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Elater and capsule wall differentiation in selected hepatics: A comparison to xylogenesis

Mankiewicz, Julie A., Ph.D.

City University of New York, 1991

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ELATER AND CAPSULE WALL DIFFERENTIATION IN SELECTED HEPATICS : A COMPARISON TO XYLOGENESIS

by

Julie A. Mankiewicz

A dissertation submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York.

1991

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Elater and capsule wall differentiation in selected hepatics : A comparison to xylogenesis

by

Julie A. Mankiewicz

Advisor: Dominick V. Basile

Hepatic elaters possess annular to helical secondary cell wall thickenings with as-yet unknown materials selectively deposited in these thickenings at maturity. These cells possess a striking similarity to higher plant protoxylem at the light microscope level. Differentiation was examined at the light and electron microscope levels, and compared to xylogenesis. Histochemical and biochemical analyses were utilized to attempt to determine whether lignin deposition occurred.

This is the first report in elaters of processes of secondary cell wall deposition nearly identical to that reported in xylogenesis, including microtubular arrays correlated with the direction of microfibril orientation in developing walls. Two phenomena strikingly different from xylogenesis were observed: severing of plasmodesmatal connections with adjoing cells prior to differentiation, and deposition of two successive waves of natively electron-dense materials during maturation of the secondary thickenings.

Histochemical and biochemical analyses of *P. epiphylla* sporangia revealed no strong evidence for the presence of lignin. While IKI-H_2SO_4 reactions were similar to that of lignified cells, Mäule and phloroglucinol tests were negative. Thioacidolysis extraction revealed a complete absence of β-0-4 lignin in all mosses and liverworts tested. While pyrolysis of *P. epiphylla* sporangia yielded breakdown products characteristic of guaicyl and hydroxyphenyl lignin, including those with vinyl and propenyl side chains, it was suspected that these results were due to phenolics other than lignin. To support this, saponification coupled with gas-chromatography revealed the presence of lignin precursors p-coumaric and ferulic acids in both ester and ether linked forms. UV microscopy demonstrated that ferulic acid was present in all primary walls but absent or obscured in the secondary thickenings. It is suggested that, rather than lignin, these ferulic and p-coumaric acids may be responsible for the putative lignin breakdown products produced during pyrolysis.

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My advisor, Dr. Dominick V. Basile, has given most generously of his time, expertise and the resources of his laboratory towards the pursual of the research described in this text. But I would like to thank him even more for sharing his perspectives on plant morphogenesis and evolution. They are the framework that made the questions and the work herein possible. He received them in part from his mentors, and made them his own. I hope to do the same, and gratefully acknowledge the gift.

The members of my committee were unfailing in their excellent advice, constructive criticism, and helpful references, often unsolicited. Would that I had come to them more often! Special thanks to Dr. Ruth Stark, for agreeing on rather short notice to replace Dr. Henry Wood on his retirement, and for offers of future collaboration.

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Dr. John R. Obst, lignin chemist at the USDA Forest Products Laboratory in Madison Wisconsin, responded to a phone call requesting advice with an offer to pyrolyse some samples. Much of the chemical analysis reported here ensued, through the mail and during two visits to FPL, with I hope still more to come. Dr. Obst's expertise, hospitality, and innovative field trips during the visits remain an inspiration. Thanks also to the Forest Products Laboratory for sponsoring the second visit, as well

as to Dr. John Hackney for assistance in thioacidolysis, much conversation, and many useful references. A gift from my parents, James and Sonja Downey, made travel to Wisconsin, and hence much of the research produced, possible.

Dr. Robert Thomas generously shared the location of his collecting site for *Pellia epiphylla*. William C. Kinsinger, an artist with both pen and computer, prepared several figures, greatly improving their clarity and presentation. Many other friends cheerfully put up with my preoccupation this summer, and pitched in as deadlines approached: John Hills, Patty Daye, and Albert Mitchell in the darkroom; Ron Hays, Meghan Brewer, Jack Donovan and Sandy Trent at the glue-stick.

The Gaia Institute at the Cathedral of St. John the Divine has provided my livelihood as well as funding for research over the past several years. I would especially like to thank the Very Reverend James Parks Morton for his vision of the collaboration of real science and real religion, and for his welcoming and nuturing of the Gaia Institute, and of Paul and me, at the Cathedral.

My recent husband and long-time colleague, Dr. Paul S. Mankiewicz, got me started on this path in the first place, keeping fine company ever since with insight, inspiration, humor and help. He gave me a luxury he never had himself — a full summer to concentrate on nothing but the completion of this work. I can only give back to him words spoken on a similar occasion: may gratitude be measured in the breadth, depth, and number of works to follow.

My grandmother, Mrs. A. Mildred Downey Fergusen, was and is an extraordinary craftswoman and teacher. During the many hours spent with a jeweler's saw trimming EM blocks, I often recollected the weekends spent with her as a small child, where we cut silver and cloth instead of epoxy. Any patience, care and joy in work comes from her tutelage and example. The flaws of workmanship in this thesis may bring tears to her eyes; nevertheless, to her I proudly and lovingly dedicate it.

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Introduction

> "One could be tempted to interpret elaters as an 'atypical' resurgence of the morphogenetic potentiality to develop tracheids, - a potentiality which normally is not expressed in bryophytes - ."
>
> Charles Hébant, 1977, The Conducting Tissues of Bryophytes

The development of xylem, defined as lignified water conducting tissue, is taken by many to be one of the key structural innovations that enabled plants to adapt to the terrestrial habitat, and the key character identifying a fossil as a land plant (Chaloner 1970, Gifford and Foster 1989). Yet the second largest group of land plants, Bryophyta, is traditionally thought to be non-vascular and non-lignified (Gifford and Foster 1989, Bold et al 1980). Liverwort, or hepatic, elaters do not conduct water, yet they look strikingly like higher land plant protoxylem tracheids: both are elongated, terminally differentiated cells, empty at maturity, with annular to helical secondary cell wall thickenings (Figure 1c, 1d:primary xylem). The cells of the sporangial walls, while not similar in shape, also display annular to helical secondary thickenings. The secondary thickenings of both of these hepatic cells turn yellowish to reddish brown at maturity, evidence of deposition of some material which has not been closely investigated.

The phylogenetic relationships between the bryophytes and higher plants, and among the bryophyte taxa themselves are still unresolved. Are phylogenetic relationships between and among the bryophytes and 'higher' land plant taxa determined by differential capacities in the synthesis of lignin and lignin-like compounds? What are the implications of the origin of lignin biosynthesis and secondarily thickened cell walls for the origin of land plants? What is the generalized or

primitive structure of the cell type ancestral to xylem, elaters and conducting cells? Finally, what is the role of lignins and phenolic compounds in the adaptation of the embryophytes to the terrestrial environment?

This study focuses on the development and differentiation of hepatic elaters and capsule walls as compared to that of xylogenesis. Xylem elements are perhaps the most studied and best known system of plant cytodifferentiation . Hepatic elaters and capsule walls, in contrast, have been studied very little . Techniques used to study higher plant xylogenesis and lignification have been used as a guide here in the study of these hepatic cell types.

Hepatic sporangia, elaters and capsule walls: Structure, function and development

Hepatic sporophytes, especially those of the subclass Jungermannidae, are remarkably uniform. They are composed of three distinct parts: foot, seta and sporangium or capsule (Figure 3c). The foot remains embedded within the parent gametophyte; just as the spores are becoming fully mature, the seta elongates to many times its original length, propelling the capsule through the surrounding calyptra and perianth and into the open atmosphere. Here the capsule opens into four valves through the rupturing of four abscission zones in the capsule wall, and the spores are dispersed by air currents (Figure 1a,b).

Ingold (1939), in his book Spore Dispersal in Plants, describes in detail various spore dispersal mechanisms in hepatics, and the key roles that the elaters and capsule walls play in many of them. Some of the work is summarized from Goebel (1895) and Kamerling (1898); much of it comes from his own original studies. As in spore dispersal in leptosporangiate ferns and certain club mosses, the mechanisms involve water distribution and water tension created by evaporation in conjunction with

different stresses produced by differential cell wall thickenings in different parts of cells and tissues. These include the following mechanisms:

1. Violent mechanisms of elater movement: The elaters behave as a "spiral spring which becomes tightened up on drying and then, when the strain is released by rupture of the tensile water inside, untwists with such violence that it springs into the air, at the same time flinging off the spores from its surface" (Figure 2a).

2. Hygroscopic mechanisms of elater movement: When the elaters are very much longer and thinner, and the spiral thickenings correspondingly weaker, violent "unspringing" of the elaters does not occur. Instead, the elaters slowly wind and unwind in response to changes in humidity, serving to "fluff up" the spore mass, and allowing the spores to either fall or be carried out gradually by gravity and/or wind, leaving the entangled mass of elaters behind (Figure 2a).

3. Spiral-spring mechanism of *Frullania* : Elaters are attached to the sporangium wall at both ends. Upon drying, the sporangium wall splits and opens, stretching the elaters and putting them under tension until the lowermost attachment breaks free. The elaters then violently contract, forming a catapult-like mechanism which flings the spores as far as 1-2 cm. The entire process often takes as little as one second (Figure 2b).

. It is worth noting that all of these mechanisms contain similarities at the cellular level. The elaters respond to changes in humidity in different ways, depending on their size, diameter, mode of attachment to capsule walls, and the relative robustness of their spiral thickenings. Nevertheless, in each case the thickenings themselves function to maintaining cell shape and integrity in the face of mechanical stresses produced during drying of the cells.

At the scale of plant organs, similar hygroscopic mechanisms utilizing patterns of secondary cell wall thickenings effect the opening of the capsule into four valves.

Secondary cell wall thickenings in the cells of the capsule wall form several different patterns (Schuster 1966), from annular rings to globular thickenings in cell corners, to sheetlike thickenings on whole inner surfaces. Capsule walls vary from being one cell thick in the Marchantiales, to several layers in many of the Jungermanniales. In general, the inner layers of the wall contain smaller cells with annular thickenings, and the outer layers containing larger cells with nodular thickenings in the corners of the cells. Schuster (1966) describes the mechanical significance of the secondary thickenings as being akin to that of a bimetallic thermostat. The larger epidermal cells, with their predominantly radial thickenings shrink more readily upon drying than the smaller interior cells, made more rigid by the annular thickenings. Exposed to the dry atmosphere, the outer layer loses water and shrinks more rapidly than the inner layers exposed to the moist spore mass. The valves therefore curve outward, separating along the four longitudinal abscission zones which have no cell wall thickenings.

Early this century and before, several studies were made of hepatic sporangial development at the light microscope level (Hofmeister 1851, LeClerc du Sablon 1885a,b, Farmer 1894 & 1895, Campbell 1895, Moore 1905, McCormick 1914), and several have been done since at the light and/or EM level (Horner et al. 1966, Suire 1970, Shertler 1979). Variation occurs between taxa, but the general course of events can be summarized as follows:

1. Early divisions variable. The development of the hepatic sporophyte begins with fertilization of the egg within the archegonium. While nearly all mature hepatic sporophytes are composed of foot, seta and capsule, like many embryological patterns, these similar outcomes are brought about by a variety of patterns of cell divisions. In general the first division is transverse. The hypobasal cell gives rise to the haustorium

and sometimes the foot as well. The epibasal cell forms the capsule, seta, and usually the foot. It often divides one to several times to form a filament.

2. Tetramerous origin of capsule and seta. At some point, two successive vertical divisions occur in the cell or cells giving rise to the capsule and seta. The tetramerous partition of this cell or cells ultimately gives rise to the four valves (four lines of abscission) of the capsule and the tetramerous arrangement of the seta in cross-section.

3. Differentiation of capsule wall or jacket. The next easily observable event distinguishes the developing capsule from that of the seta. This is the differentiation of the capsule wall from the sporogenous mass in the center. One to three or four layers thick, depending upon the species, the capsule wall or jacket divides anticlinally to maintain rectangular cells in a tightly fitting epidermal layer or layers. By contrast, the sporogenous mass divides in many directions, and the cells become more isodiametric.

4. Elaterophore differentiation - For those taxa that have an elaterophore, this is the next structure to differentiate itself from the sporogenous mass, such as in *Riccardia pinguis* (Smith 1955). The future elaterophore cells become larger than those of the sporogenous mass, eventually lengthening , with some developing secondary cell wall thickenings and approaching the morphology of the elaters.

5. Elater/spore differentiation — In nearly all liverworts, cells within the sporogenous mass finally become either elaters or spores. Elater cells stop dividing and begin to elongate. The rest of the cells may or may not divide mitotically one or more times further, and then undergo meiosis and sporogenesis. Elaters may therefore be homologous (in the sense of being derived from the same mother cell) to one spore tetrad, or to a line of several spore tetrads distributed along its length, the number of tetrads depending upon how many mitotic divisions preceded meiosis.

As the elaters elongate, and the spore mother cells begin meiosis, these cells appear to plasmolyse, that is, the cytoplasm appears to contract away from the primary cell wall, and become irregular in shape, from lobed to "amoeboid" (McCormick 1914). Campbell (1895) reports that in the Ricciaceae, "as the sporogonium increases in diameter, the central cells begin to separate and round off. Their walls become partially mucilaginous and in microtome sections stain strongly with Bismarck-brown or other reagents that stain mucilaginous membranes. With this disintegration of the division walls the cells separate more and more until they lie free within the cavity of the sporogonium."

The focus of most of these studies is on the meiosis of the spores; few describe in detail or picture what is happening to the elaters at this stage, even fewer describe the capsule wall development. Perhaps because of the apparent "plasmolysis" of the cells, and the difficulty of fixation at this stage, most authors continued following the course of sporogenesis by representing only individual spores at their different stages. Nevertheless some authors do represent and/or mention elaters during this process. Campbell (1895) shows a drawing of sporogenous as well as elaterogenous cells of *Targionia hypophylla* , retracted from their primary cells walls, before the sporogenous cells have undergone meiosis. Suire (1970) shows cells of *Pellia epiphylla* at a similar stage.

Elaters are found within the capsule distributed amongst the spores in several different patterns, which can in most cases be related to their role in spore dispersal, as described below. Representative patterns include (Ingold 1939):

1. *Frullania* -type: Elaters are attached at both ends to the capsule wall, and are oriented parallel to the longitudinal axis of the sporophyte (Figure 3a).

2. *Nowellia* -type: Elaters are attached by one end to the capsule wall, the other end is free. The elaters are oriented diagonally within the capsule (Figure 3b).
3. *Pellia* -type: A tuft of modified elaters called an elaterophore are attached at one end at the base of the capsule, and free elaters radiate from the elaterophore throughout the capsule, interspersed among the spores. Upon opening, the free elaters are dispersed with the spores, and the elaterophore remains as a tuft in the center of the four open valves of the capsule (Figure 3c).
4. *Lophocolea* -type: Elaters are unattached to the capsule wall, and appear to be randomly distributed with the capsule.

In those sporangia with regularly oriented elaters, e.g., in *Frullania* and *Nowellia* , the spores and elaters show a regular alternation of one or two files of spore tetrads between each elater.

Elaters and capsule walls themselves have seldom been used as a taxonomic character (Schuster 1966, Seissegger 1966). Tilden (1894), in a work devoted to describing the variations in annular to helical thickenings and branching in elaters of *Conocephalum conicum*, gave a review of earlier work on hepatic elaters (Dillenius 1741, Michelius 1729, Marchant 1713, Von Mohl, 1851, Kienitz-Gerloff 1874, Hedwig 1789, Kutzing 1852, Leitgeb 1874, 1877, 1879, 1881, Underwood 1884, Schacht 1850). The summary and translations below are in great part from that work.

Early writers conceived of the elater as that of a pedicel for the spores. Linnaeus (1742) described *Marchantia* elaters using the phrase "farina crinulo affixa". Marchant (1713) states that the "seeds of *Marchantia* are inserted upon elastic filaments". Von Mohl (1851) and Kienitz-Gerloff (1874) both remarked upon the

starch-grains in young elaters, which disappear when the spiral bands develop. Kutzing (1852 - as quoted by Tilden 1894) states that

> "the elaters of *Marchantia* are composed in their early stages of a gelatinous substance and contain a few chlorophyll grains which are more or less scattered about. Soon one notices that the chlorophyll grains become associated by means of very fine and delicate colorless bands, which gradually develop into the spiral bands lying on the inner wall of the cell. By further development these spriral bands lose their chlorophyll grains and the cell membrane, which until this time has surrounded them, dissolves so that finally the spiral band alone is left. These bands are very elastic, and it is by means of them that the sporogonium is opened. On staining with iodine and sulphuric acid they become first yellow, then red and at last green. They appear, therefore, to be composed of a mixture of mucilaginous and protein substances."

Leclerc du Sablon (1885c) described the development of elaters and spores in *Frullania dilatata*: "the cells with dense protoplasm are already divided into two categories; some elongate simply without dividing, the others elongate in the same manner, but divide. The first form the elaters, while the others give birth to spore mother-cells....It is interesting to remark that each elater is equivalent not only to a spore mother-cell but to a row of them." Leclerc du Sablon also compares elaterogenesis to xylogenesis: "The formation of this spiral appears to be comparable to that which has been described by Strasburger in spiral vessels."

Schuster (1966) discussed the variation and pattern of secondary thickenings and their possible, but limited, utility as a taxonomic character. Douin (1914, 1922) discussed the morphology of the sporophytes of the Marchantiae and the Cephaloziellaceae, and investigated the histochemical staining reactions of elaters and capsule walls. Secondary thickenings of *Marchantia* elaters and capsule walls were not

stained by phloroglucinol or analine sulfate, but were stained by Sudan III, ammoniacal fuchsin and "l'orcanette acétique" (Formule de Guignard). He concluded that the substances within the secondary thickenings were not lignin, but were suberin-like. Thickenings of the teeth of peristomes of *Mnium hornum* and *Atrichum undulatum* stained similarly. Douin concluded that the substance was characteristic of bryophytes, and called it "bryophytine".

The most recent work on elaters has primarily compared them to spore morphology and terminology. Suire (1970) undertook a light and electron microscopical developmental study of *Pellia epiphylla* and *Radula complanata*, including spermatogenesis, archegonial development and sporogenesis. In his discussion of elater and spore differentiation, he traced the changes in the vacuolar apparatus and suggested that the cytoplasm, through changes in vacuolisation, controlled both elongation in the elaters and lobing of the sporocyte during meiosis. He observed endoplasmic reticulum in concentric layers in developing elaters and spores, particularly near areas of actively growing walls, and two kinds of golgi-derived vesicles, one electron-dense, one electron transparent. He claimed that the electron-dense vesicles contributed to secondary cell wall deposition, while the electron-transparent vesicles seemed to be deposited in regions of the primary wall.

Taylor et. al. (1973) looked at the micromorphology of spore and elater ornamentation in *Blasia pusilla*, and concluded that granules irregularly occurring on the outside of the elaters were similar to the ornamentation of the spore in form, composition , and resistance to acetolysis, and suggested that these granules on the elaters could be composed of sporopollenin.

Bury (1984) studied the walls of the elaters of *Frullania dilatata* at the TEM level, specifically comparing them to those of the spores. Bury concluded that all three layers of the spore wall (sexine, nexine and intine) were present in the elater wall, and

that the helical secondary thickenings were another layer interpolated in between the intine and the nexine. As mentioned above, *Frullania* elaters are attached at both ends to the capsule wall, accounting for the "catapult" mechanism of spore dispersal. Interestingly, the sexine layer of the elaters (which seems to correspond to the external granules seen in *Blasia pusilla* by Taylor et. al., 1973) is not present at the juncture between the elater and the capsule wall. Bury poses the question of the origin of this layer, and whether it corresponds to a perine in the sense of Denizot (1974), or an exine in the sense of Nurit (1975): "Elle semble correspondre à la définition de la périne par Denizot (1974): 'couche déposée passivement qui provient de débris d'origine non sporale', bien que cette dernière affirme dans le même article que 'les recherches ontogéniques récentes n'ont pas mis en évidence l'existence d'une périne chez le Bryophytes' ou à celle de l'exine externe de Nurit (1975)'; Les granules formès de composès complexes libérés dans le gel inter-cellulaire, sont adsorbés à la surface des spores ou absorbés par celles-ci qui les utilisent pour constituer leur exine et ses ornementations". Bury concludes that further ontogenetic studies are necessary to resolve this question.

These recent workers have all examined elaterogenesis by comparing them to the nearby spores. In doing so, they have perhaps been predisposed to examine closely the aspects that elaters and spores have in common, for example number and characteristics of cell wall layers, with the similarity between the outer layers being of great interest. Features unique to the elaters such as the helical secondary cell wall thickenings and terminal differentiation were noted but their development was not given specific attention.

Xylem and xylogenesis: Relevent points of comparison

The structure and function of xylem is perhaps the most well known and best studied of any plant cell or tissue type. An exhaustive review is impractical, however the relevent phenomena used as major points of comparison in this study are introduced.

The earliest tracheary elements, ontogenetically and phylogenetically, bear the closest resemblance to hepatic elaters. Elongated cells with tapering endwalls, ranging from annular to one to numerous helical secondary thickenings, dead and empty at maturity, such cells are found in the earliest land plants (Chaloner 1970), and in the tissue closest to the developing meristems of root and shoot (Esau 1965).

Developmentally as well as evolutionarily, helical pattern may be the basis of other later patterns of secondary thickening, as the reticulate to scalariform patterns of metaxylem have been described as a progessive filling in of a helical pattern (Bierhorst & Zamora 1965, Fahn 1982, Falconer & Seagull 1985a). It is thought that protoxylem's predominantly annular to helical secondary thickenings are due to the fact that they differentiate in an area of the stem that is still elongating. This is correlated by investigations altering the rate of elongation through the use of X-rays and the regulation of light (Goodwin 1942; Smith and Kersten 1942) showed that with the inhibition of elongation, fewer annular and spiral tracheary elements, and more pitted elements develop.

At organismic scale, xylem's primary and probably primitive function is in conduction of water from the roots to the aerial photosynthetic tissue, with mechanical support probably later and secondary (Raven 1977). Raven calculates that a small xylem strand composed entirely of tracheids would be 10^4 times more efficient in water transport than the remaining parenchymatous tissue, and even a single file of tracheids

of the type which might be found in the early land plant *Cooksonia* would transport more water than the surrounding parenchymatous tissue.

The forces produced by the capillary action and surface tension of water transport through the open xylem conduits tend toward collapse of the cells. The lignified secondary cell wall thickenings resist the compressive forces equal and opposite to the surface tension, and thus function to keep the xylem elements open. Raven reports that investigations into the "rubbery wood" disease of apple trees, which causes decreased lignin content and dissociation of lignin from wall polysaccharides, results not only in the condition from which the name is derived, but in collapse of vessels (Beakbane and Thompson 1945). This is consistent with a role for lignification in resisting collapse of water-conducting xylem elements.

It may be noted here that, while elaters function to disperse spores, and xylem to conduct water, the secondary thickenings of each cell type have the same function in each case: that of maintaining cell shape and integrity in response to deformation caused by the surface tension of water.

The developmental sequence of xylogenesis has been studied at the TEM level by many (Hepler & Newcomb 1963,1964, Esau, et al 1966a,b; Hepler et al 1970, Hepler and Fosket 1971, Maitre and De 1971; Falconer and Seagull 1985a,b, to name just a few). Roberts (1976) and Torrey et al (1971) have reviewed the literature and summarised the process as proceeding in the following steps: elongation, patterned secondary cellulose cell wall deposition, lignification, and autolysis of the cytoplasm accompanied by selective hydrolysis of primary cell wall non-cellulosic polysaccharides. One of the major objectives of this study is to examine elaterogenesis in detail, to see if the specific ultrastructural components of xylogenesis could be observed in elater differentiation as well. Lacking in bryophyte conducting tissue,

processes involved in controlled secondary wall deposition and selective "lignification" of secondary cell walls are of special interest.

From the time of their discovery and elucidation as tubular elements (Ledbetter and Porter 1963, 1964), plant microtubules have been linked to cell wall deposition. During interphase of meristematic cells, microtubules can often be seen very close to the plasma membrane aligned in a direction parallel to the cellulose microfibrils of the wall just on the other side of the cell membrane (Ledbetter and Porter 1963). During secondary cell wall formation the correlation between the microtubules and the microfibrils is even more striking.. Microtubules underlie the developing cell wall thickenings parallel to the microfibrils in the wall. They were first seen with TEM/glutaraldehyde fixation (Hepler & Newcomb 1964, Esau et. al. 1966b, Pickett-Heaps & Northcote 1966). More recently they have been visualised with immunofluorescent staining of microtubules in whole cells (Falconer & Seagull 1985a,b, 1986).

Golgi bodies and associated vesicles have been observed in abundance in differentiating xylem cells (Esau et. al. 1966a, Maitre & De 1971). Peroxidases (Hepler et al 1972) and particle rosettes thought to be involved in cellulose microfibril deposition (Haigler & Brown 1986) have both been localized in golgi bodies, their associated vesicles, and the area of the developing secondary cell wall. Maitre & De (1971) report serial sections showing microtubules surrounding golgi-derived vesicles, and postulate that the microtubules may play a role in guiding the vesicles to the developing secondary cell walls. Endoplasmic reticula have also been observed in developing xylem cells, often in concentric layers localized over cell wall areas between the developing secondary cell wall thickenings (Hepler & Newcomb 1964, Pickett-Heaps and Northcote 1966).

As deposition of cellulosic secondary cell wall thickenings proceeds, lignification of these secondary thickenings follows closely behind. Lignification in xylem elements has been traced utilizing $KMnO_4$ staining (Hepler et al 1970, Esau et al 1966a). While the specificity of the potassium permanganate reaction has been questioned by some (O'Brien 1970, 1972), the pattern of staining is identical with lignin fluorescence resulting from ultraviolet irradiation (Roberts 1976). Lignification in *Coleus* wound vessel members (Hepler et al 1970) was initially detected in the middle lamella and primary wall directly beneath the bands of secondary wall thickening. Lignification proceeded into the secondary thickening as it developed, from primary wall inward, lagging somewhat behind the developing secondary thickening and only completing the process when new secondary wall deposition ceased.

Peroxidases appear to be the enzyme responsible for polymerisation of lignin in the cell wall (see discussion of lignin below). Staining for peroxidases with 3,3'-diaminobenzidine (DAB) in *Coleus* wound-induced xylogenesis show essentially the same pattern in the cell wall as that for $KMnO_4$ above (Hepler et al 1972), with additional staining present in the cell membrane and dictyosome membranes.

The final step in maturation of xylem elements is autolysis of the cell contents, with accompanying hydrolysis of selected cell wall components. Autolysis and hydrolysis appear to occur simultaneously (O'Brien 1970). These processes leave the cells dead and empty at maturity. Hydrolysis removes polyuronides, pectins and hemicelluloses, leaving a weakened cellulosic residue in unlignified walls (O'Brien & Thimann 1967) and producing the more open areas in pits and perforation plates. Interactions between cells seem to occur, as cell walls abutting still-living parenchyma cells are affected differently by the hydrolysis process than walls between two xylem elements (O'Brien 1970, Srivastava & Singh 1972).

This brief review of a rather voluminous literature serves to introduce the aspects of xylogenesis which will serve as points of comparison for the study of elaterogenesis, and these aspects will be discussed as appropriate in the results and discussion sections. One particular aspect of xylogenesis, that of lignin and lignification, plays a large role in this study of elaterogenesis, both because of its importance in land plant evolution and because of the interest and research already generated regarding the question of its presence or absence in bryophytes. For these reasons, a more detailed review is given below, first of lignin and lignification as it has been studied in higher plants, and then a review of the research attempting to determine whether lignin is present in bryophytes.

Lignin and lignification in higher plants

Sarkanen and Ludwig (1971) summarize the functions of lignins in the life of the plant. Lignins decrease the permeation of water across the cell walls in the conducting xylem tissue, impart rigidity to the cell walls, and act as permanent bonding agents between cells, generating a composite structure outstandingly resistant towards impact, compression and bending. They also resist attacks by microorganisms by impeding penetration of destructive enzymes into the cell wall. Their mechanical and enzymatic resistance is based on the extraordinary stability and heterogeneity of their bonds between each other and other components of the cell wall.

Lignin is generally defined (Sarkanen & Ludwig 1971) as an aromatic polymer composed of some combination of three precursor monomers (monolignols): coniferyl, sinapyl and *p*-coumaryl alcohols (Figure 4). All three monolignols are composed of a six-carbon ring with a phenolic OH, and a three carbon side chain terminated by an alcoholic OH. They differ only in the number of methoxyl (-OCH_3) groups attached to

the benzene ring: *p*-coumaryl alcohol having none, coniferyl alcohol having one, and sinapyl alcohol having two. The aromatic portions of these moieties are described as p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), and the different kinds of lignins are often described using this terminology (e.g. 'syringyl lignin').

All known higher plant lignins contain coniferyl alcohol; they differ in what, if any, other monolignols they contain. Most gymnosperm and pteridophyte lignins are composed exclusively of coniferyl alcohol and are therefore called guaiacyl lignin. Angiosperm woods are composed primarily of coniferyl and sinapyl alcohols, hence called syringyl lignin. Many monocots (especially grasses) contain *p*-coumaryl alcohol in addition to coniferyl and sinapyl alcohol. Lignin containing sinapyl alcohol is called sinapyl or syringyl lignin. Lignin containing *p*-coumaryl alcohol is called *p*-hydroxyphenyl lignin.

The three monolignols are produced from three analogous acids, which are produced along a branched biochemical pathway (Figure 5) starting from phenylalanine (and sometimes tyrosine). Phenylalanine is deaminated, and a phenolic OH added to the benzene ring to produce *p*-coumaric acid. Ferulic acid is produced from *p*-coumaric acid in a two step methoxylation process of adding first an OH, then replacing the hydrogen with a methyl group. Likewise, sinapic acid is produced from ferulic acid by another two-step addition of another methoxyl group on the other side of the phenolic OH. *P*-coumaric, ferulic and sinapic acids are transformed into their respective monolignol alcohols via three enzymatic steps utilizing a CoA-ligase, a CoA-reductase, and an alcohol dehydrogenase (Higuchi 1985).

The monolignols rarely occur in free form within the cell; rather they are attached to a glucose molecule and shipped out to the cell wall as 4-*O*-β-D-glucosides (Gross 1985). In the cell wall the glucose is removed and the monolignol is dehydrogenated by a cell wall bound peroxidase to phenoxy radicals. These radicals

then polymerize to each other and bind to cell wall components non-enzymatically. In the higher land plants, the monomers are most often linked by a β-*O*-4 ether bond (Higuchi 1985). The β-*O*-4 ether bond (Figure 6.1) links the second (β) carbon in propenyl side chain to the phenolic oxygen at the fourth carbon of the benzene ring. These β and the 4 carbons are the two most thermodynamically stable positions for the free radical. The β-*O*-4 ether bond that results from the polymerisation of monolignols with radicals in these two positions occurs in higher plants 40-60% of the time (Higuchi 1985). Other linkages include ether bonds in other positions, carbon-carbon linkages, and combinations of the two (Figure 6.2-6).

Lignins are found in intimate admixture with other cell wall components, covalently bonded to hemicelluloses and probably also to celluloses and proteins (Whitmore 1978, Higuchi 1985). They also contain varying amounts of aromatic carboxylic acids (such as *p*-coumaric and ferulic acids, the monolignol precursors mentioned above) in ester-like combinations (Higuchi 1985, Harris & Hartley 1976). Figure 7 shows a tentative structure for normal conifer wood lignin, based on modifications of the spruce lignin formulae by Freudenberg and associates in 1974 and 1975, and depicted in Sarkanen and Ludwig (1971). With some modifications (e.g. Sakakibara 1983), it is still a generally accepted model for the structure of conifer lignin *in situ*.

Regarding Figure 7, Sarkanen and Ludwig (1971) state, "To an organic chemist, the formula .. conveys a clear idea of the difficulties facing the investigator in structural research. It may be noted that most of the monomeric units are linked together by bonds of extraordinary stability. These include carbon-to-carbon linkages, either of the biphenyl type, such as between units 9 and 10, or of the alkyl-aryl type (units 17 and 18 as well as 16 and 20). Even the ether linkages, with the exception of α-aryl ether bonds (units 3 and 4) are quite resistant towards hydrolysis. On top of the

resistivity against degradation to simple molecular species comes the tendency of lignin molecules to undergo self-condensation reactions, particularly in acidic media. Structures possessing this tendency include units of the cinnamyl alcohol type (unit 14c) and those containing benzylic hydroxyl groups (unit 6)."

Lignin's stability and resistance, compounded by the tendency to become modified on extraction creates difficulties for the researcher. Many techniques have been devised for identifying and analysing lignin, each with their own problems and limitations, from histochemical staining (Jensen 1962, Gahan 1984), to chemical oxidation and extraction, to fairly recent techniques of pyrolysis coupled with gas chromatography and mass spectroscopy (Obst 1983), thioacidolysis (Lapierre et al. 1986) Raman spectroscopy (Atalla & Agarwal 1985) and in situ C^{13}-nuclear magnetic resonance (Lewis et. al. 1987). As will be seen in the next section, nearly all of them have been utilized in the attempt to detect lignin in the Bryophyta.

Bryophyte lignin research: A review of the literature

As early as 1899, Czapek commented on the widespread presence of phenolic material in the cell walls of mosses and liverworts (Czapek 1899, from Erickson & Micksche 1974a). As techniques were developed for the study of higher plant lignins, these same techniques were applied to bryophytes, often by the leading lignin researchers (Holmberg 1934, Kratzl & Eibl 1951, Freudenberg & Harken 1964, Sarkanen & Latif 1971). Lignin as it is commonly found in higher plants is clearly not present in bryophytes. The observed similarities and differences to higher plant lignins have caused investigators to reach different conclusions as to whether what they found should be called a lignin (see Table 1 for a summary). Markham and Porter (1978), in their review of the chemical constituents in the bryophytes, interpret this confusion as resulting "partly from the lack of a sound definition of lignin,

lignin's intimate association with other cell wall components and tendency to undergo secondary reactions when extracted, and the variety of isolation techniques and degradative procedures used."

A variety of histochemical tests have been used to detect lignin in higher plants. Some histochemical tests are more specific than others; none react with all lignins exclusively. The Wiesner color reaction utilizes phloroglucinol in hydrochloric acid, and turns red in the presence of coniferyl alcohol. Since all known higher plant lignins contain significant amounts of coniferyl lignin, the Wiesner stain has come to be known as the *sine qua non* indicator of lignin. However, this reaction may be weak or absent in higher plants with large amounts of syringyl lignin (Sarkanen & Ludwig 1971). The Mäule color reaction, which uses sucessive treatments of aqueous permanganate, hydrochloric acid and ammonia, and the chlorine water-sodium sulfite color reaction (Gahan 1984) both produce a red coloration with syringyl lignin, and a brown reaction with guaiacyl (coniferyl) lignin. Safranin-fast green has been cited as an indicator of lignin (Gahan 1984, Jensen 1962), but safranin is less specific, and reacts with phenolic hydroxyl groups in general, as these authors caution.

The exact chemical basis for the above staining reactions is not known in every case. The phloroglucinol in the Weisner reagent is thought to react with the coniferaldehyde groups in lignin to form a red cationic chromophore (Adler et al. 1948). The Mäule reaction with syringyl lignin is due to the formation of 5-alkyl -*o*-quinones from the syringyl and guaiacyl structures. The 3-methoxy-5-alkyl-*o*-quinone formed from the syringyl units are red-purple in color, forming the positive reaction. The 5-alkyl-*o*-quinone formed from the guaiacyl units are orange to orange-brown in color (Meshitsuka & Nakano 1979).

Various workers have tested bryophytes, and other cryptogams with these lignin histochemical indicators (Gjokic 1895, Linsbauer 1899, Douin 1914, Towers & Gibbs 1953, Gibbs 1958, Bendz et. al. 1967, Siegel 1969, Scheirer 1972, 1975, Schaffer 1985). All bryophytes have tested negatively for the syringyl lignin indicators (Mäule and chlorine sulfite tests). Most workers have reported negative results with phloroglucinol as well (Gjokic 1895, Linsbauer 1899, Douin 1914, Hébant 1974, Schaffer 1985), but there have been exceptions. Bendz et al. (1967) claimed to have observed positive phloroglucinol reactions in 20 different *Sphagnum* species after the removal of their native red coloration by extraction with methanol. Scheirer (1972) reported positive phloroglucinol and safranin staining in the lateral secondarily thickened hydroid walls, as well as in the schlerenchyma of *Dendroligotrichum* gametophytes. Siegel reported positive reactions in *Polytrichum* peristome teeth and rhizoids of "*Jungermannia* sp"(Siegel 1962) as well as in *Dawsonia* gametophytes (Siegel 1969). Schierer and Siegel have both subsequently expressed reservations regarding their assertions of positive phloroglucinol staining (Schierer 1975, Siegel, pers. comm. to Hébant reported in Hébant 1977).

Schaffer (1985) and Hébant (1974) both observe that aged phloroglucinol, as evidenced by a yellow-brown color, will stain some bryophytes a pinkish-red, whereas freshly made reagent will not. Smith (1966) noted that "apparently positive results" could be obtained with the Wiesner test as applied to thalli of *Symphyogyna circinata*. However, sections of the same thalli treated with hydrochloric acid alone also produced red and yellow coloring, leading Smith to conclude that these walls were not lignified. Sarkanen and Ludwig (1971) suggest that this may be due to the endogenous presence of phloroglucinol within the tissues.

Lignin fluoresces blue under ultraviolet light, and UV microscopy has been used to identify lignin in higher plant tissues (Saka & Goring 1985), although other

phenolics autofluoresce also (Harris & Hartley 1976). Scheirer (1972) reported UV autofluorescence in the same tissues in *Dendroligotrichum* which showed positive phloroglucinol staining. Manskaya and Koehneva (1948) did not find the blue fluorescence characteristic of lignin in *Sphagnum* or *Fucus* (a brown alga). Schaffer (1985) detected UV autofluorescence in *Anomodon attenuatus* and *Thuidium delicatulum*, but concluded that it was due to phenolics other than lignin.

Many extraction methods used in lignin analysis have been utilized with bryophyte tissue as well. These include ethanolysis (Siegel 1969, Sarkanen & Latif 1971), thioglycolic acid extraction (Holmberg 1934), dioxane (Kondrat'ev 1949, Siegel 1969, Reznikov & Novitskii 1975, Nimz & Tutschek 1977), and Klason lignin, which involves extraction of other cell wall components with concentrated sulfuric acid, leaving an insoluble lignin residue behind (Kratzl & Eibl 1951, Freudenberg 1972). The results of these extractions, and the conclusions drawn by the researchers as to whether or not lignin is present vary widely, and are summarized in Table 1. Some declare that, even though phenolics were extracted, they were not lignin because components usually found in higher plant lignin were not present (Kratzl & Eibl 1951, Sarkanen & Latif 1971, Nimz & Tutschek 1977). Others saw the differences from higher plant lignins as indicative of a different type of lignin, but still a lignin (Siegel 1962, Bland et. al. 1968, Reznikov & Novitskii 1975).

Oxidation methods developed for lignin analysis have also been used, including nitrobenzene oxidation (Kratzl & Eibl 1951), permanganate oxidation (Farmer & Morrison 1955), permanganate/cupric oxide oxidation (Erickson & Micksche 1974a,b), and cupric oxide oxidation (Nilsson & Tottmar 1967, Logan & Thomas 1985). In all cases but one (Towers 1951), some oxidation products were found that were also characteristic for *p*-hydroxyphenyl lignin. Nilson and Tottmar (1967) also

found putative guaiacyl products in *Sphagnum*; Logan & Thomas (1985) found *p*-hydroxyphenyl, guaiacyl & syringyl products in some, but not all, bryophytes tested. Interestingly, Logan and Thomas found relatively high percentages of syringic aldehyde in *Conocephalum conicum*. They found high amounts of syringic aldehyde in the fibers of many higher plants, and note that Watson (1967) reported the presence of thick-walled fibers in *C. conicum*.

Two other rather recent analytical techniques have been utilized on bryophytes. Pyrolysis coupled with gas chromatography and mass spectroscopy was performed on *Sphagnum* subfossils and peat by Smeerdijk & Boon (1987). They found many (but not all) pyrolysates characteristic of lignin. Wilson et al. (1989) examined five mosses with solid state C^{13} NMR and concluded that there was no lignin. They proposed a "tannin-like" compound with a 1,3,5-trihydroxybenzene structure that would account for the lignin-like qualities that they and others had observed.

Bland et al. (1968) put forward a hypothesis for a kind of lignin that accounts for most (but not all) of the above results of bryophyte lignin research. They proposed a type of lignin present in no higher plant — composed of primarily *p*-hydroxyphenyl units linked by carbon-carbon rather than primarily ether linkages. This hypothesis is consistent with the observed negative Wiesner and Mäule color reactions, the observed low level of methoxylation in lignin extracts, and the predominance of *p*-hydroxyphenyl derived oxidation products. Primarily C-C linked lignin would also not show Hibbert's ketones on ethanolysis, nor evidence of β-*0*-4 ether linkages between monolignols in the C^{13} NMR spectrum, the absence of these characteristics having led Sarkanen and Latif (1971) and Nimz and Tutschek (1977) to conclude that there was no lignin present in their samples.

As mentioned above, Wilson et. al. (1989), on the basis of their solid state C^{13} NMR analysis of five mosses, directly contested this hypothesis, and proposed a

non-lignin alternative that they claim to be more similar to a tannin, with a 1,3,5-hydroxybenzene structure (Figure 8).

Erickson and Micksche (1974a,b) also disagree with the Bland et. al. (1968) hypothesis. Since no purely *p*-hydroxyphenyl lignin has been found in higher plants, they synthesised it *in vitro* with *p*-coumaryl alcohol and peroxidase enzyme, and subjected it to the same oxidative degradation as the seven bryophytes they examined. Few of the oxidative degradation products obtained from the synthesised lignin were similar to those observed in the bryophytes, leading them to conclude that, rather than a *p*-hydroxyphenyl lignin, the dimethoxylated esters produced by the bryophytes as oxidative degradation products were derived from catechol structures. They tentatively suggested three different non-lignin compounds producing the "lignin-like" oxidative degradation products: A, a dibenzofuran; B, 2,2′-dihydroxy-4,4′-dialkylbiphenyl; and C, "uncondensed" *p*-hydroxyphenylalkyl structures (Figure 8). They claimed that none of these compounds occurred simultaneously in any given plant, structures A and B occurring in both mosses and liverworts, structure C occurring in *Sphagnum*.

To summarize, the lignin research in bryophytes detailed above leaves three alternative, as yet unresolved, possibilities.

l. As it is clear that neither guaiacyl nor guaiacyl/syringyl lignin as found in higher plants is present in bryophytes in other than trace amounts, the only possible lignin that could be present would be a *p*-hydroxyphenyl lignin with carbon-carbon bonds, as proposed by Bland et al. (1968). There is very little evidence for this kind of lignin except for oxidation products that could be produced by other compounds as well, e.g. tannins, flavonoids or catechols. It is a hypothesis with mainly negative evidence as support, i.e., it is the only kind of lignin possible given the absence of β-*O*-4 linkages, negative Mäule and Weisner tests, and (in general) absence of guaiacyl

and syringyl oxidation products. Since such a lignin has never been found in higher plants, no chemical or histochemical tests have yet been devised to test for a lignin of this type, and no extraction procedure has been published. Without such tests, it is very difficult to prove or disprove its existence, and it must be admitted that there is little positive evidence to support it.

2. Other compounds have been suggested as producing the putative lignin products. Logically, it is not necessary that one and the same compound occur in each and every bryophyte, and indeed several different compounds have been proposed by Erickson and Micksche (1974a,b) and Wilson et al. (1989), and are given in Figure 8.

3. It is of course within the realm of logical possibility that both *p*-hydroxyphenyl lignin and other compounds producing these "lignin" oxidation and extraction products might both be present together in the cell wall of the same bryophyte. There is as yet no clear way to determine this.

Bryophyte water-conducting cells

Despite the presence of internal conducting tissue in many bryophytes, they have traditionally been called non-vascular plants. This is because the conducting tissue, when present, has been judged to lack lignified ornamented secondary cell wall thickenings. The term "vascular" has thus been reserved for plants possessing "true" xylem, i.e. xylem that is lignified and has annular, helical or reticulate secondary cell wall thickenings. The following summarizes the range of complexity seen in bryophyte water conducting cells, focusing on similarities and differences that are useful in comparisons with xylogenesis, elaterogenesis, and the the xylem of the earliest land plants.

Charles Hébant, in The Conducting Tissue of Bryophytes (1977), defines "water conducting cell" in bryophytes as cells within a central strand which are dead and empty at maturity, as the result of a lysosomal breakdown of their protoplast occurring in the last stages of their development. The term "conducting parenchyma" is used for living cells with specializations for water and/or food conduction. These cells are present in a number of bryophytes which do not possess conducting cells empty at maturity, but will not be reviewed further here. A number of special terms have been used to designate conducting cells in mosses, the most common being "hydroid". No special terms have been used in the case of hepatics, and Hébant defends this by claiming that the conducting cells in hepatics are sufficiently different from those of mosses not to warrant the same name.

Ironically, the term "hydroid" was, according to Hébant, created by Potonié (1883), and was originally designed to describe the tracheary elements of vascular plants. It was generally adopted as a term for water-conducting tissues in mosses, probably originating from Tansley and Chick's usage of it in their 1901 paper, "Notes on the conducting tissue-system in Bryophyta". Although Hébant does not mention this, Tansley and Chick first use the term (page 8) in describing water-conducting cells in the liverwort genera *Pallavacinia* and *Moerkia* . They quote Leitgeb (1877) as saying that "the cells of the strand as being elongated with pointed ends and fairly thick walls, provided with close-set 'pores', which are arranged in spiral lines around the walls, so as to give the impression of spiral thickening on casual examination of a longitudinal section." Tansley and Chick then go on to say that "these walls, .. are distinctly brown in the unstained condition, and hold aniline stains more strongly than the surrounding tissue ... the position of these strands, and the fact that their walls though thin are lignified, agree perfectly with the strands found in the other members of the genus which do in fact conduct water. If the inference is sound, we have in this ..

plant the most primitive water-conducting tissue or 'hydrom[1]' known" (and they cite Potonié). Obviously, they are equating this tissue with higher plant conducting tissue. Just as obviously, presence of lignin is an issue from the beginning.

The above quotations present the salient features of liverwort conducting tissue, and has been borne out by more recent work. "True" water-conducting strands (in Hébant's sense of being dead and empty at maturity) occur in no liverwort sporophytes, a few leafy liverworts (*Haplomitrium* and *Takakia*) and a few thalloid liverworts, all belonging to the order Metzgeriales (e.g. *Pallavacinia*, *Moerkia* and *Symphogyna*). They originate from the innermost portion of the segments cut off from the apical cell (McCormick 1914, Campbell & Williams 1914, Smith 1966), and when first formed become rather narrow and elongated elements with a densely staining protoplasm, although in *Haplomitrium* and *Takakia* they are distinguished by the precocious degeneration of their protoplasts (Hébant 1977).

The Metzgerialian liverworts possess conducting tissue with the greatest degree of differentiation. They become quite elongated, with tapering ends, and the lateral walls become thickened. As noted by Tansley and Chick (1901), they also possess pits. Some of these pits are true perforations of the wall, and originate by digestion of wall material from around the plasmodesmata (Smith 1964,1966, studies of *Symphogyna*). Similar pores have been observed in *Pallavicinia* and *Hymenophyton* (Burr et al. 1974, Campbell et. al. 1975) and in *Haplomitrium* and *Takakia* (Hébant 1972, 1973b, Burr et al. 1974). During the last stages of their development, the protoplast breaks down, and there is a peak of strong acid phosphatase activity at that time (Hébant 1973a, 1975, Burr et. al. 1974). Hébant (1975) claims that this pattern of acid phosphatase activity is similar to that seen in the last step in differentiation of tracheary elements (Gahan & Maple 1966).

Moss water-conducting cells ("hydroids") reach their highest state of complexity in the Polytrichaceae, although water-conducting cells are of generally more widespread occurrence in mosses than in liverworts. The following summary of their development and structure at maturity comes primarily from Hébant (1977).

The differentiation of hydroids in the Polytrichaceae is accompanied by the following cytological events:

1, the nuclei become very large, and in *Polytrichum formosum* become endopolyploid (Hallet 1972), as is commonly observed in developing tracheary elements (List 1963);

2, extensive development of the vacuolar system;

3, active synthesis of wall constitutents, including golgi-derived vesicles and lomasomes and

4, final breakdown of the protoplast, with a peak of strong lysosomal acid phosphatase activity. Accompanying this is hydrolysis of the thin end walls of contact between hydroids.

Haberlandt (1866) described the mature hydroid wall and attempted to establish a comparison with the tracheids of vascular plants, as have others since (Hébant 1970, 1974, Scheirer 1973, Hébant & Johnson 1976). All of the stages listed above are similar to that of xylogenesis, with the hydrolysis of the end walls being quite similar to that reported by O'Brien (1970) in xylem. Hébant (1977) concludes his description of hydroid structure and development with the following words: "From the structural and developmental evidence summarized above, it is clear that in spite of one major difference — the lack of lignified secondary "ornamentations" — hydroids have much in common with the tracheids of primitive vascular plants."

Thus the most differentiated moss and liverwort conducting cells share much in common with xylem — elongated shape, tapering end walls, thickened lateral walls, and autolysis with selective hydrolysis of exposed primary walls. The most notable

difference between moss and liverwort conducting cells seems to be in the process of hydrolysis — the liverworts create perforations and pits originating from plasmodesmata in end and lateral walls; mosses hydrolyse the end walls (Figure 9). The two phenomena characteristic of xylem which moss and liverwort bryophyte water-conducting cells seem to lack, i.e., lignification and the process of producing secondary cell wall "ornamentations", are at the heart of this study of elaterogenesis.

METHODS AND MATERIALS

Collection

Lophocolea heterophylla (Schrad.) Dum., *Nowellia curvifolia* (Dicks.)Mitt., *Pellia epiphylla* (L.) Corda and *Porella platyphylloidea* (Schwein.)Lindb.were collected in Armonk, New York , Delaware Water Gap, Pennsylvania, and Vershire, Vt., March through May 1984-88. Plants were collected when the sporophytes were still young, placed in zip-lock bags and were either 1. refrigerated, where they developed slowly, or 2. placed in a 19^{o} +/- $1^{o}C$ culture room equipped with continous fluorescent lighting, where they would develop more rapidly. Especially when collected in a dehydrated condition, they could be stored in the refrigerater until needed, then moistened and placed in the culture room to develop.

Mature sporophytes of *Pellia epiphylla* were collected for chemical analyses along the banks of the Swift River, New Hampshire, May 1987-88. Mature exserted sporophytes were harvested by hand such that only the sporangia and setae were collected into zip-lock plastic bags. In many cases, the sporangia had already opened and dispersed their spores. Thus, only the capsule walls and elaterophores were still present. These were freeze-dried immediately upon return to the laboratory. Intact sporangia were bagged separately, and treated in one of the following ways:

1. setae were removed and sporangia and setae freeze-dried separately.
2. intact capsules were allowed to air dry over a 150 mesh grid through which the spores fell, separating them from capsule walls and elaters. Spores were freeze-dried separately from the capsule walls and elaters.

Light microscopy:

Specimens were prepared in the same way as for transmission electron microscopy (see below), thick sectioned with a glass knife, and stained on a warm hot plate with either toluidine blue or methylene blue (1% aqueous).

Electron Microscopy and DAB staining:

Immature and mature capsules were dissected out of their perianths. Very young capsules were fixed whole. Older capsules (when the sporogenous tissue began to "round up") were broken open into a drop of 3% (v/v) glutaraldehyde in phosphate buffer (.1M, pH 7.2) in small micromolds at room temperature for 1 hour. Agarose (1% w/v) was melted and subsequently cooled to 50° C. One to several drops of agarose was mixed into the drop of glutaraldehyde to stabilise the cells into small discs (following the technique described by Brown and Lemmon 1980). These disks were trimmed to approximately 2-3 mm cubes and rinsed three times in phosphate buffer.

Disks were post-stained in 1% osmium tetroxide in phosphate buffer for 1 hour, and then rinsed in distilled water for at least two hours to avoid blackening of the agar disks during dehydration. Dehydration was done slowly (at most two changes per day) in a graded series of acetone and propylene oxide, and embedded in Spurrs resin (Spurr 1969). Again, changes had to be made slowly, with incubation times of 4 to 10 days (refrigerated) for the last changes into pure Spurrs, and finally embedded into flat molds. The sporophytes were oriented either longitudinally or transversely in the molds, and readjusted with a dissecting needle under a dissecting microscope when the Spurrs had thickened, but not yet hardened.

Sections were thick sectioned with a glass knife until the desired area of tissue was reached. They were then thin sectioned with a diamond knife, stained with uranyl

acetate (Stempak and Ward 1964) and lead citrate (Reynolds 1963) and observed on a Hitachi HS-9 electron microscope.

DAB staining for peroxidases, was accomplished by modifications of the methods of Hepler et. al. (1972), and Ishida et al. (1975):

After fixation and rinsing as specified above for electron microscopy, the agarose discs were divided such that part of each individual sporophyte could be used in both control and experimental treatments. Cut surfaces of the sporophyte were stabilized with a drop of agarose when necessary. These embedded sporophyte pieces were pooled into three groups, based on the color of the capsule/sporangium:

1. green (intact capsules green, secondary thickenings in the process of forming)
2. darkening (intact capsules dark olive green to light brown, secondary thickenings well-formed, but cells still containing cytoplasm)
3. mature (intact capsules dark brown-black, elaters dead and empty and reddish brown)

These embedded sporangial pieces were incubated in 0.05 M Tris (tris(hydroxymethyl) amino methane) buffer pH 7.6 for one hour at room temperature with the following additions:

DAB: 0.05% DAB (3,3'-diaminobenzidine) and 0.01% H_2O_2 (Hepler method)
or .2% DAB and 3% H_2O_2 (Ishida method)

Controls: a. DAB only (no exogenous H_2O_2)

b. DAB and 0.002 M sodium pyruvate

c. preincubation in .01 M potassium cyanide (30 min)
followed by 60 min in standard reaction medium with K-cyanide added

d. preincubation with 0.02 M AT (3-amino-1:2:4:-triazole) and 0.01% H_2O_2 followed by 60 minutes in standard reaction medium with AT added.

After incubation, disks were postfixed in osmium tetroxide, rinsed, dehydrated and embedded as detailed above for electron microscopy. They were sectioned and examined without further staining.

Histochemistry

Fresh and/or freeze-dried sporangia of *Pellia epiphylla* were utilized for the histochemistry. Freeze-dried sporangia were placed in a flask of deionized water and placed under a slight vacuum for approximately one hour to remove air bubbles in capsule wall cells. In most cases the sporophytes and accompanying elaters were observed whole, but occasionally capsule walls were torn slightly to separate the inner from the outer capsule wall layers. Plant control tissue other than from liverworts was collected and used fresh, and hand sectioned with a razor blade when appropriate (plants/tissues used for comparison purposes are indicated in the text). See Appendix A for detailed histochemical protocols.

Eugenol enrichment experiments:(modified from Siegel, Frost & Porto 1960)

In order to determine if lignin could be formed *in muro* in hepatics as previously described in Siegel 1969, vascular bundles of celery (*Apium graveolens*) were used as a

control for the method, and were pulled from the surrounding tissue as long strands. *Pellia* sporangia were used either fresh or freeze-dried. Tissue was washed in cold M/15 phosphate buffer (pH 6.0) and used immediately.

Samples were incubated in small bottles and rotated for 15 hours at room temperature with 0.2 M KH_2PO_4 (pH 4.5) containing eugenol (0.5 mM) and H_2O_2 (1.0 mM). Control samples were run in buffer alone. Celery samples contained 100mg of vascular tissue in 25 ml of reagent; *Pellia* samples contained two intact capsules each (unweighed) in 1 ml of reagent.

After incubation, samples were rinsed in de-ionised water, followed by 100ml of cold ethanol. They were then pre-extracted with two 10 min extractions of boiling ethanol. This was followed by lignin extraction by boiling in ethanol-3% HCL for 15 minutes. After boiling, samples were left at room temperature for 30 minutes, and the extract made up to 25 ml.

UV readings were made on a Varian Series 634 scanning UV-visible spectrometer with equal aliquots of control and experimental solutions, and read from 300 to 200 nm. For the phloroglucinol color reaction, 1 ml concentrated HCl and 130 mg phloroglucinol were added rapidly and with agitation to aliquots of the sample, and the phloroglucinol color read at 540 nm after one minute.

Thioglycolic acid extraction and analysis:

extraction: (modified from Whitmore 1978)

Freeze-dried capsules of *Pellia epiphylla*, as well as fresh, air dried and freeze-dried specimens of other land plants were chopped, ground or blended into small pieces then rinsed and centrifuged 3X in deionised water, and twice more in 95% EtOH. The tissue was then heated at 100°C in .5 ml thioglycolic acid and 5 ml. 2N HCl for 4

hours. After cooling, the tissue was washed twice in distilled water, and then extracted at room temperature in 2 ml .5M NaOH for 18 hours.

After extraction the tissue was centrifuged, and the supernatant collected. The tissue was rinsed twice with a 5 ml. of dH_2O, each time centrifuging and adding the rinse to the supernatant.

Lignin was precipitated with dropwise addition of conc. HCl, centrifuged and washed three times with .1M HCl. The precipitate was then redissolved in .5M NaOH, reprecipitated, and rewashed. It was then dried under vacuum, ground with mortar and pestle, and then dried for 48 hours at 50^{o} C with P_2O_5.

The UV spectra were prepared by redissolving the precipitate in .5M NaOH, and scanned from 400-200 nm on a Varian Series 634 scanning UV-visible spectrometer. The IR spectra were prepared by a KBr disk and read on a Perkin-Elmer Infracord Model 13B infrared spectrophotometer. Proton NMR samples were prepared by dissolving approximately 2 mg. of LTGA into 0.5 ml DMSO-d_6 (Aldrich) and analysed on a QE 300 NMR spectrometer.

Analytical Pyrolysis:

Pyrolyses were performed at the USDA Forest Products Laboratory in Madison, Wisconsin under the direction of Dr. John R. Obst, with a Chemical Data Systems Pyroprobe 120. Specimens were pyrolyzed at 700^{o}C in quartz tubes using a coil probe. Products were analyzed on a Finnigan 4510 gas chromatograph-mass spectrometer. Chromatography was with a 60 meter DB-1701 fused silica capillary column (J&W Scientific), at 80^{o}C for 0.2 minutes raised to 220^{o}C at 4^{o}C/min., helium carrier gas at 1.0 ml/min and a split ratio of 20:1. Mass spectra were obtained at 70 electron volts.

Thioacidolysis: Modified from Lapierre, Monties & Rolando, 1986.

1. Treat 1.0 mg of lignin (or 10 - 150 mg. of dried plant material, according to availability) with 5.0 ml of reagent (0.2 M BF_3.etherate in 9:1 dioxane: ethanethiol) in a teflon-lined screw-cap vial under nitrogen. Heat at 100°C in oil bath for 4 hours with occasional shaking.

2. Add the cooled reaction mixture to 10 ml H_2O and adjust the pH to 3-4 with 0.4 M $NaHCO_3$. Extract three times with 10 ml $CHCl_3$. Dry over Mg_2SO_4, add tetracosane in CH_2Cl_2 (0.1 mg for lignin samples, 0.01 mg for whole plant samples), filter and evaporate to near dryness.

3. TMS derivatisation: to an aliquot, one drop each of pyridine and BSTFA. Evaporate with stream of N_2 as needed for gas chromatography or pyrolysis.

Gas-chromatography: Varian Vista 44 using a 60 meter DB-5 fused silica capillary column (J&W Scientific) at 130C for 2 minutes raised to 240C at 4C/min. Helium at 1.0 ml/min was the carrier gas, the split ratio was 40:1 and a flame ionization detector was used. The silylated saponification products were analyzed under the same conditions. Gas chromatography-mass spectrometry was as above except a 30 meter DB-1 column (J&W Scientific) programmed at 100C for 2 minutes to 250C at 20C/min. was used.

Saponification: according to John Ralph, USDA Dairy Forage Research Center (personal communication):

1. Samples of 200 mg of plant material (or less according to availability) were treated with 10 ml of NaOH (0.5 M, sparged with N_2) for 2 hours. For determination

of ferulic acid occurring as esters, treatment was at room temperature, in Teflon-lined screwcap vials under N_2. For determination of ferulic acid occurring as ether, treatment was at 150°C (oil bath), in a stainless steel bomb under N_2.

2. Acidify to pH 3-4 with HCl.
3. Extract as above with $CHCl_3$, dry over Mg_2SO_4, filter and evaporate.
4. TMS derivatisation: one drop BSTFA on dry extract, one drop pyridine.

Ferulic acid/coumaric acid quantification: (as performed by Dr. J. Obst, USDA Forest Products Laboratory, Madison, Wisconsin)

Trans-ferulic and trans-p-coumaric acids were determined by gas chromatography after derivatization with BSTFA using the external standard method (amountX = AreaX x calibration factorX). The standard solution was prepared by dissolving 4.27 mg of ferulic acid and 2.64 mg of coumaric acid in a few milliliters of pyridine and then adding chloroform to give a volume of 50.0 ml. An aliquot, 1.0 ml, was derivatized using BSTFA and then 3.0 microliters were injected to determine calibration factors. Saponification extracts were concentrated to known volume (generally 1.0 ml) and 3.0 microliters were used for gc analysis. In some cases, the chromatograms were complex and the *Pellia* samples were spiked with ferulic and coumaric acids to aid in peak identification.

UV Histochemistry:

Tissues were collected and used either fresh, freeze-dried or from dried herbarium samples. Freeze-dried and herbarium samples were rehydrated, and put under slight vacuum if necessary to remove air bubbles. When necessary, samples were hand sectioned with a razor blade. Samples were observed with either an Olympus BH2

microscope with a BH2-RLF attachment, or with a Zeiss Axioplan microscope equipped with an LP420 mercury lamp and G365 and FT395 filters.

RESULTS AND DISCUSSION

I. Developmental Analyses

Introduction

The similarities between hepatic elaters and protoxylem observed by Hébant and myself, and the evolutionary and phylogenetic implications inherent in the hypothesis that these two cell types might be homologous, have been the motivating force behind this study. To be considered truly homologous, similarities in mature form must show themselves similar in development as well. Leclerc du Sablon (1885a) studied the development of several hepatic sporangia under the light microscope and remarked that the formation of the elater spirals appeared comparable to that described by Strasburger in spiral vessels. Much more has been learned about xylogenesis since Strasburger's time. Do the ultrastructural processes of elongation, secondary cell well deposition and lignification find homologs in elaterogenesis?

While comparisons to xylogenesis were of great interest, of equal interest was the investigation of sporangial development as a whole. How might the differentiation of the three cell types within the sporangium (capsule wall, spore and elater) be coordinated and controlled? How and when does the variation of morphology and distribution of elaters in the sporangium between taxa occur?

Sporangia of *Lophocolea heterophylla*, *Nowellia curvifolia*, *Pellia epiphylla* and *Porella platyphylloidea* were fixed and embedded for electron microscopy. Some were thick sectioned and examined at the light microscope level to follow the development of the sporangium as a whole. Blocks displaying developmental stages of interest were then thin-sectioned and examined at the ultrastructural level.

Sporangial development (Light Microscopy)

This light microscopical study examines elaterogenesis in the context of the development of the sporangium. Sporangial development of *Lophocolea heterophylla* is characterized and discussed from differentiation of capsule wall and sporogenous mass to maturation of the entire sporangium. Developmental sequences of the three principal cell types in the sporangium, i.e. elater, spore and capsule wall, were compared to each other as well as to xylogenesis. In addition, variations from this pattern in *Lophocolea* are observed and discussed in *Nowellia curvifolia* and *Porella platyphylloidea*.

The following stages of sporangial development were observed in *Lophocolea heterophylla*:

1. Differentiation of the capsule wall from the inner mass of cells destined to become the spores and elaters. This inner mass of cells has been termed the "sporogenous mass" by many authors (e.g. McCormick 1914), but is here called the sporogenous / elaterogenous mass to more accurately reflect the ultimate fate of this mass of cells. The capsule wall is composed first of a single layer, but divides both anticlinally and periclinally to produce tightly adjoining rectangular cells that produce two layers (Figure 10a,) and finally three to four layers (Figures 10b). These capsule wall cells become vacuolate by the 2-layer stage.

The sporogenous / elaterogenous mass, by contrast, divide in all directions to produce more isodiametric, non-vacuolate cells with densely staining cytoplasm. This mass appears to be comprised of discrete, triangularly shaped packets of cells that in Figures 10a and b meet in the center of the sporogenous / elaterogenous mass.

The cells of the developing seta are even larger and more vacuolate than those of the capsule wall by the time the capsule wall is 2-3 layers thick (Figure 10b).

2. Rounding up of sporogenous / elaterogenous cells: When the capsule wall is 3 layers thick (Figures 10c) , the cells in the sporogenous / elaterogenous mass have begun the process of what McCormick (1914) called "rounding up", i.e. the cytoplasm of each cell seems to plasmolyse and draw inward away from the primary cell wall, and away from the other cells as well, presumably severing all plasmodesmatal connections. McCormick reports the spaces between the cells as "mucilaginous" and stainable by Bismarck Brown. The sporogenous / elaterogenous cells become quite difficult to fix and embed, and it became necessary to cut or puncture the sporangium to facilitate penetration of fixatives and embedding media.

3. Elater/spore differentiation — During the "rounding up" stage of development, sporogenous vs. elaterogenous cells cannot be readily distinguished, at least at the light microscope level. Cells in any given packet appear to be elongating and dividing in synchrony. At some point though, sporogenous cells undergo meiosis, and elaterogenous cells remain elongated (Figure 11a). Elaters lay down secondary cell wall thickenings while the cells still retain their cytoplasm (Figure 12a), but by maturity, the thickenings are darkly colored, and the cells are dead and empty (Figure 12b). At maturity, the elaters are randomly oriented within the capsule, such that any one section through a whole capsule yields different elaters in longitudinal, transverse and oblique section (Figure 11b).

The sporogenous / elaterogenous mass in Figure 10c (longitudinal section) shows evidence of the discrete packets of cells seen in Figures 10a and b. Nearby cells show evidence of similar patterns of cell division and elongation, suggesting that within these packets, cell division and elongation was coordinated. *Lophocolea* at maturity has a random arrangement of spores and elaters (Figure 11b), yet the sections in Figure 10 suggest that during development, the arrangement is more ordered.

Variations in other taxa:

Porella platyphylloidea: In these capsules it can be seen more clearly the "rounding up" of the spores and elaters, and, especially in Figures 13a and b, evidence of the primary walls remaining behind. Early literature debated whether primary walls always remained behind during this process.

While the persistence of elater primary cell walls was clearly evident in *Porella*, there was variation in other taxa. *Radula complanata* clearly showed primary walls surrounding spores, while those of the elaters were not discernable. In *Lophocolea*, primary walls were not detectable in any preparations.

Nowellia curvifolia: Figure 13d shows a sporophyte of *Nowellia curvifolia* at the "rounding up" stage where the future spores and elaters separate from one another. *Nowellia* differs from *Lophocolea* in its pattern of elater distribution. While *Lophocolea's* elaters are randomly distributed at maturity, *Nowellia's* elaters are regularly arranged, attached to the capsule wall at one end , and extending diagonally into the center of the capsule. The elaters remain parallel to one another into maturity, and spores are evenly distributed between the elaters, showing more clearly the derivation of sporogenous cell and elater as sister cells of one parent division. This regular pattern allows us to arrive at a conclusion more difficult to discern in *Lophocolea*. Figure 13d shows sporogenous cells elongated alongside the elaters before they divide further and undergo meiosis. This indicates that elongation is not a property peculiar to elater differentiation, but rather a general property of the sporogenous mass before the cells separate in their developmental paths to undergo either meiosis and sporogenesis or elaterogenesis, at least in *Nowellia curvifolia*.

Conocephalum conicum (mature elaters): *Conocephalum conicum* is unusual in the great variation in form of its mature elaters (Figure 14). Ranging in shape from thin and elongate to short and squat and even branched (Figure 14d), there is variation also in the patterns of secondary cells walls, ranging from annular to one to five helices. The central cell in Figure 14c is annular at the bottom, and helical at the top. Another very interesting variation can be seen in Figure 14a, where a unispiral thickening occurs in one half of the elater, and there are no thickenings, and still cell contents in the other half. This suggests that the development and maturation of the elaters may be polar.

Comparisons between the three cell types:

Differences and similarities between the three cell types (capsule wall, spore and elater) can be summarized as follows:

The capsule wall becomes vacuolate early in differentiation, maintaining original cell wall connections between daughter cells, while the cells of the sporogenous mass remain smaller and denser than the capsule wall, then severing connections with one another to "round up", elongate and further differentiate.

While the structure and function of mature elaters seems in many ways more similar to that of capsule walls, the elater's early development is more similar to that of the adjacent spores. Typical of sporogenesis in many higher plants as well, both future spores and elaters "round up" in that they sever connection with the surrounding cells, and pull in from their primary cell wall, apparently secreting a mucilaginous substance in the process. That elaters do this as well is in sharp contrast to the differentiation of the cells of the capsule wall as well as that of xylem. In that regard elaters are more similar in their development to spores and sporogenesis than to xylem and xylogenesis.

The developing sporogenous cells also display a property (at least in *Nowellia curvifolia*), that one might have expected only of the elaters (and can only see during development) — they elongate along with and beside the elaters prior to meiosis. It is easy to discern in *Nowellia* because of *Nowellia's* regular pattern of elater and spore distribution.

The events documented above accord very well with previously published literature on sporangial development in other hepatics. The "rounding up" process of the sporogenous mass has been previously described. While many authors described it for the sporogenous cells, Campbell (1905) and Suire (1970) presented diagrams depicting elaterogenous cells participating in this process as well. Regarding the question of persistent primary cell walls, Campbell writes that although Leclerc du Sablon (1885a) claims that at early stages of development the primary cell walls of the spores and elaters are "completely destroyed, so that the young spore mother cells and elaters are primordial cells ...a great many carefully stained microtome sections of a large number of Liverworts belonging to all the principal groups have been examined by me, and invariably the presence of a definite cell wall can be demonstrated at all stages." Comparisons between *Lophocolea*, *Nowellia* and *Porella* in this study show that the walls are more easily seen in some taxa than in others, and in some taxa more easily seen in the sporogenous cells than in the elaters.

It has also been previously documented that sporogenous cells elongate beside their elater counterparts in some taxa . In *Frullania dilatata* (Leclerc du Sablon 1885a) the sporogenous mass originally consists of a single layer of cells. As the sporogonium expands, these cells all elongate. The elaters never divide, the spores divide one to several times before undergoing meiosis. The elaters therefore extend the length of the capsule and are of different lengths, depending upon whether they are situated at the

edge or the middle of the capsule. They are thus also equivalent to one or several spore tetrads, depending upon whether the sporogenous cell divided before undergoing meiosis.

In *Nowellia curvifolia* as depicted here in Figure 10d, the elaters also appear not to divide. They are attached at one end to the outer edge to the capsule wall, and lie free on the inner side, each elater extending into the center of the capsule. Sporogenous cells in Figure 10d appear equally elongated, having not yet undergone division and meiosis.

In other taxa examined here (*Lophocolea heterophylla*, *Porella platyphylloidea*) and previously (*Porella bolanderi* and *Aneura multifida* and *pinnatifida* - Campbell 1905; *Aneura pinguis*, *Pellia epiphylla* and *Targionia hypophylla* - Leclerc du Sablon 1885a,b), the sporogenous mass divides many more times before rounding up and spore/elater differentiation occurs, such that sporogenous and elaterogenous cells lie free in the capsule, unattached to any capsule wall when elongation occurs.

Underwood (1884) noted that other hepatics rarely contained annular bands. Leitgeb (1879) pictures elaters with annular bands from *Boschia* . Josephine Tilden (1894) discusses the phenomenon of branching in *Conocephalum* , and shows many drawings of the variation in form of elaters in *Conocephalum conicum*, including 1-6 spirals, but shows no annular bands. She notes that branching most often occurs at only one end of the elater, and suggests that the branching is due to the radial arrangement of the elaters within the sporangium such that one end is crowded at the base of the sporangium, and the other extends into the center of the sporangium where it has more room to grow and expand. She likens this space-filling phenomenon to that of thyloses in the tracheæ of some higher plants plants (*Quercus*, *Sambucus*, *Canna*, palms) , and to outgrowths on cells bounding intercellular spaces in leaf mesophyll.

Comparisons with xylogenesis:

While the differentiation of an individual elater appears similar at the light microscope level to that of xylem in terms of its elongation, laying down of secondary thickenings, and autolysis, the distribution of elaters in the sporangium is quite different from that of xylem in a stem, leaf or root. Elaters are distributed singly amongst spores, not grouped together into one tissue, as is xylem. Autocatalysis in xylogenesis, where the maturation of one tracheid stimulates the differentiation of an adjacent (as is easily seen in wound tissue and induced tracheary element differentiation in e.g. lettuce pith) could thus not occur where the elaters are surrounded by and separated from one another by the developing spores.

Another significant difference is that the future elaters contract away from their primary walls ("round up") during elongation, in much the same way as spores prior to meiosis. The capsule wall cells do not do this, but seem to maintain contact with their neighboring cells, although they do undergo similar processes of patterned secondary cell wall thickening and maturation.

Elaterogenesis at the ultrastructural level

Elater differentiation in *Lophocolea heterophylla* was followed at the ultrastructural level, beginning with the undifferentiated sporogenous/elaterogenous mass, through rounding up and elongation, to secondary cell wall deposition and maturation.

The first stage of this study found ultrastructural differences between the as-yet undifferentiated sporogenous/elaterogenous mass, the developing capsule wall , and the gametophytic calyptra beyond the capsule (Figure 15). The tissue of the calyptra is much thicker-walled than either the capsule wall cells or the sporogenous/elaterogenous

mass. Chloroplasts of the calyptra are numerous and possess well developed thylakoids. The capsule wall cells have more rudimentary chloroplasts, with those of the sporogenous/ elaterogenous mass even more so. The capsule wall cells are rectangular in shape and in two to three layers. The capsule wall cells at this stage were consistently collapsed, displaying sinuous cell walls (Figure 15), while the inner sporogenous/elaterogenous mass and the surrounding calyptra remained turgid.

The cells of the sporogenous/elaterogenous mass are varied in shape, reflecting the random orientation of plane of cell division within the capsule (Figure 15). Their cytoplasm appears more electron-dense than the surrounding capsule cells. Vacuoles are present, but smaller than those found in the surrounding layers. Plasmodesmata are in evidence between the sporogenous/elaterogenous cells (Figure 16), however they appear in some areas to be disrupted by the deposition of lomasomes that form a layer of vesicles between the cell wall and the plasma membrane. These layers of vesicles appear within the sporogenous/elaterogenous mass and no where else in the sporophyte at this stage. They seem to occur first along adjacent walls forming a line extending across the sporogenous/elaterogenous mass in such a way that these vesicles extend along the walls of several cells in succession, but may not occur at all on other walls of the same cell.

Deposition of vesicles along the wall (Figure 16) appears to be the beginning of the rounding up process of the sporogenous/elaterogenous mass. Fixation became very difficult at this stage (Figure 17), as evidenced by the contorted shapes of the cells and the lack of cellular detail. The spacing between the cells is evidence of the intercellular matrix, presumably deposited by the lomasomes (Figure 16). At the early stages of rounding up there is still no indication from size or cell contents that sporogenous and elaterogenous cells are becoming differentiated from each other. It is also not easy to detect whether elongation of the elaterogenous cells has yet begun.

Elaters that are obviously elongated are densely cytoplasmic, with numerous lipid bodies, and chloroplasts with short stacks of thylakoids (Figure 18a). Subsequently, numerous starch granules develop within the chloroplasts, with thylakoids almost totally disappearing (Figure 18b).

Microtubules are numerous near the plasma membrane approximately perpendicular to the long axis of the cell . Groups of microtubules can be found in discrete groups in between which there are vesicles opening outward to the cell wall (Figure 21b). Spans of endoplasmic reticula overlay these areas in layers of two or three or more (Figure 19, 21b). The ER seem to be concentrated over the areas of vesicle deposition rather than microtubular arrays (Figure 21b), but are found over long expanses of microtubules as well (Figure 21a). While the vast majority of the microtubules are oriented parallel to the microfibrils of the cell wall, occasionally some appear at an angle to the other microtubules (Figure 20) in a manner reminiscent of that reported by Ledbetter and Porter (1963) in root cortex cells of *Phleum* .

As the process of secondary cell wall deposition begins, starch grains become less numerous within the chloroplasts, and microtubules are found closely associated with the developing thickenings in a manner reminiscent of xylogenesis (Figure 22). These secondary thickenings are relatively electron transparent, although electron dense fibrils can be observed (Figure 23). As with the previous stage of elongation (Figure 21) fibrils are oriented in the same direction as the microtubules immediately on the other side of the plasma membrane (Figures 23 and 24). There are golgi bodies present (Figures 21 and 22), as well as continued vesicle deposition into the secondary cell walls (Figure 24).

As secondary cell wall deposition proceeds, the secondary cell walls become progressively more electron dense. This process appears to begin first at the outer portion of the secondary cell wall adjacent to (but not including) the primary wall, and

proceeds inward (Figure 25). Increased vacuolation is also observed, especially between the secondary thickenings.

Chloroplasts and other organelles begin to break down as the secondary cell wall thickenings become uniformly dense (although the fibrillar nature of the wall can still be observed in cross-sections of the secondary thickenings (Figure 26). The primary wall immediately underneath the secondary thickening is less electron dense than that of the secondary thickening above it, but it is darker than the primary cell wall which occurs between the secondary thickenings . A fairly continuous but irregular layer of granules are restricted to the outside of the primary walls subtending the secondary thickenings (Figure 26).

At maturity, the elater is dead and empty, although there may be some slight residual cell contents (Figure 27), with densely staining secondary cell wall thickenings, a thin, electron transparent primary cell wall, and a layer of densely staining granules on the outside of the primary walls subtending the secondary thickenings.

While a detailed investigation of sporogenesis was not the purpose of this study, nevertheless a few key comparisons will be made between sporogenesis and elaterogenesis. At the stage of early meiosis, while elaters are elongating and building up starch deposits in their chloroplasts (Figure 14a,b), the sporogenous cells have numerous chloroplasts with well-developed thylakoids, but few starch grains. In addition to the the round, smooth lipid bodies that the elaters also possess, the sporogenous cells contain dark, irregularly shaped inclusions, as well as lipid bodies with light inclusions within them (Figure 28).

Mature spores (Figure 29) have numerous large chloroplasts completely surrounding the central nucleus. The spore wall is composed of an electron transparent primary wall with a more electron dense, lamellate outer wall (Figure 30a). In

comparison, mature elater walls (Figure 30b) also have an electron transparent primary wall, but the inner secondary thickenings and the outer granule depositions outside the primary wall have no lamellate structure, appearing much more dense and uniform than the spore's outer wall.

DAB staining for peroxidases

The pattern of darkening of the secondary cell wall thickenings began from the outside and progressed inward, reminiscent of lignification in xylogenesis (Esau et al 1966a, Hepler et al 1970). Final polymerization of lignin is believed to occur through the mediation of a peroxidase enzyme located within the lignifying cell wall (Higuchi 1985). Diaminobenzidine (DAB) has been used to detect peroxidases and catalases *in vitro* in a wide variety of plant and animal systems (Frederick 1987). Hepler et al (1972) found that lignifying wound vessel members of *Coleus* stain intensely with DAB. DAB staining was therefore used here to attempt to locate peroxidase enzymes within the developing elater secondary cell wall thickenings.

Two DAB staining procedures were used: the Hepler method (Hepler et al 1972) and the Ishida method (Ishida, Ono & Matsusaka 1985). The Ishida method utilized a roughly four-fold higher concentration of DAB and hydrogen peroxide in the incubation than the Hepler method. It had been used to localize peroxidases in the cell walls of the liverwort *Marchantia*.

Differences were found between the two methods. Using the Ishida method, the cytoplasm of the elaters of *Lophocolea heterophylla* became very darkly stained, while the cytoplasm of the nearby spore was only lightly stained (Figure 31a). The cytoplasm of the control elater incubated in buffer with no DAB or hydrogen peroxide was unstained (Figure 31b). Using the lower concentration Hepler method, the cytoplasm of the elaters were in general no more darkly stained than the spores, except for the

membranes of some of the organelles (presumably peroxisomes) which were darkly stained (Figure 32a) as opposed to the control tissue (Figure 32b).

Pellia epiphylla was also used in these DAB experiments. Similar results were obtained with the Ishida method (Figure 33), but there was no detectable staining of peroxisomal membranes with the Hepler method (Figure 34).

In course of this DAB analysis a stage of secondary cell wall maturation was discovered which did not appear in the preceding developmental study. After the secondary thickenings have become somewhat electron dense starting from the primary wall and progressing inward, a second wave of darkening of the secondary thickenings takes place, this time starting from the inward (cytoplasmic) edge of the secondary thickening, and proceeding outward (Figures 31-32). The fibrillar appearance of the wall nearly disappears during this process, taking on a much more opaque and homogenous aspect. Concomitantly, electron-dense granules are deposited on the outside of the elater primary wall opposite the secondary thickening.

This staining pattern was first found in the experimental tissue, and many control blocks were sectioned before it was discovered there as well. It was thus mistakenly identified at first as a peroxidase reaction. Subsequently the pattern was seen in control tissue (Figure 31b,32b), and even in tissue fixed in glutaraldehye alone, completely unstained (Figure 35). The deposition of this material thus exhibits an osmiophilic tendency and a natural electron density not found in lignified walls (Hepler et al. 1970).

There are thus two waves of darkening of the secondary cell wall. The first occurs from the outside in, while the elater is still elaborating further secondary cell wall tissue (Figure 25). The second occurs from the inside out, and appears to be occurring concomitantly with the breakdown of the cytoplasmic contents, as evidenced by the lack of cellular detail (Figure 32). The first wave of darkening is osmiophilic as well, as the

pattern can be observed in the control tissue for the DAB experiment (Figure 36), which was fixed with glutaraldehyde and osmium only, and unstained.

Due to the naturally osmiophilic qualities of the wall, where the control tissue was stained as well as the experimental tissue, it is therefore difficult to judge whether there was staining for peroxidases within the wall. Ironically, the DAB controls provided more useful information than the experimental tissue. Without the DAB experiment and the need for these controls, it may not have been observed.

There are many differences between the results reported here and in previous literature regarding elaterogenesis. While several authors have described the deposition of secondary cell wall thickening in elaters (Suire 1970, Heckman 1973, Bury 1984), none have shown or described the involvement of microtubules in the process. Heckman (1973) stated that no cytoplasmic organelles were observed in consistent association with the wall thickenings. Suire (1970) correlated the production of golgi-derived vesicles with the cell wall thickenings. He also described two cytoplasmic spirals which prefigure the secondary thickenings in elaters of *Pellia epiphylla.* This was not observed in *Lophocolea heterophylla..*

Heckman (1973) noted that the secondary thickenings formed internally to the thin fibrillar wall and developed osmiophilic streaks. The thickenings became increasingly osmiophilic as the cells approached maturity, but membrane-like lamellae similar to those in the spore exines were never observed. She concluded that the substance deposited was sporopollenin, and that it was deposited from the outward edge of the thickenings in streaks toward the inward edge. Heckman suggested that since the deposition occurred from the outward edge inward, the sporopollenin precursors were exterior to the elaters prior to deposition.

Suire (1970) does not mention the direction of maturation, but one of his figures (Pl. 11, Figure 5) clearly shows increased electron opacity in the inner part of the secondary thickening.

This ultrastructural study of elaterogenesis has revealed many similarities with the ultrastructure of xylogenesis. In particular, events surrounding the deposition of the secondary cell wall thickenings closely mirror that of xylogenesis, including vesicle deposition (Maitre and De 1971), parallel orientation of microtubules with wall microfibrils (Hepler and Newcomb 1964, Hepler and Fosket 1971, Falconer and Seagull 1985a), and increased staining of the secondary thickenings starting from the exterior of the secondary thickening and proceeding inward (Wooding and Northcote 1964, Esau et al 1966a).

A major difference was found from xylogenesis in that the materials deposited in the secondary cell wall thickenings are osmiophilic, and are deposited in two waves, first from the outside in, and secondly from the inside out. It thus appears that while the processes of patterned secondary cell wall deposition are exceedingly similar in xylogenesis and elaterogenesis, the chemical makeup and process of deposition differ radically.

II. CHEMICAL ANALYSES

Introduction

To reiterate, the strong resemblance between protoxylem elements and mature liverwort elaters prompted this study. The patterned annular and helical secondary cell wall thickenings, and any compounds selectively deposited within them during maturation, were of special interest. If hepatic elaters and xylem are homologous, compounds deposited within the cell wall thickenings would be expected to be lignin, or a compound very similar to lignin. Chemical techniques used to identify and characterize lignin in xylem served as a starting point and a guide to the chemical analyses of *Pellia epiphylla* sporophytes.

Histochemistry of mature elaters and capsule walls

Histochemical techniques for chemical identification are not definitive, but they can indicate functional groups or classes of compounds at specific sites in the cell or cell wall, and they necessitate only small amounts of material. An exhaustive survey of all histochemical stains used to detect lignin was not attempted. Instead, methods were chosen where the known specificity of reaction could be utilized to systematically test what kinds of lignin (or other compounds) might be present or absent, and to indicate what other compounds might be present, especially in the secondary cell wall thickenings of capsule wall and elaters.

Results are summarized in Table 2. Safranin-fast green indicated that phenolics are present, and IKI-H_2SO_4 showed that the secondary thickenings behaved similarly to lignified xylem walls in their resistance to sulfuric acid and solubility in calcium hypochlorite. Toluidine blue indicated that the secondary thickenings stain similarly to some higher plant tissues containing lignin, such as sclerenchyma tissue in the outer

cortex of *Psilotum*, but not to xylem proper (Figure 37). Phloroglucinol and Mäule tests were both negative, indicating that neither coniferyl nor syringyl lignins were present.

Two non-lignin tests (i.e. ethanolic ferric chloride for phenols and ferric chloride-HCl for tannins) selectively stained the secondary thickenings, indicating that whatever is deposited specifically into the secondary thickenings is not a lignin. However, both ferric chloride stains gave different colors from that specified in the literature, such that the identity of the compound(s) cannot be said to be phenol or tannin.

A more detailed description and discussion of each test follows.

Safranin/fast green: The thickenings of *Pellia* elaters and capsule walls turn red with safranin, which indicates that there are phenolics present in the walls. The primary walls also turn faintly red, but after counter staining with fast green, they turn green while the thickenings themselves remain red. This however may be more of a function of the thickness of the walls than a difference in composition, as if left in fast green long enough, the secondary walls will stain green as well. The safranin test, while sometimes used to indicate lignin, really only indicates presence of phenolic-OH groups (Gahan 1984). This test indicates that phenolics are present throughout the *Pellia* tissue. If non-lignin phenolics are present throughout, it is impossible to indicate with safranin whether lignin-derived phenolics are additionally present in the secondary cell wall thickenings.

Phloroglucinol: Phloroglucinol is specific for only one of the three lignin monomers, turning red in the presence of coniferyl alcohol. It has been considered the *sine qua non* for lignin because nearly all higher plant lignins contain a large proportion of this monomer.

Some hepatic elaters and capsule walls contain a red to brown pigmentation which could make it difficult to detect a change in the coloration. This is also the case with some moss tissue, such as *Dawsonia* (Siegle 1969). *Pellia* elaters are pigmented only a light golden color, hence a phloroglucinol color reaction should be detectable, should it occur. The results appear to be negative for *Pellia* , as no detectable change can be observed.

Mäule test: The Mäule test is specific for syringyl alcohol, and hence for syringyl lignin. *Pellia* tissue gives a negative (brown) reaction, as does *Psilotum nudum* and other non syringyl alcohol-containing lignins. Thus, no syringyl lignin is indicated in *Pellia* sporophytes.

Toluidine blue: The primary walls and abscission zones of *Pellia* capsule walls and elaters turn purple with toluidine blue, indicating cellulose, whereas the thickenings become blue (Figure 37). Lignin stains blue-green, and sections of *Psilotum nudum* stem were stained alongside the *Pellia* for comparison. No part of the hepatic sporophyte stained the blue-green color characteristic of lignin.

The inner and outer layers of the capsule wall show slightly different staining patterns: the outer and middle periclinal wall is purple (indicating cellulose with no additional compounds deposited), while the innermost periclinal wall is blue, as are all secondary thickenings. See Figure 38 for a diagrammatic reconstruction.

IKI/H_2SO_4: Cellulose turns bright blue in the presence of IKI/H_2SO_4 and then dissolves. This occurs as the sulfuric acid hydrolyses the cellulose into its constituent glucose monomers , freeing the glucose to react with the iodine in a characteristic iodine starch reaction. A lignin matrix surrounding the cellulose masks this reaction, as lignin

resists dissolution by sulfuric acid, thereby shielding the cellulose as well. Lignified tissue will turn yellow-green, swell, and then finally dissolve. If the tissue is first incubated overnight in Clorox (which dissolves lignin) the IKI/H_2SO_4 will turn the tissue blue immediately, reacting with the now-exposed cellulose.

This is exactly what happens with *Pellia* capsule wall and elaters (Figure 39). While described in the literature as a reaction with lignin, the question here is the specificity of the reaction. Whatever is masking the cellulose in *Pellia* secondary cell walls is resistent to sulfuric acid, and dissolved by Clorox, behaving in much the same way as lignin.

Sudan IV and Sudan Black: The Sudan stains react with lipids and waxes, and hence also stain suberins, which are a combination of lipid and lignin. Douin (1914) and Lambert (1968) reported that elater secondary thickenings were suberin-like, and that they stained with Sudan and ammoniacal fuchsin.

In treating *Pellia* sporophytes with Sudan IV, all primary walls, especially those of the seta and of the outer capsule wall, turned pink. Droplets within the cells of the setae and of the capsule wall turned intensely red. The secondary cell wall thickenings did not change color at all, and it appears that the primary walls of the elaters do not either, although this may be because they are so thin the pink color is harder to detect. Douin's (1914) report of positive staining of elater and capsule wall secondary thickenings with Sudan stains are not confirmed by the reaction seen in *Pellia epiphylla.*

Douin does not specify which liverwort elaters he tested. While *Pellia* is only pigmented a golden color, other liverwort elaters and capsule wall secondary thickenings are a brown to even reddish-brown color. In case native pigment might mask or mimic the Sudan IV stain, Sudan Black was tried as well. Again, the pattern of staining was the same as with Sudan IV, and the secondary cell wall thickenings were unaffected.

Thus, whatever the compound deposited into the secondary thickenings is, it does not appear to be a lipid.

Ferric Chloride (EtOH): This stain should turn green in the presence of polyphenols, and indeed it does in the *Pellia* seta. However, the thickenings of the elaters and capsules walls turn black, while the primary walls remain unaffected.

This, and the next ferric chloride stain, are the only two histochemical stains done in this study which gave a clearly distinct and unique reaction with the secondary thickenings only. Unfortunately, the reaction is not the one mentioned in the literature. Hence while the reaction indicates that there is something in the secondary thickenings which is not in any of the other sporophyte tissue, it is uninformative about the nature of the compound. The same phenomenon can be seen in *Pinus strobus* twigs, where the outer cortex turns greenish with $FeCl_3$ (specified in the literature as being indicative of phenols), but the pith tissue inside the xylem turns blue-black.

Ferric Chloride (HCl): Tannins are indicated by a blue-black color. The *Pellia* secondary thickenings, and only the secondary thickenings, turned reddish brown. The *Pinus strobus* pith tissue that gave a reaction similar to the *Pellia* secondary thickenings with ethanolic ferric chloride did not react with this solution at all.

What has histochemistry told us about the nature of the compounds deposited in the secondary cells walls of *Pellia* capsules and elaters?

1. It stains differently from the rest of the sporangial tissue of the seta and primary walls with toluidine blue, ethanolic ferric chloride and ferric chloride in HCl. Although all three of these staining reactions found in the secondary thickenings are also

found in tissue of other plants, the literature is not clear as to what compound (or compounds) causes these specific color reactions.

2. The secondary thickenings are, like lignin, resistant to sulfuric acid but removed by calcium hypochlorite.

These are the limited positive histochemical results on the chemical nature of elaters and other sporangial tissue. The remaining information gathered from the histochemical tests is negative in nature.

1. Material selectively deposited in the secondary cell wall thickenings is not a coniferyl or syringyl lignin. If it is a lignin at all, it is not like any lignin known in any higher plant, as it must, by process of elimination, contain only or primarily p-hydroxyphenyl units. There is no specific histochemical test for p-hydroxyphenyl (or p-coumaryl) alcohol.

2. Material selectively deposited in the secondary cell wall thickenings is not a lipid stainable by Sudan stains.

Is the compound selectively deposited in the secondary thickenings phenolic in nature? The secondary thickenings stain with safranin, indicating the presence of phenolic compounds, but so do all of the walls of the sporangium. There is no way to know if safranin is staining the unknown compound of the secondary thickenings, since it is staining other cell wall phenolics already present.

Can these histochemical tests tell us anything about the way that the secondary cell wall thickenings might contribute to the function of the capsule walls and elaters? The secondary thickenings behave identically to lignin when treated with IKI-H_2SO_4. This is significant because this test in some ways addresses the integration of chemical structure and function in the cell wall. As described in the introduction, Ingold (1939)

demonstrated that the secondary cell walls function in a similar way in both xylem and liverwort elaters in terms of resistance to mechanical stresses due to the surface tension of water and evaporation. The implications of this histochemical study is that while the functional properties of the compound (or compounds) in *Pellia* secondary cell wall thickenings may be similar to that of lignin in xylem, the chemical nature appears so far to be very different.

The diagram in Figure 38 suggests how the thickenings might function to open the capsule upon drying. If the outer cellulosic walls (stippled in the diagram) are more susceptible to shrinkage upon drying and stretching when wet, then it would function to curve the wall outward and away from the spore mass as it dried.

Schuster (1966) describes this process (quoted in the introduction) in a similar way, but he attributes the contribution of the secondary cell wall thickenings to the process of capsule opening to the fact that the secondary cell walls are physically thicker than the other walls. Evidence presented here provides several additional observations:

1. the secondary thickenings are not only physically thicker than the other cell walls, but are chemically different, as shown by the toluidine blue staining. If the depositions are both hydrophobic and a bulking agent to the cell walls (as lignin is, and as indicated by IKI-H_2SO_4), then the depositions would most likely be as important to the differential shrinkage/capsule opening process as is the thickness of the walls.

2. Schuster does not mention it, but the toluidine blue staining shows that the entire innermost wall is not thick, but it is of the same chemical nature as the secondary thickenings. If the depositions act as a hydrophobic stiffening agent, stiffening the entire inner face of the capsule wall would cause the valves to maintain their general shape upon drying, and merely curl outward, rather than completely shrivelling, as would be the case if only annular or helical thickenings were supporting the inner layer.

This is indeed what happens; even when the walls are completely dry, they do not shrivel, but maintain their original shape.

3. The inner wall is much thinner than the outermost wall, yet it is the outer wall that shrinks, causing the valve to curve outward. This evidence points to the chemical nature of the walls being more important than the thickness of the walls in determining whether it shrinks and collapses or maintains its dimensions upon drying.

Eugenol enrichment

Eugenol can be converted in many plants to coniferyl alcohol, and with the addition of hydrogen peroxide made into coniferyl (guaiacyl) lignin, even in plants that normally do not contain coniferyl lignin, such as green algae and mosses (Siegel 1962). This interesting approach of looking for synthetic potentials in biochemical pathways was applied to liverwort sporangia, to see whether they could make coniferyl (hence phloroglucinol-positive) lignin, and if they did, whether it would be preferentially put into the secondary cell walls.

Figure 40 shows the results of eugenol incubation for celery vascular tissue and freeze-dried, mature *Pellia* sporophytes. In each case, the tissue was incubated in eugenol and hydrogen peroxide, then rinsed and subjected to acid alcohol extraction. The extract was then measured for UV absorption. Lignin characteristically forms a shoulder in its UV spectrum at 280 nm. Equal amounts of celery vascular tissue showed both higher absorption in general, and also more distinct shoulders at 280 nm with greater addition of eugenol. The eugenol-treated *Pellia* tissue showed greater absorption over the control, but there was no noticeable peak at 280 nm.

Figure 41 compares fresh, maturing *Pellia* capsules with freeze-dried, mature capsules (each run consisted of three replicates each of experimental and control

samples). In each case again, the experimental tissue had consistently higher UV absorption spectra than the controls, but the shoulders are not at 280 nm. On this run acid alcohol extracts were also mixed with freshly made phloroglucinol-HCl, and absorption at 540 nm measured. Phloroglucinol turns red only with coniferyl alcohol (not eugenol), and hence its absorption at 540 nm is an indication of coniferyl lignin produced within the cell wall. There was a phloroglucinol reaction measureable in the eugenol treated fresh *Pellia* capsules, but not in the freeze-dried mature ones, indicating that the fresh, not yet mature capsules were able to produce lignin from eugenol, but mature capsules were not. To support this, subtraction of a control from the experimental spectrum in the maturing capsule left a spectral curve with a shoulder at 280 nm, indicative of lignin. The same procedure in the mature capsules yielded a shoulder at approximately 270 nm, not at 280.

If *Pellia* secondary cell wall deposition were similar to that of xylem tissue, then peroxidases would be present in the cell wall. These would be expected to be operative even in the mature tissue. A negative result in the mature tissue was surprising, as cell wall-associated peroxidases had been reported in the cultured cells of *Marchantia polymorpha* (Ishida et al. 1985). Even with the phloroglucinol-positive (fresh) capsules, phloroglucinol applied as a stain to the whole, unextracted cells was undetectable under the light microscope, and hence it was not possible to tell where the lignin had been deposited, and whether it had been selectively deposited. It is difficult to detect phloroglucinol staining, even in individual cells that are known to contain lignin. Single, isolated xylem cells of celery also appeared unstained; it was only when they overlapped one another that the staining effect could be discerned in the group as a whole.

This line of research was not pursued further. Experiments were not repeated except for the internal replications of the second run, and hence should be viewed as

indicative only . They are reported here, though, because they corroborate results obtained by subsequent research:

1. location of peroxidases: DAB (diaminobenzidine) has been used to localize peroxidases in the cell walls of lignifying xylem (Hepler et al. 1972). As reported in the developmental study above, this procedure failed to find the extracellularly located peroxidases characteristic for lignifying tissues in *Pellia epiphylla* capsule or elater secondary cell wall thickenings during maturation. Peroxidases were found, however, in the membranes of certain inclusions in the maturing, but not yet lysing, cells. Peroxidases were thus available in the fresh, maturing *Pellia* capsules to lay down phloroglucinol-positive lignin as shown in Figure 32, but were unavailable in the freeze-dried mature tissue, explaining the lack of lignin laid down in that case.

For eugenol to be polymerized in cell walls as coniferyl lignin, it must first be converted to coniferyl alcohol. It is possible that the lack of this conversion is the limiting step in the mature *Pellia* tissue, rather than the lack of peroxidase. However, lignin synthesis can be accomplished from eugenol-H_2O_2 with washed cell wall fragments in higher plants (Whitmore 1978, Siegel et al 1972). The lack of either capability, to convert eugenol to coniferyl alcohol or to polymerise coniferyl alcohol, marks a difference between *Pellia* and higher plant cell walls.

2. UV spectra of extracts: The difference in shape and size of the UV spectra between the *Pellia* and the celery tissue in Figure 40 is similar to the UV spectra produced in the thioglycolic acid extractions (Figure 42), in that the bryophyte spectra are consistently shorter (less absorptive in the short wavelengths), with little if any shoulder at 280 nm.

Ishida and coworkers (1987) reported on further work with *Marchantia* cell wall-associated peroxidases in which they measured rates of hydrogen peroxide

formation in the presence of manganese chloride by either NADH or NADPH oxidation. They reported that "this reaction was stimulated by phenols such as 2,4-dichlorophenol or *p*-coumarate, suggesting a reaction similar to that proposed for the last step of lignification in higher plant cells..." They also report that *p*-coumarate was the only naturally occurring phenol to so stimulate the cell wall-associated peroxidase; other components of the lignification pathway, and other phenolics such as ferulate, trans-cinnamate, caffeate and phenol itself had no stimulatory effect. Eugenol was not tested. If bryophyte peroxidases were only stimulated by the unmethoxylated *p*-coumarate, then this would seem to lend indirect support to the Bland et al. (1968) hypothesis that bryophytes could be capable of producing a *p*-hydroxyphenyl (*p*-coumaryl) lignin. While it would be most interesting to test this hypothesis by feeding *Pellia* capsules *p*-coumaric acid, regrettably, there is no analagous colorimetric test to phloroglucinol for *p*-hydroxyphenyl lignin. One would need to undertake labelling experiments in order to verify where the lignin, if produced, had been laid down.

Lignin thioglycolic acid extraction and analysis

Thioglycolic acid extraction was chosen from the variety of lignin extraction procedures because it has been claimed (Lai and Sarkanen 1971, Sarkanen and Ludwig 1971, Freudenberg and Neish 1968) that this extraction procedure removed the largest fraction of lignin (80-90%) in the least modified fashion, and, secondly, unlike milled wood lignins, it was amenable to working with milligram quantities of starting material.

This procedure did indeed produce a precipitate in all higher plants and bryophytes tested. This precipitate was partially purified by three rounds of solubilization in sodium hydroxide, centrifugation, and reprecipitation with hydrochloric acid. The final precipitate was resolubilized in sodium hydroxide. The colors of the

solutions ranged widely, from nearly clear in the case of privet wood, to dark reddish brown for pine, amber for *Pellia*, *Psilotum* and *Sphagnum* stems, and quite green for that of *Sphagnum* leaves indicating that the extractions still contain components other than lignin. These extracts were then subjected to UV, IR and proton NMR analysis.

UV spectra: The UV spectra of the higher plant LTGAs (lignothioglycolic acids) show the typical shoulder at 280 nm (Fig. 42), but it should be noted that there is a range, i.e., while *Psilotum* shows a marked shoulder, the shoulder for *Pinus strobus* is much smaller, and that of *Ligustrum* (privet), barely detectable. *Pellia* shows little if any peak at 280 nm. Also, the whole spectrum, which in each case runs from 400 to 250 nm, is noticeably shorter in *Pellia*. This is consistent with the UV spectra from the acid alcohol extraction after eugenol supplementation.

Infrared spectra: Infrared spectra of *Pellia*, *Pinus* and *Ligustrum* (Figure 43) show similarities to one another, and to previously published IR lignin spectra, but they also show differences. Let us first examine the known lignin-containing samples. Many of the peaks characteristic of lignin, e.g., can be seen in the *Ligustrum* and *Pinus* samples, indicating that the extraction procedure was successful in removing lignin. All of the peaks, as identified by Hergert (1971), are listed in Table 3 and identified with vertical lines in Figure 43. Peaks characteristic for syringyl lignin (marked S) can be seen in the *Ligustrum*, but are consistently absent from the *Pinus* .

The only peaks specified by Hergert that could not be found (or were so slight that their existence was in question) were all of the peaks labelled as "aromatic C-H out of plane deformation" and the one peak labelled "C-H deformation (symmetric)". These were all equally questionable in all spectra, and are shaded in Table 3.

The general shape of the *Pellia* spectrum is very much like those of *Pinus* and *Ligustrum* and some of the peaks in the *Pinus* and *Ligustrum* spectra can be matched with those in the *Pellia* spectra.

What can be deduced about possible lignins in *Pellia* sporophytes from its thioglycolic acid-extracted IR spectrum? The *Pellia* spectrum shows evidence of phenolics with aliphatic and/or methoxyl methyl groups attached, that have survived the thioglycolic acid extraction in a fashion similar to lignin. The spectrum rules out the possibility of syringyl lignin, but a hydroxyphenyl and/or a guaiacyl lignin is still a possibility. While most similar to pine, one cannot say that they are identical, and that this is a guaiacyl lignin. Hergert (1971) gives a very strong warning on this point:

> "There has been a temptation when working with polymer spectra, especially in the field of natural products chemistry, to conclude that two or more products are identical because their spectra were 'very similar'. In actuality, even slight differences in spectra, i.e., relative heights of neighboring absorption bands, the presence of weak shoulders, etc., are of considerable significance. They may signal differences in the ratio of constituent monomeric units and the order in which they are linked."

The *Pellia* and the pine spectra are clearly not identical.

Proton NMR spectra: Due to the small amounts of *Pellia* LTGA available, it was decided not to attempt to acetylate the sample first, but to obtain a proton NMR spectrum of the unacetylated extract in deuterated DMSO. While much less information is gained with unacetylated samples (all peaks for the aliphatic portion of the molecule are undetectable), the major unanswered question from the infrared analysis could nevetheless be addressed, i.e. whether the aromatic substance in question was

methoxylated or not, and hence whether *Pellia* possessed something approaching a *p*-hydroxyphenyl or a guaiacyl lignin.

The NMR spectra of pine and privet show peaks for aromatic and methoxyl groups (Figure 44b,c). As compared to the blank sample (Figure 44d) which showed only the deuterated DMSO and a water contaminant peak, the phenolic peak at approximately 6.8, and the methoxy peak at 3.7 are clearly visible. In addition, it can be seen that the privet has a larger methoxy peak relative to the phenol peak compared to the pine, indicating the extra methoxyl groups in the syringyl monomers.

Pellia (Figure 44a) shows small to no peaks at 6.8 and 3.7. Much like the UV spectrum, one cannot even say that there is evidence of phenolics, much less whether or not it is methoxylated.

The spectra from the thioglycolic acid extraction leave us with a paradox: on the one hand the UV and NMR spectra show no real evidence that there are even phenolics present, yet on the other hand, the IR spectrum, while weak, showed many of the peaks characteristic of lignin, and possibly even a guaiacyl lignin. Bland et al. (1968), in their ethanolysis of *Sphagnum*, report lack of methoxy and aromatic peaks in their pNMR spectrum.

Whole-tissue Pyrolysis:

Given the confusion generated by the LTGA analyses as to whether any phenolic was extracted, and if so how similar it was to lignin, pyrolysis coupled with gas chromatography and mass spectroscopy offered several advantages:

1. As an analysis of the whole tissue, it gave a rough estimate of the relative percentages of the products in the tissue, unobscured by non-lignin contaminants.

2. It necessitated only milligram amounts of material, making it possible to attempt with *Pellia*, and even to separately test elater, spore, capsule wall and seta fractions.

3. It was capable of distinguishing clearly between syringyl, guaiacyl and *p*-hydroxyphenyl moieties.

Objectives in this pyrolysis analysis were to attempt to identify possible lignin breakdown products (Figure 45), and assess the relative abundance within the tissue.

This analysis gave good evidence of phenolics in *Pellia* sporophytes, indeed of many of the breakdown products characteristic of guaiacyl lignin, including those with two and three carbon side chains such as vinyl guaiacol and trans-isoeugenol. In addition, analyses of separated fractions indicated that the distribution of these lignin breakdown products varied significantly between seta, spore, elater and capsule wall, with elater and capsule wall fractions being most similar to one another, and containing the largest amounts of these lignin breakdown products (Figure 46). The proportions of these products, and the concentrations within the tissues were also within the range of variation to be seen in pyrolysis of higher plant tissue (Figure 47). This however does not constitute proof of lignin being present, as other non-lignin C_6-C_3 compounds could give the same results.

Throughout this chapter pyrolysis breakdown products such as guaiacol, vinyl guaiacol etc. will be referred to as "lignin breakdown products", however it must be emphasised that, while they are breakdown products of lignin, they can be produced by the degradation of other compounds as well. Positive identification of these "typical lignin breakdown products" within *Pellia* sporophytes does not automatically mean positive identification of lignin. Indeed, this issue will become of prime importance as the investigation progresses.

Pyrolysis of *Pellia* samples was first performed in conjuction with gas chromatography. Likely peaks indicating possible lignin breakdown products were later verified with the mass spectrometer (see Appendix B). Identification of the lignin breakdown products was first performed on two fractions of *Pellia* sporophytes, one whole sporophyte sample, and another where setae and spores were removed by passing the sample through a sieve. Pyrograms of these two fractions can be compared in Figure 47. Lignin breakdown products included phenol, *p*-hydroxystyrene (vinyl phenol), guaiacol, 4-methyl guaiacol, 4-vinyl guaiacol, eugenol, cis and trans-isoeugenol (small amounts in wall+elater sample, none detected in the whole sporophyte sample), 2,6 dimethoxyphenol (in trace amounts in the wall+elater sample, not found in whole sporophyte sample), propenyl syringol (trace amounts in wall+elater sample only).

The *Pellia* samples thus contained lignin breakdown products typical of guaiacyl and *p*-hydroxyphenyl lignin in similar quantities and proportions to that found in some herbaceous higher plants (Figure 47). The syringyl compounds are in such small amounts that they were impossible to verify, and while notable, it should be acknowledged that these were field collected samples from plants underneath a mixed canopy of gymnosperms and angiosperms, and so the trace levels found could possibly be due to contamination by microscopic particles from the trees overhead. Further work would have to be done to verify their existence within the *Pellia* itself, and so are discounted here.

In addition to identifying the typical lignin breakdown products, several of the largest non-lignin breakdown product peaks were screened for an NBS Library match (Appendix B). They are identified by number in Figure 48, and are:

number	MW	scan #	NBS Library match

1	112	670	2-hydroxy-3-methyl-2-cyclopenten-1-one
2	109	965	1-hepten-3-ol
3	117	1364	1S 1H-Indole
4	179	1906	cyclododecanol
5	194	2015	1,2,3,4-cyclopentanetetrol
6	256	2385	hexadecanoic acid
7	150	2476	5-octadecene

Of great interest here is number 5, scan #2015, because it is found in abundance in the wall+elater sample, and very much less is found in the whole sporophyte sample. It is possible that it may be one of the compounds which is selectively deposited in the secondary cell wall thickenings, and hence is found in greater abundance in this fraction.

Given these observed differences between the two *Pellia* samples, more thoroughly separated fractions were prepared. Spores, elaters, setae and capsule wall fragments were separated from one another under the dissecting microscope until approximately one milligram of each was acquired. The pyrograms of these fractions can be compared in Figure 49 (The *Pellia* data in Figures 46 and 47 were from these fractions as well).

The elater and capsule wall fractions in general contain the largest percentages of lignin breakdown products. Their pyrograms are most similar in terms of lignin and non-lignin peaks. The peak at scan 2068 (as represented in the elater sample) continues to show itself as a non-lignin breakdown product most abundant in the elater and wall fractions. (This peak was at scan 2015 in the whole sporophyte and wall+elaters samples. The latter samples were run under slightly different conditions , which changed the scan numbers to some degree.) Two other peaks (1182 and 1757) are also

found primarily in the elaters and capsule wall, that were not as obvious in the comparison of wall+elater and whole sporophyte samples.

The non-lignin breakdown peaks previously given an NBS Library match are distributed in these four elater/wall/spore/seta samples in the following way:

#	scan #	NBS Library match	distribution in elater/wall/seta/spore samples
1	670	2-hydroxy-3-methyl-2-cyclopenten-1-one	off the scan range
2	965	1-hepten-3-ol	setae (1005), capsule wall?(1018)
3	1364	1S 1H-Indole	setae (1409)
4	1906	cyclododecanol	elater (1943), spores, wall, little in setae
5	2015	1,2,3,4-cyclopentanetetrol	elaters (2068), wall
6	2385	hexadecanoic acid	all (~2427)
7	2476	5-octadecene	off the scan range

The NBS Library matches are of course not a definitive identification, and would have to be verified by other means. Two of them, 2-hydroxy-3-methyl-2-cyclopenten-1-one and hexadecanoic acid (palmitic acid) were also found in all fractions of Smeerdijk and Boon's (1987) analysis of peat, subfossil *Sphagnum* leaves and ericaceous rootlets. Cyclopentanol was found in *Sphagnum* only, not in the peat or ericacious rootlets (cyclopentanetetrol, found in the elater and wall fractions of *Pellia*, is unreported). Of the lignin breakdown products found here, Smeerdijk and Boon also found phenol, vinyl phenol, guaiacol and vinyl guaiacol in *Sphagnum* leaves, in addition to other typical lignin breakdown products not reported here. They did not find any methyl guaiacol or eugenols, nor did they find any syringyl derived lignin breakdown products, with the curious exception of syringol itself.

In summary the pyrolysis analysis confirms what was suggested in the IR spectrum of *Pellia* sporophyte LTGA, namely that a lignin or compound similar to lignin was present in the tissue. Further, the compound(s) appear to be more concentrated in the capsule wall and elaters than in the other tissues. Another compound tentatively suggested as 1,2,3,4-cyclopentantetrol by NBS match appears in relative abundance in capsule wall and elaters as well, and absent or scarce in the other tissues. The abundance of these compounds in both fractions that contain secondary cell wall thickenings suggests that those compounds could be concentrated or selectively deposited there.

Thioacidolysis:

Thioacidolysis releases the lignin monomers bonded by the β-*O*-4 linkage. Requiring only small amounts of material, it is a recent technique of great sensitivity that had never been applied to bryophytes. Previous bryophyte lignin research has indicated that the only type of lignin bryophytes might contain would be a predominantly *p*-hydroxyphenyl lignin containing primarily C-C linkages. Thioacidolysis would not release C-C linked monomers. However, if bryophyte lignin contained even a small proportion of β-*O*-4 linked monomers, this technique, coupled with GC/MS would be sensitive enough to detect even trace amounts. *Bromus tectorum*, a grass, was used as one of the controls because it has significant amounts of *p*-hydroxyphenyl groups in its lignin.

Figure 50 shows a gas chromatogram of thioacidolysis products from *Pellia epiphylla* capsule wall, superimposed over that of *Bromus tectorum*, where the characteristic double peaks for *p*-hydroxyphenyl, guaiacyl and syringyl monomers are

identified. *Pellia* shows no evidence of syringyl or guaiacyl peaks, but does show a double peak in approximately the right position for a *p*-hydroxyphenyl alcohol, although of somewhat different shape. Mass spectroscopy of the same *Pellia* thioacidolysis fraction (Figure 51b) showed no *p*-hydroxyphenyl peak as compared to that of *Bromus*, demonstrating that the GC peak was indeed a different compound. The *Pellia* capsule wall therefore shows no evidence of any lignin with β-*O*-4 bonding.

Although the data is not presented here, many other bryophytes were also subjected to thioacidolysis, including *Plagiochila*, *Andreaea*, *Pohlia*, *Polytrichum*, *Sphagnum*, and *Anthoceros* . Initially, both *Sphagnum* and *Anthoceros* showed evidence of β-*O*-4 lignin , while the rest of the bryophytes displayed a complete absence of lignin products.

Because of the sensitivity of this technique, the possibility of contamination of these field collected specimens were of concern. Wilson et al (1989) showed that with ultrasonic cleaning of samples of bryophytes, lignin products were eliminated, and suggested that, especially in peat samples of *Sphagnum* (upon which much lignin extraction analysis has been performed) these samples may have had higher plant rootlets associated with them. The original *Sphagnum* sample used here was a bulk sample of field collected material from which LTGA was produced. While it was examined carefully and any visible pieces of leaf or root removed, it was not cleaned ultrasonically or even examined microscopically. Other samples of *Sphagnum* were subsequently prepared, and each branch was dissected under a dissecting microscope, and every leaf and stem washed and scraped clean of any adhering dust or debris. Neither this cleaned *Sphagnum* sample nor any axenic cultures of *Sphagnum* showed evidence of any β-*O*-4 lignin products after thioacidolysis.

Anthoceros has proved more intriguing. After the original sample proved positive, subsequent samples from herbarium specimens were cleaned by me, and the

thioacidolysis run by John Hackney and John Obst at Forest Products Laboratory. Results have been variable, but a number of carefully cleaned samples have still shown evidence of lignin. How to interpret this? All of the samples had been from the field, and grown on and become encrusted with soil. While much time and effort was taken to clean them, it is difficult to be sure that no contaminating soil remained in the numerous folds and crevices of the thallus, even after ultrasonic cleaning with detergent. Work is continuing in collaboration with Forest Products Laboratory on this question, and the next step is to grow *Anthoceros* axenically or on a lignin- free substrate in the laboratory.

No mosses or hepatics tested showed evidence of any β-*O*-4 lignin. Is it possible that they could still contain a purely carbon-carbon linked lignin? The β-*O*-4 linkage is the most common linkage in higher plant lignins, often comprising 40-60% of the total lignin . This is because the two most stable positions for the free radical produced by peroxidases are on the phenolic alcohol and the β carbon of the propyl side-chain (Higuchi 1985, Sarkanen and Ludwig 1971). Freudenberg and Neish (1968) found that higher proportions of carbon-carbon linked lignin could be produced in vitro when coniferyl alcohol was added to a peroxidase solution all at once, rather than dropwise over a long period of time. This indicates that the rate of monolignol introduction into the cell wall matrix might influence the type of bonding produced. But even in these experiments, easily detectable amounts of β-*O*-4 lignin were always observed. Is it possible to polymerize a lignin from peroxidase- produced free radicals without producing <u>any</u> of the energetically most likely bonding? If bryophytes, at least the mosses and liverworts tested here, contain lignin, this is what they must somehow be doing.

Pellia tissue shows evidence of pyrolysis breakdown products of both *p*-hydroxyphenyl and guaiacyl lignin, yet tests negative for coniferyl alcohol (the guaiacyl

lignin monomer), β-*O*-4 linkages and shows no evidence of cell wall-associated peroxidase. While it is not yet possible to absolutely rule out a C-C *p*-hydroxyphenyl lignin being present to produce the *p*-hyroxyphenyl pyrolysis breakdown products, there seems to be no way to account for the guaiacyl pyrolysis products as deriving from coniferyl lignin.

These doubts and difficulties suggested the following question: what other non-lignin compounds might account for the *p*-hydroxyphenyl and (especially) the guaiacyl lignin pyrolysis products? The following analysis addresses this question.

Saponification/Ferulic acid and *p*-coumaric acid determination:

Results of the pyrolysis of *Pellia* indicate both guaiacyl and *p*-hydroxyphenyl lignin breakdown products. The negative Weisner phloroglucinol-HCl histochemical test leads one to suspect that the guaiacyl breakdown products are not due to coniferyl lignin. What compound(s) other than coniferyl alcohol might yield these products upon pyrolysis? Similarly, are there analogous non-lignin compounds that might account for the putative *p*-hydroxyphenyl breakdown products?

Grasses and other monocots often have appreciable amounts of ferulic and *p*-coumaric acids associated with their cells walls by ester and ether linkages (Sarkanen & Ludwig 1971, Higuchi et al. 1967). As seen in Figure 5, both of these acids are produced via the lignin pathway. They are the acid analogs and the direct precursors of *p*-hydroxyphenyl and coniferyl alcohols. They have the structure necessary to produce guaiacol, phenol, vinyl-phenol and vinyl guaiacol and the eugenol isomers upon pyrolysis.

Saponification for 4 hours with 1N NaOH breaks the ester bonds of these acids, and similar saponification at 150°C breaks ether linkages as well. Figure 52 shows the presence of these acids in *Pellia* sporophytes upon gas chromatagraphy after saponification . The sample was run once, then rerun with additional *p*-coumaric and ferulic controls added to the sample. Increase in height and area under the suspected *p*-coumaric and ferulic acid peaks confirmed their identity.

Table 4 lists the amounts of ferulic and *p*-coumaric acids in various parts of *Pellia* sporophytes, as a percentage of freeze-dried weight. Sporophyte includes setae, capsule wall, spores and elaters. These figures are quite approximate, being based on only one trial. Nevertheless, except for the *p*-coumaric acid for the setae at 150°C (which seems anomalously high), they are rough indications of the amounts of these acids within the walls of these tissues. Levels of ester-linked ferulic acid in the whole sporophyte (saponified at room temperature) is roughly the same as for that of alfalfa (0.04% vs. 0.02%).

Are these acids concentrated in the secondary cell wall thickenings? It is impossible to say at this point, but this one comparison of the amounts in the sporophyte versus the setae suggest that distribution is not even throughout the sporophyte. There is ten times as much ester-linked ferulic acid in the whole sporophyte sample as there is in the setae only, and 1.5 times as much ether-linked. It is unfortunate that spores were not tested separately, because there is no way of knowing from this data whether the additional ferulic acid found in the whole sporophyte sample came from the capsule wall and/or elaters (and hence, one would suspect, from the secondary cell wall thickenings), or from the spores. *In situ* analysis for these acids would be necessary to determine this question, and the next section on UV microscopy addresses this point.

UV epifluorescence microscopy: Lignin and ferulic acid localisation

Lignin (and other phenolics) epifluoresce a yellow to yellow green (or blue, depending upon the filters used) upon being struck with UV light. Chlorophyll fluoresces a bright red. Treated with ammonium hydroxide, ferulic acid flouresces a bright green while lignin remains its original color. The green fluorescence of ferulic acid disappears when subsequently treated with sodium acetate.

Figures 54-56 demonstrate that there is ferulic acid present in the cell walls of *Pellia epiphylla* sporophytes. However, they also make plain what the saponification/gas chromatagraphy could not: the ferulic acid is to be found throughout the primary walls, not specifically concentrated in the secondary cell wall thickenings. In fact, the secondary thickenings are the only walls that do not fluoresce!

In Figure 53 (of a grass which contains both ferulic acid and lignin), the ferulic acid-containing areas that are unlignified do not fluoresce in water. Only the lignified elements fluoresce in water, and the ferulic acid only fluoresces with the addition of ammonium hydroxide. Xylem elements in the youngest leaves in the center of the stem do not fluoresce in water, yet fluoresce green with the ammonium hydroxide. Thus it appears that ferulic acid is deposited in the xylem walls before lignin.

Ultraviolet microscopy to detect ferulic acid in hepatic capsule walls and elaters is reported for the first time here. Figure 54 was photographed without the exciter and barrier filters specified for lignin and ferulic acid epifluorescence. The secondary thickenings fluoresce yellow, as does the rest of the tissue. With ammonium hydroxide, the thickenings appear orange and non-fluorescent while the rest of the tissue fluoresces, giving evidence of ferulic acid.

Figures 55 and 56 were taken with the barrier and exciter filters specified for lignin and ferulic acid epifluorescence, and the secondary thickenings appear orange and

non-fluorescent against the blue autofluorescence of the rest of the tissue in water alone. The filters block the yellow autofluorescence seen in the unfiltered set-up, allowing one to see the less intense blue coloration. With ammonium hydroxide, the secondary thickenings are again non-fluorescent.

The blue and the green fluoresence using the barrier and exciter filters show the same distribution, and are most likely due to ferulic acid, which seems not to be in evidence in the secondary cell wall thickenings. However, the yellow autofluorescence visible without the filters is distributed within the secondary thickenings as well as the rest of the tissue.

Primary walls of all the tissues examined in the *Pellia sporophyte* apparently possess ferulic acid, and fluoresce blue in water and green with ammonium hydroxide. All walls of the gamethophyte of *Lophocolea heterophylla* (Figure 58) also exibit evidence of ferulic acid. D. V. Basile (personal communication) reports that all liverwort tissue that he has ever examined with UV light are fluorescent. This study suggests the possibility that ferulic acid may be of widespread occurrence in hepatic tissue.

Ferulic acid does not seem to have as widespread an occurrence in mosses. Schaffer (1985) utilized UV microscopy to examine the cell walls of *Anomodon rostratus* and *Thuidium delicatulum*, and reported no evidence of ferulic acid in either plant. Davidson et al (1989) reported cell wall bound ferulic and *p*-coumaric acids in bryophytes, but only in the maturing tissue of the sporophytes, and suggested its occurrence there as a deterrent to herbivory by slugs, which feed readily on the immature tissue. Gametophytes of*Thuidium delicatulum* were examined here. Green fluorescence specifically in sodium acetate, characteristic of ferulic acid was not observed, although maturing leaves showed deposition of some compound that fluoresced green in both water and ammonium hydroxide. That behavior is not

indicative of ferulic acid, and application of ammonium hydroxide caused no discernable change in the fluorescence pattern in this plant. These results agree with the analysis presented by Schaffer (1985) that there is no evidence for the presence of ferulic acid in this plant.

In sum, UV microscopy confirms the presence of ferulic acid in sporophytes of *Pellia epiphylla*, as indicated by the saponification/GC analysis. However, the ferulic acid is found in all primary walls, and is either absent from or obscured in the secondary walls, not especially concentrated in them as was hypothesized in response to the pyrolysis and thioacidolysis results. Ferulic acid's occurence in gametophyte tissue of *Lophocolea heterophylla* suggests that it may be of widespread occurrence in hepatics. Previous reports, and preliminary work done here with mosses show that mosses do not contain ferulic acid in all tissues, but it may be laid down in special tissue for special purposes. It would be of great interest to examine more bryophytes with this technique, and very interesting indeed to investigate the hornworts as well.

Summary of chemical analyses

The original hypothesis motivating this study was the possible homology of hepatic elaters and higher plant xylem. Implicit in that hypothesis is the expectation that lignin or some closely allied compound would be selectively deposited in the secondary cell walls of the elaters at maturity. Has this expectation been borne out by the chemical investigation? Many differences and very few similarities have been uncovered between the chemical nature of the cell walls of *Pellia* sporophyte tissue, and what is known about higher plant lignified walls of xylem.

One of the few similarities is shown by the IKI-H_2SO_4 histochemical test, where, like lignin, the unknown compound selectively deposited in the secondary cell

wall thickenings is resistent to sulfuric acid and removed by calcium hypochlorite. Similarly to lignin, it seems to act as a binding matrix to the cellulose, perhaps resistant to the lysosomal acids which hydrolyse the exposed primary walls, and most probably restricting the ability of the secondary cell wall to expand and contract due to water tension and mechanical stress.

No test indicated that the compound deposited in the secondary cell walls at muturity was a lignin. All tests showed fundamental differences between this selectively deposited compound and all known forms of lignin. Included among these were (from the developmental study) absence of the peroxidases characteristic of lignification , compound deposition proceeding from cytoplasm outward (lignification occurs from the primary wall inward) and native electron-density. Histochemical analysis revealed red-brown staining with FeCl -HCl, blue-black staining with FeCl-EtOH (lignin shows none of these staining reactions), and non-autofluorescence under conditions when lignin fluoresces a white-blue. Although unknown in higher plant lignins, there is no reason to suspect that a C-C linked *p*-hydroxyphenyl lignin would exhibit any of these characteristics.

Extraction analyses indicate that there is no reason to suspect that a lignin occurs anywhere outside of the secondary cell wall thickenings either. The thioacidolysis analysis rules out any lignin with β-*0*-4 linkages (guaiacyl, syringyl or *p*-hydroxyphenyl). Maule and phloroglucinol histochemical tests rule out any guaiacyl or syringyl monomers regardless of linkage. The combination of these tests logically exclude any kind of lignin except C-C linked *p*-hydroxyphenyl lignin, for which there is as yet, unfortunately, no straightforward test.

CONCLUSIONS

The motivation for this study was the perceived similarity at the light microscope level between hepatic elater and higher plant protoxylem tracheary element morphology. The evolutionary and phylogenetic implications of homology between elaters and tracheary elements would imply an ancestral plant and cell type, a synapomorphy in cladistic terminology. Evidence for homology rests in similarity of structure, position and, especially, development. Of key interest in this study were two characters of tracheary elements unshared by bryophyte water conducting tissue, and hence of interest in elaters, i.e. ornamented secondary cell wall thickening, and selective deposition of lignin within those thickenings at maturity.

As a result of these investigations, the two characters have become subdivided further into three interrelated characters, with differences as to if and how they are shared by higher plant xylogenesis:

1. ornamented secondary cell wall thickenings (ultrastructural scale): The deposition of cellulosic secondary cell wall thickenings is correlated with the same ultrastructural components such as microtubules, endoplasmic reticula, and golgi bodies and derived vesicles as has been observed in xylogenesis. No significant differences have been found, leading to the conclusion that the development of secondary cell wall thickenings and central features of ultrastructural architecture are homologous in these two groups of land plants.

2. selective deposition of lignin within the secondary cell wall thickenings (macromolecular scale): Since no lignin has been found in elaters or capsule walls, this character is not homologous. Materials selectively deposited into the secondary cell walls at approximately the same period during maturation as lignification would be expected to occur appear to be aliphatic in nature, natively electron dense, and deposited within the secondary cell walls in two successive waves of opposite direction, while

lignin is polyphenolic, electron transparent and occurs in only one wave of deposition into the secondary cell walls. The thickenings at maturity appear to function similarly to thickenings in tracheary elements in terms of preventing collapse of the cells, and regulating the path of movement of water. The materials selectively deposited within the thickenings would seem to be important in terms of these functions; similarly to lignified walls, they resist dissolution in IKI-H_2SO_4, and are dissolved by calcium hypochorite. In other words, while similarly resistent to acid breakdown, and appearing to be similar in function, the identity of the compounds are quite different. At the macromolecular scale, this points to an analogous (i.e. convergent) rather than homologous relationship between the materials deposited within the secondary walls.

3. presence of lignin precursors *p*-coumaric and ferulic acids (molecular scale): An unanticipated and intriguing result of this study was the discovery of lignin precursors *p*-coumaric and ferulic acids both ether- and ester-linked to the cell walls of all portions non-thickened portions of the *Pellia* sporophyte examined, as well as all walls of the gametophyte of *Lophocolea heterophylla*. In contradistinction to the as-yet unidentified material selectively deposited into the secondary cell wall thickenings, these acids are distributed throughout the primary walls. Absent or obscured from the secondary cell wall thicknenings, they are definitely not restricted in occurrence to them, as is true of lignin in higher plant tracheary elements.

While the distribution of these lignin precursors is different from that of lignin in higher plant tracheary elements, it is similar to the distribution of these acids in some higher plants, especially that of the angiosperm taxa Graminae. The ability to make these acids, as precursors in the lignin biosynthetic pathway, are perforce a generalized trait necessary before the production of lignin. Yet their widespread occurence in primary walls is unshared by the mosses, yet shared with the angiosperm Graminae. Homology or analogy, apomorphy or synapomorphy? One possible explanation is that

widespread ferulic and *p*-coumaric acid deposition is a homology that unifies the embryophyte clade, appearing as an apomorphy in both the hepatics and the Graminiae.

Differences in ontogeny imply convergence rather than homology in comparisons among higher plant vessels (Young and Richardson 1982), as well as between elaters and tracheary elements. In addition to the characters given above, the phenomenon of rounding up early in elaterogenesis observed by others and in this study is a major difference in the early ontongeny of the cell from that of xylogenesis. Thus, while certain developmental capacities and structures within elaterogenesis such as deposition of secondary cell walls appear to be homologous to that of higher plant tracheary elements, the developmental trajectory of the cell as a whole is clearly not homologous.

As stated in the summary to the chemical analyses, this study has provided no positive evidence for lignin in the sporophytes of *Pellia epiphylla*. Given the negative phloroglucinol, Mäule and thioacidolysis results for these tissues, there appears to be no guaiacyl or syringyl lignin present, and no β-0-4 linked lignin of any kind, guaiacyl, syringyl or *p*-hydroxyphenyl. This leaves only the logical possibility of a carbon-carbon linked *p*-hydroxyphenyl lignin, the hypothesis that Bland et al. (1968) propose for lignin in *Sphagnum*. There is no positive evidence for this type of lignin in *Pellia* sporophytes, like the Bland et al. hypothesis in *Sphagnum*, it is merely the only type of lignin present that could produce the range of negative and positive results observed.

In this study, pyrolysis of *Pellia* sporophytes produced some breakdown products that are characteristic of *p*-hydroxyphenyl lignin. This was the only strong evidence produced that a *p*-hydroxyphenyl lignin could be present. However, another compound, *p*-coumaric acid, was definitely shown to exist in this tissue which could also produce these putative *p*-hydroxyphenyl lignin pyrolysis products. Is the amount

of *p*-coumaric acid sufficient to account for all of the *p*-hydroxyphenyl pyrolysis breakdown products observed? This would provide strong evidence that a *p*-hydroxyphenyl lignin was not present. Quantification of the amounts of *p*-coumaric acid and *p*-hydroxyphenyl pyrolysis breakdown products was not rigorous enough to definitively address this question. Further research would be necessary to provide a definitive answer to this elusive question.

From this study, one must conclude that, in the five different methods utilized here, there has been no evidence to indicate any kind of a lignin present in the sporophyte of *Pellia epiphylla*. In addition, preliminary evidence suggests that ferulic and *p*-coumaric acids, lignin precursors, may be producing all of the apparent *p*-hydroxyphenyl pyrolysis breakdown products present in this tissue. While it awaits rigorous quantification and confirmation, this would falsify, at least in this hepatic, the hypothesised presence of carbon-carbon linked *p*-hydroxyphenyl lignin in bryophytes. Even without lignin, however, the presence of ferulic and *p*-coumaric acid lignin precursors within these specialized hepatic cells may, with further research, provide intriguing insight into the evolution of lignin and lignification, and its role in the adaptations of the land plants to life in the terrestrial milieu.

Table 1: Summary of bryophyte lignin research

researchers	year	bryophytes studied	techniques used	conclusions
Gjokic @	1895	non-vascular plants (including mosses)	Wiesner (phloroglucinol) reaction	all negative, no lignin
Linsbauer @	1899	non-vascular plants (including mosses)	Wiesner (phloroglucinol) reaction	all negative, no lignin
Stadnikow & Baryschewa ~	1930	Sphagnum	hydrochloric acid lignin	9% hydrochloric acid lignin
Holmberg ^	1934	Polytrichum & Marchantia	lignothioglycolic acid extraction	no lignin
Manskaya & Koehneva @	1948	Sphagnum (and Fucus)	UV microscopy	no blue fluorescence characteristic of lignin
Kondrat'ev @	1949	Sphagnum	dioxane-acidolysis	no lignin isolated
Kratzl & Eibl ~	1951	Sphagnum	Klason lignin/nitrobenzene oxidation	no lignin (no vanillin, .1% methoxylated product)
Towers @	1951	Marchantia, Funaria	oxidation	no lignin (no aromatic aldehydes isolated)
Towers & Gibbs	1953	Marchantia, Funaria	Maule histochemical test	no lignin
Farmer #	1953	Sphagnum	oxidation	p-hydroxybenzaldehyde found, but not a lignin
Farmer & Morrison #	1955	Sphagnum	permanganate oxidation	4% anisic (p-hydroxybenzoic) acid, no other aromatic acids
Gibbs @	1958	non-vascular plants (including mosses)	Mäule reaction	all negative, no syringyl lignin
Siegel	1962	Polytrichum peristome, Jungermannia rhizoids	extraction with dioxane	lignin (different UV absorption than other lignins)
		Bryum, Rhodobryum, Marchantia	extraction with dioxane	no sign of lignin
Manskaya & Kodina #	1966	not described	not described	"Sphagnum lignins" derived from p-coumaryl alcohol
Nilsson & Tottmar	1967	Sphagnum	cupric hydroxide oxidation	guaiacyl and p-H products found
Bendz et al #	1967	20 spp. of Sphagnum	MeOH extraction, phloroglucinol stain	all test positive
Bland et. al.	1968	Sphagnum	vibratory ball-mill, extraction	C-C linked p-hydroxyphenyl lignin
Siegel	1969	Dawsonia, Dendroligotrichum, Polytrichum	ethanolysis, Klason lignin	low methoxyl lignin in Daw. & Dend., none in Poly.
Sarkanen & Latif	1971	Rhytidiadelphus loreus	ethanolysis	no lignin (absence of Hibbert's ketones)
Freudenberg *	1972	Polytrichum, Sphagnum	Klason lignin	high H2O, low OMe content, Poly. 38% lignin
Scheirer	1972,5	Dendroligotrichum	histochemistry, UV fluorescence	lignin present in secondary cell wall thickenings
Erickson & Micksche	1974a,b	7 mosses & liverworts	oxidation (KMnO4, cupric oxide)	no lignin, 3 alternative compounds proposed
Reznikov & Novitskii*	1975	Sphagnum	dioxan lignin, reductive degradation	p-hydroxyphenyl lignin (with ether linkages)

Table 1: Summary of bryophyte lignin research (continued)				
Nimz & Tutschek	1977	Sphagnum	ball mill/dioxane extract/C13NMR	no lignin/ p-H residues, but no b-0-4 linkages
Logan & Thomas	1985	Polytrichum, Plagiochila, Marchantia	cupric oxide oxidation	p-H, guaiacyl & syringyl products found
Smeerdijk & Boon	1987	Sphagnum subfossils, and peat	pyrolysis-GC-MS	possible lignin-derived pyrolysates
Wilson et. al.	1989	Thamnobryum, Rhizogonium, Dawsonia,	solid state C13NMR	no lignin, instead a tannin-like compound
		Leucobryum, Sphagnum	solid state C13NMR	w/ 1,3,5-trihydroxybenzene structure
* = from Markham & Porter 1978 # = from Nilsson & Tottmar 1967 ~ = from Lindberg & Theander 1952				
^ = from Erickson & Micksche 1974 @ = from Sarkanen & Hergert 1971				

Table 2: Histochemistry of *Pellia epiphylla* sporangial tissue

		reaction with *Pellia*	reaction with *Pellia*	reaction with *Pellia*
Stain	**Specific effect**	**2ary cell walls**	**primary walls**	**setae**
Phloroglucinol	coniferyl alcohol red	no reaction	no reaction	no reaction
Maule	syringyl alcohol red	brown	brown	brown
IKI/H2SO4	cellulose blue	yellow-green	blue	blue
	lignin yellow-green			
Safranin	red with phenolics	red	red	red
Toluidine blue	cellulose purple	blue	purple	purple
	lignin blue-green			
Sudan IV	fats and waxes red	no change	pink	pink
Sudan black	fats and waxes black	no change	slightly dark	black
Ferric chloride (EtOH)	phenols green	black	no reaction	green
Ferric salt (HCl)	tannins blue-black	reddish brown	no reaction	no reaction

Table 3: Infra red lignin peaks						
Position in cm-1			# on			
Guaiacyl lignin	Syringyl lignin	Band origin	Fig. 84	*Pellia*	*Pinus*	*Ligust*
3425-3400	3450-3400	OH stretching (H-bonded)	1	+	+	+
2920	2940	CH-stretch in methyl and methylene groups	2	-	-	+
2875-2850	2880	CH-stretch in methyl and methylene groups	3	+	+	+
2820	2845-2835	CH-stretch in methyl and methylene groups	4	+	+	+
1715	1715-1710	Carbonyl stretching -				
		unconjugated ketone and carboxyl groups	5	+	+	+
1605	1595	Aromatic skeletal vibrations	6A	+	+	+
1515-1510	1505	Aromatic skeletal vibrations	7A	+	+	+
1430	1425	Aromatic skeletal vibrations	8A	+	+	+
1370	1370-1365	C-H deformation (symmetric)	9	?	?	?
	1330-1325	Syringyl ring breathing with CO stretching	10S	-	-	+
1270	1275	Guaiacyl ring breathing with CO-stretching	11G	?	+	+
	1235-1230	Syringyl ring breathing with CO-stretching	12S	?	-	+
1140	1145 shoulder	Aromatic C-H in-plane deformation, guaiacyl	13G	?	+	+
	1130	Aromatic C-H in-plane deformation, syringyl	14S	-	-	+
1085	1085	C-O deformation, secondary alcohol				
		& aliphatic ether	15	?	+/-	+
1035	1030	Aromatic C-H in-plane deformation, guaiacyl				
		type, and C-O deformation, primary alcohol	16G	?	+	+
970	970	=CH out-of-plane deformation (trans)	17	?	?	?
	915	Aromatic C-H out-of plane deformation	18S	-	-	+
855	860 shoulder	Aromatic C-H out-of plane deformation	19A	?	?	?
815	835	Aromatic C-H out-of plane deformation	20A	?	?	?
750-770 shoulder	750-770 shoulder	Aromatic C-H out-of plane deformation	21A	?	?	?

Table 4:
Pyrolysis Breakdown Products Characteristic of Lignin

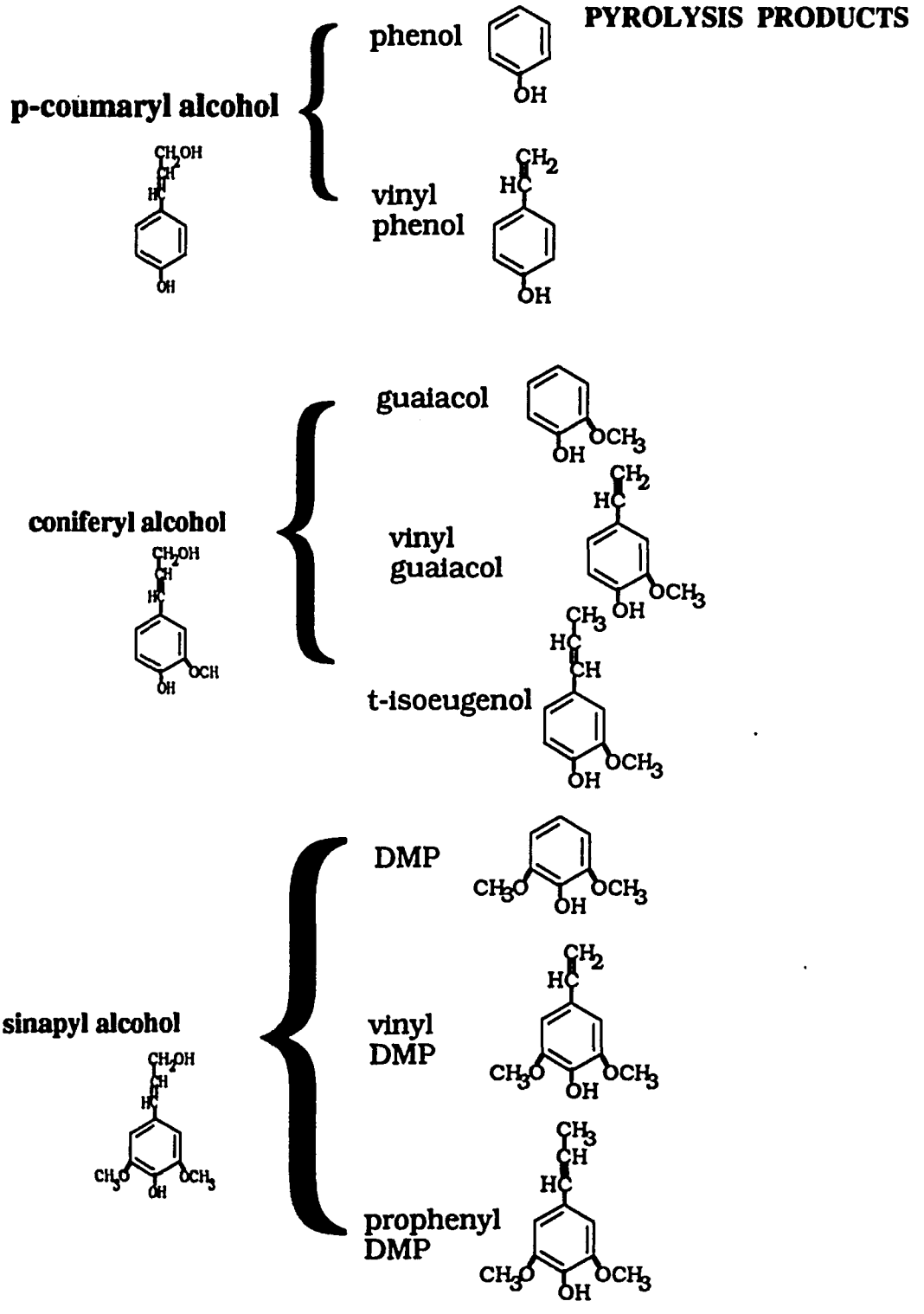


Table 5: Ferulic and p-coumaric acid quantification of *Pellia epiphylla*

	sample wt (mg)	ferulic acid	p-coumaric acid	FA/pCA
alfalfa, RT		0.02 %	0.02 %	1x
setae, RT	61.0	0.004%	0.001%	4x
sporophyte, RT	129.8	0.043%	0.005%	9x
setae, 150	52.8	0.067%	??0.237%	??
sporophyte, 150	110.1	0.100%	0.012%	8x
sporophyte/setae RT		10x	5x	
sporophyte/setae 150		1.5x	??	

Figure 1. Liverwort elaters and higher plant xylem: comparison of form and context. While their morphology is similar, liverwort elaters and higher plant xylem exist in very different contexts and have different functions in the life of the plant. Elaters occur within an ephemeral sporophyte that disperses its spores and withers away within days. The elaters function to help disperse the spores. Higher plant xylem exists within a long-lived, independent sporophyte and functions to conduct water throughout the plant body.

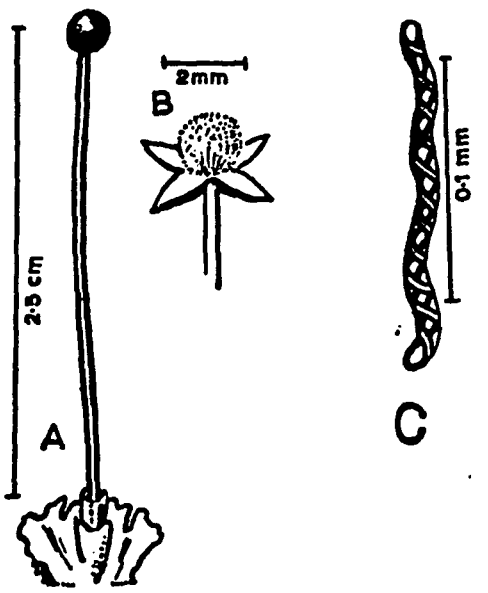
Figure 1a. Sporophyte and gametophyte of *Pellia epiphylla*. The sporophyte consists of capsule (C), seta (S) and foot (unpictured, embedded within the gametophyte (G).

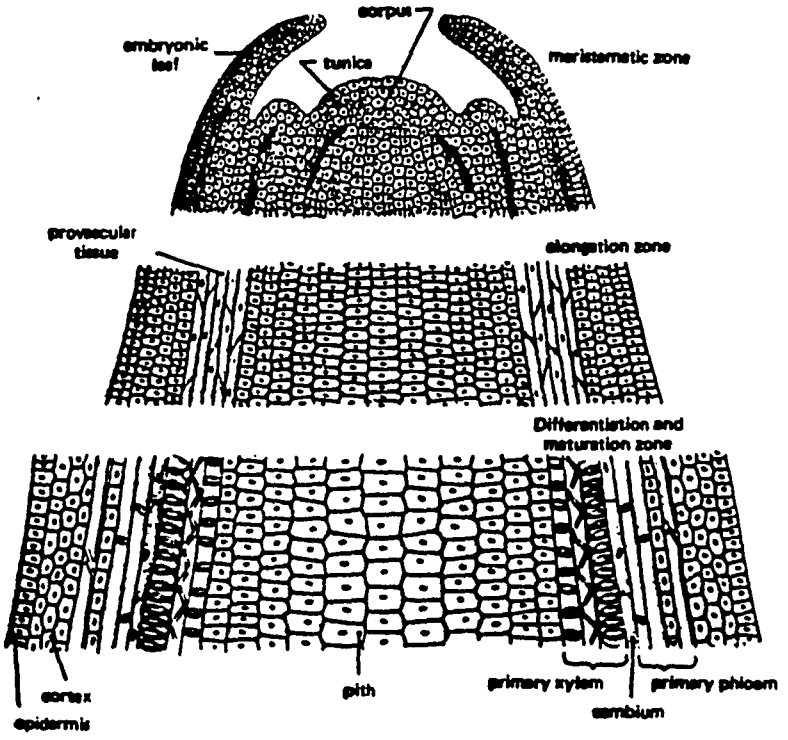
Figure 1b. Open capsule or sporangium of *P. epiphylla*, showing the capsule wall split into four valves, with the spore and elater mass in the center.

Figure 1c. One elater with two spiral secondary cell wall thickenings.

Figure 1d. Diagram of a longsection of a higher plant stem, showing the primary xylem within the differentiation and maturation zone.

(Figures 1a-c from Watson (1967), The Structure and Life of Bryophytes . Figure 1d from Bidwell (1979) Plant Physiology).


2mm
B
2·5 cm
A
0·1 mm
C


corpus
embryonic leaf
tunica
meristematic zone
provascular tissue
elongation zone
Differentiation and maturation zone
cortex
epidermis
pith
primary xylem
primary phloem
cambium

D

Figure 2. The role of elaters in spore dispersal.

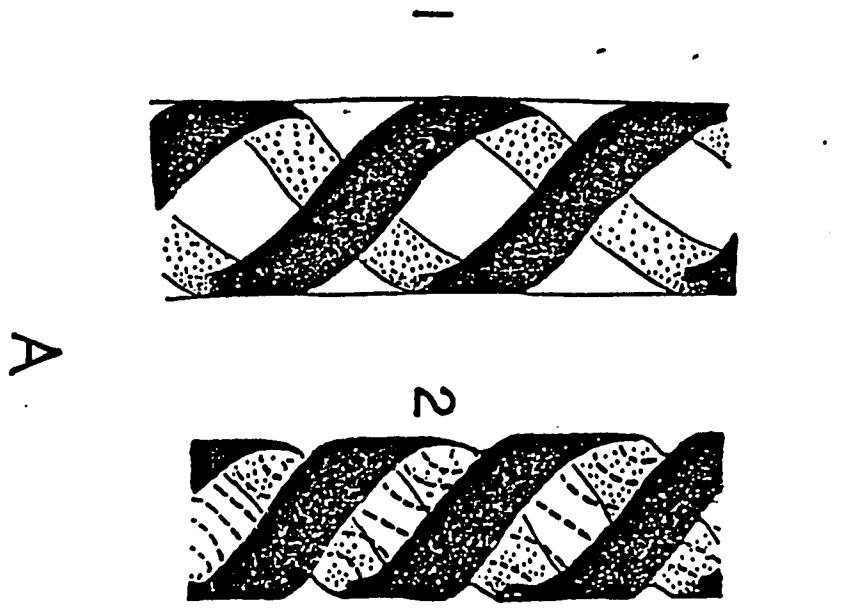
Elaters wind and unwind in response to changes in water content and humidity. This movement is utilized to disperse the spores in different ways, depending upon their size and morphology, and if and how they are attached to the capsule wall.

Figure 2a. Changes in elater morphology in response to drying. 1. hydrated elater 2. partially dried elater in which the water is under strain. Thin regions of the wall are sucked in between the coils of thickening. Depending upon the size and shape of the elaters, this movement can result in violent "spiral spring" movements which fling the spores into the air, or in gentler winding and unwinding movements which serve to simply fluff up the spore mass and disperse them gradually.

Cephalozia bicuspidata (from Ingold (1939), Spore Dispersal in Plants).

Figure 2b. The catapult method of spore dispersal in *Frullania*. 1. unopened capsule. 2. capsule valves opening, elaters still attached at both ends but stretching due to the opening of the capsule walls. 3. Elaters break loose from the base of the capsule, returning to their original size and shape in a sudden motion which flings the attached spores (not shown) into the air.

Frullania dilatata (from Ingold(1939), Spore Dispersal in Plants).


1
A
2

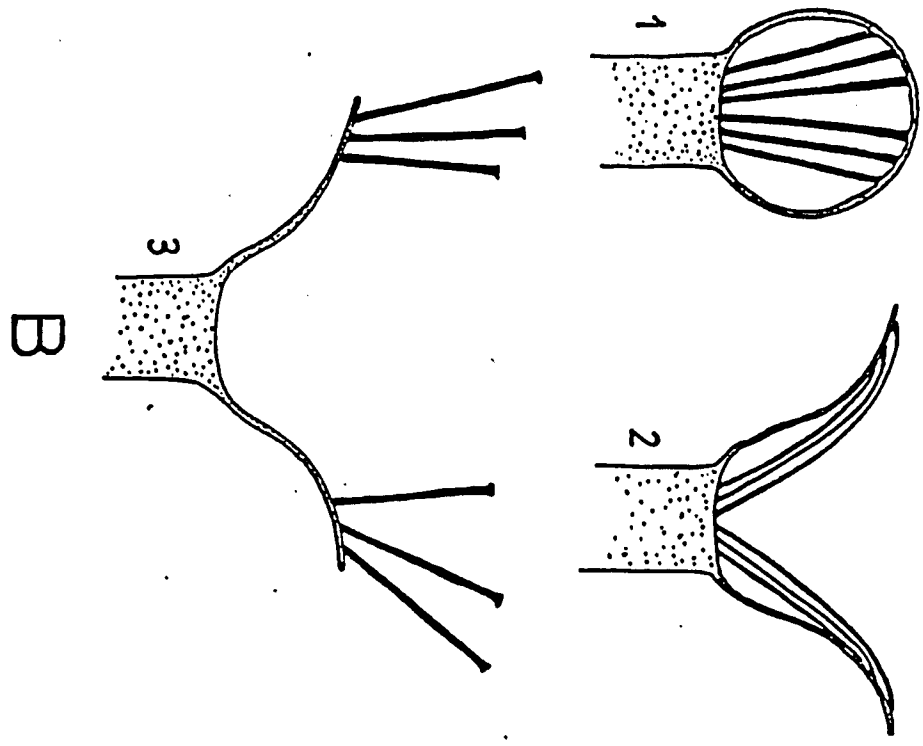

1
3
B
2

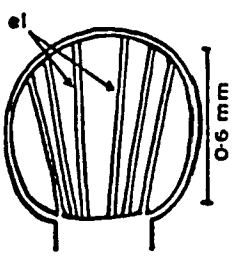
Figure 3. Elater distribution patterns in different taxa.

Figure 3a. *Frullania* type, with elaters attached at both ends to the capsule wall. (el=elater)

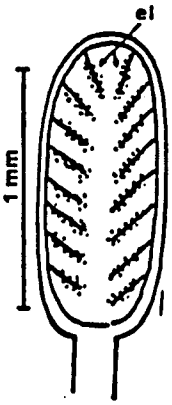
Figure 3b. *Nowellia* type, with elaters attached only at one end to the capsule wall, the other end free within the elater/spore mass.

Figure 3c. *Pellia* type, with a central mass of elaters (elaterophore), and the rest of the elaters distributed freely within the capsule. This figure also shows the parts of the hepatic sporophyte: capsule (caps), seta (s), and foot (ft).

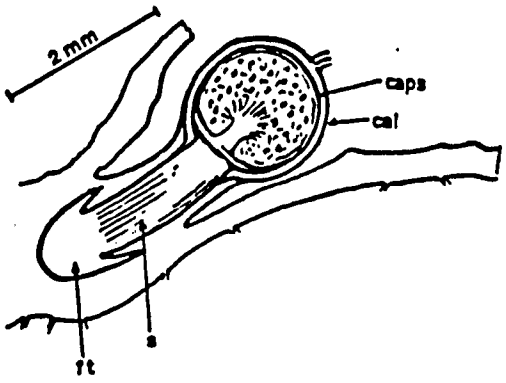
(Figures from Watson (1967) *The Structure and Life of Bryophytes*)



A



B



C

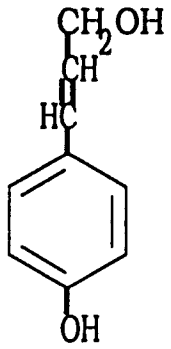
Figure 4. Lignin monolignols.

The three lignin monomers possess the same structure, i.e. a phenolic with a three carbon side chain. They differ in the number of methoxyl groups attached to the benzene ring.

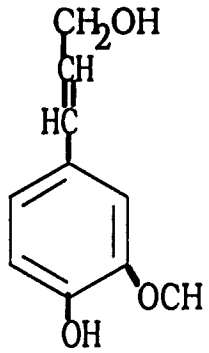
Figure 4a. *p*-coumaryl alcohol

Figure 4b. coniferyl alcohol

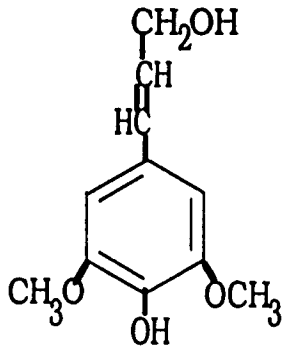
Figure 4c. syringyl alcohol



A



B



C

Figure 5. The lignin biosynthetic pathway.

Lignin is produced from phenylalanine (and in some cases tyrosine) via *p*-coumaric acid, ferulic acid and sinapic acid. The monolignols *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol are each attached to a glucose molecule when shipped out to the cell wall. Once within the cell wall, the glucose molecule is removed, and the monolignols are polymerised to lignin.

Lignin Biosynthetic Pathway

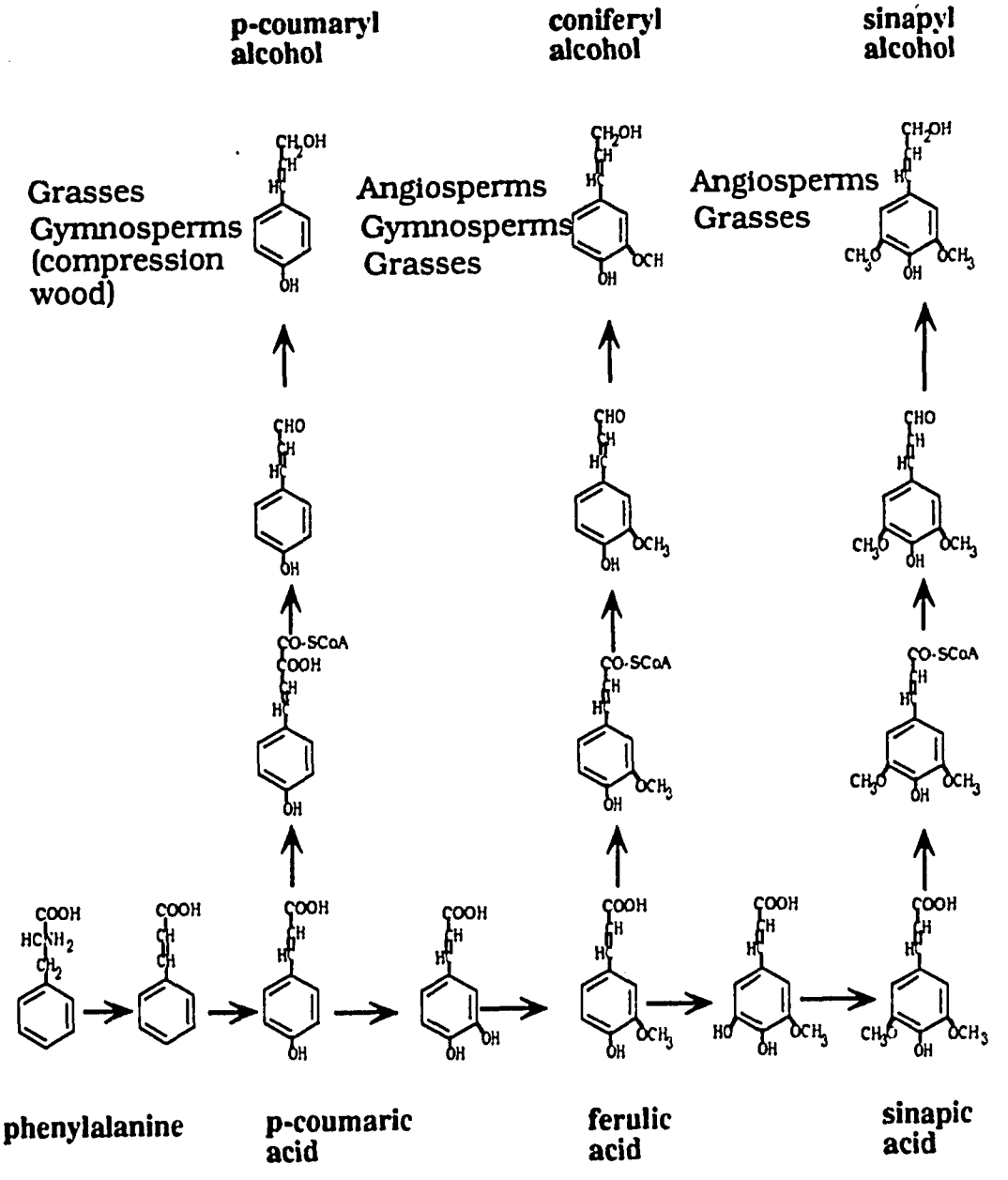
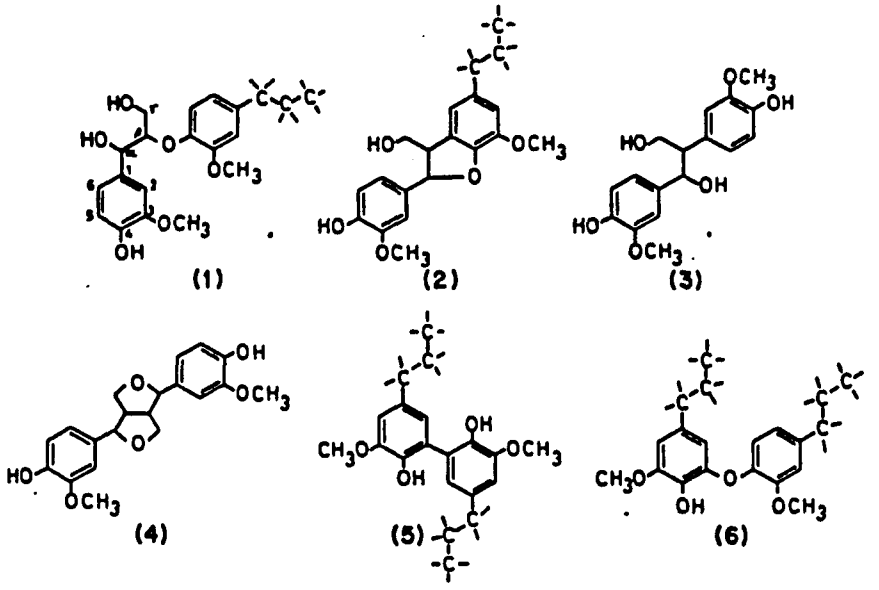


Figure 6.

Dimeric structures containing the principal linkage modes between monomeric phenylpropane units in lignin macromolecules. Note the variety of carbon-carbon and ether linkages, however most higher plant lignin is composed of 40-60% β-0-4 aryl ether linkages as pictured in (1).

(From Higuchi 1985)



[1] Dimeric structures containing the principal linkage mode between monomeric phenylpropane units in lignin macromolecules.

Figure 7. Proposed structure of spruce lignin (from Sarkanen and Ludwig 1970)
This figure illustrates the complexity and heterogeneity of the lignin molecule *in situ*.

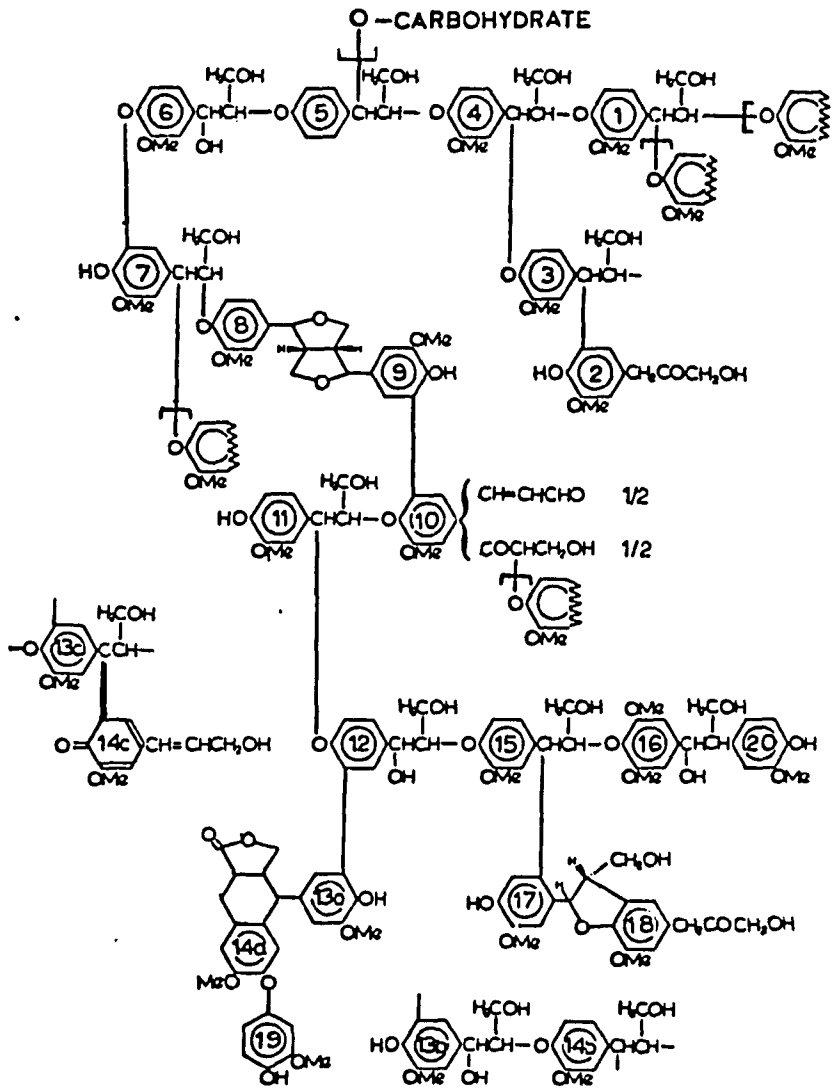

O–CARBOHYDRATE
CH=CHCHO 1/2
COCHCH₂OH 1/2

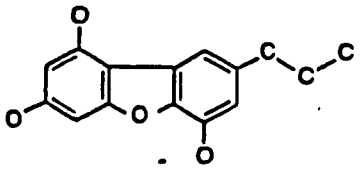
Figure 8. Proposed alternatives to bryophyte "lignin"
The following structures have been proposed by Erickson and Micksche (1974a) and Wilson et al (1989) as non-lignin compounds which are giving the "lignin-like" results in various chemical analyses of bryophytes for lignin.

Figure 8a. A dibenzofuran structure, proposed from studies of *Polytrichum commune* , but proposed to exist in both mosses and liverworts. (Erickson & Micksche 1974b).

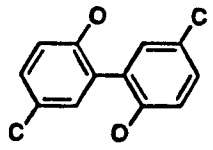
Figure 8b. 2,2′-dihydroxy-4,4′-dialkylbiphenyl structure, proposed from studies of *Ptilium crista-castrensis* and *Plagiochila asplenoides* , but proposed to exist in both mosses and liverworts (Erickson & Micksche 1974a).

Figure 8c. An "uncondensed" *p*-hydroxyphenyllkyl structure, proposed to exist in *Sphagnum* (Erickson & Micksche 1974a).

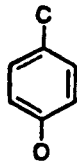
Figure 8d. A tannin-derived 1,3,5-hydroxybenzene structure, proposed from solid-state NMR studies of 5 different mosses (Wilson et al. 1989).



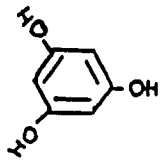
(A)



(B)



(C)



(D)

Figure 9. Bryophyte and higher plant water conducting cells.
Water conducting cells of bryophytes and higher plants have similar shapes, being elongated with tapering end walls. They differ in how the walls are rendered more porous, and how (and if) they are strengthened by secondary cell wall thickening and lignification.

Figure 9a. A liverwort conducting cell (e.g. from *Pallavacinia*) Walls are rendered porous through the enlargement of plasmodesmata-derived pores.

Figure 9b. A moss conducting cell. End walls are hydrolysed during autolysis of cell contents to become very thin and fibrillar. The lateral walls may or may not be secondarily thickened. If they are not, they too become hydrolysed.

Figure 9c. A higher plant tracheid. Secondary cell walls are produced in annular, helical or reticulated patterns. The primary walls in between the secondary walls become hydrolysed. The primary walls underneath the lignified secondary thickenings are not hydrolysed.

(From Hébant 1977)

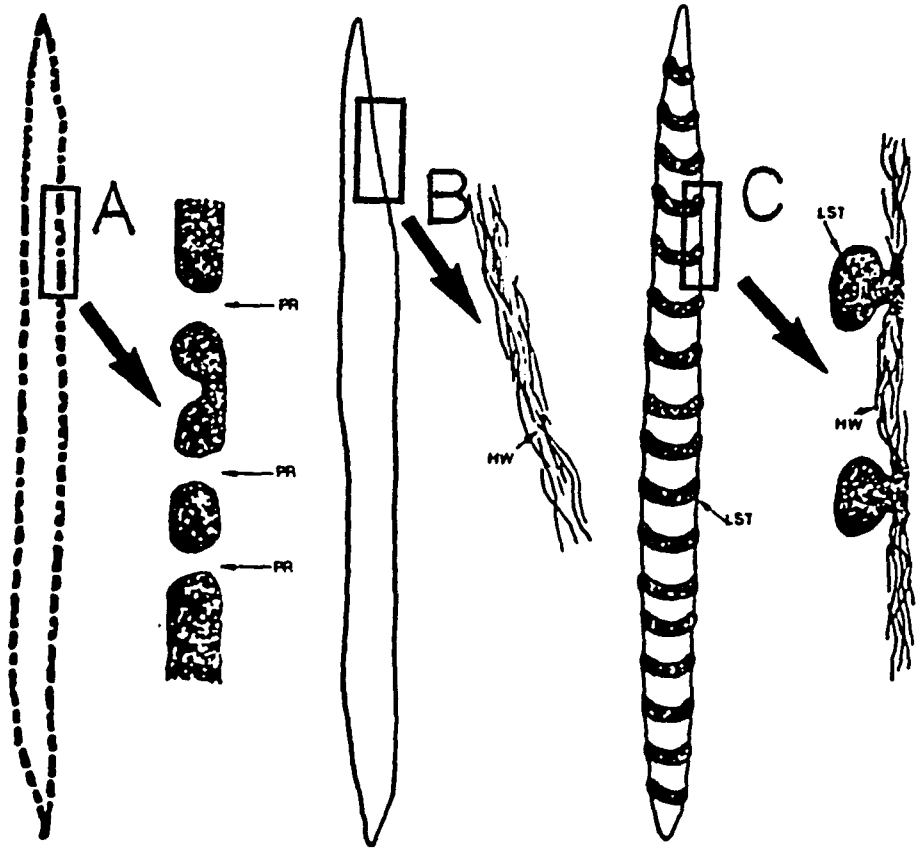

A
PR
PR
PR
B
HW
C
LST
HW
LST

Figure 10. Median longsections of *Lophocolea heterophylla* sporophytes of different ages.

The outermost layer in each case is the parent gametophyte tissue, the calyptra (C), which is derived from the archegonium which originally housed the egg. The archegonial canal (AC) seen in Figure 9c was the entrance through which the sperm swam to fertilize the egg. The sporophyte tissue visible here are capsule wall (W), sporogenous/elaterogenous mass (M) and seta (S).

Figure 10a. Capsule wall differentiated from sporogenous mass. The capsule wall is one to two layers thick, and dividing both anticlinally and periclinally. The sporogenous mass is approximately 6-8 cells wide.
magnification: 230X.

Figure 10b. Capsule wall 2-3 layers thick, sporogenous mass still undifferentiated, approximately 14-16 cells wide. Seta is now 6 cells wide and vacuolate.
magnification: 220X.

Figure 10c. Capsule wall 3-4 layers thick, the sporogenous mass has begun the process of "rounding up" and separating from one another.
magnification: 230X.

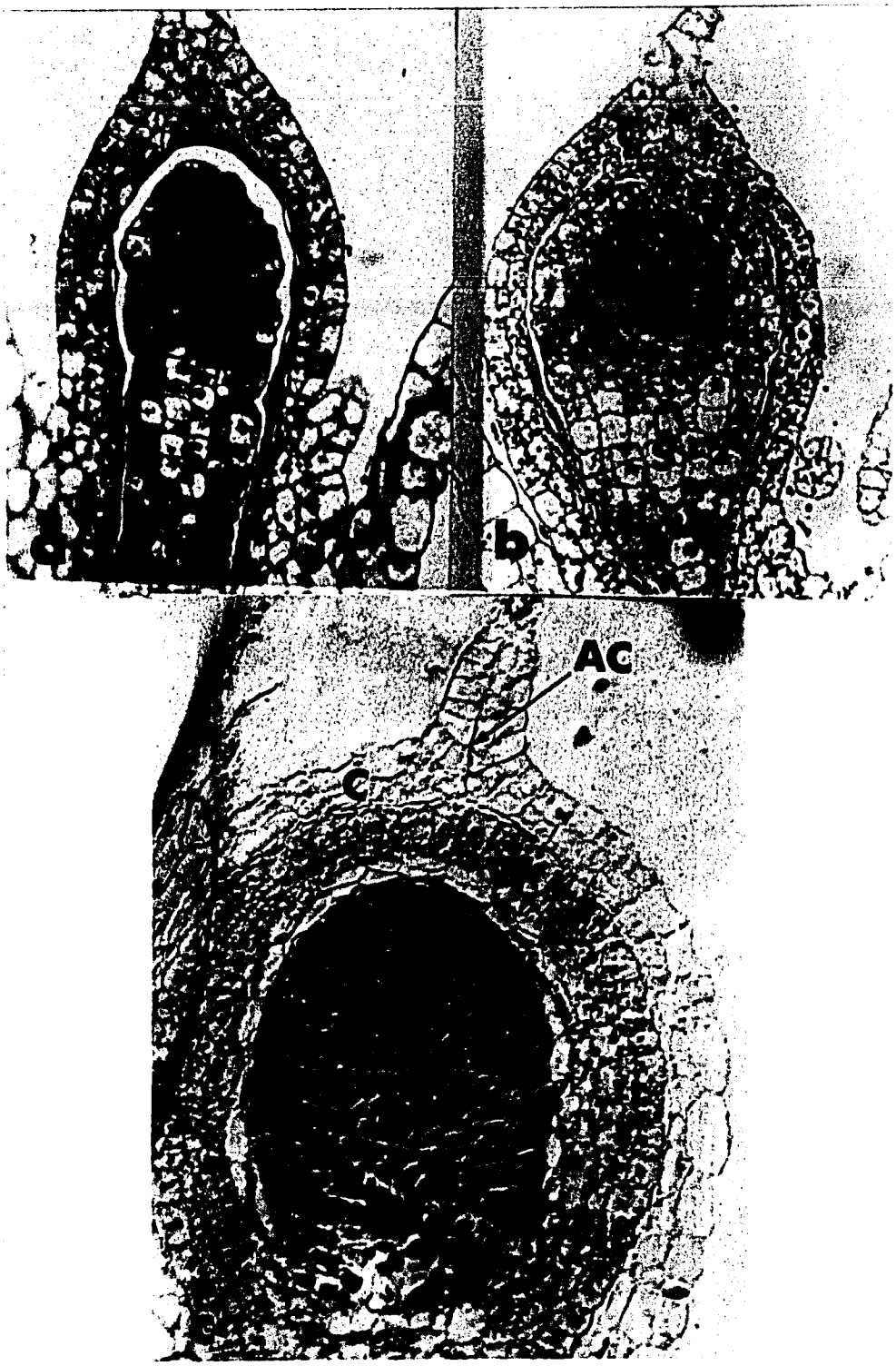

b
Ac
c

Figure 11. Older sporophytes of *L. heterophylla.*

Figure 11a. Elaters (e) are in the process of elongation. Spores (s) are beginning the process of meiosis, as can be seen from the lobing of the cells into tetrads (arrowheads). C=calyptra. W=capsule wall.
magnification: 340X.

Figure 11b. Mature sporophyte. Some of the cells of the capsule wall (W) possess secondary thickenings (st). Elaters (e) are dead and empty, visible only by the dark helical thickenings. Elaters are randomly arranged within the capsule, and hence can be seen in both longitudinal and transverse orientation in this one section (arrows). Oblique sections through the elaters cause the helices to appear as a comma with a dot in the center (arrow to left).
magnification: 275X.

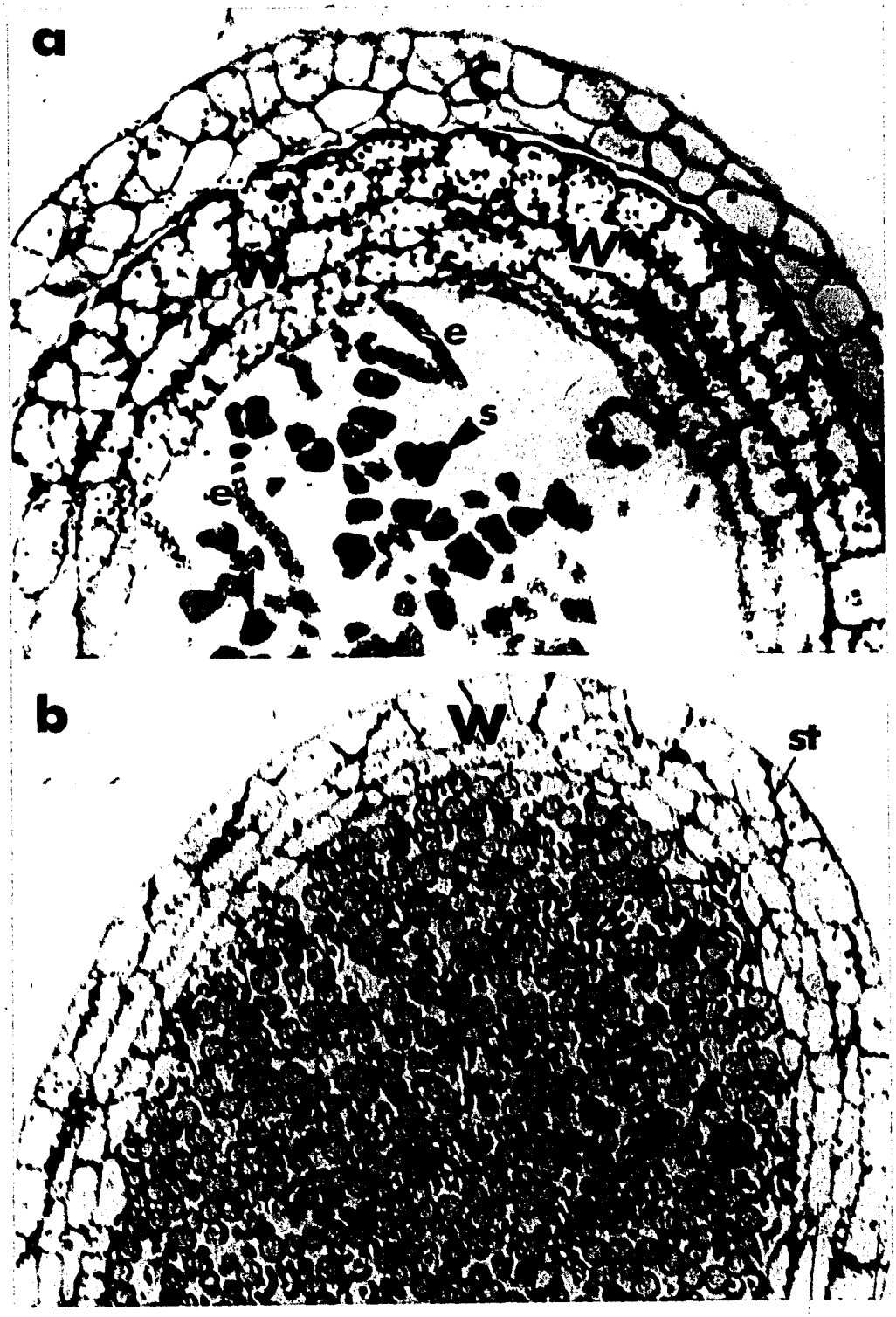

a
W
W
e
s
e
b
W
st

Figure 12. Elater maturation.

Figure 12a. An elongated elater developing secondary thickenings (st), still with live cell contents. Spores (S) have completed meiosis, but do not yet have mature walls. magnification: 1000X.

Figure 12b. Elaters are fully mature and devoid of cell contents. magnification: 700X.


st
s
a
b

Figure 13. Variations in sporangial development in other hepatics.

Figures 13a-c. *Porella platyphylloidea.*

Figure 13a. Transverse section of sporophyte. Spores within the sporogenous/elaterogenous mass (M) are undergoing meiosis, as can be seen from the formation of tetrads. Note the large amount of space between cells.
magnification: 980X.

Figure 13b. Higher magnification of Figure 13a. Note the persistent cell walls (arrows) around the spores and elaters.
magnification: 1,200X.

Figure 13c. Longitudinal section through whole sporophyte of approximately the same age. C=calyptra, M=sporogenous/elaterogenous mass, S=seta, F=foot.
magnification: 150X.

Figure 13d. *Nowellia curvifolia.* Longitudinal section through sporangium. The cells in the periphery of the sporogenous /elaterogenous mass (M) are all elongated, extending from the capsule wall (W) inward. This means that sporogenous as well as elaterogenous cells elongate at the same time. The sporogenous cells must then subsequently divide and undergo meiosis, while the elaterogenous cells remain elongated.
magnification: 760X.

C=calyptra, W=capsule wall, M=sporogenous/elaterogenous mass, S=seta, F=foot

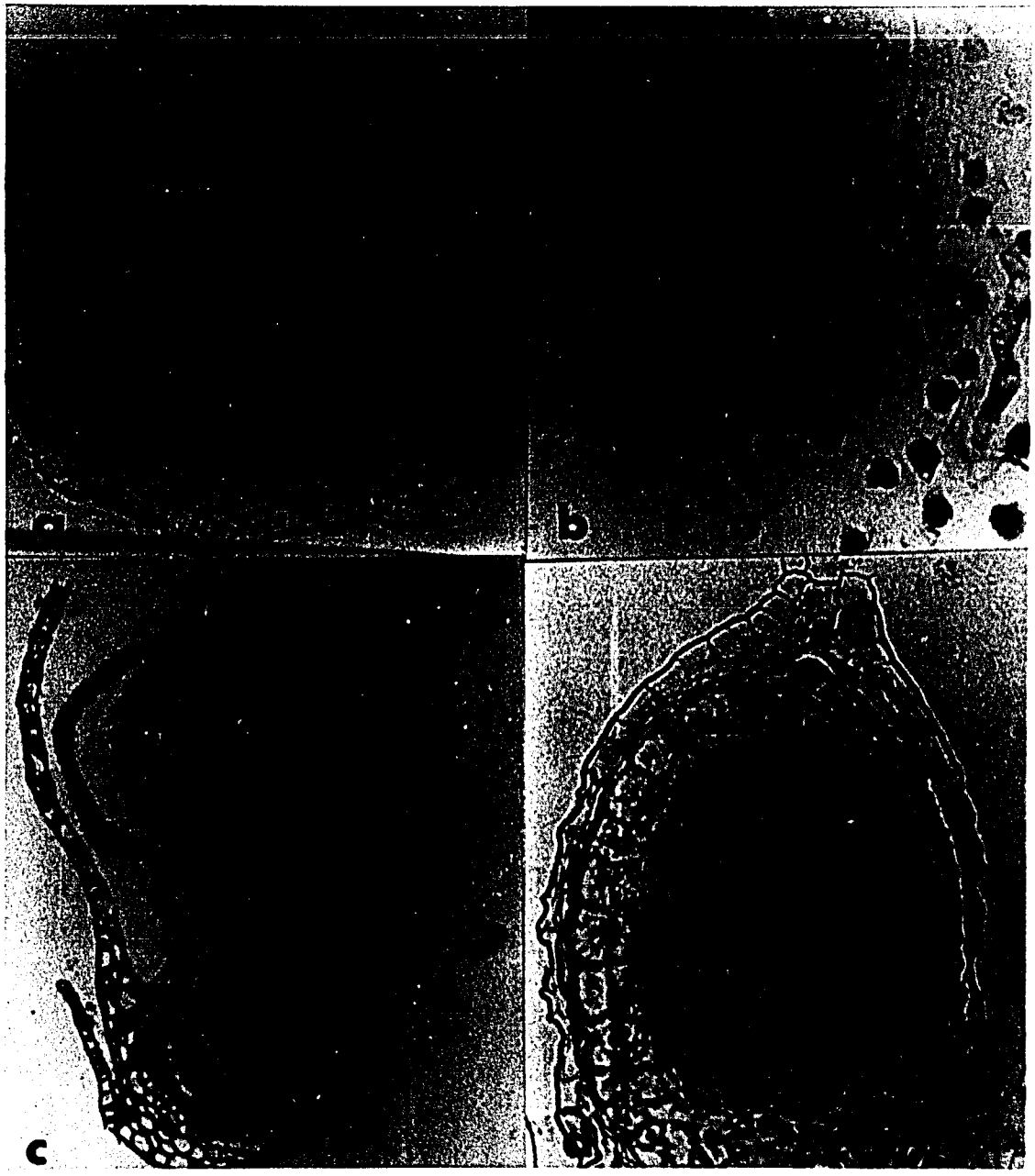

a
b
c

Figure 14. *Conocephalum conicum* elaters. The elaters of most liverwort taxa show a consistent form and pattern of thickenings. *Conocephalum* shows a wide range of morphology. These elaters were all obtained from one plant.

Figure 14a. This cell posses a unispiral helix at one end of the cell, but no secondary thickening at the other end, where there still appears evidence of cell contents (C). This suggest that the differentiation of the thickenings of the elaters may be polar.
magnification: 230X.

Figure 14b. Elaters with two, three and four helices.
magnification: 230X.

Figure 14c. The elater in the center has annular thickenings (a) on one end of the cell, helical thickenings on the other.
magnification: 230X.

Figure 14d. A branched elater.
magnification: 460X.

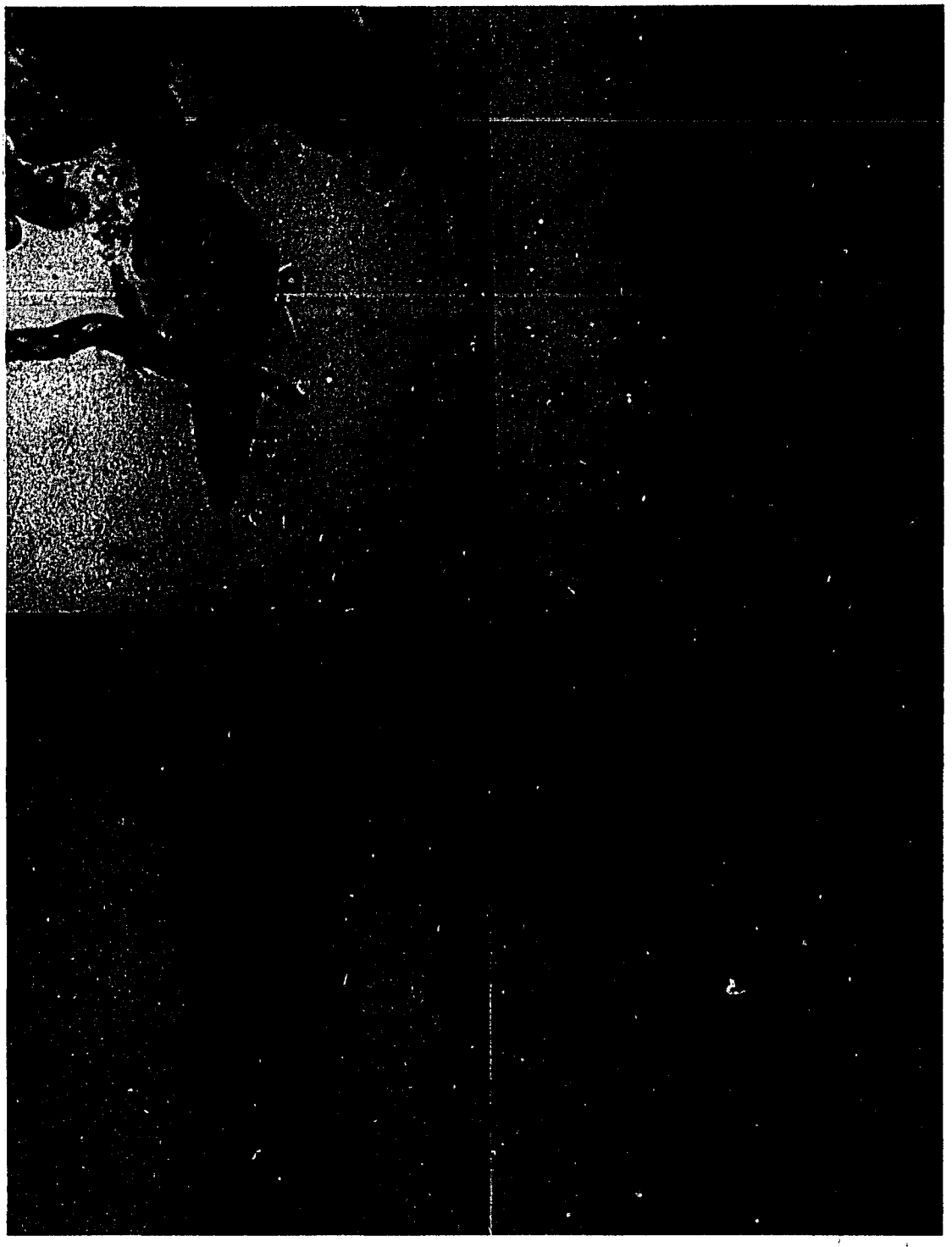


Figure 15. *L. heterophylla*, TEM young sporophyte

This sporophyte is at the stage where the capsule wall is 2-3 layers, and the sporogenous/elaterogenous mass is still undifferentiated. This figure is a section through part of the sprogenous / elaterogenous mass, the capsule wall, and part of the surrounding calyptra. The calyptra has thicker cell walls, is more vacuolate, with chloroplasts more differentiated than that of the sporophyte. The capsule wall cells appear somewhat collapsed, whereas that of the calyptra and sporogenous/elaterogenous mass are turgid. Chloroplasts of the sporogenous/elaterogenous mass (C) are rudimentary, with few thylakoids, whereas those of the capsule wall appear intermediate between that of the calyptra and the sporogenous/elaterogenous mass . The capsule wall contains numerous lipid bodies.

magnification: 960X.

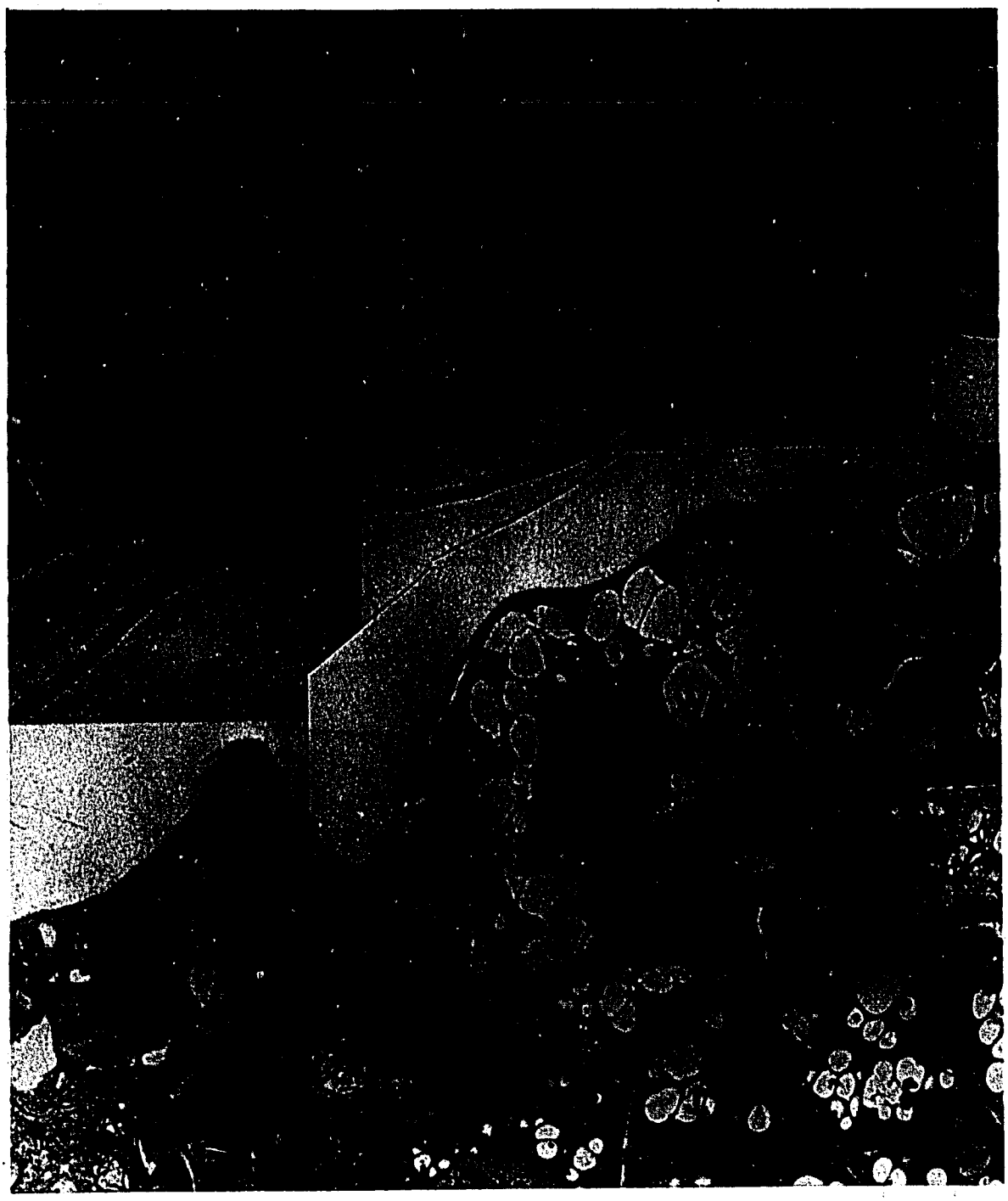


Figure 16. Sporogenous/elaterogenous mass, *L. heterophylla.*

A section through the sporogenous/elaterogenous mass of a sporophyte whose capsule wall was of 2-3 layers. These sporogenous/elaterogenous cells show deposition of vesicles (V) along some walls (arrows). The depositions accumulate along a line of cell walls, often on both sides of the wall simultaneously, whereas other walls of the same cells show no evidence of deposition. This appears to be the beginning of the rounding up process, where packets of cells separate away from each other. Plasmodesmata are obscured where this deposition occurs, whereas there are numerous plasmodesmata on the other walls of the same cell.

magnification: 2,250X.

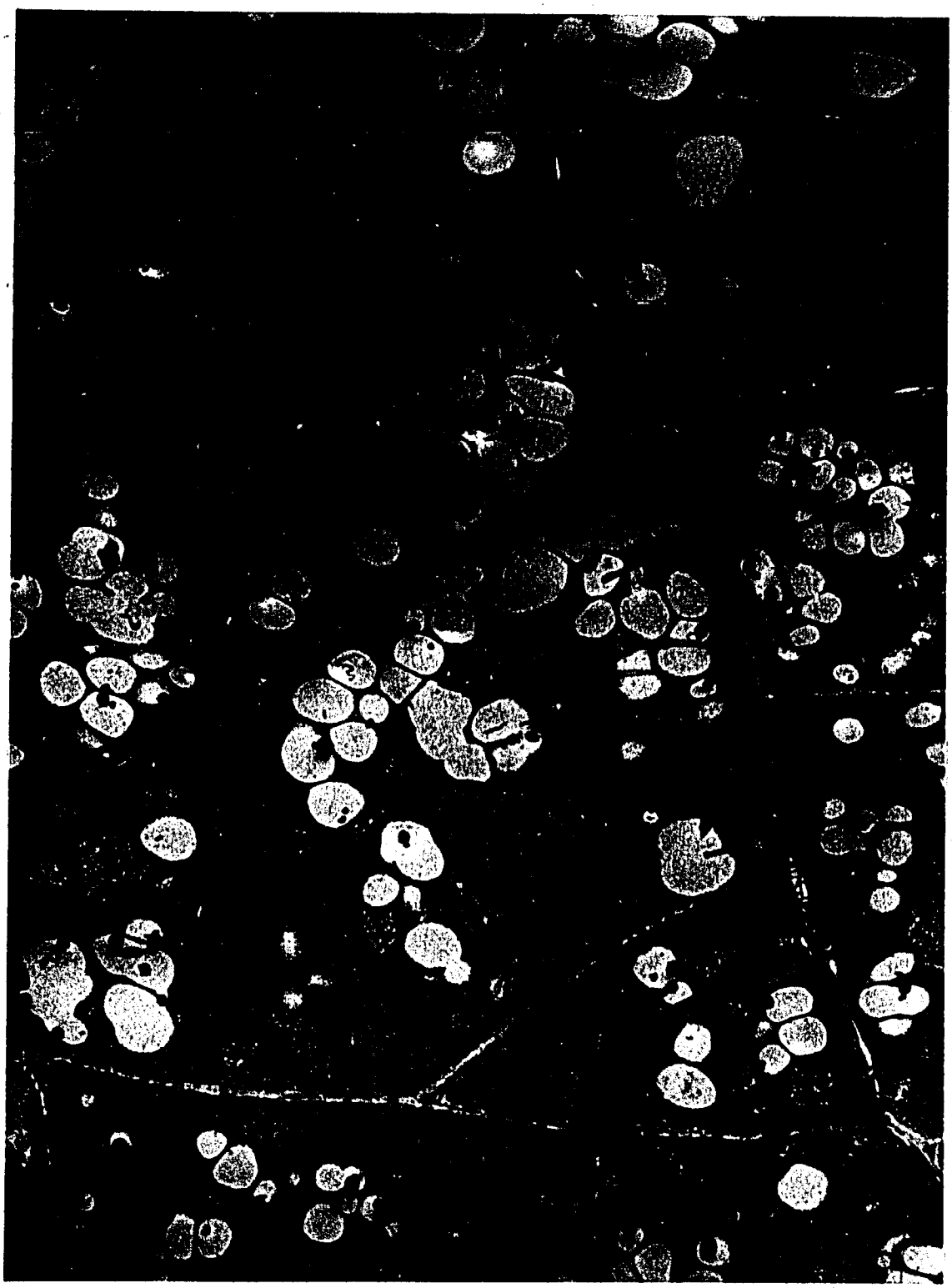


Figure 17. Rounding up of sporogenous/elaterogenous mass. *L. heterophylla.* A section through the same capsule shown in Figure10c. Each cell is clearly separated from the others by an intercellular space (IS). At this stage, the cells became very difficult to fix and embed. N=nucleus, v-vacuole, c=chloroplast. magnification: 8,000x

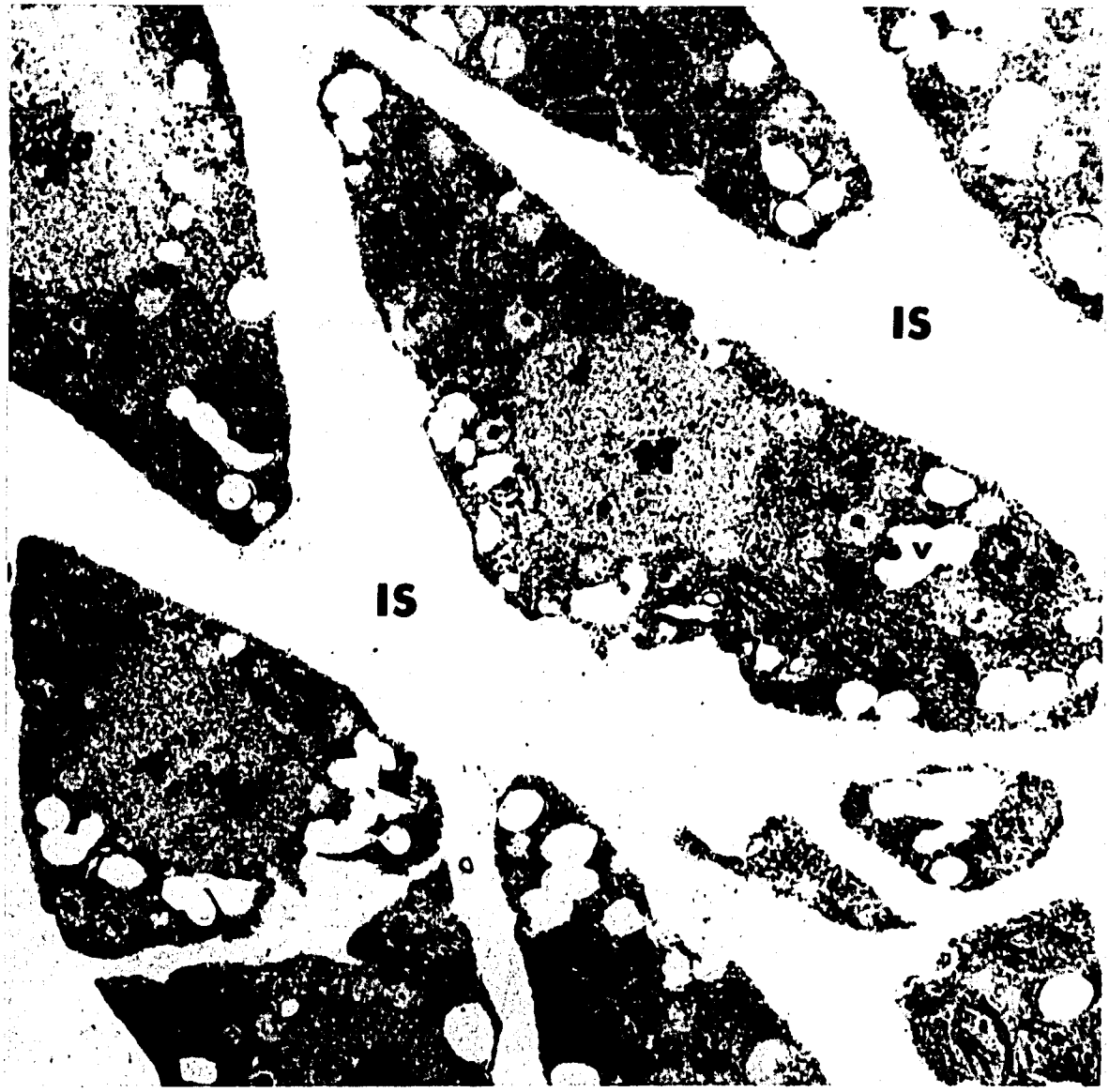

IS
IS
V

Figure 18. Early elater differentiation, *L. heterophylla.*

Figure 18a. Early differentiation (longitudinal section). Lipid bodies (L) are prevalent, chloroplasts (c) contain some thylakoids, there is little vacuolation, and no apparent secondary thickenings.
magnification: 6,000x

Figure 18b. A later stage of elater elongation and differentiation (longitudinal section). Chloroplasts have developed large starch inclusions (S), with fewer thylakoids present. Large sections of the plasma membrane have an extracellular matrix outside the plasma membrane that may be the beginnings of secondary cell wall thickenings (bars).
magnification: 9,000x

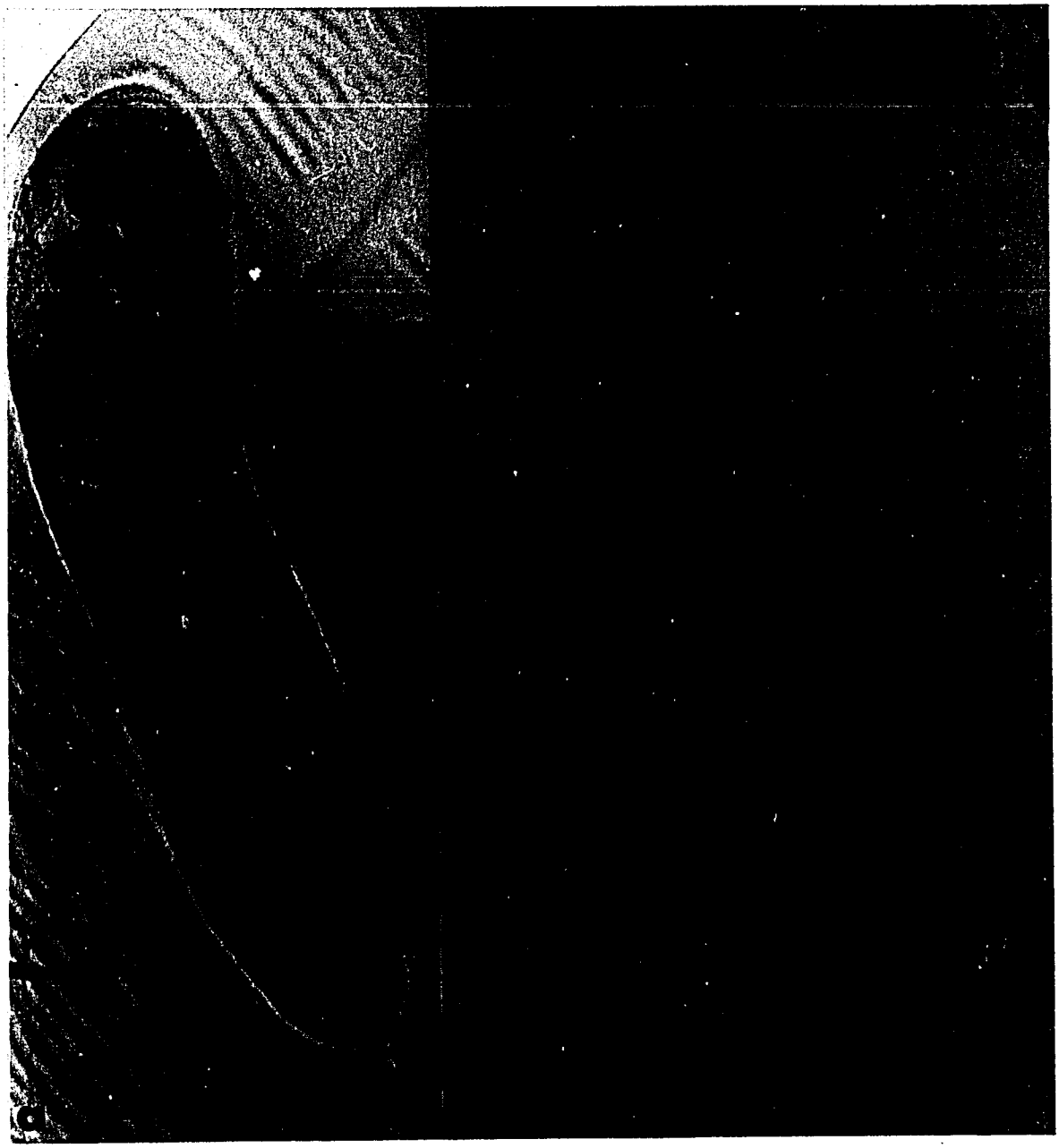


Figure 19. Cross section of an elongating elater. *L. heterophylla.*
This cell shows many signs of active wall production, including vesicle deposition into the wall (V), numerous microtubules along the cell edge (arrows), spans of endoplasmic reticula in concentric layers around the periphery of the cell (er), and golgi bodies (g) budding off vesicles. As in Figure 18a, chloroplasts (C) lack starch, and have short stacks of thylakoids. magnification: 32,000x

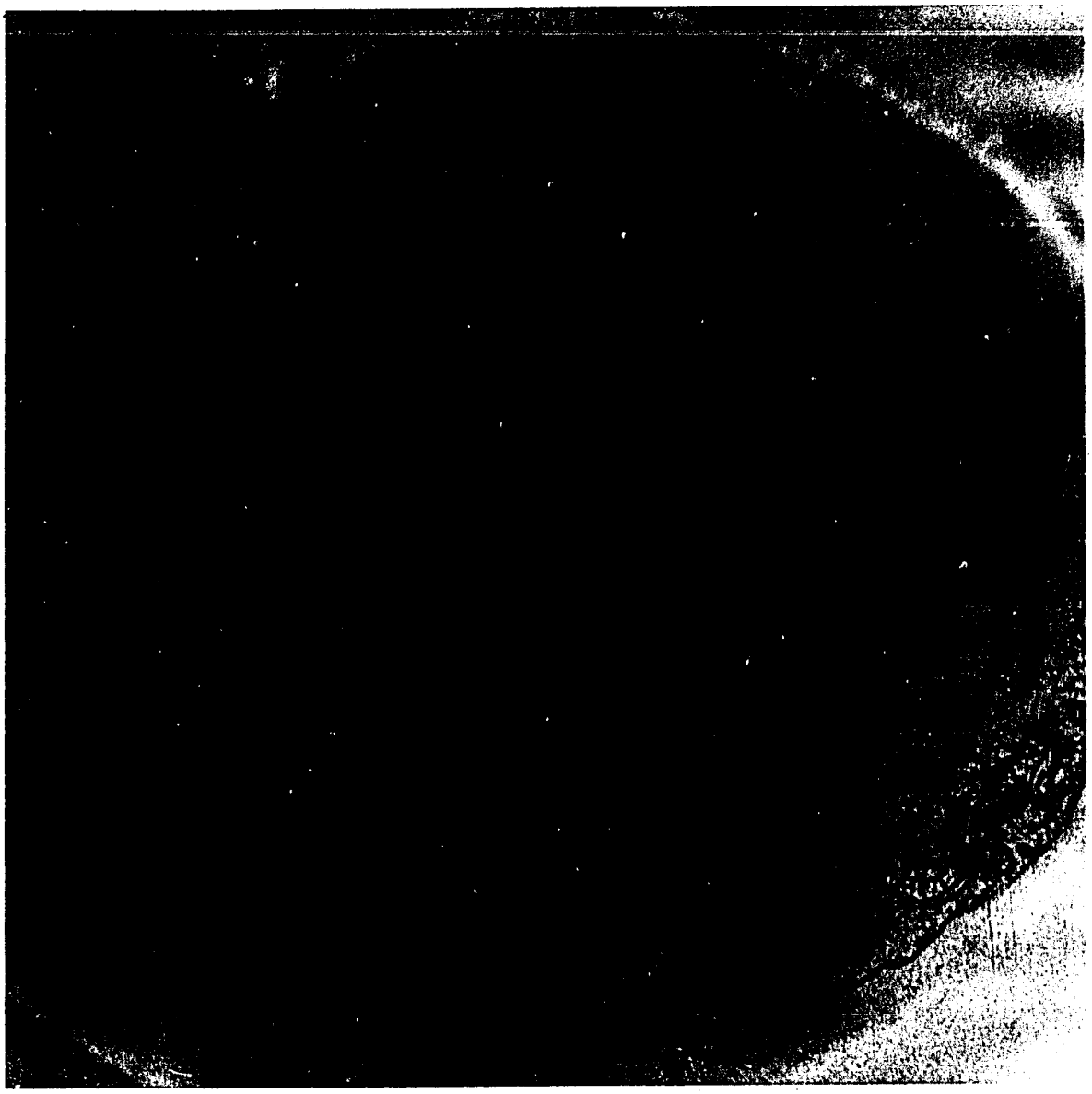


Figure 20. Elater microtubules and vesicle deposition. *L. heterophylla.*
Numerous microtubules (small arrows) extend from the edge of the cell inward, and are aligned in the same orientation as the microfibrils (mf) in the adjacent cell wall. A golgi body (g) is visible budding off a vesicle, other vesicles (v) are being deposited into the cell wall. While most microtubules are aligned in the same orientation as the cell wall microfibrils, a few (large arrows) can be seen at an angle to the rest, presenting an aspect similar to that shown in *Phleum* root cortex cells (Ledbetter and Porter 1963). magnification: 49,000X.

Figure 21. Elongating elater, microtubules in cross section. *L. heterophylla.*

Figure 21a. Microtubules in cross-section (arrow heads) adjacent to the plasma membrane, with endoplasmic reticula (er) immediately below, C=chloroplast. magnification: 12,000X.

Figure 21b. Microtubules in cross-section (arrow heads) adjacent to the plasma membrane, with vesicle deposition (v) to the cell wall interspersed among the groups of microtubules. Endoplasmic reticula (er) occur in layers immediately below, predominantly spanning across the areas of vesicle deposition, and leaving openings where microtubules occur (bar). L=lipid body.
magnification: 48,000X.

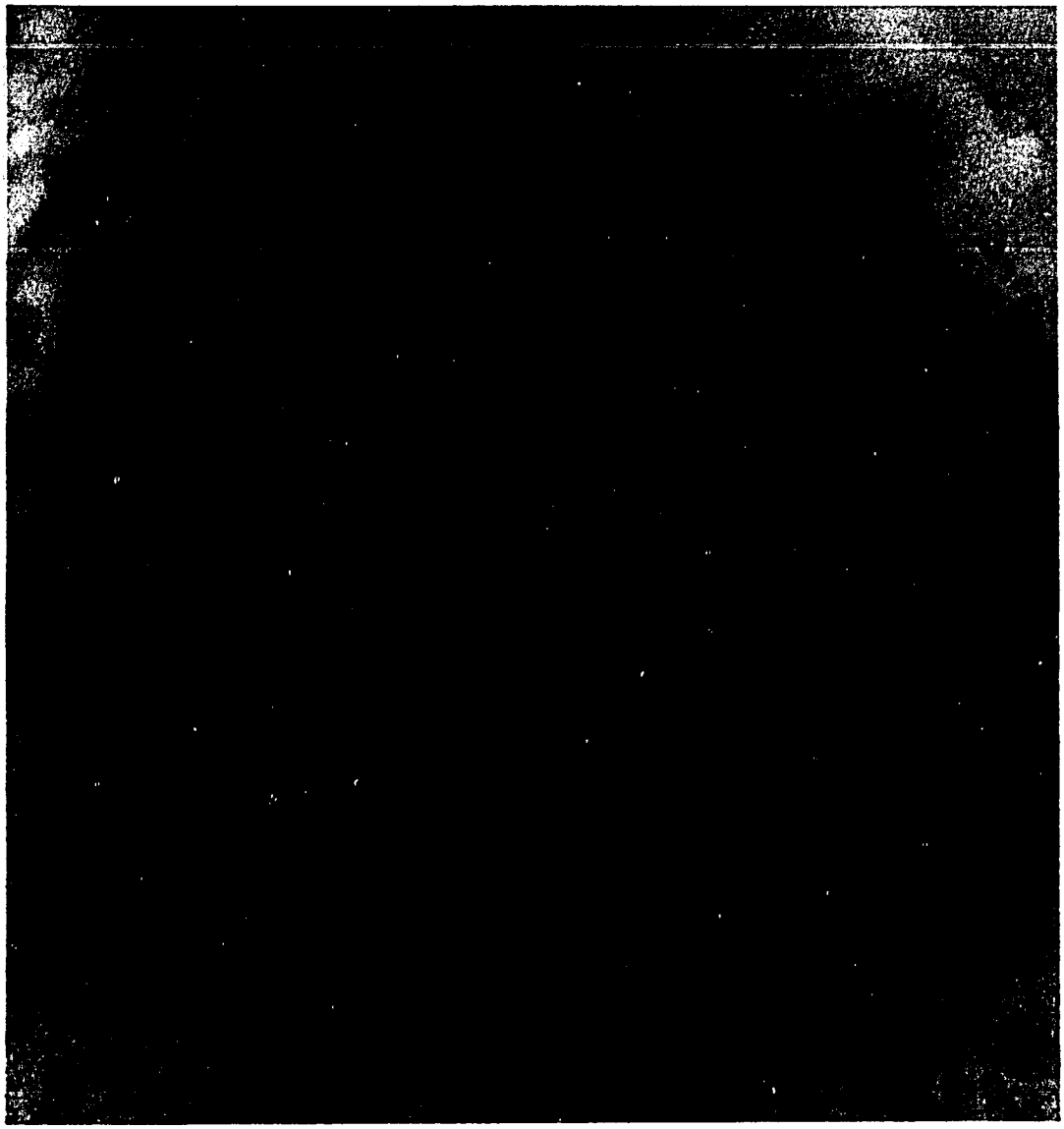


Figure 22. Microtubules and secondary thickenings. Comparison of *L. heterophylla* elater and xylogenesis.

Figure 22a. *L. heterophylla* elater. Microtubules (arrowheads) in cross section aligned adjacent to the developing secondary cell wall thickening (ST). g=golgi body. magnification: 83,000X.

Figure 22b. A similar micrograph from higher plant xylogenesis, showing microtubules (arrow) aligned along the edge of a secondary cell wall thickening. From Robards & Kidwai (1972). magnification: 99,000X.

ST
38
500 nm

Figure 23. Secondary cell wall deposition, microtubules in longitudinal section. Comparison with xylogenesis.

Figure 23a. *L. heterophylla* elater. Between two sections of the same helical secondary cell wall thickening (ST), microtubules (arrowheads) can be seen extending between the two. These microtubules are in reality directly above another part of this same thickening, which is outside of the plane of this section. These microtubules present the same orientation as the microfibrils (mf) within the secondary cell wall thickenings. magnification: 51,000X.

Figure 23b. Tobacco pith cells undergoing xylogenesis. Microtubules (Mi) extend between two secondary wall sections (SW), in the same orientation as the microfibrils (unlabelled) within the secondary cell wall thickenings. From Cronshaw (1967). magnification:

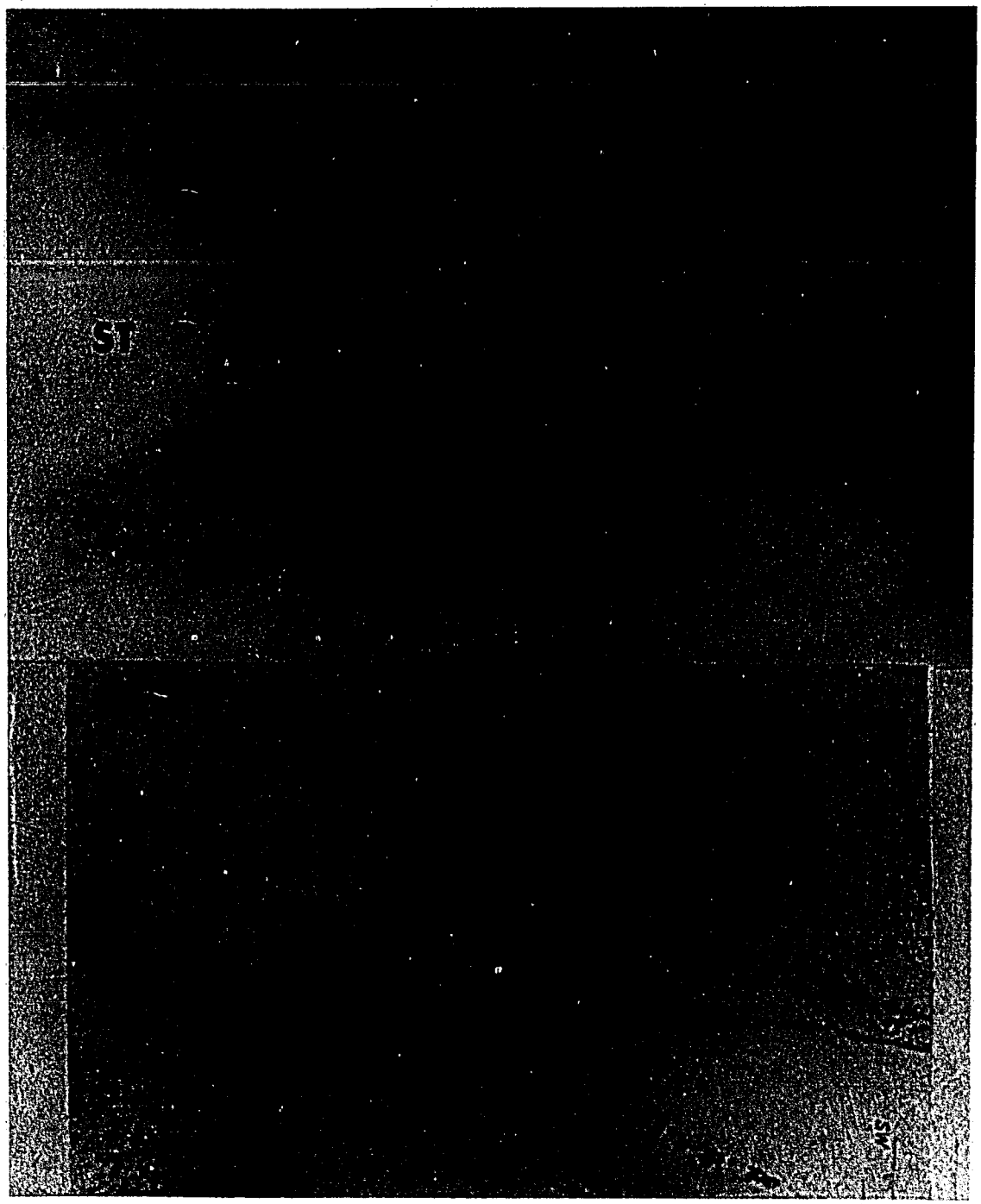

ST

Figure 24. Vesicle deposition into secondary thickenings.

Figure 24a. *L. heterophylla* elater. A vesicle (V) opens out into the base of a secondary cell wall thickening (ST). Microtubules seen in longitudinal section (arrowheads). magnification: 6,000X

Figure 24b. Vesicle deposition (arrow) into a higher plant secondary cell wall (W2). From Esau et al. 1966.

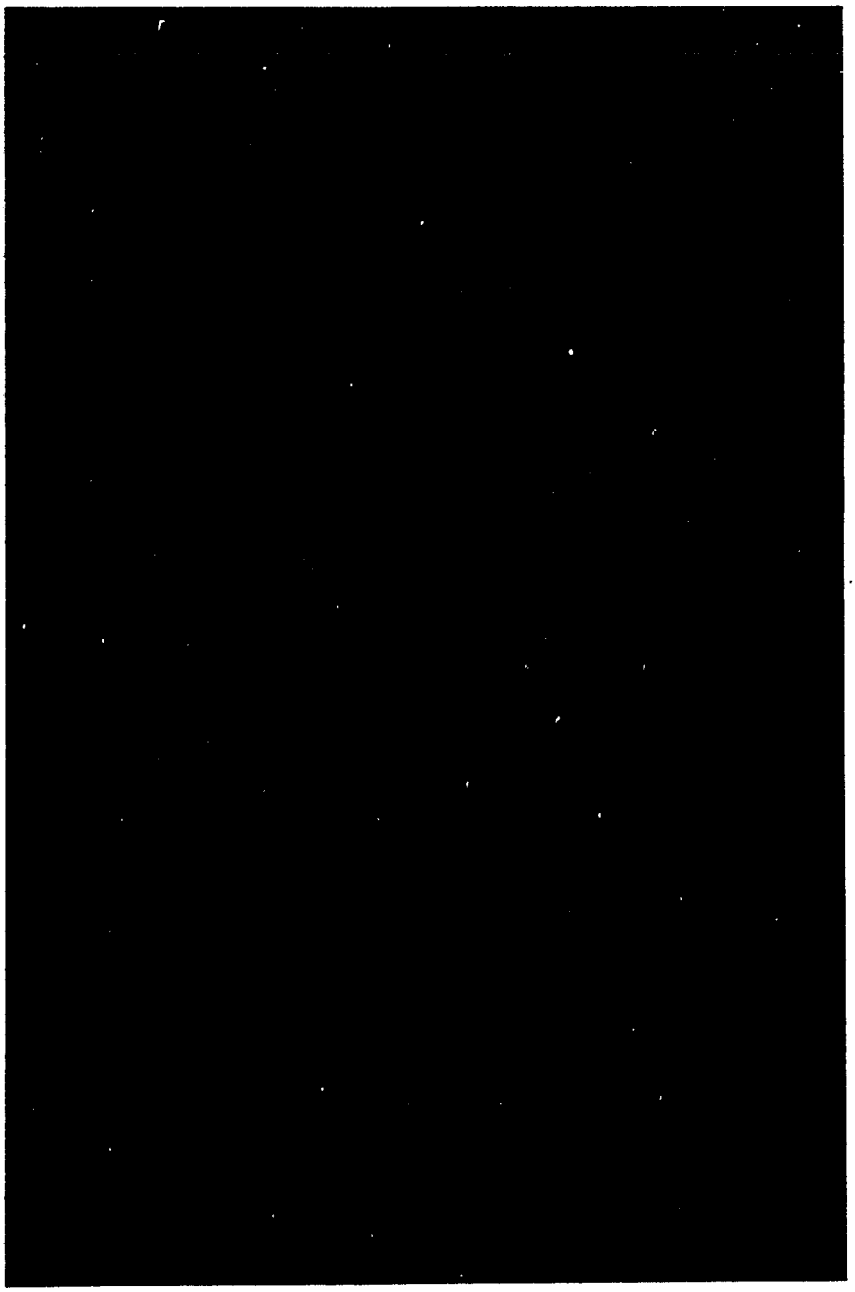


Figure 25. Early maturation of *L. heterophylla* elater.

Secondary thickenings (st) are fibrillar in nature, darker than the primary wall, and appearing to darken from the outer side in. Starch bodies in the chloroplasts (c) are fewer and smaller, vacuoles (vac) larger and coalescing, especially in between the secondary thickenings.

magnification: 13,000X

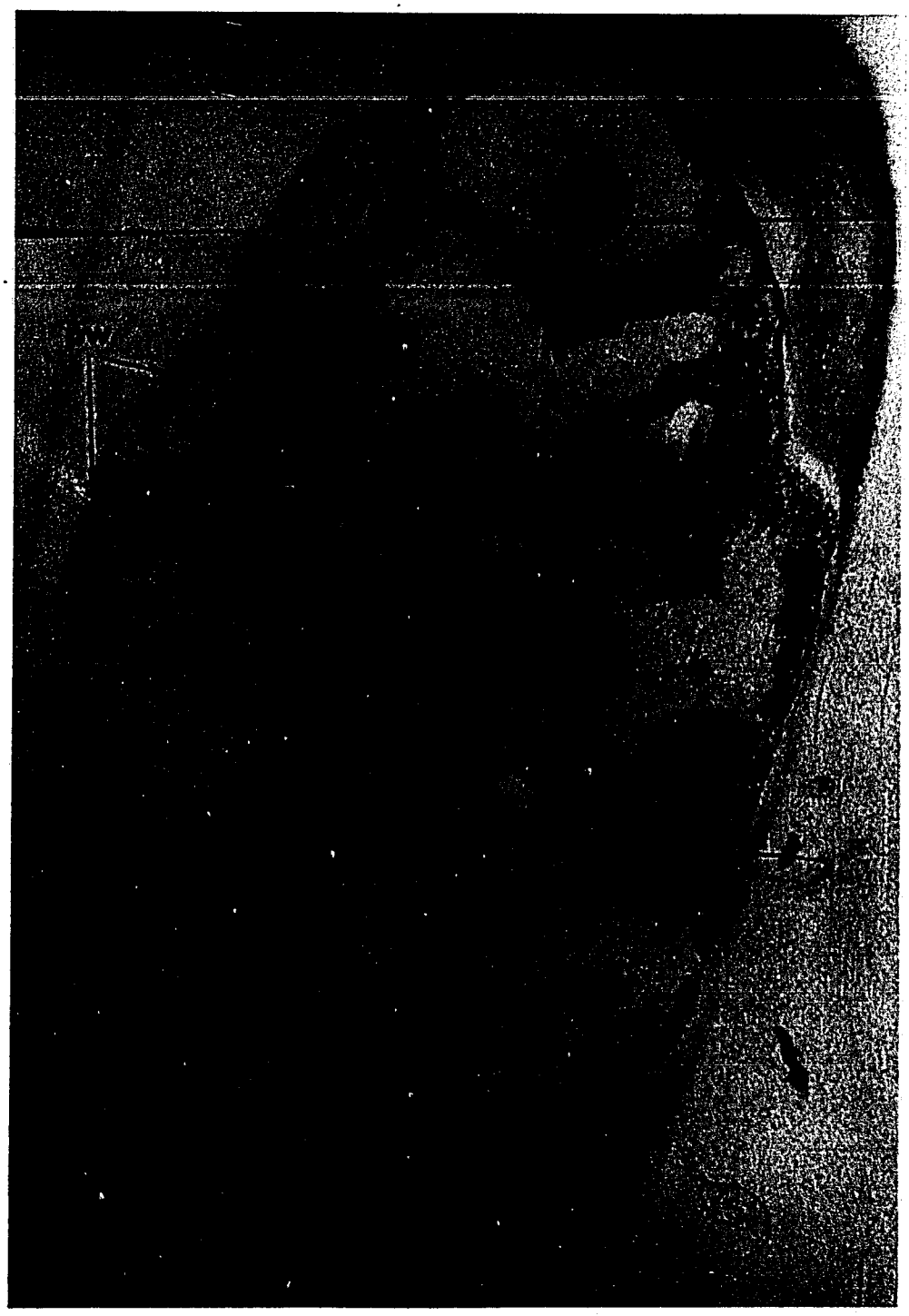


Figure 26. Later maturation of *L. heterophylla* elater.

The secondary thickenings (T) are uniformly dark, their fibrillar nature nearly obscured.The primary wall (pw) is less darkly stained. Outside of the primary wall under the secondary thickenings are irregular clusters of dark granules (g), which do not apear on the primary walls between the secondary thickenings. Lipid and starch bodies are absent to infrequent, chlorplasts (C) degenerating. Vacuoles appear to be coalescing, and the cytoplasm autolysing. m=mitochondria, Sp=spore. magnification: 21,000.

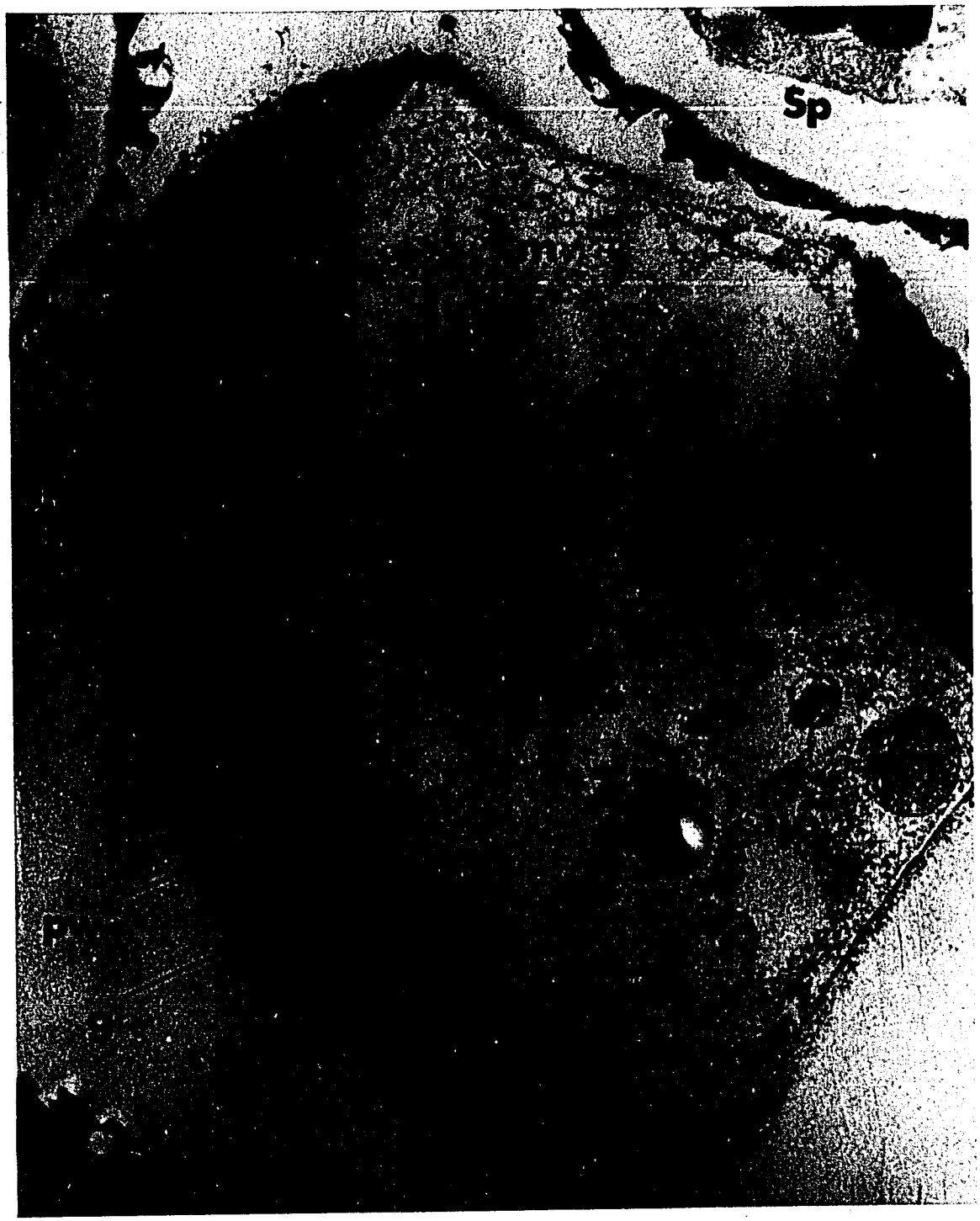

Sp
F

Figure 27. Mature elater.

The cell is dead and nearly empty, with some residual cytoplasm (r). Secondary thickenings (ST) are darkly staining, the primary wall (pw) lighter. Darkly staining granules (g) occur on the outside of the walls with secondary thickenings, but not in between.

magnification: 7,000X.

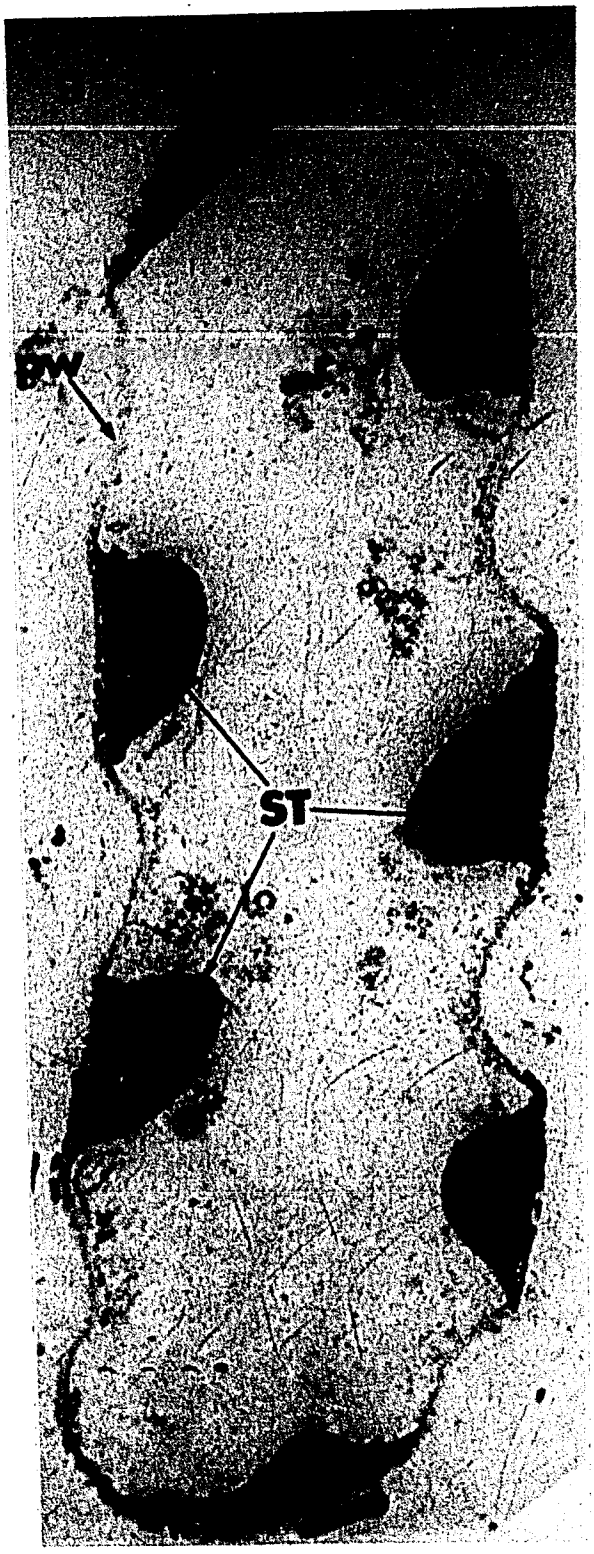

BW
ST

Figure 28. Spore mother cell undergoing meiosis.

The cell is forming a tetrad, with two furrows visible in this micrograph (arrows) and a central nucleus (N). Three different kinds of inclusions are present: a smooth lipid body (L), irregular shaped protein bodies (p), and what appears to be a smooth lipid body with a light colored inclusion (arrowhead). The latter two inclusions have not been found in the developing elaters. Numerous chloroplasts are present, with well-developed thylakoids and very few and small starch bodies.
magnification: 12,000X.

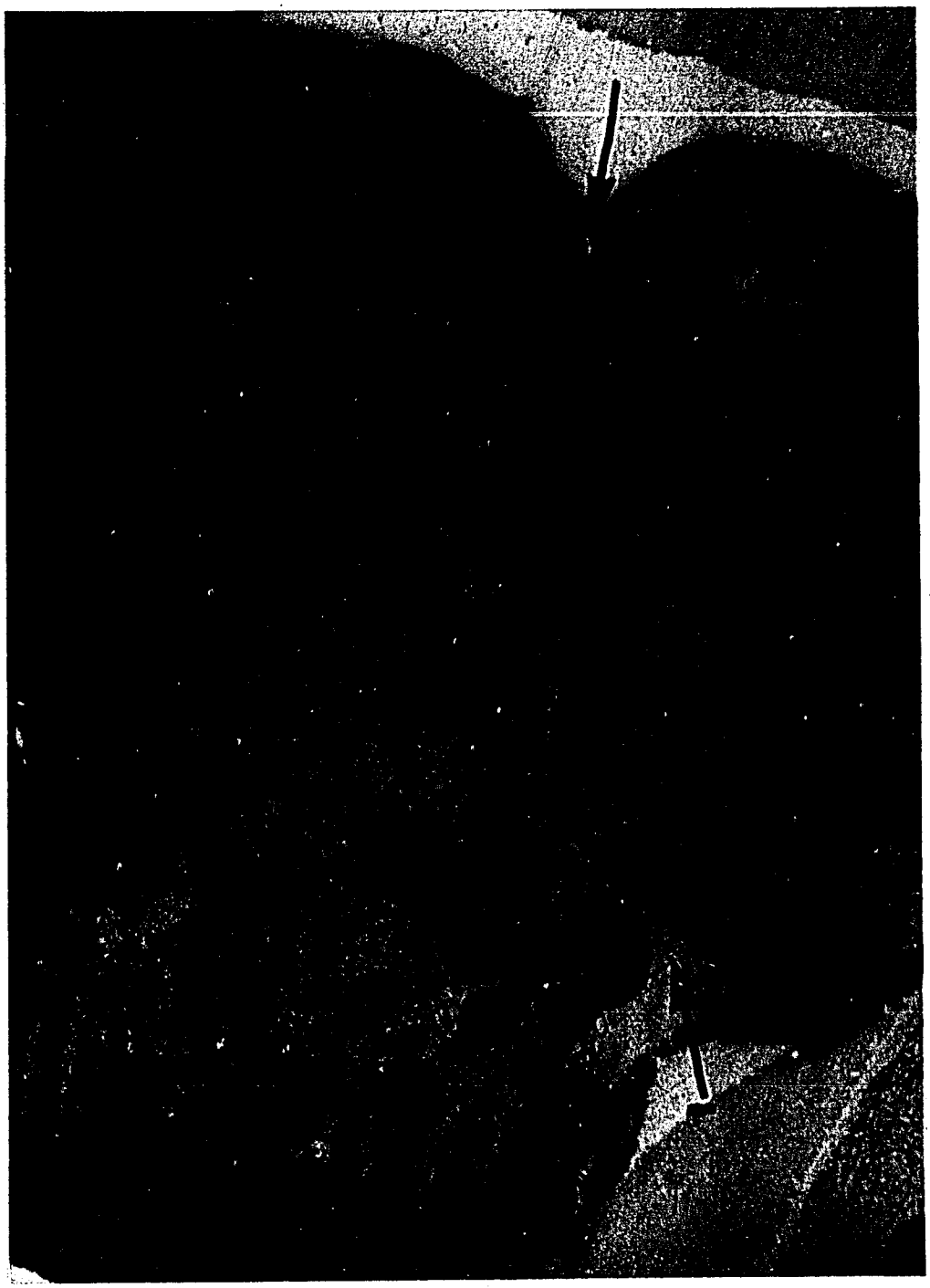


Figure 29. Mature spore.

Note the central nucleus (N) with chloroplasts (C) surrounding, lipid bodies (L), golgi (G) and mitochondria (M) outside, and mature, lamellate spore wall (W). This is in contrast to the mature elaters, which are dead and empty at maturity.

magnification: 17,500X.

Figure 30. Mature spore and elater walls.

Figure 30a. Mature elater wall. The interior of the elater (EL) is empty, the secondary thickenings (st) dark and homogenous. The primary wall (p) is unstained and fibrillar in nature. Granules (g) occur atop the primary walls adjacent to secondary thickenings only.
magnification: 25,500X.

Figure 30b. Mature spore wall. The interior of the spore (SP) is full of cytoplasmic contents, with chloroplasts (chl), mitochondria (m) and golgi bodies (g) in evidence. The wall contains two layers, a darkly staining lamellate outer layer which is usually termed an exine (e), and a lightly staining inner wall, the intine (i).
magnification: 23,000X.

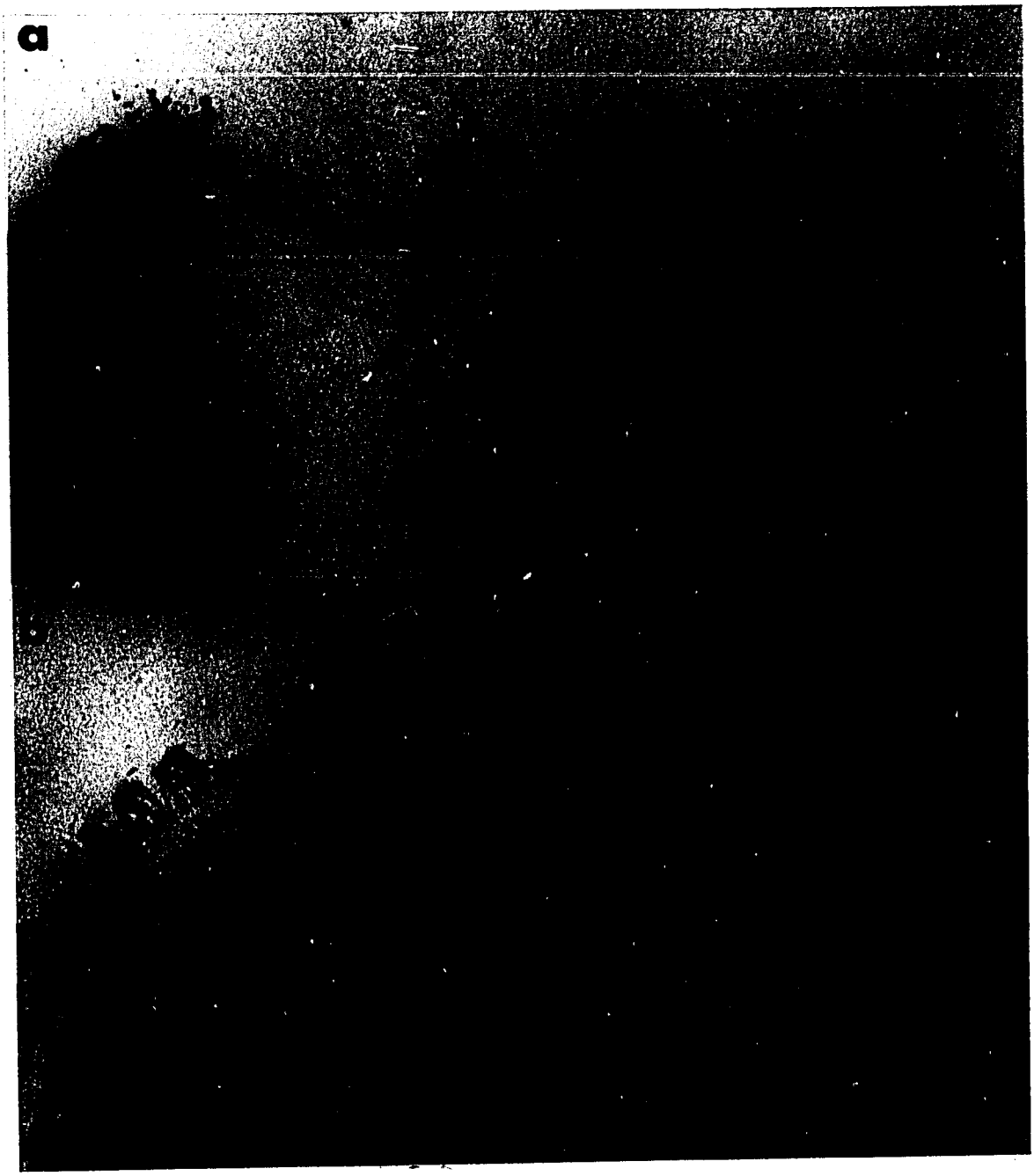

a

Figure 31. DAB staining, Ishida method. *L. heterophylla* elater.

Figure 31a. Cells incubated in DAB. Note the very dark cytoplasm of the elater (center of micrograph) as compared to that of the spore (S). The secondary cell walls are darkly stained at the interior, more lightly stained at the outside (arrowhead). v=vesicle. magnification: 11,000X.

Figure 31b. Control tissue incubated in buffer only. The cytoplasm is unstained, but note that the secondary thickenings (arrowhead) show the same staining pattern, demonstrating that the staining is not due to peroxidases in the cell wall. magnification: 11,000X.

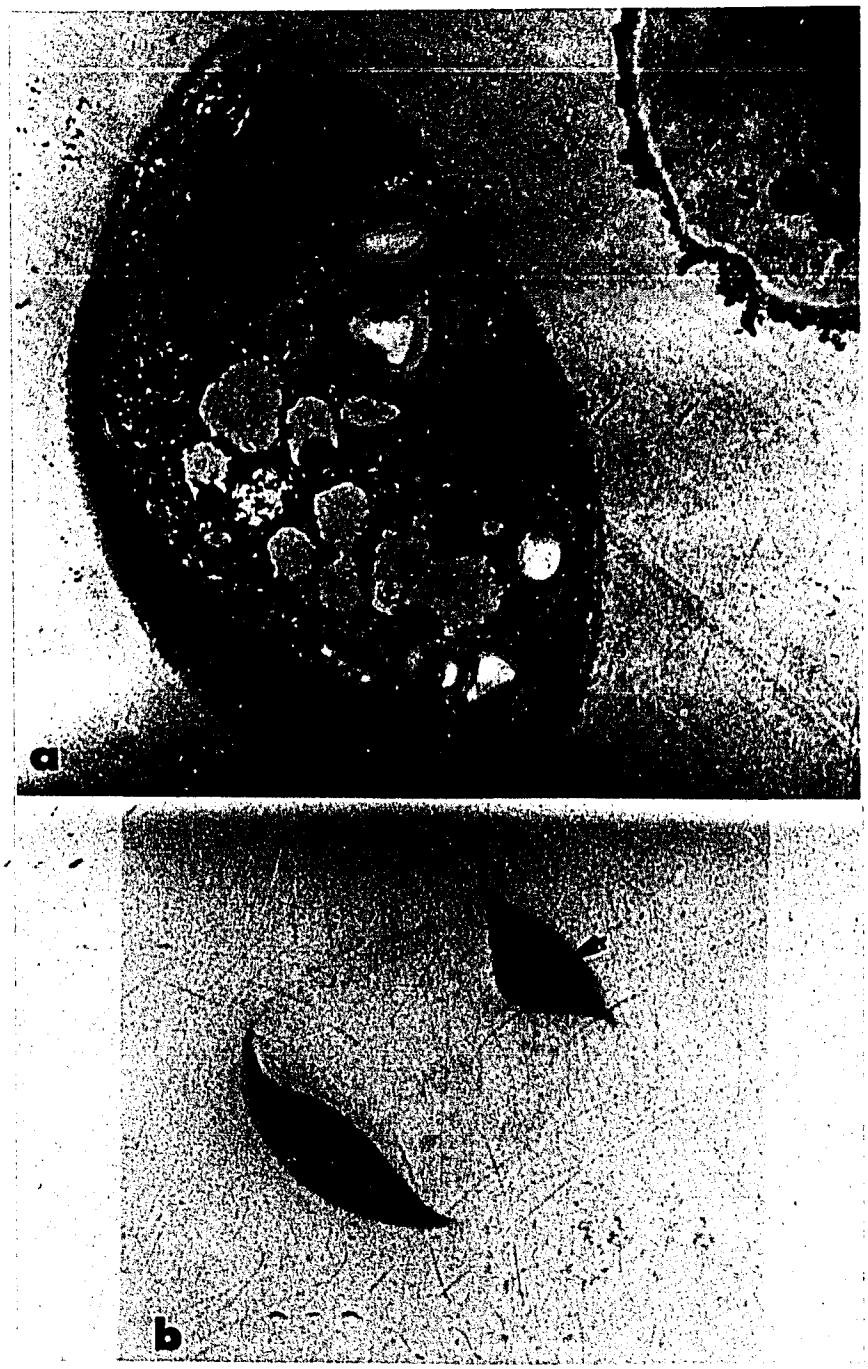

a
b

Figure 32. DAB staining, Hepler method. *L. heterophylla* elater.

Figure 32a. Cells incubated in DAB. The cytoplasm is less darkly stained than that of Figure 31a, allowing one to notice the darkly stained membranes (arrows) of some of the cell inclusions (arrows). Secondary thickenings (st) show a similar staining pattern to that of Figure 31, more darkly stained on the inner side than the outer. magnification: 6,500X.

Figure 31b. Control tissue incubated in buffer only. No cytoplasmic membranes are preferentially stained. The secondary thickenings (st) are stained here as well. magnification: 9,000X.

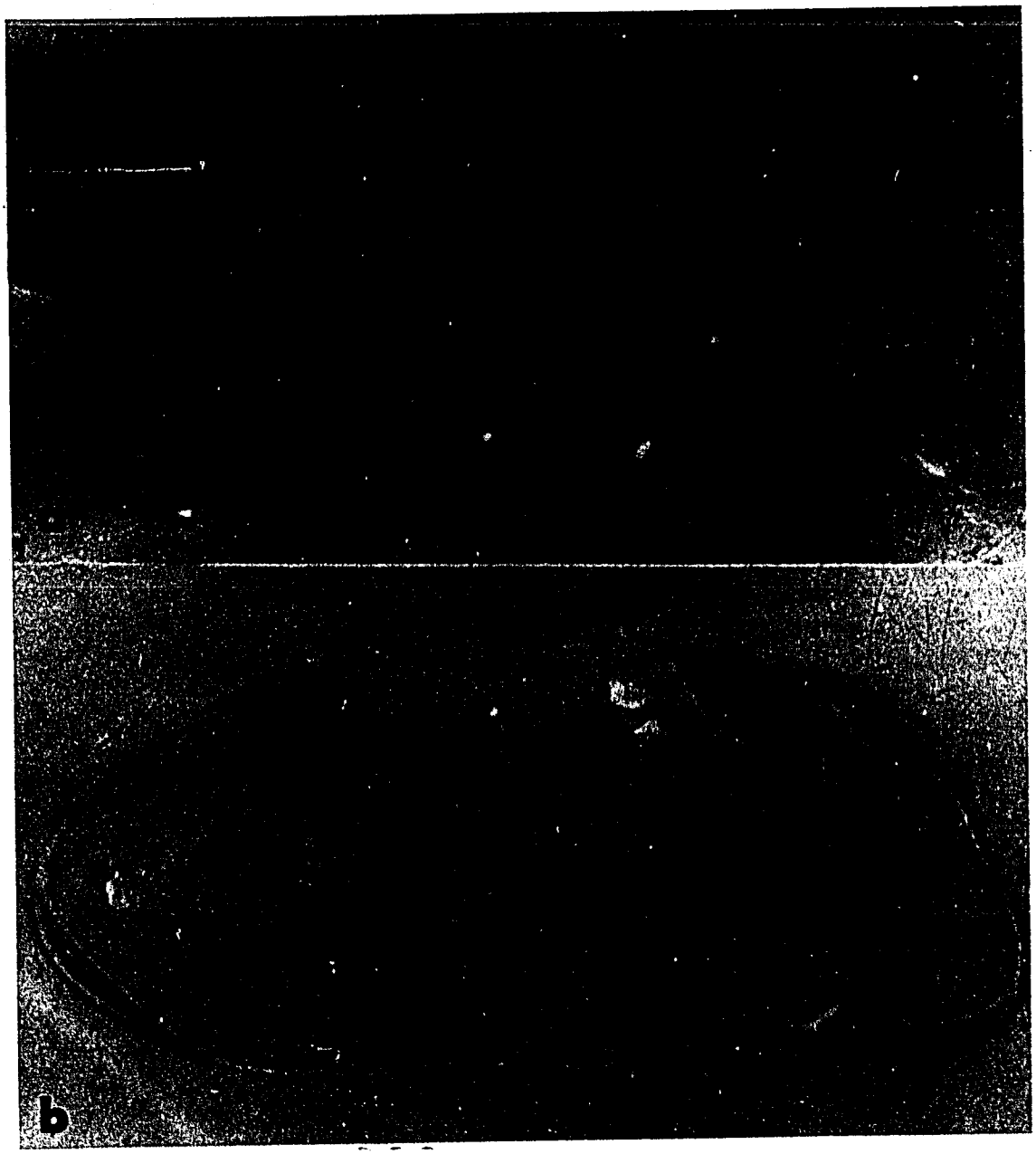

b

Figure 33. DAB staining, Ishida method. *Pellia epiphylla* spore and elater.

Figure 33a. Cells incubated in DAB. Note the very dark cytoplasm of the elater (right) as compared to that of the spore (S). Staining is less heavy in the secondary thickenings of the elater (st).
magnification: 7,000X.

Figure 33b. Control tissue incubated in buffer only. The cytoplasm of the elater is of the same staining intensity as that of the spore, indicating that the dark staining in 31a is due to peroxidase activity found in the elater cytoplasm but not the spore. The secondary thickening of the elater (st) is of approximately the same intensity as that of Figure 33a, indicating that, unlike in the cytoplasm, the staining pattern of the secondary thickenings is not due to peroxidase activity.
magnification: 7,000X.


a
b

Figure 34. DAB staining, Hepler method. *Pellia epiphylla* elater and spore.

Cells incubated in DAB. There is no discernable difference in staining intensity between the elaters (E) and that of the spore (S). There is also no discernable staining of peroxisomal membranes similar to that seen in *L. heterophylla* (Figure 32a). magnification: 6,000X.

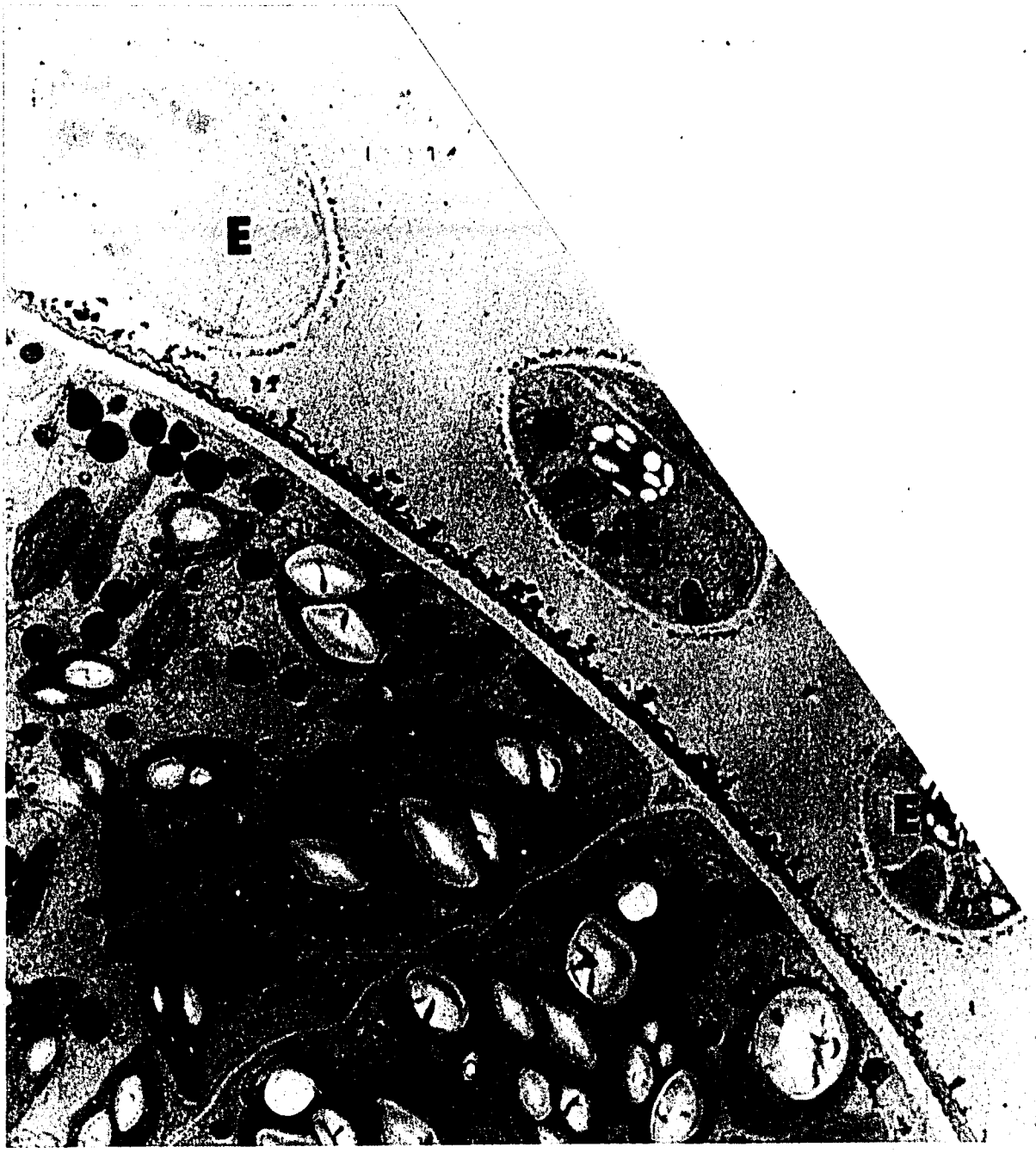

E
E

Figure 35. Spores and elaters, unstained. *L. heterophylla.*

These cells were fixed in glutaraldehyde and osmium tetroxide only, and examined unstained. The electron density of the secondary cell walls (st) of the elater (center of micrograph), therefore occurs without uranyl acetate or lead citrate staining. This is not true of lignifying xylem cells, which are electron transparent with only glutaraldehyde and osmium fixation. The fibrillar nature of the secondary thickenings can be see at the arrow. The darkening is occurring from the outside in, indicating an early stage during the first wave of deposition. An early stage of deposition is also occurring in the exine of spore (S), as parts of the exine are as yet unstained (arrows).

magnification: 4,200X.

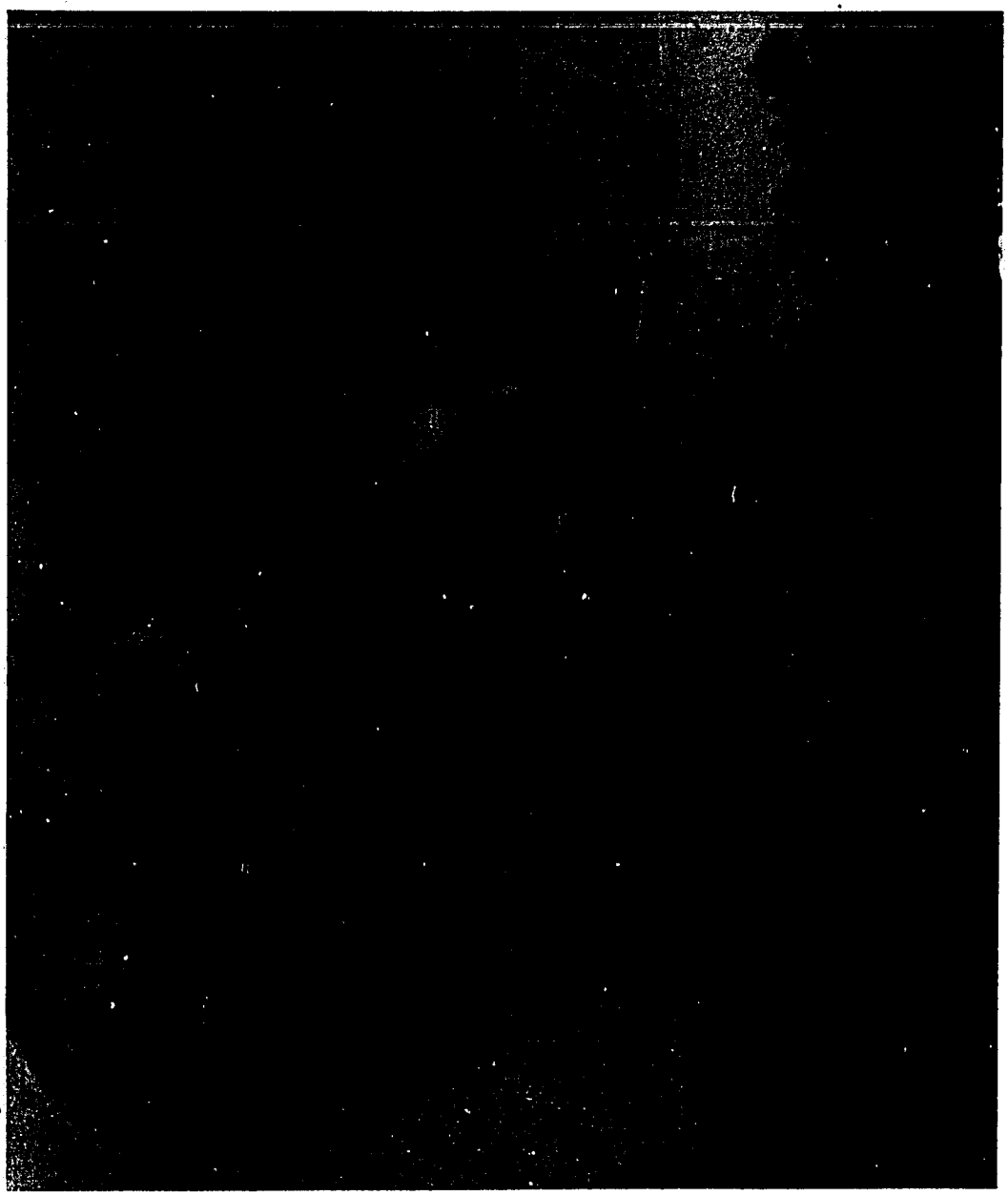


Figure 36. *L. heterophylla* elater, glutaraldehyde fixation alone.

The electron density of the secondary thickenings (st) of the elater (E) with glutaraldehyde fixation alone demonstrates that these walls are natively electron-dense. This is not true of lignin.

magnification: 7,000X.

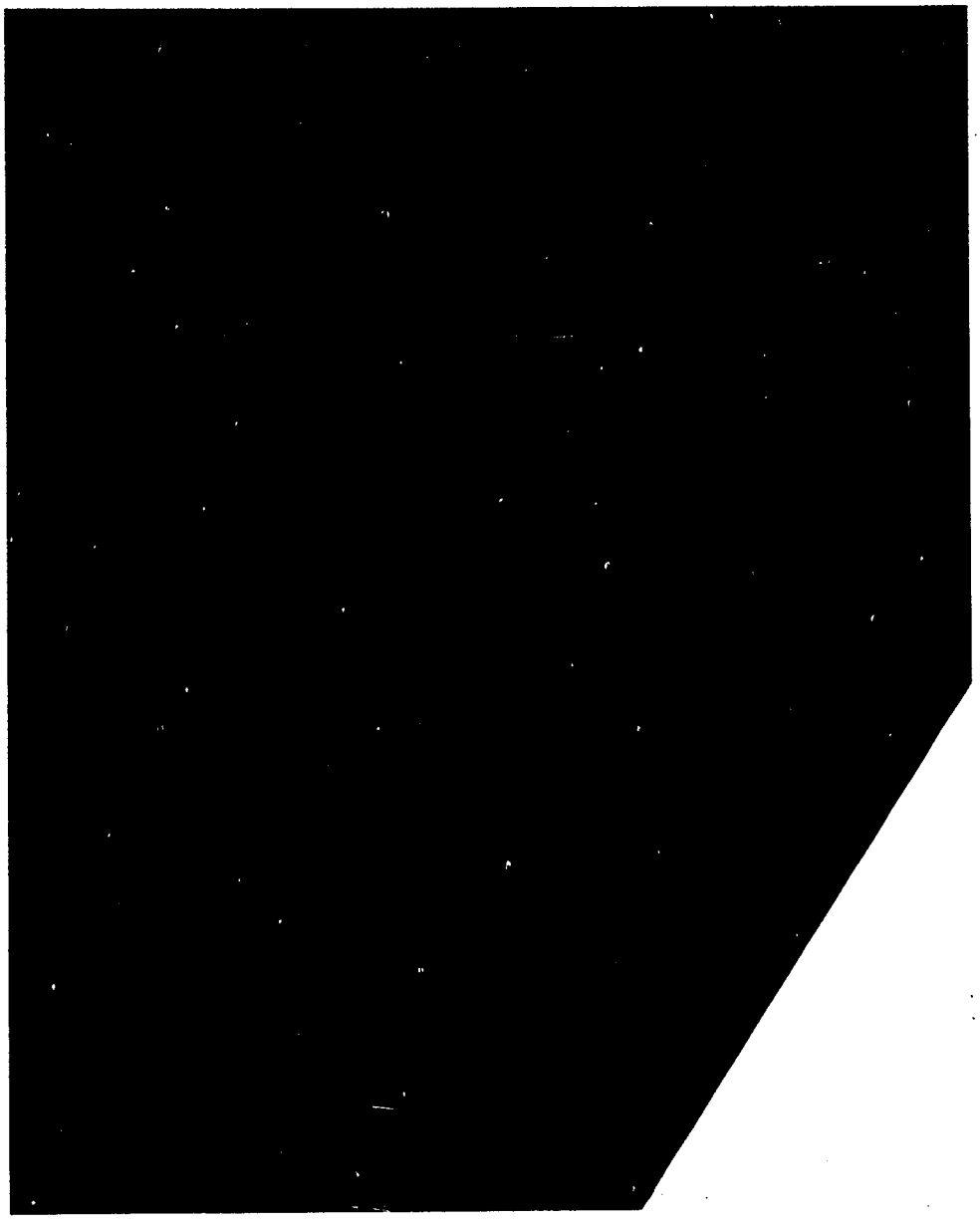
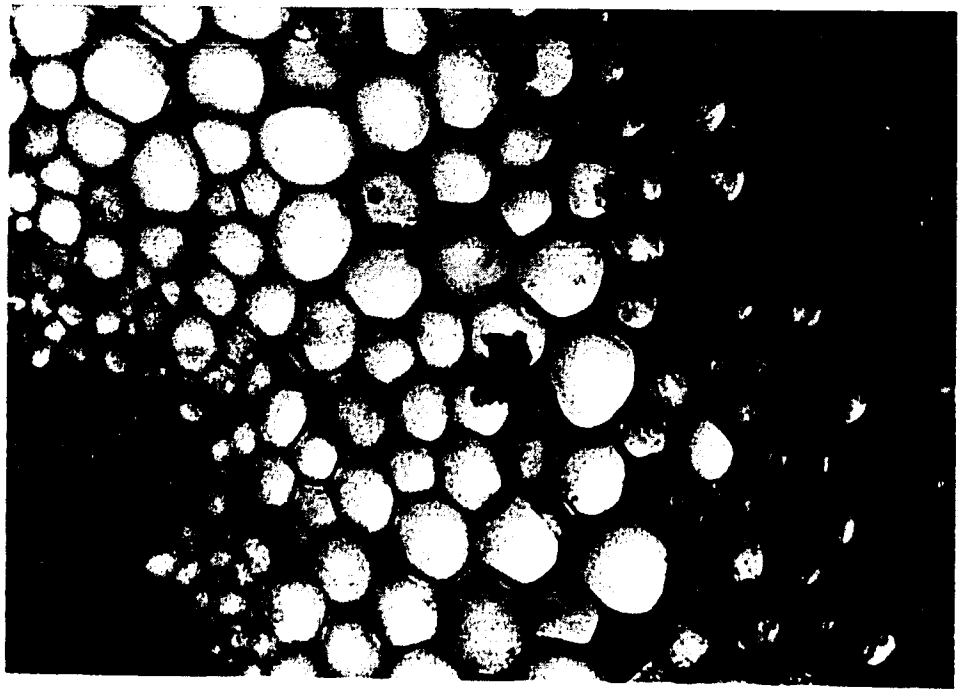


Figure 37. Toluidine blue staining

Figure 37a. *Psilotum nudum* stem cross-section. Xylem (X) light blue. Inner cortex (I) and thick outer cortex walls (O) stained purple, indicating cellulose. Middle cortex (C) stains dark blue, as opposed to the light blue of the xylem proper. magnification: 145X.

Figure 37b. Toluidine blue staining. Two *Pellia epiphylla* capsules with seta (squash mount). Seta (S) is to left, capsule wall to the right. The capsule wall is of two (to sometime three) layers, at far right the outer layer has been removed to show only the inner wall layer (IW), while a strip of the inner layer only (IW) is next to the seta (S). Seta and outer capsule wall layer stain primarily purple, indicating predominantly cellulose. The inner capsule wall layer stains a dark blue more similar to the middle cortex of *Psilotum nudum* than to *Psilotum's* xylem. The stain has not had sufficient time to diffuse into the center of the capsule (C); the light blue color seen there would in time become the same purple-blue color seen in the tissue surrounding it. magnification: 145X.



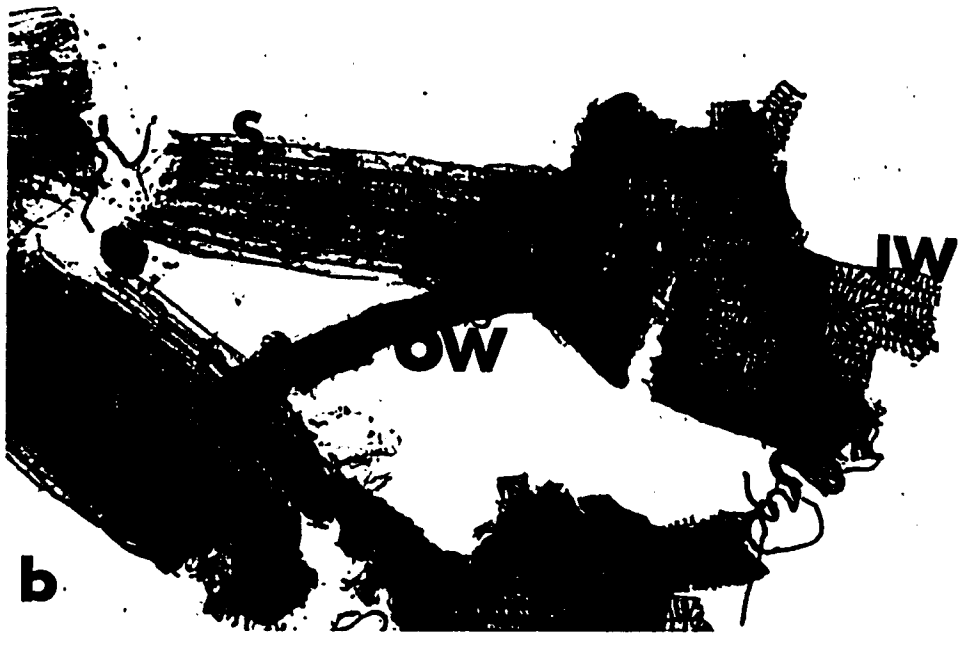

S
IW
OW
b

Figure 38. Diagram of toluidine blue staining of *P. epiphylla* capsule wall (hand cut cross-section).

The outer layer (O) of the capsule is above and the inner layer (I) is below. As evident in the surface view of Figure 37b, the outer layer of the capsule wall is a thick layer composed primarily of cellulose. It stains purple with toluidine blue and is here stippled. Thinner walls between the outer and inner layers also stain purple (stippled). Secondary thickenings on radial cell walls, as well as the innermost tangential wall stain blue (solid shading). The blue-staining secondary thickenings of the innermost layer (solid shading) appear as semi-annular bands running along the length of the cell. A middle layer is sometimes found (not shown here) which shows a pattern intermediate between the other two.
magnification: 600X.

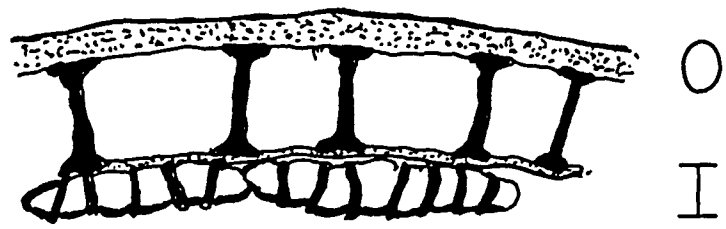

O
I

Figure 39 IKI-H_2SO_4 staining (*P. epiphylla* capsule walls and elaters).
After pretreating with IKI, upon application of 65% H_2SO_4, cellulosic walls immediately turn a bright blue, and then dissolve away. Walls impregnated with lignin turn a yellowish green and swell slightly . If tissue is treated first with commercial strength Clorox (which dissolves lignin), then treated with IKI-H_2SO_4, the cellulose within the lignified walls is exposed, turns blue and dissolves immediately.

Figure 39a. *P. epiphylla* capsule wall (unstained). Note the nodular secondary cell wall thickenings at corners of cells, and the inclusions within the cells.
magnification: 360X.

Figure 39b. *P. epiphylla* capsule wall, IKI pretreatment. Cell inclusions turn purple, indicating the presence of starch.
magnification: 700X.

Figure 39c. *P. epiphylla* capsule wall, IKI and H_2SO_4. Primary walls turning blue, secondary thickenings turning a slight greenish hue. This process takes place very quickly, the whole tissue darkening at completion such that it becomes difficult to photograph.
magnification: 700X.

Figure 39d. *P. epiphylla* capsule wall, Clorox pretreatment followed by IKI-H_2SO_4. Secondary cell wall nodules immediately turn blue, indicating cellulosic nature underneath, with resistent secondary compounds removed.
magnification: 550X.

Figure 39e. *P. epiphylla* elater from the elaterophore. IKI-H_2SO_4. The elaters (E) behave in the same way as the secondary thickenings of the capsule wall, turning greenish on application of sulfuric acid, and resisting dissolution. Note that the primary walls behave this way as well, while the primary walls of the capsule wall immediately turned blue and dissolved.
magnification: 1,450X.

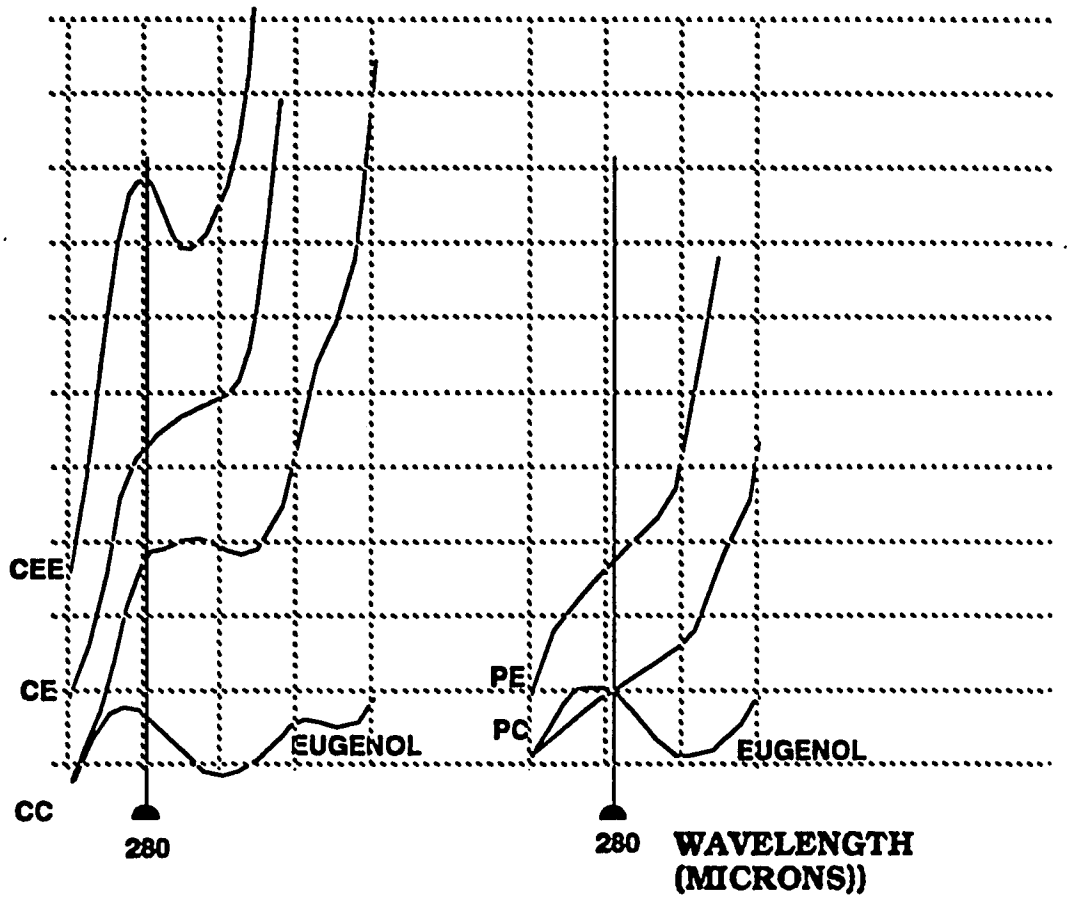

a
b


d
e

Figure 40. UV spectrum/ Eugenol incubation experiment
UV spectra (300-200 nm) of tissue incubated in eugenol and extracted in acid alcohol. 280 nm (where lignin characteristically gives a shoulder) is marked with an line. Eugenol itself gives a peak at about 285 nm.

Figure 40a. vascular strands of celery . Celery controls yield a UV spectrum with a slight peak at 280 after acid alcohol extraction due to lignins naturally present in the tissue. With incubation in increasing amounts of eugenol (CE, CEE) the peak at 280 nm is enhanced.
CC: celery control, CE: celery with eugenol added, CEE: double the amount of eugenol added.

Figure 40b. sporophytes of *Pellia epiphylla* (mature, freeze-dried). *Pellia* shows no shoulder at 280, indicating that the eugenol was not converted to coniferyl alcohol and polymerized in the cell walls. PC: control; PE: tissue with eugenol added.



a) celery

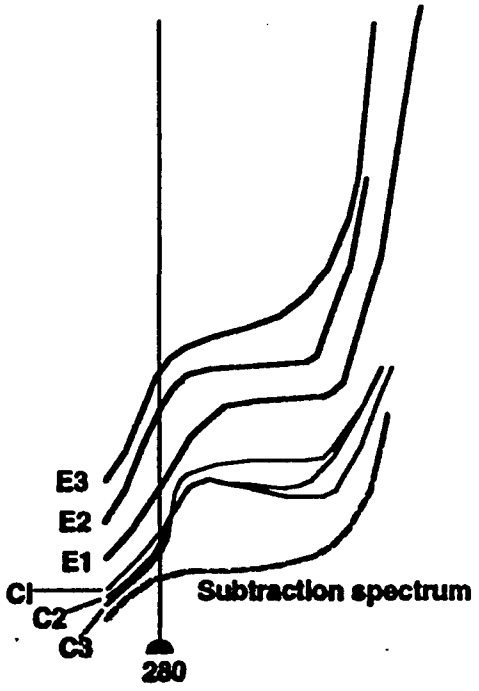
b) Pellia

Figure 41. UV spectra / Eugenol incubation experiment with young vs. mature *Pellia* capsules.

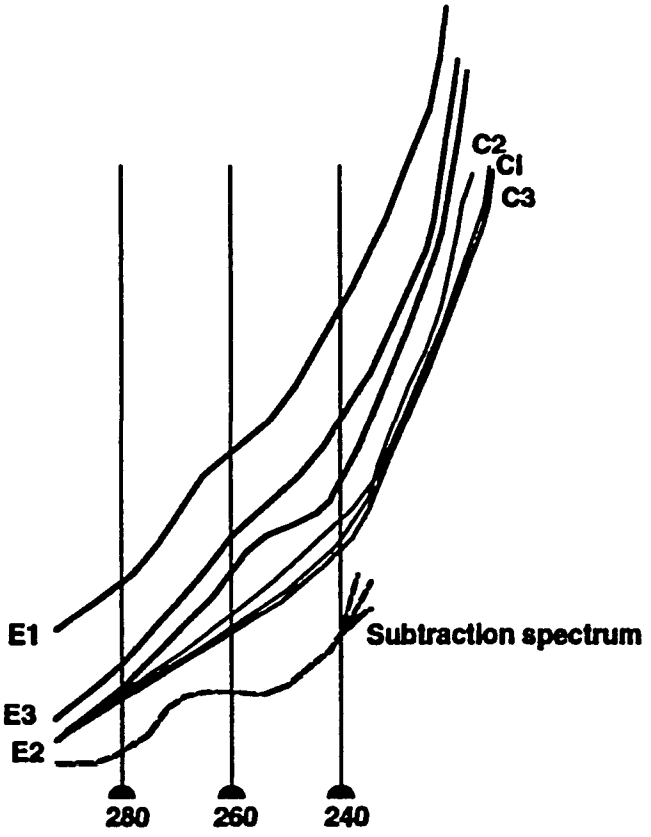
Living capsules just starting to darken were compared to mature, freeze-dried capsules. In each case, three controls (C=no eugenol added to incubation medium) and three experimental (E=eugenol added) replicates were run. UV spectra were recorded for each, and in addition, a UV specutrm of one of the the experimental replicates was run with one of the controls as the blank (labelled "subtraction spectrum").

Figure 41a. Fresh, maturing capsules. The subtraction spectrum shows a peak at 280, characteristic of lignin, indicating that the eugenol was converted to coniferyl alcohol and polymerized in the cell walls. A phloroglucinol color test, shown below the spectra, indicates that coniferyl lignin is present.

Figure 41b. Mature, freeze-dried capsules. There is no peak in the spectra at 280. The subtraction spectrum shows a peak at closer to 270. The phloroglucinol color test does not indicate the presence of lignin.



a. Fresh, maturing Pellia capsules



b. Freeze-dried, mature Pellia capsules

	Control (C)	+ Eugenol (E)	Control (C)	+ Eugenol (E)
phloroglucinol-HCl (absorption at 540 nm)	0.019	0.238	0.003-0.019	0.006-0.017

Figure 42. UV spectra of LTGAs

Thioglycolic acid extractions of *Pellia epiphylla* and three higher plants were compared. The higher plants in general showed a peak at 280 characteristic of lignin, but the peak was very slight in some. *Pellia epiphylla* showed little to no peak at 280.

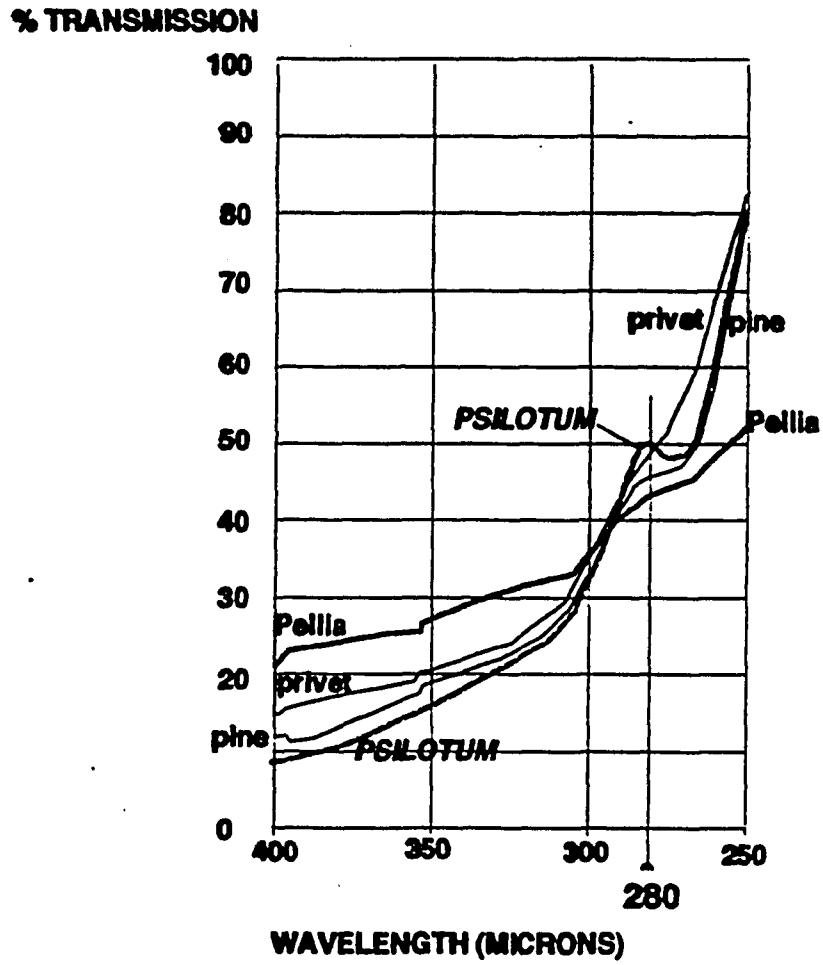

% TRANSMISSION
100
90
80
70
60
50
40
30
20
pine
0
privet
pine
PSILOTUM
Pellia
Pellia
privet
PSILOTUM
400
350
300
250
280
WAVELENGTH (MICRONS)

Figure 43. IR spectra of *Pellia*, pine and privet LTGA.

Peaks characteristic of lignin are marked with solid lines. Information is given on each peak in Table 2. Peaks characteristic of guaiacyl lignin are marked G, those characteristic of syringyl lignin are marked S. These spectra were scanned into a Macintosh computer, traced, and overlaid on one another.

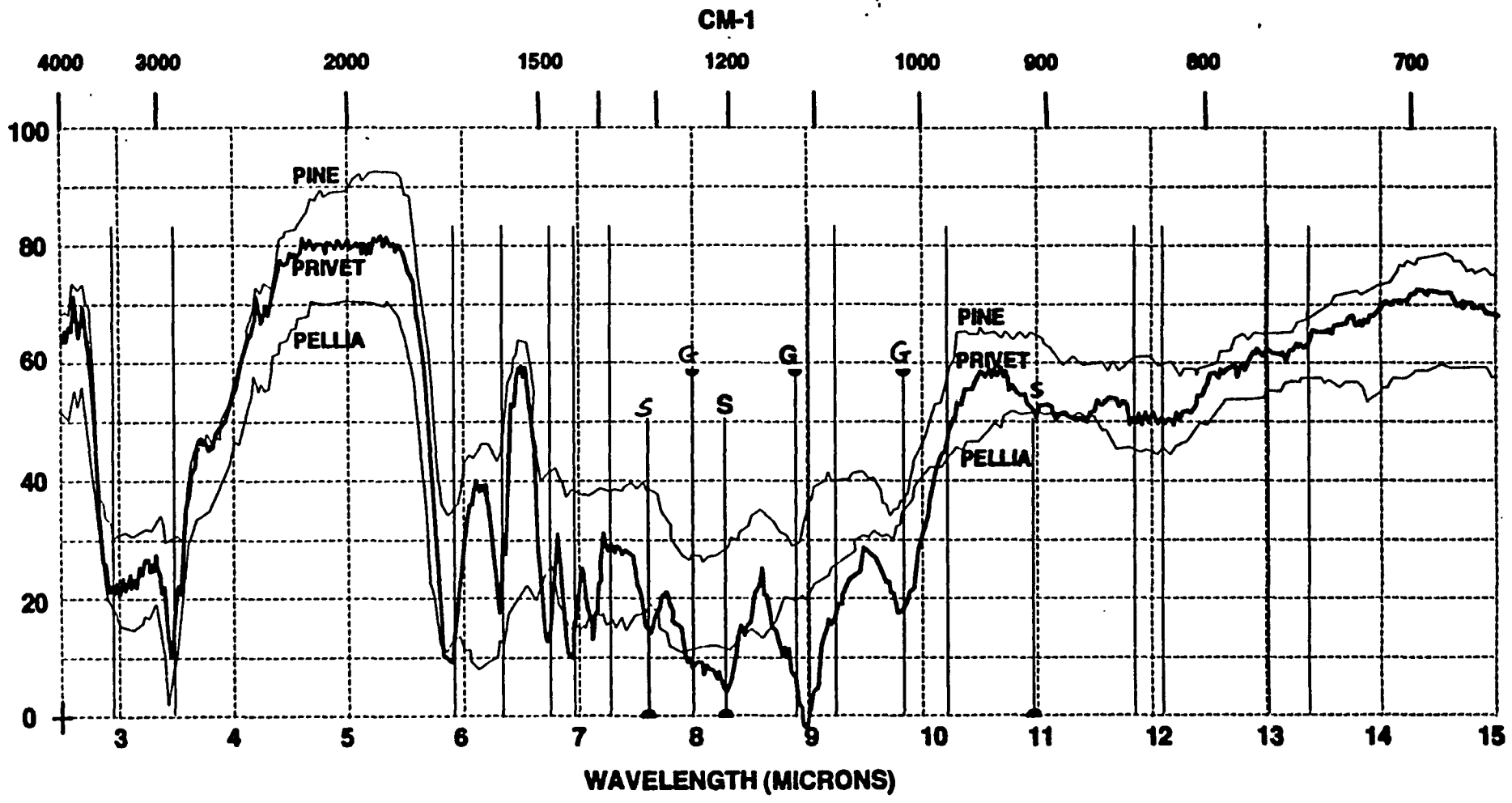

CM-1
4000
3000
2000
1500
1200
1000
900
800
700
100
80
60
40
20
0
PINE
PRIVET
PELLIA
PINE
PRIVET
PELLIA
S
S
S
G
G
G
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

Figure 44. Proton NMR spectra LTGAs.

The two major lignin peaks to be seen in the pine and privet spectra are at 6.8-7.0 for the phenolic group, and at ~ 3.7 for methoxyl groups. Privet, being an angiosperm wood with syringyl lignin, has relatively more methoxyl groups than pine, and this can be seen in the relative sizes of the methoxy peaks. Pellia has little to no peaks at either place (arrows).

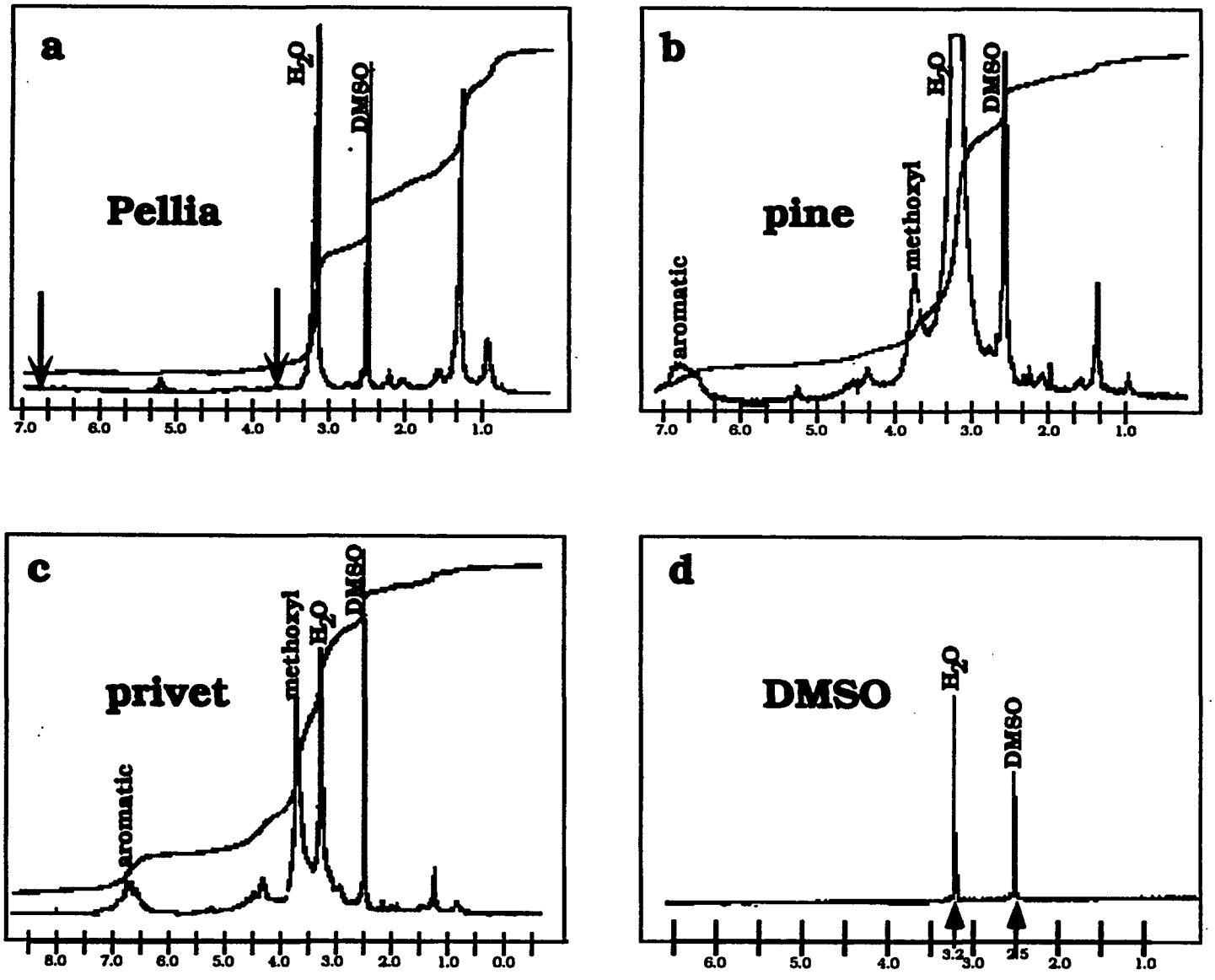

a
Pellia
H2O
DMSO
7.0
6.0
5.0
4.0
3.0
2.0
1.0
b
pine
aromatic
methoxyl
H2O
DMSO
7.0
6.0
5.0
4.0
3.0
2.0
1.0
c
privet
aromatic
methoxyl
H2O
DMSO
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
d
DMSO
H2O
DMSO
6.0
5.0
4.0
3.2
3.0
2.5
2.0
1.0

Figure 45. Structures of pyrolysis breakdown products typical of lignin.
The phenol structures are derived from *p*-hydroxyphenyl lignins, the guaiacol structures from coniferyl (guaiacyl) lignin, and the dimethoxyphenol (DMP) structures from syringyl lignin.

Pyrolysis Breakdown Products Characteristic of Lignin

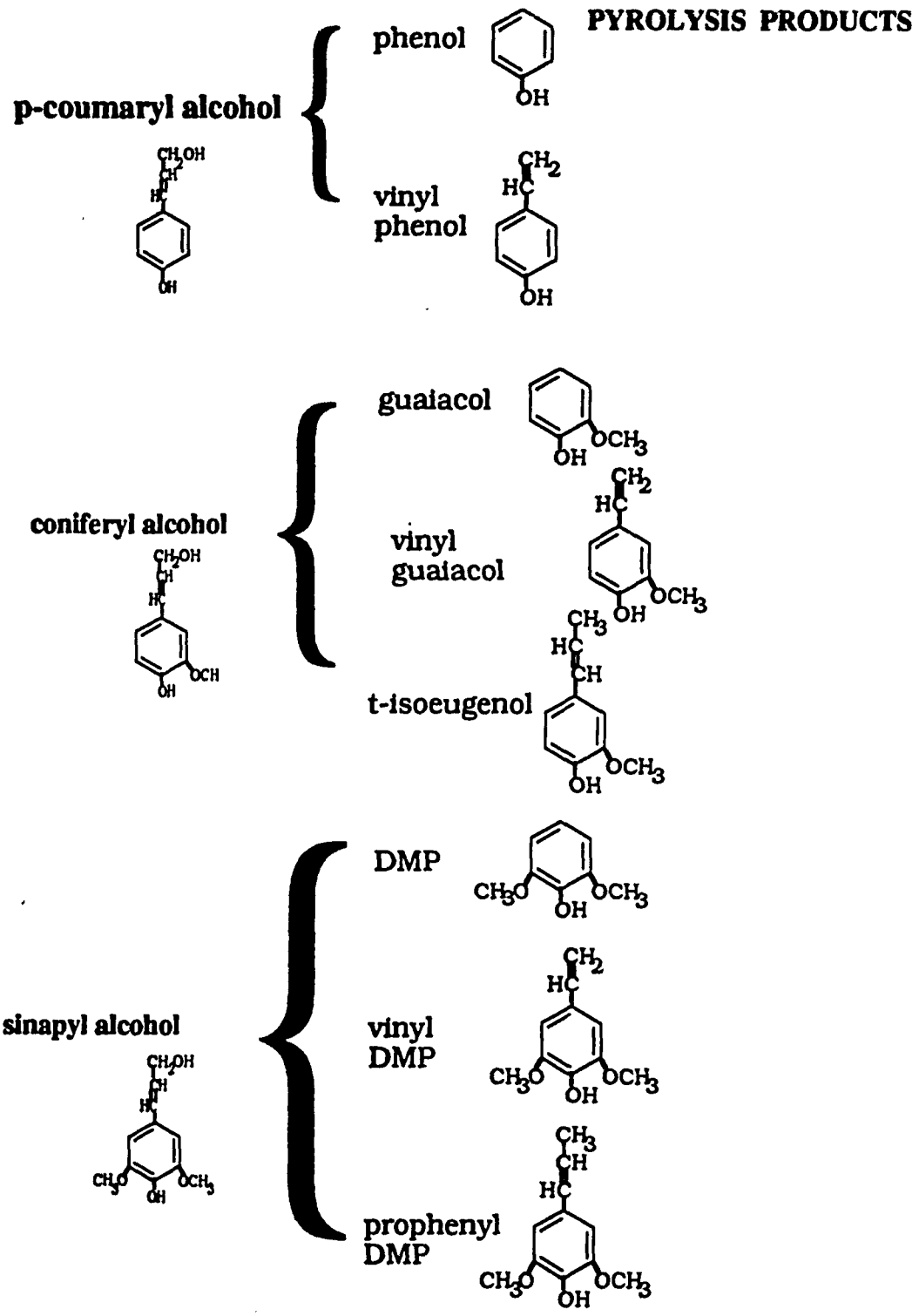


Figure 46. Abundance of pyrolysis products (*Pellia* fractions)

The elater, capsule wall and seta fractions display similar patterns (high phenol, increasing amounts of vinyl phenol, guaiacol and vinyl guaiacol. The spores show a more equal distribution of vinylphenol, guaiacol and vinyl guaiacol. Syringyl breakdown products are absent or in trace amounts, as is the guaiacyl breakdown product with all 3 carbons intact in the sidechain (t-isoeugenol). Of the elater, capsule wall and seta fractions, the elater fraction contains the highest proportion of lignin breakdown products, with the capsule wall next and the seta fraction containing the least.

Pellia pyrolysis products

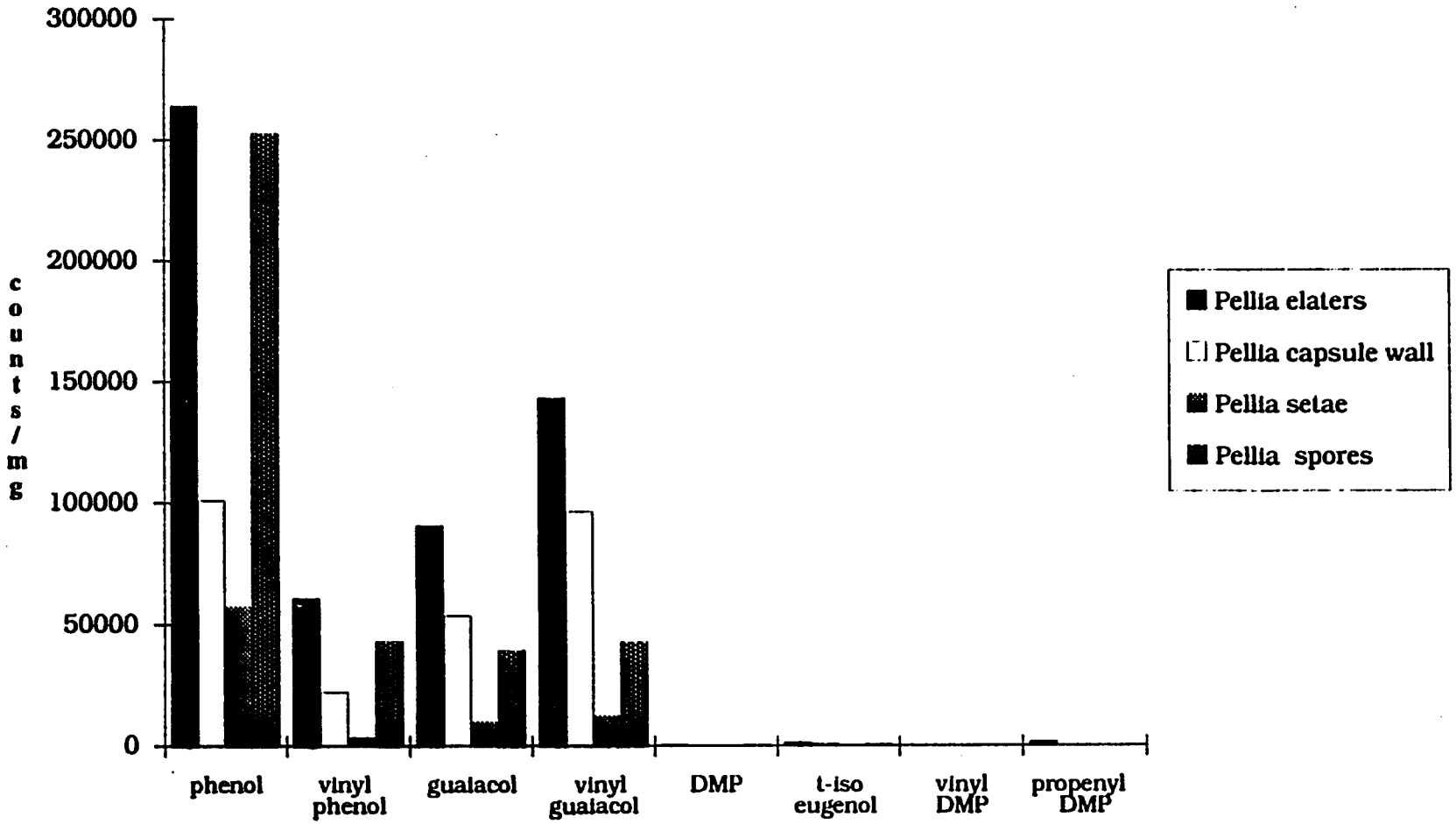


Figure 47. Abundance of pyrolysis products (*Pellia* & higher plants)
Abundance of pyrolysis products typical of lignin are compared in *Pellia* elaters, *Bromus tectorum* (a grass with *p*-hydroxhyphenyl, guaiacyl and syringyl lignin), privet (an angiosperm shrub with guaiacyl and syringyl lignin), and red pine (a conifer with primarily guaiacyl lignin). *Pellia* has a higher relative phenol content, and less t-isoeugenol than any of the higher plant lignins pictured here.

Pellia and higher plant pyrolysis products

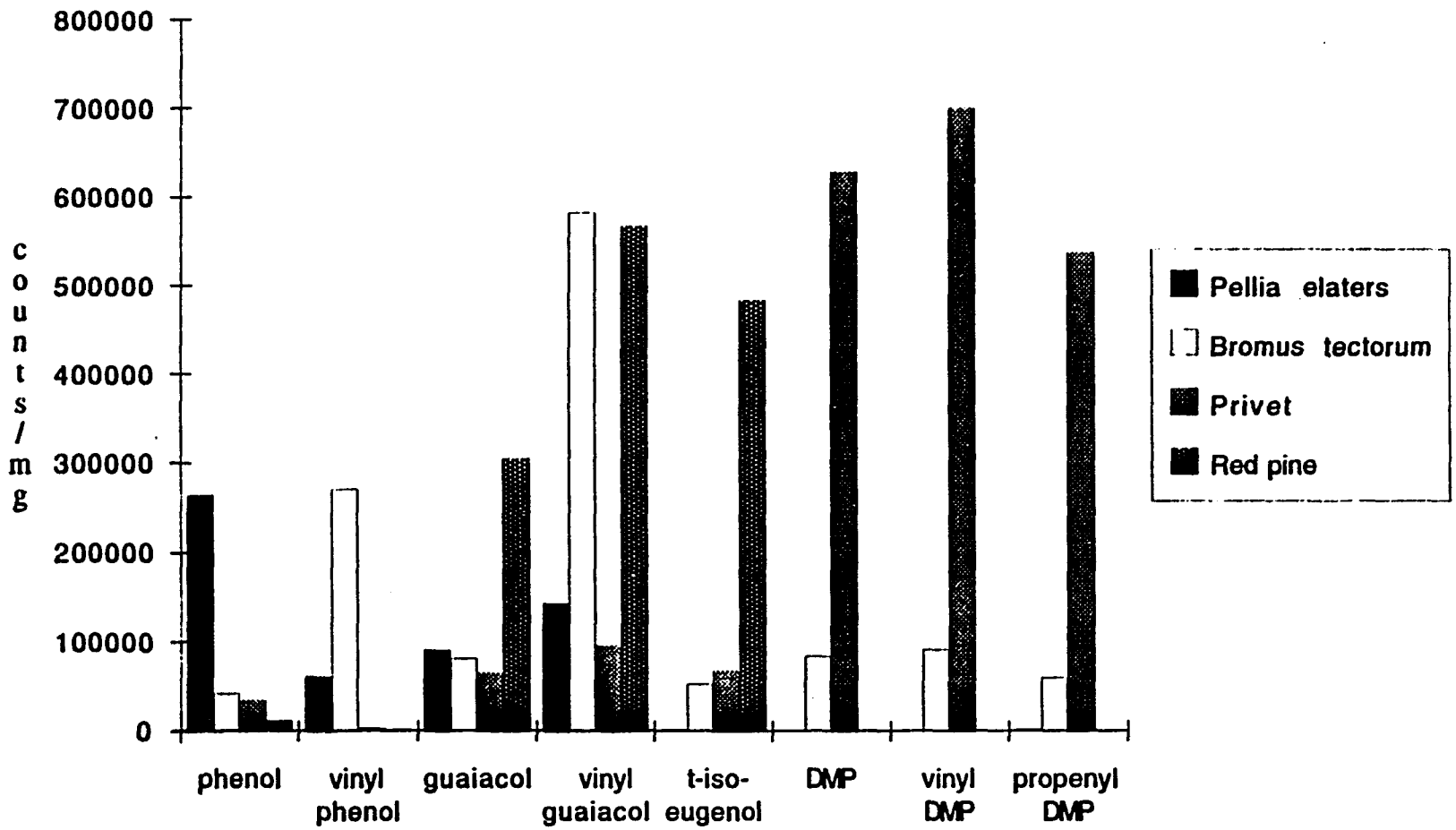
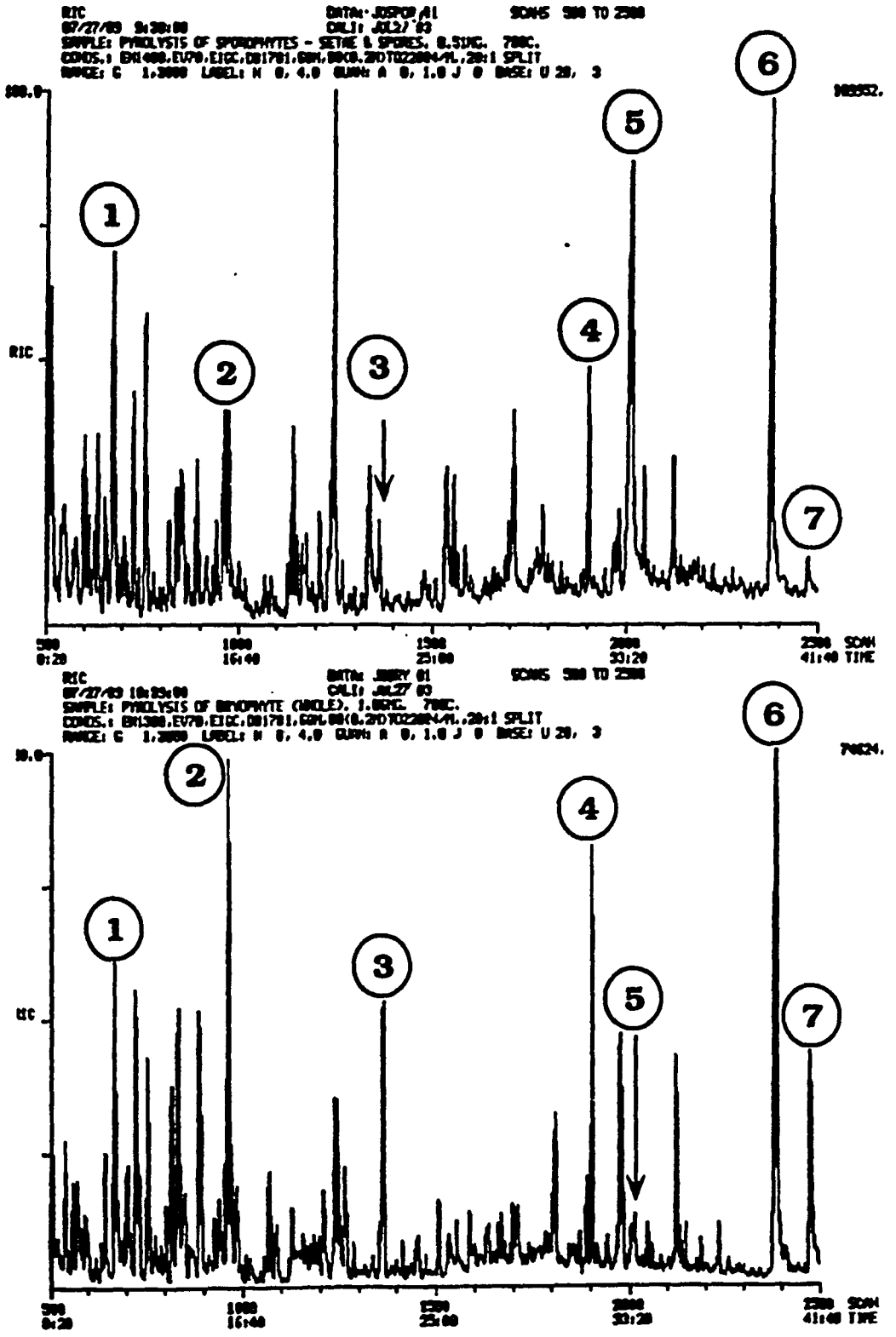


Figure 48. Pyrograms of initial *Pellia* sporophyte fractions

The suspected pyrolysis products of these first two fractions run for pyrolysis (whole sporangia with seta, and capsule walls + elaters) were confirmed with mass spectroscopy (see Appendix C). These products are labelled with a star in this figure, and identified by name in figures 49-52. The largest peaks present that were uncharacteristic for lignin were matched by a NBS match, and are labelled by number. These tentative identifications are (see Appendix C for the mass spectra):

number	MW	scan #	NBS Library match
1	112	670	2-hydroxy-3-methyl-2-cyclopenten-1-one
2	109	965	1-hepten-3-ol
3	117	1364	1S 1H-Indole
4	179	1906	cyclododecanol
5	194	2015	1,2,3,4-cyclopentanetetrol
6	256	2385	hexadecanoic acid
7	150	2476	5-octadecene

Of great interest is number 5, scan #2015, because it is found in abundance in the wall+elater sample, and very much less is found in the whole sporophyte sample. This suggests that it may be selectively deposited in the secondary cell wall thickenings, and hence found in greater abundance in the wall+elater fraction.


RIC
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.5MG. 700C.
1
2
3
4
5
6
7
RIC
SAMPLE: PYROLYSIS OF GAMETOPHYTE (GAMETE). 1.0MG. 700C.
1
2
3
4
5
6
7

Figures 49. Pyrograms of *P. epiphylla* capsule fractions

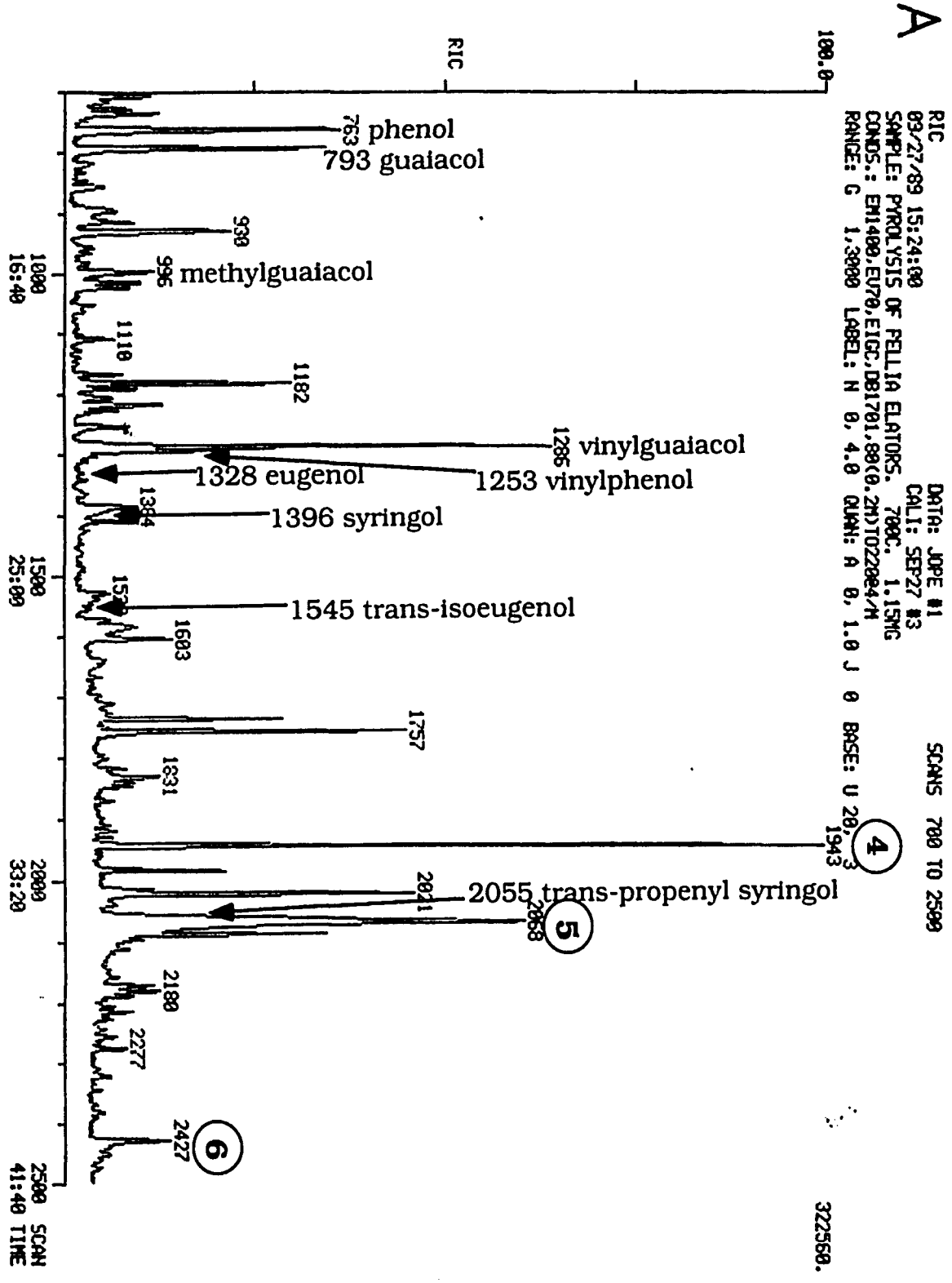
The pyrograms of these fractions identify the pyrolysis breakdown products typical of lignin, as well as the non-lignin peaks matched by the NBS (where they appear). A transparency of the elater fraction is included so that it may be overlaid on the others for comparison. Peak number 5 is shown in these fractions to be most abundant in both the wall and elater fractions, and absent in the setae and spore samples.

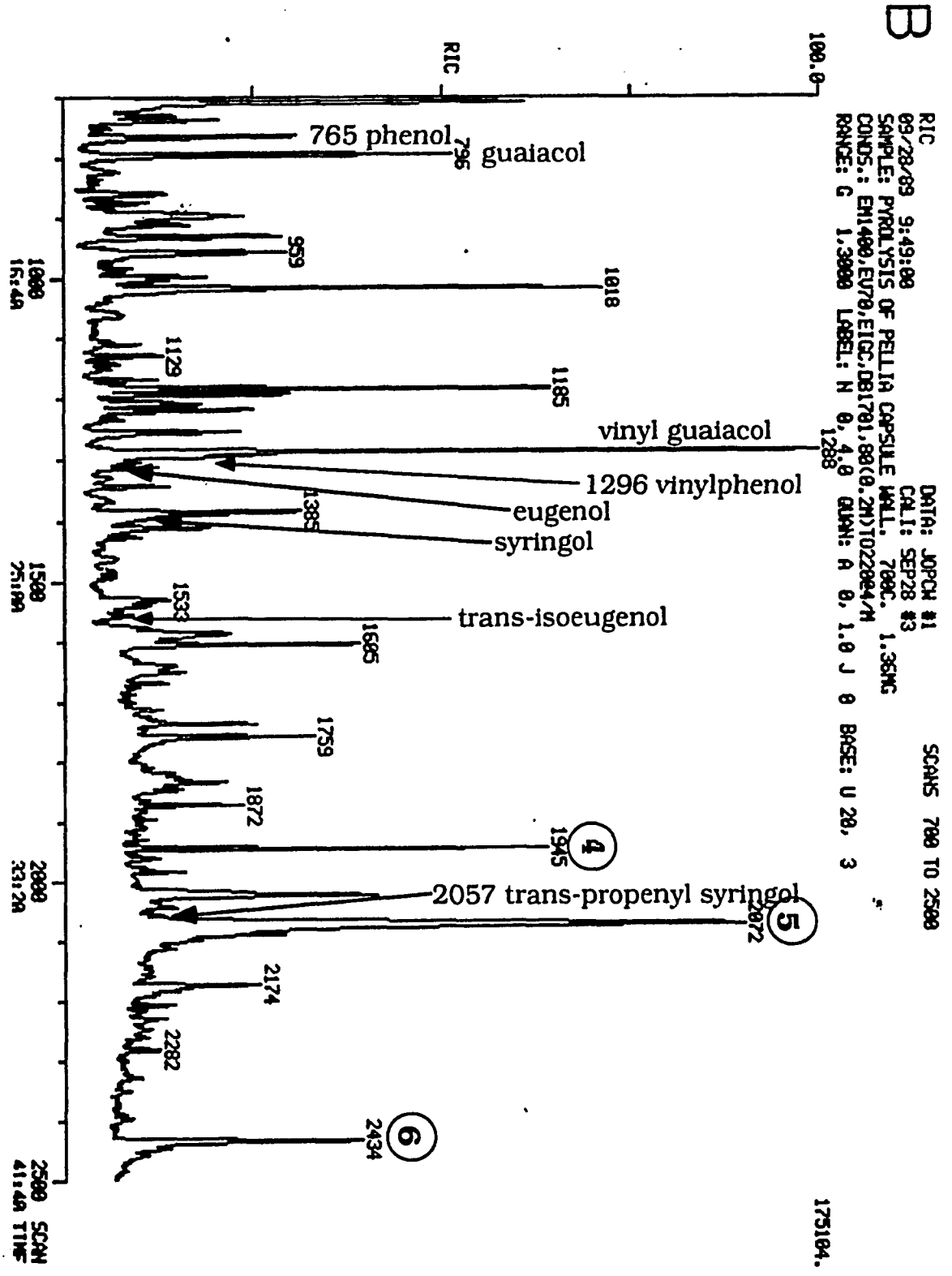
Figure 49a. Pyrogram of *Pellia* elater fraction.

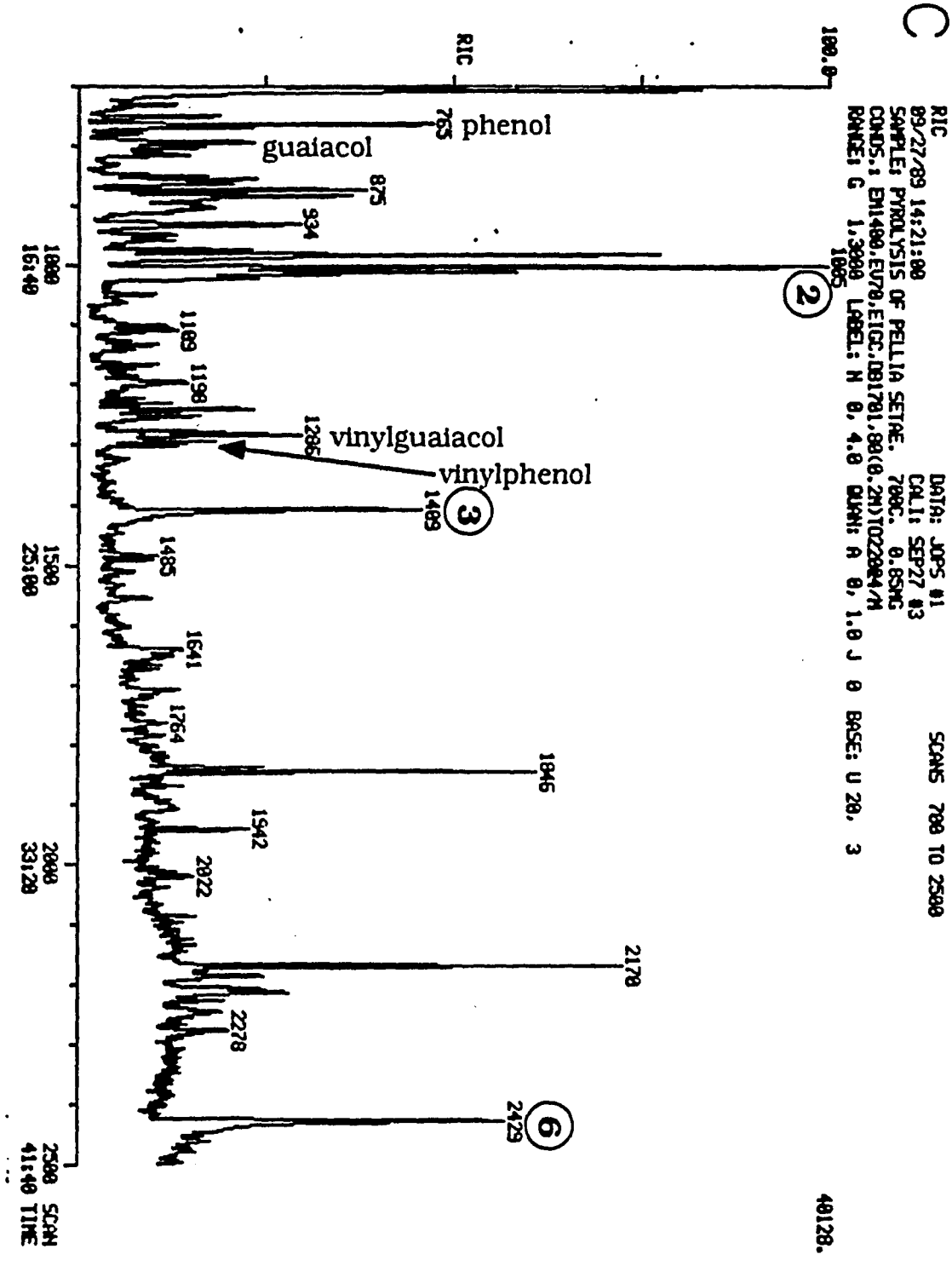
Figure 49b. Pyrogram of *Pellia* capsule wall fraction

Figure 49c. Pyrogram of *Pellia* seta fraction

Figure 49d. Pyrogram of *Pellia* spore fraction


A
RIC
03/27/89 15:24:00 DATA: JOPE #1
SAMPLE: PYROLYSIS OF FELLIA ELATORS. 700C. 1.15MG CALI: SEP27 #3
CONDS.: EM1400.EV70.EIGC.DB1701.80(0.2M)10220E4/M SCANS 700 TO 2500
RANGE: G 1,3000 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20,
100.0
322560.
783 phenol
793 guaiacol
930
996 methylguaiacol
1110
1182
1286 vinylguaiacol
1328 eugenol
1253 vinylphenol
1384
1396 syringol
1527
1545 trans-isoeugenol
1603
1757
1831
1943
2021
2055 trans-propenyl syringol
2088
2180
2277
2427
1000 16:40
1500 25:00
2000 33:20
2500 41:40 SCAN TIME


B
RIC
09/28/89 9:49:00
SAMPLE: PYROLYSIS OF PELLIA CAPSULE WALL, 700C, 1.36MG
CONDS:: EM1400,EV70,EIGC,DB1701,80(0.2M)T022004/M
RANGE: C 1.3000 LABEL: N 0, 4.0
DATA: JOPCH #1
CAL1: SEP28 #3
QUAN: A 0, 1.0 J 0
SCANS 700 TO 2500
BASE: U 20, 3
100.0
RIC
765 phenol
796 guaiacol
959
1018
1129
1185
vinyl guaiacol
1288
1296 vinylphenol
eugenol
1385
syringol
1533
trans-isoeugenol
1695
1759
1872
4
1945
2057 trans-propenyl syringol
5
2072
2174
2282
6
2434
1000
16:40
1500
25:00
2000
33:20
2500
41:40 TIME
SCAN
175104.


RIC
09/27/89 14:21:00
SAMPLE: PYROLYSIS OF PELLIA SETAE. 700C. 0.85MG
CONDS: 1 EM1400.EU70.EIGC.DB1701.88(0.2M)1022004/M
RANGE: 1 C 1.3000 LABEL: N 0, 4.0 QUANT: A 0, 1.0 J 0 BASE: U 20, 3
DATA: JOPS #1
CAL: SEP27 #3
SCANS 700 TO 2500
100.0
RIC
753
phenol
guaiacol
875
934
1005
2
1189
1198
1286
vinylguaiacol
vinylphenol
1409
3
1485
1641
1764
1846
1942
2022
2170
2278
2429
6
40128.
1000
16:40
1500
25:00
2000
33:20
2500
41:40
SCAN
TIME

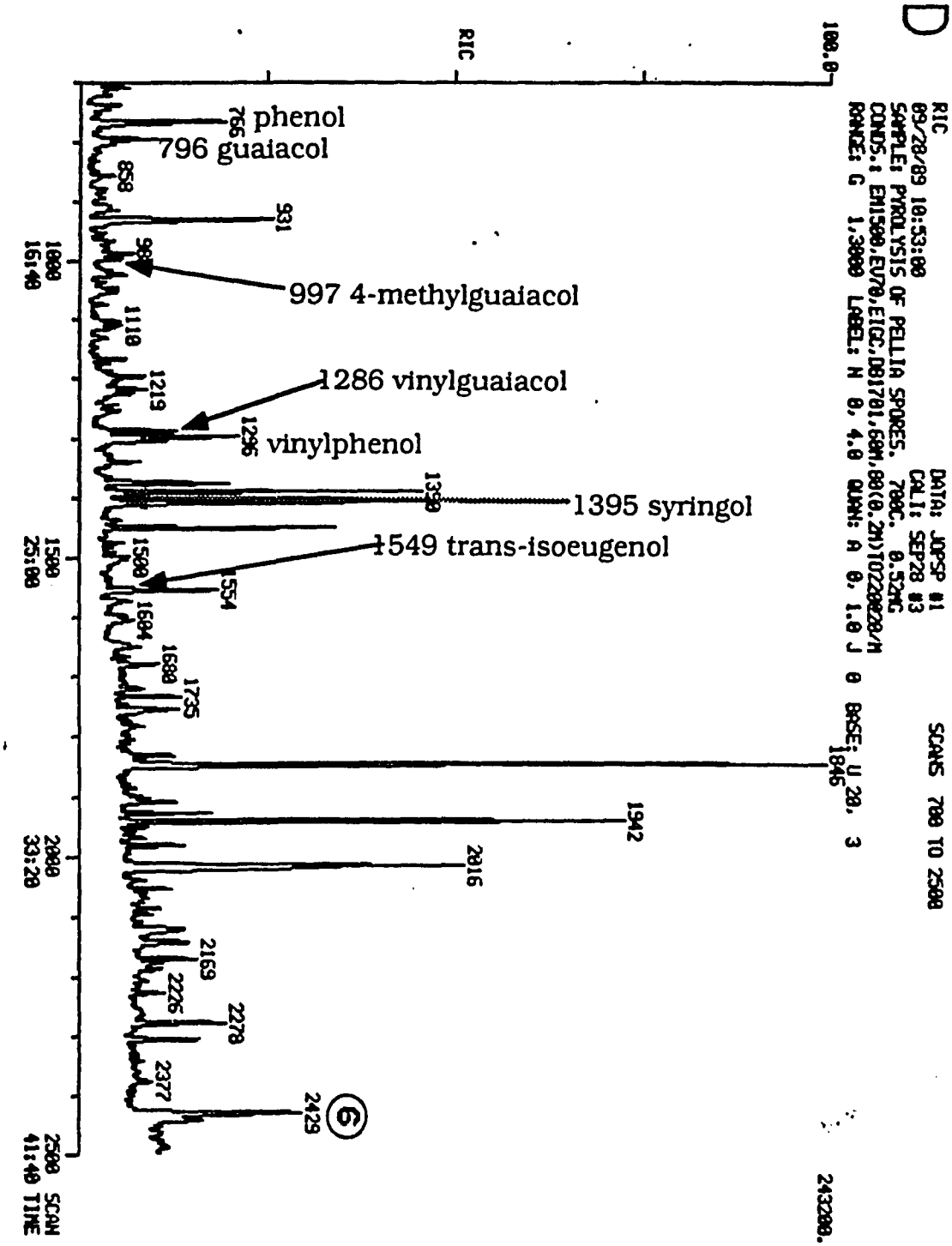

D
RIC
09/28/89 10:53:00 DATA: JOPSP #1 SCANS 700 TO 2500
SAMPLE: PYROLYSIS OF PELLIA SPORES. CAL: SEP28 #3
CONDS: 1 EM1500,EV70,EIGC,DB1701,60M,80(0.2M)10220020/M 700C. 0.52MG
RANGE: G 1.3000 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20. 3
100.0
RIC
766 phenol
796 guaiacol
858
931
997 4-methylguaiacol
1000
16:40
1110
1219
1286 vinylguaiacol
1296 vinylphenol
1339
1395 syringol
1500
25:00
1500
1549 trans-isoeugenol
1554
1604
1680
1735
1846
1942
2000
33:20
2016
2169
2226
2278
2377
2429
6
2500
41:40 SCAN TIME
243200.

Figure 50. Gas chromatogram of thioacidolysis products (*Pellia* vs *Bromus*) *Bromus tectorum* (a grass) was chosen for comparison because it contains all three lignin types, seen as double peaks in the gas chromatogram on the top line. Labelled are the double peaks for *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties, as well as the internal standard (IS), tetracosane. The gas chromatogram for *Pellia* thioacidolysis products was superimposed underneath the *Bromus* for comparison. There are no guaiacyl or syringyl peaks, but a possible *p*-hydroxyphenyl double peak, which was checked further with mass spectroscopy (Figure 51).

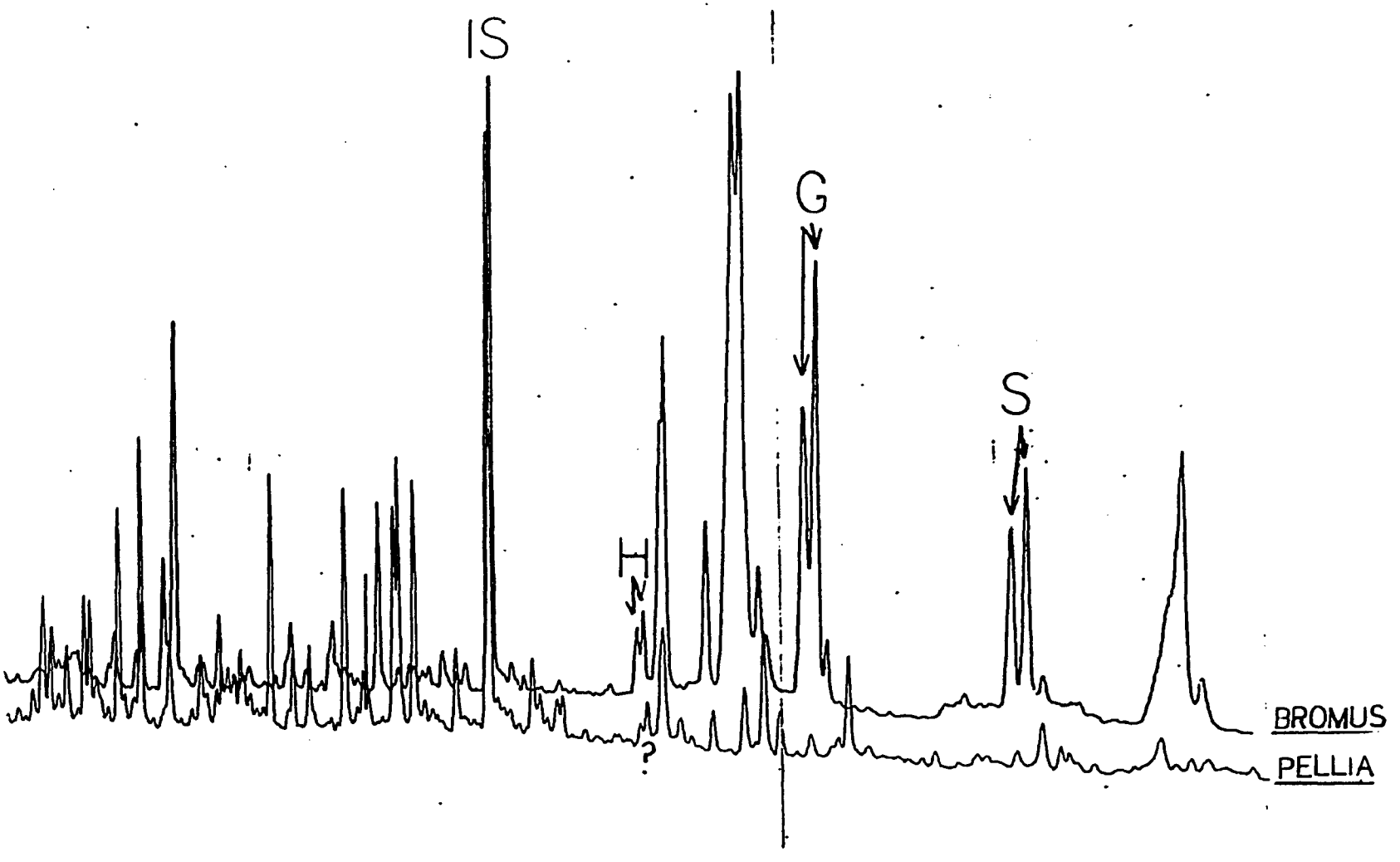

IS
G
S
BROMUS
PELLIA

Figure 51. Mass spectra of thioacidolysis products of *Bromus tectorum* and *Pellia epiphylla* capsule wall (MIDRIC + Mass chromatograms)
The mass spectrometer was used as the detector in this and the following figure. The top three lines utilized the multiple ion detector (MID) to select for specific ions: the 239 ion detects the *p*-hydroxyphenyl moieties (H, top line), the 269 ion detects the guaiacyl moieity (G, second line) and the 299 ion detects the syringyl moiety (S, third line). The bottom line is the reconstructed ion count (RIC), which records the total ions.

Figure 51a. *Bromus tectorum* thioacidolysis products. Substantial peaks can be seen for all three moieties. The RIC at bottom shows that the β-*O*-4 products are predominantly guaiacyl, with lesser amounts of syringyl and small amounts of *p*-hydroxyphenyl components.

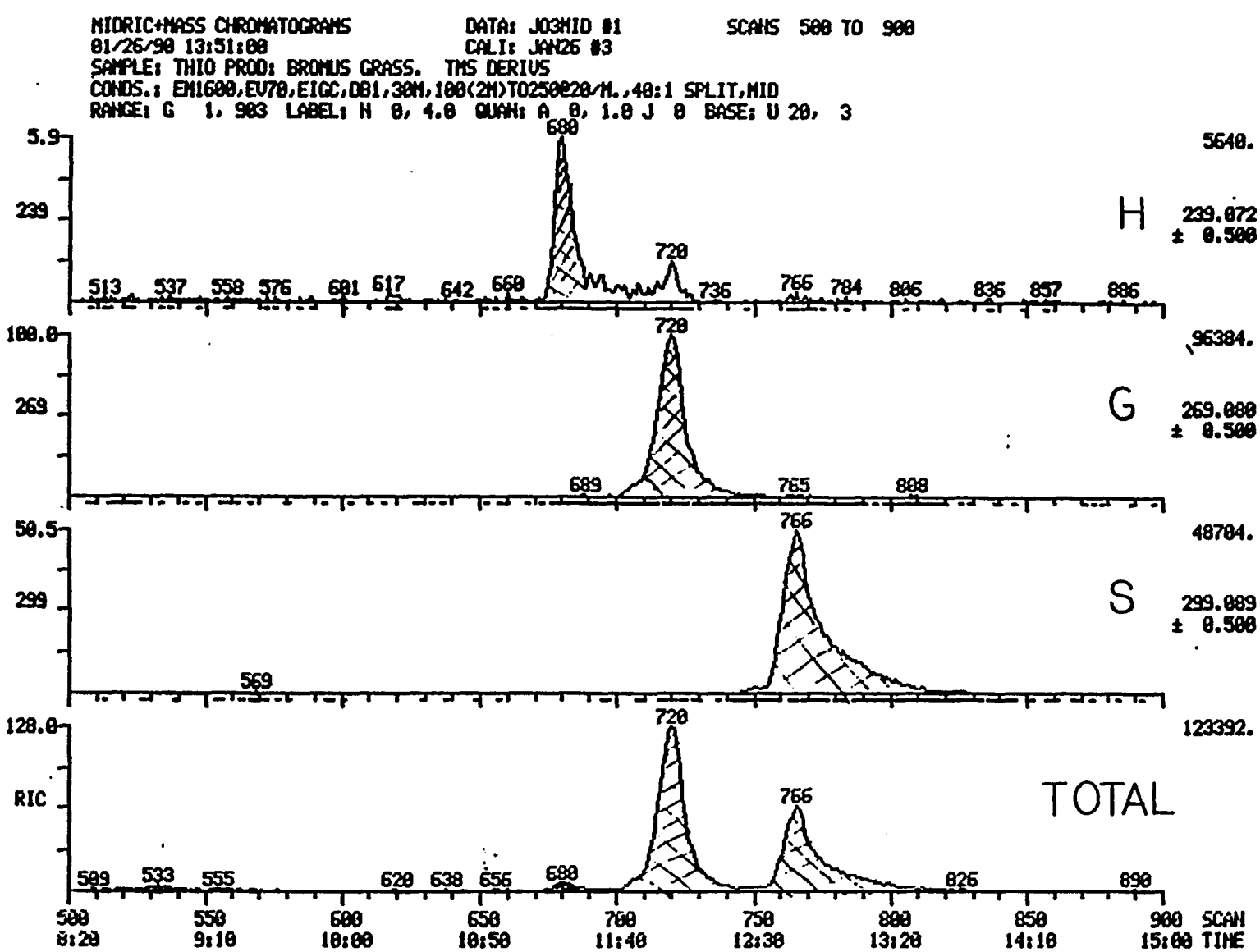

MIDRIC+MASS CHROMATOGRAMS
DATA: JO3MID #1
SCANS 500 TO 900
01/26/90 13:51:00
CALI: JAN26 #3
SAMPLE: THIO PROD: BROMUS GRASS. TMS DERIVS
CONDS.: EM1600,EV70,EIGC,DB1,30M,100(2M)T0250@20/M.,40:1 SPLIT,MID
RANGE: G 1, 903 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
5.9
239
680
720
513 537 558 576 601 617 642 660 736 766 784 806 836 857 886
5640.
H
239.072
± 0.500
100.0
269
720
689 765 808
96384.
G
269.000
± 0.500
50.5
299
766
569
48704.
S
299.089
± 0.500
128.0
RIC
720
766
509 533 555 620 638 656 680 826 890
123392.
TOTAL
500 550 600 650 700 750 800 850 900 SCAN
8:20 9:10 10:00 10:50 11:40 12:30 13:20 14:10 15:00 TIME

Figure 51b. *Pellia epiphylla* capsule wall thioacidolysis products. (See the legend on the preceding page for explication)

There are no substantial peaks for any of the lignin moieties (the *Bromus* lignin peaks have been superimposed as the cross-hatched peaks for reference), demonstrating that the possible *p*-hydroxyphenyl peak in the chromatogram in Figure 50 was not *p*-hydroxyphenyl. The low count numbers for *Pellia* (indicated above the ion number on the far right) indicate that there is little there above the noise threshold.

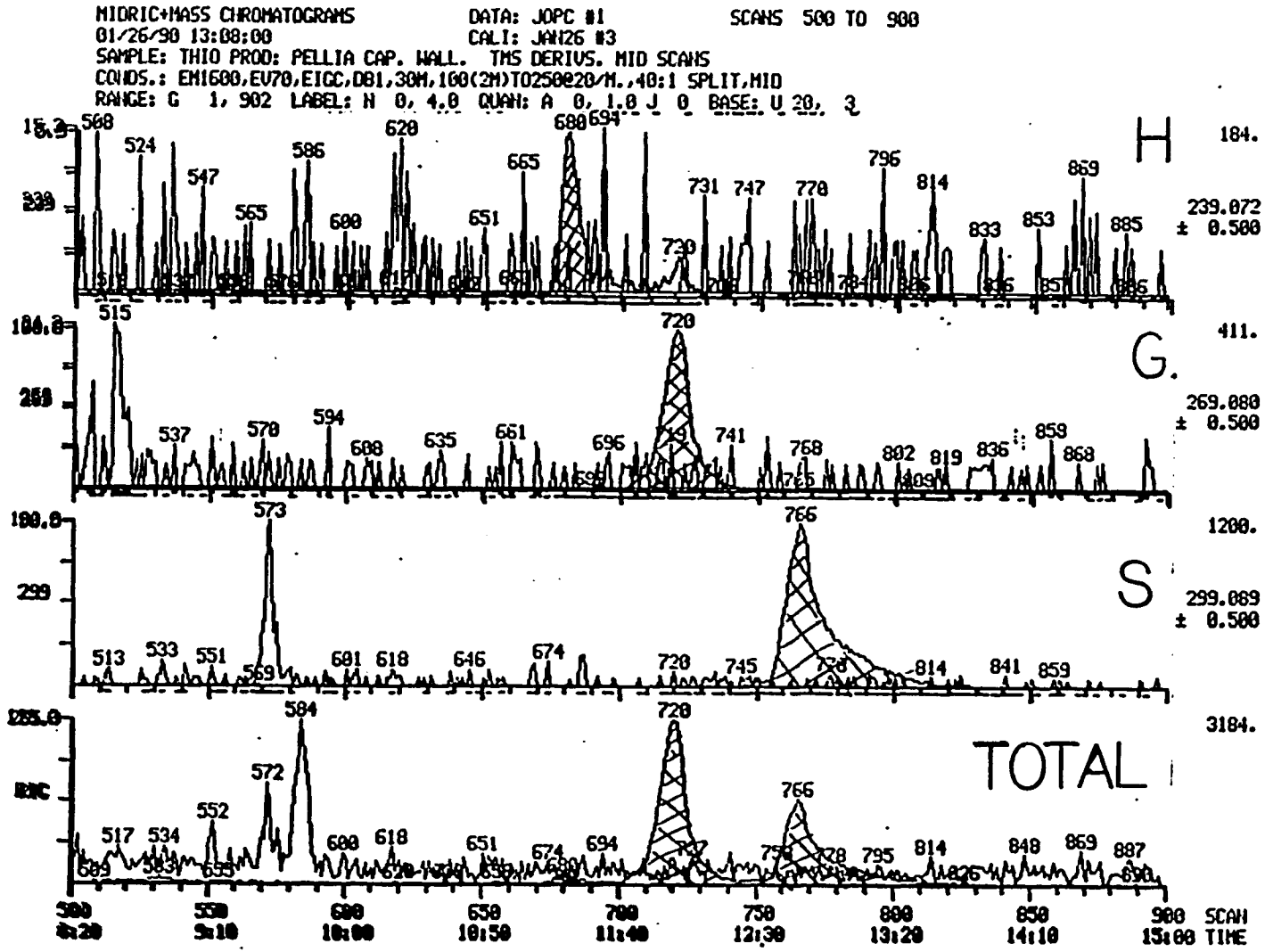
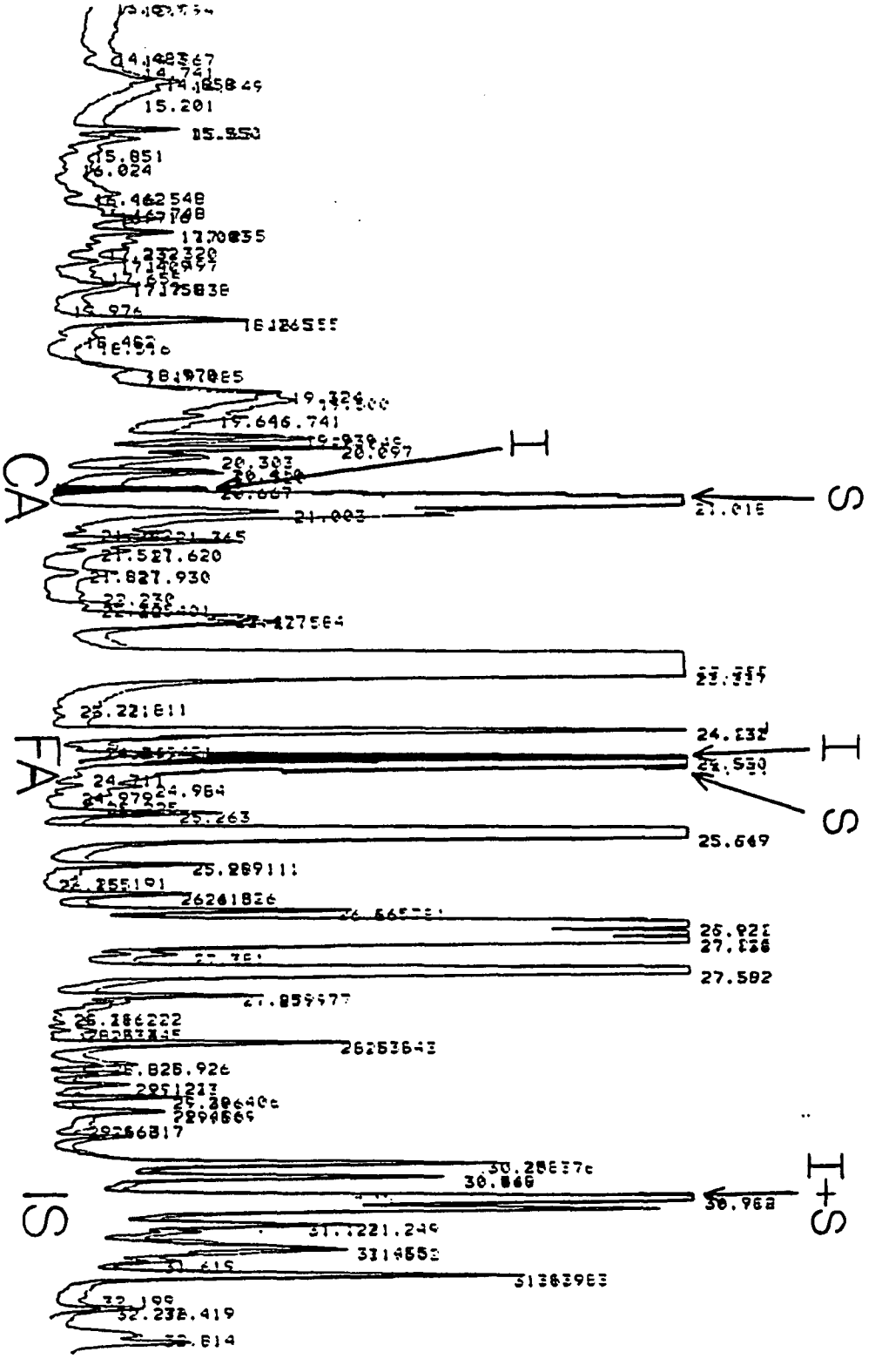

MIDRIC+MASS CHROMATOGRAMS
DATA: JOPC #1
SCANS 500 TO 900
01/26/90 13:08:00
CALI: JAN26 #3
SAMPLE: THIO PROD: PELLIA CAP. WALL. TMS DERIVS. MID SCANS
CONDS.: EM1600,EV70,EIGC,DB1,30M,100(2M)T0250@20/M.,40:1 SPLIT,MID
RANGE: G 1, 902 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
H
184.
239.072
± 0.500
G.
411.
269.080
± 0.500
S
1200.
299.089
± 0.500
TOTAL
3184.
RIC
SCAN
TIME

Figure 52. Gas chromatogram of saponification products (run at room temperature) of *Pellia epiphylla.*

A gas chromatograms were run on the saponified sample, and then rerun after being spiked with authentic ferulic (FA) and *p*-coumaric (C) acids. The first run is on the bottom line, and the spiked run is superimposed above. While the internal standard tetracosane (IS) and other peaks are all nearly the same height, the ferulic and *p*-coumaric acid peaks are substantially higher after spiking (I identifies the initial peak, S identifies the peak after spiking). This confirms the existence of these acids in *Pellia epiphylla* tissue.


CA
H
S
15.201
15.851
16.024
19.646.741
20.303
21.003
21.527.620
21.827.930
24.132
24.530
H
S
TA
24.984
25.649
25.289111
26.921
27.136
27.582
S
H+S
30.968
31.221.246
3314553
51383983
32.238.419

Figures 53-59. UV epifluorescence microscopy for lignin and ferulic acid

Briefly, lignin (and other phenolics) epifluoresce a yellow to yellow green to blue, (depending on the filters used) when struck with UV light. Chlorophyll in chloroplasts fluoresce a bright red. If treated with ammonium hydroxide, ferulic acid fluoresces a bright green, which disappears if the tissue is then treated with sodium acetate.

Figure 53. UV epifluorescence of a grass stem cross-section (Olympus set-up).
Figure 53a. Water only. Xylem tissue fluoresces a white-blue, outer leaves are chlorophyllose and flouresce red, as do some of the youngest inner leaves.
magnification: 145X.

Figure 53b. Ammonium hydroxide. The same section now flouresces bright green throughout the section, showing that ferulic acid is distributed throughout the cell walls. The xylem traces of the youngest leaves in the center, which show little evidence of lignin, clearly contain ferulic acid.
magnification: 145X.

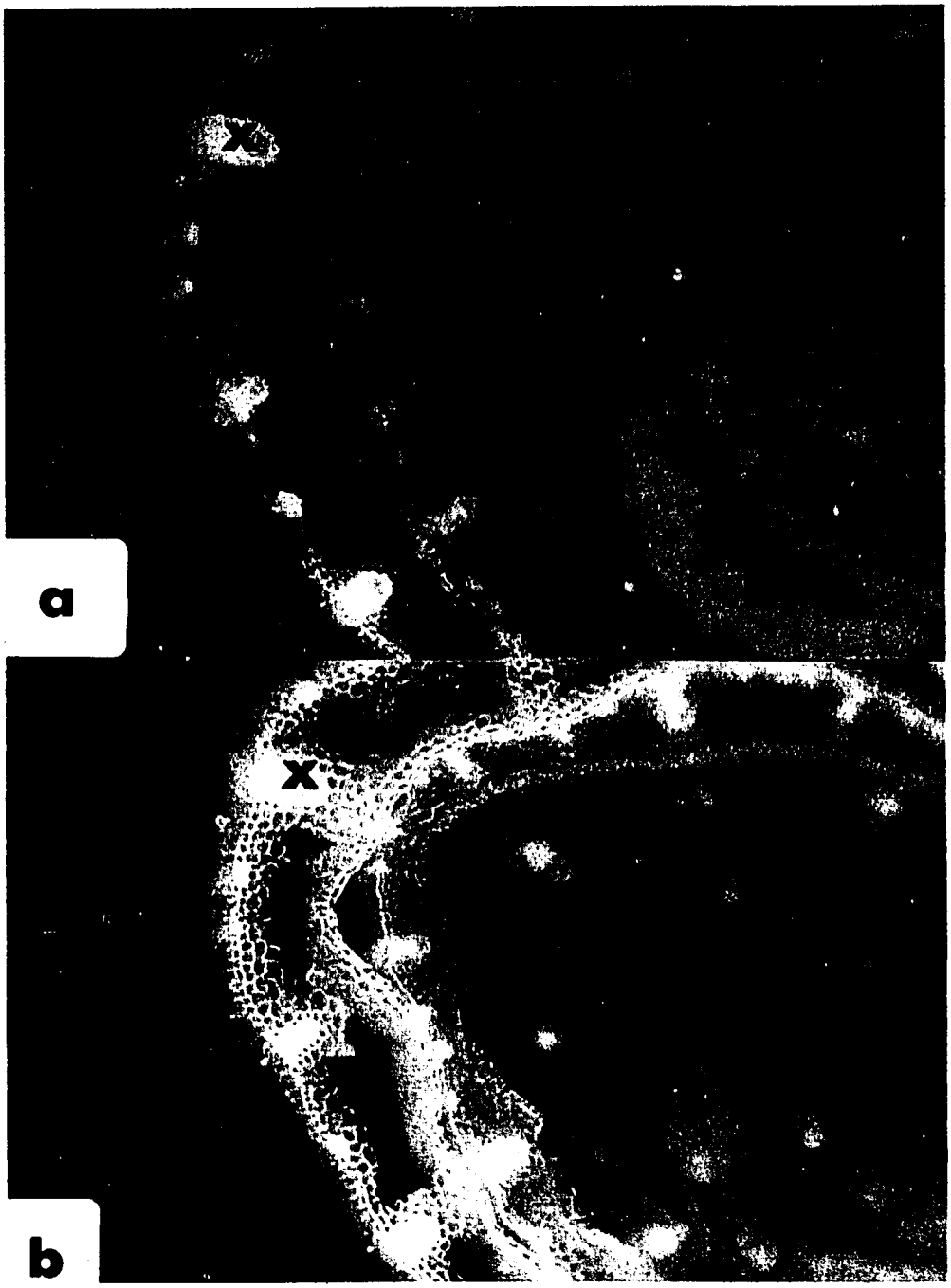

a
b

Figure 54. UV epifluorescence of *Pellia* capsule wall, spores and elaters (Olympus set-up).

Figure 54a. In water only. As compared to the ice-blue of the lignin in the grass, the *Pellia* tissue fluoresces a golden yellow, with the secondary thickenings (arrow) fluorescing brightly, and individual helical secondary thickenings of elaters (e) seen clearly. The seta (S) also flouresces. Spores (sp) are bright red due to chloroplasts within.
magnification: 145X.

Figure 54b. In ammonium hydroxide. All primary walls in seta (S) and capsule wall (CW) have turned green. The secondary thickenings (arrow) of elaters (e) and capsule wall (CW) are orange.
magnification: 145X.

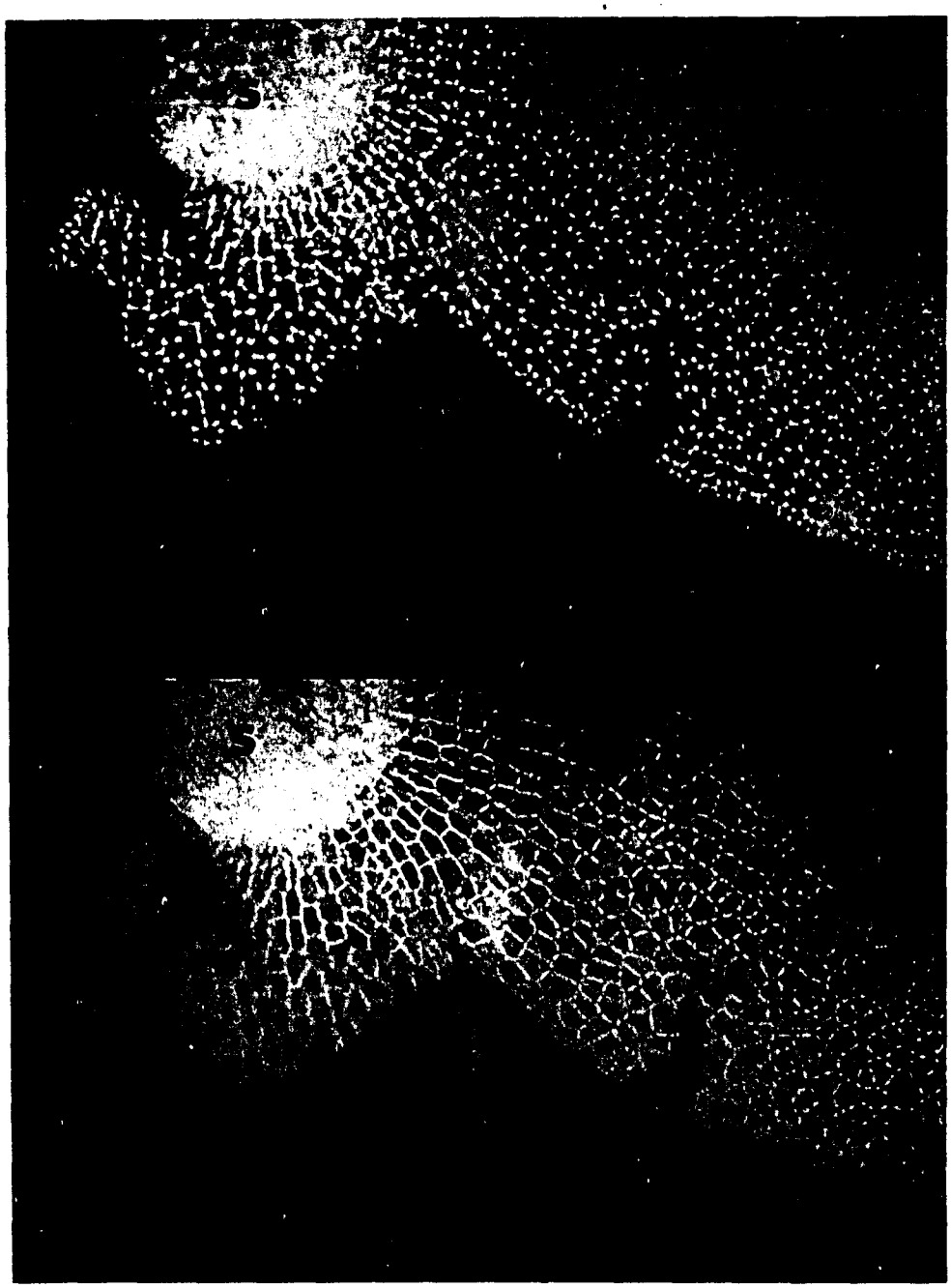


Figure 55. UV epifluorescence of *P. epiphylla* capsule wall and seta (Zeiss set-up). The Zeiss set-up utilized G365 and FT395 filters. This set-up yielded slightly different results from the Olympus set-up, which used no barrier or excitation filters.

Figure 55a. In water. The primary walls of the seta (S) and capsule wall (CW) fluoresce a ice-blue similar to that of xylem in Figure 53a. The nodules of secondary cell wall thickenings (arrow) are an orange color, which is not characteristic of lignin UV fluorescence.
magnification: 726X.

Figure 55b. In ammonium hydroxide. Compared to Figure 54a, there is a greenish cast to the primary walls, indicating the presence of ferulic acid. As in Figure 53b, the secondary thickenings are non-fluorescent.
magnification: 726X.

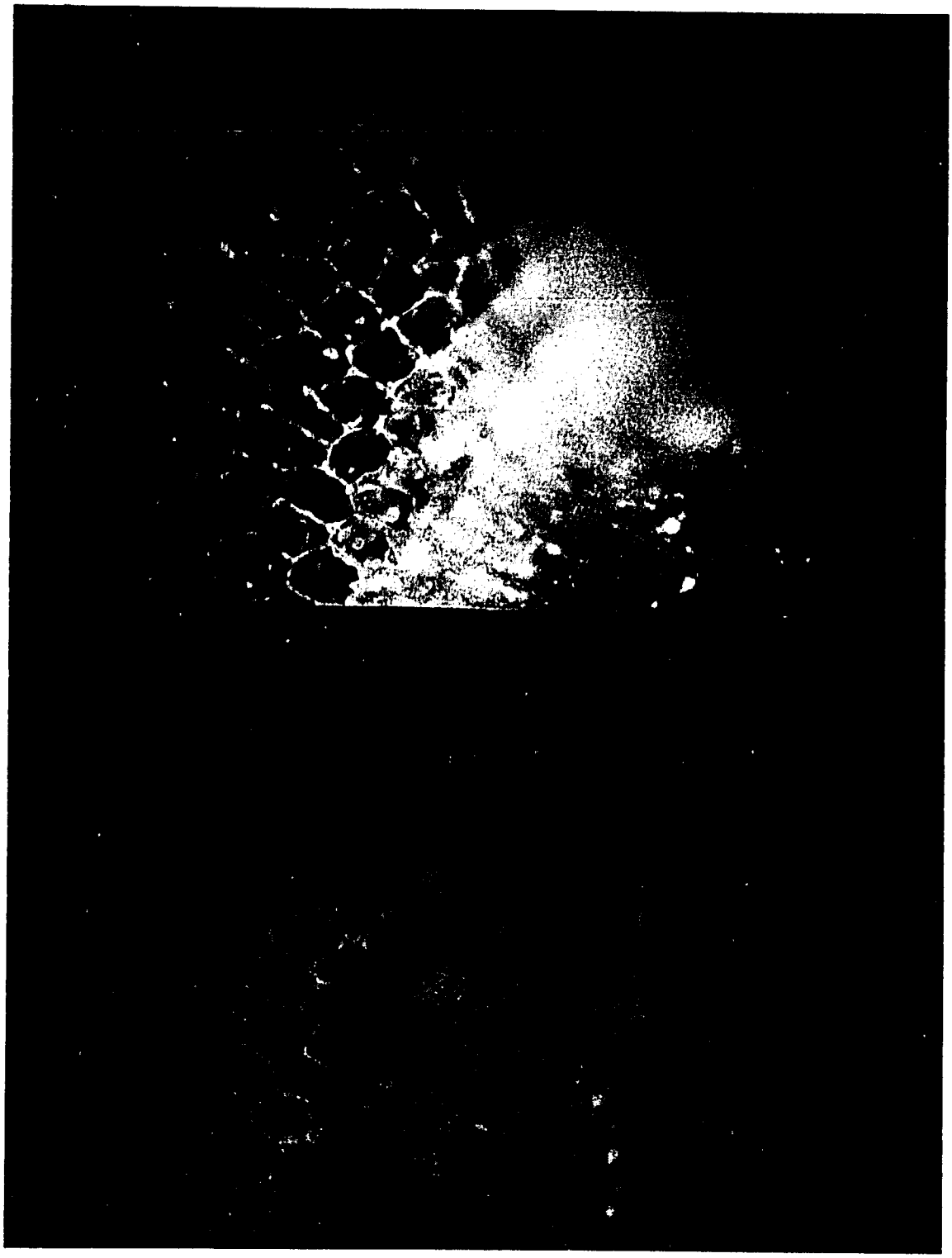


Figure 56. UV epifluorescence of *P. epiphylla* capsule wall (cross section)(Zeiss set-up).

Figure 56a. In water only. Similarly to Figure 54a, the secondary thickenings (st) are an orange color, while the radial walls (rw) fluoresce blue. This pattern is very much as observed with toluidine blue staining (Figure 28), where the secondary thickenings and inner capsule wall layer appear to differ chemically from that of the outside and central radial walls.
magnification: 1450X.

Figure 56b. In ammonium hydroxide. The secondary thickenings (st) remain orange and non-fluorescent, whereas the radial walls (rw) have taken on a greenish hue characteristic of ferulic acid autofluorescence.
magnification: 1450X.

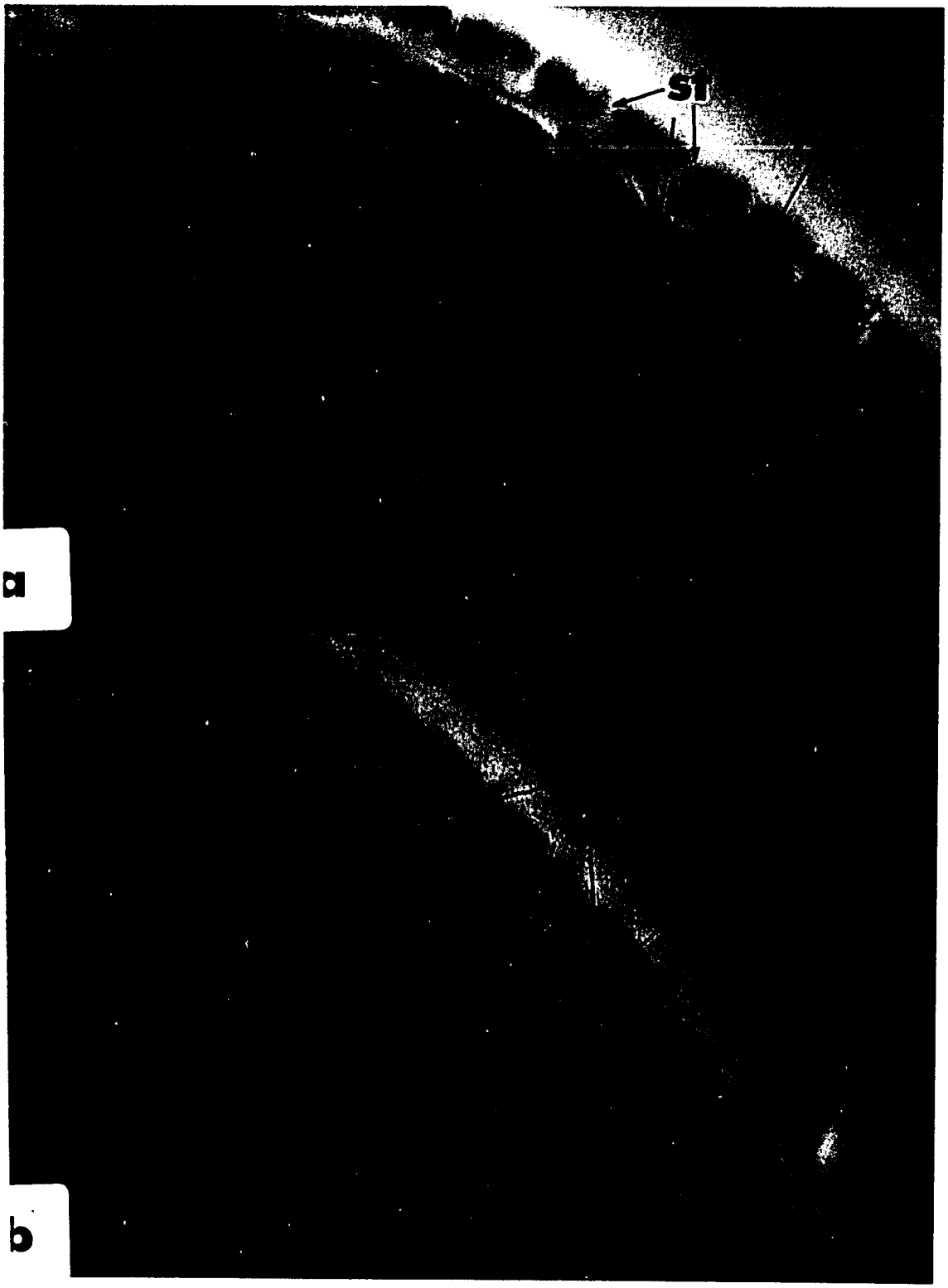

S1
a
b

Figure 57. UV epifluorescence of *P. epiphylla* capsule wall (inner and outer surface)(Zeiss set-up).

In water only. A comparison of the distribution of fluorescing (and non-fluorescing) compounds in the outer (OW) and inner (IW) layers of the capsule wall, demonstrating the very different chemical natures of these two capsule wall layers. The outer layer has nodular thickenings in the cell corners, but much greater expanses of cellulosic primary wall than the inner layer. The inner layer at bottom is a complete valve. On the edges one can see that the lines of abscission have no secondary thickenings; they are hence weaker and more likely to rip under pressure than the surrounding tissue. magnification: 360X.

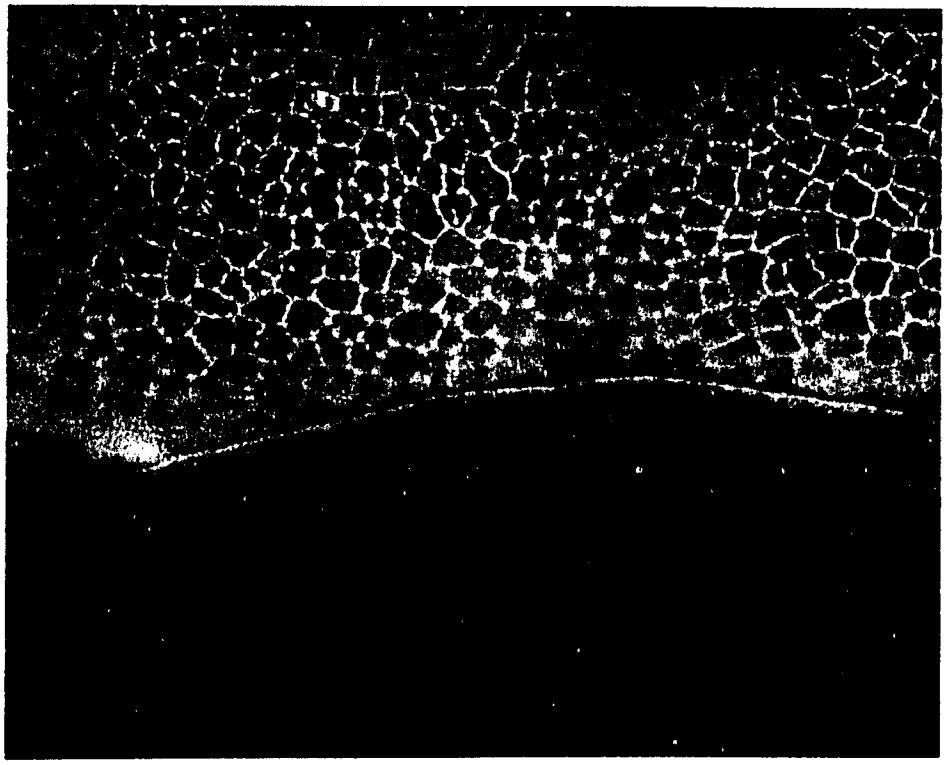


Figure 58. UV epifluorescence, *Lopholocolea heterophylla* gametophytic tissue (Zeiss setup).
This leafy liverwort gametophyte also contains abundant ferulic acid.

Figure 58a. In water, blue fluorescence is indicative of lignin and/or ferulic acid.
magnification: 360X.

Figure 58b. In ammonium hydroxide, all walls showing the green fluorescence characteristic of ferulic acid.
magnification: 360X.

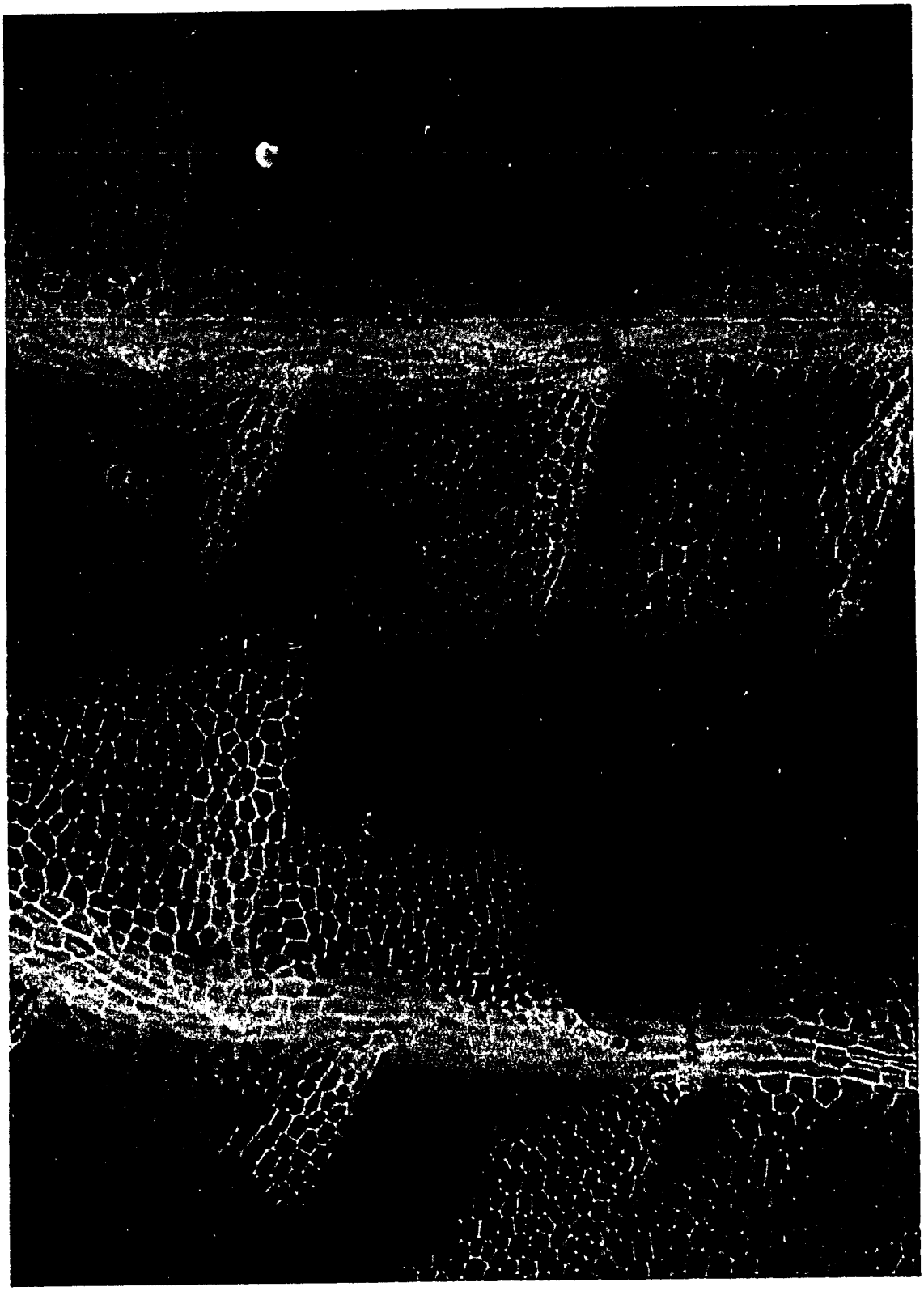
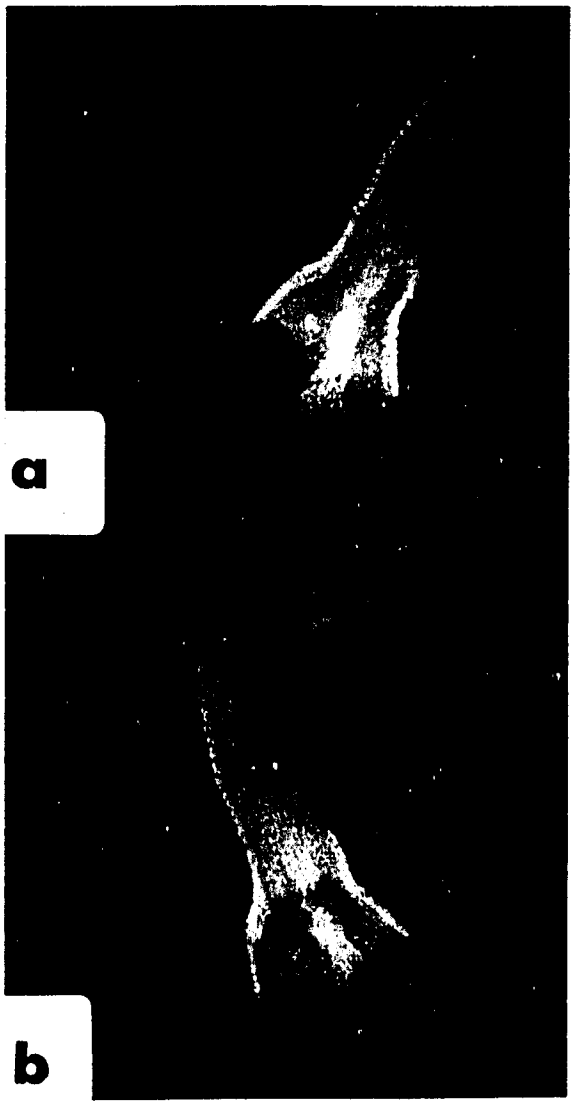


Figure 59. UV epifluorescence, *Thuidium delicatulum* gametophytic tissue (young leaves) (Zeiss setup).

Figure 59a. In water only. Note the green fluorescence in the leaf tip, while the middle is
white-blue, and the base is reddish. The green fluorescence under these conditions is not due to ferulic acid.
magnification: 360X.

Figure 59b. In ammonium hydroxide. No change can be detected with the application of ammonium hydroxide. From tip to base, the UV fluorescence changes from green, to blue-white, to the pink color of stem and primordial leaves. There is no obvious indication of ferulic acid present in this tissue. This is in accordance with the judgement of Schaffer (1985) on the same plant tissue.
magnification: 360X.


a
b

Appendix A: Histochemical stains: Recipes and Protocols for Use

Freeze-dried capsules of *Pellia epiphylla* were rehydrated and used whole unless otherwise specified. Higher plant tissues were used fresh and razor-cut unless otherwise noted.

IKI-H_2SO_4 (Jensen 1962)

1. IKI: 2g KI in 100 ml. of water, then dissolve 0.2 g of iodine in the KI solution
2. Place fresh sections in the IKI solution for 15 minutes or longer
3. Mount the section in IKI under a cover glass, add a drop of 65% H_2SO_4 at the side of the cover glass, and allow it to diffuse under the glass.

   Walls containing cellulose will stain dark blue; lignin will stain orange to yellow. As the H_2SO_4 reacts on the wall, the wall will swell, with the result taht even in lignified walls the cellulose may be seen. In the middle lamella, where the cellulose and lignin are in very close relation, a green color may be evident. After a relatively short time the H_2SO_4 will dissolve the cellulose, and the tissue will fall to pieces.

Phloroglucinol (Jensen 1962)

1. prepare freshly a saturated solution of phloroglucinol, in 20% HCl
2. Place on fresh or fixed tissue and cover with a cover slip.

   Lignin will appear red-violet. The preparation is not permanent.

Mäule reaction (Johansen 1940):

1. immerse sections in 1% neutral aqeuous potassium permanganate for 15-20 minutes
2. wash thoroughly with distilled water
3. immerse sections in 2% HCL
4. wash thoroughly with distilled water

5. add a few drops of either ammonium hydroxide or sodium bicarbonate

   Syringyl lignin will appear red-purple in color. Guaiacyl lignin will appear yellow to brown.

Toluidine Blue: aqueous 0.05%

1. place a few drops on section for 10-15 minutes (sections may be placed on a warming tray to facilitate penetration, especially with sections mounted in Spurrs)
2. wash thoroughly with distilled water

   Lignin appears blue-green. Cellulose appears purple.

Safranin O (modified from Jensen 1962)

1. immerse sections in .5% safranin in 50% ethanol for 15 minutes to 1 hour
2. wash thoroughly with 50% ethanol
3. differentiate with fast green

   Lignin will stain red. Primary cell walls and cytoplasm will stain green.

Ferric-Chloride (Gahan 1984)

Tissues: Unfixed, razor cut or cryostat sections of leaves, roots or stems of *Pinus* sp., *Tilia*, *Salix*, *Rubus*

Solution:Ferric chloride, 2%, in 95% ethanol

Method: 1. Stain for 5 min. in the ferric chloride solution

2. Rinse in 95% ethanol, hydrate and mount in Euparal or examine directly

Result: Phenols yield a green colour

Ferric Chloride-HCl for Tannins (Gahan 1984)

Solution: Ferric chloride, 1% in 0.1 N HCl

Method: React sections for 15 min.

Result: Tannin sites show blue-black colour.

Sudan IV (0.5% in 70% EtOH)

1. immerse sections for 15-20 minutes
2. rinse with 70% EtOH, then distilled water

Sudan Black (saturated solution in 70% EtOH)

1. immerse sections for 15-20 minutes
2. rinse with 70% EtOH, then distilled water

APPENDIX B:

Mass spectra of lignin pyrolysis breakdown products in *Pellia epiphylla* sporophytes

The following pyrolysis gas chromatograms (pyrograms) and mass spectra comprise the confirmation of the identities of the lignin pyrolysis breakdown products reported in the text. Samples of freeze-dried sporophytes containing primarily capsule wall and elaters (identified in the pyrograms as "minus setae and spores") were pyrolyzed at 700°C, and identified in gas chromatograms using the mass spectrometer as a detector. When necessary, peaks of suspected lignin breakdown products were compared to gas chromatograms and mass spectra of known lignins in the tropical hardwood *Drimys winteri*. Mass spectra of the major pyrolysis breakdown products present in *Pellia* sporophyte samples that are not characteristic of lignin are also presented in Figure C11. The NBS library matches for these compounds are given in the figure legend, as well as in the text.

Figure C1. RIC+Mass chromatograms: Phenol and guaiacol (*Pellia* wall+elaters).

Figure C2. RIC+Mass chromatograms: p-hydroxystyrene and 4-vinyl guaiacol (*Pellia* wall+elaters).

Figure C3. RIC+Mass chromatograms: Eugenol and isoeugenols (*Pellia* wall+elaters).

Figure C4. Mass spectrum: Trans-isoeugenol (a: *Pellia* wall+elaters, b:*Drimys winteri*).

Figure C5. MIDRIC+Mass chromatograms: Eugenol, cis & trans eugenols (a: *Pellia* wall+elaters, b: *Drimys winteri*).

Figure C6. RIC+Mass chromatograms: 2,6 dimethoxyphenol (2,6 DMP) (*Pellia* wall+elaters).

Figure C7. Mass spectra: 2,6 DMP (a: *Drimys winteri*, b: *Pellia* wall+elaters).

Figure C8. Mass spectra and NBS library matches for largest pyrolysis peaks uncharacteristic of lignin pyrolysis breakdown products (*Pellia epiphylla* whole sporangia).

Figure C1. RIC+Mass chromatograms: Phenol and guaiacol (*Pellia* wall+elaters). Phenol and guaiacol were found in both samples (only the wall+elater sample shown here). Phenol and guaiacol are typical lignin breakdown products, but could come from other sources as well.
(P on first line=phenol, G on second line=guaiacol)

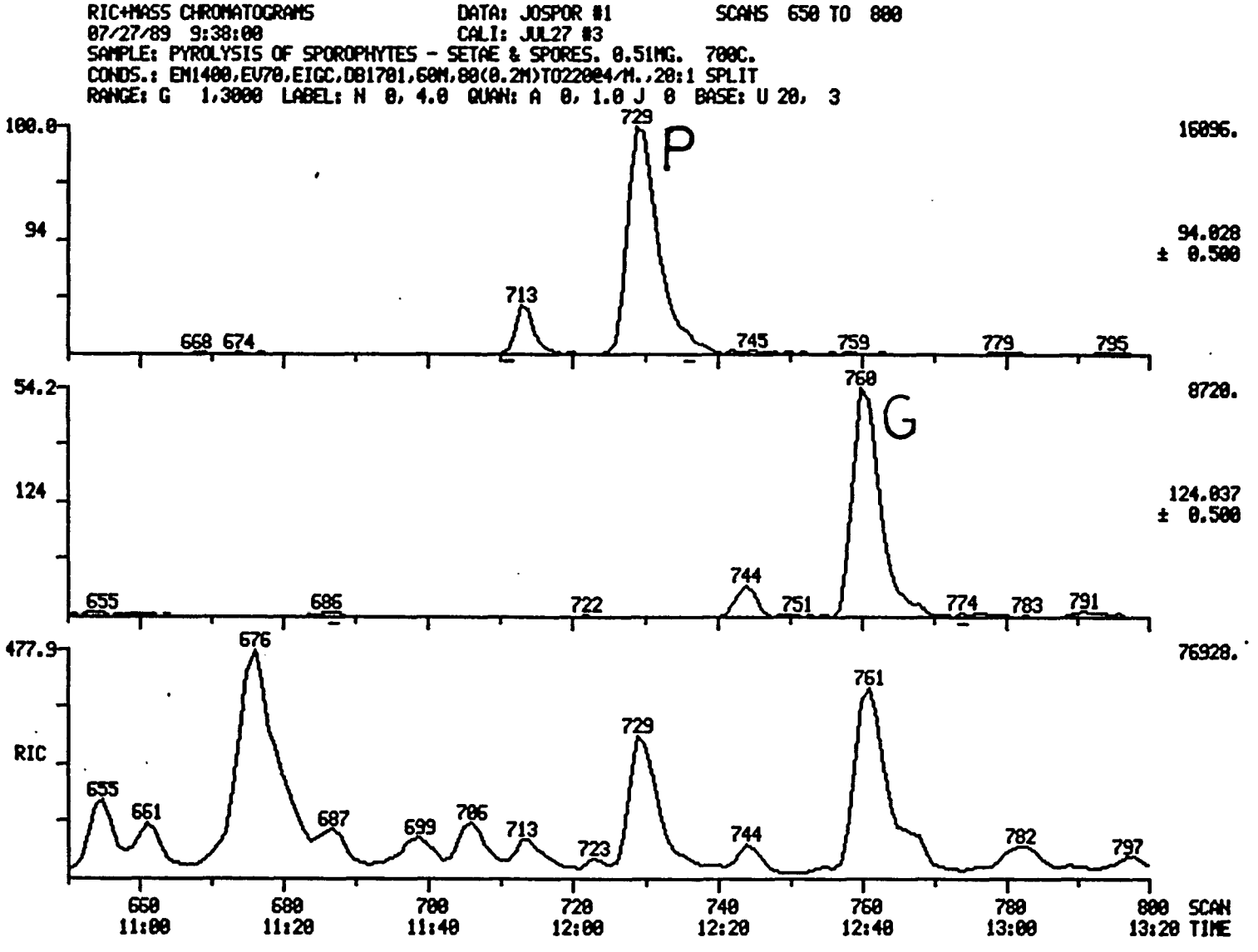

RIC+MASS CHROMATOGRAMS
DATA: JOSPOR #1
SCANS 650 TO 800
07/27/89 9:38:00
CALI: JUL27 #3
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT
RANGE: G 1,3000 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
100.0
94
729
P
16096.
94.028
± 0.500
713
668 674
745
759
779
795
54.2
124
760
G
8720.
124.037
± 0.500
744
655
686
722
751
774
783
791
477.9
RIC
676
761
729
76928.
655
661
687
699
705
713
723
744
782
797
650
11:00
680
11:20
700
11:40
720
12:00
740
12:20
760
12:40
780
13:00
800
13:20
SCAN
TIME

Figure C2. RIC+Mass chromatograms: *p*-hydroxystyrene and 4-vinyl guaiacol (*Pellia* wall+elaters). Both compounds are found in *Pellia* sporophyte tissue (H = p-hydroxystyrene on the first line, V= vinyl guaiacol on the second and third lines). The fourth line of the chromatogram is the total reconstructed ion count (RIC), which shows that the peak for p-hydroxystyrene (which, if from lignin, would come from p-hydroxyphenyl lignin units) is smaller than that of 4-vinyl guaiacol (which, if from lignin, would come from coniferyl alcohol units). This provides evidence to show that if lignin is present, it is not particularly rich in p-hydroxyphenyl units, as Bland et al. propose. The 4-vinyl guaiacol shows two peaks, indicative of two possible "isomers" being present, which is not usually seen in higher plant lignin.

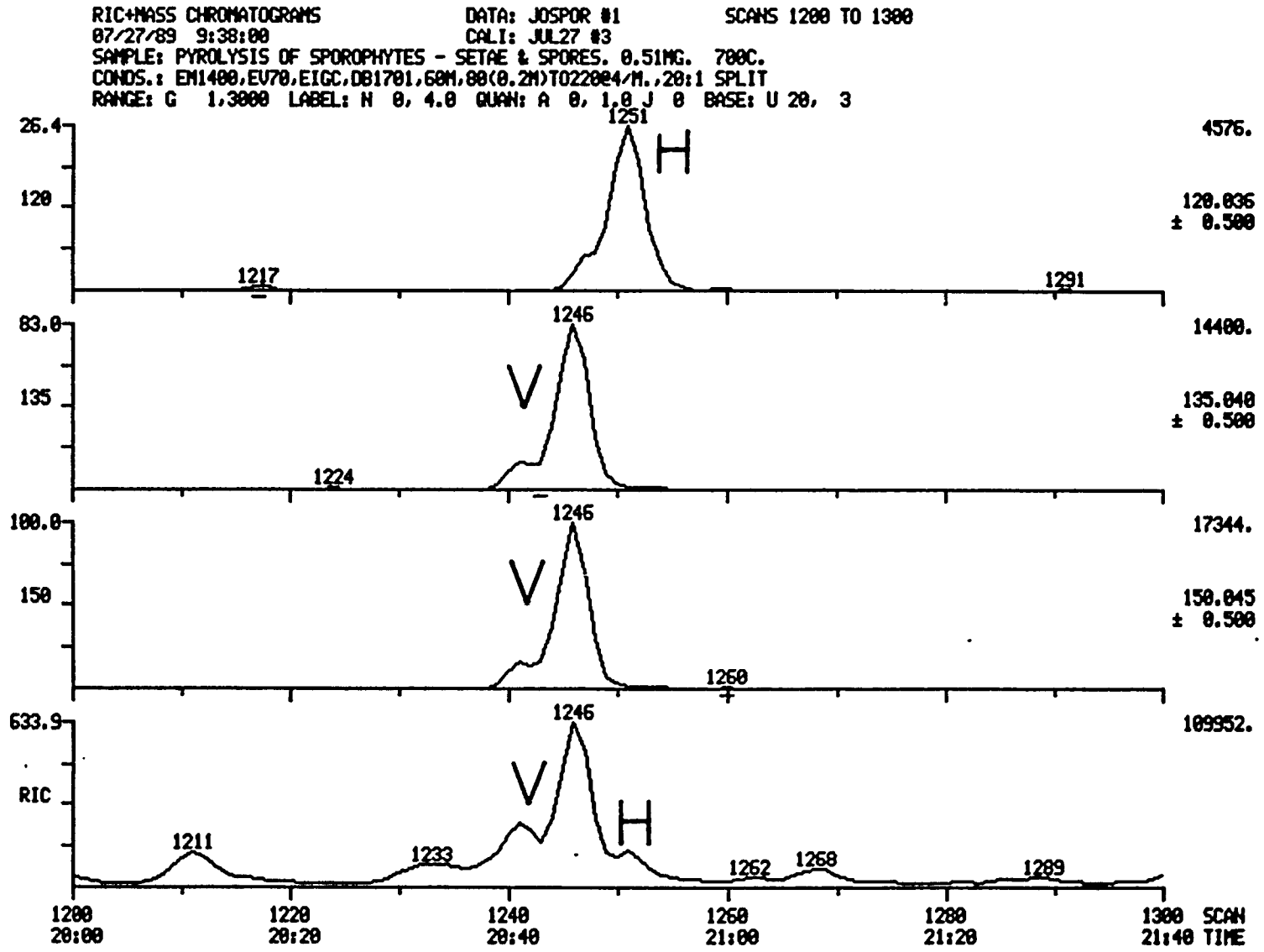

RIC+MASS CHROMATOGRAMS
DATA: JOSPOR #1
SCANS 1200 TO 1300
07/27/89 9:38:00
CALI: JUL27 #3
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)T0220@4/M.,20:1 SPLIT
RANGE: G 1,3000 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
26.4
120
1251
H
4576.
120.036
± 0.500
1217
1291
83.0
135
1246
V
14400.
135.040
± 0.500
1224
100.0
150
1246
V
17344.
150.045
± 0.500
1260
633.9
RIC
1246
V
H
109352.
1211
1233
1262
1268
1289
1200
20:00
1220
20:20
1240
20:40
1260
21:00
1280
21:20
1300 SCAN
21:40 TIME

Figure C3. RIC+Mass chromatograms: Eugenol and isoeugenols (*Pellia* wall+elaters). Eugenol, cis-isoeugenol and trans-isoeugenol occur at 1294, 1406 and 1510. This figure shows a peak at 1506, close enough to possibly be trans-isoeugenol. As the combined ion chromatogram shows, the peak is very small (348 counts compared to 109,952). For the mass spectrum, see Figure C7.

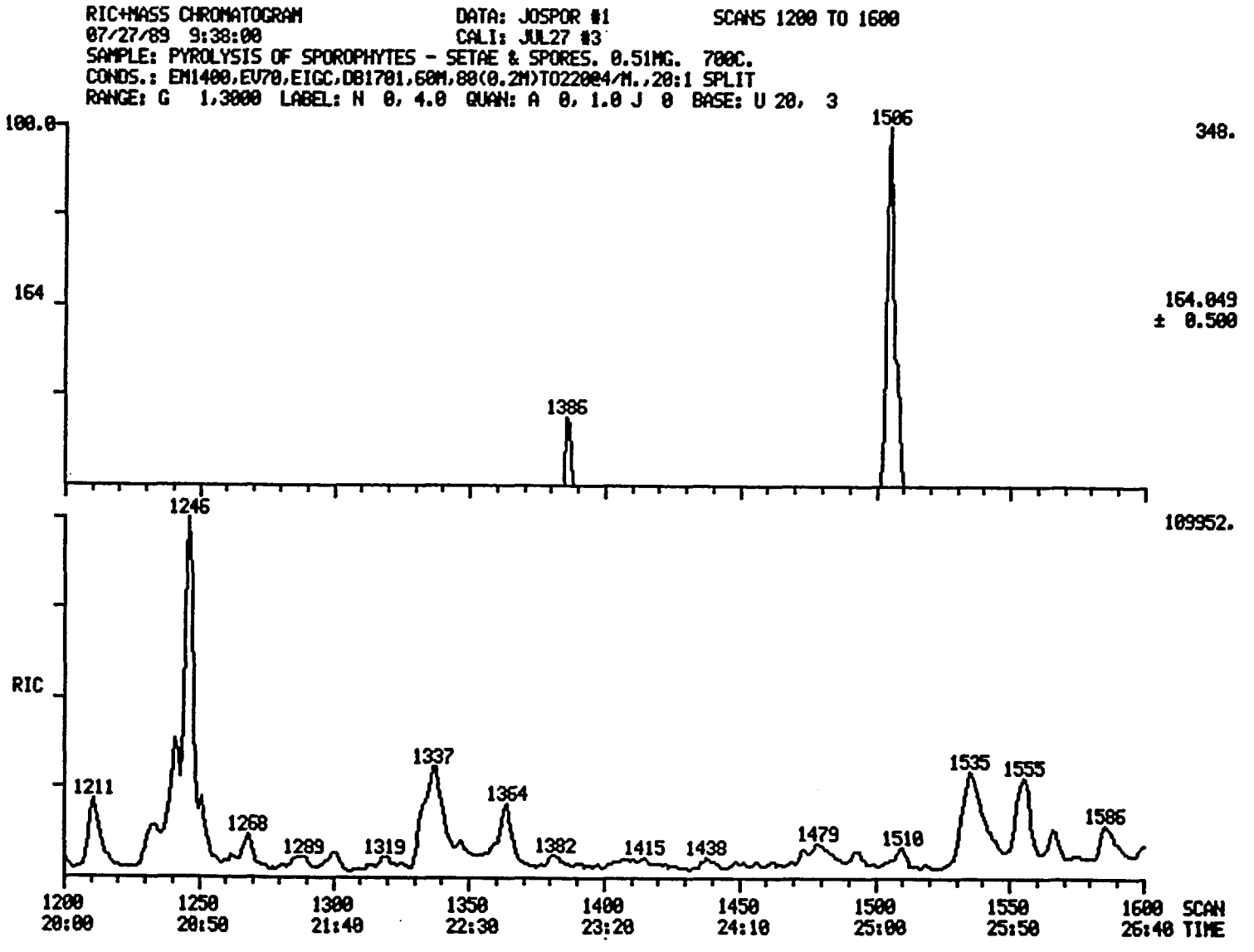
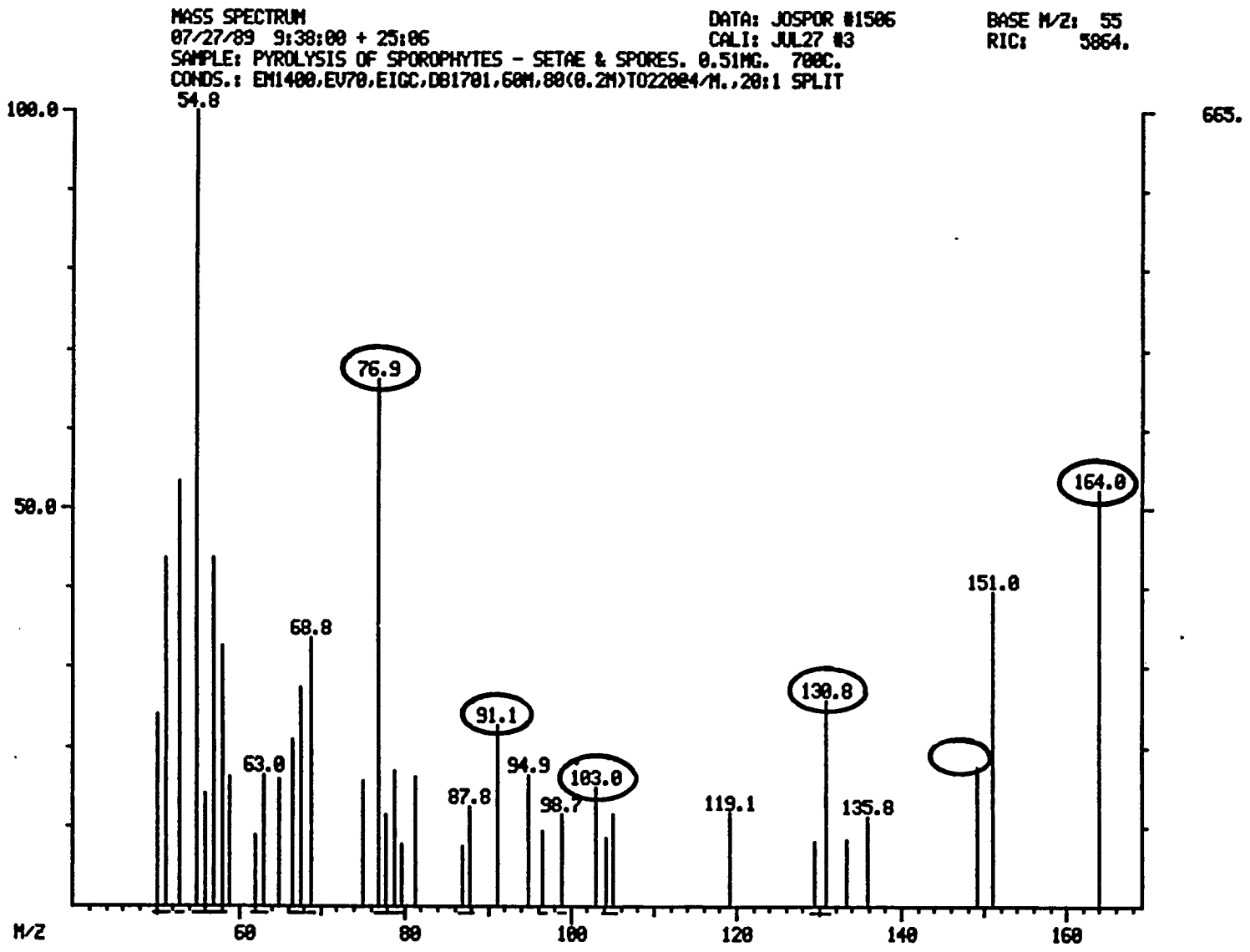

RIC+MASS CHROMATOGRAM
DATA: JOSPOR #1
SCANS 1200 TO 1600
07/27/89 9:38:00
CALI: JUL27 #3
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO22004/M.,20:1 SPLIT
RANGE: G 1,3000 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
100.0
164
348.
164.049
± 0.500
1506
1386
1246
109952.
RIC
1211
1268
1289
1319
1337
1364
1382
1415
1438
1479
1510
1535
1555
1586
1200
20:00
1250
20:50
1300
21:40
1350
22:30
1400
23:20
1450
24:10
1500
25:00
1550
25:50
1600
26:40
SCAN
TIME

Figure C4. Mass spectrum: Trans-isoeugenol (a: *Pellia* wall+elaters, b: *Drimys winteri*). The circled peaks in (a) correspond to the peaks in authentic trans-isoeugenol in *Drimys winteri* (b). Additional peaks in the *Pellia* sample indicate impurities, however, the major peaks for trans-isoeugenol are present, and indicate small amounts of trans-isoeugenol.

A


MASS SPECTRUM
07/27/89 9:38:00 + 25:06
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT
DATA: JOSPOR #1506
CALI: JUL27 #3
BASE M/Z: 55
RIC: 5864.
665.
100.0
50.0
M/Z
54.8
76.9
68.8
63.0
91.1
94.9
87.8
98.7
103.0
119.1
130.8
135.8
151.0
164.0
60
80
100
120
140
160

B

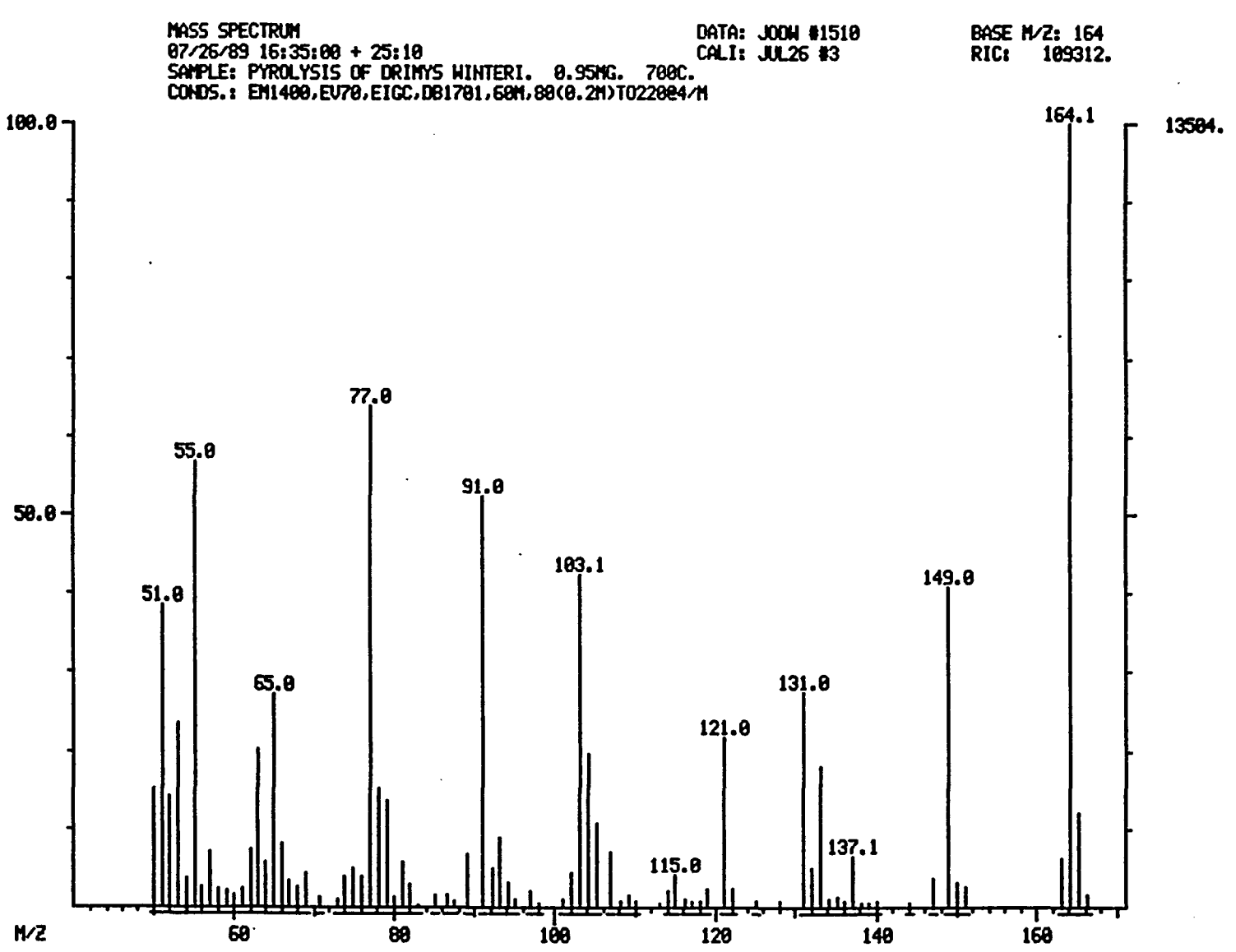

MASS SPECTRUM
07/26/89 16:35:00 + 25:10
SAMPLE: PYROLYSIS OF DRIMYS WINTERI. 0.95MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M
DATA: JOOW #1510
CALI: JUL26 #3
BASE M/Z: 164
RIC: 109312.
100.0
50.0
M/Z
60
80
100
120
140
160
164.1
13504.
77.0
55.0
91.0
103.1
51.0
149.0
65.0
131.0
121.0
115.0
137.1

Figure C5. MIDRIC+Mass chromatograms: Eugenol, cis & trans eugenols (*Pellia* wall+elaters). In this repeat scan for the eugenols, multiple ion detection (MID) was used in the ranges: 148-152, 162-166, 177-181, and 192-196. At the conditions used here, one can expect to find eugenol, cis-isoeugenol and trans-isoeugenol at approximately 1141, 1238 and 1329 respectively . All three peaks can be detected in the *Pellia* tissue at 164 (a, second line), while only trans-isoeugenol is a strong enough peak to be detected at 149.

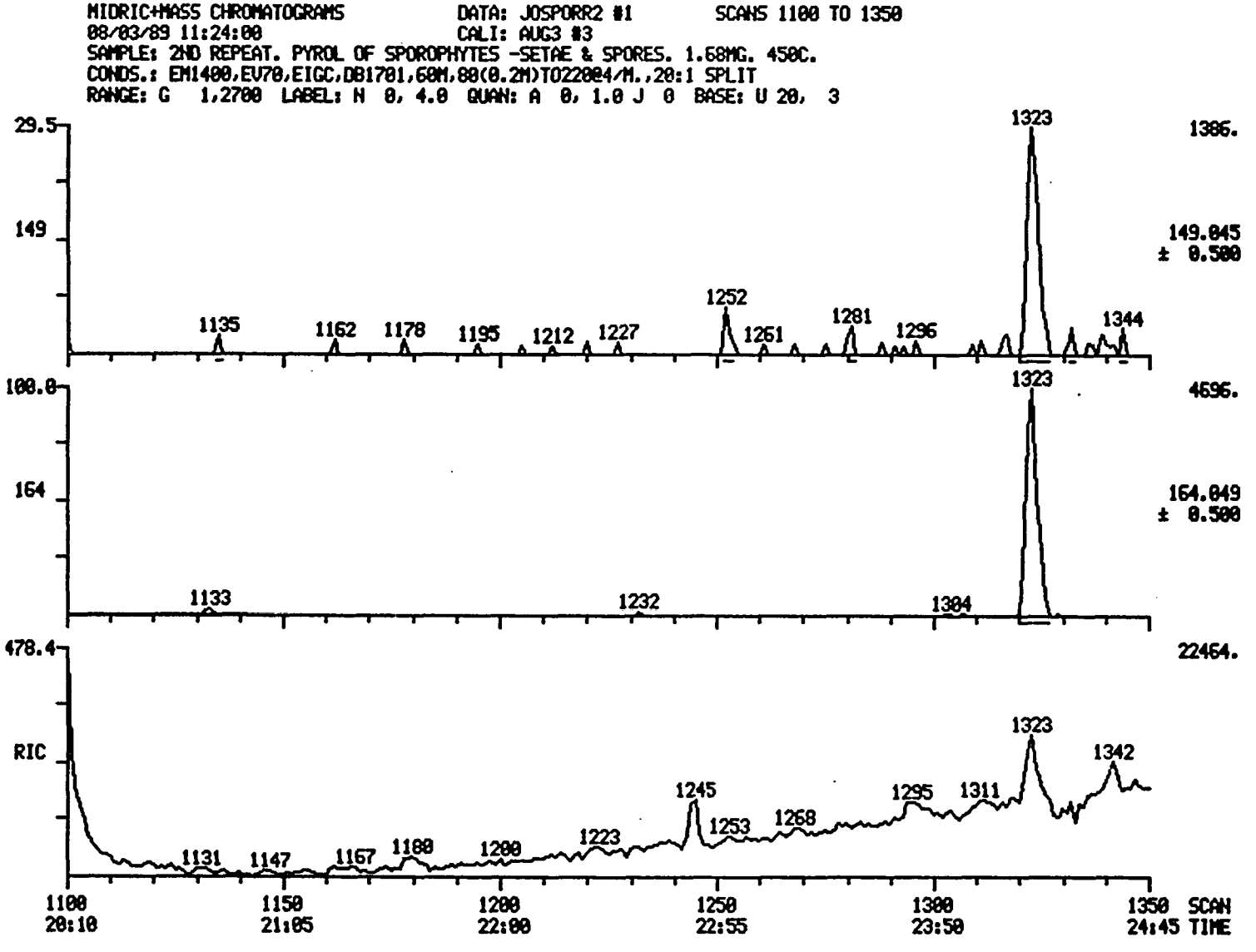

MIDRIC+MASS CHROMATOGRAMS
DATA: JOSPORR2 #1
SCANS 1100 TO 1350
08/03/89 11:24:00
CALI: AUG3 #3
SAMPLE: 2ND REPEAT. PYROL OF SPOROPHYTES -SETAE & SPORES. 1.68MG. 450C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO22004/M.,20:1 SPLIT
RANGE: G 1,2700 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
29.5
149
1386.
149.045
± 0.500
1323
1135
1162
1178
1195
1212
1227
1252
1261
1281
1296
1344
100.0
164
4696.
164.049
± 0.500
1323
1133
1232
1304
478.4
RIC
22464.
1323
1342
1245
1253
1268
1295
1311
1131
1147
1167
1180
1200
1223
1100
20:10
1150
21:05
1200
22:00
1250
22:55
1300
23:50
1350 SCAN
24:45 TIME

Figure C6. RIC+Mass chromatograms: 2,6 dimethoxyphenol (2,6 DMP) (*Pellia* wall+elaters). The peak marked D at 1352 is in approximately the right place for 2,6 dimethoxyphenol (1364). It is detected in the wall+elater sample shown here, but was not detected in the whole sprophyte sample (not shown). The second line shows the total ion chromatogram. Ion 1352 of line one is only 146 counts of the 34240 total. If it is 2,6 dimethoxyphenol, it is there in only very slight amounts.

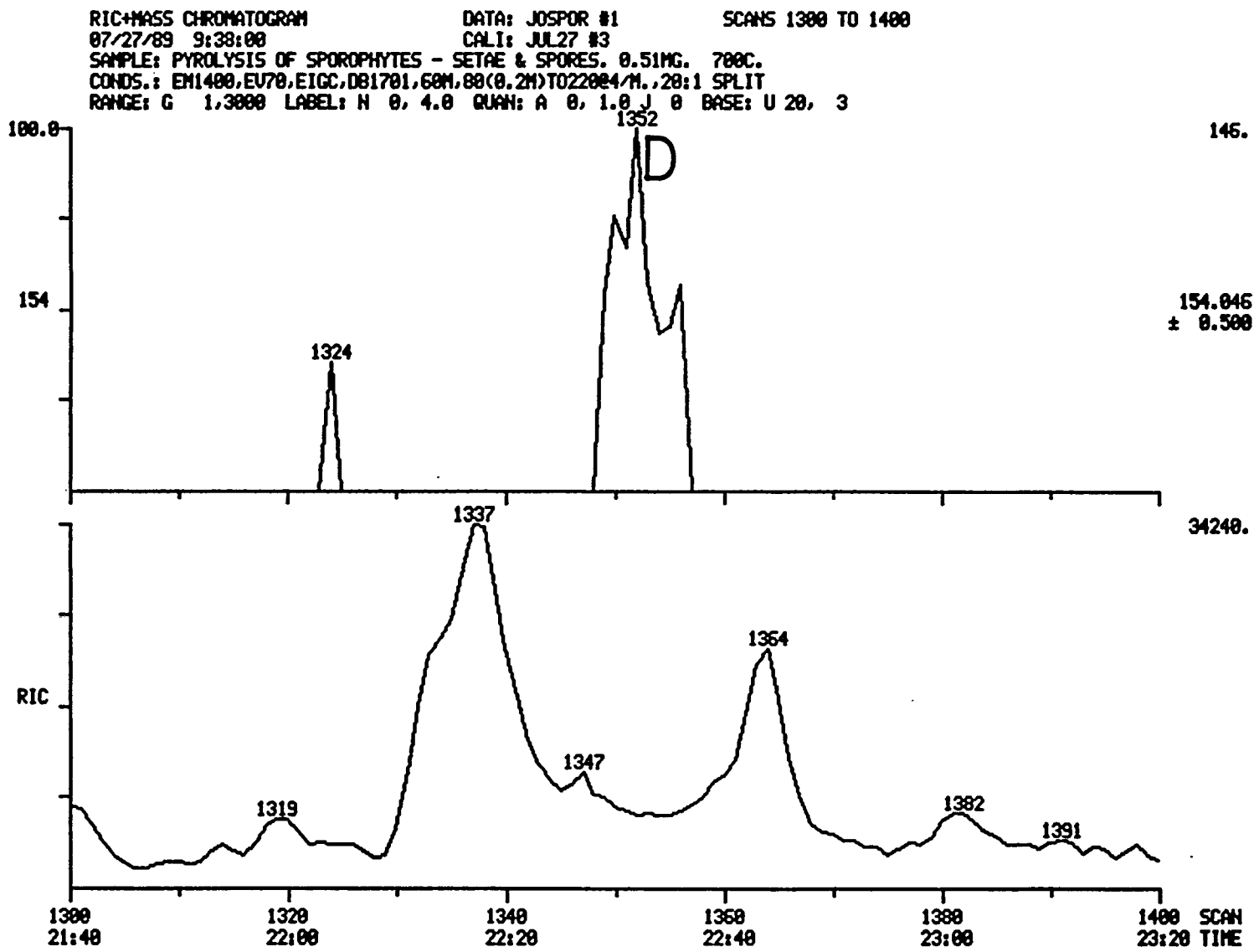
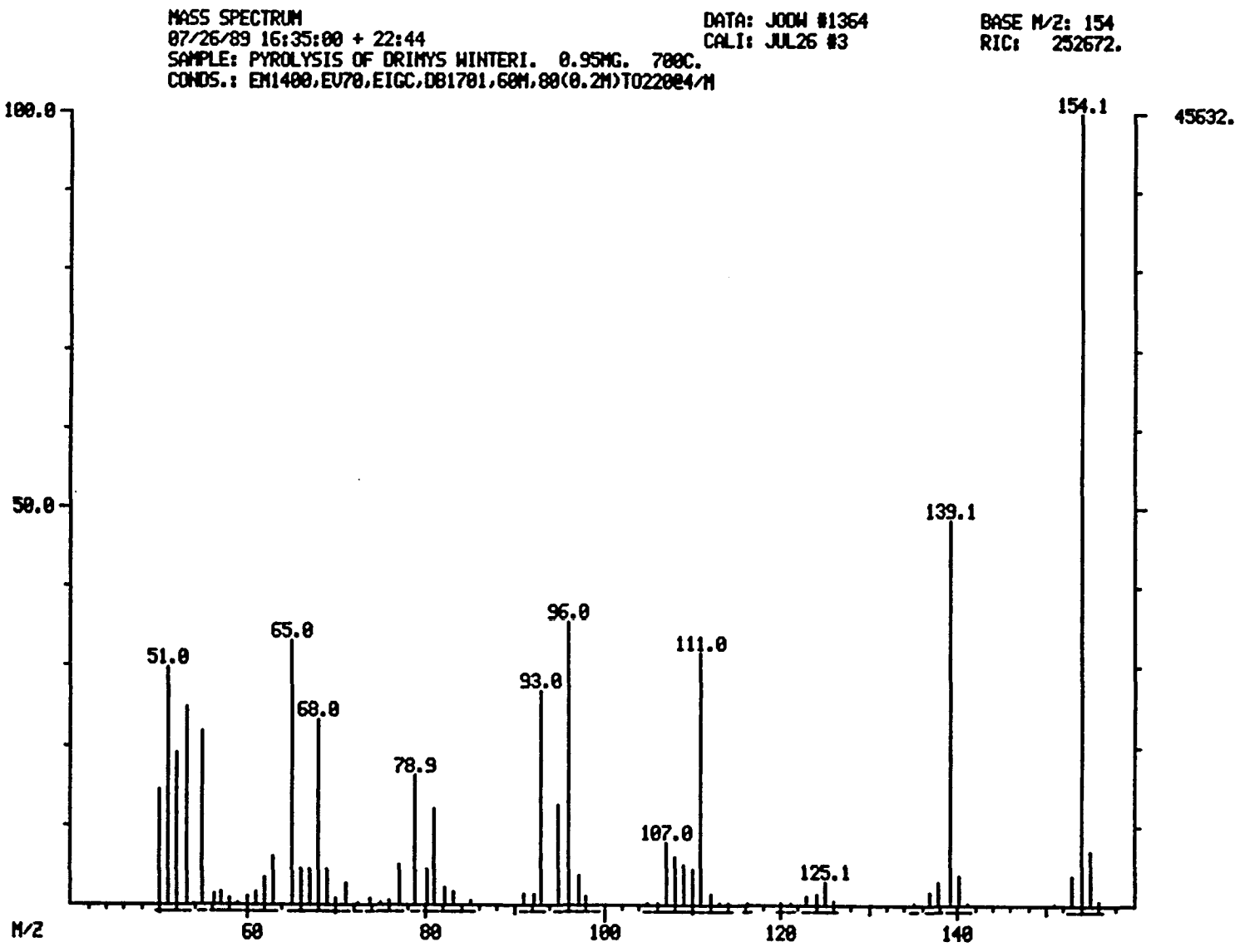

RIC+MASS CHROMATOGRAM
DATA: JOSPOR #1
SCANS 1300 TO 1400
07/27/89 9:38:00
CALI: JUL27 #3
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT
RANGE: G 1,3000 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
100.0
154
1352
D
1324
146.
154.046
± 0.500
RIC
1337
1364
1347
1319
1382
1391
34240.
1300
21:40
1320
22:00
1340
22:20
1360
22:40
1380
23:00
1400
23:20
SCAN
TIME

Figure C7. Mass spectra: 2,6 DMP (a: *Drimys winteri*, b: *Pellia* wall+elaters). Authentic 2,6 dimethoxyphenol from *Drimys winteri* has major peaks at 154, 139, 111, 96, 65 and 51. Of these, only the largest peak 154 can be observed in the spectrum of *Pellia* wall+elaters in a. It is impossible to definitively identify this product from this data. If it is 2,6 DMP, it is very weak.

A


MASS SPECTRUM
07/26/89 16:35:00 + 22:44
SAMPLE: PYROLYSIS OF DRIMYS WINTERI. 0.95MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M
DATA: JOOW #1364
CALI: JUL26 #3
BASE M/Z: 154
RIC: 252672.
100.0
50.0
M/Z
60
80
100
120
140
154.1
45632.
139.1
96.0
65.0
111.0
51.0
93.0
68.0
78.9
107.0
125.1

B

MASS SPECTRUM
07/27/89 9:38:00 + 22:34
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)T022004/M.,20:1 SPLIT

DATA: JOSPOR #1354
CALI: JUL27 #3

BASE M/Z: 51
RIC: 6936.

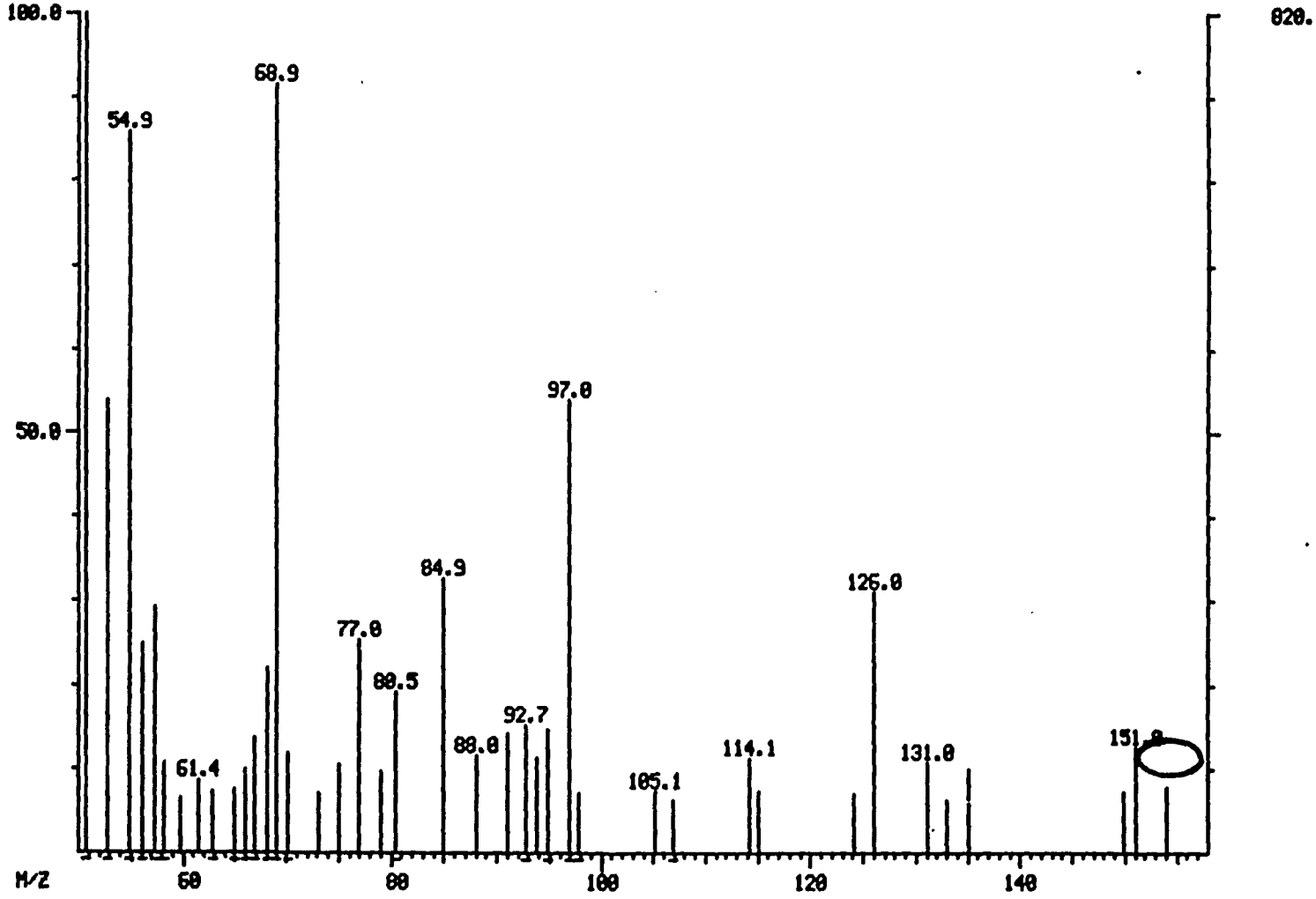


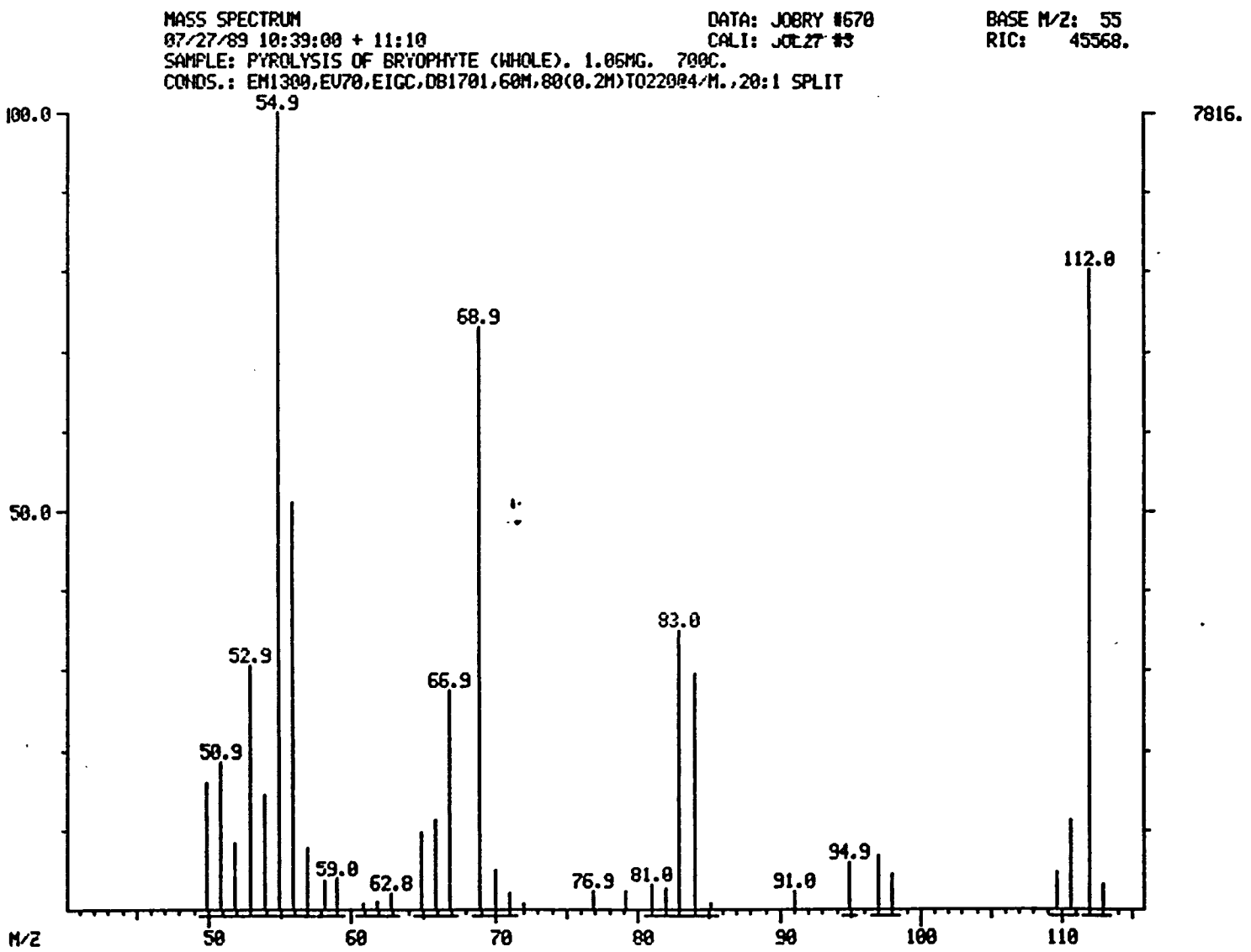
Figure C8. Mass spectra and NBS library matches for largest pyrolysis peaks uncharacteristic of lignin pyrolysis breakdown products (*Pellia epiphylla* whole sporangia).

	number*	MW	scan #	NBS Library match
Figure C11a.	1	112	670	2-hydroxy-3-methyl-2-cyclopenten-1-one
Figure C11b.	2	109	965	1-hepten-3-ol
Figure C11c.	3	117	1364	1S 1H-Indole
Figure C11d.	4	179	1906	cyclododecanol
Figure C11e.	5	194	2015	1,2,3,4-cyclopentanetetrol
Figure C11f.	6	256	2385	hexadecanoic acid
Figure C11g.	7	150	2476	5-octadecene

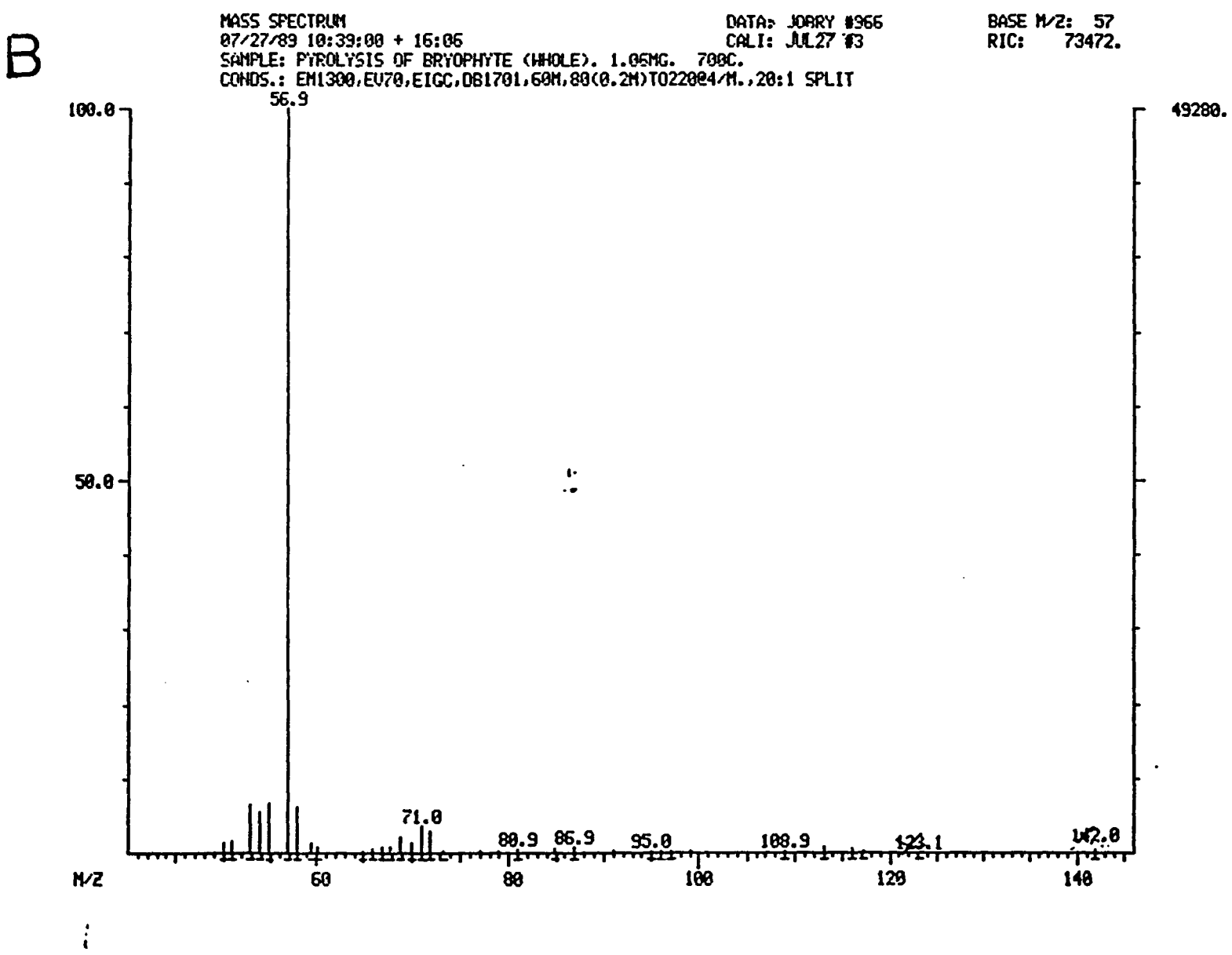
* = Number identifies the peak in Figure 48 of text.

NBS matches are not definitive. Of great interest here is number 5, scan #2015, because it is found in abundance in the wall+elater sample, and very much less is found in the whole sporophyte sample (see Figure 48). It is possible that this may be one of the compounds selectively deposited in the secondary cell wall thickenings.

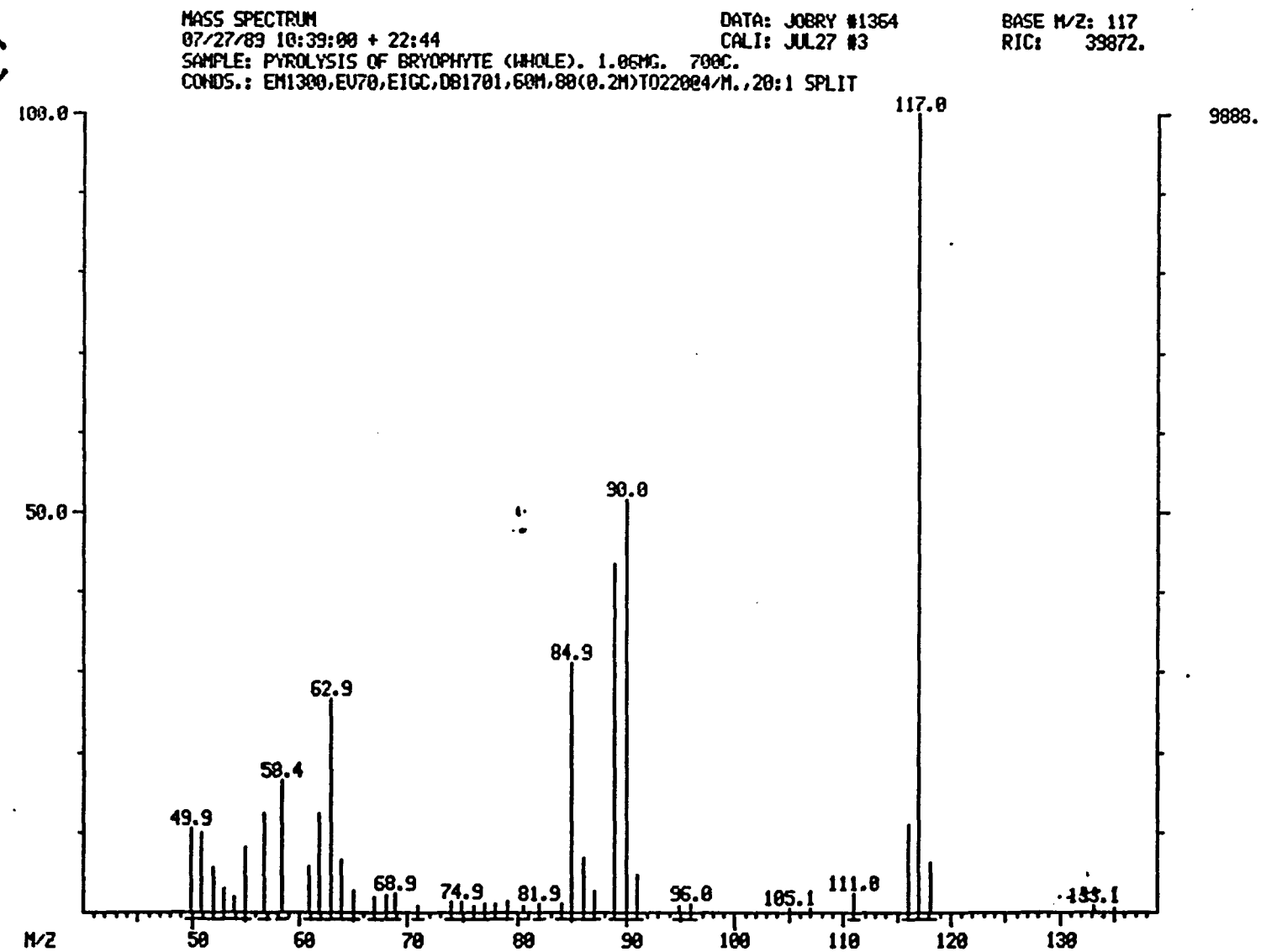
A


MASS SPECTRUM
07/27/89 10:39:00 + 11:10
SAMPLE: PYROLYSIS OF BRYOPHYTE (WHOLE). 1.06MG. 700C.
CONDS.: EM1300,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT
DATA: JOBRY #670
CALI: JOL27 #3
BASE M/Z: 55
RIC: 45568.
100.0
50.0
7816.
M/Z
50
60
70
80
90
100
110
54.9
68.9
112.0
83.0
52.9
66.9
50.9
59.0
62.8
76.9
81.0
91.0
94.9

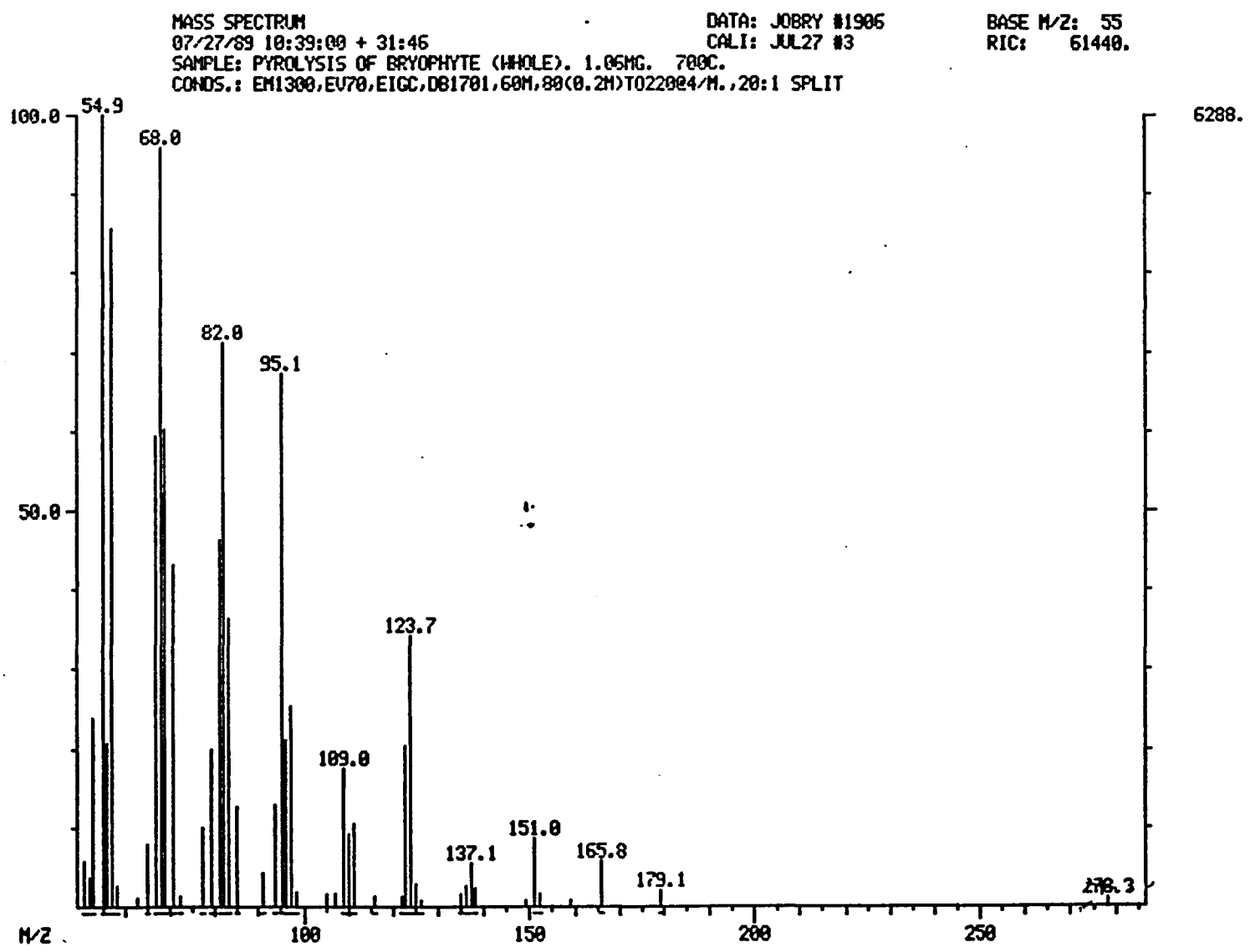
B


MASS SPECTRUM
07/27/89 10:39:00 + 16:06
SAMPLE: PYROLYSIS OF BRYOPHYTE (WHOLE). 1.05MG. 700C.
CONDS.: EM1300,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT
DATA: JOBRY #966
CALI: JUL27 #3
BASE M/Z: 57
RIC: 73472.
100.0
50.0
49280.
56.9
71.0
80.9
86.9
95.0
108.9
123.1
142.0
M/Z
60
80
100
120
140

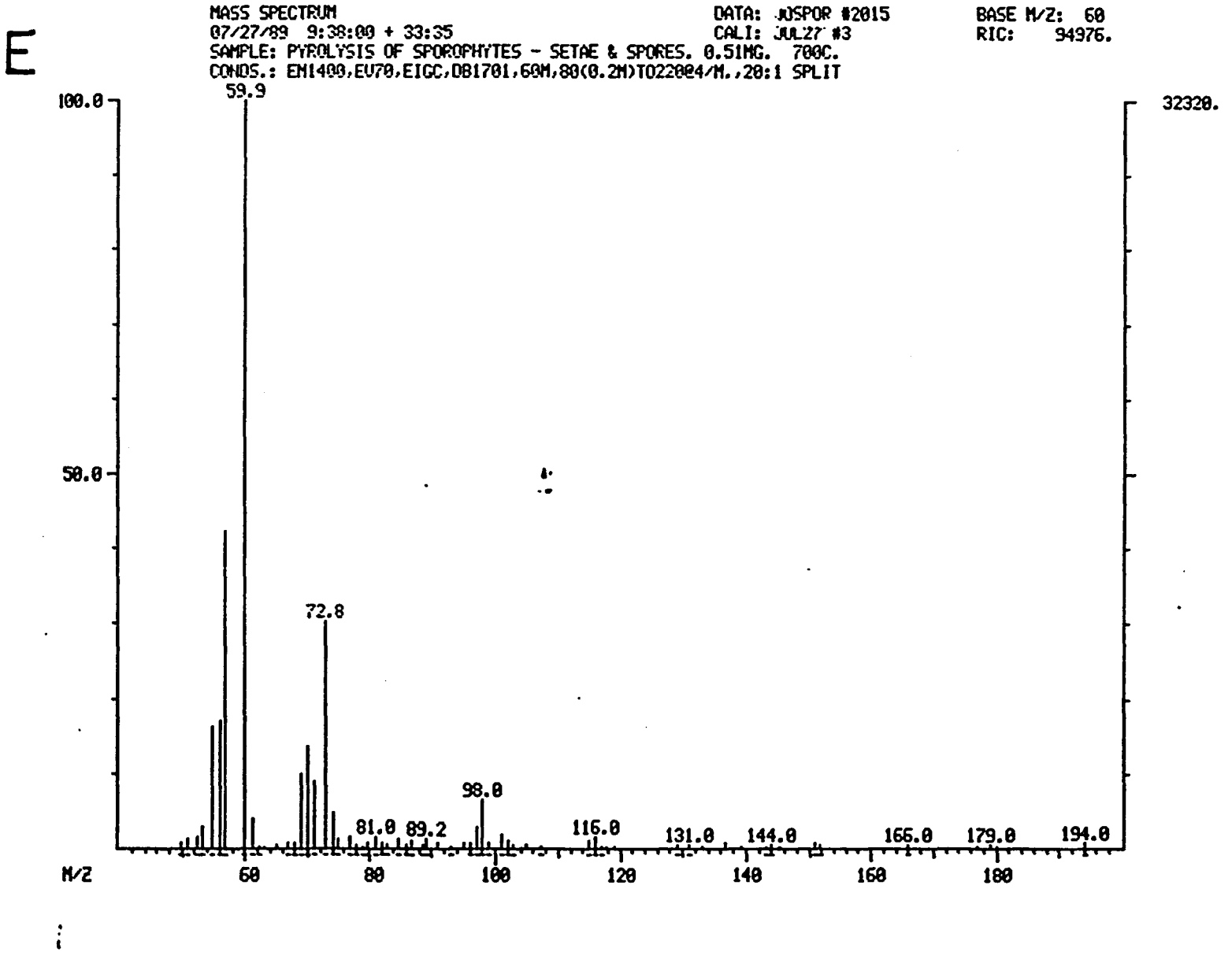
C


MASS SPECTRUM
07/27/89 10:39:00 + 22:44
SAMPLE: PYROLYSIS OF BRYOPHYTE (WHOLE). 1.06MG. 700C.
CONDS.: EM1300,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT
DATA: JOBRY #1364
CALI: JUL27 #3
BASE M/Z: 117
RIC: 39872.
100.0
50.0
M/Z
9888.
117.0
90.0
84.9
62.9
58.4
49.9
68.9
74.9
81.9
96.0
105.1
111.0
133.1
50
60
70
80
90
100
110
120
130

D


MASS SPECTRUM
07/27/89 10:39:00 + 31:46
SAMPLE: PYROLYSIS OF BRYOPHYTE (WHOLE). 1.06MG. 700C.
CONDS.: EM1300,EV70,EIGC,DB1701,60M,80(0.2M)T022004/M.,20:1 SPLIT
DATA: JOBRY #1906
CALI: JUL27 #3
BASE M/Z: 55
RIC: 61440.
100.0
50.0
6288.
54.9
68.0
82.0
95.1
123.7
109.0
137.1
151.0
165.8
179.1
M/Z
100
150
200
250

E

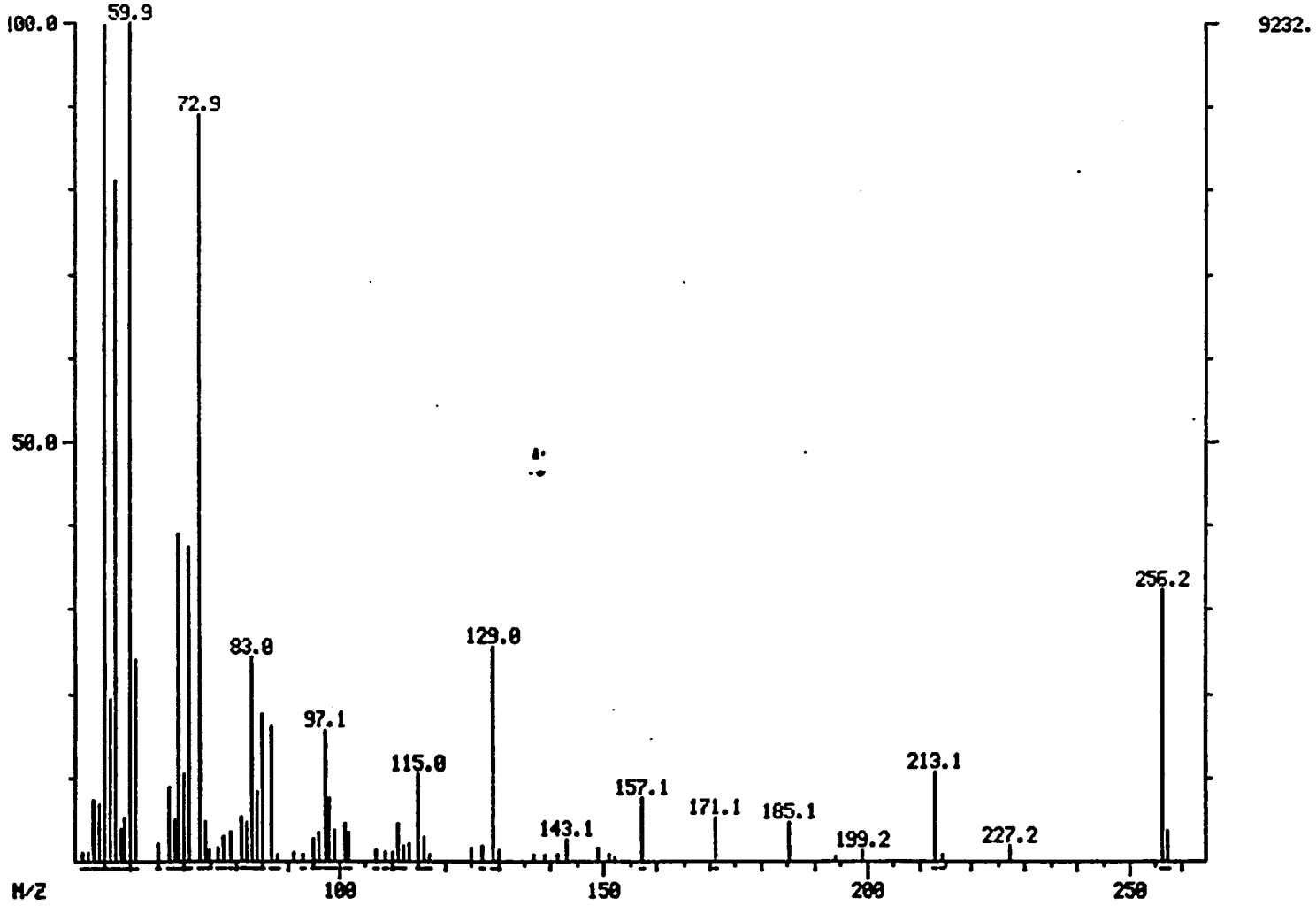

MASS SPECTRUM
07/27/89 9:38:00 + 33:35
SAMPLE: PYROLYSIS OF SPOROPHYTES - SETAE & SPORES. 0.51MG. 700C.
CONDS.: EM1400,EV70,EIGC,DB1701,60M,80(0.2M)T022004/M.,20:1 SPLIT
DATA: JOSPOR #2015
CALI: JUL27 #3
BASE M/Z: 60
RIC: 94976.
100.0
50.0
32320.
59.9
72.8
98.0
81.0
89.2
116.0
131.0
144.0
166.0
179.0
194.0
M/Z
60
80
100
120
140
160
180

F

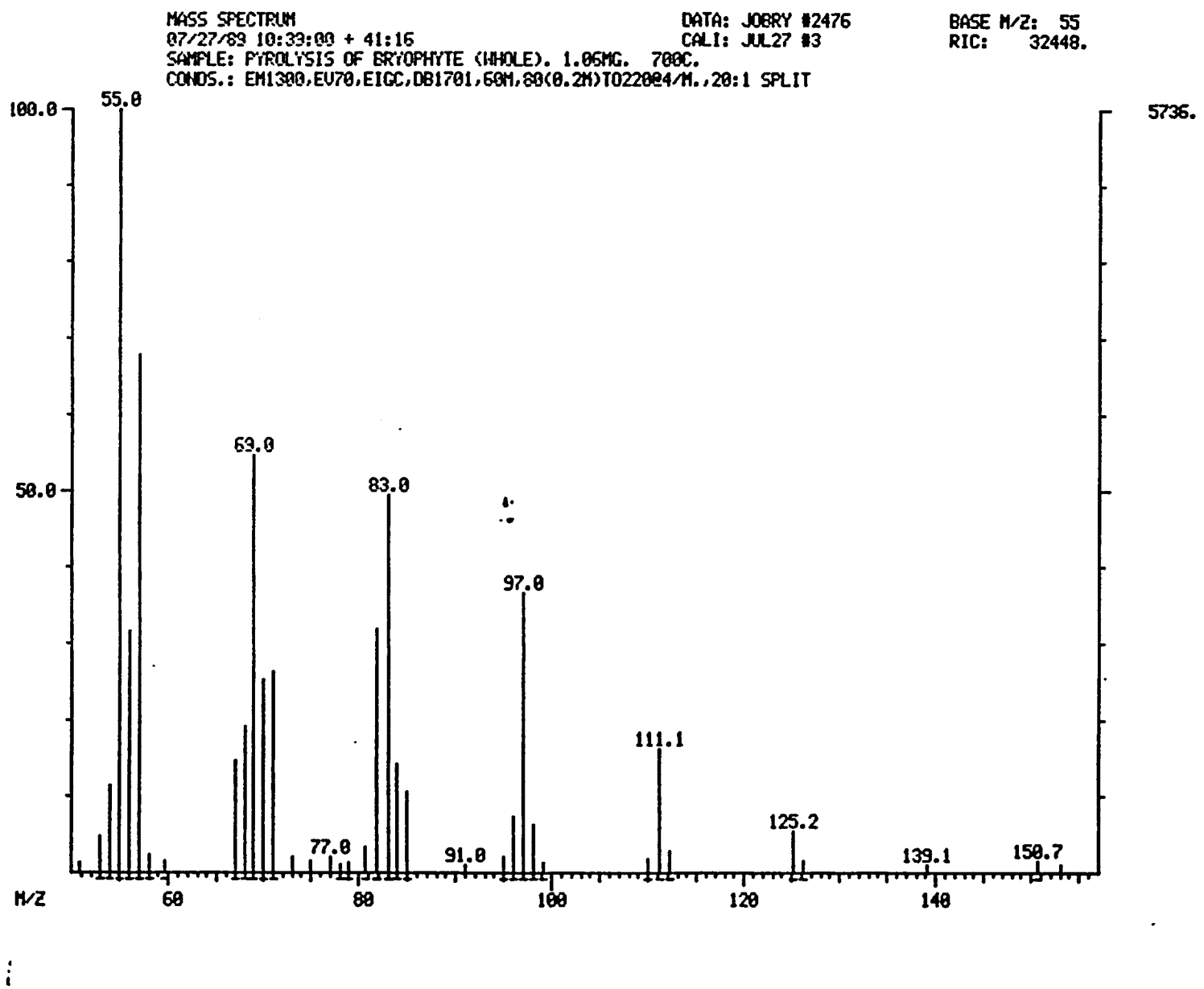
MASS SPECTRUM
07/27/89 10:33:00 + 39:45
SAMPLE: PYROLYSIS OF BRYOPHYTE (WHOLE). 1.06MG. 700C.
CONDS.: EM1300,EV70,EIGC,DB1701,60M,80(0.2M)TO220@4/M.,20:1 SPLIT

DATA: JOBRY #2385
CALI: JUL27 #3

BASE M/Z: 60
RIC: 74624.



G


MASS SPECTRUM
07/27/89 10:33:00 + 41:16
SAMPLE: PYROLYSIS OF BRYOPHYTE (WHOLE). 1.06MG. 700C.
CONDS.: EM1300,EV70,EIGC,DB1701,60M,80(0.2M)TO22004/M.,20:1 SPLIT
DATA: JOBRY #2476
CALI: JUL27 #3
BASE M/Z: 55
RIC: 32448.
100.0
50.0
5736.
55.0
69.0
83.0
97.0
111.1
125.2
77.0
91.0
139.1
150.7
M/Z
60
80
100
120
140

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