An anatomical, phytochemical and ultrastructural characterization of the 'elastic threads' of *Maytenus acuminata*

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It was found that the characteristic elastic threads obtained on breaking and subsequent pulling apart of tissue of *Maytenus acuminata* (L.f.) Loes, although always associated with vascular tissue, are not the elastic thickening of xylem elements. The threads consist of E-1,4-polyisoprene and, in both the stem and leaf, are produced from laticifer-like cells. Polyisoprene, which occurs to a greater extent in the leaves than the stem of this species, is found in the cytoplasm of most cell types including the sieve elements of the phloem.

Daar is gevind dat die kenmerkende elastiese drade wat opgemerk word wanneer weefsel van *Maytenus acuminata* (L.f.) Loes uitmekaar getrek word, alhoewel altyd aan die vaatweefsel gekoppel is, nie die gevolg is van elastiese verdikkings van die houtvate nie. Hierdie drade bestaan uit E-1,4-poli-isopreen en in beide die blare en lote kom dit in melksap-tipe selle voor. Poli-isopreen wat in groter hoeveelhede in die blare as in die lote van die plant voorkom, kom ook in die sitoplasma van meeste sel-tipes, insluitende die sif-elemente van die floëem, voor.

Keywords: Laticifers, *Maytenus acuminata*, polyisoprene

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Introduction

The production of fine silky threads on breaking and subsequent pulling apart of stem, leaf, and fruit tissue of *Maytenus acuminata* (L.f.) Loes. is an often-cited characteristic of this species (Robson 1966; Coates-Palgrave 1977; Van Wyk 1984) which occurs as a shrub or small tree in a wide band on the eastern seaboard of South Africa and extending into the northern Transvaal and Zimbabwe (Coates-Palgrave 1977). These so called 'elastic threads' (Van Wyk 1984) are suggested by Robson (1966) to be latex, while according to Van Wyk (1984) the threads are classified as gutta-percha, or occasionally are described as resin, latex or caoutchouc. In this respect it is of interest that relatively large quantities of latex (2,7% balata on a fresh mass basis) are found in this *Maytenus* species (Paterson-Jones 1983).

An alternative origin of the elastic threads is proposed by Esterhuysen (cited by Coates-Palgrave 1977) who suggests that the threads 'are the elastic thickening of the tracheids'. An anatomical and ultrastructural investigation was initiated, in conjunction with a phytochemical investigation, to determine whether the threads are the elastic thickening of xylem vessels, or whether they are latex, and in the case of the latter alternative, to characterize the chemical nature of the compound and its anatomical and ultrastructural distribution within leaf and stem tissue.

Materials and Methods

Plant material

Leaf and stem material of *M. acuminata* (L.f.) Loes. was collected from trees growing in the Doreen Clarke Nature Reserve north of Pietermaritzburg, Natal and the Royal Natal National Park. Material was processed immediately on return to the laboratory (within 20 min).

Extraction and identification of thread material

Fresh leaf and stem material was dried to constant weight at 50°C (48 h). The dried material was ground to a homogenous powder. Extraction of the dried powder (2 g) in a Soxhlet apparatus with acetone (16 h) followed by petroleum ether (b.p. 40 – 60°C, 8 h) afforded the percentage extractives, on a dry mass basis, using these two solvents. Material from the petroleum ether extract was then subjected to proton nuclear magnetic resonance spectrometry (n.m.r.) using CD_2Cl_2 as the solvent.

Scanning electron microscopy

Fresh leaf and stem material was fixed in 6% glutaraldehyde in a 0,05 mol dm^{-3} sodium cacodylate buffer (pH 7,2) and dehydrated through an ethanol series prior to critical point drying. Pieces of dried material were subsequently broken in two, gently pulled apart to produce threads, and then mounted on SEM stubs. Mounted material was sputter coated prior to viewing.

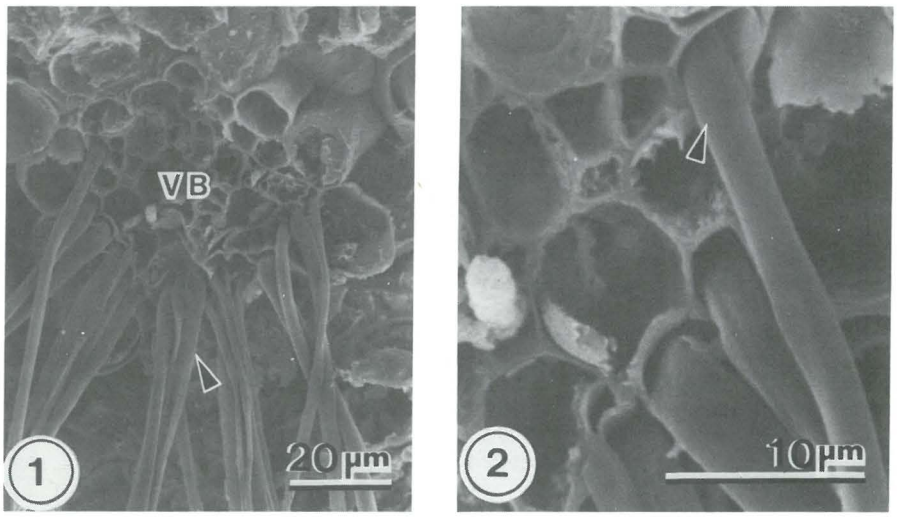
Transmission electron and light microscopy

Pieces of mature leaf tissue (2 mm^2 in area) and 2 mm slices of stem tissue were fixed in 3% glutaraldehyde in 0,05 mol dm^{-3} sodium cacodylate buffer (pH 7,2) for 22 h. After fixation the material was buffer-washed and post-fixed in 2% cacodylate-buffered osmium tetroxide. Tissue was then post-stained in 2% aqueous uranyl acetate, dehydrated through a graded ethanol series, followed by propylene oxide, and embedded in low viscosity resin (Spurr 1969). Ultra-thin transverse and longitudinal sections (approximately 0,1 µm) were collected on copper grids and contrasted with lead citrate (Reynolds 1963) prior to viewing. Thick (2 µm) transverse and longitudinal sections of leaf and stem tissue were made from resin-embedded material prepared for transmission electron microscopy. Sections were stained with Ladd's (R) multiple stain.

Results

Scanning electron microscopy

SEM showed that the elastic threads of the leaf are exclusively associated with the vascular tissues i.e. the vascular bundles and the midrib (Figure 1). However, these threads do not occur in the adaxial portion of the bundles as might be expected if they originate from the xylem tissue, but form a distinct semi-circle around the abaxial edge of each bundle (Figure 1). Furthermore, the threads seem to arise from *within*



Figures 1 & 2 **(1)** Elastic threads (▶) of the leaf occur in a semi-circle around the abaxial portion of the vascular bundles (VB). **(2)** Threads arise from within the cells (▶) and there is no evidence of tracheary element walls becoming extended to form threads.

these cells of the vascular bundles (Figure 2). Where xylem vessels were observed within the vascular bundles, there was no evidence that the wall had become extended to give rise to elastic threads (Figure 2).

SEM of the stem tissue of *M. acuminata* showed the elastic threads to be associated predominantly with the inner cortex (Figure 3).

Phytochemical data

The *M. acuminata* leaves analysed contained 11,4% acetone-extractable material and 1,4% petroleum ether-extractable material (polyisoprene), while the stems contained only 9,9% acetone extractables and 0,45% petroleum ether extractables.

The white semi-crystalline solid material (m.p. 48 – 50°C) obtained from the petroleum ether extract, and which has the properties of E-1,4-polyisoprene, gave the following elemental analysis: C, 88,23%; and H, 12,17%. C_5H_8 requires C, 88,23% and H, 11,77%. At 80 MHz the proton spectrum consists of a finely-split doublet (δ 1,55 and 1,57) for the methyl group, a singlet (1,97 δ) for the two methylene groups, and a multiplet with peaks at δ 5,01; 5,03; 5,10 and 5,11 for the vinylic proton. The ^{13}C nuclear magnetic resonance spectrum (20 MHz) of the material proved to be even more diagnostic for E-1,4-polyisoprene on account of its simplicity. In the off-resonance-decoupled spectrum, four characteristic peaks appear as a quartet at 15,41 ppm (methyl group), triplets at 26,40 and 39,42 ppm (methylene groups), a doublet of 123,95 ppm (methine group) and a singlet at 134,57 ppm (quaternary carbon) (Figure 4 shows the off-resonance-decoupled spectrum).

Anatomy of the mature leaf

The mature leaf of *M. acuminata* is hypostomatous. The mesophyll is differentiated into distinct palisade and spongy regions, with the number of cells within each tissue type being affected by environmental factors. The material used in the present study comes from shrubs growing in the forest margin. These leaves, which can thus be considered shade leaves, usually have a single palisade layer, and three to four layers of spongy tissue (Figure 5). Vascular bundles occur at regular intervals, and are situated within the spongy mesophyll, just below the palisade layer; large bundles extending almost to the abaxial epidermis (Figure 5). In addition to xylem and phloem elements, the vascular bundle comprises a crescentic layer of smaller cells which subtends the abaxial part of the bundle (Figure 5). It is from these cells that the elastic threads originate (Figure 1). Longitudinal sections show the elongated nature of the cells of the vascular bundle, including the crescentic cells (Figure 6).

Similar small cells are found around the abaxial portion of the vascular tissue of the midrib (Figure 7). In addition,

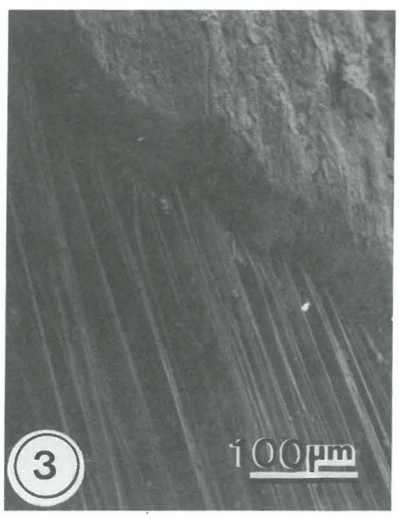


Figure 3 Threads of the stem apparently arise from cells of the cortex, at the interface of the phloem and cortex.

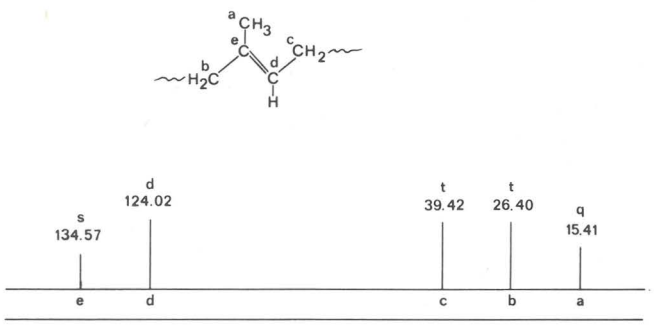


Figure 4 Off-resonance-decoupled ^{13}C n.m.r. spectrum of *M. acuminata* extract using CD_2Cl_2 solvent.

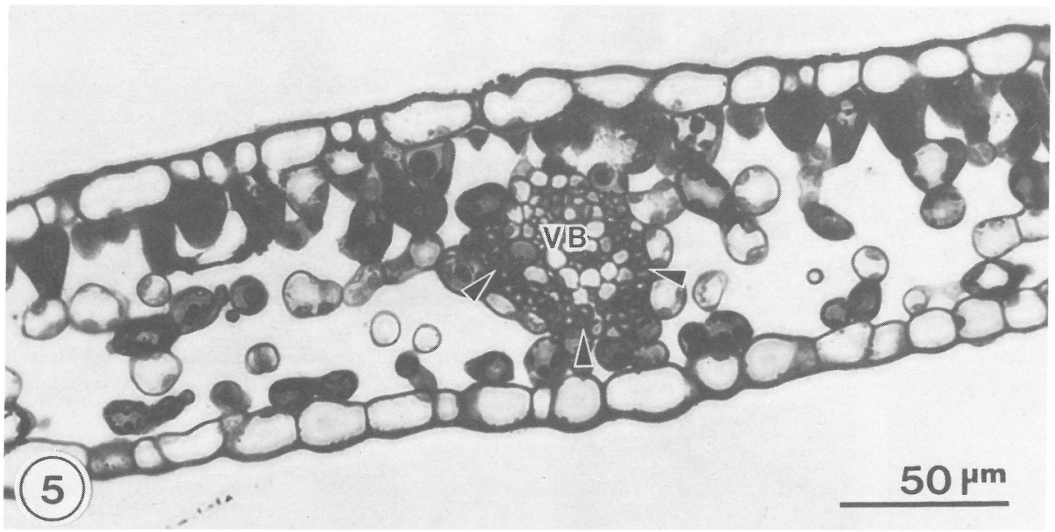


Figure 5 Transverse section of mature leaf. The crescent of cells surrounding the abaxial portion of the vascular bundle (VB) and from which the threads arise is indicated by the arrows (▶).

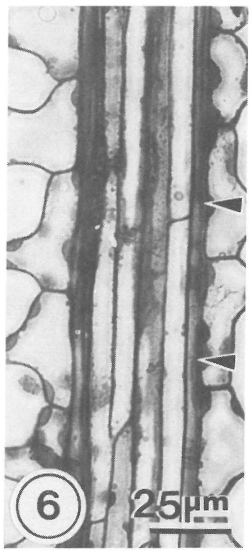


Figure 6 Longitudinal section of the leaf showing the elongated nature of cells comprising the vascular bundle (▶, cells of the abaxial portion of the vascular bundle).

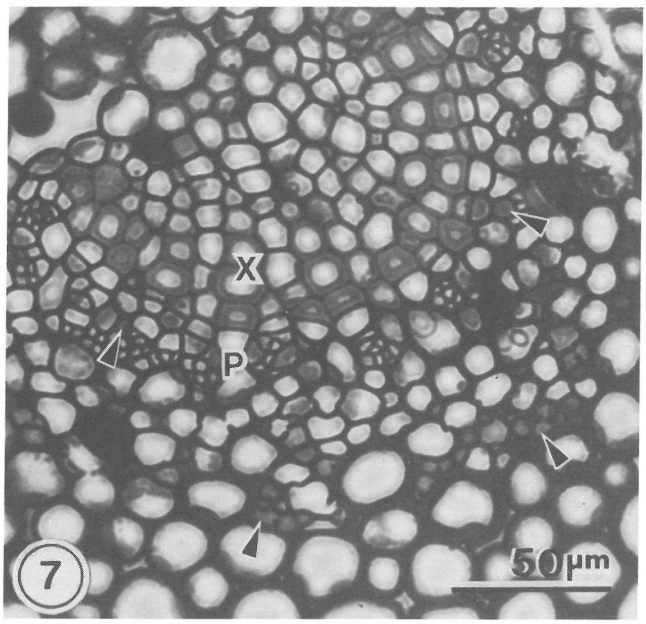


Figure 7 Transverse section of leaf midrib. Small cells with osmiophilic contents (▶) occur scattered throughout the phloem tissue. X-xylem, P-phloem.

small cells containing osmiophilic material occur scattered throughout the phloem tissue of the midrib (Figure 7). Indeed, it is not possible by the use of light microscopy alone to distinguish the phloem elements from the ground tissue, and to this end transmission electron microscopy was used.

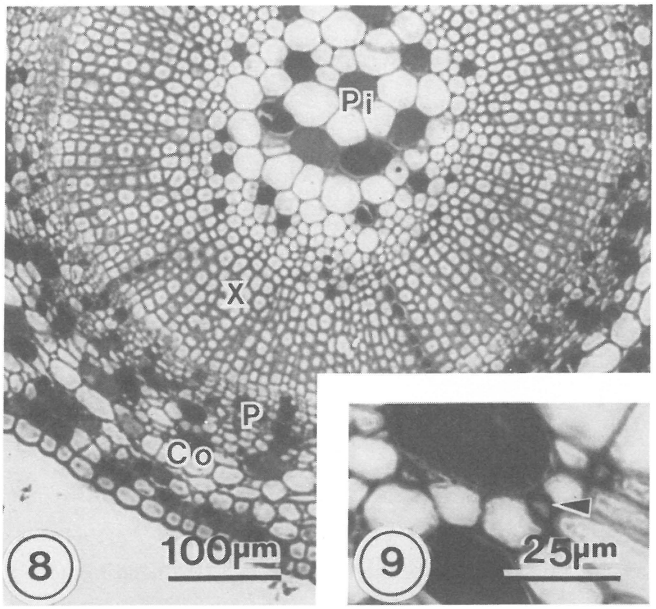
Anatomy of the mature stem

The anatomy of the stem of *M. acuminata* is illustrated in Figure 8. Small cells at the interface of the phloem and cortex (the region in the stem from which the elastic threads originate) seem similar in size and appearance to those cells comprising the crescentic layer which subtends the vascular bundles (Figure 9). Cells of both the pith and cortex contain much granular osmiophilic material (Figure 8).

Ultrastructure of the leaf

From transmission electron microscopy, it is apparent that osmiophilic material, similar in appearance to that found in the crescent of cells surrounding the vascular bundles (Figure 10), occurs within all living cell types of the leaves. There is however, a correlation between cell type and the amount of intracellular osmiophilic material. The palisade cells in particular have very little osmiophilic material, with only an occasional osmiophilic particle visible within the cytoplasm (Figure 11). The vacuoles of the palisade often contain a very densely staining, granular material (Figure 12).

In contrast to the palisade, the spongy mesophyll is relatively rich in osmiophilic material, with numerous particles occurring in the cytoplasm and partially protruding into the vacuoles (Figure 13). These osmiophilic particles often appear to be preferentially distributed around the nucleus (Figure 14). Particles also occur in close association with the chloroplasts of the spongy mesophyll and in some instances, osmiophilic material which shows the same staining density as the particles, is found within the chloroplasts (Figure 15). All particles are bounded by a dense, osmiophilic layer (Figures 13 & 14).



Figures 8 & 9 **(8)** Transverse section of stem showing cells with granular material in the pith (Pi) and cortex (Co). **(9)** Small cells in the cortex at the interface of the phloem and the cortex are similar in appearance to the cells of the leaf vascular bundles from which threads originate (▶). X-xylem, P-phloem.

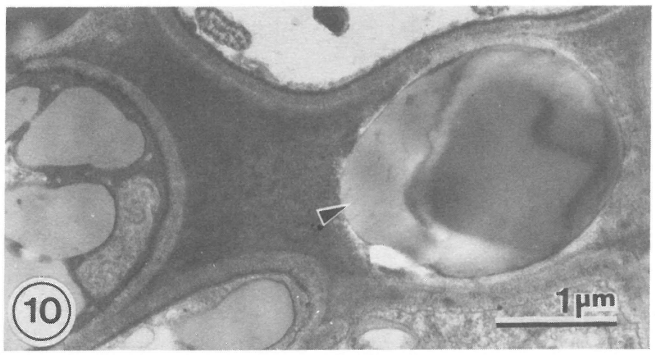


Figure 10 A thread-forming cell of the leaf vascular bundles, completely filled with dense, osmiophilic material (▶). The walls of such cells were often greatly thickened.

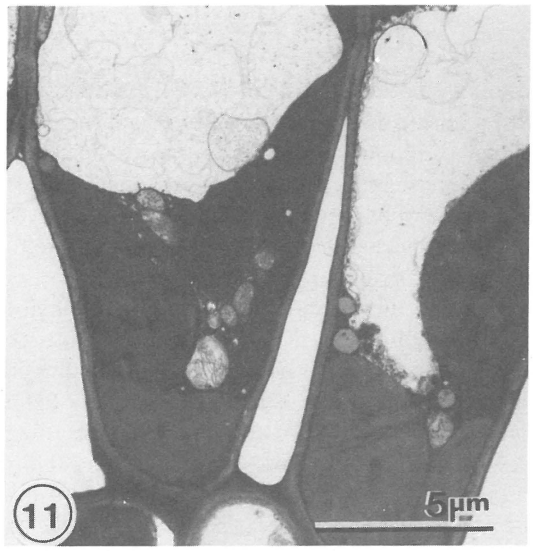


Figure 11 Palisade tissue is essentially devoid of osmiophilic particles.

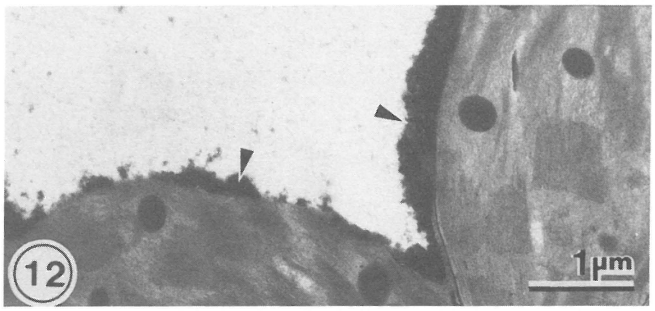


Figure 12 Intravacuolar, granular material (▶) is found in the palisade cells.

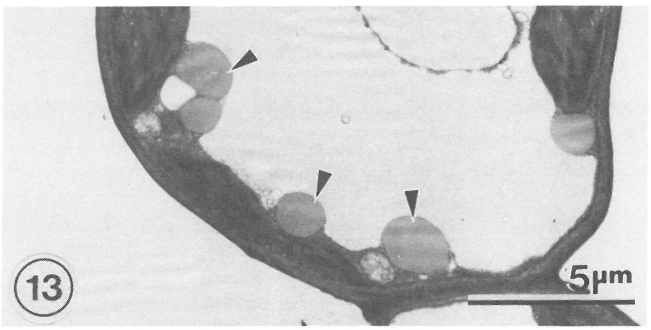


Figure 13 Larger numbers of osmiophilic particles (▶) are generally found in the cytoplasm of the spongy mesophyll cells. Note the osmiophilic boundary layer of the particles.

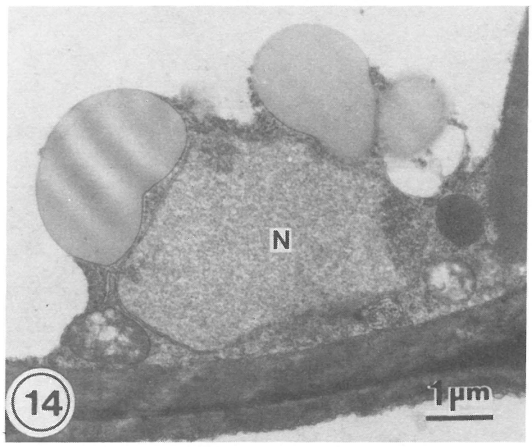


Figure 14 Osmiophilic particles often appear to be preferentially distributed around the nuclei (N) of the spongy mesophyll cells. It is not known whether such a distribution pattern occurs because there is more cytoplasm associated with the nucleus in an otherwise vacuolated cell, or whether the particles are specifically associated with the nucleus.

Particles occur in the cytoplasm of epidermal cells (Figure 16) and within the vacuoles of the guard cells (Figure 17).

Cells with large amounts of osmiophilic material are found within the vascular tissue. Indeed, the cells of the abaxial crescent of the vascular bundle appear devoid of any other cytoplasmic detail (Figure 10).

The phloem cells, particularly the sieve elements of *M. acuminata* are atypical and difficult to identify. Sieve elements can generally be distinguished by the presence of sieve plates, the presence of lateral sieve areas and the lack of vacuolar membranes. The latter criterion could not be exclusively applied in the present study, as dispersal of secondary products

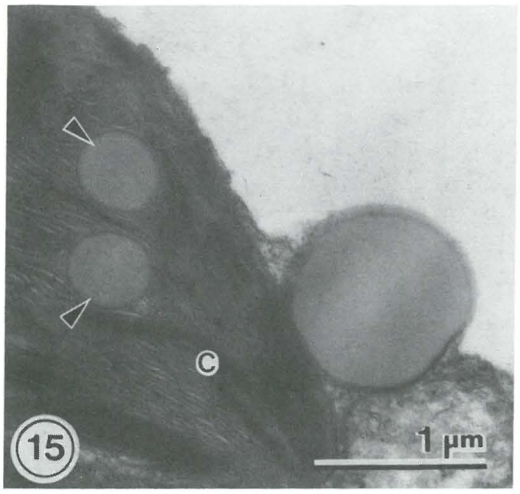


Figure 15 Material (▶) of similar staining density to the osmiophilic particles is found in the chloroplasts.

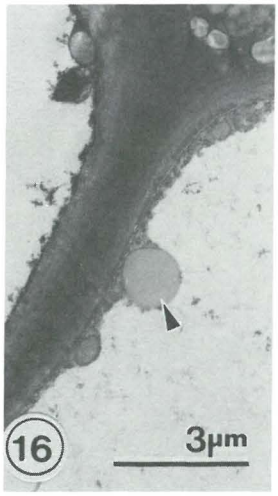


Figure 16 Osmiophilic particles (▶) are found in epidermal cells.

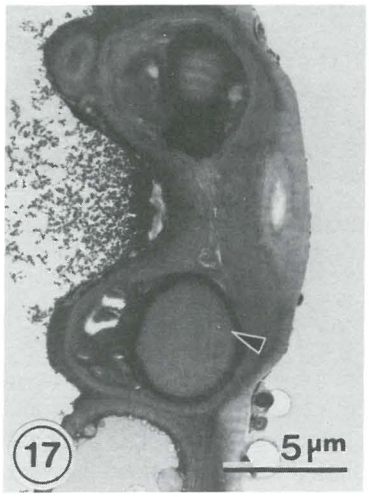


Figure 17 Large osmiophilic particles (▶) were found in the guard cells.

during fixation often obscures the tonoplast. Longitudinal sections seldom showed sieve plates. Furthermore, no plastids were observed in the sieve elements of the leaf. Some elements of the phloem were however observed to contain P-protein. Thus, for the purposes of the present study a vascular element was judged to be a sieve element if it met any of the above criteria or if P-protein was present (Figure 18). The dispersed protoplasm of these anucleate, P-protein-containing elements is dominated by P-protein and osmiophilic particles, which are in close contact with the P-protein and often almost occlude the cell (Figures 18, 19 & 20). Sieve element endoplasmic reticulum and mitochondria are found in close association with the osmiophilic particles (Figures 19 & 20).

The nucleate phloem companion cells, characterized by large numbers of mitochondria and free ribosomes within the cytoplasm also contain numerous cytoplasmic osmiophilic particles (Figure 21).

The nucleate cells of the phloem parenchyma are highly vacuolate. Endoplasmic reticulum; membrane whorls; chloroplasts (similar to those of the spongy mesophyll); peroxisomes (often with small vesicles in the matrix); mitochondria and numerous osmiophilic particles are found in the cytoplasmic region of these cells (Figure 22).

Ultrastructure of the stem

As in the leaf, numerous osmiophilic particles are found within the phloem tissue as well as in the cortex at the interface of the phloem and cortex.

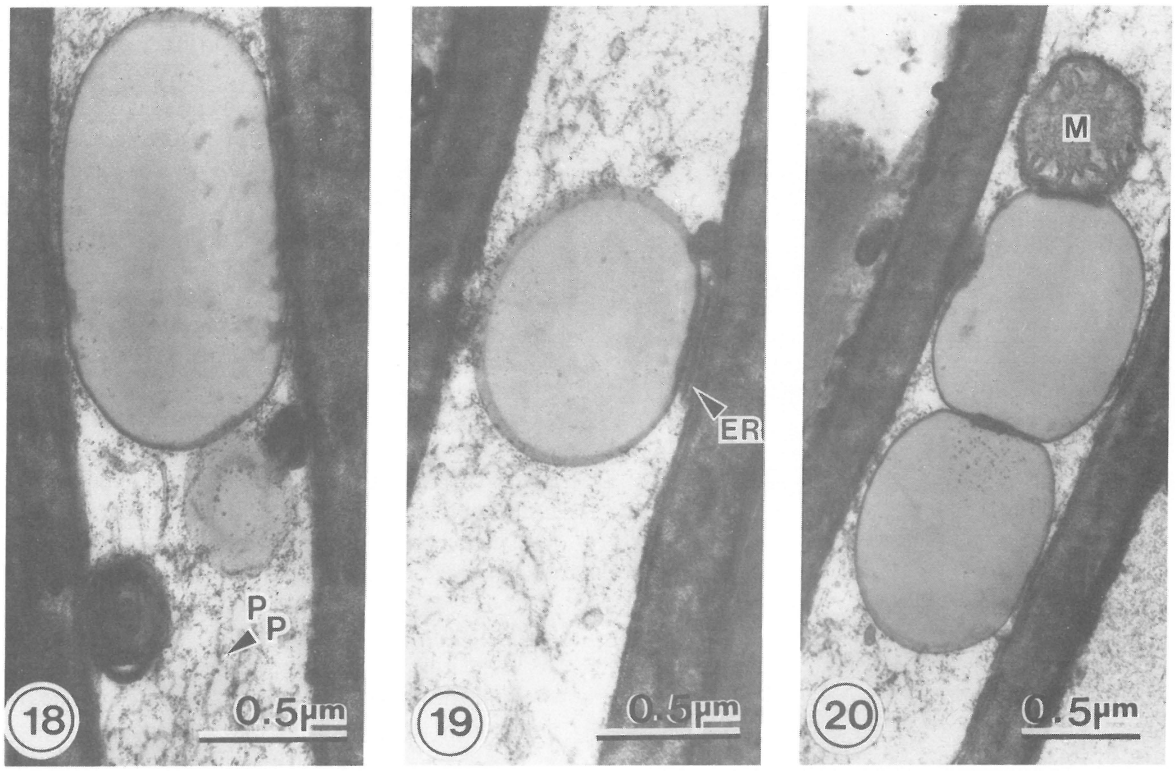
The ultrastructure of the stem phloem and the distribution of osmiophilic particles within the phloem tissue is similar to that of the leaf. Plastids are however, found in the sieve elements of the stem (Figure 23).

The cells of the cortex at the interface of the phloem and the cortex contain a large amount of very dense granular osmiophilic material. Numerous osmiophilic particles are dispersed throughout the granular matrix (Figure 24). The presence of the granular osmiophilic material in such quantities within these cells obliterates much cytoplasmic detail. However, most organelle types can be found within these cells viz. chloroplasts (often containing large starch grains), mitochondria and nuclei (Figure 24). Endomembranes cannot be discerned and it is uncertain whether the granular and particulate osmiophilic material is vacuolate or cytoplasmic. The cortical cells containing both granular and particulate osmiophilic material are elongated in longitudinal section.

Discussion

The results from SEM alone discredit the idea that the threads in the leaves and stems of *M. acuminata* are the elastic thickening of tracheids (Esterhuyzen as cited by Coates-Palgrave 1977). Although it was found that the threads are associated exclusively with the vascular bundles of the leaf, they occur only around the abaxial portion of the bundle, whereas xylem occurs in the adaxial half of the bundle. In this respect, it is also of relevance that stem threads are associated with the cortex at the interface of the phloem and the cortex and not with the xylem tissue. Thus from their spatial distribution, it seems unlikely that the threads are part of the xylem vessels. Furthermore, the threads seem to arise from within the cells of the vascular bundle, as opposed to from the walls, which again provides circumstantial evidence against the idea that the threads are the elastic thickening of the tracheids (Coates-Palgrave 1977).

This circumstantial evidence is corroborated by proton n.m.r. analysis of thread material which shows that the threads are composed of polyisoprene (E-1,4-polyisoprene). Results show that more polyisoprene is present in the leaves (1,48%) than in the stems (0,45%) of *M. acuminata*. This contrasts with the Z-1,4-polyisoprene producing shrub guayule, which



Figures 18, 19 & 20 **(18)** Longitudinal section of leaf sieve elements which are distinguished by the presence of P-protein (Pp). **(19)** Osmiophilic particles occur in the elements in close association with the P-protein, endoplasmic reticulum (ER), and mitochondria (M) **(20)**. In some instances the particles almost totally occlude the elements (18).

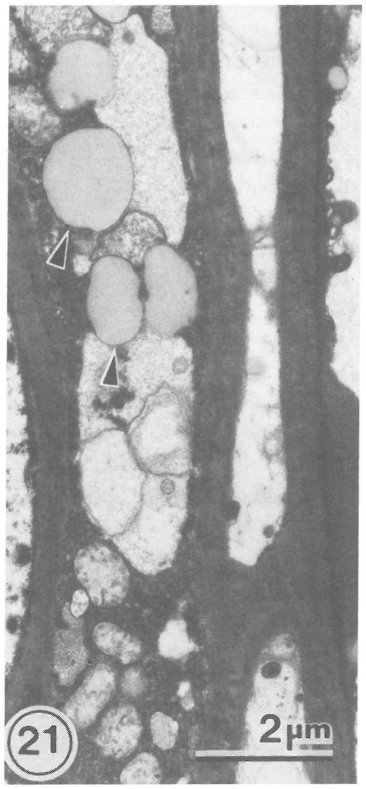


Figure 21 Osmiophilic particles (▶) are found in the phloem companion cells.

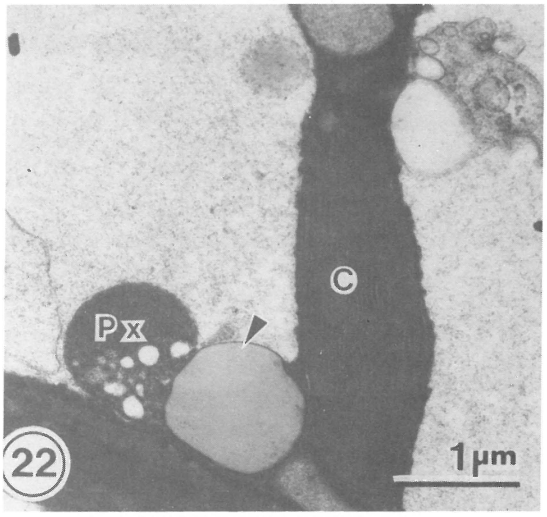


Figure 22 Osmiophilic particles (▶), chloroplasts (C), mitochondria (M), and peroxisomes (Px) can be distinguished within the phloem parenchyma.

has very little polyisoprene in the leaves (0,5%) by comparison to the stem (6,0%).

Although the polyisoprene threads in *M. acuminata* are found to be associated only with vascular tissue, transmission electron microscopy shows that polyisoprene (as suggested by the similarity in density of staining of cytoplasmic particles to the polyisoprene which occupies the abaxial cells of the vascular bundles) occurs throughout the various leaf tissue types. The formation of threads is however probably dependent not only on the occurrence of polyisoprene within the cell, but also on the quantity of polyisoprene, and the cell type. Indeed, it is suggested by Lloyd (1932) that there is no flow of latex from cut guayule stems because guayule lacks latex vessels, rather than because it lacks latex. Similarly it is suggested that in *M. acuminata* the threads originate only from cells which have firstly, a relatively high proportion of

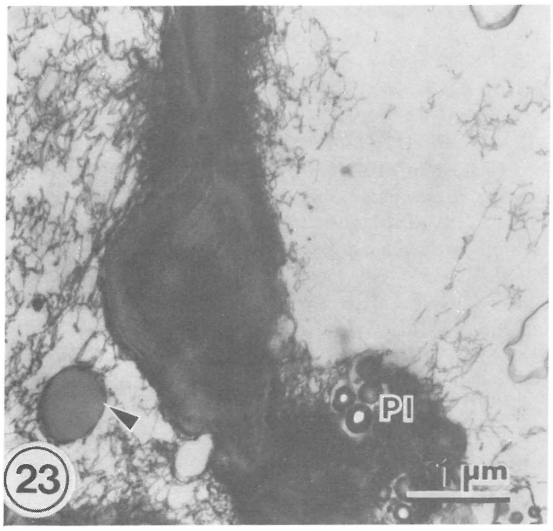


Figure 23 A sieve plate separates two adjacent stem sieve elements in which osmiophilic particles (▶) and plastids (Pl) occur.

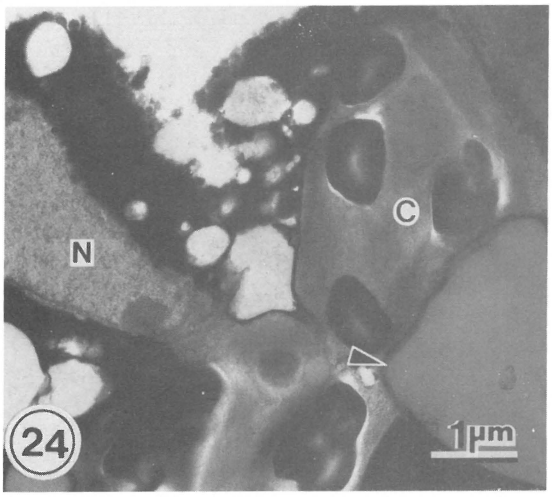


Figure 24 Dense, osmiophilic material (resin ?) in the cortical cells of the stem obliterates much cytological detail, however, nucleus (N), chloroplasts (C), and osmiophilic particles (▶) are visible within these cells.

polyisoprene, and secondly, are elongated in longitudinal section; as are the cells in the cortex at the interface of the phloem and the cortex and the cells forming the crescent around the abaxial portion of the vascular bundle.

Specialized cells, or interconnected rows of such cells, containing latex are generally termed 'laticifers' (Fahn 1979). Laticifers are found in most latex-containing species, the notable exception being guayule, where polyisoprene occurs in parenchymatous cells which are not morphologically differentiated into laticifers (Artschwager 1943). Laticifers in many species maintain a living protoplast. The nucleus remains in this protoplast on maturation of the elements, and the cytoplasm occurs as a parietal layer enclosing the vacuolar sap, or latex e.g. *Ficus* (Rachmilevitz & Fahn 1982). The laticifers in *Hevea* are formed as a result of the autolysis and anastomosis of the cytoplasm of the latex vessel cells. This results in latex that is a specialized cytoplasm containing a suspension of polyisoprene particles and intact or partially degraded organelles (Backhaus & Walsh 1983). The crescentic layer of cells subtending the abaxial portion of the vascular bundles of *M. acuminata* can, because of their high polyisoprene content and elongated nature, be defined as laticifers. There is however, no cytoplasm visible within these cells, which thus portray an atypical laticifer ultrastructure. As it is considered unlikely that rubber moves out of the cell of synthesis (Backhaus & Walsh 1983) the laticifers of the leaf vascular bundles in *M. acuminata* must therefore be the endpoint of a developmental sequence characterized by extensive rubber production. Furthermore, in the present study no partially differentiated 'laticifers' were observed associated with vascular bundles or parenchymatic elements in the cortex of the stem, which suggests that laticifer differentiation is completed at an early stage of leaf development.

The polyisoprene-containing cells of the cortex of *M. acuminata* are ultrastructurally more typical of previously described laticiferous cells (Fahn 1979) as the polyisoprene particles occur dispersed throughout the cytoplasm together with recognizable organelles e.g. chloroplasts and mitochondria. However, the presence of large quantities of dense, osmiophilic, granular material (possibly also a form of latex) makes it very difficult to determine precise ultrastructural relationships within these cell types.

The ultrastructural appearance of the E-1,4-polyisoprene in *M. acuminata* is very similar to that of the Z-1,4-polyisoprene found in *Hevea* and guayule. Particles of these three species are all irregularly shaped when they occur in the cytoplasm, but are spherical when they protrude into the vacuole, or in the case of guayule, when they occur in the vacuole (Backhaus & Walsh 1983). Furthermore, particles in all three species are bounded by an osmiophilic layer, which it is suggested comprises protein, lipids and phospholipids (Cockbain & Philpott 1963).

The site of *de novo* polyisoprene synthesis within cells is unknown. The Frey-Wyssling complexes, suggested to be highly modified plastids (Gomex & Moir 1979) are suggested to be central to this process in *Hevea* (Dickenson 1969). Furthermore, it is known that at least two of the steps required for polyisoprene biosynthesis can occur in chloroplasts (Britton & Goodwin 1971; Grumbach & Forn 1980), although it is not certain whether the formation of the long-chained polyisoprene particles within plastids is possible. In this respect the occurrence of particles very similar in staining properties to polyisoprene particles, within the chloroplasts of *M. acuminata* is of great interest as it suggests that the formation of long-chained polyisoprene particles within chloroplasts is possible. The possible role of the chloroplast in polyisoprene biosynthesis in *M. acuminata* is presently being investigated.

The function in plants of both laticifers and polyisoprene remains unknown. It has been suggested that laticifers and associated rubber may be involved with water regulation in the plant, transport of oxygen through the tissues, or protection against animals (Esau 1965). A widely accepted theory is that the laticifers form an excretory system. This interpretation stems from a commonly accepted belief that polyisoprene is a non-functional by-product of cellular metabolism (Esau 1965). The great expenditure of energy together with the high proportion of fixed carbon which must be diverted into the synthesis of polyisoprene largely negates such an argument. Furthermore, the finding in the present study that polyisoprene in *M. acuminata* occurs in the highly specialized stomatal guard cells and sieve elements (in close association with P-protein, mitochondria and endoplasmic reticulum) suggests that polyisoprene has a role to play in the functioning of these cells, even if at present the function remains obscure.

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