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ABSTRACT

Aspects of the structure/function relationships in leaves of Lolium temulentum L. were examined during their growth, ageing and senescence.

The first part of the thesis deals with the blade and sheath and demonstrates the presence of interactions within leaves (between blade and sheath) and between leaves during their extension growth. A short biochemical study of aspects of blade and sheath ageing and senescence of attached 4th leaves examined changes in fresh and dry weight, total chlorophyll, soluble protein, and nucleic acids (both quantitative and qualitative by polyacrylamide gel electrophoresis). The overall patterns of change were similar to those previously described for artificially induced grass leaf senescence but differences were found between the two organs. It was concluded that the blade was not a good model for sheath or leaf senescence. A study of the vasculature of the leaf - with particular reference to metabolite transport during ageing and senescence - revealed the presence of tyloses in the protoxylem vessels/lacunae at the blade/sheath junction of senescing leaves.

The second part of the thesis deals with the membranous ligules of L. temulentum, L. perenne L., L. multiflorum Lam., L. x hybridum Hausskn., and Festuca pratensis Huds. The structure and ultrastructure of all appeared to be the same and had the appearance of a glandular organ. Using a number of cytochemical procedures in L. temulentum, catalase, cytochrome c oxidase and succinate dehydrogenase activities were identified, along with the apparent secretion of a glycoprotein-like material by the cells of the adaxial epidermis. Aspects of the endomembrane system of cells of the latter tissue and initiation and early development of the ligule of L. temulentum were also studied. The structure/function relationships within these ligules are discussed in terms of a possible function of the membranous grass ligule. Aspects of ligule and root cap biology in L. temulentum were compared.

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Structure/Function Relationships in the Graminaceous Leaf

by

NIGEL JOHN CHAFFEY, B.Sc. (Wales)

A thesis submitted to the University of Durham for the degree of Doctor of Philosophy comprised of work carried out in the Department of Botany, University of Durham.

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Thesis
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DECLARATION

This is to certify that none of the material offered has been submitted by me for a degree in this or any other university.

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Date 13/XII/83

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ABBREVIATIONS USED IN THE PLATES, TABLES AND FIGURE 3.4

abax, ab	abaxial epidermis/epidermal cell
adax, ad	adaxial epidermis/epidermal cell
amy	amyloplast
Bl, bl	blade
cc	colourless cell
cer	cisternal endoplasmic reticulum
ci	cytoplasmic island
cl	chloroplast
cr	crista
cut	cuticle
cv	coated vesicle
cy	crystalline inclusion
cyto	cytoplasm
d	dictyosome
dc	dense chromatin
dic	diffuse chromatin
dv	dictyosome-derived vesicle
exp	extracellular product
ext	exterior
fib	fibrillar material
gn	granule
gr	granum
i	invagination
inm	inner nuclear membrane
is	inner (mestome) bundle sheath
L, lig	ligule
lac	protoxylem lacuna
lc	long cell
lf	leaf
mb	microbody
meso	mesophyll tissue/cell
mf	microfibrillar component of cell wall
ml	middle lamella
mt	mitochondrion
mx	metaxylem vessel

n	nucleus
nl	nucleolus
ol	oleosome
onm	outer nuclear membrane
os	osmiophilic globule
pap	papillate cell
pd	plasmodesma
peri	periplasmic space
ph	phloem region
pl	plasmalemma
pmb	paramural body
po	polysome
pr	peripheral reticulum
ps	parenchyma (outer) bundle sheath
pt	plastid
px	protoxylem vessel/region
(r)er	(rough) endoplasmic reticulum
ret	reticulate inner layer of cuticle
scl	sclerenchyma bundle
Sh, sh	sheath
st	starch; stoma
sv	secretory vesicle
t	tonoplast
ter	tubular endoplasmic reticulum
tr	transcellular strand
ty	tylose
v	vesicle
vac	vacuole
vb	vascular bundle
w	cell wall
x	'xylem element'
xy	xylem region

ABBREVIATIONS USED IN THE TEXT, FIGURES AND LEGENDS

A	absorbance; amp
a	tension upon ligule insertion
aniline blue	aniline blue, water soluble
ANS	8-anilino naphthalene sulphonic acid
ATP	adenosine triphosphate
aq.	aqueous
B	blade
BSA V	bovine serum albumin fraction V
C	carbon
^oC	degrees centigrade
^{14}C	carbon isotope of molecular weight 14
C_3	Calvin cycle photosynthetic cells
C_4	Hatch-Slack cycle photosynthetic cells
c-	centi-
calcofluor	calcofluor white M2R new
ch.	chapter
Chl	chlorophyll
Ci	Curie
(c/t)ER	(cisternal/tubular) endoplasmic reticulum
d	day(s)
DAB	diaminobenzidine tetrahydrochloride
DEP	diethyl pyrocarbonate
D%F	dry weight as a percentage of fresh weight
DW	dryweight
E	leaf emergence
EC	enzyme commission reference number
EDAX	energy dispersive analysis of X-radiation
eosin	eosin, water soluble, yellow shade
FAA	formaldehyde-acetic acid-ethanol
Fig.	figure
FluorM	fluorescence microscope/microscopy
FluorMgraph	fluorescence micrograph
FW	fresh weight
g	gram; gravity
GERL	Golgi apparatus-endoplasmic reticulum-lysosomal compartment

h	hour(s)
keV	thousand electron volts
kV	thousand volts
L	length; leaf
l	litre; light ray
LEZ	leaf extension zone
LM	light microscope/microscopy
LMgraph	light micrograph
Log_{10}	logarithms to the base 10
LS	longitudinal section
M	molarity; mid-vein
m	metre
m-	milli
min	minute(s)
MW	molecular weight
μ-	micro
^{15}N	nitrogen isotope of molecular weight 15
n	any number, eg of leaf
n-	nano-
NE	nuclear envelope
No.	number
ov.g.	overall growth
PA	periodic acid
PAGE	polyacrylamide gel electrophoresis
PATAg	Thiéry test for polysaccharides
PL	plasmalemma
Pl.	plate
propanediol	2-amino-2-ethyl-1,3-propane-diol
PTA	phosphotungstic acid
Q_{10}	temperature coefficient
r	correlation coefficient
(R/D)NA	(ribo-/deoxyribo-)nucleic acid
(R/D)NAse	(ribo-/deoxyribo-)nuclease
RGR	relative growth rate
RR	ruthenium red
S	sheath; Svedburg unit
SDS	sodium dodecyl sulphate

se	standard error
sec	second(s)
seg%	segment growth as a percentage of overall growth
seg.g.	segment growth
(S/R)ER	(smooth/rough) endoplasmic reticulum
S/TEM	scanning/transmission electron microscope/microscopy
S/TEMgraph	scanning/transmission electron micrograph
T	time
t	d. from 50% emergence of 4th leaf; 'mutual tensions' of tissues
TCA	trichloracetic acid
TCH	thiocarbohydraxide
TEMED	N,N,N',N'-tetramethyl-1,2-diaminoethane
triazole	3-amino-1,2,4-triazole
TS	transverse section
U	uniformly labelled
v.	verse
v/v	volume/volume
W	Watt
w/v	weight/volume
ZIO_4	zinc iodide-osmium tetroxide
-ve	negative
+ve	positive
1	Type 1 vascular bundle
2	Type 2 vascular bundle
col.	coleoptile
concn	concentration
λ	wavelength
Matt.	Gospel according to St Matthew
MV	million volts
N	nitrogen

DEDICATION

This thesis is dedicated to Mum, Dad and Chris, without whom none of this would have been possible.

CHAPTER 1 GENERAL INTRODUCTION

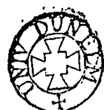
GENERAL MATERIALS AND METHODS

The work described in this thesis arose as a direct result of the observation by Lee (1979) that sheath-applied solutions of dyes and radiochemicals accumulated at the ligule region (sheath/blade junction) in ageing fescue leaves. This immediately focused attention on the ligule and interactions between blade and sheath during development of the leaf.

Although it is known that the grass leaf consists of three distinct organs - blade, sheath and ligule - equal weight in terms of their study has not been given to each. Whilst it may be accepted that the blade may be of most importance with regard to photosynthesis and hence yield, it is not the only photosynthetic organ of the leaf. The sheath must also be considered in that respect; its contribution presumably increasing higher up the culm where the ratio of blade to sheath for any given leaf usually decreases (eg Borrill, 1961). For too long the sheath has been neglected and thus any interactions between blade and sheath have been largely overlooked.

Since it is the whole leaf which may be considered the most important structural/functional unit of the grass plant, knowledge of all its constituent parts is necessary if we are to understand the whole. To this end a study of leaf growth was undertaken, particular attention being directed to identification of areas of interaction between organs within a leaf and between leaves within a plant (Chapter 2, Section a). The results from this provided the background for the more biochemically-oriented study of leaf ageing and senescence presented in Chapter 2, Section b. A direct extension of Lee's (1979) work with Festuca pratensis Huds. was provided by a study of aspects of the vasculature and metabolite transport in leaves of Lolium temulentum L. during ageing and senescence (Chapter 2, Section c). This latter investigation served to illustrate one aspect of inter-organ interaction during the course of leaf development.

The third leaf organ, the ligule, has been studied for well over a hundred years yet still it remains, 'a morphological enigma of unknown function' (Clifford and Watson, 1977). It has previously been studied from the point of view of morphology and anatomy with no more sophisticated equipment than a light microscope in the vast majority of cases. Its physiology appears to have been ignored completely. Clearly a complete understanding of the biology of the grass leaf can never be



achieved in the absence of a fuller study of the ligule. To this aim a study was begun of aspects of the structure and physiology of a membranous ligule. As a result of the ligule being the least studied of the three leaf organs, this study comprises the largest single part of the thesis and is dealt with in Chapter 3.

The choice of experimental material - Lolium temulentum L., darnel or bearded rye-grass - was dictated by a number of considerations, the most important of which was the availability of a nearly homozygous stock of grain of this species to the laboratory. This, plus the amount of information available in the literature concerning various aspects of its structure (eg Lawton, 1980), physiology (eg Evans and Wardlaw, 1966; Wardlaw, 1976) and development (eg Thomas, 1978) with which the results from this study could be compared, made it a most suitable candidate for this sort of investigation. Although L. temulentum is not a commercially valuable plant, its close phylogenetic affinity to other Lolium spp which are should mean that results obtained with darnel will have general applicability to other rye-grasses. In view of the suggestion that darnel is the 'tares' of the Bible (Matt. ch. 13, v. 24-30; eg Hastings, 1902) its use as an experimental organism may be justified on a number of historical, economic and sociological grounds as well.

The overall aim of this investigation has been to gain some understanding of the whole grass leaf in terms of its component organs and the 'functions' that take place within and between them. Certain facets of this immense subject are considered in the following Chapters.

GENERAL MATERIALS AND METHODS

Sources of Materials

Unless stated otherwise, all chemicals used were of analytical grade and obtained from either:

BDH, Poole, Dorset, UK, or,

Sigma, Poole, Dorset, UK.

Certain specialized items are listed below with the name of the supplier.

Electron Microscopy

Reagents and other materials were obtained from:
TAAB Laboratories, Reading, Berkshire, UK.
Ethanol and acetone were dried over molecular sieve type 5A obtained from BDH.
Silver dag was obtained from Acheson Colloids Ltd., Plymouth, Devon, UK.

Fluorescence Microscopy

Auramine O; eosin, water soluble, yellow shade; rhodamine 6G; aniline blue, water soluble, obtained from BDH.
8-anilino naphthalene sulphonic acid (ANS) obtained from Sigma.
Calcofluor white M2R new (calcofluor) obtained from Polyscience Inc., Pennsylvania, USA.

Nucleic Acid Determination

DNAse I obtained from Koch-Light, Colnbrook, Buckinghamshire, UK.
RNAse obtained from BDH.
E. coli RNA obtained from Sigma.

Cytochemistry

Periodic acid (PA, solid); ruthenium red (RR); sodium malonate obtained from BDH.
2-amino-2-ethyl-1,3-propanediol (propanediol); diaminobenzidine tetrahydrochloride (DAB); 3-amino-1,2,4-triazole (triazole) obtained from Sigma.
Thiocarbohydrazide (TCH) obtained from TAAB.
Silver proteinate obtained from Roques, Paris, France.

Autoradiography

(U-^{14}C)- protein hydrolysate as aq. solution with 2% ethanol obtained from Amersham International plc, Amersham, Buckinghamshire, UK.

Plant Material

Grain of Lolium temulentum L. strain Ba3081, summer annual (1973 harvest);

L. perenne L. S23;

L. multiflorum Lam. cv Trident;

L. x hybridum Hausskn. cv Augusta;

Festuca pratensis Huds. strain CvS215 were provided by the kindness of the Welsh Plant Breeding Station, Aberystwyth, Wales.

Storage of Grain

Until required for sowing, grain was stored in the laboratory at room temperature as follows:

L. temulentum in sealed plastic containers;

Other Lolium spp in the muslin bags in which they were supplied;

F. pratensis in a paper packet.

Cultural Conditions

L. temulentum: two or three grains were sown in each 10 cm diameter plastic pot containing Levington's compost; the pots were uniformly arranged in the central bench of a greenhouse. Watering was performed with tap water at ambient temperature as and when required in order to try and ensure minimally-stressed plants. Plants were grown throughout the year and supplementary heating was provided only in the winter.

Other species: grain was sown in trays containing Levington's compost; other conditions were as for L. temulentum above.

Light (LM) and Conventional Transmission Electron Microscopy (TEM)

Because of initial difficulty in orienting material embedded in opaque paraffin wax, transparent epoxy resin was used. Resin-embedded material processed for TEM was also used for correlative LM, and LM alone. In any method of preparing material for microscopy, the possibility of artefact formation is a major problem. No one procedure

can eliminate it entirely but it can be reduced to an acceptable minimum using a double aldehyde fixation step such as a mixture of formaldehyde and glutaraldehyde (eg Karnovsky, 1965). The possibility of the observed image being unrepresentative of the natural state of the specimen should always be borne in mind.

Excision and Trimming of Tissue

Leaf tissue was cut into pieces c. 1-2 mm long by c. 1 mm wide in 0.05M sodium cacodylate, pH 7.0 (cacodylate buffer) using a sharp razor blade.

Ligules were treated differently: the blade was first removed from the leaf by severing it at its base. The sheath plus attached ligule was then immersed in cacodylate buffer and three or four longitudinal cuts made with a sharp razor blade from base to apex of the ligule. The top of the sheath was then carefully flattened out and a transverse cut made across it to release ligule segments with a small piece of attached sheath tissue to aid in their orientation.

From this stage on ligule and leaf tissue were treated similarly.

Fixation, Dehydration and Embedding

The processing schedule was as follows:

1. Fixation in 2.5% (v/v) glutaraldehyde, 1.0% (w/v) formaldehyde (prepared from paraformaldehyde) in cacodylate buffer; c. 2-4 h,
2. Cacodylate buffer wash; 2 x 15 min,
3. Post-fixation in 1.0% (w/v) aq. OsO_4; c. 1-3 h,
4. Dehydration
   a. 25% (v/v) ethanol; c. 45 min,
   b. 50% ethanol; 2 x 15 min,
   c. 75% ethanol; 2 x 15 min,
   d. 100% ethanol; 15 min,
   e. 100% ethanol; 15 min,
5. Resin infiltration
   a. to tissue in 100% ethanol, 4.e an equal volume of Spurr resin was added; c. 15-30 h,
   b. to 100% Spurr resin; c. 8-24 h,
6. Embedding of tissue in 100% Spurr resin in tops of TAAB capsules,
7. Hardening of resin at c. 70°C; 1-2 d.

The times at stages 1, 3 and 5 varied and had to be found by trial and error for the tissue concerned. All stages, except 6, were performed at room temperature with constant agitation of the tissue on a rotating disc. When non-osmicated tissue was required, stage 3 was omitted.

Spurr resin (Spurr, 1969) was either prepared from TAAB premix kits or made up from the following recipe:

10 g	ERL-4206	(epoxy resin),
4 g	DER 736	(flexibilizer),
26 g	NSA	(hardener),
0.4 g	S1	(accelerator)

Sectioning

Thick sections (1 μm) for LM and ultrathin sections (gold/silver interference colours; 150-60 nm) for TEM were cut using glass knives on an LKB Ultrotome (LKB-Produkter AB, Stockholm, Sweden). The sections were collected on the surface of distilled water in a trough attached to the knife. For LM, sections were picked up in a platinum wire loop and transferred to a drop of distilled water on a microscope slide. Ultrathin sections were expanded using a heat pen before being picked up on either uncoated or formvar-coated copper grids (c. 200 mesh).

Staining for Light Microscopy

A drop of 1% (w/v) toluidine blue solution made up in 1% (w/v) sodium tetraborate was added to the sections which had been dried onto the slide over a hot plate. After warming over a hot plate for c. 30 sec, the dye solution was washed off with distilled water and the sections finally dried. Many staining procedures were experimented with but toluidine blue proved to be the best all-round stain. It did not exhibit the degree of metachromasy found in wax-embedded material (O'Brien *et al.*, 1964). However, lignified tissue was clearly distinguishable, by its green colour, from the remainder of the tissues and cellular inclusions which were various shades of blue.

The other widely used 'stain' was Sudan black B as a saturated solution in 70% ethanol for lipids and starch grains (eg Bronner, 1975). Unstained sections were dried onto the slide over a hot plate and

immersed in 70% ethanol for 2 min, before immersion in the Sudan black B solution at 60°C for 1 h. After rinsing in 70% ethanol to remove excess 'stain', a drop of 50% (v/v) glycerol was added to the sections before viewing in the LM. In this way lipids of plastids 'stained' black and the unstained, white starch grains were contrasted against them.

Viewing and photomicrography were performed using a Leitz Ortholux LM in transmitted light. Images were recorded using Ilford FP4 or Kodak XP1 black and white film. Unless stated otherwise all LMgraphs are of sectioned material stained with toluidine blue.

Staining for Conventional TEM

For general ultrastructural observations, sections were double-stained sequentially as follows:

1. saturated aq. uranyl acetate; 15-30 min,
2. thorough washing with distilled water,
3. alkaline lead citrate (Reynolds, 1963); 15-30 min,
4. thorough washing with distilled water.

Occasionally a saturated ethanolic solution of uranyl acetate was used on sections exhibiting low contrast after aqueous-staining. Dried grids were stored on filter paper in petri dishes. Grids were viewed in a Philips EM400 at 80 or 100 kV and images recorded on Kodak electon image film 4463. Unless stated otherwise all TEMgraphs are of double-stained sections.

Fluorescence Microscopy

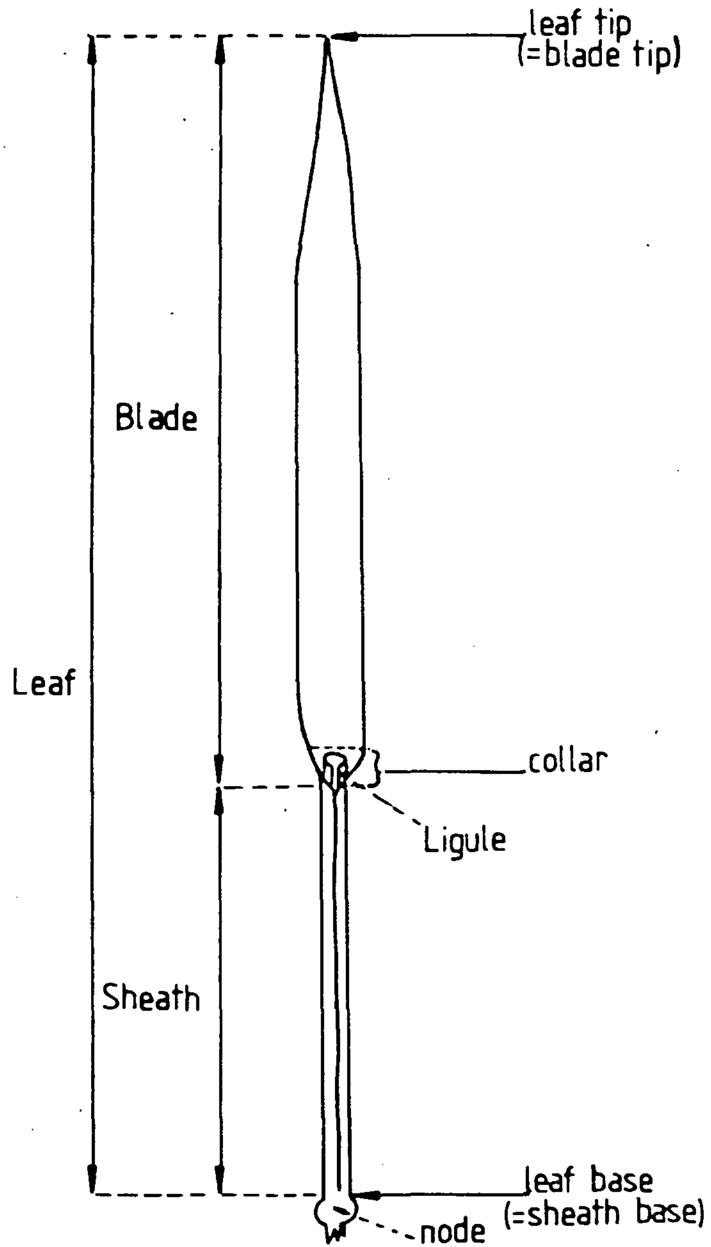
For general observations of structure, TS's of leaf tissue were cut with a razor blade and stained with 0.01 or 0.1% (w/v) aq. calcofluor. Sections were viewed in a Leitz Ortholux microscope fitted with epi-fluorescent illumination (provided by a 50W ultra high pressure mercury lamp) using excitation filter BG 3 and suppression filter K 460. Images were recorded using either Ilford HP4 or Kodak XP1 black and white film rated at 1600 ASA.

Terminology

Terminology used was as follows:

Germination	- date of appearance of the coleoptile above the surface of the soil;
Growth	- increase in length of the organ concerned with time;
Appearance	- exsertion of organ above the top of the enclosing sheath or the coleoptile;
Leaf emergence	- exsertion of the ligule;
Leaf	- main unit of construction of the vegetative grass plant consisting of three organs - blade, sheath and ligule (Fig. 1.1). The leaf tip is the tip of the blade; the leaf base is the base of the sheath. Below the base of the leaf may be a node (in the case of a culm leaf) or simply the insertion of the leaf upon the shoot (in the case of a basal leaf). The ligule is situated at the blade/sheath junction, immediately below a region of yellow-coloured tissue at the base of the blade, the collar.

Figure 1.1. Schematic diagram of a grass leaf


leaf tip
(=blade tip)
Blade
Leaf
collar
Ligule
Sheath
leaf base
(=sheath base)
node

CHAPTER 2 BLADE AND SHEATH

SECTION a. LEAF GROWTH

INTRODUCTION

The grass leaf is initiated by periclinal divisions in the adaxial epidermis of the shoot apex; these divisions spread laterally and encircle the apex. Growth then proceeds in an upwards direction, the developing leaf primordium overtops the apex and continues to grow upwards as a sleeve-like structure, joined usually only at the base. During this early stage, meristematic activity is progressively localized at the base of the leaf primordium as an intercalary meristem. Early on in leaf development this meristem splits in two - the upper gives rise to the blade, the lower gives rise to the sheath. At this time the ligule becomes initiated as a series of periclinal divisions in the adaxial epidermis between the two meristems. This event is seen as marking the end of the primordial stage of leaf growth and heralding in leaf growth proper since the three leaf organs can now all be recognized. After a short period of further meristematic activity the rest of the growth of the leaf is by elongation of cells all ready formed. (The above has been summarized from accounts in Sharman (1942), Etter (1951) and Kaufman (1959)).

Growth of the blade commences before that of the sheath and also ceases before that of the latter; the early stages of leaf growth appear to be due almost solely to blade elongation (eg Etter, 1951). As a result of elongation of its cells, the leaf is gradually exserted from within the sleeve of enclosing leaf sheaths and directly experiences the aerial environment for the first time since its initiation. As it appears it begins to unroll under the influence of light (eg Vince-Prue and Tucker, 1983). Exsertion of the blade is complete with the appearance of the ligule. The leaf is then considered to be emerged; attainment of its 'morphologically mature state' is completed with the bending of the blade in the collar region which also seems to be under the control of light (Kimura, 1977). Since physical hindrance prevents blade bending, sheath growth is prolonged just long enough to ensure that the blade is exserted clear of the enclosing sheath.

The nature of the growth of the leaf from an intercalary meristem results in a gradient of tissue differentiation from leaf tip to base. During the course of leaf growth a basipetal wave of differentiation and cell maturation can be identified (eg Sharman, 1942). At maturity this

gradient of developmental stages is replaced by a gradient of senescence (see next Section).

According to Edwards (1967) it is possible to identify three distinct phases of leaf growth:

i. primordial growth - very early stages of slow exponential growth, from initiation to the stage where it is overtopping the apex;

ii. rapid growth - after it has overtopped the apex, the young leaf grows exponentially but more quickly than in (i);

iii. declining growth - the growth rate declines as the leaf emerges and matures.

As a result most of the growth that is observable with the naked eye is not exponential at all; all growth after exsertion of tissue from the enclosing sheath or the coleoptile is decreasing in rate.

Once a certain number of leaves has been established on a shoot there appears to be a dynamic equilibrium in the number of photosynthetically active leaves, leaf production balancing loss due to senescence (eg Robson, 1967). Under certain conditions a grass plant may remain vegetative and continue producing leaves almost indefinitely. Usually, however, flowering is induced by the environment and subsequent conversion of the shoot apex to a floral apex prevents further initiation of leaves.

Leaf growth and form is affected by a number of environmental factors such as nutrient supply, temperature, daylength and light intensity (eg Evans et al., 1964). Another set of influences upon morphology are those generated within the plant. For example the marked decrease in the ratio of blade to sheath length of successive leaves up the culm (eg Borrill, 1961) suggests some interaction between blade and sheath. The timings of commencement and cessation of blade and sheath growth also suggest interaction within a leaf. Interaction between leaves is implied by the observation that only two leaves were ever seen to be growing at any one time in tall fescue (Robson, 1967) and by the dynamic equilibrium of leaf number mentioned above. Of course it is quite likely that the distinction between 'external' and 'internal' factors is meaningless since it is probable that the former may be the cause of the latter and it is the constitution of the plant itself which interprets the external environment in terms of internal changes which become translated into the pattern of growth observed.

One of the most interesting 'interactions' is the observation that those parts of the leaf exserted from within the enclosing sheath have finished growth (eg Begg and Wright, 1962). Although growth appears to have ceased by the time the tissue is exserted, actually when it finished growth does not appear to be known. In this study it was decided to try and answer this question in view of its importance to the time of attainment of 'maturity' of the blade and sheath. Two methods were used in the study of this phenomenon: measurement of leaf extension growth and determination of the zone of leaf extension.

MATERIALS AND METHODS

Measurement of Growth

1. Non-destructive

Leaf length was measured from the tip of the vertically-held leaf to the top of the next oldest sheath, or coleoptile (first leaf only). In leaves which had emerged, sheath length was measured separately from the top of the sheath concerned to the top of the next oldest sheath or coleoptile; blade length was obtained by difference between leaf and sheath length. For the first few leaves, where no internode extension occurred, leaf and sheath lengths were recalculated to 'lengths above the top of the coleoptile' by adding the lengths of all the enclosing sheaths to the leaf or sheath length concerned. Measurements were usually taken daily as near 24 h apart, or multiples thereof, as possible, either in the mid-afternoon or early morning. Leaves were numbered upwards from the base of the plant. Repeated measurements were made on groups of plants and means and standard errors computed for leaf, blade and sheath lengths.

2. Destructive

Plants were carefully removed from the soil, severed at the shoot/root junction, the individual leaves and coleoptile separated and measured. Lengths of leaves and sheaths from this type of sampling are thus lengths from the base of the coleoptile. A x10 hand lens was used to look for the ligule in each case; where it could be seen blade and sheath were measured separately. Nine plants were used for each sampling; means and standard errors were computed for leaf, blade and sheath lengths. This type of sampling was only used in addition to non-destructive sampling.

Determination of Leaf Extension Zone (LEZ)

Two methods were used: pen-marking (eg Arber, 1934) and pin-pointing (eg Davidson and Milthorpe, 1966).

1. Pen-marking

Marks were made on appearing first leaves using a 0.35 mm Rotring

pen nib and black, indelible ink at intervals measured from the leaf tips. To avoid excessively damaging the seedling the coleoptile was not removed but the leaf was marked through it. Leaf length was measured from the leaf tip to the base of the coleoptile (the soil was carefully removed to accomplish this and then replaced). After 24 h the seedlings were removed from the soil, severed at the shoot/root junction (coleoptile base), the coleoptiles removed and leaf lengths and pen marks measured. Only seedlings which appeared 'healthy' after the 24 h period were remeasured.

2. Pin-pointing

This was performed as for pen-marking using a steel needlework pin to mark the leaf.

Presentation of Data

Growth

Leaf growth data and derived data were presented as graphs; all lines were fitted by eye. The several types of graphs are briefly described below:

1. Leaf and Organ Growth

Primary data obtained from destructive and non-destructive sampling were plotted as length (L) of leaf or organ in cm against time (T) in d. Each point is the mean of from 9-70 individual measurements; standard errors were usually very small and largely omitted for clarity.

2. Absolute Growth Rate

Data were derived from the appropriate eye-fit line of organ or leaf growth and plotted as change in length of organ or leaf against time.

3. Log Leaf Growth

The primary leaf length data were transformed to Log_{10} and plotted against time. The regression line for the log-transformed points on the rising linear part of the leaf growth curve was calculated and the average relative growth rate (RGR) for this period calculated from the slope of this line.

4. Relative Growth Rate

Mean daily RGR's were calculated using the log-transformed primary leaf length data for the rising linear part of the leaf growth curve using the following equation (after Fisher, 1921):

$$RGR\ (cm.cm^{-1}.d^{-1}) = \frac{\log_{10}L_2 - \log_{10}L_1 \times 2.303}{T_2 - T_1}$$ and plotted against time.

The average RGR $\pm$ se was calculated and compared with that obtained in 3. above; in all cases the two were almost identical. The former were tested for significance using Student's t-test.

5. Germination

These data were plotted as:

$$\frac{\text{number of grain germinated}}{\text{number of grain sown}} \times 100\%$$ against time.

6. Leaf Emergence

These data were plotted as:

$$\frac{\text{number of plants with leaf 'n' emerged}}{\text{number of plants present}} \times 100\%$$ against time.

Time Scales

These took two forms:

1. d. from sowing - data were plotted relative to the date of sowing of the batch of plants from which these data were obtained;
2. d. from germination - data were plotted relative to date of germination of the individuals concerned.

LEZ

Because the positions of the marks and lengths of leaves differed between seedlings results are presented for representative individuals only. The main features of this type of analysis are shown in Fig. 2.1; positions of marks are indicated by numbers on the schematic leaf outlines.

d represents the leaf length;

x represents the segment length (from the base of the coleoptile to the first mark for $_1x$; from tip of the leaf to the highest mark for $_0x$);

Figure 2.1. Aspects of leaf growth in *Lolium temulentum*

Diagrammatic explanation of the methods for analysis of LEZ.

The equations for derivation of overall growth, segment growth, and segment growth as a percentage of overall growth are also shown.

For further explanation see text.

day 0 → day 1

d ${}_0x$ ${}_3x$ ${}_2x$ ${}_1x$ ${}_3a$ ${}_2a$ ${}_1a$ 3 2 1

d' ${}_0x'$ ${}_3x'$ ${}_2x'$ ${}_1x'$ ${}_3a'$ ${}_2a'$ ${}_1a'$ 3 2 1

$$\text{overall growth} = d' - d$$
$$= \Sigma ({}_1x' - {}_1x) + ({}_2x' - {}_2x) \cdots + ({}_nx' - {}_nx) + ({}_0x' - {}_0x)$$
$$\equiv \quad '' \quad '' \quad '' \quad + ({}_3a' - {}_3a)$$

$$\text{segment growth} = {}_nx' - {}_nx$$

segment growth as percentage of overall growth

$$= \frac{{}_nx' - {}_nx}{d' - d} \times 100\%$$

position of marks, day 0 → 1: $1, {}_1a \rightarrow {}_1a'$
$2, {}_2a \rightarrow {}_2a'$
$n, {}_na \rightarrow {}_na'$

a represents the distance between the leaf tip and the respective mark.

The leaf illustrated has three marks for convenience to illustrate the principles; $_{n}a$ and $_{o}x$ always correspond to the distance from the leaf tip to the highest mark on the leaf.

Results are presented in a tabular manner using the following abbreviations:

ov.g. - overall growth,

seg.g. - segment growth,

seg% - segment growth as percentage overall growth.

RESULTS

1. Germination

Over 90% germination was attained in all cases, indicating considerable longevity of the grain in storage. Germination began later, and occurred at a slower rate, in winter than in summer (Fig. 2.2).

2. Leaf Growth

a. General

Growth curves of the first 5 leaves of winter-grown plants are shown in Fig. 2.3a; all showed a similar pattern to each other and to summer-grown plants (results not shown). In each case a period of more or less linear growth preceded one of decreasing growth before reaching a plateau when maximum leaf length was attained. The first leaf was considerably shorter than the others which showed a more uniform stepwise increase in length.

Comparison of leaf and organ length of summer-grown plants (Table 2.1) showed that leaf and sheath length increased up the culm, whereas the ratio of blade/sheath length decreased constantly from leaf 1 to 6 (flag leaf).

Emergence curves for the first three leaves of Fig. 2.3a are shown in Fig. 2.3b; and Fig. 2.15 for the 4th leaf. Over the period of measurement, 100% emergence was not attained for any leaf; the time taken for maximal emergence to be achieved increased with increasing leaf number.

b. Non-destructive Sampling

The growth curve of the 1st leaf of winter-grown plants is shown in Fig. 2.4a and was similar to that seen in Fig. 2.3a. When blade and sheath were measurable as separate organs (at emergence), the blade had reached its maximum length; after emergence leaf growth continued as sheath growth only. Hence blade growth ceased before sheath growth.

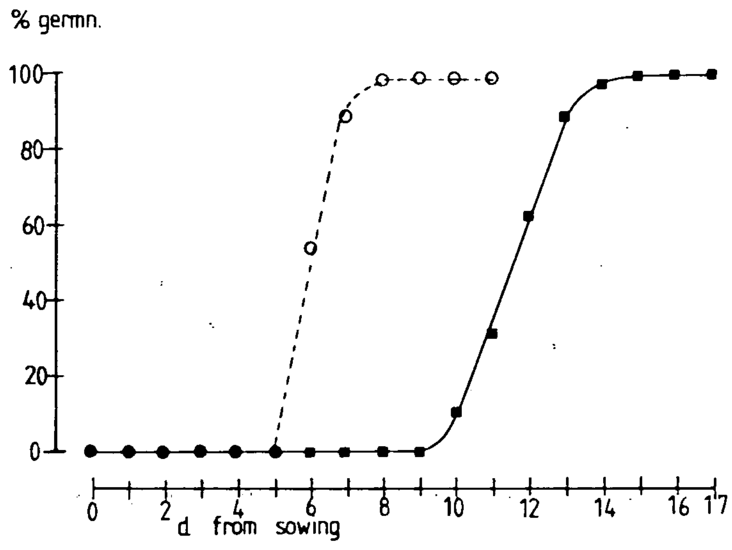
This pattern was reflected in the shapes of the growth rate curves (Fig. 2.4b); when measurable, growth rate was zero for the blade and decreasing for the sheath. Leaf growth rate continually decreased throughout the period of measurement; at no time was leaf growth

Figure 2.2. Aspects of leaf growth in *Lolium temulentum*

Germination curves of two sowings of grain;

summer (June, 1981) o- - - -o

winter (October, 1981) ■——■



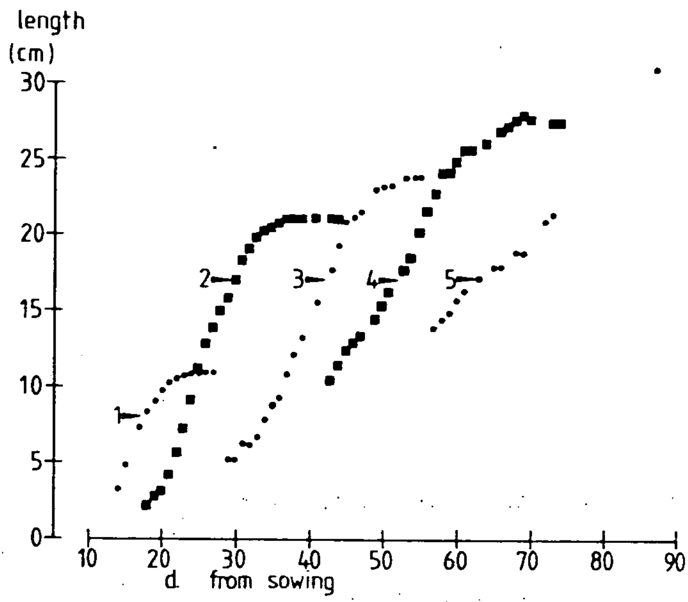
% germn.
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
17
d from sowing

Figure 2.3. Aspects of leaf growth in Lolium temulentum

a. Growth curves of the first 5 leaves; measured above top of the coleoptile by non-destructive sampling (October, 1981 - February, 1982);

b. Emergence curves for leaves 1, 2, 3, in a. above

a.



b.

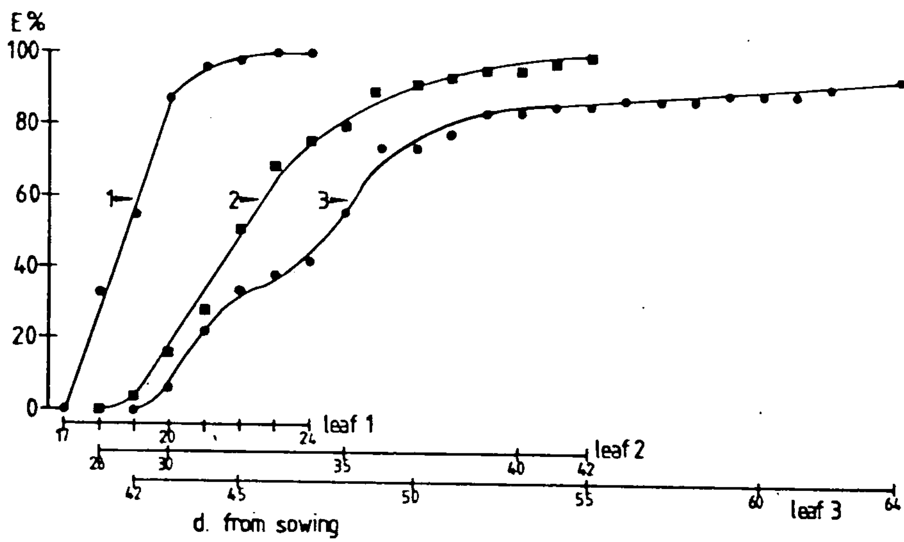


Table 2.1. Variation in length of blade, sheath and leaf up a culm of summer-grown plants of Lolium temulentum

Leaf number	Length (cm) leaf	blade	sheath	Blade/sheath ratio
1 (basal)	8.1	7.4	0.7	10.6 : 1.0
2	12.4	10.6	1.8	5.9 : 1.0
3	18.2	14.2	4.0	3.6 : 1.0
4	21.9	16.7	5.2	3.2 : 1.0
5	21.7	13.2	8.5	1.6 : 1.0
6 (flag)	28.9	14.1	14.4	1.0 : 1.0

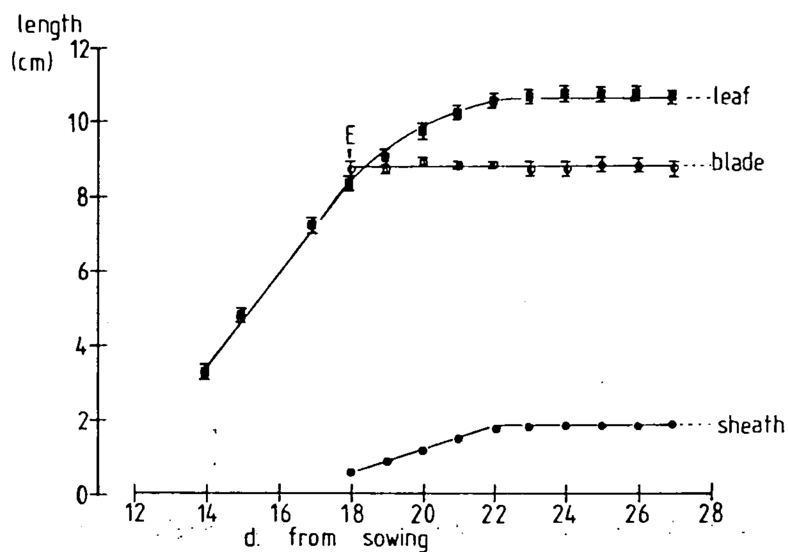
Figure 2.4. Aspects of leaf growth in Lolium temulentum

a. Leaf, blade and sheath growth curves of the 1st leaf; measured above top of the coleoptile by non-destructive sampling (October, 1981);

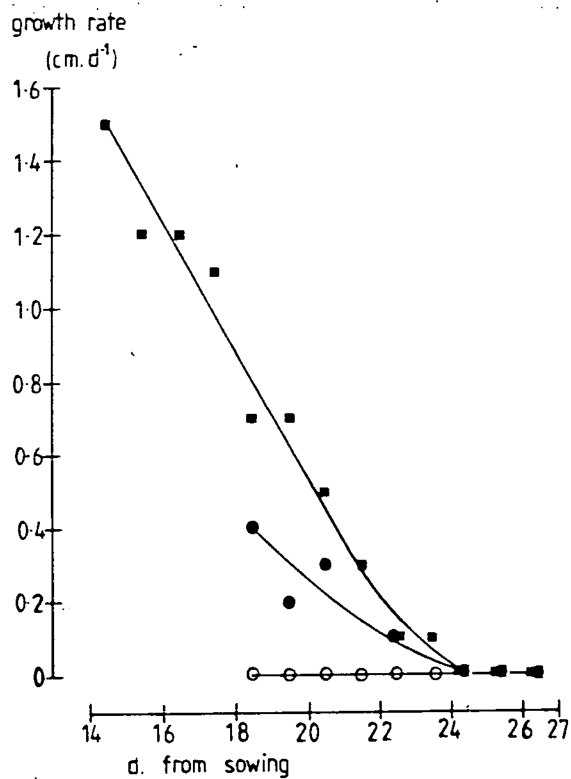
b. Absolute growth rate curves derived from data in a. above;

leaf ■———■
blade ○———○
sheath ●———●

a.



b.



exponential, as shown by the shapes of the curves of log leaf growth (Fig. 2.5a) and RGR (Fig. 2.5b).

c. Destructive and non-destructive Sampling

i. Single Leaf

The curves of 2nd leaf and organ growth of summer-grown plants are shown in Fig. 2.6a; the overall pattern of leaf growth was similar to that for other leaves (eg Fig. 2.3a). The leaf was clearly separable into blade and sheath at 14 d.; the sheath appeared to exhibit a lag period before commencing growth and it was inferred that leaf growth up to that time was solely due to growth of the blade. Leaf emergence occurred at c. 17d., by which time both blade and sheath had almost reached their maximum lengths.

The growth rate curves of the above are shown in Fig. 2.6b. Overall leaf growth rate increased to a maximum which was maintained for a few days before decreasing to zero at c. 21 d., which corresponded with cessation of leaf growth in Fig. 2.6a. Blade and sheath rate curves had similar shapes to one another and to the overall leaf rate curve; blade growth rate peaked with that of the leaf confirming that early leaf growth was due to this organ alone. Sheath growth rate, however, did not begin to increase until blade growth rate was decreasing, and peaked just before emergence. Interestingly blade growth had not ceased prior to emergence for this leaf, although it did cease before the sheath.

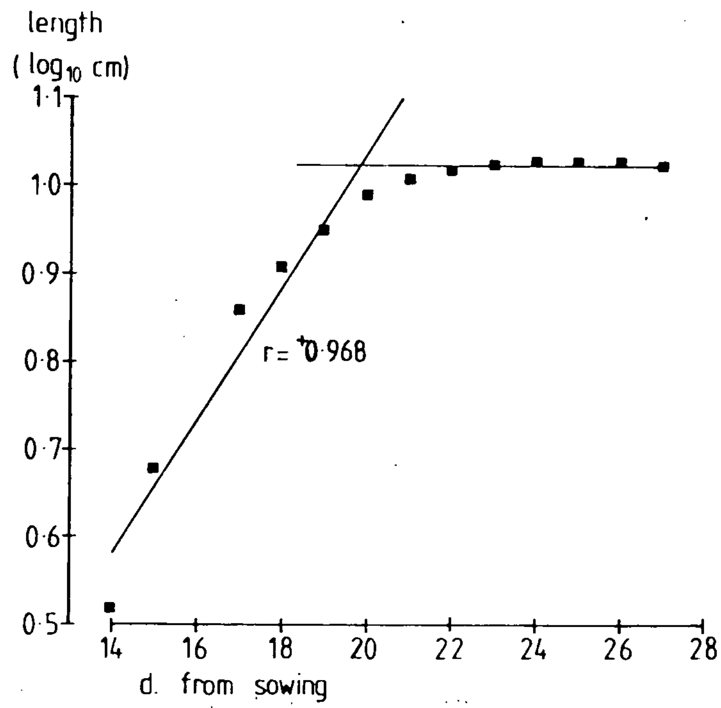
ii. Successive Leaves

Curves of leaf and organ growth of the first three leaves of summer-grown plants are shown in Fig. 2.7a. Again the overall pattern of leaf growth was similar to that seen above, and of organ growth similar to that of the 2nd leaf (Fig. 2.6a). Only two leaves were clearly growing at the same time; a lag period for beginning of 2nd leaf growth was apparent. Sheath growth of a given leaf, however, did not occur at the same time as growth of other sheaths. In each case, blade growth had finished at or by emergence. The lag in sheath growth commencement was particularly well shown for the 3rd leaf and it was clear that considerable growth took place before that of the former even when both

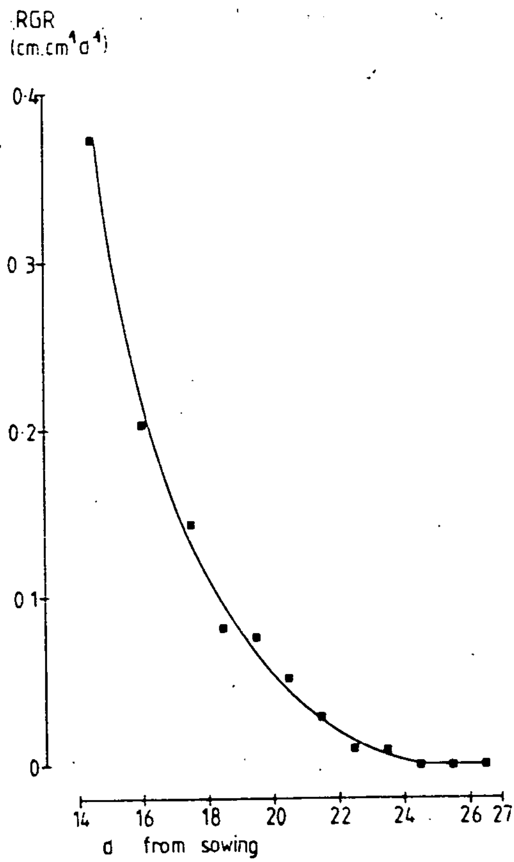
Figure 2.5. Aspects of leaf growth in *Lolium temulentum*.

a. Log_{10} growth curve of the 1st leaf in Fig. 2.4a;

b. RGR curve of the 1st leaf; derived from data in a. above



a.
length
($\log_{10}$ cm)
1·1
1·0
0·9
0·8
0·7
0·6
0·5
r = +0·968
14
16
18
20
22
24
26
28
d. from sowing



b.
RGR
(cm.cm^{-1}d^{-1})
0·4
0·3
0·2
0·1
0
14
16
18
20
22
24
26
27
d from sowing

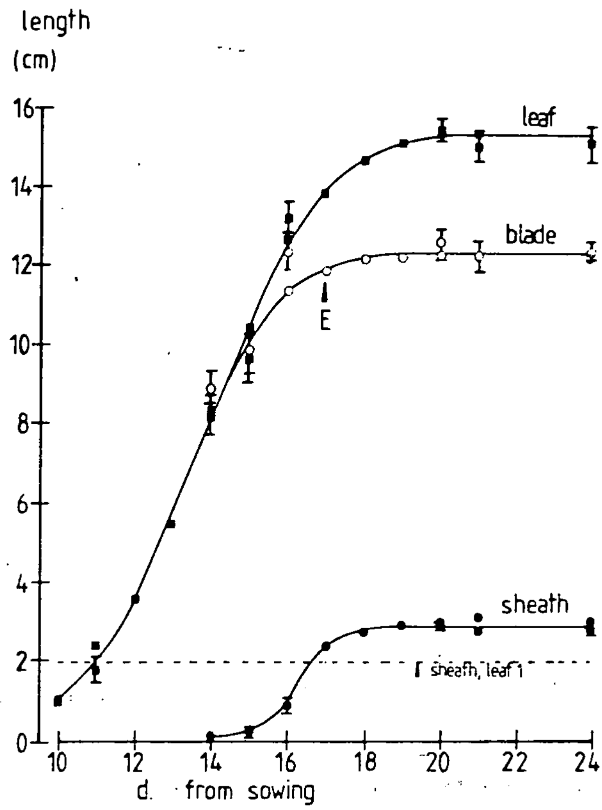
Figure 2.6. Aspects of leaf growth in Lolium temulentum

a. Leaf, blade and sheath growth curves of the 2nd leaf; measured above base of coleoptile by destructive sampling (June, 1982);

b. Absolute growth rate curves derived from data in a. above;

leaf ■——■
blade ○——○
sheath ●——●

a.



b.

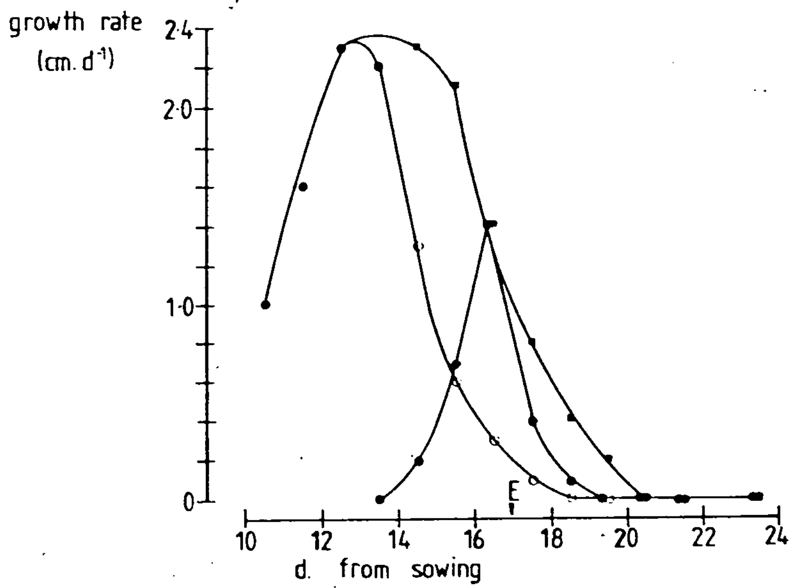
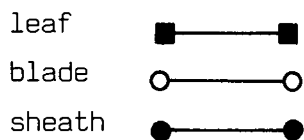


Figure 2.7. Aspects of leaf growth in _Lolium temulentum_

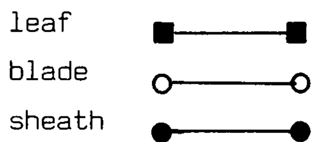
a. Leaf, blade and sheath growth curves of the first 3 leaves; measured above base of the coleoptile by destructive sampling (May, June, 1983);

leaf
blade
sheath

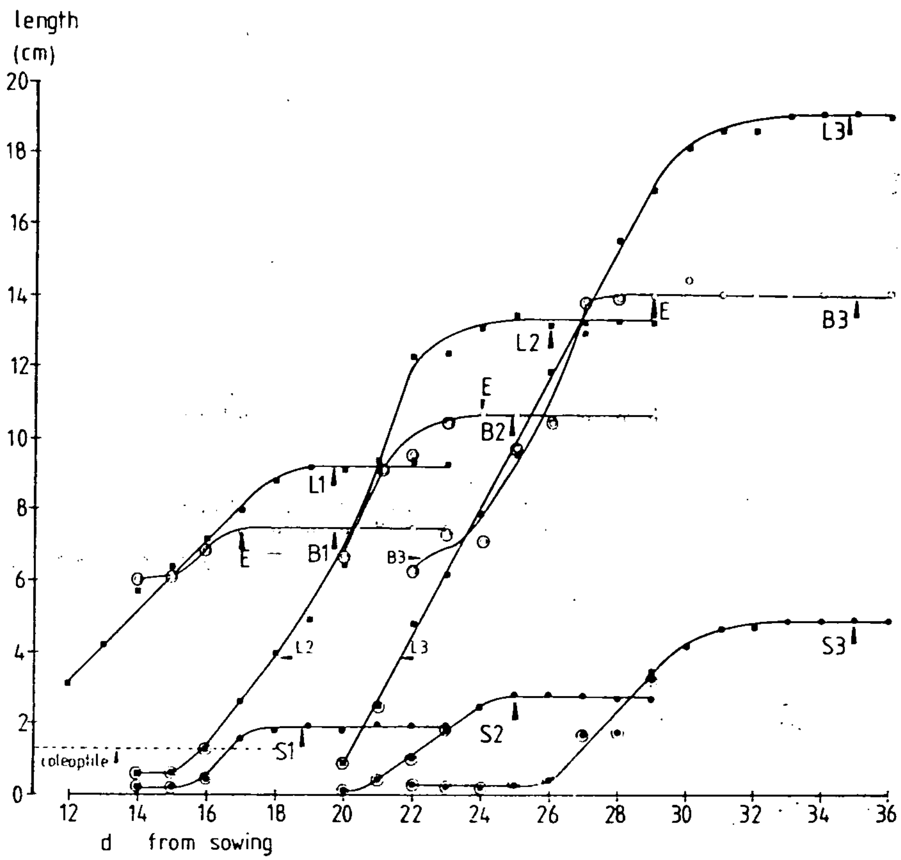


b. Absolute growth rate curves derived from data in a. above;

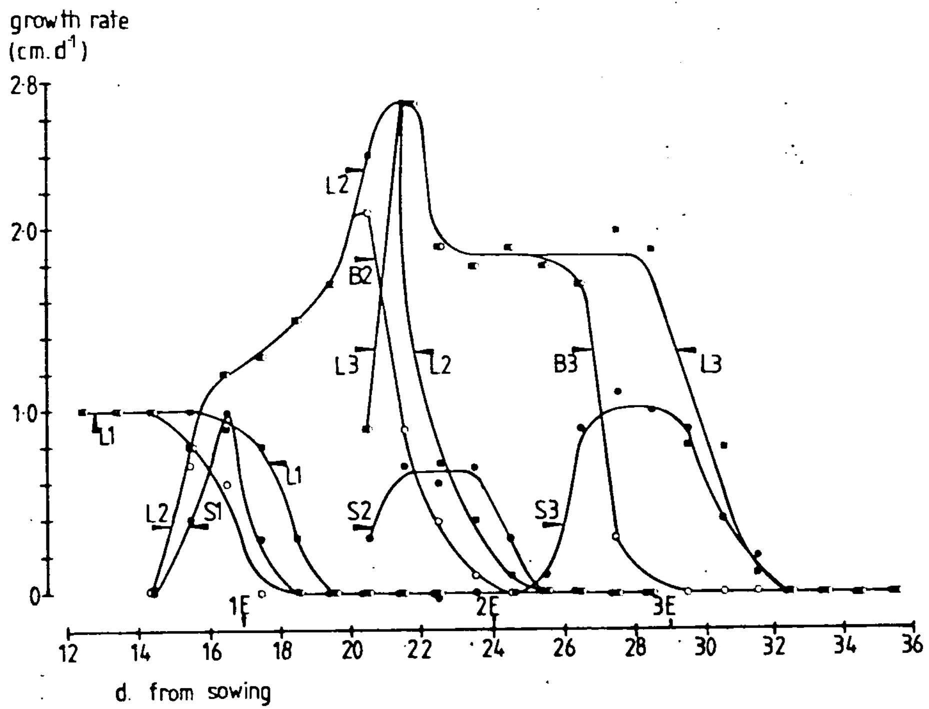
leaf
blade
sheath



a.



b.



organs were distinguishable. Each leaf - and both organs measured here - was longer than the preceding leaf.

The growth rate curves for these leaves and organs (Fig. 2.7b) were generally similar to one another and to that of the 2nd leaf (Fig. 2.6b). The rate curve for the 1st leaf differs from the others only because it was not measured early enough to include any initial lag phase. The rate curve for the 3rd leaf, however, appeared to be different from the others, showing an initial increase to a maximum, a marked decrease to a steady value held for several days followed by a final decrease to zero.

Sheath growth rates did not reach maxima until after their respective blades', and the increase in sheath growth rate did not take place until the blade growth rate was decreasing. Blade growth rates were nearly zero by the time of emergence. As expected from the growth curves, the 1st leaf growth rate maximum occurred before that of the 2nd leaf. The 2nd and 3rd leaves' growth rate maxima occurred at the same time, however, and may be related to the shape of the leaf growth curve of the former (Fig. 2.7a) which appeared to have two linear components each of differing slope.

The shapes of the log leaf growth curves of the above (Fig. 2.8a) were similar to each other and to that seen for the 1st leaf in Fig. 2.4a; nowhere did growth appear to be exponential. The RGR curves derived from these data (Fig. 2.8b) confirmed this. Although generally all three leaves showed decreasing RGR with time, the 2nd leaf exhibited a biphasic pattern which appears to be related to the two components of its growth curve and its initial lag period. Using Student's t-test on these data it was possible to show that the 2nd leaf had a significantly greater RGR than the 1st leaf but the 3rd leaf did not (Table 2.2).

d. Seedling Variability

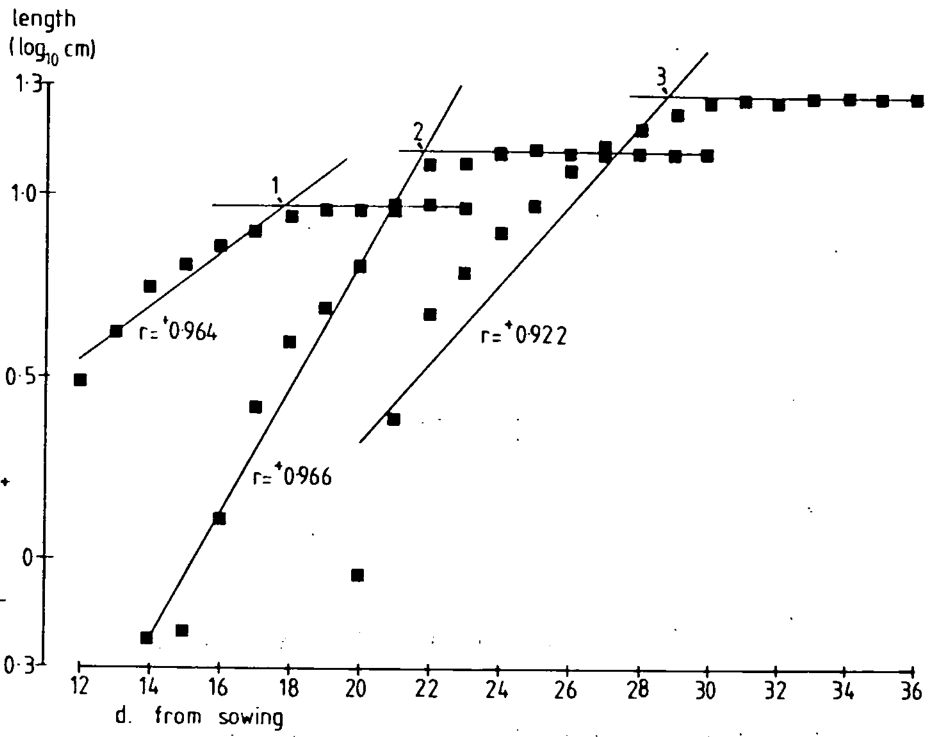
Growth of 1st leaves was recorded for each of 9 summer-grown seedlings. The growth curves were similar in shape to each other, but individual differences were marked. The mean growth curve had a wide margin of error associated with it (se bars in Fig. 2.9a), and the range of leaf lengths at each sampling point was great. Normalizing these data, with respect to germination date (Fig. 2.9b), only reduced the size of the se's in the early part of the mean growth curve.

Figure 2.8. Aspects of leaf growth in Lolium temulentum

a. Log_{10} growth curves of the first 3 leaves in Fig. 2.7a;

b. RGR curves of the first 3 leaves; derived from data in a. above

a.



b.

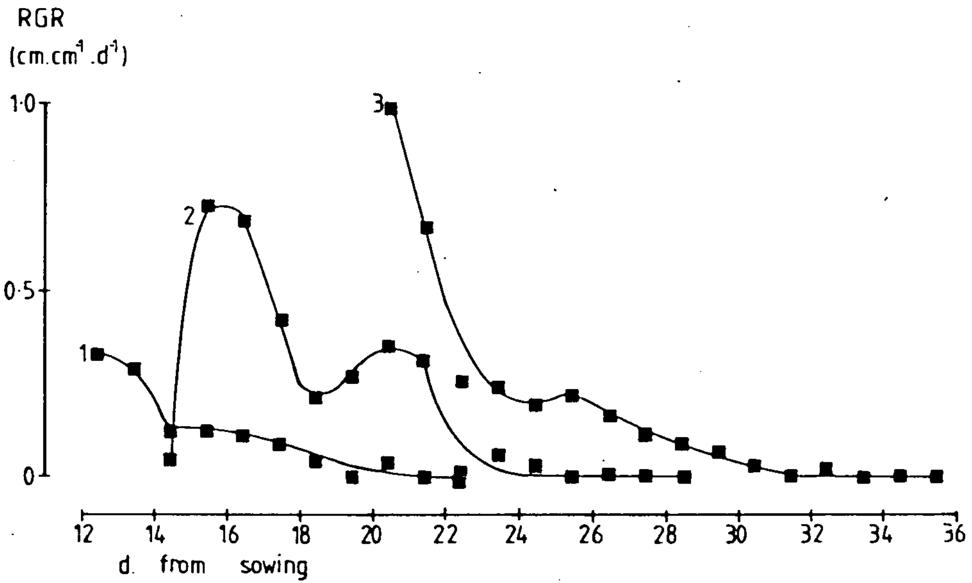


Table 2.2. A comparison of the RGR's of the first three leaves of summer-grown plants of Lolium temulentum

RGR's derived from data in Fig. 2.8a;

leaf 1, $0.18 \pm 0.043\ d^{-1}$ (mean $\pm$ se of 7 values)
leaf 2, $0.38 \pm 0.082\ d^{-1}$ (" " " 9 values)
leaf 3, $0.30 \pm 0.093\ d^{-1}$ (" " " 11 ")

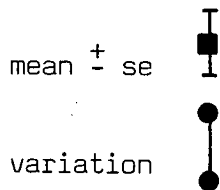
Student's t-test

	leaf 1	leaf 2	leaf 3
leaf 1	---	sig.	not sig.
	leaf 2	---	not sig.
		leaf 3	---

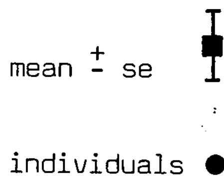
significant (sig.) or not (not sig.) at the 5% probability level

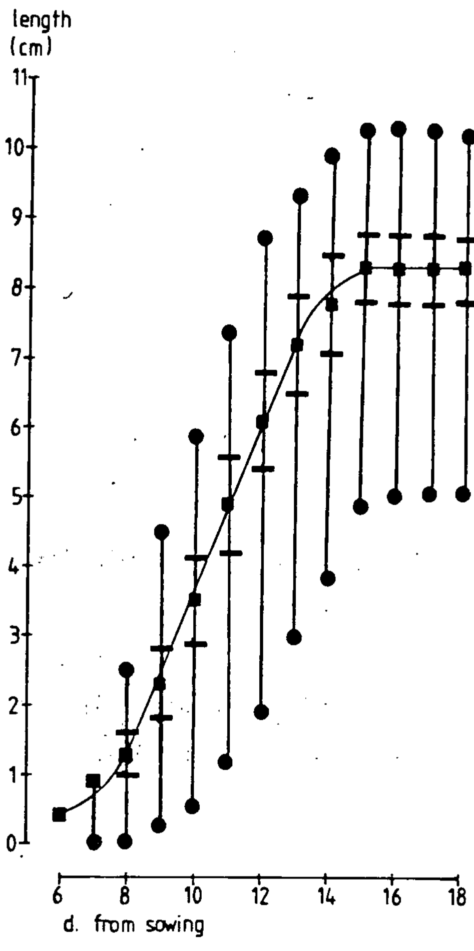
Figure 2.9. Aspects of leaf growth in Lolium temulentum

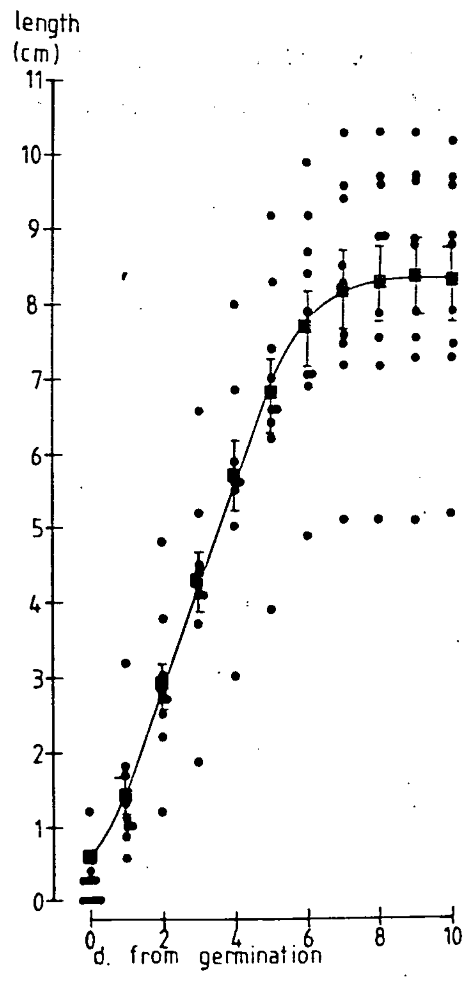
a. Mean growth curve of the 1st leaves of 9 seedlings with the limits of variation in leaf length at each sampling point shown; measured above top of the coleoptile by non-destructive sampling (June, 1983);



b. Mean growth curve of the 1st leaves of the 9 seedlings in a. above normalized with respect to date of germination of each seedling;




a.
length
(cm)
11
10
9
8
7
6
5
4
3
2
1
0
6
8
10
12
14
16
18
d. from sowing


b.
length
(cm)
11
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
d. from germination

3. Determination of the LEZ

Representative results from the use of the pen-marking and pin-pointing techniques are shown in Fig. 2.10 and 2.11 respectively for 1st leaves of summer-grown plants. Both techniques suggested that the LEZ - strictly the blade extension zone since no sheath was apparent at this stage - was confined to the lowermost part of the leaf, possibly the basal 0.1 cm (Fig. 2.11a,b).

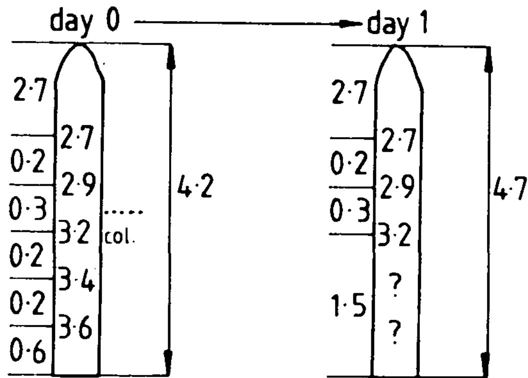
In order to assess possible effects of pin-pointing on growth, growth of the 1st leaf of pin-pointed and untreated plants was compared (Table 2.3). The results suggest that pin-pointing may reduce growth by 55-60%.

Figure 2.10. Aspects of leaf growth in Lolium temulentum

a,b. Determination of LEZ in 1st leaves of summer-grown plants by the pen-marking method

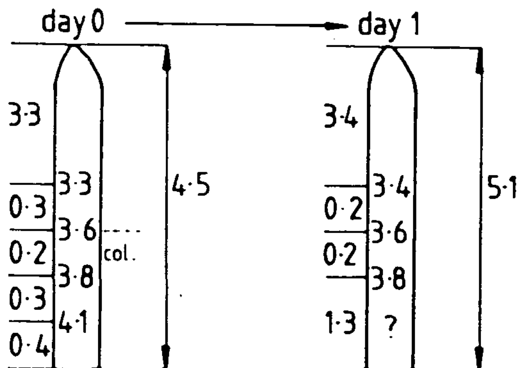
'?' - lost mark

a.



day	d	$_0x$	$_5x$	$_4x$	$_3x$	$_2x$	$_1x$	ov.g.
0	4·2	2·7	0·2	0·3	0·2	0·2	0·6	
1	4·7	2·7	0·2	0·3	1·5			
ov.g.	0·5	0 +	0 +	0 +	0·5 =			0·5
seg%		0	0	0	100			

b.

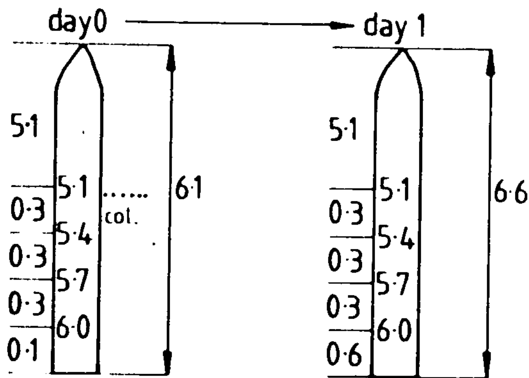


day	d	$_0x$	$_4x$	$_3x$	$_2x$	$_1x$	ov.g.
0	4·5	3·3	0·3	0·2	0·3	0·4	
1	5·1	3·4	0·2	0·2	1·3		
ov.g.	0·6	0 +	0 +	0 +	0·6 =		0·6
seg%		0	0	0	100		

Figure 2.11. Aspects of leaf growth in <u>Lolium temulentum</u>

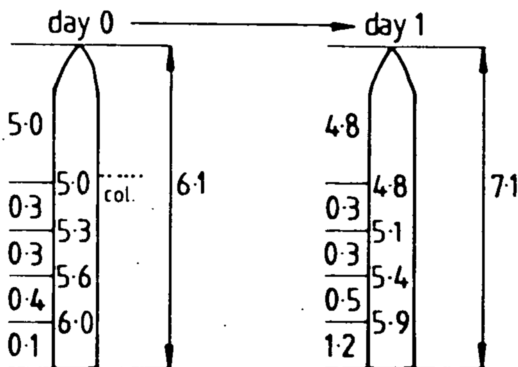
a,b. Determination of LEZ in 1st leaves of summer-grown plants by the pin-pointing method

a.



day	d	$_0x$	$_4x$	$_3x$	$_2x$	$_1x$	ov.g.
0 1	6·1 6·6	5·1 5·1	0·3 0·3	0·3 0·3	0·3 0·3	0·1 0·6	
ov.g.	0·5	0 +	0 +	0 +	0 +	0·5 =	0·5
seg%		0	0	0	0	100	

b.



day	d	$_0x$	$_4x$	$_3x$	$_2x$	$_1x$	ov. g.
0 1	6·1 7·1	5·0 4·8	0·3 0·3	0·3 0·3	0·4 0·5	0·1 1·2	
ov.g.	1·0	0 +	0 +	0 +	0 +	1·1 =	1·1
seg%		0	0	0	0	100	

Table 2.3. Comparison of growth between pin-pointed and non-treated (Control) 1st leaves of summer-grown plants of *Lolium temulentum*

Treatment	Absolute growth rate ($cm.d^{-1}$)[c] d. 0-1	d. 1-2
a) Control	1.67 ± 0.05	1.36 ± 0.11
b) Pointed	0.69 ± 0.07	0.61 ± 0.08
% reduction $\left[\frac{a-b}{a} \times 100\right]$	59	55

c = mean ± se of 10 readings

DISCUSSION

In no cases examined were the leaves measured during a phase of exponential growth - as was demonstrated by the graphs of log-transformed data and RGR. The latter continually decreased with time in a similar way to that previously recorded for blades of this species (Thomas, 1983). This ontogenetic drift of RGR may result from increased use of photosynthate in the formation of strengthening material relative to photosynthetic material and hence a decrease in observed rate of growth (eg Hunt, 1982). It thus appears that all leaves were measured during phase (iii) of growth (see Introduction).

Overall growth of leaves on a given plant showed a trend similar to that seen in the work of Robson (1973) on L. perenne. Generally it appeared that only two leaves were measurably growing at any one time. This is in agreement with the work of Robson (1967) who found that a young leaf did not expand until its predecessor on the same side of the axis had almost ceased expanding.

Attempts at analyzing the components of leaf growth was made using two different sampling procedures. Using non-destructive sampling it was possible to conclude that by emergence the blade had ceased extension growth. Leaf growth was prolonged after emergence as a result of sheath growth, with the result that successive sheaths increased in length up the culm (Table 2.1). The observation that blade extension had ceased by emergence is probably the basis for the association in the literature between leaf (blade) emergence and attainment of maturity of the leaf (blade) (eg Thiagarajah and Hunt, 1982).

Destructive sampling gave much more information regarding leaf growth because the leaf was dissected out of the enclosing sheaths or coleoptile. In this way its component organs could be measured as separate organs far earlier than with non-destructive sampling. Using this technique it was possible to show for the 2nd leaf that sheath growth commenced after blade growth, and that it ceased after blade growth ceased. Further, sheath growth did not begin until after blade growth had begun to decrease (cf also Etter, 1951; Silvy, 1982b). This observation may indicate the existence of interorgan interaction within a leaf during growth.

The concept of 'between leaf' interaction was extended by an examination of growth of the first three leaves of a seedling using destructive sampling. Here the pattern of sheath and blade growth within each leaf was similar to that observed in the 2nd leaf above. On top of this 'within leaf' interaction, a pronounced 'between leaf' interaction was evident - each successive leaf appeared to increase in growth rate as the previous one's was decreasing. It was particularly interesting that the RGR of the 2nd leaf was significantly greater than that of the 1st leaf. If the 1st leaf growth was largely dependent upon grain reserves and 2nd leaf growth used both photosynthate from the 1st leaf and itself (eg Dale and Felippe, 1972) then it is perhaps not surprising that the latter was able to sustain growth at a higher rate. This nutritional consideration may in part explain the 'between leaf' interactions on a culm.

In the two studies above making use of destructive sampling it was found that blade extension growth appeared to continue until just before or just after emergence. This suggests that emergence might be a reasonable guide to the time of cessation of blade growth and hence of its attainment of maturity. In order to try and provide an unambiguous answer to this problem, the LEZ was investigated. In agreement with Begg and Wright (1962) and Davidson and Milthorpe (1966) this study showed that all exserted parts of the blade had finished extension growth and, perhaps more importantly, that this ceased before exsertion. The LEZ appeared to occupy the basalmost 0.1 cm of the leaf. However, the two methods used to determine this had many drawbacks which make such an accurate localization of the LEZ suspect.

Pin-pointing appeared to result in a marked reduction of growth of leaves so treated (Table 2.3). This is in agreement with the work of Davidson and Milthorpe (1966) and immediately places doubt upon the validity of any results obtained with such a method. Pen-marking is a much less harmful method but the high incidence of loss of marks or inability to be certain if the enclosed leaf was marked at all made it a less useful method. The coleoptile was not pulled back to ensure marking of the enclosed leaf (as suggested by Arber, 1934) in order to avoid further damaging the leaf. Another feature of the two methods is the inaccuracy of initial positioning of the marks. Unless the pin or pen is applied exactly at right angles to the long axis of the leaf, the

marks made may be up to 0.2 cm in error. For that reason such an error margin was allowed in the analyses of LEZ results (Fig. 2.10; 2.11). When the seedling is harvested and the shoot severed at the shoot/root junction, it is possible that this does not always occur at the same place used as the zero point for initial marking. In this way the marks may be 'shifted' from their original - assumed! - positions. However, as long as we are concerned with the relative separation between points this is of little consequence but it does affect the apparent length of the LEZ. If it was thought that the lowest mark was 0.2 cm above the shoot/root junction but was actually 0.4 cm above it, and growth only occurred below this point, then the true length of the LEZ will be 0.4 cm and not the apparent 0.2 cm. Inaccuracy on the part of the investigator must also be considered a serious source of error in this sort of study.

Davidson and Milthorpe (1966)found the LEZ to be located within the lower 3 cm of leaves of cocksfoot. Ellis (1981) estimated the LEZ as being 3 cm long in primary leaves of wheat. Both these values are different to that found in this study. The observed differences may be due to species, however, since Ogawa et al. (1980) found the sheath extension zone of rice to be restricted to the basalmost 0.1 - 0.2 cm of the leaf. A more serious doubt of the LEZ of L. temulentum being 0.1 cm long is provided by the work of Kemp (1980) on wheat. This worker found that both blade and sheath extension zones could be treated as a single leaf extension zone even though they were separated by a non-expanding collar region. In L. temulentum the collar region is itself c. 0.1 cm wide and thus the true LEZ must presumably be greater than 0.1 cm here.

However, if we accept that determination of LEZ was performed entirely free from error then for the 1st leaf of L. temulentum it extends upwards from the leaf base for just over 0.1 cm. If we further assume that the LEZ is the same length for all leaves at all stages of growth we can make certain deductions about the timing of cessation of blade growth.

Since the LEZ is confined to the basalmost part of the leaf and is much shorter than the length of the next oldest sheath or the coleoptile, we can conclude that blade growth will have ceased by the time of leaf emergence. This was observed for the 1st leaf using non-destructive

sampling (Fig. 2.4a) and in determination of the LEZ. The higher the leaf is inserted on the plant, the longer the sheath and the further within the enclosing sheath the blade will be when it ceases growth. In the case of the 2nd leaf (Fig. 2.6a), blade growth should have stopped by the time the base of this organ was c. 1.9 cm below the top of the enclosing sheath (ie length of enclosing sheath (2 cm) minus length of LEZ (0.1 cm)). Since the blade final length was c. 12 cm, cessation of blade growth should have occurred at c. 15 d. from sowing (ie when the leaf was c. 12 cm long). According to the graph, it actually ceased growth at c. 18 d. from sowing.

Part of the reason for this discrepancy may be related to the fact that both blade and sheath are growing at this time and thus blade growth may actually cease when the leaf is over 12 cm long. Also, if the LEZ is longer than 0.1 cm - as seems likely in view of the reservations expressed above - blade growth will cease higher within the enclosing sheath. As a consequence, time of cessation of blade growth will get closer to time of emergence. It would thus appear that leaf-marking and leaf growth analysis give comparable results.

The problem of blade growth apparently not ceasing until after emergence remains. This may be related to the small size of populations used in each destructive sampling. If each sample was not a reliable estimate of the population from which it was obtained then its value as a method of growth analysis is diminished. In order to investigate this, sources of seedling variation were examined.

The first source of variation is the observed spread of germination date (Fig. 2.2). Even if all seedlings subsequently behaved in an identical manner this would give rise to the observed spread of leaf emergence increasing with leaf number. When the growth of 1st leaves of individual seedlings was studied (eg Fig. 2.9a), it was clear that a great deal of variation between individuals existed. Normalizing these data with respect to germination date (Fig. 2.9b) might be expected to remove any environmentally-induced variation and leave only that due to the genotype. The amount of variability was not reduced. The variability remaining was considered not to be due to genotypic variation, however, on two counts. Firstly the grain used was homozygous. Secondly since the environment was not constant from day to day or even seedling to seedling, all that was achieved by normalizing the data was

to plot them from the same point. It did not eliminate phenotypic variation between individuals because the environment experienced by a seedling germinating on day 1 may be completely different from another germinating on day 2.

Another source of variation may be related to the observation that grass embryos frequently contain 3 or 4 leaf primordia (eg Cooper, 1964). Such primordia may have been initiated under different conditions from each other and from primordia of different embryos and subsequently behave differently. Hence phenotypic variation of seedlings is not only related to the environment of post-germination growth but also to that experienced prior to grain shedding. A good example of this is provided by the work of Silvy (1982b) on barley where the length of the 1st leaf was highly correlated with the weight of the grain from which it developed.

Thus regardless of whether each grain has an identical genotype makes very little difference; it is the interaction of that genome with the environment - both external and internal - which determines growth. The more variable the growth conditions, the more variety we might expect in the plants produced. For this reason the size of destructive sampling populations was probably too small to be a reliable estimate of the growing population from which they were taken. The sample sizes for non-destructive analyses were far larger than these, and in fact used the whole growing population, and were consequently much more reliable.

This study of leaf growth began with the simple question, when does blade growth stop? In attempting to answer it we have come a long way from such 'simple' considerations and have identified several areas of complex interactions within and between leaves. The former may be postulated to take place in the small basally-located LEZ since it is here that cell number and growth is determined. Its basis, however, is not known. In considering 'between leaf' interactions we are faced with a problem of equal magnitude.

It might be suggested as a result of this investigation that we know a little about when blade growth stops; we are no nearer knowing why it stops. Begg and Wright (1962) implied that phytochrome might be involved; phytochrome appears to be implicated in extension growth of the 1st leaf of oats (Schopfer _et al._, 1982). The situation regarding

the sheath is equally unclear. Dobrynin (1960) suggested that exsertion of the ligule might cause cessation of sheath growth. This was refuted by Dudinsky and Boiko (1970) who considered the two events to be merely coincidental. The results here show that sheath growth continues after ligule exsertion; exsertion of the latter organ is neither coincident with cessation of growth of the sheath nor does it stop it. It might, however, be suggested that the ligule 'affects' sheath - or even blade - growth. We know so little about this leaf organ that all such suggestions should be given careful consideration.

SECTION 6. ASPECTS OF LEAF AGEING AND SENESCENCE

INTRODUCTION

Simon (1967) identified three types of natural leaf senescence:

i. progressive or sequential senescence - where older leaves senesced as younger leaves expanded above them;

ii. synchronous senescence - where all the leaves of a tree or shrub senesced at the same time;

iii. overall senescence - where the whole plant senesced after flowering.

He further identified two types of artificially-induced 'senescence' of excised leaves held either in the dark or the light and concluded that, 'the pattern of senescence depends to an important degree on the way in which senescence has been brought about'.

In the case of annual grasses concerned with in this Section, natural senescence takes two forms; sequential senescence of older leaves up the culm, and overall senescence of remaining living leaves and tissues after flowering. To compound further the problems of investigating these phenomena, since 1967 a number of additional types of 'artificial' senescence have been used - eg leaves attached to the plant but held in the dark (Thimann *et al.*, 1974), excised blade segments (Thimann, 1980), excised portions of culm with attached leaves (Lazan *et al.*, 1983).

Despite a great amount of work having been performed on aspects of grass leaf senescence (see reviews by eg Thimann, 1980; Thomas and Stoddart, 1980; Stoddart and Thomas, 1982), the results obtained, and the conclusions drawn, have largely been based upon work with such artificial systems. Until such time as it is established that natural and artificially-induced senescence are one and the same process, it will not be possible, 'to make full use of the existing literature on leaf senescence because this includes information about leaves caused to senesce under very different conditions' (Simon, 1967).

In view of this it is perhaps surprising that Thimann (1980) has stated that, 'to study the senescence of leaves that are still attached to the plant is to invite complications from:

1. metabolite transfer in and out of the leaves from the other organs of the plant,

2. cytokinin formation in the roots, auxin production in adjacent younger leaves, or other hormonal influences, and,
3. such unknown materials as the postulated "senescence factor" of hormonal type, which may be postulated to control senescence in the whole plant'.

Although these considerations were offered by Thimann as justification for using excised leaf systems, they seem equally to make the case against use of such artificial systems. It is the very existence of 'complications' from other organs of the plant which makes study of natural, attached leaf senescence so necessary.

While excised leaf systems may possibly give information concerning the changes that occur during senescence, it is only the attached leaf situation which can be expected to give meaningful answers regarding questions concerning the control of this process. Interactions appear to occur within and between leaves during their growth (see the previous Section). Leaves are an integral part of the plant and it is to be expected that a process as important as senescence would show some sort of correlation or interaction within and between different organs or leaves. A number of workers have recorded cases where tiller/tiller interactions (Gifford and Marshall, 1973), root/leaf interactions (Kao, 1979) and ear/leaf interactions (Feller, 1979) have affected the course and timings of senescence in grasses.

Undoubtedly, because of such interactions, attached whole leaves are more difficult to work with than blade segments floating on water in a petri dish, but they must be studied, and ideally under natural conditions. Knowledge of the course of attached leaf senescence under growth room conditions is of value in identifying possible environmental constraints upon the process. Ultimately, however, it is the way in which the plant in the field responds to its natural environment that is important eg in determining grain yield or herbage production.

Even in the few cases where attached grass leaf senescence has been studied under natural conditions (eg Hall et al., 1978), it is not always clear what is meant by 'leaf'. Usually it appears to refer to the blade only; the sheath is generally ignored. This neglect of the latter seems an undesirable practice on two counts. Firstly it overlooks

the possibility of blade/sheath interactions during ageing and senescence. Secondly it totally disregards an organ which can comprise up to half the potential photosynthetic area of the leaf (eg Thorne, 1959; Borrill, 1961). By their very nature, blades are attached to sheaths which in turn attach the leaf to the rest of the plant. If one proposes that the products of eg senescence-associated proteolysis are translocated from the blade back into the rest of the plant then it would seem important to know what happens in the sheath during ageing and senescence of the leaf. The timings of any such changes relative to those of the blade would be of relevance to determining the active life of the leaf, whether as a photosynthetic organ or a source of metabolites during senescence.

One grass which has been extensively studied from the point of view of leaf (mainly blade) ageing and senescence is L. temulentum (eg Hedley and Stoddart, 1972; Pollock and Lloyd, 1978; Thomas, 1978). The majority of this previous work has been performed using artificial systems of one kind or another. In view of this it was decided to study the course of attached leaf (blade and sheath) ageing and senescence of the same species and compare the results obtained with those available in the literature. The changes recorded for this species are very similar to those in other grasses, thus the results from the present study could be compared with 'artificial' grass leaf senescence generally. It was also hoped to undertake this study in a more natural environment than is customary in investigations of this sort. To this end various aspects of the biochemical changes occurring in the attached 4th leaf of L. temulentum were studied from cessation of leaf extension to full senescence.

MATERIALS AND METHODS

It was not possible to perform all operations on the same leaf; three separate groups of plants were thus used at each sampling point:

a. fresh and dry weights (1 leaf for each sample),

b. soluble protein and total chlorophyll (3 leaves bulked together for each sample),

c. quantitative and qualitative determination of nucleic acids (3 leaves bulked together for each sample).

In addition, the leaf was divided up into four segments lengthwise:

Ba - top half of blade;

Bb - bottom half of blade;

Sa - top half of sheath;

Sb - bottom half of sheath.

For convenience the division between blade and sheath was taken as the top of the collar; thus the ligule was included in the segment Sa.

Fresh Weight (FW)

Each leaf segment was weighed immediately after excision.

Dry Weight (DW)

Weighed leaf segments were stored individually in cellophane bags and dried at c. 70°C, in a well-ventilated oven until constant weight was achieved.

Dry weight as a percentage of fresh weight (D%F) values were computed for each leaf segment.

Total Chlorophyll (Chl)

Following weighing, leaf segments were cut up into a pestle and mortar and treated sequentially as follows:

a. homogenized in 1.0 ml extraction buffer (100 mM trizma base; 53 mM sodium acetate; 8.8 mM magnesium acetate, pH 7.2); the homogenate poured into a centrifuge tube;

b. a further 1.0 ml extraction buffer was added to the pestle and mortar; the washing was combined with the homogenate in a. above;

c. the homogenate was centrifuged at c.2000 x g for 10 min at room temperature in a bench top centrifuge (as applies to all subsequent centrifugation steps);

d. the supernatant from c. above was decanted into a centrifuge tube containing 1.0 ml, 10% (w/v) aq. TCA and stood in the dark at $4^{O}C$ for 30 min;

e. the pellet from c. above was resuspended in 90% (v/v) methanol (25 ml, blade segments; 5 ml, sheath segments) in a centrifuge tube and stood in the dark at $4^{O}C$ for 1 h;

f. the suspension from e. above was centrifuged, the supernatant decanted into a volumetric flask and made up to volume with 90% methanol;

g. the methanolic extract from f. above was scanned in a Pye Unicam SP800 spectrophotometer against a 90% methanol blank. Chlorophyll concentration was calculated from the equation of Holden (1965):

$$\text{total chlorophyll (mg. } l^{-1}) = 25.5\ A_{650} + 4.0\ A_{665}$$

A typical absorption spectrum of methanolic chlorophyll solution is shown in Fig. 2.12a.

Soluble Protein

a. the solution from Chlorophyll step d. was centrifuged, the supernatant discarded and 1.0 ml M NaOH added to the pellet;

b. following standing at $30^{O}C$ for 30 min in a water bath, the solubilized protein was determined by the method of Lowry et al. (1951) using the following reagents:

A. 2 g sodium, potassium tartrate; 100 g anhydrous Na_2CO_3 in 500 ml M NaOH; distilled water to 1 l.

B. 2 g sodium, potassium tartrate; 1 g anhydrous $CuSO_4$ in 90 ml distilled water; 10 ml M NaOH.

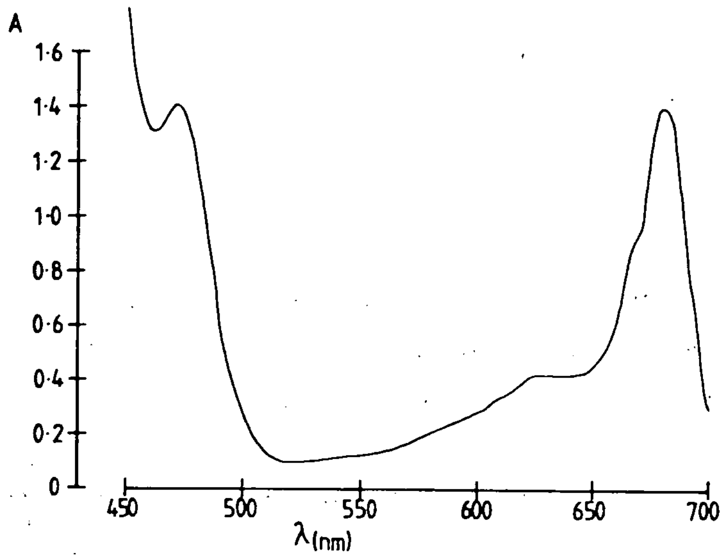
C. Folin-Ciocalteau's phenol reagent as
1 volume reagent : 15 volumes distilled water.

Figure 2.12. Aspects of ageing and senescence of the 4th leaf of *Lolium temulentum*: chlorophyll and soluble protein estimation

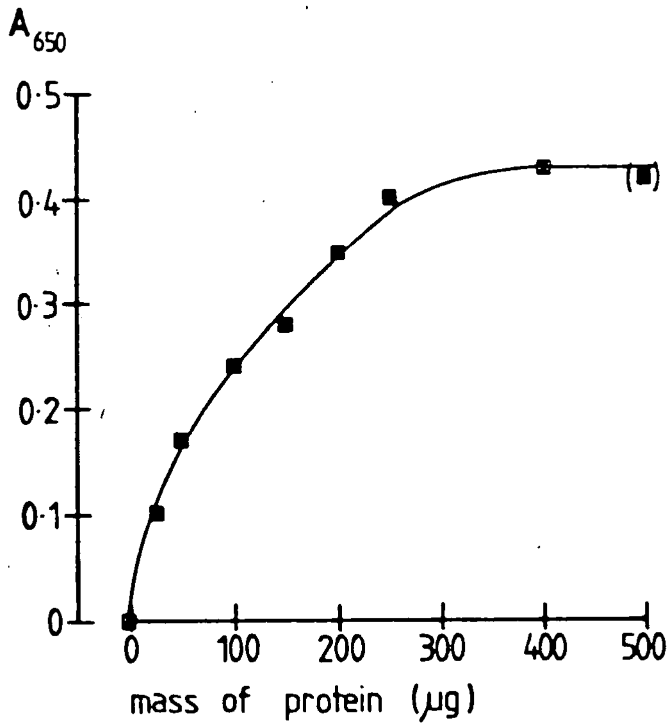
a. Absorption spectrum of methanolic chlorophyll solution from blade tissue;

b. Standard curve for soluble protein estimation

a.



b.



The procedure was as follows:

a. 0.05 and 0.1 ml samples of the protein solution were taken and made up to 1.0 ml with distilled water;

b. 0.9 ml reagent A added, the solution shaken and stood for 10 min in a 30°C water bath;

c. 0.1 ml reagent B added, the solution shaken and stood for at least 10 min at room temperature;

d. 3.0 ml reagent C added with immediate mixing using a 'Whirlimix', the solution stood for 10 min in a 30°C water bath;

e. absorbance of the blue-coloured solution was measured at 650 nm in the spectrophotometer against a blank consisting of 1.0 ml distilled water treated as the samples.

A standard curve (Fig. 2.12b) was prepared using BSA V made up in M NaOH. As a check on reproducibility of the method, 50 and 100 μg samples of BSA V were treated along with the leaf protein samples. The BSA V samples always gave absorbances close to those expected from the standard curve. Leaf protein samples were diluted with distilled water where necessary to fall on the rising part of the standard curve.

Nucleic Acids (NA's)

Following weighing, leaf segments were cut up into a pestle and mortar and treated sequentially as follows:

a. homogenized between each addition of, 0.08 ml diethylpyrocarbonate, 0.22 ml sodium dodecyl sulphate (SDS) (100 g. l^{-1}), 1.10 ml extraction buffer (see Chlorophyll step a.); the homogenate was poured into a centrifuge tube:

b. a further 1.10 ml extraction buffer was added to the pestle and mortar; the washing was combined with the homogenate from a. above;

c. NaCl to 0.1% (w/v) was added to the combined homogenate from a. and b. above and centrifuged; the supernatant was decanted into a centrifuge tube containing 7.5 ml 100% ethanol, the top sealed with parafilm and stored at -20 C overnight;

d. the following day the ethanolic NA solution was centrifuged, the supernatant discarded and the precipitate allowed to air-dry before being dissolved in 1.0 ml (blade samples) or 0.5 ml (sheath

samples) of 'E' buffer solution (40 mM trizma base; 20 mM sodium acetate; 2.5 mM magnesium acetate, pH 7.2);

e. the NA solution was further centrifuged to clear if necessary and the supernatant retained for NA determination.

Quantitative NA Determination

0.1 ml of the NA solution from e. above was made up to 3.0 ml with distilled water and scanned in a spectrophotometer against a distilled water blank. NA concentration was calculated from the following relationship:

$$(A_{260} - A_{290}) \text{ of } 1 \text{ mg.ml}^{-1} \text{ RNA} = 22.0$$

Qualitative NA Fractionation

This was performed by polyacrylamide gel electrophoresis (PAGE).

Perspex tubes (9 cm x 0.7 cm internal diameter) were arranged vertically in the electrophoresis tank. Their lower ends were sealed with distilled water-soaked dialysis membrane secured by rubber rings. Closed rubber bungs were fitted over the membranes forming an air seal to prevent the unpolymerized gel solution passing through the membrane before setting. 2.6% gel solution was prepared according to the method of Loening (1967) as modified by Jupp (1979) using the following recipe:

4.33 ml monomer solution (150 g acrylamide; 7.5 g bis-acrylamide. l^{-1}),

12.11 ml distilled water,

8.32 ml '3E' buffer (120 mM trizma base; 60 mM sodium acetate; 7.5 mM magnesium acetate, pH 7.2).

Following the addition of 0.2 ml 10% (w/v) aq. ammonium persulphate and 0.02 ml TEMED, the gel solution was carefully pipetted down the side of the perspex tubes to a depth of 7 cm. Upon polymerization of the gel, the rubber bungs were removed and both ends of the perspex tubes covered with 3E buffer diluted 1 : 2 with distilled water and containing 2 g SDS. l^{-1}. Gels were pre-electrophoresed for c. 45 min at 6 mA. gel^{-1} to equilibrate the composition of the gels with that of the buffer in the tank and thus give a continuous buffer PAGE system. The specific gravity of NA solutions from Nucleic Acids step e. above was increased

by addition of sucrose and samples containing 10 or 20 μg NA were carefully layered onto the top of the gels. Gels were then electrophoresed for c. 3½ h at 6 mA. gel^{-1} after which time the gels were removed from the tubes and allowed to soak in 7% (v/v) aq. acetic acid for up to 15 h. Gels were scanned at 265 nm in a Joyce Loebl Polyfrac linked to a potentiometric recorder upon which a trace of the scan was obtained. To confirm the identity of peaks on the scan as NA, gels were stained in 0.2% (w/v) toluidine blue; in all cases peaks on the scan accorded with toluidine blue-staining bands on the gels and vice versa.

NA Identification and MW Determination

A typical trace of blade NA is shown in Fig. 2.13a. Characteristically 5 peaks were found; peak 'X' was only observed occasionally. A similar trace was obtained for sheath tissue. In order to identify the chemical nature of the NA peaks, samples of blade NA in E buffer were incubated for 5 min with either c. 10 μg RNAse or DNAse at room temperature and then electrophoresed. In the former (Fig. 2.13c) all but peak 1 were lost and it was concluded that peaks 2-5 and X were RNA. In the case of DNAse all peaks were lost (Fig. 2.13d); it was concluded that peak 1 was DNA and that the DNAse was contaminated with RNAse.

Confirmation of the intracellular origin of the RNA's identified above was obtained by comparison of NA traces of root (eg Fig. 2.13b) with leaf tissue (eg Fig. 2.13a). Root tissue is generally devoid of chloroplasts and chloroplastic ribosomal RNA (eg Loening and Ingle, 1967). It was thus concluded that peaks 3 and 5 - only seen in leaf tissue - were chloroplastic, and peaks 2 and 4 - common to leaf and root - were cytoplasmic.

In order to determine the apparent MW's of the RNA species in leaf tissue, samples of blade NA were co-electrophoresed with ribosomal RNA's isolated from E. coli. The values of E. coli RNA MW's were taken as:

16S - 0.55×10^{6} D;

23S - 1.03×10^{6} D (Carmichael and McMasters, 1980).

Figure 2.13. Aspects of ageing and senescence of the 4th leaf of Lolium temulentum: characterization of NA species by PAGE on 2.6% gels.

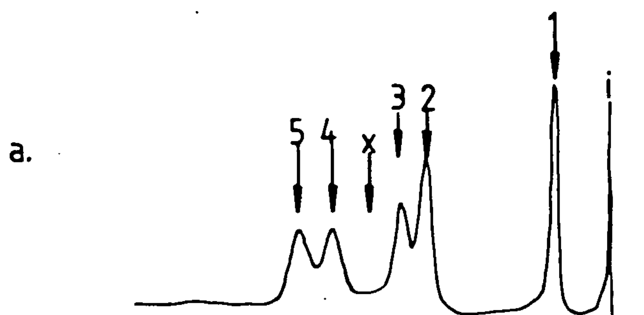
a. NA species from mature blade tissue;

b. NA species from root tissue;

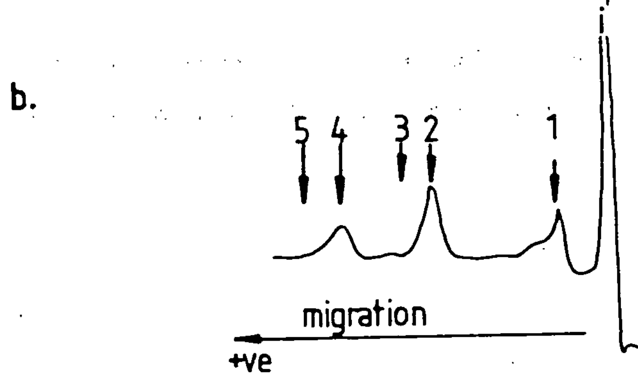
c. Blade NA after incubation with RNAse;

d. Blade NA after incubation with DNAse

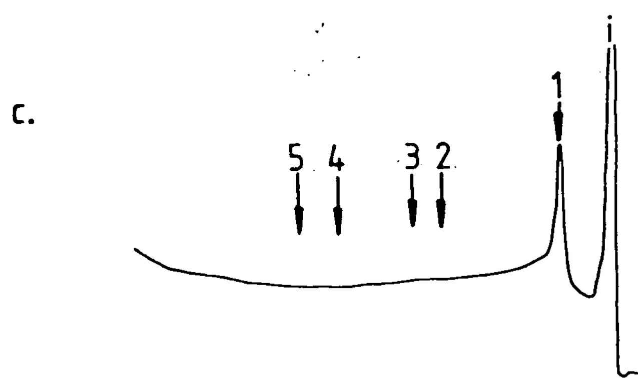
a.



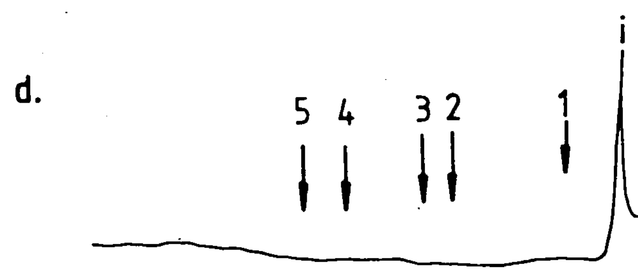
b.



c.



d.



From the relationship:

Log_{10} MW $\propto$ distance travelled in gel (eg Loening, 1969), Fig. 2.14 was drawn and MW's for leaf RNA species calculated:

peak 2 - 1.28 x 10^6 D - heavy cytoplasmic;
" 3 - 1.09 " - heavy chloroplastic;
" 4 - 0.69 " - light cytoplasmic;
" 5 - 0.53 " - light chloroplastic;
" X - 1.01 "

MW's agreed well with those obtained by Loening (1968) for a number of plant species, and Jupp (1979) for Festuca pratensis. The value for X agreed reasonably well with that obtained by Grierson (1974) for a breakdown product which appeared in a similar position in Phaseolus aureus from cleavage of the heavy chloroplastic RNA molecule. It was not consistently present and not studied further.

Presentation of Data

Time Scale

The emergence curve for the 4th leaf of the plant population used in this study (Fig. 2.15) showed a spread of 38 d. Because of this it was considered unrealistic to use the date of beginning of emergence as the starting point for the time scale against which to express the biochemical data. The date at which 50% emergence ($E_4$50%) was attained (69 d. from sowing) was used as zero time (t = 0); sampling before 69 d. from sowing has a -ve 't' value, sampling after 69 d. from sowing has a +ve 't' value.

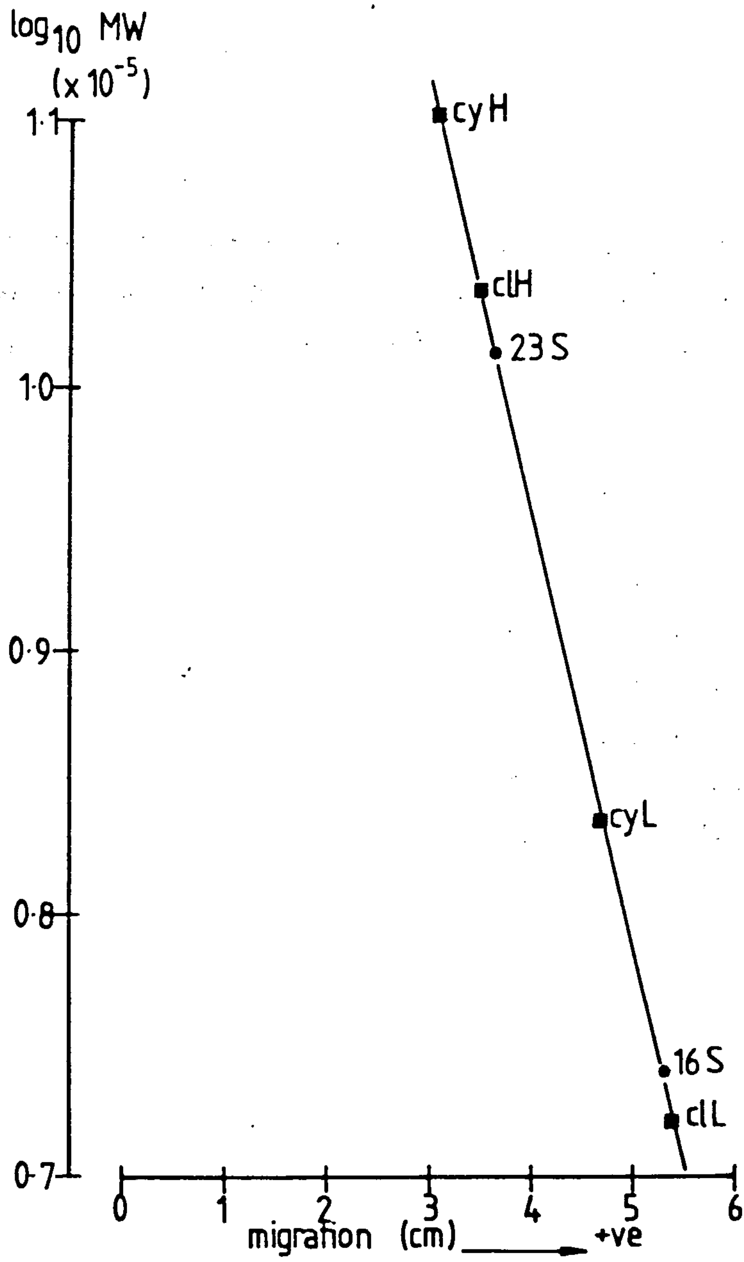
Basis for Expression of Data

At all sampling points amounts of cellular components were calculated on both FW and DW bases. It was subsequently found that FW altered during ageing; DW did not and accordingly all values are expressed on a DW basis. It was not possible to measure the DW of the leaf material used for the biochemical analyses; values were thus obtained on a FW basis for each sampling point and converted to DW basis using the D%F value at that sampling point:

Figure 2.14. Aspects of ageing and senescence of the 4th leaf of Lolium temulentum: plot of Log_{10} MW of leaf RNA species separated by PAGE on 2.6% gels against their migration distances in the gel.

E. coli RNA used as standards

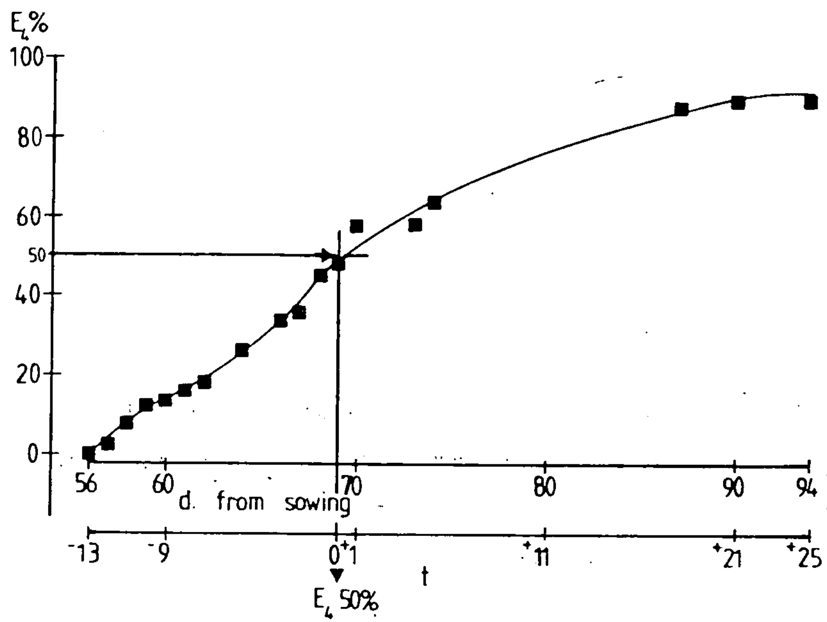
cyH heavy cytoplasmic RNA;
clH heavy chloroplastic RNA;
cyL light cytoplasmic RNA;
clL light chloroplastic RNA;
23S heavy E. coli cytoplasmic RNA;
16S light E. coli cytoplasmic RNA



log10 MW
(x 10-5)
1·1
1·0
0·9
0·8
0·7
cyH
clH
23 S
cyL
16 S
clL
0
1
2
3
4
5
6
migration (cm)
+ve

Figure 2.15. Aspects of ageing and senescence of the 4th leaf of *Lolium temulentum*: 4th leaf emergence curve

For further explanation see text.



E_4%
100
80
60
50
40
20
0
56
60
70
80
90
94
d. from sowing
-13
-9
0
+1
+11
+21
+25
t
E_4 50%

eg, t = +67, total Chl for Bb = 0.55 $mg.gFW^{-1}$,
D% F value for Bb at t = +67 is 20.74% which = c. 0.21,

$$mg.gFW^{-1} \times \frac{1}{D\%F} = mg.gDW^{-1},$$

$$\text{thus } 0.55 \times \frac{1}{0.21} = c.\ 2.62\ mg.gDW^{-1}$$

In the majority of cases, sheath values were much lower than blade values. For ease of comparison between blade and sheath it was desirable to plot data on the same graph. This was achieved by treating initial values at t = -2 (first sampling point) as being 100% and relating the other values to it.

Thus the graphs presented below take the form:

% cellular component (DW basis) against t.

DW and FW were plotted as weight of leaf segment or organ (mg) against t.

D%F values were plotted as % against t.

For cellular components two forms of graph are given:

a. the individual leaf segments plotted separately;
b. the average of (Ba + Bb) and (Sa + Sb) plotted as blade versus sheath, lines were fitted by eye.

Sampling Frequency

Sampling was performed at:
t = -2, +4, +19, +28, +39, +53, +67, +81, +94; all leaves sampled had emerged.

Ba was not sampled at t = +67 or +81.

Growing Period

Grain was sown on 9/X/81; the last sampling point (t = +94) was on 16/III/82.

Replication

At each sampling point, FW and DW was determined in quadruplicate; all other measurements were determined in triplicate. Means and standard errors were calculated at each sampling point for each leaf segment. In 'leaf segment' graphs mean values are plotted; in 'blade versus sheath' graphs the values plotted are averages of the two individual means for each half of the organ at that sampling point.

RESULTS

Leaf Growth

Growth of the 4th leaf used here was similar to that described for other leaves (see previous Section); sheath extension growth ceased c.1 d. after leaf emergence.

Dry Weight

Values of DW for segments (Fig. 2.16a) and organs (Fig. 2.16b) were more or less constant throughout the sampling period.

Fresh Weight

For segments (Fig. 2.17a), FW was initially more or less constant before beginning to decrease in a basipetal manner, at t = +40 (Ba), +50 (Bb), +65 (Sa, Sb). The values for Sa and Sb were so similar it was not possible to determine which decreased in FW first - if such a difference existed.

The average values for each organ are shown as bold lines in Fig. 2.17b. Both are similar in shape to the individual curves in Fig. 2.17a from which they were derived; decrease of blade FW beginning before that of sheath. A regression line (broken line in Fig. 2.17b) for blade values shows a -ve correlation with time from leaf emergence (significant at the 0.1% level) which can be interpreted as indicating constant loss of FW of this organ with time.

Dry Weight as a Percentage of Fresh Weight

The curves for average organ D%F values are shown in Fig. 2.17c; they are almost the inverse of those for average organ FW in Fig. 2.17b, and show constant values to begin with before marked increases beginning at t = +40 (blade) and +65 (sheath). Since the values of average organ DW were more or less constant throughout the sampling period, increase in D%F must be related to water loss from the leaf tissue. Hence the final D%F values for blade and sheath of c. 90% indicate almost total drying out of the tissue whilst still attached to the plant.

In order to analyze this situation further, D%F values were calculated for each segment, and for the averages of (Ba + Bb + Sa), (Bb + Sa + Sb) and (Ba + Bb + Sa + Sb), and plotted against 't' in

Figure 2.16. Aspects of ageing and senescence of the 4th leaf of Lolium temulentum: dry weight

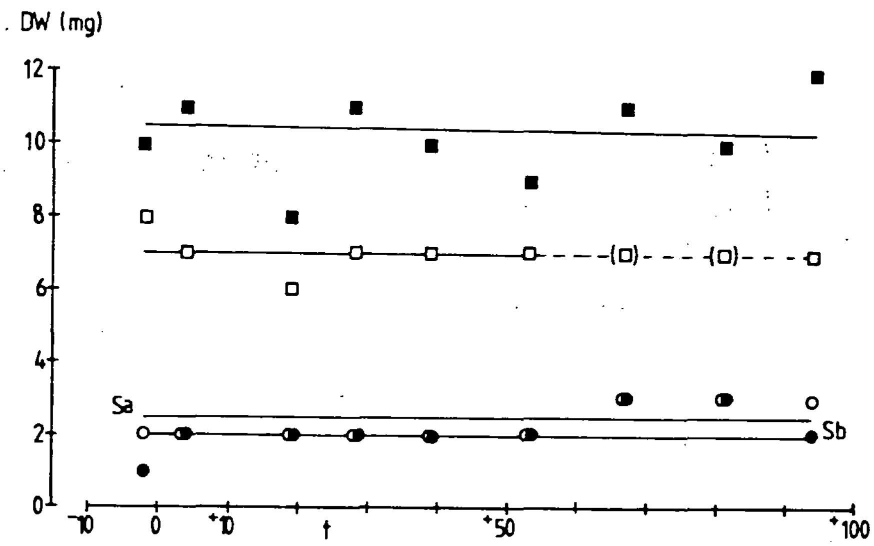
a. Individual leaf segment curves;

Ba □
Bb ■
Sa ○
Sb ●

b. Averages of blade and sheath values;

blade ■
sheath ●

a.



b.

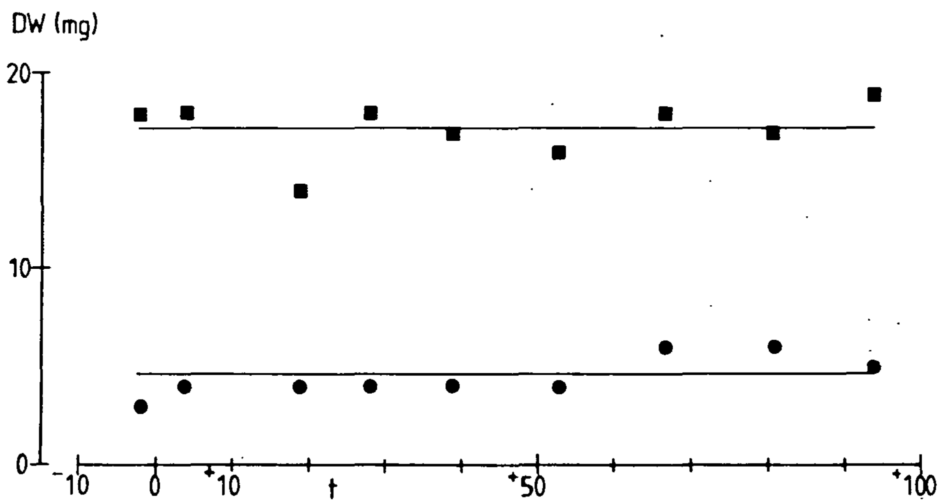


Figure 2.17. Aspects of ageing and senescence of the 4th leaf of *Lolium temulentum*: fresh weight, and dry weight as a percentage of fresh weight

a. FW of the individual leaf segments;

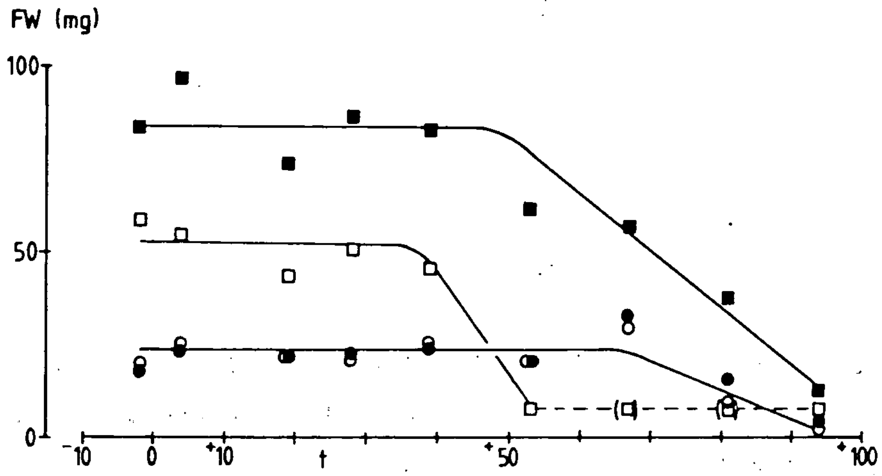
Ba □
Bb ■
Sa ○
Sb ●

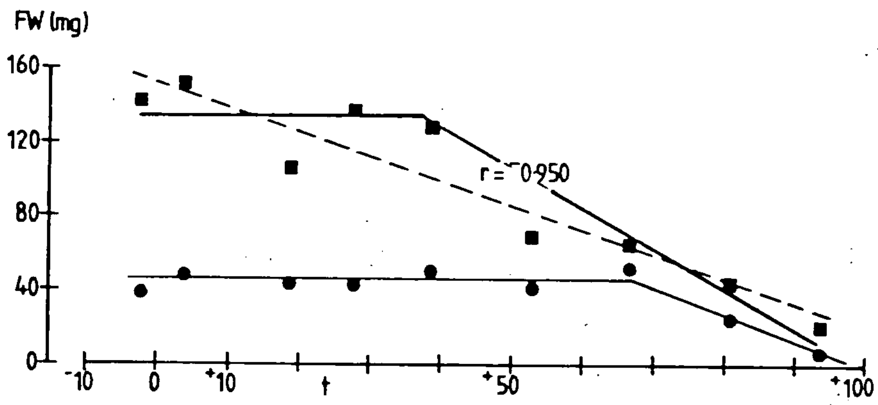
b. FW of the averages of blade and sheath values;

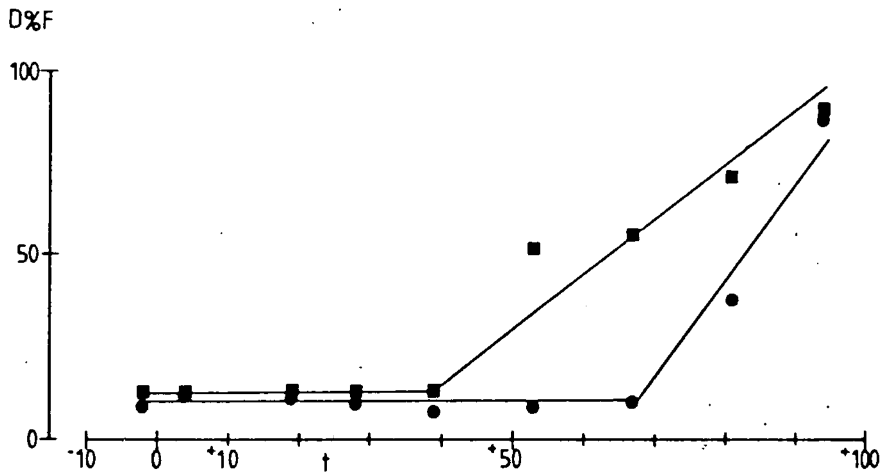
blade ■
sheath ●

c. D%F of the averages of blade and sheath values;

blade ■
sheath ●


a.
FW (mg)
100
50
0
-10
0
+10
t
+50
+100


b.
FW (mg)
160
120
80
40
0
r = -0·950
-10
0
+10
t
+50
+100


c.
D%F
100
50
0
-10
0
+10
t
+50
+100

various combinations (Fig. 2.18a-d). The shapes of the resultant curves were similar to each other and to those for the average organs in Fig. 2.17c. In each case the more apical leaf portions began to lose water (increase D%F) before the more basal leaf portions. These results are interpreted as indicating gradual loss of water from the whole leaf in a basipetal manner from leaf tip to base with no sign of disjunction between blade and sheath.

Soluble Protein

All segments followed the same overall pattern (Fig. 2.19a), with a decrease in soluble protein content with time. Ba lost protein most rapidly and it was barely detectable at t= +53. The other three segments reached minimum values at about the same time, t = +81. The only 'odd' point appears to be at t = +67 for the sheath segments and may indicate an 'extraction phenomenon'. Average organ values (Fig. 2.19b) showed patterns of loss which were very similar to each other - a gradual decrease in soluble protein content with time, reaching minimum values at t = +81. Both organs appeared to begin to decrease in soluble protein content at the same time.

Total Chlorophyll

Patterns of change for Chl were quite distinct between blade and sheath, and the individual segment curves (Fig. 2.20a) were closely paralleled by the average organ curves (Fig. 2.20b). Generally blade segment values decreased with time; Ba began to decrease before, and at a greater rate than, Bb and minimum values were reached at t = +53 and +81 respectively. In contrast, sheath segment values increased to t = +67 before a precipitous decrease to a minimum value at t = +81 (Sb) and +94 (Sa). Although both Sa and Sb showed similar patterns, the magnitude of change in the latter was much greater. It is noteworthy that both organs reached minimum values at t = +94 but the patterns of change were very different. For the blade, the pattern of Chl loss paralleled that of soluble protein (compare Fig. 2.20b with Fig. 2.19b); this was not the case for the sheath.

Nucleic Acids - Quantitative Changes

Analysis of the changes in total NA appeared to be overshadowed by the marked decrease that occurred for each segment at t = +28 (Fig. 2.21a),

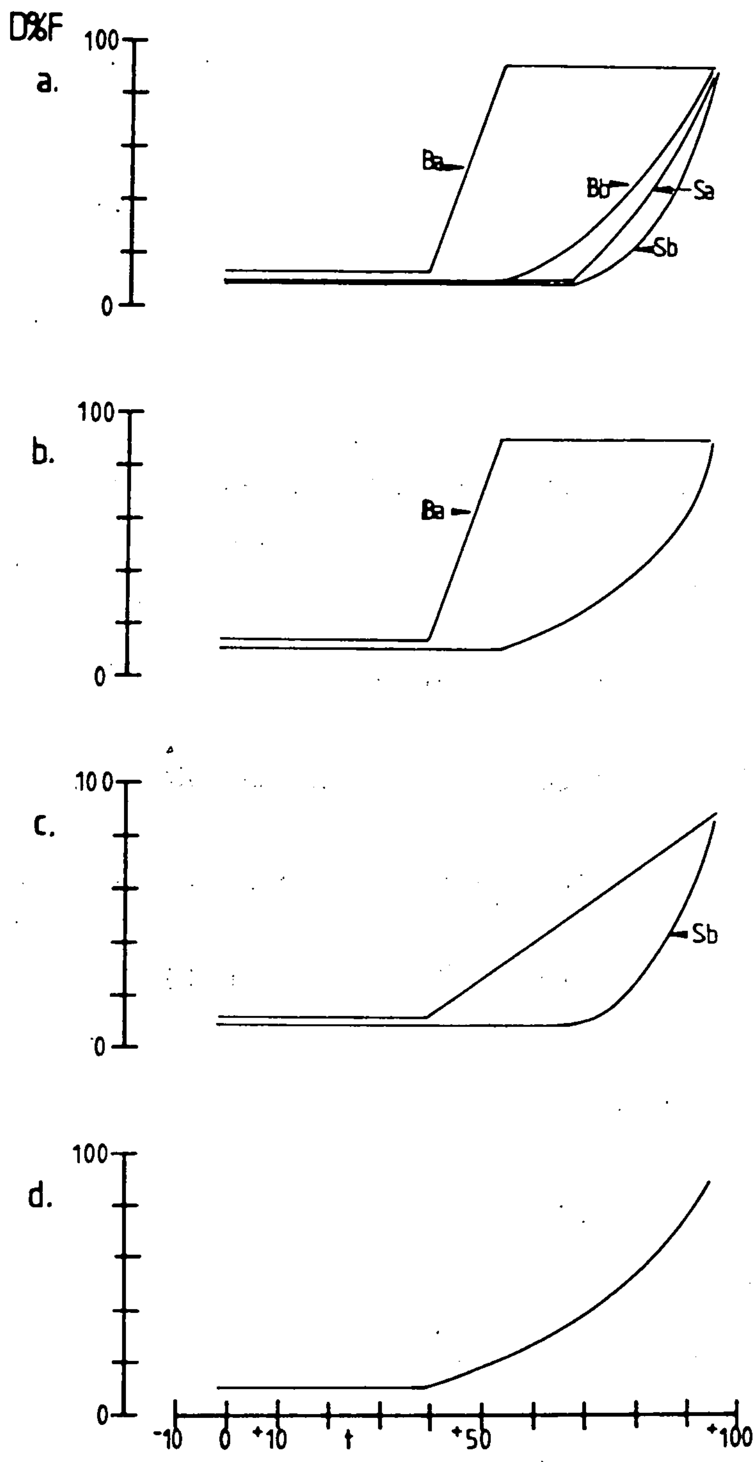
Figure 2.18. Aspects of ageing and senescence of the 4th leaf of Lolium temulentum: dry weight as a percentage of fresh weight

a. Individual leaf segment curves;

b. Values of Ba plotted against the averages of (Bb + Sa + Sb);

c. Values of Sb plotted against the averages of (Ba + Bb + Sa);

d. Curve of the averages of (Ba + Bb + Sa + Sb)



D%F
100
a.
0
Ba
Bb
Sa
Sb
100
b.
0
Ba
100
c.
0
Sb
100
d.
0
-10
0
+10
t
+50
+100

Figure 2.19. Aspects of ageing and senescence of the 4th leaf of *Lolium temulentum*: soluble protein

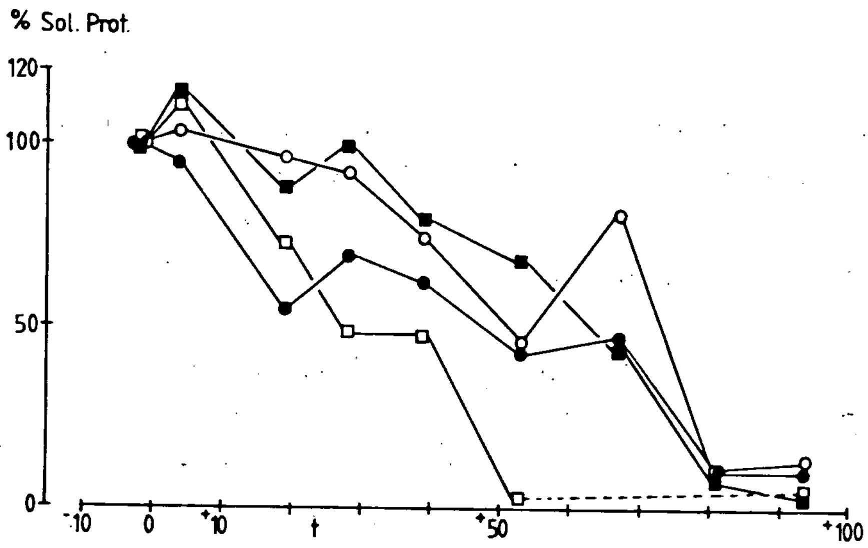
a. Individual leaf segment curves;

Ba	□	(initial	value,	t = -2:	106	$mg.gDW^{-1}$	)
Bb	■	("	"	"	54	"	)
Sa	○	("	"	"	28	"	)
Sb	●	("	"	"	40	"	)

b. Averages of blade and sheath values;

blade ■
sheath ●

a.



b.

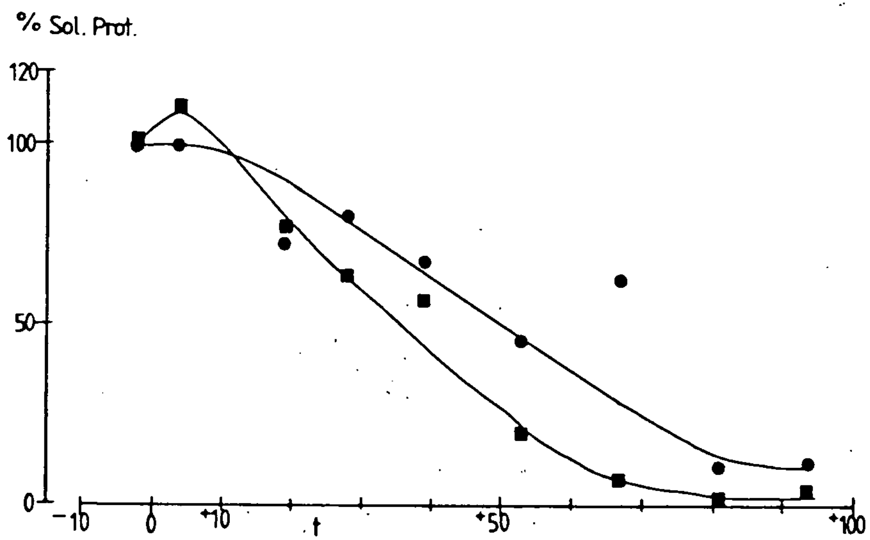


Figure 2.20. Aspects of ageing and senescence of the 4th leaf of <u>Lolium temulentum</u>: total chlorophyll

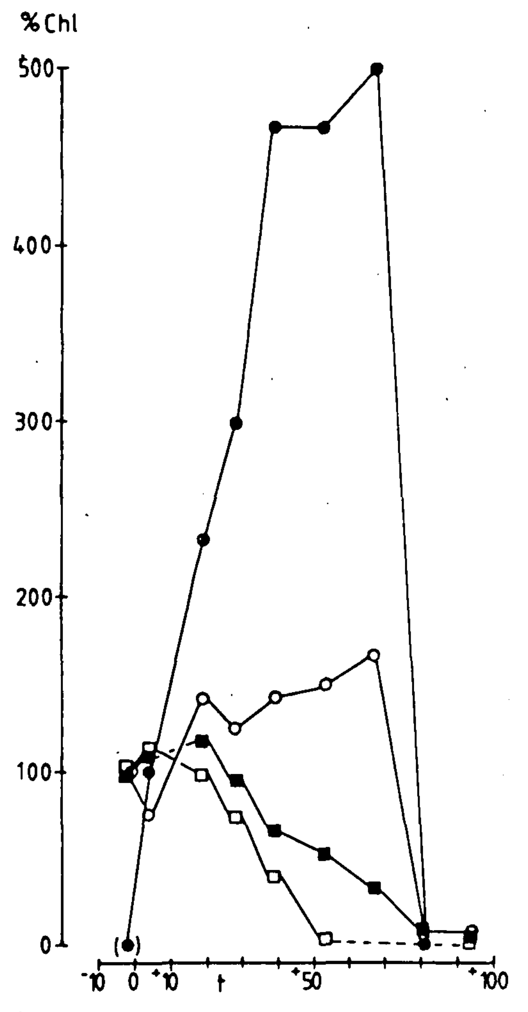
a. Individual leaf segment curves;

Ba	□	(initial value, t = -2: 13.6 $mg.gDW^{-1}$)
Bb	■	(" " " 8.3 ")
Sa	○	(" " " 1.2 ")
Sb	●	(" " t = +4: 0.3 " ; none detectable at t = -2)

b. Averages of blade and sheath values;

blade ■

sheath ●


a.
% Chl
500
400
300
200
100
0
-10
0
+10
t
+50
+100

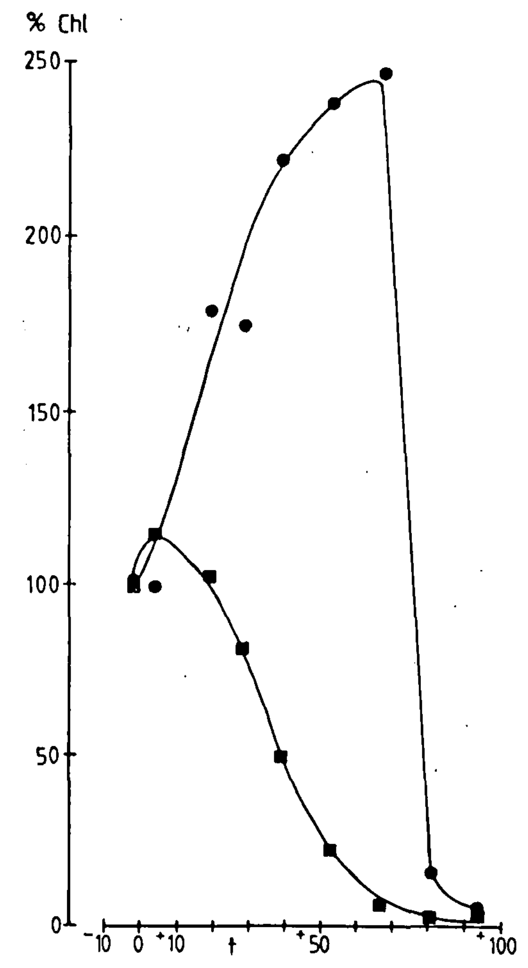

b.
% Chl
250
200
150
100
50
0
-10
0
+10
t
+50
+100

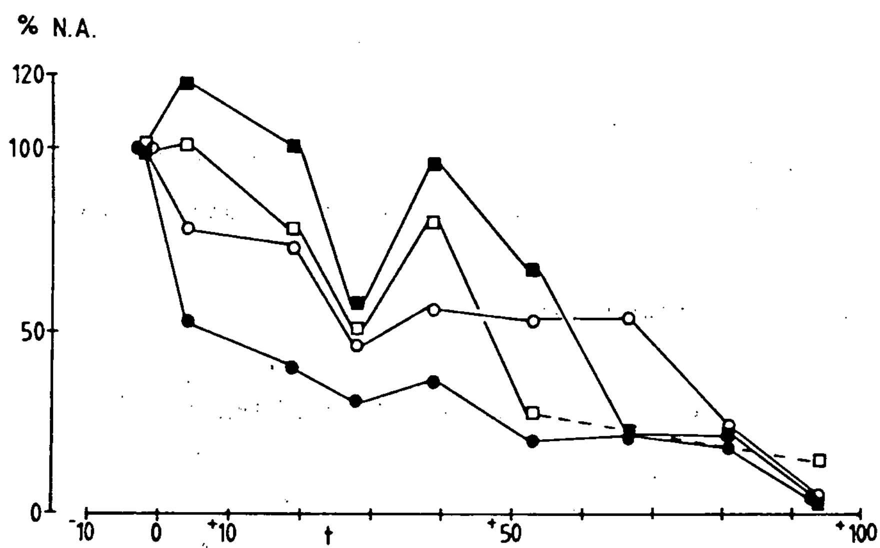
Figure 2.21. Aspects of ageing and senescence of the 4th leaf of *Lolium temulentum*: nucleic acids (quantitative)

a. Individual leaf segment curves;

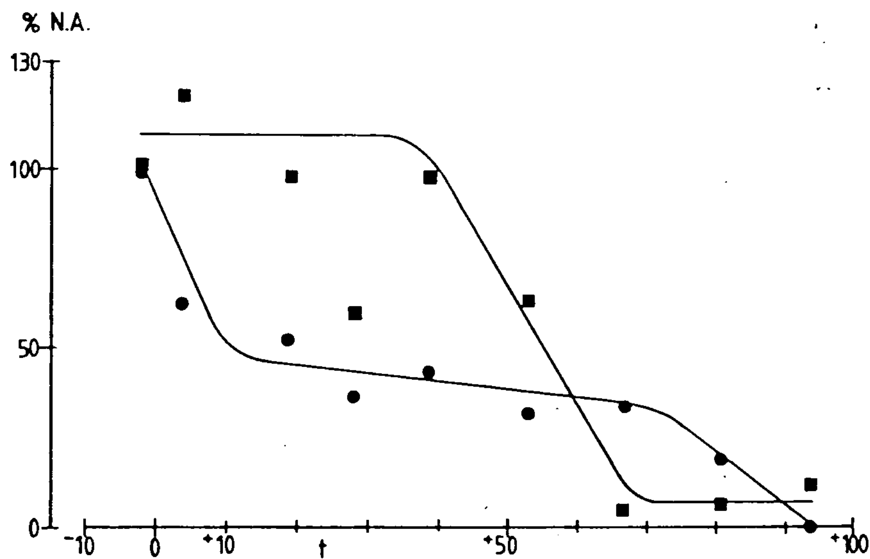
Ba	□	(initial value, $t = -2$: 11.1 $mg.gDW^{-1}$)
Bb	■	(" " " 7.6 ")
Sa	○	(" " " 5.9 ")
Sb	●	(" " " 11.3 ")

b. Averages of blade and sheath values;

blade	■
sheath	●



a.
% N.A.
120
100
50
0
-10
0
+10
t
+50
+100



b.
% N.A.
130
100
50
0
-10
0
+10
t
+50
+100

the magnitude of which decreased in the order Ba, Bb, Sa, Sb. It is not known whether this was a real or an extraction phenomenon. In general the segments showed either an initial increase (Ba, Bb) or decrease (Sa, Sb) before attaining a more or less steady value - ignoring the '+28 drop' - before a final decrease. In the blade segments, this final decrease occurred at the same time for both (t = +39) although the rate was greater for Ba. In the sheath segments, the final decrease began in Sa before Sb, although the rates appeared to be about the same. The result of this was that Bb, Sa and Sb all reached minimum values at the same time, t = +94. (It is not possible to make the same inference for Ba in view of the way this segment was sampled.)

For the average organ values (Fig. 2.21b) - and again ignoring the '+28 drop' - both show triphasic patterns of change which are almost the opposite of each other. For the blade a period of rapid decrease in NA value was sandwiched between two periods of more or less steady values. For the sheath a period of more or less steady values was sandwiched between two periods of rapid decrease of NA. Minimum NA values for the blade were reached at c. t = +70, and at t = +94 for the sheath. It is interesting to note that the beginning of the blade decrease and of the final sheath decrease in NA coincide with the timings of increase in D%F values of both organs (Fig. 2.17c).

Nucleic Acids - Qualitative Changes

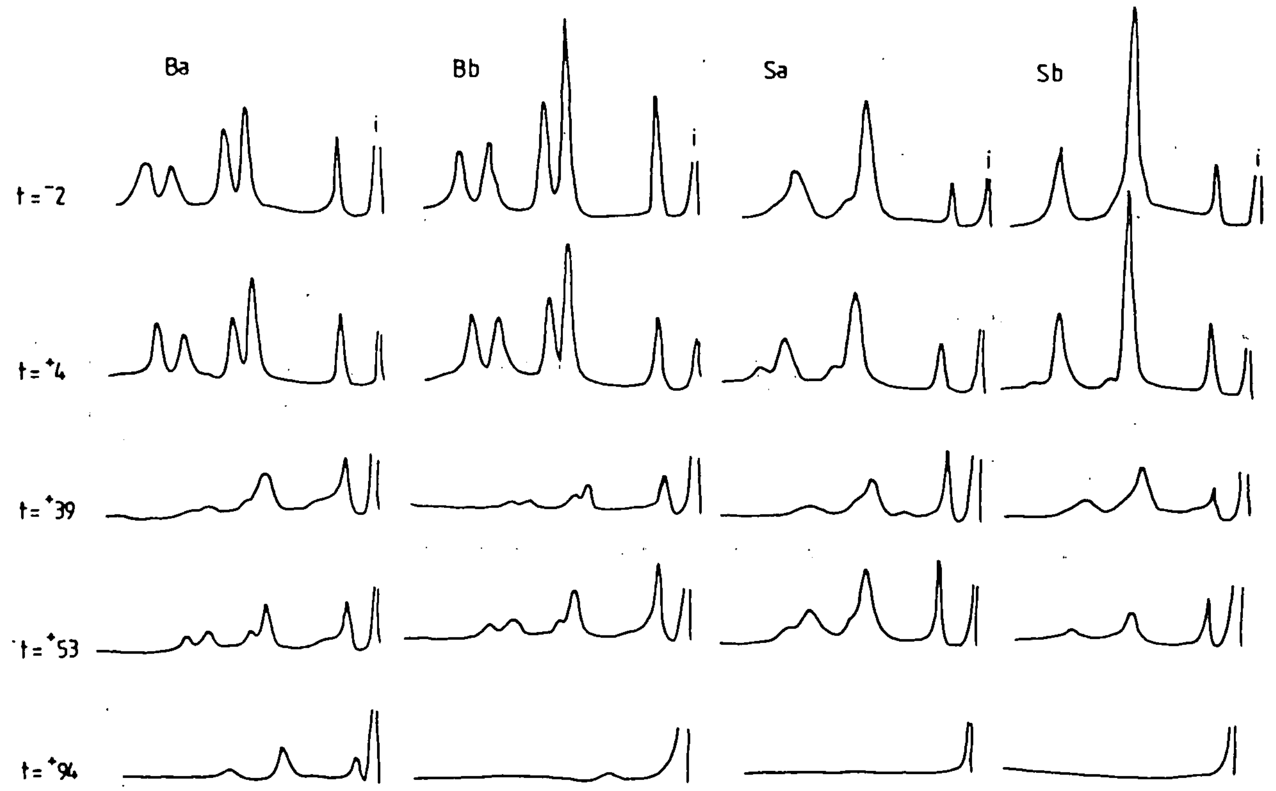
NA traces were obtained at each sampling point, only representative ones are shown here (Fig. 2.22).

Since all leaves sampled had emerged, the sequence of NA traces down the leaf at t = -2 shows the NA complement of each leaf segment at (Ba, Bb), or very near to (Sa, Sb), the end of its extension growth. Both blade segments had a full complement of DNA plus four RNA species whereas Sb only contained DNA and cytoplasmic RNA. Sa had both cytoplasmic RNA's and indications of chloroplastic RNA's as shoulders on the peaks of the former. Both sheath segments developed full NA complements with time (eg at t = +4); however, the proportions of cytoplasmic to chloroplastic RNA did not reach those of the blade segments.

In all segments, RNA content appeared to decrease with time. In the blade it is possible that chloroplastic RNA was lost preferentially; in the sheath this was not clearly seen. It was also not clear from

Figure 2.22. Aspects of ageing and senescence of the 4th leaf of <u>Lolium temulentum</u>: nucleic acids (qualitative)

Loadings of NA at t = -2, +4: 10 μg;
" " " t = +39, +53: 20 μg;
" " " t = +94: 20 μg for Ba; 15 μg for Bb; 0.4 ml NA solution for Sa and Sb (no NA detectable by spectrophotometry)


Ba
Bb
Sa
Sb
t = ⁻2
t = ⁺4
t = ⁺39
t = ⁺53
t = ⁺94
+ve
+ve
+ve
+ve
migration

the traces whether a basipetal pattern of loss of NA species occurred. It was clear, however, that even in totally senescent leaf tissue it was possible to detect DNA and cytoplasmic RNA (Ba, t = +94). Absence of NA in the other leaf segments at this sampling point may be more related to low weight of tissue rather than total loss of these cellular components. DNA appeared to be relatively stable in all segments until the very end of the life of the leaf.

DISCUSSION

The main aim of this investigation was to describe the changes in NA, Chl and soluble protein occurring in the blade and sheath of an attached grass leaf undergoing natural senescence. Before the results can be discussed in that context it is necessary to consider how 'natural' were the conditions under which the leaves senesced.

L. temulentum is an annual weed of cereal fields (Hubbard, 1976) and would be expected to grow as clumps of isolated plants rather than a sward. Sowing the grain at 3 or 4 to a pot and allowing the plants to grow up - without removing tillers - is thus akin to the natural situation. The plants received natural daylength and hence gradual changes from day to night. The situation obtaining in a growth room where the photoperiod is fixed and changes from light to dark are very sudden is a most unnatural light régime and any influence of 'end-of-day' light quality is absent. The quality of the light received within the greenhouse is believed to be largely the same as that of natural sunlight. However, the possibility of attenuation of certain wavelengths by the structure of the greenhouse must be borne in mind. Even so the light experienced here is closer to normal sunlight than any light source presently used in growth rooms.

In their natural habitat, the plants are likely to experience the effects of wind - albeit reduced by the stand of cereal plants in their midst. Wind may be predicted to aid mixing of gases within the atmosphere, enhance the rate of transpiration, cool the plant, and cause some degree of damage to it (eg Grace, 1981). By the very nature of the greenhouse, such effects of wind are largely absent because circulation of air ('wind') is minimal. As a result of this and the 'greenhouse effect', the temperature within the greenhouse will be higher than outside which will lead to an increase in rate of chemical reactions within the plant, those with a higher Q_{10} such as respiration being more affected than those with a lower Q_{10} such as photosynthesis. As a result of elevated temperatures, transpiration will be enhanced. This plus the limited degree of circulation will raise the humidity (which might offset the increase in transpiration to some extent). In the winter these problems will be exacerbated by the heating of the greenhouse to keep it above freezing. Winter-heating introduces

another problem since the morphology of the plant can be greatly affected by the balance between light and temperature (eg Mitchell and Soper, 1958). The plants used in this investigation were winter-grown.

In the early stages of the investigation the growing leaves were measured. This handling of the plants may have caused alteration in their growth or development (eg Beardsell, 1977). Since all the plants were so treated, it was assumed that they were all ffected to the same extent. Plants growing in the wild would presumably not be handled at any stage of their growth.

The spatial arrangement of the pots and hence the plants was dictated by the shape and area of the available bench space. All the pots were maintained in the centre of the greenhouse; the distance of plants to the nearest window was c. 0.5 - 2.5 m. In stands of wheat, a vertical attenuation of blue and red light has been observed (Holmes and Smith, 1977), with corresponding increase in the ratio of far red to red light deeper within the canopy. Coupled with this is the likelihood of a horizontal attenuation. Thus plants on the outside of the bench may have experienced a different light quality from those in the centre surrounded by other plants. Such considerations have an obvious relevance to the conditions of growth since it has been demonstrated by Deregibus et al.(1983) that tillering is under the control of phytochrome, and Biswal and Sharma (1976) have even proposed that phytochrome may regulate grass leaf senescence.

To try and avoid that sort of problem here the pots were moved daily to ensure that all plants experienced the same overall conditions. This became more of a liability than a help when the roots began to grow through the bottom of the pots; moving the pots frequently damaged the roots causing unknown effects to the rest of the plant.

Although the above discussion has not considered all the possible differences between the greenhouse and wild environments, it is clear that the two are different. The former falls short of the truly natural environment, but it is 'more natural' than the environment created within a growth room. Whether there is any real value in using one set of conditions which are 'more natural' than another if both fall short of the natural environment is a moot point.

However, most of the differences identified between the greenhouse and the natural situation arise as a result of the physical enclosure of the plants within the former. If such differences in environment are within the scope of the species to adapt to them then it is possible that the convenience of using a greenhouse from the point of view of the experimenter is not too far removed from the natural environment of the plant and thus a valid method of growing and studying plants.

In view of the variability of the population, how big a sample is needed to obtain statistically reliable average values for each cellular component? A statistical approach was successful for non-destructive leaf growth (previous Section) only because the sampling size was frequently over 60 plants. For the destructive samplings the results were not so reliable; 9 plants were used for each reading. Thus it seems that the means of three readings taken in the present study were not likely to be representative of the population values and a statistical analysis was not performed.

Allied to, and confounding, this is the frequency of sampling. Because of the limitations of space in which plants could be grown under similar conditions the size and frequency of sampling was limited. At each sampling point a single value for each cellular component was plotted. The shape of the curve between consecutive points, however, is not known because the samplings were so far apart and we cannot know the way the components measured may have changed between them. Thus any line drawn between the points is as good - or as bad! - as any other. All the lines in the graphs can hope to do is indicate trends - they have no validity other than this.

Any piece of leaf material is a mixture of a number of different tissues and cell types, each of which may have its own unique pattern or rate of senescence. In wheat it has been shown that bundle sheath and guard cells live longer than other cell types (Peoples _et al_., 1980). Hence the overall pattern of senescence of a given piece of material will be a composite of all the individual patterns of senescence within it. If senescence is in any way affected by the environment, each cell type might be affected differently. Added to that is the consideration that although a wave of senescence may pass basipetally down the leaf, unless it is absolutely transverse certain more basal parts of the leaf may begin to senesce before those nearer the tip.

Most of the volume of the blade of L. temulentum is occupied by photosynthetic mesophyll tissue (Harris et al., 1981), a more or less homogeneous cell type. Thus it may be reasonably assumed that changes recorded in this organ are largely those taking place in this tissue. This is almost entirely true for Chl and chloroplastic RNA (guard cell contribution is assumed to be negligible). In the sheath the proportion of photosynthetic mesophyll is much less. In both organs, however, all cell types, except xylem vessels, contain DNA, cytoplasmic RNA and soluble protein; changes for these cellular components are thus averages for all cell types.

A further series of considerations of sources of possible error is the reliability of the methods used to determine the levels of the cellular components.

The Lowry method of protein determination can give a reasonably accurate estimation of the amount of protein present in a sample (Eze and Dumbroff, 1982). Its biological validity can be improved by use of the major protein encountered in the samples analyzed as the protein standard. In mature grass leaf tissue this is probably ribulose bisphosphate carboxylase (eg Kleinkopf et al., 1970). As senescence proceeds and this enzyme is preferentially degraded (eg Wittenbach, 1978) it may not be the predominant protein in the material. We should thus probably use a different 'standard' protein at each different sampling point. This refinement is certainly worth considering for future studies of senescence. In the present study, however, the emphasis was upon using techniques previously used for study of artificial senescence so as to compare this process with that occurring in a more natural system. Hence BSA fraction V was used as the protein standard throughout the investigation.

Chl extraction and estimation suffer from a number of inaccuracies and sources of Chl loss (eg Holden, 1976), many of which can be minimized by keeping the homogenized tissue cold and in the dark before extraction takes place. Even so, Chl a and b are differentially affected during the various stages from extraction to spectrophotometry (Holden, 1976). For this reason only total Chl was recorded to avoid inaccuracy in the Chl b levels and hence distortion of derived Chl a/b ratios.

The DEP-SDS extraction method for NA's was used in view of its reliability and increased yield relative to phenol extraction methods (eg Strangeway, 1977). PAGE was probably one of the most reliable of the techniques used since a particular ribosomal RNA species is either there or is not, barring enzymatic attack during extraction. No quantitative assessment is made and the results are probably more unambiguous as a result. It would be useful to know the actual amounts of each NA species present but for the moment it is enough to try and identify any qualitative changes that may have occurred.

Given that each technique has its limitations, as long as these are appreciated it is likely that the results obtained have some relevance to the true value of the component measured in the tissue at that time. However, as long as the procedures used employ several stages of homogenization, centrifugation and decantation the possibility of loss of material before the final spectrophotometric readings are made is great.

DW was determined in the manner recommended by Evans (1972) and strictly speaking was the constant weight of the material achieved after drying at c. 70°C. Since the FW's recorded at the final stages of the investigation were almost the same as the DW's at these stages, there is a certain amount of biological validity in using DW's obtained by this procedure. FW is probably more subject to error because of water (FW) loss upon excision of the leaf segments. The greater the length of time between excision and weighing, the more inaccurate the FW obtained.

In the light of all the possible sources of error discussed above, all that can be hoped is that this preliminary study will indicate trends and highlight areas for further study. Any such trends can be investigated more rigorously once the associated methodological problems have either been minimized or overcome.

Growth of the 4th leaf used in this study was similar to that observed for other leaves of this species (see previous Section); the sheath ceased extension growth shortly after leaf emergence. Hence by the time the investigation had begun both leaf organs had more or less finished growth and could be considered 'mature' from this point of view.

Within the limits of the individual variation in size of leaf organ, it appeared that DW remained more or less constant throughout the course of ageing and senescence studied here. This may not be too

surprising if we accept that the bulk of the DW of plant material is composed of cellulose and lignin. Such materials are largely inert and not broken down during senescence and thus provide a reliable, biologically meaningful, base line against which to relate changes in the cellular components measured.

In contrast, FW showed pronounced changes with time; in leaf segments it decreased in the order Ba, Bb, Sa, Sb. This basipetal trend of FW loss may be expected from the polarity of tissue maturation within the leaf. Whilst it was clear that Ba began to decrease in FW before Bb, for the sheath segments such a distinction was not possible. This highlights yet another of the problems inherent in this study and is related to the difference in lengths of blade and sheath. In the former, the centre-to-centre distance between top and bottom halves of the organ was about four times greater than for the sheath. Hence in the latter only great differences in amounts of cellular components will be detectable. With the course of ageing and senescence taking place over such a long time it is likely that any changes will only occur slowly and the necessary magnitude of difference between the two sheath segments will probably not be found. This appeared to be the case for most measurements made, except for Chl (see below).

Another aspect of interpretation of the results is illustrated by the graph of organ FW (Fig. 2.17b). The regression line for blade showed continual loss of FW with time and in the absence of any other data this would have been a valid interpretation of the results. However, both blade segments (Fig. 2.17a) showed initial plateaux of constant FW before marked decreases which have to be reflected in the organ curve (Fig. 2.17b). This example emphasizes how valuable information can be lost if the organ graphs are considered in isolation from the data from which they were derived.

A similar situation occurred in the interpretation of the D%F data. By reference to the graph of organ D%F (Fig. 2.17c) it may have been inferred that a marked disjunction in water loss was present between the blade and the sheath. Consideration of the other graphs of D%F (Fig. 2.18) clearly showed that the leaf was losing water as a whole in a basipetal manner, with no signs of any disjunctions between its organs.

It is interesting to note that certain workers have pointed out the similarity of certain aspects of water-stress-induced metabolic changes

and those occurring during senescence (eg Brady et al., 1974). In the present investigation it seems that water loss is an effect of senescence (eg also Feller and Erismann, 1978). It should be noted that any changes in leaf dimensions and air space volume within the leaf as a result of water loss (Woolley, 1973) are likely to have profound effects upon any gas exchange between the leaf and the atmosphere that may still be taking place at this stage. Such phenomena are probably absent in leaf sections 'senescing' on solutions in petri dishes.

The observed changes in soluble protein were very similar to the pattern previously described for this grass (Thomas, 1978) and other species (eg Thomas and Stoddart, 1980). Although the leaf segments themselves appeared to show different patterns of change, interpretation of these results is confounded by the problems referred to above. When blade and sheath were compared, both curves were similar in shape and showed a loss of soluble protein with time. Blade values reached a minimum level before those of the sheath, but here the evidence for a basipetal wave of senescence was not unambiguous.

In the case of Chl content, the differences between leaf segments and organs were much more obvious. A distinct basipetal pattern of loss was seen within the blade and from this organ into the sheath. In the latter, however, it was not clear whether a basipetal pattern of loss existed; both segments appeared to increase in Chl till c. t = +67 before a marked decrease occurred. Again this may be more related to the problems of detecting differences between segments in this organ rather than to absence of basipetal loss of Chl. For the blade, the pattern of Chl loss closely paralleled that of soluble protein. This has been observed by a number of workers in different species undergoing artificial senescence.

The situation with regard to Chl in the sheath requires further investigation. The most pronounced accumulation of Chl was seen in the basal half of this organ. This part of the sheath was enclosed by several thicknesses of older leaf tissue and thus probably experienced different light conditions from the upper half of this organ and the blade. As noted above, upon passing through chlorophyllous tissue, light becomes selectively attenuated and the ratio of far red to red increases. Thus, at least initially, physiological darkness may prevail in the lower half of the sheath. As the enclosing leaves senesce their

selectivity in attenuating the light transmitted decreases (Holmes and Smith, 1977) and the light conditions experienced by the enclosed part of the sheath might become more like those of the rest of the same leaf. This may then permit development of Chl within this part of the sheath. An ultrastructural study of the leaf (results not shown) has revealed the existence of very starchy chloroplasts with poorly-developed grana in this region alongside well-developed chloroplasts. It is possible that the observed increase in Chl is due to 'greening up' of these starchy chloroplasts. A more detailed ultrastructural study is needed to answer this question.

These observations raise the problem of the attainment of 'maturity' of the blade and sheath. The blade may be considered 'mature' upon leaf emergence; the sheath when it has ceased extension growth. For the former it may be conjectured that full photosynthetic ('physiological') maturity is not achieved until it has unrolled and bent downwards and attained 'morphological maturity' (see Introduction, previous Section). For the sheath it appears likely that it does not attain its photosynthetically mature ultrastructure or biochemical complement until after cessation of its growth. This lag between attainment of maximum length/size and photosynthetic maturity has previously been remarked by Šesták (1963). In view of the many different forms of 'maturity' one can conceive of, it is advisable that its meaning should be specified when used.

It may be assumed that the loss of protein during ageing is largely due to loss of ribulose bisphosphate carboxylase (eg Wittenbach, 1978). If this is the case then, for the blade, loss of this enzyme and Chl imply loss of photosynthetic ability with time. In the sheath it may be conjectured that loss of soluble protein also implies loss of photosynthetic ability. However, the increase in Chl in this organ may in some respects offset this. Alternatively it may be an unavoidable reaction to altered light conditions which has no relevance to the photosynthetic ability of this organ. In either case a study of photosynthesis in the sheath during ageing and senescence would be valuable.

In both organs, the pattern of NA changes appeared to be triphasic, showing overall loss with time. It is possible that the early NA loss of the sheath - as for soluble protein - may be related to formation of air lacunae with consequent loss of cells (eg Kaufman, 1959).

The patterns of NA changes in the segments were largely dominated by the '+28 drop' in NA levels. This may have been a real feature or a result of some sort of 'extraction phenomenon' at that sampling point. Unfortunately, due to the nature of this sort of study, it was not possible to repeat that reading and it must thus remain of questionable significance. Generally, however, the two graphs had similar shapes; blade NA reached minimum values before the sheath indicating a basipetal pattern of NA loss. Loss of NA with time has been recorded for a number of species induced to senesce under a variety of conditions, and along with loss of protein and Chl appears to be one of the most characteristic of changes associated with ageing and senescence (eg Thimann, 1980).

PAGE gave some insight into the qualitative changes in NA underlying the quantitative changes. A number of differences were found within and between each organ. Generally blade segments had full complements of NA from the first sampling point; sheath segments largely lacked chloroplastic RNA's. This latter observation further suggests that the sheath was not 'physiologically mature' even though it had nearly ceased extension growth.

One of the features of Fig. 2.22 is the apparent decrease in all NA species at t = +39. This may be related to the '+28 drop' in NA content referred to above, or it may be due to an error in technique at that sampling point. The succeeding traces look more 'respectable' and it is difficult to accept that all NA's can be depressed and then recover their previous levels before declining again.

The NA changes in both blade segments were similar to each other and appeared to occur at the same time. However, since the methods used only gave information regarding qualitative changes, in the absence of quantitative data for each NA species it is not possible to conclude that rates and magnitudes of change were the same for each half of the organ.

The apparent greater retention of DNA relative to RNA's was seen in both organs and is presumably related to maintenance of integrity of the nucleus until late stages of senescence (eg Butler and Simon, 1971). If successful operation of the 'senescence programme' is in any way dependent upon nuclear control (eg Yoshida, 1971) it is no surprise that this organelle is amongst the last to undergo degeneration. It is also

interesting to note that even in totally senescent blade tissue it was still possible to extract DNA and cytoplasmic RNA. This suggests that the 'usefulness' of senescing leaves to the rest of the plant may be limited - not all the materials present in the leaf may be recycled back into the plant.

To a certain extent the top half of the sheath underwent changes in NA similar to those recorded for the blade - once a full complement of NA's was attained. For the bottom half of this organ the preponderance of cytoplasmic RNA over chloroplastic RNA tended to overshadow any changes in the latter. At early stages of ageing in the sheath, the increase in chloroplastic RNA paralleled the increase in Chl; thereafter Chl continued to increase but chloroplastic RNA decreased. The reasons for this are not clear but in view of its anomalous nature clearly deserves further study.

Overall the loss of NA, Chl and soluble protein followed a basipetal pattern from blade to sheath but differences were found. Both organs appeared to lose soluble protein at the same time which might suggest that senescence was initiated at the same time in both. Further study of this phenomenon is needed to establish if this is so. It does serve to emphasize that although senescence is an ordered process overall, no single aspect of this process should be taken as a 'marker' for it - cf also the case of non-yellowing mutants of fescue (eg Pearson *et al.*, 1978).

The patterns of change for the measured cellular components were similar to those reported in the literature for artificially-induced senescence in a number of species. The main difference between the two experimental systems was the greater period of time over which the changes took place in the attached leaf situation. The only anomalous feature was the behaviour of Chl in the sheath. In view of the importance of this organ as part of the pathway of translocation for degradation products from the blade to the rest of the plant, its ageing and senescence must be studied further.

Whether this study has accurately described the course of natural leaf senescence in *L. temulentum* in respect of the cellular components measured must remain doubtful in view of the reservations discussed above. This study has attempted to view the leaf as a structure consisting of blade and sheath and has succeeded in illustrating some inter-

esting features of sheath ageing. It has certainly shown that the blade cannot be considered a good model for senescence of the sheath or the leaf.

Because of the undoubted complexity of the events associated with natural leaf senescence it may be advisable to study thoroughly each aspect separately. Once that has been done it should be possible to combine all the individual facets of the 'senescence syndrome' and gain an overall impression of the process. Only when that has been achieved will it be possible to say whether natural and artificially-induced senescence are the same or comparable processes.

SECTION c. LEAF VASCULATURE AND METABOLITE TRANSPORT DURING AGEING AND SENESCENCE

INTRODUCTION

Certain aspects of interactions within and between leaves of grasses were considered in the previous two Sections. One of the most important of these involves long-distance transport of minerals, metabolites and growth regulators and is the subject of this Section.

During the early part of the life of a leaf it can be considered heterotrophic and is dependent upon older leaves for its carbon requirements. As it grows it develops the ability to photosynthesize and begins to supply its own needs for eg carbon, although still importing from older leaves to its basal tissues. Eventually it becomes totally self-sufficient and begins to export the excess photosynthate. However, it still depends upon the rest of the plant for its supply of water and nutrients (and growth regulators to some extent).

Early in its life nutrients come via the phloem from older, dying leaves; as it grows this demand increases and is met by xylem transport from the roots (Dale and Milthorpe, 1983). Once mature, the leaf acts as a source of photosynthate for younger leaves (Ryle, 1972), meristematic regions (Quinlan and Sagar, 1962) and to some extent tillers (Marshall and Sagar, 1965). During its senescence the leaf acts as a source of metabolites other than photosynthate, eg carbon and nitrogen in the form of amino acids derived from proteolysis (eg Thimann et al., 1974; Lloyd, 1982). Thus, throughout its life any one leaf is extensively integrated developmentally and nutritionally with other parts of the plant via transport of materials in the vascular system.

Such transport is performed chiefly by the longitudinally-oriented vascular bundles (or veins) which run the length of the culm or leaf. In most cases they constitute a continuous pathway from roots to leaf tips. The typical large grass vein is a collateral bundle where the phloem and xylem meet along a distinct curve. The xylem consists of one large metaxylem element either side of a protoxylem element or lacuna, and other smaller metaxylem elements (Cheadle and Uhl, 1948). Smaller bundles consist of a few xylem and phloem elements (eg Ellis, 1976).

Numerous workers have proposed schemes for classifying the different vascular bundles found within the grass leaf based on size, structural complexity, position relative to the mid-vein and time of their

initiation (eg Pée-Laby, 1898; Lewton-Brain, 1904; Artschwager, 1925; Esau, 1943; Hitch and Sharman, 1971; Patrick, 1972; Ellis, 1976). The number of types actually recognized has varied with the author and the type of investigation. Generally it is agreed that three types can usually be distinguished in most grass leaves:

I - the large bundles as described by Cheadle and Uhl (1948);

II - like Type I but lacking the large metaxylem vessels;

III - lacking large metaxylem vessels and protoxylem, usually much smaller than either Type I or II.

Although such differences seem very distinct on paper, when applied to a given leaf the often observed intergradation between Types I and II (eg Ellis, 1976) make it difficult to apply.

Transverse veins link up some of the longitudinal veins with each other (eg Kuo _et al_., 1972) both in leaves and internodes. At the nodes a complex pattern of vein fusion occurs (eg Hitch and Sharman, 1971; Patrick, 1972). Such fusion has been considered to be so extensive that few restraints to vascular transport of materials exist between the individual parts of the plant (Hitch and Sharman, 1971; Patrick, 1972).

Transport generally within the vascular system is assumed to be largely upwards from roots to leaves in the xylem, and downwards from leaves to roots in the phloem (eg Baker, 1978). The xylem, or apoplastic, pathway is essentially the transpiration stream and is responsible for carrying such materials as water, inorganic nutrients and amino acids. The phloem, or symplastic, pathway carries most importantly photosynthate and break-down products resulting from leaf senescence. No material, however, seems confined to one pathway, only carried to a greater or lesser extent by one than the other.

LM and TEM can be used to give a great deal of information about the structure of the components of the vascular system but they cannot give any indication of the route involved. The transpiration pathway has been studied by many workers using a variety of techniques including fluorochromes (Caldwell, 1925; Hughes and McCully, 1975), lead sulphide (Tanton and Crowdy, 1972), lanthanum acetate (Thomson _et al_., 1973), Prussian blue (Pizzolato _et al_., 1976) and monosilicic acid (Aston and Jones, 1976). The results obtained are in broad agreement with each other; the transpiration stream is generally confined to the xylem of

the veins but is transported via mesophyll cell walls to sites of evaporation in the epidermis.

Using both dyes and ^{14}C-labelled amino acids admitted to the transpiration stream of the leaf of Festuca pratensis, Lee (1979) found that they accumulated at the blade/sheath junction in older leaves. On the basis of a brief SEM study he suggested that this accumulation might be the result of physical restriction to transpirational flow in this region, or tyloses.

Tyloses are present throughout the vascular plants and have been recorded in ferns (eg Holden, 1925), conifers (eg Gerry, 1914) and angiosperms (eg Bowes, 1975). In the latter plant group, they occur mainly in dicotyledonous species, although their presence in grasses has been claimed by eg de Bary (1884) and Haberlandt (1914), and demonstrated in oats by O'Brien and Thimann (1967). Opinions in the literature differ as to whether they cause cessation of, or merely localized hindrance to, transpirational flow in the vessels where they occur. It is still not certain if they form in vessels which have ceased conducting or not (Zimmermann, 1979).

In view of the possibility that tyloses might form at the blade/sheath junction of senescing grass leaves, a study of the vasculature of L. temulentum was made. In this way it was hoped to extend Lee's observations to another species and provide a structure/function adjunct to the biochemical study of leaf senescence presented in the previous Section. To this aim the anatomy of the blade/sheath junction was studied by LM and TEM during leaf ageing and senescence; the number, types and course of the veins were examined as was the behaviour of sheath-applied fluorochromes and ^{14}C-labelled amino acids.

MATERIALS AND METHODS

Apoplastic Tracer Study

In an attempt to determine the pathways of apoplastic transport within the leaf, a number of fluorochromes were used:

a. eosin, 0.1% (w/v) aq. (eg Caldwell, 1925);
b. calcofluor, 0.1% (w/v) aq. (eg Hughes and McCully, 1975);
c. rhodamine 6G, 0.1% (w/v) aq.;
d. ANS, 0.1% (w/v) in McIlvaine's citrate/phosphate buffer, pH 5.0 (eg Gates and Oparka, 1982).

Tillers bearing leaves ranging in age from mature (green throughout) to senescent (blade yellow throughout), were excised below water at the shoot/root junction and the cut ends placed in a solution of one of the four fluorochromes. Following transpiration in the fluorochrome for c. 1 h in the laboratory under fluorescent white lights, the tillers were transferred to distilled water to 'chase' the fluorochrome through the leaf, for c. 1-1½ h. By the end of the 2-2½ h period, eosin and rhodamine 6G could be seen at the leaf tips - including those of senescent leaves. It was assumed that the colourless fluorochromes, calcofluor and ANS, had also reached this point. Free-hand TS's of mid-blade regions were then cut using a razor blade, mounted in water beneath a cover slip, and examined in the FluorM. The filter combination used was the same for all four fluorochromes: excitation filter BG3 and suppression filter K460.

Two 'controls' were performed:

a. since all fluorochromes gave the same image, free-hand TS's of mid-blade regions of untreated leaves were stained with the general-purpose fluorochrome calcofluor. This fluorescence image was compared with that obtained using the apoplastic tracers under the same epifluorescent conditions;
b. autofluorescence of mid-blade TS's of untreated leaves was compared with the fluorescence image obtained using the apoplastic tracers under the same epifluorescent conditions.

^{14}C Uptake and Autoradiography

Tillers bearing leaves ranging from mature to senescent were excised below water at the shoot/root junction and the cut ends immersed in 5 ml of distilled water. The tillers were allowed to transpire in the laboratory under fluorescent white lights for c. 10 min before addition of 0.1 ml (U-^{14}C)-labelled amino acid mixture (50 $\mu Ci.ml^{-1}$; specific activity 56 mCi.matom C^{-1}). After transpiring for a further 1 h, the lower 2 cm of the shoots were discarded, and the individual leaves separated and trimmed to leave c. 3 cm either side of the ligule. These leaf pieces were taped to a glass plate, labelled, another glass plate placed on top of the leaf tissue and clamped down with metal clips. The plates were placed in a drying oven at c. 70°C for 2 d to dry and kill the tissue and prevent metabolism and movement of the ^{14}C within the tissues. In a dark room, the glass plates were separated and a piece of X-ray film placed on top of the leaf tissue. The other glass plate was placed on top of the X-ray film, and the whole firmly secured with strong elastic bands and sealed in light-tight black plastic bags. This package was stored at -80°C for c. 10-15 d to expose the film. The developed, fixed and dried X-ray film was used as a negative to give a contact print which was rephotographed to give the image in Pl. 2.4.

To determine whether ^{14}C-label had reached the leaf tip, the tip-most 1 cm of leaf was excised and used for scintillation counting. To accomplish this the tissue was homogenized in a pestle and mortar with 5 ml of 5% (w/v) TCA. A 1 ml sample of this homogenate was added to 10 ml of scintillation fluid (4g 2,5-diphenyl oxazole in 1.5 l of 2:1 mixture of toluene: Triton X100) in a scintillation vial and counted in a Beckman LS 200B scintillation counter. Counts of leaf tips were always 3-4 times greater than background and thus it was assumed that label had reached the leaf tip - even in senescent leaves.

A further check was made using sheath-applied aq. eosin on tillers similar to those used for ^{14}C uptake. Here dye was found all the way along leaves, including senescent ones.

Scale Drawings of Leaf Sections

These were prepared from thin free-hand TS's of leaf tissue using a Nachet drawing tube fitted to a Vickers M75 LM.

RESULTS AND OBSERVATIONS

1. Leaf Vasculature

a. Types of Vascular Bundle

Considerable difficulty was experienced in consistently distinguishing three types of bundle because of intergradation between types. Accordingly in this study only two basic bundle types were recognized. Both were readily distinguished by their calcofluor-induced fluorescence image in TS:

Type 1 bundles showed two or three fluorescent 'bright spots' (Pl. 2.1a,b) corresponding to the phloem region and arcs of xylem-associated parenchyma cells. In the LM they were distinguished by their possession of a protoxylem vessel or lacuna between two lateral large metaxylem vessels (Pl. 2.1d). In most cases it was not clear whether the protoxylem occurred as either an 'air space' or lacuna (eg Cheadle and Uhl, 1948; Ellis, 1976) or vessel (eg Metcalfe, 1960). The lignified elements present between the meta- and protoxylem have been variously termed secondary xylem (Arber, 1934), metaxylem (Cheadle and Uhl, 1948) and tracheary elements (Ellis, 1976). In view of their uncertain nature, they are called 'xylem elements' here.

Type 2 bundles showed only one fluorescent 'bright spot' (Pl. 2.1a,b) corresponding to the phloem region. In the LM they were distinguished by their absence of large metaxylem vessels, protoxylem and associated parenchyma, and by their possession of several small metaxylem vessels at the adaxial end of the bundle (Pl. 2.1c).

Although only blade bundles are shown in Pl. 2.1c,d, the differences observed applied equally to sheath bundles (eg Pl. 2.1a). Both types of bundle were surrounded by an inner, mestome bundle sheath of small diameter cells with thickened radial and inner tangential walls, and an outer, parenchyma bundle sheath of larger diameter chlorenchymatous cells with thin walls (of Pooid type - Carolin et al., (1973). Sudan black B-staining of the inner bundle sheath (results not shown) confirmed the presence of a lipidic (suberized) layer to the inside of the cell walls

(eg O'Brien and Carr, 1970). Mid-veins could be distinguished from other veins in the lower half of the blade by the colourless cells associated with them (eg Pl. 2.2d).

The mid-vein was always observed to be a Type 1 bundle but is labelled 'M' in the Figures below as a reference aid for the location of the other bundles of the leaf. Numerous transverse veins occurred in blade and sheath joining certain longitudinal veins to one another; they were not studied in any detail here.

b. Variation in Number and Type of Bundles

The vasculature of the 4th leaf was studied.

i. Between Leaves

The number and Type of bundles at the mid-blade regions of different leaves was recorded (Fig. 2.23a). The arrangement of bundles was remarkably symmetrical either side of the mid-vein, apart from the addition of a Type 2 bundle near the mid-vein in (i) and the margin in (iv).

ii. Within Leaves

The number and Type of bundles along a leaf from tip (i) to base (ix) is represented in Fig. 2.23b. The main deductions from this sort of study may be summarized thus:

a. symmetry of vein arrangement either side of the mid-vein;
b. change of Type (intergradation) along the lengths of some veins (indicated as 2/1 in the Figure);
c. loss of some veins as the leaf tip was approached;
d. continuity of veins from sheath to blade (the reverse was not always the case; on some occasions not all blade veins continued into the sheath but stopped at the blade/sheath junction);
e. addition of a Type 2 bundle either side of the mid-vein only in the lower half of the sheath.

Similar deductions may be made from Fig. 2.24 which shows TS's taken along a different leaf from tip (a) to base (b). The number and Type of bundles was similar to that in Fig. 2.23b. The loss of veins near the tip (c to b to a) was again seen but here it carried with it the suggestion that the ratio of vascular to non-vascular tissue may be

Figure 2.23. Aspects of the vasculature of the 4th leaf of <u>Lolium temulentum</u>

a. Variation in number and Type of vascular bundle in the mid-blade region of four different plants;

b. Variation in number and Type of vascular bundle along the length of a single leaf;

i. near the tip of the blade;
ii. $\frac{1}{4}$ distance from the tip of the blade;
iii. $\frac{1}{3}$ distance from the tip of the blade;
iv. mid-blade;
v. base of the blade;
vi. blade/sheath junction
vii. top of the sheath;
viii. mid-sheath;
ix. base of the sheath

a.

i.	2	1	2	1	2	2	M	2	2	2	1	2	1	2	
ii.	2	1	2	1	2	2	M		2	2	1	2	1	2	
iii.	2	1	2	1	2	2	M		2	2	1	2	1	2	
iv.	2	1	2	1	2	2	M		2	2	1	2	1	2	2

b.

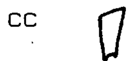
i.		2/1		1	2	2		M		2	2	1		2/1	
ii.	2	2/1	2	1	2	2		M		2	2	1	2	2/1	2
iii.	2	1	2	1	2	2		M		2	2	1	2	1	2
iv.	2	1	2	1	2	2		M		2	2	1	2	1	2
v.	2	1	2	1	2	1		M		2/1	2	1	2	1	2
vi.	2	1	2	1	2	1		M		1	2	1	2	1	2
vii.	2	1	2	1	2	1		M		1	2	1	2	1	2
viii.	2	1	2	1	2	1	2	M	2	1	2	1	2	1	2
ix.	2	1	2	1	2	1	2	M	2	1	2	1	2	1	2

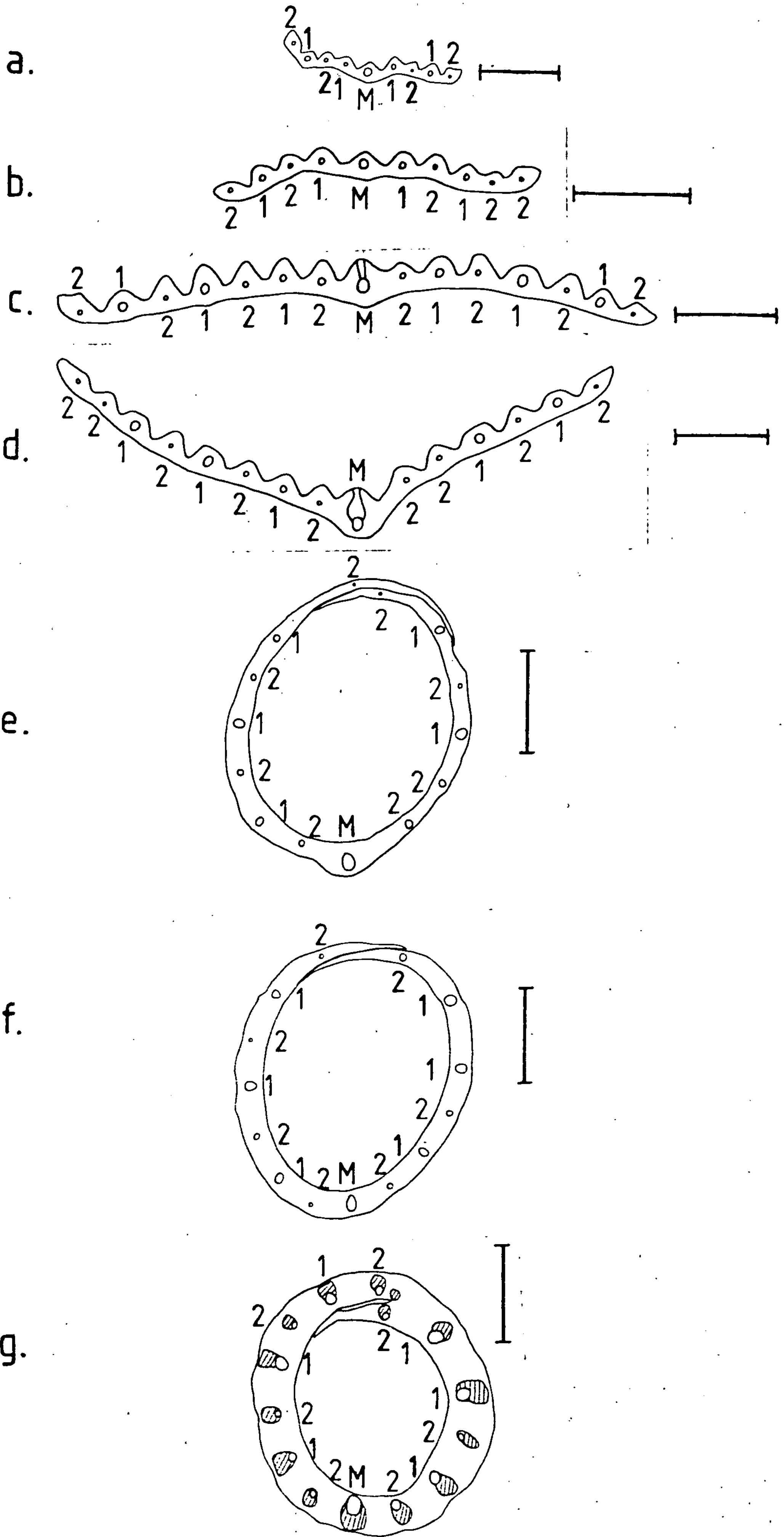
Figure 2.24. Aspects of the vasculature of the 4th leaf of _Lolium temulentum_

Scale drawings of TS's showing variation in number and Type of vascular bundles along the length of a single leaf

a. near the tip of the blade;
b. $^1/_5$ distance from the tip of the blade;
c. mid-blade;
d. base of the blade;
e. top of the sheath;
f. mid-sheath;
g. base of the sheath

Scale bar = 1 mm in each case; magnifications range from X14.5 - X20.





a.
b.
c.
d.
e.
f.
g.

more or less constant throughout the length of the leaf. The change in orientation of the veins from radial in the sheath to linear in the blade was also demonstrated.

2. Apoplastic Tracer Study

a. Sheath-applied Fluorochromes

All four fluorochromes gave similar fluorescence images. The results are summarized in Table 2.4 and illustrated for rhodamine 6G only. Generally the most intense fluorescence was associated with inner bundle sheath, lignified xylem elements and sclerenchyma; fluorescent patterns were similar for both bundle Types except that protoxylem-associated fluorescence was absent in Type 2.

Pl. 2.2a,b shows the same field of view of a Type 1 bundle from a mature blade under transmitted light (a) and epifluorescent (b) illumination. All tissues were visible in the former; in the latter fluorescence was largely confined to the inner bundle sheath, xylem elements and abaxial sclerenchyma. A Type 1 bundle of a senescent leaf is shown in Pl. 2.2c; here the intense fluorescence of the bundle made it difficult to localize the fluorescing structures but it appeared to be less intense in phloem and parenchyma tissue. Apart from the fluorescence associated with chlorenchymatous tissue - against which the chloroplasts were contrasted (white arrow-heads) - the overall pattern of fluorescence was similar to that of the mature leaf.

During short-term uptake of fluorochrome, it was often found to be largely confined to Type 1 bundles; with time it was also found in transverse veins and Type 2 bundles.

No evidence of accumulation of fluorochrome at the blade/sheath junction in ageing leaves was seen, rather uptake occurred all the way to the tips even in senescent leaves.

b. Calcofluor-induced Fluorescence of Sections

In many respects the fluorescence image observed here was the reverse of that obtained with sheath-applied fluorochrome solutions (Pl. 2.1a; 2.2d). Generally all tissues apart from lignified ones fluoresced intensely, apparently in relation to the thickness of their walls and absence of autofluorescent materials in some cases (compare epidermis with mesophyll in Pl. 2.2d).

Table 2.4. Comparison of degree of fluorescence associated with mature and senescing blade tissue of Lolium temulentum after use of four transpiration stream-applied fluorochromes

Tissue	Eosin	Rhodamine 6G	ANS	Calcofluor
abax	++	++	++	++
		(outer tangential wall mainly)		
meso	++	++	++	++
ps	-	-	-M/+S	+
is	+++	+++	+++	+++
mx	+++	+++	+++	+++
px	+	-M/+S	?	+
ph	+	+	+	+
x	+++	+++	+++	+++
xy parenchyma	+	+	+	+
cc	ND	ND	ND	ND
scl, abax	++(+)	++(+)	++(+)	++(+)
scl, adax	++	++	++	++
scl, margins	++	++	+(+)	++
adax	+(+)	+(+)	+(+)	+(+)
		(outer tangential wall mainly)		

+ = weak fluorescence

++ = moderately intense fluorescence

+++ = intense fluorescence

- = no fluorescence detected

ND = not determined

S = senescing tissue

M = mature tissue

c. Autofluorescence of Leaf Tissue

The results are summarized in Table 2.5 and illustrated in Pl. 2.3. In mature leaves the only autofluorescence recorded with the film used (and thus of relevance to the conditions under which the sheath-applied fluorochrome results were recorded) was that of lignified xylem and sclerenchyma and the inner bundle sheath (Pl. 2.3e, compare with same field of view in transmitted light in d; and f). In all cases the intensity of fluorescence was less than that of the same tissues observed after uptake of sheath-applied fluorochromes.

In senescing leaves (Pl. 2.3a), the autofluorescence pattern was similar to that of mature leaves but the beginnings of non-chlorophyll autofluorescence were seen. In senescent leaves (Pl. 2.3b,c), the intensity of this mesophyll autofluorescence was increased, although the overall autofluorescence pattern was similar to that of mature leaves.

In all cases the intensity of autofluorescence of all tissues, except the mesophyll in senescing and senescent leaves, was less than that of the corresponding structures observed after sheath-applied fluorochrome uptake.

3. ^{14}C Uptake

The autoradiograph (Pl. 2.4) suggests that ^{14}C from sheath-applied solutions of ^{14}C-labelled amino acid mixtures was accumulated at the top of the sheath in ageing leaves (increasing number). These results are very similar to those of Lee (1979) using F. pratensis.

4. Anatomical Study of the Blade/Sheath Junction

a. Light Microscopy

The general tissue distribution in this region was the same as that in the rest of the leaf - vascular bundles were surrounded by mesophyll and enclosed within abaxial and adaxial epidermes (Pl. 2.5a,b). Sclerenchyma bundles were present at the margins (Pl. 2.5a) and frequently above and below the vascular bundles (P. 2.5b).

In mature leaves no obstructions were seen in either bundle Type (eg Type 1 bundle in Pl. 2.5g). In senescing leaves, however, tyloses were seen in the protoxylem vessels/lacunae of Type 1 bundles

Table 2.5. Comparison of degree of autofluorescence associated with mature and senescing blade tissue of *Lolium temulentum*

Tissue	Senescing	Mature
abax	+(+)	+(+)
	(outer tangential wall mainly)	
meso	++(contents?)	++(Chl)
ps	(+)	(+)
is	++	+(inner tangential and radial walls)
mx	++	+
px	(+)	(+)
ph	-	-
x	++	++
xy parenchyma	-	-
cc	+	ND
scl, abax; scl, adax; scl, margins	+(+)	+(+)
adax	+(+)	+(+)
	(outer tangential wall mainly)	

\+ = weak fluorescence

++ = moderately intense fluorescence

\- = no fluorescence detected

ND = not determined

(Pl. 2.5d,e,f); none were seen in Type 2 bundles. Owing to instability of senescent material in LS, only TS's were examined, consequently it can only be concluded that the tyloses had a rounded profile (Pl. 2.5d,e). In places where this shape could not be expressed (eg Pl. 2.5f), they appeared irregularly-shaped. Some information regarding their 3-dimensional shape was obtained by the use of serial TS's (Pl. 2.5d,e) in which the upper tylose (indicated by an arrow-head) had a clearly rounded end. Whether it was spherical or sausage-shaped can only be conjectured. The structure protruding into the protoxylem lacuna of a Type 1 bundle of a senescing leaf (Pl. 2.5c) is interpreted as being a tylose at an early stage of development and suggests that they were derived from xylem parenchyma cells.

The results of a survey of tylose distribution in different regions of leaves at maturity and senescence are presented in Table 2.6. Tyloses appeared to be specifically associated with Type 1 bundles of the blade/sheath junction - or 2 mm either side of it - of senescing leaves. Profiles similar to that in Pl. 2.5c were seen in Type 1 bundles of blade/sheath junctions of mature leaves and mid-blade regions of senescing leaves and were interpreted as evidence for incipient tylose formation in those regions.

b. Transmission Electron Microscopy

Although the tyloses did have an appreciable amount of ultrastructure, this was largely confined to the periphery by the large vacuole (Pl. 2.6a). Since they appeared to be derived from xylem parenchyma cells, their structure and ultrastructure was compared with that of the latter (Table 2.7). The organelle complements were similar - both contained strands of RER, dictyosomes and highly-cristate mitochondria (Pl. 2.6b,c,e) within cytoplasm rich in free ribosomes (eg Pl. 2.6e). The main differences in organelles appeared to be the greater numbers of microbody profiles in tyloses (Pl. 2.6d) and of plastid profiles in xylem parenchyma (Pl. 2.6c). The difference in wall thickness is shown in Pl. 2.6f; the tylose wall (w) was thinner than the parenchyma wall (w'), compare 'a' with 'b'. Where the two walls were in direct contact, however, it was not possible to distinguish them as separate walls ('c') and they appeared homogeneous.

Table 2.6. Distribution of tyloses within mature and senescing leaves of Lolium temulentum

	Vascular bundles			
Location	Type 1	Type 2	Type 1	Type 2
blade tip	-	-	-	-
mid-blade	+/-	-	-	-
'collar'	+	-	+/-	-
mid-sheath	-	-	-	-
	(senescing)		(mature)	

+ = present

- = not seen

+/- = incipient tylosis?

Table 2.7. Comparison of the structure and ultrastructure of tyloses and xylem parenchyma cells of Type 1 bundles of the blade/sheath junction of senescing leaves of Lolium temulentum

Feature	Tylose	Xylem parenchyma
w	'thin'	'thick'
mt	++; highly-cristate	++; highly-cristate
mb	++	+
pt	+	++
vacuome	'large'	'small'
d	+	+
er	+; rough	+; rough
ribosomes	++	++
diameter	'large'	'small'

+,++ = relative sizes of populations of organelles concerned

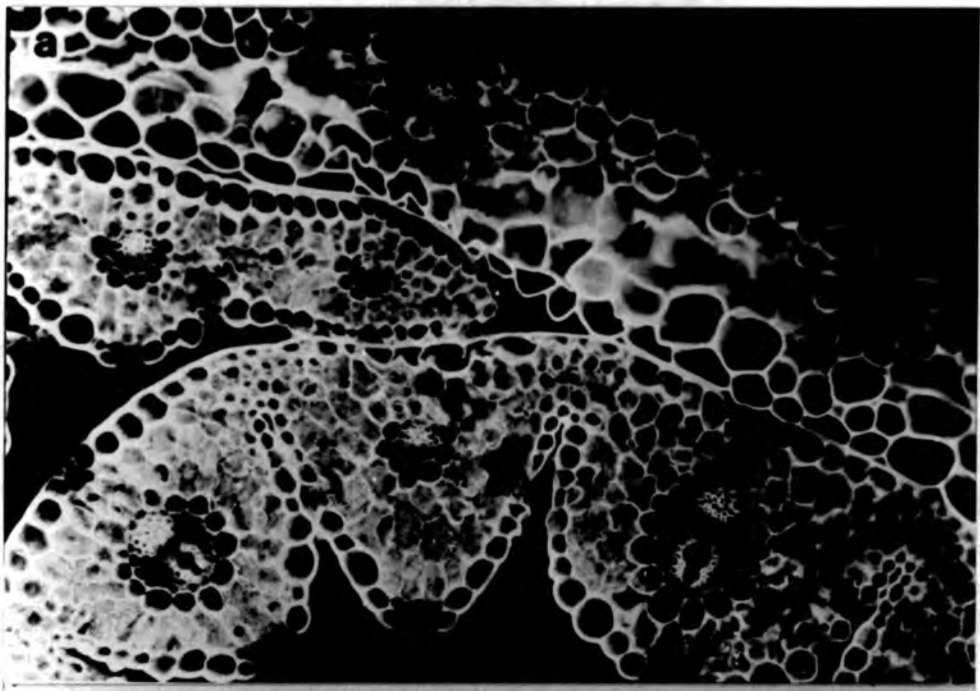
Plate 2.1. Vascular bundle Types in the leaf of Lolium temulentum

a. FluorMgraph of blade and sheath; calcofluor; X160;

b. Same micrograph as in a. above, showing the two Types of vascular bundle; X160;

c. LMgraph of a Type 2 vascular bundle from the blade; X610;

d. LMgraph of a Type 1 vascular bundle from the blade; X610.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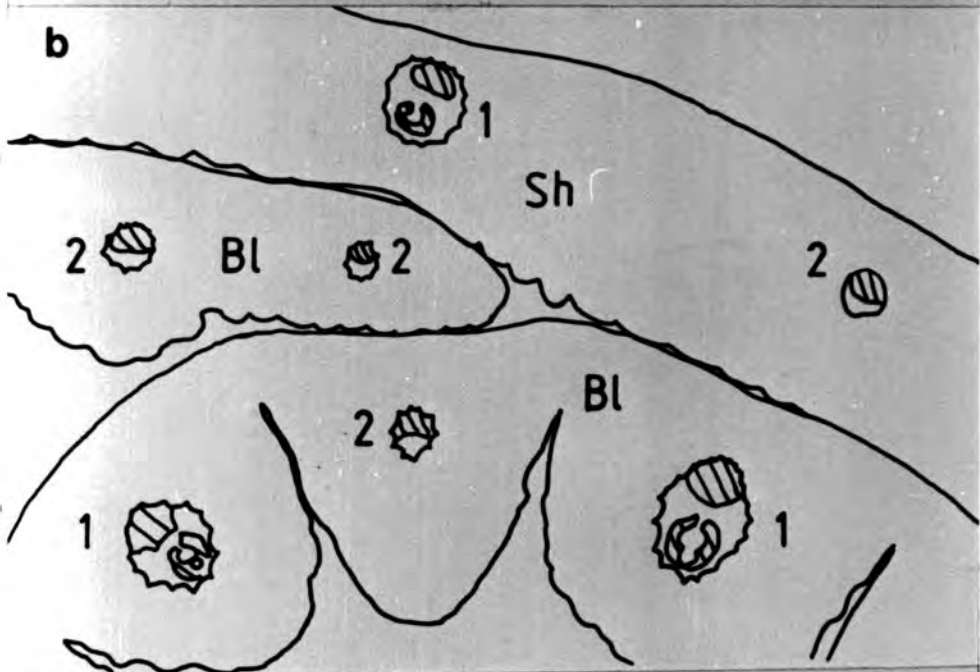
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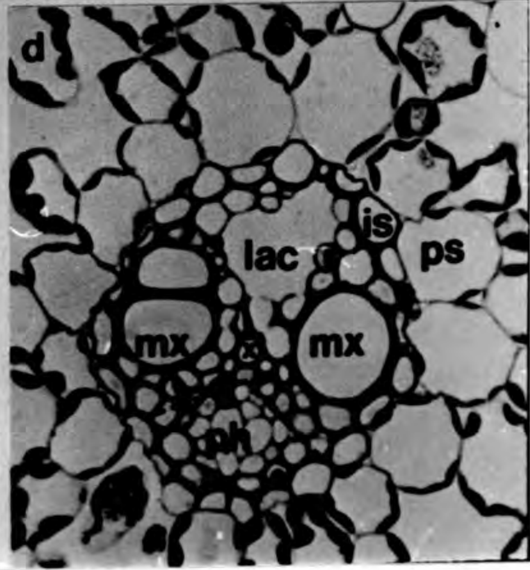
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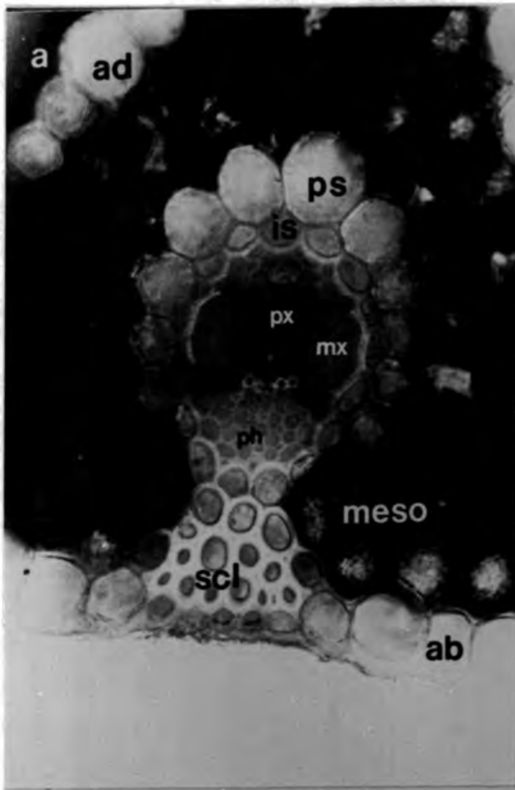
Plate 2.2. Apoplastic tracer study of leaves of Lolium temulentum

a. LMgraph showing a Type 1 vascular bundle and associated tissues of a mature leaf; transmitted light; X330;

b. FluorMgraph of section shown in a. above; rhodamine 6G, sheath-transpired; X330;

c. FluorMgraph of a Type 1 vascular bundle of a senescent leaf; white darts indicate chloroplasts; rhodamine 6G, sheath-transpired; X330;

d. FluorMgraph of mid-vein and associated tissues of a mature leaf; calcofluor, section-applied; X130.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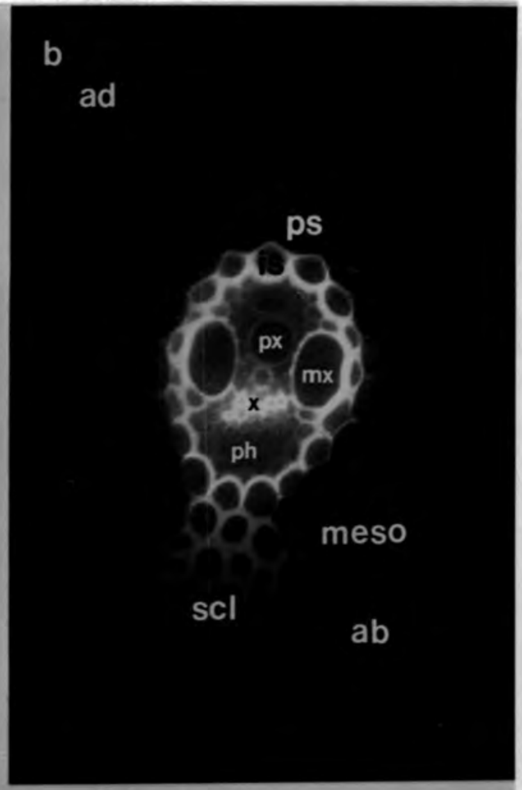
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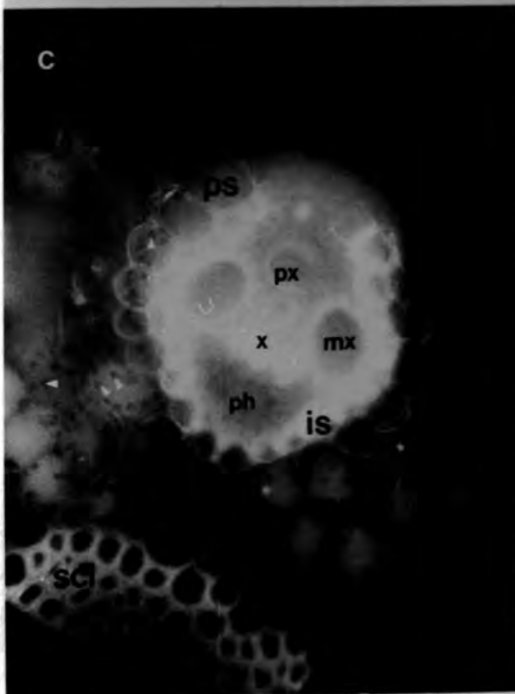
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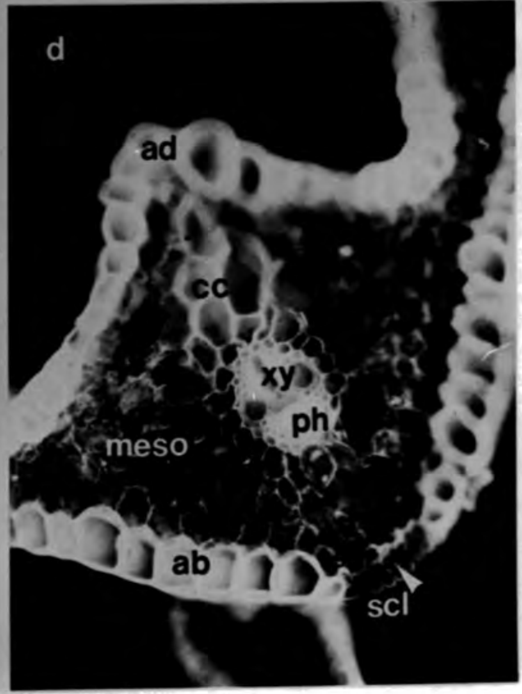
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Plate 2.3. Apoplastic tracer study of leaves of Lolium temulentum

a. FluorMgraph of a Type 1 vascular bundle and associated tissues of a leaf beginning to senesce; autofluorescence; X130;

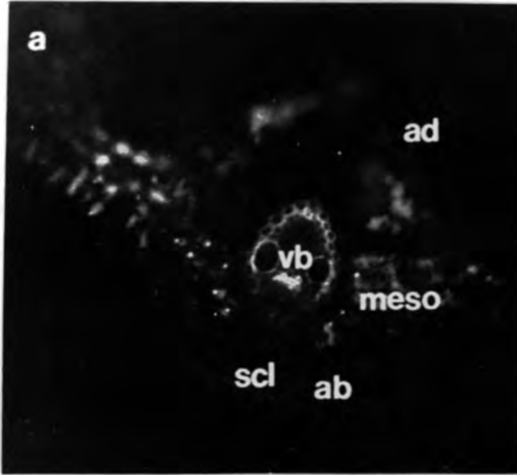
b. FluorMgraph of a Type 1 vascular bundle and associated tissues of a senescent leaf; autofluorescence; X130;

c. FluorMgraph of a Type 1 vascular bundle of a senescent leaf; autofluorescence; X325;

d. LMgraph of a Type 1 vascular bundle and associated tissues of a mature leaf; transmitted light; X130;

e. FluorMgraph of section in d. above; autofluorescence; X130;

f. FluorMgraph of a Type 1 vascular bundle of a mature leaf; autofluorescence; X325.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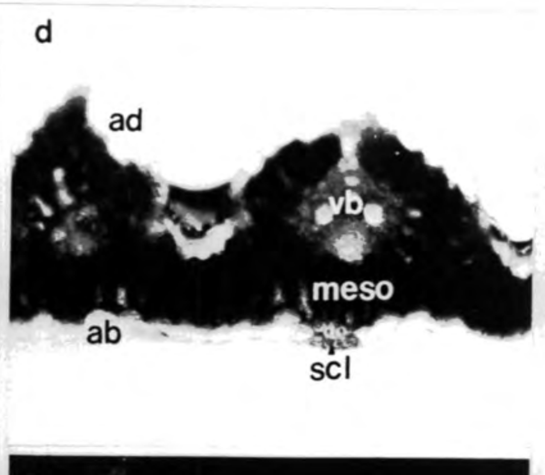
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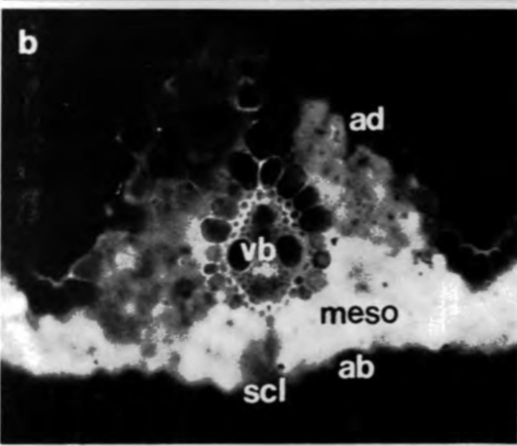
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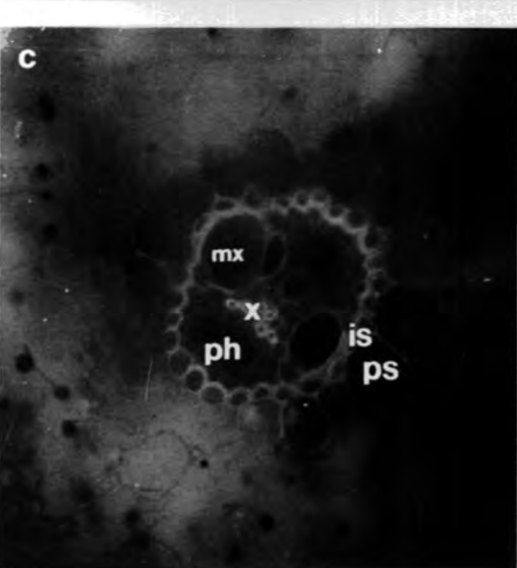
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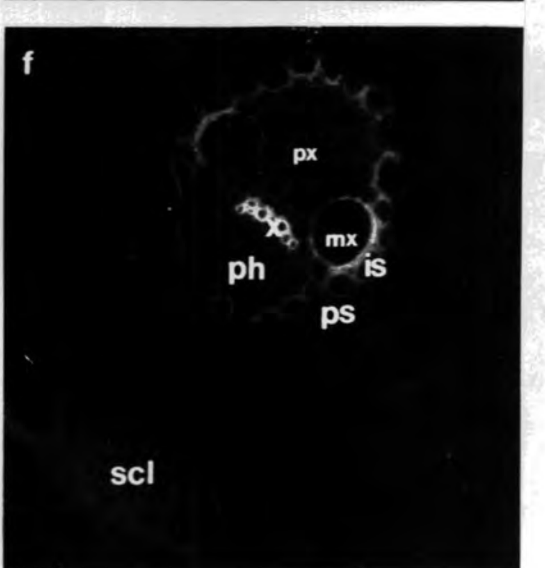
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Plate 2.4. Autoradiograph of distribution of transpiration-stream applied ^{14}C-amino acids in leaves of Lolium temulentum

The plate shows an age sequence of leaves from a single culm, from the flag leaf (1, youngest) to leaf 5, the oldest.

1. browning at leaf tip;
2. browning at leaf tip;
3. blade - brown to $\frac{1}{4}$; yellow to $\frac{1}{3}$;
4. blade - brown to $\frac{1}{2}$; yellow to $\frac{3}{4}$;
5. blade - brown throughout: sheath - brown to $\frac{3}{4}$.

X0.7

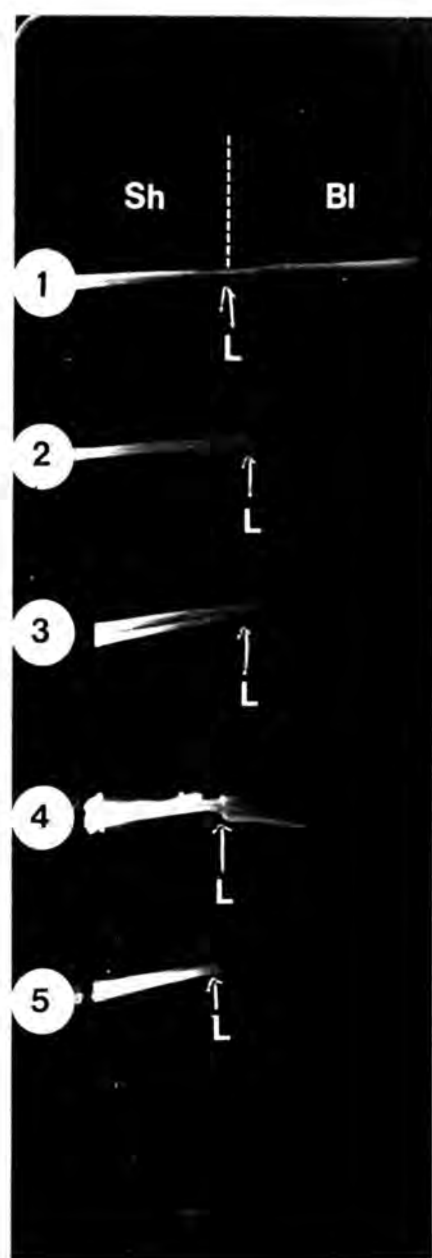
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Plate 2.5. Structure of mid-blade or blade/sheath junction of leaves of Lolium temulentum

a. Two Type 2 vascular bundles and associated tissues at the margin of the blade; the adaxial sclerenchyma bundle is indicated by a black dart; X230;

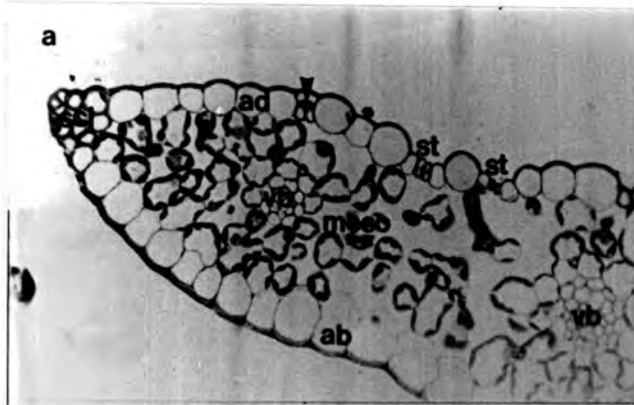
b. Type 1 vascular bundle and associated tissues of the blade; silica cells in adaxial epidermis are indicated by black darts; X230;

c. Type 1 vascular bundle from blade/sheath junction of a senescing leaf showing putative early stage in tylosis (arrowhead); X230;

d,e. Serial sections (c. 15 μm apart) through a Type 1 vascular bundle of the blade/sheath junction of a senescing leaf showing two tyloses in the protoxylem lacuna; X230;

f. Type 1 vascular bundle of the blade/sheath junction of a senescing leaf showing a single tylose in the protoxylem region; X230;

g. Type 1 vascular bundle of the blade/sheath junction of a mature leaf; X230.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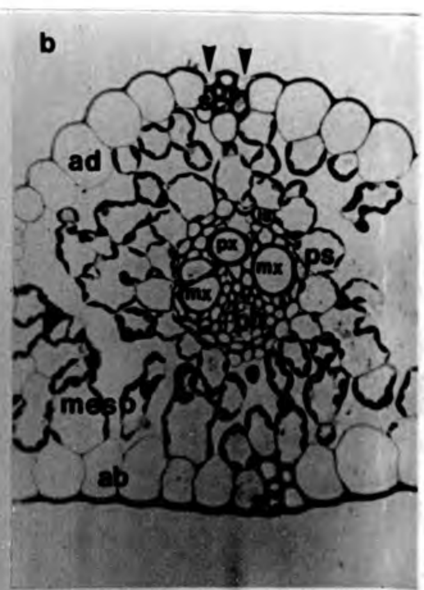
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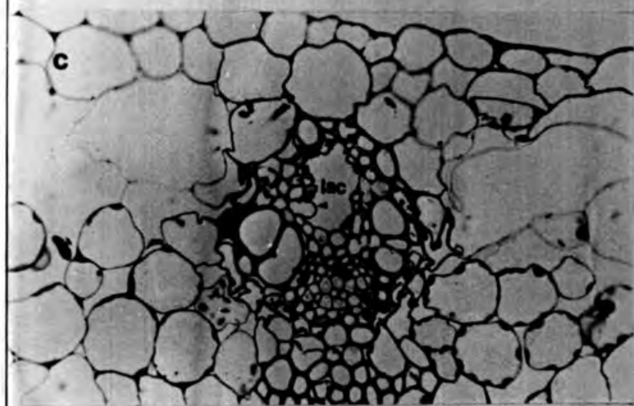
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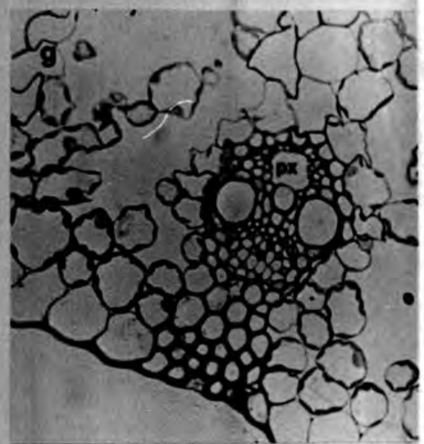
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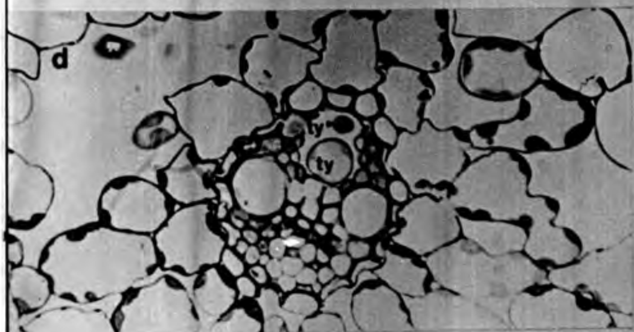
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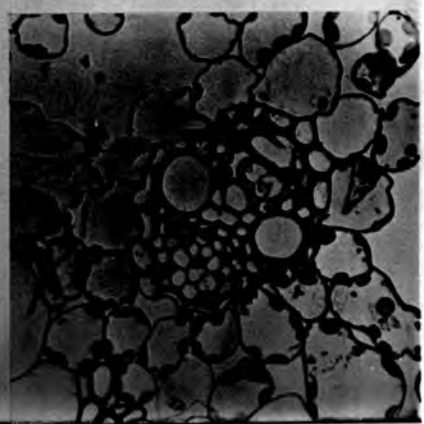


Plate 2.6. Ultrastructure of Type 1 vascular bundles of blade/sheath junctions of senescing leaves of Lolium temulentum

a. A tylose in a protoxylem lacuna; X4,750;

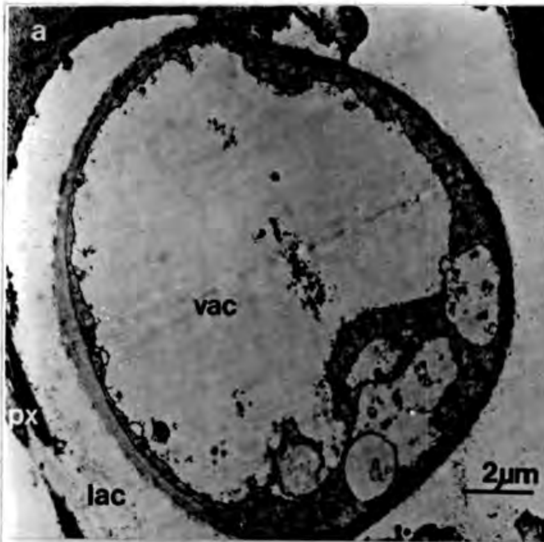
b. Parts of two adjacent xylem parenchyma cells; X38,000;

c. Parts of 4 adjacent xylem parenchyma cells; X10,250;

d. Part of a tylose; X17,000;

e. Part of a tylose; X30,000;

f. Walls of adjacent tylose (w) and xylem parenchyma cell (w'). The former ('a') is thinner than the latter ('b'), but the walls appear homogeneous where they are in direct contact ('c'); X75,000.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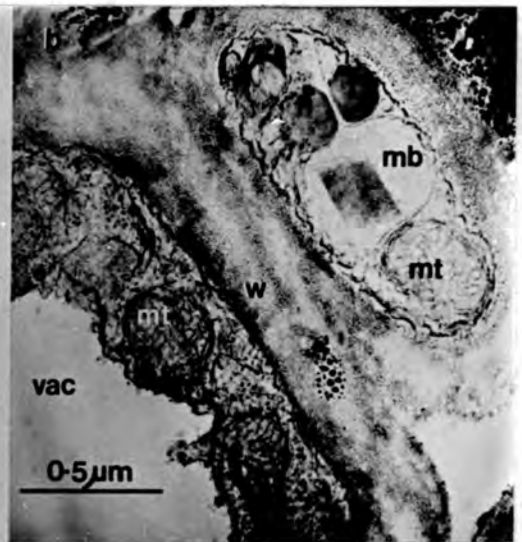
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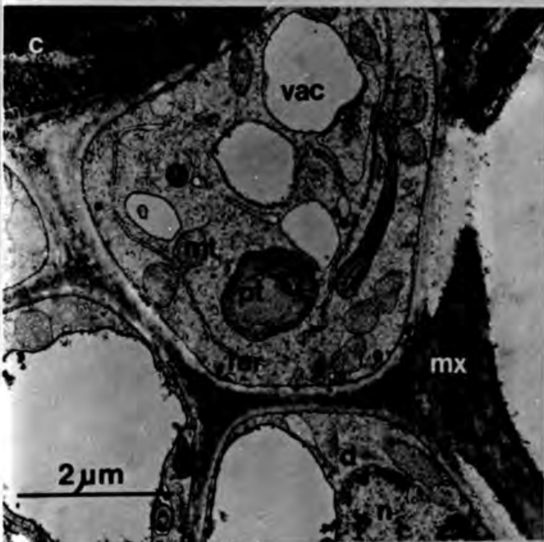
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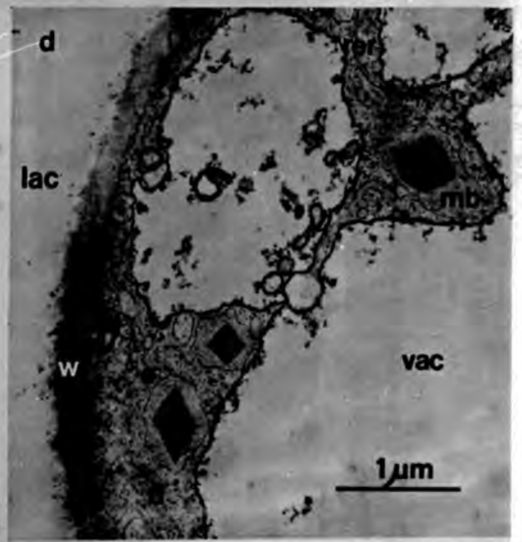
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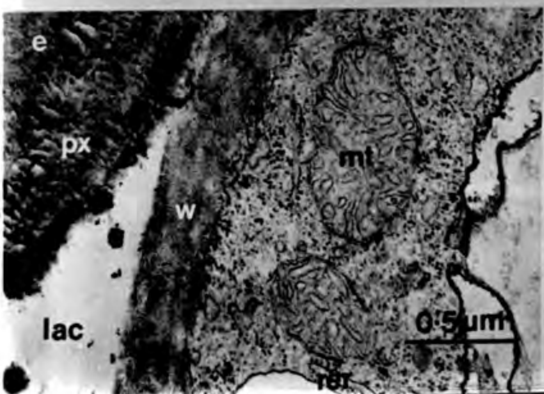
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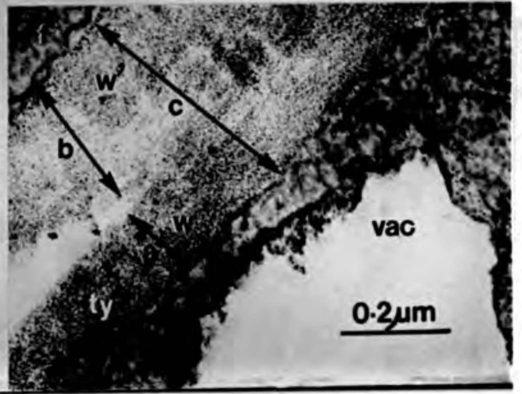
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DISCUSSION

The vascular system of L. temulentum leaves was similar to that described in the literature for other grasses: longitudinally-oriented veins bearing vascular bundles of two distinct types, interconnected by transverse veins. In all cases studied, all the veins present in the upper part of the sheath passed into the blade; the reverse was generally true. In a few cases, however, one or two blade veins stopped at the collar region. This is similar to the situation described for L. perenne (Soper and Mitchell, 1956) and illustrated as if it were a constant feature; its significance in both species is not known.

The course of the transpiration stream/apoplastic pathway was studied using pulse-chase fluorochrome application. With continuous uptake of fluorochrome, general tissue staining results as the fluorochrome diffuses from the vascular system with time. With a pulse-chase method, any general tissue staining was restricted to the leaf tip regions not the mid-blade where the sections were taken.

Evidence that such a method traced the transpiration stream came from a number of observations:

a. specific association of the coloured fluorochromes with the veins;
b. specific association of formazan reaction product with the veins when tetrazolium chloride solution was applied to the cut sheath base as for fluorochrome uptake (results not shown);
c. the speed of movement of the coloured fluorochromes through the leaf - much faster than expected by diffusion;
d. the direction of movement of the coloured fluorochromes towards the leaf tip and their accumulation there;
e. the work of others using the same or similar methods eg Caldwell (1925 - eosin), Hughes and McCully (1975 - calcofluor), Gates and Oparka (1982 - ANS), for which an apoplastic pathway was suggested.

That the fluorescence patterns observed in sections of leaves so treated was an accurate record of the tissues involved in the transpiration pathway was suggested by the following observation: all fluorochromes gave the same fluorescence image which was markedly different to that observed when the fluorochrome was directly applied

to the sections (eg calcofluor - Pl. 2.2d). Fluorescence of tissues naturally fluorescent was always much more intense following sheath-application of fluorochromes.

Thus it was concluded that the method used did trace the transpiration stream and did show which tissues were involved in conduction of water. The only reservation is in the case of senescing and, more importantly, senescent leaves where the pronounced autofluorescence of mesophyll contents may have given spurious fluorescence images by reflection within the tissues. Bearing that in mind, it is suggested that the vascular elements involved in conduction are the lignified xylem vessels and the inner bundle sheath. Both meta- and protoxylem vessels appeared to be involved but the evidence for the latter was not unequivocal. The apparent involvement of sclerenchyma bundles in conduction is in agreement with a similar observations made by G.H. Banbury (pers. comm.) in an African composite, Tridax sp., and suggests that any long series of lignified elements may conduct water provided it is subject to a 'transpirational pull'. The same pathway appeared to be involved in mature and senescing/senescent leaves.

In the previous Section, it was concluded that water was lost from the tissues of the leaf during ageing and senescence. Since the transpirational pathway appeared to function in senescing leaves, it may be suggested that either the mesophyll tissues cannot abstract water from the transpiration stream or that any water so obtained is not held within the cells. The appearance of autofluorescent material in the mesophyll cells during senescence may be related to cytomembrane degradation (eg Merzlyak et al., 1983) and is circumstantial evidence for the latter interpretation of water loss.

The xylem appeared to be open throughout its length and throughout most of the life of the leaf. However, at the blade/sheath junction of senescing leaves tyloses were found in the protoxylem vessels/lacunae of Type 1 bundles. Their specific location at this region and the observed accumulation of ^{14}C-labelled amino acids here during leaf ageing may be related. Presence of coloured fluorochromes in veins bearing Type 1 bundles before those with Type 2 bundles may indicate preferential conduction in the former. It is thus possible that the tyloses may act by selectively preventing certain materials in the transpiration stream - but not water - from entering the blade during

senescence. They may be postulated to achieve this by either, or both, of two methods - physical blockage of the protoxylem or selective uptake of metabolites.

Slatyer (1967) has shown that any additional resistance to flow in the transpiration stream - such as is assumed to be provided by the tyloses here - will only affect the overall rate of flow through the plant if the resistance so caused is a significant fraction of the total resistance to flow. The observation that coloured fluorochromes, and presumably water, reached the tips of senescing leaves at about the same rate as in mature leaves is consistent with the assumption that tyloses do not markedly affect rates of flow. However, they are likely to introduce a 'filtration resistance' (Slatyer, 1967) causing local resistance to flow only in the immediate area. These considerations presuppose the protoxylem remains conducting through the tylosed region. It is possible that transpirational flow is directed around these regions (cf the case of cuts in xylem described by Mackay and Weatherley, 1973) or that tyloses only form in vessels - or regions of vessels - which have ceased conduction (Zimmermann, 1979). Thus the case for physical blockage of the transpiration stream being the cause of the observed accumulation of ^{14}C seems weak.

Evidence for a selective uptake and accumulation of metabolites is much stronger. For the tyloses to perform such a 'filtration' rôle they do not need to be directly in the transpiration stream; materials could be abstracted from the metaxylem by the vascular parenchyma and transferred to the tyloses for storage. The highly-cristate mitochondria of both tyloses and parenchyma cells are consistent with the presence of such an energy-demanding metabolism. The large vacuoles of the tyloses may act as sites of short- or long-term storage of these materials. This view of tylose function is reminiscent of the rôle in water storage previously accorded them by eg McNicol (1908) and Haberlandt (1914); here the stored water may contain a high concentration of solutes such as amino acids.

A short study of the timing of tylose formation showed that they were present in 4th leaves which had begun to lose chlorophyll in their apical blade segment. In the absence of a more detailed study, however, it is not possible to state whether tyloses may be cause, effect or coincident with initiation of leaf senescence.

As a working hypothesis, it is proposed that tyloses affect the quality and quantity of metabolites entering the blade from the sheath in the xylem during senescence. It is assumed that translocation of eg breakdown products of proteolysis out of the blade is not affected and thus the raw materials for macromolecule synthesis are conserved within the non-senescing part of the plant as a whole. It is possible that metabolites accumulated at the blade/sheath junction may eventually be translocated back into the plant via the phloem.

Previously it has been considered that the net loss of protein observed in senescing leaves (see also previous Section) may be related to failure of synthetic reactions or loss of amino acids by translocation from the leaf (eg Simon, 1967), or enhanced proteolysis (eg Wittenbach, 1978). Tyloses offer a fourth possibility in that they may limit entry of amino acids to the blade to such an extent that only essential synthesis of senescence-associated proteins is possible. In some respects this view of tylose function brings the concept of senescence back to its old term 'starvation' (eg Yemm, 1937).

Evidence in favour of the above hypothesis of tylose function can be summarized thus:

a. Tyloses are apparently absent from mature leaves.

b. Tyloses were specifically associated with Type 1 bundles at the blade/sheath junction; such bundles contain the widest metaxylem vessels and are assumed to be the main routes for conduction of water and dissolved solutes therein (eg Ayres, 1981).

c. Tylosis is in part coincident with the observed accumulation of ^{14}C-labelled amino acids at the blade/sheath junction of senescing leaves.

d. The ultrastructure of parenchyma cells and tyloses suggests they may both be highly metabolically active. Tetrazolium chloride solution applied to the transpiration stream was rapidly converted to formazan by vein-associated tissues - assumed to be parenchyma cells (results not shown). Pate and O'Brien (1968) demonstrated the ability of pea vascular parenchyma cells to abstract and metabolize transpiration stream-applied ^{14}C-labelled amino acids. They further suggested that vacuolate parenchyma cells might store root-supplied soluble nitrogenous compounds, and have subsequently emphasized the rôle of such cells in solute retrieval (O'Brien, 1974; Pate, 1980).

e. Using ^{15}N in rice, Mae and Ohira (1981) found that entry of new N into senescing leaves was limited.

It has been suggested by Thomas and Stoddart (1980) and Stoddart and Thomas (1982) that grass leaf senescence may be initiated in an enclosing leaf sheath as a result of stress within it caused by increase in number of enclosed leaves. Although the previous Section suggested that senescence began in the blade, the changes in cellular components measured may only have been effects of this process not its cause(s). Ethylene has been implicated in inducing tylosis (eg Pegg, 1976) and in the senescence of oat leaves (Gepstein and Thimann, 1981) and may be released or formed as a result of stress. Hence tylosis in a leaf may be related to growth and development of younger, enclosed leaves. Stress need not immediately result in tylosis but may instead predispose the leaf to form tyloses at the correct place at the correct time. If in any way tylosis is related to initiation of grass leaf senescence, the original suggestion of Thomas and Stoddart (1980) that senescence might begin in the sheath seems valid. The smaller, more densely packed cells of the blade/sheath junction (eg also Soper and Mitchell, 1956) may prevent or hinder upwards diffusion of ethylene from the sheath and result in its build-up to levels sufficient to induce tylosis. It might further be speculated that any delay in initiation of stress in the sheath and subsequent tylosis may be related to time taken to form air lacunae in which upwards diffusion of ethylene takes place.

Alternatively, tylosis in the blade/sheath region may be related to stress induced here as a result of blade unrolling and bending. During the former, the radially-oriented veins become linearly-oriented in the blade; the further a vein is from the mid-vein, the more it is bent. With blade bending, the veins become secondarily bent through angles varying from near 0° to well over 90°. As a result of vein-bending it is possible that deflection of the transpiration stream in this region may alter its flow characteristics and create a 'bottle-neck'. Any such reduction in flow in this region may increase the opportunity of tyloses or parenchyma cells here to 'filter' the transpiration stream.

Tylosis in *L. temulentum* may be regarded as a physiological equivalent of leaf abscision in other, notably dicotyledonous, species, preventing egress of essential metabolites from mature parts of the plant whilst allowing maximum recovery of such from senescing parts. Although grass leaf abscission appears to be rare it does occur eg in *Neostapfia* and *Orcuttia* (Crampton, 1959) and may be more common than the data in the literature indicate because it is not a feature that is usually recorded (Clifford and Watson, 1977). In those species where it does occur it would be of interest to know if tylosis occurs at the blade/sheath junction.

Tylosis in *L. temulentum* appears to be related to senescence in some way, whether it is involved in the initiation of this process is not known. Its discovery is another example of interactions occurring between blade and sheath during development and emphasizes the statement by Woolhouse (1982) that, '...it is undeniable that if one wishes to know about the senescence of leaves one will ultimately have to work on leaves...'.

CHAPTER 3 LIGULE

'But no work of science, however passé it may subsequently be deemed to be, is lacking in some element of truth; and this we must cherish and incorporate into the summation of our knowledge.'
(C.W. Wardlaw, 1970)

LIGULE: INTRODUCTION

The grass ligule can be concisely defined as a membranous outgrowth of the adaxial leaf surface at the blade/sheath junction. Two categories are usually recognized - veined ligules which are vascularized, and membranous ligules which are not; only the latter will be considered here.

The ligule - or at least a structure occurring in the same position on the leaf and bearing this name - is not unique to the Poaceae. Examples may be found throughout the monocotyledonous plant groups, eg Palmae (Tomlinson, 1961), Cyperaceae (Metcalfe, 1971) and Liliaceae (Arber, 1925). Any relationship that might exist between these organs and the grass ligule is not known. There is, however, a certain amount of justification in suggesting analogy - or even homology - between the grass ligule and the ligule of certain lycopsids (eg Foster and Gifford, 1959). There appears to be no similarity between the grass ligule and the similarly-named structure on the florets of certain members of the Asteraceae (eg Baagøe, 1980).

At present the ligule can only be defined in terms of its position on the plant; not enough is known about either its structure or function to define it in any other way. This lack of understanding of ligule biology is in many respects surprising because the ligule has been studied for well over a hundred years (since eg Dutailly, 1878). There appear to be two main reasons for this general neglect of detailed study of grass ligules - the assumption that its function is understood and the suggestion that it has a stipular nature.

Ever since the end of the 19th century it has been largely assumed that the function of the ligule is to exclude water, dust and harmful spores from the interior of the plant (eg Kerner and Oliver, 1894). Acceptance of a stipular nature of the ligule (eg Glück, 1901) carries with it the implication that it also has a purely passive, protective function.

However, despite no apparent attempts to establish the validity - or otherwise - of these assumptions, a number of aspects of ligule biology have been studied. For example, morphology and external anatomy have been given a great deal of attention by such workers as Neumann (1938), Jacquet and Plessis (1950) and Sekine (1959). These studies have provided valuable information regarding the range of ornamentation and cell types of which the epidermes are composed and emphasized the

use of ligule structure as an aid to identification of grasses at the species level.

Aspects of the internal structure of ligules have been studied by eg Colomb (1887), Hayward (1938) and Tran (1963). The value of such studies, however, was limited by the techniques used; little information regarding types of tissue and presence or absence of chloroplasts for example can be obtained. In almost no cases were photomicrographs given.

Few attempts to study both external and internal features of the same ligule appear to have been made. Exceptions include Artschwager (1925 - sugar cane) and Bonnett (1961 - oats) but, because these workers were concerned with describing the morphology and anatomy of the whole plant, the ligule only received brief mention.

Even with the introduction of new methods of examining structure and ultrastructure, ligules appear to have been all but ignored. Only recently have SEM studies been carried out, eg Soni et al. (1970) in oats. Here, however, the emphasis was upon presence or absence of silicon in the various leaf epidermes; only brief mention was made of the ligule and very little structural detail given.

There appear to be no TEM studies reported in the literature. This lack of ultrastructural examination is surprising, especially in view of the number and variety of such studies on the analogous structure in the Lycopsida (eg Sigee, 1974; Horner et al., 1975; Kristen et al., 1982). Again it can be suggested that acceptance of the 'function question' as being settled has resulted in their fine structure being ignored. Although statements by Prat (1932) - albeit erroneous - that the ligule 'est formée seulement de deux assises de cellules épidermiques directement accolées' do not give much incentive to study these organs in the TEM.

Despite this rather negative background certain features of ligule structure seem relatively well-established and are summarized below. Ligules appear to originate from the adaxial leaf epidermis solely (Sharman, 1941) very early on in leaf development. Bugnon (1921) working with Dactylis glomerata L. and Bonnett (1961) working with Avena sativa L. have shown that the ligule consists essentially of two uniseriate epidermes enclosing a parenchymatous tissue. They appear wedge-shaped in LS (Soper and Mitchell, 1956 for Lolium perenne L.;

Bonnett, 1961 for Avena sativa), and lens-shaped in TS - widest in the middle, tapering to each edge (Bonnett, 1961). Tran (1963) showed TS's of several ligules and figured them all with cuticles over both epidermes; in sugar cane both epidermes were claimed to be suberized (Artschwager, 1925). Generally the ligule seems to be composed of elongate parenchymatous cells although numerous differentiated elements have been found in the abaxial epidermis, eg short cells and trichomes (Prat, 1932; Neumann, 1938).

Despite the fact that most reports describe the ligule as a thin, white or colourless, papery structure, such workers as Glück (1901) and Neumann (1938) stated that chlorophyll was present. Percival (1921) went further and claimed the presence of chloroplasts in some cells of the wheat ligule. The ultrastructure of any chloroplasts would be of interest, as would information concerning other organelles as an aid to defining the metabolic capabilities of the ligule.

This rather composite nature of the 'average ligule' built up above emphasizes the lack of in-depth information regarding the structure of any one species. Such a detailed study was undertaken of the membranous ligule of L. temulentum and the results described in the following Sections. It was hoped that the information so obtained would be of use in assessing the validity of the accepted view of ligule function and contribute to a more balanced view of structure and function within the whole leaf by a consideration of the third leaf organ.

SECTION a. STRUCTURE AND ULTRASTRUCTURE

INTRODUCTION

The only previous anatomical study of the ligule of L. temulentum appears to be that of Neumann (1938). This worker concluded that it was purely membranous and homogeneous throughout, although it did belong to a ligule group which occasionally bore hair-like structures (haarähnlichen Bildungen) on their margins. In view of the lack of previous study of this ligule, it was necessary to make use of a wide range of optical and electron microscopical techniques to build up as complete a picture as possible of its overall structure.

A short study was also made of the ligules of L. perenne L., L. multiflorum Lam., L. x hybridum Hausskn. (L. perenne x L. multiflorum) and Festuca pratensis Huds. in order to assess how widespread the ligule structure of L. temulentum might be. Of these four species all but L. x hybridum have been previously studied by other workers. Neumann (1938) described L. perenne ligule as bearing marginal hairs but being otherwise membranous. Jacquet and Plessis (1950) described this species and F. pratensis as being completely homogeneous, whereas they claimed presence of marginal hairs for L. italicum (L. multiflorum). Soper and Mitchell (1956) figured a LS of L. perenne ligule which demonstrated its wedge-shaped nature but made no mention of tissue types or chloroplasts within it.

MATERIALS AND METHODS

Light Microscopy

Surfaces of ligules were examined on excised material either fresh and mounted in water, or after clearing for several weeks in 75% (v/v) lactic acid (Simpson, 1929). Clearing was performed on material which had been fixed in a mixture of 5 ml, 40% formaldehyde; 7 ml, glacial acetic acid; 90 ml, 70% ethanol (FAA). Cleared material was best for viewing cell arrangement and photomicrography, but details seen in fresh material (eg chloroplasts and transcellular strands) were often lost. Both methods were used wherever possible.

Fluorescence Microscopy

Freshly-excised ligules were incubated in the fluorochrome solution on microscope slides beneath cover slips for 5-10 min before being examined in the FluorM. A number of fluorochromes were experimented with; the four for which results are presented were:

a. auramine O, 0.1% (w/v) aq., for cuticle (eg Heslop-Harrison, 1979);
b. calcofluor, 0.01 or 0.1% (w/v) aq., for 'cell walls' (eg Nagata and Takebe, 1970);
c. uvitex BOPT, 0.1% (v/v) aq., for 'cell walls' (eg Peberdy and Buckley, 1973);
d. aniline blue, 0.01% (w/v) in 0.067M K_2HPO_4, for 'callose' (eg Currier and Strugger, 1956).

Filter combinations used were:
excitation filter BG3, suppression filter K460 for calcofluor, uvitex BOPT and aniline blue;
excitation filter BG12, suppression filter K510 for auramine O.

Vascular Continuity Ligule/Leaf

Leaves were excised below water at their bases, the cut ends immersed in 0.1% aq. eosin and allowed to transpire in the laboratory at room temperature beneath fluorescent lights. Passage of the dye through the leaf was visually assessed.

Scanning Electron Microscopy (SEM)

FAA-fixed material was prepared for SEM by dehydration in a graded ethanol series (70-100%) before oven-drying at 70°C between weighted filter papers (after Stant, 1973). The filter papers were weighted by holding them between microscope slides and securing the whole with elastic bands to prevent gross distortion of shape of the material during drying. The dried material was then mounted on aluminium stubs using silver dag, coated with gold in a Polaron E5100 cool sputter coater and viewed in a Cambridge Stereoscan 600 SEM operating at 15 kV in the secondary electron mode. Images were recorded using Ilford FP4 black and white film.

No one method of specimen preparation for SEM gives entirely satisfactory preservation of structure (see eg Robards, 1978; Sargent, 1983); all current methods have their advantages and disadvantages. The method used here was employed because it gave reasonable preservation of structure as judged by the LM image.

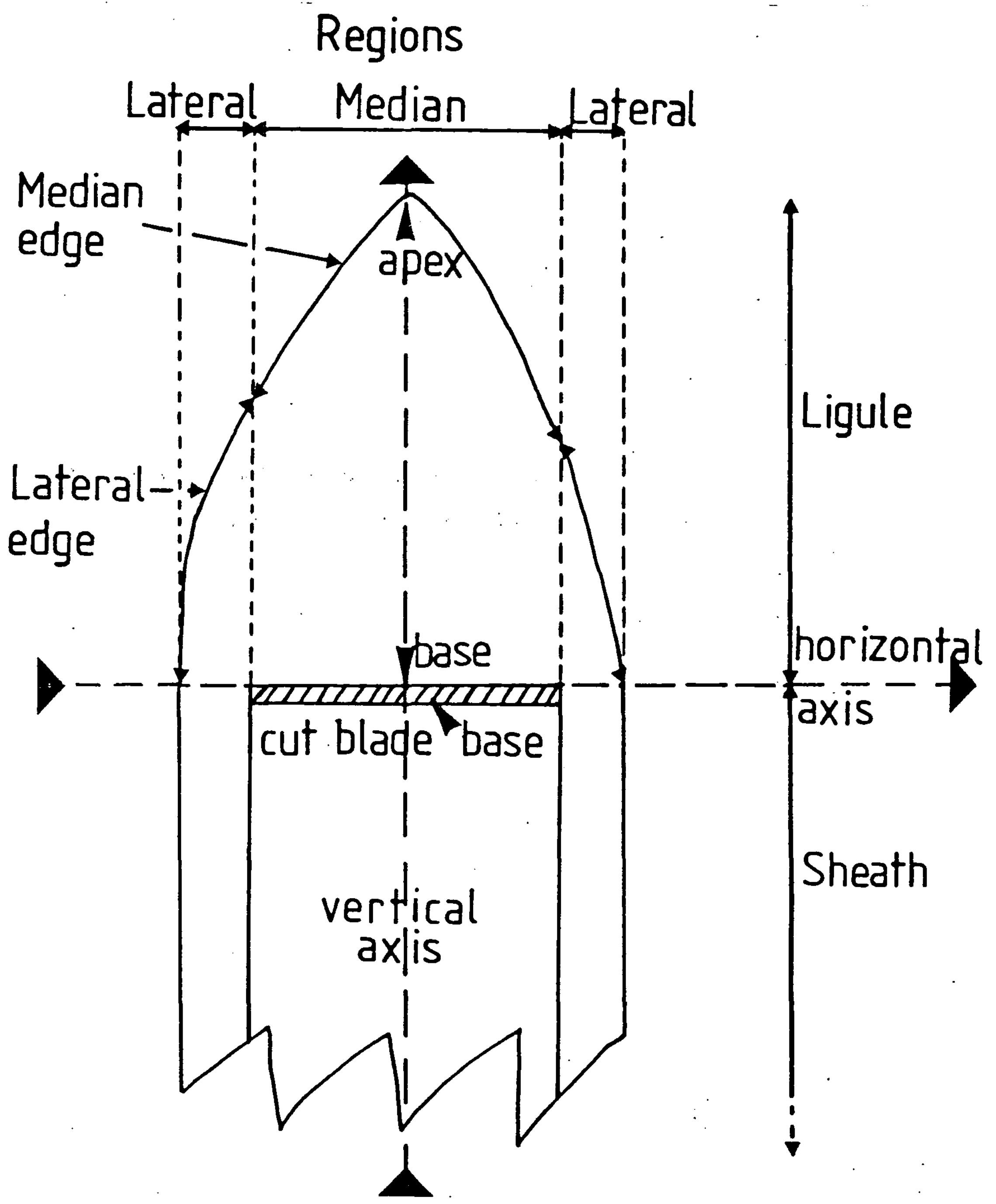
Energy Dispersive Analysis of X-radiation (EDAX)

Material prepared for SEM as above was used for EDAX of silicon in a Philips SEM 500 fitted with a Philips PV9100 EDAX system. Selected areas of abaxial surfaces of *L. temulentum* ligules were scanned over the X-ray energy range 0-20 keV with the detector (area 11 mm^2) set at 25° to the stub. Additionally ultrathin sections of ligule were analyzed by EDAX using a Philips EM 400 in the scanning/transmission mode.

Terminology

Regions of the ligule referred to in the text are diagrammatically illustrated in Fig. 3.1.

Figure 3.1. Schematic diagram of a membranous ligule showing the various regions referred to in the text


Regions
Lateral
Median
Lateral
Median
edge
apex
Ligule
Lateral
edge
base
horizontal
axis
cut blade base
Sheath
vertical
axis

OBSERVATIONS

A. L. temulentum

Morphology

The description of the ligule of this species as being 'blunt, up to 2 mm long, membranous' (Hubbard, 1976) was confirmed by examination of the plant with the naked eye. The drawing of the ligule in Hubbard (1976), however, shows it with an irregular apex which was not evident to the naked eye, but was confirmed by the LM examination below.

Anatomy

1. Externally-observed Features

a. Light Microscopy

Both surfaces - abaxial (facing the adaxial surface of the blade) and adaxial - appeared to be composed of long cells only (Pl. 3.1e). These cells were elongated parallel to the vertical axis of the ligule, had rounded/straight end walls, smooth (non-pitted) side walls and occurred in overlapping tiers. In ligules mounted in water, trans-cellular strands were seen with cyclosis moving green chloroplast-like structures and the single, larger colourless nucleus around the cell. When viewing the 'surface' of the ligule it was not easy to determine to which layer of the cells the chloroplast-like structures belonged. On several occasions regions bearing these structures were interrupted by areas where they appeared to be absent, giving the impression of green stripes in the vertical axis of the ligule. At all levels of focus, the only cell shape observed corresponded to that of typical epidermal long cells.

Towards the edges of both surfaces, the long cells became shorter. Each long cell row was terminated by either the rounded or straight end wall of a very short cell, or by a distinctly papillate cell which projected beyond the edge. These papillate cells were about the same size as the terminal long cells but their apical portions were extended to give either a conical or elongate-rounded projection (the latter only is shown; Pl. 3.1c). The irregular outline thus produced may be the

explanation for the ligule drawn in Hubbard (1976) bearing such an apex (see above).

No hairs of any type, stomata or stomata-like structures were seen in either surface, nor were any veins seen in the ligule (in agreement with Neumann, 1938).

b. Fluorescence Microscopy

Auramine O staining showed a cuticle to be present over both surfaces (Pl. 3.1f). Staining was particularly intense within the radial cell walls and might indicate the presence of cuticular pegs (eg Fig. 2 in Heslop-Harrison and Heslop-Harrison, 1982). The thread-like surface material which stained most intensely with this fluorochrome (Pl. 3.1f) may be epicuticular wax deposits.

Incubation of the ligule in aniline blue resulted in the fluorescence image seen in Pl. 3.1d. The localized areas of fluorescence either side of radial cell walls of adjacent cells in both epidermes are inferred to indicate sites of plasmodesmata, by analogy to the 'pit callose reaction' of Currier and Strugger (1956). Prolonged incubation in this fluorochrome led to general fluorescence of the contents of the whole cell, presumably as a result of increased 'callose' deposition with time.

Calcofluor gave a most unusual reaction; fluorescence was only induced in the adaxial epidermis and appeared to be confined to the cytoplasm not the cell walls (Pl. 3.1a,b). When applied to the leaf of this species only the cell walls fluoresced. The fluorescence image that resulted clearly demonstrated the decrease in size of the adaxial epidermal cells from base to apex in the ligule (Pl. 3.1a,b). Uvitex BOPT applied to the ligule gave the same fluorescence image as calcofluor (results not shown).

c. Vascular Continuity Ligule/Leaf

On no occasions were sheath-applied eosin solutions observed to pass into the ligule, although they did travel to the leaf tip.

d. Scanning Electron Microscopy and EDAX

SEM of the abaxial surface was consistent with the LM conclusion that it was composed of long cells only (Pl. 3.1g). It also showed thread-like and granular material on the surface which may be the wax deposits suggested by the use of auramine O above. EDAX of this surface (Pl. 3.1h) identified large amounts of gold (the coating metal) but no significant accumulations of silicon. EDAX of sectioned material also failed to identify significant accumulations of silicon.

2. Internal Features

a. Light Microscopy

Serial sectioning of numerous ligules from their apices to bases showed them to have a wedge-shaped structure, being 2-celled near the apex (Pl. 3.2a) and many-celled (c. 6 or 7) at the base (Pl. 3.2f). In TS near the middle of the ligule it was c. 3-5 cells thick (Pl. 3.2c) tapering to each lateral edge where it was only 1 or 2 cells thick (Pl. 3.2d). In all places where the ligule was more than two cells wide, toluidine blue-staining demonstrated a distinct polarization (Pl. 3.2c,h): a uniseriate abaxial epidermis of large-vacuolate cells; a uniseriate adaxial epidermis of densely-staining cells; a mesophyll tissue between these two epidermes which contained plastids (deemed to be chloroplasts by their green colour and red autofluorescence under calcofluor epifluorescence conditions).

The two epidermes corresponded to the surface layers of long cells in the ligule, and the mesophyll to the layer(s) bearing chloroplast-like structures. The chloroplasts were seen more clearly after Sudan black B 'staining' (Pl. 3.2b). Even where the mesophyll was present its width varied (as its number of cell layers varied) and generally appeared to have a smaller chloroplast population per cell than the blade mesophyll (Table 3.1). Some cells appeared to be devoid of chloroplasts and this might be the explanation for the stripes of chlorophyllous/non-chlorophyllous tissue referred to in 1.a. above.

Nuclei could frequently be seen in cells of all layers - never more than one per cell was observed. No intercellular spaces were seen nor any cell types other than the epidermal and mesophyll cells described. Adaxial epidermal cells - the biggest of the cell types -

Table 3.1. Comparison of number of chloroplast profiles per mesophyll cell profile between ligule and blade of Lolium temulentum

	Ligule	Blade
No. chloroplast profiles per cell[a]	2.3 ± 0.86	8.0 ± 1.81

a = mean ± se of 20 mesophyll cell profiles in transverse sections, 1 μm thick

appeared to be more or less rectangular in TS with very thick outer tangential (periclinal) walls (eg Pl. 3.2b). From the toluidine blue-staining reaction of the ligule none of the cell walls appeared to be lignified. Although both epidermes appeared to have a cuticle - as judged by auramine O staining - it did not 'stain' with Sudan black B (Pl. 3.2f, compare ligule and sheath).

At the base of the ligule its insertion upon the leaf was observed (Pl. 3.2e). However, due to its curved nature it was rare to get a LM section which showed it as clearly as the free-hand TS in Pl. 3.2g. At the insertion region, the ligule abaxial apidermis was continuous with the leaf adaxial epidermis (Pl. 3.2e); in LS continuity of both ligule epidermes with the leaf adaxial epidermis can be seen (Pl. 3.2h).

Tissue Deformation

In several of the LMgraphs presented above, deformation of cells of the abaxial epidermis and mesophyll can be seen. It is possible that this is the natural state of these tissues; against this is the undeformed image of sections of whole ligules fixed in FAA and wax-embedded (not shown), TS's of ligule near its base (Pl. 3.2f) and TS's of L. perenne ligule (below - Pl. 3.10a). Attempts were made to determine whether the deformation was the result of loss of turgor in the cells which demonstrated this feature by fixing material in aq. formaldehyde/glutaraldehyde. Both these aldehydes are believed to be not, or only weakly, osmotically active (Bernard and Wynn Gail, 1964; Bone and Ryan, 1972) and it was assumed that the cells should have been maximally turgid. The same deformation was seen as in tissue conventionally fixed in cacodylate-buffered fixative. Thus the deformation observed is attributed to removal, upon excision, of the stresses the ligule experiences when upon the leaf - tightly enclosing a leaf and being situated at the fulcrum of the blade. Whilst this deformation is inconvenient it did not appear to affect the ultrastructure of the cells concerned.

b. Transmission Electron Microscopy

The ultrastructure described and discussed below is principally that of the mid-region of ligules of mature leaves ('emerged ligules') seen in TS.

Abaxial Epidermis

The outer tangential walls of these cells were thicker than those of the inner tangential or radial walls and bore a cuticle which corresponded to Type 3 of Holloway's (1982) classification - mainly amorphous outer region, reticulate inner region next to the wall (Pl. 3.6c). Epicuticular wax deposits were not seen on this cuticle, nor on that of the adaxial epidermis. A prominent middle lamella was present around the walls; no evidence of its cutinization or cuticularization was seen. The starred region in Pl. 3.6c may, however, correspond to a cuticular peg or 'spandrel' (Holloway, 1982), the presence of which was inferred from the FluorM study.

There was no ultrastructural evidence for secondary thickening or other modifications of the walls of this, or any other tissue, and they were assumed to be largely cellulosic and primary in nature. Numerous plasmodesmata traversed the radial walls of adjacent epidermal cells (as predicted from the FluorM study) but were rarely seen between these cells and the mesophyll cells below. The epidermal cells were large-vacuolate (Pl. 3.3a,b; 3.5a); the thin parietal cytoplasm contained numerous ribosomes but few mitochondria and dictyosomes, little RER and an occasional plastid.

Mesophyll

This tissue also consisted of vacuolate cells, but the ratio of vacuole to cytoplasm volume was intermediate between that of the cells of the two epidermes (Pl. 3.4). Transcellular strands (Pl. 3.4; 3.5a) were sometimes seen, suggesting that it was this tissue which was observed, by LM, to undergo cyclosis. The prominent feature of these cells was the chloroplasts (Pl. 3.4; 3.8c) which appeared to be randomly distributed in the peripheral cytoplasm. They frequently bore starch grains, stromal ribosomes and osmiophilic globules in addition to well-developed grana (Pl. 3.8d). A prominent peripheral reticulum was often present and closely associated with the double membranes of the chloroplast envelope (Pl. 3.8d). In some cases starch grains appeared to be absent but angular/oval bodies of unknown nature were observed in the stroma ('?' in Pl. 3.8b).

The cytoplasm also contained numerous ribosomes, several mitochondria and dictyosomes, frequent strands of RER and occasional microbodies.

Numerous plasmodesmata connected these cells to one another, and to the adaxial epidermal cells below (Pl. 3.5b). The walls bore a prominent middle lamella (Pl. 3.6b) and appeared to be more or less the same thickness all the way around the cell (Pl. 3.3b).

Adaxial Epidermis

Cells of this tissue had the highest ratio of cytoplasm to vacuole volume; frequently the vacuome appeared to consist of several small, dispersed vacuoles (Pl. 3.4). The cytoplasm was densely populated with ribosomes - both free and in polysomal configurations - and abundant strands of RER (Pl. 3.7). A large population of mitochondria (Pl. 3.4) and hypersecretory dictyosomes (Pl. 3.7) was characteristically present; amoeboid plastids were common.

Plasmodesmata were common between adjacent epidermal cells (Pl. 3.5b) but tended to occur in groups in regions of the radial walls where they were thinner, suggestive of pit fields. The outer tangential wall (Pl. 3.6a) was the thickest wall of the ligule, frequently possessed a microfibrillar nature and bore a Type 3 cuticle. To the inside of this wall, within the periplasmic space, material possessing a fibrillar structure was often found (Pl. 3.6a). Vesicles, assumed to be dictyosome-derived, whose contents had a similar fibrillar structure can also be seen near the periplasmic space in Pl. 3.6a. Evidence of some degree of secretory activity was provided by the presence of numerous paramural bodies (Pl. 3.4a) observed principally in cells of this epidermis, but in all other tissues to a lesser extent.

Ultrastructural Variation in the Ligule

The ultrastructure of the tissues as described above seemed largely constant throughout the ligule; only towards the base were marked differences observed, in the abaxial epidermis. Here chloroplasts with well-developed grana and peripheral reticula (Pl. 3.8a), and microbodies bearing crystalline inclusions (results not shown) were found.

At the edges where the two epidermes were in direct contact, they maintained their ultrastructural differences and plasmodesmatal connections between them were rarely seen (results not shown).

Insertion Region

Generally the three ligule tissues in this region were as distinct as in other parts of the organ but differences were observed in the abaxial epidermis. The cuticle of this tissue appeared to be more developed, being up to 0.5 μm thick (Pl. 3.9b). The adaxial leaf cuticle was also thicker here than elsewhere on the leaf (Pl. 3.9b). Cells of the abaxial epidermis contained numerous plasmalemmal invaginations against the outer tangential wall, which occasionally contained vesicles (darts in Pl. 3.9b,c), and coated vesicles associated with the dictyosomes and plasmalemma (Pl. 3.9c,d). The adaxial leaf epidermal cells also appeared to have more cytoplasm than in other regions of the leaf and contained numerous highly-cristate mitochondria, strands of RER (Pl. 3.9b) and dictyosome-associated coated vesicles.

B. Other Species

Emerged ligules of the four species described below were taken and TS's studied with LM, FluorM and TEM. Only selected aspects of their biology are illustrated and described below.

L. perenne

The tripartite nature of the ligule is shown in Pl. 3.10a; the dome-shaped outer tangential walls of the abaxial epidermal cells are well displayed as is the gradation of toluidine blue-staining intensity across the width of the ligule. Both epidermes bore a cuticle which was generally thicker abaxially (Pl. 3.10b). Well-developed chloroplasts were present in the mesophyll (Pl. 3.10d) but were not seen in either epidermis. Adaxial epidermal cells bore numerous highly-cristate mitochondria, hypersecretory dictyosomes, sheets of RER, free ribosomes and plastids (Pl. 3.10c); vacuolation appeared to be limited to a few small vacuoles. Evidence of secretory activity was provided by the presence of paramural bodies in the adaxial epidermal cells (results not shown).

L. multiflorum

The papillate cells of the edges are shown in Pl. 3.11a. Calcofluor-induced fluorescence was restricted to the adaxial epidermis, apparently the cytoplasm not the walls (Pl. 3.11b). Well-developed

chloroplasts with grana and osmiophilic globules (white arrow-heads in Pl. 3.11c) were present in the mesophyll. Strands of RER, hyper-secretory dictyosomes, highly-cristate mitochondria and free ribosomes were abundant in the adaxial epidermal cells (Pl. 3.11d,e); plastids were also common and occasionally bore depressions which enclosed other organelles (pt' in Pl. 3.11e).

L. x hybridum

The tripartite nature of the ligule is shown in Pl. 3.12a; however, it was apparent that the mesophyll occurred in patches and probably gave rise to the vertical green stripes observed in fresh ligules. Calco-fluor-induced fluorescence was apparently confined to the cytoplasm of adaxial epidermal cells (Pl. 3.12c) and was best seen by examination of this surface directly. Examination of the abaxial epidermis under these conditions revealed a distinct banded pattern (Pl. 3.12b); adaxial calcofluor-induced fluorescence showed up as white bands ('A'), where this was masked by presence of mesophyll tissue, chlorophyll autofluor-escence was recorded as dark bands ('B'). The adaxial epidermis contained numerous plastids, highly-cristate mitochondria, hypersecretory dictyosomes, strands of RER and free ribosomes (Pl. 3.12d); a well-defined periplasmic space was present, mainly in association with the outer tangential wall, which frequently bore vesicles (Pl. 3.12d).

F. pratensis

Both epidermes were covered with a Type 3 cuticle which was generally thicker abaxially (Pl. 3.13a). Both starch grains and bodies of unknown nature ('?') were observed within the well-developed meso-phyll chloroplasts (Pl. 3.13b). Although plastids were present in cells of both epidermes (Pl. 3.13a,c), chloroplasts appeared to be absent. The degree of vacuolation decreased markedly from large, central vacuoles in abaxial epidermal cells, to few, small vacuoles in adaxial epidermal cells. The latter were densely populated with highly-cristate mitochondria, hypersecretory dictyosomes, strands of RER and free ribosomes (Pl. 3.13c); paramural bodies associated with the outer tangential walls suggested some degree of secretory activity (Pl. 3.13c).

General

Vascular tissue was absent from all four species' ligules; sheath-applied eosin solutions were not observed to pass into the ligules. The only cell types observed were long cells and papillate cells in the epidermes, and cells of similar size and shape to the former in the mesophyll. In all respects the ligules of these four species appeared to be the same as that of *L. temulentum*.

Plate 3.1. Structure of the ligule of Lolium temulentum

a,b. FluorMgraphs of the adaxial surface near the base (a) and the apex (b); calcofluor; X135;

c. LMgraph of apex; transmitted light; X260;

d. FluorMgraph of abaxial epidermis; aniline blue; X1,100;

e. LMgraph of abaxial epidermis; transmitted light; X1,100;

f. FluorMgraph of abaxial epidermis; auramine O; X400;

g. SEMgraph of abaxial epidermis; X400;

h. EDAX of region shown in g. above. Vertical scale = 3,500 counts; cursor at 1.93 keV; K_{α} line for silicon = 1.74 keV; SI = silicon; AU = gold; counting time = 61 sec.

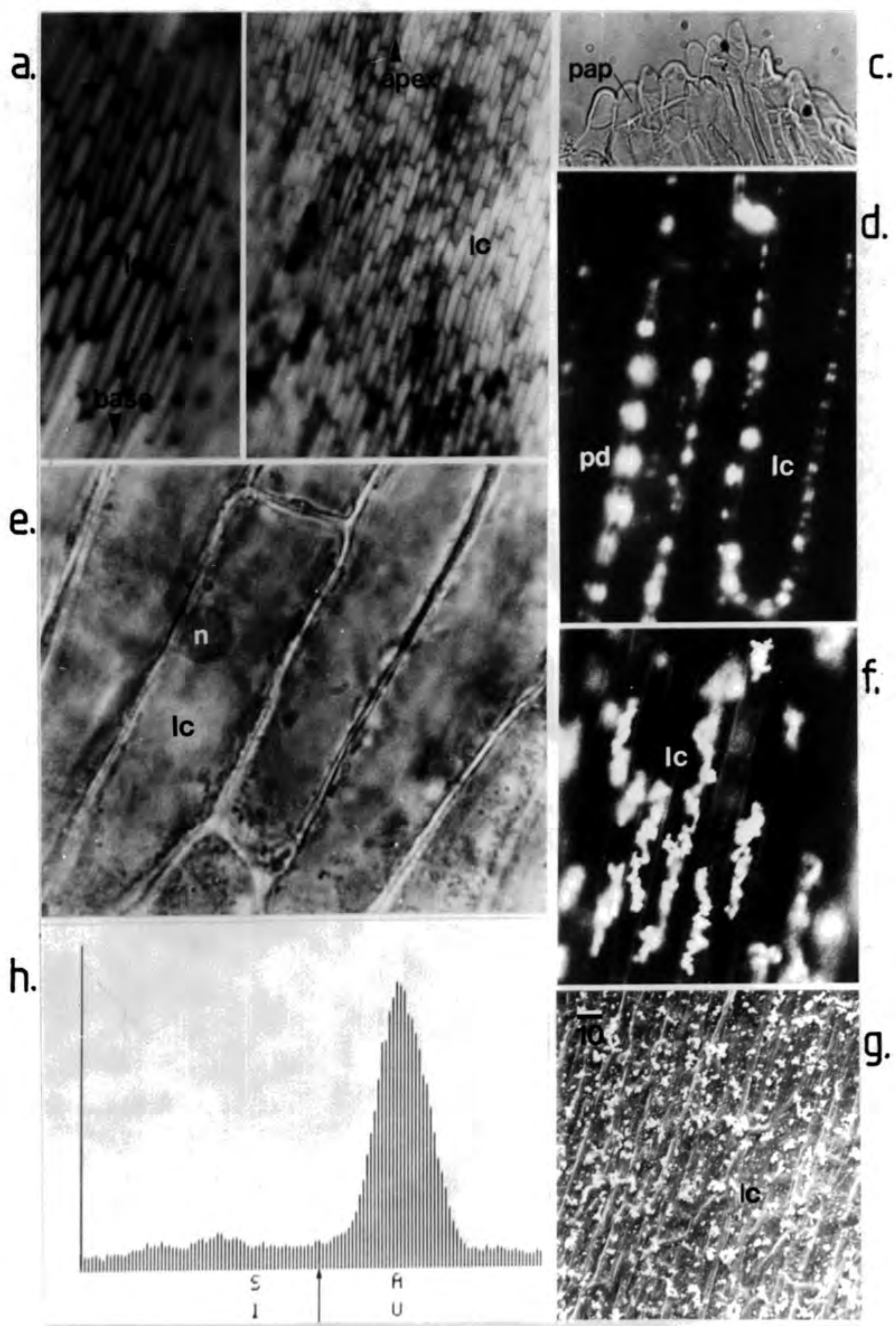


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Plate 3.2. Structure of the ligule of *Lolium temulentum*

a. LMgraph of TS near apex; X270;

b. LMgraph of TS through mid-region; Sudan black B; X1,070;

c. LMgraph of TS through mid-region; X270;

d. LMgraph of TS near the lateral edge; X270;

e. LMgraph of TS near base at insertion region; X270;

f. LMgraph of TS near base; Sudan black B; X270;

g. FluorMgraph of insertion region; calcofluor; X125;

h. LMgraph of LS through insertion region; X270.

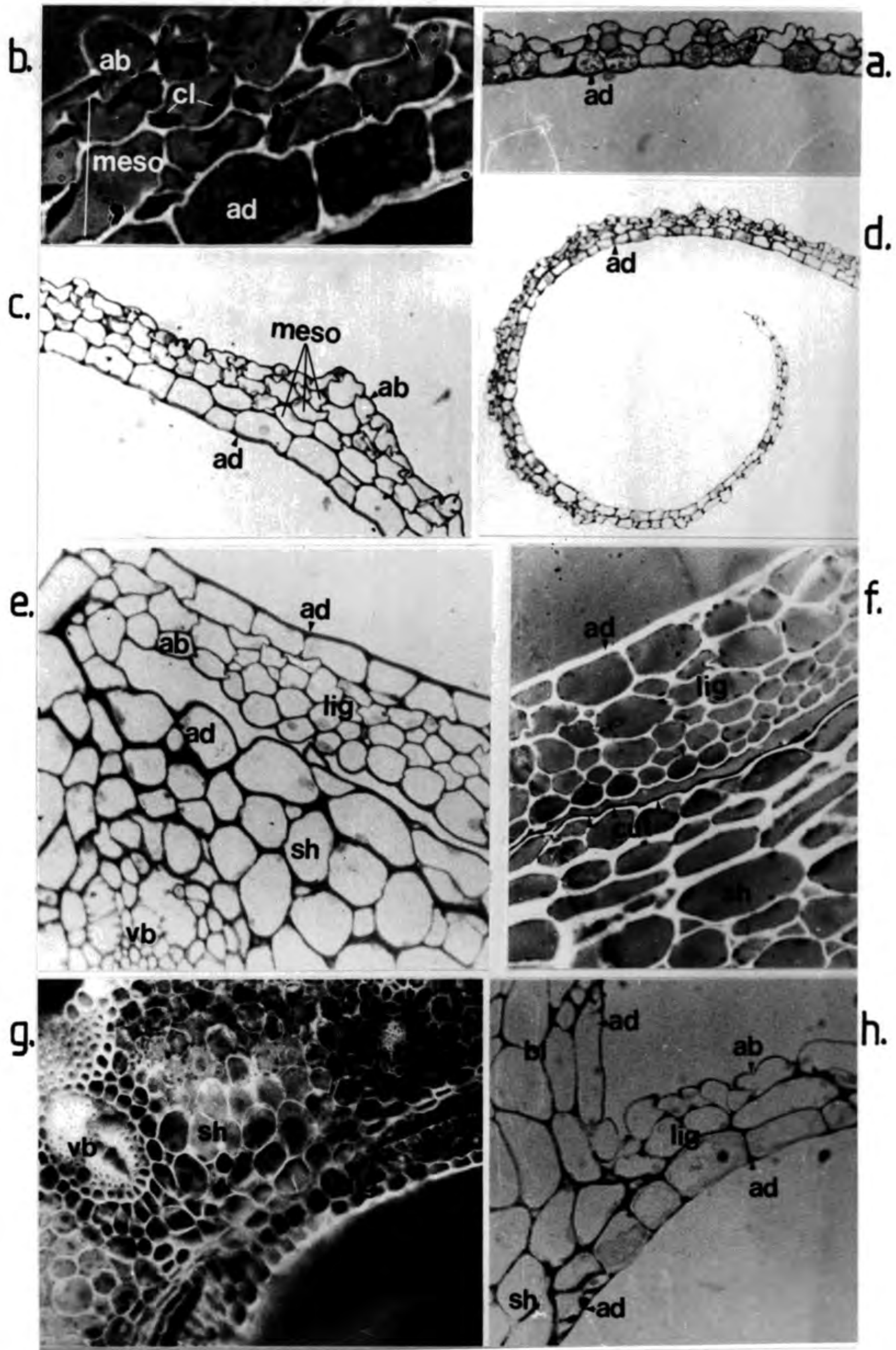
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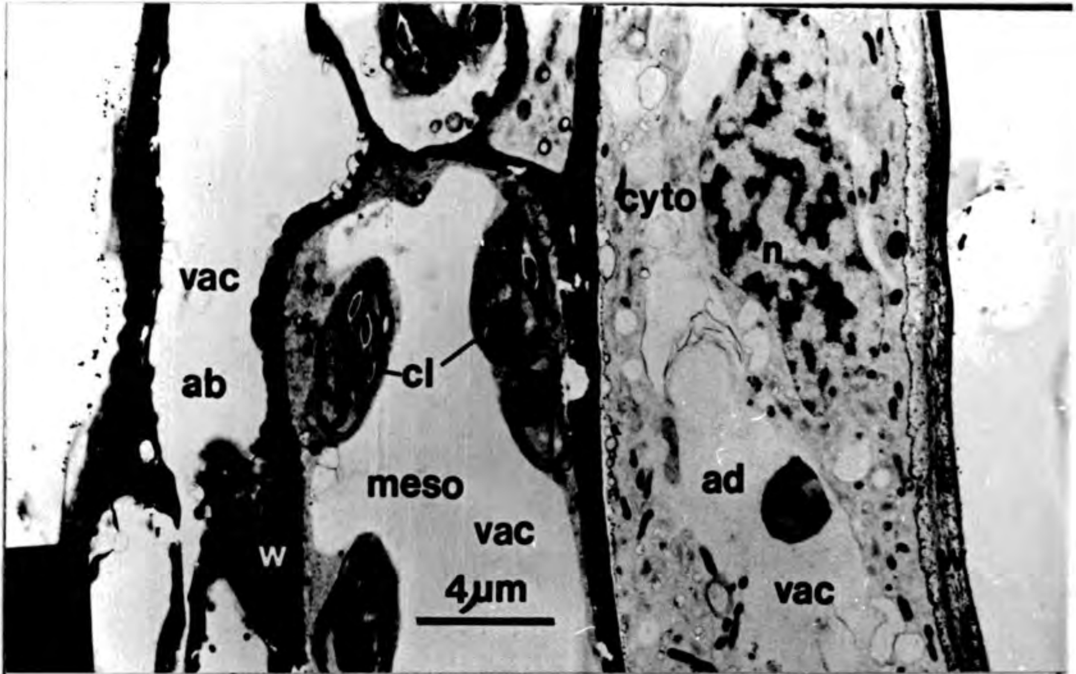
Plate 3.3. Ultrastructure of the ligule of *Lolium temulentum*

a. LS; X4,500;

b. TS; X5,000.

a.



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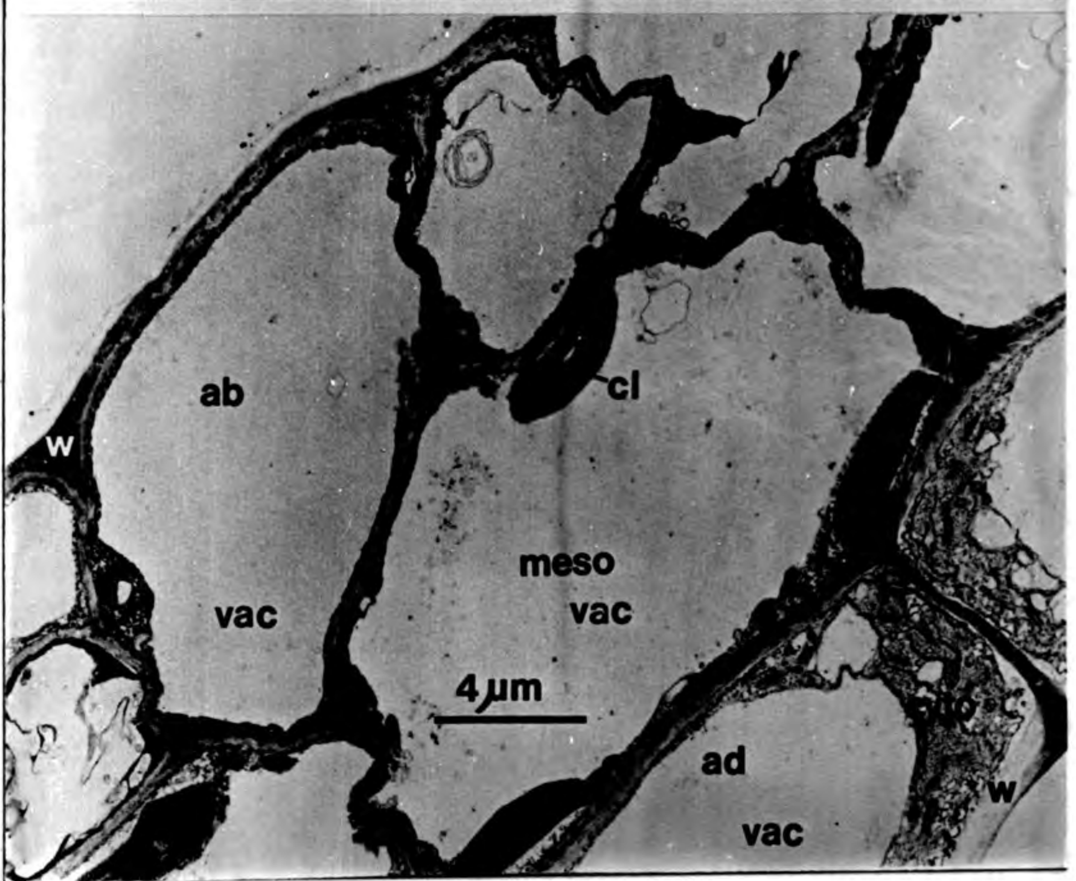

Plate 3.4. Ultrastructure of the ligule of <u>Lolium temulentum</u>

A cell of the adaxial epidermis and parts of several adjacent mesophyll cells; X4,400.

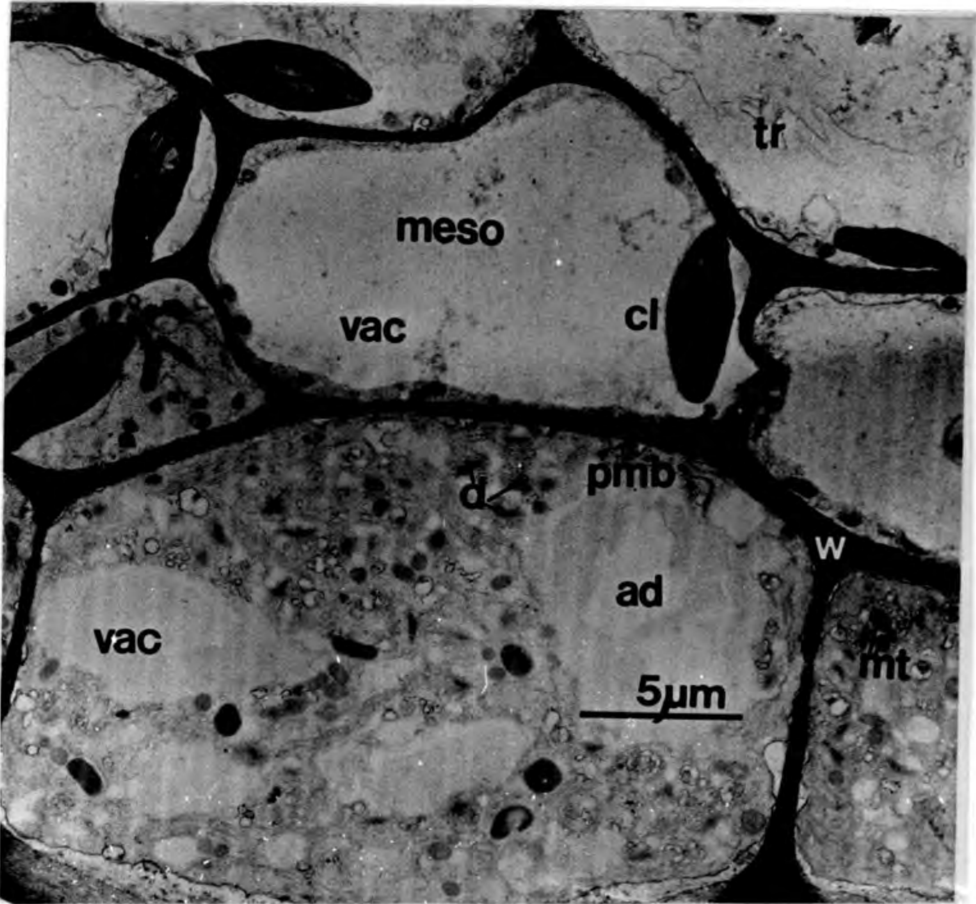

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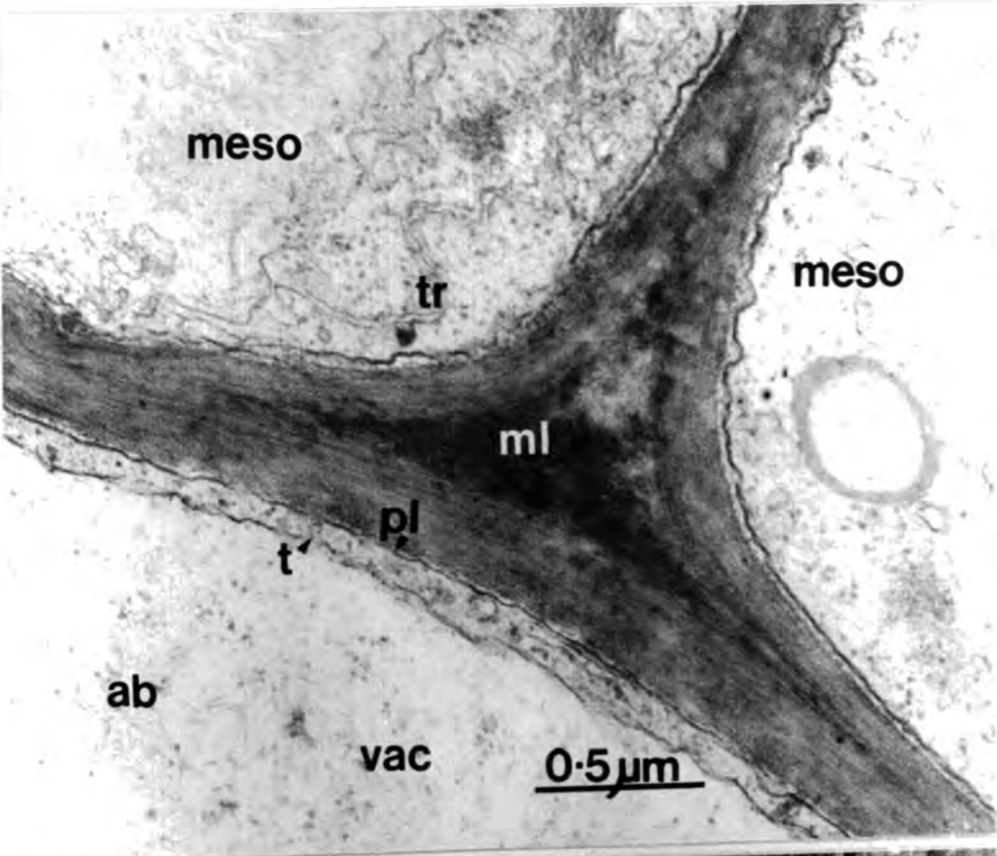
Plate 3.5. Ultrastructure of the ligule of Lolium temulentum

a. Part of an abaxial epidermal cell and parts of two adjacent mesophyll cells; X46,000;

b. Plasmodesmata in the wall between adjacent adaxial epidermal and mesophyll cells; X100,000;

c. Plasmodesmata in the wall between two adjacent cells of the adaxial epidermis; X100,000.

a.



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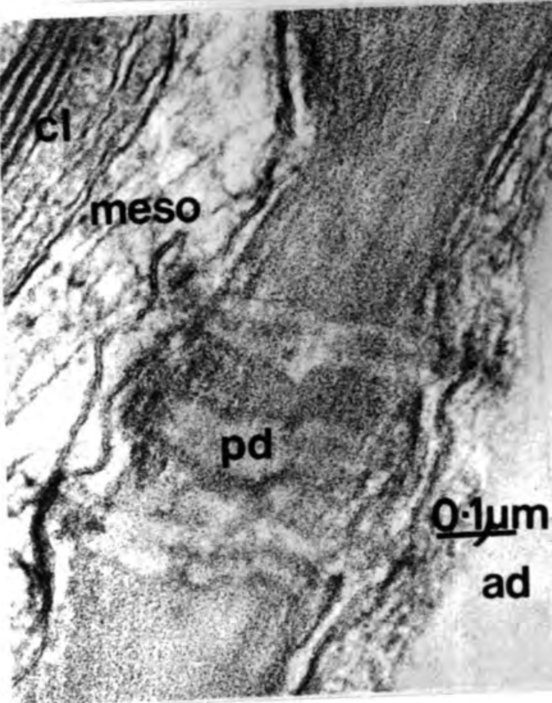

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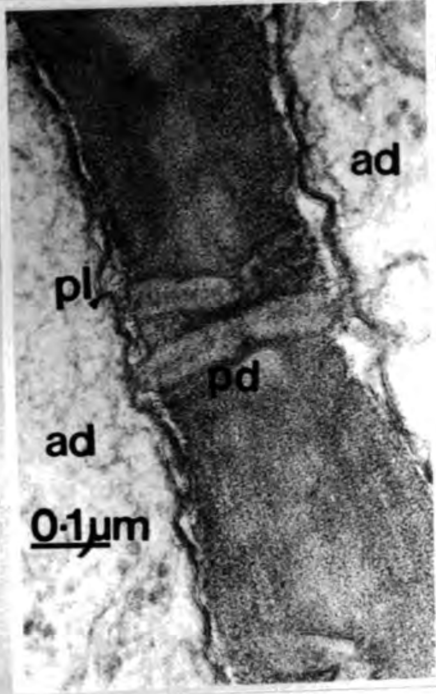


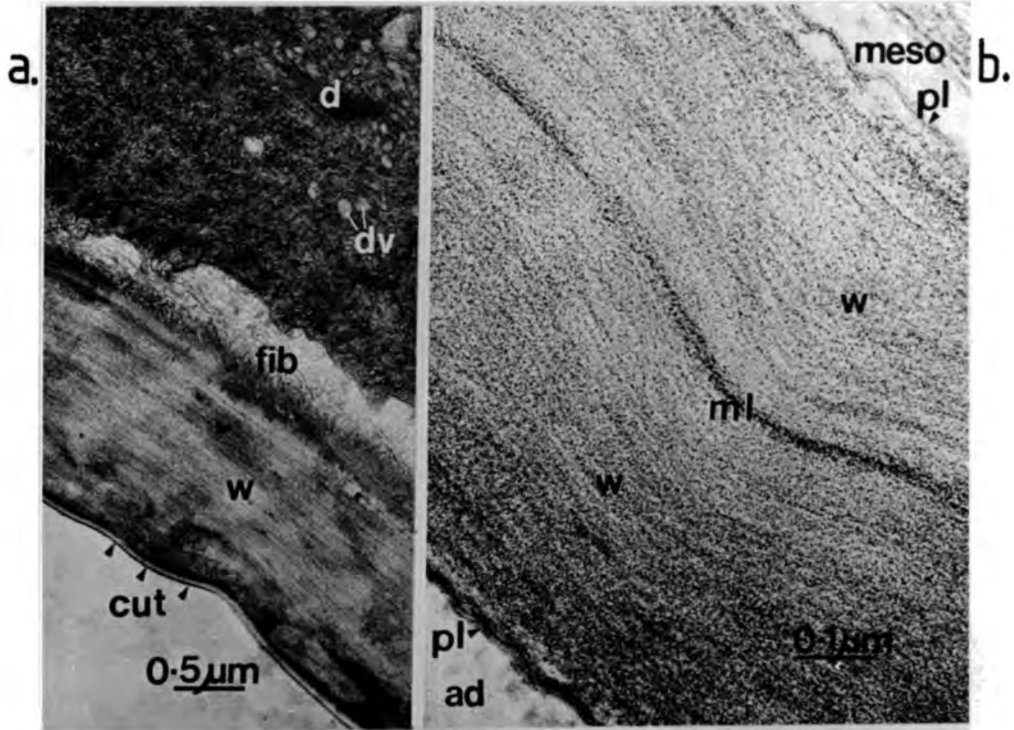
Plate 3.6. Ultrastructure and fluorescence microscopy of the ligule of Lolium temulentum

a. Outer tangential wall region of an adaxial epidermal cell; X18,000;

b. Structure of the wall between the adaxial epidermis and the mesophyll; X105,000;

c. Outer tangential wall and cuticle of two adjacent cells of the abaxial epidermis; the starred region represents the middle lamella between the two cells; X70,000;

d. FluorMgraph of adaxial epidermis; calcofluor; X150.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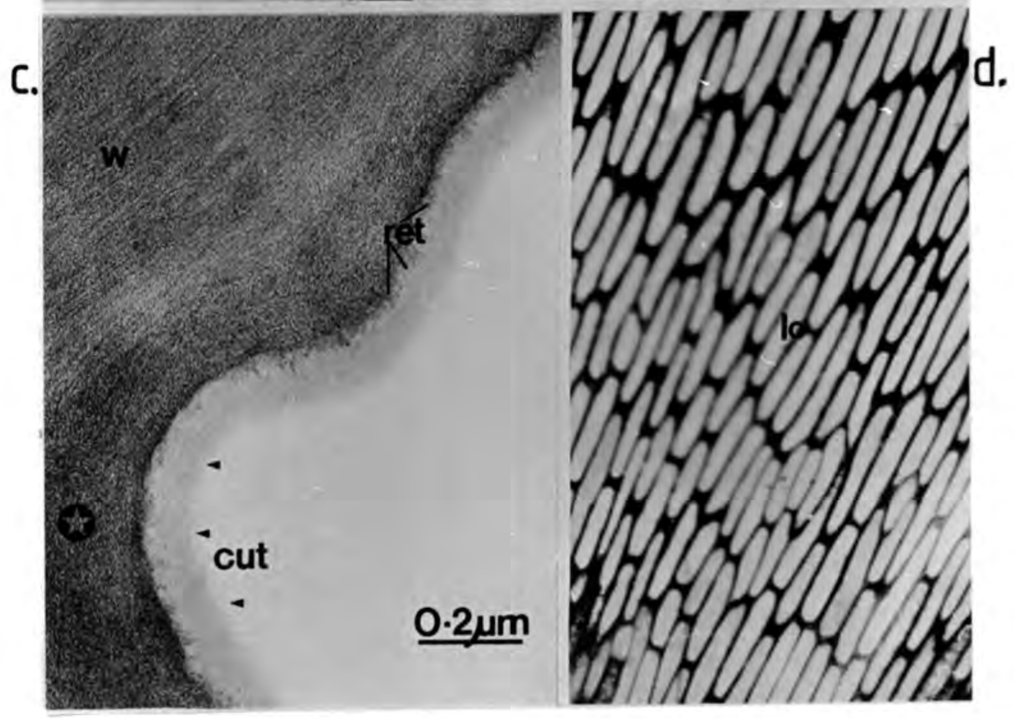
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Plate 3.7. Ultrastructure of the ligule of *Lolium temulentum*

Cytoplasm of an adaxial epidermal cell; X48,000.

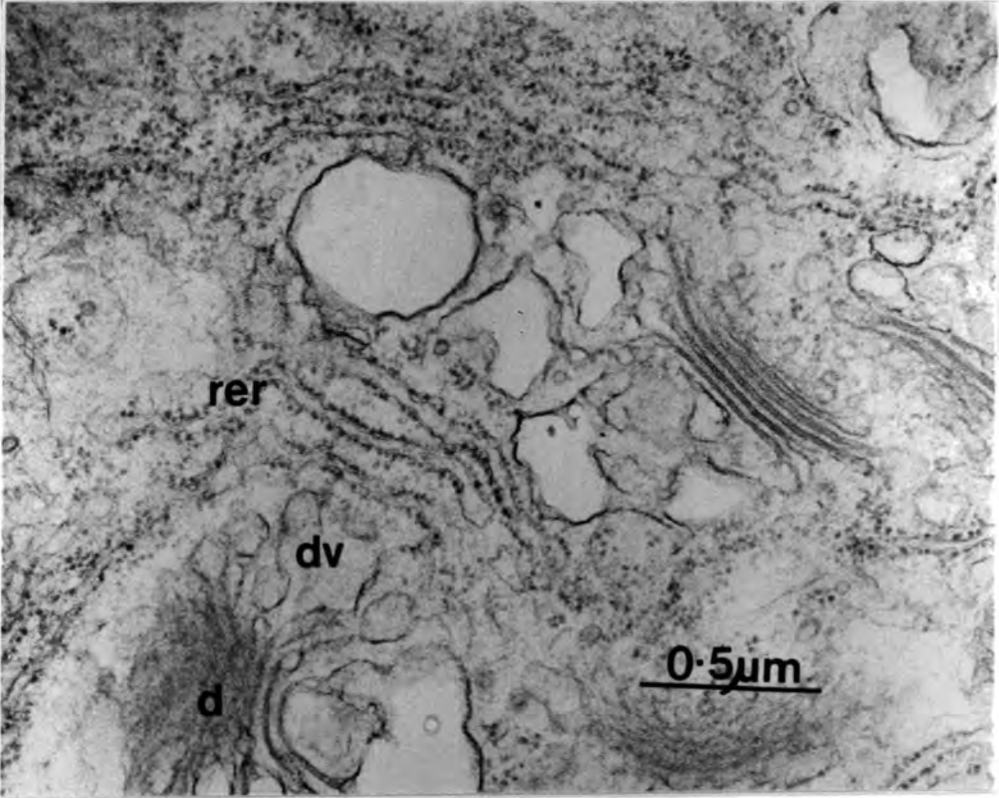
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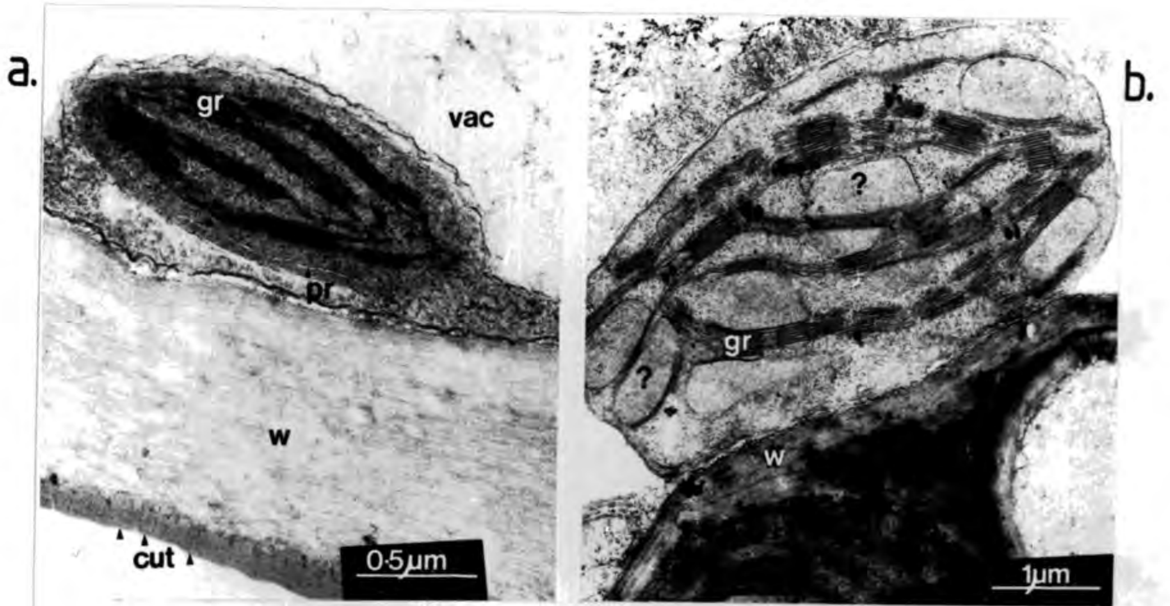
Plate 3.8. Ultrastructure of the ligule of Lolium temulentum

a. Chloroplast of an adaxial epidermal cell in basal part of ligule; X32,000;

b. Mesophyll chloroplast from mid-region of ligule; X15,000;

c. Typical mesophyll chloroplast from mid-region of ligule; X25,000;

d. Membrane systems of a typical mesophyll chloroplast; X70,000.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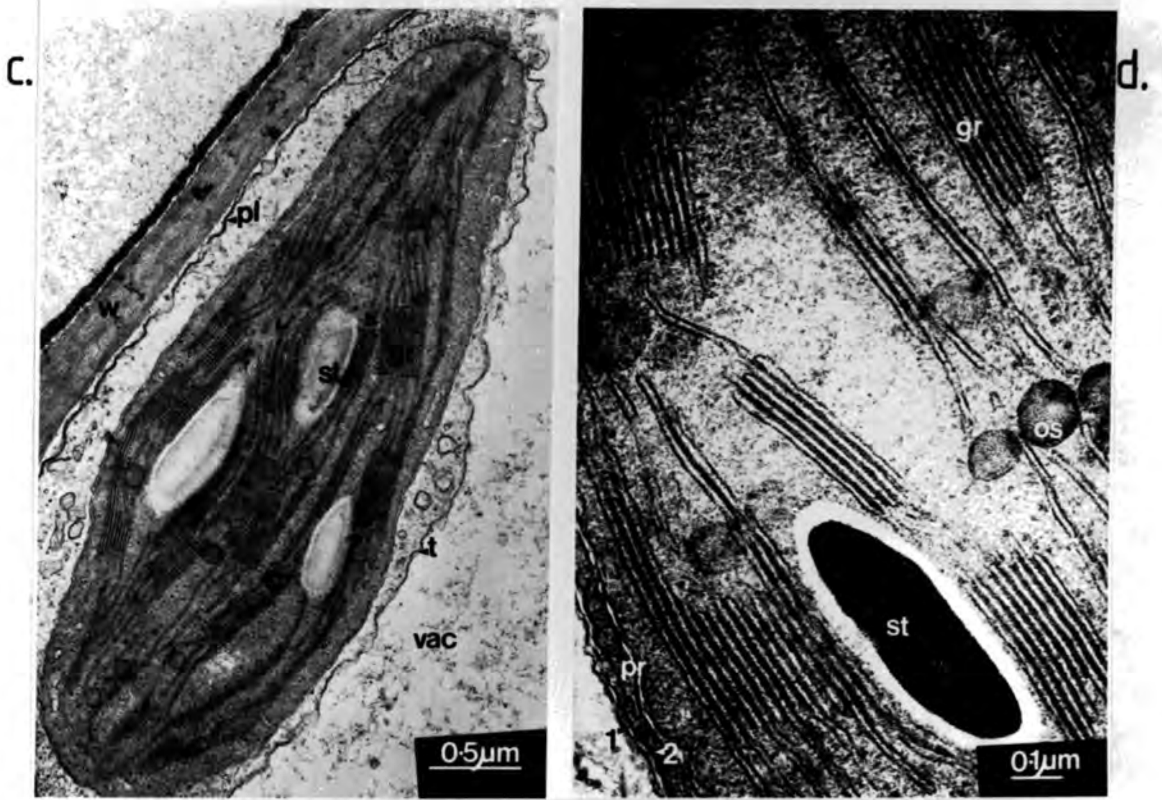
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Plate 3.9. Structure and ultrastructure of the insertion region of the ligule of *Lolium temulentum*

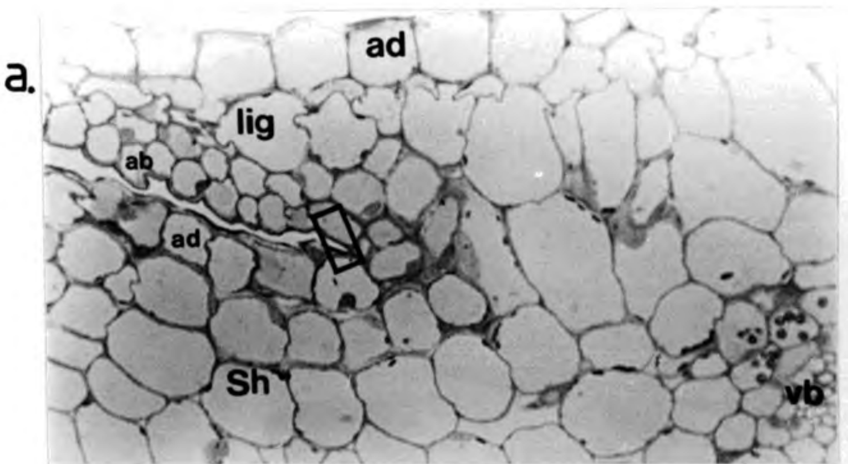
a. LMgraph of TS through insertion region; X360;

b. TEMgraph of boxed region in a. above; darts indicate invaginations of the plasmalemma; X12,500;

c. TEMgraph of boxed region in b. above; darts indicate invaginations of the plasmalemma; X46,000;

d. TEMgraph of a single coated vesicle from a ligule abaxial epidermal cell; X60,000.

Scale bars in μm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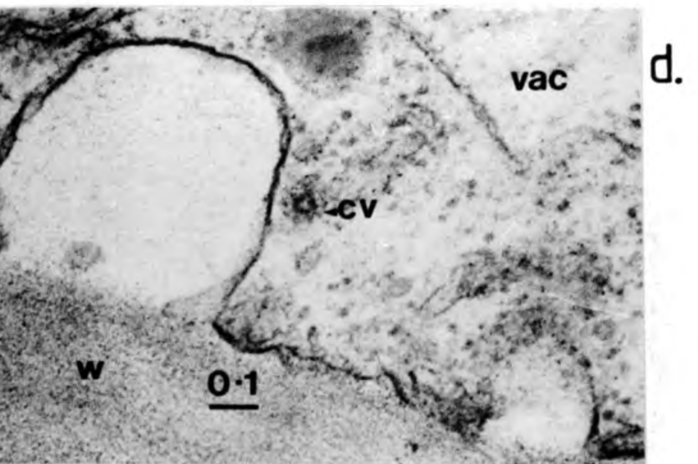
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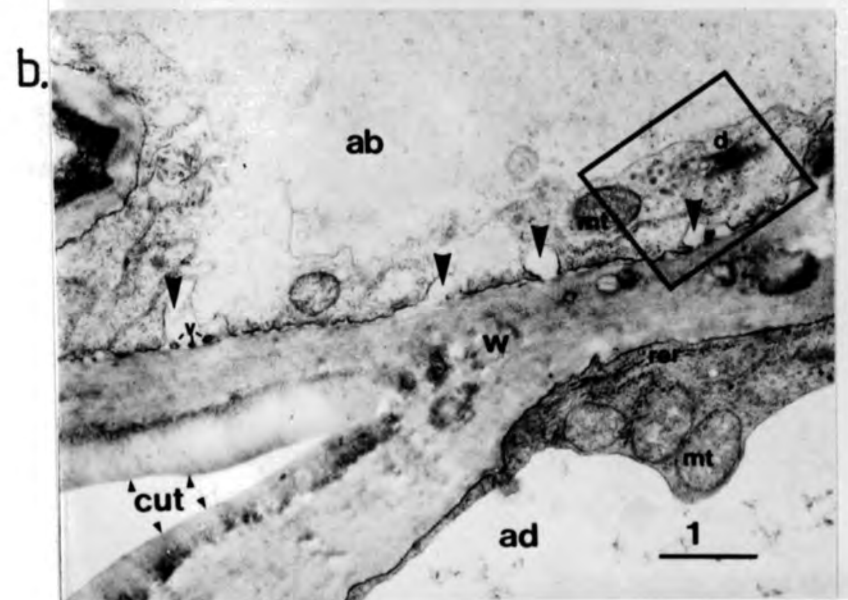
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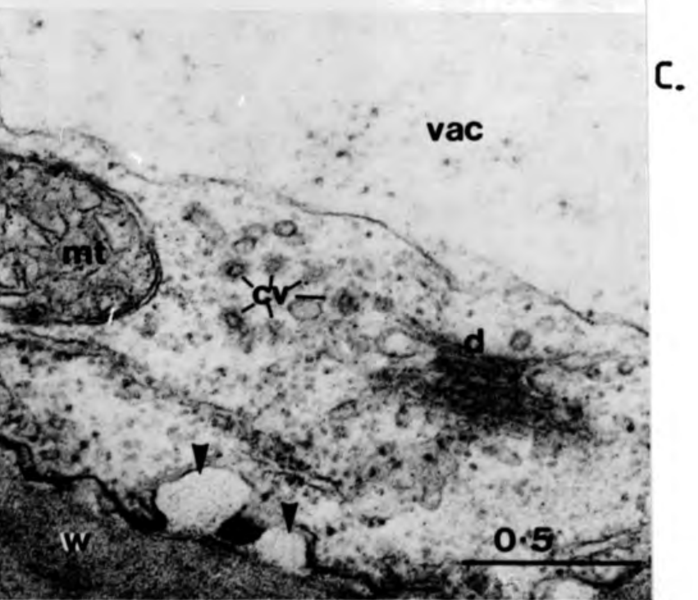
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Plate 3.10. Structure and ultrastructure of the ligule of *Lolium perenne*

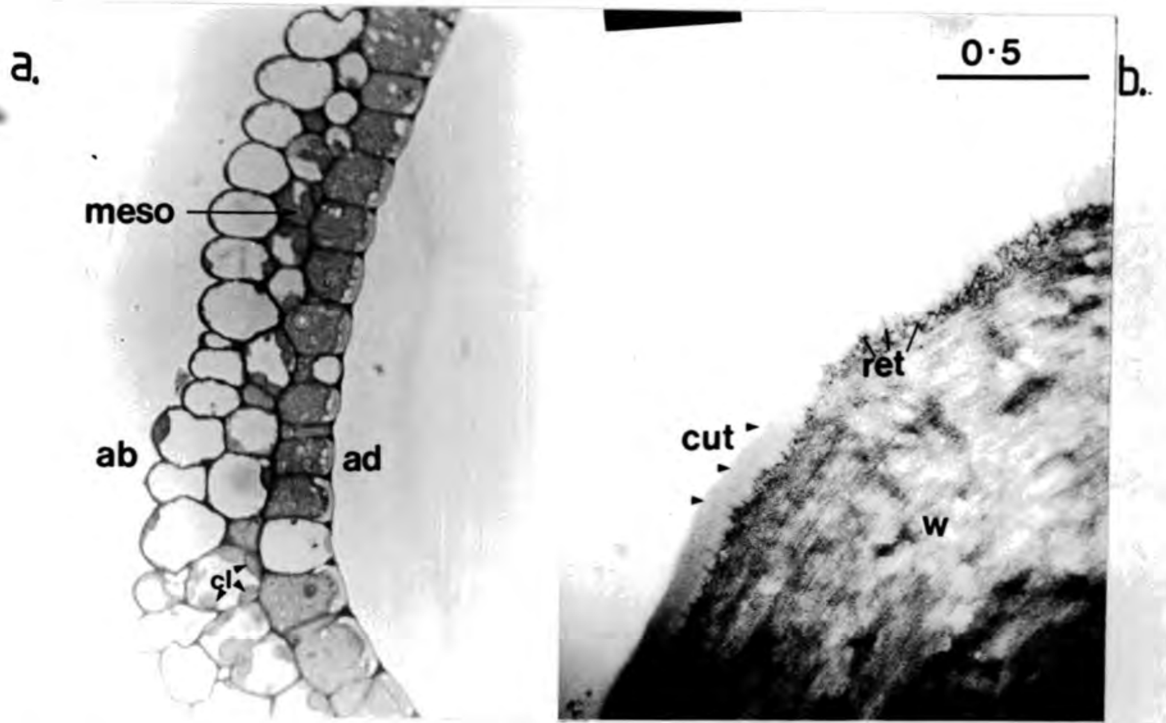
a. LMgraph of TS through mid-region of ligule; X530;

b. TEMgraph of outer tangential wall and cuticle of an abaxial epidermal cell; X42,000;

c. TEMgraph of cytoplasm of adaxial epidermal cell; X19,000;

d. TEMgraph of mesophyll chloroplast and part of adjacent adaxial epidermal cell; X18,000.

Scale bars in μm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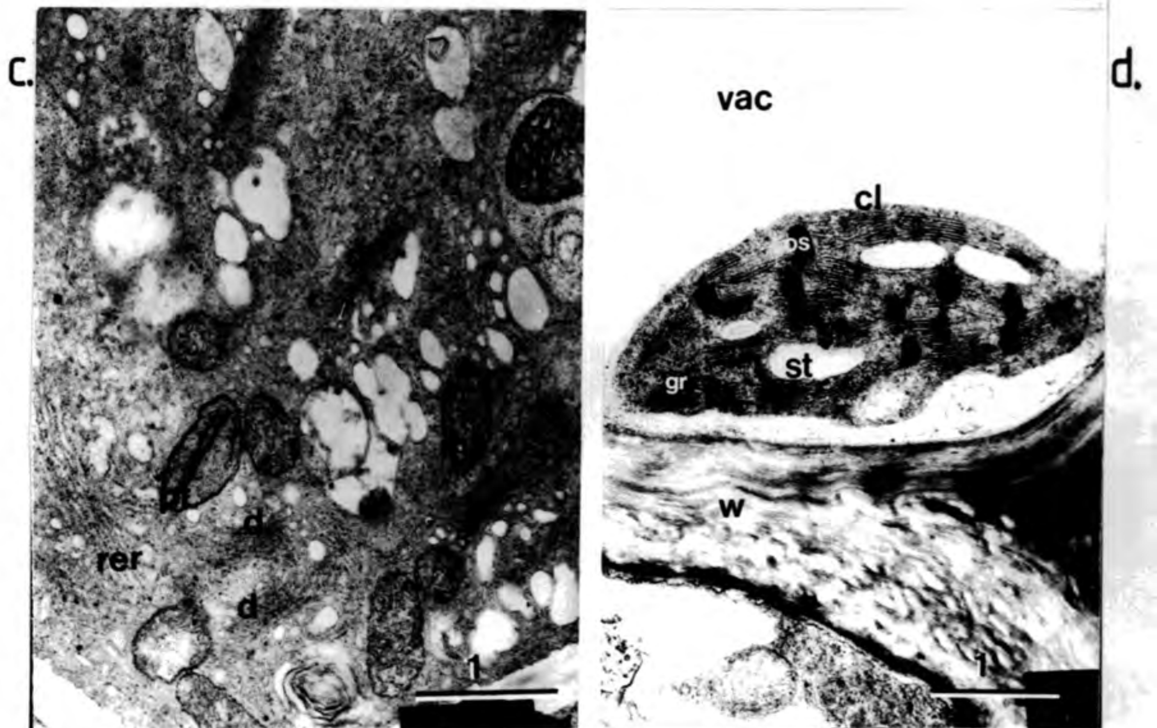
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Plate 3.11. Aspects of the biology of the ligule of Lolium multiflorum

a. LMgraph of the apex; transmitted light; X155;

b. FluorMgraph of the adaxial epidermis; calcofluor; X155;

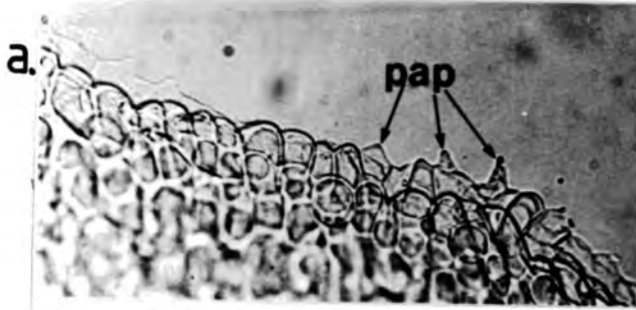
c. TEMgraph of mesophyll chloroplast; X13,000;

d. TEMgraph of cytoplasm of an adaxial epidermal cell; X21,000;

e. TEMgraph of cytoplasm of an adaxial epidermal cell; X12,500.

Scale bars in μm

a.



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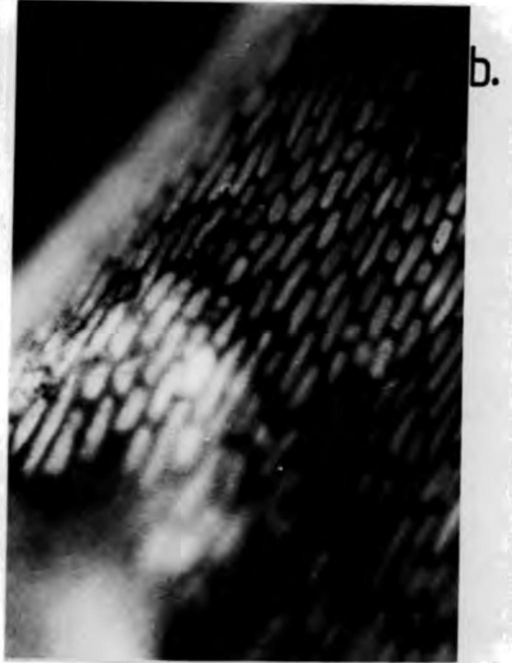

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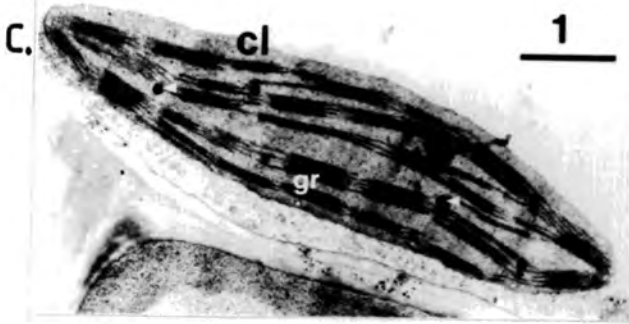

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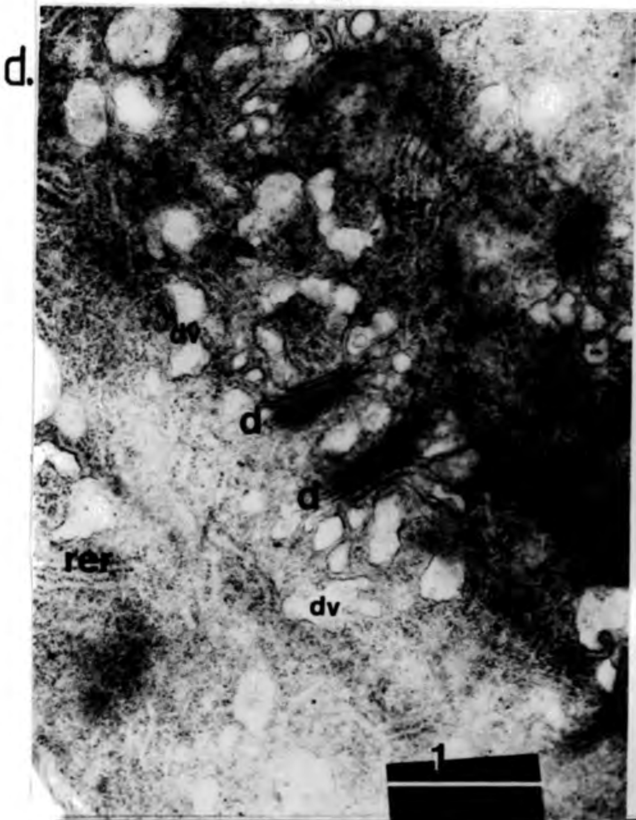


e.

Plate 3.12. Aspects of the biology of the ligule of *Lolium x hybridum*

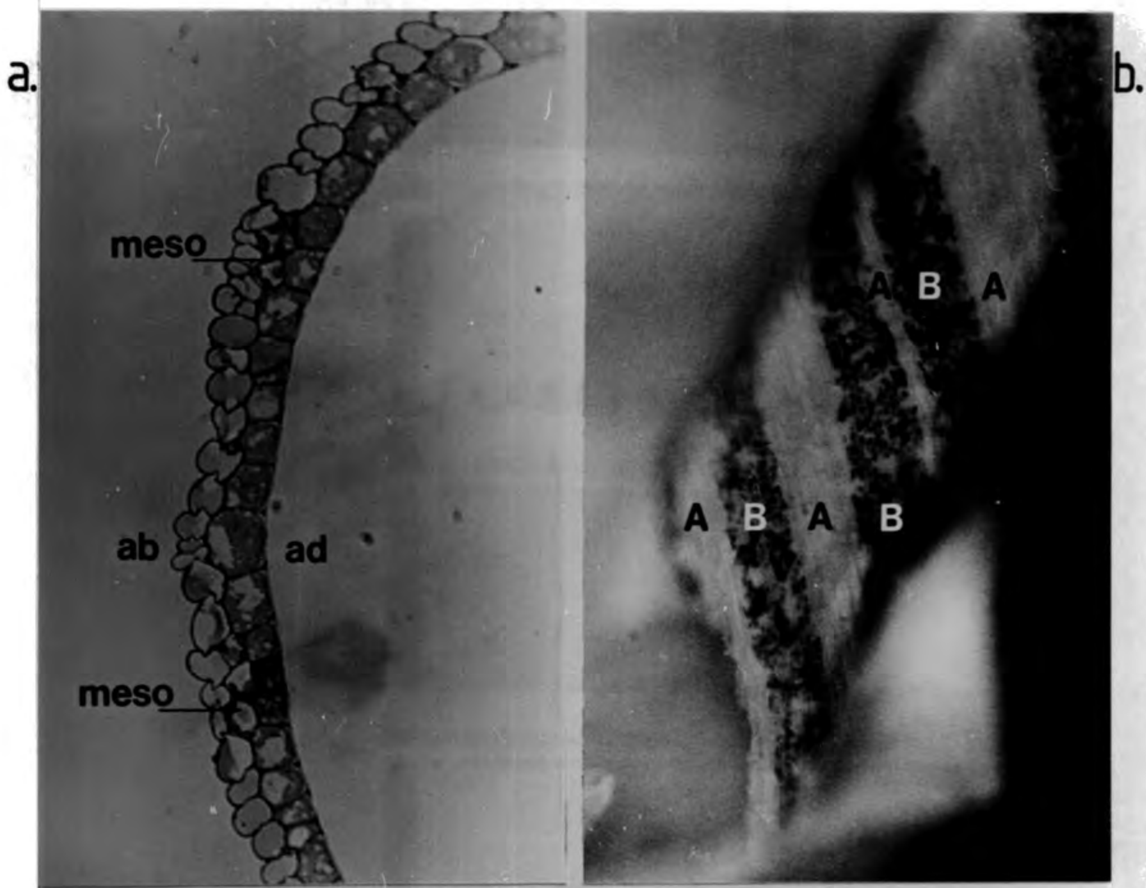
a. LMgraph of TS near mid-region of ligule; X360;

b. FluorMgraph of abaxial epidermis; calcofluor; X145;

c. FluorMgraph of adaxial epidermis; calcofluor; X145;

d. TEMgraph of parts of cytoplasm of two adjacent adaxial epidermal cells; X15,000.

Scale bars in μm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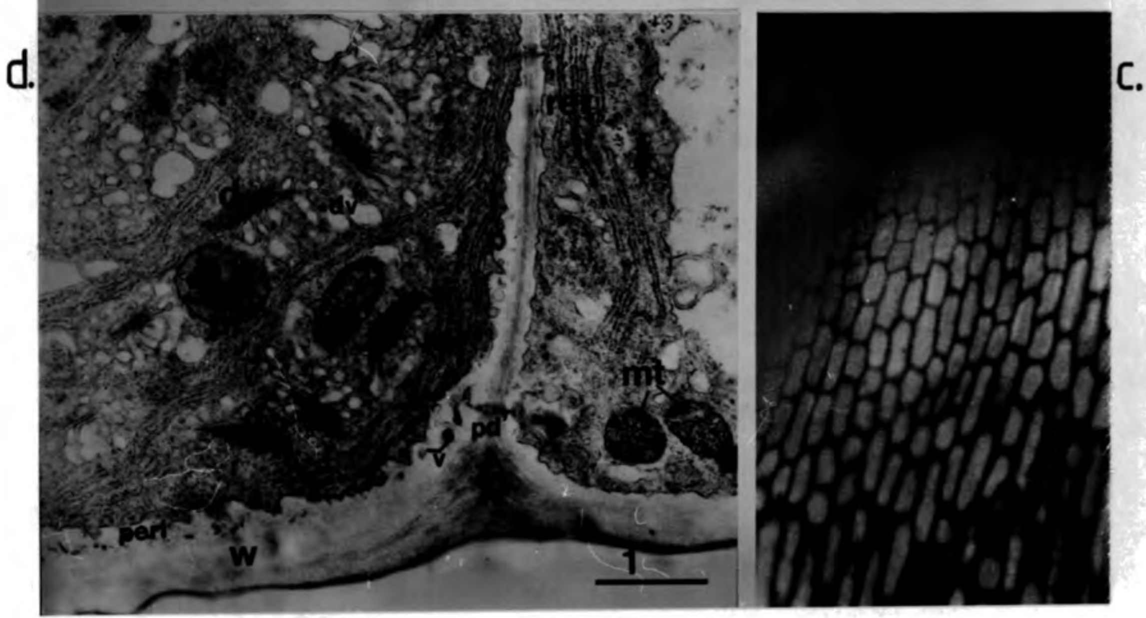
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Plate 3.13. Ultrastructure of the ligule of _Festuca pratensis_

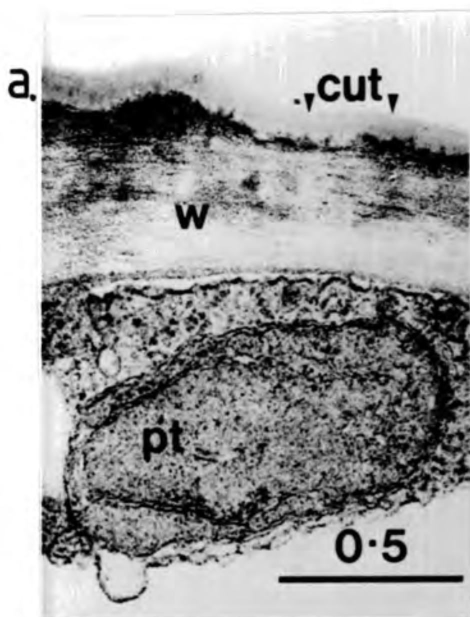
a. Outer tangential wall, cuticle and part of cytoplasm of abaxial epidermal cell; X49,000;

b. Mesophyll chloroplast and part of cytoplasm of an adjacent adaxial epidermal cell; X17,000;

c. Part of cytoplasm of two adjacent adaxial epidermal cells; X16,500.

Scale bars in μm

a.



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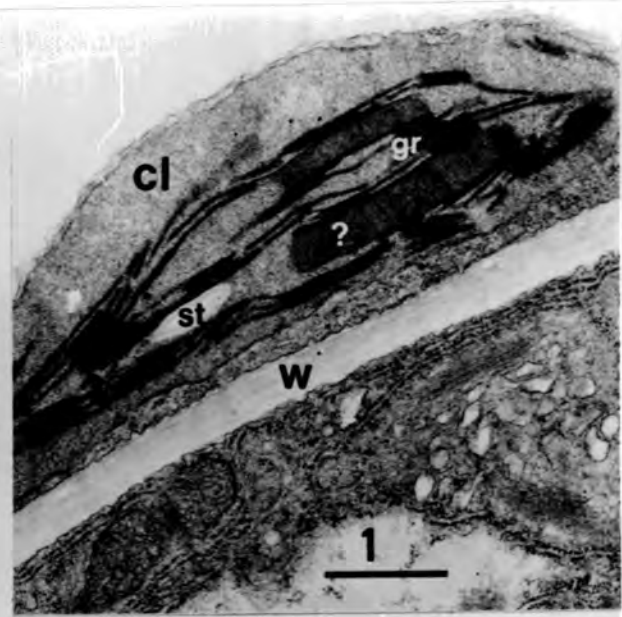

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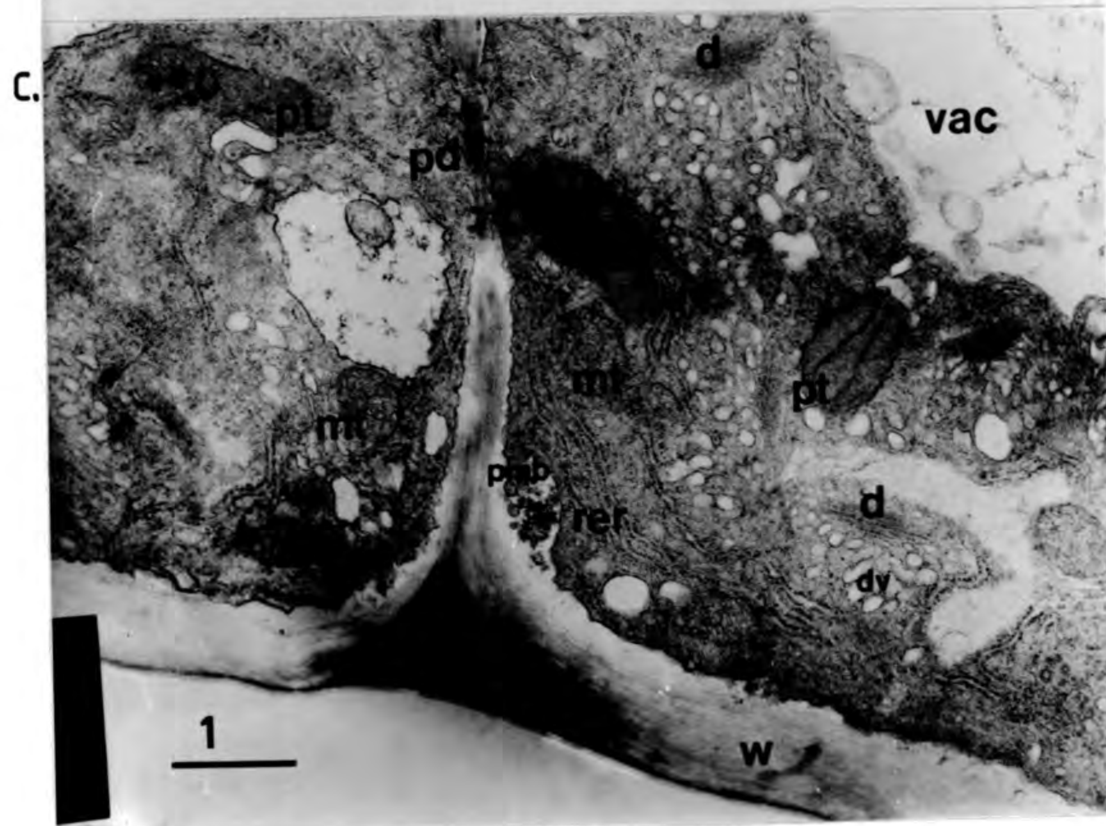


DISCUSSION

The statement by Prat (1932) that the ligule consists of two epidermes directly appressed to one another is incorrect - demonstrated in this study for membranous ligules of Lolium spp and F. pratensis. There are two epidermes in the ligules studied here but a third tissue was interposed between them. This additional tissue (designated 'mesophyll' by analogy with the tissue organization in the blade and sheath) was, however, absent from the edges where the ligule did consist of two epidermes only.

The organization of all four ligules studied here was similar in all respects. Drawings and LMgraphs of a number of other membranous ligules in the literature show structures which can be interpreted as being like those of the ligules studied here: eg Spartina townsendii H. & J. Groves (Sutherland and Eastwood, 1916), Triticum sp. (Percival, 1921), Dactylis glomerata L. (Bugnon, 1921), Saccharum officinarum L. (Artschwager, 1925), Zea mays L. (Bonnett, 1953) and Avena sativa L. (Bonnett, 1961). Although additional elements may be present in the epidermis (eg Avena fatua L. - Tran, 1963) or interior (eg Glyceria aquatica (L.) Wahlb. - Arber, 1934), it appears that the tripartite structure - uniseriate epidermes enclosing a mesophyll tissue - is the rule amongst this type of ligule. Chloroplasts have so far only been recorded for a few species, but as shown above for L. x hybridum, the mesophyll need not necessarily be chlorenchymatous throughout.

The two ligule epidermes were continuous with the leaf adaxial epidermis, which is consistent with the idea that the ligule is an outgrowth of this latter tissue (eg Sharman, 1941). The decrease in epidermal cell size from base to apex in L. temulentum ligule is presumably related to the growth of this organ; its precise explanation is not known. The absence of any trichomes in the epidermes is at present only of descriptive value; until we know what these structures 'do' in those ligules which have them (eg Agrostis gigantea Roth - Chaffey, 1982) we cannot ascribe any significance to their absence. Absence of 'stomata', however, is of interest in view of the statement that 'stomata' and veins were only found together in the ligules studied (Chaffey, 1982); absence of both in the ligules examined here is consistent with that statement.

The search for the presence of silicon in the ligule of L. temulentum using EDAX is linked with the foregoing idea, in that silicon has been found in the veined ligules of Oryza sativa L. (Chaffey, 1983) and Agrostis gigantea (results not shown) for which a transpirational pathway was assumed for its accumulation. No significant accumulation of silicon was found in the ligule of L. temulentum (nor in the membranous ligule of Avena sativa by Soni et al., 1970) and its absence is presumably related to the lack of vascular continuity between these organs and the rest of the leaf.

The papillate cells of the ligule edges studied here are of interest on two counts. Firstly it means that although essentially homogeneous the ligule does have two epidermal cell types. Secondly, they are reminiscent of similar structures in the wheat ligule (Percival, 1921; Dobrynin, 1959). They appear to be widespread amongst ligules and probably correspond to the 'hair-like structures' recorded by Neumann (1938); their significance, however, is not known.

The ultrastructure of the mesophyll chloroplasts was in agreement with that described for higher plant chloroplasts (Thomson, 1974); presence of a well-developed peripheral reticulum in those of L. temulentum was a more specialized feature. It has been recorded for chloroplasts of a number of plants with the C_4 photosynthetic pathway (eg Laetsch, 1968) and it has been considered specific to C_4 chloroplasts. However, Gracen et al. (1972) demonstrated its presence in C_3 and C_4 chloroplasts of Zea mays, and, as in the ligule chloroplasts here, it may be related to increased rate of transport between this organelle and the cytoplasm rather than the C_4 photosynthetic pathway per se. There is no unambiguous evidence in the literature that L. temulentum is a C_4 plant.

Although chloroplasts were seen in the abaxial epidermis of L. temulentum ligule, they were only present in its most basal regions and can only be considered characteristic of the mesophyll of these organs. However, their presence here does contradict the widely-held view that chloroplasts are absent from leaf epidermes.

Presence of chlorophyll, typical chloroplast ultrastructure and starch suggests that the ligule chloroplasts are photosynthetically active. One feature brought out by work on L. perenne ligule was the convex nature of the outer tangential walls of the abaxial epidermis.

It is likely that this was the case for all the ligules studied and it is possible that these cells may act as either light traps (Haberlandt, 1914; pp 120—21) or lenses (ibid.; pp 616—24). This, plus the assumed low absorbance of light by the highly vacuolate abaxial epidermal cells, would thus focus any light upon the photosynthetic chlorenchyma below (Fig. 3.3b). A similar mechanism operates in the case of window-leaved plants (Krulik, 1980).

The apparent disparity of mesophyll chloroplast density between blade and ligule in L. temulentum (Table 3.1) and the amount of mesophyll in each organ might imply that any photosynthetic capacity of the latter would be insignificant. This argument would apply if it was assumed that the ligule was acting like the blade - supplying photosynthates to the rest of the leaf and the plant. This is not suggested. What is suggested is that the ligule is a photosynthetically self-sufficient leaf organ which might undergo exchange of metabolites with the rest of the leaf.

Presence of large amounts of RER in adaxial epidermal cells suggests this layer is engaged in synthesis of protein destined for export (eg Morré and Mollenhauer, 1974; Chrispeels, 1980). The numerous hypersecretory dictyosomes further imply synthesis of large amounts of polysaccharide material, also possibly for export. The paramural bodies frequently observed are consistent with there being some degree of secretory activity associated with this tissue. Large populations of mitochondria in these cells are suggestive of a high capacity to make energy, which would be required in the proposed synthesis of protein and polysaccharide. So far no evidence for accumulation of protein within any ligule cells has been seen; evidence for polysaccharide production, however, is less circumstantial.

The fluorescence images of the ligules after incubation in calcofluor (and uvitex BOPT in L. temulentum) suggested that some part of the adaxial epidermal cells was preferentially staining. In the TEM, the only material observed which was specific to this tissue, and whose cellular distribution was consistent with the observed fluorescence image, was the material in the periplasmic space next to the outer tangential walls.

Both the fluorochromes used are diamino stilbene derivatives and are referred to as 'brighteners'. Calcofluor has been much used in studies of protoplast cell wall regeneration because of its intense fluorescence-staining of cellulose (eg Galbraith, 1981) and its non-toxic nature (eg Maeda and Ishida, 1967; Hughes and McCully, 1975). Calcofluor, however, is not specific for cellulose, but for hexa-pyranose polymers with β-linked configurations (Maeda and Ishida, 1967).

When considering the distinctive staining patterns of the ligules with these brighteners, it is important to determine to what extent differential penetration of the staining solution may have resulted in a spurious staining pattern. In this connection the observations of Harrington and Raper (1968) and Galbraith (1981) are important. The former workers stated that calcofluor is able to penetrate intact cell masses and individual cells very quickly; the latter worker concluded that the large molecule of calcofluor does not cross the plasmalemma easily. Taken together they suggest that when an excised ligule is incubated in calcofluor solution, all cell regions should be equally accessible to the fluorochrome, and that when in the cells it should be able to cross the plasmalemma, given time.

Thus there seems to be no reason why the observed fluorescence image should be considered unrepresentative of the real situation, and the distribution of fluorescing material not the same as that of the material observed in the TEM study. It is thus deduced that the material in the periplasmic space of adaxial epidermal cells was that which stained with the brighteners.

Although callose is a β-linked glucan and might be expected to stain with the brighteners used, the only report to this effect appears to be that of Hughes and McCully (1975) who suggested that calcofluor might have some affinity for callose. A number of lines of evidence suggest that this periplasmic material is not callose.

The fluorescence images with the brighteners were completely different from that seen with aniline blue in the same incubation periods. The ultrastructure of the periplasmic material was different from that described for callose by Heslop-Harrison (1979) and Noher de Halac (1980), who distinguished it by its homogeneity and non-fibrillar nature in the TEM.

The periplasmic material might be cellulose; in TS's of ligules

the walls certainly stain with calcofluor (eg Pl. 3.2g). In whole tissues it might be suggested that only the periplasmic material fluoresces because its amorphous content is higher than that of cell wall cellulose and preferentially binds it (eg Maeda and Ishida, 1967). It is interesting to note that Herth and Schnepf (1980) have shown that calcofluor stains not only cellulose microfibrils but also polysaccharides in early stages of microfibril formation - perhaps this is what was observed in the ligule. In which case the vesicles observed to have fibrillar contents similar to that of the periplasmic material (Pl. 3.6a) not only suggest dictyosome involvement in formation of the cell wall, but also that microfibril assembly begins before the polysaccharide material reaches the cell wall (cf also Wright and Northcote, 1976).

In view of the similarity of the ultrastructure of the adaxial epidermal cells to that of typical secretory tissues (eg Schnepf, 1969; Fahn, 1979), it is also possible that the periplasmic material may be a polysaccharide-containing secretory product which accumulates in this region prior to 'excretion' across the cell wall and cuticle. The work of Wright and Northcote (1976) which showed that the root cap 'slime' of maize has β -1,4-glucan linkages and stains very strongly with calcofluor is suggestive of the situation here.

From a consideration of their structure, ultrastructure and staining with calcofluor it appears that the 5 membranous ligules studied were highly active, photosynthetic leaf organs synthesizing a polysaccharide material in their adaxial epidermes.

SECTION 6. CYTOCHEMISTRY

INTRODUCTION

The previous Section showed that the ligule adaxial epidermis had the ultrastructural characteristics of a secretory tissue. FluorM identified the presence of a material having in part a β-linked hexapyranose polysaccharide content, possibly within the periplasmic space. The presence of fibrils of similar structure in vesicles and the periplasmic space provided additional evidence for secretion to the outside of these cells. However, as O'Brien (1972) has pointed out, identical electron contrast is not evidence of chemical similarity between cellular constituents unless it is backed up with good histochemical evidence. In an attempt to obtain this histochemical evidence a number of polysaccharide cytochemical procedures were used at the ultrastructural level. In order to assess how metabolically active this organ is, in respect of the postulated energy demand for polysaccharide synthesis, a number of enzyme cytochemical procedures were also used. Conventional TEM was used to correlate the cytochemical results with structure in the ligule; particular attention was paid to mitochondria, microbodies and plastids.

MATERIALS AND METHODS

Enzyme Cytochemistry

1. Catalase (EC 1.11.1.6)

After aldehyde-fixation for c. 2 h and a cacodylate buffer wash (as for conventional TEM), the tissue was incubated in the following medium (based on that of Frederick and Newcomb, 1969):
10 mg DAB,
0.1 ml freshly-made 3% (v/v) aq. H_2O_2,
4.9 ml 0.05M propanediol, pH 9.0 (propanediol buffer).
(incubation: $+H_2O_2$).

Incubation was performed in stoppered glass vials (filled with medium) maintained at 37°C in a water bath, away from direct sunlight for 1 h. After a cacodylate buffer wash for c. 1 h, the tissue was post-fixed in 1% aq. OsO_4 for c. 1 h, dehydrated in a graded acetone series (25-100%) and embedded in Spurr resin.

Three controls were used:

a. incubation in the above (complete) medium with water replacing the H_2O_2 (incubation: $-H_2O_2$);
b. incubation in the complete medium without DAB (incubation: -DAB);
c. incubation in the presence of triazole, a specific inhibitor of catalase (Margoliash and Novogrodsky, 1958). Here tissue was aldehyde-fixed and cacodylate buffer-washed before pre-incubation in 0.05M triazole in propanediol buffer for c. 20 min, followed by incubation in the complete medium with addition of 0.05M triazole (incubation: +triazole).

For all controls, post-incubation treatment was as for tissue incubated in the complete medium above.

2. Cytochrome c Oxidase (EC 1.9.1.3)

Tissue was aldehyde-fixed, cacodylate buffer-washed and incubated as for catalase above except that here the complete medium lacked H_2O_2 - water replaced it. (incubation: $-H_2O_2$)

A number of controls were used:

a. pre-incubation in propanediol buffer with addition of either 0.01M KCN (incubation: +KCN) or 0.01M NaN_3 (incubation: +NaN_3) for c. 20 min, followed by incubation in the complete medium in the presence of the respective inhibitor at 0.01M;

b. pre-incubation in 0.05M triazole in propanediol buffer for c. 20 min, followed by incubation in the complete medium with addition of 0.05M triazole (incubation: -H_2O_2 +triazole);

c. incubation in the complete medium without DAB (incubation: -H_2O_2 -DAB);

d. incubation in the complete medium in the dark (incubation: -H_2O_2 +dark).

For all incubations, post-incubation treatment was as for catalase above.

3. Succinate Dehydrogenase (EC 1.3.99.1)

Initial attempts using the method and incubation medium of Ekés (1970) proved to be unsatisfactory in view of the amount of ultrastructural damage caused and the great deal of non-specific deposition of reaction product along the cell walls. Neither symptom was alleviated by a brief formaldehyde-fixation step prior to incubation in the reaction medium. The incubation medium subsequently used was the Ekés medium altered along the lines suggested by Lewis and Knight (1977). This gave much better preservation of ultrastructure and reduced amount of non-specific staining. The improvement in organelle preservation probably resulted from the markedly lower osmolarity of the improved medium. Details of the 'improved medium' are given below alongside that of the Ekés medium for comparison.

Constituent	Improved medium (final concn mM)	Ekés medium (final concn mM)
Sodium succinate	21	140
Phosphate buffer, pH 7.6	16	16
Potassium ferricyanide	1.5	1.5
Copper sulphate	10.5	21
Sodium, potassium tartrate	60 (aq.)	360 (in 0.1M phosphate buffer)
Incubation volume	5 ml	5 ml

Tissue was fixed in 2% (w/v) formaldehyde in 0.05M phosphate buffer, pH 7.6 ('phosphate buffer') for 15 min, washed in phosphate buffer (3 x 15 min) and incubated in the reaction medium at room temperature for 30 min (incubation: +succ).

After incubation, tissue was post-fixed in 2.5% glutaraldehyde in phosphate buffer for c. 1½ h, washed in phosphate buffer (2 x 15 min) and osmicated in 1% OsO_4 in phosphate buffer for c. 1 h. Tissue was finally dehydrated in ethanol and embedded in Spurr resin.

Two controls were used:

a. incubation in the complete medium without sodium succinate - 14 mM phosphate buffer replacing it (incubation: -succ);

b. pre-incubation in 0.1M phosphate buffer, pH 7.6 with addition of 40 mM sodium malonate (Lewis and Knight, 1977) for c. 30 min, followed by incubation in the complete medium with addition of 40 mM sodium malonate (incubation: +mal).

For both controls, post-incubation treatment of tissue was as for tissue incubated in the complete medium above.

For all three enzyme cytochemical procedures, ultrathin sections were collected on uncoated copper grids and viewed in the TEM either with or without double-staining (as for conventional TEM).

Polysaccharide Cytochemistry

1. Phosphotungstic Acid (PTA) Staining

Ultrathin sections of non-osmicated tissue were collected on uncoated 200 mesh gold grids, stained for 10 min in a solution of 1% (w/v) PTA and 10% (w/v) chromic acid in distilled water, washed thoroughly in distilled water and air dried (after Syrop and Beckett, 1972). The main drawback of this technique was the low contrast of the sections - due partly to lack of osmication and partly to the low level of general tissue staining with PTA. Grids were thus viewed in the TEM at 60 or, exceptionally, 40 kV with consequent loss of resolution.

2. Thiéry Test - Periodic Acid/Thiocarbohydrazide/Silver Proteinate (PATAg) Staining

Ultrathin sections of non-osmicated tussue were collected on 200 mesh gold grids and processed sequentially by a modification of the method of Thiéry (1967):

a. Floated on 1% (w/v) aq. PA; 30 min,
b. Thorough washing in distilled water,
c. Floated on 0.2% (w/v) solution of TCH in 20% (v/v) acetic acid; 2-121 h,
d. Thorough washing in a graded acetic acid series (10-0%); c. 30 min,
e. Floated on 1% (w/v) aq. silver proteinate in the dark at 36°C; 30 min,
f. Final thorough washing in distilled water.

The dried grids were viewed in the TEM without further treatment. Steps a. - d. were performed at room temperature in the light. A control was provided by omission of the PA oxidation step a. (Courtoy and Simar, 1974); no staining was observed in control sections. All solutions were freshly prepared before use.

3. Ruthenium Red (RR) Staining

Tissue was prepared as for conventional TEM but with the addition of 0.1% (w/v) RR to both fixation stages and the intervening cacodylate buffer wash (after Dexheimer, 1976). Ultrathin sections were collected on uncoated copper grids and viewed in the TEM without further treatment. Section contrast was usually low and a certain amount of ultrastructural damage was evident.

OBSERVATIONS AND RESULTS

Enzyme Cytochemistry and Ultrastructure

Mitochondria

Deformation of cells of the outer layers of the ligule did not favour an accurate comparison of numbers of mitochondria per cell between the different tissues. It was clear, however, that they were more numerous in adaxial epidermal cells than in either of the other two tissues. Since the shape of the mitochondrion is not known, it is more correct to consider numbers of mitochondrial profiles rather than numbers of mitochondria.

The structure of the mitochondrion appeared to be the same in all tissues and consisted of a double membrane enclosing a matrix in which numerous cristae were present (Pl. 3.14a,b). Circular profiles (Pl. 3.14b) were more numerous than long profiles (Pl. 3.14a) - at least in TS's and might thus be an indication of the 'preferred' orientation of these organelles within the cell. The diameter of the former was the same order as the width of the latter suggesting the mitochondria were 'sausage-shaped' structures, c. 0.5 μm in diameter and c. 2-3 μm long. Insertion of the cristae upon the inner membrane of the mitochondrial envelope was rarely observed (eg Pl. 3.14c and inset). Electron-dense granules were frequently seen within the matrix (Pl. 3.15d) and are assumed to be deposits of eg 'calcium phosphate' (Chen and Lehninger, 1973). In all tissues, distribution of mitochondria appeared to be random, even where they were confined to the periphery of the cell by a central vacuole. Ligule mitochondria looked like those of the blade and sheath.

Cytochrome c Oxidase

Incubation of ligule material in the absence of added H_2O_2 led to staining of mitochondria in all tissues; no staining was observed in the absence of DAB. The reaction product appeared to be present on both envelope membranes and the cristae (Pl. 3.14c; 3.15g). Complete inhibition of staining was observed in the presence of KCN or NaN_3 (Pl. 3.15d); triazole neither inhibited nor decreased staining. No mitochondrial staining was observed when the tissue was incubated in

the complete medium for catalase (Pl. 3.15c) nor when incubated in the dark.

Succinate Dehydrogenase

Incubation of ligule material in unmodified Ekés medium resulted in marked ultrastructural distortion, vesiculation of the cytoplasm and large numbers of non-specific deposits associated with the cell walls (Pl. 3.16a). In the 'improved' medium, tissue preservation was best for the adaxial epidermis and results are presented largely for this tissue. Sections were routinely examined without double-staining.

Incubation in the presence of added sodium succinate resulted in the appearance of dense deposits - assumed to be reaction product - in the cytoplasm (Pl. 3.16b). The two lines bounding the sites of these dense deposits (Pl. 3.16c) were interpreted as being the membranes of the mitochondrial envelope. Reaction product thus appeared confined to the matrix of the mitochondria. An attempt to localize the reaction product further by double-staining gave the image seen in Pl. 3.16d; no reaction product was seen within the mitochondria.

Incubation in the absence of added sodium succinate or presence of sodium malonate resulted in almost total absence of reaction product within the mitochondria (Pl. 3.17b). No staining of mesophyll chloroplasts was observed in the presence of added sodium succinate (Pl. 3.17a) but was seen in certain blade mesophyll chloroplasts under similar conditions (Pl. 3.16f). In the blade, the reaction did not appear to be consistent between blocks of tissue - both Pl. 3.16e and f are of blade tissue incubated in the presence of added socium succinate - and the results of ligule and blade were not compared.

Microbodies

Using conventional TEM these organelles were only seen in mesophyll cells and abaxial epidermal cells near the ligule insertion region. They appeared as roughly circular profiles 0.5 - 1 μm in diameter, with a single membrane enclosing a granular matrix (Pl. 3.14e). The matrix usually contained a crystalline inclusion, assumed to be catalase, which distinguished these organelles from others of similar size and shape. Microbodies were frequently observed close to chloroplasts, and in all respects appeared similar to those of the blade and sheath. In the

ligule they were seen too infrequently to allow accurate estimations of their number per cell, but appeared to be much less numerous than in the mesophyll cells of the blade.

Catalase

The low numbers of ligule microbodies hampered investigations of catalase activity. However, increased microbody contrast was observed when ligule material was incubated in the complete medium (Pl. 3.15b, compare with blade mesophyll, Pl. 3.15e); no staining was seen in the absence of DAB. When incubated in the absence of added H_2O_2, microbody staining was occasionally seen, albeit less than in the complete medium (Pl. 3.15g). No staining was observed in the presence of triazole (Pl. 3.15a). The oval body in the adaxial epidermal cell ('?' in Pl. 3.15c) appears to be a microbody in respect of its structure and apparent staining in the complete medium.

General

In ligule material used for catalase and cytochrome c oxidase cytochemistry, oleosomes were observed in all tissues (Pl. 3.15f,g). Their staining image was the same in the presence or absence of DAB and appeared to be due to the natural osmiophilia of their contents. No photochemical oxidation of DAB by chloroplasts, as described by Nir and Seligman (1970), was seen.

Comparison of DAB-Staining in Ligule and Blade

The results for the ligule are summarized and compared with those of the blade mesophyll in Table 3.2. The main differences observed related to the degree of microbody staining under the various incubation conditions. Blade microbodies were generally more heavily stained in the complete medium and in the absence of added H_2O_2 and stained slightly in the presence of triazole. The low numbers of ligule microbodies made it difficult to assess the effects of incubation in KCN or NaN_3 on microbody staining in this organ. Mitochondria behaved similarly in both organs, apart from triazole-inhibition of blade mitochondrial staining.

Table 3.2. A comparison of the ability of mitochondria and microbodies of the ligule and blade of *Lolium temulentum* to oxidize DAB under the conditions stated.

Incubation medium	Ligule mitochondria	Ligule microbodies	Blade mitochondria	Blade microbodies
$+H_2O_2$	-	+	-	++
-DAB	-	-	-	-
$-DAB-H_2O_2$	-	-	-	-
$-H_2O_2$	++	+/-	++	+
+triazole	-	-	-	+
$-H_2O_2$ +triazole	+	-	-	-
+KCN	-	ND	-	?
$+NaN_3$	-	ND	-	+
$-H_2O_2$ +dark	-	ND	-	+
	(all tissues)	(mesophyll)	(mesophyll)	(mesophyll)

\+ = staining

++ = intense staining

\- = no staining detected

+/- = not all stained

ND = not determined

? = not clear

Plastids

All tissues bore plastids; the mesophyll and abaxial epidermal chloroplasts were described in the previous Section. Plastids of abaxial epidermal cells were less numerous than those of adaxial epidermal cells; this may largely be due to the disparity of amount of cytoplasm per cell between the two tissues.

Plastids of adaxial epidermal cells were chiefly (solely?) of the amoeboid type (Whatley, 1977) and exhibited a variety of shapes (Pl. 3.14d). Commonly they bore depressions at one end which, when sectioned, appeared as islands of cytoplasm surrounded by the plastid (Pl. 3.14d); these 'islands' often contained other organelles (eg Pl. 3.22a). Starch grains were occasionally seen (Pl. 3.14f) and osmiophilic globules were common (Pl. 3.14d,f). An internal membrane system could usually be seen, consisting of short-tubular elements (Pl. 3.14d).

Plastids of the abaxial epidermal cells usually appeared as oval profiles c. 0.5 μm wide and 1-2 μm long with a double membrane enclosing a matrix of greater electron density than the surrounding cytoplasm (Pl. 3.15f). They rarely appeared to contain starch grains but an internal membrane system was often present as either short-tubular or long, ribbon-like (incipient grana formation?) structures (pt and pt' respectively in Pl. 3.15f). Plastids with the former membrane system could be distinguished from mitochondria only by their lack of reaction with DAB (Pl. 3.15f). Structurally they appeared to be more highly organized than the amoeboid plastids of the adaxial epidermal cells.

Polysaccharide Cytochemistry

The results of the various staining procedures used are summarized in Table 3.3: certain aspects are illustrated in Pl. 3.18, 3.19 and 3.20 and amplified below.

Adaxial Epidermis

As expected from their polysaccharide nature, the cell walls of this, and all other, layers were stained with all three procedures (Pl. 3.18a,b,f; 3.19a,b,e,f). Fibrillar and amorphous material in the periplasmic space stained with both PATAg (Pl. 3.18a,b) and PTA (Pl. 3.19a,b). The delimiting membranes of paramural bodies and

Table 3.3. Comparison of polysaccharide staining reactions in the ligule of Lolium temulentum

Structure	PATAg-h, TCH (2	24	121)	PTA	RR
rer (all cells)	-	-	-	?	-
w (all cells)	++	++	++	++	+(ml especially)
pl (all cells)	+	+	+	+	+/?
pmb (adax)	++	++	++	++	+/?
d, dv (adax)	+	++	++	+	+/?
peri (adax)	++	++	++	++	+/?
cut (adax)	+	+	+	+	-
exp (adax)	+	++	++	++	+/?
cl (meso)	++	++	++	-	-
pmb (abax)	++	++	++	?	-
d, dv (abax)	++	++	++	?	-
cut (abax)	-	-	-	?	-
exp (abax)	-	-	-	-	+/?
pt (abax)	++	++	++	-	-

+ = staining

++ = intense staining

- = no staining detected

? = not clear

+/? = some 'staining' but specificity in doubt

their enclosed vesicles appeared to stain with all three procedures (Pl. 3.18c; 3.19c,g) as did single vesicles apparently loose in the cytoplasm or periplasmic space (Pl. 3.18a,c). The images of these structures observed after polysaccharide staining were comparable to those seen with conventional TEM (Pl. 3.18e and inset).

Dictyosomes and their associated vesicles stained with both PATAg and PTA; intensity of staining increased from the cis to the trans aspect (Pl. 3.18d; 3.19d). Intensity of PATAg-staining in most cases was increased as exposure to TCH was increased from 2 to 24 h, thereafter no further increase in intensity was observed (to 121 h). PTA-staining of the contents of vesicles - assumed to be dictyosome-derived - was occasionally seen (eg dv1 in Pl. 3.19d). The plasmalemma was clearly stained with PATAg (Pl. 3.18b,c) and PTA (Pl. 3.19a,b), as appeared to be the case in all tissues, and apparently stained with RR (Pl. 3.19g). ER did not appear to stain with PATAg (eg Pl. 3.18d) but possibly stained lightly with PTA (eg Pl. 3.19d); in the latter the results were not considered conclusive.

Staining associated with the cuticle appeared to take two forms. With PATAg and PTA a layer of staining material was observed on top of the cuticle (Pl. 3.18b; 3.19b). With all three procedures material apparently in contact with the cuticle, but extending away from it, stained; it appeared amorphous with both PATAg and PTA (Pl. 3.18a,b; 3.19b), whereas fibrillar-granular material was seen with RR (Pl. 3.19f). Breaks in the cuticle were also seen and frequently contained PATAg- and PTA-staining material (darts in Pl. 3.18b; 3.19b).

A more critical examination of conventional TEM-processed material confirmed both the presence of these 'cuticular gaps' (dart in Pl. 3.20e) and extracuticular electron-dense material (darts in Pl. 3.20d). In some cases the structure of the 'wall' beneath the cuticle appeared to be 'looser' relative to the rest of the wall (Pl. 3.18b) and may indicate accumulations of extracellular material.

Abaxial Epidermis

No cuticle staining was observed with PATAg (Pl. 3.18f), nor apparently with PTA although it did appear more electron-translucent than after conventional TEM processing. With RR-staining, fibrillar-granular material was seen extending from the cuticle, as previously

described for the adaxial epidermis (compare Pl. 3.19e with f). In some regions this material had lifted away from the cuticle but remained intact as a discrete entity, suggesting the presence of a basal layer - attached to the cuticle - from which the threads of fibrillar-granular material extended.

General staining of the cytoplasm and apparently the vacuole was observed with PATAg (Pl. 3.20c). Starch grains in plastids were intensely stained with PATAg (Pl. 3.20c) whereas the stroma stained weakly; dictyosomes were also intensely stained (Pl. 3.20c).

Mesophyll

The principal staining structures here were the chloroplasts, which stained only with PATAg. Weak staining was observed throughout the stroma; osmiophilic globules and internal membranes appeared largely unstained (Pl. 3.20a,b). In some cases, however, frets appeared to be stained on their stroma faces and within their lumina, and marked staining of interthylakoid spaces was also apparent (Pl. 3.20b). Most intense staining was associated with rounded or angular structures within the stroma ('st' in Pl. 3.20a,b). They were assumed to be starch grains by virtue of their staining reaction.

Plate 3.14. Ultrastructure and cytochemistry of the ligule of Lolium temulentum

a,b. Long (a) and circular (b) profiles of mitochondria in adaxial epidermal cells; X46,000 and X77,000 respectively;

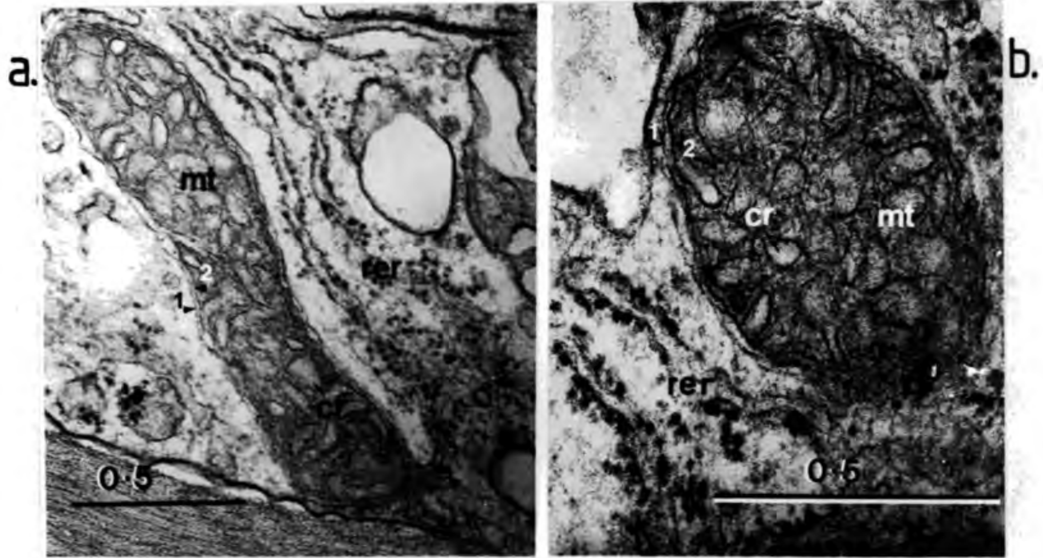
c. Mitochondrion of an adaxial epidermal cell (incubation: $-H_2O_2$); X36,000. Inset: circled region in c.; X320,000;

d. Amoeboid plastids of an adaxial epidermal cell; X9,500;

e. Mesophyll microbody with crystalline inclusion; X59,000;

f. Amoeboid plastid of an adaxial epidermal cell; X22,000.

Scale bars in µm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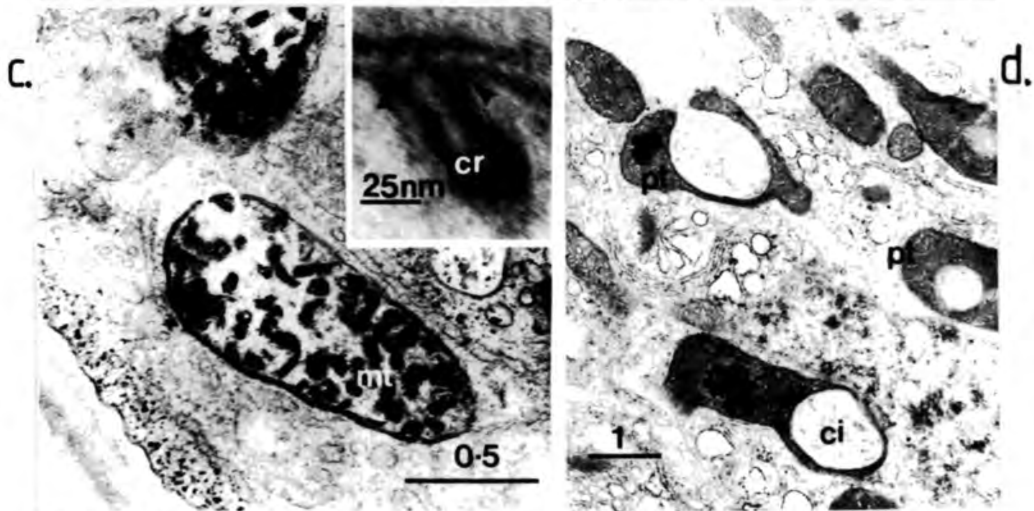
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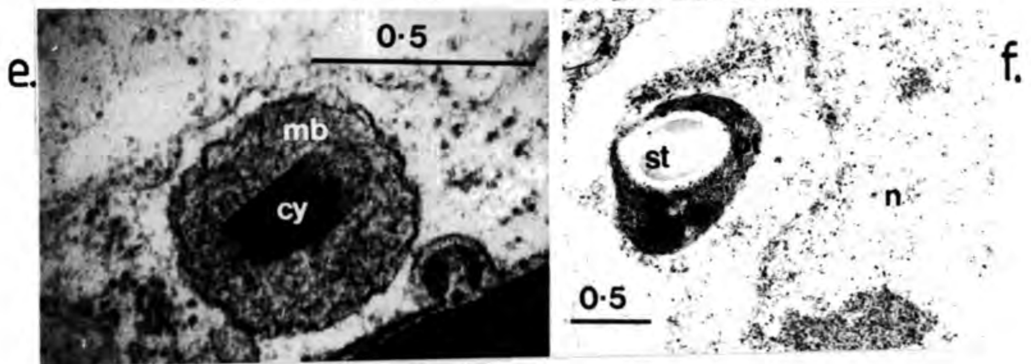
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Plate 3.15. Cytochemistry of the ligule and blade of <u>Lolium temulentum</u>

a. Dictyosome, microbody and mitochondrion of a ligule mesophyll cell (incubation: +triazole); X40,000;

b. Microbody and chloroplast of a ligule mesophyll cell (incubation: $+H_2O_2$); X40,000;

c. Mitochondrion and putative microbody ('?') in a ligule adaxial epidermal cell (incubation: $+H_2O_2$); X45,000;

d. Mitochondrion of a ligule adaxial epidermal cell (incubation: +KCN); X78,000;

e. Microbodies, mitochondrion and chloroplasts of a blade mesophyll cell (incubation: $+H_2O_2$); X17,000;

f. Portions of cytoplasm of adjacent ligule abaxial epidermal (bearing plastids) and mesophyll cells (incubation: $-H_2O_2$); X29,000;

g. Mitochondrion, microbody and oleosome of a ligule mesophyll cell (incubation: $-H_2O_2$); X65,000.

Scale bars in μm

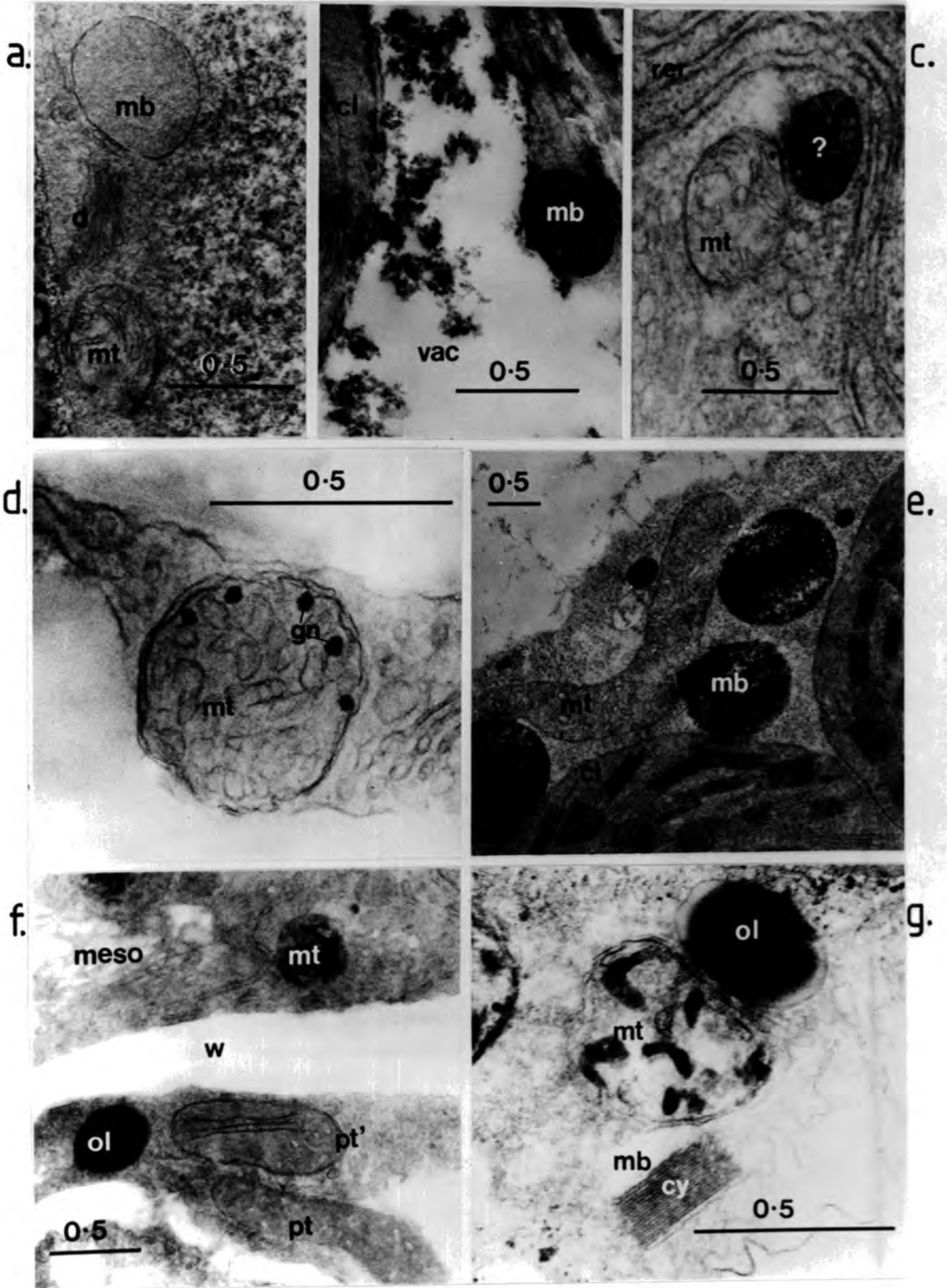

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Plate 3.16. Cytochemistry of the ligule and blade of Lolium temulentum

a. Ultrastructural distortion after incubation of ligule in unmodified Ekés medium; darts indicate wall-associated deposits; X3,600;

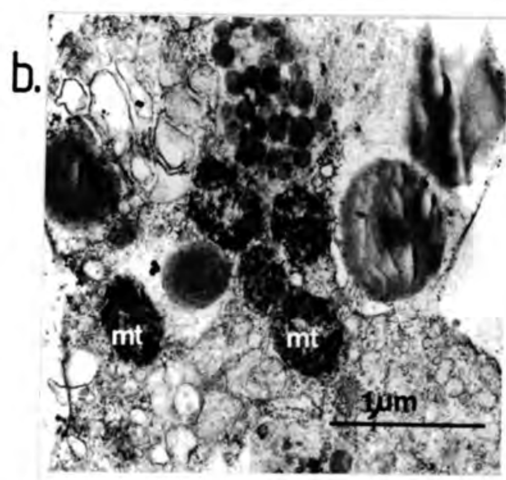
b. Discrete deposits of reaction product in cytoplasm of a ligule adaxial epidermal cell (incubation: +succ); X21,000;

c. Higher power view of part of cytoplasm in b. above; X56,000;

d. As for b. above but double-stained; X36,000;

e. Chloroplast and mitochondrion of blade mesophyll cell (incubation: +succ); X20,000;

f. As for e. above; X20,000.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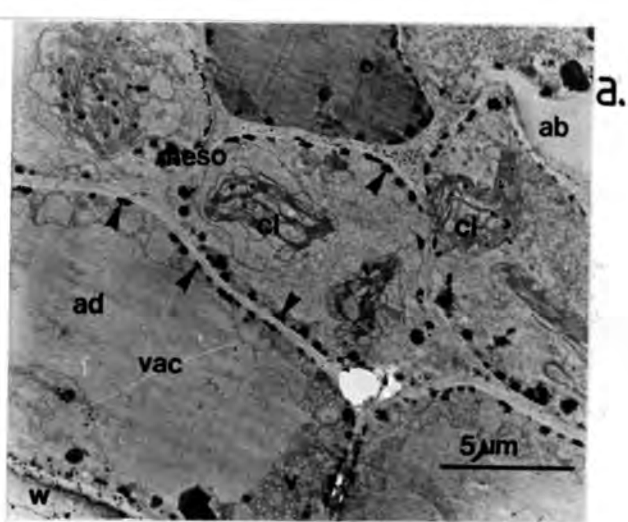
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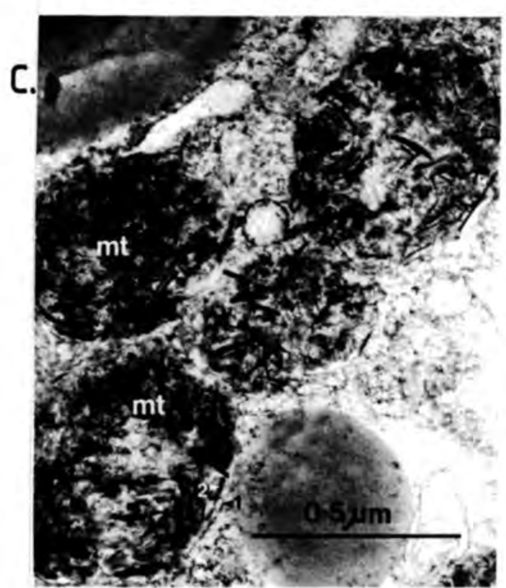
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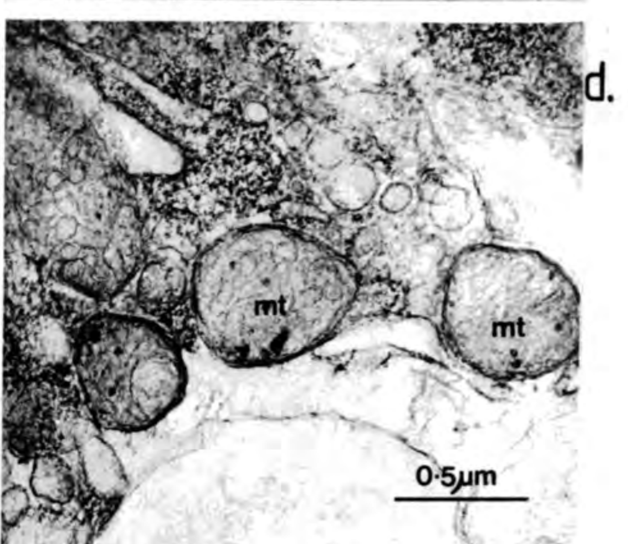
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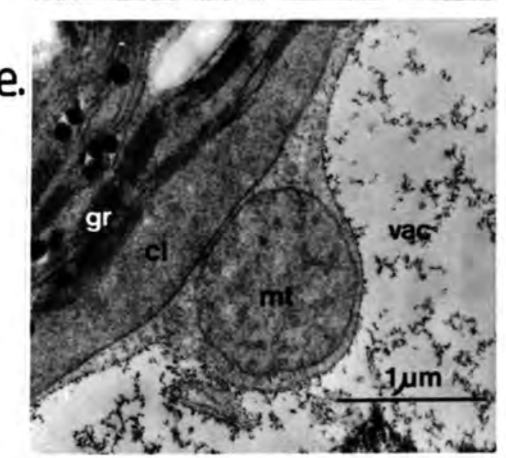
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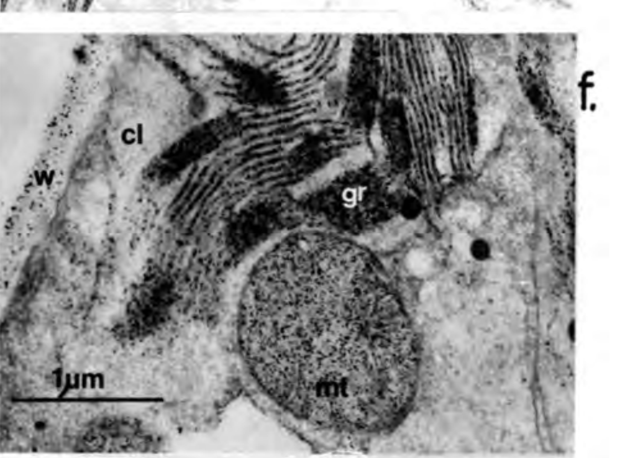
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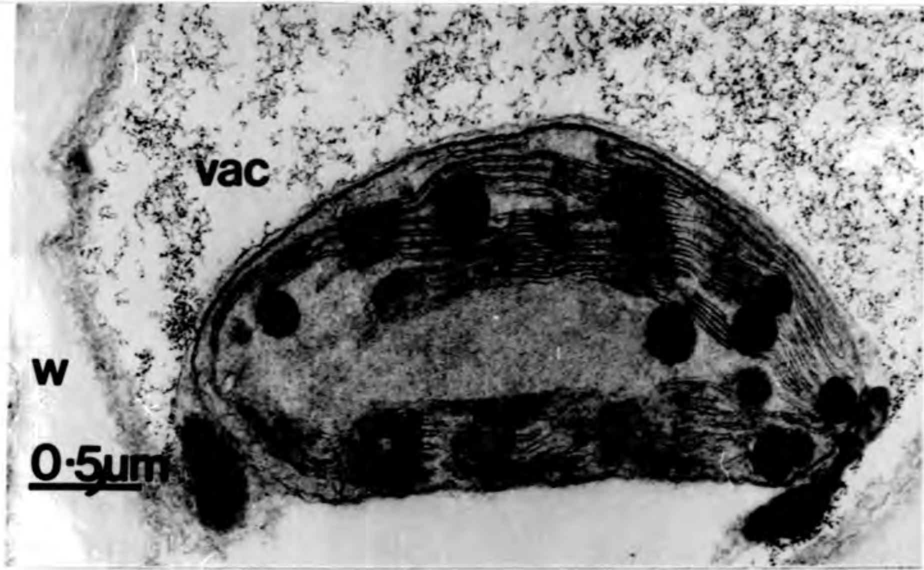
Plate 3.17. Cytochemistry of the ligule of _Lolium temulentum_

a. Mesophyll chloroplast (incubation: +succ); X30,000;

b. Part of cytoplasm of an adaxial epidermal cell (incubation: +mal); X39,000.

a.



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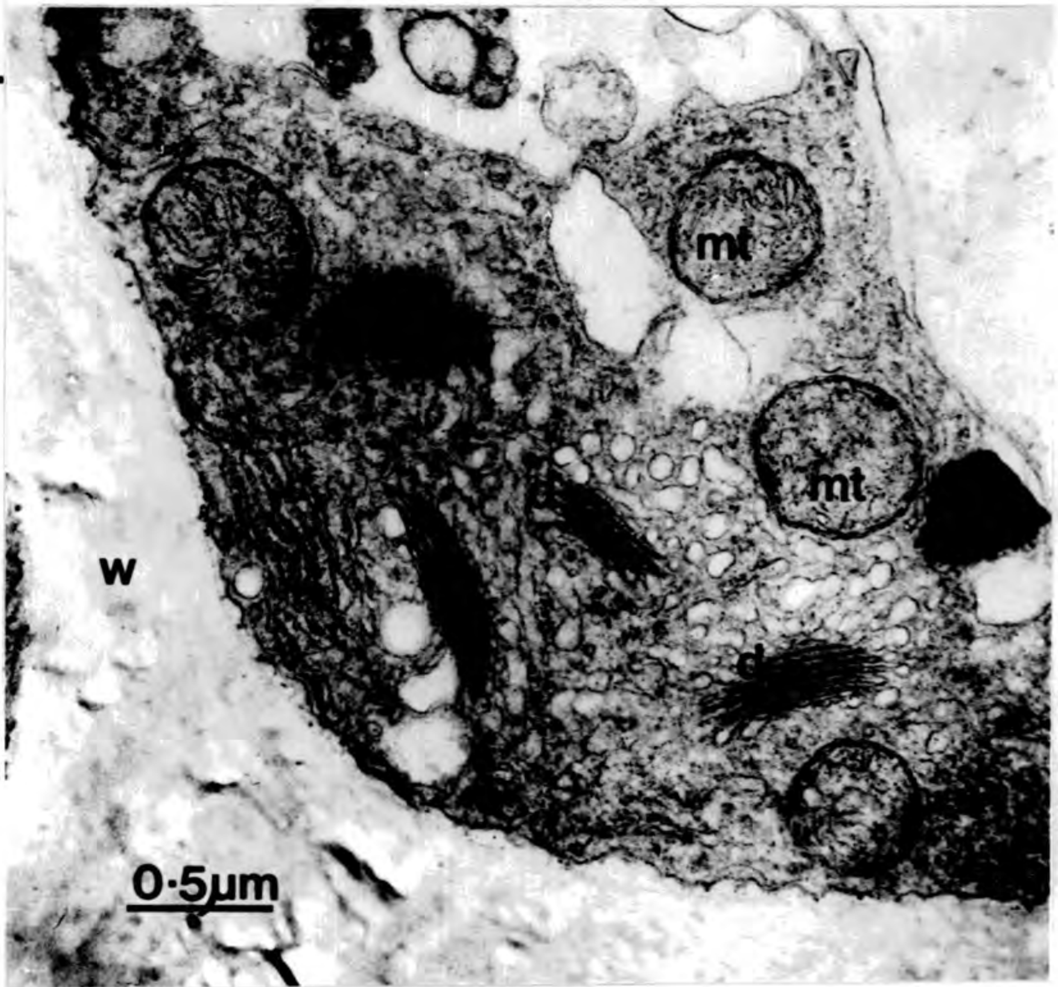


Plate 3.18. Cytochemistry and ultrastructure of the ligule of Lolium temulentum

a. Parts of cytoplasm and outer tangential walls of two adjacent adaxial epidermal cells (PATAg - 2 h, TCH); darts indicate stain on the cuticle; X23,000;

b. Region similar to a. above (PATAg - 24 h, TCH); dart indicates cuticular gap; X28,000;

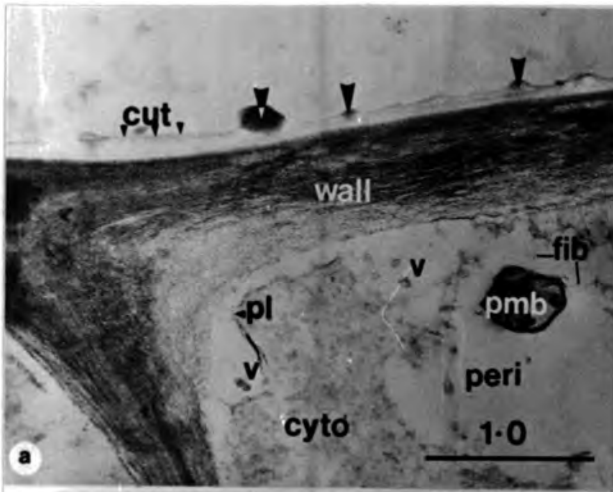
c. Part of cytoplasm of an adaxial epidermal cell (PATAg - 2 h, TCH); X48,000;

d. Part of cytoplasm of an adaxial epidermal cell (PATAg - 2 h, TCH); arrow indicates direction of increased staining intensity in dictyosome; X58,000;

e. Part of outer tangential wall and cytoplasm of an adaxial epidermal cell; X15,500. Inset: paramural body from a similar region to e.; X17,000;

f. Parts of outer tangential walls and cuticle of two adjacent abaxial epidermal cells (PATAg - 24 h, TCH); X50,000.

Scale bars in μm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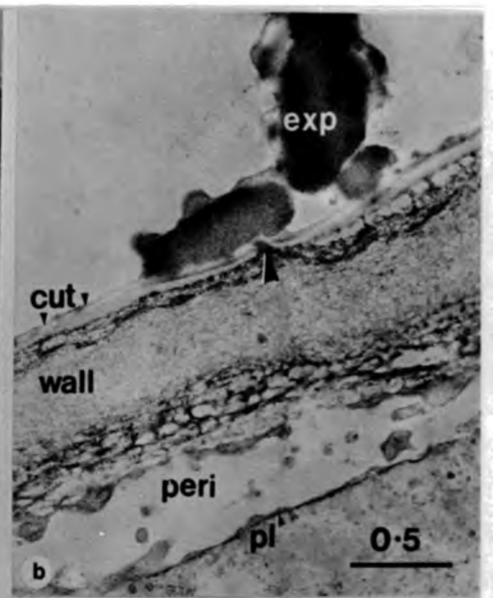
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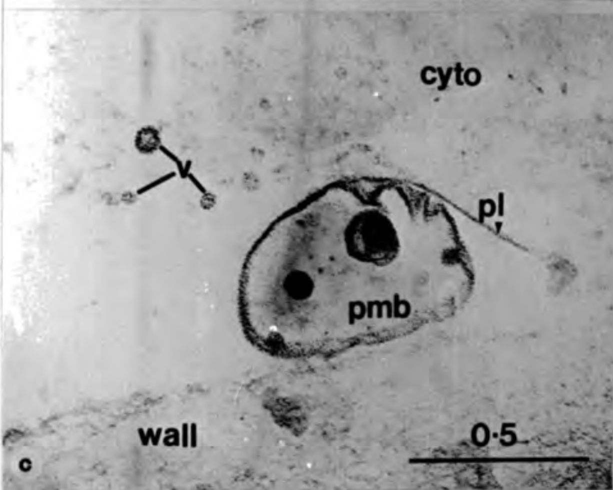
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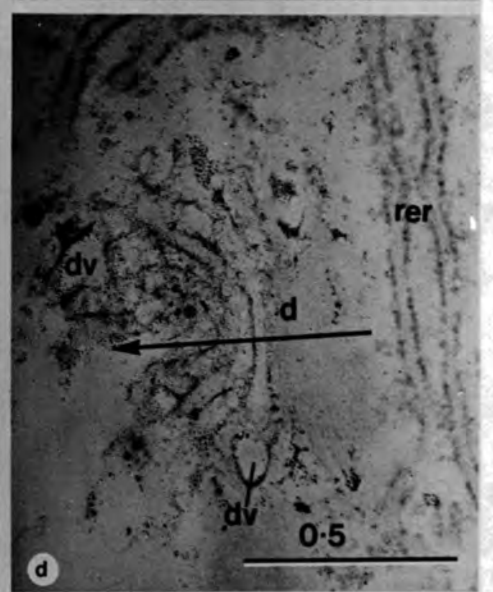
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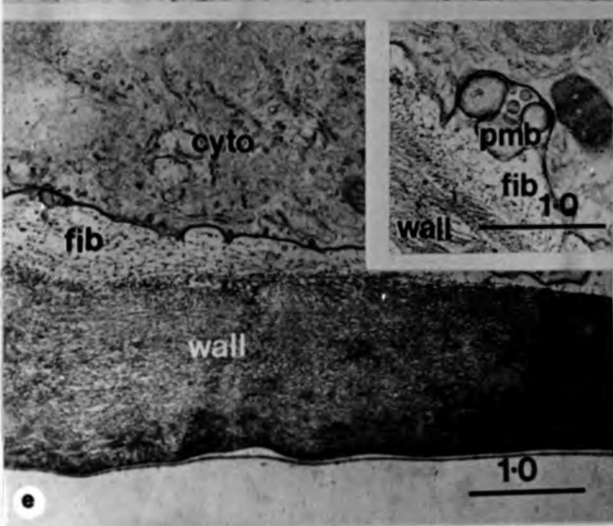
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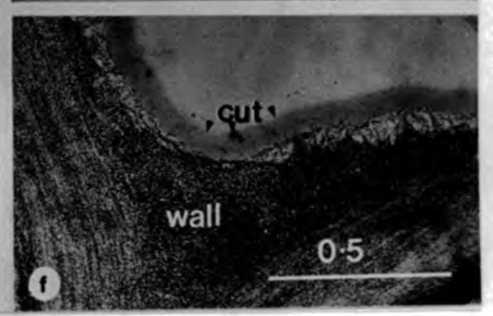
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Plate 3.19. Cytochemistry of the ligule of Lolium temulentum

a. Parts of cytoplasm and outer tangential walls of two adjacent adaxial epidermal cells (PTA); X26,000;

b. Region similar to a. above (PTA); dart indicates a cuticular gap; X36,000;

c. Part of cytoplasm of an adaxial epidermal cell (PTA); X47,000;

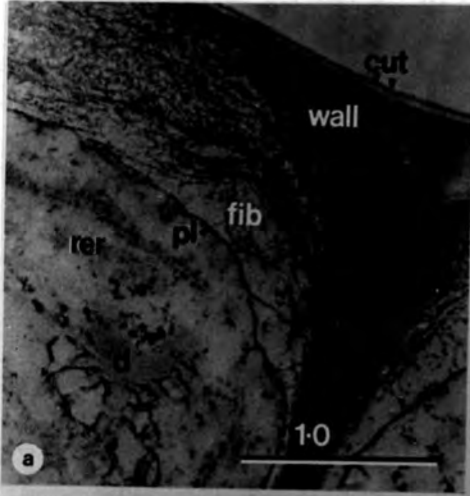
d. Part of cytoplasm of an adaxial epidermal cell (PTA); arrows indicate direction of increased staining intensity in dictyosomes; X43,000;

e. Outer tangential wall region of an abaxial epidermal cell (RR); X50,000;

f. Outer tangential wall region of an adaxial epidermal cell (RR); X72,500;

g. Part of cytoplasm of an adaxial epidermal cell (RR); X60,000.

Scale bars in μm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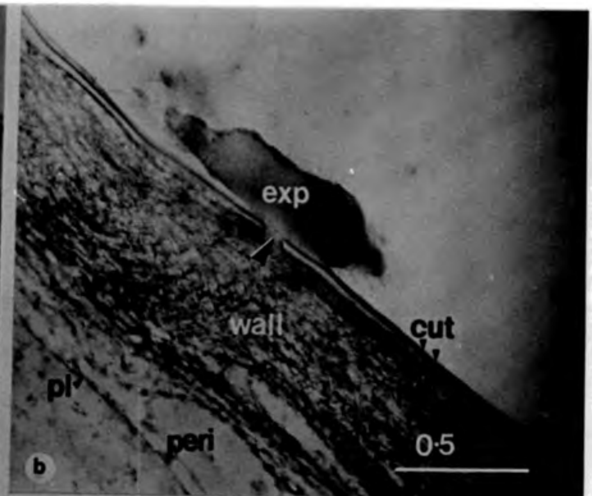
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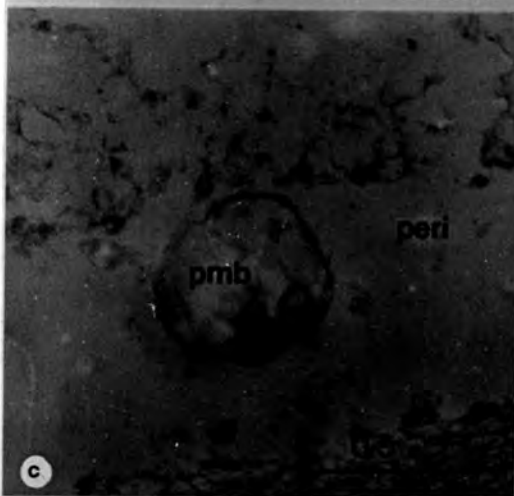
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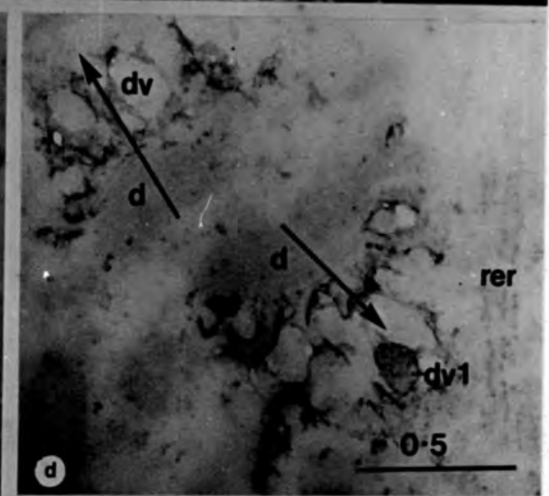
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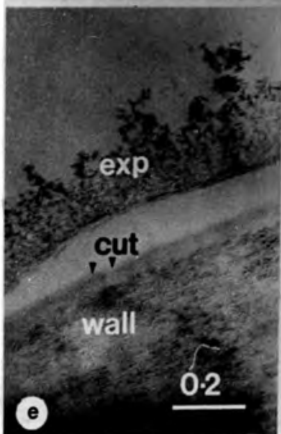
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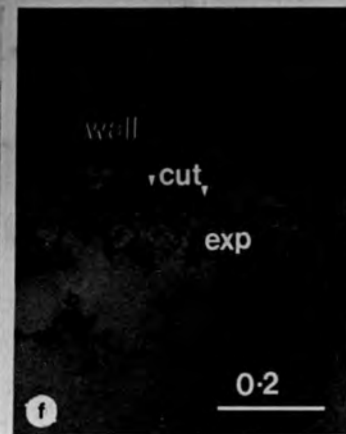
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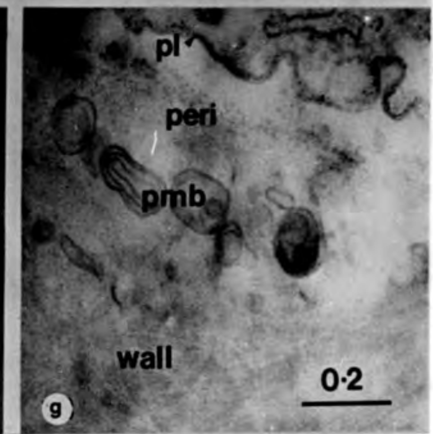
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Plate 3.20. Cytochemistry and ultrastructure of the ligule of Lolium temulentum

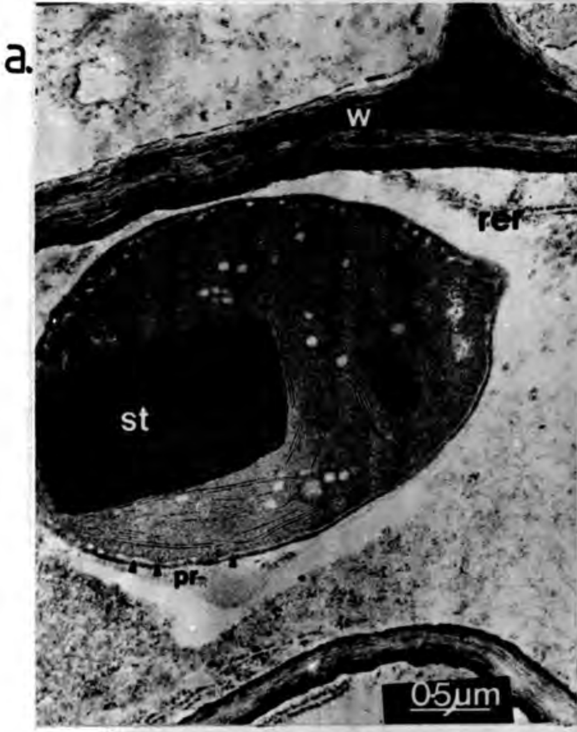
a. Mesophyll chloroplast (PATAg - 24 h, TCH); X22,000;

b. Higher power view of region similar to a. above (PATAg - 24 h, TCH); X62,500;

c. Part of cytoplasm of an abaxial epidermal cell (PATAg - 24 h, TCH); X36,000;

d. Outer tangential wall of an adaxial epidermal cell; darts indicate electron-dense material attached to the cuticle; X80,000;

e. Region similar to d. above; dart indicates a cuticular gap; X110,000.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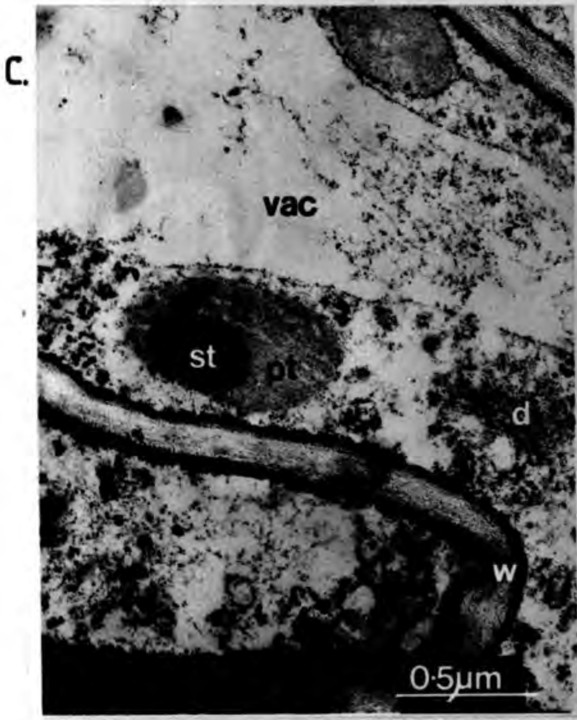
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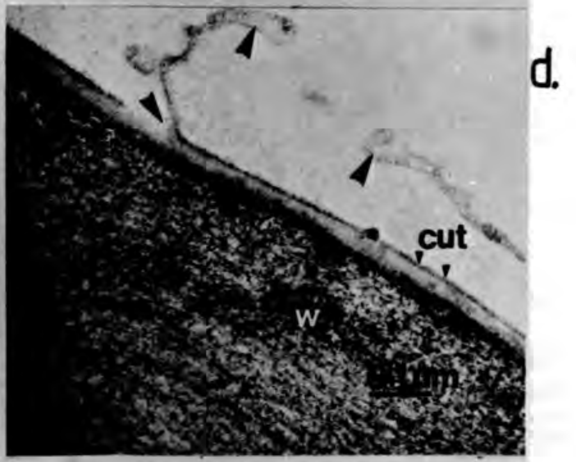
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DISCUSSION

The general ultrastructure of ligule microbodies and mitochondria was in agreement with that reported for other plant tissues by Frederick and Newcomb (1969) and Öpik (1974).

Low numbers of microbodies in the mesophyll is of interest in view of the observation that this tissue had a smaller chloroplast population than the corresponding blade mesophyll (Table 3.1). The disparity in microbody numbers appeared to be even greater and is similar to the situation in C_4 photosynthetic cells of C_4 plants (eg Hilliard et al., 1971). No data are yet available concerning photosynthetic pathways operating in the ligule, but the possibility that it may be a C_4 organ on a C_3 leaf exists.

The rarity of sightings of the junction between cristae and the inner mitochondrial envelope membrane suggests that the shape of the cristae is tubular - at least at the junction. If they were sheet-like such junctions would be observed more often, regardless of section thickness. The highly-cristate nature of the mitochondria is consistent with their being highly active in respiration (eg Öpik, 1968).

Results of enzyme cytochemical studies allow more realistic assessment of 'functionality' of such organelles.

Incubation of tissue in the presence of DAB in either the light or the dark led to the formation of a red-brown precipitate (assumed to be DAB oxide) in the vials. The apparent absence of mitochondrial staining in the dark is strong circumstantial evidence that no physical adsorption of DAB oxide occurred in these organelles. Blade microbodies did stain when incubated with DAB in the dark and this may have been due to adsorption of DAB oxide. It is, however, reasonable to assume that both mitochondria and microbodies behaved similarly in this respect and that no non-enzymatic adsorption of DAB oxide occurred in the course of this study. This observation and inference is in agreement with the work of Hall and Sexton (1972) on pea root microbodies. For succinate dehydrogenase cytochemistry the only non-specific staining of tissue appeared to be associated with the cell walls; all other staining was assumed to be enzymatic.

Staining of ligule microbodies was assumed to be due to the action of catalase in view of the apparent absence of staining in the presence of triazole, a catalase-specific inhibitor (Margoliash and Novogrodsky, 1958). Different tissues, however, differ in the degree to which triazole can inhibit DAB-staining of microbodies (eg Hall and Sexton, 1972). On this basis the reduced degree of triazole-inhibition of blade microbodies relative to ligule microbodies may still mean catalase is responsible, but the additional involvement of peroxidase is not ruled out. Although catalase is found in both peroxisomes and glyoxysomes (eg Tolbert, 1971), by analogy with the blade peroxisomes, the ligule mesophyll microbodies may be considered peroxisomes.

The observed absence of mitochondrial staining in the presence of added H_2O_2 suggests that staining of these organelles observed under other 'cytochrome c oxidase' incubation conditions was not due to the action of a mitochondrial peroxidase or catalase. Inhibition of mitochondrial DAB-staining in the presence of KCN or NaN_3 implied the involvement of a haemoprotein. The observation by Nir and Seligman (1971) that only cytochrome oxidase was stainable with DAB in maize mitochondria further suggests that in the study performed here it was cytochrome c oxidase ('cytochrome oxidase') that was being demonstrated. DAB acts as an electron donor to the enzyme and becomes oxidized to a highly osmiophilic form; thus sites of enhanced osmiophilia are assumed to be sites of the reaction and hence the enzyme. The sites observed were solely membranal; both envelope and cristal membranes stained. This pattern is similar to that recorded by Nir and Seligman (1971) in maize, and Öpik (1975) in rice.

No evidence of heterogeneity in mitochondrial DAB-staining (as recorded by Ekés, 1971) was observed; both blade and ligule mitochondria appeared to stain to the same extent and all mitochondria appeared to have stained. Although the triazole-inhibition of blade mitochondrial DAB-staining implicates the involvement of a catalase, further work is needed before this conclusion can be accepted.

Lack of mitochondrial staining in the dark, however, is puzzling. Nir and Seligman (1970) and Öpik (1975) both demonstrated DAB-staining of mitochondria in the dark. Its absence in the present study may be related to the conditions of incubation - especially the high pH and extensive aldehyde-fixation of material. It does seem likely though

that the tricarboxylic acid cycle, and hence mitochondria, is active both in the dark and the light (eg Raven, 1976) and one would expect the electron transport chain to behave similarly. Thus a supply of metabolic intermediates and energy should be available from these organelles regardless of whether the tissue is illuminated or not.

The apparent staining of ligule mitochondria in the presence of added sodium succinate, and its absence in the presence of added sodium malonate, may be interpreted as indicating the presence of succinate dehydrogenase activity in these organelles. Accurate localization of the sites of enzyme activity was not possible in view of the apparent displacement of reaction product during double-staining (cf also Ekés, 1970). Although lack of staining in the absence of added sodium succinate may suggest inability of endogenous substrate sources to sustain a 'reasonable rate' of activity, it is more likely that the processing of the tissue prior to incubation affected the material in such a way that this conclusion is not valid.

Clearly it is a big step from demonstrating presence of an enzyme in an organelle, and its activity under the artificial conditions of the assay, and proving that the enzyme and thus the organelle is functional in the plant. Microbody staining in the absence of added H_2O_2 does suggest that they can make their own H_2O_2 (presumably by the action of flavine oxidases which they contain (eg Tolbert, 1971). This implies that they may be active in the ligule. The mitochondria have the appearance of highly active organelles, both cytochrome c oxidase and succinate dehydrogenase is apparently demonstrable in them and they are assumed to be active in the ligule.

Accurate interpretation of the results of the polysaccharide cytochemical study is dependent upon knowledge of the specificity of the techniques used.

The PATAg test is the TEM equivalent of the periodic acid/Schiff's reagent technique used in LM. Here PA oxidation of polysaccharide vic glycol groups converts them to aldehyde groups (eg Roland, 1974). These aldehyde groups react with the multidentate ligand TCH to form a complex which is strongly osmio- or argentophilic (Hanker _et al._, 1966). Sites of polysaccharides are thus visualized as sites of staining with osmium or silver salts. The technique is considered to be largely specific for polysaccharides with α-1,4- or β-1,4-glucan

linkages. With increase in the degree of conjugation of the polysaccharide moiety, eg as a glycoprotein, accessibility of vic glycol sites is reduced and longer periods of reaction with TCH are required for staining. Hence an increase in staining intensity with time in TCH is indicative of a conjugated polysaccharide (eg Thiéry, 1969). Non-specific staining may be introduced by the use of aldehyde-fixatives but was not a problem in this study (cf also Pickett-Heaps, 1967).

PTA is generally regarded as being specific for complex carbohydrates (eg Pease, 1970) such as glycoproteins (eg Rambourg, 1971). It also has specificity for the plasmalemma and vesicles assumed to fuse with it (eg Roland et al., 1972; Vian and Roland, 1972). Its staining reaction parallels that of PATAg to a certain extent because of its affinity for OH groups of glycols (Pease, 1970). Both PATAg and PTA work best in non-osmicated tissue (Thiéry, 1967; Pease, 1970).

For many years RR has been used as a LM cytochemical test for pectic gums and mucilages (eg Mangin, 1893); only recently, however, have attempts been made to specify the nature of the staining reaction, by Luft (1964) and Sterling (1970). The former concluded that it had specificity for highly polymerized acidic polysaccharides of the type represented by pectin. Sterling found that staining was achieved between the RR complex and a substrate if the latter had two -ve charges in a precise spatial relationship to each other. Although the galaturonide moiety of pectin is the commonest plant material to meet these steric requirements, any suitable substance will give the same reaction.

Hence PATAg can be considered specific for α- or β-1,4-glucans; PTA probably specific for glycoproteins and RR has some affinity for pectin-like materials.

The staining patterns of PTA and PATAg were similar although starch appeared only to stain with the latter and RER only with the former. In adaxial epidermal cells the material within the dictyosomes, vesicles, paramural bodies, periplasmic space and on the cuticle is interpreted as having a glycoprotein nature by virtue of staining with PTA, and PATAg-staining increasing with prolonged TCH incubation time. In the previous Section, the FluorM study suggested that the periplasmic material in part consisted of β-linked glucan. Together with the specificity of the PATAg technique, it may be suggested that

the material within the periplasmic space is a glycoprotein with a β-1,4-linked glucan component and not cellulose as speculated in the previous Section. Whether all the sites staining with PTA and PATAg are involved in stages of synthesis or packaging of the periplasmic material is not known. It is likely that other polysaccharides are being made by these cells, but that the main product is that accumulated in the periplasmic space.

The staining pattern observed in these cells is certainly consistent with the interpretation that material made in the dictyosomes - with possible RER involvement - is the same as that which accumulates within the periplasmic space and that observed on top of the cuticle. The suggestion that the material within the periplasmic space and on top of the cuticle are the same is supported by the presence of cuticular gaps and material staining with PTA and PATAg within them. Discontinuities in the cuticle have been recorded for silica cells of this species by Lawton (1980), and for a number of other secretory systems eg Drosera (Chafe and Wardrop, 1973) and stigmas of grasses (Heslop-Harrison and Heslop-Harrison, 1982). Their mode of formation is not known.

The difference in staining pattern of the material on the cuticle and within the periplasmic space may suggest differences in structure, if not chemical composition. It may be that the fibrils are higher MW polysaccharides surrounded by a sheath of lower MW polysaccharides. The latter may not stain with the heavy metals used in conventional TEM and may even be washed out of the tissue during processing (eg Foster, 1982). The extracuticular material may be a more condensed version of this periplasmic material in which the lower MW materials have been removed during passage through the wall or washed off during processing and the individual fibrils can no longer be distinguished.

The staining pattern observed with RR was quite different to that observed with the other two techniques; a dense fibrillar-granular material was found on both cuticles. On the adaxial cuticle, material of amorphous nature, similar to that which stained with PTA and PATAg, was not stained with the RR (results not shown). These results can be interpreted as the RR staining a fibrillar component of the glycoprotein material which was removed during the numerous washing steps involved in the other techniques. On that basis it might be suggested

that the extracuticular material contains a low MW pectin-like substance. However, non-staining, by RR, of amorphous material on the adaxial cuticle; its similar 'staining' pattern on the abaxial cuticle - where no amorphous deposits were seen with PTA- or PATAg-staining or conventional TEM - and the generally poor ultrastructural preservation of material treated with RR suggest that it is an unreliable technique. The material observed on both cuticles may be precipitated RR; it does not appear to be the same material which stains with PTA and PATAg. The apparent staining of paramural bodies with RR must also be questioned since conventionally-processed material viewed without double-staining - an RR-staining control - showed a similar level of electron density.

Cells of the adaxial epidermis are apparently highly active in synthesis and secretion of a glycoprotein-like material. The numerous highly-cristate, apparently functional, mitochondria of this tissue presumably act as sources of energy for this. It may be postulated that the source of substrates for energy production and polysaccharide synthesis are the starch-bearing amoeboid plastids of this tissue. Alternatively the numerous plasmodesmata observed between this layer and the mesophyll might imply that substrates from the chloroplasts of the latter are transferred to the former.

PATAg-staining of the dictyosomes of the abaxial epidermal cells is of interest in suggesting some degree of polysaccharide synthesis in this tissue. Whether this material is destined for intracellular use or export to the cell wall is not known. The few paramural bodies seen here were also stained with PATAg which might suggest their involvement in wall turnover known to occur in mature monocotyledonous tissues (eg Labavitch, 1981). There was no indication of secretion to the outside of the ligule by this tissue. The highly-cristate nature of the mitochondria here may be interpreted as energy sources, and the starch-bearing plastids as sources of precursors, for polysaccharide synthesis. These cytochemical results certainly suggest that this tissue is more active than a strictly ultrastructural study might imply.

It is not clear to what category of plastids those of the abaxial epidermis belonged. They appeared to be more highly developed than those of the adaxial epidermis; both were more highly developed than those of newly-initiated ligules (see Chapter 3, Section d). It is

possible that both are transient stages along an as yet unfinished pathway of plastid development. Evidence for this is suggested by the presence of chloroplasts in the basal regions of the abaxial epidermis, and implies continued development of ligule ultrastructural complexity after attainment of 'maturity' of the leaf to which it is attached. This aspect of ligule biology clearly needs more study.

The presence of oleosomes in the ligule may be interpreted as an indication that the material examined here was beginning to senesce (eg Parker and Murphy, 1981). The significance of these organelles, however, can only be properly assessed when the development of the ligule has been studied.

PATAg-staining of chloroplasts was expected from their ability to synthesize starch; the shape of the presumptive starch grains was not. They appeared to be more angular than those observed by conventional TEM (eg Pl. 3.8c) and more like the chloroplast bodies previously observed whose structure and composition was not known (eg Pl. 3.8b). Pronounced staining of the interthylakoidal spaces may indicate sites of starch synthesis. General staining throughout the stroma is more likely to suggest presence of polysaccharide not yet incorporated into starch.

Functions of microbodies are many and varied (eg Tolbert, 1971). If we accept that the ligule microbodies are peroxisomes it is possible that they are involved in some degree of photorespiratory activity in the chlorenchymatous cells of this organ. In this respect it is interesting that microbodies were only found in abaxial epidermal cells near the base of the ligule which also contained chloroplasts. The possibility that they may perform other 'functions' is not excluded, however, nor is the possibility that they may be present in the adaxial epidermal cells.

From a consideration of certain aspects of the cytochemistry of the ligule we may conclude that cells of the adaxial epidermis have a high degree of respiratory activity associated with a high degree of synthetic and secretory activity of a glycoprotein-like material. This tissue may thus be considered a true gland (*sensu* Schnepf, 1974) exhibiting exogenous secretion of the granulocrine type (eg Fahn, 1979) and the ligule a secretory organ. This conclusion is of interest in

view of the recent demonstration by Kristen _et al_. (1982) that the ligule of the lycopsid, _Isoetes lacustris_ also has a secretory nature.

SECTION c. ENDOMEMBRANE SYSTEM

INTRODUCTION

The 'endomembrane system' (Morré *et al.*, 1971) denotes 'the functional continuum of membranous cell components consisting of the nuclear envelope, endoplasmic reticulum and Golgi apparatus as well as the vesicles and other structures such as annulate lamellae derived from the major components'. The tonoplast, plasmalemma (PL) and autophagic vacuoles are envisaged as end products of this system (Mollenhauer and Morré, 1980). Although the various component parts of the endomembrane system have often been treated together, before considering the whole it is necessary to give an account of its component parts.

The nucleus (Lafontaine, 1974; Hanke, 1977) can be regarded as the starting point of the endomembrane system. It is bounded by a double membrane, the nuclear envelope (NE), separated by the perinuclear space. In the region of pores these two membranes are continuous. The outer membrane may bear ribosomes, and, together with the similarity of lipid composition and enzyme activities, it is almost indistinguishable from the ER. However, in contrast to the ER, it is generally more buoyant, has slightly thinner unit membranes and bounds the nucleus. Occasionally continuity of the perinuclear space with the lumen of the ER has been observed (eg Clowes and Juniper, 1968; Morré and Mollenhauer, 1974) emphasizing the close structural relationship between NE and ER.

The ER is perhaps the most flexible and adaptable of all the parts of the endomembrane system (Clowes and Juniper, 1968) and appears to ramify throughout the cytoplasm. Because of its unique ability to synthesize protein and lipid - apart from plastids and mitochondria - it has been suggested by Hanke (1977) that all the other components of the endomembrane system are ultimately derived from ER. This worker has even termed the ER the 'meristem' of the cell. It is certainly a dynamic organelle subject to constant turn over (Chrispeels, 1980). Its structure is that of a lumen, the enchylemma, bounded by a single unit membrane. The cytoplasmic face of this membrane may bear ribosomes or polysomes - rough ER (RER), or be devoid of them - smooth ER (SER). In some cases certain regions may be rough and adjacent regions smooth, giving rise to bifacial ER. ER in the region of dictyosomes often shows a bifacial aspect, the membrane portion nearest the dictyosome cisterna being smooth.

Two forms of ER may be distinguished: cisternal (cER) which typically consists of long cisternae with associated ribosomes, seen as long sheets of parallel membranes in ultrathin sections; tubular ER (tER) which is comprised of numerous tubules and vesicles with some associated ribosomes and appears as a series of circular profiles in ultrathin sections. It seems generally accepted that RER is involved in synthesis of protein destined either for export from the cell or for intracellular deposition, for example in protein bodies (Chrispeels, 1980). The involvement of RER in polysaccharide synthesis and of SER in lipid synthesis is not unequivocal (Chrispeels, 1980).

The Golgi apparatus is probably the most studied endomembrane in view of its apparent central rôle in processing membrane between the ER and the PL. Various aspects of its structure and function have been reviewed by Morré et al., 1971; Whaley, 1975; Whaley and Dauwalder, 1979; Mollenhauer and Morré, 1980; Robinson and Kristen, 1982).

The term 'Golgi apparatus' is used to denote the dictyosomes engaged in one particular function. Any cell may have more than one Golgi apparatus; the combined Golgi apparatus of a cell have been termed the Golgi complex (Whaley, 1975). The individual unit of the Golgi apparatus, the dictyosome, is composed of a stack of 4 to several flattened cisternae or saccules. Each cisterna is single membrane-bound, devoid of ribosomes, its unit membrane thickness increases across the stack and is thicker than that of the ER. Rod-like or fibrillar intercisternal elements may be present between some of the cisternae at certain stages of development (eg Mollenhauer, 1965; Turner and Whaley, 1965). The cisternae are c. 0.5 - 1.0 μm in diameter and become fenestrated and lost as vesicles towards their edges.

Polarity is often seen in the dictyosomes with an ER-associated pole and an opposite pole which is associated with numerous vesicles. The terms used to describe these two poles are numerous and varied; the ER-associated pole has been termed, forming, proximal, convex, regenerating, immature and non-secreting; the other pole has been termed, mature, distal, concave and secretory. To avoid the implication of a 'beginning' and an 'end' to an otherwise dynamic organelle, the terms used here will be cis aspect and trans aspect for the ER-associated and opposite poles respectively (eg Rothman, 1981; Harris and Oparka, 1983).

The dictyosome has often been strongly implicated in polysaccharide synthesis (eg Mollenhauer and Morré, 1980) and, in comparison with the ER, it is well supplied with glycosyl transferases (eg Hanke, 1977). In many cases, however, it seems clear that no one organelle is responsible for synthesis of the final product. Rather all components of the endomembrane system seem to take part in the processing and elaboration of the material concerned, eg ER, dictyosomes and vesicles, and PL in the elaboration of cell wall polysaccharides (eg Robinson, 1977), and the formation of root cap slime in maize (Barlow, 1982).

In association with the various phases of cell development, a corresponding series of states can be recognized in the dictyosomes (Mollenhauer and Morré, 1966): quiescent, replicative, general and hypersecretory. The latter is associated with synthesis of cell walls and root cap slime and is characterized by the presence of hypertrophied dictyosome-derived vesicles.

The vacuole has been extensively reviewed by Buvat (1971), Matile (1974) and Marty et al. (1980). Its mode of development, either directly from the ER (eg Buvat, 1971; Matile, 1974) or from the Golgi apparatus-ER-lysosomal compartment (GERL) (Marty, 1978), clearly identifies it as a component of the endomembrane system. The external boundary of the vacuole is denoted by the tonoplast, a single unit membrane.

The external boundary of the cytoplasm is denoted by another single unit membrane, the PL. If it is assumed that the PL is continuous from cell to cell via the plasmodesmata (eg Leonard and Hodges, 1980) then it must be considered the most widespread and biggest single component of the endomembrane system. It is the thickest of the endomembranes and is c. 8-12 nm wide. It frequently exhibits asymmetry in the thickness of its component leaflets with the outermost often appearing slightly thicker than the inner. Further, selective staining procedures have demonstrated the presence of a polysaccharide-containing component in the outer leaflet (eg Roland, 1973) and hence a similarity to the glycocalyx of animal cells.

The final elements of the endomembrane system are the vesicles which occur in a variety of sizes and can be classified in a variety of ways. Probably the most meaningful distinction is based upon their external morphology - either smooth-membraned or coated. The coated vesicles are usually seen either close to the PL or to the dictyosomes

(Newcomb, 1980a,b). They are c. 85-90 nm in diameter, including their coats, and are assumed to be derived from the dictyosomes directly or via smooth-membraned dictoysome-derived vesicles. In some cases their coats may be seen on the PL and is usually interpreted as indicating fusion of coated vesicles with the PL (Franke and Herth, 1974). They are of unknown function and appear in a number of forms with different types of coat, eg spiny vesicles (Esau and Gill, 1970).

The smooth vesicles may appear throughout the cytoplasm and frequently within the periplasmic space between the PL and the cell wall. In the latter location they may appear either singly or enclosed within evaginations of the PL as paramural bodies (eg Roland, 1973). In some cases, plasmatubules for example (Harris et al., 1982), it may not be easy to distinguish between purely PL proliferations and paramural bodies per se. Smooth vesicles are assumed to be largely derived from the dictyosomes and are believed to be involved in transport of polysaccharides for secretion from the cell (eg wall synthesis).

The formulation of the 'endomembrane concept' by Morré and Mollenhauer (1974) has developed the idea of membrane transfer and transformation within and between the various components of the endomembrane system. Evidence for this had been accumulating prior to 1974; after this date it was actively sought using a wide variety of techniques. Autoradiography, membrane and organelle isolation and biochemical analyses, biophysical measurements and conventional ultrastructural examination have all provided evidence strengthening the case for the endomembrane concept (eg Morré and Mollenhauer, 1974; Hanke, 1977; Whaley and Dauwalder, 1979; Mollenhauer and Morré, 1980).

With conventional TEM of aldehyde- or permanganate-fixed tissue it has been possible to observe direct connections between the NE and ER (eg Morré and Mollenhauer, 1974). Observations by Mollenhauer et al. (1975) suggest that the ER may give rise to the dictoysomes either by direct luminal continuity, or blebbing of transition vesicles which coalesce to form cisternae (Mollenhauer and Morré, 1976).

The numerous reports of 'fusion profiles' between PL and smooth vesicles provide circumstantial evidence for membrane flow between these two components of the endomembrane system, and, indirectly, between dictyosomes and PL (eg Morré et al., 1979). In this instance staining of the vesicle membranes and the PL with PTA has shown a similarity

which has been interpreted as evidence for membrane transformation, 'capacitation' (Roland, 1973), from dictyosome-like to PL-like. Such capacitation is presumably necessary for successful fusion of vesicle membranes with the PL and is strong circumstantial evidence for this part of the endomembrane concept.

Conventional TEM techniques have not unequivocally demonstrated ER/dictyosome connections. Probably the most significant advance in this respect has been the introduction of osmium impregnation techniques - zinc iodide-osmium tetroxide (eg Marty, 1973) and 'osmium ferricyanide' (eg Hepler, 1981). These procedures, together with the use of thick (0.3-3 μm) sections, voltages up to 3 MV, high-angle tilt facility on the TEM and production of stereo pairs, have quite literally added a new dimension to the study of ultrastructure in general and of the endomembrane system in particular. Although the basis of staining with these reagents is not understood the end result is an accumulation of electron-dense deposits within the lumina between single membranes. Characteristically the intracisternal spaces of the dictyosomes, the enchylemma, the central region of plasmodesmata, and the perinuclear and perimitochondrial spaces are stained. The endomembrane components not stained are the PL, tonoplast and smooth and coated vesicles within the cytoplasm; paramural bodies quite often stain.

Because of the greatly enhanced contrast of the endomembrane components relative to the rest of the cell the distribution of the endomembrane system within the cell can be easily visualized. In addition, the greater thickness of sections that can be examined as a result of this means that direct continuity between certain components can be readily detected.

Using the zinc iodide-osmium tetroxide (ZIO) technique it is easy to distinguish the two types of ER described above; cER appears as grey sheets, the tER as black tubular structures frequently connected to the former (eg Harris and Chrispeels, 1980). The polarity of the dictyosomes shows up in the gradation of staining intensity of cisternae in ZIO-treated tissue - the cis aspect is usually more densely stained than the trans aspect (eg Dauwalder and Whaley, 1973). Marty (1978) has demonstrated the stages of vacuole genesis from the GERL using this technique; Harris and Oparka (1983) have shown the existence of numerous fine tubules emanating from dictyosomes and the extent of connections between dictyosomes and tER.

So far the only instances of direct continuity of tER and the *cis* aspect of dictyosomes appear to be in cells of Venus' fly trap which secrete protein (Juniper *et al*., 1982) and root cells of wheat (Marty, 1980); ZIO-impregnation was used in both instances. In developing wheat endosperm, using ZIO, Parker and Hawes (1982) failed to find any direct ER/dictyosome continuity, although close associations were common. Using serial section analysis, Robinson (1980) also failed to find any ER/dictyosome connections in maize root cap cells, and both he and Kristen (Robinson and Kristen, 1982) have questioned the general validity of the endomembrane concept.

In the previous Section it was concluded that the extracellular product of the ligule adaxial epidermal cells may in part be synthesized on the ER and in part in the dictyosomes. It was thus of interest to examine the endomembrane system of this tissue in an attempt to define the pathway of synthesis of the extracellular product and examine the validity of the endomembrane concept in these ligule cells. Both ZIO-impregnation and conventional TEM techniques were used.

MATERIALS AND METHODS

Zinc Iodide-Osmium Tetroxide Impregnation

Material was fixed and processed as for conventional TEM except that the osmication step was replaced with a ZIO impregnation stage. The ZnI_2 solution was freshly prepared according to the recipe of Harris (1978):

1.5 g powdered zinc,
0.5 g resublimed iodine,
10 ml distilled water.

The solution was sonicated for 1 min, stirred for a further 5 min and filtered. The ZIO solution was made by adding equal volumes of 2% (w/v) aq. OsO_4 to the ZnI_2 solution.

The tissue was allowed to impregnate in the ZIO solution for c. 2-6 h and then dehydrated and resin-embedded. Sections were cut 0.5-1 µm thick and viewed unstained in the TEM operating at 100 kV. In some cases the tilt facility of the TEM was used in the preparation of stereo pairs.

OBSERVATIONS

The various elements of the endomembrane system of ligule adaxial epidermal cells are illustrated in Pl. 3.21 and considered separately below.

Nucleus

Both inner and outer membranes of the NE could be distinguished (Pl. 3.21). Neither pores nor connections between the NE and the ER were seen in conventionally-treated or ZIO-stained tissue. The nucleus itself was generally reticulate with dense chromatin dispersed within diffuse chromatin ('dc' and 'dic' respectively in Pl. 3.21); nucleoli were occasionally observed but the number per nucleus is not known.

Plasmalemma and Tonoplast

The PL was present as a continuous boundary to the cytoplasm (eg Pl. 3.22c) and appeared to be continuous between adjacent cells via the plasmodesmata (results not shown). Occasionally its 'tramline' nature was resolved but usually it appeared as a single densely-staining line. It was generally undulate near the outer tangential wall forming pockets within which paramural bodies were common (eg Pl. 3.22c,d). The sub-structure of the tonoplast was rarely resolved; it usually appeared as a line of greater electron density than the cytoplasm, bounding the vacuoles. It was not easy, however, to decide what were vacuoles and what were large dictyosome-derived vesicles; there appeared to be much overlap in size between these two groups of structures.

Dictyosomes

Large numbers of these organelles were present within the cytoplasm, apparently randomly distributed and oriented (eg Pl. 3.23d). Generally each dictyosome consisted of c. 6 or 7 cisternae with greatly hypertrophied vesicles at its trans aspect (Pl. 3.22a, 3.23b). Intercisternal elements were present and showed two distinct configurations - rod-like profiles and linear arrays (darts in Pl. 3.23b and 3.22b respectively). Where present they were seen right across the stack. It is possible that the two configurations seen were views of the same

structure at 90° to each other (eg Turner and Whaley, 1965). The intense synthetic activity of these organelles was evidenced by the profusion of vesicles - assumed to be dictyosome-derived - within the cytoplasm (eg Pl. 3.22d). ZIO-staining of these organelles revealed a marked polarization in staining intensity of cisternae, decreasing from cis to trans aspect (Pl. 3.24a).

Endoplasmic Reticulum

Both tER and cER were distinguishable on the basis of their ZIO-staining. The former appeared as densely staining, long-tubular structures; the latter as less densely staining, irregularly-shaped regions (eg Pl. 3.24a). Occasionally sub-structure was detectable in the cER (Pl. 3.24b); the significance of this is not known. The tER radiated out from the cER (Pl. 3.24a,b) and appeared to permeate the cytoplasm; cER appeared to be more localized. On a few occasions direct continuity between tER and the cis aspect of dictyosomes was seen (eg Pl. 3.25); more often the association was very close but not direct (white arrow-head in Pl. 3.23c).

The surface of the ER was covered with either single ribosomes (Pl. 3.22d) or polysomes (Pl. 3.23a,b), although on a number of occasions it was not clear if the polysomes were attached to ER or not. Most of the ER profiles seen in ultrathin sections were probably cER since in these cases the tER would most likely occur as circular profiles with attached ribosomes; the latter were apparently rare. No obvious instances of SER were seen in these cells.

Coated Vesicles

Although these vesicles appeared to be rare, they were probably more common than suggested by their occasional association with the PL (Pl. 3.22c), since only the thinnest of ultrathin sections would show them as being more than vesicles with indistinct membranes. They consisted of a central region of c. 45 nm diameter, surrounded by a reticulate coat which brought the total diameter to c. 90 nm. Occasionally what appeared to be the coats of these vesicles were observed apparently attached to the PL (dart in Pl. 3.22c).

Paramural Bodies

These structures generally appeared as collections of vesicular profiles in pockets of the PL within the periplasmic space (eg Pl. 3.22c). They were more common in association with the outer tangential walls of these cells, although they were found all the way around the periphery. Isolated vesicles were frequently observed within the periplasmic space (Pl. 3.2d); on a few occasions vesicle-like structures were seen apparently within the outer tangential wall (Pl. 3.26).

Plate 3.21. Ultrastructure of the endomembrane system of the adaxial epidermal cells of the ligule of *Lolium temulentum*

The endomembrane system of an adaxial epidermal cell; X21,000.

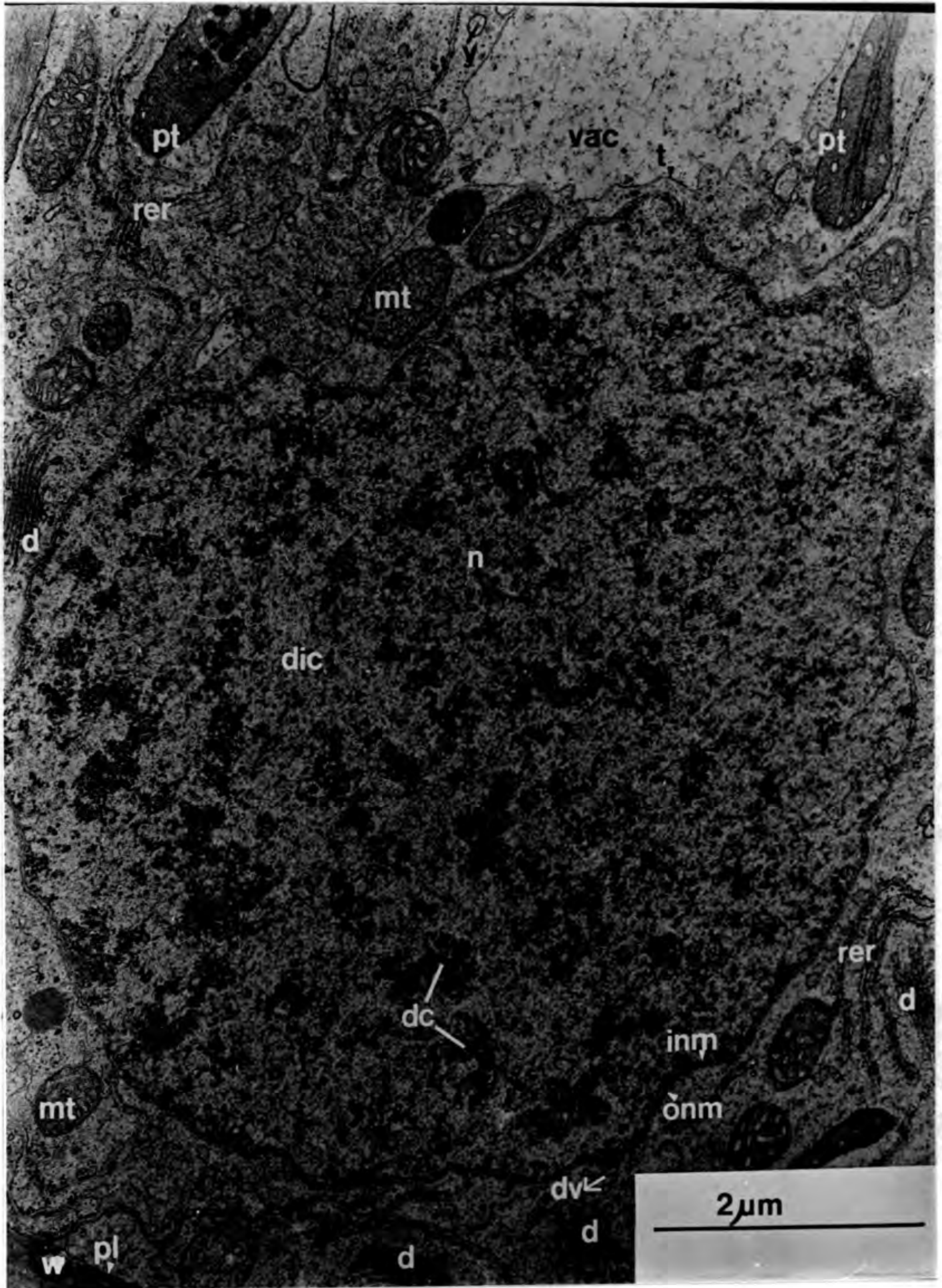

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Plate 3.22. Ultrastructure of the endomembrane system of the adaxial epidermal cells of the ligule of Lolium temulentum

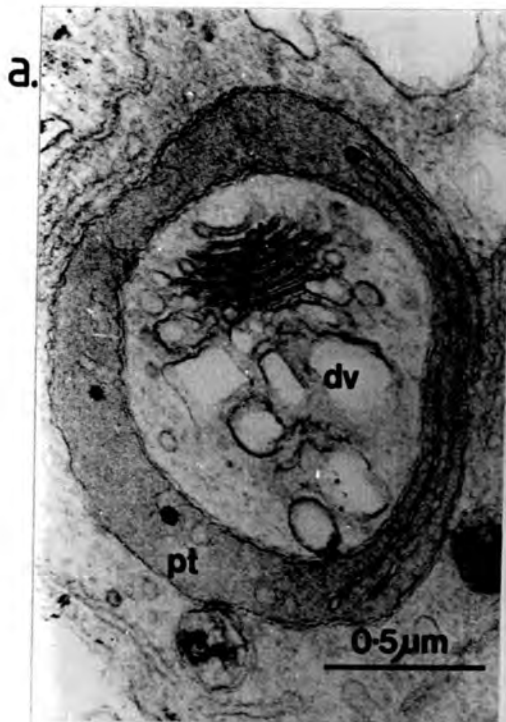
a. A dictyosome and associated vesicles within a cup-shaped depression of a plastid; X43,000;

b. Higher power view of dictyosome in a. above; darts indicate intercisternal elements; X155,000;

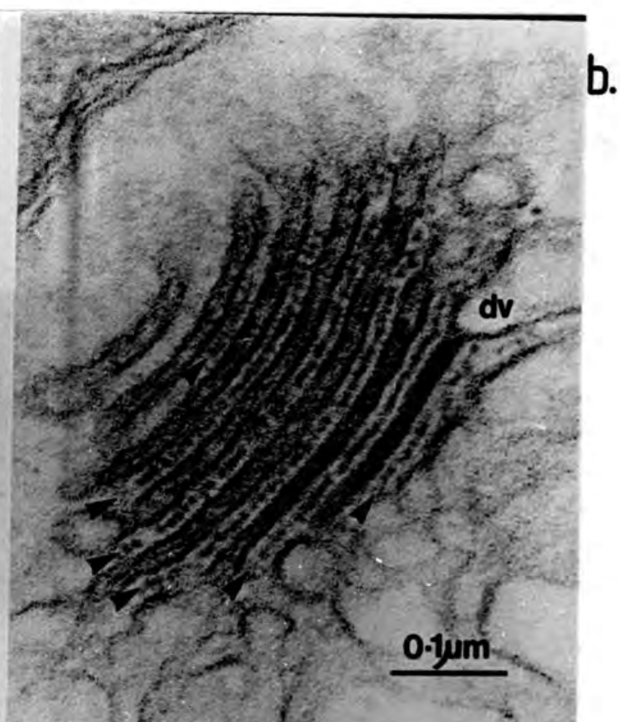
c. Outer tangential wall and part of cytoplasm; dart indicates coated vesicle reticulate coat-like structure apparently attached to the plasmalemma; X72,000;

d. Region similar to c. above; X33,000.

a.



b.



c.

d.

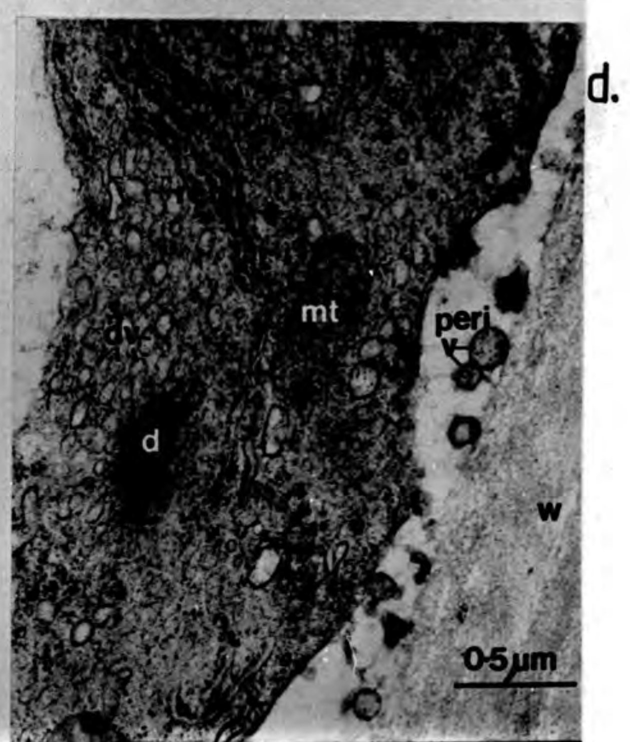


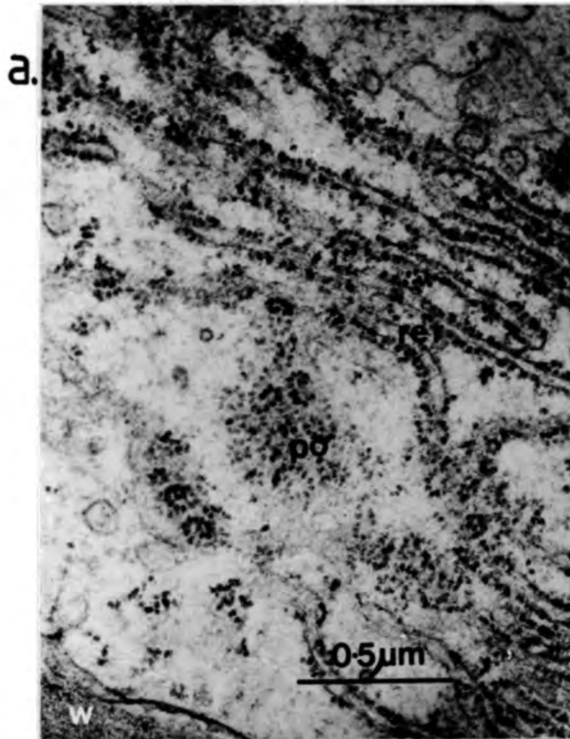
Plate 3.23. Ultrastructure of the endomembrane system of the adaxial epidermal cells of the ligule of Lolium temulentum

a. Sheets of rough endoplasmic reticulum with associated ribosomes and polysomes; X42,000;

b. Dictyosomes and apparently free polysomes; arrow-heads indicate intercisternal elements; X45,000;

c. Tubular endoplasmic reticulum and dictyosomes (ZIO); white dart indicates close association of dictyosome and tubular endoplasmic reticulum; arrows indicate direction of decreased staining intensity; X24,000;

d. Portions of cytoplasm of two adjacent cells (short-term ZIO); arrows indicate direction of decreased staining intensity; X9,250.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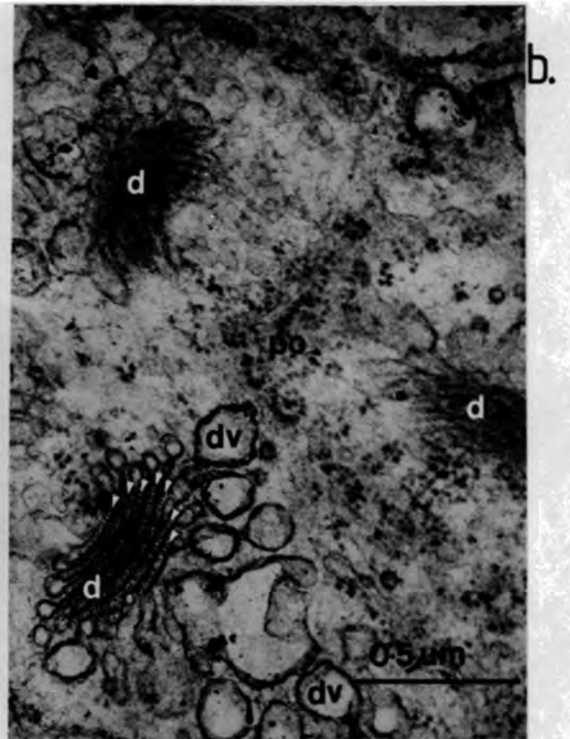
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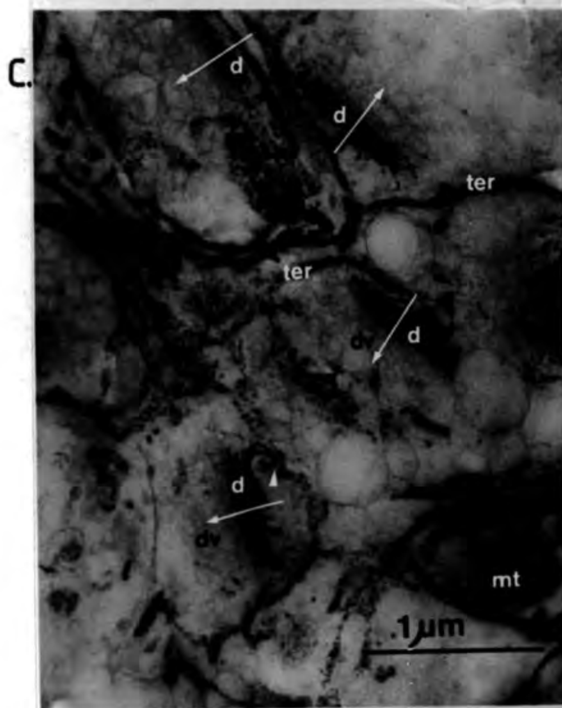
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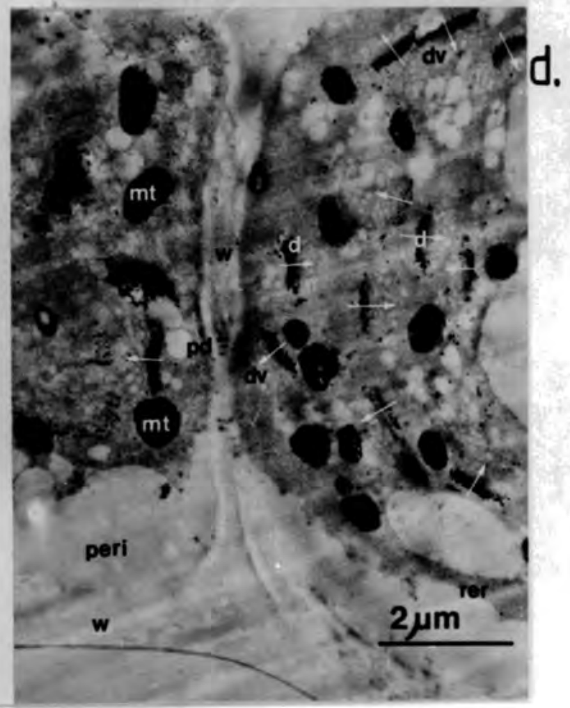
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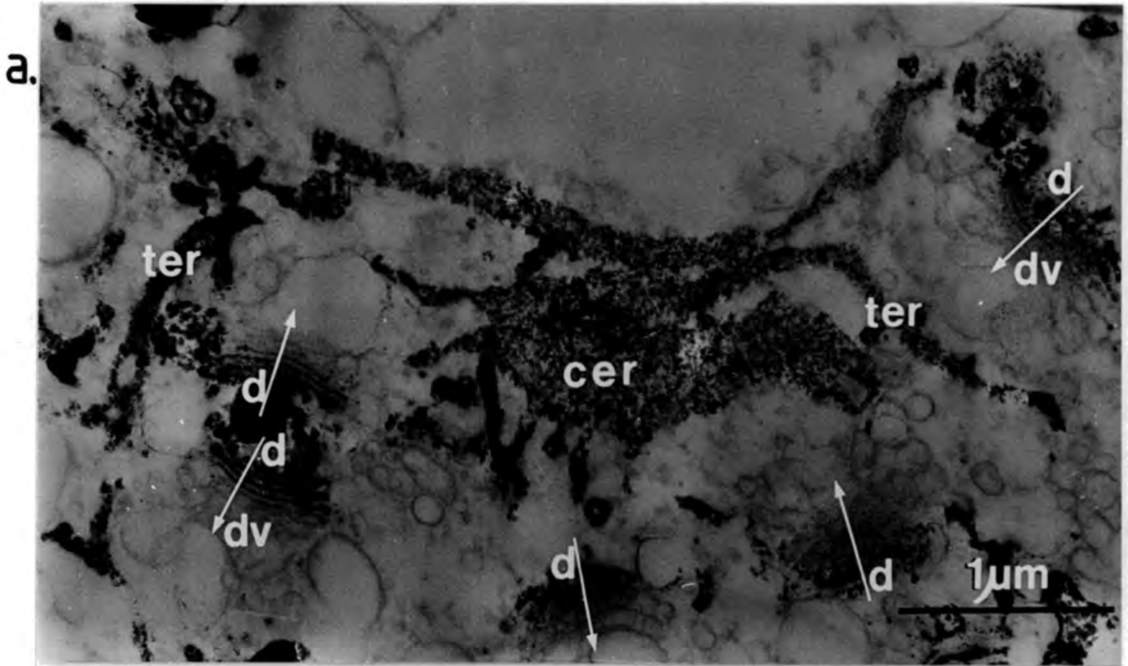
Plate 3.24. Ultrastructure of the endomembrane system of the adaxial epidermal cells of the ligule of Lolium temulentum

a. Cisternal and tubular endoplasmic reticulum and dictyosomes (ZIO); arrows indicate direction of decreased intensity of staining; X29,000;

b. As for a. above but showing sub-structure in cisternal endoplasmic reticulum; X30,500.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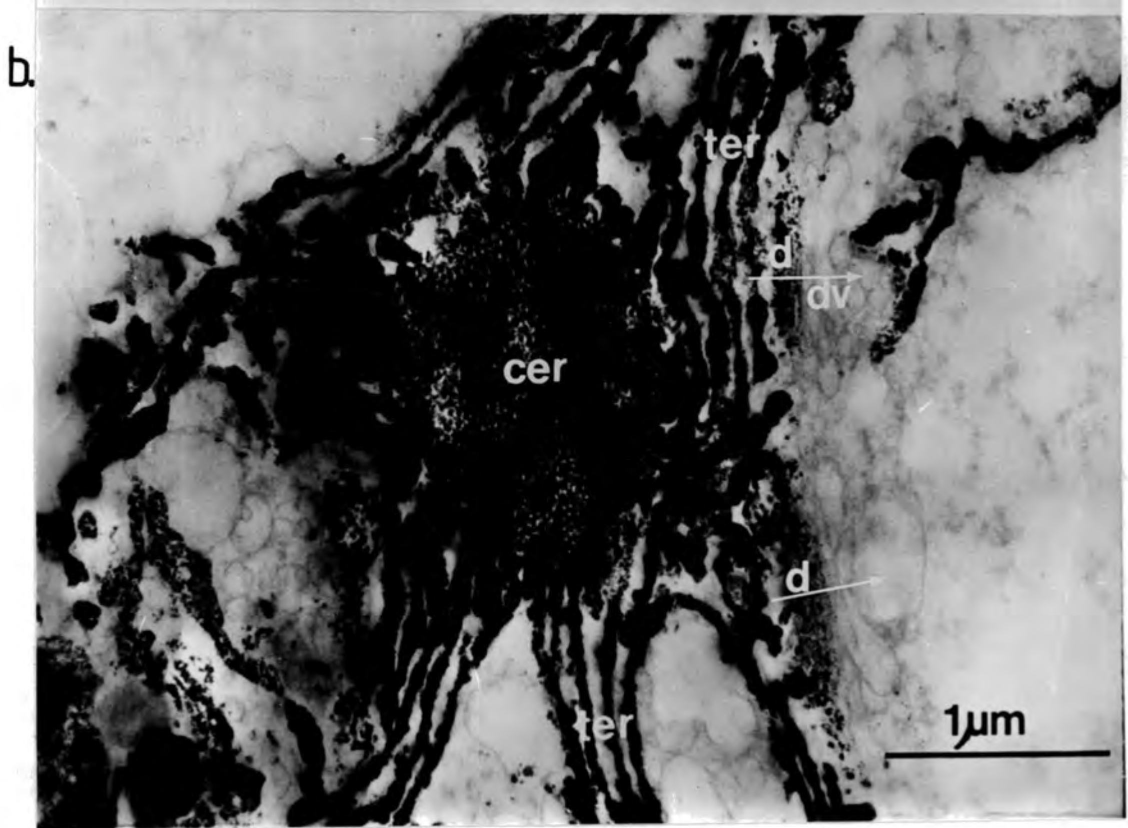
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Plate 3.25. Ultrastructure of the endomembrane system of the adaxial epidermal cells of the ligule of <u>Lolium temulentum</u>

Stereo pair showing direct connection between tubular endoplasmic reticulum and <u>cis</u> aspect of a dictyosome (dart) (ZIO); X62,000, 6°tilt.

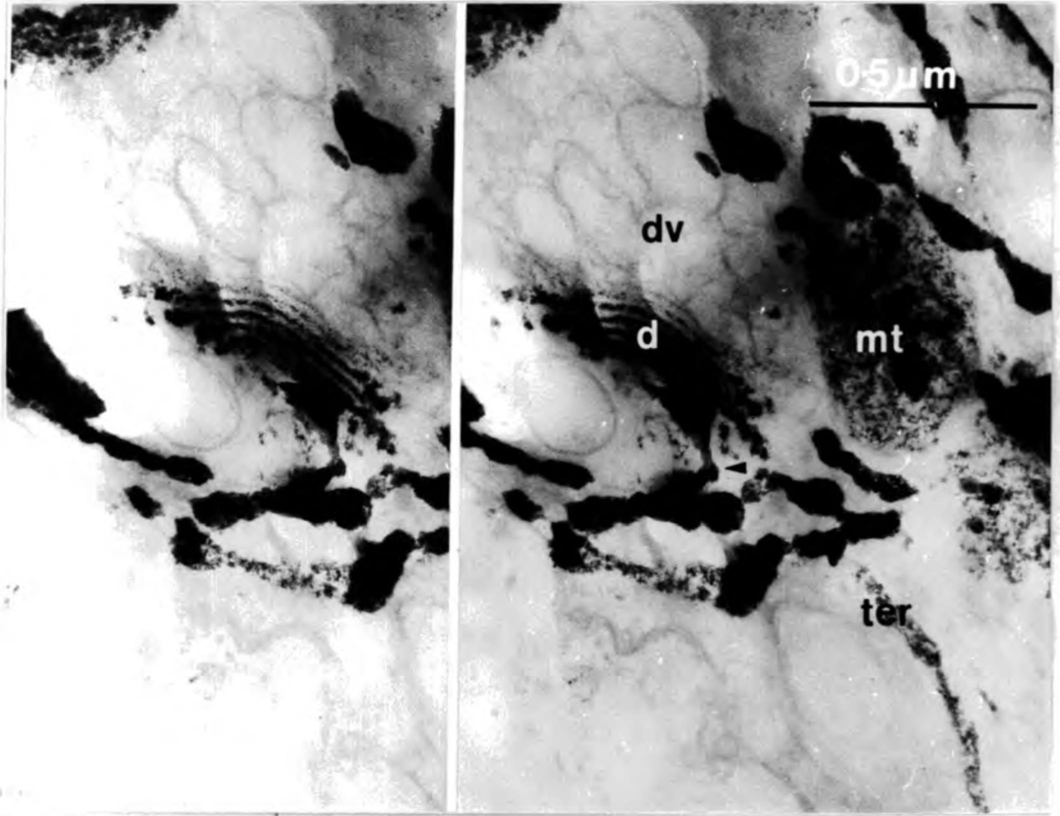
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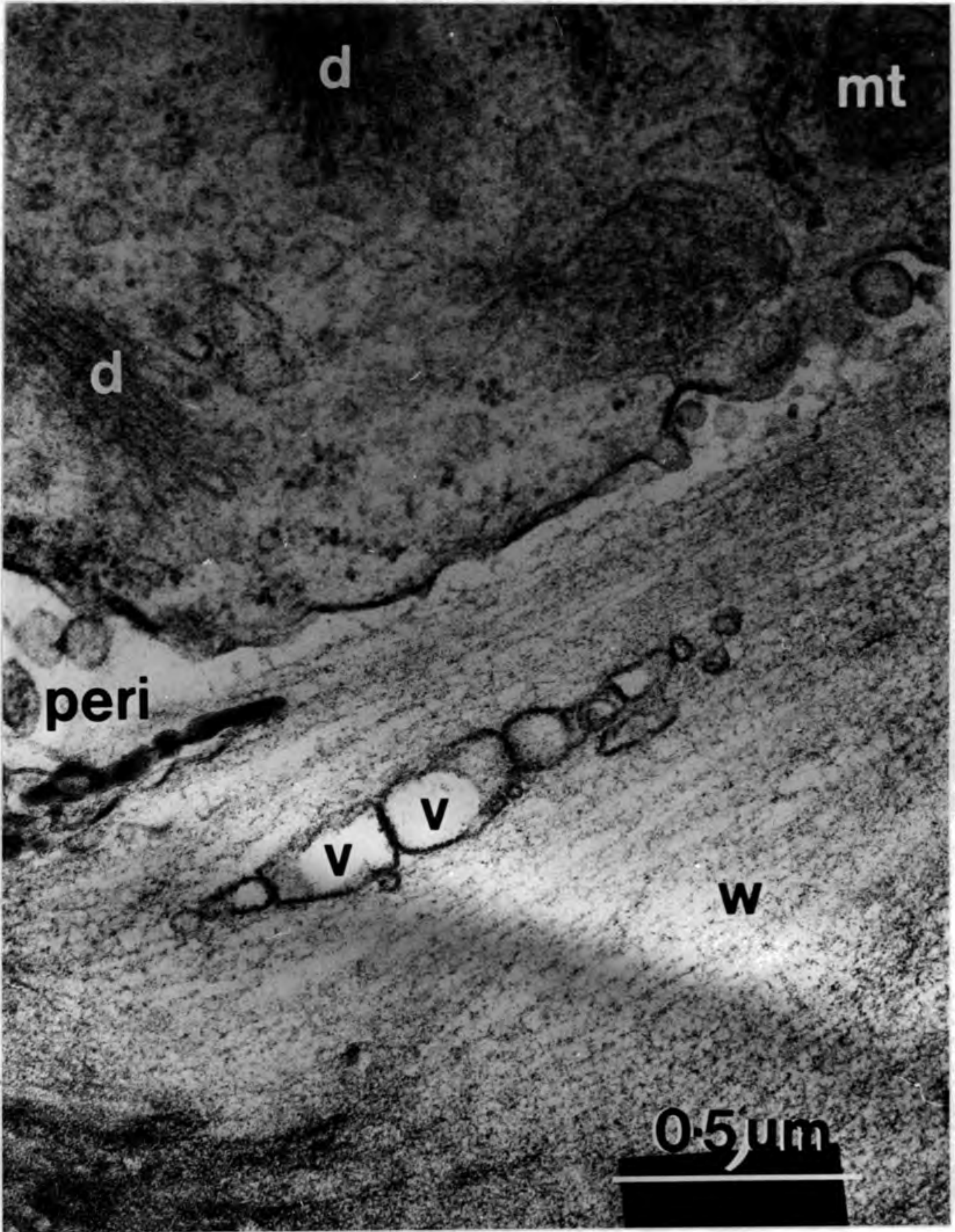
Plate 3.26. Ultrastructure of the endomembrane system of the adaxial epidermal cells of the ligule of *Lolium temulentum*

Portion of cytoplasm and outer tangential wall showing vesicular structures in the wall; X75,000.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DISCUSSION

The components of the endomembrane system of the ligule adaxial epidermal cells are the NE, RER, dictyosomes, smooth and coated vesicles, PL, paramural bodies and tonoplast.

Although no direct connections were seen between the NE and RER, this may be more a reflection of the planes of sectioning rather than their absence. The endomembrane concept would appear to be valid in the case of ER/dictyosome and dictyosome/PL compartments. The former is suggested by the occurrence of direct connections between tER and the cis aspect of the dictyosomes; the latter is suggested by the cytochemical study presented in the previous Section. Direct connections do not prove membrane transfer between the two compartments concerned - and may even have been caused by the preparation technique - but they do allow of the possibility that such may be the case.

In a recent review of the endomembrane concept, Robinson and Kristen (1982) identified three phases of membrane transfer from the ER to the PL:

i. from ER to cis aspect of the dictyosome;
ii. from the cis to the trans aspect of the dictyosome;
iii. from the trans aspect of the dictyosome to the PL.

They concluded that only the last phase was supported by the evidence then available, and they considered the concept itself to be of doubtful validity for all cells. However, they did not include an account of work performed using ZIO techniques even though this has given the best microscopical evidence for direct connections between the ER and the dictyosomes (eg Marty, 1980). By its very nature membrane transfer is difficult to prove (eg Morré et al., 1979) and reliance must be placed upon those methods which can provide the requisite circumstantial evidence, such as heavy-metal impregnation techniques. On this basis, results in the literature and the observations presented above support the possibility of both phases (i) and (iii); phase (ii) can probably only be demonstrated by more indirect, biochemical methods. Even if the endomembrane concept should prove to be untenable for some, or all, cells, the impetus it has given to use of new techniques in the study of ultrastructural problems, and the great deal of fundamental information so obtained makes it one of the most important contributions to cell biology.

The actual number of Golgi apparatus comprising the Golgi complex of the ligule adaxial epidermal cells is a moot point. If it can be established that each dictyosome has only one 'function' then the number of Golgi apparatus will be equal to the number of different functions of the dictyosomes. This information is not available, nor is it likely to be because of the inherent difficulties of obtaining such data. The number of Golgi apparatus is also likely to vary throughout the life of a cell. It may be conjectured that the Golgi apparatus concerned with the synthesis and processing of the 'secretory product' is the biggest of these cells, but more than this we cannot say.

Polarity of the dictyosomes was apparent from the gradients of ZIO, PTA and PATAg staining intensity of the cisternae and by the association of ER with one pole and of vesicles at the other (cf also Shannon et al., 1982). Thus in these cells it may be possible to speak of forming and secretory faces. Such terminology, however, tends to be too restrictive and imply a one-way flow of material through the dictyosomes. If Rothman (1981) is correct then the dictyosomes act in a more complex way than the apparent polarity suggests, with the possibility of recycling components from dictyosomes to other endomembrane components before vesicles eventually bleb off the former.

The vesicular structures apparently within the cell wall (Pl. 3.26) can be interpreted in two ways; either they are within the wall or they are not and only appear to be as a result of the plane of the section. Serial sectioning was not performed and it is thus impossible to decide between the two possibilities. If they are not within the wall, they presumably represent part of a paramural body. If they are within the wall, they may have been incorporated during wall biogenesis, or, they may represent passage of secretory vesicles through the wall from the periplasmic space to the cuticle. This latter suggestion is an intriguing one because it extends the endomembrane concept beyond the periplasmic space and symplastic compartment of the cell. In view of the lack of information concerning these structures it is unwise to speculate further.

Paramural bodies have been classified by Marchant and Robards (1968) into two categories:

i. lomasomes - derived from cytoplasmic membranes;

ii. plasmalemmasomes - derived entirely from PL.

Although the categories themselves seem quite distinct, allocation of any given paramural body to either is often very difficult. This difficulty arises from several sources. Firstly it seems likely that successful fusion between a component of the endomembrane system, such as a smooth vesicle, and the PL is dependent upon the former's membrane becoming differentiated to be PL-like. Thus the erection of two categories becomes almost a nonsense. Even in those cases where we assume no such 'capacitation', the paramural body itself may consist of cytoplasmic membrane-bound vesicles within a limiting membrane of PL. In this case the two categories merge together, or else a third one must be proposed. Because of the static nature of TEM work, in many instances we cannot even be sure where the vesicles came from or what their membranes were like. It seems more sensible to refer to such structures simply as paramural bodies. Whether this term should also include apparently isolated vesicles within the periplasmic space may be considered debatable.

The only component of the endomembrane system of these cells for which no 'function' is apparent is the coated vesicles. It has been suggested by several workers that they may be involved in wall or PL biogenesis, and thus be exocytotic (eg Bonnett and Newcomb, 1966; Franke and Herth, 1974; Newcomb, 1980a). In cells undergoing high rates of secretion (as here?), the concomitant increase in PL surface area as a result of vesicle fusion with it may be countered by coated vesicles of endocytotic origin incorporating excess PL back into the cytoplasm (eg Robinson and Kristen, 1982). Unfortunately in the situation studied here - as in all the cases cited above - these structures 'do not come armed with small arrows that tell us unequivocally in what direction they are going' (O'Brien, 1972). In view of the absence of facts concerning these vesicles, all that can be done is to note their presence and resist the temptation to speculate on their rôle in these cells.

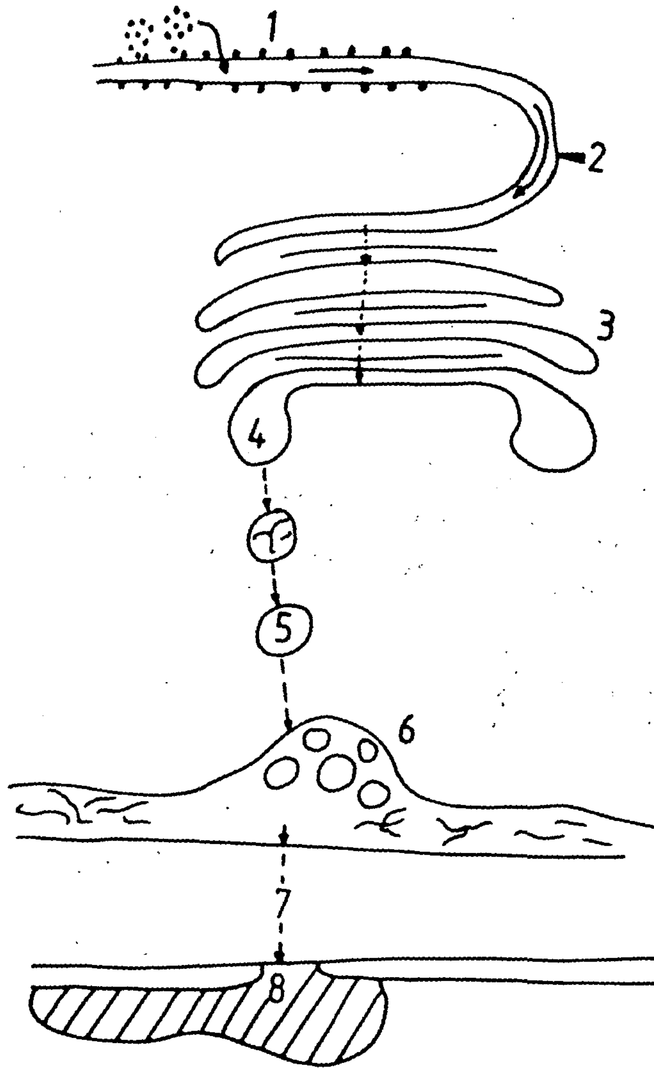
Based on the results of this and the preceding Section, it is possible to propose a pathway for synthesis, elaboration and secretion of the extracellular product of the adaxial epidermal cells of the ligule (Fig. 3.2).

The protein moiety of the glycoprotein is synthesized by ribosomes or polysomes attached to the ER (eg Chrispeels, 1980) and is secreted into the enchylemma (1) (eg Palade, 1975). The protein is then transferred to the cis aspect of the dictyosome via the direct continuity between the two (2). The main glycosylation events occur in the dictyosome (eg Morré et al., 1979) during its passage from the cis to the trans aspect (3), although it may have begun in the RER (eg Chrispeels, 1983). The possibility of refining the material at this stage exists within the stack (eg Rothman, 1981) before it is finally packaged into vesicles which bleb off the trans aspect of the dictyosome (4). These smooth vesicles are transported by an unknown mechanism to the PL (5), during which membrane capacitation takes place. At the PL they fuse and give rise to paramural bodies by reverse pinocytosis (6); the secretory product either remains within the vesicles or is released into the periplasmic space. The product is then transported across the cell wall (7) as discrete vesicles or loose, and accumulates beneath the cuticle until release from the ligule via gaps (8) formed by rupture of the cuticle.

Parts or all of the above hypothesis may be wrong; its value is that it suggests lines of study necessary to establish its validity and that of the endomembrane concept in these cells.

Figure 3.2. Proposed route of synthesis, elaboration and secretion of the extracellular product of the adaxial epidermal cells of the ligule of *Lolium temulentum*.

For explanation see text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SECTION d. INITIATION AND EARLY STAGES OF DEVELOPMENT

INTRODUCTION

A great many workers have dealt with this aspect of ligule biology since it was suggested by Dupont (1819) that the ligule, 'paroît evidemment formée par un prolongement de l'épiderme de la face interne de la gaine'. Almost all agree that it is derived solely from the adaxial epidermis of the sheath; Table 3.4 lists some of the species for which this claim has been made.

Regarding the timing of its initiation, most workers who have studied this agree that it occurs very early on in leaf development - when the leaf was 3 mm long (Trécul, 1878 - Trisetum rigidum) and c. 1 cm long (Soper and Mitchell, 1956 - Lolium perenne). If we accept the view that ligule initiation is the event which allows us to distinguish blade and sheath in the leaf primordium (eg Kaufman, 1959) then it is clear that all three leaf organs become 'initiated' together.

Although these two aspects of ligule initiation appear to be well-studied, very little is known concerning its structure at this stage and nothing regarding its ultrastructure. In order to complement the information presented in the previous three Sections on the ligule of the mature leaf ('emerged ligule'), a LM and TEM study of early stages of its development was undertaken.

Table 3.4. Grass species for which an adaxial epidermal origin has been claimed for the ligule.

Species	Reference
Dactylis glomerata L.	Bugnon, 1921
Zea mays L.	Neumann, 1938; Sharman, 1941; Bonnett, 1953
Agropyron repens (L.) Beauv.	Sharman, 1945
Secale cereale L.	de Ropp. 1946
Sorghum vulgare L.	Artschwager, 1948
Avena sativa L.	Bonnett, 1961
Hordeum vulgare L.	Silvy, 1982a

OBSERVATIONS AND RESULTS

Initiation of the Ligule

Three stages in the initiation and early development of the ligule were recognized:

I. ligule appears as a slight swelling on adaxial leaf surface (Pl. 3.27a);

II. ligule increased in cell size and number to appear as a more pronounced protuberance (Pl. 3.27b,c);

III. typical 'ligule shape' attained (Pl. 3.27d).

During all three stages, the leaf bearing the developing ligule was in a meristematic state and mitotic figures were frequently seen (results not shown).

Apparently associated with the initiation of the ligule was the appearance of a file of small cells immediately above it in the leaf adaxial epidermis (darts in Pl. 3.27f) which are believed to mark the site of the future collar region.

It has not yet been possible to accurately determine the timing of ligule initiation but it was clearly present before either blade or sheath had finished their elongation growth (Table 3.5) and initially located near the leaf base (Pl. 3.27e). The developing leaf vascular bundle (Pl. 3.27f) was further evidence that ligule initiation occurred early in leaf development.

Structure and Ultrastructure

Results for all three stages are summarized in Table 3.6 and illustrated for Stage III below.

General ligule ultrastructure was that of a meristematic tissue (Pl. 3.28a,b); a large centrally-placed nucleus - apparently dividing in Pl. 3.28c - dominated the cytoplasm which was dense and vacuolate (Pl. 3.28b). At no stage examined was it possible to distinguish distinct tissues comparable to those seen in the emerged ligule.

Both internal (Pl. 3.29c,d) and external (Pl. 3.28d,e) ligule cell walls were thinner than those of the emerged ligule and of the leaf bearing the ligule (Pl. 3.31b). The external cell walls were covered by a cuticle (Pl. 3.28d) which appeared to be unbroken. Where external

Table 3.5. Growth of 4th leaf, blade and sheath of summer-grown plants of *Lolium temulentum*

d. from sowing		Length (cm) leaf	blade	sheath
a)	24	9.16 ± 0.80^{c}	9.01 ± 0.78^{c}	0.14 ± 0.02^{c}
b)	30	26.12 ± 0.48^{d}	19.69 ± 0.26^{f}	7.27 ± 0.21^{f}
% increase $\left[\frac{b-a}{a} \times 100\right]$		185	119	5093

c = mean $\pm$ se of 9 measurements (destructive)

d = mean $\pm$ se of 31 measurements (non-destructive)

f = mean $\pm$ se of 26 measurements (non-destructive)

Table 3.6. Comparison of the structure and ultrastructure of the ligule of Lolium temulentum at early stages of development

Structure	Stage I	Stage II	Stage III
er	+; rough	++; rough	as Stage II
n		(uninucleate; division profiles)	
d	+; few cisternae	+(+); more cisternae	as Stage II
w		('thin')	
vacuome	+; small vesicles	++; vacuoles and small vesicles	as Stage II
cut	+; indistinct	as Stage I	as Stage I
pt	+; proplastids(?)	++; amyloplasts and proplastids	as Stage II
mt	+; nucleoids prominent	++; more cristate	as Stage II
ribosomes	+++; few polysomes	as Stage I	as Stage I
pd		abundant between ligule/ligule and ligule/leaf cells	
cell number	———	increasing	———→
cell size	'small'	'small'	'small' at apex; more vacuolate and larger towards base

+,++ = relative sizes of populations of organelles concerned

cell walls of ligule and leaf were in direct appression their cuticles were apparently separated by a thin electron-dense line of material (Pl. 3.31d). At the blade side of the ligule base, where ligule and leaf external cell walls were continuous, branches were seen within the wall (dart in Pl. 3.31b), apparently bounded by cuticle (Pl. 3.31c).

Microtubules were frequently observed close to ligule cell walls (arrow-heads in Pl. 3.28c; 3.29a; darts in Pl. 3.31d). They were only seen in circular profile in the LS's examined here and thus appeared to be preferentially arranged at right angles to the vertical axis of the ligule. Plasmodesmata were common between adjacent ligule (Pl. 3.29c) and leaf/ligule cells (not shown), apparently at an early stage of development in some cases (darts in Pl. 3.29d).

The cytoplasm was densely-populated with free ribosomes and polysomes but organelle numbers were low. Both mitochondria and plastids showed profiles characteristic of cells at early stages of development. The former were poorly cristate and frequently contained central regions of low electron density bearing fibrillar material which was assumed to be DNA (arrow-heads in Pl. 3.30a). Plastids were present in at least two forms - amyloplasts (Pl. 3.30c) and proplastids (Pl. 3.30b,c); the latter apparently occurring in a variety of configurations (Pl. 3.29a). Early stages of thylakoid development were evident in proplastids with centrally-placed lamellae and invaginations of the inner envelope membrane (Pl. 3.30d).

Dictyosomes appeared to exhibit the 'general' state of activity (Mollenhauer and Morré, 1966) (Pl. 3.29a,b); dictyosome-derived vesicles (Pl.3.28e) were few. Strands of RER were also few and scattered throughout the cytoplasm (Pl. 3.29b). The vacuome consisted of a few large vacuoles in basal cells and smaller but more numerous vacuoles in apical cells (Pl. 3.28a). The reticulate structures seen in some cells (darts in Pl. 3.31a) may represent early stages in vacuole formation (eg Marty, 1978).

Plate 3.27. LMgraphs of stages in the development of the ligule of *Lolium temulentum*

a. Stage I; X400;

b. Stage II; X400;

c. Stage II; X400;

d. Stage III; X400;

e. Low power view of ligule in d. above, showing the relationship between site of ligule and base of leaf; X160;

f. High power view of ligule in d. above; darts indicate cells of future collar region; X600.

Dashed line indicates blade/sheath junction.

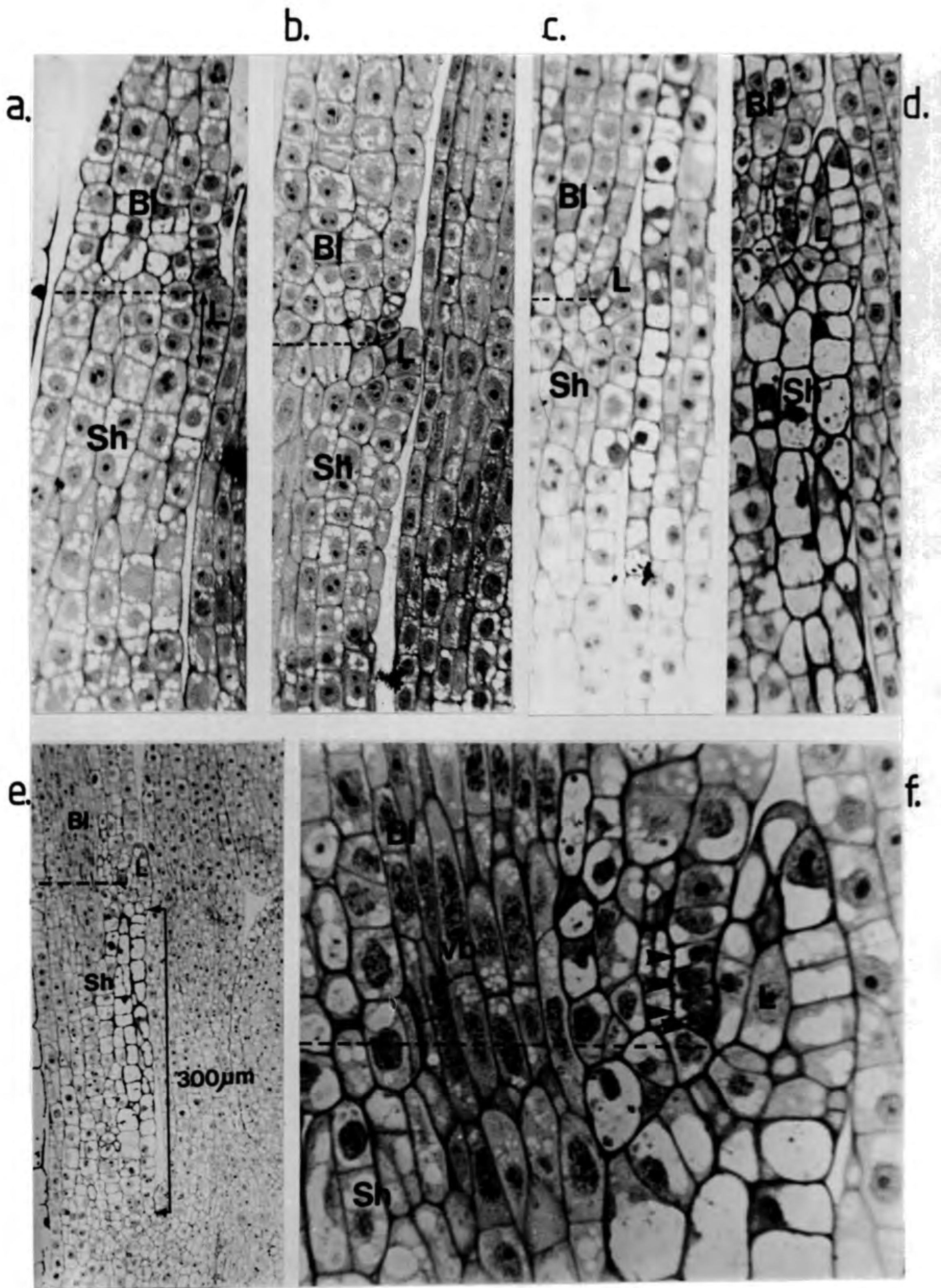

a.
b.
c.
d.
e.
f.
Bl
L
Sh
Vb
300µm

Plate 3.28. Ultrastructure of the developing ligule of *Lolium temulentum*

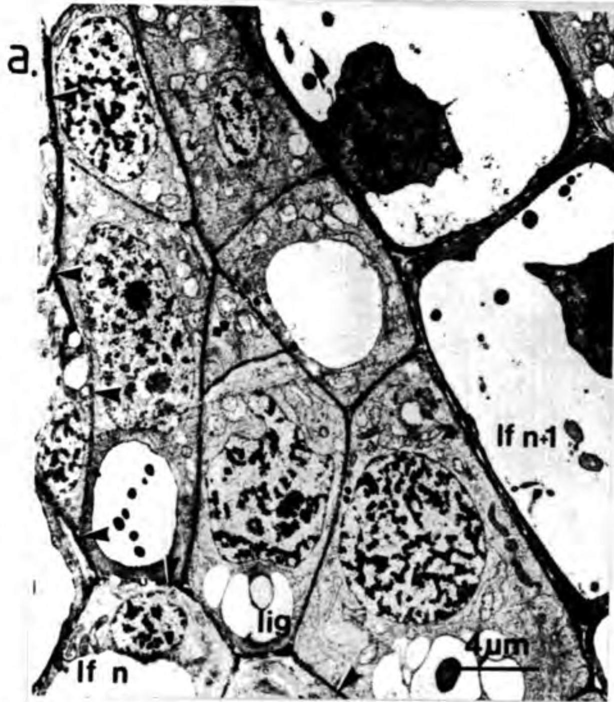
a. Low power view of a newly-initiated ligule; darts indicate boundary of ligule with the leaf bearing it (leaf n); next oldest leaf indicated by 'n+1'; X2,750;

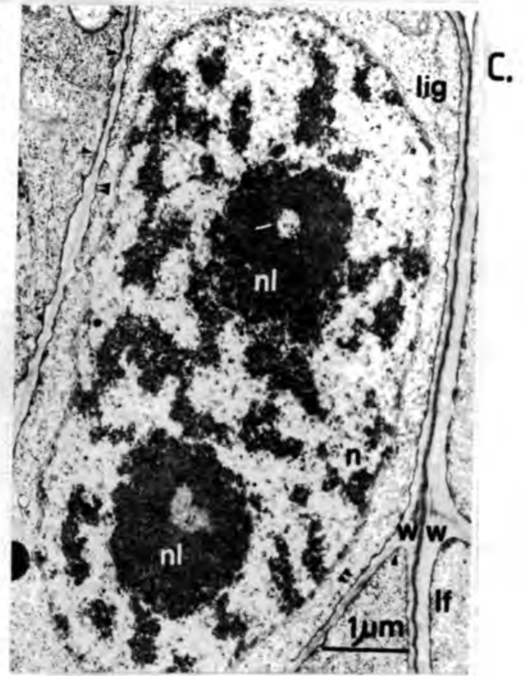
b. Parts of several meristematic cells; X5,625;

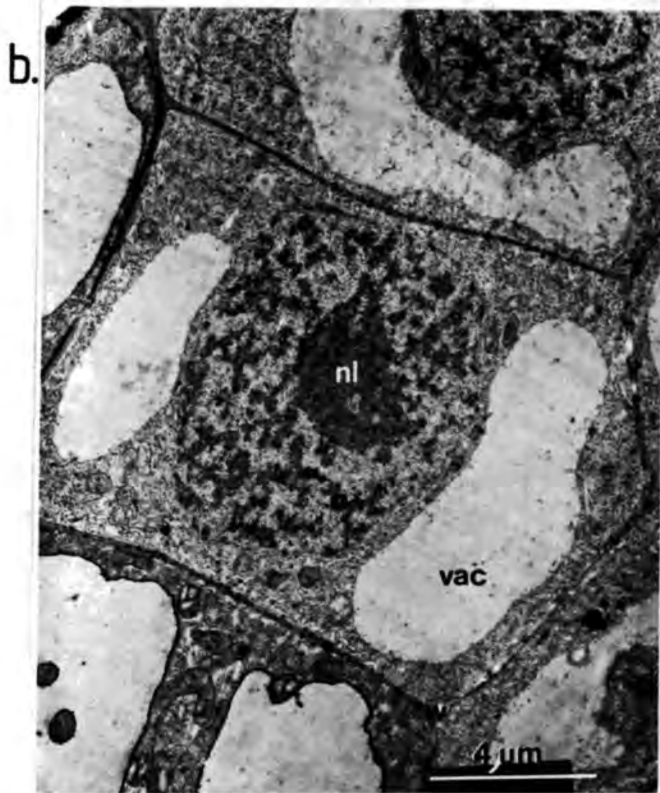
c. An apparently dividing nucleus in a cell near the apex of the ligule; arrow-heads indicate sites of microtubules; X11,500;

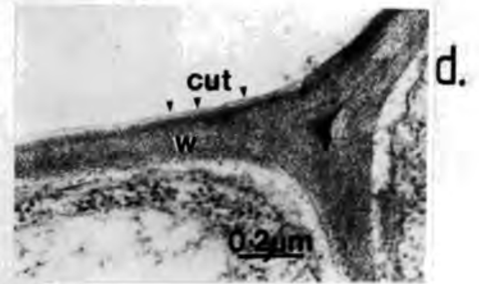
d. Outer tangential wall and cuticle; X45,000;

e. Dictyosome and associated vesicles in the cytoplasm and outer tangential wall; X85,000.


a.
lig
lf n+1
lf n
4µm


c.
lig
nl
n
nl
w w
lf
1µm


b.
nl
vac
4 µm


d.
cut
w
0.2µm

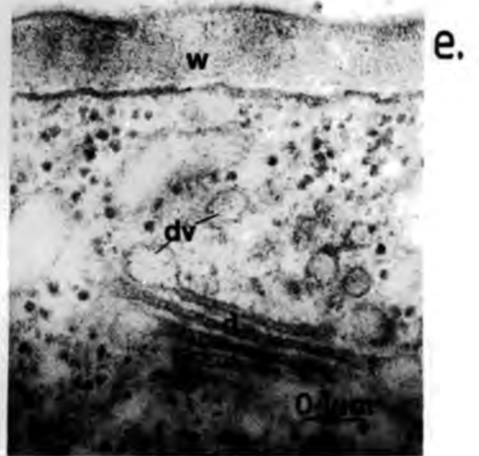

e.
w
dv

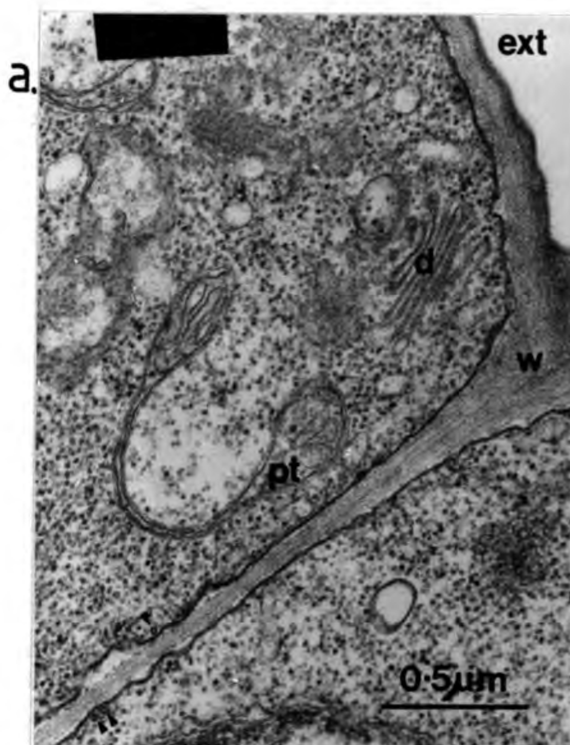
Plate 3.29. Ultrastructure of the developing ligule of _Lolium temulentum_

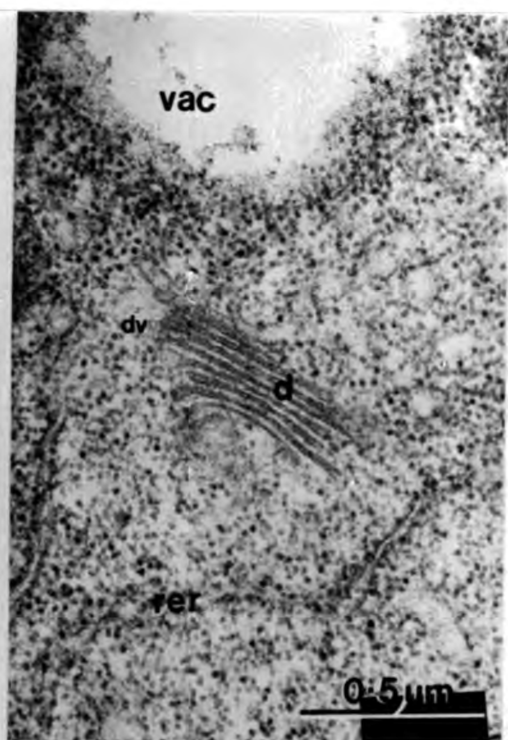
a. Parts of the cytoplasm of two adjacent cells; arrow-heads indicate sites of microtubules; X39,000;

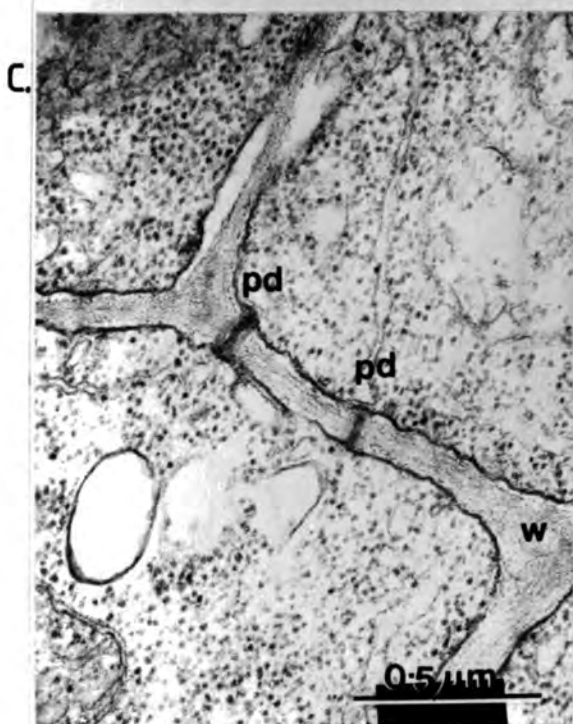
b. Part of the cytoplasm showing components of the endomembrane system; X49,000;

c. Plasmodesmata in the wall between two adjacent cells; X49,000;

d. Parts of the cytoplasm of two adjacent cells separated by a thin wall; darts indicate sites of plasmodesmata at early stages of development; X39,000.


a.
ext
d
w
pt
0·5µm


b.
vac
dv
d
rer
0·5µm


c.
pd
pd
w
0·5µm

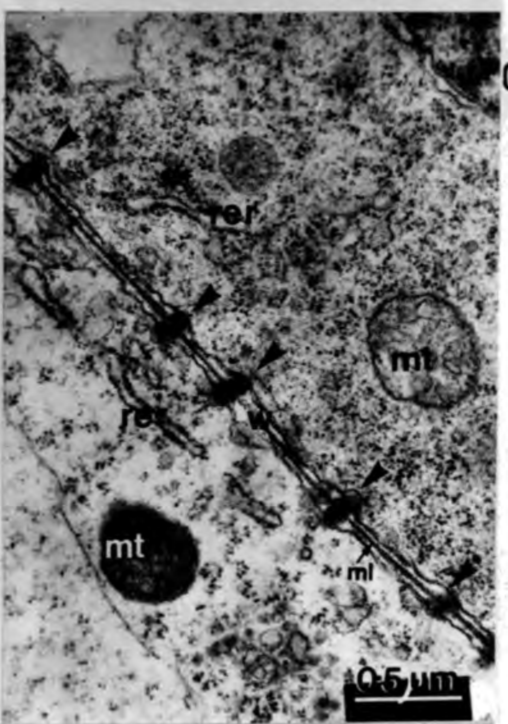

d.
rer
mt
mt
ml
0·5µm

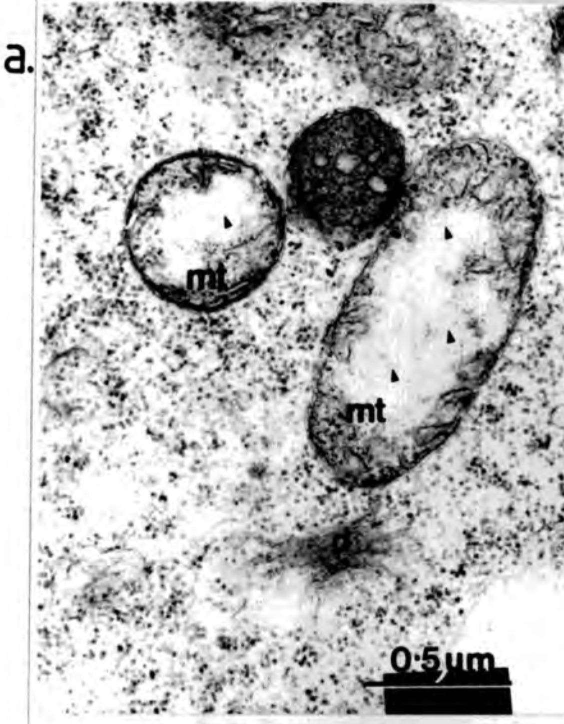
Plate 3.30. Ultrastructure of the developing ligule of Lolium temulentum

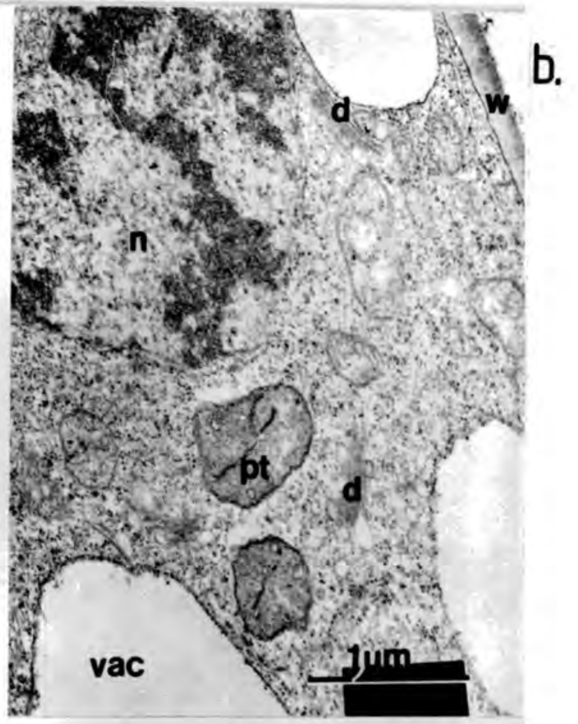
a. Mitochondrial profiles showing electron-transparent regions (nucleoids?); arrow-heads indicate fibrillar material; X38,000;

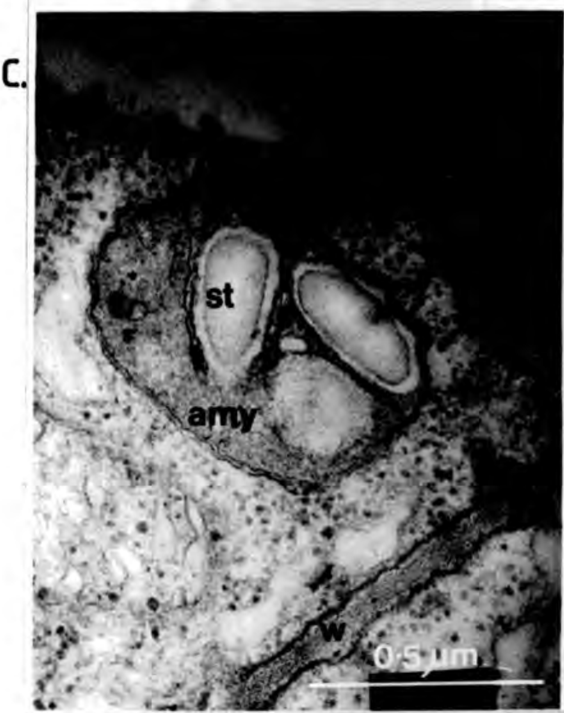
b. Part of the cytoplasm showing a nucleus and several proplastid profiles; X22,000;

c. An amyloplast; X63,000;

d. A proplastid showing early stages in the development of an internal membrane system; X63,000.


a.
mt
mt
0·5 µm


b.
d
w
n
pt
d
vac
1µm


c.
st
amy
w
0·5 µm

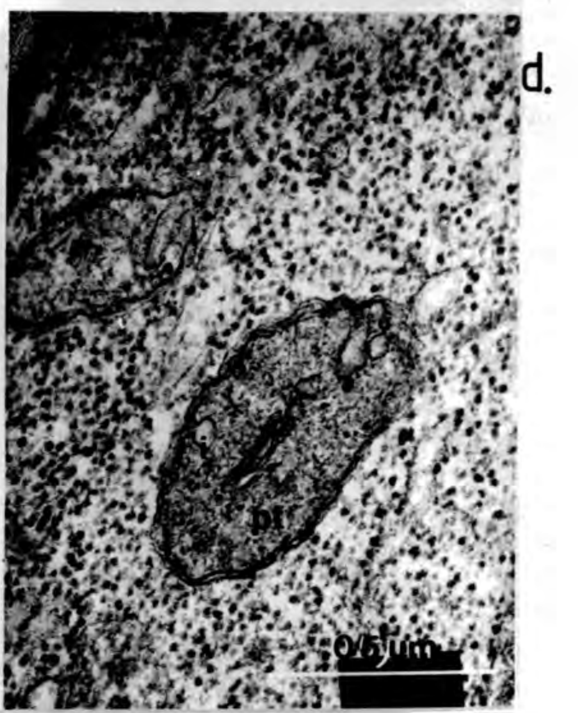

d.
0·5 µm

Plate 3.31. Ultrastructure of the developing ligule of <u>Lolium temulentum</u>

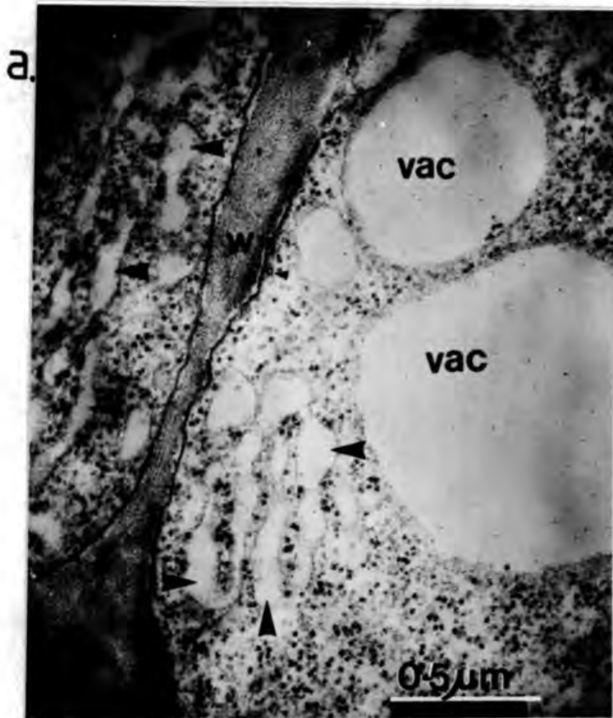
a. Parts of the cytoplasm of two adjacent cells; darts indicate tubular structures resembling distended profiles of rough endoplasmic reticulum; X40,000;

b. The junction between the ligule and the leaf; dart indicates branching of the wall; X12,000;

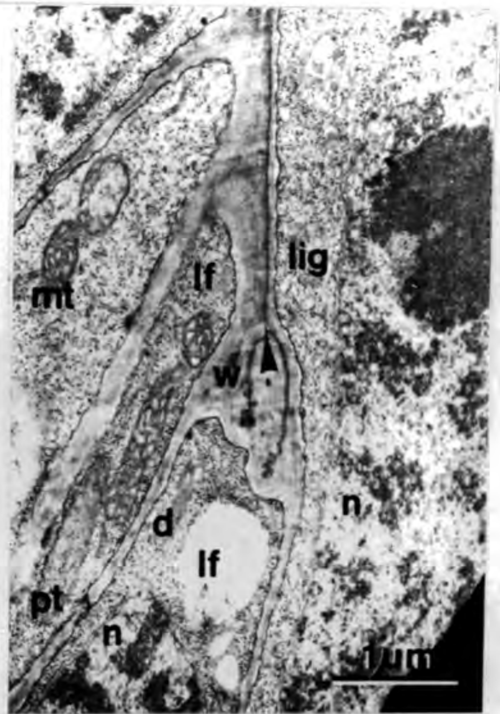
c. Higher power view of branching region in b. above; X87,500;

d. Walls of close-appressed ligule and leaf cells; the former is much thinner ('a') than the latter ('b'); the bar indicates line of electron-dense material between the two cuticles; darts indicate circular profiles of microtubules; X150,000.

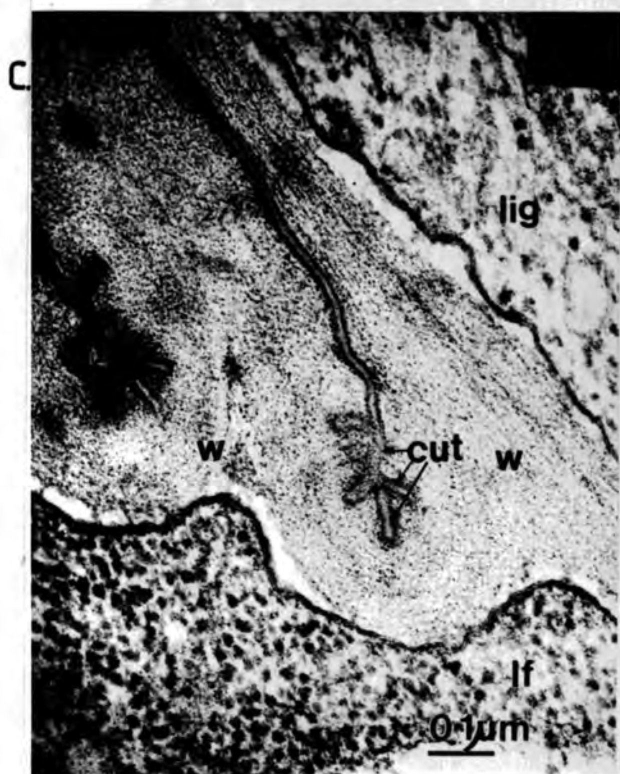
a.



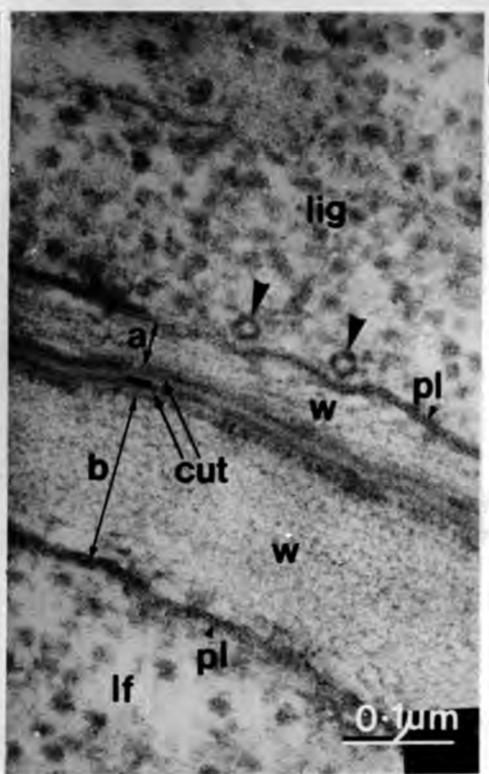
b.



c.



d.



DISCUSSION

The LMgraphs presented in Pl. 3.27a-d are consistent with the view that the ligule in this species developed solely from the adaxial epidermis. However, because of their static nature, they can at best only reflect stages along the pathway of ligule initiation and development; the process itself cannot be seen. It is possible that tissues other than the adaxial epidermis were involved in the formation of the ligule but were not detected by the techniques used to study the process.

As expected, the structure of the newly-initiated ligule was that of a typical meristematic tissue (eg Clowes and Juniper, 1964). The walls were thin, organelles were few and at early stages of development, RER was sparse, the nuclei were large and prominent, and the vacuome was usually small. No division profiles of any organelles, except nuclei, were seen.

The presence of starch grains in the amyloplasts and of vesicles associated with the dictyosomes indicated some sort of metabolic activity in these cells. The latter suggested polysaccharide synthesis was taking place, presumably in part related to wall formation. It is possible that a source of carbohydrate for this was provided by the amyloplasts, which may also have been the source of respiratory substrate for the mitochondria. In view of the poorly-cristate nature of the mitochondria it is possible that the cells may be involved in fermentative respiration - at least in part (eg Öpik, 1974) - although they may have acted as energy-sources for the presumptive polysaccharide synthesis.

The close association of microtubules with the cell walls is suggestive of a rôle in wall biogenesis (Ledbetter and Porter, 1963; Pickett-Heaps, 1974), although they may also have been involved in cell division (eg Pickett-Heaps, 1974). Their apparent absence from cells of emerged ligules is consistent with both these possibilities.

Although the tissue as a whole appeared meristematic, there was an apparent increase in the degree of vacuolation and 'maturity' of plastids and mitochondria from apex to base, and nuclear division profiles were only seen at the apex. These observations coupled with the decrease in epidermal cell size from base to apex of emerged ligules plus the apparent increase in plastid maturity in the abaxial epidermis from apex to

base there, suggest that the organ may exhibit tip-growth. Such a suggestion clearly needs to be treated cautiously in view of the basal growth of the other two leaf organs but it deserves to be followed up.

The structure and ultrastructure of the newly-initiated ligule is very different from that of the emerged ligule and supports the contention that some sort of developmental pathway exists within this organ. The course of this is not known; however, from study of nearly-emerged ligules it appears that the structural complexity that characterizes the emerged ligule is attained before exsertion. The course of this development and its timings relative to those of the blade and sheath are relevant to a consideration of function of the ligule and attainment of 'maturity' of the leaf.

The small group of adaxial epidermal cells immediately above the insertion of the ligule appeared to be formed at about the same time as ligule initiation. They are believed to mark the site of the future collar region of the blade and are probably the cells involved in the blade bending response. Upon leaf emergence these collar cells enlarge proportionately more than the corresponding cells on the abaxial epidermis (eg Ledent, 1976) resulting in bending of the blade.

The significance of the observation seems to be in relation to results obtained by workers investigating the genetics of ligule inheritance. In rice, for example, presence or absence of ligule, auricles and junctura (blade angle with sheath) are inherited together (eg Dhulappanavar, 1981). If the above interpretation is correct then it is clear why liguleless plants should be erect-leaved as well. In this connection, the statement by Emerson (1912) is important, 'Whatever the effect of the liguleless condition upon the well-being of the plant, it apparently has nothing to do with the peculiar habit of growth so characteristic of the liguleless type. It is rather the absence of the auricle that makes the leaves stand so erect.' This worker used the word 'auricle' to mean junctura. The only known exception to the association of angled leaves /liguleless appears to be the work of Lutkov (1937) who recorded the formation of a liguleless mutant of barley by X-radiation which still had a pronounced blade angle. Whether the angle obtained was simply the result of gravity acting upon the blade or a true bending response is not known.

The branching of walls and cuticle on the blade side of the ligule insertion is presumably related to the blade bending response. It may be suggested that these anatomical features actually facilitate it by acting as a zone of flexure (cf also palm leaf inception - Dengler et al., 1982). In this connection it is also of interest to note that a cleft occurs in this location on Spartina townsendii leaves and has been considered to act in a similar way (Sutherland and Eastwood, 1916).

The results from this study add little to our understanding of the siting of ligule initiation. It has been suggested by Sharman (pers. comm.) that ligules become initiated at the site of fusion of blade minor veins (Type 2 bundles) where they enter the future sheath. As shown in Chapter 2, Section c, such vein fusion does not occur in leaves of L. temulentum and is therefore unlikely to explain the siting of the ligule in this species. The occasionally-observed blade vein(s) stopping at the collar region is also unlikely to account for the siting of the ligule because of its inconstancy.

However, if we accept that the ligule is derived solely from the leaf adaxial epidermis then it is apparent that the few genes involved in ligule inheritance are not enough to code for all the differences in ultrastructure between ligule and epidermal cells. It is thus likely that all leaf adaxial epidermal cells can develop ligules but that this capability is not expressed except in that region where the ligule becomes initiated. The few genes involved may be postulated to control receipt of a 'ligule initiation signal'. It is further possible that 'spill-over' of nutrients, metabolites or growth regulators to neighbouring cells may explain the appearance of the presumptive collar cells at the same time as ligule initiation.

Although this may in some ways avoid the issue of ligule initiation, it has put it into the same category as leaf initiation. It may thus be proposed that study of the one phenomenon will yield information of relevance to the other. This view of ligule initiation also argues for a marked degree of totipotency of leaf adaxial epidermal cells.

LIGULE: DISCUSSION

The 'Exclusion Hypothesis' of ligule function mentioned above was attributed to Schlechtendahl by Hackel (1887) and Goebel (1905) and has been applied to both membranous and veined ligules. Despite no anatomical evidence apparently having been presented in its favour, it has been reiterated by such workers as Kerner and Oliver (1894), Prat (1935) and Saha (1952). 'Exclusion' is not the only function that has been suggested for the ligule; Goebel (1905) believed its rôle was to protect the 'bud' and Dobrynin (1959) considered it to have function(s) more fundamental than simply excluding water. Recently more caution has been expressed culminating in the statement by Clifford and Watson (1977) that the ligule 'is a morphological enigma of unknown function'. The present study of the grass ligule has removed a lot of the 'enigma' regarding its morphology and anatomy but the uncertainty of its 'function' remains.

In order to assess possible function(s) of membranous ligules based on the species examined in this study, the main hypotheses of ligule function will be discussed below.

Exclusion Hypothesis

Since this view of ligule function relies heavily upon close appression of the ligule against the enclosed leaf or culm, the ways in which this might be achieved will be considered below.

a. Position of the ligule at the top of the sheath: the ligule is the topmost part of the sheath which itself is more or less circular in TS and may even overlap at its edges. The ligule thus appears as a sleeve-like structure wrapped around the enclosed 'shoot'.

b. Position of the ligule at the base of the blade (Fig. 3.3c): gravity (g) acts upon the blade and pulls it downwards putting tension upon the ligule insertion at the base of the blade (a). This helps to keep the ligule closely investing the enclosed leaf or culm.

c. Curved insertion of the ligule: despite the ligule being initiated absolutely transversely (Sharman, pers. comm.), it ends up with a curved insertion (eg also Arber, 1923). This curvature - downwards to each sheath margin from its highest point opposite the mid-vein - is expected to put tension upon the ligule which is lessened by its adopting a curved transverse profile. The curved

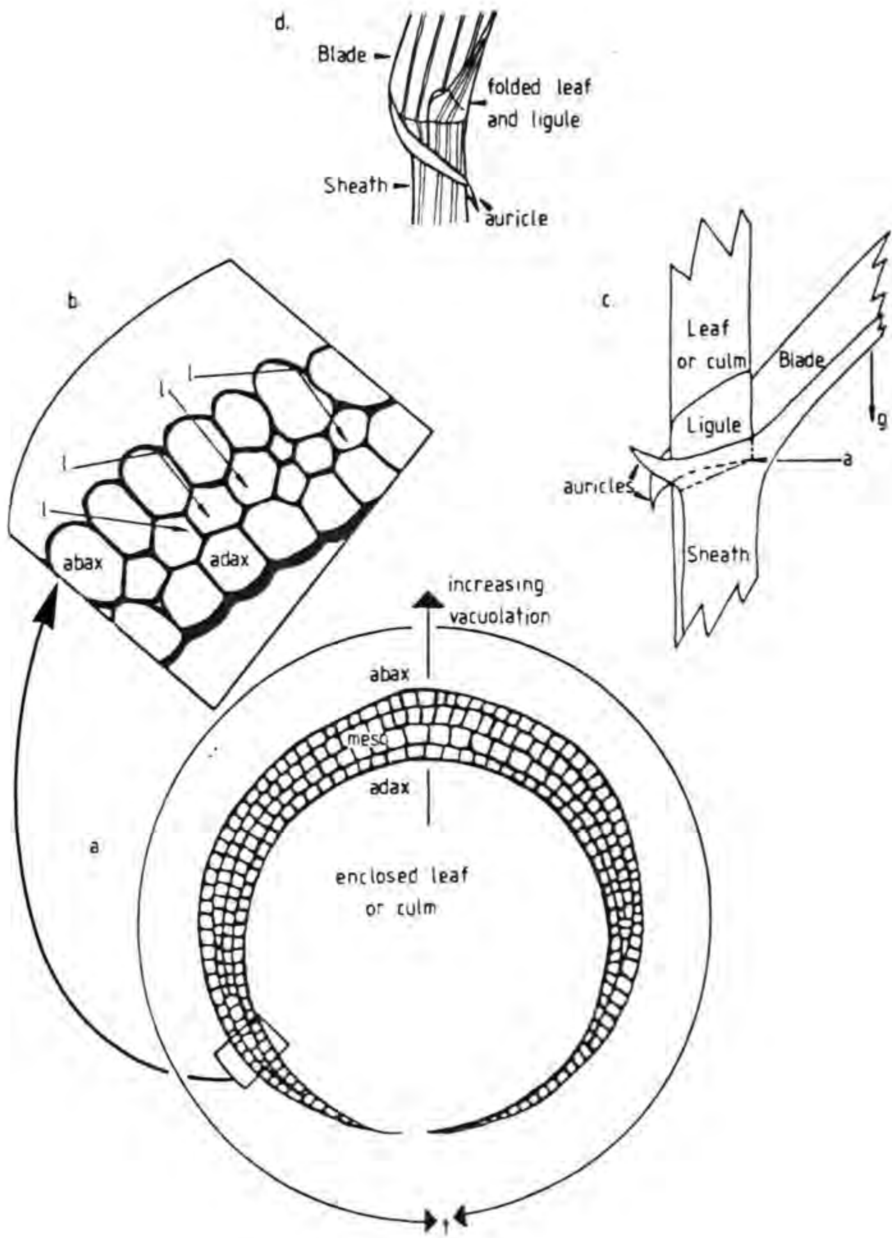
Figure 3.3. Aspects of the structure/function relationships in the membranous ligules of *Lolium temulentum*, *L. perenne*, *L. multiflorum*, *L. x hybridum* and *Festuca pratensis*

a. Schematic diagram of a TS near the mid-ligule region;

b. Part of a TS of *L. perenne* ligule; X560;

c. Diagrammatic representation of the relationship between blade, sheath, ligule and enclosed leaf or culm;

d. Diagrammatic representation of the curled ligule of the flag leaf; dextral-spiralled


d.
Blade
folded leaf
and ligule
Sheath
auricle
b.
l
l
l
l
abax
adax
c.
Leaf
or culm
Blade
Ligule
g
a
auricles
Sheath
increasing
vacuolation
abax
meso
adax
a
enclosed leaf
or culm

insertion is best seen in flag leaves (Fig. 3.3d) where the ligule may adopt a spiral transverse profile (eg also Arber, 1934 for Hordeum distichon L.). The spirals formed may be either dextral or sinistral and are believed to be so marked here because the ligules of these leaves develop in the absence of any enclosed leaf.

d. Osmotic relations within the ligule (Fig. 3.3a): the increase in degree of vacuolation from the adaxial to the abaxial epidermis is seen as aiding curvature of the ligule by the resultant gradient of turgor (t). The 'mutual tensions of tissues' produced is akin to that observable in the dandelion peduncle (eg Bower, 1939) and helps to explain the tissue deformation referred to in Chapter 3, Section a.

e. Epidermal wall structure (Fig. 3.3b): the outer tangential walls of the abaxial epidermis are convex and thus considered to aid curvature of the ligule; the corresponding walls of the adaxial epidermis are relatively straight which would presumably aid their close appression to the enclosed leaf or culm.

f. Presence of auricles (Fig. 3.3c): these appendages were present in all the species examined and may be considered to aid appression of both sheath and ligule to the enclosed leaf or culm by folding around the 'shoot' (eg also Goebel, 1905).

The only other aspect of structure which appears to be relevant to this hypothesis is the presence of cuticle over both epidermes. Abaxially it appeared to be thickest, unbroken and continuous, and of increased thickness at the base of the ligule. The adaxial blade cuticle also appeared to show pronounced thickening at the insertion region. The waterproofing properties of cuticle are well known (eg Martin and Juniper, 1970; Hadley, 1981). It would thus appear that the ligule as a whole is largely unwettable and, because of its vertical orientation, any water falling on to it would drain off to its base. The adaxial cuticle closely appressed against the abaxial cuticle of the enclosed shoot would presumably prevent entry of liquid water into the interior of the plant. In a similar way the narrow orifice resulting from the spiral flag leaf ligule would presumably hinder entry of water into the plant.

As Goebel (1905) pointed out, most of the water falling onto the blade is unlikely to reach the ligule because it will run off at the downwards-pointing tip or over the edges of this organ. Some water, however, will reach the ligule - whether from the blade or down the shoot - and it is this which is important, since the ligule can only exclude that water which reaches it. This water will drain off the ligule abaxially, to its base and be carried to the auricles by virtue of the curvature of the ligule insertion: these latter structures may act as 'drip points'. Thickening of the cuticle in the insertion region may be seen as a development in response to the likelihood that water reaching the ligule will collect here - if only transiently.

Exclusion of dust and harmful spores will presumably also be achieved by the close appression of ligule and shoot. In the case of ligules composed almost solely of hairs, such as Molinia caerules (L.) Moench and Sieglingia decumbens (L.) Bernh. (Hubbard, 1976), it is difficult to see how 'exclusion' could act unless the hairs were very close together and waterproofed in some way. However, since the structure of these ligules is so little understood, it is best to omit them from the present discussion.

Bud Protection Hypothesis

This hypothesis is attributed to Goebel (1905) but is implicit in the work of all those who regard the grass ligule as having a stipular nature (even though Goebel did not consider it to be stipular). Goebel rejected the Exclusion Hypothesis on the basis that no water would reach the ligule and hence it could not exclude water. As discussed above some water will reach the ligule and this hypothesis must thus be considered additional to the Exclusion Hypothesis not a replacement for it.

By 'bud', Goebel meant the enclosed developing leaf or floral apex and envisaged the ligule as forming a conical cap above the bud thereby protecting it until it began to appear. Even during this exsertion stage, the ligule provided additional protection and the bud tissues 'have time gradually to change in response to the claims of the outer world' (Goebel, 1905). The form of protection afforded the bud was not specified and it is difficult to see a great deal of difference between this hypothesis and the preceding one; both propose protective

functions for the ligule. Protection of the bud would seem to be best exemplified in the case of the flag leaf ligule.

It should be added, however, that Goebel did not exclude the possibility of the ligule having other functions after bud unfolding.

Active Rôle(s) of the Ligule

Both the hypotheses considered above attribute largely passive rôles to the ligule; the possibility that it may also perform more active rôles must be borne in mind. Apart from the assertion of Dobrynin (1959) that the ligule performs a more fundamental function(s) than [illegible] exclusion, evidence for an active rôle of this organ comes from the study presented in this thesis.

LM study of the ligule demonstrated the presence of starch-bearing chloroplasts in the mesophyll and thus introduced the possibility that the ligule might be an 'active' organ. The TEM study took this idea further and showed the presence of a marked ultrastructural polarization in terms of organelle quality and quantity across the width of the ligule (summarized in Fig. 3.4). The presence of large numbers of mitochondria, dictyosomes and strands of RER in the adaxial epidermal cells suggested a region of high synthetic and respiratory activity. Enzyme and polysaccharide cytochemistry provided evidence for this and suggested that this tissue and hence the ligule, had a glandular nature.

Metabolically the ligule may be considered a very active organ and a number of hypotheses may be advanced which emphasize one aspect or another of this.

It might be suggested that the extracellular product acts as a sort of adhesive aiding even closer appression of the ligule to the enclosed shoot thereby facilitating the operation of the Exclusion Hypothesis. This is reminiscent of the work of Sutherland and Eastwood (1916) on the ligule of Spartina townsendii. They stated that the abaxial surface of this ligule consisted of cells whose outer walls were 'notched, uneven, and mucilaginous'. They suggested that water was

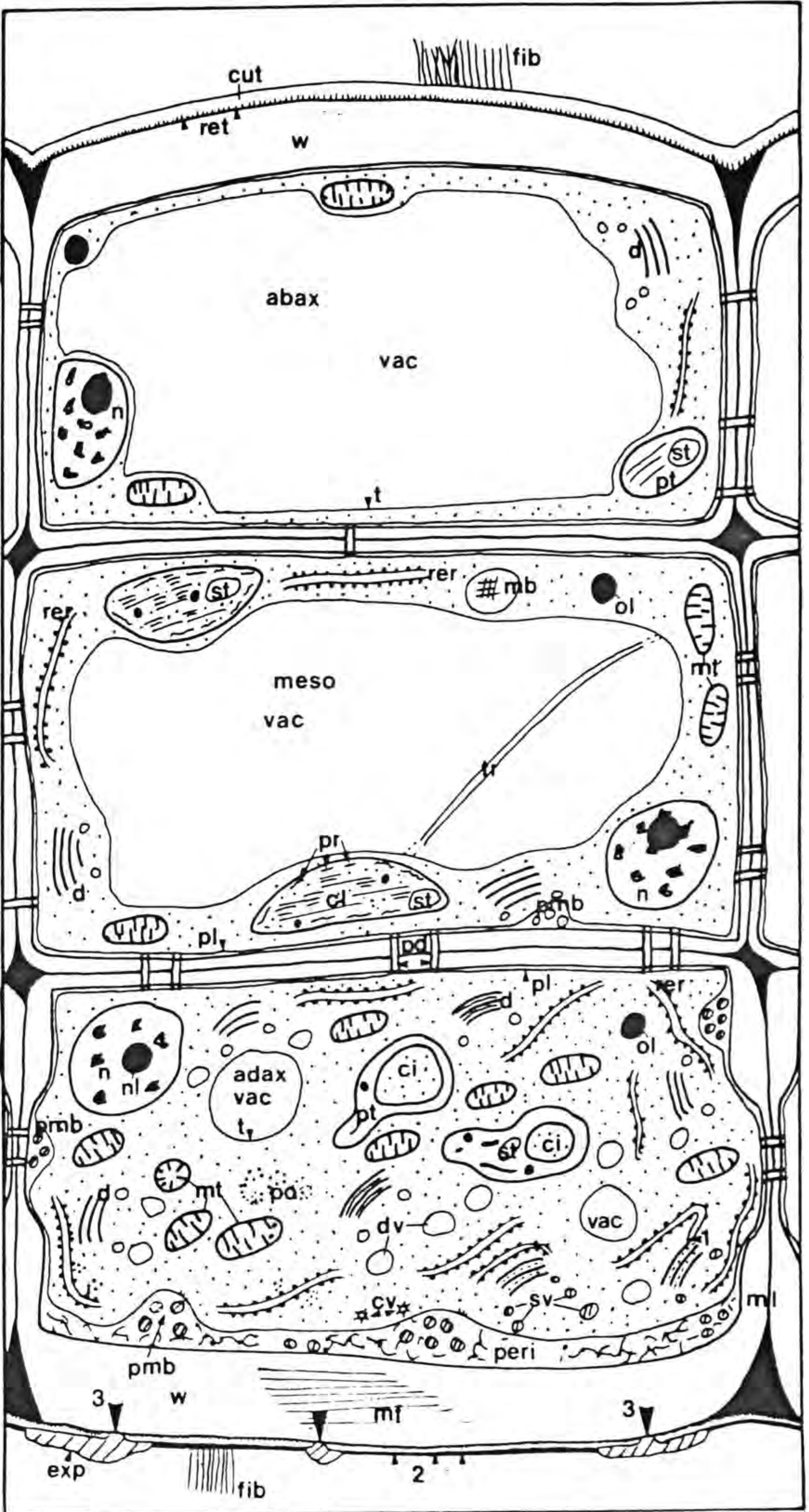
Figure 3.4. Diagrammatic TS of the emerged ligule of *Lolium temulentum* summarizing current knowledge of its structure and ultrastructure

1. Region of direct or very close contact between ER and *cis* aspect of a dictyosome;

2. Polysaccharide-containing layer on outside of cuticle;

3. Cuticular gap

All other abbreviations as for plates



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held within the cells of the body of the ligule and that this mucilage coating aided its retention even 'under unfavourable conditions'. In this way the ligule was kept more or less turgid and close-appressed to the enclosed shoot 'preventing the entrance of either water or mud'. No evidence of the cells' mucilaginous nature was presented but in view of its suggestion of structure/function within the grass ligule it is surprising that it does not appear to have been followed up by subsequent workers.

The hypothesis most favoured for the ligules studied here is that the glycoprotein-like material secreted by the adaxial epidermal cells acts as a lubricant to facilitate the exsertion of the enclosed leaf or culm. This is presumably aided by the lack of ornamentation of this epidermis (as seems to be the case for all ligules studied - results not shown), and is in direct analogy to the major function proposed for root cap slime (see Appendix). Such a rôle for the ligule carries with it the suggestion that secretory activity of this organ may be related to the extension growth of the enclosed tissues. Such an interaction deserves to be followed up.

Any function of the ligule based upon its apparent secretory activity, however, rests upon an accurate identification of the chemical nature of the secretory product: is it in fact a glycoprotein? Until we have that information it is difficult to speculate further about its rôle - and hence that of the ligule.

Just because an organ has a seemingly complicated ultrastructure and the probable existence of certain metabolic capabilities, need it necessarily have a 'function'? Further, need any postulated function bear any relationship to the known ultrastructural and physiological 'facts' regarding that organ? Williams and Barber (1961) set down four postulates which need to be satisfied before any structure can be considered as having a particular function:

i. the structure is necessary for the successful growth of the plant in competition with others;
ii. the structural provision is adequate for the requirements of the function it is supposed to serve;
iii. these requirements could not have been met with markedly greater economy by some other available means;
iv. provision is not markedly more than is necessary to fulfill the functional requirements.

How well do the two main hypotheses of ligule function - 'Exclusion' and 'Lubricant' - fulfill these postulates?

i. Regardless of the function proposed the best way to assess the value of the ligule to the plant is to consider the case of liguleless plants. Liguleless plants may occur naturally in otherwise ligulate species, be artificially induced eg by X-radiation or be the normal state for a species - eg Echinochloa crus-galli (L.) Beauv.

It has been observed for liguleless maize that 'the inside of the sheath is often more discolored, as if from incipient decay, than is the case when a well-developed ligule is present' but 'decay of the stalk or leaf sufficient to be of any material injury to the plant' was not seen (Emerson, 1912). Although it was not demonstrated that the discolouration was due to water having been admitted into the interior of the plant, it may be concluded that liguleIessness being 'disadvantageous to the plant is somewhat questionable' (Emerson, 1912).

In other cases where liguleless plants are encountered - such as crops - there appears to be no disadvantage associated with the lack of ligule. Here, however, the practice is usually to grow monocultures of a single variety or strain, under such conditions any competitive disadvantage of the liguleless condition would not be apparent. Liguleless grasses are generally rare in the wild and this may be interpreted as evidence for their being at a competitive disadvantage relative to ligulate forms.

The most serious doubt of the value of the ligule is the case of the liguleless grass species E. crus-galli. As Sharman has pointed out (pers. comm.) this grass is a notorious weed in many parts of the world and must be considered 'successful'. Are the function(s) attributed to the ligule performed by some other structure? Or is a ligule unimportant to the 'successful growth of the plant in competition with others'? Either we make a separate case for this grass or we are led to the conclusion that the ligule exists but is not of value to the plant in any way.

On the evidence available it is not possible to accept postulate (i) as being proven or disproven but it clearly emphasizes the need for further work on the rôle of the grass ligule.

ii. How much lubricant is needed to effectively facilitate leaf exsertion? If we were in a position to answer this we would be able to assess whether there is 'too much' or 'too little' structure present. For 'Exclusion' it can be reasonably assumed that a ligule 1-2 mm high would be sufficient to perform this rôle; the ligules of the 5 species examined here are about this size.

Postulate (ii) appears to hold for 'Exclusion'; for 'Lubricant' it is not possible to judge.

iii. Presumably only a ligule could exclude water, etc since no other structure is in the correct place to perform this rôle. As far as is known the ligule is the only 'glandular' structure on the leaf.

Thus postulate (iii) appears to hold for both hypotheses.

iv. For the Lubricant Hypothesis this is as difficult to assess as postulate (ii). For the Exclusion Hypothesis the range of membranous ligule heights is great - it may be up to 0.5 mm high in Vulpia bromoides (L.) S. F. Gray, and from 5-15 mm high in Glyceria fluitans (L.) R. Br. (Hubbard, 1976). Again we have to ask, how much is too much? We do not know. It is of course possible that the ligule performs both a lubricant and an exclusion function and the observed structure is more than is needed for either function alone but is just enough to fulfill both rôles together. In support of the latter the following observation is relevant. The cuticle is considered to be metabolically inert and once laid down rebuilding is not necessary (Kolattukudy, 1980). Even in totally senescent ligules of L. temulentum the cuticles appeared intact and the ligules themselves still appressed to the enclosed shoot. It would thus appear that exclusion could be performed without the degree of ultrastructural complexity found in the ligule. Teleologically speaking, there is too much structure just for exclusion. It is also possible that the ligule may have other functions not yet considered.

Postulate (iv) cannot be assessed with the available information.

Conclusion

Until such time as we know a great deal more about the biology of the grass ligule we cannot say whether it has - or had - a 'function' let alone what that function might (have) be(en).

CHAPTER 4 GENERAL DISCUSSION

A grass plant as an individual representative of a species comprises many grades of organization from the molecular to the tiller levels. Any or all of these may profitably be studied from the point of view of providing fundamental information concerning structure/function relationships. However, no one cell, tissue or organ can be considered in isolation from the other cells, tissues or organs with which they are associated as part of the larger biological entity, the plant. In the case of the vegetative grass plant concerned with in this thesis, the leaf is probably the smallest 'biologically relevant' grade of organization which can be studied.

The leaf may be considered a 'super organ' composed of three distinct organs - blade, sheath and ligule. The aim of the investigation presented here was to try and gain an appreciation of the biology of the leaf as a whole by studying aspects of the biology of its component organs. In order to achieve this it meant that each aspect was only dealt with briefly; in many cases all that has been done is to identify phenomena - some of which appear to have been previously unrecorded - which need to be studied further in order to understand their significance.

A great deal of information exists in the literature concerning various apects of the biology of blade and sheath. For that reason the emphasis here was upon trying to understand the nature of any interactions between these two organs during growth, ageing and senescence. For the ligule such basic information was not available and the emphasis accordingly was upon obtaining fundamental information concerning its structure and metabolic capabilities. Hence the difference in depth of coverage of blade/sheath on the one hand and ligule on the other.

The appearance of tyloses at the blade/sheath junction of leaves of L. temulentum during ageing in some respects typifies the 'within leaf' interaction. Whether such structures selectively accumulate xylem-borne metabolites is largely conjecture. However, whether they act in the way postulated is largely immaterial, their existence has underlined the sort of interactions that may occur between blade and sheath during ageing and senescence of the leaf. Such could not have been envisaged by use of excised blade segments alone. More information is clearly needed regarding the timing of their formation and their physiology before their association with senescence can be seen as anything more than coincidence.

In the case of the membranous ligule, we now know a great deal about its structure - and some aspects of its physiology - in 5 species. We still do not know, however, if it has, or ever had, a 'function'. All that we can reasonably conclude for the ligules studied is that they are very 'active' and make a material of incompletely understood composition which appears to be secreted but whose function is unknown. To put these results into perspective, if we accept a conservative estimate of 9,000 grass species (Clayton, 1978) both LM and TEM results are available for less than 0.1%! Yet if these organs were overlooked as being merely 'membranous outgrowths at the blade/sheath jucntion' even these few facts would have remained undiscovered.

Despite the apparent insignificance of the ligule, a number of potential interactions exist between it and the rest of the leaf. For example, of the grass genera described by Watson and Dallwitz (1982 - micro fiche, pers. comm.) for which data regarding blade disarticulation were given, c. 12% show disarticulation (blade abscission); of these over 90% have 'hairy' ligules. Whether a causal connection exists between possession of this type of ligule and blade abscission is not known. The high degree of synthetic and apparent secretory activity at the ligule insertion region in L. temulentum might suggest some sort of interorgan interaction. Both these observations serve to emphasize how poorly understood this leaf organ is, and help make the case for its further study.

A more significant interaction between the ligule and the leaf is the case of liguleless varieties of such crop plants as maize and rice. As discussed in Chapter 3, Section d, the cells responsible for the blade bending response are apparently initiated at the same time as the ligule. In view of the economic importance that has recently been attached to the use of liguleless mutants of grasses (eg Lambert and Johnson, 1978; Vaklinova and Kuen, 1978), the anatomical or physiological basis of the liguleless condition is worthy of further study.

The interactions within and between grass leaves during growth (Chapter 2, Section a) are of interest to such fields as developmental anatomy and crop breeding. It is not inconceivable that, if we understood how such interactions arose, we might be able to manipulate the plant in such a way that "high photosynthesis" blade area was in-

creased relative to "lower photosynthesis" sheath area. The potential benefits for herbage production make such a possibility highly attractive.

Similarly with study of the course of whole leaf senescence; if we understood the changes that take place and their cause(s), we might be able to delay or prevent them. In that way it might be possible to approach the much sought condition of winter greenness of grass swards (eg Borrill, 1960).

Each plant is the product of interaction between its genetic constitution and the environment. The environment can modify the plant only within the constraints imposed by the genome. No amount of variation in the environment can lead to development of such characters that are not within the scope of the latter - barring mutation. Hence a great deal of diversity exists between individuals (Chapter 2, Section a) while the species as a whole is defined in terms of the underlying unity of structure, function, growth and development. Because of this fundamental unity it should be possible to achieve an understanding of the biology of the 'average plant' if large enough samples are taken from within the heterogeneous population. This aspect of whole plant/leaf study in 'natural' environments was briefly dealt with in Chapter 2, Section b. We may try to minimize the natural heterogeneity by use of growth rooms or other artificial environments, but in so doing we are greatly compromizing the value of any information obtained. We must accept that heterogeneity exists in natural environments and that it is a normal character of any growing population. Our task therefore should be to develop methods of coping with it, not removing it. To achieve this will be no mean feat but until it has been done it will not be possible to make full use of the information derived from study of the natural system.

However, even when all this has been done we are still faced with the uncertainty of the validity of the results obtained. When dealing with aspects of ultrastructure this problem is even more acute; how can we be sure that what we see is genuine and not in some way 'created' by the methods employed to obtain this information?

Clearly even an apparently simple examination of the grass leaf is a complex problem. If we are ever to understand the processes that take place in leaves then we must study leaves, however difficult that may prove to be.

Although it may be said that the whole (the leaf) is greater than the sum of its parts (blade, sheath and ligule), we are only just beginning to understand the latter and the interactions between the parts. It is to be hoped that more studies in the future will look at the grass leaf as the sum of three distinct organs, not just of the blade. In this way such aspects of leaf biology as the rôle(s) of the ligule and tyloses should eventually be understood.

'The challenge lies, however, not in the accumulation of miscellaneous information, but in the synthesis that will give it all meaning.' (F.C. Steward, 1971)

APPENDIX COMPARISON OF ROOT CAP AND LIGULE

INTRODUCTION

The grass root cap occupies the most apical region of the root and is a site of intense cell division and synthetic activity - the root cap of maize which contains c. 10,000 cells is replaced daily (Clowes, 1971). During this rapid growth, cells are cut off near the centre and displaced towards the periphery before eventually being sloughed off and lost into the soil. Pronounced changes in both ultrastructure and function take place as the cells 'migrate'. Cells of the central region are the sites of geoperception, the statocytes (eg Barlow, 1982), and are characterized by the presence of numerous amyloplasts. As the cells near the periphery they assume a secretory rôle as their dictyosomes become hypersecretory (eg Jones *et al*., 1966).

The root cap has long been recognized as a secretory organ producing a mucilage. In view of the apparent secretory activity of the ligule of *L. temulentum*, a comparison was made between it and the root cap of the same species to try and identify differences or similarities between them. It was hoped that such a study would help provide additional support for the view that the ligule has a secretory nature, and clues to the function of the secretion produced. Aspects of root cap structure, ultrastructure and cytochemistry were examined using a number of techniques previously used with the ligule.

OBSERVATIONS

Light Microscopy

After staining TS's with toluidine blue, three distinct cell types could be identified (Pl. A.1a). They were distinguished by the number and relative staining intensity of regions within them.

Type 1 cells: the whole of the contents stained uniformly and lightly;

Type 2 cells: there was a distinction between a central, densely staining region and a much lighter peripheral region;

Type 3 cells: exhibited greatest contrast with a densely staining region, generally against the inner tangential wall, and a much lighter region which occupied the greater part of the cell.

In addition the stretched walls of destroyed cells were frequently seen at the periphery of the root cap (darts in Pl. A.1a). After staining with Sudan black B (results not shown), it was observed that amyloplast concentration appeared to decrease from cells of Type 1 to 2 to 3.

Fluorescence Microscopy

Incubation of root tips in calcofluor resulted in the staining image shown in Pl. A.3a,b. A longitudinal zonation of staining pattern was observed in which three distinct zones could be recognized.

Zone i: the extreme tip of the root; exhibited fluorescence chiefly of cell walls with some associated with cell contents (Pl. A.3b);

Zone ii: adjacent to Zone i, showed fluorescence of cell contents and cross-walls (arrow-heads in Pl. A.3b);

Zone iii (not shown): fluorescence was apparently confined to cell walls.

Transmission Electron Microscopy

General Ultrastructure

Type 1 Cells

These cells were characterized by the presence of numerous amyloplasts, dictyosomes and highly-cristate mitochondria (Pl. A.1b, A.2a). Strands of RER and numerous free ribosomes and polysomes were also present. The dictyosomes were not hypersecretory (Pl. A.2a) although synthetic activity in the form of dictyosome-derived vesicles, and secretory activity in the form of periplasmic accumulations (dart in Pl. A.1b) was evident. Frequently the dictyosomes were seen sectioned near the edge of the stack (d' in Pl. A.2a). The vacuome apparently consisted of a few small vacuoles scattered throughout the cell. These cells appeared to correspond to the statocytes mentioned in the Introduction.

Type 2 Cells

The organelle complement of these cells was similar to that of Type 1 cells but amyloplasts were fewer and large numbers of hypertrophied dictyosome-derived vesicles were present within the cytoplasm (Pl. A.1c, A.2b,c). These cells appeared to be highly active in mucilage synthesis and secretion; material could be seen within the periplasmic space right around the cell (darts in Pl. A.1c). The contents of the dictyosome-derived vesicles (Pl. A.2b,c) and the periplasmic space (Pl. A.2c, A.3d) were similar and appeared to have an amorphous-fibrillar nature. The evident reduction in cytoplasm in these cells with concomitant increase in volume of the periplasmic space presumably explains the toluidine blue-staining image referred to above - densely staining cytoplasm with lightly staining peripheral (periplasmic) region.

Type 3 Cells

The organelle complement was similar to the other two cell types but the numbers of organelles generally appeared to be lower. Secretory activity - as judged by the size of the hypertrophied dictyosome-derived vesicle population - appeared to have greatly diminished; most of the cell volume was occupied by periplasmic space (Pl. A.1d). In contrast

to the situation in Type 2 cells, here the periplasmic space was largely absent from the inner tangential wall. By reference to the toluidine blue-staining pattern of these cells, it appears that the densely staining region corresponds to the cytoplasm and the lightly staining region to the periplasmic space. Accumulations of fibrillar material - assumed to be mucilage - were often seen in the intercellular spaces between these cells (Pl. A.2d).

Polysaccharide Cytochemistry

Mucilage external to the root cap gave the same staining pattern with all three procedures used - PATAg (Pl. A.4c), PTA (Pl. A.3f), RR (Pl. A.3e) - and all were similar to that observed with conventional TEM of double-stained sections (Pl. A.2d). The mucilage appeared to be composed of fibrillar material radiating from amorphous loci in a non-staining matrix (which may originally have been water). RR-staining of material in the periplasmic space revealed the appearance of dense, amorphous deposits (Pl. A.3c). PATAg-staining of material in dictyosome-derived vesicles and the periplasmic space (Pl. A.4a) also exhibited a more amorphous nature to that observed in the external mucilage. Dictyosomes were only seen to stain with PATAg. Pl. A.4b shows the staining pattern observed at the edge of the cisternal stack; the nascent vesicles were still attached to the thread-like staining cisternae. The RER did not appear to stain with PATAg (Pl. A.4b), although slight staining was observed with PTA (results not shown). Starch grains of amyloplasts (Pl. A.4a) were only stained with PATAg; PTA treatment did, however, result in a densely-staining edge to the starch grains (results not shown).

Zinc Iodide-Osmium Tetroxide Impregnation

As expected the stain largely accumulated within elements of the endomembrane system, mitochondria (Pl. A.4e) and to some extent internal membranes of amyloplasts (Pl. A.4d). Despite the abundance of dictyosomes and tER, no direct connections were observed between them, although close associations could be seen (results not shown). Close associations were also seen between the NE and cER (results not shown). The distribution and shape of nuclear pores was clearly visible in suitably oriented sections (darts in Pl. A.4d). The starred object in

Pl. A.4d is interpreted as a form of tER and may be a transitional form between NE and more conventional tER. The frequent aggregations of material of vesicular nature seen in ZIO-treated tissue (Pl. A.4f) are interpreted as paramural bodies; however, their apparent absence in conventionally-prepared material leaves this interpretation in some doubt.

Plate A.1. Structure and ultrastructure of the root cap of *Lolium temulentum*

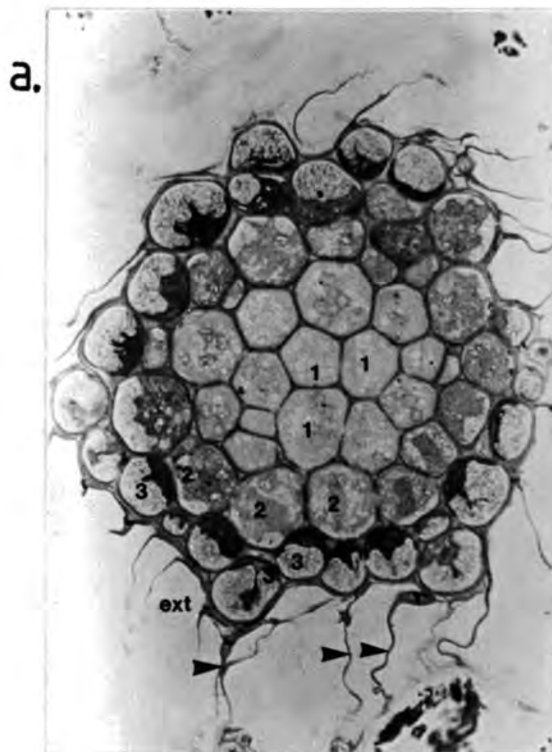
a. LMgraph of TS showing the distribution of the three cell types (1,2,3); darts indicate stretched walls; X460;

b. TEMgraph of part of a Type 1 cell; dart indicates periplasmic space; X6,500;

c. TEMgraph of part of a Type 2 cell; darts indicate periplasmic space; X4,125;

d. TEMgraph of part of a Type 3 cell; X5,250.

a.



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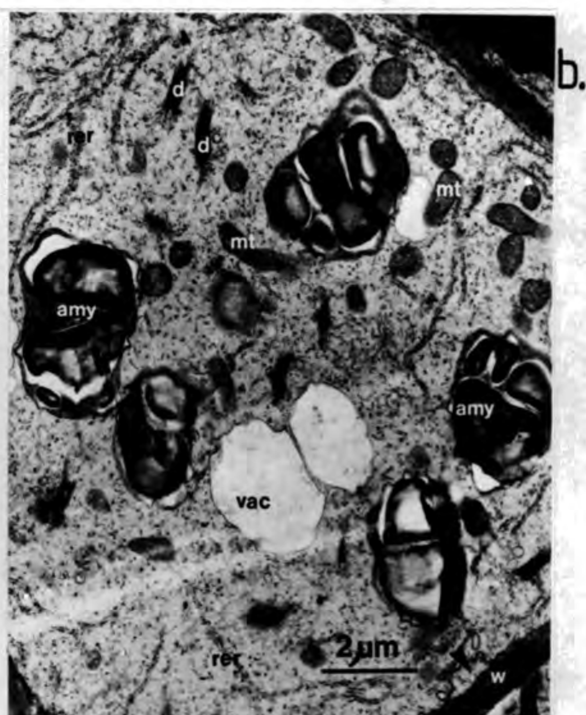
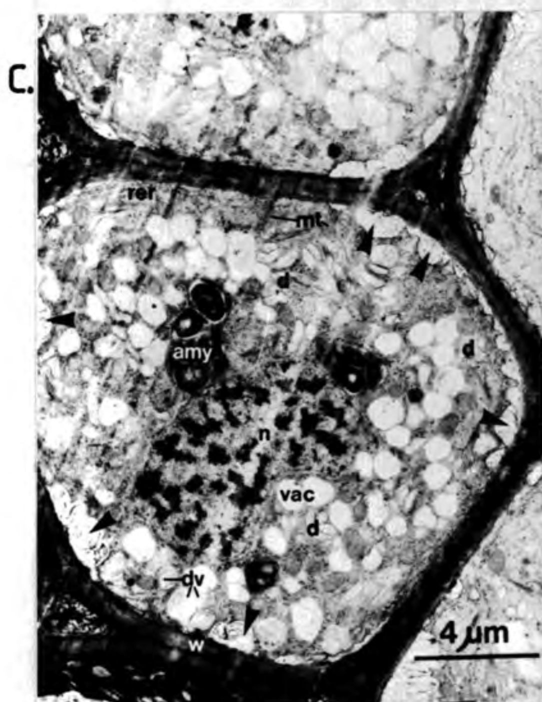


c.



d.

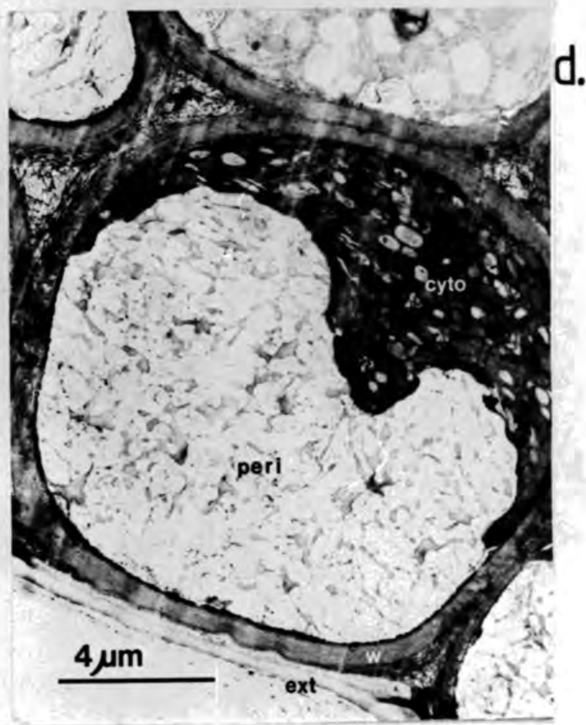


Plate A.2 Ultrastructure of the root cap of *Lolium temulentum*

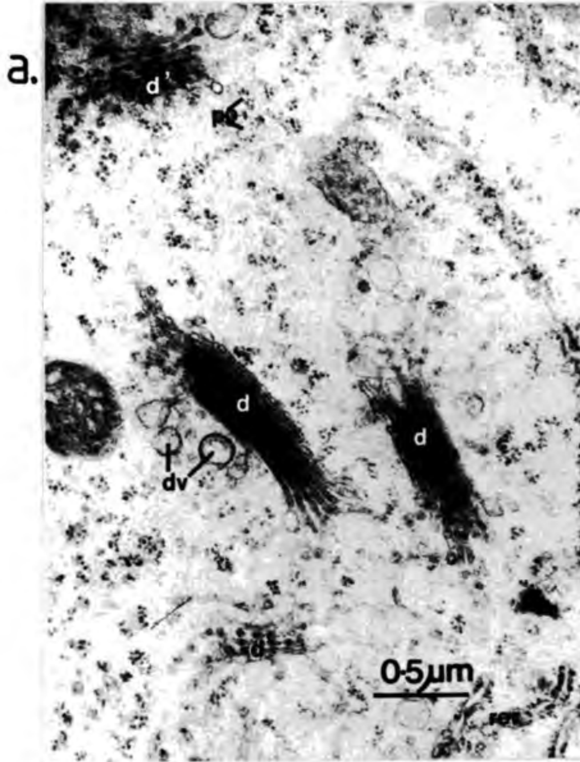
a. Part of the cytoplasm of a Type 1 cell; X25,000;

b. Part of the cytoplasm of a Type 2 cell; X35,000;

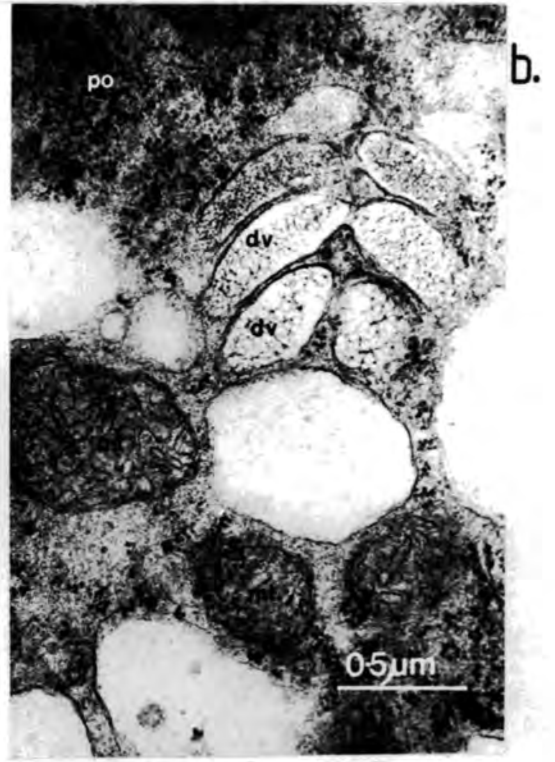
c. Part of the cytoplasm and wall of a Type 2 cell; X44,000;

d. Parts of two adjacent Type 3 cells; X25,000.

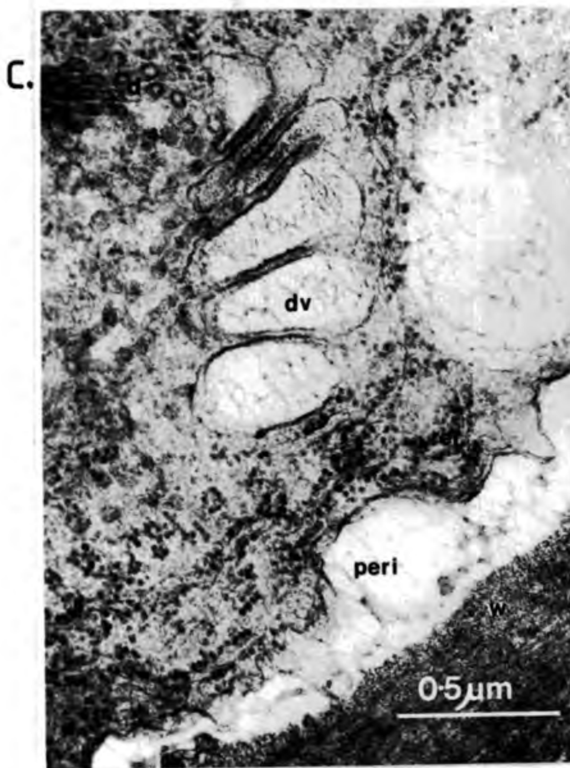
a.



b.



c.



d.

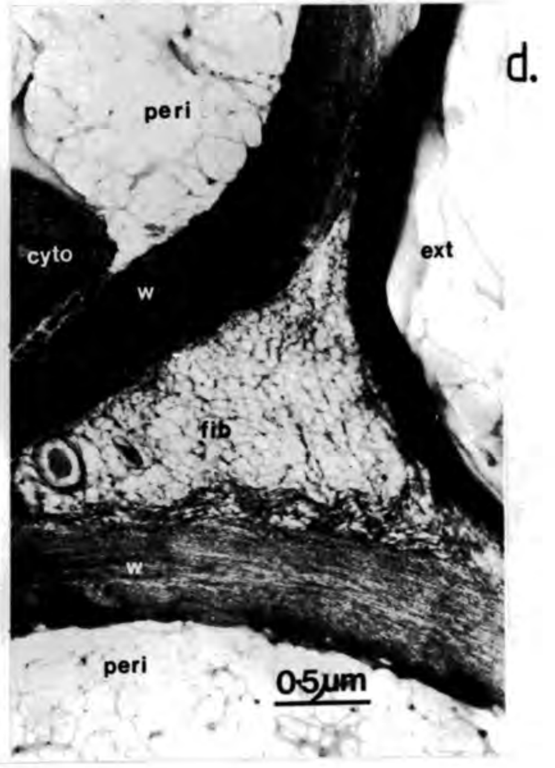


Plate A.3. Aspects of the biology of the root cap of Lolium temulentum

a. FluorMgraph of the surface of the root tip; calcofluor; X115;

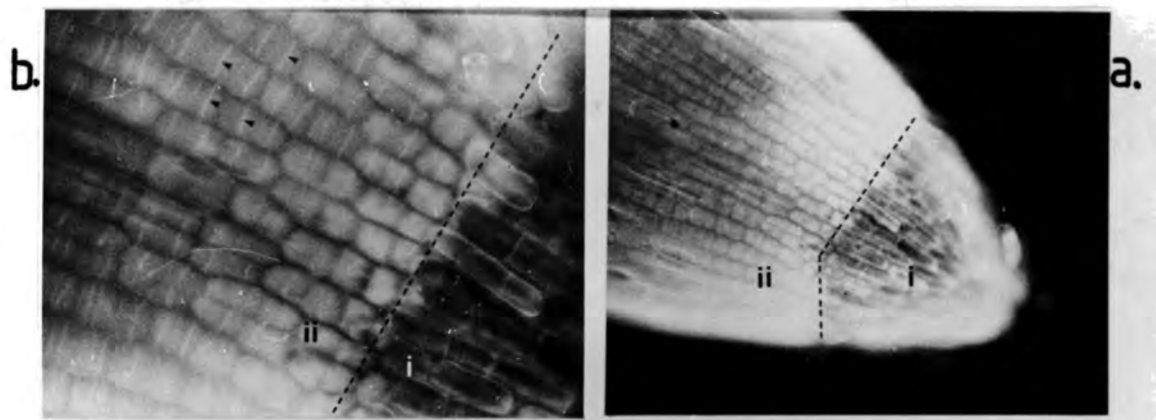
b. Higher power view of region in a. above; arrow-heads indicate cross-walls; X290;

c. TEMgraph of part of the cytoplasm and wall of a Type 2 cell (RR); X10,500;

d. TEMgraph of periplasmic space of a Type 2 cell; X57,500;

e. TEMgraph of fibrillar material external to the root cap (RR); X70,000;

f. Similar region as in e. above (PTA); X70,000.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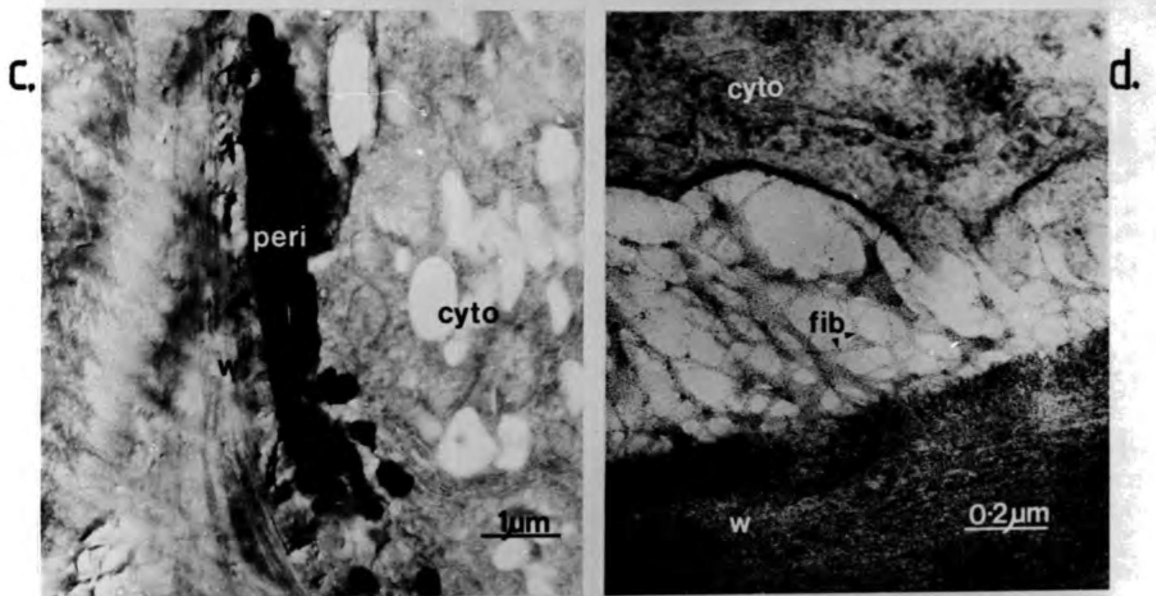
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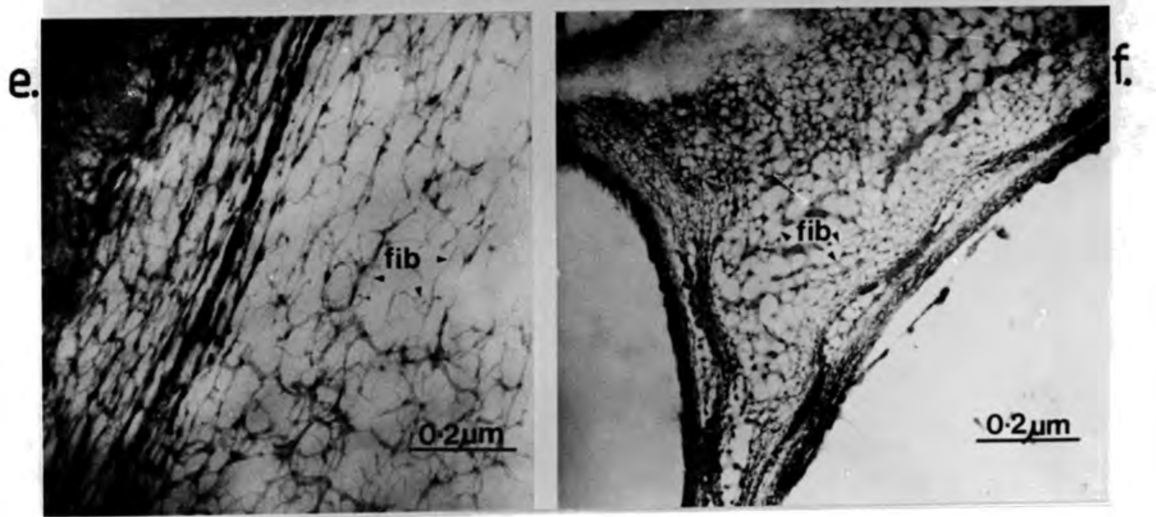
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Plate A.4. Cytochemistry and ZIO-staining of the root cap of Lolium temulentum

a. Parts of several Type 2 cells (PATAg - 2 h, TCH); darts indicate periplasmic spaces; X4,000;

b. Part of the cytoplasm of a Type 2 cell (PATAg - 2 h, TCH); X31,500;

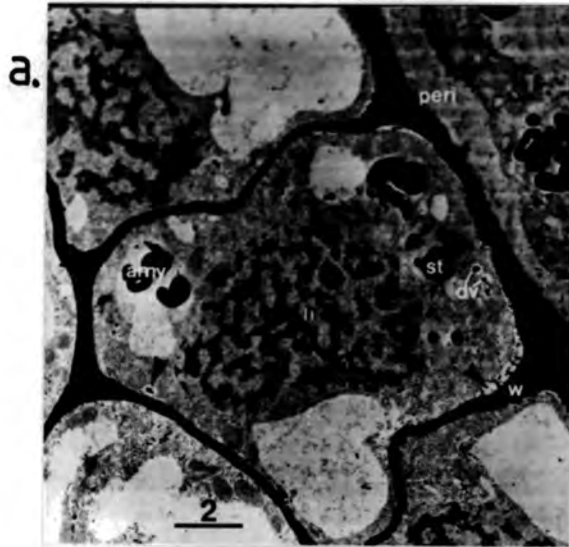
c. Parts of two adjacent Type 3 cells (PATAg - 2 h, TCH); X14,500;

d. Part of the cytoplasm of a Type 2 cell (ZIO); darts indicate nuclear pores; the star indicates a possible intermediate form of tubular endoplasmic reticulum; arrows indicate internal membranes of amyloplasts; X17,000;

e. Part of the cytoplasm of a Type 2 cell (ZIO); X13,500;

f. Putative paramural body in the periplasmic space of a Type 2 cell (ZIO); X28,000.

Scale bars in μm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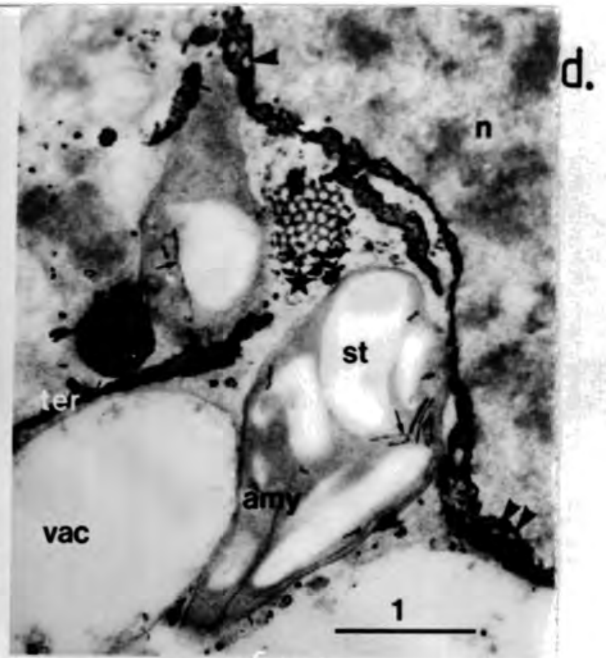
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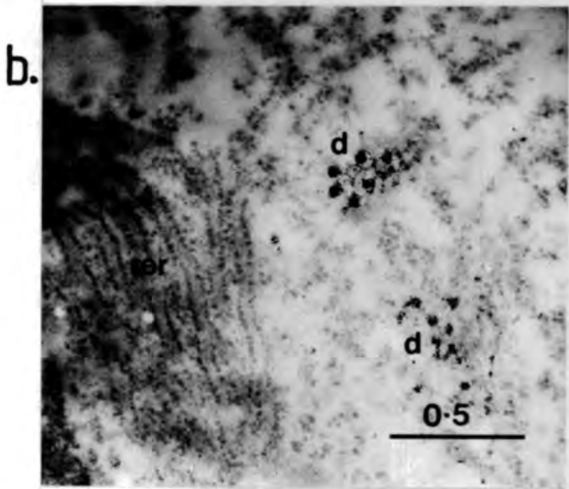
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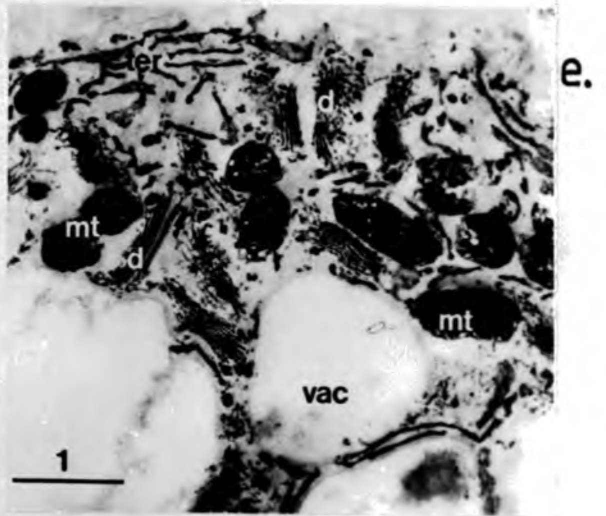
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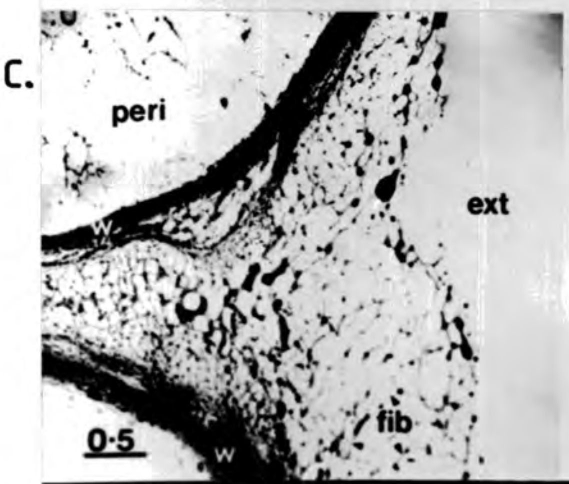
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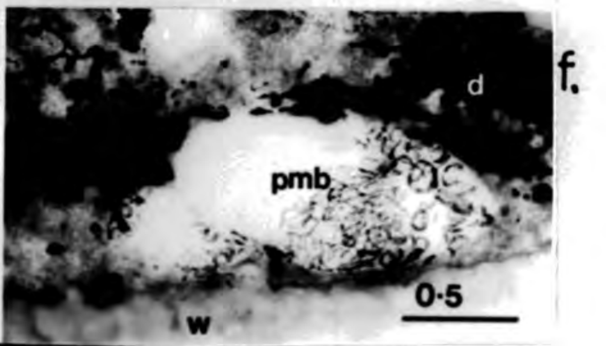
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DISCUSSION

The observations presented above were largely in accord with those shown for root caps of other grasses in the literature and will not be discussed in detail. Differences and similarities between the root cap and the adaxial epidermal cells of the ligule of L. temulentum will be considered below.

Development

The three cell types of the root cap appeared to represent stages in the course of development of a single cell from the central region to the periphery. Hence the different organelle assemblages observed in the TEM study represent stages in the differentiation of structure and function within the cell as it changes from statocytic to secretory.

In the ligule this sort of information is lacking. We know the structure of cells of the newly-initiated ligule and of the emerged ligule; we do not know the various stages through which cells of the adaxial epidermis pass to reach the secretory state.

Polysaccharide Cytochemistry

The apparent absence of toluidine blue-staining of the periplasmic material in both the root cap (Pl. A.1a) and the ligule (results not shown, but see eg Pl. 3.10) may suggest some similarity in the chemical nature of the two secretory products. Similarity of staining reactions, however, is much more satisfactory evidence and was provided by the TEM study.

All four techniques used gave the same staining image for the externally-located root cap mucilage; it appeared to be composed of fibrils radiating from amorphous loci within a non-staining matrix. In the ligule, the extracuticular material appeared as amorphous aggregates with all techniques except RR (which was considered to be unreliable).

The spatial relationships within highly-hydrated materials become altered during dehydration stages prior to embeddment. The ligule and root cap materials may have been differently hydrated to start with or differently affected by the processing undergone. Thus the differences between their staining patterns may be more apparent than real, although

it is possible that they may be fundamentally different in composition and hence behaviour.

PATAg- and PTA-staining of the root cap periplasmic mucilage gave the same image as the external mucilage. RR, however, stained it as dense amorphous aggregates which may indicate a high pectic content (eg also Wright and Northcote, 1974 for maize). The reason why RR stained periplasmic and external mucilage differently remains unclear. It may be due to differences in organization of the mucilage between the two sites, possibly caused by differences in the degree of hydration.

In the ligule, paramural bodies and fibrils stained with PATAg and PTA in the periplasmic space; only the former appeared to stain with RR. This situation is in some respects the reverse of that found in the root cap with RR-staining - fibrillar material one side of the wall, amorphous material on the other. The explanations in each case may be similar and possibly related to differential loss of low MW polysaccharides during TEM-processing, or alteration of the secretory product during its passage through the wall. The best way of tackling this problem is to extract the products from the two sides of the wall and chemically analyze them.

Apparent staining of the periplasmic material in the ligule with calcofluor and uvitex BOPT suggested that it had, at least in part, a β-linked hexapyranose composition. The three zones observed in the root tip after incubation in calcofluor make interpretation more difficult here. Comparison of the observed fluorescence image with LMgraphs of LS's of other roots (eg Clowes, 1959) suggests that Zones i and ii are both in the root cap and Zone iii is in the epidermis. The interpretation thus offered is that Zone i represents cells on the periphery of the cap which are in the process of being sloughed off; loss of most of their mucilage has resulted in staining being confined largely to cell walls. Cap cells in Zone ii were both dividing and actively synthesizing or accumulating mucilage; only the periplasmic space-located mucilage and newly-made cross-walls were stained. Epidermal cells in Zone iii were apparently not making mucilage at this stage - although they can (Miki _et al_., 1980) - consequently only the walls stained. Thus it appears that the root cap mucilage also stained with calcofluor indicating a β-linked hexapyranose content. This is in agreement with Wright and Northcote (1976) who demonstrated the presence of a central

β-1,4-glucan in maize root cap slime which stained with calcofluor.

On the basis of polysaccharide cytochemistry and calcofluor staining, ligule and root cap secretory products are very similar. A glycoprotein nature was suggested for the former; PTA-staining of the latter is evidence for it too having a glycoprotein nature (as has been demonstrated for maize root cap slime by Green and Northcote (1978)). The root cap mucilage appears to have a high pectic content but the RR-staining image for the ligule was considered too ambiguous to conclude that in this organ.

Energy and Substrate Sources

Glycosylation and phosphorylation reactions are needed in the synthesis and elaboration of polysaccharides (eg Northcote, 1979). These reactions require energy, largely in the form of ATP, and a source of substrates both to generate the ATP and to act as precursors for polysaccharide synthesis. In the case of a glycoprotein, more energy-requiring reactions are involved and the demand for substrates is greater. Both the root cap cells and the ligule have large numbers of highly-cristate mitochondria. In the ligule, enzyme cytochemistry has demonstrated the presence of succinate dehydrogenase and cytochrome c oxidase and hence the probability that the mitochondria are active. In the root cap such evidence is lacking; the mitochondria look like those of the ligule and are assumed to be active. Thus potential ATP sources exist in both secretory systems.

The decrease in starch root cap amyloplasts and the concomitant increase in hypertrophy of dictyosome-derived vesicles has been interpreted as evidence that the starch may act as a source of precursors for polysaccharide synthesis (eg Juniper and Pask, 1973). The apparent rarity of plasmodesmata between cells of the root cap (results not shown) might favour such an intracellular source of polysaccharide precursors and respirable substrates. For the ligule it was conjectured that substrates might be made within the mesophyll chloroplasts and transferred to the adaxial epidermal cells via the numerous plasmodesmata between these two tissues. In both ligule and root cap, the abundance of RER in the former and free polysomes in the latter suggests that any protein component of the secretory product may be made within the secreting cells themselves.

Thus it would appear that the root cap cells use intracellularly-generated substrates for ATP, polysaccharide and protein synthesis; in the ligule both extra- and intracellular sources may be used.

Pathway of Synthesis, Elaboration and Secretion of the Product

Work of others, largely upon maize root cap, has demonstrated the probable involvement of both RER and dictyosomes in the synthesis and elaboration of the mucilage (eg Bowles and Northcote, 1972; Green and Northcote, 1978). Direct connections between these two endomembrane components have been observed in maize root cells by Mollenhauer et al. (1975) and Mollenhauer and Morré (1976), and in wheat by Marty (1980). Using ZIO in L. temulentum root cap, direct connections between ER and dictyosomes were not seen; close associations, however, were common. PTA-staining of RER and PATAg-staining of dictyosomes and dictyosome-derived vesicles indicated involvement of these endomembrane components in synthesis of material(s), possibly the mucilage. The pathway of secretion from dictyosomes to the PL was assumed to be via smooth, dictyosome-derived vesicles. Discharge into the periplasmic space presumably took place by reverse pinocytosis of the granulocrine type (eg Dumas et al., 1974). Paramural bodies were not observed in ultrathin sections but were apparently seen in ZIO-stained material. Sections of the latter are usually 3-4 times thicker than ultrathin sections and hence presence of paramural bodies in ZIO-stained material may be related to their rarity and small size. Alternatively, in view of the large size of the hypertrophied dictyosome-derived vesicles which are assumed to fuse with the PL, paramural bodies may not form from these vesicles. The paramural bodies observed in ZIO-stained material may be derived from smaller vesicles not involved in mucilage secretion, or be an artefact of the technique.

As previously observed by Juniper et al. (1977), secretion appeared to take place all around the cell but the periplasmic material eventually accumulated next to what was - or was destined to be - the outer tangential wall (cf. cell Type 2 and 3 in Pl. A.1a). The outer tangential walls were neither cutinized nor cuticularized - as judged by Sudan black B staining (results not shown) - and were assumed not to present a barrier to secretion of the mucilage (eg also Paull and Jones, 1976). According to Paull and Jones (1976), the mucilage may be released from

the cells through the wall which has been 'loosened' by enzyme action; this was not examined here.

For the ligule a pathway was proposed (Fig. 3.2) which involved direct RER/dictyosome connections, transport of smooth dictyosome-derived vesicles to the PL and the formation of paramural bodies. Paramural bodies, and hence evidence of secretion, were commonest against the outer tangential walls of adaxial epidermal cells, as was the periplasmic material (as with the root cap). Passage through the wall was assumed to take place - possibly as discrete vesicles - culminating in release of the secretion from the ligule via cuticular gaps. Postulated formation of these latter discontinuities by turgor and accumulation of product between the wall and cuticle is akin to the mode of secretion of root cap mucilage described by Morré et al. (1967).

Thus the pathways of synthesis and secretion of the products as proposed above for the two systems are similar. The main differences appear to be the much reduced frequency of paramural bodies and absence of cuticle in the root cap.

Function

The root cap slime or mucilage has generally been considered to provide lubrication and protection to the root as it grows through the soil (eg Fritsch and Salisbury, 1938; Juniper and Pask, 1973). A variation on this theme was suggested by Esau (1953) who proposed that the mucilage might aid separation between the root cap and sloughing cells. In view of the prominence of root caps in hydrophytes, she further suggested that in these cases the cap might perform other functions. The aerial roots of Cattleya were studied by Mollenhauer (1967). He proposed that here the root cap slime might act as a water-absorbing material since a lubricant rôle was evidently not applicable.

Regardless of the different rôles proposed by these authors, they all have in common their dependence upon the high hydration capacity of the mucilage. Any chemical similarity between ligule and root cap products is not yet known and it is difficult to say anything definite about the hydration properties of the former. It was speculated in Chapter 3, however, that the ligule might secrete a 'lubricant' to ease the passage of the enclosed leaf or culm.

The secretory products of these two organs might be chemically similar and perform similar biological rôles; they might not.

Conclusion

The root cap is probably the most thoroughly studied secretory system of the grass plant. Comparison of this organ with the ligule has provided additional circumstantial evidence that the latter is also a secretory organ. Any remaining doubts as to the secretory nature of the ligule could be removed by using techniques successfully applied to the study of the root cap by other workers, such as autoradiography.

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