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# DEVELOPMENT AND DISTRIBUTION OF LATICIFERS IN PLANTS

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Submitted for the degree of Doctor of Philosophy  
Department of Biological Sciences  
University of Durham



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## ABBREVIATIONS

BF	bright field
°C	degrees Centigrade
cm	centimetre
CW	cell wall
DIC	Differential Interference Contrast
DPX	Permanent slide mountant (a mixture of distyrene, a plasticizer and xylene)
ER	Endoplasmic reticulum
Ep	epidermis
F	phloem cell
Fig.	Figure
Fs	fibre schlereid
GB	Golgi body
µg	microgram
HCl	hydrochloric acid
Lb	lipid body
Is	intercellular space
Lv	laticifers
LR	London resin
LS	longitudinal section
LV	latex vessels
M	molar
mg	milligram
ml	millilitre
mm	millimeter
mt	mitochondria
mv	midvein
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometre
n	nucleus
OsO <sub>4</sub>	osmium tetroxide
PBS	phosphate buffered saline
PM	plasma membrane
Ph	phloem
r.p.m	revolutions per minute
R.T.	room temperature
SEM	scanning electron microscope
TBS	Tris-buffered saline
TEM	transmission electron microscope
TESPA	aminopropyltriethoxysilane
Tc	tannin cell

TS	transverse section
UV	ultra-violet
V	vacuole
Vn	vein
VB	vascular bundle
v/v	volume:volume ratio
w/v	weight:volume ratio
x	xylem cell
ZIO	Zinc Iodide

### **symbols**

$\alpha$	alpha
$\beta$	beta
$^{\circ}$	degree
$\mu$	micro
$\lambda$	lambda/wavelength

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## MEMORANDUM

The work in this thesis was carried out by myself in the Department of Biological Sciences at the University of Durham. I declare that this work has not been submitted previously for a degree at this or any other University. This thesis is a report of my own work, except where acknowledged by reference. The copyright of this thesis rests with the author. No quotation should be published without his written consent, and information derived from it should be acknowledged.

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# **1. INTRODUCTION**

## 1.1 LATICIFERS

Secretory cells, canals and cavities are frequently encountered in the tissues of woody plants, often in the region around secondary xylem in stems. One such group of structures is laticifers, sometimes confused with secretory cells. The structure and distribution of laticifers have been discussed at length by Chalk (1983).

The word laticifer and its adjectival form laticiferous are derived from the word latex, meaning juice in Latin. Esau (1965) described laticifers as cells or series of fused cells containing a fluid called latex and forming systems that pass through various tissues of the plant body. The systems are usually pictured as tubular structures that are branched or unbranched, and in many species a very complex laticiferous system is formed by anastomosis between tubes.

In the past decade, research on laticifer organization has been largely overshadowed by work on the phloem system. It has been reported that laticifers and sieve elements show similar problems for microscopy studies with regard to tissue preservation. The high pressure within both types of cells is a major impediment during specimen preparation, because the moment tissues are excised the protoplasts tend to become damaged, with the contents of the cells being easily displaced, which thereafter makes any reconstruction of the overall laticifer system difficult (Fineran 1983, Fineran & Condon 1988). However, recent advances in technologies for microscopy, including preparation techniques such as cryo-preparation and low temperature electron microscopy make it possible to retain the integrity of the cells without using destructive chemicals. These methods have provided further information on laticifer architecture and cellular organization.

It is, however, very unfortunate that, until now, there has been little discussion about the specific function of the laticifers. Attention has been concentrated more on the structural studies and distribution of the laticifers in relation to their taxonomic distribution in the plants, as shown in Table 2. Altogether, the function of the laticifers was generally summarized as being a special type of storage or excretion system in plants. Another interesting observation, however, is that in almost all of the laticiferous plants, rubber or latex has been observed to have some repellent properties against insects, providing the plants with self defense against pest attack.

Studies at the ultrastructural level show that apart from the ordinary organelles that can be found in laticifers such as rubber, [vacuoles, etc.], there are some organelles that can only be found in certain genera. These distinctive organelles can sometimes contribute as a unique characteristic to a certain plant. In *Poinsettia* (Euphorbiaceae) for instance, numerous nuclei and plastids with starch grains were reported as a very prominent feature of the laticifers ( Fineran 1983, Roy & De 1992). Another example as in *Hevea brasiliensis* (Euphorbiaceae), are lutoids, the second most numerous organelle after rubber particles (Dickenson 1969, Gomez & Southorn 1969, Gomez & Yip 1975). The size of lutoids has been described as almost the same as rubber particles, which is 0.5-3  $\mu\text{m}$  (Southorn 1966, 1968 & 1969). Lutoids are very sensitive to osmotic conditions and will swell and may disrupt under hypotonic conditions (Homans *et al.* 1948).

## 1.2 STRUCTURE OF THE LATICIFERS

Laticifers are an extremely heterogeneous group of cells, not only metabolically, but also developmentally and structurally (Mauseth 1988). They are typically classified into two fundamental classes on the basis of their structure. They may be simple or compound in origin (Esau 1965). The simple laticifers can be described as single cells while the compound laticifers are derived from series of cells. In a more highly specialized state the series of cells in a compound laticifer become united by dissolution of intervening walls. Because of this junction of cells the compound laticifers are commonly called articulated laticifers, whereas the simple laticifers are called non-articulated. Both kinds of laticifers may be branched or unbranched and are often referred to as 'vessels', probably due to their resemblance in origin to that of the conducting elements. This classification, however, according to some anatomists, has no relationship to taxonomic groups and thus different types of laticifers may be found in different species of one family (de Bary 1884).

It has been reported that in several families, included the Asclepiadaceae and Euphorbiaceae, both articulated and non-articulated laticifers occurred, and in a few instances the two types occur together in the same plant, as in *Jatropha* (Dehgan & Craig 1978)

### 1.2.1 ARTICULATED LATICIFERS

Articulated laticifers are fundamentally different from the non-articulated laticifers in their development and structure. Each is actually a row or file of individual laticiferous cells. Huang and Sterling (1970) observed in *Allium*, that each cell is connected to the two adjacent laticiferous cells by plasmodesmata, but in other tissue there is usually some form of perforation of the common wall. With the variations in structure the articulated laticifers can be divided further into two subdivisions. Some of the articulated laticifers consist of long cell chains or compound tubes not connected with each other laterally; others form lateral anastomoses with similar cell chains or tubes, all combined into a net-like structure or reticulum. The former are known as articulated non-anastomosing laticifers and the latter as articulated anastomosing laticifers (Esau, 1965)

As with non-articulated laticifers, the articulated laticifers can occur in all parts of the plant body, either in young tissues or older ones, vegetative or floral. An investigation on *Taraxum kok-saghyx*, reported that the articulated laticifers are present in the seedling, at the germination stage (Bonner & Galston 1947). In *Papaver somniferum* laticifers are present in the seedling and young plant, but they are larger and most abundant in the seed capsule (Thureson-Klein 1969, Nessler & Mahlberg 1977). However, in the tree from which commercial rubber is extracted (*Hevea brasiliensis*), the most important laticifers are located in the bark from where the rubber is exuded when it is tapped (Gomez 1982).

## 1.2.2 DEVELOPMENT OF ARTICULATED LATICIFERS

The articulated laticifers develop into extensive tube-like structures by the constant addition of new primordial cells to the existing ones and not by the growth of individual cells (Esau 1965). This growth is by the continuous initiation of the nearby parenchyma cells, which are later converted to laticifer cells. As these new cells differentiate adjacent to older, existing laticifer cells, the common wall becomes perforated, and the new cells are added to the laticifer, resembling the process where new vessel elements are added in the xylem (Mauseth 1988).

There are two types of developments in articulated laticifers, depending on how the developing laticifers interrelate with the neighboring cells during the differentiation, which are the non-anastomosing articulate laticifers and anastomosing articulate laticifers. Non-anastomosing articulated laticifers occur in some species because any single laticifer, which is in a row of neighboring cells does not merge with another laticifer during its differentiation. This type of laticifer can be found in *Achras* (Sapotaceae), *Allium* (Liliaceae), *Ipomoea* (Convolvulaceae) and *Musa* (Musaceae).

In articulated anastomosing laticifers, one laticifer can fuse with others, forming an extensive three-dimensional network that permeates the entire plant. *Meconopsis*, *Papaver* (Papaveraceae); *Carica* (Caricaceae); *Cichorium*, *Lactuca*, *Taraxacum*, *Tragopogon* (Compositae); and *Hevea*, *Manihot* (Euphorbiaceae) portray this type of laticifer. To make this formation possible, the laticifers must be able to branch. However, the laticifer itself does not grow out to form the branch; instead, regular parenchyma cells that lie between two existing laticifers are induced to differentiate into latex-bearing cells (Blaser 1945, Vertrees & Mahlberg 1978).

Witler and Mauseth (1984a,b) studied a phylogenetic series of *Mammillaria* (Cactaceae) and revealed an interesting set of stages in the evolution of a complex secretory system. Amongst the articulated laticifers, *Mammillaria* has a quite unusual type of laticifer because their wide lumina are formed by the disorganization (lysis) of cylindrical cell masses several cells wide and not by the resorption of the end walls of single files of cells. The laticifers furthermore are lined by a multicellular epithelium that is quite thick. The diameter of the laticifers will increase further because the inner epithelial cells become disorganized.

In subgenus *Subhydrochylus* ("semi milky" mammillarias), the group contain cortical regions of tissue whose cells are extremely watery, contain few chloroplasts and have unusual thin walls. The cells of the outer cortex begin differentiation by complex modification of their walls. The process begins by swelling at certain sites, forming bulbous pockets that expand throughout the wall. At the same time, other flat regions arise in the wall and stain darkly. The walls are converted to large regions that are non-compact and whose loose microfibrils alternate with dense, dark regions. These walls appear to be empty epithelial cells (Mauseth 1978b). The wall ultimately breaks down to form the lumen and the first "secretion". Simultaneously, the protoplasts become modified by filling with derived vesicles; the protoplast lysis, mixing with the wall material and adding to both the lumen and the secretion. The secretion is holocrine.

However in subgenus *Mammillaria*, there is no structure formed by splitting of cells (schizogeny), and the walls are not modified as just described for subgenus *Subhydrochylus*. Instead the first step of differentiation is the production of numerous vesicles of diverse types and from diverse sources, which are from the chloroplast, endoplasmic reticulum, dictyosomes and plasmalemma. The wall becomes thinner as material is apparently removed from it, thereby forming more

vesicles. Finally, the walls rupture as digestion continues; where at this stage the protoplast has been converted to latex. Adjacent cells undergo division and create a smooth, well-defined epithelium (Mauseth 1978a).

Another study by Sheldrake (1970) on 7 species with articulated laticifers and 4 species of non-articulated laticifers, by electron microscopy, strongly suggested that the wall of the articulated laticifers is dissolved enzymatically by cellulase presence in the laticifers itself. He also suggested the high level of auxin could also help in increasing cellulase activity by weakening or loosening the structure of the cell wall. This (enzyme in the laticifers) is another fundamental enzymatic criterion in dividing the two groups of laticifers (articulate & non-articulate).

An assay study carried out on the species with articulated laticifers showed a very high content of cellulase, whereas there is hardly any evidence of cellulase in the non-articulated species. This also explains why in certain species, laticifers are abundant and concentrated, as in *Hevea brasiliensis*, where the cellulase content is 50-150 times higher than the other species (Sassen 1965; Sheldrake & Moir 1970). It has also been reported that the amount of cellulase is higher in the young tree as compared to the mature tree, where the active differentiation of cells takes place (Tracey 1950).

### **1.2.3 NON-ARTICULATED LATICIFERS**

Non-articulated laticifers originate from single cells that through continued growth develop into tube-like structures, often much branched, but typically they undergo no fusions with other similar cells. The non-articulated laticifers vary in degree of complexity in their structure. Some further develop into long, more or

less straight tubes; others branch repeatedly, each cell thus forming an immense system of tubes. Esau (1965) decided that the appropriate names for these two types of structures are non-articulated unbranched laticifers and non-articulated branched laticifers, respectively.

Non-articulated laticifers are characteristic of various species of the following families: Apocynaceae, Asclepiadaceae, Euphorbiaceae, Moraceae and Urticaceae (Fahn, 1982). Typically the non-articulated laticifers are extraordinarily long cells, often extending from the root up into the stem and leaves. However, in certain species, such as *Cryptotegia*, *Jatropha* (Dehgan & Craig 1978) and *Parthenium argentum* (Metcalf, 1967) the non-articulated laticifers are small, rather isodiametric idioblasts, somewhat resembling myosin cells. In some species they are unbranched as for instance *Cannabis* (Moraceae); *Cyclanthus* (Cyclanthaceae) (Wilder & Harris, 1982); *Urtica* (Urticaceae) and *Vinca* (Apocynaceae). In others, they branch frequently, forming an even more extensive network as in *Asclepias*, *Cerepegia*, *Cryptostegia* (Asclepiadaceae); *Broussonetia*, *Ficus*, *Maclura* (Moraceae); *Nerium* (Apocynaceae) and *Euphorbia*, *Jatropha* (Euphorbiaceae).

#### 1.2.4 DEVELOPMENT OF NON-ARTICULATED LATICIFERS

Non-articulated laticifers can occur in any part of the plant, most often in the softest regions such as the pith and cortex, but they can invade leaves and leaf gaps as well as wood and phloem (Mauseth 1988). Blaser (1945) and Mahlberg (1969) describe how the tip of the non-articulated laticifer may project into the margins of the shoot and root meristems, but as the meristem grows away, the laticifer continuously invades the newly formed stem and root tissues. In some

species, new initials are formed in new tissue and an older plant will have more laticifers than a younger plant.

As already mentioned, different forms of non-articulated laticifers exist and in certain mature plants laticiferous cells may develop into very large systems which extend throughout the different shoot and root tissues. Rosowski (1968), who worked on some *Euphorbia* species, expressed the view that their entire laticifer system is derived from a few initials that are already present in the embryo. Mahlberg (1961) found in *Nerium* that the number of initials is constant and that they can all be distinguished in the embryo where they appear in the cotyledonary node from where they send branches into the cotyledon, the hypocotyl and the radicle. In *Cryptostegia grandiflora* it has been found that the early-formed laticiferous cells in the cortex branch radially in the position of the leaf gaps and penetrate into the pith. After a period of cambial activity these branches of the laticiferous cells become surrounded by the secondary phloem and xylem (Blaser 1945).

Rachmilevitz and Fahn (1982) reported that in *Ficus carica* L., laticifers undergo a sequence of ultrastructural changes during differentiation. They noticed that vacuolar size increased by an autophagy process, which divided the cytoplasm into separated masses and follows a development of numerous vesicular structures in the cytoplasm, which subsequently are released into the vacuolar space, and the cytoplasm will disintegrate shortly after this process.

A study by Roy and De (1992) on differentiation of non-articulated laticifers of *Calotropis gigantea* (Linn.) emphasizes the anatomy, distribution, structure and ultrastructural organization of the species using both light and electron microscopy. They reported that the enlargement and fusion of the small vacuoles accompanied by the degeneration of cell components occurs and is followed by a sequential lysis of cellular components with preservation of a thin

layer of peripheral cytoplasm and the formation of central vacuole. This formation of vesicles from peripheral cytoplasm and the release of electron dense osmiophilic globules into the vacuole of the cells of *Calotropis* is comparable to that observed in *Ficus carica* (Rachmilevitz & Fahn 1982), Pea cotyledon (Hinz, *et al.* 1999) and in tobacco (Miller, *et al.* 1999).

There are other possibilities for the growth of non-articulated laticifers at their tips resulting in an intrusive manner of growth. Wilson *et al.* (1976) believed that the intrusive growth and extensive elongation of non-articulated laticifers in milkweed, *Asclepias syriaca* L., suggest a requirement for a pectolytic enzyme system. The pectinase could serve to loosen or dissolve the middle lamellae between cells and thus allow penetration of the growing tip of the laticifer. Assuming that the enzyme is synthesized in the laticifer, although the protoplasmic site of synthesis is not yet known, they concluded that the laticifer secretes pectinase ahead of the growing cell tip thereby dissolving pectic substance of the middle lamella, thus facilitating penetrations of the laticifer among other cells during its growth throughout the plant. Furthermore, such an enzyme also could loosen cell wall material distal from the tip of the laticifer to allow cell elongation. These criteria of enzyme releasing mechanism (toward the tips of the laticifers instead of in the laticifers and dissolving cell walls at any points along the laticifers) has been taken as a significant grouping criteria between articulated laticifers and non-articulated laticifers.

**Table 1: A summary of the similarities and differences between articulated and non- articulated laticifers.**

	<b>Articulated Laticifers</b>	<b>Non-articulated Laticifers</b>
Initial development	Compound cells (involves parenchyma cells)	Simple single cell
Enzyme contribution into development	Cellulase within the laticifers. Assisted by plant hormone (auxin).	Pectinase in cell wall toward the tip of laticifers.
Laticifers distribution in plants	All part of the plants	All part of the plants
Mode of development	Cell wall degradation	Intrusive growth

### 1.3 LATEX

Bonner and Galston (1947) noted that rubber frequently, but not always, occurs in plants in the form of the minute particles suspended in the liquid and in certain cases as an emulsion, with the whole forming latex. The latex is in turn contained within more or less specialized laticiferous cells or vessels. It has a chemical composition that differs in the different species of plants. The matrix may be regarded as cell sap of the laticifer. Among the suspended material are

rubber particles ( $C_5H_8$ )<sub>n</sub>, waxes, resin, proteins, essential oils, mucilages (Fahn 1982), carbohydrates, organic acids, salts, alkaloids, sterols, fats and tannin. In certain *Euphorbia* species, variously shaped starch grains can be found (Mahlberg 1975). Resin and particularly rubber are characteristic components of the latex in many plants and commonly belong to the hydrocarbon family, which includes balsams, camphors and carotenoids (Bonner and Galston 1947).

In another form, latex can also be referred to as fluids, usually with a white milky (*Asclepias*, *Euphorbia*, *Ficus*, *Lactuca*) or clear (*Morus*, *Nerium oleander*) appearance or even translucent (Fahn 1982, Metcalfe & Chalk 1989). The milky appearance is due to the suspension of many small particles in a liquid dispersion medium (matrix) with a very different refractive index. In addition to that, the colour of the latex may differ between species. Metcalfe (1967) added, for example, that it might be yellow (*Cannabis*), orange (*Papaver*), red, or even greenish in different taxa. Some investigators reported the colour may vary in different parts of a single plant, or the colour may change after the latex has exuded from the plant as observed in *Hevea brasiliensis* (Gomez 1982).

In term of size, the latex particles, as in *Hevea brasiliensis*, vary from 0.01 $\mu$ m to more than 50 $\mu$ m and can differ in diameter in various species. In addition to that, latex particles may vary in shape from spherical to pear- or rod-shaped. In *Hevea*, the size and shape of the latex particles varies depend on the age or growth stages of the plant, where mature and older plants tend to have larger and spherical shapes compared with the young plants; where the size is smaller and spherical (Gomez 1982).

In *Hevea brasiliensis*, latex is exploited commercially by a systematic excision of the external tissues of the trunk, via bark. Even though other species of plants especially in the Euphorbiaceae family, produce latex as well, they have not

being exploited commercially because of the latex production is not as great as in *Hevea brasiliensis*.

Because latex is a term that covers dozens of types of secretions, it is not surprising that their ultrastructure is extremely diverse. It is at the level of electron microscopy that structural discrepancies can be determined.

#### 1.4 LATICIFERS DISTRIBUTION IN PLANTS TISSUES

Even though cytological and differentiation studies still remain fragmentary for the vast majority of known laticiferous plants, some earlier work has shed some lights into the location and distribution of laticifers in the plant kingdom. Work done by Esau (1965), for instances, demonstrated that laticifers might occur in any plant organ and are not restricted to certain part of the plants only. This observation is further supported by other workers such as of Gomez (1982) in *Hevea*, Roy and De (1992) in *Calotropis gigantea*, Fineran (1982) in *Euphorbia pulcherrima* and Condon & Fineran (1989) in *Calystegia*.

Having said that, however, this doesn't mean that all plants in this vast plant kingdom have laticifers in their tissues or some of their tissues. In some plants the laticifers are barely present or there are none at all. In the case of known latex bearing plants the presence of laticiferous cells can sometimes be restricted or exclusively found in certain or specialized areas of plants only. Wilder and Harris (1982) have shown that in *Cyclanthus bipartitus* Poit. (Cyclanthaceae), the laticifers are restricted to certain tissues of the plants. They noticed that the laticifers were absent from roots and internal portions of rhizomes and from regions of leaf primordium. Their observation on the

occurrence of the laticifers in particular tissues of *Cyclanthus*, despite the earlier reports, suggest the possibility that this could be a point to separate the cyclanthaceous subfamilies Cyclanthoideae and Carludovicoideae merely at the level of subfamily, in their taxonomy nomenclature.

According to Metcalfe and Chalk (1989), the distribution of laticifers within the plant body varies from one species to another. As stated earlier, very often they accompany the vascular tissue, and they occur particularly in the phloem, where it is not always easy to distinguish them from sieve tubes. Sometimes they are more widely distributed in the parenchymatous tissue, e.g. in *Nerium oleander* L. In the xylem itself they are generally restricted to the rays. In many plants they pass out into the leaves and may have branches extending into the mesophyll where they sometimes reach the hypodermis (if one is present) or the epidermis itself.

In addition, Metcalfe (1967) emphasized that laticifers are not confined to plants with any one particular type of habit nor to plants from any particular type of habitat. They can also be found in herbs, including both xerophytic succulents and water plants, as well as in trees, shrubs, and lianas. In some species, as mentioned earlier, the latex is usually restricted to certain tissues, as found in the interesting *Decaisnea insignis* Hook. f. and Thomo., a shrub belonging to the family Lardizabalaceae which is related to the more familiar *Berberis* family, the Berberidaceae. Here the latex is restricted to the fruits where it is to be found in a system of canals.

## 1.5 THE DIVERSITY AND GEOGRAPHIC DISTRIBUTION OF LATEX BEARING PLANTS

Rubber bearing plants have intrigued and fascinated botanists and keen plant microscopists for their diversified and complex relationships in the plant kingdom. Work carried out by Bonner and Galston as early as 1947, mentioned that rubber formation is a property scattered through numerous families of the plant kingdom in no discernibly regular fashion. The Moraceae, Euphorbiaceae, Apocynaceae, Asclepiadaceae and Compositae are particularly well represented. All genera within any one family are not ordinarily rubber forming, and the species of one genus may differ greatly in rubber forming capacity, as for example in *Ficus* and *Euphorbia*.

A survey by Esau (1965) showed that latex occurs in 12500 species belonging to 900 genera. However, there are certain rules regarding distribution of rubber in the plants. Rubber for instance is a property confined to the dicotyledons branch of the angiosperm and other that this group (monocotyledons) only producing latex. Of late, records show that latex-bearing plants belong to rather more than 22 families, mostly of Dicotyledons; however a few monocotyledonous families are also included, together with one genus of Pteridophyte, *Regnellidium*, of the Marsileaceae (Metcalf and Chalk 1989, Ingroville 1992). The plants containing latex vary and range from such small herbaceous annuals as the spurges (*Euphorbia*) to large trees like the rubber-yielding *Hevea*.

The geographic distribution of latex bearing plants, as complex as the structures itself, is varied and fascinating. They can be found in all parts of the world, but arborescent types are most common in the tropical floras (Esau, 1965). Within a single genus, as *Euphorbia*, the tropical representatives may include numerous

species which form and accumulate significant amounts of rubber, whereas the representative of the same genus in the temperate zone may form, in general, little or no rubber (Bonner & Galston 1947).

## **1.6 THE IMPORTANCE OF THE RUBBER INDUSTRY TO MALAYSIA: A BRIEF HISTORY**

Rubber is a major commodity to Malaysia. Undoubtedly, it played a major part in contributing to the growth of the country in the past and will do so in the near future. The rubber industry helped to transform Malaysia from a once-poor and undeveloped country to one of the faster growing developing countries.

Sir Henry Wickham first introduced rubber trees into Malaya in 1877. The rubber tree quickly flourished in Malaysia; large areas of jungle were cut down and planted with rubber trees. By the end of the nineteenth century there were 2500 hectares of rubber in Asia and it reached a half million hectares by 1910 and countries of Asia became the main suppliers of rubber. During the rubber boom years in Malaysia, land used for rubber plantation grew rapidly. It reached a peak of over 2 million hectares after which the area declined to the current figure of over 1.8 million hectares. Malaysia itself is amongst the three biggest world suppliers of natural rubber, alongside Thailand and Indonesia (Ching 1977, Schultes 1978).

Realizing the importance of rubber industry to the Malayan (it was known as Malaysia after the independence in 1957) economy, the government (British

government at that time), set up and established a research station in Kuala Lumpur in 1925, striving to address the problems and matters relating to rubber. The institute was known as The Rubber Research Institute of Malaysia (RRIM) and now after 74 years of its establishment it has contributed tremendously the growth of the rubber industry in Malaysia. In 1998, RRIM, Malaysia Rubber Producer Association (a research branch in United Kingdom) and The Malaysia Rubber Research and Development Board (MRRDB) were merged together and become one corporate company known as Malaysian Rubber Board (MRB).

During the period of 1993-1997, the rubber industry's contribution to the country's Gross Domestic Product increased significantly, which accounted for 20% for world production and 13% of the world export the same years. This is also due to the rapid expansion of the downstream rubber products and furniture manufacturing industries. A good deal of Malaysia's rubber (over half) comes from thousands of privately-owned plots of land called smallholdings, which are usually about 2 hectares, The rest is grown on big estates owned by various companies; each can cover over a thousand hectares (Sooi & Sekhar 1978). These increases in productivity are mainly the outcome of innovations emanating from RRIM, complemented by contributions from the private sector, and extension and development agencies. However, the industry experienced low total production in 1993 owing mainly to reduced tapping because of low prices.

The structural changes underlying the rubber industry were manifested in the shift in importance from the upstream to the downstream sector activities. The increasing raw rubber supply needs of the rubber processing industry could not be met following the declining trend in Malaysia's natural rubber (NR) output. This has resulted in the progressive increase in rubber imports, which not only met the needs of the products industry but also bolstered Malaysia's exports. Of significance was the large volume of latex imports that were needed to

supplement the latex requirement for the latex-based glove and rubber products manufacturing sector (Allen *et al.* 1976).

Similarly, the fast expanding rubberwood furniture industry's material supply needs would depend on the long-term supply of rubberwood, which in turn would be dependent on the rate of rubber replanting programme. About 80% of the export value of wooden furniture consists of rubberwood furniture.

Research and Development (R&D) activities in the upstream sector continued to be aimed at addressing issues faced by the producers such as the needed to increase latex, land and labour productivity, improved planting materials as well as to accelerate the adoption of labor-saving technologies to overcome the labor shortage, which is the main and current problem in the industry today (Sekhar & Pee 1985).

## 1.7 OBJECTIVES

Several objectives were addressed in this project. They are listed below: -

1. To investigate several tissue preparation techniques for studies of the structure and differentiation of laticifers, and to allow application of molecular probes to advance further studies of differentiation.
2. To investigate structure changes of laticifers in different stages of development of *Meconopsis cambrica*.
3. To investigate the structure and differentiation of laticifers in different tissues of *Hevea brasiliensis*.
4. To investigate and compare laticifer development in several taxa.

**Table 2: Summary of research carried out on latex bearing plants.**

SPECIES	TECHNIQUES	AUTHORS	PAPERS
<i>Allamanda violaceae</i>	TEM	Inamdar, J.A. <i>et al.</i>	Ann. Bot. 62: 583-588, 1988
<i>Allium sp.</i>	Light & TEM	Huang, S.M. & Sterling, C.	Am. J. Bot. 57:1000-1003, 1970
75 genera of Araceae	Light mic.	French, J.C.	Bot. Gaz. 149(1): 71-81. 1988
I. <i>Asclepias syriaca</i> II. <i>Asclepias tuberosa</i>	fluorescent immunocytochemistry.	Dunbar, K.B. <i>et al.</i>	Amer. J. Bot. 73.(6): 847-851. 1986
<i>Calotropis giganteae</i>	Light mic. & TEM	Roy, A.T. & De, D.N	Ann. Bot. 70: 443-449, 1992
<i>Calystegia soldanella</i>	Fluorescence mic.	Bruni, A. <i>et al.</i>	Experientia 30, 1390-1391, 1974
<i>Calystegia silvatica</i>	Light mic., TEM & SEM (freeze-drying)	Condon, J.M. & Fineran, B.A.	Bot. Gaz. 150(3): 289-302. 1989
<i>Cannabis sativa</i>	TEM	Mesquita, J.F. & Santos Dias, J.D.	Boletim Da Sociedade Broteriana 57, 337-56, 1986
6 genera of Convolvulaceae	Freezing for SEM	Fineran, B.A. & Condon, J.M.	Can. J. Bot. 66. 1217-1226. 1988
<i>Cryptostegia grandiflora</i>	Light mic.	Blaser, H. W.	Am. J. Bot. 32, 135-141, 1945
<i>Cyclanthus bipartitus</i>	Light & stereo mic.	Wilder, G.J. & Harris, D. H.	Bot. Gaz. 143(1): 84-93. 1982.
<i>Euphorbia supina</i>	Light mic.	Rosowski, J.R.	Bot. Gaz. 129(2): 113-120. 1968
<i>Euphorbia lathyris</i>	Light mic. & TEM	Groeneveld, H.W. & Roelvink, P.W.  Groeneveld, H.W. <i>et al.</i>	Planta 154:347-351, 1982  Ann. Bot. 51, 307-315, 1983
<i>Euphorbia pulcherrima</i>	Light mic. (bright fields, phase, interference, fluorescence, Nomarsky & polarizing), TEM & SEM (freeze-fracturing)	Fineran, B.A.	Ann. Bot. 50: 207-220, 1982  Ann. Bot. 52: 279-293, 1983

I. <i>Euphorbia abyssinica</i> II. <i>Euphorbia heterophylla</i> III. <i>Euphorbia inconstantia</i> IV. <i>Euphorbia lactea</i> V. <i>Euphorbia mauritanica</i> VI. <i>Euphorbia pseudocactus</i> VII. <i>Euphorbia milii</i> VIII. <i>Euphorbia terracina</i> IX. <i>Euphorbia tirucalli</i> X. <i>Euphorbia viguieri</i>	Light mic. & SEM	Mahlberg, P. G.	Amer. J. Bot. 62(6): 577-583. 1975
<i>Euphorbia marginata</i>	Fluorescence mic.	Bruni, A. <i>et al.</i>  Bruni, A. & Tosi, B.  Bruni, A. <i>et al.</i>	Ann. Bot. 42, 1099-1108, 1978  Protoplasma 102, 343-347, 1980  Bot. Helvetica 92: 1-11, 1982
<i>Euphorbia esula</i>	Light mic.	Mahlberg, P.G. <i>et al.</i>	Bot. J. Linn. Soc. 94, 165-180, 1987
<i>Euphorbia myrsinites</i>	TEM	Biesboer, D. & Mahlberg, P.G.	Planta 143, 5-10, 1978
<i>Ficus carica</i>	Light mic. & TEM	Rachmilevitz, T. & Fahn, A.	Ann. Bot. 49: 13-22, 1982
<i>Glaucium flavum</i>	TEM	Nessler, C. L.	Can. J. Bot. 60: 561-567, 1982
<i>Hevea brasiliensis</i>	TEM  Light mic.  TEM  Light mic.	Dickenson, P.B.  Wimalaratna, S. D.  Gomez, J.B.  Hao, B.Z. & Wu, J.L.	J. Rubb. Res. Inst. Malaya 21, 543-559, 1969  Stain Technol. 48. 219-21. 1973  Proc. Int. Rubber Conf. 1-22. 1975  Ann. of Bot. 85: 37-43, 2000

<i>Hordeum sativum</i>	TEM	Buvat, R. & Robert, G.	Amer. J. Bot. 66:1219-1237. 1979
<i>Jatropha podagrica</i>	Light mic.	Cass, D. D	Phytomorphology 35, 133-40, 1985
<i>Jatropha species (37)</i>	Light mic.	Dehgan, B. & Craig, M.E.	Amer. J. Bot. 65(3): 34-352. 1978
I. <i>Mammillaria heyderi</i>	TEM	Wittler, G. H. & Mauseth, J. D.	Amer. J. Bot. 71(1):100-110. 1984
II. <i>Mammillaria guerreronis</i>	Light mic. & TEM		Amer. J. Bot. 71(8): 1128-1138. 1984
<i>Nelumbo nucifera</i>	Light mic. & TEM	Esau, K. & Kosakai, H.	Ann. Bot. 39, 713-719, 1975
<i>Nerium oleander</i>	Light mic.	Mahlberg, P. G.	Amer. J. Bot. 48: 90-99, 1961  Botanical Gazette 124, 224-31. 1963
I. <i>Pleiogynium solandri</i> II. <i>Spondias dulcis</i> III. <i>Mangifera indica</i> IV. <i>Schinus terebinthifolius</i>	Light mic.	Venning, F.D.	Amer. J. Bot. 35: 637-644. 1948
<i>Papaver bracteatum</i>	TEM	Nessler, C. L. & Mahlberg, P. G.	Amer. J. Bot. 65(9): 978-983, 1978
<i>Papaver somniferum</i>	TEM  TEM	Thureson-Klein, A.  Nessler, C.L. & Mahlberg, P. G.	Ann. Bot. 34:751-759. 1970  Bot. Gaz. 138(4):402-408. 1977
<i>Plumeria alba</i>	Light mic.	Murugan, V. & Inamdar, J. A.	Proceedings of Indian Academy of Sciences 97, 25-31, 1987

<i>Vallis solanaceae</i>	Light mic. & TEM	Murugan, V. & Inamdar, J. A.	Phytomorphology 37, 204-214, 1987
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## **2. MATERIALS AND METHODS**

## **2.1 BUFFER**

Phosphate buffer solution (PBS) pH 7.0 was used in all procedures unless otherwise stated.

## **2.2 PLANT MATERIAL**

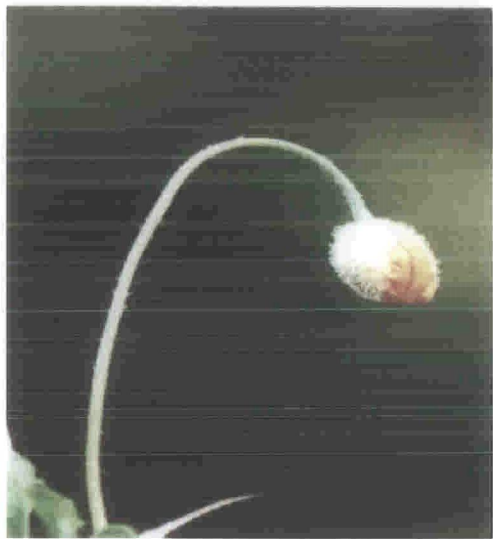
### **2.2.1 *Meconopsis cambrica* (Family = Papaveraceae)**

Initially the plant specimens were sampled from six growth stages from the early fruit capsule stage to flower senescence stage, with the interval of 38 hours between developmental stages. Later in this experiment only stage 1 and 6 were used (Plate 1). Stage 1 is the very young fruit capsule, at flower anthesis, and stage 6 is when only the fruit itself left after the petals of the flower have fallen (senescent). Prof. Nick Harris supplied the plant. The flower stems were cut with the razor blade and immediately transferred into the fixatives in a small bottle. The samples were then further cut into small blocks (app. 10mm<sup>2</sup>) in the fixative. All sampling was carried out in the morning.

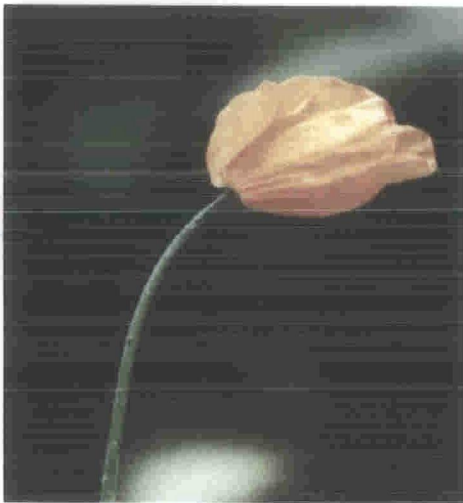
Plate 1: Six developmental stages of *Meconopsis cambrica*



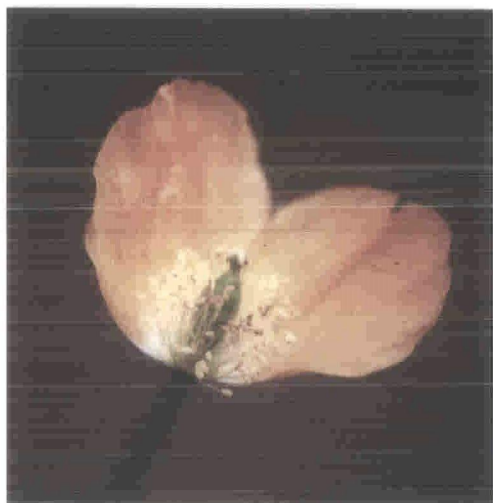
1st. Stage of development



2nd. Stage of development



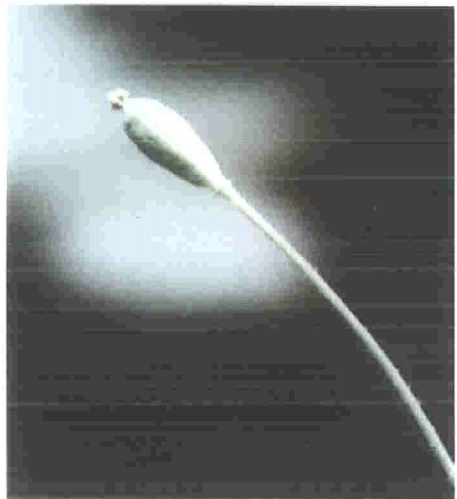
3rd. Stage of development



4th. Stage of development



5th. Stage of development



6th. Stage of development

### **2.2.2 *Papaver rhoeas*. (Family = Papaveraceae)**

The choice of developmental stages was as in *Meconopsis*. Later in this experiment only stage 1 and 6 were used (Plate 2). Stage 1 (young sample) is the very young fruit capsule, at flower anthesis, and stage 6 (old sample) is when fruit itself is left after the petals of the flower have fallen (senescent). The protocol was very similar to that used for the *Meconopsis*. Plants were collected from the waste ground next to the Department of Biological Sciences at the University of Durham.

### **2.2.3 *Musa acuminata*. (Family = Musaceae)**

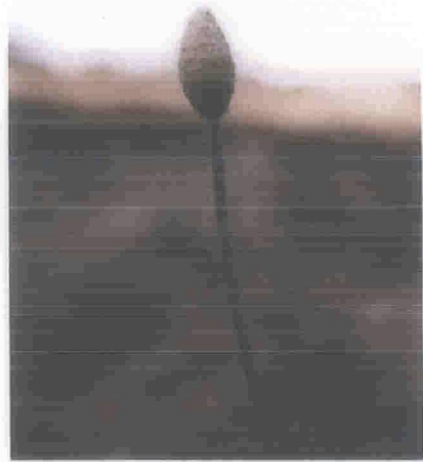
*Musa acuminata* is a very common banana species that is very popular as an ornamental greenhouse plant. There are several synonyms for this species. They are *Musa cavendishii*; *Musa chinensis*; *Musa nana*; *Musa sinensis*; *Musa zebrine*. However this banana tree is commonly known as Dwarf banana tree.

Leaf material was sampled from two plants; one from The University of Durham Botanical Garden (Plat 3) and from a pot plant supplied by Professor N. Harris. Only two stages of development were monitored, which are stage 1 and 2. Stage 1 was from a light green young leaf that had just emerged from the shoot and stage 2 was a dark green leaf which was fully expanded. The leaves were cut into approximately 1.5 cm<sup>2</sup> and immediately immersed in the fixatives. The preparation for embedding was very similar to that for other samples, except that for the electron microscopical work the samples were embedded in a flat embedding mould.

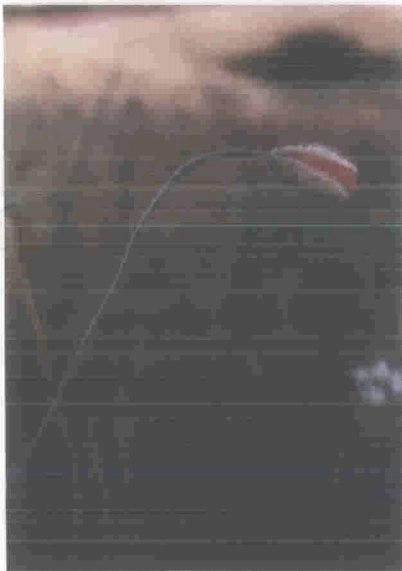
Plate 2: Six developmental stages of *Papaver rhoeas*



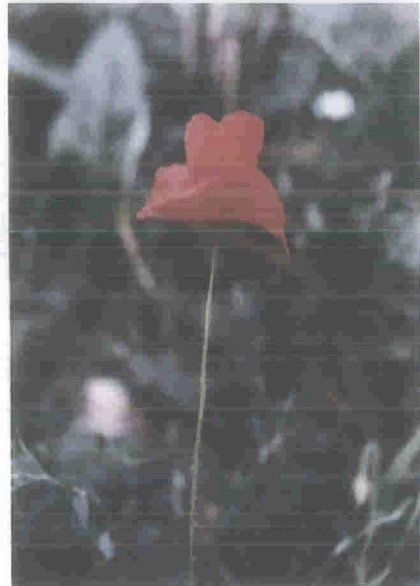
1st. Stage of development



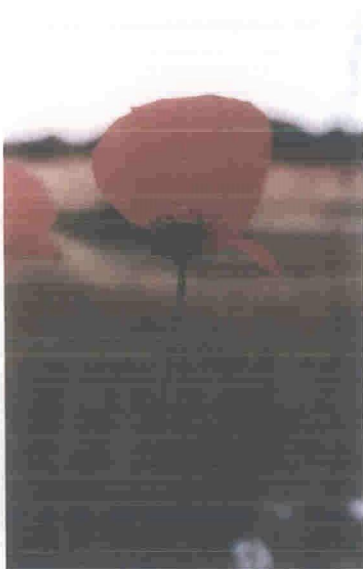
2nd. Stage of development



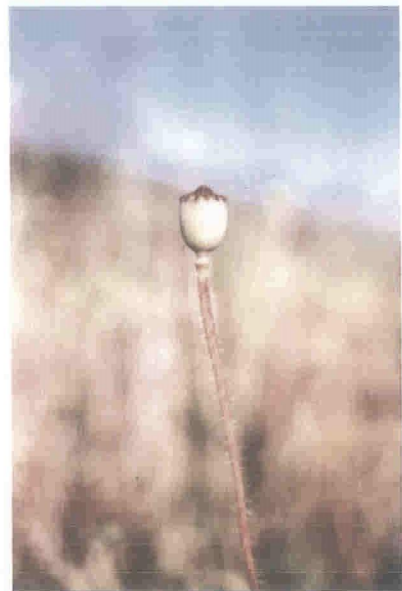
3rd. Stage of development



4th. Stage of development



5th. Stage of Development

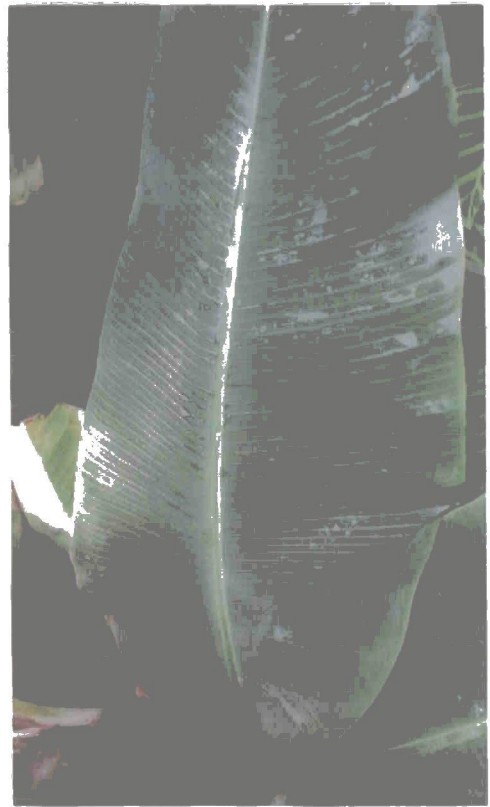


6th. Stage of development

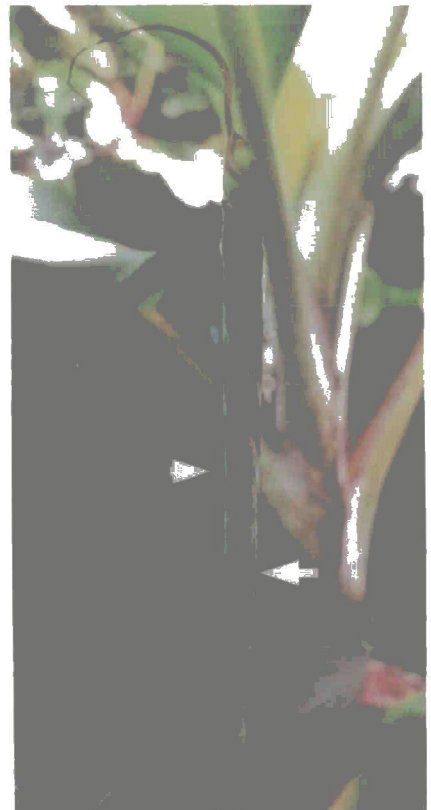
Plate 3: *Musa acuminata*



*Musa acuminata* tree from University  
of Durham Botanical Garden



Mature leaf



Young leaf

#### **2.2.4 *Hevea brasiliensis* (Family = Euphorbiaceae)**

The seeds were received from Malaysian Rubber Board (MRB). They were from the gene banks and were of different clones (mixed clones) of RRIM600, RRIM 628 and GT1.

The seeds were first washed under tap water to remove the dirt and sulphur residue (antifungus) and soaked in water overnight to soften the testa. Approximately 100 seeds were grown in the dark room for one month in Styrofoam and kept moist with a constant sprinkler.

The first samples were taken from the seedlings in the dark room. The young white shoot and the cotyledon were cut and trimmed into small cubes for further fixation treatment.

The plants were then transferred into Levingtons F2 Standard pH Compost and moved from the dark room to the growth room with a controlled temperature of 32°C. The compost was kept moist by watering for every 4-5 days. Plants were grown in 16-hour daylight (using fluorescent tube lighting). The second batches of samples were taken from the tissue of green plants (>3 months) (plate 4). The shoot apex, leaf, stem and the root were cut and immediately transferred into the fixative before being trimmed into 1 mm<sup>2</sup> (for electron microscopical work) and 1-2 cm<sup>2</sup> (histological work) blocks for further treatment. All treatments were carried out in the fume cupboard.



Plate 4: *Hevea brasiliensis*, pot-grown in the growth room. This is a one and a half year old tree.

### **2.2.5 *Mandevilla splendens* (Family = Apocyanaceae)**

Pot plant was supplied by Dr. Phil Gates; from a plant purchased at Strike's Garden Centre, Darlington, Co. Durham, United Kingdom. Matured green shoots were used (plate 5). At the time of sampling, the plant had not flowered. Sampling was performed in the morning. Cutting of approximately 2-3cm<sup>2</sup> blocks were made from the green leaves and stem. The samples were immediately transferred into the fixatives for further treatment.

### **2.2.6 *Taraxacum officinale* (Family = Compositae/Asteraceae)**

*Taraxacum officinale* (plate 6) plants were sampled from the field near the Department of Biological Sciences at University of Durham. The sampling was normally done in the morning. The leaves and the stem were cut into 3-4 cm<sup>2</sup> sections and immediately immersed in the fixative. The samples were then taken to the fume cupboard for further trimming approximately into 1-2 cm<sup>2</sup>, and other procedures.

### **2.2.7 *Euphorbia wulfenii* (Family = Euphorbiaceae)**

*Euphorbia* samples were taken from plants growing in the ornamental flowerbeds around the Department of Biological Sciences at University of Durham (plate 7). The leaves were cut whole from the stem and immediately transferred into a flask containing immunofix. The samples were taken to the lab



Plate 5: *Mandevilla splendens* tree growing in the pot.



Plate 6: *Taraxacum officinale* : Commonly known as dandelion. This plant is a very familiar sight blooming beautifully in green fields during summer. This particular one was sampled from the field near the Department of Biological Sciences at University of Durham.



Plate 7: *Euphorbia wulfenii*. This shrub was growing in the ornamental flower beds around the Department of Biological Sciences, University of Durham.

and were trimmed in the fume chamber into 2-3 cm<sup>2</sup> for further treatments. The trimmings were done while the samples were immersed in the fixative.

## **2.3 TISSUE PREPARATION FOR MICROSCOPY**

### **2.3.1 Fixation**

### **2.3.2 Immunofixative**

Excised tissues were immediately placed in a solution containing freshly prepared 3% (w/v) paraformaldehyde, 1.25% (w/v) glutaraldehyde, and 50mM phosphate buffer pH 7. Tissues were fixed for 12 hours at R.T. The tissues were agitated using a 45° rotating platform at 2 r.p.m in the fume cupboard.

### **2.3.3 Double fixation: with aldehyde/osmium tetroxide**

After the primary fixation with the immunofixative, the samples were washed in distilled water for 12 hours. The distilled water was then replaced with 2% aqueous osmium tetroxide for 8 hours on the rotator in the fume cupboard.

### **2.3.4 Triple fixation: with aldehyde/osmium tetroxide/zinc iodide (ZIO)**

According to Hawes (1994), the zinc iodide/osmium tetroxide (ZIO) technique is suitable for most plant, algal and fungal tissues. The fixative will impregnate the nuclear envelope, endoplasmic reticulum, Golgi apparatus, the tonoplast, plastid thylakoids and occasionally the stroma of mitochondria. In this experiment this procedure were applied to *Hevea* samples for ultrastructural studies.

To prepare the ZIO, 3 g of zinc powder and 1 g of resublimed iodine were added to 20 ml distilled water in a small vial. The vials were shaken vigorously by hand for 10-15 min to make sure the mixtures were mixed and reacted properly. The solution was than filtered with filter paper into another clean vial. The solution should be clear or very pale yellow: If it is too yellow this means that free iodine is still available and not usable. The filtered solution was mixed with an equal volume of 2% w/v OsO<sub>4</sub>. The solution must be used immediately for fixation. The fixing procedure is similar to that used for osmium tetroxide (2.3.3).

## **2.4 Dehydration**

After the samples had been washed in water, they were dehydrated in a graded series of alcohols, 25%, 50%, 75%, 95% (v/v) and twice in 100%, allowing 30 minutes (twice) for each step. Dehydration was carried out at room temperature on a rotator.

## **2.5 TISSUE EMBEDDING**

### **2.5.1 Embedding in LR White Acrylic Resin**

LR White medium grade resin was used. The resin was stored at 4°C and allowed to equilibrate at R.T. before opening to prevent the absorption of atmospheric water. All manipulations involving the resin were carried out in a fume hood and protective clothing was used.

An equal volume of resin (medium grade) was added to the dehydrated samples with 100% ethanol and the solutions were thoroughly mixed. Tissues were infiltrated for 12 hours at R.T., and then the solution was replaced with 100% resin for 4 hours. The resin was replaced twice daily for two days. Finally the tissues were embedded in polypropylene capsules and the resin polymerised for 12 hours at 60°C.

### **2.5.2 Embedding in Araldite resin**

The sample was infiltrated with propylene oxide:araldite in the ratio of 2:1, 1:1, 1:2 for 1 hour for each step. The specimens were then infiltrated with 100% resin and another fresh batch of pure resin for another 1 hour. The samples were orientated and embedded in a flat embedding mould for leaf, and BEEM capsules for other tissues, and left to polymerise in a 60°C oven for 48 hours.

### **2.5.3 Embedding in Spurr resin**

As Spurr resin is known to be very toxic, all procedures in handling and polymerising the resin were carried out with great care. Throughout the whole protocol, double gloves were used at all times and all manipulation was done in a fume cupboard.

The specimens were infiltrated with pure ethanol:spurr with the ratio of 2:1, 1:1, 1:2 for 1 hour for each step. The solution was replaced with pure resin for 1 hour and a batch of fresh resin for another 1 hour. Finally the samples were orientated and embedded in flat embedding mould, or BEEM capsule, and left to polymerised in 60°C oven for 48 hours.

### **2.5.4 Embedding in Paraplas wax**

An equal volume of HistoClear was added to the dehydrated samples and the solutions thoroughly mixed. Tissues were infiltrated for 2 hours then the solution was replaced with 100% HistoClear. Tissues were infiltrated in several changes of HistoClear for at least 15 hours then an equal volume of molten wax was added. Tissues were infiltrated for 12 hours at 60°C then the solution was replaced with 100% wax. Tissues were infiltrated in several changes of wax for 36 hours. Tissues were embedded in fresh wax using a metal mould. The warm moulds were submerged in a beaker of cold tap water for hardening or just left on the table at room temperature (when they took a longer time to harden).

### 2.5.6 Low temperature methods: Freeze-substitution

Two approaches were used in this method. Firstly the conventional methods were used, which utilized the cryochamber from the microtome as the freezing chamber to freeze the samples. Secondly, an instrument especially designed for the purpose was used. In the conventional method, 2% Osmium tetroxide was dissolved in cold acetone. The stem of the plants was rapidly frozen by quickly dipping into liquid nitrogen. The frozen samples were then transferred into the vial containing osmium tetroxide and kept in the freezer at  $-80^{\circ}\text{C}$  for two weeks to allow slow substitution of the medium into the tissue. The samples were transferred into the cryochamber and then were slowly brought up to room temperature over the period of 12-48 hours by adjusting the temperature of the cryochamber. The samples were then washed with acetone three times and then stained with 0.5% uranyl acetate in acetone for 2-12 hours at  $4^{\circ}\text{C}$ . The samples were again washed with acetone and followed the normal dehydration procedures [as in 2.4] except that acetone was used instead of ethanol. LR White resin was used to embed the samples.

In the second method, samples from the leaves were cut approximately  $1\text{mm}^2$  in size and immediately immersed in cryofix fixative. The samples were then stuck onto a slamming stud and cryofixed by slamming against a cold copper plate using a Leica MM90 system. The frozen samples were then quickly transferred to a Leica Automatic Freeze substitution chamber for the freeze substitution process. Tissues were first freeze-substituted in acetone at  $-50^{\circ}\text{C}$  for 3 days. The acetone was then replaced by pre-chilled ethanol at  $-50^{\circ}\text{C}$  for 24 h in three changes. The tissues were then infiltrated in increasing concentration of Lowicryl HM20 as follows: 1 hrs of 25% resin: ethanol  $-50^{\circ}\text{C}$ , 1 hrs of 50% resin: ethanol  $-50^{\circ}\text{C}$ , 1 h 75% resin: ethanol  $-50^{\circ}\text{C}$  and finally 100% pure resin for 24 h at  $-50^{\circ}\text{C}$ , three changes. Next stage was the polymerisation of the resin under the

UV light. First the resin was polymerised for 2 days at  $-50^{\circ}\text{C}$ , then another 2 days at  $-20^{\circ}$  and finally 2 days at room temperature. The blocks were ready for sectioning with normal ultramicrotome.

### **2.5.7 Microwave oven technique**

Fresh samples (stem) were cut and immediately immersed in a beaker of tap water. The beaker and the plant were then put in the microwave oven (Sanyo 650W) and then were heated for 1-5 minutes. The results were assessed by looking at the degree of latex coagulated inside the laticifers. Longer heating will make the latex burst out from the laticifers or the tissue may be damaged.

### **2.6 Sectioning resin embedded materials**

Embedded materials were sectioned using glass knives on a Sorvall MT2-B or Reichert Ultracut-S ultra microtome. The sections were floated onto a reservoir of water, which was created on the glass knives using insulating tape sealed with dental wax.

Semi-thin  $1\mu\text{m}$  sections, for light microscopy, were removed from the reservoir with a round-tip glass pipette and placed on a drop of water on TESPA coated slides. Sections were dried down on a hotplate for a few minutes until the sections had adhered to the slides.

Ultra thin 150nm sections were collected onto grids from the reservoir. Sections for morphological studies were collected onto 200 mesh copper grids.

## **2.7 Sectioning wax embedded materials**

Wax blocks were trimmed and attached on a wooden cube as a holder. The blocks were then cut at 8-10  $\mu\text{m}$  on a Leitz 1512 rotary microtome. The sections were floated onto a drop of distilled water on a preheated albumin-smear microscope slide and allowed to dry overnight on a 40°C hotplate. Sections were dewaxed by immersing the slides for 15 minutes in HistoClear, followed by 3 minutes in 80%, 50%, 30% HistoClear in ethanol, then in a decreasing order of 100% ethanol for 10 minutes and 3 minutes each in 70%, 50% and finally 30% ethanol before the staining procedure.

## **2.8 Formvar coating grids**

A thin film of 0.3% (w/v) Formvar in chloroform was floated onto distilled water and the grids carefully placed on the formvar film. The film and grids were picked up using a strip of parafilm and allowed to air dry at R.T. in a dust free environment.

## **2.9 TESPA (aminopropyltriethoxysilane) coating of microscope slides**

Microscope slides were washed in detergent then rinsed thoroughly in distilled water. The slides were immersed in a 2% solution of TESPA in acetone for 10 seconds, then rinsed in two changes of acetone and finally in distilled water. The slides were air dried at R.T. in a dust free environment.

## **2.10 SCANNING ELECTRON MICROSCOPY (SEM)**

Specimens were dehydrated as described in section 2.4 in a graded series of acetone instead of alcohol. Specimens were then critical point dried in liquid carbon monoxide in an E 3100 Jumbo Critical Point Drier, then mounted on stubs and dissected using a stainless steel blade. Specimens were gold coated and then viewed on a Hitachi JEOL JSM 848SEM at 25 kV. Images were taken both with film and stored in Iomega™ zip disks.

## **2.11 TRANSMISSION ELECTRON MICROSCOPY (TEM)**

Sections were collected on 200 mesh copper grids and were dried on a piece of filter paper. The grids were then stained on a droplet of uranyl acetate for 10 minutes and washed by dipping in fresh distilled water. The grids were immediately transferred to a droplet of lead citrate for another 10 minutes and washed in fresh distilled water. The sections were observed with a Philip EM 300 transmission electron microscope, at 100 kV. Micrographs were taken on plate film and then proceed for development and printing.

### **2.11.1 Micrographs enhancement**

Micrographs were scanned with an Agfa flatbed scanner and enhanced with Adobe™ Photoshop 5.0 software.

## **2.12 HISTOCHEMISTRY**

### **2.12.1 Calcofluor**

Fresh plant samples were cut with a razor blade and instantly put into droplets of 0.01% (w/v) aqueous solution of calcofluor. Sections were left stained for 5 minutes. Sections were rinsed in distilled water and viewed with epi-fluorescent illumination using a Nikon Diaphot equipped with an epifluorescence attachment and a Nikon ultra-violet filter block ( $\lambda = 405\text{nm}$ ).

### **2.12.2 ANS (8-anilino-1-naphthalene sulphonic acid)**

Fresh samples were cut with a razor blade and instantly put into droplets of 0.1% (w/v) ANS on a slide. The samples were left to be stained for app. 2-3 minutes and viewed with epi-fluorescent illumination using an ultra-violet filter block and with blue excitation as described by Gates & Oparka (1982).

### **2.12.3 Acridine orange**

Fresh plant samples were cut with a razor blade and instantly put into droplets of 0.01% (w/v) aqueous acridine orange on a slide. Sections were rinsed in distilled water and mounted with a cover slip. For sectioned samples, the samples were mounted in Citifluor. Samples were viewed with epi-fluorescent illumination using a Nikon blue filter block ( $\lambda = 495\text{nm}$ ) (Sanderson 1994).

#### **2.12.4 Toluidine blue**

Sections were stained for 1-3 minutes in a solution of freshly filtered 0.01% (w/v) Toluidine blue in 1% boric acid. Sections were rinsed in distilled water, air dried and mounted in DPX (Conn 1969)

#### **2.12.5 Oil Red O and Dansyl Chloride**

The samples (leaf) were cut into approximately 10 mm<sup>2</sup> before being fixed in FAA (Formalin:Acetic Acid:Alcohol) fixatives for at least 24 hours. The samples were then cleared in 1:1 hydrogen peroxide (30%): Glacial acetic acid in a test tube which was boiled on a hot plate in the fume hood for 2-3 hours. Then the samples were washed in 70% ethanol for 10 minutes followed by three change of deionised water. The samples were stained with Toluidine blue O for 2-3 minutes before the upper and the lower epidermis layers were peeled off. Then they were stained with Oil Red O for 15 minutes followed by Dansyl Chloride for 10 minutes. The samples were then washed in 1% sodium bicarbonate pH 8 and washed with deionised water before mounting onto a slide with 60% glycerine or citifluor. The samples were viewed under the fluorescent microscope with the green filter.

### **2.12.6 Safranin O and Astra blue**

This stain was used for wax sections only. The sections were stained with 30% (w/v) Safranin in ethanol for 3 minutes before rinsing in distilled water. The sections were then transferred into Astra blue (5mg/ml in 20mg/ml tartaric acid) for 10 seconds. Then the sections were rinsed with distilled water followed by 5 seconds in 30% ethanol. The sections were left on a 45°C hotplate for at least 1 hour before being transferred into 100% histoclear. The sections were finally mounted with DPX or histomount under the cover slip and were again left on the hotplate overnight (Conn 1969)

### **2.12.7. Control treatments.**

#### **2.12.7.1 Autofluorescent and staining control**

Unstained control samples were observed under the microscope to take account of autofluorescence staining artefacts in the cells.

## **2.13 IMMUNOCYTOCHEMISTRY**

### **2.13.1 Immunocytochemistry for light microscopy**

Sections were first rinsed in TBS (100mM Tris, 150mM NaCl) pH 8, and then incubated in a blocking solution containing 1% pre-immune serum, 0.01% Tween

20 in TBS for 30 minutes at R.T. Excess blocking solution was removed from the slides and then the sections were incubated in primary antibody diluted with blocking solution. Optimised incubations for different antisera ranged from 4-24 hours at 4-20°C. Sections were then washed in several changes of TBS for 15 minutes, then incubated with conjugated secondary antibody (diluted as manufacturer [Sigma-Aldrich Company] instruction).

### **2.13.2 Silver Detection**

Sections were incubated in 5nm gold-labelled secondary antibody (diluted as manufactures instructions) for 2 hours at R.T. in total darkness. Sections were sequentially rinsed in several changes of TBS, distilled water, Milli-Q (heavy metal-free) water. Sections were then silver enhanced for 1-3 minutes in darkness using Amersham IntenSEM following manufacturers instructions (the reaction was monitored for the best result). The sections were rinsed in Milli-Q water, air dried and mounted in DPX. Sections were viewed under epi-fluorescence illumination using a Nikon IGGS filter block.

### **2.14 Nomarski Imaging OR Differential Interference Contrast (DIC)**

Nomarski imaging or differential interference contrast (DIC) basically involved manipulation of two special optical components in the microscope called Wollaston prisms (Shaw & Rawlins 1994).

To adjust the microscope for DIC (Nikon Optiphot), bright field imaging was obtained with the condenser set to DIC position with the image-splitting prism out of the optical path. Next the condenser aperture was open and the polarizing filters were inserted. One was not added until the field was maximally dark and then the image-splitting prism was inserted as well and adjust until the best image (personal opinion) was achieved.

## **2.15 LIST OF SUPPLIERS**

AGAR SCIENTIFIC LTD. 66A Cambridge Road, Stansted, Essex. CM24 8DA for microscopy supplies

BDH LABORATORY SUPPLIES MERCK LTD. Hunter Boulevard, Lutterworth, Leicester. LE17 4XN for microscopy supplies and general chemicals

BIO-RAD LABORATORIES Ltd. Bio-Rad House, Maylands Ave., Hemel Hempstead, Herts HP2 7TD

BIO-CELL RESEARCH LABORATORIES, Cardiff Business Technology Centre, Senghenydd Rd, Cardiff CF2 4AY for Immunohistochemistry

EASTMAN-KODAK LTD, Acornfield Rd. Knowsley Industrial Park North, Liverpool L33 7UF, UK

LONDON RESIN COMPANY, P.O. Box 34, Basingstoke, Hants RG21 2NW, UK.

NIKON Japan, Fuji Building 2-3, Marunouchi 3-Chome, Chiyoda-Ku, Tokyo 100 Japan.

NIKON UK Ltd, Haybrook, Halesfield 9, Telford, Shropshire TF7 4EW.

POLYSCIENCES Ltd, 24 Low Farm Place, Moulton Park, Northampton NN3 1HY  
for microscopy supplies

SIGMA-ALDRICH COMPANY LTD. Fancy Road, Poole, Dorset. BH17 7NH for  
general chemicals

SPI supplies, Toronto, Canada for consumables in Electron microscopical work

TAAB LABORATORIES EQUIPMENT LTD. 3 Minerva House, Calleva Industrial  
Park, Aldermaston, Berkshire. RG7 4 QW for microscopy supplies.

ZEISS UK, PO Box 78, Woodfield Rd, Welwyn Garden City, Herts AL7 1LU, UK.  
For microscopy supply and parts.

## **3. RESULTS**

The results in this project have been divided into several sections based on the anatomy and ultrastructure of samples. The main emphasis has been placed on methodology and descriptions of latex vessels on each species, according to different treatments applied to the species.

### **3.1 *Meconopsis cambrica* (Papavaraceae)**

#### **3.1.1 Effect of different embedding media and fixatives regimes on the distribution and structure of laticifers at two developmental stages (stage 1-young and stage 2 - old).**

All samples in this experiment were stained with toluidine blue and observed under bright field light microscope, or as otherwise stated.

##### **3.1.1.1 Fresh samples (no embedding medium) stained with calcofluor**

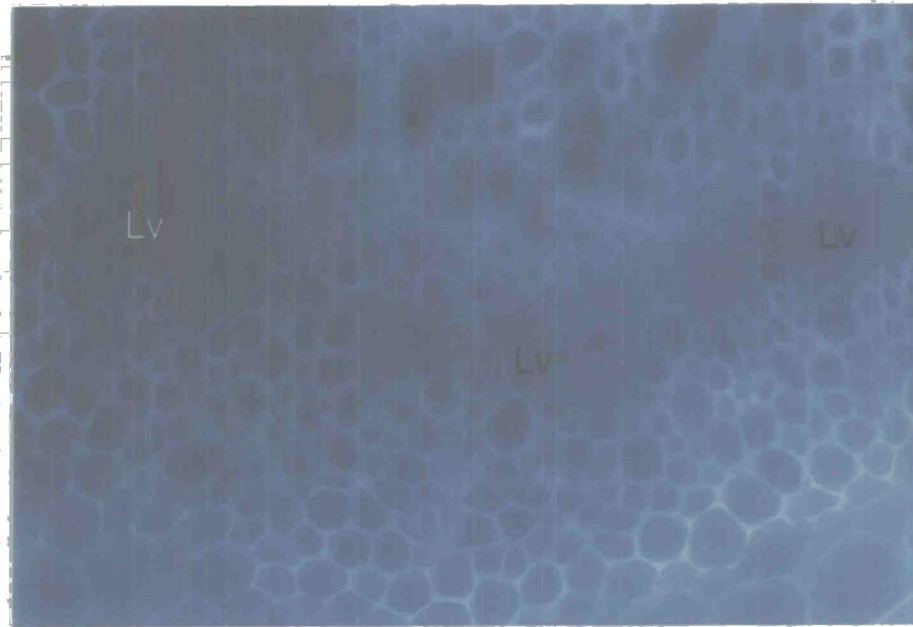
Fresh samples from the field were collected to observe the unfixed stage of the plant's cells. Both the leaves and the stem when cut, immediately produced a white and very runny liquid. The latex then turned yellowish with time.

Without any chemical fixation, the plants have the advantages in providing information on the integral and original state of the cells. Hand-cut thin sections (approximately 1mm) of *Meconopsis* flower stem were stained with calcofluor and observed with epi-fluorescent illumination. Calcofluor stain was used because it reacts with cellulose, which is the major component of the cell wall, and will make the cell walls of the tissues fluoresce blue when exposed to UV light.

Figures 1a & 1b show images of *Meconopsis* flower stem obtained using calcofluor staining alone and in combination with bright field illumination. Laticifers are clearly seen as a row of dark cloudy cells in the cambial region. The murky presentation of the cells is due to the latex oozing out during the sectioning and clogging the laticifers, hence obstructing light passing through the cell (fig. 1b). The latex, however, did not fluoresce with this stain. The technique that combines both UV and BF illumination, revealed a distinctive array of laticifers. This is a very good and fast technique to examine the location of the laticifers in fresh samples, provided the laticifers in the plant have a large amount of latex to facilitate the observation.

### **3.1.1.2 Samples embedded in LR White resin**

Figure 2 shows a transverse section (ts) of young *Meconopsis* stem. Laticifers can be identified in the cambial region of the vascular bundle, with their irregular shapes compared to other parenchyma cells. The arrangement of the laticifers resembles those in figures 1a & 1b. The laticifers also contain dense cytoplasm, which is a prominent feature of young laticifers. Fine structure



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Figure 1a

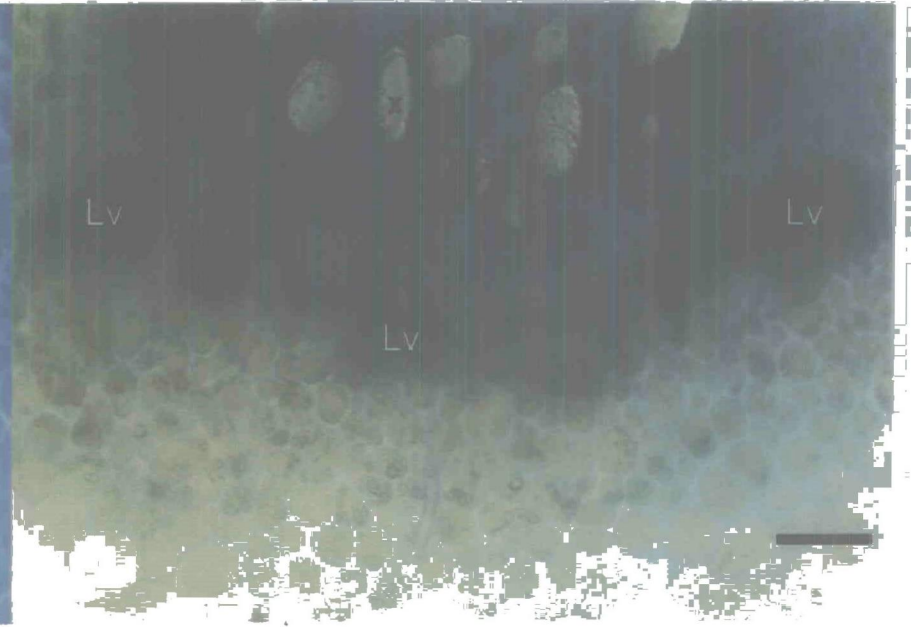


Figure 1b

Figure 1a & 1b : Cross section of fresh *Meconopsis* stem. Laticifers can be seen very clearly in both photographs, as a murky appearance in the cambial region. Figure 1a was a cross section of stem, viewed under fluorescent filter (UV filter) and figure 1b was observed when the incident light illuminate from below the section. Lv=laticifers, x=xylem. Bar=30µm.

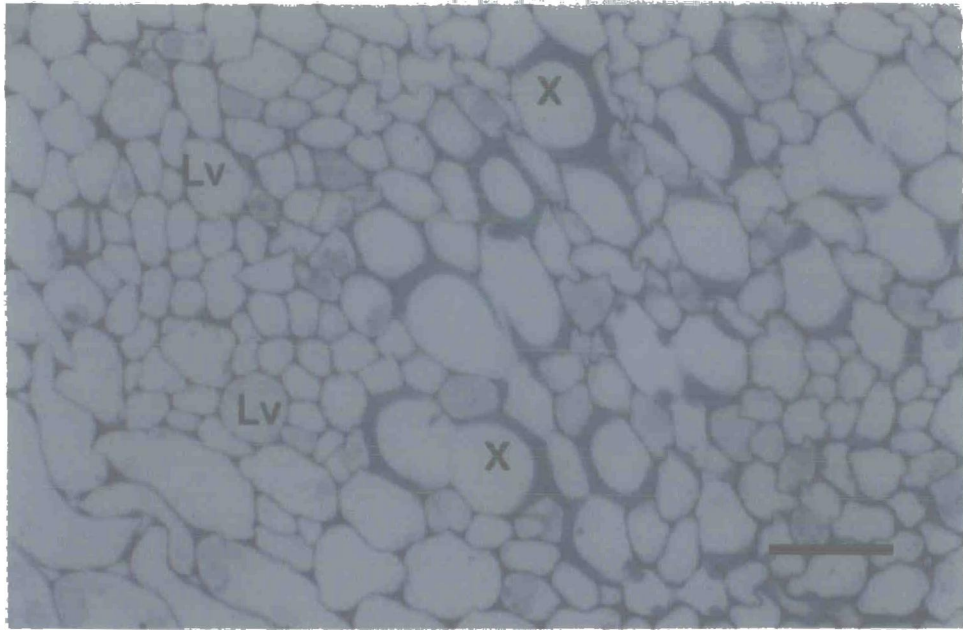


Figure 2: Cross section of young *Meconopsis* stem, embedded in LR White. Laticifers can be differentiated from other cells by their typical irregular shapes or dense cytoplasm in young samples. Xylem showed a common thickening of the wall which is very distinguishable with the stain. Lv=laticifers, x=xylem. Bar= 30µm.

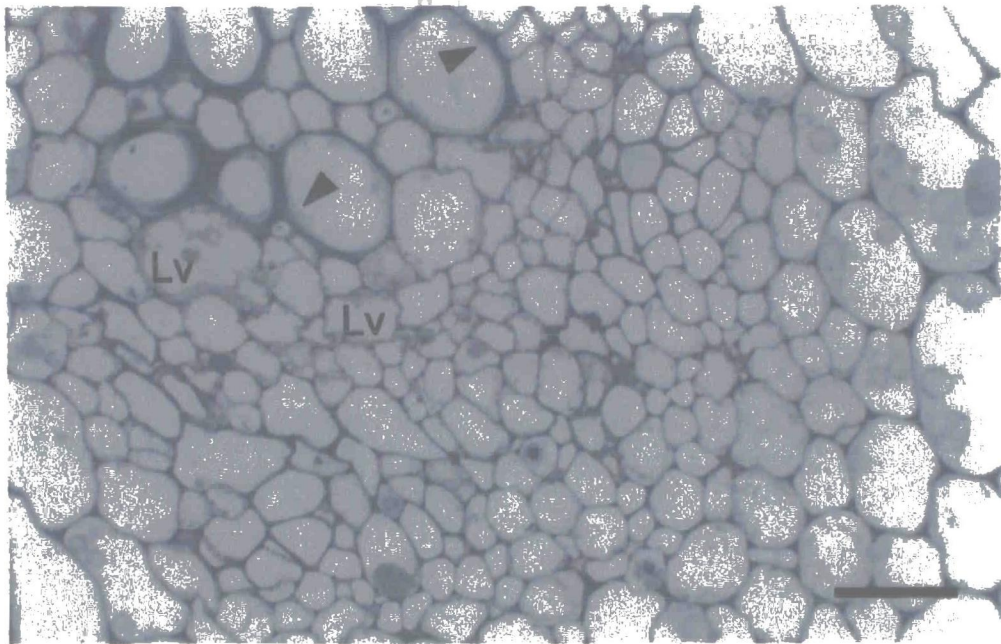


Figure 3: Cross section of old *Meconopsis* stem, embedded in LR White. Laticifers in this sample can clearly be identify by their contents, which was the vacuoles. Arrows showed the content of the xylem that look like they contained vacuoles inside them, which was sometimes confusing. Lv=laticifers, x=xylem. Bar=30µm.

appears to be well preserved where there is no cell shrinkage. In an older sample (fig. 3), vacuoles can be clearly seen in the laticifers. Lumen in xylem as shown in figure 3 can sometimes be mistaken for vacuoles (arrows).

A longitudinal section (ls) of young *Meconopsis* stem (fig. 4) showed a row of latex vessels with vacuoles. Two or more laticifers can be seen located side by side and in parallel of each other. Nuclei can clearly be seen in laticifers. Cytoplasm was quite dense in most of the laticifers. However in older plants, as shown in figure 5, vacuoles are quite scarce in laticifers. However cytoplasm appears to be quite distinguishable. Nuclei were also very scarce in the laticifers of old samples. The cell wall at certain places (black arrow) in figure 5; was observed to have disintegrated, probably due to cell lysis and the wall joining the other wall of adjacent cell to make a bigger network of laticifers.

Both in figures 4 & 5, the characteristics of laticifer cells of young and old plants are quite conspicuous, where the younger laticifer cells generally contain more cytoplasm, whereas the older plant's cell (probably due to the ageing factor) contain less cytoplasm.

### **3.1.1.3 Samples embedded in Araldite resin**

#### **3.1.1.3.1 Young and old *Meconopsis* fixed with osmium tetroxide and zinc iodide (ZIO)**

There were two regimes of fixatives used in this experiment, the first was fixed with osmium tetroxide plus zinc iodide (ZIO) and secondly the samples were fixed with standard osmium tetroxide.

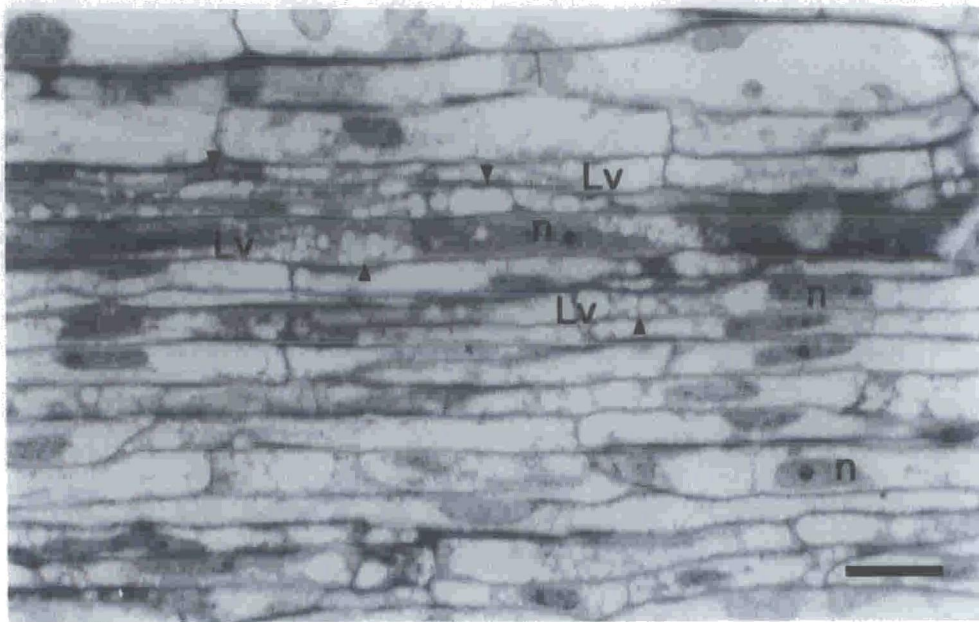


Figure 4: Longitudinal section of young *Meconopsis* stem. Laticifers contain numerous vacuoles in the cytoplasm. The cytoplasm in the laticifer was also quite dense compared with the surrounding cells, a distinguishing features and a typical character of young laticifers. Arrows indicated vacuoles. Lv=laticifers, n=nucleus. Bar=30 $\mu$ m.

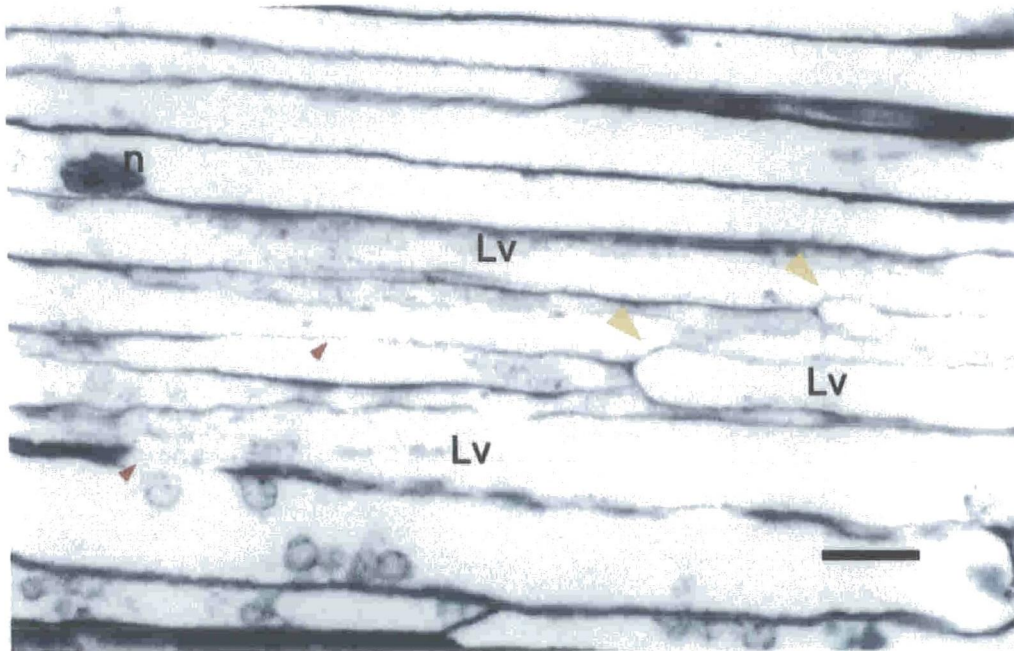


Figure 5: Longitudinal section of old *Meconopsis* stem. Laticifers can be identified and distinguished from other cells by their content of apparent cytoplasm within the cells, though vacuoles were hardly visible in the laticifers. Cell wall thinning occurred at several points (  $\rightarrow$  ) and even break down, hence connecting with the neighbouring cells. Some laticifers' end tip showed swelling and forming bulbous form (  $\blacktriangleright$  ), probably the initial step in the process of interconnecting with other adjacent laticifers. Lv=laticifer, n=nucleus. Bar=60 $\mu$ m.

The cross section from the stem of young samples fixed with ZIO did not show clear and strong evidence of the laticifers location as in other samples prepared with other fixation techniques. However, in some photographs as shown in figure 6, vacuoles in the laticifers make the cell (laticifers) quite recognisable and different from other non-laticiferous cells. From the same photograph, plasmolysis was also quite noticeable (arrows) in certain cells (parenchyma), but fortunately the plasmolysis did not seem to occur in the vascular bundle region and especially in the laticifers.

As in the younger plant, the laticifers in older plants were also quite difficult to locate. And it was even harder to verify if the suspected cells were the laticifers or not due to the lack of vacuoles in the older plants (figure 7). This photograph also shows extensive plasmolysis occurring in the parenchymatous cells; as in the young plants. The fixatives seemed to fix the cells in the vascular bundle quite well for that reason.

#### **3.1.1.3.2 Young and old *Meconopsis* fixed with osmium tetroxide**

In longitudinal section (ls) of the old *Meconopsis* stem (figure 8), fixed with  $\text{OsO}_4$ , laticifers were clearly seen and observed to be located running in parallel to xylem. Vacuoles were, however, quite prominent in the laticifers, in contrast to the previous observation. Another interesting observation was that, there were quite a few places where the cells wall seemed to swell and formed bulbous tips toward the end of laticifers (arrow). It was thought that these were actually the place where the joining of two developing neighbouring laticifers occurred. However, there was no evidence of cell plasmolysis in this particular sample.

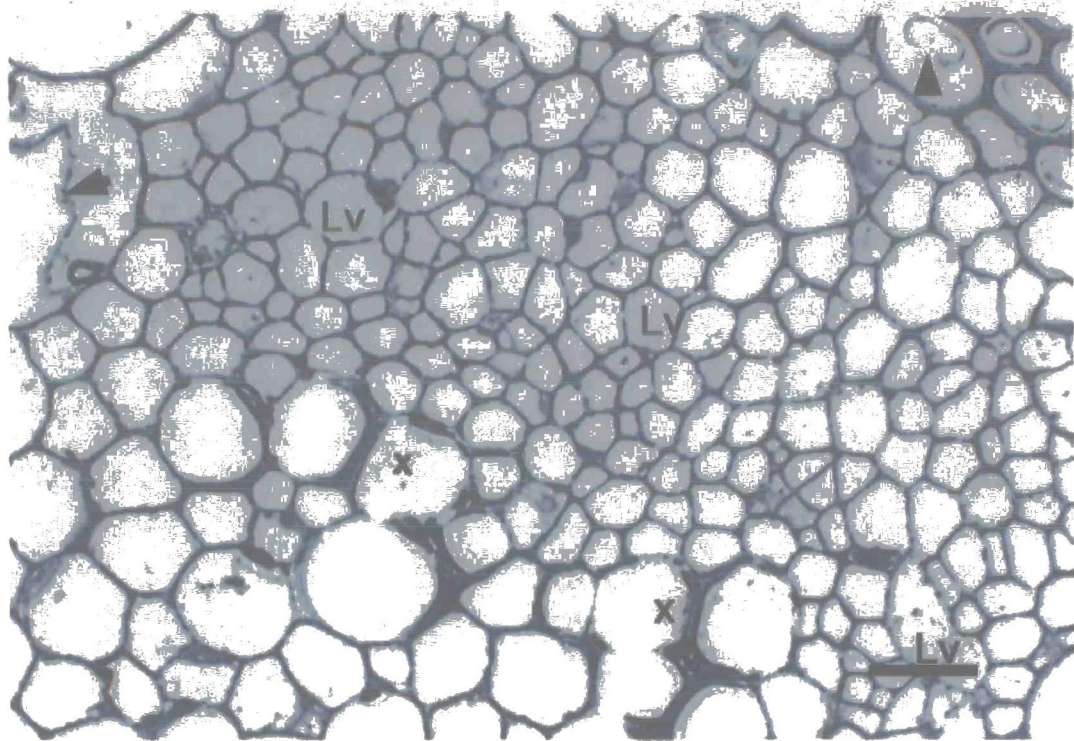


Figure 6: Cross section of young *Meconopsis* stem fixed with ZIO. Laticifers can be seen containing vacuoles. Cell plasmalemma were noticed to be shrunk inward the cells in the parenchyma cells (arrow), but were not observed within the cells of laticifers and the vascular bundles. Lv=laticifers, x=xylem. Bar=30 $\mu$ m.

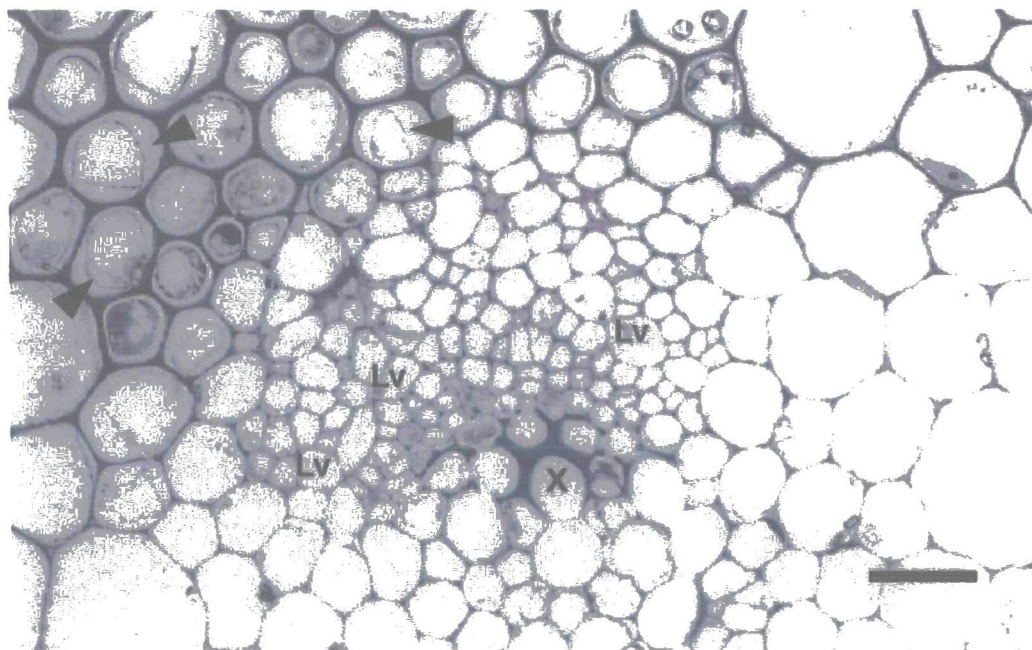
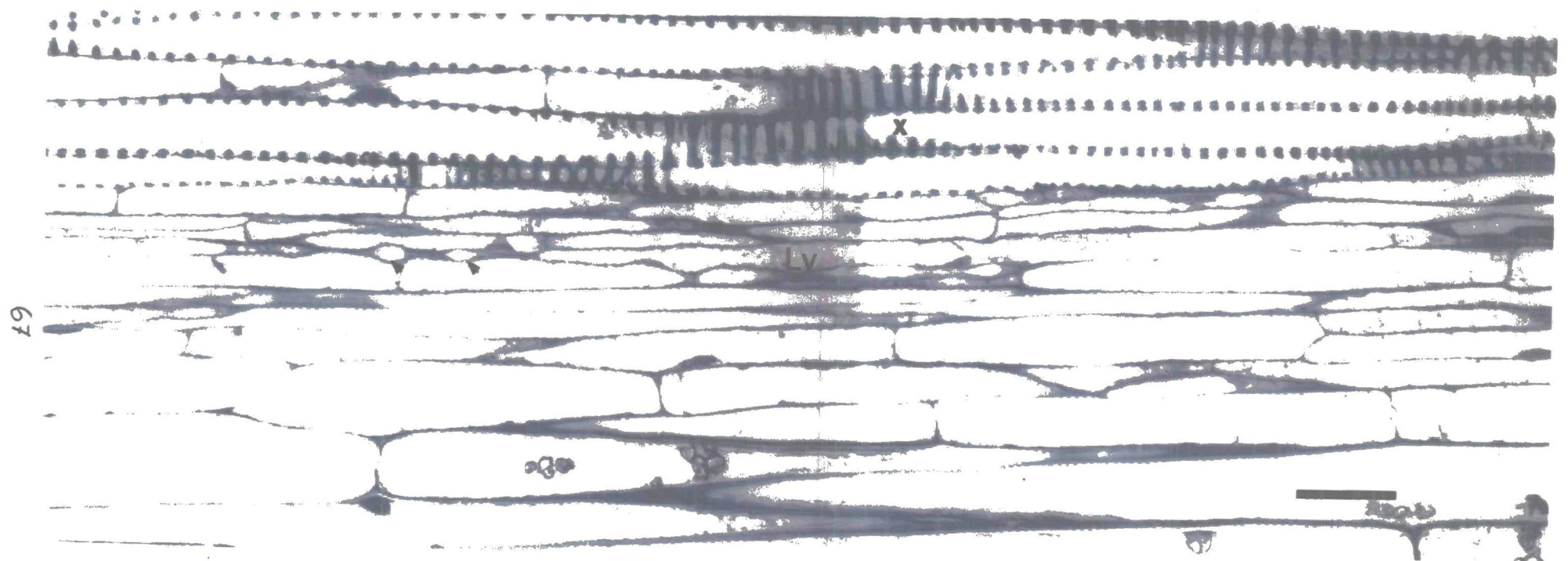


Figure 7: Cross section of old *Meconopsis* stem fixed with ZIO. Vacuoles were less visible in the laticifers. Plasmalemma in the parenchyma cells suffered quite severe shrinkage (arrow). Cell plasmolysis was not observed in laticiferous cells. Lv=laticifers, x=xylem. Bar= 30 $\mu$ m.



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Figure 8: Longitudinal section of old *Meconopsis* stem, fixed with osmium tetroxide. Laticifers seem to run in parallel with xylem. Vacuoles were quite scarce. Again, there were a few points, especially toward the end of the laticifers, where the cell wall showed swelling (arrows). Lv=laticifers, x=xylem. Bar=30 $\mu$ m.

In figure 9, a longitudinal section of young *Meconopsis* shows a stack of laticifers running in parallel with the xylem. Vacuoles could be observed in the laticifers. The structures seemed to be preserved very well, as there was no evidence of cell shrinkage.

However a cross section of young *Meconopsis* stem showed that it was rather difficult to identify the location of the laticifers, as shown in figure 10. The location of the laticifers were confirmed by comparing the fixed-embedded section with the fresh unfixed samples (Figure 1) There was no evidence of vacuoles or other organelles observed in the laticifers.

#### **3.1.1.4 Samples embedded in Spurr resin**

##### **3.1.1.4.1 Young and old *Meconopsis* fixed with osmium tetroxide/zinc iodide (ZIO)**

Results from the young *Meconopsis* stem (ls) fixed with ZIO, showed laticifers that contained numerous vacuoles (figure 11). The cell wall at the tip of the laticifers appeared to have disintegrated and joined the neighbouring laticifers, forming an extensive network of laticifers (arrows) as described previously. Cytoplasm in the laticifers was quite dense. However, in cross section (ts) of the young sample, vacuoles could hardly be seen (fig. 12). However laticifers could be identified by their irregular shape compare to other cells. Plasmolysis was seen in parenchyma cells. Fixatives seem to fix the structure of laticifers and cells in the vascular bundle well.

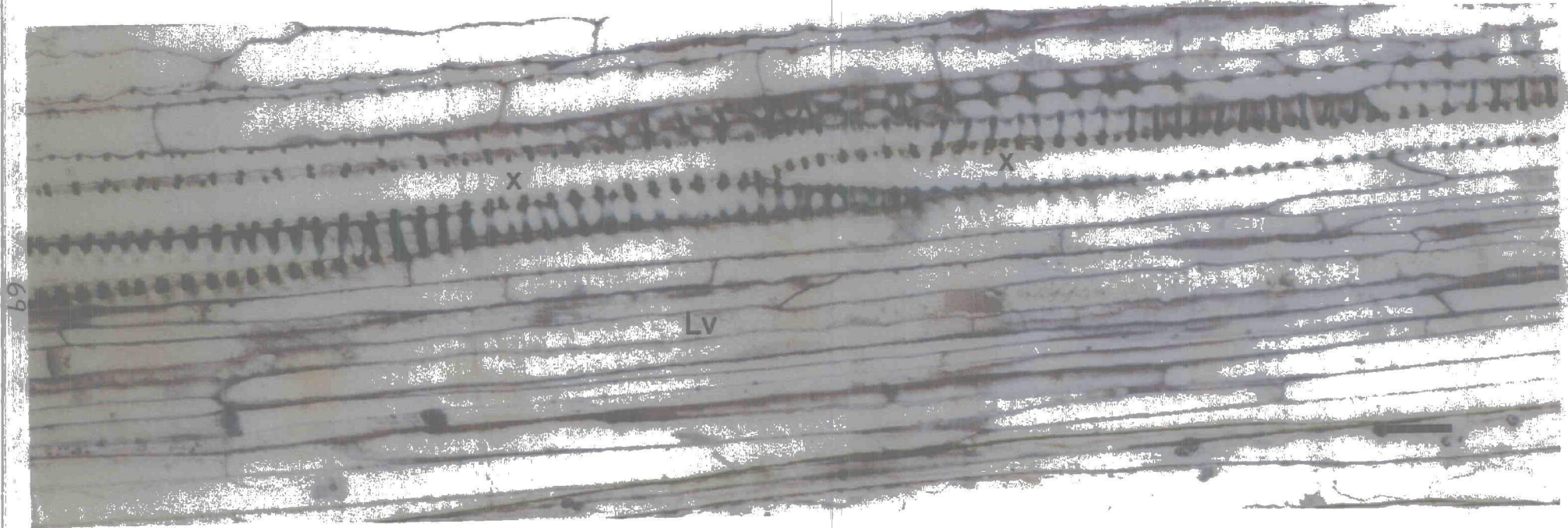


Figure 9: Longitudinal section of young *Meconopsis* stem fixed with osmium tetroxide. Laticifers were seen running in parallel with xylem. Vacuoles were visible in the laticifers. Lv=laticifers, x=xylem. Bar=30 $\mu$ m.

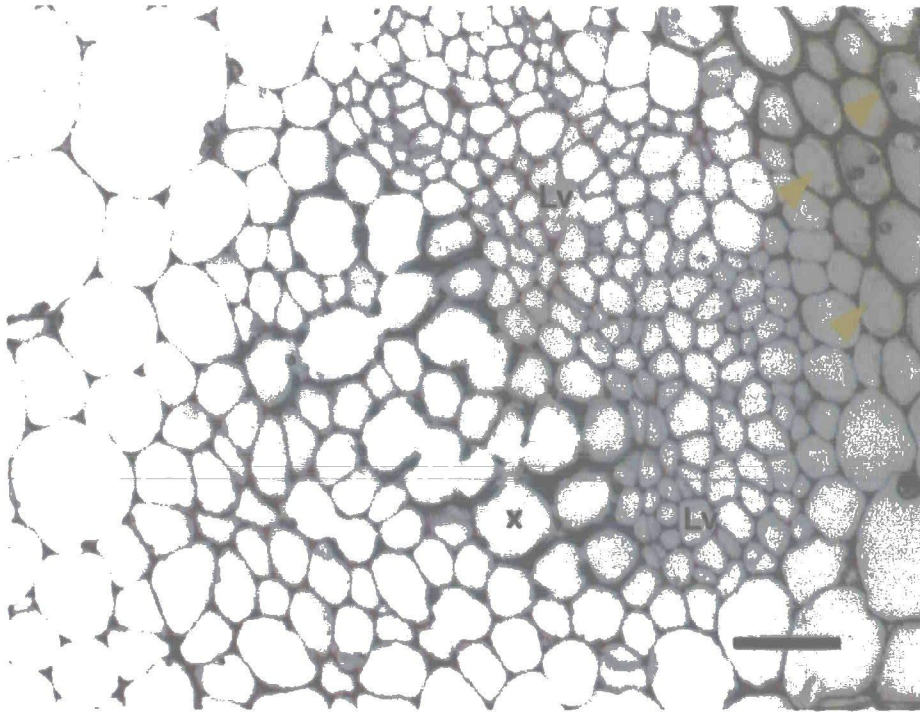


Figure 10: Cross section of old *Meconopsis* stem. No vacuoles were visible in the laticifers. Cell plasmolysis was apparent in the parenchyma cells, outside the vascular bundle (arrows). X=xylem, Lv= laticifers. Bar= 70 $\mu$ m

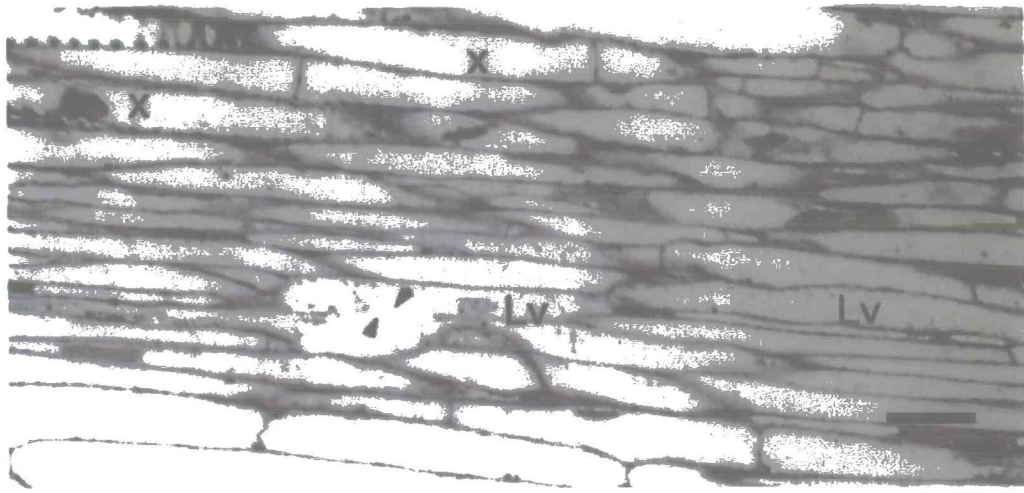


Figure 11: LS section of young *Meconopsis* stem fixed with ZIO. Vacuoles can be seen in the laticifers. Arrows showed the point where two laticifers joined together after the cell wall disintegrated. X= xylem, Lv= laticifers. Bar=30µm.

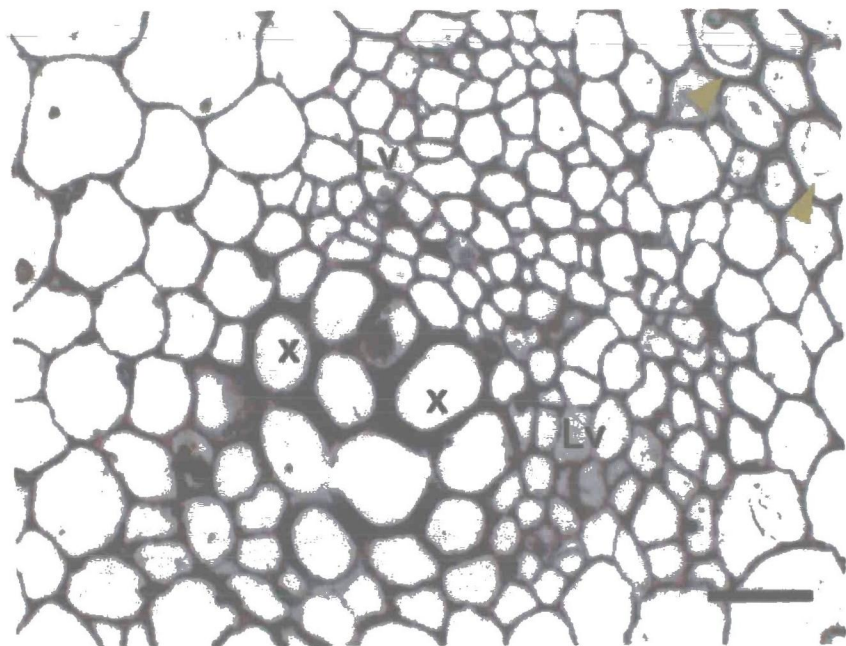


Figure 12: Cross section of young *Meconopsis* stem fixed with ZIO. Vacuoles were not visible in laticifers. Cell plasmolysis was quite evident in parenchyma cells (arrows). X=xylem, Lv= laticifers. Bar=30um.

Similarly with the young sample, the old *Meconopsis* stem, shown here in longitudinal section, exhibited cell plasmolysis in parenchyma cells. Laticifers and cells in the vascular bundle seemed to be preserved very well. Vacuoles could be observed in the laticifers (figure 13). In cross section (ts) of the old *Meconopsis* as shown in figure 14, again it was quite hard to distinguish the location of laticifers. Even though it was quite difficult to identify the laticifer cells by their normal content (vacuoles), on the other hand, their irregular shapes (caused by a few cells merging together), were easily seen. Vacuoles were not visible in the laticifers cells and cell plasmolysis was only observed in the parenchyma cells.

#### **3.1.1.4.2 Young and old *Meconopsis* fixed with osmium tetroxide**

In a longitudinal section of young *Meconopsis* stem, vacuoles could be seen clearly in laticifers (figure 15). In one particular laticifer (arrow), the cell wall had started to expand and formed a bulbous shape. However the cell wall did not disintegrate and hence did not merge with the neighbouring laticifers. The results also showed that the cell wall expanded not toward the tip of the cell, but at the middle of the cell.

It is quite interesting to note in figure 16, laticifers in young *Meconopsis* (ts) were not only forming in a single array of cells but were also in a shape of a 'flower' arrangement (\*). It was also quite obvious that some laticifers appeared to have a dense amount of cytoplasm. Vacuoles were quite apparent in the laticifers.



Figure 13: Ls of old *Meconopsis* stem fixed with ZIO. Vacuoles could be observed in the laticifers. Arrows showed cell plasmolysis in parenchyma cells. Lv=laticifer, x=xylem. Bar=30 $\mu$ m.

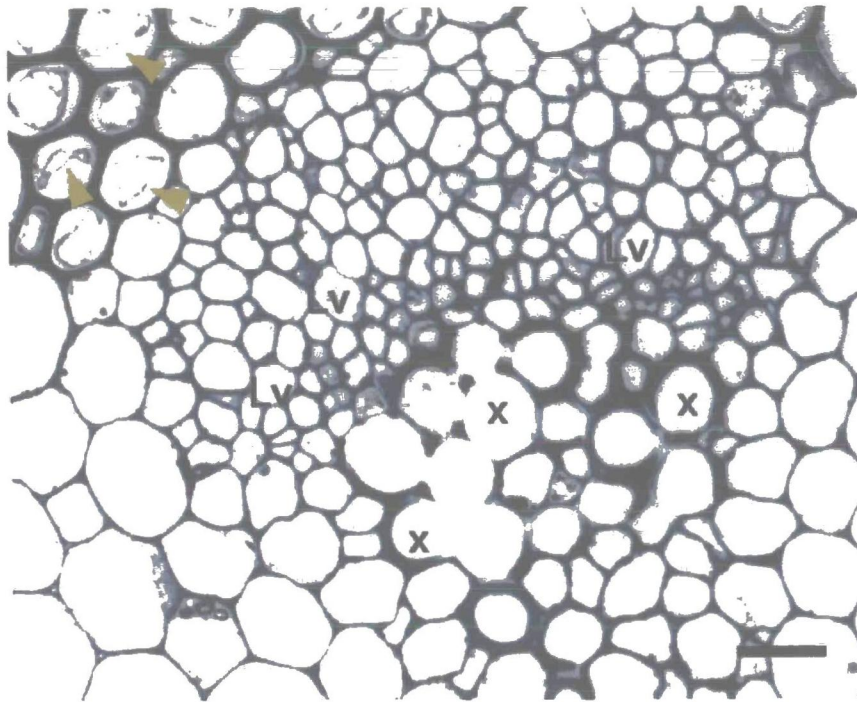


Figure 14: Ts sample of old *Meconopsis* stem fixed with ZIO. Vacuoles could not be observed in the laticifers. Cell plasmolysis were very severe in parenchyma cells (arrows). Lv=laticifer, x=xylem. Bar=30 $\mu$ m.

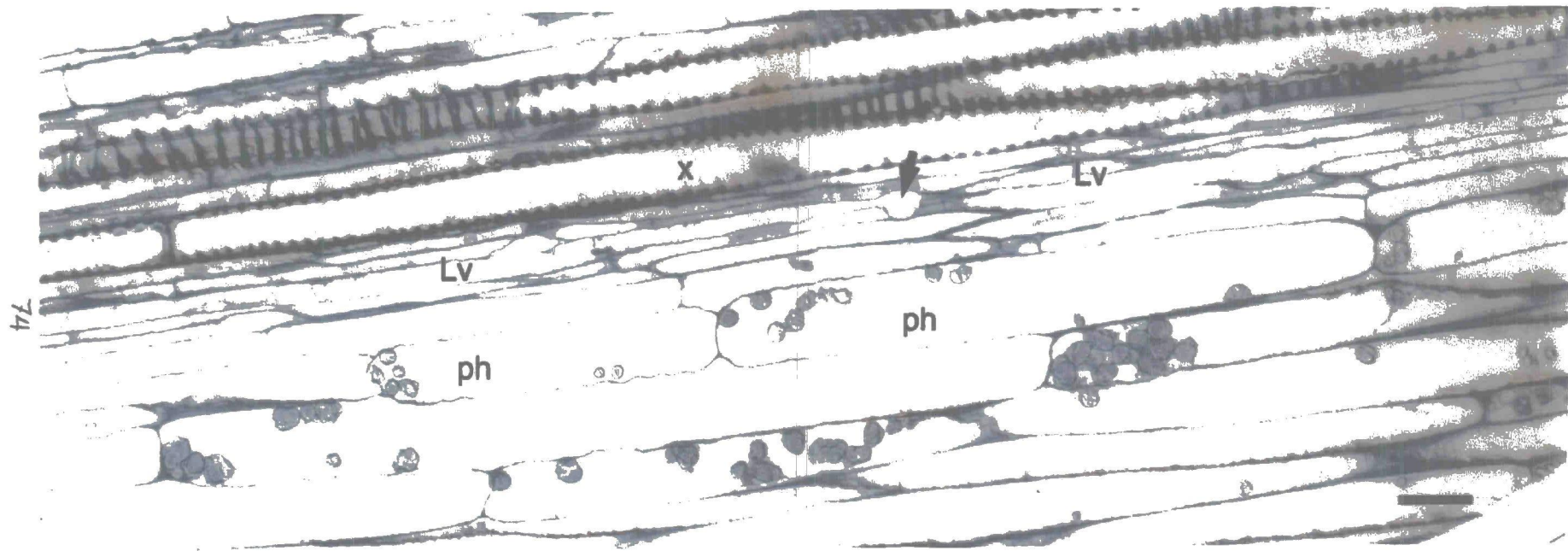


Figure 15: Longitudinal section of young *Meconopsis* stem, fixed with osmium tetroxide. Laticifers were located in between xylem and phloem. Vacuoles can be seen in the laticifers. Arrows indicated the place where the cell walls of one laticifer swelled and appeared to be blocking the neighboring laticifer. Lv=laticifers, x=xylem, ph=phloem. Bar = 30µm.

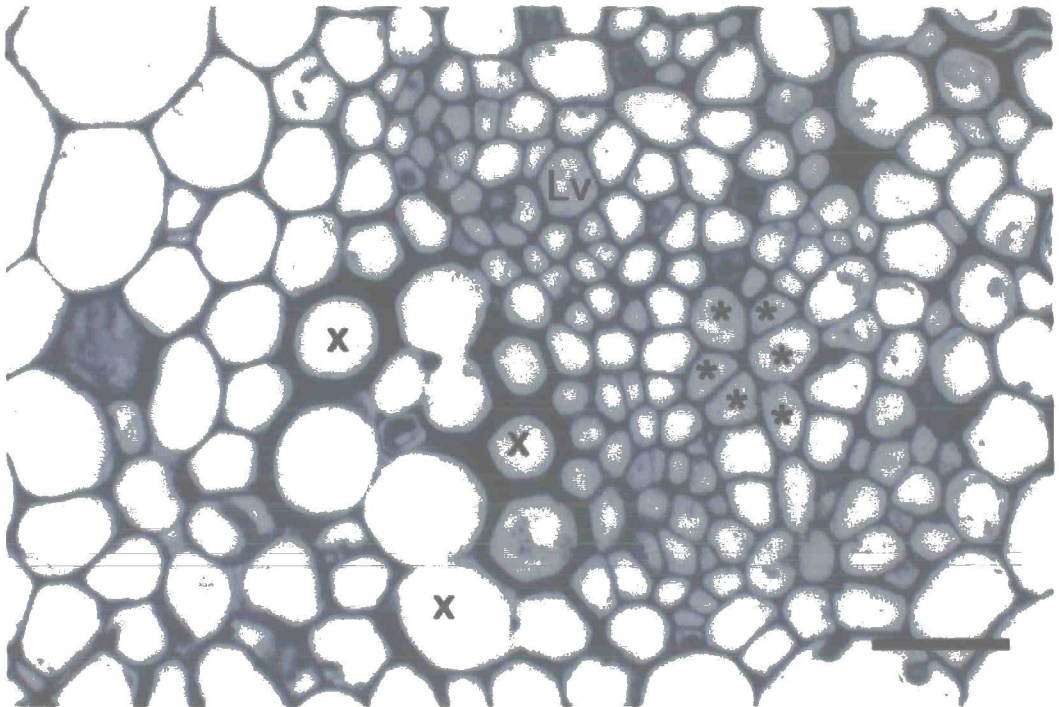


Figure 16: Cross section of young *Meconopsis* stem fixed with osmium tetroxide. Laticifers can be seen containing vacuoles. A few laticiferous cells form a conspicuous "flower" arrangement ( \* ). X=xylem, Lv= laticifers. Bar=30 $\mu$ m.

In older *Meconopsis* stem as shown in figure 17, vacuoles were visible in the laticifers. Cell walls of the laticifers were seen to disintegrate at several points and interconnected with parallel laticifers (arrow). As mentioned before, this may be an initial stage in the formation of an anastomosing network of laticifers.

### **3.1.1.5 Distribution of laticifers in young *Meconopsis cambrica* stem using the Differential Interference Contrast (DIC) Technique**

Figure 18 show an image of a cross section of young *Meconopsis* stem, observed with DIC microscopy. Results showed that the lignified cell walls of xylem were clearly visible. Laticifers could be observed located in the cambial region. However there was no vacuole visible in the laticifers. The cell wall of the laticifers could not be defined clearly as shown in figure 19 and at some point it was also clear that there were holes or gaps in between two adjacent laticifers, apparently because the cell wall ruptured (figure 20).

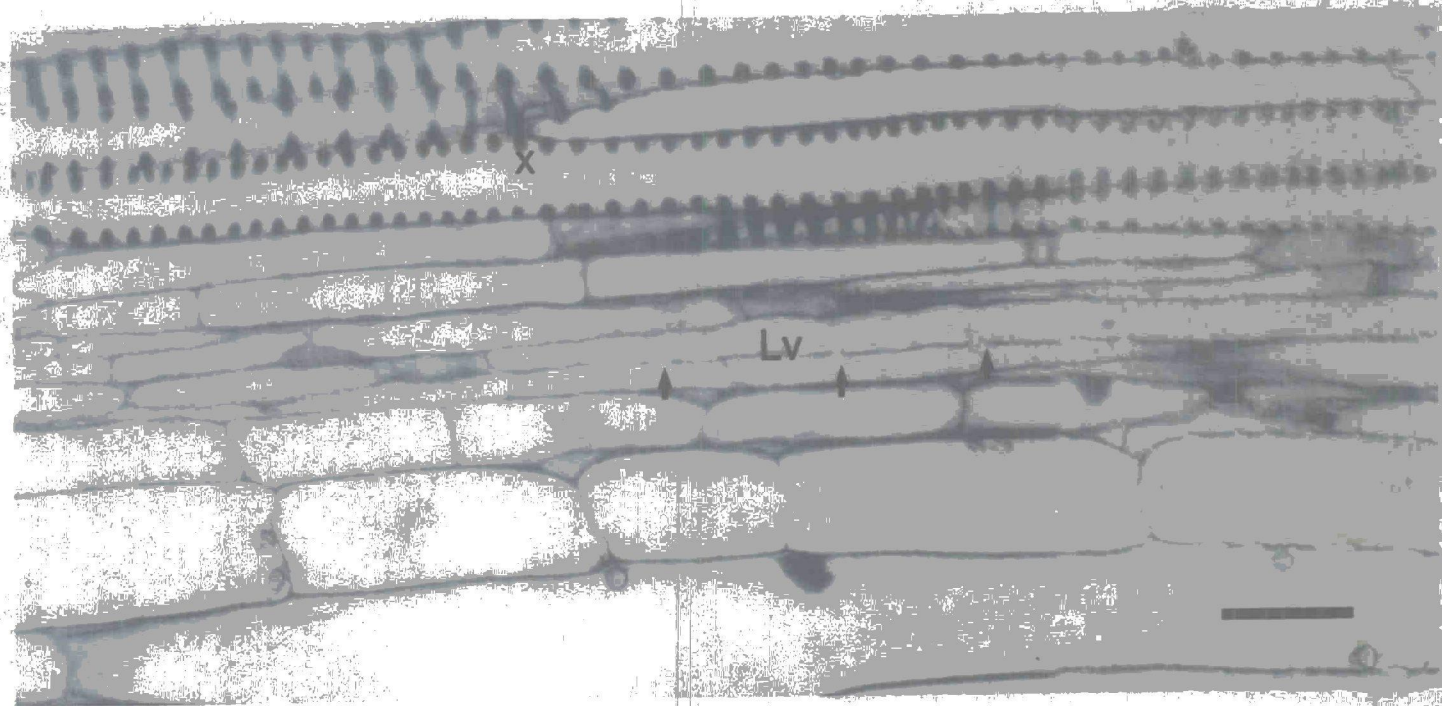


Figure 17: Longitudinal section of old *Meconopsis* stem, fixed with osmium tetroxide. Laticifers can be observed to contain vacuoles. There were a few places along the laticifers cell wall where they have disintegrated and joining the adjacent laticifers. Lv=laticifers, x=xylem. Bar=30 $\mu$ m.

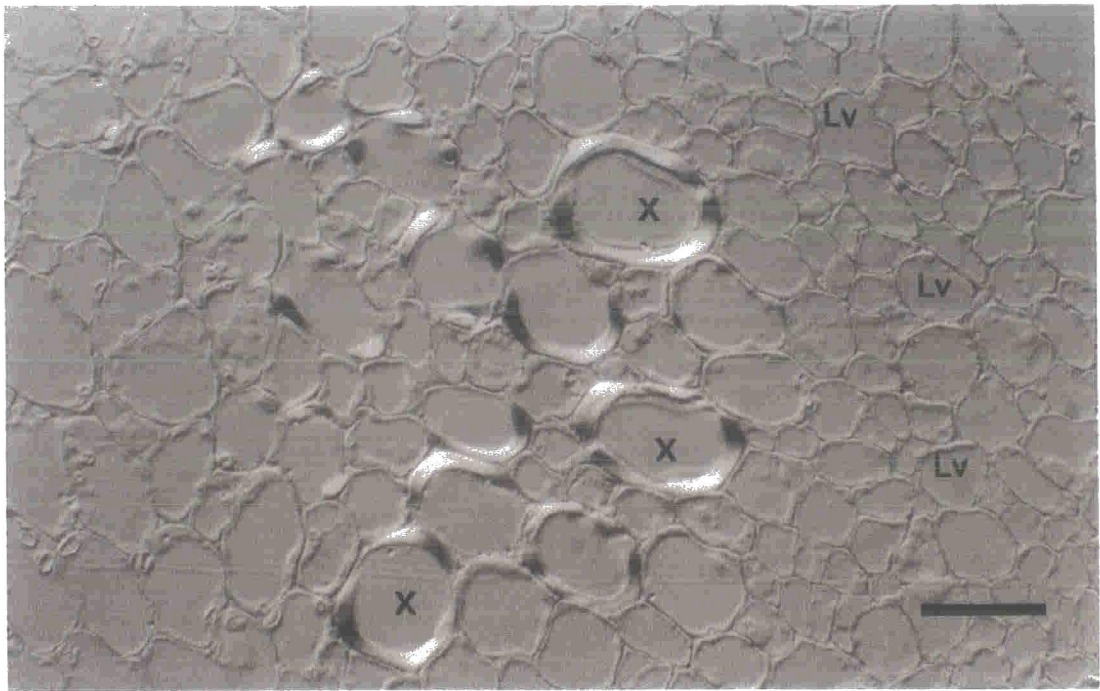


Figure 18: A Nomarski image of a cross section of young *Meconopsis* stem. Cell wall outline shown very clearly especially the wall with secondary thickening as for xylem. Laticifers were observed to be empty. X=xylem, Lv= laticifers. Bar = 30  $\mu$ m.

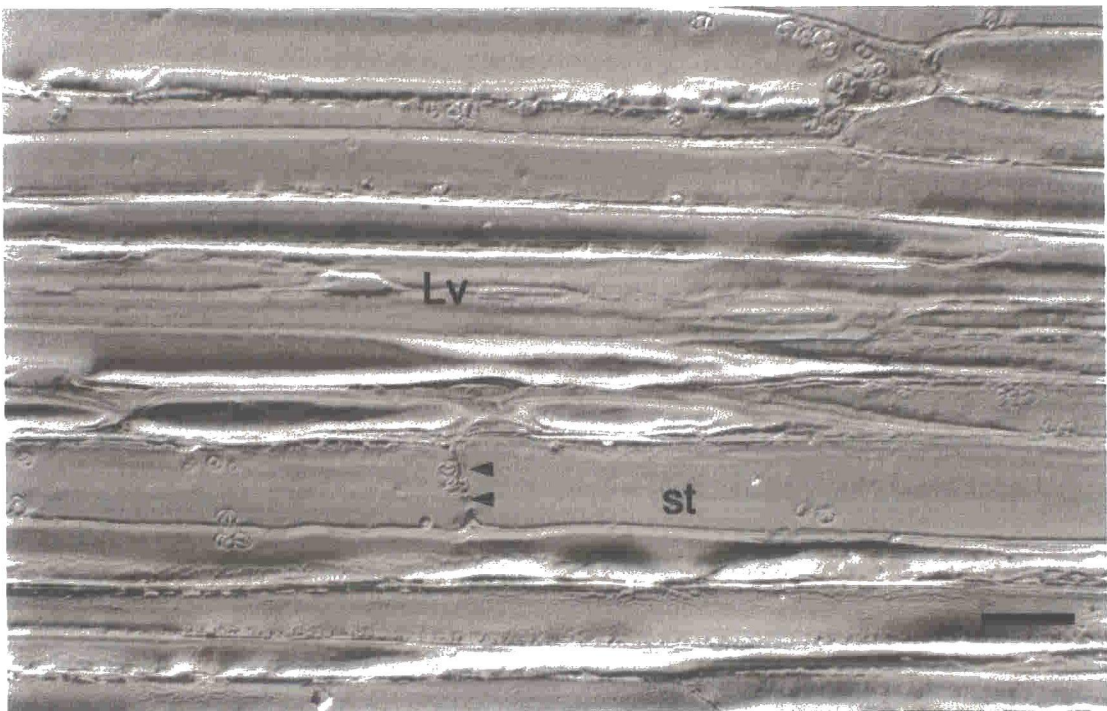


Figure 19: A longitudinal section of young *Meconopsis* stem, under Nomarski microscopy. Laticifers seem to be empty, without vacuoles. Cell walls of the laticifers were not defined well. Arrows show a sieve plate in the sieve tube. Lv= laticifers, st= sieve tube. Bar = 50 $\mu$ m.

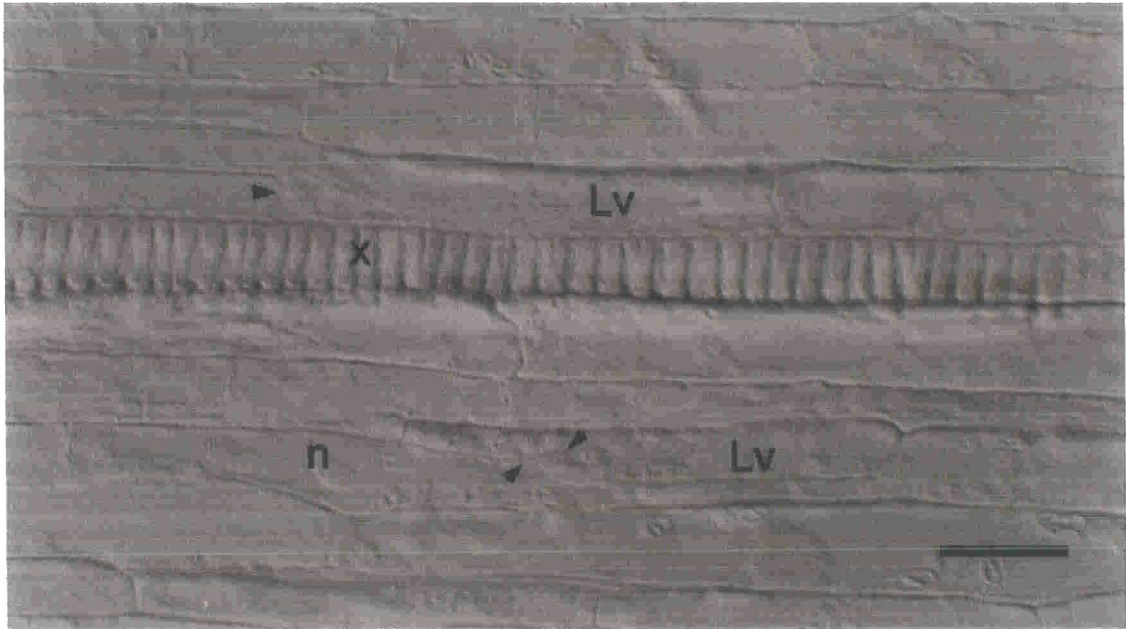


Figure 20: Nomarski image of *Meconopsis*, showed cell wall ruptures. Arrows showed cell wall of the laticifers ruptured. Laticifers were observed to be located on both side of xylem, because of the orientation during sectioning. Lv= laticifers, n= nucleus, x=xylem. Bar = 30 $\mu$ m.

### **3.1.2 Scanning Electron Microscopy (SEM) of laticifers in *Meconopsis cambrica***

Scanning electron microscopy images of *Meconopsis* showed rows of laticifers located in the cambial region of the vascular bundle (fig. 21) and numerous empty vacuoles could be seen in the laticifers. At a certain point (arrow), as seen before, the cell wall started to disintegrated and interconnected with the adjoining laticifers. It was also observed that two or more laticifers were stacked close with each other, which presumably later will merge and form a bigger laticiferous cell.

### **3.1.3 Ultrastructural study of laticifers in *Meconopsis cambrica*: Effect of different preservative regimes and embedding medium on structural preservation.**

#### **3.1.3.1 Samples fixed with OsO<sub>4</sub>, embedded in Araldite**

Electron micrograph of young laticifers in *Meconopsis* showed a large vacuole containing a net-like structure (figure 22). Whereas in contrast with the older samples as shown in figure 23, laticifers contained numerous vacuoles, packed closely together and apparently lacking a net-like structure or totally empty. Neighbouring cells appeared to be well preserved without any plasmolysis in

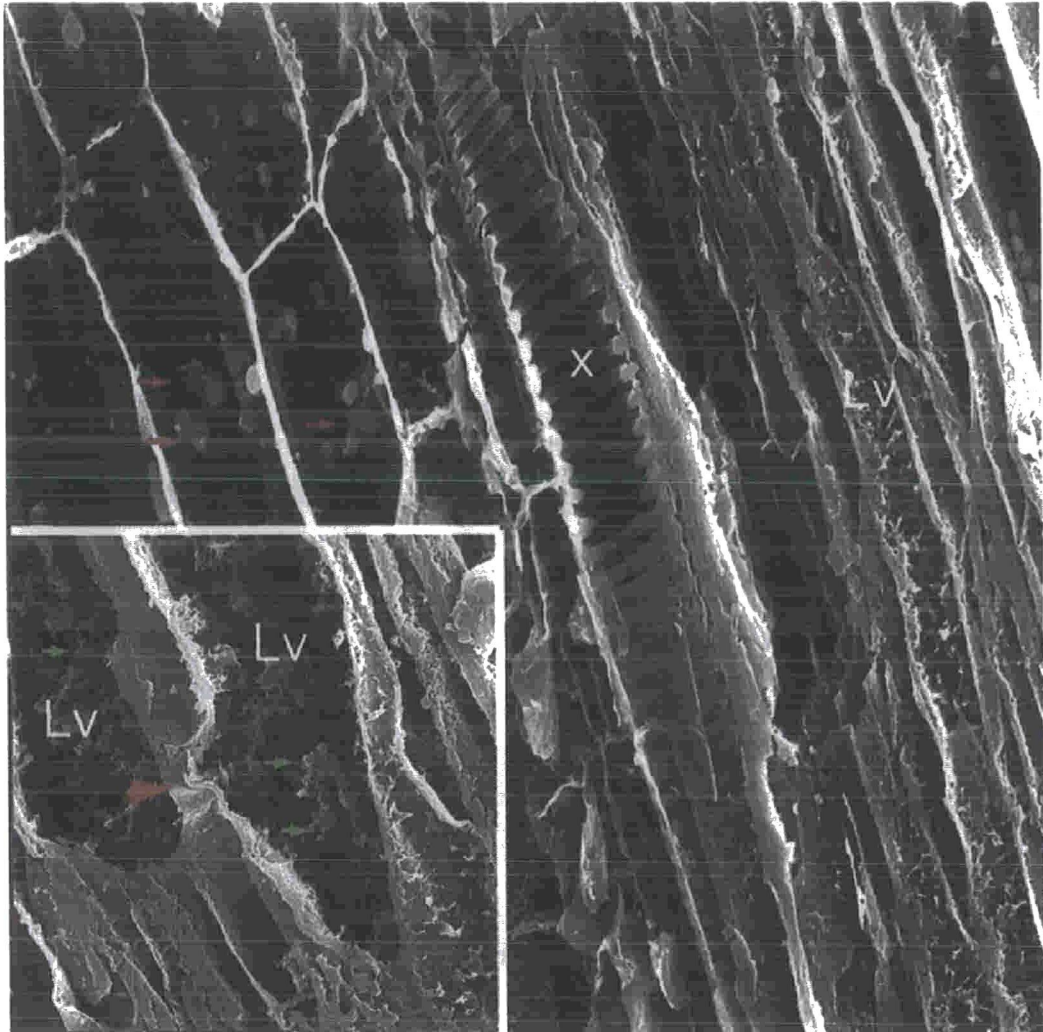


Figure 21: SEM of young *Meconopsis* stem. Laticifers were occupied with numerous amount of vacuoles. Arrows (▸) showed starch grains. Insert: showed vacuoles (➤) in the laticifers. Cell wall ruptured (➤) and merged with the adjacent laticifers. Lv=laticifers, x=xylem. Magnification = x 100 (insert magnification = x150).

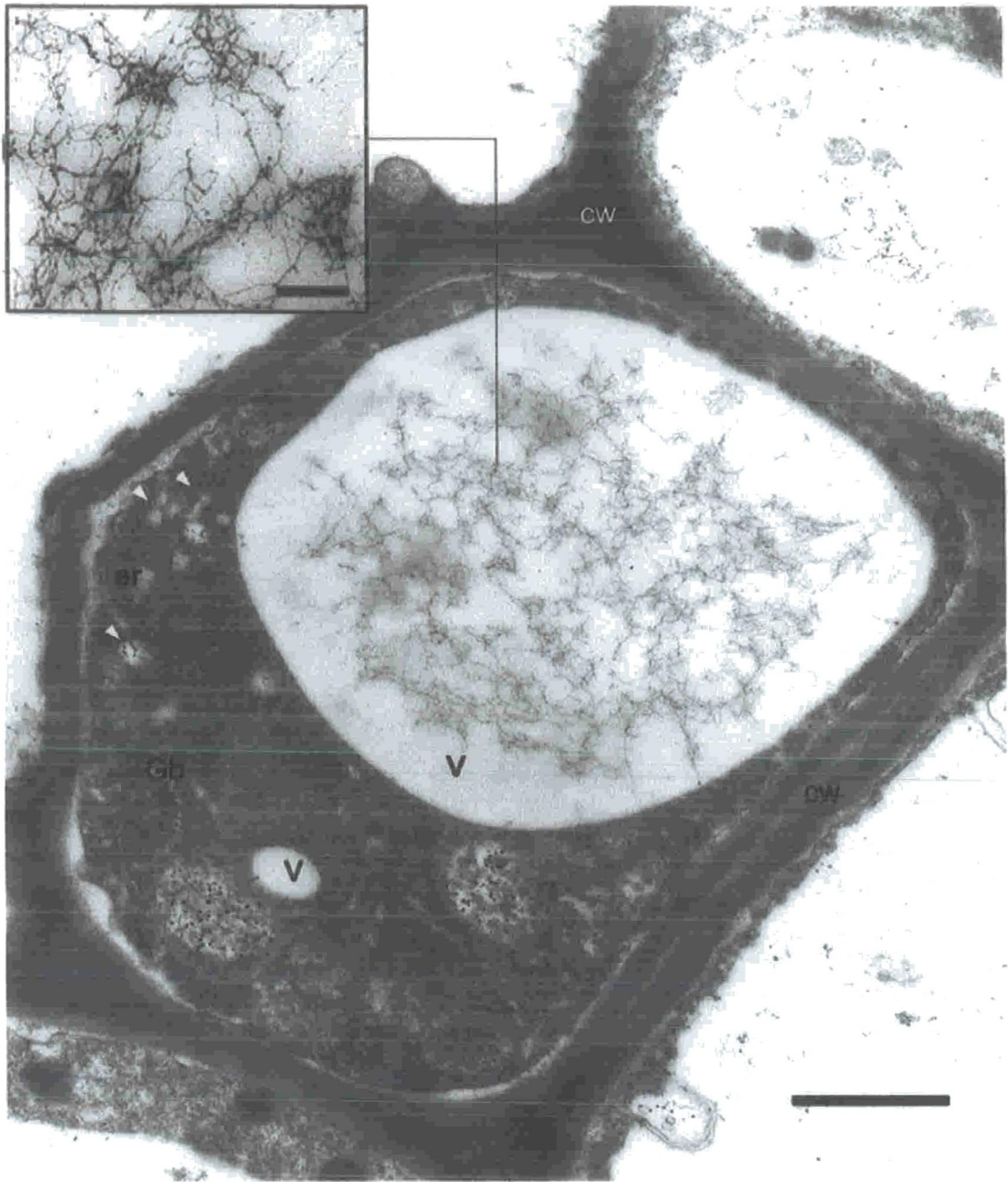


Figure 22: Laticifers in young *Meconopsis* stem with net-like structure. Vacuoles were occupied with a net-like structure, located in the centre of the laticifers (the biggest vacuole). Note that small vacuoles always associated very closely with the endoplasmic reticulum (arrows). Bar= 2.4 $\mu$ m. Small insert showed net-like structures at a higher magnification . Bar=0.3 $\mu$ m. v=vacuole, cw=cell wall, Gb=Golgi bodies, er=endoplasmic reticulum.



Figure 23: Vacuoles in laticifers of old *Meconopsis* stem, fixed with osmium tetroxide. Vacuoles were arranged closely together and very tightly packed. Net-like structures were not present in the vacuoles. Arrows showed electron dense structure in the laticifers. Symbol (\*) indicated some vacuoles in cell wall inclusion. V=vacuoles, cw=cell wall, Bar= 5.6 $\mu$ m.

young samples (figure 22). A few other organelles could be seen such as endoplasmic reticulum, vacuoles, polysomes and microtubules (fig. 24 & 25). Figure 26 shows an observation on the configuration of cell wall in the young (Figure 26a) and older sample (Figure 26b). Young sample showed microfibrils in loose arrangement whereas the older showed microfibrils in a more compact arrangement. Figure 27 showed mature laticifers occupied by empty vacuoles. Two laticifers were seen to be located adjoining to each other. Several different sizes of vacuoles were quite obvious in the laticifers, especially in the old sample. Ultrastructure information was generally very well preserved and there was hardly any structural damage or artefacts present in the samples treated with this procedure.

### **3.1.3.2 Samples fixed with ZIO; embedded in Araldite**

Apart from the vacuoles, the laticifers also contained electron dense organelles, which were quite prominent in old laticifers and this was very prominent in ZIO fixed samples, as shown in figure 28. These vacuoles had a single membrane and were packed closely to each other, particularly in the mature laticifers.

Most vacuoles were observed to be closely associated with endoplasmic reticulum and it was believed that these vacuoles originated from the dilation of endoplasmic reticulum. Vacuoles could be seen forming in different sizes and containing different structures. Some were empty and some contained electron dense material as seen in figure 29. Some ER could be seen in a tubular form, aggregate and forming multilayered cisternae. The different forms of endoplasmic reticulum observed are demonstrated very well in figure 29 of



Figure 24: TEM micrograph of laticifers in young *Meconopsis* stem. Few other organelles could be seen other than vacuoles in this laticifer. Vacuoles which originated from endoplasmic reticulum were observed near the plasmalemma (arrows) and it was presumed that this was the place where the vacuoles were synthesized before they moved toward the centre of the cell. Vacuoles vary in sizes and evolved from small to large by coalescing with the other vacuoles. V=vacuoles, M=mitochondria,er=endoplasmic reticulum, cw=cell wall. Bar=1.2 $\mu$ m.

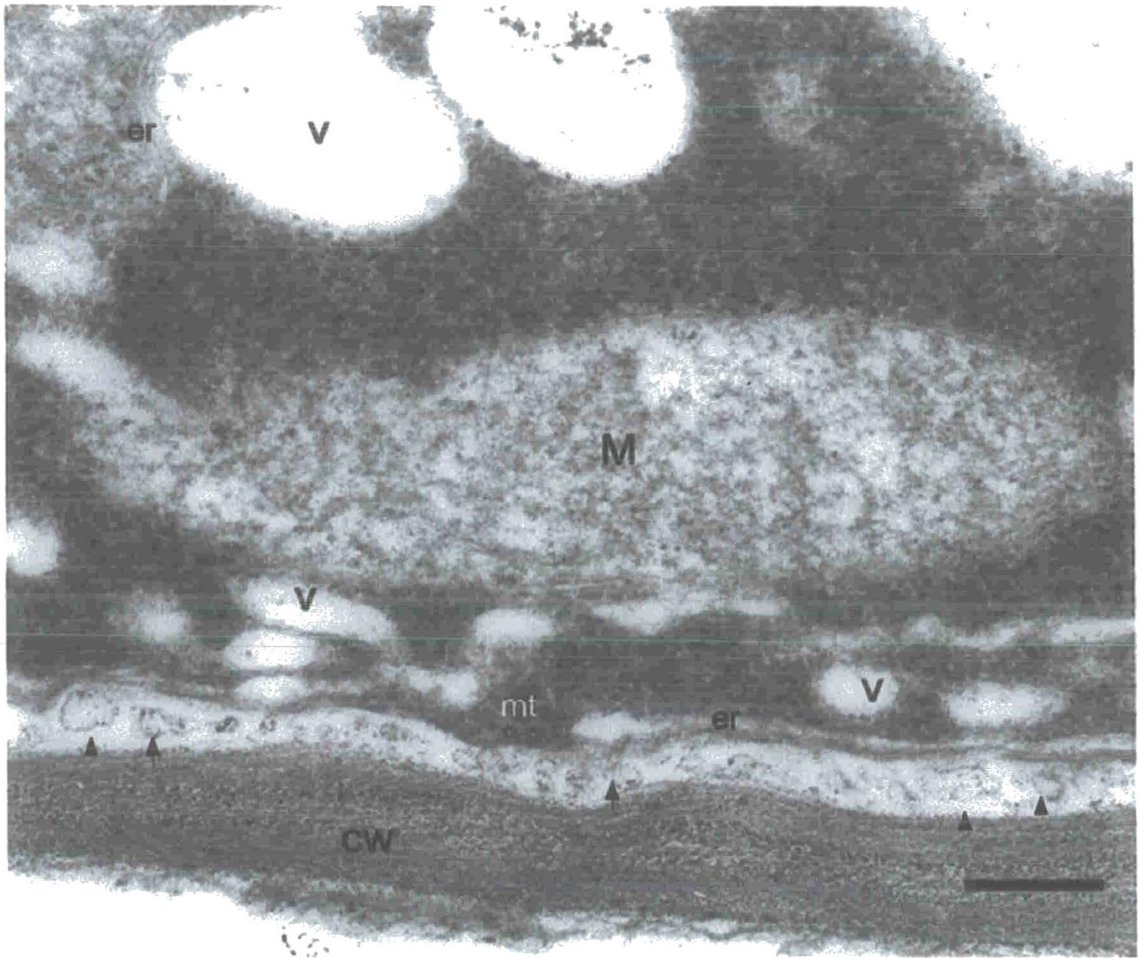
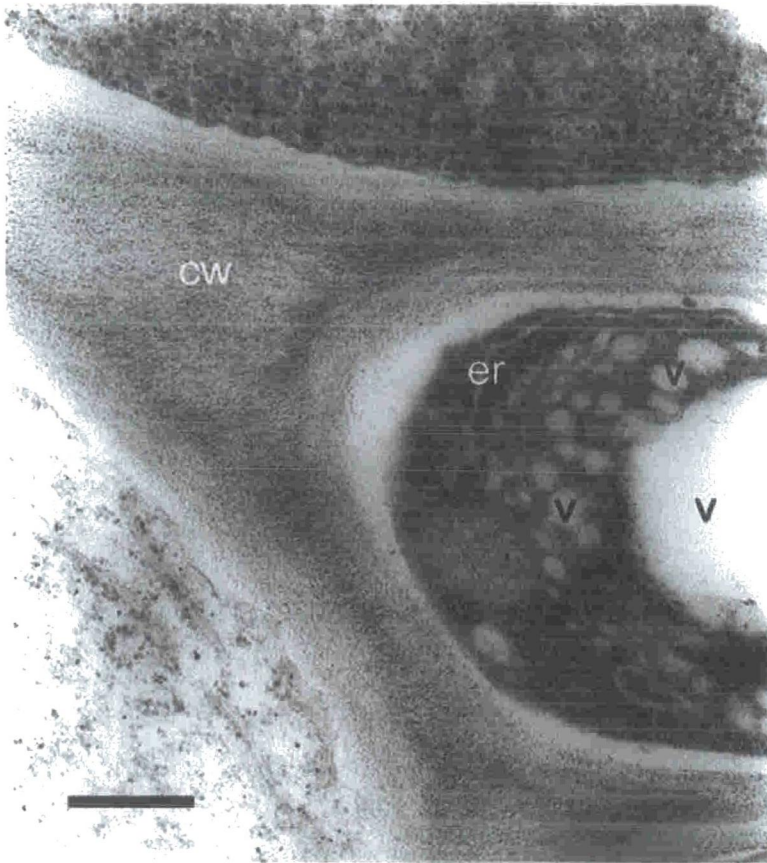


Figure 25: Organelles in laticifers of young *Meconopsis* stem. Endoplasmic reticulum is seen here, localised near the cell wall. Several vacuoles were observed near the endoplasmic reticulum. Para-mural bodies were present in between the plasmalemma and cell walls (arrows). Two microtubules were also observed (mt) near the plasmalemma. V=vacuoles, cw=cell wall, er=endoplasmic reticulum, M=Mitochondria. Bar= 0.42 $\mu$ m.

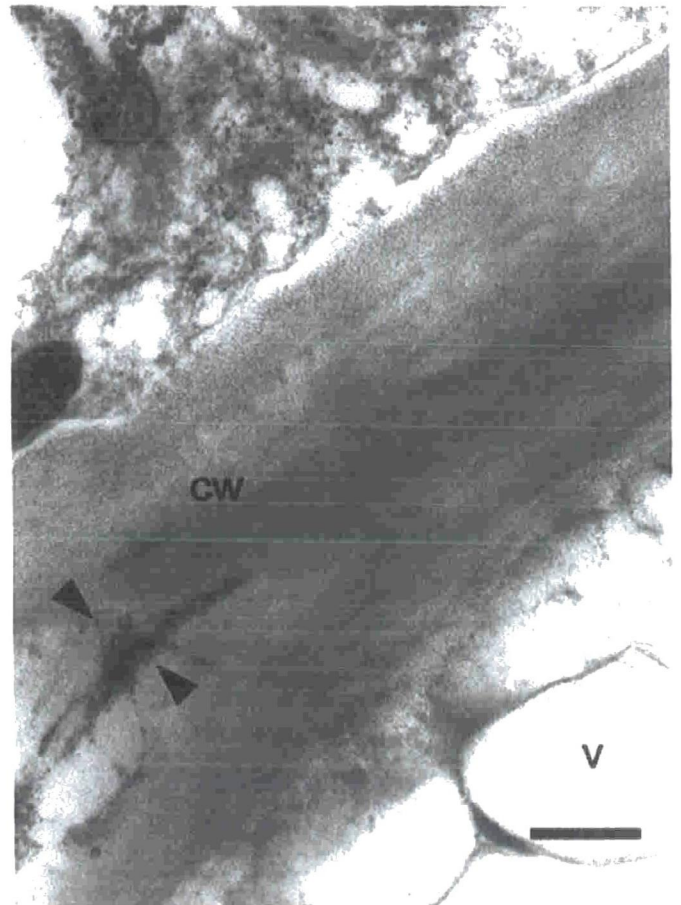


a

Figure 26 : Microfibrils arrangement in the cell wall of young and old laticifers.

A: Young laticifers cell wall shows microfibrils were arranged quite loose. CW=cell wall, er=endoplasmic reticulum, v=vacuole. Bar = 5 $\mu$ m.

B: Old laticifers cell wall shows microfibrils were arranged more compact compare to the younger laticifers (b). Arrows shows electron dense material in the cell walls. Bar = 1 $\mu$ m.



b

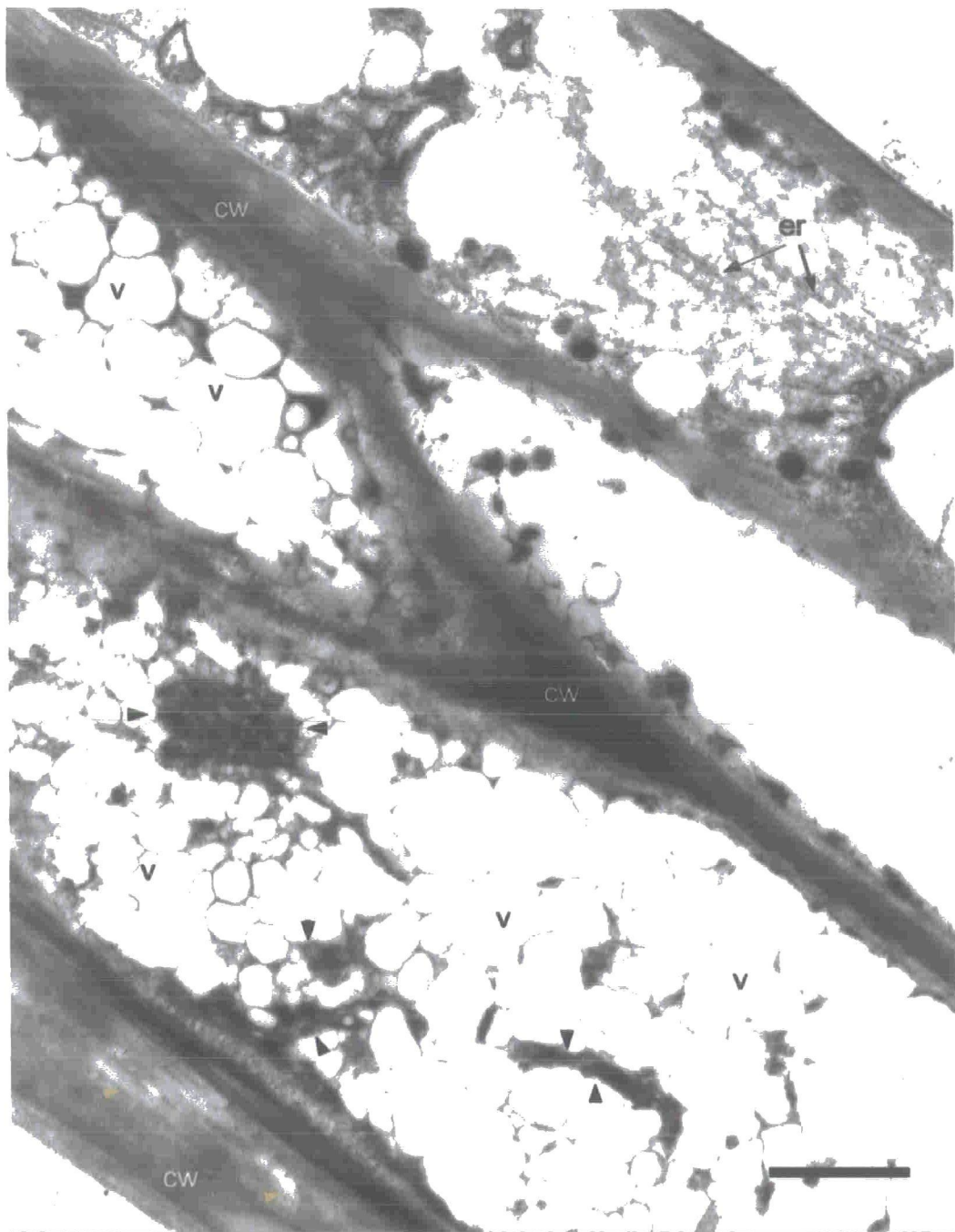


Figure 27: Electron dense organelles in old *Meconopsis* stem, fixed with  $\text{OsO}_4$ . Arrows show electron dense organelles amongst vacuoles. That could probably be part of endoplasmic reticulum and many heavily loaded small vesicles. The vacuoles were so compact that there were hardly any other organelles could be observed in the laticifers. Note that arrangement of microfibrils of the cell wall at certain places were quite loose and cell wall degradation were also noticeable. ( ). V=vacuoles, cw=cell wall,. Bar=  $4.0\mu\text{m}$ .

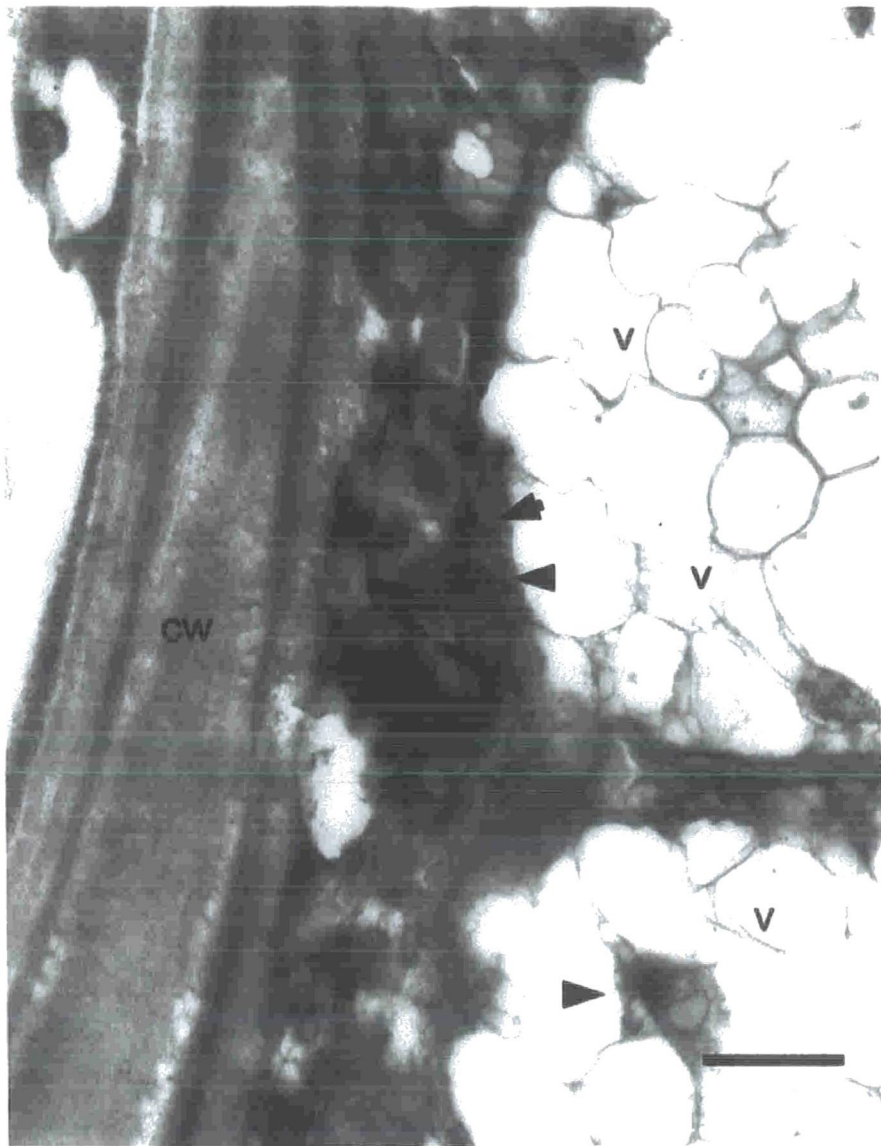


Figure 28: Laticifers in old *Meconopsis* stem, containing vacuoles and electron dense substances. Vacuoles were tightly packed to each other. Electron dense substance (arrows) was clumped near cell wall, which is most probably the endoplasmic reticulum. The cell wall (microfibrils) were quite loose. CW=cell wall, v=vacuoles. Bar=2.0 $\mu$ m.

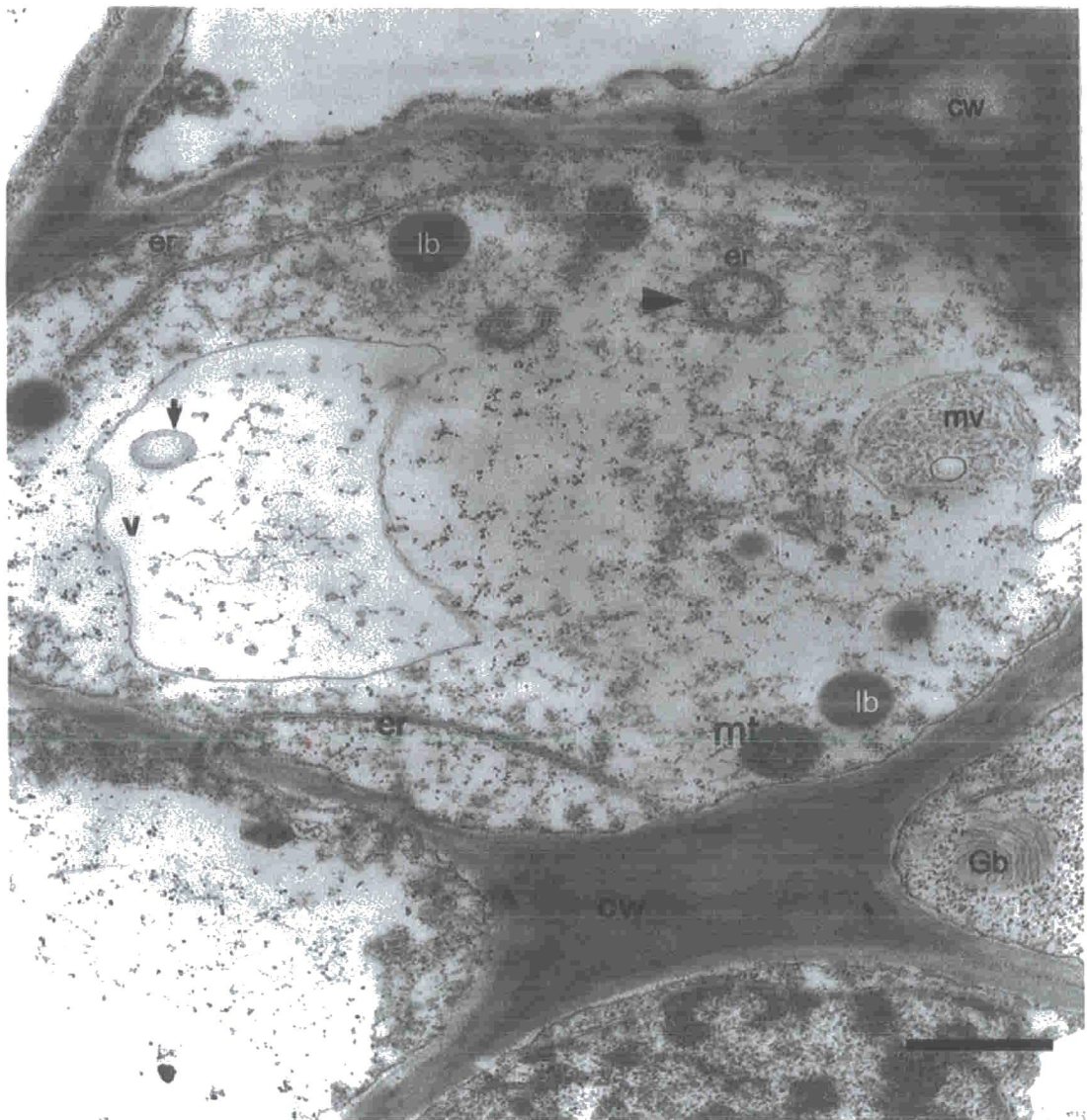


Figure 29: Laticifers of young *Meconopsis* stem fixed with ZIO. Notice one big vacuole occupying the laticifers with some organelles within it. There were net-like structure and globule (→) inside the vacuole. There was also some rough endoplasmic reticulum with a lot of ribosomes attached. One particular arrangement of er was observed (▶) in a circular form. Multivesicular body (mv) were present in cell, which probably related to loss of cytoplasmic structural integrity. V=vacuoles, er=endoplasmic reticulum, cw=cell wall, Gb=Golgi bodies, lb=lipid body, mt=mitochondria, mv=multivesicular body. Bar= 5.6µm.

young laticifers. There was also smooth endoplasmic reticulum with ribosomes still attached to them, as well as suspended in the cytoplasm. Some other organelles were also observed such as Golgi bodies (Gb), lipid bodies (lb), and mitochondria (mt).

The electron micrograph of a laticifer cell wall (figure 30) shows at least three layers of wall with different microfibril orientation. In this sample (an older sample) there was barely any para-mural vesicles observed (Figure 30) as compared to the younger sample (figure 25). The middle layer of the cell wall looked more compact compared to the outer layer, where the microfibrils were bound closely together and very densely packed. The microfibrils in the outward layer of the walls were packed loosely. Endoplasmic reticulum was stained very dark with ZIO fixative.

### **3.1.3.3 Samples fixed with Glutaraldehyde; embedded in LR White**

Both osmicated samples embedded in Araldite showed good ultrastructural preservation. However with LR White embedding medium, the quality of the structures were less than satisfactory. Figure 31a&b showed laticifers in old *Meconopsis* stem, embedded in LR White. It was very clear from the observation that the vacuole membrane seemed to be broken and looked quite porous or loose. There were no other organelles present in the laticifers. Cell walls of the laticifers were preserved quite well.

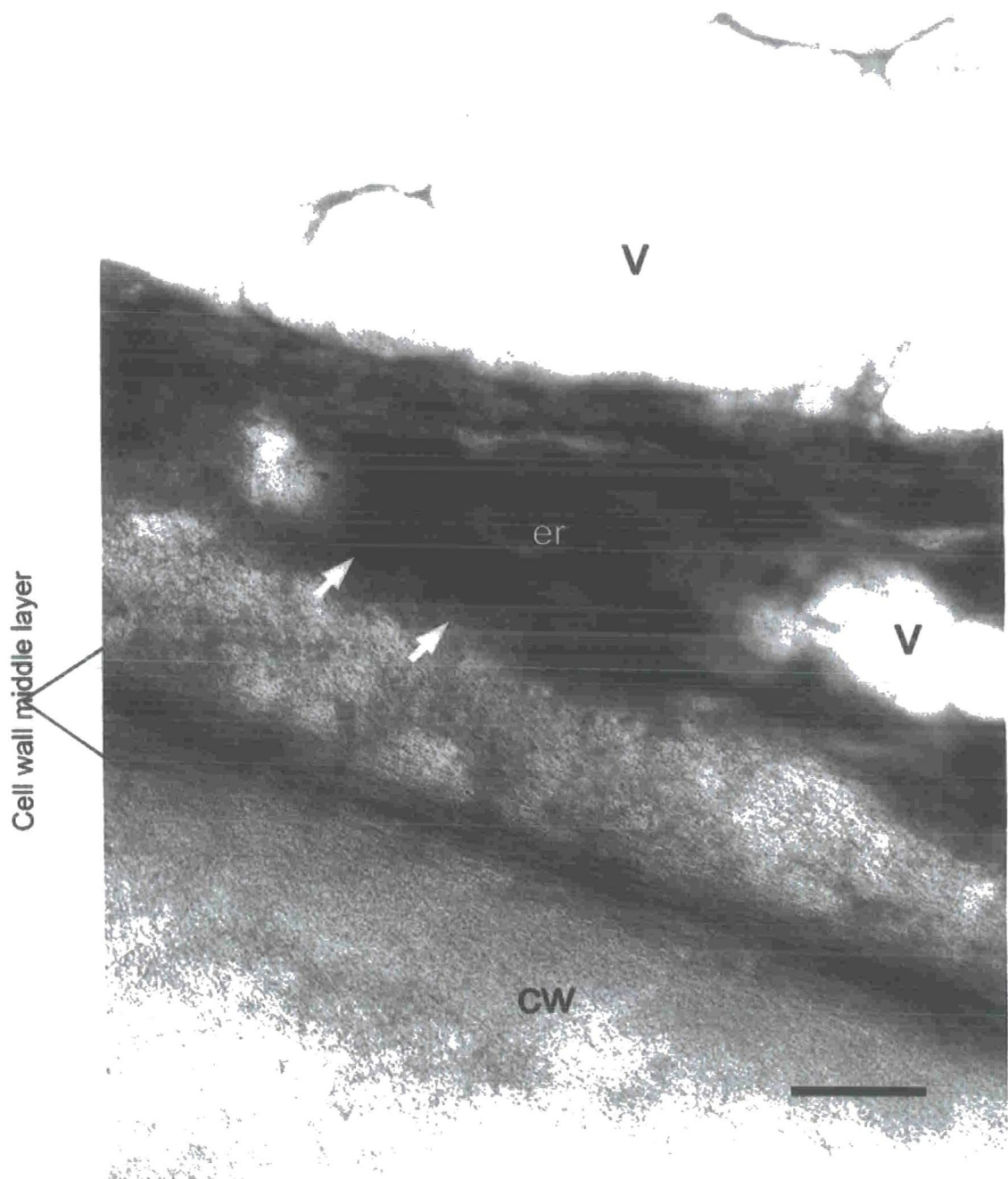


Figure 30: Cell wall of old *Meconopsis* stem fixed with ZIO. The cell wall contained microfibrils that were more compact in the middle compared to the outward region of the wall. The microfibrils in both sides (outward region) were loosely arranged. There were no para-mural vesicles in the old samples. However electron dense masses were very prominent (arrows). V=vacuoles, cw=cell wall, er=endoplasmic reticulum. Bar=0.7 $\mu$ m.

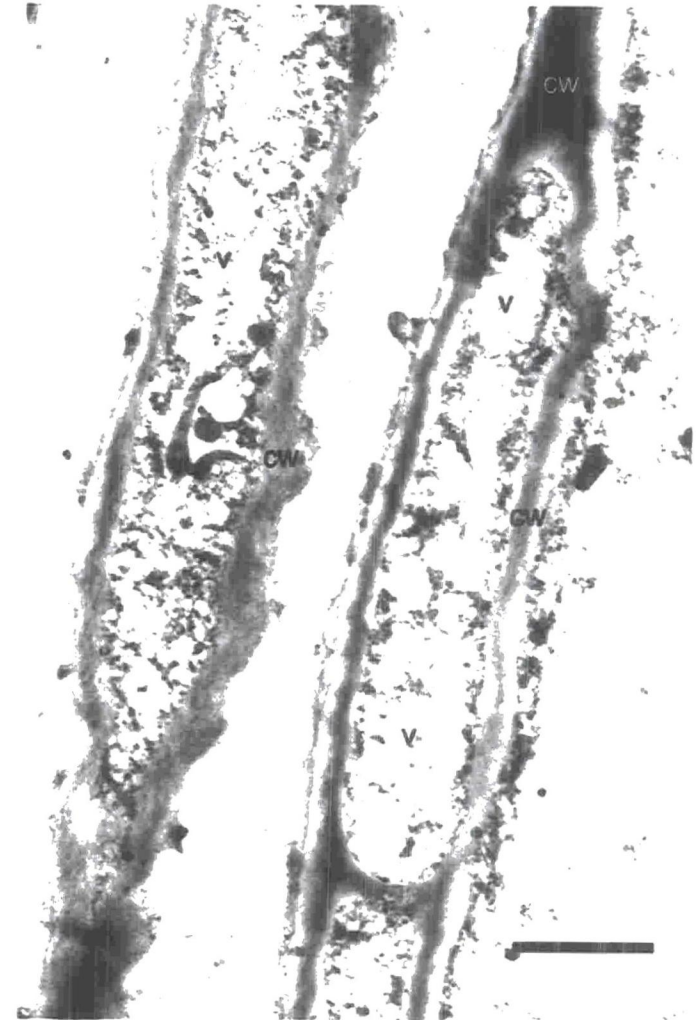
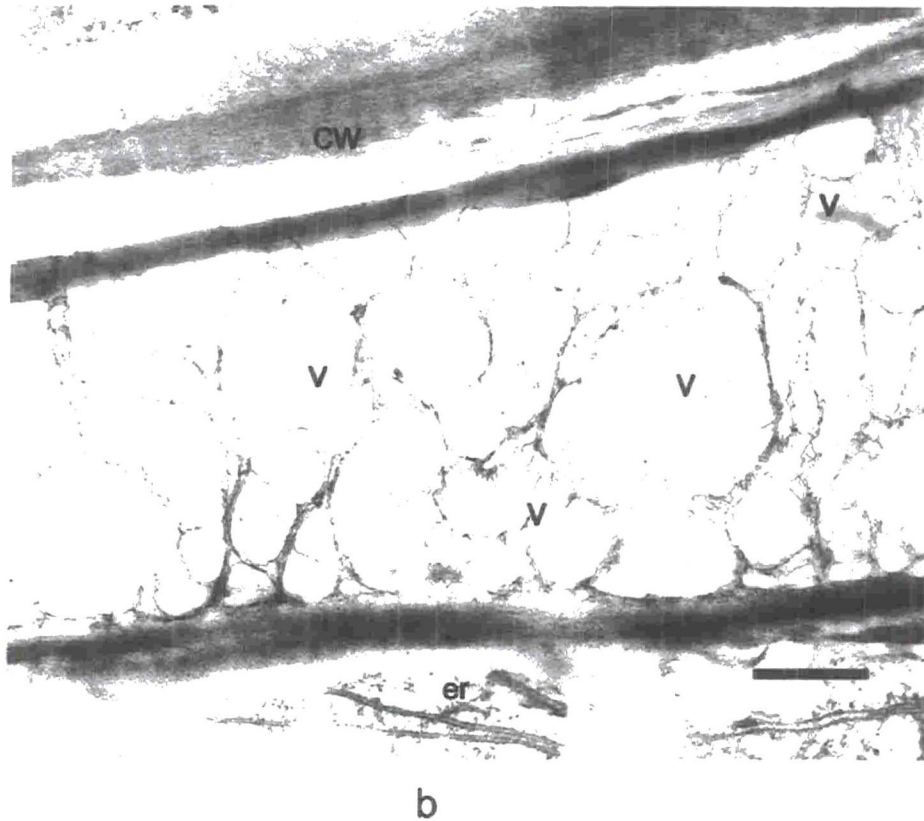


Figure 31 a&b: Laticifers structure in young & old Meconopsis stem embedded in LR White.  
 Vacuoles were observed to show some disfiguration. There were no other organelles visible. cw=cell wall, v=vacuole, er=endoplasmic reticulum  
 Bar (a) = 0.6 $\mu$ m, Bar (b) = 4.4 $\mu$ m.

a

## **3.2 *Papaver rhoeos* (Papavaraceae)**

### **3.2.1 Effect of different embedding media and fixatives regimes on the distribution and structure of laticifers**

#### **3.2.1.1 Fresh samples stained with Calcofluor**

Figure 32 showed *Papaver rhoeos* has a similar arrangement of laticifers in the stem tissue as compare to *Meconopsis*. Freshly cut flower stem also showed that latex was exuded from cells in the cambial region which, is again very similar to *Meconopsis* (figure 1). Initially it was thought that latex might possibly ooze out from the xylem. But there was no further evidence to support this assumption. This assumption was further dismissed, as xylem was fully differentiated and dead cells.

The technique on the other hand revealed the location of laticifers quite well, if not obscured by the latex smearing and smudging the xylem during cutting, hence gave a misleading observation. Laticifers were located in the cambial region of the samples and they could be seen *in situ* clearly when observed under bright field illumination.

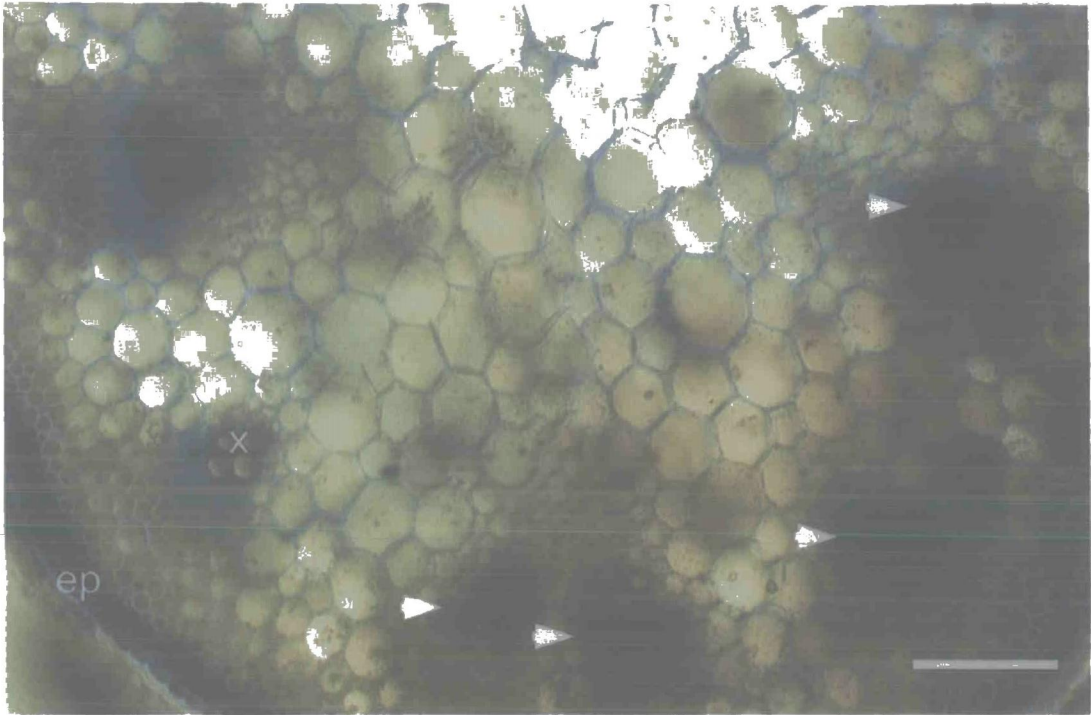


Figure 32: A hand cut section of fresh young *Papaver* stem. The sample was viewed under the light microscope (BF/UV). Laticifers were located in and around the vascular bundle region. Arrows indicated latex smears that came out from the laticifers when the section was made. The latex seemed to block the xylem cells. X=xylem, ep=epidermis. Bar = 180  $\mu$ m.

### **3.2.1.2 Samples embedded in LR White resin**

Semithin sections of young *Papaver* stem, embedded in LR White resin show a row of irregular sized cells, compared with other surrounding cells; presumed to be laticifers in the cambial region of the vascular bundle (fig. 33). Vacuoles were not visible in those cells. Even though xylem is composed of dead cells and should have no cytoplasm, few xylem vessels looked as if they had undergone cell plasmolysis (arrows). Figure 34 of a longitudinal section of an old sample, showed the laticifers extending in parallel with the xylem. In some places (arrow), the cell walls of the laticifers were seen to swell. Structural preservation seemed to be good.

### **3.2.1.3 Samples embedded in Araldite resin**

Laticifers could clearly be seen containing latex in the semithin sample of old *Papaver* impregnated in zinc iodide (Fig. 35). The laticifers were arranged in one row of cells in the cambial region of the vascular bundle. It is quite interesting to note that in this sample, particularly in a few of the xylem cells (arrow), structures resemble latex as in the laticifers were very prominent. This result is similar to the result observed in the fresh sample where latex was first thought to be exuded from the xylem. Figure 36, of the old sample fixed with osmium tetroxide further confirms the previous suspicion on the observation that latex-like structures were present in the xylem.

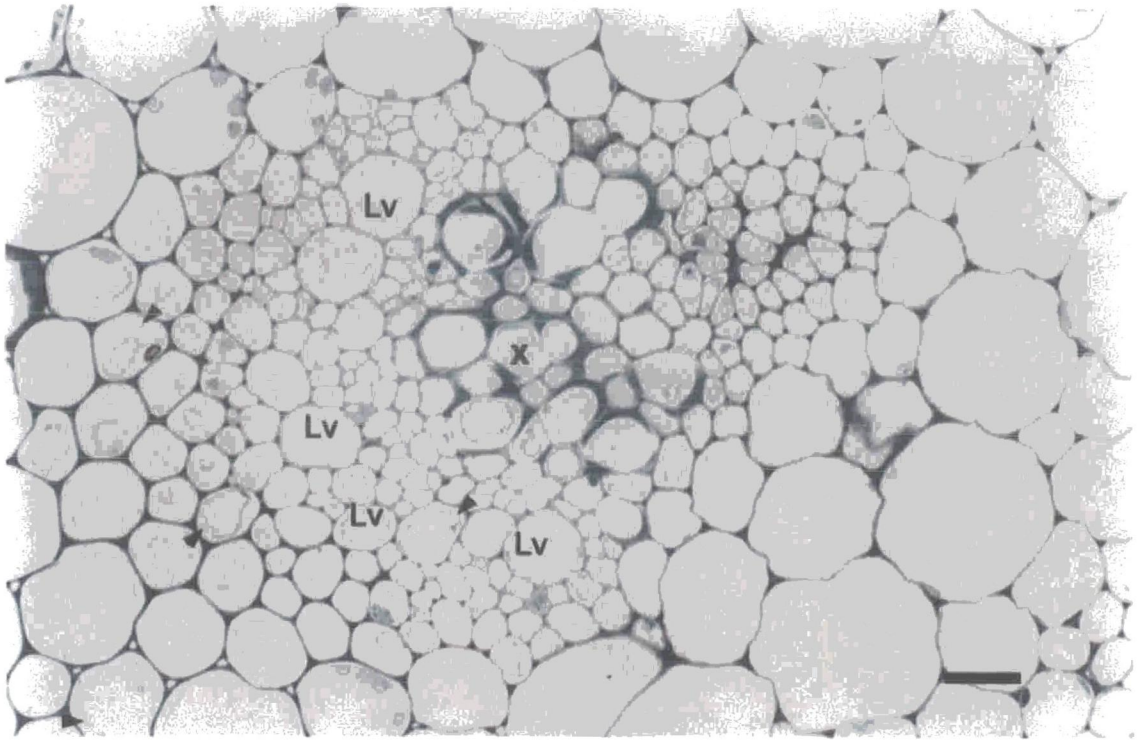


Figure 33: A cross section of young *Papaver* stem in LR White. Laticifers were located in the cambial region of the vascular bundle with distinct irregular shapes. However, the laticifers seemed to be empty. Cell plasmolysis was quite apparent in most cells including the laticifers (arrows). Lv=laticifers, x=xylem. Bar = 20  $\mu$ m

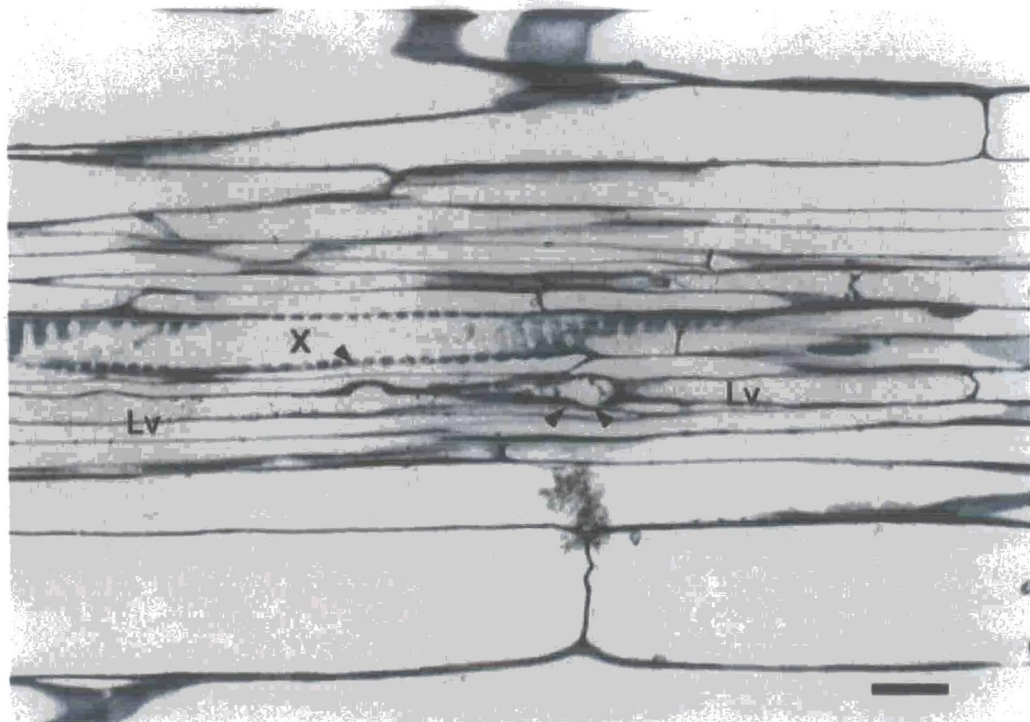


Figure 34: A longitudinal section of young *Meconopsis* stem in LR White. The laticifers run in parallel with the xylem. The cell walls of the laticifers were observed to be swollen (arrows). Lv=laticifers, x=xylem. Bar = 20  $\mu$ m.

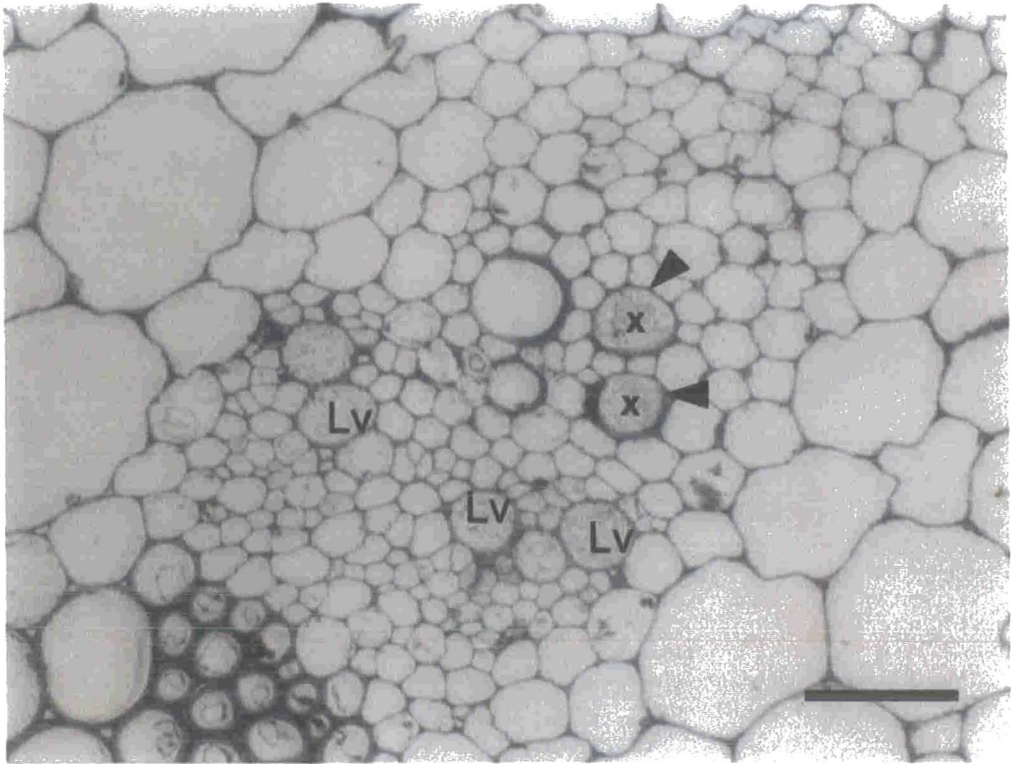


Figure 35: Semithin section of old *Papaver* stem, showing the laticifers with vacuoles. Laticifers are located in the cambial region. Two xylem cells (arrow) contained some structure that resembled vacuoles. Cells plasmolysis was quite evidence, especially in the parenchyma cell. Lv=laticifers, x=xylem. Bar = 30  $\mu$ m.

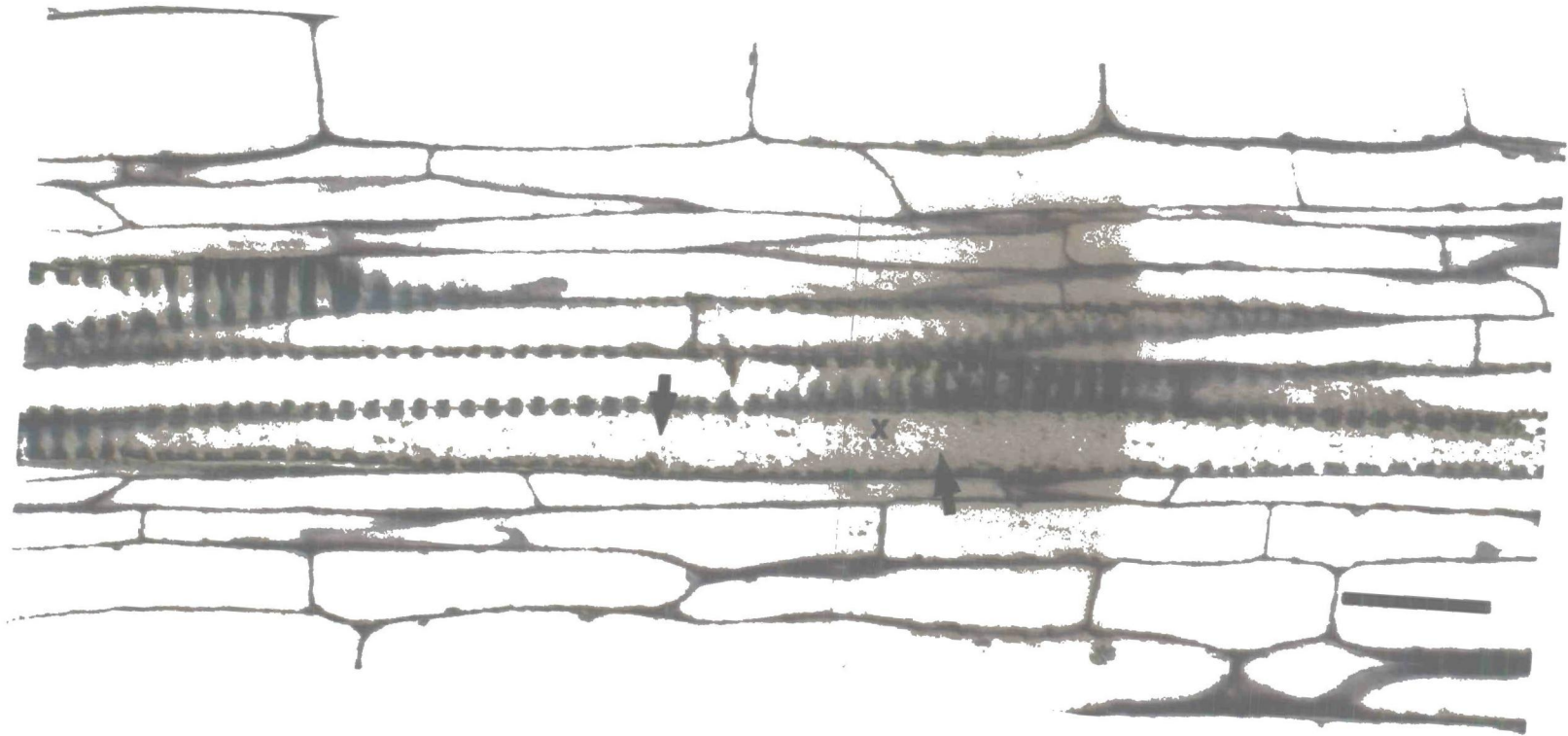


Figure 36: A longitudinal section of old Papaver stem fixed with osmium tetroxide. Xylem seems to be occupied with structures that look like vacuoles (arrows). x=xylem. Bar = 40  $\mu\text{m}$ .

#### **3.2.1.4 Samples embedded in Spurr resin**

The Spurr embedded sample showed the same evidence of granular substance present in the xylem. Figure 37, of the old sample impregnated with zinc iodide, showed that laticifers were located in a single ring around the cambial region. The photograph also showed the evidence of laticifer cells dissolved and two adjacent laticifers joining together to form a bigger laticiferous cell (arrow). Figure 38 of the longitudinal section, showing a young sample impregnated with zinc iodide supported the observation from the TS sample (figure 33), that two adjacent cells merged at a point where the cell wall disintegrated (arrows). Cell wall disintegration was also noticed in several locations along the cell walls of the laticifers cells.

#### **3.2.1.5 Ultrastructure observation on the development of the articulated laticifers in *Papaver rhoeas***

Cell wall degradation has been observed in light micrographs of both species i.e. *Meconopsis* and *Papaver*. Figure 39 shows a laticifer cell in the stem of young *Papaver*. It is quite obvious from the micrograph that the cell wall of the laticifers break down in several places (indicated by arrows). However, it is quite difficult to determine whether the process was due to enzymatic reaction or sectioning artifact. On the other hand, there was the evidence that enzyme might have involved in loosening the cell wall's microfibrils, as shown in figure 40. The cell wall looks thinned and there is a gap between the cell wall and plasmalemma (arrows). The evidence can also be seen in figure 41 & 42. It was quite interesting to note that electron dense substances were most frequently, if not

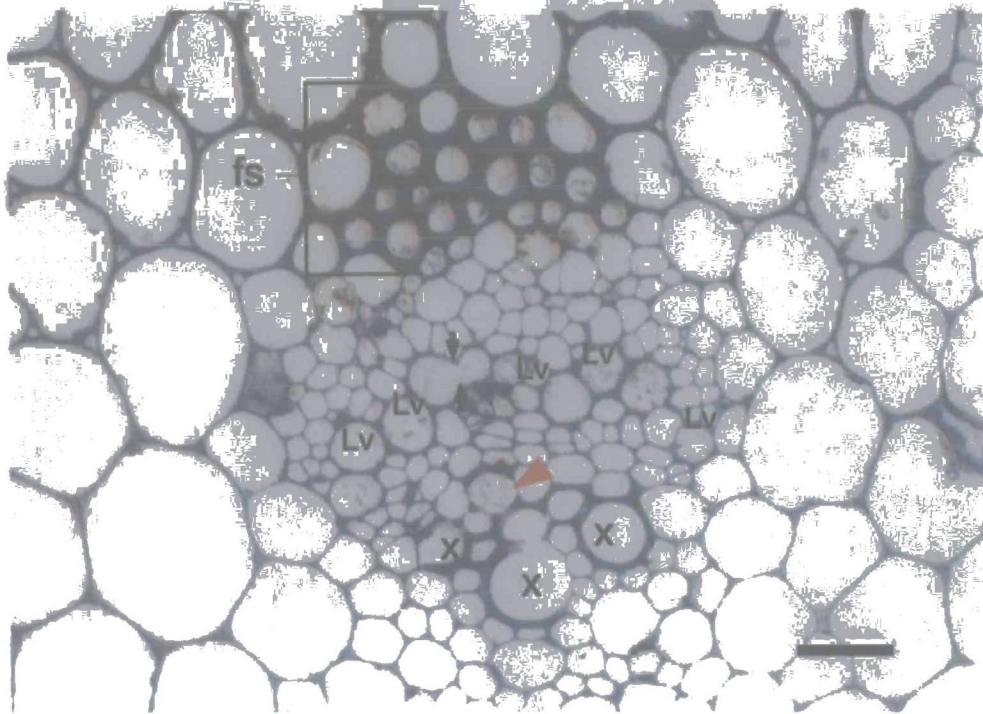
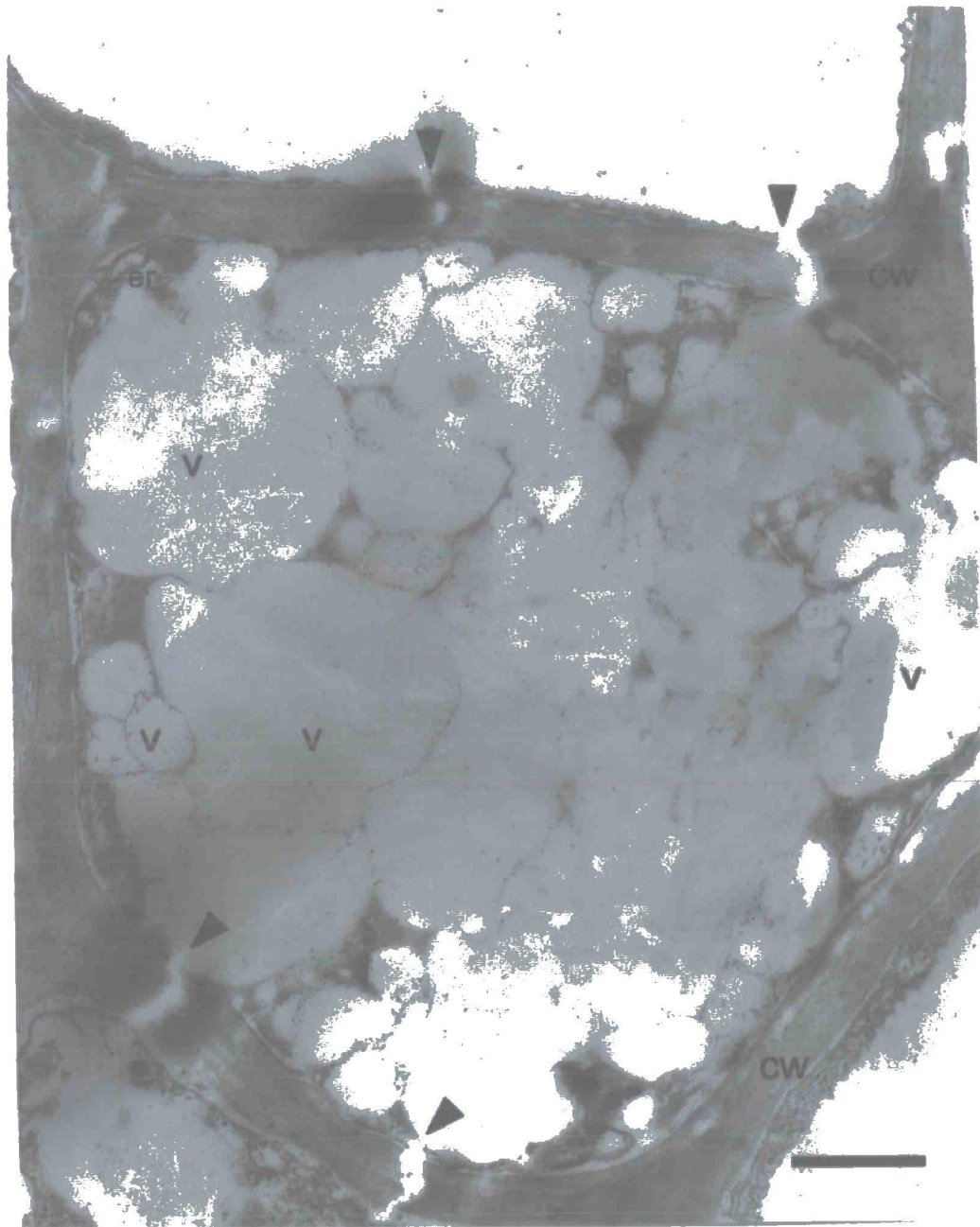


Figure 37: Laticifer arrangement in a cross section of old *Papaver* stem embedded in Spurr. Laticifers can be seen filled with vacuoles and arranged in a single row in the cambial region. Arrows show two laticifers joined after the cell wall has ruptured. Note one particular xylem vessel also contained granular substance ( ▶ ).  
Lv = laticifers, x= xylem, fs=fibre sclereid. Bar = 30  $\mu$ m.



Figure 38: Cell wall ruptured in a laticifer of young *Papaver* stem embedded in Spurr. Arrows show places where the cell walls of laticifers ruptured and joined to form bigger laticiferous cell.  
Lv= laticifers, x=xylem. Bar = 30 $\mu$ m.





**Figure 39: Laticifers in *Papaver* stem. Cell wall were broken at several places (arrows). Cw= cell wall, v= vacuoles. Bar = 2.0  $\mu$ m.**

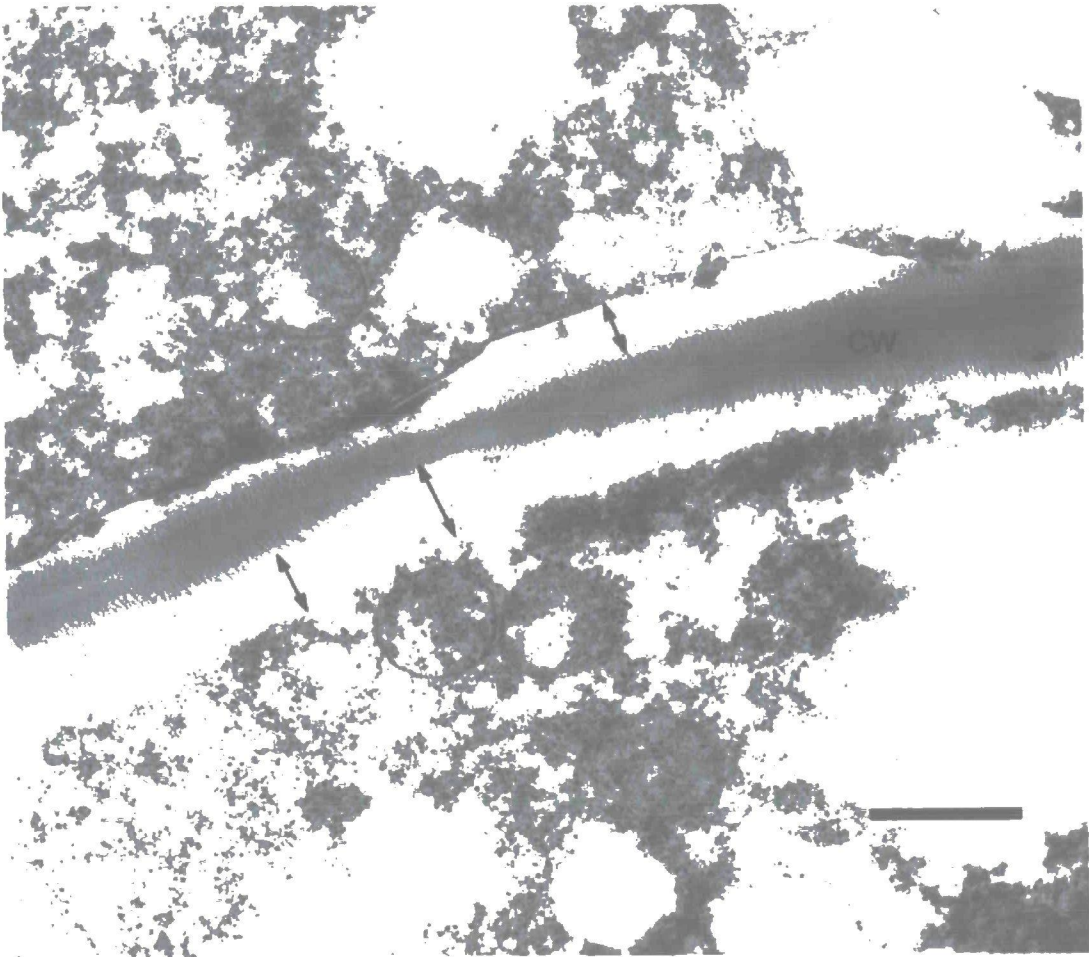
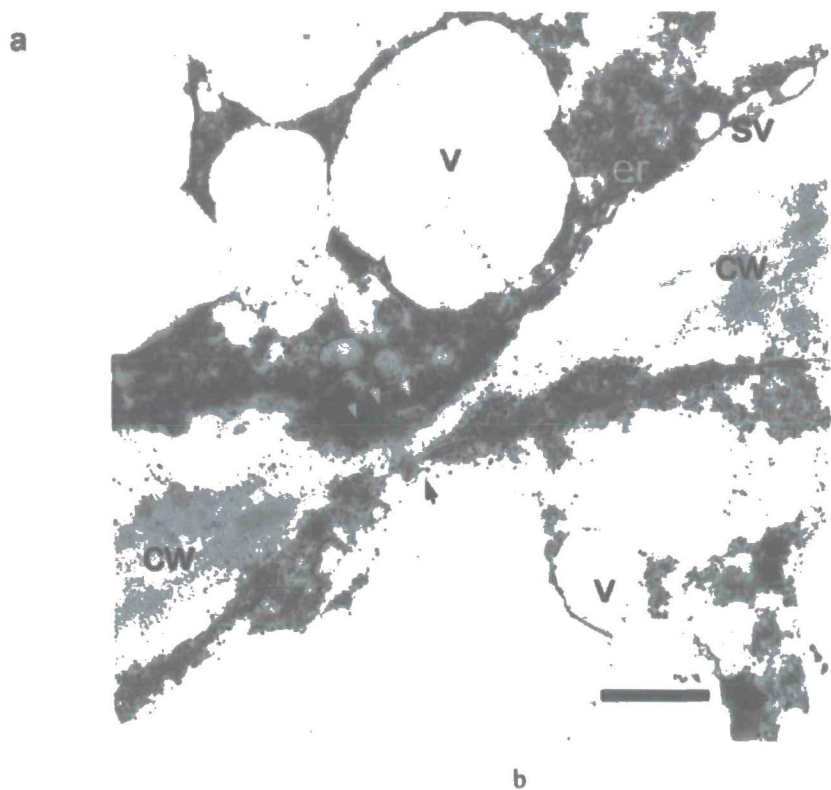
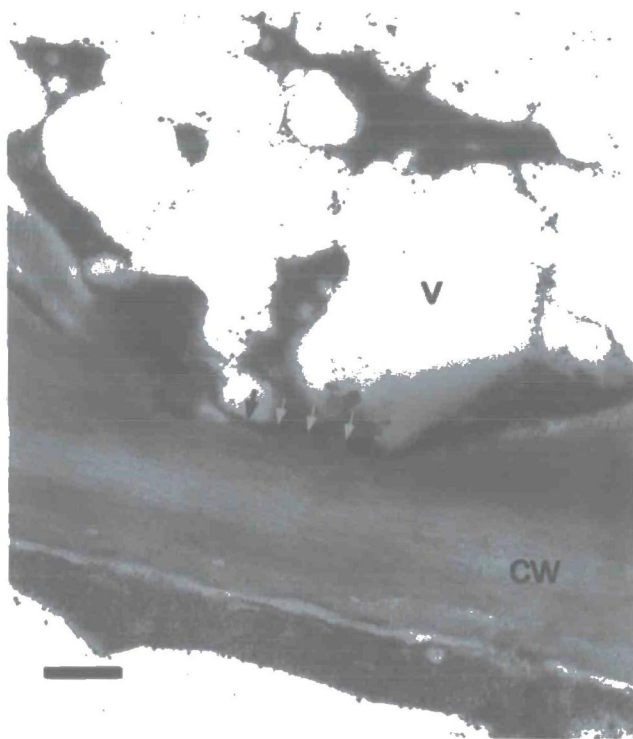


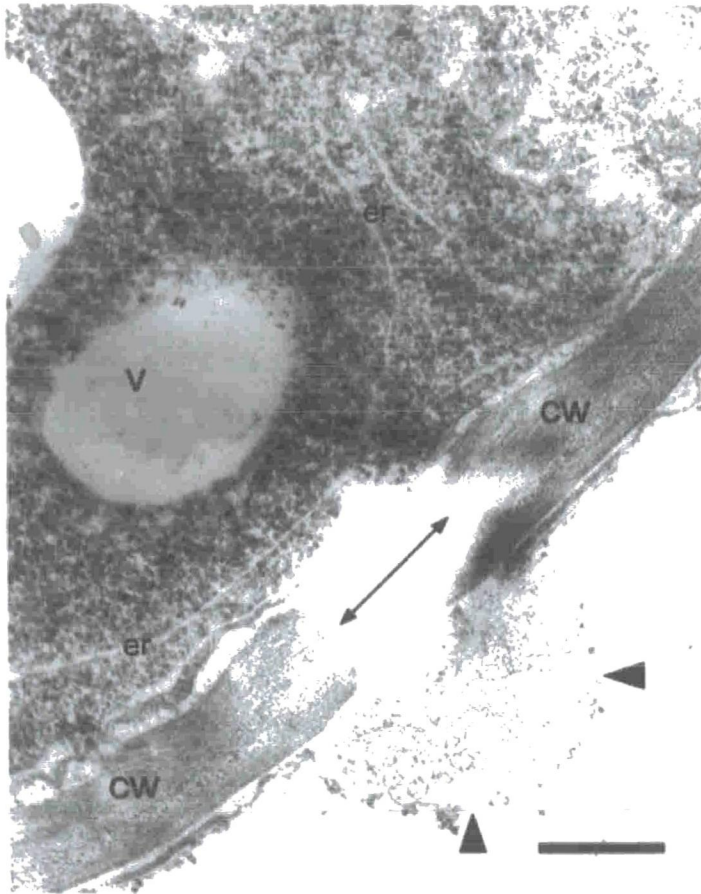
Figure 40: Gap between cell wall and plasmalemma. Arrows indicate the gap present in between the cell wall and the plasmalemma of the laticifers cw= cell wall. Bar= 2.0 $\mu$ m.



**Figure 41: Cell Wall of the Laticifers possibly becoming thinner by enzyme activities.**

**A:** Arrows indicate the place where the cell wall has become thinner. Electron dense substance is quite prominent nearby. v=vacuoles, cw=cell wall. Bar = 1.0µm

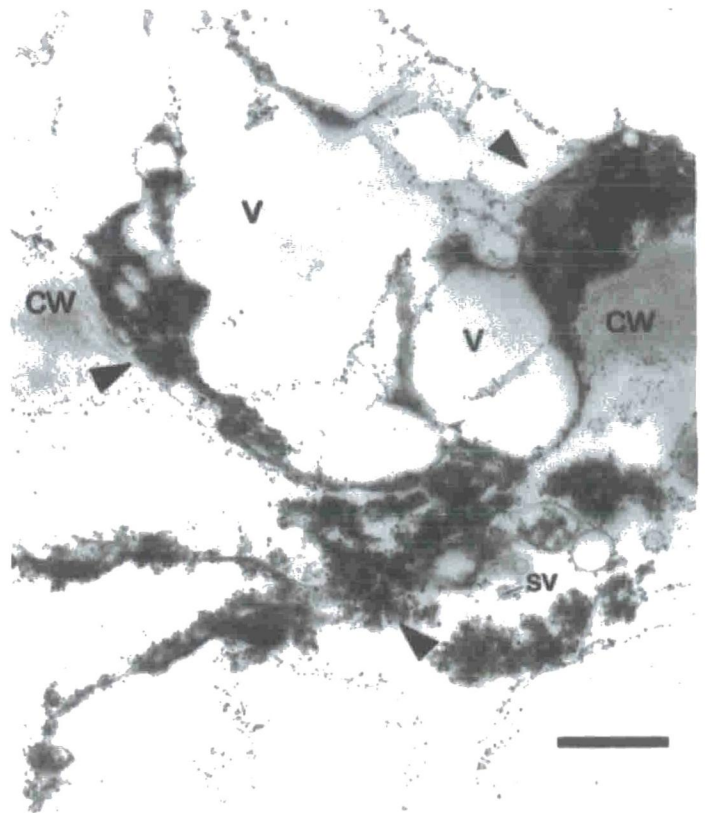
**B:** Small vacuoles concentrated near the point where the cell wall is thinnest. Arrows indicate the place where the enzymatic process possibly took place. v=vacuoles, cw=cell wall, sv=small vesicle, er= endoplasmic reticulum. Bar = 2.0µm



a

Figure 42: Cell wall of the laticifers ruptures and breaks into two sections.

A: Cell wall totally separated into two parts, leaving a big gap in between. (  $\longleftrightarrow$  ). Arrows indicate cell wall microfibrils residue. v=vacuoles, cw=cell wall, er=endoplasmic reticulum. Bar= 0.34 $\mu$ m



b

B: Electron dense substances (arrows) and vacuoles migrating into the neighbouring cell. V=vacuoles, cw=cell wall, sv=small vacuoles. Bar = 2.0 $\mu$ m

always, located and concentrated amongst with small vacuoles near the point where the cell wall became thinner.

### **3.3. *Hevea brasiliensis* (Euphorbiaceae)**

#### **3.3.1 A study of the distribution and structure of laticifers in different part or the plant using different staining reagents.**

##### **3.3.1.1 Fresh samples stained with ANS: Stem**

All parts of the plants oozed out a very viscous white and sticky latex when they were cut. The latex turned from white to yellowish colour after a period of time.

Fresh samples stained with the ANS gave a greenish colour under an epifluorescence microscopy using BV filter. Figure 43 showed fresh hand-cut sections of *Hevea* stem. There is hardly any evidence of laticiferous cells visible or fluorescing even though other tissues like xylem and fibre sclereids stain or fluoresce very well. Even though the laticifers did not stain well with this reagent, it was known that the laticiferous cell should be in between the xylem (x) and the fibre sclereids (fs). Latex or other compounds in the laticifers did not fluoresce or pick up the stain as well.

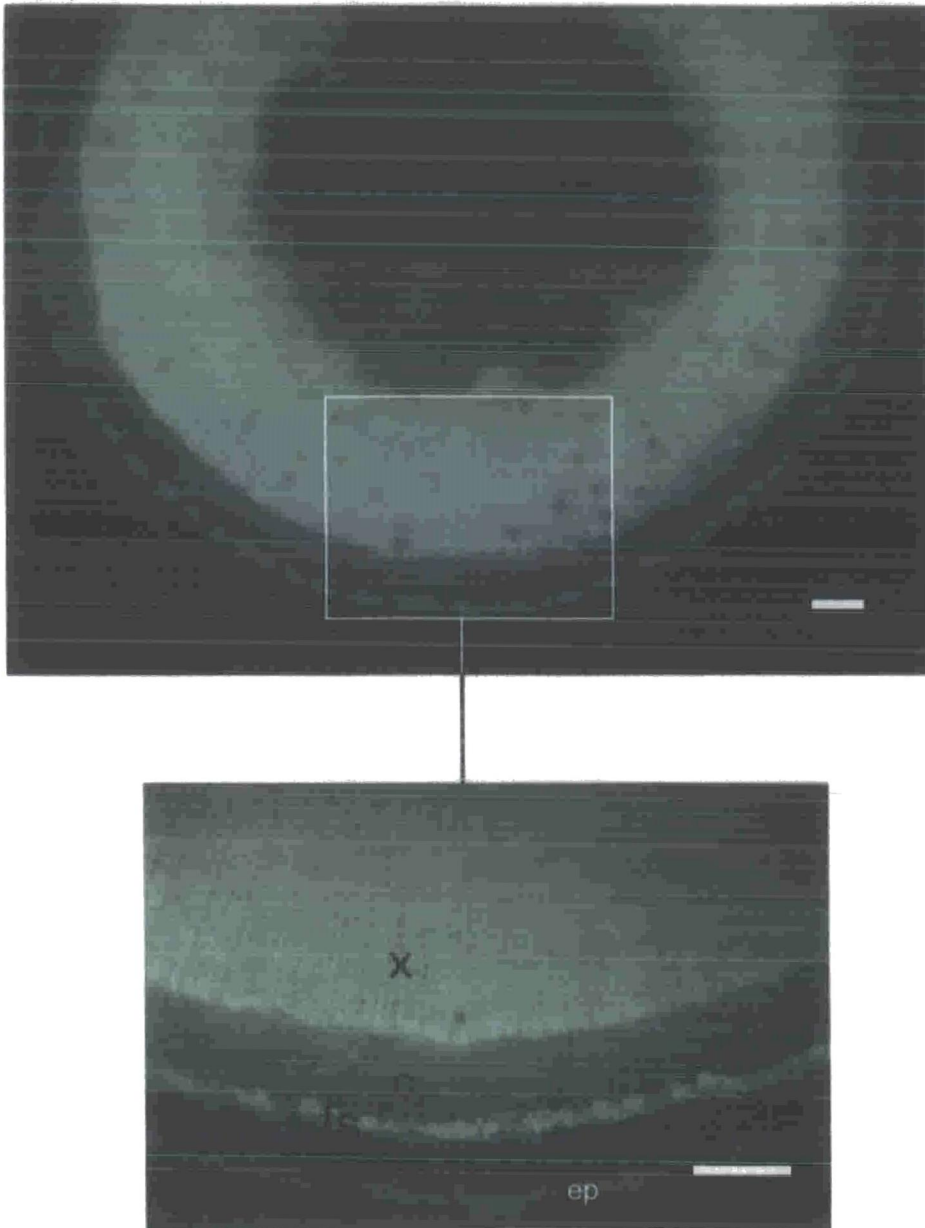


Figure 43: Cross section of hand-cut *Hevea* stem; stained with ANS. Results show that only lignified cells such as xylem and stone cells were stained well with ANS. Laticifers or latex did not stain well with this staining method. X=xylem, c=cambial region, fs=fibre sclereid, ep=epidermis Bar = 200  $\mu$ m.

### **3.3.1.2 Fresh samples stained with ANS: Leaf cotyledon**

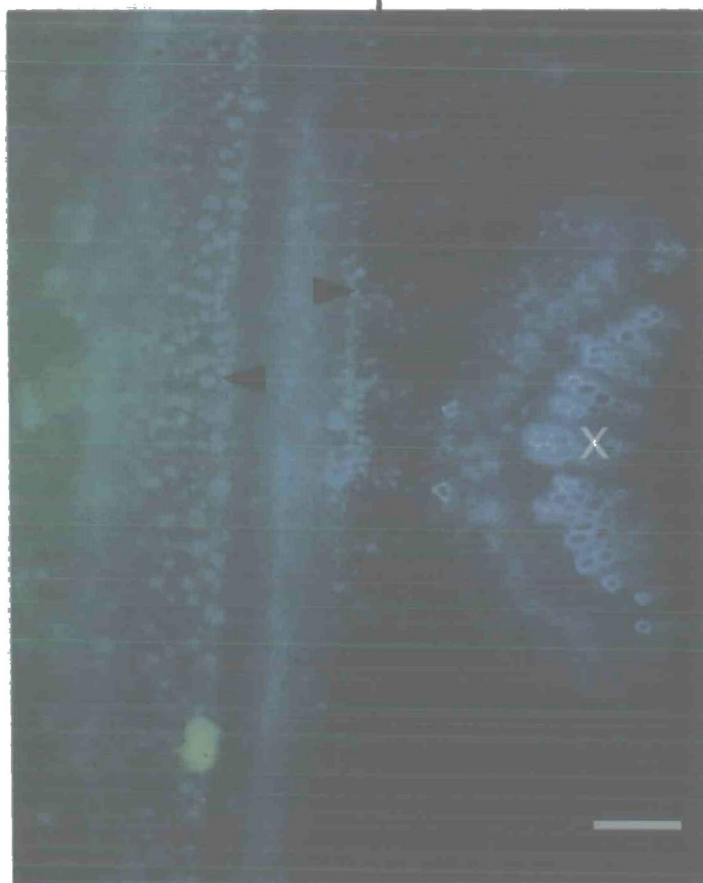
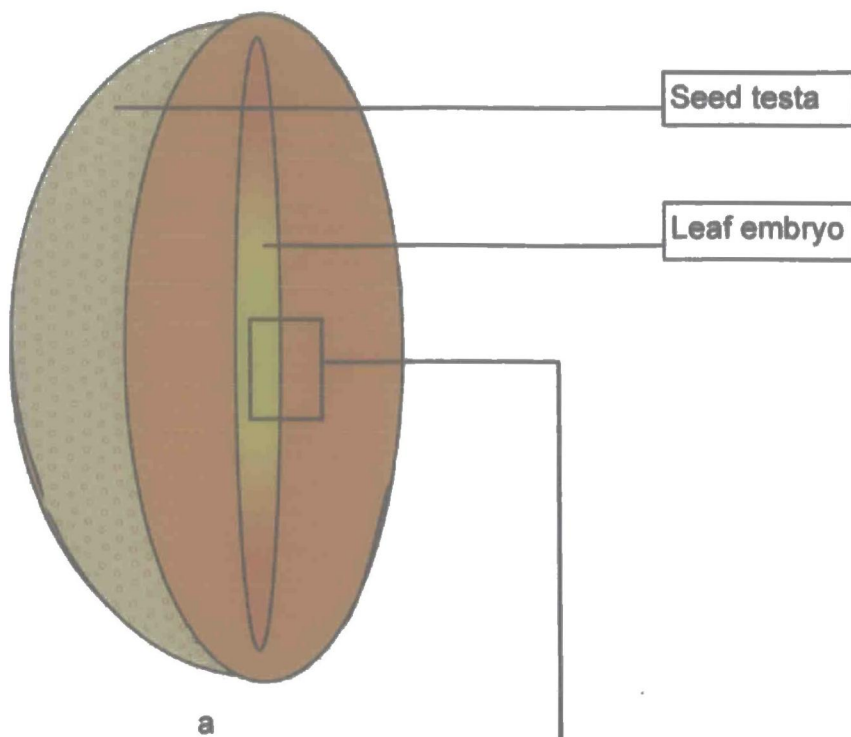
*Hevea* cotyledon showed abundant traces of latex in the laticiferous cell area (figure 44). The latex fluoresced along the vascular system of the cotyledon. The latex fluoresced as bright as the xylem vessels, compared with the other part of the samples. These results also showed that latex vessels are not distributed exclusively alongside the axis of the vascular system. There was also some distribution of the laticifers in the outer side of the vascular bundle.

### **3.3.1.3 Fresh samples stained with ANS: Root**

Hand-cut sections of fresh *Hevea* roots showed similar results to those observed in stem. Figure 45 shows that laticifers in the samples do not stain very well with the reagent. Despite that however, latex in the laticifers stained slightly (arrows) and can be seen located in the cambial region of the sample. Xylem cells were observed to fluoresce very noticeably. There was no trace of sclerenchyma cell (secondary thickening) present in the root sample obviously because of its function as opposed to the stem (supporting cells).

### **3.3.1.4 Fresh samples stained with ANS: Petiole**

Figure 46 shows a fresh, hand-cut section of *Hevea* petiole. Even though latex can be observed in the cambial region or in between the xylem and the



b

Figure 44: Fresh sample of *Hevea* cotyledon; stained with ANS. (a) Diagrammatic figure showed the place of sampling from the seed of *Hevea*. (b) Latex stained quite clearly in this sample (arrows), almost as much as the xylem vessels. It was clear that laticifers are located around the vascular bundle and on the outer side of the vascular system (in the cambial region). X=xylem. Bar = 200  $\mu$ m.

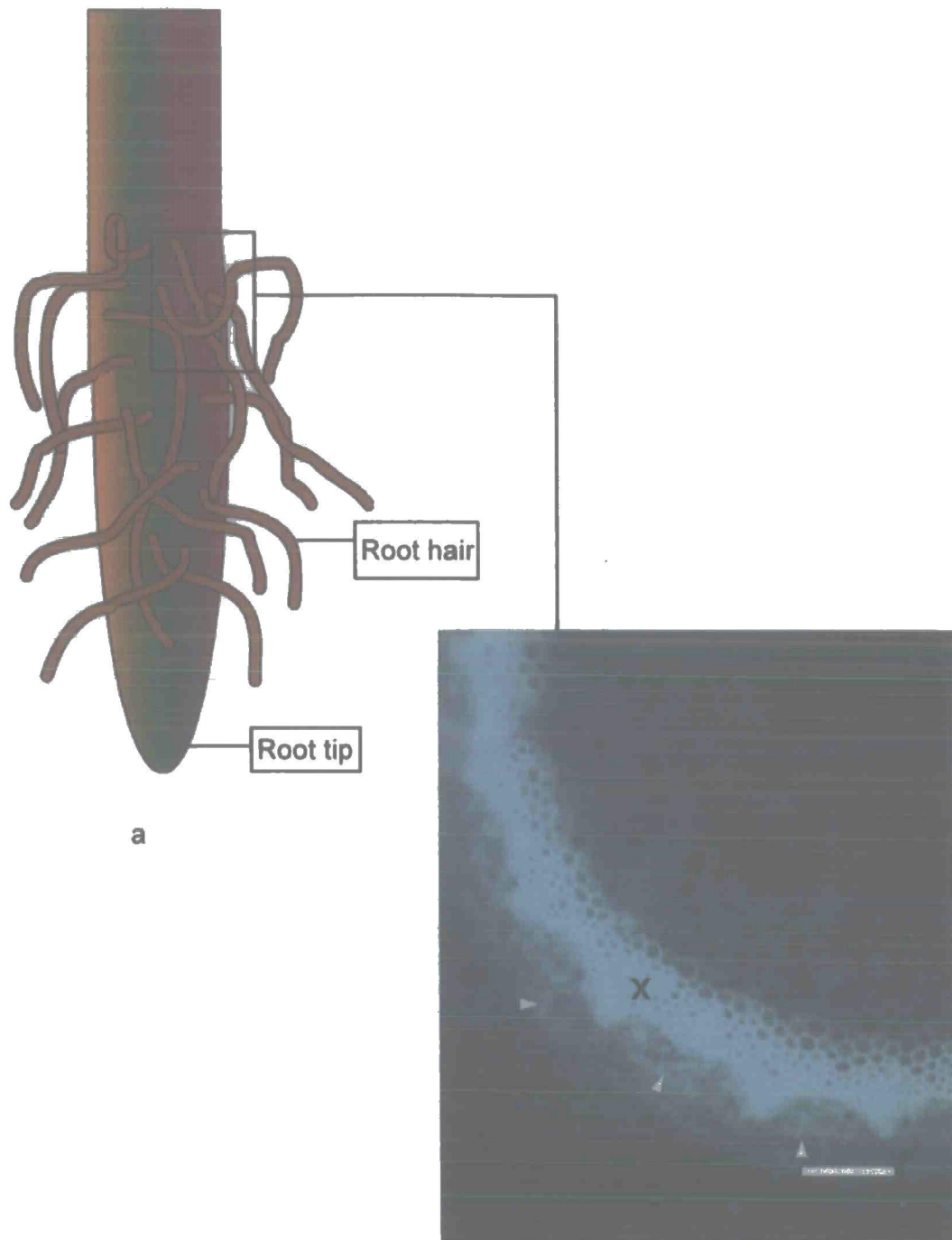


Figure 45: Freshly hand-cut *Hevea* root; stained with ANS. (a) A diagrammatic figure showed the place of sampling in *Hevea* root. (b) Xylem stained properly with ANS. Latififers were however, stained slightly (arrows) but not as bright as the xylem. X=xylem, Bar = 190  $\mu$ m.

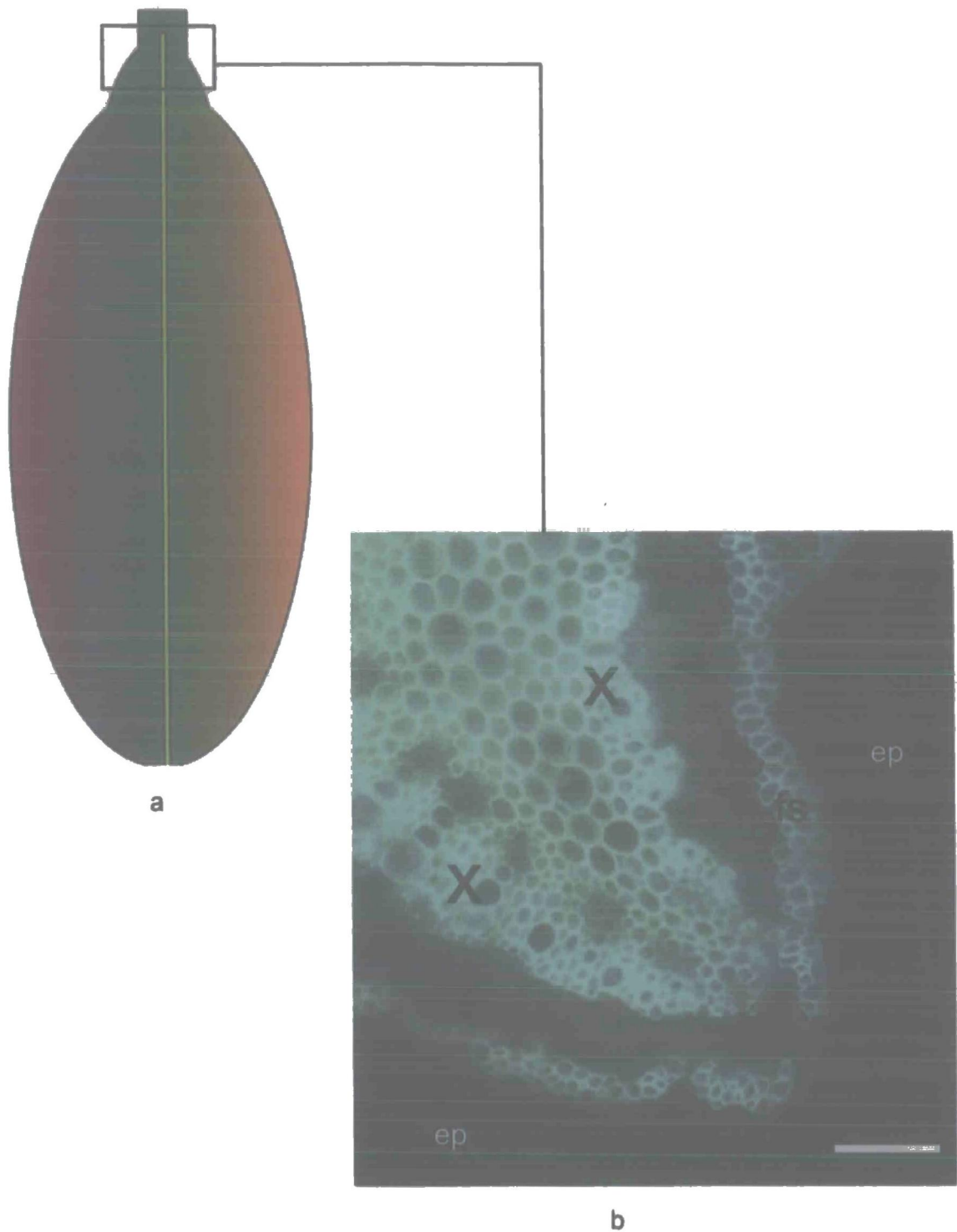


Figure 46: Cross section of *Hevea* petiole; stained with ANS. (a) A diagrammatic figure of *Hevea* leaf show the place of sampling. (b) Photograph shows that xylem and stone cells fluoresced very brightly. Laticifers and latex however were quite difficult to distinguished but pick up the stain slightly ; and fluoresced very weakly (arrows). X=xylem, fs=fibre sclereid, ep=epidermis. Bar = 200 $\mu$ m.

sclerenchyma cells, however the latex (arrows) did not fluoresce brightly as compared to the xylem and the fibre sclereids.

### **3.3.1.5 Fresh samples stained with ANS: Leaf**

Staining epidermis-cleared leaves with ANS clearly revealed the distribution of veins and veinlets in *Hevea* leaves (Figure 47). However there was no evidence of the laticiferous system or latex vessels present in the photographs, partly because either the latex itself did not stain well, or if it was stained then probably it was obscured by the xylem cells, which was presumably sandwiched the laticiferous cells, since this was not a sectioned sample.

### **3.3.1.6 Fresh samples stained with Acridine orange: Stem**

Figure 48 (a) showed a cross section of *Hevea* stem stained with acridine orange. The thickened and lignified wall of xylem and fibre sclereids fluoresced with an orangish colour. There was hardly any evidence of laticiferous cells or latex that pick up the stain. The results showed here were very similar to those samples stained with the ANS, where it was quite difficult to clearly observe the location of laticifers. Figure 48 (b), of a longitudinal section, shows sclerenchyma cells and xylem fluorescing very well. The cambial region, where the laticifers would normally be found, shows no evidence of its appearance.

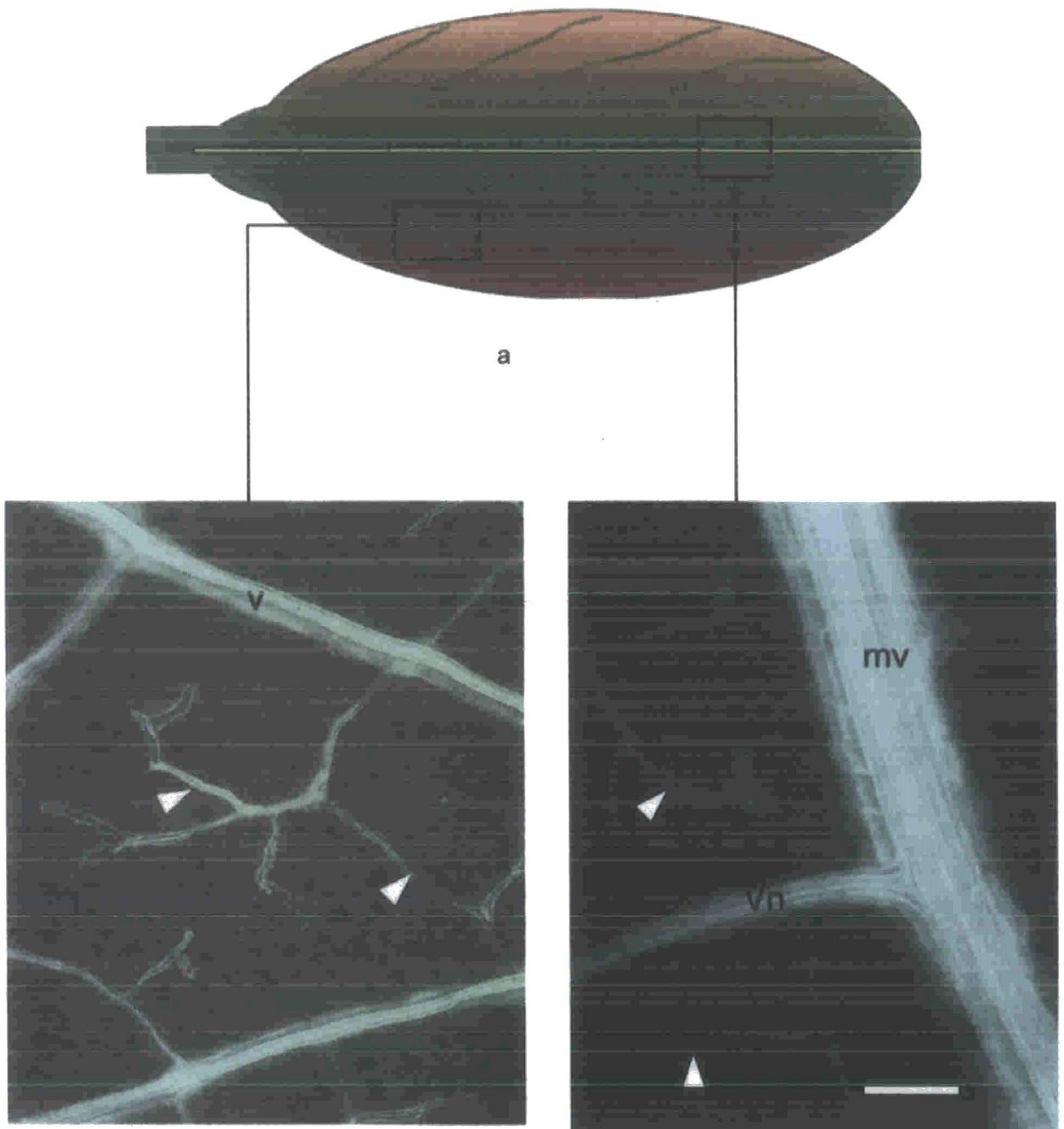
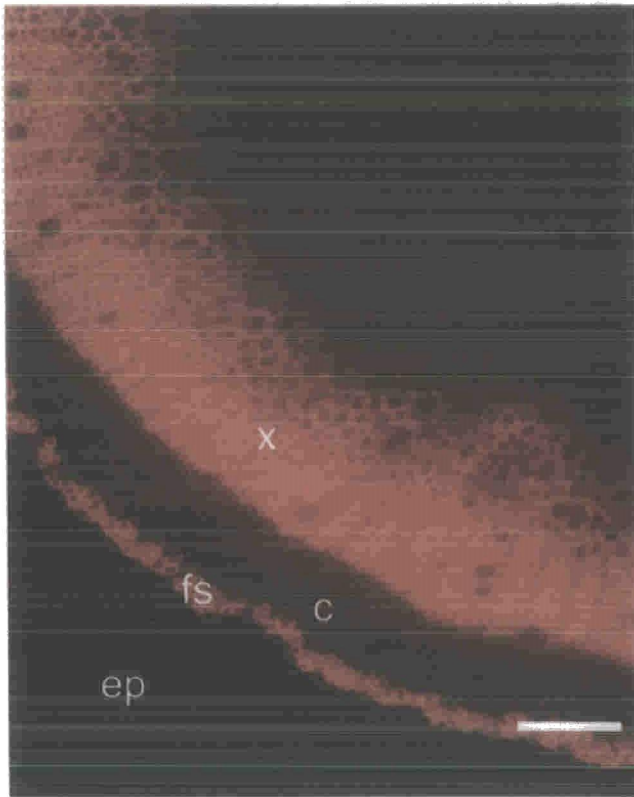


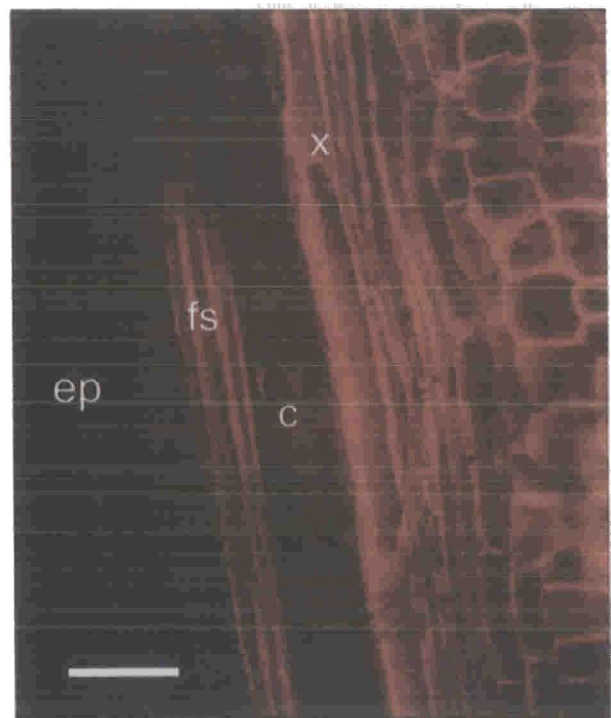
Figure 47: Cleared leaves of *Hevea* stained with ANS. (a) A diagrammatic figure of *Hevea* leaf showing the place of sampling. (b) ANS stained the vascular bundle very well. Even the small veinlets were clearly shown in the photographs. However, laticifers or latex did not stain very well in this sample. Vn=vein, mv=mivein, v=veinlet, Bar = 200  $\mu$ m.



a

Figure 48 (a): A cross section of *Hevea* stem, stained with acridine orange. Xylem vessels and fibre sclereid were stained brightly. Laticifers or latex did not pick up the stain very well. X=xylem, fs=fibre sclereid, c=cambial region, ep=epidermis. Bar = 190  $\mu\text{m}$ .

Figure 48(b) : A longitudinal section of *Hevea* stem, stained with acridine orange. Xylem and fibre sclereids were stained very well with this stain, as in figure 49. There was no laticifers or latex visible in this sample. X=xylem, fs=fibre sclereid, c=cambial region, ep=epidermis. Bar = 30  $\mu\text{m}$ .



b

### **3.3.1.7 Fresh samples stained with Acridine orange: Cotyledon**

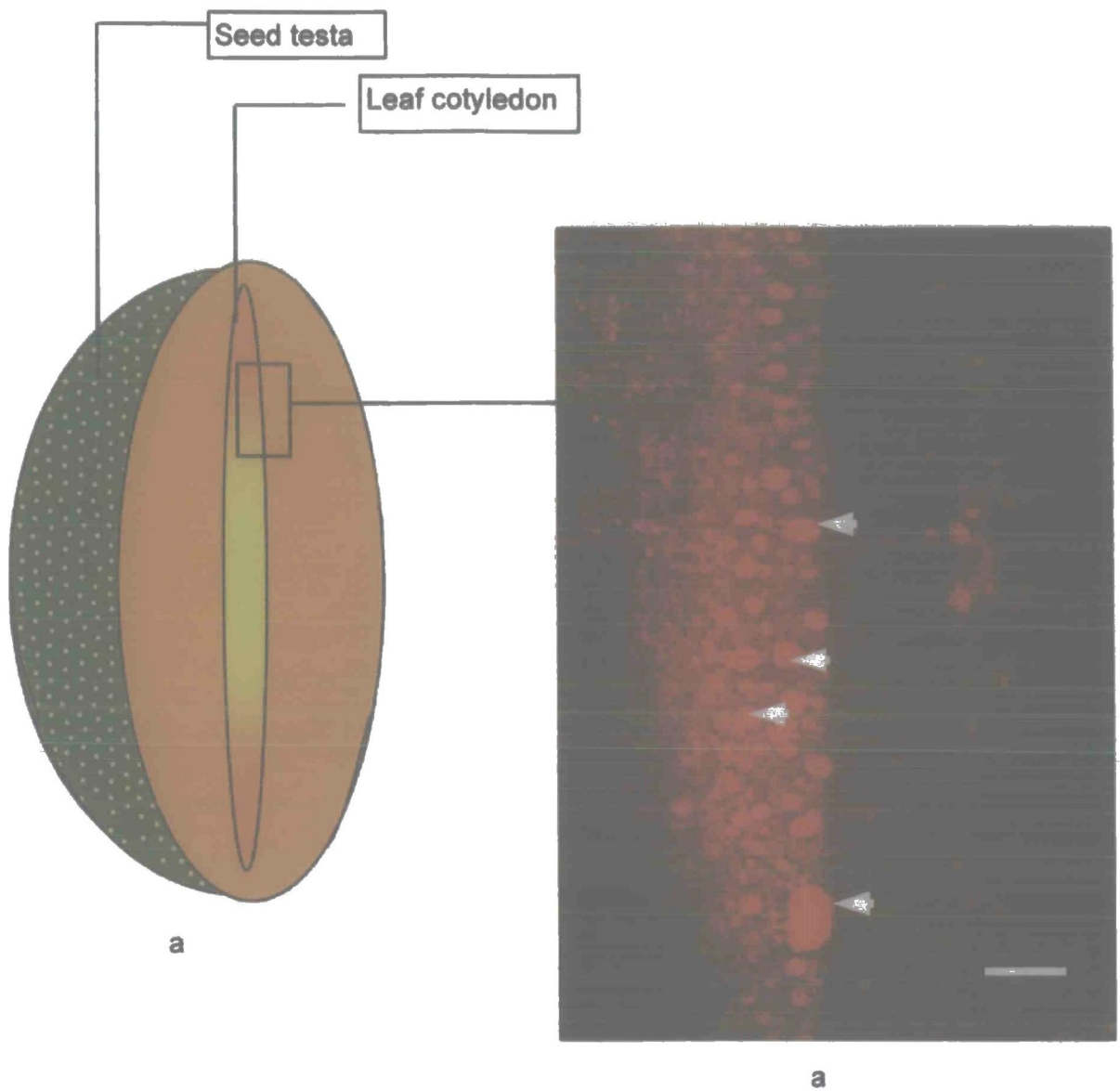
It is interesting to note that in the cross section of *Hevea* cotyledon as shown in figure 49, it appears that latex stained with Acridine orange quite well, revealed as distinguishable orangish dots along the axis of vascular tissues. This result was similar to those stained with the ANS (figure 44). It was obvious from the result that there was no other cells stained (i.e fibre sclereids).

### **3.3.1.8 Fresh samples stained with Acridine orange: Petiole**

In *Hevea* petiole, as shown in Fig. 50, latex or latex vessels did not stain well in the tissues. The tissue where the latex vessels were supposed to be located (arrows), were not stained at all. The pattern showed similar results to previous samples of *Hevea* where only lignified cell walls fluoresced.

### **3.3.1.9 Fresh samples stained with Acridine orange: Root**

Figure 51 shows a typical *Hevea* root, stained with Acridine orange. The stain created an orangish colour to the xylem area. Latex or laticifers were not stained in the tissue. The result showed here were almost identical to the one stained with the ANS (figure 45).



**Figure 49: Cross section of *Hevea* cotyledon stained with acridine orange.**  
**(a) A diagrammatic figure of *Hevea* seed showing the sampling area.**  
**(b) Even though the outline of the laticifers cells were not clear, however latex stained and fluoresced brightly. There is no lignified cell yet present in this stages. Bar = 200 $\mu$ m.**

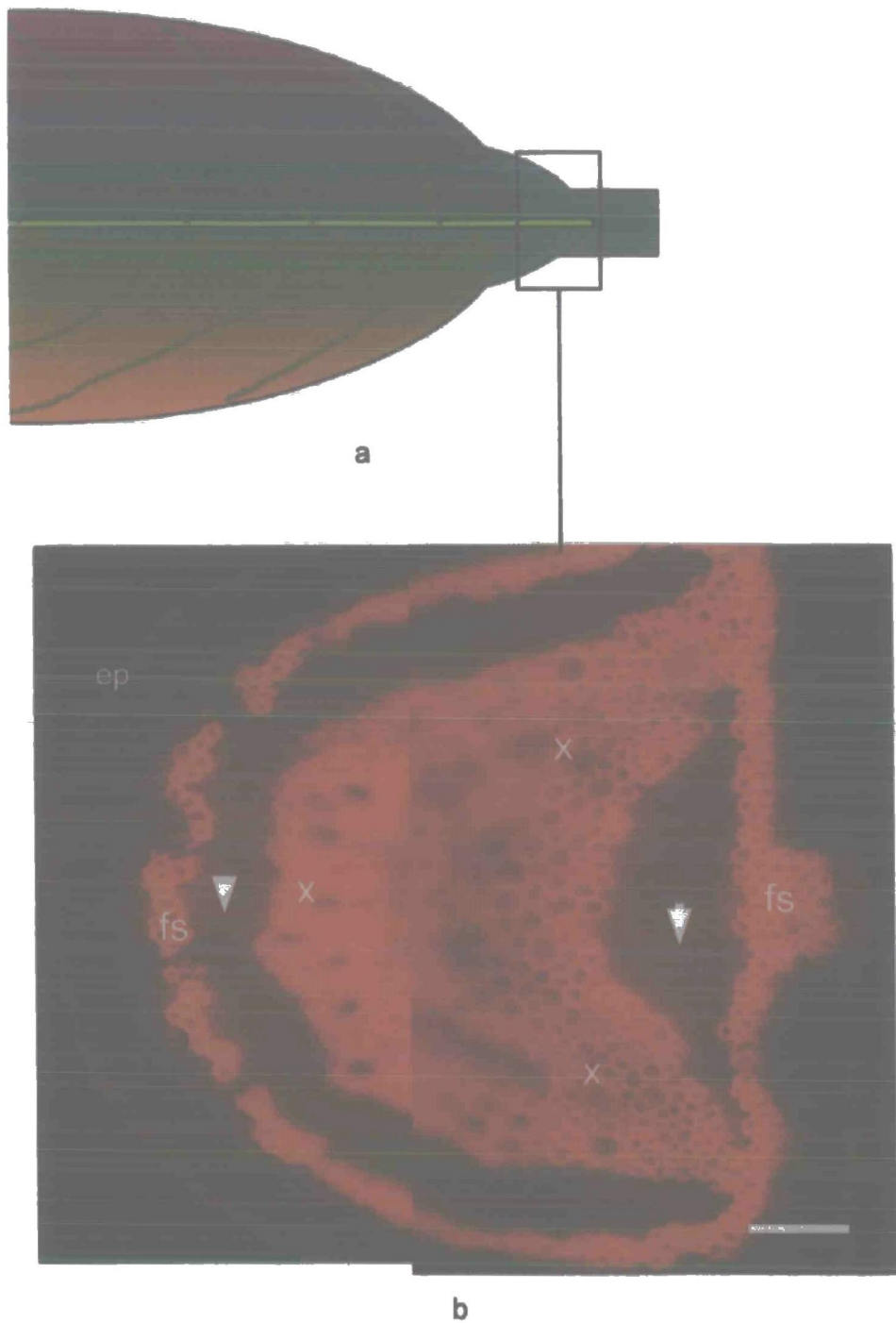


Figure 50: *Hevea* petiole stained with Acridine orange. (a) A diagrammatic figure of *Hevea* petiole, showing the place of sampling. (b) Lignified cells were stained brightly orange by the stain. Arrows indicate the location where the laticifers were expected to be present. Fibre sclereid (fs) are very prominent in the tissues. X=xylem, fs= fibre sclereid, ep=epidermis. Bar = 190 µm.

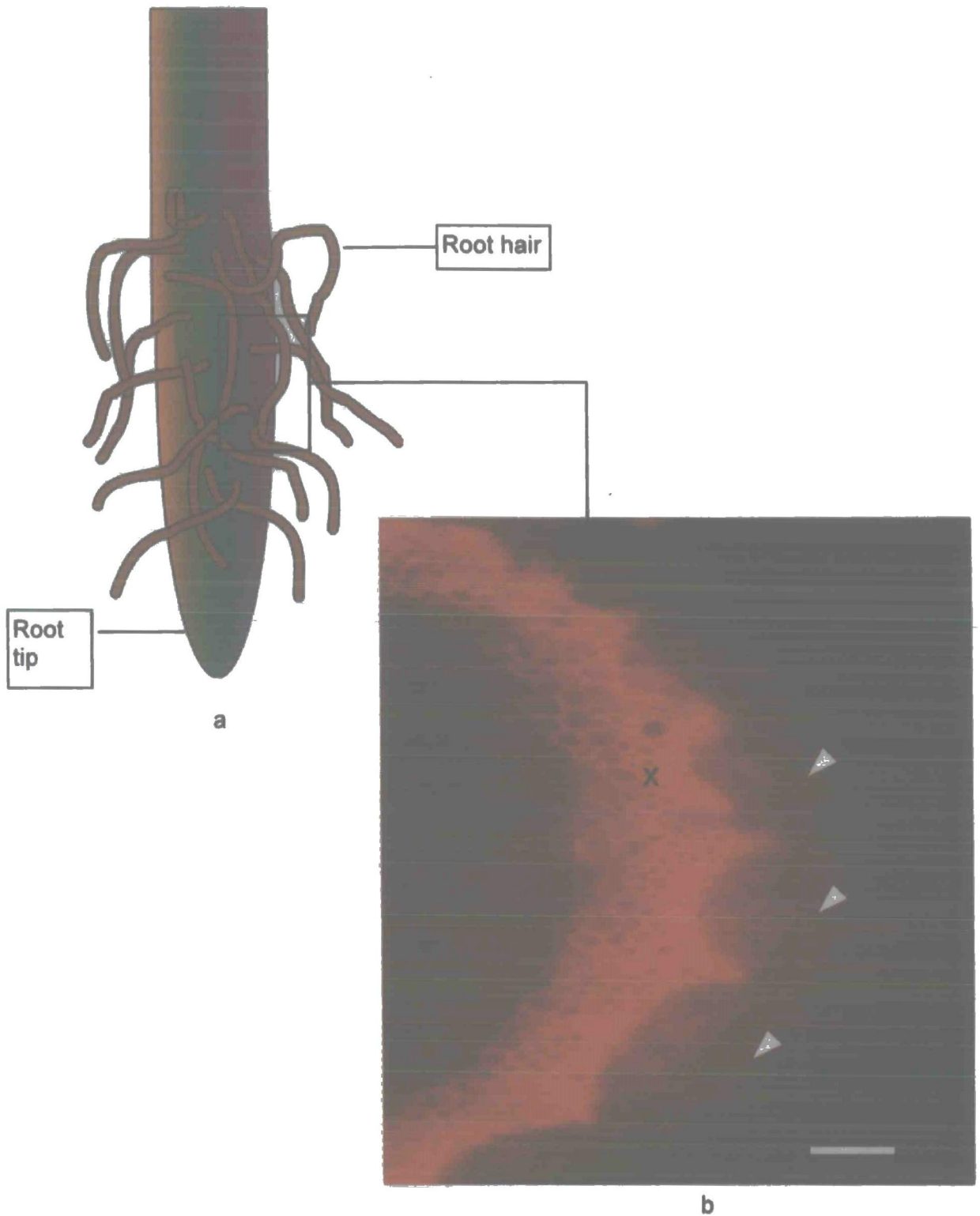


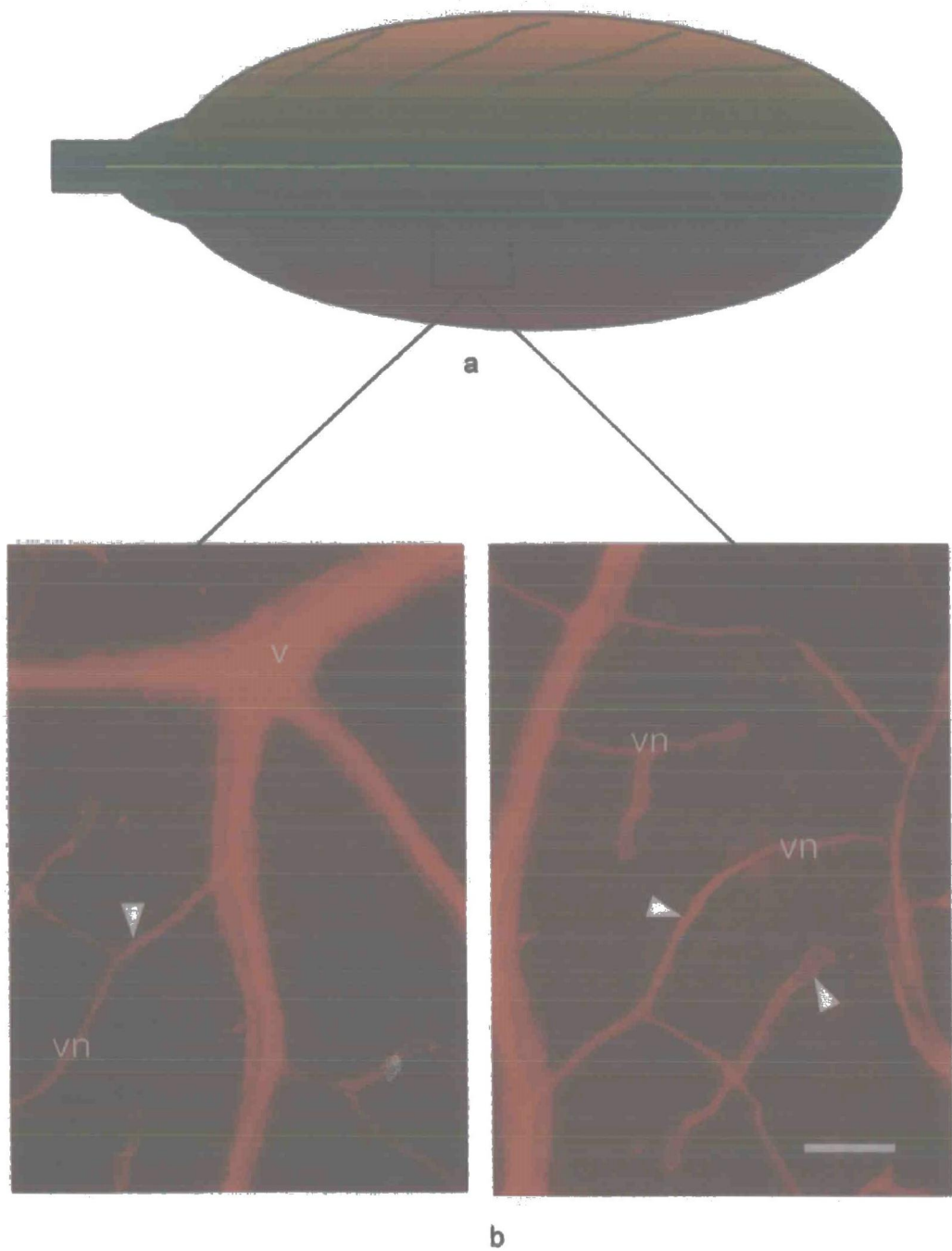
Figure 51: A cross section of *Hevea* root stained with acridine orange. (a) A diagrammatic figure of *Hevea* root showing sampling area. (b) Arrows indicate the cambial region where the laticifers were supposed to be located. Xylem fluoresced strongly. There are no other lignified cells present. X=xylem. Bar = 200  $\mu$ m

### **3.3.1.10 Fresh samples stained with Acridine orange: Leaf**

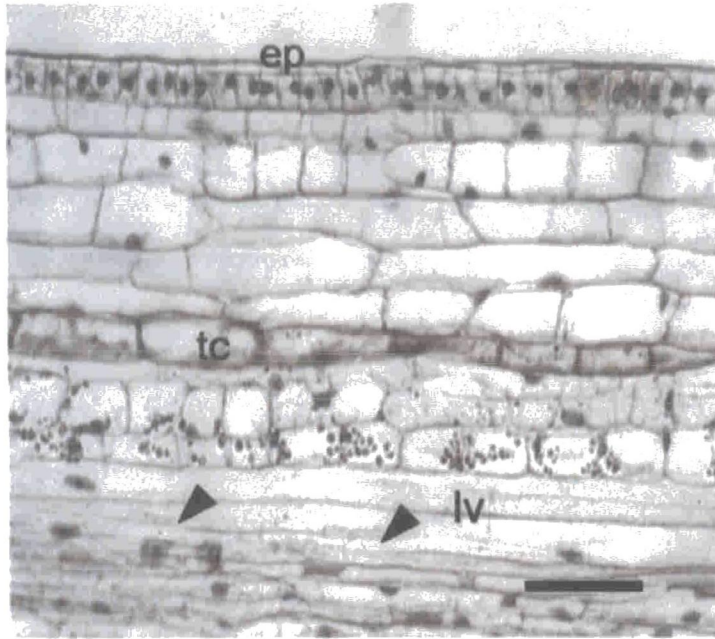
Cleared *Hevea* leaves stained with acridine orange revealed a clear outline of the vascular system (Figure 52). Laticifers or latex did not stain, as did lignified tissue in the vein. However from this result, it is clear that acridine orange can also be used as a marker to trace the distribution of vascular tissue in the leaf.

### **3.3.1.11 Samples fixed with osmium tetroxide, stained with Toluidine blue**

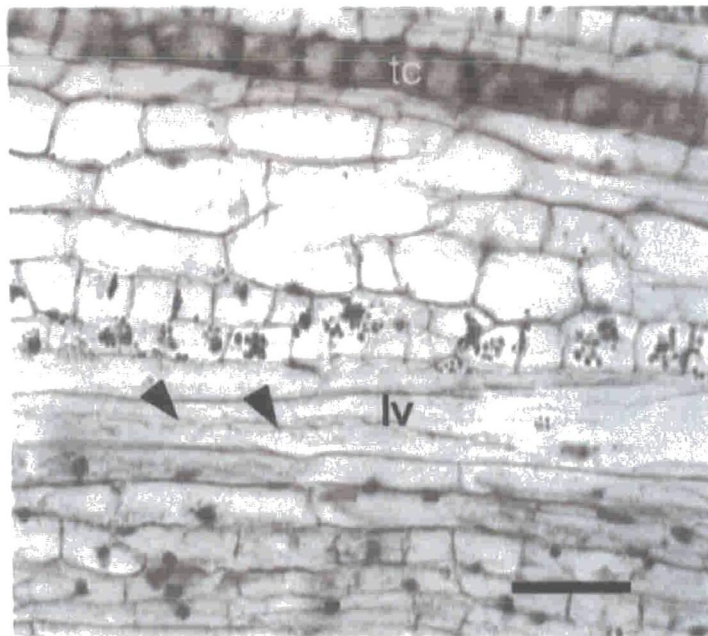
Figures 53 (a&b) showed a semi-thin section of *Hevea* stem stained in toluidine blue. This longitudinal section shows a few laticiferous cells (Lv) in the cambial region. The laticiferous cells could be distinguished from other cells by their long tube-like character, running in parallel with one or more other laticiferous cells. In this instance, the cell wall of the laticifers appears to have broken down, joining the two neighbouring laticifers to form a complex system of laticifers. In figure 53(b), it is also quite noticeable that the adjacent cell wall separating the first laticiferous cell and the second one seems to be loosened (arrows). This was probably one of the stages where the merging process of two or more laticiferous vessels happened, which later will lead into a more complex and elaborate system of tubes (anastomosis). The fixative seems to work well and no cell plasmolysis was observed.



**Figure 52: Cleared leaves of *Hevea* stained with Acridine orange. (a) A diagrammatic figure of *Hevea* leaf showing sampling area. (b) The veins and the veinlet (arrows) were stained brightly by the stain. However latex vessels were not stained in this sample. V=vein, vn=veinlet. Bar = 190  $\mu$ m.**



a



b

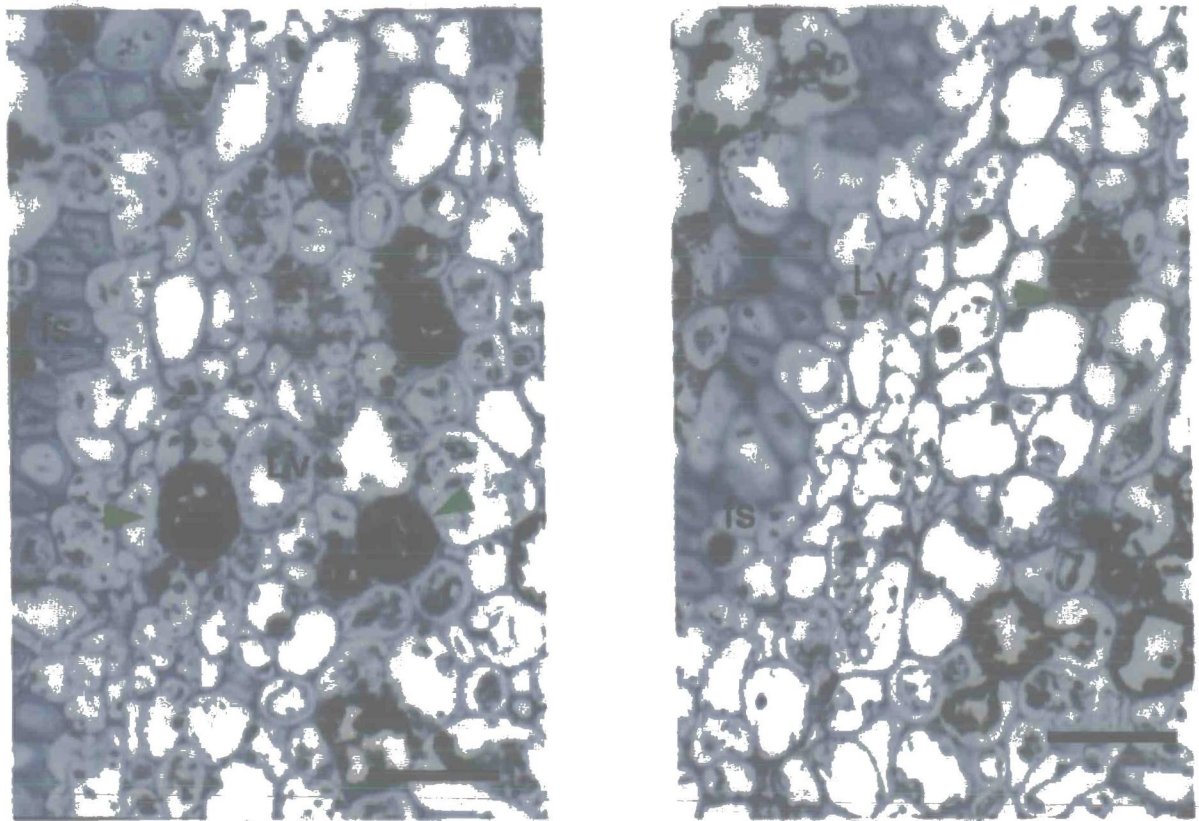
Figure 53: (a&b) Cross section of *Hevea* stem stained with Toluidine blue. Laticifers (lv) are visible amongst other cells, distinguished by their long tube-like characters. Arrows indicate the place where cell walls start to loosen or disintegrate. Lv=laticifers, tc=tannin cells. Bar = 30 $\mu$ m.

### **3.3.1.12 Samples fixed in osmium tetroxide and zinc iodide (ZIO), stained with toluidine blue**

Figures 54 (a&b) show cross sections of *Hevea* shoot apex fixed in ZIO. Some cells (presumably tannin cells) seem to be impregnated quite strongly with the stain. Laticifers were observed in figure 54(b), just outside the xylem (in the cambial region). The fixative did not impregnate the laticifers properly. However, some organelles seem to pick up the stained slightly in the laticifers. Cell plasmolysis can be seen from the results, shown in figures 54(a), where plasmalemma was observed to be pulling away (shrinking) from the cell walls, leaving an empty spaces between both plasmalemma and cell wall. This was caused by improper penetration of fixatives into the cells, and occurred quite regularly in hard and/or woody samples as in this case in the shoot apex of *Hevea*. Lignified cells (fibre sclereids) were stained blue with toluidine blue.

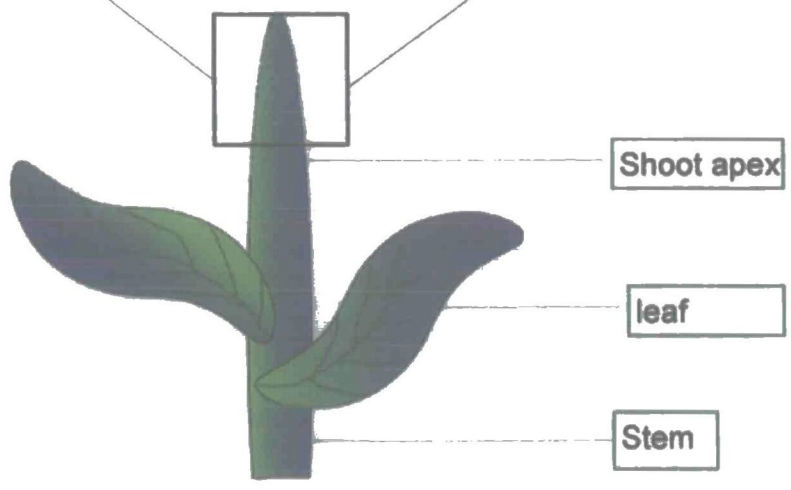
### **3.3.1.13 Samples Stained with Safranin O and Astra Blue: Stem**

Figure 55(a&b) shows a cross section of *Hevea* stem stained in Safranin O and Astra blue. Xylem stained red while other cells were stained blue. There is no clue on the location of the laticifers in the samples. However it was believed from the previous results that the laticifers were located in the cambial region of the samples, which in this case showed by the darker blue line in figure 55b (arrows).



a

b



c

Shoot apex

leaf

Stem

Figure 54: (a&b) Laticifers in *Hevea* shoot apex, fixed with ZIO. Laticifers were located in the cambial region of the sample and quite close to the fibre sclereid. Few cells (arrows), presumably containing tannin were heavily impregnated with the ZIO fixative. (c) A diagram showing the place of sampling in *Hevea* shoot apex. Ls=laticifers, fs=fibre sclereid. Bar = 30µm.

#### **3.3.1.14 Samples stained with Safranin O and Astra Blue: Root**

In root samples, as shown in figure 56, laticifers were distinguished from the other cells by their content of coagulating latex. Several laticifers were observed to run in parallel to each other. Tannin cells were stained bright red and can be distinguished from the xylem cells, which were also stained red by its content (xylem was empty) (figure 54a).

#### **3.3.1.15 Samples stained with Safranin O and Astra Blue: Shoot apex**

Crystals were found scattered in parenchyma cells in *Hevea* shoot apex. A cross section of *Hevea* shoot apex showed abundant crystals in the parenchyma cells as well as the tannin cells. The crystals were shown very clearly under Nomarski illumination (figure 57a) compared to the normal bright field illumination (figure 57b). However, there are no traces of latex in the laticifers (Lv).

#### **3.3.2 Ultrastructure of laticifers in *Hevea Brasiliensis***

Laticifers in *Hevea* show an abundance of rubber particles suspended in a matrix, as shown in figure 58(a&b). The rubber particles appeared in a dark colour, apparently impregnated by the osmium tetroxide and other heavy metals from the staining i.e. uranyl acetate and lead citrate. From the result, it was also clear that all rubber particles were spherical but they were in different sizes.

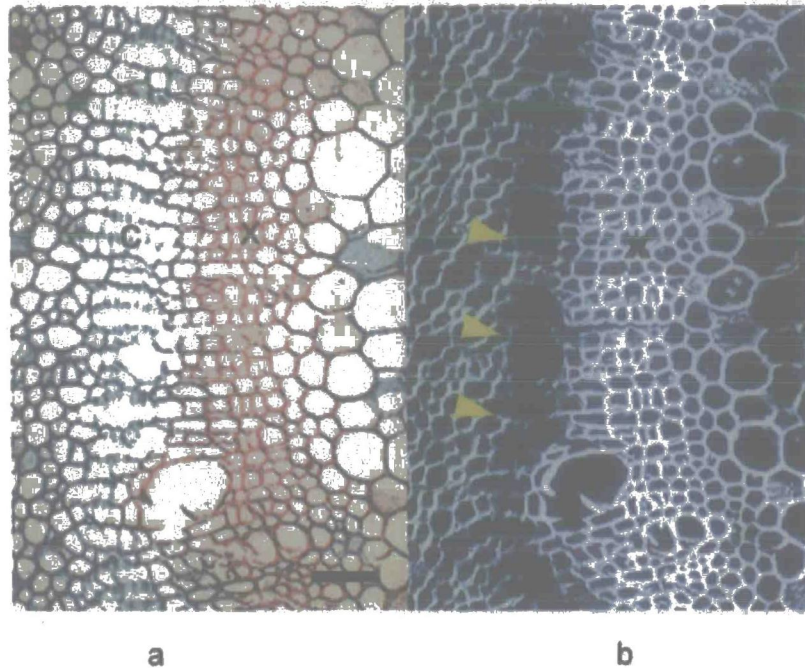


Figure 55(a&b) : Cross section of *Hevea* stem stained with Safranin O and astra blue. Figure a, with normal bright light illumination, shows no evidence of laticifers in the cambial region. Figure b of the same section view with a Nomarski illumination and shows a row of dark cells (arrows) just outside the xylem, which are believe to be laticifers. X=xylem, c=cambial region. Bar = 190  $\mu$ m



Figure 56: Longitudinal section of *Hevea* root. Latex vessels can be observed in this samples (Lv) and latex can be seen coagulating inside the vessels. Lv=laticifers, tc= tannin cell. Bar = 190  $\mu$ m

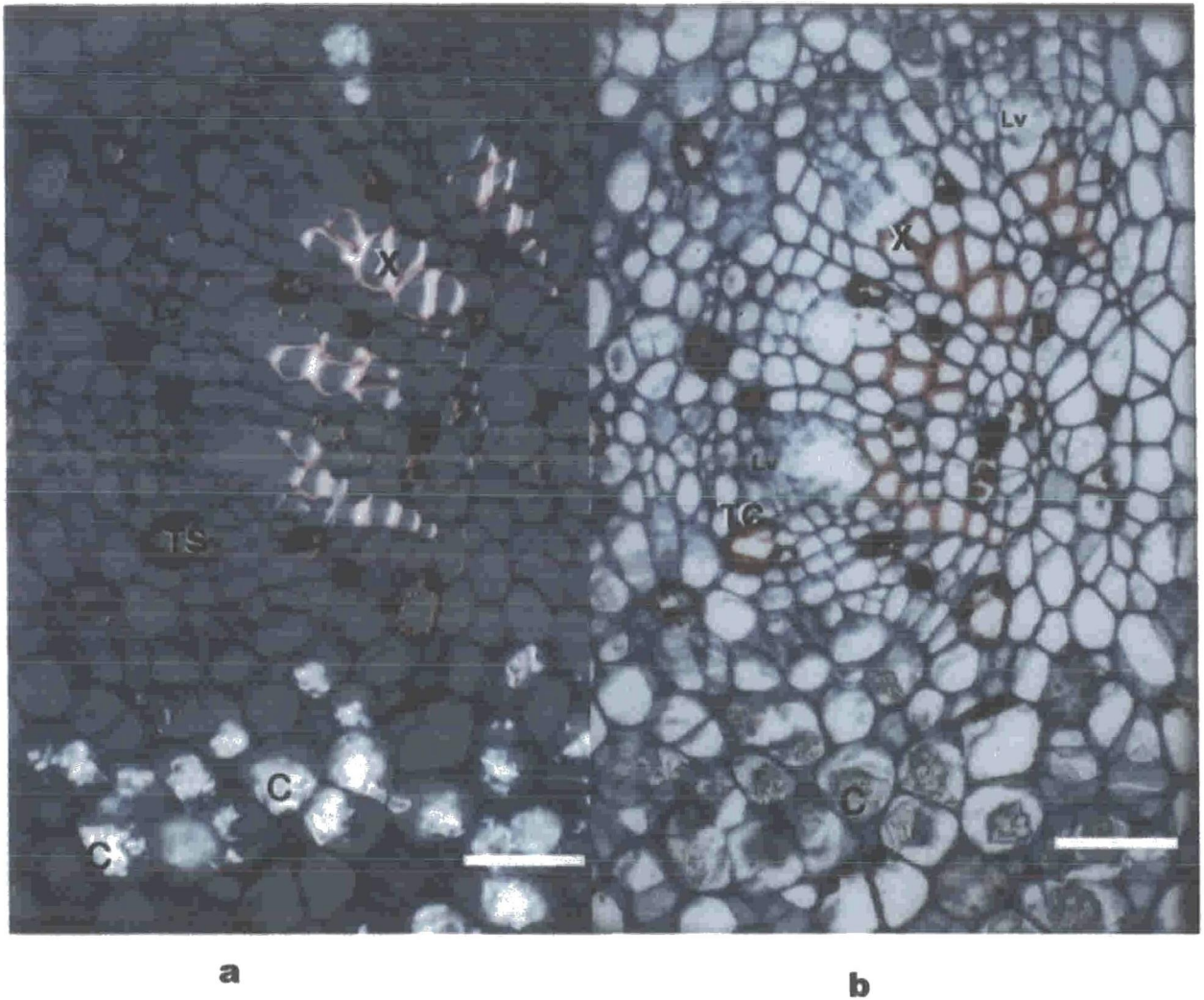
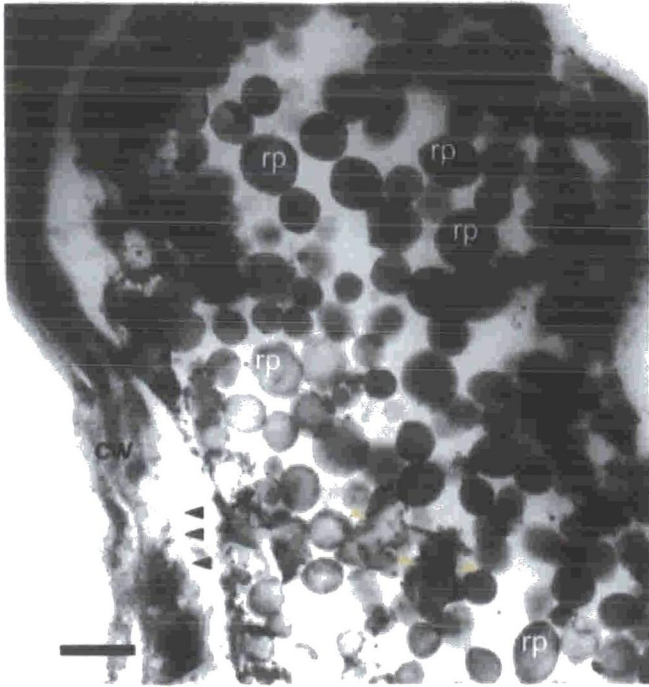


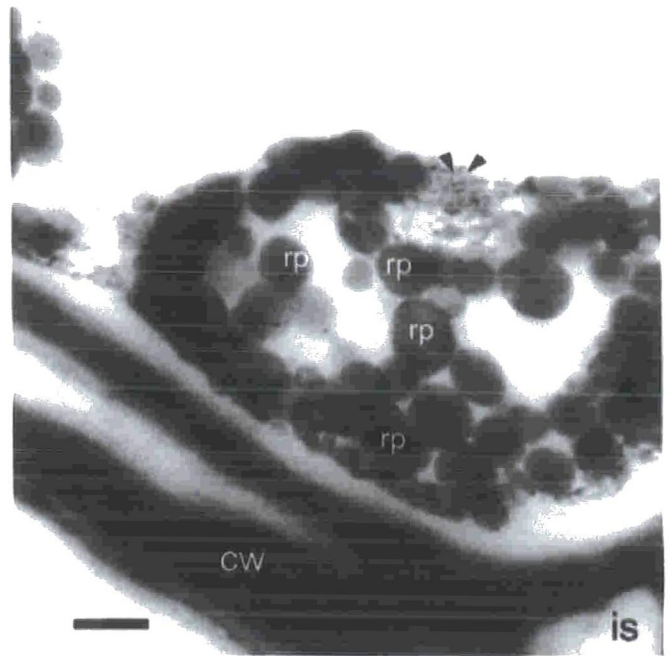
Figure 57: *Hevea* shoot apex stained with Safranin O and Astra blue. The cross sections of the sample show no traces of latex present in the laticifers (Lv). Figure (a), in Nomarski illumination, and (b) bright field illumination shows crystals are abundant in the parenchyma cells. C=crystal, Lv=laticifers, x=xylem, tc=tannin cells. Bar=30  $\mu$ m.



(a)

Figure 58 (a) : TEM image of a section through *Hevea* stem, fixed with  $\text{OsO}_4$ . Rubber particles are abundant in the laticifers. They are in spherical shapes and stained black with heavy metal. Orange arrows ( = ) show rubber particles coagulate and clump together. Cell walls show the fibres are loosening and disintegrating ( ▶ ). Cw= cell wall, rp=rubber particles, Bar = 0.10  $\mu\text{m}$ .

Figure 58 (b) : TEM image of a section through *Hevea* stem, fixed with  $\text{OsO}_4$ . Rubber particles can be seen in various sizes. Arrows ( ▶ ) show a residue of microhelices from lutoids. Rp=rubber particles, cw=cell wall, is=intercellular space. Bar = 0.10  $\mu\text{m}$ .

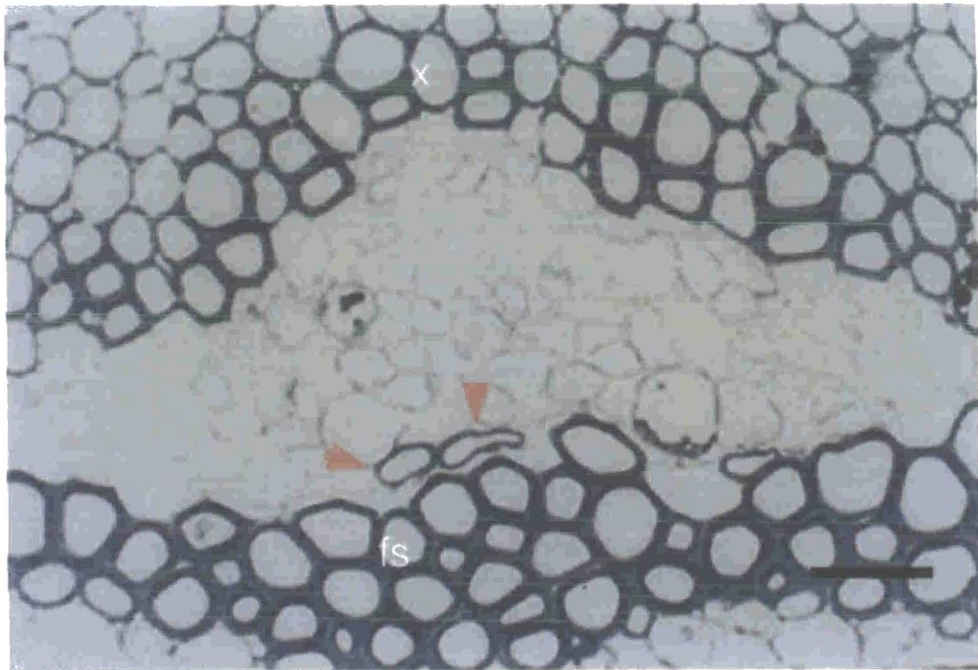


(b)

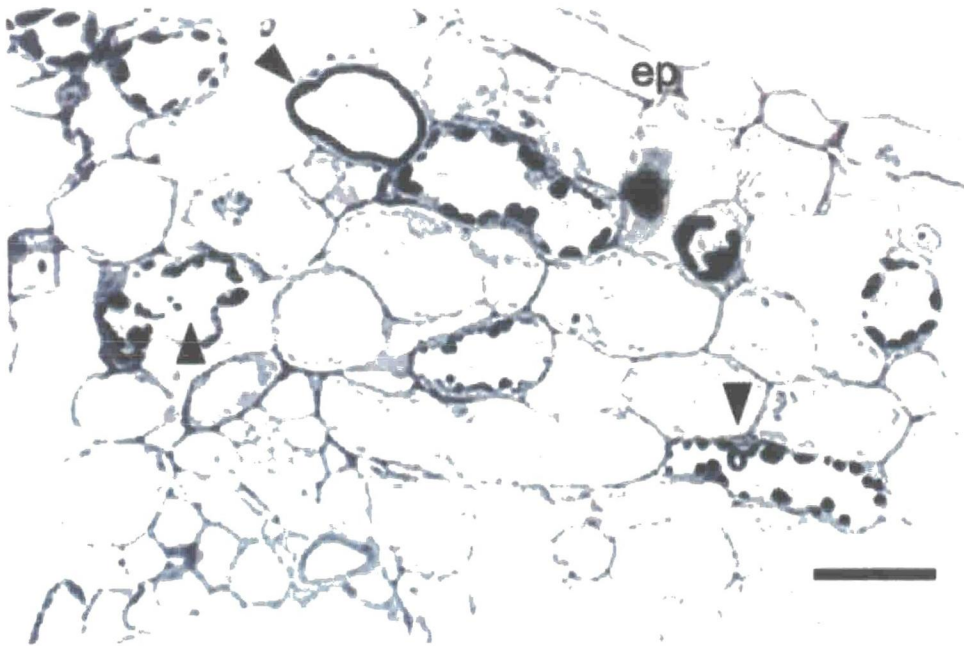
In figure 58a it was clear that cell walls of the laticifers were starting to disintegrate and the wall fibres loosened (black arrows). This has already been shown in the samples observed under light microscopy. It is obvious that the process of cell wall disintegration was a starting point of joining the two or more neighbouring laticifers together to form a more complex and bigger system of articulation of the laticifers in *Hevea*. There was also evidence of rubber particle coagulation as shown in figure 58a (yellow arrows). In figure 58b, free microhelices from the broken lutoids, an organelle exclusively found in this genus, were also observed amongst the rubber particles (arrows). Lutoids were very sensitive to osmotic pressure and it is hard to maintain the structure of the lutoids when laticifers were put under turgor pressure during sectioning.

### **3.3.2 Freeze substitution technique to preserve laticifers in *Hevea brasiliensis*.**

An attempt to use cryo preservation as an alternative way to fix laticifers was not very successful. There were no latex vessels observed from the samples that were used in the cryo technique. Figure 59a & b show cross sections of cryofixed *Hevea* leaves stained with toluidine blue. Parenchyma cells seem to be fixed quite well, where there is no cell plasmolysis and they seem structurally intact (Figure 59a). However, the penetration of the fixatives seems to be inadequate, shown by the broken cells (orange arrows). Tannin cells were preserved properly as shown in figure 59b (black arrows).



(a)



(b)

Figure 59a&b : Cross section of cryofixed *Hevea* leaf. The cryo technique seem to fix parenchyma cells quite well. However, laticifers or latex were not observed in this sample. X=xylem, fs=fibre sclereid, ep=epidermis. Bar = 40 $\mu$ m.

### **3.4 *Musa acuminata* (Musaceae)**

#### **3.4.1 Distribution and structure of laticifers**

##### **3.4.1.1 Laticifers in fresh samples of *Musa***

*Musa* leaves when cut, gave clear and sticky latex. The results of the fresh sample stained with calcofluor and viewed under bright field light microscopy shows no structural evidence of laticifers (Fig. 60). One possible reason is that the image itself is not very clear since it was very hard to erect or make the leaf blade to stand at 90° to the microscope stage plane, without it falling down during observation. Only the cell walls in the vascular region are excited with the fluorescent light.

##### **3.4.1.2 Araldite resin: Samples stained with Toluidine blue**

This is a sample fixed with OsO<sub>4</sub>. The laticifers in *Musa* are observed in the peripheral region of the vascular bundle. The distinct black osmicated cell differentiated the laticifers from other parenchyma cells (Fig. 61). The laticifers are not confine to the xylem area but also can be found in the phloem region and very close to the cells. It is also very obvious that the laticifers also stained darker compared to the other cells, which is sometime a good marker to differentiate laticifers from other cells.

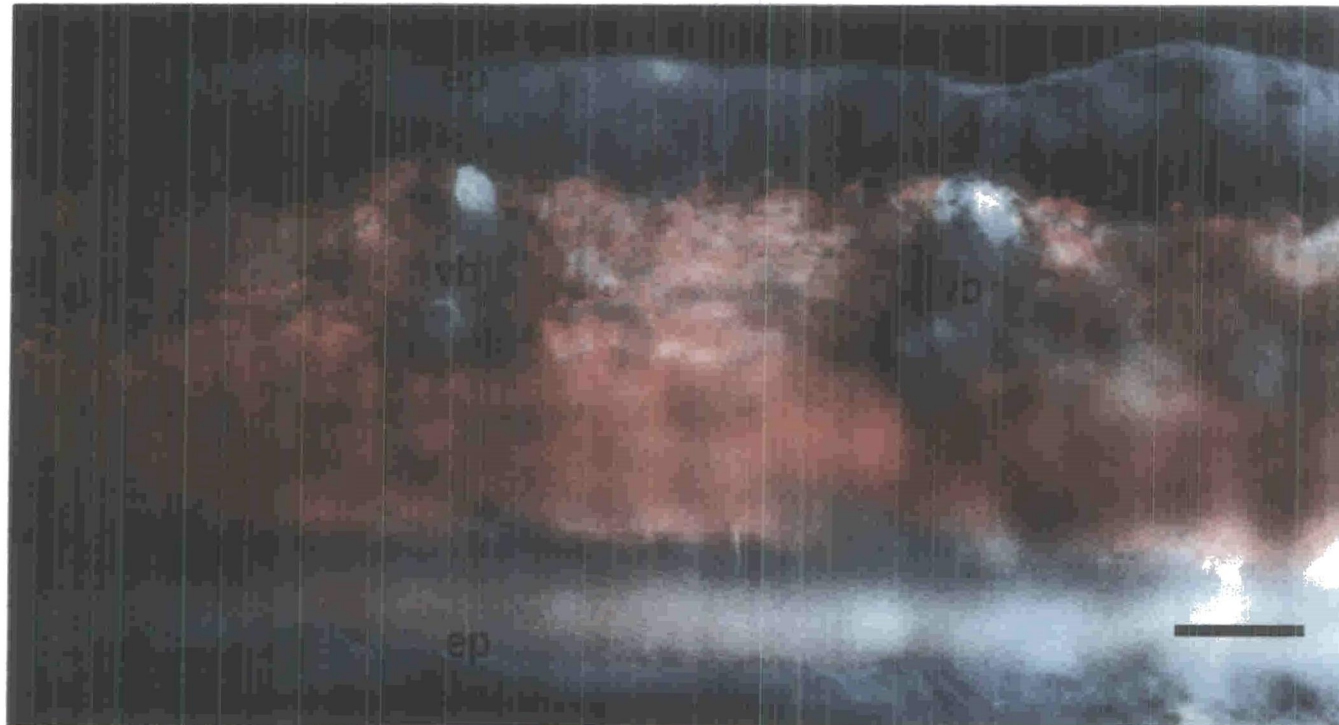


Figure 60: Fresh sample of old *Musa* leaf stained with calcofluor. The vascular bundle excited very brightly, but it is hard to tell the location of the laticifers. The red colour were excited by the chlorophyll in the parenchyma cells. Vb=vascular bundle, ep=epidermis. Bar= 190um

#### **3.4.1.3 Spurr resin: Samples stained with Toluidine blue**

Figure 62 of old *Musa* shows a similar observation to that in figure 61. The laticifers can be found in the peripheral region of the vascular bundle. However, latex in the laticifers was noticeably scarce.

#### **3.4.1.4 Wax medium: Samples stained with Safranin O and Astra blue.**

In *Musa* leaf samples, Safranin O and Astra blue stained laticifers and their content (latex) quite well. Laticifers can clearly be distinguished from other cells by bright red staining by the Safranin O reagent (figure 63 a&b). A sample viewed with Nomarski illumination showed raphide presence quite clearly (arrow), compared to the normal bright field image.

#### **3.4.1.5 Ultrastructure of laticifers in *Musa acuminata***

Ultrastructure studies of old *Musa* leaf lamina showed laticifers as cells with electron-dense contents of a granular nature (figure 64). Laticifers were located very close to the epidermis cells especially in the spongy cell area. However, a TEM image of young *Musa* leaf did not show any evidence of latex presence in the laticifers. Instead they contain numerous globular structures that occupied almost 41% of the laticifers (figure 65).

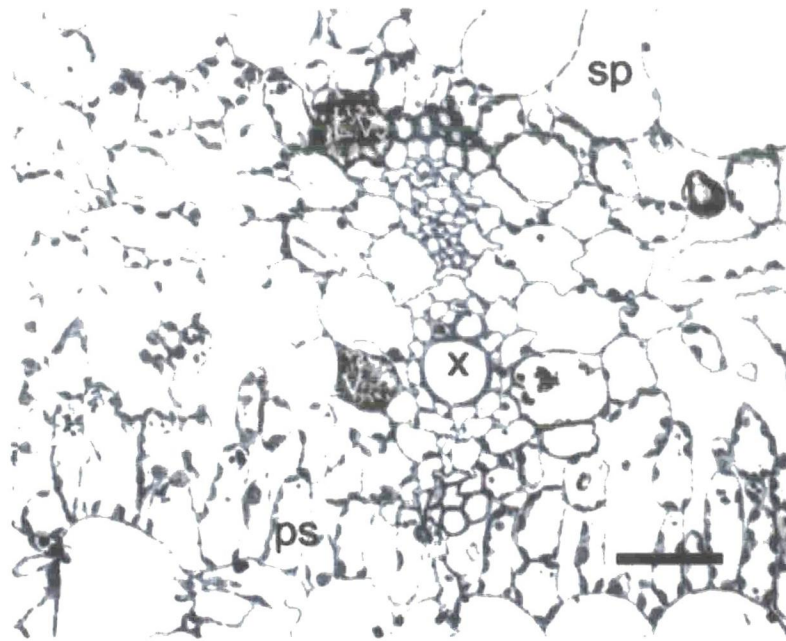


Figure 61: A cross section of *Musa* leaf, embedded in Araldite resin. Laticifers in *Musa* can be distinguished from other cells by dark latex occupying the cells. Lv=laticifers, ps=palisade cells, x=xylem, Bar = 30 $\mu$ m.

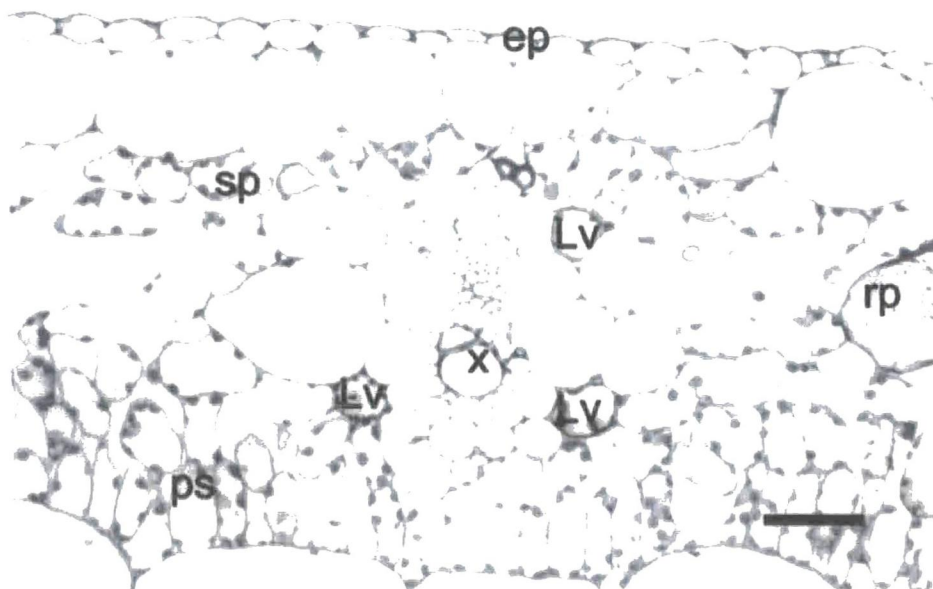
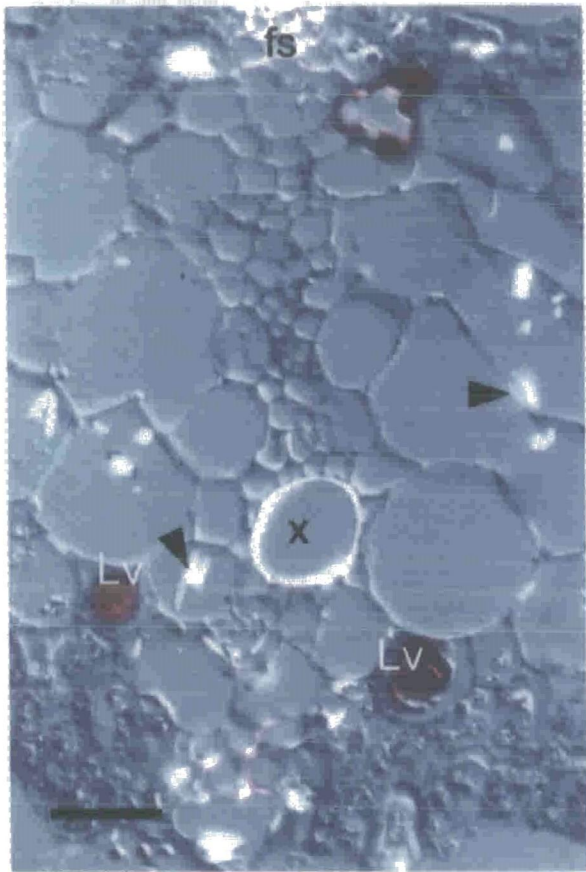
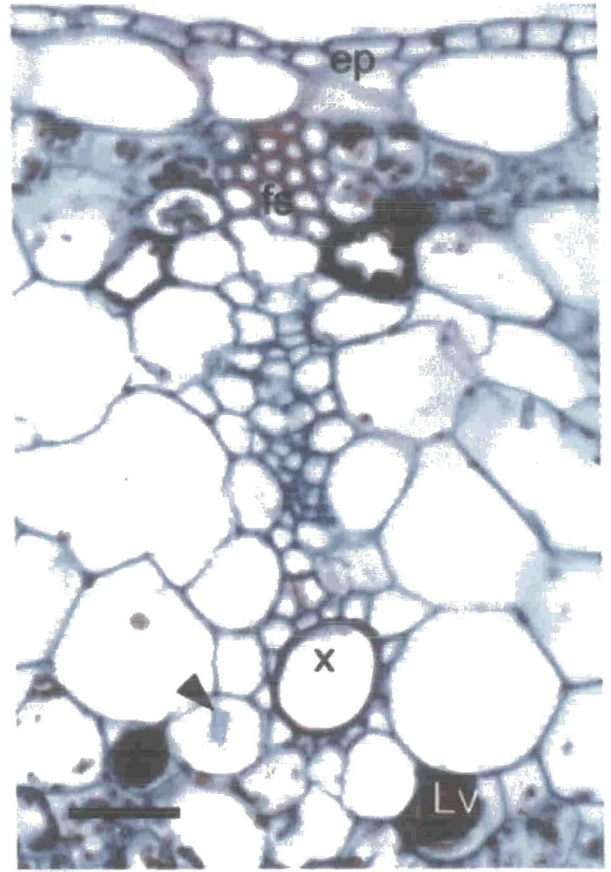


Figure 62: A cross section of old *Musa* leaf, embedded in Spurr resin. There is little latex in the laticifers. Notice raphide (rp) on the left side of the photo. Lv=laticifers, x=xylem, ps=palisade cell, sp=spongy cell. Bar= 30 $\mu$ m.

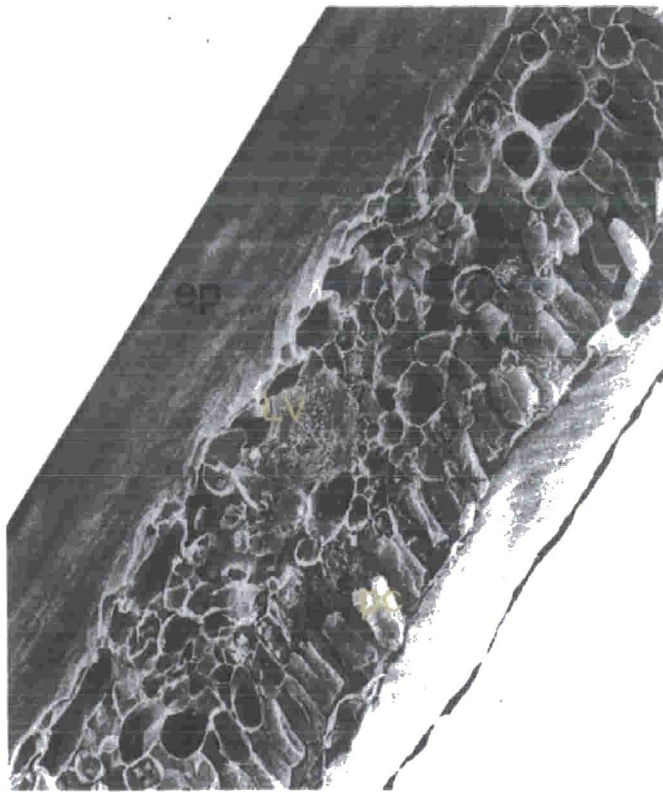


a

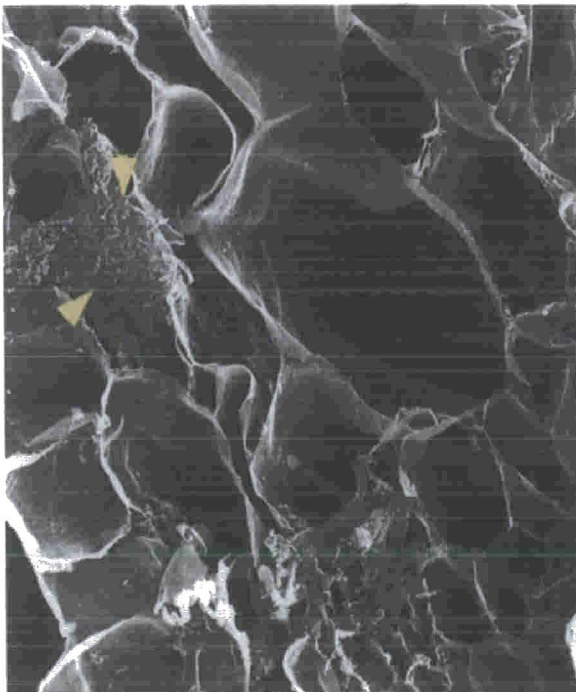


b

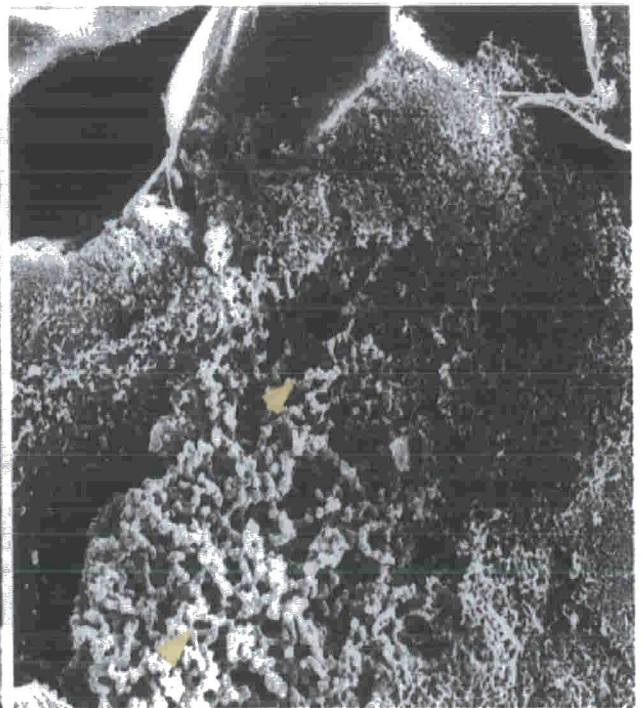
Figure 63: (a) Nomarski illumination. (b) bright field illumination. A cross section of young *Musa* leaf, stained with Safranin O and Astra blue. Laticifers were clearly stained as red. Arrows indicate the residue of raphides. Lv= laticifers, x=xylem, ep=epidermis .Bar= 30 $\mu$ m.



(a)



(b)



(b)

Figure 64: SEM image of Laticifers in Old *Musa* leaf lamina. (a) Laticifers were quite distinct from other cells with their granular content of latex (x200). (b) A higher magnification image of laticifers cell (x1000) showing the surrounding cells were almost empty, lacking organelles compared to the laticifer (arrow). (c) Latex in laticifers (arrow) They are arranged in a very compact group and clumping together. The sizes of the latex particles also varies. No other organelles were observed. (x 3500). Lv=laticifers, ep=epidermis, pc=palisade cells.

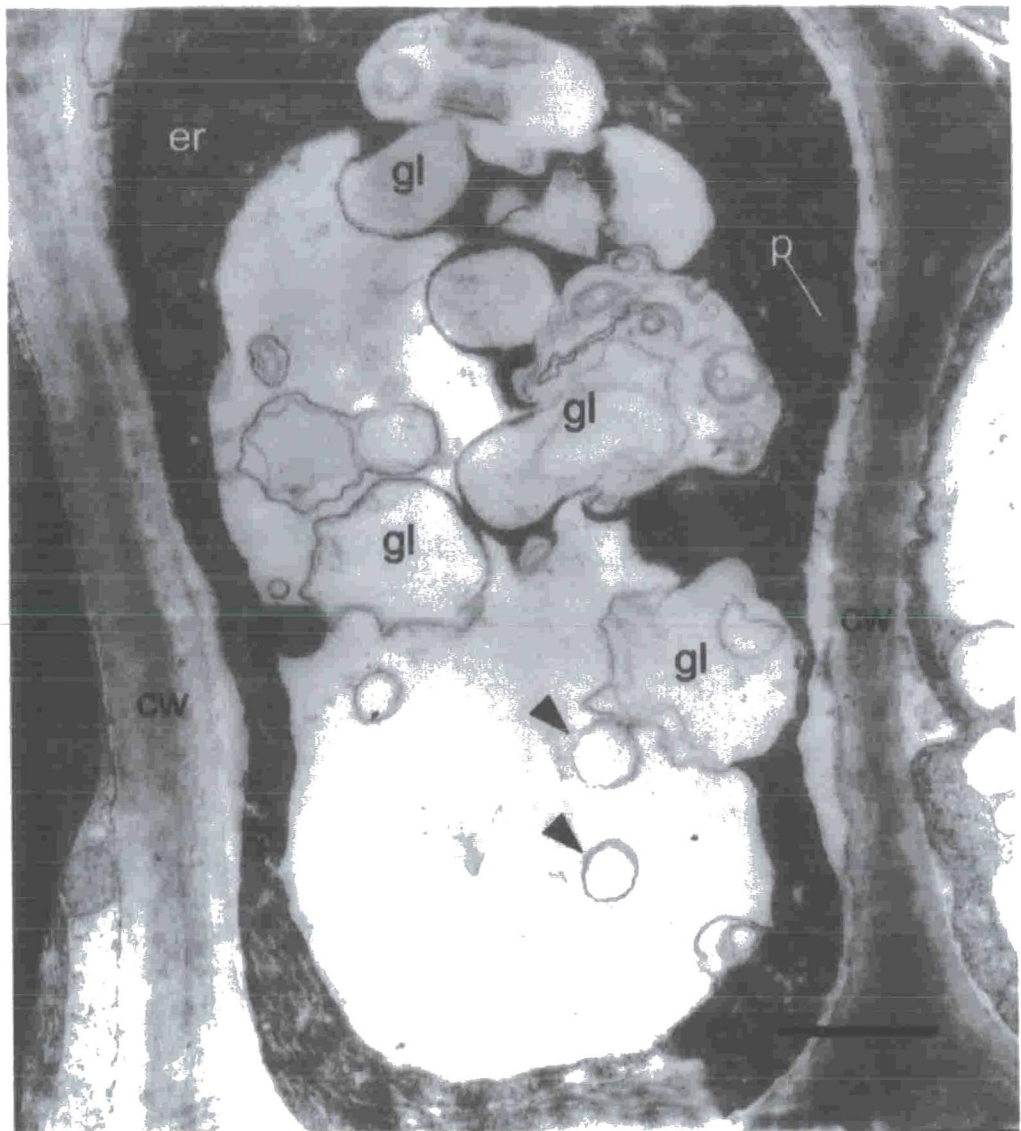


Figure 65: TEM Micrograph of laticifers in young *Musa* leaf lamina. Globular organelles were quite abundant in the laticifers. Latex particles were not observed in this stage. Arrows show small globules coalescing into large one. Cw=cell wall, gl=globule, er=endoplasmic reticulum, pl=plastid. Bar=0.4 $\mu$ m

It was also observed that latex particles were spherical in shapes (figure 64b&66) but the sizes may vary between them. Globules were abundant in the laticifers of old *Musa* leaf (figure 66). Other organelles such as Golgi bodies, endoplasmic reticulum and plastids were also present.

### **3.5. *Mandevilla splendens* (Apocynaceae)**

#### **3.5.1 Distribution and structure of laticifers**

##### **3.5.1.1 Laticifers in the leaf lamina of *Mandevilla splendens* stained with Safranin O and Astra blue.**

*Mandevilla* leaves when cut gave out white and sticky latex. Figure 67(a), shows a cross section of *Mandevilla* leaves (midvein). Laticifers were quite easily distinguishable from the other cells with their content stained brightly red by the reagent. Fibre sclereids were quite prominent in the samples. In figure 67(b), laticifers can be seen scattered in the middle section of the leaf (between palisade cells and spongy cells) and some are located quite close to the xylem. Tannin cells were very prominent in the samples and located in the palisade cells and spongy cells region. It was quite difficult sometimes to differentiate between tannin cell and the laticiferous cell in the sample since both were stained red by the reagent. The only clue come from the granular substance (latex) that occupied the laticifers, whereas the tannin cells in the cell looks more compact (figure 68).

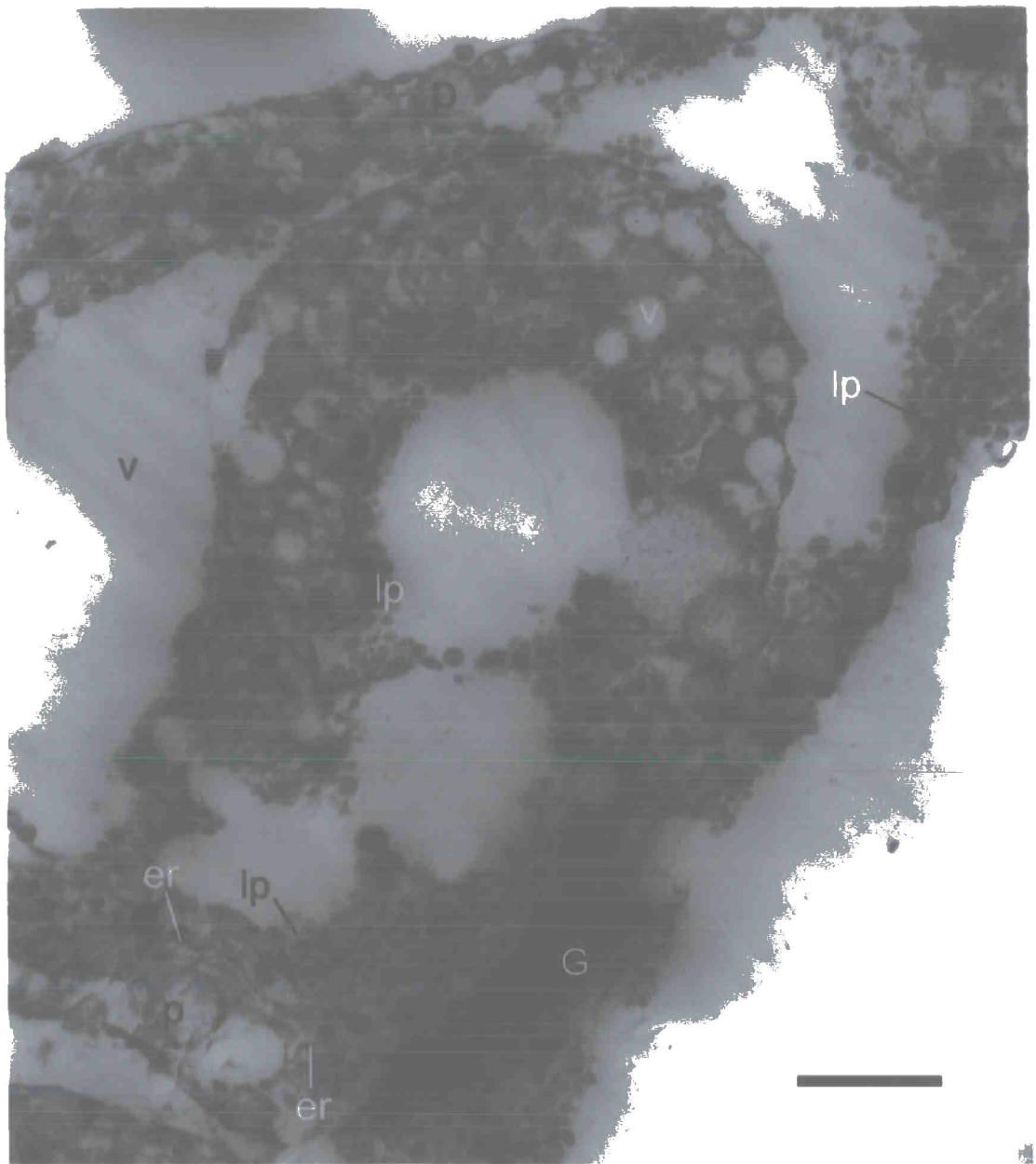
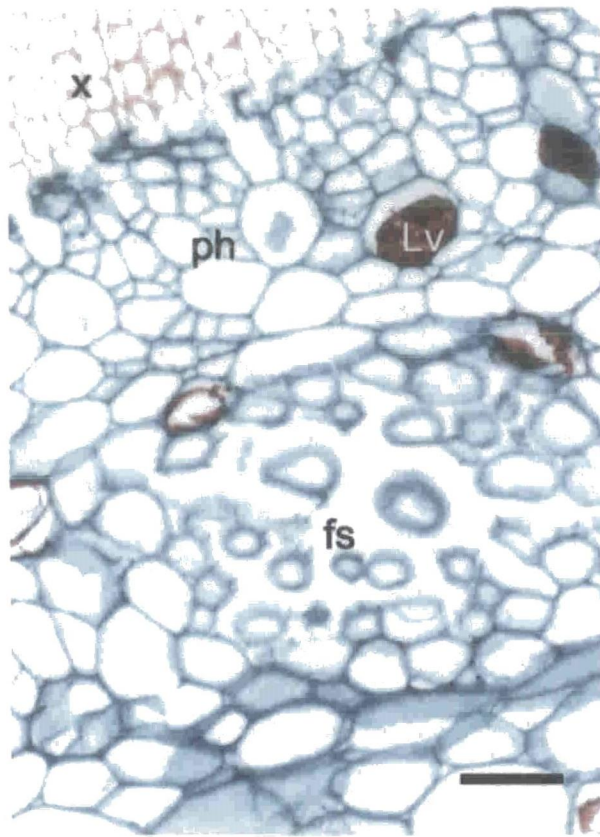
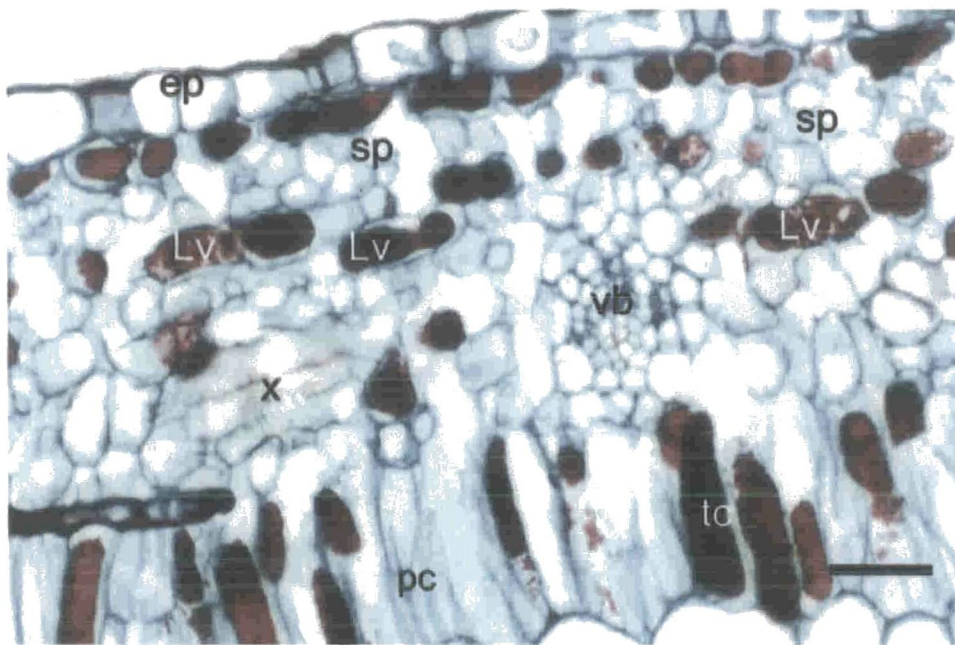


Figure 66: TEM micrograph of mature/old *Musa* leaf lamina. Latex particles were abundant. Vacuoles were quite scarce compare to the latex. Plastids were also quite abundant in the laticifers. Lp=latex particles, p=plastid, G=golgi bodies, er=endoplasmic reticulum . Bar= 3.2 $\mu$ m.



a



b

Figure 67: Cross section of *Mandevilla* leaf lamina stained with Safranin O and Astra blue. In figure 66a, laticifer was observed as bright red cell (Lv). Fibre sclereid (fs) were quite eminent showing sclerified cells with thickening cell wall. Figure 66b shows some traces of latex vessels (Lv) near xylem. Tannin cells were abundant. X=xylem, tc=tannin, pc= palisade, sp= spongy cells, vb= vascular bundle, Lv=laticifers, fs=fibre sclereid, ph=phloem, ep=epidermis. Bar= 30  $\mu$ m.

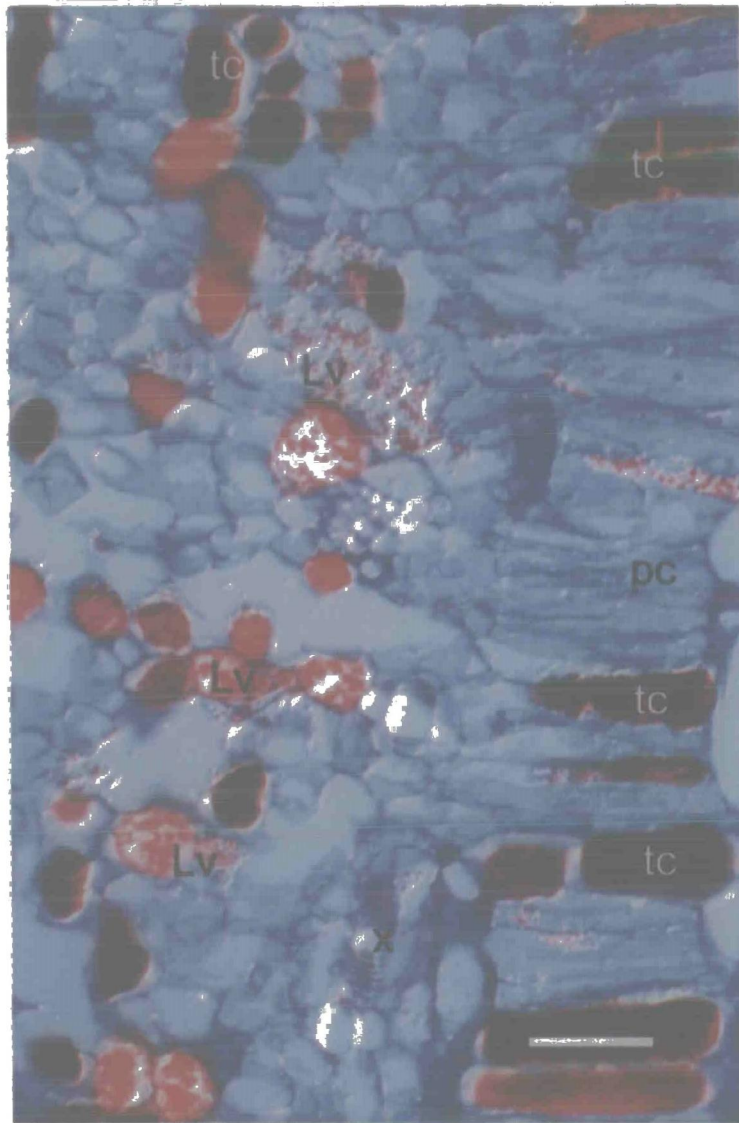
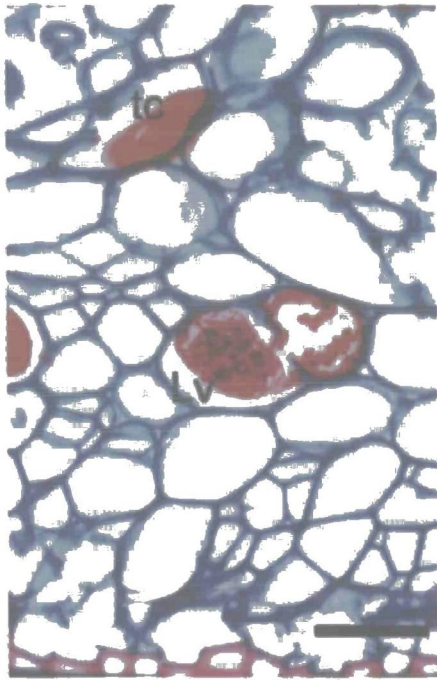


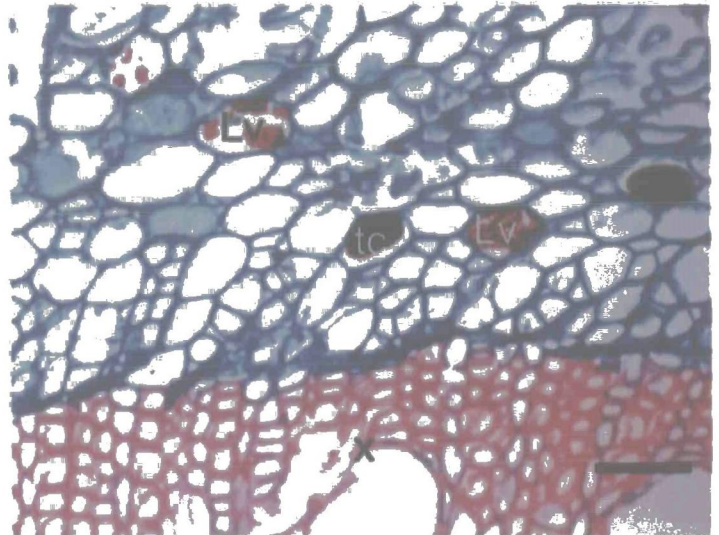
Figure 68: Laticifers in *Mandevilla* leaf lamina embedded in wax. It is clear from the photograph that tannin cells and laticifers can be confusing, because both are stained red. However, latex in laticifers looked granular compare to the compact-appearances of the tannin cells. Lv=laticifers, x=xylem, pc=palisade cells, tc=tannin cells. Bar = 190 $\mu$ m.

### **3.5.1.2 Laticifers in the stem of *Mandevilla splendens* stained with Safranin O and Astra blue.**

*Mandevilla* stem exuded white and milky latex when cut. The latex was quite runny compared with *Hevea*. Figure 69a showed laticifers were located in the cambial region of the stem. The laticifers were stained almost identically to tannin cell. The identification of laticifers can be misleading because of the same colour stained by the reagent and `mixing` in almost the same area with tannin cells. However as in the leaf, the granular appearances distinguishes between the two cells (Figure 69b). It was quite interesting to note that latex particles were also observed to occupy the cells resembling phloem cells (figure 69c). This is because of the perforation of the wall that was observed surrounding the latex in the cells.



(b)



(a)



(c)

Figure 69: Laticifers in *Mandevilla* stem. Laticifers were stained bright red and located in the cambial region of the stem (a). Granular appearance distinguishes the laticifers from tannin cells (b). Longitudinal section of the stem show latex particles coagulating and clumping together (c), and in one particular cell it looked as if the latex particles occupied and were contained in the phloem (arrows show the perforation). Lv=laticifers, x=xylem, tc=tannin cell. Bar a = 190 $\mu$ m, Bar (a&b) = 30  $\mu$ m.

### **3.6 *Taraxacum officinale* (Compositae/Asteraceae)**

#### **3.6.1 Distribution and structure of laticifers**

##### **3.6.1.1 Laticifers in the leaf of *Taraxacum officinale* stained with Safranin O and Astra blue**

*Taraxacum* leaf when cut oozed a very clear and runny sap. It was not sticky like the other species in this experiment. Figure 70(a) showed a cross section of *Taraxacum* leaf lamina stained with Safranin O and Astra blue for laticifers. It showed that laticifers and their content were stained red and located near the vascular bundle. A cross section through the leaf midvein however did not reveal any evidence of laticifers presence (Figure 70b).

##### **3.6.1.2 Laticifers in the flower stem of *Taraxacum officinales* stained with Safranin O and Astra blue**

In the flower stem of *Taraxacum*, it was difficult to identify laticiferous cells. This is probably due to the nature of the latex itself, where the laticifers could not retain the latex during the sectioning. However some laticifers were stained vaguely red and the laticifers were quite small in size (figure 71).

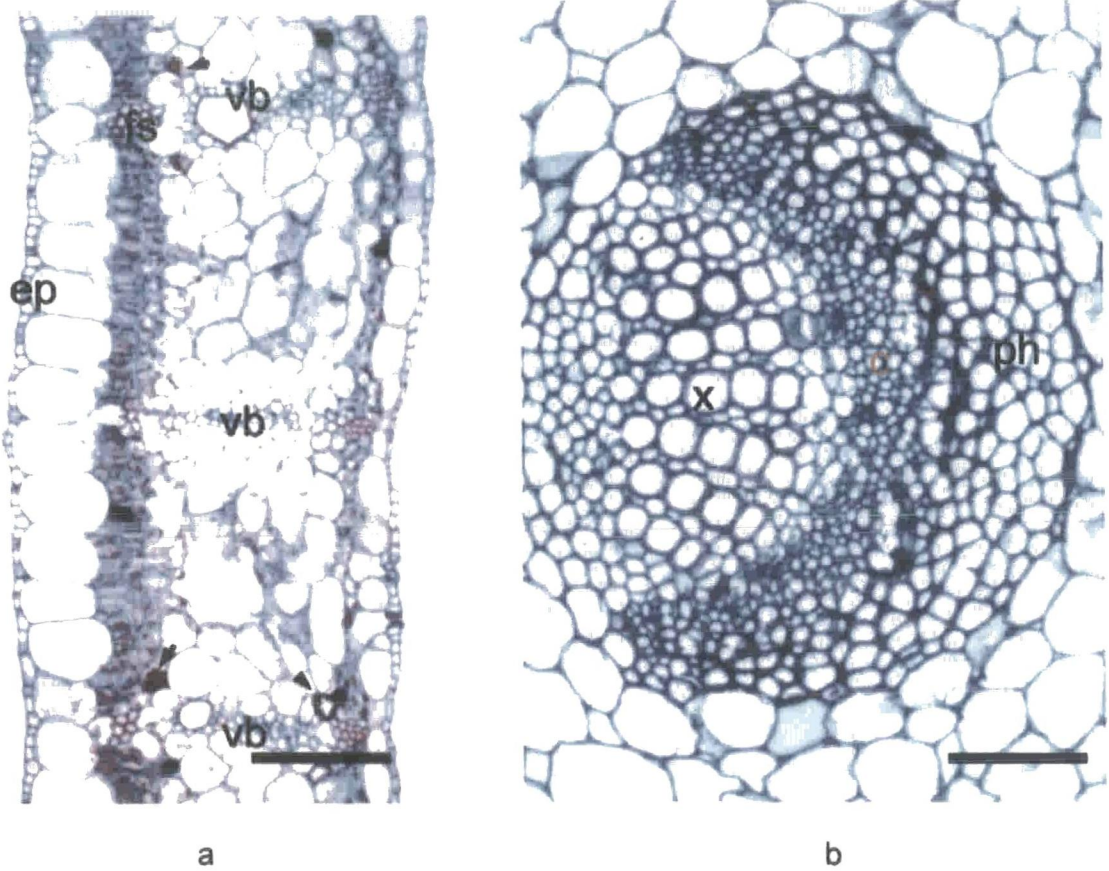


Figure 70: Laticifers in *Taraxacum* leaf. (a) A cross section through leaf lamina. Laticifers were observed near the vascular bundle (arrows). Bar=190 $\mu$ m. (b) A cross section through leaf midvein. It was very hard to locate laticifers in the sample. The image showed the laticifers or latex did not stain well with the reagent. Bar = 30 $\mu$ m. Lv=laticifers, vb=vascular bundle, ep=epidermis, ph=phloem, x=xylem, c=cambial region.

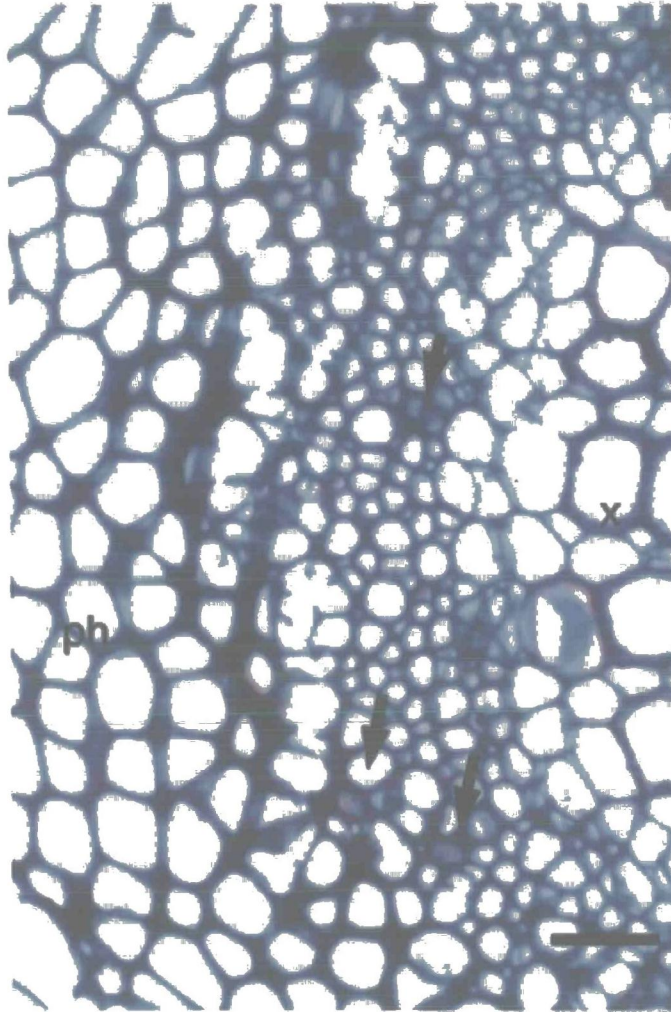


Figure 71: Laticifers in *Taraxacum* flower stem, stained with safranin O and Astra blue. Laticifers were initially quite difficult to identify. They are very tiny and almost did not stain by the reagent (arrows), and located in the cambial region of the sample. Ph=phloem, x=xylem. Bar = 30 $\mu$ m.

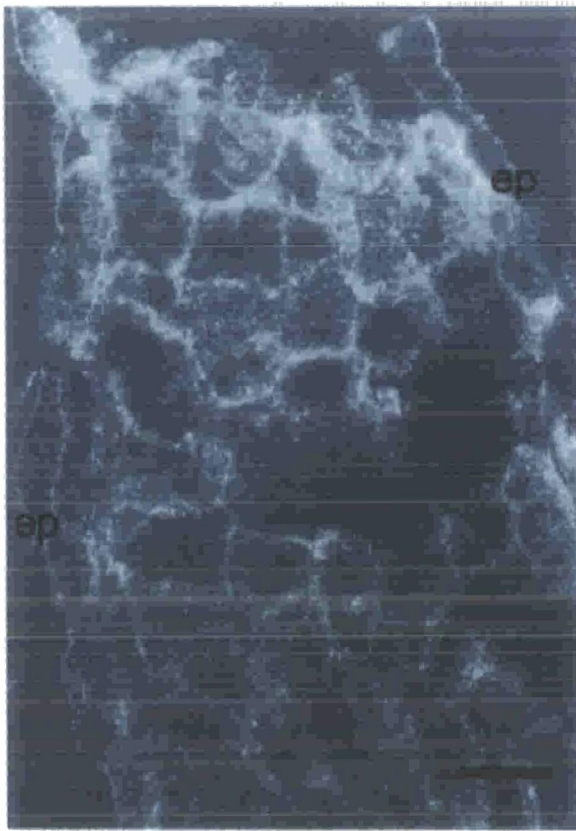
### **3.6.2 Immunohistochemistry of the cell wall protein: Localisation of JIM5 binding in *Taraxacum officinale***

Immunohistochemistry techniques may be used to localise specific proteins and polysaccharide epitopes both temporally and spatially within a cell. Work carried out at the John Innes Institute in Norwich, has successfully utilised antibodies raised against a number of plant glycoproteins, AGPs and pectins, to localise these component to specific sites within plant cells (Knox *et al.* 1989, 1990, 1991, Pennell *et al.* 1989, 1991, Pennel & Roberts 1990, Baldwin *et al.* 1993).

A number of attempts have been made to apply the immuno technique to the samples. Several antibodies were used, however the rate of specific labelling on the target was very low. Most of the time only JIM5 showed quite a prominent results.

The anti-polygalacturonic acid antibody JIM5, which recognised epitopes of un-esterified pectin, has been used to determine the spatial distribution of pectin in *Taraxacum* cell walls.

JIM5 binding was detected on wax embedded sections of *Taraxacum* leaf using gold conjugated to a secondary antibody. JIM5 binding was localised to the cell walls of the tissue (figure 72(a)). It was also noticed that the strongest binding was toward the tips of the leaf as compared to the middle portion of the leaf (Figure 72(b)).



a



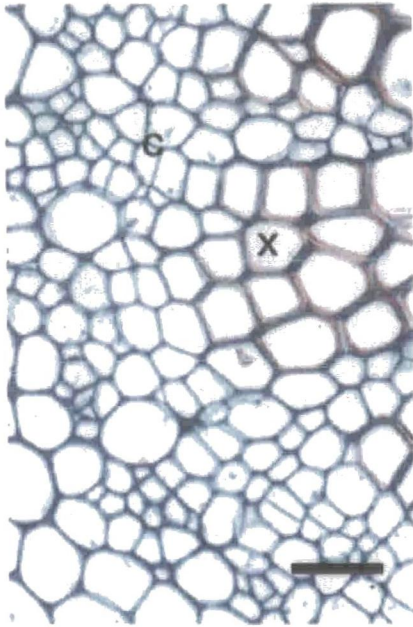
b

Figure 72: Localisation of JIM5 binding in *Taraxacum* leaf. Higher labelling intensity was observed toward the tips of the leaf (a) compared to the middle of the leaf (b). Ep=epidermis, Bar = 30 $\mu$ m.

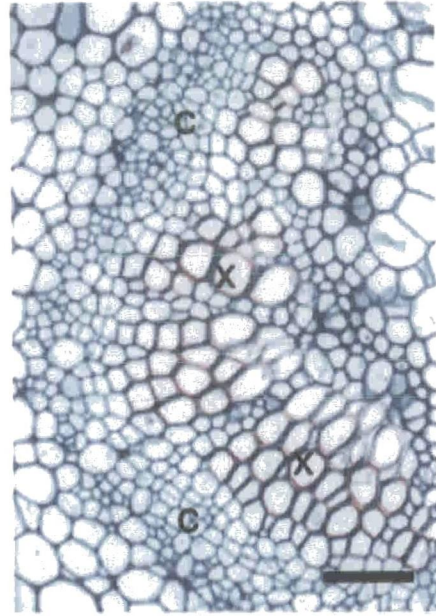
### **3.7 *Euphorbia wulfenii* (Euphorbiaceae)**

#### **3.7.1 Distribution and structure of laticifers**

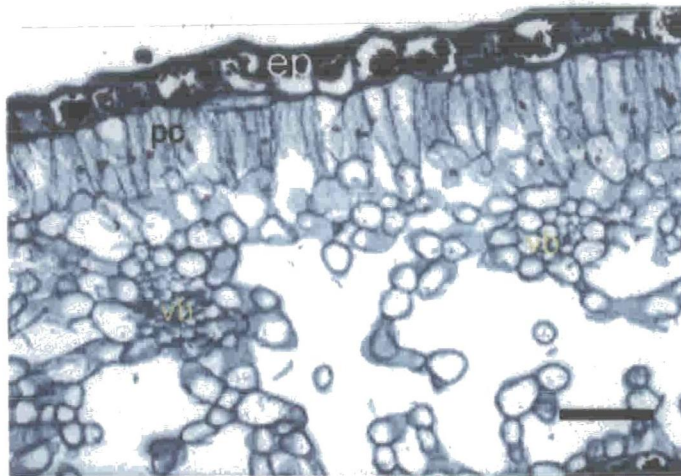
*Euphorbia* stem and leaf showed no traces of laticifers when stained with Safranin O and Astra blue (figure 73). Most of the cells in the cambial region, where the laticifers were expected to be present, were observed to be empty and did not contain any cytoplasmic materials, which supposedly explained why the reagent did not differentially stain well.



a



b



c

Figure 73: Thin section of *Euphorbia* stem (a&b) and leaf (c), stained with Safranin O and Astra blue. Laticifers were not clearly stained, thus making it very difficult to distinguish them from other cells. C= cambial region, x=xylem, vb=vascular bundle, pc=palisade cells, ep=epidermis . Bar a= 30  $\mu\text{m}$ . Bar b & c= 190  $\mu\text{m}$ .

## **4. DISCUSSION**

## 4.1 General

This study was initiated to examine and compare the development of laticifers in a range of species of latex bearing plants from five different families. Emphasis has been made on application of a range of microscopical techniques, and in particular a comparison of the effects of preparation techniques such as the use of different fixatives (including immunofix, osmium tetroxide, and osmium tetroxide plus zinc iodide), and embedding media (wax, resins and low temperature embedding) on tissue preservation for both screening purposes and for study of the development of laticifers. Additionally different staining reagents have also been compared for these purposes, including the use of histological and biochemistry stains. Optical microscopy and electron microscopy (using both scanning electron microscope and transmission electron microscopes) were used in the examination of the cell biology of developing laticifers.

Laticifer distribution in the plant kingdom has been studied quite extensively (Table 1). Most of these studies focused on the distribution and development of laticifers in a particular species. Only a few studies showed suitable technical protocols for identifying laticifers in selected latex-bearing plants (Gomez & Moir 1979, Bruni & Tosi 1980, Jayabalan *et al.* 1992). This study described in this thesis was initiated with the aim of extending understanding of this topic by presenting and comparing observations and interpretations on seven different taxa of latex bearing plants.

At the beginning of the program it had been anticipated that both antisera to specific components of latex synthesis and latex itself would be available for associated immunocytochemical studies. Similarly, it was thought that it would be likely that (in other laboratories) advances in molecular biology would provide

suitable molecular (RNA) probes for examination, by *in situ* hybridization, of the differentiation of laticifers. Unfortunately, in the short term, neither types of probes were available but the comparisons of tissue preparation methods reported here will be of particular relevance once such probes are available.

The discussion considers five main aims concerned with:

- a comparison of tissue preparation techniques for studies of the structure and differentiation of laticifers, and to allow application of molecular probes to advance further studies of differentiation.
- a comparison of tissue preparation and staining techniques for application in the screening of samples in selection programmes.
- the structure of laticifers in different stages of development of *Mecanopsis cambrica*.
- the structure and differentiation of laticifer in different tissues of *Hevea brasiliensis*.
- a comparison of laticifers development in several taxa.

#### **4.2 Different techniques and protocols to determine the location, structure and differentiation of laticifers in plants.**

The techniques chosen and examined were for the most part based on techniques that have been applied and used by others working with latex bearing tissues and that gave good consistent results (e.g. Johansen 1940, Sakai 1973, Wimalaratna 1973, Glauert 1985, Jayabalan *et al.* 1992, Mori & Bellani 1996). Recent developments in microscopical preparation techniques

however suggest that it may be possible to optimize the protocols further, and indeed some techniques used with success on other tissues had not yet been applied to *Hevea* and other taxa mentioned here.

#### 4.2.1 Fixation

Before any plant sample goes through histological or cytological study, it usually has to go through a series of preparative protocols. The first step is the fixation process that will preserve the cells in the first instance after live plant tissues have been sampled. The idea of fixing the samples is to make sure that there is little or no alteration in the subcellular integrity while observations and interpretation are being made. Any alteration or changes that happened in the cells can cause artifacts and thus will give misleading results (Glauert 1985). Fixatives used in this experiment include a single reagent or a combination of chemicals to preserve the plant tissue. A procedure involving minimal chemical association such as cryo-fixative was also used.

In looking into the most suitable fixatives, several criteria were taken into consideration. The first and main concern is to make sure that the fixatives must not in any way cause obvious disruption to the fine structure. The degree of damage can be verified by investigating the fine structure of organelles such as membranes, tubules and ribosomes etc. Ideally it is very important to have control samples where the subcellular organization is in its original stage and unaltered by any kind of chemical treatment (Harris & Oparka 1994). Unfortunately, it is quite difficult to achieve that ideal model and essentially impossible for electron microscopy studies. The closest available technique is probably the cryo-technique where the chemicals used in preparing the sample are less extensive, thus reducing the chance of chemically-induced artifact

formation, if not completely eliminating it. However problems with artifacts from the freezing process may be induced instead. Uwins *et al.* (1993) showed that there were substantial differences in potato cell integrity when fixed with different techniques. He made a comparison between the fresh samples and the samples fixed with the various fixatives including the cryo-technique and noticed artifacts present in almost all of the fixed samples.

In the experiments reported here, fresh (unfixed) samples were used as a reference or standard (figure 1, 33,43-52, 60). The fresh samples were immediately investigated under the optical microscope to observe the overall histological structure. The fresh samples showed the overall distribution of laticifers in the tissues. The identification of laticifers was made easier by the presence of coagulated latex in the laticifers, thus making the fresh samples a useful and quick reference when locating the laticifers in the sections of embedded samples later on. However, special care has to be taken with this technique. The observation could be very misleading since some latex could smear the neighboring cells and thus give false results. Several non-chemical approaches to coagulate latex *in situ* were attempted. Heating methods and even boiling in hot water were used to coagulate the latex but the tissue damage caused was at an unacceptable level for further structural examinations. Attempts with a microwave fixation method (Westcot *et al.* 1993) had mixed success. The technique was applied to various tissues but without consistent results. It is not clear whether this lack of success in coagulating latex without causing extensive damage to the tissues was because the tissue was not suitable for such an approach or because the equipment was insufficiently controllable. There are now some early reports (personal communications) suggesting that microwave fixation may be more effective and consistent if carried out in short (second) bursts using very controlled power, rather than with a typical 'domestic' or even commercial microwave oven.

Investigations in plant anatomy and cytology cannot be completed without involving the fixatives. The most common and important fixatives in electron microscopy study include aldehydes and strong oxidising agents; these are usually prepared in an ionically balanced buffer. Aldehyde fixatives [commonly formaldehyde and glutaraldehyde] work by crosslinking proteins and nucleic acids; the strong oxidizing agents, commonly osmium tetroxide or potassium permanganate, fix unsaturated (fatty acid and ethylenic) bonds (Glauert, 1985). Osmium tetroxide and potassium permanganate also act as stains for electron microscopy since they contain heavy metals.

Wilson *et al.* (1984) noted that laticifer cells, with fluid content of latex, did not fix well using only aldehyde fixatives, probably due to the high hydrocarbon concentration that is characteristic of many latexes. He then resorted to using a freezing technique. This method, however, gave very limited information on the subcellular information since the cells' integrity was not fixed properly. This may in part have been due to the lack of refinement of freezing techniques at that time. He did find however that osmium tetroxide gave a very good lipid immobilization and thus gave some fine structural information. With osmium tetroxide however, the rate of penetration of the fixative into the sample is very slow, thus hindering the optimum structural preservation.

Complementing osmium tetroxide with glutaraldehyde fixation eliminates this shortfall as glutaraldehyde penetrates faster than osmium into the cell. Very good overall fine ultrastructural preservation was achieved with this combination of fixatives (see e.g. figure 22 & 23). Most of the organelles showed good structural preservation, where the membranes looked intact, and there was no or only little occurrence of cell plasmolysis compared to the samples fixed with other fixatives. The combination of fixatives seems to fix the latex in the laticifers quite well. In semithin sections of the resin-embedded samples, laticifers showed latex as dark, granular substances inside them (figure 8 & 16). Whereas in the

ultrathin sections, latex was observed as being very dark when fixed with osmium tetroxide (figure 58) in the case of *Hevea brasiliensis*. Other work showed that in a whole –mounts of freshly exuded latex particles from *Hevea brasiliensis* were observed as dark electron dense particles under the TEM when fixed with osmium tetroxide alone (Gomez & Moir, 1979).

### **Fixation for immunocytochemistry**

It is generally found that fixation protocols involving strong oxidizing agents are not good at preserving antigenicity within samples. As it had been thought that some immunocytochemical studies would be carried out within this programme or at a later stage of my work in Malaysia, attempts were made to optimize an 'immuno fix' protocol. Cell plasmolysis was more noticeable in samples fixed with aldehyde alone (Immunofix) (figure 31 & 70). This fixative was found not to be a ideal as a general fixative, but it appeared to be sufficiently suitable for immunohistochemical studies where structural information is often compromised to retain the antigenic properties of proteins (Coetzee 1985).

Although antisera to latex components were not available, the suitability of the protocol was tested by immunohistochemistry study on plant samples fixed with immunofix and using an antibody to a cell wall protein (figure 72). The immunostaining resulted in a positive and specific reaction indicating that the sample preparation method would have wider applicability to my further studies of *Hevea laticifers*.

In further work with antibodies to *Hevea* components it may be possible to improve the structural integrity within the samples by increasing the 'strength' of aldehyde fixation [either by longer fixation or by use of a more concentrated

solution] without loss of antigenicity, and/or modifying the buffer to reduce any plasmolysis.

### **Special fixation techniques**

The site of synthesis of latex is still the subject of discussion. Suggestions for the site of synthesis include plastids, or modifications of them, and the endomembrane system (endoplasmic reticulum, Golgi apparatus, various vesicles and vacuoles (Harris, 1986), or part of it. In addition to the combination of conventional fixatives described above, special modifications have been made to the above fixatives in order to get better results and observations on certain specialized organelles. Zinc iodide was added to the osmium tetroxide to generate the fixation/stain complex ZIO. This is particularly effective in contrasting endoplasmic reticulum, the nuclear envelope, Golgi apparatus, plastid thylakoids and cristae of mitochondria (Marty, 1973 & 1978; Hawes, 1994). In this case, the samples fixed with this combination of fixatives showed endoplasmic reticulum, the tonoplast and occasionally the Golgi apparatus very well (figure 29 & 30). The image contrast was very good, making the observation of the endoplasmic reticulum quite easy because of its dark appearance. In most ZIO impregnated samples, the tonoplast appeared extremely dark, almost obscuring other organelles. This is not common in all plant tissues; often the tonoplast remains unstained. It is probable that the ZIO reacted with and precipitated a vacuolar component on to the membrane. Even on the semithin section, the dark appearance of the impregnated organelles showed up clearly in some cells against others in which organelles were not impregnated.

## **Freeze fixation**

An alternative to chemical fixation with all of its associated problems is freezing or cryo-fixation. The aim here is to fix the tissue sufficiently quickly to retain the life-like structure without the development of ice crystals. Ice crystals would inevitably cause considerable damage by their expansion, tearing through membranes and organelles. Wilson *et al.* (1984) used cryofixation of latex bearing tissues but with only limited success. Since then there have been significant advances in both freezing (to improve rates of freezing and reduce ice damage) to subsequent handling (to avoid partial thawing and/or condensation onto the specimen) and media for infiltration of the tissues after freezing.

The protocol described above, despite these advances, still gave only modest quality of preservation. This may be because the tissue pieces were relatively large; this is required to avoid cutting damage to the long laticifers, but with larger pieces of tissues the rate of freezing is slower and hence there is more ice formation.

## **Optimising fixation**

Generally all the above fixatives managed to give acceptable results for identification and distribution of laticifers in the samples, with the exception for the microwave technique. Unfixed samples could give fast and quite reliable results. Samples could be viewed and results can be achieved within the same day of sampling. Chemical fixation however involves a considerable amount of time in the preparation procedure. Aldehyde fixatives which was initially intended

for the immunostudy, showed promising results in term of ultrastructural information provided. But the most superior fixatives in this study that give good structural information, both for the optical and electron microscope were the combinations of aldehydes and osmium tetroxide, and of osmium tetroxide with zinc iodide.

The above studies optimizing the fixation protocols for the different types and needs of structural studies at optical and electron microscopy, and for immunocytochemistry in particular, will be very useful in our future work in Malaysia, on the comparative development of *Hevea* and its latex vessels.

#### **4.2.2 Embedding media**

Several embedding media have been used in this study to see which would give the best results in terms both structural and ultrastructural information. The embedding media used were wax for the light microscopy observation, and Araldite, Spurr resin and LR-White for the electron microscopy observation and light microscopy observation. Smith & Croft (1991) noted that the ideal embedding medium should have low viscosity, will polymerize uniformly without shrinkage, will permit the use of various stains, is easy to section and is stable in the electron beam. Of the resins used Araldite is the longest established. This resin is based on epoxy components that are both very viscous and hydrophobic, and so not very compatible with cells constituents. The polymerized resin is however very stable even when cut in ultra thin sections for electron microscopy. Spurr resin is also based on epoxy resins but with a formulation giving a much more fluid liquid for impregnation into tissues. It was designed specifically for work with plant tissues where infiltration rates are reduced by the presence of the plant cell walls. LR White resin is acrylic based,

very fluid in comparison to the epoxy resins and it has the major advantage of being hydrophilic. It does not however have the same high degree of stability in the electron microscope.

### **Wax embedding**

In these experiments Paraplast wax was used to embed the samples primarily for histological study. However wax has wider application and is not limited to histological study only. The medium was used extensively as an embedding medium for immunohistochemistry studies as well (Roberts 1994; Schumacher 1995; Vitha 1997). In most samples, laticifer cells appeared well-defined in terms of their shape and structure with wax embedded samples (figure 63, 67,68 & 69). The long embedding time incurred in the procedure has apparently no destructive effect on laticifer cellular integrity in the samples.

The subsequent staining procedures, which involve quite a lengthy process where samples have to be dewaxed first and taken through series of ethanol, hinder a quick evaluation/observation on the quality of the sections. However, sections after removing the wax, can be viewed unstained with Differential Interference Contrast (DIC) and gave a good, almost 3D, image. Laticifer cell walls can be observed quite clearly with this technique and in some sections cell wall breakdown was clearly seen too (figure 18,19 & 20). Laticifer cell articulation can also be observed in this instance. It was observed that this technique (DIC) was not only suitable with wax embedded samples (after dewaxed), but also showed good results with other embedding media (unstained samples).

## Epoxy resin embedding

A particular advantage of epoxy resins is that they can be polymerized uniformly with little change in volume. They are also relatively quite stable in the electron beam and thus suitable for a high contrast study. However the main disadvantage is their high viscosity, which makes the infiltration process quite lengthy compared with the other embedding media (Roberts & Hutcheson 1975). Other reports on the applicability of this resin with the plant organelles, showed that the resin has a very good crosslinking with the lipids, starch and other organelles, and thus gave good overall structural information (Kosakai 1973; Bronner 1975; Amelee 1976).

It was found that samples embedded with this resin experienced less electron damage compared to the other embedding media. In semithin sections, laticifers were observed to look normal in terms of shape and size, indicating that there was no chemical interference between the fixatives and the resin that might have an effect to the cell integrity (figure 22-30 & 35). Despite a lengthy processing procedure, that was compensated by the strong support under the electron beam and good ultrastructural information obtained from the section.

Spurr resin on the other hand has a slightly different behavior in terms of structural information provided from the samples embedded. Spurr resin is one of the two epoxy resins apart from araldite resin, used in this experiment. The difference between these two epoxy resins lies in the viscosity of the resin. Compared with Araldite, Spurr resin has a very low viscosity, which allows it to penetrate the plant samples quicker (Spurr 1969). This is found to be quite useful when time is a priority and structural information can be compromised. However, strict precautions have to be taken when handling this resin as it contains carcinogenic substances. In the laboratories it is essential to always

wear 2 pairs of gloves as an extra precaution. Several reports on the accidental skin contact with the resin have shown that it can caused immediate skin irritation (Melan 1994, Oliver 1994).

In general, laticifers were observed to be well preserved in sections of samples embedded in this resin (figure 11-17 & 37). Even though, the ultrastructure of the organelles was not so well preserved as samples embedded in the araldite, it was still considered to give a very good overall preservation of the cells. Peterson *et al.* (1978) observed that cellular preservation on dried herbarium specimens embedded with this resin was good. There were, however, a few cell artifacts where the cell walls of the laticifers had broken and the membrane of certain organelles such as the ER was not as clearly defined as in the araldite embedded samples (figure 38 & 39). Even though no immunohistochemistry study was carried out with the samples embedded with this resin, other studies on this resin showed that it is capable of giving good results with tobacco leaves (Berg *et al.* 1988; Stroobants *et al.* 1990).

### **Acrylic resin**

LR White was the least viscous compared with the Spurr and araldite resins, thus making the time taken for resin infiltration into the cell shorter. The other advantage of this resin is that it is miscible with water, so there was no need to use intermediate solvent in the procedure, although there several reports indicating that use of some alcohol as an intermediate can improve tissue structure preservation. However, it has a major disadvantage as an embedding media. Reid (1974) noted that this type of resin will polymerize with considerable shrinkage and is unstable in the electron beam. In the work reported here it was apparent that the stability of the resin depends on the amount of electron beam

energy bombarded onto the resin. The higher the energy used, the more unstable the resin will be, and the greater the chance of the resin breaking or tearing.

Histological studies carried out on the laticifers in the samples embedded with this resin showed good overall structural preservation in terms of cell integrity (figure 2 & 3). On some sections, latex in the laticifers can be seen as well. Reports on the ability of this resin to give good ultrastructural preservation have been provided by other workers (Craig & Miller 1984, Evans *et al.* 1997). Attempts were also made to do immunohistochemical study on the samples embedded with this resin. Good results were achieved with an antibody to a cell wall component.

### **Optimising embedding**

Wax embedded samples gave overall good structural information in light microscopy study. The preparation procedure was also quite user-friendly. However, from the results presented here, araldite is a good choice if good structural information is needed, both for histological or cytological study and particularly for ultrastructural studies. However, other embedding media were also suitable for some purposes. Spurr resin for example, gave good ultrastructural information as well but not as good as araldite, but for safety reasons (carcinogenic substances) it is not preferable to be routinely used in the laboratories. In the case of samples embedded in LR White resin, structural information was not as good. This embedding medium however was very good if immunocytochemistry studies were to be carried out

The above observation gave some light on the applicability of embedding media to the laticifers structural information in samples. The study will be very valuable especially for the future study on laticifers in *Hevea* that will be carried out in Malaysia.

#### 4.2.3 Staining

Staining procedures were chosen and monitored for the suitability with the lipid and cell contents of the laticiferous cells in plants. Several histological stains and cytochemical stains were used in this experiment to achieve the objective. They are toluidine blue, Safranin O and Astra blue, Calcofluor, ANS (8-anilino-1-naphthalene sulphonic acid), Acridine orange, and Oil Red O and Dansyl Chloride.

Staining of the biological sections has many purposes. It may be solely to achieve sufficient contrast for ease of studying cellular structures, either for general staining or selective staining (Lewis and Knight 1986). Selective staining is very useful for the biologist carrying out quantitative studies on cells. The other reason for the staining of biological materials is to determine the chemical composition of the cell at the structure level. In this experiment, we concentrated on observing the selective stain of several staining reagents on laticifers for qualitative studies only.

Amongst of the first stains used for trying to visualize the laticifers in resin-embedded samples was toluidine blue. Toluidine blue is a metachromatic stain, which can change its colour without changing its chemical structure (Horobin *et al.* 1990, Kiernan 1990). This is due to the molecular stacking of the dye molecules, and the phenomenon is influenced by changes in water content, concentration and solvent, pH and temperature. The stain was observed to show

the laticifers quite well. Vacuoles were also stained aqua blue with this stain (figure 2-17, 33-38). This stain showed that it would not differentially stain the laticifers from other cells organelles and structures. Xi & Burnett (1997) showed that this stain nevertheless, could differentially stain fungal structures of *Rhynchosporium secalis* from barley seed and leaf tissue. Whereas Graham & Joshi (1996) used this stain with Bismarck brown to show the delineation of cell walls of the epidermis in rose and flowering dogwood. It was also noticed that some other organelles such as starch grains were also stained dark blue (figure 15). Since the stain did not differentially stain the laticifers and other cell structure, the irregular shape and sometimes the vacuolar content of the laticifers were used to identify the cells. It is though still a good general stain for histological study. Safranin O and Astra blue, which are also metachromatic stains, showed different staining behavior. Warmke & Lee (1976) noted that cell walls were stained quite well with this stain. Laticifers were clearly shown up with this staining reagent, especially latex, where they were stained dark red (figure 63, 67-69). Even though other cells such as tannin cells were also stained red, latex could be distinguished within the resin by its granular structure compared to tannin. Other parenchyma cells were stained blue. Xylem was stained red as well but was quite easily recognised and distinguished from other cells by its thick lignified cell walls. This stain in general is a good stain for laticifer cells since it can differentially stain it from other cells. Nevertheless, this stain took longer to complete the staining procedure as compared to Toluidine blue stain.

Apart from Toluidine blue, Safranin O and Astra blue there were other types of stains that were used in this experiment for the histologies study; these were fluorescent stains. Instead of staining non-living materials (fixed-embedded samples), these stains were able to stain fresh/living cells and this gave the advantage over the other histological stains, because results can be achieved in a shorter time with less risk of artifacts from the fixation, dehydration and embedding procedures. These stains are also known as vital stains, and they

were normally applied to the sections with a very low concentrations (0.01% w/v) in order to minimize disruption to the material (Horobin 1990). In this experiment, calcofluor, ANS, Acridine orange, and Oil Red O and Dansyl chloride were used. Samples stained with calcofluor gave an overall bluish color to the cell walls when observed under the fluorescent microscope with a UV filter (figure 1a). The stain, however, did not stain the laticifers cells and latex very well. Combining UV epifluorescent illumination with bright field [trans] illumination though, gave a good indication of the laticifers' location in the sample (figure 1b, figure 33). Still the stain gave a good general outline of the cell walls and lignified cells such as xylem in the tissue (Smith & McCully 1978). In the case of ANS, it tends to bind to the hydrophobic molecules. With this stain, the overall cells in the fresh samples were observed to be greenish in colour under the epi-fluorescent microscope (figure 43-47). Coagulated latex was stained with this stain (figure 44). This stain has also been found to be a sensitive vascular stain (reviewed by Gates & Oparka 1982), especially in the cleared leaf samples (figure 47). Similar results were also observed with Acridine orange. This stain has the ability to stain intracellular RNA and DNA. This reagent stained the cell walls and lignified cells very well and gave an overall orangish colour to the samples (figure 48-52). Latex can be distinguished by bright orange dots in the cells, even though there was no cell wall outline visible (figure 49). This stain was also observed to be a good vascular stain (figure 52). Oil red O and Dansyl chloride stain also gave similar results as the Acridine orange, where only the vascular tissues were stained properly (unpublished result).

## Optimising staining

In general, almost all the stains used in this experiment showed little differential staining to the laticifers cells or latex in the samples. However, they can be used as a general stains in helping to locate the laticifer distribution in the tissue. Toluidine blue stain was a very fast stain for viewing the results especially in the resin embedded samples. The staining protocols involved a very simple and easy staining procedure. Safranin O and Astra blue stain, which were used for the wax embedded samples, involve a quite lengthy procedure. However, since this stain was a double stain, differential staining was observed in the samples, sometimes making the identification of certain cells easier.

Other stains that were used with the fluorescent microscope such as the Calcofluor, ANS and Acridine orange can be used as a general cell wall stain. Using these stains with the combination of fluorescent and bright field technique can give good and sometime interesting results on the location and distribution of laticifers in the samples.

These optimized staining technique will be used and further refined for *Hevea* study that will be carried out in Malaysia. Fluorescence stains were in particular very promising and will be fine tuned further to achieve better results.

### **4.2.4 The use of microscopy techniques to visualize the laticifers in plant tissues.**

Optical and electron microscopy have been used as supplementary tools in assisting botanists looking at plant cells. New techniques were created and

existing techniques were modified to achieve certain objective in research such as the used of microwave oven to fix plant tissues etc. Several microscopical techniques are reported here in an attempt to obtain a better view of laticifers in the plant tissues, especially in optical microscopy. The most notable success was the bright field illumination with the fresh samples where coagulated latex in the laticifers can be easily detected by a black mass in the cells (figure 1b, 33). It was a very convenient technique, where results can be viewed rapidly. However, this technique can only be applied to samples where latex has the ability to coagulate and is retained in the laticifers. Good results were obtained using this technique on *Meconopsis cambrica*, *Papaver rhoeas*, *Musa acuminata* and *Taraxacum officinale*. However, in *Hevea brasiliensis* and *Euphorbia wulfenii*, even though latex was coagulated, and some was retained in the laticifers, results were not as good as the other samples. This was because both samples (*Hevea* and *Euphorbia*) exuded so much latex when cut that the latex smeared and obscured other cells as well, not only making it difficult to view the sample but also giving misleading results as well.

The other technique that was applied was the Differential Interference Contrast (DIC) or Nomarski technique. A Nomarski technique gives the illusion of a 3D effect and improves upon the resolution of what can be seen when looking at the unstained samples, due to the optical sectioning of the specimen by the illumination. This effect is basically achieved when the light is first polarised and then passed through a prism, which splits the light up into two beams and then rotates them so they are at right angles to each other; the light then travels through the specimen and up to the objective where the beams recombine and interfere with each other. Shaw & Rawlins (1994) observed that this technique is particularly good at revealing edges in biological structures such as organelle and nuclear boundaries, cell boundaries and cell walls. Observations reported here showed that the cell walls outline was shown very nicely with this technique (figure 18,19,20). The cell wall breaking down in some of laticifers cells were noticed as well (figure 20). This technique was, however, not able to

demonstrate the outline of the latex (vacuoles) in the laticifers. Because there was no staining involved in this procedure, there was hardly any colour variation observed from the samples.

#### **4.2.5 Summary on the evaluation of light microscopy techniques for screening breeding material**

One of the aims was to identify light microscopy preparation techniques that could be used to identify laticifers numbers and distribution in breeding lines. Such a technique would need to be rapid, reliable, clear for identification and give good structural preservation. The preparation techniques and protocols used are summarized below, and the strength and weakness of each techniques and protocols analyzed.

##### **Fixation**

Unfixed and freshly cut tissues, have the ability to be used as a quick reference for the distribution of laticifers in the tissue. The time taken for the preparation protocol was very short and result can be viewed very fast. The only downside with this technique was when used with plants in which the laticifers exuded too much latex, which could smear the neighbouring cells and obscure the laticifers themselves. But this shortfall could be minimized if latex in the laticifers coagulated. The samples have to be disposed of after the investigation and could not be kept for archiving and further investigation.

An easy and relatively quick immunofix protocol was developed. Preservation of the plant cells was reasonable and results were consistent. It is anticipated that further immuno studies will be carried out with samples fixed with this fixative.

Osmium tetroxide and the combination of osmium tetroxide with zinc iodide, on the other hand, were quite cumbersome and involved a more complex preparational procedure. The structural information obtained was far better than in the samples fixed with immunofix, but considering the time involved and not so user-friendly, both osmium tetroxide and a combination with zinc iodide were only good and suitable for the purpose if details of ultrastructural information were the priority.

## **Embedding**

Wax, and Araldite and Spurr resins were all good embedding media, which were capable of giving reliable results. In terms of ease of use and the length of time taken to prepare the samples for viewing, wax has the advantage over the other two resins. Furthermore, samples embedded in wax were easier and quicker to section especially if serial sections were required. Additionally, epoxy resins were quite harmful especially Spurr's, which was carcinogenic.

## **Staining**

Staining procedure in this study can be categorized into two main groups. First were the stains for samples with no embedding media and used for fluorescent

work such as Calcofluor, ANS, Acridine orange and Oil Red O with Dansyl chloride. Secondly there were the stains for embedded samples such as Toluidine blue and Safranin O and Astra blue. All the stains for the un-embedded samples were capable of giving fast and reliable results. Even though none of the stains mentioned were very specific to the latex of laticiferous cells, however these general cell wall stains help to enhance identification. . Toluidine blue and Safranin O plus Astra blue have also shown the capability of giving good, consistent and accurate results. The only downside particularly with Safranin O and Astra blue was that they take longer in the staining procedure, where the samples has to be dewaxed, rehydrated and then stained with a two-step staining before results could be viewed under the light microscope.

On balance, the most suitable preparation technique for the rapid screening of breeding material would seem to be the examination of fresh samples and staining with fluorescent stains (which would probably require further refinement in technique). This is especially vital if a group or a big batch of samples were to be screened and a fast result is required for breeding purposes. Another major advantage is that this staining procedure does not require much technical knowledge and is very simple to use even by an inexperienced laboratory assistant. Moreover, this procedure can also be carried out in the field and in many isolated rubber estates (in a mobile lab) where usually proper facilities for anatomy study are not available.

### **4.3 Structure of laticifers in different stages of development in *Meconopsis cambrica* ; changes during tissue aging**

Tissues from plants at different stages of growth were compared, and some structural differences of cell component were found in relation to their stage of development. There are several changes in the shape, sizes and structure of organelles in the laticiferous cells that go through this developmental process. In this work particular attention was paid to some specific organelles. Emphasis was on the observation of the development and changes taking place in components of the endomembrane system e.g. vacuoles, the endoplasmic reticulum, to laticifer cell walls and to the presence or absence of plastids or plastid type structures.

#### **4.3.1 Endoplasmic reticulum**

As early as 1960s endoplasmic reticulum (ER) was discovered to be part of the secretory pathway in eukaryotic cells. In contrast to animal cells where secretion is typically extracellular, in plants the secretory pathway can target and deposit materials to both the outside of the cells and to specific organelles e.g. the vacuoles within the cells. In the secretory pathway, proteins travel from the ER through the Golgi apparatus to arrive at the cell surface or at the vacuoles (Pallade 1975, Denecke *et al.* 1990). The process involves protein biosynthesis and bi-directional protein traffic; and endocytosis of extracellular molecules as well as recycling of membranes and proteins to maintain the cell integrity (Crofts *et al.* 1999, Vitale & Denecke 1999). ER is a network of tubules, vesicles and sacs that are interconnected. They may serve specialized functions in the cell including protein synthesis, production of hormones , production and deposition

of storage compounds, and insertion of membrane proteins (Albert *et al.* 1994). There are two types of endoplasmic reticulum, the rough and the smooth. Rough endoplasmic reticulum bears the ribosomes during protein synthesis. The newly synthesized proteins are secreted into the cisternae. The system then sends the proteins via small vesicles to the Golgi complex, or, in the case of membrane proteins, it inserts them into the membrane. Rough endoplasmic reticulum may either be vesicular or tubular, or it may consist of stacks of flattened cisternae that may have bridging areas connecting the individual sheets. The ribosomes sit on the outer surfaces of the sacs (or cisternae). In plants smooth ER is associated with lipid synthesis, and synthesis and transport of e.g. nectar (Robards 1988).

Nessler (1982) observed that the differentiation of laticifer initials in *Glaucium flavum* (Papaveraceae) was characterized by the proliferation of numerous vesicles from dilation of endoplasmic reticulum. Experiments reported here show that laticifers of young developing *Meconopsis* showed the presence of numerous ER. These ER were observed to be apparently actively involved in vacuole biogenesis, where small vacuoles were seen closely associated and located very close to them (figure 24, 25). Micrographs indicate that the small vacuoles were replaced by larger ones and that these finally coalesce to form the bigger vacuoles. Golgi apparatus, which are involved in biogenesis processes (Nebenfuhr *et al.* 1999), were also present, normally close to the ER (figure 24). The dilation of ER membrane was clearly seen, which indicated the initial stage of vacuoles' synthesis. ER can be seen drawn together parallel to the longitudinal cell axis of the cell wall. In the older samples however, ER were quite scarce or absent. This might correlate with the reduced amount of vacuoles present in the older laticifers vessels. This might also suggested that at this stage (ageing stage) the biosynthesis of vacuoles was becoming slower or had entirely stopped.

### 4.3.2 Vacuoles

The vacuoles of plant cells are multifunctional organelles of plant development. (Höfte & Chrispeels 1992; Hara-Nishimura *et al.* 1998; Herman & Larkins 1999). They are lytic compartments and have a function of reservoirs for ions and metabolites, including pigments, and are crucial to processes of detoxification and general cell homeostasis. They are also the most conspicuous compartments in most plant cells. They may consist of one or more very large vesicles, which are separated from the cytoplasm by a single membrane called the tonoplast (Albert *et al.*, 1994).

In *Meconopsis cambrica* vacuoles are very prominent organelles in the laticifers, in both young and old samples. However, different compositions in the vacuoles of young and old samples were observed at two different stages of development. Francis (1999) noted that plant cell vacuoles are widely diverse in form, size, content, and functional dynamics, and a single cell may contain more than one kind of vacuole. I observed that the vacuoles in the young sample in particular, were occupied with a dense net-like structure (figure 22), which is not observed in the vacuoles of the old sample (figure 30). It was also noticed that, as the plant grew, the number of vacuoles in the laticifers increased and their arrangement became more compact, compared to the younger plant (figure 29). In most cases the vacuoles occupied more than an estimated 80% of the laticifers in the older samples (figure 23).

Vacuoles arise initially in young dividing cells by the fusion of vesicles derived from both the endoplasmic reticulum and the Golgi apparatus (Hohl *et al.* 1996; Hinz *et al.* 1999). Esau & Kosakai (1975) noted that in *Nelumbo nucifera*, laticifers contained numerous small vacuoles, or vesicles, which later merge with the central vacuole by an autophagy process. In the case of *Meconopsis*, It was

observed that both the formation of vesicles from the dilation of endoplasmic reticulum and from the Golgi apparatus, especially in the young sample (figure 24 & 25).

Even though it has been commonly assumed that all vacuoles have the same origin and belong to a common group, there are major differences amongst them morphologically (Chrispeels 1991; Okita & Rogers 1996). With improvements in cell fractionation and biochemical analyses as well as in the use of new molecular probes, it has become possible to characterize specialized vacuolar compartments in the cells from a variety of tissues (Hoh *et al.* 1995; Paris *et al.* 1996; Webb *et al.* 1999). Other than just filling the space of the cells, and transporting substances in the cells, the vacuoles also act as storage compartments in plants. In some plants, the vacuoles of certain specialized cells contain such interesting secondary plant products as rubber (as in *Hevea* and *Euphorbia*) and opium (as in the *Papaver* family) (Nessler & Mahlberg 1978).

The micrographs suggest a relationship between the ER [a possible site of pro latex synthesis] and the vacuoles [where latex is accumulated]. Specific contents of the vacuole and their substances in *Meconopsis cambrica* (and other species that having laticifers with vacuoles i.e. *Papaver rhoeas*, *Taraxacum officinale* etc.) can only be determined by doing a proper biochemical assay and molecular assessment. It is, however, notoriously difficult to prepare clean, intact vacuoles for analysis of their content, but the future availability of antibodies to latex components and enzymes involved in synthesis could, by use of immunocytochemistry, further our understanding of the process of latex deposition.

### 4.3.3 Cell walls

Attention has been paid to cell wall form, as it is apparent that during differentiation of laticifers there is, at the same time, both deposition of material to the lateral/vertical walls of the elongating tubular cells, and also breakdown of the end walls between the cells to form the extensive laticifer networks. Growing plant cells are shaped by an extensible wall that is a complex amalgam of cellulose microfibrils bonded noncovalently to a matrix of hemicelluloses, pectins, and structural proteins (Cosgrove 1997). The plant cell wall is a specialized form of extracellular matrix that is closely applied to the external surface of the plant cell plasma membrane. Moreover, the cell wall is involved in cell division, growth, cell differentiation and defense against microorganisms (Keller 1993; Showalter 1993; Müsel *et al.* 1997). The primary wall is present in all cells but a secondary wall is present in only certain cells (Mauseth 1988; Carpita & Gilbert 1993). The cell walls of young growing plant cells are thinner and are only semirigid to accommodate their future expansion (Waldron & Selvendran 1992; Stolle-Smits *et al.* 1999). Nunan *et al.* (1998) monitored the development of cell walls in grape berries and observed differences in the thickness of the cell wall from before ripening through to the final ripe berry and noticed no significant changes in the cell wall thickness, but protein content increased.

Difference of cell wall thickness in two developmental stages in *Meconopsis cambrica* are reported above. Cell walls of young *Meconopsis* showed cellulose microfibrils were arranged very loosely (figure 26a). This will accommodate the cell expansion during the growth. Whereas in the older sample, thickening of cell walls was obvious with a new deposit of more densely packed fibrils forming a secondary cell wall were observed (figure 26b). Nessler & Mahlberg (1977)

noted that in *Papaver somniferum*, the thinning of laticifer cell walls occurred progressively and simultaneously on the wall on either side of the middle lamella and this suggests that wall-degrading enzymes, rather than pressure, may be involved in the process. Most laticifers in the old sample can be seen still retaining the primary cell wall (figure 23, 27, 28, 30). Nevertheless, it is hard to say, at this point how and where the change is taking place without further biochemical study.

Microtubules were also noticed very closely associated with the cell wall in some sections (figure 25). Other workers have shown that microtubules are involved in co-ordinating mitosis, cytokinesis and the guidance of cellulose synthase complexes during cell wall deposition (Hepler & Hush 1996; Foissner & Wasteneay 1999). Similarly microtubules may play some role in the regulated programme of death in specific cells e.g. xylem and the formation of tubular transport networks (Greenberg, 1997; Whetton *et al.* 1998; Cassab 1998; Scheres & Benfey 1999).

There was some indication of cell wall breakdown occurring in the samples (e.g. figure 42). It was believed that with this action adjacent cells may merge and form a bigger laticiferous cell. Results also showed the position of cell wall breakdown not only adjacent to the laticiferous cell but close to other parenchyma cells as well (figure 41). This process could turn the joining parenchyma cells into laticiferous cells eventually. Other workers who have worked on cell wall degradation have shown that proteolytic enzymes are involved in the process (Giordani & Noat 1988; Chye & Cheung 1995; Subroto *et al.* 1996). The most notable enzyme involved in the early stages of this process was pectinase (Ward & Moo-Young 1989; Capek 1995; Ebbelaar *et al.* 1996).

#### 4.4 Laticifers distribution in different part of rubber bearing plants

Using the combination of the above-mentioned techniques, attempts were made to establish the location and distribution of laticifers in different parts of *Hevea brasiliensis*. Two staining reagents were used in accessing the results, ANS and Acridine orange were applied to the fresh samples.

ANS binds to the hydrophobic molecules; it stained the fresh samples and gave an overall greenish appearance when observed under epi-fluorescence microscope. Latex was stained well in the seed cotyledon of *Hevea* and showed as numerous bright green spots throughout the section. Latex was scattered in the outer region of the vascular bundle (figure 44). However, in the root and petiole, latex was weakly stained and could be observed located in the cambial region of the vascular bundle (figure 44 & 46). Apart from those parts of the plants that showed the distribution of latex in the cambial region, the others did not show a very constant and conclusive result on the whereabouts of laticifers in the samples. Attempts to apply the staining reagent at different dilutions and different length of time to the samples did not improve results. Leaf tissues were particularly difficult to handle, especially when it came to peeling the cuticles and trying to observe the laticifers under the microscope. Even if the laticifers were stained, the view was hindered by the position of the laticifers, which were sandwiched in-between the vascular tissues (figure 47). The vascular elements were, however, outlined very well with the staining reagent. It was concluded that, during cutting and sectioning, the latex oozed out from the laticifers as a result of turgor pressure and left no latex for the reagent to stain.

Samples stained with Acridine orange, showed almost a similar pattern of results obtained with ANS. Seed cotyledon showed latex was stained brightly with the latex marked as bright dots (figure 49). There were no apparent lignified cells present in the samples. Latex in the root was weakly stained and could be seen in the cambial region of the vascular bundle (figure 51). Vascular elements in the leaf were clearly defined and outlined (figure 52). For the same reason as the above, it was concluded that, during cutting and sectioning, the latex oozed out from the laticifers in the larger organs [leaves and roots] as a result of turgor pressure and left no latex for the reagent to stain.

In short, laticifer distribution in some parts of the plant could be determined by use of these two staining reagents. Even though most of the parts did not show very conclusive results in term of latex staining, the distribution of the laticifers cells can still be determined by other means such as the shape and relative size of the laticifers in the tissues, with the help of these stains.

## **4.5 Laticifers in several latex bearing plants**

### **4.5.1 *Meconopsis cambrica***

In general, all members of Papaveraceae have been reported to have articulate laticifers (Metcalfe & Chalk 1989). Some are articulated anastomosing and the others are articulated non-anastomosing. Laticifers in *Meconopsis* are of articulated anastomosing type as recorded by Kapoor and Sharma (1963).

It was observed that laticifers in *Meconopsis cambrica* occur in the outer region of the xylem, in both young and old samples (figure 1-20). It was often difficult

to distinguish laticifers from other cells and this is especially true with embedded samples. Unlike the fresh cut sample, some embedded samples did not retain the latex inside the laticifers, thus making the identification of the laticifers quite difficult. In the fresh samples, laticifers can be easily recognized in thick section of fresh cut whole stem, as prepared for observation by light microscopy. The presence of coagulated latex inside the laticifers helped in identifying the location of laticifers in the cells (figure 1). Examination under the electron microscopy further confirmed that these cells are laticifers (figure 21).

Another interesting observation, especially in transverse section, was that the shape of the laticiferous cells was more likely to be irregular as compare to other surrounding parenchyma cells (figure 2,3). This feature can sometimes be exploited as a quick indicator as to where the laticiferous cell is. Even though this observation was not so conclusive and precise, it helped in the elimination process during the initial investigation to locate and identify the whereabouts of the laticifers in the cells.

However this irregularity of shape of laticifers was not totally a coincidence since some work has shown that during the development of the laticifers, cell wall breakdown occurred (Ward & Moo-young 1989; Ebelaar *et al.* 1996). This process was assisted by the enzymatic activities, especially by the pectinase. As the plant grew, more cell walls broke down thus joining the neighboring cells to form a bigger cell network. The process of cell wall breaking down and several cells undergoing the similar activities, contributed in reshaping the laticiferous cell, either into irregularly shape cells or in terms of cell size, becoming much bigger than the initial cell. This process also made the laticifers anastomose throughout the entire plant.

#### **4.5.2 *Papaver rhoeas***

The distribution and pattern of laticifers in *Papaver rhoeas* shows little difference from that of *Meconopsis cambrica*. The laticifers, even though quite difficult to be distinguished from other cells, have a very distinct characteristic, which is the irregularity of their shapes (figure 33, 35, 37). They are arranged and confined to the cambial region of the vascular bundle and are almost a regular characteristic in this genus as described by Nessler & Mahlberg (1976).

However, it is noteworthy to mention that something resembling vacuoles was also observed in the xylem cells, which was not observed in *Meconopsis*. Initially when observing the fresh samples. It was thought it might be a latex smear from the neighboring laticifers, however the observation from the semithin section of the same sample verifies that there are vacuoles inside the xylem (figure 35 & 36). This might suggest that during cutting or sectioning, latex smeared into the xylem or the xylem itself might still be undergoing the process of xylogenesis.

#### **4.5.3 *Musa acuminata***

In this study, we concentrated on the leaf of the plant to see the distribution of the laticifers partly because the latex seems to be more prominent in that part. There were several difficulties in an attempt to observe laticiferous cells in *Musa* leaves, especially when trying to erect the thin lamina of the leaf onto the slide for viewing under the optical microscope. Most results from the attempts showed

either the stain did not infiltrate well thus giving less contrast than other samples, or the image itself was out of focus due to the angle of the leaf not being perpendicular to the slide surface (figure 60).

Laticifers in banana have been reported to bear many similarities to other laticifers in the plant kingdom (Fahn, 1979) and are present in almost all parts of the plant except the root (Skutch, 1932). A study by Kallarackal *et al.* (1986) showed that laticifers in *Musa* were always associated with the vascular bundle. In resin embedded samples, however, latex can be easily recognized as a dark, grainy substance in the laticifers (figure 61 & 62) and can be observed located in the middle of the leaf lamina near to the vascular bundle. The dark appearance was due to the heavy metal impregnated from the osmium tetroxide fixative. While in the wax embedded samples and stained with safranin O and Astra blue, the latex was observed quite distinctively from the other cells by its bright reddish colour (see figure 63).

At a higher magnification using a scanning electron microscope, laticifers were revealed as containing numerous small globules, packed inside the laticiferous cells (figure 64). It was also observed that the size of the latex varies. This observation was illustrated well in the transmission electron microscope (figure 65). From the micrograph, it was also noticed that some small globules coalescing with each other's to form a bigger globules.

#### 4.5.4 *Hevea brasiliensis*

It was reported that latex is produced from all major parts of *Hevea brasiliensis* (Gomez & Moir 1979; Gomez 1982). Laticifers were observed to be present in almost all parts of the plant. Fresh section from all parts of *Hevea* produced white milky latex, which turned pale to yellowish after some time. Latex will coagulate if left at room temperature. Histochemical studies of laticifers showed that latex was observed in the root (figure 45), leaf cotyledon (figure 44), petiole (figure 46) leaf (figure 47) and the stem (figure 43). Laticifers that were observed in those parts of the plants were located in the cambial region of the tissue. Even though in some of the parts, the degree of staining was not as strong as would have been expected, latex can be seen as a more intense green dot in the cells if stained with the ANS, and bright red with Acridine orange (figure 49). The locations of laticifers with the histochemical stains were confirmed by the results of the resin-embedded sections (figure 53, 54 & 56).

Latex particles in *Hevea brasiliensis* were observed to be spherical in shape and in different sizes (figure 58). Latex particle sizes were reported ranging from 0.005  $\mu\text{m}$  to 3  $\mu\text{m}$  (Dickenson 1969; Gomez & Moir 1979). Attempts to measure the particles size distribution by light microscopy gave misleading results since many of the particles lie beyond the limit of resolution (Gomez 1966). Using electron microscopy, van den Tempel (1952) found a maximum in the size frequency curve at about 0.1  $\mu\text{m}$ , so that in fact the most numerous particles is too small to be seen in the light microscope. Under the electron microscope, latex particles appeared as dark osmiophilic particles (figure 58) and some are transparent, where the shape of the latex particles was still visible even if they are situated under another latex particles. From the micrographs, latex particles can also be seen as overlapping with each other, thus making measurement of

individual particle size even more difficult. Other alternatives such as using colloidal suspensions are not so viable since after some time latex will coagulate.

#### **4.5.5 *Euphorbia wulfenii***

*Euphorbia wulfenii*, as *Hevea brasiliensis* is another latex-bearing species in the Euphorbiaceae family. Since they were sometimes used as an ornamental/garden plants, the latex is quite potentially dangerous especially to children. The latex can caused severe inflammation if contacted with the skin (Frohne & Jurgen-Pfander 1984).

Histochemistry study on fresh cut samples was quite difficult to carry out because the latex was so abundant, so the whole cut surface was smeared with latex. Embedded samples on the other hand did not show any traces of laticifers when stained (figure 73). Most of the cells and the suspected laticiferous cells looked empty.

#### **4.5.6 *Taraxacum officinale***

Although there are many studies on the pharmaceutical properties of this plant, little attention has been focused on the laticifers distribution in this species. Fresh dandelion stem and leaf when cut will produce clear transparent fluid. In

wax embedded samples of leaf lamina, laticifers were observed to be located near the vascular bundles of the cells (figure 70a). The latex was stained red with the reagent. It was quite difficult to identify the laticifers as they were comparatively quite small in size compare with the other cells (figure 71). Our observation showed that laticifers in the stem were observed in the outer region of the cambial cells.

An immunocytological study on the cell wall proteins was carried out on this species. This *ad hoc* experiment was initiated primarily to verify the immunocytochemistry technique for used on other samples, once antibodies that were thought to be suitable for latex particles or laticifers cells become available. A range of antibodies to some cell wall components was used. In many cases no antibody was observed but this is not surprising as the target molecules are minor components associated with specific stages of cell differentiation in only some species. The panel of antibodies tested did however yield one that gave some positive and specific immuno-staining, indicating that the general approach for the development of the method would be suitable at a later stage in our work once suitable antibodies are available.

#### **4.5.7 *Mandevilla splendens***

Laticifers in wax-embedded samples can be seen in the phloem area by its distinct red colour (figure 67,68,69) when stained with safranin O and Astra blue. Even though tannin cells were also stained red, the granular appearance of the latex in the laticiferous cell distinguished them both. From this instance, it was also quite obvious that a differential staining procedure especially between laticiferous cells and tannin cells is necessary in order to make a definite

judgment between these two cells. It was felt that to get an accurate results, empirical methods should have been established rather than the mere-experience-judgment.

#### 4.6 Difficulties in laticifers identification

There were several problems encountered during experiments, especially in getting the constant and correct combination of techniques to identify the laticifers in the plant tissues. The main problem was to retain latex in the laticifers during preparation and observation. It was due to the fact that, latex in the laticifers was under a great turgor pressure and the moment the laticifers were cut, latex will ooze out immediately leaving the cells to be partially or totally empty. Several preventive measures were taken, such as trying to coagulate latex in the warm water, fixing the cut samples immediately *in situ*, and the more extreme measure using a microwave technique. The last measure turned out not to be quite destructive to the cells and not very successful.

There was also some confusion during interpretation of the results especially in the ultrastructural level. A very careful approach has to be taken when observing and extracting the information from the micrographs. In figure 41 & 42 for example, it was quite difficult to come to the conclusion on whether the cell wall of the laticifers did break down or whether it is just an artifact, because of the plane of sectioning that might make the cell wall look like that. To verify the observation, several serial sections and different orientations must be taken before any conclusive observation can be made. Another method in that particular case would be to do enzymatic study or immunogold labeling on that particular protein. Immunogold labeling studies carried out on laticifers or latex were not successful. This experiment was hampered by the lack of a specific

antibody against the cells (laticiferous cells) or latex available at the time of the experiment. Perhaps in the future, work can be carried out to raised the antibodies against the laticiferous cells of the specific species, thus make the antibody available for the study to be carry out further.

#### 4.7 Conclusions

Several hypotheses were identified in the Introduction and addressed in this project. They are: -

1. To investigate several tissue preparation techniques for studies of the structure and differentiation of laticifers, and to allow application of molecular probes to advance further studies of differentiation.
2. To investigate structure changes of laticifers in different stages of development of *Meconopsis cambrica*.
3. To investigate the structure and differentiation of laticifers in different tissues of *Hevea brasiliensis*.
4. To investigate and compare laticifers development in several taxa

Comparison of tissue preparation techniques for studying structure and differentiation of laticifers were carried out. Tissue of latex bearing plants are extremely difficult to work with, hence the few previous published studies despite the very considerable commercial importance. In this study, several protocols involving fixation, embedding and staining procedures were tested. The protocols have been selected and optimized for the different types of studies on laticifers structure and distribution in seven taxa of five different families. For light microscopy study, fresh unfixed samples showed good and acceptable

structural information. This procedure was also very easy and reliable to be used as compared to other processed samples (as in fixed embedded samples). A protocol for immunohistological study was also successfully adapted and this protocol will be applied in *Hevea* study when suitable probe is available. Ultrastructural information on the laticifers achieved in this study showed that future work could be carried out for a more detail and comprehensive study on the cytology study on *Hevea* in particular of using ZIO fixatives to study the endomembrane system in the laticifers of *Hevea*. These results are vital in setting the foundation for future applications of using molecular probes to advance on understanding of control of cell differentiation. This is particularly important as many initial advances in the study of how genetics is linked to development and differentiation are now being made.

From this study, the results showed that most of the techniques in the end achieved acceptable results. For the specific purpose of screening the samples in breeding line, un-fixed fresh samples stain with fluorescent stain is therefore recommended. This preparation procedure can give a reliable, rapid and clear result, which is very vital in the breeding programme.

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## 5.0 REFERENCES

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