the stomatal cavity, and 9.5% (H₂O) and 7.4% (CO₂) for the stomatal pore contributions, respectively. The fractional contribution of the boundary layer was, however, less for the drought tolerant cultivars, being on average 3.4% (H₂O) and 6.5% (CO₂) higher for the drought sensitive cultivars.

DISCUSSION

When attempting to quantify the value of leaf pubescence, by making use of computer simulations with a leaf energy budget-photosynthesis model, Ehleringer and Mooney (1978) observed that there is always a trade off between the decline in photosynthesis caused by increased reflectance and the increase in photosynthesis caused by a decrease in leaf temperature. If we assume, as was done by Nobel (1991), that heat is conducted across the boundary layer adjacent to the leaf and then removed by forced convection in the surrounding turbulent air at low wind speeds, the drought sensitive cultivars will be better insulated whilst the drought tolerant cultivars' leaf temperature will track the ambient temperature much more closely. The higher glandular trichome frequency which typifies the adaxial leaf surface of the drought sensitive cultivars will, according to the leaf energy-budget model, result in a lower daily water loss with the photosynthesis to transpiration ratio consequently being higher in the drought sensitive cultivars. However, according to this model the less pubescent adaxial leaf surface of the drought tolerant cultivars is predicted to result in higher daily rates of carbon gain. Both these predictions have previously been experimentally validated (see Van Rensburg & Krüger, 1993). This would seem to indicate that plant fitness for tolerating drought in this mesophytic crop plant may be maximised not by minimising water loss, or by maximising the photosynthetic to transpiration ratio, but rather through maximising the carbon gaining capacity of the leaf. This can be evidenced by the diffusion resistance for CO₂ being on average ca. 48% less for the drought tolerant cultivars, which can mainly be ascribed to the differences in stomatal spacing (stomatal index) and the boundary layer resistance to CO₂, between the respective cultivars.

Cooke and Rand (1980) have extensively discussed the major role both these variables play in determining the diffusion resistance. In short this entails that for a lower stomatal frequency, as observed in the drought sensitive cultivars, a change in the boundary layer thickness results in a less marked change in conductance. Nevertheless, it must be noted that for any given boundary layer thickness, a decrease in spacing (as in the drought tolerant cultivars) increases the conductance. A possible explanation for the above mentioned adaptive value of the boundary layer can be found in the results presented by Aphalo and Jarvis (1993), which indicated that at low wind speeds the boundary layer acts as a feedback loop by altering stomatal conductance to the fraction of CO₂ and H₂O

vapour in the air outside the boundary layer, each of which include responses to changes in the internal CO₂ concentration and leaf surface water vapour saturation deficit. Furthermore, although it has been indicated that water loss *via* glandular trichomes should be large enough to lower leaf temperature by several degrees within a wide range of conditions, our data would indirectly seem to support the argument put forward by Lauter and Munns (1986) that, though the cooling benefit might be significant in a hot environment, in a dry environment glandular exudation is most unlikely to be beneficial. For then glandular exudation, well known to occur in tobacco, ought to reduce the leaf water potential for the drought sensitive cultivars even faster.

CONCLUSIONS

The results presented confirm previous findings that there is neither a set optimum leaf pubescence (Ehleringer 1980) nor a set optimal stomatal spacing (Holcomb & Cooke 1977), but that in terms of drought tolerance the advantage of a higher stomatal frequency and the role of the boundary layer is closely related to the carbon gaining capacity of a leaf. A high degree of pubescence would therefore not seem to be a prerequisite for drought tolerance, because in tobacco drought tolerant cultivars can adapt both their photosynthetic apparatus (Van Rensburg *et al.* 1996) and employ other protective mechanisms (Van Rensburg & Krüger 1994) and reactions (Van Rensburg *et al.* 1994) to cope with a high irradiation load.

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CHAPTER FOUR

DETERMINATION OF EPIDERMAL TRANSPIRATION IN FOUR CULTIVARS OF *Nicotiana tabacum* L. USING EPIDERMAL STRIPS IN A QUASI-STEADY STATE SYSTEM

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ABSTRACT

A quasi-steady state method is presented for quantifying epidermal transpiration of epidermal strips where simple relations between transmembrane fluxes and parameters of diffusibility of penetrating compounds hold. Contrary to most permeability studies, we did not make use of stomatous cuticular membranes, cuticular membranes that were enzymatically isolated, or dried cuticular membranes because these procedures are largely responsible for the problems cited in the literature. Instead, we used freshly harvested stomatous epidermal strips thus avoiding the sorption of lipids by the cuticular membranes during enzymatic isolation. Our approach allowed estimation of amounts and composition of intracuticular soluble lipids. Diffusion coefficients (D-values) were calculated with smaller associated standard deviations and an order of magnitude lower than previously reported; fresh material sorption of the compound by the membrane and hydration of the cuticular pores was greatly reduced. In the present study the hold-up time (t_e) ranged from 66.2 ± 0.3 to 110.3 ± 0.9 s. Furthermore, $0.1 \mu\text{m}$ thick membranes could be used, contrary to previous studies of water permeability that used cuticles more than $2 \mu\text{m}$ thick. Because a small but constant flow of penetrant could be detected during the first half of the steady flow to t_e , small holes probably did not influence the reported permeability. Permeability coefficients (P_d) in the order of $0.65 \times 10^{-9} \text{ ms}^{-1}$ were calculated. P_d -values in the order of $5.68 \times 10^{-3} \text{ ms}^{-1}$ could be calculated when incomplete stomatal closure occurred, while when areas of mass flow were detected, P_d values in the order of $1.26 \times 10^{-2} \text{ ms}^{-1}$ were calculated. The degree of contamination of the epidermal strips by cellular debris was quantified and expressed as the total chlorophyll content per exposed surface area of the epidermal strip, and an average of 8.7% contamination was observed compared to the total leaf chlorophyll content. Leakage from the system was calculated to approximately $0.18 \times 10^{-10} \text{ ms}^{-1}$, which represents an average 2.7% experimental variability. These results are discussed in terms of the limitations associated with using composite membranes, which are stomatous and have trichomes, for possible application in drought tolerance selection.

INTRODUCTION

A direct approach to study the effects of various factors on cuticular transpiration / penetration is the use of isolated cuticles. Such a system allows an investigation of the influence of a single factor on cuticular permeability independent of complications introduced by other factors (Glenn & Poovaiah 1985). Studies with intact detached cuticles have provided much information about the function of cuticular membranes (Geyer & Schönherr 1990); however, it is clear that the isolated cuticle does not provide a satisfactory model of the properties of the cuticles on intact leaves, and that it is difficult to determine the conductance of attached cuticles using intact leaves (Van Gardingen & Grace 1992).

Despite studies of transcuticular movement, several of which have been cited by Hoch (1975) and Larsson and Svenningsson (1986), specific experimental evidence is limited to only a few plant species, possibly because of available techniques and particular interest in those species (Schönherr & Lenzian 1981, Schönherr & Mérida 1981). Probably for the same reasons, most of the information available regarding the sorption of water by plant cuticles (Chamel *et al.* 1991), permeability of cuticles to acids (Hauser *et al.* 1993), rheological properties (Petracek & Bukovac 1995), water permeability (Schönherr *et al.* 1979), and diffusion and mobility of ions in cuticles (Tyree *et al.* 1992), has been obtained using isolated cuticles. On the other hand, Hoch (1975) demonstrated the effects of some isolation chemicals on the structural integrity of cuticles, Schönherr and Riederer (1986) showed it impossible to estimate amounts and composition of intracuticular soluble lipids using isolated cuticles, and Riederer and Schneider (1989) observed that there is no experimental evidence or theoretical reason for the assumption that chloroform or any other apolar solvent does not enter the cuticle during the extraction of cuticular lipids. In this regard it is interesting to note that after conducting a time course study of both qualitative and quantitative changes of the soluble cuticular lipids during repeated water stress, Svenningsson and Liljenberg (1986) concluded that the intra- as well as epicuticular lipids must be considered when discussing leaf surface lipids as protecting agents against water loss, when attempting to ascertain the relative importance of different lipid components for the magnitude of water loss. Larsson and Svenningsson (1986) concluded that cuticular transpiration *per se* as a selection criterion might be a useful tool in breeding for drought resistance. Similarly, epidermal transpiration has shown promise for differentiating drought resistance among wheat varieties (Araus *et al.* 1991).

In this investigation we used epidermal rather than cuticular transpiration because it is not clear whether the presumed cuticular transpiration is due in part to incomplete stomatal closure (Sinclair & Ludlow 1986) and whether its rate is a function of the epidermal conductance (Muchow & Sinclair 1989). The dual purpose of our study is to evaluate critically a quasi-steady state method used for quantification of epidermal transpiration in our laboratory and to note if the calculated permeability coefficients correlate with the known drought tolerance of four *Nicotiana tabacum* L. cultivars for possible use as drought tolerance selection criterion.

MATERIAL AND METHODS

Plant material and growth conditions

In sequence of increasing drought tolerance the four cultivars of *Nicotiana tabacum* L. included in the present investigation were TL33, CDL28, GS46 and Elsoma. These cultivars were selected because of their differing performance in the field and under controlled environmental conditions, during water stress (Van Rensburg & Krüger 1993, Van Rensburg *et al.* 1993). Young seedlings were grown under glasshouse conditions with optimal water application (watered once daily to field capacity). Prior to experimentation the plants were moved to a computerised growth room (photon flux density, 400-600 $\mu\text{mol m}^{-2}\text{s}^{-1}$; relative humidity, 35%) and allowed to acclimatise for 96 h. During the acclimatisation period plants were watered twice daily to minimise drought stress because of the slightly higher growth chamber temperatures. In the growth room, plants were exposed to 13 hours of light at 25°C followed by 11 hours dark period at 16°C. These growth conditions were critical, because epidermal conductance has been found to vary with temperature, light intensity, and especially with the degree of hydration of the leaf (Meidner 1986). Temperature and light may be closely related to epicuticular wax development (Clark & Lister 1975).

Measurement of cuticular permeability

Contrary to most studies with cuticular membranes where the cuticles used were free of trichomes and stomata (Schönherr & Schmidt 1979, Schönherr & Lenzian 1981), we used epidermal strips containing trichomes and stomata. Epidermal samples were taken from mid-lamina areas from the sixth leaf from the apex as prescribed by Weyers and Meidner (1990) for reasons discussed earlier by Van Rensburg *et al.* (1993) and / or by cleaning the abaxial side by

gently scraping away adhering mesophyll cells from the epidermis without scaring it (confirmed by light microscopy before use). Experiments were conducted in an air-conditioned room at 25°C and 35% relative humidity.

It should be noted that *N. tabacum* was also favoured by Thomas (1970) though strips from this species tend to suffer from excessive mesophyll contamination (Hudson *et al.* 1983). Contamination from adhering cellular debris has been quantified as the total chlorophyll concentration per exposed surface area of the epidermal strip (Lichtenthaler & Wellburn 1983), after epidermal transpiration quantification.

The membranes were mounted between the chamber lid and the O-ring contained in the half cells of the measuring system that was clamped together as shown in Fig. 1. Membrane area exposed to the reservoir was 19.63 mm². The apparatus, constructed from highly polished brass for optimal temperature exchange, was kept at a constant temperature of 25 ± 0.02°C by circulating water from a thermostated water bath (Haake, Hansatech, King Lyon, Norfolk). The titrated water (THO) donor solution 3x10⁻⁶ cpm ml⁻¹ (Tritium, Amersham, South Africa) in the 300 mm³ reservoir, was stirred with a spinfin magnetic stirbar to ensure mixing and eliminate nonstirred layer effects (Kerler *et al.* 1984). The air inlet protruded over the transpiration chamber and faced the upper (adaxial) epidermal surface. The morphologic inner side of the membrane (cell wall side) faced the donor solution. A constant low airflow stream (25 ± 0.02°C) was generated over the membrane with an aspirator, which avoided a positive pressure on the membrane as might be the case with systems using a pump. The air leaving the transpiration chamber was passed through a cold trap with scintillation liquid (Insta-gel, Packard, South Africa). Glass tubing was used to connect the transpiration chamber with the cold trap. The exit of the cold trap was 10 cm below the apparatus that represents the total pressure drop in the system. A quasi-steady state was obtained by approximating a constant concentration gradient between the donor and receiver solutions. Concentration in the donor solution was kept as constant as possible by making the donor reservoir as large as practically possible and replacing this solution before its concentration had decreased by 2%, limiting sorption by the membrane by only using hydrated epidermal strips, and by keeping the duration of a measurement as short as possible. On the receiver solution side the concentration was kept as low as possible by maintaining a constant airflow by using the cold trap. Radioactivity in the trap was determined by liquid scintillation spectrometry (Tri-Carb 460C, Packard, South Africa). Leakage from the system, calculated when the chamber was covered with an impermeable metal

foil and when cleaning the system, was calculated to be ca. $0.18 \times 10^{-10} \text{ ms}^{-1}$ which represents an average 2.7% experimental variability. This apparatus enabled us to simulate a system of aqueous solution (donor solution) / membrane (epidermal strips) / very dilute aqueous receiver solution (atmospheric air), which closely approximates the actual *in situ* situation (membrane as primary interface).

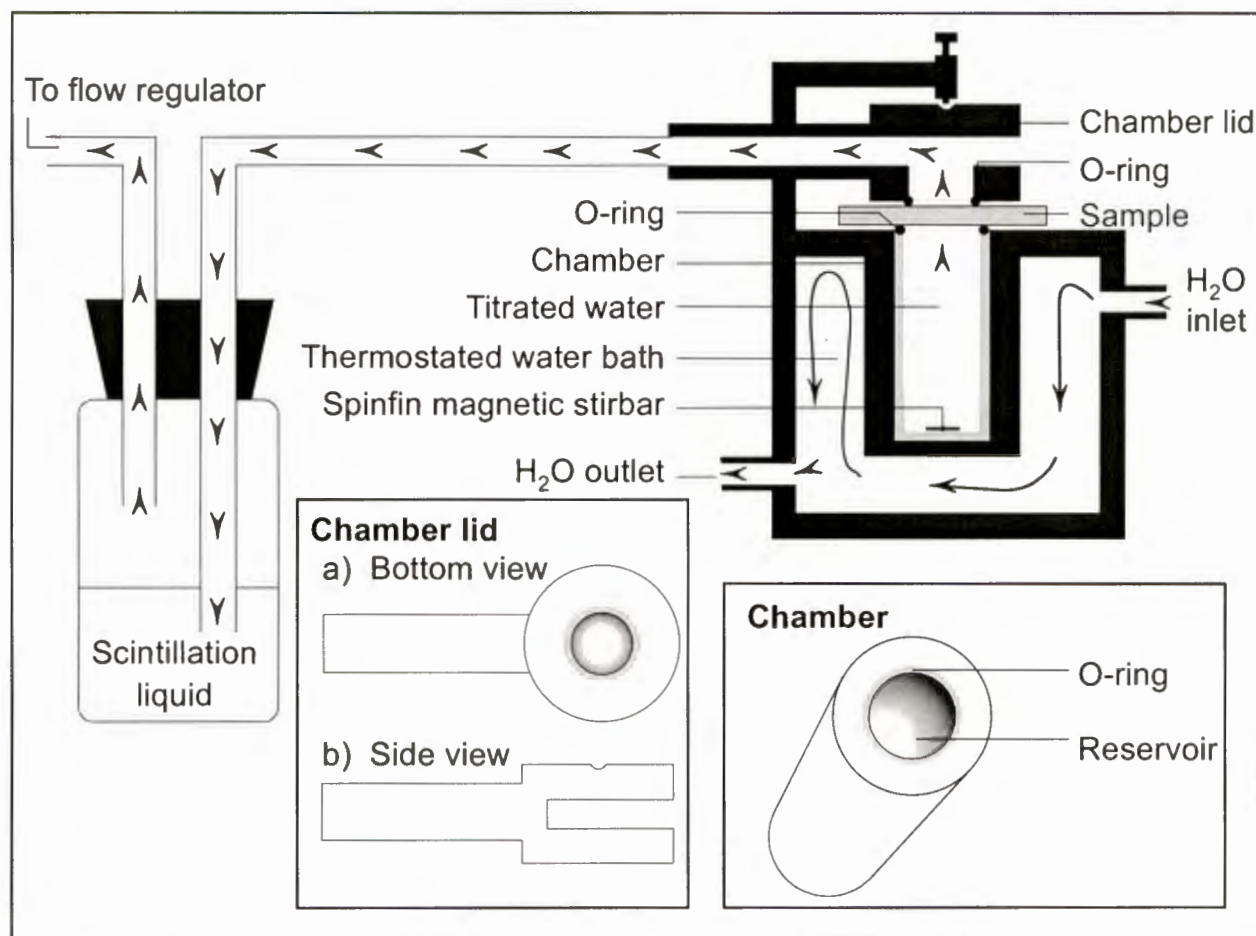


Fig. 1 Longitudinal cross section (as well as expanded view of the chamber) of the apparatus used to study cuticular permeability. Inlet and outlet arrows indicate the direction of flow of donor and collection solution. See text for more detail.

Calculation of permeability coefficients

The flow rate (F in cpm s^{-1}) was determined from the linear slope of amount penetrated versus time by the least square method, and permeability coefficient (P in cpm ms^{-1}) was calculated from the equation:

$$P_d = \frac{F}{A_m (C_{don} - C_{rec})}$$

where A_m is the exposed membrane area and C_{don} and C_{rec} are the donor and receiver concentrations (cpm ml⁻¹), respectively, as described by Kerler *et al.* (1984). Because the concentration of the donor solution greatly exceeded that of the receiver solution, $C_{don} - C_{rec}$ was replaced by C_{don} . From the intercept of the extrapolated steady-state flow with the time axis the so-called extrapolated hold up time, or time lag, t_e was obtained (Crank & Park 1968). This enabled us to calculate the diffusion coefficient, $D = (\Delta x)^2 / 6t_e$, where Δx represents the thickness of the membrane (assuming the membrane is homogeneous), which is a measure of the mobility of the solute within the membrane.

RESULTS AND DISCUSSION

Like Araus *et al.* (1991), but unlike Svenningsson and Liljenberg (1986), we did not subject the experimental plants to different drought stress cycles. We assume drought stress would not be required for the expression of genotype differences in epidermal conductance, because Clark & Romagosa (1991) pointed out that epidermal conductance is a constitutive rather than an adaptive characteristic. Also, contrary to most cuticle permeability studies, we did not use astomatous cuticular membranes (Schönherr & Lenzian 1981), enzymatically isolated cuticular membranes (Tyree *et al.* 1990) or cuticular membranes dried prior to use (Schönherr & Huber 1977, Vercher *et al.* 1994) because these procedures are largely responsible for the problems cited in the literature. Instead, we used freshly harvested stomatous epidermal strips.

We were able to avoid the complications of sorption of lipids by the cuticular membranes during enzymatic isolation (Schönherr & Riederer 1986, also see Tyree *et al.* 1990). This allows subsequent quantitative estimation of amounts and composition of intracuticular soluble lipids. Diffusion coefficients (D-values) with smaller associated standard deviations and an order of magnitude lower than those reported by Chamel *et al.* (1991) were calculated (Table 1), as with fresh cuticular membranes sorption of the compound by the membrane (Kerler *et al.* 1984) and hydration of cuticular pores (Schönherr 1976) were largely reduced. The more accurate values as discussed by Chamel *et al.* (1991), are the consequence of diffusion through the outer surface of the epidermal membrane into the gas phase, which is critical for the determination of D because it is a closer approximation of the situation *in situ*. Diffusion through the outer surface,

which is less hydrophilic than the inner one, involves a certain barrier effect, whereas in sorption experiments water can penetrate through both surfaces, especially the inner face.

According to Kerler *et al.* (1984) some penetration could occur through holes which is invisible with a light microscope, but which can be detected by the hold-up time (t_e). In the present investigation t_e also ranged from 66.2 ± 0.3 s to 110.3 ± 0.9 s (Table 1), which is higher than that reported for *Citrus aurantium* L. (Schönherr 1976) but much lower than the average of 6 h reported for *Ficus elastica* Roxb. (Kerler *et al.* 1984). Because no small, constant flow could be detected during the first half of t_e , such small holes probably did not influence the reported permeability. Furthermore, membranes of ca. $0.1 \mu\text{m}$ thick were used in the present investigation unlike earlier studies of water permeability using cuticles more than $2 \mu\text{m}$ thick (Schönherr & Mérida 1981). Since the data presented represent the permeability of composite membranes (Krüger *et al.* 1996) and epidermal wall, it is necessary to consider the possible effect of the cell wall. From results presented by Schönherr and Mérida (1981) it is clear that diffusion in the cell wall is restricted somewhat owing to the presence of cellulose and other materials, but that the cell wall has no measurable effect as long as the permeability coefficient (P_d) is at least two orders of magnitude smaller than 10^{-4} ms^{-1} , as was the case in this investigation. Thus, the permeability measured using the composite membrane reflects completely the properties of the cuticular membrane.

A problem associated with the use of stomatous epidermal strips when determining the conductance of attached cuticles using intact leaves is the assumption of stomatal closure. Hygen and Midgaard (1954) were the first investigators to warn about the dangers associated with this assumption. To overcome this obstacle, we conducted the present study in a dark room using only a green safelight when necessary and verified stomatal closure by microscopy (Van Gardingen *et al.* 1989). Alternatives could include the use of abscisic acid (Rawson & Clark 1988) or gas exchange techniques (Holmgren *et al.* 1965), though the latter usually is inadequate because of the inherently low resolving power and associated large standard deviations. Permeability coefficients of ca. $5.68 \times 10^{-3} \text{ ms}^{-1}$ could be calculated in dim light when incomplete stomatal closure occurred. This is seven orders of magnitude greater than the P_d values reported in Table 2. We, like Meidner (1986), who used stomatous *Prunus laurocerasus* L. leaves, cannot rule out the possibility that there was some residual loss through the stomata confounding the permeability measurements. However the values obtained make this unlikely. Microscopic investigation of the membranes offered the opportunity to check for sites of mass flow through

the cuticle, which would alter the measurements of the true rate of diffusion drastically (Glenn & Poovaiah 1985). When areas of possible mass flow, such as holes or cracks were detected under the light microscope, P_d -values in the order of ca. $1.26 \times 10^{-2} \text{ ms}^{-1}$ resulted (Table 2). Clearly the P_d -values in Table 2 indicate that no such alternative pathways existed in the epidermal strips used to quantify epidermal transpiration in the four tobacco cultivars.

Table 1. General properties of the leaf cuticular membranes of four *Nicotiana tabacum* L. cultivars with different drought tolerances

Cultivar	Δx (m)	t_e^a (s)	D^b (m^2s^{-1})
TL33	1.2×10^{-6}	66.2 ± 0.3	0.25×10^{-14}
CDL28	1.3×10^{-6}	84.5 ± 0.7	0.19×10^{-14}
GS46	1.5×10^{-6}	98.1 ± 0.4	0.17×10^{-14}
ELSOMA	1.4×10^{-6}	110.3 ± 0.9	0.15×10^{-14}

^a Hold up time defined as the intersection of the extension of the steady state line with the time axis of a plot of total amount diffused versus time.

^b Diffusion coefficient of water in the membrane calculated as $D=(\Delta x)^2/6t_e$ (Schöhherr, 1976).

Table 2. The differential permeability coefficients, pore area per unit diffusion path ($A_w/\Delta x$) and the fractional pore of the epidermal membranes (A_w/A_m) of four *Nicotiana tabacum* L. cultivars with different drought tolerances

Cultivar	Diffusion rate	P_d^a (ms^{-1})	$A_w/\Delta x^b$ (ms^{-1})	A_w/A_m^c
TL33	4.45 ± 0.04	0.68×10^{-9}	0.279 ± 0.112	1.42×10^{-4}
CDL28	4.27 ± 0.41	0.65×10^{-9}	0.266 ± 0.109	1.36×10^{-4}
GS46	4.21 ± 0.27	0.64×10^{-9}	0.262 ± 0.098	1.33×10^{-4}
ELSOMA	4.02 ± 0.78	0.61×10^{-9}	0.250 ± 0.078	1.27×10^{-4}

^a It has been shown experimentally that $P_d = -DA_w/\Delta x$ (Schöhherr, 1976)

^b $A_w/\Delta x = P_d/D$, where D was taken to be $2.44 \times 10^{-9} \text{ m}^2\text{s}^{-1}$ from Lakshminarayanaiah (1969), and A_w is the total area of water-filled pores.

^c $(A_w/\Delta x)\Delta x$, where $\Delta x = \text{ca. } 1.3 \times 10^{-6} \text{ m}$ and A_m is the membrane area.

The second limitation associated with the use of epidermal strips is contamination from cellular debris. We attempted to keep contamination from cellular debris to a minimum by adhering to the procedure described by Weyers and Meidner (1990) when sampling epidermal strips. Some

contamination still occurred, however, which we quantified as described by Lichtenthaler and Wellburn (1983) and expressed as the total chlorophyll content per exposed surface area of the epidermal strip. The total epidermal strip chlorophyll concentration was 0.33 ± 0.07 (TL33), 0.24 ± 0.01 (CDL28), 0.21 ± 0.04 (GS46) and 0.24 ± 0.04 (Elsoma) μgmm^{-2} . This represents 11.7, 8.3, 6.9 and 8.2% contamination, respectively, when compared to total leaf chlorophyll content. Cellular debris could also be removed with a jet of water (Schönherr & Huber 1977), or by careful washing with deionized water (Vercher *et al.* 1994).

The P_d -values calculated in the present investigation for the transport of water in *N. tabacum* epidermal strips (Table 2) agree with those reported for isolated leaf cuticular membranes of various species (Schönherr 1982), especially those of *Pyrus communis* L., *Hedera helix* L. (Schönherr & Lenzian 1981) and *Allium cepa* L. (Schönherr & Mérida 1981), all of which have comparatively thin cuticular membranes. No attempt was made to maintain defined and constant boundary layer conditions, which are possibly two orders of magnitude greater than epidermal conductance (Araus *et al.* 1991). The P_d -values reported in Table 2, therefore should include possible intercultural differences in this characteristic, representing the *in situ* situation more closely (Van Rensburg *et al.* 1994). In this regard it is interesting to note that the density of trichomes on the leaves of *Lycopersicon pennellii* (Corr) D'Arcy has been positively correlated with epicuticular lipid accumulation during the first two weeks after the onset of drought stress (Forbes *et al.* 1985), but that in *Triticum turgidum* L. durum ears the presence of trichomes was not associated with lower epidermal conductance (Araus *et al.* 1991). These results contradict the common belief that trichomes and stomata represents variables which cannot be adequately controlled when calculating epidermal transpiration, and confirm the findings (Schönherr 1976) that no significant difference in water permeability exists between isolated cuticles and the entire epidermis (Schönherr 1976).

It has been shown experimentally (Schönherr 1982) that water diffusion does not occur across the entire membrane surface, but only across the fraction that is occupied by water. In this regard it is known (Kadem & Katchalsky 1961) that the fractional pore area (A_w/A_m) is numerically equal to the fractional column of water in the membrane and is therefore a direct measure of swelling. From the data presented in Table 2, it is clear that a small fraction of the total *N. tabacum* membrane area is permeable to water, and that this correlates negatively with the known drought tolerance of the four tobacco cultivars. The data presented of the four tobacco cultivars (Table 1 and 2) confirm previous reports that genetic variability exists, pre-

drought stress in this instance, among maize, rice, oats, wheat, tea and sorghum (Jordan *et al.* 1984 and authors cited therein), and barley (Larsson & Svenningsson 1986). Positive correlation with the known drought tolerance of the cultivars, however, adds to the growing body of evidence that cuticular conductance relates positively to differences in drought tolerance among some cultivars of the above mentioned species.

If most of the water transport across the epidermal membrane takes place through structural defects in the soluble cuticular lipid barrier, as proposed by Schönherr *et al.* (1984), only small contributions of extra lipids or minor differences in lipid morphology could significantly reduce the transport through these pathways (Svenningsson 1988). This may explain our results as well as some of the inconsistencies mentioned earlier. Differences among genotypes might be too small to detect with traditional lipid analysis techniques (Svenningsson 1988), which could explain why cuticular transpiration rates are considerably altered in response to a drought stress treatment (Araus *et al.* 1991, Larsson & Svenningsson 1986), yet no clear correlation due to drought stress could be observed among changes in cuticular transpiration, soluble cuticular lipid levels (Svenningsson 1988), or epicuticular wax load (Larsson & Svenningsson 1986). Great variations in cuticular conductance have also been reported to occur within species depending on age (Viougeas *et al.* 1995), the immediate environment during growth (Jordan *et al.* 1984), different areas of the same plant and leaf (Schönherr 1982), with temperature (Schönherr *et al.* 1979), light intensity and most pronouncedly with humidity (Van Gardinger & Grace 1992).

Despite of all the uncertainties related to epidermal conductance, our results for *N. tabacum* using epidermal permeability coefficients *per se* (Larsson & Svenningsson 1986), or possibly their ability to change in response to stress (Bengston *et al.* 1978), seem to have merit as selection criteria for identifying drought tolerant genotypes. Both the intra- and epicuticular lipids (Svenningsson & Liljenberg 1986) and other leaf morphological characteristics (Araus *et al.* 1991), however, should also be taken into account when considering non-stomatally controlled epidermal factors as protecting agents against epidermal water loss.

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CHAPTER FIVE

A FE-SEM STUDY ON THE TOBACCO LEAF EPIDERMIS

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ABSTRACT

The leaf epidermis of *Nicotiana tabacum* L. was studied using high-resolution field emission scanning electron microscopy. Tobacco leaf epidermis specimens were prepared in four different ways. All preparation schedules yielded the same results. The gross morphology of the epidermis shown by FE-SEM is similar to that revealed in TEM work, namely a cuticular membrane and a cell wall. The FE-SEM revealed a globular structure for the cuticular membrane. The cell wall was found to be stratified, revealing microfibrils with a diameter of ca. 30 nm which corresponds to those of cellulose microfibrils of cell walls.

INTRODUCTION

The primary aerial parts of higher plants are covered, with the exception of the stomatal openings or glandular apertures, by a continuous extracellular lipid membrane called the cuticle (Chamel *et al.* 1991). The cuticle of plants serves many functions that are of agricultural and economical importance (Baker & Hunt 1981). Three of these important functions include: the control of water exchange with the atmosphere (Schönherr & Schmidt 1979), a barrier protecting plants against ingress by pathogens (Kolattukudy 1985), and it represents the first barrier to the penetration of foliar applied compounds. Of these, the main function ascribed to the cuticle is to minimise water loss from plants when stomata are closed (Chamel *et al.* 1991). Dynamic aspects of water vapour loss from leaves have mostly been based on variable stomatal apertures (Zeiger *et al.* 1987) with cuticular resistance usually assumed to be constant (Parkhurst 1986) or negligible (Cowan 1982). During the last decade, however, it became clear that these assumptions are questionable (Santrucek & Slavik 1990). The hydraulic conductance of the cuticle is especially relevant when estimating rates of transpiration and diffusive water vapour conductance of leaves (Weyers & Meidner 1990).

The importance of cell walls, both in terms of their usefulness to man and in the carbon economy of the biosphere, should also not be overlooked (Goodwin & Mercer 1983). The detailed structure of the cell wall must first be known before we can understand the role of the wall as a phase into which and through which transport occurs in absorption by cells and its translocation across tissues (Lüttige & Higinbotham 1979). Generally structure of the primary cell walls is composed of cellulose fibres embedded in an amorphous mixture of polysaccharides and glycoproteins. Although this structural model appears to be accurate, it lacks detail (Darvill *et al.* 1980). A more detailed description of the primary cell wall will eventually include the following: (1) isolation and identification of each of the individual macromolecular components of the cell wall; (2) determination of the primary structure of each of these macromolecules; (3) determination of the three-dimensional structure of these macromolecules; (4) determination of how and where these macromolecules are biosynthesized within plant cells; (5) determination of how these macromolecules are attached to one another or how they are interrelated; (6) determination of how the interrelated macromolecules are distributed throughout the thickness and the length of the wall; and, (7) determination of how newly synthesized macromolecules are inserted into the wall and how the wall grows (Darvill *et al.* 1980). The cell wall is biologically important for two reasons. Firstly, it largely determine the morphology and the functioning of

the cell. Secondly, since it forms the limiting envelope of the cell, it may be directly involved in regulating cell expansion (Darvill *et al.* 1980, Goodwin & Mercer 1983). The walls also represent a structural barrier to some molecules and to invading pests (Lüttige & Higinbotham 1979). Walls are involved in metabolic events such as pre-absorptive processing of nutrients and post-secretory processing of wall molecules. The ability of the wall to activate plant defense mechanisms and mimic plant hormones suggests that wall components possess enormous information potential and that cell walls are able to play an active role in cell-cell communication (Bacic *et al.* 1988). Thus, understanding the molecular detail of the biosynthesis and assembly of the plant cell wall and its components is essential for an accurate description of the physiology of plant growth (Delmer & Stone 1988).

In the quest for answers on the plant cuticle and cell wall ultrastructure three points should always be kept in mind. (1) The cell wall and cuticle of plants are heterogeneous in structure and care should be taken not to over simplify and generalise the ultrastructure of cuticles and cell walls of different plant species. (2) Plant function can ultimately be understood on the basis of the principles of physics and chemistry (Salisbury & Ross 1992). (3) In living organisms, structure and function are intimately wedded (Salisbury & Ross 1992). All these aspects should be taken into account to form an understanding of the functional aspects of the plant cuticle and cell wall. In the past researchers often studied either the cuticle or cell wall in isolation, while these composite epidermal structures should, in our view, not be studied separately in light of their integrated function and structure.

Previously scanning electron microscopy has been unable to demonstrate cuticular and cell wall fine structure due to limited resolution. Detailed information, however, is essential to understand why epidermal transpiration differs between cultivars of the same species (Van Rensburg & Peacock 1998). The present investigation was conducted in order to extend experimental evidence on transcuticular water movement. To our knowledge this is the first study of the three dimensional ultrastructure of the plant cuticle and cell wall using high resolution field emission scanning electron microscopy.

MATERIALS AND METHODS

Plant material

Four cultivars of *Nicotiana tabacum* L., TL33, CDL28, GS46 and Elsoma (listed in sequence of increasing drought tolerance), were used. 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 acclimatise for a period of 98 h. The growth chambers were lit for 13 hours at 25°C followed by 11 hours dark period at 16°C. Experiments started when the plants were approximately 90 days old (for more detail see Van Rensburg *et al.* 1993). The sixth youngest leaf represents a mature, nearly fully expanded leaf before the onset of senescence (Van Rensburg *et al.* 1993) and was used during sampling. Samples were cut with a sharp razor blade from the centre of the lamina between the main vein and the margin, avoiding large lateral veins. The adaxial side of the leaf was studied for all methods used.

Transmission electron microscopy

Samples of 1 mm² were cut from the sixth leaf in the same position as described above. The following fixation schedules were carried out at room temperature, unless stated otherwise. All material was dehydrated in an acetone series followed by embedding in epoxy resin (Spurr 1969). (1) Primary fixation in 2% OsO₄ + 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h at 4°C, post-fixation in 2% OsO₄ in the same buffer for 2 h at 4°C (Franke *et al.* 1969). Sections were contrasted with 5% aqueous uranyl acetate for 40 min and lead citrate (Reynolds 1963) for 20 min. (2) Primary fixation in 2% OsO₄ vapour for 3 h, post-fixation in aqueous 2% OsO₄ for 2 h followed by contrasting with 5% aqueous uranyl acetate for 20 min and lead citrate for 10 min. (3) Primary fixation in 2.5% formaldehyde (freshly prepared from paraformaldehyde) + 2% glutaraldehyde + 1.5 mM calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 h (Pacini *et al.* 1992), post-fixation in 0.5% OsO₄ for 1 h and contrasting with 5% aqueous uranyl acetate for 20 min and lead citrate for 10 min. The three methods were selected from Krüger *et al.* (1996). All sections were made with a Reichert-Jung Ultracut E microtome using glass knives and examined with a Phillips CM10 transmission electron microscope operated at 100 kV.

Scanning electron microscopy

Plunge freezing

Leaf samples of 6 mm² were cut from the leaf as described above and mounted upright on a graphite stub in carbon dag (Leit C, Neubauer Chemikalien). The stubs were made from spectrographic graphite rods normally used in high vacuum coating systems. The rod was turned down to a diameter of 5 mm with a lathe. Disks, 1 mm thick were cut from the rod and polished to a thickness of 0.9 mm. A very smooth surface was obtained by doing the final polishing on smooth paper. Before use, the stubs were cleaned in acetone followed by diethyl ether in an ultrasonic bath. The stub with material was plunge frozen in liquid propane at -180°C with a Reichert KF 80-plunge freezer. The time lapse between mounting of the specimen and plunging it in propane was 10 s. The stub was transferred under liquid nitrogen into a slot in a copper block where the upper part of the tissue pieces was fractured with a sharp razor blade. Tissue for cross sections was usually fractured level with the top surface of the carbon dag. This resulted in tissue fragments not higher than 0.5 mm above the stub surface. The height of the specimen was kept to a minimum because of the limited space in the specimen chamber associated with an in-lens type of microscope. This type of microscope also permits very high-resolution imaging if a thin metal coating is used. Charging is also reduced when the specimen surface is in close contact with the conductive carbon dag.

The copper block and tissue were kept immersed in liquid nitrogen (LN₂) in a plastic dish. The copper block (63 x 63 x 15 mm) was fitted with a thermocouple and heater. The surface of the copper block was lower than the rim of the plastic dish in order to keep the material in a nitrogen atmosphere to prevent moisture condensation on the tissue. The plastic dish with the copper block was transferred to a Fisons high vacuum unit and evacuation started immediately. The temperature of the copper block was typically still below -130°C when the vacuum reached 1.33 Pa (1x10⁻² Torr). The vacuum remained at this value until all nitrogen (now frozen) was sublimed from the plastic container. After about 1 h the vacuum improved to 0.133 Pa (1x10⁻³ Torr) and the temperature rose to -100°C. Freeze-drying was done overnight. After ± 15 h the vacuum was 0.0133 Pa (1x10⁻⁴ Torr) and the temperature of the copper block 0°C. The block was heated to 25°C before releasing the vacuum. Some samples were washed in both 100% chloroform and 100% methanol for 10 s before plunge freezing, to determine the influence of these solutes on the leaf surface, especially with regard to the epicuticular wax.

Nitrogen freezing

Samples of 6 mm² were cut from the leaf and washed in 100% acetone for 15 min. Nitrogen freezing was applied to determine the influence of acetone on especially the cuticle and removal of epicuticular wax. Samples were mounted in carbon dag on a graphite stub and frozen in LN₂. The tissue was fractured level with the top surface of the carbon dag and then left to air dry.

Isolation of the epidermis

Leaf disks of 5 mm diameter were enzymatically isolated by incubation in 2% (w/v) pectinase buffered at pH 3.6 with 0.2 M sodium acetate. After removal of the underlying mesophyll cell walls for 48 h at 37°C, the epidermis was recovered, washed in distilled water, and air dried in the copper loop (4 mm in diameter) used to recover the epidermis from the water. After the drying period the epidermis was cut from the loop, mounted in carbon dag on stubs and left overnight in a dessicator. This dried mount was trimmed to the level of the carbon dag with a sharp razor blade.

Removal of epoxy resin from sections

Fixation schedule 2 was used for this method as described for TEM. Cross-sections of resin embedded (Spurr 1969) leaf material (ca. 100 nm thick) were made with a Reichert-Jung Ultracut E microtome using a diamond knife. Sections were mounted on coverslips and dried on a hot plate (60°C) for 1 h. The treating solution was prepared as follows: Stock solution contained: 18-crown-6, 0.13 g (Sigma Chemical Co., Cat.No. 123H7702), DMSO, 9.9 ml and 0.1 ml distilled water. To prepare the working solution, 30% (w/w) methanolic potassium methoxide (0.3 ml) was added to 10 ml of stock solution (Iwadare *et al.* 1990). Since the activity of the working solution decreases rapidly with storage, working solutions were freshly prepared each day. Treatments with the resin removing solution were conducted for 5 - 60 min at room temperature. Slides were thoroughly rinsed with distilled water and left to air dry.

Coating and sample viewing

Samples were coated with chromium (ca. 3 - 4 nm thick) in a Gatan Ion Beam coater model 681. During coating the samples were rocked (0 - 15°) and rotated continuously. For backscatter electron imaging the samples were first coated (ca. 3 nm thick) unidirectionally with chromium

at an angle of 45° and then with carbon using the rocking and rotating parameters as described above. Material was viewed with a JEOL F6000 in-lens field emission scanning electron microscope. Before the use of the JEOL F6000 a JEOL 840 SEM was used to find specimens on the coverslips after resin was removed.

RESULTS AND DISCUSSION

Previous research on the tobacco cuticle has focused mainly on the chemical aspects (Chang & Grunwald 1980, Severson *et al.* 1984, Arrendale *et al.* 1988). Little attention has been paid to the structure of leaf cuticles of mesophytic crop plants such as *Nicotiana tabacum* L., and its relation to their water stress resistance. One of the first studies on the ultrastructure of the cuticle of *N. tabacum* and an evaluation of different preparation techniques for TEM studies of tobacco leaf cuticles, was that by Krüger *et al.* (1996). Their study showed that the leaves of *N. tabacum* exhibit a typical bilayered cuticular membrane (CM), composed of a thin outer cuticle proper (CP) and a thicker cuticular layer (CL). The CP consisted of electron-opaque lamellae alternating with electron-lucent lamellae, while a reticulate pattern was discernible in the CL. The cell wall (CW) appeared electron opaque after contrasting with uranyl acetate and lead citrate (Krüger *et al.* 1996). The terminology proposed by Sitte and Rennier (1963), Wattendorff and Holloway (1980) and Holloway (1982) will be used to describe the fine structure of *N. tabacum* cuticle as observed with the transmission electron microscope work. The basic structural concepts of the CM and CW reflected by TEM and light microscopy (LM) are applied to describe the gross morphology of the epidermis wall studied with FE-SEM.

The ultra-thin epoxy sections, from which resin was removed, revealed the basic structure of CM and CW in the FE-SEM investigation. However, the CM could not be distinguished into the CP and CL (Fig. 1), as was the case with TEM. The CW was distinctly stratified (Fig. 1). The stratification could only be faintly distinguished in the CW studied with TEM (Fig. 2). Differences between the TEM and the FE-SEM observations became evident when studying the thin resin embedded sections. Silver and gold sections were used both for TEM and FE-SEM. The only difference in preparation was contrasting of the sections for TEM and removal of resin for FE-SEM. At high magnification the CM appeared amorphous with a globular structure visible (Fig. 3) and the cell wall revealed a reticulate structure (Fig. 4). Previous results indicated that washing tobacco leaves with 100% acetone, chloroform and methanol removed both intra- and epicuticular waxes (Chu & Tso 1968, Chang & Grunwald 1980, Severson *et al.*

1981, Reed 1982, Severson *et al.* 1984, Arrendale *et al.* 1988). In this investigation the fresh leaf material was washed in 100% chloroform and then in 100% methanol and prepared with plunge freezing. Yet, after this drastic pretreatment the CM was still discernible with the globular structure (Fig. 5). The same results were obtained when fresh leaf material was washed with 100% acetone and frozen in LN₂ (Fig. 6). As acetone is an effective dehydration agent, there ought not to have been much water left in the tissue that could have resulted in ice crystal damage due to the slower freezing rate in LN₂. These harsh methods indicate that the layer described as the CM studied with the FE-SEM is not the epicuticular wax layer and that the globular structure is consistent.

Specimens for electron microscopy can be prepared without any chemical treatment by cryofixation (Ryan 1992). The aim of cryofixation is to arrest and stabilise the structure, processes and composition of biological material by rapid removal of thermal energy (e.g. by freezing) (Hall & Hawes 1991). Plunge freezing was employed in this study as it was important to preserve the epidermal wall as near to the *in vivo* state as possible and to fix specimens by different methods to minimise interpretation of data that may be artefacts caused by only one preparation procedure. The hydrocarbon propane coolant used in the present study freeze at –180°C. This liquid is an efficient coolant mainly because there is a wide range between the freezing and boiling point. The disadvantage of propane is its safety aspects as it is flammable (Barnard 1987, Ryan 1992).

Cryofixation of fresh leaf tissue gave similar results to those observed with the resin embedded sections for TEM and FE-SEM. The CM and CW were discernible but again as for the resin embedded sections studied with the FE-SEM neither the CP nor the CL was discernible at high magnification (Fig. 7). The globular structure of the CM after plunge freezing is clearly discernible in Fig. 8. The globular structure may be the consequence of freezing at -180°C as the cuticle may have lost its elasticity. This might have induced a clean fracture in the CM, when the specimens were fractured with a sharp razor blade after freezing.

As a hypothesis to explain the fracture plane, we suggest there could be a resemblance in the spatial size of the globular structure and the fibrils in the CL. Thus the fibrillar structure of the CL could be the plane where fracturing occurred after freezing. The CW again revealed a stratified appearance (Fig. 9) as also observed in Figures 1 and 2. This is more prominent in Fig.

10 where microfibrils protrude from the CW. These microfibrils had a diameter of ca. 30 nm which corresponds to that of cellulose microfibrils of cell walls (Lüttige & Higinbotham 1979). According to Salisbury & Ross (1992), about 30 to 40 pairs of long, unbranched cellulose molecules form the microfibril, while Lüttige and Higinbotham (1979) stated that about 40 up to 300 cellulose molecules are joined together in the microfibrils. If the small spots discernible in the CW (Fig. 11) with TEM work (see Krüger *et al.* 1996) are cellulose molecules (which are ca. 5 nm diameter for tobacco), it could mean that there are 36 cellulose molecules in the microfibrils of tobacco. This hypothesis is compatible with both the TEM and FE-SEM results, and shows how these techniques complement one another. The FE-SEM work revealed the gross morphology of the microfibrils while the TEM showed the cellulose molecules in the cross sections of the cell wall.

The globular structure could also be distinguished with the epidermis enzymatically isolated (Fig. 12). A globular structure manifested even though the specimen was not frozen but air-dried. In this regard, it is interesting to note that Viougeas *et al.* (1995) concluded that isolated cuticles presented no structural alteration compared to non-isolated cuticles and, therefore, isolated cuticles could be used for permeability studies. A possible explanation could be that the cuticle is elastic and tears like rubber in the same plane as the fracture planes of frozen specimens when they are trimmed. However, the cell wall appeared at low magnification to have a flaky appearance (Fig. 13) as if the strata pulled away from each other. Fig. 14 shows a clear presentation of the cell wall morphology. The stratification is discernible with some strata revealing a network of microfibrils while others reveal the matrix in which the microfibrils are embedded. The microfibrils in Fig. 13 and Fig. 14 are ca. 30 nm in diameter, which coincides with that of cellulose microfibrils (Lüttige & Higinbotham 1979). The cell wall materials of tobacco contain 34% pectin, 21.6% protein, 18.7% cellulose, 11.4% hemicellulose and 4.1% lignin (Ruben & Bokelman 1989). The matrix consists of hemicelluloses and the pectin (Lüttige & Higinbotham 1979, Goodwin & Mercer 1983). The matrix also contains glycoproteins, with the protein fraction particularly rich in amino acid hydroxyproline (Lüttige & Higinbotham 1979, Darvill *et al.* 1980). Pectin is the major component found in the primary cell walls of dicotyledonous (Ruben & Bokelman 1989, Darvill *et al.* 1980) with a galacturonic acid content of ca. 80% for tobacco pectin (Ruben & Bokelman 1989). It is not clear from the literature whether the pectin layer is an integral part of the cuticular layer, an additional layer of the CM, or a region of the epidermal wall (Holloway 1982). With the FE-SEM no layer could be identified as being the definite pectin layer. Images of plunge frozen material, imaged with

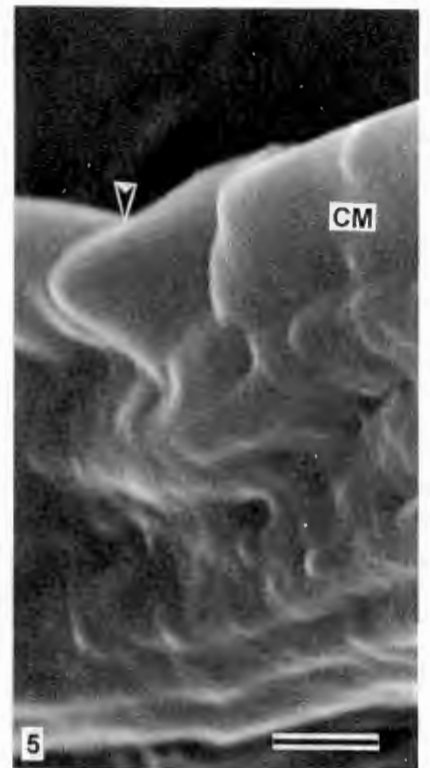
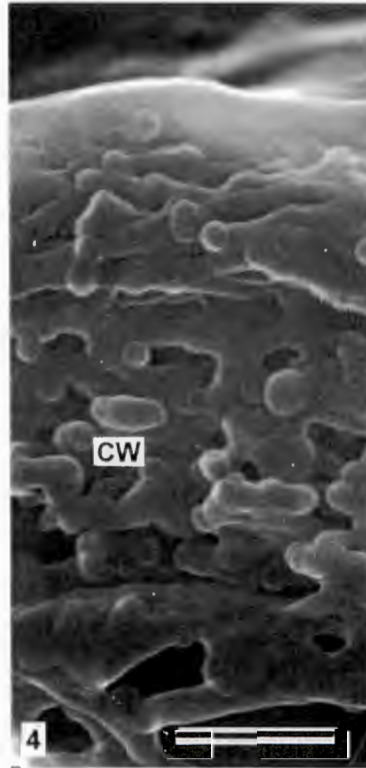
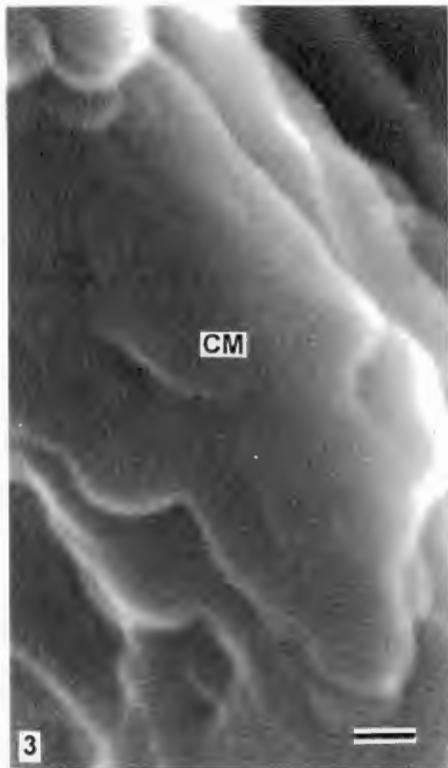
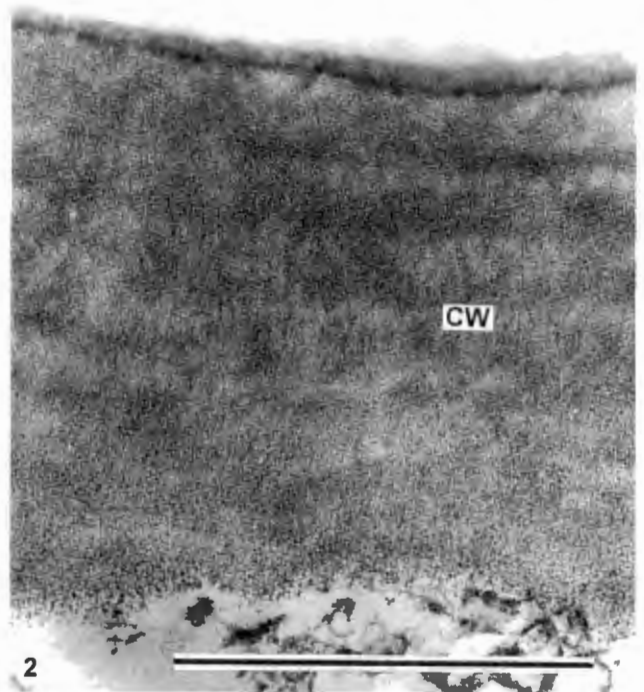
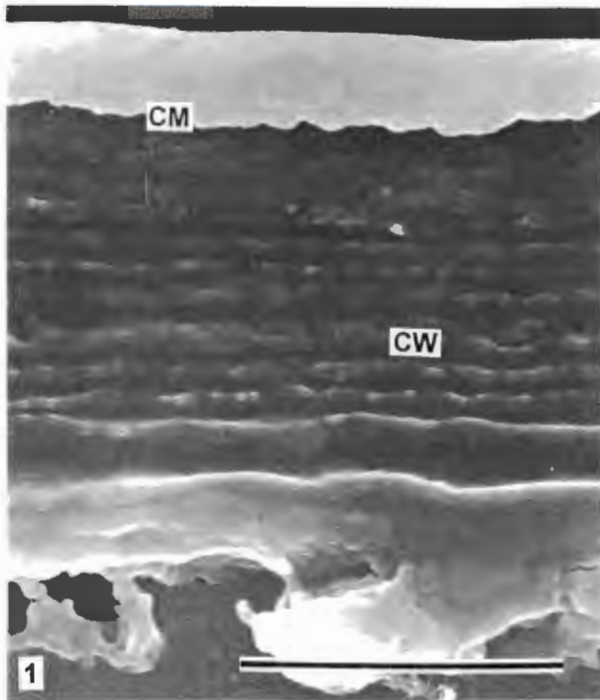


Fig. 1. Resin was removed from ultra-thin sections with 18-crown-16 and viewed with the FE-SEM. The cuticular membrane (CM) is not distinguished into the cuticle proper and cuticular layer. The cell wall (CW) reveals a stratified structure viewed with TEM. Bar = 1 μ m

Fig. 2. The cell wall (CW) displayed a faintly stratified structure viewed with TEM. The specimen was prepared with a primary fixation of 2% OsO₄ vapour and post-fixation in aqueous 2% OsO₄ and embedded in epoxy resin. Bar = 1 μ m

Fig. 3. Epoxy resin was removed from ultra-thin sections with 18-crown-16 and viewed with the FE-SEM. The cuticular membrane (CM) shows a globular structure at high magnification. Bar = 10 nm

Fig. 4. The cell wall (CW) reveals a reticulate structure after resin was removed. Bar = 100 nm

Fig. 5. Material was washed with 100% chloroform and methanol and plunge frozen. The globular structure (arrow) of the cuticular membrane (CM) was discernible. Bar = 100 nm

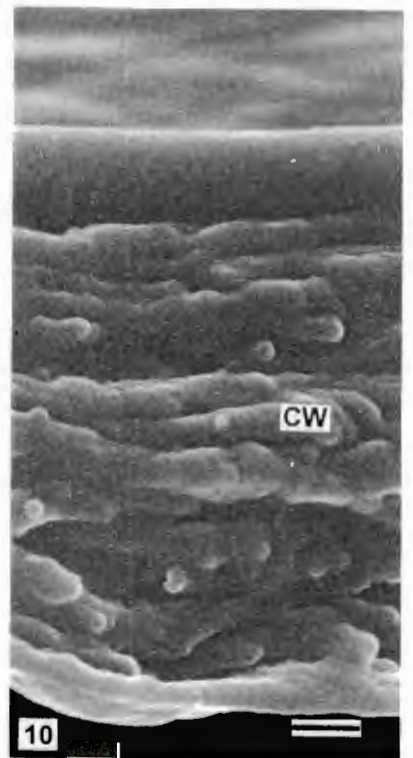
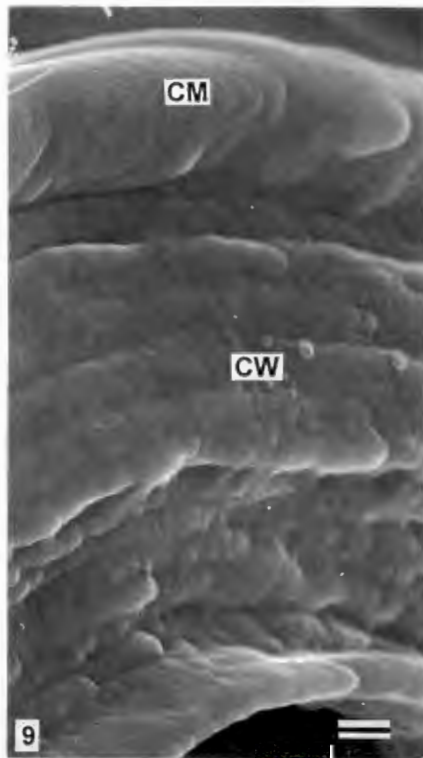
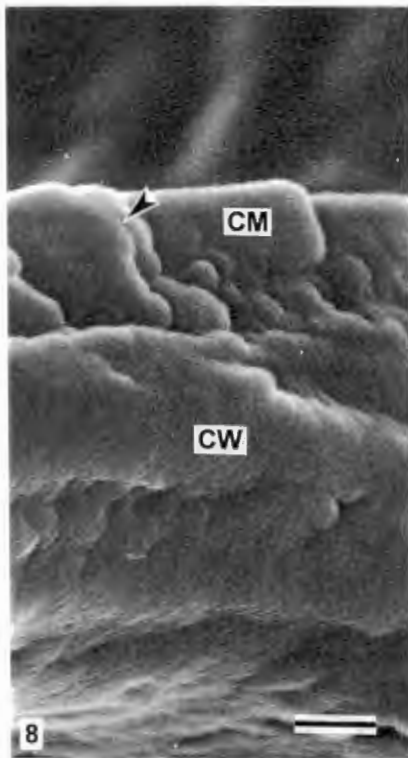
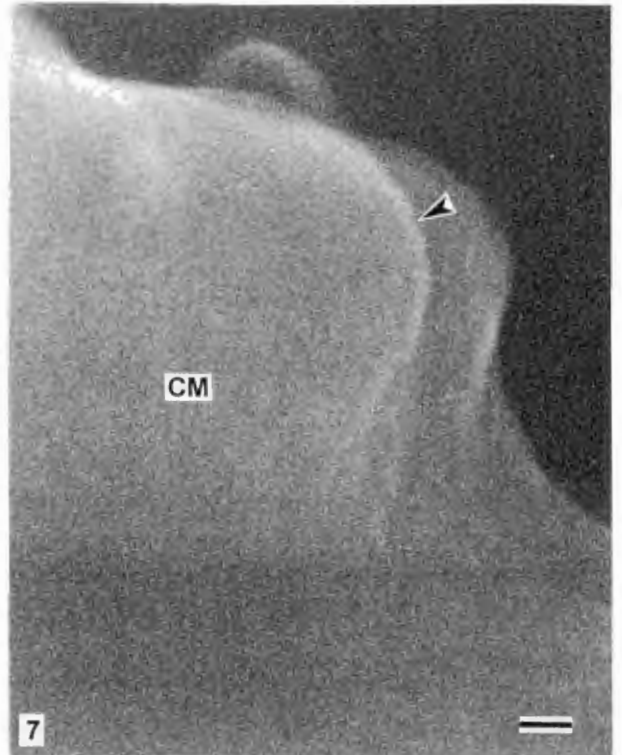
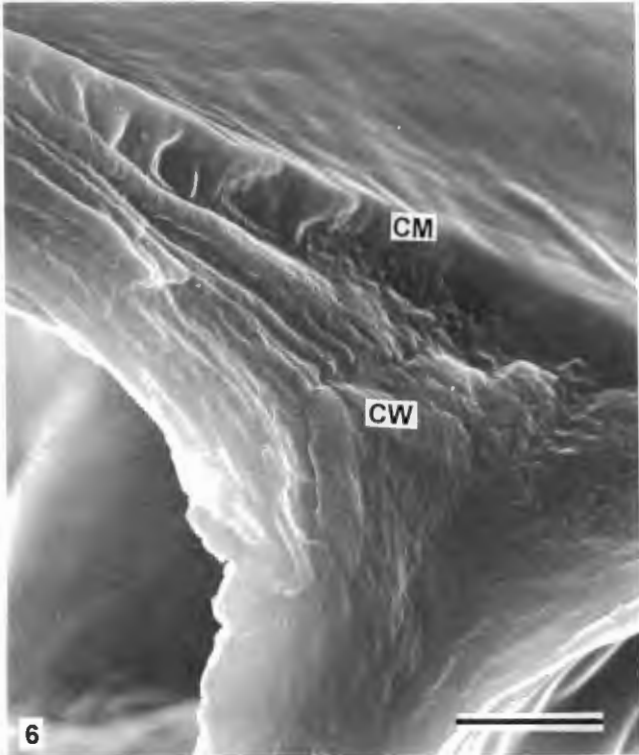


Fig. 6. The specimen was washed in 100% acetone and frozen in LN₂. The globular structure of the cuticular membrane (CM) is again evident. The CM covers the cell wall (CW) of two epidermal cells of tobacco. Bar = 1 μ m

Fig. 7. Plunge freezing showing the globular structure (arrow) of the cuticular membrane (CM) at high magnification, with no CP or CL visible. Bar = 10 nm

Fig. 8. The cell wall (CW) and cuticular membrane (CM) with the globular structure (arrow) are visible after plunge freezing. Bar = 100 nm

Fig. 9. Plunge freezing reveals a stratified cell wall (CW) and cuticular membrane (CM). Bar = 100 nm

Fig. 10. The cellulose microfibrils (ca. 30 nm) are visible in the cell wall (CW). Bar = 100 nm

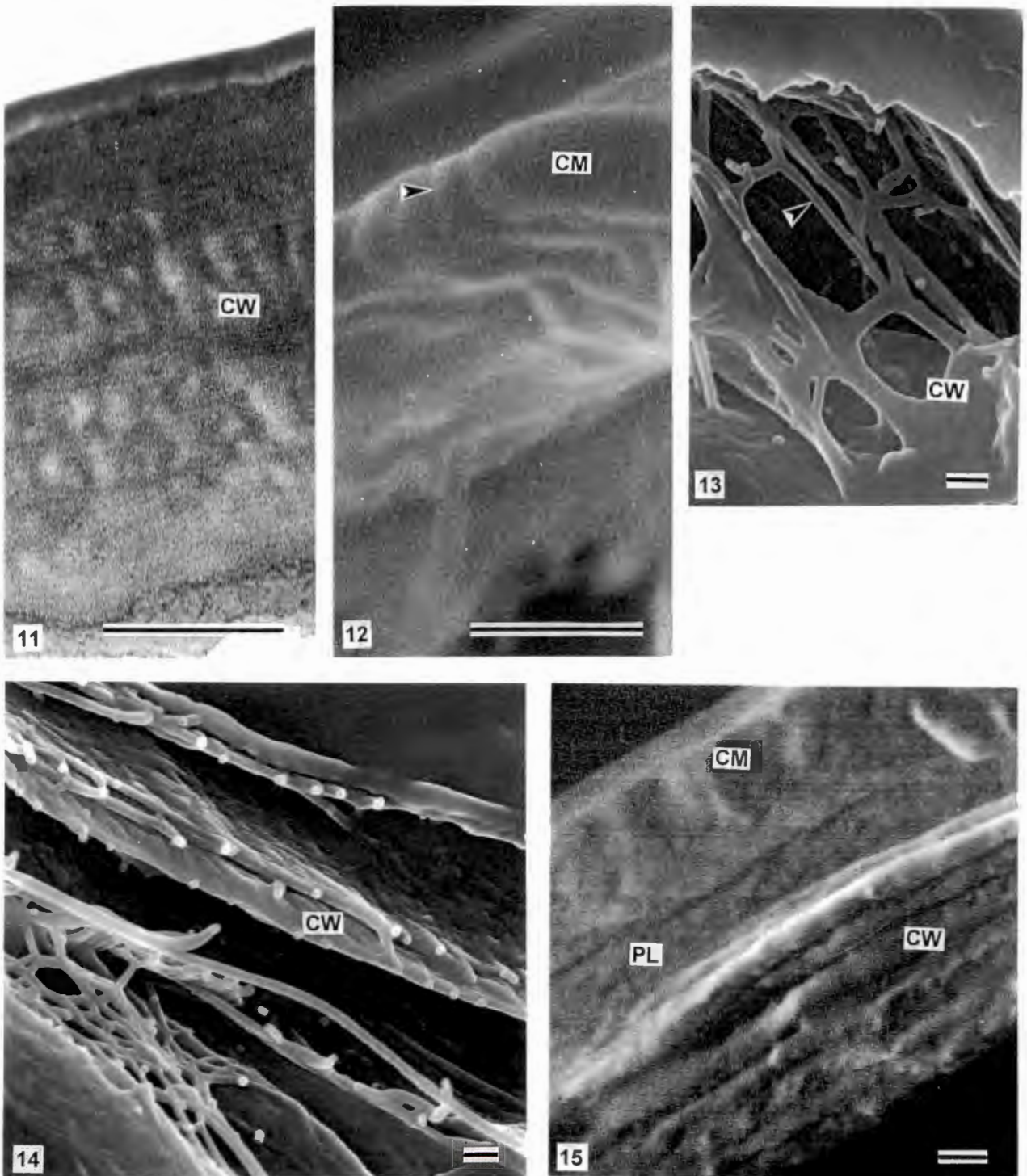


Fig. 11. The method of Pacini *et al.* (1992) was used to prepare the specimen including post-fixation with 0.5% OsO₄ for TEM. The cell wall (CW) reveals the stratified structure with electron-lucent spots, which may represent cellulose microfibrils. Bar = 500 nm

Fig. 12. A globular structure (arrow) in the cuticular membrane (CM) is visible. Bar = 100 nm

Fig. 13. The cell wall (CW) strata which pulled away from one another during enzymatic isolation display possible cellulose microfibrils (arrow). Bar = 100 nm

Fig. 14. The cell wall (CW) is differentiated into strata revealing the possible cellulose microfibrils (arrow), displaying an open network without matrix, while the first few strata reveal microfibrils with matrix. Bar = 100 nm

Fig. 15. This specimen was plunge frozen and imaged with backscatter electrons in the FE-SEM. The cuticular membrane (CM) and cell wall (CW) can be distinguished. Is it possible that the layer between the CM and CW might be the pectin 'layer' (PL). Bar = 100 nm

backscattered electrons, might reveal a pectin layer visible between the CM and CW (Fig. 15) indicating that this layer is either part of the CM or CW. Therefore, the distribution and function of pectin are no longer compatible with the idea that it is simply a gel (Roberts 1990). Future FE-SEM work with the emphasis on pectin may reveal the pectin morphology. Recent work on the pectin layer proved that boron is associated with cell wall pectins and the contingent effects of boron on cell wall extensibility suggest that boron plays a critical, although a poorly defined, role in the cell wall structure of higher plants (Hu & Brown 1994).

As with the TEM, no epicuticular wax was discernible with any of the methods used in this study. Surface wax is present even on species such as *N. tabacum* that has been classified as non-wax bearing (Hall 1967). In many species it occurs as a relatively thin structureless sheet (Hall 1967). Baker (1982) stated that the aerial surfaces of all higher plants carry a partial or continuous coverage of amorphous wax. Although epicuticular wax of tobacco is not visible, chemical analysis indicates that the epicuticular waxes exist, with major wax components being C₂₅-C₃₆ normal and branched chain aliphatic hydrocarbons, diterpenes, sucrose esters and wax esters (Severson *et al.* 1984, Arrendale *et al.* 1988). Thus even though chemical analyses indicate that tobacco epicuticular wax exists on the leaf surfaces, the high resolution FE-SEM could not reveal this thin wax layer in this study.

CONCLUSION

The TEM has provided a wealth of new structural information about the plant CM and CW, but it has not always confirmed some of its most characteristic features in the light and polarising microscopes (Holloway 1982). The FE-SEM work in which different methods were applied revealed a globular structured CM, which does not consist of wax layers, and a stratified CW, with microfibrils of ca. 30 nm in diameter. The position of pectin in the cell wall could not yet be determined in this study with the use of the FE-SEM. Pectin may have an important structural function in the cell wall and may facilitate permeability and is thus no longer compatible with the idea that it is simply a gel. Future studies are necessary to determine the position and function of pectin in the cell wall. The results obtained with the FE-SEM revealed three-dimensional structural information about the CM and CW not previously observed, which could have a direct bearing on the water transporting properties of the cuticle and cell wall. However, more detailed information is necessary on the epidermal wall ultrastructure to explain epidermal transpiration.

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CHAPTER SIX

THE ADAXIAL EPIDERMAL WALL TRANSPIRATION PATHWAY IN TOBACCO LEAVES

ABSTRACT

Understanding cuticular transpiration is hampered by the lack of knowledge regarding the ultrastructure of the cuticular membrane and of the factors contributing to the permeability resistance of the epidermal wall. The main aim of this study was to determine whether FE-SEM investigations present new information on the three dimensional ultrastructure of the epidermal wall to explain the cuticular transpiration pathway. Tobacco leaf material was studied with TEM and FE-SEM using OsO_4 and aldehyde fixation, enzymatic incubation, cryo-sectioning, plunge-freezing and a special method of coating with RuO_4 to make material conductive for SEM. RuO_4 coating rendered good results as far as microfibril dimensions were concerned. This method may be of value specifically when detailed ultrastructural studies at high resolution are required. The cuticular membrane is not amorphous, but consists of globules that are grouped together to form a membrane. The FE-SEM study of the tobacco epidermis revealed that the cuticular membrane consists of small globules grouped together within larger globules. Terminology is proposed for these structures, namely *nitis* globules for the small units and *nichrista* globules for the larger units. The fibrillar network within the cuticular membrane possibly forms the boundaries of these globules. The hypothesis is that this fibrillar network is the pathway for transport. Not only wax but also the fibrillar network may contribute to the tortuosity factor of permeability. In our opinion this model offers a potential explanation for the route of water movement through the cuticle, more so than the concept of an amorphous cuticle.

INTRODUCTION

The occurrence of cuticular transpiration (Kerstiens 1996) is an important factor for plant adaptation to drought stress in different arid parts of the world. Cuticular permeability is also of economical value with regard to foliar uptake of nutrients, growth regulators, pesticides and herbicides. One of the main problems associated with cuticular transpiration studies is the lack of a profound understanding of the physical structure of the transport-limiting barrier of the epidermal wall (Riederer & Schneider 1990). Many authors have studied the ultrastructure of the cuticular membrane (Priestly 1943, Martin & Juniper 1970, Heide-Jørgensen 1978, Wattendorff & Holloway 1980, Mérida *et al.* 1981, Riederer & Schönherr 1988, Kim & Mahlberg 1995, Krüger *et al.* 1996 and Jeffree 1996) as well as the permeability of the cuticle (Kerstiens 1996 and authors listed therein) but structure and function are not often related in these studies. Schönherr and Mérida (1981) as well as Van Rensburg and Peacock (1998) found that permeability, measured by using the composite epidermal wall, reflects the properties of the cuticular membrane including the wax layer. A logical conclusion seems that the main barrier of permeability lies within the cuticle, but this is apparently not the case. Although it may seem as if the cell wall does not offer a high resistance to permeability, the degree of resistance and the factors contributing to this resistance should be taken into account. The cell wall participates in the epidermal wall system through which water and solutes are transported from the plant to the atmosphere and *vice versa*.

The leaf surface micro-morphology (Van Rensburg *et al.* 1999) together with the epidermal wall ultrastructure also plays an interactive role in regulating the transcuticular movement of substances between the plant and the environment. The majority of the work reported in the literature focuses on either cuticle or cell wall ultrastructure and consequently ignores the integrated system of the total leaf surface that includes the epidermal wall and surface micro-morphology. The relation between function and ultrastructure has also been neglected in the past.

The plasma membrane was defined by Raven *et al.* (1992) as having several functions: (1) it mediates the transport of substances into and out of the protoplast, (2) it coordinates the synthesis and assembly of cell wall microfibrils and (3) it translates hormonal and environmental signals involved in the control of cell growth and differentiation. Important functions of the cell

wall are (1) control of morphogenesis, (2) exchange of metabolites between the protoplast and its surrounding environment, (3) maintaining of plant structure, (4) disease resistance and (5) lignin polymerisation. This implies that the plasma membrane should not be regarded only as surrounding the protoplasm, but that it lines the cell wall, making it close to what is regarded as an organelle. An organelle [Gk. Organelle, a small tool] is a specialised membrane-bound part of a cell (Raven *et al.* 1992) and each organelle in the cytoplasm is the site of specific chemical processes (Salisbury & Ross 1992). For a long time the cell wall as Fahn (1990) stated, has been considered a non-living component of the plant cell, but the relationship between the cell wall and plasma membrane was underestimated. Consequently the cell wall, in conjunction with the plasma membrane, should be regarded as an integrated specialised system that may function as an organelle.

This paper focuses on the role of trichome exudate, cuticular membrane with waxes, as well as the cell wall, in terms of how structure and function relate to permeability. In the past, emphasis has only been on the cuticular membrane transpiration. The fact that the leaf surface of plants is complex makes it even more plausible that the intracuticular wax should not be the only component to be taken into account when considering cuticular membrane resistance.

The aim of this study was to investigate and discuss (1) the three dimensional ultrastructure of the epidermal wall, (2) the possible transport pathway for water and solutes through the epidermal wall, (3) whether the permeability resistance is only a function of the wax, (4) the possible mechanism by where by cutin and wax are transported to the surface, (5) whether the ontogenetic study of the cuticle is useful for permeability studies and (6) if there is merit in using epidermal wall permeability as an aid in identification of drought tolerant genotypes.

MATERIAL AND METHODS

Plant Material

Nicotiana tabacum L. plants were grown in pots under glasshouse conditions with optimal water and standard nutrient solution application. The drought sensitive cultivar TL33 and drought tolerant cultivar Elsoma were used. Leaves from the first, third, fifth and twelfth node were selected. The leaf of the fifth node is regarded as a young mature leaf before the onset of

senescence. Material for microscopy, 1 mm² in size, was cut from the middle of the leaf, between the main vein and the margin, avoiding large side veins. A mature leaf from *Cussonia paniculata* Eckl. & Zeyh. was collected from a tree growing in natural conditions in the garden on the campus of Pretoria University, South Africa. Tissue pieces were collected from the middle of the lamina from the centre lobe of the hand shaped leaf.

Preparation for Transmission Electron Microscopy

Three fixation schedules were carried out at room temperature, unless otherwise noted. (a) Primary fixation with vapour from a 1% aqueous OsO₄ solution for 2 h followed by post-fixation with 1% aqueous OsO₄ for 30 min (Krüger *et al.* 1996). (b) Primary fixation with 2.5% glutaraldehyde or 1% formaldehyde in 0.075 M phosphate buffer (pH 7.4) for 1 h followed by post-fixation with 1% aqueous OsO₄ for 30 min. For both schedules, (a) and (b), material was rinsed in phosphate buffer after aldehyde fixation and in distilled water after OsO₄ fixation. (c) Primary fixation in 2% aqueous OsO₄ + 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 4°C and post-fixation in 2% aqueous OsO₄ for 2 h at 4°C (Franke *et al.* 1969). All material was dehydrated in a series of ethanols (30, 50, 70, 90 and 100%, for 15 min in each concentration and three times in 100% ethanol). Samples were embedded in epoxy resin (Spurr 1969) or modified Quetol 651 epoxy resin (Van der Merwe & Coetzee 1992).

Ultra-thin cross sections were made with a Reichert Jung Ultracut E microtome using a diamond knife and contrasted with 2% aqueous uranyl acetate for 10 min and lead citrate (Reynolds 1963) for 2 min. Sections were viewed with a Philips CM 10 TEM.

Enzyme extraction

Cellulase (10 mg cellulase per 1 ml buffer) dissolved in 0.075 M phosphate buffer at pH 5 was used. Small pieces of fresh material were dissected directly in the buffer. The samples were put into vials with 0.5 ml of enzyme solution for 15 min. After incubation at room temperature (ca. 23°C) the tissue was rinsed three times in the phosphate buffer (pH 5), then rinsed once in phosphate buffer (pH 7.4). Material was fixed in 2.5% glutaraldehyde + 2% formaldehyde in 0.075 M phosphate buffer (pH 7.4) for 30 min, rinsed three times in phosphate buffer, post-fixed in 1% aqueous OsO₄ for 20 min, rinsed in distilled water, dehydrated in a series of ethanols and embedded in modified Quetol 651 epoxy resin (Van der Merwe & Coetzee 1992).

Cryo-Sectioning

Small pieces of leaf material were fixed in 2.5% formaldehyde buffered with 0.075 M phosphate buffer, pH 7.4, for 1 h and then washed three times with 0.075 M phosphate buffer (pH 7.4). During fixation the abaxial epidermis was removed with sandpaper to aid the penetration of the cryo-protectant. Material was cryo-protected with 30% polyvinylpyrrolidone (PVP) for 2 h and mounted on stubs. Specimens were plunge-frozen in liquid propane at -180°C with a Reichert KF 80 plunge-freezer. Sections were made at -120°C with a cryo-microtome, picked up with a drop of 2.3 M sucrose and transferred to a carbon coated formvar grid. The grid with sections was floated on 0.075 M phosphate buffer to remove the sucrose. Sections were immersed in methyl cellulose containing 0.1% uranyl acetate for 10 min. Grids were blotted on filter paper to drain excessive methyl cellulose and dried at room temperature.

Preparation for Scanning Electron Microscopy

Plunge-Freezing

The detail of this method was described by Peacock *et al.* (1998) and will only be briefly discussed here. Leaf samples were mounted upright on a graphite stub with conductive carbon cement and was plunge frozen in liquid propane at -180°C in a Reichert KF 80 plunge freezer. The stub was transferred under liquid nitrogen to a pre-cooled copper block where the upper parts of the tissue were fractured with a sharp razor blade. The copper block was transferred to a Fisons high vacuum unit and evacuation started immediately. After ca. 1 h, the vacuum was 0.0133 Pa and the temperature of the copper block still below -120°C . Freeze-drying was done overnight. The next morning the temperature of the copper block was ca. -10°C . The block was heated to 25°C before admitting air into the unit. Samples were coated for 6 min with chromium (Cr) in a Gatan model 681 ion beam coater, or *via* a chemical coating with RuO_4 vapour for 1 - 2 h in a closed petri dish (Van der Merwe & Peacock 1998). Samples were viewed in a JEOL 6000F in-lens field emission scanning electron microscope (FE-SEM).

Enzyme extraction

- (1) Cellulase was dissolved in 0.075 M phosphate buffer at pH 5 (10 mg cellulase per 1 ml buffer). Two different fixation procedures were followed in combination with cellulase

incubation. (a) 2.5% formaldehyde fixation, followed by 20 min cellulase incubation and post-fixation in 2.5% glutaraldehyde and 1% aqueous OsO₄ for 30 min in each. (b) Unfixed material was incubated in cellulase for 20 min and then fixed with 2.5% glutaraldehyde and 1% aqueous OsO₄ for 30 min in each.

- (2) Cellulase was dissolved in 0.075 M phosphate buffer at pH 5 (10 mg cellulase per 1 ml buffer) and pectinase was dissolved in 0.075 M phosphate buffer at pH 4 (1 mg cellulase per 1 ml buffer). Material was incubated in pectinase followed by cellulase. Thereafter the material was fixed with 2.5% glutaraldehyde followed by 1% aqueous OsO₄ for 30 min in each.
- (3) Material was first fixed with 2.5% formaldehyde, followed by post-fixation in 2.5% glutaraldehyde for 30 min in each. The abaxial leaf epidermis was removed by carefully rubbing the surface with sandpaper to improve enzymatic incubation. Pectinase was prepared at a low concentration of 0.01 mg pectinase per 1 ml phosphate buffer (0.075 M) at pH 4, with a short incubation time of 5 min. Material was post-fixed with 2.5% glutaraldehyde for 30 min.

For enzymatic incubation the material was put into vials containing 0.5 ml of enzyme at room temperature. After every enzymatic incubation, the tissue was rinsed three times in phosphate buffer. After glutaraldehyde and formaldehyde fixation, material was washed in 0.075 M phosphate buffer (pH 7.4), while for OsO₄ fixation washing was done three times in distilled water. Material was dehydrated in a series of ethanols (30% - 100%) and critical point dried (CPD). Tissue pieces were mounted on graphite stubs, coated with Cr or RuO₄ vapour and viewed with a FE-SEM.

RESULTS

The terminology proposed by Sitte and Rennier (1963), Wattendorff and Holloway (1980) and Holloway (1982) is used with additions where appropriate to describe the fine structure of the tobacco leaf epidermal wall.

Cuticle Ontogeny

Procuticle

At the very earliest stages of epidermal wall development an osmiophilic procuticle (Fig. 1) was visible in leaf no. 1. A substance that may be either trichome exudate or epicuticular wax, accumulated on the young leaf surface (Fig. 1). The procuticle appeared flocculent at its transition to the cuticle proper (CP) (Fig. 2). With the transition of the procuticle to the CP as seen in leaf no. 3, only two electron-dense lamellae, separated by two electron-lucent lamellae were discernible (Fig. 2). During the CP development, an electron-dense layer bounded the CP and cell wall (CW) (Fig. 3). The CP, initially visible as a very electron-dense zone (Fig. 3), increased in thickness (Fig. 4) and became more electron-lucent compared to the procuticle (Fig. 1). Lamellae formed in later stages were not necessarily parallel to the first formed lamellae (Fig. 5). The electron-lucent lamellae exhibited a constant thickness with a width of approximately 5 nm, while the width of the electron-dense lamellae varied between 5 to 8 nm (Fig. 15). The total width of the CP of the tobacco leaf was ca. 40 nm.

Electron-dense globules with lamellae were visible within the CW, underneath the CP, enclosed in a very thin electron-dense envelope (Fig. 6), while other electron-dense globules had no lamellae but were enclosed in a thick electron-dense envelope (Fig. 9). It appeared as if the globules accumulated under the CP and if they might be responsible for transport of material to the developing cuticle proper or cuticular layer (Fig. 7). When viewed with the FE-SEM (Fig. 8) the globules appeared to be attached to the cuticular membrane, forming an integral part of the cuticular membrane. Globules without lamellae were discernible among the fibrils of the primary CW in leaf no. 3 (Fig. 4) and leaf no. 5 (Fig. 10). No globules were discernible in the old leaves (leaf no. 12) (Fig. 5).

Epicuticular Wax

A thin electron-lucent epicuticular wax layer was discernible, adcrusted to the cuticle (Fig. 5). This wax layer was visible as an electron-lucent space between the cuticle and secreted exudate (Fig. 5) and was approximately 20 nm thick in the old tobacco leaf (leaf no. 12). After cellulase incubation, the TEM (Fig. 11) and FE-SEM (Fig. 12) revealed droplets in leaves no. 3 (Fig. 12) and 5 (Fig. 11). These droplets accumulated onto the cell wall fibrillar network after cellulase

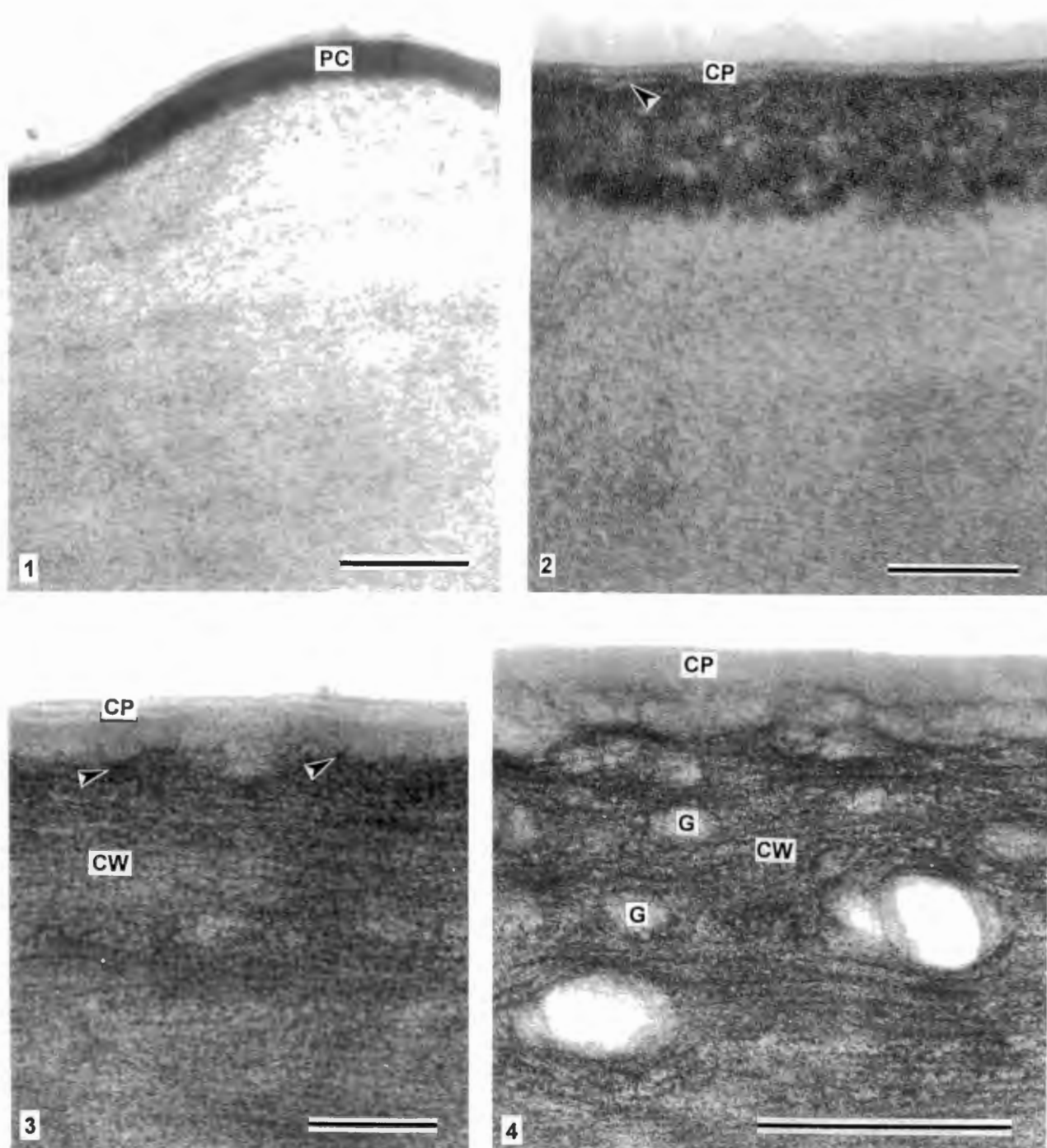


Fig. 1. Very young leaf (leaf no. 1) with electron-dense procuticle (PC) as the first development of the cuticle. Primary fixation with 1% aqueous OsO_4 vapour for 2 h followed by post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

Fig. 2. Young leaf (leaf no. 3) showing the transition of the procuticle to the cuticle proper (CP) and the first lamellae (arrow). Primary fixation with 1% aqueous OsO_4 vapour for 2 h followed by post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

Fig. 3. A young leaf (leaf no. 3) with an electron-dense layer (arrows) between the cuticle proper (CP) and cell wall (CW). Primary fixation with 1% aqueous OsO_4 vapour for 2 h followed by post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

Fig. 4. Leaf no. 3 reveals the cuticle proper (CP) that increases in thickness and is less electron-dense than the procuticle. Globules (G) are discernible within the interstices of the cell wall (CW). Primary fixation with 1% formaldehyde in 0.075 M phosphate buffer for 1 h, post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in modified Quetol 651 epoxy resin. Bar = 200 nm

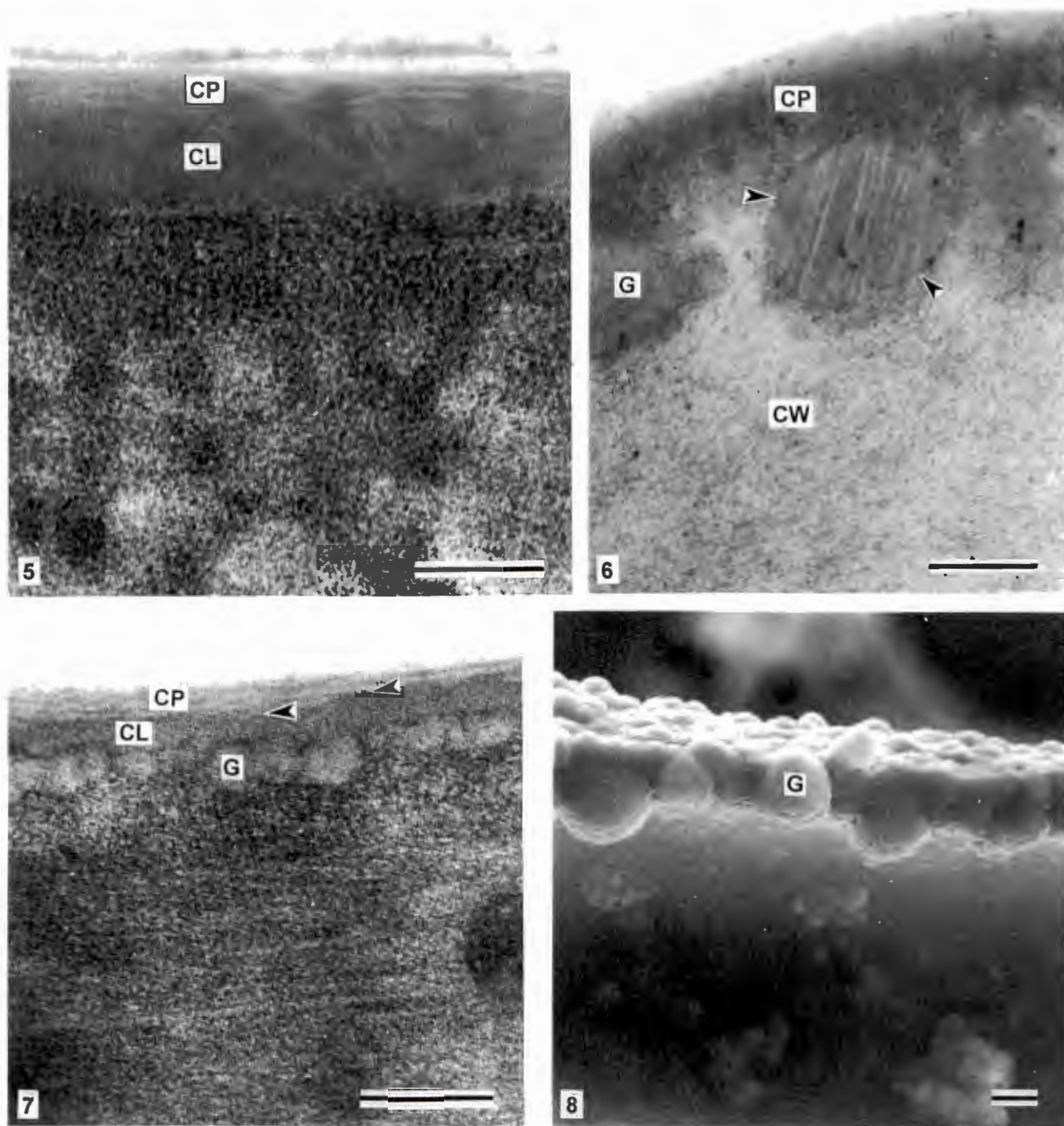


Fig. 5. Old leaf (leaf no. 12) with cuticular layer (CL) and cuticle proper (CP). Lamellae added to the cuticle proper (CP) during the later stages of development are not always oriented periclinally. Primary fixation with 1% aqueous OsO_4 vapour for 2 h followed by post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

Fig. 6. A young mature leaf (leaf no. 5) shows electron-dense globules (G) within the cell wall (CW) underneath the cuticle proper (CP). Some of the globules include electron-lucent lamellae. The globules are enclosed by a very fine electron-dense envelope (arrows). Primary fixation with 1% formaldehyde in 0.075 M phosphate buffer for 1 h, post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in modified Quetol 651 epoxy resin. Bar = 100 nm

Fig. 7. In young leaves (leaf no. 3) the globules (G) start the construction of the cuticle proper / cuticular layer zone (arrows). The lamellate cuticle proper (CP) is discernible with the first development of the cuticular layer (CL). Primary fixation with 1% aqueous OsO_4 vapour for 2 h followed by post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

Fig. 8. Globules (G) are grouped together to form the cuticular membrane in leaf no. 3. Material was incubated in pectinase followed by cellulase, 20 min each. Primary fixation after enzymatic incubation was 2.5% glutaraldehyde and post-fixation 1% aqueous OsO_4 for 30 min each. Material was CPD, coated with Cr and viewed with FE-SEM. Bar = 100 nm

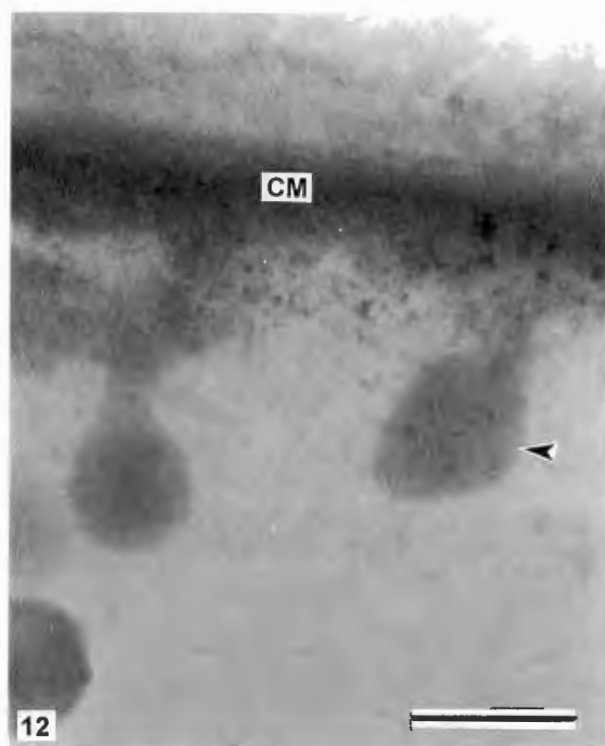
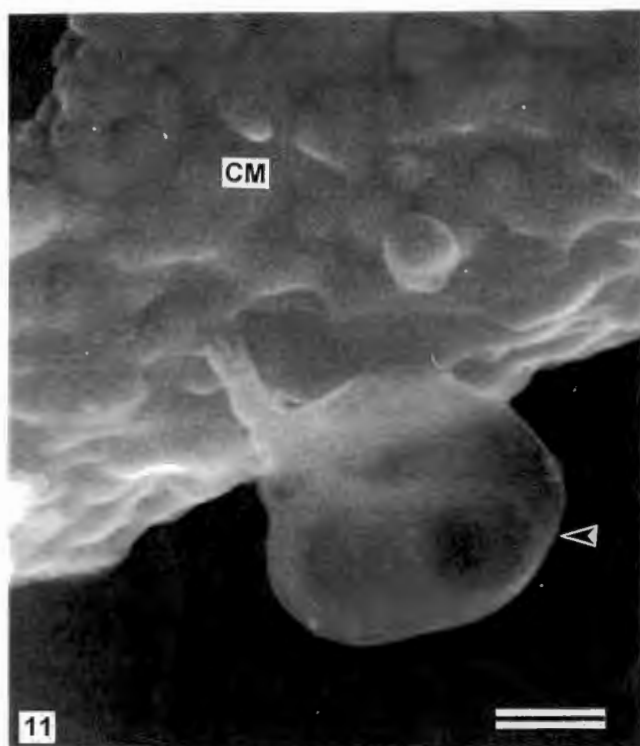
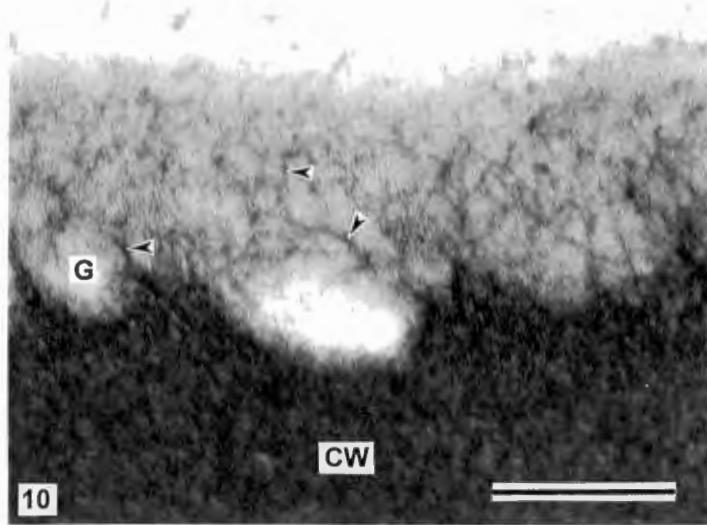
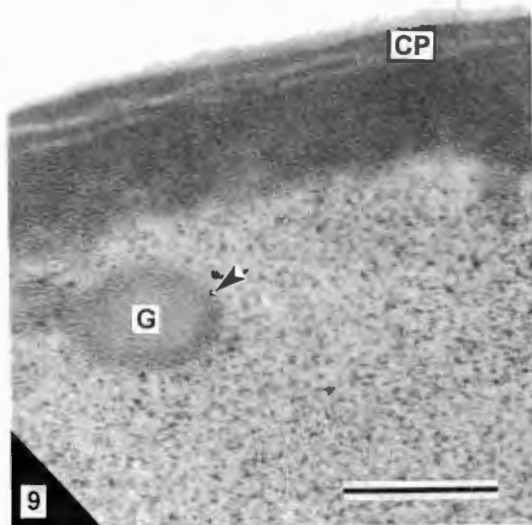


Fig. 9. Young mature leaf (leaf no. 5) reveals globules (G) that are enclosed in an electron-dense envelope (arrow). Lamellae are discernible in the cuticle proper (CP). Primary fixation with 1% formaldehyde in 0.075 M phosphate buffer for 1 h, post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in modified Quetol 651 epoxy resin. Bar = 100 nm

Fig. 10. In leaf no. 5 electron-dense fibrillar cell wall material (arrows) is held apart by the globules (G) that accumulate within the cell wall (CW). Primary fixation with 1% formaldehyde in 0.075 M phosphate buffer for 1 h, post-fixation with 1% aqueous OsO_4 for 30 min. Embedded in modified Quetol 651 epoxy resin. Bar = 100 nm

Fig. 11. With enzymatic incubation droplets (arrow) are revealed in leaf no. 5 which may be wax droplets, hanging on the inner cuticular membrane (CM). Cellulase incubation for 20 min. With post-fixation of 2.5% glutaraldehyde for 30 min. Material was CPD, coated with Cr and viewed with FE-SEM. Bar = 100 nm

Fig. 12. Droplets (arrow) are hanging from the inner cuticular membrane (CM) after enzymatic incubation of leaf no. 5. Cellulase incubation for 20 min. Primary fixation after enzymatic incubation was 2.5% glutaraldehyde and post-fixation 1% aqueous OsO_4 for 30 min each. Material was embedded in modified Quetol 651 resin. Bar = 100 nm

incubation (Fig. 13). The amorphous wax layer of the tobacco leaf was thin and not always discernible with TEM or FE-SEM. A layer, which may be the thin epicuticular wax layer, was discernible (Fig. 14) in leaf material that was naturally dried and coated only with RuO₄.

Cuticular Membrane

The CM of the young mature tobacco leaf (leaf no. 5) did not appear layered when viewed with the FE-SEM, but a bilayered structure, composed of a lamellate CP (Fig. 15) and reticulate CL (Fig. 16), was observed when viewed with the TEM. A very thin cryo-section of the cuticular membrane revealed fibrils normally not visible in resin embedded material (Fig. 17). These fibrils extended the total width of the CM (Fig. 17) and enveloped the cutin substance so that they appeared globular. Occasionally the surface of the cuticle of tobacco (Fig. 19) as well as that of *Cussonia paniculata* (Fig. 20) revealed numerous small globular structures. In the tobacco sample (Fig. 19) the lower surface of the cuticle was anchored over a void created by the removal of material by enzymatic incubation and the stress created during dehydration and CPD caused the material between the globules to shrink differentially more than the globules. These small globules (Fig. 19 and 20) are probably subunits of the larger globules seen in Fig. 18. The CM was never observed as a bilayered structure (Fig. 18) with FE-SEM, thus no differentiation could be found between a CP and CM.

Cell Wall

The primary cell wall consisted of a microfibrillar network. Different regions or layers were observed. In the mature leaf (leaf no. 5) viewed with the FE-SEM, five layers were discernible (Fig. 18). Each layer consisted of fibrils arranged perpendicular to the wall surface (Fig. 21). Some of the wall fibrils were attached to and penetrated the CM (Fig. 22). The CM was loosened from the CW by the brief treatment with cellulase to reveal the CW as consisting of fibrils (Fig. 23). Tubercular material accumulated between the CM and CW treated with cellulase (Fig. 23). Pectinase treated CW consisted only of a fibrillar network separated by spaces where a substance of the matrix, most probably pectin, was originally present (Fig. 24).

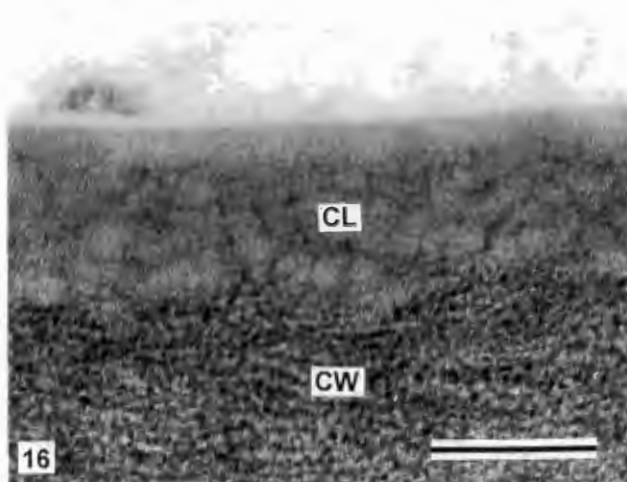
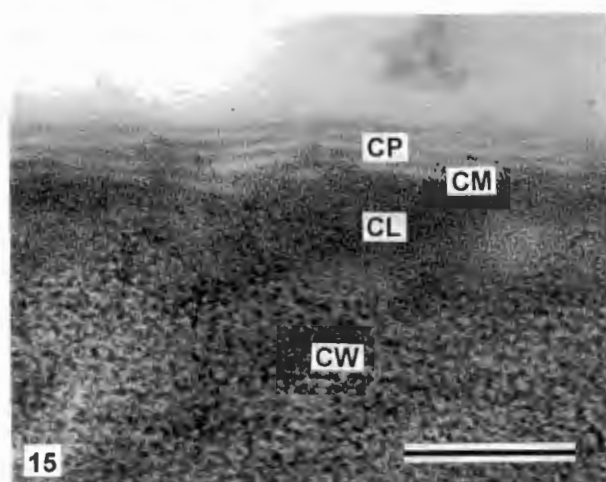
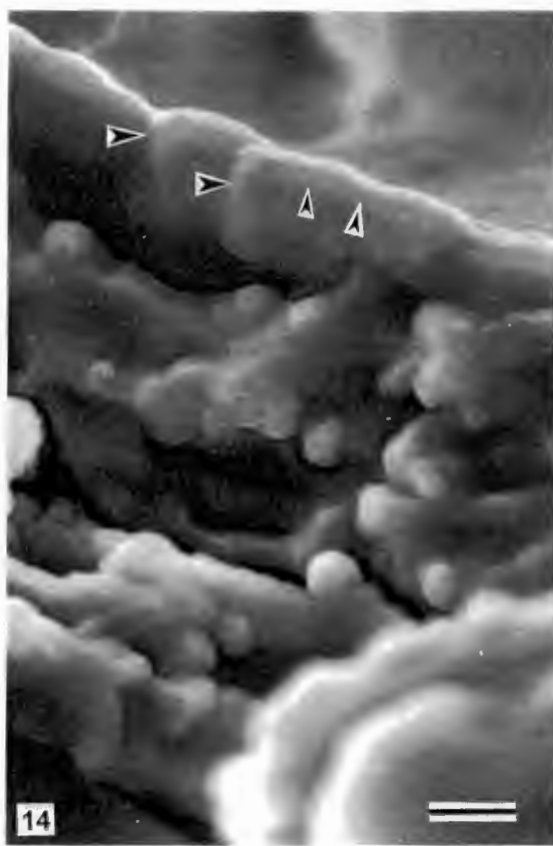
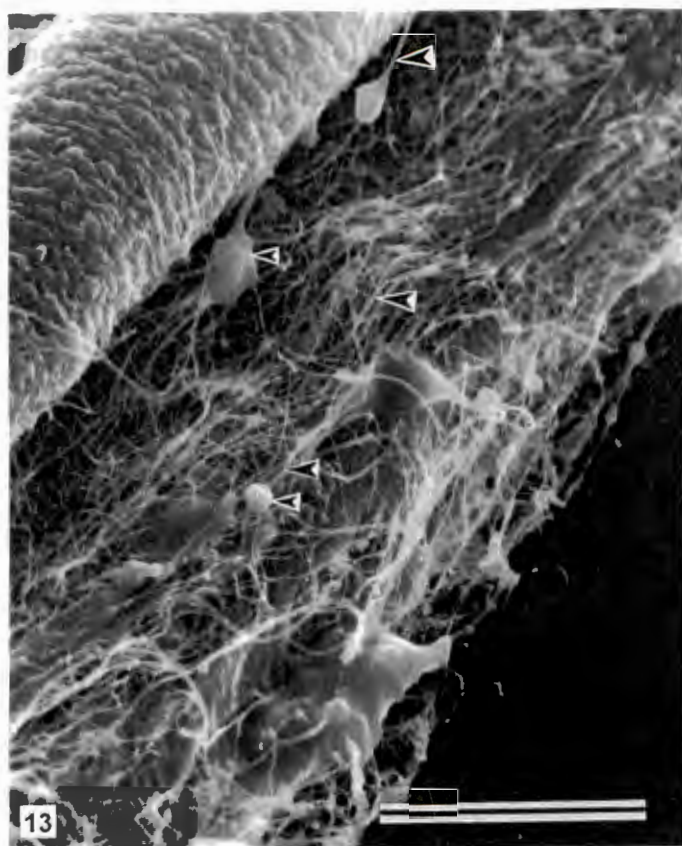


Fig. 13. With enzymatic incubation of leaf no. 5, the cell wall fibrillar network (large arrows) is pulled into a loose structureless network. Droplets (small arrows) accumulate on the fibrillar network. Fixation with 2.5% formaldehyde for 20 min followed by cellulase incubation for 20 min and post-fixation with 2.5% glutaraldehyde for 30 min. Material was CPD, coated with RuO_4 and viewed with FE-SEM. Bar = 1 μm

Fig. 14. Dead leaf material only coated with RuO_4 – no other methods of preparation. The FE-SEM reveals the possible epicuticular wax layer (small arrows) and the nichrista globules (large arrows) that are not artefacts. Bar = 100 nm

Fig. 15. The young mature (leaf no. 5) cuticular membrane (CM) and cell wall (CW). The cuticular membrane (CM) consists of the lamellate cuticle proper (CP) and cuticular layer (CL). Primary fixation in 2% aqueous OsO_4 + 4% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C, post-fixation in 2% aqueous OsO_4 in the same buffer for 2 h at 4°C. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

Fig. 16. The mature cuticular layer (CL) has a reticulate network – the fibrils protrude from the cell wall (CW) into the cuticular layer (CL). Primary fixation in 2% aqueous OsO_4 + 4% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C, post-fixation in 2% aqueous OsO_4 in the same buffer for 2 h at 4°C. Embedded in epoxy resin (Spurr 1969). Bar = 100 nm

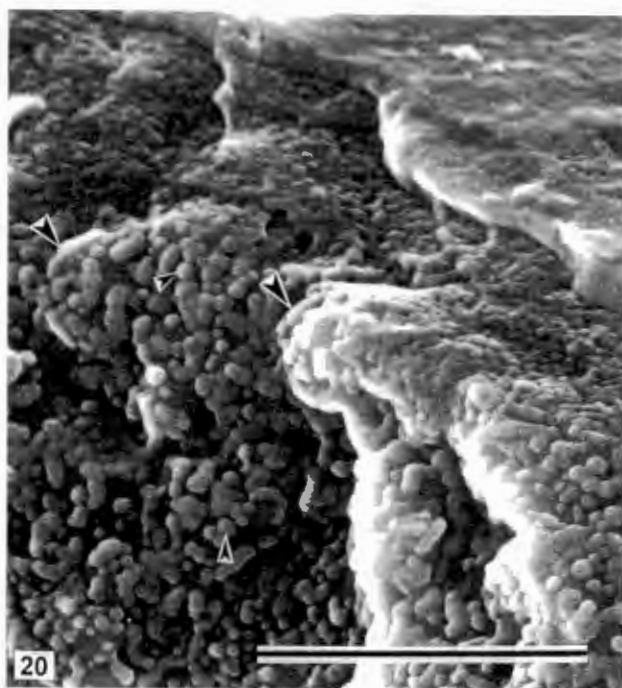
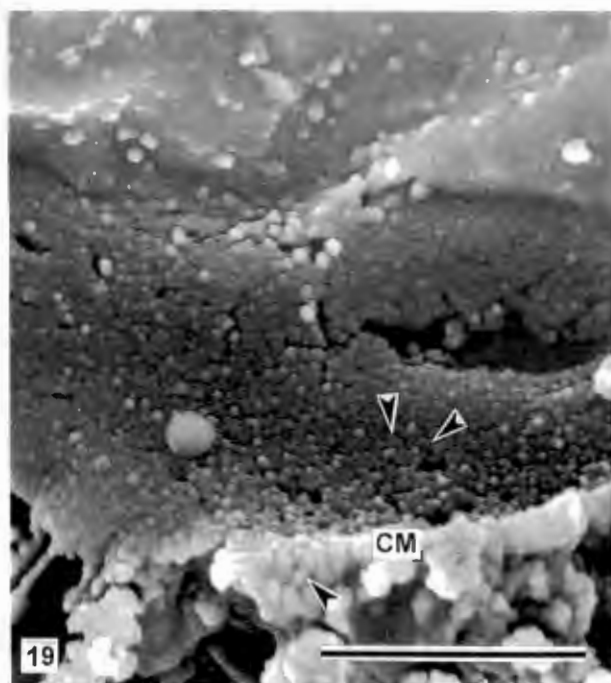
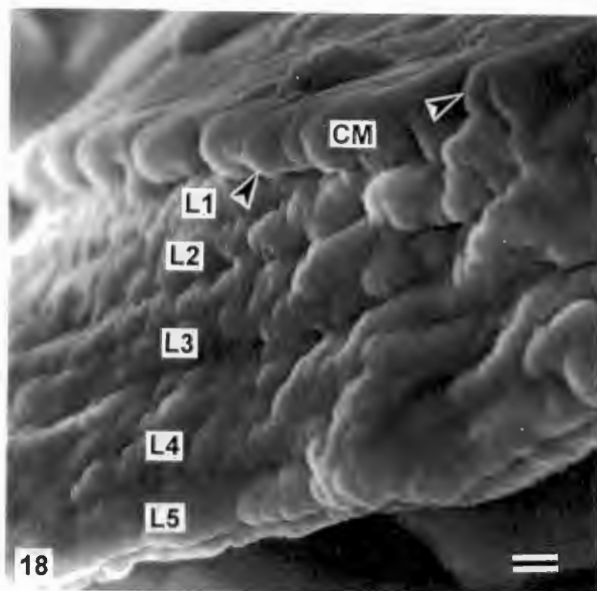
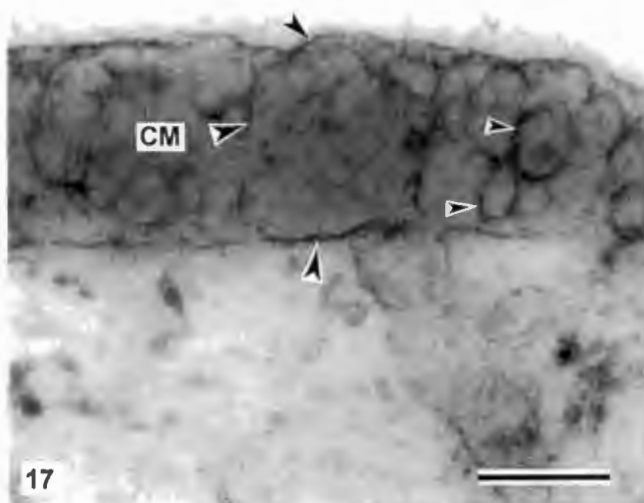


Fig. 17. A very thin cryo-section reveals that the fibrillar cell wall material protrudes the whole width of the cuticular membrane (CM) in leaf no. 5. These fibrils enclose the small nitis globules (small arrows), which group together to form the larger nichrista globules (large arrows). Fixed in 2.5% formaldehyde for 1 h. Cryo-protected with 30% PVP for 2 h. Plunge-frozen in liquid propane at -180°C and cryo-sections made at -120°C . Sections embedded in methyl cellulose, viewed with TEM. Bar = 100 nm

Fig. 18. The cell wall is lamellate with five layers (L1, L2, L3, L4, L5). The cuticular membrane (CM) reveals the larger nichrista globules (arrows). Plunge-frozen in liquid propane at -180°C . Freeze-dried, coated with Cr and viewed with FE-SEM. Bar = 100 nm

Fig. 19. Small nitis globules (arrows) of tobacco group together to form a globular cuticular membrane (CM). Cellulase incubation for 20 min. Primary fixation with 2.5% glutaraldehyde and post-fixation with 1% aqueous OsO_4 for 30 min each. Material was CPD, coated with Cr and viewed with FE-SEM. Bar = 1 μm

Fig. 20. *Cussonia paniculata* with a very thick epidermal wall reveals the large nichrista globules (large arrows) and small nitis globules (small arrows) of the cuticular membrane, showing that these globules are also found in other species than tobacco. Plunge-frozen in liquid propane at -180°C . Freeze-dried, coated with RuO_4 and viewed with FE-SEM. Bar = 1 μm

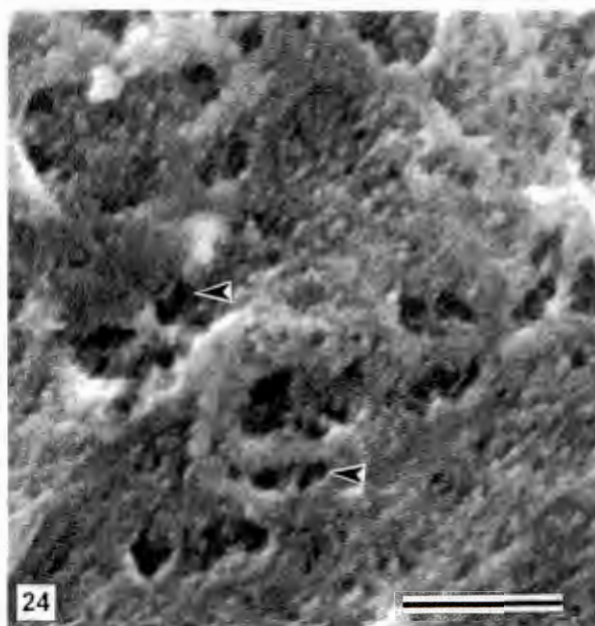
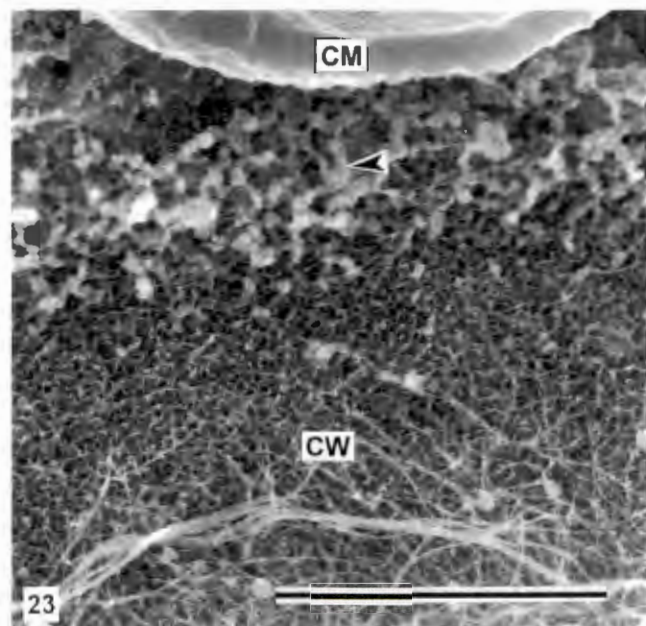
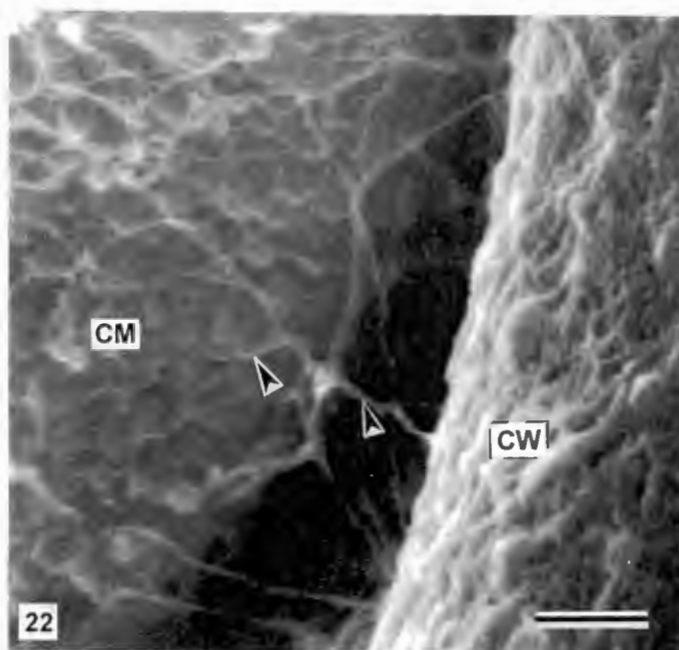
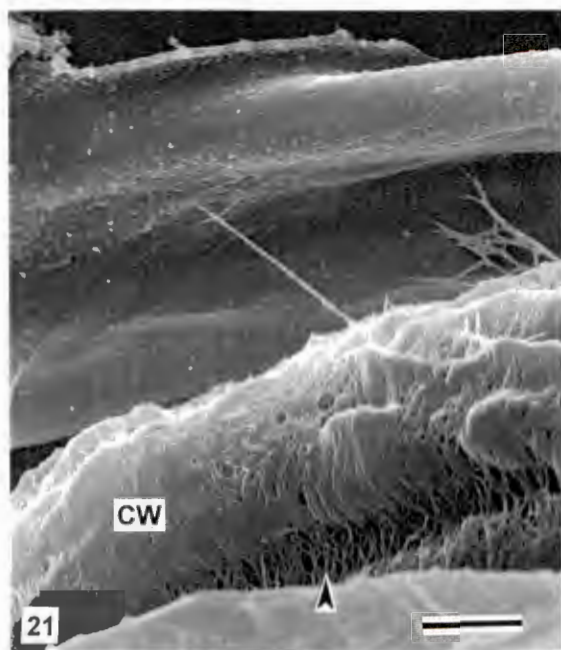


Fig. 21. The cell wall (CW) reveals layers parallel to the leaf surface. Within these layers is a network of fibrils (arrow) orientated perpendicular with the leaf surface. Cellulase incubation for 20 min. With primary fixation of 2.5% glutaraldehyde and post-fixation with 1% aqueous OsO_4 for 30 min each. Material was CPD, coated with Cr and viewed with FE-SEM. Bar = 1 μm

Fig. 22. FE-SEM reveals that the cell wall fibrils (arrows) protrude into the cuticular membrane (CM) from the cell wall (CW). Fixed with 2.5% formaldehyde for 30 min followed by post-fixation with 2.5% glutaraldehyde for 30 min. Material was sandpapered followed by a 5 min pectinase incubation, post-fixation 2.5% glutaraldehyde for 30 min. CPD, coated with RuO_4 and viewed with FE-SEM. Bar = 100 nm

Fig. 23. After cellulase incubation tubercular material accumulated (arrow) between the cuticular membrane (CM) and cell wall (CW). The cell wall fibrillar network becomes a loose woven network of fibrils. Primary fixation with 2.5% formaldehyde for 1 h followed by 20 min cellulase incubation and post-fixation with 2.5% glutaraldehyde followed by 1% aqueous OsO_4 for 30 min each. Material was viewed with FE-SEM. Bar = 1 μm

Fig. 24. After pectinase incubation openings (arrows) in the tightly woven cell wall are revealed. Fixed with 2.5% formaldehyde for 30 min followed by post-fixation with 2.5% glutaraldehyde for 30 min. Material was sandpapered followed by 5 min pectinase incubation and post-fixation with 2.5% glutaraldehyde for 30 min. CPD, coated with RuO_4 and viewed with FE-SEM. Bar = 100 nm

DISCUSSION

Cuticle Ontogeny

Leaf Surface Exudate

Glandular trichomes became visible when the leaf primordium was approximately 0.5 mm long, accounting for the high density of trichomes during earlier stages of leaf development (Avery 1933). This condition becomes less pronounced as the distance between trichomes increase, due to the expansion of the lamina (Avery 1933). Younger tobacco leaves yield more exudate per unit surface area than older leaves at lower stalk positions. This is most likely due to the greater trichome density of the younger leaves before they are fully expanded (Chang & Grunwald 1976, Gamou & Kawashima 1979). The substance present on the procuticle surface during leaf development may be trichome exudate as the glandular trichome density on young leaves is high. This substance was also more electron-dense (Fig. 1) than the electron-lucent epicuticular wax layer seen in Fig. 5, which may indicate that it is not wax, but rather trichome exudate.

Procuticle

Little information is available on the procuticle, which is present during the earliest stages of epidermal wall development of leaves. Young tobacco leaves are covered by an osmiophilic procuticle, as described by Heide-Jørgensen (1991) for *Utricularia laterifolia* as well as by Riederer and Schönherr (1988) for *Pisum sativum* L. The procuticle of the young tobacco leaf increases in thickness and reduces in electron-density to form a globular transition cuticle and thereafter transforms into a lamellate layer, the CP (Jeffree 1996).

Cuticle Proper

During the transition of the procuticle to the CP, only one pair of electron-lucent lamellae and one pair of electron-dense lamellae parallel to the leaf surface were observed in tobacco epidermal wall (Fig. 2). In *Clivia miniata* Regel however, two pairs of electron-lucent lamellae and two pairs of electron-dense lamellae were observed by Schmidt and Schönherr (1982). Jeffree (1996) reported the lamellate region to be typically about 50 – 100 nm in width in mature leaves, while that of the tobacco leaf is ca. 40 nm in width. Lamellae were already visible at the CP / CL transition stage.

Pectin Layer

During CP development, an electron-dense layer occurred between the CP and the CW in tobacco epidermal wall. According to Tenberge (1992), the first stage of development of the CL coincides with the disappearance of the pectin layer as a distinct substratum. This seems also to be true for the tobacco leaf, as the mature epidermal wall showed no sign of this when prepared according to the method of Franke *et al.* (1969). Peacock *et al.* (1998), however, reported the presence of a possible pectin layer in the tobacco leaf observed in a specimen imaged with backscattered electrons in a FE-SEM.

Cuticular Layer

Globules with electron-lucent lamellae accumulated underneath the CP. Assuming that the lamellae of the CP are not artefacts created during preparation, these globules with lamellae may be the possible mechanism whereby the electron-lucent material reaches the CP to form the lamellae. Heide-Jørgensen (1978) also reported the presence of globules, containing electron-lucent lamellae, which accumulate underneath the CP. Heide-Jørgensen (unpublished in Jeffree, 1996), observed osmiophilic globules enclosed by two different envelopes. The inner envelope is electron-lucent and ca. 5 nm in width while the outer envelope is electron-dense and ca. 2.5 nm in width. The tobacco leaf, however, revealed two different globules, one with a very thin electron-dense envelope including lamellae in the globule and globules without lamellae with a thick less electron-dense envelope. The study of Heide-Jørgensen (unpublished in Jeffree, 1996) showed that some globules also contained lamellae within the globules such as those observed in tobacco leaves. The thin layer may be an envelope of polar material, possibly protein, which may play an important role in transporting the hydrophobic globules through the aqueous environment of the apoplast (Jeffree 1996). Few globules with lamellae were observed while large numbers of globules without lamellae were present in tobacco leaves. A possible explanation for this could be that the CP constitutes the smaller area of the CM, with the electron-lucent lamellae representing the smaller percentage of the area within the CP compared to the electron-dense lamellae with often larger dimensions than those of the electron-lucent lamellae. The construction of the CL would need more cutin material, as it constitutes the larger area of the CM, thus explaining the presence of the larger number of globules without lamellae. The globules with the thick electron-dense envelope may contain chemically different components that accumulate in this manner within the globule. If these globules are part of the

transport vehicles for the construction of the CM, it might be possible that different types of globules exist, differing in contents, as the cuticular membrane is chemically heterogeneous.

Jeffree (1996) described globules as cutin-rich cystoliths, although their composition was unknown. If these globules are the vehicles transporting cutin, the process whereby they move across the CW is unknown (Tenberge 1992; Jeffree 1996). Tobacco leaf no. 5 may thus be regarded as a young mature tobacco leaf as the lamellate CP and reticulate CL were developed to the same extent as those found in the older (leaf no. 12) leaves, even though globules were still present in leaf no. 5.

The globules without lamellae accumulated beneath the CP within the tobacco epidermal CW. It may be possible that these globules construct the CL by releasing substances such as cutin. As the globules accumulated within the interstices of the cell wall, the fibrillar material of the CW blended with the cuticular material, forming what is known as the reticulate cuticular layer. According to Tenberge (1992) the first cutin-embedded fibrils of the CL are carboxylated and alkali-soluble, but not cellulosic. This could be possible if one considers the disappearance of the electron-dense layer, which may be a pectin layer, during the development of the CL. Another hypothesis concerning these fibrils in the CM is that, (1) the dimensions rather fit those of pectic substance and (2) the pectic substance would rather facilitate water and solute movement within the CM than the crystalline cellulose microfibrils.

Epicuticular Wax

The epicuticular wax layer becomes discernible on the CP during the CP / CL transition phase (Jeffree 1996). It is unknown how the epicuticular wax layer is deposited on the leaf surface. Droplets, which may be wax, accumulated within the cell wall microfibrillar network after enzymatic incubation. The network formed a loose mesh and the wax droplets, moving in a liquid phase, formed droplets that accumulated around the fibrils. Droplets suspended from the inner cuticle surface as seen with FE-SEM and TEM, may be wax droplets that form when the supporting CW structure was removed during enzymatic incubation. Such wax droplets were observed in both young and mature leaves. Wax production is influenced by the environment (Kolattukudy 1996) in young and mature plants. Schreiber *et al.* (1996b) stated that the waxy transport barrier of cuticles is a dynamic system. This is understandable from an ecological point of view, since the amount of wax deposited at the cutin / atmosphere interface may be

influenced by the plant environment. A possible hypothesis for the deposition of wax on the leaf surface is that the epidermal wall environment and leaf temperature makes it possible for wax to move in a liquid phase through the CW and CM. This implies that it would not be visible with TEM or FE-SEM unless destructive preparation methods are used. The structures observed in Fig. 11 and 12 are thus artefacts created by the method employed, but at the same time yield valuable information.

Yamada (1992) described lipid transfer proteins (LTPs) as proteins which transfer lipids between membranes by assays *in vitro*. The study of Thoma *et al.* (1993) indicated that LTPs are found in the epidermal cell walls of the leaf, stem and petiole of *Arabidopsis* Heynh., as expected for a secreted protein. LTPs have been isolated from the surface waxes of plants (Pyee *et al.* 1994). The discovery that LTPs move vectorally into the lumen of the endoplasmic reticulum and are secreted to the CW and outer surface, led to speculation that they might be involved in the transport of cuticular lipid components such as waxes and cutin monomers (Sossountzov *et al.* 1991, Sterk *et al.* 1991). It may be possible that LTPs somehow envelop the wax droplets seen in Figs. 11, 12 and 13 to accommodate transport in the aqueous CM and CW.

When LTPs appear on the surface of the CW and cuticle, an environment in which LTPs are likely to move freely, they may become part of a facilitated diffusion mechanism whereby cuticular lipids are transported from the outer layer of the plasma membrane, through the CW and CM, to the plant surface (Post-Beittenmiller 1996). When the wax globules accumulate to form the CM, an electron-dense layer, that could be LTPs, surrounds these globules. This hypothesis of secretion and deposition of extracellular lipophilic material has been argued convincingly, but has yet to be validated (Kolattukudy 1996). According to Pyee & Kolattukudy (1995) the presently available data indicates that: (1) LTPs carry acyl monomers necessary for the biosynthesis of cutin, (2) LTP gene expression and LTP gene product accumulation were detected in high levels in peripheral cell layers, including epidermis, (3) LTPs, particularly in young leaves where cutin deposition is active, are mainly concentrated in the surface wax and (4) LTPs are able to bind acyl chains.

The fact that gene expression for LTPs in tobacco leaves is higher in young leaves than in older leaves correlates with the fact that a new epicuticular wax layer must be deposited on the leaf surface of young leaves during the transition phase of the CP and CL layers. The expression of an LTP gene was found to be highest in the upper part of the tobacco plant and to decline

towards the base, which indicates that LTP genes are expressed in a developmental gradient in this plant (Fleming *et al.* 1992). Sterk *et al.* (1991) reported that the LTP gene in tobacco is cell type-specific in shoot apical meristem, particularly in the epidermis. The LTPs thus facilitate the possible transport of waxes as well as cutin. However, it seems as if wax moves as a liquid through the epidermal wall, never accumulating to form large globules, while cutin forms large globules in the interstices of the cell wall as well as underneath the CM. If one views Fig. 17 and 18, it is unlikely that these large globules move through the cell wall matrix. It might be possible that the globules are points of accumulation underneath the cuticle. However, the question remains why these globules accumulate within the CW interstices surrounded by an electron-dense border. Another interesting question is why some globules include electron-lucent lamellae. These may represent wax lipids that are intercepted during the cutin substance accumulation process.

Ultrastructure of the mature epidermal wall

Epicuticular Wax

Waxes are embedded in the cuticular membrane, which is the soluble intracuticular lipids (SCL), within the electron-lucent lamellae of the CP (Fig. 15) and the epicuticular wax layer covering the CP (Fig. 5), and are thus exposed to the environment outside the plant. Basson and Reynhardt (1988) described a molecular-level model, which had been developed for the structure of Fischer-Tropsch wax and the leaf wax of *Copernicia prunicia*. The molecular structure of solid aggregates of wax can be visualised as consisting of crystalline (solid zones) and amorphous (mobile zones) fractions. The crystalline region consists of the hydrocarbon chain with the constituents specifically packed in a perpendicular orthorhombic lattice. Due to differences in chain lengths, varying numbers of terminal $-\text{CH}_2-$ and $-\text{CH}_3$ groups will extend out of the monomolecular layer of molecules stacked side by side. Amorphous zones consisting of flexible chain ends, substituent groups and small voids in between (Basson & Reynhardt 1988) will thus separate crystalline layers. Wax studies have focused on chemical composition, but the work of Basson and Reynhardt (1988) gives a new dimension to ultrastructural studies and how permeability to water may be possible through these hydrophobic waxes. This FE-SEM study indicated that the thin epicuticular wax layer could not be observed readily in mesophytic plants, such as tobacco. Thick epicuticular

wax layers such as that of *Cussonia paniculata* could in future be utilised to study wax ultrastructure. However, if lamellae are not artefacts, they were not visible in the FE-SEM studies and it will thus be necessary to employ other methods to study the ultrastructure of SCL.

Cuticular Membrane

The cuticular membrane of tobacco leaf is composed of large units or globules (Fig. 14, 17 and 18) (also see figures in Peacock *et al.* 1998). We propose that these large globules be called nichrista globules. Observations made after material was cryo-sectioned (Fig. 17) or subjected to dehydration shrinkage (Fig. 19) indicate the presence of smaller globular subunits within the larger nichrista globules. We propose that these small subunits be called nitis globules. The nichrista globules are not regarded as artefacts as they were observed after application of different preparation methods and also in dead, dry leaf material that was only coated with RuO₄. These globules were also observed in species other than tobacco such as *Cussonia paniculata* (Fig. 20) leaves, supporting the fact that nichrista and nitis globules are not artefacts.

The presence of the nichrista and nitis globules confirm the statement that the cuticular membrane is not amorphous but has a globular structure. This globular ultrastructure of the CM does not relate to that of the CP but to that of the CL as seen with TEM. The fibrils that extend from the CW into the CL (Fig. 10) as seen with TEM relate to the fibrils as seen in cryo-sections. These fibrils were not observed with FE-SEM but may be represented by the borders of the nichrista globules as in leaf no. 1 (Fig. 19) viewed with FE-SEM. It is believed that these fibrils only extend into the CL from the CW and do not penetrate the CP (Holloway 1982; Jeffree 1996). This investigation falsifies this belief as the cryo-sections for tobacco revealed that the fibrils do extend the total width of the CM (Fig. 17). This relates with the nichrista globules discernible in naturally dried dead leaf material only coated with RuO₄ (Fig. 14) in tobacco and *Cussonia paniculata* leaves that were plunge-frozen in propane (Fig. 20).

It is not yet known how the nichrista and nitis globules are formed. A possible hypothesis is that fibrils which extend from the CW into the CM (Figs. 10, 17 and 22) may envelop the cuticular substances, forming these globules. The cuticular membrane is highly organised as the small nitis globules group together within the larger nichrista globules. It may be that pectinaceous

fibrils envelop the small nitis globules while larger protein fibrils envelop the large nichrista globules. Jeffree (1996) also referred to several authors who used different methods to enhance the lamellar structure of the CP that points to the presence of carboxyl groups. Jeffree (1996) stated that the structure of the CP might not be totally dependent either on soluble waxes or on cutin.

Cell Wall

The epidermal wall of tobacco consists of approximately five layers of microfibrils (Fig. 18) (Peacock *et al.* 1998). This is in accordance with the statement of McCann *et al.* (1992) that the thin epidermal wall of *Allium cepa* L. cannot accommodate more than about four microfibril layers. McCann *et al.* (1992) suspected that each microfibril layer could be embedded in a unique matrix environment due to the heterogeneity of the pectic substance present. The possibility exists that the microfibril layers are separated by pectic substances.

Both the pectin and cellulose-hemicellulose layers are periclinally oriented to the leaf surface, while it is possible that the protein network as well as a pectinaceous network is anticlinically arranged (Smith *et al.* 1984). Lamport and Epstein (1983) view the rodlike extensin and the cellulose microfibrils as two interpenetrating polymer systems, with cellulose periclinally and the extensin anticlinally oriented, thus creating a “wrap” and a “weft” arrangement in the wall. Their perpendicular orientation to the surface of the wall, like needles in a pincushion, would facilitate the diffusion of precursors into the wall (Smith *et al.* 1984). In Fig. 21 the different CW layers are evident with only a small portion of an anticlinally orientated network visible, that may be protein, while the rest of the cell wall is filled with matrix material. This information supports the hypothesis that it may be pectin and protein microfibrils that extend into the CM, enveloping the cuticle substances to form globules. The nature of the fibrils revealed after enzymatic incubation is unknown, but most probably includes proteins, cellulose, pectin and xyloglucans.

Discrepancies exist in the reported dimensions of cellulose fibrils. According to Salisbury and Ross (1992) cellulose microfibrils are 4.5 to 8.5 nm in width, while Lüttige and Higinbotham (1979) reported dimensions of 3 to 30 nm. Fibrils of tobacco in this study measured between 4 to 10 nm in width. Results previously reported by Peacock *et al.* (1998), show that the cellulose microfibrils in tobacco are ca. 30 nm in width. A possible explanation for these discrepancies

could be that Cr coating adds significantly to the observed dimensions of structures, while the RuO₄ coating (Van der Merwe & Peacock 1999), probably adds less than 1 nm to the fibril diameter. The RuO₄ may also ensure better conductivity of material with a very rough topography, such as cross sections of leaf material with trichomes (Van der Merwe & Peacock 1999). In our opinion the dimensions reported in this paper are close to those in the *in vivo* state.

Although the nature of the fibrils was not determined in this investigation, it is important to mention that xyloglucans and proteins play a vital role in the construction of the cell wall. Xyloglucan, a hemicellulose, is structurally similar in different plant species, but there are some differences in the side chains. In tobacco, terminal L-arabinosyl residues are α -linked to O-2 of xylosyl residues (McNeil *et al.* 1984). Xyloglucan probably has a regulatory as well as a structural function. The xyloglucan is hydrogen bonded to cellulose (McNeil *et al.* 1984). In the model proposed by Darvill *et al.* (1980) the interaction between cellulose microfibrils and matrix polysaccharides is mediated by xyloglucan. The primary cell walls of angiosperms contain various glycoproteins. At least one of these glycoproteins (i.e. extensin) is intimately associated with the cell wall *via* covalent bonds, whereas others (e.g. peroxidase) can be extracted with salts (McNeil *et al.* 1984). The model of Cooper *et al.* (1984) emphasises two independent wall polymer systems: xyloglucan-coated cellulose microfibrils intercalated through an extensin-pectin network.

Pectin Layer

From previous studies (Peacock *et al.* 1998), it is evident that a pectin layer is not a prominent feature in tobacco leaf epidermal wall layers. A tubercular substance between the CM and CW, possibly pectinaceous in nature, was observed (Fig. 23) after enzymatic incubation. A possible explanation for the accumulation of this substance could be that the substance was removed from the cell wall microfibrillar network during the brief pectinase incubation period. This leaves a muddled mesh of fibrils, as the brief enzymatic incubation did not last long enough to remove the substance, causing it to accumulate as a tubercular substance. The CW surface viewed from above where the cuticular membrane was removed (Fig. 24), revealed openings between the microfibrils after pectinase incubation. These openings may represent areas where the tubercular substance was removed from the CW. This substance may be pectin. This supports the statement of Roberts (1990) that pectin is a structural component of the cell wall and not just a glue.

Epidermal wall transpiration pathway

Leaf Surface Exudate

Jackson and Danehower (1996) reported that the leaf surface exudate of tobacco has many biological functions, including plant growth regulation, anti-microbial properties as well as effects on behaviour, development and survival of insect pests. The influence of the exudate on epidermal wall permeability has, however, not yet been clarified. The question remains whether tobacco leaf exudate has any significant influence on epidermal wall permeability. It is also important to realise that the distal portion of the leaf is relatively more mature, and the gradient from the tip to base represents a gradient from senescent to young tissue (Avery 1933). Thus the exudate and wax concentration varies on the leaf surface, which could imply that different epidermal wall permeability rates may exist in a young leaf of tobacco. Van Rensburg *et al.* (1999) found that the young mature leaves of drought sensitive tobacco cultivars have more glandular trichomes and epidermis cells (adaxial and abaxial) per leaf surface area than drought tolerant cultivars. It could thus be possible that the drought sensitive cultivars secrete a larger volume of exudate. If the trichome to epidermis cell ratio is calculated from the data of Van Rensburg *et al.* (1999), the ratios for the abaxial leaf surface of the drought sensitive and tolerant cultivars are the same. However, the ratio for the adaxial leaf surface is higher in the drought tolerant cultivar (2.90) than in the drought sensitive (1.76) cultivar. The glandular trichome density in the drought tolerant cultivar is much lower for the number of epidermis cells per leaf surface area. From these ratios it seems as if the drought sensitive cultivars probably secrete more exudate per leaf surface area, which would cover a larger leaf area with exudate, than the drought tolerant cultivars. The drought sensitive cultivars may also deposit a thicker layer of exudate on the leaf surface and this may also influence permeability. Van Rensburg and Peacock (1998) reported 11% lower epidermal wall permeability for the adaxial leaf surface of the drought tolerant cultivar, compared to the drought sensitive cultivar. This indicates that the exudate may not play an important role, as the drought sensitive cultivar with a possible higher exudate volume, had a higher permeability value. However, the exudate may play some role in very young leaves, jointly with the procuticle, contributing to the resistance to water permeability. The chemical composition of the exudate must also be taken into account. Severson *et al.* (1985) reported that drought-stressed tobacco leaves produced higher levels of α - and β -4,8,13-divatriene-1,3-diols than leaves of non-stressed tobacco plants. The diterpenoids secreted by the glandular trichomes are mainly secondary metabolites in *Nicotiana* species

(Gamou & Kawashima 1979), and these diterpenoids eventually become embedded in the wax (Chang & Grunwald 1980).

Procuticle

Neither the chemical composition of the procuticle, nor the role it plays in epidermal permeability in very young leaves, is known. Jeffree (1996) described the procuticle as a highly water-repellent and impermeable osmophilic film. As previously described in this chapter the very young tobacco leaves (Fig. 1) have not yet developed epicuticular or intracuticular wax. Thus, these young leaves are dependent on the procuticle and exudate for protection against desiccation.

Epicuticular wax

It is primarily the molecular architecture of cuticular waxes in the CP which determines cuticular water permeability and not the CM thickness, the wax load or the presence of pores, all of which have been implicated in the past (Kerstiens 1996). Waxes, which may be soluble cuticular lipids, are embedded in the cuticular membrane within the electron-lucent lamellae of the CP and in the epicuticular wax layer deposited on the CP. According to Schönherr (1976) SCL is of vital importance for water permeability of the CM. Svenningsson (1988) found that SCL most probably interacts with the surrounding lipid polymer, the cutin, forming a hydrophobic complex with large surface area. Consequently, in spite of the relatively small amount of SCL, the localisation of the lipids would make them an effective barrier for water diffusion. Because of inadequate methods for extracting and quantifying SCL and the lack of understanding of the ultrastructure of this transport-limiting barrier, results could not yet correlate secreted volumes and / or composition of cuticular waxes with the permeability of the cuticle to water and solutes (Riederer & Schneider 1990).

The crystalline phase of wax is practically inaccessible and impermeable to water molecules (Riederer & Schneider 1990). Mobilities were shown to vary considerably among plant species and decreased with increasing size of solutes (Bauer & Schönherr 1992). The amorphous wax phase would provide a tortuous diffusion path across the wax, whereas the crystalline wax phases may be regarded as excluded volumes with respect to diffusion (Schreiber *et al.* 1996a). Thus, the size of solutes, the contribution of the tortuosity of the wax (Baur *et al.* 1996) and the

fibrils in the CM (Chamel *et al.* 1991), indicate that a route with least resistance that is preferable for water and solute transport through the CM exists. Schreiber *et al.* (1996b) found that the barrier properties of *Fagus sylvatica* L. wax were enhanced in comparison to those of *Picea abies* L. (Karst.) by an increased crystallinity of the wax and the resulting higher tortuosity for the permeating solutes within the wax.

The question still remains whether the lamellae of the CP actually exist or whether they are artefacts created during sample preparation for TEM. However, the hypothesis that SCL is the important factor in determining the permeability rate may still be true, not as a thin electron-lucent layer but rather as wax filler in the cutin matrix contributing to the tortuosity. Petracek and Bukovac (1995) found that removal of waxes nearly doubled elasticity and halved the fracture force of tomato leaf cuticles, implying that wax may serve as supporting filler in the cutin matrix. The data of Baur *et al.* (1996) showed that the constituents of cuticular waxes are mobile, at least in the solid amorphous wax fraction, and that mobility rapidly decreases with increasing molar volume. It is not known how the SCLs are structurally present in the new globular CM model proposed in this investigation. In the cuticle ontogenetic study the hypothesis was presented that the wax is present as a liquid. If this is true, then the wax could be present at molecular level, thus not visible as a structural component of the CM. It still remains to determine whether these CM globules are destroyed during preparation for TEM so that the more polar cutiniferous substances rearrange as layers which alternate with the possibly non-polar waxy layers. The path of diffusion of solutes within the transport-limiting barrier of the cuticle has a high tortuosity that is significantly reduced, if not completely removed, when waxes are extracted (Schreiber *et al.* 1996a). It would be important to determine where these waxes are situated within the globular CM to determine their role in the resistance to permeability of water.

Cuticular Membrane

Cuticles act as solution-diffusion membranes for water transport since diffusion through pores does not contribute to cuticular transpiration (Kerstiens 1996). Diffusion across the cuticle is described by Lenzian and Kerstiens (1991) as water dissolving in the lipophilic medium of the cuticle at the cell wall / cuticle interface, diffusion in the solid matrix, and finally desorption from the outer surface of the cuticular membrane. Petracek & Bukovac (1995) stated that it is possible that the primary sites for water-cuticle interactions are the free hydroxyl groups of the

cutin matrix, which may form hydrogen bonds with water. Chamel *et al.* (1991) reported that sorption of water by cutin may be attributed to esters, carboxyl and hydroxyl groups.

Kerstiens (1996) stated that if microfibrils were to play a role in permeability they would have to penetrate the limiting barrier and reach the outer surface of the cuticle. The CM with the nichrista and nitis globules, possibly enveloped by the fibrils of pectinaceous and proteinaceous nature, offers a possible pathway to water and solute transport, not by means of mass flow, but only *via* diffusional passage, as it was found that these fibrils do extend the total width of the CM. Chamel *et al.* (1991) found that if the polar polysaccharides and crystalline cellulose are removed with nitric acid from a CM treated with methanol and chloroform, the water sorption capability drops dramatically. In this regard Kerstiens (1996) stated that it could be possible that polysaccharide microfibrils may, to some extent, contribute to the overall water transport pathway, although this movement may be restricted to the amorphous wax domain. According to Petrcek and Bukovac (1995) increased cuticular elasticity and fracturability due to hydration suggest that water may play a direct role in cuticular permeability. It would therefore seem that these fibrils contribute to cuticular permeability, although the magnitude of this contribution is still unquantified. If the polysaccharide microfibrils were removed, as reported in this paper, it would imply that the globular structure of the CM would be changed to an amorphous cutin structure without globules, which would hardly be permeable.

Pectic radiating strands in the reticulate CL were referred to as ‘microchannels’ in *Spartocytisus filipes* by Lyshede (1978) and in *Hakea suaveolens* R. Br. by Heide-Jørgensen (1978). However, Jeffree (1996) was of the opinion that these fibrils are denser than the matrix. This is inconsistent with the concept of open pores or channels. If the rate of movement through the CM is taken into account, open pores or channels would not make sense from a water conservation point of view. A high resistance diffusion pathway to prohibit loss of water from the moist leaf interior during drought stress would be more effective. Jeffree (1996) stated that these “pores or channels” may permanently elude observation by electron microscopy, but the novel model presented for the CM in this investigation attempts to explain the route of water and solute transport. The tortuosity of the diffusion path of water moving *via* the fibrillar network within the CM may also contribute to the transport resistance. Van Rensburg and Peacock (1998) reported P_d (differential permeability coefficient) to be $0.68 \times 10^{-9} \text{ms}^{-1}$ for a drought sensitive tobacco cultivar (TL 33) while a drought tolerant cultivar (Elsoma) had a P_d value of $0.61 \times 10^{-9} \text{ms}^{-1}$. The driving force for diffusion across the cuticle is the water vapour

concentration difference across the membrane and therefore P_d is expressed as ms^{-1} (Becker *et al.* 1986). A possible explanation for the difference between the P_d value for the drought sensitive and tolerant cultivar is that the drought tolerant cultivar may have a higher density of fibrils, thus implying a higher concentration of nichrista and nitis globules per surface CM area. This implies an increase in diffusion resistance because of the tortuosity. Schönherr (1982) found no obvious correlation between P_d and membrane thickness; permeability of the onion CM is just as low as that of *Clivia miniata* L. CM even though the latter is more than ten times as thick. The tortuosity of the fibrillar pathway in the globular CM explains why thickness is not a factor. Firstly, the CM is a heterogeneous structure and fibrils may differ in dimension and chemical composition for different species. Secondly, the nichrista and nitis globules of different species may differ both in size, number of globules per surface area and chemical composition. These factors will influence the resistance to permeability. Thirdly, the CM may have different capacities to hold water that is important for permeability, due to the globular structure of the CM. All these factors mentioned above could contribute to the fact that a thin CM may sometimes be less permeable than a thick CM.

Cell Wall

Baron-Epel *et al.* (1988) stated that pectic substances represent a major control element in defining the sieving properties of the CW. This is in agreement with our data, as shown in Fig. 24, that after pectinase incubation, spaces between the microfibrils of the CW are left open. We suggest that pectin was removed as one of the matrix substances in the cell wall. These open pores reveal the possible permeability route through the CW. These pores are, however, partially or completely filled with pectic substances. The data of Baron-Epel *et al.* (1988) and Tepfer and Taylor (1981) indicated the presence of a transport channel with a radius less than ca. 4 nm. Carpita *et al.* (1979), however, reported trans-wall channel sizes of 1.9 – 2.6 nm. This discrepancy may relate to the fact that Carpita *et al.* (1979) measured bulk transport, while Baron-Epel *et al.* (1988) measured the transport of small numbers of macromolecules. Jeffree (1996) stated that water movement most probably occurs at intermolecular level. Salisbury and Ross (1992) described the permeability of the cell wall as porous enough to allow free passage of water and dissolved substances in water. Schönherr and Mérida (1981) stated that diffusion in the cell wall will be somewhat restricted due to the presence of cellulose and other materials, but that the cell wall will have no measurable effect on cuticular permeability, as long as the permeability coefficient (P) is at least two orders of magnitude smaller than 10^{-4} ms^{-1} . Van

Rensburg and Peacock (1998) found this to be the case for tobacco leaf, indicating that the permeability of the epidermal wall directly reflects the properties of the cuticular membrane.

Pectic polysaccharides are probably the most complex class of cell wall polysaccharides (Knox *et al.* 1990) and it is not known how pectic substances facilitate water and solute movement in the CW. It constitutes approximately 34% of the CW (Ruben & Bokelman 1989). Whether pectic substances fill the cell wall pores only partially or totally, or whether water can move through pores that are totally filled by pectin, is not known. Shedletzky *et al.* (1992) reported that, with the application of the cellulose synthesis inhibitor, 2,6-dichlorobenzonitrile to tobacco cells, a Ca^{2+} -bridged pectate network is formed instead of the cellulose-xyloglucan network. These authors concluded that the Ca^{2+} -bridged pectin network, and not that of cellulose-xyloglucan, is the major determinant of wall porosity.

Bacic *et al.* (1988) reported that the primary cell walls can form pectic gels that immobilise water and, because of their open texture, small molecules may penetrate the solvent-filled interstices. An example of this is rhamnogalacturonans that have good gel-forming capabilities. In solution, the galacturonan backbone folds into a 2_1 -helix conformation, which allows calcium to bind cooperatively with carboxyl groups on unbranched, unesterified, segments of the polygalacturonosyl chains. If the pectin substances are removed by enzymatic incubation, a very porous, open, unordered network is formed. With cryo-preparation (and images reported by Peacock *et al.* 1998) an intact cell wall with no cellulose microfibrillar network is discernible as the matrix, probably pectin, fills the cellulose-xyloglucan network, giving it a smooth appearance. This corresponds with the idea of Shedletzky *et al.* (1992) that the pectic matrix is the determinant of wall porosity. Furthermore, Bacic *et al.* (1988) reported that wall porosity was dependent upon both charge, which is usually negative, and the degree of interaction of the matrix phase components. Both of these will depend on the composition of the walls. Even though the cell wall is seen as a structure that freely allows water molecules, with diameters of 0.2 nm, and solutes such as sugar molecules with diameters of 1 nm, to move through, there are still a lot of unknown aspects regarding this transport and the degree of resistance it yields to permeability.

If one takes a holistic approach concerning leaf surface permeability, the existence of aquaporins must be taken into account. Aquaporins are water channel proteins in vacuolar and plasma membranes. Plant aquaporins have six membrane-spanning domains and belong to the major intrinsic protein (MIP) superfamily of transmembrane channel proteins. The function of aquaporins would be to regulate transmembrane water transport during growth, development and stress responses of plants (Maurel 1997). Thus, as it regulates water transport, the plasma membrane plays an important role in epidermal wall permeability. The aquaporin system is sensitive to drought stress (Maurel 1997). Future studies should include the plasma membrane as part of the epidermal wall permeability complex.

CONCLUSIONS

- (1) The wax, cuticular membrane, cell wall and plasma membrane form the epidermal wall and each of these layers could be involved in the resistance to water transport and can react to drought stress.
- (2) The FE-SEM study revealed a new three-dimensional ultrastructure of the epidermal wall. The cuticular membrane consists of small nitis globules, which group together in the larger nichrista globules. The cell wall substances, possibly a polysaccharide and / or protein fibrillar network, penetrate the total width of the CM. The cell wall consists of five layers, each with fibrils with diameters ranging between 4 and 10 nm. The identity of the fibrils is not known but the components may include cellulose, hemicellulose and proteins. Pectin possibly fills the spaces between the fibrils. Each of the cell wall layers has it's own matrix.
- (3) The fibrillar network that penetrates the CM, envelops the nichrista and nitis globules and may provide a means for water and solute movement. The pectin filling the spaces in the fibrillar network possibly represents the transport pathway of least resistance. The amorphous wax areas provide the possible, and probably the only route that water and solutes could follow through the wax.
- (4) The main resistance factor is most probably the wax. However, the resistance of the crystalline cellulose microfibril network and pectin matrix should also be taken into account when epidermal wall permeability is considered. The density of polysaccharide fibrils within

the CM combined with the tortuosity that this fibrillar network has within the CM, could also be an important resistance factor. The epidermal wall permeability may be used for the identification of drought tolerant genotypes if the resistance factors differ in different genotypes.

- (5) The LTP factor has merit to be involved in the transport of cutin and wax within the epidermal wall and more investigation is needed. Wax is probably transported as a liquid phase through the epidermal wall and may accumulate as a liquid. The fact that it can form a drop hanging on the inner cuticle could imply that some protein such as LTP is holding the droplet together. The cutin is possibly transported in very small quantities, but has the tendency to accumulate as globules within the CW. Some of these globules contain electron-lucent lamellae. The exact mechanism whereby small quantities of cutin and wax move through the epidermal wall still eludes us, but this study has yielded results that open new perspectives on this aspect.
- (6) The ontogenetic study of the CM revealed the possible mode of transport of cutin and wax. By understanding step by step how the CM develops, more insight in the building blocks and the pathway of water and solutes through these units that form the epidermal wall, is gained. The development of the CM of the tobacco leaf follows the same pattern as that summarised by Jeffree (1996).

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CHAPTER SEVEN
GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION

Drought stress is a limiting factor in agriculture making it important to evaluate the leaf surface features that could be used as drought tolerance criteria. The transport properties of the epidermal wall control the exchange of water between plants and the environment. Two drought sensitive tobacco cultivars (TL 33 and CDL28) and two drought tolerant cultivars (Elsoma and GS46) were used for this study. A holistic approach was attempted by combining different aspects of the tobacco leaf epidermal wall, namely, micro-morphology of the leaf surface, ultrastructure and cuticular transpiration with the focus on drought tolerance.

Leaf surface micro-morphology

Liquid substitution is a SEM preparation method in which triethylene glycol is used to substitute the water content of the plant. Thus, an organic solvent like ethanol was not used for dehydration and critical point drying was also excluded. The triethylene glycol substitution method is simple, inexpensive and versatile with the only disadvantage that the material does not have a long lifetime in the vacuum chamber of an electron microscope. This method was found to be superior for studies on tobacco leaf surfaces for SEM.

Five trichome types were identified on the adaxial and abaxial leaf surfaces of tobacco, namely glandular trichomes with multicellular heads as well as unicellular heads, short glandular trichomes, branched glandular trichomes and non-glandular trichomes. Irrespective of cultivar, side of the leaf or drought tolerance, none of the trichome dimensions differed significantly. The average trichome frequency for the combined adaxial and abaxial leaf surface of both drought sensitive cultivars (TL33, CDL 28) was higher, when compared to that of the drought tolerant (GS46, Elsoma) cultivars. Both the stomatal fractional cavity and pore contributions to the overall entry resistance of CO₂ and H₂O were higher for the drought tolerant cultivars. The fractional contribution of the boundary layer to CO₂ and H₂O was, however, higher for the drought sensitive cultivars. From the results presented it is concluded that plant fitness for tolerating drought in this mesophytic crop plant may be maximised, not by minimising water loss by maximising the photosynthetic transpiration ratio, but by maximising the carbon gaining capacity of the leaf. This was evident from the diffusion resistance for CO₂ that was found to be lower for the drought tolerant cultivars. This can mainly be ascribed to the differences in

stomatal spacing and the boundary layer resistance to CO₂, between the respective cultivars. Trichome frequency would therefore not seem to be a prerequisite for drought tolerance.

Cuticular Transpiration

Cuticular transpiration becomes important if stomata are supposedly closed and CO₂ gaining cannot take place to withstand drought stress for a period of time. An apparatus was designed with optimal temperature exchange to study water permeability of the cuticle. This apparatus enabled us to simulate a system of aqueous solution (donor solution) / membrane (epidermal strips) / very dilute aqueous receiver solution (atmospheric air), which closely approximates the actual situation *in situ* (membrane as primary interface). Leakage from the system showed an average of 2.7% experimental variability. For this study tobacco adaxial epidermal wall strips containing trichomes and stomata were used. The abaxial side was gently scraped away. When areas of possible mass flow, such as holes or cracks, were detected under the light microscope, the permeability coefficient was much higher compared to the cuticular permeability of tobacco, showing that there were no alternative pathways. The same results were obtained with incompletely closed stomata as the value was seven orders of magnitude higher than the permeability coefficient reported for the tobacco epidermal wall when stomata were open, thus making it unlikely that there was residual loss through the stomata conforming the permeability measurements. The degree of contamination of the epidermal strips by cellular debris was quantified and expressed as the total chlorophyll content per exposed surface area of the experimental wall strips, and an average of 8.7% contamination was observed compared to the total leaf chlorophyll content. In this study the term epidermal rather than cuticular transpiration is used, as it is not clear whether the presumed cuticular transpiration is due in part to incomplete stomatal closure and whether transpiration rate is a function of epidermal conductance.

Contrary to most permeability studies, we did not use astomatous, enzymatically isolated, or dried cuticular membranes. We used freshly harvested epidermal strips, avoiding the sorption of lipids by the cuticular membranes during enzymatic isolation. Diffusion coefficients were calculated with smaller associated standard deviations and an order of magnitude lower than previously reported. The fresh material sorption of the diffusing compound by the membrane and hydration of the cuticular pores was greatly reduced.

The fractional pore data of the epidermal membrane, which is a calculation of the total area of water filled pores derived by membrane area, showed that a small fraction of the total tobacco membrane area is permeable to water, and that this correlates negatively with the known drought tolerance for the four tobacco cultivars. The data presented for the four tobacco cultivars confirmed previous reports that genetic variability exists, pre-drought stress in this instance, as the drought tolerant cultivar Elsoma had a permeability coefficient of 11% lower than that of the drought sensitive cultivars. This adds to the growing body of evidence that cuticular conductance relates positively to differences in drought tolerance of some cultivars of this species. These results for tobacco, using epidermal permeability coefficient *per se*, or possibly the ability of the plant to change in response to stress seems to have merit as selection criteria for identifying drought tolerant genotypes.

Ultrastructure

From the cuticular transpiration study it became evident that it is important to study the ultrastructure of the cuticular membrane, but also the ultrastructure of the epicuticular wax layer and the cell wall. Studying the ultrastructure of the epidermal wall is important as it could reveal the small fraction of the total membrane that is permeable and supply reasons why this correlates negatively to drought tolerance while the permeability coefficient seems to correlate positively. The FE-SEM study opened a new window through which we could view the three-dimensional ultrastructure of the epidermal wall, yielding new information that may correlate structure to function.

The ontogenetic development of the cuticular membrane indicated a possible mode of transport for cutin and wax. From this ontogenetic ultrastructural study it became evident that determining the cuticular transpiration of young leaves could contribute to cuticular transpiration studies. It seems as if only exudate was present on the leaf surface, without an epicuticular wax layer to protect the leaf from dehydration. Cuticular transpiration studies on very young leaves yield new information, not only on the procuticle, but also on the permeability rate of the mature cuticular membrane. At the procuticle phase the cuticle proper with lamellae had not yet developed. Determination of the transpiration rate when stomata are closed at different ontogenetic stages (procuticle, cuticle proper, cuticular layer) could reveal new information. If a difference in the permeability coefficient is detected between transpiration rates at the stage when the cuticle proper is present and the stage when the cuticular layer is developed, it may indicate the

difference in the permeability resistance of the epicuticular wax and soluble intracuticular lipids in the cuticle proper compared to the fibrils in the cuticular layer. It might also be interesting to measure the permeability coefficient at different development stages to determine whether a cuticle proper with two electron-lucent lamellae has a different transpiration rate than a cuticle proper with 10 electron-lucent lamellae in leaves of the same species.

Globules that accumulate underneath the developing cuticular membrane were discernible within the cell wall. If these globules are the vehicle to transport cutin and / or wax, the process whereby they move across the cell wall is unknown. In our opinion it is unlikely that these large globules move through the cell wall matrix, but it may be that the globules are points of accumulation underneath the cuticular membrane. However, the question remains why these globules accumulate within the cell wall interstices and are enveloped by an electron-dense border, which may be lipid transfer proteins (LTP). The globules that include electron-lucent lamellae may represent wax lipids that are intercepted during the cutin substance accumulation process. What is evident from these globules that accumulate underneath the cuticular membrane is that there is some transport mechanism that exists to transport substances such as cutin to the leaf surface. It is proposed that wax move as a liquid through the epidermal wall as droplets that may consist of wax were discernible after enzymatic incubation with cellulose. However, these wax droplets could not be observed when a method such as cryo-fixation, that keeps the epidermal wall intact, was employed. This study emphasised the importance of methods that not only reveal plant structures in the intact form, but also dissect plant structures, thus revealing the building blocks.

A new model for the cuticular membrane and cell wall is proposed (Fig. 1). Previous models described the cuticular membrane as an amorphous membrane. The new model suggests that it has a globular structure.

The FE-SEM study of tobacco epidermis revealed that the cuticular membrane consisted of small globules, referred to as *nitis* globules, that are grouped together within the larger globules referred to as *nichrista* globules. The fibrillar membrane within the cuticular membrane as seen in TEM cryo-sections envelops the globules. The model offers a potential explanation for the transport pathway of water movement through the cuticle, more so than the concept of an amorphous cuticle.

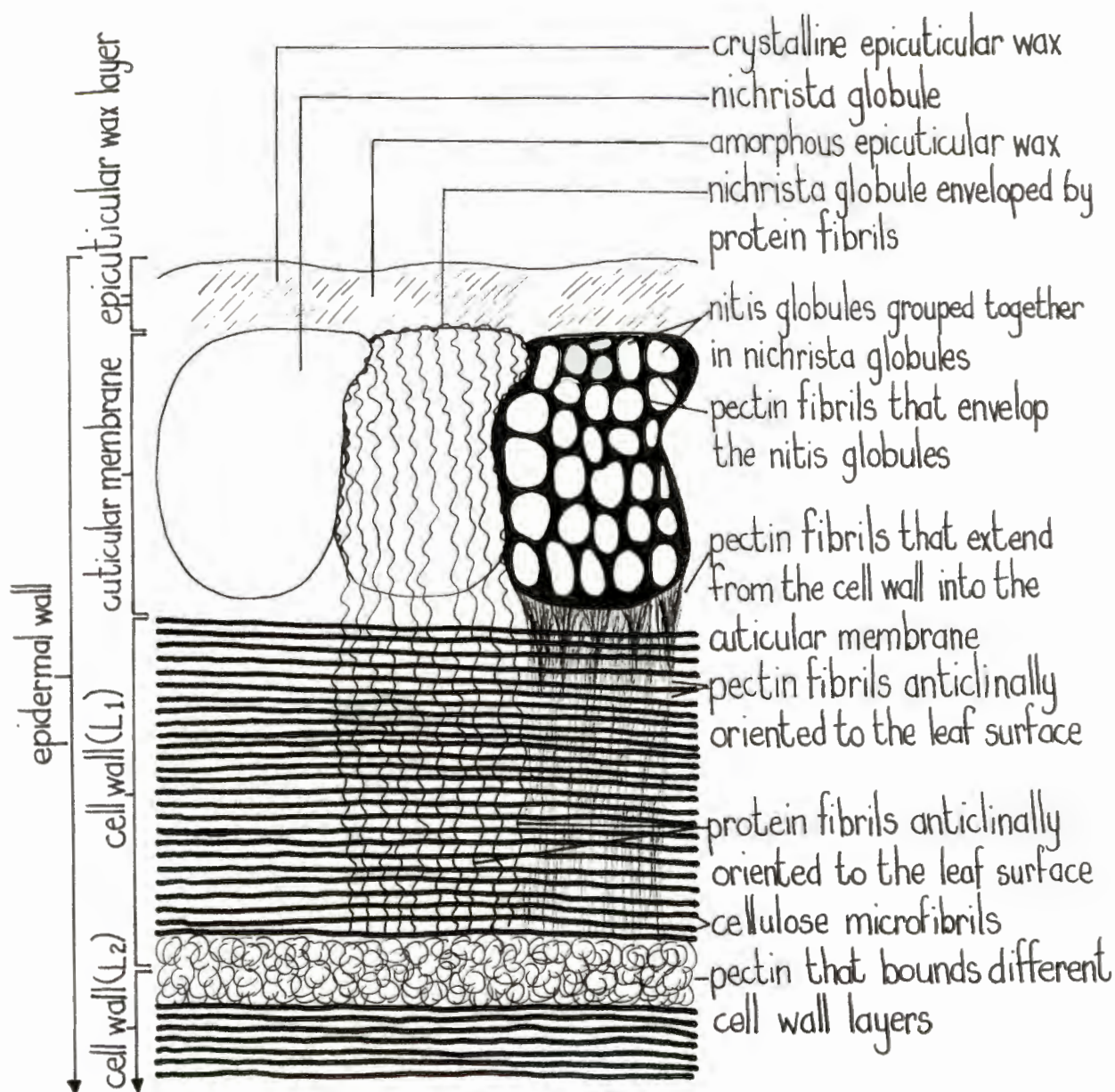


Fig. 1. The new proposed epidermal wall model of the tobacco leaf. The cuticular membrane consists of nictis globules grouped together within the nictista globules. The epicuticular wax layer has crystalline and amorphous areas that facilitate water movement. The cell wall consists of five layers with cellulose microfibrils that extend periclinally, while protein and pectin fibrils extend anticlinally towards the leaf surface. Pectin is present between each cell wall layer as well as between cellulose microfibrils. Hemicellulose bounds the cellulose microfibrils.

The cuticular membrane of the tobacco leaf studied with FE-SEM did not reveal a cuticle proper with lamellae, but does resemble the cuticular layer with fibrils. The existence of the lamellae is questioned. It seems possible that the belief in the existence of lamellae has led to our misunderstanding of the role of wax lipids and that they may not be important factors contributing to water resistance permeability.

The cuticular membrane model with nitis and nichrista globules enveloped by fibrils, indicates a possible pathway for water and solute transport, not by means of mass flow, but only *via* diffusional passage, explaining why water diffusion through a stomatal opening would be much higher than diffusional transpiration through the cuticle. Cryo-sections used in this study revealed that these fibrils extend the total width of the cuticular membrane and thus also the cuticle proper. If water transport is restricted to the fibrillar area in the globular cuticular membrane, it would make sense, as previously reported in this chapter that only a small fraction of the total membrane surface area of tobacco leaves is permeable to water. A possible explanation for the difference in permeability coefficient value for drought tolerant and drought sensitive cultivars could be that the drought tolerant cultivars may have a higher density of fibrils, implying thus a higher concentration of nichrista and nitis globules per surface area in the cuticular membrane. This indicates an increase of the diffusion resistance due to higher tortuosity.

The epidermis cell wall of tobacco consists of approximately five layers of microfibrils. The chemical composition of the microfibrils has not been identified in this study but proteins, pectins, cellulose and xyloglucans are probably present. The fibril width measured to be between 4 – 10 nm after RuO₄ coating, while, after chromium coating, the measured width was 30 nm. Unlike chromium, RuO₄ coating does not influence the structural dimensions, while yielding optimal results for conductivity and contrast in the SEM. After pectinase incubation openings were present between the microfibrils of the cell wall. It is proposed that pectin may have been removed and that pectin, rather than cellulose microfibrils may facilitate water movement through the cell wall. A fibrillar network, that may be proteinaceous, arranged anticlinally to the leaf surface, was observed. A possible hypothesis is that the protein and pectin fibrils extend into the cuticular membrane, thus facilitating water transport through the cuticular membrane. It is also a suggestion that the protein fibrils envelop the large nichrista globules, while pectin fibrils envelop the small nitis globules.

GENERAL CONCLUSION

Maximising the carbon gaining capacity of the leaf for periods of drought when stomata are open has merit as selection criterion for identifying drought tolerant genotypes. However, when drought stress becomes severe and stomata close, a low permeability coefficient for cuticular transpiration has merit as selection criterion for identifying drought tolerant genotypes. The

ultrastructural study led to a new understanding of the three-dimensional structure of the epidermal wall. Even though this ultrastructural study raised more questions than answers, it still yielded results indicating a cuticular membrane with a globular structure that offers a better explanation of cuticle permeability than an amorphous membrane. Structure and function relations are basic to understanding nature.

ANNEXURE 1

Conference Contributions

Person who delivered poster or paper marked by *

KRÜGER, H.*, BREYTENBACH, J. & VAN RENSBURG, L. 1994. Cuticle ultrastructure of *Nicotiana tabacum* L. (In Electron Microscopy Society of South Africa, vol. 24, poster delivered at the 33rd annual conference of EMSSA held 29 November to 2 December at Port Elizabeth)

BREYTENBACH, J.*, KRÜGER, H., HAMILTON-ATTWELL, V.L., VAN RENSBURG, L. & KRÜGER, G.H.J. 1995. Preparation of epidermis of *Nicotiana tabacum* L. leaves for SEM by liquid substitution with triethylene glycol. (In South African Association of Botanists, poster delivered at the 21st annual conference of SAAB held 9 to 12 January at Bloemfontein)

Workshop: Chlorophyll Fluorescence, R.J. Strasser, Laboratoire de Bioenergetique, University of Geneva, Switzerland.

PEACOCK, J.*, KRÜGER, H., VAN DER MERWE, C.F. & VAN RENSBURG, L. 1995. A liquid substitution method for the preparation of tobacco leaf epidermis for SEM. (In Microscopy Society of Southern Africa, vol. 25, poster delivered at the 34th annual conference of MSSA held 28 November to 1 December at Warmbaths)

PEACOCK, J.*, VAN DER MERWE, C.F., KRÜGER, H. & VAN RENSBURG, L. 1996. Correlative high resolution FE-SEM and TEM study of the epidermis of *Nicotiana tabacum* L. leaf. (In Microscopy Society of Southern Africa, vol. 26, paper delivered at the 35th annual conference of MSSA held 4 to 6 December at Durban)

Award: Wirsam Scientific Student Prize : for the best paper on light or electron microscopy in the Life Sciences and Physical Sciences.

Workshop: Low - Temperature Microscopy and Analysis Workshop, P. Echlin, Multi-Imaging Center, School of Biological Sciences, University of Cambridge, Cambridge, United Kingdom.

PEACOCK, J.*, VAN RENSBURG, L., VAN DER MERWE, C.F. & KRÜGER, H. 1997. A study of the tobacco leaf epidermis with high resolution field emission scanning electron

microscopy. (*In* The South African Association of Botanists, paper delivered at the 23rd annual conference of SAAB held 13 to 17 January at Alice)

VAN RENSBURG, L.* & PEACOCK, J. 1997. Epidermal transpiration in four cultivars of *Nicotiana tabacum* L. which differ with regard to their drought tolerance, using epidermal strips in a quasi - steady state system. (*In* The South African Association of Botanists, paper delivered at the 23rd annual conference of SAAB held 13 to 17 January at Alice)

VAN RENSBURG L.* & PEACOCK, J. 1997. Epidermal transpiration in four cultivars of *Nicotiana tabacum* L. which differ with regard to their drought tolerance: - Using epidermal strips in a quasi-steady state system. (*In* Scandinavian Society for Plant Physiology XVIII, poster delivered at SPPS held June at Uppsala, Sweden)

PEACOCK, J.*, VAN DER MERWE, C.F., PRETORIUS, W. & L. VAN RENSBURG. 1997. Cuticle ontogeny and epidermis ultrastructure of the tobacco leaf. (*In* Microscopy Society of Southern Africa, vol. 27, paper delivered at the 36th annual conference of MSSA held 3 to 5 December at Belville)

PEACOCK, J.*, VAN DER MERWE, C.F., VAN RENSBURG, L. & PRETORIUS, W. 1998. Ultrastructure of tobacco leaf epidermis. (*In* International Congress on Electron Microscopy, vol. IV, poster delivered at the 14th conference held 31 August to 4 September at Cancun, Mexico.

Workshop: 3-D Information in the Biological Sciences: What Image Analysis can do for us. J.M. Carazo, Centro Nacional de Biotechnologia-CSIC, Campus Universidad Autonoma de Madrid, Madrid, Spain.

PEACOCK, J.*, VAN RENSBURG, L. & VAN DER MERWE., C.F. 1999. The ultrastructure of the epidermal transpiration pathway in the tobacco leaf. (*In* Royal Microscopical Society – International Botanical Meeting, paper delivered at 6th conference held 25 to 29 March at St Andrews, Scotland)

VAN DER MERWE, C.F.* & PEACOCK, J. 1999. Some new sample preparation techniques for high-resolution FE-SEM study of the epidermal cell wall. (*In* Royal Microscopical Society –

International Botanical Meeting, paper delivered at 6th conference held 25 to 29 March at St Andrews, Scotland)

VAN DER MERWE, C.F.* & PEACOCK, J. 1999. Enhancing conductivity of biological material for SEM. (*In* Microscopy Society of Southern Africa, vol. 29, paper delivered at the 38th annual conference of MSSA held 1 to 3 December at Bloemfontein)

PEACOCK, J.*, VAN DER MERWE, C.F. & VAN RENSBURG, L. 2000. A new perspective on the cuticular membrane model. (*In* The South African Association of Botanists, paper delivered at the 26th annual conference of SAAB held 10 to 14 January at Potchefstroom)

Workshop: Screening of higher plants for vitality, stress and adaptation, based on the fast fluorescence rise and conventional modulated techniques. R.J. Strasser, Laboratoire de Bionérgétique, University of Geneva, Switzerland.

Workshop: Writing scientific papers. C.H. Bornman, Department of Plant Physiology, Lund University, Sweden.

VAN DER MERWE, C.F.* & PEACOCK, J. 2000. Material preparation and high resolution SEM. (*In* The South African Association of Botanists, paper delivered at the 26th annual conference of SAAB held 10 to 14 January at Potchefstroom)

VAN RENSBURG, L.*, PEACOCK, J. & KRÜGER, G.H.J. 2000. Boundary layer, stomatal geometry, and –spacing, in relation to drought tolerance in *Nicotiana tabacum* L. (*In* The South African Association of Botanists, poster delivered at the 26th annual conference of SAAB held 10 to 14 January at Potchefstroom)

ANNEXURE 2

Publications

KRÜGER, H., VAN RENSBURG, L. & PEACOCK, J. 1996. Cuticular membrane fine structure of *Nicotiana tabacum* L. leaves. *Annals of Botany*, 77:11-16.

PEACOCK, J., VAN RENSBURG, L., VAN DER MERWE, C.F. & KRÜGER, H. 1998. A FE-SEM study on the tobacco leaf epidermis. *South Africa Journal of Botany*, 64:361-667.

PEACOCK, J., VAN RENSBURG, L., KRÜGER, H. & VAN DER MERWE, C.F. 2000. Liquid substitution, a procedure without drying or coating: an ideal method for SEM leaf surface studies. *Scanning Microscopy*, (in press).

VAN RENSBURG, L. & PEACOCK, J. 1998. Determination of epidermal transpiration in four cultivars of *Nicotiana tabacum* L. Using epidermal strips in a quasi-steady state system. *Biotechnic & Histochemistry*, 73:97-106.

VAN RENSBURG, L., PEACOCK, J. & KRÜGER, G.H.J. 1999. Boundary layer, stomatal geometry and -spacing, in relation to drought tolerance in four *Nicotiana tabacum* L. cultivars. *South African Journal of Plant and Soil*, 16:44-49.

Other Publications

Micrographs published in:

JEFFREE, C.E. 1996. Structure and ontogeny of plant cuticles. (In Kerstiens, G., ed. *Plant Cuticles, an integrated functional approach*. Oxford : Bios Scientific Publishers. p. 33-75.)

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